

# Fluorescence Intensity Fluctuation Analysis of Protein Oligomerization in Cell Membranes

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Fluorescence fluctuation spectroscopy (FFS) encompasses a bevy of techniques that involve analyzing fluorescence intensity fluctuations occurring due to fluorescently labeled molecules diffusing in and out of a microscope's focal region. Statistical analysis of these fluctuations may reveal the oligomerization (i.e., association) state of said molecules. We have recently developed a new FFS-based method, termed Two-Dimensional Fluorescence Intensity Fluctuation (2D FIF) spectrometry, which provides quantitative information on the size and stability of protein oligomers as a function of receptor concentration. This article describes protocols for employing FIF spectrometry to quantify the oligomerization of a membrane protein of interest, with specific instructions regarding cell preparation, image acquisition, and analysis of images given in detail. Application of the FIF Spectrometry Suite, a software package designed for applying FIF analysis on fluorescence images, is emphasized in the protocol. Also discussed in detail is the identification, removal, and/or analysis of inhomogeneous regions of the membrane that appear as bright spots. The 2D FIF approach is particularly suited to assess the effects of agonists and antagonists on the oligomeric size of membrane receptors of interest. © 2022 Wiley Periodicals LLC.

**Basic Protocol 1:** Preparation of live cells expressing protein constructs

**Basic Protocol 2:** Image acquisition and noise correction

**Basic Protocol 3:** Drawing and segmenting regions of interest

**Basic Protocol 4:** Calculating the molecular brightness and concentration of individual image segments

**Basic Protocol 5:** Combining data subsets using a manual procedure (Optional)

**Alternate Protocol 1:** Combining data subsets using the advanced FIF spectrometry suite (Optional; alternative to Basic Protocol 5)

**Basic Protocol 6:** Performing meta-analysis of brightness spectrograms

**Alternate Protocol 2:** Performing meta-analysis of brightness spectrograms (alternative to Basic Protocol 6)

**Basic Protocol 7:** Spot extraction and analysis using a manual procedure or by writing a program (Optional)

**Alternate Protocol 3:** Automated spot extraction and analysis (Optional; alternative to Protocol 7)

**Support Protocol:** Monomeric brightness determination

Keywords: fluorescence-based imaging • fluorescence fluctuation spectroscopy • oligomerization • protein-protein interactions

### How to cite this article:

Killeen, T. D., Rahman, S., Badu, D. N., Biener, G., Stoneman, M. R., & Raicu, V. (2022). Fluorescence intensity fluctuation analysis of protein oligomerization in cell membranes. *Current Protocols*, 2, e384. doi: 10.1002/cpz1.384

## INTRODUCTION

Interactions between membrane receptors play a vital role in many biological processes (Farran, 2017; Palczewski, 2010). Detailed studies of these interactions would have profound implications for understanding signaling mechanisms and their dysfunction in diseases. However, the functional roles of many such complexes have remained elusive, as characterization of the oligomeric state of many membrane receptors has turned out to be a substantial experimental challenge, due in part to the fact that the receptors may exist as mixtures of monomers and variedly sized oligomers whose proportion may change as a function of physiological conditions. Since identifying the size and supramolecular structure of protein oligomers in living cells can yield important insights into the biological consequences of oligomerization, advanced methods for probing the association of cellular receptors in cells are of great importance (George, O'Dowd, & Lee, 2002). Drug discovery efforts would particularly benefit from such information, especially in light of recent studies proving that affinity between a receptor and a ligand is not enough and ligand binding may depend on the oligomeric size of the receptor complex or vice versa, possibly leading to biased signaling (or biased agonism) (Ahmed, Zapata-Mercado, Rahman, & Hristova, 2021; Karl, Paul, Pasquale, & Hristova, 2020; Paprocki, Biener, Stoneman, & Raicu, 2020; Stoneman et al., 2019).

The majority of methods for studying membrane receptor interactions in living cells are fluorescence-based. Typically, the receptors of interest are labeled with one or more fluorescent markers, and cells expressing the labeled receptor are imaged using a specialized fluorescence microscope designed to implement the desired technique. For example, Förster resonance energy transfer (FRET)-based approaches are particularly effective for probing whether two or more macromolecules form molecular associations (or complexes) and determining the spatial distribution of such complexes in living cells. Standard FRET-based approaches for quantifying protein-protein interactions rely on plotting the average FRET efficiency against the concentration of donors and/or acceptors (King, Raicu, & Hristova, 2017; Raicu, 2019; Raicu, Jansma, Miller, & Friesen, 2005) and then fitting a model derived from the kinetic theory of FRET (King et al., 2017; Raicu, 2007, 2019) to the experimental data. Unfortunately, this method only works well if the number of protomers within an oligomeric complex and the geometry of the oligomer are already known (Raicu, 2007). An alternative method—FRET spectrometry (Mishra et al., 2016; Paprocki et al., 2020; Raicu et al., 2009; Stoneman, Raicu, Biener, & Raicu, 2020)—provides information on the oligomer geometry but not on the proportion of oligomers with different sizes.

Methods based on fluorescence fluctuation spectroscopy (FFS; Qian & Elson, 1990), such as Photon-Counting Histogram (PCH) analysis (Chen, Muller, So, & Gratton, 1999; Herrick-Davis, Grinde, Cowan, & Mazurkiewicz, 2013), Spatial Intensity Distribution Analysis (SpIDA; Godin et al., 2011; Moller et al., 2018; Pediani, Ward, Marsango, & Milligan, 2018), and Number and Brightness (N&B) analysis (Digman, Dalal, Horwitz, & Gratton, 2008; Nagy, Claus, Jovin, & Arndt-Jovin, 2010; Unruh & Gratton, 2008) have been proposed for determining the size of protein oligomers. In such approaches, the signal fluctuations caused by the diffusion of fluorescently labeled molecules are analyzed with statistical methods to reveal numerous properties of the molecules. The

core of the FFS-based techniques specializing in quantifying molecular interactions is the relation between the molecular brightness, defined as the average of the fluorescence signal of a single fluorescent molecule over a given time, and the amplitude of the intensity fluctuations in ensembles of associated molecules (i.e., oligomeric complexes), which is extracted from the width of distributions of such intensities. The molecular brightness of a complex of fluorescently labeled proteins scales linearly with the size of the complex; e.g., a dimer has twice the molecular brightness of a monomer. Therefore, the molecular brightness-based FFS techniques have been very effective for determining the size at which a protein complex forms (Chen, Wei, & Muller, 2003; Digman, Wiseman, Choi, Horwitz, & Gratton, 2009; Godin et al., 2015; Pediani et al., 2018). However, previously developed intensity fluctuation-based approaches typically only provide average values of the oligomer size over mixtures of oligomers with different sizes. Therefore, detailed information on the relative abundances of each of the differently sized oligomers is essentially washed out using these approaches.

We have recently developed an FFS-based technique termed Two-Dimensional Fluorescence Intensity Fluctuation (2D FIF) Spectrometry (Stoneman et al., 2019), which provides quantitative information on the size and stability of variously sized protein oligomers as a function of receptor concentration. The 2D-FIF spectrometry method relies on the analysis of spatial-intensity fluctuations obtained from fluorescence images of cells expressing the receptor of choice fused to a fluorescent marker protein. Regions in the fluorescence images where cells are present are divided into smaller sub-regions, which are referred to as segments. The intensity fluctuations across the pixels in a given segment are used to calculate the molecular brightness of each segment. The molecular brightness values from thousands of segments are divided, based upon receptor concentration, into various brightness histograms, otherwise known as spectrograms. The brightness distributions can be decomposed to determine the relative abundance of monomers and variously sized protein oligomers. Analysis of these brightness distributions, as opposed to averaging brightness values over large regions, is advantageous because the spectrograms preserve information regarding fluctuations in oligomer size occurring across the membrane. Also critical to 2D FIF is the extraction of individual protomer concentrations within an ensemble of oligomers, enabling one to sort the molecular brightness spectrograms as a function of protomer concentration. From this concentration sorting, the monomeric and oligomeric relative abundances can be plotted as a function of the total concentration of molecules. The method is simple and robust, and may be used in conjunction with both confocal microscopes and two-photon excitation microscopes, which may or may not need spectral resolution depending on whether or not additional fluorescent markers are present in the cell.

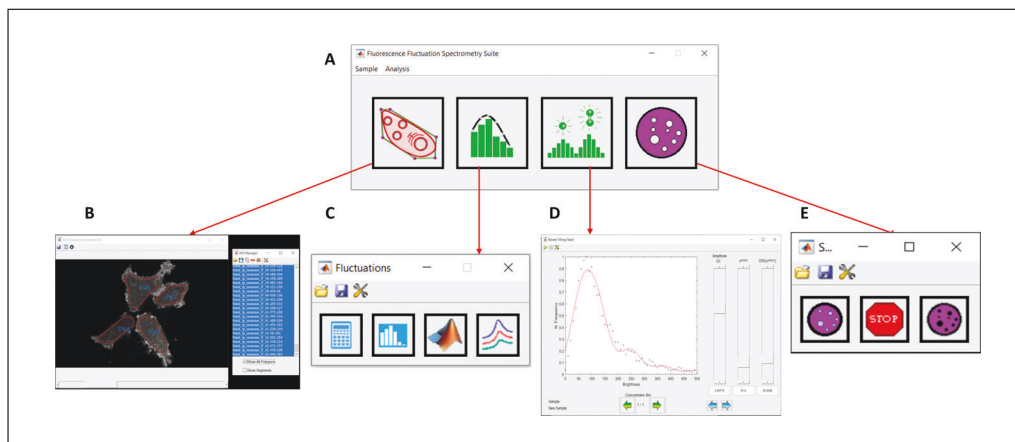
A dedicated software package, the *FIF Spectrometry Suite*, has been developed for the purpose of applying the entire 2D-FIF analysis procedure to fluorescence images of cells expressing fluorescently labeled proteins. The software consists of four modules, each responsible for implementing a different part of the FIF analysis. The four separate modules are: (i) ROI Manager, (ii) Fluctuation Data Assembly, (iii) Model Fitting, and (iv) Spot Identification and Analysis. A free version of the FIF Spectrometry Suite is available for download from FIF Spectrometry Software (<https://figshare.com>) or <https://sites.uwm.edu/raicu-research-group/software/>. Additional useful features are available in an advanced version of the suite. The advanced version is available with full functionality for a limited time, after which it requires a license that can be purchased from the UWM Research Foundation. The limited time trial of the advanced version of the software suite can be obtained by emailing [vraicu@uwm.edu](mailto:vraicu@uwm.edu). A set of tutorial videos that give step-by-step instructions for using each of the four software modules can be seen at the FIF Spectrometry Suite YouTube channel ([https://www.youtube.com/watch?v=M\\_S1rPd8jjk&list=PLVxMLgWQeTpNeO\\_gRoZanZHGb-uJKeevG](https://www.youtube.com/watch?v=M_S1rPd8jjk&list=PLVxMLgWQeTpNeO_gRoZanZHGb-uJKeevG)).

This article provides detailed instructions for determining oligomeric sizes of membrane receptors and their relative abundance in cells using 2D-FIF spectrometry. Basic Protocols 1 and 2 detail the sample preparation and image acquisition steps of this technique. Basic Protocols 3-7 describe steps for the subsequent analysis of the collected fluorescence images using the freely available version of the FIF Spectrometry Suite, sometimes supplemented with certain manual procedures or short codes for computer-based analysis. This article also contains three Alternate Protocols 1, 2, and 3 (alternative to Basic Protocols 5, 6, and 7, respectively) that detail the analogous steps for fluorescence image analysis when using the advanced version of the FIF Spectrometry Suite. Therefore, if implementing FIF using the advanced version of the FIF Spectrometry Suite, please follow Alternate Protocols 1, 2, and 3 instead of the corresponding Basic Protocols 5, 6, and 7, respectively.

In Basic Protocol 1, we describe how to culture cells for imaging, specifically cell lines expressing fluorescent protein–labeled transmembrane receptors and cell lines expressing monomeric forms of the fluorescent protein. Stable cell lines may be replaced by transient transfection if the user desires. In Basic Protocol 2, we present information about the equipment needed to collect fluorescence images to be used in the FIF procedure, and outline the critical instrumental parameters needed to collect the images. The 2D-FIF approach can be implemented using standard laser-scanning fluorescence microscopes, including confocal, two-photon excitation, and total internal reflection microscopes (TIRF). The protocol also gives detailed instructions on correcting background and camera noise. Basic Protocol 3 presents the instructions for drawing Regions of Interest (ROIs) in each cell and using an automated segmentation feature in the FIF Spectrometry Suite to generate small ROI segments so that thousands of brightness and concentration data points are obtained from hundreds of imaged cells. Basic Protocol 4 provides detailed instructions for analyzing the intensity fluctuations occurring for individual segments, i.e., extracting the molecular brightness and protomer concentration from each and creating multiple types of plots (volcano graphs and brightness spectrograms) for visualization of the relationship between molecular brightness and concentration. Basic Protocol 5 provides users with instructions on combining data from multiple days of experimentation. Alternate Protocol 1 includes directions on how to use an advanced feature of the FIF Suite to combine multiple subsets of data after the segmentation step has been performed. In Basic Protocol 6, we detail how to fit the brightness spectrograms with a model consisting of multiple Gaussian curves, extract species fraction information from the fitting, and save the fitting results. Alternate Protocol 2 includes analogous steps to Basic Protocol 6, with an additional advanced feature for creating a species fraction plot. In Basic Protocol 7, we present a method to identify high-intensity spots, i.e., small groups of pixels whose average intensity is significantly higher than the surrounding region, within fluorescence images. Instructions are also given for either removing the bright spots from the fluorescence images once they are identified or grouping the spots into clusters and performing the analysis detailed in Basic Protocol 6 and Alternate Protocol 2 on the clusters. Alternate Protocol 3 includes directions on applying the algorithm described in Basic Protocol 7 using the advanced version of the FIF Suite, which has the spot identification/analysis algorithms built into the program. The Support Protocol details the analysis of fluorescence images of monomeric constructs to extract the effective monomeric brightness value used in fitting spectrograms of the receptor of interest.

