Quantifying membrane protein oligomerization using two-dimensional fluorescence intensity fluctuation spectrometry

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Abstract

Current methods for determining membrane protein association in cells are either very time consuming, require complicated procedures, or lack the sensitivity needed to assess the effect of concentration or ligand binding on the observed oligomerization. To overcome these limitations, we provide a detailed protocol for quantifying the relative abundance and stability of oligomers of differing sizes using two-dimensional fluorescence intensity fluctuation (2D-FIF) spectrometry. The approach can be implemented using a standard laser scanning fluorescence microscope along with custom written software for image analysis. This method may be applied to evaluate the extent of oligomerization as a function of receptor concentration and is particularly suited to assess the effects of agonists and antagonists on the oligomeric size of membrane receptors of interest.

Introduction

Characterization of the oligomeric state of membrane receptors continues to be a substantial experimental challenge. Methods based on fluorescence fluctuation spectroscopy (FFS) [1], such as Photon-Counting Histogram (PCH) analysis [2,3] Number and Brightness (N&B) analysis [4-6], and Spatial Intensity Distribution Analysis (SpIDA)[7-9] are promising approaches for studying protein interactions. In FFS-based approaches, the signal fluctuations caused by fluorescently labeled molecules diffusing in and out of a microscope focal region are analyzed with statistical methods to reveal properties of the molecules. Originally applied to quantify the diffusion of fluorescently labeled molecules in solution, FFS based approaches have been improved in recent decades to study the abundance and interactions of biomolecules in solutions as well as in living cells, and thereby offer a robust means for measuring both oligomer size and dynamics. However, current FFS-based approaches typically only provide average values of the oligomer size and concentration over mixtures of oligomers with different relative abundances and sizes.

This protocol describes the steps needed for using two-dimensional fluorescence intensity fluctuation (2D-FIF) spectrometry, a new method that can quantify the relative abundance and concentration dependence of oligomers of differing sizes in the presence or absence of ligands. The 2D-FIF spectrometry method relies on collecting fluorescence images of cells expressing the receptor of choice fused to a fluorescence marker protein. Regions in the fluorescence images where cells are present are divided into smaller subregions, or segments. The intensity fluctuations across the pixels in a given segment are used to calculate an “effective molecular brightness,” ε_{eff}, of each segment. The average intensity of the segment is divided by the effective brightness of a single protomer, ε^{proto}, to determine the average concentration of receptors in that segment. By scanning a large number of cells, tens of thousands of segments are collected, and an effective brightness and concentration is calculated for each. From a 2D-map assembled from all the effective brightness-concentration pairs, effective brightness distributions can be generated for a number of different protomer concentrations ranges. Each brightness distribution, or spectrogram, is comprised of contributions from different sized oligomers and

can be decomposed to find the relative contributions, or abundance, of each of the different oligomer sizes present across the measured sample. To extract this relative abundance information from each spectrogram, an effective brightness distribution must be generated from measurements on cells expressing a monomeric form of the fluorescent marker as well as cells expressing a tandem-dimer of the fluorescent marker. From the measured monomer and dimer brightness distributions, the brightness distribution for any oligomer of size *n* can be predicted, as the peak value of the size *n* oligomer distribution will be simply scaled by the size of the oligomer, i.e. $n\varepsilon^{proto}$. Each composite spectrogram is then “unmixed”, with the unmixing components consisting of the various predicted oligomer brightness distributions. The relative abundance of each oligomer species is then computed as the fraction of the total area underneath the composite spectrogram that it occupies. After unmixing the spectrograms obtained from a number of concentration ranges, the trajectory of these relative abundances is plotted as a function of concentration. Along with evaluating the extent of oligomerization as a function of receptor concentration, the method is also can be applied to assess the effects of ligand-inducing shifts in the oligomeric size of membrane receptors of interest.

The 2D-FIF approach can be implemented using standard laser scanning fluorescence microscopes, including confocal, two-photon excitation, and total internal reflection microscopes. A software package, titled the FIF Spectrometry Suite, is available at https://figshare.com/s/acfd94b21b1105317f56 and can be used by following the steps listed in the detailed protocol below. The presented workflow describes the application applied to fixed cells, but the method can be used just as easily to probe protein oligomerization *in vivo*.

