CHAPTER 1

INTRODUCTION

1.1 Intracellular transport
The inner world of a eukaryotic cell is very busy, dynamic and crowded with moving proteins, nucleic acids and organelles. The mitochondrion, the energy factory of cells, is an important organelle in animals and plants cells and supplies adenosine triphosphate (ATP) molecules as the energy currency for various cellular processes. The energy stored in ATP molecules is, among others, consumed by the active transport system. Passive transport based on Brownian motion, diffusion processes, works effectively for short-range transport of ions and biomolecules within cells. To perform long-range and directed motion within cells, certain molecular engines, known as motor proteins, drive transport at the nanoscale along the relatively rigid proteinaceous filaments of the cytoskeleton [1].
The cytoskeleton (Fig.1.1), is a complex network of microtubule, actin and intermediate filaments. Distinct motor proteins of the microtubule-based dynein and kinesin superfamilies and actin-based myosin superfamily work collectively to perform a wide range of cellular processes, such as cell-division, intracellular transport, maintaining cell-shape, cell-migration, and organelle positioning. In the following sections, an overview is presented of microtubules, the molecular motor Kinesin-1 and its consensus chemomechanical cycle, and the Kinesin-2 family motors OSM-3 and Kinesin-II.

1.2 Microtubules

In many cells, microtubules originate from the Microtubule Organizing Center (MTOC) that is positioned close to the nucleus and organize into a star-like fashion protruding radially outward organized themselves into highly dynamic microtubule networks. This system enables long-range directed intracellular transport and appropriately positions cellular organelles throughout the cytoplasm. They also facilitate to determine cell shape and in a variety of cell movements and the separation of chromosomes during mitosis [3,4,5].

Microtubules are highly dynamic structures that constantly undergo growth and shrinkage phases. This confers microtubules a unique ability to perform a wide variety of tasks as stated above. Microtubules are rigid hollow tubular proteins approximately 25 nm in diameter that generally consist of 13 linear protofilaments assembled around a hollow core (Fig. 1.2). The protofilaments, which are composed of head-to-tail arrays of tubulin heterodimers, α-tubulin and β-tubulin, with a total molecular weight of ~110 kDa, Fig. 1.2, are arranged in parallel. Microtubules are self-assembled dynamic biological polymers that undergo ‘dynamic instability’ driven by guanosine triphosphate (GTP) hydrolysis. Tubulin heterodimers are loaded with two GTP
1.2 Microtubules

molecule. Tubulin dimers have a preference to be added to the plus-ends of growing microtubules. Once the dimers are assembled, they hydrolyze GTP to guanosine diphosphate (GDP). When the stabilizing cap of GTP tubulin is lost, in that case, microtubule biopolymers become metastable and rapidly start to shrink. In solution, tubulin dimers quickly exchange GDP for GTP and can be added to the growing plus-ends of microtubules again [7, 8]. Various microtubule associated proteins (MAPs) bind to the microtubules and facilitate in regulating microtubule dynamics. [9].

Figure 1.2: **Microtubule and its subunits.** (A) The crystal structure of the tubulin dimer, which consists of α and β subunits. The β-tubulin has tightly bound GTP, while the GTP in the α-subunit can be hydrolyzed. (B) The dimers form polar protofilaments (C) Parallel alignment of 13 protofilaments form closed hollow tubes. Polar conformation of microtubules with one end (-) of slow growth and another end (+) more dynamic. (D) and (E) Electron micrographs of a microtubule cross section and segment. Image taken from [6].
1.3 Biological molecular motors

Numerous cytoskeletal molecular motor proteins of the myosin, dynein and kinesin superfamilies perform various functions in eukaryotic cells. These motor proteins specifically attach to the filaments and walk along the actin (myosin V) and microtubule (kinesin and dynein) tracks while transporting cargoes along cytoskeleton filaments, Fig. 1.3. Over the years, 24 different classes of myosins, 14 different families of kinesins and two groups of minus-end directed dyneins have been discovered with distinct structure, functions and motility properties to accomplish their versatile cellular tasks.

Figure 1.3: Microtubule-based motor proteins intracellular transport in (a) neuronal cell, (b) non-neuronal cell and (c) cilia. Image taken from [10].
These motor proteins can be considered as nano-engines because of the common feature of converting chemical energy, in the form of ATP hydrolysis, into mechanical work. With the advent of advanced techniques in biochemistry and biophysics, our knowledge of the mechanisms and kinetics of motor proteins have vastly increased over the last decades. However, there are many open questions to be answered. Such as: how exactly do different kinds of motors walk on the filament under traffic-jam-like or crowded situations created by macromolecules and nearby motors? How do the different motility and kinetic properties of an individual motor affect collective dynamics? How is the opposite polarity of motors acting on one and the same cargo is controlled in vivo?

