Chapter 6

Probing Protein Distribution Along the Nuclear Envelope In Vivo by Using Single-Point FRAP

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Abstract

Determining the locations of nuclear envelope transmembrane proteins and their concentrations across the outer and inner nuclear membranes has been a challenging and time-consuming process. Typically, this required the week-long process of fixing and immunogold staining of cells prior to analysis by electron microscopy. Here, we describe a method, single-point fluorescence recovery after photobleaching (spFRAP), which is able to quickly determine the localization and distribution of nuclear membrane proteins along the double nuclear envelope membranes with a precision of 10–15 nm in a matter of 10–20 min the day after transfection.

Key words Nuclear envelope transmembrane proteins, Fluorescence recovery after photobleaching, Single molecule, Super-resolution

1 Introduction

The nuclear envelope (NE) is comprised of three distinct membrane systems: the outer nuclear membrane (ONM) that is continuous with the endoplasmic reticulum, the inner nuclear membrane (INM) where many membrane proteins that organize and regulate the nucleus reside, and the pore membrane where ONM and INM are connected and nuclear pore complexes (NPCs) are inserted. Determining the distribution of NE transmembrane proteins (NETs) between these distinct membrane compartments has historically been challenging, requiring the counting of immunogold-labeled NETs by electron microscopy [1, 2], which yields no dynamic information and could be undercounted due to epitope masking or steric factors. NETs play many important roles in the organization and structure of the NE, nucleus, and cytoskeleton [3–10]. Mutations in these proteins have been linked with a variety of debilitating diseases [11–14]. Therefore, determining the localization and concentration of transmembrane proteins to the INM is extremely important. Recently, the groundbreaking

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techniques of single-molecule tracking and super-resolution light microscopy are revolutionizing the field of molecular biology by providing unprecedented spatial resolutions. These techniques are able to break the diffraction-limit barrier of conventional light microscopy by localizing single molecules or reducing point spread function of fluorophores [15]. Fluorescence recovery after photobleaching (FRAP) is a widely used technique where a cellular membrane, which contains membrane proteins tagged with a fluorophore, is photobleached with a high-powered laser and the recovery of fluorescence, through the diffusion of the protein of interest, is recorded [16]. Here, we combine single-molecule tracking and FRAP techniques to determine the spatial distribution of NETs in live cells. In general, we first pre-photobleach fluorescently tagged proteins in a small detection area (~0.5 μm), then track fluorescent intact single protein molecules moving into this photobleached area, and finally reconstruct the locations of these protein molecules to form their super-resolution spatial distributions in the NE in vivo. In this way, the distribution of NETs along the NE was determined in vivo with a precision of 10–15 nm in just minutes on the day after cell transfection. Furthermore, by calculating the diffusion coefficient of the NETs in question and using FRAP to determine the immobilized fraction of NETs on the INM, the absolute concentration of NETs on both the ONM and INM can be quantified

2 Materials

All materials are prepared using deionized water and sterile techniques. We also provide specific details of our microscope setup and the detailed approach to achieve the resolution stated here.

2.1 Microscope Components

1. Microscope: Olympus IX81 equipped with a 1.4 NA 100× oil immersion objective (UPLSAPO 100XO, Olympus).
2. CCD camera: On-chip multiplication gain charge-coupled device camera (Cascade 128+, Roper Scientific).
3. Lasers: 35 mW 633-nm He-Ne laser (Melles Griot), 50 mW solid state coherent 488-nm He-Ne laser (Obis).
4. Lamp: Mercury lamp with GFP filter set up.
5. Filters: Dichroic filter (Di01-R405/488/561/635-25x36, Semrock) and an emission filter (NF01-405/488/561/635-25X5.0, Semrock), two neutral density filters (Newport).
6. Optical chopper (Newport) to generate an on-off mode of laser excitation (*see* **Note 1**).
7. Data acquisition and processing: Slidebook software package (Intelligent Imaging Innovations).

