D2.3: Demonstration of force measurement on single molecule systems using optical tweezers

Nature: Report

Dissemination level: Public

Lead Beneficiary:	UNIVDUN		
Author(s):	Q. Tyrell Davis		
Work Package:	WP2	**Task:**	ESR6
Version:	1:00	**Last modified**	April 19 2016
Status:	Released		
Approved by:	Kees Weijer	**Date**	April 19 2016

Disclaimer:

Copyright Notice:

Table of Contents

1 Introduction

1.1 Single-molecule biophysics

Life is built out of structured nanomolecular machines: the biological macromolecules performing catalysis, storing information, and forming the mechanics of the cell. Optical microscopy has allowed the curious to investigate high-level outcomes of this activity for centuries, but much of the fundamental details of these intricate machines remain obscured by the diffraction limit. Imaging can be used to determine a molecule's general locality, but does not tell us anything about its mechanics in general. Investigating the fundamental building blocks of life on an individual basis requires circumventing some of the very challenges faced by the macromolecules themselves.

Ernst Schrödinger's seminal venture into biology *What is Life?* [8] describes the difficulty of precisely measuring extremely small forces. To relate an example from Schrödinger, the deflection of an extremely thin needle can serve to measure small forces, but as the magnitude of the measurements decrease the mass of the needle must also, until the instrument's inertia is so slight that it reacts strongly to the thermal noise of the system. In this way Brownian motion begins to dominate the signal of a sufficiently sensitive force measurement.

Instrumentation in low-force regimes, down to the measurement of single molecules, lends insight to the environment experienced by the macromolecules. The same thermal noise that dominates the signal from a sufficiently sensitive instrument will also be felt by the single molecules comprising the structure of life. The physics of biology occur in wet and warm conditions, typically at room or body temperature, and biological macromolecules must work in a mechanically noisy environment, continuously buffeted at all sides by thermal motion.

How does the intricate biological machinery of life, comprised of flexible macromolecules of scripted chains, manage to extract order in so much chaos? How can a single molecule in a noisy, analogue, environment make discrete decisions? These are the fundamental questions in biology investigated by single-molecule biophysics. Indeed, the continuous exportation of entropy is a necessary (though not sufficient) prerequisite for life to occur.

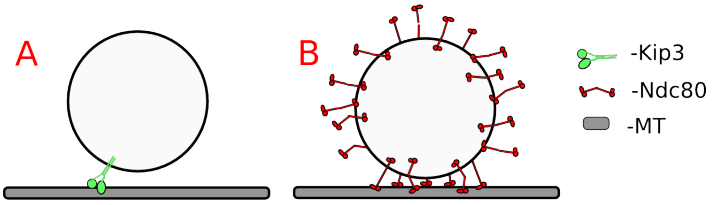


Fig. 1: **Single-molecule and small-cohort experiments**
Examples of single molecule and small-cohort experiments. **(A)** Kip3, in green, is a highly processive molecular motor, capable of maintaining an association with a microtubule while travelling for several micrometers [7]. **(B)** is a cartoon of conditions described in [9], requiring an estimated 7 available Ndc80 complexes to maintain an association to a microtubule.

1.2 Defining and validating single-molecule biophysics

Single-molecule experiments allow researchers to investigate the fundamental activity of biological building blocks that give rise to life processes. Unlike ensemble or small- cohort measurements, single-molecule investigations may measure discrete and transient events, such as steps, slipping, or conformational changes. Although the definition of single-molecule measurements is self-explanatory, there can be some confusion, particularly when the techniques are applied across a broad variety of disciplines. In a single-molecule measurement, the discrete contribution of an individual molecule must be resolved from that of its partners. The cartoon in Figure 1 gives examples of both single-molecule and small-cohort experiments.

In many cases, reconstituting life-like conditions for in vitro assays actually requires small-cohort conditions. For example, the Ndc80 complex, a component of the outer kinetochore made up of 4 proteins that mediates connections to microtubule (MTs), reportedly requires at least 7 Ndc80 complexes to negotiate a measurable attachment to an MT. Optical tweezers and total interference reflection fluorescence (TIRF) microscopy experiments show that lone Ndc80 complexes loses the ability to maintain a connection to a microtubule. Therefore, cooperation between multiple Ndc80 complexes is required to overcome random motion.

To determine single-molecule conditions, one must estimate the number of molecules expected at the location of interest and validate the low-density of active molecules being measured. Therefore tuning a single-molecule experiment entails guess- and-check methodology. In practice this means that in a single-molecule optical trapping experiment, most of the beads will not be active [2].

The directed stepping motility of a kinesin motor protein is a classic example of a single-molecule measurement. Dimers of conventional kinesin undergo directed 8nm steps (corresponding to the size of a tubulin subunit) on microtubules, and these steps can be identified above the thermal noise of an experimental system.

