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Assessment of membrane lipid state at the natural killer cell immunological synapse

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The plasma membrane is a fluid structure that protects cells as one of their first barriers and actively participates in numerous biological processes in many ways including through distinct membrane sub-regions. For immunological cells, highly organized sub-compartments of plasma membranes are vital for them to sense and react to environmental changes. This includes a varying spectrum of lipid order in the plasma membrane which signifies or enables cellular functions. Thus, comprehensive analyses of the plasma membrane can facilitate understanding of important cell biological elements which include insights into immune cells. Here, we describe two methods that can be used to assess membrane lipid state at the natural killer cell immunological synapse via high-resolution live cell imaging techniques.

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Materials

Disposables
• Flasks and multi-well plates (polystyrene, 75 or 25 cm2 for flasks and 6 or 12 wells for plates)
• Automatic pipette and 10 ml serological pipettes
• Micropipettes and tips (1–20µl, 20–200µl, 200–1000µl)
• 8-well chamber slides (Lab-Tek II Chambered Coverglass #1.5 Sterile)

Equipment (parentheses state what was specifically used in our work)
• Cell culture incubator (Thermo Scientific 3020 Water Jacketed CO2 Incubator)
• Benchtop centrifuge (Eppendorf 5424R Centrifuge)
• Rotator and shaker (Thermo Scientific Tube Revolver / Rotator)
• Sonication bath (Branson M1800 Ultrasonic Cleaner with Mechanical Timer)
• Isothermal plate (Thermo Scientific Cimarec Stirring Hot Plate)
• Confocal microscope (Zeiss Axio Observer Z1 inverted microscope outfitted with a Yokogawa CSU-W1 spinning disc, with a 63×/1.4 NA objective)
• TIRF microscope (GE DeltaVision OMX-SR microscope using a 60×/1.42 PlanApoN objective)
• Image processing tools (Fiji package of ImageJ, version 1.53c)

Reagents
• Culture medium: RPMI medium 1640 (Invitrogen), 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco), 20 mM HEPES (Gibco), Sodium pyruvate (Gibco). Adjust pH to 7.0.
• Imaging medium: Phenol red-free RPMI medium 1640 (Invitrogen), serum (FBS), 2 mM L-glutamine (Gibco), 20 mM HEPES (Gibco), Sodium pyruvate (Gibco). Adjust pH to 7.0.
• Phosphate buffered saline, without calcium and magnesium (1x PBS, Gibco)
• Poly-L-lysine solution, 0.1 % (w/v) in H2O (Sigma-Aldrich)
• Di-4-ANEPPDHQ (1 mM solution in DMSO, Invitrogen)
• CellMask Deep Red (1 mM solution in DMSO, Invitrogen)
• Common chemicals (Ethanol, Acetone, Sodium hydroxide)

Cell lines
• While many different types of cells can be used for this work, we have focused on using two different human NK cell lines and their corresponding classical target cells listed below.
• NK cell line YTS and corresponding target cell line 721.221 are maintained in standard culture conditions (at 37°C, under 5% CO2) using complete R10 medium: RPMI medium 1640 (Invitrogen), 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco), 20 mM HEPES (Gibco), Sodium pyruvate (Gibco) adjusting pH to 7.0.
• NK cell line NK92 and corresponding target cell line K562 are maintained in standard culture conditions (at 37°C, under 5% CO2). For the K562 cell line, complete R10 medium is used. For NK92 cells Myelocult 5100 (Stemcell) with 100u/ml of IL-2 is used.

1 Introduction

The plasma membrane is composed of a complex collection of phospholipids, sterols and membrane proteins which is dynamic in its constitution. It provides cells basic protections against external stress and regulates the exchange between the intra- and extra-cellular environments. It also actively participates in various biological processes through distinct membrane sub-regions, which are quite relevant in immunological cells (1-3).

