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Studying ion exchange in solution and at biological membranes by FCS

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Abstract

By FCS, a wide range of processes can be studied, covering time ranges from sub-nanoseconds to seconds. In principle, any process at equilibrium conditions can be measured, which reflects itself by a change in the detected fluorescence intensity. In this review, it is described how FCS and variants thereof can be used to monitor ion exchange, in solution and along biological membranes. Analysing fluorescence fluctuations of ion sensitive fluorophores by FCS offers selective advantages over other techniques for measuring local ion concentrations, and in particular for studying exchange kinetics of ions on a very local scale. This opens for several areas of application. The FCS approach was used to investigate fundamental aspects of proton exchange at and along biological membranes. The protonation relaxation rate, as measured by FCS for a pH-sensitive dye can also provide information about local accessibility/interaction of a particular labeling site and conformational states of biomolecules, in a similar fashion as in a fluorescence quenching experiment. Beyond protonation kinetics, the same FCS concept can also be applied to ion exchange studies using other ion sensitive fluorophores, and by use of dyes sensitive to other ambient conditions the concept can be extended also beyond ion exchange studies.

1. Introduction

Fluorescent indicators, are since long well established and useful tools to monitor concentrations of physiologically important ions within living cells (Lakowicz, 2006), (Demchenko, 2009). These indicators typically respond with changes in fluorescence intensities and/or lifetimes, or with shifts in emission or excitation spectra. Such shifts allow ratioing between different excitation or emission wavelengths (Demchenko, 2010), (O'Connor, Silver, 2007), whereby differences in dye concentrations, optical pathlengths or other parameters that affect the fluorescence output are cancelled out. In this chapter, an overview will be given of how FCS can provide an alternative method to these approaches to monitor ion concentrations, offering some specific advantages, in particular with respect to local ion concentrations, and for studying exchange kinetics of ions on a very local scale.

Fluorescence Correlation Spectroscopy (FCS) is one out of several techniques exploiting the concept of number fluctuation analysis, which have in common that a fluctuating number of particles within a fixed volume is analyzed in terms of its time dependence. This concept was introduced already a long time ago (Svedberg, 1911), (von Smoluchowski, 1914), (Chandrasekhar, 1943). Preceding FCS, several other techniques were developed providing indications of number fluctuations, i.e. the dynamic light scattering technique, exploiting the light scattering intensity from the particles of interest (Schaefer, 1943), (Berne and Peccora, 1975), and the voltage clamp approach (Hodgkin *et al*, 1949), where fluctuation analysis of electrical currents over sections of cellular or artificial membranes is performed (with the later developed patch clamp technique (Neher and Sakmann, 1976) as its single-molecule counterpart).

The theory and first experimental realizations of the FCS technique, utilising fluorescence intensity as the fluctuating quantity, was originally introduced during the years 1972-1974 (Magde *et al*, 1972), (Elson and Magde, 1974), (Magde *et al*, 1974), (Ehrenberg and Rigler, 1974). Although showing great potential, the applicability of FCS was strongly reduced at this time due to high background light levels and low detection quantum yields. However, the introduction of small, diffraction-limited observation volumes in FCS measurements, confocal epi-illumination, highly sensitive avalanche photodiodes for fluorescence detection and very selective band-pass filters to discriminate the fluorescence from the background, made it possible to improve signal-to-background ratios in FCS-measurements by several orders of magnitude (Rigler and Widengren, 1990), (Rigler *et al*, 1992), (Rigler *et al*, 1993).

In a FCS measurement, in its most simple realization, fluorescence fluctuations arise from translational diffusion, as the fluorescent molecules are diffusing into and out of the confocal detection volume (figure 1A). From the fluorescence fluctuations, information can be retrieved about the translational diffusion coefficients and the average number of molecules residing simultaneously in the detection volume. In the absence of any other kinetic process other than translational diffusion affecting the fluorescent molecules the time-dependent normalized intensity autocorrelation function (ACF) can be written:

$$G(\tau) = \lim_{T \rightarrow \infty} \frac{1}{T} \int_0^T \frac{F(t+\tau)F(t)}{\langle F \rangle^2} dt = \frac{1}{N} \left(\frac{1}{1+4D\tau/\omega_1^2} \right) \left(\frac{1}{1+4D\tau/\omega_2^2} \right)^{1/2} + 1 \quad (1)$$

Here, N denotes the mean number of fluorescent molecules within the sample volume element, and D the translational diffusion coefficient of the molecules. Brackets denote time average. ω_1 and ω_2 signify the distances in the radial and axial dimensions, respectively at which the detected fluorescence per unit volume has decreased by a factor of e^2 .

