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Quantifying spatial and temporal variations of the cell membrane ultra-structure by bimFCS

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1. INTRODUCTION

The cell membrane is a mosaic of phospholipids, cholesterol, and embedded or attached proteins. Many membrane components interact weakly with their neighbors which transiently localizes them into compartments [1]–[4]. A filamentous protein meshwork underlying the membrane also causes heterogeneity by anchoring components within the membrane or by posing steric diffusion barriers [5]–[9]. These membrane structures provide essential regulation for cell signaling, either keeping reaction partners together long enough to signal, or in other cases, separating reaction partners to suppress erroneous signaling.

However, as the forces creating the domains are weak and the sizes of the domains are smaller than the optical diffraction limit, it is very challenging to directly visualize these structures without perturbing them. In particular, studying their function during signaling requires a non-destructive non-perturbing method suitable for live cell imaging. Super-resolution imaging techniques (such as PALM, STORM, and STED [10]–[12]) had limited success resolving these domains as each domain may only contain two to three copies of a protein at any given time, and their residence time in the domains is very short. Instead, a large part of our knowledge about the properties of various membrane domains in living cells has been deduced from studies of membrane protein and lipid diffusion. Early diffusion measurements of receptors and lipids in the cell membrane reported a wide range of diffusion coefficients depending on the temporal resolution of the measurement, from 1 μ s [13] to 33 ms Hz (summarized in [14]). As the molecules diffuse quickly, they traverse a larger membrane area during the slower acquisition. In a homogeneous medium, the area, $\langle r^2 \rangle$, traversed by a freely diffusing molecule grows linearly with time, t , elapsed: $\langle r^2 \rangle = 4Dt$, with diffusion coefficient D . However, in heterogeneous cell membranes, protein diffusion deviates from Einstein's diffusion equation. Some cell membrane components become transiently trapped in nanodomains, and hence diffuse more slowly over short distances. Others interact sterically with cytoskeleton fences and diffuse more slowly over long-distances as they 'hop' over the fences, a process termed hop-diffusion [15], [16].

Hence, measuring diffusion coefficients over a range of different length scales permits deducing information about the membrane ultrastructure. In 2005, Wawrezynieck et al. showed that multi-length scale diffusion measurements identify whether the diffusion molecule is being transiently trapped in nanodomains, moves freely, or diffuses slower over long distances due to interaction with the cell membrane cortex [17]. To measure diffusion on multiple length scales, Wawrezynieck et al. used spot variation fluorescence correlation

spectroscopy (svFCS) a variant of confocal FCS in which the excitation volume is changed between measurements [18], [19]. Confocal FCS records fluorescence intensity fluctuations of fluorophores entering and exiting a 3-D focal volume. The temporal autocorrelation of the fluctuations is calculated, and the half decay time is the average transit time (τ_D) of the fluorophores through the observation spot:

$$g(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D} \right)^{-1} \left(1 + \frac{\tau}{S^2 \tau_D} \right)^{-1/2}$$

Here S is the ratio between azimuthal and transverse Gaussian beam waist values of focal detection volume. In svFCS the observation volume is varied to measure transit times at several spot sizes, and the transit time is plotted versus detection area ω^2 and fit with line [19], [20]:

$$t_D = t_0 + \frac{1}{4D_{eff}} \omega^2$$

with D_{eff} being an effective diffusion coefficient, and t_0 being a measure for the strength of the association with the sub-diffraction nanoclusters (Fig. 1 H). This analysis, termed FCS diffusion law, quantifies different diffusion modes by the intercept t_0 : free diffusion slowed by transient interactions with nano-clusters or -domains corresponds to $t_0 > 0$, free Brownian diffusion to $t_0 = 0$ and hop diffusion to $t_0 < 0$. Subsequently, svFCS was extended below the diffraction limit by stimulated emission depletion (STED) FCS, which confirmed the heterogeneity of the cell membrane down to 20 nm length scale [21]. A major limitation of these spot-FCS experiments is that different sizes of spots were measured sequentially over minutes as the size is size by changing the excitation beam. Recently, Vicidomini et al. showed that it is possible to measure the diffusion through various spot sizes simultaneously using fluorescence lifetime correlation spectroscopy (FLCS) STED [22].

An elegant implementation of multi-length scale diffusion measurements is the combination of FCS with a spatially resolved detector, such as a camera, and two-dimensional extended excitation profile [23]. Analyzing fluorescence fluctuations not only from individual pixels, but also from binned neighboring pixels, this binned-imaging (bimFCS) measures diffusion on multiple length scales simultaneously and uniquely permits applying of the FCS diffusion law on short movie sequences from individual cells [24], [25]. Here we discuss how imaging FCS, in conjunction with well characterized membrane protein probes, provides a powerful method to quantify dynamic changes of membrane ultrastructure in living single cells over extended time periods while perturbing the cell [26]–[29].

