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Measurements of Molecular Transport in Small Systems

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ABSTRACT. Three methods based on optical microscopy are available for measuring the lateral transport of molecules in small systems such as individual living cells. Two of these, Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Photobleaching Recovery (FPR), measure changes in the number ("occupation number") of fluorescence labelled molecules in a small open subregion of the sample. In an FCS measurement, the system rests in equilibrium while the occupation number undergoes microscopic spontaneous fluctuations. In an FPR measurement, a macroscopic concentration gradient is generated by photobleaching a fraction of the fluorophores in the observation region. A third method, Single Particle Tracking (SPT), determines the trajectory of individual particles visible in the light microscope. FCS and SPT measure microscopic changes and so require that an extensive record of occupation number fluctuations or particle trajectories be analyzed statistically to provide accurate measurements of diffusion coefficients. FPR measurements, based on macroscopic concentration changes, can yield valid diffusion coefficient values from a single recovery transient. In this paper we describe these three methods, discuss the validity of the measurements, and illustrate applications.

1. Introduction

Measurements of molecular transport in biological systems can provide important information about both physiological mechanisms and physical in-

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teractions that drive and constrain molecular motions. For example, according to a simple model of membrane organization, cell surface proteins are embedded in a fluid lipid bilayer and should diffuse freely, limited mainly by the viscosity of the bilayer (Singer and Nicolson, 1972). It has also been proposed that systematic rearward transport of cell surface particles is driven by a current of lipid that sweeps along with it slowly diffusing membrane protein aggregates (Bretscher, 1984). As described below, measurements of diffusion and systematic transport of cell surface proteins have shown that these models cannot account for the transport properties actually observed.

Methods for measuring the rates of random lateral diffusion and of systematic drifts or flows of proteins in individual cells have been based on optical microscopy. One approach is to measure the number of fluorescence labelled molecules in a small open subregion of the system. The number of molecules (the "occupation number") can change due to either systematic motion or diffusion. The rate of change of this number depends on the size of the observed subregion and the rate of molecular motion. Therefore, once the size of the observation region is known, measurements of the rate of change of the occupation number yield the rates of molecular motion, e.g., diffusion coefficients and drift velocities. Two methods have been developed based on this idea (Elson, 1985). One, called "Fluorescence Correlation Spectroscopy" or FCS, measures the spontaneous fluctuations of the occupation number about its constant mean value as the system rests in thermodynamic equilibrium or in a steady state condition of diffusion and flow. The other, called "Fluorescence Photobleaching Recovery" or FPR[1], measures the rate of relaxation of a macroscopic concentration gradient generated by an intense pulse of excitation light that irreversibly photolyzes a fraction of the fluorescent molecules in the observation region. In FCS many spontaneous fluctuations must be analyzed statistically to determine the desired diffusion coefficients and velocities of transport. In FPR a single macroscopic transient is sufficient in principle to yield these quantities.

A different approach tracks the position of one or a small cluster of molecules linked to a small particle that is visible by video enhanced contrast optical microscopy (Geerts et al., 1987) and that can be localized with high precision (Gelles et al., 1988). In this "Single Particle Tracking" or SPT method, diffusion coefficients and rates of systematic transport are determined from a statistical analysis of the trajectories of individual particles (Gross and Webb, 1988; Sheetz et al., 1989; Qian et al., 1991). Like FCS, the SPT method does not require a perturbation of the system to establish a macroscopic concentration gradient but does require the analysis of an extensive record of particle motion.