In a confocal epi-illuminated FCS arrangement, the total detected fluorescence intensity is given from:

$$F(t) = Q \int_{-\infty}^{\infty} CEF(\bar{r}) I_{\text{exc}}(\bar{r}) C(\bar{r}, t) dV \quad (2)$$

Here, $C(\bar{r}, t)$ is the concentration of fluorescent molecules at position $\bar{r}$ and time t . $CEF(\bar{r})$ is the collection efficiency function of the confocal microscope setup and $I_{\text{exc}}(\bar{r})$ denotes the excitation intensity. $Q = q\sigma_{\text{exc}}\Phi_f$, is a brightness coefficient, where σ_{exc} is the excitation cross section of the fluorescent molecules under study, Φ_f is their fluorescence quantum yield, and q denotes the efficiency of fluorescence detection, when emitted from the center of the laser focus. The parameter q includes the solid angle of light collection, the transmission of the microscope optics and the spectral filters, as well as the detection quantum yield of the detector.

Even for a standard FCS instrument with a single-point, stationary detection volume, a wide range of processes can be studied, spanning a time range from sub-nanoseconds to seconds. In principle, any process at equilibrium conditions, which reflects itself by a change of the detected fluorescence $F(t)$, can be measured. In figure 1, the three most widely applied FCS-based approaches to extract information from molecules undergoing or participating in dynamic processes or reactions are outlined.

When monitoring fluctuations in the detected fluorescence intensity, $F(t)$, within one emission wavelength range, there are two criteria, of which at least one needs to be fulfilled in order to gain information about a reaction process by FCS:

A. The reaction must either lead to a change of the diffusion coefficient, D , of the fluorescent species under investigation (figure 1B). The typical duration of the fluorescence bursts will then change due to the longer passage times of the fluorescent molecules through the detection volume. This is a typical strategy followed for ligand-receptor interaction studies by FCS (Rauer *et al*, 1996).

B. The reaction leads to change in the fluorescence quantum yield, Φ_f , or the excitation cross-section, σ_{exc} , of the reactants (figure 1C). This changes the fluorescence emission per molecule, and the fluorescence brightness coefficient Q of the studied molecules. This strategy, has been exploited to monitor inter- or intra-molecular dynamics, influenced by fluorescence quenching (Bonnet *et al*, 1998), (Chattopadhyay *et al*, 2002), a range of photo-physical processes inherent to the fluorophore marker molecules, including triplet state formation (Widengren *et al*, 1995), photo-isomerisation (Widengren and Schwille, 2000), and photo-induced charge transfer (Widengren *et al*, 2007), but also other processes influencing fluorescence blinking, such as ion exchange involving ion-sensitive fluorophores, as discussed in this review.

If there is no significant change in either diffusion properties or in the fluorescence brightness as a consequence of the reaction taking place, a cross-correlation approach can be applied, as an additional alternative (figure 1D). In Fluorescence cross-correlation spectroscopy (FCCS), the fluorescence intensity fluctuations from two spectroscopically (typically spectrally) separate species are correlated (Schwille *et al*, 1997). Here, $G(\tau)$ is calculated as in eq. 1, but with $F(t)$ and $F(t+\tau)$ separately detected. In dual-color FCCS the fluorescence intensity fluctuations from two spectrally separate detection channels are correlated. The degree of correlation in the fluctuations provides a measure of the extent of interaction (concerted diffusion) between two spectrally separate molecular species (figure 1D), even if the species display no particular change in the diffusion or brightness properties as a consequence of the interaction.

Figure 1

In this chapter, an overview will be given of how single-point FCS, with a stationary detection volume can be used to monitor ion exchange in solution, as well as at biological membranes. The concepts are mainly based on the strategy B above (figure 1C), but it will also be shown how additional specificity may be obtained by incorporating a FCCS approach in the measurements (figure 1D).

2. Ion exchange monitoring by FCS – basic approach

There exist a range of fluorescent indicators to monitor concentrations of physiologically important ions, which are used not the least for live cell studies (Lakowicz, 2006), (O'Connor and Silver, 2007), (Demchenko, 2010). These indicators display different responses upon ion binding, including changes in fluorescence intensities and/or lifetimes, or shifts in emission or excitation spectra. FCS can exploit these properties in an alternative fashion to monitor not only ion concentrations, but also to extract information about local buffering properties and ion exchange kinetics (Widengren and Rigler, 1997), (Widengren *et al*, 1999).

