Chapter 22

Fluorescence Microscopy Methods in the Study of Protein Structure and Function

Heather Jensen-Smith, Benjamin Currall, Danielle Rossino, LeAnn Tiede, Michael Nichols, and Richard Hallworth

Abstract

As more and more proteins specific to hair cells are discovered, it becomes imperative to understand their structure and how that contributes to their function. The fluorescence microscopic methods described here can be employed to provide information on protein-protein interactions, whether homomeric or heteromeric, and on protein conformation. Here, we describe two fluorescence microscopic methodologies applied to the outer hair cell-specific membrane protein prestin: the intensity and fluorescence lifetime (FLIM) variants of FRET (Fluorescence Resonance Energy Transfer), used in the study of protein-protein interactions, and the Scanning Cyanine Accessibility Method (SCAM), used for the determination of protein conformation. The methods are readily adaptable to other proteins.

Key words: Prestin, FRET, FLIM, SCAM, confocal microscopy, cyan fluorescent protein, yellow fluorescent protein.

1. Introduction

An abundance of new proteins has been revealed in hair cells by biochemical and genetic strategies (1–3). Frequently, the mechanisms and functions of these new proteins are as yet unclear. Here, we describe fluorescence microscopic methods applied to the study of a novel protein specific to the outer hair cell, prestin (4). This membrane protein is believed to be the agent of outer hair cell motility, which in turn mediates the outer hair cell contribution to cochlear amplification (5). In the first method, hypotheses concerning putative multimerization by prestin are tested using Fluorescence (sometimes, Förster) Resonance Energy Transfer
(FRET) (6). FRET is the non-radiative exchange of energy between fluorophores and occurs only when two fluorophores with overlapping emission and excitation spectra, referred to as the donor and the acceptor, are sufficiently close (in practice, about 5 Å). If FRET is occurring, excitation of the donor leads not only to donor photon emission but also to acceptor emission by the acceptor in its wavelength range. The two modified fluorescent proteins (FPs), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), make an excellent FRET pair and can be introduced into a cell-based expression system as FP constructs of the proteins of interest (7). In our experiments, FRET between prestin-CFP and prestin-YFP constructs in co-transfected HEK 293 cells is used to demonstrate multimeric self-association of prestin (8,9). Two variants of FRET are used, an intensity-based procedure that separates CFP and YFP emissions by linear un-mixing (10), and a Fluorescence Lifetime Imaging (FLIM) method (11-13) that examines the lifetimes of excited CFP fluorophores. For both procedures, the acceptor photobleach method is used, which reduces concerns about cross-talk between donor and acceptor channels (9,14). In the second method, hypotheses concerning the structure of prestin in the plasma membrane (PM) are examined. Competing and mutually incompatible 10 and 12 transmembrane (TM) models for prestin have been advanced (15,16). We test these models using the Scanning Cysteine Accessibility Mutagenesis method (SCAM) (17). Prestin, coupled C-terminal to enhanced GFP (eGFP), is expressed in HEK 293 cells. Cysteines, whether naturally occurring or introduced by mutagenesis, are alkylated by a membrane impermeable thiol-reactive reagent (maleimide) coupled to biotin in living cells. Binding to the biotin by streptavidin coupled to a fluorophore (in this case, Alexa Fluor 568, chosen because its emission band is easily separable from that of eGFP) is used to determine whether a given amino acid position is present on the extracellular surface of prestin.

2. Materials

2.1. Cell Culture and Transient Transfection (FRET/FLIM, SCAM)

1. Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biomedics, Burlington, CA).
2. 0.25% trypsin, IX.
3. Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA).
4. 25 cm² cell culture flask for cell culture maintenance.
5. 35 mm glass-bottomed culture dishes (MatTek, Ashland, MA) for experiments.
6. Human embryonic kidney (HEK) 293 cell line (ATCC, Manassas, VA)
7. eGFP-N2 plasmid (Clontech)
8. Cre-lucian-CFP (cCFP) plasmid, obtained by material transfer agreement from Dr. D. Piston laboratory, Vanderbilt University (18)
9. Venus-YFP (vYFP) plasmid, obtained by material transfer agreement from the A. Miyawaki laboratory, Brain Science Institute, RIKEN, Saitama, Japan (19)

