Detection of Actin Assembly by Fluorescence Energy Transfer

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ABSTRACT Fluorescence energy transfer was used to measure the assembly and disassembly of actin filaments. Actin was labeled at cysteine 373 with an energy donor (5-iodoacetamidofluorescein) or an energy acceptor (tetramethylrhodamine iodoacetamide or eosin iodoacetamide). Donor-labeled actin and acceptor-labeled actin were coassembled. The dependence of the transfer efficiency on the mole fraction of acceptor-labeled actin showed that the radial coordinate of the label at cysteine 373 is ~35 Å, which means that this site is located near the outer surface of the filament. The distance between a donor and the closest acceptor in such a filament is 58 Å. The increase in fluorescence after the mixing of actin filaments containing both donor and acceptor with unlabeled filaments showed that there is a slow continuous exchange of actin units. The rate of exchange was markedly accelerated when the filaments were sonicated. The rapid loss of energy transfer caused by mechanical shear probably resulted from an increase in the number of filament ends, which in turn accelerated the exchange of monomeric actin units. Energy transfer promises to be a valuable tool in characterizing the assembly and dynamics of actin and other cytoskeletal and contractile proteins in vitro and in intact cells.

A fundamental problem in cell motility is to define the state of assembly and interaction of the major cytoskeletal and contractile proteins both in vitro and in vivo. One example is the role played by actin assembly and disassembly during motile events. To date, actin assembly has usually been assayed indirectly both in vitro and in vivo. Experiments using viscosity, light scattering, and electron microscopy yield results that could be interpreted in several ways (2, 10, 12, 13, 20, 44; see Taylor and Condeelis [32] and Uyemura and Spudich [37] for reviews). Furthermore, more direct measurements of assembly using sedimentation assays (3, 25) can only be applied in vitro. Therefore, techniques must be developed that would yield direct evidence for the state of actin assembly and interaction with specific molecules even in living cells.

Fluorescence spectroscopy has already generated some information in vitro on the assembly of actin (4, 6, 40), the structure of actin (14, 15, 19, 21, 31), and the interaction of actin with associated proteins (6, 23, 24).[1] Recently, the technique of molecular cytochemistry (33, 40; see Taylor and Wang [39] for a review) has opened the possibility of making spectroscopic measurements of fluorescently labeled actin in living cells. Actin labeled with 5-iodoacetamidofluorescein has been studied initially, because the site of labeling is known, the extinction coefficient is high, and it is excited by a relatively long wavelength of light (495 nm), which minimizes autofluorescence and cellular damage (40).

Fluorescence energy transfer (7, 28) is a powerful technique for studying the structure and dynamics of molecular assemblies.[2] Electronic excitation energy can be efficiently transferred between a fluorescent energy donor (such as fluorescein) and a suitable energy acceptor (such as eosin) over distances of the order of 50 Å. This transfer process depends on the inverse sixth power of the distance between the donor and acceptor (11, 18, 29). Hence, we expected that a donor-labeled actin would transfer energy to an adjacent acceptor-labeled actin but not to a more distant one. We show here that fluorescence energy transfer is in fact a valuable method for monitoring the assembly and turnover of actin filaments.

MATERIALS AND METHODS

Materials

5-iodoacetamidofluorescein (IAF) and eosin-5-iodoacetamide (IAE) were purchased from Molecular Probes, Inc., Plano, Tex. Tetramethylrhodamine iodoacetamide (IAR) was obtained from Research Organics, Inc., Cleveland, Ohio. Dithiothreitol (DTT), ATP, and Sephadex G-25 were purchased from Sigma Chemical Co., St. Louis, Mo. DEAE-cellulose (DE-52) was obtained from Whatman, Inc., Clifton, N. J.

Methods

The preparation and labeling of skeletal muscle actin, which was used in all the experiments reported here, was performed as described previously (40). The

[1] Dr. P. Detmers kindly provided D. L. Taylor with a copy of her manuscript (6) before publication.

[2] A similar approach has been used by Berlin and colleagues in investigations on microtubules (9).

