Fluorescence Correlation Spectroscopy: Past, Present, Future

Elliot L. Elson*
Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Washington University in St. Louis, St. Louis, Missouri

ABSTRACT In recent years fluorescence correlation spectroscopy (FCS) has become a routine method for determining diffusion coefficients, chemical rate constants, molecular concentrations, fluorescence brightness, triplet state lifetimes, and other molecular parameters. FCS measures the spatial and temporal correlation of individual molecules with themselves and so provides a bridge between classical ensemble and contemporary single-molecule measurements. It also provides information on concentration and molecular number fluctuations for nonlinear reaction systems that complement single-molecule measurements. Typically implemented on a fluorescence microscope, FCS samples femtoliter volumes and so is especially useful for characterizing small dynamic systems such as biological cells. In addition to its practical utility, however, FCS provides a window on mesoscopic systems in which fluctuations from steady states not only provide the basis for the measurement but also can have important consequences for the behavior and evolution of the system. For example, a new and potentially interesting field for FCS studies could be the study of nonequilibrium steady states, especially in living cells.

INTRODUCTION AND HISTORICAL BACKGROUND
During the almost 40 years since its introduction (1), fluorescence correlation spectroscopy (FCS) has evolved from an esoteric and difficult measurement to a technique routinely used in research and technology (2,3). Yet, the value of FCS for the physical and biological sciences consists not only in the measurements it makes possible but also in the concepts that it illustrates and that form its basis. FCS provides a window on the mesoscopic world and is one progenitor of the field of single-molecule measurements (2). Two groups, one at Cornell University (Ithaca, NY) and the other at the Karolinska Institute (Stockholm, Sweden) simultaneously developed the FCS approach. The Ithaca group focused on lateral diffusion and chemical reaction kinetics (1,4–6) and the Stockholm group, on rotational diffusion (7,8). This minireview describes some of the motivations for the development of FCS, current applications, and suggests some new directions for the future.

One of the original motivations for FCS in Ithaca was to study the mechanism of DNA untwisting (9). An elegant model had been proposed to account for the untwisting of the DNA strands after a perturbation of state sufficiently large to release all basepair interactions (10). Still open was the question of how the strands untwisted when only a fraction of the basepairs was melted. Because of the differences in AT and GC basepair stability, regions of the DNA molecule rich in AT melt at lower temperatures than those rich in GC, forming interior loops. To test a kinetic model that accounts for this conformational heterogeneity, it was desirable to measure the kinetics of melting in steps that included the least number of interior loops. Thus a small perturbation, e.g., temperature jump, is essential, but the smallest perturbation is no perturbation at all. The initial plan, therefore, was to measure the kinetics of fluctuations of helicity of DNA molecules maintained in equilibrium within the helix-random coil transition region. The hypothesis of ultraviolet absorbance that results from the unstacking of the nucleotide basepairs was to be the indicator of fluctuations of helicity (11). Fortunately, studies then being carried out by Bresloff and Crothers (12) suggested that a useful tune-up experiment would be to measure fluorescence fluctuations caused by binding and unbinding of ethidium bromide (EB) to DNA. Eventually this preliminary experiment led to the development of FCS, and it was clear that the original idea of measuring helix fluctuations by hypochromicity was utterly impractical.

The aim of the EB-DNA studies was to demonstrate that the kinetics of chemical reactions could be measured via spontaneous fluctuations of reaction progress in a system resting in equilibrium, and this was accomplished (1,4,5). The origins of this idea are rooted in chemical relaxation kinetics (13) and in dynamic light scattering (14–17) on the experimental side and on Onsager’s regression hypothesis on the theoretical side. Although elegant theoretical treatments of measurements of chemical reaction kinetics in equilibrium systems by dynamic light scattering had been developed (18,19), experimental measurements were unsuccessful. Dynamic light scattering is an excellent approach for measuring molecular transport in highly purified, relatively concentrated systems. It is less useful for measuring chemical reaction progress; the polarizability that determines the magnitude of scattering typically changes very little because of chemical transformation. This realization led to the idea of using more chemically sensitive optical parameters such as fluorescence, optical

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*Correspondence: elson@wustl.edu
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absorbance, or optical rotation as indicators of reaction progress. Of these, fluorescence has some important advantages. Not only can it provide a sensitive indicator of reaction progress in favorable systems, fluorescence measurements are 1), simple and rapid; 2), have a low background due to the Stokes shift of the fluorescence emission away from the excitation wavelength; 3), can be measured with high sensitivity down to the nanomolar and even single-molecule range; and 4), have a selectivity that allows for the measurement of specific fluorescent molecules in systems that contain high concentrations of molecules that are not fluorescent in the same spectral range. Furthermore, fluorescence microscopy enables the facile application of FCS to small systems, especially biological cells.

The original FCS experiment was set up on an optical bench with the EB-DNA sample contained in a flat thin-walled cell with 150- or 25-µm path-length. A long-focal-length lens focused the laser beam through the sample so that the beam was effectively uniform (5.7-µm radius) through the sample. This lack of variation along the optical (z) axis provided an effectively two-dimensional system (5). Immediately after the first successful demonstration of FCS with the EB-DNA system, the experiment was performed on a confocal microscope (20) for the study of the mobility of molecules on cell surfaces. At that stage of its development, however, FCS was no longer suitable. To obtain a statistically adequate fluctuation record required excessively long times for measurements on labile systems like live cells. It became clear that fluorescence photobleaching, which had previously been introduced by Peters et al. (21) and Edidin et al. (22), would be more useful. Our group developed a general fluorescence photobleaching recovery method (20,23), and applied it to study the mobility of membrane proteins (24–28) along with many other laboratories (29). The advantage of fluorescence photobleaching recovery over FCS for these studies arose from the fact that, being a measurement of a macroscopic fluorescence recovery, the signal is larger, and a single recovery transient is sufficient to characterize the diffusion rather than a record of many fluctuations that each required a comparable time. Although FCS and photobleaching recovery seem rather different, their implementation on a confocal microscope (20) and their theoretical interpretation are fundamentally quite similar (30).

**CONCEPTUAL BASIS OF FCS**

**Fluorescence fluctuation autocorrelation function**

FCS provides information about both kinetic and thermodynamic properties of fluorescent molecules in solution. The temporal relaxation of the measured fluorescence fluctuations provide the former; the amplitudes of the fluctuations, the latter. Conventional methods for measuring diffusion coefficients and chemical reaction rate constants rely on establishing an initial state displaced from equilibrium and then measuring the rate of the system’s relaxation back to equilibrium. For example, diffusion coefficients can be measured by observing the dissipation of an initial concentration gradient (31) and chemical rate constants by observing the rate of return of system to equilibrium after a temperature jump (13). FCS determines these coefficients in systems that remain in a steady state, either equilibrium or nonequilibrium.

Although the average concentrations in these systems remain constant in space and time for long (in principle infinite) time-periods, they spontaneously fluctuate locally within the system due to Brownian motion in space and as a Poisson process for chemical reaction. Thus, molecules constantly diffuse into and out of a local subvolume of the system, causing fluctuations of their local concentrations and therefore, if the molecules are fluorescent, fluctuations in the fluorescence measured from the subvolume. Similarly, the concentrations of the reactants in a chemical reaction system fluctuate about their equilibrium values, which, if the reaction causes a change in fluorescence, also produce fluorescence fluctuations. These fluctuations are stochastic and differ one from another over time and position. Therefore, it is not possible to determine accurately a transport coefficient, e.g., for diffusion or electrophoretic mobility, or a chemical rate constant by measuring a single fluctuation. Nevertheless, the rates at which the fluctuations dissipate are determined on average by the same phenomenological transport coefficients and rate constants that govern macroscopic dynamic processes (32).

