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Determination of Membrane Protein Distribution on the Nuclear Envelope by Single-Point Single-Molecule FRAP

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Abstract

Nuclear envelope transmembrane proteins (NETs) are synthesized on the endoplasmic reticulum and then transported from the outer nuclear membrane (ONM) to the inner nuclear membrane (INM) in eukaryotic cells. The abnormal distribution of NETs has been associated with many human diseases. However, quantitative determination of the spatial distribution and translocation dynamics of NETs on the ONM and INM is still very limited in currently existing approaches. Here we demonstrate a single-point single-molecule fluorescence recovery after photobleaching microscopy technique that enables quick determination of distribution and translocation rates for NETs *in vivo*.

Keywords

Cellular imaging; membrane biophysics; super-resolution; transmembrane protein; FRAP

INTRODUCTION

In eukaryotic cells, the nuclear envelope (NE) is a double-membrane system composed of the outer nuclear membrane (ONM) which folds in on itself to form the inner nuclear membrane (INM). The ONM is continuous with the endoplasmic reticulum (ER) and faces the cytoplasm, whereas the INM is lined with lamina and faces the nucleoplasm. In both the INM and ONM reside many different types of transmembrane proteins, collectively referred to as nuclear envelope transmembrane proteins (NETs). These membrane proteins are major components of the NE, and it is vital to understand their spatial location on the NE and their translocation rates between the two membranes in order to fully understand their role in genome architecture, epigenetics, transcription, nuclear structure, organization, and positioning (Arib a& Akhtar, 2011; Burns & Went, 2012; Dauer & Worman, 2009; de las Heras et al., 2013; Gruenbaum, Margalit, Goldman, Shumaker, & Wilson, 2005; Heessen & Fornerod, 2007; Hetzer & Went, 2009; Méndez-López & Worman, 2012; Schreiber & Kennedy, 2013; Wilson & Foisner, 2010; Worman & Dauer, 2014; Zuleger, Kerr, & Schirmer, 2012; Zuleger, Korfali, & Schirmer, 2008).

Determining the distribution NETs on the NE has historically been a challenging and time-consuming process, requiring the counting of immunogold-labeled NETs by electron microscopy (EM) (Ellenberg et al., 1997; Wilhelmsen et al., 2005). However, this EM-based method yields almost no dynamic information about NETs, and, furthermore, epitope masking or steric factors may lead to undercounting (Huang, Bates, & Zhuang, 2009).

Recently, the groundbreaking techniques of single-molecule tracking and super-resolution light microscopy are revolutionizing the field of biological or biomedical imaging by providing unprecedented spatial resolutions. The techniques generally fall into two broad categories: optics-based super-resolution techniques, which generate sub-diffraction illumination volume due to nonlinear optical response of fluorophores in samples, and algorithm-based super-resolution techniques, which utilize mathematical functions to localize the centroids of fluorophores and then reconstitute these centroids to form super-resolution images. Additionally, 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 (Axelrod, Koppel, Schlessinger, Elson, & Webb, 1976). Here, we combine single-molecule tracking, super-resolution imaging, and FRAP techniques to determine the spatial distribution of NETs in live cells (Mudumbi, Schirmer, & Yang, 2016). In general, we first pre-photobleach fluorescently tagged proteins in a small detection area ($\sim 0.5 \mu\text{m}$), after which we track fluorescently intact single protein molecules moving into this photobleached area. Finally, we 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 has been determined *in vivo* with a precision $< 10 \text{ nm}$ in a matter of 10 to 20 min 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, as well as the translocation rate of NETs from one membrane to the other.

BASIC PROTOCOL 1

TISSUE CULTURE AND PREPARATION OF CELLS FOR SINGLE-MOLECULE AND CONFOCAL MICROCOPY MEASUREMENTS

This protocol will discuss the preparation of cells for both single-molecule and confocal microscopy measurements. Once HeLa cells are grown, plated, and transfected, they must be incubated with transport buffer (see recipe) to reduce both background fluorescence, as well as cell and nuclear envelope movements prior to microscopy experiments.

