

## High- and Super-Resolution Microscopy Imaging of the NK Cell Immunological Synapse

Emily M. Mace and Jordan S. Orange

### Abstract

Recent advances in imaging technology have enabled significant advances in the study of NK cell cytotoxic effector function through quantitative analysis of the NK cell immunological synapse. This can include the use of high- and super-resolution microscopy to quantify dynamics of cytoskeletal elements and the role they play in the regulation and execution of NK cell directed secretion. Here we describe a protocol for the recapitulation of the NK cell lytic synapse on glass, the acquisition of microscopy images, and suggested approaches for the processing and analysis of microscopy data.

**Key words** Natural killer cell, Immunological synapse, Super-resolution microscopy, Confocal microscopy, Image analysis

---

### 1 Introduction

Natural killer (NK) cells provide critical human host defense against malignancy and viral infections, particularly through the directed secretion of specialized secretory lysosomes termed lytic granules. This targeted lysis is a tightly regulated process as NK cells largely identify susceptible cells through germline encoded receptors. Therefore, their innate ability is harnessed and only released following passage through multiple regulatory checkpoints [1]. Key control of the cytotoxic process is contributed largely by cytoskeletal components, namely microtubules and F-actin. At each of the main stages of function, recognition, effector and termination, the cytoskeleton modulates NK cell responsiveness through the immunological synapse formed between it and the target cell.

The study of these processes, their regulation, and their contribution to disease has been driven largely by cell biological techniques. In particular, the use of high- and super-resolution microscopy to dissect the molecular contribution of cytoskeletal elements has been very productive. While detailed description of these techniques and their application is beyond the scope of this

introductory targeted primer, perspectives on the use of microscopy to study the immunological synapse have been well reviewed elsewhere [2, 3]. This protocol focuses specifically on the application of confocal microscopy or stimulated emission depletion (STED) microscopy to the study of an NK cell activated on glass coated with antibody to activating receptors.

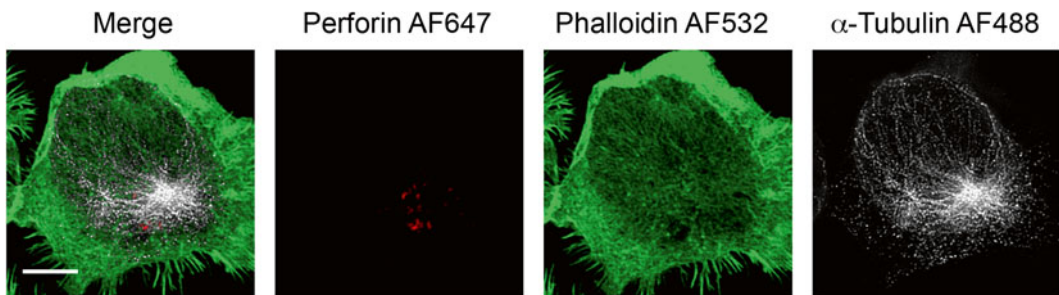
The use of glass coated with antibody (or recombinant protein) has been shown to recapitulate the target cell interface while still allowing for high-resolution examination of the synapse. In addition, it allows for the selective ligation of receptors of interest. Under activating conditions, specifically the engagement of an adhesion receptor and an activating receptor, the NK cell will undergo degranulation at the plane of the glass, accompanied by the molecular events associated with exocytosis, including the expansion of the actin meshwork to enable or facilitate lytic granule passage, the exposure of CD107a (LAMP1) to the outer membrane and the release of lysosomal contents, namely perforin, granzymes and esterases [4, 5]. The choice of which receptor(s) to engage is dependent upon the cell origin (primary or cell line) and, if a cell line, the expression of receptors on the cell surface. The primary adhesion molecule on all NK cells is LFA-1, the  $\alpha$ L $\beta$ 2 integrin (CD11a/CD18). Ligation of LFA-1 can occur through the use of a non-blocking anti-CD18 antibody, or through the use of recombinant ICAM-1, LFA-1's most common physiological ligand. We use anti-CD18 (clone 1B4), which is commercially available in purified form from several suppliers. For activation of primary NK cells, we most commonly use anti-NKp30 in combination with anti-CD18, which is sufficient for NK cell activation and degranulation. Alternatively, anti-NKG2D may be used [4]. For cell lines, the choice is more dependent upon the line. For example, the NK-92 cell line [6] expresses the natural cytotoxicity receptor NKp30, and engagement of NKp30 in combination with LFA-1 results in robust activation and degranulation. Engagement of CD28 on YTS or YT cells has a similar effect, whereas for the NKL cell line NKG2D or CD16 engagement is commonly used [4].