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As a heterogeneous surface, the plasma membrane has highly organized sub-compartments that are vital for immunological cell to interact with and generate function in response to its environment. One way in which membrane sub-compartments can be created is via the ordering of the lipids themselves. Specifically, the phospholipid tails can be packed tightly owing to interspersed sterols imparting specific properties to the membrane including the ability to collect glycophosphoinositol linked proteins.Such regions of packed lipid membranes are also known as lipid rafts.These have had specific function ascribed to them in immune cells.For example, lipid rafts, can recruit, maintain and regulate multiple signaling complexes during the activation of NK cells (1, 4-6). In addition to generating a signaling platform, certain membrane domains also interact with the actin cytoskeleton to serve a role in cell motility and intracellular mechanics. NK cells and cytotoxic T lymphocytes (CTLs) can dynamically alter their membrane properties, such as packing density (7), viscosity (8) and surface tension (9), to affect a variety of functions. Recently these properties have been demonstrated as important in ensuring cytotoxic lymphocyte self-survival while allowing for the elimination of target cells (7, 10). Collectively, composition, distribution, interaction of membrane domains holds significant information, and their analysis can promote understanding of important cell biological questions in immunology.

Benefiting from the substantive progress in chemical biology, a wide range of fluorescent membrane probes have become commercially available and can be applied to investigate membrane properties. When combined with high-resolution live cell microscopy, these probes can enable precise observations of lipid composition, phase separation, and lipid-protein interaction, providing information on the lateral heterogeneity of and alterations in plasma membranes (11).Furthermore, some environmentally-sensitive probes can change their fluorescence intensities (12) or emission spectra (13) in response to changes in their local environment (polarity, viscosity, and packing density etc.). These sophisticated tools allow for the real-time capture of membrane dynamics, and hence greatly expand the dimensionality for increased assessment and understanding of membrane biology as it relates to cellular function.

Here, we provide two detailed protocols refined in our laboratory that can be used to assess membrane lipid state at the NK cell immunological synapse via high-resolution microscopy. In developing methods for studying the NK cell immunological synapse, we utilized live cell imaging techniques, which allows measuring biological events within optical sections of interest in living cells without interference of artifacts from fixation procedures. A model immunological synapse can be formed between an NK cell and its susceptible target carrying the ligands that trigger NK cell activation. It can be also induced between NK cells and functionalized surfaces (14). And we utilized two different imaging modalities, confocal and total internal reflection fluorescence (TIRF) microcopy, to analyze immunological synapses generated by these two approaches. Confocal microscopy has advantages in global comparison between the synaptic region and non-synaptic region, while TIRF microscopy can provide *en face* images of the immunological synapse with higher spatial resolution. Other imaging approaches are also relevant, but we focus on these two given how they were applied in our own laboratory. To provide a basic experimental process for discerning lipid state, we describe how to quantitatively assess lipid packing of the NK cell

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presynaptic membrane using the Di-4-ANEPPDHQ probe (15). Di-4-ANEPPDHQ is a potentiometric styryl dye that shows green and red emission in the liquid-ordered and liquid-disordered phases of lipid membrane, respectively. We can use it to image phase-separated membrane domains and their dynamics. These protocols, however, are compatible with other ratiometric probes, such as Laurdan (13), to measure lipid packing.Given some of the characteristics of Di-4-ANEPPDHQ (16), it is important to not rely solely upon this probe in considering lipid membrane biology. The experimental approach, however, can also be applied to observe other membrane properties such as lipid composition and distribution (with suitable molecular probes) and of course in other cell types and using other imaging modalities.

The outcomes of these approaches will provide quantitative measurements of the membrane order via ratiometric calculation between images taken through two distinct channels. The packing density of membrane lipid will be quantified as general polarization (GP) values. Pseudo-colored images using GP values can allow for the intuitive visualization of the distribution and dynamics of membrane microdomains of the NK cell presynaptic membrane which can help illustrate membrane biology.

2 Common procedures

Preparing glass surface: deep-cleaning
Due to the lipophilic nature of membrane probes, a clean and fully hydrated glass surface is critical for minimizing probe non-specific binding and background noise. To prepare a high-quality glass surface for use with lipid probes:

1. Add 1 ml acetone to each well of 8-well chamber slides for 30 min (see **Note 1**).
2. Rinse chamber slides using fresh deionized water at least five times (see **Note 2**).
3. Add 2 ml NaOH solution (1 M) to each well of the 8-well chamber slides.
4. Place chamber slide on 60 °Cisothermal plate for 45 min (see **Note 3**).
5. Rinse the chamber slides using fresh deionized water at least five times.
6. Rinse the chamber slides with 100% ethanol and let air dry.
7. Store the chamber slides in a clean container and use within 3 days.

