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REVIEW

Recent Advances in Fluorescence Cross-correlation Spectroscopy

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Abstract Fluorescence cross-correlation spectroscopy (FCCS) is a method that measures the temporal fluorescence fluctuations coming from two differently labeled molecules diffusing through a small sample volume. Cross-correlation analysis of the fluorescence signals from separate detection channels extracts information of the dynamics of the dual-labeled molecules. FCCS has become an essential tool for the characterization of diffusion coefficients, binding constants, kinetic rates of binding, and determining molecular interactions in solutions and cells. By cross-correlating between two focal spots, flow properties could also be measured. Recent developments in FCCS have been targeted at using different experimental schemes to improve on the sensitivity and address their limitations such as cross-talk and alignment issues. This review presents an overview of the different excitation and detection methodologies used in FCCS and their biological applications. This is followed by a description of the fluorescent probes currently available for the different methods. This will introduce biological readers to FCCS and its related techniques and provide a starting point to selecting which experimental scheme is suitable for their type of biological study.

Keywords Dual-color · Two-photon excitation · Single-wavelength excitation · Pulsed interleaved excitation · Multiple focal spot excitation · Total internal reflection

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Introduction

Fluorescence correlation spectroscopy (FCS) monitors the fluorescence intensity fluctuations of single molecules moving in and out of a confined illuminated volume. The method provides information that lie hidden in the fluctuating signal such as dynamic processes, chemical kinetics, or molecular interactions. Correlation functions of the intensity fluctuations are calculated to give the number of particles and the average residence time spent in the detection volume. FCS was first introduced by Elson, Magde, and Webb in the 1970s [1]. The theory was established to use intensity fluctuations of fluorescent particles diffusing through a focused laser beam to characterize translational diffusion coefficients and chemical rate constants [2–5]. The improvement of this technique to single-molecule sensitivity was achieved by using a confocal microscope system with a high numerical aperture objective and single-photon counting avalanche photodiodes (APDs) as detectors [6, 7]. Since then, it has become an increasingly popular technique for the study of dynamics at thermodynamic equilibrium. Besides the ability to determine the concentration, diffusion characteristics [8], rotational diffusion [9–12] and various processes such as flow [13] and chemical reactions [14, 15], FCS has also been used to measure receptor–ligand interactions in solution and on cell membranes [16–18].

The concept of FCS is based on the correlation analysis of fluorescence fluctuations in a confined observation volume. The sensitivity of this technique to detect binding of two or more components depends on the relative change in

mass upon binding. For a multi-component system consisting of reactants and products labeled with the same fluorescent dye, the only way of differentiating the product from the reactant is when the product has a molecular mass that differs from the reactants by at least a factor of 4 [19]. This in turn shifts the correlation curve to longer diffusion times by at least a factor of 1.6, given by the Stokes–Einstein equation ($\tau_D \sim M^{1/3}$). By separately labeling the reactants with differently emitting fluorophores, the probes can be simultaneously excited with two different laser lines and detected in separate channels. The signals from both detector channels are cross-correlated and the doubly labeled products can be easily distinguished from the singly labeled reactants, independent of their mass. Earlier cross-correlation systems have made use of light scattering or a combination with fluorescence to measure their cross-correlation functions and determine rotational diffusion and association–dissociation kinetics [20, 21]. Although the concept of dual-color fluorescence cross-correlation spectroscopy (FCCS) has been proposed for biotechnological applications [22], it was first experimentally realized by Schwille and co-workers to measure nucleic acid hybridizations [23]. In the following years, applications of FCCS were brought into live cells and new experimental schemes to perform FCCS were set up. The application of FCCS to different biological or biomimetic systems has been reviewed extensively [24, 25]. This review presents an overview of the different optical setups used in FCCS. This includes the differences in excitation and detection modes (shown in Fig. 1), their recent applications to in vivo measurements and their various strengths and weaknesses for each approach. This is followed by a discussion of acceptable fluorescent probes for the different FCCS schemes.

Excitation Sources

Dual Laser Excitation

Fluorescence cross-correlation spectroscopy with dual laser excitation involves the use of two spectrally distinct fluorophores each of them excited by one of the lasers. Their fluorescence emission is separated into two different detection channels using dichroic mirrors and filters. The fluorescence signals from both channels are correlated with each other in time to give a cross-correlation function that contains information of the dual-colored complexes. This allows the dual-colored complexes to be distinguished from the single-colored molecules in the auto-correlation functions. The first dual-color FCCS (DC-FCCS) setup was demonstrated by Schwille and co-workers using two different wavelength laser beams (488 nm and 647 nm) aligned to the same focal volume for one-photon excitation [23]. They investigated the kinetics of the hybridization of Cy5 and rhodamine green-labeled complementary DNA oligonucleotides. The double-stranded hybrid produces positive cross-correlation signals while the auto-correlation function of each color contains signals from both the hybrid and the single strands. When the concentrations of reactants are constant, the amplitude of the cross-correlation function is then directly proportional to the concentration of the dual-color complexes formed with time. This easily distinguishes the products from the free reactants via the amplitude of the cross-correlation function, as compared with the weak dependence of the auto-correlation function with the mass of the complex formed. Assuming that there is no cross-talk between both detectors, the general theory of FCS and FCCS is depicted in Fig. 2. The comparison between the auto- and cross-correlation functions is shown in Fig. 2A–C.