Reagents

1. Chinese hamster ovary (CHO) cells growth medium: Hams F-12 Nutrient Mix (Gibco^{TM}, 11765054) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, #S10350), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Gibco^{TM}, #15140-122), and 100 mg ml^{-1} zeocin (Gibco^{TM}, # R25001).

2. Cell fixation solution: 4% w/v paraformaldehyde (Sigma-Aldrich #158127-5G) in phosphate buffered saline (PBS, Gibco^{TM}, #14040-133).

3. Sub-diffraction sized fluorescent microspheres for determining the laser beam waist size of microscope. We have used the following two types of microspheres:

i. 100 nm Tetraspeck fluorescent microspheres (Invitrogen, # T14792)

ii. 170-nm PS-Speck Microscope Point Source Kit fluorescent microspheres (Invitrogen, # P7220)

Equipment

1. Humidified incubator with 95% air and 5% CO2 kept at a temperature of 37°C

2. Poly-D-Lysine (Sigma-Aldrich, P7280-5mg) coated chambered #1.5 cover glass. We have used chambered coverslips in the following two forms:

i. Lab-Tek 4-well-chambered cover glasses (Thermo Fisher Scientific, # 155382)

ii. 35-mm culture dishes containing a 14-mm diameter microwell (MatTek Corp.)

3. Inverted laser scanning fluorescence microscope for image acquisition. The imaging can be performed on any inverted fluorescence microscope capable of image sectioning. Therefore either single-photon excitation in combination with a confocal pinhole or two-photon excitation may be used. The single (i) and two-photon (ii) excitation systems we have used are described below.

i. Zeiss LSM 510 PASCAL EXCITER laser scanning head coupled to a Zeiss Axiovert 200M inverted microscope equipped with a plan apochromat oil immersion objective (63×, NA=1.4¸Carl Zeiss Microscopy). A long-pass beam splitter with center wavelength 490 nm along with a long pass emission filter with a wavelength of 505 nm were chosen to efficiently collect monomeric enhanced green fluorescent protein (mEGFP) emission signal.

ii. Two-photon optical micro-spectroscope[10,11] comprised of a Zeiss Axio Observer inverted microscope stand equipped with an infinity-corrected, C-Apochromat, water-immersion objective (63×, NA=1.2; Carl Zeiss Microscopy), and an OptiMiS detection head from Aurora Spectral Technologies, LLC. A mode-locked laser (MaiTai^{TM}, Spectra Physics) was used for fluorescence excitation at 960 nm. FIF Spectrometry Suite. Image analysis software which has been deposited on the *Figshare* digital repository and is accessible from: https://figshare.com/s/acfd94b21b1105317f56

Procedure

A. Cell Preparation Protocol

1. Generate cell lines stably expressing membrane receptors of interest and cells expressing monomeric or dimeric forms of the fluorescent marker protein; see previously described protocol[12,13]

2. Maintain a healthy cell culture on T-25 flask. If cells are being revived from deep freeze, allow for at least two cell passages. Subculture cells if they are more than 85% confluent on flask. Subculturing of cells should be performed in a sterile environment using standard cell culture procedures

3. Lift cells from T-25 flask and plate on poly-d-lysine coated chambered cover glass. Seed between 500-1000• cells/mm2 of coverslip growth area onto poly-d-lysine coated chambered #1.5 cover glass. The amount of cells seeded will ultimately depend on the doubling time of the cell line being studied. Allow the cells to grow for 36-48 hours in humidified incubator in order to spread out on the glass surface.

4. Prepare cells for imaging

i. Aspirate cell growth medium

ii. Wash cells twice with PBS

iii. If exposing cells to ligand, add ligand solution to cells for desired time. If no ligand is being used, simply add PBS to cells for same amount time.

iv. Remove ligand solution or PBS solution and add 1.5 mL of 4% formaldehyde fixing solution. Leave cells in fixing solution for 20 minutes. Leave room lights off for duration of time

v. Remove fixing solution

vi. Resuspend cells in PBS; cells now ready for imaging.

B. Image Acquisition Protocol

1. Set the pixel dwell time for the measuring system to be at least a factor of five times shorter than the two-dimensional characteristic diffusion time, τ_D, of the receptor under study. The characteristic diffusion time is the average time a receptor molecule will reside within the measurement volume before diffusing out and is related to the laser beam waist, ω, and the diffusion coefficient, D, through the following relation $\tau_D=\omega^2/4D$. The laser beam waist is defined as the distance from the center of the focused beam to where the intensity of the beam drops to e^{-2} its maximum value.