This FCS-approach in its basic form is based on the second strategy above (figure 1C), and the analysis of fluorescence fluctuations arising as a consequence of ions binding to and dissociating from the fluorophores. In general, for a fluorescent species undergoing a reaction influencing the fluorescence, and at the same time diffusing into and out of the detection volume by Brownian diffusion, the normalized autocorrelation function of the detected fluorescence fluctuations can not be expressed in an analytical form. However, for a broad range of reactions simplifications are possible, and this is also the case for ion exchange reactions influencing the fluorescence of ion sensitive fluorophores. In general, if diffusion is much slower than the chemical relaxation time(s) and / or the diffusion coefficients of all fluorescent species are equal, then the time-dependent fluorescence correlation function can be separated into two factors (Palmer and Thompson, 1987). In the ACFs, the first factor, $G_D(\tau)$, then depends solely on transport properties (diffusion or flow) and the second, $R(\tau)$, depends only on the reaction rate constants:

$$\underline{G(\tau) = G_D(\tau)R(\tau) + 1} \quad (3),$$

In its general form, for M species participating in a chemical reaction and with T_{ij} denoting the corresponding matrix of the kinetic rate coefficients, the second factor $R(\tau)$ is given by:

$$R(\tau) = \frac{\sum_{i,j=1}^M Q_i Q_j X_{ij}(\tau)}{\sum_{i=1}^M Q_i^2 \bar{C}_i} \quad (4)$$

Here, Q_i is the fluorescence brightness coefficient of state i and $X_{ij}(\tau)$ is the solution to the following set of differential equations and initial conditions:

$$\begin{aligned} dX_{ik}(\tau)/d\tau &= \sum_{j=1}^M T_{ij} X_{jk}(\tau) \\ X_{ik}(0) &= \bar{C}_i \delta_{ik} \end{aligned} \quad (5)$$

$X_{ij}(\tau)$ describes the probability of finding a molecule in state j at time τ , given that it was in state i at time 0.

As mentioned, a rather broad range of chemical reactions fulfills one or both of the criteria for eqs. 3-5. Moreover, for a reaction that under standard conditions does not fulfill these criteria, the conditions can often be modified so that the criteria can be fulfilled. Dwell times in the detection volume can be retarded with respect to the chemical relaxation times by expanding the observation volume (figure 1A), or by speeding up the reactions under study (figure 1C), for instance by using higher concentrations of unlabelled reactants.

In its most simple realisation, the FCS approach for ion exchange studies exploits the strong contrast in the fluorescence brightness coefficient Q (eqs 2 and 4) that follows from ions binding to or dissociating from an ion-sensitive fluorophore. For the case of pH-sensitive dyes there are several different dyes commercially available. Taking the pH-sensitive dye Fluorescein Isothiocyanate (FITC) in a buffered aqueous solution in a close to neutral pH range as an example, the following three equilibria need to be considered:

In the equations above, HFl^- and Fl^{2-} denote the protonated anion and the dianion forms of FITC. BH and B^- denote the protonated and the unprotonated forms of the buffer, which are active in the proton exchange with FITC. The ratios $\text{HFl}^-/\text{Fl}^{2-}$ and BH/B^- for a given pH are

determined by the pK_a of FITC and the buffer, respectively. For FITC, the proton exchange will not notably affect the diffusion properties of the dye. Typically, the proton exchange rate also takes place on a time scale at least an order of magnitude faster than that of translational diffusion of the dye molecules through the observation volume in an FCS experiment. Consequently, in the context of an FCS experiment one or both of the prerequisites are fulfilled to separate the contribution from proton exchange into a separate factor in the correlation function (eq. 3). This protonation dependent factor can then be described by:

$$R(\tau) = \frac{1}{1-P} (1-P + P \exp(-k_p \tau)) \quad (9)$$

Assuming that the protonated form of FITC is non-fluorescent, i.e. that $Q_{HFI^-} = 0$, P corresponds to the fraction of protonated FITC, i.e. $P = [HFI^-] / ([FI^{2-}] + [HFI^-])$. k_p is the protonation rate constant given by:

$$k_p = k_+ [H^+] + k_- + ([B^-] + [BH]) \frac{k_1 [H^+] / K_a + k_{-1}}{[H^+] / K_a + 1} \quad (10)$$

Here, K_a is the acidity constant of the buffer.