8. GLIMPSE software (Gelles lab).
9. ThunderSTORM software [17].

2.2 Tissue Culture

1. Cell line: we use wild-type (WT) HeLa cells.
2. Media: DMEM, high glucose, GlutaMAX™ Supplement (Life Technologies), 10 % fetal bovine serum, 1 % penicillin-streptomycin. DMEM with 1 % penicillin-streptomycin and no FBS was used for transfections (as per the specific manufacturer's protocol; adapt according to whichever transfection reagent is used).
3. 0.25 % trypsin EDTA.
4. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH adjusted to 7.4 with HCl.
5. Flasks and dishes: 25 cm^2 flasks were used to grow WT HeLa cells. Glass bottom dishes (e.g., MatTek Corporation) were used for transfection and for recording single-molecule events after. If other dishes are used, they should have at least equivalent optical properties.
6. Plasmid preparation: Plasmids for transfection were encoding NETs fused to GFP, although other fluorescent protein fusions can be used with appropriate adjustments in laser wavelength and bleaching parameters. Plasmids were prepared using the plasmid miniprep (alkaline lysis) method [18].
7. Transfection: In our case, HeLa cells were transfected using the lipofection technique as specified by Mirus Bio TransIT-LT1 Transfection Reagent protocol (*see* **Notes 2** and **3**). If other transfection reagents are used, they should be certain to not leave autofluorescent material adhered to the glass as this background will interfere with imaging.
8. Transport buffer: 20 mM Hepes, 110 mM KOAc, 5 mM NaOAc, 2 mM MgOAC, 1 mM EGTA, pH adjusted to 7.3 with HCl.

3 Methods

3.1 Tissue Culture and Transfection

1. At least 1 week in advance, using sterile techniques, start a fresh culture of your cell line from a frozen stock (−80 °C) by thawing at 37 °C and putting in a 25 cm^2 culture flask with 5 mL DMEM media (at 37 °C). Place cells into an incubator and incubate at 37 °C with 5 % CO_2 for 24 h in the first instance and split the culture at least three times over the week for the cells to be at optimal health for transfection and optimal optical conditions for live cell microscopy.
2. Using sterile technique, 2 days before the single-molecule experiments, split the cells and plate them onto a glass

bottom dish. Return to the incubator for at least 12 h of growth (*see* **Note 4**).

3. Using the protocol provided by the manufacturer and sterile techniques, transfect cells (in our case, using LT1 transfection reagent) with plasmids coding for the protein of interest. In our case, serum-free DMEM media with the LT1 and plasmid complex was added dropwise to the glass bottom dishes and then gently mixed by moving the dishes side to side so that the solution can distribute homogeneously. Return glass bottom dishes to the incubator and grow for 18+ h at 37 °C with 5 % CO_2 so that the NET-fluorescent protein fusions are expressed at reasonable levels at the time of the experiment.
4. The following day, using sterile technique, remove the DMEM media from the glass bottom dishes. Wash twice using 1 mL of PBS warmed to 37 °C. Following the second wash, add 2 mL of pre-warmed (to 37 °C) transport buffer to the dishes. Note that it is important to use the transport buffer and to allow the cells to equilibrate in it (approximately 45 min) to reduce fluorescent backgrounds for single-molecule work: the phenol red in DMEM interferes with experiments and even some of the media specially designed to reduce background in FRAP experiments yield too high backgrounds for single-molecule work.

3.2 spFRAP Experiment

1. Prior to imaging the sample, both the 488-nm and the 633-nm lasers need to be aligned so that their light paths overlap exactly when they reach the microscope objective (*see* **Note 5**). Generate a diffraction-limit illumination volume (alternatively named illumination point spread function) of the 488-nm excitation laser (≈210 nm in the x and y directions and ≈500 in z direction) by focusing the laser through a high-NA microscope objective (1.4 NA in this particular setup) (Fig. 1).
2. Using a 100× oil immersion objective, visualize the cells using a mercury lamp with the filter setting adjusted for GFP visualization.
3. Only cells with good nuclear morphology (*see* **Note 6**) should be targeted. Adjust the focus so that the equator of the cell is targeted and such that the laser focus is on the left or right edge of the NE (tangent to the edge of NE that is being targeted).
4. Using the lamp, set the 500-ms exposure time to take a "before" photobleaching image of the cell while avoiding overexposure and saturation of the image.
5. Close the port to the mercury lamp and switch to the lasers. First, using the 633-nm laser, align the laser to the intended area of photobleaching. Close the port to the 633-nm laser and open the port to the 488-nm laser to begin photobleaching.