2. MATERIAL AND METHODS

2.1. Multi-length scale FCS as tool to characterize the membrane heterogeneity

Binned-imaging fluorescence correlation spectroscopy (bimFCS) [24], [26] and internal reflection-fluorescence correlation spectroscopy (ITIR-FCS) [28], [29] use total internal reflection fluorescence microscopy (TIRF) to illuminate the bottom membrane of surface

grown, intact cells, and a fast, high quantum yield, ultra-low noise camera to acquire intensity data and treats pixels and binned pixels of the camera as observation areas (Fig. 1 A, B). The method requires stable laser TIRF with homogenous illumination across the region, and a fast, sensitive, low-noise camera. This study used a custom-built system including an inverted microscope (AxioObserver, Zeiss) equipped with high NA objective lens (Zeiss, 100× oil, NA=1.45). Excitation light for mGFP (488 nm, Argon-Krypton laser, Innova 70C, Coherent) and for mCherry (561 nm, CoboltLaser) is coupled through a single-mode optical fiber for spatial filtering into the back port of the microscope. This arrangement permits to separately adjust the TIRF angle, TIRF spot position, and the focus onto the back-focal plan of the imaging objective.

Typically, fluorescence signals from the bottom membrane of the cells (or lipid bilayer) are collected by the objective, passed through emission filters (Semrock), and acquired by an EMCCD (Andor iXon+ 897). Detecting the fluorescence intensity fluctuations caused by individual GFP molecules entering the detection area, requires a camera combining maximal quantum efficiency with lowest read-out noise and fast acquisition, typically a back-illuminated cooled EMCCD. The latest generation of sCMOS cameras is approaching the low noise and high quantum efficacy required, but earlier versions did not. Dual-wavelength imaging was done by inserting a beamsplitter module equipped with filter sets for GFP and mCherry between microscope and camera (DualView, Optical Insights).

The EMCCD camera collects the fluorescence signal at 600 frames per second. These data are binned according to the camera pixels into larger size $n \times n$ super-pixels, providing simultaneous acquisition over various length scales (Fig. 1 D, E). After bleach correction (see section 2.5), the temporal autocorrelations (AC) in each pixel and super-pixel are performed (Fig. 1 F, G). Fitting these curves with an appropriate diffusion model (see Method section 2.6 and 2.7.) provides a transit time t_D through each detection area size ω^2 , and the number of fluorophores in each ω^2 . The number of fluorophores calculated from the fit is used to compute a molecular area concentration, which in turn is used to determine a molecular brightness. Stable molecular concentration, neither too high nor too low, and sufficient brightness are indicators of high quality data.

The transit time t_D may be plotted against the differently sized areas and fit with a straight line:

$$t_D = t_0 + \omega^2 / 4D_{eff}$$

with D_{eff} being an effective diffusion coefficient, and t_0 being a measure for the strength of the association with the sub-diffraction nanoclusters (Fig. 1 H). This analysis, termed FCS diffusion law, quantifies the different diffusion modes by the intercept t_0 : free diffusion slowed by transient interaction with nanodomains corresponds to $t_0 > 0$, free diffusion to $t_0 = 0$ and hop diffusion $t_0 < 0$. This analysis reliably quantifies the effect of various treatments on the diffusion, when used in combination with a well characterized probe. However, the FCS law curve and D_{eff} provide more information on the diffusion and should be reported. The value of t_0 is very robust within one system, but comparisons across systems may be

affected by microscope and camera parameters, cell type, cell cycle, protein probe used, temperature and laser power. In addition, many proteins are affected by both, transient trapping in nanodomains and hop-diffusion, which leads to confusing results. Hence, for proteins diffusion by hop-diffusion it is preferred to fit the temporal autocorrelations with two time constants t_{free} and t_D , whereby t_{free} is the free related to the free diffusion within each membrane fence corral, while t_D is a result of the hop-diffusion. However, this requires recording some free diffusion within the corral, which is only possible if the membrane fence spacing leading to hop-diffusion is not more than two-fold smaller than the resolution limit (more details in the method section).