The potential of this technique to effectively measure biomolecular interactions has expanded its applications to detecting PCR complexes [26, 27], quantification of mRNA expression levels [28] or expressed gene copies [29], monitoring enzyme kinetics [30, 31], measuring protein–DNA interactions [32], and the binding of ATP-utilizing chromatin assembly and remodeling factor (ACF) to four DNA-duplexes [33]. Assays for DNA recombination [34] and monitoring of topoisomerase II action [35] have been developed based on FCCS. FCCS has also been applied in artificial model membranes [36] and live cell measurements to probe the endocytic pathway of bacterial cholera toxin labeled with Cy2 and Cy5 dyes on different subunits of the same holotoxin [37]. See also below for in vivo applications using fluorescent proteins.

Dual-color FCCS has the highest count rates per molecule (cpm) when compared to other forms of laser excitation. It gives the highest signal-to-noise (S/N) ratio since different fluorophores can be excited with lasers having excitation wavelengths that match the fluorophores' absorption maxima. Fluorophores can also be chosen to have widely separated emission wavelengths to minimize cross-talk. A disadvantage of early setups was the difficulty to maximize and maintain the overlap of the two laser foci stable enough over time since artifacts could be introduced otherwise. This disadvantage has been reduced with newer commercial instruments available.

Although the dual-laser excitation approach improves the detection sensitivity of interacting particles compared with FCS, the requirement of matching two laser beams to the same focal spot makes it experimentally challenging. The setup has to be first calibrated to maximize the overlap

