Cargo Transport on Engineered Surfaces Powered by Molecular Motors

Christian Brunner
Cargo Transport on Engineered Surfaces Powered by Molecular Motors

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Sciences
(Dr. sc. ETH Zürich)

presented by
Christian J. Brunner
Dipl. Werkstoff-Ing. ETH
born on September 11, 1977
citizen of Laupersdorf (SO)

accepted on the recommendation of
Prof. Dr. Viola Vogel, examiner
Prof. Dr. Vahid Sandoghdar, co-examiner
Prof. Dr. Marcus Textor, co-examiner
Dr. Christian Wahnes, co-examiner

August 27, 2007
FOR MY PARENTS
L’urgent, c’est fait!
Je gère l’impossible,
Pour les miracles y’a un petit délai!...

Gaston Lagaffe (by André Franquin)
Acknowledgements

The first big thank you goes to Viola Vogel for giving me the chance to pursue my doctoral thesis in her research group. I very much appreciate her enthusiasm and open mind for science, as well as her great ability to always find alternative ways to eventual drawbacks.

I would like to thank the board of co-referees Vahid Sandoghdar, Marcus Textor, and Christian Wahnes for continuous support and valuable discussions during my thesis. Special thanks to my lab supervisor Christian Wahnes for his consistent optimism and precious chemical background that kept me motivated and focused in the past few years.

During my stay in Seattle for almost one year in the beginning of my work, I met a vivid and very diverse group, active in many different research areas. I am grateful to Henry Hess and John Clemmens, who introduced me to the world of molecular shuttles, and Karl-Heinz Ernst for fruitful collaboration and nice lunch breaks in Seattle. Special thanks to Henry Hess for outstanding support and generously providing kinesin motor proteins. I am also thankful to Manu Forero, who provided shelter in his apartment during the first weeks of my Seattle experience.

Back in Zürich, we started to collaborate with the Nano-Optics group of Vahid Sandoghdar and could benefit from their great experience in optical imaging. Many thanks to Volker Jacobsen for showing me very small gold particles and unlabeled microtubules.

In the same time, Didier Falconnet from the lab of Marcus Textor introduced me to the secrets of the MAPL technique. Thanks also to Brigitte Städler, Christoph Huwiler, and Thomas Blättler for sharing their experience in surface science and technology, and Samuele Tosatti and Stefan Zürcher for providing PLL-g-PEG molecules.

The FIRST center for micro- and nanoscience is a great interdepartmental institution at ETH Hönggerberg for micro- and nanofabrication. Many thanks to Otte Homan for fantastic support and patient help in all photolithographic-, RIE-, and SEM-related work performed in the cleanroom. Thanks also to Christoph Widmeier, who evaporated numerous gold layers onto our substrates.

I would also like to thank all the Vogel group members for scientific discussions and several demanding 3D simulations. In particular, I would like to thank Sheila Luna and Michael Smith for being cool office mates. You almost made me find my body balance.
Thanks also to Kris Kubow, Vesa Hytönen, and Heike Hall for answering many biological and biochemical questions. Margrit Zeller and Carmen Ruesch are acknowledged for their continuous administrative efforts and support.

Many thanks to the semester-, bachelor-, and masterstudents Norma Graf, Josias Wacker, and Philipp Spycher for their great work and interest in the topic of molecular shuttles.

Thanks to Martin Halter, Diana Trentin, Michael Smith, and Jens Möller for careful proof-reading parts of the present thesis.

Very special thanks to Martin Halter, Thomas Frey, Tobias Künzler, Pascal Jud, and Martin Schuler for a great time during the whole time at ETH. I owe you guys countless cheerful moments and not so scientific discussions.

Thanks also to Diana Trentin for many cultural and culinary field trips.

Last but not least, I would like to express my deepest gratitude to my parents and my two sisters and their families for endless support during all these years, which crystallized in the recurrent question: “Are you done yet?”

Financial support from the DARPA Biomolecular Motors Program and ETH Zürich are gratefully acknowledged.
Abstract

Biomolecular motors, such as myosin and kinesin, are the workhorses in nature’s protein toolbox. They are not only responsible for macroscopic movements such as muscle contraction (myosin), but also account for active intracellular transport along a network of protein filaments called microtubules, protein polymers assembled from many tubulin subunits. Bioengineers have begun to exploit this transport system for technological purposes. Based on the concept of molecular shuttles—microtubule filaments propelled by surface-adsorbed kinesin motors—various types of applications, such as sensoric, nanomechanical or information processing devices, have been proposed. This thesis purposes to develop new, and to expand existing concepts and tools towards an in vitro transport system based on molecular shuttles. More specifically, we address important design criteria of a future device, i.e. (1) the expected lifetime of a final device, (2) suitable methods of visualizing transport within the device, and (3) the specific binding, transport and pick-up of cargo on plain and micro-engineered surfaces.

The lifetime of a molecular motor-based device depends on the stability of its most fragile component. In order to define a benchmark for the loss of performance over time, we investigated the stability of molecular shuttles operating in flowcells consisting of different synthetic materials. In this study, we could demonstrate that microtubules are most prone to degradation, and exhibit a lifetime of several hours under stable conditions. This lifetime, however, is dramatically decreased if casing materials, such as PDMS or PMMA, which promote the effusion of oxygen into the solution, are applied. Under such conditions and in combination with intense illumination, microtubule filaments degrade within seconds. On the other hand, kinesin motor proteins were active for at least 1–2 days independent of environmental conditions.

Fluorescent Microscopy is the most common method for live-imaging of microtubule shuttles. However, due to photobleaching and possible destabilizing effects of intense fluorescent illumination, alternative methods are sought to visualize microtubule filaments, protein assemblies, and even biological samples, which may serve as cargo. We therefore expanded the range of application of an interferometric optical imaging technique—originally developed for the detection of nanometer-sized gold colloids—to visualizing unlabeled microtubules. In motility assays, the filaments could be detected with sufficient contrast of 3–5% against the background.
Specific binding of cargo—key in a transport system—was investigated using biological interactions such as biotin–streptavidin, antibody–antigen, and DNA–DNA. By these means, cargo ranging in size from tens of nanometers to several micrometers could be specifically adsorbed and transported by molecular shuttles. However, due to the random orientation of adsorbed kinesins on a surface, microtubule shuttles move in a random, directionless manner. Therefore, to optimize the guidance of shuttle movement, new fabrication processes for open micro-channels featuring adjustable channel-overhangs were developed. Characterization of the transport of 40 nm gold particles by microtubule filaments inside these structures revealed a correlation between increased cargo-loss and increased curvature of the channels.

The loading of functionalized microtubules with cargo dispersed in solution is straightforward and effective, but lacks the spatial separation of the origin of loading and the destination of the load. We therefore aimed to fabricate cargo loading stations, which are spatially confined on a surface. In addition, these loading stations were shown to permit release of cargo upon contact to passing microtubule shuttles. Successful pick-up of 40 nm gold particles could be achieved by using DNA–DNA interactions to tether the cargo to the surface. In addition, we could demonstrate that co-existence of motors and cargo inside the loading stations was crucial for efficient pick-up, even if the average length of microtubule filaments exceeded the width of the loading stations by a factor of 4. Finally, we integrated loading stations into a micro-engineered channel system to combine shuttle guiding as well as pick-up and transport of cargo. The feasibility of cargo pick-up from loading stations inside the channels could be demonstrated. However, the average transport distance of cargo after pick-up (∼16 µm) was found to be below the requirements of an envisioned in vitro transport device. A functional and efficient integrated design therefore depends on further optimization and development.

Overall, we were able to provide valuable engineering concepts and principles for the further development of a future cargo transport device based on molecular shuttles. The integration of the independently developed key elements, i.e. pick-up and transport of cargo and the guidance of shuttles, into a final system as presented in this work is an important step towards this goal.
Zusammenfassung


Fluoreszenzmikroskopie ist die populärste Methode, molekulare Shuttles in Echtzeit zu beobachten. Aufgrund von Photobleaching (das Ausbleichen der FluoreszenzfARBSTOFFE) und potentiell destabilisierender Effekte, ausgelöst durch intensives Fluoreszenzlicht,


in dieser Umgebung gelangen. Allerdings war die durchschnittliche Transportlänge der Ladung nach dem Auflesen mit ungefähr 16 \( \mu \text{m} \) noch unterhalb der Anforderungen eines angestrebten \textit{in vitro} Transportsystems. Ein funktionierendes und effizientes Komplett-design eines solchen Transportsystems benötigt in Zukunft deshalb weitere Optimierung und Entwicklung.

Zusammenfassend konnten wir verschiedene Designkonzepte und -prinzipien erarbeiten, die für die Weiterentwicklung eines auf molekularen Shuttles basierenden Transportsystems wertvoll sind. Die Kombination der Schlüsselfelemente Auflesen und Transport von Ladung sowie Kontrolle der Bewegung der Shuttles, wie sie hier präsentiert wurde, ist ein wichtiger Schritt in Richtung eines Gesamtdesigns.
List of Abbreviations

Ab Antibody
ADP adenosine 5′-diphosphate
ATP adenosine 5′-triphosphate
bp base-pair
BRB80 80 mM PIPES, 2 mM MgCl₂, 1 mM EGTA, pH 6.85 with KOH
BRB160 160 mM PIPES, 4 mM MgCl₂, 2 mM EGTA, pH 6.85 with KOH
CATO 460 µl BRB80, 5 µl taxol, 10 µl casein (10 mg ml⁻¹), 5 µl MgATP, 5 µl D-glucose, 5 µl glucose oxidase, 5 µl catalase, 5 µl dithiotreitol
CLSM Confocal Laser Scanning Microscope
DMSO dimethylsulfoxide
DNA deoxyribonucleic acid
DTT dithiotreitol
EGTA ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EHT extra high tension
EVOH ethylene-vinyl alcohol copolymer
GDP guanosine 5′-diphosphate
GTP guanosine 5′-triphosphate
HMDS hexamethyldisilazane
IPA ispropyl alcohol
MT(s) microtubule(s)
MW molecular weight
NA numerical aperture
NMP 1-methyl-2-pyrrolidinone
nt nucleotide
OD optical density
PDMS poly(dimethylsiloxane)
PEG poly(ethyleneglycol)
PIPES piperazine-N,N′-bis(2-ethanesulfonic acid)
Piranha solution mixture of H₂SO₄ and H₂O₂ at a ratio 3:1
PLL poly(L-lysine)
PLL-g-PEG poly(L-lysine)-graft-poly(ethylene glycol)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMGI</td>
<td>poly(methylglutarimide) polymer with proprietary solvent blends (MicroChem)</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>PS</td>
<td>poly(styrene)</td>
</tr>
<tr>
<td>PTFE</td>
<td>poly(tetrafluoroethylene)</td>
</tr>
<tr>
<td>PU</td>
<td>poly(urethane)</td>
</tr>
<tr>
<td>RIE</td>
<td>Reactive Ion Etching</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SA</td>
<td>streptavidin</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>WD</td>
<td>working distance</td>
</tr>
</tbody>
</table>
Contents

1 Scope of the Thesis
  1.1 Background and Motivation ........................................... 2
  1.2 Objectives ..................................................................... 3
  1.3 Overview ....................................................................... 4

2 General Introduction—Motors and Machines
  2.1 Synthetic Motors and Machines ....................................... 8
  2.2 Biological Motors and Filaments ...................................... 8
    2.2.1 Microtubule Filaments ............................................. 9
    2.2.2 Kinesin Motors ..................................................... 11
    2.2.3 A Few Words About Intracellular Transport ............... 11
  2.3 From Filaments to Shuttles ............................................ 15
    2.3.1 Standard Motility Assay ........................................ 15
    2.3.2 Molecular Shuttles—The Inverted Motility Assay ....... 15
    2.3.3 Characteristics of Shuttle Movement ....................... 18

3 Lifetime of Microtubules in Hybrid Bio-Nanodevices
  3.1 Introduction ................................................................. 23
  3.2 Materials and Methods .................................................. 26
    3.2.1 Experimental Setup ............................................... 26
    3.2.2 Kinesin and Microtubules Preparation ...................... 26
    3.2.3 Motility Assay ...................................................... 26
    3.2.4 Kinesin Stability Experiments .................................. 27
    3.2.5 Microtubule Stability Experiments .......................... 27
  3.3 Results .......................................................................... 28
    3.3.1 Kinesin Stability ................................................... 28
    3.3.2 Microtubule Stability .............................................. 28
    3.3.3 Bleaching ........................................................... 32
  3.4 Discussion .................................................................... 33
  3.5 Conclusions and Outlook ............................................... 37
4 Visualizing Molecular Shuttles 39
  4.1 Introduction .................................................. 41
  4.2 Fluorescence Microscopy ........................................ 42
    4.2.1 Overview ................................................. 42
    4.2.2 Imaging Microtubule Filaments ............................ 44
  4.3 Confocal Laser Scanning Microscopy ............................ 44
  4.4 Confocal Interference Microscopy ............................... 45
    4.4.1 Overview ................................................. 45
    4.4.2 Principles ................................................. 46
    4.4.3 Materials and Methods .................................... 47
    4.4.4 Results and Discussion ................................... 48
    4.4.5 Conclusions .............................................. 51

5 Cargo Transport Driven by Kinesin Motors 53
  5.1 Introduction .................................................. 55
  5.2 Materials and Methods ........................................ 57
    5.2.1 Microtubule Self-assembly ................................ 57
    5.2.2 Transport of Polystyrene Microspheres .................... 58
    5.2.3 Transport of Hybridized DNA ............................... 59
    5.2.4 Transport of Antibody-coated Au Nanoparticles ............ 59
  5.3 Results and Discussion ........................................ 59
    5.3.1 Microtubule Self-assembly ................................ 59
    5.3.2 Loading of Polystyrene Microspheres ....................... 60
    5.3.3 Cargo Transport via DNA Hybridization ..................... 62
    5.3.4 Loading of 40 nm Anti-biotin Conjugated Au Colloids ...... 64
  5.4 Conclusions and Outlook ....................................... 66

6 Cargo Transport in Microlithographic Channels 69
  6.1 Introduction .................................................. 71
  6.2 Materials and Methods ........................................ 73
    6.2.1 Fabrication of Micro-channels ............................. 73
    6.2.2 Cargo Transport Assays .................................. 74
    6.2.3 Gold Colloid Assembly .................................... 76
  6.3 Results and Discussion ........................................ 77
    6.3.1 Micro-channel Fabrication ................................ 77
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3.2</td>
<td>Microtubule Movement and Cargo Transport Inside Microfabricated Channels</td>
<td>79</td>
</tr>
<tr>
<td>6.3.3</td>
<td>Gold Assembly Inside the Spiral Center</td>
<td>83</td>
</tr>
<tr>
<td>6.4</td>
<td>Conclusions and Outlook</td>
<td>83</td>
</tr>
<tr>
<td>7</td>
<td>Cargo Pick-up from Engineered 2D Surfaces</td>
<td>87</td>
</tr>
<tr>
<td>7.1</td>
<td>Introduction</td>
<td>89</td>
</tr>
<tr>
<td>7.2</td>
<td>Materials and Methods</td>
<td>92</td>
</tr>
<tr>
<td>7.2.1</td>
<td>Substrate Preparation</td>
<td>92</td>
</tr>
<tr>
<td>7.2.2</td>
<td>Cargo Pick-up Assays</td>
<td>93</td>
</tr>
<tr>
<td>7.2.3</td>
<td>Image Acquisition and Data Analysis</td>
<td>95</td>
</tr>
<tr>
<td>7.3</td>
<td>Results and Discussion</td>
<td>96</td>
</tr>
<tr>
<td>7.3.1</td>
<td>Loading Stations</td>
<td>96</td>
</tr>
<tr>
<td>7.3.2</td>
<td>Pick-up Assays</td>
<td>99</td>
</tr>
<tr>
<td>7.4</td>
<td>Conclusions and Outlook</td>
<td>109</td>
</tr>
<tr>
<td>8</td>
<td>Cargo Pick-up in Engineered Micro-Channels</td>
<td>111</td>
</tr>
<tr>
<td>8.1</td>
<td>Introduction</td>
<td>113</td>
</tr>
<tr>
<td>8.2</td>
<td>Materials and Methods</td>
<td>114</td>
</tr>
<tr>
<td>8.2.1</td>
<td>Pattern Design</td>
<td>114</td>
</tr>
<tr>
<td>8.2.2</td>
<td>Fabrication of Gold Line Patterns</td>
<td>114</td>
</tr>
<tr>
<td>8.2.3</td>
<td>Fabrication of Spiral Patterns</td>
<td>114</td>
</tr>
<tr>
<td>8.2.4</td>
<td>Pick-Up Assays in Micro-Channels</td>
<td>117</td>
</tr>
<tr>
<td>8.2.5</td>
<td>Microscopy and Analysis of Pick-up</td>
<td>119</td>
</tr>
<tr>
<td>8.2.6</td>
<td>Preparation of SEM Samples</td>
<td>120</td>
</tr>
<tr>
<td>8.3</td>
<td>Results and Discussion</td>
<td>120</td>
</tr>
<tr>
<td>8.3.1</td>
<td>Fabricating Loading Stations inside Micro-Channels</td>
<td>120</td>
</tr>
<tr>
<td>8.3.2</td>
<td>Motility Assays in Microfabricated Channels Featuring Loading Stations</td>
<td>123</td>
</tr>
<tr>
<td>8.4</td>
<td>Conclusions and Outlook</td>
<td>129</td>
</tr>
<tr>
<td>9</td>
<td>Summary</td>
<td>133</td>
</tr>
<tr>
<td>10</td>
<td>Outlook</td>
<td>137</td>
</tr>
<tr>
<td>10.1</td>
<td>Optimization of Developed Platforms</td>
<td>138</td>
</tr>
<tr>
<td>10.2</td>
<td>Further Development and New Directions</td>
<td>139</td>
</tr>
</tbody>
</table>
1

Scope of the Thesis
1.1 Background and Motivation

The intriguing ability of cells to self-organize and adapt to different environments has fascinated both scientists and engineers ever since their functional principles and mechanisms have started to elucidate. Not long ago, cytoskeletal motors have emerged as a key organizing principle of biology as they actively actuate positioning and transport processes within and between cells. These motors not only support mitosis and cell division, but are also responsible for the transport of structural elements, vesicles, organelles, and even mitochondria to specific target locations inside the cell. Even large scale motions, such as muscle contraction, are powered by protein motors working cooperatively.

These biological nanomotors are able to directly convert chemical energy, derived from ATP hydrolysis, into mechanical work, thereby exhibiting high energy conversion efficiency of about 50%. Not long after discovering first molecular details of these protein motors, bioengineers became interested in exploiting these systems for technological use and investigation of biological molecular motors in a device-oriented context [1–4]. One approach uses surface-adsorbed kinesin motors that propel microtubules (MTs), which are polymerized from asymmetric tubulin proteins into structures with a diameter of 24 nm and a length of several micrometers. Concepts were developed to adopt these self-driven molecular shuttles, e.g. for sensing devices [5], nano-mechanical applications [6], or even information storage and processing [7, 8].

Many strategies have been investigated to enable micro- and nanoscale transport of cargo by molecular shuttles in vitro, e.g. for the application in a sensor device. In particular, motional control of the shuttle movement in micro-engineered environments and the specific loading of cargo were extensively studied [9–13]. Yet, most key elements of such a transport system could only be demonstrated separately in proof-of-principle experiments. For example, shuttle movement was studied in engineered micro-channels, but no data of cargo-carrying shuttles in micro-channels have been acquired yet. Also, little is known about the biocompatibility of biomolecular shuttles in man-made, micro-engineered devices.

Hence, there is critical need for quantifying the performance of molecular shuttles in synthetic environments, the development of new concepts for cargo loading and transport, and finally, the integration of these key elements into one device.
1.2. Objectives

In this thesis, we focus on finding and defining design criteria and strategies for the development of a shuttle-based transport system applicable for a biosensoric device, as illustrated in Fig. 1.1. Overall, we target the integration of the necessary key elements into one autonomously functioning system. For this purpose, we investigate the following topics:

- **Lifetime of molecular shuttles in synthetic environments**: We aim to investigate the influence of light and the use of different packaging materials on the lifetime and stability of kinesin motors and microtubule filaments.

- **Label-free imaging of molecular shuttles**: In order to prevent the use of fluorophores and concomitant problems such as photobleaching and illumination-based damaging, we seek after alternative label-free detection and imaging methods to visualize microtubule filaments.

- **Screening of appropriate cargo binding strategies**: We intend to find appropriate biological interactions to specifically capture and transport cargo by molecular shuttles.
• **Specific transport of cargo in micro-engineered channels:** We further develop the fabrication of micro-engineered channels to guide molecular shuttles and investigate the transport of cargo inside these structures.

• **Specific pick-up of cargo from loading stations:** The goal is the fabrication of spatially confined cargo pick-up zones (loading stations) and their integration into micro-fabricated channels. Such an integrated design is designated to combine the key elements of a final device (Fig. 1.1), *i.e.* pick-up and transport of cargo by molecular shuttles guided in an engineered environment.

### 1.3 Overview

In **Chapter 2**, biological motors, in particular the motor kinesin and the corresponding microtubule filaments, are introduced. Furthermore, intracellular transport is briefly reviewed. At the end of the chapter, the use of biomolecular motors and filaments for technological purposes and the concept and characteristics of molecular shuttles are described.

The lifetime of a hybrid bio-nanodevice is depending on the stability of its most fragile component. In **Chapter 3**, the compatibility of kinesin motors and microtubule shuttles in different synthetic environments is tested. In addition, the effect of light on the stability of the components was investigated. Acquired data allowed us to evaluate a benchmark for the lifetime of a molecular shuttle based device.

**Chapter 4** reviews various methods for the visualization of microtubule filaments. Besides the popular and well-established Fluorescent Microscopy, a novel interferometric imaging scheme—originally developed for the detection of small Au nanoparticles—is presented for label-free visualization of microtubules.

In **Chapter 5**, various strategies of how to specifically bind cargo to functionalized microtubules are described. Biotin–streptavidin interaction is used to demonstrate microtubule self-assembly and transport of polystyrene microsphere agglomerates and hybridized DNA. Also, transport of nanoscale gold particles *via* biotin–antibiotin interaction is shown.

Cargo transport in micro-fabricated channels is described in **Chapter 6**. A novel fabrication process of overhang micro-channels is introduced that provides full control
over the dimensions of the undercut geometry. The transport of 40 nm gold particles is characterized with respect to the curvature of the micro-channels.

Chapter 7 presents the first approach to pick-up surface-adsorbed cargo by molecular shuttles. Cargo loading stations, resistant to thermal activation, are fabricated by using contemporary self-assembly techniques. The influence of different linker chemistries on the pick-up efficiency is investigated.

In Chapter 8 cargo loading stations are integrated into micro-fabricated channels. We describe the fabrication process of such surfaces and investigate the pick-up of 40 nm gold particles by functionalized microtubule filaments guided inside the channels.

Chapter 9 summarizes the most important findings of the presented work. Further developments, as well as future directions are discussed in Chapter 10.
2

General Introduction—Motors and Machines
2.1 Synthetic Motors and Machines

The development of molecular motors and machines to control the motion of matter on a molecular level has become a research area of great interest. Just like molecular machinery evolved by nature (see next section), synthetic machines are envisioned that allow the translational and rotational movement of nanometer-scale objects for the fabrication of self-assembly or self-repairing structures. By definition, a machine is based on a motor—a device that converts energy (chemical, thermal or light) into kinetic energy in a controlled manner [15]. In the past, a plethora of different synthetic motors have been developed, i.e. brownian rotors, unidirectionally rotating molecular motors, linear molecular motors and also multicomponent mechanical machines [15]. Most of these motors are chemically or thermally activated and fueled. These different endeavors can be separated into approaches based on adapting mechanical principles from the macroscopic world to nanoscale motors, such as the molecular scissors [16] or nanocars [17], and approaches seeking to adapt principles of chemistry to achieve controllable motion at the molecular level, such as the rotaxane-based linear motor systems [18]. It was found that these synthetic motors are able to perform work and move objects. In addition, it could be shown that rotatory and linear synthetic molecular motors can be attached to surfaces without the loss of their function. Synthetic motors and machines have been reviewed and discussed extensively in two recent review articles by Kay et al. [16] and Browne et al. [15]. Even though synthetic motors and machines in principle work, their development is still in its infancy.

2.2 Biological Motors and Filaments

In the past 100 years, a vast amount of new knowledge in biology, biochemistry, and bioengineering sciences has been accumulated. This includes the identification of many molecular key players in the organization of cells. Only about 80 years ago, microtubule spindles were discovered to take part in cell mitosis. After the pioneering work on muscle physiology by Fenn and Hill between 1910 and 1940, A. F. Huxley introduced in 1957 first evidence for the existence of molecular motors (although nomenclature was different). Until today, many different motors have been identified, such as the linear molecular motors myosin, dynein, and kinesin [19, 20], the flagella motors that drive bacterial movement [21, 22] and rotating motors, such as ATP synthase [23, 24]. Linear
motor proteins specifically bind and walk on the corresponding filaments, \textit{i.e.} actin and microtubules, while transporting cargo. A common feature of biomolecular motors is the direct conversion of chemical energy into mechanical work, mediated by ATP hydrolysis. As knowledge and methods in biochemistry and biophysics have advanced, many details about the mechanisms and functions of these motors have been revealed. However, there still remain many open questions, such as how exactly a motor walks on the filament or how the binding of the motor to a specific cargo is controlled \textit{in vivo}.

Here, we focus on the linear motor kinesin and the corresponding microtubule filaments. In the following three sections, structure, function and relevance \textit{in vivo} of microtubules and kinesin are briefly reviewed. In addition, their role in the technological concept of molecular shuttles is introduced.

2.2.1 Microtubule Filaments

Microtubules (MTs) are rigid filaments, which are formed by dynamic protein aggregation (polymerization), and are essential for cell transport and cell division in all eukaryotic cells. In example, MTs form the mitotic spindle apparatus that mediates chromosome segregation during mitosis. Also, long-distance transport of organelles, vesicles, RNA and protein complexes is controlled along MT filaments. An MT consists of a hollow cylinder of tubulin subunits (mainly $\alpha$, $\beta$ tubulins) allowing the specific binding of various proteins, notably MT-binding motors, such as kinesin and dynein, and MT-associated proteins (MAP), such as MAP1, MAP2, and tau \cite{25}. The tube-like structure has an outer diameter of 25 nm and an inner diameter of 15 nm \cite{26}. MTs vary in length from fractions of micrometers to several tens of micrometers, and are, together with actin filaments, the largest known atomic structures of any biomolecules \cite{27}.

The heterodimeric subunits of MTs, composed of $\alpha$- and $\beta$-tubulin, have a molecular mass of $\sim$100 kDa and dimensions of $4 \times 5 \times 8$ nm$^3$ (Fig. 2.1 A). During MT polymerization, $\alpha\beta$-tubulin dimers associate head-to-tail forming protofilaments that later assemble \textit{via} lateral interactions between adjacent protofilaments into the hollow cylindrical MT polymers. The core structure of MTs \textit{in vivo} consists of typically 13 or 14 protofilaments that form a hollow tube \cite{30} \cite{31}. The assembly of the dimeric subunits into the protofilaments is driven by the high affinity of the tubulin-GTP dimer to the end of the MT. Due to the polarity of the $\alpha\beta$ heterodimer, MTs have a polarity resulting in different polymerization rates at both ends: the faster growing end is referred to as plus-end and the
Figure 2.1: Polymerization of tubulin dimers into MT filaments: (A) αβ-tubulin heterodimer as seen in the wall of the protofilament. The originally bound GTP inside the β-tubulin has been hydrolyzed during polymerization. Protofilaments assemble via head-to-tail association of dimeric tubulin. The polymerization is driven by GTP hydrolysis. Protofilaments assemble via lateral interactions between protofilaments into the hollow cylindrical structure of MTs. Typically, MTs consist of 13 protofilaments resulting in a outer diameter of the MT of 25 nm. Due to the different polymerization rates at the ends, MTs have a polarity: the faster growing end is referred to as the plus end, the slower growing end as the minus end (B). Dynamic instability is characterized by the coexistence of polymerizing (B) and depolymerizing (C) MTs [25]. Figure adapted from Howard et al. [28] and Steinberg [29].

slower growing one as the minus-end. During polymerization, the hydrolysis of the GTP bound to the β-tubulin is activated as soon as the α-tubulin of the incoming dimer docks to it (Fig. 2.1A). The resulting GDP-MT is very unstable and is able to depolymerize spontaneously. This property is known as dynamic instability: MT filaments are able to elongate until they stochastically switch to shrinkage (catastrophe, Fig. 2.1C). The complete disappearance of MTs, however, is often avoided by a subsequent MT elongation, an effect termed as rescue (Fig. 2.1C) [29]. The catastrophe and rescue transitions are believed to be associated with the loss and reformation of a GTP-cap at the plus end of the MT [32]. The switch between growing phase and shrinkage can happen on a time-scale of minutes, emphasizing the highly non-equilibrium mechanism underlying this process. By this means, capture of the kinetochore during the prophase of mitosis is facilitated [27]. MTs can also be considered as molecular machines themselves, since force can be generated during the dynamic assembly and disassembly process (e.g. force generation at the MT-kinetochore interface) [33–35]. These mechanisms can be disabled by stabilizing the structure of the MT polymers. This can be achieved in example by the use of non-
hydrolizable GTP analogues [36] or the adsorption of molecules, such as paclitaxel (taxol) [37, 38], into the MTs that block the normal dynamics of assembly and disassembly.

### 2.2.2 Kinesin Motors

Molecular motors known as the kinesin superfamily proteins are responsible for the transport of organelles, protein complexes and mRNA along the MT network in the cell [39]. A force-generating protein involved in MT-based motility was first discovered by Vale et al. [40] in 1985 and named kinesin. Conventional kinesin (kinesin-1) is a processive plus-end directed motor that takes 8 nm steps per hydrolyzed ATP [41, 42] exerting a force of ~6–7 pN [43]. The motor domain of the protein, consisting of two heavy chains and two light chains (Fig. 2.2 B), is followed by a short neck linker leading to the coiled-coil stalk (Fig. 2.2 A); the coiled-coil is associating with the cargo binding domain [27, 44–46]. The step size of 8 nm is the distance between consecutive binding sites along the MT protofilament, i.e. the length of the αβ tubulin dimer. These steps are driven by the hydrolysis of ATP, which causes a structural change in the kinesin motor that drives motility [47] (Fig. 2.3 i.–viii.): after the motor head has collided with the binding site on the MT protofilament (i.), ADP is released from the bound motor head (ii.). The subsequent binding of ATP to the bound forward head causes the mentioned structural change (neck linker docking), whereupon the rear head moves towards the plus-end where it promotes binding at the next site on the protofilament (iii–vi.). Upon ATP hydrolysis in the rear head (vi.) and following phosphate release (vii.), the rearward head detaches (viii.) and the motor has taken one step [48]. Current opinion supports an asymmetric hand-over-hand walking mechanism of the motor [49–51]. The rate of ATP hydrolysis is on the order of ~100 s⁻¹ (about 50 s⁻¹ per head), resulting in a speed of 500–1000 nm s⁻¹ [52]. Single kinesin motors move over a distance of 1–2 µm along the MT before dissociating [53]. In the native situation, this limitation is overcome by the attachment of multiple motors per cargo, increasing the travel distance up to ~1 m (as necessary in the longest axons) if only 10 motors cooperatively drive the movement [54].

