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Fluorescence corelation microscopy: Probing molecular interactions inside living cells

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Certain scenarios are common in the laboratory: The scientist has obtained clear images of living cells using confocal laser scanning microscopy with green fluorescence protein (GFP)-labeled receptors spotted as small, green dots within the cell membrane. Colocalization experiments indicate that the assumed ligand is close to its receptor. But is there really specific interaction? What are the rate constants? At that point, fluorescence correlation microscopy (FCM) could be of assistance.

FCS analyzes the diffusion times of molecules and their differences, if they have bound together.

Another scenario: Finally, after weeks of hard work, the laboratorian has purified some micrograms of proteins. The goal is to quickly determine the interaction properties between them using a homogenous, free solution assay at equilibrium. In this situation, fluorescence correlation spectroscopy (FCS) could be the method of choice.

This article details a method that works better when using a low concentration. It also enables valuable information to be extracted out of random noise generated by Brownian motion.

Technique description

FCS analyzes the diffusion times of molecules and their differences if they have bound together. This is done by fluctuation analysis of fluorescence-labeled molecules within a well-defined volume element of a liquid sample or cell. In most experiments, Brownian motion drives the fluctuation. The volume element is the confocal volume defined by the excitation spot of a well-focused laser beam and the selected emission region defined by the properly aligned pinhole of the detection optics (*Figure 1*).

The fluctuations are analyzed by treating the measured photon counts with mathematical methods called correlation functions (*Figure 2*). If the two interacting molecules are of different size, only the smaller one has to be labeled using fluorescent dyes. This method is called autocorrelation. If the diffusion constants of both partners are similar, they are both labeled with different dyes and cross-correlation is used.

Because of the tiny size of the confocal measurement volume and its nature, the measurement can be carried out, in principle, in every area that is reachable by light and that is not smaller than an *E.coli* bacterium (approx. 0.2 fL). In particular, measurements can be done inside living cells or on cell membranes. In order to be able to place the measurement volume at its proper place, it is advantageous to combine FCS with powerful light microscopy, e.g., confocal laser scanning microscopy (LSM). This combination is called fluorescence correlation microscopy (FCM). A commercially available implementation of this combination is the ConfoCor 2 with the LSM 510 (Carl Zeiss Microscopy, Jena, Germany).

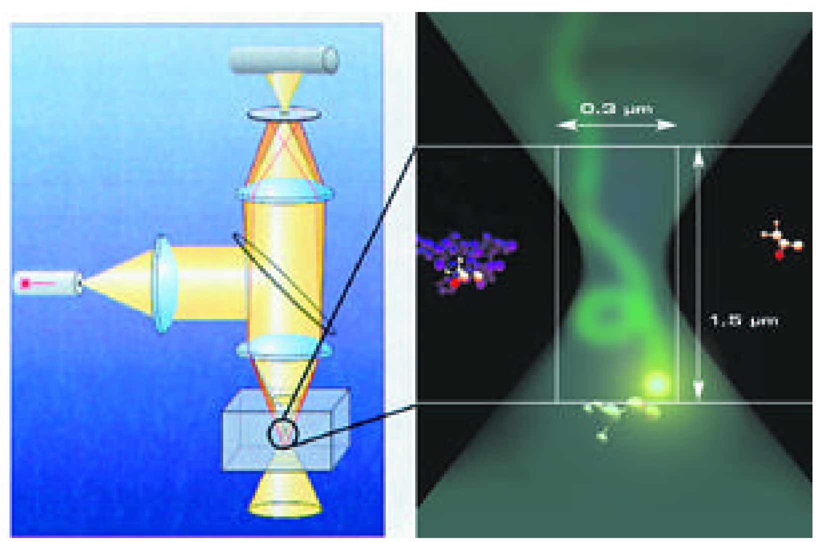

Figure 1 *Advanced confocal optics defines a measurement volume in the size of an* E.coli *bacterium (approx. 0.2 fL). Confocal optics comprises a laser spot focused to its diffraction limit (beam waist of approx. 300 nm) and a pinhole in the detection optics, rejecting all light with an origin outside the confocal slice of approx. 1.5 µm.*

