

Quantitative Optical Studies Using
Supported Lipid Bilayers - From
Molecular Mobility to Collective
Organization

Inaugural-Dissertation

zur Erlangung des Doktorgrades der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Luisa Coen

aus Dorsten

Düsseldorf, Juni 2026

Aus dem Institut für Experimentelle Medizinische Physik
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

Berichterstatter:

1. Prof. Dr. Cornelia Monzel
2. Physikalisches Institut
Universität Stuttgart
2. Prof. Dr. Thomas Heinzel
Institut für Festkörperphysik
Heinrich-Heine-Universität Düsseldorf

Tag der mündlichen Prüfung: **25.08.2026**

Abstract

Biological membranes constitute highly dynamic two-dimensional systems in which diffusion, intermolecular interactions, and external forces jointly determine molecular transport and spatial organization. While these processes are central to many cellular functions, their quantitative description remains challenging due to the complexity of biological environments. This work approaches this problem from a physical perspective by combining simplified model systems with advanced microscopy and quantitative image analysis.

Supported lipid bilayers are employed as controllable model membranes that retain essential features such as lateral fluidity while allowing systematic variation of experimental parameters. A central methodological aspect of this dissertation is the development and application of analysis strategies that convert fluorescence microscopy data into measurable quantities, including diffusion coefficients, molecular densities, transport velocities, and spatial organization metrics. To enable reproducible and scalable data analysis, dedicated image analysis software was developed. In this way, the work establishes a direct link between experimentally accessible imaging data and the underlying physical mechanisms governing membrane dynamics.

The results demonstrate that membrane behavior can be consistently described in terms of a small set of quantitative observables. It is shown that the presence of mobile and immobile obstacles leads to pronounced deviations from ideal Brownian motion. These findings highlight the strong influence of spatial heterogeneity and confinement on transport processes in two-dimensional systems. By applying externally controllable forces mediated by magnetic nanoparticles, complex spatial patterns of biomolecules in and on membranes can be generated and stabilized in a controlled manner. This establishes a new experimental approach for magnetic molecular pattern formation and demonstrates for the first time that externally applied forces mediated by magnetic nanoparticles can be used to generate and stabilize complex molecular patterns of biomolecules in and on membranes, revealing how the interplay between intrinsic membrane properties and external driving forces can give rise to tunable non-equilibrium organization. The quantitative analysis of receptor organization at cell-membrane interfaces shows that complex molecular pattern formation emerges when cells adhere to biomimetic membrane interfaces. Using the medically relevant example of the immunological synapse, it is demonstrated that molecular phase separation and clustering within the adhesion zone are not only influenced by active cellular processes, but are also significantly shaped by passive molecular dynamics on the interacting cell and model membrane interfaces as well as by molecular concentration. This reflects collective behavior of biomolecules in the form of emergent, phase-separated organization. In this work, a detailed quantitative analysis of chimeric antigen receptor engineered natural killer cell synapse organization is presented, providing one of the first systematic insights into their structural organization at the nanoscale.

In particular, spatial synapse formation and synapse size, rather than absolute receptor concentrations, are strongly influenced by externally defined ligand conditions. Furthermore, even non-signaling chimeric antigen receptors co-localize within the immunological synapse, indicating that receptor organization can arise from physical membrane processes independently of any cellular active process.

Taken together, these results show that diffusion, active transport, and receptor organization can be understood as interconnected manifestations of membrane dynamics governed by physical principles. The work emphasizes that quantitative image analysis is essential for transforming microscopy from a descriptive tool into a method for extracting reproducible physical observables and for developing and testing theoretical models. By combining controlled model systems with rigorous data analysis, this dissertation contributes to a unified physical framework for describing transport and organization in two-dimensional and non-equilibrium membrane systems.

Eidesstattliche Versicherung

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Hein-Universität Düsseldorf“ erstellt worden ist.

Düsseldorf, 22.06.2026
Ort, Datum


Luisa Coen

Erklärung zur Nutzung von KI-gestützten Hilfsmitteln

In der vorliegenden Arbeit wurden KI-gestützte Tools (z. B. ChatGPT, Google Gemini, Illustrae) ausschließlich zur sprachlichen Überarbeitung einzelner Textpassagen , zur Strukturierung von Formulierungen sowie zur Erstellung von Abbildungen verwendet.

Die inhaltliche Konzeption, Datenauswertung und wissenschaftliche Interpretation wurden eigenständig durchgeführt. Sämtliche Inhalte wurden von mir geprüft und verantwortet.

Contents

Abstract	iii
Eidesstattliche Versicherung	v
Erklärung zur Nutzung von KI-gestützten Hilfsmitteln	vi
List of Figures	ix
List of Abbreviations	xi
1 Introduction	1
1.1 Motivation	1
1.2 Outline	4
1.3 Scientific Results	6
1.3.1 Articles	6
1.3.2 Presentations	7
2 Fundamentals: Physical Framework for Molecular Dynamics on Membrane Surfaces	9
2.1 Optical Microscopy for Molecular Interaction and Dynamic Analysis	10
2.1.1 Continuous Photobleaching	11
2.1.2 Fluorescence Correlation Spectroscopy	13
2.1.3 Total Internal Reflection Fluorescence Microscopy	17
2.2 Transport and Organization in Two-Dimensional Membranes	21
2.2.1 Physical Properties of Lipid Bilayers	22
2.2.2 Supported Lipid Bilayers as Model Systems	22
2.2.3 Diffusion in Two-Dimensional Fluids	25
2.2.4 Diffusion in Membranes: Saffman-Delbrück	26
2.2.5 Obstacle and Anomalous Diffusion in Membranes	27
2.2.6 Phase Behavior and Membrane Heterogeneity	29
2.3 Force-Mediated Transport and Pattern Formation on Membrane Surfaces .	31
2.3.1 Superparamagnetic Nanoparticles in Soft Matter Systems	32

2.3.2	Magnetic Forces at the Nanoscale	36
2.3.3	Field-Driven Transport in Two-Dimensional Fluids	36
2.3.4	Pattern Formation under External Fields	38
2.3.5	Coupling Between Membrane Phase State and Particle Mobility . .	38
2.3.6	Particle-Induced Ligand Redistribution	39
2.4	Active Cellular Membrane Interfaces and Receptor Organization	39
2.4.1	Receptor-Ligand Interactions at Membrane Interfaces	40
2.4.2	Spatial Organization and Cluster Formation	43
2.4.3	Recruitment Dynamics at Membrane Interfaces	44
2.4.4	Quantification of Molecular Density via Fluorescence	46
2.4.5	Influence of Ligand Density and Mobility	47
2.4.6	Model Membranes for Studying Synapse Formation	48
2.5	Quantitative Image Analysis and Extraction of Physical Observables	50
2.5.1	Fluorescence Image Analysis	50
2.5.2	Quantitative Intensity Analysis and Calibration	52
2.5.3	Correction of Imaging Artifacts	54
2.5.4	Extraction of Spatial Organization and Clustering	57
2.5.5	Analysis of Transport Dynamics	58
2.5.6	Software and Implementation	60
2.6	Aim and Scope of the Dissertation	62
3	Publications	65
3.1	Publication I	67
3.2	Publication II	93
3.3	Publication III	113
3.4	Publication IV	140
4	Conclusion and Outlook	189
	Bibliography	193
	Danksagung	199

List of Figures

1.1	Conceptional overview of the dissertation.	5
2.1	Schematic illustration of optical microscopy techniques and their respective data outputs.	11
2.2	Structure and manufacture of SLBs.	23
2.3	Schematic illustration of membrane phase behavior.	29
2.4	Magnetic manipulation of membrane-bound nanoparticles and induced membrane reorganization.	37
2.5	Natural and CAR-mediated immune cell synapse formation.	44
2.6	Key biophysical parameters governing immune synapse formation.	49

List of Abbreviations

B7-H3 B7 homolog 3. 41

C6 complement component 6. 41, 44

CAR chimeric antigen receptor. ix, 2, 9, 21, 39–45, 47, 50–53, 60, 63, 66, 113, 190–192

CD19 cluster of differentiation 19. 41

CD28 cluster of differentiation 28. 41, 44

CD3 ζ cluster of differentiation 3 zeta chain. 41, 44

CD8 cluster of differentiation 8. 41, 44

CPB continuous photobleaching. 2, 9–13, 60, 62, 65, 67

cSMAC central supramolecular activation cluster. 43, 44

dSMAC distal supramolecular activation cluster. 43, 44

EGFR epidermal growth factor receptor. 41

FCS fluorescence correlation spectroscopy. 2, 9–11, 13–15, 17, 46, 52, 53, 60

FRAP fluorescence recovery after photobleaching. 11, 12

GaAsP gallium arsenide phosphide. 54

ICAM-1 intercellular adhesion molecule 1. 43, 44

LFA-1 lymphocyte function-associated antigen 1. 43

MHC major histocompatibility complex. 40–44

MNP magnetic nanoparticle. 2, 10, 32, 33, 37, 59–61, 65, 93

MSD mean square displacement. 25

N3 human nerve growth factor receptor-derived hinge N3. 41, 44

NA numerical aperture. 19, 20

NK natural killer. 39–45, 63, 66, 190

PMT photomultiplier tube. 54

pSMAC peripheral supramolecular activation cluster. 43, 44

ROI region of interest. 11, 12

scFv single-chain variable fragment. 41, 44

SLB supported lipid bilayer. ix, 1–5, 9–11, 13, 14, 17, 19–25, 27, 31, 32, 35–37, 47–50, 53, 57, 59, 60, 62, 63, 65–67, 93, 113, 189–191

TCR T-cell receptor. 40, 41, 43

TIRF total internal reflection fluorescence. 2, 9–11, 17–21, 24, 48, 60

Chapter 1

Introduction

1.1 Motivation

Cells constitute the fundamental units of life. Their interior is formed by cytoplasm, in which numerous organelles are spatially organized and enclosed by the plasma membrane. Far from acting as a passive boundary, the cell membrane is a highly dynamic and functional structure that plays a central role in communication with the environment and interactions with neighboring cells [1][2][3]. Many essential processes take place within or at the membrane, including lateral diffusion, molecular transport, spacial organization and specific intermolecular interactions, which can give rise to collective behavior in the form of phase separation and clustering [1][2][4]. These processes ultimately regulate cellular behavior and signaling pathways [5][6]. Understanding how such complex behavior emerges from comparatively simple physical interactions remains one of the central challenges of modern membrane biophysics.

While the biological interpretation of membrane-associated phenomena belongs to biology and life science, physics contributes by identifying, defining and quantitatively measuring observables that describe the underlying mechanism. Gaining insights into the principles governing transport, organization and interactions in low-dimensional systems is a central topic of modern physics, particularly in membrane-biophysics and soft matter physics. Two-dimensional fluid systems provide a unique platform in which fundamental processes such as diffusion and force-driven transport can be studied under controlled and experimentally accessible conditions [7][8]. They, therefore, offer the possibility to connect biological function with universal physical principles.

By reducing the structural and biochemical complexity of native three-dimensional cell membranes to a quasi-two-dimensional system, supported lipid bilayers (SLBs) offer excellent accessibility for optical microscopy and enable the isolation of individual physical mechanisms that are often difficult to disentangle in living cells [7][9][10]. At the same time, they preserve key membrane properties such as lateral fluidity and molecular mobil-

ity [11][12]. As such, SLBs serve as an ideal model system for investigating fundamental questions of transport and organization in two-dimensional systems [7][8]. This reduction of complexity is not merely a technical simplification, but a prerequisite for quantitative insight.

Optical fluorescence microscopy has become an indispensable tool for probing dynamic processes in membranes [13]. A variety of complementary techniques are available to extract quantitative information on molecular dynamics. Lateral diffusion, a key parameter in membrane systems, can be characterized using methods such as fluorescence correlation spectroscopy (FCS) or continuous photobleaching (CPB) [14][15][16]. In addition, fluorescence time-laps microscopy enables the direct observation of dynamic reorganization processes over time [13], while total internal reflection fluorescence (TIRF) microscopy selectively illuminates the contact interface between two membranes and is thus particularly suited for studying interfacial interactions [17]. The combination of these methods enables membrane dynamics to be addressed across spatial and temporal scales.

Beyond passive diffusion, force-driven transport represents another fundamental process in membrane systems. In living cells, receptor motion and membrane reorganization are often actively controlled by biochemical forces generated through the cytoskeleton or molecular motors [18][19]. In model systems, magnetic manipulation offers an attractive physical alternative for exerting controlled forces on membrane components. Magnetic fields are non-invasive, penetrate biological tissue efficiently and can be applied with high spatial and temporal control [20][21]. The development of highly biocompatible magnetic nanoparticles (MNPs), which can be coupled to specific proteins or ligands, enables targeted forces to be applied to membrane constituents and allows the active organization of membranes under well-defined conditions [21][22][23]. This creates a powerful route to study non-equilibrium membrane phenomena under externally tunable conditions.

Such active and passive membrane processes are particularly relevant in the context of membrane self-organization during cellular communication. A prominent example is the immunological synapse [18][24]. These systems exhibit collective behaviour manifested as phase separation and nanoscale clustering of receptors at the cell-membrane interface, which determines synapse organization. Here the spatiotemporal arrangement of receptors and ligands controls signaling between immune cells and their targets. Despite major progress, important differences between natural immunological synapses and engineered chimeric antigen receptor (CAR)-mediated immune synapses remain incompletely understood [25][26][27]. This highlights the need for well-controlled model systems in which individual parameters can be tuned independently, which SLBs successfully provide [28][10]. In combination with quantitative fluorescence microscopy, they enable direct links between externally defined molecular conditions and emergent receptor organization.

Finally, the increasing amount and complexity of microscopy data require robust strategies for quantitative evaluation. A central challenge is to extract meaningful physi-

cal observables from seemingly simple image data in a reliable and reproducible manner. Quantitative image analysis, which utilizes computational methods to extract quantitative information from microscopy data, provides the tools to convert large sets of microscopy images and time series into measurable parameters such as diffusion coefficients, absolute molecular densities, transport velocities or spatial correlation metrics. In this way, modern microscopy combined with quantitative image analysis enables visual information to be transformed into rigorous physical insights into membrane dynamics and organization. Without such quantitative analysis, advanced imaging remains descriptive rather than analytical [29].

This dissertation addresses the question of how optical measurement techniques can be employed to extract quantitative physical observables that characterize membrane-associated processes. The central objective is to develop and apply experimental approaches that translate dynamic fluorescence microscopy data into measurable parameters, thereby enabling a quantitative description of molecular motion, force-induced rearrangements, emergent spatial organization and collective behavior of biomolecules in membrane systems.

A particular focus lies on combining complementary optical methods to access different aspects of membrane dynamics across spatial and temporal scales. By using techniques sensitive to molecular mobility, local concentrations, transport behavior, and collective pattern formation, this work aims to establish a comprehensive experimental framework for investigating two-dimensional membrane systems under controlled conditions. In this way, the dissertation pursues not a single isolated measurement problem, but a broader strategy for quantitatively characterizing complex membrane dynamics.

Rather than focusing primarily on biological complexity itself, this dissertation uses biologically inspired model membranes as simplified and tunable platforms to study general physical mechanisms. SLBs allow individual parameters to be varied independently, making it possible to isolate the contributions of diffusion, intermolecular interactions, confinement, and externally applied forces.

In this way, the present work is positioned at the interface of physics and biology: biological systems provide the motivation and relevant phenomena, while the scientific approach is rooted in quantitative physical analysis. Consequently, the results contribute not only to the understanding of membrane-associated processes, but more broadly to the fundamental question of how transport, interactions, and external driving forces give rise to structured organization in low-dimensional and non-equilibrium systems. This combination of biological relevance and physical generality forms the central motivation of the present dissertation.

1.2 Outline

This dissertation is structured as follows: It begins with a chapter on the biophysical foundations relevant to the work presented. This part establishes the theoretical and methodological background required for the subsequent studies and introduces the physical concepts underlying transport, interactions, and organization in membrane-associated systems.

Section 2.1 presents the principal optical techniques used to investigate membrane processes. Particular emphasis is placed on fluorescence-based microscopy methods that enable the quantitative observation of molecular mobility, transport dynamics, and spatial organization in two-dimensional membrane environments. These methods form the experimental basis for all studies presented in this dissertation.

Section 2.2 focuses on transport and organization in two-dimensional membranes, with particular relevance to Publication I. In this study, lateral diffusion in SLBs is systematically investigated using quantitative fluorescence microscopy. Special attention is given to the influence of mobile and immobile obstacles, providing insights into how heterogeneities lead to deviations from ideal Brownian motion. The results contribute to a broader understanding of diffusion in crowded and structurally complex membrane systems.

Section 2.3 addresses active transport and pattern formation on membrane surfaces. Introducing controlled external forces enables not only the manipulation of individual membrane-associated components, but also the generation of collective spatial organization. In Publication II, this concept is explored by combining SLBs with superparamagnetic nanoparticles, thereby enabling force-driven transport within a two-dimensional fluid environment. External magnetic field gradients induce directed motion and controlled particle accumulation, resulting in tunable spatial patterns. Importantly, the interplay between external driving forces and intrinsic membrane properties, such as lipid phase behavior, provides an additional level of control over the dynamics and stability of these patterns. This establishes a model framework for studying non-equilibrium organization in membrane systems.

Section 2.4 is concerned with active cellular membrane interfaces and receptor organization. Such interfaces are central to many biological communication processes, where receptor distributions and local molecular organization determine signaling outcomes, in particular the immunological synapse. In Publication III, these phenomena are investigated using SLBs with controlled ligand densities as model target membranes. Quantitative fluorescence microscopy is employed to determine absolute receptor concentrations and to analyze the formation of spatial patterns and receptor clusters. This enables a direct connection between externally defined boundary conditions and the emergent organization at the cell-membrane interface. Publication IV extends this approach to multicomponent receptor systems, in which distinct receptor species are simultaneously

recruited to the interface, allowing more complex interaction scenarios to be examined.

Section 2.5 focuses on the quantitative image analysis performed to determine quantitative physical observables from fluorescence image data. Different fully or half automated analysis pipelines are introduced to gather reliable and reproducible membrane-associated parameters.

Following the introductory and fundamentals chapters, the four publications that form the core of this cumulative dissertation are presented in full. For each publication, the individual contributions of the author are explicitly stated.

The dissertation concludes with a general conclusion and outlook. In the final chapter, the interrelations between the individual studies are examined and their combined

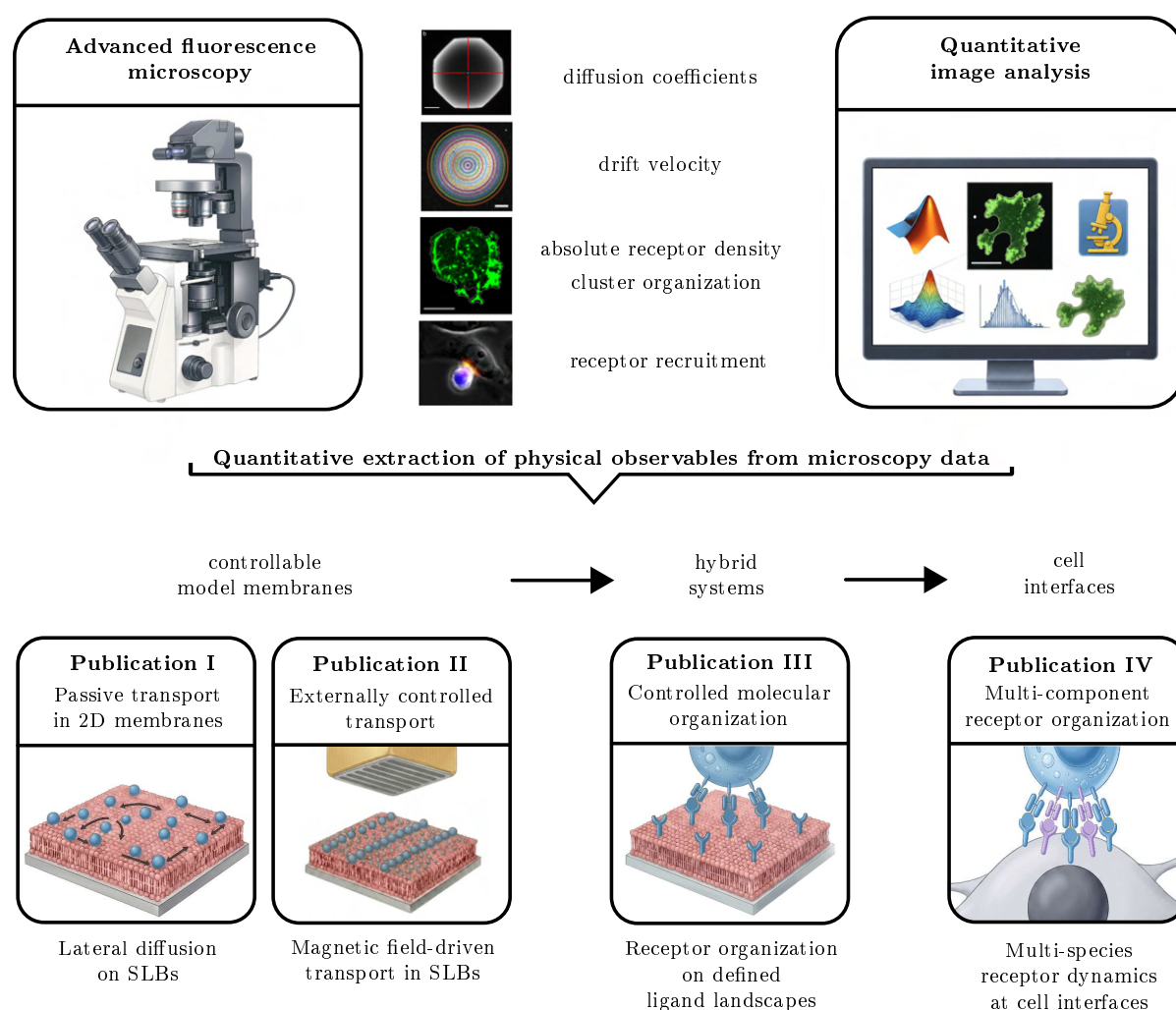


Figure 1.1: Conceptual overview of the dissertation. Controlled SLBs serve as tunable two-dimensional model systems to investigate membrane-associated processes using advanced fluorescence microscopy. Quantitative image analysis enables the extraction of physical observables such as diffusion coefficients, drift velocities, receptor densities, and spatial organization metrics from microscopy data. The four publications address increasing levels of complexity, ranging from passive diffusion and force-driven transport in model membranes to receptor organization at biologically relevant cell-membrane interfaces.

contribution to a common physical framework is discussed, allowing broader insights to be drawn. Future perspectives for the quantitative investigation of membrane-associated systems are also outlined.

1.3 Scientific Results

1.3.1 Articles

The work presented focuses on the development of quantitative measurement approaches based on advanced fluorescence microscopy to characterize dynamic membrane-associated processes such as diffusion, active transport and spatial organization. This research has resulted in four publication, comprising two accepted article, one submitted manuscripts and one manuscript in preparation (see figure 1.1).

Article *Mobile and Immobile Obstacles in Supported Lipid Bilayer Systems and Their Effect on Lipid Mobility*

Luisa Coen, Daniel Alexander Kuckla, Andreas Neusch and Cornelia Monzel

Colloids and Interfaces (2024), Volume 8, Issue 5

Status | Published

<https://doi.org/10.3390/colloids8050054>

Article *Superparamagnetic Biomembranes for Dynamic Nanoparticle and Molecular Patterning*

Luisa Coen, Jan Lange, Andreas Neusch, Thomas Heinzel and Cornelia Monzel

Nano Letters (2026)

Status | Accepted

<https://doi.org/10.1021/acs.nanolett.6c00639>

Article *Ligand-Defined Phase Space Predicts CAR-NK Synapse Architecture and Cytotoxic Organization.*

Luisa Coen, Miriam N. Tappel, Lara Hölzle, Maren Soldierer, Nicole Sander, Constanze Wiek, Helmut Hanenberg, Cornelia Monzel

Nature Communications

Status | In Preparation

Article *Co-expression of Non-Signaling CARs Enhances/Boosts EGFRvIII-Specific CAR-T Cell Cytotoxicity Against Head and Neck Squamous Cell Carcinoma*

Kathleen Grueter, Nicole Sander, Luisa Coen, Saskia Hüsken, Eileen Kleinfelder, Corinna Haist, Franziska Blaesche, Kathrin Scheckenbach, Cornelia Monzel, Helmut Hanenberg, Constanze Wiek

BMC Cancer

Status | Submitted

1.3.2 Presentations

In addition, parts of this work were presented at national and international conferences in the form of contributed talks and poster presentations.

Flash Talk & Poster *Towards Understanding the Molecular Composition of the Immunological Synapse in CAR-NK Cells,*

Lara Hölzle, Michael-Nicholas Reifenscheid, Luisa Coen, Constanze Wiek, Maren Soldierer, Helmut Hanenberg and Cornelia Monzel

From Single Molecules to Cell Function in Biomembranes Symposium (2025), HHU Düsseldorf

Flash Talk & Poster *Dynamically tunable Pattern of Magnetic Nanoparticles on a Supported Lipid Bilayer*

Luisa Coen, Jan Lange, Thomas Heinzl and Cornelia Monzel

Emerging Trends in Physics of the Cell (2025), TU Munich

Chapter 2

Fundamentals: Physical Framework for Molecular Dynamics on Membrane Surfaces

Here the physical and methodological foundations required for the interpretation of the experimental results are provided. The systems investigated here, ranging from SLBs and synthetic model interfaces to active immunological synapses formed by CAR-engineered effector cells, are governed by a combination of membrane physics, molecular transport processes, receptor–ligand interactions, and force-driven non-equilibrium dynamics. A quantitative understanding of these systems therefore requires a unified framework that connects optical observables obtained from fluorescence microscopy with the underlying physical parameters of molecular motion and organization.

To this end, the following sections introduce the essential concepts and experimental techniques used throughout this work. First, the optical microscopy methods employed for probing molecular dynamics and interactions are discussed, including FCS, CPB, and TIRF microscopy. These techniques yield complementary access to diffusion, molecular densities, and interfacial processes at membrane surfaces.

Subsequently, the physical properties of lipid bilayers and their usage as model systems for biological membranes are outlined, together with the fundamental principles governing two-dimensional diffusion, membrane hydrodynamics, and anomalous transport behavior. Building on this, the role of external forces and active manipulation, particularly magnetic field-driven transport using superparamagnetic nanoparticles, is introduced as a means to control and probe non-equilibrium pattern formation in membrane systems.

The fourth section extends these concepts to active cellular interfaces, where receptor–ligand interactions, adhesion processes, and cytoskeletal coupling give rise to spatial organization, clustering, and dynamic remodeling of the immunological synapse. In this context, quantitative fluorescence measurements serve as a direct link between molecular-

scale processes and experimentally accessible observables such as receptor densities and spatial distributions.

Finally, the methodological framework of quantitative image analysis is presented. This includes the conversion of fluorescence intensity into physical quantities, the correction of imaging artifacts, the extraction of spatial organization and transport dynamics, and the computational implementation of the analysis pipelines. Together, these components establish the basis for translating fluorescence microscopy data into physically meaningful descriptions of molecular organization and dynamics on membrane surfaces.

2.1 Optical Microscopy for Molecular Interaction and Dynamic Analysis

Optical Microscopy constitutes a central methodological pillar for inquiring molecular organization, transport processes and interaction dynamics in biomimetic membrane systems. In contrast to purely structural techniques, fluorescence-based microscopy provides simultaneous spatial and temporal access to membrane-associated processes under physiologically relevant conditions. This is particularly important for SLBs, where the lateral mobility of lipids, proteins and ligand-functionalized MNPs determines both equilibrium organization and externally induced non-equilibrium pattern formation.

By selective fluorescent labeling of membrane constituents, optical microscopy enables quantitative observation of diffusion, clustering, recruitment and interface formation over length scales ranging from nanometers to entire cell-surface contact zones. Depending on the imaging modality, different aspects of membrane behavior can be resolved. Time-resolved approaches are especially valuable for extracting dynamic parameters such as diffusion coefficients, whereas high-contrast surface-sensitive methods permit direct visualization of membrane interfaces and cell synapses.

In the context of this dissertation, several complementary fluorescence techniques were employed (see figure 2.1). CPB was used to quantify lateral diffusion over large membrane areas on SLB model systems. FCS provided access to molecular mobility inside the cell, by discriminate between different diffusion species. TIRF microscopy allows selective imaging of processes occurring in close proximity to the SLB, particularly during cell-membrane interactions. In addition, widefield, epifluorescence, and confocal imaging were applied for structural characterization, overview imaging, large time scale experiments and three-dimensional visualization.

Together, these methods establish a versatile toolbox for linking molecular-scale diffusion and transport processes with macroscopic membrane organization and cellular function.

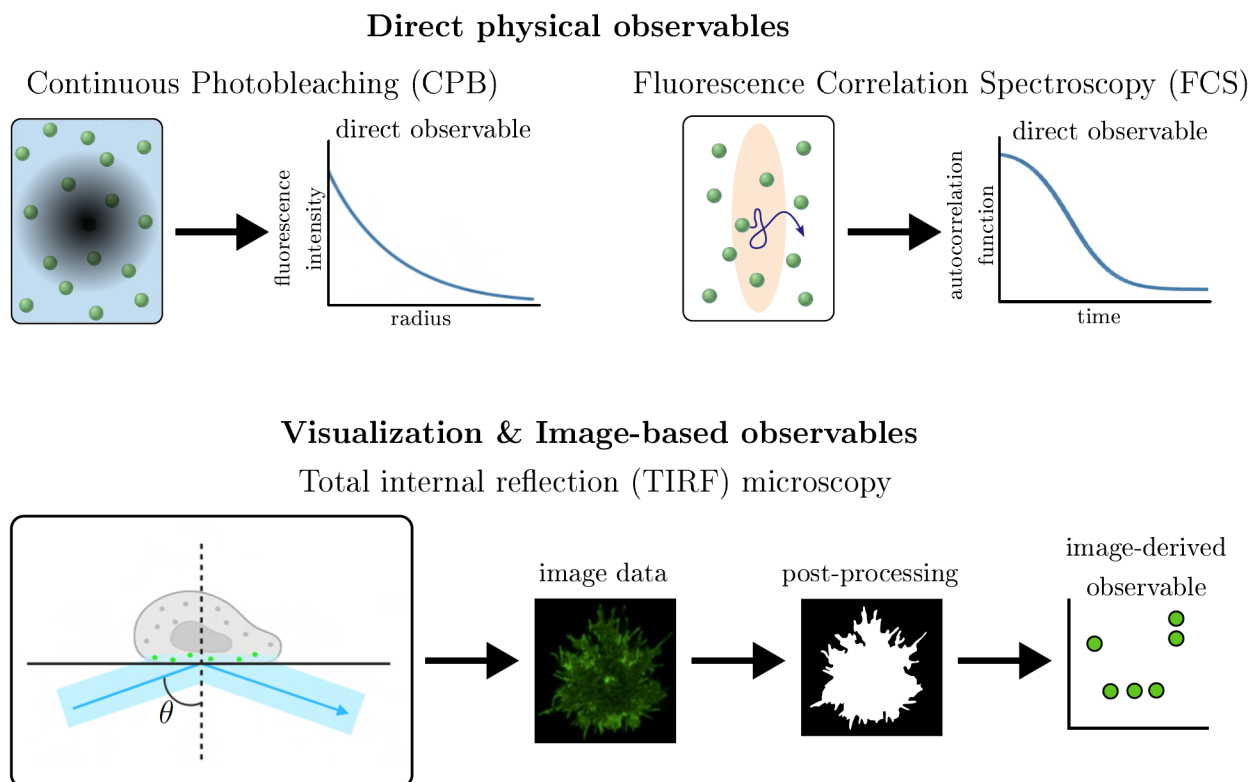


Figure 2.1: Schematic illustration of optical microscopy techniques and their respective data outputs. Under Direct physical observables, CPB analyzes steady-state fluorescence profile generated by ongoing bleaching of a defined area and diffusion of unbleached molecules from the periphery to extract spatial diffusion properties, while FCS quantifies temporal fluorescence intensity fluctuations within a small observation volume to distinguish different diffusion populations. Under Visualization and image-based observables, TIRF microscopy selectively excites fluorophores in close proximity to the glass-sample interface via an evanescent field generated by total internal reflection, enabling surface-sensitive visualization of membrane-associated structures. Acquired TIRF image data can subsequently be processed to extract quantitative image-derived observables such as synapse size, morphology, or spatial receptor organization.

2.1.1 Continuous Photobleaching

CPB (see figure 2.1) is a robust fluorescence-based method for determining lateral diffusion coefficients in two-dimensional membrane systems. The technique is particularly well suited for SLBs because it probes diffusion over comparatively large membrane areas and does not rely on an instantaneous high-intensity bleaching pulse, as required in fluorescence recovery after photobleaching (FRAP), another often used fluorescence-based technique to determine diffusion constants on SLBs. As a result, CPB can be applied to a broad range of fluorophores and often yields diffusion coefficients that are more representative of the global sample behavior [16][Publication I].

The principle of CPB is based on the competition between irreversible photobleaching and lateral molecular diffusion. During the experiment an approximately circular region of interest (ROI) is continuously illuminated with sufficient intensity to bleach fluorescent

molecules within this area. Experimentally, this ROI can be defined by partially closing the field diaphragm of the microscope, thereby restricting the illuminated region to a fraction of the total field of view [16][Publication I].

Fluorophores located inside the ROI gradually lose their fluorescence due to continuous excitation and photobleaching. In the central region of the illuminated area, molecules are exposed longest and the fluorescence intensity decreases strongly, ideally approaching background level. At the same time, unbleached molecules from the surrounding, not illuminated membrane regions laterally diffuse into the illuminated region and replace bleached molecules, while bleached molecules diffuse outwards. This continuous exchange generates a stationary non-equilibrium concentration profile governed by the balance between bleaching and diffusion [16][Publication I].

As a consequence, the fluorescence signal is typically lowest in the center of the ROI and increases toward its boundary, where replacement of bleached molecules is highest. The resulting radial intensity profile contains direct information about the diffusion coefficient: faster diffusion replenishes bleached regions more efficiently and leads to shallower gradients, whereas slower diffusion produces steeper spatial decay [16][Publication I].

Two complementary observables are commonly analyzed. First, the temporal fluorescence decay at the center of the ROI is recorded during illumination. This intensity trace primarily reflects the local bleaching kinetics and can be approximated by an exponential decay toward a background offset:

$$I(t) = I_0 e^{-Bt} + I_{Bg} \quad ,$$

where I_0 is the initial fluorescence intensity, B is the effective bleaching rate constant, t the time and I_{Bg} is the background intensity [16][Publication I].

Second, after reaching steady state, the spatial fluorescence profile from the ROI boundary toward the center is evaluated. Here, the intensity follows:

$$I(x) = I_0 e^{-x\sqrt{\frac{B}{D}}} + I_{Bg} \quad ,$$

where x donates the distance from the edge of the illuminated region, D is the lateral diffusion coefficient and B the bleaching rate determined from the temporal fit. Since the bleaching rate is known independently, the diffusion coefficient can be extracted directly from the spatial decay profile [16][Publication I].

To improve robustness, experimental studies often rely on averaging procedures. For example, the temporal trace may be obtained from a pixel region in the center of the ROI, instead of a single pixel and several line profiles from the steady state are examined. Independent fits of multiple profiles allow calculation of mean diffusion coefficients and associated statistical uncertainties [Publication I].

Compared with FRAP, CPB offers several practical advantages. Since bleaching is

continuous rather than brief, the method is less sensitive to imperfect bleaching behavior. It also samples diffusion over a larger effective area, which reduces sensitivity to local membrane heterogeneity. Thus CPB is particularly valuable for characterizing phase-dependent lipid and protein diffusion in SLBs [16]. The method therefore provides an important quantitative bridge between fluorescence imaging and membrane diffusion physics. In the context of this dissertation CPB was employed to quantify how lipid composition, phase state and obstacle occurrence affect the lateral mobility of membrane components [Publication I].

2.1.2 Fluorescence Correlation Spectroscopy

FCS (see figure 2.1) is a fluctuation-based single-molecule sensitive method that enables quantitative analysis of molecular diffusion, concentration, and photophysical state dynamics from spontaneous fluorescence intensity variations within a small observation volume. In contrast to image-based approaches, FCS does not primarily rely on spatially resolved pictures, but on the temporal analysis of fluorescence signals originating from a statistically small ensemble of molecules. This method is thus especially powerful for probing transport processes in living cells and biomimetic membrane systems at nanomolar concentrations and on microsecond-to-second timescales [30][31][32].

The physical basis of FCS is straightforward: fluorescent labeled molecules continuously enter and leave the illuminated detection region due to Brownian motion. Since the number of emitters inside this region changes over time, the measured fluorescence intensity $F(t)$ exhibits stochastic fluctuations around its mean value $\langle F \rangle$. The characteristic amplitude and temporal persistence of these fluctuations contain direct information about molecular concentration and mobility. Fast diffusion generates rapidly decaying fluctuations, whereas slow diffusion leads to longer correlation times [30][32].

To extract this information quantitatively, the normalized (converges to 0) temporal autocorrelation function is calculated:

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \quad ,$$

with

$$\delta F(t) = F(t) - \langle F(t) \rangle \quad .$$

The autocorrelation function measures how strongly the fluorescence signal at time t resembles the signal after a delay τ . At short lag times, molecules have not yet significantly redistributed and the correlation is high. At long lag times, positional memory is lost, as diffusive motion randomizes molecular positions over length scales exceeding the observation volume, and the correlation decays toward zero. The shape of this decay defines the characteristic diffusion time through the detection region [32].

Confocal Observation Volume and Effective Detection Area

For most biological applications, FCS is implemented on a confocal microscope. A focused laser beam excites fluorescence only in a diffraction-limited region, while a pinhole suppresses out-of-focus emission. The resulting detection profile can be approximated by a three-dimensional Gaussian function $W(r, z)$. This defines the confocal observation volume, typically in the femtoliter range, which is sufficient small that only a few fluorescent molecules are present simultaneously [30].

The detection volume is commonly parameterized by a lateral beam waist w_0 and an axial radius z_0 :

$$W(r, z) = \exp\left(-\frac{2r^2}{w_0^2}\right) \exp\left(-\frac{2z^2}{z_0^2}\right) \quad .$$

The ratio

$$\kappa = \frac{z_0}{w_0}$$

describes the structural parameter of the confocal volume and is typically determined by calibration with reference dyes of known diffusion coefficient [30].

For membrane measurements, the relevant geometry changes fundamentally. If the focal plane intersects a thin plasma membrane or SLB, fluorophores are effectively restricted to a two-dimensional plane whose thickness is negligible compared with the axial extent of the confocal volume. In that case, diffusion occurs within the effective confocal ground area rather than throughout the full three-dimensional volume. The relevant area is approximately

$$A_{\text{eff}} = \pi w_0^2 \quad .$$

Thus, membrane FCS converts the confocal volume into a laterally confined sampling area, while cytoplasmic fluorophores continue to diffuse in the full three dimension geometry.

This distinction is highly relevant for the experiments presented here. When the confocal focus is positioned at the plasma membrane of a cell, the measured signal generally contains two contributions:

1. a **membrane-associated fraction**, diffusing laterally in two dimensions, and
2. a **cytoplasmic fraction**, diffusing in three dimensions through the convocal volume above the membrane plane.

Consequently the measured autocorrelation function in membrane-focused FCS contains at least two diffusion components that can be separated by appropriate model fitting.

FCS Diffusion Models for Two and Three Dimensions

For freely diffusing species in solution or cytoplasm, the standard confocal FCS model assumes three-dimensional Brownian motion through a Gaussian detection volume. The

corresponding correlation function is:

$$G_{3D}(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{\kappa^2 \tau_D}\right)^{-1/2},$$

where N is the average number of fluorescent particles in the observation volume and τ_D is the characteristic correlation time due to diffusion. The diffusion coefficient follows as:

$$D = \frac{w_0^2}{4\tau_D}.$$

This model explicitly describes normal Brownian motion, whereas in intracellular, cytoplasmic measurements molecules have to diffuse through a crowded three-dimensional environment. For live-cell data, extended formulations that include triplet-state kinetics or slow intensity drifts are often preferable [32][30]. This corresponds to the **Triplet Extended (3D)** model [14] frequently used in commercial fitting software.

For membrane-bound molecules, diffusion is laterally confined to a plane. Thus the autocorrelation function can be simplified to the two-dimensional form [30]:

$$G_{2D}(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1}.$$

This description forms the basis of the **Triplet Extended (2D)** approach[15] used for fluorophores diffusing in artificial or cellular membranes.

Triplet-State Extension and Photophysics

Many fluorophores transiently enter non-emissive dark states, as the triplet-state. These fast blinking events generate additional short-time correlations unrelated to translational diffusion. To account for this, a multiplicative triplet term is introduced:

$$G_T(\tau) = 1 + \frac{T}{1-T} \exp\left(-\frac{\tau}{\tau_T}\right),$$

where T is the triplet fraction and τ_T the corresponding relaxation time. The full model is then given by:

$$G(\tau) = G_{\text{diff}}(\tau) G_T(\tau).$$

Including this corrections substantially improves fits for organic dyes and fluorescent proteins, especially at microsecond lag times [32].

Separation of Membrane and Cytoplasmic Contributions

A central application of FCS in this work is the quantitative separation of mixed fluorescence signals when the confocal focus is positioned at the plasma membrane. In this

measurement geometry, the detection volume intersects both the membrane plane and the adjacent cytoplasmic region. Consequently, fluorescence fluctuations originate from two physically distinct molecular populations: a membrane-associated fraction undergoing lateral two-dimensional diffusion and a cytoplasmic fraction diffusing three-dimensional through the confocal volume [Publication III].

As both populations contribute simultaneously to the measured intensity trace, the resulting autocorrelation curve contains overlapping dynamic components with different characteristic timescales. In general, the total correlation function can be described as the superposition of a membrane-related two-dimensional contribution and a cytoplasmic three-dimensional contribution:

$$G(\tau) = a G_{2D}(\tau) + b G_{3D}(\tau) \quad ,$$

where a and b represent the relative amplitudes of the two populations [Publication III].

To obtain robust membrane diffusion parameters, a sequential fitting strategy was employed. First, measurements were performed exclusively in the cytoplasm, away from the membrane interface. These data were analyzed using the triplet-extended three dimensions diffusion model, yielding the characteristic cytoplasmic diffusion time and corresponding photophysical parameters of the freely diffusing intracellular fraction [Publication III].

Second, measurements acquired with the focus positioned on the plasma membrane were analyzed using a two-component model. Here, the cytoplasmic three-dimensional component was kept fixed at the previously determined value, while only the membrane-associated diffusion time and the relative amplitudes of both components were fitted. This procedure substantially reduces parameter covariance and improves the reliability of the extracted cellular diffusion coefficients [Publication III].

Physically, the membrane-associated component generally exhibits longer correlation times due to reduced mobility in the viscous two-dimensional membrane environment, additional crowding effects, and possible transient interactions with other membrane constituents. In contrast, cytoplasmic molecules usually display faster diffusion unless incorporated into larger complexes or hindered by intracellular crowding [Publication III].

This sequential decomposition strategy is particularly valuable for transmembrane receptor systems that are dynamically transported from intracellular compartments to the plasma membrane. Beyond the determination of diffusion coefficients, it enables quantitative extraction of the relative fractions of membrane-associated and cytoplasmic populations. This provides direct information on receptor localization, membrane recruitment, and redistribution dynamics, while simultaneously resolving the mobility of the biologically active surface-associated fraction [Publication III].

Relevance for the Dissertation

Within this dissertation, FCS serves primarily as a quantitative tool to distinguish membrane-associated fluorescence from cytoplasmic background contributions in confocal measurements of living cells. When the focal plane is positioned at the plasma membrane, a substantial fraction of the detected signal may still originate from fluorescent molecules diffusing in the adjacent cytoplasm. Without correction, this overlap would lead to systematic overestimation of membrane-localized receptor densities.

By separating the two-dimensional membrane component from the three-dimensional cytoplasmic component, FCS enables determination of the relative membrane fraction of the total detected fluorescence signal. This information is essential for all subsequent confocal measurements aimed at absolute concentration quantification, as it defines which proportion of the measured intensity truly arises from membrane-localized molecules and should therefore be included in surface density calculations, and which proportion originates from the cytoplasmic pool and must be excluded.

In this way, FCS provides a critical calibration step linking fluorescence intensity measurements to physically meaningful membrane concentrations. The simultaneously obtained diffusion coefficients of the membrane-bound and cytoplasmic fractions offer additional insight into molecular mobility and receptor dynamics, but represent a secondary benefit relative to the central objective of accurately quantifying membrane fractions.

2.1.3 Total Internal Reflection Fluorescence Microscopy

TIRF microscopy (see figure 2.1) is a surface-selective fluorescence imaging technique that enables highly specific excitation of fluorophores located within a thin optical layer adjacent to an interface between two media of different refractive index. In contrast to conventional epifluorescence microscopy, where fluorophores throughout a substantial sample depth are excited, TIRF restricts excitation to a nanometric region close to the substrate. This strongly suppresses background fluorescence from the sample bulk and provides excellent signal-to-noise ratio for membrane-associated processes. TIRF is therefore particularly well suited for studying SLBs, cell adhesion, receptor recruitment, and contact interfaces between cells and functionalized surfaces [17].

In the present work, TIRF microscopy was employed to image the contact zone between living immune cells and SLBs formed on glass coverslips. In this geometry, total internal reflection occurs at the interface between the high-index glass substrate and the thin aqueous layer underneath the supported membrane. The evanescent excitation field selectively illuminates fluorophores located in the immediate vicinity of this interface, such that the cell-SLB contact region can be visualized with high contrast, while fluorescence from the upper cell body is mainly suppressed.

Optical Principle and Snell's Law

The physical basis of TIRF is refraction at an interface between two media. When light passes from a medium of refractive index n_1 into a second medium of lower refractive index n_2 , the propagation direction changes according to Snell's law:

$$n_1 \sin \theta_1 = n_2 \sin \theta_2 \quad ,$$

where θ_1 is the incident angle and θ_2 the refracted angle, both measured relative to the surface normal [17].

As the incident angle increases, the refracted beam bends progressively toward the interface. At a specific limiting angle, the refracted beam propagates parallel to the interface ($\theta_2 = 90^\circ$). This defines the critical angle for total internal reflection:

$$\sin \theta_c = \frac{n_2}{n_1} \quad ,$$

and therefore

$$\theta_c = \arcsin \left(\frac{n_2}{n_1} \right) \quad .$$

For incidence angles larger than θ_c , no propagating wave can enter the lower-index medium, and the excitation beam is reflected completely back into the glass [17].

Critical Angle in the Present Experimental System

For a typical glass coverslip or immersion oil objective system, the refractive index is approximately

$$n_1 \approx 1.518 \quad ,$$

whereas the aqueous buffer below the SLB is approximately

$$n_2 \approx 1.333 \quad .$$

Thus the theoretical critical angle is:

$$\theta_c = \arcsin \left(\frac{1.333}{1.518} \right) \approx 61.4^\circ \quad ,$$

confirming that the chosen experimental angle ($\theta \approx 64^\circ$) fulfills the total internal reflection condition. This suggests that excitation does not arise from a propagating wave penetrating the sample, but is instead governed by an evanescent field confined to the membrane interface.

Evanescent Wave and Exponential Decay

Although no propagating beam enters the aqueous phase above the critical angle, the electromagnetic boundary conditions require continuity of the optical field across the interface. As a result, a non-propagating near-field is generated in the low-index medium: the evanescent wave [17].

This field propagates parallel to the interface but decays exponentially in the direction normal to the surface:

$$I(z) = I_0 \exp\left(-\frac{z}{d_p}\right) \quad ,$$

where I_0 is the intensity directly at the interface and d_p is the penetration depth.[17]

For an excitation wavelength λ , the penetration depth is given by [17]:

$$d_p = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2 \theta - n_2^2}} \quad .$$

Using parameters representative of the used setup:

- $\lambda = 488 \text{ nm}$
- $n_1 = 1.518$ (glass/immersion oil objective)
- $n_2 = 1.333$ (aqueous buffer)
- $\theta = 64^\circ$

the theoretical penetration depth is:

$$d_p \approx 134 \text{ nm} \quad . \tag{2.1}$$

Thus, only fluorophores located within roughly the first 100 – 150 nm above the glass-supported membrane are efficiently excited. This shallow excitation layer is ideally suited for imaging cell-SLB contact zones, because fluorescence from the cell interior is strongly suppressed while signals originating from the membrane interface remain highly selective.

Objective Lens Method

In this dissertation, TIRF illumination was implemented using the objective lens method (through-the-objective TIRF). In this configuration, the excitation beam is introduced through a high numerical aperture (NA) objective and focused off-axis in the back focal plane. This causes the beam to exit the front lens element at a highly oblique angle and strike the glass sample interface above the critical angle [17].

The maximum achievable illumination angle is determined by the objective NA [17]:

$$\text{NA} = n_1 \sin \theta_{\max} \quad .$$

For the oil immersion objective used in the context of this dissertation with

$$\text{NA} = 1.49 \quad ,$$

the maximum theoretical angle is:

$$\theta_{\text{max}} = \arcsin\left(\frac{1.49}{1.518}\right) \approx 79.9^\circ \quad .$$

Thus, the objective provides a sufficiently large angular range to exceed the critical angle and to tune the penetration depth reproducible. High-NA objectives are essential for objective-based TIRF, since lower NA optics may not reach the critical incidence angles [17].

Advantages and Experimental Considerations

Compared with epifluorescence illumination, TIRF offers greatly improved contrast for surface-near processes. However, quantitative interpretation requires careful consideration of several factors:

- the penetration depth depends sensitively on incidence angle and refractive indices,
- local refractive index variations near cells can modify the evanescent field,
- only fluorophores close to the interface are excited, meaning vertical cell movements alter signal intensity,
- alignment of the off-axis beam in the back focal plane is critical for stable TIRF conditions.

Despite these considerations, TIRF remains one of the most powerful methods for analyzing dynamic membrane interfaces with high temporal resolution and minimal background fluorescence [17].

Relevance for Cell-SLB Contact Imaging

For the experiments described in this dissertation [Publication III], the most relevant fluorescent structures are located at the interface between a cell membrane and a SLB. This contact zone typically lies within less than a few ten nanometers of the glass surface and is therefore ideally matched to the evanescent excitation field. As a consequence, TIRF selectively visualizes:

- receptor accumulation at the cell-SLB contact site,
- ligand redistribution within the supported membrane,

- formation of adhesion zones and immune synapse
- receptor clustering and membrane reorganization

At the same time, fluorescence from intracellular compartments deeper inside the cell is strongly reduced. This is particularly advantageous for studies of CAR-immune cell interactions with SLBs, where signaling and receptor reorganization occur predominantly within the basal contact plane.

Relevance for the Dissertation

Within this dissertation, TIRF microscopy constitutes a key tool for directly observing the active contact interface between cells and SLB. Its nanometric axial selectivity makes it possible to isolate the biologically relevant interaction plane where receptor binding, ligand redistribution, and synapse formation occur. TIRF therefore provides the essential experimental link between membrane biophysics, molecular reorganization, and functional cellular responses at engineered membrane surfaces [Publication III].

The experimental investigation of membrane systems requires quantitative imaging techniques capable of resolving spatial and temporal variations in molecular distributions. In the following, the physical properties of two-dimensional lipid membranes and their implementation as model systems are introduced, providing the basis for studying transport and organization in confined membrane systems.

2.2 Transport and Organization in Two-Dimensional Membranes

Two-dimensional lipid membranes constitute a class of soft matter systems in which transport processes, interactions, and spatial organization can be investigated under well-defined physical boundary conditions. Their quasi-two-dimensional geometry leads to fundamentally different transport behavior compared to bulk fluids, making them an important model system for studying diffusion and non-equilibrium transport phenomena.

From a physical perspective, lipid membranes can be described as viscous, thermally fluctuating two-dimensional fluids embedded in a three-dimensional environment [4]. Their behavior is governed by the interplay of thermal energy, hydrodynamic coupling, intermolecular interactions, and structural heterogeneity. These properties make them specifically suitable for studying how microscopic interactions give rise to emergent spatial and dynamical organization.

In this context, SLB provide experimentally accessible realizations of such two-dimensional systems. They combine lateral fluidity with mechanical stability and optical accessibility,

enabling quantitative analysis of diffusion and transport processes at the molecular scale.

2.2.1 Physical Properties of Lipid Bilayers

Lipid bilayers are self-assembled structures formed by amphiphilic lipids that spontaneously organize in aqueous environments. This organization is driven by the hydrophobic effect, which minimizes the free energy of the system by shielding hydrophobic lipid tails from water while exposing hydrophilic head groups to the aqueous phase [1][2].

From a physical perspective, lipid bilayers represent nanometer-thick two-dimensional fluids with finite bending rigidity and lateral viscosity. Their mechanical and dynamical properties are determined by lipid composition, temperature, molecular charge and structure. These parameters define the effective elasticity, thickness, and transport properties of the membrane [1][2][33].

The lateral mobility of membrane components is a central feature of lipid bilayers and forms the basis for spatial reorganization processes such as clustering, phase separation, and signal transduction in biological systems.

2.2.2 Supported Lipid Bilayers as Model Systems

SLB are artificial membrane systems formed on solid substrates such as glass or silica. A thin hydration layer (~ 1 nm) separates the bilayer from the substrate, allowing partial decoupling of the membrane from the solid support (see figure 2.2A).

This geometry provides two key advantages: mechanical stabilization and optical accessibility. At the same time, it introduces an additional physical parameter in the form of substrate-induced friction, which can influence lateral transport, particularly in the lower leaflet [34].

Several preparation methods exist for SLB formation, including vesicle fusion [35], solvent-assisted bilayers [36], and Langmuir–Blodgett [37]/Langmuir–Schaefer [38] transfer. Among these, the Langmuir–Blodgett/Langmuir–Schäfer method provides the highest degree of control over lipid composition and leaflet asymmetry and was therefore used for all SLBs in this work.

Formation Methods of SLBs

The vesicle fusion method relies on the adsorption and rupture of small unilamellar vesicles on hydrophilic surfaces, leading to bilayer formation. While experimentally simple, this approach offers limited control over leaflet composition and may result in structural defects [35][39].

Solvent-assisted methods involve deposition of lipids from organic solvents followed by gradual solvent exchange with water, enabling bilayer formation. Although this method

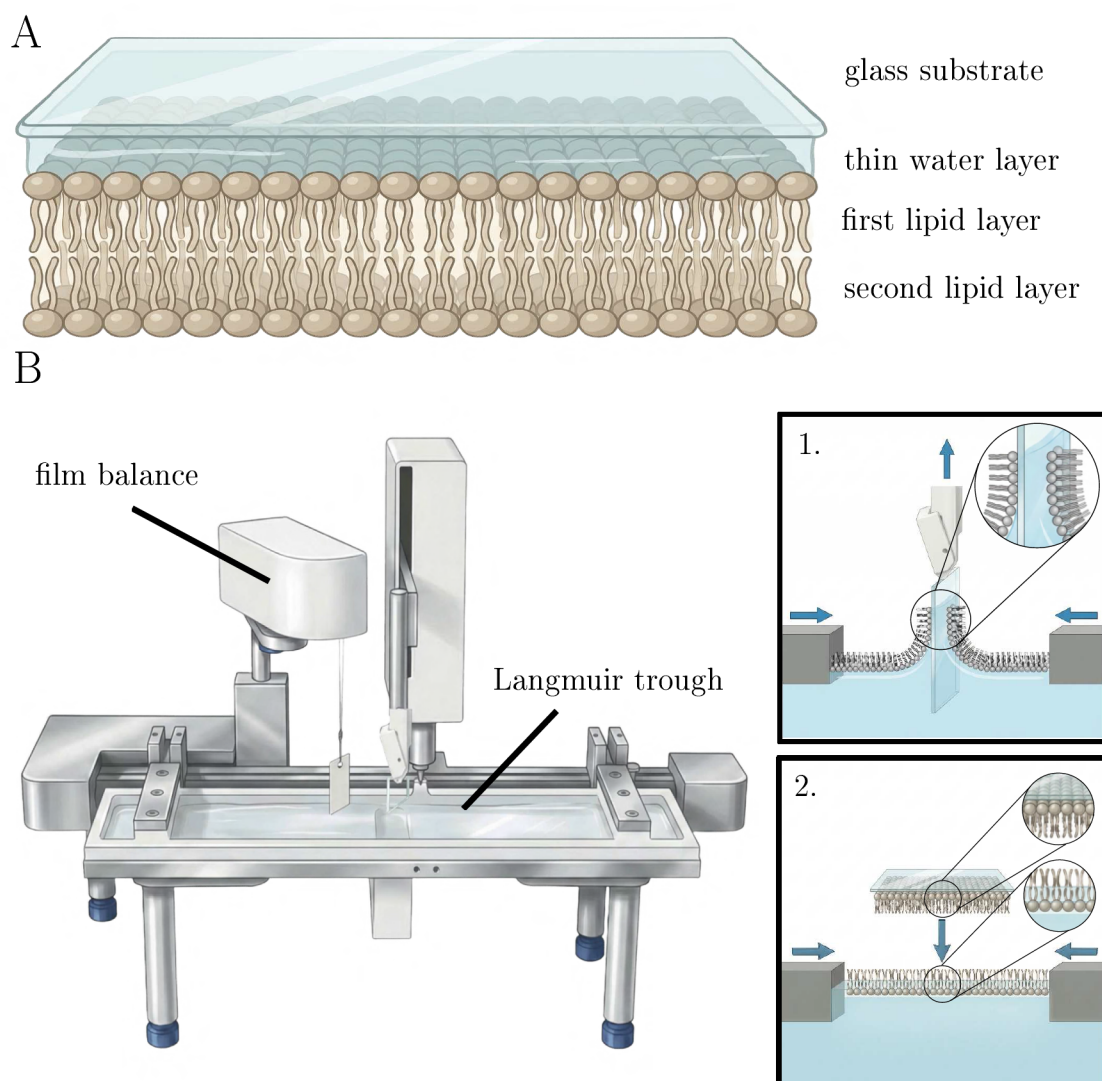


Figure 2.2: Structure and Manufacture of SLBs. A: SLBs are defined as lipid bilayers on a solid substrate. Here the solid substrate is thickness corrected glass. Glass and lipid bilayer are separated by a thin water layer. B: Langmuir trough with film balance for SLB manufacturing. The first lipid layer is produced via Langmuir-Blodgett transfer (1. inlet) the second lipid layer is added via the Langmuir-Schäfer transfer (2. inlet).

facilitates incorporation of certain proteins, residual solvent effects can influence membrane properties [36][40].

In the Langmuir-Blodgett/Langmuir-Schäfer transfer method (see figure 2.2B), a lipid monolayer is formed at the air-water interface in a Langmuir trough. The lipid monolayer is compressed to a defined density using movable barriers. In the Langmuir-Blodgett technique, the substrate is pulled vertically through the surface and picks up a lipid layer. In the Langmuir-Schäfer technique, the substrate is placed horizontally on the monolayer. A combination of both methods results in a complete SLB. This method is the most labor-intensive and requires special equipment, such as a Langmuir trough with a

film balance. Nevertheless, the advantages outweigh the additional time required. The Langmuir-Blodgett/Langmuir-Schäfer transfer provides the best control over the membrane structure, as lipid composition and density can be tuned with high precision. Arbitrary lipid mixtures can be prepared prior to deposition, and the composition of the spreading solution is reliably transferred to the monolayer, resulting in a direct one-to-one transfer of the desired lipid ratios to the membrane. Furthermore, only this method allows for the generation of different lipid compositions in the upper and lower lipid layers [37][38]. Owing to these advantages, all SLBs examined in the publications corresponding to this dissertation were produced using the Langmuir-Blodgett/Langmuir-Schäfer transfer.

Physical Advantages and Limitations

SLBs provide a highly controlled system for investigating two-dimensional transport processes. Their planar geometry makes them ideally suited for fluorescence-based microscopy techniques such as epifluorescence, TIRF, and confocal imaging [7].

Another major advantage of SLBs is the high level of control. SLBs offer the possibility to define the exact lipid composition and specific amounts of, for example, proteins or cholesterol, leading to an extremely high reproducibility. [41] Although the more time-consuming Langmuir-Blodgett/Langmuir-Schäfer transfer was chosen for SLB preparation, the production of SLBs is relatively straightforward and compared to living systems, faster, more flexible and more cost-effective

As already mentioned above, SLBs can mimic key properties of cell membranes, for example the lateral mobility of lipids and proteins, allowing for examination of diffusion and domain assembly. In addition, with the Langmuir-Blodgett/Langmuir-Schäfer transfer, the asymmetry of cell membranes can be recreated. Furthermore, SLBs are highly simplified systems whose reduced complexity allows for the targeted investigation and deeper understanding of individual mechanisms in cell membranes. They are therefore particularly well-suited for fundamental research[16].

However, the coupling to the solid substrate introduces additional frictional interactions that can modify diffusion behavior, particularly for membrane components in the lower leaflet. These effects depend on substrate roughness, surface chemistry, and lipid composition. Polymer cushions or thickness-corrected substrates can be used to reduce these interactions [42][43].

It was further shown, that also the material of the substrate and the formation method of the SLB influence the mobility. However, for glass substrates, using the Langmuir-Blodgett deposition technique (as employed in the context of this work), both leaflets of the bilayer have the same dynamics [44].

Due to the absence of a cellular environment, membrane proteins often lose their natural orientation and function. Furthermore, active processes such as metabolism, en-

ergy supply and signaling cannot be replicated, which means that their influence on the membrane is completely lost using SLBs as model systems. In contrast to biological membranes, SLBs also lack natural membrane curvature. Consequently, processes such as vesicle formation, endocytosis and membrane fusion cannot be studied or can only be studied to a limited extent. In particular, when it comes to membrane curvature, giant unilamellar vesicle provide more suitable model systems [45]. While SLBs represent powerful and well-controlled model systems, they cannot fully replicate the complexity of biological membranes, making experimental comparisons with living systems both necessary and valuable.

Despite these limitations, SLBs remain one of the most widely used model systems for quantitative studies of membrane physics due to their reproducibility, stability, and compatibility with optical techniques [7].

2.2.3 Diffusion in Two-Dimensional Fluids

Transport in lipid membranes is dominated by Brownian motion constrained to two dimensions. The mean square displacement (MSD) in such systems is given by:

$$\langle r^2 \rangle = 4Dt \quad ,$$

where D is the diffusion constant and t the time [46].

Theoretical descriptions of membrane diffusion must account for both the reduced dimensionality and the hydrodynamic coupling between the membrane and the surrounding fluid.

Brownian Motion and Fick's Laws

The stochastic motion of particles in membranes arises from thermal fluctuations and can be described using Fick's laws of diffusion. The diffusion flux J is given by:

$$J = -D \frac{\delta c}{\delta x}$$

and the temporal evolution of concentration follows [47]:

$$\frac{\delta c}{\delta t} = D \frac{\delta^2 c}{\delta x^2} \quad .$$

For two-dimensional systems, this results in the diffusion equation [9]:

$$\frac{\delta c}{\delta t} = D \left(\frac{\delta^2 c}{\delta x^2} + \frac{\delta^2 c}{\delta y^2} \right) \quad .$$

The diffusion constant D is a central parameter for describing the mobility of particles

and has the dimension $[\text{m}^2 \cdot \text{s}^{-1}]$. For spherical particles in viscous medium, D can be described by the Einstein-Stokes relation, as the spherical approximation is commonly used to describe membrane inclusions with an effective hydrodynamic radius:

$$D = \frac{k_B T}{6\pi\eta a} \quad ,$$

where k_B is the Boltzmann constant, T the temperature, η the viscosity of the medium and a the particle radius [46].

For membrane systems, diffusion coefficients are typically on the order of $\mu\text{m}^2/\text{s}$ but vary strongly with lipid composition, phase state, and molecular interactions.

2.2.4 Diffusion in Membranes: Saffman-Delbrück

While the basic description of diffusion assumes motion in a homogeneous three-dimensional fluid, diffusion in biological membranes is fundamentally different due to their almost two-dimensional nature. A theoretical framework to describe lateral diffusion in lipid bilayers was developed by Saffman and Delbrück [4].

In this model, the lipid membrane is considered to be an infinitely extended, thin, viscous fluid layer embedded in a less viscous, surrounding medium. Membrane proteins are approximated as cylindrical objects that span the membrane and diffuse laterally within the membrane plane. Based on this hydrodynamic description, the diffusion constant is given by:

$$D = \frac{\overline{r^2}}{4t} = \frac{k_B T}{4\pi\eta h} \cdot \left(\log_{10} \left(\frac{\eta h}{\eta' a} \right) - \gamma \right) \quad ,$$

where η denotes the membrane viscosity, η' the viscosity of the surrounding fluid, h the membrane thickness, a the radius of the diffusion particle and γ the Euler-Mascheroni constant [4].

Although the model is based in simplifying assumptions, it provides an important theoretical framework for understanding lateral diffusion in lipid membranes. Notably, it highlights the strong influence of membrane viscosity and the coupling between the membrane and the surrounding fluid on the mobility of embedded molecules [4].

As a first approximation, the model can also be applied to lipid diffusion, although deviations are expected due to molecular complexity and membrane heterogeneity [Publication I].

Supported Membranes: Evans-Sackmann

The Saffman-Delbrück model describes lateral diffusion in free-standing membranes, but does not take into account any additional frictional effects due to the solid substrate. For

SLBs, however, an influence of the substrate on the mobility of the membrane components is to be expected.

To characterize this additional friction, Sackmann and Evans developed an extended hydrodynamic model. In this approach, the membrane is still defined as a viscous two-dimensional fluid, but an additional frictional force between the membrane and the substrate is introduced. This frictional force is parameterized by the friction parameter b , which describes the coupling between membrane and substrate [48].

Taking this additional friction into account, the diffusion constant is as follows:

$$D = \frac{k_B T}{\lambda} = \frac{k_B T}{4\pi\eta_m} \left(\frac{1}{4}\epsilon^2 + \epsilon \frac{K_1(\epsilon)}{K_0(\epsilon)} \right)^{-1},$$

where η_m is the membrane viscosity, K_0 and K_1 are modified Bessel functions of the second kind and ϵ is the dimensionless particle radius, defined as:

$$\epsilon = a \cdot \sqrt{\frac{b}{\eta_m}}.$$

Here a denotes the radius of the diffusion particle and b the friction parameter between the membrane and the substrate. The model indicates that diffusion of membrane-spanning proteins in SLBs is significantly influenced by the interaction with the underlying substrate. In the limiting case of low friction, the model by Sackmann and Evans shifts towards the Saffman-Delbrück description, whereas in the case of high friction, the diffusion constant is much more dependent on the size of the diffusing object. The Evans-Sackmann model thus offers an important extension to the description of lateral diffusion in supported membrane systems [48][4].

The consideration of these frictional effects is specifically important for experimental systems with SLBs, as they directly influence the observed mobility of the lipids and proteins. To minimize these substrate-induced effects, the systems investigated in this work were specifically designed to include only lipids and proteins in or coupled to the upper leaflet of the bilayer, thereby reducing substrate-induced friction.

In addition to the interaction with the substrate, lateral diffusion can be further influenced by the presence of obstacles within the membrane, which is considered in the following section.

2.2.5 Obstacle and Anomalous Diffusion in Membranes

Transport in lipid membranes deviates from ideal Brownian motion due to the presence of spatial heterogeneities such as protein complexes or lipid domains. These structures act as obstacles that modify diffusion behavior.

Mobile Obstacles: Saxton Model

A commonly used approach for describing diffusion in the presence of mobile obstacles was developed by Saxton. In this model, diffusion is described as random motion on a two-dimensional grid, with both lipids (tracers) and obstacles distributed across the grid and moving at different speeds [49].

A key parameter here is relative mobility, described by the ratio of the jump rates of the tracers and obstacles. Additionally, diffusion depends on the concentration of obstacles, which is specified as a membrane area fraction. For quantification, a relative diffusion constant is defined as:

$$D^*(c) = \frac{D(c)}{D(0)} \quad ,$$

where $D^*(c)$ is the diffusion constant of the tracers in the presence of obstacles, while $D(0)$ is the diffusion constant of the tracers in the absence of obstacles [49].

This model reveals that diffusion decreases as the concentration of obstacles increases. For mobile obstacles, long-range diffusion is generally maintained, since the obstacles themselves are also in motion. The exact dependence of the diffusion constant is determined through analytic approximations and numerical simulations [49].

Immobile Obstacles: Nieuwenhuizen Model

In contrast, Nieuwenhuizen's model describes diffusion in the presence of stationary obstacles. In this approach, the diffusing particles are restricted by fixed barriers, which can limit their movement to defined regions of the membrane [50].

For low obstacle concentrations, the diffusion constant can be derived as a function of the obstacle concentration. Diffusion decreases sharply as the number of stationary obstacles increases. In contrast to the case of mobile obstacles, long-range diffusion can be completely suppressed at high concentrations due to the formation of continuous barriers [50].

Physical Implications

Both models demonstrate that lateral diffusion is not only determined by thermal motion but also by spatial constraints and interaction landscapes. The nature and mobility of obstacles determine whether diffusion remains homogeneous or becomes spatially heterogeneous and constrained.

In real biological cell membranes as well as in biomimetic model systems, diffusion is not a global membrane parameter, but is governed by local membrane organization, specifically phase behavior and domain formation. Consequently, it must be considered a local observable that depends on lipid composition and fluidity.

2.2.6 Phase Behavior and Membrane Heterogeneity

Lipid membranes are thermodynamically complex two-dimensional systems occurring in biological and artificial systems that can exist in distinct structural phases with markedly different physical properties. The most relevant states for lateral transport are the liquid-disordered phase (L_α) and the gel phase (L_β) (see figure 2.3A) [1][8][5].

In the gel phase, lipid molecules are tightly packed and exhibit predominantly extended hydrocarbon chains. This results in high structural order, increased membrane thickness, and strongly reduced lateral mobility. In contrast, the liquid-disordered phase is characterized by increased configurational entropy of the lipid tails, leading to reduced packing density, enhanced molecular fluctuations, and significantly higher lateral diffusion coefficients [1][8][5].

The transition between these two phases is governed by the thermodynamic control parameter temperature. At a characteristic transition temperature T_m , the system undergoes a first-order phase transition between the ordered and disordered states. Importantly, T_m is not a universal constant but depends on molecular parameters such as chain length, degree of saturation, and cholesterol content, which determine the balance between enthalpic packing interactions and entropic contributions [1][8][5].

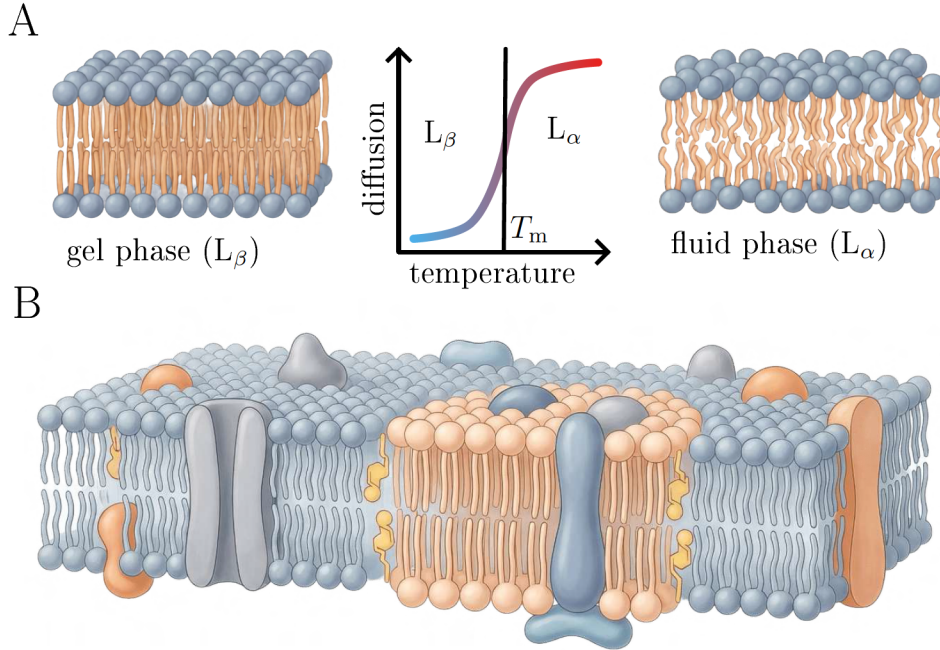


Figure 2.3: Schematic illustration of membrane phase behavior. A: Lipid organization in the gel (L_β) and fluid (L_α) phase. Temperature-dependent phase transition characterized by the transition temperature T_m , directly influences membrane lateral diffusion. B: Phase separation leading to the formation of membrane domains with distinct physical properties (lipid rafts). This phase separation can arise either from (i) energetic demixing of different lipid species within the same thermodynamic phase due to differences in molecular structure and charge, or (ii) coexistence of lipids in distinct thermodynamic phases (gel and fluid) at temperatures below or near the phase transition regime.