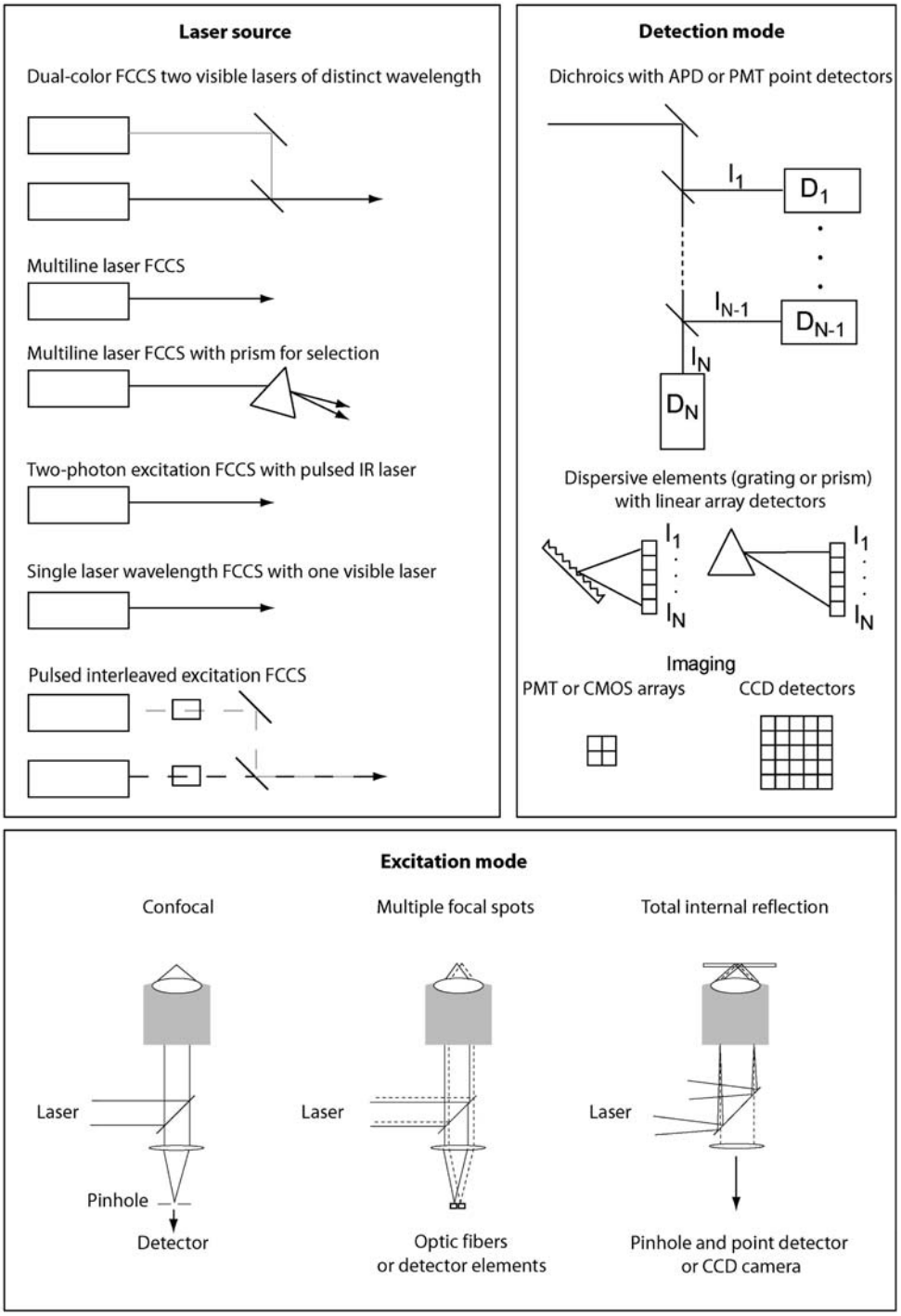

Fig. 1 An overview of various laser sources, excitation, and detection modes used for fluorescence cross-correlation spectroscopy

between the two excitation volumes and detection volumes. Laser misalignment or chromatic aberrations of the optics lead to displacement shifts in the observation volumes. This causes the cross-correlation function to decrease its amplitude and shift towards a slower decay [38, 39]. This led others to develop new methods of aligning two laser beams to the same excitation volume using a prism [40] and alternative excitation methods using a multiline laser [41].

Single Laser Wavelength Excitation

A less expensive and simpler optical setup has been proposed called single wavelength FCCS (SW-FCCS). This instrumentation scheme uses a single laser line for one-photon excitation of two fluorophores with similar excitation but different emission characteristics [31, 42]. However, the realization of such a system was only achieved later with fluorophores that could be excited at one

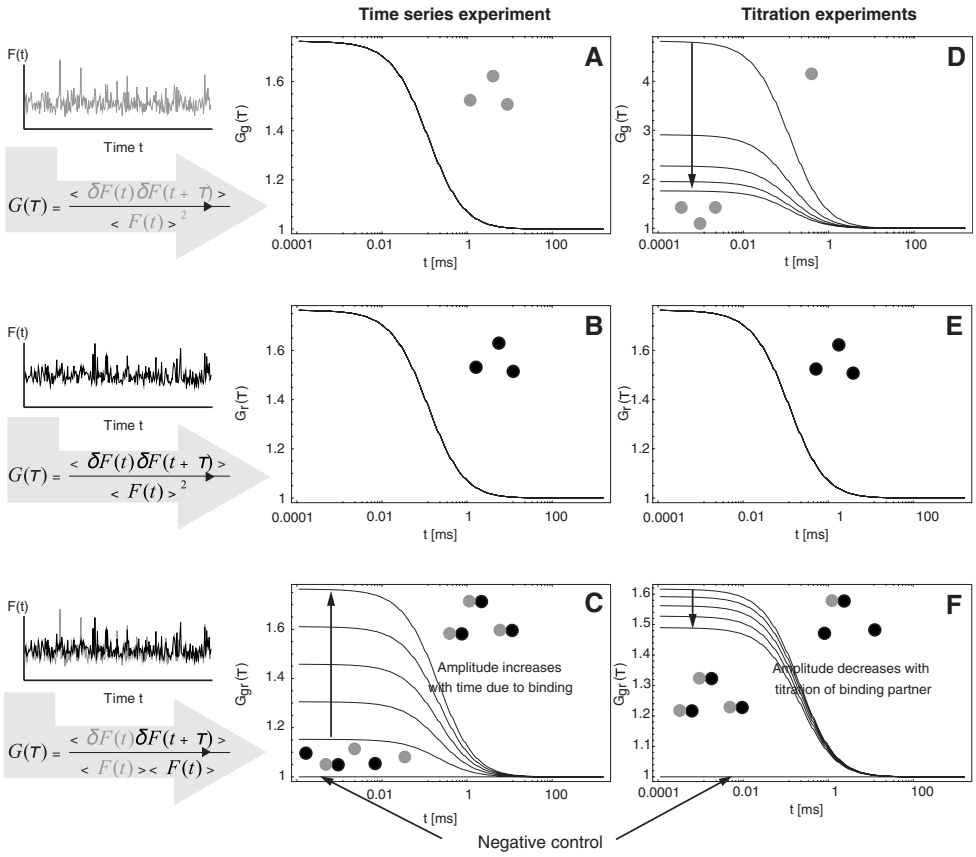

Fig. 2 Autocorrelation and cross-correlation functions of green and red fluorescence signals are depicted on the left. (**A**–**C**) Kinetic measurements with equimolar binding partners. (**D**–**F**) Equilibrium measurements with increasing concentration of titrated binding partner. For further details, see text

