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Measurement of lytic granule convergence after formation of an NK cell immunological synapse

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Summary

Natural killer (NK) cells contain specialized lysosome-related organelles termed lytic granules allowing them to mediate cytotoxicity against tumorigenic or virally infected target cells. NK cells polarize their lytic granules towards a target cell via the microtubule-organizing center (MTOC). Prior to that, however, lytic granules converge to the MTOC along microtubules utilizing minus-end directed microtubule motors. Herein we describe how to visualize and quantify lytic granule convergence using confocal microscopy to gain quantitative insight into NK cell cytotoxicity and its regulation.

Keywords

Immunological synapse; MTOC; lytic granules; convergence; confocal microscopy; mathematical algorithm

1. Introduction

The interface between an NK cell and its target, where the NK cell obtains activating and inhibitory inputs through its germline encoded receptors, is a form of an immunological synapse (IS). IS formation is an essential prerequisite for target cell contact-dependent NK cell function, most notably cytotoxicity. The formation of a mature lytic NK cell IS (NKIS) is a complex process which requires numerous steps to promote cellular function (reviewed in (1,2)). One of the characteristic features of lytic NKIS formation is polarization of the microtubule-organizing center (MTOC) to the IS along with the lytic granules followed by exocytosis of the lytic granule contents onto the target cell to trigger target cell death. By quantitatively studying the dynamics of the NKIS the advancement of lytic granules towards their directed secretion can be utilized as a precision indicator of commitment to and effectiveness of NK cell cytotoxicity. However, this demands an in-depth understanding of the process, access to high-resolution microscopy, and application of unbiased quantitative analyses of images paired with appropriate statistical evaluations. Performed rigorously, the

NKIS can be measured and linked to effectiveness in cytotoxicity and other related NK cell functions.

In developing methods for studying the NKIS, we have focused on the use of confocal microscopy, which provides the advantage of measuring events within a given optical section in a single cell without interference of signals from elsewhere in the cell. This increases the specificity of the data, particularly when the NK cell is still conjugated to its target. Using this approach, optical sections taken through a vertical axis of the conjugate makes it possible to capture the entire biology in three-dimensions (3D). Additionally, confocal microscopy allows for the imaging of living cells to create four-dimensional sequences (3D imaging over time). Live cell imaging allows individual NK cells in conjugates to be evaluated throughout the entire range of IS formation and function including lytic granule convergence, MTOC polarization, granule exocytosis, killing and detachment. Quantitative algorithms have been developed for precise measurement of each of these steps. Because of the large amount of information that can be collected from a single live cell imaging sequence, confocal microscopy remains one of the most effective and affordable means of obtaining highly quantitative information regarding the kinetics and dynamics of NKIS formation.

Herein we focus specifically on methods for measuring one important feature of the NKIS: the positioning of lytic granules relative to the MTOC. Specifically their characteristic behavior of converging lytic granules to the MTOC which happens rapidly after NKIS formation (3,4). We refer to this process as granule convergence (5). The same process of convergence has recently also been identified in cytotoxic T cells (6) and is thus likely a general feature of certain types of activation in cytotoxic lymphocytes. In NK cells engaged in the destruction of a susceptible target cell, the granules move along individual microtubules towards the MTOC (in a minus-end-directed manner) using dynein motors (3). They aggregate around the MTOC and then polarize along with the MTOC towards the IS. Once polarized, they are capable of degranulation upon the presence of an appropriate signal (5,7,8). Aside from the activation of motor function, numerous adaptors and signals have been defined that are needed for granule convergence (4,5,7,9–11). The ability to precisely quantify this process is essential in measuring the effectiveness of signaling for and preparedness of lytic granules for directed secretion (5). A generalizable protocol for precisely measuring lytic granule positioning and convergence is provided.

2. Materials

1. Culture medium: RPMI 1640, supplemented with 10% Fetal Bovine Serum (FBS), 10mM HEPES, 1× Penicillin/Streptomycin, 100 µM MEM nonessential amino acids, 1 mM MEM sodium pyruvate, 2mM L-glutamine (R10). Phenol Red-free RPMI is used for live-cell imaging. Myelocult H5100 (Stemcell), supplemented with 1× Penicillin/Streptomycin and 100 U/ml of IL-2. All cells were cultured in R-10 medium except for the NK-92 cells which were cultured with the Myelocult medium.