Materials—

- HeLa cells (American Type Culture Collection)
- Complete DMEM medium (see recipe)
- TransIT-LT1 Transfection Reagent (Mirus Bio, see manufacturer's protocol)
- Serum-free DMEM medium (see recipe)
- Transport buffer (see recipe)
- 0.25% trypsin/EDTA
- 1× PBS (see recipe)
- 25-cm² culture flasks

37°C, 5% CO₂ humidified incubator

Glass bottom dishes (MatTek Corporation)

1. At least 1 week in advance, start a fresh culture of your cell line from a frozen stock (−80°C) by thawing at 37°C and putting in a 25-cm² culture flask with 5 ml of 37°C complete DMEM medium. Place cells into an incubator and incubate at 37°C with 5% CO₂ for 24 hr. Split the cells at this time, and continue to split the culture at least three times over the week when the cells reach 60% to 80% confluency.

Sterile technique must be used during the initial thawing and plating process. This will ensure that the cells will be at optimal health for transfection and optimal optical conditions for live cell microscopy imaging.

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 hr of growth.
3. Using the protocol provided by the manufacturer and sterile techniques, transfect cells using the LT1 transfection reagent with the plasmids coding for the protein of interest. Use a 3:1 ratio of reagent to plasmid. Add serum-free DMEM medium with the LT1 and plasmid complex dropwise to the glass bottom dishes and then gently mix by moving the dishes from side to side so that the solution can distribute homogeneously. Return glass bottom dishes to the incubator and grow for at least 18 hr at 37°C with 5% CO₂ 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 medium from the glass bottom dishes. Wash cell monolayer 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.

It is important to allow the cells to equilibrate in the transport buffer approximately 45 min. This will help reduce background fluorescence for single-molecule work, since 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.

BASIC PROTOCOL 2

SINGLE-POINT SINGLE-MOLECULE FRAP EXPERIMENT

The NETs of interest are visualized by a light-diffraction-limited illumination spot (point spread function (PSF)) with the use of high numerical aperture (NA) objective. GFP tagged NETs are then photobleached in a 0.5-μm area on the NE through the use of a high laser power (488-nm excitation laser in the case of GFP) (Fig. 21.11.1A-H). Once the area is sufficiently bleached, the laser beam with two-fold less power is used to image and track

single fluorescently tagged NETs with intact fluorescence, which diffuse into the photobleached area from the neighboring regions outside the photobleached area (See Fig. 21.11.1I).

Materials—

Glass slide

Immersion oil

Microscope: Olympus IX81 equipped with a 1.4 NA 100× oil immersion objective (UPLSAPO 100XO, Olympus)

CCD camera: On-chip multiplication gain charge-coupled device camera (Cascade 128+, Roper Scientific)

35 mW 633-nm He-Ne laser (Melles Griot)

50 mW solid state coherent 488-nm laser (Obis)

Mercury lamp with GFP filter set up

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)

Optical chopper (Newport)

Slidebook software package (Intelligent Imaging Innovations).

GLIMPSE software (Gelles lab)

ThunderSTORM software

1. Prior to imaging the sample, 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 100× oil immersion objective in this particular setup).
2. 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. If needed, further slightly adjust the lasers so that the focal points of both lasers are aligned on top of each other at the focal plane.
3. Add immersion oil onto the 100× oil immersion objective and place the glass bottom dish with the NETs of interest on the objective.
4. Cells were visualized using a mercury lamp with the filter setting adjusted for GFP visualization.
5. Only cells with good nuclear morphology, where the NE is round and in interphase and the fluorescently tagged protein expresses in abundance, should be targeted (Fig. 21.11.1A). 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).

6. Using the lamp, set the exposure time to ~500-ms to take a "before" photobleaching image of the cell while avoiding overexposure and saturation of the image.
7. 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. Note that the 633-nm laser is used solely for alignment purposes since it cannot excite for GFP tagged NETs. For NETs tagged with a fluorophore that can be excited by the 633-nm laser, use a different wavelength for alignment. This allows us to target the desired area and avoid photobleaching until the appropriate time.
8. Close the port to the 633-nm laser and open the port to the 488-nm laser to begin photobleaching.
9. Using high laser power (10–20 mW), photobleach a small region of the NE for about 30 sec. The bleached region should be around 0.5 μ m in diameter.
10. For subsequent detection, reduce the laser power by approximately tenfold using the neutral density filter between the laser and the sample.
11. Engage the optical chopper at 2-Hz rotation speed with an on time of 1/10 of the total frames recorded.
12. Record videos at a 0.4-ms frame rate for 30 s. Five to ten total videos are taken consecutively. We used the Slidebook software package for recording images and videos.
13. 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.

