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Myosin monomer density and exchange in synthetic thick filaments investigated using fluorescence microscopy with single molecule sensitivity

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The number of myosin molecules in synthetic thick filaments, prepared by dialysis at 0.12 M NaCl and pH 7.0, was estimated to be between 400 and 800 molecules per micrometre under conditions appropriate for *in vitro* motility assays. This estimate was based on a number count of Cy3-labelled myosin molecules incorporated into filaments at a nominal ratio of 1:1000. At this dilution, single fluorescent spots were resolved corresponding to individual labelled myosins. The spots usually bleached with a one- or two-step profile but, in around 30% of the cases, fluctuations were observed indicating that additional photophysical or photochemical events had occurred. Myosin molecules were shown to exchange between filaments in suspension on a time-scale of several hours at 4 °C, but the reaction was only 75% complete after 48 h, suggesting a non-exchangeable core. However, myosin exchange does not appear to be the predominant source of the fluctuations in fluorescence intensity in the single molecule assays.

Keywords: total internal reflection fluorescence; Cy3 fluorophore; nucleotide analogue; photobleaching; *in vitro* motility

1. INTRODUCTION

Synthetic myosin filaments prepared by dialysis or dilution of soluble myosin to physiological ionic strengths have long been used as a model for thick filaments of muscle (Huxley 1963). Although the myosin molecule contains some intrinsic properties which direct the assembly to give bipolar structures with the correct 14.3 nm axial stagger between myosin heads, the length-determining mechanism is not completely understood. Under some *in vitro* conditions, the length distribution of synthetic filaments can be remarkably narrow, but it is likely that, *in vivo*, accessory proteins (e.g. myosin binding protein C and titin) play a role in the precise structure of native thick filaments (Davis 1988). Despite these limitations, synthetic thick filaments continue to provide a useful experimental preparation for both establishing the nature of the intermolecular interactions within the filament and providing arrays of orientated myosin heads for *in vitro* motility assays (Yamada & Wakabayashi 1993; Ishijima *et al.* 1996). With respect to the latter, it is important to characterize the properties of such filaments under the conditions of the assay (i.e. when the filaments are adhered to a glass–silica substrate at low protein concentrations).

Previous work has established that synthetic filaments produced by dialysis or dilution of vertebrate skeletal muscle myosin at pH 8.0 tend to be shorter than native filaments (*ca.* 1 μm cf. 1.6 μm) but have a narrow length distribution (Davis 1988). Investigation of the kinetics of filament assembly and disassembly led Davis (1993) to surmise that their length was under kinetic control such that the association rate constant was independent of length, but the dissociation rate constant increased exponentially with length. Thus, pH 8.0 filaments are dynamic and in equilibrium with a free myosin pool. Computer simulations indicated that, if this monomer pool is removed, the entire filament would disassemble within *ca.* 5 s. However, if the pool is maintained, monomer exchange would occur rapidly (milliseconds-to-seconds time-scale) at the filament ends, whilst the probability of exchange in the central third is practically zero.

In contrast, synthetic filaments produced at pH 7.0 tend to be longer with a broader length distribution (up to 10 μm) and generally are wider than native filaments. Bare zones can sometimes be resolved in an electron microscope (Kaminer & Bell 1966) and *in vitro* motility assays support the idea that there is a switch in polarity (or at least in the predominant polarity) across the centre of the filament (Yamada & Wakabayashi 1993; Ishijima *et al.* 1996). Saad *et al.* (1991) reported that all myosin monomers are free to exchange within several hours, with relatively little heterogeneity along the length of the filament. This study was offered in support of the dynamics of thick filaments *in vivo* during growth and differentiation of muscle cells. However, this conclusion was challenged by Davis (1993) who considered that the exchange may be limited to an adventitious coating of myosin which may predominate under these pH 7.0 conditions.

The development of fluorescence microscopy techniques with single-molecule sensitivity offers an opportunity of re-examining this question. Furthermore, it is now possible to resolve single turnovers of myosin ATPase at the level of single molecules by examining the lifetime of fluorescent ATP analogues bound to the myosin active site (Funatsu *et al.* 1995; Conibear *et al.* 1998; Ishijima *et al.* 1998; Oiwa *et al.* 1999). However, fluctuations in fluorescence can arise via photochemical or photophysical mechansims (Moerner & Orrit 1999). In addition, in the case of filament-based assays, the myosin monomers may undergo reversible dissociation on a seconds-to-minutes time-scale and the contribution of such a process to the observed fluctuations must be determined.

