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Quantifying membrane protein oligomerization with fluorescence cross-correlation spectroscopy

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ABSTRACT

Fluorescence cross-correlation spectroscopy (FCCS) is an advanced fluorescence technique that can quantify protein-protein interactions *in vivo*. Due to the dynamic, heterogeneous nature of the membrane, special considerations must be made to interpret FCCS data accurately. In this study, we describe a method to quantify the oligomerization of membrane proteins tagged with two commonly used fluorescent probes, mCherry (mCH) and enhanced green (eGFP) fluorescent proteins. A mathematical model is described that relates the *relative cross-correlation value* (f_c) to the degree of oligomerization. This treatment accounts for mismatch in the confocal volumes, combinatoric effects of using two fluorescent probes, and the presence of non-fluorescent probes. Using this model, we calculate a ladder of f_c values which can be used to determine the oligomer state of membrane proteins from live-cell experimental data. Additionally, a probabilistic mathematical simulation is described to resolve the affinity of different dimeric and oligomeric protein controls.

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1. Introduction

Fluorescence correlation spectroscopy (FCS) is an established technique with many applications in biophysics. In live-cell experiments, fluorescence fluctuations are measured as fluorescently tagged protein moieties diffuse in and out of a diffraction limited excitation volume. Correlation of these fluctuations ($G(\tau)$) gives information about concentration ($\langle N\rangle/V_{eff}$), mobility (τ_D), and oligomerization (η). A complimentary technique to FCS is fluorescence cross-correlation spectroscopy (FCCS), which correlates the diffusion of two spectrally separated species as they move through the excitation volume, denoted green for the shorter wavelength species and red for the longer wavelength species. In dual-color FCCS, excitation is typically achieved with two concentric beams, for instance from a 488 nm laser and a 561 nm laser. FCCS measures the concentration and mobility of the red-green co-diffusing species, which is indicative of the degree of association. The principle of FCCS has been described in several excellent reviews [1–8]. The focus of this paper is on the factors that affect the relative amplitude of the cross-correlation function. Several of these factors have been dealt with in earlier reports that will be reviewed below. The goal of this paper is to combine each of the factors into a simple model that can be adapted to a variety of experimental conditions.

For applications of FCCS that use fluorescent proteins, their wide absorbance and emission spectra lead to spectral cross-talk which contaminates the cross-correlation data. In 2005, Müller et al. introduced pulsed interleaved excitation (PIE) as a modification to remove spectral crosstalk [9]. In PIE, picosecond laser pulses are temporally separated so that the fluorescence signals counted at each detector are gated corresponding to each excitation beam. In PIE-FCCS the cross-correlation function (CCF) is calculated from photons counted by detector A after 561 nm excitation with photons counted by detector B after 488 nm excitation. The red and green auto-correlation functions (ACFs) are calculated for fluorescence signal at detector A after 561 nm excitation and at detector B after 488 nm excitation respectively. Additional information can be obtained by measuring the donor fluorescence lifetime to determine the Förster resonance energy transfer (FRET) efficiency, which is accessible in PIE-FCCS with picosecond pulsed excitation and time-correlated single photon counting. We have used this technique to study dimerization of visual opsins, plexinA4 and

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other membrane receptors [10–12]. Recently, we used PIE-FCCS to study the organization of EGFR and found that cross-correlation can be used to resolve simple dimerization from the formation of larger multimers [13].

Special considerations must be made when interpreting the results of live cell measurements with fluorescent protein labels. Dual color excitation produces two wavelength-dependent volumes. Knowledge of the overlap and size of these volumes is essential for accurate determination of concentrations. Additionally, several factors lower (or dilute) the apparent cross-correlation which can lead to a misinterpretation of the data. The emergence of fluorescent protein (FP) fusions has contributed profoundly in the advancement of *in vivo* imaging, but incomplete maturation and dark states leads to a significant population of receptors labeled with non-fluorescent FP. Non-fluorescent FP fusions can associate with other FP fusions, lowering the apparent concentration of co-diffusing species. When investigating homo-dimerization, same-color FP fusion dimers can bias the data because they do not contribute to the CCF. Incomplete dimerization also lowers the apparent cross-correlation. Furthermore, protein oligomerization beyond simple dimerization leads to the formation of multiple diffusing species, which complicates the interpretation. Several approaches have been reported in the literature to deal with these experimental factors [14]. Rippe used DNA duplexes labeled with either fluorescein or rhodamine to investigate the NtrC-enhancer complex in solution [15]. It was noted that the duplex compositions follow a binomial distribution, which lowers the maximum binding ratio from 1 in the case of complete 1:1 binding of red and green probes to $\frac{1}{3}$ with binomial distribution. In a similar way, the binomial distribution was used to describe the integer accumulation of randomly-selected, singly-labeled molecules into a vesicle [4]. A thorough quantification of ligand binding with FCCS has also been detailed [16], including further corrections that account for the impact of the volume mismatch [17,18]. While several groups have used FCCS to quantify heterodimeric interactions of cellular components [19–23], only a handful have used corrections for non-fluorescent labels and/or volume mismatch to quantify the apparent dissociation constant ($K_{D,app}$) [3,14,24–26]. Several recent studies have attempted to use FCCS data to quantify $K_{D,app}$ in the membrane; however, none of these papers have included corrections for the effective volume, the presence of non-fluorescent labels, and a binomial distribution due to homo-oligomerization [27–31]. In this publication, we collect all these corrections into a single model as it applies to a system with homo-oligomerization.

