Single-protein motion on microtubules and in cell membranes
This thesis was reviewed by:

prof.dr. P.R. ten Wolde       VU University Amsterdam, the Netherlands
dr. J. Luiirink              VU University Amsterdam, the Netherlands
prof.dr. T. Schmidt          Leiden University, the Netherlands
dr. Stefan Diez              Max Planck Institute of Molecular Cell Biology and Genetics, Germany

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Single-protein motion
on microtubules and in cell membranes

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Siet Martinus Joannes Lucas van den Wildenberg

geboren te Goirle
promotoren: prof.dr. C.F. Schmidt
prof. dr. S.M. van der Vies

copromotor: dr.ir. E.J.G. Peterman
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1. General introduction

Abstract

Since the identification of the cell as the smallest unit of life, numerous biological studies have been focused on the understanding of the cell and all it contains. Living systems such as the cell are constantly changing and a large variety of dynamic processes occur continuously inside the cell. In order to understand what the world inside the cell is like, how small particles behave and how they move, individually or on average, knowledge about parameters such as rates and velocities is necessary. Biophysics provides the techniques and methodology to quantitatively determine these parameters using physical principles. This thesis focuses on different dynamic processes in vitro and in vivo studied using such approaches. In the following introduction chapter, I will present the physical concepts of the dynamics and the biological systems that are the subjects of this thesis.
1. General introduction

1.1 Introduction to motion

In our macroscopic world, dynamics come in a number of different forms. A type of dynamics that occurs often in our daily life is directed motion. Let us consider a man walking home from work. If all the steps that he makes are of equal size and always straight, in the direction of his home, then the displacement of the man will increase linearly in time, proportional to a velocity \( v \) that depends on the step size and step rate. A different type of dynamics is the random walk (figure 1.1A). Consider that the man stops at a bar and that, after a night of drinking, he leaves the bar and tries to walk home. Let us assume that he makes steps of the same, fixed size. The direction of these steps, however, will be random and his stepping will result in a random walk. A characteristic for a random walk is that after a number of steps the man might return to his starting position, resulting in a trajectory without net displacement. If we now divide a trajectory in time windows and determine the net displacement within such a window, a distribution of displacements will be obtained that has a Gaussian shape centered around zero. The Gaussian width increases with the length of the time window (figure 1.1B).

Interestingly, the dynamics found in the macroscopic world also occur in the microscopic world of molecules in cells. All organisms consist of cells, which are typically in the order of a few micrometers. Cells in turn contain many different particles such as proteins, lipids, ions, organelles, which all undergo some kind of motion. In fact, in the microscopic world, everything, alive or not, that is immersed in an aqueous environment (for example the cell) is moving due to thermal fluctuations. This discovery was made in the 1820s by Robert Brown who noticed the random motion of pollen particles suspended in solution. The observed random motion of the particles, sometimes also referred to as Brownian motion or diffusion, is the consequence of the thermal force. This thermal force results from temperature fluctuations that make solvent molecules move around randomly and collide with the particles (1). For micrometer-sized (or smaller) particles in solution the thermal forces are of the same order as the deterministic forces that might act on the particle, such as electrostatic interactions.

1.2 Diffusion

To gain insight in diffusion, we will consider the phenomenon from the point of view of a single particle performing a random walk on a flat plane (figure 1.1A). The diffusive motion of this particle can be described by the two-dimensional diffusion equation (1):

\[
\frac{\partial p}{\partial t} = D \left( \frac{\partial^2 p}{\partial x^2} + \frac{\partial^2 p}{\partial y^2} \right)
\]
1. General introduction

Figure 1.1 Characteristics of a random walk.

(A) Trajectory obtained from a 2-dimensional random walk. A characteristic of such walk is that the average displacement of the particle after a certain time interval will be zero. (B) Cartoon of the displacement distributions that can be obtained from a random walk for increasing time intervals (1<2<3). For simplicity the displacements of a one-dimensional random walk were plotted. The obtained distributions are all Gaussian and centered around zero, the width of the distribution increases for increasing time lag, proportional to the diffusion constant. (C) Mean square displacement (MSD) as a function of time lag for two particles with distinct types of motion. The MSD, for a particle undergoing a random walk, increases linear in time proportional to a characteristic diffusion constant (black squares). The MSD, for a particle undergoing a biased random walk, increases quadratically in time (grey circles).

with $D$ is the diffusion constant and $p$ the probability. The solution of this equation is the well-known Green’s function:

$$p(x, y, \tau) = \frac{1}{4\pi D\tau} \exp \left( -\frac{x^2 + y^2}{4D\tau} \right)$$

Green’s function is a Gaussian distribution centered around zero with a width equal to the square root of $(4D\tau)$, where $\tau$ is the time lag. This function can also be written as the probability density function (PDF), $p(x, y, \tau)dx\,dy$ giving the probability for finding the particle at time $t$ in the rectangular area between $x$ and $x+dx$ and $y$ and $y+dy$ given that the particle started at position $0,0$ at time $0$.

When the positions of the particle in time (its trajectory) are known, its diffusivity can be determined in one of three ways (i) determining the cumulative probability distribution of finding a particle outside a radius $R$ at time $\tau$ (ii) calculating the mean square displacement as a function of time interval (iii) determining the width of the distribution of displacements as a function of time interval (variance).
1.2.1 Cumulative probability distribution

The probability \( P \) that at time \( \tau \) the particle is within a circle with radius \( r \) from its starting position (the origin) can be obtained by transforming Green’s function to polar coordinates and subsequent integration:

\[
P(r, \tau) = \int_0^{2\pi} d\theta \int_0^r p(R, \theta, \tau) dR = \frac{2\pi}{4\pi D\tau} \int_0^r RdR \exp \left( -\frac{R^2}{4D\tau} \right) = 1 - \exp \left( -\frac{r^2}{4D\tau} \right)
\]

\( P(r^2, \tau) \) can be readily obtained from the trajectories by scoring displacements less or equal to \( r^2 \) and normalizing by all the displacements found for a particular time lag (\( \tau \)). The diffusion constant can then be obtained from an exponential fit of the cumulative probability distribution (CPD), which is equal to \( 1 - P(r^2, \tau) \), plotted as a function of \( r^2 \) (2).

1.2.2 The mean square displacement

A more widely used approach to extract the diffusive behavior from experimental data is by calculating the mean square displacement (MSD) for different time lags. The mean square displacement can be obtained by integrating over all the possible square displacement each weighted by its probability as follows.

\[
\langle r^2 \rangle = \int_{-\infty}^{\infty} r^2 \frac{1}{4\pi D\tau} \exp \left( -\frac{r^2}{4D\tau} \right) dr = 4D\tau
\]

This equation shows that the diffusion constant can be extracted from experimentally obtained trajectories by determining the MSD for different time lags and performing a linear fit (3).

1.2.3 Variance of distribution of displacement for a diffusing particle

As can be seen from Green’s function the variance of the Gaussian distribution, which corresponds to the area probed by the diffusing particle, can be written as:

\[
\sigma^2 = \langle r^2(\tau) \rangle - \langle r(\tau) \rangle^2 = 4D\tau
\]

From this equation an important property of diffusion becomes evident, namely that the width of the distribution of displacements increases with the square root of time. In other words the probability that the particle undergoes large displacements increases only slightly even for larger time intervals. This property of diffusion shows that diffusion is a slow and inefficient way for particles to move over large distances.
1.3 Biased random motion

Another kind of motion is the biased random walk, which is a combination of directed motion and diffusive motion. Biased random motion occurs when the probability to step in a certain direction is larger than probability to step in any other direction. The resulting directional component greatly enhances the time-efficiency of translocation, as can be seen from the mean displacement which increases linear in time in contrast to what we have seen before for diffusive motion alone (where the mean displacement is 0, Paragraph 1.2). For a particle undergoing a biased random walk the MSD gets an additional quadratic term (figure 1.1C) (1):

$$\langle r^2(\tau) \rangle = v^2 \tau^2 + 4D\tau$$

The width of the distribution of displacements is, however, still governed by the diffusion constant of the particle.

1.4 Motion in biology

Characterizing the motion of biomolecules is important, since it may directly relate to their function, or give insight in possible interactions or heterogeneities of their surroundings. In this thesis I will address the movement of biomolecules in the bacterial cell membrane and proteins interacting with the cytoskeleton. These two important cellular structures are described in more detail below.

1.4.1 Biological membranes

a. Introduction

Membranes are essential cellular structures that separate the cell body from the surroundings and form the boundaries of sub-cellular compartments like the nucleus, Golgi apparatus, mitochondria and others (figure 1.2). Membranes allow the passage of specific molecules and block the passage of others. A membrane consists for a large part of phospholipids that are partly hydrophobic and partly hydrophilic and spontaneously form a bilayer in aqueous solution, minimizing the exposure of their hydrophobic parts to the solution. Based on this homogeneous structure, the first model of the membrane was a simple two-dimensional fluid with an occasional, embedded protein (fluid-mosaic). Later on, the discovery that the membrane was composed of a diversity of lipids and embedded proteins led to a more complex model (figure 1.2, box 2). In this heterogeneous model, local interactions cause the formation of subdomains that can distort the thickness of the membrane (4).
Membranes are present in all organisms from very simple unicellular (such as bacteria) to complex multicellular ones (such as humans). In fact, mainly based on the presence of a nuclear membrane organisms can be divided in two groups: Prokaryotes and Eukaryotes. Eukaryotic cells have their genetic material compartmentalized by a nuclear membrane, prokaryotes lack this membrane. Furthermore, eukaryotic cells contain many other specialized membranes such as the plasma membrane, the mitochondrial membrane, etc. These membranes differ in structur, in the concentrations of different lipids (e.g. phospholipids, cholesterol) (4) and the embedded proteins.

In contrast, bacterial cells rarely contain organelles. Gram negative bacteria, such as *E.coli* have an outer membrane and an inner membrane delimiting the periplasm. These two membranes differ in lipid composition e.g., only the outer membrane contains lipopolysaccharide (4).
b. Protein translocation across the membrane of *E. coli*

It is important to the cell that its membrane is kept intact and that leakage of ions and proteins essential to the cell is prevented. This is achieved by the lipid bilayer, which is impermeable to large molecules and has different intrinsic permeabilities to a variety of small molecules. The permeability for water is relatively high resulting in the spontaneous equilibrium of water on the two sides of the lipid bilayer. In contrast, the permeability for potassium ions is several orders of magnitude lower resulting in an ion gradient across the membrane (1). However, at the same time, the cell has to be able to take up nutrients and excrete its waste products. To be able to fulfill these contradicting features the cell membrane is equipped with specialized translocation systems that allow the passage of specific molecules through the membrane in a strictly regulated matter. Protein translocation through the inner cell membrane of *E. coli* is regulated by two dedicated translocation systems (figure 1.3A): (i) the general secretory pathway (SEC), which pushes unfolded proteins through a narrow pore in the membrane in an ATP-dependent manner (5), and (ii) the Twin Arginine Transports (Tat) system, which translocates fully folded proteins across the inner membrane using the proton motive force (pmf) as a driving force. It is hypothesized that the Tat system has to be flexible in size, since it is able to translocate folded proteins of different size (sometimes in oligomeric state) without the leakage of ions (6).

c. Protein translocation by the Tat system

The observation that the translocation of a subset of proteins in chloroplasts is ATP-independent and relies only on the presence of a proton gradient, led to the discovery of the Tat pathway (7). The name Tat, for Twin-arginine translocation, was coined later, when Berks found that substrates targeted for transport by the Tat system shared a signal peptide containing a double arginine motif (8). The Tat system is found in many bacteria and in chloroplasts. The minimal set of components required for Tat translocation in *E. coli* are three integral membrane proteins: TatA, TatB, and TatC, present *in vivo* in a 20:1:1 stoichiometry (6). To date the accepted model for translocation of substrate by the Tat system is the polymerization model. In this model the substrate protein first binds to a TatBC complex (9). This binding event triggers, in the presence of a proton gradient, the recruitment and polymerization of TatA to form the Tat pore(10). The substrate is subsequently translocated through the TatA pore and the Tat system disassembles (figure 1.3B) (6).
1. General introduction

Figure 1.3 Two membrane translocation pathways of *Escherichia coli*.

(A) The two membrane translocation pathways in the *E.coli* cell membrane providing the transport of proteins through the inner membrane. The Sec system allows the translocation of unfolded proteins through the inner membrane while hydrolyzing ATP. The Tat system transports fully folded proteins and uses the proton gradient (proton motive force (pmf)) as a driving force. (B) The polymerization model for substrate translocation by the Tat system. First the signal peptide of a substrate binds to a complex of TatB together with TatC. Subsequently TatA units are recruited and polymerize to form the pore in the membrane. The recruitment of TatA to substrate bound TatB/C is thought to be dependent on the pmf. After translocation of the substrate the Tat complex disassembles.
1.4.2 Cytoskeleton

a. Introduction

Because of its physical flexibility the membrane cannot offer structure to the cell. In eukaryotic cells, the structural integrity is provided by the cytoskeleton that supports the cell membrane. Besides providing the structural integrity of the cell, the cytoskeleton also guides intracellular traffic of organelles and other biological cargo from one part of the cell to another and coordinates the cell division. The cytoskeleton is made up of three filamentous proteins: (i) actin filaments, which determine the shape of the cell and are necessary for cell locomotion (4) and intracellular transport (11, 12), (ii) intermediate filaments which provide mechanical strength and resistance to shear (4), and (iii) the microtubules (MTs), which are involved in the positioning of organelles and direct intracellular transport. MTs are hollow protein tubes formed by linear protofilaments consisting of a heterodimeric subunits of an α- and a β-tubulin monomer (figure 1.2, Box 1) (13). Due to this arrangement of subunits the “minus” and “plus” end of an MT are structural different and have different polymerization/depolimerization properties. Molecular motor proteins exploit this polarity to generate directed motion along the MTs.

b. Microtubule-based motor proteins

In 1985 Vale et al. (14) discovered kinesin-1, a protein that is responsible for the intracellular directed transport of organelles and membrane vesicles along MTs in vivo (11). Kinesin-1 consists of two identical heavy chains necessary for motility and two identical light chains that are involved in cargo binding (figure 1.4A). Each heavy chain has three different domains: (i) a globular domain that can hydrolyze ATP and bind to a microtubule also referred to as the motor domain (15), (ii) a stalk domain that together with the stalk of another heavy chain forms a coiled coil dimer, and (iii) a tail domain that is involved in cargo-binding and self inhibition (16). When bound to an MT, Kinesin-1 moves towards the “plus” end in a highly processive fashion, meaning that it makes many steps (~100) before detaching from the MT. Furthermore, kinesin-1 motor domains make 16 nm steps in a “hand-over-hand” matter, moving the centre of mass of the dimer 8 nm and hydrolyzing one ATP per step (17-19). Since the discovery of this kinesin-1 many other kinesin-like proteins have been identified based on the presence of a homologous globular domain. All members of the kinesin superfamily are involved in a wide range of intracellular processes including intracellular transport of cargo, but also in MT organization during cell division and mitosis (20, 21). During cell division the replicated genetic material is divided equally over the two daughter cells. To accomplish this, microtubules are organized, amongst others, by microtubule-based motor proteins, such as kinesin-5, in a specific structure called the mitotic spindle (figure 1.4B). The mitotic spindle is a bipolar structure in which the
microtubules at the middle of the spindle are oriented antiparallel. Its assembly strongly depends on the activity of kinesin-5 (e.g. Eg-5 in human and *Xenopus leavis* and KLP61F in *Drosophila melanogaster*) (22).

Figure 1.4: Microtubule binding proteins and molecular motors.

(A) Schematic representation of kinesin-1 that coupling a vesicle and moving towards the plus-end of the MT. Kinesin-1 walks “hand-over-hand” and makes about 100 steps before detaching from the MT. (B) Cartoon of the mitotic spindle that is assembled to segregate the genetic material over two daughter cells. The key players in spindle assembly are depicted in this cartoon: the microtubules, microtubule-based motors and the microtubule associated proteins (MAPs) Ase1p. The molecular motor kinesin-5 is necessary to establish the bipolarity of the spindle by sliding the antiparallel microtubules in the spindle midzone and thereby contributing to the separation of the spindle poles. MAPS, such as Asep, do not have motor activity but are thought to be responsible for formation of the midzone.
It has been shown in vitro that homotetrameric Eg5 from Xenopus can crosslink microtubules and that it is a slow (~20 nm/s), plus-end directed motor that can move processively over both cross-linked microtubules (23). It has therefore been suggested that Eg5 can exert forces on the spindle and drive pole-pole separation. The mode of action of individual Eg5 tetramers was shown to be rather peculiar: in in vitro experiments with ionic strength close to cellular conditions, Eg5 tetramers diffuse on a single MT and only become directional upon cross linking with a second MT (24).

c. Microtubule-associated proteins

Apart for molecular motors, formation and maintenance of the mitotic spindle depends on the dynamics of the microtubules themselves and on a group of non-motor microtubule-associated proteins (MAPs) (figure 1.4B). These MAPs are able to bundle microtubules, but do not have ATP hydrolysis activity and thus do not show directional motion. A detailed in vitro study of the MAP Ase1p from S. pombe, has revealed that dimeric ase1p diffuses on single microtubules. When two microtubules are close together Ase1p can bind to both of them and crosslink them, while it remains diffusing, but with a lower diffusion coefficient (25, 26). Furthermore, in between two microtubules, Ase1p was shown to form immobile multimers in a cooperative manner (25). Although the behavior of Ase1p is well characterized it remains to be seen whether other MAPs, such as PRC1, the human homologue of Ase1p show similar behavior.

1.5 Outline of the thesis

The work in this thesis focuses on the motion of different cellular proteins in vitro and in vivo. I have investigated the mobility of two different molecular motors. The first motor is Kinesin-1, which provides the intracellular transport. The second motor, KLP61F, is involved in microtubule organization during spindle assembly. Also the dynamics of the non-motor protein PRC1 binding to either single or bundled microtubules in vitro was examined. The last part of the thesis focuses on the dynamics of a trans-membrane translocation complex, Tat, in E. coli cells. To explore the motion of all these different proteins I have made use of high-resolution microscopy.

Chapter 1 and 2 contain a description of the biological systems and single-molecule fluorescence microscopy, respectively. An overview on the different cellular proteins and processes is given as well as a description of the equipment necessary to build a high-resolution and high-sensitivity microscope. In chapter 2 are also described the different parameters of biomolecules that can be measured with this technique.

Chapter 3 presents a study of mobility properties of a fluorescently labeled kinesin-1 using a single-molecule fluorescence microscope. This chapter discussed how this relatively simple
technique can be used to determine the number rate limiting transitions underlying the stepping of kinesin-1. In addition, a novel way was developed to determine the lengths of the runs that kinesin-1 makes over a microtubule.

To test whether KLP61F, the kinesin-5 from Drosophila, can induce microtubule-microtubule sliding, experiments on fluorescently labeled microtubules in the presence of this motor protein are presented in Chapter 4. Results are presented on the crosslinks and sliding of microtubules by KLP61F.

In Chapter 5 the change in the dynamics of microtubule-bound PRC1 in the presence of a second microtubule is discussed. A comparison is made between the observed dynamics of PRC1 on single and double microtubules and the earlier observations of Ase1p. Although both MAP proteins are from different biological systems, they nevertheless show striking similar behavior.

A study on the Tat system in the membrane of E.coli is presented in Chapter 6. In this study fluorescently labeled TatA molecules were tracked in vivo. The results indicate that the dynamics of TatA depends on the expression level of the Tat substrate and the presence of the proton motive force that drives the Tat-dependent translocation. Based on these observations we present a new model for the dynamics of the Tat system.

In Chapter 7 I have tested how the tracking of proteins in the bacterial membrane can lead to seemingly complex diffusive behavior. Computer simulations were performed to address potential causes of complexity.

References


2. A brief introduction to single-molecule fluorescence methods

Siet M.J.L. van den Wildenberg, Bram Prevo, Erwin J.G. Peterman

Abstract

One of the more popular single-molecule approaches in biological sciences is single-molecule fluorescence microscopy, which will be the subject of the following section of this thesis. Fluorescence provides the sensitivity required to study biological systems at the single-molecule level. It allows the determination of useful parameters on time and length scales, relevant for the biomolecular world. In this chapter, a general overview of single-molecule fluorescence microscopy is presented. We start with the phenomenon of fluorescence in general and the history of single-molecule fluorescence microscopy. Next we review fluorescent probes in more detail and the equipment required to visualize them on the single-molecule level. This chapter finishes with a description of parameters that can be determined, like counting and tracking of proteins, measurement of distances with Förster Resonance Energy Transfer and of orientations with fluorescence polarization.
2. Introduction to single-molecule fluorescence

2.1 Introduction

2.1.1 A brief introduction to fluorescence spectroscopy

The name fluorescence was first used by Sir George Gabriel Stokes in his seminal 1852 paper "On the Change of Refrangibility of Light" (1), where he describes this phenomenon in many different materials, following in the steps of Herschel's studies on quinine solutions (2, 3) and Brewster's work on fluor-spar (fluorite) (4). In a note Stokes states: "I am almost inclined to coin a word, and call the appearance fluorescence, from fluor-spar, as the analogous term opalescence is derived from the name of a mineral". By now, fluorescence spectroscopy has become an indispensable technique, in particular for biomolecular research (5). Fluorescence is defined as light emitted by a molecule after absorption of light by the same molecule and involves a spin-allowed, singlet-singlet electronic transition. As an example, in figure 2.1A an imaginary absorption and fluorescence emission spectrum is shown. The energy levels involved in absorption and fluorescence are usually depicted in a Jabłoński diagram (figure 2.1B). The electronic ground state and first and second excited singlet states are designated $S_0$, $S_1$, and $S_2$, respectively. The thin horizontal lines represent vibronic levels, involving in addition to electronic, vibrational excitation. Transitions between the levels are depicted as vertical arrows, straight ones involving radiative transitions, wavy ones radiation-less ones. From the ground state $S_0$ a molecule can absorb a photon, leading to an excited state, in figure 2.1B to $S_2$. Usually, excitation from a higher vibronic state is followed by fast (typical time scale: $\sim 10^{-12}$ s) radiation-less relaxation to the lowest vibrational level of $S_1$, a process called internal conversion, leading to the generation of heat. From $S_1$, the excited molecule can usually relax to the ground state in one of three ways. (i) The molecule can return to the ground state while emitting a photon, fluorescence ($\sim 10^{-8}$ s). (ii) The molecule can get rid of the excitation energy via internal conversion, without emitting a photon (IC, $\sim 10^{-8}$ s). Finally, (iii) the electrons in the molecule can undergo a spin conversion to a triplet state, a process called intersystem crossing (ISC, $\sim 10^{-8}$ s). The resulting triplet state ($T_1$) can decay to the ground state in a radiation-less manner via either internal conversion or while emitting a photon, phosphorescence, which usually takes place on a much longer time scale than fluorescence, since it involves a spin-forbidden transition. The time scales mentioned are typical values and vary substantially among different molecules and the solvent environment. An important property of fluorescence of molecules in condensed phases is the so-called Stokes' shift: the energy of the emitted photons is generally lower than that of the absorbed photons (figure 2.1A). The most important reason, as depicted in figure 2.1B, is fast relaxation of the excited state to the lowest vibrational level of $S_0$, from which transitions can occur to vibrationally excited states of $S_0$. In addition, in the liquid state, solvent effects can contribute to the energy shift (5).
2. Introduction to single-molecule fluorescence

**Figure 2.1 Properties of fluorescence**

(A) Absorption (Abs) and fluorescence emission (Em) spectrum of an imaginary molecule. The maximum of the emission spectrum is typically shifted towards the red (higher wavelength) with respect to the maximum of the absorption spectrum, a phenomenon called the Stokes’ shift. (B) Jabłoński diagram. The electronic states ($S_0$, $S_1$, $S_2$) and their vibronic states are depicted by horizontal lines. The straight arrows indicate radiative transitions, the wavy ones non-radiative transitions (internal conversion (IC) and intersystem crossing (ICS)).