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2. METHODS

(a) *Proteins and nucleotides*

Myosin was prepared from rabbit skeletal muscle as described by Margossian & Lowey (1982) without column purification. Synthetic filaments were prepared by dialysing solubilized myosin as described in the figure legends. Cy3-EDA-ATP and Cy5-EDA-ATP were synthesized from 2′(3′)-*O*-[aminoethyl carbamoyl]-adenosine triphosphate (EDA-ATP) using commercial carbocyanine fluorolink dyes (Amersham Life Sciences, Amersham, UK) as outlined (Bagshaw 1998). Myosin was labelled with these fluorophores by trapping the nucleoside diphosphate at the active site with excess Na_3VO_4 (Wells & Bagshaw 1984) or AlF_4 (Conibear *et al.* 1996) prior to dialysis.

(b) *Total internal reflection fluorescence microscopy*

Fluorescently labelled myosin filaments were examined by total internal reflection fluorescence (TIRF) microscopy to achieve the low background required for single-molecule imaging (Funatsu *et al.* 1995). Our optical set-up has been described in detail (Conibear *et al.* 1998; Bagshaw & Conibear 1999). Cy3 fluorophores were excited with a frequency-doubled Nd-YAG laser (μGreen 4301–050, Uniphase) and Cy5 was excited with an HeNe laser (05LHR927, Melles Griot). TIRF was induced in filaments adhered to a Spectrosil slide coupled to a 15 mm cubic Spectrosil prism (TSL, Wallsend, UK). Fluorescence was selected using Omega interference filters (580DF30 for Cy3 and 670DF40 for Cy5). The laser power was *ca.* 5 mW at the prism. Fluorescence images were captured on a Gen III-intensified CCD camera (IC-300, Photon Technology International, Surbiton, UK) and analysed using NIH Image software (available at (http://rsb.info.nih.gov/nih-image/)).

3. RESULTS

(a) *Monomer density in synthetic filaments*

Myosin filaments, prepared by dialysis at pH 7.0 from Cy3-ADP.Vi-trapped myosin pre-mixed in ratios of up to 1:10 with unlabelled ADP.Vi-trapped myosin, were observed to have intensity profiles that were even along their lengths. With mixing ratios of between 1:10 and 1:100 the intensity–length profile became more uneven and above 1:100 the images were distinctly punctate. Above a ratio of 1:300, individual fluorescent spots became spaced increasingly farther apart without significant change in their average intensity. This provides strong evidence that the latter correspond to single labelled myosin molecules. Further support comes from the observation of quantized photobleaching (see figure 2*b*,*c*). In this regime, the number of such spots per unit length for a known ratio of Cy3-labelled to non-labelled myosins provides a measure of the myosin density. A typical experiment is shown in figure 1 with a nominal mixing ratio of 1:1000. A second label (Cy5) was present at a higher ratio to image each filament in order to determine its location and length. The Cy3 ratio was low enough that spots would rarely overlap by chance yet sufficiently high to ensure that randomly located spots, arising from other sources, make an insignificant contribution. The latter was ascertained by the observation that the majority of spots co-aligned with the Cy5-labelled filaments. The average filament length was 5.7 μm (2.7 μm s.d. and $n = 23$). The average number of spots per unit length was 0.56 μm^{-1}, corresponding to a myosin density of 560 ± 70 molecules per micrometre, subject to any correction for incomplete trapping of the labelled myosin stock, nucleotide dissociation during dialysis, photobleaching prior to image capture and the accuracy of mixing the fluorescent and non-fluorescent