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polymerizability of the labeled actins was assayed as discussed previously and is identical to unlabeled actin (40). Unlabeled and labeled actins were stored as F-actin (3.0–9.0 mg/ml) on ice as described by Uyemura et al. (36). Aliquots of F-actin were pelleted in a Beckman airfuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.), depolymerized, repolymerized, and finally depolymerized and clarified (25). The resulting G-actin was dialyzed vs. buffer G (3.0 mM Tris-HCl, 0.2 mM ATP, 0.2 mM DTT, 0.02% sodium azide, pH 7.5) until used in an experiment (within 8 h of preparation). Polymerization of actin was initiated by the addition of KCl to 100 mM and $MgCl_2$ to 2.0 mM.

The concentration and dye-protein ratios of the various actins were determined according to published procedures (40). The extinction coefficients (ϵ) of the labeled actins are as follows: IAF-labeled actin (AF-actin), $\epsilon_{495} = 6.0 \times 10^4$ cm^{-1} M^{-1}, IAE-labeled actin (AE-actin), $\epsilon_{530} = 7.0 \times 10^4$ cm^{-1} M^{-1}, and IAR-labeled actin (AR-actin), $\epsilon_{555} = 2.4 \times 10^4$ cm^{-1} M^{-1}.

A Cary-14 spectrophotometer and a Spex Fluorolog fluorometer (Spex Industries, Inc., Metuchen, N. J.) were used for absorption and fluorescence measurements, respectively. 2 mm × 2 mm or 1 mm × 1 mm quartz cuvettes were used and the ODs of all solutions were maintained below 0.05 at the peak absorption for all probes used in the experiments. Under these conditions, actin concentrations as high as 0.5 mg/ml can be investigated without inner filter effects. 10-mm slits were used for excitation and emission. A fluorescence lifetime (τ) instrument was used either with the 2 mm × 2 mm cuvettes in the standard configuration (43), or coupled to a Zeiss Universal Microscope fitted with epifluorescence optics.[3]

Calculations and measurements required to determine the Forster critical distance (R_0) and the efficiency of energy transfer (E) were performed as described previously (7, 28).

$$R_0 = (9.7 \times 10^3 \text{ Å}) (J K^2 \Phi_D N^{-4})^{1/6}$$

The overlap integral (J) was calculated for each donor-acceptor pair with corrected spectra. The orientation factor was calculated assuming that $K^2 = 2/3$. Nanosecond emission anisotropy measurements showed that the energy donor rotated quite freely during its excited-state lifetime. The quantum yield of the donor (AF-actin) was determined by comparison with a standard fluorescein solution (10^{-6} M sodium fluorescein in 0.01 N NaOH, pH 12.0 $\phi = 0.79$).

The distance (r) between the donor and acceptor was calculated as follows:

$$r = R_0(E^{-1} - 1)^{1/6}$$

The efficiency (E) of energy transfer was determined by measuring the donor quenching and decrease in fluorescence lifetime (τ) of the donor-acceptor complexes compared with the donor control.

Energy transfer in actin filaments formed by the coassembly of AF-actin and either AR-actin or AE-actin was measured with various molar ratios of donor: acceptor:unlabeled actin. The probability of donors in contact with acceptors was determined by calculating the polynomial distribution. In some experiments the molar ratios were adjusted to ensure that 90% of all donors were in contact with at least one acceptor-labeled subunit. The measured efficiency of energy transfer was subsequently corrected for the critical concentration of actin by substracting the donor fluorescence remaining in the supernate after pelleting the actin filaments in an airfuge.

Chaos carolinensis was microinjected with AF-actin according to the published protocol (35).