To extract these commonly used coefficients it is necessary to carry out a statistical analysis of the fluctuation data. A fluorescence fluctuation autocorrelation function, \( G(\tau) \), accomplishes this for systems in which fluorescent molecules participate in dynamic processes. \( G(\tau) = \langle \delta F(t) \delta F(t+\tau) \rangle \) (31). The symbols \( \langle \cdot \rangle \) imply averaging over a long (in principle, infinite) time-period. The second equality emphasizes that the system is stationary and can be understood as an average over many randomly selected intervals \( \tau \) or as an ensemble average. The fluorescence fluctuation is \( \delta F(t) = F(t) - \langle F \rangle \), where \( \langle F \rangle \) is the average value of \( F(t) \). That \( G(\tau) \) is independent of \( t \) arises from the fact that the system is in a steady state. Except for nonequilibrium systems, e.g., oscillating systems, the average product of a fluctuation amplitude at some time, \( 0 \), and a later time, \( \tau \), \( \langle \delta F(0) \delta F(\tau) \rangle \) is a decreasing function of \( \tau \) as the fluctuation regresses to the steady state.

The fluctuations of fluorescence report on fluctuations of concentration in the measurement subvolume. The fluorescence photons emitted by molecules of a single fluorescent component, say the \( i \)th component, from a subvolume \( dV \) at position \( r \) and time \( t \) is \( dF_i(r,t) = g_i(r)c_i(r,t)dV \), where \( I(r) \) is the intensity of the excitation laser beam and \( c_i(r,t) \) and \( g_i \) are, respectively, the concentration and a factor that
accounts for absorbance and quantum yield of the \(i^{th}\) component. The number of photons detected by the photodetector (typically, a photomultiplier or avalanche photodiode) is 
\[dF(r,t) = cef(r),\]
where \(cef(r)\) is the collection efficiency function that specifies the fraction of the emitted fluorescence intensity that is registered (33). Thus, concentration and fluorescence fluctuations are related for the \(i^{th}\) fluorescent component as 
\[\delta F_i(t) = \frac{\int_{-\infty}^{\infty} I(r) cef(r) \delta c_i(r,t) dV}{\text{volume of the entire system}} ,\]
with the integral taken over the volume of the entire system. For simplicity in the following, we will suppose that \(cef(r) = 1\). Finally, for a system that has \(M\) fluorescent components,
\[F(t) = \sum_{i=1}^{M} F_i(t); \delta F(t) = \sum_{i=1}^{M} \langle F_i \rangle \]
and so
\[G(\tau) = \frac{\sum_{i=1}^{M} \sum_{j=1}^{M} \langle \delta F_i(0) \delta F_j(t) \rangle}{\langle \sum_{j=1}^{M} \langle F_j(t) \rangle \rangle^2}
= \frac{\sum_{i=1}^{M} g_i \int_{-\infty}^{\infty} I(r) I(r') \langle \delta c_i(r,0) \delta c_j(r',\tau) \rangle dVdV'}{\left[ \sum_{i=1}^{M} g_i \langle c_i \rangle \right]^2} \tag{1} \]
where the laser excitation power is 
\[P = \int_{-\infty}^{\infty} I(r) dV .\]
Experimental measurements are a sequence of numbers of photons counted during time intervals or bins of duration specified to suit the experimental system. The duration of the time bin should be short compared to the shortest characteristic time of interest, but, to minimize the shot or detector noise, no shorter. For the \(i^{th}\) time bin of duration \(dt\), the experimentally measured photon count is \(\hat{F}(idt)\). (The accent \(\hat{\cdot}\) indicates an experimental measurement of \(F(t)\).) Then, the experimentally measured correlation function is defined operationally as
\[\hat{G}(\tau) = \lim_{N \to \infty} \left( \frac{1}{N} \right) \sum_{i=1}^{N} \delta \hat{F}(idt) \delta \hat{F}(idt + \tau) / \langle \hat{F} \rangle^2 \]
\[= \lim_{N \to \infty} \left( \frac{1}{N} \right) \sum_{i=1}^{N} \hat{F}(idt) \delta \hat{F}(idt) = \hat{F}(idt) - \langle \hat{F} \rangle . \tag{2} \]

**Dynamics**

The phenomenological rate parameters, diffusion coefficients, chemical kinetic rate constants, etc., are derived from the experiment by comparison with analytical models of the dynamic processes in the system. For example, a system containing \(M\) chemical components in which diffusion, convection, and chemical reaction are all taking place would be described with equations of the form
\[\frac{\partial \delta c_i(r,t)}{\partial t} = D_i \nabla^2 \delta c_i(r,t) - V_i \frac{\partial \delta c_i(r,t)}{\partial x} + \sum_{j=1}^{M} T_{ij} \delta c_j(r,t) . \tag{3} \]
The first two terms on the right-hand side of Eq. 3 refer, respectively, to diffusion with diffusion coefficient \(D_i\) and convection along the \(x\) axis at constant velocity \(V_i\) of the \(i^{th}\) component. The third term represents chemical reaction kinetics with \(T_{ij}\) being the rate coefficient for the transformation of component \(j\) to component \(i\). Note that \(T_{ij}\) contains chemical kinetic rate constants and, for nonlinear reaction steps, also steady-state reactant concentrations. Each kind of process yields a different characteristic relaxation rate. For simplicity, consider a two-dimensional system, e.g., a cell membrane, interrogated by a Gaussian laser excitation intensity profile: \(cef(r)I(r) = I_0 \exp(-2r^2/h^2)\), where \(r^2 = x^2 + y^2\) with \(x\) and \(y\) being coordinates in the plane of focus of the microscope. Table 1 shows the correlation functions for common dynamic molecular processes. The characteristic times each have a different dependence on the beam radius \(w\). The correlation functions for these different processes are readily distinguishable in theory, but data of high quality are required to accomplish this in practice. Examination of the initial slope of the correlation function can provide useful information about the mechanism of fluctuation relaxation.

For simple diffusion and diffusion plus convection, the initial slope is 
\[\lim_{\tau \to 0} \frac{dG(\tau)}{d\tau} = -\tau_{D}^{-1} \]
where \(\tau_{D} = w^2/4D\), the initial slopes for these processes vary as \(w^{-2}\), which can be tested by changing the objective lens or by using a variable beam expander to vary \(w\) (34). For simple convection, the initial slope vanishes. The correlation times for chemical reactions are independent of \(w\) and vary with reactant concentrations for reactions of order higher than first. For coupled diffusion and reaction, the dependence of the initial slope on \(w\) and concentrations is more complicated, as can be seen from the analysis of the simple isomerization reaction (4). Sometimes it is important to use different methods to verify transport mechanisms that have been deduced from FCS measurements (119).

