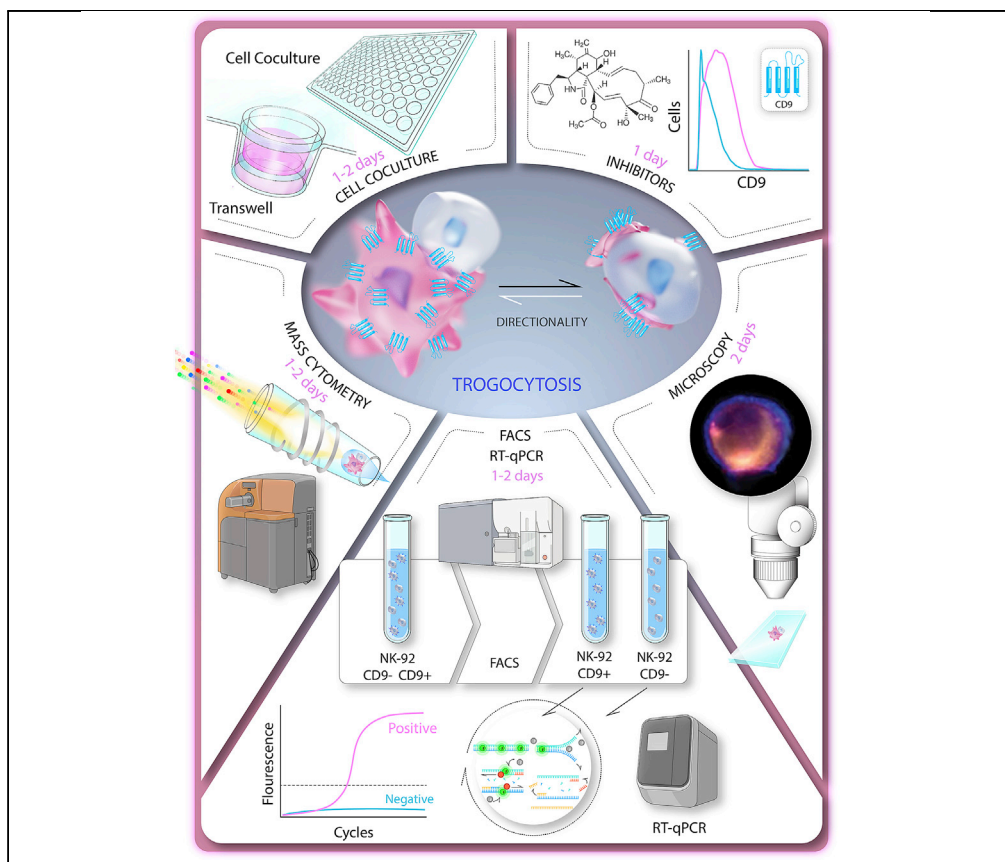


## Protocol

# Measuring trogocytosis between ovarian tumor and natural killer cells



Trogocytosis is an active transport mechanism by which one cell extracts a plasma membrane fragment with embedded molecules from an adjacent cell in a contact-dependent process leading to the acquisition of a new function. Our protocol, which has general applicability, consolidates and optimizes existing protocols while highlighting key experimental variables to demonstrate that natural killer (NK) cells acquire the tetraspanin CD9 by trogocytosis from ovarian tumor cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Antonio Delgado-Gonzalez, Ying-Wen Huang, Ermelinda Porgiglia, Kenyi Donoso, Veronica D. Gonzalez, Wendy J. Fantl

wjfantl@stanford.edu

**Highlights**  
Protocol to measure contact-dependent transfer of plasma membrane fragments

Conditions optimized for ovarian tumor and NK cell cocultures and single-cell analysis

Use of a membrane barrier and cytoskeletal inhibitors to block trogocytosis

Flourescence microscopy and flow cytometry to visualize transfer of membrane fragments

Delgado-Gonzalez et al., STAR Protocols 3, 101425  
June 17, 2022 © 2022 The Author(s).  
<https://doi.org/10.1016/j.xpro.2022.101425>



## Protocol

## Measuring trogocytosis between ovarian tumor and natural killer cells

Antonio Delgado-Gonzalez,<sup>1,2,6</sup> Ying-Wen Huang,<sup>1,2,6,8</sup> Ermelinda Porpiglia,<sup>2,5</sup> Kenyi Donoso,<sup>1</sup> Veronica D. Gonzalez,<sup>2,7</sup> and Wendy J. Fantl<sup>1,3,4,9,\*</sup>