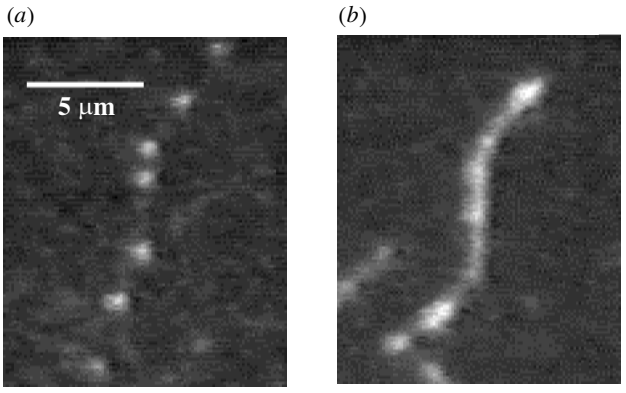

Figure 1. Dual images of a synthetic myosin filament co-labelled with Cy3 and Cy5 fluorophores. Separate soluble myosin preparations (5 μM heads) were labelled by incubating with either Cy3-EDA-ADP or Cy5-EDA-ADP (10 μM) and Na_3VO_4 (100 μM) for 12 h at 4 °C in a buffer comprising 0.5 M NaCl, 1 mM $MgCl_2$ and 20 mM imidazole (corrected to pH 7.0 at 20 °C). Unlabelled myosin was prepared similarly with ADP. Synthetic filaments were prepared by pre-mixing the Cy3-, Cy5- and ADP-trapped myosin preparations in a ratio of 1:10:1000 and dialysing for 12 h at 4 °C against a large excess of 0.12 M NaCl, 1 mM $MgCl_2$, 100 μM Na_3VO_4 and 20 mM imidazole (corrected to pH 7.0 at 20 °C). The co-filament suspension was diluted to 200 nM (myosin heads) in the dialysis buffer and a 15 μl droplet applied to a 75 mm × 25 mm silica slide and dispersed between the slide and a 22 mm × 40 mm no. 0 glass cover-slip. Paper spacers (< 30 μm thick) were present at each corner to make a flow cell. The sample was perfused with 200 μl of dialysis buffer plus glucose (2.5 mg ml^{-1}), catalase (180 μg ml^{-1}) and glucose oxidase (43 μg ml^{-1}). After removal of the spacers, the flow cell was collapsed to *ca.* 10 μm thickness and observed by TIRF microscopy. (*a*, *b*) The images observed using the Cy3 and Cy5 filter sets and excitation at 532 and 633 nm, respectively. All spots in the Cy3 image mapping onto filaments as observed by Cy5 imaging were counted. The total lengths of the filaments were determined using the NIH Image software, including the few short ones in which no Cy3 spots were observed. The degree of labelling of the filament preparation with Cy3 was determined as follows. In order to remove any free fluorophore, the dialysed myosin filaments were diluted fourfold, centrifuged at 10 000 *g* in an MSE Micro Centaur and the pellet resuspended in a buffer containing 0.5 M NaCl, 1 mM $MgCl_2$ and 20 mM imidazole at pH 7.0 and 20 °C. The myosin concentration was determined by absorbance using $A_{280} = 259\,000\ M^{-1}\ cm^{-1}$. The Cy3-EDA-ADP concentration was determined from the fluorescence emission spectrum measured with an SLM 48000 fluorimeter, calibrated against standard solutions (using $A_{552} = 150\,000\ M^{-1}\ cm^{-1}$). The standard solutions also contained myosin at the same concentration as the sample and 4 M urea was used to prevent protein–nucleotide interaction. This gave a ratio of 1:400 Cy3 labels:myosin molecules (i.e. 1:800 labelled myosin molecules: total myosin molecules if both heads are labelled).