To use these methods with confidence it is necessary to assess the accuracy of the results they yield. For the occupation number methods there are uncertainties due to the random emission of fluorescence photons, i.e., "shot" noise. In SPT the analogous source of uncertainty is in the determination of the position of the observed particle tag. For both SPT and FCS there is a

[1] This method is also called "Fluorescence Recovery After Photobleaching" or FRAP.

more intrinsic uncertainty due to the stochastic character of random diffusion. Even if the time course and amplitude of a single spontaneous occupation number fluctuation could be measured with infinite accuracy, this would not accurately determine the phenomenological diffusion coefficient (Onsager, 1931). Rather many fluctuations must be analyzed statistically. Similarly, even if the position of a particle could be determined with infinite accuracy, a short record of its trajectory is insufficient to determine its diffusion coefficient. The statistical analyses are most conveniently accomplished in terms of temporal correlation functions computed from the measurements. In FCS, the diffusion coefficients are derived from a fluorescence fluctuation autocorrelation function, which corresponds to an occupation number autocorrelation function and which is computed from a time sequence of fluorescence intensity measurements (Elson and Magde, 1974). In SPT, diffusion coefficients are calculated from the mean square displacement of the observed particle, which is closely related to the correlation function of particle positions (Qian et al., 1991). Because they are based on the relaxation of macroscopic concentration gradients, FPR measurements are not influenced by these kinds of stochastic uncertainties.

2. Occupation Number Measurements

The fluorescence signal $f(t)$ measured in FPR and FCS is related to the concentration of fluorophores $c(\mathbf{r},t)$ at position $\mathbf{r}$ and time t and to the excitation intensity profile $I(\mathbf{r})$ as

$$f(t) = Q \int I(\mathbf{r}\,) c(\mathbf{r},t)\; d^n\mathbf{r}$$

where $n(= 1, 2, 3)$ is the dimensionality of the system. In an FPR experiment the time course of relaxation to the initial equilibrium concentration $< c >$ is

$$\Delta f(t) = Q \int I(\mathbf{r})\; \Delta c(\mathbf{r},t)\; d^n\mathbf{r}$$

where $\Delta f(t) = f(t) - < f >$ and $\Delta c(\mathbf{r},t) = c(\mathbf{r},t) - < c >$ represent macroscopic deviations from the equilibrium values and Q takes account of the absorption and emission characteristics of the fluorophore and the optical excitation and losses in the measurement system. In an FCS experiment the statistical analysis of the fluorescence fluctuations is carried out in terms of a fluorescence fluctuation autocorrelation function $G(\tau)$ where

$$G(\tau) = \lim_{T\to\infty} (1/T) \int_0^T \delta f(t)\delta f(t+\tau) dt$$

$$= Q^2 \int I(\mathbf{r}) I(\mathbf{r}') < \delta c(\mathbf{r},0)\delta c(\mathbf{r}',\tau) > d^n\mathbf{r} d^n\mathbf{r}'$$

Here δ represents a microscopic fluctuation, $< \ldots >$ denotes a time or ensemble average, and we have supposed that the system is stationary. The phenomenological equation that governs concentration change due to diffusion with diffusion coefficient D and drift with velocity V along the x-direction is

$$\partial \delta c(\mathbf{r},t)/\partial t = D\nabla^2 \delta c(\mathbf{r},t) - V\partial \delta c(\mathbf{r},t)/\partial x$$

To describe multicomponent mixtures, this equation is readily extended into a system of equations that can account, in addition, for chemical reactions among the components. The analysis of these equations for the interpretation of FPR and FCS measurements has been discussed in detail (Elson, 1985).

Due to the random character of fluorescence emission, the number of photons emitted by a fluorophore will fluctuate about its mean value according to a Poisson distribution. Hence, the accuracy of both FCS and FPR measurements will be influenced by the magnitude of these "shot noise" fluctuations. The magnitude of the shot noise will be determined by the duration, ΔT, of the individual intensity measurements and the mean number of photons produced per unit time, $\lambda I(\mathbf{r})$. Denoting $x(\mathbf{r}) = \lambda I(\mathbf{r})\Delta T$, the probability that the photocount p measured from a fluorophore at $\mathbf{r}$ in an interval ΔT should equal the number k is Prob $\{p = k\} = x(\mathbf{r})^k \exp(-x(\mathbf{r}))/k!$. More generally, if the probability density that the fluorophore is at $\mathbf{r}$ is $P(\mathbf{r})$, then

$$\text{Prob}\,\{p = k\} = \int [x(\mathbf{r})^k \exp(-x(\mathbf{r}))/k!] P(\mathbf{r}) d^n\mathbf{r}$$