The central focus of this thesis is on the kinesin motor proteins homodimeric Kinesin-1, homodimeric OSM-3 and heterodimeric Kinesin-II (both from the Kinesin-2 family). In the following sections, I will describe the structural and functional importance of these three motor proteins in more detail.

1.3.1 Kinesin-1: A conventional Kinesin motor protein from the Kinesin-1 family

The kinesin protein was partially purified in 1985 for the first time and named after the Greek word ‘kinein’, which means ‘to move’. It was identified as a microtubule-based motor protein with a force-generating ATPase activity in axonal transport in the giant axon squid [11,12]. It is also known as conventional kinesin, Kinesin-1, KIF5 and is the founding member of the kinesin superfamily (KIF) [12,13]. After a decade, the structure of the truncated constructs derived from human and rat conventional kinesin enzymatic active was determined by X-ray crystallography. [14,15]. Since its discovery, the biochemical and biophysical properties of Kinesin-1 have been studied extensively. There is a high degree of sequence similarity in the motor domains
of various kinesin motor proteins, whereas the other parts show substantial diversity [14,16].

The prototypical Kinesin-1 is a heterotetramer comprised of two identical kinesin heavy chains (KHC) and two kinesin light chains (KLC). The two heavy chains of conventional kinesin (110-140 kD) are twice as heavy as the two associated light chains (60-80 kD) [17,18]. The heavy chains are required for kinesin’s motility, whereas the light chains provide regulatory and cargo-binding functions [19,20], Fig. 1.4.

![Structure of tetrameric Kinesin-1 motor proteins](image)

**Figure 1.4:** Structure of tetrameric Kinesin-1 motor proteins. Image adapted from [21].

Structurally, the kinesin heavy chain consists of three main domains, Fig. 1.5: (1) the N-terminal globular *motor domains*, which contain the microtubule and nucleotide binding sites [14,22], followed by a ~15 amino acids long *neck linker*. This domain is responsible for force-generation [23]. (2) The *stalk domain* facilitates dimerization of two motor domains and a flexible hinge domain joins the neck linker to the stalk and (3) the C-end *tail domain* is a globular domain with regulatory function and it binds to cargoes either directly or via the tetratricopeptide repeat (TPR) motif of the KLC. The tail domain has the ability to fold back if it is not bound to cargo and mask the motor domains avoiding ATP molecules unnecessary consumption [24]. This structural change restricts the motor’s stepping, and thereby regulating transport [25].
1.3 Biological molecular motors

**Figure 1.5: Structure of a Kinesin-1 heavy chain dimer.** The motor domains are available as crystal structures; the structures of stalk and tail are inferred from electron microscopy and coiled-coil prediction analyses. Image taken from [26].

*The chemomechanical cycle: A hand-over-hand mechanism*

Kinesin-1 is a highly processive motor and undergoes around 100 steps along the plus-end of microtubules before releasing. Intrinsically, these motor proteins are chemomechanical nano-engines with ATPase enzymatic activity that have the ability to convert chemical energy stored in the phosphate bonds of ATPs into mechanical energy during the ATP hydrolysis; these motor proteins walk along the cellular tracks, microtubules, in a hand-over-hand fashion, with a step size of 8.2 nm that can exert a force of ~6–7 pN [27, 28, 29, 30, 31, 32].

The consensus model for the Kinesin-1 chemomechanical cycle, Fig. 1.6 [33, 34, 35] suggests that during the stepping cycle, each kinesin head (motor domain) transitions between strongly bound states (the ATP-containing state, and also the no-nucleotide state), and weakly bound states to the microtubules (the ADP-containing state or ADP). The kinesin stepping cycle starts from the one-head-bound (1-HB) ATP-waiting state, shown as state-A, it can be characterized by the first step of strongly bound, nucleotide-free (Ø) front head (dark violet) and an unbound, ADP-containing tethered head (light violet). State-B contains one or more transition sub-states, an ATP molecule
binding induces a force-dependent transition that leads to partial Neck Linker (NL) docking, shifting the tethered head past the bound head (B1), followed by ATP hydrolysis, at the front head (ADP-Pi), that completes NL docking and facilitates tethered-head binding to the microtubule (B2). In this step, the binding gate promotes binding of the tethered head at the forward microtubule binding site while opposing release of the bound head, $k_{AB}$ is the rate of transitioning state A to B. After this state B, the tethered head reaches the forward microtubule binding site and dissociates ADP and enters the two-heads-bound (2-HB) state [State C], $k_{BC}$ is the rate of transition from state B to C. However, kinesin may dissociate from the microtubule and enter to an [Off] state due to premature phosphate release from the bound head. The rear-head releases P_i and returns the dimer to the ATP-waiting state [A], $k_{CA}$ is the rate of transition from state C to A. After this cycle, kinesin motor has moved forward by 8.2 nm. (Fig. 1.6). Single kinesin motors move over a distance of 1–2 μm along the microtubule before dissociating.