2. Human immortalized NK cell line NK-92 (ATCC Cat # CRL-2407) as an example cytotoxic cell utilized here.
3. K562 (ATCC Cat # CCL-243), an erythroleukemic cell line, was used as target cells for NK-92 cells.
4. Poly-L-lysine- or silane-coated glass slides (Electron Microscopy Sciences Cat # 63410-01 or 63411-01 respectively).
5. Coverslips 22 mm square #1.5.
6. 8-well culture slides (Fisher Scientific Cat # 354108)
7. Hydrophobic ink pen.
8. Fixative and permeabilization agent (Cytofix/Cytoperm, BD) containing 0.1% (v/v) Triton X-100.
9. Phosphate-buffered saline.
10. PBS containing 1% bovine serum albumin.
11. PBS containing 1% BSA and 0.1 % saponin.
12. Rabbit anti- α -tubulin antibody (Abcam Cat # ab18251).
13. Goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 647 conjugate (Thermo Fisher Cat # A21245).
14. Alexa Fluor 488 anti-human perforin antibody, clone dG9 (Biolegend Cat # 308108).
15. Mounting medium: Vectashield (Vector laboratories Cat # H-1000) or ProLong Gold antifade mountant (Thermo Fisher Cat # P36930).
16. 8-well chambered coverglass (Thermo Scientific Cat # 155411).
17. LysoTracker Red DND-99 (Thermo Fisher Cat # L7528).
18. SiR-tubulin (Spirochrome Cat # SC002). Dissolve the content of the vial in anhydrous DMSO to make a 1 mM stock solution. Store in the dark at -20°C .
19. Verapamil. Dissolve the content of the vial in pure ethanol to prepare a 10 mM stock solution. Store at 4°C .
20. CellTrace CFSE cell proliferation kit (Thermo Fisher Cat # C34554). Dissolve the content of the vial in DMSO to make a 5 mM stock solution. Store in the dark at -20°C .
21. Clear nail polish.
22. General lab equipment:
 - a. Tissue culture biocontainment hood, incubator for cell culture.
 - b. Hemocytometer or other suitable device for cell enumeration.
 - c. Tweezers.

- d. Leica Laser Scanning Confocal Microscope SP8 (*see* Note 1), or equivalent.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Visualizing Lytic Granules and Microtubules in fixed cells

3.1.1. Preparation of NK and Target Cells for Fixed Cell Imaging

1. Culture NK cells and target cells in appropriate culture medium (*see* Section 2) at a low density, preferably under 5×10^5 /ml, to enable optimal cellular function (*see* Note 2). As an alternative *ex vivo* NK cells freshly isolated by negative selection using the human NK cell isolation kit (Miltenyi Biotec) or the RosetteSep human NK cell isolation reagent (StemCell Technologies) may be used.
2. Wash NK and target cells once with RPMI medium prior to use.
3. Resuspend NK and target cells with culture medium at the density of 1×10^6 /ml and 2×10^6 /ml, respectively, to enable a 1:2 effector to target ratio.
4. Take 100 μ l of NK and target cell suspension and mix them in a 15 ml polypropylene conical tube (*see* Note 3).
5. Allow conjugates to form at 37 °C for approximately 10 min. Note that lytic granule positioning can be measured in non-conjugated NK cells as well as NK cells that are activated by another means such as by soluble stimulants (such as cytokines), antibody coated on glass or planar lipid bilayers (*see* Note 4) (12). The conjugate protocol is offered here as an example.

3.1.2. Slide Preparation

1. On poly-L-lysine or silane-coated slides, use a hydrophobic pen to draw an enclosed section. Squared regions with sides of 1 cm should hold up to 200 μ l of liquid (*see* Note 5).

¹The microscope setup described here can be replaced with any other confocal system equipped with similar capacities in term of excitation laser lines and detection: Leica SP8 inverted laser scanning confocal microscope (Leica microsystems) with Leica APO PL 63 $\times$ objective (NA = 1.47) or another high numerical aperture oil immersion objective. The excitation laser is a white light laser configured to provide discrete illumination from 470 nm to 670 nm and controlled LAS AF software for image acquisition (supplied with the microscope). Emitted light is collected by PMT or HyD detectors, following selective optical plan transmission via a variable pinhole. The microscope stand is enclosed in a heated chamber for live experiments.

²To enable optimal cellular function and activity, it is recommended to split the cells or supplement the culture with fresh culture medium the day before experiments.