The different physical properties of the two lipid phases have significant biological consequences. Sufficient fluidity is essential for the function of membrane proteins, for transport processes and for signal transduction. Although a high fluidity is indispensable for most biological processes, a certain degree of structural stability is also necessary to ensure the integrity of the membrane. Cells are therefore able to actively control their membrane composition to ensure optimal fluidity. This phenomenon is referred to as homeoviscous adaptation. Overall, the equilibrium between the gel and the liquid phase is a central requirement for the functionality of biological membranes [8][5][1].

Temperature as Control Parameter for Transport

From a physical perspective, temperature acts as a direct control parameter for both phase state and transport properties in lipid membranes. Below the transition temperature ($T < T_m$), the system resides predominantly in the gel phase, where lateral diffusion is strongly suppressed due to reduced free volume and increased energetic barriers for molecular rearrangements. Above T_m , thermal energy overcomes these barriers, and the membrane transitions into a fluid regime with significantly enhanced lateral mobility [1][8][5].

This coupling between phase state and diffusion leads to a direct and experimentally exploitable relationship (see figure 2.3A):

- $T \uparrow \Rightarrow D \uparrow$
- $T \downarrow \Rightarrow D \downarrow$

Thus, temperature does not only affect molecular kinetics but effectively tunes the dimensional transport properties of the entire membrane system.

Phase Coexistence and Spatial Heterogeneity

In biological membranes, there is usually not only one global lipid phase, but they organize themselves into regions with different physical properties. Thus, membranes do not have homogeneous structures, but aggregate into so-called lipid domains (see figure 2.3B). These lipid domains represent local regions of varying flexibility and fluidity within an otherwise largely continuous membrane. Importantly, the formation mechanisms of such domains can differ: they may either result from structural phase transitions of a single lipid species between gel (L_β) and fluid (L_α) states, or from multicomponent phase separation of chemically distinct lipid species. Lipid domains are highly dynamic, meaning they can continuously form, grow larger or smaller, or dissolve entirely. Due to their fluid properties, lipid domains can diffuse as a whole within the membrane. Despite this, they are often stabilized by proteins or even anchored to the cytoskeleton, which severely restricts the lateral mobility of the lipid domains [8][5][1].

Within such heterogeneous systems, transport is no longer spatially uniform. Instead, diffusion becomes position-dependent, with locally varying diffusion coefficients depending on the phase environment. Interfaces between coexisting phases introduce additional energetic barriers that can further modulate molecular mobility and induce partial confinement effects [1][8][5].

Relevance for Controlled Membrane Systems

In model systems such as SLBs, phase behavior can be externally tuned with high precision. By controlling temperature and lipid composition, it is possible to switch between regimes of high and low mobility or to stabilize intermediate coexistence states [51].

This tunability is of central importance for experimental approaches that rely on controlled transport and pattern formation. In particular, the ability to modulate diffusion coefficients via phase state enables stabilization or relaxation of spatial patterns, depending on whether the system is operated above or below T_m [Publication II].

Overall, membrane phase behavior provides a direct thermodynamic handle on diffusion properties in two-dimensional lipid systems. Temperature-dependent phase transitions couple molecular organization to macroscopic diffusion behavior, allowing controlled switching between high-mobility and low-mobility regimes. This coupling plays a central role in the regulation of spatial organization in both model membranes and more complex biological membrane systems.

While transport in two-dimensional membrane systems under equilibrium conditions is governed by diffusion and interaction-induced constraints, many relevant physical and biological processes operate far from equilibrium and are subject to external driving forces. In the following section, the influence of magnetic fields on transport and spatial organization in membrane systems is introduced, enabling controlled manipulation of membrane-associated components and the generation of defined spatial patterns.

2.3 Force-Mediated Transport and Pattern Formation on Membrane Surfaces

Magnetic manipulation provides a powerful strategy to actively control spatial organization in membrane systems. In contrast to purely diffusive membrane dynamics, external magnetic field gradients enable directed transport of membrane-bound entities and thereby programmable pattern formation on lipid interfaces. The resulting steady states emerge from the interplay between deterministic magnetic forces and thermal fluctuations, placing these systems intrinsically in a non-equilibrium regime.

2.3.1 Superparamagnetic Nanoparticles in Soft Matter Systems

MNPs present a unique interface between condensed-matter physics, colloid science, and biomimetic membrane systems. Their relevance in soft matter arises from the combination of nanoscopic size, Brownian mobility, surface functionalization, and strong responsiveness to external magnetic fields. In contrast to passive colloids, MNPs can be actuated remotely without mechanical contact, making them especially suitable for SLBs and other membrane-based model systems.

In this work, MNPs are coupled to lipid bilayers via specific linker chemistry such as biotin–streptavidin binding. Once attached, they behave as laterally mobile inclusions confined to an effectively two-dimensional viscous fluid. External magnetic field gradients can therefore be converted into directed in-plane transport [Publication II].

Classification of Magnetic Materials

A microscopic understanding of this behavior requires a classification of magnetic materials based on the origin and collective organization of their magnetic moments.

Paramagnetic materials consist of atoms or ions with unpaired electron spins that give rise to permanent magnetic moments on the atomic scale. In the absence of an external magnetic field, these moments continuously change due to thermal fluctuations, resulting in zero net magnetization. Upon application of a field, the moments partially align along the field direction, leading to a positive, typically small magnetic susceptibility. In weak fields, this response is approximately linear, reflecting the competition between Zeeman energy and thermal disorder. However, this alignment is not stable: once the field is removed, thermal agitation randomizes the moments again, and no remanent magnetization persists [20].

In contrast, magnetically ordered materials exhibit collective behavior arising from quantum-mechanical exchange interactions between neighboring spins. Depending on the sign and symmetry of these interactions, different types of long-range magnetic order emerge below a critical temperature [20].

In **ferromagnetic** materials, exchange interactions favor parallel alignment of neighboring spins, leading to a macroscopic spontaneous magnetization below the Curie temperature T_C . This ordered state is characterized by a large magnetic susceptibility and the ability to sustain magnetization even in the absence of an external field. However, a real ferromagnet does not form a uniformly magnetized single crystal. Instead, it minimizes its total free energy by subdividing into regions of homogeneous magnetization, known as magnetic (Weiss) domains. Within each domain, spins are aligned parallel, but the orientation of magnetization varies between domains. This domain structure reduces magnetostatic energy at the cost of domain wall energy. When an external magnetic field is applied, domains aligned with the field grow at the expense of oppositely oriented ones

through domain wall motion and spin rotation, leading to a net increase in magnetization. This mechanism underlies hysteresis and remanence in bulk ferromagnets [20].

Antiferromagnetic materials arise when exchange interactions favor antiparallel alignment of neighboring spins. This leads to the formation of two or more sublattices with equal, oppositely oriented magnetic moments, resulting in a vanishing net magnetization in the ideal case. Long-range antiferromagnetic order exists only below the Néel temperature T_N , above which thermal fluctuations destroy the ordered state and the material behaves paramagnetically. Despite the absence of a macroscopic moment, antiferromagnets exhibit well-defined magnetic structure and collective excitations [20].

Ferrimagnetism can be understood as an intermediate case between ferro- and antiferromagnetism. Here, antiparallel alignment of magnetic sublattices persists, but the magnitudes of the opposing moments differ, resulting in a finite net magnetization. This situation is characteristic of many iron oxides, most notably magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$), which are highly relevant for MNPs. On a microscopic level, these materials exhibit complex crystal structures with inequivalent lattice sites that host ions with different valence states and magnetic moments. The resulting incomplete compensation of sublattice magnetizations gives rise to strong magnetic response similar to ferromagnets, including domain formation and hysteresis in the bulk state [20].

A key concept connecting these classes of magnetic materials to nanoscale systems is the existence of magnetic domains. In bulk ferro- and ferrimagnetic materials, domain formation is energetically favorable because it reduces long-range stray fields. However, the formation of multiple domains requires the presence of domain walls, which carry an associated energy cost. As the size of a magnetic particle decreases, the relative importance of domain wall energy increases. Below a critical size, it becomes energetically unfavorable to sustain multiple domains, and the particle transitions into a single-domain state. In this regime, the entire particle behaves as a single, coherently magnetized entity characterized by a uniform magnetic moment [20].

This size-dependent transition has profound consequences. While bulk materials exhibit hysteresis and stable remanent magnetization due to domain dynamics, single-domain particles lack internal domain walls and therefore cannot reduce their magnetostatic energy through domain formation. Instead, their magnetic stability is governed by the competition between magnetic anisotropy, which favors alignment along specific easy axes, and thermal fluctuations, which tend to randomize the magnetization direction [20].

When the particle size is further reduced such that thermal energy becomes comparable to or exceeds the anisotropy energy barrier, spontaneous stochastic reversal of the particle's magnetization occurs. In this regime, the magnetic moment fluctuates rapidly in time and averages to zero in the absence of an external field. This phenomenon is referred to as superparamagnetism and represents the central magnetic regime for nanoparticles used in soft matter and membrane-based systems [20].

Superparamagnetism at the Single-Particle Level

In the single-domain regime, strong exchange coupling enforces a coherent alignment of all atomic spins within the nanoparticle, such that the particle behaves as a single magnetic macrospin. The resulting magnetic moment is given by:

$$\mu = M_s V \quad ,$$

where M_s is the saturation magnetization and V the magnetic core volume. The absence of internal domain walls implies that magnetization reversal can only occur via coherent rotation of this macrospin [21].

The stability of the magnetic moment is governed by magnetic anisotropy, which defines energetically preferred orientations (easy axes) of the magnetization. The corresponding energy landscape is characterized by an anisotropy barrier

$$E_K = KV \quad ,$$

with K being the effective anisotropy constant (including magnetocrystalline, shape, and surface contributions). This energy barrier separates equivalent minima of the magnetization direction [21].

At finite temperature, thermal fluctuations can induce stochastic switching of the magnetic moment between these minima. This process is described by Néel relaxation, with a characteristic time scale

$$\tau_N = \tau_0 \exp\left(\frac{KV}{k_B T}\right) \quad ,$$

where τ_0 is an attempt time on the order of 10^{-9} – 10^{-12} s, $k_B T$ the Boltzmann constant and T the temperature. The Néel relaxation time defines the temporal stability of the particle's magnetization [21].

A crucial consequence is the dependence on the experimental observation time τ_{obs} . If $\tau_{\text{obs}} \ll \tau_N$, the magnetic moment appears quasi-static, and the particle behaves similarly to a blocked ferromagnet with a well-defined magnetic orientation. In contrast, if $\tau_{\text{obs}} \gg \tau_N$, repeated thermally activated reversals lead to a vanishing time-averaged magnetic moment in zero field. In this regime, the particle exhibits no remanence and no coercivity despite possessing a large instantaneous magnetic moment. This defines the superparamagnetic state [21].

In fluid suspensions, an additional relaxation channel arises from Brownian rotation of the entire particle, characterized by the time scale

$$\tau_B = \frac{3\eta V_h}{k_B T} \quad ,$$

where η is the dynamic viscosity and V_h the hydrodynamic volume. The effective magnetic relaxation is then given by [21],

$$\tau_{\text{eff}}^{-1} = \tau_N^{-1} + \tau_B^{-1} \quad .$$

However, in the present system, nanoparticles are immobilized with respect to rotational degrees of freedom by their coupling to the lipid bilayer via molecular linkers. As a consequence, Brownian rotation is strongly suppressed, and magnetic relaxation is dominated by internal Néel processes. This distinction is essential for membrane-bound particles, as it decouples magnetic moment fluctuations from hydrodynamic rotation.

The competition between thermal fluctuations and field-induced alignment leads to an equilibrium orientational distribution governed by Boltzmann statistics. The resulting ensemble magnetization follows the Langevin model:

$$M = n\mu\mathcal{L}(\alpha) \quad , \quad \mathcal{L}(\alpha) = \coth(\alpha) - \frac{1}{\alpha} \quad , \quad \alpha = \frac{\mu B}{k_B T} \quad .$$

where n is the particle number density [21].

Thus, superparamagnetic nanoparticles combine two key properties: a large magnetic moment enabling strong coupling to external fields, and complete reversibility due to thermally driven moment fluctuations. In membrane-bound systems, where rotational motion is constrained, this behavior is governed primarily by Néel relaxation, making the magnetic response fast, reversible, and well-suited for controlled manipulation without residual magnetization.

Synomag[®]-CLD-redF Nanoparticles

In this work, commercially available superparamagnetic nanoparticles (Synomag[®]-CLD-redF, Micromod Partikeltechnologie GmbH, Rostock, Germany) are used.

These particles consist of a superparamagnetic iron oxide core embedded in a polymeric, bio-compatible coating. The hydrodynamic diameter is approximately 70 nm, and the particles are fluorescent labeled, enabling optical quantification of spatial distributions on SLBs [52].

A key functional feature is streptavidin functionalization, allowing specific binding to biotinylated lipids within the membrane. This interaction is effectively irreversible under physiologically relevant conditions and standard experimental forces, and ensures stable coupling of nanoparticles to the lipid bilayer. Under sufficiently strong magnetic field gradients, however, mechanical forces can overcome this binding and induce detachment [Publication II].

The polymer coating minimizes nonspecific interactions with biological systems and ensures compatibility with subsequent cell-based experiments [22].

2.3.2 Magnetic Forces at the Nanoscale

A homogeneous magnetic field mainly exerts torque and aligns particle moments (see figure 2.4A). Translational motion, however, requires spatial field inhomogeneity (see figure 2.4B), as only gradients in the magnetic field result in a net force acting on the particle, whereas homogeneous fields solely induce a torque. The interaction between magnetic fields and nanoparticles is fundamentally described by the dipole approximation. For a magnetic moment $\boldsymbol{\mu}$ in a magnetic field $\mathbf{B}$, the interaction energy is

$$U = -\boldsymbol{\mu} \cdot \mathbf{B}.$$

The resulting force is given by

$$\mathbf{F} = \nabla(\boldsymbol{\mu} \cdot \mathbf{B}) \quad .$$

For superparamagnetic particles in moderate fields, the induced moment approximately aligns with the local field direction, so particles migrate toward regions of higher field magnitude [53].

For membrane-bound nanoparticles, this coupling has direct consequences for transport. Since the particles are confined to a quasi-two-dimensional fluid, only the in-plane components of $\mathbf{F}$ contribute to lateral motion, while the out-of-plane component modulates membrane attachment and linker tension. The stochastic nature of $\boldsymbol{\mu}$ implies that even in the presence of a deterministic force, particle trajectories remain diffusive on short time scales, with magnetic forces introducing a directional bias that becomes apparent in ensemble averages or long-time dynamics.

In this way, superparamagnetism provides the microscopic foundation linking thermal fluctuations, field-induced alignment, and effective forces, thereby enabling controlled and reversible manipulation of membrane-bound nanoparticles in spatially structured magnetic fields.

2.3.3 Field-Driven Transport in Two-Dimensional Fluids

Once bound to a SLB, nanoparticle motion is confined to a quasi-two-dimensional viscous environment. For membrane-bound particles, forces can be decomposed into vertical and lateral components (see figure 2.4C):

$$F_z = \mu \frac{dB_z}{dz} \quad , \quad F_{xy} = \mu \sqrt{\left(\frac{dB_{xy}}{dx}\right)^2 + \left(\frac{dB_{xy}}{dy}\right)^2} \quad .$$

The vertical component governs membrane attachment, detachment, and linker tension, whereas the lateral component drives in-plane transport along the membrane surface (see figure 2.4D) [Publication II].

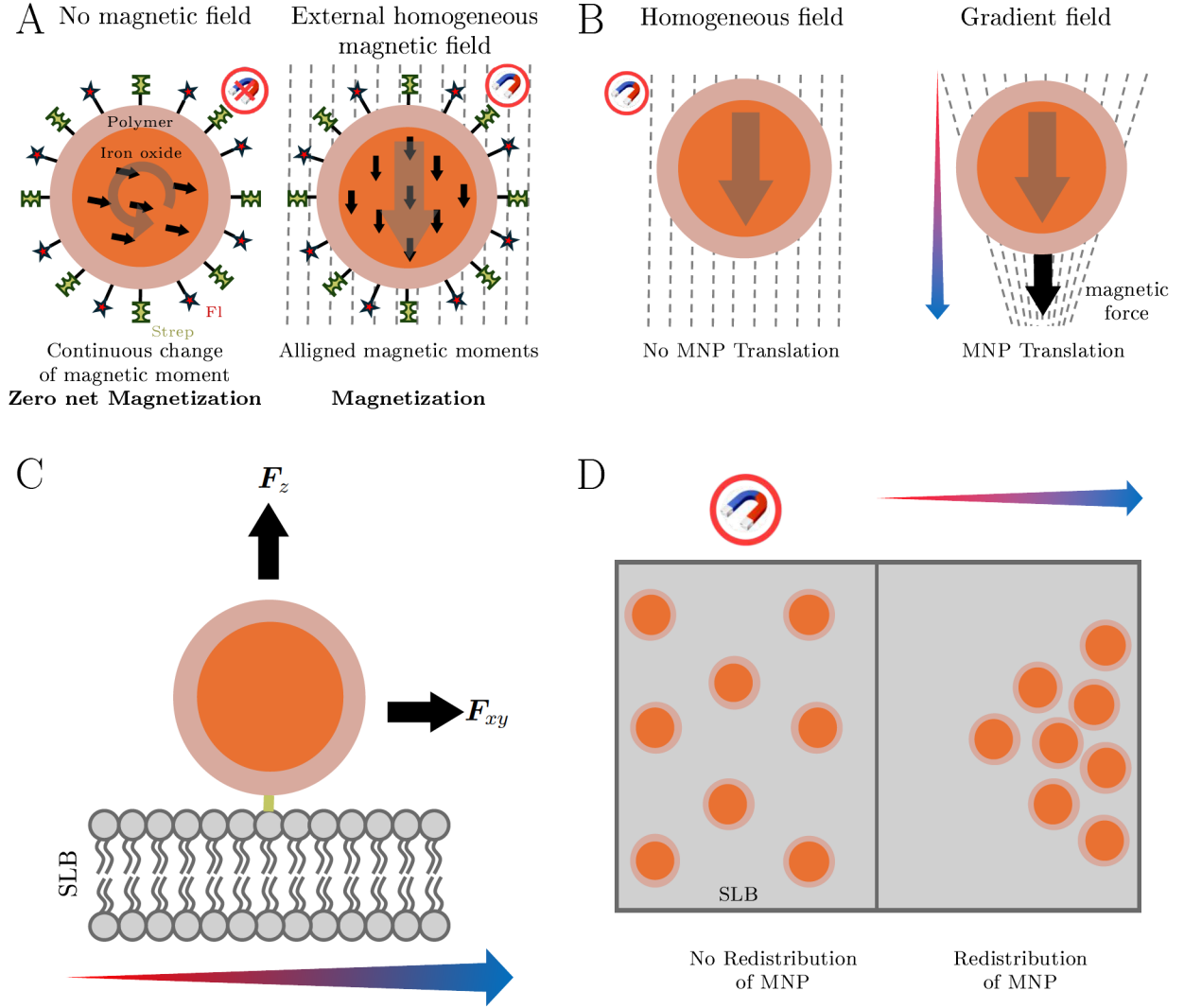


Figure 2.4: Magnetic manipulation of membrane-bound nanoparticles and induced membrane reorganization. (A) Superparamagnetic nanoparticle with a field-dependent magnetic moment. In the absence of an external magnetic field, all atomic moments are aligned, but thermal fluctuations lead to a continuous change of the particle magnetic moment resulting in zero net magnetization over time. Upon application of a magnetic field, the moments align along the field direction, resulting in a finite, reversible magnetization without remanence. The polymer shell of the MNPs is coated with Streptavidin (Strep) and fluorophores (FL) for coupling to the SLB and visualization, respectively. (B) Comparison of homogeneous magnetic fields and magnetic field gradients. In a homogeneous field, magnetic dipoles align but do not experience a net translational force. In contrast, spatially inhomogeneous magnetic fields generate forces proportional to the field gradient, leading to directed particle motion toward regions of higher magnetic flux density. (C) Forces acting on membrane-bound nanoparticles on SLBs. A vertical force component (F_z) influences particle-membrane interactions such as binding and detachment, while lateral force components (F_{xy}) drive directed in-plane motion within the membrane. This decoupling enables independent control of membrane attachment and lateral transport. (D) Magnetically induced reorganization of membrane components. Application of magnetic field gradients leads to accumulation of nanoparticles in defined regions of the membrane, which in turn redistributes other membrane-associated components, resulting in inverse concentration gradients and spatial pattern formation.

Compared with three-dimensional suspensions, hydrodynamic dissipation in membranes depends on membrane viscosity, leaflet coupling, substrate friction, and linker geometry. Thus, the same magnetic force can produce markedly different drift speeds depending on membrane state and coupling architecture.

2.3.4 Pattern Formation under External Fields

Spatially varying fields generate nonuniform drift velocities and therefore nonuniform steady-state particle densities. Particles accumulate where drift flux is balanced by back-diffusion. Thus, stronger gradients or lower diffusion coefficients sharpen accumulation zones.

Beyond simple enrichment, interacting particles can generate collective phenomena such as crowding fronts, excluded-area effects, and field-induced chaining (if dipolar interactions become relevant). In membrane systems, steric packing often dominates before true magnetic aggregation occurs, especially for polymer-coated superparamagnetic particles [23].

Dynamic protocols, such as switching fields on/off, translating magnets, or applying oscillatory gradient, allow time-dependent pattern writing. This transforms the membrane into a programmable non-equilibrium surface.

2.3.5 Coupling Between Membrane Phase State and Particle Mobility

A decisive feature of lipid membranes is that transport properties depend strongly on phase state. In the liquid-disordered phase, lipids and membrane-bound particles exhibit comparatively high lateral mobility. In liquid-ordered or gel-like phases, viscosity increases and diffusion slows dramatically [1][8][5].

Close to a phase transition, small temperature changes can strongly alter mobility. This creates an experimentally powerful control parameter: magnetic forces may remain constant while transport changes through thermal tuning alone [Publication II].

Increasing temperature generally promotes chain disorder, lowers packing density, and enhances lateral diffusion. Decreasing temperature increases chain order and can kinetically arrest previously generated particle distributions. Consequently, field-written patterns may be dynamically created in a fluid state and subsequently stabilized by cooling into a less mobile phase [Publication II].

This coupling between force induced transport and high control of phase behavior is central for programmable membrane structuring.

2.3.6 Particle-Induced Ligand Redistribution

Magnetically transported nanoparticles do not act as isolated tracers; they modify the local membrane environment. If particles occupy finite lateral area or are linked to specific lipid species, their accumulation redistributes surrounding components through steric exclusion, entropic demixing, and co-transport of bound lipids [Publication II].

As a consequence, enrichment of nanoparticles can produce depletion zones for other ligands, whereas particle-poor regions become relatively enriched in mobile receptors or adhesion molecules. Such inverse gradients are highly relevant for receptor-ligand systems, where local density determines binding probability and signaling thresholds [Publication II].

If nanoparticles are functionalized with capture molecules, they may additionally recruit specific ligands into dense macroscopic clusters. This would enable active writing of receptor landscapes that mimic biological membrane heterogeneity.

For cell-interface studies, these effects are particularly powerful because immune synapses respond not only to mean ligand density but also to nanoscale spacing, cluster size, and local mobility. Magnetic nanoparticles therefore provide a route from passive membrane models toward actively reconfigurable cellular interfaces.

2.4 Active Cellular Membrane Interfaces and Receptor Organization

Cell-cell contacts in the immune system are highly dynamic membrane interfaces in which recognition, signaling, and force generation are tightly coupled in space and time. Rather than being passive adhesion zones, these interfaces act as active reaction platforms where receptors and ligands diffuse, bind, cluster, and are continuously reorganized by cytoskeletal processes. Their functionality therefore emerges from the interplay between molecular affinity, membrane mechanics, lateral mobility, and active transport.

Such interfaces are particularly relevant for cytotoxic lymphocytes, including T cells and natural killer (NK) cells, which must rapidly decide whether a contacted cell should be tolerated or eliminated. In both cases, signaling is initiated at a specialized contact zone commonly referred to as the immunological synapse. However, the architecture and regulation of this synapse differ substantially between receptor systems and cell types.

In engineered immune cells, such as CAR-T and CAR-NK cells, synthetic receptors partially replace endogenous recognition pathways and thereby alter the physical principles governing synapse formation. As a result, the membrane interface becomes an experimentally tunable system in which receptor density, ligand mobility, and spatial organization directly determine activation efficiency.

2.4.1 Receptor-Ligand Interactions at Membrane Interfaces

The first step in immune cell activation is the formation of receptor-ligand bonds across two opposing membranes. These interactions occur within an intermembrane cleft of only a few tens of nanometers, where the separation distance between both cells must match the extracellular domain length of the interacting molecules. Productive signaling therefore depends not only on biochemical affinity, but also on geometric compatibility [6].

For natural T cells (see figure 2.5A), recognition is mediated by the T-cell receptor (TCR), which binds peptide-loaded major histocompatibility complex (MHC) on antigen-presenting or target cells. TCR binding is highly specific and sensitive, allowing activation by very low numbers of agonistic ligands. However, TCR signaling additionally requires co-receptors, adhesion molecules, and proofreading mechanisms that discriminate between weak and strong ligands [6].

NK cells (see figure 2.5A) follow a fundamentally different strategy. Instead of relying on one dominant receptor, they integrate signals from multiple activating and inhibitory receptors. Activating receptors detect stress-induced ligands, whereas inhibitory receptors recognize self-markers such as MHC. The balance of these signals determines whether cytotoxicity is initiated. This distributed sensing mechanism enables NK cells to eliminate abnormal cells that have lost MHC expression, a principle referred to as missing-self recognition [54].

Many tumor cells exploit this recognition system by downregulating or entirely losing MHC expression, thereby evading TCR-mediated detection by T cells. Although this loss of “self” can render them susceptible to NK cell-mediated killing via missing-self recognition, immune evasion often persists due to additional inhibitory mechanisms and the heterogeneity of tumor environments. To overcome these limitations, CARs were developed as synthetic receptors that redirect immune cells toward defined surface antigens independent of MHC presentation. Initially engineered for T cells, CAR-based approaches are now also being actively explored in NK cell engineering [55][56].

Chimeric Antigen Receptors as Synthetic Recognition Modules

CARs are engineered membrane receptors that enable immune cells to recognize pre-defined surface antigens independently of natural antigen-recognition pathways. They combine extracellular antibody-based target binding with intracellular immune signaling domains and thereby convert antigen binding directly into cellular activation [26][27].

The term *chimeric* refers to their modular composition, as CARs are assembled from domains originating from different proteins. A typical CAR consists of four functional elements (see figure 2.5B) [26][27]:

- **Extracellular binding domain:** Usually a single-chain variable fragment (scFv) derived from an antibody. This domain determines antigen specificity and binds directly to surface molecules such as cluster of differentiation 19 (CD19), B7 homolog 3 (B7-H3) or epidermal growth factor receptor (EGFR).
- **Hinge or spacer region:** Provides flexibility and defines the distance between the immune cell membrane and the target surface. Spacer length can strongly influence accessibility of membrane-proximal epitopes and synapse geometry (here complement component 6 (C6) or human nerve growth factor receptor-derived hinge N3 (N3)).
- **Transmembrane domain:** Anchors the receptor in the plasma membrane and contributes to receptor stability (here cluster of differentiation 28 (CD28) or cluster of differentiation 8 (CD8)).
- **Intracellular signaling domain:** Contains activation motifs that initiate downstream signaling after antigen binding. Primary signaling domains (e.g., cluster of differentiation 3 zeta chain (CD3 ζ)) initiate T cell activation upon antigen recognition, whereas co-stimulatory domains (e.g., CD28) enhance activation strength, proliferation, and persistence.

Different CAR generations have been developed. First-generation CARs contain only CD3 ζ , second-generation CARs additionally include one co-stimulatory domain, and later generations integrate multiple signaling modules or inducible cytokine systems [26][27].

Upon multivalent antigen binding, CARs cluster within the membrane and trigger phosphorylation cascades that induce cytotoxicity, cytokine secretion, proliferation, or survival depending on the host cell type. In contrast to the TCRs, CARs recognize intact surface antigens directly and therefore do not require peptide presentation by MHC molecules [26][27].

As already mentioned above, CAR immune cells are able to overcome a major tumor immune-evasion strategy, the downregulation of MHC molecules. By recognizing tumor-associated antigens in an MHC-independent manner, CAR-engineered effector cells bypass the requirement for classical antigen presentation and thereby restore effective immune recognition of malignant cells. This has led to the development of a highly promising therapeutic approach, which has already demonstrated considerable clinical success, particularly in hematological malignancies, where CAR-T cell therapies have achieved durable remissions in a subset of patients [57].

In parallel, CAR-engineered NK cells are emerging as a complementary effector platform, showing encouraging preclinical and early clinical results [55][56]. A key advantage of CAR-NK cells lies in the integration of engineered antigen specificity with the preservation of their innate activation pathways. In contrast to CAR-T cells, CAR-NK cells

retain their natural receptor repertoire, enabling target cell recognition not only via the CAR construct but also through endogenous activating and inhibitory receptors. This includes the ability to detect so-called “missing-self” signals resulting from reduced MHC expression, thereby providing an additional layer of tumor surveillance. As a consequence, CAR-NK cells may remain functionally active even in cases where tumor cells downregulate the CAR-targeted antigen, reducing the risk of complete immune escape variants.

Another important distinction is the safety profile. While both CAR-T and CAR-NK approaches are effective immunotherapeutic strategies, CAR-NK cells are generally associated with a reduced risk of severe systemic toxicities. CAR-T cell therapies can be accompanied by cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome, which are linked to strong pro-inflammatory cytokine secretion. In contrast, NK cells produce a distinct cytokine profile, including interferon- γ and tumor necrosis factor- α , which is associated with a lower incidence and severity of systemic inflammatory toxicity [26][27][56].

Furthermore, CAR-NK cells offer practical advantages in manufacturing and availability. Unlike CAR-T cells, which often require autologous production to avoid graft-versus-host disease, NK cells can be safely derived from allogeneic sources such as peripheral blood, umbilical cord blood, or induced pluripotent stem cells. This enables the development of standardized “off-the-shelf” products that can be manufactured in advance and administered to multiple patients [26][56].

At the same time, the generally shorter in vivo persistence of NK cells compared to T cells represents both a potential limitation and an opportunity, as it may reduce long-term adverse effects but can also necessitate repeated dosing to maintain therapeutic efficacy [27][56]. Additionally, NK cells intrinsic capacity to home to inflamed tissues provides a promising basis for targeting solid tumors, where infiltration remains a major challenge for many cellular immunotherapies [55].

Collectively, CAR-NK cells represent a highly promising and complementary extension of CAR-based immunotherapy, combining engineered specificity with innate immune flexibility and favorable safety and manufacturing properties, while CAR-T cell therapies remain a clinically established and highly effective modality, particularly in hematological malignancies.

Another important engineered concept is the non-signaling CAR (see figure 2.5B). These receptors contain an extracellular antigen-binding domain but lack intracellular signaling motifs and therefore do not directly trigger cytotoxic activation [Publication IV].

At membrane interfaces, non-signaling CARs act as adhesive or targeting modules that increase the local avidity of immune cells toward antigen-positive surfaces. This is particularly relevant when tumor-associated antigens are expressed at low levels and are insufficient to reach the activation threshold.

Non-signaling CARs can stabilize and prolong cell–cell contacts through recognition of

highly expressed (but not necessarily tumor-specific) surface markers, thereby facilitating effective engagement of separate activating CAR constructs. Importantly, this modular separation of binding and signaling ensures that activation only occurs when a true activation signal is present, while binding alone on healthy tissue does not trigger cytotoxicity [Publication IV].

2.4.2 Spatial Organization and Cluster Formation

Single receptor-ligand bonds are typically insufficient to induce robust activation. Instead, signaling emerges through the formation of nanoscale and microscale receptor clusters that concentrate kinases, exclude phosphatases, and stabilize cooperative interactions.[6]

In classical T cell synapses, receptor complexes reorganize into a characteristic concentric pattern consisting of (see figure 2.5C):

- **central supramolecular activation cluster (cSMAC):** enriched in TCR-MHC complexes and signaling assemblies,
- **peripheral supramolecular activation cluster (pSMAC):** peripheral ring dominated by adhesion molecules such as lymphocyte function-associated antigen 1 (LFA-1)/intercellular adhesion molecule 1 (ICAM-1),
- **distal supramolecular activation cluster (dSMAC):** containing dynamic actin-rich structures and large excluded proteins.

This architecture reflects self-organization driven by diffusion, binding, steric sorting, and actomyosin flow [6][18][58].

NK cell synapses are generally less symmetric and more heterogeneous. Because NK cells integrate multiple receptor classes simultaneously, activating and inhibitory microclusters often coexist within the same contact zone. Their synapses are therefore more dynamic and decision-oriented than the highly structured T cell synapse. In cytotoxic NK synapses, granule secretion sites form in regions where actin is locally cleared, allowing directed release of perforin and granzyme [54].

CAR-T synapses frequently display faster but less canonical organization than natural TCR synapses. Strong CAR clustering can rapidly generate signaling domains without requiring the full mature bulls-eye structure [25].

CAR-NK synapses combine both principles: engineered CAR clusters generate dominant activating hotspots, while native NK receptors continue to modulate adhesion, inhibition, and serial killing behavior [24][54].

In the schematic representation in figure 2.5A, CAR-mediated synapses are therefore depicted in a generalized form, reflecting the shared principle of CAR-driven antigen clustering rather than cell-type-specific synaptic architectures.

2.4.3 Recruitment Dynamics at Membrane Interfaces

Membrane interfaces are open non-equilibrium systems in which molecules continuously enter, leave, bind, unbind, and are actively transported. The resulting receptor composition is therefore governed by recruitment dynamics rather than static stoichiometry. These principles apply broadly to immune synapses formed by T cells, NK cells, as well as engineered CAR-T and CAR-NK cells, where similar dynamic processes govern receptor

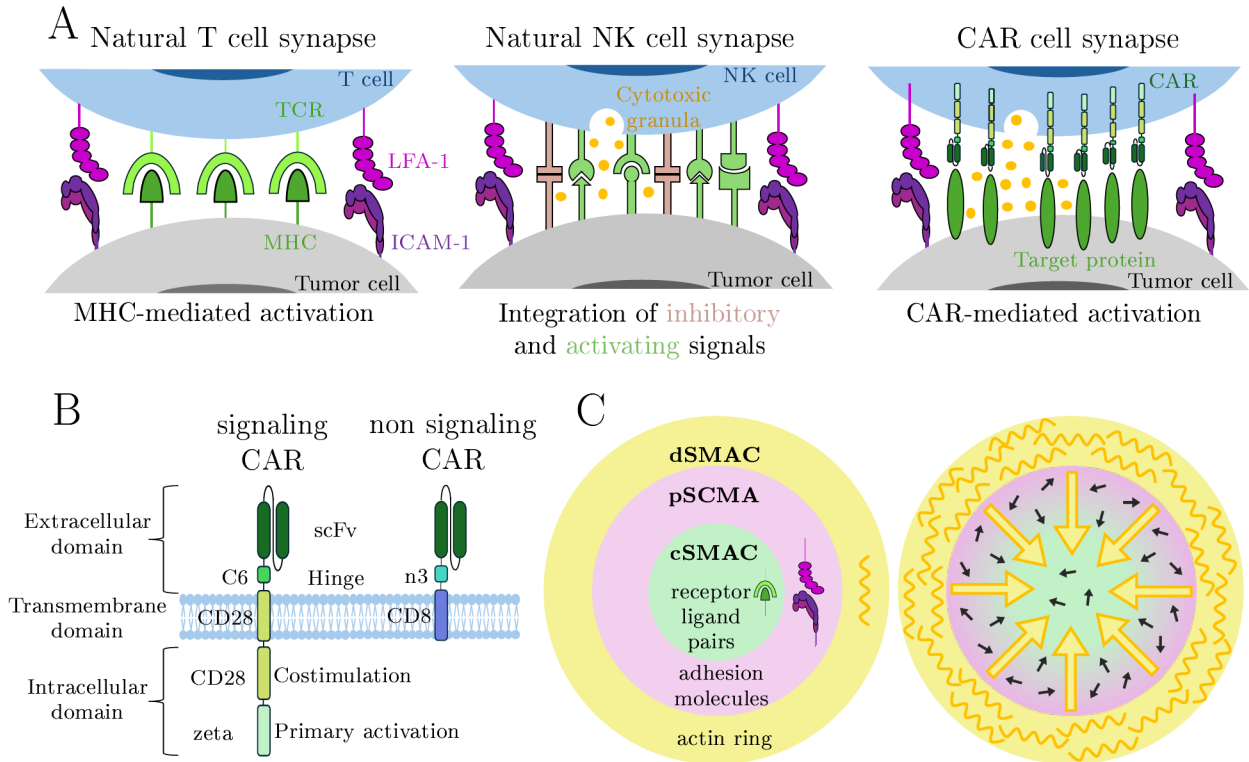


Figure 2.5: Natural and CAR-mediated immune cell synapse formation. (A) Comparison of a natural T cell synapse, natural NK cell synapse, and a generalized CAR-mediated synapse representing engineered immune cell synapses. Natural T cells synapse formation is MHC mediated, natural NK cells integrate signals from multiple activating and inhibitory receptors, resulting here in activation. The CAR synapse illustrates the common functional principle of CAR-driven antigen recognition, which is shared by both CAR-T and CAR-NK cells, despite differences in their intracellular signaling machinery. (B) Schematic structure of a signaling CAR and a non-signaling CAR as used in Publication III and Publication IV of this dissertation. The signaling CAR consists of an extracellular scFv, a C6 hinge region, a CD28 transmembrane domain, and intracellular signaling domains, CD28 (co-stimulation) and CD3 ζ (primary signaling). The non-signaling CAR consists of an extracellular scFv, a N3 hinge and a CD8 transmembrane domain. (C) left: Schematic top view of the immunological synapse showing its characteristic concentric organization into the cSMAC, enriched in receptor-ligand complexes, the pSMAC, dominated by adhesion molecules such as ICAM-1, and the dSMAC, containing dynamic components and actin cytoskeleton structures. right: Dynamic self-organization processes within the synapse. Lateral diffusion enables molecular encounters, while actin-driven centripetal flow transports receptor-ligand complexes from the periphery toward the center, leading to cluster formation and signal amplification [6][18][58][19].

organization and signaling across different cellular contexts.

After first contact, adhesion receptors increase encounter lifetime between both cells. This prolonged contact allows additional receptors to diffuse into the interface and bind available ligands. Once signaling begins, local phosphorylation events further enhance recruitment by stabilizing receptor residence times and attracting adaptor proteins [54].

A central mechanism is centripetal transport driven by retrograde actin flow (see figure 2.5C). Receptor clusters formed near the periphery are transported inward toward the synapse center. Actin polymerization at the periphery drives membrane spreading and enlarges the contact zone. Myosin-mediated contraction generates inward transport of receptor clusters. In cytotoxic lymphocytes, microtubule reorientation polarizes secretory vesicles toward the synapse, enabling directional release of lytic granules [19]. During this movement, clusters may grow by fusion, lose components by dissociation, or undergo signal maturation [19].

In addition, size-based segregation plays an important role: small receptor-ligand pairs accumulate in regions where the membranes of immune and target cell are closely apposed, typically in the center, whereas larger molecules, like adhesion molecules are sterically excluded from this regions and localized in the peripheral zone. Next to steric effects, also other receptor-ligand interactions further stabilize the synapse clusters. Differences in binding affinity and local concentration contribute to the selective enrichment of components in defined synaptic regions. This principle is described by the kinetic segregation model, in which steric effects and binding kinetics jointly determine spatial organization [59].

At the center of the synapse, signaling is consolidated and receptor-ligand complexes are eventually degraded or internalized, contributing to signal termination. Overall, the interplay between diffusion, binding kinetics, steric constraints and cytoskeletal forces generates a dynamic structure that is tightly coupled to signaling efficiency [18][60].

The kinetics differ strongly between cell types:

- **Natural T cells:** slower but highly sensitive recruitment with sustained signaling [25].
- **Natural NK cells:** rapid scanning contacts with reversible recruitment depending on activating/inhibitory balance [54].
- **CAR-T cells:** strong recruitment once antigen threshold is exceeded, often followed by persistent signaling [25].
- **CAR-NK cells:** rapid recruitment with efficient detachment and re-engagement, favoring serial killing [56].

Thus, immune synapses are not only organized by passive diffusion but are actively remodeled through forces generated by the actin cytoskeleton, membrane tension, and

receptor pulling. Cells mechanically probe their targets while simultaneously reorganizing the interface.

2.4.4 Quantification of Molecular Density via Fluorescence

To understand membrane interface organization quantitatively, methods are required that convert fluorescence intensity into local molecular surface density. This is particularly relevant for determining ligand thresholds, receptor recruitment efficiencies, and cluster enrichment factors. Consequently, quantitative knowledge of receptor or ligand density is often more informative than fluorescence intensity alone, since biological responses are governed by molecule numbers per unit area rather than arbitrary detector counts.

Fluorescence microscopy offers a powerful route to access such densities because fluorescent labels convert molecular abundance into optically detectable signals with high spatial resolution. In the simplest approximation, the detected intensity is proportional to the number of fluorophores within the observation area. However, this proportionality is only meaningful after consideration of instrumental factors such as excitation power, collection efficiency, detector gain, pixel dwell time, and fluorophore photophysics. Raw fluorescence images therefore provide relative contrast, whereas calibrated measurements can yield absolute molecular surface densities [Publication III].

For membrane systems, the relevant quantity is typically the areal concentration

$$\sigma = \frac{N}{A} \quad ,$$

where N is the number of molecules contained within a membrane region of area A . Spatially resolved determination of σ allows construction of molecular density maps across interfaces, revealing enrichment zones, depletion regions, gradients, or clustered states [Publication III].

In cellular systems, additional complexity arises from intracellular background fluorescence. Therefore, the fluorescence measured at membrane-focused positions most likely contain contributions not only from the plasma membrane but also from fluorophores located in the adjacent cytoplasm [Publication III].

For this reason, reliable determination of membrane molecular density often requires decomposition of total fluorescence into membrane-bound and non-membrane fractions. Dynamic methods such as FCS are particularly useful in this context, since two-dimensional membrane diffusion and three-dimensional cytoplasmic diffusion exhibit distinct characteristic timescales. By exploiting these differences, the membrane-associated contribution to the total signal can be identified and used for corrected density estimation (see section 2.1.2) [Publication III].

Once the membrane-specific signal is isolated, conversion onto molecular number requires knowledge of single fluorophore count rates in the present microscopy setup. This

can be obtained by independent calibration using purified fluorophores or reference samples of known concentration. Thereafter, fluorescence images can be transformed from arbitrary count maps into quantitative distributions of molecules per square micrometer [Publication III].

Such quantitative density measurements provide direct access to biologically relevant observables. Examples include the number of receptors recruited into a contact zone, ligand density required for activation thresholds, enrichment factors relative to surrounding membrane regions, and temporal changes during synapse formation. In engineered immune interfaces, these quantities are essential for understanding how receptor abundance and spatial organization regulate signaling performance [Publication III].

Within this dissertation, fluorescence-based density quantification is used to determine the abundance of membrane-associated CAR-receptor populations in cell-SLB and cell-cell interfaces. Particular emphasis is placed on distinguishing the truly membrane-resident receptor fraction from intracellular fluorescence contributions, thereby ensuring that only the functionally accessible surface population enters the quantitative analysis [Publication III].

In this way, fluorescence microscopy becomes not merely an imaging modality but a methodological tool for extracting absolute molecular densities at dynamic membrane interfaces. This establishes a direct connection between optical observables and the physical parameters governing molecular recognition, transport, and collective receptor organization.

2.4.5 Influence of Ligand Density and Mobility

Besides receptor properties, the physical state of the opposing membrane strongly influences signaling outcomes in both living cells and artificial membranes. Two key parameters are ligand surface density and lateral mobility.

At low ligand density, receptor encounters are rare and cluster nucleation may remain below the signaling threshold [61]. Increasing ligand density raises rebinding probability, promotes cooperative interactions, and stabilizes synapse formation. Many immune responses therefore show initially threshold-like behavior followed by a more graded increase with ligand number once the initial threshold is exceeded (see figure 2.6A) [62][63].

Ligand mobility is equally important. Mobile ligands can be collected by receptors into clusters, whereas immobilized ligands resist reorganization. Highly mobile ligands frequently promote larger signaling domains and more efficient synapse maturation than rigidly anchored ligands [28].

Membrane phase state provides a direct physical control parameter for mobility and has therefore an immediate influence on synapse formation (see figure 2.6B):

- disordered membrane phase $\rightarrow$ higher diffusion coefficient $\rightarrow$ stable synapse organization
- ordered membrane phase $\rightarrow$ reduced diffusion coefficient $\rightarrow$ weak or dispersed synapse engagement

Thus, temperature-dependent phase transitions modulate whether ligands reside in fluid or ordered membrane environments, which in turn affects receptor transport and clustering in synapse formation [64].

2.4.6 Model Membranes for Studying Synapse Formation

To systematically disentangle all these parameters important for synapse formation, reductionist model systems such as SLBs have been developed to mimic and control key features of the target membrane. SLBs enable the presentation of defined ligands with precisely tunable density and lateral mobility, allowing systematic analysis of key parameters.

Using SLBs, it has been demonstrated that the organization and dynamics of receptor-ligand interactions are strongly influenced by the physical properties of the target membrane. Furthermore, these systems allow targeted variation of individual parameters, facilitating the identification of causal relationships [10].

Key advantages of SLBs studying immunological synapses are (see figure 2.6C):

1. **Precise control of lateral mobility:** Membrane viscosity can be tuned, enabling the presentation of freely diffusing ligands that immune cells can reorganize without constraint.
2. **Defined ligand density:** Ligand concentration can be accurately adjusted, allowing a quantitative determination of the activation threshold.
3. **Compatibility with advanced microscopy:** The planar geometry positions the synapse always within the focal plane, enabling optical imaging, like TIRF or others.
4. **Geometric manipulation:** Spatial organization can be controlled using artificial barriers or ligand patterns, enabling the study of self-organization under constrained conditions.

A key limitation of SLBs in immune synapse studies is the lack of mechanical responsiveness. Immune cells exert mechanical forces on the ligands to probe their mechanical anchoring and properties. Rigid glass substrates, as used in classical SLBs, do not deform accordingly. To address this, enhanced SLB systems with polymer gels or elastomeric substrates have been developed to better mimic the mechanical properties of the target cells [10].

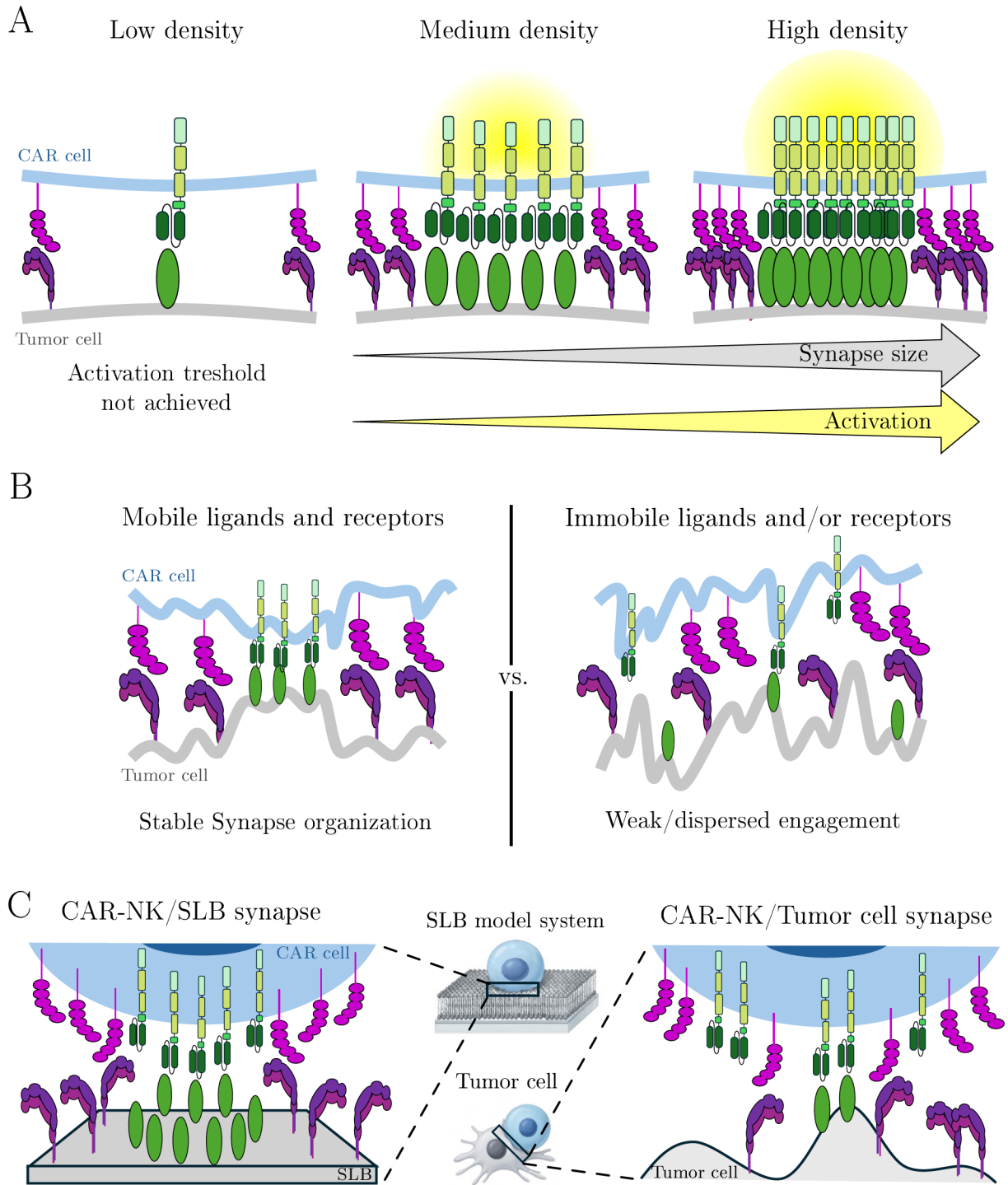


Figure 2.6: Key biophysical parameters governing immune synapse formation. (A) Effect of antigen density on synapse formation. Low antigen density results in limited receptor engagement, which can not exceed the activation threshold. Increasing antigen density promotes receptor clustering, enlargement of the contact interface, enhanced synapse stability and induces signaling [61][62][63]. (B) Influence of ligand mobility on synapse formation. Mobile ligands can be reorganized into clusters upon receptor binding, promoting stable synapse formation, whereas immobilized ligands impair clustering and reduce signaling efficiency. (D) Comparison of model membrane systems and tumor cells. SLBs provide a planar and controllable platform with defined ligand density and mobility, while tumor cells exhibit heterogeneous and spatially variable antigen expression. Legend for the molecules is given in figure 2.5.

Furthermore, SLBs do not provide a functional cellular response, preventing direct observation of downstream effects such as target cell killing and thereby limiting the correlation between synapse formation and effector function.

2.5 Quantitative Image Analysis and Extraction of Physical Observables

Fluorescence microscopy delivers access to spatial and temporal information on molecular distributions within biological systems. While qualitative imaging allows direct visualization of spatial heterogeneities and dynamic processes, it does not by itself provide access to quantitative physical parameters. In contrast, quantitative image analysis enables the extraction of physically meaningful observables such as molecular densities, diffusion coefficients, transport velocities, and measures of spatial organization.

In this sense, quantitative image analysis transforms fluorescence microscopy from a primarily descriptive imaging technique into a quantitative tool that bridges experimental observation and physical modeling. By converting intensity distributions and time-resolved image sequences into calibrated molecular and spatial observables, microscopic data can be directly related to underlying biophysical processes such as membrane organization, receptor transport, interaction-driven clustering, and active transport.

2.5.1 Fluorescence Image Analysis

Fluorescence microscopy generates spatially and temporally resolved image data in which the measured intensity distribution reflects the localization of fluorescent labeled molecular species. To transform such image series into quantitative observables, biologically relevant structures must be identified and converted into analyzable regions of interest. In the context of this dissertation, fluorescence image analysis is primarily used to characterize immune-cell contact interfaces, receptor redistribution during synapse formation, and time-dependent cytotoxic responses.

A central step of the analysis is the segmentation of cellular structures and contact regions. For experiments involving CAR effector cells interacting either with SLBs or tumor target cells, the contour of the effector cell defines the relevant analysis region. Automated mask-generation routines were therefore used to determine the projected cell boundary from fluorescence images. From these masks, geometric quantities such as contact-zone size, and synapse morphology can be extracted [Publication III].

Beyond the overall cell contour, particular emphasis is placed on the identification of localized receptor enrichment zones within the contact interface. These regions correspond to spatially confined areas of enhanced fluorescence intensity and indicate local accumulation of receptors at the immunological synapse. Such enrichment domains were

segmented by threshold-based or intensity-relative criteria within the previously defined contact mask. Their area, position, integrated intensity, and fractional coverage of the synapse provide quantitative measures of receptor reorganization [Publication III].

Mask-based quantification enables direct extraction of intensity-derived observables from biologically defined compartments. Once the cell contour and enrichment zones are known, mean fluorescence intensity, integrated signal, local receptor density, and enrichment ratios between synaptic and non-synaptic membrane regions can be calculated. A commonly used quantity is the enrichment factor

$$E = \frac{\langle I \rangle_{\text{contact}}}{\langle I \rangle_{\text{outside}}} \quad ,$$

where $\langle I \rangle_{\text{contact}}$ denotes the mean fluorescence intensity inside the contact zone and $\langle I \rangle_{\text{outside}}$ the corresponding intensity in non-contact membrane regions. Values $E > 1$ indicate preferential receptor recruitment towards the synapse [Publication III].

For calibrated confocal measurements, these intensity values can further be converted into absolute molecular surface densities (see section 2.4.4), allowing quantitative comparison of receptor abundance between different cells, synapse morphology, or experimental conditions [Publication III].

To analyze spatial receptor organization in a rotational averaged manner, radial averaging procedures were applied. These profiles reveal whether receptor accumulation occurs centrally, peripherally, or in pattern-like arrangements and permit direct comparison between different synapse classes independent of local angular irregularities. If brightness calibration is available, the same procedure yields absolute radial concentration profiles [Publication III].

Time-resolved fluorescence image sequences provide additional access to dynamic cellular processes. During formation of the immune synapse, labeled receptors often undergo pronounced spatial redistribution from an initially diffuse membrane distribution toward the contact interface. By analyzing the temporal evolution of fluorescence intensity along the effector cell membrane, the kinetics of receptor recruitment and accumulation can be analyzed [Publication IV].

In cytotoxicity assays, time-lapse epifluorescence microscopy further enables determination of target-cell death dynamics. Such curves report the temporal efficiency of CAR-mediated cytotoxicity and allow comparison between different effector-to-target ratios or receptor constructs [Publication III].

Time-lapse analysis also provides access to serial killing behavior of individual effector cells. By tracking repeated contacts of a single CAR-effector cell with several target cells, the number of sequential killing events per effector cell can be determined. This quantity characterizes functional persistence and cytotoxic capacity beyond single-cell endpoint measurements [Publication III].

Overall, fluorescence image analysis transforms microscopy recordings into quantitative descriptors of cellular organization and function. Through segmentation, mask-based quantification, time-resolved event detection, and spatial profile analysis, optical image data become a source of physically meaningful observables such as receptor enrichment, recruitment kinetics, contact geometry, and cytotoxic performance.

2.5.2 Quantitative Intensity Analysis and Calibration

Fluorescence microscopy provides highly sensitive spatial information, but raw image intensity is not directly equivalent to molecular number. The measured signal depends on excitation power, detector efficiency, optical alignment, dwell time, fluorophore quantum yield, and local environment. Hence, quantitative conversion of fluorescence counts into absolute molecular densities requires calibration procedures that relate detected photons to individual fluorophores under defined imaging conditions [65].

A key quantity in this context is the **molecular brightness**, i.e. the average detected photon count rate contributed by a single fluorescent molecule. Molecular brightness links image intensity to molecular abundance and is particularly powerful when fluorescent proteins are used as genetically encoded stoichiometric labels. Once determined experimentally, it allows fluorescence images to be interpreted on an absolute scale rather than only comparatively.

For the systems investigated in this dissertation, calibration was performed using purified mNeonGreen protein, the fluorescent reporter fused to the CAR-receptor of interest. Serial dilution series of known concentration provide a direct relationship between fluorophore concentration and detected count rate under confocal excitation. To ensure absolute concentration accuracy, independent spectrophotometric determination of protein concentration is required. Measurements over multiple laser powers additionally verify the linear response regime of the optical system.

To extract molecular brightness reliably, the confocal detection volume must first be known. This is commonly determined by FCS using reference dyes with established diffusion coefficients, such as Rhodamine 110. From the measured correlation time and known diffusion constant, the effective confocal volume can be obtained. The calibrated volume then converts concentrations into absolute molecule numbers inside the observation region.

If the fluorescence count rate is normalized by excitation power, one obtains an experimental brightness parameter B (in kHz/μW). Its dependence on fluorophore concentration is linear in the absence of saturation effects. The slope b of this relation can be transformed into the single-molecule brightness

$$m_B = \frac{b}{N_A V} \quad ,$$

where N_A is the Avogadro constant and V the effective confocal volume. The quantity m_B consequently represents the fluorescence contribution of one molecule under the chosen optical conditions [Publication III].

Once molecular brightness is known, fluorescence images can be converted into local molecular densities. For a pixel with integrated intensity I , dwell time t and excitation power P , the number of fluorescent molecules contributing to that pixel is given by:

$$N_{pix} = \frac{I}{t P m_B} \quad . \quad (2.2)$$

Division by the effective confocal area A_{eff} yields a surface density in molecules per μm^2 . In practice, this enables direct quantification of receptor abundance within cell-cell or cell-SLB contact regions [Publication III].

For membrane proteins, an additional correction is required because confocal imaging at the membrane plane also captures fluorescence from nearby cytoplasmic molecules within the axial detection volume. For this reason, the total intensity in a membrane-focused image is generally a superposition of membrane-derived and cytoplasmic contributions. If uncorrected, this leads to systematic overestimation of receptor surface density.

To resolve this problem, the membrane fraction determined independently by FCS (see section 2.1.2) is incorporated into the intensity analysis. By separating the slowly diffusing two-dimensional membrane component from the faster three-dimensional cytoplasmic component, the relative membrane-associated signal fraction f_{mem} is obtained. Only this fraction is then used for molecular counting:

$$I_{mem} = f_{mem} I_{tot} \quad .$$

This procedure ensures that only receptor residing in the plasma membrane are counted, whereas soluble intracellular fluorescence is excluded [Publication III].

Such membrane-fraction correction is particularly relevant for CAR constructs, since a significant intracellular pool may coexist next to the functional surface population. The biologically relevant parameter for interface formation and signaling is the number of receptors available at the membrane, not the total cellular fluorescence. Combining brightness calibration with FCS-based fractionation therefore enables direct determination of functional receptor densities within contact zones.

Beyond receptor counting, calibrated intensity analysis also permits determination of integrated receptor number per synapse, spatial heterogeneity, and enrichment factors relative to non-contact membrane regions. It thereby transforms fluorescence microscopy from a qualitative imaging method into a quantitative tool for extracting physically meaningful observables of molecular organization.

2.5.3 Correction of Imaging Artifacts

Quantitative fluorescence microscopy requires accurate measurement of image intensities and spatial information in order to reliably represent the underlying molecular distribution. In practice, however, optical detection systems introduce systematic distortions that can bias localization, co-localization, and intensity-based quantification if left uncorrected. Such artifacts arise from wavelength-dependent imaging errors, detector asymmetries, polarization sensitivity, imperfect alignment of optical components, and nonuniform detection efficiencies.

Because the present work relies on precise determination of receptor densities, membrane contact geometries, and multi-color spatial organization, correction procedures constitute an essential step of the image-analysis pipeline. Depending on the experimental modality, different corrections become relevant. In multi-color imaging, chromatic shifts and geometric distortions must be compensated to ensure accurate overlay of fluorescence channels. In polarization-resolved confocal detection, unequal detector sensitivities must be corrected before channel combination and intensity quantification.

The following sections summarize the physical origin of these effects and the corresponding correction strategies used to obtain quantitatively reliable fluorescence images.

Chromatic Aberration Correction

In dual-color confocal microscopy, precise spatial co-registration of spectral separated image channels is essential for reliable quantitative analysis. However, residual wavelength-dependent imaging errors frequently lead to geometric misalignment between channels recorded at different emission wavelengths. In the present setup, fluorescence signals centered at 450 nm and 525 nm were detected in two independent channels using a conventional photomultiplier tube (PMT) and a gallium arsenide phosphide (GaAsP) PMT, respectively. While both channels were well aligned near the optical axis, an increasing lateral displacement was observed toward the periphery of the field of view. This behavior is characteristic of residual lateral chromatic aberration combined with channel-specific relay-optical distortion, commonly appearing as pincushion-type warping.

The physical origin of chromatic aberration lies in the dispersive nature of optical materials, for which the refractive index depends on the wavelength,

$$n = n(\lambda) \quad .$$

As a consequence, fluorescence emitted at different wavelengths propagates through the imaging system with slightly different refraction angles and effective magnifications. Although modern apochromatic microscope objectives strongly suppress these effects, small residual errors remain, particularly at larger field angles away from the image center [13].

The resulting lateral misregistration can be described by a wavelength-dependent radial displacement field,

$$\Delta r(r) = r_{525}(r) - r_{450}(r) \quad ,$$

where r denotes the radial distance from the image center and r_{525} and r_{450} are the measured positions of identical emitters in the respective channels. In agreement with the experimentally observed pincushion distortion, the displacement increases with increasing radial distance and is negligible near the optical axis.

To compensate this artifact, multicolor fluorescent fiducial markers (TetraSpeck microspheres) were used as spatial calibration standards. Since these beads emit simultaneously in multiple spectral channels, they provide identical point-like reference positions throughout the field of view. Bead images were acquired under identical imaging conditions as the experimental data, and the relative displacement between channels was determined from the measured bead coordinates. Based on these data, a spatial transformation map was calculated and applied to all recorded images,

$$(x', y') = T(x, y) \quad ,$$

where T denotes the calibration-derived coordinate transformation used to map one spectral channel onto the reference channel.

This procedure compensates residual chromatic distortion and restores accurate pixel-wise correspondence between both channels. Such correction is particularly important for comparison of spatial arrangements of different receptor-ligand pairs in the immune synapse labeled with different fluorophores.

Polarization-Resolved Detection and G-Factor Correction

In high-sensitivity confocal microscopy, fluorescence emission is frequently separated into orthogonal polarization components before detection. This configuration is particularly common when using avalanche photodiodes, since polarization splitting can improve detection efficiency and enables additional analysis of fluorescence anisotropy or polarization-dependent signals. In such systems, the emitted fluorescence is decomposed into components parallel and perpendicular to a defined optical axis and recorded in two independent detector channels.

Even for completely unpolarized fluorescence emission, the measured count rates in both channels are generally not identical. This discrepancy does not primarily reflect physical properties of the sample, but instead originates from unequal detector quantum efficiencies, differences in optical transmission, polarization-dependent beam splitter characteristics, or small alignment asymmetries. Without correction, direct addition of both channels would therefore produce biased total intensities.

To compensate for this systematic imbalance, a detector-specific scaling factor, commonly referred to as the G-factor, is introduced. It is defined as the ratio of the effective detection efficiencies of the two channels,

$$G = \frac{\eta_{\parallel}}{\eta_{\perp}} \quad ,$$

where $\eta_{\parallel}$ and $\eta_{\perp}$ denote the overall sensitivities of the parallel and perpendicular detection paths, respectively. Experimentally, the factor is determined from calibration measurements using reference samples with known or isotropic polarization behavior. In the context of this dissertation Rhodamin 110 is used for calibration, offering a fast rotational diffusion in solution, its emission is practically depolarized over measurement time-scales [Publication III].

After calibration, the total fluorescence image is reconstructed by weighted channel addition,

$$I_{tot} = I_{\parallel} + 2GI_{\perp} \quad ,$$

where $I_{\parallel}$ and $I_{\perp}$ are the recorded intensities of the two polarization channels. The weighting term compensates for the unequal response of the perpendicular channel and restores a signal proportional to the total emitted fluorescence [Publication III].

The factor of two arises from the polarization geometry of the detection scheme. The emitted fluorescence is decomposed into two orthogonal polarization components within the detection plane. While the parallel channel directly measures one defined polarization axis, the perpendicular channel represents only a single of two equivalent orthogonal directions contributing to the emission in that plane. As a result, the measured perpendicular signal accounts for only half of the total intensity associated with the perpendicular polarization manifold. Consequently, a factor of two is required to recover the full contribution of this component to the overall fluorescence signal.

For the applications considered in this dissertation, G-factor correction is particularly important because subsequent analyses rely on absolute fluorescence intensities. Examples include determination of molecular surface densities in membrane contact zones, comparison of receptor enrichment between conditions, and extraction of concentration-dependent spatial profiles. Any systematic detector imbalance would propagate directly into these quantities.

More generally, polarization-channel correction illustrates an important principle of quantitative microscopy: raw detector counts are not identical to physical fluorescence intensity. Instead, detector-specific response functions must first be removed before reliable biological or biophysical observables can be inferred.

2.5.4 Extraction of Spatial Organization and Clustering

A central objective of fluorescence-based image analysis in the presented work is the characterization of how receptors and adhesion molecules are spatially organized within the immunological synapse. Beyond average intensity or total receptor numbers, the key physical information lies in how molecules redistribute into distinct enrichment zones at the cell–cell or cell–SLB interface. These spatial patterns reflect the underlying balance between antigen engagement, adhesion strength, and membrane organization.

To describe such spatial organization in a consistent and quantitative manner, regions of enhanced fluorescence intensity within the synapse are identified relative to the surrounding membrane background. In this context, an enrichment zone is defined as a connected region of the contact interface in which the local receptor density exceeds the mean intensity of the adjacent non-enriched membrane. This definition ensures that enrichment is always interpreted relative to the same cellular reference state, rather than as an absolute intensity value. As a consequence, enrichment zones represent physically meaningful deviations from the baseline receptor distribution.

Based on this criterion, different synaptic morphologies can be classified according to the spatial arrangement of enrichment zones within the contact area. Importantly, this classification is not intended as a discrete biological taxonomy, but as a phenomenological description of recurring spatial organization patterns observed under different ligand and adhesion conditions.

The classification into enrichment-based morphologies is performed manually based on the spatial distribution of intensity within the contact region. This approach is motivated by the intrinsic variability of synapse shapes and the limited robustness of fully automated segmentation approaches for complex cellular interfaces. The resulting categorization is therefore phenomenological and intended to guide interpretation of spatial organization trends rather than to impose strict discrete states.

To quantitatively capture these spatial arrangements, radial intensity profiles are used as a central analytical tool. Fluorescence intensity is averaged over concentric annuli as a function of radial distance from the center. This yields a radial profile of receptor density that is independent of local angular variations and provides a compact representation of synaptic organization. Differences in spatial organization are then directly reflected in the shape of these radial profiles, such as centrally peaked distributions, ring-shaped maxima, or broadened plateau-like regions.

These radial profiles serve as a robust bridge between image data and physical interpretation, as they reduce complex two-dimensional spatial patterns to a one-dimensional representation while preserving the essential structural information. In particular, they allow comparison of synapses formed under different ligand densities or on different target cells without requiring point-by-point spatial alignment.

Overall, this framework provides a consistent way to describe how receptor distributions reorganize within the immunological synapse. By combining a clear definition of enrichment zones with radial intensity profiling, spatial organization can be quantified in a way that is directly comparable across experimental conditions while remaining grounded in the underlying physical distribution of membrane-associated receptors.

2.5.5 Analysis of Transport Dynamics

Many biologically relevant membrane processes are inherently dynamic and cannot be fully understood from static images alone. Molecular recruitment, directed transport, diffusion, cluster growth, and relaxation toward equilibrium all involve characteristic timescales that encode the underlying physical mechanisms. Quantitative analysis of transport dynamics therefore aims to extract kinetic parameters such as velocities, diffusion coefficients, drift forces, and relaxation times from time-resolved fluorescence microscopy data.

In fluorescence imaging experiments, the measured intensity field $I(\mathbf{r}, t)$ can often be interpreted as a proxy for the local concentration of labeled molecules or particles. Time-dependent changes in this field then directly reflect redistribution processes on the membrane surface. By analyzing how spatial intensity profiles evolve in time, it becomes possible to reconstruct the transport laws governing the system.

A particularly important distinction is that between **passive diffusion** and **directed drift**. Diffusion arises from stochastic thermal motion and tends to homogenize concentration gradients, whereas drift results from deterministic external forces such as electric, hydrodynamic, or magnetic fields. In many membrane systems, both processes occur simultaneously and the observed dynamics reflect their superposition.

This behavior is commonly described by the drift–diffusion equation

$$\frac{\partial c}{\partial t} = D \nabla^2 c - \nabla \cdot (c \mathbf{v}) \quad ,$$

where $c(\mathbf{r}, t)$ is the local surface concentration, D the diffusion coefficient and $\mathbf{v}$ the drift velocity field. The first term accounts for the thermal spreading, whereas the second term describes force-driven transport [66].

For radially symmetric accumulation processes, such as particles attracted toward a localized force center, the problem simplifies substantially. If fluorescence intensity is proportional to particle concentration, the radial signal $I(r, t)$ can be analyzed as a function of distance r from the force center and time t . In this representation, the inward migration of particles generates characteristic temporal intensity increases at fixed radii. Fitting these local rise kinetics yields the radial drift velocity $v(r)$, which quantifies how rapidly particles are transported toward the accumulation center. A convenient phenomenological

description of the temporal intensity increase is given by:

$$I(r, t) = I_{\max}(t) \left[1 - \exp\left(-\frac{r}{v(r)t}\right) \right] \quad ,$$

where $I_{\max}$ is the asymptotic local intensity and $v(r)$ is the effective inward transport velocity at radius r [Publication III].

Once the drift velocity is known, the underlying force can be obtained:

$$F(r) = \gamma v(r) \quad ,$$

where γ is the effective friction coefficient. For SLB-coupled MNP, this friction contains contributions both from the surrounding aqueous medium and from lateral dissipation within the membrane itself:

$$\gamma = \gamma_{3D} + \gamma_{mem} \quad .$$

The first contribution is commonly approximated by Stokes friction of the nanoparticle in solution, whereas the second arises from two-dimensional membrane hydrodynamics and can be described within the Saffman–Delbrück framework [Publication III].

This principle is especially powerful for magnetically manipulated MNPs on SLBs. Here, a magnetic field gradient generates directed lateral transport toward the field maximum. The temporal increase in fluorescence at different radii reflects the local drift speed and therefore the radial magnetic force distribution within the membrane plane. Since the force cannot be measured directly at the nanoscale during imaging, extraction from transport dynamics provides a practical and physically robust alternative.

Complementary information is obtained during the relaxation phase after removal of the external force. Once the driving field is switched off, accumulated particles redistribute spontaneously under thermal motion and concentration gradients. In the absence of drift, the system approaches the pure diffusion limit:

$$\frac{\partial c}{\partial t} = D \nabla^2 c \quad .$$

For an initially localized concentration profile, the temporal intensity decay can be approximated by a Gaussian diffusion form,

$$I(r, t) = I_0(t) \exp\left(-\frac{r^2}{4D(r)t}\right) \quad ,$$

where $I_0(t)$ is a time-dependent amplitude and $D(r)$ is an apparent diffusion coefficient extracted at radius r . Fitting the relaxation dynamics therefore yields direct access to the lateral mobility of the membrane-bound particles [Publication III].