2.2. Suitable samples and experiments

Combined with TIRF, bimFCS is applicable to flat surfaces near the glass water interface, although it has been shown that imaging FCS may be adapted to light sheet illumination [30]. TIRF based bimFCS can be applied to (polymer) supported lipid bilayers, living cells, cell membrane sheets, or vesicles. Polymer supported lipid bilayers and vesicles are preferred over clean glass supported ones because the strong interaction of the lipids with the supporting glass may mask their true dynamics. Different cell types vary in how smoothly they adhere to the substrate, as some form very distinct adhesion zones leaving the rest of the membrane further away from the glass, while others adhere smoothly over large areas. We find PtK2, RBL-2H3, TM12, MCF7, and C2C12 well suited for bimFCS, while using HEK293, TM12T, MDA-MB 231 and NRK cells may require some surface optimization. In addition, the glass surface coating (clean, poly-L-Lys or extracellular matrix proteins, such as laminin or collagen) affects the cell - surface interactions. Cells are grown in serum containing media following standard cell culture protocols. Three to four days before an experiment, they are plated on glass coverslips. The next day they are transfected with the plasmids to express the probe proteins using Lipofectamine 3000 (Thermo Fisher Scientific). Depending on the membrane protein of interest the cells are imaged 20–72 hours post-transfection.

Ideally, the microscope is equipped with an incubation chamber for temperature and humidity control, and optionally gas control. As for all live cell experiments, care needs to be taken to use a buffer suitable for fluorescent microscopy and for long-term cell health. Here, we have imaged the cells in physiological salt solution (ingredients in mM: CaCl_2 , NaCl 151, MgCl_2 , KCl 5, HEPES 10, Glucose 10 pH 7.3).

The method is ideally suited to study the effect of treatment or perturbation on the membrane structure. A computer driven stage permits repeatedly revisiting the same ROI on the same group of cells and such obtaining statistically significant data within a single acquisition. Due to bleaching the number of reliable measurements on the same ROI and cell is limited to three to ten, depending on the cell size, fluorophore used and precision required. For continuous time series studies the samples should contain a large area not exposed to the excitation laser so the bleaching pool of diffusing markers can be replenished. This limits studies of small vesicles or membrane sheets.

In all cases it is important that the probe is tightly bound to the membrane and does not diffuse away from the membrane or is still being delivered to the membrane during the

measurement. In addition, the vast majority of molecules of the studied protein or lipid need to be mobile on the time-scale of the FCS experiments. This time-scale can be adjusted over about an order of magnitude but is limited at the one end by the acquisition speed of the camera and at the other by bleaching (see acquisition requirements below).

In the experiments studying the effects of transient Calcium influx, the ionophore Ionomycin (Sigma-Aldrich) was used. To control the amount of Ca^{2+} ions entering the cytosol, the free Ca^{2+} in the imaging buffer was adjusted using EGTA and Ca^{2+} .

2.3. Useful probes and fluorescent markers

Any membrane protein or lipid to be studied by bimFCS needs to be labeled covalently with a fluorescent probe at a fix ratio, typically one-to-one, so that each molecule adds a fixed fluorescent intensity when entering the detection spot. This probe should not affect the behavior of the protein or lipid. Especially for lipids, the latter is very challenging to achieve because the lipids are small and neutral or only weakly charged, while many fluorophores are charged and prefer the hydrophilic environment [31], [32]. Therefore, studies of lipids in living cells often use lipid binding proteins [33]. For proteins, one can either use genetically encoded labels, such as variants of the green fluorescent protein (GFP) or genetically encoded tags [34]. Which are then labeled with a synthetic fluorophore. GFP variants such as monomeric GFP (with A206K mutation, mGFP) and mCherry provide very good bimFCS data as they remain monomeric even when tethered to the membrane, are bright, fold efficiently and bleach slowly [35], [36]. A reason we prefer mCherry over other variants of RFP is its minimal crossing with green emission channel besides its high photostability [37].

FCS analysis requires only a limited number of fluorescent molecules within the observation volume. Too many molecules drown-out the fluctuations by the constant intensity whereas presence of too few results in data acquisition to take a long time or background noise blacks out the signal. For GFP labeled proteins on living cell membranes, the useful expression range is from a few hundreds to less than ten thousand molecules per square micron. Overexpression should be avoided as it may perturb the interaction of the probe with the membrane structures of interest. Using a bright synthetic fluorophore, the lower limit can be brought down to 100 molecules per square micron permitting the study of endogenous proteins.

2.4. Detection: Camera, pixel size and acquisition speed and duration

The camera pixel size should be chosen to oversample the microscope's point spread function to permit reasonable dense sampling of the increasing detection areas. In the set-up used here a $2.5\times$ magnification in front of the camera translates the EMCCD camera pixel edge length to 64 nm in the sample. The useful super-bins are then 128 nm, 192 nm, 256 nm, 320 nm, 384 nm, 448 nm and 512nm wide, small enough that diffusion times are affected by heterogeneities on the sub-100nm length scale. To increase signal to noise for weakly fluorescent probes, the pixels are binned 2×2 on the camera chip, resulting in 128nm pixel size.