<sup>1</sup>Department of Urology, Stanford University School of Medicine, Stanford, CA 94305, USA

<sup>2</sup>Baxter Laboratory for Stem Cell Biology, Department of Microbiology & Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA

<sup>3</sup>Stanford Cancer Institute, Stanford University School of Medicine, Stanford, CA 94305, USA

<sup>4</sup>Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, CA 94305, USA

<sup>5</sup>Department of Biomedicine, Aarhus University, 8000 Aarhus, Denmark

<sup>6</sup>These authors contributed equally

<sup>7</sup>Present address: 10x Genomics, Pleasanton, CA 94588, USA

<sup>8</sup>Technical contact

<sup>9</sup>Lead contact

\*Correspondence: [wjfantl@stanford.edu](mailto:wjfantl@stanford.edu)  
<https://doi.org/10.1016/j.xpro.2022.101425>

## SUMMARY

Trogocytosis is an active transport mechanism by which one cell extracts a plasma membrane fragment with embedded molecules from an adjacent cell in a contact-dependent process leading to the acquisition of a new function. Our protocol, which has general applicability, consolidates and optimizes existing protocols while highlighting key experimental variables to demonstrate that natural killer (NK) cells acquire the tetraspanin CD9 by trogocytosis from ovarian tumor cells.

For complete details on the use and execution of this protocol, please refer to Gonzalez et al. (2021).

## BEFORE YOU BEGIN

This protocol details steps from several orthogonal methods to demonstrate that human NK cells acquire CD9 from ovarian tumor cells by trogocytosis (Gonzalez et al., 2021). While we use a human system, the methods are equally applicable to studies in other species. Each method requires establishing cocultures between NK and tubo-ovarian high grade serous carcinoma (HGSC) cell lines. Most frequently we use the OVCAR4, and NK-92 cell lines. However, we have performed these methods with other HGSC ovarian tumor cell lines (Kuramochi and TYK-nu), as well as with cell lines derived from other malignancies (colon (HCT116 and CaCo), epidermoid/skin (A431) and breast (MCF7)). In addition, we have used the NKL cell line and primary NK cells from peripheral blood mononuclear cells (PBMCs). Technologies used include mass cytometry (Cytometry by Time-Of-Flight (CyTOF)), fluorescence-based flow cytometry and fluorescence microscopy. For each technology steps to prepare and analyze samples are provided. The methods have been developed from previous work by us and others and enable a researcher to demonstrate the transfer by trogocytosis of a protein of interest between two different cell types in both human and murine systems (Daubeuf et al., 2010; Gary et al., 2012; Gonzalez et al., 2021; Hamieh et al., 2019; Li et al., 2019; Reed and Wetzell, 2019; Schriek et al., 2022; Tilburgs et al., 2015; Uzana et al., 2012; Vanherberghen et al., 2004; Hasim et al., 2022).



**Note:** The system chosen for this protocol has specific relevance to the field of immunoncology. However, it is widely applicable to many cell types and thus facilitates the investigation of trogocytosis in other systems.

### Institutional permissions

Peripheral blood samples from anonymous healthy donors were obtained from the Stanford Blood Center (Palo Alto, CA, USA).

### Culturing NK cell lines

⌚ **Timing:** 1.5–2 weeks

The protocol below describes specific steps for culturing human NK cell lines (NK-92 and NKL) from frozen stocks. It includes media preparation, cell thawing, counting, and viability determination (Gonzalez et al., 2021, 2022). Both cell lines use the same culture conditions.

1. Preparing IL-2 stock solution ( $2 \times 10^6$  IU/mL).
  - a. Reconstitute recombinant human IL-2 powder (50  $\mu$ g, equivalent to  $1 \times 10^6$  IU) in 500  $\mu$ L sterile 100 mM acetic acid containing 0.1% bovine serum albumin (BSA).
  - b. Aliquot and store at  $-80^\circ\text{C}$ . Use within 3 months.

**⚠ CRITICAL:** Successful growth of NK cell lines is highly dependent on the quality of IL-2 used in the culture media. The activity of IL-2 can change between lots. The activity of the IL-2 we use as calibrated by the manufacturer has an  $\text{ED}_{50}$  of 0.05–0.25 ng/mL in a murine T cell proliferation assay. Its specific activity is  $9.1 \times 10^6$  IU/mg when calibrated against recombinant IL-2 WHO International Standard. Please consult vendors' specifications using the parameters described as a guide. We have found R&D Systems to be a reliable source of recombinant human IL-2 protein.

**Note:** IL-2 is critical for both NK cell viability and proliferation and is added fresh to media used for NK cell cultures (Kotzur et al., 2022).