### 2.2.3 A Few Words About Intracellular Transport

In cells, the active transport system based on motors and filaments is responsible to deliver synthesized proteins encapsulated in vesicles and various types of organelles to specific
target regions \[56, 58\]. Starting from the centrosome, the main MT organizing center (MTOC) close to the nucleus of a cell, MTs spread into the cell providing tracks for motor proteins from the kinesin and dynein family (Fig. 2.4A). The cargo-binding motifs of these motors specifically bind to ligands incorporated in the surface layers of the cargo. However, few is known about the receptors and mechanisms that target specific types of cargo. It has been proposed that the existence of different isoforms of a particular motor, e.g. kinesin-1, allows binding of the same motor to many different cargos \[45\]. There is evidence, that the variable C-terminal region of the kinesin light chain plays an important role not only in the activation of folded inactive kinesin-1, but also in targeting different cellular structures \[45\]. While conventional kinesin is a plus-end directed motor (Fig. 2.4B), mainly transporting cargo from the cell center to the periphery (Fig. 2.4A), dynein motor proteins are responsible for minus-end directed transport. In example, pigment granule positioning in fish melanophores or lipid droplet movement in \textit{Drosophila melanogaster} are controlled by the cooperation of different motors \[59\]. However, the presence of different motors causes the cargo to move bi-directionally, reversing course every few seconds \[56\]. Different models have been proposed to describe activation and inactivation of plus- and minus-end directed motors on the same cargo. Very likely is the coexistence of different motors on one cargo, and that opposite polarity of the motors is coordinated to avoid interference with each other’s function \[56\]. The mechanism of such coordination is still unknown. Malfunctioning intracellular transport might be linked to diseases such as Alzheimer’s, ciliary dyskinesias or retinitis pigmentosa \[60\]. Furthermore, viruses are able to hijack the transport system: viral cores utilize the MT network to be transported to the vicinity of the MTOC, where they set up replication, and later
2.2. BIOLoGICAL MoToRS AND FiLiMENtS

Figure 2.3: Stepping model for conventional kinesin along a MT protofilament: binding of the motor to the MT (i.) causes the release of ADP (ii.), leaving space for ATP to bind and cause neck linker docking (iii.). After a diffusional search (iv.), the second ADP is released, forming an intermediate with both heads tightly bound to the protofilament and the neck linkers strained (v.). Before ATP binding on the forward head can occur again, ATP hydrolysis and product release precede (vi. and vii.). The motor has taken one step and is back to its original position (viii.). Figure adapted from Valentine and Gilbert [44].
Figure 2.4: (A) Scheme of a cell, showing the radial organization of the MT network originating from the MT organizing center (MTOC) near the cell nucleus. Also, some examples of bi-directionally moving cargo are shown (reprinted from Gross [56]). (B) Electron micrograph of kinesin transporting an organelle along a MT (reprinted from Hirokawa [57]). The scheme highlights the situation as observed in the electron micrograph. Most kinesin motor proteins (such as conventional kinesin) move from the minus- to the plus-end of the MTs at a maximum speed of about 1 µm s⁻¹.
2.3 From Filaments to Shuttles

2.3.1 Standard Motility Assay

Standard \textit{in vitro} motility assays transfer the native transport system based on molecular motors into a synthetic environment. This setup not only qualifies for biotechnological use, but also for biophysical studies of the motor proteins. In the assay, MT filaments are immobilized on functionalized (glass) substrates, and motors bound to cargo bind and start to walk on them (Fig. 2.5A). Different techniques are applied to anchor MTs to a substrate: besides direct adsorption to the substrate, tethers such as PLL [62], polysaccharides [63] or also kinesin [64–66] are used. Often, especially if kinesin is involved in tethering, the protein structures are chemically fixed, \textit{e.g.} glutaraldehyde [63], while motility is preserved. For technological use, assays were developed to allow unidirectional transport of cargo. For that purpose, MT filaments were aligned in an inverted motility assay (see next section) by a flow field and then chemically fixed and used as tracks for motor-coated cargo [62, 64–66]. These types of assays could for example mimic the transport and positional control of pigments, as proposed by Doot \textit{et al.} [67].

2.3.2 Molecular Shuttles—The Inverted Motility Assay

As implied by the name, the inverted motility assay turns the natural system upside down, \textit{i.e.} kinesin motor proteins are immobilized onto a surface and MT filaments are thereupon actively propelled, inevitably reminiscent of a nanoscale train system (Fig. 2.5B) [68–70]. From a technological point of view, this system has at least two significant advantages over the standard motility assay: (1) As the motor density present on the surface is of the order of \(\sim 100 \mu \text{m}^{-2}\), detachment rates of micrometer long MTs are considerably lower than in standard motility assays where motors are attached directly to the cargo. This ensures low loss-rates of shuttles. (2) Directional control of shuttle movement can be easily achieved by microengineering the surface and is thereby an intrinsic property of the system. In standard motility assays, surface-immobilized polar MTs have to be first properly aligned to enable cargo transport into the desired direction.
2. GENERAL INTRODUCTION—MOTORS AND MACHINES

2.5. Inverted Motility Assay

A. Motility Assay

- Cargo
- Motor protein (kinesin)
- Filament (microtubule)
- Surface anchors
- Substrate surface

~25 nm

B. Inverted Motility Assay

- Flow cell
- Spacer
- Substrate surface
- Casein
- Motor protein (kinesin)
- Filament (microtubule)

~25 nm

Figure 2.5: (A) In standard motility assays, MTs are immobilized on a substrate surface, either directly or via suitable surface anchors, i.e., crosslinked kinesin motors or other specific interactions with functionalized MTs. MTs can be aligned and directed by using flow [71] or electric fields [72, 73]. Cargo can then be transported by attached kinesin motors along the MT filaments. (B) Inverted motility assay: MT filaments are propelled by surface-adsorbed kinesin. A passivating casein layer prevents denaturation of the kinesin motors on the surface. MTs (also referred to as molecular shuttles) move at maximum speeds of \(~800–900\) nm s\(^{-1}\). Typically, MTs are labeled with fluorescent dyes (e.g., rhodamine) for visualization in fluorescent microscopy. Alternative methods for MT visualization are described in Chapter [4]. Tubulin building blocks of the filaments can also be functionalized with biological recognition sites, e.g. biotin.
Components are sequentially injected into a flowcell, consisting of a bottom glass slide, double-coated tape as spacer, and the substrate surface as top (Fig. 2.5 B). A primarily adsorbed layer of casein proteins prevents the motor proteins form denaturing on the substrate surface, which would result in a loss of motility. Finally, MTs are injected into the cell and start being propelled by the kinesins. The distance between substrate surface and kinesin head bound to MT filaments was determined to be $17 \pm 2 \text{ nm}$ [74].

MTs, being the most fragile component of the system (see Chapter 3), are usually stabilized using paclitaxel[2], a drug used in the treatment of cancer. Paclitaxel stabilizes MTs by binding to the $\beta$-subunits of the heterodimeric tubulin building blocks of MTs, counteracting the effects of GTP hydrolysis on the other side of the monomer [37]. Stabilized MTs utilized in inverted motility assays are usually stable for a couple of hours (again, see Chapter 3) [75, 76].

As outlined before, directional control over the movement of MT filaments is crucial for the realisation of a transport system destined to move cargo from point A to point B. In first attempts, kinesin motors were adsorbed in a linear arrangement onto mechanically deposited PTFE films [77, 78]. This caused a mainly linear movement of MT filaments along the adsorbed kinesin. While this is a simple method to guide MT movement by means of confinement of kinesin, it does not allow unidirectional movement of the shuttles. In addition, filaments guided in this manner are prone to lose their contact to the motor when heading towards motor-depleted areas. Recent research is therefore focusing on the development of more appropriate ways to unidirectionally guide and concentrate molecular shuttles by using micro-patterning approaches (see Chapter 6 for a more detailed review) [5, 10, 79–83].

Chemical modification of the MTs allows to specifically bind targeted cargo. In first studies, biotin–streptavidin (SA) interaction was used to specifically bind SA-coated microspheres to biotinylated MTs [6, 14]. More advanced systems use antibody–antigen interaction to load and transport viruses [84]. Of particular technological interest are the possible interactions between cargo-carrying shuttles, as they could limit length and directionality of transport, both crucial parameters in a final device [11, 85]. MT-based cargo transport and pick-up will be discussed in more detail in Chapters 5, 6, 7, and 8.

---

1determined for kinesin-1
2commercial product name: Taxol®
2.3.3 Characteristics of Shuttle Movement

Kinesin motors attached to a surface interact with MT filaments suspended in the motility solution. Once attached to a motor, the MT filament starts to move at a speed that depends on the ATP concentration in the solution (Fig. 2.6A). Apparently, one kinesin motor is sufficient to move an MT for several micrometers as could be shown by Howard et al. [69]. This hypothesis is consistent with the finding that MT speed is independent on the kinesin density on the surface over a wide range (Fig. 2.6B). The study also showed that a threshold density (∼5000 motors µm$^{-2}$) of kinesin on the surface is needed for MTs to attach and move, when kinesin was directly adsorbed. If the surface was pretreated with tubulin and cytochrome c (a small 12 kDa protein), no threshold was observed and MTs attached at much lower motor densities. Consequently, it has to be assumed that pretreatment prevents denaturation of kinesin motors on the surface, meaning that already at low motor densities enough active motors are present to bind and propel MT filaments. In our experiments, surfaces are pretreated with casein proteins that likewise prevent denaturation of kinesin motors on a surface.

As the motors are statistically distributed on the surface and because the head-linkers of the motors are very flexible, the resulting motion of filaments attached to the kinesins is of statistical nature, too. At high motor density, MTs are propelled over several micrometers before eventually unbinding. Surprisingly, MT filaments are even unidirectionally moved when motor proteins of opposite polarity, i.e. plus-end (kinesin) and minus-end di-
rected motors (dynein), are co-adsorbed on the surface [86], thereby continually switching between kinesin-directed and dynein-directed transport. Occasionally, the uniform gliding of the filaments is disrupted by defects in motion. For example, MTs get pinned at their leading ends to the surface. This causes the MT to buckle and, due to the motors pushing the MT towards the point-defect, to rotate around the pinned end. Although, these spiral defects have been studied [87][88], it is unclear what causes them. Potentially, surface roughness and inactive motors that remain in rigor play a role in this phenomenon. With increasing motor density on the surface, the diameters of these rotating MTs is decreasing down to only several microns. Compared to the persistence length of MTs in solution (∼5 mm [89]), these small radii of curvature illustrate the co-operating force that motor proteins can exert to the MTs (each kinesin exerts ∼5 pN of force) compelling the filaments to bend.
3

Lifetime of Microtubules in Hybrid Bio-Nanodevices
Abstract

Prolonging the lifetime of biomolecules in their functional states is critical for many applications where biomolecules are integrated into synthetic materials or devices. A simplified molecular shuttle system, which consists of fluorescently labeled MTs propelled by kinesin motor proteins bound to the surface of a flowcell, served here as a model system to probe the lifetime of a hybrid device. In this system, the functional decay can easily be assayed by utilizing optical microscopy to detect motility and disintegration of MTs. We found that the lifetimes of these hybrid systems were mainly limited by the stability of MTs, rather than of kinesin. To determine the biocompatibility of polymers widely used in microfabrication, we assembled flowcells with glass bottom surfaces and covers fabricated from glass, PU, PMMA, PDMS, and EVOH. Without illumination, only PU had a substantial negative impact on MT stability, while PMMA, PDMS and EVOH showed stabilities comparable to glass. Under the influence of light, however, the MTs degraded rapidly on PDMS or PMMA, even in the presence of oxygen scavengers. A similar effect was observed on glass if oxygen scavengers were not added to the medium. Strong bleaching of the fluorophores was again only found on the polymer substrates and photobleaching coincided with an accelerated depolymerization of the MTs [75].

3.1 Introduction

Interfacing biological molecules and supramolecular assemblies with the synthetic world is critical to many applications in nanotechnology [90–92]. A particularly exciting class of such hybrid devices utilizes biomolecular motors, which can add active, chemically powered force generation and movement to the functionality of the device. Applications of devices based on biomolecular motors have been explored for nanoscale transport systems (molecular shuttles) [1, 77, 93], surface imaging [7], force measurements [6], single molecule manipulation [94], and lab-on-a-chip systems [82]. These studies have proven the feasibility of utilizing motor proteins, such as kinesin, and the specific “roads” supporting their movements, such as MTs, in synthetic environments for a variety of technological purposes. A number of studies have discussed specific fabrication approaches and designs [10, 14, 66, 79, 80, 95–99] and determined critical parameters of performance [71, 72, 100–103]. Here we investigated how motor proteins and their associated filaments, serving as models of biological nanomachines and supramolecular structures, cease to function over time in the presence of several synthetic polymer materials. Furthermore, since the read-out schemes of micro- and nanodevices are often based on optical detection, we studied their functional decay in the presence or absence of light.

The longevity of biological components in synthetic environments is crucial for the commercialization of hybrid devices and materials, but biomolecules function only in a narrow band of environmental conditions. The degradation of a protein and its concomitant loss of function can be induced, for example, by temperature changes, the action of protease contaminants, oxidation, and interaction with synthetic surfaces. Additional stresses can be exerted by intense illumination, which is often used to detect fluorescence signals. Motor proteins, such as kinesin, myosin, or F1-ATPase, seem particularly prone to degradation, since they are assembled from multiple subunits, have to undergo large conformational changes, and achieve specific binding states as they move in steps along their supporting filaments [19]. The supporting filaments, polymerized from thousands of individual proteins, have evolved to exhibit a fine-tuned balance between assembly and disassembly within cells [104]. Various strategies exist to slow the rate of disassembly in vitro, either by addition of inhibitors [105], polymerization with non-hydrolyzable GTP analogues [106], or chemical fixation [63], however these complex supramolecular structures remain sensitive to depolymerization.

In hybrid devices, the synthetic materials themselves often introduce additional challenges for proteins. Immediate loss of motor protein function upon adsorption has been
reported for a number of surfaces, and a significant effort has been devoted to finding surfaces that permit micropatterning but also support motor function after adsorption \([2, 9, 78, 107-109]\). Now a number of suitable photoresists, which can be used to pattern a glass surface covered with active motor proteins, have been identified.

However, not only the material of the surface directly in contact with the motor protein, but also the material properties of surfaces in contact with the buffer solution can affect the lifetime of the proteins. For example, PDMS, which is widely used as a biocompatible material \([37]\), has been reported to be incompatible with motor protein activity \([110]\). Because polymers may release substances into the motility assay which can affect its performance, even if the motor proteins or the MTs have no direct contact with their surfaces, the performance as housing material has to be tested. A crucial example is oxygen effusion from the material into the solution, as will be shown here.

The aims of this study were: (1) to define a benchmark for the lifetime of a hybrid device integrating kinesin motor proteins and MTs, and (2) to test a variety of polymeric materials for their compatibility with kinesin motor proteins and MTs. For the definition of a benchmark, we have chosen an all-glass synthetic environment as widely used in biophysical experiments for gliding motility assays \([69]\). For the materials screen, we used poly(dimethylsiloxane) (PDMS), poly(methyl-methacrylate) (PMMA), a photocurable poly(urethane) (PU), and an ethylene-vinyl alcohol copolymer (EVOH) (Fig. 3.1).

Our results show that the typical lifetime of a hybrid device integrating kinesin motors and MTs is on the order of hours, and determined by progressive depolymerization of MTs. In contrast, kinesin motor activity can be observed over 1–2 days. The depolymerization of fluorescently labeled MTs is accelerated by exposure to intense light. While the tested polymer materials perform almost as well as glass in the absence of intense illumination exciting the fluorescent labels of the MTs, housings made from PMMA and in particular PDMS cause rapid depolymerization of MTs under light exposure, even in the presence of oxygen scavengers.
3.1. INTRODUCTION

Figure 3.1: Experimental setup for evaluation of the lifetime of hybrid nanodevices that integrate kinesin motor proteins and MTs with different synthetic environments. Kinesin motors are dimeric proteins that are organized into two MT-binding “heads” and a cargo binding “tail”. MTs are directional polymeric structures assembled from thousands of asymmetric tubulin proteins into tubes with an outer diameter of 30 nm and a length of many micrometers. Kinesins hydrolyze ATP to walk towards the so-called “plus” end of the MT while generating a pulling force of several piconewtons. In our devices, kinesin motors are adsorbed to a surface pre-coated with casein with a density of several hundred motors per $\mu$m$^2$. MTs functionalized with fluorescent labels (rhodamine fluorophore) glide across this kinesin carpet with a maximum velocity of 800–900 nm s$^{-1}$. The flowcell consists of a glass coverslip serving as an optically transparent bottom surface, spacers with a height of 100 $\mu$m, and a glass or polymer cover.
3. Materials and Methods

3.2.1 Experimental Setup

As illustrated in Fig. 3.1, all setups were assembled using an optically transparent glass cover slip as bottom surface and the respective cover surface (glass or one of the four different polymers). Double-coated tape—ca. 100 µm in thickness—was used as spacer to build the flow channel. PDMS plates were made by mixing Sylgard®184 base:curing agent at a ratio of 10:1, degassing the mixture under vacuum and curing it for 6 h at 70°C. UV-curable PU was polymerized for 24 h at a wavelength of 365 nm using a Spectroline Q-22SNF UV lamp. PMMA was obtained from CYRO Industries. The EVOH with an ethylene content of 29 mol% was generously provided by Soarnol. The thickness of the polymer plates ranged from 3–4 mm. For all-glass flowcells, Fisher Scientific slides replaced the polymer cover.

3.2.2 Kinesin and Microtubules Preparation

The expression and purification of the wild-type D. melanogaster conventional kinesin gene construct with a histidine C-terminal tag and its purification by Ni²⁺ affinity column are described elsewhere [42]. Tubulin was polymerized as follows: BRB80 buffer (80 mM PIPES, 2 mM MgCl₂, 1 mM EGTA, pH 6.85 with KOH) containing 4 mM MgCl₂, 1 mM GTP and 5% DMSO was added to a 20 µg aliquot of rhodamine labeled tubulin to yield a final tubulin concentration of 3.2 mg ml⁻¹. After polymerization for 30 min at 37°C, the MTs were diluted 100-fold in BRB80 containing 10 µM taxol as stabilizer.

3.2.3 Motility Assay

If not stated otherwise, motility assays were prepared as follows: A flowcell was filled with a solution of 0.5 mg ml⁻¹ casein in BRB80 buffer to pre-coat the surfaces with

---

1Fisherfinest™, Premium Cover Glass, No. 1, Fisher Scientific
2Scotch, 3M, St. Paul, MN
3Dow Corning
4NOA 73, Norland Products, Cranbury, NJ
5Spectronics, Westbury, NY
6Soarus LLC, Arlington Heights, IL
7Fisher-finest premium slides
8TL331M, Cytoskeleton, Denver, CO
3.2. MATERIALS AND METHODS

protein and to reduce kinesin denaturation. After 5 min, the casein solution was exchanged against a kinesin solution (∼40 µg ml⁻¹ kinesin, 0.2 mg ml⁻¹ casein, 1 mM ATP in BRB80). Five minutes later a motility solution, containing 3.2 µg ml⁻¹ rhodamine labeled MTs (1–20 µm in length), 1 mM ATP and stabilized by 10 µM taxol with oxygen scavenging additives (20 mM D-glucose, 20 µg ml⁻¹ glucose oxidase, 8 µg ml⁻¹ catalase, 1% dithiotreitol) was added. To allow casein and kinesin to adsorb to the surface, the first two solutions were sequentially kept for 5 min in the flow channel before the motility solution was filled in. Images (1 s exposure) of rhodamine-labeled MTs gliding along the surface were captured using an epi-fluorescence microscope with a 100 W Hg lamp, a 60 × oil objective (NA = 1.4) and a cooled CCD camera. Reduced light intensity measurements were performed using two neutral density filters (ND-4, ND-8). All measurements were carried out at room temperature.

3.2.4 Kinesin Stability Experiments

In order to investigate the lifetime of the kinesin, the preparation of the motility assay was slightly modified. After pre-coating the flow channel with casein (0.5 mg ml⁻¹ in BRB80) for 5 min, a kinesin solution (∼40 µg ml⁻¹ kinesin, 0.2 mg ml⁻¹ casein, 1 mM ATP stabilized by 10 µM taxol with oxygen scavenging additives (see Section 3.2.3 in BRB80) was added. Flowcells were thereafter stored at room temperature in a sealed Petri dish for 6, 16, 24, and 37 h. After the respective time, the motility solution was added and the sample was characterized under the microscope as described above. Kinesin stability studies were only carried out in the all-glass flowcell setup.

3.2.5 Microtubule Stability Experiments

Motility assays for MT stability studies were prepared as described in Section 3.2.3. A new flowcell was assembled for each time point (0.5, 1, 2, 4, 6, 8 and 16 h) and cover material (glass, PDMS, PU, EVOH, PMMA). The flowcells were sealed with immersion oil to prevent evaporation and kept in a dark, humid environment. Since the immersion oil and the polymer spacer cover less than one percent of the buffer/flowcell interface, we assume that they do not affect the lifetime of the device. Time-lapse movies of the samples

9 Eclipse TE200, Nikon
10 Roper Cascade
were recorded in the following sequence: (1) On both surfaces—glass and polymer—time-lapse movies were recorded to check the motility of the MTs. In order to minimize the influence of the fluorescent illumination, 10 frames were recorded with a time interval of 5 s and an exposure time of 1 s for each frame using the automatic shutter. Data were recorded at three different spots in the flowcell. (2) To investigate the influence of the light on the MTs motility, the field of view on the glass cover slip surface was continuously illuminated. A maximum of 241 frames was recorded in time intervals of 5 s (equal to 20 min of exposure). For image analysis, the moving MTs on the glass surface were counted manually and their lengths were measured. Only moving MTs completely in the field of view were considered for counting and measurement of length. The fluorescence intensity was analyzed via “line-plot” analysis determining the MT intensity and the background intensity in a line scan perpendicular to the MT. The background, which scaled with the MT intensity decrease, amounted to about 60% of the absolute MT intensity. The intensity for every data point was obtained by the average of four different line analyses of the same time frame image. In order to test the system under absence of the antifade system, experiments were performed without D-glucose and glucose oxidase in the motility solution.

3.3 Results

3.3.1 Kinesin Stability

The stability of the kinesin protein was tested in the glass setup only. Even after 37 h, the MTs showed excellent motility. Under a continuous light exposure for 20 min following the dark storage, no increase in degradation or further decrease in motility has been observed.

3.3.2 Microtubule Stability

MT stability was investigated in flowcells with a glass bottom surface and covers fabricated from glass or four different polymers. At first, all flowcells were stored without exposure to light but under full operational conditions, e.g. the MTs were mobile in the presence of ATP. We compared the stability of the MTs in these different environments by analyzing characteristic properties, such as the changes of number and length of MTs, as
3.3. RESULTS

well as the decrease of fluorescence activity as a function of storage time. Furthermore, we investigated the impact of the light exposure, unavoidable in fluorescence microscopy, on these properties, \textit{i.e.} number of MTs, length, and fluorophore bleaching. Fig. 3.2 shows the number of MTs in the field of view over time for samples stored in the dark. On glass, the number of moving MTs initially increased, reaching a maximum after 2–4 h, and then decreased to less than 20% of the maximal count after 16 h (Fig. 3.2a). After 2–4 h, substantially more short MTs (1–5 $\mu$m in length) were observed with respect to the count after 30 min, thus explaining the initial increase of MTs. The decay in MT number on EVOH, PMMA, and PDMS was comparable to glass (Fig. 3.2b, c, and d), but only PMMA showed the initial increase. On PU practically no MTs were observed after 4 h dark storage time (Fig. 3.2e).

The impact of light illumination on the moving MTs was studied for flowcells which have been previously kept in the dark for one hour to permit sufficient time for the initial adsorption of MTs from the solution to the surface. Fig. 3.3 and 3.4 illustrate the dependence of the MT-numbers on continuous irradiation for all setups. Plotted in Fig. 3.3 is the function of the MT-number over time, while Fig. 3.4 shows the corresponding microscopy images. The decay curve for the PDMS setup in Fig. 3.3 was acquired by using a 32-fold lower light intensity. At full intensity, no motility was observed and complete depolymerization occurred after 30 s (Fig. 3.4 bottom row). Also plotted in Fig. 3.3 is the number of MTs as a function of time for the glass assay at this reduced light intensity, but without using the oxygen scavenger substances D-glucose and glucose oxidase. Omitting these substances led to complete depolymerization of all MTs in the field of view within $\sim$40 s at full intensity (100 W mercury arc lamp) of the fluorescent excitation (not shown here). At reduced light intensity, motility was observed and degradation slowed (movie), \textit{i.e.} about 90\% of all MTs stopped moving after 60 s and started to depolymerize until complete depolymerization occurred after 150 s (Fig. 3.3). Fig. 3.3 also shows that illumination does not affect MTs in flowcells assembled from glass, EVOH, or PU for at least the first 20 min, but causes the number of MTs to decrease rapidly in flowcells with PMMA covers and even faster in flowcells with PDMS covers (PDMS movie). The striking similarities of the decay for the glass setup without oxygen scavengers provides a hint on the origin for this dramatic effect on PDMS and will be discussed further below. Pre-cleaning of the PDMS samples in deionized water or hexane did not improve the motility and stability of the MTs in the PDMS flowcells. By rinsing the flowcell with BRB80 buffer, a slight improvement of the stability of the remaining MTs could be observed, \textit{e.g.} the time of complete depolymerization increased from $\sim$30 s to $\sim$120 s.
Figure 3.2: Decay of the number of MTs for the five different flowcell setups with dark storage time. MTs were counted on the glass surface of all flowcells. Two separate data sets for each setup at identical conditions are plotted (different symbols in plots). The error bars represent the square-root values of Poisson-distributed MT numbers. With the exception of PU (e), all setups showed similar rates of MT loss. An initial increase in MT number was observed for the glass (a) and PMMA (c) setups and is attributed to the breaking of longer MTs moving on the surface. Lines to guide the eye.
3.3. RESULTS

Figure 3.3: The number of MTs as function of illumination time in flowcells with different cover materials. The number of MTs is normalized to the initial value at 0 s (lines to guide the eye). While in the flowcells with glass, EVOH, and PU cover MTs are not damaged due to the exposure of light from a 100 W mercury arc lamp, MTs in flowcells with a PMMA cover disintegrated within 400 s. MTs in flowcells with a PDMS cover, or flowcells with glass covers and disabled oxygen scavenging system showed no movement and extremely rapid disintegration. In order to count moving MTs in these flowcells, the light intensity was reduced 32-fold using neutral density filters.

For PMMA, complete depolymerization occurred under continuous illumination after 5–10 min for all storage times (Fig. 3.4). Many events have been observed in which MTs stopped moving before they fell apart. However, events were observed where MTs collapsed and the fragments were still moving on the surface. Fig. 3.5 illustrates such an event after an exposure time of 6 min. A MT, moving from the lower left corner into the field of view, broke apart into two proceeding fragments. In a second event, both fragments were cleaved again into overall four fragments that were still moving (movie). Interestingly, the breaking events are correlated with the change of direction of the particular moving MT on the surface.

The MT stability results are summarized in Table 3.1. The half-life values for MT stability without any light exposure were taken from Fig. 3.2. Under illumination, the stability was good for glass, EVOH and PU, but depolymerization occurred on PDMS and PMMA. It was also observed that the stability of moving MTs was very similar on the glass and the polymer surface for the PU, EVOH, and PMMA. The motility of the MTs on the glass, PU, PMMA, and EVOH surfaces of all flowcells was good in the absence and presence of light. For PDMS, the motility was zero under intense illumination, but initially good on the glass surface when filters were used to lower the light intensity by a factor of 32.
3.3.3 Bleaching

Fig. 3.6 shows the fluorescence intensity as a function of light exposure. The left graph shows the decay of the MT-unstable setups PDMS, PMMA, O$_2$-scavenger-free glass, and for comparison the fit obtained for the decay of the “MT-stable” setups: all-glass, PU, and EVOH (Fig. 3.6 right). For glass, PU and EVOH, the decays are very similar, showing a half-life of about 300 s. As mentioned above, PDMS, and PMMA show total MT-disintegration after 30 s and 300 s, respectively. However, the fluorescence decreases also faster than on the ‘MT-stable’ setups, indicating a link between bleaching and depolymerization. In addition, we note that the timescale for fluorophore photobleaching of the O$_2$-scavenger-free glass setup is comparable to the bleaching timescale on the PDMS setup.
3.4 Discussion

The lifetime of a device is determined by the lifetime of the most fragile component, and similarly the biocompatibility of a material depends critically on its interaction with the least stable biological component. Hybrid nanodevices that integrate extremely fragile biological nanomachines do not only require the determination of the biocompatibility of typical synthetic materials for their own sake, but can also serve as test systems (“canaries in the coalmine”) for the assessment of biocompatibility of materials in a wide range of technological and biomedical applications.
3. Lifetime of Microtubules in Hybrid Bio-Nanodevices

Table 3.1: Stability and motility of MTs in different flowcell environments. Except for the motility on the polymer surfaces (last column), all observations were made on the glass cover slip surface of the setups. Flowcells with PDMS cover showed no motility under full illumination, but MTs were moving on the glass surface under 32-fold reduced light levels during the first minute of illumination. At this low illumination, it was impossible to visualize MTs on the surface of the PDMS cover.