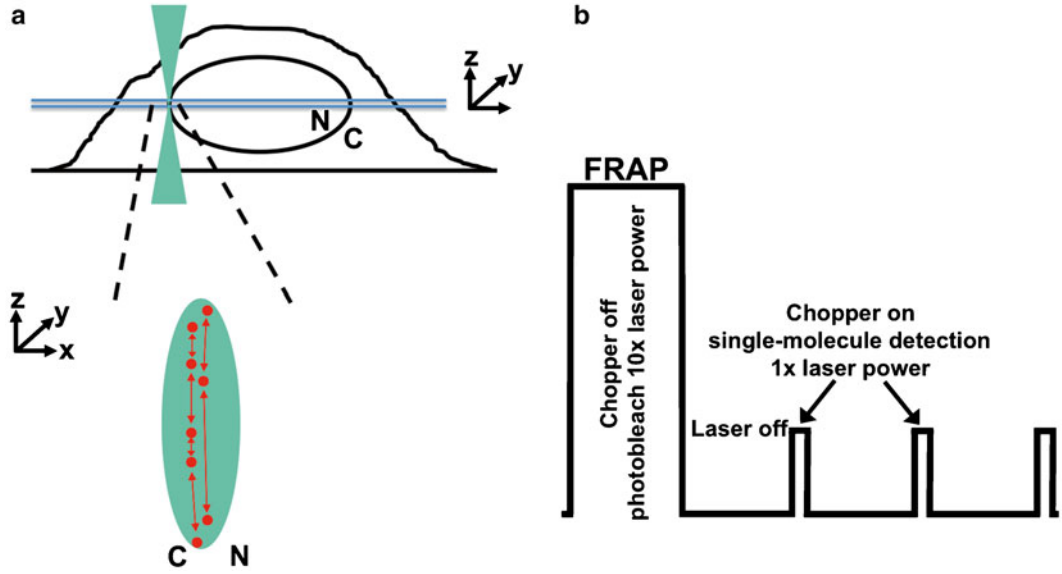

Fig. 1 Single-point illumination and FRAP used to detect transmembrane proteins on the NE. (**a**) Imaging of single-molecule events of NETs along the NE by single-point spFRAP microscopy. The focal plane is between the *two light blue lines. C* cytoplasm; *N* nucleus. (**b**) A laser power with tenfold difference was used to photobleach and detect in the experiments. An optical chopper was used to regulate the laser to have an on-off mode. The longer off time allowed GFP-NETs outside the photobleached area to have enough time to diffuse into the detection area

6. Using high laser power (*see* **Note 7**), photobleach a small region of the NE. The bleached region should be around 0.5 μm in diameter.
7. For subsequent detection, lower the laser power by at least tenfold using the neutral density filter between the laser and the sample (*see* **Note 8**).
8. Engage the optical chopper at 2-Hz rotation speed with an on time of 1/10 of the total frames recorded.
9. Record videos at a 0.4-ms frame rate for 30 s. Ten total videos were taken consecutively. In our setup, we use Slidebook software for recording images and videos.
10. Once recording is finished, switch back to the mercury lamp. Take an "after" picture while avoiding overexposure and oversaturation of the image and compare with the "before" picture to make sure the NE did not shift during our measurements (*see* **Note 9**).

3.3 Bulk FRAP Experiment

1. Prior to imaging the sample, both the 488-nm and the 633-nm lasers need to be aligned so that their light paths overlap exactly when they reach the microscope objective (*see* **Note 5**). Generate a 5-μm illumination area in the focal plane by inserting a defocus lens before the objective.