In general, the solution of systems of equations in the form of Eq. 3 is complicated. Significant simplification is possible, however, if all the reaction components have the same diffusion coefficients (or convection rates). Then the autocorrelation function can be expressed as a simpler product of a function that depends only on the transport coefficients and a function that depends only on the chemical kinetic rate constants and equilibrium reactant concentrations (35).
TABLE 1 Some of the processes that can be measured by FCS

<table>
<thead>
<tr>
<th>Process</th>
<th>( \frac{\delta c_i}{\delta t} )</th>
<th>( \frac{G(\tau)}{G(0)} )</th>
<th>Parameter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion</td>
<td>( D_i \nabla^2 c_i )</td>
<td>( 1 + \frac{\tau}{\tau_d} )</td>
<td>( \tau_d = \frac{w^2}{4D_i} )</td>
<td>(5)</td>
</tr>
<tr>
<td>Convection</td>
<td>(-V_i \nabla c_i )</td>
<td>( \exp \left[ -\frac{\tau}{\tau_v} \right] )</td>
<td>( \tau_v = \frac{w}{V_i} )</td>
<td>(6)</td>
</tr>
<tr>
<td>Diffusion + convection</td>
<td>( D_i \nabla^2 c_i - V_i \nabla c_i )</td>
<td>( 1 + \frac{\tau}{\tau_d} )</td>
<td>As above</td>
<td>(6)</td>
</tr>
<tr>
<td>Chemical reaction</td>
<td>( \sum_{j=1}^{M} T_{ij} \delta c_i )</td>
<td>( \sum_{j=1}^{N} A_j \exp \left[ -\frac{\tau}{\tau_j} \right] )</td>
<td>( T_{ij}, A_r, ) and ( \tau_r ) are functions of the rate constants and equilibrium concentrations</td>
<td>(5)</td>
</tr>
</tbody>
</table>

For simplicity, we suppose a two-dimensional system. There are \( M \) chemical components with concentration \( c_i \) and \( N \) chemical reactions. For the \( k \)th component, the diffusion coefficient and convection velocity are \( D_i \) and \( V_i \), respectively. For coupled diffusion and reaction typical for an FCS measurement in an open chemical reaction system (Eq. 3), the relaxation modes are often complicated functions mixing diffusion (and convection) and chemical reactions (5).

Amplitudes

Although the time dependence of \( G(\tau) \) yields a system’s dynamic characteristics such as transport and chemical kinetic coefficients, the correlation function amplitude, \( G(0) \), is a thermodynamic property that provides information about the concentrations and brightnesses of the fluorescent molecules in the system. For a simple illustration, suppose that \( n(t) \) is the number of molecules at time \( t \) in an observation area or volume exposed to uniform excitation intensity and that the fluorescence is proportional to \( n \): \( F(t) = q n(t) \) and \( \delta F(t) = q(n(t) - \langle n \rangle) \), where \( q \) is the brightness of the molecules, i.e., the number of photon counts emitted per second by a molecule observation region. The normalized amplitude of the correlation function is \( G(0) = \langle (\delta F(0))^2 \rangle / \langle F \rangle^2 = q^2 \langle (\delta n)^2 \rangle / \langle q(n) \rangle^2 \). FCS is almost universally carried out on systems in which the concentrations of fluorescent molecules are very low, in the nanomolar range. Hence, the fluorescent molecules interact negligibly with each other and so, as an ideal system, the number of molecules in the observation volume can be described by a Poisson distribution for which \( \langle (\delta n)^2 \rangle = \langle n \rangle \) (36). Then, \( G(0) = \langle n \rangle^{-1} \). Hence, we have the remarkable result that \( G(0) \) yields the absolute number of the molecules of the fluorophore in the observation region, and, knowing the volume of the observation region, their concentration in solution. The mean fluorescence, \( \langle F \rangle \), then provides the brightness, \( q \), of the fluorophore: \( q = \langle F \rangle / n = G(0) / (F) \). These results can be derived directly from Eq. 1, noting that as a consequence of ideality and the Poisson distribution,

\[
\langle \delta c_i(r, 0) \delta c_i(r, 0) \rangle = \langle c_i \rangle \delta(r-r') \delta_{ij} \tag{4}
\]

(4,37), where \( \delta_{ij} \) is the Kronecker delta and \( \delta(r-r') \) is the Dirac delta function. For a system with \( N \) species of fluorescent molecules, the result is more complex,

\[
G(0) = \frac{1}{\pi w^2} \sum_{i=1}^{N} \frac{\langle c_i \rangle q_i^2}{\left( \sum_{i=1}^{N} \langle c_i \rangle q_i \right)^2}.
\]

as can readily be demonstrated using Eqs. 1 and 4. Adapting this concept to laser scanning microscopy provides the basis of the useful number-and-brightness (N&B) method for characterizing fluorophores on cell surfaces (38).

One way to obtain information about the distribution of the concentrations and brightnesses of a mixture of fluorophores \( \{c_i, q_i\} \) is to evaluate the higher moments of the fluorescence fluctuations (39,40). In a system containing \( M \) distinct components, with the concentration and brightness of the \( k \)th component being \( c_k \) and \( q_k \), respectively, the cumulants of the measured fluorescence fluctuations are related to moments of the brightness, \( \sum_{k=1}^{M} c_k q_k^n, n = 1, 2, \ldots \) (A probability distribution can be reconstructed from its moments (41). Cumulants are related to moments. Just as moments can be derived from a Taylor’s expansion of a moment-generating function, cumulants can be derived from a cumulant-generating function that is the logarithm of the moment-generating function. Compared to moments, photocount cumulants have the advantage that they properly account for detector (shot) noise.) For example, the brightness and concentrations in a two-component system can be determined from the first three cumulants of the fluorescence fluctuations. An alternative is to evaluate the zero-time amplitudes of high-order correlation functions, e.g.,

\[
\frac{\langle \delta F^n(0) \delta F^n(0) \rangle}{\langle F \rangle^{2n}} - \langle \delta F^n \rangle \langle \delta F^n \rangle
\]

(42,43). This approach has recently been simplified and extended (44). The practical applicability of this latter intriguing method remains to be demonstrated in practical applications.
More effort has been devoted to developing and applying methods based on the photon count histogram (PCH) to provide information about the distribution of molecule concentrations and brightnesses (45,46). As indicated above, the data record for an FCS measurement is a sequence of time bins each of which contains some number, \( n \), of photons including the possibility that \( n = 0 \). The PCH is the probability, \( P(n) \), that a time bin contains \( n \) photons. To determine the values of the \( \{n_i,q_i\} \) of the fluorescent particles from an experimental measurement it is necessary to postulate an analytical model that has a given number of particle species with specified values of \( n_i \) and \( q_i \). A good fit, e.g., using a nonlinear least-squares method, of the measured PCH to one derived from the model corroborates the adequacy of the model and provides values of \( \{n_i,q_i\} \). The model is either based on prior knowledge of the composition of the system or else is a minimal model just sufficient to account for the measurements.

To construct the model PCH one accounts for the probabilities that \( m \) molecules are in the observation volume and that these \( m \) molecules produce \( n \) photons. For a single component, \( P(n) = \sum_{m=0}^{\infty} P_m(m)P_n(n|m) \), where \( P_m(m) \) is the probability that \( m \) molecules are in the laser-illuminated observation volume and \( P_n(n|m) \) is the conditional probability that \( n \) photons are emitted from the \( m \) molecules (45). Both \( P_m \) and \( P_n \) are Poisson probability distributions. The PCH for a multicomponent system can be expressed as convolutions of the PCHs of the various system components (47) or as a product of the generating functions, \( H(\xi) \) (where \( H(\xi) = \sum_{n=1}^{\infty} P(n)\xi^n \)) for the PCHs of each component (45).