Suppose that the ideal, shot noise free fluorescence intensity is $\Phi(\mathbf{r}) = \lambda I(\mathbf{r})\Delta T$. Since $P(\mathbf{r})dr = \text{Prob}\{\Phi = x\}dx$, we have

$$\text{Prob}\{p = k\} = \int [x(\mathbf{r})^k \exp(-x(\mathbf{r}))/k!]\, \text{Prob}\,\{\Phi = x(\mathbf{r})\} d^n\mathbf{r}$$

This illustrates that the probability distribution of the detected photocounts p is a Poisson transformation of the distribution of Φ, independent of the detailed form of Prob $\{\Phi = x\}$ (Qian, 1990a). In principle, it is possible to carry out an inverse Poisson transformation to filter out the shot noise contribution (Qian, 1990b). It is also interesting to note that the Poisson transformation is a special example of Hidden Markov Models, which have recently received increasing attention in studies of signal processing (Rabiner and Juang, 1986).

To account for the statistical uncertainty in FCS measurements, we begin by noting that the fluorescence intensity, P, from a system of M particles is simply the sum of the intensities measured from the individual particles, $P = \sum p_i$. If we measure P at a series of N times, then the mean fluorescence signal is $S_1 = \sum P_i/N$. In an FCS experiment we calculate

$$S_2(m) = \sum_{k=1}^{N} (P_k P_{k+m})/N - \left[\sum_{k=1}^{N} P_k/N\right]^2$$

to yield the correlation function $< S_2(m) >= G(m\Delta T) =< P(0)P(m\Delta T) > - < S_1 >^2$, where ΔT is the interval between measurements. The statistical uncertainty is represented by the variance of S_2, $< (\Delta S_2(m))^2 >$. Then the signal to noise ratio, Ξ, is $\Xi =< S_2(m) > /[< (\Delta S_2(m))^2 >]^{\frac{1}{2}}$. Expressions for $< S_2(m) >$ and $< (\Delta S_2(m))^2 >$ have been presented (Qian, 1990a). Analysis of the signal to noise ratio is complex and leads to two limiting cases (Qian, 1990a):

(1) Under conditions in which q, the measured intensity per fluorophore, is high and m, the number of fluorophores in the observation region, is large, Gaussian occupation number statistics prevail, and the contribution from shot noise is small; then Ξ is independent of m and q and is limited only by the stochastic nature of the fluctuation measurements and is governed by N, the number of **independent** intensity measurements. (Note that successive intensity measurements will not be independent if, as is usually true, the dwell time ΔT is less than the correlation time, τ, for the observed process, e.g., diffusion across the observation region. When $\Delta T < \tau$, Ξ varies as $(T/\tau)^{-1/2}$, i.e., as (number of independent measurements)$^{-1/2}$ [cf. Qian et al., 1991]).

(2) If m is small, Poisson rather than Gaussian statistics prevail, and Ξ will depend also on m. If q and m are both small, the contribution of shot noise is also significant. Then Ξ depends on both q and m as well as N. If q is large, but m is small, the shot noise contribution is small, and Ξ depends only on m and N.

3. Single Particle Tracking

SPT measurements provide the trajectory of a particle, $\mathbf{r}(t) = [x(t),\ y(t),\ z(t)]$, from a sequence of position measurements. Values for diffusion coefficients and drift velocities are conveniently obtained from the mean squared displacement of the particle, $\rho(t)$, as a function of time:

$$(1) \qquad \rho(t) =< |\mathbf{r}(t) - \mathbf{r}(0)|^2 >= \int\int P(\mathbf{r}')|\mathbf{r} - \mathbf{r}'|^2 P(\mathbf{r}|\mathbf{r}', t) d^n\mathbf{r} d^n\mathbf{r}'$$