2. Using a 100× oil immersion objective, visualize the cells using a mercury lamp with the filter setting adjusted for GFP visualization.
3. Only cells with good nuclear morphology (*see* **Note 6**) should be targeted. Adjust the focus so that the equator of the cell is targeted and such that the illumination area is on the left or right edge of the NE.
4. Using the lamp, set the 500-ms exposure time to take a "before" photobleaching image of the cell while avoiding overexposure and saturation of the image.
5. Close the port to the mercury lamp and switch to the lasers. First, using the 633-nm laser, align the laser to the intended area of photobleaching. Close the port to the 633-nm laser and open the port to the 488-nm laser to begin photobleaching.
6. Using high laser power (*see* **Note 7**), photobleach a fraction of the NE. The bleached region should be around 5 μm in diameter.
7. Imaging parameters may be adjusted for a particular set of proteins being investigated based on their recovery times. In general, these should be based on obtaining images with a high-intensity signal output from the start of the time-lapse pictures to the end FRAP recovery. It is important to select an exposure time that will give a high-intensity signal output without causing too much photobleaching and a decrease in the overall signal by the end of the recovery phase (*see* **Note 10**).
8. After photobleaching, switch from the laser to the mercury lamp and take an "after" photobleaching picture. Compare the "before" and the "after" pictures to make sure there is no significant shift of NE during the detection.
9. Convert time-lapse data into individual .tiff files using Slidebook and then analyze them using the ImageJ plugin FRAP Profiler as described in Subheading 3.4.2.

3.4 Data Analysis

1. Convert images from videos generated with Slidebook into multiple .tiff files.

3.4.1 Analysis of spFRAP Data

2. Open the files using the ImageJ plugin ThunderSTORM and filter the raw data for a high signal-to-noise ratio (SNR) with parameters including single-molecule intensity (>2000) and Gaussian width (between 0.5 and 1.5) of single-molecule spots.
3. From the data prefiltered by ThunderSTORM, determine the average x and y pixel positions to find the appropriate ROI to be covered when further running the GLIMPSE software (*see* **Note 11**).
4. In GLIMPSE, use nine ROIs covering all the possible x and y pixel positions determined in the previous step and select just

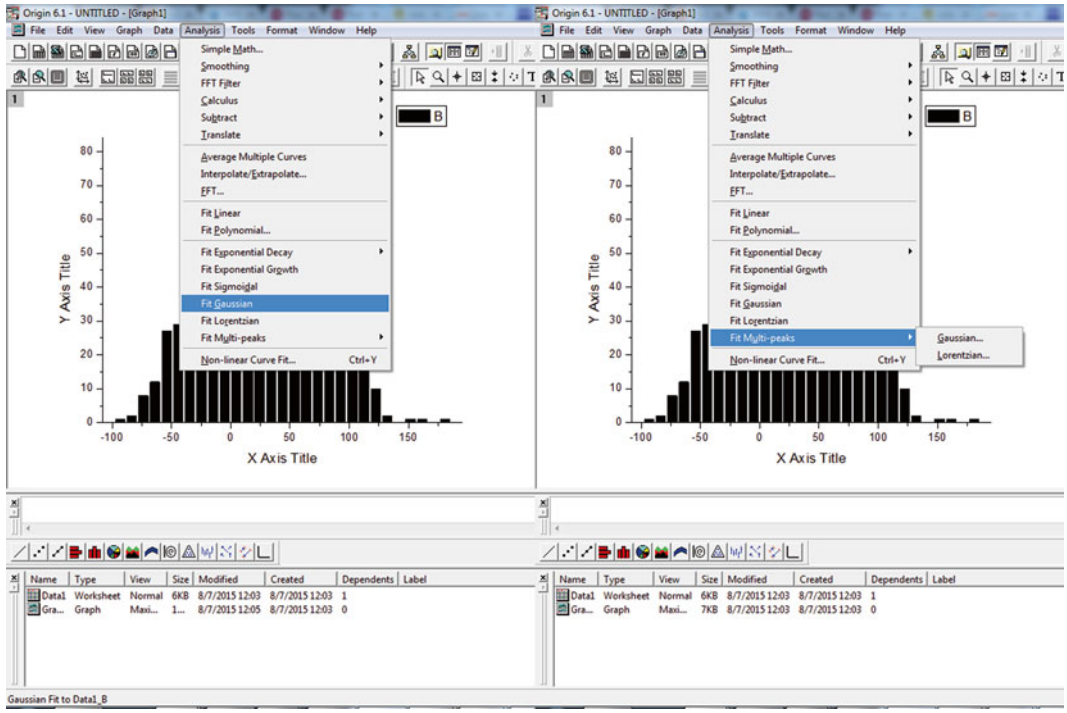

Fig. 2 Screen shots showing how to fit histogram data with single and two-peak Gaussian functions

