Fluorescence Correlation Spectroscopy

I.   Introduction--Fluctuation Spectroscopies

1. **Historical Background**: FCS as a member of a family of fluctuation correlation methods
    - A. Thermodynamic Equilibrium → stochastic (thermal) fluctuations → Statistical Analysis
    - B. DLS —Dynamic Light Scattering.
    - C. Motivations of FCS
        - a. Choose an optical parameter that reports chemical reaction progress.
        - b. Combination of fluctuations and transient relaxation methods
        - c. Number fluctuations→ fluorescence fluctuations. (Note that fluorescence is incoherent light even when excited by a laser.)
    - D. Single Channel methods.

2. **Renaissance of Interest**
    - A. Difficulties in old measurements→ lack of photon sensitivity → need to measure many molecules→ small relative fluctuations.
    - B. Relatively large illuminated volumes → long measurement times. Requirement for sample stability.
    - C. Need to measure many fluctuations to gain adequate statistical characterization of dynamics.
    - D. The advance that made FCS a routinely practical method arose mainly from improved sensitivity that permits measurements down to the single molecule level. This yields much larger relative fluctuations signals. Smaller illuminated volumes reduce measurement time. Reduction of time for individual fluctuations yields reduction in measurement time.
3. **Fluorescence Photobleaching Recovery (FPR or FRAP)**
4. **Advantages of FCS**
    - A. Minimal perturbation→ analysis of states closely spaced in free energy. But many macromolecular conformational processes are highly cooperative.
    - B. Very small sample region
    - C. Fluorescence detection→ molecular specificity
    - D. Wide dynamic range: $<\mu s^{-1}$ to $>s^{-1}$.
    - E. Amplitude information
    - F. Cross-correlation methods.
5. **FCS and Single Molecule Studies**

II.   How FCS works

1. Schematic of the simplest FCS measurement—translational diffusion.
2. Fluorescence fluctuations: $\delta F_j(t) = Q_j \int I(r) \, \delta c_j(r,t) \, d^3r$
3. Typically a Gaussian laser beam is used: $I(x) = I_0 \exp(-2x^2/w^2)$

4. Statistical analysis: the correlation function

$$G(\tau) = <\delta F(0)\delta F(\tau)> = \lim_{T \longrightarrow \infty} (1/T) \int_0^T \delta F(t)\delta F(t+\tau)dt$$

5. Diffusion fluctuations.

$$\frac{\partial c(r,t)}{\partial t} = D\nabla^2 c = D(\frac{\partial^2 c}{\partial x^2} + \frac{\partial^2 c}{\partial y^2} + \frac{\partial^2 c}{\partial z^2})$$

6. Restrict to two dimensions for simplicity:

$$c(x,y,t) = \frac{C_0}{4\pi Dt} \exp[\frac{[(x-x_0)^2 + (y-y_0)^2}{4Dt}]$$

7. Diffusion correlation function

$$G(\tau) = \int I(x)I(x') < \partial c(x,0)\partial c(x',\tau) > dx dx' = \frac{G(0)}{1+\tau/\tau_D}$$

Where $\tau_D = w^2/4D$.

8. More generally FCS experiments can take account of convective motion and chemical reactions as well as photophysical effects, e.g., triplet formation.

$$\frac{\partial \delta c_j}{\partial t} = D_j \nabla^2 \delta c_j - V_x \frac{\partial \delta c_j}{\partial x} + \sum_k T_{jk}\delta c_k$$

III. Amplitude Effects

1. Poisson Distribution

$P(n) = <n>^n \exp(-<n>)/n!$

2. Variance of the Poisson Distribution

$Var(n) = <[n-<n>]^2> = <(\delta n)^2> = <n>$

3. Correlation function amplitudes, e.g., for a single component.

$$G(0) = \langle(\delta F)^2\rangle = Q^2 \int I(r)I(r')\langle\delta c(r,0)\delta c(r',0)\rangle d^3r\,d^3r'$$

But $\langle\delta c(r,0)\delta c(r',0)\rangle = \langle c\rangle\delta(r-r')$.