3. Monitoring of local ion concentrations and exchange in solution

The basic features of the FCS-based ion-exchange monitoring approach are illustrated in figure 2A-B for the pH-sensitive fluorophore FITC. The approach is also applicable to other ions than hydrogen, for instance for the monitoring of local calcium ion exchange, as previously shown (Widengren and Rigler, 1997). However, in the following we restrict ourselves to the monitoring of hydrogen ion exchange, and the use of pH sensitive dyes.

Figure 2A shows a series of FCS curves, recorded from FITC in a non-buffered aqueous solution at different pH. The curves were fitted to equation 3 using equation 9, with the addition of a second factor to the correlation function describing singlet-triplet transitions (having a relaxation time in the range of 1-2 μ s) (Widengren *et al*, 1995). In a first approximation, the protonation kinetics of the dye can be considered independent to these singlet-triplet transitions, and later investigations by other groups using the same FCS approach also confirm that the excitation intensity dependence of k_p and P , for the dyes pyranine (Wong and Fradin, 2011) and the xanthene dye TG-II (Paredes *et al*, 2011), is very small. One can note from the correlation curves, and with reference to eqs 6 and 9, that the fraction, P , of non-fluorescent, protonated FITC molecules increases, and the protonation

relaxation time, $1/k_p$, decreases with lower pH, and that the fitted P and k_p are well compatible with a one-step protonation with a pK_A of FITC of 6.5 (figure 2, inset).

From the FCS measurements the amplitude, P , and with knowledge of the fluorophore pK_A , also the pH can be determined. Moreover, from k_p information is obtained about the local exchange kinetics of protons to and from the fluorophores. Fig. 2B demonstrates the effect of buffer strength on the correlation curves of FITC at a fixed pH. In figure 2B (inset) the relaxation rate of ion exchange, k_p , as measured by FCS, can be seen to increase linearly with the concentration of an added phosphate buffer. For comparison, graphs of HEPES, and citric acid buffers at the same pH have been added to illustrate the general influence on k_p by an added buffer and the difference observed from one buffer to another. The slope of the graphs in the inset of figure 2B provides a measure of the buffer strength of the different buffers under the conditions of the measurements. The buffer strength depends on several parameters, including how close the pK_A of the buffer is to the ambient pH and the diffusion coefficient of the buffer molecules, determining the collisional rate between buffer and fluorophore molecules. The slopes can also be influenced by the ionic strength of the solution. Depending on the mutual charges of the buffer molecules and the fluorophores, charge screening through increase of the ionic strength of the solution may either increase (if the buffer molecules and the fluorophores are all equally charged) or decrease the protonation rates (Wang and Fradin, 2011). In analogy with this, similar charge screening effects have been analysed by FCS for protonation kinetics at biological membranes (Sandén *et al*, 2010) and for fluorescence quenching of fluorophores close to dielectric interfaces (Blom *et al*, 2010). However, since this charge screening affects the interaction between buffer molecules and fluorophores, no particular effect of ionic strength on k_p is seen in the absence of a buffer, as illustrated in the inset of figure 2B, showing the relaxation rates measured at different concentrations of sodium chloride, with no buffer added. Evidently, an effect on k_p can also be expected if the fluorophore is shielded from the buffer molecules with which it can exchange protons. From this point of view, the k_p parameter value, as measured by FCS for a pH-sensitive dye can provide information about local accessibility/interaction of a particular labeling site and conformational states of biomolecules, in a similar fashion as in a fluorescence quenching experiment. Possible advantages of monitoring local accessibility via k_p rather than by fluorescence quenching is that the rate of quenching can be much lower, and can be scaled by the buffer concentration. Thereby, the quenching rate can be adapted for maximum sensitivity to the kinetics of any dynamic process that is to be studied.

As an illustration of how the local accessibility may influence the protonation kinetics is given by green fluorescent proteins (GFPs). A set of correlation curves of the Green Fluorescent protein (GFP) mutant (F64L, S65T) is shown in figure 2C. This protein displays a similar pH-dependence as that for Fluorescein. However, much higher buffer concentrations were required in order to significantly increase the relaxation rate of the proton transfer kinetics, and the relation between the measured k_p rate for GFP and the buffer concentration was non-linear (inset, figure 2C). X-ray structures of GFPs (Ormö *et al*, 1996) have revealed that the fluorescently active part (residues 65-67) of the GFP molecule is surrounded by a very tight barrel, formed by an 11-stranded β -barrel. Most likely, this barrel not only slows the exchange of protons within the microenvironment of the GFP molecule, but also physically prevents buffer molecules and protons from directly reaching the fluorescently active residues in the interior of the barrel. Hence, the active residues are only affected indirectly, where changes in fluorescence are a secondary effect mediated intramolecularly, following a proton exchange at some exterior part of the molecule. This two-site model for protonation of the chromophore part of GFPs has subsequently been supported by other experimental and theoretical data (see (Bizzarri *et al*, 2009) for a review).

