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Confocal microscopy of intracellular components within NK cells

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ABSTRACT

NK cells are small cells and their organelles e.g. mitochondria are consequently harder to visualize than those in larger cells. We have found that imaging NK cells that are stretched out across fibronectin-coated coverglass spreads out their organelles across a wider area and makes it easier to resolve differences e.g. their mitochondrial surface area and volume.

There are many possible options for chamber slides, fixatives, permeabilization agents and stains. The protocol here gives specific instructions for a particular nuclear and mitochondrial staining, but the principle can be applied to many different types of stain.

GUIDELINES

Selection of staining reagents

This protocol can be applied for the visualization of various intracellular components. When staining with dyes, make sure the dyes are fixable for microscopy e.g. Mitotracker Deep Red probe, but not MitoTracker Green. Make sure they fluoresce in separate channels e.g. MitoTracker Deep Red FM and Wheatgerm Agglutinin AlexaFluor 647 fluoresce in the same channel on the Nikon A1Rsi microscope described, so should not be used together.

Optimal cell density

Cell density per well depends on the size of the cell when it is spread out on the glass, how many you tend to knock off with your wash steps and what power you are imaging at. You generally want a higher density if imaging at 60x and a lower density if imaging at 10x. If adding directly to fibronectin coated surfaces 200,000-400,000 pNK/cm^2 should be about right. If staining in the well, consider doubling the number when plating initially. For experiments where NK cells are cultured at 300,000 cells/200 µL in 96 well U-bottom plate format, we find transferring 2 wells from the U-bottom into a single chamber slide well one day before the end of culture works well.

MATERIALS

Necessary components

Matrix coating e.g. Fibronectin bovine plasma (Sigma-Aldrich, Cat. F1141)
Chamber slides e.g. µ-Slide 8 well polymer bottom 1.5 (Ibidi, Cat. 80826)
Fixative e.g. Paraformaldehyde (Electron Microscopy Sciences, Cat. 15710)
Permeabilization agent e.g. 0.1% Triton X in PBS (Sigma, Cat. 100261-4890)

Variable components

Nuclear stains e.g. DAPI (NucBlue fixed cell stain ReadyProbes; ThermoFisher Scientific, Cat. R37606)
Mitochondrial stains e.g. Mitotracker DeepRed (ThermoFisher Scientific, Cat. M22426)
Membrane dyes e.g. CellTrace reagents or WheatGerm Agglutinin AlexaFluor647 (ThermoFisher Scientific Cat. W32466)
Early endosome marker e.g. rabbit anti-EEA1 (Cell Signaling, Cat. 3288T)

Others

DMSO (Cat. BP231-100, Fisher Bioreagents)

Different chamber slide dimensions

µ-Slide 8 well polymer bottom 1.5 (Ibidi, Cat. 80826)

- Culture area - 1cm^2
- Minimum volume - 300µL
- Maximum volume - 600µL
- Lid seals tightly - leave off inside a large covered petridish when culturing cells; seal when storing in the fridge to avoid evaporation.

Lab-Tek I 8 well (ThermoFisher, Cat. 155411PK)

- Culture Area - 0.8cm^2
- Minimum volume - 200µL
- Maximum volume - 400µL
- Lid seals loosely - leave on when culturing cells; place inside a petri dish with damp tissue ('moist box') if storing in the fridge.

Lab-Tek II 8 well (ThermoFisher, Cat. 155409PK)

- Culture Area - 0.7cm^2
- Minimum volume - 200µL
- Maximum volume - 500µL
- Lid seals loosely - leave on when culturing cells; place inside a petri dish with damp tissue ('moist box') if storing in the fridge.

Coat a plate with fibronectin

1 Coat chambered coverglass (µ-Slide 8 well polymer bottom 1.5, Ibidi, Cat. 80826) with fibronectin (10µg/mL in PBS, Sigma-Aldrich, Cat. F1141) to allow NK cells to stretch out across the coverglass.

Coating options:
- 30 min at 37°C
- 45 min at room temperature
- overnight at 4°C

2 When ready to continue, quickly rinse wells three times with PBS to remove excess fibronectin.

Allow NK cells to adhere to coverglass

3 Add NK cells onto the coverglass and allow them to adhere.

- A minimum of 20 min at 37°C is required.
- Overnight culture at 37°C is often the simplest option.
- See *guidelines* for NK cell density e.g. 750,000 cells/well.

Stain the cells

4 **MitoTracker Deep Red FM (Thermo Fisher Scientific, Cat. M22426)**
- The stock is resuspended in DMSO (Cat. BP231-100, Fisher Bioreagents) and diluted to 200 nM in RPMI (Gibco Cat. No. 2240-089).
- Add 400 µL of this solution into each well
- Stain for 30 min at 37°C
- Gently rinse once with RPMI.

5 **Fix the cells**
- Add 400 µL 4% paraformaldehyde/RPMI for 15 min at 37°C *(do not prepare this solution in advance)*.
- Gently rinse once with PBS.

6 **Permeabilize**
- 400 µL 0.1% Triton-X/PBS for 5 min at room temperature.
- Gently rinse twice with PBS

7 **Counterstain**
- Add 400 µL DAPI (2 drops/mL PBS)

- Incubate for 20 min at room temperature.
- Proceed to imaging (no need to wash DAPI or phalloidin stains).

Imaging

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- If no azide is included in the media, proceed to imaging soon after staining is complete (store at 4 °C until required).
- Chamber slides can be imaged with water immersion objectives.
- Within our University Imaging Center, Jackson Hall, there is a Nikon A1Rsi HD confocal microscope with 60x 1.27 NA water immersion objective suitable for this kind of microscopy.
- DAPI is excited by the 405 nm laser and MitoTracker Deep Red FM is excited by the 640 nm laser.
- There are 4 PMT detectors, of which two are highly sensitive GaAsP detectors and a transmitted light detector.

Analysis

9 **Denoise**

e.g. Nikon Elements Fourier transform.

10 **Model**

e.g. Imaris Spot detection can model mitochondria. Membrane and nuclear dyes help model the whole cell structure around these mitochondria.