Other methods to recapitulate the synapse include the use of supported lipid bilayers, which allows for imaging at the plane of the glass, yet is restricted to live cell imaging. The use of target cells has also been described, although it can be difficult to keep these oriented for imaging in the XY dimension at the synapse and the added depth of the target precludes imaging at the plane of the glass, a requirement for such techniques as total internal reflection microscopy (TIRF). While all systems have their advantages and drawbacks, the recent report of similar behavior of T cells on lipid bilayer and glass lends confidence to the consistency of each system to study synaptic events [7].

Close examination of the NK cell lytic synapse has revealed some unexpected findings and underscored the importance of

rigorous biophysical analysis. These findings include the interaction between activating and inhibitory receptors in microclusters, which shows that microclusters of inhibitory receptors at the NK cell synapse undergo reorganization following activating receptor ligation [8]. The movement of lytic granules prior to degranulation at the synapse has been described by TIRF yet is incompletely understood [9]. Finally, our group and others recently described the presence of an actin mesh throughout the lytic synapse [4, 5]. This F-actin network creates granule-permissive clearances in response to activating signal, and its opening specifically requires the depolymerization of F-actin [10]. These findings were possible only because of the parallel use of multiple high- and super-resolution techniques and, again, underscore the importance for such type of analysis.

In this protocol we will describe the activation of NK cells on glass and the subsequent steps to the acquisition and preliminary processing of data obtained using confocal or STED microscopy (*see* example in Fig. 1). In this case, the technique described utilizes a commercially available Leica SP8 laser scanning confocal microscope with STED capabilities. STED exploits the use of a high energy, toroidal shaped depletion beam that selectively darkens the photons produced around a single fluorophore and thus allows for sub-diffraction limited imaging [11]. In the case of the Leica system, this beam is positioned at 592 nm and/or 660 nm, potentially enabling depletion in the 488–630 nm range and allowing use of commercially available fluorophores. A further advantage of this system is the tunable white light laser and tunable PMTs or detectors. This allows for manipulation of both excitation and detection of emission. While this protocol describes the use of this system, the techniques described will be germane to the application of other confocal microscopes.



**Fig. 1** Representative image of the cytoskeleton of an NK cell activated on glass. NK92 cells were activated on glass then fixed, permeabilized and stained for F-actin,  $\alpha$ -tubulin and perforin as described in the protocol. Images were acquired on a Leica SP8 using both confocal (perforin) and STED ( $\alpha$ -tubulin, F-actin) modules. Images were deconvolved with Huygens (SVI) software. Scale bar = 5  $\mu$ m

---

## 2 Materials

1. ProLong anti-fade reagent (Life Technologies).
2. Antibodies for coating the coverslips for activation: purified anti-NKp30, anti-CD18, anti-NKG2D, anti-CD16, etc. (as required by experimental setup).
3. Antibodies for staining sample: anti-perforin, phalloidin, anti- $\alpha$ -tubulin, etc. (as required by experimental setup).
4. Bovine serum albumin (BSA) (Sigma-Aldrich).
5. Phosphate buffered saline (PBS).
6. Cotton tipped applicator.
7. Super PAP pen (hydrophobic barrier pen) (Life Technologies).
8. BD Cytotfix/Cytoperm (BD Biosciences).
9. Triton X-100 (Electron Microscopy Sciences).
10. Laboratory tissue wipers.
11. Thermo Scientific™ Colorfrost™ Plus Slides (Fisher Scientific).
12. #1.5 coverslips.
13. NK-92 cell line (ATCC).
14. Ficoll-Paque (for isolation of primary NK cells).
15. RosetteSep (STEMCELL Technologies) for enrichment of primary NK cells.
16. NK92 medium: Myelocult (STEMCELL Technologies) supplemented with 100 U/ml IL-2 and penicillin–streptomycin.
17. NK cell medium: RPMI 1640, 10% FBS, penicillin–streptomycin, HEPES, nonessential amino acids, sodium pyruvate, L-glutamine.
18. Staining buffer: 1% BSA PBS with 0.1% Saponin.
19. Cytotfix/Cytoperm buffer: BD Cytotfix/Cytoperm with 0.1% Triton X-100.
20. Leica SP8 laser scanning confocal microscope with STED module.
21. Volocity software (PerkinElmer).
22. Huygens software (Scientific Volume Imaging).
23. 50 ml sterile conical tubes.
24. Saponin (Sigma-Aldrich)