wavelength and efficiently separated in emission due to distinct Stokes shifts. For this, a single laser wavelength of 488 nm was used for the excitation of a pair of fluorescent probes with overlapping excitation spectra, but with spectrally distinct emission spectra. The detection path remains the same as that for a dual-color FCCS setup. SW-FCCS was used to measure the K_d and stoichiometry of equilibrium binding of biotin-fluorescein and streptavidin labeled with either quantum dots or tandem dyes [43]. The resolution of SW-FCCS was investigated with the binding of streptavidin-biotin labeled with spectrally similar dyes, fluorescein and tetramethylrhodamine. Limits for the measurement of K_d in dependence on sample concentration, impurities, labeling ratios, and spectral cross-talk were presented [44]. SW-FCCS was further extended from dual-color detection to triple-color detection. Using two dichroic mirrors to split the detection path into three channels, green-emitting fluorescein, yellow-emitting R-phycoerythrin, and red-emitting Alexa 647-R-phycoerythrin fluorescence signals were simultaneously separated for cross-correlation [45]. By adding a third color to cross-correlation analysis, higher order interactions such as from molecular assemblies could be studied. Recently, a new long Stokes shift fluorescent protein has been developed for SW-FCCS [46] (see below). This new development of fluorescent proteins brings SW-FCCS measurements into live cells.

The SW-FCCS theory is essentially the same as that for FCCS (Fig. 2). Two experimental schemes can be distinguished. First, in the case where an equimolar reaction is measured with FCCS, the autocorrelation amplitudes remain the same with constant concentration of reactants (Fig. 2A, B). The cross-correlation function increases in amplitude as more double-labeled complexes are formed with time (Fig. 2C). In the second case when FCCS measurements are taken at equilibrium binding, the autocorrelation amplitude decreases with increasing concentration of the titrated binding partner (Fig. 2D). Furthermore, an increase in unbound reactants contributes as background and increases the denominator of the cross-correlation function more than the increase of the numerator due to the complex formation. This overall decreases the cross-correlation amplitude with increasing binding (Fig. 2F). The K_d and complex concentrations can then be fitted from the binding curve plotted from amplitude versus concentration of the titrated sample.

Single wavelength FCCS has the advantage that a single laser line is used for one-photon excitation eliminating the problems of chromatic aberrations and focal volume overlap. This not only simplifies the setup considerably, it also eliminates the potential artifacts that arise from non-ideal overlap of excitation volumes. However, artifacts to the cross-correlation function can still arise due to

chromatic displacements and size differences in the wavelength-dependent detection volumes. Resolution of SW-FCCS depends largely on the S/N ratio of the system. Resolution could be improved by increasing the S/N ratio through maximizing cpm and minimizing detector cross-talk. This is influenced by a combination of factors including fluorophores' brightness, spectral characteristics, and matching filter sets. Currently, the main limitation using single laser excitation is the lack of bright fluorophores with a wide range of Stokes' shifts excited by a single laser line. SW-FCCS has higher detector cross-talk and the S/N ratio is relatively lower than for DC-FCCS. Depending on the sample and whether chromatic aberrations play a role (e.g., in tissue measurements), either technique can be advantageous.

Two-photon Excitation

Two-photon excitation FCCS (TP-FCCS) uses as well a single laser line for the simultaneous excitation of several fluorophores. Thus, two-photon excitation laser sources have been used to overcome the difficulty of aligning two laser beams to the same excitation volume. Two-photon excitation of fluorescent molecules involves the simultaneous absorption of two photons of half the energy for a transition to the excited state. Femtosecond-pulsed IR lasers, e.g., titanium sapphire systems, are commonly used to excite fluorescent probes in the blue or green spectral range. Excitation probability in two-photon absorption is proportional to the mean square of the photon intensity produced by the laser. The pulsed laser intensity drops as the square of the distance from the focal plane, decreasing the excitation probability of a fluorophore with the fourth power of the distance from the focal plane. This provides an optical sectioning that confines the excitation of fluorophores at the focal plane, eliminating the need for a pinhole. Out-of-focus background light is strongly suppressed and photobleaching of fluorophores in the sample is reduced. Two-photon excitation reduces scattering in cells and tissues and allows much deeper penetration depths in tissues compared with one-photon excitation methods.

TP-FCCS has found several applications in solution measurements of proteolytic cleavage [42]. Increased axial resolution from a more confined focal spot reduces background fluorescence and photobleaching making it suitable for in vivo studies [24, 47, 48] such as intracellular calmodulin and calmodulin-kinase II binding [49, 50] and interactions between antigen-stimulated IgE receptors and Lyn kinase [51]. Recently, two-photon excitation has achieved the excitation of up to three dyes simultaneously to perform triple-color coincidence analysis [52].

Despite the advantages of two-photon excitation, the cpm is in general lower than for DC-FCCS and SW-FCCS. Also, the high cost of a high-power femtosecond laser source can be an important factor in the decision of which method to use. Furthermore, TP-FCCS requires fluorophores with large overlapping two-photon absorption cross-sections at the laser line and with different emission spectra. While such fluorophore pairs are readily available as compared with the large Stokes' shift fluorophores for SW-FCCS, DC-FCCS offers the widest selection of fluorophore pairs.