Fig. 1A illustrates experimentally measured PCH curves for a preparation of allophycocyanine. The PCH is most simply interpreted in terms of a two-component system with one component at low concentration that is 15-fold brighter than the component at higher concentration. Fig. 1B shows the fluctuation autocorrelation function measured for the same solution. For spherical particles, the ratio of diffusion coefficients should vary inversely as the cube root of the molecular weight. Hence, if the aggregate were approximately spherical, the diffusion coefficients of the two components should be in the ratio of \((1/1.5)^{1/3} = 2.5\). The FCS data are well fitted by fixing the correlation times in the ratio of 2.5 although the best fit to the correlation function yields a ratio of 4.5. These graphs are meant to illustrate the relationship between the PCH and the diffusion correlation function for an aggregation system. Because a range of parameters will fit both PCH and FCS curves, however, these data alone are insufficient to define the brightness and numbers of the components of the system. A proper PCH/FCS analysis of this system requires more measurements.

In principle, this approach can supply the values of \( \{n_i,q_i\} \) for an arbitrary multicomponent system. In practice, however, to identify the values even of only a few components...
requires an extensive fluctuation data record. One source of ambiguity is the fact that a dim particle near the center of the Gaussian laser excitation profile appears similar to a bright particle farther from the beam center. The ability of the PCH approach to distinguish between models has been explored (46) and the approach has been extended to the time domain and the use of several fluorescence colors (e.g., (48–52)).

**Single-molecule aspects of FCS**

Although our formulation of FCS theory uses conventional continuum chemical concentrations $c(r,t)$, FCS is essentially a single-molecule measurement. Due to the ideality of systems investigated by FCS, fluorescent molecules do not significantly interact with one another, and so the motion of each fluorescent molecule is correlated only with itself and is uncorrelated with that of all the others. FCS measures the behavior of many individual molecules without dwelling on any of them. In this sense, it differs from current approaches that identify individual single-molecules and characterize their behavior one at a time (e.g., (53–55)). Nevertheless, FCS can be regarded as a precursor of these kinds of measurements and is capable of single-molecule sensitivity (56,57).

The improvement of sensitivity to the single-molecule level, which was crucial in transforming FCS from an esoteric and difficult to a routinely applied measurement (3), resulted from a variety of technological advances including improvements in lasers, correlators, and microscopes. The reduction of the laser-illuminated sample region to a diffraction-limited volume reduced background fluorescence and was an essential step in enabling single-molecule sensitivity (56). Moreover, the reduction of the sample volume and therefore also the diffusion fluctuation correlation time accelerated the acquisition of statistically significant correlation functions. Nevertheless, the use of diffraction-limited confocal optics can raise problems in precisely defining the shape of the laser-defined observation volume. Diffraction effects can distort the shape of the sample volume enough to have a substantial effect on FCS autocorrelation functions (58). Using two-photon excitation (59–61) diminishes the magnitude of these effects (58).

Finally, an interesting application of the principle that a molecule’s motion correlates only with itself is the pair correlation function approach, recently demonstrated for tracking populations of single fluorescent molecules, typically on cell surfaces (62). Because of the exclusive self-correlation of a molecule with itself, cross correlation from two regions of an object will reveal the extent to which a molecule can move from the first to the second region. This approach is useful for mapping barriers to diffusion on cell surfaces that define regions of the surface that are inaccessible to the diffusing molecule and has been applied to study transport through nuclear pores (63). (For an instructive explanation of the pair correlation method, see Digman and Gratton (64).) Earlier implementation of the pair correlation concept involved cross correlation of fluorescence from two laser foci separated by a fixed distance (e.g., (65–67)). An advantage of the approach described above is that position pairs are sampled over the entire scan range rather than only at the two foci of the split laser beam (64).

**FCS measurement error**

To understand the level of significance of FCS measurements, it is important to understand and evaluate the contributions of various error sources including both systematic errors and random noise. Systematic errors are consistent from measurement to measurement and can result, for example, from misalignment of the optical system resulting in distortions of the measurement volume and therefore in errors in diffusion measurements. Random errors can result both from photon (shot) noise and from finite character of the fluctuation record. Shortly after the introduction of FCS, Koppel (68) provided a pioneering analysis of the variance and signal/noise ratio of FCS measurements. This was then extended by several authors (69–71). These articles provide analytical calculations of the theoretical variance of FCS measurements that can be used to optimize experimental measurements and to assess their validity.

**FCS measurements below the optical resolution limit**

To measure diffusion by FCS, the diffusing fluorescent particles must be able to move between regions of high and low excitation intensity. Hence, the volume of the laser-excited observation region, the detection volume, must be smaller than the volume in which the particles are confined. The minimum detection area in the focal plane, set by the optical diffraction limit, can be gauged by the full width at half-maximum (FWHM) of the point spread function (PSF) of the microscope objective: $d_{\text{xy}} = \text{PSF}_{\text{FWHM}} \approx 0.61 \lambda_o / \text{NA}$, where $\lambda_o$ is the excitation wavelength and NA is the numerical aperture of the objective (72). Also, $d_z \approx n \lambda_o / \text{NA}^2$, where $n$ is the refractive index of medium (72). For currently available high NA objectives, $\text{PSF}_{\text{FWHM}}$ values are in the range 200–250 nm in the focal plane and 500–700 nm along the optical axis. It could be useful to measure diffusion in compartments smaller than this size range (for example, in endocytic vesicles or to test small-scale heterogeneity on cell membranes). Furthermore, because the amplitude of the correlation function varies inversely as the number of fluorescent molecules in the observation volume, smaller observation volumes would allow FCS measurements at higher concentrations. Recently, several approaches to shrink the observation volume have emerged (72) that are applicable to FCS (73).

Among these is stimulated emission depletion microscopy, a method developed for superresolution fluorescence
microscopy that shrinks the excitation laser diameter by using a second, ring-shaped laser intensity profile to deplete the fluorescence excited by the peripheral regions of the excitation beam (74). For example, stimulated emission depletion FCS measurements have shown that cholesterol-rich nanodomains <20 nm in diameter transiently trap glycophosphatidylinositol-anchored cell membrane proteins (75). Methods based on evanescent radiation fields provide another way to reduce observation volume. Of these, the best known is total internal reflection fluorescence microscopy (TIRFM) in which the evanescent field illuminates a thin layer of the object above its interface with a glass substrate. Applications of TIRFM to FCS and to photobleaching recovery date back 30 years (76) and continue to be actively pursued (e.g., Lieto et al. (77), Ohsugi et al. (78), and Vobornik et al. (79)). Although TIRFM strongly reduces the illuminated dimension, special measures are needed to obtain a small spot in the x,y plane.

Using a parabolic mirror objective yields a detection volume <5 attoliters (<5×10⁻⁸ liter), well below a typical value for conventional confocal microscopy (~100 attoliters) (80). By using this approach, it was possible to perform FCS measurements at concentrations as high as 0.2 μM (80). Zero-mode waveguides or nanometric apertures allow FCS to be performed with even smaller detection volumes in the zeptoliter range (10⁻²¹ liter) so that samples are in the single-molecule regime even at concentrations as high as 200 μM (81). FCS with zero mode waveguides has been used to measure single-molecule motion on cell membranes (82). Before this approach can quantitatively determine diffusion coefficients, however, the shape of the effective detection volume must be better defined. Near-field optical microscopy provides another way to reduce observation volume (83) that can be applied to study membrane transport (79). A study using near-field scanning optical microscopy of transport through nuclear pores provides an interesting illustration of this approach (84).

SELECTED APPLICATIONS

FCS has become a practical measurement method with applications ranging from photophysics, e.g., triplet state dynamics (85,86) and photon antibunching (87,88), to polymer physics (89–93) and to biology and medicine including studies of living cells (2,3,64,94–96). An extensive survey over this wide range is beyond the scope of this minireview. Studies in two areas—molecular interactions and chemical kinetics, respectively—illustrate how FCS concepts have been extended in many directions.