RESULTS

The fluorescence intensity of fluorescein covalently attached to actin decreased by 15% over several minutes when monomeric actin was polymerized by the addition of KCl and $MgCl_2$ (Fig. 1). Viscosity measurements confirmed that this fluorescence change was attributable to the assembly of actin filaments. The degree of fluorescence quenching was larger when AF-actin was coassembled with actin monomers containing a prospective energy acceptor than with unlabeled actin. The extent of quenching in filaments with 0.91 mole fraction AR-actin was 28%. More quenching (37%) was observed in filaments containing 0.74 mole fraction AE-actin. The efficiencies of energy transfer, calculated by taking into account the quenching caused by filament formation in the absence of an energy acceptor, are 16% for filaments containing AR-actin and 27% for those with AE-actin (Table I). As expected, eosin is a more

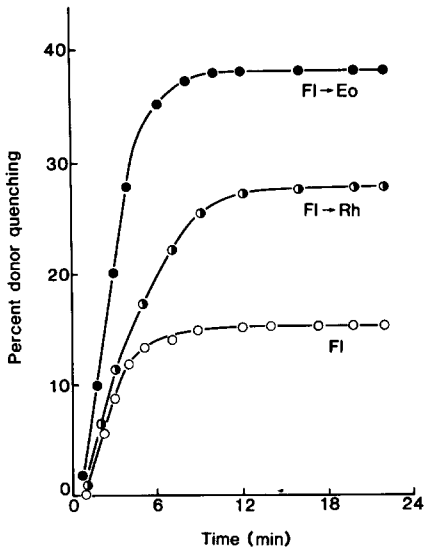

FIGURE 1 Donor-actin (AF-actin) quenching during coassembly with either AR-actin or AE-actin and unlabeled actin. The molar ratios are of donor:acceptor:unlabeled actins. (○) 1:0:10, AF-actin exhibits self quenching during assembly (41); (◑) 1:10:0, using AR-actin as acceptor; (●) 1:25:8, using AE-actin as acceptor. Polymerization was initiated by adding KCl to 100 mM and $MgCl_2$ to 2.0 mM, vortexing the mixtures of actin monomers and salts, and then placing the solution (0.45 mg/ml actin) in the cuvette. The first possible time-point under the present conditions is at ~1 min.

TABLE I

Transfer Efficiencies in Coassembled Filaments

Acceptor	Molar ratios D:A:U*	Acceptor mole fraction	Transfer efficiency
			%
Rhodamine	1:12:18	0.39	10
	1:1:0	0.50	11
	1:65:30	0.67	12
	1:10:0	0.91	16
Eosin	1:25:8	0.74	27
	1:19:14	0.56	21

* Donor:acceptor:unlabeled.

effective energy acceptor than rhodamine (R_0 = 45.6 Å compared with 37.4 Å) because its absorption spectrum more closely matches the emission spectrum of fluorescein.

Nanosecond fluorescence measurements (Fig. 2) provided additional evidence for the occurrence of energy transfer in filaments containing AF-actin and AE-actin. The mean excited state lifetime of AF-actin coassembled with AE-actin (0.67 mole fraction) was 2.7 ns, compared with 3.5 ns for AF-actin in filaments containing unlabeled actin. This shortening of the excited state lifetime corresponds to 23% energy transfer.

The observed transfer efficiencies provide information concerning the locations of the covalently attached chromophores in the actin filament helix. F-actin can be approximated as a 13/6 helix (13 monomers in 6 turns of a single-start left-handed helix); each successive monomer (~50 Å in diameter) is translated axially 27.5 Å, rotated by 166°, and comes into contact with four adjacent monomers in the filaments, which are 70 Å in diameter. Thus the coordinate of a particular group in unit n of an actin helix are: $x_n = a \cos (n \times 166°)$, $y_n = a \sin (n \times 166°)$, and $z_n = n \times 27.5$, where a is the radial coordinate of

[3] Reidler, J., and L. Stryer. Manuscript in preparation.

