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COMMUNICATION

Active motor proteins can couple cargo to the ends of growing microtubules

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Abstract
In living cells, dynamic microtubule ends interact with specialized protein complexes located on microtubule targets such as chromosomes and the cell cortex. A significant role in coupling microtubule ends to these complexes has been attributed to motor proteins, which are thought to provide a physical link while at the same time allowing for microtubule growth or shrinkage. In the past, motor-coated beads have been shown to be able to follow the ends of depolymerizing microtubules, in a direction opposite to their natural walking direction. Here we show that beads coated with plus-end-directed motors can also stay attached for several seconds to the ends of growing microtubules. Upon arrival at the microtubule end, fast-moving beads reduce their velocity to the microtubule growth velocity. We show that the tendency to stay attached depends on the initial bead velocity and that the microtubule growth velocity is unaffected by the presence of the bead.

Introduction

Molecular motors are proteins that use energy available from the hydrolysis of ATP to move actively along tracks provided by cytoskeletal polymers such as microtubules (MTs) and actin filaments [1]. MT motor proteins are found in all eukaryotic cells, where they perform a wide variety of cellular functions [2, 3] ranging from the transport of vesicles along the long axons of neuronal cells to controlling the spatial organization of complex MT networks such as the mitotic spindle [4]. In many situations, MTs play primarily a passive role and the force generating capabilities of motor proteins are sufficient to carry out these cellular functions. MTs are however inherently dynamic structures themselves that constantly switch between growing and shrinking states through a process termed dynamic instability [5]. In fact, molecular motors are often found in molecular complexes that interact specifically with the dynamic plus ends of MTs [6]. A clear example of this is the kinetochore, the specialized protein complex that takes care of the dynamic coupling between chromosomes and MTs in the mitotic spindle [7, 8], but similar complexes are also found at the cell cortex [9]. In these situations, the MT end and the motor proteins together form a sophisticated machine. Forces generated by both MT dynamics and the motor proteins appear to be needed to, for example, correctly position chromosomes during mitosis [10, 11].

The directed motion of motor proteins along stabilized MTs has been studied extensively *in vitro* over the last two decades [3]. This has resulted in extensive knowledge about the biophysical properties of the different classes of MT motor proteins, such as their directionality (plus- or minus-end directed), processivity, speed, force–velocity relation, etc. Few experiments, however, have focused on the behaviour of motor proteins at the ends of (dynamic) MTs. As a result, relatively little is known about the specificities of

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motor-mediated cargo-coupling to dynamic MT ends and about the molecular mechanisms that allow motors to stay attached to the ends of growing and shrinking MTs. A key set of experiments was performed by Lombillo *et al*, who studied the interaction of chromosome fragments and motor-coated microspheres with depolymerizing MTs [12, 13]. These studies showed that beads coated with multiple plus-end-directed motor proteins were able to stay attached to the ends of shrinking MTs, both in the presence and absence of ATP.

Here we investigate the kinesin-mediated coupling of cargo to the ends of growing MTs. Specifically, we show that beads coated with plus-end-directed kinesin motor proteins that move along the MT with speeds exceeding the MT growth velocity are able to stay attached to the ends of growing MTs, without affecting the growth velocity of the MTs. Since these experiments are performed under conditions where the dynamic instability of MTs occurs normally, we sometimes observe beads interacting with shrinking MTs as well.

Materials and methods

All reagents were purchased from Sigma, unless stated otherwise.

Microtubule nucleation seeds

Short stabilized biotin-labelled MTs, called seeds, were assembled in the presence of the non-hydrolyzable GTP-analogue GMPCPP (a gift from T J Mitchinson, Harvard Medical School, Boston, MA) essentially as described [14]: a solution of biotin-labelled tubulin (10 μM, Cytoskeleton, Inc, Denver, USA) and unlabelled tubulin (40 μM, Cytoskeleton) in 10 μl MRB80 buffer (pH 6.8, 80 mM K-PIPES, 1 mM EGTA, 4 mM $MgCl_2$) was mixed with 0.5 μl GMPCPP (10 mM) (the tubulin obtained from Cytoskeleton was first cleaned from remnants of glycerol and GTP using dilution in buffer and subsequent concentration using a Microcon 30 microconcentrator). This mixture was left for 45 min at 35 °C in order to grow seeds. A solution with a total volume of 50 μl containing tubulin (0.62 μM, Cytoskeleton), GMPCPP (0.4 mM) and 2 μl MT nucleation seeds in MRB80 buffer was prepared and stored in liquid nitrogen in aliquots of 2.5 μl. Before use 2.5 μl of this mixture was left for 10 min at 35 °C to grow short ($\sim$1 μm) unlabelled GMPCPP ends to the seeds. These unlabelled ends enhanced the nucleation efficiency of the seeds when they were attached to a streptavidin-coated surface.