## STRATEGIC PLANNING

The protocols below list detailed steps for implementing 2D FIF spectrometry. The FIF Spectrometry suite can be used to expedite the analysis process. Several additional useful features are available in an advanced version of the suite. The software layout is illustrated in Figure 1, which will be referenced in a number of the protocols below.



**Figure 1** Four modules of the FIF Spectrometry Suite, a software package for applying 2D FIF to fluorescence images. **(A)** The Main Window of the FIF Spectrometry Suite, which contains icons responsible for launching one of the four modules of the suite. Each module is responsible for implementing a different part of the FIF spectrometry analysis. The four modules are entitled: **(B)** ROI Manager, **(C)** Fluctuation Data Assembly, **(D)** Model Fitting, and **(E)** Spot Identification and Analysis. Image is generated using Advanced FIF Spectrometry Suite.

## PREPARATION OF LIVE CELLS EXPRESSING PROTEIN CONSTRUCTS

Basic Protocol 1 provides the detailed steps for culturing mammalian cells expressing either fluorescently labeled proteins of interest or monomeric/dimeric calibration standards for cell imaging. All procedures should be performed in a sterile environment using standard procedures. Whenever possible, we recommend generating stable cell lines for this purpose. However, transiently expressing cells have also been used effectively in the FIF method and are a viable option when stable cell lines are not feasible. The presented workflow describes the preparation of samples that are fixed using paraformaldehyde (Stoneman et al., 2019), but the method can also be used to probe protein oligomerization in living cells. While this protocol focuses on how to culture CHO cells stably expressing a secretin receptor attached to a monomeric enhanced green fluorescent protein (secR-mEGFP), any other cell line may be used in which the fluorescently labeled receptor can be expressed. The final product of this protocol is a coverslip-bottom dish that contains a single layer of adherent cells between 40% and 70% confluent.

### Materials

- Flp-In T-REx 293 cells (Invitrogen™, cat. no. R78007)
- Dulbecco's Modified Eagle Medium (DMEM, Gibco™, cat. no. 11965092)
- Ham's F-12 Nutrient Mix (Gibco™, cat. no. 11765054)
- Fetal bovine serum (Atlanta Biologicals, cat. no. S11550)
- Penicillin/Streptomycin (pen/strep; Gibco™, cat. no. 15140122)
- Zeocin (Invitrogen™, cat. no. 46-0509)
- Blastidicin (Invivogen, cat. no. ant-bl)
- Hygromycin (Invivogen, cat. no. ant-hg)
- Trypsin-EDTA (0.25%), phenol red (Gibco™, cat. no. 25200056)
- Poly-d-lysine (Sigma-Aldrich, cat. no. P7280-5 mg)
- Plasmids:
  - PM-1-mEGFP (Ward, Pediani, Harikumar, Miller, & Milligan, 2017)
  - PM-2-mEGFP (Ward, Pediani, Harikumar, Miller, & Milligan, 2017)
  - Wild-type hSecR-mEGFP (Ward, Pediani, Harikumar, Miller, & Milligan, 2017)
- DNA Transfection Kit (Lipofectamine 3000, Invitrogen™)
- Qiagen Plasmid Kit

## BASIC PROTOCOL 1

Dulbecco's phosphate-buffered saline with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (DPBS, Gibco™, cat. no. 14040-133)

4% formaldehyde solution (Sigma-Aldrich, cat. no. 158127-5G)

Biological Safety Cabinet (Thermo Scientific, cat. no. 1300 Series A2.)

Humidified incubator with 95% air and 5%  $\text{CO}_2$  kept at a temperature of  $37^\circ\text{C}$

25- $\text{cm}^2$  (T-25) cell culture flasks (Thermo Scientific, cat. no. 156340)

0.1-mm deep hemacytometer (Hausser Scientific)

35-mm glass-bottom dish with 14 mm bottom well and #1 or #1.5 cover glass (Cellvis, cat.no. D35-14-1.5-N)

Micropipettor (2-20  $\mu\text{l}$  range, 20-200  $\mu\text{l}$  range, and 200-1000  $\mu\text{l}$  range)

Micropipette tips

Plastic serological pipettes (10 and 5 ml)

15-ml and 50-ml conical centrifuge tubes

Additional reagents and equipment for basic cell culture techniques, including trypsinization (see Current Protocols article: Phelan & May, 2015)

### **Cell culture**

1. Generate Flp-In T-REx 293 cells stably expressing monomeric (e.g., PM1-mEGFP) or dimeric forms (e.g., PM2-mEGFP) of the fluorescent protein (e.g., mEGFP) (Ward, Pediani, Harikumar, Miller, & Milligan, 2017). Similarly, generate cells expressing membrane receptors of interest, e.g., secretin receptor attached to a fluorescent protein (secR-mEGFP).
2. Grow cells in a T-25 flask in DMEM supplemented with 10% FBS and other ingredients as described below at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .

*Prepare cell culture medium for Flp-In T-REx 293 cells using DMEM (high glucose) supplemented with 10% (v/v) fetal bovine serum, 100  $\mu\text{g}/\text{ml}$  penicillin, and 0.1 mg/ml streptomycin. The cell culture medium for the CHO cells should be prepared using Ham's F-12 Nutrient Mix supplemented with 10% (v/v) fetal bovine serum, 100  $\mu\text{g}/\text{ml}$  penicillin, and 0.1 mg/ml streptomycin. For stable cell lines, the appropriate selection agents must also be added to the medium. For example, cell culture medium for CHO cells expressing secretin receptor-mEGFP required 500 mg/ml zeocin. Cell culture medium for Flp-In™ T-REx™ 293 cells required 10 mg/ml blasticidin and 200 mg/ml hygromycin.*

*Maintain growth in log phase by sub-culturing the cells every 2 to 3 days.*

*Use 5 ml of cell culture medium per flask.*

*Subculture cells if they are more than 85% confluent on the flask.*

*If cells are being revived from deep freeze, allow for at least two cell passages before use in transfection or imaging.*

3. Lift cells from T-25 flask using 0.5 ml trypsin (see Phelan & May, 2015) and plate on a poly-d-lysine-coated glass-bottom dish.

*Seed between 500 and 1000 cells/ $\text{mm}^2$  on coverslip growth area of each poly-d-lysine-coated glass-bottom dish. The number of cells seeded will ultimately depend on the doubling time of the cell line being used. Allow the cells to grow for 36-48 hr in a humidified incubator in order for cells to spread out on the glass surface.*

*If glass-bottom dishes are not already coated with poly-d-lysine, coat them with poly-d-lysine according to the instructions from the manufacturer. Dishes should be coated a minimum of 2 hr prior to the addition of cells to the coverslip surface.*

4. *Optional:* Transfect cells (e.g., HEK-293 or CHO) with plasmid construct using Lipofectamine 3000 Kit according to manufacturer's protocol.

*Transient transfection should only be done if cell line is not stably expressing the protein of interest.*

*Cells should be transfected after 24-48 hr of seeding on glass-bottom dish, such that they are firmly attached and spread out over the surface.*

*Cells should not be more than 70% confluent on the day of transfection.*

*After 12-16 hr, remove transfection medium and replace with growth medium. At this time, cells can be taken for imaging (see steps 5-12 below). Longer transfection times should be avoided in order for the cells to remain sparse on the coverslip surface.*

### **Cell preparation for imaging**

5. Aspirate growth medium from glass-bottom dish.
6. Wash cells twice with 2 ml of DPBS.
7. If exposing cells to ligand, add the desired amount of ligand solution to cells for desired time.

*For initial measurements on a receptor/ligand pair, we recommend adding ligand at a concentration at least 100 times the value of the dissociation constant of the pair.*

*If a no-ligand sample is being compared to ligand-treated samples, simply add DPBS to cells for the same amount of time as the ligand-treated cells are exposed to ligand.*

8. Remove ligand solution or DPBS solution and add 1.5 ml of 4% formaldehyde fixing solution. Leave cells in fixing solution for 20 min at room temperature; leave room lights off during this time.
9. Remove fixing solution by aspirating.
10. Add 2 ml of DPBS to dish; cells are now ready for imaging.

*DPBS is used for imaging to avoid autofluorescence from the phenol red and serum in cell culture medium.*

## **IMAGE ACQUISITION AND NOISE CORRECTION**

Basic Protocol 2 provides details regarding fluorescence data acquisition from cells expressing a particular receptor, key acquisition parameters of the imaging system, and detector calibration measurements needed to separate fluorescence intensity fluctuations due to the molecules of interest from detector noise. The fluorescence images used in the FIF protocol may be acquired using any fluorescence microscope capable of image sectioning, including single-photon excitation in combination with a confocal pinhole, two-photon excitation, or TIRF. The single and two-photon excitation systems we have primarily used to carry out the method are described below.

As we want to capture the intensity fluctuations due to molecules diffusing in and out of the laser excitation spot (or voxel), it is crucial to set an exposure time for the detector significantly shorter than the average time a receptor molecule will reside within the measurement volume before diffusing out, i.e., the characteristic diffusion time.

Finally, to accurately extract the molecular brightness from a measured intensity histogram, the distribution of intensities must be corrected for the variance in the signal due to the detector. To this end, one must first determine the noise characteristics of the detector from measurements of a light source with uniform and constant intensity (i.e., no temporal or spatial fluctuations), such that any fluctuations in detected signal are attributed to the detector readout. The relationship between the detector variance and intensity is linear and therefore can be fit with a straight line of slope  $S$  and intercept  $\sigma_o^2$ .

## Materials

Cells expressing labeled proteins of interest attached to coverslip (see Basic Protocol 1)

Immersol™ immersion oil (Carl Zeiss™, cat. no. 444960-0000-000)

100 nm Tetraspeck fluorescent microspheres (Invitrogen, cat. no. T14792)

170-nm PS-Speck Microscope Point Source Kit fluorescent microspheres (Invitrogen, cat. no. P7220)

Laser-scanning fluorescence microscope capable of image sectioning, e.g.:

Single excitation system: 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)

Two-photon excitation system: 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

Image-acquisition software (Image J)

Microsoft Excel

## Image acquisition procedure

1. Turn on the excitation laser at least 1 hr before imaging to allow for warm-up.
2. Obtain fluorescence images of sub-diffraction sized fluorescent microspheres for determining the laser beam waist size.

*Fluorescence images should be obtained for at least 15 different fluorescent microspheres. A single field of view can contain multiple microspheres.*

*See the section Determine Laser Beam Waist Size (steps 33-40) below for detailed steps describing the extraction of the laser beam waist ( $\omega_0$ ) of the fluorescence microscope.*

3. 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,  $\tau_D$ , of the receptor under study.

*The characteristic diffusion time is related to the laser beam waist,  $\omega_0$ , and the diffusion coefficient,  $D$ , through the relation  $\tau_D = \omega_0^2/4D$ . The laser beam waist is defined as the distance from the center of the focused beam to the location in the focused beam where the intensity drops to  $e^{-2}$  of its maximum value.*

4. Turn off overhead lights and reduce ambient light in the imaging facility as much as possible.

*This may include turning off the computer monitor while a fluorescence scan of the cells occurs.*

5. Mount the dish containing cells expressing the labeled receptor of interest.

*Place a light-tight chamber on the microscope stage over the sample dish to prevent remaining ambient light from passing through the objective and reaching the detector.*

6. Obtain fluorescence images from several hundred cells expressing the receptor of interest.

*Search for cells using a reduced power or much shorter exposure time (preview scan) compared to the settings used for the actual data collection (fluorescence scan), to avoid exposing the cells to too much laser light, which causes photobleaching.*

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

*Take a fluorescence scan of the focused cells.*

7. *Optional:* If comparing the oligomerization properties of the receptor of interest in the presence and absence of a particular ligand, obtain fluorescence images from several hundred cells expressing the receptor of interest that have been exposed to ligand.

*If cells are not fixed, most likely multiple dishes will be needed that have been exposed to the ligand, as any effect on the oligomerization properties of the receptors due to ligand will be time-sensitive, and imaging time on a given dish will have to remain short.*

8. Obtain fluorescence images from several hundred cells separately expressing monomeric forms of the fluorescent marker fused to the plasma membrane. Apply the same imaging parameters used to collect images of cells expressing the fluorescently labeled receptor of interest.

*The monomeric plasma membrane-bound fluorescent markers (or other monomeric standards) are necessary to determine the monomeric brightness, a key component in the analysis process. Instructions for analyzing images expressing the monomeric constructs to determine the monomeric brightness are given in the Support Protocol.*

9. *Optional:* Obtain fluorescence images from cells separately expressing dimeric forms of the fluorescent marker fused to the plasma membrane. Apply the same imaging parameters used to collect images of cells expressing the fluorescently labeled receptor of interest.

10. Obtain measurements from a light source with uniform and constant intensity (i.e., no spatial or temporal fluctuations). Signal from a constant light source can be obtained from a number of different methods:

*Focusing the laser spot on a mirror slide and scanning a portion of the mirror's surface.* Remove the filters that are used to prevent laser light from striking the detector from the detection pathway. Acquire scans for a range of laser powers.

*Using a transmitted light illuminator during acquisition.* Use the same integration time settings as were applied to the fluorescence scans. Acquire scans with a range of transmitted light intensity levels.

### ***Background and camera noise correction***

In order to properly calculate the molecular brightness values from each ROI segment, two critical parameters indicative of the noise of the detector must be determined from calibration measurements: (i) the average background intensity of the fluorescence images and (ii) the variance arising due to the detector. All fluorescence images must be corrected for background noise 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. The variance arising due to the detector must be corrected for in order to ensure that the fluctuations in intensity are solely due to that of molecules diffusing in and out of the beam.

### ***Determine average background intensity of fluorescence images***

11. Open the fluorescence images of cells using the open-source software package Image J.
12. Choose 'Set Measurements' from the 'Analyze' toolbar and check the 'Mean Gray Value', 'Standard Deviation', and 'Area' boxes.
13. Use the polygon tool to demarcate a region in one of the fluorescence images where no cells/fluorophores are present.
14. Press 'Analyze' and choose 'Measure'.