In membrane systems, the apparent diffusion coefficient may depend on local particle

density. At high surface coverage, steric hindrance, excluded-volume interactions, or transient clustering can slow transport substantially. As a consequence, diffusion extracted from densely packed central regions may be lower than the true single-particle diffusion coefficient measured at larger radii or lower densities [Publication III].

Within this dissertation, dynamic fluorescence analysis is used to quantify the motion of SLB-bound MNP under applied magnetic gradients and during field-free relaxation. From the accumulation kinetics, lateral drift velocities and corresponding force distributions are obtained. From the subsequent relaxation dynamics, effective diffusion coefficients are extracted. Together, these observables establish a direct connection between measured fluorescence movies and the physical parameters governing transport on membrane surfaces [Publication III].

Thus, time-resolved image analysis transforms microscopy data into quantitative information on nanoscale forces, mobilities, and non-equilibrium transport processes that are otherwise inaccessible from static endpoint measurements alone.

2.5.6 Software and Implementation

The quantitative analysis presented in this dissertation relies on a combination of commercially available microscope control software, dedicated image-processing tools, and custom-written analysis routines. All core image analysis procedures were implemented in MATLAB (MathWork Inc.), which served as the central environment for processing, quantification, and statistical evaluation of fluorescence microscopy data. The majority of scripts used for image analysis were written specifically for this work, while some routines were adapted from previously established code within the research group to ensure consistency with earlier studies.

Image acquisition was performed using the corresponding manufacturer software provided with the respective microscopy platforms. Epifluorescence data (including time-laps videos of CAR-effector cells, CPB and magnetic manipulation analysis) were recorded using Olympus imaging systems, controlled via cellSense Dimensions acquisition software. For confocal measurements, two different microscope setups were utilized, depending experimental requirements. For confocal imaging that was converted into absolute receptor concentrations an Abberior setup with the corresponding imInspector software was employed, while for TIRF and multi-color confocal imaging a Nikon setup with the corresponding Nis-Elements software was facilitated. For FCS, data acquisition and initial correlation analysis were performed using SymPhoTime software (PicoQuant).

For image inspection and intermediate processing steps, Fiji (ImageJ) was used as a complementary tool. In particular, Fiji was employed for visualization of raw image data, manual inspection of segmentation quality, and correction of chromatic shifts in multicolor confocal images. These preprocessing steps ensured that subsequent quantitative analysis

was performed on spatially aligned and visually verified datasets.

A central component of the workflow consists of fully automated analysis pipelines implemented in MATLAB. These pipelines include CPB and field-driven MNP transport evaluation, mask generation for defining cellular and synaptic regions, radial averaging of fluorescence intensity or concentration distributions, and conversion of calibrated fluorescence signals into absolute surface density values.

Beyond fully automated routines, the analysis framework also includes hybrid workflows that combine computational processing with manual classification steps. This is particularly relevant for the identification of spatial organization categories within the immunological synapse, where enrichment patterns and morphological classes are assigned based on visual inspection of intensity distributions. A similar hybrid approach is used in time-resolved analyses of cytotoxicity assays. Here, the time point of target-cell death is determined manually from image sequences, defined by morphological cell collapse. In addition, serial killing behavior is quantified by manually tracking individual effector cells across time-lapse recordings and counting successive killing events. To minimize user-dependent variability, all datasets that are directly compared within a given analysis are evaluated by the same trained operator, ensuring internal consistency and reducing inter-analyst bias.

Reproducibility of the quantitative analysis is ensured through standardized processing procedures. Identical thresholds and parameter sets are applied across all datasets belonging to a given experimental series. All analyses are performed using the same MATLAB scripts without modification between conditions. This approach guarantees that differences between experimental groups reflect biological variation rather than changes in data processing.

Statistical evaluation is performed on populations of cells rather than individual measurements, ensuring that reported values reflect biologically relevant variability. Depending on the size and distribution of the dataset, results are typically reported as either mean values with standard deviation or as median values with median absolute deviation. For smaller or non-normally distributed datasets, median-based statistics are preferred, whereas mean and standard deviation are used for larger populations where distributional assumptions are more appropriate. Statistical significance between probes of same experimental conditions or between different experimental conditions are assessed using either wilcoxon-rangsum test or two-way analysis of variance, followed by Bonferroni correction to account for multiple comparisons. This framework allows systematic comparison across multiple ligand conditions, cell types, and experimental parameters.

Overall, the software and implementation framework integrates instrument-specific acquisition software, standardized image processing tools, and custom quantitative analysis pipelines into a coherent workflow. This combination enables reproducible extraction of physical observables from complex fluorescence microscopy datasets while maintaining

flexibility for diverse experimental modalities ranging from static imaging to dynamic single-cell time-lapse measurements.

Quantitative fluorescence image analysis thus bridges microscopy and statistical physics by transforming optical contrast into densities, spatial correlation functions, transport coefficients, and force-dependent observables.

2.6 Aim and Scope of the Dissertation

A central challenge in biophysics is the quantitative description of dynamic processes in complex membrane-associated systems. In particular, molecular transport, spatial organization, and receptor redistribution at biological interfaces emerge from the interplay of diffusion, interactions, and external constraints. Direct investigation of these processes in living cells is often limited by structural complexity, heterogeneous composition, and restricted experimental control. This creates a need for simplified and tunable model systems that retain essential membrane properties while allowing precise quantitative measurements. SLBs provide such a platform, combining biological relevance with well-defined geometry, controllable composition, and compatibility with high-resolution optical microscopy.

Against this background, the central aim of this dissertation is to establish and apply SLB-based systems as quantitative experimental platforms for investigating membrane dynamics and interfacial organization using fluorescence microscopy. Particular emphasis is placed on the systematic extraction of physical observables from imaging data, including diffusion coefficients, transport behavior under external driving forces, molecular concentrations, and spatial redistribution of receptors at cell interfaces. The dissertation therefore addresses the broader question of how optical microscopy can be combined with controllable membrane model systems to resolve dynamic processes that are difficult to access in native cellular environments.

To answer this question, the work is structured along three connected objectives.

1. Fundamental transport processes in model membranes are investigated. This includes the characterization of lateral diffusion of lipids and proteins in SLBs and the influence of obstacles or crowding on molecular mobility. CPB is used as a quantitative tool to determine diffusion coefficients in laterally structured membrane environments. Special emphasis is placed on how molecular mobility is modulated by protein binding lipids, electrostatic interactions, and spatial crowding.
2. The dissertation explores how membrane organization can be actively manipulated in a controlled non-equilibrium setting. For this purpose, a magnetic actuation platform was developed in which membrane-bound magnetic nanoparticles are displaced

by externally applied field gradients. This approach enables directed transport and reversible spatial patterning of membrane-associated components, allowing to study the role of lipid phase behavior and membrane mechanics in active transport processes.

3. The developed methodologies are extended toward biologically relevant interfaces. Functionalized SLBs are employed as controllable surrogate surfaces to mimic selected properties of target-cell membranes. Within this framework, quantitative confocal fluorescence microscopy is used to determine absolute concentrations of CARs on CAR-NK cells during synapse formation. By comparing synapses formed between effector cells and SLBs with those formed between effector cells and tumor cells, the transferability of observations from model to cellular systems is assessed. In a further step beyond planar model membranes, multicolor fluorescence microscopy is applied to analyze the redistribution of different CAR constructs within immune synapses formed between CAR-T cells and tumor cells.

The dissertation is therefore positioned at the interface of soft-matter physics, quantitative imaging, and biomembrane science. Rather than focusing primarily on biological outcome measures, the work emphasizes the development and application of optical methodologies to isolate measurable physical parameters from complex image data. In this way, the presented studies demonstrate how controllable membrane model systems and advanced fluorescence microscopy can be combined to investigate diffusion, active transport, and molecular self-organization at biologically relevant interfaces.

Chapter 3

Publications

The research conducted within this dissertation resulted in four publication, comprising two accepted article, one submitted manuscripts and one manuscript in preparation. The common focus of these works is the quantitative investigation of dynamic processes in biomimetic membrane systems and immune cell interfaces using advanced fluorescence microscopy. From a physics perspective, the dissertation emphasizes the isolation of specific observables and the application of optical methods to characterize transport phenomena, molecular organization, and receptor redistribution in complex soft-matter systems.

A central theme throughout this work is the lateral mobility of proteins and lipids in SLBs. In Publication I, lateral diffusion was quantified using CPB, enabling the determination of molecular mobility and the influence of membrane obstacles. It is shown that lipid and protein mobility is strongly dependent on protein concentration, the concentration and charge of membrane-coupled binding partners, and the degree of molecular crowding. In particular, immobile obstacles reduce diffusion significantly more strongly than mobile obstacles, demonstrating the dominant role of static spatial heterogeneities in governing transport in two-dimensional membranes.

Publication II extended this approach toward driven non-equilibrium transport, where MNPs coupled to SLBs were displaced by external magnetic field gradients to investigate active transport processes and the role of lipid phase behavior. By tracking the velocity of individual membrane-bound magnetic nanoparticles under controlled field gradients, the acting magnetic forces on the particles could be quantitatively determined, revealing forces in the femtonewton regime. These forces were sufficient to locally displace and reorganize membrane-associated MNPs, enabling controlled mechanical patterning of the membrane surface. Furthermore, such force-induced patterns could be stabilized by tuning the temperature across lipid phase transitions, effectively allowing “freezing-in” of non-equilibrium spatial organization. Importantly, it was observed that cells respond to these externally imposed molecular patterns, demonstrating functional biological sensitivity to engineered membrane organization.

The later publications increasingly connect these physical model systems to biolog-

ical applications. In Publication III, confocal fluorescence microscopy was employed to determine absolute receptor concentrations of CARs on CAR-NK cells interacting with functionalized SLBs and to relate these findings also in CAR-NK cells engaging with tumor cells. Different structural classes of CAR-NK synapses were identified and systematically classified based on receptor distribution patterns. This enabled the formulation of a predictive framework linking ligand concentration and adhesion molecule density to the resulting synapse morphology and organizational state.

Finally, Publication IV moved completely beyond planar model membranes toward native cellular interfaces. Using multicolor fluorescence microscopy, the spatial redistribution of different CAR constructs within the immune synapse formed between CAR-T cells and tumor cells was investigated. It is shown that even non-signaling CAR variants contribute to synapse organization through purely adhesive interactions. Moreover, these constructs enhance cellular killing efficiency by stabilizing receptor–ligand contacts, indicating that receptor architecture can modulate immune function independently of intracellular signaling domains.

Overall, the publications demonstrate how physical methods and quantitative fluorescence microscopy can be used to address fundamental questions of diffusion, active transport, and molecular self-organization in biologically relevant membrane systems.

3.1 Publication I: Mobile and Immobile Obstacles in Supportel Lipid Bilayer Systems and Their Effect on Lipid Mobility.

Autors: Luisa Coen, Daniel Alexander Kuckla, Andreas Neusch and Cornelia Monzel

Journal: *Colloids and Interfaces*

Date of publication: 24. September 2024

DOI: <https://doi.org/10.3390/colloids8050054>

Contribution

The doctoral candidate (LC) made the primary contribution to this work, accounting for approximately 80% of the overall effort. This includes the design and preparation of SLB systems, execution of diffusion measurements using CPB, and systematic investigation of protein-induced obstacle effects. The doctoral candidate further carried out the data analysis and contributed substantially to the interpretation of the results.

In addition, the doctoral candidate was the main contributor to the manuscript preparation, including drafting and revising. The remaining contributions, including supervision, conceptual input, and additional experimental or analytical support, were provided by the co-authors as detailed in the original publication.

Article

Mobile and Immobile Obstacles in Supported Lipid Bilayer Systems and Their Effect on Lipid Mobility

Luisa Coen, Daniel Alexander Kuckla, Andreas Neusch  and Cornelia Monzel *

Experimental Medical Physics, Heinrich-Heine University, 40225 Düsseldorf, Germany; luisa.coen@hhu.de (L.C.); andreas.neusch@hhu.de (A.N.)

* Correspondence: cornelia.monzel@hhu.de; Tel.: +49-(0)21181-12893

Abstract: Diffusion and immobilization of molecules in biomembranes are essential for life. Understanding it is crucial for biomimetic approaches where well-defined substrates are created for live cell assays or biomaterial development. Here, we present biomimetic model systems consisting of a supported lipid bilayer and membrane coupled proteins to study the influence of lipid–lipid and lipid–protein interactions on membrane mobility. To characterize the diffusion of lipids or proteins, the continuous photobleaching technique is used. Either Neutravidin coupled to DOPE-cap-Biotin lipids or GFP coupled to DOGS-NTA lipids is studied at 0.005–0.5 mol% concentration of the linker lipid. Neutravidin creates mobile obstacles in the membrane, while GFP coupling results in immobile obstacles. By actin filament coupling to Neutravidin-lipid complexes, obstacles are crosslinked, resulting in lipid mobility reduction along with the appearance of a membrane texture. Theoretical considerations accurately describe lipid diffusion changes at high obstacle concentration as a function of obstacle size and viscous effects. The mobility of membrane lipids depends on the concentration of protein-binding lipids and on the concentration and charge of the coupled protein. Next to diffusion and friction coefficients, we determine the effective obstacle size as well as a charge-dependent effect that dominates the decrease in lipid mobility.

Keywords: supported lipid bilayer (SLB); diffusion; (Im-)mobile obstacle; GFP; Neutravidin; actin; Continuous Photobleaching



Citation: Coen, L.; Kuckla, D.A.; Neusch, A.; Monzel, C. Mobile and Immobile Obstacles in Supported Lipid Bilayer Systems and Their Effect on Lipid Mobility. *Colloids Interfaces* **2024**, *8*, 54. <https://doi.org/10.3390/colloids8050054>

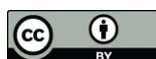
Academic Editors: Georgi G. Gochev and Eduardo Guzmán

Received: 13 July 2024

Revised: 2 September 2024

Accepted: 20 September 2024

Published: 24 September 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Changes in lipid and protein mobility in the plasma membrane are a hallmark of many vital biological functions [1], such as adhesion [2,3], recognition [4], or transport [5], and contribute to the formation of biomolecular condensates [6]. Next to the understanding of biological membranes, mobility changes in synthetic systems may further be harnessed for biotechnological applications [7–9]. On a molecular scale, a breakdown of mobility in the cellular membrane has been attributed to the combined effect of molecular crowding on the cell surface, hydrodynamic drag, molecular interactions, or attachment of peripheral proteins, such as the cytoskeleton [10,11]. Most recently, it was shown that drug treatment influences the diffusion of transmembrane proteins [12]. Biological and biophysical studies have probed each of these effects in cells [13–18]; however, in cellular studies, it is difficult to quantify and separate their respective contributions.

To study the influence of individual membrane components on the local membrane dynamics, simplified synthetic membranes, such as supported lipid bilayers (SLB) [19–23], or giant unilamellar vesicles [24–31] proved to be powerful systems. While SLBs are particularly suitable for examination under the microscope due to their planarity, other aspects such as membrane curvature can only be examined with vesicles [32,33]. Lipid diffusion in the membrane was shown to be affected by introducing discrete obstacles, such as non-diffusing protein clusters, membrane adhesion sites, or artificial barriers [34]. For example, (i) membrane-associated proteins (coupled or reconstituted into the membrane) that

oligomerize, reduced lipid mobility within the membrane [19,22,35]; (ii) specific adhesion of membranes to a solid surface inevitably led to an immobilization of bound receptors and associated lipids [36–39]; and (iii) micropatterned barriers have been demonstrated to inhibit free lipid diffusion [40]. Another prominent example is the cortical cytoskeleton, a dense fiber network of actin and spectrin, connecting to the plasma membrane via lipid-binding proteins, transmembrane proteins, or membrane-attached proteins [41–44]. These examples show that obstacles may appear in different geometries, in a mobile or immobile form, and may consist of several molecules due to multivalent coupling or (non-)specific interactions. The model membranes mentioned exhibit rich structural and dynamical behavior in the presence of obstacles; yet, systematic and quantitative studies on how obstacles alter membrane molecular mobility are sparse [45,46].

It is extremely important to know the influence of each membrane component (different lipids and proteins, etc.) on membrane mobility for the future design of these systems for biotechnological applications. For example, these mimetic models serve as well-defined substrates to understand and modify cell adhesion and signaling processes [47,48] and, to this end, must be precisely understood. Other diffusion-specific examples include the following: (1) Diffusion controls the frequency of random protein–protein encounters, which underlies other cellular processes [49]; (2) it has been shown in bacteria that diffusion and membrane viscosity have an influence on the rates of respiratory metabolism and cell growth [50]; and (3) diffusion of transmembrane proteins can be reduced by drug treatment [12]. In addition, (4) SLBs with the surface glycoprotein hemagglutinin can serve as model systems for the viral envelope of Influenza A H1N1 and can give insights into the stability of the virus at low humidity [23]. (5) Furthermore, SLBs can be used as highly tunable substrates to let cells adhere to them. For example, CAR T cells were placed on a SLB in order to precisely control which proteins are presented to the receptor of the CAR T cells [51].

A breakdown of mobility can arise from specific molecular interactions, Coulomb or van der Waals forces, as well as physical forces due to friction within the membrane [52], at the membrane–buffer or the membrane–substrate interface [53]. For the diffusion of lipids in a bilayer, different theoretical models of increasing complexity have been developed and are applied in this work: (1) a basic model by Saffman and Delbrück [54], which was later verified by Axelrod [55], where cylindrical objects diffuse thermally within a two-dimensional homogeneous membrane (for larger membrane inclusions, the model was further extended [56]); (2) an extended model by Evans and Sackmann [57], which was verified experimentally by Merkel et al. [58] and others [59] to describe a supported lipid bilayer where the embedded object exhibits an increased hydrodynamic drag due to interaction with the membrane–substrate interface; and (3) the theory by Saxton, which, along with other models, deals with the variable nature of the membrane components where tracers diffuse around immobilized or mobile lipid or protein obstacles [60,61]. Here, we use these models and test Saxton’s theory to describe lipid tracer mobility in the presence of mobile or immobile obstacles in the membrane.

In this work, we present a systematic experimental study on how lipids within the membrane, or proteins coupled to the membrane, give rise to lipid mobility changes in a supported lipid bilayer. To this end, we use an *in vitro* model system consisting of a SLB doped with an increasing concentration of linker lipids and couple either GFP or Neutravidin proteins to the membrane surface. Next to these lipid and lipid–protein obstacles, an actin network is used to crosslink lipid–protein complexes (see Figure 1). Expanding on previous studies [62–65], our system enables the direct quantification of diffusion constant changes and friction in the different membrane systems. We observe significant lipid mobility changes already when different linker lipid concentrations are used, further reduced by the coupling of proteins to the membrane, and find that obstacle crosslinking by actin gives rise to a membrane texture.

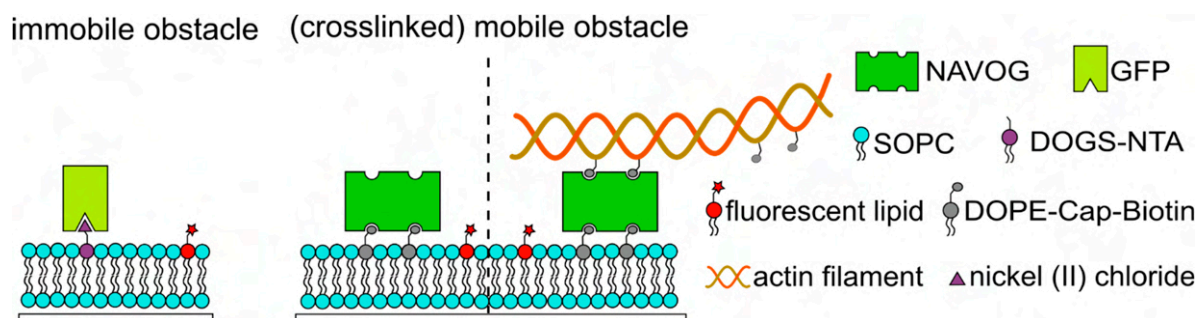


Figure 1. Sketch of model systems consisting of SLB and coupled proteins. Left: immobile obstacle system with GFP and DOGS-NTA. Middle: mobile obstacle system with NAVOG and DOPE-Cap-Biotin. Right: mobile obstacles that are crosslinked by actin filaments coupling to NAVTMR on a DOPE-Cap-Biotin doped membrane. Middle and right system separated by dashed line. See text for details and abbreviations.

2. Materials and Methods

2.1. Materials

All lipids: SOPC (1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine), DOPE-NBD (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)), NBD-DOPE (1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphoethanolamine), DOPE-Cy5 (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 5)), DOPE-cap-biotin (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl)), and DOGS-NTA (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)) were purchased from Avanti Polar Lipids (Birmingham, AL, USA) and used without further purification.

The Green Fluorescent Protein (GFP) with a 6-Histidine tag on the N-terminus was purchased from Merck (Darmstadt, Germany; UniProt ID: P42212). To ensure that protein samples were free from small oligomers, GFP was reconstituted in PBS (140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.5) and ultracentrifuged at 214,880 rcf at 4 °C for 2 h. For GFP coupling to DOGS-NTA, nickel(II) chloride (NiCl₂·6H₂O, Sigma-Aldrich, St. Louis, MO, USA) was used. Neutravidin covalently linked to the fluorescent dye Oregon Green (488 conjugate), termed NAVOG, or linked to the fluorescent dye tetramethylrhodamine, termed NAVTMR, was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). NAVOG and NAVTMR were treated equally to GFP. Preformed actin filaments from rabbit skeletal muscles (Cytoskeleton Inc., Denver, CO, USA) were bound to NAVTMR via Biotin-XX Phalloidin (PromoCell, Heidelberg, Germany). The actin filaments were stained with Acti-Stain 488 Phalloidin (Cytoskeleton Inc., Denver, CO, USA). Thickness-corrected glass coverslips ($d = 170 \pm 5 \mu\text{m}$, Paul Marienfeld, Lauda-Königshofen, Germany) were cleaned as follows: ultrasonication in a 2% Hellmanex solution (Hellma, Müllheim, Germany) for 15 min followed by two cycles of ultrasonication for the same duration in ultrapure water. Ultrapure water was produced by a water purification system (Milli-Q Gradient A10, Merck Millipore, Burlington, MA, USA). After each ultrasonic treatment, the coverslips were rinsed 10 times with ultrapure water.

2.2. Sample Preparation

Supported lipid bilayers (SLBs) were prepared with a film balance (Biolin Scientific, KSV Nima, Gothenburg, Sweden) using the Langmuir–Blodgett [66,67] technique for the first layer and a modified Langmuir–Schäfer technique for the second layer. As a modification for the second layer, the glass substrate with the first layer was pressed onto the second lipid layer and into the subphase. The subphase was ultrapure water. The first membrane layer consisted of 2 mg/mL pure SOPC. In the second layer, next to the matrix lipid SOPC, 2.5 mol% of a fluorescent lipid was added: DOPE-Cy5, DOPE-NBD, or NBD-DOPE. In addition, the protein-anchor lipids DOGS-NTA or DOPE-Cap-Biotin were

included in concentrations of 0.5 mol%, 0.05 mol%, or 0.005 mol% for DOGS-NTA, and 10 mol%, 5 mol%, 0.5 mol%, 0.05 mol%, 0.025 mol%, or 0.005 mol% for DOPE-Cap-Biotin. All SLB layers were prepared at a transfer pressure of 25 mN/m and were constantly kept underwater. In order to passivate the SLB surface and to diminish nonspecific binding, SLBs were passivated with BSA (Bovine Serum Albumin Fraction V, ITW Reagents, Monza, Italy) dissolved in PBS (5 mg/mL) for 15 min. For SLBs containing DOGS-NTA, the sample was incubated with nickel(II) chloride in large excess at a final concentration of at least 5 mM for 45 min at 37 °C to prepare the NTA binding site for GFP coupling via its His-tag. Thereafter, GFP was incubated in large excess with a concentration of at least 1 mg/mL for 48 h at 5 °C. After each step, excess nickel(II) chloride or GFP was removed by a tenfold exchange of PBS. To prepare the Biotin–Neutravidin system, the SLB containing DOPE-Cap-Biotin was incubated after BSA passivation with NAVOG in large excess at a final concentration of at least 50 µg/mL (400 µL total volume) for 1.5 h at room temperature. Excess proteins were removed using ten washing steps as before. To produce the Biotin–Neutravidin–Actin system, SLBs containing DOPE-Cap-Biotin were passivated with BSA and NAVTMR as described in the case of NAVOG coupling. Afterwards, the SLB was incubated with 2 µM (300 µL) Biotin-XX Phalloidin for 40 min at room temperature and thereafter with 35 µg/mL (300 µL) of preformed actin filaments for 20 min at room temperature. Finally, the sample was incubated with 1 µM (300 µL) actin-stain 488 phalloidin for 20 min at room temperature. In between and after the last incubation step, the sample was washed ten times with PBS. Prior to imaging, SLBs were scanned extensively to ensure their homogeneity.

2.3. Image Acquisition

All movies were recorded with the epifluorescence IX73 microscope from Olympus (Olympus Corporation, Tokyo, Japan), equipped with a 60x NA 1.25 objective (Olympus Corporation, 60x Planfluor PH3 UPLFLN60XOIPH/1.25, Tokyo, Japan). All samples were illuminated via the solid-state light source SOLA SE 395 light engine (Lumencor, Beaverton, OR, USA). Appropriate excitation (482/18 (green), 563/9 (red), 563/18 (far red)) and emission filters (525/39 (green), 595/31 (red), 655+ (far red)); all from AHF Analysentechnik AG, Tübingen, Germany) were used to only excite and detect light from the fluorophore of interest. Furthermore, illumination intensities were chosen to observe the bleaching process in a reasonable amount of time (~5–15 min). Illuminated regions of interest were chosen large enough to ensure complete bleaching of diffusing lipids once they reached the center of the bleached area. Each movie consisted of 300 images, corresponding to a measurement time of 5 to 15 min depending on the exposure time. Movies were recorded at room temperature (21 °C) in different locations on each SLB to obtain robust average diffusion constants.

2.4. Measurements of Diffusion Constants

The diffusion constant is determined here using a technique called continuous photo-bleaching (CP) [68]. It is applicable to a wide range of fluorophores since it is not dependent on the instantaneous bleaching of fluorophores like in fluorescent recovery after photo-bleaching (FRAP). In addition, CP has the advantage in determining the diffusion constant over a large measuring area, making the diffusion constant more robust and representative of the whole sample. In CP, the diffusion constant of proteins or lipids is derived from continuously bleaching a region of interest (ROI) on the SLB (see Video S1). To select the ROI, the field diaphragm of the microscope is closed to about 1/3 of the field of view. Thus, fluorescent lipids that enter or are present within the ROI are continuously bleached, causing the intensity at the center to vanish completely. Due to lipid or protein diffusion in the membrane, lipids and proteins located at the edge of the field diaphragm can be replaced by unbleached ones, giving rise to a bright fluorescent signal at the edge of the diaphragm (see Figure 2). This fluorescent signal exponentially decays toward the center of the ROI. At the center, the fluorescence vanishes over long time scales. During the entire process of bleaching and diffusion, a bleaching curve is recorded at the center of the ROI.

This is also referred to as the temporal intensity profile. After reaching a steady state, the spatial intensity profile is recorded and evaluated at a fixed time point.

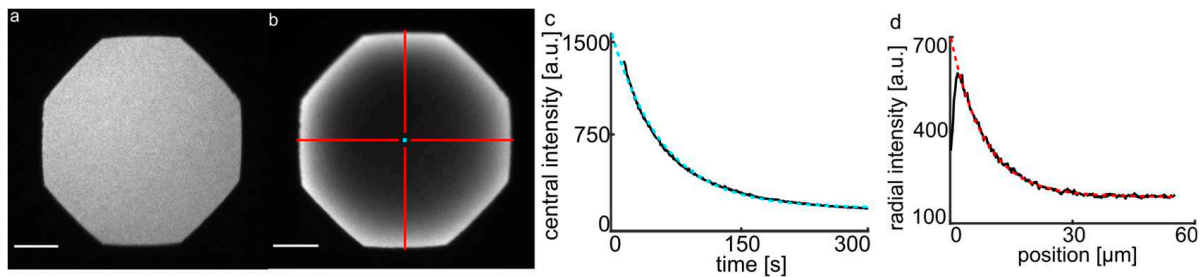


Figure 2. Continuous photobleaching. (a) SLB before bleaching. (b) SLB after 250 s of bleaching. Intensities within the square at the center and along four lines of the ROI were used to determine the temporal and spatial intensity profile, respectively. Scale bar in (a,b) 25 μm. (c) Central intensity profile and fit (dashed line) according to Equation (1). (d) Spatial intensity profile along one direction and fit (dashed line) according to Equation (2).

The temporal intensity profile at the central position hence depends only on the bleaching rate B of the fluorophore [19]:

$$I(t) = I_0 \cdot e^{-Bt} + I_{Bg} \quad (1)$$

Here, I_0 is the intensity at the center of the area at the beginning of the recording, t is the time, and I_{Bg} is the background intensity. The spatial intensity profile from the edge of the ROI toward the center follows a simple exponential function that depends on the bleaching rate and the diffusion constant [19]:

$$I(x) = I_0 \cdot e^{-x(\sqrt{B/D})} + I_{Bg} \quad (2)$$

where x is the spatial coordinate from the edge toward the center. Experimentally, the temporal intensity profile at the center was derived by averaging the intensity over a 5×5 pixel square. The spatial intensity profile was derived from the last acquired frame from 4 lines, which were averaged over a width of 5 pixels. A diffusion constant was determined from the fit to each line and an average value from these 4 lines (see Figure 2) was calculated. Errors are standard deviations of intensities or errors in fit parameters. For an automated evaluation, self-written routines in Matlab (R2018a, The Math Works, Natick, MA, USA) were used.

2.5. Statistical Analyses

Statistical analyses were performed using Matlab (R2018a-R2021a). All samples prepared and measured under identical conditions were combined in one boxplot. A Wilcoxon rank-sum test was used to identify significance levels of diffusion constant changes between samples. Significance levels p are defined as follows: n.s.: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

2.6. Theory

There are various models for describing diffusion in lipid membranes. The first and simplest is the theory of Saffman and Delbrück [54], which describes the diffusion of a lipid in a homogeneous fluid. The theory of Evans and Sackmann [57] further includes frictional effects that occur in the SLBs. Mobile obstacles are taken into account in Saxton's theory [60]. These models are applied to the systems used in this study. A detailed explanation of the theories can be found in the Supplementary Materials.

3. Results and Discussion

3.1. General Characterization of the SLB

In this study, SLBs are chosen as an appropriate model system due to the high control of lipid composition and packing density, along with the easy production process. With SLBs not only can the molecular composition be controlled, but also other important parameters such as the membrane phase, the lipid lateral density, and their biofunctionalization. In addition, SLBs are suitable for various microscopic imaging techniques (TIRF, RICM, FCS, CP, FRAP, etc.) to allow for the quantification of system parameters (e.g., cell–bilayer interactions, as in [48,51]). Furthermore, in SLBs, it is possible to compose the two lipid layers differently, which we used here to minimize the effects of the membrane–substrate interaction. Nevertheless, the limitations of using SLBs must also be recognized. Primarily, this due to the limited lipid or protein mobility, which leads to an underestimation of the diffusion constant compared to free-standing membranes (black-lipid membranes or GUVs) [69–71]. However, our measured diffusion constants compare well to those measured in cell membranes ($0.8\text{--}3.7\ \mu\text{m}^2/\text{s}$ for different membrane tracers), and based on the previously mentioned advantages, SLBs are the most suitable model system for the present study.

Prior to the probing effects of protein–lipid interactions, we determined lipid diffusion in a simplified SLB, consisting only of a matrix lipid and doped with a tracer lipid. We tested whether these measurements compare well with those of other publications and whether the theoretical approximation allows assuming unhindered diffusion in an obstacle-free system. SLBs were fabricated at a final lateral pressure of $25\ \text{mN/m}$, where lipids cover a $63\ \text{\AA}^2$ area per molecule. This value was derived from the measured isotherm, which further verified a homogeneous fluid membrane phase [72]. To verify unconstrained lipid mobility, we first determined the diffusion constant of the fluorescent tracer lipid DOPE-NBD in an otherwise pure SOPC SLB. Here, a diffusion constant of $(2.4 \pm 0.2)\ \mu\text{m}^2/\text{s}$ was obtained ($N = 46$ measurements, $S = 5$ independent samples). This value is, within error margins, identical to the diffusion constant of SOPC measured by Fenz et al. $((2.5 \pm 0.2)\ \mu\text{m}^2/\text{s})$ [19], also using NBD as tracer fluorophore, as well as measured by Horton et al. $((2.3 \pm 0.4)\ \mu\text{m}^2/\text{s})$ [35], using TR-DPPE as a tracer lipid [35]. Since the SLB here consisted only of tracer lipids and SOPC, but no additional components, the Saffman/Delbrück theory is readily applied to theoretically calculate the diffusion constant in an obstacle-free membrane, which amounted to $1.9\ \mu\text{m}^2/\text{s}$. Here, the lipid radius $a = 4.5\ \text{\AA}$ (from the measured lipid area) and the lipid membrane height $h = 4\ \text{nm}$ were used [73]. $\eta = 0.1\ \text{kg}/(\text{m}\cdot\text{s})$, $\eta' = 1\cdot 10^{-3}\ \text{kg}/(\text{m}\cdot\text{s})$, and $\chi = 0.5772$ were taken from the original publication by Saffman and Delbrück [54]. The theoretical model is in high agreement with the experimentally determined tracer diffusion constant, with the experimental values slightly exceeding the theoretical estimate. It should be noted that recent lipid molecule tracking experiments find that an extended Saffman–Delbrück model must be used, when measurements on a single-molecule scale are performed, to correctly describe the membrane hydrodynamics [74]. For our microscale measurements, however, the venerable Saffman/Delbrück theory (Equation (S1)) applies. Hence, our diffusion constants are typical of free lipid diffusion in a membrane [75], corroborating our conclusion of unconstrained diffusion in an obstacle-free system for this membrane configuration.

We then tested whether the position of the lipid fluorophore label has a significant impact on the diffusion constant and found that headgroup-labeled lipids generate fewer friction effects. Using a SLB composition as before, with only the headgroup-labeled DOPE-NBD exchanged with the NBD chain-labeled DOPE (NBD-DOPE), the diffusion constants amounted to $(1.9 \pm 0.1)\ \mu\text{m}^2/\text{s}$ ($N = 9$, $S = 2$) in comparison to $(2.4 \pm 0.2)\ \mu\text{m}^2/\text{s}$ in the case of DOPE-NBD (see Figure S1). Thus, the chain labeling causes a larger friction in the system. For the following experiments, we decided to use headgroup-labeled lipids in order to minimize any friction effects arising from the lipid label. Since the NBD spectrum overlaps with the green fluorescence of the coupled proteins, the lipid DOPE with headgroup-coupled fluorophore Cy5, i.e., DOPE-Cy5, was tested as a tracer molecule in the following

experiments. To ensure comparability throughout the measurements, identical illumination settings were used, and lamp intensities were chosen just high enough to ensure bleaching of all fluorophores once they reached the center of the selected ROI.

3.2. Immobile Obstacles

GFP turned out to be an immobile obstacle using CP (see Figure S2), i.e., it exhibits a diffusion constant that is at least two orders of magnitude slower than lipid diffusion, which is the estimated detection limit of CP. With CP, no bright ring at the edge of the ROI could be detected (see Figure S2), indicating no diffusion. Nevertheless, it is possible that GFP diffuses very slowly, which is not detectable with CP. Other methods have indeed shown that GFP is mobile when coupled to the SLB via a His-Tag, albeit very slowly ($0.026\text{--}0.009\text{ }\mu\text{m}^2/\text{s}$) [23]. Since the lipids will not ‘perceive’ the movement of GFP, given these values, the NTA-GFP system thus served as an example to effectively study immobile obstacles in the context of lipid diffusion in membranes. First, the diffusion constants of tracer lipids were measured in SLBs with linker concentrations of 0 mol%, 0.005 mol%, 0.05 mol%, and 0.5 mol%. Diffusion constants were measured before and after passivation with bovine serum albumin (BSA), a protein used to passivate the bilayer against nonspecific interactions, as well as after GFP coupling, whose His-Tag specifically binds to DOGS-NTA. In all of the latter experiments, GFP was confirmed to be immobile on timescales of lipid diffusion.

Both an increase in the linker concentration and coupling of GFP led to a drop in the diffusion constant of the tracer lipid. Figure 3 summarizes the recorded diffusion constants of the NTA-GFP system. Intriguingly, increasing the linker concentration yielded a systematic decrease of the tracer lipid diffusion constant (see Figure 3). Thus, replacing already five out of one thousand SOPC lipids with DOGS-NTA led to a decrease in the diffusion constant by 34%. Such a decrease in diffusion constant was also observed when a lipid tracer with a chain label was used (see Figure S1). Besides the decrease in lipid mobility with increasing tracer lipid concentration, mere passivation of the control sample with BSA also resulted in a reduced diffusion constant by 18%. Even though BSA simply rests on the membrane and is used to cover up potential defects on the SLB, the hydrodynamic friction at the bilayer-BSA/buffer interface increases. GFP coupling further decreases the lipid diffusion constant on average by 69%.

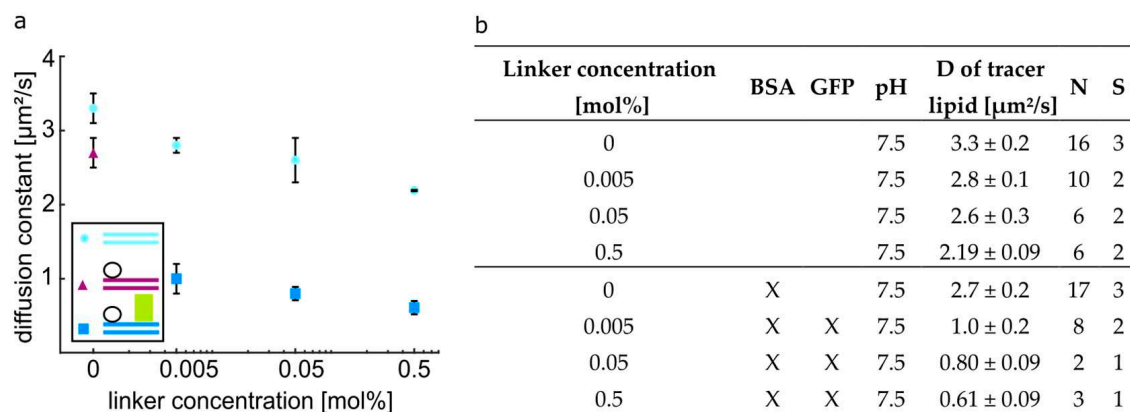


Figure 3. Measured diffusion constants for the NTA-GFP system. DOGS-NTA concentrations of 0 mol%, 0.005 mol%, 0.05 mol%, and 0.5 mol% were used. (a): SLB alone (circle), SLB after BSA passivation (triangle), SLB after BSA passivation, and GFP coupling (box). Data points are median $\pm$ median absolute deviation (MAD). (b): Table of diffusion constants for the NTA-GFP system at pH 7.5. Diffusion constants are measured for different linker concentrations before and after incubation with GFP. N: Number of measurements, S: Number of independent samples, X: Passivation/coupling with the protein. The diffusion constants are measured at pH 7.5 (for changes at pH 6.4, see Figure 4b). Diffusion constants are given as median $\pm$ MAD.

A drastic increase in the friction parameter after GFP coupling indicates a high influence of friction at the protein-membrane interface. For the NTA-GFP system, we hence considered the theoretical model from Evans/Sackmann (Equations (S2) and (S3)) to calculate the friction parameter b (see Figure S3). The Saffman/Dellbrück model is no longer applicable, since an unconstrained diffusion after the introduction of linker lipids and the coupling of GFP no longer exists. The friction coefficients for SLBs doped with DOGS-NTA are in the $0.4 \cdot 10^8$ – $2 \cdot 10^8$ Pa·s/m range and change only slightly with increasing amounts of DOGS-NTA. These friction coefficients are in the same range as values derived by Fenz et al. [19] and Purucker et al. [59] using continuous photobleaching. They are also consistent with friction coefficients obtained using other techniques [58,76]. Interestingly, after GFP-coupling on the lipid head group, the friction coefficients dramatically increased to values $\geq 1 \cdot 10^9$ Pa·s/m. This change by an order of magnitude cannot be explained by an obstacle consisting of lipids alone, since friction coefficients within the membrane would only change slightly in this case. Instead, b appears to be largely influenced by friction at the membrane-protein interface. This is supported by the diffusion constant change of tracer lipids after passivation with BSA, since BSA can only interact with the lipids at the protein-membrane interface. Nevertheless, it is important to keep in mind that the model only considers weak membrane-substrate interactions [57] by an effective friction coefficient and does not consider individual contributions such as hydration forces or surface roughness. Thickness-corrected coverslips are used as the substrate to diminish surface roughness effects.

In addition to friction, charge-dependent effects could cause the immobility of GFP as well as a significant drop in tracer lipid diffusion after GFP coupling. GFP is known to carry a negative surface charge at pH 7.5, which was used in this study [77]. According to Wang et al. [78], such negatively charged molecules can lead to nonspecific interactions between SOPC—the main component of SLBs in this study—and the charged molecule. This is justified by the structure of the lipid SOPC, which exhibits a zwitterionic headgroup whose orientation highly influences lipid mobility: The phosphocholine consists of a positively charged choline group and a negatively charged phosphate group. This headgroup reorients depending on charges in the molecular environment. When the positively charged choline group is facing the surface, the head group is longitudinally oriented, resulting in a higher packing density of the lipids. The lipid is then in the gel phase, which limits its mobility. When the negatively charged phosphate group faces the surface, the head group is kinked. In this case, lipids are less densely packed in a liquid phase and are consequently more mobile [78]. It appears likely that the presence of the negatively charged GFP leads to interactions with the positively charged choline group of SOPC and its transition to a densely packed gel-phase. This is consistent with our observation of a decrease in the lipid diffusion constant.

In the following, it is shown that an increase in pH results in a smaller drop in the diffusion constant of the tracer lipid after GFP coupling. To test if the membrane and GFP mobility changes when the protein effective charge is changed, experiments with the NTA-GFP system at pH 6.4 were performed. This pH was chosen as it is closer to the pH where GFP is neutral (isoelectric point 5.7, calculated with Prot pi, Version 2.2.29.152) while avoiding the pH where histidine fused to the GFP becomes increasingly protonated (pKa of 6) and less likely to bind to Ni(II)-NTA. Figure 4 illustrates the direct comparison of lipid diffusion constants before and after GFP coupling at buffer pH 6.4 (see Figure 4) and 7.5 (see Figure 3).

To test if the change in pH has an effect only when GFP is coupled, we first determined the diffusion constants of the tracer lipid before GFP coupling at pH 6.4. Figure 4 (circles) shows that the tracer mobility perfectly agrees with the values measured at pH 7.5; therefore, in the absence of GFP, no effect arises, as desired. Interestingly, after the coupling of GFP, the diffusion constant of the tracer lipid decreases, but significantly less compared to the measurements at pH 7.5. Specifically, instead of an average decrease of 69% at pH 7.5, diffusion constants decrease by only 31% at pH 6.4. The faster diffusion of the tracer lipid

can be attributed to the following: The charge-dependent interaction between GFP and the SOPC headgroup is diminished; therefore, lipid headgroups remain in the kinked orientation, keeping the lipid in the liquid phase. A second indication for the charge dependent interactions is that at pH 6.4, the more neutral GFP leads to only half as many charge-dependent interactions as the more negatively charged GFP at pH 7.5 (see Figure S4). The mobility of GFP itself, however, was not affected by this change, and GFP remained immobile at pH 7.5 and pH 6.4. Of note, BSA is also more negatively charged at pH 7.5 than at pH 6.4 [79]; therefore, BSA charge effects may also contribute to the higher diffusion constant at pH 6.4

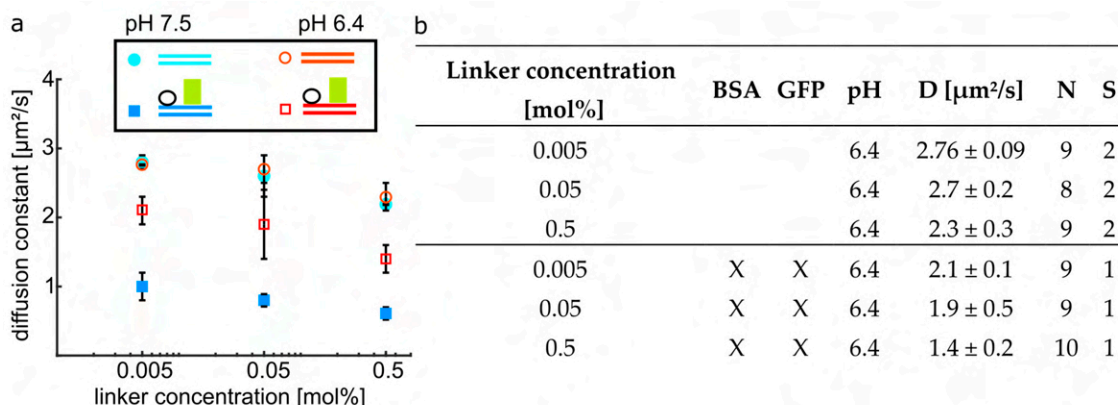


Figure 4. Diffusion constants for the NTA-GFP system. DOGS-NTA concentrations of 0.005 mol%, 0.05 mol%, and 0.5 mol% were used. (a): pH of the buffer: 7.5 or 6.4. SLB alone (filled circles (pH 7.5) or unfilled circles (pH 6.4)) and after BSA passivation and GFP coupling (filled box (pH 7.5) or unfilled box (pH 6.4)). Data points are median $\pm$ MAD. (b): Table of diffusion constants for the NTA-GFP system at pH 6.4 (for changes at pH 7.5 see Figure 3b). N: Number of measurements, S: Number of independent samples, X: Passivation/coupling with the protein. Diffusion constants are measured for different linker concentrations before and after incubation with GFP. Diffusion constants are given as median $\pm$ MAD.

3.3. Mobile Obstacles

As an example of a mobile obstacle, the protein NAVOG was investigated. NAVOG, together with the SLB containing SOPC, DOPE-Cy5, and DOPE-Cap-Biotin lipids, form the Biotin-Neutravidin system. In this case, in addition to the diffusion constants of the tracer lipid, the diffusion constants of NAVOG were determined (see Figure 5).

Both the diffusion constant of the tracer lipid before and after protein coupling and the diffusion constant of NAVOG exhibited a decrease with increasing DOPE-Cap-Biotin concentration. Qualitatively, these results are in accordance with the study by Vaz et al. [80], who report the direct dependency of the lateral diffusion of lipids and proteins on the protein concentration in the membrane. Hence, the presence of the linker at the highest concentration in comparison to the linker-lipid-free membrane led to a drop in the diffusion constant of the tracer lipid by 22%. Coupling of NAVOG resulted in an additional average decrease in the tracer lipid mobility by 31%.

As for the NTA-GFP system, the tracer lipid diffusion here also exhibits a linker concentration dependency, which strongly decreases once the protein is coupled. For NAVOG, a nearly linear dependency between linker and protein concentration can be observed (see Figure S6). Therefore, the diffusion constant of the tracer lipid decreases with the amount of bound protein. This suggests a change in friction that occurs in a similar manner, independent of the system. Interestingly, NAVOG coupling resulted in the same average breakdown as the coupling of GFP at pH 6.4 (31%), corresponding to a comparable effective friction acting on the membrane lipids. The similar drop in membrane mobility may be attributed to a similar membrane-surface interaction of the proteins, albeit some differences can arise from the different protein sizes (28 nm² for NAVOG; 5 nm² for GFP).

In this comparison, both proteins should be only slightly negatively charged (NAVOG at pH 7.5 with pI 6.3 [81]; GFP at pH 6.4 with pI 5.7), which should result in only a slight distortion of the matrix lipids (SOPC) into the gel phase. NAVOG turned out to be a mobile obstacle for all the recorded conditions, with measured diffusion constants reported in Figure 5. For linker concentrations of 0.025 mol% and 0.005 mol%, the number of binding sites and, hence, the number of NAVOG were too small to have a measurable intensity gradient with continuous photobleaching; therefore, no NAVOG diffusion constant could be determined. On average, the diffusion constant of the protein NAVOG was 66% lower than the diffusion constant of the lipid with bound NAVOG.

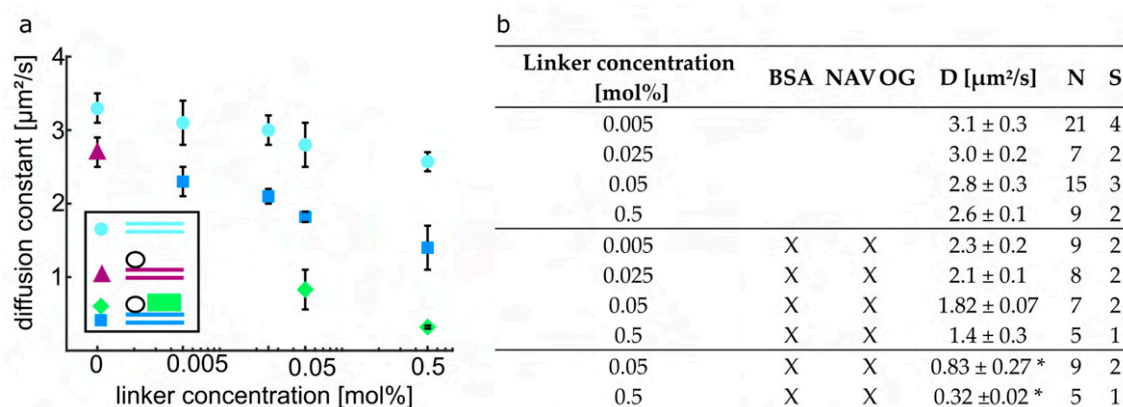


Figure 5. Measured diffusion constants for the Biotin–Neutravidin system. DOPE–Cap–Biotin concentrations of 0 mol%, 0.005 mol%, 0.025 mol%, 0.05 mol%, and 0.5 mol% were used. The diffusion constants are measured at pH 7.5. (a): SLB alone (circle), SLB after BSA passivation (triangle), SLB after BSA passivation and NAVOG coupling (box), and NAVOG diffusion constant (diamond). Data points are median $\pm$ MAD. (b): Diffusion constants are measured for different linker concentrations before and after incubation with NAVOG. N: Number of measurements, S: Number of independent samples, X: Passivation/coupling with the protein. Diffusion constants are given as median $\pm$ MAD. * D of protein NAVOG (not the tracer lipid).

As for the NTA–GFP system, the friction parameter b was calculated for the Biotin–Neutravidin system from Equations (S2) and (S3) (see Figure S5). Here, the membrane surface viscosity, $\eta_m = 0.16 \cdot 10^{-9}$ Pa·s/m, from Fenz et al. [19] and the hydrodynamic radius of Neutravidin, $a = 3$ nm, were used. The latter value is based on a theoretical calculation of protein diameters from PDB files according to the HydroPro algorithm [82]. For both the lipid and the protein, friction coefficients increased with increasing DOPE–Cap–biotin concentration. Friction coefficients for the tracer lipid alone were in the range of $1 \cdot 10^8$ Pa·s/m, which is similar to those in the NTA–GFP system and on the same order of magnitude as reported by Fenz et al. [19]. After NAVOG coupling, the values strongly increased from $4 \cdot 10^7$ Pa·s/m to $6 \cdot 10^8$ Pa·s/m with increasing linker density. Here, the effective friction is significantly smaller in comparison to the friction generated by GFP coupling in the NTA–GFP system at pH 7.5, i.e., $>10^9$ Pa·s/m.

All absolute diffusion constants of the tracer lipids measured in this study are in the range of $\sim 1 \mu\text{m}^2/\text{s}$. Previous studies in the adhesion zone of cells have shown that diffusion constants are of the same order of magnitude [2]. This demonstrates that the SLB model system is well suited to mimic diffusion processes in cells close to an interface.

Membrane curvature was also shown to influence diffusion. For example, Kumar et al. [32] showed in MD simulations that the diffusion constant drops with increasing membrane curvature ($1/\text{radius} > 0.8 \text{ nm}^{-1}$). However, this only holds for high curvatures, whereas small curvatures do not cause this effect ($1/\text{radius} < 0.01 \text{ nm}^{-1}$) [33]. Since the curvature of the cell membrane in adherent regions is negligibly small (roughness around 20 nm [83]), as it is the case for the planar SLB on the substrate, any curvature effect in this study should be negligible. It should be kept in mind, though, that in case of

non-adherent cells or vesicle model systems, diffusion constants can be altered at high membrane curvature.

For the following study, where the influence of cytoskeleton filament coupling to the membrane is probed, and in order to enable a dense filament network formation, even higher linker concentrations, up to 10 mol%, were used. Additionally, in the case of higher linker concentrations, a declining trend in the diffusion constant is obtained. Figure 6 shows the change in the diffusion constant as a function of linker concentration for the tracer before and after coupling of NAVTMR.

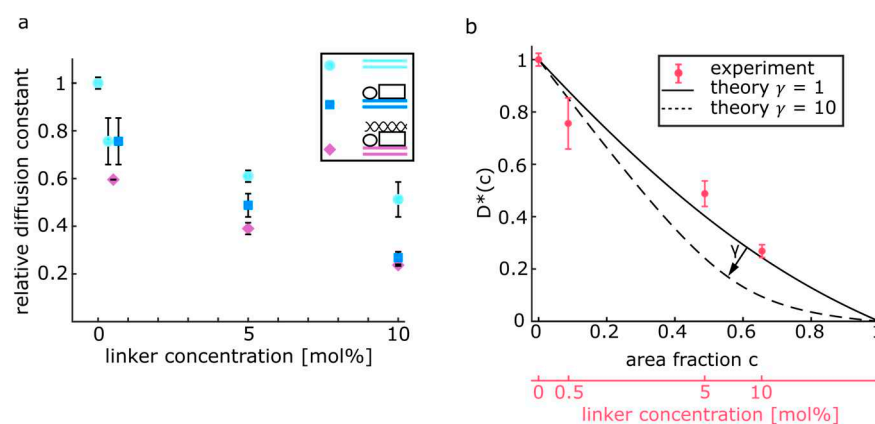


Figure 6. Effect of higher linker concentration on diffusion constants in the Biotin–Neutravidin system. (a) Change in diffusion constant relative to SLB alone (circle) for the SLB after NAVTMR coupling (box) and the SLB after NAVTMR and actin coupling (diamond). Data points are median $\pm$ MAD. (b) Comparison of experiment (Biotin–Neutravidin system) with the theory of Saxton for mobile obstacles with $\gamma = 1$ and $\alpha = 0.2820$ (solid line) or $\gamma = 10$ and $\alpha = 0.2820$ (dashed line). Fit of Saxton's theory to the experimental data yields the effective obstacle size $a_0 = 12 \text{ nm}^2$, corresponding to 19 lipids, $\gamma = 1$, and $\alpha = 0.2820$. Data points are median $\pm$ MAD.

Here, NAVTMR is used instead of NAVOG to allow for the subsequent imaging of green-labeled actin (see Section 3.4). Since the diffusion constant exhibited a dependency on the initial SLB fabrication pressure (see Figure S7), we plot the relative diffusion constant here. To this end, we corrected the initial SLB fabrication pressure of the Biotin–Neutravidin–Actin system (12 mN/m) for the initial pressure used in the Biotin–NAVOG system (8 mN/m) and normalized the diffusion constants to the value of the linker-free SLB. For all experimental conditions (SLB, after NAVTMR coupling, and after actin filament coupling), a decrease in membrane mobility was observed when the linker concentration in the SLB was increased. At 10 mol% linker concentration, the diffusion constant dropped by 49% compared to the linker-free case. After NAVTMR coupling, the lipid diffusion constant decreased, in the case of 5 mol% and 10 mol% linker concentrations, by 20% to 48%, respectively, in comparison to the SLB without NAVTMR.

For higher linker concentrations, we derived a measure of the effective obstacle size of $a_0 = 12 \text{ nm}^2$ from Saxton's theory for the case of mobile obstacles. To this end, a triangular lattice was used as grid model (as stated in the SI section “1.3 Friction due to mobile obstacles”). From the data, the jump rate γ was extrapolated for negligible c to $\gamma = 1$, where jump rates of tracers and obstacles are identical. Figure 6 shows the comparison of experimental data with Saxton's theory (Equations (S4) and (S5)) for different jump rates $\gamma = 1$ and 10. We then used Saxton's theory as a fit function and set a_0 as a free parameter to determine the effective size of the lipid complex. The best fit was found for an effective obstacle size of $a_0 = 12 \text{ nm}^2$, which was less than $\frac{1}{2}$ of the area the protein covers on the lipid membrane ($5.6 \text{ nm} \cdot 5 \text{ nm} = 28 \text{ nm}^2$ [84]). Since this effective obstacle size corresponds to 19 lipids that are restricted in mobility, the theory suggests that next to the two linker lipids bound to the protein, several neighboring lipids in the membrane plane are part of the obstacle. Interestingly, Saxton's theory accurately represents the experimentally

determined values at concentrations >0.5 mol%, but it underestimates the decrease in tracer lipid mobility in the 0.005 mol% to 0.5 mol% linker lipid regime. Since deviations could not be explained by a higher γ or a larger effective obstacle size, an extended theoretical framework would be desirable that correctly describes the diffusion dynamics in the low obstacle concentration regime as well as for the limit of immobile obstacles.

3.4. Filament to Mobile Obstacle Coupling

We then studied the influence of cytoskeleton filament coupling on the mobility of the membrane, since the anchoring of actin filaments to SLBs was recently shown to sort and phase-separate lipids in the membrane—in a similar manner as reported here, but using the highly different ternary lipid composition consisting of a saturated phospholipid, an unsaturated phospholipid, and cholesterol [85]. In the Biotin–Neutravidin–Actin system of this study (see Figure 1), phalloidin-biotin was coupled to Neutravidin on the SLB, and, thereafter, actin filaments were bound to the Phalloidin-protein complexes. Due to the substantial actin filament length of ~ 1 – 10 μm and the high phalloidin abundance on the substrate, actin filaments could crosslink several of the mobile Neutravidin obstacles. Since coupling effects were determined to become significant at linker concentrations ≥ 0.5 mol%, linker concentrations up to 10 mol% were used. A representative image of actin filaments bound to the SLB is shown in Figure 7. Note that in this system, NAVOG was replaced by NAVTMR to avoid spectral overlap with the actin-stain 488 phalloidin.

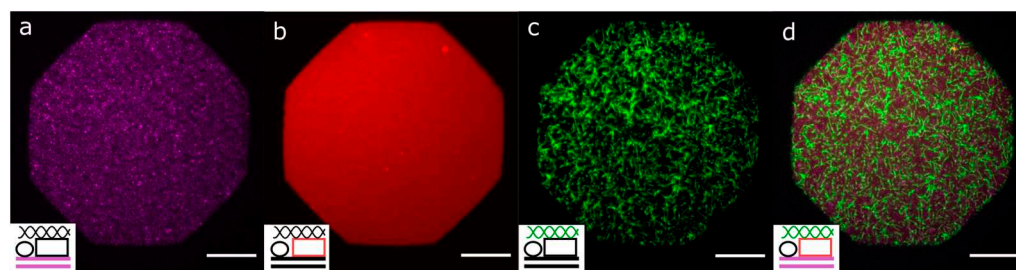


Figure 7. Effect of actin-coupling on diffusion constants in the Biotin–Neutravidin system. Representative images of the SLB tracer lipid (purple) (a), of coupled NAVTMR (red) (b), and actin (green) (c) after the last actin coupling step at 10 mol% linker concentration. (d) Overlay of (a–c). Scale bar in (a–d) 25 μm .

Interestingly, actin filament coupling decreased lipid mobility (see Figure 6). In the case of 0.5 mol%, 5 mol%, and 10 mol% linker concentration, the additional diffusion constant drops amounted to 22%, 20%, and 11%, respectively. The 22% drop at 0.5 mol% suggests that NAVTMR is crosslinked via actin filaments (length of ~ 1 – 10 μm) and creates obstacles of larger effective size that significantly affect the tracer lipid mobility. At 5 mol% and 10 mol% linker concentrations, the diffusion constants decrease less with actin filament crosslinking. This appears to result from the a priori high density of protein–lipid obstacles, such that the actin filament crosslinking, resulting in a larger obstacle size, is barely detectable. Overall, actin coupling created lipid–protein complexes, yielding a higher effective friction for tracer lipids within the SLB. A rough estimate of this obstacle size can be made using the theory of Petrov and Schwille [56] (Figure S8). Intriguingly, in addition to detecting changes in lipid diffusion constants, a significant change in the apparent SLB homogeneity occurred after NAVTMR and actin coupling: The SLB appeared homogeneous when NAVTMR alone was present or when actin alone without NAVTMR was incubated on the sample (see control measurements in Figure S9). However, after the actin coupling, the SLB exhibited a granular appearance. This finding matches previous studies [85] in which lipid phase separation in the presence of actin filaments was observed. In the cited case, linker lipids would colocalize with actin structures, whereas matrix lipids were expelled from these regions. However, the ternary lipid–cholesterol mixture used in the cited work is well-known to phase separate at particular temperatures and is therefore highly different from

the lipid composition used in this study. Since the control measurement (see Figure S9) excludes any nonspecific interaction of actin with the membrane and since DOPE-Cy5 did not colocalize with actin filaments, we reasoned that the membrane texture arises from the steric expulsion of tracer lipids from the locations of protein–lipid complexes. This observation is of interest for more comprehensive study in a subsequent work.

4. Conclusions

Here, we investigated the effects of immobile and mobile obstacles on lipid mobility in supported bilayer systems using continuous photobleaching. GFP coupled to DOGS-NTA lipids served as an immobile obstacle system and exhibited a 69% decrease in the lipid tracer diffusion constant in comparison to the obstacle-free system. Part of this breakdown could be attributed to SOPC–GFP interactions, where the negative GFP charge would switch SOPC from the liquid into the more densely packed gel phase. Repeated experiments at a pH where GFP is neutral (but still immobile) resulted in a smaller 31% decrease in diffusion constants. Neutravidin coupled to DOPE-cap-Biotin lipids in the bio-membrane served as a mobile obstacle system. Interestingly, the coupling of Neutravidin to the bilayer resulted in the same average decrease of the tracer lipid diffusion constant as for GFP at the pH where it is neutral, suggesting a substantial friction effect at the membrane–protein interface. Additional coupling of actin filaments to Neutravidin at high linker concentrations was used as a potential crosslinker of obstacles and led to a maximum 53% breakdown of tracer lipid mobility in comparison to the obstacle free case. The decrease in lipid mobility is well described by the theory of Saxton at high linker lipid concentrations. Here, the obstacle consisted of 19 membrane lipids associating with the initially formed lipid–protein complex. This study highlights the dependency of lipid membrane mobility on linker lipid concentration as well as on the concentration, charge, and type of the coupled protein. We determined diffusion constants and friction parameters for the systems. We further found that complex lipid–protein obstacles consisting of the linker lipid and Neutravidin crosslinked by actin filaments can give rise to a visible change in membrane texture. This comprehensive study of mobility changes in biological membranes provides new insights into our present understanding of membrane mobility [1,2,15,16] and may further be harnessed in semisynthetic systems or cell studies to create well-defined biomaterials with tunable properties [7–9].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/colloids8050054/s1>. Theory, Figure S1: Fatty acid labeled tracer; Figure S2: SLB with bound protein after bleaching; Figure S3: Calculated friction parameter for the NTA–GFP system; Figure S4: GFP intensity changes at 0 mol% linker concentration, as a function of pH; Figure S5: Calculated friction parameter for the Biotin–Neutravidin system; Figure S6: NAVOG intensity change, as a function of linker concentration of the Biotin–Neutravidin system; Figure S7: Calibration data for initial pressure correction for the Biotin–Neutravidin–Actin system; Figure S8: Diffusion constant as a function of obstacle radius; Figure S9: Representative images of the SLB before and after coupling of proteins; Supplementary references; Video S1: Continuous Photobleaching. Additional References are provided in the supporting information [86–88].

Author Contributions: The manuscript was written through contributions of all authors. Conceptualization: C.M.; Methodology: L.C.; Software: L.C. and D.A.K.; Validation: L.C. and C.M.; Formal analysis: L.C. and D.A.K.; Investigation: L.C., D.A.K., A.N. and C.M.; Resources: C.M.; Data Curation: L.C. and D.A.K.; Writing—Original Draft: L.C. and C.M.; Writing—Review and Editing: L.C., C.M., D.A.K. and A.N.; Visualization: L.C.; Supervision: C.M.; Project Administration: C.M.; Funding Acquisition: C.M. All authors have read and agreed to the published version of the manuscript.

Funding: C.M. acknowledges financial support by the DFG Collaborative Research Center 1208 “Identity and dynamics of biological membranes” (project ID 267205415), the DFG Collaborative Research Center 1535 “MiBiNet” (project ID 458090666), Volkswagen Foundation “Freigeist Fellowship”, and Fonds der Chemischen Industrie. We acknowledge the DFG and the state NRW for establishing the Abberior STED microscope (at the Molecular Physical Chemistry, HHU) with the funding ID (INST 208/741-1 FUGG) within the Major Research Instrumentation program as per Art. 91b GG.

Data Availability Statement: All data supporting the conclusions are included in this article. Any raw data will be made available by the authors upon request.

Acknowledgments: We thank the Institute of Physical Chemistry at HHU for providing the Langmuir-Blodgett trough. We thank Martin Schramm (Molecular Physical Chemistry, HHU) for upgrading the Langmuir-Blodgett trough by a motorized sample holder.

Conflicts of Interest: The authors declare no conflicts of interests.