Step annotations

ALTERNATE PROTOCOL 2 (optional)

ALTERNATE PROTOCOL TITLE

Introductory paragraph

Materials

Protocol steps

Step annotations

SUPPORT PROTOCOL 2 (optional)

SUPPORT PROTOCOL TITLE

Introductory paragraph

Materials

Protocol steps

Step annotations

BASIC PROTOCOL 3, etc. (add additional protocols as needed, optional)

BASIC PROTOCOL 3

BULK FRAP IMAGING WITH CONFOCAL MICROSCOPY

Bulk FRAP is a technique typically employed to determine the diffusion coefficient and immobilized fraction of membrane proteins (Axelrod et al., 1976). Here, HeLa cells transfected with the fluorescently tagged NET of interest are used to determine the immobilized fraction of NETs on the NE. Similar to the smFRAP step, cells must be incubated for 45 min with transport buffer prior to performing bulk FRAP experiments. Next, cells with a good NE morphology that express the GFP fused NETs are selected and photobleached at a high laser power before time lapse images are taken at a lower power to analyze the return of fluorescence to the photobleached area.

Materials—

Immersion oil

Leica DM IRE2 confocal microscope

TCS SL software package

ImageJ

FRAP Profiler ImageJ plugin

1. Using a 100× oil immersion objective, visualize the cells using a 488-nm argon laser at 20% laser power. Only cells with a good nuclear morphology that express the GFP fused NETs well should be used in experiments. Adjust the focus so that the equator of the nuclear envelope is in focus.
2. Take a "before" photobleaching image of the cell while avoiding overexposure and saturation of the image.
3. Select a region of interest (ROI) on the nuclear envelope and adjust the argon laser to 100% power and photobleach an area of $\sim 5 \mu\text{m}^2$ for 5 sec (or until the region selected no longer has any fluorescence).
4. Return the argon laser to 20% power and take time lapse images of the nuclear envelope every 5 sec for 5 min (or until fluorescence recovery reaches a plateau).
5. Convert the data to .tiff files and open in ImageJ starting with the "before" photobleach image and ending with the final image of the time lapse.
6. Using the ImageJ, open up the ROI manager (Analyze/Tools/ROI Manager). Select two regions of interest: 1) The region that was photobleached, and 2) The nuclear envelope which must be selected using the freehand selection tool.

7. In ImageJ, run the FRAP Profiler plugin and use the normalized output to determine the immobilized fraction.
8. To determine the immobilized fraction on the ONM and INM, use the equation provided in Supplemental Fig. 6 by Mudumbi et al., 2016 (Mudumbi et al., 2016)

Step annotations

BASIC PROTOCOL 4

SINGLE-MOLECULE DATA ANALYSIS

This section will discuss the process of running both ThunderSTORM and GLIMPSE to analyze the data generated from the single-point smFRAP technique discussed in Basic Protocol 2. Furthermore, it will illustrate the data selection methods used to obtain the most precisely localized NETs.

Materials—

ThunderSTORM Plugin for ImageJ (Ovesný, Křížek, Borkovec, Švindrych, & Hagen, 2014)

Glimpse software package (Gelles Lab)

Origin 6.1

1. Convert images from videos generated with Slidebook (smFRAP data generated from Basic Protocol 2) into multiple .tiff files.
2. In ImageJ, run the plugin ThunderSTORM using the .tiff files generated in the previous step.
3. 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.
4. From the data prefiltered by ThunderSTORM, determine the average x and y pixel positions to find the appropriate ROIs that need to be covered when running the GLIMPSE software.
5. In GLIMPSE, use nine ROIs to cover all the possible x and y pixel positions determined in the previous step. Next, select just the frames during which the optical chopper was "open" and the NE was exposed to the laser.
6. Run GLIMPSE software using nine ROIs to localize each point in the detection area. The Raw data output from GLIMPSE is filtered using a high SNR. This is determined by using the intensity of the 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.