*The mean gray value, standard deviation, and area (in number of pixels) of the polygon are printed to the Image J results array.*

*The polygon area should be a minimum of 500 pixels.*

*The quick key for 'Measure' (in the 'Analyze' tab) is Ctrl+M.*

15. Repeat 13-14 for multiple regions in the fluorescence images where no cells/fluorophores are present.

*Typically ~50 regions are drawn.*

16. Copy the values of the mean and standard deviation from the results array to an Excel spreadsheet.

17. Compute the average mean value from all regions.

*The value computed should be input in the average background intensity field of the Fluctuation Data Assembly Module Settings Window.*

18. Compute the average standard deviation value from all regions and square the value to obtain the average variance from all regions.

*The average variance value should be input in the intercept field of the Fluctuation Data Assembly Module Settings Window.*

***Determine slope and intercept,  $S$  and  $\sigma_o^2$ , respectively, for a plot of variance vs intensity obtained from constant light source measurements***

19. Obtain images of a constant-intensity light source using the same imaging parameters used for obtaining fluorescence images of cells.

*The constant-illumination measurements can be obtained by laser-spot scanning the surface of a mirror slide in the plane of focus of the microscope, or by turning on a transmitted light illuminator during acquisition.*

*Images should be collected for a range of intensities (i.e., various illumination powers) starting from images taken with the constant light source turned off and increasing in increments until the amount of signal measured is approximately equal to the maximum signal level detected in ROIs drawn on cells expressing the receptor of interest.*

20. Open the fluorescence images obtained of the constant intensity light source using the open-source software package Image J.

*Open the entire range of intensity measurements as a single stack of images.*

*Choose 'File', 'Import', and Image Sequence to import images as a stack.*

21. Open the ROI manager tool by selecting 'Analyze', 'Tools', and 'ROI Manager'.

22. Use the polygon tool to demarcate a region in one of the images.

*The polygon size should be a minimum of 500 pixels.*

*Look for regions in the image where the only fluctuations in intensity are from pixel to pixel. If there are any intensity gradients in the image, avoid these regions.*

23. Press *Add* in the ROI Manager module to save the ROI.

24. Move the polygon drawn in step 22 to a new location, and once again press *Add* in the ROI Manager module.

*Repeat this step until you have ~30 ROIs saved in the ROI Manager module.*

25. Choose 'Analyze' and 'Set Measurements' options from the 'Analyze' toolbar and check the boxes labeled 'Mean Gray Value' and 'Standard Deviation'.

26. Select the first ROI in the ROI Manager module and press 'MultiMeasure'.

*Image J outputs a table where two of the columns in the table are the mean and standard deviation of the pixels within the selected polygon region. The rows of the table represent the mean and standard deviation within the ROI from different slices in the stack of images.*

27. Copy the mean and standard deviation values from each slice to an Excel spreadsheet in the same two-column form.
28. Repeat 27-28 for each ROI saved in the ROI Manager module.
29. Subtract the *average background intensity* value, determined using the **Determine average background intensity of fluorescence images** steps given above, from each of the values in the mean column of the Excel spreadsheet.
30. Create an additional column tabulating the variance of each ROI by squaring each of the values in the standard deviation column.
31. Construct a scatter plot of the variance vs. background-subtracted average intensity from all the regions measured.
32. Insert a linear trendline on the plot and display the equation on the plot.

*Set the intercept of the linear trendline to be the average background variance obtained from regions in fluorescence images where no cells/fluorophores are present (see step 18).*

*The slope,  $S$ , of the linear trendline equation should be input in the slope field of the Fluctuation Data Assembly Module Settings Window.*

#### **Determine laser beam waist size**

33. Open a fluorescence image, using the open-source software package Image J, obtained from a scan of the fluorescent microspheres.
34. Draw a rectangular ROI (45 pixels wide by 3 pixels high) in the image using the rectangular selection tool.

*Center the rectangular ROI on one of the fluorescent microspheres.*

35. Obtain a plot of average intensity vs. column number within the ROI by pressing 'Analyze' from the menu bar and choosing 'Plot Profile'.

*The quick key for 'Plot Profile' is Ctrl+K.*

*A new figure window will pop up, displaying a plot of the average intensity over the three rows in a given column vs. column number within the ROI.*

36. Copy the plot profile intensity values by pressing 'More > Copy Data' in the newly created 'Plot Profile' window.
37. Paste the intensity values into a single column in an Excel spreadsheet.
38. Insert the corresponding laser beam position (relative to the center of the ROI) in the cell to the left of each intensity value.

*Each row in the spreadsheet corresponds to a different pixel location along the longer side of the rectangular ROI.*

*The distance increment between each row corresponds to the pixel size (laser movement step size) of the corresponding measurement (on the sample plane).*

39. Fit the intensity ( $I$ ) vs laser beam position ( $x$ ) data using the function  $I(x) = A \exp\left[-\frac{2(x-\bar{x})^2}{\omega_0^2}\right]$ .

*Adjust  $A$ ,  $\bar{x}$ , and  $\omega_0$  to obtain the best fit of  $I(x)$  to the measured intensity profile.*

*The value of  $\omega_o$  is an approximate value of the laser beam waist size determined from a single microsphere.*

40. Repeat steps 33-39 for at least 15 different microspheres. The laser beam waist size to be used in further analysis is the average  $\omega_o$  value obtained over all the microspheres that were measured.

### **BASIC PROTOCOL 3**

#### **DRAWING AND SEGMENTING REGIONS OF INTEREST**

The first step in the analysis process is to demarcate ROIs in the fluorescence images containing pixels to be included in subsequent analysis. The ROI Manager module of the FIF Spectrometry Suite provides a convenient graphical user interface (GUI) for quickly drawing and saving ROIs on fluorescence images of cells. The ROI Manager module also features an automated procedure for dividing the area inside ROIs into smaller sub-regions, or segments. One reason for segmenting the large ROIs into smaller segments is that calculating the brightness and concentration from large pixel areas has the effect of averaging out fluctuations of concentration and oligomer sizes present across the ROI, because only a single brightness value is obtained for each pixel. An additional advantage of analyzing small segments rather than large ROIs is that molecular brightness values calculated for regions containing high-intensity spots skew high. However, when brightness values are calculated from smaller segments, the brightness values from the segments containing these high-intensity spots are skewed so high that they are effectively filtered out from subsequent analysis (see Basic Protocol 6 and Alternate Protocol 2). Below are the instructions on using the FIF Spectrometry Suite to prep the fluorescence images for further analysis. A tutorial video showing step-by-step instructions for using the ROI Manager Module can be seen at the following link: [https://www.youtube.com/watch?v=M\\_S1rPd8jjk&t](https://www.youtube.com/watch?v=M_S1rPd8jjk&t).

1. Open FIF Spectrometry Suite.
2. Load 2D fluorescence images in the ROI Manager Module.
3. Demarcate ROIs on fluorescence images of cells expressing the monomeric fluorescent marker.

*Create ROIs using the polygon tool. Draw the polygon such that it encompasses the majority of the region inside the cell boundary but excludes the higher-intensity periphery of the cell.*

*Multiple ROIs can be drawn for each loaded image.*

4. Segment each ROI into smaller segments.

*The segmentation procedure is fully automated; the only user input is the maximum size of each segment.*

*We advise using a segment area between 200 and 500 pixels<sup>2</sup>.*

5. Save the ROI polygon information and the segment information by pressing the save button located in the ROI Manager Module.
6. Repeat steps 2-5 until ROIs and segments have been generated for all groups of cells imaged in Basic Protocol 2. The following groups of images should now have ROIs:  
Cells expressing the receptor of interest tagged with the fluorescent marker.  
Cells expressing plasma membrane-bound monomeric fluorescent markers.  
*Optional:* Cells expressing plasma membrane-bound dimeric fluorescent markers.

## CALCULATING THE MOLECULAR BRIGHTNESS AND CONCENTRATION OF INDIVIDUAL IMAGE SEGMENTS

The second step in the FIF analysis process is to calculate molecular brightness and concentration values for each segment generated using the ROI Manager module. The Fluctuation Data Assembly Module of the FIF Spectrometry Suite contains all the necessary routines for calculating the segment-level brightness and concentration values. First, a histogram is created from the intensity values of the pixels located within the segment for each segment analyzed. The intensity histograms are fitted by a Gaussian curve to obtain the mean and standard deviation of the histogram. The molecular brightness and concentration of the segment are then calculated from the parameters of the fitted Gaussian. The molecular brightness values determined for segments that fall within a particular concentration range are grouped to create a histogram, otherwise known as a “brightness spectrogram.” Multiple spectrograms can be created from various concentration ranges and plotted as a “wire-stack” graph. A 3-D “volcano graph” can also be created, which plots the frequency of occurrence of  $\epsilon_{eff}$  for each concentration (in protomers  $\times \mu\text{m}^{-2}$ ). A tutorial video showing step-by-step instructions for using the Fluctuation Data Assembly Module can be seen at the following link: <https://www.youtube.com/watch?v=-cN7m00FAFI>.

**NOTE:** The Support Protocol must be completed before proceeding with this protocol.

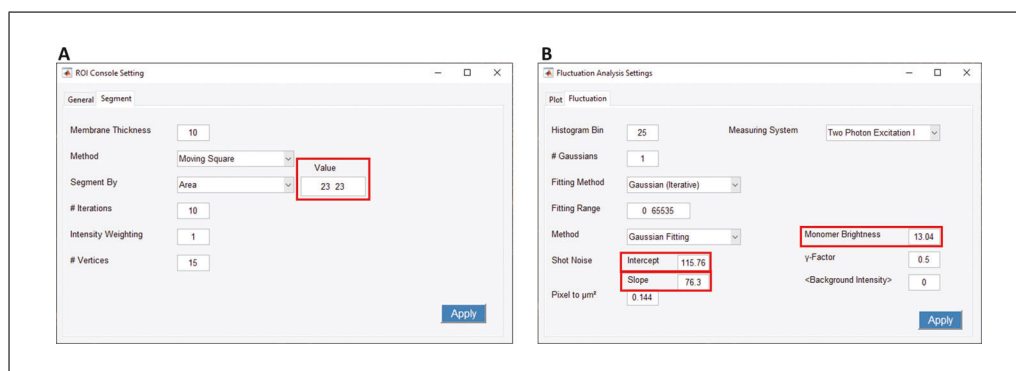
1. Calculate the molecular brightness for each segment generated in the images of cells using the Fluctuation Data Assembly Module.

*In the Fluctuation Data Assembly settings window (see Figure 2B), input the values for average background intensity (<Background Intensity>), Slope (S), and intercept ( $\sigma_0^2$ ). Detailed steps for determining these values are given in Basic Protocol 2. Also input the value for the Monomer Brightness ( $\epsilon^{MONO}$ ), determined in the Support Protocol. Text fields for these parameters are indicated by the red rectangles drawn in Figure 2B.*

2. Generate a 3-D surface plot of the concentration and brightness pairs.

*Adjust the Concentration Bin value for the Concentration axis to be 2 while viewing the Surface Plot.*

*Adjust the Concentration Max value for the Concentration axis until all the data are captured in the Surface Plot. Regenerate the Surface Plot every time the Concentration Max value is changed.*



**Figure 2** FIF Spectrometry Suite Settings windows. **(A)** Settings window of the ROI Manager Module, which allows the user to adjust a number of parameters related to the segmentation of the ROI. A key parameter is the size of the segment regions; the highlighted area indicates where the user can define the length (left number entry) and width (right number entry) of the segment area by number of pixels. **(B)** Settings Window of the Fluctuation Data Assembly Module. The *fluctuation* tab of the Settings Window allows the user to modify how the brightness and concentration for each segment are calculated. The highlighted areas are critical parameters, and their significance are described in Basic Protocol 4.

3. Generate a wire-stack plot consisting of brightness spectrograms for various concentrations.

*Adjust the Concentration Bin value for the Concentration axis such that brightness points are as evenly distributed as possible between each of the individual spectrograms and that none of the spectrograms have a low-frequency count.*

4. *Optional:* Save the processed fluctuation data in .xls or .xlsx format.

### **COMBINING DATA SUBSETS USING A MANUAL PROCEDURE (OPTIONAL)**

Experiments involving the assessment of protein complex size may require multiple days of data acquisition sessions, necessitating the analysis of images across multiple acquisition days. One way to accomplish this is to acquire all fluorescence images prior to starting image analysis, create an image stack with images from all measurement days, and proceed with image analysis using the FIF Spectrometry suite starting with Basic Protocol 3. For this route, the FIF Spectrometry Suite can be used to complete the entire analysis procedure. The user might also choose to conduct the analysis of the fluorescence images throughout the experiment and not wait for multiple days of acquisition to occur before starting the analysis process. Analysis of images along this route can still make use of the ROI Manager and Fluctuation Data Assembly Modules of the FIF Spectrometry Suite. However, once the brightness and concentration values are calculated, the user will need to combine all brightness and concentration pairs from multiple days of acquisition and then proceed with the rest of the FIF analysis procedure outside of the standard FIF Suite. Below are the instructions for performing the FIF image analysis across multiple acquisition days using this second analysis route.

#### ***For each day of acquisition***

1. Follow Basic Protocol 3 for ROI generation and segmentation.
2. Follow Basic Protocol 4 for fluctuation data analysis.

*Be sure to save the processed fluctuation data in .xls or .xlsx format.*

3. Repeat steps 1-2 for each day of measurement.

#### ***Combining data sets***

4. Combine brightness and concentration values from all Excel files created in step 2 into a single file.
5. Create brightness spectrograms using the assembled data from step 4.

*These spectrograms can be created for each concentration range by splitting the list of brightness and concentration values into different concentration range sub-lists.*

*For each sub-list, create a spectrogram. Users familiar with Excel, for example, can use the histogram tool from Excel. However, any program that can create histograms can be used here.*

6. Fit each individual brightness spectrogram with a sum of Gaussian functions, each of which represents an oligomer with a particular size (e.g., monomer, dimer, etc.).