---

## 3 Methods

### 3.1 NK Cell Culture

1. NK-92 cell line should be maintained in Myelocult media (STEMCELL Technologies) with 100 U/ml IL-2.
2. Cells should be passaged 1:10 every 2–3 days and maintained at a density of  $1\text{--}3 \times 10^5$  cells/ml.

### **3.2 Primary NK Cells Isolation and Purification**

1. Enrich and isolate peripheral blood mononuclear cells (PBMCs) from peripheral blood by Ficoll-Paque method.
2. Incubate 15 ml fresh peripheral blood with 750  $\mu$ l RosetteSep (STEMCELL Technologies) for 20 min.
3. Dilute with 15 ml of PBS and carefully layer on 15 ml of Ficoll-Paque in a 50 ml tube.
4. Spin the cells at  $400\times g$  for 20 min with no brake.
5. Carefully recover the PBMCs from the Ficoll-Paque interface.
6. Wash NK cells once with complete NK cell medium and resuspend at a concentration of  $1\times 10^6$ . In our experience it is recommended to use cells immediately after isolation (*see Note 1*).

### **3.3 Coating Slides with Activating Antibody**

1. Use a Super PAP pen to mark a circle or square (approximately 1.5 cm diameter) on one 22 mm $\times$ 22 mm #1.5 coverslip per condition to be tested (including single stained controls), in order to create a shallow hydrophobic barrier, to contain cells and staining buffer.
2. Resuspend primary antibody against receptors of interest (i.e., CD18, NKp30, NKG2D) (*see Note 2*) at 5  $\mu$ g/ml in phosphate buffered saline (PBS). Vortex briefly to mix. Prepare approximately 200  $\mu$ l per condition.
3. Gently pipette 100–200  $\mu$ l of coating antibody on to coverslip inside the hydrophobic barrier (may be done under non-sterile conditions) and incubate at 37 °C 5% CO<sub>2</sub> for 30 min.
4. Just prior to seeding NK cells, gently dip the coverslips in a 50 ml conical tube of PBS to wash off unbound activating antibodies. Coverslips should be used immediately following coating (*see Note 3*).

### **3.4 Preparing NK Cells and Activation on Coverslips**

1. Harvest  $2\times 10^5$  NK cells (primary or cell lines) from culture per condition (per each coverslips prepared in Subheading 3.3).
2. Wash NK cells once with prewarmed NK cell medium, centrifuge at  $400\times g$ , remove the supernatant, and resuspend in NK cell medium at a density of  $1\times 10^6$ /ml.
3. Gently pipette 100–200  $\mu$ l cells onto coverslips over the immobilized antibodies and incubate at 37 °C 5% CO<sub>2</sub> for desired time (*see Note 4*).
4. Gently dip coverslips in a 50 ml conical tube containing PBS to remove unbound cells.
5. Gently pipetting 200  $\mu$ l of prewarmed Cytofix/Cytoperm buffer, to fix and permeabilize the cells on coverslips.
6. Incubate for 10–30 min in humidified chamber (e.g., slide box with moist paper towel) in the dark at room temperature.