Pulsed Interleaved Excitation

Instead of reducing cross-talk via the emission path, spectral cross-talk between fluorophores can be prevented by discriminating its excitation. Pulsed interleaved excitation FCCS (PIE-FCCS) involves the use of two lasers that are pulsed alternatively in the range of nanoseconds to allow for complete fluorescence decay before the next pulse. Photons are detected using time-correlated single-photon counting (TCSPC) and their arrival times are recorded with respect to their excitation pulse. Off-line cross-correlation analysis of photons collected after green and red excitation pulses produce cross-correlation functions that are free of cross-talk. This increases the sensitivity of FCCS to detect fluorescent probes with similar emission characteristics such as CFP- and YFP-connexin fusion proteins that were measured in the membranes of live HeLa cells [53]. With PIE-FCCS, fluorescence resonance energy transfer (FRET) efficiency could be quantified from FCCS measurements and single-particle FRET measurements could also be performed [54]. Although PIE-FCCS can remove cross-talk and improve the sensitivity of FCCS, other forms of background light such as scattering and autofluorescence from biological samples can reduce the signal-to-background ratio. Pulsed laser excitation also decreases molecular brightness due to limited excitation cycles, thus reducing the S/N ratio.

Multiple Focal Spot Excitation

In dual-beam fluorescence cross-correlation spectroscopy, two polarized beam splitters are used to split the laser light into two beams and rejoin them slightly displaced. The two parallel beams that are coupled into the back aperture of the microscope objective created two spatially separated focal spots. The fluorescence emission from each volume element is collected back by the objective and focused onto two closely placed optic fibers for cross-correlation. This setup has been applied to characterize flow properties in microstructured channels [55, 56] and capillary electrophoresis [57, 58]. Folding kinetics and conformations of DNA hairpins have been investigated with dual-beam FCCS [59, 60]. Diffractive optical elements have also been

used to create multifocal spots of 2 × 2 [61, 62] and 4 × 1 [63] array for parallel fluorescence detection and auto/cross-correlations. This allows the simultaneous excitation and detection of four foci that can be used in biochip microarrays and multiplexed detection. Scanning two-foci FCCS was performed on model and cell membranes to measure slow membrane dynamics and is not affected by positioning of the membrane in the detection volume [64]. Another setup configuration for spatial FCCS is using only a single laser beam and cross-correlating signals from two spatially shifted detection volumes [65]. This opens up the possibility of extending FCCS to measure the spatial distribution of molecules by using multifocal excitation. Another approach to improve the spatial resolution of FCCS is via the detection mode (see below).

Total Internal Reflection

The spatial resolution of a FCS setup is determined by the numerical aperture of the microscope objectives and the size of the confocal pinhole. Axial resolution of a confocal setup is typically in the order of a few μm, but it can be further improved using total internal reflection illumination. Total internal reflection FCS (TIR-FCS) involves coupling a laser beam into a prism [66] or a high NA oil-immersion objective [67] and the spatial filtering with a pinhole at the image plane. TIR illumination creates an evanescent wave on a glass–solution interface. As the intensity of the evanescent wave decreases exponentially with the axial distance from the glass surface, an axial resolution of less than 100 nm is achieved. This reduces background fluorescence and the cpm was reported to be higher than FCS with objective-type TIR-FCS [68]. The axial confinement of the excitation depth on a glass surface makes it ideal for the study of diffusion or receptor–ligand binding on cell membranes or supported lipid bilayers immobilized on the glass substrate [69–71]. Recently, dual-color TIR-FCCS has been achieved with an objective-type setup [72]. With higher collection efficiency and S/N ratio coupled with enhanced sensitivity, TIR-FCCS could provide a valuable tool to probe weak molecular interactions on cell or model membranes that are not accessible with conventional FCCS.

The decision of which FCCS technique to use will be influenced by several factors. The highest cpm is achieved with DC-FCCS and this would be the method of choice in cell measurements. When measuring in tissues and high-penetration depths of more than 100 μm, TP-FCCS is the better choice. In tissues where measurements are performed in the first 100 μm, SW-FCCS can be equally applicable. Similar to TP-FCCS, SW-FCCS does not suffer from chromatic aberrations from mismatch of excitation volumes that may be caused by focusing into thick cell layers. For measuring cell membranes with a thickness of at most 10 nm, TIR-FCCS has higher axial resolution compared to confocal setups. Illuminating only fluorophores within 100 nm from the glass–solution interface, background fluorescence from freely diffusing species is reduced. This makes TIR-FCCS an ideal technique to study molecular interactions and dynamics on cell or model membranes. The availability of instruments has also to be taken into account such as in the case of TP-FCCS where expensive pulsed laser systems are used. Another important factor for the decision of any of the FCCS techniques is the availability of the appropriate fluorophores for the different techniques.

Detection Modes

Dispersive Optics

Fluorescence cross-correlation spectroscopy setups use conventional instrumentation including dichroic mirrors and emission filter sets to select the desired emission wavelengths or separate them into different detection channels. For multiple wavelength detection, multiple dichroic and emission filters will have to be used. Not only does this complicate the setup it also amplifies the intensity losses due to non-ideal transmission principally from surface reflections through each optical component. Commercially available emission filters and dichroics have usually broad spectral bandwidths and rise/fall bandwidths, respectively. Unless each detection window of the filter is customized to overlap with the emission spectra of the probes, the difficulty of balancing between optimizing signal detection and reducing spectral cross-talk will augment with each additional detection channel.

