Studies on the structure of actin gels using time correlation spectroscopy of fluorescent beads

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ABSTRACT Fluorescence correlation spectroscopy (FCS) has been used to measure the diffusion of fluorescently labeled beads in solutions of polymerized actin or buffer. The results, obtained at actin concentrations of 1 mg/ml, show that small beads (0.09 μm in diameter) diffuse nearly as rapidly in the actin gel as in buffer, whereas the largest beads tested (0.5 μm in diameter) are immobilized. Measured autocorrelation times for motions of beads with intermediate sizes show that the diffusion is retarded (relative to buffer) and that the time behavior cannot be represented as a single diffusive process. In addition to the retarded diffusion observed over distances > 1 μm, 0.23-μm beads also show a faster motion over smaller distances. Based on the measured rate of this faster motion, we estimate that the beads may be constrained within a cage ~0.67 μm on a side, equal to a filament length of ~250 subunits. Fluorescence correlation spectroscopy measurements made in the same small spot (radius of 1.4 μm) of the gel vary over time. From the variations of both the autocorrelation functions and the mean fluorescence, we conclude that, corresponding to a spatial scale of 1.4 μm, the actin gel is a dynamic structure with slow rearrangement of the gel occurring over periods of 20–50 s at 21–22°C. This rearrangement may result from local reorganization of the actin matrix. Data for the retardation of beads by the actin gel are consistent with a detailed theory of the diffusion of particles through solutions of rigid rods that have longitudinal diffusion coefficients much less than that of the particles (Ogston, A. G., B. N. Preston, and J. D. Wells. 1973. *Proc. R. Soc. Lond. A.* 333:297–316).

INTRODUCTION

It generally is believed that actin filaments, organized in various states such as random networks and filament bundles by regulatory actin binding proteins (Stossel et al., 1985; Pollard and Cooper, 1986), help to determine a host of physiological characteristics of living cells, ranging from their shapes to their motility (Bray and White 1988; Elson, 1988). In vitro, polymerized actin forms a gel due to interactions among the filaments (Korn, 1982; Frieden, 1985). Although the structure of the gel is not well characterized, it may be considered as a partly liquid, partly solid viscoelastic material or as a liquid held in a fibrous network of polymers by the extremely large friction between the liquid and the polymer (Tanaka, 1981). It has been proposed that the dynamics of the gel are controlled either by adhesive interactions among the actin filaments (Sato et al., 1985) or by steric interactions that impede the diffusion of the long rod-like actin filaments (Zaner and Stossel, 1983; Janmey et al., 1986). Certainly the latter interactions must exist, but it has not yet been verified definitively that this minimal model is sufficient to account for the gel dynamics (cf. Elson, 1988).

Because of their physiological and structural importance, actin gels have been the focus of various biophysical and biochemical studies to determine the characteristics of the filaments and their interactions (Fujime, 1972; Doi and Frieden, 1984; Sato et al., 1985; Janmey et al., 1986; Newman et al., 1989; Schmidt et al., 1989; Hou et al., 1990*a, b;* Seils et al., 1990; Janmey et al., 1991). One approach that has yielded useful information is to measure the diffusion of inert fluorescent particles by fluorescence photobleaching recovery (FPR) to probe the matrix structure of the gels. Physical chemical models for diffusion of inert tracers in various complex media, such as concentrated macromolecular solutions, rigid gel networks, and entangled filament networks, have been discussed by Luby-Phelps et al. (1988). The FPR measurements applied to small regions of the gel occupied by few fluorescent particles have inherent problems, however, primarily due to the stochastic character of diffusion. When only a few fluorescent particles are in the observed region, the recovery curves can vary substantially from one to another for purely statistical reasons, and this can obscure variations due to changes in the properties of the gels over space and time (C. Frieden, unpublished data). Averaging the measurements from several observation regions loses information concerning the spatial inhomogeneity of the actin gels (Luby-Phelps et al., 1988; Frieden, unpublished data). In this work, we use fluorescence correlation spectroscopy (FCS) (Elson and Webb, 1975; Elson, 1985; Elson and Qian, 1989) for in vitro studies to provide information complementary to that obtained by FPR. As will be discussed, FCS specifically overcomes the problems associated with FPR measurements and accommodates the requirement for a low concentration of fluorescent particles as well. Furthermore, FCS measures the diffusion of the fluorescent probe over very small distances (on the order of several microns) and thus can characterize a small region of the gel. This work can be extended further to studies of the interaction between actin and its binding proteins that

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may play important roles in cell motility, intracellular transport, and protein movement on the cell surface.

Using FCS, we show that actin gels are dynamic. The retardation of the diffusion of inert probe particles in a defined region of the gel may change either due to local flexion of the filaments or to global rearrangement of the matrix. We demonstrate that, on a microscopic scale, the actin gel is spatially inhomogeneous but that the local organization persists over only a limited time frame. Over a time scale that is long compared with the lifetime of fluctuations in the gel porosity, however, the inhomogeneities in the gel average out. Therefore, whether the gel is considered static or dynamic must be addressed within a given time frame. We also demonstrate that FCS can be used to obtain useful information that could not be obtained by other techniques, such as FPR or dynamic light scattering (DLS) (Luby-Phelps et al., 1988; Newman et al., 1989).

In addition to establishing the methods for measurement, analysis, and data interpretation, the purpose of the present work is twofold. First, we wanted to demonstrate the application of FCS measurements to particle diffusion and the related data analysis and interpretation to study the properties of a dynamic gel matrix and, second, to understand particle diffusion inside a gel, a subject of interest because of its general applicability to the diffusion of cytoplasmic and membrane proteins (Jacobson et al., 1987).

MATERIALS AND METHODS

Monomeric actin was isolated and purified according to the method of Spudich and Watt (1971) with the gel filtration modification of MacLean-Fletcher and Pollard (1980). The actin was stored lyophilized in the presence of sucrose according to the procedure described earlier (Tellam and Frieden, 1982). When needed, it was dissolved in 2 mM tris(hydroxymethyl)-aminomethane-HCl buffer, pH 8, containing 200 μM adenosine triphosphate, 200 μM $CaCl_2$, 1.5 mM NaN_3, dialyzed against the same buffer for $\geq$15 h, and centrifuged. Polymerization was induced by Mg^{2+}, usually 2 mM (Doi and Frieden, 1984).

Fluorescent beads were purchased from Polysciences (Warrington, PA). Actin solutions containing fluorescent beads were polymerized in glass chambers of 1.5 cm diameter and 0.25 cm depth covered with a coverslip. A large sample volume was used to minimize the surface area to volume ratio. Both chambers and microscope coverslips were coated with 1 mg/ml bovine serum albumin to prevent absorption of the actin and beads to the glass surface.