<table>
<thead>
<tr>
<th>Setup</th>
<th>Half-life of MT decay (no light)</th>
<th>MT stability under illumination</th>
<th>Motility on glass</th>
<th>Motility on polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>glass</td>
<td>10</td>
<td>stable</td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td>PMMA</td>
<td>8</td>
<td>unstable</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>PDMS</td>
<td>8</td>
<td>extr. unstable</td>
<td>yes*</td>
<td>-</td>
</tr>
<tr>
<td>EVOH</td>
<td>8</td>
<td>stable</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>PU</td>
<td>2</td>
<td>stable</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

*depending on light intensity

In our study, we first assessed the degradation mechanisms of motor protein-driven transport of MTs in a flowcell assembled only from glass. Glass is a material with a long history of application in in vitro reconstitution of kinesin-driven transport of MTs on surfaces, and serves here as a reference material. Kinesin motors can be bound to a glass surface pre-coated with a layer of casein. Our experiments show that the kinesin carpet on the surface is able to transport freshly added MTs for a period of at least 37 h, even when the flowcell is stored at room temperature (Fig. 3.1). From these observations, it is difficult to determine the lifetime of an individual motor, since the initial motor density is on the order of 1000 motors per $\mu m^2$ (assuming complete binding of the motors in the kinesin solution), while the presence of only one functional motor per $\mu m^2$ can lead to observable binding [112].

However, the stability of the fluorescently labeled MTs, which are transported by the kinesin motors, is even smaller and thus limiting the device’s lifetime. In a dark glass flowcell, the number of MTs moving on a kinesin-coated surface increases in the first four hours after introduction of the MTs into the flowcell and then decreases to half the maximum number after 10 h, and to less than 20% of the maximum number after 16 h (Fig. 3.2). The ability of a hybrid molecular shuttle system to efficiently transport cargo will be therefore severely impaired by the loss of MTs rather than by the degradation of...
Figure 3.6: Fluorescence intensity (after background subtraction) of rhodamine-labeled MTs under full illumination as a function of time. MTs in flowcells with glass, PU, and EVOH covers show a similar rate of photobleaching, losing 50% of the initial brightness within $\sim 300$ s. In the flowcells with PMMA and PDMS covers, photobleaching occurred at a much higher rate. Lines are to guide the eye.

The initial rise and subsequent decay in the number of taxol-stabilized MTs indicates that more than one of the degradation mechanisms “breakage”, “shrinkage”, and “catastrophic disintegration” is acting. We attribute the initial rise to breakage of MTs moving on the surface. This is supported by the observation of actual breakage events (Fig. 3.5), and by measuring the length distribution of MTs 0.5 h and 4 h after introduction into the flowcell. The average length of MTs drops from $5.6 \mu m$ after 0.5 h to $4.0 \mu m$ after 4 h, indicating that a 50% increase in number is accompanied by a similar reduction in average length. “Catastrophic disintegration”, meaning simultaneous breaking at multiple sites and rapidly vanishing fragments is frequently observed as the endstage of photodamage, but does not necessarily account for the reduction in the number of MTs in the dark. More likely is a combination of “breakage”, which reduces the average length of MTs, and “shrinkage” at the ends, which leads to the disappearance of very short MTs, to account for the reduction in MT number.

Added stress can be exerted on the fluorescently labeled MTs by illuminating them through a high-powered objective ($60 \times$ magnification, $NA = 1.4$) with the intense light emanating from a 100 W mercury arc lamp. Vigers et al. [113] have shown previously that the excitation of the fluorophore leads to rapid dissolution of the MTs. We could reproduce this observation if the oxygen scavenging mixture in our solution was disabled.
Table 3.2: Tabulated oxygen permeabilities $P$ (taken from different sources) of the polymers used in this study.

<table>
<thead>
<tr>
<th>Material</th>
<th>$T$ [°C]</th>
<th>$P \times 10^{13}$ [cm$^2$s$^{-1}$Pa$^{-1}$]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS</td>
<td>35</td>
<td>700.0</td>
<td>[115]</td>
</tr>
<tr>
<td>PU</td>
<td>25</td>
<td>0.9</td>
<td>[116]</td>
</tr>
<tr>
<td>PMMA</td>
<td>34</td>
<td>0.1</td>
<td>[115]</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.07</td>
<td>[115]</td>
</tr>
<tr>
<td>EVOH</td>
<td>20</td>
<td>0.0002</td>
<td>[117]</td>
</tr>
</tbody>
</table>

(Fig. 3.3). In contrast, removal of dissolved molecular oxygen through the enzyme-based oxygen scavenger significantly extended the lifetime of the MTs and the fluorescent labels under illumination. After 20 min of constant illumination, the brightness of the MTs decreased by roughly a factor of ten (Fig. 3.6) but the number of MTs was unchanged (Fig. 3.3), demonstrating that the oxygen scavenger succeeds in reducing photobleaching of the dye and eliminating the stress on the MT structure in the all-glass flowcell.

While an all-glass flowcell assembled from a glass coverslip and a glass cover has been the setup of choice for many successful biophysical studies, the emergence of microfluidics inspires a new experimental approach, which combines a patterned glass coverslip controlling motor-driven movement of MTs and a cover with microfluidic channels for the controlled delivery of fluids. The low-cost fabrication of the cover and straightforward integration with the coverslip into a flowcell is best achieved by soft-lithography methods [114], requiring a polymeric material. PDMS, the current default choice for such a material, has been reported to be incompatible with kinesin-driven MT movement, and it has been speculated that the high oxygen diffusivity and solubility of PDMS is responsible for this [110]. We therefore set out to compare the biocompatibility of PDMS with the biocompatibility of PMMA, PU (two popular materials for microfabrication), and EVOH, a polymer with extremely low oxygen diffusivity (commonly used for food packaging). The oxygen permeabilities of these materials, compiled from different sources, are listed in Table 3.2.

In the absence of intense light, flowcells with a glass coverslip and a PDMS, PMMA, or EVOH cover supported MT motility almost as well as an all-glass flowcell. The lifetime of MTs had decreased only slightly compared to a flowcell with a glass cover, and the kinesin ‘carpet’ still supported the smooth movement of the remaining MTs. The
Conclusions and Outlook

Cover surface was typically covered with only a tenth of the MTs adsorbed to the glass coverslip, but the movement of the adsorbed MTs was unaffected. This situation seems advantageous for the envisioned devices, where MT movement is desired primarily on the patterned coverslip surface, but a permanent immobilization of MTs on the cover surfaces should be avoided.

The presence of the fluorescence illumination changed the situation dramatically. Even though the oxygen scavenger was active, illumination lead to rapid photobleaching of the fluorescent labels (Fig. 3.6) and complete disintegration of the MTs (Fig. 3.4) in the PDMS flowcell. This is surprising, since PDMS has been previously used in microfluidic chips, which integrate fluorescence measurements [118, 119], and has been shown to have excellent biocompatibility in cell patterning and protein stamping applications [120]. The observations in the PDMS flowcell parallel the observations in an all-glass flowcell without oxygen scavenging, confirming that molecular oxygen released from the PDMS into the buffer solution is the main culprit for the accelerated disintegration of MTs. The alternative explanation that release of PDMS oligomers into the solution is responsible can be excluded, since Lee et al. [121] showed recently that the oligomer release from PDMS into aqueous solution is negligible. In addition, pre-cleaning of PDMS with hexane, which has been identified as effective procedure to further reduce oligomer release in the same study, had no effect in our experiments. The utilization of PDMS as a material is therefore restricted to devices, which do not integrate fluorescence detection, but utilize differential interference contrast (DIC) microscopy or other mechanisms to follow MT movement. PMMA, a possible alternative to PDMS due to the availability of microfabrication techniques, promotes photobleaching and MT disintegration to a lesser extent but is still significantly inferior to glass as cover material. EVOH, a polymer with low oxygen permeability, roughly matches the performance of glass, providing further support for the hypothesis that removal of oxygen from the buffer stabilizes the MTs. PU, which is another traditional microfabrication material (it can be photopolymerized from a precursor solution), performed equally well as EVOH or glass when the flowcell is exposed to light, with low photodamage to the fluorescent labels and slow disintegration of MTs.

3.5 Conclusions and Outlook

Overall we have shown that a number of polymers can replace glass as packaging material for hybrid nanodevices integrating kinesin motors and MTs, but that special care has to
be taken when intense illumination is needed, for example, for fluorescence imaging. PDMS and PMMA, two widely used materials in micro- and nanofabrication, cause rapid disintegration of MTs under exposure to light, presumably via release of oxygen into the solution. This demonstrates that materials, which are judged to be biocompatible in less demanding applications, do not necessarily integrate well with fragile biological supramolecular assemblies. The polymers EVOH and PU are compatible with kinesin and MTs under illumination, but for PU the advantage of straightforward microfabrication by soft-lithography comes at the cost of reduced MT lifetime in the absence of illumination.

The presented data provide a benchmark for the lifetime of motor protein-based bio-nanodevices, which utilize glass as the primary synthetic material, and test the impact of a variety of polymer materials on the longevity of MTs, the most fragile biological structure in the device. Strategies to further stabilize MTs exist—using non-hydrolysable GTP analogs and chemical cross-linkers—and can now be compared against the taxol stabilization method. In addition, we can explore the stability of other supramolecular assemblies, such as actin filaments or DNA nanotubes, to achieve a unified perspective on engineering challenges in the design of hybrid devices. This study also demonstrates that our definition of biocompatibility evolves, as we progress towards architectures engineered on a molecular level, which integrate multimeric proteins and protein assemblies.
Visualizing Molecular Shuttles
Abstract

Fluorescence-based imaging is the most common microscopy method to visualize biological samples. Specific labeling and staining is a very efficient and straight-forward way to detect and observe regions of interest. MT filaments (25 nm in diameter) can be fluorescently labeled and imaged—using chemicals that prevent oxidative degradation of fluorophores and filaments—for a reasonable amount of time without losing significant amount of signal. However, there is a high need of dye-independent imaging of biological samples, as effects such as photo-induced damage can be prevented. In the following, we thus introduce a novel imaging technique, *i.e.* Confocal Interference Microscopy, originally developed for the visualization of nanometer-sized gold colloids [122]. The method has proven to be also applicable for the imaging of proteins assemblies, such as MT filaments and viruses, with reasonable signal-to-noise ratio. For example, single MTs can be detected, showing a normalized intensity of 3–5%, which is comparable to the contrast of single gold nanoparticles with a diameter of 10–20 nm. Furthermore, protein assemblies and metallic nanoparticles can be easily distinguished from each other by performing a two-color experiments [123]. Because of the high sensitivity of the method in z-direction, very small relative changes in z-position can be detected.

4.1 Introduction

The invention of the optical microscope sometime in the 16th century revolutionized the scientific work by allowing insight into the microscopic structure of objects. Already in the 17th century the optical microscope has drawn biologist’s attention. However, compound microscopes as known today were not assembled till the beginning of the 19th century. In 1889, Ernst Karl Abbe (1840–1905) developed the first microscope featuring oil immersion objective, condenser, and eyepiece. It was also Ernst Abbe who postulated that the light’s wavelength gives a measure of the image resolution. The far-field resolution limit \( d_{\text{min}} \) for an optical microscope (known as "diffraction limit") is today known as the Rayleigh criterion:

\[
d_{\text{min}} = 0.61 \frac{\lambda}{n \sin \theta} = 0.61 \frac{\lambda}{NA},
\]  

(4.1)

where \( \lambda \) is the wavelength of the light and \( n \sin \theta \) is the numerical aperture (NA), defined by the refractive index \( n \) and the angular resolution \( \theta \). Assuming light with a wavelength of \( \sim 550 \) nm and according to eq. 4.1, the minimal resolvable feature size using an optical microscope is thus \( \sim 250 \) nm. In practical terms, this means that the smallest biological entities that can be clearly discerned by light microscopy are bacteria and mitochondria. Special optical techniques derive image by interference (Differential Interference Contrast, DIC), differences in phase (Phase Contrast Microscopy) as the light travels through matter with different optical density or by detection of scattered light (Dark-Field Microscopy). Using DIC, even MT filaments can be visualized, with the disadvantage, however, that the diffraction limit still remains, which makes it impossible to discern single filaments from bundles. Recent approaches try to break the diffraction limit by using anisotropic materials to fabricate optical lenses (superlens) \([124, 125]\). These materials or metamaterials feature negative refractive indices in the visible frequency range. Optical hyperlenses \([126]\) magnify a sub-diffraction-limited image and project it into the far-field, resolvable by standard optical microscopy. These approaches are all based on plasmon-assisted microscopy and lens materials therefore include metallic layers (Ag or Au) that feature special surface plasmon polariton properties when using light in the visible range.

Beside optical imaging techniques, other types of microscopes—such as Atomic Force Microscopy (AFM) or Scanning Electron Microscopy (SEM)—are commonly used to image biological samples at higher spatial resolution than the optical microscope. AFM
imaging is very useful for imaging at a resolution of few nanometers\(^1\) and performing force spectroscopy to study specific biochemical interactions e.g. between proteins. However, AFM imaging only provides topographical or frictional information of a scanned surface, limiting it for studying biological samples as for example different organizational units of a cell can not be distinguished. Similar limitations apply to SEM imaging: besides tiresome sample preparation, submicrometer resolution can only be achieved in ultra-high vacuum requiring chemical fixation and drying of biological samples. In addition, in order to reduce charge effects, dielectric biological probes need to be sputtered with a conducting metal layer (usually 2–3 nm of Pt or Au) to render the surface electrically conductive.

Indeed, another optical imaging technique has been established in lifesciences, i.e. Fluorescence Microscopy. Since its invention in the early 20\(^{th}\) century, Fluorescence Microscopy has become a widely used tool for biologists, biochemists, and bioengineers. In the following two sections, we briefly review Fluorescence Microscopy for imaging of MTs and present a novel confocal interference-based method to visualize MT filaments.

### 4.2 Fluorescence Microscopy

#### 4.2.1 Overview

Fluorescence is a property of some atoms and molecules to absorb light of a specific wavelength and, after a certain amount of time (fluorescent lifetime), to emit light at a higher wavelength. Electrons in fluorescent molecules (fluorophores) absorb the energy of the exciting light and jump to a higher energy level. Soon, the excited electron drops back to its ground state by losing energy in the form of an emitted photon at a lower energy (higher wavelength) than the exciting light (Stokes shift). In fluorescence microscopy, the sample itself becomes the light-source. This way, molecules can be selectively and specifically detected at low concentrations (even down to single molecules) with good signal-to-background ratios. However, the excitation light illuminates a large area of the sample, causing out-of-focus fluorescence. In addition, fluorophores are prone to photobleaching, especially in presence of free oxygen radicals (also compare Chapter\(^3\)), causing a loss of signal over time. Moreover, loss of fluorescence can also occur due to quenching, i.e. absorption of emitted fluorescence by adjacent molecules. The latter

\(^1\)spatial resolution limited by the tip geometry
4.2. **Fluorescence Microscopy**

Effect is also used to perform fluorescence spectroscopy, such as Fluorescence Resonance Energy Transfer (FRET) [127].

Epi-Fluorescence Microscopy (Fig. 4.1A) has become a widely used technique in lifesciences. In this setup, the sample is illuminated through the objective of the microscope. This way, only the reflected and the fluorescent light originating from the sample, respectively, is collected causing a signal enhancement. Specific parts of biological samples (e.g., cells) can be labeled with fluorophores—such as fluorescein isothiocyanate (FITC) or rhodamine—that exhibit different excitation and emission wavelengths.

In the past decade, the development of genetically encoded fluorophores, such as the green-fluorescent protein (GFP), and the invention of Two-Photon Microscopy have further revolutionized Fluorescence Microscopy [128]. The GFP gene can be expressed in any species that can be genetically modified. It is commonly used to fluorescently label proteins, which allows the detection of the resulting fusion protein. In Two-Photon Microscopy, two photons at a wavelength of \(\sim 700–1000\,\text{nm}\) excite a fluorophore with an excitation wavelength in the range of 400–500 nm. The occurrence of this energy transfer...
and the concomitant fluorescence has very low probability. Thus, a high flux of excitation photons is needed (usually provided by femtosecond laser pulses). As the photon density in the focus is highest, fluorescence is exclusively generated in the focal volume, allowing high resolution imaging.

4.2.2 Imaging Microtubule Filaments

MT filaments can be easily imaged using fluorescently labeled tubulin building blocks (e.g. see Fig. 3.4). Rhodamine-labeled tubulin is commercially available in lyophilized form\(^2\). Biotin functionalized MTs are usually co-polymerized with a low percentage (∼10–20%) of rhodamine-labeled tubulin. Even at a low fraction of fluorescent tubulin of 10%, MTs are easily detectable (see for example Fig. 7.8B). Photo-bleaching during motility assays is reduced by using an oxygen scavenging system (D-Glucose, Glucose Oxidase, Catalase, and DTT). However, as the scavenging system is used up over time, the fragile MT protein assemblies are subject to photo-bleaching and degradation as discussed in section 3.3.3 especially in presence of oxygen-permeable surfaces.

4.3 Confocal Laser Scanning Microscopy (CLSM)

Even though the principle of confocal imaging was patented by the genius Marvin Minsky already in 1952, commercial confocal microscopes are available only since about 30 years. In CLSM, a scanning laser beam is used to excite fluorescence molecules only in a very small focal volume of the sample (Fig. 4.1B). The emitted fluorescent light is then reflected by a dichromatic mirror and passed through a confocal pinhole before being detected in a photomultiplier tube (PMT). The pinhole aperture blocks any fluorescent light emanating from close vicinity of the focal volume, thereby providing further attenuation of out-of-focus interference. These characteristics contribute to a slight resolution enhancement if compared to standard fluorescence microscopy. But moreover, the use of in-focus illumination significantly reduces the photo-bleaching of the sample.

Besides acquiring 2D images in the focal plane of the beam, CLSM also has the potential to produce 3D images by changing the relative z-position of the beam. The penetration depth of the beam, defining the maximum possible depth of the 3D image,

\(^2\) TL 331-M, Cytoskeleton, Denver, CO, USA
4.4. Confocal Interference Microscopy

4.4.1 Overview

Label-free detection of nm-sized objects by-passes chemical modification of target proteins with e.g. fluorescent dyes and concomitant problems such as photobleaching. Particularly in MT-based motility assays, photoreactions between fluorophore and tubulin lead to the disintegration of MTs in various synthetic environments (see Chapter 3). The integrity of the transport system, however, is essential to enable pick-up and long-distance transport as photo-induced breakdown of the shuttles directly limits the lifetime of an active transport device and facilitates the turn-around of MT fragments in channels. Therefore, fluorescence-free imaging of nano-objects using far-field optics is highly desirable as it prolongs the lifetime of any photosensitive biological assays.

The presented Confocal Interference Technique was developed in Vahid Sandoghdar’s lab for the detection of very small gold particles, down to a diameter of \( \sim 5 \text{ nm} \). In collaboration with the Nano-Optics group, the imaging technique was applied to visualize both MT filaments and transported gold colloids (see Chapter 7). In contrast to DIC, this technique allowed us to exploit the possibilities of our commercial CLSM, without the need of additional equipment.

---

3 Nano-Optics Group, Laboratory of Physical Chemistry, Swiss Federal Institute of Technology (ETH), CH-8093 Zurich, Switzerland
4.4.2 Principles

The scattered field ($E_s$) of the particle is interfered with the reflected field ($E_r$) from the glass/water interface (Fig. 4.2). The image contrast is controlled by the relative phase of the interference cross term under observation, whereby a Gouy phase shift (longitudinal phase delay) of $-\frac{\pi}{2}$ has to be considered for the reflected Gaussian beam.

The scattered field at the detector can be expressed by $E_s = sE_i$ ($E_i$: electric field of the incident laser beam), and $s$ is proportional to the polarizability $\alpha$ of the particle [122]:

$$s(\lambda) = \eta \alpha(\lambda) = \eta \varepsilon_{\text{med}}(\lambda) \frac{\pi D^3}{2} \frac{\varepsilon_{\text{part}}(\lambda) - \varepsilon_{\text{med}}(\lambda)}{\varepsilon_{\text{part}}(\lambda) + 2 \varepsilon_{\text{med}}(\lambda)},$$

where $\eta$ is the detection efficiency and $\varepsilon_{\text{part}}(\lambda), \varepsilon_{\text{med}}(\lambda)$ are the complex dielectric constants of the particle and the medium, respectively. The measured intensity $I_m$ at the detector can be written as:

$$I_m = |E_r + E_s|^2 = |E_i|^2 \left\{ r^2 + |s|^2 - 2r|s|\sin\phi \right\}.$$

In eq. 4.3, $r^2$ denotes the background intensity originating from the reflected field at the interface between sample surface and the immersion oil. Whereas $r^2$ is constant for a given illumination, the term $|s|^2$ strongly depends on the particle diameter (proportional to $D^6$) as can be verified in eq. 4.2. For small particle diameters (<30 nm), this term becomes negligible and the interference cross-term $2r|s|\sin\phi$ gains importance as it only
scales with $D^3$. For this reason, small particles appear dark against a white background, whereas bigger particles have higher intensity than the background.

The contrast of nanoparticles is significantly enhanced for metal colloids that feature plasmon resonance at the visible frequencies of light. Scattering of colloidal gold reaches its maximum at $\sim 530$–$560$ nm, also depending on the particle’s diameter. Resonance is reached if $\varepsilon_{\text{part}}(\lambda) = -2\varepsilon_{\text{med}}(\lambda)$, as can be seen in eq. 4.2. While this is true, e.g., for Au, protein assemblies such as MTs do not contribute to an enhanced scattered field and therefore provide less contrast towards the background (see Sandoghdar et al. [132] and section 4.4.4).

The contrast of the detected particles and/or protein assemblies is also strongly depending on the relative $z$-position towards the background surface, as phase differences between reflected and scattered field cause alternating constructive and destructive interference. Upon a change in $z$-position, particles may change their contrast from dark to bright towards the background (see Jacobsen et al. [131] and section 4.4.4).

### 4.4.3 Materials and Methods

**Preparation of Motility Assays**

MTs were assembled from biotinylated tubulin\(^4\) (without the addition of rhodamine-labeled tubulin) and stabilized using taxol. Flowcells consisting of a $20 \times 20$ mm\(^2\) glass coverslip bottom and a $10 \times 10$ mm\(^2\) glass coverslip as top (separated by double-coated tap) were filled sequentially with 0.5 mg ml\(^{-1}\) casein solution (in BRB80), a kinesin solution ($\sim 40$ µg ml\(^{-1}\) kinesin, 0.2 mg ml\(^{-1}\) casein, 0.01 mM ATP in BRB80), and a motility solution (3.2 µg ml\(^{-1}\) biotin-labeled MTs in CATO containing 0.01 mM ATP), for 5 min each. At the used ATP concentration, MTs move at an average speed of $\sim 100$ nm s\(^{-1}\). After allowing the MTs to adsorb for 5 min, the flowcell was rinsed with CATO solution. Then, a solution of 40 nm anti-biotin-functionalized gold colloids\(^5\) (1000× diluted in CATO solution) was added and incubated for 10 min. Finally, the flowcell was rinsed again with CATO solution and mounted on the sample holder for confocal imaging.

\(^4\)T 333-B, Cytoskeleton, Denver, CO, USA
\(^5\)Array MAB 40, Batch 6862, BB International, Cardiff, UK
Confocal Setup for Au Colloid and Microtubule Visualization

Experiments performed in Vahid Sandoghdar’s lab were performed on a custom-built piezoceramic motion stage scanning setup based on a commercial inverted microscope (Zeiss Axiovert 100). Laserlight at a wavelength of 532 nm (close to the peak of the plasmon resonance of a 40 nm gold colloid [122]) and 488 nm was used to image gold colloids and MTs (see Jacobsen et al. [123] for more details). A photomultiplier tube (PMT) was used to detect the signal.

Equivalent experiments could also be performed on a commercial confocal microscope using an HeNe 543 nm laser for illumination of the sample, a 60× oil immersion objective (NA = 1.35) and a pinhole diameter of 120 µm. For interference detection, a dichromatic mirror was used to collect wavelengths below 560 nm in one PMT. Wavelengths above 560 nm were used to detect rhodamine fluorescence of the labeled MTs after passing an interference filter using a second PMT. The sensitivity (HV, gain, offset) of the PMTs was adjusted to obtain optimal contrast in interference and fluorescence. Image sequences were recorded at a resolution of 800×800 px² (pixel resolution: 132 nm) and a pixel dwell time of 10 µs px⁻¹ resulting in a scan rate of ~7.4 s per frame. If not stated otherwise, images were acquired using these settings.

4.4.4 Results and Discussion

Microtubule and Gold Particle Visualization

The ability of the confocal interference technique to detect Au particles down to diameters of 5 nm has been discussed well by Lindfors et al. [122]. The 40 nm anti-biotin coated gold colloids used to bind to MT filaments (Fig. 4.3A) showed a normalized intensity σ of ~25% (Fig. 4.3B), which compares well with normalized intensities determined by Jacobsen et al. [123]. The normalized intensity σ (see Fig. 4.3B) was calculated using the following equation:

\[ \sigma = \frac{I_{\text{meas}} - I_r}{I_r}, \]  

(4.4)

where \( I_{\text{meas}} \) is the measured intensity of the particle or MT, respectively, and \( I_r \) is the intensity of the background originating from reflected light without the particle. Due to

---

4.4. Confocal Interference Microscopy

Figure 4.3: (A) Contrast of MTs and Au particles in the interference-based imaging technique at two different time points at a wavelength of 532 nm. Moving directions of MTs are indicated by arrows. Scale-bar is 4 µm. (B) Normalized intensity (according to eq. 4.4) plots of a MT filament and a 40 nm Au colloid taken from the lines marked in (A). While the 40 nm Au colloids give a contrast of ~25%, MT filaments only differ ~3-5% from the background. The measured contrast decreases with decreasing Au particle diameter. Thus, 5 nm particles (normalized intensity change of ~0.3% [123]) might only be distinguishable from MT filaments by using laser light at two different wavelengths (see Fig. 4.4).

the destructive interference between the reflected and scattered fields, single Au colloids appear dark against the background. However, as soon as two or more particles are in close proximity (closer than the diffraction limit of the microscope) and therefore increase the nominal diameter of the particle, the contrast may switch from dark to bright, as can be seen in Fig. 4.3 A at 0 s below the upper arrow. A similar effect can be observed if the Au nanoparticle is changing its relative z-position (Fig. 4.3 A at 60 s). This is discussed in more detail in the next section.

Astonishingly, also MT filaments could be easily imaged using the interference-based technique. In our experiments, MTs showed a normalized intensity of 3–5% (Fig. 4.3 B). Obviously, the refractive index ($\varepsilon \sim n^2$) of polymerized MTs (in the range of 1.36–1.55 [133]) is sufficiently different from the surrounding medium ($n_{buffer} \approx 1.35$) to add to the intensity of the scattered field (eq. 4.2 and 4.3). Since gold particles as small as 5–10 nm only provide a contrast of <5%, they can not be distinguished anymore if bound to MT filaments. In order to overcome this limitation, a two-color experiment can be performed: as the gold particles exhibit a maximum scattering cross-section at ~530 nm due to their plasmon resonance (Fig. 4.4 (a)), illuminating the sample at a wavelength of 532 nm and at 488 nm, respectively, and subsequent subtraction of the two obtained images can separate the signal of the nanoparticles from the protein assembly (Fig. 4.4 (b)–(d)) [123].

On gold surfaces, 40 nm Au nanoparticles exhibited about 10% lower contrast compared to glass surfaces (see Chapter 7). This is still remarkable considering the high
reflectivity of the Au metal layer and imaging through a 100 µm thick flowchannel filled with buffer solution. However, MT filaments could not be resolved in this case.

**Gold Colloid Blinking**

The image in Fig. 4.5 shows three filaments, two of them carrying gold colloids. The derived pixel intensity is a sensitive measure for the position change of the Au particle in z-direction: elevation of a colloid introduces a path difference between the scattered and reflected light and thus causes a change in the overall phase of the interference (from destructive to constructive) [131]. As a consequence, the particle may appear bright with respect to the background. This makes it possible to even probe the relative z-positions of two crossing MTs. As shown in Fig. 4.5 the MT transporting the cargo is crossing above the horizontally moving shuttle. The same effect also reveals the swiveling leading end of a MT moving along a surface before binding to the next kinesin motor.
Because of the high sensitivity in z-direction changes, the confocal interference imaging could be used in the future to detect blocked binding sites on MT filaments that cause stalling of molecular motors, such as kinesin or dynein. MT-associated proteins (MAPs) such as Tau are suspected to play a role in blocking kinesin-mediated transport along MTs, which is a possible reason for diseases such as Alzheimer’s. Experiments that aim to mimic this process are currently investigated in our lab [134].

### 4.4.5 Conclusions

Fluorescence microscopy is a reliable and still the most common method to image MT filaments. Because of the large protein-assembled structure of MTs consisting of many labeled tubulin subunits, only a small fraction of fluorescent tubulin is required to detect the filaments. Therefore, photo-bleaching of labeled MTs is slow (especially in combination with an oxygen scavenging system) and image sequences can be easily recorded over at least 5–10 min without a significant loss of fluorescent signal.

Fluorescence-free visualization of protein assemblies enables long-term studies of scientific samples circumventing otherwise restrictive surface bleaching or photodamage. The interferometric detection scheme, which allows to directly observe the metallic nanoparticles as well as the organic MTs, might be particularly significant in the study of other biomolecular assemblies whose functionality might be imparted by photodamage. The non-covalent bonds that stabilize MTs, for example, are broken by light in the presence of oxygen if tubulin is labeled with fluorophores (Chapter [3] [75]). In the interference-based technique, MT filaments show a contrast of ~3–5% against the background if visualized on a transparent substrate, such as glass. This is comparable to the contrast of single gold nanoparticles with a diameter of 10–20 nm and more than sufficient...
for imaging. Compared to other interference methods, such as DIC, MT bundles can be distinguished from single filaments via increase of contrast.

Moreover, gold colloids that could be used as markers in biological samples can be easily distinguished from protein assemblies by performing a two-color experiment. This way, specifically labeled regions of biological probes can be exposed and distinguished from background. Also, the strong dependence of the normalized intensity of particles on the $z$-coordinate relative to the surface could provide interesting data on molecular interaction between MT filaments and corresponding motors.
Cargo Transport Driven by Kinesin Motors
Abstract

The specific attachment and subsequent transport of cargo by MT filaments is a key feature of a future molecular motor based biosensoric device. Various possible types of interactions between cargo and MT filament, including biotin–SA, DNA hybridization and antibody–antigen, were investigated within the scope of this study. Using biotin–SA interactions, dynamic self-assembly of MT filaments could be demonstrated, characteristically starting with the formation of linearly extended MTs and finally resulting in micrometer-sized assembled ring structures held together by multiple biotin–SA bonds [135]. Attachment of hybridized DNA via SA to MT filaments could be demonstrated and in addition, photo-induced bleaching could be used to release the hybridized complementary DNA strand by preserving the motility of the shuttles. This could be used in the future as light-controlled method for the discharge of cargo. Moreover, the transport of cargo significantly differing in their dimensions could be shown: 1 µm PS beads (and even agglomerates thereof) could be successfully transported. Likewise, attachment of anti-biotin coated 40 nm Au colloids to and subsequent transport by molecular shuttles could be demonstrated.