2.1.2 The history of single-molecule fluorescence microscopy

A key cause for the popularity of fluorescence to study biomolecules is its sensitivity. The sensitivity is such that, using the appropriate instrumentation, the fluorescence emitted by a single fluorophore can be readily detected. An important reason for the sensitivity is the Stokes’ shift, which allows, after proper filtering, detection of the fluorescence signal against a black background. Over the last decades researchers have pushed the detection limit further and further. In the 1970s, Hirschfeld observed single antibodies, labeled with ~100 fluorescein molecules (6). Later in the 1980s, single phycoerythrin labeled proteins, also containing multiple fluorophores, were detected (7, 8). In 1989, Moerner and coworkers succeeded in detecting, at liquid helium temperatures, the absorption of single dye molecules embedded in organic crystals (9). At these low temperatures and in crystalline environment, the absorption line of an individual molecule is extremely narrow but very strong, making the detection of a single molecule possible. Orrit and coworkers detected, for the first time, using the same molecular system, the fluorescence of a single fluorophore (at low temperature) (10). For most biological applications more ambient conditions are
required: room temperature and solutions in water. Such conditions lead to different spectral properties (absorption and emission bands are often tens of nanometers wide) and quite different instrumentation is required. In 1990, Keller and coworkers were able to detect single Rhodamine-6G molecules flowing through a small detection volume (11). This discovery paved the way for the new advancements in fluorescence methodology that have made single-molecule fluorescence microscopy to a successful tool to study biomolecules (5, 12, 13).

2.2 Materials

2.2.1 Important properties of fluorescent molecules used for single-molecule microscopy

Single-molecule fluorescence microscopy at ambient conditions relies on the accurate detection of photons emitted by one or more fluorophores attached to a single molecule while, at the same time, limiting the background signal using advanced microscopy techniques. The higher the signal-to-background ratio, the more detailed and clear the information is that can be obtained. Optimization of the signal-to-background ratio is therefore an essential element in single-molecule fluorescence microscopy (14). Generally speaking, two approaches can be used. (i) Increasing the signal, by creating optimal conditions for the fluorophore to emit photons and by increasing the sensitivity and efficiency of photon collection and detection. (ii) Decreasing the background, by using advanced microscopy techniques that probe only small volumes around the molecule of interest. Under optimized conditions, the signal from a single fluorophore such as Rhodamine-6G (R6G) can be detected (11). To provide an idea about the expected fluorescence intensity due to a single R6G, we will make a rough estimation of the number of photons that can be detected when a laser (\( \lambda = 532 \) nm), with an intensity of 100 W cm\(^{-2} \), illuminates a single R6G molecule suspended in water. First we have to calculate how many photons are absorbed by the molecule, using its absorption cross section, \( \sigma \), which can be determined from the molecular extinction coefficient, \( \varepsilon \), of R6G (~100,000 L mol\(^{-1} \) cm\(^{-1} \) at 532 nm) using:

\[
\sigma = \frac{2.303 \varepsilon}{N_A}
\]

(1)

where \( N_A \) is Avogadro’s constant. Use of this equation yields a cross section of \( 3.8 \times 10^{-16} \) cm\(^2 \) for R6G (5). Next, we calculate the energy of a single photon:
\[ E = \frac{hc}{\lambda} \]  

(2)

with \( h \) the Planck’s constant, \( c \) the velocity of light and \( \lambda \) the excitation wavelength resulting in \( 3.7 \times 10^{19} \) J per photon. This means that with an illumination intensity of \( 100 \) W cm\(^{-2} \) the sample is bombarded by \( 2.7 \times 10^{20} \) photons s\(^{-1} \) cm\(^{-2} \). Multiplying the photon flux per cm\(^2 \) \( (q_p) \) with the absorption cross section of R6G:

\[ q_a = q_p \cdot \sigma_{R6G} \]  

(3)

we find that every second \( 1.0 \times 10^5 \) photons are absorbed \( (q_a) \) by a single R6G molecule! From \( S_0 \), the molecule can relax to the ground state in one of three ways and thus not every photon that is absorbed by R6G leads to fluorescence. The fluorescence quantum yield \( \Phi_f \) is the ratio between photons absorbed and photons emitted. For R6G the fluorescence quantum yield is about 0.45 in water, resulting in an emission rate of \( 4.5 \times 10^4 \) photons per second for a single R6G under the conditions defined above (15). Even with fully optimized instrumentation only about 12% of the emitted photons can be counted by the detector (16). Thus, one can expect a fluorescence photon flux of \( \sim 5 \times 10^3 \) photons s\(^{-1} \) from a single R6G fluorophore illuminated with an intensity of \( 100 \) W cm\(^{-2} \). R6G is a water-soluble, synthetic fluorophore with properties comparable to other fluorophores widely used in single-molecule microscopy. Below we will discuss the key properties of a fluorophore relevant for making a proper choice which fluorophore to use for what experiment.

### 2.2.2 Important characteristics of fluorophores

Nowadays, an array of different fluorophores exists that can be detected simultaneously to study different single molecules at the same time. Four different classes of fluorescent labels can be distinguished: (i) synthetic dyes such as Cy3, Cy5, Rhodamine 6G and fluorescein isothiocyanate (FITC), (ii) semiconductor nanocrystals such as quantum dots (QDs), (iii) Proteins that contain a chemical structure with fluorescent properties like the enhanced Green Fluorescent Protein (eGFP) and yellow fluorescent protein (YFP), and (iv) natural occurring fluorophores such as flavin and chlorophyll. Every fluorophore has its own advantages and disadvantages. To determine which fluorophore to use for a certain experiment one has to look at the different fluorophore characteristics. Besides the fluorescence quantum yield and the molecular extinction coefficient, which were already described above, several other characteristics should be considered. Fluorophore excitation and emission wavelengths are the most important and determine the choice of the excitation source and filters, since not all colors are equally apt for single-molecule measurements. Wavelengths below \( \sim 450 \) nm are generally speaking problematic since detectors can be relatively insensitive, microscope optics are often not optimized in this
range of the spectrum, and these colors often result in high background signals due to impurities in glass or sample. Another key fluorophore characteristic is the rate of photobleaching. In general, a fluorophore does not survive infinite absorption/emission cycles. In many cases, there is a certain probability that an absorption/emission cycle leads to an irreversible modification of the fluorophore, resulting in an abrupt loss of its ability to fluoresce called photobleaching. The propensity of a fluorophore to photobleach is expressed in the average number of photons a fluorophore can emit. Photostable synthetic dyes, such as Cy3 and Cy5 can emit $10^5$-$10^6$ photons before photobleaching (17), QDs are orders of magnitude more photostable. Fluorescent proteins such as eGFP are usually slightly less stable than their optimized, synthetic equivalents (18). Photobleaching is a probability process; in general the rate of bleaching decreases linearly with decreasing excitation intensity. An important cause of photobleaching is molecular oxygen, which can react with the fluorophore’s triplet to form singlet oxygen. Singlet oxygen in turn is very reactive and can readily react with the fluorophore or surrounding molecules (13). Adding an oxygen scavenger system (a mixture of glucose, glucose oxidase and catalase is often used) to the sample decreases the concentration of molecular oxygen and can help to increase the lifetime of the fluorophores. Addition of antioxidants like Trolox® or ascorbic acid can have additional effect. Other problems with fluorophore photostability include triplet blinking, which can be a problem when the triplet lifetime is rather long (>ms). Certain fluorophores show different on-off blinking behavior, for example, fluorescent proteins are known to undergo cis-trans isomerization and intramolecular proton transfer, both resulting in long-lived dark states (18). Another example is the photoblinking of QDs, which is caused by ejection of electrons from the semiconductor core (19). Considering all the fluorophore characteristics mentioned above the ideal single-molecule fluorophore (i) has high fluorescence quantum yield and molecular extinction coefficient, (ii) has well defined excitation and emission wavelengths, (iii) shows steady emission intensity (20), (iv) can be followed for a long time using high illumination intensity, (v) does not affect the natural behavior of the single molecule, (vi) shows no blinking behavior, (vii) can be easily attached to the molecule of interest, (viii) is soluble in buffers used (21), (ix) and its characteristics are well described.

2.2.3 Fluorophores used for single-molecule research

Let us take a closer look at the different classes of fluorescent labels and compare and discuss some of their characteristics important for single-molecule research (figure 2.2). (i) Synthetic dyes have been around for decades, are commercially available and constructed to suit the means of use. They are constructed in a way that they contain different reactive groups such as maleimides or succinimidyl esters, which can be used for attachment to a
protein or biomolecule of interest. Succinimidyl esters react with free amino groups, which are available in large quantities on the surface of most proteins. Maleimides or other sulfhydryl reactive compounds can be used for more specific labeling of cystein residues, which are generally less abundant in proteins. Because synthetic dyes have been around for a long time, their characteristics have been optimized and labeling protocols are widely available. Their small size (~0.5 nm) minimizes the chance of causing steric hindrance to the labeled molecule. Cyanine and rhodamine dyes (figure 2.2A) are most often used for in vitro single-molecule research, in particular Cy3, Cy5, Alexa555 of the cyanine family and Rhodamine 6G and Texas Red of the rhodamine family (20). (ii) QDs are very bright fluorophores with a very wide range of absorption wavelengths, narrow (about 10 nm) and symmetric emission bands and quantum yields close to 90% (figure 2.2C) (22, 23). QDs in general consist of a CdSe, CdTe, InP or InAs core and a ZnS shell. Their size, shape and structure can be controlled precisely, in order to tune the emission from visible to infrared wavelengths (24, 25). For biological applications, QDs are normally coated to make them hydrophilic or modified for specific attachment to the biomolecule of interest (26). Compared to organic dyes, QDs are brighter (molecular extinction coefficients between $10^5$ – $10^6$ L mol$^{-1}$ cm$^{-1}$). They are also more photostable and therefore can be followed longer and their position can be determined with higher accuracy (21). However, their size varies between 6-60 nm (with coating), which is relatively large in comparison to the organic dyes (~0.5 nm). As mentioned above, they can suffer from on/off blinking over a wide range of time scales (19). (iii) Green Fluorescent Protein (GFP, figure 2.2B) is an autofluorescent protein from the jellyfish Aequorea Victoria and is very well suited for (but not restricted to) in vivo applications. A key advantage of GFP is that this fluorescent label is genetically encoded, does not require a cofactor and can be genetically fused to the gene that encodes the protein of interest (27). Another advantage is that they are less sensitive to their surroundings than many synthetic dyes (28). Disadvantages of GFP are that it is rather large (27 kDa, ~4 nm in diameter), its emission shows blinking behavior and its photostability is substantially less than synthetic dyes (18). By now, many different variants with different colors, optimized for different organisms have been developed on basis of the Aequorea Victoria protein and related proteins in other organisms (29). A very exciting recent development is the generation of photoactivatable and photoswitchable fluorescent proteins that are very well suited for superresolution methods like PhotoActivation Localization Microscopy (PALM) (30). (iv) Biological materials also contain naturally occurring fluorescent molecules. Many of these (such as tryptophan in proteins and the NADH metabolite), are widely used in bulk fluorescence measurements. However, they are not photostable enough and absorb and emit too far in the ultraviolet to allow single-molecule detection. Protein cofactors such as chlorophyll and flavin can be very fluorescent and have been used for studying conformational changes of the photosynthetic peripheral light-
harvesting complex of type 2 (LH2) and enzymatic turnovers of single cholesterol oxidase molecules in vitro respectively (31, 32). Their occurrence is however limited to a small subset of proteins, which hinders general application.

2.2 Structure, size and spectra of different fluorescent probes used in single-molecule fluorescence microscopy.

(A) Chemical structure and absorption/emission spectrum of the synthetic dye rhodamine 6G (R6G). (B) Structure (Protein Databank entry 1S6Z (58)) and absorption/emission spectrum of enhanced Green Fluorescent Protein (eGFP). (C) Schematic representation of a functionalized QD consisting of a core and different shells and corresponding absorption/emission spectrum.

2.3 Methods

2.3.1 The microscopic detection of single fluorophores

Single, fluorescently labeled particles can be detected using a fluorescence microscope. The main components of such a microscope are an illumination source for
excitation of the fluorophores, filters to extract light of specific wavelength and to suppress “unwanted” light, an objective to direct the excitation light and efficiently collect the emission light, and a detector. Since much of the emitted light is lost in the detection pathway and the total number of photons that a fluorophore can emit is restricted due to photobleaching, it is imperative in single-molecule fluorescence studies to use optimal components in each part of the instrument. In the next section we will discuss the different parts of a fluorescence microscope in more detail.

a. The light sources

To maximize the signal obtained from a single fluorophore, it is essential to excite it with a wavelength close to its absorption maximum. In bulk fluorescence microscopy often broad-band sources such as metal halide and mercury arc lamps are used (33). A key advantage of these sources is that they are broad-band and contain several intense spectral lines. This allows them to be used to excite spectrally distinct fluorophores, with the proper excitation filters to suppress unwanted lamp light. Relatively new on the market are Light Emmiting Diode (LED) sources, which are more monochromatic than lamps with spectra of a band width of several tens of nanometers. LEDs are much more energy efficient and thus produce less heat. In single-molecule applications, in most cases, lasers are used for fluorescence excitation since they emit monochromatic and collimated light, allowing better separation of excitation from fluorescence light and a more straightforward construction of complex optical paths using mirrors, lenses, filters, polarizers etc. In addition, collimated laser beams can be focused to diffraction limited spots (see below), which is indispensable for confocal fluorescence microscopy. Apart from high financial costs, there are two key disadvantages of lasers. (i) Laser beams are Gaussian, which can result in uneven excitation intensity profiles. (ii) Lasers are in many cases monochromatic meaning that for each spectrally distinct fluorophore an additional laser needs to be purchased and installed, which is expensive and labor intensive to set up.

b. Optical filters and dichroic mirrors

Optical filters are used to separate fluorescence light from scattered excitation light and other background signals. On basis of their transmission spectra two classes of filters can be discerned: edge filters and band-pass filters. Edge filters transmit light above (long-pass) or below (short-pass) a specific wavelength, and block the other light. Band-pass filters transmit only a narrow range of wavelengths and block wavelengths on either side of this range. The performance of the filters depends on three aspects: the percentage of transmission of the desired light, the optical density in the blocked region of the spectrum and the steepness of the edges between the transmitted and blocked regions (14). In the past, filters were based on stained glass, which often suffered from considerable
autofluorescence. Later, thin film interference filters, consisting of repetitive, thin layers of material with differing refractive index evaporated on a surface were developed with substantially better performance. Recently, new technologies to make precise thin layers based on ion-beam sputtering have further improved filter performance. Nowadays, filters are available that are designed for simultaneous excitation and/or detection of several, spectrally distinct fluorophores.

A typical fluorescence microscope consists of three optical filters. (i) The excitation filter that selects one line or band from the excitation source to illuminate the sample. In many cases use of an excitation filter is not required when using lasers. (ii) A dichroic beam-splitting mirror that reflects excitation light in the direction of the objective and transmits fluorescence light collected by the objective. (iii) The emission filter that is used to select an emission band and blocks any residual excitation light. In principle, for each lightsource and dye combination a separate combination of these filters needs to be used.

c. Detectors in single-molecule fluorescence microscopy

In single-molecule fluorescence methods the number of photons emitted is limited, putting forward strong demands on the quantum efficiency and noise characteristics of detectors used. Broadly speaking, two distinct classes of detectors can be used, depending on the imaging modality (see below) (14, 34). (i) Point detectors such as avalanche photodiodes (APD) and photomultipliers do not provide position information but are capable of counting single photons with high time resolution. APDs have high quantum efficiency, but a small active area, which makes alignment tedious. Photomultipliers usually have a lower quantum efficiency and worse dark noise characteristics, but a larger active area. For single-molecule applications, photomultipliers are usually only used in the blue part of the spectrum, where APDs perform poorly. In general, point detectors are used in confocal instruments or when time resolution is important. Key applications are in burst analysis of diffusing molecules for example in fluorescence correlation spectroscopy (FCS) (35, 36) or in FCS-like experiments (37). (ii) Array detectors, such as Charged Coupled Devices (CCD) are the most widely used detectors in wide-field fluorescence microscopy. CCD detectors are two-dimensional array detectors that can be read out in one of three ways, full-frame, frame-transfer or interline-transfer. The latter two are fast and allow for continuous detection. Limitations of CCD detectors are read-out noise due to analog-to-digital conversion (a problem solved in modern electron multiplying CCDs) and the relatively slow speed: an entire frame is integrated and read out. For single-molecule detection frame rates up to ~100 s\(^{-1}\) can be reached. Key advantages are the almost unity quantum efficiency (in the visible) and the very low dark currents. These detectors are, therefore, optimally suited in conditions when acquisition times down to ~10 ms are sufficient (38).
d. Microscope objectives

The key optical element in a fluorescence microscope is the objective. The objective concentrates the excitation light in the sample and collects the emitted light. In principle, an objective is nothing more than a strong lens, but in order to fulfill its tasks it needs to be highly corrected for optical aberrations, which can only be achieved by complex designs involving multiple optical elements. Important properties with respect to single-molecule experiments are the magnification, which determines the size of the field of view and the number of pixels over which a single fluorescent particle is imaged and the numerical aperture (NA). The NA is a measure of the angle over which photons can be detected, defined as (33):

$$NA = n \cdot \sin \theta_{\text{max}}$$

(4)

Where $n$ is the refractive index of the medium between the sample and the objective, and $\theta_{\text{max}}$ is the maximum half-angle of the collection cone of the objective. A fluorophore emits light in all directions and to maximize the collected light the NA of the objective should be as high as possible. This can be achieved by using an objective designed to be immersed in a high-$n$ medium (14, 39). Furthermore, the NA is not only important for collection efficiency, but it also determines the resolution of the optical system, as is discussed in the next section.

e. Resolution of a fluorescence microscope

In general, a fluorescent molecule is much smaller than the wavelength of visible light and after excitation the molecule emits photons in a random direction. By using a single microscope objective it is impossible to collect light emitted in all directions and consequently only a fraction of the emitted photons can be collected. The circular apertures of the microscope optics (in particular the objective) will result in the diffraction of the transmitted light, which causes the fluorescent particle not being imaged as an infinitely small point, but as an Airy disc, with a finite width and side lobes. A three-dimensional representation of this diffraction pattern is referred to as the point spread function (PSF). The diameter ($d$) of the PSF only depends on the NA of the objective and the wavelength of the light ($\lambda$) (33):

$$d = \frac{1.22\lambda}{2NA}$$

(5)

The width of the PSF is also a measure of the resolution of the optical system: when the distance between two closely spaced point sources is less than $d$, the images of the sources overlap and their peaks cannot be resolved. This definition of the resolution is called
2. Introduction to single-molecule fluorescence

the Rayleigh criterion (33). For a typical fluorescence microscope (\(NA = 1.4, \lambda = 505\text{ nm}\)), the resolution is \(~220\text{ nm}\).

2.3.2 Key imaging modalities in fluorescence microscopy

a. Confocal fluorescence microscopy

In confocal fluorescence microscopy the sample is illuminated with a diffraction limited spot and an image is acquired by moving this spot over the sample (figure 2.3A). To this end, a collimated laser beam is coupled in the microscope objective, resulting in a tightly focused spot with a diameter of typically 200-300 nm, ruled by the same effects of diffraction as discussed above. The resulting fluorescence is collected by the objective, separated from the excitation beam by a dichroic mirror and further spectrally filtered by an emission filter. In addition, the fluorescence light is spatially filtered with a pinhole before being detected with a point detector (APD or photomultiplier). The pinhole increases the spatial resolution, but its key purpose is to suppress the out-of-focus background signal, allowing optical sectioning. To create an image, scanning of the beam (using galvanic mirrors) or the sample (using a piezo stage) is required. In single-molecule experiments, excitation intensities need to be reduced to avoid saturation or limit photobleaching, resulting in rather long image acquisition times. Consequently, the most important single-molecule application of confocal microscopy is to study molecules freely diffusing in and out the confocal spot and FCS.

b. Wide-field epi-fluorescence microscopy

In wide-field epi-fluorescence microscopy, the excitation beam is not tightly focused to a diffraction-limited spot in the sample plane, but illuminates a substantially larger area (figure 2.3B). The fluorescence arising from this illuminated area is detected with an array detector such as a CCD camera. Uniform illumination throughout the sample is obtained by focusing the excitation beam in the back-focal plane of the objective. In case of a laser this results in a collimated, Gaussian-shaped beam illuminating the sample. Filters and dichroic mirrors are applied in exactly the same way as described above for confocal fluorescence microscopy. Single-molecule wide-field fluorescence microscopy is particularly useful either when molecules in the sample are moving or when a time resolution of \(~100\text{ Hz}\) is sufficient. As discussed above, modern CCD cameras are superior with respect to dark noise and quantum efficiency, compared to point detectors used in confocal microscopy. In addition, wide-field fluorescence microscopes are optically simpler and often require less frequent and precise alignment (14).
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c. Total internal reflection fluorescence microscopy (TIRF)

Wide-field approaches have, in certain cases, advantages compared to confocal approaches, at the cost of time resolution and worse suppression of out-of-focus background fluorescence than in confocal microscopy. This latter problem is addressed in TIRF microscopy, in which the evanescent wave resulting from a totally internally reflected laser beam is used for fluorescent excitation. The evanescent wave, generated by reflection of a glass/water interface, penetrates into the medium with lower refractive index (the water) and its intensity \( I \) drops exponentially with distance \( z \) into the low-index medium (40):

\[
I(z) = I_0 \cdot \exp(-z/d)
\]  

(6)

With \( I_0 \) being the intensity at the surface and \( d \) the decay constant. The decay constant of the intensity of the evanescent wave is in the order of 100 nm, depending on the angle of incidence and the refractive indices of glass and medium. To obtain total reflection and generate an evanescent wave, the angle of incidence of the laser beam impinging on the glass/water interface needs to be larger than the critical angle \( \theta_c \):

\[
\theta_c = \arcsin \left( n_1/n_2 \right)
\]

(7)
where $n_1$ and $n_2$ are the refractive indexes of water and glass respectively. At the glass-water interface the internal reflection is achieved at an $\theta_c$ of $61^\circ$. These high angles of incidence are usually obtained in one of two ways: (i) by using a prism, coupled to the coverslip on the side of the sample opposite to the objective. (ii) By using an ultra-high NA objective (> 1.45) and coupling in the laser beam off axis, on the edge of the entrance pupil (41) (figure 2.3C). The key advantage of TIRF microscopy is that only fluorophores close to the interface will be excited and fluorophores deeper in the sample will not, resulting in a reduced background. Disadvantages of TIRF microscopy are that the polarization of the evanescent wave is complex and the excitation intensity strongly depends on the depth in the sample, making comparison of fluorescence intensities difficult.

2.4. Measurables

2.4.1 Counting the number of fluorescent molecules within a diffraction-limited spot

So after the instrument is built and a sample prepared, how does one know one is looking at a single molecule? Observation of a single diffraction-limited spot is not enough: the Rayleigh criterion tells us that when particles are too close they cannot be resolved and will be imaged as a single spot. The most straightforward signature of fluorescence arising from a single fluorophore is step-wise photobleaching: the intensity is rather constant for a while, until photobleaching occurs and the signal abruptly drops to the background level. Another signature is the intensity of the fluorescence, which should be constant from molecule to molecule. However orientation and polarization effects can substantially modulate fluorescence intensity (see below). If the intensity of a fluorescent spot is due to more than one fluorophore, the number of fluorophores can be determined in two ways. (i) By comparing the fluorescence intensity of the spot to the average value of single fluorophores (figure 2.4A). To correct for possible bleaching within the first frame the intensity at $t=0$ can be extrapolated by fitting an exponential decay to the fluorescence intensity profile over time (42). (ii) One can also determine the number of fluorophores by counting the number of bleaching steps. This approach has been used to determine the number of Ase1p dimers incorporated in multimers bound to microtubules (43). Under conditions where photobleaching is negligible changes in numbers of molecules due to association or dissociation can be measured. This approach has been used to determine the number of Rad51 monomers disassembling from DNA in a single burst (44).

2.4.2 Localization of single molecule

We have seen above that the resolution of a fluorescence microscope is limited, by diffraction, to about half the wavelength of the emitted light. The resolution is a measure for
how close two point sources can be while still being resolvable (figure 2.4B), it does not restrict the accuracy with which the location of a single point source can be determined. By fitting the resulting image with the PSF (often an approximation with a Gaussian function is sufficient), the location of the maximum of the image can be determined with far greater accuracy than the width of the PSF. This method is frequently used in single particle tracking (SPT) (38, 45). Given the noise encountered in most single-molecule experiments a PSF of the microscope with a full width at half maximum of ~1.5-2 pixel yields the best results for the accuracy (46). The uncertainty in the localization of a point source ($\Delta x$) depends on the size of the pixels ($a$), the number of photons ($N$), the background noise ($b$), the standard deviation of the point spread function ($s$) (46, 47):

$$
\langle (\Delta x)^2 \rangle = \frac{s^2}{N} + \frac{(a^2/12)}{N} + \frac{8\pi^4 b^2}{a^2 N^2}
$$

(8)

The first term represents the photon counting noise ($s^2/N$), the second term represents pixilation noise arising from the uncertainty of where in the pixel the photon arrived ($a^2/12N$). The final term is due to background. Under typical single-molecule fluorescence conditions, position accuracies down to about 2 nm can be achieved (47).