where $P(\mathbf{r})$ is the steady-state distribution of particle position and $P(\mathbf{r}|\mathbf{r}', t)$ is the probability that a particle originally at $\mathbf{r}'$ will be at $\mathbf{r}$ after a time period t. Values of $\rho(t)$ are calculated from particle positions measured sequentially at time intervals ΔT: e.g.,

$$\rho_x(n\Delta T) = \sum_{i=0}^{N} (x_{i+n} - x_i)^2/(N+1) \ .$$

If the particle is both randomly diffusing with diffusion coefficient D and drifting with velocity $\mathbf{V}$, then in a two-dimensional system appropriate for analysis of diffusion on a cell membrane, $P(\mathbf{r}|\mathbf{r}', t) = (1/4\pi Dt) \exp[-|\mathbf{r} - \mathbf{r}' - \mathbf{V}t|^2/4Dt]$ and $\rho(t) = 4Dt + |\mathbf{V}t|^2$. Hence, the contributions of drift and diffusion are readily discerned and quantitatively characterized in a plot of $\rho(t)$ versus t (Sheetz et al., 1989).

As with FCS, even with perfectly precise measurements of particle positions, the values of $\rho(t)$ calculated from experimental data will have statistical variances due to the stochastic character of diffusion. This variance will diminish as the number of position measurements increases. To assess the validity of experimentally determined diffusion coefficients, it is essential to determine the dependence of the variance on the number of measurements. We first consider independent measurements of the squared displacement at time t, $\xi_t = |\mathbf{r}(t) - \mathbf{r}(0)|^2$. The probability distribution of ξ_t considered as a random variable is $\text{Prob}[z \leq \xi_t \leq z + dz] = (1/4Dt)\exp(-z/4Dt)dz$. Suppose that we have K independent measurements $\xi_t(i)$ $(i = 1, ..., K)$. Because $\xi_t(i)$ is a random variable, the sum of independent measurements of ξ_t and therefore the mean $\rho(t) = [\xi_t(1) + \xi_t(2) + \cdots + \xi_t(K)]/K$ are also random variables. The probability distribution of ρ can then be derived as successive convolutions of $\text{Prob}[z \leq \xi_t \leq z + dz]$ with itself to yield $\text{Prob}[z \leq \rho(t) \leq z + dz] = [(1/4Dt)^K \exp(-zK/4Dt)K^K z^{K-1}/(K-1)!]dz$ (Qian et al., 1991). Using this one may show that the relative error in the determination of ξ is $(< (\Delta\rho)^2 > / < \rho >^2)^{1/2} = K^{-1/2}$.

In actual SPT measurements the value of $\rho(t)$ is determined from a sequence of N *consecutive* positions $\mathbf{r}$ at time intervals of ΔT. Then $\rho_n = \rho(n\Delta T)$ is obtained by averaging over $N - n + 1$ measurements, and so the successive determinations of $\rho(t)$ are not independent, due to the overlap between the measurements. This leads to an increase in the relative error depending on n. Calculation of the relative error is complex (Qian et al., 1991). When $N \gg n$, $[< (\Delta\rho_n)^2 > / < \rho_n >^2]^{1/2} = [(2n^2 + 1)/3n(N - n + 1)]^{1/2} \approx [2n/3(N-n+1)]^{1/2} \approx [2n/3N]^{1/2}$. Hence, the variance increases with increasing n because the larger n, the smaller the number of statistically independent samples of displacement within the interval $N\Delta T$. This analysis is extended to account for measurements of the velocity of systematic drift by Qian et al., (1991).

Uncertainties in measurements of the positions of particles are typically small and vary randomly from one video frame to the next (Gelles, 1988). The contribution of this type of uncertainty is analogous to shot noise; it should appear in $\rho(t)$ only at $t = 0$. Therefore, the major uncertainty in the measurement of diffusion and drift by SPT arises from the stochastic character of random particle motion (Qian et al., 1991).