³To optimize conjugation efficiency by increasing the chances of cell-cell encounter, it is recommended to reduce the volume of NK-target cell mixture, preferably ranging from 100 μ l to 200 μ l with a total number of $1-2 \times 10^6$ cells. It is also recommended to have a 1:2 or even 1:3 effector to target ratio.

⁴To activate NK-92 cells or human primary NK cells, pre-coat the chambered coverglass or glass slides with anti-CD18 (Clone TS1/18) and anti-NKp30 (Bio Legend Cat # 325204). For antibodies; to activate YTS cells, pre-coat the glass with anti-CD18 and anti-CD28 antibodies. In each case there are numerous other alternatives that can be utilized. All coating antibodies should be diluted to a concentration of 5 μ g/ml with PBS and allowed to adhere to the coverglass for at least 30 min at 37°C or overnight at 4°C.

⁵Culture slides with removable chambers can be used as an alternative measure. This is especially useful when the number of effector cells is limited (*e.g.* patient primary NK cells).

2. Prepare a humidified covered chamber containing moistened paper towels for slide incubation during the staining process.
3. Keep the glass slides in the humidified chamber at 37 °C to pre-warm the slides enabling optimal binding efficiency of cells.
4. Prepare buffers for the staining process. Fixative and permeabilization (fix and perm) buffer: 0.1% Triton X-100 in Cytofix/Cytoperm buffer. Staining/Washing buffer: 1% BSA and 0.1% saponin in PBS (for the evaluation of living cells *see Section 3.2*).
5. Prepare antibodies at desired concentration in the staining/washing buffer (*see Note 6*).
6. Following the conjugation process (*see Section 3.1.1*), gently resuspend the conjugates by pipetting slowly until no cell aggregates are observed.
7. Transfer the conjugate suspension onto the slides within the hydrophobic enriched region.
8. Allow cells to adhere to the slides in the humidified chamber for approximately 20 min at 37 °C.
9. Use a 20–200 µl pipette, gently lay the tip against the ink and point the tip towards the constrained liquid to carefully withdraw liquid from the hydrophobic enriched region. Be sure not to touch the cells or disrupt the hydrophobic ring. The cells should now appear as a thin cloudy layer on the slides.
10. Add 150 µl of fix and perm buffer slowly to the hydrophobic enriched area. Be careful not to disrupt the cells on the slides.
11. Withdraw buffer from the slides as per **Step 9**.
12. Add 200 µl of staining/washing buffer to the ink enclosed area to rinse off excess fix and perm buffer.
13. Repeat the washing **Steps 11–12** twice.
14. Aliquot 150 µl of primary antibody solution to the staining region and incubate in the humidified chamber for 30–60 min. If using a fluorophore directly conjugated antibody, skip to **Step 18**.
15. Repeat **Steps 11–13** to remove residual primary antibody.

⁶Antibody titration is essential to ensure efficient fluorescent detection of a molecule of interest while preventing unspecific staining that will contribute to a high fluorescent background and a reduced signal-to-noise ratio. Guidelines for antibody titration are as follows:

- a. Each primary antibody should be titrated individually and compared to an isotype control.
- b. Each secondary antibody should be titrated using a fixed concentration of a primary antibody with known effectiveness.
- c. Utilize a wide range of antibody concentrations to determine the ideal concentration that provides efficient fluorescence at reasonable microscopic settings and good signal-to-noise ratio.
- d. The signal-to-noise ratio can be measured for each antibody dilution by calculating the mean fluorescence intensity (MFI) of 10 objects (at least) and divide it by the MFI of the same number of similarly sized regions of the background. Ideally, this ratio should be above 3.

16. Add 150 μ l of fluorophore-conjugated secondary antibody solution to the hydrophobic ink region and incubate in the humidified chamber for 30–60 min.
17. Repeat **Steps 11–13** to wash off the residual secondary antibody.
18. Add directly conjugated fluorescent antibody that recognize other molecules of interest and incubate for 30–60 min in the humidified chamber.
19. Repeat **Steps 11–13**.
20. Perform **Steps 14–19** until all molecules of interest are stained subsequently.
21. Add one drop of mounting medium to each staining region (*see* Note 7).
22. Place a coverslip on the slide using forceps. Gently press on the coverslip to remove excess mounting medium and dry with tissue paper.
23. Keep slides in the dark overnight with the coverslip facing upwards.
24. Seal all sides of the coverslip with nail polish.