7. Using the Origin6.1 software, select the data ± 40 -nm from the highest peak and mask it so that it does not contribute to upcoming Gaussian fitting step. Then, fit the remaining data from GLIMPSE with a single Gaussian function to remove the background noise (Fig. 21.11.2).
8. Next, further fit the subtracted data (the previously masked or excluded data is now included at this point) with a two-peak Gaussian to determine the distribution of the NET along the NE (Fig. 21.11.2).
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 (see Mudumbi et al. 2016 for the relevant formulae).

SUPPORT PROTOCOL 4

FORMULAE USED FOR DIFFUSION COEFFICIENT, SINGLE-MOLECULE LOCALIZATION PRECISION, SPATIAL CONCENTRATION DISTRIBUTION AND TRANSLOCATION RATE FOR NETs

This section will list the various formulae used to determine single-molecule localization precision, diffusion coefficient, as well as the formulae used to correct for diffusion based bias in smFRAP and determine the translocation rate of NETs. More detailed explanations can also be found in Mudumbi et al. 2016.

1. Diffusion coefficients were calculated using the following two equations:

$$\text{MSD} = 4Dt \quad (1)$$

$$\rho(\delta, t, D) = \left(\frac{\delta}{2Dt} \right) e^{\left(-\frac{\delta^2}{4Dt} \right)} \quad (2)$$

Equation 1, mean square displacement (MSD) was used consist of multiple frames (>6). Equation 2, frequency distribution probability function, was used if there are at least two, but less than six consecutive frames for the tracked single-molecule (Zuleger et al., 2011). In these equations, δ , t and D are the displacement between consecutive frames, the interval time and the diffusion coefficient respectively. Finally, an averaged diffusion coefficient was determined for each NET.

2. The following formula was used to determine the localization precision of diffusing single-molecules (σ) imaged during smFRAP:

$$\sigma = \sqrt{F \left[\frac{16(s^2 + a^2/12)}{9N} + \frac{8\pi b^2(s^2 + a^2/12)^2}{a^2 N^2} \right]} \quad (3)$$

Here, F is equal to 2, N is the number of collected photons, a is the effective pixel size of the detector, b is the standard deviation of the background in photons per pixel, and $s = \sqrt{s_0^2 + \frac{1}{3}D\Delta t}$, s_0 is the standard deviation of the point spread function in the focal plane, D is the diffusion coefficient of substrate on the membrane of interest (INM or ONM) and t is the image acquisition time (21–24) [*CE: Reference 21–24 are not cited in the reference list.]. We suggest to only spatially localize and superposed targeted molecules with > 2000 signal photons and in-focus Gaussian widths (0.5–1.0 pixel, corresponding to molecule locations in the focal plane) to obtain a precise super-resolution image of the NETs on the NE.

3. To correct the effects of diffusion based bias on the concentration of NETs on the NE, the following formulae were used:

$$G(i, D, t) = \frac{1}{\sqrt{4\pi Dt}} * e^{-\frac{i^2}{4Dt}} \quad (4)$$

$$f(D, t) = \int_R^{R_{max}} 2\pi x dx \int_{x-R}^{x+R} \frac{2R}{2\pi i} G(i, D, t) * di \quad (5)$$

$$V(D) = \int_{t1}^{t1+30} f(t) dt \quad (6)$$

$$\frac{N_{ONM}}{N_{INM}} = \frac{V_{ONM} * C_{ONM}}{V_{INM} * C_{INM}} \quad (7)$$

Where $G(i, D, t)$ represents the probability of finding a randomly diffusing particle at location i after diffusion with a diffusion constant of D within time t ; $f(D, t)$ refers to the probability of observing the particles moving into the detection area in two dimensions from the entire area; $V(D)$ is the total area that a particle covered from $t1$ to $t1 + 30$ s; and V (either ONM or INM) is calculated from the previous function and N (ONM or INM) is determined from the original INM:ONM ratio (Fig. 21.11.3).