Interactions, aggregation, polymerization

In biology and chemistry, it is often desirable to learn whether molecular species can form heterologous complexes or polymerize (or aggregate). Archetypal examples include clustering of cell membrane proteins and amyloid polymerization. The former phenomenon is central for the activation of growth factor receptors, e.g., Yarden and Schlessinger (97), and the latter, for the formation of pathogenic amyloid fibers (98). FCS enables study of these phenomena at high dilution and in small systems like biological cells or in nonbiological nanostructures.

Fluorescence cross-correlation spectroscopy (FCCS) detects interactions between molecules that have fluorophores with different fluorescence emission wavelengths, e.g., λa and λb (99,100). The fluorescence intensities of the two colors, Fa(t) and Fb(t), detected in the same observation volume, are separately registered by two different detectors. If the a-colored and b-colored molecules move independently, their fluctuations are uncorrelated, and so the cross-correlation function, Gab(τ) = ⟨δFa(0)δFb(τ)⟩ = 0. If, however, the a-colored and b-colored molecules are linked so that they diffuse into and out of the fluorescence detection volume together, then Gab(τ) ≠ 0, and its initial amplitude, Gab(0), depends on the fraction of the molecules that are linked (101). Measurement of FCCS, using standard one-photon excitation, requires precise alignment of the two excitation lasers that excite the two fluorescence colors to maximize their coincidence, with calibration to assess cross talk between the detection channels and supply an accounting of photobleaching (102). Using two-photon excitation circumvents the alignment difficulties. Because different selection rules pertain to one-photon and two-photon excitation, it is possible to simultaneously excite spectrally distinct fluorophores with a single two-photon excitation wavelength, eliminating the need for coincident alignment of two separate one-photon excitation lasers tuned to different wavelengths (103). It is also possible with a favorable choice of fluorophores to carry out FCCS measurements with a single one-photon excitation laser (104). Pulsed interleaved excitation or alternating laser excitation provides a way to reduce cross talk between two fluorophores (105–107). The lasers that excite the two different fluorophores are alternatively pulsed at intervals that allow complete decay of the fluorescence intensity of one color before the excitation of the other. This allows uncontaminated detection of the photons emitted from the two fluorophores. Several applications of FCCS have been described in a recent review (99).

Aggregation and interaction can also be detected through their effects on molecular frictional coefficients and on brightness. An early approach was to measure the decrease in the diffusion coefficients of the reactant molecules due to their formation of larger and therefore more slowly diffusing structures (3). Although this approach was applied successfully, it is relatively insensitive because diffusion depends weakly on the size of a compact diffusing particle. The diffusion coefficient of a sphere varies inversely as its radius and therefore as the cube root of its molecular weight, i.e., as the cube root of the number of subunits in an approximately
spherical aggregate. In contrast, fluorescence brightness can be much more sensitive to aggregation. Suppose that each monomer or subunit in an aggregate or polymer has a fluorescent label and that the fluorophores in the aggregate do not interact electronically, i.e., they do not quench or enhance each other. Then, the brightness of the aggregate will be directly proportional to the number of subunits (6). As we have seen, the average molecular brightness is readily available in a FCS measurement from \( G(0)/F \).

The accuracy of brightness measurements increases with the number of observed fluorescence fluctuations. For methods such as standard FCS or PCH fluorescence intensity distribution analysis (FIDA), the rate at which fluctuations are observed depends on the diffusion rate of the fluorescent molecules or particles. If the lifetime of the fluctuations, \( \tau_d \), is large, an excessively long time may be required to collect enough fluctuations. For solution samples, one could accelerate the accumulation of fluctuation data by flowing the sample past the laser beam. This acceleration will occur if \( \tau_f < \tau_d \), where \( \tau_f \) and \( \tau_d \) are the characteristic times of flow and diffusion (Table 1) (6). A simpler approach, called scanning FCS (sFCS), avoids the need to account for velocity gradients in the flow pattern by scanning the laser beam over the sample. sFCS is most readily applicable to measurements on stable structures and in particular to cell surface measurements of membrane protein aggregation (108). One obtains a record of the fluctuations of fluorescence intensity at positions along the scan line to yield a correlation function of the form

\[
G(\xi) = \frac{\langle \delta F(x) \delta F(x + \xi) \rangle}{\langle F(x) \rangle^2},
\]

or, operationally, if the fluorescence is measured at points \( k = 1, 2, ..., N \) along the scan,

\[
\hat{G}(k) = \frac{1}{N_k} \frac{1}{N} \sum_{j=1}^{N} \hat{F}(j) \hat{F}(j + k),
\]

where \( N_k = N - k \). Both FCS and sFCS detect series of independent samples of particle brightness, the former as particles diffuse into and out of the sample volume, the latter as the laser beam is scanned across the object. Hence, for sFCS as well as for FCS, \( G(0) = 1/N \), and so both methods can yield average particle brightness and therefore aggregation.

This approach, based on spatial rather than temporal auto-correlation, can be further generalized to an analysis of fluorescence fluctuations in images acquired by confocal scanning laser microscopy, an approach called image correlation spectroscopy (ICS) (109). ICS is the progenitor of a series of image correlation methods summarized below and in Table 2 that have been admirably reviewed (110). These methods are used mainly to obtain information about aggregation of or interactions among cell surface proteins. They have two important advantages: 1) large quantity of fluorescence fluctuation data is simultaneously and rapidly acquired in individual images, and 2) the morphological information in the image can be correlated with the measurements of aggregation.

ICS extends the sFCS approach to planar objects, e.g., cell surfaces, in the form of a two-dimensional spatial correlation function,

\[
G_{ICS}(\xi, \eta) = \frac{\langle \delta F(x, y) \delta F(x + \xi, y + \eta) \rangle}{\langle F(x, y) \rangle^2},
\]

and

\[
\hat{G}(\xi, \eta) = \frac{1}{NM} \sum_{k=1}^{N} \sum_{j=1}^{M} \hat{F}(j, k) \hat{F}(j + \xi, k + \eta)
\]

Using a confocal or two-photon laser scanning microscope, the excitation laser intensity profile, determined by the point spread function of the microscope objective lens, integrates the emission intensity detected from the object area (pixels) that it spans at each scan location. Given the usual Gaussian intensity profile, \( I(x, y) = I_0 \exp\left(-2(x^2 + y^2)/w^2\right) \),

\[
G_{ICS}(\xi, \eta) = G_{ICS}(0, 0) \exp\left[-\frac{2(\xi^2 + \eta^2)}{w^2}\right]
\]

(110). Fitting this Gaussian function then yields \( G_{ICS}(0, 0) \).