the frames during which the optical chopper was "open" and the NE was exposed to the laser.

5. Run GLIMPSE software using nine ROIs to localize each point in the detection area.
6. Raw data from GLIMPSE is then filtered using a high SNR, determined by using the intensity of single-molecule spot and by selecting for points in the focal plane of the microscope by using the width of the Gaussian fittings from GLIMPSE (height and width respectively). Generally, data with a GLIMPSE-generated height value higher than 2000 and width between 0.5 and 1.5 is used (*see* **Note 12**).
7. Fit the resultant data with a single Gaussian function to remove the background noise (*see* **Note 13**) (Figs. 2 and 3).
8. Next, further fit the subtracted data with a two-peak Gaussian to determine the distribution of the NEt along the NE (Figs. 2 and 3).
9. By using the full width at half maximum (FWHM) as determined by data analysis software (a built-in function in Origin 6.1 is used here), the range between where points can lie along either side of the NE can be determined for further analysis such as diffusion coefficient and protein concentration along the NE.

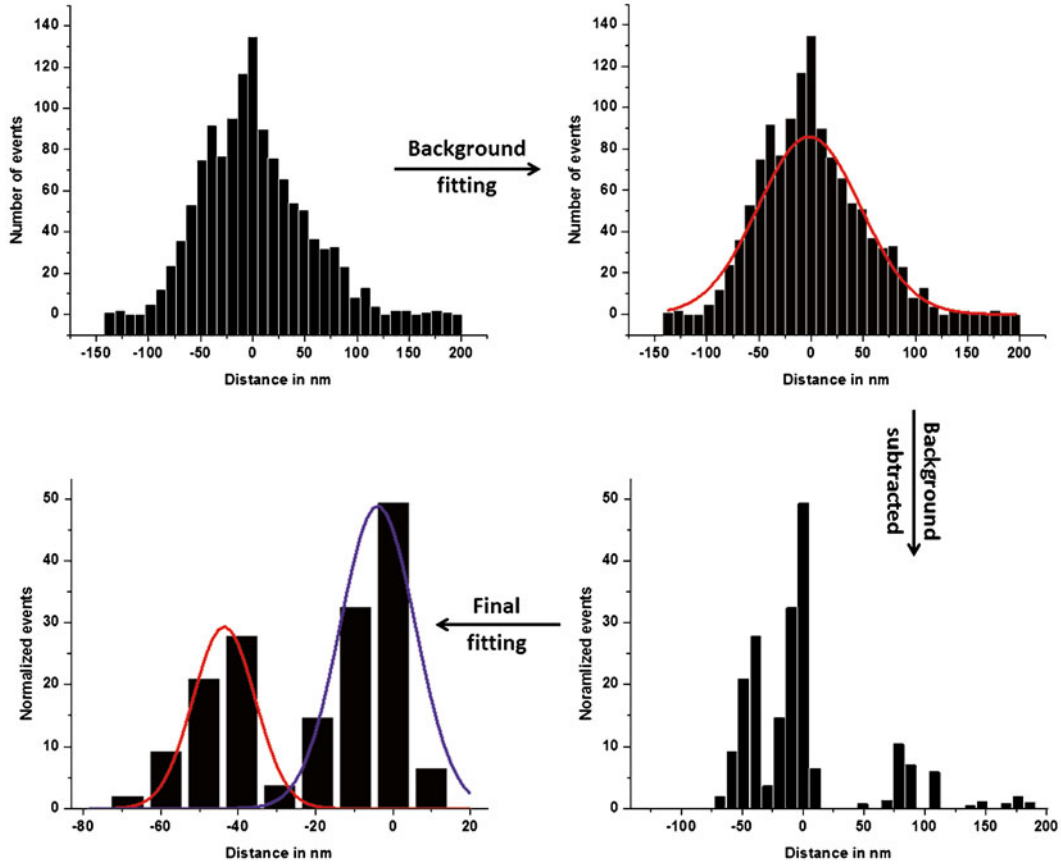

Fig. 3 Histograms were prepared from the raw data. The background noise (without including the data of two peaks) was fit with a single Gaussian function (shown in *red*). Next, the background was subtracted from the raw data to generate a histogram of the normalized data with two clear peaks. Finally, the resultant data was then fit with a two-peak Gaussian function to determine the localization of NETs on the NE (INM shown in *red* and ONM shown in *purple*)

3.4.2 Analysis of Regular FRAP Data

1. Open the FRAP recovery images in sequence starting with the "before" photobleach picture, followed by the "after" photobleach picture, and then the sequence of time-lapse images of recovery.
2. Open up the ROI manager (Analyze/Tools/ROI Manager) and select the two regions of interest. The first ROI is the region that was photobleached and the second ROI is the nuclear envelope, which must be carefully outlined with the freehand selection tool.
3. Run the FRAP Profiler plugin and use the normalized output to determine the immobilized fraction.

4 Notes

1. The speed of the chopper was set to 2 Hz rotation with an on time of 1/10 of the total frames recorded.
2. A 3:1 ratio of LT1 transfection reagent to plasmid resulted in the best transfection efficiencies for our HeLa cells.
3. Electroporation techniques were also used for transfection, resulting in similar final spFRAP results.