To overcome these problems, a dispersive element such as a diffraction grating or a prism could be used to spectrally separate the emission light. Multicolor fluorescence imaging using a single-laser wavelength excitation has been applied on quantum dots for co-localization studies [73]. Commercial confocal microscopes now utilize parallel detection channels for multicolor imaging. Zeiss LSM510 Meta uses a grating for the spectral dispersion of the signal onto an array and the Leica TCS SP2 AOBS uses a prism as a spectral dispersion element and a scanning detector [74–77]. On the other hand, the only reported FCS systems that used dispersive elements were conceived for filtering the scattered excitation light and Raman scattering of water using a prism monochromator for rotational FCS experiments [12]; and a grating-based detection setup consisting of a fiber array coupled to individual APDs. The grating-based setup was developed for simultaneously measuring autocorrelations of four distinct quantum dots [78].

More recently, a prism-based spectrometer combined with a SW-FCCS system has been demonstrated [79]. A prism was used for its cost effectiveness and higher efficiency as compared with diffraction gratings, which can lose part of the light due to multiple diffraction orders. Dispersion by the prism spectrometer caused a wavelength-dependent deflection angle such that the fluorescence signals were focused on well-separated spots for the spectral ranges of interest. An optic fiber coupled to an APD was scanned through these foci to select different spectral ranges for detection and autocorrelation analysis. For cross-correlation, an optic fiber array was aligned to the image spots to detect signals from the binding of green- and red-labeled biotin and streptavidin. As compared with a typical FCS setup, a lower cpm was recorded for the spectrometer. This could be due to several reasons: the narrower spectral range collected by the optic fiber and light losses due to scattering and reflection from the prism, lenses, and optical fibers. Detection of wavelength ranges could be improved by continuous detection elements such as a photomultiplier tube (PMT) array or a high-speed CCD camera. Nevertheless, the prism-based FCCS setup gave a higher cpm relative to the grating-based setup.

These new setups establish a wavelength tunable and filter-free technique for multicolor correlation spectroscopy. This is an important step towards multiplexing technologies for high throughput screening of molecular interactions.

Single-photon Counting Devices

Avalanche photodiodes (APDs) are typically used as detectors for FCS and FCCS. They have high quantum efficiency, low noise levels, and a dead time of about 50 ns. This enables the APDs to measure fast photophysical and biophysical processes in the μs–ms timescale. With the advancement towards multicolor detection and multiplexing, there is a greater need for the development of the FCS/FCCS setup to have the capability of simultaneously detecting multiple species or have parallel detection. Multifocal excitation and detection was developed for parallel FCS [61] and FCCS [62] using multiple optic fibers or fiber array for coupling the fluorescence signal into four separate APDs or an APD array. However, this requires the complex alignment of the detection stage or individual fibers to each image spot. A new APD array has been developed using complementary metal-oxide semiconductor (CMOS) technology and was applied to multifocal FCS. Despite the lower detection efficiency, they were reported to have similar dead time with lower dark count and no afterpulsing [80]. With the low manufacturing costs involved, CMOS detectors may prove to be a valid alternative to their more expensive counterpart. PMT arrays, although commercially available with up to 32 channels, are so far not developed for photon counting purposes.

Charge-coupled Device (CCD) Camera

Avalanche photodiodes and PMTs are single point detectors and have limitations in multicolor and multiplexing applications. The electron-multiplying CCD (EMCCD) camera is an alternative option for detectors used in FCS/FCCS. Although the temporal resolution is limited by the frame readout time of several ms, it has been shown that it is still feasible to perform FCS and FCCS by software correlation of intensity data collected from the EMCCD. FCS and FCCS were performed on an epi-fluorescence microscope using an EMCCD to achieve the simultaneous measurement of multiple spots at a resolution of 4 ms for 20×20 pixel areas [81]. Kannan et al. demonstrated the first time use of EMCCD for FCS measurements of mRFP fused EGFR diffusion on cell membranes. Adjacent pixels were cross-correlated to calculate the flow rate and direction of fluorescent probes in a microchannel. The time resolution of EMCCD was further enhanced to 20 μs for line measurements [82]. The spatial resolution of FCS was improved dramatically by using a spinning disk confocal microscope that uses rotating disks of an array of microlens and pinholes to image onto an EMCCD [83]. Spatial maps of diffusion coefficients and flow velocities of 10,000 spots can be measured simultaneously. The EMCCD camera has a clear advantage over point detectors on the flexibility of selecting and combining pixels (binning) for multicolor or multiplexed detection and providing spatial information, especially in probing localized regions in live cells. The high-quantum efficiency and spatial resolution of EMCCD camera is expected to increase in speed in the near future, thus enhancing their advantage over CLSM-based image correlation or cross-correlation spectroscopy (ICS/ICCS).