Figure 1.6: Chemomechanical cycle of Kinesin-1 motor proteins, adapted from [35].
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1.3.2 Kinesin-2 family

Kinesin-2 family motors are involved in cellular transport ranging from mammals (respiratory cells, olfactory cells, oviduct, epithelium cells, sperm cells) to nematodes (sensory cilia of *Caenorhabditis elegans*). They are also present in frog (in melanophores and unfertilized eggs) and squids. Members of the Kinesin-2 family are homodimeric and heterotrimeric plus-end directed. In this thesis, I worked specifically on *C. elegans* sensory ciliary motor proteins: OSM-3 and Kinesin-II.

*C. elegans* sensory ciliary motor proteins: OSM-3 and Kinesin-II

*C. elegans* intraflagellar transport (IFT) is one of the remarkable examples of cooperation between kinesin-2 family motor proteins with distinct motor properties while transporting the same cargos. Two anterograde intraflagellar transport (IFT) motors, heterotrimeric kinesin-II, KLP11/KAP1/KLP20, (a homologue of vertebrate KIF3AB) and homodimeric OSM-3 (a homologue of vertebrate KIF17), cooperate to move cargo along *C. elegans* ciliary axonemes and building and maintenance of ciliary structures.

* C. elegans* ciliary axonemes consist of a 1-μm–long transition zone (TZ) followed by 4-μm–long proximal (also known as middle) segment (PS), from which 2.5μm–long, 9 singlet microtubules extend to form the distal segment (DS), Fig. 1.7. Kinesin-II motors work as the cargo-loaders and transport the IFT proteins at the base to the transition zone of the cilium. Kinesin-II and OSM-3 together transport IFT trains along the PS to deliver axoneme subunits for PS assembly, whereas DS assembly depends on the long-range transport of tubulin precursors driven by OSM-3 alone [36, 37, 38, 39, 40]. From *in vitro* and *in vivo* single-molecule fluorescence experiments, it is well established that kinesin-II generates short (observed only *in vitro*) and slow runs (~0.2 μm at ~0.5 μm/s) and OSM-3 generates longer (observed only *in vitro*)
vitro) and faster runs (~2 μm at ~1.5 μm/s, [40, 41, 42, 43] at physiological conditions. Recent studies on *C. elegans* IFT transport have increased our understanding of different Kinesin-2 motors in a broad range of cilia. Through *in vivo* single-molecule fluorescence experiments, it has been demonstrated that less processive kinesin-II imports IFT trains into the cilium and OSM-3 does the long-range transport, thereby optimizing IFT delivery [40]. However, several advanced and different techniques are required to unravel the need of two very different motor proteins for the cilia maintenance.

**Figure 1.7: Schematic presentation of cilium and the IFT machinery of *C. elegans*.** Components of the IFT machinery and ciliary cargo assemble at or near the transition zone. These cilia consist of middle segment with microtubule doublets and distal segment with microtubule singlet. Two Kinesins-2 motors, heterotrimeric Kinesin-II and homodimeric OSM-3 bind IFT particle A and B respectively and transport these particles together with IFT-dynein along the middle segment in the anterograde + direction. Image taken from [36].
After having discussed the proteins that are the protagonists of this thesis, I will briefly introduce the microscopy technique -Total Internal Reflection Fluorescence (TIRF) microscopy- and the biophysical assays -the motility assays- used in this thesis.

1.4 Total internal reflection fluorescence microscopy

The *in vitro* motility properties of motors investigated in this study were performed using fluorescence-microscopy techniques. The imaging of single fluorescent motor proteins with standard epi-illumination is cumbersome due to the high background fluorescence of the fluorophores in solution, since not only fluorophores in the focal plane are excited, but also out-of-focus fluorophores. Wide-field epi-illumination fluorescence microscopy is the best suited for samples where the majority of fluorophores is located in a relatively thin layer, avoiding problems with out-of-focus fluorescence.