Motor-coated beads

The motor protein we used was a genetically modified *Drosophilia* kinesin with a biotinylated domain [15]. The protein was expressed in *E. coli* and purified as described [15, 16]. The silica beads we used had a diameter of 1.25 μm (a gift from C van Kats of the Utrecht Colloid Facility). A solution of these beads ($4 \times 10^{-7}\mu$M) and streptavadin (42 μM) was left for 10 min at room temperature, rotating at 900 rpm by use of a vortexer. Since streptavadin was present in an excessive amount, we assumed that the beads were maximally covered with streptavadin. The unbound streptavadin was removed by one round of centrifugation and washing. To bind kinesin, 1 μl of streptavadin-coated beads was mixed with 5 μl biotinylated kinesin (5 μM) and left for 15 min at room temperature, rotating at 700 rpm.

Sample preparation

A surface with randomly located, strongly attached MT nucleation seeds was created by use of a flow cell. Vacuum grease lines were drawn along the long sides of the objective slide with 25-μm-thick metal wires as spacers to get a fixed volume of 25 μl. The microscope slide was coated before use with agarose (4 mg ml^{-1}) to prevent proteins from sticking to the glass. After flow cell assembly, the cover slip was coated with a layer of biotin-labelled BSA (2.5 mg ml^{-1} in sodium acetate buffer with pH 5.2, which is near the isoelectric point of BSA) and then a layer of streptavadin (10 μM in MRB80 buffer). 25 μl of seeds (100$\times$ diluted from stock) was introduced into the flow cell, which resulted in a few attached seeds per field of view of the microscope (35 μm $\times$ 26 μm). The flow cell was then filled with a solution containing 14–20 μM tubulin, variable amounts of GTP and ATP, an oxygen scavenging system [17], 0.8 mM free biotin, 10 mg ml^{-1} BSA, 10 mM phosphocreatine, 80 μg ml^{-1} phosphocreatine kinase, and kinesin-coated beads (1.3 μM). Biotin was added to block vacant streptavadin binding sites on either the beads or the coverslip. Afterwards the cell was closed at the open sides with hot candle wax. The levels of ATP (0, 0.0125, 0.025 or 1 mM) and GTP (0, 0.5 or 1 mM) were varied to control the walk velocity of the kinesin-coated beads. Because the chosen ATP concentration was sometimes very low, a phosphocreatine ATP-regenerating system was added to keep the ATP level constant. The experiments without GTP were done with taxol-stabilized microtubules, which were non-specifically bound over their full length to the surface. All samples were thermostated yielding a constant sample temperature of 23 °C.

Experimental set-up

Samples were observed with an inverted microscope equipped with DIC optics (Leica, DMIRB/E) and a 100$\times$ oil-immersion objective (numerical aperture 1.3). The images, acquired by a CCD camera (Kappa), were contrast enhanced (Argus, Hamamatsu), and taped with a video recorder (JVC). To place individual beads on MTs, we used a simple optical trap consisting of a laser beam (Spectra Physics, Nd:YVO_4, λ = 1064 nm) introduced via a dichroic mirror placed below the microscope objective. One of the two lenses forming a 1:1 telescope was mounted on an x, y, z stage outside the microscope to allow for manual manipulation of the trap position in the sample.

Data analysis

The video images were digitized at a rate of two images per second. A manual (mouse-clicking) position detection