5.1 Introduction

Specific active transport of cargo by the movement of motor proteins along filaments inside the cell cytoplasm has evolved over millions of years. Harnessing this potential for the use in hybrid bionanodevices has attracted the interest of bioengineers since the discovery of the kinesin molecular motor in 1985 by Vale et al. [40]. With the concept and realization of molecular shuttles—MT or actin filaments propelled by surface-adsorbed kinesin or myosin motors, respectively—appropriate binding mechanisms had to be developed to specifically attach cargo to functionalized MTs. In the past decade, various types of cargo, ranging from proteins [135] to DNA molecules [94, 136] and protein assemblies [12, 13] to microspheres [6, 85], quantum dots [11], gold wires [137] and silicon microchips [95], were successfully adsorbed to MT filaments using specific interactions such as biotin–SA, DNA hybridization, and antibody–antigen.

Biological bonds between ligands and receptors are typically based on non-covalent interactions such as hydrogen-bonds and hydrophobic interactions. While these interactions are highly specific, meaning that ligand and receptor have to match geometrically and/or chemically, they are also reversible implicating limited lifetime, especially if external forces are applied. Because of these properties—high specificity and reversibility—these types of interactions are qualified for the use in the molecular shuttle system, as the shuttles can be exclusively loaded with the targeted cargo and, in addition, the reversibility leaves the option to release the cargo at a desired location. Fig. 5.1 A–C shows a selection of possible interaction types that can be used to load molecular shuttles with cargo:

- **Biotin–SA** The vitamin biotin (MW=244 Da) specifically adsorbs into one of the four binding pockets of a SA-tetramer (MW=60 kDa). The highly specific and strong interaction (bond energy of 5–30 $k_B T$ [138] and a binding affinity $K_A \geq 10^{14} M$ [139]) is widely used in biotechnology and methods and commercial products are easily available. The biotin–SA interaction has been used in various molecular shuttle applications, such as binding of microspheres [6] or proteins [12].

- **Antibody–Antigen** Antibodies are glycoproteins that consist of subunits containing two identical light chains ($\sim$200 amino acids) and two identical heavy chains (ca. twice as long as the light chains) and together form a Y-shaped structure, consisting of an Fc part and two ligand binding Fab parts. Antigens, such as proteins, polysaccharides, DNA and RNA or other molecules, specifically bind to the two arms of
Figure 5.1: Possible strategies to link cargo to molecular shuttles. (A) Microspheres bind to MTs via biotin-streptavidin interaction. This system was already experimentally tested for cargo transport [14] (B) DNA single strands hybridize to a double-strand DNA helix. The single strands can be chemically modified and functionalized. (C) Antibodies cross-linked with molecular shuttles can bind and transport antigens such as viruses or bacteria.

• **DNA Hybridization** Single DNA strands specifically bind to the complementary strand via base-pairing. The interaction is based on hydrogen bonds between adenine (A) and thymine (T), and between guanine (G) and cytosine (C). The bond energy of a single AT pair is \( \sim 2.3 k_B T \). The binding strength of the DNA is sequence-dependent and can be adjusted by using specifically designed DNA strands. The sequence-dependent formation of the hybridization makes DNA a very versatile and specific linker. DNA can be end-functionalized or labeled with various types of molecules, such as biotin, thiol groups or fluorescent dyes. As the melting temperature of the DNA hybridization is also salt- and temperature-dependent, various options are offered in order to tune the binding strength. In a recent study, Taira et al. attached DNA strands via SA and hybridization to biotinylated MTs [136].
In an inverted motility assay, where MT filaments are actively propelled by kinesin motors, the distance between filament and substrate was recently determined to be on the order of 17 nm [74]. Considering possible supertwisted MTs [140] that cause a rotational movement of the shuttles along the kinesin motors, this finding might have implications on the possible cargo size. The transport system might therefore also be limited by the dimensions and surface-properties of the cargo (e.g. brush-like structures could cause entanglements and hinder motility of the shuttles). Despite these apprehensions, various types of cargo could be successfully transported (see above). In future devices, virus particles or even bacteria are potential targets for the transport by antibody-functionalized MTs (Fig. 5.1 C).

In our experiments, we focused on the use of potential cargo linkers, such as biotin–SA, DNA-DNA, and antibody–antigen interactions, and demonstrated capture and transport of various model cargo, including e.g. PS microspheres, DNA strands and nanometer-sized gold colloids. In addition, by using the biotin–SA interaction, self-assembly of MT shuttles into extended filaments, bundled MTs, and ring structures could be observed [135].

5.2 Materials and Methods

5.2.1 Microtubule Self-assembly

Flow cells were assembled from glass slides, stripes of double-sided tape, and glass cover-slips (Fisher’s Finest, Fisher Scientific Inc.). The flow cells were filled with kinesin solution (~10 nM kinesin, 10 µM ATP, 0.02 mg ml⁻¹ casein) for 3 min, followed by two washes for 1 min (20 µl volume, 10 µM ATP) and one wash for 3 min (20 µl volume, 10 µM MgATP, 0.02 mg ml⁻¹ casein). Then a MT solution (20 µl volume, 20 µg ml⁻¹ biotinylated MTs, 10 µM taxol, 10 µM ATP, 0.02 mg ml⁻¹ casein) was injected for 10 min, followed by a wash step (20 µl volume, 10 µM taxol, 10 µM ATP, 0.02 mg ml⁻¹ casein). One minute later, a solution of fluorescently labeled SA (20 µl volume, 10 µg ml⁻¹ Alexa488-SA, 10 µM taxol, 10 µM ATP, 0.02 mg ml⁻¹ casein) was injected for 3 min, followed by two wash steps separated by 1 min (20 µl volume, 10 µM taxol, 10 µM ATP, 0.02 mg ml⁻¹ casein). Finally, 1 min after the last wash an ATP solution with an antifade system was injected into the flow cell (20 µl volume, 10 µM taxol, 1 mM ATP, 0.02 mg ml⁻¹ casein).
5. CARGO TRANSPORT DRIVEN BY KINESIN MOTORS

0.02 mg ml\(^{-1}\) casein, 20 mM D-glucose, 0.02 mg ml\(^{-1}\) glucose oxidase, 0.008 mg ml\(^{-1}\) catalase, 20 mM DTT)\(^{[135]}\).

### 5.2.2 Transport of Polystyrene Microspheres

**Beads Preparation**

Biotin-labeled, yellow-green fluorescent (505/515 nm) polystyrene (PS) beads with a diameter of 1 \(\mu\)m were purchased from Molecular Probes (F-8768). Before use, the stock suspension was vortexed and sonicated for 2 min to break agglomerates. The solution was then 10-fold diluted in 200 \(\mu\)l BRB80. Subsequently, 10 \(\mu\)l of SA stock solution\(^2\) was added to 190 \(\mu\)l of the diluted biotinylated PS beads, vortexed and sonicated for 2 min. SA-coated beads were centrifuged at 13000 rpm for 20 min. After centrifugation, the supernatant was pipetted off and beads were resuspended in 100 \(\mu\)l CATO solution (at 10 \(\mu\)M ATP). This suspension was vortexed and sonicated until all beads had redispersed. The final suspension was then directly injected to the motility assay (see following section).

**Motility Assay**

Motility assays were started at low ATP concentration (10 \(\mu\)M) in order to reduce MT movement to a minimum. After assembling the glass flowcells, motility assays were prepared according to standard protocol: a casein solution (0.5 mg ml\(^{-1}\) in BRB80), kinesin motors (in BRB80 containing 0.25 mg ml\(^{-1}\) casein and 10 \(\mu\)M ATP) and a mix of rhodamine-labeled biotinylated MTs\(^3\) in motility solution (0.25 mg ml\(^{-1}\) casein, 10 \(\mu\)M taxol, 10 \(\mu\)M ATP and oxygen scavenging additives in BRB80) were incubated for 5 min each. MTs not bound to the surface-adsorbed kinesins were rinsed out using motility solution (10 \(\mu\)M ATP) and subsequently the prepared PS microsphere solution (see section 5.2.2) was adsorbed to the MTs for 20 min. Finally, the flowcell was rinsed with CATO solution at full ATP concentration in order to wash out excessing microspheres dispersed in solution. Before imaging, the channel ends of the flowcell were sealed using immersion oil.

\(^2\)1 mg ml\(^{-1}\), Molecular Probes, MP S-11223

\(^3\)50% biotinylated and 50% rhodamine-labeled tubulin
5.2.3 Transport of Hybridized DNA

Motility assays to attach DNA oligonucleotides to biotinylated MTs via SA were prepared in a similar way as described above for PS-bead transport. Assays were performed at low ATP concentration (10 µM). After injecting casein, kinesin and MT solutions, the flowcell was rinsed with CATO. Then, a solution of biotinylated DNA (5' biotin CCC CCA TGG AAT CGT AA 3') and green-fluorescent SA (premixed at a ratio of 1:1 for 10 min) in CATO (20 µg ml⁻¹ SA, 0.7 µM DNA) was added to the assay and incubated for 10 min. After a CATO rinse, the complementary rhodamine-labeled DNA (5' thiol CCC CCT TAC GAT TCC AT rhodamine 3') was injected and incubated for 10 min. Before imaging using CLSM, the flowcell was rinsed again with CATO solution and immersion oil was used to seal the channel ends. Photobleaching experiments were performed at a wavelength of 488 nm (1024 iterations).

5.2.4 Transport of Antibody-coated Au Nanoparticles

Loading of 40 nm anti-biotin coated Au colloids was performed in standard motility assay at reduced ATP concentration (10 µM) using biotinylated MTs as described above. Gold colloids were diluted (100- or 1000-fold) in CATO solution prior to injection to the flowcell. The colloids were allowed to adsorb to the MT filaments for 5 min and finally, the flowcell was rinsed using motility solution and prepared for imaging.

5.3 Results and Discussion

5.3.1 Microtubule Self-assembly

Formation of extended biotinylated MT filaments could be achieved by partial coverage of SA. Assays were started at low ATP concentration in order to inhibit the self assembly process during the adsorption steps of the motility assay. The critical parameter in the self-assembly was to adjust the SA concentration to reach ~50% coverage of

---

4 MedProbe, Oslo, Norway
5 Alexa488 labeled, Molecular Probes
6 Zeiss LSM 510, Zeiss Germany
7 Array MAB 40, Batch 6862, BB International, Cardiff, UK
8 particle concentration of stock solution (as indicated by the manufacturer): ~2 × 10¹¹ particles per ml
biotinylated MTs. At higher SA coverage, the majority of free biotin molecules on the filaments were inactivated by adsorbed SA resulting in hardly any assembled MTs. The assembly process usually started with the formation of MT bundles bound together via strong biotin–SA interactions (Fig. 5.2 A), which is reflected in above average length and increased fluorescent intensity of the bundled MTs. After a few minutes, circularly bundled MT filaments started to appear (Fig. 5.2 C and D). As MT shuttles glided along the kinesin motors, they eventually wind up when encountering surface impurities or binding to an inactive motor. Due to the partial coverage with SA, the filaments are able to bind to their own tail as they wind up, being forced into the formation of a ring. The diameters of the rings ranged from 1–10 µm. These rings are held together via many biotin–SA bonds and are therefore exceedingly stable, at least for 30 min. After many rings have formed on the surface, most MTs were trapped by the ring structure, depleting the surface of linearly assembled and single MTs over time. Efforts to avoid the formation of spools, e.g. by orienting MT movement along a friction-transferred PTFE layer [78, 141] (Fig. 5.2 B), were not successful. After a certain time, ring structures appeared and extended filaments (Fig. 5.2 B, right side in image sequence) were consumed by the spools. Surprisingly, despite of the multiple formation of biotin–SA bonds (each exhibiting a bond energy of \( \sim 30 k_B T \)), kinesin motors were able to disassemble bundled MTs (Fig. 5.2 B at 30 s). Few events were observed where even ring structures unspooled (see reference [135]), moving on as a linear MT bundle.

The "nanospools" can be considered as self-assembled ordered non-equilibrium structures, that could be used as templates for the fabrication of micro- and nanometer sized synthetic structures. However, for the fabrication of linear extended structures, e.g. for the use as templates for metallization [142, 143], the surface has to be prepared in a way to avoid the formation of ring structures. In example, shuttle movement could be restricted to linear channels at a width smaller than the minimum diameter of the spools, i.e. \( \leq 1 \mu m \).

5.3.2 Loading of Polystyrene Microspheres

The transport of micrometer-sized PS beads by MT shuttles served as a proof-of-principle study for the loading of cargo to MTs from solution. Two differently functionalized microspheres were sampled—1 µm PS beads directly coated with SA (Fig. 5.3 A i.) and 1 µm biotinylated PS beads that were coated with SA (Fig. 5.3 A ii). While SA-coated
Figure 5.2: (A) Example of MT assembly and formation of extended filament structures: Rhodamine-fluorescent MTs functionalized with biotin and partly covered with SA spontaneously combine and form bundles of filaments (arrowhead at 20 s) (B) The formation of extended MTs on an oriented thin film of PTFE \[78, 141\] to some extent allowed the assembly of oriented filaments, such as the one in right part of the images (note the higher fluorescence intensity, indicating multiple MTs bundled together). However, the development of "nanospools" \[135\] (C and D) also occurred on structured surfaces impeding extended "nanowires" over time. In addition, bundled MTs eventually "unwire" (arrowhead in B at 30 s), (D) The formed ring structures are rotating, clock- and also counterclockwise \[movie\]. Scale-bars are 10 µm in (A) and (C), and 5 µm in (B) and (D).
beads did not bind to biotinylated MTs even after long incubation times, biotinylated microspheres coated with SA attached successfully to biotinylated shuttles and were transported after increasing the ATP concentration in the assay. Preparation of SA-coated biotinylated microspheres required to mix the beads with an excess concentration of SA and subsequent centrifugation in order to separate SA-enriched solution from the coated particles. After taking off the supernatant, the beads were re-dispersed in motility solution supported by ultrasonic treatment. In case of uncareful mixing and re-dispersion, agglomerates of several particles formed and, upon addition to the motility assay, attached as bundles to the MT shuttles (Fig. 5.3B). Surprisingly, these agglomerates were readily transported by the MTs showing no loss of motility. Agglomerates of up to ten 1 µm-sized particles are transported by single MTs over a distance of several micrometers. Cargo-transporting molecular shuttles interacted with crossing MTs, for example cargo transfer, filament bending or joining could be observed as described by Boal et al. [85]. In addition, the reversible binding properties of the biotin–SA interaction were reflected in the slipping of a microsphere on a moving MT shuttle as shown in Fig 5.3C. The transported particle slipped over a distance of ∼2–3 µm on the moving MTs without desorbing from the filament.

5.3.3 Cargo Transport via DNA Hybridization

Single stranded biotinylated DNA oligonucleotides (17 nucleotides) were adsorbed via green-fluorescent SA to biotinylated MTs (Fig. 5.4A). After rhodamine-labeled complementary DNA strands were injected into the flow chamber, the DNA spontaneously hybridized (Fig. 5.4B, C). A low ATP concentration prevented crosslinking of MTs in the early stage of the experiment by slowing down MT movement. While Fig. 5.4B shows the fluorescence of Alexa488-labeled SA bound to the MTs, the fluorescent signal detected in Fig. 5.4C originates from the rhodamine dye attached to the complementary strand of the DNA. Both signals colocalize well, thus furnishing proof for successful hybridization of the DNA strands. The motility of MTs was unaltered at ∼100 nm s⁻¹ (10 µM ATP) after decoration with DNA oligonucleotides. The retained motility is an interesting result considering the spiky properties of DNA oligonucleotides bound to MT shuttles. Hybridized DNA has a persistence length of ∼50 nm, rendering the MT surface covered with stiff obstacles. Obviously, kinesin motors are not significantly hindered by the DNA strands in finding the tubulin binding pockets. The effects of different molecules attached to MT filaments are discussed in more detail elsewhere [134]. As the fusion of MAPs with MT
Figure 5.3: Loading of 1 µm PS-beads to MTs via biotin/streptavidin interaction. (A) Scheme of the specific adsorption of functionalized microspheres to biotinylated MTs (not to scale): (i.) Microspheres functionalized with SA are directly attached to biotinylated MTs. (ii.) Biotinylated microspheres bind via SA to biotinylated MTs. (B) Agglomerates of 1 µm PS-beads transported by MTs. The biotinylated microspheres are attached via green-fluorescent SA to the shuttles, according to scheme (A ii). (C) Single microsphere transported by a MT: surprisingly, the bead is changing its relative position on the shuttle without falling off, mirroring the reversible property of the biotin–SA interaction. Scale-bars are 10 µm in (B) and 5 µm in (C).
filaments might have severe implications to the cargo transport system in vitro, the results obtained from this model system could be of extensive interest beyond the consequences for the technological use.

Transport systems functioning on the micro-scale are exceedingly interesting for the use in future (bio)sensoric or self-assembly devices. Together with cargo-loading, the discharge of the load is a key feature of such a system. We present a cargo-loading strategy based on DNA hybridization and show that unloading of the cargo occurs upon illumination with laser-light at a specific wavelength. As shown, single-stranded DNA cross-link via biotin–SA interaction with MTs. The complementary DNA strands spontaneously hybridize and are transported. If areas of the MT filaments were photo-bleached at 488 nm, disintegration of the DNA hybridization or biotin–SA interaction was initiated and caused unbinding of the rhodamine-labeled complementary strand (Fig. 5.4 D and E). Thereby, the motility of the affected MT was preserved (see movie). Evidence for the hypothesized disintegration is partly delivered by the absent fluorescent signal of the rhodamine dye attached to the complementary DNA strand (see Fig. 5.4 E). This effect was investigated in detail by Städler et al. [144] and disintegration of DNA hybridization as well as biotin–SA interaction could be verified.

The obtained results demonstrate a possible way of light-controlled cargo discharge with (sub-)micrometer precision (limited by the dimensions of the focal points of the laser). In combination with the light-controlled MTs using caged ATP [14], movement could be initiated by UV light, shuttles could pick-up cargo, transport it to a defined location, and release of cargo could finally controlled by a specific pulse of light.

5.3.4 Loading of 40 nm Anti-biotin Conjugated Au Colloids

40 nm anti-biotin coated Au particles could be readily attached from solution to biotinylated MTs. Even at short incubation times (5–10 min) and low particle concentration (∼2×10⁸ particles per ml), at least one colloid adsorbed per filament (Fig. 5.5 B). As can be seen in Fig. 5.5 A, MT shuttles were covered with multiple gold colloids after 5 min (≥5 particles per µm MT), when incubated with more concentrated particle dispersions (∼2×10⁹ particles per ml). At high colloid coverage, MT movement slowed down to about 50% of the speed of unloaded MTs (depending on ATP concentration). The fast coverage of MTs with anti-biotin functionalized colloids compared to SA-coated particles could be due to higher on-rate of the antibody to the biotin attached to the tubulin
Figure 5.4: (A) Scheme showing loading of MTs with DNA oligonucleotides. (B) Biotinylated MTs are loaded with streptavidin-bound biotinylated single-stranded DNA that hybridizes with its complementary DNA strand. (C) Green fluorescent (Alexa 488) SA bound to the MTs and biotinylated single-stranded DNA (left) and rhodamine-labeled complementary DNA (right). (D) Bleached region in the 488 nm channel. (E) Region bleached at a wavelength of 488 nm shown at 540 nm (rhodamine fluorescence of complementary DNA). Obviously, the loss of fluorescence signal in (D) is not only due to bleaching of the Alexa488-conjugated SA, but causes either the SA to unbind from biotinylated MTs or damages DNA hybridization [144]. Time between images is approximately 30 s (movie). Scale-bars are 10 µm.
5. Cargo Transport Driven by Kinesin Motors

Figure 5.5: Adsorption of anti-biotin coated 40 nm gold colloids to biotinylated MTs same incubation time but different particle concentration (confocal interference images): (A) MTs are covered with several gold colloids per µm MT when incubated for 5 min in a 100 fold diluted Au colloid dispersion (stock concentration: $\sim 2 \times 10^{11}$ particles per ml). Bright spots originate from particles close together (see section 4.4). MTs are still propelled by kinesin motors at this colloid coverage. (B) At 1000 fold dilution of Au particles, single colloids bound to the MTs can be discerned. Scale-bars are 2 µm in (A) and 4 µm in (B).

building blocks of the filaments. Especially as the biotin might be attached only via a short linker to the tubulin, adsorbing to the deep binding pocket of SA might be hindered (compare section 7.3.1).

Gold particles down to a diameter of $\sim 20$ nm can be easily imaged and distinguished from MT filaments using the interference-based detection scheme discussed in Chapter 4, allowing label-free detection of cargo-transport. The dimension of the colloids is comparable to the size of viruses ($\sim$30–300 nm), which are possible cargos of interest in a future biosensoric device.

5.4 Conclusions and Outlook

In this chapter, we presented various interaction types to bind different types of cargo to molecular shuttles. The dimensions of the attached cargo significantly varied from a few nanometers up to several micrometers. The investigated linker strategies are all based on biological bonds, such as receptor–ligand or DNA–DNA interactions. These
interactions have proven to be highly specific and stable for a reasonable amount of time, 
\textit{i.e.} exceeding the lifetime of an envisioned molecular shuttle based sensor device.

We demonstrated the self-assembly of MTs using biotin–SA interactions and showed
that ordered circular structures emerge after a couple of minutes. While these "nanorings"
are detrimental for the formation of long linear extended MT structures, the formed tens
of nanometers thick rings with diameters between 1–10 \(\mu \text{m}\) could be interesting to use as
templates for the fabrication of inorganic microstructures. Moreover, the synergetic effect
of many kinesin motors propelling an MT filament could be visualized, as the motors are
able to exert enough force to break multiple biotin–SA bonds when linear or circular
assemblies of MTs disassemble.

Biotinylated PS microspheres functionalized with SA could be successfully attached
to and transported by biotinylated MT shuttles. Even agglomerates of several 1 \(\mu \text{m}\) PS
beads were transported without loss of motility. However, hindered by slow diffusion of
the relatively large particles and particle assemblies, rather long incubation times were
needed (\(~20\) min) to adsorb a reasonable amount of microspheres to the MT filaments. In
contrast, the small 40 nm anti-biotin coated gold particles adsorbed at significantly shorter
incubation times to the shuttles. At increased particle concentration during incubation,
several colloids per \(\mu \text{m}\) filament could be attached, yet causing decreased motility of
the MT shuttles. In combination with the interference based detection of gold colloids
(Chapter 4), the transport of nanometer-sized Au particles provides a useful model system
before moving to technologically relevant types of cargo, such as viruses.

Functionalization of molecular shuttles with DNA and DNA oligonucleotides pro-
vides a means for very specific sequence-dependent pick-up and loading of cargo
equipped with the complementary DNA strand. Our experiments demonstrate the attach-
ment of hybridized DNA oligonucleotides \textit{via} SA bound to biotinylated MTs. Moreover,
DNA hybridization or biotin–SA interaction could be disintegrated upon photo-bleaching
with a laser at a wavelength of 488 nm [144]. This resulted in the loss of the comple-
mentary strand and could thus constitute a light-controlled method for the discharge of
cargo.

Future studies aim for the detection and transport of biotechnological relevant cargo,
such as viruses,—as shown in a proof-of-principle experiment by Bachand and coworkers
[13]—bacteria or even cells. Evidently, as these biological samples significantly differ
from model cargo in terms of chemical complexity, surface properties and overall stability,
the efficient long-distance transport of such cargo will be challenging.
Cargo Transport in Microlithographic Channels
Abstract

A consignment cannot not depend on a random process, if the freight to be delivered should reach its destination within reasonable amount of time. Likewise, cargo transport enabled by molecular shuttles is directionless and unoriented if the movement of MT filaments is not controlled. Indeed, MT shuttles can be efficiently guided in open micro-channels featuring overhangs as could be for example demonstrated by Hess et al. [5]. In this study, we developed a process to fabricate under-cut channels based on two layers—PMGI and PMMA—that allows full control over the geometry of the overhang. Moreover, we tested the effect of the channel walls on the transport of 40 nm anti-biotin coated Au particles transported by biotinylated MTs. Results indicate increased cargo loss of MTs moving in curved channels constantly being in contact with the channel walls. Moreover, MTs loaded with high density of colloids could be efficiently concentrated in the center of a spiral pattern, potentially providing an elegant means for the active self-assembly of Au nanowires.


6.1 Introduction

Controlled active transport in cells has evolved to mere perfection. Motor proteins carry cargo, *i.e.* vesicles, organelles, and even mitochondria, along a network of protein filaments that spans the entire cell [59]. Polarity of the filaments guarantee directional control over the movement of the motors and enable bidirectional transport of cargo from the center of the cell (cell nucleus) to the cell periphery and backwards. By this means, cargo can be transported up to the distance of one meter (organelle transport along axons) [145], facilitated by the cooperation of several motors [54]. Even though it has been shown *in vitro* that increasing viscosity reduces the velocity of MTs propelled by kinesin motors [146], the movement of the motor-cargo complex through the viscous cytoplasm [1] *in vivo* does not seem to significantly reduce the efficiency of the transport system. Specific interactions between motor and cargo allow a sophisticated cargo recognition process.

With such a powerful toolbox at hand, it is not surprising, that harnessing molecular motors and their corresponding filaments for technological use has become an appealing and interdisciplinary field of research [1, 2]. For this purpose, the natural system was turned upside down: motors attached to a surface to propel filaments. Filaments—referred to as (molecular) shuttles—can be functionalized to specifically bind targeted cargo (see Chapter 5). Mainly, two molecular shuttle systems were investigated, *i.e.* myosin/actin and kinesin/MT. For both systems, strategies were elaborated to gain positional control over filament movement on motor-coated surfaces. In example, shuttle movement could be successfully confined in micro- and nano-structured channels [5, 9, 10, 79, 82, 108, 109, 148-150] and directionality of the movement could be attained by using appropriate pattern-geometries [79]. The high persistence length of MTs (∼5 mm) [89]) is beneficial for the guidance in micro-channels that can be fabricated with less technological effort than nano-channels. Likewise, transport of cargo by molecular shuttles could already be shown previously on plain surfaces, *e.g.* transport of micro- and nanoparticles [11, 85, 94, 95, 137, 151] and protein assemblies [12, 13]. Future biosensor devices are expected to feature both, MT-based cargo transport as well as MT guidance in micro-channels. In this perspective, it is important to know what effect the guided movement of the shuttles has on the transported cargo. Previous studies on plain surfaces discuss interactions between cargo-transporting MTs [11, 85]. There is, however, no knowledge about how channel walls may affect transport of cargo.


\[1\] Cytoplasm shows highly non-Newtonian properties, exceeding the viscosity of water by at least 4 orders of magnitude [147].
In this study, we analyzed how cargo transport is affected by the micro-channels. Unlike previously published by Hess et al. [5, 82] and Doot et al. [67], we fabricated micro-channels featuring overhangs using a novel two-layer approach (Fig. 6.2). Sequentially, poly(methylglutarimide) (PMGI), poly(methyl methacrylate) (PMMA), and photoresist were spin-coated onto a glass substrate. The pattern fabricated on the photoresist then served as a mask during an oxygen plasma etch where the mask-pattern was transferred into the PMGI/PMMA layers. Finally, the overhang geometry was produced by dissolving PMGI but not PMMA. PMMA as well as PMGI exhibit good refractive index match with glass at wavelengths of interest. These patterns were then used to run inverted motility assays. Briefly, surface-bound kinesin motors propel MT filaments that carry 40 nm Au colloids (Fig. 6.1). In order to assess the influence of channel walls on the transported cargo, different parameters such as average transport length and loss-rates in dependence of radius of curvature of the channels, were analyzed. We compare acquired data with possible dimensions of a future lab-on-a-chip device. This data will hopefully contribute to a better understanding of how to design a final device.

Furthermore, significantly increasing the gold colloid concentration in the above mentioned experiments, can lead to fully decorated MTs. By applying appropriate mi-
Figure 6.2: Fabrication process of undercut micro-patterns: (A) PMGI, PMMA and photore sist (ma-N 1410) are sequentially spin-coated onto a 35 mm diameter glass coverslip and subsequently the photoresist was illuminated and developed. (B) In an oxygen plasma step, the photoresist and underlying PMGI and PMMA layers are ablated. (C) To produce the undercut, the patterned surface is developed for 1 min in IPA and 2 min in standard photoresist developer solution. (D) Zoom-in from (C) to demonstrate dimensions of the layers.

cropatterns, these MTs can be concentrated and eventually used either directly or as templates for Au nanowires [142,152,153], which arouse increasing interest for the detection of proteins and protein assemblies (such as viruses) [154,155].

6.2 Materials and Methods

6.2.1 Fabrication of Micro-channels

Glass coverslips were Piranha cleaned for 10 min and extensively rinsed in nanopure water. In order to enhance adhesion of the following layers, HMDS was spincoated (5000 rpm for 45 s) onto the clean glass substrates and baked for 5 min at 100°C. As a first layer, PMGI \(^2\) was spincoated at 3000 rpm for 45 s. After cleaning the sample edge with

\[^2\]PMGI S8, 8% in cyclopentanone and tetrahydrofurfuryl alcohol, MicroChem, Newton (MA), USA
Table 6.1: RIE settings for the plasma etching procedures in the micro-channel fabrication process (RF = radio frequency, DC = direct current)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>O₂ etch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas</td>
<td>O₂</td>
</tr>
<tr>
<td>Gas flow (sccm)</td>
<td>50</td>
</tr>
<tr>
<td>Pressure (mTorr)</td>
<td>50</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>19</td>
</tr>
<tr>
<td>RF power (W)</td>
<td>97</td>
</tr>
<tr>
<td>DC bias (V)</td>
<td>~340</td>
</tr>
<tr>
<td>Process time (s)</td>
<td>450</td>
</tr>
</tbody>
</table>

acetone (to get rid of resist bulge, repeated after each coating step), the PMGI was baked at 180°C for 3 min. Secondly, PMMA (950K, undiluted) was spincoated at 4000 rpm for 45 s on top of the PMGI layer, and subsequently baked at 180°C for 30 min. Finally, ma-N 1410 negative photoresist was spincoated at 3000 rpm for 30 s. After baking at 95°C for 150 s, the photoresist was exposed for 55 s at 8.5 mW cm⁻² (≈ 500 mJ cm⁻²). The photoresist layer was then developed in ma-D 376XP developer solution for 150 s.