Comparison of FCS/FCCS and ICS/ICCS

Just as FCS and FCCS were developed as temporal correlation techniques, ICS and ICCS (image correlation spectroscopy and image cross-correlation spectroscopy) were developed as spatial correlation techniques. A confocal laser scanning microscope (CLSM) is used to image the sample and the spatial auto-correlation function is calculated from the correlation analysis of the pixels, giving access to the spatial distribution of molecules [84]. A time series of images are also collected with the CLSM enabling the measurements of large molecules with slow dynamics such as membrane protein transport [85]. ICCS has been used to measure the co-localization of two different labeled proteins [86]. ICS and ICCS were limited in

their temporal resolution since for each measurement a full picture had to be scanned by PMTs and thus the frame rate of CLSM would limit the time resolution of these methods. On the other hand, FCS and FCCS despite their good time resolution were limited in multiplexing and could only measure 1–4 spots simultaneously (see multiple focal spot excitation).

A convergence of these techniques came in the last few years when a new ICS technique, k-space ICS could reach a frame acquisition rate of 50 ms using an EMCCD on a TIRF microscope [87]. Although this time resolution is not sufficient to determine diffusion coefficients on the scale of the optical resolution it is sufficient to measure active transport on cell membranes. A compromise between the two techniques was found with Raster Image Correlation Spectroscopy (RICS) [88] and Space-Time Image Correlation Spectroscopy (STICS) [89] where the spatial and temporal information within a confocal picture was used simultaneously to calculate spatial and temporal correlations. STICS has been applied to protein velocity mapping in living cells and RICS extends the temporal resolution of measuring biomolecular dynamics from μs to s by using different scanning modes. These new methods will allow the user to selectively probe the regions of interest in a cell and measure the dynamics and interactions that range from fast diffusion species to immobile structures that is not easily accessible by FCS/FCCS.

Fluorescent Probes

Fluorescent probes play an important role in distinguishing the target molecule from the background light such as scattering or autofluorescence. FCCS applications require probes with high quantum yields and long-term photostability. Suitable probes have to be selected and calibrated to determine their excitation and emission characteristics for the mode of FCCS they need. For instance, probes used for TP-FCCS and SW-FCCS need to have similar two-photon and one-photon excitation spectra, respectively. This is so that multiple probes can all be excited with a single laser line, be it in the IR or UV/VIS region. It is also advantageous for dyes to have narrow emission spectra for minimal cross-talk as red-shifted dyes tend to have broader emission spectra. Possible probes for use with FCCS include small organic fluorophores, quantum dots, tandem dyes, and fluorescent proteins for in vivo measurements.

Organic Probes

Organic fluorophores have long been the favored fluorescent labels for FCCS. Fluorophores such as xanthene and cyanine dyes are small, photostable, and have high-molecular brightness. They are readily available with reactive groups for labeling to target biological molecules. Besides commonly used for dual-color FCCS or even SW-FCCS [44], they have overlapping two-photon excitation spectra and could be simultaneously excited with the same IR laser line. Recently, long Stokes shift organic probes with small molecular weight called Megastokes dyes [90] have been introduced with chemical modifications for labeling. Although these dyes have lower count rates [45], their small sizes offer promising applications in labeling biological molecules.

Quantum Dots

With the recent advent of long Stokes shift fluorophores such as quantum dots, multicolor imaging using a single laser wavelength for excitation has been achieved [73]. Quantum dots are semiconductor nanocrystals that are further coated with a polymer shell or other ligands [91] that allow the materials to be conjugated to biological molecules. Quantum dots have the unique optical property of size-dependent emission wavelengths [92]. Other benefits of quantum dots include long-term photostability, high quantum yield, multiple labeling with several colors, and single wavelength excitation for all colors. Due to its long Stokes shift, multicolor FCS experiments [78] have been performed to detect heterogeneities in lipid bilayer membranes [93], combined with submicrometer fluidic channels for isolation and detection [94] and to measure the binding constants of quantum dot-labeled streptavidin-biotin with SW-FCCS [43] and two-photon excitation FCCS [95]. Although quantum dots have been used in fluorescence imaging of live cells and even whole organisms, single-molecule experiments with quantum dots have been limited. This is due to its blinking characteristics, aggregation tendency, and large size, which affect the mobility (hence possibly function) of the target molecule [96]. These factors will have to be taken into account when applied to FCCS. Nevertheless because of its intense brightness, low photobleaching rate and tunable emission wavelengths with broad adsorption spectra, quantum dots prove to be promising fluorescent probes for multicolor detection in cell biology. A new development, quantum rods, recently published by the Alivisatos group, have shown to be biocompatible, linearly polarized, and brighter than Quantum dots; although size of the labels will remain an issue [97].