4. Relationship of the Methods

FCS, FPR, and SPT can all be interpreted in terms of an elementary theory of molecular transport represented in terms of a stochastic (Markov and Gaussian) process with the transition probability distribution $P(\mathbf{r}|\mathbf{r}', t)$. In SPT measurements the positions of a particle are weighted equally in calculating the mean square displacement $\rho(t)$. As indicated above [Eq. (1)], the mean square displacement of a particle can be expressed in terms of $P(\mathbf{r}|\mathbf{r}', t)$. It is also straightforward to show that for motion within a finite space there is a close connection between the positional correlation function of a particle, $g_r(t) =< r(t)r(0) >$, and its mean square displacement (Qian et al., 1991): $g_r(t) =< r^2 > +\rho(t)/2$.

In contrast to SPT, in FCS and FPR measurements the position of the particle is weighted by the excitation intensity profile, $I(\mathbf{r})$, according to its location relative to the center of the excitation laser beam and relative to the focal plane of the microscope. For example, the fluorescence fluctuation autocorrelation function can be represented as

$$G(t) = P^2 \int\int I(\mathbf{r})I(\mathbf{r}')P(\mathbf{r}')P(\mathbf{r}|\mathbf{r}',\tau)d^n\mathbf{r}d^n\mathbf{r}'$$

in which the position of the fluorophores at positions $\mathbf{r}$ and $\mathbf{r}'$ are weighted by the excitation light intensities $I(\mathbf{r})$ and $I(\mathbf{r}')$. The distribution of the excitation light intensity in FCS and FPR measurements imposes a characteristic distance on the measurement. In the simplest spot photobleaching measurements, that characteristic distance is the radius, w, of the Gaussian excitation laser light intensity: $I(r) = I_0 \exp(-2r^2/w^2)$. Then, simple diffusion with diffusion coefficient D has a characteristic time, τ_D, set by this distance, $\tau_D = w^2/4D$, which plays an important role both in FCS and FPR measurements of diffusion (Elson, 1985). Similarly, in dynamic light scattering (DLS) the weighting function is $I(\mathbf{r}) = \exp(i\mathbf{r} \cdot \mathbf{q})$ where $\mathbf{q}$ is the scattering vector and $1/|q|$ is the characteristic distance imposed on the diffusion measurement (Cummins et al., 1969; Qian et al., 1991). Analogous to the characteristic time $w^2/4D$ for FCS, the charactersitic time for DLS is $1/Dq^2$. The relationship between characteristic time and distance in FPR and FCS can be used to accommodate measurements to experimentally accessible ranges. For example, in measurements of very fast diffusion, the value of w can be increased to bring the τ_D into a range convenient for measurement.

In contrast to FPR, FCS, and DLS, there is no characteristic distance in an SPT measurement. To measure diffusion with high relative precision it is merely necessary to allow the particle to move to a large mean square displacement. Of course, systematic errors in the measurement could impose additional limits.

5. Discussion and Illustration

5.1. Two- and Three-Dimensional Systems. Application of these methods to quasi-two-dimensional systems such as cell membranes is straightforward because the plane of focus of the microscope can be made to coincide with the plane in which the particle motion is to be measured. Extension to three-dimensional systems can be difficult because the measured fluorescence intensity emitted from a particle or molecule diminishes the further it is from the focal plane. To account for the effects of this variation on FCS measurements it is necessary to analyze how the excitation intensity and the collected emission intensity vary with distance from the focal plane. This is accomplished in terms of the point spread function of the microscope, which relates the intensity of light transmitted to a point $\mathbf{r}'$ in the microscope from a position $\mathbf{r}$ in the sample. It is also necessary to take account of a field aperture placed before the photomultiplier that reduces the off-focus intensity collected. This problem has been discussed in detail for the confocal microscope optics typically used for FCS (Koppel et al., 1976; Qian and Elson, 1991). In SPT

measurements differential interference contrast (DIC) or darkfield microscope optics are typically used with high levels of incident illumination. Therefore shot noise is usually not a problem. An advantage of DIC optics is a very narrow depth of focus so that particles remain visible only within a short distance of the focal plane. In SPT measurements the observed particle can therefore disappear by leaving the focal plane, and, even if it remains in view, its displacement from the focal plane is difficult to measure precisely.