2. Obtain fluorescence images from several hundred cells for each type of experimental condition.

i. Search for cell/cells using scanning parameters with a reduced power or much shorter exposure time (preview scan) compared to the settings used for the actual data collection (fluorescence scan) so as not to expose cells to too much laser light.

ii. Focus on the basolateral membrane of a cell or group of cells using the preview scan.

iii. Take fluorescence scan of the focused cell/cells.

iv. Repeat steps *i-iii* until fluorescence images have been acquired from several hundred cells

3. Obtain fluorescence images from cells separately expressing monomeric and tandem dimer forms of the fluorescent marker which has been fused to receptor of interest. Apply the exact same imaging conditions which were used to collect images of cells expressing fluorescently labeled receptor of interest.

4. Obtain fluorescence images of sub-diffraction sized fluorescent microspheres for determining the laser beam waist size.

5. Obtain measurements from a light source with constant intensity (i.e. no temporal fluctuations). Signal from a constant light source can be obtained from a number of different methods:

i. Laser spot scanning the surface of a mirror slide in the plane of focus of the microscope. Remove filters used to eliminate laser light from striking the detector from the detection pathway. Acquire scans for a range of laser powers.

ii. Transmitted light illuminator turned on during acquisition. The same integration time settings can be used as was applied to the fluorescence scans. Acquire scans with a range of transmitted light intensity levels

C. Analysis Protocol

1. Determine average background intensity of fluorescence images. All fluorescence images must be corrected for background prior to computing brightness values. Contributions to intensity levels due to background include the electronic offset (i.e. bias level) added to the output signal of the detector as well as dark noise. Determine this contribution by measuring the mean intensity in multiple ~10,000 pixel subregions of the acquisitions where there are no cells/fluorophores present and averaging the results from these multiple subregions.

2. Determine slope and intercept for a plot of variance vs intensity for constant light source measurements.

i. Fluorescence intensity traces must be corrected for fluctuations in intensity arising due to the detector[5,7].

ii. Measure both the variance as well as mean intensity from small subregions of the scans taken of the constant light source.

iii. Construct a scatter plot of the variance vs average intensity from all of the subregions measured. The relationship between the detector variance and intensity is linear and therefore the scatter plot can be fit with a straight line of slope S, and intercept, σ^2.

3. Use FIF Spectrometry Suite to demarcate regions of interest (ROI) on fluorescence images of cells separately expressing monomeric and tandem dimer forms of the fluorescent marker

i. Load 2D fluorescence images for a given sample

ii. Select ROIs using a polygon tool. Draw the polygon such that it encompasses the majority of the region inside the cell boundary. Multiple ROIs can be selected for each loaded image.

iii. Segment each ROI into smaller segments. The segmentation procedure is fully automated, the only user input is the maximum size of each segment. We have used a maximum segment size of 500 pixels.

4. Use FIF Spectrometry Suite to calculate the effective brightness for each segment generated in the images of cells expressing monomeric and dimeric fluorescent protein constructs. Make sure to input the values for average background intensity, S, and σ^2. determined in steps C1 and C2 above.

i. Determine the monomeric effective brightness value, ε^{proto}, from the distribution of monomer and dimer brightness values calculated in C4

ii. Simultaneously fit the two spectrograms with Gaussian functions

iii. Set the Gaussian peak position simulating the monomer distribution to be ε^{proto} and the Gaussian peak position simulating the dimer peak position to be $2\varepsilon^{proto}$. Use the same standard deviation for both Gaussian functions, but allow it to vary as a fitting parameter. Use the best fit value of ε^{proto} as the monomeric effective brightness value.

5. Repeat step C3 for images of cells expressing the receptor of interest under appropriate experimental conditions.

i. Use FIF Spectrometry Suite to extract brightness and concentration from each segment generated in C6

ii. Calculate the brightness and concentration of each segment using the mean and standard deviation obtained from the corresponding intensity histograms. The entire procedure of fitting starting at intensity histogram calculation and ending in the calculation of effective brightness and concentration of each of the segments is performed after a click of a single button. Be certain to input the values for ε^{proto}, average background intensity, S, and σ^2 determined in steps C1, C2, and C5 above.

iii. Create a two-dimensional surface plot of the frequency of occurrence of each effective brightness-concentration pair for visual analysis of the effective brightness distribution

iv. Generate brightness spectrograms for various concentration ranges. The brightness spectrogram is a partitioning of the surface plot into one dimensional brightness spectrograms for a chosen concentration range.