3.2 Preparing glass surface: coating
To prepare an adhesive surface for efficient cell attachment:

1. Add 0.5 ml 0.01% Poly-L-lysine solution (diluted with deionized water) at room temperature (18~26 °C) to each well of 8-well chamber slides for 1 hour.
2. Rinse the chamber slides with fresh deionized water three times.
3. Dry the chamber slides in air and use them within 24 hours.

To prepare an activating surface for immunological synapse formation:

1. Under room temperature (18~26 °C), add 0.25 ml antibody solution (5 mg/ml, see **Note 4**) to each well of 8-well chamber slides for 1 hour.
2. Rinse the chamber slides three times using PBS.
3. Cover the glass surfaces with imaging medium and use the chamber slides in 24 hours.

3 Assessment of membrane lipid state via confocal microscopy

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1. Prepare clean adhesive glass surfaces (i.e. PLL coated) in chamber slides and pre-warm to 37°C.
2. Pre-warm the imaging medium (5-10 ml) and PBS (50-100 ml) to 37°C (see **Note 5**).
3. Pre-warm microscope stage, objectives and environmental control chamber to 37°C (see **Note 6**).
4. Prepare NK cells (e.g. YTS cells) and target cells (e.g. 721.221 cells) by centrifuging at 300 x g, at room temperature, for 5 min.
5. Gently decant supernatants and wash cell pellets with 10 ml pre-warmed imaging medium.
6. Centrifuge both NK and target cells again at 300 x g, at room temperature, for 5 min.
7. Resuspend both NK and target cells with 1~3 ml pre-warmed imaging medium.
8. Add Di-4-ANEPPDHQ (recommended working concentration: 2 mM) and CellMask deep red (recommended working concentration: 1 mM) to the NK cell suspension and incubate at 37 °C, for 30 min.
9. Gently wash NK cells with 10 ml pre-warmed imaging medium.
10. Centrifuge NK cells again at 300 x g, at room temperature, for 5 min.
11. Repeat step 9 and 10.
12. Resuspend NK cells with 0.5 ml pre-warmed imaging medium.
13. Count both NK and target cells and adjust the concentrations of them to 0.5 x 10^6/ml using pre-warmed imaging medium.
14. Mix target cells and NK cells at the desired ratio (e.g. E:T=1:1 or 2:1).
15. Load 200 ml of the cell suspension into the chamber wells.
16. Incubate the chamber slides at 37°C for 20 min to allow the NK cells and target cells to form conjugates.

17. Drip immersion liquid (usually oil) on top of the lens.
18. Mount the chamber slide with cells onto the pre-warmed stage.
19. Raise the objective to touch the bottom surface of chamber slide.
20. Use transmitted light or differential interference contrast (DIC) to identify cells.
21. After finding the optimal focal plane, identify cell conjugates using differential interference contrast (DIC) or transmitted light imaging for downstream analysis.
22. Switch to the florescent channel for CellMask deep red (or other similar cell membrane dye) to confirm that a conjugate contains an NK cell (CellMask or similar positive) and target cell (CellMask or similar alternatives).
23. Adjust z-position to correspond to the z-plane crossing the center of the immunological synapse (usually indicated by approximately the largest contact region).
24. Capture images in the DIC channel, the two ratiometric channels for Di-4-ANEPPDHQ and the CellMask deep red channel (see **Note7**). 488 nm laser excites Di-4- ANEPPDHQ, and the detection ranges of the two channels are set to 500–580 nm and 620–750 nm. CellMask deep red is excited by 647 nm laser, and the detection range is set to 620-750 nm.
25. An example of this approach is provided in Figure 1