3.1.3. Imaging

1. Initialize the confocal microscope system according to the manufacturer's instructions and allow the lasers to reach stable working temperature and output power. The results of the experiment can be acquired using any of the two most common setups to achieve confocal imaging: either a confocal laser scanning microscope (CLSM) or a confocal spinning disk microscope (CSDM). One typical CLSM system is detailed in Note 1 but this protocol is applicable to any confocal system with appropriate laser lines (CLSM) or laser line and excitation/emission filter sets (CSDM).
2. Select an apochromatic, high magnification objective (60 $\times$ or higher) with a high numerical aperture (above 1.2) and add a drop of immersion oil.
3. Secure the sample on the stage and adjust the focus to be able to clearly identify cells using the eyepiece and bright field illumination for transmitted light.
4. Adjust the specific illumination parameters for the acquisition of images of the best resolution possible while maintaining the photobleaching of the dye to a minimum and removing all potential chromatic spillover between the fluorescent dyes imaged:
 - a. Confocal scan head settings: rate of 600 Hz for an intermediate image size of 512×512 pixels (*see* Note 8) and a line averaging value of 6–8

⁷Several commercially available mounting media can be used to preserve the samples for storage and to reduce photobleaching. ProlongGold is a hard-setting antifade mounting solution specifically designed for long-term storage of samples stained by Alexa Fluor dyes. Its optical properties evolve over time and the sample should only be imaged after 20 hours minimal to allow the increase of refractive index from 1.42 to 1.45. VectaShield is another excellent choice which has better optical properties immediately after use (higher refractive index of 1.45) and, as a non-hardening medium, helps to preserve three dimensional structures.

⁸All the recent line scanning confocal microscopes are equipped with resonant scanners to allow acquisition at very high frequency (above 10000 Hz). Although this is a substantive advantage for fast live acquisition while minimizing photodamage, it comes at a cost of an increase in noise. Therefore, a higher value for line averaging needs to be used to average out the electronic noise in the system. Routinely, the regular scanning method is preferred for fixed samples imaging where resolution is more important than speed and sample preservation.

passages. The pinhole should be set to 1 Airy unit to maintain high confocality and Z resolution precision.

- b. Excitation laser lines are set to coincide with the maximal excitation wavelength of the fluorescent dye used in the experiment (*see* Note 9).
 - c. The spectral gates for each detector for each fluorescent reporter are set to collect photons at wavelength overlapping with the maximum emission peak of the fluorescent dye (*see* Note 9).
 - d. For conventional PMT detectors (Photo Multiplier Tube), the adjustment gain is set between 600 and 800 V. For the high sensitivity and low noise GaAsP (Gallium Arsenide Phosphide) detectors, the common range is 100–250 % (*see* Note 10).
5. Adjust the Z position of the sample and, if 3D imaging is performed, define the bottom and the top limits of the volume encompassing the entire cell or conjugate. Set the step size according to the recommendation of the acquisition software.
 6. Start the acquisition and proceed similarly for at least 20 cells.
 7. Analyze the preliminary results as described below before performing a power analysis test to evaluate the required sample size. Acquire additional data as necessary.

3.2. Visualizing Lytic Granules and Microtubules in live cells

3.2.1. Preparation of the Imaging Chambered Coverglass

1. Pre-coat the chambered coverglass with a monoclonal antibody at the concentration of 5 µg/ml in PBS that will specifically recognize the target cells and not the NK cells for the purpose of immobilizing the target cell (*see* Note 11, Figure 1). Allow the antibody to adhere to the coverglass at 37 °C for at least 30 min or at 4 °C overnight. Alternatively, if target cells are not used, pre-coat the chambered coverglass with antibodies that will engage the adhesion and/or activation receptors on the surface of NK cells to induce formation of an “artificial” IS as an activating surface (*see* Note 4, Figure 1).

⁹Point scanning confocal microscopes allow the user to precisely set the spectral gates for each detector, unlike the spinning disk confocal systems which are based on a set of pre-determined chromatic filters and beam splitters. Although the former are more flexible than the latter, they require precise knowledge of the optimal absorption and emission wavelengths of each dye used in the experiment. This information is typically provided with the consumables and must be carefully studied to organize the collection of emitted photons between the detectors in different sequences. Usually, the spectral gates are all set to collect photons at wavelengths matching the maximum emission peak of each dye and then split into different sequential illumination sequences to suppress spillover between dyes with overlapping spectra. Finally, the segregation of the different signals must be confirmed by acquiring all while disabling each laser line successively. The result should be a disappearance of the signal specific to the disabled excitation wavelength with little to no change in the intensity of the other wavelengths acquired.