2.2. Mutagenesis (SCAM)

1. QuickChange II XL Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA)
2. Template DNA - cDNA (gerbil preS1), obtained as a gift from Jian Zuo, cloned into plasmid vector EGFP-N2 (Clontech, Mountain View, CA) to generate a gerbil preS1-EGFP fusion protein (see Note 1)
3. Mutagenic primers are designed according to Stratagene primer guidelines. Primers should be 25-45 base pairs in length, not directly overlapping. The mutated base pairs should also be located in the middle of the primers. Primers should be phosphorylated at the 5’ end.
4. MyCycler Personal Thermal Cycler (Bio-Rad, Hercules, CA)

2.3. Maleimide Labeling (SCAM)

1. Dulbecco’s Phosphate Buffered Saline (DPBS) (Invitrogen)
2. NADH-maleimide-propargyl) biocytin (bionin maleimide) (Molecular Probes, Eugene, OR). Prepare a 1–10 mM stock solution in dimethyl sulfoxide (DMSO)
3. 2-mercaptoethanol
4. Phosphate Buffered Saline (PBS): 22 mM NaH2PO4, H2O, 78 mM anhydrous Na2HPO4, 1 L distilled water, 0.1 g thimerosal (as an antimicrobial)
5. Paraformaldehyde (PFA): Prepare at a fresh 4% (w/v) solution in PBS
6. Wash buffer: 0.1% bovine serum albumin (BSA) in PBS
7. Streptavidin-Alexa Fluor 568 conjugate (Molecular Probes): Prepare a 1:200 working solution in wash buffer from a 1:1 stock in distilled water.

2.4. Confocal Microscopy (FRET/FLIM, SCAM)

1. A Zeiss LSM 510 META NLO scanning confocal microscope (Zeiss Microscoping, Thornwood, NY) with a 63X/1.4 numerical aperture oil objective, modified by addition of a 1280U photomultiplier detector (Hamamatsu, Bridgewater, NJ) coupled to a single photon counter board (SPC; Becker & Hickl, Berlin, Germany)
2. A META detector (available as a component of the confocal microscope), consisting of a band of 32 narrow band (10.2 nm) detectors covering the range 360–700 nm
3. Methods

3.1. Construct Generation for FRET and FLIM

1. Generate constructs containing protein coding sequences for the specific protein constructs of interest. Also, construct or obtain from another investigator a plasmid vector expressing a construct consisting of cCFP and vYFP linked by a short linker sequence (e.g., GGPGG) containing a central proline. This construct will bring cCFP and vYFP into proximity and will thereby serve as a positive control for FRET. As a negative control, consider a fusion construct of vYFP and a related protein not found in the same tissue.

2. Human embryonic kidney cells (HEKs) are plated onto 35 mm glass-bottomed culture dishes. Once cells reach 80-90% confluency (2-3 days) they are transfected with various cCFP and/or vYFP coupled constructs using Lipofectamine™ 2000 reagent. Our optimum transfection efficiency is achieved with a DNA: Lipofectamine™ 2000 ratio of 4 μg (total):10 μl respectively (see Note 2). After an 18 h incubation period, the medium is replaced with DMEM.
culture medium (see Section 2.1, step 1). Transfected cells are ready for fixation 24–48 h post addition of the transfection reagents.

3. HEK cells are fixed with 4% PFA in PBS for 30 min, mounted using MMB, and covered with glass coverslips. To preserve fluorescence, dishes are sealed with rubber cement (see Note 3) and stored at 4°C.

1. Linear unmixing is used to separate spectra of overlapping fluorophores when both are excited by the same excitation wavelengths. Both CFP and YFP are excited by 458 nm. Spectra are acquired at each pixel of an image using the META detector. The spectra at each pixel is considered to consist of the weighted sum of the spectra of the two fluorophores present, i.e.,

$$S(\lambda) = A \times \text{Donor}(\lambda) + B \times \text{Acceptor}(\lambda),$$

for wavelengths $\lambda = \lambda_1, \lambda_2, \ldots, \lambda_n$.

2. Donor and acceptor spectra are obtained from cells transfected with the CFP construct only or the YFP construct only, using the 458 nm laser line and the META detector set to collect 472–643 nm fluorescence (see Note 5).