Hence $G(0) = Q^2 \langle c\rangle \int I^2(r)d^3r$

It is convenient to define a normalized correlation function, $G'(\tau) = G(\tau)/\langle F\rangle^2$

Then $G'(0) = 1/\langle n\rangle$, where $\langle n\rangle$ is the average number of particles in the illuminated volume, defined as πw^2 for a 2-dimensional "volume", i.e., a planar system such as a biological membrane.

4. Example: an aggregation equilibrium: $nA \rightarrow A_n$

Suppose $n = 10$ and that there are 100 A molecules in the illuminated volume. In the absence of the aggregation reaction $G(0) = 1/100$; if the reaction goes far toward completion, $G(0) = 1/10$, a ten-fold increase in amplitude. In contrast, if the monomer and aggregate are approximately spherical, $D \sim M^{1/3}$, and so the diffusion coefficient increases by only a factor of $\sim 10^{1/3} \sim 2.2$, and the advantage in sensitivity of the amplitude versus the diffusion coefficient measurement increases as $M^{2/3}$. Hence, measurement of the fluctuation amplitude provides more sensitive information about aggregation and polymerization than does measurement of translational diffusion. (Note, however, that the rotational diffusion coefficient varies as M. In principle measurements of polarization of fluorescence could provide a sensitive indicator of particle size, but these measurements are limited to relatively small particles and are less straightforward to interpret than are those of FCS amplitudes.) Supposing that the aggregation process has no effect on the spectroscopic properties of the fluorophores, then the brightness, i.e., the product of the absorbance and fluorescence quantum yield, of an n-mer is n-fold the brightness of a monomer. This is readily confirmed experimentally by dividing the average fluorescence by the average number of molecules in the illuminated region as determined from G(0).

5. Photon Count Histogram (PCS) and Fluorescence Intensity Distribution Analysis
(FIDA) Fluorescence fluctuation measurements can also yield information about the distribution of aggregate sizes or the composition of mixtures of different kinds of fluorophores. One approach derives this information from an analysis of the distribution of fluorescence fluctuation amplitudes. Let us suppose initially that the excitation intensity in the illuminated volume is uniform and that the brightness of an n-mer is n times the brightness of a monomer. If the mean number of photons emitted by a single fluorophore during a measuring time T is λ, then the probability $P_P(n)$ of recording n photons is governed by the Poisson distribution $P_P(n) = \lambda^n \exp(-\lambda)/n!$. The number of fluorophores in the open illuminated volume fluctuates, however, and is also governed by the Poisson distribution $P_N(m)$. Hence,

$$P_P(n) = \sum_{m=0}^{\infty} (\lambda m)^n exp(-\lambda m) / n! \, P_N(m) \qquad = \sum_{m=0}^{\infty} (\lambda m)^n exp(-\lambda m) / n! \, \mu^m \, exp(-\mu) / m!$$

where μ is the mean number of fluorophores in the illuminated volume (1, 2). If there are more than one fluorescent species in the system, then the joint probability that accounts for all contributing species can be obtained by convolutions of the individual photocount distribution functions (3-5). A more compact approach is to develop the generating function, $G(\xi)$, for the photon count distribution (1, 2):

