

## Minireview

## Single molecule biochemistry using optical tweezers

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**Abstract** The use of optical trapping to create extremely compliant mechanical probes has ushered in a new field of biological inquiry, the mechanical and kinetic study of proteins at the single molecule level. This review focuses on three examples of such study and includes methods of extracting parameters of interest from the raw data such experiments generate.

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**Key words:** Optical trap; Single molecule; Myosin; Kinesin; Titin

## 1. Introduction

Optical trapping allows non-destructive manipulation of dielectric particles in solution via focused laser beams (cf. [1]). Particles of uniform shape, such as spherical beads, can be tracked with high spatial and temporal resolution [2] and constrained by optical traps rendered compliant enough to yield under forces produced by single protein molecules. Biomolecules can be fixed upon such beads, which are then trapped for use as handles to move attached biomolecules into a desired experimental geometry. The beads also act as probes to monitor movement or tension reflecting biochemical behaviour. Here, we outline applications of optical trapping to study three classes of proteins: processive motors, non-processive motors, and proteins experiencing significant mechanical strain.

## 2. Processive motor proteins

A processive enzyme undergoes multiple productive catalytic cycles per diffusional encounter with its binding partner. A widely studied example is kinesin, a two-headed motor observed to transport vesicles along microtubules. A single kinesin molecule can move along its microtubule track for micrometres before dissociating [3,4]. The stepwise character of this movement, and the distance between dwell positions ('step size'), were observed by Svoboda et al. [5] by attaching kinesin at low density to silica beads, capturing such a bead in an optical trap, and moving it into contact with a microtubule fixed upon a microscope coverslip (Fig. 1). The beads were observed with nm and ms resolution to move along the microtubules with dwell positions spaced around 8 nm apart [5]. In some cases, step transitions could be identified visually in filtered records. Even when noise obscured such transitions, the step distance could be extracted indirectly from bead position records by constructing a distribution of pairwise posi-

tion differences and taking its power spectrum. A peak in this spectrum appears at the reciprocal unitary step distance [5].

In these experiments, the optical trap is used to place the bead on a microtubule and reduce bead diffusion such that stepwise movement can be detected, a feat attempted without success using high resolution microscopy alone [6]. The kinesin will pull an optically trapped and thus elastically loaded bead until it no longer can, at which point the optical load provides the 'stall force' of the molecule [7]. The bead eventually detaches and falls back to trap centre before the kinesin again finds its microtubule track and begins pulling anew, yielding bead position records that resemble a series of staircases.

New methods of data analysis were devised to examine the kinetic scheme underlying kinesin movement and, by extension, the coupling of chemical energy to mechanical work (Fig. 2). Many of these 'staircase' records, each reflecting a single diffusional encounter of a kinesin with its track, were compiled and analysed at the ensemble level [8,9]. We define 'contact' as the first evident step transition in a given staircase, the earliest point at which kinesin binding to the microtubule becomes evident.

One can extract the distribution of dwell times between step transitions by computing the mean and variance of bead position across the position record ensemble as a function of time after contact. The mean should advance monotonically with time, the slope being the average kinesin velocity. The ensemble variance, however, contains information regarding the number of rate limiting processes preceding each mechanical step. If the intervals between steps vary randomly, the ensemble variance will also rise monotonically with time and at a pace affected by the kinetics of stepping. If stepping is a Poisson process, meaning there is only one rate-limiting process per mechanical step, or, equivalently, the probability the molecule will step at any given time is independent of its history, the ensemble variance will rise at a rate roughly equal to the velocity times the step size. If each rate-limiting process leads to two mechanical steps, the variance will rise at twice that rate, and if there are two comparably rate-limiting processes per mechanical step, the variance will rise at half that rate [8]. If a large number of processes are all rate-limiting in each mechanical step, the dwell time between steps should be constant. In this case, ensemble variance should remain nearly zero. Such measurements do not require identifying step transitions in the data, and thus can be applied when the stepping rate is fast or the data noisy. This method has been used to show that, under saturating ATP conditions, each mechanical step likely follows two rate-limiting processes [8]. Moreover, under limiting ATP conditions, most or all mechanical steps are rate-limited by only one process, ATP binding [9]. This observation demonstrates that at any ATP concentration, all