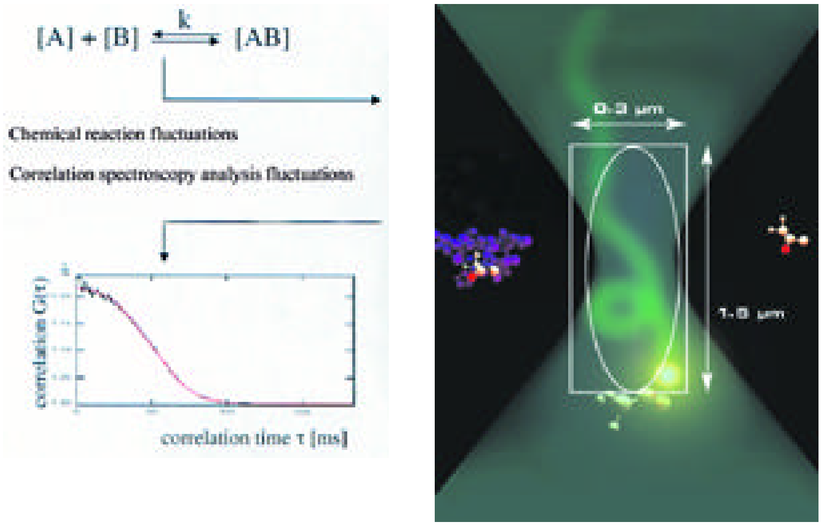

Figure 2 *Inside the measurement volume defined by confocal optics, fluorescence-labeled molecules are fluctuating. The source of the fluctuation is Brownian motion dependent on molecule size. Chemical reactions change the size of the molecules involved and thus their fluctuation properties. The fluctuations are analyzed by detecting the photons emitted from the dyes attached and using correlation functions. From these data diffusion constants, concentrations, and thus rate constants of the molecules involved are determined.*

Applications

A good overview about current applications in FCS was given during the International Carl Zeiss Workshop on Fluroescence Correlation Spectroscopy and Related Methods, held March 2000 in Jena, Germany. About 140 scientists from all over the world discussed new developments and applications in this new field. (Several workshop abstracts are available at www.zeiss.de/fcsevents and some of the talks will be published as a highlight issue of *Biological Chemistry*.) Compared to similar events during the last few years, the number of pure application-oriented talks had increased. In 1998, more than half of all talks discussed mainly methodical issues; now about two-thirds focus on applications. This reflects the fact that FCS is not only part of the domain of biophysical-oriented physicists but is also on its way to becoming an accepted method in biology. A similar symposium will be held October 25–26, 2000, at the Washington University Conference Center, St. Louis, MO. (www.zeiss.com/micro/ fcs-event.shtml).

The most challenging task is the determination of diffusion properties, concentrations, and rate constants within living cells.

Several groups have investigated oligonucleotide interactions with each other and with proteins or polymers. A group at the Karolinska Institute (Stockholm, Sweden) studied the first steps of polymerase chain reactions (PCR) with differently labeled DNA. The results are important for developments in the field of single-molecule DNA sequencing. To understand the regulation of specific gene products, groups in Belgium combined their knowledge with new methods developed in Urbana, IL, and Leuven, Belgium, and studied the entry of oligonucleotides into the cytoplasm or nucleus, thereby measuring the interaction between oligonucleotides and polycationic polymers. Researchers based in Leipzig, Germany, used FCS for in vitro selection of nucleic acid receptors (aptamers) produced by combinatorial chemistry. In addition to the use of fluroescent labels instead of radioactive labels, a major advantage of FCS over other methods used for concentration and interaction measurements containing DNA is that the investigation is noninvasive and can be carried out in homogeneous assays, enabling researchers to use the identical sample after the FCS measurements for subsequent experiments. In addition, the small sample amount necessary to carry out an investigation (less than nanoliters with picomolar concentrations) makes FCS a suitable tool to investigate the first steps of PCR. In Frankfurt, Germany, and Geneva, Switzerland, FCS was used to characterize drug delivery systems comprising oligonucleotides and cationic peptides or nanoparticles.