The temporal resolution $\tau_{\min}$ required to measure diffusion coefficients from FCS should be two thirds of the transit time of the probe for a single pixel [38]. Lipid anchored probes in

the cell membrane at 37 °C, diffuses through a diffraction limited area in 2 – 3 ms, so camera exposure and frame acquisition time should be time at most 1.6 ms. Due to the faster diffusion of lipids in supported lipid bilayers, twice shorter acquisition times can be necessary, which are typically achievable with the higher brightness of synthetic fluorophores. Slower diffusing transmembrane proteins require three- to five-fold longer exposure times, which should be coupled with reduced excitation laser power to permit proportionally longer recordings. The required high frame rates limit the maximum region of interest (ROI) in EMCCDs to a few tens of pixel rows.

The total measurement duration should be about 1,000 times longer than the expected correlation time, here the expected diffusion time through the largest binned-pixel. In practice, we find 20 seconds data sufficient for lipid anchored proteins at physiological temperatures. In our experience, it is necessary to discard the first 10,000 frames of each recording while the EMCCD output stabilizes.

2.5. Data Analysis for bimFCS

As bimFCS acquires the data on all length scales simultaneously, the experiment is quick and simple. However, with TIRF illumination a larger area of the cell membrane is exposed to the excitation light and there is more photobleaching than in spot variation FCS. Before the temporal autocorrelations can be calculated and curve fitted, bleach correction is required. Bleaching of fluorophores while transiting the detection volume would shorten the measured correlation time and such bleaching has to be minimized by adjusting laser power, frame rate and choosing a suitable fluorophore. However, in bimFCS the excitation volume defined by the TIRF illumination spot, is significantly larger than the detection volume, defined by the camera pixel. Hence, bleaching occurs even when no bleaching is detectable in the detection area. This reduction of diffusing fluorophores is easily corrected before calculating the autocorrelation function. The intensity value of each individual pixel is then corrected by subtracting the spatial average intensity of each camera frame, which corrects the effects of photo-bleaching and laser fluctuations. Figure 1 F shows data presented from a supported lipid bilayer with 0.025% Rhodamine labelled phosphatidylethanolamine (PE).

2.6. The detection volume is a square detector convoluted with the point-spread function

A key feature of bimFCS analysis is the simultaneous calculation of multiple auto-correlations by binning the bleach corrected intensity in each camera pixel into super-pixels covering a range of sizes. In our study, 12 auto-correlations are calculated from bin1 (1×1) up to bin 12 (12×12). The auto-correlation functions for the same bin-size are averaged across a single ROI with some pixel overlap or, in some cases, analyzed for multiple ROIs as spatially separate images.

The membrane area from which the data in each pixel stems is defined by the convolution of the point spread function (PSF) on the microscope used and the camera pixel (or super-pixel) area. The PSF depends on the objective lens, the excitation and emission wavelengths and should be experimentally be determined using sub-diffraction limit sized fluorescent beads. In confocal or svFCS, the detection volume is a 3-D Gaussian volume in the laser beam with beam waist ω . The bimFCS software calculates from the input parameters PSF

and camera pixel size, an effective observation spot size, ω^2 , corresponding to the size of the detection profile $L_{xy} = L_x(x)L_y(y)$. The square pinhole convoluted with the system point spread function (PSF) in 2D is mathematically expressed in one axis as [27]

$$L_x(x) = \frac{1}{a} \int_0^a \text{PSF}(x - x_0) dx_0 = \frac{1}{2a} \left(\text{erf}\left(\frac{a-x}{\sqrt{2}\sigma}\right) + \text{erf}\left(\frac{x}{\sqrt{2}\sigma}\right) \right)$$

where the PSF is approximated by a Gaussian. For each bin, the distance from the center of the binned pixel to the position where the detection efficiency drops to $1/e^2$ of the central value is calculated and averaged with angular step size of $1/1000$ radians to obtain the corresponding w value.

2.7. Double-component square-pinhole FCS function for hop-diffusion

Membrane proteins undergoing hop-diffusion exhibit free diffusion on short length and time-scale, but on longer time-scales, show greatly reduced diffusion due to the low hopping possibility to pass through cytoskeleton meshwork. As a result, FCS curves should be interpreted as temporal autocorrelation from two populations of molecules with two separate diffusion coefficients. Concentration of molecules can be written as the sum of these two populations:

$$\frac{\partial C(x, y, t)}{\partial t} = D_1 \nabla^2 C_1(x, y, t) + D_2 \nabla^2 C_2(x, y, t)$$

where $C = C_1 + C_2$. The concentration correlation function will become

$$\langle \delta C(\vec{r}, t) \cdot \delta C(\vec{r}', t + \tau) \rangle = \sum_{i=1,2} \langle C_i \rangle \frac{1}{4\pi D_i \tau} \exp\left(-\frac{(x-x')^2 + (y-y')^2}{4D_i \tau}\right)$$

The time average of fluorescence signal will be rewritten as

$$\langle F(t) \rangle = \mu(\langle C_1 \rangle + \langle C_2 \rangle)A$$

where A is the detection area.