*The mean position of each Gaussian is fixed at a multiple of the monomeric brightness value monomer (calculated using the Support Protocol) where the multiple corresponds to the particular oligomeric size the Gaussian represents, e.g., a multiple of two for the dimer, three for a trimer, four for a tetramer, etc.*

*Each Gaussian has the same preset standard deviation, which is calculated using the Support Protocol.*

*For more details about fitting a spectrogram, see Background Information in the Commentary section below.*

7. Once the spectrograms are fit, the user can extract species fraction information using the area underneath each fitted Gaussian (Stoneman et al., 2019).

### **COMBINING DATA SUBSETS USING THE ADVANCED FIF SPECTROMETRY SUITE (OPTIONAL; ALTERNATIVE TO BASIC PROTOCOL 5)**

**ALTERNATE  
PROTOCOL 1**

Fluorescence images are usually acquired over a span of multiple days in order to confirm that results are repeatable. The analysis of the data typically starts with analyzing the datasets on a day-by-day basis. In other words, a set of images for a particular day are loaded into the FIF software, ROIs drawn, and segments generated. Ultimately, if the data prove to be consistent from day to day, the data from similarly treated samples can be combined over multiple days. This combined analysis of multiple datasets can be performed without redrawing the ROIs and generating the segments using a feature offered in the advanced version of the FIF Spectrometry Suite, as detailed below.

1. Press the sample menu on top of the Fluorescence Fluctuation Spectrometry Suite main window (see Fig. 1A).

*A window will pop up with the title Sample List.*

2. Add a sample by pressing the green plus (+) icon in the Sample List window.

*A sample includes all the images acquired of cells expressing the same type of receptor, prepared under the same protocol, and imaged using the same imaging parameters*

*The sample name can be changed by selecting the sample, for which a table will show The user should write the desired name in the Name field.*

*To add an image set to a particular sample, press the yellow plus (+) icon in the Sample List window after selecting the sample to which the set will be added.*

3. Launch the ROI manager.
4. Press the folder icon in the ROI manager window and load the image set.  
*The image set window will pop up and the image set name will change in the tree presented on the left side of the Sample List window.*
5. Load the ROI and Segment list (if it exists) or draw ROIs as required. The user may also load the segment list file or segment the newly drawn ROIs.
6. Repeat steps 2-5 for each sample.
7. Proceed with rest of FIF analysis using the FIF Spectrometry Suite, starting with Basic Protocol 4.

### **PERFORMING META-ANALYSIS OF BRIGHTNESS SPECTROGRAMS**

**BASIC  
PROTOCOL 6**

The spectrograms that were generated in the Fluctuation Data Assembly Module are potentially composed of contributions from monomers and different-sized oligomers, and may be decomposed to find the fraction of total protomers, or relative abundance for each of the different sized oligomer species relative to the total number of protomers in the sample. This deconvolution is accomplished by fitting each spectrogram with an array of Gaussians, as seen in Equation 6 in the Background Information section below, whose mean values are multiples of the monomeric molecular brightness. Fitting can be accomplished using the Model Fitting Module of the FIF Spectrometry Suite. The relative abundance, or species fraction, for a particular oligomer size is computed as the fraction

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of area that its corresponding Gaussian occupies underneath the composite spectrogram. Species fraction graphs, which are plots of the relative abundance of each oligomer size vs. concentration, are a handy tool when comparing measurements taken before and after adding a ligand, as they clearly show if there is a significant change occurring as a result of ligand addition. A tutorial video showing step-by-step instructions for performing meta-analysis of brightness spectrograms using the FIF Spectrometry Suite can be seen at the following link: <https://www.youtube.com/watch?v=wLHBwZlgbMM&t>.

1. Launch the Model Fitting Module.

*The brightness spectrograms generated in step 3 of Basic Protocol 4 will automatically be loaded when launching the Model Fitting Module.*

*One can switch between spectrograms by pressing the large left and right green arrows at the bottom of the Model Fitting Module Window (see Figure 1D).*

2. Adjust the numerous settings related to the fitting of the spectrograms in the Model Fitting Module Settings Window.

*Change the value of the first Gaussian (i.e., the one with the smallest mean) to that obtained from fitting the spectrogram of the monomeric standard,  $\epsilon^{MONO}$  (See Support Protocol).*

*Change the value of the Gaussian standard deviation to that obtained from fitting the spectrogram of the monomeric standard (see Support Protocol).*

*Uncheck the check buttons labeled “ $\mu$  Adjustable” and “ $\sigma$  Adjustable,” which represent the mean and standard deviation of the Gaussian used to fit the monomer.*

3. Manually fit the brightness spectrograms generated for each of the concentration ranges with a model consisting of an array of Gaussian functions.

*Adjust the amplitude of each of the Gaussians for each of the concentration ranges until a coarse fit is achieved. The adjustments are done by moving the amplitude bar up or down for each Gaussian. The user can switch between the Gaussians by clicking the left or right blue arrows under the adjustment bars.*

*A satisfactory coarse fit is obtained when there are no points along the curve where there is an extreme mismatch (>20% difference) between the sum of Gaussians (solid red line) and the measured data points (blue circles).*

*If the fitted line does not fit the spectrogram for large brightness values, increase the number of Gaussians used to fit the spectrogram in the Model Fitting Module Settings Window.*

4. Refine the coarse fit using the automatic fit refinement tool of the FIF Spectrometry Suite

*The automatic fit refinement is implemented by pressing the green arrow in the icon bar of the Model Fitting Module window (see Figure 1D).*

*One may need to press the auto-fit button multiple times to ultimately arrive at the best fit. Continue to press the auto-fit button until the fit is no longer changing (roughly 5 times).*

5. After fitting each spectrogram, the relative abundance of each oligomeric size over all concentration ranges can be saved.

*The user can save the spectrograms' information in the following formats: .txt, .xls, .xlsx.*

*The saved data includes information on the species fraction of each oligomer (e.g., monomer, dimer, tetramer, etc.) as a function of concentration.*

*Use your favorite plotting software and plot species fraction information against concentration to create a kinetic curve. Kinetic curves are a simple tool used to determine the oligomeric sizes contained in the measured samples.*

## PERFORMING META-ANALYSIS OF BRIGHTNESS SPECTROGRAMS (ALTERNATIVE TO BASIC PROTOCOL 6)

The spectrograms that were generated in the Fluctuation Data Assembly Module are potentially composed of contributions from monomers and different-sized oligomers, and may be decomposed to find the fraction of total protomers, or relative abundance for each of the different-sized oligomer species relative to the total number of protomers in the sample. This deconvolution is accomplished by fitting each spectrogram with an array of Gaussians, as seen in Equation 6 in Background Information, below, whose mean values are multiples of the monomeric molecular brightness. Fitting can be accomplished using the Model Fitting Module of the FIF Spectrometry Suite. The relative abundance, or species fraction, for a particular oligomer size is computed as the fraction of area that its corresponding Gaussian occupies underneath the composite spectrogram. Species fraction graphs, which are plots of the relative abundance of each oligomer size vs. concentration, are a handy tool when comparing measurements taken before and after adding a ligand, as they clearly show if there is a significant change occurring as a result of ligand addition. A tutorial video showing step-by-step instructions for performing meta-analysis of brightness spectrograms using the FIF Spectrometry Suite can be seen at the following link: <https://www.youtube.com/watch?v=wLHBwZlgbMM&t>.

### 1. Launch the Model Fitting Module.

*The brightness spectrograms generated in step 3 of Basic Protocol 4 will automatically be loaded when launching the Model Fitting Module.*

*One can switch between spectrograms by pressing the large left and right green arrows in the bottom of the Model Fitting Module Window (see Figure 1D).*

### 2. Adjust the numerous settings related to the fitting of the spectrograms in the Model Fitting Module Settings Window.

*Change the value of the first Gaussian (i.e., the one with the smallest mean) to that obtained from fitting the spectrogram of the monomeric standard,  $\epsilon^{MONO}$  (see Support Protocol).*

*Change the value of the Gaussian standard deviation to that obtained from fitting the spectrogram of the monomeric standard (see Support Protocol).*

*Uncheck the check buttons labeled “ $\mu$  Adjustable” and “ $\sigma$  Adjustable,” which represent the mean and standard deviation of the Gaussian used to fit the monomer.*

### 3. Manually fit the brightness spectrograms generated for each of the concentration ranges with a model consisting of an array of Gaussian functions.

*Adjust the amplitude of each of the Gaussians for each of the concentration ranges until a coarse fit is achieved. The adjustments are done by moving the amplitude bar up or down for each Gaussian. The user can switch between the Gaussians by clicking the left or right blue arrows under the adjustment bars.*

*A satisfactory coarse fit is obtained when there are no points along the curve where there is an extreme mismatch (>20% difference) between the sum of Gaussians (solid red line) and the measured data points (blue circles).*

*If the fitted line does not fit the spectrogram for large brightness values, increase the number of Gaussians used to fit the spectrogram in the Model Fitting Module Settings Window.*

### 4. Refine the coarse fit using the automatic fit refinement tool of the FIF Spectrometry Suite.

*The automatic fit refinement is implemented by pressing the green arrow in the icon bar of the Model Fitting Module Window (see Figure 1D).*

*One may need to press the auto-fit button multiple times to ultimately arrive at the best fit. Continue to press the auto-fit button until the fit is no longer changing (roughly 5 times).*

5. After fitting each spectrogram, the relative abundance of each oligomeric size over all concentration ranges can be saved.

*The user can save the spectrograms' information in the following formats: .txt, .xls, .xlsx.*

*The saved data includes information on the species fraction of each oligomer (e.g., monomer, dimer, tetramer, etc.) as a function of concentration.*

6. Create a kinetic curve using the plot button in the advanced version of FIF Spectrometry Suite.

*The user may wish to adjust concentration bin sizes to increase or decrease the number of points on the x axis. To do this, the user must return to Basic Protocol 4 and create new wire-stack graphs with a different concentration bin size, then proceed to fit the resulting spectrograms using Alternate Protocol 2.*

## **BASIC PROTOCOL 7**

### **SPOT EXTRACTION AND ANALYSIS USING A MANUAL PROCEDURE OR BY WRITING A PROGRAM (OPTIONAL)**

In some instances, fluorescence images may contain an abundance of “spots,” i.e., small groups of pixels whose average intensity is significantly higher than the surrounding region. It may be desirable to identify and separate the high-intensity spots, either for further analysis or to completely remove them from images before calculating segment level brightness values. The user can use the ROI manager module of the FIF Spectrometry Suite to carefully draw polygons while avoiding spots in all images; however, there are generally many spots per cell, so this method is quite tedious and generally results in loss of data. Likewise, if users are interested in analyzing the spots in their images, it is possible to draw ROIs that only include the bright spots; however, due to the high number of polygons and significant time investment needed for this method, it is inadvisable to do so. A computer algorithm that identifies and either removes or collects spots for further analysis is recommended. In this protocol, we provide general instruction on how to program a spot identification tool to identify and remove spots if necessary. The algorithm for identifying and removing spots includes two parts. The first part is the segmentation of the region of interest using simple linear iterative clustering (SLIC), and the second part is the thresholding portion where segments are identified as spots if they pass a certain threshold. This protocol also gives detailed steps on how to group the spots into clusters such that the same meta-analysis performed in Basic Protocol 6 can be performed on the clusters. The pseudo-code for developing a spot identification program is found in the supplementary materials of Biener, Stoneman, & Raicu (2021).

#### ***Description of SLIC-based segmentation of the ROI***

1. Split the ROI into identical square segments of length  $d$ .

*We tend to choose the size of the segment to be as close as possible to the average size of the spots.*

2. Define a matrix that stores the distance (see Equation S27 from Stoneman et al., 2019) of each pixel from the closest segment center.

*We identify this matrix as the distance matrix.*

*The distance matrix has the same dimensions as the image.*

*The weighting factor  $m$  defines the weight a pixel's intensity has on the calculated distance.  $M$  can be constant or dependent on the segment's intensity distribution as well as*

on the positions of the pixels compared to the center of the tested segment (Biener et al., 2021).

3. Define a second matrix which stores the segment number that each pixel belongs to.

*The pixel belongs to the segment with the center that is closest to the pixel.*

4. Move a square a segment length  $d$  from left to right and from top to bottom where the square size is  $x$  times larger than the segment size  $d$ .

*$x$  needs to be larger than 1 but smaller than 3.*

5. Calculate the distances of all the pixels from the center of the square defined in step 4, and compare each pixel's distance to the corresponding pixel value in the *distance matrix*.

*If the distance is smaller, update the distance in the distance matrix for the corresponding pixel and update the label matrix by changing the pixel's value to the segment number whose center is currently overlapping with the center of the square.*

6. Calculate the center of mass of all the new segments by using the updated segment numbers in the *label matrix*.

*For each pixel having the same segment number in the label matrix, record the  $x$  and  $y$  coordinates.*

*Average all the  $x$  coordinate values of the selected pixels and perform the same averaging for the  $y$  coordinate.*

*The resulting  $\langle x \rangle$  and  $\langle y \rangle$  are the center of mass coordinates of the specific segment.*

*Repeat the center of mass calculation for all segments.*

7. Repeat steps 4 and 5 using the centers computed in step 6, but set the *distance matrix* and *label matrix* to their initial values, i.e., infinity for each pixel in the *distance matrix* and 0 for each pixel of the *label matrix*.

8. Terminate the process if the number of iterations reaches a chosen maximum value, or the centers of the segments do not move any more.