PMGI/PMMA layers were etched in an O₂ plasma for 7.30 min using the parameters listed in Table 6.1. After the etching process, samples were developed for 1 min in IPA and 2 min in ma-D 376XP developer solution to generate the undercut. Samples were quality-checked under the microscope after each critical step, i.e. photolithography and undercut formation. The channel height was determined by profilometry.

6.2.2 Cargo Transport Assays

Restriction of Microtubule Movement to Micro-channel Bottoms

In order to restrict MT movement to the glass channel bottoms, it was tried to render the PMMA surface protein repellent [156]. Therefore, acrylamide hydrogels were soaked in a 1 mg ml⁻¹ PLL-g-PEG solution (in DI water) for 20 min and then stamped onto the

---

3% in chlorobenzene, MicroChem, Newton (MA), USA
4MicroResist Technology, Berlin, Germany
5Oxford Instruments RIE 80+, Oxfordshire, UK
6Surface Step Profiler, Tencor Alpha-Step 500, San Jose (CA), USA
plasma-cleaned (15 s) micro-channel substrates according to Ochsner et al. [157]. Samples were then used to assemble flowcells (see below) and before starting the motility assay rinsed extensively with BRB80 buffer to remove unbound PLL-g-PEG.

Alternatively, micro-channel surfaces were treated with HMDS to achieve hydrophobic glass bottom surfaces while leaving the PMMA surface partly hydrophilic, accessible for PLL-g-PEG adsorption. Microfabricated substrates were incubated in a petri-dish including a Kimwipe tissue soaked with a few drops of HMDS for 1 h. Samples were then used to assemble flowcells for motility assays. Before injecting the protein solutions (see below), the flowcell was rinsed with an 0.05 mg ml$^{-1}$ PLL-g-PEG solution (in nanopure water). After 30 min of incubation, the flowcell channel was rinsed extensively with BRB80 buffer solution.

**Motility Assay**

Prepared 35 mm glass wafers (see above) provided 4 samples for flow cell assembly. The wafers were cut into 4 pieces using a diamond cutter, blown off with filtered air and subsequently air plasma treated for 10 s to render the surface hydrophilic. The flow cell was then assembled by putting double-coated tape (Scotch) onto an air plasma cleaned $24 \times 50 \text{mm}^2$ glass coverslip bottom and by placing the patterned surface on top. The channel was then filled with a 0.5 mg ml$^{-1}$ casein solution to reduce kinesin denaturation. After 5 min, kinesin ($\sim 40 \mu g \text{ml}^{-1}$ kinesin, 0.5 mg ml$^{-1}$ casein, 20 $\mu M$ ATP in BRB80) was adsorbed for 5 min and then replaced by a motility solution (3.2 $\mu g \text{ml}^{-1}$ MTs$^7$, 0.2 mg ml$^{-1}$ casein, 10 $\mu M$ taxol, 20 $\mu M$ ATP, 20 mM D-glucose, 20 $\mu g \text{ml}^{-1}$ glucose oxidase, 8 $\mu g \text{ml}^{-1}$ catalase, 1% DTT in BRB80) for 5 min. After MT adsorption, the flow cell was rinsed with a motility solution not containing MTs. Antibiotin-coated 40 nm Au colloids (diluted in motility solution) were then injected for 5 min. Finally, the flow cell was rinsed again with motility solution, sealed with immersion oil and mounted onto the sample holder for confocal imaging.

**Image Acquisition and Data Analysis**

As model cargo, we chose 40 nm gold(graft-anti-biotin) colloids allowing fluorescence-free detection and tracing using an interference-based confocal scheme as reported earlier.

---

$^7$80% biotinylated, 20% rhodamine-labeled
6. CARGO TRANSPORT IN MICROLITHOGRAPHIC CHANNELS

(see references [122], [123] and Chapter 4.4 for details). Both the interference signal from the gold colloids and the fluorescent light originating from rhodamine-labeled MTs (using an interference filter omitting wavelengths below 560 nm) were detected simultaneously. Image sequences at 800×800 px² resolution (105.6×105.6 µm² field of view resulting in a pixel resolution of 132 nm) were acquired using a pinhole width of 120 µm and a pixel dwell-time of 10 µs px⁻¹ resulting in a scan rate of ~7.4 s per frame. Image sequences were acquired from three different pattern regions: (1) spiral center, (2) curved spiral entrance-channels, and (3) straight micro-channels. Colloids were tracked manually using ImageJ and the transport distance was calculated from obtained coordinates of the particles. In cases where cargo could not be tracked continuously (e.g. because not visible underneath the overhang), frames were skipped.

6.2.3 Gold Colloid Assembly

As the spiral centers of the pattern trap MT filaments due to the chosen geometry, shuttles accumulate over time. At higher Au colloid density on MTs, the formation of Au nanowires was expected inside the spiral center. Motility assays as described in section 6.2.2 were therefore prepared using higher Au colloid concentration during incubation (~4×10⁹ particles per ml). Particles were adsorbed to fully biotinylated MTs for 10 min at low ATP concentration (10 µM) in the motility assay. After incubation, the ATP concentration was increased to 100 µM and the assay was run for 1 h to allow MTs to accumulate inside the center of the spirals. Assays were then either imaged using confocal microscopy or prepared for SEM imaging. Samples for SEM were prepared using semi-adhesive tape as spacer to assemble the flowcell. This tape allowed easy disassembly of the flowcell after sample preparation. SEM samples were fixed using 1% glutaraldehyde solution (in motility solution) for 5 min and, after disassembly of the flowcell, the samples of interest were extensively rinsed in nanopure water and dried in filtered air.

—

8Polyolefin Plastomer Affinity EG8150G, Dow PLastic
6.3 Results and Discussion

6.3.1 Micro-channel Fabrication

Micro-channels with overhang geometry were successfully fabricated by applying the above mentioned two-layer approach. The two-layer approach allows high versatility in adjusting the geometry of the overhang: the thickness of the spin-coated layers can be easily controlled via the spinning speed and viscosity of the resist. Hence, overhang height can be increased to heights exceeding the 200 nm as shown by Hess et al. [5]. This offers a broader range in the choice or type of transported cargo. The chosen geometry of the pattern is shown in Fig. 6.3 A. HMDS treatment of the samples significantly increased the adhesion of the PMGI to the substrate surface. This way, delamination of the PMGI layer could be prevented. PMGI and PMMA layers were spincoated at thicknesses of ∼500 nm, each. The O₂ plasma etching of the PMGI/PMMA layers using the developed photoresist layer as mask proved to be well reproducible. PMGI and PMMA were etched down to the glass substrate. Possible PMGI residues were dissolved in the subsequent overhang formation, performed by wet-etching the PMGI in developer solution. At an etch-rate of ∼250 nm min⁻¹, micro-channels with overhangs of 500–600 nm were produced (Fig. 6.3 B). Due to the overhang, the final channel width at the interface to the glass substrate was approximately 3 µm (Fig. 6.3 B).

The low auto-fluorescence of PMGI and PMMA at the excitation wavelength of ∼540 nm allowed proper visualization of rhodamine-labeled MTs. In addition, PMGI and PMMA exhibit refractive indices very close to glass at the wavelength of interest. This reduced undesirable optical effects during the interference-based imaging to a minimum. However, light-scattering at the edges of the resist layers still complicated optical detection of gold colloids in the channels, as can be seen in Fig. 6.4 B and 6.6 A. While MTs moving under the PMMA overhang could be easily resolved by fluorescence, they could be hardly imaged using the interference method. This complicated the tracking of the 40 nm gold colloids, as they were only visualized by interference. The use of fluorescent cargo (e.g. fluorescently labeled particles, quantum dots, etc.) will be inevitable in future experiments, especially when automated particle tracking is considered for data analysis.
Figure 6.3: Microfabrication of patterns based on the design shown in (A). Channel width is 2 µm and arrowheads are \( \sim 10 \times 10 \) µm². (B) SEM image of the overhang geometry. As illustrated in (C), an overhang of \( \sim 500 \) nm was achieved by dissolving the PMGI layer. (D) and (E) show the spiral center and the arrow geometry of the patterns. SEM images were acquired at an acceleration voltage of 5 kV and a working distance of 4 mm. Scale-bars are 2 µm in (B), 4 µm in (D), and 6 µm in (E).
6.3.2 Microtubule Movement and Cargo Transport Inside Microfabricated Channels

In previous works [5, 9, 10, 79, 82], guidance and unidirectional movement of MTs in microfabricated channels were already extensively studied. Open microfabricated channels featuring overhang geometry have proven to be suitable for the effective guidance of MTs without the need of chemical contrast to restrict shuttle movement [5]. Not only unidirectional movement of \( \geq 90\% \) of MTs could be achieved by incorporating special geometrical features such as side-arms [82] or arrowheads [9], but also concentration of MTs inside the center of a spiral could be realized [82]. We combined these features, i.e. overhang, arrowheads and spiral geometry, into one pattern.

MTs in our assays moved at an average speed of \( \sim 200–300 \text{nm s}^{-1} \) (30 \( \mu \text{M} \) ATP), and were successfully guided inside the microfabricated channels (Fig. 6.4). Restriction of MT movement to the channel bottom could not be realized by chemical means: samples prepared by stamping PLL-g-PEG or HMDS evaporation (as described in section 6.2.2) to prevent the adsorption of kinesin motors to the PMMA surface exhibited very low MT density and almost no motility on the glass surface of the channel bottom [156]. However, the adsorption of MT shuttles on PMMA only manifests in complicated analysis of cargo transport if cargo is loaded from solution. A future device collecting cargo dispersed in solution could even profit from this effect, since shuttles moving on the PMMA eventually enter the channels and therefore contribute to the accumulation of cargo in the center of the spiral. As can be seen in Fig. 6.4A, a significant percentage of MTs is trapped inside the spiral center after 30 min. MTs continuously transport cargo picked up from solution towards the spiral, as demonstrated in Fig. 6.4B and C. Thereby, shuttle movement in the wrong direction is effectively adjusted by the arrowheads (not quantified), shown in example in Fig. 6.4C at 60 s.

40 nm anti-biotin coated Au particles were picked up from solution by biotinylated MTs and transported over several micrometers as illustrated in the image sequence in Fig. 6.4B. The concentration of particles dispersed in the motility solution was adjusted to separate single particles attached to MT filaments. On average, one particle per \( \mu \text{m} \) MT was transported. However, as multiple MTs glide along the channel walls, transported colloids often overlay and therefore complicate analysis of transport (see for example Fig. 6.4B at 120 s close to channel junction). In addition, as already mentioned in section 6.3.1, the confocal interference based imaging scheme hardly detects colloids moving underneath the PMMA overhang. For this reason, Au colloid movement could
Figure 6.4: (A) Images of the rhodamine fluorescence of MTs moving in engineered micro-channels at the beginning of imaging (0 min) and after 30 min: most of the MTs that moved in the arrowhead regions of the pattern reached the center of the spiral after 30 min. Due to the chosen geometry of the spiral center and due to the rigidity of the MT filaments, a high percentage of shuttles is trapped. Image sequences (B) and (C) show a magnified part of the pattern. Confocal interference (B) reveals the transport of various gold particles (one highlighted by red arrow). MTs are reflected by arrowheads and redirected into the desired direction (C, red arrowhead). In many cases, the rhodamine fluorescence of the MTs (C) provided a valuable control, as shuttles shuttles that move close along the pattern walls are hardly visible in the interference images. Scale-bars are 10 µm in (A) and 2 µm in (B) and (C).
Figure 6.5: The travel distances of transported cargo till loss was determined by manual tracking. (A) Cargo transport length inside spiral geometry: more than 50% of tracked colloids are transported no further than 100 µm. MTs are regularly touching the walls of the micro-channels when moving inside the spirals, thus probably causing increased loss of particles. (B) Particle loss inside straight channels: max. distance is $\sim$105 µm (field-of-view). A total of 16 particles did not fall off during the length of the image sequence and moved out of the field-of-view (arrow). (C) Reasons for the loss of cargo are diverse: in many cases, cargo spontaneously dissociated, and in 12 out of 31 cases, the reason of loss was unclear, because the particle was positioned under the overhang and therefore not visible [156].
often not be followed continuously. At given MT speed and duration of imaging (25 min), the maximal theoretical transport distance of the cargo amounts to $\sim 400 \, \mu m$. In the actual assay, the transport distance depends on the chosen section of the observed pattern, i.e. spiral or straight lines. In order to evaluate the influence of channel walls and MT interaction during cargo transport, the transport length of 25 particles in the spiral and 23 particles moving in the straight channels (including arrowheads) were analyzed and the reasons for loss of cargo were determined (Fig. 6.5). The average transport distance inside the spiral is $\sim 130 \, \mu m$ (Fig. 6.5A), but many particles are already lost after a maximum transport distance of $80 \, \mu m$ (11 out of 25). In comparison to the transport length in the straight channels (Fig. 6.5B), where almost 70% of the tracked cargo was transported further than the field-of-view ($\sim 105 \, \mu m$), more cargo was dropped inside the spiral. This could be an indication for the influence of the channel walls on the transported cargo. If the limited transport length proves true in additional future experiments, the dimensions of the pattern, in example the length of the spirals, have to be adjusted accordingly.

The reasons of loss were analyzed for 30 colloids (Fig. 6.5C). Many particles were lost due to spontaneous dissociation, which can be related to the reversible character of the anti-biotin–biotin interaction(s). For almost a third of the analyzed cargo, though, the reason for loss could not be determined, as MTs and the transported colloids were situated underneath the PMMA overhang. Since MTs contacting the walls during movement is more likely to happen in curved channels (such as the spiral), the visualization of the transported cargo in the undercut will be crucial for further investigation regarding the influence of the channel walls on the transport length. This could be solved by using fluorescently labeled cargo, such as quantum dots. Single events also show cargo loss or transfer via MT–MT interactions as described by Boal et al. [85]. These effects are probably even more prominent in the confined channels, especially at high shuttle concentration. Merging of two cargo-carrying MTs, however, can be considered favorable, since that way the number of cargo–MT interactions are increased, which results in increased stability of the cargo–shuttle complex. This was mainly observed in the spiral center, where MT concentration was increased over time. Furthermore, we analyzed cargo movement through curved channels with differing radii (Fig. 6.4B, C) and could not find a correlation between increased particle loss with decreasing radius of curvature of the channel. The time, MTs are in contact with the walls in these areas of the pattern might be too short to see any effect on the transported cargo. Given the small amount of statistical data, more experiments have to be performed and analyzed to be able to draw significant conclusions on how the microchannels influence cargo transport.
6.3.3 Gold Assembly Inside the Spiral Center

In order to analyze cargo transport in micro-channels, it was important to adsorb only a discrete number of gold colloids onto MTs. This way, single colloids could be tracked and followed. By increasing the gold colloid concentration in the motility solution (up to \( \sim 4 \times 10^9 \) particles per ml), MTs were loaded with a considerably higher amount of Au colloids (see also Fig. 5.5 A). The anti-biotin coated 40 nm Au colloids were picked up out of solution and transported towards the spiral centers of the micro-pattern to form high colloid density Au nanowires (see Fig. 6.6 A and the according movie). The interference image (Fig. 6.6 A) reveals the gold particles transported in a clockwise movement in the spiral center. Single particles appeared dark compared to the background, particles closer than the optical resolution of \( \sim 250 \) nm change contrast and appeared bright compared to the background. Rhodamine fluorescence of MTs served as control for the colocalization of MTs and gold colloids.

However, the use of this system for nanowire assembly remains questionable. The images acquired by confocal interference (Fig. 6.6 A) simulate higher colloid density on the MT filaments due to the optical resolution limit. Therefore, SEM samples were prepared to verify the actual particle density. As can be seen in Fig. 6.6 C, the "real" density of colloids attached to MT filaments is on the order of \( \sim 5 \) particles per \( \mu \)m. This density is too low to form a continuous wire, even when increasing the particle diameter via gold enhancement [144]. In order to increase the particle density on the MT filaments in future experiments, the biotin density on the tubulin building blocks of the MTs needs to be increased. This can be achieved in example by functionalizing the filaments with branched PEG-biotin as suggested by Wahnes [158]. However, since MTs assembled into bundles (as shown in Fig. 6.6 C), the thickness of the resulting nanowires might vary significantly, depending also on the diameter of the used Au colloids. As wires with defined thickness and electrical conductivity are required for the use as biosensors [154, 155], the Au assembly based on molecular shuttles might be challenging.

6.4 Conclusions and Outlook

For the fabrication of a future sensor device based on molecular shuttles, it is important to establish a reproducible and flexible way of producing efficient patterns to gain control over the movement of the shuttles. In previous studies by Hess and Clemmens et al. [5]...
Figure 6.6: Loading of 40 nm anti-biotin functionalized gold particles (50-fold diluted in motility solution) leads to colloid concentration in the center of the spiral pattern. (A) Interference image revealing the gold particles. Single particles appear dark compared to the background, multiple particles close together (closer than optical resolution of $\sim250$ nm) appear bright compared to the background. (B) Fluorescent equivalent showing the rhodamine labeled MTs. The movie illustrates the movement of the colloids bound to the MTs. (C) SEM images reveal the "real" density of colloids attached to the MTs in the spiral center. Scale-bars are 5 $\mu$m in (A) and (B), and 1 $\mu$m in (C).
it could be demonstrated that topographical patterns featuring an overhang geometry provide an efficient means to guide shuttle movement. However, their photolithographic approach only allows limited control over the dimensions of the overhang. Here, we present a two-layer approach, based on a first layer of PMGI and a second layer of PMMA, to generate overhang geometry. MTs were efficiently guided in fabricated channels at a PMGI thickness of \( \sim 600 \) nm and an overhang height of \( \sim 600 \) nm. By adjusting the layer thickness and the development time after patterning of the underlying PMGI layer, the dimensions of the overhang could be easily controlled. This way, design parameters for future devices can be adjusted to the size of cargo transported by the molecular shuttles. In the future, the somehow intricate fabrication process via reactive ion etching of the PMGI/PMMA pattern using the negative photoresist as mask could be circumvented by using deep UV illumination to directly illuminate and develop the PMMA layer.

40 nm antibiotin-coated gold particles were successfully attached to biotinylated MTs moving in micro-fabricated channels and imaged using the interference-based technique discussed in Chapter 4. Visualization of gold colloids moving underneath the overhang, however, was limited, and particle movement could not always be followed. This flaw needs to be considered in future studies. Most likely, fluorescent cargo, such as quantum dots or fluorescently-labeled particles reduce imaging problems close to the channel walls. For the same reason, the analysis of how contact with the channel walls influences the transport length of the cargos, was complicated. In the spiral geometry of the pattern, cargo was transported on average over a length of \( \sim 130 \) µm. This is less (at higher speed) if compared to cargo loss on unpatterned surfaces (see Chapter 7), suggesting influence of the micro-channel walls. Indeed, in many cases, cargo was lost during abidance of the shuttle in the undercut. However, exact causes for the loss could not be determined for the above mentioned reasons. Limited transport length will have implications on the final dimensions of a device. Based on our results, travel distances (defined by the chosen dimensions of the pattern) should preferably be reduced to \(< 100 \) µm, in order to achieve reasonable yield of cargo in the MT concentrator region. However, even with the limited amount of statistical data, it can be said that another reason for the loss of cargo, i.e. spontaneous dissociation, plays a comparably important role, occurring for approximately a third of the analyzed particles. This problem can be approached by using different linker strategies or by increasing the multiplicity of interactions between shuttle and cargo. Significant conclusions on how the channel walls affect cargo transport in micro-fabricated channels will require more statistical data in the future. This will be inevitable, since only reliable data can predict the feasibility and performance of an envisioned sensor device.
Due to the special geometry of the spiral center, MTs transporting Au particles could be trapped and accumulated over time. From optical images it was expected to achieve a colloid density sufficient for the fabrication of gold nanowires via gold enhancement. However, as shown by SEM studies, the particle density is not higher than \( \sim 5 \) particles per \( \mu \mathrm{m} \), and are not perfectly linearly aligned, since multiple cargo-transporting MTs contribute to the overall density. Moreover, large density fluctuations are observed that make it nearly impossible to achieve a continuous, and thus electrically conductive, metal wire. Nevertheless, higher colloid density on MT shuttles could be achieved by increasing the number of functional units on the shuttle, e.g. by using branched, dendritic end-functionalized PEG chains. If successful, more appropriate patterns could be used, such as simple wells, to collect MT–cargo complexes. This would be an elegant, self-driven way for the assembly of colloidal gold into mesoscopic, nanometer thick metal wires.
Cargo Pick-up from Engineered 2D Surfaces
Abstract

Exploiting biological motors \textit{ex vivo} to transport and distribute cargo with high spatial control, as done by cells, requires that we learn how molecular shuttles (MTs propelled by kinesins) can pick up cargo from defined surface regions (loading stations). The main challenge of building micro-fabricated cargo loading stations is to adjust the sum of non-covalent interactions such that the station stably holds on to the cargo under static conditions, but allows for transfer when a gliding MT collides with station-bound cargo and starts to pull on it. Successful pick-up of cargo could be observed using biotin–anti-biotin interactions and hybridized oligonucleotides. The effect of different tethering chemistries on the efficiency of cargo pick-up was tested \cite{159}.

7.1 Introduction

In the macroscopic world, complex objects are typically mounted piece-by-piece on assembly lines. In contrast, building complex systems at the nanoscale *ex vivo* still depends on mainly two approaches, either the highly parallel process of molecular assembly [160–162], or the time consuming sequential structuring or manipulation of matter by man-made nanotools [163–168]. While highly desirable, no analogues to macroscopic assembly lines exist at the nanoscale that enable the sequential assembly of diverse nanoparts into complex systems. Yet, cells utilize biological motors for active transport of cargo along their respective filaments to specific destinations in order to regulate local concentrations of macromolecules and sequential assembly processes with high spatial precision.

Analogous to the macroscopic world, it is intriguing to drive directed transport along engineered tracks by nanoscopic motors. Due to the lack of appropriate synthetic motors that perform well if loaded [15], biological motors have been integrated in the past into microfabricated environments (Fig. 7.1). Feasibility of the following concepts was demonstrated in proof-of-principle experiments: controlling the unidirectional movement of motor-driven transport along engineered tracks [5, 9, 67, 79, 81, 82, 149, 169], adjusting the speed of the transport system on user demand by light [14, 170], and specifically attaching cargo from solution [12, 13, 53, 68, 84, 151]. Due to the high bending rigidity of MTs, which reduces their chance of turning around in microchannels, MT filaments propelled by surface-anchored kinesin motors have been most frequently exploited as cargo carrier systems [2, 95, 171].

Current developments aimed at utilizing the kinesin/MT system for active transport tasks in engineered devices. They focused on various aspects which include (a) active steering of MTs by external electric or magnetic fields [73, 172, 173], (b) the physical manipulation of surface-bound objects by moving MTs, *i.e.* the stretching of molecules [94, 174], and utilizing cantilevered MTs to probe receptor-ligand interactions subjected to tensile force [6], (c) speed control of the gliding MTs by light [14, 170, 175] and by thermoresponsive polymers [176], and (d) the loading and transport of biomedically relevant [13, 84, 177] or engineered cargo [62, 66, 95, 178].

Engineering effective cargo loading stations requires that the cargo is bound sufficiently stable to the surface for the lifetime of the device, yet is picked up efficiently by passing MTs. Successful cargo pick-up thus depends on fine-tuning the strengths and/or number of the bonds involved (Fig. 7.1B). An added advantage of picking up the cargo
from surface-bound stations rather than from solution, as frequently done in the past [11–13, 84, 85, 137, 151, 178], is that cargo-pick-up and its subsequent utilization can be spatially separated. A sufficiently high cargo density has to be achieved within the loading stations to allow for frequent collisions between passing shuttles and cargo without the MTs getting stuck or being reflected upon entry. Finally, since the MTs are propelled by the surface anchored and tilted kinesins, thus gliding roughly 17 nm above the surface [74], the pick-up efficiency of cargo is further increased if it is not directly bound but tethered to the surface via a sufficiently long linker as illustrated in Fig. 7.1B.

Based on these demands, we designed a few alternative cargo tether systems that reversibly link anti-biotin functionalized gold colloids to microfabricated surface patterns by using reliable and widely known self-assembly techniques [179] (Fig. 7.2): (1) Biotinylated Poly(L-lysine)-graft-Poly(ethylene glycol) (PLL-g-PEG-biotin) [180] binds to negatively charged surfaces via its cationic PLL backbone chain, whereas biotin—bound to 16 respectively 50% of the ends of PEG side chains—is recognized by the anti-biotin antibodies on the cargo surface (Fig. 7.2A). Tethers are flexible PEG chains with a contour length of approximately 15 nm in this case. (2) Alternatively, 3′-thiolated single stranded deoxyribonucleic acid (DNA) oligonucleotides micropatterns were produced on gold micropatterns. If hybridized with a biotinylated complementary DNA strand, cargo can again be immobilized via recognition of the cargo-bound anti-biotin antibodies (Fig. 7.2B). In this case, the tether has approximately the same length, but is stiff and equipped with flexible linkers on both ends. The advantage of this second approach is that the binding strength of DNA-based surface tethers can in principle be tuned by changing the number of overlapping base pairs [181]. Further tuning of bond strength can also be achieved by switching the symmetry of pairing the oligonucleotides: if hybridized in parallel (as done here), the bonds between all nucleotides have to be broken all at once (shearing), while they are broken in a zipper-like fashion for the antiparallel arrangement—requiring far less force [182].

The proof-of-principle for how to link the cargo to the surface tethers was given for a common antibody-antigen interaction, i.e. anti-biotin–biotin, which resists stronger forces than the hybridized double-stranded DNA fragments [181–183]: Anti-biotin-conjugated 40 nm gold nanoparticles were used here as model cargo as they are in the size-range of inorganic quantum structures and virus particles. A technical advantage of particles in this dimension is that they neither cause significant friction/drag forces [13] nor a problem of MT bending and severing upon shuttle crossings [85], both of which may play a role for the transport of larger particles.
Figure 7.1: (A) Motility assay: in contrast to intracellular transport, where cargo is bound to motor proteins (e.g., kinesins or dyneins) and transported along MT filaments, the inverted assay uses surface-adsorbed kinesin motors to move functionalized microtubules—molecular shuttles—along a surface [69]. Kinesin motors bind to the tubulin building blocks of the MTs, each exerting up to 5 pN of drag force [52], and thus propel the movement of the filament. Casein passivates the substrate surface and prevents the denaturation of the kinesin motors, thus preserving their function. (B) Illustration of cargo pick-up from loading stations by molecular shuttles. Functionalized MTs move into cargo-immobilized areas (loading stations) to (1) pick-up and (2) transport the load into cargo-free areas. Cargo is immobilized via reversible linkers to allow the passing shuttle to bind to the cargo and break the surface tether (1). The scheme is roughly to scale: tethers keep the cargo in a favorable position for pick-up.
7.2 Materials and Methods

7.2.1 Substrate Preparation

PLL-g-PEG-biotin based cargo-binding areas were produced by adsorbing PLL(20)-g[3.5]-PEG(2)-biotin with 50% (PPB50) and 16% (PPB16) biotinylation into the photoresist pattern on a glass coverslip. Glass coverslips were cleaned in Piranha solution for 10 min and extensively rinsed in de-ionized water. Surface patterns were created using standard photolithography: Shipley S1818 positive photoresist was spin-coated (4000 rpm, 100 s) onto pre-baked (180°C, 5 min) surfaces, baked at 110°C for 2 min and subsequently exposed to 405 nm UV light at 7.5 mW cm−2 for 20 s using a Karl Süss MJB3 mask aligner. Samples were then developed (1:1 developer concentrate:water mix) for 60 s, rinsed in water and dried under a nitrogen stream. Glass coverslips with photoresist patterns were rinsed in de-ionized water, dried using filtered air and then air plasma-cleaned for 10 s. PPB50 and PPB16, respectively, were dissolved in BRB160 (160 mM PIPES, 4 mM MgCl2, 2 mM EGTA, pH 6.85 with KOH) at a concentration of 0.05 mg ml−1 and adsorbed for 15 min onto the prepared surfaces. After adsorption, samples were rinsed in water and dried with filtered air. In order to strip the photoresist, the surfaces were sonicated for 5 min in 1-methyl-2-pyrrolidinone, rinsed for 5 min in water and finally dried using filtered air [179]. Flow cells were assembled using an air plasma-cleaned (30 s) coverslip slide as bottom, double-coated tape as spacer and the treated sample as top. Casein protein (1 mg ml−1 in BRB160) was injected and adsorbed for 5 min in order to block free glass surface. Followed by a 20 µl buffer rinse, anti-biotin conjugated 40 nm gold colloids at 20-fold dilution of the stock solution were added to the flow cell and adsorbed for 1 h. Unbound colloids were washed out with 100 µl buffer solution.

DNA based loading areas were fabricated analogous to the PLL-g-PEG-biotin system. All oligonucleotides were purchased from MedProbe. 3’-thiol-modified 44 nucleotide (nt) single-stranded DNA (thDNA) (5’ AGT ACT CAG TTG CTT GCA AAT ATC TAA TAC CTT AGC ATT CCC CC SH 3’) was adsorbed overnight at a concentra-
7.2. MATERIALS AND METHODS

7.2.1 Preparation of DNA Arrays

7.2.1.1 Surface Immobilization and Hybridization with Complementary DNA Strands

A solution of 8 µM (in BRB160) into the photoresist pattern on gold-coated glass coverslips (2 nm Cr and 50 nm Au). After photoresist lift-off (see above), flow cells were assembled and the surface was incubated with casein solution (0.5 mg ml⁻¹) for 5 min to block the free gold surface between the thDNA-patterned areas. Upon rinsing, the complementary 3'-biotinylated DNA strands—44 nt (5' AAT GCT AAG GTA TTA GAT ATT TGC AAG CAA CTG AGT ACT CCC CC biotin 3') and 17 nt (5' CAA CTG AGT ACT CCC CC biotin 3'), respectively—were injected into the flow cell at a concentration of 8 µM and hybridized at 4°C for 2 h resulting in a hybridization length of 39 bp DNA setup and 12 bp DNA setup, respectively. After a buffer rinse, anti-biotin conjugated 40 nm gold colloids were adsorbed for 1 h. Unbound colloids were extensively washed out with buffer solution. In addition to hybridized DNA, single stranded 49 nt, 3'-thiol-modified and 5'-biotinylated DNA strands (49 nt DNA) (5' biotin CC CCC AGT ACT CAG TTG CTT GCA AAT ATC TAA TAC CTT AGC ATT CCC CC SH 3') were used in a control experiment. Except for the hybridization step, samples were prepared as described before.