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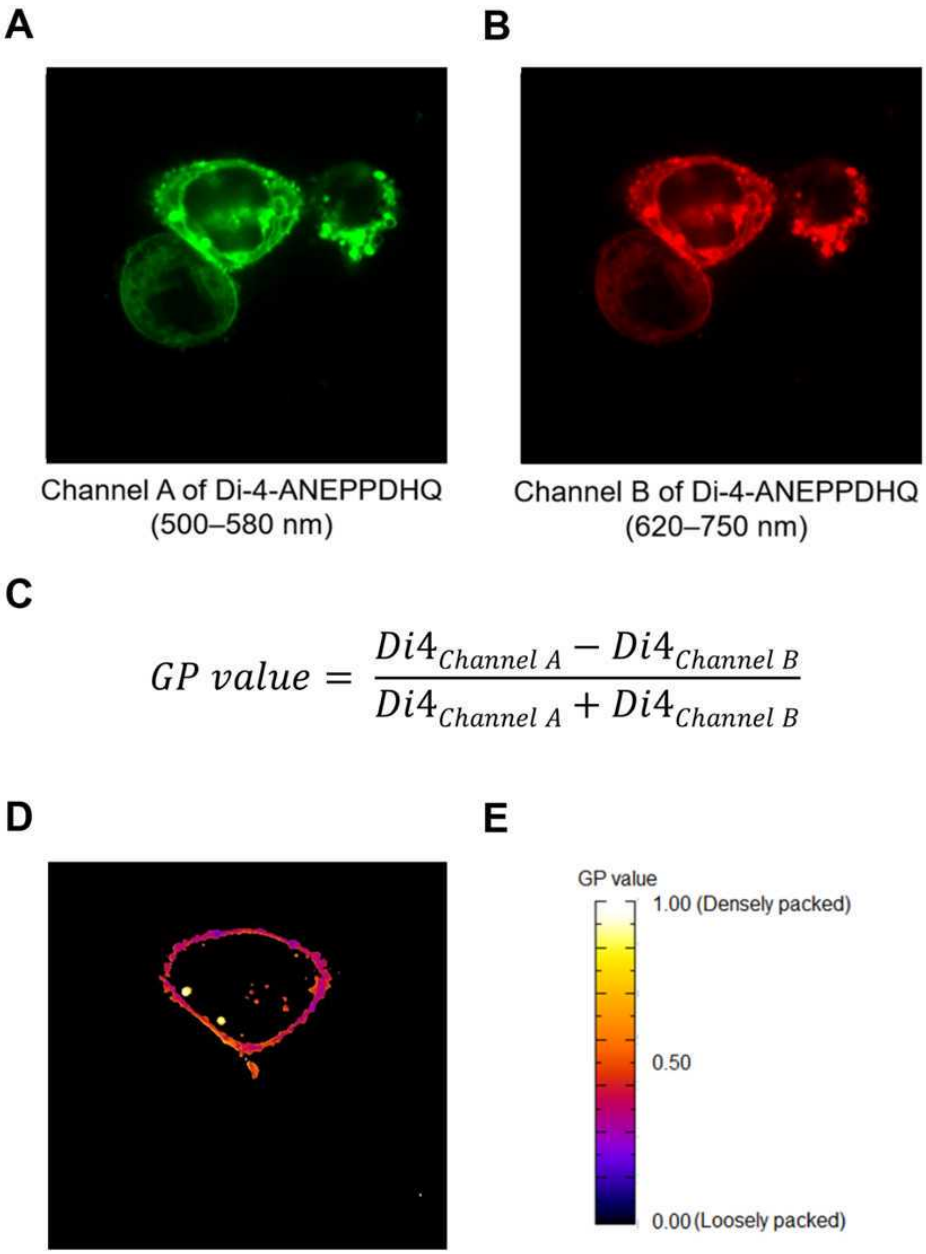

Figure 1. The lipid packing of NK cell membranes in Di-4-ANEPPDHQ labeled YTS conjugated with 721.221 target cells, is measured using live cell confocal microscopy. Two ratiometric channels (panel A and B) for Di-4-ANEPPDHQ are captured to quantify membrane lipid density as a general polarization (GP) value. The GP value is calculated using the formula shown (panel C) and the image (panel D) is overlaid with a pseudocolor scale (panel E) to allow for the visualization of packing differences.Please note that the target cell can be seen in the 4ANEPPDHQ channels A and B (see **Note 9** for additional details)