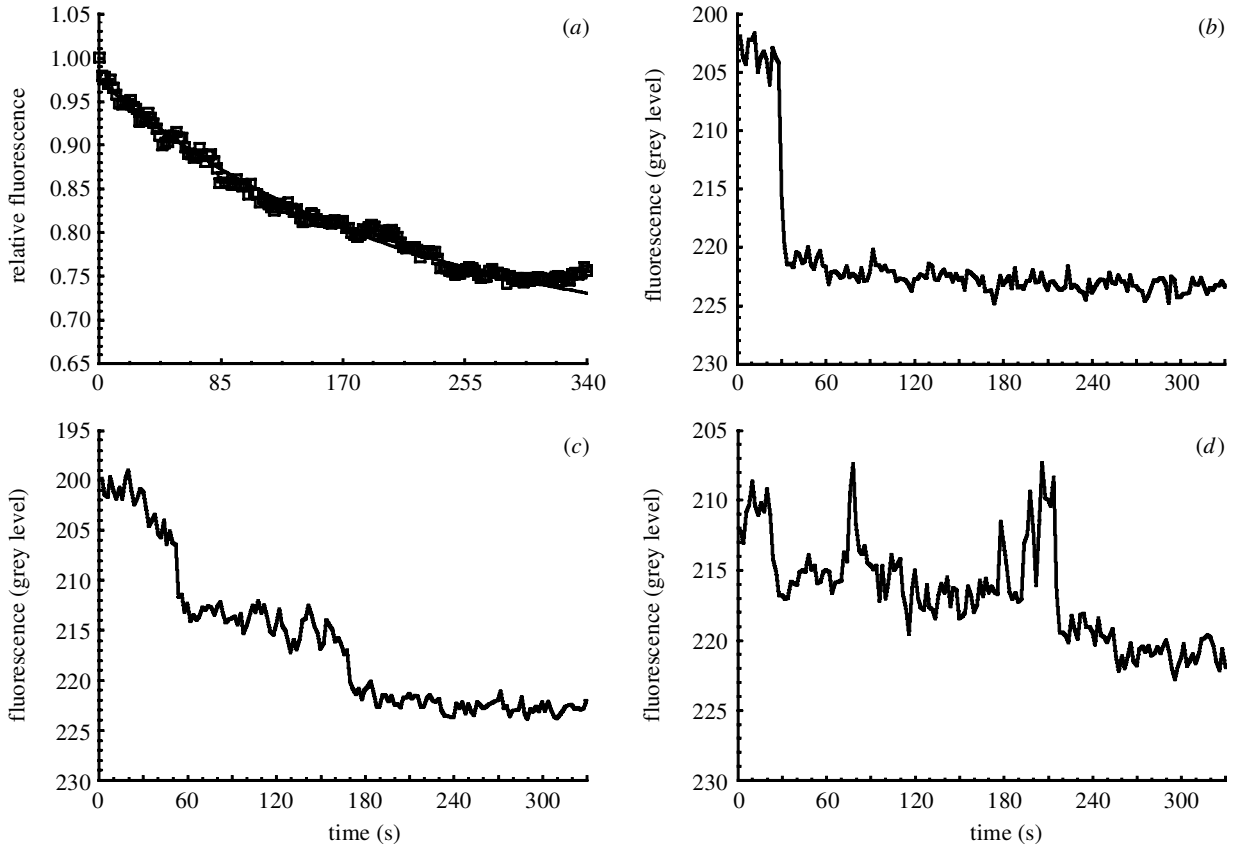

Figure 2. Kinetics of the fluorescence intensity changes of Cy3-labelled myosin in synthetic filaments during prolonged illumination. The sample was prepared as described in figure 1. (*a*) Composite assay: the time-course of the normalized summed intensity of 23 spots distributed over three filaments and fit to a single exponential yielding a rate constant of $0.005\,s^{-1}$. (*b–d*) Selected single-molecule bleaching profiles: spots (area $= 0.15\,\mu m^2$) within a filament were selected and the fluorescence intensity computed for each successive frame using the NIH Image software (where white = 0 and black = 255). (*b*) One-step photobleaching, (*c*) two-step photobleaching and (*d*) blinking.

myosin stocks (such corrections are discussed below). The value obtained is to be compared with that of native filaments which have a crown of three myosins at 14.3 nm intervals, thus giving a number count outside the bare zone of 210 molecules per micrometre.

(b) *Photobleaching kinetics*

Upon prolonged illumination of the samples, such as those in figure 1, the number of spots falls to near zero due to photobleaching (figures 2 and 3). Under the conditions of figure 1, the intensity averaged over 23 filament-based spots decayed to the background level in an approximately exponential manner with a rate constant of $0.005\,s^{-1}$ (figure 2*a*). Individual spots showed quantized behaviour with either of the three types of profile: one-step decay (figure 2*b*), as expected for bleaching of a labelled myosin with just one head containing a fluorescent label, two-step decay (figure 2*c*), as expected for a myosin with both heads fluorescently labelled and more complex behaviour with quantized reversals in intensity, which is termed 'blinking' (figure 2*d*).

Blinking may represent reversible photobleaching chemistry (Moerner & Orrit 1999), temporary quenching due to interaction of the fluorophore with a quenching centre (e.g. the silica surface or another bleached fluorophore) (Vanden Bout *et al.* 1997) or myosin dissociation–reassociation. The latter possibility is raised by the frequent observation of new spots appearing briefly at random positions across the field, as previously reported in non-filamentous samples (Conibear *et al.* 1998). However, these were rarely observed to occur on the filaments themselves, consistent with the relatively small proportion of the surface that the filaments occupied. The blinking observed within filaments was usually localized where spots were observed initially (e.g. figure 3). This behaviour is more indicative of photochemical or photophysical mechanisms (cf. Pierce & Vale 1999).

The data in figure 2 also have implications with regard to the errors in the estimate of the myosin density outlined above. The spectroscopic assay (figure 1 legend) yields a Cy3 content high enough to suggest that all myosin heads derived from the labelled stock must contain a Cy3 label and gives a final ratio of labelled myosin to total myosin in the filament preparation of 1:800. The difference from the nominal 1:1000 mixing ratio is within the accuracy of transferring small volumes of the labelled myosin stock ($\pm 20\%$). The images obtained for measuring the myosin density were captured within 20 s of illumination. Based on the measured photobleaching rate (figure 2*a*), $<10\%$ of the Cy3 label would be bleached and thus $>99\%$ of the labelled myosin molecules would retain at least one fluorophore and remain visible, i.e. the photobleaching correction is negligible. On the basis of a 1:800 labelling ratio, the myosin density