References

- Bolmatov, D.; Soloviov, D.; Zhernenkov, M.; Zav'Yalov, D.; Mamontov, E.; Suvorov, A.; Cai, Y.Q.; Katsaras, J. Molecular Picture of the Transient Nature of Lipid Rafts. *Langmuir* **2020**, *36*, 4887–4896. [[CrossRef](#)] [[PubMed](#)]
- Kleusch, C.; Monzel, C.; Sridhar, K.C.; Hoffmann, B.; Csiszár, A.; Merkel, R. Fluorescence Correlation Spectroscopy Reveals Interaction of Some Microdomain-Associated Lipids with Cellular Focal Adhesion Sites. *Int. J. Mol. Sci.* **2020**, *21*, 8149. [[CrossRef](#)] [[PubMed](#)]
- Burk, A.S.; Monzel, C.; Yoshikawa, H.Y.; Wuchter, P.; Saffrich, R.; Eckstein, V.; Tanaka, M.; Ho, A.D. Quantifying Adhesion Mechanisms and Dynamics of Human Hematopoietic Stem and Progenitor Cells. *Sci. Rep.* **2015**, *5*, 9370. [[CrossRef](#)]
- Knight, J.D.; Lerner, M.G.; Marciano-Velázquez, J.G.; Pastor, R.W.; Falke, J.J. Single Molecule Diffusion of Membrane-Bound Proteins: Window into Lipid Contacts and Bilayer Dynamics. *Biophys. J.* **2010**, *99*, 2879. [[CrossRef](#)] [[PubMed](#)]
- Stieger, B.; Steiger, J.; Locher, K.P. Membrane Lipids and Transporter Function. *Biochim. Biophys. Acta (BBA)—Mol. Basis Dis.* **2021**, *1867*, 166079. [[CrossRef](#)]
- Cheng, X.; Ullo, M.F.; Case, L.B. Reconstitution of Phase-Separated Signaling Clusters and Actin Polymerization on Supported Lipid Bilayers. *Front. Cell Dev. Biol.* **2022**, *10*, 932483. [[CrossRef](#)]
- Banger, A.; Pasch, P.; Blawitzki, L.C.; Weber, S.; Otten, M.; Monzel, C.; Schmidt, S.; Voskuhl, J.; Hartmann, L. Detection of Lectin Clustering in Self-Assembled, Glycan-Functionalized Amphiphiles by Aggregation-Induced Emission Luminophores. *Macromol. Chem. Phys.* **2023**, *224*, 2200314. [[CrossRef](#)]
- Urban, P.; Pritzl, S.D.; Ober, M.F.; Dirscherl, C.F.; Pernpeintner, C.; Konrad, D.B.; Frank, J.A.; Trauner, D.; Nickel, B.; Lohmueller, T. A Lipid Photoswitch Controls Fluidity in Supported Bilayer Membranes. *Langmuir* **2020**, *36*, 2629–2634. [[CrossRef](#)]
- Ulmefors, H.; Nissa, J.; Pace, H.; Wahlsten, O.; Gunnarsson, A.; Simon, D.T.; Berggren, M.; Höök, F. Formation of Supported Lipid Bilayers Derived from Vesicles of Various Compositional Complexity on Conducting Polymer/Silica Substrates. *Langmuir* **2021**, *37*, 5494–5505. [[CrossRef](#)]
- Tolentino, T.P.; Wu, J.; Zarnitsyna, V.I.; Fang, Y.; Dustin, M.L.; Zhu, C. Measuring Diffusion and Binding Kinetics by Contact Area FRAP. *Biophys. J.* **2008**, *95*, 920–930. [[CrossRef](#)]
- Zhu, D.M.; Dustin, M.L.; Cairo, C.W.; Golan, D.E. Analysis of Two-Dimensional Dissociation Constant of Laterally Mobile Cell Adhesion Molecules. *Biophys. J.* **2007**, *92*, 1022–1034. [[CrossRef](#)] [[PubMed](#)]
- Kure, J.L.; Karlsson, T.; Andersen, C.B.; Lagerholm, B.C.; Loitto, V.; Magnusson, K.E.; Arnspang, E.C. Using KICS to Reveal Changed Membrane Diffusion of AQP-9 Treated with Drugs. *Membranes* **2021**, *11*, 568. [[CrossRef](#)] [[PubMed](#)]
- Kusumi, A.; Suzuki, K. Toward Understanding the Dynamics of Membrane-Raft-Based Molecular Interactions. *Biochim. Biophys. Acta Mol. Cell Res.* **2005**, *1746*, 234–251. [[CrossRef](#)] [[PubMed](#)]
- Kusumi, A.; Nakada, C.; Ritchie, K.; Murase, K.; Suzuki, K.; Murakoshi, H.; Kasai, R.S.; Kondo, J.; Fujiwara, T. Paradigm Shift of the Plasma Membrane Concept from the Two-Dimensional Continuum Fluid to the Partitioned Fluid: High-Speed Single-Molecule Tracking of Membrane Molecules. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 351–378. [[CrossRef](#)] [[PubMed](#)]
- Lommerse, P.H.M.; Spaink, H.P.; Schmidt, T. In Vivo Plasma Membrane Organization: Results of Biophysical Approaches. *Biochim. Biophys. Acta Biomembr.* **2004**, *1664*, 119–131. [[CrossRef](#)] [[PubMed](#)]
- Adkins, E.M.; Samuvel, D.J.; Fog, J.U.; Eriksen, J.; Jayanthi, L.D.; Vaegter, C.B.; Ramamoorthy, S.; Gether, U. Membrane Mobility and Microdomain Association of the Dopamine Transporter Studied with Fluorescence Correlation Spectroscopy and Fluorescence Recovery after Photobleaching. *Biochemistry* **2007**, *46*, 10484–10497. [[CrossRef](#)] [[PubMed](#)]
- Dietrich, C.; Yang, B.; Fujiwara, T.; Kusumi, A.; Jacobson, K. Relationship of Lipid Rafts to Transient Confinement Zones Detected by Single Particle Tracking. *Biophys. J.* **2002**, *82*, 274–284. [[CrossRef](#)]
- Jacobson, K.; Mouritsen, O.G.; Anderson, R.G.W. Lipid Rafts: At a Crossroad between Cell Biology and Physics. *Nat. Cell Biol.* **2007**, *9*, 7–14. [[CrossRef](#)]
- Fenz, S.F.; Merkel, R.; Sengupta, K. Diffusion and Intermembrane Distance: Case Study of Avidin and E-Cadherin Mediated Adhesion. *Langmuir* **2009**, *25*, 1074–1085. [[CrossRef](#)]
- Mossman, K.; Groves, J. Micropatterned Supported Membranes as Tools for Quantitative Studies of the Immunological Synapse. *Chem. Soc. Rev.* **2007**, *36*, 46–54. [[CrossRef](#)]
- Sackmann, E. Supported Membranes: Scientific and Practical Applications. *Science (1979)* **1996**, *271*, 43–48. [[CrossRef](#)] [[PubMed](#)]
- Yamazaki, V.; Sirenko, O.; Schafer, R.J.; Groves, J.T. Lipid Mobility and Molecular Binding in Fluid Lipid Membranes. *J. Am. Chem. Soc.* **2005**, *127*, 2826–2827. [[CrossRef](#)] [[PubMed](#)]
- Iriarte-Alonso, M.A.; Bittner, A.M.; Chiantia, S. Influenza A Virus Hemagglutinin Prevents Extensive Membrane Damage upon Dehydration. *BBA Adv.* **2022**, *2*, 100048. [[CrossRef](#)] [[PubMed](#)]

24. Baumgart, T.; Hess, S.T.; Webb, W.W. Imaging Coexisting Fluid Domains in Biomembrane Models Coupling Curvature and Line Tension. *Nature* **2003**, *425*, 821–824. [[CrossRef](#)] [[PubMed](#)]
25. Limozin, L.; Sackmann, E. Polymorphism of Cross-Linked Actin Networks in Giant Vesicles. *Phys. Rev. Lett.* **2002**, *89*, 168103. [[CrossRef](#)]
26. Smith, A.S.; Sengupta, K.; Goennenwein, S.; Seifert, U.; Sackmann, E. Force-Induced Growth of Adhesion Domains Is Controlled by Receptor Mobility. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 6906–6911. [[CrossRef](#)]
27. Fenz, S.F.; Sengupta, K. Giant Vesicles as Cell Models. *Integr. Biol.* **2012**, *4*, 982–995. [[CrossRef](#)]
28. Dimova, R.; Aranda, S.; Bezlyepkina, N.; Nikolov, V.; Riske, K.A.; Lipowsky, R. A Practical Guide to Giant Vesicles. Probing the Membrane Nanoregime via Optical. *J. Phys. Condens. Matter* **2006**, *18*, S1151. [[CrossRef](#)]
29. Monzel, C.; Schmidt, D.; Kleusch, C.; Kirchenb  chler, D.; Seifert, U.; Smith, A.S.; Sengupta, K.; Merkel, R. Measuring Fast Stochastic Displacements of Bio-Membranes with Dynamic Optical Displacement Spectroscopy. *Nat. Commun.* **2015**, *6*, 8162. [[CrossRef](#)]
30. Monzel, C.; Sengupta, K. Measuring Shape Fluctuations in Biological Membranes. *J. Phys. D Appl. Phys.* **2016**, *49*, 243002. [[CrossRef](#)]
31. Drabik, D.; Drab, M.; Peni  , S.; Igli  , A.; Czogalla, A. Investigation of Nano- and Microdomains Formed by Ceramide 1 Phosphate in Lipid Bilayers. *Sci. Rep.* **2023**, *13*, 18570. [[CrossRef](#)] [[PubMed](#)]
32. Kumar, J.; Chng, C.P.; Huang, C. Curvature-Enhanced Membrane Asymmetry Slows down Protein Diffusion. *Extrem. Mech. Lett.* **2024**, *66*, 102114. [[CrossRef](#)]
33. Urban  i  , I.; Brun, J.; Shrestha, D.; Waithe, D.; Eggeling, C.; Chojnacki, J. Lipid Composition but Not Curvature Is a Determinant of a Low Molecular Mobility within HIV-1 Lipid Envelope. *bioRxiv* **2018**, 315168. [[CrossRef](#)]
34. Domanov, Y.A.; Aimon, S.; Toombes, G.E.S.; Renner, M.; Quemeneur, F.; Triller, A.; Turner, M.S.; Bassereau, P. Mobility in Geometrically Confined Membranes. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 12605–12610. [[CrossRef](#)] [[PubMed](#)]
35. Horton, M.R.; Reich, C.; Gast, A.P.; R  dler, J.O.; Nickel, B. Structure and Dynamics of Crystalline Protein Layers Bound to Supported Lipid Bilayers. *Langmuir* **2007**, *23*, 6263–6269. [[CrossRef](#)]
36. Monzel, C.; Schmidt, D.; Seifert, U.; Smith, A.S.; Merkel, R.; Sengupta, K. Nanometric Thermal Fluctuations of Weakly Confined Biomembranes Measured with Microsecond Time-Resolution. *Soft Matter* **2016**, *12*, 4755–4768. [[CrossRef](#)]
37. Monzel, C.; Fenz, S.F.; Giesen, M.; Merkel, R.; Sengupta, K. Mapping Fluctuations in Biomembranes Adhered to Micropatterns. *Soft Matter* **2012**, *8*, 6128–6138. [[CrossRef](#)]
38. Fenz, S.F.; Merkel, R.; Sengupta, K. Inter-Membrane Adhesion Mediated by Mobile Linkers: Effect of Receptor Shortage. *Biophys. J.* **2010**, *98*, 665a. [[CrossRef](#)]
39. Fenz, S.F.; Bihr, T.; Schmidt, D.; Merkel, R.; Seifert, U.; Sengupta, K.; Smith, A.S. Membrane Fluctuations Mediate Lateral Interaction between Cadherin Bonds. *Nat. Phys.* **2017**, *13*, 906–913. [[CrossRef](#)]
40. Groves, J.T.; Ulman, N.; Cremer, P.S.; Boxer, S.G. Substrate-Membrane Interactions: Mechanisms for Imposing Patterns on a Fluid Bilayer Membrane. *Langmuir* **1998**, *14*, 3347–3350. [[CrossRef](#)]
41. Saarikangas, J.; Zhao, H.; Lappalainen, P. Regulation of the Actin Cytoskeleton-Plasma Membrane Interplay by Phosphoinositides. *Physiol. Rev.* **2010**, *90*, 259–289. [[CrossRef](#)] [[PubMed](#)]
42. Mangeat, P.; Roy, C.; Martin, M. ERM Proteins in Cell Adhesion and Membrane Dynamics. *Trends Cell Biol.* **1999**, *9*, 187–192. [[CrossRef](#)] [[PubMed](#)]
43. Janmey, P.A.; Lindberg, U. Cytoskeletal Regulation: Rich in Lipids. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 658–666. [[CrossRef](#)] [[PubMed](#)]
44. Janmey, P.A. The Cytoskeleton and Cell Signaling: Component Localization and Mechanical Coupling. *Physiol. Rev.* **1998**, *78*, 763–781. [[CrossRef](#)]
45. Deverall, M.A.; Gindl, E.; Sinner, E.K.; Besir, H.; Ruehe, J.; Saxton, M.J.; Naumann, C.A. Membrane Lateral Mobility Obstructed by Polymer-Tethered Lipids Studied at the Single Molecule Level. *Biophys. J.* **2005**, *88*, 1875–1886. [[CrossRef](#)]
46. Lin, W.C.; Blanchette, C.D.; Ratto, T.V.; Longo, M.L. Lipid Asymmetry in DLPC/DSPC-Supported Lipid Bilayers: A Combined AFM and Fluorescence Microscopy Study. *Biophys. J.* **2006**, *90*, 228–237. [[CrossRef](#)]
47. G  lc  ler Balta, G.S.; Monzel, C.; Kleber, S.; Beaudouin, J.; Balta, E.; Kaundl, T.; Chen, S.; Gao, L.; Thiemann, M.; Wirtz, C.R.; et al. 3D Cellular Architecture Modulates Tyrosine Kinase Activity, Thereby Switching CD95-Mediated Apoptosis to Survival. *Cell Rep.* **2019**, *29*, 2295–2306.e6. [[CrossRef](#)]
48. Grakoui, A.; Bromley, S.K.; Sumen, C.; Davis, M.M.; Shaw, A.S.; Allen, P.M.; Dustin, M.L. The Immunological Synapse: A Molecular Machine Controlling T Cell Activation. *Science (1979)* **1999**, *285*, 221–227. [[CrossRef](#)]
49. Levental, I.; Lyman, E. Regulation of Membrane Protein Structure and Function by Their Lipid Nano-Environment. *Nat. Rev. Mol. Cell Biol.* **2023**, *24*, 107–122. [[CrossRef](#)]
50. Budin, I.; de Rond, T.; Chen, Y.; Chan, L.J.G.; Petzold, C.J.; Keasling, J.D. Viscous Control of Cellular Respiration by Membrane Lipid Composition. *Science (1979)* **2018**, *362*, 1186–1189. [[CrossRef](#)]
51. Gudipati, V.; Rydzek, J.; Doel-Perez, I.; Gon  alves, V.D.R.; Scharf, L.; K  nigsberger, S.; Lobner, E.; Kunert, R.; Einsele, H.; Stockinger, H.; et al. Inefficient CAR-Proximal Signaling Blunts Antigen Sensitivity. *Nat. Immunol.* **2020**, *21*, 848–856. [[CrossRef](#)] [[PubMed](#)]

52. Okamoto, Y.; Motegi, T.; Morita, K.; Takagi, T.; Amii, H.; Kanamori, T.; Sonoyama, M.; Tero, R. Lateral Diffusion and Molecular Interaction in a Bilayer Membrane Consisting of Partially Fluorinated Phospholipids. *Langmuir* **2016**, *32*, 10712–10718. [CrossRef] [PubMed]
53. Nissen, J.; Gritsch, S.; Wiegand, G.; Rädler, J.O. Wetting of Phospholipid Membranes on Hydrophilic Surfaces—Concepts towards Self-Healing Membranes. *Eur. Phys. J. B* **1999**, *10*, 335–344. [CrossRef]
54. Saffman, P.G.; Delbrueck, M. Brownian Motion in Biological Membranes. *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 3111–3113. [CrossRef]
55. Axelrod, D.; Koppel, D.E.; Schlessinger, J.; Elson, E.; Webb, W.W. Mobility Measurement by Analysis of Fluorescence Photobleaching Recovery Kinetics. *Biophys. J.* **1976**, *16*, 1055–1069. [CrossRef]
56. Petrov, E.P.; Schwille, P. Translational Diffusion in Lipid Membranes beyond the Saffman-Delbrück Approximation. *Biophys. J.* **2008**, *94*, L41. [CrossRef]
57. Evans, E.; Sackmann, E. Translational and Rotational Drag Coefficients for a Disk Moving in a Liquid Membrane Associated with a Rigid Substrate. *J. Fluid. Mech.* **1988**, *194*, 553–561. [CrossRef]
58. Merkel, R.; Sackmann, E.; Evans, E. Molecular Friction and Epitactic Coupling between Monolayers in Supported Bilayers. *J. Phys.* **1989**, *50*, 1535–1555. [CrossRef]
59. Purucker, O.; Förtig, A.; Jordan, R.; Sackmann, E.; Tanaka, M. Control of Frictional Coupling of Transmembrane Cell Receptors in Model Cell Membranes with Linear Polymer Spacers. *Phys. Rev. Lett.* **2007**, *98*, 078102. [CrossRef]
60. Saxton, M.J. Lateral Diffusion in an Archipelago. The Effect of Mobile Obstacles. *Biophys. J.* **1987**, *52*, 989–997. [CrossRef]
61. Saxton, M.J. Anomalous Diffusion Due to Obstacles: A Monte Carlo Study. *Biophys. J.* **1994**, *66*, 394–401. [CrossRef] [PubMed]
62. Liu, A.P.; Fletcher, D.A. Actin Polymerization Serves as a Membrane Domain Switch in Model Lipid Bilayers. *Biophys. J.* **2006**, *91*, 4064–4070. [CrossRef] [PubMed]
63. Heinemann, F.; Vogel, S.K.; Schwille, P. Lateral Membrane Diffusion Modulated by a Minimal Actin Cortex. *Biophys. J.* **2013**, *104*, 1465–1475. [CrossRef] [PubMed]
64. Subramaniam, A.B.; Guidotti, G.; Manoharan, V.N.; Stone, H.A. Glycans Pattern the Phase Behaviour of Lipid Membranes. *Nat. Mater.* **2012**, *12*, 128–133. [CrossRef]
65. Vogel, S.K.; Petrasek, Z.; Heinemann, F.; Schwille, P. Myosin Motors Fragment and Compact Membrane-Bound Actin Filaments. *eLife* **2013**, *2*, e00116. [CrossRef] [PubMed]
66. Langmuir, I. The Constitution and Fundamental Properties of Solids and Liquids. II. Liquids. *J. Am. Chem. Soc.* **1917**, *39*, 1848–1906. [CrossRef]
67. Blodgett, K.B. Films Built by Depositing Successive Monomolecular Layers on a Solid Surface. *J. Am. Chem. Soc.* **1935**, *57*, 1007–1022. [CrossRef]
68. Dietrich, C.; Merkel, R.; Tampé, R. Diffusion Measurement of Fluorescence-Labeled Amphiphilic Molecules with a Standard Fluorescence Microscope. *Biophys. J.* **1997**, *72*, 1701–1710. [CrossRef]
69. Beckers, D.; Urbancic, D.; Sezgin, E. Impact of Nanoscale Hindrances on the Relationship between Lipid Packing and Diffusion in Model Membranes. *J. Phys. Chem.* **2020**, *2020*, 124–1487. [CrossRef]
70. Przybylo, M.; Sykora, J.; Humpolíčová, J.; Benda, A.; Zan, A.; Hof, M. Lipid Diffusion in Giant Unilamellar Vesicles Is More than 2 Times Faster than in Supported Phospholipid Bilayers under Identical Conditions. *Langmuir* **2006**, *22*, 9096–9099. [CrossRef]
71. Thomas, F.A.; Visco, I.; Petrášek, Z.; Heinemann, F.; Schwille, P. Introducing a Fluorescence-Based Standard to Quantify Protein Partitioning into Membranes. *Biochim. Biophys. Acta* **2015**, *1848*, 2932–2941. [CrossRef] [PubMed]
72. Tamm, L.K.; McConnell, H.M. Supported Phospholipid Bilayers. *Biophys. J.* **1985**, *47*, 105–113. [CrossRef]
73. Kučerka, N.; Nieh, M.P.; Katsaras, J. Fluid Phase Lipid Areas and Bilayer Thicknesses of Commonly Used Phosphatidylcholines as a Function of Temperature. *Biochim. Biophys. Acta (BBA)—Biomembr.* **2011**, *1808*, 2761–2771. [CrossRef] [PubMed]
74. Schoch, R.L.; Brown, F.L.H.; Haran, G. Correlated Diffusion in Lipid Bilayers. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2113202118. [CrossRef] [PubMed]
75. Wolfe, C.A.; James, P.S.; Mackie, A.R.; Ladha, S.; Jones, R. Regionalized Lipid Diffusion in the Plasma Membrane of Mammalian Spermatozoa. *Biol. Reprod.* **1998**, *59*, 1506–1514. [CrossRef] [PubMed]
76. Evans, E.; Yeung, A. Hidden Dynamics in Rapid Changes of Bilayer Shape. *Chem. Phys. Lipids* **1994**, *73*, 39–56. [CrossRef]
77. Gurunathan, S.; Woong Han, J.; Kim, E.; Kwon, D.N.; Park, J.K.; Kim, J.H. Enhanced Green Fluorescent Protein-Mediated Synthesis of Biocompatible Graphene. *J. Nanobiotechnol.* **2014**, *12*, 41. [CrossRef]
78. Wang, B.; Zhang, L.; Sung, C.B.; Granick, S. Nanoparticle-Induced Surface Reconstruction of Phospholipid Membranes. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 18171–18175. [CrossRef]
79. Servagent-Noinville, S.; Revault, M.; Quiquampoix, H.; Baron, M.H. Conformational Changes of Bovine Serum Albumin Induced by Adsorption on Different Clay Surfaces: FTIR Analysis. *J. Colloid. Interface Sci.* **2000**, *221*, 273–283. [CrossRef]
80. Vaz, W.L.C.; Goodsaid-Zalduondo, F.; Jacobson, K. Lateral Diffusion of Lipids and Proteins in Bilayer Membranes. *FEBS Lett.* **1984**, *174*, 199–207. [CrossRef]
81. NeutrAvidin Protein. Available online: <https://www.thermofisher.com/order/catalog/product/de/de/31000> (accessed on 6 August 2024).
82. García De La Torre, J.; Huertas, M.L.; Carrasco, B. Calculation of Hydrodynamic Properties of Globular Proteins from Their Atomic-Level Structure. *Biophys. J.* **2000**, *78*, 719. [CrossRef] [PubMed]

83. Zhang, Y.; De Mets, R.; Monzel, C.; Acharya, V.; Toh, P.; Chin, J.F.L.; Van Hul, N.; Ng, I.C.; Yu, H.; Ng, S.S.; et al. Biomimetic Niches Reveal the Minimal Cues to Trigger Apical Lumen Formation in Single Hepatocytes. *Nat. Mater.* **2020**, *19*, 1026–1035. [[CrossRef](#)] [[PubMed](#)]
84. Pugliese, L.; Coda, A.; Malcovati, M.; Bolognesi, M. Three-Dimensional Structure of the Tetragonal Crystal Form of Egg-White Avidin in Its Functional Complex with Biotin at 2.7 Å Resolution. *J. Mol. Biol.* **1993**, *231*, 698–710. [[CrossRef](#)] [[PubMed](#)]
85. Honigsmann, A.; Sadeghi, S.; Keller, J.; Hell, S.W.; Eggeling, C.; Vink, R. A Lipid Bound Actin Meshwork Organizes Liquid Phase Separation in Model Membranes. *eLife* **2014**, *3*, 1671. [[CrossRef](#)] [[PubMed](#)]
86. Sackmann, E.; Tanaka, M. Supported Membranes on Soft Polymer Cushions: Fabrication, Characterization and Applications. *Trends Biotechnol.* **2000**, *18*, 58–64. [[CrossRef](#)]
87. Pace, H.; Simonsson Nyström, L.; Gunnarsson, A.; Eck, E.; Monson, C.; Geschwindner, S.; Snijder, A.; Höök, F. Preserved Transmembrane Protein Mobility in Polymer-Supported Lipid Bilayers Derived from Cell Membranes. *Anal. Chem.* **2015**, *87*, 9194–9203. [[CrossRef](#)]
88. Bartels, N.; van der Voort, N.T.M.; Opanasyuk, O.; Felekyan, S.; Greife, A.; Shang, X.; Bister, A.; Wiek, C.; Seidel, C.A.M.; Monzel, C. Advanced Multiparametric Image Spectroscopy and Super-Resolution Microscopy Reveal a Minimal Model of CD95 Signal Initiation. *Sci. Adv.* **2024**, *10*, 3238. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Mobile and Immobile Obstacles in Supported Lipid Bilayer Systems and Their Effect on Lipid Mobility

Luisa Coen, Daniel Alexander Kuckla, Andreas Neusch and Cornelia Monzel *

Experimental Medical Physics, Heinrich-Heine University, 40225 Düsseldorf, Germany
* Correspondence: cornelia.monzel@hhu.de; Tel.: +49-(0)21181 - 12893

SUPPORTING INFORMATION

1. Theory

1.1 Diffusion in Biological Membranes

The first theoretical model to calculate the diffusion constant of proteins in lipid membranes, proposed by Saffman and Delbrück, models the lipid membrane as an infinitely flat sheet of a homogeneous, viscous fluid. The model ignores the complex nature of the bilayer, which is naturally composed of various phospholipids, glycolipids, cholesterol, and proteins. Membrane proteins are represented as cylinders embedded in this viscous fluid with the symmetry axes pointing perpendicular to the membrane surface and without any molecular parts extending above or below the membrane. The diffusion constant of the membrane fluid is, in this case, logarithmically dependent on the size of the membrane embedded moiety [54]:

$$D = \frac{\overline{r^2}}{4t} = \frac{k_B T}{4\pi\eta h} \cdot \left(\log\left(\frac{\eta h}{\eta' a}\right) - \gamma \right), \quad (\text{Eq. S1})$$

where $\overline{r^2}$ is the mean square displacement, t the time, k_B the Boltzmann constant, T the temperature, η the viscosity of the fluid representing the membrane, η' the viscosity of the surrounding fluid, h the height of the lipid membrane, a the radius of the diffusing particle, and γ the Euler constant. As a first approximation, **Eq. S1** can also be used to calculate the diffusion constant of lipids. One extension of this theory is given by Petrov and Schwill. They expanded the Saffman and Delbrück model to consider larger membrane inclusions as obstacles [56].

1.2 Friction Between SLB and Substrate

In the case of SLBs, the interaction between the solid and the membrane results in an additional source of friction, which reduces the mobility within the membrane. Here, the theory of Evans and Sackmann introduces inertia-lacking equations of motion for objects in a fluid SLB and assumes proportionality between interfacial shear stress and membrane velocity. In this model, the friction coefficient of the moving object, b , is the key parameter to calculate the diffusion constant [57]:

$$D = \frac{k_B T}{4\pi\eta_m} \left(\frac{1}{4} \varepsilon^2 + \frac{\varepsilon K_1(\varepsilon)}{K_0(\varepsilon)} \right)^{-1}. \quad (\text{Eq. S2})$$

Here, η_m is the membrane surface viscosity, and K_1 and K_0 are the modified Bessel functions of second kind and first or zeroth order. ε is the dimensionless particle radius given by [57]:

$$\varepsilon = a \cdot \sqrt{\frac{b}{\eta_m}}, \quad (\text{Eq. S3})$$

with a as the real particle radius, and b as the friction parameter.

For low friction, the Saffman/Delbrück result is recovered, whereas for high friction, the diffusion coefficient depends strongly on the size of the diffusing particle. In our study, both the tracers and the proteins can be interpreted as diffusing particles, which is considered a disc-like particle, without further assumption. For this reason, the Evans/Sackmann model is well-suited to compare friction parameters between the different model systems. Like Saffman/Delbrück, the Evans/Sackmann description ignores the discrete nature of the bilayer.

It should be noted that the Saffman/Delbrück and Evans/Sackmann models consider membrane-spanning probes, which were not present in our sample. Instead, lipids or lipids with proteins coupled to the membrane were present in the upper membrane leaflet. This setup was chosen on purpose to allow for an accurate estimate of obstacle concentrations. Moreover, membrane-spanning probes were shown to contribute additional friction due to interactions with the substrate [86,87] and would require the use of a polymer cushion beneath the SLB. Since a polymer cushion would increase the system complexity and, since for polymer cushions, the tethering was reported to yield an immobile fraction [45], this approach was not considered in this work. Nonetheless, a previous study points to an epitactic coupling between the lipids in both membrane layers [58]; therefore, some membrane-spanning effects may also arise in our case.

1.3 Friction Due to Mobile Obstacles

To deal with the variable nature of the membrane components, theories accounting for friction effects arising from mobile obstacles within the membrane have been developed [60,61]. The obstacles can be individual proteins or protein clusters on the membrane surface that diffuse more slowly with increasing size, thereby influencing the diffusion of the lipids in the membrane. In Saxton's theory, diffusion is considered as a random motion on a two-dimensional grid. Obstacles and particular lipids in the membrane, so-called tracers, are randomly distributed on the grid and can jump to adjacent free places. The relative jump rate γ is defined as the ratio of the jump rate of the tracers T to the jump rate of the obstacles H : $\gamma = \gamma_T/\gamma_H$. In addition, the diffusion constant of the lipids depends on the concentration of the obstacles c , which is given as an area fraction defined by: $\left(1 + \frac{a_1}{a_0} \frac{n_1}{n_0}\right)^{-1}$, with a_1 as the area of the lipid, a_0 the effective area of the obstacle, and n_1/n_0 , the molar ratio of lipid to obstacle. The relative diffusion constant $D^*(c) = D(c)/D(0)$ is defined with $D(c)$ and $D(0)$ as the diffusion constants of the tracer lipid in the presence and absence of obstacles, respectively. Next to its dependence on obstacle concentration, the relative diffusion constant is also a function of the relative jump rate [60]:

$$D^*(c, \gamma) = (1 - c) \cdot \frac{\{[(1-\gamma)(1-c)f_0+c]^2 + 4\gamma(1-c)f_0^2\}^{1/2} - [(1-\gamma)(1-c)f_0+c]}{2\gamma(1-c)f_0}. \quad (\text{Eq. S4})$$

and

$$f_0 = \frac{1-\alpha}{1+(2\gamma-1)\cdot\alpha}. \quad (\text{Eq. S5})$$

Here, α is a lattice-dependent constant. For a square lattice $\alpha = 1 - \frac{2}{\pi}$, for a triangular lattice $\alpha = 0.282$, and $\alpha = 0.500$ for a honeycomb lattice. Using Monte Carlo simulations, Saxton was able to show that the relative diffusion constant is independent of the choice of the lattice; therefore, a triangular lattice was used in this study [60].

2. Supplementary Figures

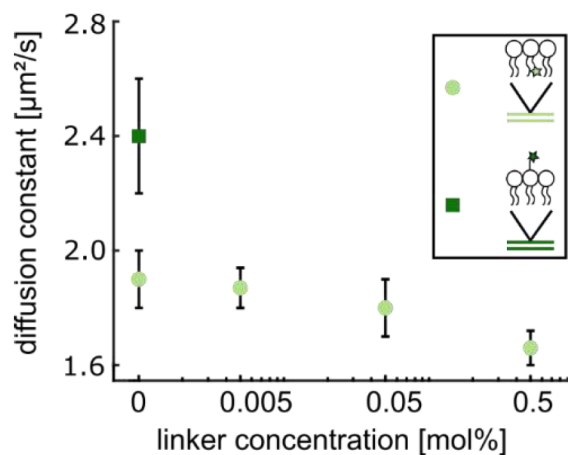


Figure S1. Fatty acid-labeled tracer. Diffusion constants for the NTA-GFP system. DOGS-NTA concentrations of 0 mol%, 0.005 mol%, 0.05 mol%, and 0.5 mol% were used. SLB with fatty acid-labeled tracer (circle), SLB with headgroup-labeled tracer (box). Data points are median $\pm$ MAD.

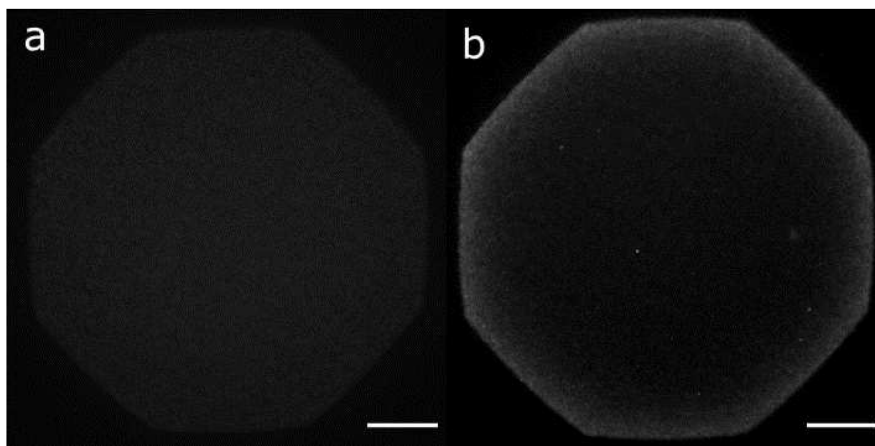


Figure S2. SLB with bound protein after bleaching. (a) NTA-GFP system. The missing bright ring at the aperture edge after bleaching indicates the immobilization of GFP. (b) Biotin-NAVOG system. The bright ring at the aperture edge after bleaching indicates the mobility of NAVOG. Scale bar in (a) and (b): 25 μm .

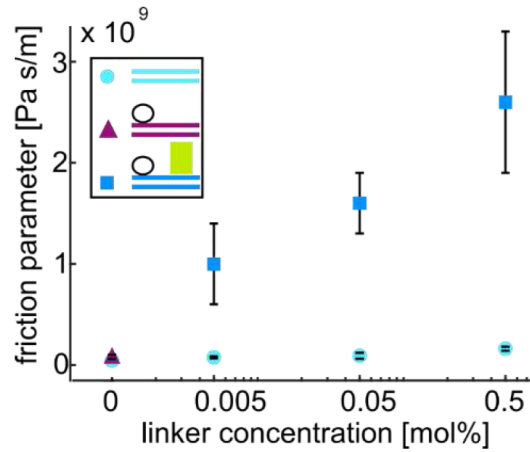


Figure S3. Calculated friction parameter for the NTA–GFP system. DOGS-NTA concentrations of 0 mol%, 0.005 mol%, 0.05 mol%, and 0.5 mol% were used. SLB alone (circle), SLB after BSA incubation (triangle), SLB after BSA passivation and GFP coupling (box). Data points are median $\pm$ MAD.

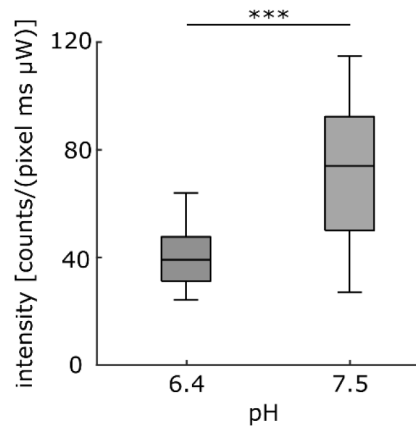


Figure S4. GFP intensity changes after incubation on the SLB at 0 mol% linker concentration as a function of pH. The intensity increase from pH 6.4 to pH 7.5 is an indicator of the charge-dependent interaction between GFP and SLB. Significance was tested via Wilcoxon rank-sum test with n.s.: $p > 0.05$, * : $p \leq 0.05$, ** : $p \leq 0.01$, ***: $p \leq 0.001$. Confocal images of the SLB were taken with an Abberior Confocal Microscope (Abberior Instruments GmbH, Göttingen, Germany) equipped with a 100x objective with NA 1.4 from Olympus. The system has previously been described in detail [88].

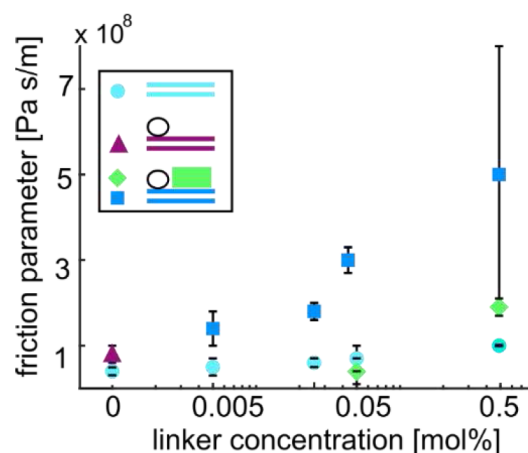


Figure S5. Calculated friction parameter for the Biotin–Neutravidin system. DOPE-Cap-Biotin concentrations of 0 mol%, 0.005 mol%, 0.05 mol%, and 0.5 mol% were used. SLB alone (circle), SLB after BSA incubation (triangle), SLB after BSA passivation and NAVOG coupling (box), NAVOG (diamond). Data points are median $\pm$ MAD.

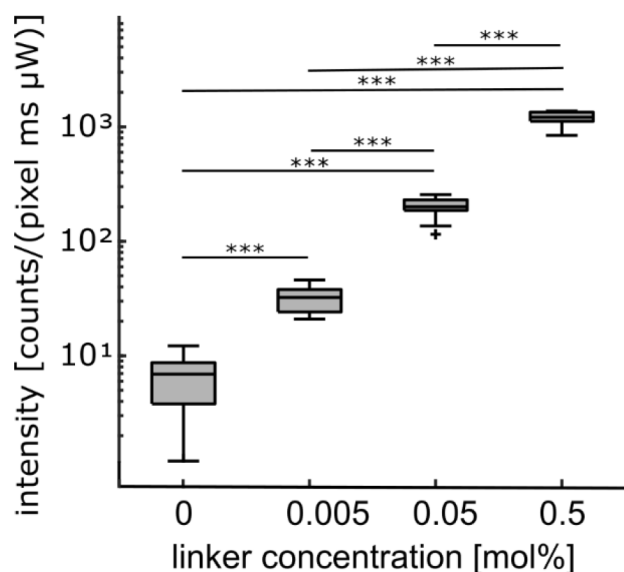


Figure S6. NAVOG intensity changes as a function of the linker concentration of the Biotin–Neutravidin system. DOPE-Cap-Biotin concentrations of 0 mol%, 0.005 mol%, 0.05 mol%, and 0.5 mol% were used. Significance was tested via Wilcoxon rank-sum test with n.s.: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. The linear intensity detection with increasing fluorophore concentration was checked. Confocal images of the SLB were taken with an Abberior Confocal Microscope (Abberior Instruments GmbH, Göttingen, Germany) equipped with a 100x objective with NA 1.4 from Olympus. The system has previously been described in detail [88]. Here, a near linear increase was found with a tenfold linker concentration increase and a corresponding sixfold NAVOG intensity increase.

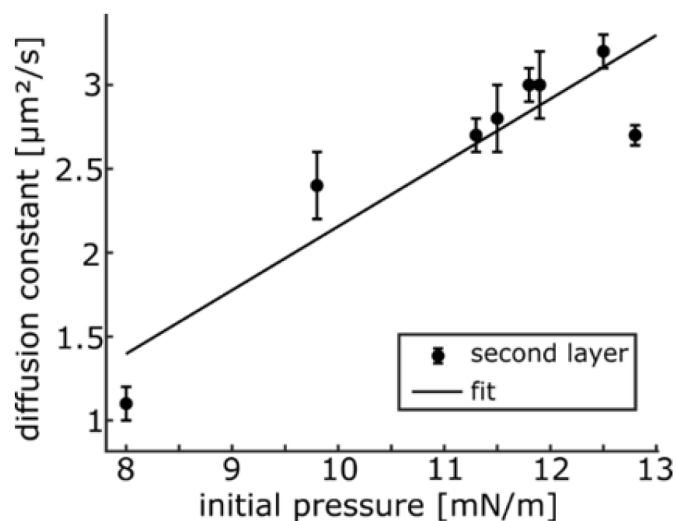


Figure S7. Calibration data for the initial pressure correction for the Biotin–Neutravidin–Actin system. Data in the graph were acquired from a linker-free SLB with 2 mg/ml SOPC + 2.5 mol% Cy5. Linear fit with y-intercept = -1.6 and slope = 0.4. The diffusion constant exhibited a dependency on the initial pressure of the second layer of the SLB. The first layer did not exhibit any pressure dependency. For this reason, diffusion constants were corrected to an initial lateral pressure value of 8 mN/m, which was the SLB fabrication pressure of the second layer in the Biotin–Neutravidin system. Data points are median $\pm$ MAD.

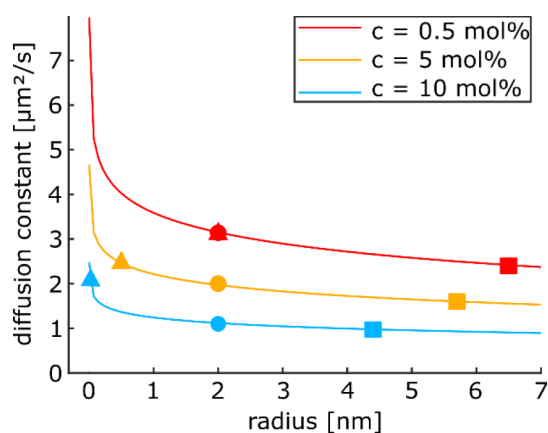


Figure S8. Diffusion constant as a function of obstacle radius for linker concentrations of 0.5 mol% (red), 5 mol% (yellow), and 10 mol% (blue). Lines represent the theory of Petrov and Schwille [56]. The theory depends on the membrane viscosity and the particle radius as parameters. To fix the membrane viscosity, the obstacle size derived from Saxton's theory ($a_0 = 12 \text{ nm}^2$, i.e. radius = 1.95 nm) for the SOPC + NAVTMR model system and all concentrations is used. The diffusion constants of the matrix lipid for pure SOPC (triangle), SOPC + NAVTMR (circle), and SOPC + NAVTMR + actin (square) are inserted into the graph to yield the obstacle radii. The theory gives reasonable obstacle radii for actin-coupled obstacles of 4.4 nm to 6.5 nm size. However, it should be noted that these values are a rough estimate based on the result of Saxton's theoretical approach.

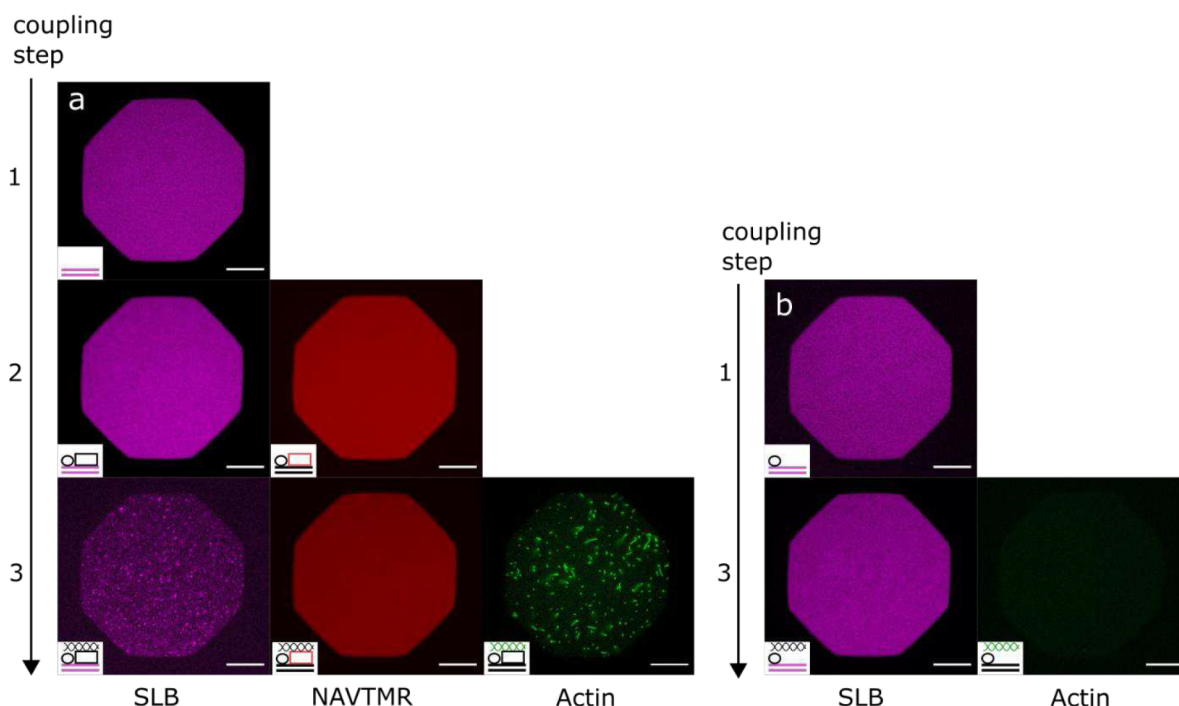


Figure S9. Representative images of the SLB before and after coupling of proteins. Different coupling steps: (1) before coupling, (2) after coupling of NAVTMR, and (3) after coupling of actin. (a) Experiment with all incubation steps; (b) control measurement without NAVTMR but with actin incubated. Linker concentration in (a) and (b): 5 mol%. Scale bars: 25 μm .

SUPPLEMENTARY REFERENCES

45. Deverall, M.A.; Gindl, E.; Sinner, E.K.; Besir, H.; Ruehe, J.; Saxton, M.J.; Naumann, C.A. Membrane Lateral Mobility Obstructed by Polymer-Tethered Lipids Studied at the Single Molecule Level. *Biophys J* **2005**, *88*, 1875–1886, doi:10.1529/biophysj.104.050559.
54. Saffman, P.G.; Delbrueck, M. Brownian Motion in Biological Membranes. *Proc Natl Acad Sci USA* **1975**, *72*, 3111–3113, doi:10.1073/PNAS.72.8.3111.
56. Petrov, E.P.; Schwille, P. Translational Diffusion in Lipid Membranes beyond the Saffman-Delbrück Approximation. *Biophys J* **2008**, *94*, L41, doi:10.1529/BIOPHYSJ.107.126565.
57. Evans, E.; Sackmann, E. Translational and Rotational Drag Coefficients for a Disk Moving in a Liquid Membrane Associated with a Rigid Substrate. *J Fluid Mech* **1988**, *194*, 553–561, doi:10.1017/S0022112088003106.
58. Merkel, R.; Sackmann, E.; Evans, E. Molecular Friction and Epitactic Coupling between Monolayers in Supported Bilayers. *Journal de Physique* **1989**, *50*, 1535–1555, doi:10.1051/JPHYS:0198900500120153500.

60. Saxton, M.J. Lateral Diffusion in an Archipelago. The Effect of Mobile Obstacles. *Biophys J* **1987**, *52*, 989–997, doi:10.1016/S0006-3495(87)83291-5.
61. Saxton, M.J. Anomalous Diffusion Due to Obstacles: A Monte Carlo Study. *Biophys J* **1994**, *66*, 394–401.
86. Sackmann, E.; Tanaka, M. Supported Membranes on Soft Polymer Cushions: Fabrication, Characterization and Applications. *Trends Biotechnol* **2000**, *18*, 58–64, doi:10.1016/S0167-7799(99)01412-2.
87. Pace, H.; Simonsson Nyström, L.; Gunnarsson, A.; Eck, E.; Monson, C.; Geschwindner, S.; Snijder, A.; Höök, F. Preserved Transmembrane Protein Mobility in Polymer-Supported Lipid Bilayers Derived from Cell Membranes. *Anal Chem* **2015**, *87*, 9194–9203, doi:10.1021/ACS.ANALCHEM.5B01449.
88. Bartels, N.; van der Voort, N.T.M.; Opanasyuk, O.; Felekyan, S.; Greife, A.; Shang, X.; Bister, A.; Wiek, C.; Seidel, C.A.M.; Monzel, C. Advanced Multiparametric Image Spectroscopy and Super-Resolution Microscopy Reveal a Minimal Model of CD95 Signal Initiation. *Sci Adv* **2024**, *10*, 3238, doi:10.1126/sciadv.adn3238.

3.2 Publication II: Superparamagnetic Biomembranes for Dynamic Nanoparticle and Molecular Patterning.

Autors: Luisa Coen, Jan Lange, Andreas Neusch, Thomas Heinzl and Cornelia Monzel

Journal: *Nano Letters*

Date of acceptance: 29. May 2026

DOI: <https://doi.org/10.1021/acs.nanolett.6c00639>

Contribution

The doctoral candidate (LC) made the primary contribution to this publication, accounting for approximately 80% of the overall work. This included the preparation of all SLBs and the execution of the majority of the experimental work, in particular magnetic tip preparation, as well as the systematic investigation of force-induced MNP transport and cell experiments. Experimental tasks not performed by the doctoral candidate comprised transmission electron microscopy imaging and fabrication of the master mould.

Furthermore, the doctoral candidate carried out the data curation, software implementation, formal data analysis, and contributed substantially to the interpretation and validation of the results. LC was also the main contributor to the manuscript preparation, including drafting the original manuscript, generating the visualizations, and revising. The remaining contributions, including supervision, conceptual input, resources, project administration, funding acquisition, and additional methodological or experimental support, were provided by the co-authors as specified in the original publication.

Superparamagnetic Biomembranes for Dynamic Nanoparticle and Molecular Patterning

Luisa Coen, Jan Lange, Andreas Neusch, Thomas Heinzl, and Cornelia Monzel*



Cite This: <https://doi.org/10.1021/acs.nanolett.6c00639>



Read Online

ACCESS |

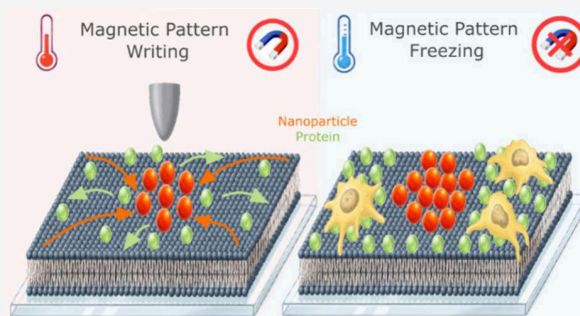
Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: We introduce superparamagnetic biomembranes as a novel, actively tunable hybrid platform. The superparamagnetic biomembranes are supported lipid bilayers (SLBs) with defined lipid composition and site-specific coupling of proteins and superparamagnetic nanoparticles (MNPs). Application of magnetic field gradients induces rapid and fully reversible lateral transport of lipid-anchored MNPs with forces in the femtonewton range. Using single magnetic tips as well as multiple magnetic tips and line structures, we achieve parallelized and spatially defined MNP patterning. Transition of the SLB from the liquid-crystalline phase to the gel phase enables reversible immobilization of the patterned MNP distributions. Local MNP accumulation displaces lipids and membrane-associated proteins, including Neutravidin, mEGFP, and CD19, thereby generating inverse molecular gradients that are recognized by living cells. This biocompatible substrate with dynamically controllable nanoparticle and molecular organization provides a versatile framework for applications in bio- and nanotechnology.

KEYWORDS: Superparamagnetic nanoparticle, Supported lipid bilayer (SLB), Synomag, Magnetic forces, Molecular gradient, Molecular pattern



Dynamically tunable patterning of magnetic nanoparticles (MNPs) underpins a broad range of applications, including the fabrication of magnetic–nonmagnetic composites,^{1,2} the development of responsive sensing systems,³ and the controlled activation of biological signaling pathways.^{4–6} A variety of assembly strategies have been developed to position colloids from the nanometer to the micrometer scale into predefined architectures,^{7–10} including complex three-dimensional structures and multicomponent lattices.¹ However, the realization of fully reversible and dynamically reconfigurable nanoparticle patterns remains challenging. Moreover, only a limited number of existing approaches are compatible with biological environments or enable direct biofunctionalization for biomedical applications.^{11,12}

A promising strategy addressing several of these limitations is creating dynamically changeable molecular gradients and patterns via remote magnetic nanoparticle redistribution, where magnetic nanoparticles are coupled to biomolecules and remotely manipulated using magnetic fields in a contact-free manner.^{13–15} Significant progress has been made in the synthesis and biofunctionalization of MNPs exhibiting high monodispersity, stability, and tailored magnetic responses.^{16–19} Magnetic actuation has been exploited to (i) induce a localized nanoparticle redistribution and accumulation,²⁰ (ii) to generate mechanical forces,^{21–23} or (iii) produce localized thermal effects.^{17,24–27} When combined with signaling-relevant biomolecules, these stimuli have been shown to modulate

cellular processes such as polarization,²⁸ neuronal activation,^{29–31} cell differentiation,²¹ or growth.³² Yet, magnetic nanoparticle functionalized substrates that allow for a remote, dynamic redistribution of nanoparticles and associated molecules, are hitherto missing. Previous studies highlight the potential of supported lipid bilayer (SLB)-based magnetic model systems for studying membrane-associated transport processes on the single-particle level.³³ While here the dynamics of individually tracked particles were analyzed, the present work establishes dynamically reconfigurable collective nanoparticle and biomolecular patterning on SLBs with active control over molecular gradients as well as a membrane phase-dependent immobilization.

Here, we couple superparamagnetic nanoparticles to SLBs³⁴ to establish a two-dimensional, dynamically tunable platform for MNP patterning across nanometer-to-micrometer length scales. The resulting substrates are readily fabricated, biocompatible, and allow simultaneous functionalization with MNPs and biomolecules. Magnetic field gradients enable rapid,

Received: February 6, 2026

Revised: May 21, 2026

Accepted: May 29, 2026

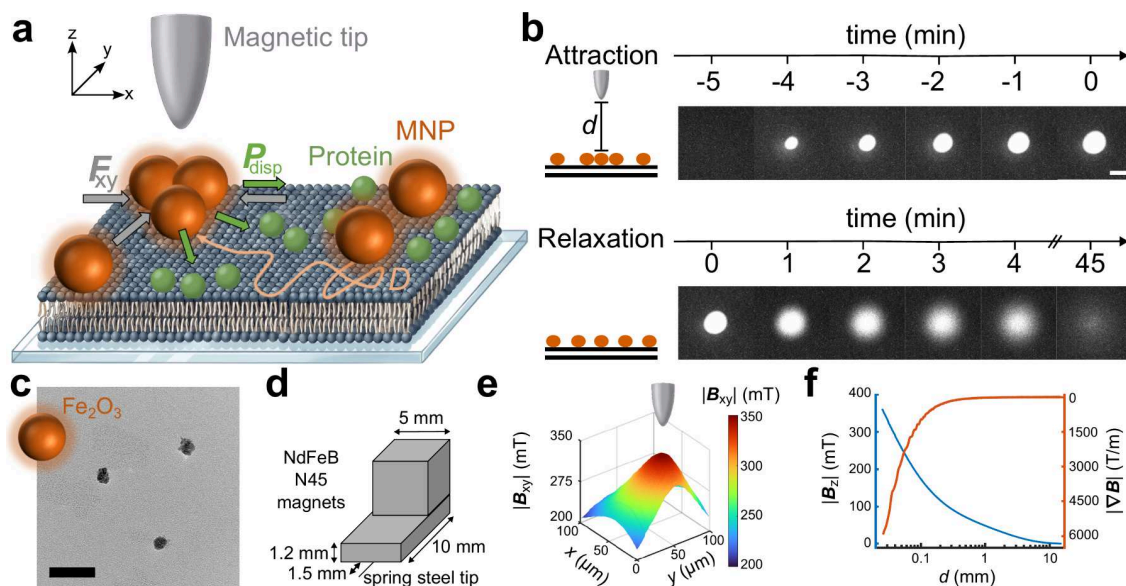


Figure 1. Superparamagnetic biomembranes and system components. (a) Schematic of the superparamagnetic biomembrane. Superparamagnetic nanoparticles (MNP) are coupled to the supported lipid bilayer (SLB) via specific lipid headgroup linkers. Similarly, proteins (Neutravidin, mEGFP, or CD19) are coupled to a different lipid headgroup linker. A vertically approached magnetic tip attracts the MNPs in the horizontal plane. Proteins are displaced from the MNP accumulated regions. Lipid coupled MNPs exhibit a velocity v in the force field F_{xy} (gray arrows) and diffuse freely (lateral diffusion of lipid–MNP complex) with diffusion constant D in the absence of the magnetic field. Lateral motion of the MNPs induces protein displacement (P_{disp}), indicated by green arrows. (b) Micrographs of fluorescent MNPs during magnetic attraction and field free ‘relaxation’. During attraction, the magnetic tip was placed at a distance $d = 20 \mu\text{m}$ above the SLB and at $d = 2000 \mu\text{m}$ during relaxation. Scale bar: $50 \mu\text{m}$. (c) TEM image of MNPs. The iron oxide (Fe_2O_3) core exhibits a flower-like multicore shape. The MNP cores are surrounded by a dextran shell. For the SLB coupling via biotinylated lipids, MNPs are coated with streptavidin. Scale bar: 100 nm . (d) Schematic of the magnetic tip. (e) Horizontal scan of the magnetic field of the tip, $|B_{xy}|$, at a distance $d = 25 \mu\text{m}$. (f) Magnetic field profile, $|B_z|$, at the center of the tip along the vertical direction over several magnitudes of distances (blue). Calculated magnetic field gradient $|\nabla B_z|$ (orange).

fully reversible, and contact-free redistribution of MNPs into complex spatial patterns. Displacement of membrane-associated biomolecules by MNP accumulation generates inverse chemical gradients that are recognized by living cells, establishing superparamagnetic biomembranes as a versatile platform for dynamic bio- and nanotechnological applications. In order to create a biocompatible, dynamically tunable substrate, a SLB is used. This simplified biomembrane combines the advantage of a 4 nm flat, homogeneous sheet with the control of the fluidity state via both lipid composition and temperature. Moreover, doping the membrane with headgroup modified lipids—henceforth called linker lipids—in the upper leaflet of the SLB, enables to couple MNPs or proteins to the SLB (see Figure 1a). Biotinylated lipids served as anchors for streptavidin coated MNPs with highest biological affinity ($K_D \sim 10^{-15} \text{ M}$). Proteins were either coupled competitively with the MNP to the biotinylated lipids (e.g., Neutravidin–Oregon Green (NAVOG)) or the SLB was additionally doped with nitrilotriacetic acid (NTA) headgroup modified lipids to anchor proteins via the His-Tag– Ni^{2+} –NTA interaction ($K_D \sim 10^{-5} \text{ M}$), thus avoiding competitive coupling (e.g., for monomeric Enhanced Green Fluorescent Protein (mEGFP), Cluster of Differentiation 19 (CD19)). A fast and fully reversible attraction of MNPs was observed, when a magnetic tip was vertically approached at 10 – $100 \mu\text{m}$ distances d above the SLB. Figure 1b shows an example measurement, with the magnetic tip positioned at $d = 20 \mu\text{m}$ above the SLB. MNP are rapidly attracted into a densely packed disc, with intensities starting to saturate within the first 5 min (see also Video S1). When the magnetic tip was positioned at large distances (e.g., $d = 2000 \mu\text{m}$) no influence

on the MNP motion was detected, in line with the measurement of magnetic forces, F_{xy} , vanishing at $d \geq 1000 \mu\text{m}$ (see Figure S1). In this case, the MNPs fully redistributed over the SLB surface on diffusion limited time scales ($\sim 1 \text{ h}$). As MNPs, dextran coated iron oxide (Fe_2O_3) cores exhibiting a flower-like multicore shape were chosen. These so-called Synomag MNPs are commercially available (see Materials and Methods) and are individually synthesized with a fluorescence or streptavidin coating. In our previous study, these MNPs showed to be superparamagnetic at ambient temperatures, which is important to maintain their monodispersity, maintaining colloidal stability while minimizing any induced dipolar interactions or irreversible chain formation.²⁰ Figure 1c shows a transmission electron microscopy (TEM) image of the iron oxide cores of the MNPs exhibiting a narrow size distribution with average core diameter of $34 \pm 8 \text{ nm}$ (see also Novoselova et al.²⁰ for a more detailed characterization). The fully functionalized streptavidin-coated particles exhibit a hydrodynamic diameter of approximately 70 nm . The custom-fabricated magnetic tip consists of two NdFeB magnets, cube and cuboid, and an attached spring steel tip (see Figure 1d and Materials and Methods for a more detailed description). The latter concentrates the magnetic field lines in the tip, which emerge as a strongly divergent field - throughout the manuscript characterized as gradients of the magnetic flux density B in z direction and within the xy plane. Together with the magnetic moment $|m| = 2.65 \times 10^{-19} \text{ Am}^2$ of the MNP (derived from fitting the Langevin equation to the magnetization curve, see Figure S2), these gradients give rise to the measured vertical and horizontal force components $F_z = m \cdot \frac{dB_z}{dz}$

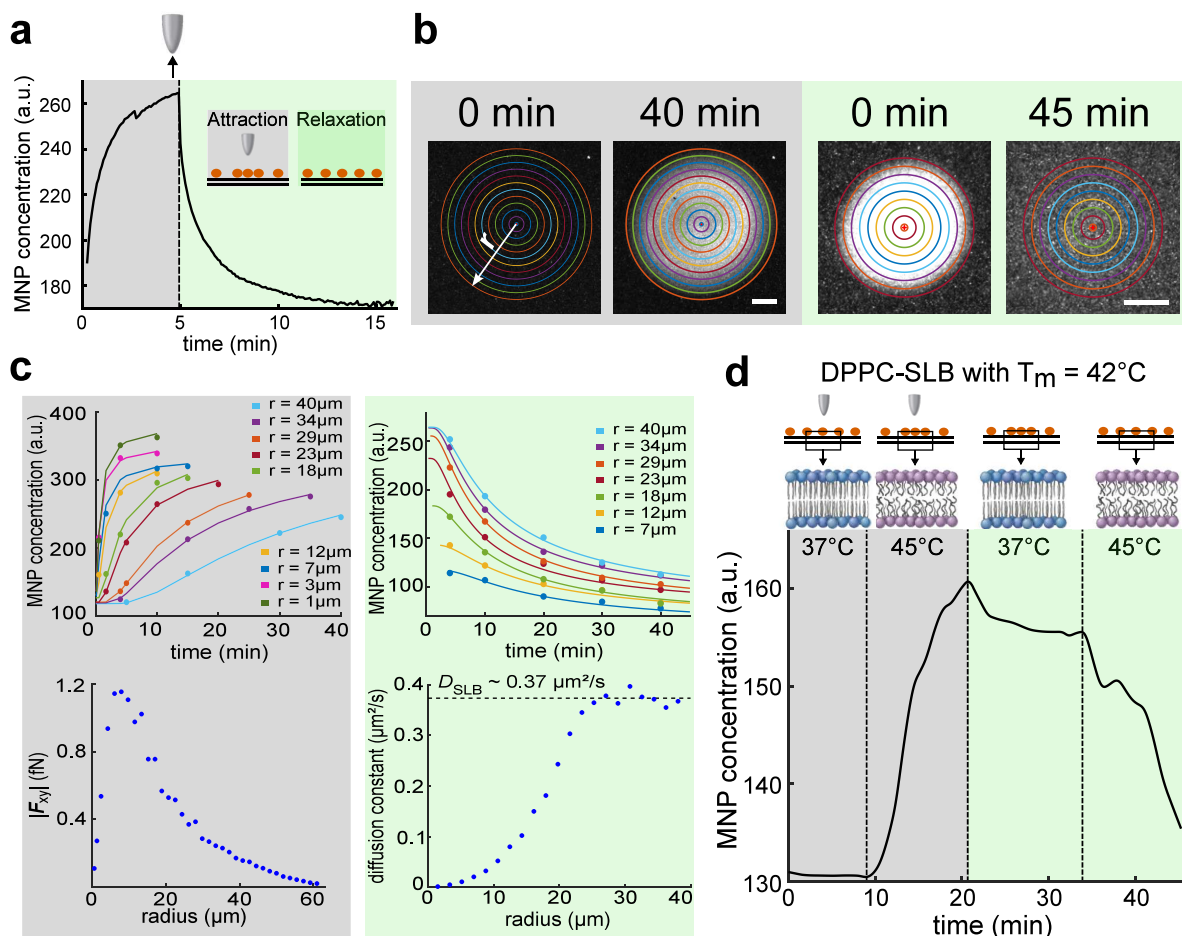


Figure 2. System dynamics: Switching between dynamic and static MNP distributions. (a) Measured magnetic force induced MNP attraction and field free relaxation dynamics. After 5 min of MNP attraction the intensity starts to saturate, corresponding to a dense MNP packing. Under field free conditions, MNPs redistribute. The accumulation is fully reversible. (b) Selected time points of fluorescent MNP recordings during magnetic field attraction and field free relaxation. Intensities are averaged over circular lines and evaluated as MNP concentration along the radial direction r in the following analysis. Scale bar: 20 μm . (c) Temporal change of MNP concentration for selected radii and time points (dots = data points, lines = fit) with magnetic tip at $d = 20 \mu\text{m}$ above the SLB. Attraction and field free relaxation dynamics are fitted using $I(r, t) = I_{\text{max}}(t) \cdot \left(1 - \exp\left(-\frac{v(r) \cdot t}{r}\right)\right)$, as a solution of the first-order differential equation $\frac{dI}{dt} = k \cdot (I_{\text{max}} - I(t))$, with $k = \frac{v(r)}{r}$ and $I(r, t) = I_0(t) \cdot \exp\left(-\frac{r^2}{4D(r)t}\right)$, the 2D time-dependent solution of Ficks law, to determine v and D , respectively. The magnetic force field $F_{xy}(r)$ is calculated from v according to the equation $F_{xy}(r) = v(r) \cdot (\gamma_{\text{MNP}} + \gamma_{\text{mem}})$. $I(r, t) = I_0(t) \cdot \exp\left(-\frac{r^2}{4D(r)t}\right)$ is correctly applied only at $r \geq 20 \mu\text{m}$, where D saturates and MNPs diffuse freely (lateral diffusion of lipid–MNP complexes). (d) An immobilization and release of MNP pattern is realized by switching a DPPC–lipid-based SLB, with main transition temperature $T_m = 42^\circ\text{C}$, from its fluid-crystalline phase to its gel phase and vice versa. Magnetic tip distance $d \approx 40 \mu\text{m}$.

and $F_{xy} = m \cdot \sqrt{\left(\frac{dB_{xy}}{dx}\right)^2 + \left(\frac{dB_{xy}}{dy}\right)^2}$, respectively. The lateral force F_{xy} drives the motion of the MNP within the horizontal plane of the SLB, whereas F_z strongly increases toward the center of the tip, where it can give rise to MNP detachment at very small distances $d \leq 20 \mu\text{m}$. To quantify the magnetic flux density B_{xy} in the plane of the SLB, Scanning Hall Probe Microscopy³⁵ was performed at a tip distance of $d = 25 \mu\text{m}$ over a $100 \times 100 \mu\text{m}^2$ area, yielding a maximum value of $|B_{xy}| = 350 \text{ mT}$ (Figure 1e). From a vertical scan of the magnetic flux density B_z above the tip center, a value of $\left|\frac{dB_z}{dz}\right| \sim 6000 \text{ T/m}$ at small d was obtained (see Figure 1f, right). Thus, magnetic forces of $|F_z| \sim 1.6 \text{ fN}$ are applied to each MNP along d

directly below the tip and similar forces are obtained from the magnetic flux density gradient within the horizontal plane of the SLB (see Figure S1).

To first characterize the motion of the MNPs alone, only the MNPs and no additional proteins were coupled to the SLB. Video S2 demonstrates that the MNPs can be actively pulled to different positions on the SLB by the single magnetic tip. In addition, a scratch was intentionally created in the SLB. Upon magnetic redistribution, the MNPs were observed to accumulate exclusively at the edge of the scratch, while no particles were detected within the scratched area, demonstrating that MNPs are indeed specifically coupled to the SLB rather than freely diffusing on the substrate. Figure 2a shows the magnetic field-induced attraction and field-free relaxation dynamics of the MNPs. The measured fluorescence intensities

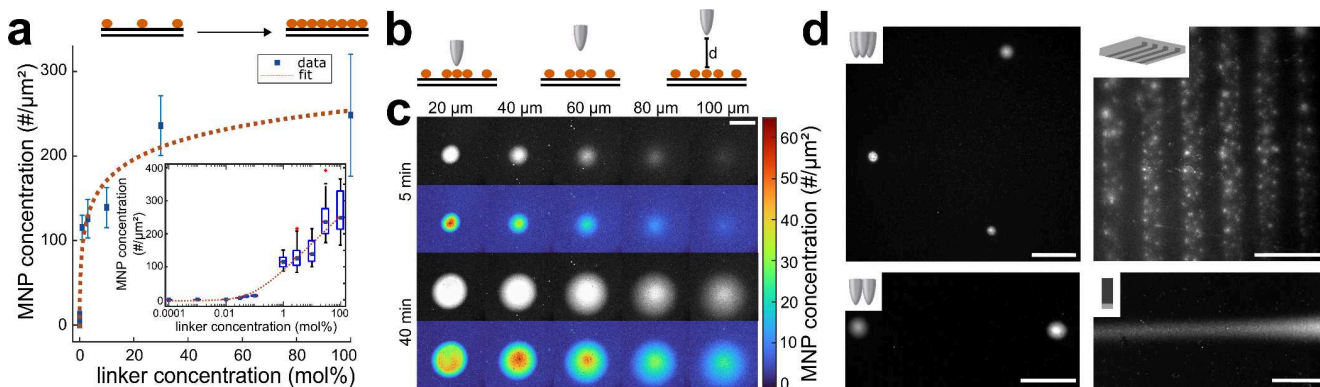


Figure 3. Variation of MNP concentration and pattern formation. (a) MNP concentration as a function of lipid-linker concentration in the SLB for homogeneously distributed MNPs in the absence of magnetic fields. At high lipid-linker concentrations, MNPs are densely packed and the MNP concentration saturates. Insert: Semilog representation of the same data. The data are best described by the fit of $c(x) \sim a + b \cdot \log(x)$. (b) Schematic of magnetic force-induced MNP accumulation at different magnetic tip distances d . (c) Micrographs of accumulated MNPs after 5 and 40 min with color maps, showing the calculated MNP concentrations. The color scale was limited to exclude the highest and lowest 1% of intensities, while preserving absolute concentration values. Magnetic tips were positioned at 20, 40, 60, 80, and 100 μm . The MNP lipid linker concentration was 0.1 mol %. Scale bar: 50 μm . (d) MNP pattern formation on SLBs using a double tip (scale bar: 100 μm), triple tip (scale bar: 100 μm), razor blade (scale bar: 20 μm), or soft ferro-/diamagnetic composite stencil (scale bar: 100 μm). Micrographs show the MNP pattern after magnetic attraction.

are taken to be directly proportional to the MNP concentration. After 5 min, a spot of approximately 50 μm is accumulated ($d = 20 \mu\text{m}$) and the mean intensity starts to saturate. For longer accumulation times (e.g., over 40 min), the intensity per pixel at the spot center saturates and the spot size diameter slowly increases further (see Figures 2b and 3c). Here, removal of the tip (i.e., placement at $d = 2000 \mu\text{m}$) initiates the system relaxation, where lipid-MNP complexes diffuse over the SLB until they are homogeneously distributed. Figure 2b shows fluorescence images at selected time points showing the attraction and complete redistribution of MNPs. For the following analysis, the radial system symmetry is utilized, and the average intensity per radius over time is evaluated. Figure 2c shows the radial coordinate system and selected circles. For the case of magnetic attraction, the MNP velocity toward the magnetic tip, $v(r)$, scales with the exponential intensity increase as

$$I(r, t) = I_{\text{max}}(t) \cdot \left(1 - \exp\left(-\frac{v(r) \cdot t}{r}\right)\right).$$

From this, the attraction force $F_{xy}(r) = v(r) \cdot (\gamma_{\text{MNP}} + \gamma_{\text{mem}})$ is calculated, accounting for the hydrodynamic drag acting on the membrane-coupled MNP arising from the surrounding aqueous medium and the lipid membrane. Here, $\gamma_{\text{MNP}} = 6\pi\eta a$ denotes the Stokes friction coefficient of the MNP with hydrodynamic radius $a = 35 \text{ nm}$ in the aqueous buffer of viscosity $\eta = 1 \times 10^{-3} \text{ Ns/m}^2$. The membrane contribution γ_{mem} was calculated using the Saffman-Delbrück hydrodynamic expression for a membrane-coupled object, where $\gamma_{\text{mem}} = \frac{4\pi\eta_{\text{mem}}}{\ln(l_{\text{SD}}/R_C)}$. Here, $\eta_{\text{mem}} = 2 \times 10^{-9} \text{ Ns/m}^2$ is the 2D area-related viscosity, l_{SD} the Saffman-Delbrück length and R_C the radius of the coupled object of approximately 10 nm, consistent with reported values for supported lipid bilayers.³⁶

Under these conditions, the resulting attraction force is in the femtonewton regime and increases gradually with decreasing distance from the magnetic tip, reaching a maximum value of approximately $|F_{xy, \text{max}}(r)| = 1.2 \text{ fN}$ at $|r| = 8 \mu\text{m}$ ($d = 20 \mu\text{m}$). Directly below the tip, the force decays to 0, since the total force vector $F(r)$ continuously changes its orientation with decreasing r toward the magnetic tip,

eventually pointing perpendicular away from the substrate (at $|r| = 0$). For the case of field free relaxation and assuming an unconstrained MNP motion, the MNPs follow the diffusion

equation, with $I(r, t) = I_0(t) \cdot \exp\left(-\frac{r^2}{4D(r)t}\right)$. Fitting the temporal intensity decay, yields the r -dependent diffusion constant $D(r)$, which at large radii r yields the true diffusion constant $D(|r| \rightarrow \infty) \sim 0.37 \mu\text{m}^2/\text{s}$. At smaller radii, i.e. $|r| \lesssim 20 \mu\text{m}$, the MNP motion is inhibited by the densely packed MNPs of the accumulated spot. Note, the MNP dynamics changes depending on the accumulated spot size, since larger spots exhibit higher MNP concentrations at larger radii. Hence, care must be taken to determine the true D from the limit behavior ($|r| \rightarrow \infty$). The obtained diffusion coefficient is lower than that of freely diffusing lipids in fluid SLBs and likely reflects the coupled motion of a larger membrane-associated complex. In particular, multivalent interactions between the streptavidin-coated MNPs and multiple biotinylated linker lipids are expected to increase the effective hydrodynamic coupling radius and membrane drag. This interpretation is consistent with the effective coupling radius of approximately 10 nm estimated from the Saffman-Delbrück formalism. Still, the measured diffusion coefficient remains within the expected range for membrane-coupled nano-objects and proteins.³⁷ Next, we sought to test in how the membrane phase affects the MNP dynamics. To this end, we fabricated a SLB with DPPC matrix lipid. Since DPPC features a main phase transition temperature of $T_m = 42 \text{ }^\circ\text{C}$, switching the sample temperature between 37 and 45 $^\circ\text{C}$ changes the SLB phase from a gel to a liquid crystalline state and vice versa. Figure 2d shows the MNP intensity change below the magnetic tip, when the SLB switches from the gel to a liquid crystalline phase. In the gel phase, the SLB bound MNPs are immobilized and are only attracted toward the tip, after heating the membrane to $\sim 45 \text{ }^\circ\text{C}$. When the magnetic tip is removed and the temperature drops to $\sim 37 \text{ }^\circ\text{C}$, the accumulated MNP pattern is 'frozen'. Subsequent heating to $\sim 45 \text{ }^\circ\text{C}$ releases the MNPs to redistribute on the SLB. Video S3 shows the corresponding

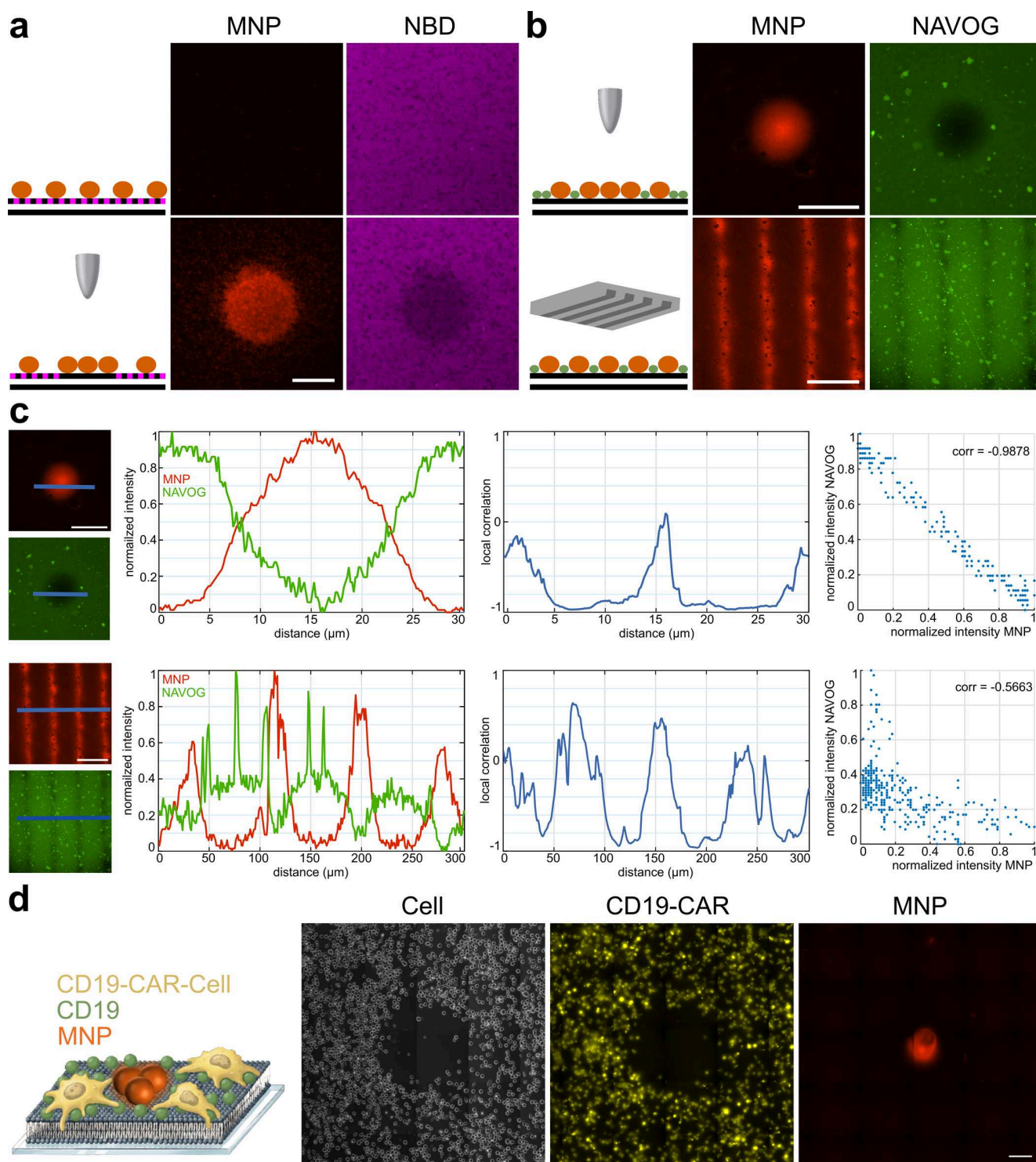


Figure 4. MNP create inverse molecular gradient pattern of either lipids or proteins as bioactive substrates. (a) Fluorescent NBD-lipids in the SLB are displaced by lipid coupled MNPs. Thus, an artificially, locally phase separated SLB is obtained. Scale bar: 10 μm . (b) Neutravidin–Oregon Green (NAVOG) coupled to biotinylated lipids is displaced by the MNPs. Next to a single MNP spot free of NAVOG (scale bar: 20 μm), a regular line pattern of MNP and NAVOG (scale bar: 100 μm) is created. Premanipulation NAVOG fluorescence images in Figure S5. (c) Quantitative analysis of single-spot MNP accumulation geometries. (i) Representative fluorescence images of MNP and NAVOG distributions from b with evaluated line profile positions indicated. (ii) Corresponding fluorescence intensity line profiles demonstrating the inverse spatial distribution of MNPs and NAVOG. (iii) Local spatial correlation analysis along the evaluated line. (iv) Pearson correlation analysis shown as scatter plot, revealing a strong spatial anticorrelation between MNP and NAVOG distributions ($\text{corr} = -0.9878$ for single spot accumulation and $\text{corr} = -0.5663$ for stripe geometries). (i) to (iv) from left to right. (d) Clinically relevant natural killer cells, expressing a Chimeric Antigen Receptor (CAR) against Cluster of Differentiation 19 (CD19) are seeded on a CD19 functionalized SLB. After MNP accumulation, CD19-CAR cells recognize regions of high CD19 concentration, while avoiding CD19 depleted regions. Scale bar: 100 μm .

experiment, serving as a proof-of-principle to dynamically 'freeze and release' created MNP patterns.