Figure 2

4. Monitoring of proton exchange at biological membranes by FCS

The FCS-based approach for ion exchange studies above does not provide any significant advantages for monitoring ion concentrations over extended three-dimensional volumes. However, it can offer selective advantages over other techniques for measuring local ion concentrations, and in particular exchange kinetics of ions on a local scale. Transport of ions across biological membranes is of central importance for a range of cellular processes such as nerve conduction, energy metabolism, and import of nutrients into cells.

Proton gradients across biological membranes act as driving forces for many energy-consuming cellular processes, not the least ATP synthesis by ATP synthase in the mitochondria. To generate the gradients, proton transport at and across membranes is required and involves a series of membrane-spanning proteins in the inner membranes of the mitochondriae. The underlying mechanisms for this proton transport has been subject to extensive research, but is nonetheless not completely understood. One of the key questions concerns the nature of coupling between proton generators, such as cytochrome C oxidase

(Cyt_cO), pumping protons across the membrane, and proton consumers, such as ATP synthase, using the proton gradients across the membrane to drive the ATP synthesis (Medvedev and Stuchebrukhov, 2011). Both the outlet of the generator and inlet of the consumer proteins are located on the same side of the membrane, but the proteins are spatially separated. A major question is how the generated protons get to the consumers before they are dissociated from the membrane surface. A major experimental technique for these molecular proton exchange studies is the laser-induced proton pulse approach (Gutman, 1986) and other relaxation techniques. Basically, a light flash directed to one site of a membrane releases protons from caged compounds in the membrane, and the response from pH-sensitive fluorophores is then monitored on another site of the membrane. The FCS approach above can offer complementary angles of view to these relaxation techniques. In particular, in FCS measurements protons associating to and dissociating from pH-sensitive fluorophores are observed at equilibrium conditions. No perturbation into some, often strongly unphysiological, initial condition is required. Moreover, while most other fluorescence-based techniques for ion concentration monitoring require dye concentrations of the order μM or more, FCS is typically performed at about 1000-fold lower concentrations. Thereby, the influence of buffering effects due to ions binding to the fluorophores themselves, or to any light absorbing proton emitter, can be avoided. Finally, the proton exchange seen in the FCS measurements reflects the immediate environment around the fluorescent probes, and is not influenced by the conditions in the media the protons pass on their way from a light absorbing proton emitter.

Based on these selective advantages of the FCS approach we have investigated the principal role of biological membranes for proton uptake of membrane incorporated proteins (Brändén *et al*, 2006).

Observations have been made that certain membrane incorporated proteins can receive protons at a rate faster than that limited by proton diffusion in water (Ädelroth and Brzezinski, 2004). The proton uptake has been proposed to be facilitated by “proton-collecting antennae” composed of surface-accessible negatively charged and protonatable groups close to the entry points of the proton-conducting pathways of the proteins (Riesle *et al*, 1996), (Checover *et al*, 1997). Theoretical studies have also indicated that the membrane itself can contribute to an increased proton uptake by membrane proteins in a similar fashion, i.e. yielding a protonation cross-section larger than the physical size of the membrane proteins (Georgievskii *et al*, 2002), (Smondyrev *et al*, 2002). However, the occurrence and mechanisms of such proton-collecting antennae or localized proton circuits at the surface of biological membranes had

previously not been directly verified experimentally. The FCS approach above was applied to investigate these phenomena, offering the advantages of studying the protonation kinetics at the local level of individual surface proton acceptors/donors on the membrane, at physiologically relevant conditions, and at thermodynamic equilibrium.