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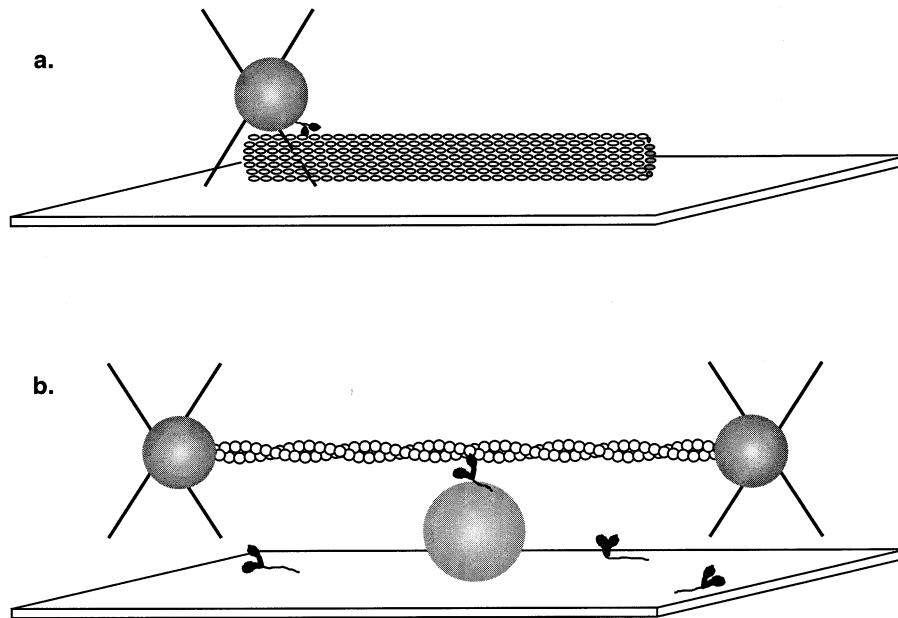


Fig. 1. a: Schematic illustration of a processive motor experiment. The motor protein is linked to an optically trapped bead, and the bead is tracked with nm and ms resolution. The motor moves along its polymer track, pulling the bead behind it. b: Schematic illustration of a non-processive motor experiment. The polymer track is linked to optically trapped beads on either end. The motor binds to and moves the polymer, and thus also the attached bead, before dissociating.

or nearly all of the 8 nm advances are tightly coupled to single ATP binding events, a result repeated using a different method [10].

Since the optical trap also serves as a force transducer, one can extract the kinesin velocity as a function of load [11] or observe kinesin behaviour in response to force jumps, either along or against the direction of protein movement [12], experiments not described here.

### 3. Non-processive motor proteins

A non-processive enzyme will undergo only one productive catalytic cycle per diffusional encounter with its binding partner, or, almost equivalently, spend most of its cycle time detached from the partner. Typifying this, conventional myosin II, the primary force generator in skeletal muscle contraction, drives actin filament movement only when large numbers of myosin molecules are present (cf. [13,14]). If only a single molecule were bound, the actin would diffuse away when the myosin dissociates after a single catalytic cycle. Hence, in a hypothetical analogue to the Svoboda et al. experiment described above for kinesin, with a single myosin fixed upon an optically trapped bead encountering actin fixed to a coverslip surface, the myosin would bind the actin filament, pull upon it once, and then release it. A single such stroking event would likely rotate the trapped bead, obscuring from view some of the displacement it causes. To avoid this problem, Finer et al. [15] devised a different experimental geometry for studying non-processive motors. Two optically trapped beads are attached to either end of a single actin filament in solution. This filament is moved into close proximity of surface-fixed silica beads decorated sparsely with myosin molecules. The myosin will then bind to and pull upon the filament, pulling one bead away from its trap centre and allowing the other bead to move closer to its trap centre (Fig. 1).

A long-standing controversy, in large part motivating the early optical trap experiments, concerned the distance a single myosin moved an actin filament per ATP hydrolysed. Estimates ranged from around 10 nm to over 100 nm. Pioneering experiments showed the optically trapped bead moved near 10 nm in a given actomyosin encounter, within acknowledged resolution limits [15]. Subsequent work focused on a key difference of data analysis between these and the processive motor experiments, the randomising effect of thermal diffusion [16].