¹⁰The gain value of the detectors should be very carefully adjusted. Typical PMT display a linear conversion range between 600 to 800 V. If it needs to be lowered below 600 V, then the laser line intensity must be lowered instead; conversely it needs to be increased if the gain setting reaches above 800 V. The newest generation of detectors (GaAsP) is not so sensitive to noise and displays a linear amplification across a much broader range (50 % to 300 % from our experience). Consider lowering the laser line intensity if the amplification is below 100 %.

¹¹To capture and immobilize the K562 and 721.221 cells on the chambered coverglass for increased stability and hence higher live imaging quality, pre-coat the wells with antibodies against CD58 and CD48 antibodies, respectively.

2. Rinse the antibody-coated chambered coverglass 3 times with PBS prior to use.

3.2.2. Preparation of NK and Target Cells for Live Cell Imaging

1. Prepare NK and target cells separately, washing each once with RPMI 1640 by spinning at $225 \times g$ for 5 min.
2. Add 0.5 μ l of SiR-tubulin and 1 μ l of verapamil stock solution to 1×10^5 NK cells in 1 ml of R-10 medium (500 nM and 10 μ M, respectively) and incubate for 1 h at 37 °C before continuing to **Step 3**. Alternatively, a cell expressing a fluorescent protein-conjugated microtubule biosensor may be utilized (while there are many possibilities examples include FP- α -tubulin and FP-MAP4) in which case the entirety of this step would be skipped.
3. Add 10 μ l of LysoTracker Red DND-99 to 1×10^5 cells NK cells in 1 ml of R-10 medium (10 μ M) and incubate for another 30 min at 37 °C. During incubation, proceed to **Step 4** for target cell preparation.
4. Add 1 μ l of CFSE stock solution to 2×10^5 target cells in 1 ml of R-10 (5 μ M) and incubate for 5 min at 37 °C.
5. Following incubation, wash NK and target cells 3 times with R-10 by spinning at $225 \times g$ for 5 min.
6. Resuspend NK and target cells with dye-free R-10 medium supplemented with verapamil (10 μ M) at the density of 1×10^5 /ml and 2×10^5 /ml, respectively, to enable a 1:2 effector to target ratio.
7. Aliquot 100 μ l of target cell suspension to the pre-coated and PBS-rinsed well of the 8-well chambered coverglass. Allow the target cells to adhere to the coverglass for approximately 10 min at 37 °C.
8. Add 100 μ l of NK cell suspension to the target cells after having transferred the sample on the microscope stage.

3.2.3. Imaging

1. To ensure the stability of the sample during live imaging and avoid axial drifting, it is absolutely paramount to equilibrate the temperature of the microscope chamber with the sample.
2. Adjust the confocal scanning head settings and illumination settings according to **section 3.1.3**. In order to reduce the phototoxicity, some parameters should be set to conservative values if available as follows:
 - a. Set the laser scanning mode to resonant in order to achieve faster laser scanning rates (14000 Hz).
 - b. To compensate for the faster rate, increase the line averaging to 16–32 passages per line. It is not recommended to use any of the accumulation functions as they result in loss of time resolution.

- c. In practice, increasing the Z-spacing between the optical sections to 1 μm might be considered as a fair compromise for reducing photo damaging of the cell (and in light of the mean size of lytic granules and special resolution of a confocal microscope).
3. Using the eyepiece and using transmitted light, identify isolated cells or conjugates (depending on your experiment). Using fluorescence and the camera or photomultiplier tube captured image, set the Z position of the sample to define the bottom and the top limits of the volume encompassing the entire object of interest.
4. Acquire one single stack and review your illumination setting accordingly.
5. Visually identify 5 objects of interest and start the acquisition of the time lapse with 5 min interval between each time point, for a total duration of 2 h (see Note 12).
6. Repeat the experiment to collect at least 20 cells and perform a power analysis test as described in **3.1.3 step 7**.

3.3. Measuring Lytic Granule Convergence to the MTOC in fixed cells

The degree of lytic granule convergence is demonstrated by the mean distance of individual granules to the MTOC in an NK cell. The more scattered the granules are within an NK cell, the larger the numerical value becomes. The values may vary among NK cell lines and primary NK cells due to their size differences. Therefore, it is critical to always include control groups to enable objective comparisons. To calculate granule to MTOC distance, it is required to obtain coordinates of the centroids of the lytic granules and the MTOC. For this purpose, multiple software platforms are available to the scientific community. While the use of other platforms are also viable, we offer below the analysis workflow for two currently available software platforms: Volocity (Perkin Elmer) and Fiji (13,14).