Figure 3

On this basis, and in a first round of experiments, we used FCS to investigate the particular role of the biological membrane for proton uptake and transport by monitoring the protonation dynamics at the surface of liposomes with well-defined compositions. In each liposome (approximately 30 nm in diameter), only one of the lipid head groups was covalently labelled with fluorescein (figure 3A). FCS curves recorded from these fluorescein-labelled liposomes in unbuffered solutions were obtained at different pH. In figure 3B, a set of FCS curves are shown, measured from liposomes, composed of the lipid DOPG (1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]). The corresponding protonation rates, k_P , obtained by fitting the curves to eqs. 3, 9, and 10 are shown in the inset. At low proton concentrations, and from the intercept and the slope of k_P versus the proton concentration, the protonation association and dissociation rates of liposome-associated fluorescein, k_+ and k_- could be determined. While the proton dissociation rate of fluorescein in the liposomes was found to be indistinguishable from that of free fluorescein in aqueous solution, the association rate was measured to be two orders of magnitude faster. Changing the lipids of the liposomes from DOPG (negatively charged head group) to DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), with a zwitterionic head group slowed down the association rate k_+ , but it still remained about two orders of magnitude larger than that of fluorescein free in solution. The increased association rates can thus not be attributed to electrostatic effects. Instead, the fact that the fluorescein is surrounded by a lipid membrane generates a proton-collecting antenna, dramatically accelerating proton uptake from water to the membrane-anchored proton acceptor of fluorescein. This suggests that proton migration along the surface can be significantly faster than the diffusion of the lipid molecules in the membrane and can efficiently compete with the dissociation rate of protons from the membrane surface to the surrounding water phase. If a specific proton acceptor (in this case a dye molecule) at a surface is surrounded by protonatable groups (lipid heads groups) its proton uptake rate, can increase dramatically (figure 3C). Both DOPG and DOPC are protonatable. However, their pK_a values are in the range of 2, and the lipids are therefore very sparsely protonated themselves. On the other

hand, addition of the lipid DOPA (1,2-dioleoyl-*sn*-glycero-3-phosphophate), with a pK_a close to that of fluorescein (around 7), into the DOPC/DOPG liposomes results in a further significant increase of the measured protonation relaxation rate, k_p . The DOPA lipid head groups can store membrane-localized protons that are in rapid equilibrium with the fluorescein proton acceptor. The increase in k_p is linearly dependent on the added DOPA concentration, in a similar fashion as observed for buffers in solution (inset, figure 2B), and can be described by eq. 10. In this sense, DOPA acts as a two-dimensional buffer.

As an extension to the experiments above, the proton association and dissociation rates of fluorescein were also studied with the fluorescein labelled to the membrane protein Cyt c O, and in the presence or absence of a membrane surrounding the protein (Öjemyr *et al*, 2009). As for lipid-labeled fluorescein (Brändén *et al*, 2006), it was found that the proton association rate, k_{+I} , was more than two orders of magnitude larger for fluorescein-Cyt c O reconstituted into lipid vesicles (DOPG), than for detergent-solubilized fluorescein-Cyt c O, which in turn was slightly larger (by a factor of two) than the k_{+I} rate measured for free fluorescein. The minor increase of k_{+I} for the detergent-solubilized fluorescein-Cyt c O, in the absence of a membrane, compared to that of free fluorescein indicates that the particular site of labelling of fluorescein on Cyt c O was not surrounded by a proton-collecting antenna on the protein surface. However, the dramatic increase of k_{+I} upon membrane incorporation indicates that the protein surface is in protonic contact with the membrane surface, providing a pathway for proton transfer between proton transporter and proton “consumer” proteins residing within the same membranes in living cells.

Extending the range of pH over which the protonation kinetics of fluorescein-labelled lipids in liposomes were measured, two different proton exchange regimes could be identified (Sandén *et al*, 2010). At high pH (>8) the k_{+I} rate strongly increases with increasing proton concentrations, whereas at low pH (<7) the increase of the k_{+I} rate with increasing bulk $[H^+]$ is far smaller and comparable to the rate of protonation of the fluorophore expected to take place directly from the bulk solution. The enhancement of the protonation observed for high pH, but not for low pH, can be explained by the liposome membrane acting as a proton-collecting antenna at low proton concentrations, whereas at high proton concentrations the membrane surface is saturated and only direct flux from the bulk can yield a higher k_{+I} upon increase of bulk $[H^+]$.

Taken together, ion translocation across membranes and between the different membrane protein components is a complex interplay between the proteins and the membrane itself. Our recent studies indicate that the membrane, or the ordered water layer close to the membrane,

can act as a proton-conducting link between membrane-spanning proton transporters, provide a proton-collecting antenna effective in particular at lower proton concentrations in the bulk, and can be modulated by the ionic strength of the bulk and the lipid composition of the membrane. FCS is well suited to investigate these phenomena and can be applied further to a broad range of investigations of membrane-associated ion-exchange, also involving other membrane proteins and the proton exchange between them, as well as exchange of other ions than hydrogen.