Then, the autocorrelation function of the fluorescence signal gives:

$$g(\tau) = \sum_{i=1,2} \frac{\langle C_i \rangle}{(\langle C_1 \rangle + \langle C_2 \rangle)^2} \left(\frac{2}{a^2 \sqrt{\pi}} \sqrt{\sigma^2 + D_i \tau} \left(\exp\left(-\frac{a^2}{4(\sigma^2 + D_i \tau)}\right) - 1 \right) + \frac{1}{a} \text{erf}\left(\frac{a}{2\sqrt{\sigma^2 + D_i \tau}}\right) \right)^2$$

with amplitude at $\tau = 0$

$$\begin{aligned}
g(0) &= \frac{\langle C_1 \rangle}{(\langle C_1 \rangle + \langle C_2 \rangle)^2} \\
&+ \frac{\langle C_2 \rangle}{(\langle C_1 \rangle + \langle C_2 \rangle)^2} \times \left(\frac{2\sigma}{a^2\sqrt{\pi}} \left(\exp\left(-\frac{a^2}{4\sigma^2}\right) - 1 \right) + \frac{1}{a} \operatorname{erf}\left(\frac{a}{2\sigma}\right) \right)^2 \\
&= \frac{1}{(\langle C_1 \rangle + \langle C_2 \rangle)_A} = \frac{1}{N_1 + N_2}
\end{aligned}$$

where a is the pixel size, and σ this point spread function. Still, there are four fit parameters, D_1 , D_2 , C_1 , C_2 so fitting the double exponential decay requires better S/N than the single exponential fit used for nanodomains. Additional constraint of the system enable robust fitting: The free diffusion between the membrane fences, D_1 , is the same for all pixel sizes. Hence, first only free diffusion in the smallest three pixel sizes is fit to obtain an estimate for $D_1 = D_{free}$. Then a global fit of all autocorrelation curves with the double-diffusion coefficient function is performed with D_1 constraint to be the same for all curves. In addition, the number of free diffusing molecules, N_1 , is set to increase linearly with increasing detection area. Under these constraints the fit is robust as long as the acquisition duration was sufficiently long, at least 100-fold of the corral confinement times, and the bleaching was minimized (see results 3.3.).

3. Results demonstrating some of the strengths of bimFCS

3.1. Spatially resolved quantification of membrane ultrastructure

Being camera based, bimFCS is ideally suited to study spatial variations of the diffusion of membrane protein over the surface of a cell. Compared to other spatial image correlation approaches [39], spatial bimFCS possess a temporal autocorrelation recording diffusion. This advantage permits direct correlation between cell membrane structures in live. Figure 3 displays the spatial correlation of t_0 with the density of actin cortex in living PtK2 cells for three different proteins, mGFP-GPI probing lipid nanodomains in the external, Lck-mGFP probing such domains in the internal membrane leaflet, and mGFP-GT46 probing membrane cytoskeleton [26], [40]. The data 300×20 pixel acquisition ROIs are subdivided in smaller 1.5-μm wide regions, 50×20 pixel sized sub-ROIs, in which the bimFCS analysis is performed. The minimal ROI size is the one that delivers sufficient statistics for reproducible results. While the bimFCS parameters are highly reproducible in one ROI of a cell, we found a much wider spread between cells and also across the surface of single cells. To understand the origin of the spatial variations within single cells, we correlated bimFCS parameters of mGFP-GPI with the actin membrane cytoskeleton density as recorded by α-Actinin-mCherry fluorescence intensity. BimFCS data were collected at 600Hz acquisition rate under 488 nm laser TIRF excitation with 580 W/m² power in the sample plane. Data is recorded in a ROI of 300 × 20 pixels for 75,000 frames, and then analyzed as one ROI and also in overlapping sub-ROIs (50 × 20 pixels), which were then moved across the surface of single PtK2 cells (Fig. 5a). The mGFP-GPI molecules are homogeneously distributed across the entire cell surface (Fig. 5b), as confirmed by the molecular concentration measurement (Fig. 5c). The detected spatial variations of the concentrations are within our measurement