The laser microscope (Zeiss Universal, Thornwood, NY) optical system is as described previously (Tait and Frieden, 1982). Unless specified otherwise, all measurements were performed using a 40×/0.75 objective lens for which ω, the in focus e^{-2} radius of the Gaussian excitation laser beam, is 1.4 μm. The photomultiplier signal was interfaced to a computer (LeCroy 3500; Kinetic System, Oak Park, IL). The data were then transferred to a micro-VAX computer for further data analysis. All the software on the micro-VAX was written in FORTRAN using the IMSL mathematical library subroutines.

Data analysis

For simple diffusing fluorescent particles, the autocorrelation function of fluorescence fluctuations in FCS is expected to vary as $1/(1 + t/\tau_d)$, where $\tau_d = \omega^2/4D$, the characteristic time constant for the particle diffusing through the characteristic distance ω (Elson and Magde, 1974).

The diffusion of fluorescent beads in an actin gel is not simple, and, when using FCS to study processes that occur over a range of time scales, it is important to consider the time range over which FCS is applicable. Each FCS measurement requires that three time parameters be chosen to suit the kinetic characteristics of the process being studied. These are (*a*) the time interval for acquisition of a single data point of fluorescent intensity (T_D, the dwell time), (*b*) the time window used to calculate the time correlation function (T_W, a time that should be at least two- to threefold longer than the correlation time (τ_d) of the major kinetic processes of interest), and (*c*) the total run time over which data are acquired (T_R). In general, $T_D < \tau_d < T_W < T_R$. To obtain an accurate measurement, the total data acquisition run time, T_R, must be much longer than τ_d. According to a rough estimate, the relative error in the measurement is on the order of $N^{-1/2}$, where $N = T_R/\tau_d$ (Koppel, 1974; Qian, 1990).

Processes that occur on different time scales can influence a measured correlation function in different ways. For a process with a correlation time of the same order as the data acquisition run time, T_R, there is not enough statistical sampling in each FCS measurement to yield an accurate characterization. Hence, a process in this time range will contribute a slow kinetic phase to the correlation function with great stochastic variation from measurement to measurement. A still slower process with a correlation time much greater than T_R will not be detected in the FCS correlation function at all because the data acquisition time is too short to record even a single fluctuation event. Even though this latter process will not contribute detectably to the time correlation, its relative contribution to the overall amplitude of the fluctuation signal can be estimated from an analysis of higher moments of the equilibrium distribution of fluctuation amplitudes. This estimate is embodied in a factor γ (see Table 2) that is analogous to an immobile fraction in a FPR measurement (Qian and Elson, 1990). For a sample of fluorophores all of which are mobile within the time range of the measurement, $\gamma = 0.5$. On the other hand, a kinetic process that occurs over a time short compared with the dwell time also cannot be resolved in the FCS correlation function. Nevertheless, a very rapid process will contribute to the measured shot noise. Even though its characteristic time cannot be obtained, the amplitude of this process can still be estimated, again, by moment analysis (Qian and Elson, 1990). A similar approach can be found in studies of ion channel kinetics (Heinemann and Sigworth, 1991).

Therefore, choosing the optimal dwell time as well as the optimal data acquisition time is important in experiments dealing with processes that occur in different time ranges. For example, to characterize a process with a 1-s correlation time from a measurement with T_R = 100 s, two types of calculations can be used. One is the standard calculation of the correlation function out to T_W = 3 s, for example, from the

TABLE 1 Diffusion of beads of different sizes in actin gels

	Bead diameter	τ_d/τ_0*
	μm	
Actin polymerized with	0.13	2.3
1 mM Mg^{2+}	0.3	9.0
Actin polymerized	0.09	1.4
with 2 mM Mg^{2+}	0.23	5.4
	0.28	6.0
	0.5	∞‡

Experimental conditions: 1 mg/ml actin, 2 mM Tris/Cl, pH 8, 200 μM Ca^{2+}, 200 μM ATP, 1.5 mM NaN_3, 21–22°C. * τ_0 is the correlation time under the same conditions in unpolymerized G-actin. ‡Essentially immobile as measured by video microscopy.

entire data record. In the other, the 100-s interval is separated into 10 portions of 10 s each. The 3-s time correlation function is calculated for each of the 10 data segments and then averaged to yield an overall time correlation. The second calculation serves as a low frequency filter, biased against the slow kinetic component.[1]

RESULTS AND DISCUSSION

The general features of the diffusion of beads of different sizes in actin gels are summarized in Table 1, which provides data on the processes of major correlation amplitude for each of the beads. The table lists the ratio of the average diffusion correlation times, τ_d, of beads in the gels relative to beads in unpolymerized G-actin. Since τ_d is inversely proportional to the diffusion coefficient D, the ratio of correlation times is related inversely to the ratio of diffusion coefficients (cf. Hou et al., 1990*b*). The diffusion coefficients measured in buffer for each bead size are consistent with expectation from Stokes law, i.e., $\tau_d = \omega^2/4D = 3\omega^2\pi\eta d/kT \simeq 1.1 \times d$ s, where d is the diameter of the bead in microns, and $\eta \simeq 10^{-3}$ kg m^{-1} s^{-1} is the viscosity of water. In our experimental measurements, τ_d equals 0.1 and 0.24 s for 0.09 and 0.23 μm diameter beads in G-buffer, respectively. Furthermore, the scatter of the measurements of the diffusion of beads in G-buffer is within the expected statistical uncertainty, <5%. The presence of the actin gel only slightly retards the diffusion of the smallest (0.09 μm diameter) beads examined but immobilizes the largest (0.5 μm diameter).

More detailed examination reveals that the diffusion of beads is complex; several kinetic phases can be observed. In addition to the major process characterized in Table 1, attributed to the diffusion of particles over a range of microns hindered by the actin filament matrix, there are also faster and slower components in the measured correlation functions. As discussed below, the faster component is attributed to the rapid motion of a bead trapped within small cavity within the matrix. We also have seen a much slower, somewhat variable contribution to the correlation functions, which we attribute to a slow stochastic rearrangement of the gel structure. This last process could be complicated and involve several time scales in accord with the recent suggestion by Nagle (1992). Our analysis is not sufficiently accurate, however, to investigate the detailed mechanism of the observed slow processes.