3.3.1. Volocity

1. Create a new library in Volocity. Drag the image files onto **Library** to open the data set.
2. Select **Measurements** tab to initiate the features for image analysis.
3. Go to **Properties** under **Edit**. Set the calibrated X, Y and Z pixel dimensions (measured) according to the microscope used for image acquisition.
4. Create a measurement protocol to determine the coordinates of each and every lytic granules in individual NK cells:
 - a. Drag and drop the **Find Objects** onto the tasks panel. Select the appropriate channel in which perforin is to be identified. Replace the default name Population 1 with Lytic Granules. Click on the gear icon

¹²The length of acquisition depends on the aim of the experiment. Image for 30–60 min if only observation of convergence/polarization is required; image for 1–2 h if death of the target cell is also of interest.

in the protocol to access the dialog for intensity thresholding. Select **Threshold** using **Intensity** and drag the red bar to include voxels that define perforin and remove noise (*see* Note 13). Lastly, set the **Minimum object size** as $0.03 \mu\text{m}^3$ (*see* Note 14).

- b. Drag and drop the **Clip to ROIs** into the Find objects protocol.
 - c. Drag and drop the **Separate Touching Objects** into the existing protocol. Set Object size guide at $0.01 \mu\text{m}^2$ to separate lytic granules in close proximity.
 - d. Drag and drop **Compartmentalize** into the tasks panel and select to **Divide** Lytic Granules **Between** ROIs. This separates the lytic granules within different ROIs (NK cells).
 - e. Click **Measure** at the bottom of the protocol and select **Centroid**.
 - f. Select **Save Protocol...** from the Measurements menu. Name and save the protocol as Lytic granules to allow rapid application for image analyses.
5. Create a second measurement protocol to determine the coordinates of the MTOC in NK cells:
 - a. Drag and drop the **Find Objects** onto the tasks panel. Select the appropriate channel in which α -tubulin or pericentrin is to be identified and name the population MTOC.
 - b. Click on the gear icon in the protocol to access the dialog for intensity thresholding. Set the **Minimum object size** as $0.1 \mu\text{m}^3$. Select **Threshold** using **Intensity** and drag the red bar to include voxels that define one single MTOC in each NK cell.
 - c. Drag and drop the **Clip to ROIs** into the Find objects protocol
 - d. Click **Measure** at the bottom of the protocol and select **Centroid**.
 - e. Select **Save Protocol...** from the Measurements menu. Name and save the protocol as MTOC.
 6. Click on the image to be analyzed in the library.
 7. Use **Freehand** or **Circle** tool to draw individual regions that include the NK cells in the conjugates that are to be analyzed. Each enclosed region is accounted as one ROI (NK cell), separated from the others in the same image field and thus

¹³To allow for unbiased comparisons, it is essential to apply the same threshold setting for images within one experiment and ideally for images from both the control and experimental groups. It is also recommended to acquire all images on the same day. To apply thresholding using intensity (fixed cell) or SD (live cell) in Volocity, drag the red bar along the x-axis of the histogram of intensities until Volocity picks up all the signals that define the molecule of interest in the image. The goal is to include the desired signals while diminishing unwanted (but quantitatively determined) background.

¹⁴The size of lytic granules in NK-92 cells, as determined by super resolution microscopy, ranges from 100–650 nm in diameter with the majority of them ranges from 250–400 nm (15).

will be analyzed individually. Be sure to exclude any part of the target cells from the marked regions.

8. Click on the saved protocols Lytic Granules and MTOC under **Saved protocols** in the tasks panel to apply all settings to the image under analysis.
9. Select **Make Measurement Item...** from the Measurements menu. Create a new file or add the results to an existing measurement table.
10. Repeat **Steps 6–9** until all desired images are analyzed.
11. Click on the measurement item created in the library. Go to **Columns** under **Raw** to select the data wish to be displayed (e.g. Item Name, Group ID, Centroid X and Y).
12. Select Lytic Granules from the dropdown menu of **Display**.
13. Save the X and Y coordinates for Lytic Granules to an Excel sheet.
14. Repeat **Steps 12–13** for MTOC measurements.
15. Go to **Section 3.3.3** for calculations and data plotting.

