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Molecular Motors: Single-Molecule Recordings Made Easy

Dispatch

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A fusion protein of kinesin and gelsolin binds a short actin filament which can be visualized using a standard fluorescence microscope. This technique has provided new insight into the mechanism of kinesin action, and in principle it can be extended to allow single-molecule assays of any protein.

Although a number of single-molecule detection methods have emerged over recent years, reliable imaging has remained challenging because the requisite high optical sensitivity has demanded a sophisticated experimental setup. This problem has now been circumvented by Yajima *et al.* [1] in their efforts to image individual kinesin molecules walking on microtubules. By fusing kinesin to gelsolin, they have created a construct that binds fluorescently labeled actin filaments; the short actin filaments shine brightly enough to be seen by conventional video microscopy. Using this technique, the authors reliably measured an important parameter of kinesin action — its run length at low mechanical load.

Observing single molecules at work has been a long-standing goal of biologists. The first time-resolved recordings from individual proteins were made in 1976 by Neher and Sakmann [2], who measured the tiny currents passing through single ion channels in membranes. The subsequent refinement of the patch-clamp technique made single-channel electrical recordings routine, and has led to an explosion of information about the structure and function of ion channels [3]. But the spectacular success of the patch-clamp technique relied on the unique electrical properties of ion channels — a single channel can pass ~10^6 ions per second when open — and there was no way to generalize it to other proteins. Thus alternative approaches had to be found.

Motor proteins offered a promising avenue for such studies because the cytoskeletal filaments along which they move are huge polymeric molecules, and even the narrow, 6 nm diameter actin filaments could be imaged by darkfield [4] or fluorescence [5] microscopy. This led to the development of 'up-side-down' motility assays in which motors were bound to a surface and the movement of filaments across the surface followed by video microscopy [6]; by reducing the density of motors on the surface it was even possible to record from individual motor proteins [7]. In order to visualize the movement of the motor rather than the filament, however, the motor protein itself has to be labeled. This was first done by binding the motor to a large, micron-diameter bead. One advantage of this preparation over the upside-down assay is that the bead (with attached motor) can be held in an optical trap, making possible measurements of single-molecule forces and steps [8–10]. But the disadvantages are that binding exactly one motor molecule to one bead is not straightforward, and the method will not be generally applicable to other proteins or for use within cells.

A crucial step towards the development of a general method for imaging single proteins was made by Funatsu *et al.* [11]: they pushed the sensitivity of the fluorescence microscope to the limit of being able to visualize individual myosin molecules labeled with the fluorophores Cy3 or Cy5. Using total internal reflection fluorescence microscopy, the processive movement of individual kinesin molecules along microtubules was visualized by labeling the motor with Cy-3 [12] or with the green fluorescent protein (GFP) [13]. Fusing GFP to the protein of interest offers several advantages: it avoids difficult labeling procedures needed for conventional dyes; expression of GFP-tagged proteins is possible inside cells; and there is an exact one-fluorophore to one-protein labeling ratio. The problems with GFP as a single-molecule marker are that it blinks on and off [14] and has low photostability.

In general, the price paid to use a single fluorophore or GFP-tagged molecule for labeling is a limited observation time, typically about 10 seconds or less because only ~10^5 fluorescent photons are emitted before photobleaching occurs and the intensity abruptly drops to zero [15]. Certainly, this limits the applicability of the method to measuring the processivity of a motor enzyme — the number of steps that it takes along its track — as premature photobleaching of the fluorescent marker cannot be distinguished from dissociation of the motor from the track.

Yajima *et al.* [1] have developed an elegant method that renders long-time imaging of single motor molecules possible. By linking rhodamine-phalloidin-stained actin filaments — mean length 88 nm, about 39 actin monomers and 38 fluorophores — to kinesin–gelsolin constructs, they created fluorescently marked kinesin dimers (Figure 1) that are bright enough to be detected by conventional epi-fluorescence microscopy and that do not show discrete photobleaching. The advantages of this method are tremendous: the labeling procedure is applicable to any protein and reliable long-time imaging of single molecules is possible with high signal-to-noise ratio. The ability to use a conventional video-microscope means that the experiments can be done in any molecular biology lab.