Variation of gel properties over time and space

The rate of bead diffusion varies significantly both with time and with position in the gel. Fig. 1 (*top*) and Table 2 demonstrate the variability of measurements of the dif-

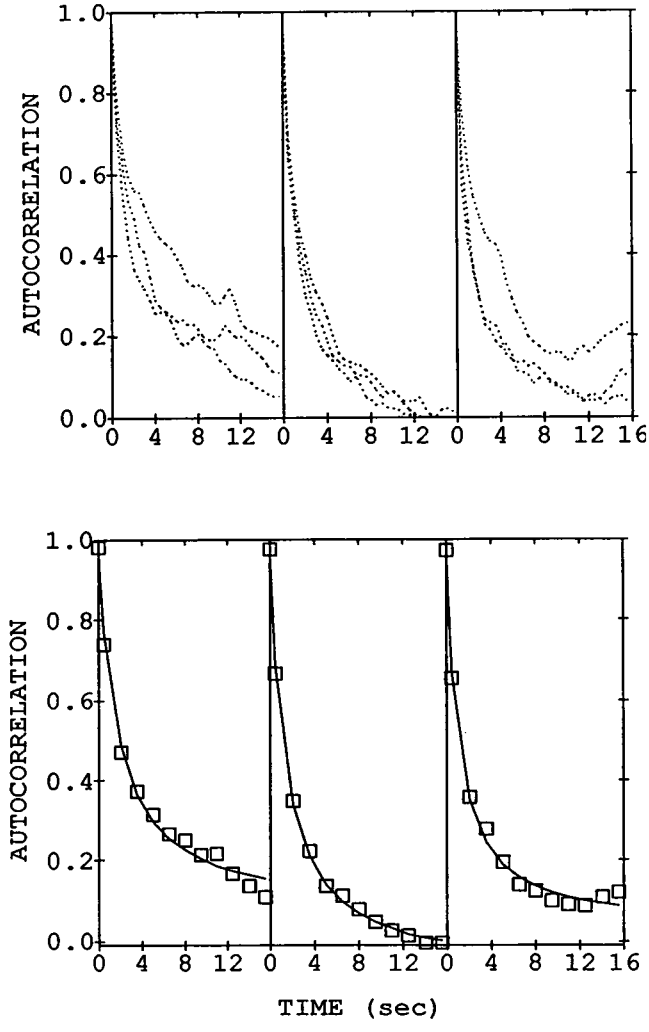

FIGURE 1 Normalized FCS autocorrelation functions for diffusion of 0.23-μm diameter fluorescence microspheres in an actin gel (1 mg/ml actin polymerized with 2 mM Mg^{2+}). The three dotted curves in each top panel are repeat measurements on a same spot, with a data acquisition time of 16.7 min for each measurement. Different panels present measurements performed on different regions of the gel. The bottom panels present the correlation functions obtained by averaging the correlation functions in corresponding top panels (□). The curves result from fitting to $\alpha/(1 + t/\tau_d) + (1 - \alpha)$. Experimental conditions and values of fitted parameters are listed in Table 2.

[1] It is instructive to describe the difference between averaging methods in a little more detail. Consider two consecutive identical measurements, each consisting of a sequence of fluorescence intensity readings. When there is no slow process, each sequence has the same mean fluorescence, and calculation of the correlation function by the two different methods yields identical results. If there is a systematic change of the mean fluorescence intensity from one sequence to the next due to a slow kinetic process, the two methods of calculation will, however, yield different results. According to the first method, treating both sequences as a single data set, the mean fluorescence is $\langle i \rangle = (\langle i \rangle_1 + \langle i \rangle_2)/2$, where i is the intensity or number of photocounts per dwell time (T_D); $\langle i \rangle$ denotes an average over the run time, T_R; $\langle i \rangle_1$ and $\langle i \rangle_2$ are the mean intensities for the first and second sequence, respectively. Further, $G(t) = [G_1(t) + G_2(t) + \langle i \rangle_1^2 + \langle i \rangle_2^2]/2 - \langle i \rangle^2$, where $G(t) = \langle i(t)i(0) - \langle i \rangle^2$ is the correlation function calculated for the entire set of data and $G_1(t)$ and $G_2(t)$ are the correlation functions calculated for the first and second sequences. According to the second calculation method, $G(t) = G_1(t)/G_1(0) + G_2(t)/G_2(0)$. The former method does not bias against slow processes, whereas the latter one does.

TABLE 2 **FCS of 0.23-μm-diam beads in an actin gel***

	Spot No.									
	I			II			III			
	1	2	3	1	2	3	1	2	3	Buffer
$\langle i \rangle$‡	10.3	8.58	14.4	17.8	18.3	21.5	18.9	21.2	24.4	
$\langle \Delta i \Delta i \rangle$§	195	173	333	277	395	510	627	410	653	
γ‖	0.35	0.35	0.58	0.38	0.50	0.57	0.35	0.30	0.33	
τ_d¶	1.35	1.53	3.28	1.67	1.48	1.09	1.66	0.84	0.86	
α**	0.96	0.91	0.86	1.06	1.08	1.05	0.93	0.99	0.99	
$\langle i \rangle$		11.1 ± 27%			19.2 ± 10%			21.5 ± 13%		63.92
τ_d (s^{-1})		1.72			1.33			1.02		0.24
α		0.91			1.06			0.96		1.0

* Roman numerals I, II, and III represent three different regions of an actin gel. Columns 1–3 associated with each Roman numeral present repetitive measurements carried out on that region. There was a 2- to 3-minute period between the end of one measurement and the beginning of the next during which data was transferred from the correlator to the computer. Figure 1 depicts this experiment. Experimental conditions: 1 mg/ml actin, 2 mM Mg^{2+}, 2 mM Tris/Cl, pH 8, 200 μM Ca^{2+}, 200 μM ATP, 1.5 mM NaN_3, 21–22°C. Optics: 40×/0.75NA obj, ω_0 = 1.4 μm, 250-μm-diam field plane aperture (to discriminate against off-focus intensity). Bead dilution: 1:1000 of a 2.5% solids solution. Dwell time: 20 ms. Data acquisition time: 16.7 min.