2.4.3 Detection of motion of single molecules

Given this high localization accuracy, the positions of an emitting fluorophore can be determined in each image from a time stack of images and subsequently a trajectory can be constructed by connecting the positions. Using this approach, the motion of single-molecules can be accurately determined. Care has to be taken that the motion of the molecules is not too large within the acquisition time of an image, since this can smear out the Gaussian intensity profile, complicating fitting. This problem can be avoided by using short acquisition times and increasing the excitation intensity, at the cost of enhanced photobleaching. It is important in single-molecule tracking to find the proper balance between movement of a particle within the acquisition time, and the total number of time points (frames) over which the particle is observed (48). Motion of biomolecules can be directional (for example driven by motor proteins) or diffusive (like membrane proteins). To analyze the precise nature of mobility, the mean square displacement (MSD) is often calculated as a function of time. Motion with constant speed (and direction) yields an MSD that increases with the square of time, while diffusive motion results in a linear increase of the MSD with time. The localization uncertainty leads to a constant offset in the MSD, due to its time-independence (38, 49). The MSD analysis was for example used to show that, depending on the exact conditions, the motor protein kinesin-5 can switch between different modes of motility, diffusion and directed motion (50).
2. Introduction to single-molecule fluorescence

Figure 2.4 Measurable parameters in single-molecule fluorescence microscopy.

(A) The number of fluorophores within a fluorescent spot, even though they are not resolvable, can be deduced from the intensity of that spot. (B) Localization of single fluorophores. The fluorophores are imaged as Airy disks, which can be approximated with a 2D Gaussian function. The limit in which two neighboring fluorophores can still be resolved is described by Rayleigh criterion. (C) Colocalization can be observed by using fluorescent labels with different colors. By imaging both color channels simultaneously on a CCD camera the precise localization of both fluorophores can be determined. (D) FRET reports on the distance between two spectrally distinct dyes, and can be used to study for example intra-molecular conformational changes. When the distance between the dyes is in the order of tens of Å (20 Å < r < 90 Å), the energy can get transferred from the donor, D, to the acceptor, A, causing an increase in acceptor signal. When the distance between the dyes is large (r > 90 Å) no energy transfer occurs and the acceptor signal will be low. (E) Fluorescence polarization reports on orientation or orientational dynamics. Circular polarized light can be used to excite dyes in all orientations. Subsequently, the emitted light is filtered for a specific polarization.
2. Introduction to single-molecule fluorescence

2.4.4 Colocalization of fluorescent molecules

One of the key interests in (cell) biology is to resolve which proteins interact and how. To this end, proteins of interest can be labeled with differently colored fluorophores (51-53). Subsequently the different dyes can be excited by the appropriate lasers and the fluorescence signal can be separated in two or more wavelength channels and detected independently using different cameras or side-by-side on one. In this way, different biomolecules can be tracked simultaneously and their motion can be correlated to resolve whether they move independently or interact (part of the time) (figure 2.4C). High-resolution colocalization was applied to show myosin V’s alternating heads while it walked hand-over-hand along an actin filament (53).

2.4.5 Förster Resonance Energy Transfer (FRET)

Positions and distances of single fluorophores can be determined with an accuracy that is substantially smaller than the diffraction limit using PSF fitting (see above). This approach is very powerful, but has its limitations, in particular in its poor time resolution and its inability to resolve multiple molecules that are closer than the optical resolution, without photobleaching them. An excellent method to measure relative distances and changes on a length scale of ~2-9 nm is FRET (figure 2.4D). In order to observe FRET two spectrally distinct fluorophores are needed. One, with the highest energy excited state, is excited and serves as donor, the other as acceptor. When the two fluorophores are physically close and their dipoles are oriented favorably, dipole-dipole coupling can occur and excitations can be transferred from donor to acceptor. The distance dependency of the FRET efficiency ($E$) is:

$$E = \frac{1}{1 + (R/R_0)^6}$$

(9)

With $R$ the distance between donor and acceptor and $R_0$ the Förster distance. The Förster distance is defined as the distance at which half the fluorescence of the donor is transferred to the acceptor. The Förster distance depends on the overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of donor and acceptor dipole moments and the fluorescence quantum yield of the donor. It has a typical value of about 5 nm (5). FRET has proven to be a valuable tool to study conformational dynamics in nucleic acids and proteins. Examples are the folding of ribozymes (54) and the observation of conformational dynamics in kinesin-1 (55).
2.4.6 Fluorescence polarization

Another way to measure conformational changes of single biomolecules is to use the polarization of the fluorescence signal. Absorption and emission are governed by the interaction of the absorption and emission transition dipole moments of the chromophore, which are vectors, with the electric component of the electromagnetic light field, also a vector. By using polarized light for excitation and/or a polarizer in the emission path the orientation and dynamics of the transition dipole moment can be obtained. Care needs to be taken that the fluorophore is not free to rotate with respect to the biomolecule of interest, but that its orientation is tightly linked to that of the biomolecule. This can be achieved by using bisfunctional fluorophores that are connected with two chemical links to the protein or nucleic acid of interest (56). One way of determining dipole orientations at the single-molecule level is to excite with circular polarized light and to split the resulting fluorescence in two perpendicular linear polarized signals, detected with two APDs or side-by-side on a CCD chip (figure 2.4E). Another way is to detect without polarizers and to use alternating (linear) polarization of the excitation light. If one combines polarized excitation with polarized detection, a separation of the depolarization due to rapid fluorophore orientation (on the nanosecond scale) and much slower conformational changes can be obtained. Polarization methods have for example been applied to study the conformational changes occurring during stepping of the kinesin-1 motor protein (57).

2.5 Concluding remarks

Here we have provided an overview of the wealth of single-molecule fluorescence approaches and their backgrounds. These tools have become indispensible in the study of the diverse properties of biomolecules such as active and diffusive motion, conformational changes and assembly and disassembly. In the following chapters, we have used single-molecule, wide-field epi-fluorescence microscopy to investigate different biological systems. This technique was employed to follow individual MT-binding proteins in vitro (Chapters 3 and 5) and to track individual membrane protein complexes in the bacterial cell membrane in vivo (Chapter 6). We also used this technique to investigate how MTs are oriented and moved by the molecular motor KLP61F (Chapter 4).

References


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3. Novel ways to determine Kinesin-1's run length and randomness using fluorescence microscopy

Sander Verbrugge*, Siet M.J.L. van den Wildenberg* and Erwin J.G. Peterman

Department of Physics and Astronomy and Laser Centre, VU University, De Boelelaan 1081, 1081 HV, Amsterdam, the Netherlands.

* these authors contributed equally

Abstract

The molecular motor protein Kinesin-1 drives intracellular transport of vesicles, by binding to microtubules and making hundreds of consecutive 8 nm steps along them. Three important parameters define the motility of such a linear motor: velocity, run length (the average distance traveled) and the randomness (a measure of the stochasticity of stepping). We used total internal reflection fluorescence microscopy to measure these parameters under conditions without external load acting on the motor. First, we tracked the motility of single motor proteins at different ATP concentrations and determined both velocity and, for the first time using single-molecule fluorescence assays, randomness. We show that the rate of Kinesin-1 at zero load is limited by two or more exponentially distributed processes at high ATP concentrations, but that an additional, ATP-dependent process becomes the sole rate-limiting process at low ATP concentrations. Next, we measured the density profile of moving Kinesin-1 along a microtubule. This allowed us to determine the average run length in a new way, without the need to resolve single-molecules and to correct for photobleaching. At saturating ATP concentration, we measured a run length of 1070 ± 30 nm. This value did not significantly change for different ATP concentrations.
3. Kinesin-1’s run length and randomness

3.1 Introduction

Kinesin-1 is a motor protein that drives intracellular transport of vesicles and organelles in many eukaryotic organisms (1). To fulfill this task, Kinesin-1 (called kinesin hereafter) steps along microtubules (MTs), with a step size of 8 nm (2, 3). Each step is driven by the hydrolysis of one ATP molecule into ADP (3). Both kinesin’s velocity and ATPase rate are dependent on the ATP concentration and follow Michaelis-Menten kinetics (4). Kinesin has two identical motor domains that transiently bind to the MT and contain the active site for ATP-hydrolysis. These two motor domains are kept out of phase and thus work together such that the motor can make hundreds of processive steps along the MT (5).

Over the last years, the technical approaches that allow for the resolution of single steps have tremendously increased our insight in kinesin’s mechanism. Optical trapping experiments have taught us that kinesin’s center of mass makes 8 nm steps at an average step time of about 10 ms (at saturating ATP concentration) (2), that one ATP molecule is hydrolyzed per step (3) and that kinesin slows down when a load is applied and stalls at a counteracting force of about 5 pN (2, 5). Single steps have also been discerned in wide-field optical microscopy experiments. Traveling-wave tracking (6) has allowed for determination of step sizes and rates with microsecond time resolution and sub-nanometer spatial-resolution without external load (7). In wide-field fluorescence microscopy also a localization accuracy of several nanometers has been achieved by fitting the point-spread function (8-10). This approach has been applied to kinesin with a fluorescent label attached to only one of the motor domains, directly demonstrating that each individual motor domain makes steps of 16 nm (11), confirming that the motor domains move in a hand-over-hand fashion (12, 13).

Although these techniques have provided fundamental insight in kinesin’s stepping mechanism, many key properties of motor motility do not require the resolution of single steps and can thus be determined faster, with less complex and more widely available instrumentation. This is important in light of the many motor proteins that have been studied to a lesser extent than Kinesin-1, such as dynein and Eg5 (14, 15). or numerous other ones that have not been studied at all on the single-molecule level. We see in particular three parameters as important for defining a motor protein’s motile properties: the run length, the velocity and the so-called randomness (3) and all three as a function of ATP concentration.

(i) The run length is the average distance a motor protein travels along its track before it releases and describes how processive a motor protein is. The run length is in most cases measured using wide-field single-molecule fluorescence microscopy on labeled motors. For kinesin, typical values on the order of a
micrometer (depending on the exact conditions) have been obtained, indicating that it makes more than one hundred of 8 nm steps before it releases from the MT (16, 17). Single-molecule fluorescence measurements of the run length are complicated by photo bleaching of the fluorophores, which requires a correction of the measured run length for photobleaching and hence determination of the fluorophore bleaching rate. Here, we demonstrate another way of determining the run length using TIRF microscopy that does not require the observation of single motors and can thus be performed under illumination conditions at which photo bleaching is negligible. In our approach, we measure the fluorescence of many motors walking along a MT and derive the run length from the motor-concentration profile on the minus end of the MT.

(ii) The velocity of a motor protein is another key motility parameter. The two most widely applied approaches to measure the velocity of a kinesin are surface-gliding assays and single-motor walking assays, detected with single-molecule fluorescence. The former approach is a multi-motor assay, in which the motors are bound to a surface and the motility of the MT track driven by these bound motors is measured using fluorescence or other microscopy methods (4). In the latter, single-molecule approach, the MTs are bound to the surface and the motion of single motors is followed (1, 2, 18). Although it requires more advanced instrumentation, we have a preference for the latter method since it allows for monitoring of the multimeric state of the motor proteins, it is less prone to undesired interactions with the surface, and can resolve potential heterogeneous behavior. By performing the measurements at different ATP-concentrations the Michaelis-Menten parameters can be obtained. These parameters describe the intrinsic catalytic rates and the affinity of the active site for ATP. Typically, for Kinesin-1 a maximal velocity around 900 nm s⁻¹ and a $K_m$ (the substrate concentration at which half the velocity is reached) around 30 μM has been determined (19).

(iii) The randomness of motility ($r$) of a motor protein can be obtained from time-displacement trajectories ($x(t)$) of individual motors using Eq. 1 (20), once the step size, $s$, is known,

$$r = \lim_{t \to \infty} \frac{\langle x(t)^2 \rangle - \langle x(t) \rangle^2}{s \langle x(t) \rangle} = \frac{1}{s} \frac{d}{dt} \frac{\langle x(t)^2 \rangle - \langle x(t) \rangle^2}{s \langle x(t) \rangle}$$  (1)

With $v$ the velocity of the motor. The randomness is a measure of the stochasticity of the time between steps (dwell time). If the motor were to be a
perfect clock (i.e. the duration between steps would be constant), there would be no variance in the dwell times and as a consequence the randomness would be zero. If, on the other hand, the stepping would be determined by a single exponentially-distributed, rate-limiting process, the randomness would be one. In the case of two sequentially occurring exponentially-distributed processes with (on average) equal duration, the randomness would be one half. For Kinesin-1, the randomness has been measured using traveling-wave tracking (6) and optical tweezers (3, 20, 21). In the most elaborate studies using optical tweezers, at different ATP concentrations and a load of 1.05 pN (21), it was found that the randomness is about one half at saturating ATP, decreases with ATP-concentration to one third (around $K_m$) and then increases to one at even lower ATP-concentrations. This behavior was explained by Kinesin-1’s mechanism consisting of two rate-limiting, ATP-concentration independent exponential processes and one ATP-dependent process. At lower ATP-concentrations ATP-binding takes longer and longer, first leading to a decrease of the randomness (three processes become rate limiting) and subsequently, at concentrations below $K_m$, to an increase to unity (only ATP-binding is rate limiting). In a later study, a more complex data set, involving force dependence of the randomness, was described with a more elaborate model, involving 5 states, one reversible ATP-concentration dependent, one reversible ATP-independent transitions. Of these transitions, 4 were required to be force dependent. Here, we show that the randomness of kinesin motility can also be measured using single-molecule fluorescence microscopy, which has the advantage of less complicated and more widely available instrumentation and of measuring without applying a load to the motor. Our data can be well described on basis of models with one ATP-dependent rate and at least two additional ATP-independent rates.

3.2 Methods

3.2.1 Experimental setup

Assays were performed at 21°C using an epi-illuminated wide-field fluorescence microscope. Excitation light was coupled into a TIRF-objective (TIRF Plan Apo 100x/1.45 oil, Nikon, Amstelveen, The Netherlands) with a polychromatic dichroic mirror (Z488RDC/532/633RPC, Chroma, Rockingham, VT) that allowed for combined excitation with a 635 nm (IQ1C10(LD1338)G3H5, Power Technology Inc., Little Rock, AR) and a 532 nm (Compass 215M-20, Coherent, Santa Clara, CA) laser. To induce TIRF the excitation beam was coupled off-axis into the objective creating an evanescent wave in the sample. Fluorescence was collected with the objective and filtered using HQ610/75M or a
HQ700/75M band-pass filter (Chroma). The two spectral bands were imaged sequentially on a CCD camera (MicroMax 512FTB, Princeton Instruments, Trenton, NJ).

3.2.2 Microtubule preparation

Tubulin was purified from pig brain and labeled with Cy5 (14); biotinylated tubulin was purchased (027T333-A, Cytoskeleton, Denver, CO). To polymerize MTs, tubulin was incubated in PEM 80 (80 mM H2PIPES, 1 mM EGTA, 2 mM MgCl2, pH 6.8, adjusted with NaOH) with GMPCPP (200 μM, Jena Bioscience, Jena, Germany) at 37 °C for 20 minutes. Subsequently, the MTs were stabilized with 10 μM Taxol and stored at room temperature.

3.2.3 Experimental assay for kinesin density profile determination

To determine the motor density profile along a MT, a chamber was prepared by attaching plasma-cleaned dimethyl-dichlororsilane-treated slides to cover slips using double-stick tape. The chambers were incubated for 5 minutes with BSA-biotin at 0.1 mg ml⁻¹ (A2289, Sigma-Aldrich, Zwijndrecht, Netherlands) in PEM80, washed with buffer and incubated for 5 minutes with streptavidin at 0.1 mg ml⁻¹ (8587, Sigma-Aldrich). The surface was blocked by incubating with 0.2 % (w/v) Pluronic F108 (BASF, Ludwigshafen, Germany) for five minutes. Next, the chamber was incubated with biotinylated Cy5-labeled MTs for 5 minutes. After rinsing with buffer, the chambers were flushed with Alexa555-labeled human kinesin at a concentration of roughly 20 nM (truncated human kinesin of 391 amino acids with a single surface-exposed cysteine at amino acid position 217 (hKin391- T217C) labeling stoichiometry ~1 Alexa555/ kinesin dimer (determined from the absorbance ratio at 280 nm and 555 nm), kind gift from Günter Woehlke) in motility buffer containing PEM80 with 4 mM DTT, 25 mM glucose, 20 μg ml⁻¹ glucose oxidase, 35 μg ml⁻¹ catalase, ATP and an ATP-regeneration system was added as previously described (22). The exposure time during the measurements of the motor density on the MT was 1 second and the excitation power was 9 μW (more than ten times lower than the power required for single-molecule imaging). To obtain an intensity profile of many kinesin motors on a single MT over one hundred frames were averaged. Stacks of images were acquired using Winview (Princeton Instruments) and analyzed with routines written in Labview 8.2 (National Instruments, Austin, TX).

3.2.4 Experimental assay for velocity and randomness determination

Cover slips were cleaned by plasma cleaning for 20 min. A positively charged surface was created by amino-silanization with DETA (3-[2-(2-aminoethylamino)ethyl-amino]propyl-trimethoxysilane, Aldrich). Sample chambers were first incubated with Cy5 labeled MTs followed by 5 minutes incubation with 0.2 mg ml⁻¹ casein. Next, the chambers were flushed with Alexa555-labeled kinesin at a concentration below 1 nM in the motility buffer.
In these experiments a truncated (at amino acid 560) human kinesin construct containing only a single native cysteine residue at position 421 (hKin560-421C) was used. At 4 µM ATP an ATP-regeneration system was added.

3.2.5 Calculation of velocity and randomness

From stacks of images, traces were extracted using kymographs as described elsewhere (14). A trace was defined as the discretely sampled trajectory of a single motor between appearance and disappearance of a fluorescent spot on a MT. The x-y-coordinates of a moving spot were determined by fitting a 2D-Gaussian to the observed fluorescence intensity profile in each frame (9). Only traces with a minimal length of three time points were used. In the rare events that a motor stalled, traces were discarded or only the beginning segment was used. The mean displacement \( MD(\tau) \) was calculated from the traces for all independent (non-overlapping) time lags \( \tau \) in all trajectories at a given ATP concentration. The error was estimated by calculating the standard error of the mean. The motor velocity, at each ATP concentration, was determined from a weighted linear fit to \( MD(\tau) \) \( MD(\tau) = v \tau \). Next, the variance of the MD \( var(\tau) \) was calculated for each time lag \( \tau \) with:

\[
\text{var}(\tau) = \left\langle x^2(\tau) \right\rangle - \left\langle x(\tau) \right\rangle^2
\]

The errors in velocity and randomness were calculated from the standard error of the mean of the mean displacement and the standard error of the mean of the variance using standard error propagation rules. The variance of the mean displacement at a certain time lag has a time-dependent contribution due to the randomness of stepping (20) and a time-independent offset due to the localization uncertainty of fluorescent spots (23). The offset was approximately constant in all experiments \( (540 \pm 30 \text{ nm}^2) \), implying a localization uncertainty of \( \sim 23 \text{ nm} \). The randomness (Eq.1), was determined by a weighted linear fit to the variance at the first 10 time lags (corresponding to a traveled distance of about 130 nm, (24)) for every ATP concentration.

3.2.6 Modeling the ATP-concentration dependence of the randomness

The ATP-concentration dependence of the randomness was modeled with three distinct models (3). All three consist of a single ATP-concentration-dependent process (that dominates at low ATP concentrations). The duration of this process \( T_{ATP} \) is calculated using:

\[
T_{ATP} = \frac{K_m}{k_{cat}[ATP]}
\]

with \( k_{cat} = \frac{v_{max}}{S} \) and \( K_m \) the Michaelis-Menten parameters determined in the velocity experiments. The three models differ in the number of ATP-independent exponential
3. Kinesin-1’s run length and randomness

processes: 1, 2, or 3, all with the same duration \( T_n = 1/(n k_{cat}) \) (\( n \) representing the number of additional ATP-independent processes).

The randomness is then calculated using:

\[
\rho = \left( \frac{T_{ATP}}{T_{tot}} \right)^2 + \sum_{i=1}^{n} \left( \frac{T_i}{T_{tot}} \right)^2
\]

with

\[
T_{tot} = T_{ATP} + \sum_{i=1}^{n} T_i = \frac{K_m}{k_{cat}[ATP]} + \frac{1}{k_{cat}}
\]

the total stepping time.

In addition to these three models, we compared our data to a fourth, more complex model consisting of 5 states (25), the first transition being ATP-dependent and only the first and second transitions being reversible. This reaction scheme was modeled using stochastic simulation (100,000 transitions, and the transition times were obtained from exponential distributions with fixed transition rates). The values for \( k_{cat} \) and \( K_m \) used by Block and coworkers (25) differ from the values we observed, which might reflect the different source of the protein (human versus squid). To account for this, we adjusted the transition rates in the 5-state model to fit our Michaelis-Menten parameters, while keeping the ratios the same. Note that all models applied do not contain fitting parameters.

3.3 Results

3.3.1 Determination of kinesin’s velocity and randomness using TIRF microscopy

In order to determine the randomness of Kinesin-1’s motility at zero load using TIRF microscopy, we tracked single, fluorescently labeled kinesin motors binding to and moving unidirectionally along MTs (figure 3.1). The illumination conditions were optimized for signal-to-noise and an average displacement of at most 20 nm was allowed within the integration time of one frame. From stacks of images obtained in this way (figure 3.1B) time trajectories of individual kinesin motors (position as function of time) were extracted (figure 3.1C). For each ATP concentration we obtained typically ninety trajectories (except at 4 µM ATP where 44 were obtained). From these trajectories, we calculated the mean displacement and the variance of the mean displacement over time for each ATP concentration (figure 3.2A and B) and found that both increase linearly with time as expected. The velocities were determined from the slopes of mean displacement time traces, yielding for example (figure 3.2A) a velocity of 143 ± 1 nm s\(^{-1}\) at an ATP concentration
of 10 μM. The average velocities obtained at different ATP concentrations can be fit well with the Michaelis-Menten model, yielding an apparent Michaelis-Menten constant ($K_m$) of 42 ± 6 μM and a maximum velocity at an infinite ATP concentration ($v_{\text{max}}$) of 925 ± 33 nm s$^{-1}$ (figure 3.2C), in agreement with literature values (26, 27). From the velocity, the variance of the mean displacement, and a step size of 8 nm, we determined the randomness of motility for each ATP concentration using Eq. 1 (figure 3.2D).

![Figure 3.1 Kinesin-1 moving over a microtubule](image)

(A) Schematic representation of an experimental assay. Labeled MTs are fixed to the surface and labeled Kinesin-1 and ATP are added to the solution. The surface is blocked to prevent non-specific kinesin binding. MTs and motors are visualized using TIRF microscopy. (B) Several frames from a time-laps recording showing an individual Alexa-555-labeled Kinesin-1 (hKin560-421C) moving along a MT at 10 μM ATP (exposure time 90 ms, frames displayed are 450 ms apart). The MT position is indicated in the first frame by the dashed line, black scale bar: 1 μm. (C) Eleven representative single-molecule trajectories of Kinesin-1 at 10 μM ATP, obtained from stacks of images like in (B).

The values we obtained using TIRF microscopy at zero load are similar to those obtained with an optical tweezers force clamp at a load of 1.05 pN (21). We determined that the
randomness at 2 mM ATP (saturating concentration) is $0.45 \pm 0.06$ confirming that at saturating ATP two or more exponential processes are rate limiting (Figure 3.2D) (20). At ATP concentrations near $K_m$ the randomness decreases slightly, suggesting that an additional process becomes rate limiting. At concentrations below $K_m$, the randomness increases and becomes close to unity ($0.8 \pm 0.2$ at 4 µM ATP), indicating that at very low ATP concentrations a single exponential process is rate limiting, namely the binding of ATP. For comparison, we also plotted three curves that represent simple models with one ATP-dependent exponential process, representing ATP binding and with one, two, or three ATP-independent exponential processes of equal duration (dashed lines). The ATP-dependence of the randomness as obtained with TIRF microscopy is best explained by a model in which the kinetics of kinesin stepping are governed by two ATP-independent Poissonian processes with equal duration and a third ATP-dependent one (20). A different, more complex model for kinesin mechanochemistry was proposed before by Block et al (25) and consists of 5-states. In this model the first (ATP-dependent) and second transitions are reversible, the three other transitions are irreversible and not equal. We adapted the model from the published version in order to correspond to the $K_m$ and $k_{cat}$ we measured for human Kinesin-1 at zero load. A comparison between this model and our data (figure 3.2D) shows that this 5-state model could be a good description of our measurements, although additional information (like the force dependence presented in (25)) would be required to discriminate between this 5-state model and the simple 3-state model presented before.