5. Approach for ion exchange monitoring incorporating dual-color cross-correlation (FCCS)

Although the FCS-based approach to monitor local ion exchange is straightforward and relatively robust, the fluorescence fluctuations due to ion exchange may overlap in time with other fluorescence fluctuation processes, in particular with singlet-triplet transitions (Widengren *et al*, 1995). For the case such overlap may be a problem, and as a general means to better discriminate the proton exchange dynamics, we introduced proton exchange monitoring, based on dual-color FCCS applied to ratiometric pH-sensitive dyes (Persson *et al*, 2009). For the dye studied (NK 138, ATTO-Tec GmbH, Siegen Germany), its excitation as well as emission spectra changes as a function of pH. In the FCCS measurements, the fluorescence signal from the predominant emission wavelength range of the protonated form of NK 138 is cross-correlated with that of the deprotonated form. At the same time, by the excitation scheme above, the protonated and the deprotonated form of the dye are alternatingly predominantly excited. We show that this combination of alternating excitation and FCCS allows a dramatic reduction in cross-talk. The principle of the approach and the overall instrumental design is illustrated in figure 4A.

Figure 4

Alternating excitation, introduced some years ago under the acronyms ALEX (Alternating Laser EXcitation) (Kapanidis *et al*, 2004) and PIE (Pulsed Interleaved Excitation) (Müller *et al*, 2005), can provide information about which excitation source has generated each detected fluorescence photon. Thereby, spectral cross-talk can be strongly suppressed, significantly improving the sensitivity by which the interaction between two spectrally distinct interaction partners can be characterized, on a single-molecule level or by FCCS. Depending on the time-scale of the molecular dynamics to be investigated ALEX and PIE need to be implemented

with different rates of alternating excitation, to avoid frequency overlaps with the dynamic process to be studied. However, using a recently developed concept to perform FCS with modulated excitation (Persson *et al*, 2008) allows retrieval of full time-scale correlation curves from FCS measurements with arbitrary time-scales of modulation and with arbitrarily low fractions of active excitation. Thereby, we could apply an alternating excitation scheme for FCCS without pre-knowledge and concern regarding the time-scales of the molecular dynamic processes to be studied.

In figure 4B and 4C, some correlation curves are shown, illustrating the main features in the auto-correlation and cross-correlation curves recorded for the dye NK 138. Cross-correlating the fluorescence signals originating from the protonated and deprotonated species of the pH-sensitive fluorophore makes the protonation-deprotonation process appear in the fluorescence correlation curve as an anti-correlating component. The prerequisite for this is that both species are fluorescent and that the signals originating from different species can be separated. A shift of both absorption and emission spectra upon protonation makes it possible to both excite and detect the species selectively. Because it is generally not possible to either excite or detect one species exclusively, the combination of alternating excitation and two-color detection provides a significant improvement in contrast.

The results show that alternating excitation and gating allows strong reduction of spectral cross-talk in FCCS measurements of ratiometric dyes. A further contrast enhancement may be obtained by separating the different dye species also by their possibly different fluorescence lifetimes, using Fluorescence Lifetime Correlation Spectroscopy (FLCS) (Böhmer *et al*, 2002). FLCS allow discrimination of fluorescence from different emitters with different fluorescence lifetimes by applying temporal filters based on the fluorescence decay. Recently, FLCS was used to discriminate and selectively extract the auto-correlation functions of a protonated and non-protonated form of a pH-sensitive dye, regardless of the spectral overlap between the two forms (Paredes *et al*, 2011). Nonetheless, our generally applicable approach for alternating excitation/detection already drastically improve the contrast, and turning the protonation component of the correlation curve into an anti-correlation by cross-correlation makes it substantially easier to separate from the diffusion component or photophysical processes in the same time range, which makes the curve fitting more robust. The presented approach is not limited to protonation studies but could also be applied to ratiometric dyes sensitive to other ambient conditions and ions.