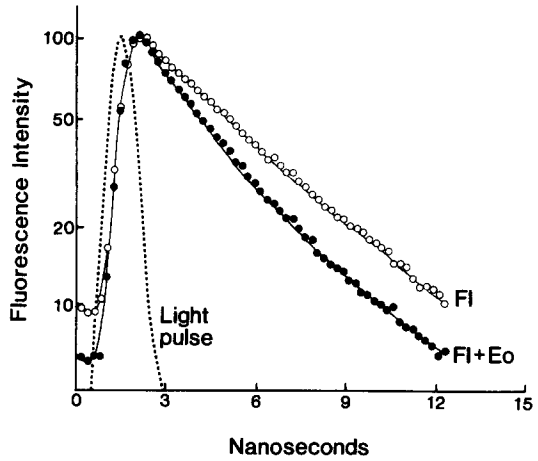

FIGURE 2 Lifetime of AF-actin fluorescence. A Spectra-Physics 171 mode-locked argon-ion laser tuned to 4579 Å was used to excite fluorescent molecules in solution. A 3-70 long-pass filter (Corning Glass Works, Science Products Div., Corning, N. Y.) and 5200-Å narrow-pass interference filter (Optical Thin Films, North Andover, Mass.) were combined to eliminate laser light and acceptor fluorescence. Fluorescein fluorescence was collected using single photon counting techniques in a fast, sensitive phototube (no. 8850; RCA Solid State, Somerville, N. J.) and analyzed according to standard techniques (44). AF-actin coassembled with unlabeled actin in a molar ratio of 1:26 served as the control (○) and was compared to AF-actin coassembled with AE-actin and unlabeled actin (●) in a molar ratio of 1:18:8. The final actin concentration was 0.50 mg/ml. The average lifetime (τ) of the AF-actin coassembled with AE-actin is decreased by 23% compared with the AF-actin control.

the group (i.e., its distance from the helix axis). The energy donor and acceptors used in this study were attached to cysteine 373. Hence, the distance r_n between a donor on actin unit O and an acceptor on unit n is given by

$$\begin{aligned} r_n &= [(\Delta x)^2 + (\Delta y)^2 + (\Delta z)^2]^{1/2} \\ &= [a^2(1 - \cos(n \times 166°))^2 \\ &\quad + a^2 \sin^2(n \times 166°) \\ &\quad + (n \times 27.5)^2]^{1/2}. \end{aligned}$$

This equation shows that the donor-acceptor distance is markedly dependent on the radial coordinate. For example, if cysteine 373 were on the helix axis ($a = 0$), the distance between chromophores one and two units apart would be 27.5 and 55 Å, respectively. On the other hand, if cysteine 373 were near the outside of the filament (a = 35 Å), the corresponding distances would be 74.7 and 57.5 Å. Thus, the smallest possible distance between a donor and an acceptor would be 27.5 Å for groups located on the helix axis, compared with 57.5 Å if they were positioned on the outside of the filament (Fig. 3). This large difference makes it feasible to experimentally determine the radial coordinate of the attached groups. The calculated dependence of the transfer efficiency on the radial coordinate of the groups and on the mole fraction of energy acceptor is shown in Fig. 4. The observed transfer efficiencies best fit the lines calculated for large radial coordinates ($a \cong 35$ Å). Thus, the energy donor and acceptors attached to cysteine 373 are located near the outside of the actin helix. Consequently, much of the energy transfer is to an acceptor on the actin unit that is directly above or directly below the one containing the donor (i.e., if the donor is in actin unit *0*, the acceptor is in unit *2* or *−2*; see Fig. 3).

Having defined the structural basis of the energy transfer process, we proceeded to use this fluorescence technique to monitor rearrangements of actin filaments. Suppose that filaments containing both donors and acceptors are mixed with an excess of filaments consisting only of unlabeled actin. If these two types of filaments are stable, no change in fluorescence