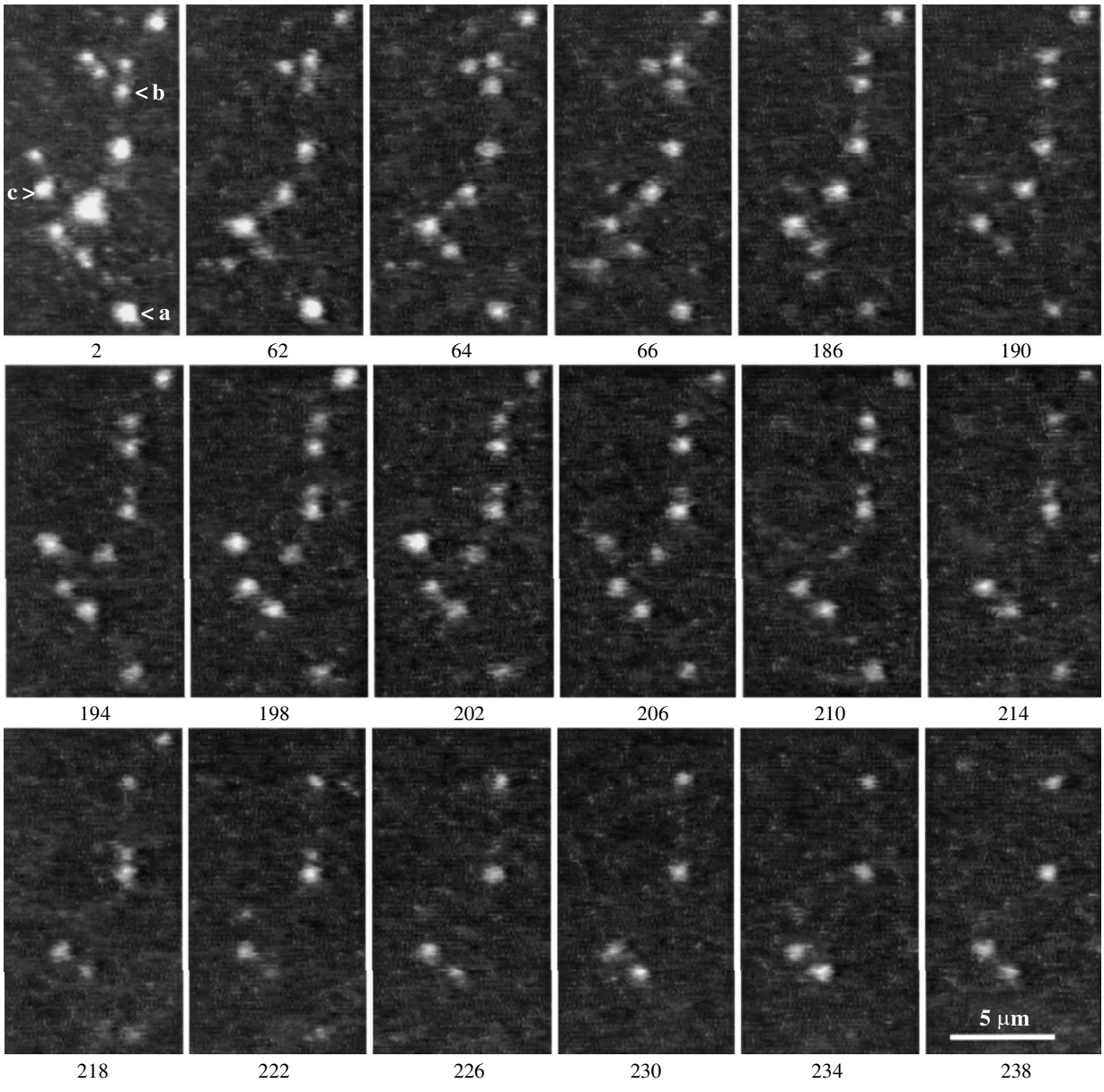

Figure 3. Video montage showing the fluorescence intensity of individual Cy3-labelled myosin molecules within synthetic filaments. The sample was prepared as in figure 1. The images were recorded with 32 frame averaging and captured at 2 s intervals. The time of capture (s) is shown below each panel. The 'a' indicates a spot which bleaches in two steps (one between 62 and 64 s and another between 222 and 226 s). The 'b' indicates a spot which bleaches in a single step (between 210 and 214 s). The 'c' indicates a spot which blinks (i.e. it is absent at 62 s but reappears between 190 and 194 s and disappears again between 206 and 210 s).

is 450 ± 50 molecules per micrometre. However, uncertainties remain in the value used for the absorption coefficients. In this case, the corrected value may be an underestimate of the myosin density.