3. A pre-acceptor photobleaching CFP/YFP image from a cell with both CFP and YFP labeled protein(s) is acquired under the above acquisition parameters.

4. Linear un-mixing. The CFP and YFP specific portions of this image are assigned to separate profiles using Zeiss LSM software or Excel to unmix the overlapping spectra using the spectra acquired in Section 3.2, step 2 (Fig. 22.A–C).

5. Acceptor (YFP) photobleaching using a 514 nm laser line and band pass filter of 535–590 nm is performed in one or more regions of interest (ROIs) containing CFP/YFP fluorescence. Consecutive scans are used to reach at least an 80% decrease in YFP intensity.

6. A post-acceptor photobleaching CFP/YFP image is acquired and unmixed as described above (see Section 3.2, step 3) (Fig. 22.1D–F).

7. FRET is detected as an increase in CFP (donor) fluorescence intensity after photobleaching of YFP (acceptor). FRET
Fig. 22.1. Intensity-based measurements of spFRET in a specific ROI (dashed line) of cells transfected with plasmids expressing prelin-FP C-terminal constructs. Prelin-CFP (A1) and prelin-YFP (A2) fluorescence intensity before (A, B) and after (C, D, E) prelin-YFP photobleaching (F, F merged image). The observed increase in prelin-CFP fluorescence after acceptor photobleaching indicates intermolecular FRET between prelin molecules and, in turn, indicates prelin multimerization. Scale bar = 20 μm.

Efficiency $E$ is calculated as:

$$E = \left( \frac{(A_1 - A_0)}{A_0} \right) \times 100\%,$$

where $A_1$ and $A_0$ are respectively the cCFP fluorescence intensities after and before YFP photobleaching in the ROI. cCFP fluorescence intensities of non-photobleached ROIs in the same scan window are used to calculate potential baseline shifts in cCFP intensity occurring during the imaging process. These values can be used to normalize the data set.

1. A pre-acceptor photobleaching cCFP fluorescence lifetime image is acquired with 800 nm fs-duration pulses from a tunable Chameleon ULTRA Titanium:Sapphire laser with a pulse repetition rate of 80 MHz using the Zeiss confocal microscope (Fig. 22.2A). Emitted photons are filtered by a 580 nm short-pass filter and detected with a R3809U photomultiplier detector. A model SPC-40 board, synchronized with the laser pulses and the confocal scan signal, is used to measure photon emission latency. Photons are accrued for 60-120 s (see

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Note 6): Photon count rates are kept well below 1 photon per pulse to avoid double counting artifacts.

2. Acceptor (vYFP) photobleaching is performed as previously described in Section 3.2, step 3 (Fig. 22.2B).

3. A post-acceptor photobleaching lifetime image is acquired as previously described in Section 3.3, step 1 (Fig. 22.2C).

4. Photon latency histograms, displaying accumulated pixel information from specific ROIs before and after acceptor photobleaching, are generated for each pixel in the ROI with SPCImage. Mean lifetime distributions (in picoseconds) weighted by intensity for each ROI are fit with two time constants with a $\chi^2$ value between 1.0 and 2.0. FRET efficiency $E$ is calculated as:

$$E = \left[1 - \frac{\tau_{DA}}{\tau_D}\right] q_i$$

where $\tau_D$ and $\tau_{DA}$ are the lifetimes of the donor alone (post-vYFP photobleaching) and the donor in the presence of the acceptor (pre-vYFP photobleaching), respectively.

3.4. Mutagenesis

1. A predicted extracellular loop residue in the protein of interest should be mutated to a cysteine residue with the QuickChange II XL Site Directed Mutagenesis kit. A mutation to a cysteine residue in a predicted intracellular loop can act as a negative control (see Note 7).

3.5. Preparation of Samples for SCAM

1. HEK 293 cells are maintained in DMEM supplemented with 10% FBS on 25 cm$^2$ tissue culture flasks. After cells reach 80% confluence, they are removed from the cell culture flask with trypsin and split 1/10 on 35 mm culture dishes for the experimental procedure. A 1/10 split will provide experimental dishes that are ready for transfection in 48 h.