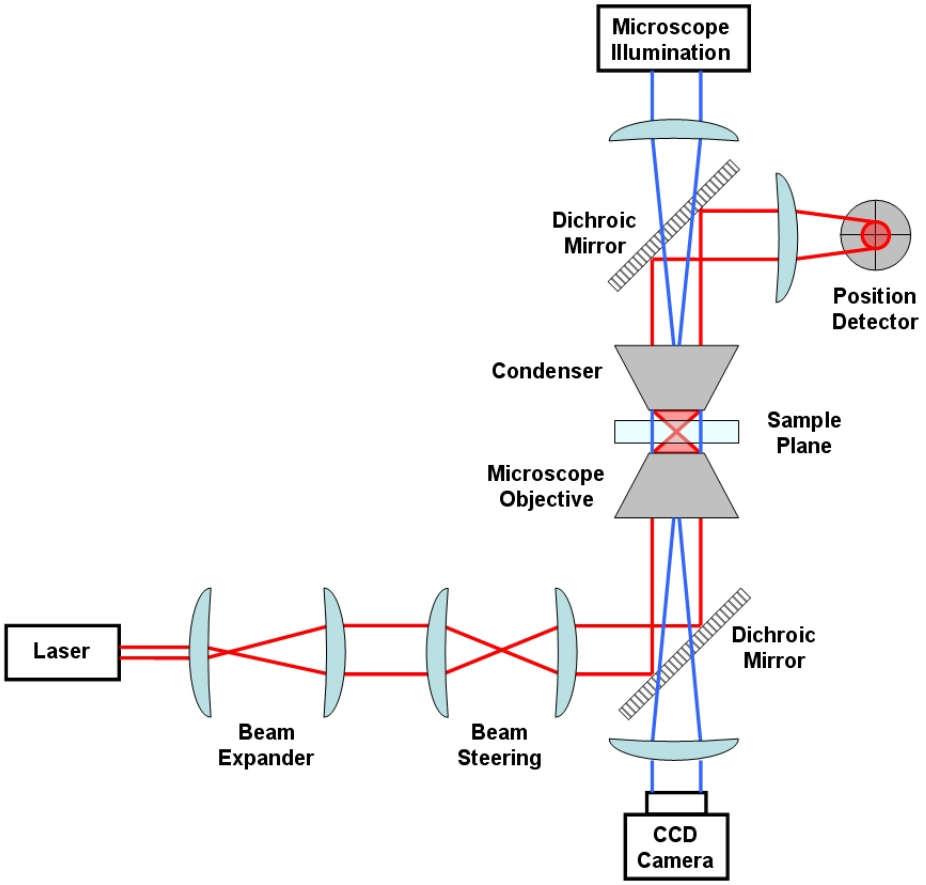


Fig. 2: **Cartoon of a typical optical tweezers setup.**
In a typical optical trap, a laser is focused by a high numerical aperture microscope objective, which constrains the motion of dielectric particles such as polystyrene beads. The trapping laser is then collected, partially scattered by any trapped objects, and the back focal plane of the collecting objective (condenser in this cartoon) is imaged onto a position detector such as a QPD. Image courtesy of Wikipedia user RockyRaccoon (Public Domain).

1.3 Optical Tweezers

Optical tweezers leverage the momentum of light to exert force, and by measuring the deflection of the trapping beam this force can be quantified. In the purest treatment, the deflection of the light beam can be considered a direct measurement of the change in momentum of the light, and thus the force imparted to the experimental system[10]. In practice, light is typically detected on a quadrant photodiode (QPD, ”position detector” in Figure 2), and converted to a voltage corresponding to displacement on an x,y coordinate system, and these x,y voltages must be calibrated to a physical displacement of the trapped object (Equation 1, V_n are voltage values from each of the four photodiode quadrants). Those values are in turn calibrated to yield force values in a region where the trapping potential functions as a Hookean spring, that is, the displacement of the trapped object is linearly proportional to the returning force [12].

$$V_x = V_A + V_B - V_C - V_D$$

$$V_y = V_A - V_B + V_C - V_D$$

$$V_z = V_y + V_x \tag{1}$$

It is worth noting that the linearity of the relationship between the raw voltage signal and the force exerted by the trap extends beyond the region for which the same is true of the displacement in relation to the voltage signal [10, 14]. The relationship between the QPD voltage and actual displacement is only linear over a short range of a few 100s of nanometres, but the relationship between the QPD voltages and force is often linear over a much longer range of about a micron, depending on bead size. As a result, the linear relationship between the product (α in pN/V) of the displacement calibration constant (typically β, in nm/V) and the calibration constant for force (κ, in pN/nm) is valid over a comparatively large distance from the trap centre [14].

$$F_x = \kappa[\frac{pN}{nm}] \cdot \beta[\frac{nm}{V}] \cdot V_x = \alpha[\frac{pN}{V}] \cdot V_x \tag{2}$$

Optical tweezers can therefore be considered primarily a force measurement and manipulation technique, and secondly a technique for measuring displacement. The scales that optical tweezers operate at are well-suited to the forces and displacements of active biological macromolecules, on the order of picoNewtons and nanometres [11, 2]. A cartoon of a typical basic optical tweezers setup is shown in Figure 2.