Single molecule experiments demand extremely compliant optical traps. In consequence, trapped beads and attached actin filaments experience diffusion spanning many tens of nm. A surface-mounted myosin can bind the actin filament anywhere in this diffusion range, meaning that the positions to which it will move the filament will also be spread over tens of nm (Fig. 3). Unlike the processive case, one observes the endpoints of myosin strokes without observing the corresponding start points. However, the distribution of start points is well defined, as the position density of the diffusing actin filament before myosin binds. Hence, the distribution of the end points should be similarly defined [16], perhaps broader if myosin step distances vary for other reasons.

Since such a range of predicted dwell positions may encompass some not displaced significantly from baseline centre, myosin binding must be detected using methods other than visual inspection of filtered bead position data. Among those developed to date include observing a decrease in bead thermal diffusion amplitude when myosin binds [16] and observing a loss of statistical correlation between diffusion at the two filament ends when myosin binds [17] (Fig. 3). Building upon the first method, Guilford et al. have discarded the time information and instead looked only at the mean and variance of bead position, constructing a 3D histogram showing the amount of time spent at given pairs of mean and variance

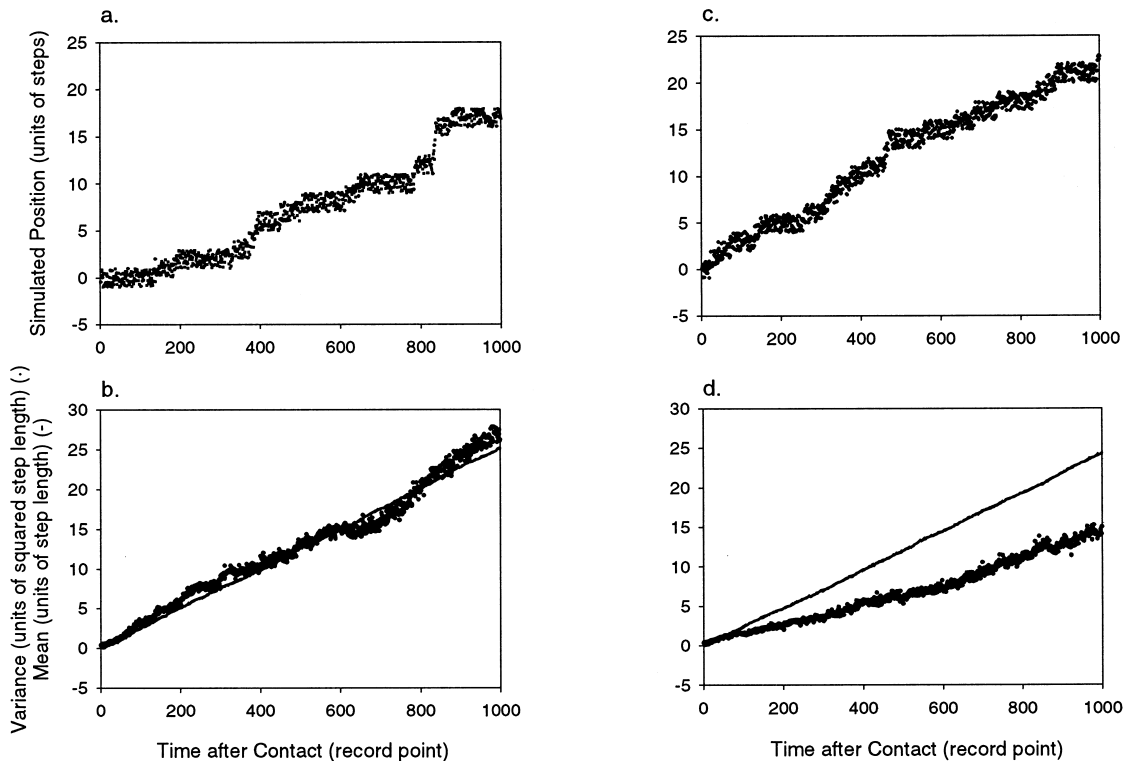


Fig. 2. Simulated data illustrate the method used to examine the kinetic scheme underlying kinesin stepping [8,9]. a: Simulated steps reflecting a single rate-limiting process per step. Of 1000 sequential record points, each has a 2.5% probability of advancing by one unit. Random noise of two units (peak-to-peak) has been superimposed. b: Mean and variance across an ensemble of 100 such staircases, as a function of time after contact. For each record index ('time'), abscissa points at that index in each of the 100 staircases are used to compute the mean and variance. The mean rises linearly with the record count, increasing by 25 over the entire record. The variance increases at the same rate as the product of the mean and the step length, meaning each mechanical step follows a single rate-limiting process. Svoboda et al. [8] and Schnitzer et al. [9] define a randomness parameter  $r = v/(d \times m)$  where  $v$  is the ensemble variance,  $d$  is the step distance, and  $m$  is the ensemble mean. In this case,  $r \approx 1$ . In practice,  $r$  often exceeds this value, probably due to a minority of steps exhibiting aberrant behaviour [9]. An observed  $r$  just over 1 at limiting ATP demonstrates that most or all of the mechanical steps are tightly coupled to single ATP binding events, this conclusion holding at all ATP concentrations [9]. c: Simulated steps reflecting two rate-limiting processes