‡$\langle i \rangle$ and $\langle \Delta i \Delta i \rangle$ are the mean fluorescence and the second moment of the fluorescence fluctuations. §The values of $\langle \Delta i \Delta i \rangle$ have been corrected to remove shot noise by extrapolation to zero time (cf. Qian and Elson, 1990). ‖$\gamma = \langle \Delta i \Delta i \rangle^2 / \langle i \rangle \langle (\Delta i)^3 \rangle$, used in the present work as an empirical parameter (see text). The value of γ may be related to a contribution of "immobile" fluorophores to the FCS measurement (Qian and Elson, 1990). ¶The values in the upper portion of the table have been obtained by fitting the correlation functions for the individual measurements. The three correlation functions obtained at each region in the gel were then averaged and refitted to determine the values, representative of each region, in the lower portion of the table. As explained above, averaging the separately normalized correlation functions biases against the detection of very slow processes (cf. Fig. 2). **α is the fraction of the correlation amplitude that is fit by a single component relaxation function.

fusion of 0.23-μm-diameter beads made at successive times at three different locations in the gel. As indicated in Table 1, the average correlation time (τ_d) measured in the three regions of the gel is five- to sixfold longer than the value of 0.24 s obtained for beads of this size in G-buffer. This retardation of bead diffusion is comparable with that observed by FPR under similar conditions (Frieden, unpublished results). The correlation times, however, vary over approximately a twofold range among the individual measurements, whereas the mean fluorescence, $\langle i \rangle$, an indication of the number of beads in the observation region, also varies but to a smaller extent. As indicated above, the relative error in determining the correlation time should be approximately given by $(\tau_d/T_R)^{1/2}$. Using a value of 1.36 s for the correlation time (the average for the 3 regions), the error in the measurement accumulated over a 16.7-min period (T_R) should be of the order of $100\times(1.36/1{,}002)^{1/2} = \sim 4\%$ (Koppel, 1974). Hence, the differences observed at different times and among the different regions are well beyond the level expected from stochastic uncertainty. That the characteristics of particle diffusion can remain relatively constant over a 30-min period and then appear substantially different at a later time suggests that a slow rearrangement of the filaments is occurring in the actin gel.[2]

The time course of any slow rearrangement of the gel can be very roughly estimated from the measured amplitudes of the fluorescence fluctuations. The relative error in determining the mean fluorescence should also be approximately given by $(\tau_d/T_R)^{1/2}$, which yields a 4% fluctuation, the same as computed above for the correlation times. The fluctuations of the mean fluorescence ($\langle i \rangle$) observed in the three different regions of the gel (shown in Table 2), however, ranged from 10% to 27%. If we assume that the fluctuations of mean fluorescence are ~15%, then a process with a characteristic time in the range of $\tau_d = T_R(0.15)^2 \simeq 20$ s is needed to account for this observed magnitude of fluctuation.[3]

Measurements carried out using a longer period for data acquisition (for example, by combining the three data sets for each region in Fig. 1) show less heterogeneity among the different observation regions in the gel, presumably due to the averaging out of regional differences in the gel over the longer observation period by dynamic processes (Fig. 1, *bottom*, Table 2). The estimated 20-s process, attributed to gel rearrangement, is

[2] It is also possible that the microscope stage drifted during the measurement period. We have checked this with a stationary source, however, and found that the drift is on the order of 1 μm in 1,500 s. Hence, this effect is not significant in these measurements.

[3] Note that only a fraction of the beads might be contributing to this correlation decay, and so the magnitude of the fluctuation relative to the fluorescence of the fraction of the beads that participate in this slow process could be larger than the estimated 15%. Hence, our estimate of 20 s is a lower limit to the correlation time for this process.

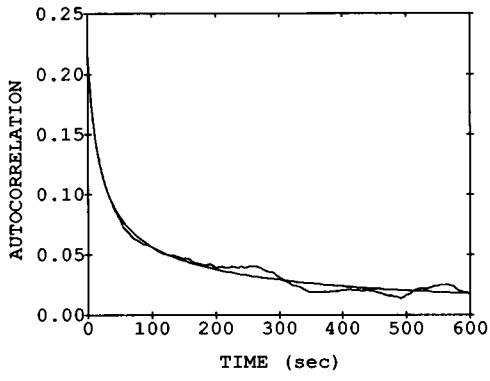

FIGURE 2 Experimental and fitted (*smooth curve*) FCS autocorrelation functions for diffusion of 0.30-μm-diam beads in actin gels, 1 mg/ml actin polymerized with 1 mM Mg^{2+}. Dwell time 1 s, total data acquisition time 13.8 h, 16×/0.35 objective lens with ω_0 = 4.5 μm. Hence, from Stokes law, 0.30-μm beads in unpolymerized G-actin would have correlation time of ~3 s with this objective lens. The fitted curve is $0.175/(1 + t/18.0) + 0.041/(1 + t/269.2)$, and so the correlation times are 6- and 90-fold that of the same beads in buffer. For a correlation time of 269.2 s, a 13.8 h data acquisition period should provide statistical accuracy better than 10%.

~15-fold slower than the 1.36-s component, attributed to diffusion of beads retarded by the gel matrix, and is ~80 times slower than the 0.24-s correlation time observed for the 0.23-μm beads in aqueous solution (G-buffer) using the 40×/0.75 NA objective lens.

The process responsible for the fluctuations of the mean fluorescence of 0.23-μm beads observed in Fig. 1 and Table 2, which we have estimated to have a correlation time of ~20 s, is too slow to have been observed clearly in the measurements described by correlation functions with T_W = 16 s (in the presence the 1.36-s diffusional relaxation) but should be observable in correlation functions calculated to a longer T_W for measurements carried out over a longer period. Fig. 2 shows another measurement carried out on 0.30-μm beads with a total data acquisition time of 13.8 h and with the correlation time calculated out to T_W = 600 s. The non-normalized correlation function in Fig. 2 could be fitted with two components, representing ~80% and 20% of the measured correlation decay with correlation times of ~18 and ~270 s, respectively. This correlation function was obtained with a 16× objective to produce a relatively large focused laser spot size to minimize the effects of stage drift. Assuming that the correlation decay results from random diffusion, we can compare with the measurements of Fig. 1 and Table 2 by multiplying the time constants of Fig. 2 by $(16/40)^2$ (to correct for the magnification of the microscope objective) to a yield time constant of ~3 s for the faster process, ~10-fold slower than for diffusion in G-buffer (Table 1). The slower process, also assuming that it scales with ω^2, would have a time constant on the order of ~43 s for a 40× objective lens, and so would be 15-fold slower than the fast phase. The ratio of time constants is consistent with the ratio of the 20- and 1.3-s time constants discussed above for the 0.23-μm particles. The existence of a slower process that scales as a diffusive process has been pointed out previously (Schmidt et al., 1989). They attributed the slow process that they observed by dynamic light scattering to cooperative motions of the ensemble of filaments. In contrast, due to the different spatial characteristics of the two measurements, we have attributed the slow process that we have observed by FCS to dynamic rearrangement of the actin matrix.