Furthermore, in order to convert measured MNP intensities into concentrations, we performed a calibration measurement, where the MNP linker lipid doping of the SLB was systematically increased (see Figure 3a). After MNP coupling to the SLB, the average fluorescence intensity of the homogeneously distributed MNPs was determined. While for low linker concentrations between 0.0001 mol % and 0.1 mol % a near linear dependency of the MNP intensity was obtained, above 30% linker concentration, the MNP fluorescence signal was saturating. The latter arose from the dense coverage of the whole SLB surface by MNPs corresponding to a concentration of about 240 MNPs/ μm^2 (for a hydrodynamic MNP diameter of 70 nm). This start and limit behavior enabled to convert MNP intensities into absolute concentrations, where the data was best described by the relation $c(x) \sim a + b \cdot \log(x)$, with linker concentration x , MNP concentration c , as well as fit parameters $a = 87$ MNPs/ μm^2 and $b = 36$ MNPs/ μm^2 . This relation can be used for future experiments to estimate MNP concentrations in advance. It should be noted, that all experiments in this article were performed in the 0.05 mol % or 0.1 mol % lipid-linker regime, as here MNP concentrations are low enough to minimize particle–particle collisions while maintaining a considerable fluorescence signal. To further investigate the MNP concentration dependency on the magnetic tip–SLB distance and the time course of the MNP accumulation, MNPs were attracted at different distances d of the magnetic tip (20, 40, 60, 80, 100 μm) and the measured intensity was converted into MNP concentrations (see Figure 3b). Figure 3c shows example images of accumulated MNPs after 5 and 40 min (for all images see Figure S3). Utilizing the intensity–concentration conversion relation, colormaps of the MNP concentration distribution are calculated. For all d , a significant MNP accumulation is observed. Fluorescence spots at $d = 20$ μm exhibit high, local MNP concentrations within the accumulation region with sharp edges, whereas at $d = 100$ μm a lower MNP concentration with shallow intensity increase toward the center is observed (see also Figure S4). Intriguingly, MNPs are only stably attracted toward the magnetic tip up to a finite radius from the center. In the present system, this transition is observed at radii $r \geq 60$ μm (see Figure 3c), where F_{xy} approaches the thermal fluctuation force F_{therm} . Here the MNP motion is diffusion dominated, the thermal force prevails over the magnetic force. Only for radii $r > 60$ μm , the magnetic force governs the motion of the MNPs. Small magnetic tip–SLB distances of $d = 20$ μm exhibit steep concentration gradients of up to 70%, whereas at $d = 100$ μm only 20% concentration changes occur across the accumulated spot. Hence, variation of d and time enables to systematically modulate the local MNP concentrations between 10–80 MNPs/ μm^2 . Accordingly, the applied magnetic attraction forces are changing (see Figure 1f and Figure S1). In some cases, for $d \leq 20$ μm , forces in the vertical direction directly below the tip were high enough to pull MNPs off the membrane. As a consequence, MNP densities in the center of the accumulated spot partially decreased (e.g., Figure 3c, $d = 20$ μm after 40 min).

Finally, to verify the variability of MNP pattern formation on SLBs, other magnetic stimuli were created. It should be noted, that the modulation of magnetic flux densities on the μm -scale with gradients of $\sim 10^3$ T/m is difficult to realize, wherefore

new magnetic stimuli had to be designed. Among them are a double tip, triple tip, razor blade, or soft ferro-/diamagnetic composite stencil (see also Material and Methods). Figure 3d verifies that the magnetic field generated by them results in equivalent MNP pattern on the SLB. By using a soft ferro-/diamagnetic composite stencil attached to the cubic magnet, stripe-like MNP patterns could be generated (Figure 3d). Owing to the lower effective magnetization and the larger structural dimensions of the PDMS composite compared to the piano-wire tips, the resulting magnetic field gradients are expected to be lower in magnitude and more spatially extended. Both the magnetic field gradient and the corresponding magnetic force were estimated based on simulations using the model by Yang et al.³⁸ for comparable geometries, yielding gradient and force values in the range of $\sim 10^4$ T/m and ~ 0.3 fN, respectively. Still, the obtained gradients were sufficient to induce robust and highly reproducible MNP distribution over large-areas on the SLB. Local intensity maxima within the stripe patterns likely arise from small spatial variations in the magnetic field distribution and transient local increases in MNP density rather than irreversible nanoparticle aggregation. Interestingly, when SLBs were functionalized with fluorescent lipids or proteins in addition to the MNP coupling, molecular gradient pattern of either lipids or proteins inverse to the MNP pattern could be created (see Figure 4). Importantly, this redistribution was observed both for competitively coupled proteins and for orthogonally anchored His-tagged proteins coupled via Ni2+-NTA lipids (see Video S4), demonstrating that the effect is not restricted to competitive binding to biotinylated linker lipids. First, fluorescent NBD-lipids were integrated into the SLB membrane and showed to be displaced by the lipid coupled MNPs. Thus, a local lipid phase separation³⁹ in the SLB is actively induced (Figure 4a). Second, when NAVOG was coupled to the lipids, the accumulation of MNPs displaced the proteins. Minor fluorescence intensity heterogeneities were already present prior to magnetic actuation, likely originating from local variations in protein surface density or fluorescence intensity fluctuations within the SLB system (see Figure S5). Cluster removal by ultracentrifugation will be tested in future. Here, next to a single MNP spot free of NAVOG, also regular line patterns of alternating MNPs and NAVOG were created (Figure 4b). Quantitative fluorescence intensity line profile analysis confirmed the spatial anticorrelation between MNP accumulation and protein distribution (Figure 4c). For single-spot accumulation geometries, the MNP and protein fluorescence distributions exhibited an almost perfect inverse spatial correlation (Pearson correlation coefficient: $\text{corr} = -0.9878$), whereas stripe-like patterns generated using the soft ferro-/diamagnetic composite stencil showed a broader but still pronounced anticorrelation ($\text{corr} = -0.5663$). Finally, a CD19 functionalized SLB was produced in order to test, how cells would recognize the actively generated protein gradients (Figure 4d). Prior to cell addition, the patterned MNP distributions were immobilized by cooling the DPPC-based SLB below its phase transition temperature, thereby suppressing further lateral MNP redistribution. The magnetic tip was subsequently removed before cell seeding, ensuring that cells were exposed only to static molecular patterns in the absence of active magnetic actuation. Note that DPPC is chosen as matrix lipid here, allowing the structures of the displaced CD19 to be 'frozen'. Consequently, no magnetic tip is required during cell experiments, thereby avoiding potential artifacts in

cellular behavior induced by the magnetic tip. As an example, we used the clinically relevant natural killer cells expressing a Chimeric Antigen Receptor (CAR) against Cluster of Differentiation 19 (CD19), which are recently used in the field of CAR-therapy in cancer research.^{40,41} Following MNP accumulation, CD19-CAR cells selectively recognized regions with high local CD19 concentration, while avoiding areas depleted of CD19. Notably, the cell-depleted region extended beyond the densely packed MNP core, indicating that the laterally displaced CD19 gradient extends over larger distances than the central MNP accumulation spot visible in fluorescence microscopy. Since CAR NK cells spatially integrate receptor–ligand interactions across their entire contact area, even moderate local reductions in CD19 density surrounding the MNP-rich region may influence cell positioning. We envisage further applications of local protein accumulation and patterning for activating cell signals, which are dependent on absolute protein concentrations and clustering.^{42,43} No significant uptake of MNPs by the cells was observed under the experimental conditions used here, indicating that the observed cellular behavior originated from the CD19 pattern on the substrate rather than nanoparticle internalization. Potential effects associated with cellular MNP uptake have been investigated by us before in detail.²⁰ Notably, the cell-depleted region extended beyond the densely packed MNP spot. We attribute this observation to a slight increase in MNP concentration in the vicinity of the central accumulation spot compared to the background MNP density on the SLB. Even this moderate local increase appears sufficient to reduce the local CD19 density below the threshold required for CAR NK cell adhesion. Since various types of cells are capable of sensing molecular concentration gradients on the order of 1–10% across the cell,⁴⁴ the molecular gradients created here would suffice to induce a biological reaction. Examples of molecular gradients triggering specific cell signals include cell polarization, cell reorientation and morphogenesis⁴⁵ or directed cell motion (Haptotaxis).⁴⁶

In summary, we introduce superparamagnetic biomembranes as a novel and actively tunable hybrid platform. We demonstrate fully reversible spatial control of magnetic nanoparticles (MNPs) on supported lipid bilayers (SLBs) and introduce quantitative assays that characterize system components with nano- to micrometer-scale precision. The platform is biocompatible and enables biological functionality, including the active redistribution of biomolecules and the modulation of cell adhesion through spatially controlled molecular densities. The presented platform may therefore provide a versatile tool for the spatial organization of biomolecules and the investigation of biological responses to dynamic molecular gradients. In particular, the use of structured soft ferromagnetic templates may enable the generation of complex and spatially extended biomolecular landscapes over large areas, which could become useful for future studies on directed cell migration, cell guidance, and spatially resolved receptor signaling. Beyond biotechnological patterning of MNPs and biomolecules, this approach provides a versatile framework for nanotechnological applications in which MNPs are remotely repositioned and magnetized or locally heated to generate condensed-matter stimuli.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.6c00639>.

Detailed description of materials and methods for SLB preparation and protein coupling, magnetic tip(s) and stencil fabrication, TEM imaging, mammalian cell culture, fluorescence microscopy, bioimage data analysis. (PDF)

Video S1: Video of MNP attraction (5 min) and relaxation (45 min) on the SLB. This video corresponds to the data shown in Figure 1b, Figure 2b right (green), and Figure 2c right (green). (MP4)

Video S2: Video of MNP attraction to different positions on the SLB. To provide evidence of the MNP coupling to the SLB, the SLB is scratched with the magnetic tip, and MNPs are accumulated at the edge of the scratched region. (MP4)

Video S3: Video of MNP immobilization and release by thermally switching a DPPC–lipid-based SLB from fluid-crystalline phase to gel phase and vice versa. This video corresponds to the data shown in Figure 2d. (MP4)

Video S4: Video of MNP attraction causing protein (GFP) displacement. (MP4)

Video S5: Video of MNP attraction on the SLB during the stepwise approach of the magnetic tip from $d = 100$ to $20 \mu\text{m}$ in $20 \mu\text{m}$ steps. This video corresponds to the data shown in Figure S4. (MP4)

■ AUTHOR INFORMATION

Corresponding Author

Cornelia Monzel – *Experimental Medical Physics, Heinrich-Heine University Düsseldorf, 40225 Düsseldorf, Germany*; Present Address: Second Institute of Physics, University of Stuttgart, 70569 Stuttgart, Germany; orcid.org/0000-0003-0892-3427; Email: cornelia.monzel@pi2.uni-stuttgart.de

Authors

Luisa Coen – *Experimental Medical Physics, Heinrich-Heine University Düsseldorf, 40225 Düsseldorf, Germany*; Present Address: Second Institute of Physics, University of Stuttgart, 70569 Stuttgart, Germany

Jan Lange – *Solid State Physics, Heinrich-Heine University Düsseldorf, 40225 Düsseldorf, Germany*

Andreas Neusch – *Experimental Medical Physics, Heinrich-Heine University Düsseldorf, 40225 Düsseldorf, Germany*

Thomas Heinzel – *Solid State Physics, Heinrich-Heine University Düsseldorf, 40225 Düsseldorf, Germany*;

orcid.org/0000-0001-5149-4379

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.nanolett.6c00639>

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Conceptualization: C.M. Methodology: L.C., J.L., A.N. Software: L.C. Validation: L.C., C.M. Formal analysis: L.C., A.N. Investigation: L.C., J.L., A.N. Resources: C.M., T.H. Data Curation: L.C. Writing—original draft: L.C.,

473 C.M. Writing—review and editing: L.C., A.N., C.M. Visual-
474 ization: .L.C. Supervision: C.M., T.H. Project administration:
475 C.M. Funding acquisition: C.M.

476 Notes

477 The authors declare no competing financial interest.

478 ■ ACKNOWLEDGMENTS

479 C.M. and J.L. acknowledge financial support by the DFG
480 Collaborative Research Center 1535 ‘MiBiNet’ (project ID
481 458090666). C.M., L.C., and A.N. acknowledge financial
482 support by the ‘Freigeist-fellowship’ of VolkswagenFoundation
483 and CM and AN by the ‘Momentum’ (Projekt-Nr.:0072536-
484 00) funding line of VolkswagenFoundation. The authors
485 acknowledge the Deutsche Forschungsgemeinschaft (DFG,
486 German Research Foundation) and the state of North Rhine-
487 Westphalia for funding the cryo-TEM (INST 208/749-1
488 FUGG) hosted by the Centre of Advanced Imaging (CAi,
489 Heinrich-Heine University), for the custom MFIS-SSTR
490 Nikon microscope (A1HD25-NSTORM) within the program
491 Major Research Instrumentation as per ART.91b GG with the
492 funding ID INST 208/797-1 FUGG to Claus Seidel
493 (Molecular Physical Chemistry, HHU) and C.M.. We thank
494 Gorky Shaw for performing Scanning Hall Probe imaging and
495 Nora Dempsey for discussions at Institut Néel (CNRS,
496 Grenoble). We gratefully acknowledge Maren Soldner,
497 Helmut Hanenberg, and Constanze Wiek from the department
498 of Pediatrics of the University Hospital Essen and the
499 Department of Otorhinolaryngology of the University Hospital
500 Düsseldorf for providing the CAR NK cells used in this study.
501 C.M. thanks Udo Seifert (II. Institute of Theoretical Physics,
502 University of Stuttgart) for useful discussions. C.M. dedicates
503 this article to the memory of Maxime Dahan (formerly,
504 Physico-Chimie, Institut Curie, Paris), who introduced her to
505 the fascinating science of magnetic nanoparticles and single-
506 molecule tracking.

507 ■ ABBREVIATIONS

508 SLB, supported lipid bilayer; MNP, magnetic nanoparticle;
509 NAVOG, Neutravidin–Oregon Green; CD19, Cluster of
510 Differentiation 19; mEGFP, monomeric Enhanced Green
511 Fluorescent Protein

512 ■ REFERENCES

513 (1) Demirörs, A. F.; Pillai, P. P.; Kowalczyk, B.; Grzybowski, B. A.
514 Colloidal Assembly Directed by Virtual Magnetic Moulds. *Nature*
515 **2013**, *503* (7474), 99–103.
516 (2) Tao, Q.; Lu, J.; Dahlqvist, M.; Mockute, A.; Calder, S.;
517 Petruhins, A.; Meshkian, R.; Rivin, O.; Potashnikov, D.; Caspi, E. N.;
518 Shaked, H.; Hoser, A.; Opagiste, C.; Galera, R. M.; Salikhov, R.;
519 Wiedwald, U.; Ritter, C.; Wildes, A. R.; Johansson, B.; Hultman, L.;
520 Farle, M.; Barsoum, M. W.; Rosen, J. Atomically Layered and Ordered
521 Rare-Earth i-MAX Phases: A New Class of Magnetic Quaternary
522 Compounds. *Chem. Mater.* **2019**, *31* (7), 2476–2485.
523 (3) Shipway, A. N.; Katz, E.; Willner, I. Nanoparticle Arrays on
524 Surfaces for Electronic, Optical, and Sensor Applications. *CHEM-*
525 *PHYSICHEM* **2000**, *1* (1), 18–52.
526 (4) Gemperle, J.; Liße, D.; Kappen, M.; Secret, E.; Coppey, M.;
527 Gregor, M.; Menager, C.; Piehler, J.; Caswell, P. Live-Cell Magnetic
528 Manipulation of Recycling Endosomes Reveals Their Direct Effect on
529 Actin Protrusions to Promote Invasive Migration. *Science Advances*
530 **2025**, *11* (27), 6361.
531 (5) Heschem, S. A.; Chiang, P. H.; Gregurec, D.; Moon, J.;
532 Christiansen, M. G.; Jahanshahi, A.; Liu, H.; Rosenfeld, D.; Pralle, A.;
533 Anikeeva, P.; Temel, Y. Magnetothermal Nanoparticle Technology

Alleviates Parkinsonian-like Symptoms in Mice. *Nat. Commun.* **2021**, *12* (1), 5569.
(6) Latypova, A. A.; Yaremenko, A. V.; Pechnikova, N. A.; Minin, A. S.;
Zubarev, I. V. Magnetogenetics as a Promising Tool for
Controlling Cellular Signaling Pathways. *J. Nanobiotechnology* **2024**,
22 (1), 327.
(7) Spatz, J. P.; Mößmer, S.; Möller, M. Mineralization of Gold
Nanoparticles in a Block Copolymer Microemulsion. *Chem.—Eur. J.*
1996, *2* (12), 1552–1555.
(8) Marć, M.; Wolak, W.; Drzewiński, A.; Mudry, S.; Shtablayvi, I.;
Dudek, M. R. The Concept of Using 2D Self-Assembly of Magnetic
Nanoparticles for Bioassays. *Applied Sciences* **2024**, *14* (5), 1906.
(9) Bui, T. Q.; Oberdick, S. D.; Abel, F. M.; Donahue, M. J.;
Quelhas, K. N.; Dennis, C. L.; Cleveland, T. E.; Liu, Y.; Woods, S. I.
Magnetodynamics of Short Nanoparticle Chains. *Sci. Rep.* **2025**, *15*
(1), 43507.
(10) Gentili, D.; Ori, G. Reversible Assembly of Nanoparticles:
Theory, Strategies and Computational Simulations. *Nanoscale* **2022**,
14 (39), 14385–14432.
(11) Dubrova, A.; Cavaniol, C.; Van de Walle, A.; Mathieu, P.;
Fusilier, Z.; Yaacoub, N.; Lalatonne, Y.; Descroix, S.; Wilhelm, C.
Magnetite Nanoparticle Photothermal Therapy in a Pancreatic
Tumor-on-Chip: A Dual-Action Approach Targeting Cancer Cells
and Their Microenvironment. *ACS Nano* **2025**, *19* (21), 19790–
19805.
(12) Gavilán, H.; Rizzo, G. M. R.; Silvestri, N.; Mai, B. T.;
Pellegrino, T. Scale-up Approach for the Preparation of Magnetic
Ferrite Nanocubes and Other Shapes with Benchmark Performance
for Magnetic Hyperthermia Applications. *Nat. Protoc.* **2023**, *18* (3),
783–809.
(13) Shin, W.; Lee, Y.; Lim, J.; Lee, Y.; Lah, J. D.; Lee, S.; Lee, J.; Yu,
R.; Lee, P. H.; Lee, J.-H.; Kwak, M.; Cheon, J. Nanoscale Magneto-
Mechanical-Genetics of Deep Brain Neurons Reversing Motor
Deficits in Parkinsonian Mice. *Nano Lett.* **2024**, *24*, 270–278.
(14) Hajiali, H.; Rotherham, M.; El Haj, A. J. Remote Activation of
Mechanotransduction via Integrin Alpha-5 via Aptamer-Conjugated
Magnetic Nanoparticles Promotes Osteogenesis. *Pharmaceutics* **2024**,
16 (1), 21.
(15) Lee, J. uk; Shin, W.; Lim, Y.; Kim, J.; Kim, W. R.; Kim, H.; Lee,
J. H.; Cheon, J. Non-Contact Long-Range Magnetic Stimulation of
Mechanosensitive Ion Channels in Freely Moving Animals. *Nat.*
Mater. **2021**, *20* (7), 1029–1036.
(16) Liße, D.; Monzel, C.; Vicario, C.; Manzi, J.; Maurin, I.; Coppey,
M.; Piehler, J.; Dahan, M. Engineered Ferritin for Magnetogenetic
Manipulation of Proteins and Organelles Inside Living Cells. *Adv.*
Mater. **2017**, *29* (42), 1700189.
(17) Neusch, A.; Wiedwald, U.; Novoselova, I. P.; Kuckla, D. A.;
Tetos, N.; Sadik, S.; Hagemann, P.; Farle, M.; Monzel, C.
Semisynthetic Ferritin-Based Nanoparticles with High Magnetic
Anisotropy for Spatial Magnetic Manipulation and Inductive Heating.
Nanoscale **2024**, *16* (32), 15113–15127.
(18) Hadjidemetriou, M.; Kostarelos, K. Evolution of the Nano-
particle Corona. *Nat. Nanotechnol.* **2017**, *12* (4), 288–290.
(19) Bushra, R.; Ahmad, M.; Alam, K.; Seidi, F.; Qurtulen; Shakeel,
S.; Song, J.; Jin, Y.; Xiao, H. Recent Advances in Magnetic
Nanoparticles: Key Applications, Environmental Insights, and Future
Strategies. *Sustainable Materials and Technologies* **2024**, *40*,
No. e00985.
(20) Novoselova, I. P.; Neusch, A.; Brand, J.-S.; Otten, M.; Safari, M.
R.; Bartels, N.; Karg, M.; Farle, M.; Wiedwald, U.; Monzel, C.
Magnetic Nanoparticles for Spatio-Mechanical Manipulation in Single
Cells. *Nanomaterials* **2021**, *11*, 2267.
(21) Seo, D.; Southard, K. M.; Kim, J. W.; Lee, H. J.; Farlow, J.; Lee,
J. U.; Litt, D. B.; Haas, T.; Alivisatos, A. P.; Cheon, J.; Gartner, Z. J.;
Jun, Y. W. A Mechanogenetic Toolkit for Interrogating Cell Signaling
in Space and Time. *Cell* **2016**, *165* (6), 1507–1518.
(22) Master, A. M.; Williams, P. N.; Pothayee, N.; Pothayee, N.;
Zhang, R.; Vishwasrao, H. M.; Golovin, Y. I.; Riffle, J. S.; Sokolsky,
M.; Kabanov, A. V. Remote Actuation of Magnetic Nanoparticles For

- 603 Cancer Cell Selective Treatment Through Cytoskeletal Disruption.
604 *Sci. Rep.* **2016**, 6 (1), 33560.
- 605 (23) Jeong, S.; Shin, W.; Park, M.; Lee, J. U.; Lim, Y.; Noh, K.; Lee,
606 J. H.; Jun, Y. W.; Kwak, M.; Cheon, J. Hydrogel Magnetomechanical
607 Actuator Nanoparticles for Wireless Remote Control of Mechano-
608 signaling in Vivo. *Nano Lett.* **2023**, 23 (11), 5227–5235.
- 609 (24) Kuckla, D. A.; Brand, J. S.; Czech, B.; Asharion, A.; Jüttner, J.
610 V.; Novoselova, I. P.; Neusch, A.; Hagemann, P.; Getzlaff, M.;
611 Monzel, C. An Efficient Magnetothermal Actuation Setup for Fast
612 Heating/Cooling Cycles or Long-Term Induction Heating of
613 Different Magnetic Nanoparticle Classes. *J. Phys. D Appl. Phys.*
614 **2023**, 56 (50), 505002.
- 615 (25) Attanayake, S. B.; Nguyen, M. D.; Chanda, A.; Alonso, J.; Orue,
616 I.; Lee, T. R.; Srikanth, H.; Phan, M. H. Superparamagnetic
617 Superparticles for Magnetic Hyperthermia Therapy: Overcoming
618 the Particle Size Limit. *ACS Appl. Mater. Interfaces* **2025**, 17 (13),
619 19436–19445.
- 620 (26) Ma, X.; Liang, X.; Li, Y.; Feng, Q.; Cheng, K.; Ma, N.; Zhu, F.;
621 Guo, X.; Yue, Y.; Liu, G.; Zhang, T.; Liang, J.; Ren, L.; Zhao, X.; Nie,
622 G. Modular-Designed Engineered Bacteria for Precision Tumor
623 Immunotherapy via Spatiotemporal Manipulation by Magnetic Field.
624 *Nat. Commun.* **2023**, 14 (1), 1606.
- 625 (27) Monzel, C.; Vicario, C.; Piehler, J.; Coppey, M.; Dahan, M.
626 Magnetic Control of Cellular Processes Using Biofunctional Nano-
627 particles. *Chem. Sci.* **2017**, 8 (11), 7330–7338.
- 628 (28) Tseng, P.; Judy, J. W.; Di Carlo, D. Magnetic Nanoparticle-
629 Mediated Massively Parallel Mechanical Modulation of Single-Cell
630 Behavior. *Nat. Methods* **2012**, 9 (11), 1113.
- 631 (29) Nguyen, T.; Gao, J.; Wang, P.; Nagesetti, A.; Andrews, P.;
632 Masood, S.; Vriesman, Z.; Liang, P.; Khizroev, S.; Jin, X. In Vivo
633 Wireless Brain Stimulation via Non-Invasive and Targeted Delivery of
634 Magnetolectric Nanoparticles. *Neurotherapeutics* **2021**, 18 (3),
635 2091–2106.
- 636 (30) Dhillon, K.; Aizel, K.; Broomhall, T. J.; Secret, E.; Goodman,
637 T.; Rotherham, M.; Telling, N.; Siaugue, J. M.; Ménager, C.; Fresnais,
638 J.; Coppey, M.; El Haj, A. J.; Gates, M. A. Directional Control of
639 Neurite Outgrowth: Emerging Technologies for Parkinson's Disease
640 Using Magnetic Nanoparticles and Magnetic Field Gradients. *J. R. Soc.*
641 *Interface* **2022**, 19 (196), 20220576.
- 642 (31) Liu, Y.; Zhang, Y.; Huang, Y.; Bi, X. Mechanisms and Research
643 Progress of Magnetic Nanoparticles in Modulating Neural Plasticity
644 for Neuroregeneration. *Nanotechnology, Science and Applications* **2025**,
645 18, 553–572.
- 646 (32) Abdel Fattah, A. R.; Kolaitis, N.; Van Daele, K.; Daza, B.;
647 Rustandi, A. G.; Ranga, A. Targeted Mechanical Stimulation via
648 Magnetic Nanoparticles Guides in Vitro Tissue Development. *Nat.*
649 *Commun.* **2023**, 14 (1), 5281.
- 650 (33) Li, J. H.; Santos-Otte, P.; Au, B.; Rentsch, J.; Block, S.; Ewers,
651 H. Directed Manipulation of Membrane Proteins by Fluorescent
652 Magnetic Nanoparticles. *Nat. Commun.* **2020**, 11 (1), 4259.
- 653 (34) Safinya, C.; Rädler, J. *Handbook of Lipid Membranes: Molecular,*
654 *Functional, and Materials Aspects*, 1st ed.; CRC Press, 2021.
- 655 (35) Shaw, G.; Kramer, R. B. G.; Dempsey, N. M.; Hasselbach, K. A
656 Scanning Hall Probe Microscope for High Resolution, Large Area,
657 Variable Height Magnetic Field Imaging. *Rev. Sci. Instrum.* **2016**, 87
658 (11), 113702.
- 659 (36) Faizi, H. A.; Dimova, R.; Vlahovska, P. M. A Vesicle
660 Microrheometer for High-Throughput Viscosity Measurements of
661 Lipid and Polymer Membranes. *Biophysj* **2022**, 121, 910–918.
- 662 (37) Coen, L.; Kuckla, D. A.; Neusch, A.; Monzel, C. Mobile and
663 Immobile Obstacles in Supported Lipid Bilayer Systems and Their
664 Effect on Lipid Mobility. *Colloids and Interfaces* **2024**, 8 (5), 54.
- 665 (38) Yang, Z.; Wei, J.; Gizynski, K.; Song, M. G.; Grzybowski, B. A.
666 Interference-like Patterns of Static Magnetic Fields Imprinted into
667 Polymer/Nanoparticle Composites. *Nat. Commun.* **2017**, 8 (1), 1564.
- 668 (39) Rathe, V.; Kuckla, D.; Monzel, C. Phase Separation in
669 Biological Membranes: An Overview with Focus on Experimental
670 Effects of Illumination and Osmotic Pressure Changes. *Advances in*
671 *Biomembranes and Lipid Self-Assembly* **2021**, 34, 31–66.
- (40) Haist, C.; Schulte, E.; Bartels, N.; Bister, A.; Poschinski, Z.; 672
Ibach, T. C.; Geipel, K.; Wiek, C.; Wagenmann, M.; Monzel, C.; 673
Scheckenbach, K.; Hanenberg, H. CD44v6-Targeted CAR T-Cells 674
Specifically Eliminate CD44 Isoform 6 Expressing Head/Neck 675
Squamous Cell Carcinoma Cells. *Oral Oncol.* **2021**, 116, 105259. 676
- (41) Soldierer, M.; Bister, A.; Haist, C.; Thivakaran, A.; Cengiz, S. 677
C.; Sendker, S.; Bartels, N.; Thomitzek, A.; Smorra, D.; Hejazi, M.; 678
Uhrberg, M.; Scheckenbach, K.; Monzel, C.; Wiek, C.; Reinhardt, D.; 679
Niktoreh, N.; Hanenberg, H. Genetic Engineering and Enrichment of 680
Human NK Cells for CAR-Enhanced Immunotherapy of Hemato- 681
logical Malignancies. *Front. Immunol.* **2022**, 13, 847008. 682
- (42) Bartels, N.; van der Voort, N. T. M.; Opanasyuk, O.; Felekyan, 683
S.; Greife, A.; Shang, X.; Bister, A.; Wiek, C.; Seidel, C. A. M.; 684
Monzel, C. Advanced Multiparametric Image Spectroscopy and 685
Super-Resolution Microscopy Reveal a Minimal Model of CD95 686
Signal Initiation. *Sci. Adv.* **2024**, 10 (35), eadn3238. 687
- (43) Neusch, A.; Siepe, C.; Zitzke, L.; Fux, A. C.; Monzel, C. 688
Semisynthetic Ferritin Nanocages for Flexible, Site-Specific Targeting 689
and Ligand-Free Activation of Membrane Receptors. *Nano Lett.* **2025**, 690
25 (52), 18006–18015. 691
- (44) Brandley, B. K.; Shaper, J. H.; Schnaar, R. L. Tumor Cell 692
Haptotaxis on Immobilized N-Acetylglucosamine Gradients. *Dev. Biol.* 693
1990, 140 (1), 161–171. 694
- (45) Ben-Reuven, L.; Reiner, O. Toward Spatial Identities in Human 695
Brain Organoids-on-Chip Induced by Morphogen-Soaked Beads. 696
Bioengineering **2020**, 7 (4), 164. 697
- (46) Dlamini, M.; Kennedy, T. E.; Juncker, D. Combinatorial 698
Nanodot Stripe Assay to Systematically Study Cell Haptotaxis. 699
Microsyst. Nanoeng. **2020**, 6 (1), 1–12. 700

Superparamagnetic Biomembranes

for Dynamic Nanoparticle and Molecular Patterning

*Luisa Coen^{1,2}, Jan Lange³, Andreas Neusch¹, Thomas Heinzel³, and Cornelia Monzel^{1,2} **

*corresponding author: cornelia.monzel@pi2.uni-stuttgart.de

¹ Experimental Medical Physics, Heinrich-Heine University Düsseldorf, 40225 Düsseldorf, Germany

² Present Address: 2nd Institute of Physics, University of Stuttgart, 70569 Stuttgart, Germany

³ Solid State Physics, Heinrich-Heine University Düsseldorf, 40225 Düsseldorf, Germany

SUPPLEMENTARY INFORMATION

Materials and Methods

All lipids: SOPC (1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine), DOPE-cap-biotin (1,2-dioleoyl-sn-glycero-3-phosphoethano-lamine-N-(cap biotiny), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and DOGS-NTA (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)) were purchased from Avanti Polar Lipids (Birmingham, AL, USA) and used without further purification. Synomag[®]-CLD-redF (surface: streptavidin, diameter: 70nm) were produced upon request by Micromod Partikeltechnologie GmbH (Rostock, GER) and were used as superparamagnetic nanoparticles. Thickness-corrected glass coverslips ($d = 170 \pm 5 \mu\text{m}$, Paul Marienfeld, Lauda-Königshofen,

GER) were cleaned as follows: ultrasonication in a 2% Hellmanex solution (Hellma, Müllheim, GER) for 15 min followed by two cycles of ultrasonication for the same duration in ultrapure water. Ultrapure water was produced by a water purification system (ELGA Purelab Flex, Veolia, High Wycombe, UK). After each ultrasonic treatment, coverslips were rinsed 10 times with ultrapure water. Supported Lipid Bilayers (SLBs) were prepared with a film balance (Biolin Scientific, KSV Nima, Gothenburg, SWE) using the Langmuir-Blodgett technique¹ for the first layer, and a modified Langmuir-Schäfer technique for the second layer. As modification for the second layer, the glass substrate with the first layer was pressed onto the second lipid layer and into the subphase. The subphase was ultra-pure water. The first membrane layer consisted of 2 mg/ml pure SOPC or DPPC. In the second layer, SOPC or DPPC were used as matrix lipid combined with different concentrations of the linker lipids DOPE-cap-biotin and DOGS-NTA. All SLB layers were prepared at a transfer pressure of 25 mN/m and constantly kept under water. After preparation, the water was exchanged for phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM, KH₂PO₄, pH 7.5 with 1% Penicilin/Streptomycin (P/S) (Sigma-Aldrich, St. Louis, MO, USA)) In order to passivate the SLB surface and to diminish unspecific binding, SLBs were incubated with BSA (Bovine Serum Albumin Fraction V, ITW Reagents, Monza, ITA) dissolved in PBS (0.5 mg/ml) for 15 min. Afterwards SLBs were incubated with synomag[®] MNPs (16 µg/ml) for 1h and rinsed 10-times with PBS. For competitive protein coupling, MNP and NAVOG (Thermo Fisher Scientific, Waltham, MA, USA) were incubated simultaneously at a MNP to protein ratio of 1:5. In contrast, mEGFP and CD19 (Sino Biological, Beijing, CHN) were not competitively coupled. Instead, they were attached via His-tag-mediated binding to the DOGS-NTA lipids in the SLB. mEGFP (10 µg/ml) or CD19 (0.9 µg/ml) was incubated on the SLB overnight. After ten washing steps with PBS, MNPs were added as described above.

Fluorescence images were acquired in widefield epi-fluorescence mode using either the IX73 (for manual imaging) or IX83 (for automated imaging) microscope systems from Olympus (Olympus Corporation, Tokyo, JPN) equipped with a 60x, NA 1.25 objective (Olympus Corporation, 60x Planfluor PH3 UPLFLN60XOIPH/1.25, Tokyo, JPN) was used. SLBs were examined at room temperature, 37 °C, or 45 °C. Heating was controlled by the Tempcontroller 2000-2 (PeCon GmbH, Erbach, GER) with corresponding heating stage.

The single-tip configuration consisted of a cube magnet ($5 \times 5 \times 5 \text{ mm}^3$, NdFeB), a cuboid magnet ($10 \times 4 \times 1.2 \text{ mm}^3$, NdFeB), which were magnetically coupled and used as a single magnetic unit, and a custom-fabricated tip (piano wire of key C8 (88th key), which was heated in the flame of a Bunsen burner and manually pulled apart). For the double- and triple-tip configuration, two or three custom-fabricated tips, respectively, were mounted onto the magnetic unit. Alternatively, a commercially available razor blade was cut to size and attached to the magnetic unit to obtain the razor blade configuration. For the soft ferro-/diamagnetic composite stencil configuration, a master mold was first fabricated prior to PDMS casting and subsequent attachment to the cubic magnet. The fabrication procedure is described in detail below: For fabrication of photoresist master molds, prime grade 4" Si wafers with thickness of 0.5 mm (Nova Electronic Materials, Flower Mound, TX, USA) were laser-diced into 30 mm substrates using a laser cutting device. The substrates were cleaned by sequential puddle rinsing with acetone, isopropanol and water, followed by drying with nitrogen flow. Photoresist master molds for PDMS casting were fabricated using the negative-tone photoresist AZ 125nXT-10B (MicroChemicals GmbH, Ulm, GER). The resist was spincoated (WS-400B-6NPP/LITE, Laurell Technologies Corporation, Lansdale, PA, USA) with final spin speed of 1600 rpm to 3000 rpm for 12 s to reach a film thickness of approximately 20 μm to 50 μm . Edge bead removal was performed using AZ EBR solvent (MicroChemicals GmbH). After soft baking at 130 °C for 13 min, the samples were exposed for 128 s through a chromium photomask (parallel

stripes with a width of 40 μm and equal spacing of 40 μm) using a mask aligner (MJB3, SUSS MicroTec SE, Garching, DE). Development was carried out with AZ 726 MFI developer (MicroChemicals GmbH) for 60 s to 90 s, followed by rinsing with ultrapure water and nitrogen drying. To fully crosslink the photoresist and improve mechanical stability for repeated PDMS replication, the patterned master molds were flood-exposed for 256 s without a mask. Soft ferro-/diamagnetic composite stencils were fabricated using the master molds described above. For the “magnetic” layer, a PDMS-iron composite was prepared by mixing iron (Fe) micron powder (grain size 5 μm ; Nanografi Nano Teknoloji A.Ş., Ankara, TUR) with PDMS prepolymer and curing agent at a mass ration of 0.5 g iron powder, 1.5 g prepolymer and 0.2 g curing agent (SYLGRADTM 184 Silicone Elastomer Kit, DOW Silicones Deutschland GmbH, Wiesbaden, GER). The mixture was dispensed onto the master mold and spin-coated (100 rpm, 60 s, 200 rpm s⁻¹, Spin150, Semiconductor Production Systems, Putten, NLD), and afterwards cured overnight at 65 °C. After curing, the PDMS-iron layer was carefully peeled off the master mold. The resulting structured PDMS-iron layer, containing the negative replica of the master mold, was placed with the patterned side facing upward on a glass substrate. A conductive PDMS-graphite composite was then prepared by mixing graphite micron powder (grain size 5-10 μm , Nanografi Nano Teknoloji A.Ş.) with PDMS prepolymer and curing agent (0.18 g graphite powder, 1.82 g prepolymer and 0.2 g curing agent). This mixture was dispensed onto the structured surface and spin-coated (3000 rpm, 60 s, 200 rpm s⁻¹). The assembly was cured overnight at 65 °C. After curing, the completed PDMS stamp was carefully released from the glass substrate, trimmed to size and mounted onto the cubic magnet for subsequent use as a soft ferro-/diamagnetic composite stencil. In this configuration, the diamagnetic graphite layer is important to concentrate the magnetic field lines within the iron-rich PDMS phase, thereby enhancing the local magnetic field gradients sufficiently to enable the formation of stripe-like MNP patterns on the SLB.

For all experiments, the respective magnetic tip configuration was controlled using a micromanipulator (Inject man 4, Eppendorf SE, Hamburg, GER).

For Transmission electron microscopy (TEM), MNP in buffer solution (0.05 mg/ml) were dropped onto Formvar coated nickel grids (200 mesh, S162N-100, Plano GmbH, Wetzlar, Germany) and left to sediment for 1 min. Solution remaining on the grid was removed between each step using filter paper. After sedimentation, the sample was negatively stained twice for 3 to 5 s and 30 s by subsequently placing the grid onto two separate drops of 2 % uranyl acetate. The remaining solution was removed and the grid was left to air dry. Imaging was performed on a Jeol JEM-2100Plus (Akishima, Tokyo, Japan), operating in bright field mode at an acceleration voltage of 80 kV.

For Scanning Hall Probe Microscopy, the magnetic flux density field of the magnetic tip was determined by scanning a motorized Hall element above the tip, using a self-made setup.² The scan parameters were: pixel size = $1 \times 1 \mu\text{m}^2$, scan area = 100×100 pixel, probe size = $5 \mu\text{m}$, bias current = $7 \mu\text{A}$, sensitivity = $200 \pm 0.5 \text{ ohm/T}$.

The NK92 cell line used in this study was generated at the Department of Pediatrics at the University Hospital Essen, by stable lentiviral transduction with mNeonGreen (NG) genetically fused to the Chimeric Antigen Receptor (CAR) against the respective protein of interest, here CD19. This resulted in the stable CAR NK Cell Line: NK92 CD19 NG. Cells were maintained in 9 ml RPMI (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) medium, supplemented with 10 % FCS (Fetal Calf Serum, Gibco), 1 % P/S and 250 IU Interleukin-2 (Proleukin® S 18x106 IU, Clinigen Healthcare, Schiphol, NDL). Cultures were maintained in T25 flasks at 37°C with 5% CO₂, passaged and additionally diluted with fresh medium weekly. For experiments 1×10^5 cells were seeded onto SLBs. Prior to cell addition, the PBS on the SLBs was exchanged with L-15 Medium (Gibco) supplemented with 10 % FCS, 1 % P/S and 250 IU Interleukin-2. For magnetic pattern generation in the cell experiments, DPPC-based SLBs were

heated well above the lipid phase transition temperature to approximately 56 °C in order to induce membrane fluidity and enable lateral MNP redistribution. A magnetic tip positioned at a distance of approximately 40 µm from the SLB surface was used to accumulate MNPs for approximately 30 min. Subsequently, the system was cooled to 37 °C while maintaining the magnetic tip position in order to prevent field-free MNP redistribution during membrane solidification. After complete cooling and stabilization of the SLB for approximately 1 h at 37 °C, the magnetic tip was removed prior to cell addition. Fluorescence images of the cells were acquired approximately 20 min after cell seeding. Importantly, cells were therefore exposed only to immobilized MNP patterns in the absence of active magnetic field gradients during incubation and imaging. No obvious indications of substantial nanoparticle internalization were observed on experimental timescales.

Custom-written Matlab (Version R2021a, The MathWorks Inc., Natick, MA, USA) scripts were used for quantitative image analysis. For the force calculation from MNP accumulation, fluorescence images were acquired at time points 0, 5, 30, 100, 240, 300, 600, 900, 1200, 1500, 1800, 2100, and 2400 s. The image corresponding to the final time point, exhibiting the highest MNP accumulation, was used to identify the central reference point of the accumulated spot. Fluorescence intensity, serving as a measure of MNP concentration, was evaluated as a function of time for each radius. For the analysis of the diffusion coefficient from the relaxation of the MNP, an image acquired at the end of the accumulation phase was used to determine the center of the MNP distribution. Concentric radii up to a distance of 40 µm from the center were defined. Fluorescence intensity, serving as a measure of MNP concentration, was evaluated over time for each radius. Images were acquired every 20 s for a total duration of 45 min. The velocity and diffusion coefficient of MNPs were determined as described in the main text. From the velocity the magnetic force distribution F_{xy} was obtained.

SUPPLEMENTARY IMAGES

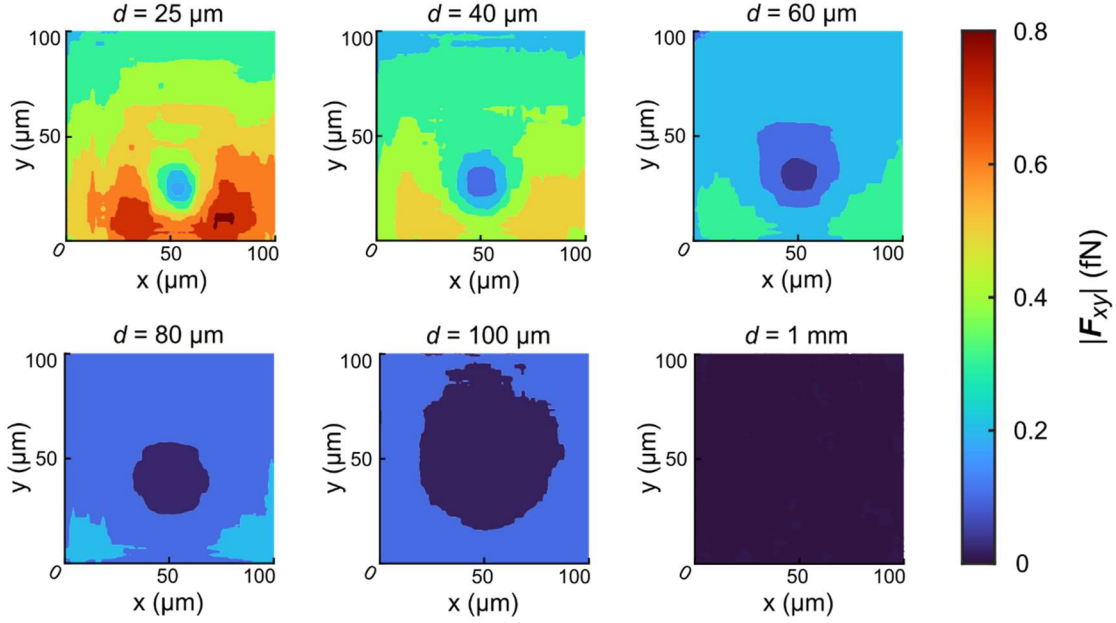


Figure S1: Magnetic force profiles of single magnetic tip at different distances d . Lateral magnetic force F_{xy} calculated from lateral scans of the magnetic flux density of the tip at distances $d = 25 \mu\text{m}$, $d = 40 \mu\text{m}$, $d = 60 \mu\text{m}$, $d = 80 \mu\text{m}$, $d = 100 \mu\text{m}$, and $d = 1 \text{ mm}$.

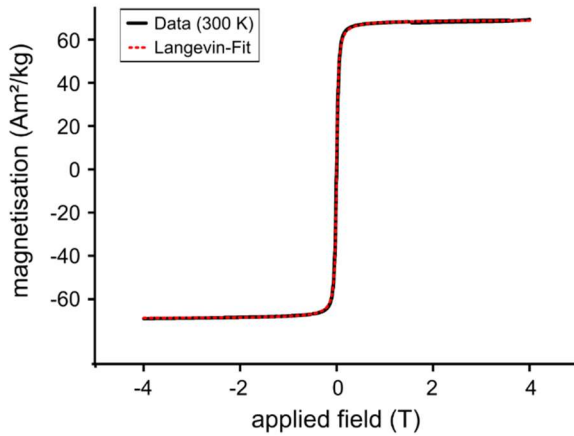


Figure S2: Magnetization curve of MNP at 300 K. The Langevin function $M = M_0 L(\beta m H) = M_0 \left[\coth(\beta m H) - \frac{1}{\beta m H} \right]$ is used as fit function (red line) to determine the magnetization of the MNPs. Here M is the magnetization, M_0 the saturation magnetization, H the applied magnetic field and $\beta = \frac{1}{k_B T}$, with k_B the Boltzmann constant and T the temperature.

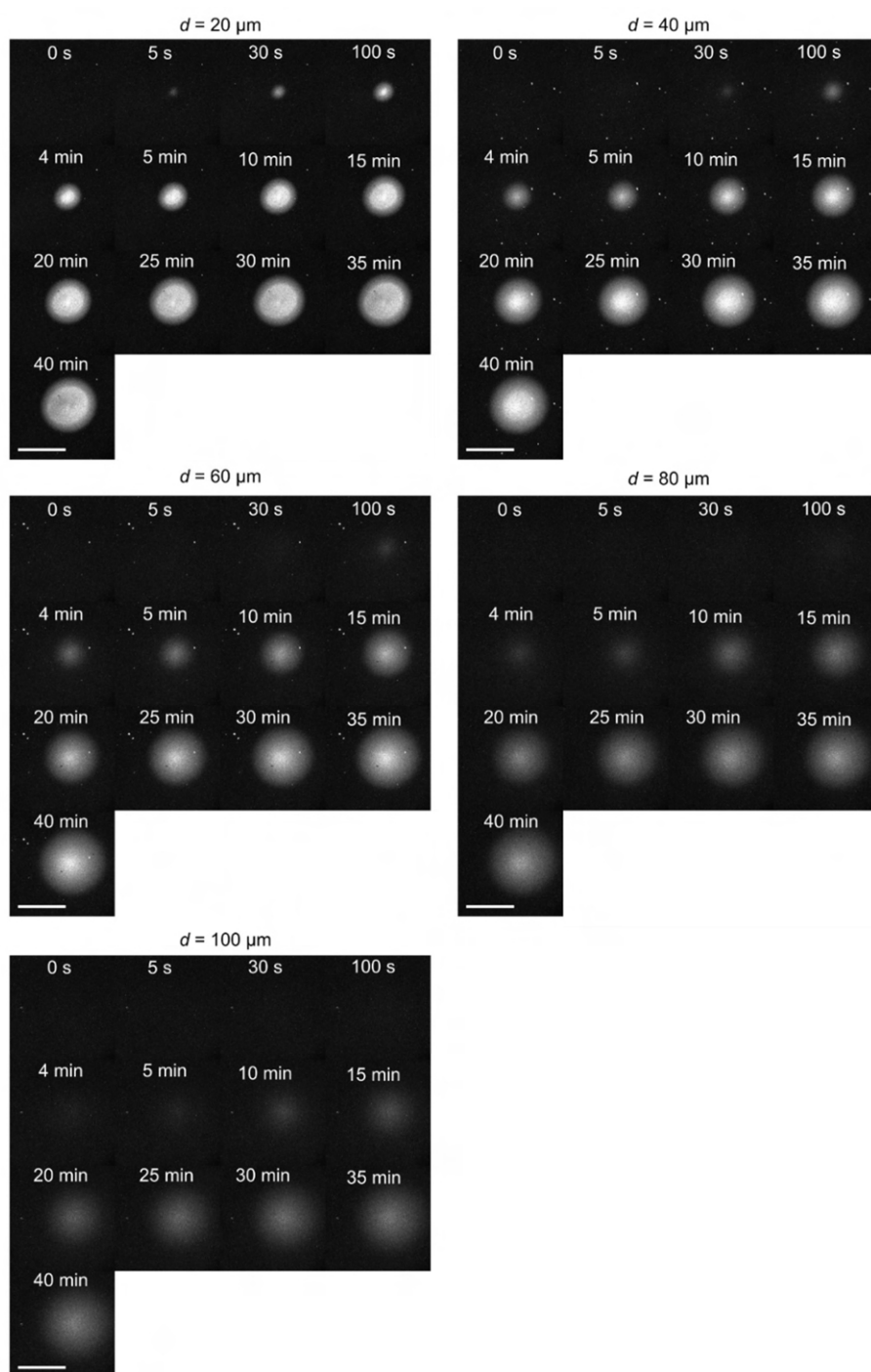


Figure S3: Micrographs of fluorescent MNPs during magnetic attraction. During attraction, the magnetic tip was placed at distances $d = 20 \mu\text{m}$, $40 \mu\text{m}$, $60 \mu\text{m}$, $80 \mu\text{m}$, $100 \mu\text{m}$ above the SLB. Scale bars 100 μm . Fluorescence intensity was globally normalized across all images. Micrographs at distance $d = 20 \mu\text{m}$ are further analysed for Figure 2b left (gray), Figure 2c left (gray). Micrographs for all distances at 5 min and 40 min are further analysed for Figure 3c.

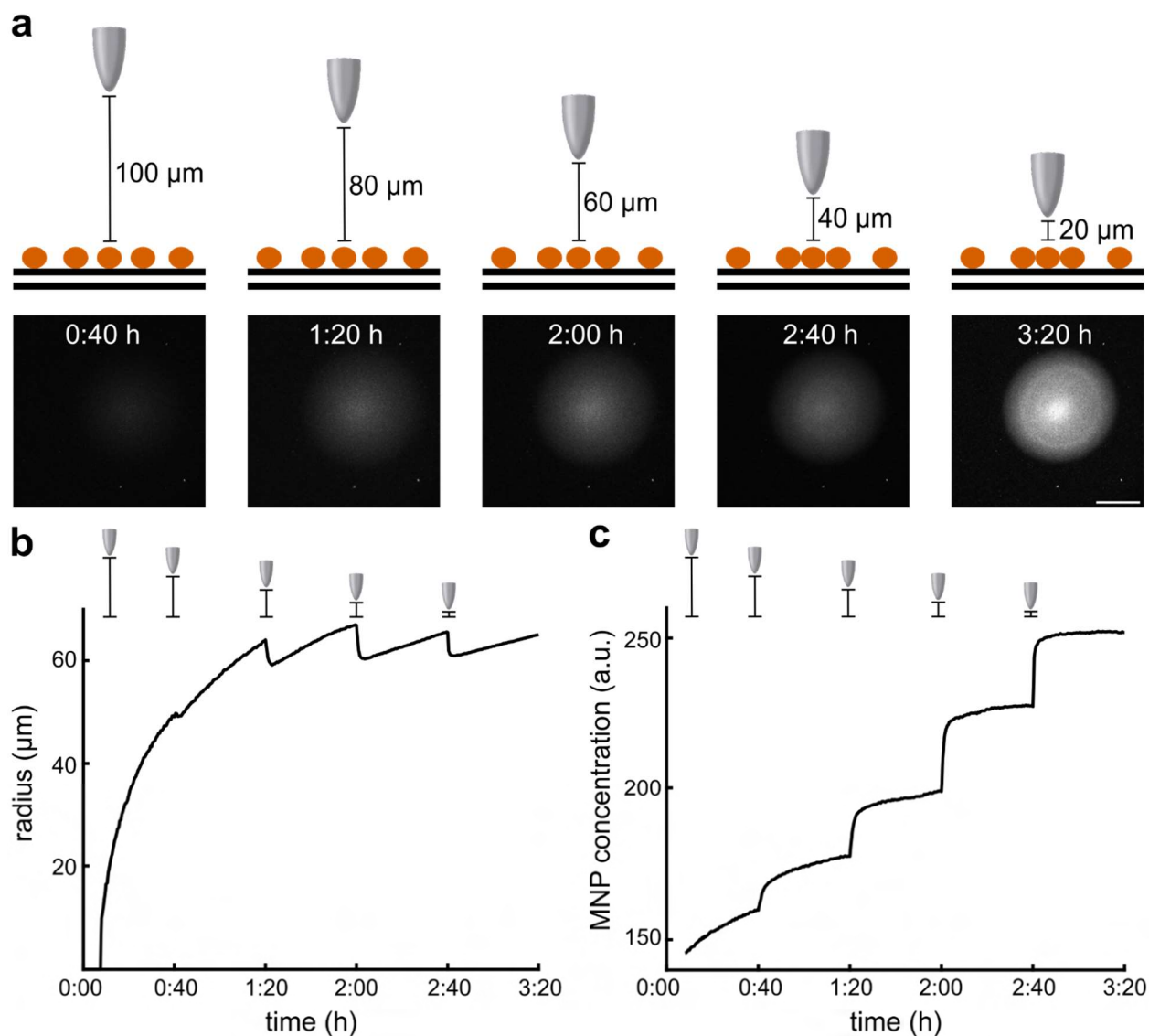


Figure S4: Dynamics of MNP attraction as a function of d . a: During magnetic attraction of fluorescent MNPs, the magnetic tip was approached to the MNP-SLB in steps of 20 μm , placing the tip at distance $d = 100 \mu\text{m}$, 80 μm , 60 μm , 40 μm and 20 μm . Scale bar 100 μm . At each position, the tip resided for 40 min until a new equilibrium state was (nearly) achieved. b: Change of MNP accumulated spot radius over time. c: Change of MNP concentration over time. See also **Video S5**.

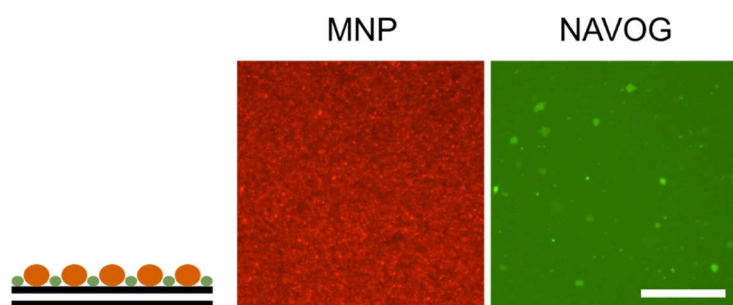


Figure S5: Fluorescence images of NAVOG-functionalized SLBs prior to magnetic manipulation. Local fluorescence intensity heterogeneities and bright spots are already present before magnetic MNP accumulation, indicating that these features do not originate from magnetic nanoparticle-induced protein redistribution. Scale bar: 20 μm .

SUPPLEMENTARY REFERENCES

- (1) Blodgett, K. B. Films Built by Depositing Successive Monomolecular Layers on a Solid Surface. *J. Am. Chem. Soc.* **1935**, 57 (6), 1007–1022.
<https://doi.org/10.1021/JA01309A011>.
- (2) Shaw, G.; Kramer, R. B. G.; Dempsey, N. M.; Hasselbach, K. A Scanning Hall Probe Microscope for High Resolution, Large Area, Variable Height Magnetic Field Imaging. *Review of Scientific Instruments* **2016**, 87 (11).
<https://doi.org/10.1063/1.4967235/363115>.

3.3 Publication III: Ligand-Defined Phase Space Predicts CAR-NK Synapse Architecture and Cytotoxic Organization.

Authors: Luisa Coen, Miriam N. Tappel, Lara Hölzle, Maren Soldierer, Nicole Sander, Constanze Wiek, Helmut Hanenberg, Cornelia Monzel

Journal: *Nature Communications*

In Preparation

Contribution

The doctoral candidate (LC) made a major contribution to this publication, accounting for approximately 50% of the overall work. This included the preparation of all SLBs and the execution of the confocal experiments for the analysis of absolute CAR concentration, as well as the complete data evaluation associated with these measurements. In addition, the doctoral candidate extensively validated the biomimetic system through numerous preliminary experiments to ensure the reliability and robustness of the quantitative approach. Multicolor and time-laps imaging experiments were predominantly performed by other co-authors.

Furthermore, the doctoral candidate contributed substantially to the interpretation of the data and played a leading role in deriving the central conclusions of the study. The doctoral candidate was also the main contributor to the manuscript preparation, including drafting the majority of the manuscript text and creating approximately half of the figures. The remaining contributions, including complementary experiments, additional analysis, supervision, and conceptual input, were provided by the co-authors as specified in the original publication.

Ligand-Defined Phase Space Predicts CAR-NK Synapse Architecture and Cytotoxic Organization

Authors

Luisa Coen^{1,2}, Miriam N. Tappel², Lara Hölzle¹, Maren Soldierer⁴, Nicole Sander³, Constanze Wiek³, Helmut Hanenberg^{3,4}, Cornelia Monzel^{1,2*}

Affiliations

- 1 Experimental Medical Physics, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany
- 2 present address: 2nd Physics Institute, University Stuttgart, 70569 Stuttgart, Germany
- 3 Department of Otorhinolaryngology (ENT), University Hospital Düsseldorf, 40225 Düsseldorf, Germany
- 4 Department of Pediatrics, University Hospital Essen, 45147 Essen, Germany

Abstract

CAR-engineered natural killer (CAR-NK) cells are promising effectors for solid tumor immunotherapy, yet the organizational principles governing their immune synapses remain poorly understood. Here, we establish a biomimetic supported lipid bilayer (SLB) platform to systematically investigate how antigen and adhesion ligand density shape CAR-NK synapse architecture. Using B7 Homolog3 (B7-H3)-specific CAR-NK cells derived from NK-92, KHYG-1 and primary human NK cells, we quantitatively compare synapses formed on defined SLBs with those established on head and neck squamous cell carcinoma (HNSCC) and hepatocellular (HCC) cells.

We identify multiple distinct CAR synapse morphologies, including multifocal, edge-ring and centralized receptor organizations, demonstrating that CAR-NK cells do not adopt a single stereotypic synaptic architecture. Instead, synapse topology emerges as a ligand-dependent organizational continuum governed by the combined availability of B7-H3 and ICAM-1. Mapping these morphologies across defined ligand combinations revealed a predictive “synapse phase space” that links target-cell ligand composition to emergent CAR interface states.

Importantly, specific SLB conditions accurately reproduced the synapse architectures observed on UM-14C and UM-17A tumor cells, demonstrating that complex tumor-associated interfaces can be reconstructed using reductionist ligand environments. Together, our findings establish a predictive framework for CAR-NK synapse organization and provide mechanistic insight into how antigen and adhesion landscapes shape engineered immune-cell interfaces.

1. Introduction

Cancer remains one of the leading causes of mortality in industrialized countries, including Germany¹ and the United States², despite major advances in surgery, radiotherapy, and chemotherapy^{3,4}. In recent years, immunotherapies have significantly improved clinical outcomes by harnessing the patient’s immune system to selectively eliminate malignant cells^{5–7}. Among these approaches, chimeric antigen receptor (CAR)-

engineered immune cells have emerged as a powerful strategy for redirecting cytotoxic effector functions toward tumor-associated antigens⁸.

CAR T-cell therapies have demonstrated remarkable efficacy in hematological malignancies^{8–12}. However, their translation to solid tumors remains limited by multiple biological and physical barriers, including immunosuppressive tumor microenvironments^{13–15}, heterogeneous antigen expression^{14,16,17}, restricted tumor infiltration^{18,19}, and on-tumor/off-target toxicities^{20,21}. These limitations have motivated the exploration of alternative effector cell types with improved intrinsic anti-solid-tumor properties.

Natural killer (NK) cells therefore represent a particularly promising platform for CAR engineering, as they are considered intrinsically better suited for targeting solid tumors than conventional CAR-T cells^{22,23}. In addition to their innate ability to recognize and eliminate transformed cells in a non-MHC-restricted manner, NK cells can promote pro-inflammatory immune responses and retain endogenous cytotoxic activity even in the absence of CAR target recognition, thereby enabling dual-mode tumor elimination²⁴. Importantly, CAR-NK cell therapies have been associated with a reduced risk of cytokine release syndrome compared to CAR-T cells, highlighting their potential safety advantage for clinical translation^{25–27}. Clinically relevant NK cell sources include primary NK cells²⁸ as well as established cell lines such as NK-92^{29,30} and KHYG-1^{31,32}, which enable “on-the shelf” products.

A central determinant of CAR-NK cell function is the formation of the immune synapse, which serves as the physical and molecular interface for target recognition, receptor organization, and signal integration²⁶. Despite its importance, the structural principles governing CAR synapse formation, particularly in NK cells, remain incompletely understood. This is especially relevant in solid tumor settings, where spatial heterogeneity of antigen expression and adhesion molecule availability may critically influence synapse architecture and cytotoxic output¹⁸.

The transmembrane glycoprotein B7 Homolog3 (B7-H3 or CD276) represents a clinically relevant CAR target that is frequently overexpressed in head and neck squamous cell carcinoma (HNSCC) and other epithelial malignancies, while exhibiting limited expression in most healthy tissues^{33,34}. This expression profile makes B7-H3 an attractive antigen for CAR-based immunotherapy, particularly in the context of solid tumors where target accessibility and specificity are critical determinants of therapeutic efficacy.

Supported lipid bilayers (SLBs) provide a powerful biomimetic platform to systematically dissect immune cell–target interfaces under defined molecular conditions. As planar, compositionally tunable membranes, SLBs allow precise control over antigen and adhesion ligand density, thus allowing the reduction of complex cellular interfaces to defined molecular parameters. Previous studies have established SLBs as robust models for studying T cell immunological synapses^{35–38}; however, their potential to recapitulate CAR-NK cell synapse organization and function remains less explored.

In this study, we establish a defined SLB system functionalized with B7-H3 and intracellular adhesion molecule 1 (ICAM-1) to investigate how ligand density governs CAR-NK cell synapse architecture. Using B7-H3-specific CAR-NK cells derived from NK-92, KHYG-1, and primary human NK cells, we systematically compare biomimetic interfaces formed on SLBs with immunological synapses established on B7-H3-expressing tumor cell lines representing distinct epithelial malignancies. Beyond descriptive characterization of synapse morphology, we identify a ligand density-dependent organizational regime in which CAR and lymphocyte function-associated antigen 1 (LFA-1) distribution patterns are encoded by the combined availability of antigen (B7-H3) and adhesion molecules (ICAM-1). Strikingly, this relationship facilitates the construction of a quantitative “synapse phase space”, in which distinct synaptic architectures can be predicted based on local B7-H3 and ICAM-1 expression levels. This framework allows the synaptic organization formed on heterogeneous tumor cells to be mapped onto a defined ligand landscape derived from SLB measurements, thereby enabling prediction of CAR-NK cell interface architecture directly from target cell surface composition.

2. Results

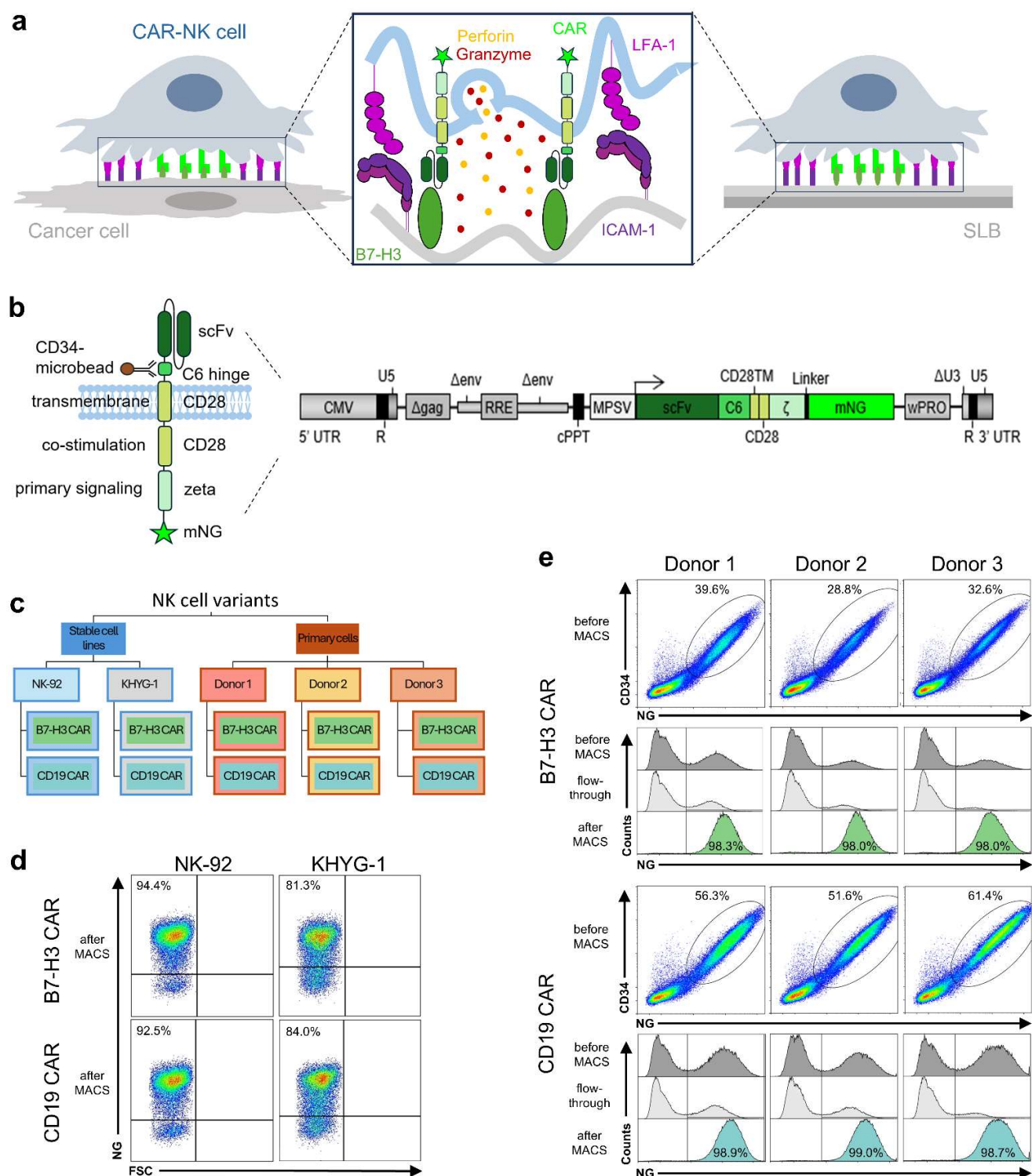


Figure1: Generation and enrichment of B7-H3- and CD19-specific CAR-NK cells for SLB synapse assays. **a**, Schematic illustration of the experimental system used to investigate CAR-NK cell immunological synapse formation on SLBs. CAR-NK cells interact either with antigen-presenting cancer cells or with biomimetic SLBs functionalized with B7-H3 and ICAM-1 ligands. **b**, Design of the second-generation lentiviral CAR construct. The CAR backbone comprises a CD8 leader peptide, and antigen-specific scFv, a CD34-derived C6 hinge region enabling magnetic enrichment with anti-CD34 microbeads, the CD28 transmembrane and intracellular signaling domains, and the CD3 ζ activation domain. A

fluorescent mNeonGreen (mNG) reporter was included downstream the CD3 ζ domain to identify transduced cells. **c**, Overview of CAR-NK cell populations generated in this study. Two NK cell lines (NK-92 and KHYG-1) and primary human NK cells from three independent donors were transduced with either B7-H3-specific or CD19-specific CAR constructs, yielding 10 experimental cell populations. **d**, Flow cytometry plots of NK-92- and KHYG-1- derived CAR-NK cells after MACS, showing high frequencies of mNG-positive CAR-expressing cells in both B7-H3-CAR and CD19-CAR populations. **e**, Flow cytometric analysis of primary human NK cells from three donors before and after MACS enrichment. Top panels show B7-H3-CAR-transduced cells and bottom panels CD19-CAR-transduced cells. Histograms demonstrate efficient enrichment of mNG-positive CAR-expressing cells to >98% purity following MACS sorting.

Robust generation of highly enriched B7-H3-specific CAR-NK cell populations for biomimetic synapse studies

To investigate CAR-NK cell synapse architecture on tumor cells and SLBs (**Figure 1a**), we generated a panel of CAR-expressing NK cell populations using a second-generation lentiviral CAR construct containing a CD8 leader peptide, a CD34-derived hinge region (C6), CD28 transmembrane and intracellular domains, and the CD3 ζ signaling chain (**Figure 1b**). The extracellular scFv was directed either against B7-H3 or, as a specificity control, against CD19. All constructs additionally encoded the fluorescent reporter mNeonGreen (mNG) downstream of CD3 ζ .

Using these vectors, we transduced the NK cell lines NK-92 and KHYG-1 as well as primary human NK cells from three independent donors, generating ten distinct CAR-NK populations (**Figure 1c**). CAR-positive cells were enriched by CD34-based magnetic sorting as described previously³⁹. Flow cytometry revealed CAR expression frequencies of 81–84% in enriched NK-92 and KHYG-1 populations (**Figure 1d**). In primary NK cells, transduction efficiencies increased from 29–40% before enrichment to >98% after sorting (**Figure 1e**). Forward scatter profiles indicated preserved cell integrity across all populations. These experiments yielded highly enriched CAR-positive NK-92, KHYG-1, and primary NK cell populations for subsequent synapse imaging analyses.