per step. Of 1000 record points, each has a 5% probability of occurring simultaneously with a given process. A one unit advance results from every other process. Random noise of two units (peak-to-peak) has been superimposed. d: Mean and variance across an ensemble of 100 such staircases, computed as described above in b. The mean rises just as it did for the Poisson process, but the variance rises at half the rate, indicating the stepping is less 'random' than a Poisson process.  $r \approx 0.5$  here, just as in kinesin traces at saturating ATP, suggesting that each step is rate-limited by two kinetically comparable processes under such conditions [8].

values. The unitary displacement is extracted from regions of this histogram remaining after the baseline portions have been 'subtracted' statistically [18]. All optical trap measurements to date place the unitary displacement in the 5–15 nm range, although experiments with needle probes have yielded 20–30 nm estimates [19,20].

#### 4. Proteins experiencing significant strain

The above experiments seek to decipher the motor's mechanical activity without perturbing it excessively. Even when loads become high, they should not distort the fold of the molecule. In other applications, optical traps have been used to perturb the fold deliberately. Pioneering experiments with titin, a structural element in muscle, have demonstrated that reversible domain unfolding in single molecules can be induced by mechanical stress and observed. Three studies, two involving optical tweezers [21,22] and the third atomic force microscopy (AFM) [23], demonstrated that at low tensions a single titin molecule acts as an entropic spring, meaning it resists an extension that constrains its range of accessible

states. At higher tensions, constituent immunoglobulin domains unfold.

Rief et al. observed sawtooth-like structures in force-extension curves generated from a stiff AFM cantilever attached to one end of a titin molecule, the other end of which was anchored to the slide surface. They interpreted each 'tooth' to reflect stochastic unfolding of an immunoglobulin domain. Such data could be repeated for the same molecule, although many trials led eventually to apparently irreversible unfolding of some domains [23].

Optical trapping led to different observations with similar conclusions. Kellermayer et al. stretched a titin molecule bound to a micropipette on one end and an optically trapped bead on the other. Instead of observing discrete, apparent unfolding events, they found hysteresis in a force-extension curve. When stretched, the molecule at first behaves as an entropic spring. The molecule deviates from this behaviour at high tension. When relaxed from an experimental peak tension, the molecule would at first follow entropic spring behaviour and then deviate at low tensions (Fig. 4). Interpreting this, the authors argued that during the stretch, the mol-