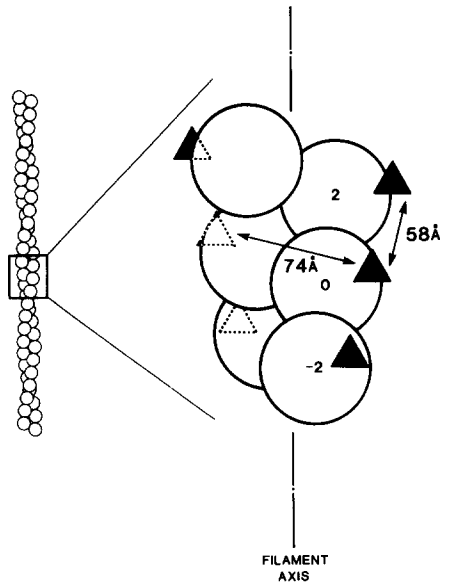

FIGURE 3 Diagram of F-actin indicating one possible relationship between the residue, cysteine 373, in adjacent monomers of a filament. Each monomer is in contact with four other monomers in a left-handed single-start helix. This diagram depicts cysteine 373 (▲) on the surface of the filament. Under these conditions, energy transfer could occur from the monomer labeled *0* to two possible acceptors (*2* and *−2*).

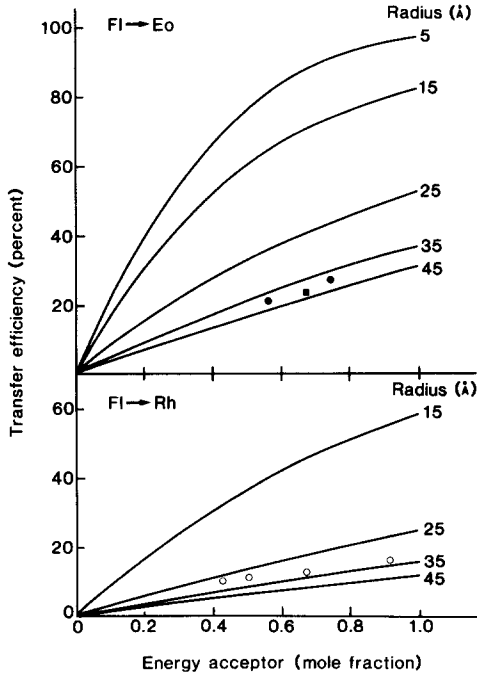

FIGURE 4 The solid lines depict the calculated dependence of the transfer efficiency on the mole fraction of energy acceptor for different radial coordinates of the labeled site. The experimental points are: ○, steady-state measurements of fluorescein-to-rhodamine energy transfer; ●, steady-state measurements of fluorescein-to-eosin energy transfer; ■, excited-state lifetime measurement of fluorescein-to-eosin energy transfer.

intensity will be observed. On the other hand, if extensive disassembly and reassembly take place, there will be a decrease in the efficiency of energy transfer because the mole fraction of acceptor in the newly formed filaments will be low (Fig. 5). As shown in Fig. 6*A*, there was a very slow change in fluorescence intensity when these two types of filaments were gently mixed. The solution was not stirred in this period. In contrast, sonication of the solution (see arrows in Fig. 6*A*) led to a nearly complete loss of energy transfer. On the other hand, sonication of a solution of a single type of filament containing both donors and acceptors had no effect on its fluorescence intensity (Fig. 6*B*). The simplest interpretation of these experiments is that mechanical shear markedly accelerated the turnover of actin filaments so that unlabeled actin units became intimately intermixed with the labeled ones, as depicted in Fig. 5.

An intriguing possibility emerges from these studies. Quantitative analysis of energy transfer between labeled actin monomers introduced into live cells could yield crucial information on the dynamics of actin filaments in vivo. Preliminary measurements of the lifetime of AF-actin fluorescence in living cells (Fig. 7) indicates that the cytoplasm of *C. carolinensis* does not quench the fluorescence. Thus, it may be possible to detect energy transfer between actin molecules in living cells without the complication of nonspecific quenchers.