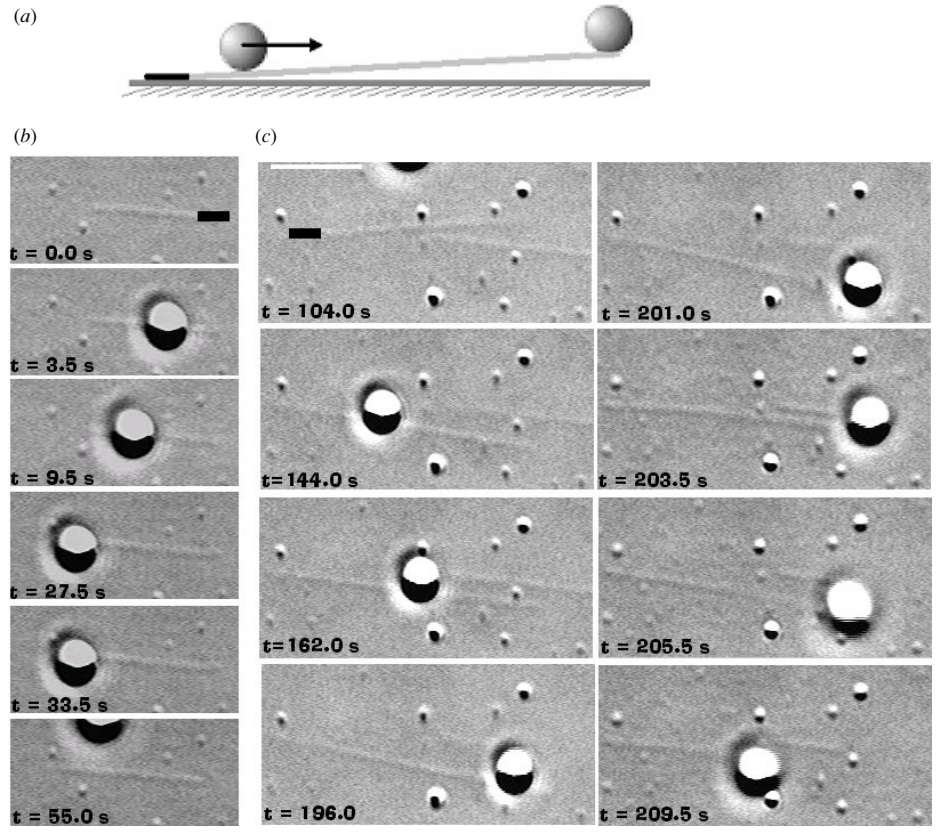

Figure 1. (*a*) Schematic picture of the *in vitro* assay. An MT nucleation seed (black) is attached to a coverslip, from which an MT (light grey) is growing that moves freely in solution. A silica bead (diameter 1.25 μm), coated with multiple kinesin molecules, walks towards the dynamic plus end of the MT. (*b*) and (*c*) DIC images from the *in vitro* assay. White scale bar equals 5 μm. (*b*) A silica bead is placed onto the growing MT with the optical trap (at 3.5 s), the trap is turned off and the bead starts walking towards the MT end. When the bead reaches the MT end, the bead stays attached for 6.0 s (from 27.5 s to 33.5 s). (*c*) A silica bead is placed on the growing MT with the optical trap (at 144.0 s), the trap is turned off and the bead starts walking towards the MT end. Just before the bead reaches the MT end, the MT switches from a growing to a shrinking phase (at 203.5 s). The bead encounters the end of the shortening MT and stays attached for 6.0 s (from 203.5 s to 209.5 s).

program (home-written) was used to measure the positions of the beads (with a precision of 0.08 μm; standard deviation over 3 traces of same run) and the positions of the—less clearly visible—MT ends (with a precision of 0.35 μm) over time. Individual walk velocities of beads and MT growth and shrinkage velocities (plus their errors) were obtained from single linear fits to position sequences (see, e.g., figure 2(*a*)). Average velocities were obtained by weighted averages of the individual velocities (weighted by the duration of the event). Averages are given together with the standard deviation. The behaviour of a freely diffusing bead could be clearly distinguished from a bead attached to an MT by eye. In this way, the moments of bead attachment and detachment to MTs could be determined with an accuracy of one image or 0.5 s.

Results and discussion

In order to investigate whether motor proteins can couple cargo to growing MTs, we studied the behaviour of beads coated with multiple kinesin molecules at the dynamic ends of MTs. For this purpose, the *in vitro* assay shown schematically in figure 1(*a*) was used. Dynamic MTs were allowed to grow from stabilized nucleation seeds, which were attached to a glass surface using biotin–streptavadin links. Glass beads, coated with kinesin molecules, were brought in contact with the seed-region of MTs using a simple optical trap. After turning off the trap, the beads walked towards the plus ends of the dynamic MTs, and their movement as well as the growth dynamics of the MTs was observed. In addition, a few experiments were performed with preformed taxol-stabilized MTs that were aspecifically attached to the surface along their whole length.