precision. Deff varies about 2 σ of control measurements, but these variations are uncorrelated to the α -actinin-mCherry density (Fig. 5d). However, the t_0 values correlate positively with α -actinin-mCherry density (Fig. 5e). Unusually high t_0 values (greater than the 75 percentile of the data obtained across all cells) are found in regions of very high α -actinin-mCherry density (top 25%). Pearson and Spearman coefficients for t_0 vs. ROI intensity are calculated to be $\rho_p = 0.85 \pm 0.06$ and $\rho_s = 0.85 \pm 0.08$ ($N = 4$), confirming a strong correlation. The data is fitted well by a quadratic function ($X^2 = 0.9$, a linear fit gives $X^2 = 1.3$) with a significant positive offset in the absence of α -actinin-mCherry ($t_{0, \text{zero actinin}} = 4.1 \text{ ms}$). The brightness of the co-expressed α -actinin-mCherry is taken as maker for the actin cortex density. The association of mGFP-GPI in the outer leaflet of the membrane with cholesterol stabilized nanodomains correlates positively with the actin cortex density (Figure 3 A, B), consistent with a model in which cholesterol stabilized nanodomains are pinned to the underlying cytoskeleton, yet the positive intercept ($t_0 = 4 \text{ ms}$) shows presence of lipid nanodomains even in the limit of no detectable cytoskeleton (α -actinin intensity).

A small amount of lipid nanodomains persist apparently without the actin network, as also validated in vesicles (data not shown). Figure 3 C and D compare similar data for the inner leaflet, tested by Lck-mGFP. A positive correlation between association with lipid nanodomains and α -actinin density is found, see Figure 3 C and D. When using mGFP with an inert transmembrane domain, the intercept t_0 is negative due to cytoskeletal interaction. The magnitude of that negative t_0 correlates with the cortical cytoskeleton density (Figure 3 E, F). The bimFCS approach is ideal to study spatial comparisons within one system as this approach eliminates many sources of noise from using different cell types, cell ages, probes, and other factors [26].

3.2. Resolving the diffusion barriers causing hop-diffusion

Many diffusing transmembrane proteins encounter filaments of the underlying cytoskeleton as steric hindrance causing corralled or hop-diffusion [15], [16], [41]. Such diffusion will cause the autocorrelation curves to contain two time-constants, one for the free diffusion within corrals and one for hop-diffusion (see method section). Figure 4 A shows example bimFCS data of mGFP-GT46 in Normal Rat Kidney (NRK) epithelial cells. NRK have been previously show to transiently confine the diffusion of transmembrane proteins in corrals with about 220 nm edge length [16]. To obtain good bimFCS data on the slower diffusing transmembrane protein mGFP-GT46, the frame rate was reduced to 200Hz and the excitation light intensity to 250 W/m² power. Sequences of 55,000 frame were recorded and analyzed. Fitting the decay with a two-diffusion component square pinhole FCS function yields an estimate of the average spacing between membrane fences and of the strength or confinement (Fig. 4 B) [40], [42]. These parameters vary widely between cell types, even closely related cells lines, such TM12 and TM12T, a daughter-mother breast cancer cell line pair where one is aggressive and the other is not (Fig. 4 C) [43]. Further, mechanical, chemical and chemical environmental factors modulate these parameters as well [44].

3.3. Time-course studies of membrane ultrastructure

During cell signaling, some activated receptors change their association with other membrane components forming transient clusters. It has been shown in several systems that this clustering is crucial for successful signaling [45]–[47]. A bimFCS time-series permits to analyze the association of these receptors with nanodomains or the cytoskeleton in single cells during the entire process, from before the signal is active, during ligand binding to after the signal has decayed. Figure 5 shows examples for this type of application. It is possible to tune the timewindow of observation by choice of fluorophore, excitation power and time-lapse from a few minutes to days. The maximal temporal resolution is several seconds.