**3.3.2 Kinesin’s density profile along a MT reveals the average run length**

Next, we set out to determine the run length of our Kinesin-1 construct. The kinesin motility traces we used above are not well suited for this purpose, since photo bleaching severely limited the observed length of the runs. We performed single-molecule measurements with longer integration times and lower excitation intensity and estimate a run length of $950 \pm 100$ nm after correction for photo bleaching (at 2 mM ATP, figure S1, Supporting Material). To circumvent both the need to correct for photo bleaching and to work at the single-molecule level, we developed another approach to determine the run length. We noticed that at kinesin concentrations too high for clearly resolving single motors, the motor density profile on the minus-end of the MT was clearly different from that on the plus-end. Our hypothesis was that this was caused by the motors’ finite run length. In Figure 3.3A a TIRF microscopy image (integration time about three minutes) is shown of a Kinesin-1 coated MT. In this experiment the Alexa-555-labeled hKin391–T217C concentration was 25 times higher than with the single-molecule measurements and the ATP concentration was saturating (2 mM). Due to the long integration time, the motility of individual kinesins is smeared out.
3. Kinesin-1's run length and randomness

Figure 3.2 The velocity and randomness of Kinesin-1 depends on the ATP concentration.

(A) Mean displacement of Kinesin-1, at 10 μM ATP, calculated for all independent time intervals in the trajectories of all runs measured. Error bars represent standard error of the mean. Solid line is a weighted fit: \( MD = vt \) (with \( v = 143 \pm 1 \text{ nm s}^{-1} \)).

(B) Variance of the mean displacements calculated in 3.2a. Error bars represent standard error of the mean. Solid line is a weighted fit to \( \text{varMD} = C \tau + \text{offset} \) (offset reflects the localization uncertainty of 23 nm, the slope \( C = 620 \pm 80 \text{ nm}^2 \text{ s}^{-1} \)).

(C) Average velocity of Kinesin-1 at different ATP concentrations (calculated from a weighted fit of the mean displacement). Data is fitted with the Michaelis-Menten model \( \nu = \frac{\nu_{\text{max}} [\text{ATP}]}{[\text{ATP}] + K_m} \) yielding \( K_m = 42 \pm 6 \mu \text{M} \) and \( \nu_{\text{max}} = 925 \pm 33 \text{ nm s}^{-1} \).

(D) Randomness as a function of ATP concentration, calculated from a weighted fit of the time trace of the variance (2B), an 8 nm step size and the average velocity (Eq. 1). The three thin black curves are models of the ATP dependence of the randomness assuming 1 (\( \chi^2 = 132.41 \)), 2 (\( \chi^2 = 7.15 \)), or 3 (\( \chi^2 = 26.19 \)) ATP-independent exponential processes (thin solid, thin dashed, thin dotted curve respectively). The randomness is best described by a model with 2 ATP-independent exponential processes. The thick, solid curve represents a complex, 5-state model with first two reversible transitions followed by three irreversible transitions (\( k_{\text{on}} = 3.5 \mu \text{M}^{-1} \text{s}^{-1}, k_{\text{off}} = 236 \text{ s}^{-1}, k_2 = 2573 \text{ s}^{-1}, k_3 = 7 \text{ s}^{-1}, k_4 = 1084 \text{ s}^{-1}, k_5 = 325 \text{ s}^{-1}, \chi^2 = 4.00 \)).

The intensity profile along the long axis of the MT (figure 3.3A), is clearly asymmetric: the intensity increases steeply, within about 350 nm on the plus-end of the MT, while it increases more gradually on the minus-end of the MT. Similar, asymmetric intensity profiles were measured for all the other MTs in the sample. If, on the other hand, the non-
hydrolysable analogue AMPPNP was used instead of ATP, the intensity profiles became more symmetric and the intensity rise at both ends of the MT was sharp, within 350 nm (figure 3.3B). This was to be expected since, in the presence of AMPPNP, kinesin motors are fixed on the MT, the run length is zero and therefore the intensity profile should drop sharply at both ends of the MT. The resulting intensity profile should be the convolution of a uniform distribution of kinesin along the MT and the point-spread function of our microscope. The point-spread function of our microscope is well approximated by a Gaussian with width 310 ± 10 nm. The convolution of such a Gaussian and a step function (resulting in an error function) provides a good description of the AMPPNP data (figure 3.3B), indeed indicating that the kinesin density is constant along a MT and drops sharply to zero beyond the length of the MT. This is in contrast to the gradual fluorescence incline that is observed on one side of kinesin-coated MTs in the presence of ATP.

To quantitatively understand the intensity profile in the presence of ATP, we modeled the density profile of kinesin binding to a MT from solution and walking unidirectionally along it. Assuming equilibrium between MT-bound kinesins and kinesin in solution (i.e. the average number of kinesins detaching equals the average number of kinesin attaching to a MT) and assuming that the kinesin binding sites on the MT do not saturate (the motor concentration should be adjusted accordingly), a steady-state differential equation can be expressed as:

\[
v \frac{dn(x)}{dx} = kn_0 - k'n(x) - k'_\text{bleach} n(x)
\]

where \( v \) is kinesin’s velocity, \( n_0 \) its concentration in solution, \( k \) the attachment rate, \( k' \) the detachment rate of MT-bound motors, \( k'_\text{bleach} \) the rate of photo bleaching, and \( n(x) \) the kinesin number on the MT as a function of the position \( x \). The minus end of the MT is the \( x=0 \) position (figure 3.1A). Here we assume a homogenous field of illumination, such that the rate of photo bleaching is position independent. To solve this differential equation, we set the kinesin number on the minus-end of the MT to zero and find:

\[
n(x) = \frac{l_{\text{app}} kn_0}{v} (1 - e^{-x/l_{\text{app}}})
\]

where \( l_{\text{app}} \) is the apparent run length, defined as follows (with \( l \) the real run length):

\[
l_{\text{app}} = \frac{v}{k' + k'_\text{bleach}} = \frac{l}{1 + \frac{k'_\text{bleach}}{k'}}
\]

The intensity profile on the minus end of the MT is described by Eq. 3. It does however not describe the intensity drop-off at the plus end of the MT.
To fully describe the intensity profile along the entire length of the MT Eq. 3 has to be convoluted with the point-spread function (approximated by a Gaussian), yielding fit equations as described in the Supporting Material. The resulting fit of the entire intensity profile along the MT figure3.3A, yields an apparent run length of 908 nm. The apparent run lengths obtained from fitting the intensity profiles of 32 MTs observed under the same conditions form a Gaussian distribution, with an average of 1070 ± 30 nm (s.e.m.) (figure 3.3C). This apparent run length could in principle deviate from the real one, as described by Eq. 4, if the rate of detachment was not substantially larger than the rate of photo bleaching. We determined that under our experimental conditions the rate of photobleaching is less than 0.001 s⁻¹, by measuring the rate of single fluorophore bleaching on MT-bound kinesins in the presence of 1 mM AMPPNP (data not shown). The average detachment rate of the motor was more than an order of magnitude larger, 0.05 s⁻¹ (v/l, at the lowest velocity measured: v = 60 nm/s at 5 µM ATP, see below), indicating that photo bleaching did not affect our determination of the run length, within the uncertainty of the measurements. The run length determined from the kinesin density on the minus end of the MT, 1070 ± 30 nm, is in good agreement with the value we determined in the traditional way (figure S1, Supporting Material) and values reported before for similar Kinesin-1 constructs at saturating ATP concentrations (16, 17, 26).

3.3.3 The run length of kinesin at low ATP concentrations.

Next, we set out to measure the run length of our Kinesin-1 construct at lower ATP concentrations. At low ATP concentrations kinesin spends an appreciable amount of its total cycle time in an ATP waiting state, an amount that increases with decreasing ATP concentration. If the ATP waiting state is a weakly bound state with a relatively high probability for detachment, one would expect the run length to be ATP concentration dependent. If, on the other hand, the ATP waiting state were to be a strongly bound state, with a low probability of detachment (compared to the other states in the cycle) the run length would not be affected by the concentration of ATP. Experimental determinations of the ATP-dependence of the run length have yielded conflicting results (19, 28), but have been used for modeling of Kinesin-1 motility (28, 29). When we apply the intensity profile approach to samples with a low ATP concentration, we observe that the run length is independent of ATP concentration (figure 3.3D), confirming the results of an earlier report (19). Our results are consistent with models with an ATP-waiting state that is tightly MT bound and has a very low off rate (30).
3. Kinesin-1’s run length and randomness

Figure 3.3 Run length of Kinesin-1 obtained from its intensity profiles on a MT.

(A) Time-integrated intensity profile of a MT with moving Alexa-555 labeled Kinesin-1 (hKin391-T217C). The corresponding integrated image of the MT is shown below the profile. The profile was determined summing over the width of the image of the microtubule (three pixels). This profile was constructed by integrating 200 frames with one second exposure time each. The solid line represents a fit of Eq. S1 to the data points with the same position range as the fit curve. Fit parameters are $A = 7261$, $d = 9670$ nm, $x_0 = 65$ nm, $r = 290$ nm, $l_{app} = 908$ nm, $B = 10220$. (B) Time-integrated intensity profile of a MT decorated with AMPPNP fixed kinesins. The MT is shown below the profile and was illuminated with a threefold higher intensity than the MT in 3A. Each point in the profile is calculated in the same manner as described in 3A, the $z$-scale has the same relative scale, the integration was 1 second and 30 frames were summed. The solid line represents a fit of a convolution of a block function with a Gaussian with a fixed width, $A(\text{Erf}(2(x-x_{c1})/\text{FWHM}) + \text{Erf}(2(x-x_{c2})/\text{FWHM})) + I_0$ (Erf being the error function, and $x_{c1}$ and $x_{c2}$ are the locations of the MT ends). Fit values are $A = 3310$, $x_{c1} = 2576$ nm, $x_{c2} = 7759$ nm and $I_0 = 2298$. The FWHM was set to 310 nm. (C) Distribution of run lengths obtained from individual MTs decorated with moving Kinesin-1 at an ATP concentration of 2 mM. The solid line represents a Gaussian fit with a center position at $1070 \pm 30$ nm. (D) Average run length of Kinesin-1 at different ATP concentrations. Averages and their s.e.m. are determined from the Gaussian fit on the distribution of average run lengths of single MT’s (2 mM: 32 MTs; 20 µM: 25 MTs; 5 µM: 18 MTs). The solid line, to guide the eye, represents the average of the three values $1030 \pm 40$ nm.
3.4 Discussion

Single-molecule fluorescence microscopy is a well-established technique to measure the motility of single, fluorescently labeled motor proteins such as kinesin under biochemical conditions (ATP concentration and ionic strength) in vitro. In particular, it has been widely applied to determine the motor's velocity and run length (19, 31) and it has been used to measure the step sizes of individual motor domains (11). Here we have shown that TIRF microscopy can also be used to measure the randomness of the motility. The randomness is a parameter that provides information on the stochastic nature of the underlying motility process, in particular the number of rate-limiting exponential processes that underlie motility.

Furthermore, we demonstrated a novel way of determining the run length of kinesin from the intensity profile of many fluorescent kinesins walking along a MT. We observed that on the MT minus-end the intensity increases more gradually compared to the plus-end of the MT. Indeed, the intensity increase on the minus-end follows exponential saturation, with an exponential constant equal to the run length. Run lengths have before been determined from single-molecule tracking measurements using optical tweezers (2) or fluorescence microscopy (31). The former approach can only be performed under load, the latter suffers from complications due to photo bleaching. Our approach has the key advantages that it provides data averaged over many motors obtained in a single measurement. It hardly suffers from photo bleaching and does not require single-molecule observations. Our method does, however, require homogeneous illumination, a good batch of proteins (without 'dead' motors that remain stuck on the MT), and a run length that is at least as long as the point spread function of the instrument, ~300 nm.

We have shown here that TIRF microscopy, which is far less complex than other methods such as optical tweezers and is available in many labs, can be used to reliably determine the key motility parameters run length, velocity and randomness, all three as a function of ATP concentration. An additional advantage of TIRF microscopy is that using this method no load is applied to the motors. We envision that the approaches presented here can be applied by many labs to various other motor proteins from the kinesin and other super families that still remain to be characterized.

Using these approaches, we have shown here that the run length of Kinesin-1 is 1070 ± 30 nm, independent of ATP concentrations. Our observations, which are not hampered by the effects of photo bleaching, settles a discrepancy between previous experimental and modeling studies (19, 28, 29). In addition, we have measured the ATP-dependence of the randomness, at zero load. Our results show that the rate of Kinesin-1 is limited by two or more exponentially distributed processes at high ATP concentrations, but that an additional,
ATP-dependent process becomes the sole rate-limiting process at low ATP concentrations. These results will be of importance for a detailed understanding of Kinesin-1's mechanism and kinetics.

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**References**

3. Kinesin-1’s run length and randomness

Supporting Material

S.3.1. Determination of Kinesin-1 run length using single-molecule tracking

Figure S1: Run length determination of Kinesin-1 using the traditional single-molecule tracking approach. **Left:** cumulative distributions of Kinesin-1 runlengths measured at 700 and 400 µW. Straight lines: single exponential fits yielding an average run length of 475 ± 12 and 675 ± 13 nm, respectively. **Right:** linear extrapolation of the run length determined at different excitation power to estimate the run length in the absence of photo bleaching (extrapolated to zero power). Here, it is assumed that motors only contain a single fluorophore, such that terminations of runs due to photobleaching and dissociation are both exponential processes. We estimate that the run length is 950 ± 100 nm.
3. Kinesin-1’s run length and randomness

S.3.2. Fitting function of the kinesin intensity profile along the microtubule axis

The observed intensity profile along the MT axis is a convolution between the point-spread function of the microscope and the actual distribution of labeled kinesin along the MT. The point-spread function can be approximated by a Gaussian function, yielding the following equation for the intensity profile:

$$ I(x) = \int_{0}^{d} dx' n(x') \cdot PSF(x-x') = A' \int_{0}^{d} dx' \left[ 1 - e^{-\frac{x'}{l_{app}}} \right] \cdot e^{-\frac{(x-x')^2}{r^2}} $$

with $d$ the length of the microtubule, $n(x')$ the motor density, $r$ half width $(1/e^2)$ of the point-spread function, and $l_{app}$ the apparent run length. Choosing $x = x_0 - x$, with $x_0$ the beginning of the MT, this yields the following equation for the intensity profile:

$$ I(x) = \left( e^{\frac{r^2 - 8l_{app}(x-x_0)}{8l_{app}^2}} \right) \left[ G(r,l_{app},x) - H(d,r,l_{app},x) \right] + I(r,x) + J(d,r,x) A + B $$

[S1]

with:

$$ G(r,l_{app},x) = \text{Erf} \left[ \frac{r^2 - 4l_{app}(x-x_0)}{2 \sqrt{2l_{app}}} \right] $$

$$ H(d,r,l_{app},x) = \text{Erf} \left[ \frac{4d \cdot l_{app} + r^2 - 4l_{app}(x-x_0)}{2 \sqrt{2l_{app}}} \right] $$

$$ I(r,x) = \text{Erf} \left[ \frac{\sqrt{2}(x-x_0)}{r} \right] $$

$$ J(d,r,x) = \text{Erf} \left[ \frac{\sqrt{2}(d-x-x_0)}{r} \right] $$

Where, $B$ is the offset due to background and Erf the error function. To obtain an accurate value for $r$, we fitted a Gaussian to the intensity profile perpendicular to the MT every 5 pixels (over the length of the MT). The average value obtained in this way was used in Eq. S1 to fit the intensity profile and extract the apparent run length.
4. The Homotetrameric Kinesin-5, KLP61F, Preferentially Crosslinks Microtubules into Antiparallel Orientations

Siet M.J.L. van den Wildenberg,*¶ Li Tao,†¶ Lukas C. Kapitein,*§ Christoph F. Schmidt,‡ Jonathan M. Scholey,† and Erwin J.G. Peterman.*

*Department of Physics and Astronomy and Laser Centre, VU University Amsterdam, The Netherlands, †Department of Molecular and Cell Biology, University of California at Davis, Davis, CA 95616, and ‡Department of Physics, Georg August University Göttingen, Germany. §Present address: Department of Neuroscience, Erasmus MC, Rotterdam, The Netherlands.
¶These authors contributed equally.

Abstract

During mitosis, the segregation of the genetic material is coordinated by the mitotic spindle. The mechanism of action of the spindle depends upon the polarity patterns of its constituent microtubules (MTs). Homotetrameric mitotic kinesin-5 motors are capable of crosslinking and sliding adjacent spindle MTs, but it is unknown if they contribute to the establishment of these MT polarity patterns. Here we explored if the Drosophila embryo kinesin-5, KLP61F, which is thought to crosslink both parallel and anti-parallel MTs, displays a preference for the parallel or anti-parallel orientation of MTs. In motility assays, KLP61F was observed to crosslink and slide adjacent MTs, as predicted. Remarkably, KLP61F displayed a three-fold higher preference for crosslinking MTs in the antiparallel, relative to the parallel orientation. This polarity preference was observed in the presence of ADP or in ATP plus AMPPNP, but not with AMPPNP alone, which induces instantaneous rigor binding. Also, a purified motorless tetramer containing the C-terminal tail domains displayed an antiparallel orientation preference, confirming that motor activity is not required. The results suggest that, during the morphogenesis of the Drosophila embryo mitotic spindle, the crosslinking and sliding activities of KLP61F could facilitate the gradual accumulation of KLP61F within antiparallel interpolar (ip) MTs at the equator, where the motor could then generate force to drive poleward flux and pole-pole separation.
4. KLP61F Crosslinks microtubules into antiparallel orientations

4.1 Introduction

Separation of the genetic material during mitosis is coordinated by the mitotic spindle, whose mechanism of action depends upon the polarity patterns of its constituent microtubules (MTs) (1, 2). Although, homotetrameric mitotic kinesin-5 motors are capable of crosslinking and sliding adjacent spindle MTs (3-11), it is unknown if they contribute to the establishment of these MT polarity patterns. In this study we explored if the Drosophila embryo kinesin-5, KLP61F, which is thought to crosslink both parallel and anti-parallel MTs (7, 12), displays a preference for the parallel or anti-parallel orientation of MTs. First, KLP61F was observed to crosslink and slide adjacent MTs. Interestingly, KLP61F displayed a three-fold higher preference for crosslinking MTs in the antiparallel, relative to the parallel orientation. Unlike in the presence of ADP or ATP plus AMPPNP, where this polarity preference was observed, in the presence of AMPPNP alone no polarity preference was found. Also, a purified motorless tetramer containing the C-terminal tail domains displayed an antiparallel orientation preference. Thogheter these results suggest that, by its sliding activities and its binding preference, KLP61Ft could gradually accumulate within antiparallel interpolar (ip) MTs at the spindle equator, where the motor could generate force to drive pole-pole separation.

4.2 Methods

4.2.1 Protein preparation and characterization

Three different constructs corresponding to full length KLP61F, headless KLP61F, and KLP61F stalk were generated as described previously (7). After verification by sequencing, the recombinant constructs were used to generate recombinant Baculovirus (Invitrogen Baculovirus Expression System). Amplified virus was used to infected sf9 cells. The proteins were purified from cell lysates with a Ni-NTA affinity column (Qiagen) followed by superose 6 gel-filtration FPLC (GE Pharmacia). Tubulin and polarity-marked MTs were prepared as described before (4, 7, 21). In short, rhodamine-labeled biotin was purchased from Cytoskeleton Inc. Fluorescent (and biotinylated) MTs were polymerized from a mixture of 0.1 μM Cy5- or rhodamine-labeled tubulin, (0.8 μM biotin-labeled tubulin) and 10 μM unmodified tubulin in the presence of 1 mM GpCpp (Jena Bioscience) and 2 mM DTT at 35 °C for 25 minutes. To construct polarity-marked MTs, MTs were further incubated in the presence of a mixture of 0.4 μM NEM-tubulin, 0.1 μM rhodamine-labeled tubulin and 0.4 μM unmodified tubulin for 30 minutes. After stabilization with 10 μM paclitaxel (Sigma-Aldrich), MTs were centrifuged through a glycerol cushion (50% (v/v), using a Beckman Coulter Airfuge Ultracentrifuge (operated at at a pressure of 25 psig) to remove free tubulin, and were subsequently resuspended.
4. KLP61F Crosslinks microtubules into antiparallel orientations

4.2.2 MT-bundling assay and hydrodynamic assays

Both assays were performed exactly as described previously for purified full length KLP61F (7). In the case of KLP61F subfragments, instead of partially purified proteins from a Ni-NTA affinity column, pure motor-less, and stalk subfragments were used after FPLC purification. From hydrodynamic assays, the full length KLP61F homotetramer had Rs = 16.7 nm, S-value = 7.4 S, native MW = 520 kDa; the motorless KLP61F homotetramer (K354 – 1066) had Rs = 16.4 nm, S-value = 5.5 S and native MW = 378 kDa; and the KLP61F stalk homotetramer (K354-923) had Rs = 13.3 nm, S-value = 4.9 S and native MW = 272 kDa.

4.2.3 Fluorescence Microscopy

MT sliding and orientation experiments were performed at 21°C using a custom-built widefield fluorescence microscope described previously (4, 14) using a 100x Nikon S-Fluor objective (NA = 1.3). For simultaneous observation of rhodamine and Cy5, the sample was simultaneously illuminated with 635 nm (Power Technology Inc., IQ1C10(LD1338)G3H5) and 532 nm (Coherent, Compass 215M-20) laser light. The emission was first filtered with a triple bandpass filter (Z488/532/633M, Chroma), then separated with a dichroic beam splitter (565DCXR, Chroma) and finally redirected onto the tube lens at slightly different angles, resulting in two separate images on the camera chip (Micromax, Roper Scientific). Images were taken at a frame rate of 1 s⁻¹, typical laser intensities used were 10 W cm⁻².

4.2.4 Relative MT sliding assays

Tubulin and polarity-marked MTs were prepared as described above and before (4). Cover slips were treated with dimethyl-dichlororsilane (4), and chambers were prepared by attaching the cover slips to microscope slides using double-stick tape. Chambers were incubated for 5 minutes with BSA-biotin (Sigma-Aldrich, 0.1 mg ml⁻¹) in PEM80 (80 mM K₂PIPES, 1 mM EGTA, 2 mM MgCl₂, pH 6.8, set with HCl), washed with buffer and incubated for 5 minutes with streptavidin (Biochemika, 0.1 mg ml⁻¹). The surface was blocked by incubating with a watery solution of Pluronic F108 (0.2 %(w/v), BASF) for five minutes. Next, the chambers were incubated with biotinylated Cy5-labeled MTs (5min). After rinsing with buffer, the chambers were flushed with 1 nM KLP61F, 2 mM ATP and rhodamine labeled MTs in motility buffer (PEM80, pH 6.8, 2mM MgCl₂, 4 mM DTT, and 25 mM glucose, 20 μg ml⁻¹ glucose oxidase, 35 μg ml⁻¹ catalase).

4.2.5 Assays to determine the orientation of MTs bundled by KLP61F

In order to determine the crosslinking preference of KP61F, we used cover slips that were positively charged by silanization with 0.1 % (V/V) DETA (3-(2-(2-
aminoethylamino)ethyl-amino)propyl-trimethoxysilane, Aldrich) in water (incubated for 10 minutes, subsequently washed in water). Sample chambers were incubated with a mixture of polarity marked MTs, 20 nM KLP61F, 2 mM nucleotide (AMPPNP, ATP/AMPPNP, or ADP) in motility buffer. Fluorescent images were taken, and for all observed bundles consisting of two MTs of which the polarity could be unambiguously assigned, the relative orientation was determined. For each experiment, control experiments without KLP61F and with KLP61F stalk subfragments were performed to exclude the possibility that MT bundling occurred non-specifically.