### ***Identifying spots via brightness thresholding***

The SLIC-based algorithm outlined in steps 1-8 will result in segmented ROIs. Next, the algorithm must decide which of the newly generated segments are defined as “spots” and which are defined as “flat membrane,” a procedure we call thresholding. In order to identify the segments containing spots, the segments' average intensity is either compared to the area surrounding the segment or to the entire ROI where the segment resides. The method using the area around the segment (dubbed Local Thresholding) is outlined in Option 1 below, while the method using the entire ROI (dubbed Global Thresholding) is described in Option 2 below. Global Thresholding tends to remove all spot-related pixels, including some low-intensity pixels adjacent to the spot, while Local Thresholding tends to only remove pixels on the interior of the spots. To analyze the spots themselves, Local Thresholding is recommended to avoid incorporating low-intensity pixels from adjacent membrane areas into the spot intensity analysis. By contrast, Global Thresholding is recommended when analyzing the basolateral membrane after spot removal, to avoid including any remaining high-intensity pixels in the membrane intensity distribution.

#### ***Local thresholding (Option 1)***

9. Average the segments' intensity values and replace each pixel within a segment with the segment's average intensity value.

10. Extract an area of a certain thickness surrounding the segment and calculate the average and standard deviation of the intensities from the surrounding area's pixels.
11. Identify the segment as a spot if its average intensity is equal to or higher than the average intensity found in step 10.
12. Create a table of spot intensities ordered from lowest average intensity to highest average intensity.
13. *Optional:* Remove the spots from the image by simply putting zero values in place of the segments identified as spots.
14. *Optional:* Analyze the spots separately.

*Once spot identification has been performed, the spots are grouped together into clusters of five.*

*A cluster of spots is created by consolidating the intensity values from the pixels of five consecutive individual spots in the table created in step 12.*

*Analyze the clusters using Basic Protocols 4-6 (or corresponding Alternate Protocols).*

#### **Global thresholding (Option 2)**

15. Calculate the average intensity at each segment resulting from the SLIC algorithm.
16. Generate a histogram of average intensities.
17. Fit a Gaussian to the histogram and find mean and standard deviation of the fitted Gaussian.
18. Calculate the threshold intensity by summing the mean and the standard deviation multiplied by a constant threshold value (TH).
19. For each segment, check whether its average intensity is higher than or lower than the intensity threshold calculated in step 18. If the intensity is higher, the segment is considered to be a spot.
20. Create a table of spot intensities ordered from lowest average intensity to highest average intensity.
21. *Optional:* Remove the spots from the image by simply putting zero values in place of the segments identified as spots.
22. *Optional:* Analyze the spots separately.

*Once spot identification has been performed, the spots are grouped together into clusters of five.*

*A cluster of spots is created by consolidating the intensity values from the pixels of five consecutive individual spots in the table created in step 12.*

*Analyze the clusters using Basic Protocols 4-6 or corresponding Alternate Protocols.*

#### **ALTERNATE PROTOCOL 3**

#### **AUTOMATED SPOT EXTRACTION AND ANALYSIS (OPTIONAL; ALTERNATIVE TO BASIC PROTOCOL 7)**

In some instances, fluorescence images may contain an abundance of "spots," i.e., small groups of pixels whose average intensity is significantly higher than the surrounding region. It may be desirable to identify and separate the high-intensity spots, either for further analysis or to completely remove them from images before calculating segment level brightness values. In this protocol, we provide general instructions on how to program a spot identification tool to identify and remove spots if necessary. The algorithm for the identification and removal of spots includes two parts: the first part is the

segmentation of the region of interest using SLIC, and the second part is the thresholding portion where segments resulting from the first part are identified as spots if they pass a threshold. Also given in this protocol are detailed steps on how to group the spots into clusters such that the meta-analysis in Basic Protocol 6 (or Alternate Protocol 2) can be performed on them. These procedures are described in detail in Biener et al. (2021). Below, the user will find a description of how the SLIC-based segmentation process and the brightness thresholding process were designed. After the descriptions of the processes, instructions are provided on how to navigate the Spot Module and perform spot identification using the Advanced FIF Spectrometry Suite. A tutorial video showing step-by-step instructions for performing automated spot extraction and analysis using the FIF Spectrometry Suite can be seen at the following link: <https://www.youtube.com/watch?v=ZYmgoUR-goI&t>.

### **Description of SLIC-based segmentation of the ROI**

1. Split the ROI into identical square segments of length  $d$ .

*We tend to choose the size of the segment to be as close as possible to the average size of the spots.*

2. Define a matrix that stores the distance (see Equation S27 from Stoneman et al., 2019) of each pixel from the closest segment center.

*We identify this matrix as the distance matrix.*

*The distance matrix has the same dimensions as the image.*

*The weighting factor  $m$  defines the weight a pixel's intensity has on the calculated distance. the value of  $m$  can be constant or dependent on the segment's intensity distribution as well as on the positions of the pixels compared to the center of the tested segment (Biener et al., 2021).*

3. Define a second matrix which stores the segment number that each pixel belongs to.

*The pixel belongs to the segment with the center that is closest to the pixel.*

4. Move a square a segment length  $d$  from left to right and from top to bottom where the square size is  $x$  times larger than the segment size  $d$ .

*$x$  needs to be larger than 1 but smaller than 3.*

5. Calculate the distances of all the pixels from the center of the square defined in step 4, and compare each pixel's distance to the corresponding pixel value in the *distance matrix*.

*If the distance is smaller, update the distance in the distance matrix for the corresponding pixel and update the label matrix by changing the pixel's value to the segment number whose center is currently overlapping with the center of the square.*

6. Calculate the center of mass of all the new segments by using the updated segment numbers in the *label matrix*.

*For each pixel having the same segment number in the label matrix, record the  $x$  and  $y$  coordinates.*

*Average all the  $x$  coordinate values of the selected pixels and perform the same averaging for the  $y$  coordinate.*

*The resulting  $\langle x \rangle$  and  $\langle y \rangle$  are the center of mass coordinates of the specific segment.*

*Repeat the center of mass calculation for all segments.*

7. Repeat steps 4 and 5 using the centers computed in step 6, but set the *distance matrix* and *label matrix* to their initial values, i.e., infinity for each pixel in the *distance matrix* and 0 for each pixel of the *label matrix*.
8. Terminate the process if the number of iterations reaches a chosen maximum value, or the centers of the segments do not move any more.

#### ***Description of how the algorithm identifies spots via brightness thresholding***

The SLIC-based algorithm outlined in steps 1-8 will result in segmented ROIs. Next, the algorithm must decide which of the newly generated segments are defined as “spots” and which are defined as “flat membrane,” a procedure that we call thresholding. In order to identify the segments containing spots, the segments’ average intensity is either compared to the area surrounding the segment or to the entire ROI where the segment resides. The method using the area around the segment (dubbed Local Thresholding) is outlined in Option 1 below, while the method using the entire ROI (dubbed Global Thresholding) is described in Option 2 below. Global Thresholding tends to assign more pixels from surrounding membrane regions to the spot (i.e., the spot regions tend to be comparatively larger), while Local Thresholding tends to only remove pixels on the interior of the spots (resulting in smaller spots for analysis). To analyze the spots themselves, Local Thresholding is recommended, in order to avoid incorporating lower-intensity pixels from adjacent membrane areas into the spot-intensity analysis. By contrast, Global Thresholding is recommended when analyzing the basolateral membrane after spot removal to avoid including any remaining high-intensity pixels in the membrane intensity distribution.

#### ***Local thresholding (Option 1)***

9. Average the segments’ intensity values and replace each pixel within a segment by the segment’s average intensity value.
10. Extract an area of a certain thickness surrounding the segment and calculate the average and standard deviation of the intensities from the surrounding area’s pixels.
11. Identify the segment as a spot if its average intensity is equal to or higher than the average intensity found in step 10.
12. Create a table of spot intensities ordered from lowest average intensity to highest average intensity.
13. *Optional:* Remove the spots from the image by simply putting zero values in place of the segments identified as spots.
14. *Optional:* Analyze the spots separately.

*Once spot identification has been performed, the spots are grouped together into clusters of five.*

*A cluster of spots is created by consolidating the intensity values from the pixels of five consecutive individual spots in the table created in step 12.*

*Analyze the clusters using Basic Protocols 4-6 (or corresponding Alternate Protocols).*

#### ***Global thresholding (Option 2)***

15. Calculate the average intensity at each segment resulting from the SLIC algorithm.
16. Generate a histogram of average intensities.
17. Fit a Gaussian to the histogram and find mean and standard deviation of the fitted Gaussian.

18. Calculate the threshold intensity by summing the mean and the standard deviation multiplied by a constant threshold value (TH).
19. For each segment, check whether its average intensity is higher than or lower than the intensity threshold calculated in step 18. If the intensity is higher, the segment is considered to be a spot.
20. Create a table of spot intensities ordered from lowest average intensity to highest average intensity.
21. *Optional:* Remove the spots from the image by simply putting zero values in place of the segments identified as spots.
22. *Optional:* Analyze the spots separately.

*Once spot identification has been performed, the spots are grouped together into clusters of five.*

*A cluster of spots is created by consolidating the intensity values from the pixels of five consecutive individual spots in the table created in step 12. Analyze the clusters using Basic Protocols 4-6 (or corresponding Alternate Protocols).*

*Analyze the clusters using Basic Protocols 4-6 (or corresponding Alternate Protocols).*

### **Using FIF Spectrometry Suite's spot module**

The use of SLIC and thresholding makes the spot identification and removal a time-consuming process. Therefore, before actually extracting the spots from the images, the user is advised to tune the module parameters. For that purpose, first test the choice of parameters and tune them using a single ROI. If the image after spot removal for the particular ROI is satisfactory, the user can de-spot the entire set of ROIs and images using the selected parameters.

23. Start the FIF Spectrometry suite.
24. Click the ROI manager button and open the ROI manager.
25. Load the image or image set of interest as well as the ROI list.
26. If ROI list does not exist, draw ROIs using the polygon tool in the ROI manager window.
27. Once the image set is loaded as well as the ROI list, open the Spots Identification module window by pressing the far right button in the FIF Spectrometry Suite main window (See Figure 1E).
28. Select one of the ROIs from the ROI list in the ROI manager window.
29. Press the setting button in the Spots Identification module window, and a window pops up with the Spots Identification module parameter names and values.

*The user can select a thresholding method for spot determination. Local thresholding compares the tested segment with pixels in the close surrounding area, while global thresholding uses the overall intensity of the ROI to determine spots, which results in a more aggressive approach to spot identification.*

*The settings window also includes an 'Accumulate Spots' check box. If parameter testing is in progress, make sure to uncheck that box. If multiple initial segment sizes are required, uncheck this box only after testing the entire sequence of initial segment sizes while making sure the box is checked in-between segment sizes executions.*

30. Select the initial choice of parameters and press Apply. The parameters window will close.

31. Press the spot identification button in the Spots Identification module window (the far left button).

*While waiting for the calculation to complete, the progress bar might not change (as it is a time-consuming process), but eventually it will close. Once the calculation is over, a window will pop up displaying the image set that include the de-spotted ROI. If the image looks satisfactory and most of the blackened regions overlap with the locations of spots, then keep the parameters and use them for further analysis; otherwise change the parameters one at a time and, at each change, run step 31. Some of the spots might not be removed, but the few spots left will be disregarded by simply using the FIF spectrometry analysis, which has a built-in filtering ability.*

32. Once the parameters are selected, the user needs to select all the ROIs in the ROI list and run from step 31. The resulting image set with the de-spotted ROIs can be saved in a .tiff format using the Save icon in the de-spotted image display window.

#### ***De-spotting and spot analysis with multiple segment sizes***

The Spots Identification module provides the user with the ability to de-spot or extract spots while using multiple segment sizes. This procedure can be performed in two different ways.

33. Starting from step 30, the user can choose an individual initial segment size and run through step 32.
34. Make sure the 'Accumulate Spots' checkbox is checked and repeat step 33 with a different initial segment size (preferably smaller than the former choice).
35. Repeat steps 33-34 as many times as required to remove as many of the spots as possible.

## **SUPPORT PROTOCOL**

### **MONOMERIC BRIGHTNESS DETERMINATION**

In order to (i) calculate the protomer concentration for each segment and (ii) deconvolute brightness spectrograms, the molecular brightness of the fluorescent marker in monomeric form,  $\epsilon^{MONO}$ , must be determined. This protocol describes how to determine  $\epsilon^{MONO}$  from fluorescence images taken of cells expressing the fluorescent marker in monomeric form. This protocol relies on concepts detailed in Protocols 3, 4, and 6.

1. Implement Basic Protocol 3 to demarcate and segment ROIs on fluorescence images of the cells expressing a monomeric form of the receptor.
2. Open the Fluctuation Data Assembly Module in FIF Spectrometry Suite and calculate the effective brightness for each segment generated.

*Make sure to input the values for average background intensity,  $S$ , and  $\sigma_o^2$  determined in Basic Protocol 2.*

*The program creates intensity histograms for each segment and fits them with a Gaussian. The mean and standard deviation of the Gaussian are used to calculate the brightness for each particular segment. The brightness values collected from each segment are combined to form a brightness spectrogram which can be fitted with a sum of Gaussians to calculate the monomeric effective brightness value.*

*To perform the effective brightness calculation, press the calculator button.*

3. Set the Concentration Max and Bin values for the Concentration axis in surface and wire-stack plots to be equal.

*The purpose of this step is to include data of all concentrations into a single spectrogram.*

*Set the Max to a value under which the intensity value of the majority of selected segments fall.*

*Generate a 3-D surface plot of the concentration and brightness pairs to gauge if the Concentration max is high enough to include the majority of segments*

4. Generate a Wire-Stack of the brightness spectrogram.

*See Fluorescence Fluctuation Spectrometry Suite User Guide for more detailed instructions on generating these two plots using the FIF Spectrometry Suite.*

5. Launch the Model Fitting Module to load the newly generated brightness spectrogram to the module.

6. Adjust the fit settings in the Model Fitting Module Settings Window.

*Enter the number of Gaussians used to fit the spectrogram; we typically use 4 Gaussians for fitting the monomeric control data.*

*Click the buttons labeled “ $\mu$  Adjustable” and “ $\sigma$  Adjustable,” which represent the mean and standard deviation of the Gaussian used to fit the monomer. Checking these boxes allows for the mean,  $\epsilon^{MONO}$ , and standard deviation,  $STD(\epsilon^{MONO})$ , to be adjustable during the fitting.*

7. Manually fit the brightness spectrogram generated for the monomeric protein construct with a model consisting of an array of Gaussian functions.