DISCUSSION

We have measured energy transfer in actin filaments containing monomers labeled at cysteine 373 (cys 373) with energy donors and acceptors. These experiments show that cys 373 is near the surface of the filament. A number of other observations are in agreement with this conclusion. Cys 373 is not located at the sites of actin-actin interaction during assembly, as demonstrated by its accessibility during labeling of F-actin and the absence of effect on polymerizability after modification (37). However, cys 373 is within 12 Å of a neighboring monomer, as judged by cross-linking experiments (16). There is also evidence that cys 373 is close to, but not part of, the active site for actin-myosin interactions (19, 26, 30). In addition, large

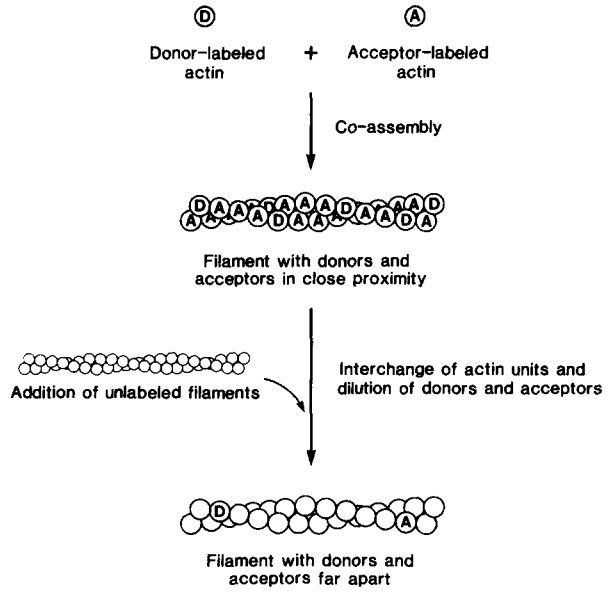

FIGURE 5 Experimental strategy for using energy transfer to monitor filament turnover. Interchange of actin units leads to a loss of energy transfer because donors and acceptors become separated.

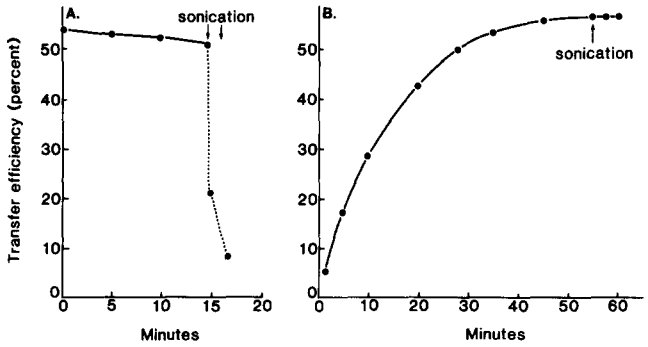

FIGURE 6 Effect of mechanical disruption of labeled filaments on donor quenching in the presence and absence of unlabeled filaments. (*A*) AF-actin, AE-actin, and unlabeled actin monomers were mixed at final ratios of 1:13:5 and coassembled by the addition of KCl and $MgCl_2$. The total actin concentration was 0.25 mg/ml. Unlabeled actin at 1.0 mg/ml was assembled separately by the addition of KCl and $MgCl_2$, and then the labeled filaments and unlabeled filaments were mixed at time = 0 min by gentle rolling in a test tube. The final ratio of donor:acceptor:unlabeled actin was 1:13:45. Nearly all of the unlabeled actins were localized in separate actin filaments immediately after mixing. A continuous decrease in donor quenching of ~5%/h occurred when the filaments were not perturbed. Filament disruption by sonication (two separate bursts denoted by the arrows) caused an essentially complete loss of energy transfer. (*B*) Control experiment for the filament disruption assay. AF-actin, AE-actin, and unlabeled actin monomers were mixed at ratios of 1:13:5. The actins were coassembled by the addition of KCl and $MgCl_2$. The extent of energy transfer increases with filament assembly. The total concentration of actin was 0.25 mg/ml. The extent of energy transfer was unaffected by sonication. The time between sonication and the next measurement was ~1.0 min.