B7-H3 CAR-NK cells mediate antigen-specific cytotoxicity and serial tumor cell killing

To validate the functionality of the generated CAR-NK populations, we analyzed synapse formation and cytotoxicity against B7-H3-positive UM-14C tumor cells. Confocal imaging demonstrated stable contacts between B7-H3 CAR-NK cells and UM-14C targets across NK-92, KHYG-1, and primary NK cell populations (**Figure 2a**). Pronounced CAR-derived fluorescence accumulated at the contact interface, consistent with directed receptor recruitment and mature synapse formation. In contrast, CD19 CAR-expressing control cells failed to establish stable tumor cell contacts.

Live-cell imaging further revealed dynamic CAR-NK-mediated cytotoxicity (**Figure 2b**). Shortly after initial contact, multiple UM-14C cells accumulated around localized killing regions, followed by detectable apoptosis after ~75 min and extensive tumor cell elimination during prolonged incubation. After 16 h, NK-92 B7-H3 CAR-NK cells eliminated ~80% of UM-14C target cells.

Quantitative analysis revealed marked differences in cytotoxic potency between CAR-NK populations (**Figure 2c,d**). NK-92 B7-H3 CAR-NK cells showed the strongest anti-tumor activity, reaching ~50% target cell death within 2 h and a maximal apoptosis fraction of 74%. In contrast, KHYG-1 B7-H3 CAR-NK cells displayed slower killing kinetics and a maximal apoptosis fraction of 24%. Neither NK-92 nor KHYG-1 CD19 CAR controls induced substantial killing, confirming antigen specificity.

Primary B7-H3 CAR-NK cells also mediated efficient cytotoxicity with a maximal apoptosis fraction of 46% (**Figure 2c**). To minimize CAR-independent killing, experiments with primary NK cells were performed in the presence of a blocking antibody cocktail. Despite analysis at a higher effector-to-target ratio (3:1 versus 1:1 for cell lines), primary B7-H3 CAR-NK cells induced substantial tumor cell

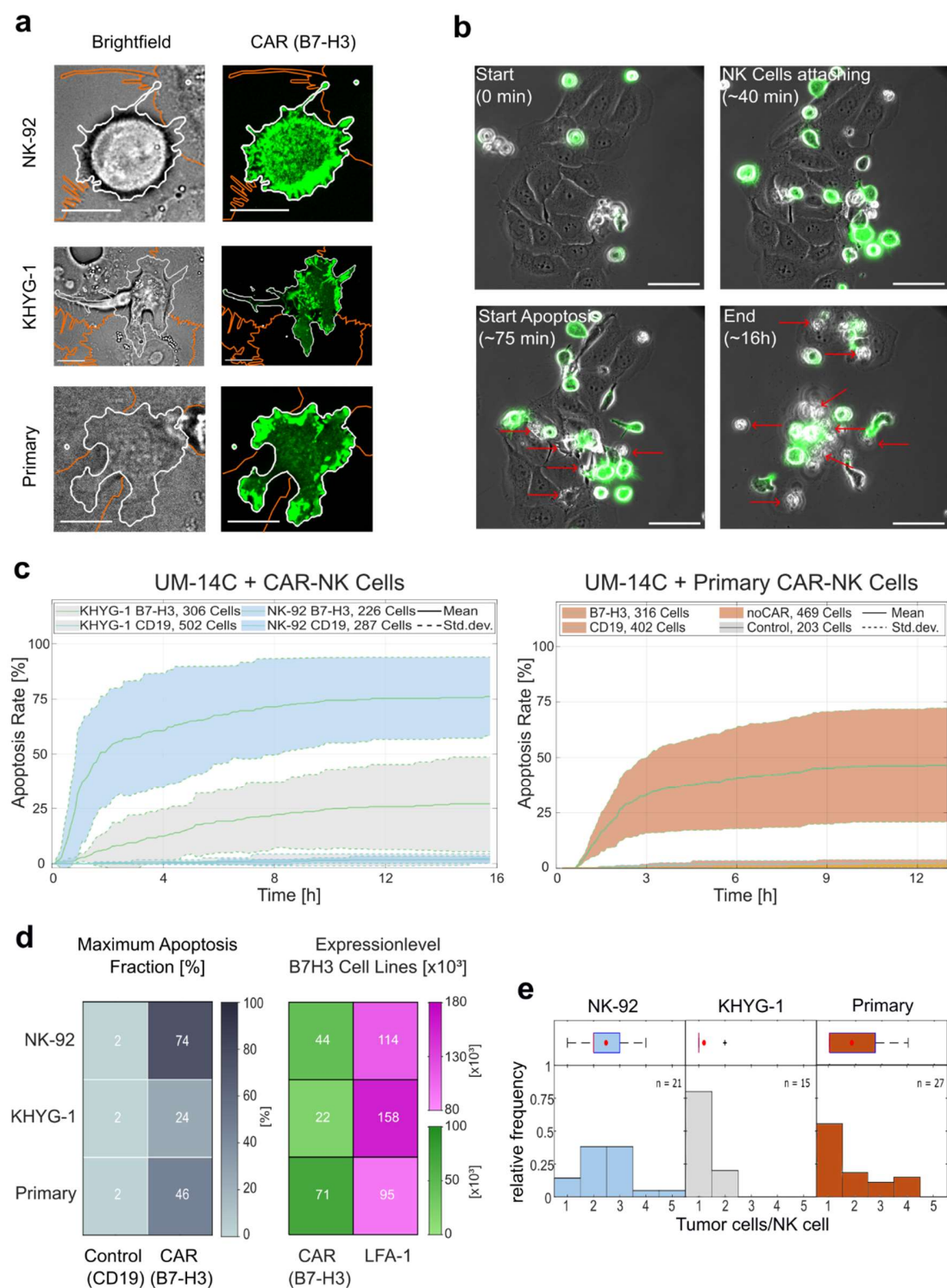


Figure 2: Cytotoxic activity of (primary) CAR-NK cells against UM-14C tumor cells. **a**, Brightfield and confocal microscopy images showing immunological synapse formation between different CAR-NK cell types and UM-14C cells. CAR-NK cells are masked in white, while UM-14C cells are shown in orange. Scale bar: 10 μ m. **b**, Representative merged images of PH and EPI of NK-92 B7-H3 CAR-NK cells (green) on UM-14C (E:T 1:1) during a killing assay. Scale bar: 50 μ m. **c**, Killing efficiency of different CAR-NK cells at effector-to-target ratios of 1:1 (cell lines) and 1:3 (primary cells) against UM-14C cells. **d**, Maximum apoptosis fraction induced by different CAR-NK cell lines and primary CAR-NK cells, including target-specific CARs and CD19 CAR controls. **e** Serial killing analysis showing the number of tumor cells

eliminated per individual CAR-NK cell. Box plots indicate median and mean numbers of kills per effector cell: NK-92 B7-H3 NG (median = 2.0, mean = 2.5), KHYG-1 B7-H3 NG (median = 1.0, mean = 1.2), and primary NK cells (median = 1.0, mean = 1.9).

elimination, whereas untransduced and CD19 CAR-expressing primary NK cells showed minimal cytotoxicity.

To relate cytotoxicity to receptor expression, surface levels of B7-H3 CAR and LFA-1 were quantified across all populations (**Figure 2d**). Primary NK cells exhibited the highest CAR expression, followed by NK-92 and KHYG-1 cells. Among the engineered cell lines, higher CAR expression correlated with stronger cytotoxicity in NK-92 cells. In contrast, LFA-1 expression was highest in KHYG-1 cells, indicating that killing efficiency was not solely determined by adhesion receptor abundance.

Additional killing assays were performed using UM-17A and HLE tumor cells (**Figure S1**). HLE cells expressed very low B7-H3 levels, whereas UM-14C and UM-17A cells showed overexpression (**Figure S2**). Overall, CAR-mediated killing was substantially more efficient against UM-14C and UM-17A cells than against HLE cells, indicating that sufficient antigen availability is critical for efficient CAR-NK cytotoxicity.

Despite comparable B7-H3 expression on UM-14C and UM-17A cells, killing efficiency was markedly lower on UM-17A cells, where NK-92 and KHYG-1 B7H3 CAR-NK cells reached maximal apoptosis fractions of ~25% and ~15%, respectively. In contrast, both CAR-NK populations eliminated UM-14C cells substantially more efficiently, suggesting additional tumor-intrinsic factors beyond B7-H3 abundance. On HLE cells, overall cytotoxicity remained weak. Under these conditions, KHYG-1 B7-H3 CAR-NK cells showed the strongest activity (~20% apoptosis), whereas NK-92 B7-H3 CAR-NK cells induced minimal killing. NK-92 CD19 CAR cells exhibited low but detectable cytotoxicity (~10%), while all remaining populations remained below 5% apoptosis induction. These findings indicate that under low-antigen conditions, additional cellular properties may contribute to tumor cell elimination.

Single-cell tracking further revealed pronounced differences in serial killing capacity (**Figure 2e**). NK-92 B7-H3 CAR-NK cells showed the strongest multi-killing phenotype, eliminating up to five tumor cells per effector cell during the 16 h observation period. KHYG-1 B7-H3 CAR-NK cells killed a maximum of two target cells, whereas primary B7-H3 CAR-NK cells eliminated up to four. Together, these findings identify NK-92 B7-H3 CAR-NK cells as the most potent and persistent cytotoxic population among the investigated NK cell models. NK-92 and primary B7-H3 CAR-NK cells were therefore selected for subsequent comparative synapse analyses.

Multicolor mapping of CAR and LFA-1 organization identifies distinct synaptic interface states reproduced by defined biomimetic SLBs

To investigate the spatial organization of antigen recognition and adhesion receptors during CAR-NK synapse formation, we performed multicolor confocal imaging of B7-H3 CAR-NK cells interacting with tumor cells and analyzed CAR and LFA-1 distribution at the contact interface. Across all tumor cell conditions, six recurrent synapse categories were identified based on characteristic CAR and LFA-1 organization patterns (**Figure 3a**).

LFA-1 distributions could be grouped into three principal morphologies. Category 1 showed a relatively homogeneous interface distribution without pronounced enrichment. Categories 3, 4, and 6 displayed radial intensity gradients decreasing from the synapse center toward the periphery, whereas Categories 2 and 5 exhibited reduced intermediate intensity combined with an additional peripheral maximum, indicating preferential outer-interface accumulation. CAR organization displayed greater structural diversity. Categories 1, 2, and 4 showed pronounced peripheral CAR enrichment resembling edge-ring (ER) morphologies, Category 3 exhibited a broad multipattern (MP)-like organization, Category 5 combined central (CP) and peripheral enrichment characteristic of ER+CP architectures, and Category 6 represented an intermediate ER+MP hybrid state.

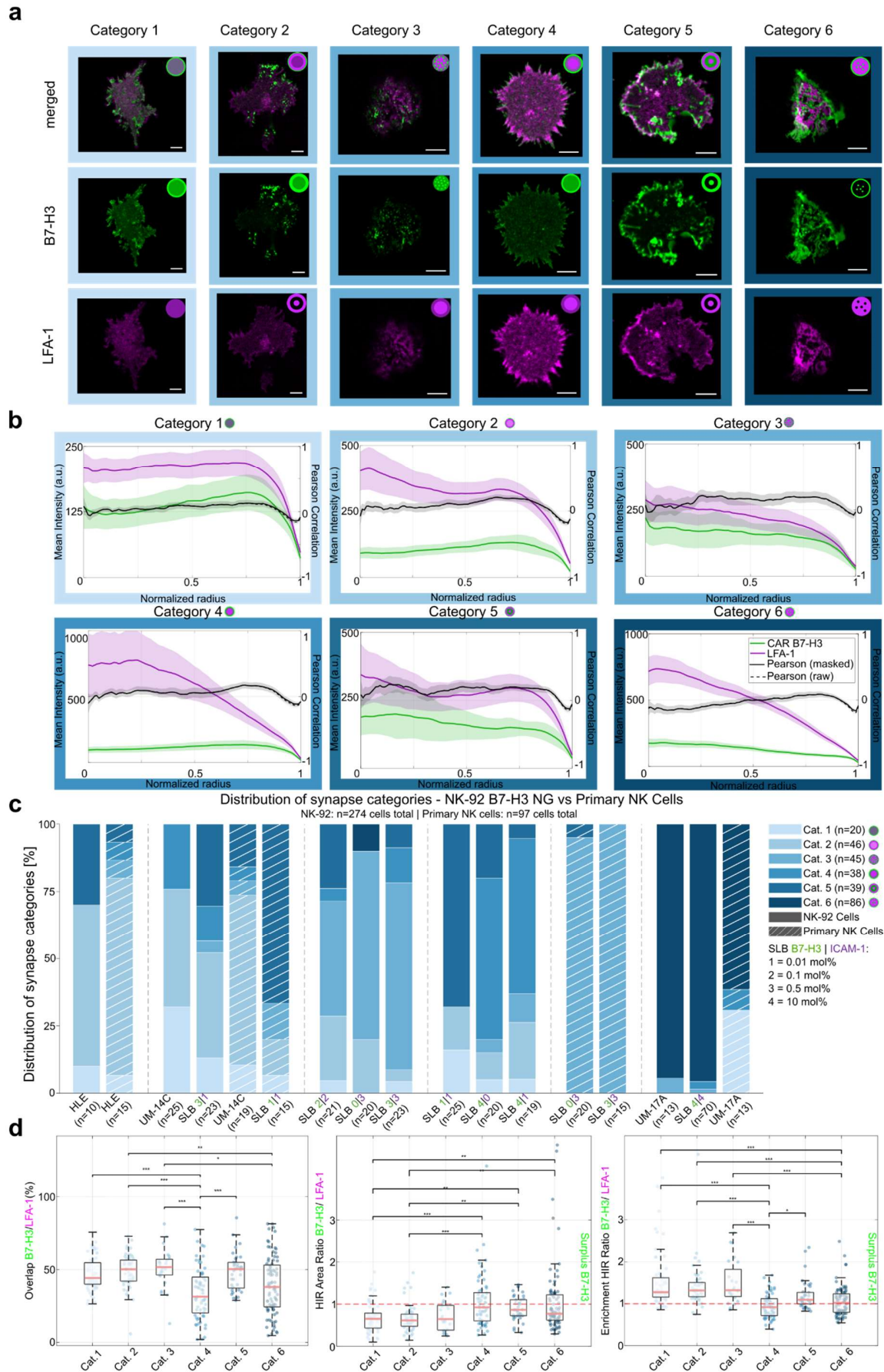


Figure 3: Multicolor analysis of CAR-B7-H3 and LFA-1 at varying target cell concentrations.

Multicolor mapping of CAR and LFA-1 organization identifies distinct synaptic interface states reproduced by defined biomimetic SLBs. **a**, Representative confocal images of six synapse categories defined by structural differences. The first row shows CAR-B7-H3 NG, the second row LFA-1 staining (antibody-labelled), and the third row the corresponding merged images. Scale Bar: 5 μ m. **b**, Radial intensity and Pearson correlation profiles for each category, shown as mean $\pm$ 95% confidence interval. Radial distances are normalized to the distance from the cell center, and fluorescence intensities are normalized to the mean cellular intensity. **c**, Distribution of synapse categories across different experimental conditions for NK-92 B7-H3 NG and primary NK cells, grouped by similar distribution patterns. **d**, Quantification of the ratio of enrichment in high enrichment region (HIR), the ratio of HIR area between B7-H3 and LFA-1, and the overlap of B7-H3 HIR with LFA-1 HIR for each category.

Radial intensity analysis confirmed the distinct spatial signatures of all six synapse categories (**Figure 3b**). Despite partial co-localization, Pearson correlation analysis revealed no significant global correlation between CAR and LFA-1 distributions, indicating largely independent receptor organization within the interface.

To establish a reductionist biomimetic model, we next analyzed CAR-NK cell interactions with SLBs functionalized with defined combinations of B7-H3 and ICAM-1. B7-H3-containing SLBs supported robust adhesion and stable contact zone formation, whereas control bilayers lacking antigen failed to induce stable interactions (**Figure S3a,c**). CAR engagement additionally induced pronounced lateral redistribution of fluorescent membrane lipids within the bilayer, indicating active interface remodeling during synapse formation (**Figure S3b**).

Three-dimensional confocal imaging further revealed distinct redistribution behaviors of CAR and LFA-1 on both tumor cells and SLBs (**Figure S4**). CAR molecules showed strong and nearly complete recruitment into the contact interface, whereas LFA-1 displayed only moderate enrichment and remained broadly distributed across the cell surface, indicating fundamentally different organizational principles for antigen recognition and adhesion receptors.

We next examined whether defined SLB ligand compositions could reproduce the synapse morphologies observed on tumor cells (**Figure 3c**). Distinct tumor cell lines induced characteristic synapse category distributions. HLE cells predominantly induced Category 2 synapses together with lower frequencies of Categories 1 and 5 in NK-92 CAR-NK cells, whereas primary CAR-NK cells additionally displayed Categories 3 and 4. UM-14C cells similarly induced mainly Category 2 synapses accompanied by Categories 1 and 4, while primary cells again showed additional Categories 3 and 5. In contrast, UM-17A interactions were dominated by Category 6 with low frequencies of Category 4, and occasional Category 1 synapses in primary CAR-NK cells.

Importantly, defined SLB conditions closely reproduced the tumor-associated synapse distributions observed for both UM-14C and UM-17A cells. For NK-92-derived CAR-NK cells interacting with UM-14C cells, SLBs containing 0.5 mol% B7-H3 and 0.01 mol% ICAM-1 most accurately matched the tumor-associated morphology distribution, whereas primary CAR-NK cells showed highest similarity to SLBs containing 0.01 mol% B7-H3 and 0.01 mol% ICAM-1. For NK-92-derived CAR-NK cells interacting with UM-17A cells, SLBs containing 10 mol% B7-H3 and 10 mol% ICAM-1 most closely reproduced the observed synapse distribution. These findings demonstrate that defined antigen and adhesion ligand densities are sufficient to recapitulate complex tumor cell-induced synaptic architectures in a minimal biomimetic system.

Finally, quantitative comparison of CAR and LFA-1 high-intensity regions (HIRs) revealed category-specific differences in receptor overlap and enrichment behavior (**Figure 3d**). Across most categories, ~50% of CAR-enriched regions overlapped with LFA-1-positive domains, whereas Categories 4 and 6 showed lower overlap values (~30%), indicating stronger spatial segregation. In Categories 4–6, HIR area and enrichment ratios were close to one, indicating comparable HIR size and enrichment for both receptors. In contrast, Categories 1–3 displayed HIR area ratios below one and enrichment ratios above one,

demonstrating that CAR formed smaller but more densely enriched assemblies, whereas LFA-1 occupied broader and less enriched interface regions. Together, these findings establish that CAR and LFA-1 form partially overlapping but spatially distinct supramolecular assemblies whose organization is strongly shaped by the ligand environment and can be reconstructed using defined biomimetic SLBs.

Ligand-defined phase space of CAR patterning predicts synaptic interface architecture in NK-92-derived and primary CAR-NK cells

We next investigated how antigen availability and adhesion strength shape CAR organization in biomimetic and tumor cell-derived synapses formed by B7-H3 CAR-NK cells. Fluorescence imaging identified four recurrent CAR organization patterns at the contact interface: multipattern (MP), edge ring (ER), central point (CP), and edge ring plus central point (ER+CP) morphologies (**Figure 4a**). In all configurations, CAR molecules accumulated in spatially confined membrane regions distinct from surrounding non-enriched areas.

Radial CAR intensity analysis confirmed distinct spatial signatures for each morphology class (**Figure 4b**). CP interfaces showed strong central CAR accumulation followed by a monotonic peripheral decrease, whereas MP interfaces displayed a broad central plateau gradually declining toward the interface edge. ER morphologies exhibited pronounced peripheral enrichment, while ER+CP interfaces combined peripheral CAR accumulation with an additional central density maximum. Analysis of high-intensity region (HIR) geometry further revealed an inverse relationship between normalized HIR area and receptor density, with smaller HIRs displaying stronger CAR enrichment (**Figure 4b, insert**).

We next analyzed how defined ligand environments regulate CAR organization in NK-92-derived (**Figure 4c**) and primary human B7-H3 CAR-NK cells (**Figure 4d**). CAR-NK cells were incubated either on SLBs containing defined combinations of B7-H3 and ICAM-1 or with tumor cell lines exhibiting distinct endogenous ligand profiles. HLE cells expressed very low B7-H3 levels, whereas UM-14C and UM-17A cells showed intermediate B7-H3 expression. ICAM-1 expression was low on HLE and UM-14C cells and high on UM-17A cells (**Figure S2**). Under all conditions, CAR-NK cells formed stable synapses on both SLBs and tumor cells.

Increasing either B7-H3 or ICAM-1 density progressively enhanced interface spreading and increased CAR-enriched region size, indicating cooperative regulation of synapse expansion by antigen recognition and integrin-mediated adhesion. In contrast, CAR density in non-enriched membrane regions remained relatively constant at ~ 600 CARs μm^{-2} across all conditions, whereas CAR-enriched regions reached local densities of 800–1800 CARs μm^{-2} .

Distinct interface topologies emerged under defined ligand-density regimes. In the absence of ICAM-1 or at very low ICAM-1 concentrations (<0.01 mol%), MP morphologies predominated. At intermediate ICAM-1 densities (0.01–1 mol%), ER and ER+CP architectures became increasingly frequent, whereas pure CP morphologies occurred exclusively at the highest tested B7-H3 and ICAM-1 densities (10 mol% each), indicating that centralized CAR accumulation requires strong simultaneous antigenic and adhesive stimulation.

Despite similar ligand-dependent organizational trends, primary CAR-NK cells formed structurally distinct interfaces compared with NK-92-derived CAR-NK cells. Primary cells generated smaller CAR-enriched regions, exhibited fewer ER+CP morphologies, and more frequently formed simple ER structures. In addition, maximal contact areas reached ~ 500 μm^2 in NK-92-derived CAR-NK cells but rarely exceeded 200 μm^2 in primary NK cells, likely reflecting their smaller overall cell size.

Interestingly, NK-92-derived CAR-NK cells displayed lower overall CAR surface density but stronger local receptor accumulation within the interface. In contrast, primary CAR-NK cells exhibited high constitutive CAR abundance with only modest additional enrichment at CAR-enriched regions (**Figure S5**), suggesting a more limited dynamic range for further receptor recruitment.

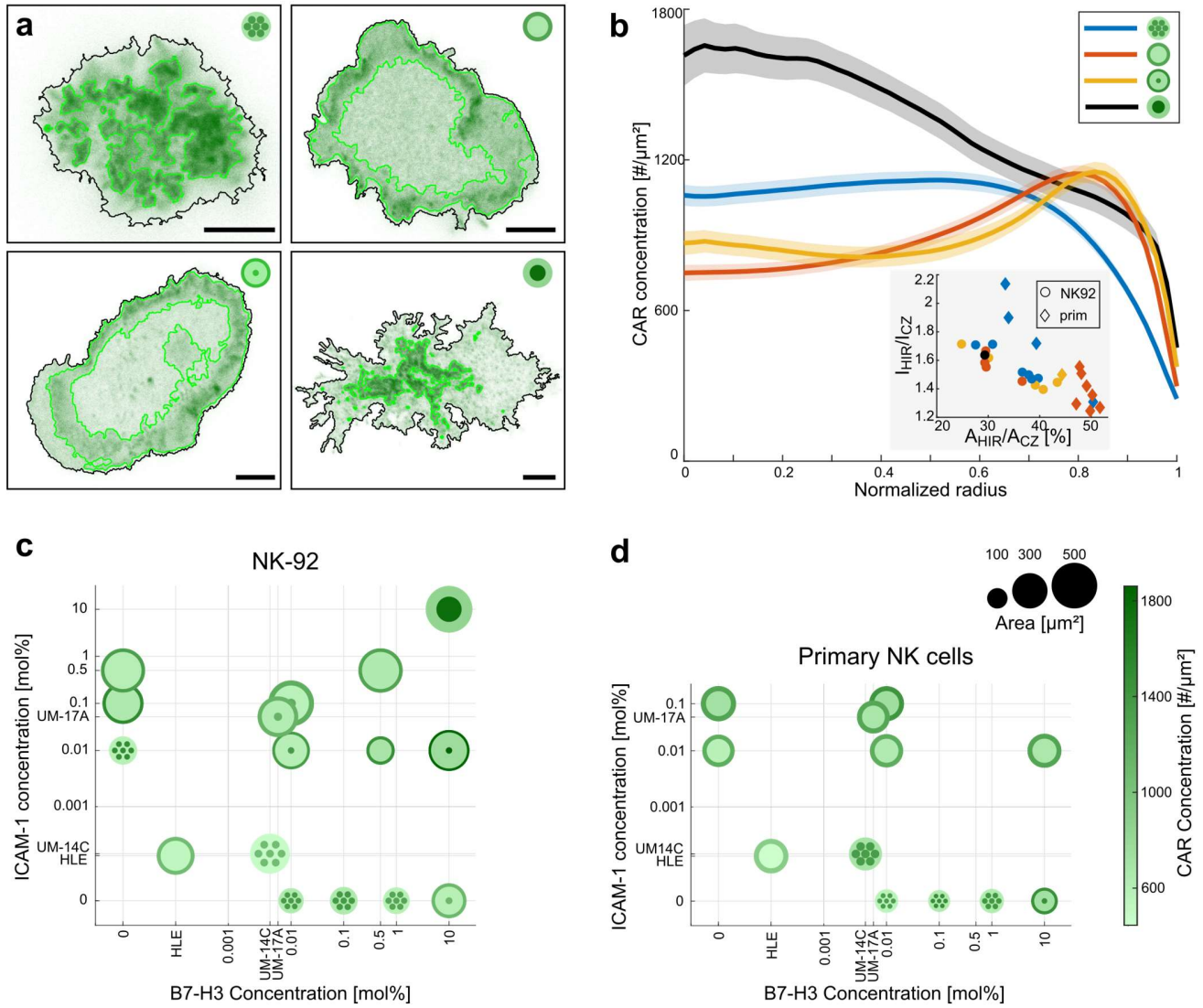


Figure 4: Antigen and ICAM-1 densities cooperatively regulate CAR patterning and synaptic interface organization in NK-92-derived and primary CAR-NK cells. **a**, Representative fluorescence images illustrating four recurrent CAR organization patterns observed at CAR-NK cell contact zones (CZ): multipattern (MP, top left), edge ring (ER, top right), central point (CP, bottom right) and edge ring plus central point (ER+CP, bottom left). Black outlines indicate the total contact area and green outlines delineate CAR-enriched regions used for quantitative segmentation. Scale bars: 5 μm . **b**, Radial CAR density profiles corresponding to the four interface classes shown in a. Multipattern interfaces display broad central enrichment ($n=492$), ER interfaces peripheral accumulation ($n=705$), CP interfaces strong receptor enrichment at the center with declining peripheral density ($n=57$), and ER+CP interfaces combine central and peripheral maxima ($n=389$). Solid lines represent the mean profile, shaded areas indicate the 95% confidence interval (mean $\pm$ 1.96 SEM). Insert, inverse relationship between normalized HIR density ($I_{\text{HIR}}/I_{\text{CZ}}$) and relative HIR area ($A_{\text{HIR}}/A_{\text{CZ}}$) for NK-92-derived and primary CAR-NK cells. **c**, Quantitative analysis of CAR-enriched contact interfaces formed by B7-H3 CAR-NK-92 cells on SLBs containing defined B7-H3 and ICAM-1 densities, or on tumor cell lines (HLE, UM-14C and UM-17A) expressing endogenous ligand levels. Bubble size indicates contact area (μm^2), and color intensity denotes mean CAR surface density within enriched and non-enriched membrane regions. Each bubble represents at least $n \geq 49$ cells per condition. **d**, Corresponding quantitative analysis for primary human B7-H3 CAR-NK cells. Primary NK cells formed overall smaller CAR-enriched contact regions but displayed

similar ligand-dependent trends in interface expansion and receptor enrichment. Marker size and color coding are identical to c. $n \geq 55$ for each bubble.

Mapping synaptic morphologies across all B7-H3 and ICAM-1 combinations revealed a continuous ligand-dependent organization landscape in which discrete CAR interface states emerged as a function of combined antigenic and adhesive input. This defines a two-dimensional phase space of CAR-NK synapse architecture, allowing tumor cells and biomimetic SLBs to be positioned according to their ligand composition and enabling prediction of synaptic organization directly from target-cell surface properties.

Together, these findings establish ligand density-dependent CAR patterning as a central organizational principle governing CAR-NK synapse architecture.

3. Discussion

CAR-engineered immune cells have emerged as powerful effectors for cancer immunotherapy^{8,9,12,40}, yet the structural principles governing CAR synapse organization and their relationship to functional outcomes remain incompletely understood. Here, we establish a quantitative and mechanistic framework for CAR-NK cell interface formation using a reductionist SLB system and complementary tumor cell models. Our data show that CAR-NK synapse architecture is not stochastic but follows a ligand-density-dependent organizational landscape defined by antigen (B7-H3) and adhesion molecule (ICAM-1) availability. This phase space enables prediction of synaptic topology directly from target-cell ligand composition.

Ligand-dependent synaptic organization observed in SLBs closely recapitulated morphologies on UM-14C and UM-17A tumor cells. Across all regimes, we observed strong structural concordance between artificial and cellular interfaces, indicating that SLBs capture essential biophysical determinants of CAR-NK synapse formation. This allowed mapping of tumor cell states onto defined antigen–adhesion combinations within the phase space.

Robust quantitative analysis of CAR-NK synapses requires homogeneous effector populations. However, primary NK cells are difficult to engineer due to low and variable transduction efficiency, limited persistence, and toxicity-associated constraints. Lentiviral transduction in particular leads to heterogeneous CAR expression and reduced reproducibility⁴¹. These limitations necessitate robust enrichment strategies for quantitative imaging studies.

We previously established a CD34-based magnetic enrichment strategy for CAR-NK purification³⁹. Here, this approach consistently yielded highly enriched CAR-positive populations across primary NK cells and NK-92 and KHYG-1 lines. CAR positivity exceeded 98% in primary cells, enabling reliable comparative synapse quantification. This homogeneity was essential, as CAR expression variability strongly influences clustering and downstream signaling.

NK cell platforms differ substantially in their biological properties. NK-92 and KHYG-1 cells provide standardized, reproducible systems and are widely used for CAR studies due to stable expansion and cytotoxicity^{41,42}. In contrast, primary NK cells exhibit higher physiological relevance but increased donor variability and signaling heterogeneity⁴¹. Consistent with this, we observed distinct functional and expression profiles across all platforms despite comparable enrichment efficiency.

Together, these systems provide complementary advantages: NK cell lines enable controlled mechanistic studies, while primary NK cells preserve physiological relevance. This standardized framework allows direct comparison of synapse formation and cytotoxic function across effector types.

Functionally, B7-H3 CAR-NK cells formed productive, antigen-specific cytotoxic synapses characterized by CAR accumulation at the effector–target interface and efficient tumor cell killing. CD19 CAR controls failed to form stable synapses or induce cytotoxicity, confirming antigen specificity. CAR redistribution was highly organized and activation-dependent rather than passive.

This spatial organization aligns with known immune synapse principles. Classical T cells form SMAC structures with central TCR accumulation⁴³, while natural NK cells exhibit coordinated receptor and granule polarization⁴⁴. Inhibitory NK synapses show distinct integrin-centered architectures with reduced

cytoskeletal dependence⁴⁵. CAR-T synapses, in contrast, often display fragmented receptor clusters rather than classical SMAC organization⁴³. Our data indicate that CAR-NK synapses combine features of natural NK and engineered CAR interfaces as suggested before²⁶.

B7-H3 CAR-NK cells also mediated efficient cytotoxicity across HNSCC models (UM-14C, UM-17A), consistent with previous reports in multiple tumor entities^{46–48}. We frequently observed collective killing events, where multiple effectors engaged single or multiple targets simultaneously, a phenomenon also described for CAR-T cells⁴⁹. Such cooperative behavior may enhance killing efficiency in dense tumor microenvironments⁵⁰.

Cytotoxic efficiency depended strongly on antigen density. Low B7-H3 expression (e.g., HLE cells) resulted in weak susceptibility, consistent with antigen threshold effects⁵¹. Nevertheless, synapses still formed even at low antigen levels, suggesting high sensitivity of CAR systems. Residual ICAM-1/LFA-1 interactions likely contribute to stabilization under low antigen conditions. Prior work suggests that even ~200 antigen molecules may be sufficient for CAR activation⁵². Experimental studies in CAR-T cells have suggested that relatively low antigen densities may already trigger cytotoxic activation, whereas substantially higher antigen abundance is required for maximal downstream signaling and cytokine release^{52,53}.

Importantly, CAR density alone did not predict cytotoxicity. NK-92 cells showed stronger killing than KHYG-1 cells despite lower or comparable CAR expression, while primary NK cells exhibited high CAR levels but intermediate killing. This indicates that receptor abundance alone is insufficient, and that synaptic organization and signaling competence are additional key determinants.

Serial killing capacity varied across systems, with NK-92 cells showing the highest activity, KHYG-1 the lowest, and primary cells intermediate. Serial killing is a hallmark of NK biology and reflects synapse stability and efficient detachment⁵⁴. Similar behavior has been described for CAR-T cells and is associated with high overall therapeutic efficacy⁵⁵. These differences likely reflect intrinsic variations in synaptic dynamics and re-engagement efficiency.

Adhesion signaling further modulated function. KHYG-1 cells expressed high LFA-1 levels and showed strong ICAM-1-dependent spreading, also reported in other studies⁵⁶, yet weak cytotoxicity, highlighting that integrin abundance alone does not guarantee functional potency. In natural T cells, LFA-1 lowers activation thresholds⁵⁷, while in natural NK cells LFA-1 alone is sufficient to initiate activation⁵⁸, promote granule polarization⁵⁹ and supports actin remodeling for secretion⁶⁰. Beyond its mechanical adhesive role, ICAM-1 recognition has therefore been proposed to function as a contextual cue enabling NK cells to distinguish pathogen-infected or transformed cells from freely circulating particles lacking stable adhesive contacts⁶¹.

Referring to its early activating role in natural NK cells, we observed partly related behavior in our experiments. LFA-1 engagement alone partially induced CAR organization patterns such as ER and MP structures, suggesting a role in early synaptic patterning. However, as early signaling was not directly measured, this remains speculative. Integrin signaling has also been shown to modulate CAR-T toxicity without impairing efficacy⁶², supporting a regulatory role in engineered systems.

Our analysis revealed a predominant synapse architecture characterized by central enrichment of LFA-1 in conjunction with B7-H3 clustering at either the peripheral ring or the central zone of the CAR-NK immunological synapse. When interpreted in the context of our observed killing efficiency data, this synapse configuration may represent a functionally superior architecture for the induction of target cell lysis, consistent with principles established for CAR-T cells, where morphological Synapse Quality predicts effectiveness⁶³. that IS quality is predictive of cytotoxic effectiveness. However, given the inherent differences between CAR-T and CAR-NK cell biology, as well as the distinct target antigen (B7-H3 vs. CD19), direct comparisons should be made with caution. Further characterization of activation markers, including degranulation markers and downstream signaling molecules, will be required to fully validate these findings in the context of CAR-NK cell-mediated cytotoxicity.

To dissect ligand dependence, we used SLBs with defined B7-H3 and ICAM-1 densities. SLBs faithfully reproduced tumor cell synapse morphologies, including MP, ER, CP and combinational states, validating

their physiological relevance. CAR engagement induced receptor redistribution and ligand reorganization, confirming active synaptic remodeling.

Synapse spreading was strongly dependent on combined ligand availability. Low antigen or low adhesion alone resulted in reduced interface size, whereas combined low-level ICAM-1 and B7-H3 synergistically increased spreading. Thus, coordinated antigen recognition and integrin engagement are required for physiological synapse expansion.

The observed behavior aligns with theoretical models of immune synapse self-organization⁶⁴, where receptor size differences and adhesion energies drive spatial segregation. Depending on binding energy and adhesion strength, systems transition between multifocal clusters, ring-like structures, and centralized assemblies. Active cytoskeletal transport reverses this organization from centralized adhesion with peripheral signaling receptors to centralized signaling receptors with a peripheral adhesion ring, producing classical SMAC structures⁶⁴.

Natural immune synapses reflect these regimes: activating natural T cells show centralized TCR organization⁴³, natural activating NK cells exhibit polarized granule secretion and peripheral integrin enrichment⁶⁰, while inhibitory NK synapses resemble passive adhesion-dominated, characterized by central integrin accumulation and comparatively limited dependence on cytoskeletal-driven receptor transport⁴⁵.

Thymocyte synapses show multifocal architectures consistent with intermediate multipattern regimes⁶⁵. Our observations indicate that CAR-NK synapses adopt multiple organizational states rather than a single fixed architecture. Unlike physiological TCRs, CAR receptors differ in signaling properties and ligand recognition geometry, and CAR-T synapses have previously been described as fragmented multifocal receptor clusters with limited classical SMAC organization⁴³. Consistent with this, CAR-NK cells predominantly formed fragmented cluster-like receptor assemblies under low ICAM-1 availability, resembling intermediate passive self-organization states. Increasing ICAM-1 density promoted peripheral edge-ring structures, either alone or together with central CAR accumulations (ER+CP morphologies), indicating that enhanced adhesive engagement promotes increased spatial ordering. Pronounced centralized CAR accumulation emerged only under very high ICAM-1 and B7-H3 densities, consistent with highly stabilized synaptic states requiring both strong antigenic and adhesive input.

Unlike classical models predicting strict spatial separation between antigen receptors and LFA-1/ICAM-1 domains, LFA-1 in CAR-NK synapses did not segregate into mutually exclusive peripheral adhesion zones. Instead, LFA-1 exhibited morphology-dependent radial distributions across the synapse. In most organizational states, LFA-1 declined progressively from the center toward the periphery, although the extent of this decay varied between morphologies. In edge-ring architectures (categories 2 and 5), this decline was accompanied by peripheral LFA-1 enrichment, producing composite distributions with both central and ring-like features. Particularly striking were perforated low-LFA-1 regions within dense central adhesion domains in category 6, resembling perforated LFA-1 patterns previously reported in thymocyte synapses⁶⁵. Together, these findings suggest that CAR-NK synapses deviate from strict receptor segregation paradigms of classical T-cell synapse models and instead exhibit hybrid organizational regimes in which CAR signaling and adhesion remain partially interwoven across the synaptic interface.

SLBs were dynamic and underwent ligand reorganization upon CAR engagement, indicating active feedback between receptor binding and membrane structure. While powerful for controlled analysis, SLBs lack tumor-intrinsic features such as mechanical heterogeneity and additional costimulatory signals. This imposes limitations on physiological interpretation.

Additional limitations include: (i) uncertain absolute ligand densities due to differing coupling chemistries (His-tag vs streptavidin), requiring empirical optimization; (ii) high technical complexity limiting large-scale screening; and (iii) time-resolved imaging constraints, as each channel reflects dynamic processes captured at slightly different states.

Future work should employ higher temporal resolution and simultaneous multichannel imaging to resolve dynamic synapse evolution.

In conclusion, we define a quantitative phase space governing CAR-NK synapse organization as a function of antigen and adhesion density. CAR-NK cells do not adopt a single synaptic architecture but instead

occupy multiple ligand-dependent regimes spanning fragmented, ring-like, and centralized states. These morphologies are conserved between SLBs and tumor cells, supporting the predictive power of the model. Our findings show that CAR synapse topology emerges from cooperative interactions between CAR signaling, integrin adhesion, and membrane self-organization. CAR-NK synapses thus represent hybrid structures combining features of natural NK synapses and engineered receptor systems.

Most importantly, the identified ligand-dependent phase space enables prediction of synapse architecture from tumor ligand composition, providing a framework for rational targeting and CAR design. More broadly, immune synapse topology emerges as a tunable, emergent property of engineered immune cells linked directly to target-cell molecular composition.

4. Materials and Methods

Reagents

All lipids: SOPC (1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine), DOPE-Cy5 (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 5)), DOPE-cap-biotin (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl), and DOGS-NTA (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)) were used without further purification. Recombinant proteins included bovine serum albumin (BSA), human B7-H3 (CD276, His-tagged extracellular domain), biotinylated human ICAM-1 (CD54 extracellular domain (His & AVI Tag), Biotinylated), and streptavidin.

Cell culture media (RPMI 1640, DMEM, L-15, DPBS) were supplemented with 10% FCS and 1% penicillin-streptomycin unless otherwise stated. RPMI was additionally supplemented with 250 U/mL IL-2 (Proleukin® S 18x10⁶ IU).

All Antibodies for multicolor staining and FACS analysis were used according to manufacturers instructions: Atlantic Blue™ Anti-Human CD11a (Proteintech Group, Rosemont, IL, USA), CD276 PE-conjugated monoclonal antibody (Miltenyi Biotec, Bergisch-Gladbach, GER), CD34 PE-conjugated monoclonal antibody (clone QBEND/10; Invitrogen, Regensburg, GER; MA1-10205), PE-conjugated anti-human CD54 (ICAM-1) Antibody (Miltenyi Biotec, Bergisch-Gladbach, GER), PE anti-human CD11a Antibody (BioLegend, San Diego, CA, USA).

CAR Construct Design and CAR-NK Cell Generation

Second-generation CAR constructs were optimized for human codon usage containing a scFv specific for either B7-H3 (clone 376.96)⁴⁶ or CD19 (clone FMC63)³⁹, a CD34-derived hinge (C6)⁶⁶, a CD28 transmembrane and co-stimulatory domain, and a CD3ζ signaling domain and are expressed under the control of the MPSV promotor as described previously⁶⁷. Using a flexible linker, mNeonGreen (mNG)⁶⁸ was fused downstream of the CD3ζ signaling cassette at the C-terminal of the CAR to enable quantitative detection of CAR-expressing cells.

Replication-deficient lentiviral particles were pseudotyped with baboon endogenous retroviral envelope lacking the R-peptide (BaEV-Rless), and produced in HEK293T cells as previously described³⁹. After 48 h, viral supernatant was filtered (0.45-μm), concentrated (10,000 × g, 2 h, 4°C), and stored at -80°C. Transduction of the activated primary human NK-cells or the NK-92 and KHYG-1 cell lines was performed on RetroNectin® (Takara)-coated plates as described^{39,69}. Transduction rates between 12-27% for the cell lines NK-92 and KHYG-1 and 29-62% for the primary NK-cells were selected to perform MACS enrichment via the C6-hinge to enrich for CAR-positive cells⁶⁶.

CAR NK cells

The NK cell lines KHYG-1 and NK-92 were obtained from DSMZ (Braunschweig, Germany) and were expanded in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS-HI), 1% penicillin/streptomycin (P/S), and 250 U/ml IL-2 under standard culture conditions. Primary human NK-cells were isolated from healthy donors from leukoreduction system chambers (LRSCs) provided by the

Institute of Transfusion medicine at the University Hospital Essen, approved by the local ethics committee (24-12172-BO). After Ficoll density gradient centrifugation (Ficoll® Paque Plus Cytiva, Sigma Aldrich), peripheral blood mononuclear cells (PBMCs) were co-cultured with K562/41BBL/mb15/mb21 feeder cells⁷⁰, kindly provided by Winfried Wels (Frankfurt), at a PBMC to feeder cell ratio of 3:2 in RPMI-1640 medium supplemented with 10% FCS-HI, 1% P/S, and 40 U/ml IL-2 for one week until transduction of NK-cells.

Tumor Cell Lines

UM-14C (UM-SCC-14C), UM-17A (UM-SCC-17A), and HLE (hepatocellular carcinoma-derived epithelial line) were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin at 37°C and 5% CO₂. Cells were passaged every 2–3 days using trypsin-EDTA and used within defined passage ranges (tumor cells: 50–100).

Cells were selected based on differential endogenous expression of B7-H3 and ICAM-1, as validated by flow cytometry (**Figure S2**).

Tumor cells are seeded into 8-well glass-bottom slides at a density of 2×10^4 cells per well for time-laps measurements on the IX83 setup, or at a density of 2×10^4 – 6×10^4 cells per well for TIRF and Confocal imaging on the Nikon or Abberior setup (see Imaging section).

Supported Lipid Bilayers (SLB)

SLBs were generated using a Langmuir-Blodgett/Langmuir-Schäfer deposition system (KSV Nima, Biolin Scientific). Bilayers consisted of SOPC as matrix lipid with controlled incorporation of DOGS-NTA and DOPE-cap-biotin as functional linker lipids at defined molar fractions (0.01–10 mol%).

Fluorescent DOPE-Cy5 was included for visualization when required. Bilayers were formed at a surface pressure of 25 mN/m and transferred onto cleaned glass coverslips under aqueous conditions.

SLBs were passivated with BSA (0.5 mg/mL, 15 min) prior to functionalization. Sequential protein coupling was performed as follows:

(i) B7-H3 (0.7 µg/mL, 14 h) via Ni-NTA linkage, (ii) streptavidin (0.3 µg/mL, 45 min) and (iii) ICAM-1 (0.4 µg/mL–7 ng/mL, 1 h)

For dual-functionalized SLBs, B7-H3 coupling was performed first, followed by streptavidin and ICAM-1 in separate steps. Between all steps, BSA passivation was repeated to minimize nonspecific binding.

All functionalization steps were performed in PBS. Prior to imaging, PBS was exchanged for L-15 medium. Approximately 2×10^4 CAR-NK cells were added per SLB.

Ligand densities are reported as mol% of linker lipid (0.01–10 mol%).

Imaging (TIRF, Confocal, Polarization-Resolved Microscopy)

TIRF imaging was performed on a Nikon Eclipse Ti2 system equipped with a 100x oil immersion objective (NA 1.49) and a 488 nm excitation laser. Emission was collected at 525/45 nm. The penetration depth of the evanescent field was adjusted to ~150 nm.

Confocal images (except of the ones for determination of the CAR concentration and area of contact zone) were also acquired with the same setup using the 405 nm and 488 nm laser lines for excitation and 525/50 nm and 452/45 nm emission filter respectively.

All confocal imaging required for calibration and analysis of contact zones was performed on an Abberior Expert Line system with a 60x water-immersion objective (NA: 1.2) and polarization-sensitive detection. Excitation was achieved with 488 nm pulsed laser, followed by 500–550 nm emission. For polarization-resolved detection, parallel and perpendicular emission channels were recorded and corrected using a G-factor calibration. Total intensity was reconstructed according to: $I_{\text{total}} = I_{\text{parallel}} + 2 \cdot G \cdot I_{\text{perpendicular}}$, with G the fraction of the detection efficiencies of the parallel and perpendicular channel.

All live-cell imaging was conducted at 37°C using environmental control systems.

Images were processed using Fiji (v1.54f), Omero (v5.29.2) and MATLAB (vR2021a).

Killing Assays and Single-Cell Dynamics

Time-lapse imaging was performed on an Olympus IX83 inverted microscope equipped with a 60x oil-immersion objective (NA 0.65-1.25) and a temperature-controlled stage (37°C). CAR-NK cells were added to tumor cells at effector-to-target ratios between 1:1 and 1:3.

Prior to co-culturing with target cells, primary NK-cells were incubated with monoclonal REAfinity™ antibodies against NKG2D, DNAM-1, NKp30, NKp44, NKp46, and NKp80 (Miltenyi Biotec) to block the activating NK-cell receptors as previously established³⁹.

Images (PH and excitation: 470/40 nm + emission: 525/50 nm) were acquired every 5 min over 16 h or every 6 min over 13 h for the primary NK cells. Target cell death was determined based on morphological features consistent with apoptosis (membrane blebbing, fragmentation).

Single-cell killing analysis included only CAR-NK cells remaining in the field of view for the entire experiment. Cells without killing events were excluded from serial killing quantification.

Molecular Brightness Measurement

Absolute quantification of CAR surface molecules was performed using molecular brightness calibration of mNeonGreen fused to the CAR construct.

Recombinant mNeonGreen was expressed in E. coli BL21(DE3) and purified via Ni-NTA affinity chromatography. Protein purity and concentration were verified spectrophotometrically.

FCS was used to determine the confocal volume of the Abberior setup using Rhodamine 110 ($D = 430 \mu\text{m}^2/\text{s}$) as a reference standard.

Molecular brightness (m_B) was defined as photon count rate per molecule under defined optical conditions and calculated as: $m_B = \frac{b}{N_A \cdot V}$, where b is the slope of the intensity–concentration relationship, N_A is Avogadro's constant, and V is the confocal volume. The calibrated brightness was $4.2 \pm 0.3 \text{ kHz}/(\mu\text{W} \cdot \text{molecule})$.

Quantitative Image Analysis and CAR Density Mapping

Fluorescence intensities were converted into absolute molecule numbers using: $\frac{I/(t \cdot P)}{m_B}$, where I is pixel intensity, t dwell time, P laser power, and m_B molecular brightness.

CAR densities were calculated by normalizing molecule numbers to the effective pixel area ($A_{eff} = \pi \omega_0$, ω_0 : lateral beam waist). Synaptic interfaces were segmented using a custom MATLAB pipeline based on intensity thresholding and morphological reconstruction.

Only membrane-localized CAR signal (corrected by FCS-derived membrane fraction, see below) was used for quantitative analysis.

FCS-Based Separation of Membrane and Cytoplasmic CAR Populations

FCS was used to deconvolve membrane-associated and cytoplasmic CAR fractions at the plasma membrane plane.

When the confocal volume intersects the membrane, fluorescence fluctuations originate from both: (i) 2D lateral diffusion of membrane-bound CAR and (ii) 3D diffusion of cytoplasmic CAR.

The correlation function was modeled as: $G(\tau) = f_{mem} G_{2D}(\tau) + f_{cyt} G_{3D}(\tau)$. Cytoplasmic diffusion parameters were first determined from measurements performed in the cytosol and subsequently fixed during membrane fits to reduce parameter covariance.

This sequential fitting approach yields the membrane-associated fraction f_{mem} , which was used to correct intensity-based quantification: $I_{mem} = f_{mem} I_{total}$. Across all measurements, the membrane-associated fraction was determined to be $f_{mem} = 0.82$ (see **Figure S6**). This correction ensures that only plasma membrane-resident CAR molecules contribute to surface density measurements.

Correction for Non-Fluorescent Fraction of mNeonGreen

To account for non-fluorescent mNG molecules resulting from incomplete chromophore maturation or improper protein folding, an additional correction factor was applied. The fraction of fluorescent mNG

molecules was determined spectrophotometrically from absorbance measurements at 280 nm, reporting total protein concentration, and at 506 nm, reporting mature fluorescent mNG.

Concentrations were calculated according to the Beer-Lambert law, $A_\lambda = \epsilon_\lambda \cdot c \cdot l$, where A is the absorbance, ϵ the molar extinction coefficient, c the concentration and l the optical path length. The fluorescent fraction of mNG was obtained from the ratio $R_{mNG} = \frac{c_{bright}}{c_{total}} = \frac{A_{506} \cdot \epsilon_{280}}{A_{280} \cdot \epsilon_{506}}$, using extinction coefficients of $\epsilon_{280} = 44,475 M^{-1} cm^{-1}$ and $\epsilon_{506} = 116,000 M^{-1} cm^{-1}$. This analysis yielded a fluorescent fraction of $R_{mNG} = 0.67$ (see **Figure S6**), indicating that approximately 67% of mNG-tagged CAR molecules were fluorescent under the experimental conditions.

Consequently, membrane-associated CAR numbers were corrected for the non-fluorescent population according to $N_{CAR} = \frac{N_{mem}}{R_{mNG}} = \frac{N_{mem}}{0.67}$. This correction accounts for CAR molecules carrying non-fluorescent mNeonGreen and provides an estimate of the total CAR abundance independent of fluorophore maturation efficiency.

The final CAR abundance was obtained by correcting the measured signal for both the membrane-associated fraction determined by FCS ($f_{mem} = 0.82$) and the fluorescent fraction of mNG ($R_{mNG} = 0.67$) according to:

$$N_{CAR} = N_{total} \cdot f_{mem} \cdot \frac{1}{R_{mNG}}$$

Flow Cytometry (FACS)

Flow cytometry was performed at the Core Facility Flow Cytometry of the Heinrich-Heine-Universität, Düsseldorf, German, using a CytoFLEX-S system. On CAR-NK cells CAR and adhesion molecule expression was detected via CD34-PE and anti-LFA-1 staining. Tumor cell ligand expression was analyzed using anti-B7-H3 and anti ICAM-1 antibodies. For detachment of tumor cells, Accutase was used to minimize proteolytic cleavage of surface proteins and ensure accurate quantification of cell surface B7-H3 and ICAM-1 expression. Quantification was performed using Quantibrite PE beads for calibration.

Statistical Analysis

Statistical analyses were performed in MATLAB. The multicolor analysis was carried out using the Kruskal-Wallis test. For metrics yielding a significant result ($p < 0.05$), pairwise post-hoc comparisons were conducted using Dunn's test with Bonferroni correction for multiple comparisons (15 pairs). Comparisons between same SLB conditions was performed using Wilcoxon rank-sum tests. Significance levels were defined as $p > 0.05$ (n.s.), ≤ 0.05 (*), ≤ 0.01 (**), and ≤ 0.001 (***)

Use of AI-assisted tools in manuscript preparation

The authors used ChatGPT (OpenAI, 2025) to support improvements in language clarity, spelling, and readability. Elicit was used as a supportive tool for literature search and identification of relevant publications. Claude (Anthropic) was used to assist with programming-related tasks and code optimization during data analysis and visualization.

All scientific interpretations, experimental design decisions, data analyses, and conclusions presented in this manuscript were made independently by the authors, who take full responsibility for the content of the work.

References

1. Häufigste Todesursachen in Deutschland | Statista. <https://de.statista.com/statistik/daten/studie/158441/umfrage/anzahl-der-todesfaelle-nach-todesursachen/>.
2. USA - Sterberate nach Todesursache bis 2023 | Statista. <https://de.statista.com/statistik/daten/studie/1224004/umfrage/sterberate-in-den-usa-nach-todesursache/>.
3. Liu, B., Zhou, H., Tan, L., Siu, K. T. H. & Guan, X. Y. Exploring treatment options in cancer: Tumor treatment strategies. *Signal Transduct. Target. Ther.* 9, (2024).
4. DeVita, V. T. & Rosenberg, S. A. Two Hundred Years of Cancer Research. *New England Journal of Medicine* 366, 2207–2214 (2012).

5. Abdelazeem, B. *et al.* The role of immunotherapy plus chemotherapy versus chemotherapy alone as first-line treatment for advanced non-small cell lung cancer: an updated systematic review and meta-analysis of randomized controlled trials. *Expert Rev. Anticancer Ther.* 22, 1127–1140 (2022).
6. Chen, Y.-W. *et al.* Improved overall survival of metastatic cancers in the United States across all age groups in the immunotherapy era: Implications for considering elderly patients (age ≥ 75) for immune checkpoint inhibitors (ICIs). *Journal of Clinical Oncology* 40, (2022).
7. Foster, C. C. *et al.* Overall survival according to immunotherapy and radiation treatment for metastatic non-small-cell lung cancer: A National Cancer Database analysis. *Radiation Oncology* 14, (2019).
8. Lu, J. & Jiang, G. The journey of CAR-T therapy in hematological malignancies. *Mol. Cancer* 21, (2022).
9. Drokow, E. K. *et al.* Survival outcomes and efficacy of autologous CD19 chimeric antigen receptor-T cell therapy in the patient with diagnosed hematological malignancies: A systematic review and meta-analysis. *Ther. Clin. Risk Manag.* 15, 637–646 (2019).
10. Wang, Y. *et al.* CD19-Specific CAR-T Cell Treatment of 115 Children and Young Adults with Acute B Lymphoblastic Leukemia: Long-term Follow-up. *Cancer Res. Treat.* 56, 945–955 (2024).
11. Song, F. *et al.* Safety and efficacy of autologous and allogeneic humanized CD19-targeted CAR-T cell therapy for patients with relapsed/refractory B-ALL. *J. Immunother. Cancer* 11, (2023).
12. Mitra, A. *et al.* From bench to bedside: the history and progress of CAR T cell therapy. *Front. Immunol.* 14, (2023).
13. Beatty, G. L. *et al.* Activity of Mesothelin-Specific Chimeric Antigen Receptor T Cells Against Pancreatic Carcinoma Metastases in a Phase 1 Trial. *Gastroenterology* 155, 29–32 (2018).
14. O'Rourke, D. M. *et al.* A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci. Transl. Med.* 9, (2017).
15. Liu, G., Rui, W., Zhao, X. & Lin, X. Enhancing CAR-T cell efficacy in solid tumors by targeting the tumor microenvironment. *Cell. Mol. Immunol.* 18, 1085–1095 (2021).
16. Lin, H. *et al.* Antigen escape in CAR-T cell therapy: Mechanisms and overcoming strategies. *Biomedicine and Pharmacotherapy* 178, 117252 (2024).
17. Chen, N., Li, X., Chintala, N. K., Tano, Z. E. & Adusumilli, P. S. Driving CARs on the uneven road of antigen heterogeneity in solid tumors. *Curr. Opin. Immunol.* 51, 103–110 (2018).
18. Chen, T., Wang, M., Chen, Y. & Liu, Y. Current challenges and therapeutic advances of CAR-T cell therapy for solid tumors. *Cancer Cell Int.* 24, (2024).
19. Deng, C. *et al.* The Vascular Disrupting Agent CA4P Improves the Antitumor Efficacy of CAR-T Cells in Preclinical Models of Solid Human Tumors. *Molecular Therapy* 28, 75–88 (2020).
20. Morgan, R. A. *et al.* Case report of a serious adverse event following the administration of t cells transduced with a chimeric antigen receptor recognizing ERBB2. *Molecular Therapy* 18, 843–851 (2010).
21. Thistlethwaite, F. C. *et al.* The clinical efficacy of first-generation carcinoembryonic antigen (CEACAM5)-specific CAR T cells is limited by poor persistence and transient pre-conditioning-dependent respiratory toxicity. *Cancer Immunology, Immunotherapy* 66, 1425–1436 (2017).
22. Huntington, N. D., Cursons, J. & Rautela, J. The cancer–natural killer cell immunity cycle. *Nat. Rev. Cancer* 20, 437–454 (2020).
23. Böttcher, J. P. *et al.* NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. *Cell* 172, 1022–1037 (2018).
24. Dagher, O. K. & Posey, A. D. Forks in the road for CAR T and CAR NK cell cancer therapies. *Nat. Immunol.* 24, 1994–2007 (2023).
25. Zhang, X. *et al.* Cytokine Release Syndrome After Modified CAR-NK Therapy in an Advanced Non-small Cell Lung Cancer Patient: A Case Report. *Cell Transplant.* 31, (2022).
26. Zhang, L., Meng, Y., Feng, X. & Han, Z. CAR-NK cells for cancer immunotherapy: from bench to bedside. *Biomark. Res.* 10, 1–19 (2022).
27. Rezvani, K., Rouce, R., Liu, E. & Shpall, E. Engineering Natural Killer Cells for Cancer Immunotherapy. *Molecular Therapy* 25, 1769–1781 (2017).

28. Liu, E. *et al.* Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. *New England Journal of Medicine* 382, 545–553 (2020).
29. Zhang, C. *et al.* Chimeric antigen receptor-engineered NK-92 cells: An off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity. *Front. Immunol.* 8, (2017).
30. Biggi, A. F. B. *et al.* IL-27-engineered CAR.19-NK-92 cells exhibit enhanced therapeutic efficacy. *Cytotherapy* 26, 1320–1330 (2024).
31. Stikvoort, A. *et al.* CD38-specific Chimeric Antigen Receptor Expressing Natural Killer KHYG-1 Cells: A Proof of Concept for an ‘off the Shelf’ Therapy for Multiple Myeloma. *Hemasphere* 5, (2021).
32. Nakazawa, T. *et al.* KHYG-1 cells with EGFRvIII-specific CAR induced a pseudoprogression-like feature in subcutaneous tumours derived from glioblastoma-like cells. *Anticancer Res.* 40, 3231–3237 (2020).
33. Lin, W. *et al.* Multi-Omics Data Analyses Identify B7-H3 as a Novel Prognostic Biomarker and Predict Response to Immune Checkpoint Blockade in Head and Neck Squamous Cell Carcinoma. *Front. Immunol.* 12, (2021).
34. Kontos, F. *et al.* B7-H3: An attractive target for antibody-based immunotherapy. *Clinical Cancer Research* 27, 1227–1235 (2021).
35. Torres, A. J., Contento, R. L., Gordo, S., Wucherpennig, K. W. & Love, J. C. Functional single-cell analysis of T-cell activation by supported lipid bilayer-tethered ligands on arrays of nanowells. *Lab Chip* 13, 90–99 (2012).
36. Choudhuri, K. *et al.* Polarized release of TCR-enriched microvesicles at the T cell immunological synapse. *Nature* 507, 118 (2014).
37. Kaizuka, Y., Douglass, A. D., Varma, R., Dustin, M. L. & Vale, R. D. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc. Natl. Acad. Sci. U. S. A.* 104, 20296–20301 (2007).
38. Groves, J. T. Spatial mutation of the T cell immunological synapse. *Curr. Opin. Chem. Biol.* 10, 544–550 (2006).
39. Soldierer, M. *et al.* Genetic Engineering and Enrichment of Human NK Cells for CAR-Enhanced Immunotherapy of Hematological Malignancies. *Front. Immunol.* 13, (2022).
40. Li, R., Ma, C., Cai, H. & Chen, W. The CAR T-Cell Mechanoimmunology at a Glance. *Advanced Science* 7, 2002628 (2020).
41. Vahidian, F. *et al.* The tricks for fighting against cancer using CAR NK cells: A review. *Mol. Cell. Probes* 63, 101817 (2022).
42. Murakami, T. *et al.* Novel human NK cell line carrying CAR targeting EGFRvIII induces antitumor effects in glioblastoma cells. *Anticancer Res.* 38, 5049–5056 (2018).
43. Davenport, A. J. *et al.* Chimeric antigen receptor T cells form nonclassical and potent immune synapses driving rapid cytotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* 115, E2068–E2076 (2018).
44. Orange, J. S. Formation and function of the lytic NK-cell immunological synapse. *Nat. Rev. Immunol.* 8, 713–725 (2008).
45. Davis, D. M. *et al.* The human natural killer cell immune synapse. *Proc. Natl. Acad. Sci. U. S. A.* 96, 15062–15067 (1999).
46. Du, H. *et al.* Antitumor Responses in the Absence of Toxicity in Solid Tumors by Targeting B7-H3 via Chimeric Antigen Receptor T Cells. *Cancer Cell* 35, 221–237 (2019).
47. Choe, J. *et al.* ATRT-10. ANTI-B7-H3 CHIMERIC ANTIGEN RECEPTOR NK CELLS SHOW ANTIGEN SPECIFIC CYTOTOXICITY AGAINST ATYPICAL TERATOID/ RHABDOID TUMORS IN VITRO AND IN VIVO. *Neuro. Oncol.* 26, (2024).
48. Yang, S. *et al.* Targeting B7-H3 Immune Checkpoint With Chimeric Antigen Receptor-Engineered Natural Killer Cells Exhibits Potent Cytotoxicity Against Non-Small Cell Lung Cancer. *Front. Pharmacol.* 11, (2020).
49. Liadi, I. *et al.* Individual motile CD4⁺ T cells can participate in efficient multikilling through conjugation to multiple tumor cells. *Cancer Immunol. Res.* 3, 473–482 (2015).

50. Newick, K., O'Brien, S., Moon, E. & Albelda, S. M. CAR T Cell Therapy for Solid Tumors. *Annu. Rev. Med.* 68, 139–152 (2017).
51. Walker, A. J. *et al.* Tumor Antigen and Receptor Densities Regulate Efficacy of a Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase. *Molecular Therapy* 25, 2189–2201 (2017).
52. Watanabe, K. *et al.* Target Antigen Density Governs the Efficacy of Anti-CD20-CD28-CD3 ζ Chimeric Antigen Receptor-Modified Effector CD8⁺ T Cells. *The Journal of Immunology* 194, 911–920 (2015).
53. Majzner, R. G. *et al.* Low CD19 Antigen Density Diminishes Efficacy of CD19 CAR T Cells and Can be Overcome By Rational Redesign of CAR Signaling Domains. *Blood* 132, 963–963 (2018).
54. Bhat, R. & Watzl, C. Serial killing of tumor cells by human natural killer cells - Enhancement by therapeutic antibodies. *PLoS One* 2, (2007).
55. Davenport, A. J. *et al.* CAR-T cells inflict sequential killing of multiple tumor target cells. *Cancer Immunol. Res.* 3, 483–494 (2015).
56. Suck, G. *et al.* KHYG-1 and NK-92 represent different subtypes of LFA-1-mediated NK cell adhesiveness. *Frontiers in Bioscience - Elite* 3, 166–178 (2011).
57. Bachmann, M. F. *et al.* Distinct roles for LFA-1 and CD28 during activation of naive T cells: Adhesion versus costimulation. *Immunity* 7, 549–557 (1997).
58. Barber, D. F., Faure, M. & Long, E. O. LFA-1 Contributes an Early Signal for NK Cell Cytotoxicity. *The Journal of Immunology* 173, 3653–3659 (2004).
59. Anikeeva, N. *et al.* Distinct role of lymphocyte function-associated antigen-1 in mediating effective cytolytic activity by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* 102, 6437–6442 (2005).
60. Orange, J. S. *et al.* The mature activating natural killer cell immunologic synapse is formed in distinct stages. *Proc. Natl. Acad. Sci. U. S. A.* 100, 14151–14156 (2003).
61. Brown, A. C. N., Dobbie, I. M., Alakoskela, J. M., Davis, I. & Davis, D. M. Super-resolution imaging of remodeled synaptic actin reveals different synergies between NK cell receptors and integrins. *Blood* 120, 3729–3740 (2012).
62. Wang, H. *et al.* Triple knockdown of CD11a, CD49d, and PSGL1 in T cells reduces CAR-T cell toxicity but preserves activity against solid tumors in mice. *Sci. Transl. Med.* 17, (2025).
63. Xiong, W. *et al.* Immunological Synapse Predicts Effectiveness of Chimeric Antigen Receptor Cells. *Molecular Therapy* 26, 963–975 (2018).
64. Weikl, T. R. & Lipowsky, R. Membrane adhesion and domain formation. *ArXiv* <http://arxiv.org/abs/0709.3759> (2007).
65. Hailman, E., Burack, W. R., Shaw, A. S., Dustin, M. L. & Allen, P. M. Immature CD4⁺CD8⁺ thymocytes form a multifocal immunological synapse with sustained tyrosine phosphorylation. *Immunity* 16, 839–848 (2002).
66. Bister, A. *et al.* A novel CD34-derived hinge for rapid and efficient detection and enrichment of CAR T cells. *Mol. Ther. Oncolytics* 23, 534–546 (2021).
67. Roellecke, K. *et al.* Optimized human CYP4B1 in combination with the alkylator prodrug 4-ipomeanol serves as a novel suicide gene system for adoptive T-cell therapies. *Gene Therapy* 23:7 23, 615–626 (2016).
68. Shaner, N. C. *et al.* A bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum*. *Nature Methods* 2013 10:5 10, 407–409 (2013).
69. Hanenberg, H. *et al.* Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nature Medicine* 1996 2:8 2, 876–882 (1996).
70. Oberoi, P. *et al.* Directed Differentiation of Mobilized Hematopoietic Stem and Progenitor Cells into Functional NK Cells with Enhanced Antitumor Activity. *Cells* 2020, Vol. 9, 9, (2020).

Acknowledgments

We thank the healthy donors for providing peripheral blood for the studies.

Funding sources

CM, LC and MT acknowledge financial support from the Freigeist Fellowship of the Volkswagen Foundation. CM further acknowledges financial support from the Momentum program of the Volkswagen Foundation. The authors also acknowledge funding by DFG (German Research Foundation) and the state of North Rhine-Westphalia for the acquisition of the custom MFIS-SSTR Nikon microscope (A1HD25-NSTORM) within the Major Research Instrumentation program (Art. 91b GG), funding ID INST 208/797-1 FUGG, awarded to Claus Seidel (Physical Chemistry, Heinrich Heine University Düsseldorf) and CM and for the MFD-STED microscope within program Major Research Instrumentation as per Art. 91b GG with the funding ID (INST 208/741-1 FUGG), awarded to Claus Seidel.

Authors contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conceptualization: CM, CW, HH.

Methodology: LC, MT, MS.

Software: LC, MT.

Validation: LC, MT, CM.

Formal analysis: LC, MT, MS, LH, NS.

Investigation: LC, MT, MS, LH, NS.

Resources: CM, CW, HH.

Data Curation: LC, MT, MS, LH, NS.

Writing—original draft: LC, MT, MS, CM.

Writing—review and editing: LC, MT, CM.

Visualization: LC, MT.

Supervision: CM, CW, HH.

Project administration: CM.

Funding acquisition: CM.

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

Primary human NK cells were isolated from peripheral blood of healthy adult volunteers who provided written informed consent prior to participation. The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee of the University Hospital Essen (24-12172-BO).

SUPPLEMENTS

Ligand-Defined Phase Space Predicts CAR-NK Synapse Architecture and Cytotoxic Organization

Authors

Luisa Coen^{1,2}, Miriam N. Tappel², Lara Hölzle¹, Maren Soldierer⁴, Nicole Sander³, Constanze Wiek³, Helmut Hanenberg^{3,4}, Cornelia Monzel^{1,2*}

Affiliations

- 1 Experimental Medical Physics, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany
- 2 present address: 2nd Physics Institute, University Stuttgart, 70569 Stuttgart, Germany
- 3 Department of Otorhinolaryngology (ENT), University Hospital Düsseldorf, 40225 Düsseldorf, Germany
- 4 Department of Pediatrics, University Hospital Essen, 45147 Essen, Germany

Supplementary Figures

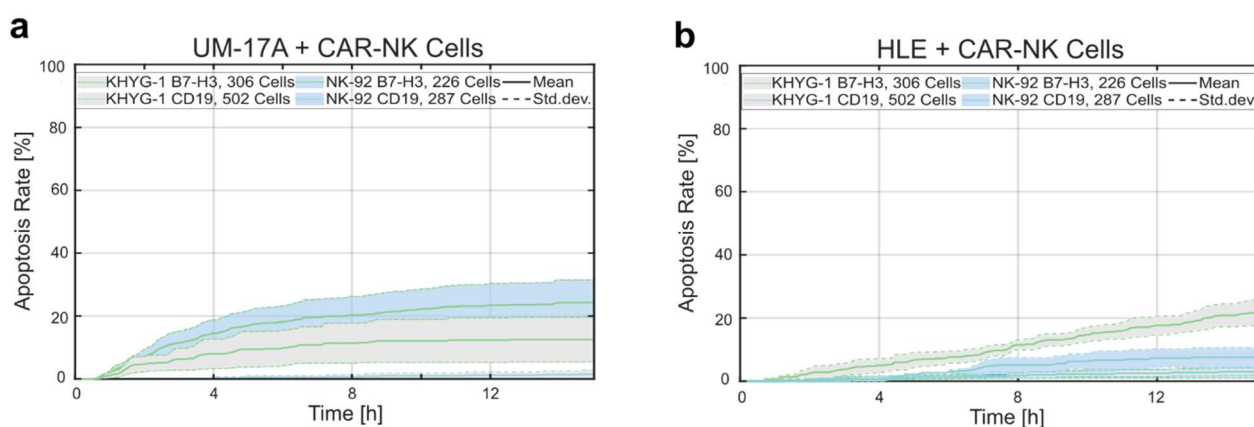


Figure S1: Killing efficiency of different CAR-NK cell lines against tumor cells. Cytotoxic activity of NK-92 and KHYG-1 cells engineered to express either a B7-H3-specific CAR (targeting construct) or a CD19-specific CAR (control construct) against tumor target cells. **a**, Cytotoxicity against UM17A cells. **b**, Cytotoxicity against HLE cells. Killing efficiency was determined under the indicated experimental conditions. Effector-to-target ratios of 1:1.