Protein–protein interactions measured in solution, however, outnumber DNA measurements by far. In order to avoid separation of free and bound antibodies, Japanese and Swiss biologists used FCS to probe lysobisphoshatidic acid (LBPA) domains found in internal membranes of endosomes. In Jena, Germany, the dynamics of microtubule assembly was investigated. In Düsseldorf and Martinsried, Germany, prion aggregation was under focus. A group based in Wageningen, The Netherlands, investigated the photophysical and structural properties of flavins and flavoproteins. Proteins may be as large as whole phages (as studied by the Leipzig group). But subunits of proteins are under investigation as well, such as the subunit δ of adenosine triphosphate (ATP) synthase probed by researchers based in Onsabrück, Germany. The speed of these measurements, in the range of seconds to tens of seconds, makes it obvious why several major pharmaceutical companies and research groups have adapted FCS as a detection tool for screening applications.

Although so far most of the experiments described have dealt with biochemical interactions, researchers from Potsdam, Ger-

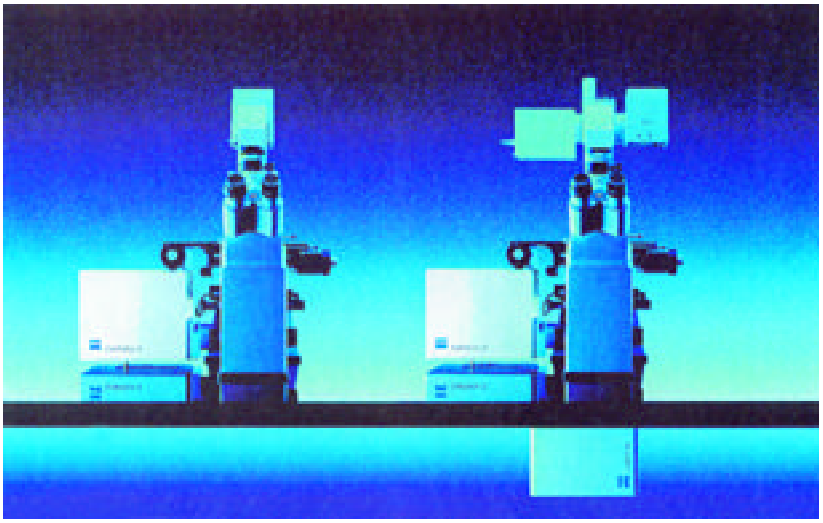

Figure 3 *The ConfoCor 2 highly automated FCS measures biochemical interactions within tiny sample volumes. The standalone ConfoCor 2 (left) is optimized for auto and cross-correlation measurements in liquids. The ConfoCor 2/LSM 510 combi (right) combines the sensitivity of FCS with the 3-D imaging capabilities of the LSM 510 confocal laser scanning microscope.*