4.3 Results and discussion

4.3.1 KLP61F can crosslink and slide adjacent MTs

We first tested if purified, full length KLP61F (figure 4.1A, lane 1), like its vertebrate ortholog, Eg5, is able to facilitate MT-MT sliding, using fluorescence microscopy-based MT-MT sliding assays (4). To this end, biotinylated Cy-5 labeled MTs were specifically attached to a glass surface. Subsequently, the surface was blocked with the amphiphilic block copolymer Pluronic F108 to prevent non-specific binding of MTs and KLP61F to the surface. Purified KLP61F and rhodamine-labeled MTs were added together with ATP. We then acquired time series of images which showed clear movement of rhodamine-labeled MTs over immobilized Cy5-labeled MTs (figure 4.1B). Rhodamine MTs did not land or slide on regions of the surface where no MT was immobilized. This excludes the possibility that MTs were driven by KLP61F directly attached to the glass surface. In most of the recorded events we observed crosslinked, non-aligned MTs, with a crossover point moving relative to both filaments with an average velocity of $11.0 \pm 3.1 \text{ nm s}^{-1}$ (s.d., $n = 18$) (figure 4.1C, D), which was independent of the crossing angle. Occasionally, as shown in figure 4.1B and C, the sliding MT rotated into alignment with the immobilized MT, whereupon the two relative velocities of sliding added up to about twice the individual velocities indicating that these MTs all ended up aligned anti-parallel (4). In some of the recorded events the sliding MT had already been aligned. The average velocity we measured for all aligned, sliding MTs was $26.7 \pm 4.5 \text{ nm s}^{-1}$, ($n = 16$) (see figure 4.1D). These observations suggest that KLP61F can crosslink MTs in either parallel or antiparallel orientation and that it moves with a well-defined velocity along both crosslinked MTs, largely independent of their relative orientation. Thus KLP61F can mediate the sliding of parallel and antiparallel MTs, just like its *Xenopus* ortholog, Eg5 (4).

However, the question remains if either of these kinesin-5 motors preferentially crosslinks MTs into parallel or antiparallel polarity patterns.
4. KLP61F Crosslinks microtubules into antiparallel orientations

Figure 4.1 Purified KLP61F can crosslink and slide adjacent MTs.

(A) Characterization of purified recombinant full length (FL)-KLP61F, headless KLP61F, and KLP61F stalk used in these studies. The coomassie-blue-stained SDS-polyacrylamide gel shows the purity of recombinant proteins after gel-filtration (Superose 6 FPLC, GE Pharmacia). Lane 1 shows FL-KLP61F, Lane 2 shows headless KLP61F (stalk + tail), Lane 3 shows KLP61F stalk. (B) Frames from a time-lapse recording showing a relatively short rhodamine-labeled MT sliding sideways (down and left) along a surface-attached Cy5-labeled MT. After 120 s the sliding MT rotates and aligns with the immobilized MT. The two velocities now add, indicating anti-parallel orientation. Scale bar, 1 μm. (C) Displacement of the hindmost interaction point of the rhodamine labeled MT along the immobilized MT axis in (B) is plotted versus time. A linear fits reveal two sliding velocities. (D) Histograms of velocities of all measured MTs in aligned and non-aligned configurations shown together with Gaussian fits.
4.3.2 KLP61F homotetramers preferentially crosslink MTs into antiparallel bundles

As a prelude to assaying kinesin-5’s MT crosslinking polarity preference, we used standard bundling assays to test the MT bundling activity of the following constructs: (i) purified full-length KLP61F (a 520 kDa tetrameric holoenzyme), (ii) a tetrameric 272 kDa native MW "stalk" fragment lacking both the N-terminal motor and the C-terminal tail domains, (iii) a tetrameric 378 kDa MW native "motorless" fragment (figure 4.1A). As expected, highly purified motorless KLP61F, like the full-length protein, displayed robust MT-bundling activity, whereas the purified stalk subfragment displayed no detectable bundling activity (figure 4.2A), supporting the idea that KLP61F homotetramers must contain either N-terminal motor domains or C-terminal tail domains to be capable of bundling MTs (7).

To determine if KLP61F has a preference for crosslinking MTs into either parallel or antiparallel bundles, polarity-marked MTs and purified KLP61F ((7); figure 4.1A) were mixed for 1 minute in assay buffer containing nucleotides, and were subsequently introduced into a microscope chamber with an amino-silanized glass surface which led to a fixation of the relative orientation of MTs upon attachment. After rinsing the sample, the parallel and antiparallel MT bundles attached to the surface were counted to determine their relative abundance (figure 4.2B). In these assays, unlike the more routine bundling assays shown in Fig 2a, the relative concentration of KLP61F and MTs was optimized to generate bundles consisting of two MTs and not more.

We observed that, in saturating concentrations of the non-hydrolyzable ATP analog AMPPNP, equal numbers of parallel and anti-parallel MT pairs were formed (figure 4.2C). We reason that this occurred because AMPPNP facilitates the strong binding of KLP61F motor domains to the MT tracks, immediately locking them in place in a tight binding state. In other words, AMPPNP freezes the on/off kinetics of the motors and will not allow potential differences in binding affinity of either the motor domains or the binding domains in the tails between antiparallel and parallel MTs to establish a preferred polarity pattern. The result further suggests that each individual KLP61F motor has considerable rotational flexibility (consistent with figure 4.2B-D) since the pairs of motor domains at opposite ends of the stalk domain must be capable of rotating by 180° in order to crosslink MTs oriented in either parallel or antiparallel configurations. It should also be noted that, even if the orientational preference of a single crosslink was small compared to thermal energies, several motors could still collectively cause a strong orientational bias over time if the crosslinking is transient.
Figure 4.2 KLP61F has a preference for crosslinking MTs into antiparallel orientations.

(A) Pure full-length (FL)-KLP61F and motorless KLP61F, but not KLP61F stalk, can crosslink and bundle MTs in 1mM ATP. Fluorescence microscopy shows that headless KLP61F and FL-KLP61F have obvious bundling activity. KLP61F stalk without motor and tail domains, however, did not bundle MTs under the same conditions. Scale bar: 10 μm.  

(B) Image showing crosslinked pairs of polarity marked MTs. The minus end of the MT is indicated in red, the plus end in green. When two MTs are bundled, the fluorescence intensity doubles. Based on the relative fluorescence intensity and the location of the polarity marks, the orientation of crosslinking can be determined, as indicated. White scalebar: 2 μm  

(C) Histogram showing the orientation of crosslinking by FL-KLP61F in the presence of AMPPNP (n = 124), ATP/AMPPNP (n = 60), and ADP (n = 122), as well as by the motorless KLP61F (n = 49). The errors indicated were calculated from the propagation of the counting errors (square root of the number of counts in each category).
In principle, the two sets of binding sites, on the motor domains and on the tails, could each cause an orientational bias, the bias could be equal or opposite, or just one set could cause the bias. The following experiments were designed to differentiate between the various scenarios. The existence of a bias implies a certain degree of mechanical torsional stiffness in the tetramers. Note that a bias caused by only one set of binding sites allows one to roughly localize flexibility in the molecule. To avoid the initial “orientation quench” caused by AMPPNP on the motor domains, which appears to lock KLP61F-MT complexes in a random initial tight binding configuration, we modified the assay. We first incubated MTs and KLP61F in the presence of ATP for one minute to allow the system to equilibrate. This time is appropriate since it exceeds the residence time of individual kinesin-5 motors on MTs, but is short enough to prevent sliding to the end of travel, whereupon kinesin-5 reaches the ends of “sorted” MTs. When this is allowed to occur, complicating events (e.g. “snap-backs” of dangling MTs etc) can introduce artifacts into the assays (discussed in ref. (4)). Following incubation, the crosslinked MTs were attached to the glass surface and AMPPNP was flushed in to lock the KLP61F motors in an immotile state. Under these conditions we observed three times more anti-parallel MT pairs than parallel ones, indicating that the full length KLP61F has a preference for generating anti-parallel MT pairs in the presence of ATP (figure 4.2D).

Earlier studies had shown that the KLP61F homologue, Eg5 can diffuse axially along the MT polymer lattice in the presence of ADP (13) which presumably does not involve specific and strong binding states of the motor domains, but likely depends on interactions with the C-terminal tail domains instead (7, 14). To examine the MT-bundling behavior of KLP61F in this "diffusive mode" where the binding via the motor domains is likely switched off, we tested MT-MT crosslinking in the presence of KLP61F and ADP. We again observed three times more anti-parallel versus parallel MT crosslinking under these conditions. All results thus suggest that the tail binding sites are responsible for the bias. To entirely exclude the possibility that the motor domains are required, we tested whether KLP61F’s C-terminal MT-binding domains alone can cause the orientational preference of these kinesin-5 motors. We determined the orientation of MTs bundled by motorless constructs (which were already shown to bundle MTs (figure 4.2A) in the presence of ADP. We again observed three times more anti-parallel than parallel MT bundles (figure 4.2D). KLP61F thus has an approximately 3-fold preference for bundling antiparallel MTs over parallel ones. This preference is preserved when the motor domains are totally absent as was the case for the motorless subfragment, or if they are switched off in a weakly and dynamically bound MT-binding state in the absence of ATP and in the presence of ADP.

Taken together, our results demonstrate that the homotetrameric kinesin-5, KLP61F, like its homolog Eg5, can crosslink and slide MTs. Our findings further suggest that kinesin-5
motors display a preference for crosslinking MTs into antiparallel bundles. It may be reasonable to assume that the bipolar structure observed for *Drosophila* KLP61F (5) and the MT-MT sliding activity demonstrated for *Xenopus* Eg5 (4) are shared by all members of the kinesin-5 family. However, kinesin-5 motors appear to be deployed to play different roles in spindles from different systems (15-20) which could be correlated with system-specific differences in the molecular architecture and mechanism of action of kinesin-5 motors. KLP61F is, to our knowledge, the first member of the kinesin-5 family explicitly shown to display both a bipolar ultrastructure (5) and MT-MT sliding activity (this report), both of which underlie the proposed kinesin-5-dependent “sliding filament” mechanism.

We do not know the molecular mechanism by which KLP61F preferentially crosslinks MTs into antiparallel orientations. This is a fascinating problem that merits further detailed analysis. The observation that tetramers of both ADP-bound full-length KLP61F and motorless KLP61F subfragments preferentially crosslink MTs into antiparallel orientations shows that the mechanochemical activity of the motor domains is not essential for the antiparallel polarity preference. In this context, it is interesting to note that MT crosslinking is also brought about by the non-motor MT binding protein, Ase1p which displays a similar antiparallel orientation preference (21). Note that the antiparallel MT orientation preference of motorless KLP61F suggests that the C-terminal tail domains may control the polarity preference of full length KLP61F, but we cannot exclude the possibility that active KLP61F motor domains (in contrast to those trapped in the presence of AMP-PNP) could contribute as well. We also note that the tail domains contain the cdk-dependent phosphorylatable bimC box which may target kinesin-5 to spindle MTs (12, 22), so it is tempting to speculate that the phosphorylation state of the bimC box influences the polarity preference of kinesin-5. To address the above issues, detailed structure-function studies of the MT crosslinking polarity preference of headless and tailless, phosphorylated and non-phosphorylated KLP61F constructs are planned.

Based on the results of the relative sliding experiments (figure 4.1) together with the absence of any MT crosslinking orientation preference in the presence of AMPPNP (figure 4.2), it is apparent that full-length KLP61F is flexible enough to crosslink MTs in any orientation. However, to explain the orientation preference that is observed in the presence of ADP and ATP, we imagine that some part of the tetramer must have sufficient torsional rigidity to form and maintain the antiparallel MT orientation. This apparent contradiction is resolved if one assumes that the stalk between the opposing tail domains is relatively rigid, that the C-terminal tail domains specifically interact with a MT, resulting in an antiparallel orientation preference, and that the flexibility of the motor domains resides in the neck and/or neck linker. An improved understanding of the torsional rigidity of different domains of the KLP61F homotetramer would therefore be illuminating.
4. KLP61F Crosslinks microtubules into antiparallel orientations

What are the implications of kinesin-5’s antiparallel polarity preference for the mechanism of mitosis? At present, there is considerable interest in the mechanisms of establishment of MT polarity patterns within mitotic spindles and in other MT-based structures such as axons and dendrites (21, 23, 24). In astral mitotic spindles such as those in the early Drosophila embryo, spindle MTs are organized into two overlapping radial arrays, with their minus ends located at the centrosomes, and their plus ends facing the equator of the spindle (12). Consequently MTs around and near the centrosomes are oriented parallel, whereas MTs overlapping with their plus ends at the equator are likely to encounter antiparallel neighbors. These antiparallel pairs are crucial for generating forces between the spindle poles. In some spindles such as Drosophila embryo mitotic spindles, motor-dependent crosslinking and relative sliding of antiparallel MTs at the spindle equator is thought to underlie poleward flux within interpolar MT (ipMT) bundles and pole-pole separation during anaphase spindle elongation (15, 25-28). It is plausible that antiparallel ipMT-MT crosslinking and sliding by kinesin-5, acting in concert with non-motor MT-associated proteins and with nucleated MT assembly around centrosomes and chromosomes could play significant roles in establishing the MT polarity patterns found in spindles (21, 24, 29).

To our knowledge, the specific MT-orientation preference of KLP61F motors is so far unique amongst mitotic sliding motors. The fact that purified kinesin-5 motors all appear to be slow, plus-end-directed bipolar homotetramers, capable of crosslinking adjacent MTs, is consistent with the idea that kinesin-5 homotetramers serve as dynamic MT-MT crosslinks that both bundle parallel MTs and drive antiparallel MT sliding (12, 25, 30) and that this is their main contribution to mitotic spindle morphogenesis and function. Our results suggest that in the Drosophila spindle, KLP61F could initially bind and crosslink MTs of either polarity throughout the spindle, thereby "zipping" together parallel MTs to form MT bundles. This might be aided by an additional "stickiness" caused by the tail domains. Then via on/off kinetics or after moving towards crosslinked MT plus ends, the antiparallel preference mediated by the tails would cause KLP61F to accumulate in the overlap region of antiparallel ipMTs at the spindle equator to efficiently slide them apart, thereby contributing to poleward flux and spindle elongation (12, 15).

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5. Dynamics and multimerization of PRC1 on single and bundled microtubules

S.M.J.L. van den Wildenberg\textsuperscript{1}, W. Jiang\textsuperscript{2} and E. J.G. Peterman\textsuperscript{1}

\textsuperscript{1}Department of Physics and Astronomy and Laser Center, VU University Amsterdam.
\textsuperscript{2}The Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA.

Abstract

To separate genetic material, microtubules (MT) inside the cell are reorganized into a structure called the mitotic spindle. Formation and evolution of this mitotic spindle consists of many crucial steps, one of which is the establishment of the spindle midzone. In the spindle midzone the overlapping MTs are bundled antiparallel, which is important for molecular motors that can use this geometry to drive pole-pole separation. Major players in stabilizing the spindle midzone are the microtubule-associated proteins (MAPs) of the PRC1/Ase1p/MAP65 family. In the current \textit{in vitro} study we track single molecules mCherry-labeled PRC1 using fluorescence microscopy and show that at low concentrations PRC1 dimers bind to individual MTs and diffuse along them. The PRC1 concentration dependence of MT binding is not linear, but obeys a power law, suggesting that binding of PRC1 to MTs is cooperative. In agreement with this notion, we observe, at higher PRC1 concentrations, static multimers of PRC1 bound to MTs. Furthermore, even at low concentrations, PRC1 is able to bundle MTs. We observe that in between two bundled MTs the diffusion constants of PRC1 dimers is substantially decreased. These findings suggest that PRC1, just like its fission yeast homologue Ase1p, can accumulate on overlapping MTs via a complex mechanism consisting of diffusion along MTs and multimerization.
5. Dynamics and multimerization of PRC1

5.1 Introduction

Microtubules (MTs) are polar polymer rod-like structures consisting of alpha and beta tubulin, which can be organized in specific MT-polarity patterns. The regulation of these patterns is directly related to the function of the MT network in the cell. One of the most interesting examples of the MT polarity patterns is seen when a cell divides. In order to segregate the duplicated chromosomal DNA over the two daughter cells, the MTs are organized in a distinct structure called the mitotic spindle, in which three different types of MTs can be recognized (i) the kinetochore MTs are attached to the chromatin via the kinetochore and form a link between the chromatin and the centrosome, (ii) the astral MTs position the spindle in the cell by attaching their outward pointing plus-ends to the cell cortex, and (iii) the interpolar MTs (ipMTs), which form the spindle midzone (1-3). In the spindle midzone the MTs are oriented antiparallel with their plus ends overlapping. This configuration allows molecular motors to slide the MTs apart (3, 4), generating the force necessary to derive pole-pole separation (figure 1.4B).

The formation of these antiparallel bundles is a crucial step in the formation and evolution of the mitotic spindle during cell division. The assembly of the central spindle is mediated by a variety of microtubule-associated proteins (MAPs), molecular motors and mitotic kinases. A key component is the MAP protein regulator of cytokinesis 1 (PRC1), which is highly conserved in eukaryotic cells (Ase1p in S. cerevisiae (5) and S. pombe (6), MAP65 in plants (7), KLP3A in D. melanogaster (8)).

In vitro, PRC1 was shown to interact with MTs and bundle them (9). In vivo, the localization of PRC1 to the spindle midzone depends on interactions with a kinesin 4 motor, KIF4A. Additionally, the presence of a domain of PRC1 that mediates its oligomerization is also necessary for spatial control of the spindle midzone (9-11). The fission yeast homologue Ase1p has been demonstrated to bundle MTs with a preference for antiparallel orientations. In addition MT binding of Ase1p was observed to be cooperative, resulting in the formation of immobile multimers at high Ase1p-concentrations (12, 13). At lower concentrations, Ase1p dimers were observed to diffuse on a MT lattice and this diffusion substantially slowed down in the presence of a second MT (13). Based on these results a model was proposed in which the specific localization of Ase1p to regions of antiparallel MT overlap is driven by a mechanism involving diffusion of Ase1p dimers along the MT lattice and incorporation of dimers into immobile Ase1p multimers with high preference between the overlapping MTs. It remains unclear whether these properties of Ase1p are shared by the other MAPs, in particular since in some cases localization in zones of antiparallel MT overlap has been shown to depend on the presence of a molecular motor (9). Here we explore the dynamics of fluorescently labeled human PRC1 on MTs in vitro using single-molecule, wide-
field epifluorescence microscopy. We determined the mobility of PRC1 on single MTs and bundled MTs and found that the mobility is more than 5 times slower on bundled MTs. Furthermore, we demonstrate that the binding of PRC1 to MTs in the absence of Kif4 is cooperative due to the formation of multimers.

5.2 Methods

5.2.1 Binding of PRC1 on single MTs

To investigate the binding behavior of PRC1 on single MTs, MTs were grown from a mixture of biotinylated tubulin together with Cy5 labeled tubulin and non labeled tubulin at 37°C (all tubulin was isolated from pig brain). These MTs were specifically attached to the dimethyl-dichlorosilane-treated glass coverslips via biotinylated BSA at 0.1 mg ml⁻¹ (A2289, Sigma-Aldrich, Zwijndrecht, the Netherlands) and 0.1 mg ml⁻¹ streptavidin (8587, Sigma-Aldrich, Zwijndrecht, the Netherlands). Next, the exposed glass surface was covered with Pluronic F108 (BASF, Ludwigshafen, Germany) to prevent nonspecific binding of the PRC1 (for more details see (14)) and PRC1-mCherry in sample buffer (PEM80 (80 mM K₂Pipes, 1 mM EGTA, 2 mM MgCl₂, pH 6.8 with HCl) supplemented with 4 mM DTT, 25 mM glucose, 20 ug ml⁻¹ glucose oxidase and 35 ug ml⁻¹ catalase) was added to the sample. PRC1-mCherry was expressed and isolated from SF9 cells (Wei Jiang, personal communication). Single PRC1-mCherry complexes moving on MTs were imaged at 21°C using a custom-built epifluorescence microscope (14), a time stack of images was acquired using WinView (Princeton Instruments) and analyzed using routines written in Labview 8.2. To determine the binding dynamics, the PRC1-mCherry concentration in the sample was varied and for every concentration the intensity of the PRC-mCherry coated MTs was calculated and normalized by the length of the MTs.

5.2.2 Motion of PRC1 on MTs

To determine the movement of PRC1-mCherry on a Cy5-labeled MT, we nonspecifically immobilized MTs to a glass surface that had been positively charged with DETA (3-[2-(2-aminoethylamino)ethyl-amino]propyl-trimethoxysilane, Aldrich). Subsequently, exposed glass surface was blocked with casein to prevent aspecific binding of PRC1-mCherry to the glass. PRC1-mCherry in sample buffer was added to the sample and movies of PRC1-mCherry complexes moving on MTs were acquired. To determine the diffusion on bundled MTs, a low concentration of MTs was added to the sample buffer. The acquired movies were analyzed with custom written tracking programs (Labview 8.2). In short, the position of PRC1-mCherry in a single frame was obtained from a 2D-Gaussian fit to the intensity profile (15), the obtained positions were connected to form a trajectory from which the displacements for
5.3 Results and discussion

5.3.1 PRC1 dimers bind to a single MT and show motion along the MT

First, we investigated the MT-binding activity of PRC1 in vitro. To this end, we immobilized MTs on a glass surface and added a low concentration of PRC1-mCherry. Using single-molecule fluorescence microscopy, we observed PRC1-mCherry molecules binding to MTs and moving along them, confirming that PRC1 has MT affinity. In order to quantify the observed mobility, we tracked individual fluorescent spots due to PRC1-mCherry, with sub-pixel resolution by fitting a 2D Gaussian to the intensity profile in each frame. A well-established method for quantification of motion is to calculate the mean square displacement (MSD) for different time lags and average these over all trajectories (16). For a particle performing a one-dimensional random walk, the MSD increases linear with time (MSD = 2Dt + offset), with a slope proportional to the diffusion constant (D) (16). We determined that the mobility of PRC1-mCherry on single MTs can be well described by a random walk with a one-dimensional diffusion constant of $13.1 \pm 0.5 \cdot 10^4$ nm$^2$ s$^{-1}$ (figure 5.1A).

![Figure 5.1 Stochastic motion of single molecule PRC1-mCherry on MTs.](image)

(A) The MSD calculated from tracks of PRC1-mCherry complexes moving along single MT (dots). The data was fitted with MSD = 2Dt + offset, yielding diffusion constant, D, of $13.1 \pm 0.5 \cdot 10^4$ nm$^2$ s$^{-1}$ (s.e.) (offset $-700 \pm 1500$ nm (s.e.)). (B) Initial intensity distributions of the tracked PRC1-mCherry complexes. The distribution can be fitted with a Gaussian yielding a mean intensity $\pm$ s.e.m. $= 137 \pm 2$ (n= 89) moving over a single MT. The inset shows for comparison the intensity of isolated mCherry molecules on the glass surface under the same illumination conditions. The intensity of the PRC1-mCherry complexes is twice that of a single mCherry, indicating that it moves as a dimer over MTs.
Next, we analyzed the oligomeric state of PRC1 as several studies have indicated that Ase1p from fission yeast forms dimers (13, 17). It is, however, unknown what the oligomeric state is of PRC1, which differs substantially in sequence from Ase1p. The average intensity of the tracked PRC1-mCherry over the first 4 frames was determined from the product of the amplitude and the width of the two dimensional Gaussian fitted to the intensity profile for position determination. At low PRC1-mCherry concentrations, the mean intensity of the moving fluorescent spots was $137 \pm 2$ (s.e.m) (n= 89) (figure 5.1B). This intensity is about twice that of single mCherry molecule stuck to the cover glass (mean intensity ± s.e.m.= $63 \pm 2$) (n= 60) (figure 5.1B inset), indicating that the tracked PRC1-mCherry molecules are dimers.

5.3.2 Binding and oligomerization of PRC1 on single MTs

We further tested the binding behavior of PRC1 to MTs by exploring its concentration dependency. For different concentrations, the average fluorescence intensity of MT-bound PRC1-mCherry, normalized to MT length, was determined (13). In case binding is not cooperative, the intensity on the MT due to PRC1-mCherry is expected to increase linearly with increasing protein concentration. We found, however, that the intensity increase is not linear, but instead increases with the square of PRC1-mCherry concentration, signifying that MT-binding of PRC1-mCherry is cooperative (figure 5.2A).