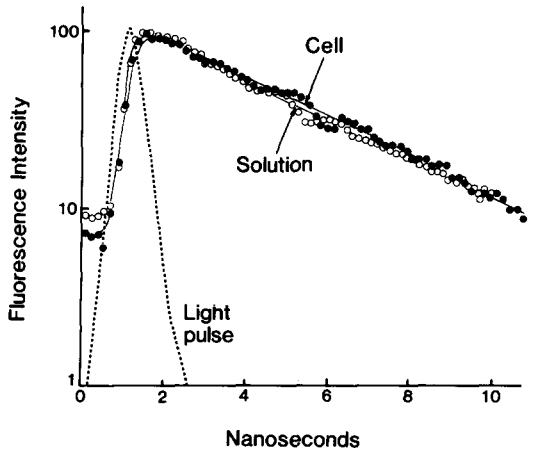

FIGURE 7 The lifetime (fluorescence intensity vs. time) of AF-actin fluorescence measured through a microscope.[3] (○) Lifetime fit for AF-actin coassembled with unlabeled actin in a 1:10 molar ratio and placed on microslides (0.1-mm microslides; Vitro Dynamics, Rockaway, N. J.). (●) Lifetime fit for AF-actin coassembled with unlabeled actin in a 1:10 molar ratio and microinjected into living *C. carolinensis* (~4 nl of 0.5 mg/ml actin was injected into the cell).

sulfhydryl reagents (i.e., IAF and 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid) labeled predominantly cys 373, where smaller reagents (i.e., iodoacetamide) can label up to three cysteines (24). Finally, quenching studies also suggest that cys 373 is at or near the surface of the filament (31).

The results in Fig. 1 demonstrate that energy transfer can be used as a sensitive assay for actin assembly. The large spectral

overlap integral for AF-actin to AE-actin makes this the optimum donor-acceptor pair studied so far. Under the present conditions, there appears to be no time lag in energy transfer after initiation of assembly, which is different from the results obtained in viscosity assays (20; footnote 4). However, the data are consistent with OD_{232} measurements (5; footnote 4), which suggest that no lag occurs in the conformational changes associated with assembly. Further studies on this point will involve additional steps to purify actin monomers free of oligomers (nuclei for assembly) and possibly the use of a rapid mixing chamber to study the first 30 s of assembly. It should be noted that newer batches of eosin iodoacetamide and eosin maleimide have consistently yielded higher transfer efficiencies (see Fig. 6). These eosins appear to be less brominated than the earlier samples. An altered spectral overlap integral could account for the observed differences.

Actin filaments exhibit an ATP-driven continuous exchange of monomers under the ionic conditions used here, and this exchange involves primarily the ends of the actin filaments (25, 41. We report here the rapid loss of energy transfer in a mixed population of doubly labeled and unlabeled filaments upon mechanical shear by sonication (Fig. 6). This loss of transfer would be explained if actin filaments rapidly disassembled and reassembled, as depicted in Fig. 5. These results are consistent with the large increase in actin ATPase activity when filaments are sheared in the same fashion (1, 22). The simplest explanation of the present results is that the number of ends, and therefore the monomer exchange rate, is increased significantly by mechanical shear.

The technique of molecular cytochemistry has been developed in order to study both the distribution and the interaction of specific molecules in vivo (such as the polymerization of actin) (33–35, 38–40). The early applications of molecular cytochemistry indicate that fluorescently labeled molecules can be incorporated into cellular pools. AF-actin has been characterized biochemically and spectroscopically (40) and microinjected into living cells. *Physarum* (33), *C. carolinensis* (35), and sea urchin eggs (39) all exhibit distributions of fluorescent actin that indicate a normal incorporation in the cellular actin pool. Furthermore, various controls, particularly in *C. carolinensis*, suggest the specific association of labeled actin with endogenous cytoskeletal and contractile proteins. Recent applications of molecular cytochemistry to tissue culture cells also indicate that contractile proteins including actin become distributed as predicted by immunofluorescence (8, 17, 42).

It has been suggested that molecular cytochemistry could yield its greatest value when used in conjunction with microspectrofluorometric techniques (33, 34, 40). Preliminary measurements of the lifetime of AF-actin fluorescence in living cells (Fig. 7) indicate that the cytoplasm of *C. carolinensis* does not quench the fluorescence. Therefore, it might be possible to detect energy transfer of actin in living cells without the added complication of nonspecific quenchers. Quantitative molecular cytochemistry could then yield information on both the distribution and interaction of specific molecules in living cells (34).

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