### **3.5 Antibody and Phalloidin Staining**

1. Following fixation and permeabilization, wash cells gently in staining buffer (by gently dipping coverslips in 50 ml conical tube).
2. Prepare antibodies for staining in staining buffer. Include single stained controls (*see Note 5*).
3. Stain first with primary antibody to structure of interest (i.e., microtubules or perforin) for 30–60 min.
4. Rinse gently in staining buffer and dab edges of hydrophobic region with cotton swab.
5. Stain with secondary antibody for 30–60 min and wash as in **step 1** in Subheading 3.5 (*see Note 6*).
6. Repeat for subsequent antibody stains.
7. Stain for phalloidin to detect F-actin last (if phalloidin staining is desired).
8. Maintain humidity during staining by performing all stains in humidified chamber.

### **3.6 Mounting Coverslips on Slides**

1. Use mounting media that is compatible with the imaging technique (*see Note 7*).
2. Gently pipette approximately 10–20  $\mu$ l of mounting media on each glass slide, avoiding formation of bubbles. Should bubbles form, gently pipette these off.
3. Gently lay the coverslip down on the slide, so that the surface on which the cells are fixed is in contact with the mounting media.
4. Allow coverslips to set for 24–48 h and then seal edges of coverslips with nail polish.

### **3.7 Image Acquisition**

1. Prior to acquiring images turn on the Leica SP8 confocal microscope. Initialize LASAF software and allow lasers to warm up for 30–60 min.
2. Place slide on microscope and adjust focus with eyepieces.
3. Acquire first channel and set laser power for optimal signal without pixel saturation. Adjust gain and, if necessary, exposure time (*see Note 8*).
4. Repeat setup for each channel to be imaged. Acquire single stained controls using each setting and adjust as necessary to reduce spectral overlap into other channels.
5. Acquire images. For quantitative imaging it is recommended to acquire at least 20 cells per condition, although this can be best dictated by the experiment in question and using traditional appropriately powered statistical sample size calculations. To preserve fluorescence during sequential scan it is best

to acquire images with longer excitation wavelengths first. For best resolution of those components at the synapse, focus finely to ensure imaging at the plane of the glass.

6. Should the application of STED be desired, align the depletion laser prior to beginning STED imaging using automatic STED alignment in LASAF software.
7. Apply STED laser by activating laser to 50% to begin with and determine conditions that give best resolution. Variables to be adjusted at this stage may include laser power, gain, STED laser power and time gating (where applicable). A visual step-wise demonstration of this process has been published and common pitfalls are shown in Fig. 2 [12].
8. Save and export images as .LIF files.

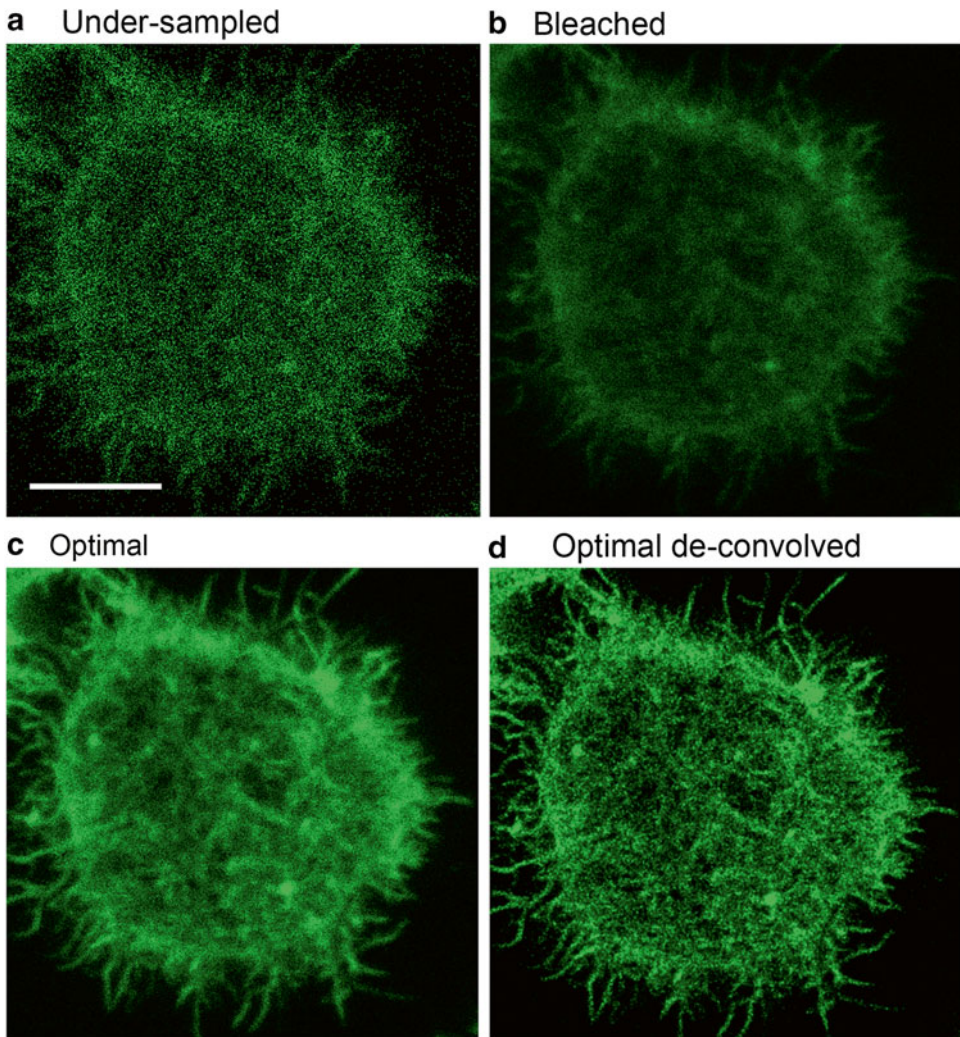
### **3.8 Processing and Analyzing Images (See Note 9)**