The MTs that were growing from the plus-ends of the seeds showed normal dynamic instability behaviour (almost no growth was observed from the minus-ends of the seeds). Their individual growth velocities ranged from 0.016 ± 0.004 μm s^{-1} (1.0 ± 0.3 μm min^{-1}) to 0.026 ± 0.003 μm s^{-1} (1.6 ± 0.2 μm min^{-1}) depending on the sample studied (see, e.g., figure 2(*a*)). The average catastrophe time (time between the initiation of growth and the abrupt switch to a shrinking state) of these MTs was 490 ± 100 s ($n = 26$), and no rescues were observed. These dynamics are comparable to data reported earlier [14].

We varied the velocity of the beads towards the MT ends by changing the concentration of nucleotides present in the

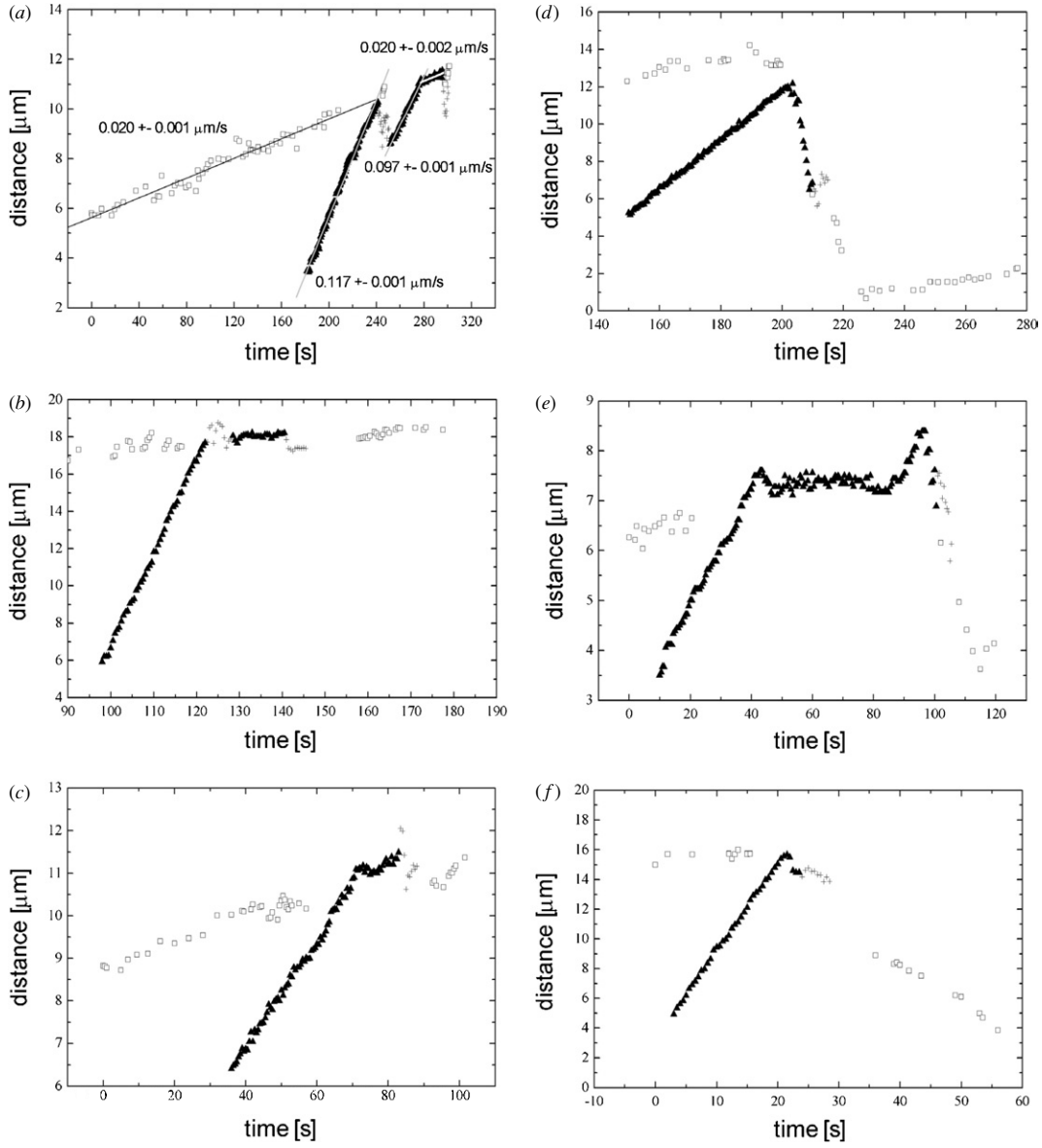

Figure 2. Bead and MT dynamics. The positions of the beads (closed triangles when attached to the MT; crosses when diffusing freely) and the ends of the MTs (open squares) relative to the MT nucleation seeds are plotted as a function of time. Some beads stay attached to the ends of growing MTs ((*a*), (*b*), (*c*)) and other beads stay attached to the ends of shrinking MTs ((*d*), (*e*), (*f*)). (*a*) The bead moves with velocity $v_B = 0.117 \pm 0.001\ \mu m\ s^{-1}$, arrives at the end of the growing MT ($v_{MT} = 0.020 \pm 0.001\ \mu m\ s^{-1}$), detaches (at 241.5 s), diffuses and attaches to the MT again (at 251.5 s). When it arrives again at the end (at 278.0 s), it stays attached to the end of the growing MT for 7.0 s. During this time the bead velocity is $0.020 \pm 0.002\ \mu m\ s^{-1}$. (*b*) The bead arrives at the end of the growing MT ($v_B = 0.505 \pm 0.003\ \mu m\ s^{-1}$), detaches (at 122.0 s), diffuses and attaches to the end of the MT again (at 128.5 s). The bead stays attached to the end of the growing MT for 12.0 s. (*c*) The bead arrives at the end of the growing MT (at 71.5 s; $v_B = 0.134 \pm 0.002\ \mu m\ s^{-1}$) and stays attached for 11.5 s. (*d*) The bead encounters the end of a shortening MT (at 203.5 s; $v_B = 0.132 \pm 0.001\ \mu m\ s^{-1}$) and is pulled in the minus end direction for 5.5 s over 5.7 μm. (*e*) The bead arrives in the vicinity of the MT end (at 40.0 s; $v_B = 0.121 \pm 0.002\ \mu m\ s^{-1}$), sits still for 41.5 s and then starts walking again. It reaches the end of the MT, which starts to shrink at that time. The bead is pulled along in the minus-end direction over 1.5 μm. (*f*) The bead arrives at the end of the MT (at 21.5 s; $v_B = 0.586 \pm 0.003\ \mu m\ s^{-1}$), which starts to shrink. The bead is pulled in the minus-end direction over an apparent 1.2 μm. Nucleotide concentrations: (*a*) [ATP], [GTP] = 0 mM, 0.5 mM, ((*b*), (*f*)) 1 mM, 1 mM, ((*c*), (*d*), (*e*)) 0 mM, 1 mM.

sample. In most cases the samples contained both GTP, which is necessary for tubulin polymerization, and ATP. ATP is normally used as a source of energy in kinesin motility assays [3], but kinesin is also able to move by hydrolyzing GTP, albeit less efficiently [18]. Beads walking along MTs in the presence of 1 mM ATP alone (along taxol-stabilized MTs) moved with an average velocity of $0.78 \pm 0.10\ \mu m\ s^{-1}$ ($n = 17$; ±SD), which is comparable to previous results [3]. With only GTP present the average walk velocity was much lower ($0.14 \pm 0.03\ \mu m\ s^{-1}$, $n = 36$ for 1 mM GTP and 0.085 ± 0.014, $n = 43$ for 0.5 mM GTP), as was previously observed [19, 20]. Intermediate velocities were obtained by

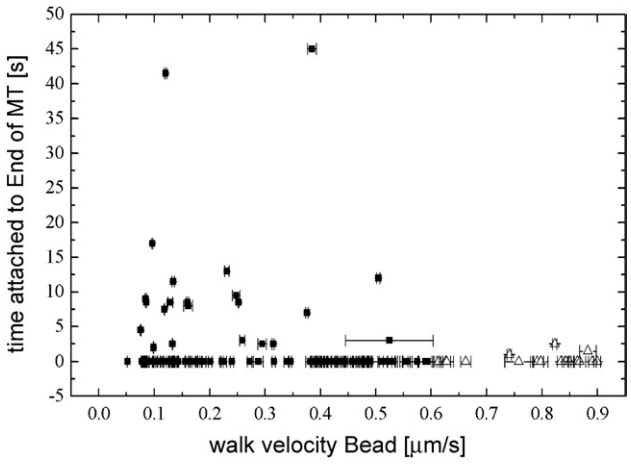

Figure 3. Relation between the time of bead-attachment to a growing MT end and the initial walk velocity of the bead (squares). The initial bead velocity was varied by changing the combination of [ATP] and [GTP] present. The data indicated with open triangles were taken in the absence of GTP with taxol-stabilized MTs. All data are shown (total of 148 events), where $t = 0$ s is plotted for beads that detached immediately upon arrival at the end of the growing MT. The figure shows that attachment of beads to the ends of growing MTs can occur under all conditions, but that it occurs more often at low bead velocities.