To compare the model with experimental data, we use a control system based on membrane-bound coiled-coil dimerization motif (GCN4). This control system has been reported in published work from our lab and includes a monomer control, a dimer control, and a multimer control [12,13]. Here, we also constructed a new control system consisting of membrane anchored chimeras of the FK506 binding protein (FKBP), which dimerize upon stimulation of synthetic dimerizer. Using the model below we identify not only the oligomer state of these control systems, but also their binding affinity in the live cell plasma membrane. These results demonstrate the ability of PIE-FCCS to identify homo-oligomerization and quantify the affinity of membrane protein-protein interactions in live cells.

2. Material & methods

2.1. PIE-FCCS instrument

Fluorescence measurements were collected on a customized inverted microscope (Eclipse Ti, Nikon, Tokyo, Japan), which was described previously [10] (Fig. 1A). The 488 nm and 561 nm lasers beams were produced by a supercontinuum fiber laser with a repetition rate of 10 MHz (SuperK NKT Photonics, Birkerød, Denmark). Selection of the two beams from the white light source was achieved through a series of dichroic mirrors and clean-up filters (LL01-488-12.5, Semrock, Rochester, NY/LL02-561-12.5, Semrock, Rochester, NY). The 488 nm light was coupled into a 5 m single mode optical fiber while the 561 nm light was sent through a 15 m identical-core fiber, which introduced a time delay of about 50 ns. An additional dichroic (LM01-503-25, Semrock, Rochester, NY) was used to overlap the laser beams after exiting their respective fibers. A laser filter cube (zt488/561 rpc and zet488/561 m, Chroma Technology, Bellows Falls, VT) reflected the overlapped excitation light into the sample through a 100× oil-immersed objective (NA 1.49, Nikon, Tokyo, Japan). Fluorescence signal was collected as it passed back through the objective and was directed to one of the detection ports of the microscope. A 50 µm pinhole was positioned at the image plane, after which the light was collimated and then spectrally separated by a dichroic mirror (FF560-FDi01-25 × 36, Semrock, Rochester, NY). A final set of bandpass filters (FF01-520/44-25, Semrock, Rochester, NY/FF01-621/69-25, Semrock, Rochester, NY) were placed before two single photon avalanche diodes (SPAD, Micro Photon Devices, Bolzano, Italy). We assign the label **detector A** to the red signal (621 ± 34 nm) and **detector B** to the green signal (520 ± 22 nm). Single photons were time-tagged and time-resolved by a four-channel routed device (Picoharp 300, PicoQuant, Berlin, Germany) with a 32 ps timing resolution.

Time-correlated single-photon counting (TCSPC) data were time-gated such that photons at detector A within the 40 ns after the 561 nm pulse were assigned to $\mathbf{F_R(t)}$ and photons at detector B within 40 ns after the 488 nm pulse were assigned to $\mathbf{F_G(t)}$ (Fig. 1B). A multi-tau algorithm was used to calculate the auto and cross-correlation functions.

$$G_{ij} = \frac{\langle \delta F_i(t) \cdot \delta F_j(t+\tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle} \tag{1}$$

where i and j are either R or G so that for the two auto-correlations $i = j$ and for cross-correlation $i \neq j$ [32,33]. Individual curves are averaged before fitting to an appropriate diffusion model using a non-linear least squares fitting algorithm.

2.2. Data collection and analysis

Transfected Cos-7 cells were grown on 35 mm uncoated #1 glass bottom dishes (P35G-1.0-20.C, MatTek Corporation, Ashland, MA) and changed to Opti-MEM I without phenol red (31985070, ThermoFisher Scientific, Waltham, MA) right before imaging. For the dimerizer assay, cells were incubated with AP20187 (HY-13992, MedChemExpress, Monmouth Junction, NJ) for 1 h at a final concentration of 100 nM before imaging. The stage of the microscope is adapted with a stage-top incubator (Chamlide IC, Quorum Technologies, Guelph, Ontario) to keep cells at 37 °C. The laser spot was focused on single cells in membrane areas so that we simultaneously measured the top and bottom membranes of the cell. For cell measurements, the 488 nm laser was set to a power of 300 nW while 561 nm was 800 nW as was measured before entering the back of the microscope. Data was collected at 5 × 10 s intervals for each cell and then averaged together for fitting. Single cell data were binned at 10 µs. The correlation data was fit to a single component 2D Brownian diffusion model with triplet blinking.

$$G_i(\tau) = G(0) \cdot G_{Trip}(\tau) \cdot G_{Diff}(\tau)$$
$$G_i(\tau) = \frac{\gamma}{\langle N_i \rangle} \cdot \frac{1 - F + Fe^{\frac{-\tau}{\tau_T}}}{1 - F} \cdot \frac{1}{(1 + \tau/\tau_D)} \tag{2}$$

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