![Figure 5.2 Cooperative binding and multimerization of PRC1-mCherry on a single MT.](image)

(A) Plot of the average fluorescence intensity due to PRC1-mCherry per unit MT length as a function of PRC1-mCherry concentration. The increase of MT-bound PRC1-mCherry increases clearly nonlinear with increasing PRC1-mCherry concentration, indicating cooperative binding (the fit is a power law fit yielding a power of 2). (B) Image of bright and immobile multimers that are formed on a single MT at higher PRC1-mCherry concentrations (5.5 µg/ml). Scale bar, 0.8 µm. (C) Examples of a kymograph where a multimer releases or incorporates a PRC1 dimer. Scale bar, 0.9s.
Similar cooperative behavior was previously observed for Ase1p. In that case, also static MT-bound Ase1p-multimers formed at higher concentrations (13). Such multimers were also observed for PRC1-mCherry, at high concentrations (figure 5.2B/C). These PRC1-multimers appeared immobile (within the position accuracy) and occasionally we observed the incorporation or release of PRC1 dimers into or from oligomers (figure 5.2C). Taken together these results indicate that MT-binding of PRC1 is cooperative, resulting on single MTs and at high PRC1 concentrations in the formation of static PRC1 multimers.

Figure 5.3 Localization and multimerization of PRC1-mCherry at low concentrations on overlapping MT sites.

(A) Image of Cy5-labeled MTs. The MTs in the middle of the image are partly bundled (white rectangular box), at the bundled region the intensities of both MTs are summed. Scale bar, 0.8 µm. (B) Kymograph of the MTs shown in (A). In the first part of the kymograph the intensity of the Cy5 labeled MTs is shown (as indicated by the double arrow) with a clear intense part over the length of the MT overlap. After a couple of frames (as indicated by the arrow) PRC1-mCherry is visible. PRC1 is located at the MT overlap and mostly as multimers with limited mobility. Scale bar, 3 s.

5.3.3 Microtubule crosslinking by PRC1

We next investigated the MT-bundling properties of PRC1. In the experiments discussed before, we noted that MTs occasionally overlapped, as observed from a doubling of the intensity observed in the Cy5 channel. We further noticed that PRC1 multimerization occurred more frequently on these MT overlap zones than on single MTs (figure 5.3). Multimerization on bundles occurred even at concentrations at which none or only few multimers form on single MTs, demonstrating that the presence of a second MT increases the probability of multimerization. To test whether also the mobility of PRC1 is affected by
5. Dynamics and multimerization of PRC1

the presence of a second cross-linked microtubule we repeated the experiments with a low concentration of MTs added to the sample mix, such that these MTs could become bundled by PRC1 to surface-attached MTs. We tracked PRC1-mCherry moving on bundled MTs and found the MSD to increase linear in time, with a diffusion constant of $D = 2.4 \pm 0.3 \cdot 10^4 \text{ nm}^2 \text{ s}^{-1}$ (figure 5.4 A), about five times smaller than on single MTs. A similar decrease in diffusion constant, about four times, was observed for Ase1p (13). Taken together, these results show that dimeric PRC1 diffuses between bundled MTs at a slower rate than on single ones. In addition, the formation of multimers appears to occur at substantially lower concentrations between bundled MTs then on single ones.

![Figure 5.4](image)

**Figure 5.4 Stochastic motion of single molecule PRC1-mCherry on the overlap of bundled MTs.**

(A) The MSD calculated from tracks of PRC1-mCherry complexes moving on bundled MTs (squares). A linear fit of the MSD(t) yields $D = 2.4 \pm 0.3 \cdot 10^4 \text{ nm}^2 \text{ s}^{-1}$ (s.e.) and offset $3700 \pm 1100 \text{ nm}$ (s.e.). The motility of PRC1-mCherry dimers slows down by a factor of 5 when compared to the motility on single MTs. (B) Initial intensities of tracked PRC1 complexes on bundled MTs. The distribution of intensities has a mean intensity $\pm$ s.e.m. = $157 \pm 3$ (n= 62) corresponding to about twice the intensity of a single mCherry.

**5.4 Conclusion**

In summary, PRC1 dimers were observed to bind to and diffuse along MTs. Binding experiments revealed that PRC1 binds cooperatively to MTs, resulting in the formation of static multimers at higher concentrations. The concentration at which these multimers formed was lower in the presence of a second MT. In addition, the diffusion constant of PRC1 dimers moving along bundled MTs was fivefold lower than the one found for PRC1 dimers on single MTs. This decrease in mobility of PRC1 when bound on two MTs, in addition to the increased propensity to form (static) multimers could be an important contribution to PRC1’s ability to localize on overlapping MTs, such as it occurs in the spindle midzone. Overall, the behavior of PRC1 is similar to that observed for the related yeast protein Ase1p.
(13). This is remarkable for two reasons. (I) The central region of Ase1p and PRC1 share 40% amino acid sequence similarity, beyond this region they do not share any significant sequence homology (18). (II) In vivo experiments in human cells have indicated that PRC1 relies on the motor protein KIF4A for proper localization to the spindle midzone (9), while Ase1p in yeast does not require motor proteins for localization on antiparallel MT bundles in interphase cells (12). The precise nature and function of the interaction between PRC1 and motor proteins such as KIF4 and why it is necessary for the in vivo recruitment of PRC1 to the spindle midzone remains to be determined. Detailed in vitro experiments to determine the mobility and MT bundling activity of PRC1 in combination with KIF4A will shed light on this matter.

References

5. Dynamics and multimerization of PRC1


Epilogue to chapter 4 and chapter 5

The spindle is a complex structure and a variety of motors and non-motor MAPs play important roles in crosslinking the MTs in the spindle. Recently, after compiling this thesis, new studies on MAPs, in particular on PRC1, in combination with motors and studies on motors alone have shed new light on the formation of the spindle and the crosslinking of its MTs.

In this thesis, I have shown that human PRC1 crosslinks MT. Recently a structural study of PRC1 has brought new information on the mechanisms required for MT binding. Using X-ray crystallography, it was shown that the central region of PRC1 that mediates MT binding has similarities to a spectrin domain, a motif also found in proteins associated with the actin cytoskeleton. It was proposed that this domain binds MTs via a cluster of basic amino acids. Moreover, the C-terminal domain was suggested to play a role in the cooperative binding of PRC1 to MTs (1).

In vitro, PRC1 forms dimers that bind stronger in the overlap zone of antiparallel oriented MTs than on single MTs or on parallel MT pairs (10 fold higher on bundles than on single MTs (1), 28 fold higher on antiparallel than on single MTs or parallel MT pairs (2). The preference for antiparallel MT bundling could be facilitated by the ordered spectrin domain and communicated through the partly rigid structure of dimeric PRC1 molecules (1).

Besides these new insights in the MT binding activity of PRC1, these recent studies have successfully achieved an interesting challenge: combining PRC1 with a molecular motor. First, by adding the molecular motor Eg-5 (a human Kinesin-5) to PRC1-crosslinked MTs, it was observed that PRC1 crosslinks do not resist the sliding of filaments by the Kinesin-5 (1). Another study has addressed the role of Kinesin-4, Xklp1 from Xenopus leavis. In contrast to Kinesin-5 that can itself bind and crosslink MTs, Xklp1 does not bind directly to MTs. Instead it is recruited by PRC1 bound in the MT overlap zone. This physical interaction of the two proteins results in an inhibition of the MT growth, thereby controlling the overlap length (2).

Molecular motors are also involved in the MT organization in the spindle region independently of MAPs. For example, as we showed in chapter 4, Kinesin-5 KLP61F (D.melangolester) preferentially crosslinks antiparallel MTs and slides them apart. The action of Kinesin-5 is counteracted by another molecular motor, Kinesin-14. Kinesin -14 Ncd (Drosophila melanogaster), in contrast to Kinesin-5, has no clear MT orientation preference (3). Moreover, Kinesin-14’s Ncd and Klp2p (S. pombe) interact with one MT with their motor domains and another MT with their tail domains (3, 4), whereas kinesin-5 binds with its motor domains to both crosslinked MTs. The antagonist effect of these two motors results from the fact that Kinesin-14 locks together parallel MT pairs and slides apart antiparallel MT
pairs in the opposite direction of Kinesin-5 (3, 4). How the balance between the actions of these two motors is achieved is not yet elucidated but is of major interest.

References


5. Dynamics and multimerization of PRC1
6. The diffusion of Twin Arginine Translocation complexes in bacterial membranes is substrate and membrane-potential dependent

Siet M.J.L. van den Wildenberg, Kah Wai Yau, Froukje Franken, Holger Lill, Erwin J.G. Peterman and Yves J.M. Bollen

Abstract

The Twin arginine translocation (Tat) system transports fully folded proteins across the inner membrane of bacteria. The energy for translocation is provided by the electrochemical gradient across the inner membrane, called proton motive force. The Tat system is located in the inner membrane and consists of three essential components, TatA, TatB and TatC. TatA is the most abundant Tat protein and is thought to be responsible for pore formation. Here, we tracked eGFP-labelled TatA complexes moving in the membranes of living bacteria under various conditions. For this purpose, TatA-eGFP was expressed at low, sub-physiological concentrations in Escherichia coli. The cells were immobilized and fluorescent complexes were tracked with sub-diffraction accuracy using laser-illuminated wide-field fluorescence microscopy. Displacement data was analyzed in terms of cumulative distributions, and computer simulations were used for the determination of diffusion constants. TatA is found to form at least two different types of complexes, which contain a similar number of TatA molecules yet have a largely different diffusion coefficient. Overexpression of a substrate protein leads to a larger contribution of the slowly diffusing complexes, whereas an uncoupler, which dissipates the proton motive force, results in more complexes that diffuse rapidly. Under all three conditions, the fluorescence intensities of the particles reflect that they consist of ~20 TatA subunits. The diffusion constant of the rapidly diffusing fraction appears to agree with a size corresponding to around 20 TatA subunits, whereas the slowly diffusing complexes are expected to be substantially larger. In our interpretation this is due to the docking TatA complex on a hetero-oligomeric TatB-TatC-substrate complex.
6.1 Introduction

The inner membrane of Gram-negative bacteria is an ion-tight phospholipid bilayer that separates the cytoplasm from the periplasm. The cytoplasm and the periplasm have different electrochemical potentials, resulting in an electrochemical gradient across the inner membrane called the proton motive force (pmf), which provides the driving force for many cellular processes including flagellar motion and ATP synthesis. It is essential for the viability of the bacterium that the permeability barrier of the membrane is kept intact at all times. At the same time, however, signaling molecules nutrients and even macromolecules need to be able to cross the inner membrane, which raises a potential challenge for the intactness of the membrane. In particular, one eighth of the proteins synthesized in the cytoplasm of the Escherichia coli (E.coli) are either active in the periplasm or the outer membrane (1). These proteins are targeted for post-translational translocation across the inner membrane via N-terminal signal sequences. Dedicated protein transport systems have evolved to transport proteins across the inner membrane while keeping the pmf intact. Like most Gram-negative bacteria, E. coli contains two different machineries for post-translational protein translocation (2). One machinery is the general secretory (Sec) system, which pushes protein substrates in an unfolded conformation through a narrow pore in the membrane while hydrolyzing ATP (3). The other machinery is the Twin arginine transport (Tat) system (4, 5), which uses the pmf as driving force (6, 7) for the transport of fully folded proteins and in some cases even oligomeric protein complexes over the inner membrane (8, 9).

The essential components of the E. coli Tat system are the integral inner membrane proteins TatA, TatB and TatC. TatB and TatC form large complexes (440 – 600 kDa) containing several copies of both proteins in a 1:1 ratio (10). These TatBC complexes interact with the N-terminal signal sequences of Tat substrates and most likely serve as the substrate receptor (11, 12). TatA is thought to be the pore-forming component of the Tat system. Detergent-solubilized TatA forms large, homo-oligomeric complexes of variable size (13, 14). Furthermore, cryo-electron microscopy in combination with image reconstruction has revealed that these complexes have a ring-like shape with an internal cavity that is large enough to facilitate the passage of a folded protein (15). Recently, single-molecule imaging in living bacteria was used to show that an E. coli cell contains around 100 freely diffusing TatA monomers and approximately 15 TatA-complexes (16). The complexes had a wide distribution of sizes, with on average 25 TatA monomers per complex. In the absence of TatBC, no large TatA complexes were observed; instead TatA was either monomeric or tetrameric. These and other observations (12, 17) have led to the so-called TatA polymerization model (16). According to this model, substrate binding to TatBC triggers the recruitment and subsequent polymerization of TatA monomers or small multimers. In these
large TatABC complexes, the TatA proteins form a pore, which allows the passage of the substrate to the periplasmic side of the membrane. In this model, leakage of ions through the Tat-pore is limited by adjusting the number of TatA monomers such that the diameter of the pore matches the size of the substrate. After translocation, the TatABC complexes disassemble.

Here we describe single-molecule tracking experiments of fluorescently labeled TatA complexes in living bacteria. Careful regulation of the expression levels allows reduction of the number of complexes to only one or two per cell, which can be tracked during typically several seconds. Analysis of the resulting trajectories reveals heterogeneous diffusion behavior, which is drastically altered by overexpression of substrate proteins or in the presence of uncoupler. In contrast to the diffusion constants, the number of TatA proteins per complex does not appear to be altered.

6.2 Materials and methods

6.2.1 Generation of expression plasmids

The tatABC operon of E. coli strain MC4100 was amplified by Polymerase Chain Reaction (PCR), fragmented with EcoRI and BamHI restriction and transferred to vector pUC18 resulting in plasmid pFF001. The operon was then transferred into the pBAD33 expression plasmid (18). It was amplified from pFF001 by PCR using forward primer FF24 (5’-GCATGCCTGCAGTCGTTACCCAGGAGGAATTCCATTGGGTGGATC-3’), which encodes a KpnI restriction site (shown in boldface) followed by the Shine-Dalgarno box (which is not encoded on pBAD33) and the 5’-end of the tatA gene, and reverse primer FF21 (5’-CAGGTCCGATCCATTCTAGATTATTCTTCAGTTTT-3’), which encodes the 3’-end of the tatC gene and an XbaI restriction site (shown in boldface). The PCR product and vector pBAD33 were digested with KpnI and XbaI and ligated, resulting in plasmid pFF013.

A TatA-eGFP fusion in the operon was generated as follows. The tatA gene was amplified from pFF001 by PCR. The forward primer FF01 (5’-AGCTTGATCGGCTGCAGGTGAATTCATGGGTGGATC-3’) encodes the EcoRI restriction site and the first 15 bases of the tatA gene, whereas the reverse primer FF02 (5’-TTCTCCTTACTCATCACCCTGCTCTTTATC-3’) removes the stop-codon from the tatA gene and adds the first 15 bases of gfp. The gfp gene was amplified by PCR, using the forward primer FF03 (5’-GATAAAGAGGAGGTAGTAAAGGAGAA-3’), which adds the last 15 bases of the tatA gene (excluding the stop-codon) to the 5’-end of the gfp gene, and the reverse primer FF04 (5’-ACCGATATCACAATTTTTATTTGTTATGTC-3’), which adds the first 15 bases of the tatB gene downstream of gfp. Subsequently, the two PCR products were fused in a third PCR reaction, making use of the mutually complementary parts. pFF001 and the fused PCR
products were cleaved with EcoRI and EcoRV. The latter enzyme cleaves after the ninth base of the tatB gene. The cleaved fragments were fused, resulting in plasmid pFF002, which encodes a tatABC operon in which gfp is fused behind tatA, as was verified by sequencing. Subsequently, the tatAGFPBC operon was transferred into pBAD33 as described above for tatABC, resulting in plasmid pFF014.

To obtain plasmid pCL-SufI, sufl was amplified by PCR using pBAD18-SufI (37) as a template and the primers PstI-RBS-SufI forward (5’-cagtcatactgacgttatggacacatg-3’; PstI site in boldface) and SufI-FL-BamHI reverse (5’-acgcggatatctacgtacgcggagctg-3’; BamHI site in boldface). The resulting PCR fragment was transferred into vector pCL1920 (38) using the PstI/BamHI restriction sites, yielding pCL-SufI. Expression of SufI is under control of the tac promoter.

6.2.2 SDS sensitivity assay

Over-night cultures of E. coli strain DADE (MC4100 ΔtatABCDE)(21) transformed with pFF013, pFF014 or an empty pBAD33 vector were transferred into fresh YT media containing 0.34 µg/ml chloramphenicol (Fluka 23275). Cells were grown, at 37°C until mid-log phase (OD₆₀₀ = 0.5). Subsequently, expression of Tat proteins was induced by the addition of 0.02% arabinose for 2 hours. Then cells were transferred onto YT-agar plates containing 0.34 µg/ml chloramphenicol, 0.02% arabinose and 2% SDS. Plates were incubated overnight and examined for growth.

6.2.3 Sample preparation for microscopy

The E. coli strain DADE (21) was transformed using pFF014 plasmid. DADE cells containing pFF014 were made electro-competent and transformed with the pCL-SufI plasmid and plated on double-selective plates containing both chloramphenicol (34 µg/ml) and spectomycin (100µg/ml).

Bacteria were grown overnight at 180 rpm and 37°C in YT media containing 34 µg/ml chloramphenicol. For cells containing pCL-SufI, 100µg/ml spectomycin was also present in the media. Of the overnight culture 500µl was added to 4500µl fresh YT media containing the appropriate antibiotics and supplemented with 0.2 % glucose (w/v). Cells were grown for 90 minutes at 180 rpm and 37°C. Cells were collected by centrifugation for 10 minutes and resuspended in minimal media (1.2% (w/v) Na₂HPO₄.2H₂O, 0.6% (w/v) KH₂PO₄, 0.2% (w/v) NH₄Cl, 0.1% (w/v) NaCl, 0.002 M MgSO₄, 0.6 % (w/v) glucose, 0.0001 M CaCl₂) containing the appropriate antibiotics. In some experiments 100mM IPTG was added to the minimal media in order to induce SufI expression.
Sample chambers were created by fixing coverslips on top of microscope slides (both plasma-cleaned) using double-sticky tape. Sample chambers were incubated with 0.03\% (w/v) poly-L-Lysine for 45 minutes, and then washed with minimal media. Subsequently, the bacteria are applied and allowed to bind to the surface for 10 minutes. Unbound or loosely bound bacteria are removed by thorough washing with minimal media containing the appropriate amounts of antibiotics, glucose and IPTG as required. In some experiments carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was also added to the media used for washing, at a concentration of 10µM. Finally, the sample chambers are sealed with vacuum grease.

6.2.4 Wide field fluorescence microscopy

Immobilized bacteria were imaged at 21 °C using an epi-illuminated wide-field fluorescence microscope (39). A 488nm laser (Coherent Sapphire 488-20) was focused in the back focal plane of the objective (Plan Apo TIRF 100x 1.45 oil, Nikon) via a polychromatic dichroic mirror (Chroma Z488RDC/532/633RPC), with an intensity of 0.13Wcm\(^{-2}\). Fluorescence was collected by the same objective and filtered using a HQ510/75M band-pass filter (Chroma) and imaged on a CCD camera (Roper Scientific, MicroMax 512FTB). Time series of images with 60 ms integration times were acquired using Winview software (Roper Scientific).

6.2.5 Confocal microscopy

Confocal images were recorded on a Biorad 2000 confocal scanning light microscope using the Lasersharp 2000 software. A 488 nm Argon laser was used for excitation; GFP emission was selected using a HQ515/30 emission filter. A Nikon Plan Apo 60x 1.40 oil immersion objective was used.

6.2.6 Data analysis

The trajectories of individual fluorescent particles were obtained and analyzed using home-written routines in Labview (National Instruments). For each frame \(i\) the \((x_i, y_i)\) coordinates of a fluorescent spot were resolved by fitting a 2-dimensional Gaussian function to the observed fluorescence intensity profile (26). For trajectories of more than 10 time points, the squared displacement per time lag \(r^2(\tau)\) was calculated for all independent non-overlapping time lags \(\tau\). The diffusion constant can be derived from the squared displacements using a cumulative distribution analysis (29, 30) as follows. Provided that the population of diffusing particles is homogeneous, the probability \(P\) that a molecule starting at a given position will be found within a distance \(r\) from the starting position after time lag \(\tau\) is given by:
\[1 - P(r^2, \tau) = A \exp \left( -\frac{r^2}{4D\tau + 4\sigma^2} \right)\]  \hspace{1cm} [1]

where \(D\) is the diffusion constant and \(\sigma\) the localization inaccuracy. For an inhomogeneous population of diffusing particles the probability distribution is given by a sum of the contributions of each subspecies.

6.2.7 Simulations

Diffusion of a particle over a bacterium was simulated in Labview 8.6 (National Instruments), in a similar way as described by Deich et al. (31). The simulations will be described in detail elsewhere (Chapter 7 of this thesis). In short, the bacterium was represented as a cylinder capped with two semi-spheres. A particle was placed at a random starting position on the cylinder and allowed to take 1800 steps over the surface with a fixed step size and a random angle. Every step is assumed to represent 1 millisecond. The position of the particle was averaged over 60 milliseconds and projected onto a plane parallel to the long axis of the cell. A position inaccuracy was introduced by adding a value from a Gaussian distribution centered around zero with a standard deviation of 34 nm corresponding to our experimental position inaccuracy (see below). The simulation was repeated 100 times with different starting positions, and a probability distribution plot of the observed displacements was made. The procedure was repeated \(10^6\) times with different step sizes, which represent different diffusion constants.

Heterogeneity was introduced into the simulations by randomly combining the displacements from two simulations, each with a different step size, into a single cumulative distribution plot. The relative contribution of each of the two diffusion constants was varied. The obtained probability distribution plots, which are a function of two diffusion constants and corresponding amplitudes, were compared to the experimental data, in order to find the two diffusion constants and corresponding amplitudes that best describe the experimental results.

To quantify the positional accuracy (\(\sigma\)), we performed simulations using Labview 8.2. We generated an image of an immobile point source that was randomly placed in a field of view (40). The image was convoluted with a point-spread function of the microscope (approximated by a Gaussian curve with a FWHM of 300 nm). The amplitude of the Gaussian curve was obtained from the average intensities of the tracked fluorescent spots, and determined to be 120 counts. Photon counting noise was introduced by adding a random value from a normal distribution centered at zero with standard deviation equal to the amplitude at that coordinate to the value of the pixel. The background noise with a standard deviation of 5 counts, obtained from the experimental data, was added to the generated...
image. In this way 1000 images were generated with random variation of the position of the point source with regard to the pixel array. Subsequently, these images were analyzed in the same way as the experimental data. The difference between the input coordinates and the coordinates obtained from a two dimensional Gaussian fit yielded a mean positional deviation of 34 nm in each dimension.

6.3 Results

6.3.1 Fluorescently labeled TatA is functional

Two plasmids were created containing the tat operon, both based on the pBAD33 vector, which allows quantitatively controlled expression; expression can be induced by arabinose and blocked by glucose (18, 19). Plasmid pFF013 contains the unmodified tatABC genes, whereas pFF014 contains enhanced Green Fluorescent Protein (GFP) gene as a translational fusion at the C-terminal end of the tatA gene.

First, we tested whether the TatA-GFP fusion protein is functional. We made use of the fact that cells with a functional Tat system can grow in the presence of 2% of the detergent SDS, whereas cells without an active Tat system cannot (20). Plasmids pFF013 and pFF014 were used to transform the Tat-free E. coli strain DADE (21). Cells were grown in liquid media to mid-log phase and expression was induced by the addition of arabinose. Subsequently, cells were transferred onto agar plates containing arabinose as well as 2% SDS. DADE cells expressing either an unmodified tatABC operon or the fluorescently labeled tatA<sup>GFP</sup>BC operon were able to grow on these plates, whereas DADE cells containing an empty vector were not (results not shown). In addition, cells expressing tatA<sup>GFP</sup>BC were examined using confocal fluorescence microscopy. Bacteria showed up as fluorescent rings, indicating that TatA-GFP localizes to the membrane (figure 6.1). In addition, cells were lying separate and had a normal shape, whereas cells without a functional Tat system are known
to suffer from elongated growth and chain formation (22, 23). Together, these results demonstrate that the function of TatA is not hampered by fusion to GFP, in accordance with earlier observations (16, 24, 25).

6.3.2 TatA forms complexes of variable size

The fluorescence intensity in bacteria with induced TatA-GFP expression was too high to resolve and track individual protein complexes (figure 6.1). In order to reduce the number of Tat-complexes sufficiently, we diluted cells from an uninduced overnight culture into fresh YT media supplemented with 0.2% glucose, which completely inhibits expression from the pBAD33 vector (18, 19). As a result, Tat proteins that were made during overnight growth were divided over the daughter cells. After 90 minutes, cells were harvested, washed and immobilized on glass slides as described in Materials and Methods. Three different samples were prepared: (i) bacteria that expressed TatA<sup>GFP</sup>BC; (ii) bacteria that expressed TatA<sup>GFP</sup>BC and overexpressed the Tat-substrate SufI; and (iii) bacteria that expressed TatA<sup>GFP</sup>BC and overexpressed SufI to which the uncoupler FCCP had been added to remove the pmf the driving force of the Tat-system. In all these experiments, cells had an ellipse-like shape (figure 6.2A), which suggests that the very low number of Tat complexes in these cells is sufficient for viability.