6. Use FIF Spectrometry Suite to perform meta-analysis of brightness spectrograms for various concentration ranges and obtain oligomer species fraction plots as a function of protomer concentration

i. Fit the individual brightness spectrogram for a single concentration range with an array of Gaussian functions, $S(\varepsilon_{eff})=\sum A_n exp\hat{}[-(\varepsilon_{eff}-n\varepsilon^{proto})^2/(2\sigma^2)]$ The mean value of each Gaussian function, $n\varepsilon^{proto}$, corresponds to the peak brightness value from a particular oligomer size n, and are all linearly related to a multiple of the monomeric molecular brightness, ε^{proto}

ii. Calculate the area underneath each Gaussian (relative to the area underneath the entire histogram). This area reflects the relative abundance of the particular oligomer size corresponding to that Gaussian.

iii. Repeat fitting of spectrogram for all concentration ranges and obtain relative abundance values for each oligomer size

Troubleshooting

1. Problem: Simultaneous fitting of spectrogram of monomeric and dimeric constructs is poor due to broad distribution and the peak not well defined.

- Allow additional Gaussian functions.

- For the monomer distribution, allow n=1,2,3,4 while for the dimer distribution, remove odd size constructs, i.e. n=2, 4, 6. This addresses issues in step C5 in the procedure section.

2. Problem: The brightness values obtained from samples expressing monomeric fluorescent markers present concentration dependence. This may be a result of poorly estimated values for slope and intercept describing the linear relationship between detector variance and intensity levels.

- Restrict the variance vs. intensity plot to the range of intensities sampled in fluorescence measurements of cells.

- Do not include intensities much larger than sampled in cells, as the detector response can be non-linear at high signal levels, and could result in an inaccurate correction in regions with low intensities. This addresses issues in step C5 and step C2 in the procedure section.

3. Problem: Poor fit to the brightness spectrogram, or overfitting of brightness spectrogram (i.e., non unique set of parameters minimizing fit).

- Perform fitting with a number of different models, each with a different number of Gaussians used to simulate the spectrogram.

- Choose the model which best fits the data with the least amount of input parameters needed. This addresses issues in step C8 in the procedure section.

Time Taken

A. Cell preparation

- Cells will need to be seeded and cultured on coverslip dishes no less than 36 hours before measurement, starting from an already active cell culture.

B. Image Acquisition

- Expect to collect fluorescent images on viable cells at a rate of ~2 cells/minute.

- It is recommended to collect images on at least 300 different cells, therefore for each type of receptor or ligand treatment, imaging should take on the order of 2.5 hours.

- Images must also be collected on cells expressing monomeric and tandem dimer forms of the fluorescent marker which has been fused to the receptor of interest, each taking on the order of 1.5 hours.

- Finally, collecting images from a light source with constant intensity will take ~0.5 hours.

- The time taken to collect a complete set of data would therefore be (3.5+2.5x) hours where x represents the number of different receptors or ligand treatments being studied.

C. Image analysis

- Analysis of the cells expressing either monomeric or tandem dimer forms of the fluorescent marker to obtain values for the monomeric brightness takes 4 hours.

- Demarcating the boundaries of several hundred cells will take ~2 hours.

- Automated image segmentation and brightness and concentration calculation for several hundred cells will take ~30 minutes.

- Therefore image analysis takes (4+2.5x) hours, where x represents the number of different receptors or ligand treatments being studied. However, the time needed for interpretation of the final results depends on the experience of the researcher and the complexity of the experiment.

Anticipated Results

Fluorescence images and ROI files used to generate a number of example FIF spectrograms are posted on *Figshare* digital repository and is accessible from the following link: https://figshare.com/s/77b90d060901fa8b4cb3.

For the expected output from each of the data sets, refer to Figures 2 and 3 of the associated publication (cited below).

Each dataset on the digital repository is named with its corresponding figure number in the manuscript.

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