The new data from Yajima *et al.* [1] on the 'run length' of kinesin — the distance moved by a single kinesin molecule before it dissociates from a

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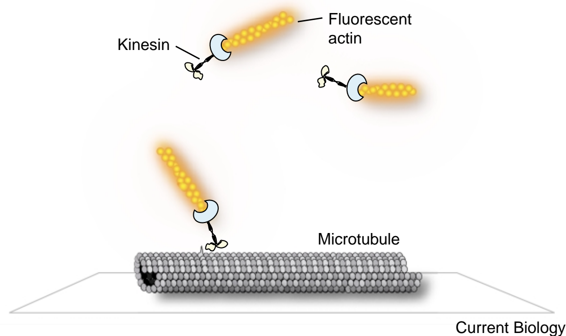

Figure 1.

Shining bright for long-time imaging: rhodamine–phalloidin-labeled actin filaments are linked to individual kinesin molecules via gelsolin. See text for details. (Image courtesy of Rob Cross.)

microtubule— challenge the status quo. Previously, the most comprehensive data on kinesin run length was obtained using an optical trap which both resisted kinesin's forward progress and pulled kinesin away from the microtubule [10]. At low load, Schnitzer *et al.* [10] found that kinesin run lengths are shorter at low ATP concentrations than at high ATP concentrations. To the contrary, Yajima *et al.* [1] found the run length to be independent of the ATP concentration.

One possible explanation for these different results is that they are due to the use of different kinesins or different buffer conditions. The run length at low ATP concentration is probably determined by the rate of detachment of the motor from the microtubule when the motor is in the rigor state (without bound nucleotide): perhaps the rat kinesin used by Yajima *et al.* [1] detaches from the microtubule in the rigor state at a far lower rate than the squid kinesin used by Schnitzer *et al.* [10]. If this is the case, then extending the run length measurements of Yajima *et al.* [1] to even lower ATP concentrations might reveal a decrease similar to that found by Schnitzer *et al.* [10] But if this is not the case, then the discrepancy suggests that detachment from the rigor state has a strong load dependence — this possibility needs to be followed up with force experiments.

In addition to stirring up a new controversy over kinesin processivity, Yajima *et al.* [1] have brought fresh evidence to bear on an older debate over the sequence of nucleotide states through which kinesin cycles as it moves. As kinesin moves unidirectionally along a microtubule, its two heads must undergo synchronized changes in their conformations and binding strengths to the microtubule, these changes being driven by the processing of ATP in each head. In the quest to understand this intricate choreography, a major goal is to determine the sequence of mechanochemical states of the two heads. Yajima *et al.* [1] obtained new data relevant to the nucleotide state of the rear head when it detaches. They found that kinesin's run length and velocity decline with added ADP, and propose that detachment of the kinesin dimer occurs in the state when both heads have ADP bound to them. Previous studies have shown that ADP-containing heads bind weakly to microtubules in the absence of ATP (for example, see [16]), but the data of Yajima *et al.* [1] demonstrate for the first time that ADP causes dissociation while kinesin is moving and hydrolyzing ATP.

The debate in the field has been whether the rear head detaches in the K·ADP or the K·ADP·P_i state [17], but unfortunately Yajima *et al.* [1] are not able to settle this once and for all. The dwell time data of Yajima *et al.* [1] indicate that the detachment rates of the K·ADP and K·ADP·P_i states are similar, in agreement with previous work [18]. Furthermore, Yajima *et al.* [1] find that P_i alone has no effect on the run length or velocity at high ATP, but that is the expected result whether the rear head releases in the K·ADP or K·ADP·P_i state. It is likely that a final resolution of this question will require measurement of phosphate release kinetics.

A key open question is how the two heads move as kinesin dances down the microtubule. The use of an elongated fluorescent structure such as an actin filament may make it possible to observe the changes in orientation of the individual heads, and this would be an exciting development. This is precisely the approach that Noji *et al.* [19] took to show that the F1-ATPase is a rotary engine, though this work used the more cumbersome streptavidin–biotin linkage to attach the actin filament.

The holy grail of single-molecule research is to track individual molecules in living cells. This poses many problems, not least of which is overcoming autofluorescence: an assembly containing some ten fluorophores will probably be necessary in order to give reliable detection (though less in the cell nucleus and at the periphery). A promising new approach is the use of colloidal semiconductor nanocrystals, also termed quantum dots [20]. Such nanocrystalites, which might become commercially available this year, are small spheres with diameters of 2–5 nm and have very low rates of photobleaching. But getting them into cells will be difficult. That is why the fusion protein approach of Yajima *et al.* [1], or possibly a tandem GFP tag, is probably the way forward.

As more and more labs make technical contributions, the future of single-molecule recordings looks brighter and brighter.

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