4. Translocation rates were determined by using the following formulae:

$$TR = \frac{N_T * a_2 * A}{a_3 * 2\tau_{1/2}} \quad (8)$$

$$a_1 * F_{mi} = A(a_2 * F_{mo}) + B(a_1 * F_{mi}) \quad (9)$$

$$\frac{A}{B} = \frac{a_2 * F_{mo} * D_o}{a_1 * F_{mi} * D_i} \quad (10)$$

Where N_T is the total NET molecules, a_1 , a_2 and a_3 are the INM value, the ONM value and the added INM and ONM value from the INM:ONM ratio, respectively. The variable A represents the fraction of NETs in the ONM that translocate into the INM after FRAP experiments and B is the fraction of NETs in the INM that diffuse into the photobleached area after FRAP. F_{mi} is the mobile fraction on the INM, F_{mo} is the mobile fraction on the ONM. D_o and D_i are the diffusion coefficients on the ONM and INM respectively as determined by single-molecule experiments, and finally, $\tau_{1/2}$ is the time it takes for half the fluorescence recovery during FRAP experiments

REAGENTS AND SOLUTIONS

Use ultrapure water in all recipes and protocol steps.

Complete DMEM medium

DMEM medium, high-glucose (Life Technologies) containing:

1× GlutaMax supplement (Life Technologies)

10% fetal bovine serum (FBS)

1× penicillin/streptomycin (Life Technologies)

Store up to ?? at ??°C

1×PBS

137 mM NaCl

2.7 mM KCl

10 mM Na₂HPO₄

2 mM KH₂PO₄

pH adjusted to 7.4 with HCl

Store at 4°C

Serum-free DMEM medium

DMEM medium, high-glucose (Life Technologies) containing:

1× GlutaMax supplement (Life Technologies)

1× penicillin/streptomycin (Life Technologies)

Store up to ?? at ??°C

Transport Buffer

20 mM Hepes

110 mM KOAc

5 mM NaOAc

2 mM MgOAc

1 mM EGTA

pH adjusted to 7.3 with HCl.

Store at 4°C

COMMENTARY

Background Information

Immunogold-label electron microscopy has been used to determine the localizations of a small set of NETs along the ONM and INM (Ellenberg et al., 1997; Wilhelmsen et al., 2005) (25)[*CE: Reference 25 is not cited in the reference list.]. This approach is very labor intensive, and is, therefore, very impractical to apply in determining the localization and distribution of the hundreds of NETs now identified (de las Heras et al., 2013). More recently, several super-resolution microscopy techniques (STORM, PALM and RESOLFT/STED) have been employed to obtain sub-diffraction images in live cells (26)[*CE: Reference 26 is not cited in the reference list.]. However, since most of these techniques were shown to provide approximately a 50-nm imaging resolution *in vivo*, they are unlikely to distinguish the real-time localizations of NETs on the INM and ONM, since the two membrane bilayers are separated by a 40-nm perimembrane space (26).

Fluorescence recovery after photobleaching (FRAP) was developed to mainly study cell membrane diffusion and protein binding (Axelrod et al., 1976). However, over the past years this technique has been modified many times, and is now being widely applied to study various membrane protein dynamics on the lipid bilayer (Ellenberg et al., 1997) (25, 27–29) [*CE: Reference 27–29 are not cited in the reference list.], including the lateral diffusion of NETs on the NE (Ellenberg et al., 1997) (25). Here we have further developed the FRAP technique by adapting a diffraction-limit photobleaching area and recording the recovery of single NETs on the INM and ONM with super-high spatiotemporal resolutions in live cells (Mudumbi et al., 2016).

Through the combination of single-point illumination and smFRAP, we have been able to distinguish the spatial localizations of NETs on the INM and ONM spatial resolution of <10-nm in real-time. Moreover, through measuring the diffusion coefficients and the immobilized fractions of these NETs, we further determine the *in vivo* translocation rates and concentrations of NETs along the ONM and INM (Mudumbi et al., 2016).