Laser-illuminated wide-field epi-fluorescence microscopy was applied to record series of images with 60 ms integration time. For further image analysis, immobilized bacteria with a single fluorescent spot were selected. In each frame, the centre of the fluorescent spot was determined with sub-resolution accuracy by fitting a two-dimensional Gaussian function to the intensity profile (26) and trajectories were constructed (figure 6.2B). For each experiment, between 75 and 150 trajectories of 10 or more time steps were further analyzed.

First, we analyzed the intensities of the tracked fluorescent spots. Since all tat-genes are absent from the genome of the used strain, each TatA protein is covalently coupled to a GFP molecule and hence the fluorescence intensity of a spot is a direct measure for the number of TatA proteins in a membrane protein complex. Intensities were obtained from the product of the amplitude and the width (standard deviation) of the fitted two-dimensional Gaussian. In order to minimize photobleaching effects, we considered only the initial intensity (the average intensity of a particle in the second, third and fourth frame in which the particular particle was observed). A histogram of the initial intensities for all tracked spots shows that the intensities are widely distributed (figure 6.2C), suggesting that TatA forms complexes of variable size. Remarkably, the intensity distributions are very similar for the three different experimental conditions, indicating that the size distribution of
TatA complexes is independent of the presence of an overexpressed substrate or on the presence or absence of a pmf.

To quantify the actual size of the complexes, we immobilized purified eGFP on glass cover slips and measured its fluorescence under the same conditions. The histogram of the initial fluorescence intensities (figure 6.2C, inset) shows that the average fluorescence intensity of a single GFP molecule is 4.8 ± 0.1. This implies that the tracked particles in all three experiments consist on average of 20 TatA-GFP molecules, in agreement with previous results obtained on bacteria expressing TatA-YFP at - significantly higher - physiological levels (16).

Figure 6.2 Tracking of fluorescent particles in *E. coli* cells expressing TatA<sup>GFP</sup>BC.

(A) Series of frames showing first the bacterium via autofluorescence, and subsequently a moving fluorescent TatA particle. Each frame is averaged over 60 ms. (B) Reconstructed trajectory of a fluorescent particle (part of which is shown in (A)). The trajectory starts at the black dot and ends at the triangle. (C) Histogram of the initial fluorescence intensities of tracked complexes. Light grey: cells expressing TatA<sup>GFP</sup>BC; dark grey: cells expressing TatA<sup>GFP</sup>BC and over-expressing the Tat substrate SufI; black: cells expressing TatA<sup>GFP</sup>BC and over-expressing the Tat substrate SufI in the presence of the uncoupler FCCP. Inset: intensity histogram of immobilized GFP molecules fitted with a Gaussian curve.
6.3.3 TatA displays a complex diffusion behavior

The trajectories of the fluorescent complexes contain information on their diffusive behavior. The most widely used method to extract diffusion constants from trajectories is to calculate the mean squared displacement as a function of time lag and perform a linear fit. An important disadvantage of this method is that heterogeneity, for example due to complexes with different diffusion constants, is averaged out (27,28). The wide distribution of fluorescence intensities indicates that TatA complexes vary in size, which might result in heterogeneous diffusion. Therefore, we chose another approach to analyze the trajectories, namely by calculating cumulative probability distribution (CPD) of squared displacements. The CPD is equal to 1-P(r^2, τ), with P the probability that a particle remains within a circle with radius r during time-lag τ (28, 29). In case of a single, homogeneously diffusing particle, 1-P is an exponentially decaying function of r^2 (29, 30), with 1/4Dτ as decay constant (Eq. 1, see Materials and Methods). In Figure 3 the CPD is plotted as a function of r^2 for bacteria expressing TatA\textsuperscript{GFP}BC. The graph clearly shows that TatA diffusion cannot be described by simple diffusion with a single diffusion constant.

![Figure 6.3 Cumulative probability distribution (CPD).](image)

(A) Schematic of the probability of finding a particle outside a circle of radius r from its starting position (1-P (r^2, τ)). (B) the cumulative distribution of the probability depicted in A (CPD) for the first time lag of TatA-eGFP complexes in the membranes of bacteria. The distributions are shown for TatA-eGFP from three independent experiments (light grey), the distributions for two experiments in cells that over-express SufI (dark grey), and the distributions from two independent experiments in cells that over-express SufI and that are uncoupled with FCCP (black). The CPDs of TatA-eGFP in cells expressing excess SufI show a clear shift towards smaller displacements indicating that the complexes move slower compared to the other experiments.
In addition to heterogeneous diffusion, the shape of the bacterial membrane could also complicate the CPD. In our experiments we measure a 2-dimensional projection of the 3-dimensional trajectories and it is well-known that this can severely affect the determination of the diffusion constants (31). To determine the effect of the projections on the CPDs we performed computer simulations, which are described in detail elsewhere (Chapter 7 of this thesis). The key result of these simulations is that the 2-dimensional projection of the 3-dimensional trajectory leads to non-exponential CPDs. This effect is, however, not sufficient to explain the experimental data, indicating that the measured diffusion is heterogeneous. The experimental data could be explained satisfactory assuming two populations of particles with clearly distinct diffusion constants. Simulations were performed varying the diffusion constants and relative contributions and a best fit of the experimental CPDs of the first 5 time lags was selected based on the lowest $\chi^2$ (figure 6.4A). In this analysis it is assumed that the diffusion coefficients and their relative contributions are time independent, which seems warranted given the quality of the fits at different time lags (figure 6.4A). Additionally, when the experimental CPDs of the first seven time lags are fitted with a model in which the fraction is a global variable (i.e., independent of time lag) and the diffusion coefficients are local variables (i.e., they are independent for each time lag), the fitted diffusion coefficients are similar for every time lag, except for the first one (figure 6.4B). In the first time lag the lower diffusion coefficient yields a displacement (40 nm) that is similar in size to the error in the position determination (34 nm) making it impossible to obtain reliable values for both diffusion coefficients (Fig 4B). At larger time lags this effect decreases since the displacement increases with time lag ($r = \sqrt{4Dt}$), whereas the position uncertainty is time independent. In order to obtain reliable values for both diffusion coefficients we used the first global fitting procedure described above throughout this work, in which the CPDs of the first five time lags were fitted against CPDs generated in simulations with two populations of particles, each of them with a distinct diffusion constant. The two diffusion coefficients and their relative contribution were treated as global, time-independent variables.

In the absence of SufI and uncoupler, three independent experiments were evaluated yielding TatA-GFP diffusion constants of $0.179 \pm 0.009 \, \mu m^2 s^{-1}$ (average $\pm$ standard deviation; relative amplitude $0.58 \pm 0.02$) and $0.009 \pm 0.003 \, \mu m^2 s^{-1}$ (relative amplitude $0.42 \pm 0.02$; Table 1). This result suggests that TatA diffusion is heterogeneous and consists of at least two distinct components.

**6.3.4 An excess of substrate slows down the diffusion of TatA complexes**

Next, the diffusion data for bacteria over-expressing the Tat-substrate SufI from the plasmid pCL-SufI were analyzed. In this condition, the CPD is clearly shifted to smaller
displacements, implying that TatA diffusion slows down significantly in the presence of excess SufI substrate. The diffusion of TatA in cells containing an empty pCL1920-plasmid was very similar to that in cells without this plasmid, indicating that the slowing down of diffusion is not an artifact caused by the presence of either the plasmid or the additional antibiotic (spectomycin). As before, simulations were used to describe the experimentally obtained CPDs. The CPDs of TatA diffusion in the presence of excess substrate during the first five time lags could again be well described by two differently diffusing populations (figure 6.4C). Interestingly, the diffusion constants were similar to the ones found in the absence of SufI, but with a larger contribution of the slowly diffusing component (Table 1).

### 6.3.5 TatA diffuses faster in the absence of the proton motive force

To find out whether TatA diffusion in cells expressing excess SufI substrate is influenced by the pmf (the driving force of Tat transport), we analyzed the effect of the uncoupler FCCP, which dissipates the pmf. Addition of FCCP shifted the CPDs towards larger displacements, i.e. diffusion was sped up and became comparable to or even faster than the diffusion in the absence of SufI overexpression (figure 6.3). The variation between different FCCP experiments was large compared to other conditions, most likely due to the strong, time-dependent effect of the uncoupler on the bacterial membrane, which appeared to vary from cell to cell. Nevertheless, the data clearly show that the decrease in diffusion coefficient of TatA does not only depend on saturation with substrate, but also requires an intact pmf. The CPDs of the first five time lags are well described by our model with two populations of TatA particles (figure 6.4D, Table 1).

### 6.4 Discussion

We have introduced a procedure to grow bacteria in a way that they contain as few as only one fluorescently labeled Tat-complex that still appears to function normally: cells have a healthy shape, and the number and distribution of TatA proteins per complex is similar to that observed with wild-type expression levels (16). The low expression levels allowed us to track individual TatA complexes for relatively long periods of time while they diffused in the inner membrane of a living bacterium. We analyzed single-complex trajectories by calculating cumulative probability distributions, an approach that allowed us to unravel the heterogeneity in TatA diffusion. A model with two populations of TatA complexes with diffusion constants that differ by a factor of 20 was the simplest that explains the observed data. We can, however, not exclude that more than two populations or a continuum are responsible for the observed heterogeneity, since our approach does not allow for reliable quantification of more than two components. In addition, complexes might switch between faster and slower diffusion within one trajectory. Notwithstanding these limitations, the
experiments clearly show that co-expression of a large excess of substrate proteins results in a substantial slowing down of TatA-complex diffusion, without altering the number of TatA proteins per complex. When the pmf, the driving force for Tat protein translocation, is artificially removed, the average diffusion constant (Table 6.1) increases, occasionally even beyond that obtained in cells that do not express an excess of substrate. Also under uncoupled conditions, no change in the number of TatA monomers per complex was observed.

Figure 6.4 CPDs for five time lags of squared displacements of TatA-eGFP complexes in the membranes of living bacteria.

Upper left, CPDs of TatA-eGFP in vivo (black) are best described by a model (grey) in which 59% of the complexes have a diffusion coefficient of 0.169 µm²s⁻¹ and 41% have a diffusion coefficient of 0.007 µm²s⁻¹ (χ² = 59). Note that these values are fits to CPDs obtained from a single experiment. Upper right, shows the independency of the diffusion coefficient with respect to the time lag. The CPDs of the first seven time lags of TatA-eGFP complexes are described well by a model in which the fraction is taken to be a global variable and the diffusion coefficients are local variables. Shown are the two diffusion coefficients for the different time lags. Bottom left, CPDs of TatA-eGFP in cells that over-express SufI in vivo (black) are best described by a model in which a fraction (42%) has a diffusion coefficient of 0.163 µm²s⁻¹ and another (58%) with a diffusion coefficient of 0.011 µm²s⁻¹ (χ² = 36) (model shown in grey). Bottom right, CPDs of TatA-eGFP in cells that over-express SufI to which FCCP is added (black) are best described by a model (grey) having a fraction (55%) with a diffusion coefficient of 0.22 µm²s⁻¹ and a slow moving fraction (45%) with a diffusion coefficient of 0.017 µm²s⁻¹ (χ² = 69) (model shown in grey).
6. Diffusion of Twin Arginine Translocation complexes

The most likely explanation for our observations is that TatA is already present as a homo-oligomer of about 20 proteins in the 'resting' state of the Tat system's activity cycle, prior to substrate binding and translocation. This TatA-oligomer then associates with a TatBC complex to form an active translocon as soon as a substrate molecule is bound to TatC. The assembly of the full TatABC translocon appears to be pmf-dependent, since removing the pmf results in faster diffusion of TatA-GFP. After translocation of the substrate, the translocon dissociates into two parts: a TatBC complex and the TatA oligomer. According to our data, the TatA oligomer does not readily fall apart into monomers or smaller oligomers as predicted by the polymerization model (16). A new model for the activity cycle of the Tat system that explains our data is shown in Figure 6.5.

![Figure 6.5 Schematic presentation of the association-dissociation cycle of the Tat system.](image)

In the resting state (1) the Tat proteins are divided into a TatBC complex (the two dark greys) and an oligo-TatA complex (light grey) of on average 20 subunits. (2) Upon substrate binding to TatC, the two complexes associate to form an active pore (3). After translocation of the substrate the pore dissociates into the two initial complexes. The TatA oligomeric complex does not dissociate further into smaller oligomers.

It would be interesting to extract the various sizes of the observed TatA-complexes from the diffusion data. Lateral diffusion of a transmembrane protein is primarily determined by the diameter of the transmembrane region, and not by the molecular mass of the complex, as is the case for water-soluble proteins (32). In the last few years there has been some debate about the validity of this Saffman-Delbrück theory for larger proteins and protein complexes in living cells, as well as about the exact parameterization of the theory. Recently, Guigas and Weiss (33) used mesoscopic simulations to make a thorough
quantification of the size-dependence of transmembrane protein diffusion. According to their model, the rapidly diffusing TatA-complexes (D = ~0.18 μm²s⁻¹) that we observe in the inner membrane of *E. coli* cells would correspond to complexes with a radius of approximately 7 nm, corresponding well to the 3-6 nm radius observed using cryo-electron microscopy for ring-like complexes of purified TatA (15). The slow diffusing TatA complexes (D = ∼ 0.01 μm²s⁻¹) would correspond to complexes with a radius of around 50 nm, which seems to be rather large for a single pore, considering that the largest known Tat substrate in *E. coli* has a diameter of nearly 7 nm (34). There is some evidence that one TatBC complex can bind more than one substrate molecule (35). If each substrate recruits a TatA ring, this might lead to a TatABC-substrate complex with a very irregular shape that appears to be much larger than one would expect. In addition, there could be other factors than size that influence the diffusion of a transmembrane complex. For example, during the translocation of a substrate molecule the TatA proteins might undergo a conformational change that results in a mismatch between the length of the hydrophobic part of the protein and the thickness of the membrane bilayer. Such hydrophobic mismatch is known to influence the diffusion of transmembrane proteins (36). More experiments are needed in order to explain in particular the slow diffusion of TatA complexes.

In summary, we have introduced a method to reduce the number of fluorescently labeled protein complexes per cell, such that complexes can be tracked for long periods of time. Analyzing the distribution of displacements combined with computer simulations revealed a strong heterogeneity in the diffusion of fluorescently labeled TatA complexes. The data suggests an association/dissociation mechanism, in which preformed TatA complexes dock onto substrate-containing TatBC complexes in a pmf-dependent manner.

**Acknowledgement**

Plasmid pCL-SufI was a kind gift of Dr. W.S. Jong. We are grateful to T. Antonisse for writing the LabView routine for two-dimensional particle tracking.
### Table 6.1 Diffusion constants (D) and corresponding amplitudes (A) that best describe the diffusive behavior of TatA-eGFP under various conditions. Values are average ± standard deviation obtained from two or three independent experiments.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$D_1$ ($\mu$m$^2s^{-1}$)</th>
<th>$A_1$</th>
<th>$D_2$ ($\mu$m$^2s^{-1}$)</th>
<th>$A_2$</th>
<th>Weighted average $D$ ($\mu$m$^2s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TatA-eGFP ($N = 3$)</td>
<td>0.18 ± 0.01</td>
<td>0.58 ± 0.02</td>
<td>0.009 ± 0.003</td>
<td>0.42 ± 0.02</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>TatA-eGFP + Sufi ($N = 2$)</td>
<td>0.16 ± 0.01</td>
<td>0.37 ± 0.07</td>
<td>0.012 ± 0.001</td>
<td>0.63 ± 0.07</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>TatA-eGFP + Sufi + FCCP ($N = 3$)</td>
<td>0.22 ± 0.01</td>
<td>0.50 ± 0.14</td>
<td>0.02 ± 0.01</td>
<td>0.50 ± 0.14</td>
<td>0.12 ± 0.03</td>
</tr>
</tbody>
</table>

**References**

6. Diffusion of Twin Arginine Translocation complexes

6. Diffusion of Twin Arginine Translocation complexes
7. Protein complexes diffusing through the bacterial membrane: computer simulations addressing heterogeneity and 2-dimensional projection

Siet M.J.L van den Wildenberg\textsuperscript{1}, Yves J.M. Bollen\textsuperscript{2} and Erwin J.G Peterman\textsuperscript{1}

\textsuperscript{1} Department of Physics and Astronomy and Laser Centre, VU University, Amsterdam, 1081HV Amsterdam, The Netherlands.

\textsuperscript{2} Department of Molecular Cell Biology and Laser Centre, VU University, Amsterdam, 1081HV Amsterdam, The Netherlands.

Abstract

In order to quantify the dynamics of the Twin-arginine translocation (Tat) system present in the inner membrane of \textit{E.coli}, single-protein complexes were tracked using fluorescence microscopy. The diffusive behavior of eGFP-tagged TatA complexes was analyzed using cumulative probability distributions (CPDs) obtained from trajectories of single complexes. CPDs were non exponential, indicating that the diffusive behavior of TatA is complex and heterogeneous. To test how the diffusion within the bacterial membrane can result in the experimentally obtained CPDs, we performed computer simulations incorporating various potential causes of heterogeneity. From these simulations we conclude that the complex diffusive behavior observed for TatA \textit{in vivo} is caused by two effects: the planar projection of the three-dimensional trajectories (following the shape of the bacterium), and the presence of two mobile populations, each with its own diffusion coefficient.
7. Membrane diffusion: heterogeneity and 2-D projection

7.1 Introduction

In the aqueous solutions where most biology takes place, thermal energy causes constant, random collisions of solvent and biomolecules, resulting in motion of biomolecules in random directions, also called Brownian motion (1). Also proteins embedded in membranes undergo such random walks, within the membrane. Diffusion of membrane proteins has attracted substantial attention since it is functionally important and deviations from pure Brownian motion can report on either the exact (local) composition of the membrane, or interactions with other biomolecules and with the cytoskeleton (2, 3). To quantify diffusion of membrane proteins several technical approaches have been developed, including fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS), and single particle tracking of particles labeled with antibodies, microspheres or single fluorescent molecules (2, 4, 5).

In many studies single-particle tracking data have been analyzed by determining the mean squared displacement (MSD) from the observed trajectories. In case of ideal diffusion, the MSD as a function of time lag results in a straight line, with a slope proportional to the diffusion coefficient (4, 6). In order to address heterogeneity between particles, the trajectories should be long enough such that diffusion coefficients can be determined for individual particles. In practice, however, the observation time of particles is often severely limited by for example photobleaching (7). To obtain statistically reliable numbers, the MSD can be determined from the displacements of all trajectories taken together, under the assumption that all particles diffuse in the same way, averaging out any potential heterogeneity. In other words, using this approach it is not possible to discriminate between populations of particles with different diffusion coefficients. When trajectories are short and heterogeneity is suspected, a more informative way of analyzing diffusion is to calculate the cumulative probability distribution function (CPD) of finding the particle within a circle with radius \( r \) at a given time lag \( \tau \). For a homogeneous population of diffusing particles, the probability \( P \) that a particle starting at the origin will be found within a distance \( r \) from the origin after a time lag \( \tau \) is a single exponential distribution (8, 9):

\[
P(r^2, \tau) = 1 - \exp\left(-\frac{r^2}{4D\tau + 4\sigma^2}\right),
\]

(1)

with \( D \) representing the diffusion coefficient and \( \sigma \) the localization inaccuracy. The presence of an additional population of particles, with different diffusion coefficient will lead to an extra exponential, which can be resolved (in contrast to the MSD approach) using (multi) exponential curve fitting.
Recently we have measured the diffusion behavior of the Tat system, which transports intact, folded proteins across the inner membrane of *E.coli*. The system consists of three components: the transmembrane proteins TatA, TatB and TatC (10). It is thought that a substrate protein first binds to a mixed TatB/C complex (11). Subsequently TatA is recruited and a pore is formed through which the substrate is transported across the membrane. We have tracked single, eGFP-tagged TatA complexes moving through the membrane and calculated CPDs (figure 7.1).

![Figure 7.1 Tracking of single TatA-eGFP complexes in E. coli cells.](image1)

(A) series of fluorescence frames from a time-laps recording. In the first frame the bacterium is visible due to autofluorescence, in subsequent frames a single fluorescent TatA complex can be seen to move. The integration time of each frame was 60 ms. (B) Trajectory of the fluorescent particle in (A); the positions of the particle were obtained by fitting a 2D Gaussian to the intensity profile in every frame. (C) CPD of squared displacements obtained from 100 trajectories like in (B). The CPD cannot be well described by a single exponential (dashed line) as expected for Brownian motion. At least 3 exponentials are required for a multi-exponential fit (grey solid line).

The experimental approach and the biological consequences are described in detail elsewhere (Chapter 6 of this thesis). We observed that the CPDs are clearly non exponential, and can be altered by quenching the proton motive force (the driving force of Tat-dependent protein translocation) or by over-expressing substrate protein (Chapter 6 of this thesis). In order to find out the cause of this complex and apparently heterogeneous diffusive behavior, we have performed the computer simulations described here.
In these computer simulations we test different reasons for heterogeneity. The results show that the complex diffusive behavior of TatA in vivo is caused by two effects: the presence of two populations with distinct diffusion coefficients and projection of the three-dimensional trajectories (following the shape of the bacterium) in a two-dimensional image plane.

7.2 Methods

Simulations were performed using Labview (National Instruments, Labview 8.6) as follows, in a similar way as described by Deich et al (12). The bacterium was depicted as a cylinder with a length of 1 μm capped by two half spheres with a diameter of 1 μm, resulting in a total length of 2 μm (similar to the E. coli cell dimensions observed in the experiments), unless stated differently. At time zero a particle was positioned at a random location and allowed to make 1800 steps of a fixed step size (δ) in a random direction, each step representing a time interval (Δ) of 1 ms. Steps were performed in a cylindrical or spherical coordinate system depending on the position on the cell. To further approximate the fluorescence microscopy measurements, which were taken with an acquisition time of 60 ms, the positions during 60 steps (of 1 ms) were averaged. Next, the averaged coordinates were converted to Cartesian coordinates. The localization inaccuracy of our experiments (which we estimate to be 34 nm, Chapter 6 of this thesis), caused by the intrinsic noise of the measurements, was taken into account by adding a noise contribution randomly picked from a Gaussian distribution centered around zero with a width of 34 nm. In this way, for each condition, ~100 trajectories were generated, comparable to the amount of experimental data acquired. From each simulated trajectory the displacements for non-overlapping time lags (τ) were calculated. These displacements were further analyzed in two alternative ways. (i) They were used to determine cumulative probability distributions of non overlapping displacements, \( P(r^2, \tau) \), by counting the number of squared displacements in all trajectories smaller than or equal to \( r^2 \), normalized by the total number of data points. (ii) They were used to calculate the MSD for all non-overlapping time intervals within a trajectory for all trajectories.

7.3 Results

7.3.1 2-Dimensional projection of the 3-dimensional trajectories

The experimentally recorded trajectories of TatA particles are projections of 3-dimensional diffusion over the curved bacterial surface on the 2-dimensional image plane, parallel to the long axis of the bacterium. Deich et al. have shown how a 2-dimensional projection of this 3-dimensional geometry results in substantial changes in MSD [12]. To appreciate the effect of projection, consider a particle located at the edge of the bacterium,
7. Membrane diffusion: heterogeneity and 2-D projection

where the membrane is oriented perpendicular to the image plane. When this particle diffuses in the direction perpendicular to the image plane the projected displacement will be less than the actual displacement. In contrast, the displacement of a particle diffusing along the bacterial long axis (and in the image plane) is not distorted by the projection and the observed displacement will be equal to the actual displacement. Here, we address how the CPD of squared displacements is affected by the projection. We chose to perform simulations of particles diffusing along the surface of a bacterium. To test our simulation algorithm, we first generated trajectories in 3 dimensions, without projection. From these trajectories, we calculated the CPD of finding the particle outside a circle with radius \( r \) on the bacterial surface after a time lag \( \tau \). The CPDs calculated for the first five time lags decay exponentially, as expected for a homogenously diffusing population of particles (figure 7.2A). From these CPDs, a diffusion coefficient \( (D) \) of 151 nm\(^2\)ms\(^{-1}\) was determined, by globally fitting Eq.1 to all CPDs. This corresponds well to the input step size (25 nm corresponding to \( D = 156 \) nm\(^2\)ms\(^{-1}\)), indicating that our diffusion algorithm is sound and statistically solid. In addition, the contribution of localization inaccuracy (which we estimate to be 34 nm) is small compared to the average motion within a single frame (190 nm). We next performed the same simulation implementing a planar projection of the trajectories by discarding the position coordinate perpendicular to the image plane. The resulting CPDs could be less well described by single exponentials (figure 7.2B). This deviation of the CPD of projected trajectories from single exponential is more evident at longer time lags (figure 7.2C, time lag 180 ms), in contrast to the CPD of the three-dimensional trajectories. A global fit of the CPDs obtained from projected trajectories yielded a \( D \) of 96 nm\(^2\)ms\(^{-1}\), substantially less than the diffusion coefficient that was used as input parameter (156 nm\(^2\)ms\(^{-1}\)). Taken together, these simulations show that projection of the 3-dimensional trajectory of a particle along the bacterial surface on a 2-dimensional image plane leads to a distorted distribution of displacements, resulting in an underestimation of the diffusion coefficient.