Effect of transient calcium influx on PIP₂ clusters in the inner membrane leaflet with actin cortex density—Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) is a minor phospholipid component of cell membranes, but an important effector in many signaling processes [47]–[50]. PIP₂ is negatively charged and can be clustered by electrostatic interaction with free calcium to form observable PIP₂ enriched nanodomains [51]–[54]. bimFCS enables investigating the formation, electrostatic stabilization and the following disassembly of Calcium-induced PIP₂ domains in intact living cells. Using the PIP₂ binding protein pH-PLC δ -mGFP as bimFCS probe resolves clusters on the inner membrane leaflet that are Calcium and PIP₂ dependent [55], [56] (recording parameters: 600 Hz frame rate, 580 W/m² power 488 nm laser TIRF excitation, 420s total recording time on single cell, analyzed in 20s sliding window). Ionomycin, a Calcium ionophore shuttling Ca²⁺ across the membrane, and extracellular buffering of free Ca²⁺ were used to generate transient Ca²⁺ influx. To monitor the intracellular Ca²⁺ levels, the inner leaflet anchored red calcium indicator Lck-RCaMP1h was co-expressed with pH-PLC δ -mGFP in PtK2 cells (Fig. 5 A). Dual-color recording using a DualView splitter to record the mCherry signal in the same camera frame as the GFP FCS signal, enables continued quantification of Ca²⁺ levels near the inner membrane leaflet while recording the mobility of PIP₂ in the inner membrane leaflet (Fig. 5 B). Upon addition of Ionomycin, the brightness of Lck-RCaMP1 h increased rapidly, but as the cell counter-regulates the Calcium influx, the levels return to baseline within one minute. The bimFCS analysis of pH-PLC δ -mGFP measures a transient aggregation whose kinetics largely follows the Calcium kinetics, albeit lagging behind (Fig. 5 C). The process is depending on Calcium concentration and the presence of PIP₂ [55], [56].

4. Discussion and Conclusions

The FCS diffusion law, plotting the diffusion coefficient as function of observation area has been value has been used to study membrane organization and various receptor proteins [17], [55], [56]. This method report summarizes how implementing the FCS law idea with TIRF microscopy and camera based FCS creates a versatile tool for multi-color, spatial and temporal analysis of the dynamics of cell membrane organization. The approach enables performing spatial and temporal comparisons within one experiment. The cell membrane ultrastructure changes of the cell cycle, is affected by the extracellular matrix, mechanical and chemical cues, changes of the cell cycle as well as temperature. Therefore, dynamic studies within one cell can be significantly more quantitative and sensitive than studies comparing cohorts of cells.

The spatial analysis of 20×50 pixel ROIs across the cell membrane confirmed significant spatial variations in t_0 for three proteins, mGFP-GPI, Lck10-GFP and mGFP-GT46 (Fig. 3). Their molecular density per area and diffusion (D_{eff}) are more homogenous spatially (data not shown) [26]. Using dual-color TIRF to measure the density of α -actinin-mCherry, we confirmed that the spatial inhomogeneity of t_0 correlated with the density of the actin bundling protein. Such correlation was expected with mGFP-GT46 undergoing hop diffusion as an increased number of diffusion barriers will cause stronger confinement. The results for the two markers for cholesterol stabilized nanodomains, mGFP-GPI for the external membrane leaflet and Lck10-GFP for the inner leaflet, are consistent with data and models from several groups suggesting that many cholesterol nanodomains form around pinning points on the membrane cytoskeleton [21], [59]–[62].

However, these spatial variations emphasize a major challenge to comparing FCS-law results across cell cohorts. Even within the same cell, during the same experiment, the t_0 value can vary four- to five-fold. The cause would be hidden unless using a marker for adhesion zones or cytoskeleton simultaneously. One way to reduce the variation, is to choose similar regions midway between nucleus and cell edge with low to average amount of adhesion zones. Better would be to design the study so that FCS-law analysis can be performed in the same cell and the same region repeatedly and provide an internal control.

Next, we showed the sensitivity of bimFCS and the FCS-law to detect small transient changes in the membrane ultra-structure when performing the experiments continuously in the same cell. We used the cytosolic protein pH-PLC δ -mGFP which is recruited to the inner leaflet of the membrane by the binding of its pH-domain to PIP₂ to study the dynamics of PIP₂ domains continuously over six minutes in the same cell. Using simultaneously the red Calcium indicator Lck-RCaMP1h permitted correlating any changes of the PIP₂ clusters with changes in free Calcium near the membrane. Transient influx of Calcium is measured by a relative increase in Lck-RCaMP1h brightness, which is quickly followed by a relative increase of t_0 for pH-PLC δ -mGFP indicating the PIP₂ was clustered. When the free Calcium is reduced, also the PIP₂ clusters break up. The kinetics of both processes appear to be purely diffusion driven, the details are subject of further investigation.

Furthermore, we showed that for transmembrane proteins interacting with the membrane cytoskeleton, the bimFCS analysis may be expanded past the simple FCS diffusion law analysis. Under conditions providing sufficient statics (see methods), the autocorrelation curve for transmembrane proteins may be best fit with a double-diffusion coefficient fit. The fast diffusion coefficient corresponds to the free diffusion within one corral, while the slow one corresponds to hop-diffusion across the membrane fences. The benefit of this analysis is that it provides an estimate for the spacing between membrane fences and the confinement strength, the ratio between the free diffusion coefficient and the hop-diffusion coefficient. This confinement strength crucially modulate the interaction rate of dimerizing receptors, such as EGF-R, and hence affect the length of their signaling bursts [63]–[66].