5.2. Advantages and Disadvantages of the Methods. An advantage in principle of FCS is that the system rests unperturbed in equilibrium during the measurement. In an FPR measurement the system is perturbed from equilibrium by photobleaching in a localized area of the system. It is assumed in most analyses of FPR measurements that the photobleaching can be described as an irreversible process that is first order in the concentration of fluorophores and with a rate proportional to the incident light intensity. These assumptions are rarely tested and their failure could lead to errors in the evaluation of FPR measurements (Elson, 1985). For example, if the photobleaching process were reversible, some of the measured fluorescence recovery could be due to this process rather than to diffusion or drift of unbleached fluorophores into the observation region. FCS, however, has the disadvantage of requiring the measurement of a long sequence of microscopic fluorescence changes. This imposes a stringent requirement for stability on the measured system, which usually makes this method unsuitable for application to living systems. Any motion of a cell, ruffling or locomotion, for example, might produce a fluorescence change, which would overwhelm the small fluorescence changes due to occupation number fluctuations. In contrast FPR requires only a single macroscopic recovery transient to characterize transport properties within the limits of precision of the measurement. Major advantages of the SPT method are its high spatial resolution, which enables measurements of motions within very small domains, and the ability to detect motions exhibited by only a small fraction of the labelled molecules. A disadvantage of SPT in studies of the motion of cell surface molecules is that the marker beads are polyvalent for binding to the surface molecules. The number of molecules bound to the beads is difficult to determine and the polyvalent binding could itself influence the dynamic state of the bound molecules. The effective crosslinking of these molecules could activate dynamic cellular processes such as capping or other forms of systematic molecular transport. Another disadvantage of SPT is that, like FCS, an extensive record of random motion is required for a statistically accurate characterization of diffusion coefficients.

5.3. Examples:

5.3.1. FCS Measurements of the Motions of Beads in Actin Gels. Actin filaments form random viscoelastic networks that have an important role in determining cell shape and mechanical consistency and in driving cellular dynamic processes (Elson, 1988). One approach to characterizing the mechanical properties of actin gels is to measure the motion of particles embedded in gels reconstituted from filaments polymerized *in vitro* from purified actin. FPR and DLS have been used to study the mesh size and other microscopic characteristics of reconstituted actin filament networks (e.g., Luby-Phelps et al.,

1988; Hou et al., 1990a, 1990b; Newman et al., 1989; Seils et al., 1990; and Schmidt et al., 1989). In a recent study FCS was used to measure the motion of fluorescence labelled beads of various sizes in reconstituted actin gels (Qian et al., 1992). In these measurements the number of beads in the observation region was small, typically less than ten. FPR measurements on this system were difficult because the recovery curves varied substantially from one to another for statistical reasons, as described above. This variability made it difficult to discern the spatial and temporal properties of the gel that were of interest. By applying FCS instead of FPR, the large occupation number fluctuations due to the small number of observed particles is converted from a disadvantage to an advantage. In this study the FCS measurements indicated that the fluorescent beads were constrained in small cages formed by the gel and that the gel had a dynamic structure that varied over time and from one region of the gel to another. These measurements show the applicability of FCS to stable systems in which the motions of highly labelled particles present at low concentration yield large fluctuation signals.