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This fitting process evades a shot noise artifact at \( \xi = 0 \),

<table>
<thead>
<tr>
<th>Name</th>
<th>Correlation function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>STICCS</td>
<td>( G_{ab}(\xi, \eta, \tau) = \frac{\langle \delta F_a(x, y, \tau) \delta F_b(x + \xi, y + \eta, \tau) \rangle}{\langle F_a(x, y, \tau) \rangle \langle F_b(x + \xi, y + \eta, \tau) \rangle} )</td>
<td>(110)</td>
</tr>
<tr>
<td>ICS</td>
<td>( G(\xi, \eta, 0) = \frac{\langle \delta F(x, y) \delta F(x + \xi, y + \eta) \rangle}{\langle F(x, y) \rangle^2} )</td>
<td>(109)</td>
</tr>
<tr>
<td>TICS</td>
<td>( G(0, 0, \tau) = \frac{\langle \delta F(x, y, \tau) \delta F(x, y + \tau) \rangle}{\langle F(x, y, \tau) \rangle \langle F(x, y + \tau) \rangle} )</td>
<td>(138)</td>
</tr>
<tr>
<td>ICCS</td>
<td>( G_{ab}(\xi, \eta) = \frac{\langle \delta F_a(x, y) \delta F_b(x + \xi, y + \eta) \rangle}{\langle F_a(x, y) \rangle \langle F_b(x + \xi, y + \eta) \rangle} )</td>
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</tr>
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<td>(110)</td>
</tr>
<tr>
<td>STICS</td>
<td>( G(\xi, \eta, \tau) = \frac{\langle \delta F(x, y, \tau) \delta F(x + \xi, y + \eta, \tau) \rangle}{\langle F(x, y, \tau) \rangle \langle F(x + \xi, y + \eta, \tau) \rangle} )</td>
<td>(113)</td>
</tr>
</tbody>
</table>

The most general space-time correlation approach is STICCS. The other methods correlate subsets of spatial and temporal correlations (110). Abbreviations: ICS, image correlation spectroscopy; STICCS, spatiotemporal image cross-correlation spectroscopy; TICS, temporal image correlation spectroscopy; ICCS, image cross-correlation spectroscopy; TICCS, temporal image image cross-correlation spectroscopy; STICS, spatiotemporal image correlation spectroscopy.
We have seen, diffusion into and out of the sample volume, convective flow, or chemical reaction. For example, as measured surface are correlated over a time sequence of fluctuations of fluorescence among area elements in the fluorescence image. As before, one can determine the mean brightness of the fluorescent particles as \( \langle q \rangle = G_{ICS}(0,0) \langle F \rangle \) and the mean aggregate size from \( G_{ICS}(0,0) \langle F \rangle / q_m \), where \( \langle F \rangle \) and \( q_m \) are the mean fluorescence over the measured surface and the monomer brightness, respectively. In practice, it is essential to take into account background fluorescence from the cell surface (110). Because FCS measures fluctuations from particles that diffuse into and out of the sample volume, aggregates are detected as dynamically linked. This is not so for ICS and sFCS for which the sampling process is independent of the dynamics of the particles in the system. ICS can also be implemented using a high-sensitivity charge-coupled device camera to view a full-field microscope image. For example, TIRFM has been used with ICS to study IgE clustering on cell surfaces (111).

For temporal image correlation spectroscopy (TICS), fluctuations of fluorescence among area elements in the measured surface are correlated over a time sequence of images (Table 2) (112). Fluorescent particles can enter or leave a particular area element on the cell membrane, \( \delta x \delta y \), by a number of dynamic mechanisms including diffusion, convective flow, or chemical reaction. For example, as we have seen, diffusion into and out of \( \delta x \delta y \) yields a characteristic correlation function \( -(1 + \pi/\tau_D)^{-1} \). If diffusion is homogeneous across the surface, then

\[
G_{TICS} = \frac{G(0,0)}{\tau} + G_{\infty},
\]

where \( G_{\infty} \) is a long-time offset and, as before, \( \tau_D = w^2/4D \).

Image cross-correlation spectroscopy (ICCS) is the straightforward extension of FCCS to an imaging mode (Table 2) and, as with FCCS, ICCS can detect the association of molecules that have been labeled with fluorophores of different fluorescence colors. Note that for FCCS the cross-correlation signal requires the associated molecules to move together into and out of the observation area. In contrast, ICCS provides a measure of colocalization within areas defined by the PSF of the microscope but does not indicate a dynamic linkage. Temporal image cross-correlation spectroscopy (TICS), the cross-correlation analog of TICS, dispenses with spatial cross correlation but does provide this dynamic link (Table 2); \( G_{ab}(0,0,\tau) \neq 0 \) only if \( a \)-colored and \( b \)-colored particles move together.

Spatiotemporal image correlation spectroscopy (STICS) includes both spatial and temporal correlation of image fluorescence and reveals the direction and velocity of systematic motion, e.g., convective flow, of fluorescent particles (Table 2). The spatial autocorrelation provides a Gaussian peak that moves away from its initial center (\( \tau = 0 \)) as the time-lag increases. Tracking this peak yields direction and speed of the systematic motion (113).

Transformation of the STICS correlation function by a two-dimensional spatial Fourier transform allows an easy extraction of the desired dynamic contributions to the autocorrelation function from interfering dynamic processes such as photobleaching and fluorophore blinking. This is the aim of \( k \)-space image correlation spectroscopy (kICS) (114). Space-dependent dynamic processes such as diffusion and flow depend on the Fourier variable, \( k \). The correlation functions for diffusion and flow vary as \( k^2 \) and \( k \), respectively. Photobleaching and blinking do not depend on spatial variables and so are independent of \( k \). Hence, plots of the logarithm of the correlation function versus \( k \) or \( k^2 \) extract diffusion and flow rates from blinking and photobleaching. Of course, the situation becomes more difficult if space-independent processes such as chemical reaction kinetics are to be measured and the time constants of the interfering and the desired processes are similar. A further advantage of kICS is that it can be used without requiring a calibration of the PSF of the optical system (114).

A scanning microscope sequentially scans the object field line-by-line to form a rectangular grid of pixels that compose the image. This raster scanning occurs on three distinct timescales: First, pixels along a line are rapidly scanned with a dwell-time on the order of a few microseconds per pixel. Second, scanning the entire line of pixels requires a time on the order of a few milliseconds. Finally, the full quota of lines required to produce the entire image requires a time on the order of a second. Hence, correlating fluorescence fluctuations along a single line, across a range of lines, and from image to image yields information about dynamics, e.g., diffusion, that occurs on the microsecond, millisecond, and second timescales. This is the basis of raster ICS (115). Advantages of this approach include the wide time-range over which dynamics can be measured, the ability to relate the dynamic information to the structural information in the image, and that readily available commercial scanning confocal microscopes suffice for the measurements. (Note, however, that using line or circular orbit scanning may have some advantages over raster scanning: the former have millisecond temporal and submicron spatial resolution, whereas the latter has microsecond time-resolution but spatial resolution in the range of micrometers (115).) A recent review presents a brief survey of applications of ICS and related methods (110).

Measuring the clustering on cell membranes of growth factor receptors has become a test case for fluorescence fluctuation and other methods. For example, ICS was initially applied to demonstrate clustering of platelet-derived growth factor receptors on human fibroblasts (109,116). A recent article provides a survey of the application to this subject of various fluorescent methods and also an analysis of...
clustering of ErbB receptors using the number-and-brightness (N&B) method (117). The ErbB proteins are a family of tyrosine kinases of which ErbB1, the EGF receptor, is the prototype. The N&B approach is based on the statistics of fluorescence fluctuations in individual pixels of a sequence of microscope images, taking into account not only the fluctuations due to fluorescent molecules entering and leaving the pixel but also the detector noise (38). The basic principles of the N&B approach follow directly from our earlier discussion of fluctuation amplitudes. The variance of the fluorescence, \( \sigma_n^2 \), due to fluctuations in the number of particles in the illuminated sample volume is equivalent to the nonnormalized correlation function defined as above as
\[
q(n) = \langle (\delta F(0))^2 \rangle = q^2 \langle (\delta n)^2 \rangle = q^2(n) = \sigma_n^2, \quad \text{where} \quad q \text{ is the particle brightness.}
\]