Critical Parameters and Troubleshooting

It is vitally important that during the single-molecule imaging step that the laser center is aligned directly on region of the NE that will be studied. If the laser is mis-aligned, the resultant data will be biased and unsuitable for data analysis. It is important that only healthy cells in interphase are also used for single-molecule imaging, as they will provide the most accurate data for the distribution of the NET in question. Furthermore, the expression of the GFP tagged NET might vary from cell to cell, in which case it may be necessary to extend the photobleaching step in the smFRAP protocol. Also, the laser power used during single-molecule imaging for smFRAP should be adjusted so that nearly the maximum number of photons can be collected from each GFP tagged NET for the best precision.

During bulk FRAP experiments, it is crucial that the initial photobleaching step completely photobleaches the region of interest (ROI) of the nuclear envelope where FRAP measurements will be made. Furthermore, the suggested 20% power for time lapse imaging may also be adjusted so that the time lapse images are crisp without introducing too much imaging induced photobleaching.

Anticipated Results

Once smFRAP data is obtained and analyzed, one can expect to see higher ratios on the ONM than the INM prior to correcting the ratios by considering the diffusion effect and the immobilized fraction. 5–10 cells transfected with the same GFP fused NET will provide enough data to determine the localization of NETs on the NE, as well as the concentration of the NET on the ONM or INM. Furthermore, NETs on the endoplasmic reticulum studied through bulk FRAP experiments typically can be used as a reference to show full recovery once the time course of the time lapse imaging is finished.

Time Considerations

HeLa stock cell lines should be thawed at least one week prior to experiments. If using LT1 for transfection, cells need to be plated on glass bottom dishes 2 days prior to performing smFRAP experiments. If an electroporation technique is used, cells can be plated on glass bottom dishes the day before smFRAP experiments. On the day of smFRAP experiments, data can be acquired, and analyzed in 10–20 min once the protocol has been mastered.

Acknowledgments

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Figure 21.11.1.

Single-point illumination and smFRAP used to detect transmembrane proteins on the NE.

(A) HeLa cell transfected with GFP tagged NET used to visualize the NE. The purple circle indicates the usual 5- μm illumination area used in bulk FRAP experiments and the red circle indicates single-point illumination area up to 0.5 μm used in this study. (B) Both the INM and ONM of the NE are studded with NETs fused to GFP. (C) Using single point illumination, a small, 0.5- μm area of the NE is targeted, and GFP fused NETs in this area are excited using a high laser power. (D) NETs in the laser excitation area, as well as those that diffuse into the area are photobleached. (E, F, and G) Once the area is completely

photobleached, diffusion events of freshly incoming GFP-NETs occur at the single-molecule level and can be precisely localized. **(H)** Localized single-molecule events from the ONM and INM are compiled and the data is fitted with Gaussian functions to determine 2D distribution of NETs along the NE. **(I)** 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. This figure was included in our recent research article (Mudumbi et al., 2016) and is re-used here with the permission of the publisher.

Figure 21.11.2.

Background subtraction and fitting of smFRAP data. Histograms were prepared from the raw data. The background noise (without including the data ± 40 -nm from the highest peak) 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). This figure was included in our recent research article (Mudumbi et al., 2016) and is re-used here with the permission of the publisher.

Figure 21.11.3.

Method used to correct the ONM:INM ratios by including the effect of molecular diffusion coefficient as determined by single-molecule trajectories. This calculation takes into account the differing two dimensional diffusion coefficients of transmembrane proteins along the nuclear envelope of the cell as they enter the detection area (shown in gray), and corrects the distribution ratio to reflect the actual transmembrane protein concentrations along the nuclear envelope. The outer ring (R_{max}) represents the entire circumference of the nuclear envelope and the detected molecule (shown in green) can come from any locations with the distance X from the center of the photobleached area (indicated by the second inner ring). (A) $G(i, D, t)$ represents the probability of finding a randomly diffusing particle at location i after diffusion with a diffusion constant of D within time t . (B) The probability that molecules starting at i (shown in green) eventually diffuse into the detection area (gray). (C) $f(D, t)$ refers to the probability of observing the particles moving into the detection area in two dimensions from the entire area(R_{max}). This figure was included in our recent research article (Mudumbi et al., 2016) and is re-used here with the permission of the publisher.