adding different amounts of ATP to samples containing 1 mM GTP. Note that when both 1 mM ATP and 1 mM GTP were present in the sample, the beads moved with a lower average velocity (0.46 ± 0.04 μm s^{-1}; $n = 68$) than expected from a simple competitive Michaelis–Menten model for the hydrolysis of both ATP and GTP, judging from the published data for $V_{\max}$ and K_{M} [19, 21].

Most beads (192 out of 213) walked all the way to the MT end without detaching from the MT, corresponding to a typical run length exceeding 10 μm (figures 1(*b*) and (*c*)). The other 21 beads detached before they reached the end of the microtubule. This implies that multiple kinesin molecules were interacting with the MT, since single kinesin molecules only have run-lengths of about 1 μm [3]. When a bead arrived at the growing plus end of an MT, several types of behaviour were observed (figures 1 and 2). Most beads that encountered a growing plus end (109 out of 136) detached from the MT immediately after reaching the end (judged from a sudden change in Brownian motion, see methods). However, a significant fraction of the beads (22 out of 136) reduced their walk velocity abruptly upon arrival at the MT plus end (figures 1(*b*) and 2(*a*)–(*c*)) and prolonged their attachment for several seconds. This shows that active motor proteins can couple cargo to the ends of growing MTs. Beads seemed more likely to stay attached when the walk velocity of the beads before reaching the MT end was low (see figure 3): below 0.3 μm min^{-1} 16 out of 69 beads (23%) stayed attached, whereas above 0.3 μm min^{-1} only 4 out of 60 (7%) stayed attached (excluding the two longest events, see below). The average attachment time ($\pm$SD) for all 20 beads was 7 ± 4 s. Figure 3 also shows that 3 out of 17 fast-moving beads reaching the ends of taxol-stabilized MTs stayed attached for 1 or 2 s. Since the velocities in figure 3 were varied by changing the nucleotide composition, it is in principle possible that the nucleotide composition determines how likely the beads are to stay attached to MT ends. We cannot strictly distinguish between these two possibilities, but it is clear that MT end-attachment is possible under all circumstances: with both ATP and GTP present and with one of them absent.

The growth velocity of an MT did not seem to be affected by the presence of a bead at its end. The ratio between the velocity of the bead while it was attached to the MT end and the MT growth velocity before the bead arrived at the tip (which could be measured for 15 events) was on average 1.01 ± 0.08 ($\pm$SE). In a few cases (5 out of 136), an MT experienced a catastrophe apparently at the exact moment that the bead arrived at its end (catastrophes were, however, never observed during prolonged bead attachment to the end of a growing MT). In addition, a walking bead sometimes encountered an already disassembling MT (19 events). In both situations beads either detached immediately or stayed attached to the ends of the shortening MTs for a few micrometres (8 out of 24 events, figures 1(*c*) and 2(*d*)–(*f*)). It is interesting to note that in figure 2(*d*), the velocity of the bead that is attached to a shrinking MT (1.03 μm s^{-1} or 62 μm min^{-1}) appears much faster than the normal shrinkage velocity observed under these conditions ($\sim$18 μm min^{-1}), an effect that was predicted by Peskin and Oster [22] and previously observed for a different motor protein by Lombillo *et al* [12].

Our observations confirm that plus-end-directed motor proteins can couple cargo to the ends of growing MTs, just as was previously shown for shrinking MTs. Our data give us no reason to believe that the presence of a motor-coated bead affects the growth velocity of an MT. In one exceptional case, we observed that a bead reduced its velocity to zero apparently upon reaching the end of the MT (figure 2(*e*)). After some time the bead started walking again at its original velocity, suggesting that the bead had simply stayed behind, possibly because the MT that formed in front of it did not immediately assemble into a closed cylinder but formed an open sheet first [23]. This special situation may have caused the exceptionally long apparent end-attachment time of this bead (more than 40 s, see figure 3), and quite likely there is a similar reason for the second long event.