The total photocount variance, \( \sigma_d^2 \), also includes a shot (detector) noise contribution: \( \sigma_d^2 = q(n) \). (The shot noise is described by a Poisson distribution, and so its variance is proportional to its mean.) Hence, the total variance is
\[
\sigma^2 = \sigma_n^2 + \sigma_d^2 = (n)q(q + 1).
\]
Similarly, the mean photon count is \( \langle k \rangle = q(n) \). An apparent brightness is defined as \( B = \sigma^2 / \langle k \rangle = q + 1 \) and an apparent particle number as \( N = \langle k \rangle / \sigma^2 = qn/(q + 1) \). If the fluorescent molecules are entirely immobile, \( \sigma_n = 0 \), and \( B = 1 \). A useful feature of the N&B method is that \( N \) and \( B \) values can be mapped onto the object, e.g., a cell. The \( B \) parameter is therefore useful to distinguish regions of a cell surface in which, for example, fluorescent membrane proteins are entirely immobile (\( B = 1 \)) from those in which they are mobile (\( B > 1 \)). Note also that the mobile contribution to \( B \) increases as the laser excitation power increases while the immobile contributions remains at \( B = 1 \), independent of laser power. Finally, it is straightforward to see that \( q = (\sigma^2 - \langle k \rangle) / \langle k \rangle \) and \( \langle n \rangle = (\langle k \rangle)^2 / (\sigma^2 - \langle k \rangle) \). In most applications, especially to biological objects like cells, it is also necessary to correct for a constant background fluorescence and also slow photobleaching. This is accomplished in a straightforward manner as described in Digman et al. (38).

Using N&B, Nagy et al. showed that members of the ErbB family differed in their tendency to cluster and that this tendency also depended on the concentration of the receptor on the cell surface (117). Their results agreed with earlier work using FIDA (118). Although FIDA holds the promise of yielding the size distribution of receptor clusters, this method requires exceptionally high-quality data that may be difficult to obtain on living cells. N&B supplies only the mean values of the brightness and occupation number, but it obtains a large volume of data from the many pixels in an image. Different N&B values can be mapped to different regions of the cell surface.

**Chemical reaction kinetics**

To determine the rate of a chemical reaction, the reaction must cause some change in a measurable property of the reaction system. For FCS, this would typically be a change in fluorescence intensity, i.e., the reaction quenches or enhances the fluorescence of the reactants. Cross-correlation measurements could also detect changes in fluorescence color. FCS can be used to measure kinetics of chemical reactions that are slow compared to the time required to collect a sufficient fluctuation data set (minutes to tens of minutes) by taking serial FCS measurements to determine the relative concentrations of the reactants and products over time. In this instance not only changes in fluorescence but also changes in transport rates, e.g., diffusion or convection, could be used to measure reaction progress. In most cases, however, transport coefficients are relatively insensitive indicators of reaction progress. For reactions that are fast compared to transport (diffusion or convection), the reaction kinetics are uncoupled from transport rates and so can be measured directly via fluorescence change as early relaxation components in the fluctuation autocorrelation function.

When transport and reaction rates are comparable, the extract of the kinetic parameters is more complex. In general, the solutions to Eq. 3 take the form of eigenmodes in which chemical reaction and transport are coupled in a complicated fashion (4). Independent measurements of the diffusion coefficients of the reactants and, for reactions of order two or higher, the dependence of the correlation decay rates on reactant concentrations are essential (e.g., Magde et al. (5)). For systems in which the diffusion coefficients and/or convection rates are the same for all components, expression of the correlation function as a product of correlation functions that separately describe reaction kinetics and transport is an important simplification that allows the extraction of the correlation function for reaction kinetics using independent measurements of transport (35). Any study of chemical kinetics requires a reaction model that is the simplest possible to describe the experimental data. Finally, it is always helpful to test the model by obtaining kinetic data by several different experimental approaches (119).

Although the original motivation for FCS was to develop a method that could measure the kinetics of chemical reaction systems in equilibrium, there have been far fewer applications to this kind of problem than to measurements of transport rates and brightness. Conformational fluctuations of proteins and other polymers is one kind of reaction kinetics that has been studied by FCS (e.g., (54,120–122)). FCS has also been used to measure the kinetics of DNA hairpin helices, a subject that has recently received an excellent review (123). Studies of hairpin helix formation date back to work from the 1960s and 1970s on oligomers of the alternating poly(deoxyadenylate thymodylate) copolymer (dAT) (124–126). Because of their alternating self-complementary structure, dAT oligomers display numerous intermediate helical conformations that complicate the interpretation of experimental measurements. Nonalternating oligonucleotides provide simpler systems with which to study hairpin
helix formation. The first application of FCS to this subject used oligonucleotides with a fluorophore and a quencher attached to the 5' and 3' ends, respectively (127).

The nucleotide sequences at the two ends were 5'-CCCAA and 3'-TTGGG. Intervening were the loop nucleotides composed of varying lengths of T or A. In the hairpin helix conformation, the fluorescence was quenched. When the stem helix melted, the fluorophore and quencher were no longer held in apposition and the fluorescence increased. At temperatures near the helix opening transition, FCS measurements reflected both diffusion (τD ~150 μs) and the relaxation time for the opening and closing of the hairpin helix (τR = 5 μs – 1 ms). Assuming that the diffusion coefficient of the oligonucleotide was sufficiently insensitive to its conformation, the correlation function could be expressed as \( G(τ) = G_D(τ)G_R(τ) \), and \( G_D(τ) \) could be measured with control oligonucleotides that did not produce fluorescence fluctuations from the conformational process. Then \( G_R(τ) = G(τ)/G_D(τ) \) provides τR. An important assumption is that the transition between open (O) and closed (C) conformations is a single-step process: \( O \overset{k_+}{\rightleftharpoons} C \), with rate constants \( k_+ \) and \( k_- \) and the equilibrium constant \( K = k_+/k_- \). Then,

\[
G_R(\tau) = 1 + \frac{1 - p}{p} \exp(-\tau/\tau_R),
\]

where \( p \) is the fraction of the oligonucleotides in the open conformation and \( 1/\tau_R = k_+ + k_- \). By measuring the open-closed (melting) equilibrium (using simple fluorometry) at various temperatures one can determine \( K(T) \) independently, and then \( k_+ = 1/\tau_R(1 + K) \) and \( k_- = k_+K \).

Thus, the rate constants are obtained from the FCS results and the activation energies for opening and closing can be determined from the dependence of \( k_+ \) and \( k_- \) on temperature (127).

Despite its elegant demonstration of how FCS can be used to dissect a chemical reaction system, an important flaw in this analysis appears to be its assumption of a one-step opening-closing transition. Additional studies using both FCS and laser temperature-jump for kinetics and PCH to determine the relative concentrations of open and closed states revealed that the one-step model was insufficient to describe the data. A three-state mechanism was proposed in which the unfolded oligonucleotide forms an intermediate with closed loop and quenched fluorescence but incomplete helical stem (123). The FCS measurements describe this process that occurs in the time-range of tens of microseconds. Formation of the complete hairpin helix is a slower process requiring milliseconds or longer. Further studies that define the energy landscape for hairpin helix formation and characterize kinetic traps that slow the formation of the native hairpin are summarized by Van Orden and Jung (123).

An essential requirement for direct measurements of kinetics of chemical reactions (or conformational changes) by FCS is that the chemical relaxation rate be fast or, at least comparable, to the correlation times for diffusion. If diffusion is fast compared to reaction then the reaction components diffuse into or out of the detection volume before the effects of the reaction can be detected. It is also essential that the reaction cause a substantial change in fluorescence. Both criteria are satisfied in the hairpin measurements described above. It may also be useful to note that transport depends on the radius of the detection volume, \( w \), but chemical kinetics do not. Therefore, varying this radius can help to disentangle contributions from transport and reaction kinetics.