A further indication of dynamic rearrangement of the actin filament matrix is seen in changes of the form of the diffusional correlation function over time. The correlation functions that we have measured sometimes behave as expected for simple diffusion of a single component but at other times show more complex behavior. The former instances, in which $g(t) \sim 1/(1 + t/\tau_d)$ (i.e., as in G-actin samples), show very small standard deviations and can serve as internal controls for the measurement uncertainties. For the more complex correlation functions, we can characterize the contributions of faster and slower processes by comparing the initial rate of correlation decay with the behavior of the entire measured correlation function. Analysis of the initial portion of the correlation function has two advantages: (*a*) the measurement accuracy for the initial portion of the correlation function is higher, simply due to more statistical averaging (cf. Qian, 1990), and (*b*) the slower processes contribute less to the initial portion. Fig. 3, for example, shows a plot both of the initial portion and long time behavior of the correlation function for 0.23-μm beads in one region of an actin gel. The standard deviation of the experimental data from the best fit function for diffusion of a single component is <10% for the initial portion and ≃10% for the long time behavior. Fitting the data both from initial slope and the time course over 15 s yield correlation times of 0.83 and 0.98 s, respectively. Therefore, this correlation function conforms fairly well to expectation for diffusion of a single component. For a process with a 1-s correlation time and using a total data acquisition time of 16.7 min, the stochastic uncertainty in the data should be ≃5%, on the same order as observed (Fig. 3 *B*).

In contrast, at a neighboring region of the gel, the dynamic behavior was quite different (Fig. 4 and Table 3). The mean fluorescence intensity decreases from 124 (T_D = 20 ms) to 34 over 1 h (Table 3). Furthermore, this region appears to display two distinct processes, with correlation times differing by about an order of magnitude (Fig. 4) and with the faster correlation time much faster than expected for the diffusion of the bead in buffer. Fitting the data to a function with two terms of the form $1/(1 + t/\tau)$ may be ambiguous because of the uncertainty due to the "long tail" of the hyperbolic function. That is, the function $\alpha/(1 + t/\tau_1) + \beta/(1 + t/\tau_2)$ can

have multiple sets of α, β, τ_1, and τ_2 that yield similarly good fits over a reasonable time range. Nevertheless, for purposes of illustration, it is possible to represent the data in Fig. 4 in terms of two processes with constant correlation times and with variable relative amplitudes. The longer correlation time was chosen to be $\tau_2 = 0.83$ s from the previous experiment in which only a single correlation time was observed (Fig. 3 *A*). The data were then fitted to the function $\alpha/(1 + t/\tau_1) + (1 - \alpha)/(1 + t/\tau_2)$ to yield a consistent value of the shorter correlation time ($\tau_1 = 0.055$ s) and a variable α, the amplitude of the faster process. This yields values of α that decrease to zero over the observation period, finally leaving only the slower process (Fig. 4, Table 3). Other fitting procedures, e.g., constant α and variable τ_1 or τ_2, would also be possible. The data are insufficient to specify the parameters of a more complicated function, e.g., τ_1, τ_2, and α, in which no variable is held constant. Although uncertainties about the quantitative analysis of Fig. 4 remain, it is appropriate to suppose that at least two dynamic processes contribute to the observed behavior, that they differ by approximately 10-fold in rate, and that their relative contributions change over the observation period. It is interesting to note that 0.23-μm beads have a correlation time of ~0.24 s in buffer solution, whereas the fast time constant measured in Fig. 4 is one fifth of this value. This can be explained by supposing that the bead is constrained to a limited volume (cage) in which it can diffuse. For example, when the diffusant in a two-dimensional (planar) system is limited to a finite square region smaller than the size of the laser beam ω, the FCS correlation time is no longer $\tau_d = \omega^2/4D$ but rather $\tau_d = s^2/4D$, where s is the length of the side of the square area available for diffusion (Elson and Qian, 1989).[4] Using the value of the fast time constant, a rough estimate of s yields a value of ~0.67 μm for the side of the box equal to ~250 subunits of the actin filament. By a similar argument, 0.5-μm beads should have a correlation time of

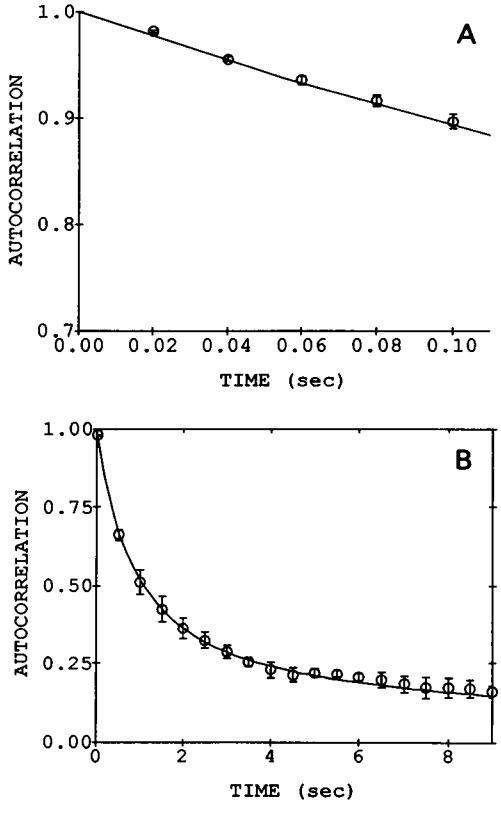

FIGURE 3 FCS autocorrelation function for 0.23-μm-diam beads in an actin gel as described in Table 3. (*A*) The initial slope, and (*B*) the long time behavior. Circles are data and lines are fitted functions, respectively, that yielded correlation times of 0.83 and 0.98 s for *A* and *B*, respectively. The total data acquisition time in these measurements is 16.7 min. The data were obtained with a 40×/0.75NA objective.

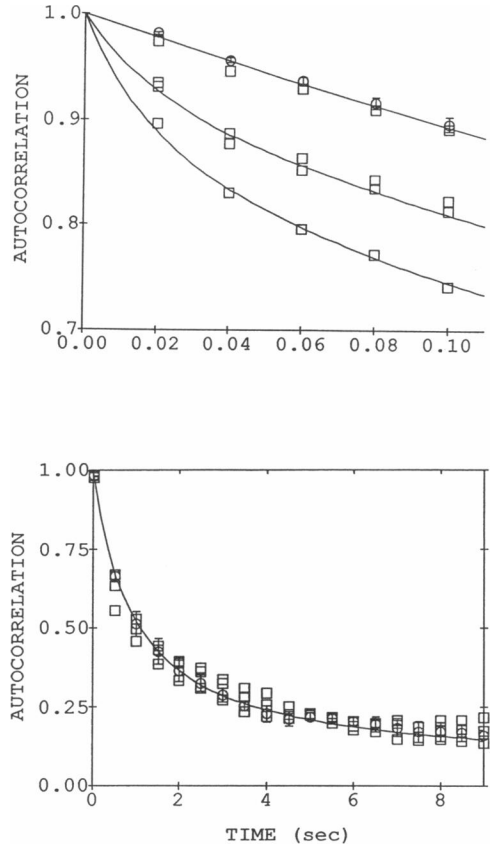

FIGURE 4 Measurements were carried out as in Fig. 3 but on a different region of the gel. There are great variations in each measurement. Squares are new data and circles are taken from Fig. 3 for the purpose of comparison. Experimental conditions and values of parameters obtained from fitting the data (*solid curves*) are listed in Table 3. The upper frame represents initial slopes of the measured correlation functions; the lower frame, the longer time behavior.