1.4 Kip3, a kinesin-8 motor protein

Kip3 is described as a highly-processive, weak molecular motor [7]. Unlike conventional kinesin (kinesin-1 family proteins), Kip3 probably does not move cargo throughout the cell, but is instead an important regulator of microtubule dynamics. Kip3 catalyses depolymerisation of microtubules in a density dependent manner: an accumulation of Kip3 at microtubule tips results in increased MT depolymerisation [4]. This is thought to be mediated by the increased lateral surface area of larger MTs: providing increased opportunities for Kip3 to bind, allowing them to subsequently travel to MT tips and catalyse depolymerisation once there.

2 Methods

2.1 Polystyrene bead functionalisation

Typical biophysics experiments require an optically trappable handle for manipulating single biological macromolecules. For many experiments, including the one described here, functionalised spherical micro-beads are suitable. The functionalisation used here is based on anti-eGFP antibodies, and so the beads can be used with a variety of proteins of interests that contain this tag in the desired orientation. The main aim of this small experiment was to determine the feasibility of omitting a polymer linker to decrease latency between bead motion and protein activity. I used stage motion to simulate 30 nm steps, and compared the stage displacement to the displacement measured at the bead in Figure 3.

The protocol we used to functionalise beads is as described in [8], with the exception that heterobifunctional PEG, containing a COOH group for binding antibodies, is omitted. Insted, functionalisation relies on a competition between monofunctional PEG (containing no carboxyl group for further binding of antibodies) and the antibodies themselves for pre-existing carboxyl groups on the microsphere surface. Heterobifunctional PEG can span about a hundred nanometres when stretched, and coupling antibodies directly to the bead surface presumedly would reduce the latency in the bead's experience of protein motion. Kip3 is a very weak motor protein (stalling at forces of about 1 pN or less[7, 3]), and it is difficult to resolve steps in this low force regime.

Omitting heterobifunctional PEG limits the total number of molecules of interest per bead to the availability of available antibodies bound to the bead surface, and so is not particularly sensitive to protein dilution (as compared to the original protocol). This makes it easier to reach single-molecule conditions, but may result in unwanted free protein in the experiment. In the case of Kip3, which works cooperatively at MT ends to depolymerise MTs, this is an unwanted hindrance that would hinder investigations into the MT-regulating activity of a single protein.

The modified protocol for bead functionalisation, based on[13], can be found in the appendix of this document.

2.2 ASWAD optical tweezers system

Experiments with Kip3 were conducted with ASWAD, a custom optical tweezers system for biophysics experiments. It employs a single optical trapping laser with force and displacement signals detected on a QPD according to back focal plane interferometry described in [12] and video-enhanced differential interference contrast (VE-DIC) with LED illumination [15]. Actuated mirrors can be used to maintain functionalised beads at clamped trap positions (and thus maintain desired forces) in 2 or 3 dimensions. ASWAD is named for the pattern of keys on the left-hand side of a typical QWERTY keyboard, and has been described previously [5].

2.3 Force/displacement calibration

To convert intensity signals in volts to useful data describing displacement and force, the trapped bead-fluid medium system can be perturbed in a number of ways to probe