The single-molecule kinetic data provide an independent means of correction which takes account of both the Cy3 content of the myosin derived from the labelled stock and the extent of photobleaching. Assuming no cooperativity between the two heads in nucleotide binding, dissociation or photobleaching, the fraction of total molecules from the labelled stock which retain at least one fluorophore can be determined on statistical grounds from the ratio of the number of observed molecules which contain one label to that of molecules containing two. It is difficult to determine the latter ratio from the spot intensities because the distribution of the intensities is relatively broad. This may be expected because an evanescent field strength varies across the *x*-, *y*- and *z*-planes and the 2′- and 3′-*O*-Cy3-EDA-ATP isomers show different enhancements when bound to myosin (Oiwa *et al*. 1999). However, the ratio of interest can be obtained from the relative proportions of spots bleaching in one or two steps. Out of 14 records which showed clean profiles eight (57%) were double and six (43%) were single steps. Thus, 93% of the total myosin molecules from the labelled stock would have retained at least one fluorophore. After allowance for the likely error associated with mixing myosin stocks, this leads to a corrected myosin density of between 400 and 800 molecules per micrometre. This statistical approach may tend to overestimate

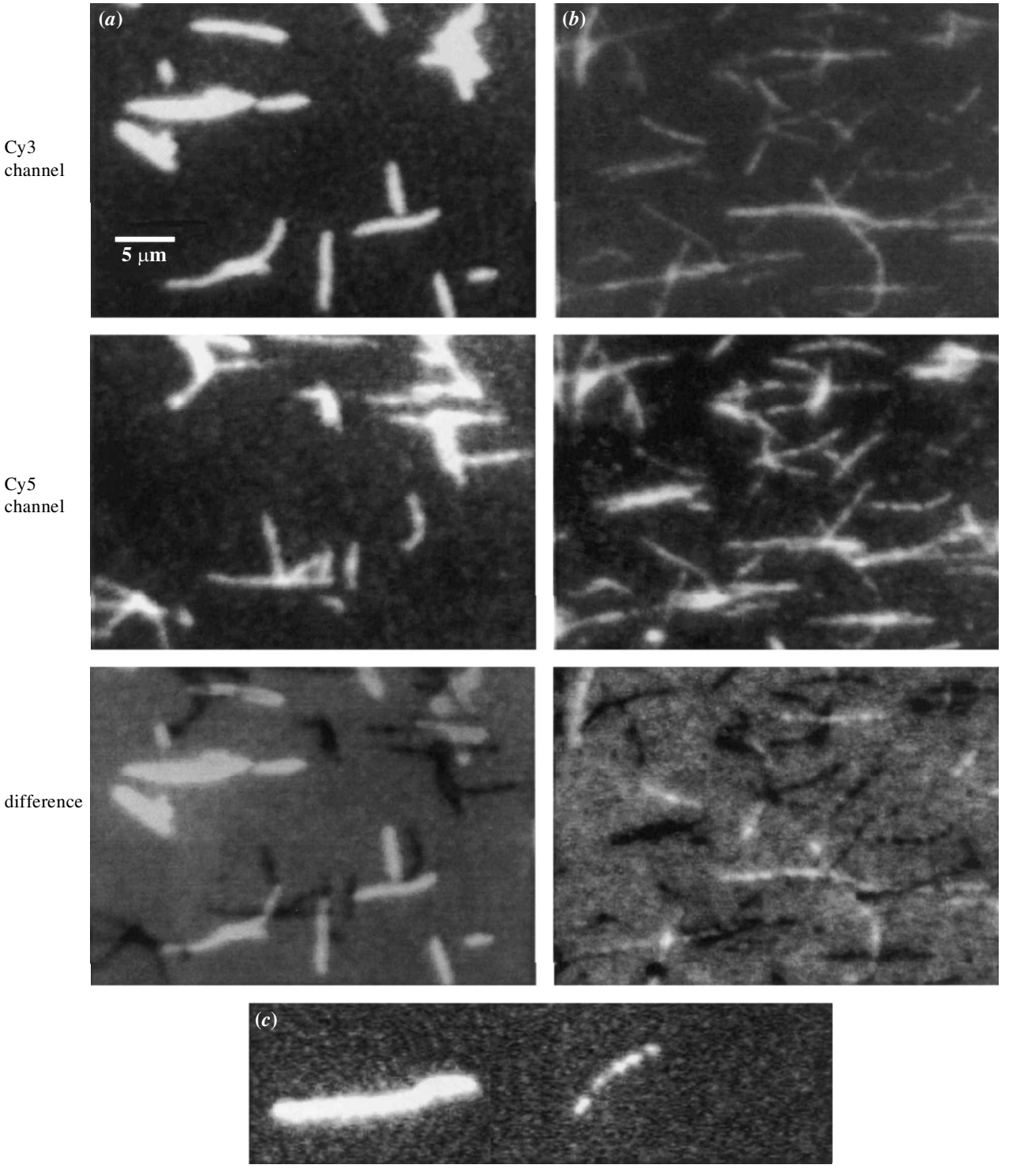

Figure 4. Kinetics of the exchange of myosin between filaments. Synthetic filaments labelled with either Cy3 or Cy5 were prepared as follows. Solubilized myosin (4.6 μM heads) was incubated at 20 °C for 4 h with either Cy3-EDA-ADP or Cy5-EDA-ADP (10 μM) and AlF_4^- (0.1 mM $AlCl_3$ and 5 mM NaF) in a buffer comprising 0.5 M NaCl, 1 mM $MgCl_2$ and 20 mM imidazole (corrected to pH 6.8 at 20 °C). Unlabelled myosin was also prepared containing trapped ADP (10 μM). Cy3-, Cy5- and unlabelled-trapped myosin preparations were separately dialysed for 12 h at 4 °C against a large excess of a buffer comprising 0.12 M NaCl, 1 mM $MgCl_2$, 100 μM ADP and 20 mM imidazole (corrected to pH 6.8 at 20 °C). The excess ADP was present in the exchange reaction mixtures to prevent any fluorescent nucleotide from rebinding should it dissociate from the active site. The Cy3- and Cy5-trapped myosin filaments were mixed in a 1:1 ratio and incubated at 4 °C: (*a*) incubation for 1 min and (*b*) incubation for 48 h. Aliquots were removed at the time-intervals shown, diluted with dialysis buffer to 200 nM (myosin heads) and observed as described in figure 1. Difference images were computed using the NIH Image software (bottom panels). White filaments represent those having a high Cy3 intensity whereas black filaments represent those having a high Cy5 intensity. In the case of (*b*), the difference image was contrast enhanced to emphasize the two classes of filaments, although the separate Cy3 and Cy5 images show that each filament contains both labels. (*c*) Synthetic filaments were labelled with Cy3 as above and were incubated with unlabelled filaments to allow exchange for 2 h before loading into the flow cell.

