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FCS, Then and Now

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Fluorescence Correlation Spectroscopy (FCS), inspired by dynamic light scattering and chemical relaxation kinetics, was developed initially as a method for studying conformational fluctuations of biological macromolecules and for measuring the rates of dynamic processes in cells. In the simplest FCS experiment a laser beam focused by a microscope generates an illuminated observation volume in a solution of fluorescent molecules. As the molecules diffuse into and out of the observation volume they correspondingly increase or decrease the fluorescence intensity. The duration of the fluctuations depend on the rate of diffusion. Similarly, if the molecules fluctuate among chemical states that differ in fluorescence, the fluorescence changes due to chemical fluctuations, yield chemical reaction rates. FCS measures microscopic molecular fluctuations that occur in systems resting in equilibrium. Because these fluctuations are stochastic, many must be analyzed statistically. In contrast, conventional methods for measuring diffusion and chemical relaxation are based on measurements of the relaxation toward equilibrium of macroscopic concentration gradients. These methods provide the phenomenological rate parameters, e.g., diffusion coefficients and chemical rate constants, from a single relaxation transient to an accuracy determined by the measurement accuracy.

The initial work on FCS demonstrated its ability to measure both diffusion and chemical reaction rates [1]. The experiments were difficult and slow because the fluctuation amplitudes were small, and many fluctuations had to be measured to obtain accurate parameter values. Fluorescence photobleaching recovery (FPR) was developed as an alternative to FCS for studies on cells [2-4]. In an FPR measurement an intense but brief laser pulse irreversibly photolyzes a fraction of the fluorophores in the illuminated observation volume. Since FPR measures the relaxation of a macroscopic concentration gradient, a single transient is sufficient to obtain rate parameters within the measurement accuracy. FPR was, therefore, more suited for studies of unstable living cells. Both the experimental measurement approach and the theoretical basis of FCS and FPR are very similar [5, 6].

In recent years advances in microscopy and electronics have greatly facilitated FCS measurements [7, 8]. The size of the observation volume can now be much smaller than before. Hence, diffusive fluctuations are faster and so the time to accumulate a sufficient fluctuation record is correspondingly diminished. Moreover, reduction of the observation volume diminishes the background interference, thereby enabling measurement of the fluorescence of single molecules [9, 10]. Decreasing the average number of fluorescent molecules within the observation volume increases the relative magnitude of the fluctuations. Hence, FCS can now be used to measure diffusion in cells [9, 11, 12]. The sensitivity of FCS measurements is now sufficient to allow measurements of conformational fluctuations as well, e.g., in DNA hairpins and oligomers [13, 14]. Small fluctuations in the native state of the intestinal fatty acid binding protein have also been observed [15].

Two additional applications of FCS have great potential for studying molecular interactions. One of these is two-color cross-correlation FCS [16-18]. The other is based on analysis of fluctuation amplitudes [19]. Originating in work by Qian [20], this idea has been extended to provide methods for determining the distribution of molecular brightness values and therefore of the degrees of aggregation of fluorophores within a complex mixture [21, 22].

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