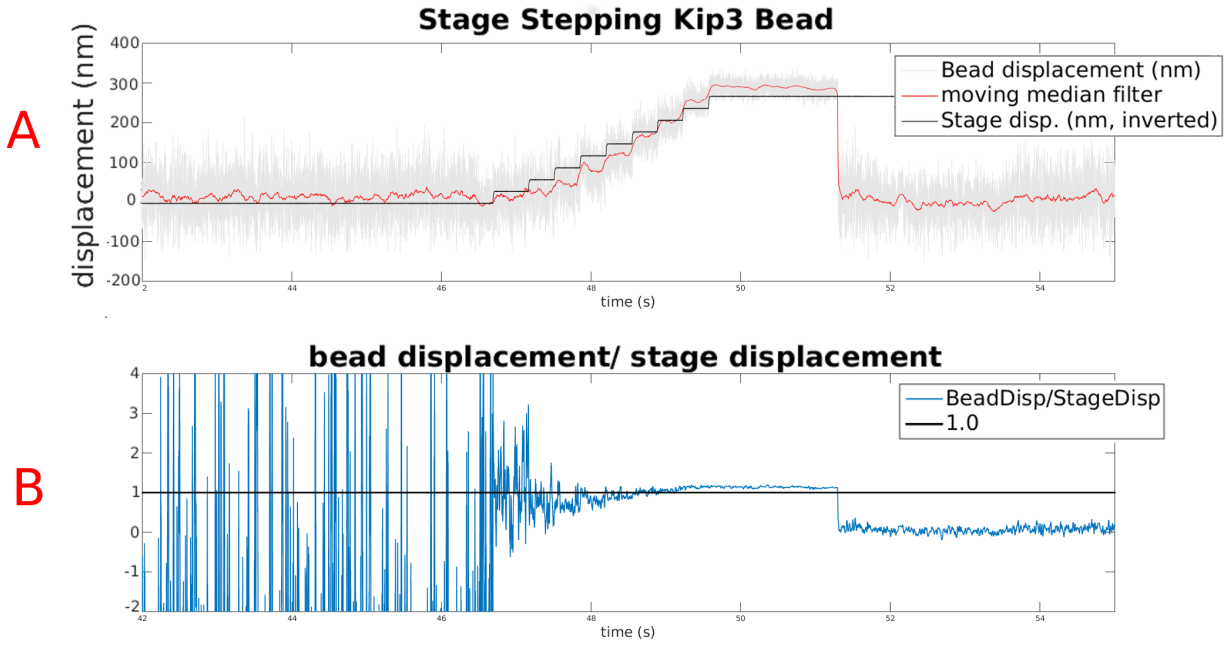


Fig. 3: **Comparison of measured bead displacement to stage actuation.** **(A)** These measurements are of a bead coupled to a coverslip-attached microtubule via a Kip3 motor protein. This figure corresponds to a stationary optical trap restraining the bead relative to an actuated stage. **(B)** Comparison of the measured bead displacement to stage displacement highlights the breakdown in linearity of the displacement calibration factor β at some distance from the centre of the trap. Below a displacement of about 175 nm, bead displacement is underestimated. Further than about 175 nm from the centre of the trap measured bead displacement is an overestimate. The discrepancy between the bead- measured displacement and the measured stage displacement (lower blue trace) is likely due to a combination of the stretched connection of the bead to the microtubule via antibody and Kip3 protein, and the nonlinearity of the voltage-displacement relationship further from the optical trap centre. The blue trace in **(B)** is a unitless ratio of $[\frac{nm}{nm}]$, while **(A)** is the raw (gray) and median-filtered (red) QPD signals calibrated in $[nm]$