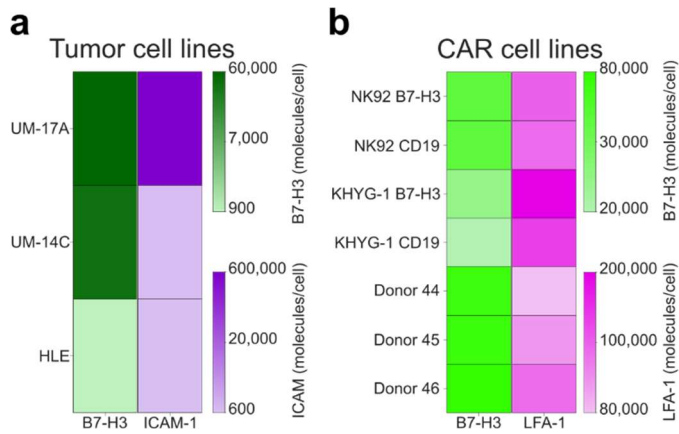


Figure S2: Quantitative surface protein densities determined by flow cytometry. a, Absolute surface abundances of B7-H3 (green) and ICAM-1 (purple) on the tumor cell lines UM17A, UM14C, and HLE, as determined by quantitative flow cytometry. Color intensity scales with the measured number of surface proteins per cell. Based on B7-H3 expression levels, UM17A and UM14C are classified as intermediate-target tumor cell lines, whereas HLE displays low target antigen expression. **b,** Absolute surface abundances of CAR (light green) and LFA-1 (pink) on the indicated CAR-NK cell lines. Color intensity corresponds to the quantified number of proteins present on the cell surface.

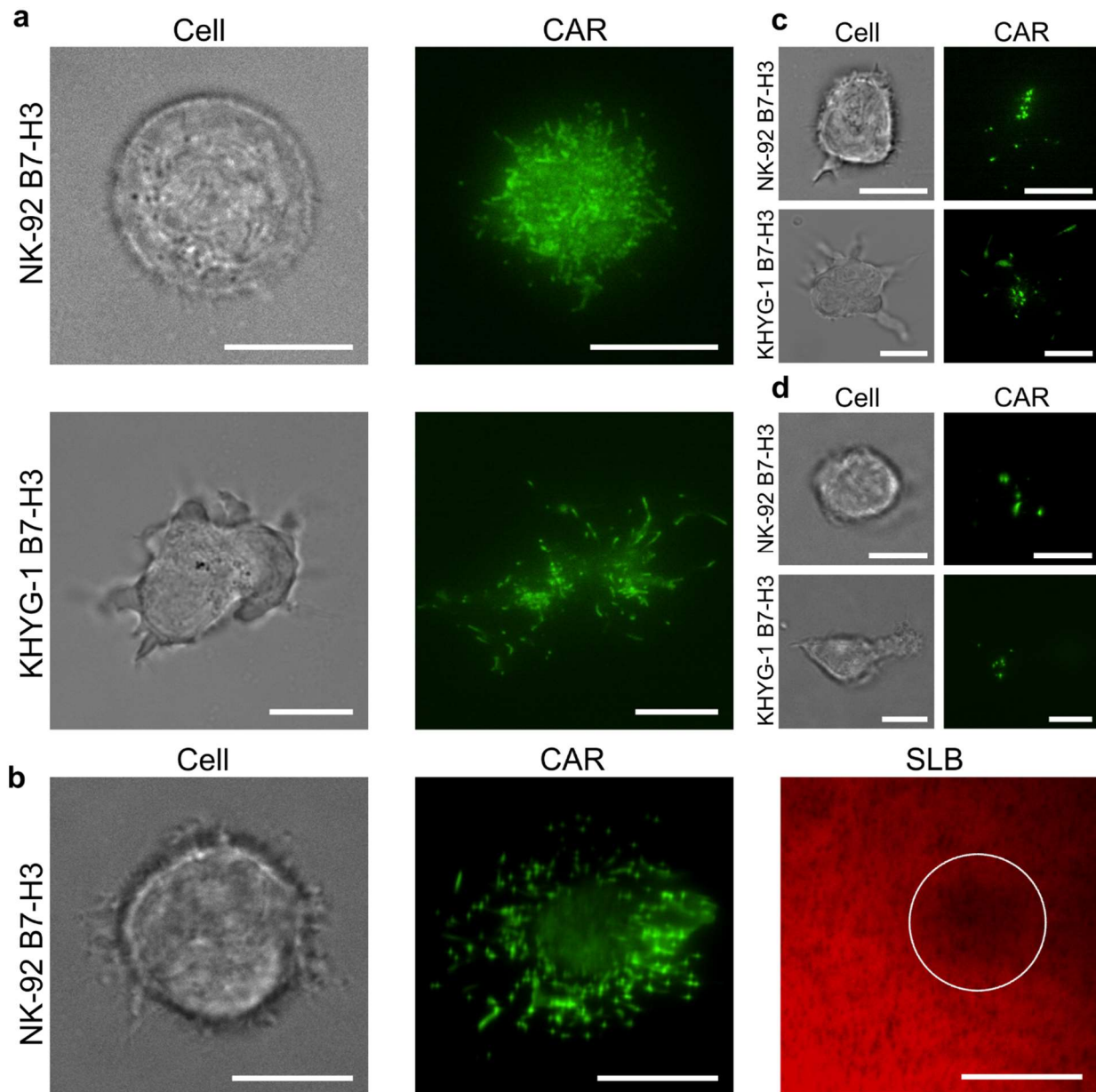


Figure S3: Antigen-specific formation of CAR-NK cell contact zones on simplified tumor-mimetic SLBs. Brightfield and total TIRF microscopy images of CAR-NK cells interacting with SLBs serving as a minimal model of the tumor cell membrane. **a**, SLBs functionalized with B7-H3, the target antigen of the CAR construct, promote robust adhesion of B7-H3-CAR NK cells and the formation of stable cell-bilayer contact zones. **b**, Engagement of B7-H3-CAR NK-92 cells with B7-H3-functionalized SLBs induces lateral reorganization of fluorescent lipids within the bilayer, indicating active remodeling of the membrane interface. **c**, B7-H3-CAR NK cells fail to establish stable contacts with unfunctionalized SLBs. **d**, No stable binding is observed on SLBs functionalized with the irrelevant control antigen CD19, demonstrating that contact formation is specifically mediated by recognition of B7-H3. Scale bars: 10 μm .

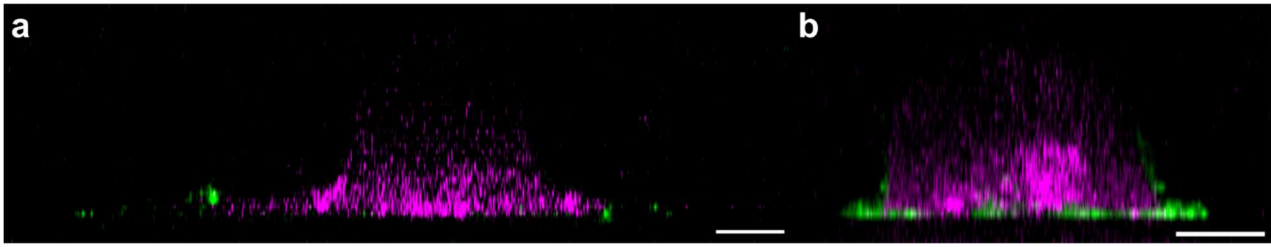


Figure S4: Differential redistribution of CAR and LFA-1 during immune synapse formation on supported lipid bilayers. Representative confocal z-stack of a B7-H3-CAR NK-92 cell. **a**, building an immunological synapse with a UM-14C tumor cell. **b**, the same recruitment of receptors is observed on a SLB functionalized with 10 mol% B7-H3 and 10 mol% ICAM-1. The B7-H3 CAR is shown in green and LFA-1 in magenta. B7-H3 CAR molecules are strongly enriched at the cell–bilayer interface, indicating pronounced recruitment into the immune synapse, whereas LFA-1 remains distributed across the cell surface with only modest accumulation at the contact site. Scale bar: 5 μ m. Corresponding three-dimensional rendering shown in **Video S1** (B7-H3-CAR-NK92 cell on UM-14C tumor cell) and **Video S2** (B7-H3-CAR-NK92 cell on SLB) illustrating the distinct spatial localization of CAR and LFA-1 during synapse formation.

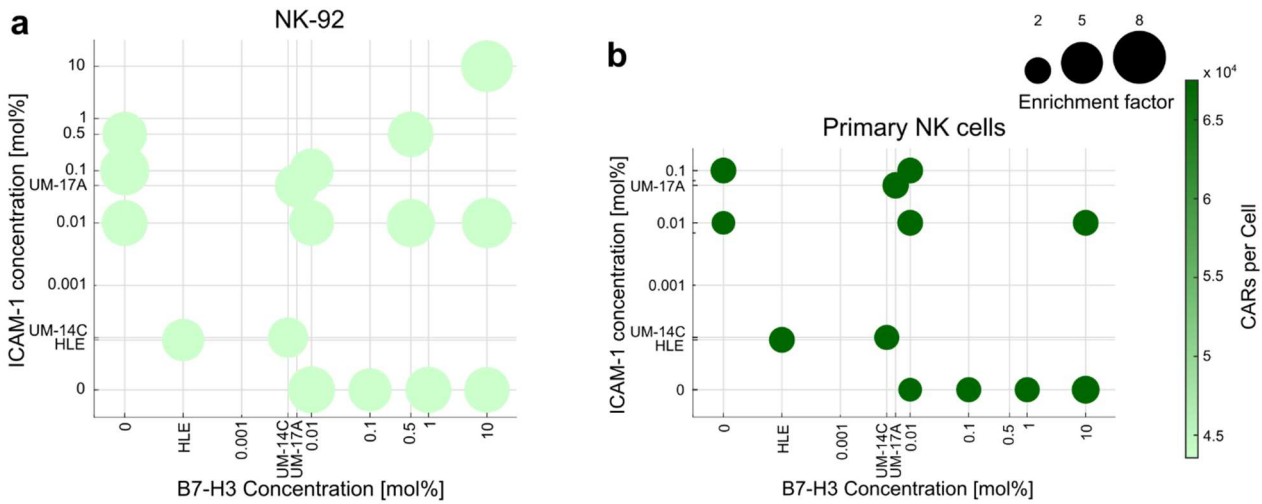


Figure S5: Antigen-dependent enrichment of B7-H3 CAR at the contact interface on SLBs and tumor cells. Enrichment factor of B7-H3 CAR within the cell–bilayer contact zone formed on SLBs containing varying densities of B7-H3 and ICAM-1. **a**, CAR-NK92 cells. **b**, Primary CAR-NK cells. Color scale indicates the overall CAR surface density on non-engaged cells, as determined by quantitative flow cytometry, whereas circle size represents the degree of CAR enrichment within the contact zone, determined by the fraction of intensity in the HIR divided by the intensity in the remaining CZ. CAR-NK92 cells exhibit lower overall surface receptor densities but show pronounced CAR accumulation at the synaptic interface. In contrast, primary CAR-NK cells display high constitutive CAR surface abundance accompanied by only modest additional enrichment at the contact zone, consistent with a limited dynamic range for further receptor recruitment.

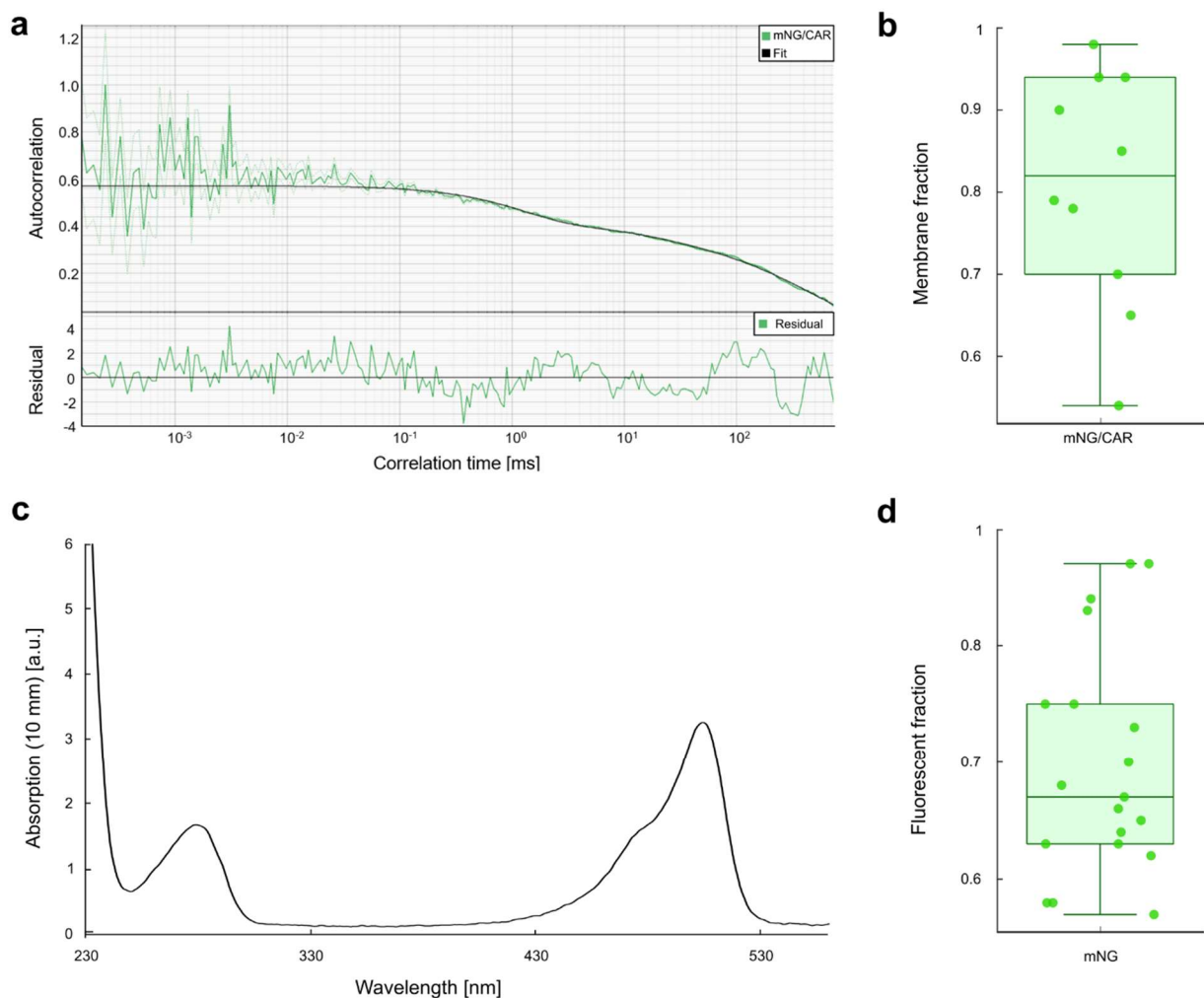


Figure S6: Determination of correction factors for membrane localization and fluorophore maturation.

a, Representative FCS measurement acquired with the confocal volume positioned at the plasma membrane. The autocorrelation curve was fitted using a two-component diffusion model comprising a membrane-associated (2D) and a cytoplasmic (3D) CAR population. The cytoplasmic diffusion time was fixed to the value determined from cytosolic measurements ($\tau_{cyt} = 26$ ms). Residuals of the fit are shown below. **b,** Membrane-associated CAR fraction (f_{mem}) obtained from the FCS analysis shown in panel a. Each data point represents an independent measurement ($n=10$). The median membrane-associated fraction was 0.82. **c,** Absorbance spectrum of purified mNeonGreen used to determine the fraction of fluorescent molecules. Absorbance at 280 nm and 506 nm was used to quantify total and fluorescent protein concentrations, respectively. **d,** Fluorescent fraction of mNeonGreen (R_{mNG}) determined from absorbance measurements according to the Beer–Lambert law. Each data point represents an independent measurement ($n=19$). The median fluorescent fraction was 0.67.

3.4 Publication IV: Co-expression of Non-Signaling CARs Enhances/Boosts EGFRvIII-Specific CAR-T Cell Cytotoxicity Against Head and Neck Squamous Cell Carcinoma.

Authors: Kathleen Grueter, Nicole Sander, Luisa Coen, Saskia Hüsken, Eileen Kleinfelder, Corinna Haist, Franziska Blaesche, Kathrin Scheckenbach, Cornalia Monzel, Helmut Hanenberg, Constanze Wiek

Journal: *BMC Cancer*

Submitted

Contribution

The doctoral candidate (LC) made a minor contribution to this publication, accounting for approximately 5% of the overall work. This contribution comprised the acquisition of multicolor fluorescence imaging data and the corresponding visualization of experimental results.

In addition, the doctoral candidate provided advisory input during the course of the study and contributed comments and revisions to the completed manuscript draft. The remaining contributions, including conceptualization, the main experimental work, data analysis, manuscript preparation, and supervision, were carried out by the co-authors as specified in the original publication.

Co-expression of Non-Signaling CARs Enhances EGFRvIII-Specific CAR T-Cell Cytotoxicity Against Head and Neck Squamous Cell Carcinoma

Kathleen Grueter^{1,2,3,4}, Nicole Sander^{1,5}, Luisa Coen^{5,6}, Saskia Hüsken¹, Eileen Kleinfelder^{1,7}, Corinna Haist¹, Franziska Blaesche^{2,3,8}, Kathrin Scheckenbach¹, Cornelia Monzel^{5,6}, Helmut Hanenberg^{1,7}, Constanze Wiek^{1*}

¹Department of Otorhinolaryngology, University Hospital Düsseldorf, Medical Faculty of Heinrich-Heine University, Düsseldorf, Germany.

²Hopp Children's Cancer Center Heidelberg (KiTZ), Heidelberg, Germany.

³German Cancer Research Center (DKFZ), Heidelberg, Germany.

⁴Faculty of Biosciences, Heidelberg University, Heidelberg, Germany.

⁵Department of Experimental Medical Physics, Heinrich-Heine University, Düsseldorf, Germany.

⁶2nd Institute of Physics, University of Stuttgart, Stuttgart, Germany

⁷Department of Pediatrics III, University Children's Hospital Essen, University Duisburg-Essen, Essen, Germany.

⁸Department of Pediatric Oncology, Hematology and Immunology, Heidelberg University Hospital, Heidelberg, Germany.

*Correspondence:

Constanze Wiek, PhD

Constanze.wiek@med.uni-duesseldorf.de

Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) remains difficult to treat due to heterogeneous antigen expression and an immunosuppressive tumor microenvironment. The epidermal growth factor receptor variant III (EGFRvIII) is a tumor-specific splice form without detectable protein expression in normal tissues. Nevertheless, its low and often heterogeneous expression in HNSCC limits its usability as target antigen for immunological therapy with chimeric antigen receptor (CAR) T-cells. As full-length EGFR is robustly/consistently (over)expressed in HNSCC and frequently co-expressed with EGFRvIII, we investigated whether EGFR could serve as a secondary target for a non-signaling CAR (nsCAR), thereby potentially enhancing target cell recognition and cytotoxicity while minimizing *off-target* effects.

Methods: Primary human T-cells were engineered to express EGFRvIII-specific CARs alone or in combination with EGFR-directed nsCARs. Functional activity was evaluated using *in vitro* cytotoxicity assays, time-resolved killing dynamics, and fluorescence microscopy to assess immunological synapse (IS) formation in HNSCC cell lines with defined EGFRvIII expression levels.

Results: EGFRvIII CAR T-cells exhibited clear target antigen density-dependent cytotoxicity. Co-expression of EGFR nsCARs specifically enhanced tumor cell killing under low antigen conditions and accelerated killing kinetics, while the nsCAR alone was non-cytotoxic. Fluorescence imaging revealed co-localization of CAR and nsCAR proteins at the IS.

Conclusions: EGFR nsCAR co-expression provides a modular strategy to enhance EGFRvIII CAR T-cell efficacy in low-antigen solid tumors by potentially stabilizing IS formation and thereby increasing cytotoxicity. This dual-targeting approach may improve cellular immunotherapy in HNSCC and other solid cancers and warrants further investigation regarding *in vivo* efficacy, persistence, and broader applicability.

Keywords: HNSCC, EGFRvIII, CAR T-cell therapy, non-signaling CAR, tumor-specific antigen (TSA), immunotherapy, antigen density, dual-targeting.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common cancer worldwide with 890,000 cases diagnosed and 450,000 deaths per year [1, 2]. Treatment modalities for HNSCC primarily comprise combinations of surgery, radiation therapy, chemotherapy, and targeted therapies, including antibody therapies with the epidermal growth factor receptor (EGFR)-antibody Cetuximab and immune checkpoint inhibitors (ICIs) such as Nivolumab or Pembrolizumab, targeting the immunosuppressive PD-1/PD-L1 interaction between the cytotoxic T-lymphocytes and the malignant cells within the tumor microenvironment (TME) [3-5]. Still, the 5-year overall survival of HNSCC remains poor, as up to half of patients with locally advanced disease develop locoregional recurrence and/or distant metastases [3, 4].

Targeted personalized therapies with chimeric antigen receptors (CARs) represent a new powerful immunotherapeutic strategy to redirect autologous T-cells against antigens expressed on malignant or non-malignant cells. When expressed on T-cells, CARs enable efficient recognition and elimination of antigen-positive cells in an HLA-independent manner [6]. While CAR T-cell therapy has achieved major clinical breakthroughs in hematological malignancies of the B-cell lineage, its translation to solid tumors remains limited due to substantial challenges (reviewed in [7]), including (i) heterogeneity of target antigen expression among malignant cells; (ii) restricted trafficking and infiltration of CAR T-cells into tumors, which comprise a complex

milieu of malignant cells, stromal fibroblasts, extracellular matrix, vasculature, and immunosuppressive cells; (iii) a hostile tumor microenvironment characterized by hypoxia, nutrient deprivation, and elevated immunosuppressive mediators; and (iv) limited persistence and functional fitness of CAR T-cells within tumor tissue. Nevertheless, some solid tumors have shown responsiveness to CAR T-cell therapy, particularly when a tumor-associated and relatively homogeneously expressed target antigen is available. Notable recent examples are the disialoganglioside GD2 in neuroblastoma or glypican-3 in several tumor entities, where recent clinical studies have demonstrated feasibility and specific activity of target antigen-directed CAR T-cells [8, 9]. Building on this concept, other tumor-specific antigens (TSAs) are being investigated to enable more selective targeting approaches in solid tumors [10].

One of the most frequently altered signaling pathways in HNSCC involves the epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase that plays a crucial role in regulating cell proliferation, survival, and differentiation in epithelial tissues [11]. Overexpression or aberrant activation of EGFR has been detected in the majority of HNSCC cases and contributes to tumor progression and therapy resistance [12]. Among EGFR variants, the mutant form EGFRvIII is of particular interest because it is tumor-specific and lacks detectable protein expression in normal tissues. EGFRvIII arises from an *in-frame* deletion of exons 2 to 7 in the extracellular domain of EGFR, removing 267 amino acids and resulting in a shortened protein that is capable of constitutive, ligand-independent signaling [13-16]. Although the kinase activity of EGFRvIII is weaker than the enzyme activity of ligand-activated full-length EGFR, its residual constitutive activity is nevertheless sufficient to confer a survival advantage to tumor cells of various origins [17, 18]. Specifically, expression of EGFRvIII promotes cell proliferation, invasion,

and tumor progression in different model systems and can indirectly enhance neoangiogenesis through upregulation of pro-angiogenic factors such as VEGF via downstream signaling pathways [19-21].

Importantly, the deletion generates a novel tumor-specific junctional epitope that is absent in full-length EGFR, thereby enabling selective targeting. This unique neoepitope, together with the restricted expression of EGFRvIII in tumor tissue, has made it an attractive candidate for immunotherapeutic strategies. Indeed, EGFRvIII CAR T-cells have demonstrated selective recognition of EGFRvIII-expressing tumor cells and have shown safety and feasibility in early-phase clinical trials in patients with recurrent glioblastoma (GBM); however, clinical efficacy has been limited, potentially due to antigen heterogeneity and antigen loss under therapeutic pressure [22, 23]. More recently, dual-targeting strategies have been explored to overcome these limitations. In a small cohort of patients with recurrent GBM, treatment with EGFRvIII CAR T-cells engineered to secrete a bispecific T-cell engager (BiTE) targeting EGFR and CD3 ϵ resulted in encouraging initial responses and manageable toxicity following a single intraventricular administration. However, despite these promising early outcomes, disease progression ultimately occurred, likely reflecting the ongoing challenges of antigen escape and tumor heterogeneity in this setting [24, 25].

CAR T-cells are highly sensitive to target antigen density on tumor cells [26-28]. Their function can diminish considerably when target antigen expression levels fall below a critical threshold which varies depending on the specific target and the affinity and avidity of the single chain variable fragment (scFv) in the CAR construct [26, 29-31]. In addition, considering the tumor heterogeneity and the specific down-regulation or loss of target antigen expression, different

strategies have been proposed to improve tumor recognition, including the co-expression of a second CAR in bicistronic constructs or the use of tandem CAR designs targeting multiple tumor-associated antigens [32-35]. However, the functional consequences of simultaneously engaging two distinct antigens – particularly with respect to CAR T-cell activation, persistence, and susceptibility to antigen-loss variants – are complex and remain poorly understood.

Here, we explored a dual-targeting approach based on the co-expression of a 2nd generation CAR with CD28 costimulation, specifically recognizing EGFRvIII, alongside a CAR without any signaling domains (non-signaling CAR, nsCAR) that targets a second antigen or epitope. The nsCAR is designed to enhance target cell recognition without directly triggering T-cell activation, thereby providing an additional layer of target engagement and potentially improving specificity while minimizing *off-target effects*. We hypothesized that this modular strategy will improve CAR T-cell performance under conditions of heterogeneous antigen expression by increasing functional avidity and reducing vulnerability to antigen-loss mechanisms.

Methods

Cells culture

HEK293T and DK-MG cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). HNSCC cell lines were obtained from the University of Michigan (UM, USA), the University of Turku (UT, Finland), or from the University of Düsseldorf (UD, Germany). Primary human fibroblasts (Fibro) from a healthy donor were described previously [36]. Several EGFR knockout (KO) cell lines, UM-14C^{KO}, UT-24A^{KO} and UM-10B^{KO}, were previously generated in our laboratory by using lentiviral CRISPR/Cas9 technology [37]. All adherent cells were cultured

in DMEM GlutaMAX medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Thermo Fisher Scientific, Germany).

Primary human T-cells were collected from peripheral blood of healthy adult donors who gave informed consent according to the protocols approved by the local ethics committee of the University Hospital Düsseldorf. Peripheral blood mononuclear cells (PBMCs) were separated by density-gradient centrifugation using Ficoll-Paque (Cytiva Europe, Germany) and cultured in IMDM (Sigma-Aldrich, Germany) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (all from Thermo Fisher Scientific), and 100 U/mL interleukin-2 (IL-2 (Proleukin; Novartis, Switzerland) on 6-well plates pre-coated with CD3 (Thermo Fisher Scientific) and CD28 antibodies (BD Biosciences, USA) to activate and expand T-cells, as described before [37]. All cells were maintained at 5% CO₂, 95% humidity, and 37 °C.

RT-PCR Analysis for EGFRvIII identification

Total RNA was extracted from 5×10^6 cells using the NucleoSpin® RNA Kit (Macherey-Nagel, Germany) following manufacturer's instructions. cDNA was synthesized via two-step reverse transcription-PCR. First-strand cDNA was generated from 1 µg total RNA using Random Hexamer primers (Thermo Fisher Scientific), and M-MuLV Reverse Transcriptase (New England Biolabs, USA). For *EGFRvIII* detection, a subsequent PCR was performed using gene-specific primers flanking the deletion of exons 2 to 7, as described by Sok et al. [38]. PCR amplification was performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs). The thermal cycling conditions consisted of denaturation at 94 °C, annealing at 55 °C, and extension at 72 °C for 1 min each, repeated for a total of 38 cycles. Amplification products were analyzed by agarose

gel electrophoresis. For confirmatory cDNA sequencing, DNA fragments corresponding to *EGFR* and *EGFRvIII* were excised from the gel and subjected to sequencing by SeqLab-Microsynth (Germany).

Lentiviral Expression Constructs

All lentiviral vectors are derivatives of the puc2CL6 vector [39] and were generated using standard cloning techniques.

Constructs used for the comparative analysis of different promoters were generated by PCR amplification of the promoters from donor plasmids and subsequently cloned into our standard enhanced green fluorescent protein (GFP) vector (p2CL6EGwo). The myeloproliferative sarcoma virus (MPSV), human phosphoglycerate kinase 1 (hPGK), spleen focus-forming virus (SFFV), and human elongation factor 1- α (hEF1 α) promoter constructs were also previously published [40-42]. Human promoters derived from *Fanconi anemia C* and *G* (*FANCC* and *FANCG*, not published, personal communicated by Prof. Dr. Detlev Schindler, Würzburg) genes and in addition, a murine promoter from *phosphoglycerate kinase* (*mPGK*) gene were included in the promoter analysis [43].

EGFR cDNA was cloned out of pBABEpuro-ERBB2 (a gift from Matthew Meyerson (Addgene plasmid # 40987) [44]) in our lentiviral vector and then the EGFRvIII splice form was generated by removing exons 2–7 in the EGFR gene via overlap PCR. EGFR protein sequence information corresponds to UniProt accession P00533. EGFRvIII corresponds to the natural EGFR variant VAR_066493 (deletion of amino acids 30–297). To express EGFRvIII and neomycin

phosphotransferase *npt II* (NEO) genes separated by a ribosome entry site (IRES) in different levels, three promoters were chosen (MPSV, hEF1 α and hPGK).

We used CARs directed to EGFRvIII based on scFvs derived from two mAbs: murine 3C10 (CAR 9 referred to CAR EGFRvIII^A) and humanized murine 3C10 (CAR 6 referred to CAR EGFRvIII^B; CAR 8 referred to CAR EGFRvIII^C; CAR 1 referred to CAR EGFRvIII^D) from Novartis patent US2017/0008963A1 [45] and humanized 139 scFv (referred to CAR EGFRvIII^E) from patent WO2012/138475A1 [46]. All EGFRvIII scFv sequences (CAR EGFRvIII^{A-E}) were codon optimized for human using the internal algorithm of Thermo Fisher GeneArt and cloned into our self-inactivating lentiviral vector expressing a CD19 CAR with CD8 leader and a c6 hinge off a viral MPSV U3 promoter [47]. For expression of two transgenes all second-generation CARs were inserted behind tagBFP (referred to as BFP) or mCherry in front of the T2A site [41, 42]. The control FMC63-based CD19, Cetuximab-based EGFR, DRB2-based CD33, and BIWA8-based CD44v6 CAR constructs were previously published [37, 42, 47, 48]. The FMC63-based CD19 and Cetuximab-based EGFR scFv were used for nsCAR design with the human Ig heavy chain leader peptide under control of the MPSV U3 promoter and equipped with a NGFR-derived hinge region (n3), CD8 transmembrane, and without costimulatory domains [49].

The codon-optimized firefly luciferase/GFP fusion (LucGFP) vector was previously published [47, 50].

Lentiviral production and cell transduction

Lentiviral particle production in HEK293T cells was carried out as previously described [41, 42, 50]. For pseudotyping, either vesicular stomatitis virus glycoprotein (VSV-G; 6 μ g per transfection)

or codon-optimized baboon endogenous virus envelope lacking the R-peptide (BaEV-Rless; 1 µg per transfection) was used. Cell culture supernatants containing replication-deficient lentiviral particles were collected 48 hours post-transfection, filtered through a 0.45-µm filter, and either used directly or concentrated 5-fold by centrifugation at 10,000 × g for 2 hours at 4 °C. Viral supernatants were applied fresh or stored at –80 °C until use. Transduction of HNSCC cell lines and CD3/CD28-prestimulated primary human T-cells was performed in the presence of 10 µg/mL protamine sulfate (Sigma-Aldrich). 72 hours after expansion and transduction, CAR T-cells were enriched with the MACS system (magnetic cell sorting, Miltenyi Biotec, Germany) by CD34 microbeads or CD271/LNGFR MACSelect microbeads as described in Bister et al. [47, 49], following the manufacturer's protocol.

Flow cytometry analysis and sorting

To assess EGFR and EGFRvIII surface expression, HNSCC cells were stained with anti-EGFR (PE or APC Conjugate, Miltenyi Biotec) and EGFRvIII Recombinant Rabbit Monoclonal Antibody (clone RM419, Invitrogen, USA) and 2nd antibody Anti-rabbit IgG (H+L) F(ab')₂ Fragment (PE Conjugate, Cell Signaling Technology, USA) following manufacturer's instructions. For the detection of CARs or nsCARs on T-cells in the three fractions (pre-MACS, flow-through, and post-MACS), staining was performed using anti-CD34 (clone QBend-10, Thermo Fisher Scientific) or anti-CD271 antibodies (clone ME20.4, Miltenyi Biotec). After staining and washing, cells were resuspended in MACS buffer containing either PI (Thermo Scientific) or DAPI (Miltenyi Biotec) for live/dead cell discrimination. Data were acquired on either a MACSQuant X flow cytometer (Miltenyi Biotec) or

CytoFLEX S flow cytometer (Beckman Coulter, USA), and analysed using FlowJo software (BD Biosciences).

Fluorescence-activated cell sorting was performed at the Core Facility Flow Cytometry at the Heinrich-Heine University Düsseldorf, Germany. For sorting of EGFRvIII-positive cells, 5×10^6 cells were stained with an anti-EGFR-PE antibody (Miltenyi Biotec), washed and sorted. Sorted cells were washed again, resuspended in fresh culture medium and further cultured under standard conditions.

Cytotoxicity assay

CellTiter96® AQueous One Solution Cell Proliferation Assay

To assess the cytotoxicity of effector T-cells, the CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega, USA) was used. Target cells (2×10^4 target cells per well) were seeded in tissue culture-treated 96-well plates and incubated overnight at 37 °C. On the following day, MACS-selected transduced T-cells were added at different effector to target cell (E:T) ratios ranging from 3:1 to 0.01:1. Co-cultures were incubated for 16–20 hours at 37 °C, unless stated otherwise. After incubation, wells were washed three times with PBS to remove non-adherent T-cells and dead target cells. The CellTiter96® reagent was added according to the manufacturer's instructions. The number of viable, adherent target cells was quantified by measuring absorbance at 490 nm using a Sunrise™ microplate reader (TECAN, Switzerland).

Luciferase Luminescence Cytotoxicity Assay

The cytotoxicity of CAR T-cells on target cell lines (UM-14C^{KO}+EGFRvIII^{high} and DK-MG), which were successfully transduced with the luciferase transgene, was measured via luciferase cytotoxicity assay. Target cells (2×10^4 target cells per well) were seeded in white TCT 96-well plates with a transparent bottom (Nunclon 96 Flat Bottom White Polystyrol LumiNunc FluoroNunc, Thermo Fisher Scientific) and cultured overnight at 37 °C. On the following day, MACS-selected transduced T-cells were added at different effector to target cell (E:T) ratios ranging from 3:1 to 0.01:1. Co-cultures were incubated for 16–20 hours at 37 °C. After incubation, the medium from each well was removed, and 100 µL of cell culture medium supplemented with D-luciferin (OZ Biosciences, France) at a final concentration of 0.75 µg/mL was added. The number of viable tumor cells was determined by measuring the luminescence with the Infinite M200 PRO plate reader (TECAN).

For both cytotoxicity assays the percent lysis was calculated as: $100\% - (\text{absorption of target cells incubated with T-cells} / \text{absorption of target cells only} \times 100)$.

Microscopy

For visualization of the IS, 2×10^4 UM-14C^{KO}+EGFRvIII^{high} cells were seeded in 8-well glass bottom chamber slide (Ibidi, Germany). On the following day, doubly transduced CAR/nsCAR T-cells were stained using an anti-CD34-FITC antibody (Qbend-10, Thermo Fisher Scientific) (labelling the c6 hinge region of the CAR) and an anti-NGFR-PE-Vio770 antibody (Miltenyi Biotec) (labelling the n3 hinge region of the nsCAR), according to manufacturer's protocols.

Subsequently, stained CAR/nsCAR T-cells were co-cultured with the target cells, and IS formation was analyzed by fluorescence microscopy using an IX83 microscope system (Olympus, Japan)

equipped with a 60× oil-immersion objective (NA 0.65–1.25) and CellSense Dimensions software (Olympus, version 1.18). In addition, cells were monitored with a confocal *Nikon ECLIPSE Ti2* microscope (Nikon, Japan), equipped with a 100× oil-immersion objective (NA 1.49) and NIS-Elements AR-SP software (Nikon). CAR and nsCAR expression were visualized using CD34^{FITC} (green) and NGFR^{PE-Vio770} (red), respectively. BFP-positive T-cells appeared in blue, and UM-14C^{KO}+EGFRvIII^{high} cells were visualized in phase contrast (PH, grayscale). Contrast settings (channels: PH, 377/447 nm, 470/525 nm and 545/620 nm) were adjusted to maximize the visibility of the IS. Analysis was performed with Omero software (Open Microsoft Environment, version: 5.30.1).

Results

EGFRvIII expression profiles of primary HNSCC cell lines

To investigate EGFRvIII (**Figure 1A**) expression at mRNA levels in HNSCC, a panel of established HNSCC cell lines was analyzed by RT-PCR using primers flanking the deletion of exons 2 to 7. This primer design allowed discrimination between the truncated EGFRvIII transcript (236 bp) and the full-length EGFR transcript (1037 bp) [38]. As shown in **Figure 1B**, full-length EGFR mRNA (1037 bp) was detected in all HNSCC cell lines, in primary human fibroblasts (Fibro), and the EGFRvIII-positive glioblastoma line DK-MG [51]. Clear EGFRvIII PCR products were observed in DK-MG, UM-14C and UT-24A cells and the specificity of the bands was confirmed by sequencing. Flow cytometric analysis (**Figures 1C–D**) demonstrated surface expression of EGFR (blue bars) across all HNSCC cell lines with variable intensity, whereas EGFRvIII (red bars) was not

reliably detected on cells, including the three PCR-positive cell lines, as detected using a previously described monoclonal antibody targeting the deletion-specific amino acids [52].

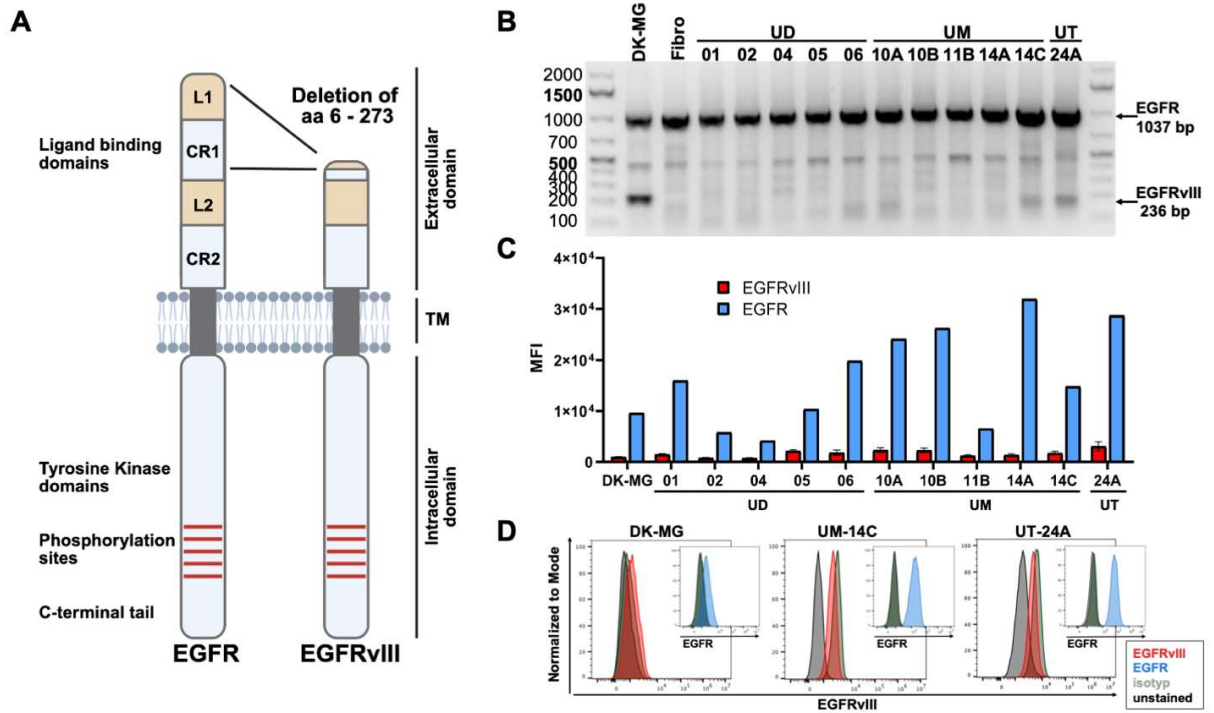


Figure 1: EGFRvIII expression in WT HNSCC cell lines. (A) Schematic structure of EGFR and EGFRvIII. The EGFRvIII variant is characterized by an in-frame deletion of exons 2–7 in the full-length EGFR gene. The domains of EGFR are shown, labeled domains L1 (also known as domain 1), CR1 (domain 2), L2 (domain 3) and CR2 (domain 4), respectively (adapted from Gan et al. 2009 [53]). **(B) RT-PCR analysis of EGFRvIII mRNA expression.** EGFRvIII mRNA levels were assessed in HNSCC cell lines by RT-PCR. A representative ethidium bromide-stained agarose gel shows PCR products for EGFR (1037 bp) and EGFRvIII (236 bp). Primary human fibroblasts (Fibro) served as negative control for EGFRvIII, while DK-MG cells were used as a positive control. Multiple HNSCC cell lines were analyzed. **(C+D) Flow cytometric analysis of EGFRvIII protein expression.** Cell lines were stained using a recombinant rabbit monoclonal anti-EGFRvIII antibody followed by a PE-conjugated anti-rabbit IgG (H+L) F(ab')₂ secondary antibody. EGFR expression was detected using APC- or PE-conjugated anti-human EGFR antibodies. **(C)** Relative mean fluorescence intensity (MFI; y-axis) of EGFRvIII and EGFR expression in HNSCC cell lines. EGFRvIII data represent the mean $\pm$ SEM of three independent

experiments. **(D)** Representative histograms of EGFRvIII (red), EGFR (blue), isotype staining with secondary antibody or isotype alone (dark green), and unstained cells (black). Normalized cell count is plotted against the fluorescence intensity.

Generation of HNSCC cell lines with differential EGFRvIII expression

To reliably test EGFRvIII CAR T-cells, we sought to generate HNSCC cell lines lacking the full-length EGFR and overexpressing EGFRvIII at defined levels. For this purpose, we cloned seven internal promoter elements into a self-inactivating (SIN) lentiviral vector expressing the green fluorescent protein (GFP) in the previously described EGFR CRISPR/Cas9 knockout (KO) cells UM-14C^{KO} and UT-24A^{KO} [37] (**Figure S1A**). These included two viral U3 promoters derived from the spleen focus-forming virus (SFFV) and the myeloproliferative sarcoma virus (MPSV), four human promoters derived from the human *elongation factor-1 alpha* (*hEF1α*), *phosphoglycerate kinase* (*hPGK*), and the *Fanconi anemia C and G* (*FANCC* and *FANCG*) genes as well as one promoter derived from the murine *phosphoglycerate kinase* gene (mPGK) [40-43]. Transduction of EGFR KO cells at limiting dilutions with VSV-G-pseudotyped recombinant lentiviral particles enabled to directly compare the strength of the different promoters by measuring the transgene expression levels in flow cytometry. As shown in **Figure S1B**, the viral promoters SFFV and MPSV drove the strongest GFP expression levels at gene transfer efficiencies <25% in both, UM-14C^{KO} and UT-24A^{KO} cells. The lentiviral vectors with the mPGK, hPGK and hEF1α promoters achieved similar GFP expression levels, that were lower than those of the viral promoters. The expression levels of GFP in the cells transduced with the vectors containing the FANCC and FANCG promoters were very low. Based on these findings, SFFV was selected as promoter to achieve high EGFRvIII

transgene expression levels, whereas the hEF1 α and hPGK promoters were chosen to generate intermediate and low expression levels, respectively.

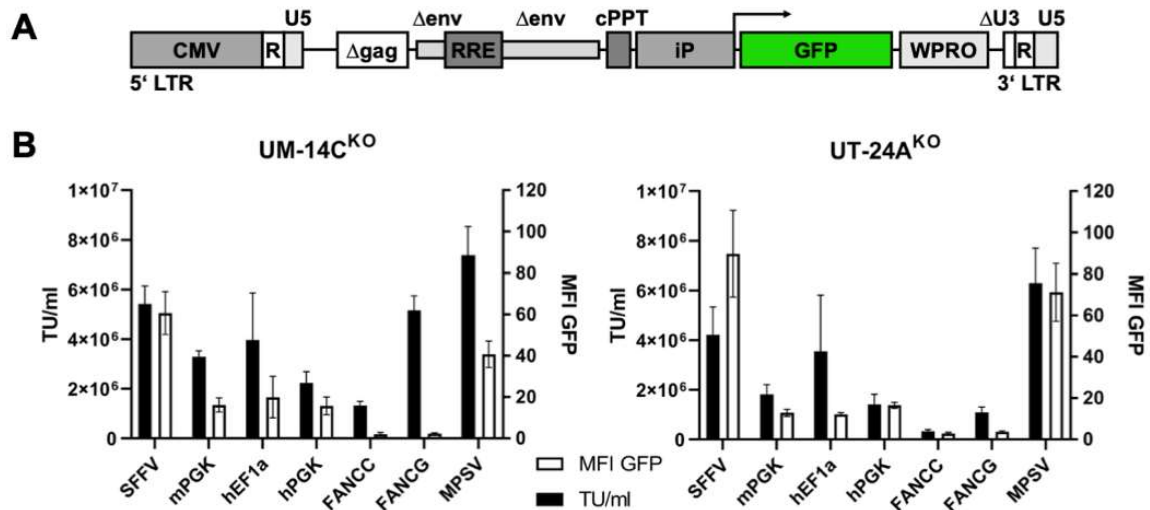


Figure S2: Comparison of the strength of different internal promoters in a SIN lentiviral vector to drive GFP as a marker gene. (A) Schematic representation of the LV expression vector. The internal promoters drive the expression levels of the GFP transgene. **(B) Evaluation of infectious titer (TU/mL) and GFP mean fluorescence intensity (MFI) of LV vectors in EGFR KO cell lines UM-14C^{KO} and UT-24A^{KO}.** Cells were analyzed for GFP expression 5 days after transduction by flow cytometry. Only samples containing fewer than 25% GFP-positive cells were included in the flow cytometry analysis. Means $\pm$ SEM from three independent experiments are shown.

We next generated EGFRvIII expression vectors with an IRES-neoR cassette incorporating these three internal promoters (**Figure 2A**). VSV-G lentiviral particles were used to transduce UM-14C^{KO} and UT-24A^{KO} cells at limiting dilutions, followed by Geneticin selection until all non-transduced cells were eliminated. EGFRvIII expression was detected using a commercially available directly conjugated anti-EGFR antibody targeting domain III of the extracellular region,

which selectively recognizes EGFRvIII in EGFR KO cells [54]. Importantly, the anti-EGFR staining in WT cells detected both EGFR isoforms.

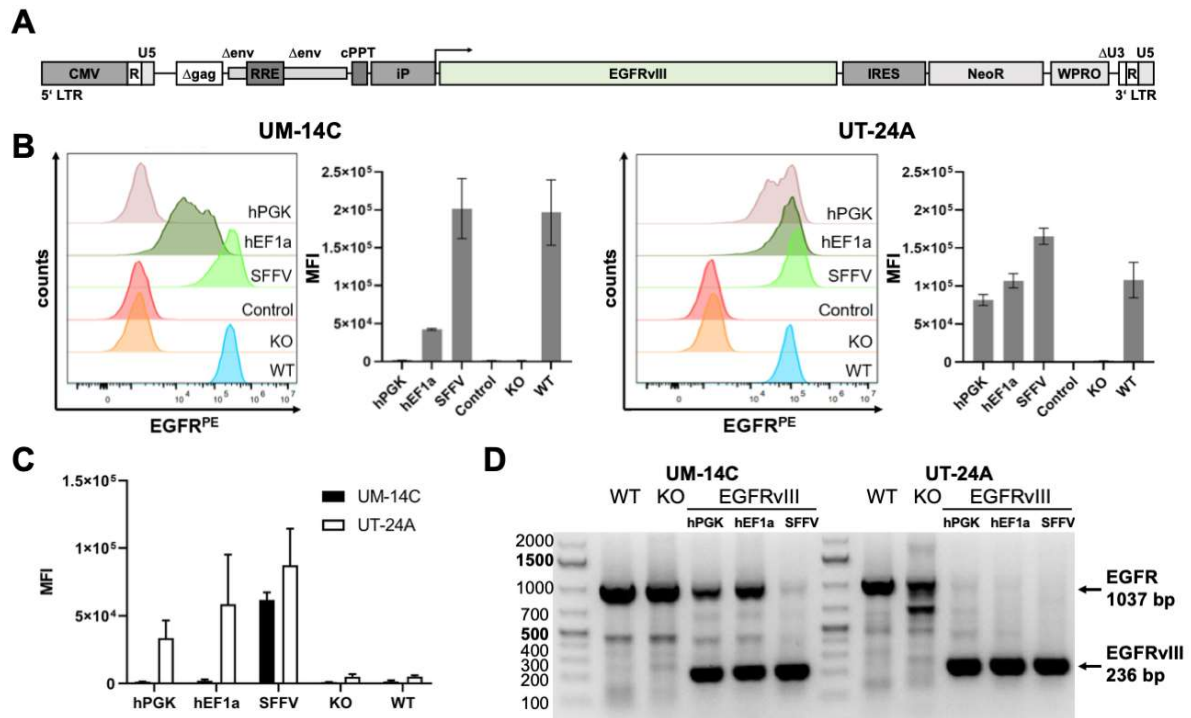


Figure 2: Artificial overexpression of EGFRvIII at different levels in HNSCC cell lines. (A) Schematic representation of the lentiviral EGFRvIII expression vector. The internal promoters (iP: hPGK, hEF1 α and SFFV) drive expression of the EGFRvIII transgene and via an IRES site also the Geneticin resistance gene *neor*. **(B-D)** UM-14C^{KO} and UT-24A^{KO} cells were transduced with LV EGFRvIII expression vectors. EGFRvIII-positive EGFR^{KO} cells were further purified by flow sorting, generating new EGFRvIII-expressing cell lines. **(B+C)** Flow cytometry analysis of EGFRvIII protein expression after sorting. UM-14C and UT-24A cells were stained with **(B)** an anti-EGFR^{PE} antibody for EGFR and EGFRvIII expression. Left: EGFR expression is shown by histogram. Right: Means $\pm$ SEM of MFI values of EGFRvIII-positive EGFR^{KO} UM-14C and UT-24A cells as well as the corresponding UM-14C and UT-24A KO, WT and control cells transduced with an empty vector (data from three independent experiments) are shown. **(C)** EGFRvIII expression was additionally determined using an EGFRvIII-specific recombinant rabbit monoclonal antibody and a PE-conjugated anti-rabbit IgG (H+L) F(ab')₂ secondary antibody. Data are presented as mean $\pm$ SEM from two independent

experiments. **(D) RT-PCR analysis of EGFRvIII mRNA expression.** EGFRvIII mRNA levels were assessed in HNSCC cell lines artificially overexpressing EGFRvIII by RT-PCR. A representative ethidium bromide-stained agarose gel shows PCR products for EGFR (1037 bp) and EGFRvIII (236 bp). WT cell lines (UM-14C^{WT} and UT-24A^{WT}), their corresponding EGFR^{KO} derivatives, and the three EGFRvIII-overexpressing cell lines generated using different internal promoters were analyzed.

As shown in **Figure 2B**, EGFR KO cells transduced with vectors containing the viral SFFV promoter exhibited high levels of EGFRvIII expression, whereas cells transduced with vectors utilizing the hEF1 α promoter showed intermediate EGFRvIII expression levels. These findings were further confirmed by staining with an EGFRvIII-specific antibody (**Figure 2C**). Subsequent experiments therefore used cells where the EGFRvIII cDNA was driven by either the SFFV or hEF1 α promoters, hereafter referred to as EGFRvIII^{high} and EGFRvIII^{mid}, respectively.

RT-PCR analysis with primers flanking the deletion of exons 2 to 7 confirmed the overexpression of the truncated EGFRvIII transcript in the EGFRvIII overexpressing HNSCC cell lines (**Figure 2D**). Interestingly, despite efficient EGFR knockout on protein level (assessed by flow cytometry), full-length EGFR transcripts remained detectable in both WT and KO cells, consistent with potential transcription of CRISPR/Cas9-induced mutant EGFR mRNA(s) that prevent stable protein expression. EGFRvIII overexpression led to a marked reduction in the full-length EGFR amplicon alongside a strong increase in the EGFRvIII-specific product, probably due to preferential amplification of the shorter fragment under competitive PCR conditions. The effect was most pronounced in UM-14C cells, where the increasing EGFRvIII expression levels led to diminished or almost undetectable full-length EGFR signal.

Characterization of EGFRvIII specific CAR T-cells

To characterize different EGFRvIII-specific CAR T-cells, five scFv sequences targeting EGFRvIII were obtained (see Materials and Methods), optimized for human codon-usage and cloned into our second generation CAR lentiviral backbone (**Figure 3A**), containing a CD8 leader peptide, a CD34-derived hinge (C6), the CD28 transmembrane and cytoplasmic regions and the CD3 ζ -chain, all of human origin [37]. An EGFR-specific CAR derived from the monoclonal antibody cetuximab served as a positive control, while CD19 CAR T-cells were used as a negative control [37]. In the bicistronic CAR vectors, the TagBFP fluorescent protein was included upstream of the T2A sequence as a reporter for genetically modified cells. Primary human T-cells were transduced with the respective CAR vectors and enriched for CAR expression by the MACS technology utilizing CD34 microbeads as published previously [47]. Analysis of BFP-expressing cells demonstrated that the percentage of CAR positive T-cells ranged from 42–54% before and exceeded 92% after MACS enrichment (**Figure 3B**). Only samples with >90% CAR T-cells were used for cytotoxicity assays.

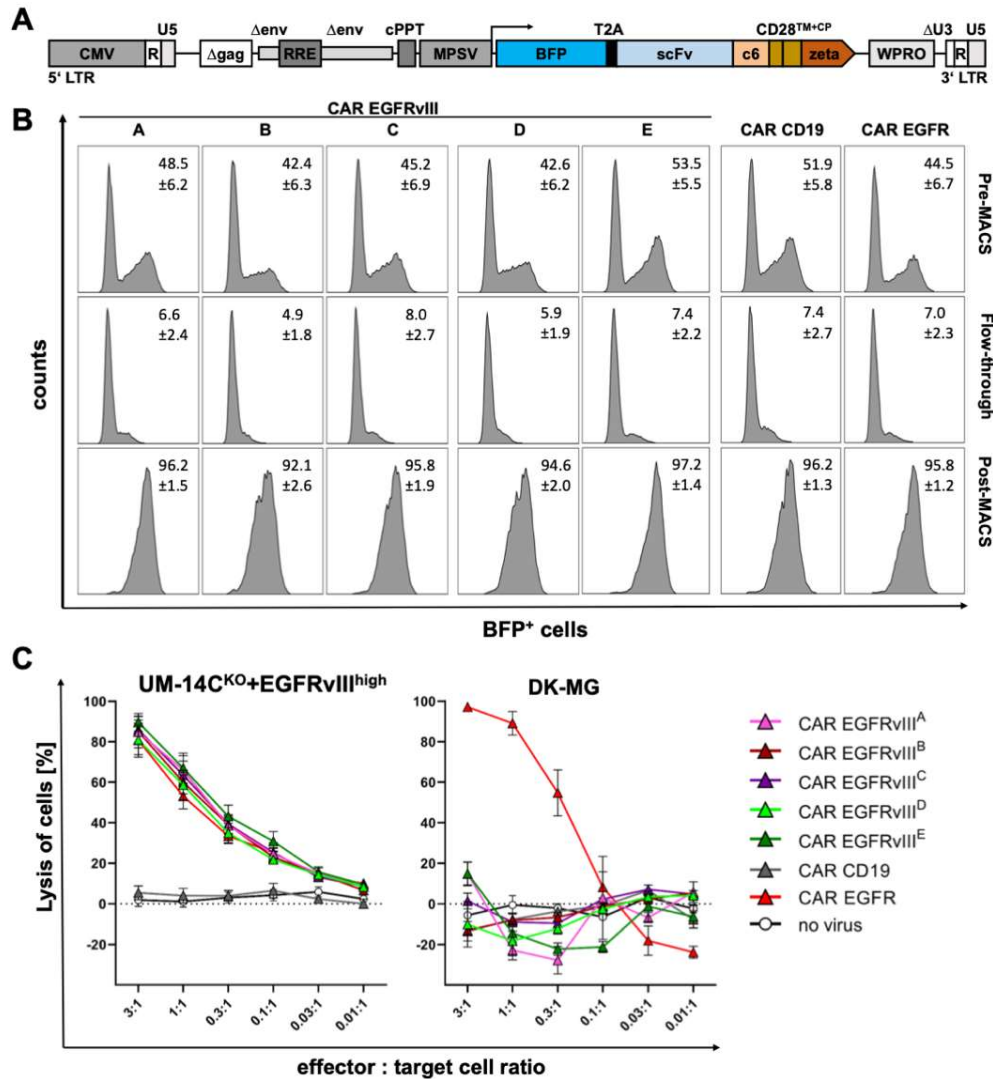


Figure 3: Design, enrichment, and cytotoxicity of CAR-positive T-cells. (A) Lentiviral CAR expression vector expressing the blue fluorescent protein TagBFP and a second-generation CAR construct. The vector contains either CD19, EGFR, or EGFRvIII^{A-E}-specific scFvs, followed by the CD28 transmembrane (TM) and cytoplasmic (CP) domains and the CD3 ζ signaling chain. A BFP reporter gene is co-expressed via a T2A sequence. Transgene expression is driven by the MPSV promoter. **(B) Transduction and enrichment efficiency of CAR-positive T-cells.** Transduced T-cells were analyzed by flow cytometry before magnetic separation (Pre-MACS), in the flow-through fraction and after MACS selection (Post-MACS). CAR-positive cells were quantified based on BFP co-expression. Representative histograms show CAR expression (x-axis) plotted against normalized cell count (y-axis). Percentages of CAR-positive cells represent mean $\pm$ SEM from at least three independent experiments. **(C) CAR T-cell cytotoxicity.** The target cell lines

UM-14C^{KO}EGFRvIII^{high} and DK-MG were co-cultured with CD19, EGFR and EGFRvIII^{A-E} CAR T-cells or untransduced T-cells (no virus) for 16 to 20 hours. Target cell lysis according to the E:T ratio (x-axis) is plotted against the percentage of lysed target cells (y-axis). Data represent mean $\pm$ SEM of at least three independent experiments.

To assess functional differences between the EGFRvIII CAR constructs, cytotoxicity was evaluated using UM-14C^{KO}EGFRvIII^{high} and the EGFRvIII expressing DK-MG cells (**Figure 3C**). All EGFRvIII CAR T-cell variants efficiently lysed UM-14C^{KO}EGFRvIII^{high} cells with comparable activity, while showing very limited activity against DK-MG cells. Based on these results, two constructs, EGFRvIII^D and EGFRvIII^E CAR T-cells were selected for further characterization.

EGFRvIII CAR T-cells exhibit antigen level-dependent cytotoxicity

To evaluate the cytotoxic potential of EGFRvIII-specific CAR T-cells, we performed co-culture killing assays using WT cells (UD-06, UD-05, UM-14C, UT-24A) or their EGFR^{KO} counterparts (UM-14C^{KO}, UT-24A^{KO}). Additionally, we also used the latter two cell lines engineered to express intermediate or high levels of EGFRvIII for cytotoxicity assays. Cells were also transduced with empty vectors as control. As shown in **Figure 4**, EGFR CAR T-cells killed all four WT EGFR-positive cell lines but did not exert any detectable cytotoxic activity against the UM-14C^{KO} and UT-24A^{KO} cells. Both EGFRvIII CAR T-cell variants showed only very limited activity against WT cells, but in the UM-14C^{KO} cells overexpressing EGFRvIII, the cytotoxic activity of EGFRvIII^D and EGFRvIII^E CAR T-cells correlated nicely with the EGFRvIII target antigen expression levels: almost 60% of UM-14C^{KO}+EGFRvIII^{mid} cells and roughly 80% of UM-14C^{KO}+EGFRvIII^{high} cells were lysed at effector-to-target ratios of 3:1. For the UT-24 set of cells, the killing efficiency was generally lower and the

differences in the cytotoxicity against UT-24^{KO}+EGFRvIII^{mid} und UT-24^{KO}+EGFRvIII^{high} cells were not as pronounced (**Figure 4**).

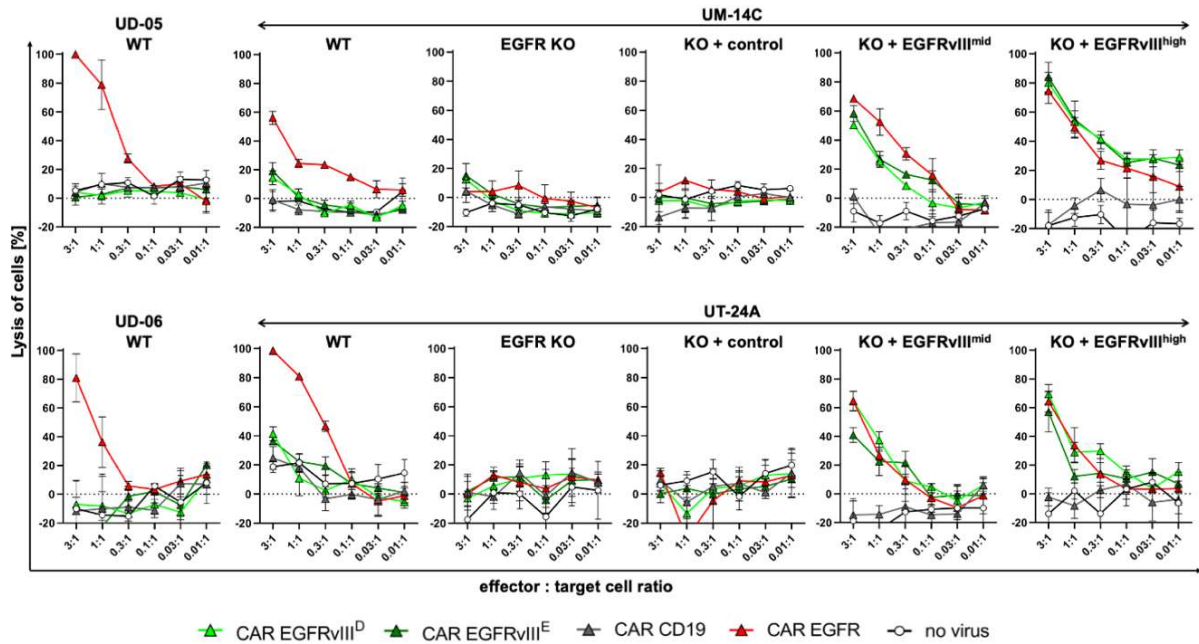


Figure 4: CAR T-cell cytotoxicity against different HNSCC cell lines. Different WT and genetically engineered UM-14C/UT-24A cells were co-cultured with CD19, EGFR, EGFRvIII^D or EGFRvIII^E CAR T-cells or untransduced (no virus) T-cells for 16 to 20 hours. Target cell lysis at different E:T ratios (x-axis) is plotted against the percentage of lysed target cells (y-axis). Data represent mean $\pm$ SEM of at least three independent experiments.

CARs and non-signaling CARs (nsCARs) can be co-expressed on the same T-cell

Unlike classical CARs, nsCARs consist only of an antigen-binding domain, a hinge, and a transmembrane region and therefore cannot mediate activation and killing of T-cells [55]. In order to enhance the weak cytotoxic activity of EGFRvIII CAR T-cells against cells with low antigen levels, we co-expressed 2nd generation CARs and nsCARs on the same T-cell (**Figure 5A**). To this end, we generated mCherry-labeled CAR constructs targeting CD33 [47], EGFRvIII (D and E variants), and

CD44v6 (a splice variant frequently expressed in HNSCC [42]). These standard CAR constructs were paired with nsCARs directed against CD19 (negative control) or EGFR (positive control) (**Figure 5B**). To minimize the risk of homologous recombination between the two CARs during lentiviral integration, we employed a leader sequence derived from the human Ig heavy chain (R) for the nsCARs - instead of the CD8 leader (J) used in the CAR vectors, and replaced the CD28 transmembrane domain with the counterpart of CD8. To enable flow cytometric detection and magnetic-activated cell sorting (MACS) of nsCAR-expressing cells, we incorporated an NGFR-derived hinge region (n3) instead of the CD34-derived c6 hinge [47, 49]. Standard cytotoxicity assays revealed that the T-cells expressing the three complete CARs, namely CD44v6, EGFRvIII^D and EGFRvIII^E, efficiently and specifically killed HNSCC cell lines, while the negative control CAR against CD33, as well as nsCARs directed against EGFR and CD19, did not mediate cytotoxicity (**Figure 5C**).

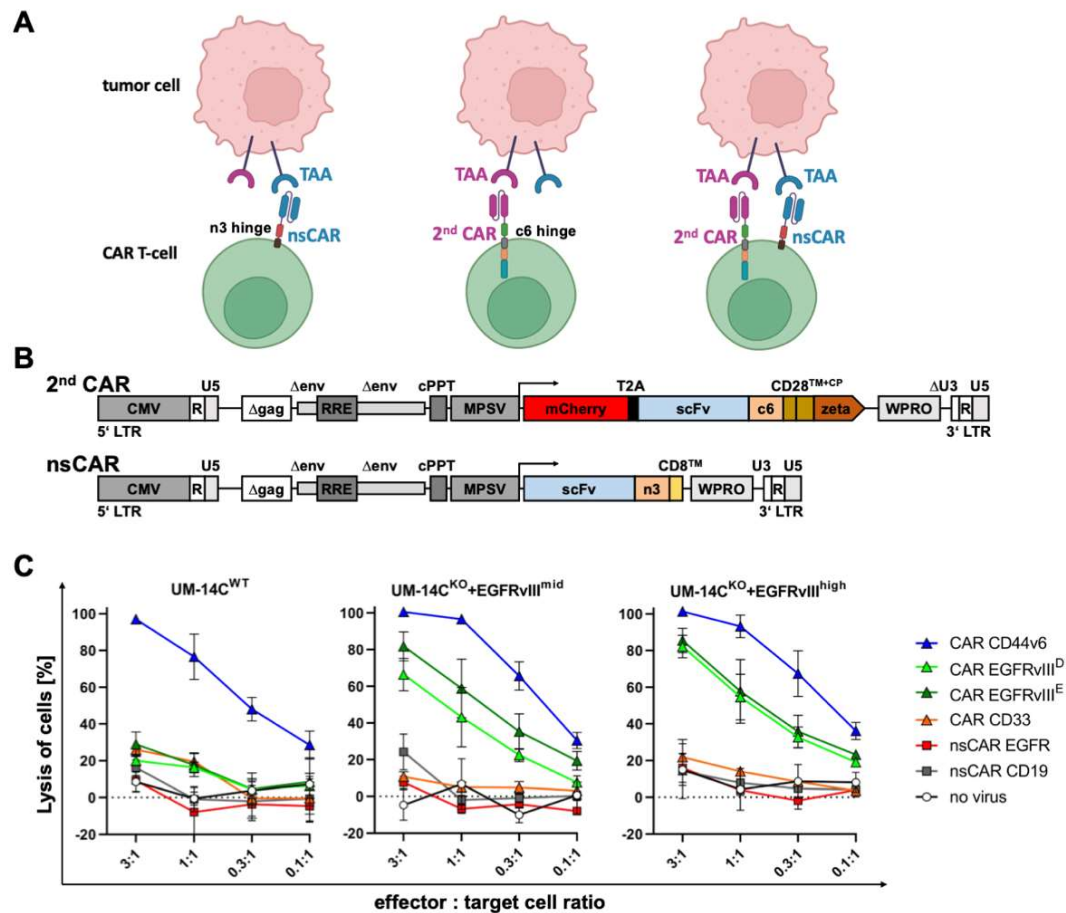


Figure 5: Concept, design and cytotoxicity of CAR and nsCAR T-cells. (A) Schematic representation of dual-targeting CAR T-cell strategies. Second generation CARs (c6 hinge, CD34-derived) were combined with non-signaling CARs (nsCAR; n3 hinge, NGFR-derived), which lack signaling domains and bind a second tumor-associated antigen epitope. **(B)** Lentiviral expression vectors encoding 2nd generation CARs or nsCARs. The 2nd generation CAR vector contains either CD44v6, CD33, or EGFRvIII^{D-E}-specific scFvs, the c6-hinge, CD28 transmembrane and cytoplasmic regions, and the CD3 ζ chain. An mCherry reporter was co-expressed via a T2A sequence. nsCAR contains EGFR- or CD19-specific scFvs, the n3-hinge, and the CD8 transmembrane domain. **(C)** Killing efficiency of CAR and nsCAR T-cells against WT and genetically engineered UM-14C cells. Primary human T-cells were singly transduced with lentiviral constructs encoding 2nd generation CAR or nsCARs. Cells were CAR or nsCAR positive T-cells (>90%) were co-cultured with UM-14C cells for 16 to 20 hours at varying E:T ratios. Target cell lysis at different E:T ratios (x-axis) is plotted against the percentage of lysed target cells (y-axis). Data represent mean $\pm$ SEM of at least three independent experiments.

To enable efficient co-expression of both constructs in the same T-cells, we generated lentiviral vectors pseudotyped with VSV-G for the CAR and BaEV-Rless for the nsCAR. After titration, we co-transduced primary human T-cells with both vectors, thereby avoiding competition between the viral particles for the same cellular entry receptor and ensuring higher percentages of double-positive cells [56]. As the shorter nsCARs consistently exhibited much higher transduction efficiencies (72–79%) than the CARs (6–19%) (**Figure S2**), we enriched the double-positive T-cells using CD34 microbeads only, thus yielding a CAR-positive T-cell population with >90% purity. Co-expression analysis demonstrated that up to 79% of T-cells co-expressed both CAR and nsCAR when EGFR nsCARs were used (**Figure S2**). NGFR microbead-based enrichment was performed for cells transduced with nsCARs alone. Only MACS-enriched T-cells were used in subsequent functional analyses.

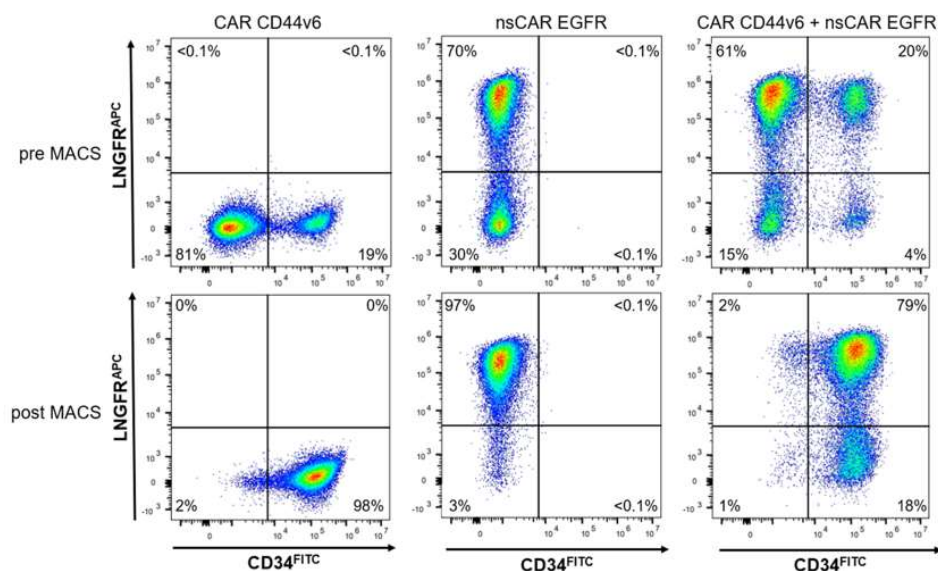


Figure S2: Enrichment of T-cells co-expressing CAR and nsCAR constructs. Dot plots show CD34 expression (x-axis) versus NGFR expression (y-axis). Primary human T-cells expressing only the EGFR nsCAR were enriched with NGFR microbeads, whereas cells expressing only the CD44v6 CAR or the CD44v6 CAR together with the EGFR nsCAR were

enriched using CD34 microbeads. Before and after MACS separation, gene transfer efficiency was assessed by staining with CD34-FITC and NGFR-APC antibodies. Representative experiments are shown. Numbers indicate the percentages of cells within each quadrant.