5.3.2. Random Diffusion and Systematic Drift on Cell Surfaces. Systematic rearward transport of particles on locomoting cells has been attributed to a current of membrane lipid that was thought to sweep large particles or membrane protein aggregates along with it. An alternative hypothesis is that the rearward motion results from the operation of motor proteins that link surface molecules to the underlying cortical cytoskeleton and drag them toward the back of the cell. It is difficult to test the dynamic character of randomly diffusing and systematically transported molecules using FPR because many particles in both dynamic states contribute to the measured signal. In particular the motion of systematically transported particles is frequently difficult to discern against the background of randomly diffusing particles that are usually present in greater proportion. SPT provides a detailed characterization of the motion of both randomly diffusing and systematically transported individual particles. Studies of the motions of 40 nm particles linked to the surface of mouse bone-marrow macrophage by the lectin concanavalin A showed that the particles could undergo rapid reversible transitions between random diffusion and systematic transport and that the diffusion coefficient of particles being systematically transported was markedly reduced compared to the randomly diffusing particles (Sheetz et al., 1989). These results are difficult to reconcile with a lipid flow model, but are consistent with the concept that crosslinked cell surface proteins link reversibly to cytoskeletal motors and are drawn by them toward the back of the cell. SPT was also used to measure the motion of randomly diffusing particles on rapidly locomoting fish epidermal cells (Kucik et al., 1990). It was shown that the diffusing particles experienced no systematic drift relative to the cell carrying them and therefore that effects of lipid currents could not be detected in the motions of these particles.

These measurements show how SPT is especially useful in characterizing the motions of particles that can exist in and can undergo transitions among different dynamic states.

5.3.3. Voltage-Dependent Sodium Channels Exist in Different Dynamic States in Different Domains of Neurons. Voltage-dependent sodium channels

are essential for the propagation of action potentials in nerve cells. It is therefore interesting to determine the distribution and mobility of these channels in different regions of neurons. The channels could be labelled with specific fluorescent tags derived from well characterized toxins. Using these specific labels it was possible to show that nearly all labelled channels diffuse freely and rapidly in the membrane of the cell body (Angelides et al., 1988). In contrast, on the axon hillock (the initial portion of the axon as it emerges from the cell body) the lateral diffusion of the channels is restricted. On this region of the cell the channels diffuse much more slowly. Moreover, there is a barrier that prevents diffusion of channels between the hillock and the cell body. This is a pattern of organization specific to the voltage-dependent sodium channel and is not shown either by a lipid probe or by a relatively nonspecific lectin label.

FPR and other fluorescence microscopy methods have been used to characterize the development and organization of the cell membrane into different domains in which, in addition to voltage-dependent channels, molecules such as acetylcholine receptors and specific sperm antigens are either constrained or are free to diffuse randomly (e.g., Dubinsky et al; 1989; Cowan et al., 1987; cf., Elson, 1993). The proper spatial and dynamic organization of these domains is presumably essential for the transmission of signals or for other interactions within and among cells.

5.3.4. Diffusion in Small Systems. Because of their high spatial resolution, methods such as FPR and SPT can be used to characterize small domains (e.g., Angelides et al., 1988; Yechiel and Edidin, 1987; Edidin et al., 1991; Vaz, 1992; Glaser, 1992; Edidin, 1992; Wolf, 1992). In measurements of the diffusion of molecules constrained to remain within domains, it is important to take account of the effects of the domain size on the apparent diffusion rate. Mathematically, this corresponds to considering the domain boundaries when solving the diffusion equation. This subject has been analyzed for FPR measurements (Qian and Elson, 1988; Elson and Qian, 1989). It was shown that when the fluorescent probe molecules are confined to a small area, both the size of the area and its shape can influence the apparent rate of recovery. As the area available for diffusion decreases, the apparent diffusion rate increases relative to that for an infinite sample area. This increase in small areas is due to the absence of the contributions from remote points in large domains that contribute relatively slow recovery components. Furthermore, as the sample area becomes more elongated, the diffusion rate appears slower than for compact sample domains with the same area. The apparent fraction of mobile molecules also depends on the area available for diffusion. If the area of the bleached spot is comparable to the available area, the photobleaching pulse could eliminate a substantial fraction of the fluorophores that can participate in recovery. This would cause the fraction of the fluorescence that recovered after photobleaching to be less than unity, even if all the fluorophore were mobile. These effects are relatively simple to account for (Qian and Elson, 1988; Elson and Qian, 1989) and can be useful in the interpretation of experiments, as, for example, in assessing the size of "cages" in actin gels

from the enhancement in the rate of diffusion of fluorescent beads that they contain (Qian et al., 1992).

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