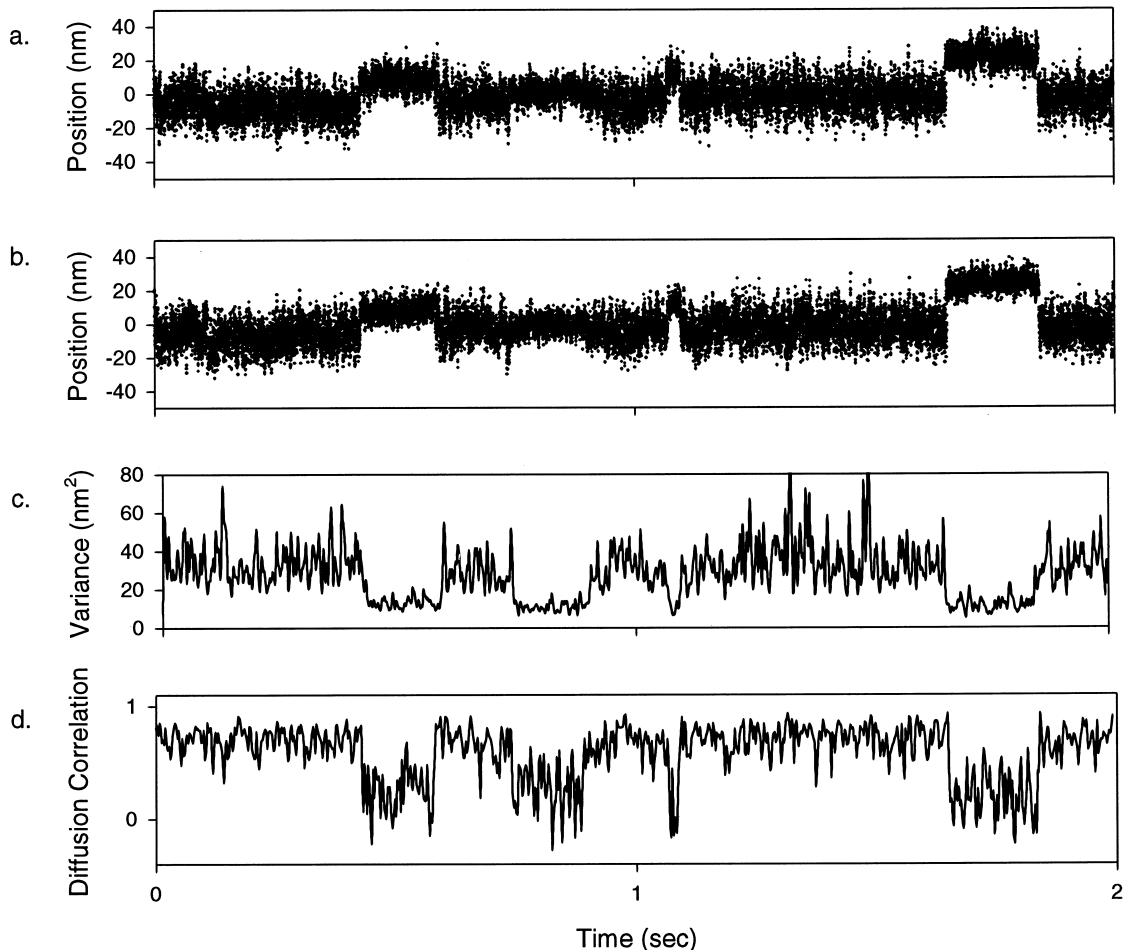


Fig. 3. Sample data reflecting actomyosin interactions, examined using the Finer et al. geometry [15]. a, b: Position of the two beads parallel to the long axis of the actin filament as a function of time. c: Running measure of bead diffusion amplitude from b. The variance of each 10 sequential data points (1 ms) was computed and the resulting sequence was filtered (second order Butterworth,  $f_c$  100 Hz) to clarify binding events. Since each point is computed using only 1 ms of data, abscissa numbers underestimate the actual variance characterising diffusion of the bead. However, they still provide a clear signature of myosin binding, which will more tightly constrain bead diffusion than the optical traps at the baseline [16]. The four transient drops in variance are interpreted to reflect myosin binding. d: Running measure of linear correlation coefficients between position records of the two beads, one computed every 5 ms. At the baseline, movement of each bead affects the other through the intervening filament and compliant connections. Hence, diffusion of the two beads is strongly correlated. When myosin binds the actin, it effectively decouples diffusion of the two beads, causing the diffusion correlation measure to fall and providing another signature of the binding event [17]. Judging from c or d, four myosin binding events occur through the given traces. The first event and last two events correspond to visually pronounced bead displacement from the baseline position. The second event presumably reflects myosin binding when the filament is displaced by thermal fluctuation from baseline centre. Myosin then pulls the filament back to the baseline centre [16]. These events make necessary the observation of signatures for myosin binding, such as position variance or diffusion correlation.

ecule reaches a tension where the equilibrium favours unfolding, but it remains kinetically trapped in the folded state while tension continues to increase. Transcending such a kinetic barrier may involve laterally directed extension, something not accelerated by the axial tension applied. Domains unfold eventually, at higher tension. During the relaxation, refolding will not occur until after equilibrium again favours folding, at a tension lower than that present when unfolding occurred [21]. If one could move the probes at a slow rate and reduce the size of strain fluctuations relative to the changes in strain that significantly affect unfolding probability, one would expect the force-extension curve to reflect equilibrium conditions and thus the energetics of folding, a proximal goal in current and future experiments (S.B. Smith and C. Bustamante, personal communication).