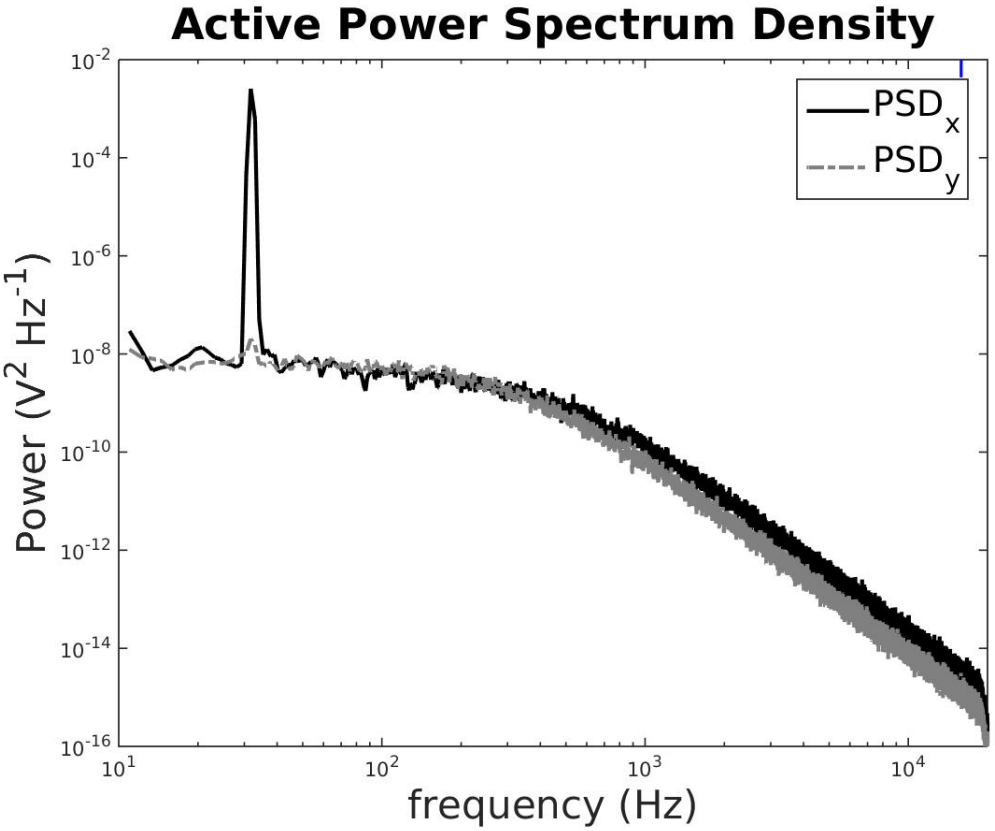


Fig. 4: **Active power spectrum density of a polystyrene bead.**
A piezo stage is used to actuate the sample chamber with a motion of 975 nm, visible in the spike at 32 Hz in the PSD. The relationship between the power content of the spike and the flat portion of the PSD yields the calibration factor for displacement, , and the frequency at the PSD elbow describes the trap stiffness. For more accurate results, a PSD for each individual bead should be considered. This PSD corresponds to the bead used for motility events presented in Figure 5.

the effects of Brownian motion and viscous drag on the bead. For the Kip3 experiments described here, an active measurement of power spectrum density (PSD, Figure 4) provided a means of ascertaining Brownian motion damping by the optical trap. A sinusoidal motion of the experiment chamber was actuated by a piezo stage to probe the drag forces acting on the bead as described in [6]. The PSD in Figure 4 is the calibration PSD for the bead used to record the subsequent motility events in Figure 5 and 6 (but a different bead than used to compare bead-measured and stage displacement in Figure 3).

The key elements of the active PSD (Figure 4) are the corner frequency f_c and the relationship between the power contained in the spike and the damped plateau of the spectrum (measurements contained in $W_e x$).

$$\beta = \sqrt{\frac{W_{th}}{W_{ex}}}$$

$$W_{th} = \frac{A^2}{2(1 + \frac{f_c^2}{f^2})} \tag{3}$$

Where A is the displacement amplitude of the stage, driven by a sine wave function. f_c is the corner frequency of a best-fit to a Lorentzian function of the form $\frac{D}{\pi^2(f^2+f_c^2)}$ (where D is the diffusion coefficient). W_ex is the power (V^2) measured in the spike above the power at the same position interpolated from the surrounding. To find the PSD corner frequency I used custom software written in Python by Steve Simmert, Universität Tübingen (not yet published). This software accounts for hydrodynamic effects to fit a Lorentzian function to the measured PSD, yielding the corner frequency. I computed W_{ex} and W_{th} manually in MATLAB according to [6].

3 Results

3.1 Summary

In 4 experiments a total of 55 functionalised beads were tested for MT interaction. Of these, 38 beads showed no discernible interaction with microtubules. The remaining beads appeared to have some interaction with MTs, but in some cases this may have been do to non-specific surface interactions and crowding. Upon closer inspection of the QPD voltage traces, 3 beads showed binding to MTs, verified by either stretching the Kip3 molecule via artificial, stage-generated steps (Figure 3) or motility, and of these 1 showed repeated runs of motility with a stall force of about 1 pN (Figure 5). A few (2-3) promising traces were not saved and at least 6 beads were lost from the trap before they could be measured. In a fifth experiment I used functionalised liquid crystal birefringent beads (14 additional beads) but was not able to discern any clear instances of motility.

The low number of beads generating any discernible interaction with MTs putatively validates that active Kip3 was present on the beads in single-molecule concentrations. In addition, visible interactions with microtubules occurred with a similar frequency regardless of Kip3 dilution of 10X to 1000X, which does not disagree with a limiting number of antibody binding sites.

Date	Bead Dia.	Interacting/total	Comments
2016/01/19	(590 nm)	8/20	Did not save QPD voltage traces
2016/01/20	(590 nm)	4/12	2 MT-connections supported tension
2016/01/27	(590 nm)	4/13	Bead B012 showed motility
2016/01/27	(320 nm)	1/10	Some questionable association with MTs

3.2 Force measurements

Of the 55 beads tested, only 1 showed motility (out of 3 showing reliable interaction with microtubules upon close inspection). In each motility run Kip3 pulls the bead away from the centre of the trap until motor activity stalls at about 1 pN, near literature-reported values [7, 3]. After stalling, the bead/Kip3 system sometimes showed backward slippage before contact was totally lost and the bead returned to the centre of the trap. These motility events occurred three times during data capture and are shown in Figure 5. When viewed next to the stage traces (Figure 6), we can see that the motility events in Figure 5 are unaccompanied by stage actuation.