1. Open images in Volocity (direct import) or FIJI (using Bioformats plug-in) for image analysis and processing.
2. Determine threshold based on background fluorescence (*see Note 10*). In general, it is best to analyze data in its most unmanipulated form possible.
3. Following selection of objects based on threshold, the mean fluorescence intensity and area of these objects can be calculated using the Measurement feature in both Volocity and FIJI software.
4. Choose representative images for display and export as .TIF files at 300 dpi (*see Note 11*).

---

## **4 Notes**

1. Isolate primary NK cells from peripheral blood prior to the experiment. For imaging experiments it is best to use a negative selection kit such as RosetteSep (STEMCELL Technologies). Primary NK cells should not be maintained or expanded in cytokines if the cytoskeleton is to be studied (unless this is the purpose of the experiment) as these have been shown to influence signaling pathways following activation [13].
2. Ensure that if primary-secondary antibody combinations are used, they were not raised in the same species as the antibody used to coat the coverslip.
3. Coverslips can be coated overnight at 4 °C but should be prewarmed at 37 °C prior to use. It is preferable to coat immediately prior to the experiment but the user may prefer to save time by precoating.
4. The duration of activation should be chosen based on the function of interest. For example, F-actin accumulation will



**Fig. 2** Acquisition by STED imaging and common pitfalls. NK92 cells were activated on anti-CD18 and -NKp30 coated glass for 20 min then fixed, permeabilized and stained for F-actin with Phalloidin Alexa Fluor 488. **(a)** An example of loss of image information due to under-sampling. **(b)** An example of loss of resolution due to bleaching/over-sampling **(c)** conditions optimized **(d)** optimized conditions lead to greater improvement in resolution with deconvolution. Scale bar = 5  $\mu$ m. Image reproduced with permission: “Mace, E. M., Orange, J. S. Visualization of the Immunological Synapse by Dual Color Time-gated Stimulated Emission Depletion (STED) Nanoscopy. *J. Vis. Exp.* (85), e51100, doi:[10.3791/51100](https://doi.org/10.3791/51100) (2014)”

occur relatively quickly (5–10 min), where as complete polarization of lytic granules may take 25–30 min.

5. We recommend staining each antibody sequentially, although phalloidin may be combined with the final secondary antibody. Single stained controls should be prepared in order to ensure lack of spillover of signal into other channels of interest. Antibody concentration will require titration depending on



the sample and the antibody. For many antibodies dilutions of 1:100 or 1:200 are suitable but titration of primary antibodies in particular is highly recommended. Generally, 30 min of staining time per antibody is sufficient. In the case of low avidity antibodies staining time may be increased to 1 h.