the myosin density given the possibility of blinking behaviour preferentially obscuring two-step profiles and of photobleached fluorophores quenching unbleached fluorophores in their vicinity (Vanden Bout *et al.* 1997). Nevertheless, both correction procedures suggest that synthetic filaments contain more myosin than native filaments with an excess of two- to fourfold.

(c) *Exchange of monomers between filaments*

The exchange of myosin molecules between filaments was followed by incubating preformed myosin filaments containing trapped Cy3-ADP with filaments containing trapped Cy5-ADP. Within 1 min of mixing, the individual Cy3- and Cy5-labelled filaments were easily distinguished and there was no evidence of significant exchange between them (figure 4*a*). However, the dynamic range of the camera was insufficient to detect the limited exchange (i.e. at the level of a single molecule) when fully labelled filaments were in the same field of view. After a 1 h incubation at 4 °C, the Cy3 and Cy5 filaments remained distinct. In contrast, after 24 h incubation, all filaments were labelled with both fluorophores indicating significant exchange between them. However, the exchange was not complete. Filaments which were bright in the Cy3 channel were usually relatively dull in the Cy5 channel and vice versa. This situation was still apparent after a further 24 h (figure 4*b*). Thus, the bright filaments were assumed to reflect the label initially present in the filament before mixing, while dull filaments corresponded to those that acquired the other fluorophore by exchange. The separate origins of the filaments could be clearly demonstrated by computing a difference image from the individual Cy3 and Cy5 images (figure 4*b*). The observation of distinct and continuous dark or light filaments in the difference image, randomly distributed across the field, rules out an artefact due to uneven or offset excitation by the two lasers.

In an attempt to visualize an early phase of the exchange, Cy3-ADP-trapped filaments were mixed with unlabelled ADP-trapped filaments (to avoid the potential problems of fluorescence cross-talk between the Cy3 and Cy5 labels) and incubated for 2 h. The images contained continuous bright filaments as well as dull punctate ones (figure 4*c*). The integrated intensity of the punctate filaments showed the number of labelled myosins to be 20–30% of the bright filaments and, thus, the highlights correspond to clusters of tens of molecules rather than single molecules.