6. Conclusions

Based on concepts of experimental fluctuation methods described about one decade ago, invented 40 years ago, and having its major breakthrough about 20 years ago, the FCS technique and related fluorescence fluctuation methods are today still under strong development. In this review, it is described how FCS and variants thereof can be used to monitor ion exchange, in solution and along biological membranes. FCS, applied to the analysis of fluorescence fluctuations of ion sensitive fluorophores offers selective advantages over other techniques for measuring local ion concentrations, and in particular exchange kinetics of ions on a very local scale. Measurements are performed at equilibrium conditions and at low fluorophore concentrations, which reduces perturbations to an absolute minimum. This offer opportunities to investigate fundamental aspects of proton exchange at and along biological membranes, and new experimental means to investigate the theory of how dimensionality affects molecular transport and reaction processes (Adam and Delbrück, 1968), (Berg and Purcell, 1977). The protonation relaxation rate, as measured by FCS for a pH-sensitive dye can also provide information about local accessibility/interaction of a particular labeling site and conformational states of biomolecules, in a similar fashion as in a fluorescence quenching experiment. By changing the buffer concentration, the quenching rate can be adapted for maximum sensitivity to the kinetics of the particular dynamic process to be studied. The presented approach is not limited to protonation studies but could also be applied to exchange studies of other ions using other ion sensitive fluorophores, or by use of ratiometric dyes sensitive to other ambient conditions the same concept can also be extended beyond ion exchange studies.

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Figures

Figure 1:

Major categories of molecular dynamic processes or reactions that can be analyzed by a FCS instrument with a single-point, stationary detection volume. A: Translational diffusion of fluorescent species through the confocal detection volume of an FCS instrument, yielding an ACF given by eq. 1. B: Reactions that lead to a significant change in the translational diffusion coefficient, D , of the fluorescent reaction partner. C: Molecular dynamic processes or reactions that change the fluorescence brightness, Φ_f , of the studied molecules, yielding an ACF of the form given in eq. 3. D: FCCS (here, dual-color FCCS), e.g. of reaction partners labelled with green (G) and red (R) emitting fluorophores that upon association move in concert and generate correlated fluctuations in the green and red emission range.

Figure 2.

Examples of ion exchange, monitored by FCS. A: FITC measured at different pH. Decays of the correlation curves in the 1 ms, 2-80 μs , and 1 μs time range are attributed to translational diffusion, proton exchange and single-triplet state transitions of the fluorophores, respectively. Inset: Measured fraction, P , of fluorescein molecules in their protonated, anion state (solid squares), and the protonation relaxation rate (open squares) as a function of $[\text{H}^+]$. B: FITC at pH6, with different concentrations of phosphate buffer. Inset: Measured k_p versus concentration of buffer / salt at pH6. C: FCS curves of GFP (S65T) at different pH. Inset: Fraction of non-fluorescent GFP molecules, Y (solid squares), and the protonation relaxation rate (open squares) as a function of $[\text{H}^+]$. Figures 2A, 2B and 2C reprinted from (Widengren et al, 1999) with permission from Elsevier.

Figure 3:

A:

Principal design of FCS experiments to study proton exchange kinetics at biological membranes. Liposomes were labelled with one pH-sensitive fluorophore undergoing fluorescence fluctuations due to protonation/deprotonation. B: Set of FCS curves recorded from the vesicles at different pH. The FCS curves reflect singlet-triplet transitions in the μs time range, protonation kinetics in the 10-100 μs time range and translational diffusion in the ms time range. Inset: measured protonation relaxation rates versus proton concentration C: Principle of the proton collecting antenna effect for a proton acceptor in a biological membrane. Modified from (Brändén et al, 2006). Copyright (2006) national Academy of Sciences, USA.

Figure 4:

A: Setup used for FCCS with alternating excitation. Two lasers, emitting at 488nm and 594nm respectively, are modulated by acousto-optical modulators and serve as excitation sources. The setup has two separate detection pathways. The upper gray box shows how the blue laser preferably excites the protonated species of the fluorophore which is mainly detected in the green detection channels and the yellow laser almost exclusively excites the deprotonated species which is detected in the red detection channels. The lower gray box shows examples of how the switching between the protonated and deprotonated states contributes to the correlation in the case of autocorrelation (upper red curve) and gives rise to an anticorrelation when cross-correlating the signals from the two spectral detection ranges (lower black curve). B-D: Measurements on NK 138 in 500 μM HEPES buffer at a pH of 7.7. B: Autocorrelation curves from measurements with CW excitation and detection of either the deprotonated (upper curve, 594 nm excitation) or the protonated species (lower curve, 488 nm excitation). C: Cross-correlation of the signals from the two spectral ranges in a measurement with alternating excitation. Gating was used to remove cross-talk. D: Cross-correlation without gating. The solid lines in C and D represents model fits, using eq. 3, with $R(\tau) = 1 - H \exp(-k_p \tau)$ where H is a constant between 0 and 1. H decreases with increasing cross-talk. Figure in modified form from (Person et al, 2009), reproduced by permission of the PCCP Owner societies.