A key mechanistic question is whether individual motors, upon reaching the MT end, actually slow down the kinetics of their attachment/detachment cycle to keep up with MT growth. This could be the case if the detachment probability of individual motor heads is lowered at MT ends, which has in fact been postulated before [24]. It would explain why motor proteins that are turning over nucleotides fast (corresponding to fast initial motor velocities) have a harder time staying attached, since their intrinsic detachment rate will be higher. Another possibility is that the motors keep the same attachment/detachment cycle but that the attachment to the MT is 'handed over' from one motor to the next, which should result in a backwards rotation of the bead and a reduced bead velocity compared to the individual motor velocity. Again, this could be consistent with fast-moving beads falling of more easily, since they would have to rotate the bead backwards faster.

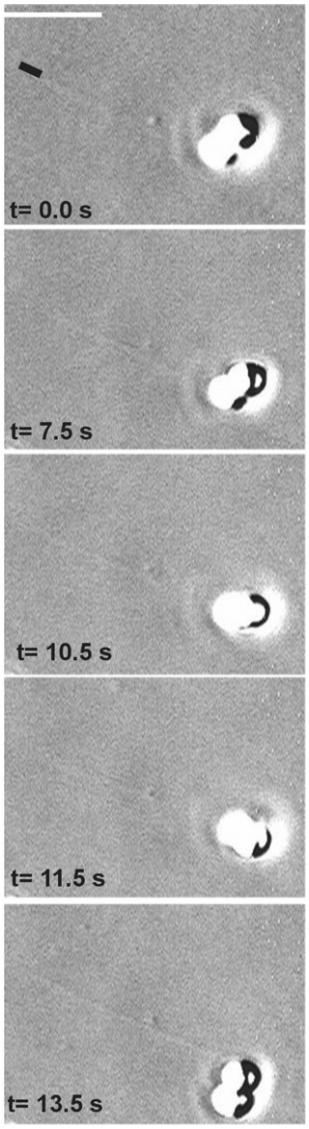

Figure 4. A double bead walking towards the end of a growing MT. Scale bar equals 5 μm. An MT is growing from an MT nucleation seed (black). One bead walks to the end of the MT, with another bead attached to it. At the end of the MT the walking bead rolls over perpendicular to the MT, which is detected by the motion of the second bead.

To distinguish between these two mechanistic possibilities, one may try to use asymmetric beads or beads with a marker on them to follow the potential rotational motion of the bead. How this may work is illustrated in figure 4, where a double instead of a single bead is observed to move along the microtubule lattice. The relative position of the second bead is not changing during the majority of the run suggesting that a (small) number of motors are transporting the cargo along always the same protofilament(s). When the beads reach the MT end, the second bead rolls over to the side before both beads detach completely (which in this case happened immediately, before any backwards rotation could be detected). The rolling over to the side may be due to the motor proteins switching to (a) different protofilament(s), the detachment of some motors leading to different steric constraints on the bead, or a change in MT structure near the tip.

In conclusion, we have presented an *in vitro* assay in which we investigate cargo-coupling by motor proteins to MT ends that are undergoing their natural dynamic behaviour. For the particular motor protein studied (kinesin), we find that beads can stay attached to the ends of growing MTs, although not for very long times. This attachment capability seems dependent on the speed of the hydrolysis cycle of the motor proteins since at low nucleotide concentrations, or unfavourable combinations of nucleotides, the tendency to stay attached is higher.

In the future, it will be interesting to study the coupling capability of motor proteins that are known to localize to relevant structures such as kinetochores [7, 8] or that are known to specifically interact with MT ends [6, 25]. The assay presented here can be used for these purposes, where the use of optical tweezers may be extended to apply forces to end-attached beads. Alternatively, one may study MT end interactions with static barriers coated with relevant (motor) proteins in an assay similar to the one that has been used to study MT pushing forces [14, 26, 27]. Our results may be of interest not only for motor proteins involved in coupling macroscopic cargo to dynamic MTs, but also to situations where motor proteins are involved in the targeting of regulatory proteins to the ends of growing MTs. For example, in both budding [28] and fission [29] yeast, kinesin-like proteins have been shown to be involved in the transport and subsequent MT-end-tracking of proteins involved in the regulation of MT organization.

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