7.2.2 Cargo Pick-up Assays

MT shuttles consisting of 80% biotinylated and 20% rhodamine-labeled tubulin (in the following simply referred to as MTs) are propelled by surface-bound kinesin motor proteins (expression and purification of the wild-type D. melanogaster conventional kinesin gene are described elsewhere [42]) through loading stations prepared to pick-up cargo. MTs were prepared by polymerizing mixtures of biotin- and rhodamine-labeled tubulin at a mass ratio of 80:20. BRB80 buffer (80 mM PIPES, 2 mM MgCl₂, 1 mM EGTA, pH 6.85 with KOH) containing 4 mM MgCl₂, 1 mM GTP and 5% DMSO was added to a 20 µg aliquot of rhodamine-labeled tubulin⁷ and to a 20 µg aliquot of biotinylated tubulin⁸ to yield tubulin concentrations of 3.2 mg ml⁻¹, each. Both tubulin solutions were mixed together, vortexed and put on ice for 5 min to achieve a homogeneous mixture of differently labeled tubulin proteins. After polymerization for 30 min at 37°C, the biotin-rhodamine-labeled MTs were diluted 100-fold in BRB 80 containing 10 µM taxol as stabilizer. Motility assays were started immediately after flow cell assembly in order to probe the suitability for cargo pick-up by molecular shuttles of both PLL-g-PEG-biotin and DNA based systems. A solution of 0.5 mg ml⁻¹ casein in BRB 160 buffer was injected into the assembled flow cell to passivate the surface and to reduce kinesin denaturation. After

⁷TL 331-M, Cytoskeleton, Denver, CO, USA
⁸T 333-B, Cytoskeleton, Denver, CO, USA
Fabrication process of loading stations for cargo pick-up: cargo-patterned areas are produced by combining photolithography and self-assembly [179]. (A), (B) Photoresist-patterned surfaces were fabricated using standard photolithographic methods. The photoresist on the glass and the 50 nm Au-coated surface served as a mask for the adsorption of PLL-g-PEG-biotin (I.) and thiol-modified oligonucleotides (i.), respectively. After adsorption, the photoresist is stripped in both systems using 1-methyl-2-pyrrolidinone (NMP) (ii., II.). Following the photoresist lift-off, casein proteins are used to backfill and passivate the free surfaces in both approaches (iii., IIIa.). The complementary biotinylated DNA strands) are hybridized in the DNA-based setup (IIIb.) before 40 nm Au-(anti-biotin) conjugates are adsorbed (iv., IV.). Schemes are not to scale.
5 min, the casein solution was exchanged for a kinesin solution (∼40 µg ml$^{-1}$ kinesin, 0.5 mg ml$^{-1}$ casein, 20 µM ATP in BRB 160). Five minutes later, a motility solution (3.2 µg ml$^{-1}$ MTs, 0.2 mg ml$^{-1}$ casein, 10 µM taxol, 20 µM ATP and oxygen scavenging additives 20 mM D-glucose, 20 µg ml$^{-1}$ glucose oxidase, 8 µg ml$^{-1}$ catalase, 1% DTT in BRB160) passed through a 30 G needle (to shorten MTs) was added to the flowcell and incubated for 5 min to let MTs adsorb to the kinesin motors. Subsequently, the flowcell was rinsed with 20 µl buffer only containing taxol, ATP and oxygen scavenging additives. The shuttle speed was adjusted to ∼80–100 nm s$^{-1}$ by lowering the ATP concentration (max. MT speed at 1 mM ATP ≈ 800–1000 nm s$^{-1}$). This ensured traceability of the transported particles. To prevent quick evaporation of the solution, channel ends were sealed using immersion oil. All experiments were performed at RT.

7.2.3 Image Acquisition and Data Analysis

For both approaches, we chose 40 nm gold(graft-anti-biotin) colloids as model cargo allowing fluorescence-free detection and tracing using an interferometric confocal scheme as reported earlier [122, 123]. Both the interference signal from the gold colloids and the fluorescent light originating from rhodamine-labeled MTs (using an interference filter omitting wavelengths below 560 nm) were detected simultaneously. Image sequences at 800×800 px$^2$ resolution (105.6×105.6 µm$^2$ field of view resulting in a pixel resolution of 132 nm) were acquired using a pinhole width of 300 µm and a pixel dwell-time of 10 µs px$^{-1}$ resulting in a scan rate of ∼7.4 s per frame. For each setup, sequences of 100 frames were recorded at three different positions of the according sample.

Image sequences acquired by confocal microscopy were prepared for particle tracking using ImageJ$^9$. After contrast adjustment images were inverted as the feature point tracking algorithm [184] used to analyze colloid movement is searching for local intensity maxima. Due to a limited optical resolution, tracking of particles inside the patterned areas was not possible. Consequently, paths of tracked particles were inevitably cut by the loading zones resulting in an increased number of events. This effect, however, was considered a systematic error equal for all analyzed setups. Parameters listed in Table 7.1 were used for the automated particle tracking.

All tracks were normalized to the total MT length per area (field of view) in each setup. MT lengths were determined by thresholding rhodamine-labeled MT sequences.

---

Table 7.1: Parameter settings for the algorithm in the feature point tracking software [184] used for the analyzed setups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DNA setups</th>
<th>PPB setups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle radius [px]</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Intensity percentile [%]</td>
<td>4.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Cutoff score [-]</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Maximum step length [px]</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Link range [frames]</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

skeletonizing the resulting images and calculating the stack histogram of the sequence using ImageJ. The total number of black pixels from the resulting histogram yielded the total MT length.

7.3 Results and Discussion

7.3.1 Loading Stations

In order to fabricate reactive micropatterns, the lift-off process—originally developed by Falconnet et al. [179] using PLL-g-PEG—was successfully extended to DNA oligonucleotides. The passage of MTs through microfabricated loading stations in the shape of stripes was analyzed. Presenting the cargo on stripe patterns has the advantage that the path length of crossing shuttles depends mainly on the angle of entrance in contrast to e.g. a pattern of dots. Stripes in our experiments were 2 µm broad (Fig. 7.3 A, B), thus significantly smaller than the average length of moving MTs (Fig. 7.6 A shows a typical length distribution of MT filaments with \( l_n = 9 \mu m \)). In a sequential step, these regions were functionalized with DNA oligonucleotides or PLL-g-PEG-biotin [179]. The cargo density was controlled by adjusting the incubation time of the colloids resulting in \( \sim 25 \) particles per \( \mu m^2 \) for the DNA and \( \sim 30 \) particles per \( \mu m^2 \) for the PLL-g-PEG-biotin surface tethers. SEM images demonstrate the high specificity of binding at the designated areas and were used to determine the cargo densities (Fig. 7.3 A and B). These loading stations thus show long-term stability, by far exceeding the maximum lifetime of MTs in hybrid environments [75]. The particle density on patterned areas after one week of storage remained at 25–30 particles per \( \mu m^2 \). Even brief ultrasonication as used during the lift-off process of the photoresist in NMP did not change the final colloid density.
7.3. Results and Discussion

Figure 7.3: SEM images reveal the high specificity of the adsorbed anti–biotin functionalized 40 nm Au particles as well as the particle density in the loading stations. Cargo densities are \( \sim 25 \) particles per \( \mu \text{m}^2 \) for the DNA (A ii.) and \( \sim 30 \) particles per \( \mu \text{m}^2 \) for the PLL-g-PEG-biotin (B ii.) surface tethers. Histograms in (A iii.) and (B iii.) represent nearest neighbor distances of colloids determined from images as shown in (A ii.) and (B ii.). Scale-bars are 20 \( \mu \text{m} \) in (A i., B i.) and 2 \( \mu \text{m} \) in (A ii., B ii.).

**Choice of Nanoparticles: Streptavidin vs. Anti-biotin Functionalization**

Qualitative results of patterns fabricated with Ab- and SA-conjugated gold colloids are shown in Fig. 7.4 (a)–(c). At same incubation times of the adsorption steps (overnight thDNA, 2 h bDNA hybridization, and 1 h Au colloid adsorption), patterns of significantly different colloid density emerged. Dense patterns could be fabricated using Au–anti-biotin colloids and Au surfaces (Fig. 7.4 (a)). The density of these patterns corresponds to the density determined using SEM, shown in Fig. 7.3, i.e. \( \sim 30 \) particles per \( \mu \text{m}^2 \). Adsorption of 40 nm Au–anti-biotin colloids on indium tin oxide (ITO) surfaces—ITO has interesting properties such as transparency in the optical range and electrical conductivity—
resulted in decreased colloid density (Fig. 7.4(b)). Presumably, the thDNA density on the ITO surface is lower than on Au causing a less dense layer of hybridized DNA oligonucleotides and thus biotin functions.

As an alternative to anti-biotin coated colloids, 40 nm SA-conjugated Au particles\(^\text{10}\) were tested for the formation of colloid patterns. Yet, colloid patterns emerged at significantly lower density on gold surfaces (see Fig. 7.4(c)), even when prolonging the adsorption time of the colloids by a factor of 10. This could be caused by the different structural conformations of anti-biotin and SA proteins: unlike the globular SA featuring deep biotin binding pockets, anti-biotin molecules are more flexible, thus potentially resulting in increased on-rates \(^{[185]}\).

As a consequence, all pick-up experiments were performed using 40 nm Au–anti-biotin colloids providing the most reasonably dense patterns. In all experiments involving DNA oligonucleotides, Au surfaces were used to bind thiol-modified DNA single strands.

\(^{10}\)Array STP 40, Batch 6863, BB International, Cardiff, UK
7.3. Results and Discussion

Casein Passivation

Casein protein is an essential ingredient when setting up motility assays, reportedly preventing kinesin motors from denaturing on the underlying surface. During the preparation of the colloid loading stations, it is necessary to passivate free surface area between pattern lines to render it colloid repellent to guarantee high specificity of colloid adsorption only in the PLL-g-PEG-biotin and bDNA functionalized areas, respectively. As technical grade casein\textsuperscript{11} may contain vitamins such as biotin that could interact with our tethering system, we tried vitamin-free casein\textsuperscript{12} to passivate the surfaces. However, it turned out that the specificity of gold colloid adsorption decreased when substituting technical grade casein with vitamin-free casein. An SDS gel comparing vitamin-free and technical grade casein (Fig. 7.5) showed that vitamin-free casein contains a large fraction of very small fragments (average molecular mass: 2.3 kDa). Technical grade casein, in contrast, reveals several distinct high molecular mass peaks. Apparently, the passivating and colloid repelling effect of casein is maximized by proteins with higher molecular mass. Intriguingly, vitamin-free casein used in standard inverted motility assays showed no decreased motility of MTs propelled by adsorbed kinesin motors.

7.3.2 Pick-up Assays

Analysis: Particle Tracking

For technological applications as envisioned in the introduction, it is essential to ensure that: (1) directed transport dominates cargo diffusion through solution, (2) transport of cargo does not significantly slow down shuttle movement, and (3) only negligible amounts of cargo dissociates from the shuttle on the way to their target locations. We thus tracked and analyzed the movement of all detected colloids using automated particle tracking software [184]. Fig. 7.6C shows the representative trajectories of colloid movement for one sequence of the 12 bp DNA tether in a cutout of 50×50 µm\textsuperscript{2}. The dashed lines in the figure indicate the positions of the loading zones. Analysis of particle movement included the calculation of moments of displacement \(\langle |r|^v \rangle\) (\(v\): order of displacement). As described by Sbalzarini et al. [184], the slope of the moment scaling spectrum (MSS) is a good measure for the type of the observed motion. Slopes of 0 correspond to stationary

\textsuperscript{11}Sigma C7078
\textsuperscript{12}Sigma C3400
Figure 7.5: Gel of technical grade and vitamin free casein. Notice the large fraction of small proteins in the vitamin free casein. The average molecular mass of the large peak is 2.3 kDa. Nevertheless, the vitamin free protein still worked well to run MT motility assays. A Western Blot (data not shown) showed no detectable biotin content, neither in the vitamin free nor in the technical grade casein.

objects, values between 0 and 0.5 indicate (confined) diffusion, whereas MSS slopes between 0.5 and 1 represent directed motion as observed for active transport. The histogram of MSS slopes shows that more than 90% of the values lie within 0.5 and 1 confirming directed motion of the tracked particles (Fig. 7.6D). This analysis was especially useful in case of MTs transporting particles on the gold surfaces, as in these setups the filaments could not be resolved using the interference based detection.

The average velocity of MTs appeared to be not affected by the number of attached colloids. The MT transporting six gold particles in Fig. 7.8 moves at a speed of ~90 nm s$^{-1}$, whereas the average cargo velocity in the whole field-of-view image sequence was ~80 nm s$^{-1}$ (Fig. 7.6B). In addition, we confirmed that the velocity distributions of tracked gold particles (N = 306) and manually tracked MTs (N = 132) widely match (Fig. 7.6B). Small discrepancies in histograms at lower speeds can be attributed
to temporary stuck and incorrectly tracked particles, which is partially reflected in the intermediate regime of MSS slopes between 0 and <0.5 (Fig. 7.6 D).

The average transport time of cargo reflects the longevity of the non-covalent bond(s) between cargo and shuttle during active transport. As the automated tracking software fractionated the shuttle path either when the respective shuttles leaving the field of view or by crossing the contrast-rich loading station, we determined the cargo loss-rate manually for the data set shown in Fig. 7.6: twenty MTs were selected, exhibiting a path with a start and end inside the field of view. These 20 shuttles carried 39 particles, 11 of which got lost during the 12.3 min of our experiment (100 frames). This corresponds to a total loss of 28% and a loss-rate of $\sim2.3\%\ \text{min}^{-1}$ per number of transported particles. Another 10 particles were transferred to other shuttles during transport. Cargo transfer, however, does not change the overall transport efficiency of a putative device. The average distance a particle was transported after pick-up was 167 $\mu$m. Considering that the speed of MTs in our assay is only 10% of the maximum possible, this average transport length seems reasonable if compared with common dimensions of microfabricated devices. For applications demanding longer cargo-transport times, there are numerous possibilities to strengthen MT–cargo interaction, e.g., by increasing bond multiplicity. Finally, as expected, the average velocity of MTs is not affected by the number of attached gold nanoparticles [11]. The MT transporting six gold particles (Fig. 7.8), for example, moved at a speed of $\sim90\ \text{nm s}^{-1}$, coinciding with the average cargo velocity in the whole field-of-view image (Fig. 7.6 B).

### Visualizing the Pick-up of Antibody-functionalized Gold Nanoparticles Immobilized on DNA-based Loading Stations

In proof-of-principle experiments, we will now demonstrate that cargo pick-up from the loading stations (Fig. 7.3 A, B) is possible. As the average particle-to-particle distance (histograms in Fig. 7.3 A, B) is below the optical resolution, individual gold particles could not be imaged spatially resolved by the interference based contrast imaging method [122, 123, 132] (Fig. 7.7 B and Fig. 7.8 A, movie 1, movie 2). It was thus only possible to directly detect pick-up events within the loading stations in a few spots with below average cargo densities. Exemplary individual pick-up events are presented in Fig. 7.7 and 7.8 where colloids were immobilized via double-stranded DNA (dsDNA) containing a 39 bp overlap. The interference based visualization technique reveals the pick-up locations of the cargo in the pattern (Fig. 7.7 B), arrowheads indicate the movement of the
Figure 7.6: Analysis of MT and cargo movement (example from the 12 bp DNA assay) illustrating the correlation between tracked cargo and shuttle movement: (A) Length distribution of MTs used in this assay determined from one 100×100 µm² field-of-view frame (N = 123). The numerical average length of MTs is \( l_n = 9 \mu m \). (B) Velocity distributions of the tracked particles (N = 306) and manually tracked MTs in the same sequence for the first 20 frames (N = 132). In the case of colloid tracking, the peaks at lower velocities are caused by temporary stuck and incorrectly tracked particles, which is partially reflected in the intermediate regime of MSS slopes between 0 and <0.5 (see subfigure D). (C) 50×50 µm² cutout showing paths of tracked gold colloids. Dashed lines indicate the position of the loading stations. (D) Slopes of the moment scaling spectrum (MSS) determined by the method described by Shalzarini et al. [184] of all tracked particles (N = 306) in one sequence of the 12 bp DNA setup. Values of MSS slopes between 0.5 and 1 refer to strongly self-similar diffusion and a MSS slope of 1 can be assigned to uniform and directed motion. The results demonstrate strong correlation between tracked particles and the actively driven motion of MT shuttles. Experiments were performed at room temperature, an ionic strength of 160 mM and an ATP concentration of 20 µM.
7.3. Results and Discussion

particles. The supplementary movie shows an overlay of the images in Fig. 7.7B and the rhodamine–labeled MTs (Fig. 7.7C) to demonstrate the correlation between particle and MT movement. Even multiple particles can be picked up by a single MT, as shown in Fig. 7.8. Attachment of more than 4 separated gold particles to single MTs was exclusively observed from hybridized DNA loading stations. To ensure that the majority of transported particles is picked up in loading stations, we determined the number of particles picked up from solution at the opposite bare glass surface of the flow cell as an internal control. Less than 5% of events were detected compared to the surface of interest (on average 5–10 transported particles vs. 230–400 events on the patterned side).

Observation of direct pick-up events requires fast sampling of images taken at highest magnification to resolve the MTs and nanoparticles, which limits the number of events per view and thus hampers an extensive statistical analysis. In addition, a majority of pick-up events could not be quantified, because they happened during the first minutes of MTs landing on the surface before we could start taking data. As the total path length of shuttles at a speed of 90 nm s$^{-1}$ is in the range of 75 µm, a significant part of the detected MTs had also left the field of view (105.6 × 105.6 µm$^2$) without passing a loading station. Future attempts to integrate loading stations into microfabricated channels that guide the motion of MTs will significantly increase the throughput of MTs through loading stations.

**Evaluating Different Linker Strategies**

Various parameters affect the pick-up efficiency, including the speed of MTs, the mean free path length of shuttles in the loading stations, the width of the loading station, and the overall strength of the bonds formed between cargo and loading station. In addition to biotin–anti-biotin, a variety of other common non-covalent bonds (e.g. biotin–streptavidin, antibody–antigen, DNA hybridization) is available to tune the binding strength to the loading station. Forces of these bonds differ over more than one order of magnitude [181, 183, 186]. Also, bond lifetimes of receptor-ligand complexes decrease with the tensile force applied and their rupture forces depend on the pulling rate [187]. While individual biotin–anti-biotin complexes require unbinding forces of 60 ± 10 pN [183] at pulling speeds of ~400 nm s$^{-1}$, hybridized oligonucleotides break at 15–40% lower forces [181], depending on the number of base pairs. To gain a sense by how much the pick-up efficiency can be tuned by decreasing the length of hybridization from 39 to 12 base pairs in the shearing geometry, we normalized the number of transported particles in all experiments to the average density of MTs and compared the resulting numbers of
Figure 7.7: Examples of pick-up events from DNA-based cargo loading stations (39 bp DNA assay): (A) Images as acquired by confocal microscopy, showing the interference contrast (top) and the rhodamine fluorescence (bottom) taken from the same surface spot. The interference-based imaging technique resolves the 40 nm gold particles. (B) Time sequence of interference images showing two pick-up events of Au colloids (indicated by black and white arrowheads). The MTs involved in the pickup are outlined. Arrows in the first frame (0 s) show the direction of their movement. (C) Same sequence as in (B) showing the MTs involved in cargo pick-up in color. Arrowheads point to the positions of the picked-up cargo, black dotted lines represent the outer boundaries of the loading station. Scale-bars are 2 µm. See also movies [movie 1](#) and [movie 2](#).
7.3. RESULTS AND DISCUSSION

Figure 7.8: Multiple pick-up from DNA-based cargo loading stations (39 bp DNA assay). (A) Image sequence demonstrating multiple cargo pick-up and transport. Black arrowheads indicate the colloid positions. (B) White arrowheads and red circles show the position of the cargo on the corresponding rhodamine labeled MT. Dashed lines represent the position of the loading station. The velocity of the MT carrying multiple cargos is $90 \text{ nm s}^{-1}$ and thus not slower than the average cargo velocity (Fig. 7.6B). Scale-bars are 2 $\mu$m.

all systems, normalizing to the least efficient system (PPB50, Fig. 7.9). Obtained values were defined as pick-up efficiency. Considering the large standard deviations from image sequence to image sequence, our data show a trend but not a significant major increase in pick-up efficiency, when shortening the base pair overlap from 39 to 12 bp (Fig. 7.9). The deviations originate in part from the parameters described above, as well as from the fact that some cargo might not bind to the loading station via one single bond. These data were collected for an average MT speed of $\sim 90 \text{ nm s}^{-1}$ (Fig. 7.6B), whereby their speed was purposely reduced by ATP depletion in order to facilitate tracking of the transported gold particles.

Since the tethers used in the dsDNA-based loading stations contain two type of serial bonds that can potentially be broken—the DNA-DNA interactions and the terminal interaction of biotin with the cargo-bound anti-biotin antibody—we compared the pick-up efficiency of dsDNA with that of single-stranded 49 nucleotides long DNA (ssDNA). If only antibody–antigen interactions can be broken, our results shows a trend towards decreased pick-up efficiencies of about 50% (ssDNA compared to 12 bp dsDNA, Fig. 7.9).
The Pick-up Efficiency is Significantly Reduced if the Cargo is not Co-localized with Kinesin in Loading Stations.

How crucial is the presence of motor proteins inside the loading stations to assure efficient pick-up? To investigate this question, we prepared loading stations based on PLL-g-PEG \[188\] (Fig. 7.2 A), since PEGylated surfaces are known to significantly reduce protein adsorption. To verify that these loading stations were indeed depleted of any surface-adsorbed proteins, rhodamine-labeled casein was adsorbed. Fluorescence intensity in the loading areas was significantly lower in all setups, but virtually zero in the case of PLL-g-PEG-biotin. In the performed experiments, 60% of the MTs were longer than 5 \(\mu\)m.
(Fig. 7.6A) and should thus be able to bridge a pattern of 2 μm width, even if they enter a PEG–cargo stripe at an angle of 25°. Nevertheless, the complete absence of motors in the loading stations lead to immobilization of the majority of MTs (Fig. 7.10 movie 3). In both PLL-g-PEG-biotin loading stations, with biotin on 16% (PPB16) or 50% (PPB50) of the PEG chains, the front end of a MT moving into a stripe gets immobilized once it binds to antibody-coated gold particles (Fig. 7.10). As the MTs are further pushed into the cargo area by the kinesin motors outside the loading station, they start buckling (see colored MTs in Fig. 7.10B) as expected when their fronts are immobilized. While passing through these loading stations, most MTs thus get derailed from their otherwise mostly linear trajectories, then accumulate in the loading zones (Fig. 7.10C), and partially align with the pattern (Fig. 7.10A, movie 4). A 4.4 μm long taxol-stabilized MT is known to buckle above a critical load of 0.7 pN [189, 190]. This means that—despite a persistence length in the mm-range [89]—MTs are not stiff enough to exert enough force on the cargo to detach it from the surface if there is no motor in direct proximity. The pick-up efficiency from the PLL-g-PEG-biotin loading stations was thus considerably lower than that of the ssDNA station (49 nucleotides with terminal biotin) (Fig. 7.9). To compare pick-up efficiencies between loading stations made from different chemistries, we tried to match the biotin densities within the DNA- and PLL-g-PEG loading stations. Assuming a surface coverage of thiol-DNA nucleotides of about 10^{12} to 10^{13} per cm^{2} [191], about 6–60 biotin molecules are present on an area of 600 nm^{2}. This corresponds to the occupied surface area of an individual PLL-g-PEG molecule [192], which presents on average 20 (PPB50) or 6–7 (PPB16) biotins.

Cargo Loss-rate Outside of Loading Stations

For technological applications, knowledge about the cargo loss rate for the given tether chemistry is needed. We thus tracked and analyzed the movement of all detected colloids using automated particle tracking software [184] and determined the cargo loss rate manually for the data set shown in Fig. 7.7 and 7.8: twenty MTs were selected, exhibiting a path with a start and end inside the field of view. These 20 shuttles carried 39 particles, 11 of which got lost during the 12.3 min of our experiment (100 frames). This corresponds to a total loss of 28% and a loss-rate of ~2.3% min^-1 per number of transported particles. Another 10 particles were transferred to other shuttles during transport. Cargo transfer, however, does not change the overall transport efficiency of a putative device. The average distance a particle was transported after pick-up was 167 μm. Considering that the speed
**Figure 7.10**: MTs interact with the cargo and get stuck on kinesin motor-depleted PLL-g-PEG-biotin (50% biotinylated) loading stations. (A) Biotinylated MTs (rhodamine-labeled) move into the 2 µm wide cargo loading zones (indicated by dashed lines) and get stuck resulting in ineffective cargo removing and shuttle-decorated loading stations after 30 min as shown in (C). (B) MTs are color-labeled to demonstrate the immobilization process. Note the green- and red-colored MTs, buckling and finally winding into the cargo pattern until they are completely stuck. Scale-bars are 2 µm in (A) and (B), 25 µm in (C). See also movies [movie 3](#) and [movie 4](#).
of MTs in our assay is only 10% of the maximum possible, this average transport length seems reasonable if compared with common dimensions of microfabricated devices. For applications demanding longer cargo-transport times, there are numerous possibilities to strengthen MT–cargo interaction, e.g. by increasing bond multiplicity. Finally, as expected [11], the average velocity of MTs is not affected by the number of attached gold nanoparticles. The MT transporting six gold particles (Fig. 7.8), for example, moved at a speed of $\sim 90 \text{ nm s}^{-1}$, coinciding with the average cargo velocity in the whole field-of-view image (Fig. 7.6B).

### 7.4 Conclusions and Outlook

The work presented here is, to the best of our knowledge, the first attempt to specifically harvest functional objects from micropatterned surfaces. The pick-up of functionalized cargo from defined surface regions is a major milestone on the way to directed assembly on the micro- and nanoscale. As our experiments utilized common molecular recognition complexes for surface tethering, the developed methods can be easily expanded to other cargos, from quantum systems to biomolecules, viruses and even larger objects [84]. A plethora of molecular recognition complexes can be exploited to adjust the binding strength to other specifications. For example, surface tethers consisting of short DNA oligonucleotides hybridized in an antiparallel manner (unzipping geometry) can be ruptured by at least 50% less force [182] than in the parallel arrangement, yet both geometries have similar thermal stability of deposited cargo. While demonstrated here for one cargo, arrays or patterns of stations loaded with different cargos can be fabricated in future developments, which would open the door for sequential assembly of diverse nanoscale objects. The bonds chosen here to tether cargo to the loading stations provide sufficient long-term stability of the cargo in a surface-bound state. Yet, when colliding with the biotinylated MTs, the surface-links can break within seconds, which is needed for efficient cargo pick-up. This interplay between thermodynamic stability under equilibrium and bond-lifetime shortening when attaching to the moving MTs [135, 187], thus comes to an advantage when engineering loading stations. Future challenges towards functional lab-on-a-chip devices or other integrated systems will be to combine the main components of a transport system: pick-up of cargo from defined locations, guided transport, and controlled discharge of the cargo at the final destination.
Cargo Pick-up in Engineered Micro-Channels
Abstract

The development of an assembly line or sensor application based on molecular shuttles requires the combination of pick-up, directed transport, and concentration of cargo on a single micron-sized device. In this work, we present a first attempt to integrate loading stations into micro-fabricated channels. Experimental conditions could be adjusted to be fully compatible with the biomolecular shuttle system. Pick-up of 40 nm gold colloids specifically adsorbed via hybridized 39 bp DNA to a gold surface could be observed approximately every 2 min. However, cargo was only transported over an average distance of 16.5 µm after pick-up, which imposes more careful adjustment of experimental conditions in the future. The chosen pattern geometry of the channel system contains the essential key elements of a final device, i.e. MT landing zone, cargo pick-up and subsequent guided transport, and finally concentration in the center of a spiral. Valuable information necessary for the development of a shuttle-based biosensor could be collected. This knowledge will hopefully contribute to a better understanding of the required design criteria of a final device.

8.1 Introduction

In the past, suitable strategies were developed that employ molecular motors in synthetic environments to specifically transport and assemble nano- and microscopic building blocks. Advances in biochemistry allowed the coupling of functions—e.g. biotin or synthetic polymers such as PEG [158]—to MT filaments. By this means, various types of cargo could be specifically picked up from solution and transported by molecular shuttles (Chapter 5) [13, 151]. Additionally, pick-up of surface-immobilized cargo could be demonstrated, as discussed in Chapter 7 [159]. Not least, the expansion of photo- and soft-lithographic techniques into the field of bio- and lifesciences paved the way towards functional bio-hybrid devices [114]. Thus, different patterning approaches, such as replica molding, standard photolithography and e-beam techniques, lead to successful motional control of MT filaments [9, 10, 82, 95, 150]. Although, transport of cargo and efficient guiding of shuttles could be demonstrated in numerous independent studies, no attempts have been made yet to combine these key elements in one single device.

In order to combine pick-up and guidance, we integrated the loading stations inside open microfabricated channels. This strategy combines the introduced chemistry—immobilization of nanometer-sized cargo via DNA oligonucleotides (Chapter 7)—with the guidance in open microfabricated channels as discussed in Chapter 6. The substrates are thus fabricated in two steps: first, gold lines are evaporated onto glass substrates and, second, the micro-channels featuring overhang geometry are aligned on top of them (Fig. 8.1). Hence, effective guiding can be combined with the already established chemistry to surface-immobilize the cargo. Unlike in previous studies [5, 67, 82], micropatterns were fabricated using a two-layer process based on standard photolithography and subsequent plasma etching of the PMGI and PMMA resists (Chapter 6). This way, long and costly e-beam writing times could be circumvented and the low autofluorescence of PMGI and PMMA reduced problems in combination with fluorescent imaging of MTs.