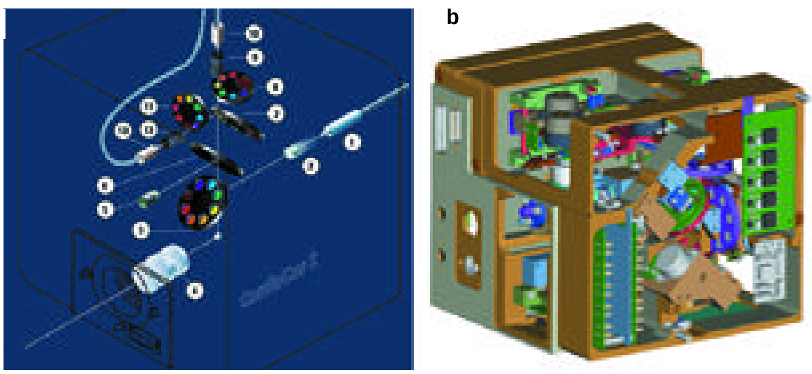

Figure 4 *The detection head of the ConfoCor 2 FCS is equipped with two independent detection channels to perform autocorrelation and cross-correlation measurements. a) (1) optical fiber from laser module, (2) motorized collimator, (3) main beamsplitter wheel, (4) lens, (5) CCD detector, (6) camera mirror, (7) secondary beamsplitter wheel, (8) emission filter wheel channel 1, (9) pinhole channel 1 (motorized in* x,y, *and* z*), (10) detector channel 1: fiber-coupled avalange photo detector (APD), (11) emission filter wheel channel 2, (12) pinhole channel 2 (motorized in* x,y, *and* z*), (13) detector channel 2: fiber-coupled APD. The detection head is fully motorized b) to ease adjustment and enable automated and reproducible measurements.*

many, used FCS to probe the mobility of ultrathin films. They analyzed the diffusion and optical properties of tracer dyes incorporated into polymer matrixes. Such experiments rely on diffusion properties being a key source of fluctuations determined by FCS and the possibility of positioning the extremely small measuring spot virtually everywhere on sample surfaces or inside the transparent sample.

Nevertheless, the most challenging task is the determination of diffusion properties, concentrations, and rate constants within living cells. At the Max-Planck-Institute (Göttingen, Germany) much work was done to adapt FCS to cell measurements. Inspiring results were seen within cells; however, the group pointed out the importance of careful selection of dyes that do not interact with cell structures (e.g., the superiority of GFP dyes over Cy5). GFP-fused proteins were also the choice of a group of researchers from Wageningen, The Netherlands. The group monitored signal transduction processes in vivo. At the Karolinska Institute, Sweden, the confocal measurement spot was placed at the cell membrane of epidermal growth factor (EGF)-receptor expressing cells using the light microscope section of an FCS setup. The interaction with fluorescence-labeled EGF could be investigated without the need to separate bound and unbound EGF. Thus, rate constants of interactions with half-life times faster than typical separation procedures can be determined. Using competition experiments, it was possible to probe the specificity of the interaction. A group from London, U.K., also performed such measurements with mutant EGF. These applications will be pushed further with the spread of recently introduced combinations of FCS and confocal laser scanning microscopy instruments. These instruments allow the 3-D imaging of living cells, selection of a point of interest within these images, and measurement of the biochemical and physical properties of diffusing molecules at this spot. This may even lead to high-throughput screening applications within living cells, as studied by researchers based in Tübingen, Germany.

While most of the measurements described above were performed with the previous ConfoCor instrument (Carl Zeiss and Evotec, Hamburg, Germany), a second-generation ConfoCor 2 system is available. The system comes as a standalone unit optimized for measurements in liquids and as the ConfoCor 2/LSM 510 combi (*Figure 3*). The combi system integrates FCS into a confocal laser scanning microscope. Its intended use is mainly cell-based investigation. The system makes it easy to capture a 3-D representation of a sample and perform FCS measurements inside the nucleus or on top of the cell membrane.

The ConfoCor 2 is a fully automated system that can be used for autocorrelation and cross-correlation measurements (*Figure 4*). The software controls all settings, performs automated adjustment routines, and analyzes the data. The ConfoCor 2/LSM 510 combi system uses only one piece of software for FCS and LSM. The system is equipped with up to five excitation wavelengths covering all important dyes in the visible range.

Because FCS is a new, rapidly developing method, the ConfoCor 2 is designed as an open system to enable researchers to develop solutions optimized for their demands. Researchers have access to the raw data representing each single photon detected as well as to intensities, correlation data, and fitting results. However, the software is designed not only to have open interfaces, but users can also easily attach detectors (e.g., spectrometers) to the detection head using the fiber coupling interface.

Summary

As indicated by the examples shown, FCS is a fast-spreading method used to determine the concentration, diffusion, and interaction properties of molecules and other particles. The method works with very small sample amounts in homogeneous assays. It is fast and does not require time-consuming preparation steps. Its most promising property is the possibility of locating areas of interest, e.g., inside living cells, by using confocal laser scanning microscopy and obtaining the biochemical data immediately.

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