I also made displacement measurements of artificial steps generated with a piezo stage, displayed in Figure 3 These steps are larger (30 nm) than the 8 nm forward steps of kinesin (corresponding to the length of a tubulin subunit). A closer look at the QPD signal associated with these stage movements reveals that the first few steps are not readily visible above noise. Individual steps only become visible over thermal motion when the bead-antibody-Kip3-microtubule link is under tension, constraining Brownian motion. If we look at the ratio of the bead displacement measurement to the stage displacement, we see that the position tends to be slightly underestimated below $\sim$ 175 nm and overestimated at values above 175 nm. This may be due to a combination of additional stretching of the bead-antibody-Kip3 connection to the microtubule, and the

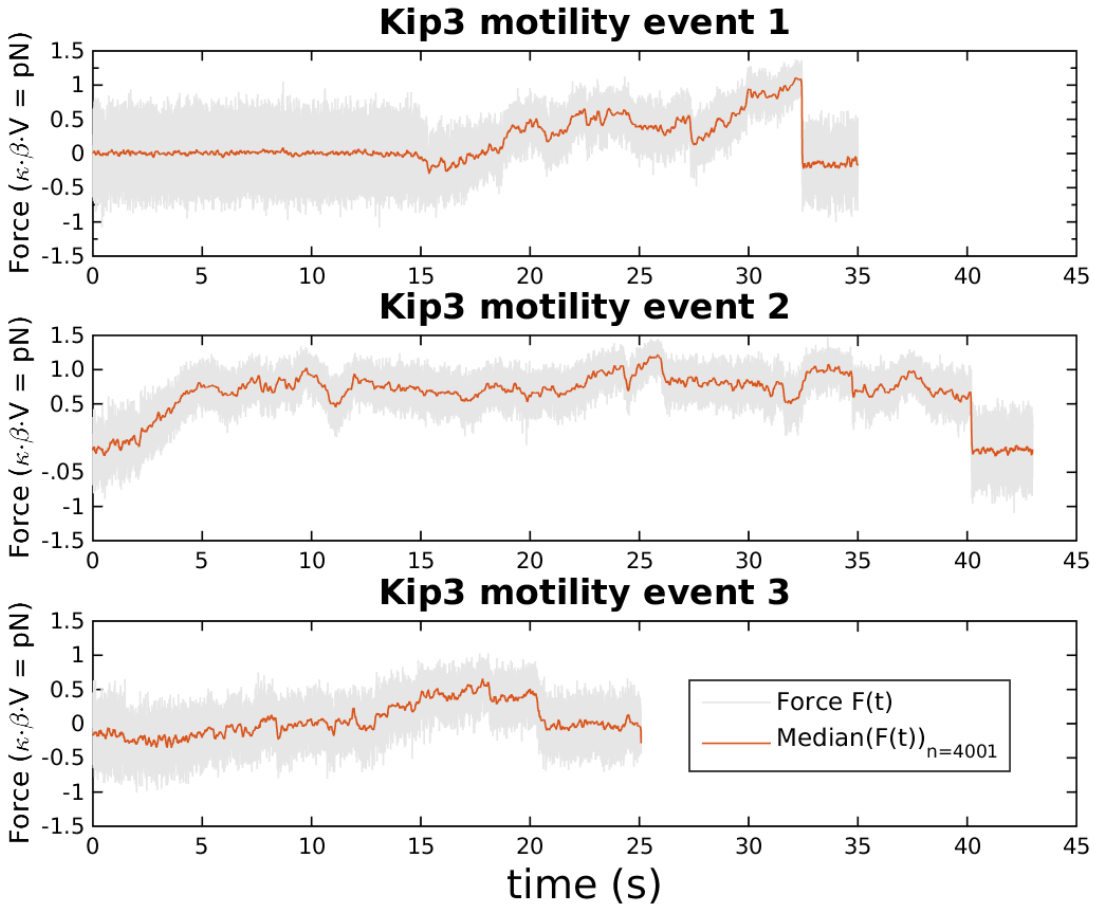
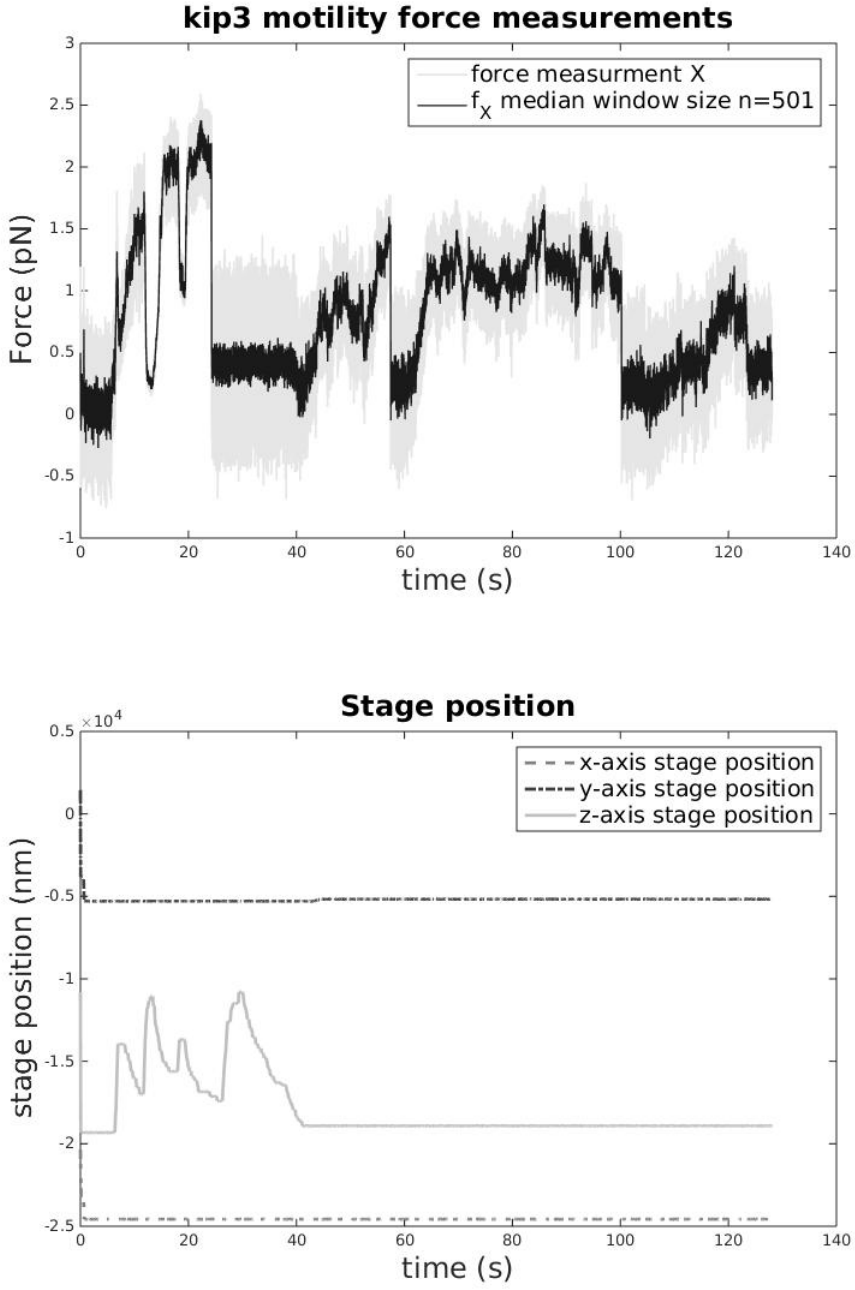