One experimental dish is sufficient for each wild type and
mutant construct. Make two dishes for negative controls (see Note 8).
2. Transfect each dish with 4 μg of either WT or mutated DNA using 10 μl Lipofectamine 2000™ according to Invitrogen’s recommendations. Check cells for GFP synthesis in 48 h.
Mock transfect (Lipofectamine only, no DNA present) the negative control dishes.
3. Rinse cells three times with DPBS. The medium must be completely aspirated.
4. Incubate the cells with biotin maleimide (obtained from the 1-10 mM stock solution in DMSO) dissolved in PBS to a final concentration of 50 μM for 10 min at room temperature. The concentration of DMSO in PBS must not exceed 1% (v/v).
5. Treat cells for 1 min with 2-mercaptoethanol to a final concentration of 14 mM to remove excess maleimide.
6. Aspirate the solution from the cells. Rinse cells thoroughly with PBS three times for 5 min each.
7. Fix cells in fresh 4% PFA for 30 min at room temperature.
8. Remove the PFA and rinse cells in wash buffer three times for 5 min each.
9. Under light restricted conditions at room temperature, incubate cells in streptavidin-Alexa Fluor 568 (1:200) for 1 h.
10. Discard the streptavidin-Alexa Fluor 568 and rinse the cells with wash buffer three times for 5 min each.
11. Mount the cells with MMA and cover with a round glass coverslip. Remove excess mounting media. Seal the cover slip with rubber cement. Store dishes at 4°C in the dark.

3.6. Confocal Microscopy for SCAM

1. The samples are viewed on a Zeiss LSM 510 META NLO scanning confocal microscope equipped with a 63X oil objective. GFP is excited using a 488 nm laser line and collected with a band pass filter of 500-530 nm. Streptavidin-Alexa Fluor 568 is excited using a 543 nm laser line and collected with a band pass filter of 565-615 nm.
2. For integral membrane proteins such as prestin, the PMs of 10-12 cells are imaged in each experimental group to determine the optimal imaging parameters. These parameters are averaged within and between each experimental group, and are used to acquire all of the subsequent images. For each experimental group, images of 20 transfected cells are acquired for data analysis.

3.7. Analysis of Fluorescence for SCAM

1. Raw pixel intensity data exported from GFP (green channel) and biotin maleimide-streptavidin-Alexa Fluor 568 (red channel) are used to compare fluorescence intensities within and between experimental groups.
2. Fluorescence intensities are compared by averaging peak fluorescence amplitudes from two or more representative PM regions using ImageJ software (Fig. 22.3). (See Note 9).

3. The ratio of Alexa Fluor 568 intensity to GFP intensity (see Note 10) is compared between experimental groups. Addition of a cysteine to the extracellular loop should increase the ratio of Alexa Fluor 568 fluorescence intensity to GFP fluorescence intensity, while additions to the intracellular loops or transmembrane regions will not.

4. Notes

1. Gerbil (or rat) prestin are the preferred prestin models. Mouse prestin, for unknown reasons, does not reliably incorporate into the membrane of HEK 293 cells.

2. Optimum transfection efficiency may require alteration of the DNA (μg): Lipofectamine™ 2000 ratio.

3. Use of acetone-based sealants will reduce or eliminate fluorescence from fluorescent proteins.
4. A third spectrum, collected from fixed untransfected cells, may be used for background subtraction by unmixing.
5. Detector settings and fluorophore absorptions will vary with the FRET pair.
6. Although increasing photon collection can increase the data available for calculating lifetimes, prolonged imaging can also lead to fluorophore bleaching.
7. Mutated proteins must be functional (structural effects are minimal).
8. The first negative control is incubated with biotin maleimide and then streptavidin-Alexa Fluor 568, in the absence of plasmid transfection. The second negative control is transfected but incubated with streptavidin-Alexa Fluor 568 alone.
9. In the first negative control, in which biotin maleimide and streptavidin-Alexa Fluor 568 is added and there is no plasmid transfection, there should be minimal fluorescence. The first negative control consists of the background fluorescence of native HEK 293 integral membrane proteins with cysteines in their extracellular loops. In the second negative control (Streptavidin-Alexa Fluor 568 alone), there should be no fluorescence in the red channel.
10. The GFP intensities are compared between groups to ensure there are no significant differences in protein insertion in the PM between experimental groups.

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