3.3.2. Fiji

1. Open your raw images by dragging their icon onto the main task bar.
2. If more than a cell is present in the frame, use the **Freehand tool** to circle one and select **Clear outside** to restrict all the channels to one cell only.
3. If running this analysis for the first time, select the desired measurements to be captured in the **Set Measurements** menu. Tick the options for Area, Centroid, Center of Mass and Stack Position.
4. This analysis needs to be done for each channel sequentially (Figure 2A).
5. Adjust the brightness of each fluorescence channel using the **Brightness/Contrast** tool to be able to distinguish the subtle details surrounding the objects of interest.
6. Using the **Threshold** panel, turn on the checkbox for **Dark background** and, using the sliders, highlight the population of pixels that form the object of interest based on the fluorescence intensity displayed in the pixel intensity histogram. To measure the localization of the MTOC from a microtubule straining (α -tubulin instead of pericentrin), make sure to only include the brightest portion of your signal to limit the detection to one object per cell.
7. Optionally, if a high level of background is present in the image, it is possible to reduce it using a **Rolling Ball** algorithm found under **Process > Subtract Background...** and set to a radius of 2–4 times the size of the object under analysis to avoid removing any object of interest (usually a 20 pixels wide radius is a safe choice).

8. Click **Apply** to transform the fluorescence image into a binary image. If the granules are contiguous or overlapping, a **Watershed** segmentation algorithm can be applied and is found under the **Process > Binary** menu.
9. Go to **Analyze > Analyze Particles...** menu and set a filter for the granule size from $0.05 \mu\text{m}^2$ to $2 \mu\text{m}^2$ or $0.1 \mu\text{m}^2$ to $1 \mu\text{m}^2$ for the MTOC. Select the following options: **Display Results** and **Show Outlines, Exclude on Edges**. Turn on the checkbox **Clear Results** to not add this set of measurement to the previously analyzed image.
10. Visually inspect the accuracy of the detection using the outlines drawn on the image after detection (Figure 2A). If too many objects are missed, return to **Step 4** and adjust the values of the threshold until a satisfactory coverage is obtained.
11. **Save** the spreadsheet containing all the positions measured in the current image and proceed to the next channel or image. The position of each granule is under the column X, Y and Z if applicable.

3.3.3. Calculations and data plotting

1. Measure lytic granule convergence using the MTOC to granule distance (MGD) formula, where x , y and x_i , y_i are centroid coordinates for the MTOC and the individual lytic granules, respectively. The MGD measures the shortest length between centroid of granules and MTOC as depicted in Figure 2A. “ n ” indicates the number of granules, in a given frame, selected by Volocity or Fiji based on the intensity threshold used.

$$\text{MGD} = \frac{(\sum_{i=1}^n \sqrt{(x - x_i)^2 + (y - y_i)^2})}{n}$$

2. For images involving 3D reconstructions of multiple z-axis planes, the centroid coordinates for MTOC and lytic granules should be measured in the same way as described above using the formula provided below:

$$\text{MGD} = \frac{(\sum_{i=1}^n \sqrt{(x - x_i)^2 + (y - y_i)^2 + (z - z_i)^2})}{n}$$

3. Calculate the physical distance of each and every granule from the MTOC as described in **Step 1** or **Step 2** (2D or 3D). Take the mean of all calculated distances in individual NK cells and plot them as single dots. In the dot plots, x-axis contains different sample populations (e.g. control vs. patient, vehicle- vs. drug-treated, Figure 2B) and y-axis shows the mean lytic granule to MTOC distances (Figure 2C). Each dot represents how dispersedly lytic granules are localized within one NK cell conjugated with a target cell. The more converged the lytic granules are, the smaller the number will become, ranging from close to zero (all granules converged tightly around the MTOC) to approximately the radius of the NK cells (all granule at the periphery of the cell).

3.4. Measuring Lytic Granule Convergence to the MTOC/Granule Centroid in live cells

Fixed cell confocal microscopy allows for high-resolution images and the convenience of collecting sample sizes that are large enough to achieve a desired power with biologically relevant differences. Meanwhile, live cell confocal microscopy provides temporal resolution that is critical in exploring the dynamics in the biological processes involved in NK cell cytotoxicity such as synapse maturation. For instance, whether a genetic defect or drug treatment affects lytic granule convergence in NK cells can be rapidly determined by fixed cell imaging analysis, while live cell imaging can further resolve the potential temporal delay or the instantaneous velocity and directionality of lytic granule movements. Therefore, fixed and live cell imaging analyses are both complementary and equally important in studying NK cell cytotoxicity.