We also present the first attempt of developing an integrated device featuring shuttle landing zones, loading stations, shuttle guiding in microfabricated channels, and, finally, concentration in a spiral center—all in all a first step towards a sensor prototype (Fig. 8.2 A). Conditions required for full compatibility of synthetic and biological components were determined in motility assays. Furthermore, we analyzed and compared the pick-up characteristics, as well as cargo transport of the first working sequences with the results obtained from 2D pick-up experiments (Chapter 7) and transport in micro-channels (Chapter 6), respectively.
8.2 Materials and Methods

8.2.1 Pattern Design

With prospect of a future prototype biosensor device, the design of the micro-pattern was adjusted accordingly. As sketched in Fig. 1.1, a possible final device should consist of a MT loading area (1), analyte capture (2), tagging (3) and detection (4). With the pattern design shown in Fig. 8.2 A we cover at least three parts of the aimed prototype, i.e. MT holding area, analyte tagging (loading stations) and analyte detection area (center of spiral).

8.2.2 Fabrication of Gold Line Patterns

In order to produce a chemical contrast on the substrate material, gold line patterns were evaporated onto glass coverslips. The gold lines are later used as loading stations. The procedure described in the following, was established at the FIRST clean room facility. Glass coverslips (No. 1, ø35 mm) were cleaned in Piranha solution for at least 10 min and extensively rinsed in nanopure water. To increase the adhesion of the photoresist to the glass substrate, a layer of HMDS was spin-coated (5000 rpm, 45 s) onto the samples and thereafter put on a hotplate at 100°C for 5 min to evaporate NH₃ that forms upon the reaction of the glass surface with the silazane. Samples were then spin-coated (3000 rpm for 30 s) with a ∼1 µm layer of negative ma-N 1410 photoresist. After coating, edges were cleaned using acetone and samples were baked on the hotplate at 95°C for 100 s. Exposure of the photoresist layer was performed on a MJB 3 mask aligner at 500 mJ cm² (∼55 s at 8.5 mW cm⁻²). The samples were then developed in ma-D 376XP for 150 s. The height of the channel walls (∼1.2 µm) was verified by profilometry. After that, 2 nm Cr (or 2 nm Ti) followed by 50 (or 20 nm) Au were evaporated into the pattern (Fig. 8.1 A). Photoresist lift-off in NMP and acetone was performed in an ultrasonic bath for 5 min. The samples were then again cleaned in Piranha solution for 10 min.

8.2.3 Fabrication of Spiral Patterns

Spiral patterns were fabricated as described in Section 6.2.1. Glass coverslips were Piranha cleaned for 10 min and extensively rinsed in nanopure water. Before spin-coating

---

¹MicroResist Technology, Berlin, Germany
Table 8.1: RIE settings for the plasma etching procedures in the micro-channel fabrication process (RF = radio frequency, DC = direct current)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>O₂ etch</th>
<th>Ar etch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas</td>
<td>O₂</td>
<td>Ar</td>
</tr>
<tr>
<td>Gas flow (sccm)</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Pressure (mTorr)</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>RF power (W)</td>
<td>97</td>
<td>50</td>
</tr>
<tr>
<td>DC bias (V)</td>
<td>~340</td>
<td>~245</td>
</tr>
<tr>
<td>Process time (s)</td>
<td>450</td>
<td>120</td>
</tr>
</tbody>
</table>

the different resist layers, the samples were put on hotplates at 190°C for 30 min to evaporate the surface-adsorbed water. PMGI was spincoated first at 3000 rpm for 45 s onto the glass/gold lines surface. After cleaning the sample edge with acetone (to get rid of resist bulge, repeated after each coating step), the PMGI was baked at 180°C for 3 min. Secondly, PMMA (950K, undiluted) was spincoated at 4000 rpm for 45 s on top of the PMGI layer, and subsequently baked at 180°C for 30 min. Finally, ma-N 1410 negative photoresist was spincoated at 3000 rpm for 30 s. After baking at 95°C for 150 s, the photoresist was exposed for 55 s at 8.5 mW cm⁻², resulting in ~500 mJ cm⁻² (Fig. 8.1C). Alignment of the spiral patterns with the Au lines was achieved by making use of the position control features on the mask. The photoresist layer was then developed in ma-D 376XP developer solution for 150 s. PMGI/PMMA layers were etched in an O₂ plasma for 7.30 min (Fig. 8.1D) using the parameters listed in Table 8.1.

After the etching process, samples were developed for 1 min in IPA and 2 min in ma-D 376XP developer solution to produce the undercut (Fig. 8.1E). Samples were quality-checked under the microscope after each critical step, i.e. photolithography and undercut formation. The channel height was determined by profilometry.

---

2PMGI S8, 8% in cyclopentanone and tetrahydrofurfuryl alcohol, MicroChem, Newton (MA), USA
34% in chlorobenzene, MicroChem, Newton (MA), USA
4Oxford Instruments RIE 80+
5Surface Step Profiler, Tencor Alpha-Step 500, San Jose (CA), USA
Figure 8.1: Fabrication process of undercut micro-patterns featuring loading stations: (A) Evaporation of a 50 nm gold layer into a ma-N 1410 photoresist layer. (B) Lift-off of the photoresist leaves the glass substrate surface with gold features (lines). (C) The view of the cross-section is rotated by 90°: PMGI, PMMA and photoresist (ma-N 1410) are sequentially spin-coated onto a 35 mm diameter glass coverslip and, subsequently, the photoresist was illuminated and developed. (D) In an oxygen plasma step, the photoresist and underlying PMGI and PMMA layers are ablated. (E) To produce the undercut, the PMGI layer is developed for 2 min in standard photoresist developer solution. See text for more details. (F) Pattern detail: PMGI and PMMA are \(~500–600\) nm thick, depending on chosen parameters. Scheme is not to scale.
8.2.4 Pick-Up Assays in Micro-Channels

Several procedures to prepare functioning motility assays in the integrated device were tried (see screened parameters in Table 8.2). For the setup of the loading stations inside the microfabricated channels, the system based on 3’-thiol DNA (thDNA, 44 nt, 5’ AGT ACT CAG TTG CTT GCA AAT ATC TAA TAC CTT AGC ATT CCC CC SH 3’) and 3’-biotinylated DNA (44 nt, bDNA, 5’ AAT GCT AAG GTA TTA GAT ATT TGC AAG CAA CTG AGT ACT CCC CC biotin 3’) resulting in 39 bp hybridization was applied for the tethers between surface and Au colloids (see Chapter 7). Different protocols were investigated to run motility assays in the integrated device (see Table 8.2). In the following, the experimental survey of the different parameters are described in detail:

1. **Standard** The standard preparation of motility assays refers to the same procedures as used in the experiments described in Chapter 7. Glass coverslips were cleaned in Piranha solution, coated with HMDS, and gold lines consist of a 2 nm Cr and 50 nm Au layer. PMGI/PMMA undercut microchannels were fabricated as described in Chapter 6. Microfabricated surfaces were then cut into ∼0.5×1 cm² pieces for flow cell assembly. After an air plasma treatment for 15 s (both bottom coverslip slide and sample), the flow cell was assembled using double-adhesive tape. The thDNA was then adsorbed for 1 h at a concentration of 8 µM (in BRB160) onto the sections of exposed gold lines inside the microchannels. After thDNA adsorption, the surface was passivated for 5 min with 1 mg ml⁻¹ casein solution. Complementary biotinylated DNA (bDNA) was then injected and allowed to hybridize for 2 h at RT. After 2 h, the flowcell was rinsed using casein solution. Finally, a 20-fold dilution of 40 nm anti-biotin-coated Au colloids in a 1 mg ml⁻¹ casein solution (in BRB80) was added to the flow cell and adsorbed for 1 h. After the gold colloid adsorption, the flowcell was rinsed using casein solution. Kinesin and motility solution (20 µM ATP) were sequentially added as described in section 7.2.2. Flowcells were then rinsed with motility solution not containing MTs and finally sealed with immersion oil to prevent drying-up. The sample was immediately transferred to the microscope.

2. **ATP concentration** Standard assay, but increased ATP concentration, from 20 µM to 1 mM.

---

6MedProbe, Oslo, Norway
7MAB 40, BBI International
3. **Kinesin concentration** Standard assay, but increased kinesin motor concentration (double amount \( \cong 200 \text{nM} \) kinesin)

4. **Ar etch** Microfabricated samples were etched in Ar plasma for 120 s (see Table 8.1). Motility assay were then performed according to standard protocol (see above).

5. **SDS rinse after DNA adsorption** Flowcells were filled with thiolDNA (incubated for 1 h), rinsed with SDS solution (0.2% SDS in 10 mM Tris, 100 mM NaCl), and, before the adsorption of anti-biotin-coated Au colloids, extensively rinsed with BRB160 buffer containing 1 mg ml\(^{-1}\) casein. Motility assay was then performed according to standard protocol.

6. **DNA in Tris buffer** For the adsorption of thDNA and subsequent hybridization with the complementary bDNA, the BRB160 buffer was replaced by Tris buffer including high salt concentration (10 mM Tris, 1 mM NaCl). Further steps were performed using BRB160 buffer.

7. **Thinner Au layer** Standard motility assay protocol was applied to surfaces coated with only 20 nm Au layer and using 2 nm titanium as adhesion-promoting layer (instead of Cr).

8. **DNA in Tris buffer plus MgCl\(_2\)** Adsorption of thDNA dissolved in Tris buffer (10 mM Tris, 1 mM NaCl) onto 20 nm Au layers (1 h incubation). Complementary biotinylated DNA was hybridized in the same Tris buffer for 2 h. In further steps, Tris buffer was replaced by BRB160 buffer, and motility assays were performed according to standard protocol.

9. **HMDS adsorption from gas phase** Microfabricated surfaces with 20 nm thick Au lines were plasma treated for 15 s. The sample was then put into a petri dish together with a Kimwipe tissue soaked with several droplets of HMDS. The petri dish was sealed with parafilm and the sample was incubated overnight. Further steps according to standard protocol.

10. **Prehybridization of DNA** Before flowcell assembly, DNA oligonucleotides were prehybridized: 8 \( \mu \text{M} \) solution of thDNA and complementary bDNA in Tris buffer (10 mM Tris, 1 mM NaCl) were hybridized for 2 h at RT. The flowcell was then filled with the prehybridized thiol-biotin-DNA. After 1 h of incubation at RT, the free
8.2. MATERIALS AND METHODS

Surface was passivated by injecting a 1 mg ml$^{-1}$ casein solution (in BRB160) for 5 min. Further steps according to standard protocol.

8.2.5 Microscopy and Analysis of Pick-up

As described in Chapter[7] we used 40 nm gold(graft-anti-biotin) colloids as model cargo allowing fluorescence-free detection using the interferometric confocal scheme developed in Vahid Sandoghdar’s lab [122, 123] and discussed in Chapter[4]. Both the interference signal from the gold colloids and the fluorescent light originating from rhodamine-labeled MTs (using an interference filter omitting wavelengths below 560 nm) were detected simultaneously using an Olympus Confocal Laser Scanning Microscope (CLSM). Best image contrast results were obtained by using the following settings: Interference channel: HV (225), Offset (100%); Rhodamine channel: HV (500), Offset (0), Gain (2x). Image sequences at 800×800 px$^2$ resolution (105.6×105.6 µm$^2$ field of view resulting in a pixel size of 132 nm) were acquired using a pinhole width of 120 µm and a pixel dwell-time of 10 µs px$^{-1}$ resulting in a scan rate of ~7.4 s per frame. The formation of gold colloid patterns was checked before starting the motility assay. For this purpose, the surface was imaged through the flowchannel (see sketch in Fig. 8.5 A). To image MT movement, colloid pick-up and colloid transport, the sample was directly imaged through the PMMA/PMGI pattern (upside down compared to sketch in Fig. 8.5 C).

Pick-up was evaluated and analyzed for an area with four consecutive 2×3 µm$^2$ loading stations, with four parallel channels resulting in 16 regions of interest. Events of pick-up were determined by comparing shuttles before and after the loading stations, and cross-checking with the fluorescent signal of the labeled MTs. The total amount of time MTs spent inside the loading stations was calculated by multiplying the number of frames MTs were observed inside the area of interest by the time between frames (7.4 s). The transport length of colloids till loss after pick-up was manually tracked using ImageJ and the Manual Tracking plugin[10].

---

[8] Olympus FV1000
8.2.6 Preparation of SEM Samples

SEM samples were prepared by using semi-adhesive polymer foil\textsuperscript{11} (see also section 8.2.4) that could be used to build leak-proof flowcells and could be easily removed to restore the microfabricated surface after adsorption of DNA oligonucleotides and gold colloids. After the adsorption of gold colloids, the flowcell was washed with casein solution and then crosslinked using an 0.5% glutaraldehyde solution (in BRB80). Flowcells were disassembled in nanopure water, and the surface of interest was rinsed several times in nanopure water to get rid of buffer salts. Finally, the surface was dried in a filtered air stream. Samples were coated with \(~\text{3 nm}\) layer of Pt to render the surface conductive. Images were acquired using a Zeiss Ultra 55 at an acceleration voltage (EHT) of 3 kV.

8.3 Results and Discussion

The integration of the loading stations into the micro-engineered environment probably presents the first attempt to combine directed shuttle movement and cargo pick-up and transport. Not unexpected, the way to a working device was a stony one, not only from a microfabrication point-of-view, but mainly due to combining the synthetically derived surfaces with the biochemical system. The exposure to various interfaces and chemical compounds, \textit{i.e.} PMMA/PMGI, glass, and gold surfaces as well as the DNA oligonucleotides, challenged the functionality of kinesin motors and, as a consequence, the motility of the MT shuttles.

8.3.1 Fabricating Loading Stations inside Micro-Channels

The design of the integrated loading stations inside micro-channels aimed to cover the main parts of potential final device, which are shuttle landing zone, pick-up and transport channels ending in a concentrator spiral. As shown in Fig. 8.2 A, one functional unit (total length of \(~\text{260 }\mu\text{m}\)) consists of a MT landing zone that funnels the shuttles into 2 \(\mu\text{m}\) wide channels, cargo loading stations inside the channels, and a concentrator spiral with a total diameter of \(~\text{70 }\mu\text{m}\). The pathlength for MTs entering the spiral until the concentrating center is \(~\text{370 }\mu\text{m}\). Loading stations (gold lines) were fabricated at different widths ranging from 2–10 \(\mu\text{m}\). Fig. 8.2 B shows a section of the fabricated pattern array.

\textsuperscript{11}\textit{Polyolefin Plastomer Affinity EG8150G, Dow Plastics}
The micro-channels could be easily aligned on top of the pre-adsorbed gold lines using a mask aligner.

The developed fabrication process of overhanging walls with defined geometry (as discussed in Chapter 6) could be successfully combined with the integration of evaporated, underlying gold lines that served as chemical contrast for the final adsorption of cargo. As can be seen in Fig. 8.3B ii., the gold lines integrated well inside the microchannels. Results of SEM image analysis revealed a similar colloid density (∼30 particles per µm²) on the gold surfaces if compared to the 2D approach presented in Chapter 7 and if prepared according to process number 1 listed in section 8.2.4. However, colloid density increased to ∼100 particles per µm² if DNA oligonucleotides were prehybridized for 2 h and then adsorbed for 1 h onto the gold (according to process number 10 in section 8.2.4, see Fig. 8.5B). The reason for the higher colloid density at same incubation times could be a result of more complete hybridization in solution and thus potentially caused a higher biotin density on the surface. For reasons discussed in the following section 8.3.2, motility assays had to be performed using the latter protocol. More detailed characterization in the future should focus on the determination of the actual biotin density in the loading stations to be able to directly compare the results obtained from studies on plain surfaces without micro-channels.

Photolithography on thin glass substrates (100–150 µm thick coverslips) in combination with negative photoresist was challenging even though the dimensions of the pattern (smallest features are 2 µm) are well within the resolution of the used wavelength for illumination (405 nm). Bringing the flexible substrate in close contact with the mask in order to achieve best resolution of the final pattern required a minimum amount of surface impurities in the spin-coated resist layers. Scattering of light at the edges of the mask features resulted in crosslinking of the underlying negative photoresist if not in direct contact. This caused incomplete development of the pattern structures and may be part of the reason for gold layer delamination as shown in Fig. 8.4. For the same reasons, the thickness of the evaporated gold layer was reduced from 50 nm to 20 nm, as a thicker layer might contribute to reduced adhesion of the PMGI layer in proximity of the gold lines (visible in Fig. 8.4A and B).
A. Pattern Design

Figure 8.2: (A) Pattern design: microchips consist of arrays of the shown spiral featuring different gold line widths. MTs land in the landing zone, move into the channels, pick up cargo from the loading stations, and are concentrated inside the spiral. (B) Light microscope image (transmission) of a part of the array of micropatterns. Dark lines are gold lines that can be functionalized with thiolated DNA oligonucleotides. Scale-bar in (B) is 50 µm.
8.3. RESULTS AND DISCUSSION

8.3.2 Motility Assays in Microfabricated Channels Featuring Loading Stations

As described above, arrays of micro-engineered channels incorporating Au lines serving as loading stations could be successfully fabricated. The developed process allowed high flexibility in terms of pattern geometry, especially dimensions of overhang geometry and position and width of the Au lines. However, the chemical and physical properties of the engineered surface and the used fabrication process require compatibility with the biological components of the molecular shuttle system in order to be fully functional. The following section delivers important results for the future assembly of a molecular
motors based device. Several physical and chemical characteristics could be detected that have detrimental effects on the delicate members of the motility assay, *i.e.* casein, kinesin, and MTs.

**How to Encourage Microtubule Shuttles to Move**

In a first run, the motility assays in the integrated device were prepared in a similar way as performed on plain surfaces (Chapter [7]). While the preparation of the patterns and the adsorption of the anti-biotin functionalized 40 nm Au colloids performed successful, the attempt to implement the molecular shuttles has emerged to be not as straightforward as expected. First experiments prepared according to standard protocol (see Table [8:2]...
and section 8.2.4 for details) showed very low binding of MTs inside the channels of the pattern and hardly any motility, whereas the motility of MTs on the PMMA layer was unharmed (\(\sim 200 \text{ nm s}^{-1}\) at 50 \(\mu\text{M}\) ATP). Additionally, the PMGI/PMMA layers detached in various regions of the pattern array causing MTs to get stuck and trapped underneath the resist layers (Fig. 8.4 B and C). While the latter problem could be solved by decreasing the thickness of the evaporated Au layer down to 20 nm, the lacking motility of MTs, however, could first not be related to any parameters in combination with the microstructures. Increasing ATP and kinesin concentration did not have a positive effect on the low motility of the shuttles. Any negative factors emerging from the fabrication process of the microstructures, such as reduced casein adsorption due to pretreatment of the glass surface with HMDS or the use of Cr as adhesion promoting layer for the evaporated Au lines, could be excluded, since the performance of the motility assays in microchannels without Au lines (Chapter 6) was not affected.

Thus, evidence pointed to the DNA adsorption steps as potential source for the reduced motility of MTs inside the channels. Presumably, the highly negative charged oligonucleotides strongly bind to the negatively charged glass surface \textit{via} divalent cations (such as Mg\(^{2+}\)) \[193\] which are present in BRB160 buffer solution. As casein is quite hydrophobic, its adsorption onto the unspecifically adsorbed oligonucleotides is possibly reduced, thus leading to denaturation of kinesin motors and, as a consequence, low motility of MTs.

Different attempts—including SDS rinse after thDNA adsorption and subsequent hybridization in Tris buffer (10 mM Tris, 100 mM NaCl) with and without the addition of MgCl\(_2\)—were made to reduce DNA binding to the surface. Unfortunately, all of these efforts only had little or no positive effect on MT motility. Also, the approach to render the surface hydrophobic by treating the bottom surfaces of the microchannels with HMDS from the gas phase to reduce DNA adsorption was not feasible as the microchannels could not be wetted anymore in the flowcell setup (even if supporting the first rinse with detergent).

In a final try, DNA oligonucleotides were prehybridized in Tris buffer at high salt concentration (1 M NaCl) for 2 h and then directly adsorbed for 1 h onto the gold lines inside the channels. Even after a buffer change to BRB160 for the remaining adsorption steps (motors, MTs), the motility of the shuttles was surprisingly unhindered (\(\sim 280 \text{ nm s}^{-1}\) at 50 \(\mu\text{M}\) ATP) and normal on both glass bottom and PMMA surfaces as can be seen in the \textit{movie}. Smooth MT movement indicated high motor density and activity on the surface.
Table 8.2: Effect of different parameters on the motility of MTs in 3D pick-up assays. Parameters are discussed in detail in section 8.2.4. Protocol number 10 showed best compatibility with the molecular shuttle system.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Motility in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Standard</td>
<td>HMDS, 2 nm Cr, 50 nm Au, PMGI/PMMA</td>
</tr>
<tr>
<td>2 ATP conc.</td>
<td>20 µM (\rightarrow) 1 mM</td>
</tr>
<tr>
<td>3 Kinesin conc.</td>
<td>double</td>
</tr>
<tr>
<td>4 Ar etch(^*)</td>
<td>remove HMDS</td>
</tr>
<tr>
<td>5 SDS rinse after DNA adsorption</td>
<td>remove nonspecifically bound DNA from glass</td>
</tr>
<tr>
<td>6 DNA in Tris buffer</td>
<td>avoid divalent cations</td>
</tr>
<tr>
<td>7 Thinner Au layer</td>
<td>HMDS, 2 nm Ti, 20 nm Au, PMGI/PMMA</td>
</tr>
<tr>
<td>8 DNA in Tris buffer plus MgCl2</td>
<td></td>
</tr>
<tr>
<td>9 HMDS adsorption from gas phase</td>
<td></td>
</tr>
<tr>
<td>10 Prehybridization of DNA</td>
<td>in 10 mM Tris, 1 M NaCl</td>
</tr>
</tbody>
</table>

\(^*\)see Table 8.1 for etching parameters
A Closer Look to the Working Setup

In order to verify the adsorption process of the prehybridized DNA oligonucleotides to the gold lines, samples were qualitatively checked by CLSM. Therefore, the flowcell was placed upside down to visualize the emerged colloid pattern on the Au lines (Fig. 8.5 A). As can be seen in the image, areas appear dark where gold particles adsorbed (e.g. red circle). The contrast observed in Fig. 8.5 A corresponds to a colloid density of \( \sim 100 \) particles per \( \mu m^2 \), as shown in Fig. 8.5 B. Compared to loading stations prepared by hybridizing the biotinylated DNA strands to preadsorbed thiol-modified DNA (as described in Chapter 7 and standard preparation in Table 8.2), the achieved cargo density was ca. three times higher. For imaging during motility assays, the sample was placed with the patterned surface facing down (Fig. 8.5 C), being in direct contact to the objective. While in this setup gold colloids could not be detected inside the loading stations (because of the high reflectivity of the evaporated gold), it improved image quality of the transported particles in the channels, since the sample could be directly imaged through the coverslip.

As mentioned above, motility assays performed best on samples prepared according to the protocol involving prehybridized DNA (Table 8.2, nr 10). Smooth MT shuttle movement was a proof for high motor activity on the bottom of the micro-channels. The average speed of MTs moving in this environment at an ATP concentration of 50 \( \mu M \) was 280\( \pm \)20 nm s\(^{-1}\). Guided by the side-walls, MTs moved through the loading stations, picking up cargo tethered via 39 bp DNA to the gold surface (Fig. 8.6 B and C). A total number of 150 pick-up events in a field-of-view (105\( \times \)105 \( \mu m^2 \)) containing four channels and 16 loading stations (3 \( \mu m \) wide) could be observed within the time of the recorded sequence (740 s). Due to the spatial confinement of the loading stations, the identification of "actual" pick-up events was significantly easier as on the surfaces without micro-channels (Chapter 7). During the time of the sequence, 315 MT shuttles crossed the loading stations, thereby spending 277 min inside the loading stations. Compared to the total number of pick-up events, this corresponds to ca. 1 pick-up every 2 min.

However, the performance of the setup is not only given by the mere number of pick-up events, but also depends on the transport length of the load after pick-up. By analyzing the transport length of all 150 picked-up particles till loss, we found that more than 65% of the particles are dropped after very short transport of only \( \sim 20 \mu m \) after pick up (Fig. 8.6 A) resulting in an average transport distance after pick-up of only 16.5 \( \mu m \) corresponding to an average lifetime of the MT–cargo complex of \( \sim 1 \) min. More than 90% also compare Fig. 8.3 B iii.
Figure 8.5: CLSM images of the successfully prepared micropatterns with loading stations and attached 40 nm gold colloids. All images were acquired at a wavelength of 543 nm through the bottom slides of the flowcells (see sketch) to image the gold particles. (A), (C), and (D) highlight one of nine loading stations in this particular field-of-view. (A) Interfer-ence image as seen if imaged through the flow-channel (at reduced PMT sensitivity) to vi-sualize the areas where gold colloids are attached (dark segments in bright lines). (B) SEM image of the actual colloid density in prehybridized samples (∼100 particles per µm²). (C) Interference image as seen directly through the sample surface (at elevated PMT sensi-tivity) to show the glass channel bottom of the micropattern. White lines are overexposed gold lines. (D) Fluorescence image revealing the faint autofluorescence of the PMMA (area between arrowhead pattern). Scale-bars are 10 µm in A, B, and C and 500 nm in B.

of these lost particles spontaneously dissociated (see also Chapter 6) or disappeared after having contact with the side-walls of the channels. Compared to pick-up on plain surfaces (Chapter 7), where cargo transport distances of >100 µm could be observed after pick-up, the short lifetime of the cargo–shuttle complex in the present experiment might be caused by the higher shuttle speed—∼280 nm s⁻¹ compared to ∼100 nm s⁻¹ (see Chapter 7)—resulting in a decreased number of formed bonds between shuttle and cargo during the pick-up process.

Only a small fraction of colloids left the channels with the shuttle being able to es-cape. Cargo transported farther than 10 µm often got trapped and lost in the subsequent loading station. It is well possible that the higher cargo density of ∼100 particles per µm² in these samples caused more interactions with the passing colloid-carrying shuttle resulting in increased likelihood for loss of cargo. Only very few MT shuttles managed to transport the load pass the loading stations towards the spiral center of the pattern. It is therefore not surprising that only very few particles (>10) could be observed being trapped
inside the spiral center after 30 min of the experiment. While transport lengths were quite short, the directionality of the colloid transport induced by the arrowhead-shaped channels was excellent. Less than 10% colloids were transported in the wrong directions and in most cases efficiently redirected.

The high number of spontaneous dissociation of cargo evidently is a major detriment for the performance and efficiency of the system. Accordingly, the number of lost particles could be reduced by increasing the lifetime of the cargo–MT complex, e.g. by increasing the multiplicity of interactions between shuttle and cargo, however, at the expense of partly losing the reversible characteristics of the interaction, which could be disadvantageous for the controlled discharge of cargo.

A yet unaddressed problem is the movement of MTs on top of the PMMA surface. As discussed in Chapter 6, unrestricted movement of MTs is favorable if as much dispersed cargo as possible shall be captured from solution and concentrated inside the spiral center. However, in a device envisioned for the controlled sequential assembly of nanoscale objects, this is definitely not desired. A simple solution in this case could be the use of closed micro-fabricated channels as demonstrated e.g. by Huang et al. [148].

8.4 Conclusions and Outlook

The combination of MT guidance, cargo pick-up and transport is an essential step towards a final design of a molecular motor based sensor or assembly line. Here, we present a first attempt to integrate loading stations (Chapter 7) into micro-fabricated channels as developed in Chapter 6. 40 nm anti-biotin functionalized Au particles were specifically adsorbed via 39 bp DNA tethers to small gold sections inside the micro-channels. The fabrication of patterns and integrated cargo loading stations were straightforward, only requiring application and optimization of standard photolithography methods, ion etching and colloid adsorption. However, performing motility assays in the synthetic environment has proven to be challenging. The delicate biological components, namely casein, kinesin, and MTs, demanded fine-tuning of environmental parameters during experiments. In most cases, prepared surfaces omitted successful adsorption of active kinesin motors, resulting in very low shuttle density inside the micro-channels showing hardly any motility. These problems could be solved by prehybridization of DNA strands and subsequent adsorption of the hybridized double strands to the gold surfaces. This procedure—and especially the change from BRB160 to Tris buffer during the DNA adsorption step—most likely
Figure 8.6: (A) Histogram of the distance cargo was transported after pick-up. The average transport distance amounts to 16.5 µm corresponding to a lifetime of \( \sim 1 \) min for the cargo–shuttle complex. (B, C) Example of pick-up events in microfabricated channels: (B) Interference-based images acquired by CLSM reveal pick-up of 40 nm anti-biotin coated gold particles by biotinylated MTs (white arrowheads). The colloids were immobilized via DNA oligonucleotides (39 bp hybridization length) on the 3 µm wide gold lines (white). (C) Rhodamine fluorescence of MTs of the same sequence. Arrows in the first frame indicate the moving direction of the MTs. See the (movie). Scale-bar is 3 µm.
reduced the nonspecific adsorption of DNA to the glass surface of the channels allowing sufficient casein adsorption and finally resulting in high kinesin motor activity. These developed design and fabrication principles may contribute to a better understanding how to set up a final prototype.

Proof-of-principle of cargo pick-up from loading stations inside the micro-channels could be demonstrated. During an image sequence of 740 min featuring 16 loading stations (field-of-view: $105 \times 105 \, \mu m^2$) 150 pick-up events could be identified, corresponding to approximately 1 picked up particle every 2 min. However, analysis of the transport distances of cargo after pick-up showed a disappointing average distance of $16.5 \, \mu m$. This transport distance is certainly not sufficient in an envisioned final device, even if the dimensions of the micro-fabricated channels are significantly reduced. Compared to pick-up from plain surfaces (Chapter 7), where average transport distances of $>100 \, \mu m$ were spotted, this short-term transport might be a direct cause of the higher velocity of MTs ($\sim 280 \, nm \, s^{-1}$ compared to $\sim 100 \, nm \, s^{-1}$). Potentially, the formation of bonds between surface-tethered cargo and functionalized MT is impaired at higher shuttle speed, resulting in a reduced number of interactions between cargo and MT filament. Further studies testing pick-up at lower speeds are required to verify this possible effect.