The authors argue that single unfolding events could be observed by Rief et al. and not in their trapping experiment

due to differences in probe stiffness. A domain unfolding event will extend the molecule by around 20 nm, causing system tension to relax by the product of this with probe stiffness. A stiff scanning probe will experience a significant tension drop, decreasing the probability that yet another domain will unfold. Such anti-cooperative behaviour renders singular unfolding events visible. A flexible trap yielding in response to one domain unfolding will experience a small tension drop only. Chances of further unfolding are not affected significantly, meaning that unfolding events occur in clusters that obscure the single events from view (S.B. Smith and C. Bustamante, personal communication).

Tskhovrebova et al. used a much stiffer optical trap to observe titin behaviour in response to a sudden jump in force. A single titin molecule was linked to a coverslip surface on one end and to an optically trapped bead on the other. The molecule was subjected to a sudden increase in tension, re-

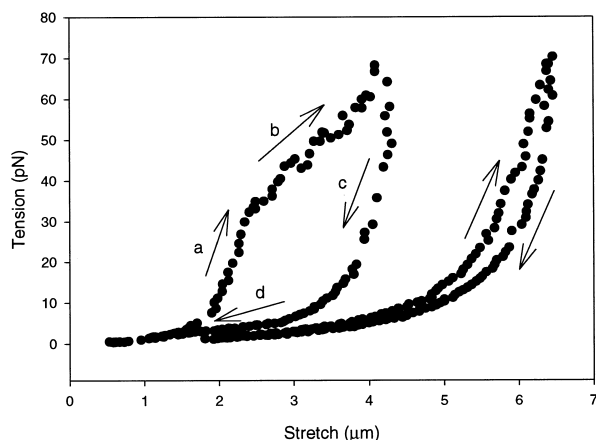


Fig. 4. Force-extension curves of single titin molecules, provided by S. Smith and C. Bustamante. The leftmost trace illustrates the reversible stretching of a single molecule, before most of the constituent immunoglobulin appears to unfold irreversibly. At low tension, the molecule resists extension as an entropic spring (a), after which it begins to show deviations from such behaviour (b). Such deviations are presumed to reflect protein domain unfolding events. After reaching peak tension, the molecule is allowed to relax. It follows the path (c), reflecting entropic spring relaxation and thus indicating that refolding is not occurring at the tension levels where unfolding occurred. Finally, in (d), it deviates from entropic spring behaviour, interpreted to reflect protein refolding. Such hysteresis shows the observed domain unfolding is likely not an equilibrium process but, rather, domains remain kinetically trapped in the folded state during the stretch, even after equilibrium favours unfolding. At the far right is a force-extension curve after addition of 3 molar guanidinium hydrochloride as a denaturant to prevent domain refolding. A similar effect is seen after many cycles of stretching and relaxing a given molecule. The domains appear to unfold irreversibly [21–23] and the hysteresis largely vanishes [21].

sponding with immediate strain and relaxing to an apparent equilibrium after 1 s. This decay to equilibrium occurred in discrete steps, interpreted to reflect single domain unfolding events. At the mean tension levels observed, stochastic domain unfolding events occurred with rate constant 1/s, and thus individual events were separated enough to identify visually in the records [22]. A more flexible optical trap could in principle be used to observe such events, by fixing tension at a level such that unfolding rates are fast enough for discrete events to be observed but slow enough for them to be clearly separated, even if they are not anti-cooperative.

The above enumeration, far from exhaustive, represents the nascent and growing application of optical trapping to study single molecule biochemistry. In the first two experiments, optically trapped beads were used as handles to move proteins and as probes to track protein-driven motion. Such methods are being extended to study of motor proteins moving along DNA [24], where sequence-specific behaviour provides a further level of complexity to decipher. Probe thermal diffusion, significant on size scales relevant to protein dimensions, can randomise and obscure probe deflections caused by interacting proteins. On the other hand, extensive thermal diffusion provides a key advantage, removing the daunting task of moving the proteins precisely into binding positions. Instead, like any skilled matchmaker, the experimentalist need only position the trapped beads approximately and allow the proteins to find each other.