3.4.1. Volocity—Considerations for measuring lytic granule convergence in live cell imaging are fairly similar to those outlined for fixed cells in **Section 3.3.1** with several variations.

1. After opening the data file, use the **Rectangle** or **Freehand** tool to draw and encircle one NK-target cell conjugate that is to be analyzed first.
2. Go to **Actions** and select **Crop to Selection**. A new image file will now appear underneath the original file.
3. Perform the convergence analyses as per **Steps 2–19 in Section 3.3.1** with a few modifications:
 - a. Lytic granules and the MTOC are defined by the fluorescent channels encompassing LysoTracker Red and SiR-tubulin, respectively.
 - b. Step 4: Instead of thresholding using intensity, select **Threshold** using **SD** to adjust for the potential decay of fluorescent intensity over time in time-lapse experiments (*see Note 13*).
 - c. Step 7: After marking the region, which confines the NK cell in the initial time frame, go through the following time frames to ensure that the enclosed area will contain all the lytic granules within the NK, cell. Be sure to exclude the target cell or any other NK cells in close proximity at all time points. If the NK-target cell conjugate underwent substantive movement over time, mark the area that confines the NK cell individually for each time frame.
 - d. Step 11: When making a measurement item, select **Selected timepoint(s)** or **All time points** depending on **Step 7**.
4. If SiR-tubulin staining was not performed, centroid of the MTOC can be replaced by centroid of all the lytic granules to determine the variance in relative positioning of lytic granules:
 - a. Click on the measurement item containing the coordinates of lytic granules.

- b. Select all the data within a single time frame.
- c. Go to **Raw** and select the **Join Objects**.
- d. The coordinates of the centroid of all granules will appear as a new object in the measurement table. This can then be used to determine the distance from each granule.

3.4.2. Fiji—Measuring lytic granules convergence in time lapses instead of still images is virtually identical and the procedure is identical to the one described above in 3.3.2. The measurements are performed on all time points present in the current image sequence. The reference to the time point in the final spreadsheet is given by the Stack Position column. Only two points need particular attention as follows:

- a. The NK cell must remain inside the region of interest during the entire duration of the time lapse. Expand slightly the ROI drawn on the first frame to accommodate all its positions in the subsequent frames.
- b. If only the staining for the lytic granules was successful, the position of the centroid of the MTOC over time can be replaced by the center of mass of all the granules detected in the binary image (after **Step 6 in 3.3.2**): select the option **Analyze > Measure...** The positions will be registered in a table under the columns XM, YM and ZM if applicable.

3.4.3. Calculations and data plotting—Perform measurement of lytic granule convergence as described in **section 3.3.3** for each time frame and plot the temporal data from each NK cell as one single line. In the line plots, x-axis shows all time points during the live imaging process and y-axis indicates mean granule to MTOC distance (Figure 2D).

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Figure 1.

Diagram of different approaches for analyzing lytic granule convergence in NK cells. The left panel shows the diffusely distributed lytic granules in the cytoplasm **before** formation of the immune synapse (IS). The right panel demonstrates how lytic granules are tightly clustered around the MTOC **after** IS formation. Within each panel, the left-hand side depicts formation of an artificial IS between an NK cell and the glass surface coated with activating antibodies against the adhesion and activation receptors for NK cells. On the right-hand side of each panel, it shows IS formation between an NK cell and its susceptible target carrying the ligands that trigger NK cell activation.

Figure 2.

Images extracted from the analysis workflow to illustrate the different critical steps in a sequential way (A): adjustment of brightness and contrast, analysis performed on each channel independently, segmentation of the objects and finally measurement of the distance between the centroid of each granule to the centroid of the MTOC (assumed to be the brightest object within the α -tubulin staining shown here). Examples of the confocal images (B) and plots (C and D) displaying the measurement of lytic granule convergence on an activating surface (C) and in effector-target cell conjugates (D). NK cells are labeled with SiR-tubulin and LysoTracker Red to identify the positions of the MTOC and lytic granules.

The dot plot shows the degree of lytic granule convergence in NK cells using fixed cell confocal microscopy. Each dot indicates the average granule to MTOC distance within a single NK cell (C). The line plot shows the average granule to MTOC distance over time using live cell confocal microscopy. Each line represents one NK cell. In the control group, the gradual decrease in distances indicates the real-time converging process of lytic granules toward the MTOC (D).