4 Assessment of membrane lipid state via total internal reflection fluorescence microscopy

1. Prepare clean activating glass surfaces (i.e. antibody coated) in chamber slides and pre-

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warm to 37°C

2. Pre-warm the imaging medium (5-10 ml) and PBS (50-100 ml) to 37°C (see **Note5**).
3. Pre-warm microscope stage, objectives and environmental control chamber to 37°C (see **Note6**).
4. Prepare NK cells (e.g. YTS cells) by centrifuging at 300 x g at room temperature for 5 min.
5. Gently decant supernatants and wash cell pellets with 10 ml pre-warmed imaging medium.
6. Centrifuge cells again at 300 x g at room temperature for 5 min.
7. Re-suspend cells with 1~3 ml pre-warmed imaging medium.
8. Add Di-4-ANEPPDHQ (recommended working concentration: 2 mM) and CellMask deep red (recommended working concentration: 1 mM) to cell suspension and incubate at 37 °C, for 30 min.
9. Gently wash cells with 10 pre-warmed imaging medium.
10. Centrifuge cells again at 300 x g, room temperature, for 5 min.
11. Repeat step 9 and 10.
12. Re-suspend cells with 0.5 ml pre-warmed imaging medium.
13. Count cells numbers and adjust the concentrations of NK cells to 0.2 x 10^6/ml using pre-warmed imaging medium.
14. Load 200 ml of cell suspension to chamber well.
15. Incubate chamber slides at 37°C for 15 min to allow for immunological synapse formation.
16. Drip immersion liquid (usually oil) on top of the lens.
17. Mount the chamber slide with cells onto the pre-warmed stage.
18. Raise the objective to touch the bottom surface of chamber slide.
19. Identify fully spread NK cells using differential interference contrast (DIC) or transmitted light imaging.
20. Switch to florescent channel of CellMask deep red (or other similar cell membrane dye) to confirm cell integrity.
21. Adjust z-position and illumination depth to focus on the presynaptic membrane of NK cell and to engage total internal reflection.
22. Reduce the illumination depth or increase angle of incidence (depends on the microscope configuration, see **Note 8**), until there is a dramatic loss of fluorescence signal inside the cell. This loss of signal indicates the establishment of total internal reflection status of illumination light.
23. Increase the illumination depth or reduce the angle of incidence until the signal reappears. This is TIRF with the smallest penetration depth. As the angle of incidence is reduced, the penetration depth will increase until a certain point where total internal reflection is disrupted and TIRF transitions to epifluorescent imaging.
24. Between the maximum and minimum illumination depth or angle of incidence, choose a setting that balances the intensity of signal and specificity of signal.
25. Capture images in DIC channel, the two ratiometric channels of Di-4-ANEPPDHQ and CellMask deep red channel Di-4- ANEPPDHQ is excited by 488 nm laser, and the detection ranges of the two channels are set to 500–580 nm and 620–750 nm. CellMask deep red is excited by 647 nm laser, and the detection range is set to 620-750 nm.
26. An example of this approach is provided in Figure 2