Fig. 5: **Force measurements for 3 Kip3 motility events.**
Force/displacement measurements of 3 subsequent Kip3 motility events on the same microtubule and bead. In each case, Kip3 motility pulls a bead out of the centre of the optical trap until stalling at a force of $\sim$ 1.0 pN. Some slippage seems to occur during stalling, before Kip3 finally loses the connection to the MT and the bead returns to the centre of the optical trap. In this low force regime, individual steps are occasionally visible in the median-filtered (*e.g.* just before the 15s mark during motility event 3.



ht

Fig. 6: **Motility validation.**
Comparison of measured bead motility to stage movements. Force experienced by the bead on the left hand of the trace is due to z-axis actuation of the piezo stage, as is apparent in the stage monitor traces in the lower figure. During the 3 motility events that follow (displayed individually in Figure 4), the stage remains stationary.

nonlinear relationship between displacement and back focal plane intensity beyond a limited range, which we discussed in Section 1.3.

4 Conclusion

Motor proteins such as the non-conventional kinesins play important roles beyond intracellular cargo transport. For example, Kip3 plays an interesting role in regulating MT dynamics[4]. The $\sim$8 nm steps of a strong motor such as a conventional kinesin-1 (exerting forces of several pNs) is apparent above Brownian motion as tension constrains bead motion. Many important activities in the cell, such as the individual steps of small sideward movements Kip3 may use to facilitate MT depolymerisation at the MT ends [3, 4], are more difficult to resolve with current techniques. A primary objective of these experiments was to determine the efficacy of direct coupling of antibodies to the COOH groups on the bead surfaces, omitting a heterobifunctional 3 kDa PEG linker. Although the experiment did yield Kip3 motility, this modified bead functionalisation hinders titration of the number of active molecules on a bead. A likely byproduct is an unknown number of free Kip3 molecules in the reaction mix. Further optimisation of the ratio of monofunctional PEG to antibodies is needed if this approach is to prove useful in future experiments, elucidating the intricate mechanics of MT depolymerisation by Kip3.