*Adjust the monomeric brightness and standard deviation of the Gaussians along with the amplitude of each of the Gaussians in the Fitting Adjustment Window.*

8. *Optional:* Improve the fitting by initiating the automatic fit routine of the FIF Spectrometry Suite.

9. Record the mean and standard deviation obtained from the best fit of the monomeric control data.

*The mean and standard deviation are used as  $\epsilon^{MONO}$  and  $STD(\epsilon^{MONO})$ , respectively, moving forward.*

10. *Optional:* If measured, use the distribution of brightness values of the dimeric constructs along with the monomeric brightness distribution to determine  $\epsilon^{MONO}$ .

*Set the Gaussian peak position simulating the dimeric distribution to be  $2\epsilon^{MONO}$ . Use the same standard deviation for both Gaussian functions but allow it to vary as a fitting parameter. Use the best fit value of  $\epsilon^{MONO}$  as the monomeric effective brightness value.*

*This simultaneous fitting of monomeric and dimeric standards using a common  $\epsilon^{MONO}$  cannot be done in the FIF Spectrometry Suite.*

## COMMENTARY

### Background Information

#### **Fluorescence fluctuation spectroscopy**

FFS-based approaches rely on statistical analysis of fluorescence intensity fluctuations arising due to fluorescently labeled molecules diffusing in and out of a microscope focal region; such analysis reveals critical properties of the molecules, such as the diffusion coefficient or aggregation state. The original application of FFS was to determine the diffusion coefficient of biomolecules in solution by analyzing the correlations between the intensity fluctuations (Elson, 2011). FFS-based approaches have been extended in

recent decades to allow quantification of the abundance and interactions of the molecules, not only in solutions, but in living cells as well. This advancement was made possible by analyzing the moments of the fluorescence intensity distribution. FFS techniques that rely on moment-based analysis of intensity fluctuations include Photon-Counting Histogram (PCH; Chen et al., 1999; Herrick-Davis et al., 2013), Number and Brightness (N&B; Digman et al., 2008; Nagy et al., 2010; Unruh & Gratton, 2008), and Spatial Intensity Distribution Analysis (SpIDA; Godin et al., 2011; Pediani et al., 2018).

The core of the moment-based techniques is the relation between the molecule's brightness and the amplitude of the intensity fluctuations. The molecular brightness is defined as the average detected signal from the molecule over a given time. Therefore, the molecular brightness scales linearly with the size of the complex, e.g., a dimer has twice the molecular brightness of a monomer. For this reason, the set of moment-based FFS techniques, such as those listed above, are very effective for determining the size of protein complexes (Chen et al., 2003; Digman et al., 2009; Godin et al., 2015; Pediani et al., 2018). However, when the relative proportion of the various states of a particular protein complex varies, e.g., as a function of concentration, these traditional FFS-based approaches only provide average values of the oligomer size over the entire population. Therefore, detailed information, such as changes to the binding strength of the complex as a result of ligand binding, may be washed out when the sampled concentration range is extensive.

### Fluorescence Intensity Fluctuation (FIF) spectroscopy

In order to extract more detailed information regarding the oligomerization properties of a particular receptor over various conditions, we have developed a moment-based FFS technique called Fluorescence Intensity Fluctuation (FIF) Spectrometry (Stoneman et al., 2019). FIF is a spectrometric method that provides entire probability distributions (i.e., spectrograms) of molecular brightness. The molecular brightness distributions are sorted according to individual protomer concentration, and are deconvoluted to determine the percentage of units comprised by each of the oligomeric structures present in the population. Therefore, FIF spectrometry offers more detailed information than the moment-based FFS methods referenced in the previous paragraph, which provide average molecular brightness values over large concentration ranges.

The FIF spectrometry method relies on collecting fluorescence images of cells expressing the receptor of choice fused to a fluorescent 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,"  $\varepsilon_{eff}$ , of each segment. The molecular brightness,  $\varepsilon$ , for each

segment is calculated from average intensity and variance by using the following relation:

$$\varepsilon_{eff} = \frac{\sigma^2 - \sigma_D^2}{\gamma I_s},$$

#### Equation 1

where  $\varepsilon_{eff} \equiv G\varepsilon$  and  $G$  is the analog gain of the fluorescence microscope detector in digital levels/photon,  $I_s$  is background-corrected intensity,  $I_s = I_{meas} - I_{back}$ , and  $\sigma_D^2$  is the variance arising due to the detector,  $\sigma_D^2(I_s) = \sigma_o^2 + SI_s$ .  $\gamma$  is a shape factor which depends on the shape of the laser's point-spread function (PSF) as well as the geometry of the sample (Chen et al., 1999).

The total number of protomers,  $N_{proto}$ , can be found from the measured intensity, as follows:

$$N_{proto} = \frac{I_s}{\varepsilon^{MONO}}$$

#### Equation 2

where  $\varepsilon^{MONO}$  is the molecular brightness of a single protomer determined from separate measurements on samples expressing a monomeric form of the fluorophore, using the same imaging conditions that were used to collect images of cells expressing the fluorescently labeled receptor of interest. The concentration of protomers,  $C$ , is determined by the following equation:

$$C = \frac{I_s}{\varepsilon^{MONO} V_{PSF}}$$

#### Equation 3

Here,  $V_{PSF}$  is a volume-like quantity calculated by integrating over the PSF of the focused laser beam:

$$V_{PSF} = \iiint PSF^x(x, y, z) dx dy dz$$

#### Equation 4

The exponent  $x$  is 2 for single-photon excitation and 4 for two-photon excitation. By assuming a Gaussian-Lorentzian spatial profile for the laser's PSF and measuring the  $e^{-2}$  laser beam waist at the focal plane ( $\omega_0^2$ ), the volume integral of Equation 4 can be evaluated.  $V_{PSF}$  accounts for the non-uniform intensity profile of the laser focal spot, which is crucial for the correct calculation of concentration of molecules using the average background-corrected intensity.

For fluorescence measurements performed on the basolateral membranes of cells expressing membrane proteins, we assume that all the fluorescent molecules are bound to the membrane and are together positioned within the

focal plane of the laser beam at  $z = 0$ . Under this condition, the value of the shape factor,  $\gamma$ , needed for the calculation of  $\varepsilon_{eff}$  in Equation 1, becomes 0.5. The concentration of the molecules in the membrane,  $C_m$ , then becomes the number of molecules per unit area, and is given by:

$$C_m = \frac{I_s}{\varepsilon^{MONO} A_{PSF}}$$

**Equation 5**

where  $A_{PSF} = \iint PSF^x(x, y, 0) dx dy$ . For a Gaussian-Lorentzian PSF,  $A_{PSF}$  can be evaluated analytically, with  $A_{PSF} = \frac{\pi \omega_0^2}{2}$  for single-photon excitation and  $V_{PSF} = \frac{\pi \omega_0^2}{4}$  for two-photon excitation.

The effective brightness and concentration are calculated for each demarcated segment. From a 2D map assembled from all the effective brightness-concentration pairs, effective brightness distributions can be generated for a number of different protomer concentration ranges. Each brightness distribution, or spectrogram, is composed 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. FIF spectrometry has the inherent ability to filter out inhomogeneities attributable to, e.g., endocytic vesicles, by applying two-level filtering. The first level of filtering occurs when fitting the intensity histograms, because high intensities will not follow the fitting model (a Gaussian). The second level occurs in the assembly and presentation of the brightness spectrograms; at this level, oligomers with negligible abundance values will be disregarded.

In order 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. From the measured monomer distribution, the brightness distribution for any oligomer of size  $n$  can be predicted, as the peak position of the distribution of an  $n$ -sized oligomer will be simply scaled by the size of the oligomer, i.e.,  $n\varepsilon^{MONO}$ . Each composite spectrogram is then “unmixed,” with the unmixing components consisting of the various predicted oligomer brightness distributions.

$$\varepsilon_{eff} = \sum_n A_n \exp \left[ -\frac{(\varepsilon_{eff} - n\varepsilon^{MONO})^2}{2\sigma^2} \right]$$

**Equation 6**

where  $\sigma$  represents the standard deviation, labeled  $STD(\varepsilon^{MONO})$  in the FIF Spectrometry Suite, of the fitting Gaussians determined from measurements of a monomeric standard (see Support Protocol). The relative abundance of each oligomer species is then computed as the quotient of the area underneath the specific species distribution curve and 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, a plot which is dubbed a kinetic curve. By plotting the extent of oligomerization as a function of receptor concentration, the method can assess the effects of ligand, as the ligand might induce shifts in the oligomeric size of membrane receptors of interest as observed by the kinetic curve.

## Critical Parameters

### Background-corrected intensity

The background-corrected intensity,  $I_s$ , should be obtained for each measurement by subtracting the intensity read out from the detector when there is no sample present,  $I_{back}$ , from the total signal read out from the detector in the region of interest,  $I_{meas}$ , as follows:

$$I_s = I_{meas} - I_{back}$$

**Equation 7**

Contributions to  $I_{back}$  include the electronic offset (i.e., bias level) added to the output signal of the detector as well as dark noise.  $I_{back}$  is determined by measuring the mean intensity in multiple  $\sim 10,000$ -pixel subregions of the acquisition where no cells/fluorophores were present. If the sample measured is uniformly fluorescent, e.g., a solution of fluorescent molecules, the mean background intensity was measured from images acquired when the laser is turned off.

### Detector variance characteristics

The parameters  $S$  and  $\sigma_0^2$  should be determined from measurements of a light source with constant intensity (i.e., no temporal fluctuations), such that any fluctuations in detected signal are attributed to the detector readout. The relationship between the detector variance and intensity is linear and therefore could be fit with a straight line of slope  $S$  and intercept  $\sigma_0^2$ . From these two parameters,  $\sigma_D^2$  can be calculated for any value of  $I_s$  and subtracted from the variance of the intensity distribution, as

seen in Equation 1. It should be noted that the intensities measured from the constant light source must still be corrected for background signal, as shown in Equation 7.

### **Imaging monomeric constructs**

When imaging monomeric constructs for standard brightness calibration (see Basic Protocol 2 and Support Protocol), it is important to use the same instrument settings that will be used in the rest of the measurements. Ensure that the monomeric constructs are the same fluorescent marker that is fused to the receptor of interest.

### **Determination of beam waist**

In order to determine the laser beam waist size, fluorescence images of sub-diffraction-sized microspheres must be obtained. We have used two different types of microspheres in our experiments for this purpose: 100 nm Tetraspeck fluorescent microspheres (Invitrogen, #T14792) and 170-nm PS-Speck Microscope Point Source Kit fluorescent microspheres (Invitrogen, #P7220). 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  $e^{-2}$  of its maximum value.

### **Pixel dwell time**

The pixel dwell time for the measuring system should be shorter by at least a factor of five than the two-dimensional characteristic diffusion time,  $\tau_D$ , 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,  $\omega_0$ , and the diffusion coefficient,  $D$ , through the following relation:  $\tau_D = \omega_0^2/4D$ .

### **SLIC parameters for spot identification**

i. **Maximum number of iterations**—The spot removal algorithm uses SLIC in order to identify spots, and SLIC is an iterative algorithm. Therefore, one needs to choose the maximum number of iterations for which the SLIC algorithm will stop in case it does not converge fast enough.

ii. **Initial segment size**—This parameter is used in order to determine the first step distance between segments. A smaller segment size will result in more segments to manipulate.

iii. **Search window multiplication factor**—Search window denotes the square that the algorithm uses in order to scan the sample, and search window multiplication factor is the ratio between the search window

size and the initial segment size. The search window size needs to be larger than the segment size. However, extremely large windows will slow down the spot identification process and, in most cases, it will not be justified. Also, if the search window is larger than two segment sizes, multiple spots might combine into a single spot. This issue is the direct result of the SLIC algorithm property of calculating distance (Biener et al., 2021).

iv. **Surrounding width**—This parameter is part of the algorithm responsible for thresholding. The parameter defines the thickness of the ring-shaped area outside the segment of interest. The ring-shaped areas are used to calculate the average intensity of the surroundings, which is used for the local threshold calculation.

v. **Threshold**—This parameter is a constant multiplying the standard deviation of the surrounding intensity added to the mean. A segment will be considered as a spot if its average intensity is larger than the threshold parameter.

vi. **Minimal segment size**—This parameter determines the minimum segment size, in pixels a segment must be in order to be considered for further analysis.

vii. **Number of pixels in a cluster**—This parameter determines the approximate number of pixels required for a cluster of spots in order to consider the cluster reliable for further analysis. This parameter has no effect on the spot identification process.

### **Troubleshooting**

1. **Problem:** Simultaneous fitting of the brightness spectrogram of monomeric constructs is poor due to a broad distribution with poorly defined peaks.

*Allow additional Gaussian functions to be used in the fitting.*

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  $S$  and intercept  $\sigma_0^2$  describing the linear relationship between detector variance and average intensity values.

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

*Do not include intensities much larger than the values sampled in cells, as the detector response can be non-linear at high signal levels because of, e.g., saturation, and could result in an inaccurate correction in regions with low intensities.*

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 that best fits the data with the least amount of input parameters needed.*

4. **Problem:** If brightness values are negative, then the detector noise parameters are incorrect and need to be remeasured (see problem 2).

