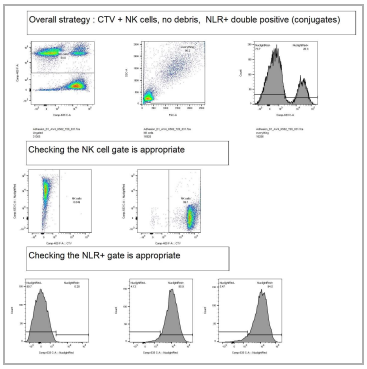

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We use this protocol and it's working

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Adhesion assay

In 1 collection

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ABSTRACT

Adapted from Natural Killer Cell Protocols 2009 p89-96

Enriched effector cells and tumor targets are labelled with different fluorescence. They are allowed to interact over a time-course and samples are taken and fixed at regular intervals. These samples are assessed by flow cytometry for the proportion of firmly adhered NK-tumor conjugates (double positive fluorescent events) at each timepoint.

GUIDELINES

This method does not involve a live/dead stain therefore NK cells and tumor targets must have good viability (>90%) at the start of the assay for results to be meaningful.

If a drug is being used to increase adherence e.g. Rituximab, consider including the same cell line without the drug to evaluate the contribution of the drug.

We have tested this assay using K562 as a readout of natural cytotoxicity and Raji with Rituximab (Genentech) as a readout of antibody dependent cellular cytotoxicity.

Effector:target ratios will impact the frequency of adherence. A 1:2 ratio used here gives NK cells a high chance of encountering a tumor cell. The experimental readout is the proportion of NK cells in conjugates. Increase the proportion of tumor cells to maximize the likelihood of NK cells encountering a tumor cell (and therefore the limiting factor is its ability to adhere to a target cell).

Effector cells will be able to adhere to different target types at different rates. When working with a new target cell or effector, optimize the effector:target cell ratio and timing for maximum sensitivity to experimental perturbation.

MATERIALS

- 2% PFA chilled on ice.
- Fluorescent effector cells e.g. CellTrace Violet (Cat. No:C34557, Thermo Fisher) labeled NK cells
- Fluorescent target cells e.g. Raji-NucLight Red (See Cell line information for details)
- Appropriate cell culture media
- Flow cytometry tubes
- Timer
- Waterbath at 37˚C

BEFORE START INSTRUCTIONS

Duplicates of each condition are necessary to check the reproducibility of results. Timing is key to reproducibility, so have the bench setup with ice-cold PFA, a vortex, timer and waterbath, all in close proximity.

Calculate the required number of cells for this assay ahead of time
e.g.

- 8 tubes per target cell per donor (4 timepoints in duplicate): 0, 10min, 20min, 30min.
- 3 target cells: K562, Raji + Rituximab, Raji alone
- 2 culture conditions: 20% oxygen v. 1% oxygen

= 48 tubes per NK cell donor
x 100,000 NK cells/tube = 4.8 million NK cells/donor

Set aside cells for flow cytometer setup

1 The following controls will be required to setup the gates on a flow cytometer:
1. Unlabeled cells for an unstained control
2. Single color control (effector) e.g. CellTrace Violet labeled NK cells.
3. Single color control (target) e.g. K562-Nuclight Red.

2 Add 200 µl of ice cold 2% paraformaldehyde to individual control samples and store on ice.

Prepare the cells

3 Obtain fluorescent effector and target cells and prepare them in fresh pre-warmed media:

3.1 Resuspend effector cells at 1 × 10^6 live cells/ml. *If this is not possible, resuspend at Xmillion/mL where X is the concentration of the least abundant condition.*

3.2 Resuspend tumor cells that do not require drugs *e.g. K562,* in assay medium at 2X *e.g. 2 × 10^6 live cells/ml if effector cell numbers are not limiting.*

3.3 Resuspend tumor targets that require drugs *e.g. Raji,* in assay medium at 4X *e.g. 4 × 10^6 live cells/ml if effector cell numbers are not limiting.* Prepare drugs *e.g. Rituximab* at 2X.

4 In **duplicate** - for each desired time point *(e.g. 0, 10 min, 20 min and 30 min)* aliquot 100 µl of target cells or target cells and drugs *(50 µL of each)* into flow cytometry tubes.

Add effectors to target cells

5 *Guidance: For this section, work in batches with small numbers of tubes so that targets and effector cells are not together in the same tube for uncontrolled periods of time. Keeps cells warm, by storing tubes in the incubator until needed.*

6 Add 100 µl of effector cells. Mix gently, but consistently - *e.g. two flicks/tube*

7 Spin tubes at 20 × *g* for 1 min.

8 Incubate at 37°C in a water bath for desired time points.

Guidance: Put them all in together then take them out one at a time at the appropriate timepoint e.g. after 10 min. Do not use an incubator here. For the 'time zero' control, there is no incubation - proceed immediately to step 9.

9 At the end of each time point, vortex sample at high speed for 3 s.

Guidance: This step can introduce a lot of variation. Have a stopwatch in front of you for precise timing. It is the reason you have to do everything in duplicate to start off with!

10 Immediately fix by adding 200 µl of ice-cold 2% paraformaldehyde.

11 Store samples at 4°C protected from light until analysis.

Run on a flow cytometer

12 Run samples and controls on a flow cytometer.

Guidance:

- *Use single color effector and target cells to set up the forward and side scatter parameters and set an acquisition gate to collect at least 20,000 cell events (not debris).*
- *Be sure to use a wide forward scatter gate to capture conjugates, which may have higher forward scatter (see an example in the description image).*
- *Use untreated labeled cells to set up the sensitivity of detectors and compensation for the fluorescent channels.*
- *We have checked cells stored in the fridge for 5 days and this gave the same result as freshly fixed cells, so you can store them if necessary.*

13 Analyze for the proportion of effector cells in conjugates.

- Set up the analysis leaving a generous margin for single color events when setting the quadrants.
- Calculate the proportion NK cells in conjugates = 2-color events/(2-color events + effector only events) × 100%.
- If desired, the background value (proportion of effectors in conjugates at time zero) can be subtracted from each experimental point. For some effector/target cell types, this can be a significant value.