IV. Chemical Kinetics

Although it is now mainly used for measurements of diffusion, a major motivation of the development of FCS was to study the kinetics of chemical reaction systems in equilibrium. In contrast to scattered light, fluorescence can provide a sensitive indicator of chemical reaction progress. Therefore, in contrast to DLS, in favorable cases FCS can provide a direct readout of chemical reaction rates (38-40). Because the illuminated volume is open, however, the chemical kinetics are coupled to diffusion. This requires a somewhat more involved analysis than for chemical kinetic measurements in closed systems (3). The presence of the chemical reaction influences the apparent diffusion behavior of the reactants. Consider a system in which a small rapidly diffusing molecule, B, which is fluorescent, binds reversibly to a large slowly diffusing molecule, A, which is not fluorescent, to form the large slowly diffusing fluorescent complex, C, with the association and dissociation rate constants, k_f and k_b. The chemical relaxation rate is $R = k_f(C_A + C_B) + k_b = \tau_c^{-1}$, where τ_c is the characteristic time for the chemical reaction. If the diffusion coefficient of component B is D_B, then characteristic diffusion time for component B is $\tau_d(B) = w^2/4D_B$. If $\tau_d(B) \ll \tau_c$, a B molecule will be either free or complexed with A as it diffuses across the illuminated volume, but it will rarely either associate or dissociate during that time. Then the FCS measurement will detect two components, rapidly diffusing B and slowly diffusing C (Figure 1A). If $\tau_c \ll \tau_d(B)$, then B will react with A many times as it diffuses across the illuminated volume. If A is in molar excess, only one diffusing component will be detected and it will have a diffusion coefficient, D_e, intermediate between D_B and D_C. $D_e = f_C D_C + f_B D_B$, where f_C and f_B $f_C = KC_A/[1 + KC_A]$, $f_B = 1 - f_C$, and $K = k_f/k_b$, the equilibrium constant for the reaction. This is illustrated in Figure 1 B,C. Hence, a direct readout of chemical kinetics is possible only when $\tau_c \ll \tau_d(B)$, but in the limit of slow kinetics the reaction progress might be monitored by observing the decrease in the fast diffusing component and increase in slow component as B bound to A, e.g., (41).

New Directions

FCS on Cells

The large increase in speed of acquisition of FCS measurements has greatly improved the feasibility of applications to cells. Even with a sensitive instrument in hand, however, potential interfering factors such as variable autofluorescence, photobleaching and the tendency of living cells to move and thereby generate large artifactual fluorescence fluctuations must be considered in interpreting results (6, 7).

Two-photon FCS

Two-photon (2PE) microscopy is a nonlinear optical technique in which two long-wavelength (red) photons combine in the region of high photon density in the focal volume of a microscope to produce a single photon at half the original wavelength (8, 9). This photon excites fluorescence only within the focal volume and has the advantages of very high spatial resolution in the absence of off-focal background fluorescence and photobleaching. 2PE microscopy has been extended to FCS (10, 11).

Two-color Cross-correlation FCS

Two-color cross correlation experiments provide a sensitive way of detecting molecular interactions. The two interacting molecules are labeled with different fluorescence colors, say red and green. Then the fluctuations of the red and green fluorescence are measured in separate detectors. If the two colors are on molecules that move independently, the fluctuations of red and green fluorescence are also independent and do not correlate with each other. If, however, some of the red and green fluorophores are in the same complex and so move together, then a fraction of the red and green fluctuations will be coincident. This coincidence is assessed quantitatively via the cross correlation function: $G_{rg}(\tau) = <\delta F_r(0)\delta F_g(\tau)>/<F_r><F_g>$, where $\delta F_r(t)$ and $\delta F_g(t)$ are fluctuations of the red and green fluorescence occurring at time t, respectively. The utility of this method has been demonstrated in several studies (12, 13). When two lasers are used separately to excite the red and green fluorescence, it is essential that the volumes illuminated by the two lasers coincide (14). This condition is ensured by using 2PE to excite both colors with a single laser (15). It is also important to take into account detector cross-talk, i.e. the registration of red light in the detector for green fluorescence and vice versa. (13, 14)

FCS and Single Molecule Studies

The reduction in the illuminated volume and increase of the sensitivity of fluorescence detection has brought single molecule measurements into the reach of FCS measurements (16, 17). These experiments are carried out in very dilute solution so that the probability of more than one molecule diffusing through the beam at a time is low. Since conventional FCS and single molecule measurements both deal with systems in which thermally driven molecular fluctuations are fundamental, the basic analytic approach is similar. A recent paper addresses the interference from background fluorescence and the statistical significance of the results (18).

V.   Examples.

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