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Figure 1. Instrumentation and analysis for bimFCS.

(A) Two laser lines, 561 nm to excite mCherry and 488 nm to excite mGFP, are combined through an optical fiber and coupled into an objective type TIRF microscope. The fluorescence emission is split by color in a DualView beamsplitter and recorder side-by-side using an EMCCD camera. (B) The TIRF illumination angle is adjusted so that only fluorophores in the bottom cell membrane are excited. (C) Contour plot for square detection profile of 5x5 binned super-pixel size of $a = 512$ nm with PSF Gaussian width of $\sigma = 108.8$ nm. Dashed circle shows $1/e^2$ efficiency belt, from which ω is computed to be 396.4 nm. (D) Using a camera enables us to bin camera pixels into super-pixels before calculating the autocorrelation curves for increasing membrane areas. (E) Increased S/N is

obtained by averaging the data from pixels and super-pixels of same sized within a user defined region of interest. **(F)** Fluorescence intensity data before (top) and after (bottom) bleach correction. **(G)** Fitting autocorrelation curves of bleach corrected intensity data provides a transit time t_D through each detection area ω^2 and average number of fluorophores in each ω^2 . The number of fluorophores calculated from the fit is used to compute a molecular area concentration. **(H)** The transit times t_D are plotted against the different area sizes, and fit with a straight line. This graph, also referred to as FCS law, allows to extract an intercept with the time axis for zero area, t_0 , and an effective diffusion constant, D_{eff} , the reverse of the slope.

Figure 2. User interface and flow bimFCS analysis in Fiji.

(A) Flowchart of the bimFCS analysis. (B) User interface during processing, showing the data files, correlation set up and brightness per molecule as control parameter. (C) Overview of output windows showing autocorrelation curves, bimFCS law plot, spatial analysis of diffusion and more.

Figure 3. Spatial correlation of bimFCS results with membrane cytoskeleton.

(A) Tiled TIRF fluorescent images of a PtK2 cell expressing α -Actinin-mCherry as marker for membrane cytoskeleton density. To cover most of the cell, multiple shifted images were acquired. (B) mGFP-GPI expressed in the same cells was used for bimFCS analysis of nanodomains at the extracellular leaflet of cell membrane. The t_0 obtained from bimFCS analysis for the numbered and color-coded ROIs plotted versus the α -Actinin-mCherry fluorescence intensity in that same ROI. (C) Fluorescence image of α -Actinin-mCherry in a PtK2 cell, which also expressed Lck10-mGFP (anchored at the intracellular leaflet of cell membrane). (D) The t_0 obtained from bimFCS analysis of Lck10-mGFP for the marked ROIs plotted versus the α -Actinin-mCherry fluorescence intensity. (E) Fluorescence image of α -Actinin-mCherry in PtK2 cell co-expressing transmembrane protein mGFP-GT46. (F) The t_0 obtained from mGFP-GT46 bimFCS plotted versus the α -Actinin-mCherry fluorescence intensity in that same ROI. The t_0 showing the confinement in corrals is more negative in areas of higher cytoskeleton density.

Figure 4. bimFCS analysis to study hop-diffusion.

(A) Two-diffusion component square pinhole FCS function fits the autocorrelation curves computed from mGFP-GT46 diffusion in NRK cells (multiple observation areas from single to 12×12 binned-pixels). (B) FCS law plot of transit time values calculated from the fit parameters exhibiting a kink in the linear trend due to transmembrane protein-cytoskeleton interactions. (C) Comparison of bimFCS hop-diffusion data obtained for the same molecule, mGFP-GT46, expressed in different cells types, NRK, TM12, TM12T, PtK2 and RBL. These cells are known to have different membrane cytoskeleton spacing.

Figure 5. Time-course analysis of dynamic changes of the membrane structure.

(A) Schema showing how to study the effect of transient calcium influx on PIP₂ nanodomains in the inner leaflet of intact cells: Ptk2 cells co-expressing Lck-RCaMP1 h (membrane bound calcium indicator) and GFP-PHPLCδ (PIP2 marker); Ionophore used to shuttle Ca^{2+} across the membrane. (B) Dual-color TIRF microscopy enables observation of both fluorescent proteins simultaneously. Over-lapping time-windows were analyzed with the bimFCS approach. (C) Time- course of Lck-RCaMP1h intensity changes (Ca^{2+} concentration) and bimFCS t_0/t_0 changes of GFP-PHPLCδ (PIP2 clustering) (Average of 5 cells, percentage change of Lck-RCaMP1h calculated as $F/F_0(\%)$, percentage change of GFP-PHPLCδ calculated as $t_0/t_0(\%)$).