[4] We consider the FCS measurements to be two-dimensional as a rough approximation because of the relatively long depth of field compared with the radius of the laser spot in these measurements (cf. Qian and Elson, 1991).

TABLE 3 **Variation of diffusional correlation function and number of measured particles (0.23-μm diameter) in one region of the gel over time**

	$\langle i \rangle$	n^*	γ	α	τ_1	$1-\alpha$	τ_2
					s		*s*
(*a*)	124	10	0.50	0.39	0.055	0.61	0.83
(*b*)	79	10	0.42	0.28	0.055	0.72	0.83
(*c*)	59	8	0.26	0.28	0.055	0.72	0.83
(*d*)	34	3	0.31	0.0		1.0	0.83
(*e*)	18	1	0.54	0.0		1.0	0.83
(*f*)	±11%	±11%	±14%				

Rows *a–d* represent successive measurements in the same region of an actin gel. The integration time of each measurement was T_R = 8.3 min and each measurement (after the first) was begun 15–20 min after the beginning of the previous measurement. $\langle i \rangle$ and γ are defined in the legend of Table 2. Also, see Fig. 4. These results are obtained by fitting the data to a two component correlation function: $G(t) = \alpha/(1 + t/\tau_1) + (1 - \alpha)/(1 + t/\tau_2)$. The value of τ_2 = 0.83 s is taken from Fig. 3 in which only a single phase is observed (row *e* provides analogous data from Fig. 3, whereas row *f* gives the standard deviation). The inclusion of an additional fast phase is suggested by the difference in the apparent time constants obtained by fitting the early portion of the correlation function compared to fitting the whole correlation function. The fitting of the initial phase consistently yielded a value of 0.055 s. Then, the values of τ_1 = 0.055 s and τ_2 = 0.83 s were fixed to obtain the variation in α, the fractional amplitude of the faster correlation component. Experimental conditions as in Table 2 except as follows: Beads dilution: 1:500 of a 2.5% solids solution. Dwell time: 10 ms. Data acquisition time: 8.3 min. Bead diameter 0.23 μm. This Table and Figs. 3 and 4 describe the same experiment.

* Mean number of particles in laser beam: $\langle i \rangle^2/\langle \Delta i \Delta i \rangle$.

~0.007 s. Fig. 5 shows several correlation functions of 0.5-μm beads in an actin gel that demonstrate good agreement with this prediction (Elson and Qian, 1989) (see legend to Fig. 5). When these beads are observed by videomicroscopy, they appear to be essentially immobile. Hence, the FCS correlation functions shown in Fig. 5 result from local motions of the beads. Also consistent with the constrained motion of the beads is the failure of the correlation functions to decay to zero. Based on experiments using gelsolin to control filament length, it is noteworthy that the 0.67 μm length deduced for the cage dimension corresponds to the size of actin filaments (~250 actin subunits) at which the actin filament diffusion begins to be retarded by mutual interactions. Smaller filaments diffuse without retardation; larger filaments are more severely retarded (Doi and Frieden, 1984).

Complex diffusion behavior is also observed for the smallest beads examined. Fig. 6 shows a sequence of FCS autocorrelation functions for 0.09-μm beads. The data indicate that two kinetic components are present and can be fitted with correlation times in the range of 100 ms and 10 s with considerable variation in the relative amplitudes of the two components (Table 4). The slow process here is on the same order as observed for other size beads, and so we suggest that they result from similar mechanisms.

Retardation of diffusion by the actin matrix

Table 1 demonstrates that the diffusion of 0.3-μm beads is severely retarded and that of 0.5-μm beads is prevented within the limits of our experimental measurements. Similarly, Hou et al. (1990*a*) have shown that diffusion of 0.51-μm beads is prevented in actin gels with concentrations of 0.65–0.70 mg/ml.

One might attempt to relate the retardation of the diffusion of a particle in a polymer matrix to the volume fraction occupied by the polymer. This effect is minimal in the systems that we have studied because the volume fraction occupied by 1 mg/ml actin is <0.2%. Therefore, a recently developed model to account for diffusion in a polymer solution also is not applicable to our measurements since it emphasizes the hydrodynamic drag caused by the presence of a substantial polymer surface area (Yam et al., 1988).

A simple theory by Ogston (1958) provides a rough estimation of the mean pore size of a matrix of interpenetrating actin filaments. This theory estimates average pore size to be $\frac{1}{2}(L^{-1/2})$, where L is the total filament length per unit volume. For 1 mg/ml actin, L = 39.2 μm filament/μm^3, leading to an average pore size of ~0.08 μm. This is substantially smaller than the apparent limiting pore size suggested by our experimental data (Table 1) and that of Hou et al. (1990*a*). The inhomogeneity of

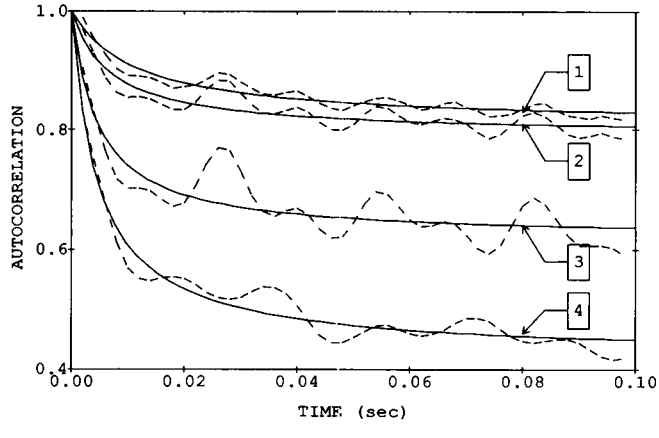

FIGURE 5 Four FCS autocorrelation functions for 0.5-μm-diam beads diffusion in actin gels, 1 mg/ml actin polymerized with 2 mM Mg^{2+}. The beads are essentially immobile judging from digitized video microscopy (data not shown), but some local motions can be detected by FCS. If bead motion corresponds to a relatively fast process of relative amplitude α and correlation time τ_d and a much slower component (effectively immobile) with relative amplitude $1 - \alpha$, the data (*dashed line*) are fitted by $\alpha/(1 + t/\tau) + (1 - \alpha)$ to yield α and τ_d as follows: curve *1*, α = 0.19, τ_d = 0.012; curve *2*, α = 0.21, τ_d = 0.007; curve *3*, α = 0.38, τ_d = 0.005; and curve *4*, α = 0.58, τ_d = 0.005. The dwell time for these measurements is set at 2 ms to reveal the fast process.