In the third application, optically trapped beads were used as handles to pull upon a protein and as probes to track their resistive force. In principle, such a method can be extended to unfold better characterised proteins and address more general questions. Probe fluctuations are more pernicious here, since they complicate the task of equilibrium measurements by making strain levels ill defined over time. Nonetheless, such problems can be overcome by reducing the fraction of probe strain fluctuations that affect a given protein domain, for instance by attaching the domains to probes using long linkers [C. Bustamante, personal communication].

The range of applications for optical trapping experiments continues to expand. Integration with single fluorophore detection has enabled simultaneous observation of mechanical stepping and ATP binding in the cases of myosin [25] and kinesin [26], experiments certain to inspire followers. Continued development and application of optical trapping promises to maintain a flow of discoveries regarding the mechanical and kinetic properties of motors and other proteins.

## References

- [1] Ashkin, A. (1998) *Methods Cell Biol.* 55, 1–27.
- [2] Simmons, R.M., Finer, J.T., Chu, S. and Spudich, J.A. (1996) *Biophys. J.* 70, 1813–1822.
- [3] Howard, J., Hudspeth, A.J. and Vale, R.D. (1989) *Nature* 342, 154–158.
- [4] Vale, R.D., Funatsu, T., Rombergk, L., Pierce, D.W., Harada, Y. and Yanagida, T. (1996) *Nature* 380, 451–453.
- [5] Svoboda, K., Schmidt, C.F., Schnapp, B.J. and Block, S.M. (1993) *Nature* 365, 721–727.
- [6] Malik, F., Brillinger, D. and Vale, R.D. (1994) *Proc. Natl. Acad. Sci. USA* 91, 4584–4588.
- [7] Kuo, S.C. and Sheetz, M.P. (1993) *Science* 260, 232–234.
- [8] Svoboda, K., Mitra, P.P. and Block, S.M. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11782–11786.
- [9] Schnitzer, M.J. and Block, S.M. (1997) *Nature* 388, 386–390.
- [10] Hua, W., Young, E.D., Fleming, M.L. and Gelles, J. (1997) *Nature* 388, 390–393.
- [11] Svoboda, K. and Block, S.M. (1994) *Cell* 77, 773–784.
- [12] Coppin, C.M., Pierce, D.W., Hsu, L. and Vale, R.D. (1997) *Proc. Natl. Acad. Sci. USA* 94, 8539–8544.
- [13] Spudich, J.A. (1994) *Nature* 372, 515–518.
- [14] Howard, J. (1997) *Nature* 389, 561–567.
- [15] Finer, J.T., Simmons, R.M. and Spudich, J.A. (1994) *Nature* 368, 113–119.
- [16] Molloy, J.E., Burns, J.E., Kendrick-Jones, J., Tregear, R.T. and White, D.C.S. (1995) *Nature* 378, 209–212.
- [17] Mehta, A.D., Finer, J.T. and Spudich, J.A. (1997) *Proc. Natl. Acad. Sci. USA* 94, 7927–7931.
- [18] Guilford, W.H., Dupuis, D.E., Kennedy, G., Wu, J., Patlak, J.B. and Warshaw, D.M. (1997) *Biophys. J.* 72, 1006–1021.
- [19] Ishijima, A., Kojima, H., Higuchi, H., Harada, Y., Funatsu, T. and Yanagida, T. (1995) *Biophys. J.* 70, 383–400.
- [20] Kitamura, K., Tokunaga, M., Iwane, A.H. and Yanagida, T. (submitted).
- [21] Kellermayer, M.S.Z., Smith, S.B., Granzier, H.L. and Bustamante, C. (1997) *Science* 276, 1112–1116.
- [22] Tskhovrebova, L., Trinick, J., Sleep, J.A. and Simmons, R.M. (1997) *Nature* 387, 308–312.
- [23] Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J.M. and Gaub, H.E. (1996) *Science* 276, 1109–1112.
- [24] Yin, H., Wang, M.D., Svoboda, K., Landick, R., Block, S.M. and Gelles, J. (1995) *Science* 270, 1653–1656.
- [25] Ishijima, A., Kojima, H., Funatsu, T., Tokunaga, M., Higuchi, H., Tanaka, H. and Yanagida, T. (1998) *Cell* 92, 161–171.
- [26] Funatsu, T., Harada, Y., Higuchi, H., Tokunaga, M., Saito, K., Vale, R.D. and Yanagida, T. (1996) *Biophys. J.* 70, A6.