On the basis of the relative fluorescence intensities of the filaments, we concluded that the exchange proceeds on a time-scale of several hours to an extent of around 75% of the total myosin after 48 h (at 4 °C) and further exchange, if it occurs at all, is even slower. This result contrasts with Saad *et al.* (1991) who concluded that nearly 100% exchange of myosin monomers between filaments occurred within 3 h (at 20 °C) on the basis of indirect fluorescence resonance energy transfer (FRET) measurements.

4. DISCUSSION

As found in previous studies, synthetic myosin filaments prepared at pH 7.0 have a higher molecular density than native filaments (Kaminer & Bell 1966; Emes & Rowe 1978). We showed that this holds for myosin filaments which have been immobilized on a silica substrate and perfused with myosin-free buffer for several minutes (i.e. the excess myosin does not rapidly dissociate when the monomer pool is reduced). Davis (1993) argued that the additional myosin represents an adventitious layer around a more stable core structure. On this basis, Davis (1993) considered that the exchange of myosin between filaments reported by Saad *et al.* (1991) might be restricted to this adventitious layer because his own studies indicated filaments prepared at pH 8.0 showed exchange at the filament ends only. This explanation is not compatible with Saad *et al.*'s (1991) conclusion that monomer exchange between filaments was nearly 100% complete within 3 h. However, the latter was based on a FRET assay involving multiple labelling sites and, thus, susceptible to nonlinear errors. Indeed, in an alternative assay using electron microscopy and biotin probes, Saad *et al.* (1991) showed that, when long and short filament preparations were mixed, the exchange of monomers occurred over 1 h but the filaments retained their original length distribution. We have observed a similar phenomenon by mixing short Cy5-labelled filaments with long Cy3 filaments. Exchange occurred between the fluorescent myosins but the filaments maintained their original length distribution. This result would seem unlikely unless the filaments retained a stable core. Our data (figure 4) is more compatible with Davis' (1993) conclusion that only the adventitious layer is exchangeable and the core is stable for >48 h. Although we observed partial exchange throughout the length of the filament (figure 4*c*), we did not note preferential exchange at the ends where monomer addition and release from the core might be expected (Davis 1993). It is possible that, during the dilution procedure and initial perfusion of the flow cell, this potentially exchangeable region of a myosin filament undergoes net dissociation. Once adhered to the silica, the dynamic behaviour of the filament, as envisaged in the Davis (1993) model, might be inhibited and only surface adventitious myosin would be available for exchange.

Funatsu *et al.* (1995) used intensity fluctuations to determine nucleotide binding and release kinetics at the level of single myosin molecules. Oiwa *et al.* (1999) employed a similar approach using myosin filaments, with the advantage that spots within a linear array can be more reliably identified as being derived from myosin itself rather than artefacts. The multipoint attachments of myosin filaments to the surface also ensures they are well immobilized and less likely to detach and reattach during the assay than monomeric myosin (cf. Conibear *et al.* 1998). Although myosin monomers may dissociate and re-bind to immobilized filaments, given that exchange clearly occurs between filaments in suspension, we calculated that the expected rate is in the order of one molecule per minute per micrometre of filament and, therefore, at low labelling ratios (e.g. 1:1000), the observed number of fluctuations arising from monomer release and reincorporation would be very low. Nevertheless, even with myosin filaments we have observed fluctuations in trapped fluorophore intensity on a seconds time-scale (figures 2*d* and 3). Any such fluctuations occurring in single-molecule

ATPase assays would tend to reduce the average residence time and may account for the generally higher values obtained for the catalytic cycle rate compared with bulk assays (Oiwa *et al*. 1999).

One of the reasons for characterizing the myosin density of synthetic myosin filaments on silica surfaces concerns their use in *in vitro* motility assays. While we have noted F-actin sliding on such preparations, when sliding is initiated from the rigor state by photolysis of caged ATP, the most common result is that the actin dissociates rather than slides (Bagshaw & Conibear 1999). On the other hand, if the same myosin filament preparation is applied at high density so that there are many filament cross-overs or the filaments are clumped by reducing the ionic strength to 40 mM prior to loading the flow cell, then actin tends to slide along the whole length of the filament aggregate. The axial distance between myosin heads in single synthetic filaments ($\leqslant$14.3 nm depending on the arrangement of the adventitious myosin) appears at least as small as the minimum nearest-neighbour distance which supports motility in conventional assays using random arrays of myosin (Uyeda *et al*. 1990). Thus, the dimensionality of the surface also appears to be important. In the case of single myosin filaments, it is possible that the flexibility of F-actin causes the latter to lose contact and bend at its leading end and, having done so, the lack of any off-axis myosin heads would result in complete dissociation. In random assays or on aggregated myosin filaments however, the actin may continue to slide in a new direction or on a new filament.

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