### Statistical Analysis

Statistical errors regarding the fractions of oligomeric species that were present in pixel-level mixtures of oligomers have been estimated using the bootstrapping method (Efron, 1979). We suggest producing 500 ‘bootstrapped’ sets of images by randomly selecting, with replacement, images from a particular sample. These images are analyzed using the techniques described above; the resulting  $\varepsilon_{eff}$  distributions can then be fitted with three different  $\varepsilon^{MONO}$  values that were determined from (1) the peak of the plasma-membrane-targeted mEGFP monomeric construct  $\varepsilon_{eff}$  distribution, (2) the peak of the dimeric construct  $\varepsilon^{MONO}$  distribution, and (3) a simultaneous fit of the monomeric and dimeric  $\varepsilon_{eff}$  distributions. It should be noted that to perform this error determination, measurements must be performed on a dimeric form of the fluorescent receptor, a step which is listed as optional in Basic Protocol 2 above. Fitting 500 different datasets with Gaussians whose centers (i.e., means) were determined from three different  $\varepsilon^{MONO}$  values should result in 1500 relative fraction values for each oligomer size and protomer concentration. Each data point in the species fraction graphs can be obtained by taking the mean of the 1500 relative fraction values, and the error bar for each data point can be represented by  $\pm 1$  S.D. of the same set of values (Stoneman et al., 2019).

### Understanding Results

All figures in the following sections are obtained from the FIF Spectrometry Suite.

#### **Determination of monomeric brightness**

The value of the monomeric brightness,  $\varepsilon^{MONO}$ , is critical to the FIF method both for determining protomer concentration and deconvoluting brightness spectrograms. Shown

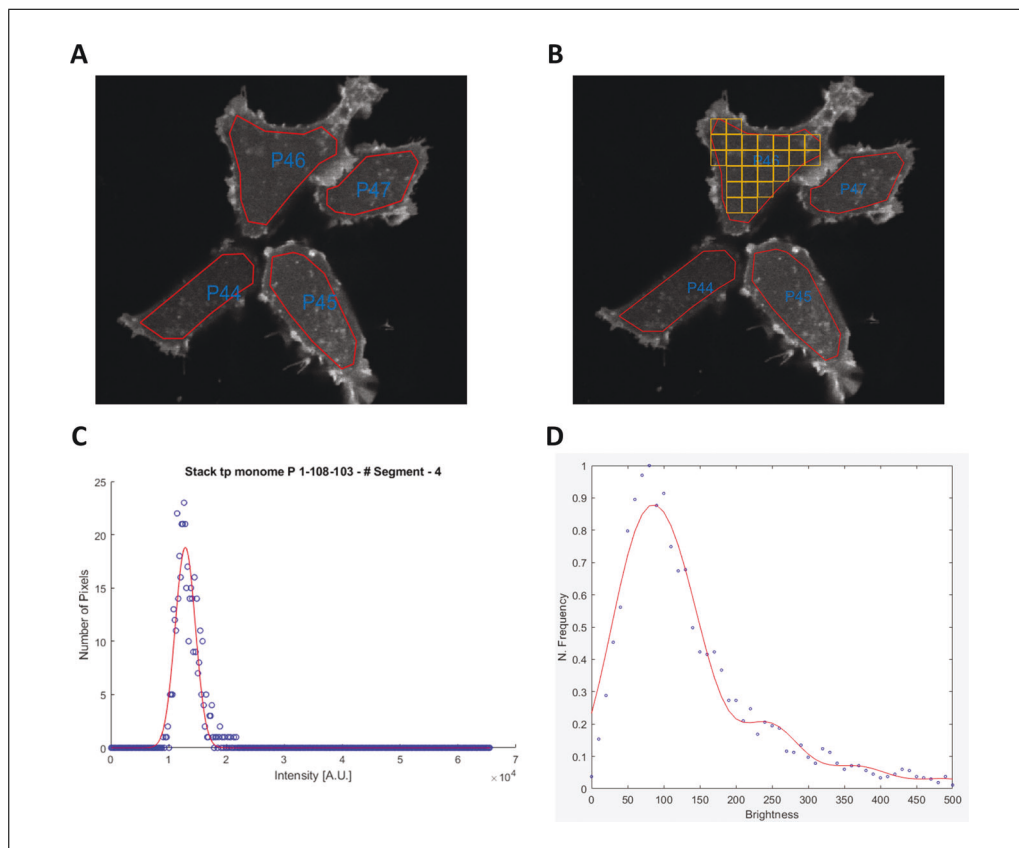
in Figure 3 are example images of the various steps needed to extract  $\varepsilon^{MONO}$  from fluorescence images. The fluorescence intensity histogram of each segment should be fit with a Gaussian curve (solid red line), as exemplified in Figure 3C. If there is not enough data to accurately distinguish a Gaussian curve, the segmentation size may need to be increased or the intensity bin size should be increased. Using the Model Fitting Module of the FIF Spectrometry Suite, one should fit the brightness distribution to determine the values of  $\varepsilon^{MONO}$ , and the standard deviation,  $STD(\varepsilon^{MONO})$ , used in Equation 6, to fit the brightness spectrograms of the receptor of interest.

#### **Brightness and concentration extraction from cells expressing receptors of interest**

Figure 4 provides example images illustrating the workflow involved in assembling brightness spectrograms. We first load the images and draw ROI polygons, as shown in Figure 4A. Next we segment each ROI, Figure 4B, and fit each segments intensity distribution with a Gaussian (Figure 3C) to extract the mean and standard deviation in order to calculate brightness and concentration. The FIF spectrometry software provides visualization tools to allow the user to assess the results obtained from the collection of segment-level brightness and concentration values. These tools include the plotting of the “volcano graph,” Figure 4D, which is a surface plot of the distribution of brightness values as a function of brightness and concentration. Using this tool, we can see whether there is any dependence of the brightness distribution on concentration, i.e., whether increasing the concentration will result in an increase of the brightness. The second visualization tool is the “wire-stack,” Figure 4E. This plot provides multiple 2D curves plotting the brightness histogram, or spectrogram, for a number of concentration ranges; the individual spectrograms are displayed stacked on top of one another. Figure 4 summarizes the results of analyzing cells expressing the secretin receptor in the absence of ligand.

#### **Determination and analysis of kinetic curves**

Each brightness spectrogram is potentially composed of contributions from different-sized oligomers, and it may be decomposed to find the fraction of total protomers, or relative abundance, present in each of the different-sized oligomer species. This deconvolution process can be implemented using the Model



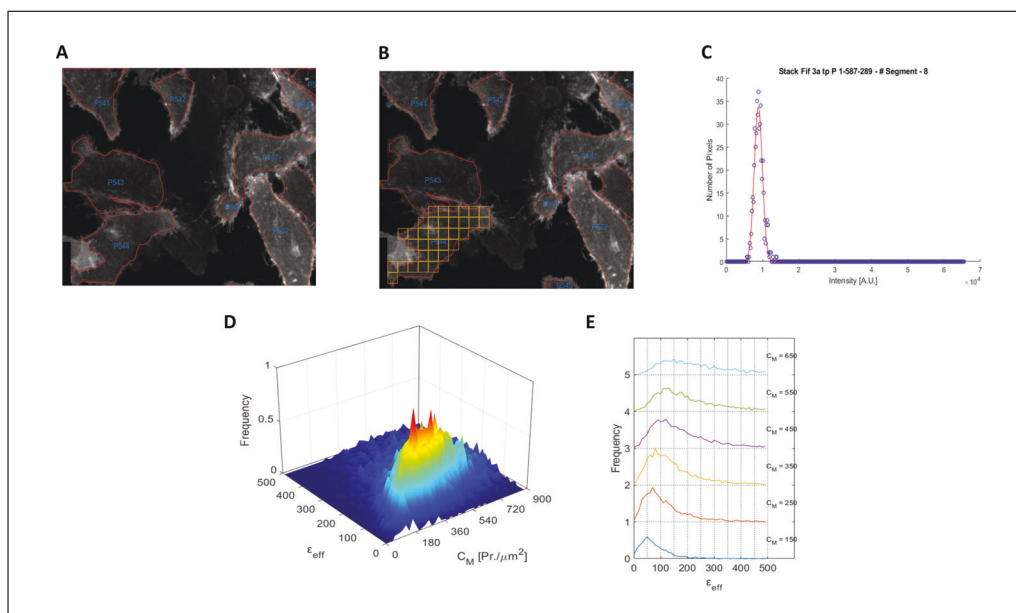
**Figure 3** Demonstration of the data reduction process in two-dimensional FIF spectrometry using two-photon excitation of cells expressing plasma-membrane-targeted mEGFP construct (PM-1-mEGFP). **(A)** Representative fluorescence image obtained from two-photon excitation of Flp-In™ T-REx™ cells expressing fluorescently labeled plasma-membrane-targeted mEGFP construct (PM-1-mEGFP), and polygons (P44, P45, P46, P47) indicating a region of interest (ROI) which comprises a patch of the basolateral membrane of each cell. **(B)** Software-generated image segmentation of the ROI (P46) using the moving-squares method. **(C)** A fluorescence intensity histogram of a single segment selected at random. The mean and width of the fitted Gaussian (solid red line) are used to calculate the brightness ( $\epsilon_{eff}$ ) and concentration for each segment. **(D)** Normalized frequency distribution obtained from several cells expressing monomeric (PM-1-mEGFP) mEGFP constructs was fit to find the brightness of single mEGFP protomers,  $\epsilon^{MONO}=61.4$ .

fitting module of the FIF Spectrometry Suite. Each curve within the wire-stack, Figure 4E, is fitted with multiple Gaussians, as given by Equation 6, where the mean of each Gaussian corresponds to an oligomeric species with a specific size,  $n$ . An example of such a fit can be found in Figure 5A. The blue circles represent the calculated distribution while the red line represents the fit. The Gaussians are characterized with a mean value of  $n\epsilon^{MONO}$ , where  $n$  is the oligomer size and  $\epsilon^{MONO}$  is the molecular brightness of the monomeric sample (see Figure 3). The amplitudes of each of the Gaussians ( $A_n$ ) are adjusted by the user and can be refined by pressing the ‘auto-fit’ button in the software. The software calculates the species fractions of the oligomeric species using the area underneath each of the Gaussians. The species fractions can be found

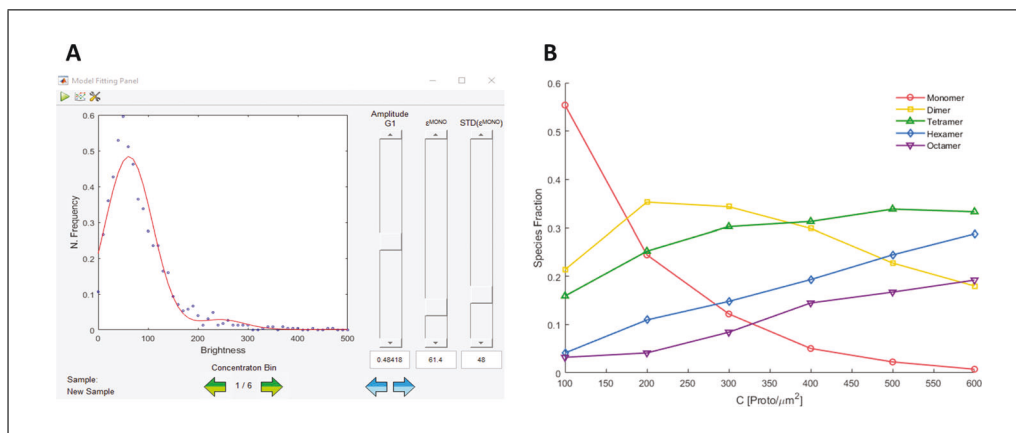
for each concentration that appear in the wire-stack plot. The resulting graph plotting the species fraction values versus the concentration, denoted as a “Kinetic Curve,” is shown in Figure 5B.

#### Spot identification and removal

Displayed in Figure 6 is an example of the spot identification algorithm applied to a single image using the steps described in Basic Protocol 7 or Alternate Protocol 3. To generate Figure 6B, we used the Spot Removal Module of the FIF Spectrometry Suite on the image shown in Figure 6A. The de-spotted image and clusters of the spots which were removed can be analyzed using Basic Protocols 4-6 or their corresponding Alternate Protocols. In case the user is interested in the analysis of the de-spotted images, Figure 7 provides an example of that analysis.



**Figure 4** Illustration of the data-reduction process in two-dimensional FIF spectrometry using two-photon excitation of cells expressing wild-type secretin receptor tagged with mEGFP in the absence of agonist ligand. **(A)** Fluorescence image of cells and associated polygons indicating Regions of Interest (ROI) drawn by the 1<sup>st</sup> module of the FIF Spectrometry Suite. **(B)** Software generated image segmentation of the ROI using the Moving Square method (area of segment squares set to be  $23 \times 23$  pixels). **(C)** Fluorescence intensity histogram of an image segment selected at random. **(D)** Surface plots of the frequency of occurrence of  $\epsilon_{eff}$  for each concentration. **(E)** Frequency of occurrence vs. effective brightness for different concentration ranges. The average concentration for each range (in *protomers*  $\mu\text{m}^{-2}$ ) is indicated to the right of each plot.



**Figure 5** Determination of Species fraction plot by fitting each brightness spectrogram using the Model Fitting module. **(A)** Model fitting panel used to fit effective brightness spectrograms, generated in the Fluctuation Data Assembly Module, for different concentration ranges using Equation 6. **(B)** Species fraction values for oligomers of size  $n = 1, 2, 4, 6,$  and  $8$ , obtained by performing the spectrogram deconvolution procedure shown in **(A)** for a number of concentration ranges. Image generated by using advanced version of FIF Spectrometry software.