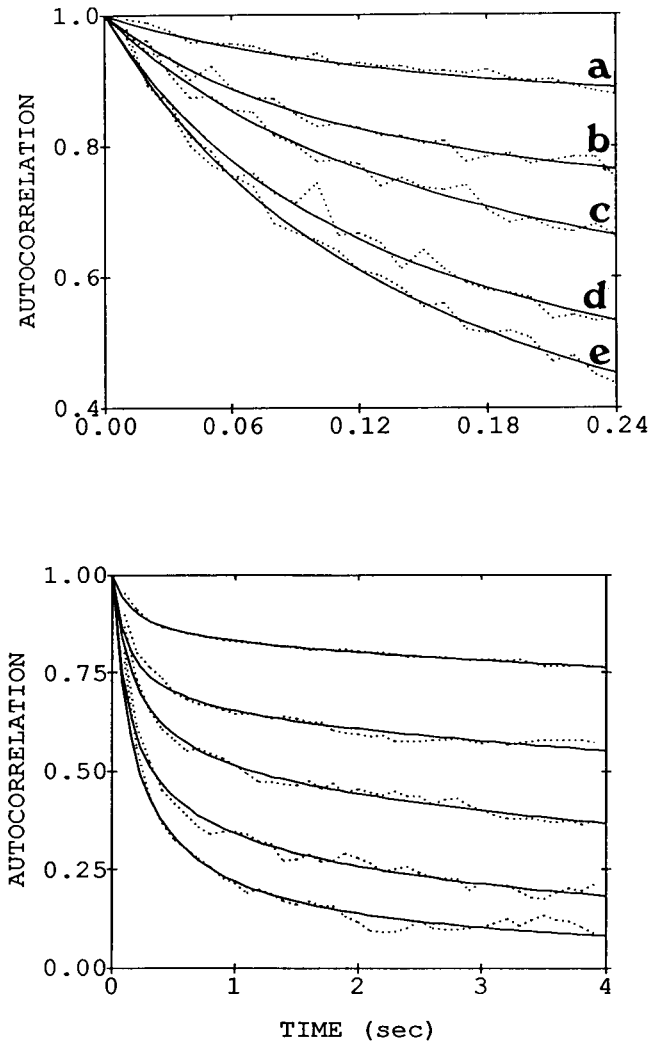

FIGURE 6 Normalized FCS autocorrelation functions for diffusion of 0.09-μm-diam fluorescent beads in an actin gel (1 mg/ml actin polymerized with 1 mM Mg^{2+}). The data for each of the autocorrelation functions were taken in different regions of the same actin gel. Top panels are for short-time and bottom panels are the corresponding long-time correlations. The total data acquisition time in these measurements is 8.33 min. Experimental conditions and values of fitted parameters are listed in Table 4.

the actin gels may contribute to this discrepancy between the measurements and prediction. Hou et al. (1990*a*) have observed that the diffusion of particles is enhanced when the actin filaments were bundled by the cross-linking protein filamin.

A more detailed theory of the diffusion of particles through solutions of rigid, immobile, or very slowly diffusing rod-like polymers developed by Ogston et al. (1973) also can be applied. This theory yields the ratio of the diffusion coefficient of a particle in the polymer solution, D, to that in water, D_0, as $D/D_0 \simeq \exp[-r(\pi L)^{1/2}]$, where r is the hydrodynamic radius of the particle and L is the length of the polymer chain per volume (Ogston et al., 1973). For example, the predicted ratios of the diffusion coefficients, D_0/D, for the 0.28-, 0.13-, and the 0.09-μm beads in a 1 mg/ml actin matrix are 4.71, 2.05, and 1.65 compared with the corresponding values observed in Table 1: 6.0, 2.3, and 1.4, respectively. Hence, this theory accounts fairly well for the experimental data.

Although the mechanism of the major fluctuation relaxation process in the 1-s time range (Tables 2 and 3) can be reasonably interpreted as the diffusion of beads in rigid random networks as predicted by Ogston et al. (1973), the mechanism for the much slower process in the range of 20–50 s remains to be determined. We can estimate local flexion of actin filaments. According to Oosawa (1980), the rigidity of F-actin is $\sim 1.7 \times 10^{-17}$ dyn cm^2. The mean flexion fluctuation and longest relaxation time for a 600-nm filament with two ends fixed are 15.3 nm and 0.03 ms (Oosawa and Asakura, 1975). Therefore, if we suppose that the fluctuation amplitudes are normally distributed, we can calculate that on average it takes $\sim$590 s for a fluctuation of 0.09 μm, and so rod flexion is too slow (and with too small a fluctuation amplitude) to account for the 20- to 50-s process.

The polymerization rate of actin is markedly influenced by Mg^{2+}, suggesting a possible dependence of the gel structure on the concentration of this ion, but we did not observe a substantial effect on the diffusion of particles due to varying the Mg^{2+} concentration in which the actin filaments were polymerized. Fig. 7 shows FCS measurements of 0.3-μm beads in gels polymerized from 1 mg/ml actin at different concentrations of Mg^{2+} ion. Measurements carried out over a fairly long time scale indicate that over the range from 1 to 4 mM the diffusion of the beads does not depend on Mg^{2+} concentration.

A likely mechanism for the observed relaxation of the local heterogeneity in the diffusion properties of beads in actin matrices is a continuous reorganization of the matrix due to fluctuating interactions among the actin fila-

TABLE 4 FCS of 0.09-μm diameter beads in actin gels*

	$\langle i \rangle$	$\langle \Delta i \Delta i \rangle$	τ_1	$1-\alpha$	τ_2
			s		s
	21.9	6.68	0.139	0.33	3.92[d]
	24.9	12.4	0.107	0.61	25.9
	20.5	7.91	0.121	0.29	3.58
	20.3	8.47	0.165	0.16	1.73[c]
	22.8	8.24	0.185	0.47	10.7[c]
	25.6	30.6	0.180	0.82	46.0[a]
	24.4	14.4	0.137	0.64	22.2[b]
	21.4	7.53	0.106	0.41	1.67
	22.5	7.47	0.122	0.35	1.96
Mean	22.7 ± 1.9	11.5 ± 7.6	0.14 ± 0.03	0.45 ± 0.2	13.1 ± 15
Buffer			0.10	1.0	

* Experimental conditions as in Table 3. Values designated [a–c] refer to the correspondingly indicated correlation functions in Fig. 6. $1-\alpha$ is the relative amplitude of the slow phase.