### Possible future application to nonequilibrium steady states

In biological cells, some types of molecules (e.g., genes, mRNA molecules, and regulatory proteins) may be present in concentrations so low that spontaneous fluctuations can influence physiological behavior. These fluctuations can cause functional epigenetic differences among isogenic cells (128,129) and could contribute to evolutionary adaptation and disease (130). Single-molecule approaches have proved very useful to analyze stochastic effects in gene expression (131,132). It seems likely that measurements of the dynamics and concentrations of sparse molecules in cells (e.g., some transcription factors and regulatory kinases) by FCS could also help us to investigate mechanisms of cellular stochastic variation. The field of nonequilibrium steady states (NESSs) is both central to cell behavior and relatively untouched by FCS. The possibility of using FCS to study stochastic effects on NESSs in living cells is worth considering (133).

A major virtue of FCS is that it provides measurements of dynamic parameters for systems that remain unperturbed in equilibrium; however, FCS is also suitable for measurements in systems in NESSs. In both NESS and equilibrium, the concentrations of the reaction participants are statistically stationary in time, but, in contrast to equilibrium steady states, energy must be dissipated to maintain a NESS. Consider the following simple first-order system:

\[
A \xleftarrow{k_1} \; B \xrightarrow{k_2} \; C \xrightarrow{k_3} \; D. \tag{5}
\]

If this system is in equilibrium, then the net flux for each reaction must vanish, e.g., \( k_2\overline{C}_B = k_3\overline{C}_C \), where \( \overline{C}_x \) is the equilibrium concentration of \( x \) and for the system overall,

\[
\frac{\overline{C}_D}{\overline{C}_A} = \frac{k_1k_2k_3}{k_1k_3k_1}.
\]

In a NESS, however, there can be a net flux either to the left or right. For example, if \( k_2\overline{C}_B > k_3\overline{C}_C \) and \( \overline{C}_x \) is the
NESS concentration of x, there is a net flux to the right, and A is continuously transformed into D. To maintain the steady state, the concentrations of A and D must be held constant (guaranteeing that B and C are constant), and so A must be continually supplied to, and D continually withdrawn from, the system—processes that expend energy. (In a test-tube experiment, this can be achieved by a regenerating system.)

In cells, networks of metabolic and signaling reactions drive physiological functions. When a cell is in a stable functional quiescent state, e.g., as an interphase fibroblast or endothelial cell, its underlying biochemical networks are in stable NESSs. An important goal for systems biology is to characterize these NESSs in molecular terms, i.e., to determine the concentrations and reaction fluxes that support the NESSs. If appropriate fluorescent reaction components can be found, FCS can contribute substantially to achieving this goal. As we have seen, concentrations can be obtained directly from \( G(0) \), e.g., \( G_{B0}(0) = \langle N_B^{0s} \rangle^{-1} \).

Because reaction constituents in a NESS have constant concentrations, conventional chemical kinetic methods that rely on measuring rates of macroscopic concentration change are not useful in small systems (although perturbation-relaxation kinetics methods can be used for macroscopic NESS systems). FCS, however, can measure reaction fluxes in steady states without perturbing the system (133,134). Consider the four-component system, Eq. 5. When the system is in equilibrium, the two-color cross-correlation functions \( G_{BC}(\tau) = G_{CB}(\tau) \), as guaranteed by the principle of detailed balance (4). In a NESS, however, \( G_{BC}(\tau) \neq G_{CB}(\tau) \). Moreover, as can be seen from an analysis of the simple one-step isomerization reaction (4), the flux from B to C and the flux from C to B can be obtained with appropriate normalization by taking the limit as \( \tau \to 0 \) of the appropriate cross-correlation function divided by the time, 

\[
\text{flux}_{B \to C} = \lim_{\tau \to 0} \left[ \frac{G_{BC}(\tau)}{\tau} \right]
\]

and

\[
\text{flux}_{C \to B} = \lim_{\tau \to 0} \left[ \frac{G_{CB}(\tau)}{\tau} \right]
\]

respectively (133). For this approach to work, B and C must have sufficiently different fluorescent colors and sufficiently high fluorescence intensities to supply a substantial two-color cross-correlation signal.

One possible approach is Förster resonance energy transfer (FRET) (135). When energy is transferred from a donor to an acceptor, the fluorescence of the donor is quenched, and the fluorescence of the acceptor is enhanced. Because the donor emits at a lower and the acceptor at a higher wavelength, it would be possible to obtain a two-color cross-correlation function by cross correlating at the donor and acceptor emission wavelengths. The measurement would require that the chemical reaction change the energy transfer efficiency, perhaps due to a large conformation change or a redox reaction. To carry out this type of measurement would require finding an appropriate system in which the chemical relaxation rate was fast compared to diffusion and the donor and acceptor fluorophores were both bright and experienced a large change in FRET efficiency due to the chemical reaction. Note, however, that unless the FRET efficiency, \( E \), of the donor acceptor pair changes from nearly \( E = 0 \) in one state to nearly \( E = 1 \) in the other, the extraction of the needed cross-correlation function is complicated by the fact that for both \( B \) and \( C \), both donor and acceptor fluorescence is detected. The application of FRET to conformational change and reaction kinetics has been perceptively discussed (136,137).

CONCLUSIONS

Over the past four decades, FCS has evolved from an esoteric and difficult experiment to a routine measurement used in chemistry, biology, biophysics, and biotechnology. Not only is it a useful measurement method, its conceptual basis also provides an introduction to the behavior and characterization of mesoscopic systems. It is a bridge between measurements on macroscopic systems and on single molecules. FCS is itself a type of single-molecule method, in that the signal results from self-correlation of individual molecules although many molecules (rather than only one) provide the signal. This information is extremely important for fluctuating nonlinear chemical reaction systems and is not available in data on state transitions within an individual molecule. When the method was first introduced, it would have been difficult to predict the variations and extensions of FCS that have been developed in fluctuation amplitude analysis, imaging, and diverse types of optical approaches such as two-color cross correlation and multiphoton excitation. Similarly, we may also presume that the future will bring further unexpected developments. Some of these may be in the areas of nonequilibrium steady states and stochastic behavior of individual cells, each of which is a biochemical reaction system.

The development and application of FCS at Cornell and beyond resulted from the work of many people. I can list only a few whose contributions seem to me notably conspicuous. All of the work at Cornell benefitted greatly from the wisdom, enthusiasm, and experimental expertise of Watt Webb. The initial demonstration of FCS could not have happened without the persistence and technical know-how of Douglas Magde. Dennis Koppel and Daniel Axelrod established the microscope-based versions of FCS and developed our version of fluorescence photobleaching recovery. Joseph Schlessinger was the prime mover in the early applications of these methods to cells. In later work, Hong Qian provided a number of important contributions including, among others, analyses of the FCS optical system, measurement noise, and the high moment approach to measuring molecule number and brightness. Saveez Saffarian extended the analysis of measurement noise and carried out studies of the aggregation of EGF receptors and of a matrix metalloproteinase Brownian ratchet. I am also grateful to Rudolf
Rigler for the work that he and his colleagues have done that led to the establishment of FCS as a widely accessible measurement method as well as for the many interesting and wide-ranging applications that they have provided. Thanks also to K. M. Pyse for supplying Fig. 1 and to Hong Qian for suggestions about the text.

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