## Time Considerations

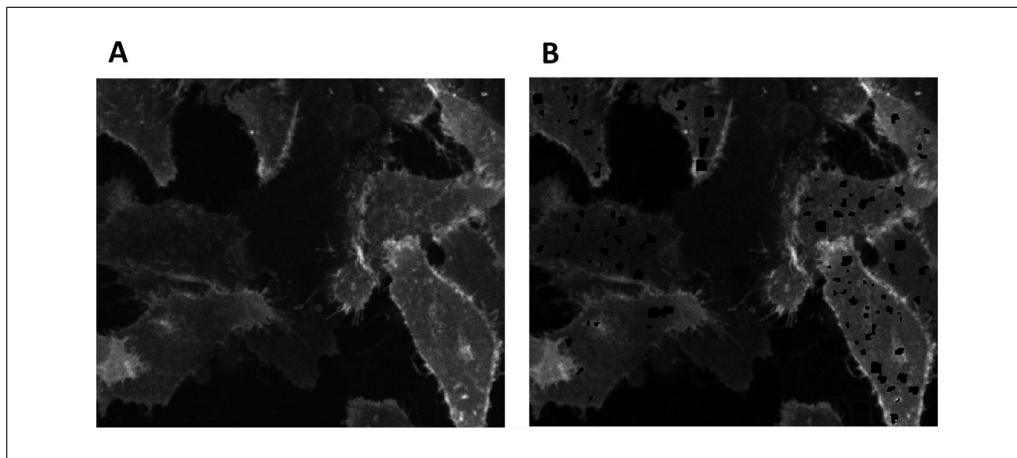
### Cell preparation

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

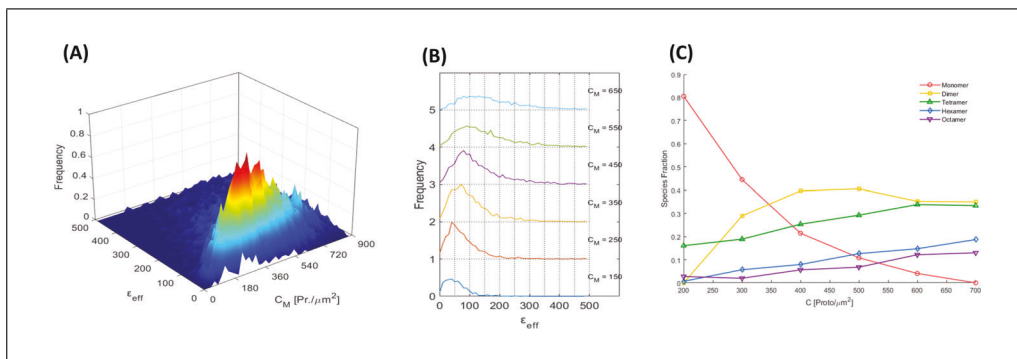
### Image acquisition

Expect to collect fluorescent images on viable cells at a rate of  $\sim 2$  cells/min.

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 hr. Demarcating



**Figure 6** Fluorescence images of cells expressing wild-type SecR, before and after spot removal. **(A)** Fluorescence image of the whole basolateral membranes of cells before spot removal from the image. **(B)** Image of basolateral membranes of the cells after spot removal. Image generated by using advanced version of FIF Spectrometry software.



**Figure 7** FIF spectrometry analysis of basolateral membrane patches of cells expressing wild-type SecR after spot removal (i.e., de-spotted membrane analysis). **(A)** Surface plot of the  $\epsilon_{eff}$  occurrence frequency versus receptor concentration of protomers. **(B)** Stacks of cross sections of surface plots in **(A)**, representing brightness spectrograms for different receptor concentration ranges. The median receptor concentration for each cross section is given to the right of the corresponding curve in *protomers*  $\times \mu m^{-2}$ . **(C)** Species fraction values vs. total concentration of protomers, as derived from the fitting of the curves in **(B)** with the different Gaussian components representing different oligomeric species. Image is generated using Advanced FIF Spectrometry Suite.

the boundaries of several hundred cells will take approximately 4 hours.

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

Finally, collecting images from a light source with constant intensity will take  $\sim 0.5$  hr.

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 being studied multiplied by the number of different treatments (e.g., ligands).

#### 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 hr.

Demarcating the boundaries of several hundred cells will take  $\sim 4$  hr.

- Automated image segmentation and brightness and concentration calculation for several hundred cells will take  $\sim 30$  min.

- Therefore, image analysis takes  $(4+4.5x)$  hr, 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.

#### Acknowledgements

This work was partly supported by grants from the National Science Foundation (grant

DBI 1919670) as well as the University of Wisconsin–Milwaukee Research Growth Initiative (101×396).

### Author Contributions

**Thomas Killeen:** data curation, formal analysis, investigation, validation, writing original draft, writing review and editing; **Sadia Rahman:** formal analysis, validation, writing original draft; **Dammar Badu:** validation, writing original draft; **Gabriel Biener:** Added features to the FIF Spectrometry Suite, formal analysis, methodology, supervision, writing review and editing; **Michael Stoneman:** data curation, investigation, methodology, supervision, writing original draft, writing review and editing; **Valerica Raicu:** conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing original draft, writing review and editing.

### Conflict of Interest

The authors wish to declare that the advanced version of the FIF Spectrometry Suite is licensed and distributed through the UWM Research Foundation.

### Data Availability Statement

Fluorescence images and ROI files used to generate the FIF spectrograms in this study are accessible from <https://figshare.com/s/77b90d060901fa8b4cb3> and described in detail at <https://doi.org/10.1038/s41592-019-0408-9>.

### Literature Cited

Ahmed, F., Zapata-Mercado, E., Rahman, S., & Hristova, K. (2021). The biased ligands NGF and NT-3 differentially stabilize Trk-A dimers. *Biophysical Journal*, *120*(1), 55–63. doi: 10.1016/j.bpj.2020.11.2262.

Biener, G., Stoneman, M. R., & Raicu, V. (2021). Fluorescence intensity fluctuation analysis of receptor oligomerization in membrane domains. *Biophysical Journal*, *120*(15), 3028–3039. doi: 10.1016/j.bpj.2021.06.01ssss5.

Chen, Y., Muller, J. D., So, P. T., & Gratton, E. (1999). The photon counting histogram in fluorescence fluctuation spectroscopy. *Biophysical Journal*, *77*(1), 553–567. doi: 10.1016/S0006-3495(99)76912-2.

Chen, Y., Wei, L. N., & Muller, J. D. (2003). Probing protein oligomerization in living cells with fluorescence fluctuation spectroscopy. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(26), 15492–15497. doi: 10.1073/pnas.2533045100.

Digman, M. A., Dalal, R., Horwitz, A. F., & Gratton, E. (2008). Mapping the number of molecules and brightness in the laser scanning microscope. *Biophysical Journal*, *94*(6), 2320–2332. doi: 10.1529/biophysj.107.114645.

Digman, M. A., Wiseman, P. W., Choi, C., Horwitz, A. R., & Gratton, E. (2009). Stoichiometry of molecular complexes at adhesions in living cells. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(7), 2170–2175. doi: 10.1073/pnas.0806036106.

Efron, B. (1979). Bootstrap methods: Another look at the Jackknife. *The Annals of Statistics*, *7*(1), 1–26. doi: 10.1214/aos/1176344552.

Elson, E. L. (2011). Fluorescence correlation spectroscopy: Past, present, future. *Biophysical Journal*, *101*(12), 2855–2870. doi: 10.1016/j.bpj.2011.11.012.

Farran, B. (2017). An update on the physiological and therapeutic relevance of GPCR oligomers. *Pharmacological Research*, *117*, 303–327. doi: 10.1016/j.phrs.2017.01.008.

George, S. R., O’Dowd, B. F., & Lee, S. R. (2002). G-protein-coupled receptor oligomerization and its potential for drug discovery. *Nature Reviews Drug Discovery*, *1*(10), 808–820. doi: 10.1038/nrd913.

Godin, A. G., Costantino, S., Lorenzo, L. E., Swift, J. L., Sergeev, M., Ribeiro-da-Silva, A., ... Wiseman, P. W. (2011). Revealing protein oligomerization and densities in situ using spatial intensity distribution analysis. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(17), 7010–7015. doi: 10.1073/pnas.1018658108.

Godin, A. G., Rappaz, B., Potvin-Trottier, L., Kennedy, T. E., De Koninck, Y., & Wiseman, P. W. (2015). Spatial intensity distribution analysis reveals abnormal oligomerization of proteins in single cells. *Biophysical Journal*, *109*(4), 710–721. doi: 10.1016/j.bpj.2015.06.068.

Herrick-Davis, K., Grinde, E., Cowan, A., & Mazurkiewicz, J. E. (2013). Fluorescence correlation spectroscopy analysis of serotonin, adrenergic, muscarinic, and dopamine receptor dimerization: The oligomer number puzzle. *Molecular Pharmacology*, *84*(4), 630–642. doi: 10.1124/mol.113.087072.

Karl, K., Paul, M. D., Pasquale, E. B., & Hristova, K. (2020). Ligand bias in receptor tyrosine kinase signaling. *Journal of Biological Chemistry*, *295*(52), 18494–18507. doi: 10.1074/jbc.REV120.015190.

King, C., Raicu, V., & Hristova, K. (2017). Understanding the FRET signatures of interacting membrane proteins. *Journal of Biological Chemistry*, *292*(13), 5291–5310. doi: 10.1074/jbc.M116.764282.

Mishra, A. K., Gragg, M., Stoneman, M. R., Biener, G., Oliver, J. A., Miszta, P., ... Park, P. S. H. (2016). Quaternary structures of opsin in live cells revealed by FRET spectrometry. *Biochemical Journal*, *473*, 3819–3836. doi: 10.1042/Bcj20160422.

- Moller, T. C., Hottin, J., Clerte, C., Zwier, J. M., Durroux, T., Rondard, P., ... Kniazeff, J. (2018). Oligomerization of a G protein-coupled receptor in neurons controlled by its structural dynamics. *Scientific Reports*, 8, 1–15. doi: 10.1038/s41598-018-28682-6.
- Nagy, P., Claus, J., Jovin, T. M., & Arndt-Jovin, D. J. (2010). Distribution of resting and ligand-bound ErbB1 and ErbB2 receptor tyrosine kinases in living cells using number and brightness analysis. *Proceedings of the National Academy of Sciences of the United States of America*, 107(38), 16524–16529. doi: 10.1073/pnas.1002642107.
- Palczewski, K. (2010). Oligomeric forms of G protein-coupled receptors (GPCRs). *Trends in Biochemical Sciences*, 35(11), 595–600. doi: 10.1016/j.tibs.2010.05.002.
- Paprocki, J. D., Biener, G., Stoneman, M. R., & Raicu, V. (2020). In-cell detection of conformational substates of a G protein-coupled receptor quaternary structure: Modulation of substate probability by cognate ligand binding. *Journal of Physical Chemistry B*, 124(45), 10062–10076. doi: 10.1021/acs.jpbc.0c06081.
- Pediani, J. D., Ward, R. J., Marsango, S., & Milligan, G. (2018). Spatial intensity distribution analysis: Studies of G protein-coupled receptor oligomerisation. *Trends in Pharmacological Sciences*, 39(2), 175–186. doi: 10.1016/j.tips.2017.09.001.
- Phelan, K., & May, K. M. (2015). Basic techniques in mammalian cell tissue culture. *Current Protocols in Cell Biology*, 66, 1.1.1-1.1.22. doi: 10.1002/0471143030.cb0101s66.
- Qian, H., & Elson, E. L. (1990). On the analysis of high-order moments of fluorescence fluctuations. *Biophysical Journal*, 57(2), 375–380. doi: 10.1016/S0006-3495(90)82539-X.
- Raicu, V. (2007). Efficiency of resonance energy transfer in homo-oligomeric complexes of proteins. *Journal of Biological Physics*, 33(2), 109–127. doi: 10.1007/s10867-007-9046-z.
- Raicu, V. (2019). Ab initio derivation of the FRET equations resolves old puzzles and suggests measurement strategies. *Biophysical Journal*, 116(7), 1313–1327. doi: 10.1016/j.bpj.2019.02.016.
- Raicu, V., Jansma, D. B., Miller, R. J., & Friesen, J. D. (2005). Protein interaction quantified in vivo by spectrally resolved fluorescence resonance energy transfer. *Biochemical Journal*, 385(Pt 1), 265–277. doi: 10.1042/BJ20040226.
- Raicu, V., Stoneman, M. R., Fung, R., Melnichuk, M., Jansma, D. B., Pisterzi, L. F., ... Saldin, D. K. (2009). Determination of supramolecular structure and spatial distribution of protein complexes in living cells. *Nature Photonics*, 3(2), 107–113. doi: 10.1038/Nphoton.2008.291.
- Stoneman, M. R., Biener, G., Ward, R. J., Pediani, J. D., Badu, D., Eis, A., ... Raicu, V. (2019). A general method to quantify ligand-driven oligomerization from fluorescence-based images. *Nature Methods*, 16(6), 493–496. doi: 10.1038/s41592-019-0408-9.
- Stoneman, M. R., Raicu, N., Biener, G., & Raicu, V. (2020). Fluorescence-based methods for the study of protein-protein interactions modulated by ligand binding. *Current Pharmaceutical Design*, 26(44), 5668–5683. doi: 10.2174/1381612826666201116120934.
- Unruh, J. R., & Gratton, E. (2008). Analysis of molecular concentration and brightness from fluorescence fluctuation data with an electron multiplied CCD camera. *Biophysical Journal*, 95(11), 5385–5398. doi: 10.1529/biophysj.108.130310.
- Ward, R. J., Pediani, J. D., Harikumar, K. G., Miller, L. J., & Milligan, G. (2017). Spatial intensity distribution analysis quantifies the extent and regulation of homodimerization of the secretin receptor. *Biochemical Journal*, 474(11), 1879–1895. doi: 10.1042/BCJ20170184.

### Internet Resources

- <https://figshare.com/s/acfd94b21b1105317f56> or <https://sites.uwm.edu/raicu-research-group/software/>
- Free FIF Spectrometry Software.*  
[https://www.youtube.com/watch?v=M\\_S1rPd8jjk&t](https://www.youtube.com/watch?v=M_S1rPd8jjk&t)
- FIF Spectrometry Suite: ROI Manager Tutorial.*  
<https://www.youtube.com/watch?v=cN7m00FAFI>
- FIF Spectrometry Suite: Fluctuation Data Assembly Tutorial.*  
<https://www.youtube.com/watch?v=wLHBwZlgbMM&t>
- FIF Spectrometry Suite: Meta-Analysis of Brightness Spectrograms Tutorial.*  
<https://www.youtube.com/watch?v=ZYMgoUR-goI&t>
- FIF Spectrometry Suite: Spot Extraction and Analysis Tutorial.*