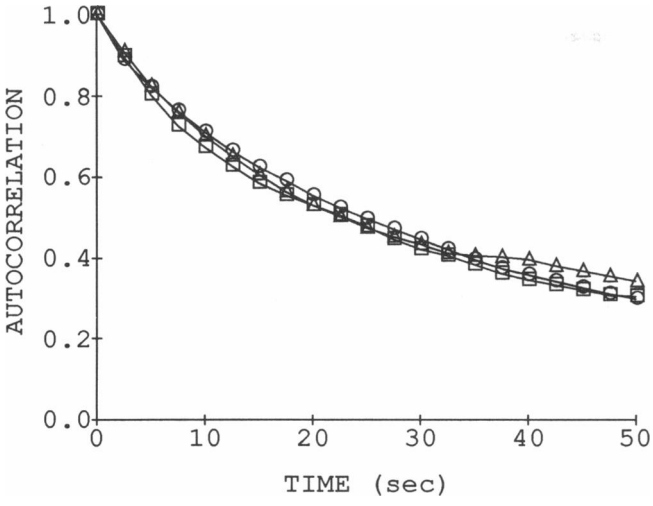

FIGURE 7 FCS measurements of the diffusion of 0.3-μm beads in actin gels polymerized in different concentrations of Mg^{+2}. The actin (1 mg/ml) was polymerized by Mg^{+2} at 1 (○), 2 (□), and 4 mM (△), respectively. The data acquisition time was 81.9 min for each measurement using an 16×/0.35NA objective.

ments in a process with a characteristic time in the range of 20–50 s. It is reasonable to suppose that this relaxation would influence all or most of the beads. It is also possible, however, that the process observed in this time range is due to another mechanism. For example, a small fraction (20%) of the beads might be caught in denser regions of the actin matrix that more strongly retard their diffusion so that the characteristic diffusion correlation time is in the 20- to 50-s range. Even if this occurred, however, these hypothetical actin filament density fluctuations also must dissipate as demonstrated by the time-dependent changes observed in Fig. 5. It is important to point out that the motions of the actin gel responsible for this reorganization cannot be macroscopic. FPR measurements on fluorescent labeled actin filaments have not detected recovery over distances of the order of 1 μm using monitoring periods of several minutes (Tait and Frieden, 1982; Loftus, 1988). Hence, more localized motions (i.e., $< \sim 1$ μm) must be responsible for releasing the constraints on the embedded fluorescent beads to permit them to diffuse over macroscopic distances. We have observed, however, that occasionally there are especially large fluctuations of the fluorescence intensity. Sometimes these rare events can overwhelm the overall time correlation function, causing the function to appear to have a much longer correlation time. It has been demonstrated that when these rare events are excluded from the record, the resulting correlation time returns to its expected value (data not shown). Therefore, it is possible that the rearrangement of actin gels is dominated by these rare events. These rare events are not due to the formation of bead aggregates since the diffusion of beads of larger size (0.5 μm) has not been observed.

Applicability of FCS

This work has demonstrated the utility of FCS for characterizing the diffusion of particles through a matrix. Like DLS, FCS is a fluctuation measurement that does not perturb the sample (Elson, 1985). For this kind of measurement, the relative fluctuation amplitude, and therefore the signal, increases as the concentration of the labeled probe particles decreases. Therefore, in contrast to FPR, low concentrations of the fluorescent diffusant provide optimal conditions for the measurement. Since FCS measures fluorescence emission instead of scattered light, the actin gel contributes no background to the measurement. Thus, this measurement is more specific than DLS in which the light scattered from the dynamic density fluctuations of the gel makes a significant contribution (Fujime, 1972; Tanaka et al., 1973). Hence, in DLS measurements it is necessary to introduce a large number of probe particles to overcome scattering from the gel itself (Newman et al., 1989).

DLS and FCS also differ in their spatial resolution. Although DLS measures diffusion over a characteristic distance on the order of the wave length of light, the volume from which the scattered light is acquired, and therefore over which the diffusion behavior is averaged, is usually on the order of a cubic millimeter. In contrast, for our FCS measurements the sample volume is in the range of tens of cubic microns. Therefore, FCS can detect spatial fluctuations in gel porosity (in terms of heterogeneity of diffusion) over much smaller distances than can DLS. For this reason, the slow processes observed by DLS (Schmidt et al., 1989) and by FCS (this work) may not be comparable.

FCS has the disadvantage of requiring relatively long periods for acquisition of data and so can be applied only to relatively stable systems. Systems that exhibit rapidly changing properties are unsuitable for study by this approach.

Our results are qualitatively consistent with the earlier DLS measurements by Newman et al. (1989), who showed that 0.27-μm polystyrene latex spheres in an actin gel at 1 mg/ml concentration experienced approximately a fivefold reduction in diffusion coefficient. By comparison, we have observed a sixfold reduction in the diffusion coefficient of 0.28-μm beads and a ninefold reduction of 0.30-μm beads under comparable conditions (Table 1). Hence, qualitatively similar diffusion behavior in actin gels is observed for FCS measurements with a characteristic diffusion distance in the range of 2 μm, FPR measurements with a characteristic distance of 10 μm (Hou et al., 1990*b*), and for DLS measurements with a characteristic distance in the range of 0.5 μm. Our results are also consistent with the FPR measurements of Hou et al. (1990*a*), who have found that long range diffusion of 0.51-μm polystyrene latex particles was curtailed at an F-actin concentration of 0.65–0.70 mg/ml.

SUMMARY

(*a*) FCS measurements provide a useful method for probing the diffusion of fluorescence labeled particles in polymer matrices. In contrast to DLS, FCS has the advantage that there is no background due to gel scattering. Furthermore, the spatial resolution for probing regional differences in the matrix is higher, although the characteristic distance over which diffusion is measured is smaller for DLS than for FCS. FCS has the additional advantage that large fluctuations in the measured fluorescence due to fluctuations in the particle "occupation number" (Elson and Webb, 1975) contribute to the measurement rather than distort it as they do in FPR measurements.

(*b*) The macroscopic lateral diffusion of particles of various sizes embedded in actin filament matrices is retarded to different extents due to interaction with the matrix. This retardation can be understood qualitatively in terms of simple steric models developed previously (Ogston, 1958; Ogston et al., 1973).

(*c*) The constraints on particle diffusion fluctuate over time and from position to position in the gel. The characteristic time for relaxation of these constraints is in the range of 20–50 s. We have supposed that this relaxation results from a local reorganization of the actin matrix due to fluctuations in the interactions among the actin filaments. This reorganization is compatible with both models for the dynamic behavior of the actin filament matrix. Hence, further work is required to determine whether interference of filament diffusion due to steric interfilament interactions is sufficient or whether it is necessary to suppose the existence of additional cohesive interactions among the filaments to account for the rates of filament reorganization.

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