The cytotoxicity of EGFRvIII CAR T-cells can be enhanced by additional nsCARs

Next, we co-cultured CAR/nsCAR double-positive T-cells with UM-14C^{WT}, UM-14C^{KO}+EGFRvIII^{mid} and UM-14C^{KO}+EGFRvIII^{high} target cells and assessed the cytolytic activity in standard killing assays. As shown in **Figure 6**, no cytotoxicity occurred with CD33 CAR T-cells, independent of the nsCARs. In contrast, CD44v6 CAR T-cells killed the target cells very efficiently, thus no influence of the co-expression of nsCARs was detectable. Consistent with previous observations, neither EGFRvIII CAR T-cells alone nor in combination with nsCARs induced measurable cytotoxicity against UM-14C^{WT} cells. However, the limited cytotoxicity of EGFRvIII^D CAR T-cells against UM-14C^{KO}+EGFRvIII^{mid} cells was clearly enhanced by EGFR nsCAR co-expression, increasing the killing efficiency from ~65% to nearly 100% at the 3:1 E:T ratio, and from ~40% to ~80% at a 1:1 ratio. In UM-14C^{KO}+EGFRvIII^{high} cells, EGFRvIII^D CAR T-cells already exhibited high cytotoxicity, which was slightly enhanced by co-expression of EGFR nsCARs. A similar trend was also observed for EGFRvIII^E CAR T-cells when using UM-14C^{KO}+EGFRvIII^{mid} cells as targets.

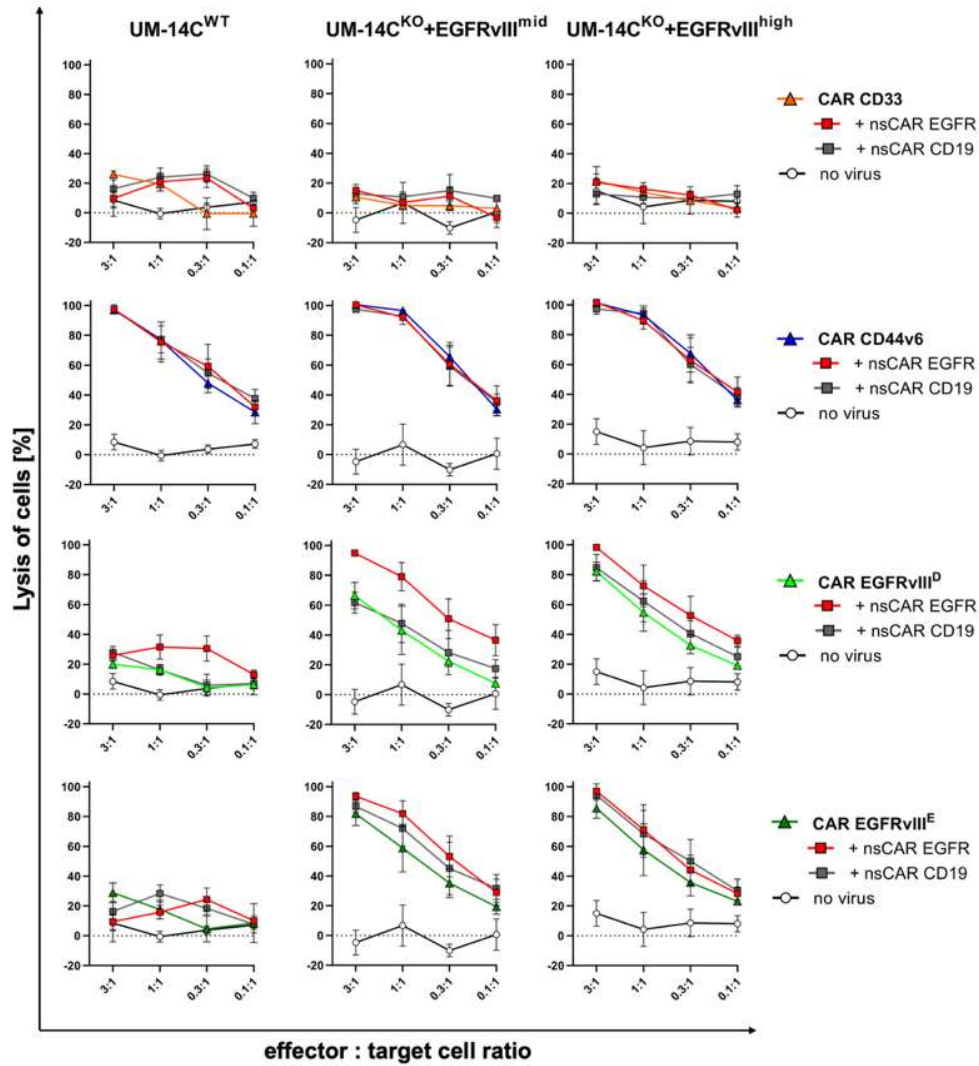


Figure 6: Killing efficiency of CAR/nsCAR T-cells. Primary human T-cells were singly or doubly transduced, and CAR- or CAR/nsCAR double positive cells were enriched using CD34 microbeads. Cells were co-cultured with UM-14C cells for 16 to 20 hours at varying E:T ratios. Target cell lysis at different E:T ratios (x-axis) is plotted against the percentage of lysed target cells (y-axis). Data represent mean $\pm$ SEM of at least three independent experiments.

Co-expression of CARs and nsCARs accelerates and enhances target-cell killing

To understand the killing dynamics of singly positive CAR T-cells and CAR/nsCAR co-expressing T-cells, MACS-enriched T-cells were co-cultured with UM-14C^{KO}+EGFRvIII^{mid} cells and the

cytotoxicity measured after 6, 10 and 20 hours, respectively. As shown in **Figure 7**, nsCAR co-expression did not further enhance the antitumor activity of EGFRvIII^E or CD44v6 CAR T-cells at any time point. In contrast, EGFRvIII^D CAR/EGFR nsCAR T-cells exhibited moderately increased cytotoxicity at early time points compared with EGFRvIII^D CAR T-cells alone. At 10 hours, EGFRvIII^D CAR T-cells lysed ~25% of target cells, whereas CAR/nsCAR co-expressing T-cells achieved ~50% lysis. After 20 hours, the additive effect of the nsCAR expression became more pronounced: ~75% of target cells were lysed by EGFRvIII^D CAR / EGFR nsCAR T-cells compared with <40% by EGFRvIII^D CAR T-cells alone. This enhancement was also observed at an E:T ratio of 1:1.

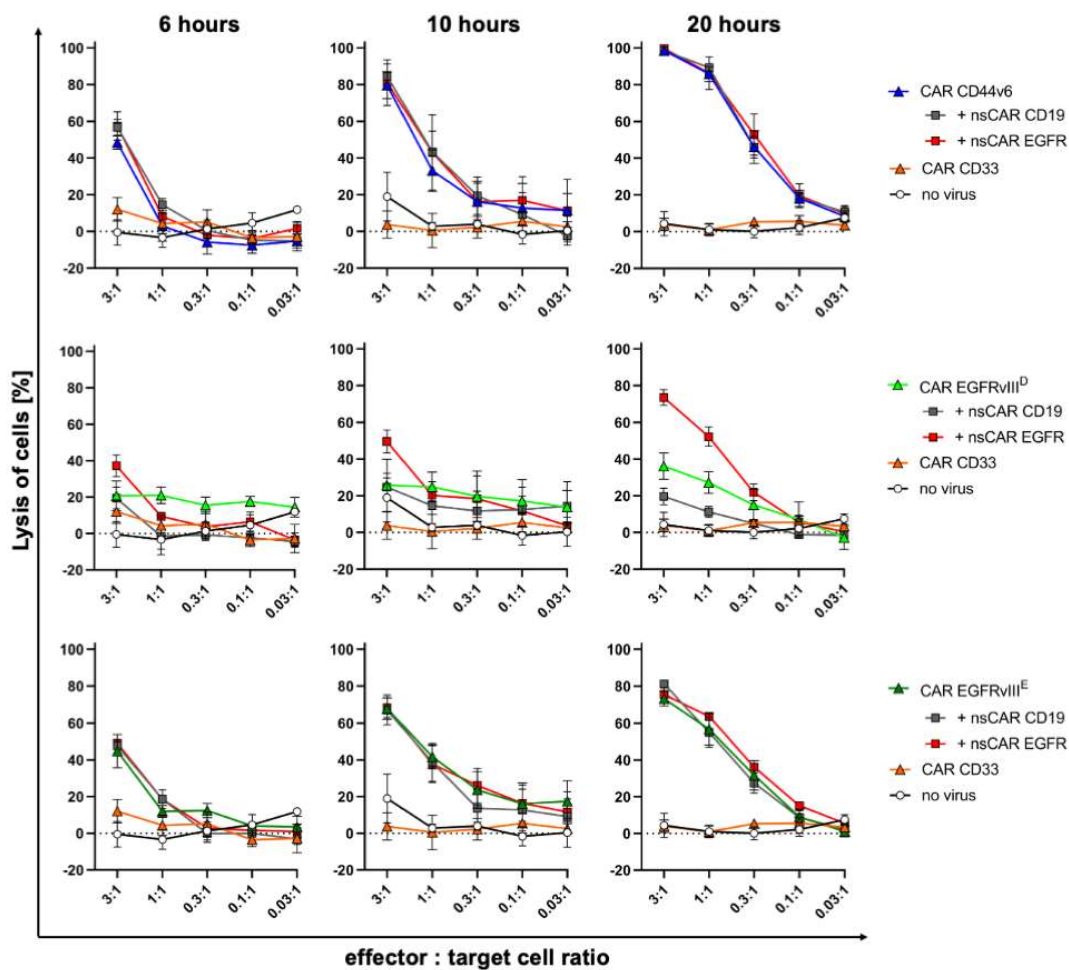


Figure 7: Killing dynamics of CAR/nsCAR T-cells. Primary human T-cells were either singly transduced with an EGFRvIII^{D-E} or CD44v6-specific CAR, or doubly transduced with additional EGFR- or CD19-specific nsCAR constructs. CAR- and/or nsCAR-positive cells were enriched using CD34 microbeads and co-cultured with UM-14C^{KO}+EGFRvIII^{mid} cells for 6, 10, and 20 hours at varying E:T ratios. Target cell lysis at different E:T ratios (x-axis) is plotted against the percentage of lysed target cells (y-axis). Data represent mean $\pm$ SEM of at least three independent experiments.

Differential recruitment of CAR and nsCAR at the immunological synapse

To investigate whether enhanced cytotoxicity of CAR/nsCAR T-cells can be linked to IS formation, we used fluorescence microscopy to visualize the CAR and nsCAR protein distribution during the T-cell–tumor cell interactions. For these experiments, CAR/nsCAR T-cells were stained with hinge-specific antibodies (CD34^{FITC} for CAR, NGFR^{PE-Vio770} for nsCAR) and co-cultured with UM-14C^{KO}+EGFRvIII^{high} cells. Additionally, CAR T-cells were identifiable by BFP expression. Without target cell contact (**Figure 8, No IS**), both CAR (green) and nsCAR (red) proteins were evenly distributed across the surface of CAR/nsCAR T-cells (blue). Upon target recognition (**Figure 8, CAR/nsCAR IS**), both EGFRvIII^D CAR and EGFR nsCAR proteins accumulated at the IS, indicating coordinated recruitment of both constructs to the contact zone between the target and the effector cell. EGFRvIII^D CAR /CD19 nsCAR T-cells also formed synapses with the target cells (**Figure 8, CAR IS**). However, in contrast to CAR proteins, which localized exclusively to the IS, the nsCAR proteins targeting CD19, which was not expressed on these tumor cells, did not localize to the IS. These results demonstrate that, in double-positive T cells, CAR and nsCAR proteins are co-recruited to the same IS when both target antigen epitopes are present on the tumor cell, thereby strengthening the interaction between effector and target cells.

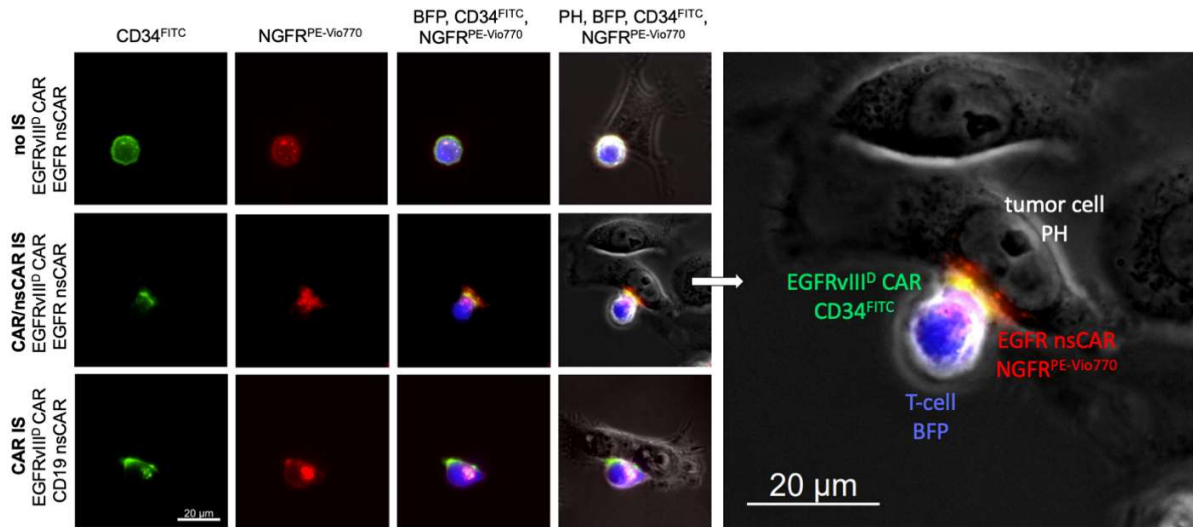


Figure 8: Visualization of the IS. The IS was analyzed by fluorescence microscopy, visualizing the EGFRvIII^P CAR (CD34^{FITC} antibody, **green**) and EGFR or CD19 nsCAR (NGFR^{PE-Vio770} antibody, **red**) proteins on MACS-enriched CAR/nsCAR T-cells (BFP expression, **blue**) in relation to UM-14C^{KO}+EGFRvIII^{high} cells (**phase contrast**). Contrast settings (channels: PH, 377/447 nm, 470/525 nm and 545/620 nm) were adjusted to maximize the visibility of the IS.

Discussion

CAR T-cell therapy has revolutionized the treatment landscape for hematologic malignancies of the B-cell lineage, yet its role in solid tumors remains markedly limited due to the scarcity of tumor-specific antigens, the heterogeneous target antigen expression profiles, the suppressive tumor microenvironment, restricted trafficking and infiltration of CAR T-cells into tumors and limited persistence and functional fitness of CAR T-cells [7, 57].

EGFRvIII as a target antigen in HNSCC

As EGFRvIII is one of the few target antigens well established in glioblastoma with remarkable tumor-specificity [15] and also described in HNSCC [38, 58, 59], our aim/primary goal in the

present study was to characterize the cytotoxic activity of five distinct EGFRvIII scFvs used as targeting moieties in 2nd generation CAR constructs on human T-cells. However, analysis of EGFRvIII expression on human HNSCC cell lines revealed that only two of eleven cell lines, UM-14C and UT-24A, were positive at the mRNA level by PCR. While protein expression on these two lines using a previously described EGFRvIII-specific antibody [51] in flow cytometry could not be reliably detected, the overexpression of EGFRvIII confirmed the specificity of the commercial antibody. Detailed analysis of the HNSCC literature indicates that EGFRvIII expression frequencies were described as high as ~40% in early studies [38], whereas later analyses using larger cohorts and more stringent molecular detection methods (e.g., qRT-PCR, RNA-sequencing) consistently find EGFRvIII expression to be rarely detectable [60-62]. One study even described variant protein expression in only 2 of 638 tumors (~0.3%) [62]. We therefore also included DK-MG cells, a glioblastoma cell line widely used as EGFRvIII-positive reference cells [51, 63], in our studies. However, cytotoxicity assays with the five EGFRvIII CAR T-cells against all three positive cell lines (UM-14C, UT-24A, DK-MG) did not reveal any killing activity, suggesting that the endogenous EGFRvIII protein levels are insufficient to trigger robust T-cell activation by 2nd generation CAR constructs. To systematically investigate the impact of antigen density on EGFRvIII CAR T-cell activation, we overexpressed the EGFRvIII cDNA in WT EGFR-KO HNSCC cell lines, using lentiviral vectors with different internal promoters. Employing these engineered HNSCC cell lines with defined EGFRvIII expression levels as target cells allowed us to demonstrate that all five EGFRvIII-specific CAR constructs (EGFRvIII^{A-E}) mediated robust cytotoxicity when EGFRvIII was present at sufficient levels and that all five EGFRvIII CAR constructs were specific, as they did not recognize the parental EGFR KO cells.

EGFRvIII has previously been explored as a target for immunotherapeutic approaches. Peptide-based vaccination strategies, particularly the EGFRvIII-specific vaccine rindopepimut (CDX-110), which targets the tumor-specific junctional epitope created by the exon 2–7 deletion, demonstrated robust immunogenicity with signs of clinical benefits in early-phase clinical trials in patients with glioblastoma [64]. However, the randomized phase III trial (ACT IV ,NCT01480479) failed to improve overall survival of patients, with subsequent analyses suggesting that antigen loss and tumor heterogeneity may have contributed to the lack of clinical benefit [65]. After initial signs of activity in Glioblastoma, antibody–drug conjugates targeting EGFR - including EGFRvIII-expressing tumors – such as depatuxizumab mafodotin (ABT-414) – failed in phase II/III clinical trials (NCT02343406, NCT01800695) due to lack of significant survival benefit and ocular toxicity [66, 67]. Preclinical studies demonstrated that EGFRvIII CAR T-cells can selectively recognize and eliminate EGFRvIII-expressing tumor cells [45, 68, 69], and a first-in-human phase I/II clinical trial in patients with recurrent GBM (NCT02209376) showed that intravenous infusion of autologous EGFRvIII CAR T-cells following tumor resection was safe and feasible, and demonstrated CAR T-cell trafficking to tumor sites [22]. However, no objective tumor responses were observed and tumor recurrence occurred in all cases, with findings suggesting that antigen heterogeneity and treatment-associated antigen loss may have contributed to the limited efficacy [22]. Also a second clinical trial of anti-EGFRvIII CAR T-cells against recurrent GBM (NCT01454596) showed safety and transient efficacy but did not result in durable therapeutic responses, with findings indicating potential contributions of antigen escape and adaptive resistance mechanisms [23].

Enhancing the cytotoxicity of EGFRvIII CAR T-cells for low EGFRvIII expression

Different strategies have been developed to enhance the killing efficiency of CAR T-cells in solid cancers [57, 70, 71]. Interestingly, Choi *et al.* have genetically modified EGFRvIII CAR T-cells to secrete BiTEs that recognize EGFR and CD3 ϵ [25]. This strategy was later translated into the clinic using related T-cell-engaging antibody molecules (TEAMs), where a phase I/II study employing a single intraventricular administration in a small cohort of patients with recurrent glioblastoma resulted in encouraging initial responses and manageable toxicity. However, despite these promising early outcomes, disease progression ultimately occurred, likely reflecting the ongoing challenges of antigen escape and tumor heterogeneity in this setting [24]. As almost all HNSCC cells are positive for WT EGFR, we decided to co-express a cetuximab-based non-signaling CAR (nsCAR) against domain 3 (L2) of EGFR on the EGFRvIII CAR T-cells, thus potentially increasing the binding affinity and thereby the strength of the activation between target cells and CAR T-cells in the IS [37].

Co-expression of the EGFR nsCAR did not enhance the cytotoxic activity of EGFRvIII^E CAR T-cells, as this CAR construct alone already mediated highly efficient killing of both, intermediate and high EGFRvIII-expressing cells, comparable to the cytotoxic activity of the control CD44v6 CAR T-cells. In contrast, when combined with the EGFR nsCAR, EGFRvIII^D CAR T-cells exhibited markedly improved cytotoxicity in our standard overnight assays, with maximal killing increasing from approximately 65% to near complete target clearance at the 3:1 effector-to-target ratios. Compared with EGFRvIII^D CAR T-cells alone, the killing curve of EGFRvIII^D CAR/EGFR nsCAR T-cells was parallel-shifted across all effector-to-target cells ratios (**Figure 7**), suggesting that additional factors such as T-cell exhaustion or target cell detachment kinetics may also influence the cytotoxic activity. Time-resolved cytotoxicity assays further revealed that EGFRvIII^D CAR/EGFR

nsCAR T-cells initiated tumor cell lysis earlier than CAR-only controls (**Figure 8**), indicating accelerated engagement and synapse formation of the combination. Imaging experiments further demonstrated that both EGFRvIII CAR and EGFR nsCAR molecules strongly co-localized at the same IS, suggesting that dual-epitope binding may stabilize the T-cell–tumor cell interface. This could potentially increase dwell time and promote efficient granule polarization, although these parameters were not directly assessed in this study [72]. In control experiments, nsCARs alone did not mediate cytotoxicity. Moreover, a CD19 nsCAR, which does not bind to HNSCC cancer cells, was not recruited to the IS and did not enhance cytotoxicity of EGFRvIII^D CAR T-cells, confirming that the enhancement by the nsCAR constructs depends on cooperation with a signaling-competent CAR.

The differences in the cytotoxic activity between EGFRvIII^D and EGFRvIII^E CARs likely depend on multiple parameters, including the target antigen density in combination with the specific affinity and avidity of the scFvs used [73, 74], characteristics of the hinge region within the CAR construct [75, 76], the antigen accessibility and location in the target cell membrane [77], or the epitope positioning on the extracellular domain [77]. High-affinity CARs or membrane-proximal epitopes can enhance effective receptor engagement and promote IS formation, particularly under conditions of low target antigen density [78, 79]. In addition, the observation that the EGFR nsCAR did not influence the already strong cytotoxic activity of CD44v6 CAR T-cells may at least partially be explained by differences in membrane localization: CD44v6 is preferentially found in lipid-raft-associated areas, while EGFR is organized in nanoclusters or microdomains whose signaling depends on the local lipid environment [80–82]. Here, it would be interesting to evaluate in the future whether a CD44v6 CAR construct with less affinity/avidity

can profit from co-expression of EGFR nsCAR, as both antigens probably need to co-locate in close proximity in the cell membrane/same synapse for improved killing. Furthermore, evaluating alternative CAR/nsCAR combinations can be important to better define the impact of co-expressing nsCARs on the killing efficiency of CAR T-cells. In this context, several TAAs, which are often abundantly present on malignant cells, may represent promising targets for nsCARs when combined with TSAs like EGFRvIII in the signaling-competent CAR construct [83].

Enhancing the cytotoxicity of CAR T-cells further

Modulating signaling pathways in CAR T-cells is an interesting possibility to improve their killing activity in case of low target antigen expression and limited CAR T-cell persistence/activity. For example, the CD19 CAR T-cell killing efficiency of CD19-low tumor cells was vastly improved *in vitro* and *in vivo* by using CD38 chimeric costimulatory receptors (CCRs), that contained CD28, 4-1BB, or both as costimulatory domains, but did not induce tonic signaling due to the absence of a CD3 ζ domain [84]. In addition, utilizing 4-1BB/CD137 agonists in a BiTE format, targeting either an antigen on the tumor cells or FAP in the TME, might become another promising *off-the-shelf* therapy to improve the basic cytotoxic activity of both BiTE and CAR T-cell therapies (reviewed in [85]). Finally, strengthening the IS is increasingly recognized as a powerful strategy to overcome the barriers imposed by low antigen density and the solid TME [72], e.g. by increasing LFA-1 or ICAM-1 interactions [86, 87] or by adding the thalidomide derivative lenalidomide that led to enhanced immunological synapses between EGFRvIII CAR T-cells and glioblastoma targets as determined by CD11a and F-actin polymerization [88]. High-quality IS formation is characterized by elevated F-actin accumulation, efficient lytic granule polarization, and robust polarization of

key signaling molecules within the synapse [72]. In this context, the nsCAR approach fits within a broader framework of strategies that modulate receptor architecture to enhance CAR–tumor interactions, while offering notable conceptual simplicity.

Conclusion

The application of nsCAR in solid cancers such as HNSCC requires further investigation to determine whether nsCAR-mediated synapse augmentation might also translate into improved persistence, trafficking, and antitumor activity in the context of stromal barriers and TME-mediated suppression. The impact of nsCAR engagement on cytokine secretion, T-cell exhaustion, and memory formation warrants detailed studies using longitudinal functional and transcriptional profiling. In addition, the current study evaluated a limited panel of CAR architectures and tumor models, and broader validation of CAR architectures and different tumor target antigens is necessary to determine the generalizability of nsCAR strategies across diverse antigen landscapes. Here, deeper mechanistic investigations using quantitative imaging approaches – including F-actin accumulation assays, granule-polarization metrics, and high-resolution synapse biophysics [72, 89, 90] – will be informative to directly link nsCAR-mediated enhancements to defined structural and functional properties of the IS.

Declarations

Ethics approval and consent to participate

Primary human T-cells were isolated from peripheral blood of healthy adult volunteers who provided written informed consent prior to participation. The study was conducted in accordance

with the Declaration of Helsinki and approved by the local ethics committee of the Universitätsklinikum Düsseldorf (reference numbers #2019-623).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

FB has received research awards from Gilead, Kite, and the Bristol Myers Squibb Foundation (Immuno-Oncology). The remaining authors declare that they have no competing interests.

Funding

This work was supported in part by the Deutsche Forschungsgemeinschaft DFG (grant number GI WI 5377/2-1) awarded to CW. HH is grateful for support from the Deutsche Krebshilfe e. V. (70114844), the Bild hilft e. V. – ein Herz für Kinder e.V. (PÄ-44217 2025), and die Brigitte und Dr. Konstanze Wegener-Stiftung (A2025I #23). CM and LC acknowledge financial support from the Freigeist Fellowship of the Volkswagen Foundation. CM further acknowledges financial support from the Momentum program of the Volkswagen Foundation. The authors also acknowledge funding by DFG and the state of North Rhine-Westphalia for the acquisition of the custom MFIS-

SSTR Nikon microscope (A1HD25-NSTORM) within the Major Research Instrumentation program (Art. 91b GG), funding ID INST 208/797-1 FUGG, awarded to Claus Seidel (Physical Chemistry, Heinrich Heine University Düsseldorf) and CM. The FB Lab is supported by the DKFZ-MOST Cooperation Program, the Dr. Rolf M. Schwiete-Stiftung, the EU CAN KIDS program, and the International Human Frontier Science Program Organization (RGEC26/2025). Further support is provided by DFG (PF 691/6-1, SFB 1709/1 – 533056198, SFB 1479 – 441891347, and Germany's Excellence Strategy EXC-3018/1 – 533587280), the Carl Zeiss Foundation through the Seed Funding Program of the Center for Synthetic Genomics, the Childguard-Stiftung, the Dr. Erich Neuy und Margrit Howe-Neuy Stiftung, the Kirstin-Diehl-Stiftung, and private donors. KG is supported by the EU CAN KIDS program.

Authors' contributions

KG, HH, CW: Conceptualization, Writing – original draft.

KG, NS, LC, SH, EK: Investigation, Data curation, Interpretation of data.

CH, FB, KS, CM, HH, CW: Supervision, Interpretation of data, editing, Funding acquisition.

All authors contributed to writing – review & editing and approved the final manuscript.

Acknowledgements

We thank the healthy donors for providing peripheral blood for the studies. We thank Prof. Dr. Jörg Schipper for his support of this project. We are grateful to Prof. Dr. Homey and Anke van Lierop (Department of Dermatology, Heinrich Heine University Düsseldorf) for providing access to the CytoFLEX S flow cytometer and the Infinite M200 PRO reader. We also thank PD Dr. Michèle

Hoffmann-Massier (Department of Urology, Heinrich Heine University Düsseldorf) for access to the MACSQuant system.

Authors' information (optional)

References

1. Sun, H., et al., *Global burden of head and neck cancer: Epidemiological transitions, inequities, and projections to 2050*. Front Oncol, 2025. **15**: p. 1665019.
2. Barsouk, A., et al., *Epidemiology, Risk Factors, and Prevention of Head and Neck Squamous Cell Carcinoma*. Med Sci (Basel), 2023. **11**(2).
3. Machiels, J.P., et al., *Squamous cell carcinoma of the oral cavity, larynx, oropharynx and hypopharynx: EHNS-ESMO-ESTRO Clinical Practice Guidelines for diagnosis, treatment and follow-up*. Ann Oncol, 2020. **31**(11): p. 1462-1475.
4. Borcoman, E., G. Marret, and C. Le Tourneau, *Paradigm change in first-line treatment of recurrent and/or metastatic Head and Neck Squamous Cell Carcinoma*. Cancers (Basel), 2021. **13**(11).
5. Ruffin, A.T., et al., *Improving head and neck cancer therapies by immunomodulation of the tumour microenvironment*. Nat Rev Cancer, 2023. **23**(3): p. 173-188.
6. June, C.H. and M. Sadelain, *Chimeric Antigen Receptor Therapy*. N Engl J Med, 2018. **379**(1): p. 64-73.
7. Uslu, U. and C.H. June, *Beyond the blood: expanding CAR T cell therapy to solid tumors*. Nat Biotechnol, 2025. **43**(4): p. 506-515.
8. Locatelli, F., et al., *GD2-targeting CAR T cells in high-risk neuroblastoma: a phase 1/2 trial*. Nat Med, 2025. **online**(10.1038/s41591-025-03874-6).

9. Steffin, D., et al., *Interleukin-15-armoured GPC3 CAR T cells for patients with solid cancers*. Nature, 2025. **637**(8047): p. 940-946.
10. Guo, J., et al., *Challenges and breakthroughs: current landscape and future prospects of CAR-T cell therapy clinical trials for solid tumors*. Front Oncol, 2025. **15**: p. 1652329.
11. Arteaga, C.L. and J.A. Engelman, *ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics*. Cancer Cell, 2014. **25**(3): p. 282-303.
12. Yao, Y., et al., *Clinical utility of PDX cohorts to reveal biomarkers of intrinsic resistance and clonal architecture changes underlying acquired resistance to cetuximab in HNSCC*. Signal Transduct Target Ther, 2022. **7**(1): p. 73.
13. Huang, H.S., et al., *The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling*. J Biol Chem, 1997. **272**(5): p. 2927-35.
14. Guo, G., et al., *Ligand-Independent EGFR Signaling*. Cancer Res, 2015. **75**(17): p. 3436-41.
15. Wikstrand, C.J., et al., *Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas*. Cancer Res, 1995. **55**(14): p. 3140-8.
16. Voldborg, B.R., et al., *Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials*. Ann Oncol, 1997. **8**(12): p. 1197-206.
17. Moscatello, D.K., et al., *Transformational and altered signal transduction by a naturally occurring mutant EGF receptor*. Oncogene, 1996. **13**(1): p. 85-96.
18. Batra, S.K., et al., *Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene*. Cell Growth Differ, 1995. **6**(10): p. 1251-9.
19. Katanasaka, Y., et al., *Epidermal growth factor receptor variant type III markedly accelerates angiogenesis and tumor growth via inducing c-myc mediated angiopoietin-like 4 expression in malignant glioma*. Mol Cancer, 2013. **12**: p. 31.

20. Bonavia, R., et al., *EGFRvIII promotes glioma angiogenesis and growth through the NF-kappaB, interleukin-8 pathway*. *Oncogene*, 2012. **31**(36): p. 4054-66.
21. Feng, H., et al., *EGFRvIII stimulates glioma growth and invasion through PKA-dependent serine phosphorylation of Dock180*. *Oncogene*, 2014. **33**(19): p. 2504-12.
22. O'Rourke, D.M., et al., *A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma*. *Sci Transl Med*, 2017. **9**(399).
23. Goff, S.L., et al., *Pilot Trial of Adoptive Transfer of Chimeric Antigen Receptor-transduced T Cells Targeting EGFRvIII in Patients With Glioblastoma*. *J Immunother*, 2019. **42**(4): p. 126-135.
24. Choi, B.D., et al., *Intraventricular CARv3-TEAM-E T Cells in Recurrent Glioblastoma*. *N Engl J Med*, 2024. **390**(14): p. 1290-1298.
25. Choi, B.D., et al., *CAR-T cells secreting BiTEs circumvent antigen escape without detectable toxicity*. *Nat Biotechnol*, 2019. **37**(9): p. 1049-1058.
26. Ramakrishna, S., et al., *Modulation of Target Antigen Density Improves CAR T-cell Functionality and Persistence*. *Clin Cancer Res*, 2019. **25**(17): p. 5329-5341.
27. Walker, A.J., et al., *Tumor Antigen and Receptor Densities Regulate Efficacy of a Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase*. *Mol Ther*, 2017. **25**(9): p. 2189-2201.
28. Fry, T.J., et al., *CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy*. *Nat Med*, 2018. **24**(1): p. 20-28.
29. Watanabe, K., et al., *Target antigen density governs the efficacy of anti-CD20-CD28-CD3 zeta chimeric antigen receptor-modified effector CD8+ T cells*. *J Immunol*, 2015. **194**(3): p. 911-20.
30. Majzner, R.G., et al., *Tuning the Antigen Density Requirement for CAR T-cell Activity*. *Cancer Discov*, 2020. **10**(5): p. 702-723.
31. Barisa, M., et al., *Functional avidity of anti-B7H3 CAR-T constructs predicts antigen density thresholds for triggering effector function*. *Nat Commun*, 2025. **16**(1): p. 7196.

32. Schneider, D., et al., *A tandem CD19/CD20 CAR lentiviral vector drives on-target and off-target antigen modulation in leukemia cell lines*. J Immunother Cancer, 2017. **5**: p. 42.
33. Ruella, M., et al., *Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies*. J Clin Invest, 2016. **126**(10): p. 3814-3826.
34. Hegde, M., et al., *Combinational targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma*. Mol Ther, 2013. **21**(11): p. 2087-101.
35. Fernandez de Larrea, C., et al., *Defining an Optimal Dual-Targeted CAR T-cell Therapy Approach Simultaneously Targeting BCMA and GPRC5D to Prevent BCMA Escape-Driven Relapse in Multiple Myeloma*. Blood Cancer Discov, 2020. **1**(2): p. 146-154.
36. Virts, E.L., et al., *AluY-mediated germline deletion, duplication and somatic stem cell reversion in UBE2T defines a new subtype of Fanconi anemia*. Hum Mol Genet, 2015. **24**(18): p. 5093-108.
37. Haist, C., et al., *Engineering a single-chain variable fragment of cetuximab for CAR T-cell therapy against head and neck squamous cell carcinomas*. Oral Oncol, 2022. **129**: p. 105867.
38. Sok, J.C., et al., *Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting*. Clin Cancer Res, 2006. **12**(17): p. 5064-73.
39. Nakano, M., et al., *CYP4V2 in Bietti's crystalline dystrophy: ocular localization, metabolism of omega-3-polyunsaturated fatty acids, and functional deficit of the p.H331P variant*. Mol Pharmacol, 2012. **82**(4): p. 679-86.
40. Yu, X., et al., *Lentiviral vectors with two independent internal promoters transfer high-level expression of multiple transgenes to human hematopoietic stem-progenitor cells*. Mol Ther, 2003. **7**(6): p. 827-38.
41. Roellecke, K., et al., *Optimized human CYP4B1 in combination with the alkylator prodrug 4-ipomeanol serves as a novel suicide gene system for adoptive T-cell therapies*. Gene Ther, 2016. **23**(7): p. 615-26.

42. Haist, C., et al., *CD44v6-targeted CAR T-cells specifically eliminate CD44 isoform 6 expressing head/neck squamous cell carcinoma cells*. Oral Oncol, 2021. **116**: p. 105259.
43. Qin, J.Y., et al., *Systematic comparison of constitutive promoters and the doxycycline-inducible promoter*. PLoS One, 2010. **5**(5): p. e10611.
44. Greulich, H., et al., *Functional analysis of receptor tyrosine kinase mutations in lung cancer identifies oncogenic extracellular domain mutations of ERBB2*. Proc Natl Acad Sci U S A, 2012. **109**(36): p. 14476-81.
45. Johnson, L.A., et al., *Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma*. Sci Transl Med, 2015. **7**(275): p. 275ra22.
46. Morgan, R.A., et al., *Recognition of glioma stem cells by genetically modified T cells targeting EGFRvIII and development of adoptive cell therapy for glioma*. Hum Gene Ther, 2012. **23**(10): p. 1043-53.
47. Bister, A., et al., *A novel CD34-derived hinge for rapid and efficient detection and enrichment of CAR T cells*. Mol Ther Oncolytics, 2021. **23**: p. 534-546.
48. Rossig, C., et al., *Adoptive cellular immunotherapy with CD19-specific T cells*. Klin Padiatr, 2005. **217**(6): p. 351-6.
49. Bister, A., et al., *Optimized NGFR-derived hinges for rapid and efficient enrichment and detection of CAR T cells in vitro and in vivo*. Mol Ther Oncolytics, 2022. **26**: p. 120-134.
50. Soldierer, M., et al., *Genetic Engineering and Enrichment of Human NK Cells for CAR-Enhanced Immunotherapy of Hematological Malignancies*. Front Immunol, 2022. **13**: p. 847008.
51. Del Vecchio, C.A., et al., *EGFRvIII gene rearrangement is an early event in glioblastoma tumorigenesis and expression defines a hierarchy modulated by epigenetic mechanisms*. Oncogene, 2013. **32**(21): p. 2670-81.
52. An, Z., et al., *EGFR and EGFRvIII coopt host defense pathways promoting progression in glioblastoma*. Neuro Oncol, 2025. **27**(2): p. 383-397.

53. Gan, H.K., A.H. Kaye, and R.B. Luwor, *The EGFRvIII variant in glioblastoma multiforme*. J Clin Neurosci, 2009. **16**(6): p. 748-54.
54. Makabe, K., et al., *Anti-EGFR antibody 528 binds to domain III of EGFR at a site shifted from the cetuximab epitope*. Scientific Reports, 2021. **11**(1): p. 5790.
55. Fleischer, L.C., et al., *Non-signaling Chimeric Antigen Receptors Enhance Antigen-Directed Killing by gammadelta T Cells in Contrast to alphabeta T Cells*. Mol Ther Oncolytics, 2020. **18**: p. 149-160.
56. Pollok, K.E., et al., *High-efficiency gene transfer into normal and adenosine deaminase-deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments*. J Virol, 1998. **72**(6): p. 4882-92.
57. Lamplugh, Z.L., et al., *Microenvironmental regulation of solid tumour resistance to CAR T cell therapy*. Nat Rev Immunol, 2026. **26**(3): p. 230-248.
58. Wheeler, S.E., et al., *Epidermal growth factor receptor variant III mediates head and neck cancer cell invasion via STAT3 activation*. Oncogene, 2010. **29**(37): p. 5135-45.
59. Zhang, H.D., et al., *[Preliminary study on the expression of EGFRv III and CXCR4 in head and neck squamous cell carcinoma]*. Lin Chuang Er Bi Yan Hou Tou Jing Wai Ke Za Zhi, 2017. **31**(11): p. 817-821.
60. Melchers, L.J., et al., *Head and neck squamous cell carcinomas do not express EGFRvIII*. Int J Radiat Oncol Biol Phys, 2014. **90**(2): p. 454-62.
61. Thomas Koch, D., et al., *Epidermal growth factor receptor variant III in head and neck squamous cell carcinoma is not relevant for targeted therapy and irradiation*. Oncotarget, 2017. **8**(20): p. 32668-32682.
62. Khattri, A., et al., *Rare occurrence of EGFRvIII deletion in head and neck squamous cell carcinoma*. Oral Oncol, 2015. **51**(1): p. 53-8.

63. Stec, W.J., et al., *Cell line with endogenous EGFRvIII expression is a suitable model for research and drug development purposes*. *Oncotarget*, 2016. **7**(22): p. 31907-25.
64. Gedeon, P.C., et al., *Rindopepimut: anti-EGFRvIII peptide vaccine, oncolytic*. *Drugs Future*, 2013. **38**(3): p. 147-155.
65. Weller, M., et al., *Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial*. *Lancet Oncol*, 2017. **18**(10): p. 1373-1385.
66. Lassman, A.B., et al., *Depatuxizumab mafodotin in EGFR-amplified newly diagnosed glioblastoma: A phase III randomized clinical trial*. *Neuro Oncol*, 2023. **25**(2): p. 339-350.
67. van den Bent, M., et al., *Efficacy of depatuxizumab mafodotin (ABT-414) monotherapy in patients with EGFR-amplified, recurrent glioblastoma: results from a multi-center, international study*. *Cancer Chemother Pharmacol*, 2017. **80**(6): p. 1209-1217.
68. Zhu, H., et al., *EGFRvIII-CAR-T Cells with PD-1 Knockout Have Improved Anti-Glioma Activity*. *Pathol Oncol Res*, 2020. **26**(4): p. 2135-2141.
69. Miao, H., et al., *EGFRvIII-specific chimeric antigen receptor T cells migrate to and kill tumor deposits infiltrating the brain parenchyma in an invasive xenograft model of glioblastoma*. *PLoS One*, 2014. **9**(4): p. e94281.
70. Li, J., et al., *Optimizing CAR T cell therapy for solid tumours: a clinical perspective*. *Nat Rev Clin Oncol*, 2025. **22**(12): p. 953-968.
71. Tu, Z., et al., *Barriers and solutions for CAR-T therapy in solid tumors*. *Cancer Gene Ther*, 2025. **32**(9): p. 923-934.
72. Liu, D., et al., *The Role of Immunological Synapse in Predicting the Efficacy of Chimeric Antigen Receptor (CAR) Immunotherapy*. *Cell Commun Signal*, 2020. **18**(1): p. 134.
73. Greenman, R., et al., *Shaping Functional Avidity of CAR T Cells: Affinity, Avidity, and Antigen Density That Regulate Response*. *Mol Cancer Ther*, 2021. **20**(5): p. 872-884.

74. Mao, R., W. Kong, and Y. He, *The affinity of antigen-binding domain on the antitumor efficacy of CAR T cells: Moderate is better*. Front Immunol, 2022. **13**: p. 1032403.
75. Guest, R.D., et al., *The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens*. J Immunother, 2005. **28**(3): p. 203-11.
76. Hudecek, M., et al., *Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells*. Clin Cancer Res, 2013. **19**(12): p. 3153-64.
77. Hanssens, H., et al., *The antigen-binding moiety in the driver's seat of CARs*. Med Res Rev, 2022. **42**(1): p. 306-342.
78. Xiong, Y., K.A. Libby, and X. Su, *The physical landscape of CAR-T synapse*. Biophys J, 2024. **123**(15): p. 2199-2210.
79. Harrer, D.C., et al., *Fine-tuning the antigen sensitivity of CAR T cells: emerging strategies and current challenges*. Front Immunol, 2023. **14**: p. 1321596.
80. Balbis, A. and B.I. Posner, *Compartmentalization of EGFR in cellular membranes: role of membrane rafts*. J Cell Biochem, 2010. **109**(6): p. 1103-8.
81. Ghatak, S., et al., *Chemotherapy induces feedback up-regulation of CD44v6 in colorectal cancer initiating cells through beta-catenin/MDR1 signaling to sustain chemoresistance*. Front Oncol, 2022. **12**: p. 906260.
82. Oliferenko, S., et al., *Analysis of CD44-containing lipid rafts: Recruitment of annexin II and stabilization by the actin cytoskeleton*. J Cell Biol, 1999. **146**(4): p. 843-54.
83. von Witzleben, A., et al., *HNSCC: Tumour Antigens and Their Targeting by Immunotherapy*. Cells, 2020. **9**(9).
84. Katsarou, A., et al., *Combining a CAR and a chimeric costimulatory receptor enhances T cell sensitivity to low antigen density and promotes persistence*. Sci Transl Med, 2021. **13**(623): p. eabh1962.

85. Claus, C., C. Ferrara-Koller, and C. Klein, *The emerging landscape of novel 4-1BB (CD137) agonistic drugs for cancer immunotherapy*. MAbs, 2023. **15**(1): p. 2167189.
86. Gao, Y., et al., *Optimization of T Cell Redirecting Strategies: Obtaining Inspirations From Natural Process of T Cell Activation*. Frontiers in Immunology, 2021. **12**.
87. Espie, D. and E. Donnadieu, *New insights into CAR T cell-mediated killing of tumor cells*. Frontiers in Immunology, 2022. **13**.
88. Kuramitsu, S., et al., *Lenalidomide enhances the function of chimeric antigen receptor T cells against the epidermal growth factor receptor variant III by enhancing immune synapses*. Cancer Gene Therapy, 2015. **22**(10): p. 487-495.
89. Banerjee, P.P. and J.S. Orange, *Quantitative measurement of F-actin accumulation at the NK cell immunological synapse*. J Immunol Methods, 2010. **355**(1-2): p. 1-13.
90. Jang, J.H., et al., *Imaging of Cell-Cell Communication in a Vertical Orientation Reveals High-Resolution Structure of Immunological Synapse and Novel PD-1 Dynamics*. J Immunol, 2015. **195**(3): p. 1320-30.

Chapter 4

Conclusion and Outlook

The overarching aim of this dissertation is the quantitative description of membrane-associated physical processes, including diffusion, force-driven transport, and spatial organization. A central premise is that membrane mechanics can be formulated in terms of experimentally accessible physical observables. Advanced fluorescence microscopy, combined with quantitative image analysis, enables the robust extraction of such observables, thereby providing a direct link between imaging data and physical parameters governing membrane dynamics.

SLBs constitute the central model system of this work. They preserve essential physical properties of biological membranes, in particular lateral fluidity, while allowing systematic control over composition and boundary conditions [11][12]. In Publication I, it is shown that lateral diffusion in SLBs is strongly affected by structural heterogeneities such as mobile and immobile obstacles. These constraints lead to pronounced deviations from ideal Brownian motion, in agreement with established theoretical [48][49][50] and experimental descriptions[16][67] of anomalous diffusion in crowded two-dimensional systems. This demonstrates that even in simplified membrane environments, transport properties are highly sensitive to spatial disorder and confinement effects.

Beyond structural heterogeneity, lipid phase behavior provides an additional mechanism that governs membrane dynamics. Initial evidence for the influence of phase-related effects on diffusion is already observed in Publication I. In Publication II, this aspect is extended by actively exploiting phase properties as a functional control parameter. In combination with externally applied forces, this enables the controlled generation and stabilization of spatial patterns in the membrane plane. In particular, this work demonstrates that externally driven molecular pattern formation can be coupled to lipid phase transitions in order to “write” and subsequently thermodynamically stabilize spatial molecular patterns via phase freezing. This represents one of the first experimental realizations of controlled pattern formation and fixation in a biomimetic membrane system using phase-dependent transitions. This transition from passive to actively controlled systems highlights the interplay between intrinsic membrane properties and external driving forces,

which can be used to tune non-equilibrium spatial organization. In this context, SLBs also serve not only as passive model systems but as functional interfaces capable of mediating and modulating interactions with cells, as evidenced by cellular responses to externally imposed patterns.

Publication III and Publication IV extend these physical principles to biologically more complex systems, focusing on receptor organization at membrane interfaces relevant to immunological synapses. In Publication III, SLBs are employed as well-defined target membranes, enabling precise control over ligand density and spatial distribution. Previous studies have demonstrated that structural barriers and heterogeneities in target membranes can strongly influence synapse formation and receptor organization[68][28]. Building on this, the present work systematically investigates how controlled variations in ligand concentration affect receptor accumulation and spatial pattern formation at the synaptic interface. Importantly, this work provides one of the first quantitative molecular-level descriptions of CAR–NK synapse organization, a system that has so far been only sparsely characterized compared to conventional immunological synapses or CAR-T synapses. Different classes of synaptic organization are identified and described in a quantitative framework, enabling a systematic link between ligand conditions and emergent receptor organization. In this context, receptor redistribution and clustering are interpreted as manifestations of collective behaviour of biomolecules, arising from phase separation-like organization.

In Publication IV, this framework is extended to multi-receptor systems, allowing the simultaneous analysis of different receptor species with distinct structural and functional designs. A key observation is that even non-signaling CARs are recruited into the immunological synapse, indicating that spatial organization can emerge independently of active cellular processes. This highlights the dominant role of physical membrane organization principles in determining receptor distribution. From a broader perspective, these findings provide a physical basis for understanding receptor organization in engineered immune systems and may contribute to the rational optimization of CAR-based immunotherapies.

A further key methodological achievement of this dissertation is the development of dedicated quantitative image analysis software, which enables robust extraction of physical observables from fluorescence microscopy data. This software was designed to be reusable and extensible, and is intended for dissemination within the research group and the broader scientific community, thereby supporting reproducible and standardized analysis of membrane dynamics.

Taken together, the results of this dissertation demonstrate that membrane-associated phenomena across both simplified model systems and biologically relevant interfaces can be consistently described within a unified framework of physical observables. Diffusion, externally driven transport, and receptor organization are shown to be interconnected man-

ifestations of underlying membrane mechanics, which can be systematically controlled, measured, and interpreted using quantitative optical microscopy approaches.

To conclude, this dissertation demonstrates that membrane-associated processes such as diffusion, force-driven transport, and receptor organization can be consistently described using a unified set of physical observables. Across different experimental systems, SLBs present a controlled platform that enables systematic variation of structural and dynamic parameters governing membrane behavior.

Overall, the results show that membrane dynamics and molecular pattern formation are not solely determined by biochemical complexity, but emerge from the interplay of thermal fluctuations, spatial constraints, intermolecular interactions, and external forces. These principles apply both to simplified model systems and biologically relevant membrane interfaces.

The results of this dissertation open several directions for future research at the interface of membrane physics, quantitative imaging, and cellular systems.

A first natural extension concerns the combination of force-engineered SLBs with CAR-based receptor systems in living immune cells. By designing increasingly complex spatial ligand patterns on the substrate, it becomes possible to systematically investigate how individual cells interpret and respond to defined molecular landscapes. In particular, the generation of nanoscale and microscale ligand patterns would enable a quantitative analysis of how spatial information is translated into receptor organization and signaling responses at the single-cell level.

Building on this concept, a particularly promising direction is the introduction of dynamically reconfigurable model membranes. Using external control parameters such as magnetic manipulation or phase-dependent patterning, the substrate could be actively switched between different spatial configurations. This would allow the same immune cell to be exposed to alternating conditions, for example between patterned and homogeneous ligand distributions, or between different pattern geometries. Such a system would enable the direct investigation of how immunological synapses adapt to time-dependent boundary conditions, including questions such as the timescales of synapse reorganization, stability of receptor clusters, and memory effects in cellular signaling. This approach effectively transforms static model interfaces into active membranes, providing a dynamic experimental platform for studying non-equilibrium cell–surface interactions.

In this context, combining ligand patterning with controlled membrane phase behavior offers an additional level of control over ligand mobility and spatial stability. Phase-separated membrane regions can be used to regulate the effective diffusivity of ligands, thereby introducing mobility as an independent tuning parameter in addition to density

and spatial geometry. This enables a more comprehensive control over receptor–ligand interactions and their resulting spatial organization.

Building on the observations that both signaling and non-signaling CAR constructs are recruited into the immunological synapse, future studies could further investigate how receptor architecture, mechanical coupling, and membrane organization jointly determine synapse formation and stability under dynamic conditions. In particular, applying time-dependent substrate reconfiguration could help disentangle the roles of biochemical signaling and purely physical organization principles in immune cell activation.

From a methodological perspective, future advances in advanced fluorescence imaging and real-time quantitative analysis will further expand the range of accessible physical observables, including transient interaction kinetics and local rearrangement dynamics. Combined with dynamically controllable membrane substrates, this could ultimately enable a systematic exploration of non-equilibrium organization principles in living cell–membrane interfaces under externally programmed boundary conditions.

Bibliography

- [1] William Stillwell. *An Introduction to Biological Membranes: Composition, Structure and Function*. 2nd ed. Elsevier, 2016, pp. 1–597. ISBN: 9780444637727. DOI: 10.1016/C2015-0-06226-8.
- [2] Stephen H. White, Gunnar von Heijne, and Donald M. Engelman. *Cell boundaries: How membranes and their proteins work*. 1st ed. Garland Science, 2022, pp. 1–564. ISBN: 9780429341328. DOI: 10.1201/9780429341328.
- [3] Rob Phillips et al. *Physical Biology of the Cell*. 2nd ed. Garland Science, 2012, pp. 1–1088. ISBN: 9780429168833. DOI: 10.1201/9781134111589.
- [4] P. G. Saffman and M. Delbrueck. “Brownian motion in biological membranes”. In: *Proceedings of the National Academy of Sciences of the United States of America* 72 (8) 1975, pp. 3111–3113. DOI: 10.1073/pnas.72.8.3111.
- [5] Bruce Alberts et al. *Molecular biology of the cell*. 7th ed. Wiley VCH, 2022, pp. 1–1776. ISBN: 9783527353644.
- [6] Kenneth Murphy and Casey Weaver. *Janeway Immunologie*. 9th ed. Springer Spectrum, 2018. ISBN: 9783662560037. DOI: 10.1007/978-3-662-56004-4.
- [7] Edward T. Castellana and Paul S. Cremer. “Solid supported lipid bilayers: From biophysical studies to sensor design”. In: *Surface Science Reports* 61 (10) 2006, pp. 429–444. DOI: 10.1016/j.surfrep.2006.06.001.
- [8] Cyrus R. Safinya and Joachim O. Rädler. *Handbook of Lipid Membranes Molecular, Functional, and Materials Aspects*. 1st ed. CRC Press, 2021, pp. 1–354. ISBN: 9781466555730.
- [9] Dzmitry Afanasenkau. “Supported lipid bilayer as a biomimetic platform for neuronal cell culture”. PhD thesis. 2013. ISBN: 978-3-89336-863-1. URL: <https://publications.rwth-aachen.de/record/209271/files/4540.pdf> (visited on May 12, 2026).
- [10] Jay T. Groves and Michael L. Dustin. “Supported planar bilayers in studies on immune cell adhesion and communication”. In: *Journal of Immunological Methods* 278 (1-2) 2003, pp. 19–32. DOI: 10.1016/S0022-1759(03)00193-5.

- [11] L. K. Tamm and H. M. McConnell. “Supported phospholipid bilayers”. In: *Biophysical Journal* 47 (1) 1985, pp. 105–113. DOI: 10.1016/S0006-3495(85)83882-0.
- [12] Roxanne Glazier and Khalid Salaita. “Supported lipid bilayer platforms to probe cell mechanobiology”. In: *Biochimica et Biophysica Acta - Biomembranes* 1859 (9) 2017, pp. 1465–1482. DOI: 10.1016/j.bbamem.2017.05.005.
- [13] Volodymyr Nechyporuk-Zloy. *Principles of Light Microscopy: From Basic to Advanced*. 1st ed. Springer International Publishing, 2022, pp. 1–327. ISBN: 9783031044779. DOI: 10.1007/978-3-031-04477-9.
- [14] Malte Wachsmuth, Waldemar Waldeck, and Jörg Langowski. “Anomalous diffusion of fluorescent probes inside living cell investigated by spatially-resolved fluorescence correlation spectroscopy”. In: *Journal of Molecular Biology* 298 (4) 2000, pp. 677–689. DOI: 10.1006/jmbi.2000.3692.
- [15] Laure Wawrezinieck et al. “Fluorescence correlation spectroscopy diffusion laws to probe the submicron cell membrane organization”. In: *Biophysical Journal* 89 (6) 2005, pp. 4029–4042. DOI: 10.1529/biophysj.105.067959.
- [16] Susanne F. Fenz, Rudolf Merkel, and Kheya Sengupta. “Diffusion and intermembrane distance: Case study of avidin and E-cadherin mediated adhesion”. In: *Langmuir* 25 (2) 2008, pp. 1074–1085. DOI: 10.1021/la803227s.
- [17] Nikon Instruments Inc. *Total Internal Reflection Fluorescence (TIRF) Microscopy*. URL: <https://www.microscopyu.com/techniques/fluorescence/total-internal-reflection-fluorescence-tirf-microscopy> (visited on Apr. 20, 2026).
- [18] Michael L. Dustin. “The immunological synapse”. In: *Cancer immunology research* 2 (11) 2014, pp. 1023–1033. DOI: 10.1158/2326-6066.CIR-14-0161.
- [19] Jason Yi et al. “Actin retrograde flow and actomyosin II arc contraction drive receptor cluster dynamics at the immunological synapse in Jurkat T cells”. In: *Molecular Biology of the Cell* 23 (5) 2012, pp. 834–852. DOI: 10.1091/mbc.E11-08-0731.
- [20] Mathias Getzlaff. *Fundamentals of magnetism*. 1st ed. Springer, 2008, pp. 1–387. ISBN: 9783540311508. DOI: 10.1007/978-3-540-31152-2.
- [21] Frank Wiekhorst et al. “Magnetorelaxometry assisting biomedical applications of magnetic nanoparticles”. In: *Pharmaceutical Research* 29 (5) 2012, pp. 1189–1202. DOI: 10.1007/s11095-011-0630-3.
- [22] K. Riahi et al. “Magnetic performance of synomag nanoparticles in various environments”. In: *International Journal on Magnetic Particle Imaging* 6 (2) 2020, pp. 1–3. DOI: 10.18416/IJMPI.2020.2009022.

-
- [23] Jordi Faraudo, Jordi S. Andreu, and Juan Camacho. “Understanding diluted dispersions of superparamagnetic particles under strong magnetic fields: A review of concepts, theory and simulations”. In: *Soft Matter* 9 (29) 2013, pp. 6654–6664. DOI: 10.1039/c3sm00132f.
- [24] Zheng Chao et al. “Immunological synapse: structures, molecular mechanisms and therapeutic implications in disease”. In: *Signal Transduction and Targeted Therapy* 10 (254) 2025, pp. 1–36. DOI: 10.1038/s41392-025-02332-6.
- [25] Rui Li et al. “The CAR T-Cell Mechanoimmunology at a Glance”. In: *Advanced Science* 7 (24) 2020, p. 2002628. DOI: 10.1002/advs.202002628.
- [26] Leisheng Zhang et al. “CAR-NK cells for cancer immunotherapy: from bench to bedside”. In: *Biomarker Research* 10 (12) 2022, pp. 1–19. DOI: 10.1186/s40364-022-00364-6.
- [27] Lei Peng et al. “CAR-T and CAR-NK as cellular cancer immunotherapy for solid tumors”. In: *Cellular and Molecular Immunology* 21 (10) 2024, pp. 1089–1108. DOI: 10.1038/s41423-024-01207-0.
- [28] Kaspar Mossman and Jay Groves. “Micropatterned supported membranes as tools for quantitative studies of the immunological synapse”. In: *Chemical Society Reviews* 36 (1) 2007, pp. 46–54. DOI: 10.1039/b605319j.
- [29] Johannes Schindelin et al. “Fiji: An open-source platform for biological-image analysis”. In: *Nature Methods* 9 (7) 2012, pp. 676–682. DOI: 10.1038/nmeth.2019.
- [30] Machán Radek and Martin Hof. “Fluorescence Correlation Spectroscopy (FCS)”. In: *Practical Manual for Fluorescence Microscopy Techniques*. Ed. by PicoQuant GmbH. 2016.
- [31] S. Rüttinger et al. “Determination of the confocal volume for quantitative fluorescence correlation spectroscopy”. In: *Optics InfoBase Conference Papers*. 2007, pp. 1–8. ISBN: 9780819467713. DOI: 10.1117/12.728463.
- [32] PicoQuant GmbH. *Introduction to Fluorescence Correlation Spectroscopy*. 2024. URL: https://www.tcspc.com/doku.php/general:fluorescence_correlation_spectroscopy-_a_short_introduction (visited on Apr. 20, 2026).
- [33] Bo Wang et al. “Nanoparticle-induced surface reconstruction of phospholipid membranes”. In: *Proceedings of the National Academy of Sciences of the United States of America* 105 (47) 2008, pp. 18171–18175. DOI: 10.1073/pnas.0807296105.
- [34] James A. Goodchild, Danielle L. Walsh, and Simon D. Connell. “Nanoscale Substrate Roughness Hinders Domain Formation in Supported Lipid Bilayers”. In: *Langmuir* 35 (47) 2019, pp. 15352–15363. DOI: 10.1021/acs.langmuir.9b01990.

- [35] A. A. Brian and H. M. McConnell. “Allogeneic stimulation of cytotoxic T cells by supported planar membranes”. In: *Proceedings of the National Academy of Sciences of the United States of America* 81 (19) 1984, pp. 6159–6163. DOI: 10.1073/pnas.81.19.6159.
- [36] Seyed R. Tabaei et al. “Solvent-assisted lipid bilayer formation on silicon dioxide and gold”. In: *Langmuir* 30 (34) 2014, pp. 10363–10373. DOI: 10.1021/la501534f.
- [37] Katharine B. Blodgett. “Films Built by Depositing Successive Monomolecular Layers on a Solid Surface”. In: *Journal of the American Chemical Society* 57 (6) 1935, pp. 1007–1022. DOI: 10.1021/ja01309a011.
- [38] Irving Langmuir and Vincent J Schaefer. “Activities of Urease and Pepsin Monolayers”. In: *Journal of the American Chemical Society* 60 (6) 1938, pp. 1351–1360. DOI: 10.1021/ja01273a023.
- [39] Ralf P. Richter, Rémi Bérat, and Alain R. Brisson. “Formation of solid-supported lipid bilayers: An integrated view”. In: *Langmuir* 22 (8) Apr. 2006, pp. 3497–3505. DOI: 10.1021/la052687c.
- [40] Joshua A. Jackman and Nam Joon Cho. “Supported Lipid Bilayer Formation: Beyond Vesicle Fusion”. In: *Langmuir* 36 (6) 2020, pp. 1387–1400. DOI: 10.1021/acs.langmuir.9b03706.
- [41] Nikoleta Ivanova and Hassan Chamati. “The Effect of Cholesterol in SOPC Lipid Bilayers at Low Temperatures”. In: *Membranes* 13 (3) 2023, p. 275. DOI: 10.3390/membranes13030275.
- [42] Motomu Tanaka and Erich Sackmann. “Polymer-supported membranes as models of the cell surface”. In: *Nature* 437 (7059) 2005, pp. 656–663. DOI: 10.1038/nature04164.
- [43] E. Sackmann. “Supported membranes: Scientific and practical applications”. In: *Science* 271 (5245) 1996, pp. 43–48. DOI: 10.1126/science.271.5245.43.
- [44] C. Scomparin et al. “Diffusion in supported lipid bilayers: Influence of substrate and preparation technique on the internal dynamics”. In: *European Physical Journal E* 28 (2) 2009, pp. 211–220. DOI: 10.1140/epje/i2008-10407-3.
- [45] Yee Hung M. Chan and Steven G. Boxer. “Model membrane systems and their applications”. In: *Current Opinion in Chemical Biology* 11 (6) 2007, pp. 581–587. DOI: 10.1016/j.cbpa.2007.09.020.
- [46] Rüdiger Bessenrodt. “Brownsche Bewegung: Hundert Jahre Theorie der wichtigsten Brücke zwischen Mikro- und Makrophysik”. In: *Physikalische Blätter* 33 (1) 1977, pp. 7–16. DOI: 10.1002/phbl.19770330104.

-
- [47] W Jost and K Hauße. *Diffusion: Methoden der Messung und Auswertung*. Dr. Dietrich Steinkopf Verlag, 2013. ISBN: 9783642852824.
 - [48] Evan Evans and Erich Sackmann. “Translational and rotational drag coefficients for a disk moving in a liquid membrane associated with a rigid substrate”. In: *Journal of Fluid Mechanics* 194 1988, pp. 553–561. DOI: 10.1017/S0022112088003106.
 - [49] Michael J. Saxton. “Anomalous diffusion due to binding: A Monte Carlo study”. In: *Biophysical Journal* 70 (3) 1996, pp. 1250–1262. DOI: 10.1016/S0006-3495(96)79682-0.
 - [50] Th M Nieuwenhuizen, P. F.J. Van Velthoven, and M H Ernst. “Diffusion and long-time tails in a two-dimensional site-percolation model”. In: *Physical Review Letters* 57 (20) 1986, pp. 2477–2480. DOI: 10.1103/PhysRevLett.57.2477.
 - [51] Nikoleta Ivanova and Hassan Chamati. “Phase Behavior of Binary Mixtures of SOPC and Cholesterol”. In: *ACS Omega* 10 (19) 2025, pp. 19235–19242. DOI: 10.1021/acsomega.5c01375.
 - [52] *synomag®-CLD-F – micromod Partikeltechnologie GmbH*. URL: <https://micromod.de/produkt-kategorie/fluoreszente-magnetpartikel/synomag-cld-f/> (visited on Apr. 8, 2026).
 - [53] Wolfgang Nolting. *Theoretical Physics 3: Electrodynamics*. Springer International Publishing, 2016, pp. 1–659. ISBN: 9783319401683. DOI: 10.1007/978-3-319-40168-3.
 - [54] Jordan S. Orange. “Formation and function of the lytic NK-cell immunological synapse”. In: *Nature Reviews Immunology* 8 (9) 2008, pp. 713–725. DOI: 10.1038/nri2381.
 - [55] Sahar Balkhi et al. “CAR-NK cell therapy: promise and challenges in solid tumors”. In: *Frontiers in Immunology* 16 2025, p. 1574742. DOI: 10.3389/fimmu.2025.1574742.
 - [56] Katayoun Rezvani et al. “Engineering Natural Killer Cells for Cancer Immunotherapy”. In: *Molecular Therapy* 25 (8) 2017, pp. 1769–1781. DOI: 10.1016/j.ymthe.2017.06.012.
 - [57] Junru Lu and Guan Jiang. “The journey of CAR-T therapy in hematological malignancies”. In: *Molecular Cancer* 21 (194) 2022, pp. 194–. DOI: 10.1186/s12943-022-01663-0.
 - [58] Arash Grakoui et al. “The immunological synapse: A molecular machine controlling T cell activation”. In: *Science* 285 (5425) 1999, pp. 221–227. DOI: 10.1126/science.285.5425.221.

- [59] Simon J. Davis and P. Anton van der Merwe. “The kinetic-segregation model: TCR triggering and beyond”. In: *Nature Immunology* 7 (8) 2006, pp. 803–809. DOI: 10.1038/ni1369.
- [60] Sašo Čemerski et al. “The Balance between T Cell Receptor Signaling and Degradation at the Center of the Immunological Synapse Is Determined by Antigen Quality”. In: *Immunity* 29 (3) 2008, pp. 414–422. DOI: 10.1016/j.immuni.2008.06.014.
- [61] Darrell J. Irvine. “Function-specific variations in the immunological synapses formed by cytotoxic T cells”. In: *Proceedings of the National Academy of Sciences of the United States of America* 100 (24) 2003, pp. 13739–13740. DOI: 10.1073/pnas.2536626100.
- [62] Peter S. Andersen et al. “A Response Calculus for Immobilized T Cell Receptor Ligands”. In: *Journal of Biological Chemistry* 276 (52) 2001, pp. 49125–49132. DOI: 10.1074/jbc.M109396200.
- [63] D T Kim et al. “Quantitative analysis of T cell activation: role of TCR/ligand density and TCR affinity”. In: *The Journal of Immunology* 156 (8) 1996, pp. 2737–2742. DOI: 10.4049/jimmunol.156.8.2737.
- [64] Chih Jung Hsu et al. “Ligand mobility modulates immunological synapse formation and T cell activation”. In: *PloS one* 7 (2) 2012. DOI: 10.1371/JOURNAL.PONE.0032398.
- [65] J. M. Zwier et al. “Image calibration in fluorescence microscopy”. In: *Journal of Microscopy* 216 (1) 2004, pp. 15–24. DOI: 10.1111/j.0022-2720.2004.01390.x.
- [66] Pieter Wesseling. *Principles of Computational Fluid Dynamics*. Vol. 29. Springer, 2001. ISBN: 3540678530 9783540678533. DOI: 10.1007/978-3-642-05146-3.
- [67] Daniel Beckers, Dunja Urbancic, and Erdinc Sezgin. “Impact of Nanoscale Hindrances on the Relationship between Lipid Packing and Diffusion in Model Membranes”. In: *Journal of Physical Chemistry B* 124 (8) 2020, pp. 1487–1494. DOI: 10.1021/acs.jpcc.0c00445.
- [68] Jay T. Groves. “Spatial mutation of the T cell immunological synapse”. In: *Current Opinion in Chemical Biology* 10 (6) 2006, pp. 544–550. DOI: 10.1016/j.cbpa.2006.10.021.

Danksagung

An dieser Stelle möchte ich allen danken, die mich in den letzten vier Jahren fachlich und persönlich unterstützt haben.

Zuallererst gilt mein besonderer Dank meiner Doktormutter, Prof. Dr. Cornelia Monzel. Liebe Cornelia, vielen Dank für die Möglichkeit, meine Doktorarbeit in deinem Institut anzufertigen. Ich bin dir sehr dankbar für die hervorragende Betreuung – sowohl in fachlicher Hinsicht als auch darüber hinaus. Du schaffst eine Arbeitsatmosphäre, in der gegenseitige Unterstützung und Zusammenhalt selbstverständlich sind, in der auf Augenhöhe kommuniziert wird und so wertvolle Beiträge zur Forschung entstehen. Bereits als Bachelorstudent habe ich mich in deiner Arbeitsgruppe sehr wohlfühlt, und auch meine Zeit als Doktorand werde ich in bester Erinnerung behalten.

Außerdem danke ich Prof. Dr. Thomas Heinzel herzlich für die Übernahme des Zweitgutachtens meiner Dissertation.

Mein Dank gilt ebenso allen Studierenden, die an den Projekten dieser Arbeit mitgewirkt haben. Besonders hervorheben möchte ich Kathlen Grüter, Nicole Sander, Lara Hölzle und Michael-Nicolas Reifenscheid. Vielen Dank für euren Einsatz und eure Motivation. Gemeinsam ist es uns gelungen, das CAR-Projekt von physikalischer Seite voranzubringen.

Ich danke allen aktuellen und ehemaligen Mitgliedern der AG Monzel für die vielen anregenden fachlichen Diskussionen sowie für die aufmunternden Worte. Nina, Daniel, Andi, Xiaoyue und Phillip – vielen Dank für die herzliche Aufnahme in die Arbeitsgruppe. Gerade im ersten Jahr habt ihr mir viel gezeigt und mich auf meinem Weg vom Studenten zum Doktoranden begleitet. Danke Siegrun und Soner für eure Unterstützung bei technischen und organisatorischen Belangen. Ein besonderer Dank geht an Miriam und Rio für die schöne Zeit im Büro 30 und für die daraus entstandenen neuen und alten Freundschaften.

Ebenso danke ich unseren Kollaborationspartnern aus der HNO, insbesondere Prof. Dr. Helmut Hanenberg, Dr. Constanze Wiek und Maren Soldierer. Vielen Dank für die zahlreichen Zellen, die ihr für uns hergestellt habt, und für die hervorragende Zusammenarbeit, durch die wir das CAR-Projekt gemeinsam aufbauen konnten.

Ein besonderer Dank gilt außerdem Jan Lange und der AG Heinzel für die Unterstützung bei den Lithographieverfahren. Jan, in den ersten beiden Jahren meiner Promotion waren wir Lab-Buddys und es hat mich sehr gefreut, dass wir unsere Zusammenarbeit auch später noch fortsetzen konnten. Zusätzlich danke ich Noah Salama für die Unterstützung am Abberior, besonders in Momenten, in denen scheinbar nichts mehr funktionierte.

Ich danke meinen Freunden, die mir stets zur Seite standen und sich nicht nur einmal meine Beschwerden über nicht funktionierende Mikroskope anhören mussten. Vielen Dank, dass ihr immer für mich da seid.

Zuletzt gilt mein herzlichster Dank meiner Familie – meinen Eltern Claudia und Frank sowie meinem Bruder Sebastian. Ihr habt mich mit eurer Liebe geprägt, mir die wichtigsten Werte vermittelt und mir stets das Vertrauen gegeben, an mich selbst zu glauben. Ihr habt mich immer ermutigt, neue Herausforderungen anzunehmen und über mich hinauszuwachsen. Ohne eure Unterstützung und die Möglichkeiten, die ihr mir eröffnet habt, wäre mein Studium und damit auch dieser Weg nicht möglich gewesen. Dank euch stehe ich heute hier. Dafür bin ich euch von Herzen dankbar.