4. The cells plated here should be about 50 % confluent after the 12 h growth period in order to achieve optimal transfection results. If the cells from a 25 cm^2 dish are ~90–95 % confluent, trypsinize the cells and resuspend in 5 mL media before adding 300 μL of the suspended HeLa cells to the glass bottom dish.
5. Only the 488-nm laser that is being used to excite the fluorophore needs to be precisely focused, whereas the other laser, 633-nm, will be used only to align the 488-nm for single-molecule measurements. The 633-nm laser is used for alignment because it does not excite the GFP-tagged NET and maybe used as a reference point to know the alignment of the 488-nm laser on the NE in the region of interest. While a 633-nm laser is used in these experiments, any other laser that similarly doesn't excite GFP can be used. A glass slide coated with Alexa Fluor 647-labeled GFP is used to focus and align the lasers.
6. Only cells in interphase should be selected. Cells that seem to be in the middle of mitosis or cells with NEs that are not circular in shape should be avoided.
7. A laser power of 20 mW was used to photobleach the NE for 30 s.
8. You must select the best power for your system in order to get a high signal with reduced background noise. A range between 2 mW and 50 μW can be used in these experiments with our setup.
9. "Before" and "after" pictures can be overlapped quite easily and used to compare the movement of the NE during the imaging time. Ideally, there should be no movement of the NE during the video acquisition. Data from cells that exhibit obvious movements during data collection should be discarded. These pictures are taken with the mercury lamp to obtain wide-field epifluorescent images.
10. It is best to try and use a set exposure time for each group of NETs studied in an experiment. For instance, if three different NETs are being studied and compared, one should use the same exposure time for the time-lapse imaging of each NET. The authors have found that a 2 s exposure time with an 18 s delay between images works best.

11. The filtering in Subheading 3.4.1 **step 2** by using the ImageJ plugin ThunderSTORM was done so that the locations with good S/N ratios from which single-molecule data was collected by spFRAP can be determined. The GLIMPSE software uses ROIs in the region of data prefiltered by ThunderSTORM to further determine the detailed XY distribution with high spatial localization precision.
12. It has been determined that GLIMPSE-generated data with Gaussian width between −0.5 and 0.5 is noise and should be eliminated.
13. The Origin software package was used to perform data analysis, but any powerful data analysis software may be used in its place (refer to Fig. 3).

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