We next compared our simulations to those of Deich et al. [12], who determined the effect of projection on the MSD. For all trajectories (without and with planar projection) we calculated the MSD for the first 10 time lags (figure 7.2D). Subsequently, the diffusion coefficients were obtained by performing a linear fit to the MSD as function of lag time, resulting in a \( D = 141 \) nm\(^2\)ms\(^{-1}\) without projection and \( D = 92 \) nm\(^2\)ms\(^{-1}\) with projection. These results are in line with the values obtained using CPD analysis and are similar to what was observed before [12]. It is important to note that in case of the planar projection the functional form of MSD(\( \tau \)) is still a straight line and cannot be discriminated from the expected behavior (albeit that the slope is different). In the case of CPD analysis the projection not only results in altered fitted diffusion coefficients, but also in a functional form that deviates from the expected exponential behavior (figure 7.2C).
7. Membrane diffusion: heterogeneity and 2-D projection

Figure 7.2 Projection of diffusion in three dimensions on a 2-dimensional image plane affects the CPD.

Comparison of CPDs obtained from 3-dimensional and projected simulated trajectories (input step size: 25 nm \((D = 156 \text{ nm}^2 \text{ ms}^{-1})\); length of cylindrical part of bacterium: 1000 nm; diameter: 1000 nm). (A) CPD obtained from the 3-dimensional simulated traces (black squares). The CPDs are shown for the first 5 time lags (60, 120, 180, 240 and 300 ms). The grey lines represent a global fit of Eq.1 to the five CPDs, resulting in a diffusion constant of 151.0 nm² ms⁻¹ \((\chi^2 = 1.2)\). (B) CPD obtained from the projected simulated traces (black circles). A global fit with Eq. 1 describes the data less well \((D = 96 \text{ nm}^2 \text{ ms}^{-1}; \chi^2 = 5.2)\) than in (A). (C) Zoom of the third time lag of the CPDs shown in A (open black squares) and B (black circles) both fitted with exponential decays (grey line). (D) MSD as a function of time lag \((\tau)\) obtained from 3-dimensional (squares) and projected (circles) simulated trajectories. Both curves are well described by a linear fit \((\text{MSD}(\tau) = 4D\tau, \text{ weighted with the s.e.m.})\), resulting in diffusion coefficients of 141 nm² ms⁻¹, respectively 92 nm² ms⁻¹.

7.3.2 Cell dimensions hardly affect the projected random walks

As shown above, planar projection causes the distribution of squared displacements to be non exponential. This effect, however, is not sufficient to explain the experimental data. We investigated whether this discrepancy could be caused by variations in \(E.\ coli\) cell dimensions. In our \(E.\ coli\) samples the cell diameter is relatively constant, the cell length, however, varies considerably, depending on, for example, the stage of the cell in the division
cycle. We tested the effect of variations in cell length by performing simulations as presented above but with different cell lengths. From the simulated, projected trajectories we calculated both the MSD and CPD of squared displacements (figure 7.3). As anticipated the effect of the planar projection is largest when the surface is completely spherical ($l = 0 \mu m$), since in this case the projection distorts steps in all directions. Indeed, the MSD shows that, for shorter cylindrical parts of simulated surface the $D = 85 \text{nm}^2\text{ms}^{-1}$, obtained from a weighted linear fit (figure 7.3A), deviates more from the $D$ that was used as input parameter in the simulation ($156 \text{nm}^2\text{ms}^{-3}$). The CPDs of the simulations are non-exponential, which is most pronounced when a spherical shape is considered (figure 7.3B). The deviation from single exponential behavior decreases with increasing length of the cylindrical part (figure 7.3C). However the CPD is never exponential, illustrating that the impact of planar projection is largely due to the cell diameter. Moreover, the effect obtained by altered geometry of the cell is too small to account for the measured discrepancy with exponential behavior of the CPD of squared displacements.

**7.3.3 Immobile particles in combination with position inaccuracy**

To explain the experimental data we next increased the complexity of the simulation by adding an extra population of particles. In a first approach, we took these particles to be immobile. In combination with the inaccuracy of position determination, such an immobile fraction leads to a population with an effective diffusion coefficient equal to $\sigma^2/(4\tau)$, with $\sigma$ the standard deviation of the position determination. In the CPD of the squared displacements, such an additional population will lead to an extra exponent in Eq.1, with a decay constant independent of time lag, in contrast to the fraction of diffusing particles. The relative amount of immobile particles and the diffusion constant (of the moving fraction) describing best the experimental data was determined by a global analysis of the CPDs of the first 5 time lags. The best fitting simulation was selected by calculating the $\chi^2$ between the experimental and simulated distributions. This yielded a fraction of 42% immobile particles and 58% particles moving with a diffusion constant of 169 $\text{nm}^2\text{ms}^{-1}$. Close inspection of the measured and simulated distributions of squared displacements in the first time lag indicates that the data is well described assuming an immobile fraction. At larger time lags, however, there is a clear deviation of data and simulations at short displacements. In the simulations, the slope at small displacements (which is mainly caused by the immobile fraction) is similar for the distributions at all time lags shown. In the experimental distributions, this slope changes with time lag, indicating that an additional population might be present, but that this population is mobile, with a different diffusion constant than the major diffusing fraction.
7. Membrane diffusion: heterogeneity and 2-D projection

7.3.4 Description of experimental data by two populations of diffusing particles

Next we added a second fraction of diffusing particles. Again we performed simulations, this time by randomly varying the two fractions of particles characterized by different diffusion constants. A million conditions were probed by independently changing the three independent parameters and building 100 traces per condition. We compared the simulated CPDs for the first five time lags of the square displacements with the experimental values.

Figure 7.3 Effect of cell length on projected displacements.

The length of the cylindrical part varies from 0 nm (resulting in a spherical bacterium, black squares) to 1000 nm (open circles), and 10 000 nm (open triangles). (A) MSD as a function of time lag. Black lines represent weighted linear fits yielding values for $D$ of 85 nm$^2$/ms$^{-1}$ ($l = 0$ nm), 101 nm$^2$/ms$^{-1}$ ($l = 1000$ nm) and 117 nm$^2$/ms$^{-1}$ ($l = 10000$ nm). (B) CPDs. All CPDs deviate from a single exponential decay (grey lines). (C) Zoom of the CPD for spherical ($l = 0$ nm) and elongated ($l = 10000$ nm) shapes in B). These plots show that the effect of projection is less for $l = 10000$ nm (open triangles) and this curve can be fitted with an exponential, although the fit is still not optimal (grey lines are exponential fits).
ones by calculating the $\chi^2$. In this way, a best fitting simulation was obtained, the one with the lowest $\chi^2$, characterized by a fraction (0.41) of particles with a $D$ of 7 nm$^2$ms$^{-1}$ and a fraction (0.59) with a $D$ of 169 nm$^2$ms$^{-1}$. Comparison of the experimental curves with those of the best fitting simulation (figure 7.4B) shows that this model of two fractions of diffusing particles, each characterized by a different diffusion coefficient, describes the experimental CPDs of the squared displacement well, also at larger time lags. We conclude, therefore, that at least two major populations of TatA particles are present in the E. coli cell membrane, each with distinct diffusion coefficient.

**Figure 7.4 Assessment of simulations with an additional immobile or mobile fraction.**

(A) Comparison of experimental CPD and CPD obtained from simulated trajectories assuming a mobile and an immobile fraction of particles. Simulations were used to fit the experimental CPDs (black), with the fraction of immobile particles and the step size (of the mobile particles) as fit parameters. Shown (grey) is the best fit of the first five time lags: $D = 169$ nm$^2$ ms$^{-1}$; fraction of immobile particles = 0.42 ($\chi^2 = 79$). (B) Comparison of experimental CPD and CPD obtained from simulated trajectories assuming two mobile fractions of particles. Simulations were performed by randomly varying both diffusion coefficients and their relative contributions. The experimental CPDs of the first five time lags (black) are best described assuming 59% of the particles to diffuse with a diffusion coefficient of 169 nm$^2$ms$^{-1}$ and 41% with 7 nm$^2$ms$^{-1}$ ($\chi^2 = 59$) (grey).

**7.4 Conclusion**

In summary, we have used the CPD of squared displacements to analyze the dynamics of TatA diffusing in the membrane of E. coli. This approach has two important advantages with respect to the more commonly used MSD method. First, it allows to resolve heterogeneity in the sample due to populations with distinct diffusion coefficients, since this results in multi exponentially decaying CPDs [9]. Second, in the CPD a projection of a 3-dimensional random walk results in a distortion of the distribution, which can be readily
obtained using simulations. Therefore, the shape of the CPD can be used to investigate effects due to cell shape and curvature.

To explain the CPD obtained from single-molecule tracking data of single TatA in vivo we performed simulations, which revealed that the projection of 3-dimensional walks on a 2-dimensional plane by itself is not enough to explain the CPD. In addition, at least two mobile populations with distinct diffusion coefficients are necessary to explain the experimental data. Assuming two components, 41% of the particles moved with a diffusion coefficient of 7 nm²ms⁻¹, while the rest moved substantially faster with a diffusion coefficient of 169 nm²ms⁻¹.

References

Supporting Material

Figure S1 validation of the quality of the fitting procedure:

A) CPDs of TatA diffusion (black) together with the simulation (grey) that describes the data best: consisting of two mobile fractions; a population of 59% moving with a diffusion constant of 0.169 $\mu$m$^2$s$^{-1}$ and a second population moving with 0.009 $\mu$m$^2$s$^{-1}$ (see text, identical to fig 6.4A).

B) For comparison, a fit of the experimentally obtained CPDs by a simulation in which the parameters are fixed at the optimal values (like in A), except for the diffusion constant of the fast moving population, which is fixed at 0.135 $\mu$m$^2$s$^{-1}$, 20% off from A. This simulation clearly results in a less optimal description of the data.

C) For comparison, a fit of the experimentally obtained CPDs by a simulation in which the parameters are fixed at the optimal values (like in A), except for the diffusion constant of the slow moving population, which is fixed at 0.0072 $\mu$m$^2$s$^{-1}$, 20% off from A. This simulation clearly results in a less optimal description of the data.

D) For comparison, a fit of the experimentally obtained CPDs by a simulation in which the parameters are fixed at the optimal values (like in A), except for the fraction of slow moving particles, which is fixed at 32.8% (20% less than in A). This simulation clearly results in a less optimal description of the data.
7. Membrane diffusion: heterogeneity and 2-D projection
Summary
The title of this thesis is “Single-protein motion on microtubules and in cell membranes”. As this title implies, the central theme of this thesis was motion, a change of position in time. We can distinguish two main types of motion: random motion and directed motion. An example to illustrate these types of motion is the walk of a man on the street. Let us consider first that, after work, the man walks straight home. He will thus make all his steps in the same direction and the distance he will cover will increase linearly in time. This is a typical illustration of directed motion. Let us now imagine that the man stops on his way in a bar and gets drunk. After leaving the bar, he will have equal probability to step either home or away from home, resulting in a random walk, which will take longer to bring him home. The types of motion described above are also present in biology, from the level of the cell to the level of the molecule. The first scientific observation of random motion was made in 1828 by the British botanist Robert Brown while studying pollen particles in water. Later on, this phenomenon was explained by the random collision of pollen particles with water molecules and was named Brownian motion.

The cell can be viewed as a small compartment delimited by a membrane and crowded with proteins and biomolecules. All these molecules undergo some kind of motion that can be directly related to their function. The shape and the structure of the cell are supplied by the cytoskeleton. A major component of the cytoskeleton are the microtubules (MT), which, besides providing mechanical structure, are also crucial for protein transport and cell division. MTs are long hollow tubes that display a polarity (− end and + end). Molecular motors, such as Kinesin-1, take advantage of this polarity to transport cargo from one side of the cell to another. Kinesin-1 “walks” over the MT towards the + end using ATP as an energy source and it can make more than hundred steps before it detaches from the MT. The velocity of this “walk” is about 1 µm/s, making this mode of cargo transport more effective and faster over µm distances than diffusion (2s to directional transport of cargo across a cell of 2 µm long vs. 8s for diffusion with a diffusion constant of 10 nm² s⁻¹). Chapter 3 deals with Kinesin-1 walking over MTs without transporting cargo. We describe novel methods to successfully determine 3 important parameters that define the directional motion of such a molecular motor: the velocity, the run length (the average distance travelled) and the randomness (a measure of the stochasticity of stepping).

Besides its role as a “track” for molecular motors, MTs are also involved in the separation of the genetic material between the two daughter cells during the cell division. This segregation is coordinated by a combination of molecular motors and a specific MT structure, namely the mitotic spindle. The mechanism of action of the mitotic spindle depends on the polarity patterns of its constituent MTs. In Chapter 4, we studied one of the MT-based molecular motor, KLP61F. We show that KLP61F is capable of crosslinking and sliding adjacent spindle MTs and that it displays a preference for crosslinking MTs in the
antiparallel orientation. These results explain its crucial role in the formation of the mitotic spindle.

The architecture of the cytoskeleton depends on the interplay of the aforementioned molecular motors, which can crosslink and move directionally along MTs, and of non-motor proteins which can also crosslink MTs but do not drive directional motion. In Chapter 5, we investigated such a non-motor protein, PRC1, and show that it binds to individual MTs and diffuses along them. This diffusive motion slows down upon binding of PRC1 to a second MT, leading to an accumulation of PRC1 on overlapping MTs and thus to the formation of stable MT bundles.

Proteins can also diffuse within the cell membrane. This is the case of the Tat system (twin arginine translocation), a protein complex present in the cell membrane of the bacterium E.coli. The Tat complex consists of 3 different proteins, TatA, TatB and TatC, and translocates proteins over the inner membrane. In Chapter 6 we measured the number of TatA present in single Tat complexes and its diffusive motion. We showed that the motion of the complex depends on the concentration of the protein that is transported and on the presence of the energy source needed for the translocation. In Chapter 7 we perform computer simulations to explain the motions of the Tat complexes obtained in Chapter 6. The simulations reveal the existence of two distinct types of complexes: complexes formed by only TatA and much larger ones, likely formed by a combination of TatA, TatB and TatC.

Almost everything in biology is in motion; either in passive motion (e.g. the diffusion of a protein in the cell membrane due to the constant collisions with surrounding molecules), or active motion (e.g. the directional motion of Kinesin-1, which requires the energy released by ATP hydrolysis) or a combination of both. To be able to quantitatively and qualitatively describe the motion of a particle, one has to be able to visualize the particle and accurately determine its position at different times to reconstitute its trajectory. The tool of choice for this challenge is single-molecule fluorescence microscopy, as introduced in Chapter 2. Indeed, it allows the specific visualization of biomolecules labelled with fluorescent dyes – numerous procedures are available for fluorescent labelling- against a dark background. In combination with the use of specific optics and high speed camera’s this yields to a high position accuracy as well as high time resolution. Single-molecule fluorescence microscopy is thus an excellent tool with spatial and temporal resolution required to unravel all kinds of motion in biology.
Nederlandse samenvatting
De titel van mijn proefschrift luidt, in het Nederlands: “de beweging van enkele eiwitten over microtubules en in het cel membraan”.

Zoals de titel al aangeeft is de rode draad door dit proefschrift *beweging*, de verandering van positie in de tijd. In dit proefschrift onderscheiden we twee soorten beweging: directionele beweging en willekeurige beweging. Een illustratief voorbeeld van deze bewegingen is een man die op straat loopt. Laten we eerst kijken naar deze man als hij van zijn werk naar zijn huis loopt. In dit geval zet de man alle stappen in dezelfde richting, namelijk die van zijn huis, en de afstand die de man aflegt groeit rechttevenredig met de tijd (als gevolg van de constante snelheid waarmee de man loopt). Dit is een typisch voorbeeld van de directionele beweging. Laten we nu eens kijken wat er gebeurt als de man op weg naar huis stopt in een café en dronken het café verlaat. Wanneer hij dronken naar huis loopt is de kans dat hij een stap in de richting van zijn huis zet even groot als een stap in een willekeurige andere richting. Dit heeft tot gevolg dat de wandeling die deze dronken man maakt volkomen willekeurig is. In de natuur, op het niveau van cellen en moleculen, komt deze dronkemanswandeling ook voor. De eerste wetenschappelijke observatie dateert uit 1828 toen de Britse botanicus Robert Brown zag dat stuifmeel in water een zelfde dronkemanswandeling ondergaat. Later werd dit gedrag verklaard door de willekeurige botsingen van stuifmeel deeltjes met water moleculen en deze beweging werd diffusie (of Brownse beweging) genoemd.

De cel wordt vaak gezien als de bouwsteen van het leven. Een gesimplificeerde voorstelling van de cel is een compartiment dat is gevuld met eiwitten en biomoleculen welke omringd is door een membraan. Al deze deeltjes zijn voortdurend in beweging en die beweging kan vaak direct gerelateerd worden aan de functie van het deeltje binnen de cel. De vorm en de structuur van de cel wordt bepaald door het cytoskelet, waarvan een belangrijke component de microtubels (MTs) zijn. MTs zijn niet alleen belangrijk voor de vorm van de cel, maar ook voor het transport binnen de cel en voor de deling van de cel. MTs zijn lange holle buizen die door hun specifieke bouw directionaaliteit (-eind en +eind) hebben, die directionaaliteit wordt gebruikt door moleculaire motoren, zoals Kinesin, om hun vrachtdoelen binnen de cel te brengen. Kinesin-1 “loopt” over de MT in de richting van het plus einde terwijl het ATP (brandstofmolecuul) verbrandt en het kan onherinnerd stappen maken voor het de MT loslaat. De snelheid waarmee Kinesin-1 beweegt is ongeveer 1 μm/s (1 miljonoende meter per seconde) wat dit transport vele malen efficiënter maakt dan diffusie (Kinesin-1 doet er ongeveer 2 seconde over om van een kant van een cel van 2μm naar de andere kant te lopen, terwijl diffusie 8 seconde zou duren (met een typische diffusie constante van 10 nm²/s)). Hoofdstuk 3 van dit proefschrift gaat over de Kinesin-1 dat over MTs loopt, we beschrijven een nieuwe manier voor het bepalen van drie belangrijke eigenschappen die de directionele beweging beschrijven, namelijk: de
snelheid, de gemiddelde afgelegde afstand en het aantal snelheidslimiterende processen wat ten grondslag ligt aan een enkele stap.

MTs zijn niet alleen belangrijk voor het transport in de cel, maar ze spelen ook een belangrijke rol tijdens de deling van een cel wanneer het genetisch materiaal (DNA) gelijk moet worden verdeeld tussen de twee dochter cellen. Deze verdeling wordt gecoördineerd door een combinatie van moleculaire motoren en een specifieke MT spoelstructuur waarvan de werkzaamheid nauw samen hangt met de directionaliteit van de MTs. In hoofdstuk 4 hebben we de MT-bindende moleculaire motor, KLP61F bestudeerd en we hebben aangetoond dat deze motor in staat is om MTs te binden en bundelen om ze vervolgens over elkaar heen te laten bewegen. Ook hebben we laten zien dat KLP61F een preferentie heeft om MTs in een antiparallele orientatie (de directionaliteit in de tegenovergestelde richting) te bundelen. Samen verklaren deze resultaten de cruciale rol van KLP61F in de vorming en de evolutie van de spoelstructuur.

De structuur van een MT netwerk wordt dus bepaald door de hierboven genoemde directionaliteit van de MTs en door de MT-bindende moleculaire motoren, maar ook door MT-bindende niet-motor eiwitten. Deze eiwitten kunnen dus wel MTs binden en bundelen, maar ze kunnen in tegenstelling tot de motoren niet directioneel over een MT kunnen bewegen. In hoofdstuk 5 bestudeerden we PRC1, een niet-motor eiwit wat wel aan MTs bindt. Wij hebben aangetoond dat dit eiwit MTs bindt om er vervolgens over heen te diffunderen. Wanneer PRC1 vervolgens ook een tweede MT bindt wordt de diffunderende beweging langzamer, met als gevolg dat PRC1 ophoopt tussen gebundelde MTs waar het de bundels kan stabiliseren.

Een andere plaats in de cel waar diffusie kan worden waargenomen is in het cel membraan waar eiwitten in bewegen. Een voorbeeld is het Tat systeem (Twin arginine translocation), dat bestaat uit verschillende eiwitten en diffundeert in het cel membraan van de bacterie E.coli. Het Tat systeem vormt complexen die zijn opgebouwd uit een combinatie van drie eiwitten namelijk TatA, TatB en TatC, met als functie om andere eiwitten door het cel membraan te transporteren. In hoofdstuk 6 hebben we gemeten hoeveel TatA eiwitten voorkomen in een Tat complex en hoe deze complexen in het cel membraan bewegen. Verder hebben we aangetoond dat de beweging afhankelijk is van de hoeveelheid te transporteren eiwit en van de beschikbare energie. Vervolgens hebben we in hoofdstuk 7 de beweging van de Tat complexen verklaard met behulp van computer simulaties. De simulaties lieten zien dat de complexen uit hoofdstuk 6 kunnen worden verdeeld in twee groepen, meest waarschijnlijk zijn dat complexen gevormd uit alleen TatA en grotere complexen gevormd uit zowel TatA, TatB en TatC.

Vrijwel alles in de biologie is in beweging; het zij passieve beweging (bijvoorbeeld de diffusie die een eiwit in het cel membraan ondergaat door de constante botsingen met andere
moleculen in het cel membraan), het zij actieve beweging (bijvoorbeeld de directionele beweging van kinesin-1, die de benodigde energie voor deze beweging haalt uit de verbranding van ATP), het zij een combinatie van deze twee. Om de beweging te beschrijven en te kwantificeren is het belangrijk dat men het bewegend deeltje zichtbaar kan maken zodat de positie van het deeltje op verschillende tijden kan worden bepaald en vervolgens de gelopen weg kan worden gereconstrueerd.

Een prima techniek voor deze uitdaging is enkele-molecuul fluorescentie microscopie dat we in detail uitleggen in hoofdstuk 2. Deze techniek maakt het mogelijk om specifiek biomoleculen zichtbaar te maken tegen een donkere achtergrond door er een fluorescente kleurstof aan te hangen. In combinatie met geoptimaliseerde filters en lenzen en snelle en gevoelige camera’s kan de positie van de gelabelde biomoleculen dan nauwkeurig worden bepaald. Enkele-molecuul fluorescentie microscopie is dus een techniek waarmee zowel een plaats als tijd resolutie kan worden bereikt die nodig is om de verschillende bewegingen in de biologie te bestuderen.
List of publications

Novel Ways to Determine Kinesin-1’s Run Length and Randomness Using Fluorescence Microscopy

Microtubule-Driven Multimerization Recruits ash1p onto Overlapping Microtubules

The Homotetrameric Kinesin-5 KLP61F Preferentially Crosslinks Microtubules into Antiparallel Orientations

A Brief Introduction to Single-Molecule Fluorescence Methods

The diffusion of Twin Arginine Translocation Complexes in Bacterial Membranes is Substrate and Membrane-Potential Dependent
S.M.J.L. van den Wildenberg, K.W. Yau, F. Frankena, H. Lill, E.J.G. Peterman, Y. J.M. Bollen (manuscript in preparation)

How to Quantify Protein Diffusion in the Bacterial Membrane
S. M.J.L. van den Wildenberg, Y.J.M. Bollen, E.J.G. Peterman (accepted in: Biopolymers)

*Joint first authors
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