Tandem Dyes

Phycobiliprotein-based tandem dyes have also been used for FCCS and were first applied in flow cytometry and cell sorting in fluorescence immunoassays [98]. Phycobiliproteins, a class of light-harvesting proteins that enhances the

efficiency of photosynthesis are found in many species of algae [99]. Phycobiliproteins have high extinction coefficients and quantum yields. The molecular sizes can be large, with R-phycoerythrin (RPE) at 240 kDa containing 34 bilin fluorophores. With its high molar absorption coefficient at a broad range of absorbance wavelengths between 470 nm and 550 nm, phycoerythrin (PE) can be coupled as an energy donor to a range of potential acceptor molecules, including Allophycocyanine (APC, λ_{em} = 660 nm) [99, 100], Cyanine dyes (Cy5, λ_{em} = 670 nm or Cy7, λ_{em} = 767 nm) [101], and Alexa Fluor dyes (Alexa Fluor 647, λ_{em} = 667 nm) [102]. When excited at an excitation wavelength of 488 nm, energy transfer of the tandem dyes produces large Stokes shifts with emission wavelengths that can be easily resolved from PE (λ_{em} = 575 nm) or fluorescein (λ_{em} = 518 nm) [103]. However, tandem dyes have lower photostability than quantum dots. They have higher photobleaching rates and an observed loss of FRET efficiency with longer storage time. In addition, the non-negligible emission signal from the phycobiliprotein (phycoerythrin at 550–600 nm) contributes to cross-talk and increases background. Although it has been commonly used for cell sorting in flow cytometry, its large size could as well deter biophysicists from using tandem dyes as labels for FCCS. In spite of this, with its high quantum yield and long-wavelength emission, tandem dyes have shown to be valuable probes for applications in SW-FCCS [43, 45].

Fluorescent Proteins

The green fluorescent proteins (GFP) and its mutants have become versatile tools for fluorescence imaging and spectroscopy in cell biology. GFP can be genetically tagged to target proteins and expressed in living cells. This allows biologists to study structural and dynamic processes of subcellular compartments, which were previously not possible with organic probes. In vivo applications of FCCS have been demonstrated with fluorescent proteins as probes. A typical FP pair for dual-color FCCS would be enhanced green fluorescent protein (EGFP) and DsRed variants, monomeric red fluorescent protein (mRFP). Cross-correlation analysis in live cells has been demonstrated where GFP was fused to mRFP with a caspase-3 recognition linker. Caspase-3 activation was detected through the decrease of the cross-correlation amplitude when the cells undergo apoptosis and protease cleavage [104]. On the other hand, the cross-correlation amplitudes were unaffected for a caspase insensitive linker [105]. Protein–protein interactions of transcription factors Fos and Jun [106] and between transcription regulating proteins from plants [107] have been measured in HeLa cells. CFP/YFP has also been used to work with FCCS by using pulsed interleaved excitation to remove cross-talk. Recently, a new fluorescent variant of a protein from the stony coral Montipora has been developed by Miyawaki and coworkers [46]. This long Stokes shift fluorescent protein called Keima absorbs at 440 nm and emits at 620 nm. It was coupled with CFP and applied with SW-FCCS to detect proteolysis by caspase-3 and the association of calmodulin and calmodulin-dependent enzyme. SW-FCCS has also most recently been applied to study the tertiary structures of ErbB family receptors in vivo by fusing the receptors with the widely used fluorescent proteins GFP and mRFP. The majority of EGFR and ErbB2 have been found to exist in dimeric forms on cell surface before ligand activation. The study raises a new possibility for anti-cancer drug design, i.e., to develop anti-cancer drugs acting at the receptor dimer interface [108]. With the development of more FPs for multicolor detection, FCCS will unravel an exciting area of biological applications

Summary

Fluorescence cross-correlation spectroscopy is a rapidly expanding field and new developments are reported in fast succession. New excitation and detection schemes, such as pulsed interleaved excitation and dispersive element-based detection, combined with advances in detector technology, e.g., CMOS detectors and EMCCDs, have significantly improved the capabilities of FCCS. The combinations of the different excitation, illumination, and detection techniques presented here open up a plethora of new possibilities from which the researcher can choose his customized system according to his needs. Particularly, with the introduction of camera-based detection schemes, FCCS can now be efficiently multiplexed and will soon be applied for FCS/FCCS imaging. The increase of spatial resolution in FCCS will initiate the merging of FCCS and ICCS related techniques, giving researchers access to a vast range of analysis methods.

Fluorescence cross-correlation spectroscopy is a powerful method to detect molecular interactions; however it has limitations to accurately resolve molecular oligomerizations. Other fluorescence fluctuation techniques could then be used to provide additional information. Photon counting histograms (PCH) [109–111], fluorescence intensity distribution analysis (FIDA) [112], and fluorescence resonance energy transfer (FRET) techniques [113] could be combined with FCCS to reveal molecular properties such as molecular brightness and conformational dynamics. Further reviews of fluorescence fluctuation spectroscopy describe these techniques more in detail [114, 115]. New developments in fluorescent probes that target the problems encountered in single-molecule spectroscopy;

such as brightness, Stokes' shift and photostability, have significant contributions to the applications of these techniques. Therefore, the unison of different excitation and detection modes, fluorescent probes and other fluorescence fluctuation techniques will pave the way to achieve higher resolution and to detect higher order molecular interactions in biological systems.

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