References

[1] Svoboda, K. & Block, S. M. Force and velocity measured for single kinesin molecules. Cell 77, 773-784 (1994).

[2] Svoboda, K., Schmidt, C. F., Schnapp, B. J. & Block, S. M. Direct observation of kinesin stepping by optical trapping interferometry. Nature 365, 721-727 (1993).

[3] Schäffer, E., Bugiel, M. & Bo, E. Article The Kinesin-8 Kip3 Switches Protofilaments in a Sideward Random Walk Asymmetrically Biased by Force. 108, (2015).

[4] Varga, V. et al. Yeast kinesin-8 depolymerizes microtubules in a length-dependent manner. Nat. Cell Biol. 8, 957-962 (2006).

[5] Mahamdeh, M. & Sch, E. Optical tweezers with millikelvin precision of temperature-controlled objectives and base-pair resolution. 17, 17190-17199 (2009).

[6] Tolic-Norrelykke, S. F. et al. Calibration of optical tweezers with positional detection in the back focal plane. Rev. Sci. Instrum. 77, 103101 (2006).

[7] Jannasch, A., Bormuth, V., Storch, M., Howard, J. & Schäffer, E. Kinesin-8 is a low-force motor protein with a weakly bound slip state. Biophys. J. 104, 2456-2464 (2013).

[8] Schrödinger, E. What is life? The physical aspect of the living cell. Trinity College viii, 91 p. (1944). doi:10.1002/ajpa.1330050116

[9] Powers, A. F. et al. The Ndc80 Kinetochore Complex Forms Load-Bearing Attachments to Dynamic Microtubule Tips via Biased Diffusion. Cell 136, 865-875 (2009).

[10] Farré, A. & Montes-Usategui, M. A force detection technique for single-beam optical traps based on direct measurement of light momentum changes. Opt. Express 18, 11955-11968 (2010).

[11] Bowman, R. W. & Padgett, M. J. Optical trapping and binding. Rep. Prog. Phys. 76, 026401 (2013).

[12] Gittes, F. & Schmidt, C. F. Interference model for back-focal-plane displacement detection in optical tweezers. Opt. Lett. 23, 7-9 (1998).

[13] Jannasch, A. et al. Versatile microsphere attachment of GFP-labeled proteins with preserved functionality Microsphere preparation. J. Biol. Methods 2, 1-12 (2015).

[14] Jahnel, M., Behrndt, M., Jannasch, a., Schäffer, E. & Grill, S. W. Measuring the complete force field of an optical trap. Opt. Lett. 36, 1260-2 (2011).

[15] Bormuth, V., Howard, J. & Schäffer, E. LED illumination for video-enhanced DIC imaging of single microtubules. J. Microsc. 226, 1-5 (2007).

Appendix: anti-eGFP functionalisation of polystyrene beads.

Modified from Bugiel et al.. Journal of Biological Methods. 2015 [13]

1. **Activation**
   (a) Suspend 12.5 μl 590 nm COOH-polystyrene beads (Bangs Labs) in 0.500 ml MES buffer. The same for 320 nm COOH-PS beads. Spin down at 5500 g (about 8700 rpm on a 6.5 cm radius rotor) for 5 minutes and repeat resuspension/centrifuge steps for a 2x wash. Vortex and sonicate for 2-4 minutes to resuspend pellet fully.
   (b) Dissolve about 10 mg each of sulfo-NHS and EDC in 100 μl MES. Add 0.77 mg NHS and 0.68 mg EDC to each 320 and 590 nm bead tubes (calculate corresponding volume). Add NHS first.
   (c) Incubate 15 min at 37°C and 600 rpm on thermomixer.
   (d) Wash 2x in 250 μl MES using the same spin speed and time from above. Vortex and sonicate for about 10 s each time.
   (e) Resuspend bead pellet in 125 μl PBS. Vortex and sonicate for about 180 s
2. **PEG coupling (monofunctional PEG only)**
   (a) Dissolve 9.36 mg 2kDa NH2-PEG-CH3O in PBS with 10 μl (3 mg/ml) anti-eGFP. Note: pre-mix PEG and antibodies before adding them to beads for consistent results. This step is modified to omit 3kDa, heterobifunctional PEG
   (b) Incubate for 120 minutes at 37°C and 600 rpm on thermomixer.
   (c) Wash 5x in 0.250 ml PBS with centrifuge settings as used earlier in this protocol.
   (d) Resuspend beads in 125 μl PBS.
   (e) Sonicate 90 s
   (f) Store at 4°C