New and Notable

Fluorescence Correlation Spectroscopy: New Methods for Detecting Molecular Associations

Keith M. Berland
Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599 USA

Molecular associations are of great importance in biology, including interactions such as protein oligomerization, nucleic acid hybridization, protein-nucleic acid interactions, and receptor-ligand or antibody-antigen recognition. Accurate detection and quantitation of these associations is important for elucidating biological and biophysical mechanisms and for relating biomolecular structure and function. One attractive approach to quantifying molecular associations is to use fluorescence correlation spectroscopy (FCS) (Elson and Magde, 1974). FCS is used to measure hydrodynamic properties and number concentrations (number of independent molecules per unit volume) by monitoring time-dependent spontaneous equilibrium fluctuations in fluorescence intensity from a small sample volume. Fluctuations may arise from phenomena such as Brownian diffusion, flow, and chemical reactions. The fluctuations obey Poisson statistics, and the amplitude of the average fluctuation is proportional to $\sqrt{N}$, where N is the average number of molecules in the volume. Using FCS, molecular associations can be detected by their corresponding changes in number concentration. For example, the number concentration of a solution of protein monomers is twice the number concentration of the same solution after reacting to form dimers. The strength of this approach is its ability to quantify the degree of association directly, as opposed to relying on more subtle signatures of interactions, such as changes in hydrodynamic properties. FCS measurements exploit the high efficiency and specificity of fluorescence detection and can detect and quantify molecular associations with very dilute sample concentrations that may not be accessible by other methods. Typical sample concentrations are in the nanomolar range, and measurements with much lower concentrations are possible. Although for many years these measurements were difficult to perform, the true potential of FCS is now beginning to be realized. The improvements in the method have largely been based on the ability to measure fluorescence signals from very small sample volumes (about 1 fl) using either confocal or two-photon microscopes and highly efficient fluorescence detection, which can achieve single-molecule sensitivity (Rigler et al., 1992; Mertz et al., 1995).

In this issue of *Biophysical Journal*, Schwille et al. (1997) introduce an exciting new variation to the FCS approach, called dual-color fluorescence cross-correlation spectroscopy. Their method uses separate lasers to excite two different color probe molecules, and independent red and green detector channels are used for fluorescence detection. The correlation between the two channels is then measured, as opposed to the autocorrelation of a single channel in standard FCS measurements. In the experiment described, two complementary single-stranded DNA molecules have each been fluorescently tagged at one end, one strand with rhodamine green and the other with Cy-5. The hybridization reaction kinetics are followed using cross-correlation FCS analysis. The measurement described is highly optimized for detection of the hybridization in this case and of molecular associations or reactions in general. The main advantage of the dual-color FCS approach is that there is no correlation between the two channels unless individual molecules produce both red and green fluorescence signals. In other words, only the DNA molecules that have associated with their complementary strand produce a correlated signal. The amplitude of the cross-correlation signal is thus directly proportional to the concentration of hybridized DNA, and the hybridization kinetics can be interpreted directly as changes in the time 0 amplitude of the measured cross-correlation signal. This is in contrast to a one-color FCS measurement (not shown in this article) in which the single- and double-stranded DNA molecules would both contribute to the amplitude of the correlation signal, requiring some analysis to discern the equivalent information.

FCS can be applied to study molecular interactions between virtually any combination of biomolecules provided they can be fluorescently labeled, and FCS measurements allow quantitation of molecular associations over a wide range of experimental conditions, varying parameters such as concentration, temperature, presence or absence of ligand, pH, and pressure. Measurements can often be completed in a few seconds to a few minutes, and thus are both easy to perform and amenable to kinetic studies. In addition, only small sample quantities are generally required. These attributes will facilitate experiments that may not be possible to perform using other methods. The applications in which the dual-color technique will be most advantageous are those in which a small number of associated molecules must be detected among a larger number of nonassoci-

Received for publication 27 January 1997 and in final form 27 January 1997.

0006-3495/97/04/1487/02 $2.00

ated molecules or in other experimental conditions in which a large background fluorescence signal would normally impede the detection of associated species using standard one-color FCS. Some potential applications cited by Schwille et al. (1997) include using the method for molecular diagnostics with two-color antibody assays and the possibility of more specifically recognizing two DNA probes binding to their complementary DNA sequences. This method may also prove quite useful in cellular systems, where detection of interactions can be complicated by autofluorescence. One important constraint on these measurements is that it must be possible to label the individual molecular species with different color fluorescent probes. Also, although the cross-correlation signal is directly proportional to the concentration of associated molecules, the presence of nonassociated molecules does reduce the absolute correlation signal amplitude, limiting the smallest concentration of associated molecules that are detectable among large concentrations of nonassociated molecules. Nonetheless, the increased product specific sensitivity achieved with the dual-color cross-correlation approach is a very important development, and many exciting results are sure to follow.

REFERENCES

Elson, E. L., and D. Magde. 1974. Fluorescence correlation spectroscopy. I. Conceptual basis and theory. *Biopolymers.* 13:1–27.

Mertz, J., C. Xu, and W. W. Webb. 1995. Single-molecule detection by two-photon excited fluorescence. *Optics Lett.* 20: 2532–2534.

Rigler, R., J. Widengren, and U. Mets. 1993. Interactions and kinetics of single molecules as observed by fluorescence correlation spectroscopy. *In* Fluorescence Spectroscopy. O. S. Wolfbeis, editor. Springer Publishing Co., New York. 13–24.

Schwille, P., F. J. Meyer-Almes, and R. Rigler. 1997. Dual-color fluorescence cross-correlation spectroscopy for multicomponent diffusional analysis in solution. *Biophys. J.* 72: 1878–1886.