6. Recommended secondary antibodies for STED include Alexa Fluor 488, tetramethylrhodamine, Alexa Fluor 532, and Horizon V500. For STED, avoid streptavidin–biotin combinations.
7. The use of hard-set medias is preferred, namely ProLong, ProLong Gold or ProLong Diamond. Vectashield is not compatible with STED. Mowiol is acceptable. 2, 2 thiodioethanol cannot be used in combination with phalloidin.
8. Pixel size is a function of several variables on a laser scanning confocal microscope. These include the image format (number of pixels contained in the image), the amount of zoom used and the objective. Data collected as part of a single experiment should be obtained with the same pixel size and acquisition settings throughout. For STED, a pixel size of less than 40 nm is highly recommended.
9. The analysis performed will be dependent upon the experiment. However, common measurements may include mean fluorescent intensity and area as these can reflect the relative density of protein of interest. In general, image processing for visualization should be limited to the application of a threshold to remove background signal. All forms of nonlinear thresholding ( $\gamma$ ) should be avoided. The threshold should be chosen based on fluorescent intensity of the background and should generally be fixed at this intensity for all images and analyses.
10. For STED images, deconvolution can dramatically improve image quality and enable more accurate analysis. Deconvolution recovers information lost through imaging and can be performed mathematically through software. Huygens (SVI) is provided with Leica LASAF software and uses the theoretical point spread function estimated by the parameters used in your experiment to deconvolve both confocal and STED data. We generally use default parameters for deconvolution although optimizing the signal to noise ratio may be necessary.
11. Images portrayed should reflect the mean of values obtained and be processed in the same way as during analysis. If possible, each channel should be shown separately and together as a merge. Scale bars should be shown. It is recommended that in an aggregate data summary the representative image be specifically denoted as to where it resides within the aggregate.

## References

1. Mace EM, Dongre P, Hsu HT et al (2014) Cell biological steps and checkpoints in accessing NK cell cytotoxicity. *Immunol Cell Biol* 92:245–255
2. Lagrue K, Carisey A, Oszmiana A et al (2013) The central role of the cytoskeleton in mechanisms and functions of the NK cell immune synapse. *Immunol Rev* 256:203–221
3. Mace EM, Orange JS (2012) New views of the human NK cell immunological synapse: recent advances enabled by super- and high-resolution imaging techniques. *Front Immunol* 3:421
4. Brown AC, Oddos S, Dobbie IM et al (2011) Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. *PLoS Biol* 9, e1001152
5. Rak GD, Mace EM, Banerjee PP et al (2011) Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol* 9, e1001151
6. Gong JH, Maki G, Klingemann HG (1994) Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia* 8:652–658
7. Comrie WA, Babich A, Burkhardt JK (2015) F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse. *J Cell Biol* 208:475–491
8. Pigeon SV, Cordoba SP, Owen DM et al (2013) Superresolution microscopy reveals nanometer-scale reorganization of inhibitory natural killer cell receptors upon activation of NKG2D. *Sci Signal* 6:ra62
9. Mace EM, Wu WW, Ho T et al (2012) NK cell lytic granules are highly motile at the immunological synapse and require F-actin for post-degranulation persistence. *J Immunol* 189: 4870–4880
10. Mace EM, Orange JS (2014) Lytic immune synapse function requires filamentous actin deconstruction by Coronin 1A. *Proc Natl Acad Sci U S A* 111:6708–6713
11. Toomre D, Bewersdorf J (2010) A new wave of cellular imaging. *Annu Rev Cell Dev Biol* 26:285–314
12. Mace EM, Orange JS (2014) Visualization of the immunological synapse by dual color time-gated stimulated emission depletion (STED) nanoscopy. *J Vis Exp*
13. Orange JS, Roy-Ghanta S, Mace EM et al (2011) IL-2 induces a WAVE2-dependent pathway for actin reorganization that enables WASp-independent human NK cell function. *J Clin Invest* 121:1535–1548