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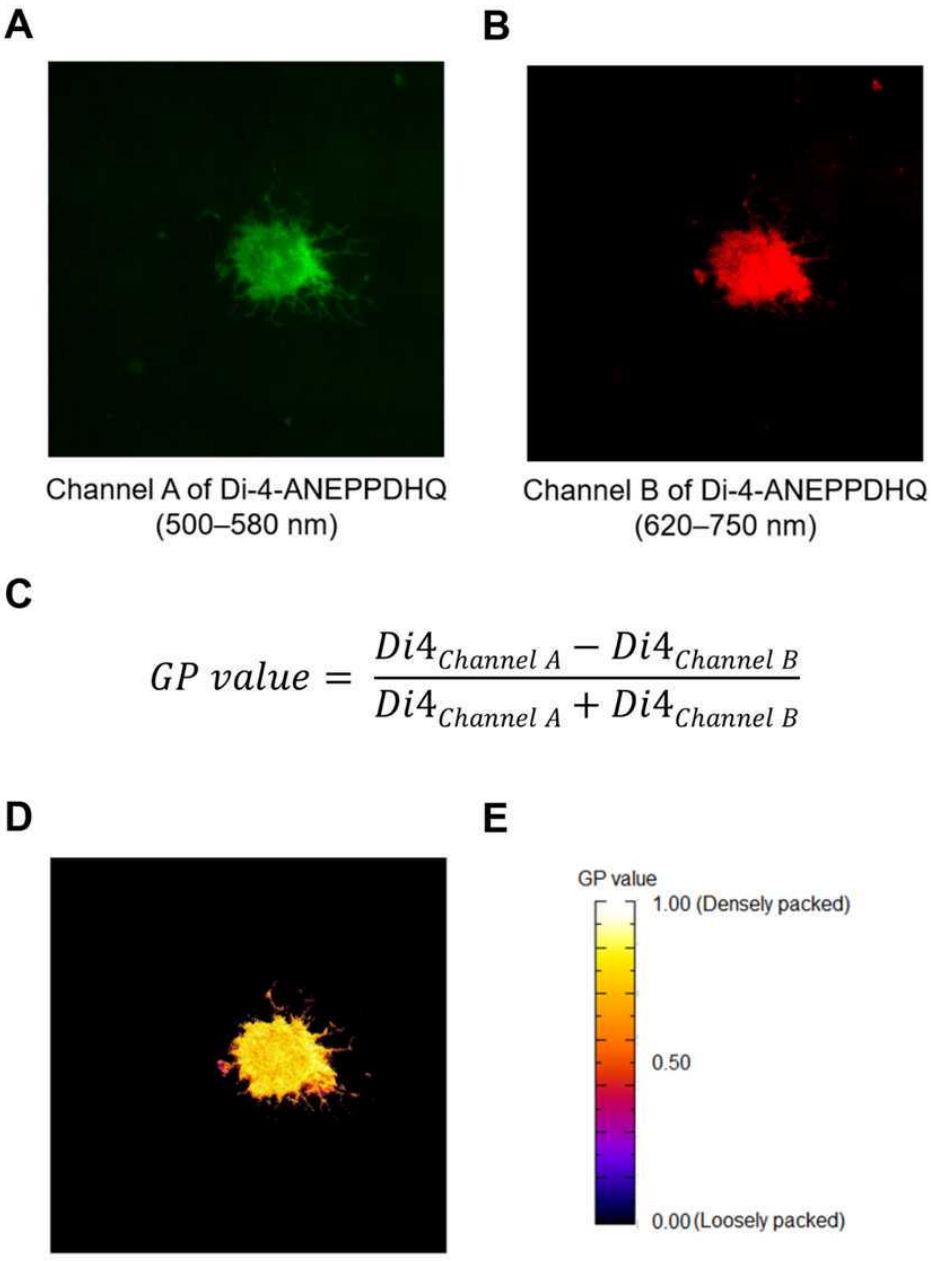

Figure 2. The lipid packing of presynaptic membranes in Di-4-ANEPPDHQ labeled YTS is measured using TIRF microscopy. Two ratiometric channels (panel A and B) for Di-4-ANEPPDHQ are captured to quantify membrane lipid density as a general polarization (GP) value. It is calculated using the shown formula (panel C) and the images (panel D) are then overlaid with a pseudocolor scale (panel E) to allow for visualization of packing differences.