Despite the discussed problems, we were able to successfully develop a first precursor of a future sensor device based on molecular motors. Furthermore, several design principles could be formulated that have to be considered in further developments. Future assays should aim at adjusting fabrication parameters (e.g. geometry and dimensions of the micro-channels), as well as tuning experimental conditions that assure full compatibility of the biological components in the synthetic environment. In order to be able to define the performance of a future device, more quantitative data—i.e. pick-up rate, transport distance, entry-rate of cargo inside the (spiral) concentrator—should be acquired. From an engineering perspective, accounting of other possible types of cargo, such as viruses or bacteria, is only advisable after detailed knowledge of the maximum performance of the system.
Summary
In the present thesis, new technology was developed to employ biomolecular motor driven transport of cargo in microengineered environments. For the first time, key elements of molecular shuttle-based transport were finally combined and integrated into a single system. Methods from surface technology and micro- and bio-engineering were exploited to fabricate precursor prototypes of a possible molecular shuttle-based micron-scale sensor device. The characteristics and performance of the designed platforms were analyzed by common techniques, such as Fluorescence Microscopy, CLSM, or SEM. In many cases, experimental conditions needed to be adjusted to guarantee compatibility of the biological components with synthetic platforms.

In this very moment, tremendous number of organelles and vesicles are transported in each cell of our body. Over billions of years, biomolecular motors such as kinesin, dynein, and myosin have evolved to inherit active transport along protein filaments, such as microtubules (MTs) and actin. In Chapter 2 we briefly reviewed the current knowledge of structure and function of protein motors and corresponding filaments. Furthermore, we presented a short overview of the adoption of these proteins for technological use. The basics of the Inverted Motility Assay and the use of molecular shuttles—MT filaments propelled by surface-adsorbed kinesin motors—in hybrid bionanodevices were introduced.

Exploiting the functionality of biomolecular motors and filaments for technological applications, requires biocompatibility of the biological components with the synthetic environment. In order to screen the stability of motors and MT shuttles under different environmental conditions, their lifetime in inverted motility assays was determined. In the course of this study presented in Chapter 3 it could be demonstrated that MT filaments are the most fragile components of the system, exhibiting a maximal lifetime of several hours until complete depolymerization. In contrast, kinesin motors were active for at least 1–2 days. The disintegration of fluorescently labeled MTs was strongly dependent on the casing material of the assembled flowcell: common materials used in microlithography, i.e. PDMS and PMMA, initiated MT depolymerization in seconds and minutes, respectively, especially in combination with fluorescent illumination. Other types of materials, such as glass, PU, and EVOH, did not tend to destabilize MT shuttles, and might be applicable for the packaging of a final device setup. Increased oxygen effusion from the critical materials (PDMS, PMMA) into solution caused light-activated oxidation and concomitant depolymerization of the protein assemblies.

Fluorescent Microscopy is a widely used technique in life sciences in order to image
labeled compartments of biological samples. MT filaments can be fluorescently labeled and—because of the large number of fluorescent dyes present in the MT structure—easily visualized, even at low illumination intensities. Even though straightforward and well-established, Fluorescent Microscopy holds the disadvantage of limited photostability of fluorescent dyes causing photobleaching and thus reduced signal intensity. Moreover, biological probes often need to be biochemically modified to become fluorescently detectable. In the work presented in Chapter 4 we expanded the application of a label-free confocal interference-based imaging technique—originally developed for the detection of nm-sized Au particles—to visualize the 25 nm thick MT filaments. We could demonstrate that MTs exhibit a contrast of \( \sim 3–5\% \) against the background (comparable to the contrast of 10–20 nm Au particles), sufficient for label-free imaging of molecular shuttles. The high sensitivity of the technique in \( z \)-direction could potentially enable experiments to study the stepping mechanisms of kinesin on MAP-decorated MTs.

The ability to specifically bind cargo must be one of the key features in a transport system. In Chapter 5 we demonstrate molecular shuttle-based transport of cargo, ranging in size from nanoscale Au particles to PS microsphere agglomerates of several \( \mu \text{m} \) diameter. Linker systems such as the common biotin–SA, antibody–antigen, or DNA–DNA interactions were successfully employed to link cargo dispersed in solution to the functionalized MT shuttles. By adjusting the total coverage of biotinylated MTs with SA to \( \sim 50\% \), self-assembly of the MT shuttles could be observed, over time assembling to circular mesoscopic ring-structures. Furthermore, a possible way of controlled cargo unloading induced by photo-activated disintegration of DNA hybridization or biotin–SA complex could be shown.

In Chapter 6 we conveyed transport of cargo from plain surfaces into microfabricated channels. A novel process allowed us to fabricate undercut microstructures with maximal geometrical control over the geometry of the overhang. The adjustable geometry permits the transport of cargo of variable size. Microstructures were produced in a two-layer process, consisting of PMGI and PMMA. Patterns were created by reactive ion etching of PMGI/PMMA, using a photoresist pattern as mask. A chemical etch of the soluble PMGI layer provided the desired undercut. The transport of 40 nm Au particles by molecular shuttles was analyzed inside straight and curved channels. Loss-rates of cargo increased in curved channels due to more frequent contact of MT shuttles with walls. Furthermore, cargo could be effectively accumulated inside the center of the spiral concentrator of the pattern.
Spatial control over the transport of cargo from point A to point B is essential for a future assembly or sensor device. For this reason, we developed strategies to confine the pick-up of cargo by molecular shuttles to specific areas, *i.e.* loading stations, on a surface. In Chapter 7, we presented a first attempt to attach 40 nm gold colloids to a surface via tethers that resist thermal activation but break upon collision with a passing molecular shuttle. Pick-up was most successful if the cargo was attached via DNA oligonucleotides to the surface. An important finding within this study was that cargo needs to be co-adsorbed with kinesin motors to guarantee efficient pick-up. If loading stations were depleted of motors, as in the case of PLL-g-PEG-biotin as surface tether, MT shuttles got stuck inside the loading zones, even if the average length of MTs exceeded the width of the cargo stripe.

In the work presented in Chapter 8, various key elements of a controlled cargo transport system based on molecular shuttles as discussed in Chapters 5, 6, and 7 are combined in one device. Loading stations were successfully integrated into micro-fabricated channels. 40 nm gold colloids were specifically adsorbed via 39 bp DNA oligonucleotides to spatially confined regions of evaporated gold inside the micro-channels. Approximately one particle was picked up every two minutes, but only transported over an average distance of 16.5 μm. While the proof-of-principle could be demonstrated and important engineering and design parameters could be identified, the observed transport distance is certainly not sufficient for the use in a future device and requires further optimization.

Overall, we have presented different tools and techniques of how to integrate molecular motors and shuttles into synthetic, micro-engineered systems. The presented advances in the design of appropriate cargo pick-up and transport strategies, as well as their integration into a micro-fabricated channel system, will be helpful for the further development of sensor prototype.
10.1 Optimization of Developed Platforms

In the presented work, we have developed tools and concepts to realize various key features of a future molecular shuttle-based sensor device. Many of these approaches—in particular the design of efficient loading stations and the integration of loading stations into a micro-engineered environment—are still in the early development stage and thus require further optimization.

In proof-of-principle experiments, we could demonstrate pick-up of nanometer-sized cargo tethered via DNA to a surface. DNA hybridization can be basically broken in two different ways (Table 10.1): by shearing, as done in our experiments, base-pairs have to be broken all at once to separate the two complementary strands. The required force is thus depending on the length of hybridization \(^{[181]}\). However, if the hybridized DNA is separated in a zipper-like fashion, rupture forces are independent on the total length of the strand. Hence, required forces are much lower and only depend on single base-pairs, \(\sim 9\) pN for A-T, and \(\sim 20\) pN for G-C pairs \(^{[182]}\). In this manner, pick-up efficiencies could be increased by conserving the thermal stability of the tethers, thus still ensuring the stability of the loading zones if not stressed by passing molecular shuttles.

Since MTs can be deformed when being propelled by surface-adsorbed kinesin motors, their persistence length in the inverted motility significantly differs from the persistence length of MTs in solution (\(\sim 5\) mm \(^{[89]}\)). Consequently, the path of an MT through a loading station is not necessarily straight. This fact complicates theoretical estimates of shuttle–cargo interactions and consequential predictions of pick-up efficiencies. Simulation of the shuttle movement through these areas could help to adjust the optimal cargo density inside the loading station by means of theoretical models. These models could be based on previous efforts to simulate the movement of molecular shuttles as proposed by Nitta \emph{et al.} \(^{[194, 195]}\).

Further improvements of pick-up rates could be achieved by labeling MT filaments with branched, end-functionalized PEG chains. This way, the multiplicity of bonds between shuttle and cargo can be increased, thus increasing the ratio of shuttle–cargo/cargo–surface-tether interactions. Moreover, PEG molecules are flexible chains (persistence length is on the order of the monomer length) and to a big extent subject to Brownian motion. Therefore, the chance of the functionalized end of the PEG chain to reach out to the surface-tethered cargo is significantly enhanced. First experiments in this direction
10.2 Further Development and New Directions

The developed tools and concepts presented in this thesis are only first steps towards a complete implementation of the molecular shuttle system into arrays of microchips allowing controlled transport of nanoscale objects. By pursuing this goal, future milestones will manifest in (1) the development of cargo-unloading stations, (2) the fabrication and characterization of a prototype device (Fig. 1.1), and (3) the transport and tagging of biomedically relevant cargo (such as viruses, bacteria, etc.).

Concepts used for the fabrication of loading stations inspire the design of future unloading stations. By increasing the ratio of cargo–surface-tether/cargo–shuttle interac-
tions, loaded MT shuttles strip off their cargo. This way, cargo can be delivered without losing the shuttle, as in the case of the spiral concentrators. In preliminary experiments [158], PLL-g-PEG-biotin with increased grafting ratio g—allowing kinesin adsorption inside the unloading station—were tested as unloading tethers. While single unloading events could already be observed in this setup, it could possibly be further improved by using branched PEG chains to increase the possible number of interactions between carried cargo and surface-tethers. Future directions consider the use of surface-tethers that exhibit different rupture force characteristics towards the same type of cargo [196]. This will potentially enhance the specificity of cargo loading and unloading process, respectively.

In a future device as proposed in Chapter 1, analyte (cargo) tagging is required for the detection of transported load (Fig. 1.1). This requires the pick-up of marker molecules specifically binding to the transported cargo, which will imply an even more careful balance of involved bonds. Moreover, adequate methods have to be developed to specifically bind biomedically or technologically relevant cargos, such as viruses [13, 84] or bacteria, to accordingly functionalized MT filaments. Previous efforts, where antibodies were bound to MTs via biotin–SA interaction [13, 84], could be simplified by directly functionalizing MT filaments with receptor molecules (e.g. antibodies).

Not least, the interferometric optical detection scheme expanded to the imaging of protein complexes, as shown for MT filaments (Chapter 4) and viruses [197], could provide exciting information about the stepping behavior of motors along the corresponding filaments. By this means, for example the effect of MAPs on kinesin motility could be investigated and eventually compared to the performance of other motors, such as dynein. Obtained data could be valuable for the better understanding of neurodegenerative diseases, such as Alzheimer’s [198, 199].
A

Standard Protocols

A.1 Preparation of Solutions

BRB80 buffer

BRB80 compounds

- PIPES acid form (MW=302.4 g mol\(^{-1}\)), Sigma P6757
- EGTA: 0.5 M stock solution, Sigma E4378
- KOH pellets (MW=56.1 g mol\(^{-1}\)), Fluka 60375
- Magnesium Chloride MgCl\(_2\): 1 M stock solution (purity of liquid = 4.9 mol l\(^{-1}\))

Preparation

EGTA  Make up stock solution by adding 18.02 g EGTA to 70 ml ddH\(_2\)O. Adjust the pH with 1 M or 2 M NaOH to 7.0 (EGTA will not go into solution until it gets close to pH 7). Bring up to volume (in this case 100 ml) with ddH\(_2\)O. Sterile filter (0.2 µm).

MgCl\(_2\)  MgCl\(_2\)·6H\(_2\)O (FW=203.31 g mol\(^{-1}\)). Dissolve 20.33 g in 80 ml H\(_2\)O. Stir until dissolved. Bring up to 100 ml, autoclave.

BRB80 (1 l)  Composition:

- 80 mM PIPES
• 1 mM MgCl₂

• 1 mM EGTA

Add:

• 24.2 g PIPES acid form

• 3.1 g KOH pellets (can easily be a bit more; this way you save some time when adjusting the pH)

• 800–900 ml H₂O

Make a stock solution of KOH and add dropwise while stirring above solution to increase the pH and get more PIPES into solution (PIPES will not go into solution until the pH ≥ 6). Continue adjusting pH to 6.9. After it is all in solution add:

• 2 ml of 0.5 M EGTA

• 1 ml of 1 M MgCl₂

Bring up to volume (1000 ml) with ddH₂O and aliquot into many 15 ml falcon tubes (and some 50 ml falcon tubes). Store in refrigerator or freezer. It can also keep for quite a while at RT (if kept at RT for a longer time, it should be filtered before used). For a double concentration BRB80 (BRB802x or BRB160) just add double amounts of PIPES, MgCl₂, and EGTA.

**Casein**

Casein is the magical ingredient in motility assays that alters the glass surface to achieve maximum kinesin activity after surface adsorption. Most problems with novices learning the motility assay arise from casein problems, so it is important to get a good casein stock in the 20 mg ml⁻¹ range. Casein can be tested in the motility assay by comparing the landing rate at a low motor density using casein known to work and newly prepared casein.
A.1. Preparation of Solutions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>about 20 mg ml(^{-1}) in BRB80</td>
</tr>
<tr>
<td>Uses</td>
<td>surface alteration to increase kinesin activity</td>
</tr>
<tr>
<td>CAS</td>
<td>9000-71-9</td>
</tr>
<tr>
<td>Source</td>
<td>Sigma C7078</td>
</tr>
<tr>
<td>Purity</td>
<td>technical grade, contains vitamins</td>
</tr>
</tbody>
</table>

**Preparation**

1. Add 3 g casein to empty 50 ml falcon tube
2. Add 30 ml BRB80
3. Rotate for >1 hour at 4°C (until solution develops thick consistency; significant portion won’t dissolve)
4. Centrifuge at highest speed (at 4°C) for 20–30 min (balance!)
5. Carefully transfer supernatant using disposable transfer pipette to 15 ml falcon tube (about 9 ml). Sacrifice supernatant near pellet.
7. Filter first with 0.4 µm syringe filters (important: *do not use much force during filter steps or face losing material!*)
8. Filter with 0.2 µm syringe filters
9. Assay protein concentration:
   
   (a) Dilute filtrate 1:25 in BRB80 (add 40 µl filtrate to 960 µl BRB80 in tube)
   
   (b) Transfer to cuvette (no bubbles!)
   
   (c) Blank against BRB80
   
   (d) Measure absorbance at 280 nm (3–5 times)
   
   (e) Calculate casein concentration using conversion: 1 mg ml\(^{-1}\)=A\(_{280}\) of 0.67
10. Dilute to 20 mg ml\(^{-1}\) in BRB80
11. Assay new casein concentration as in previous step
12. Dilute again if necessary
13. Aliquot (50 µl) into 500 µl tubes and store at -20°C
Guanosine-5′-triphosphate, disodium salt (GTP)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>25 mM in ddH₂O</td>
</tr>
<tr>
<td>Uses</td>
<td>microtubule polymerization</td>
</tr>
<tr>
<td>CAS</td>
<td>56001-37-7</td>
</tr>
<tr>
<td>Source</td>
<td>Roche Diagnostics, 106399, MW=567.1 g mol⁻¹</td>
</tr>
<tr>
<td>Purity</td>
<td>76% GTP (enzymatic), 6% GDP (enzymatic)</td>
</tr>
<tr>
<td>Concentration</td>
<td>25 mM GTP in ddH₂O</td>
</tr>
</tbody>
</table>

Weigh out dry powder and dissolve in ddH₂O in a higher concentration than the final. pH balance with NaOH to just less than pH 7. Bring up to volume with ddH₂O. Aliquot into small tubes, 25 µl per tube. Store at -20°C.

Make a 1 ml dilution (1:1000 in ddH₂O). Put 1 ml into 1 cm cuvette, blank against ddH₂O. Measure absorbance at 260 nm (ε=11.7×10³ M⁻¹ cm⁻¹).

DMSO

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uses</td>
<td>to dissolve taxol and in microtubule polymerization</td>
</tr>
<tr>
<td>CAS</td>
<td>67-68-5</td>
</tr>
<tr>
<td>Source</td>
<td>ChemStore at ETH</td>
</tr>
<tr>
<td>Purity</td>
<td>&gt;99.9% ACS reagent</td>
</tr>
</tbody>
</table>

Open bottle and make 500 µl and 50 µl aliquots. This keeps exposure to the air to a minimum and there is less oxidation/contamination. Store at -20°C.

Paclitaxel (Taxol)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>TX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>1 mM in DMSO</td>
</tr>
<tr>
<td>Uses</td>
<td>stabilizes microtubules</td>
</tr>
<tr>
<td>CAS</td>
<td>33069-62-4</td>
</tr>
<tr>
<td>Source</td>
<td>Sigma T7191, MW=853.91 g mol⁻¹</td>
</tr>
<tr>
<td>Purity</td>
<td>≤0.5% paclitaxel degradation products</td>
</tr>
<tr>
<td>Notes</td>
<td>Hydrolyzes in aqueous solutions</td>
</tr>
</tbody>
</table>

Weigh out and dissolve in DMSO under fume hood. Aliquot into small tubes, 25 µl per tube. Store at -20°C.
Adenosine-5'-triphosphate (ATP)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>MA100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>100 mM ATP, 100 mM MgCl₂</td>
</tr>
<tr>
<td>Uses</td>
<td>motility assay, microtubule fuel</td>
</tr>
<tr>
<td>CAS</td>
<td>987-65-5</td>
</tr>
<tr>
<td>Source</td>
<td>Roche Diagnostics 10519987001, MW=605.2 g mol⁻¹</td>
</tr>
<tr>
<td>Purity</td>
<td>disodium salt, crystals, special quality</td>
</tr>
</tbody>
</table>

Weigh out dry powder and dissolve in ddH₂O. Add as much 1 M MgCl₂ (stock solution) as needed for 100 mM concentration in end-volume, bring up to volume. Don’t filter nucleotides. Store at -20°C. To check concentration, dilute 1:1000 in ddH₂O, put 1 ml into 1 cm cuvette, blank against ddH₂O and measure absorbance at 260 nm \((ε=15.4×10³ \text{ M}^{-1}\text{cm}^{-1})\)

D-Glucose

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>DG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>2 M in ddH₂O</td>
</tr>
<tr>
<td>Uses</td>
<td>motility assay, anti-fade solution</td>
</tr>
<tr>
<td>CAS</td>
<td>50-99-7</td>
</tr>
<tr>
<td>Source</td>
<td>Sigma G7528, MW=180.16 g mol⁻¹</td>
</tr>
<tr>
<td>Purity</td>
<td>99.5%</td>
</tr>
</tbody>
</table>

Weigh out dry sugar and dissolve in ddH₂O, usually heating mildly quickens the dissolving process.

Glucose oxidase

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>GO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>2 mg ml⁻¹ in BRB80</td>
</tr>
<tr>
<td>Uses</td>
<td>motility assay, anti-fade solution</td>
</tr>
<tr>
<td>CAS</td>
<td>9001-37-0</td>
</tr>
<tr>
<td>Source</td>
<td>Sigma G7141, Glucose Oxidase Type X-S from Aspergillus niger, MW=160 kDa, isoelectric point (IEP)=4.2</td>
</tr>
</tbody>
</table>

Dissolve in BRB80, don’t filter. Store aliquots at -20°C. It may not be necessary to OD the GO, it just needs to be in excess. The important thing here is to make sure that the units/mg protein are the same.
Catalase

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>0.8 mg ml(^{-1}) in BRB80</td>
</tr>
<tr>
<td>Uses</td>
<td>motility assay, anti-fade solution</td>
</tr>
<tr>
<td>CAS</td>
<td>9001-05-2</td>
</tr>
<tr>
<td>Source</td>
<td>Sigma C9322, from bovine liver, MW=240 kDa</td>
</tr>
<tr>
<td>Activity</td>
<td>2000–5000 units mg(^{-1}) protein</td>
</tr>
</tbody>
</table>

For scale accuracy, make a 1.6 mg ml\(^{-1}\) stock solution = 12 mg catalase in 7.5 ml BRB80. Take the absorbance to double-check and dilute to final concentration of 0.8 mg ml\(^{-1}\). Store aliquots at 4°C (do not freeze!). To check concentration dilute 10× in BRB80, blank against BRB80 and measure absorbance at 276 nm and 406 nm.

\[
A_{276} = x
\]

\[
x \left( \frac{\text{mol}}{31.4 \times 10^4} \right) \left( 240 \times 10^3 \frac{\text{g}}{\text{mol}} \right) (10) = y \frac{\text{mg}}{\text{ml}}
\]

where: \(A(1\%, 1 \text{ cm})_{276} = 12.9; \ OD = 31.4 \times 10^4; \ MW = 240 \text{kDa}\)

\[
A_{406} = x_2
\]

\[
x_2 \left( \frac{\text{mol}}{16.6 \times 10^4} \right) \left( 240 \times 10^3 \frac{\text{g}}{\text{mol}} \right) (10) = y_2 \frac{\text{mg}}{\text{ml}}
\]

where: \(A(1\%, 1 \text{ cm})_{276} = 6.9; \ OD = 16.6 \times 10^4\)

Dithiothreitol (DTT)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>1 M in ddH(_2)O</td>
</tr>
<tr>
<td>Uses</td>
<td>motility assay, anti-fade solution</td>
</tr>
<tr>
<td>CAS</td>
<td>27565-41-9</td>
</tr>
<tr>
<td>Source</td>
<td>Sigma D0632, MW=154.1 g mol(^{-1})</td>
</tr>
<tr>
<td>Purity</td>
<td>99% by titration</td>
</tr>
</tbody>
</table>

Dissolve in ddH\(_2\)O (1 M stock solution), aliquot and store at -20°C. DTT has limited lifetime in aqueous solution (oxidizes).
A.2 Inverted Motility Assay

Microtubules Preparation

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
<th>Concentration</th>
<th>Marking</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 μl</td>
<td>MgCl₂ (100 mM)</td>
<td>~4 mM</td>
<td>MgCl or Mg</td>
</tr>
<tr>
<td>1.0 μl</td>
<td>GTP (25 mM)</td>
<td>~1 mM</td>
<td>GTP</td>
</tr>
<tr>
<td>1.2 μl</td>
<td>DMSO</td>
<td>~5%</td>
<td>DMSO</td>
</tr>
<tr>
<td>21.8 μl</td>
<td>BRB80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mix up in small tube. Add 6.25 μl of solution to a 20 μg aliquot of labeled tubulin (Cytoskeleton #TL331M-A). Grow for 30 min at 37°C. Then add 5 μl microtubules to 495 μl warmed BRB80T (see below). Label these MT100, these microtubules (0.32 μM) are long: 5–20 μm.

Mixed Microtubules

Mixed MTs—rhodamine- and biotin-labeled—are prepared by mixing solutions of tubulin aliquots (see above). In example, 80% biotinylated and 20% rhodamine-labeled MTs are prepared as follows:

1. Dissolve rhodamine- and biotin-labeled tubulin aliquots, respectively, in 6.25 μl GTP-solution, as described above (Microtubules Preparation).
2. Take out 1.25 μl from the biotin-labeled tubulin solution and add 1.25 μl of the rhodamine-labeled tubulin solution
3. Vortex and put on ice for 5 min
4. Incubate for 30 min at 37°C
5. Add 5 μl of the solution to 495 μl warmed BRB80T (see below)

Flow Cell

- microscope slide (Fisher Finest, not frosted)
- #1.5 coverglass (Corning)
• #0 spacer (Gold Seal) (100 µm thickness)

• white grease (Dow, high vacuum)

• width ~5–7 mm, volume ~10–15 µl, double sticky tape also works well as spacer

### Standard Solutions

Make up 0.5 ml of each:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRB80T</td>
<td>BRB80 + 10 µM taxol [a]</td>
</tr>
<tr>
<td>BRB80CS0.5</td>
<td>BRB80 + 0.5 mg ml⁻¹ casein</td>
</tr>
<tr>
<td>BRB80CA</td>
<td>BRB80 + 0.2 mg ml⁻¹ casein + 1 mM MgATP</td>
</tr>
<tr>
<td>BRB80CT</td>
<td>BRB80 + 0.2 mg ml⁻¹ casein + 10 µM taxol [a]</td>
</tr>
</tbody>
</table>

[a] add 1 mM taxol (in DMSO) to a vortexing solution

### Motility Solution (MT1000)

<table>
<thead>
<tr>
<th>µl</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
<td>BRB80CT</td>
<td>CT</td>
</tr>
<tr>
<td>1.0</td>
<td>MgATP (100 mM)</td>
<td>1 mM</td>
</tr>
<tr>
<td>1.0</td>
<td>D-glucose (2 M)</td>
<td>20 mM</td>
</tr>
<tr>
<td>1.0</td>
<td>glucose oxidase (2 mg ml⁻¹)</td>
<td>0.02 mg ml⁻¹</td>
</tr>
<tr>
<td>1.0</td>
<td>catalase (0.8 mg ml⁻¹)</td>
<td>0.008 mg ml⁻¹</td>
</tr>
<tr>
<td>1.0</td>
<td>DTT</td>
<td>10 mM</td>
</tr>
<tr>
<td>10</td>
<td>MT100 (0.32 µM)</td>
<td>~32 nM</td>
</tr>
<tr>
<td>100</td>
<td>final volume</td>
<td>final conc</td>
</tr>
</tbody>
</table>

If you run a motility assay on a patterned surface: Shear by passing twice through 30 G (not 26 S) needle (no bubbles!) at flow rate of ~100 µl s⁻¹ – final length 1–5 µm.

### Run an Assay

20 µl BRB80CS0.5, 5 min

20 µl kinesin (0.05–5 µg ml⁻¹, dilute in BRB80CA), 5 min

20 µl motility solution

Wick solutions with Whatman #4 filter paper (Kimwipes also work). Look straight away under microscope: 60×/100× objective, 25°C, low viscosity immersion oil.
Notes

1. Make new motility solution after 1.5 h—antifade dies.

2. Keep MT100, motility solution, taxol, and BRB80CT at room temperature, others on ice. NEVER put microtubules on ice, they will depolymerize in a matter of minutes.

3. Make sure casein is soluble and right concentration—this is the cause of most problems.

4. Make up stock solutions (GTP, DMSO, MgATP, taxol, etc) ahead of time, divide into multiple 10 µl aliquots and store at -20°C.
Mask Design

A 3” mask was designed using L-Edit physical layout software\textsuperscript{1} available at the FIRST Center for Micro- and Nanoscience, ETH Zürich and fabricated in Jena, Germany\textsuperscript{2}. The main area of the mask consists of variable dot geometries used by the bacteria group at the Laboratory for Biologically Oriented Materials at ETH Zürich.

Spiral patterns and lines used for the fabrication of micro-channels in this study are situated in the center of the mask. Spiral patterns are aligned in parallel arrays, alternately with and without arrowheads. The dimension of the pattern are shown in Fig. 8.3A. Arrays of lines used for the production of gold lines integrated as loading stations inside the micro-channels exhibit line widths of 2, 3, 5, and 10 $\mu$m. The mask includes features to help align the spiral patterns on top of the lines with positional precision of $\sim$500 nm.

The mask is stored in the photolithography lab of the FIRST clean-room center.

\textsuperscript{1}Tanner EDA, Monrovia (CA), USA
\textsuperscript{2}ML&C GmbH, Jena, Germany
### Figure B.1: Mask plan, see Fig. 8.3 A for detailed dimensions of spirals and line patterns.
References


Curriculum Vitae

**Personal**

Name: Christian Brunner  
Date of Birth: September 11, 1977  
Nationality: Swiss, Citizen of Laupersdorf (SO)  
Present Address: Seebacherstrasse 171  
CH-8052 Zürich  
Switzerland  
e-mail christian_j.brunner@alumni.ethz.ch

**Education**

2003–2007  
Doctoral student at the Laboratory for Biologically Oriented Materials, Department of Materials, Swiss Federal Institute of Technology (ETH) Zürich.

1997–2003  
Studies of Material Sciences at the Swiss Federal Institute of Technology (ETH) Zürich. Graduation with the degree Dipl. Werkstoff-Ing. ETH

Gymnasium in Solothurn (SO), Switzerland, Matura Typus B

**Presentations**

2007  
Cargo transport on engineered surfaces powered by molecular motors (*poster presentation*)  
**C. Brunner**, C. Wahnes, V. Vogel

ESF-EMBO Symposium: Biological Surfaces and Interfaces, July 1–6, 2007, Sant Feliu de Guixols, Spain

Cargo transport on engineered surfaces powered by molecular motors (*poster presentation*)  
**C. Brunner**, C. Wahnes, V. Vogel
Synthetic Biology 3.0, June 24–26, 2007, Zürich, Switzerland

Cargo transport on engineered surfaces powered by molecular motors (*oral presentation*)

**C. Brunner**, C. Wahnes, V. Vogel
Colloquium D-MATL, April 11, 2007, ETH Zürich, Switzerland

---

2006  
Cargo Pick-up from Engineered Loading Stations by Kinesin Driven Molecular Shuttles (*oral presentation*)

**C. Brunner**, C. Wahnes, V. Jacobsen, V. Sandoghdar, V. Vogel
MRS Fall Meeting, Symposium D, November 27–December 1, 2006, Boston MA, USA

Cargo transport on engineered surfaces powered by molecular motors (*oral presentation*)

**C. Brunner**, C. Wahnes, V. Vogel
Sino-German Forum on Nanoscience and Biomedicine, October 10–15, 2006, Beijing, China

---

2005  
Molecular Shuttles: Transport Systems on the Micro- and Nanoscale (*oral presentation*)

**C. Brunner**, C. Wahnes, K.-H. Ernst, H. Hess, V. Vogel
First EMPA PhD Symposium, October 20, 2005, Dübendorf, Switzerland

Hybrid Nanodevices based on Biomolecular Motors: A Lifetime Study (*oral presentation*)

**C. Brunner**, K.-H. Ernst, H. Hess, V. Vogel
MRS Spring Meeting, Symposium M, March 28–April 1, 2005, San Francisco CA, USA

---

2004  
Molecular Shuttles: Biomimetic Transport Systems for Bio-Nano Hybrid Devices (*poster presentation*)

**C. Brunner**, K.-H. Ernst, H. Hess, V. Vogel
Nanofair, September 14–16, 2004, St. Gallen, Switzerland
Lifetime of biomolecules in polymer-based hybrid nanodevices (*poster presentation*)

**C. Brunner**, K.-H. Ernst, H. Hess, V. Vogel
SSBE (Swiss Society for Biomedical Engineering) Meeting, September 2–3, 2004, Zürich, Switzerland

**Publications**