In the quest of high sensitivity and accuracy, Total Internal Reflection Fluorescence (TIRF) microscopy method was developed by Daniel Axelrod and coworkers in 1981 [44]. It is a widely used method providing a sub-diffraction limited illumination volume in the axial direction. The phenomenon of total internal reflection occurs at the interface between two optical media with different refractive indices, e.g. glass-water. Total internal reflection fluorescence (TIRF) microscopy is a near-field method that employs a fluorescence excitation laser beam incident on the glass/water interface (between cover slip and sample) with an angle that is larger than the critical angle, $\theta_c$. Since the angle of incidence is larger than critical, the incident light reflects back into the glass, but also generates, in the aqueous sample, a profile of the evanescent electromagnetic field decaying exponentially from the interface, $E(z) = E_0 e^{-z/d}$. The penetration depth (d) depends on the
wavelength, the angle of incidence and the ratio of refractive indices and is typically about 100 nm for a glass/aqueous interface at visible wavelengths and a 66° incidence angle (Fig. 1.8). Because of the exponentially decaying excitation field, only fluorescent molecules near the surface will be excited, which drastically reduces the background fluorescence from molecules in solution compared to epifluorescence illumination. The fluorescence emitted by the illuminated area is typically imaged on an EMCCD camera.

![Figure 1.8: Objective-type TIRF microscope.](image)

The incident light is guided through the objective, refracted by the high numerical aperture objective lens and brought to a critical angel to the glass-water interface. The reflected light goes back through the objective to the dichroic mirror. Image taken from [45].

1.5 Single-motor motility assays

Standard in vitro motility assays mimic the cellular transport system driven by molecular motors into a synthetic environment. They are a biophysical tool to study motor proteins behavior and dynamics in a minimal and well-controlled system. In single-molecule motility assays, microtubules are immobilized on a functionalized (glass) substrates using for example anti-β tubulin antibodies. Upon landing on a microtubule, active motor proteins start to walk on them, consuming ATP from solution in the flow chamber. The moving, fluorescently
labeled motor proteins are imaged using TIRF microscopy employing an EMCCD camera, Fig. 1.9.

![Figure 1.9: Schematic of the *in vitro* motility assay.](image)

Molecular motors walk along the microtubules attached to the glass slide. The fluorescently labeled motor proteins are excited by a suitable laser and imaged using TIRF microscopy.

### 1.6 Outline of this thesis

My doctoral research has two main topics: I first focus on motor-protein dynamics in crowded conditions and next focus on the temperature dependence of motor-protein dynamics. I specifically study three motor proteins: (1) Homodimeric Kinesin-1 of *D. melanogaster* facilitates long distance intracellular cargo transport along microtubules in a wide variety of eukaryotic cells, (2) Homodimeric OSM-3 and (3) heterodimeric Kinesin-II (KLP11/20) of *C. elegans* play key roles in IFT transport in *C. elegans*.

Molecular motor proteins drive essential transport processes in cells by carrying cargo while walking along the cytoskeleton. Just like traffic jams affect vehicular traffic on highways, similar effects are believed to affect the motion of these dense motor proteins. But it is unclear how motor proteins traffic affects motor transport properties. While the motion of individual motors has been
studied in great detail, their behavior under crowded, traffic-jam conditions remains experimentally challenging to study.

In chapter 2, the crowding dynamics of Kinesin-1 and OSM-3 have been studied using a novel correlation image analysis technique to analyze image sequences and accurately measure motor velocities and run lengths at high motor densities, allowing a quantitative assessment of jamming effects in motor protein transport. By comparing experimental results with model predictions, I arrive at a clear understanding of the collective motor protein transport under crowding, governed by motor-specific strategies.

In chapter 3, crowding dynamics of Kinesin-II have been studied using an approach similar to chapter 2. Studies from chapter 2 and chapter 3 show that Drosophila Kinesin-1 is more of a lone walker, whereas OSM-3 and Kinesin-II from C. elegans are team players.

In chapter 4, I present the systematic study of the impact of temperature on the motility parameters of Kinesin-1, OSM-3 and Kinesin-II at the single-molecule level using single-motor motility assays. The current understanding of temperature dependency on motor stepping is limited and to the some extend incomplete. Several multi-motor gliding assays-based studies reported the impact of temperature on motor proteins, primarily on Kinesin-1. However, the consequences of temperature change on motor-mechanochemistry and therefore, their motility properties, run length and velocity, have not been studied at the single-molecule level. This chapter provides new insights on temperature-dependent mechanochemistry of motor proteins and how some kinetic steps are more temperature sensitive than the others.
References


URL:http://images.nigms.nih.gov/index.cfm?event=viewDetail&imageID=2428


Chapter 1 Introduction