5 Data analysis

To manually quantify lipid ordering of presynaptic membranes using the Fiji version of ImageJ:

1. Download and open software (https://imagej.net/software/fiji/).

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2. Import and open acquired images with Fiji (at least including three channels: two ratiometric channels for Di-4-ANEPPDHQ probe and one for CellMask deep red).
3. Generate binary masks to identify cell membrane region from images of CellMask deep red channel via the Fiji “Threshold” function (via “Image › Adjust › Threshold” or “Ctrl+Shift+T”).
4. Calculate GP values from images of the two ratiometric channels via the Fiji “image calculator” function (via “Image › Image Calculator…”). The equation used to calculate the GP value is:
5. Multiply the resulting images with corresponding binary masks to remove background and only preserve the GP values of the membranes of interest via the Fiji “image calculator” function.
6. To visualize GP values, choose and apply a lookup table in Fiji and set the display scale manually to “0 to 1” in the “Brightness and Contrast” window. Select a lookup table and range that is consistent with the dynamic range of the GP values to emphasize
7. To quantitatively analyze of GP values, obtain measurements via the “Analyze ›Measure” function of Fiji.
8. For time-lapse imaging series apply this procedure individually to each frame.

To perform batched imaging analysis automatically using the Fiji version of ImageJ:

1. Download and open software (https://imagej.net/software/fiji/).
2. Import and open acquired images with Fiji (at least including three channels: two ratiometric channels for Di-4-ANEPPDHQ probe and one for CellMask deep red).
3. Rename the images from 500–580 nm and 620–750 nm channels as “Di4_A_1, Di4_A_2, Di4_A_3 etc.” and “Di4_B_1, Di4_B_2, Di4_B_3 etc.”, respectively.
4. To run auto-analysis script for batch processing, paste and run the source code below via the “Process › Batch › Macro” function of Fiji. User also needs to specify the input directory in the opened dialog window as the location where all Di4 images are located.

Source code of auto-analysis script:

```
selectWindow("Di4_A.tif");
run("8-bit");
selectWindow("Di4_B.tif");
run("8-bit");
imageCalculator("Subtract create 32-bit stack", " Di4_A.tif"," Di4_B.tif");
selectWindow("Result of Di4_A.tif");

imageCalculator("Add create 32-bit", "Di4_B.tif","Di4_A.tif");
selectWindow("Result of Di4_B.tif");
imageCalculator("Divide create 32-bit", " Result of Di4_A.tif","Result of Di4_B.tif");
selectWindow("Result of Di4_A.tif");
close();
```

6 Notes

1. Only use glassware to transfer acetone.Ensure adequate ventilation via the use of a fume hood.
2. For extra cleaning measures, place the chamber slide in a beaker of deionized water (the

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slide should be completely immersed) and place the beaker in a sonication bath for 30 min.

3. For antibodies used to create activating surfaces we prefer anti-CD18 and anti-NKp30 to activate NK92 cells, and anti-CD18 and anti-CD28 for YTS cells.We have utilized the former for ex vivo NK cells as well.
4. Lipid rafts and the cytoskeleton can be extremely temperature sensitive from a biological standpoint. Make sure the cells are maintained at 37°C during labeling and imaging.
5. Lipids and membranes are also temperature sensitive. Again, ensure cells are maintained at 37°C and always use pre-warmed materials and reagents.
6. When using an immersion objective and microscope stage that have direct contact with the glass surface, be aware that heat conduction between them can greatly affect imaging quality, cell activity and membrane properties. Pre-warming the objective and stage to 37°C can be achieved using a thermal sleeve or environmental chamber.
7. For long-term imaging like a time-lapse experiment (see also **Note 10**), it is vital to adjust laser power and exposure to minimize photo bleaching and toxicity, User is highly recommended to titrate laser power in test-runs before formal acquisition (17).
8. TIRF microscopes can have a variety different configurations and user interfaces. Please note that the illumination depth and angle of incidence are inversely correlated parameters that determine the range of fluorescence excitation.
9. Uptake of lipid dye can be seen to some degree in target cells and certainly at levels that are lower than in NK cells.The likely reasons for this are: trans-difusion, cis-diffusion and some degree of autofluorescence.For this reason it is important that lysotraker, Calcein, CellMask, or the likes used to definitively discern the NK cell in a conjugate and also allow for the generation of a binary mask for NK cell identification.
10. After NK cells establish contacts with activated surface or target cells, it takes about 8~15 min for presynaptic membrane of NK cell to accumulate high density membrane. Then it will maintain a very stable GP value for 30~45 min, until the detachment phase of NK cell cytotoxicity.

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