2. Three different RPMI-1640 media preparations are used throughout.
  - a. "Unsupplemented RPMI-1640" contains no added supplements.
  - b. "Supplemented RPMI-1640" contains 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin (PS).
  - c. "Supplemented RPMI-1640 + IL-2" contains IL-2 (200 IU/mL) added before each use.

**Note:** For supplemented RPMI-1640 + IL-2: freshly thawed IL-2 stock solution is added immediately before each use and all subsequent media changes for NK cells in culture should likewise be with supplemented RPMI-1640 containing freshly thawed IL-2.

**Note:** Supplemented RPMI (without IL-2) can be stored at  $4^\circ\text{C}$  for up to 3 months.

3. Thawing NK-92 cells.
  - a. NK-92 cells are stored at  $5 \times 10^6$  cells/mL in freezing media (FBS + 10% dimethylsulfoxide (DMSO)) in liquid nitrogen.
  - b. Thaw vial of frozen NK-92 cells by with gentle agitation in a  $37^\circ\text{C}$  water bath.
  - c. After thawing, wipe outside of vial with 70% ethanol.
  - d. Immediately transfer cells dropwise into a 15 mL conical tube containing 9 mL pre-warmed ( $37^\circ\text{C}$ ) supplemented RPMI-1640 + IL-2.
  - e. Centrifuge, 300g, 5 minutes (min), room temperature ( $20^\circ\text{C}$ – $26^\circ\text{C}$ ) and discard supernatant to remove DMSO;

f. Resuspend cells in 1 mL unsupplemented RPMI-1640.

**Note:** Good Practice per Environmental Health and Safety at Stanford, the lab room temperature should be maintained at 20°C–26°C.

**Note:** Cells are thawed and diluted quickly into supplemented media + IL-2 to minimize toxic effects of DMSO.

**Note:** Keep time that NK cells are in unsupplemented RPMI-1640 to an absolute minimum.

#### 4. Counting cells and determining their viability.

- Transfer 10  $\mu$ L cell suspension from step 3.f. to microfuge tube or well of 96-well plate.
- Add 10  $\mu$ L 0.4% trypan blue to the tube or well and mix thoroughly.
- With the coverslip placed over the hemocytometer, fill each chamber with trypan blue-cell suspension.
- Aim to complete cell count within 3–5 min after mixing cells with trypan blue.
- Count cells (clear (viable) and blue (dead)) within the 1 mm<sup>2</sup> center square and within the four 1 mm<sup>2</sup> corner squares in chamber 1 of the hemocytometer and repeat for chamber 2.
- Calculate the number of viable cells: average cell count per square  $\times$  dilution factor  $\times 10^4$ /mL. Viability is the percentage of trypan blue negative cells out of the total number of cells counted.

**Note:** For accurately distinguishing live from dead cells, it is essential to use unsupplemented media as trypan blue binds strongly to serum proteins.

**Note:** The dilution factor may vary depending on the concentration of the harvested cells.

**Optional:** When counting cells, an automatic cell counter can be used instead of a hemocytometer.

#### 5. Placing NK cell lines in culture.

- Centrifuge cells from step 3.f. (300g, 5 min, 20°C–26°C) and resuspend in supplemented RPMI-1640 + IL-2 in a T25 flask at an initial seeding density of  $0.3\text{--}0.4 \times 10^6$  cells/mL and up to a volume of 15 mL.
- Place T25 flask in an upright position so that cells are in close contact with each other at the bottom of the flask.
- After seeding the viability declines by 20% and can take up to 2 weeks to recover. During the first week, change media every 2–3 days. Take care not to disturb cells, which will have settled at the bottom of flask, by gently aspirating media and leaving a residual  $\sim 2$  mL. Add supplemented RPMI-1640 + IL-2 to restore the original volume in T25 flask.
- During the second week check cell viability. Centrifuge cells (300g, 5 min, 20°C–26°C), resuspend in supplemented RPMI-1640 + IL-2 in a 15 mL conical tube and take a small aliquot for counting with trypan blue.
- If cell viability is below 85%–90%, transfer cells to a new T25 at a density of  $0.2\text{--}0.3 \times 10^6$  cells/mL keeping flask in upright position. Change media every 2–3 days until viability reaches 85%–90%.
- When cell viability is 85%–90% resuspend NK cell pellet in supplemented RPMI-1640 + IL-2 (20–40 mL) at a density of  $0.2\text{--}0.3 \times 10^6$  cells/mL in a T75 flask. Continue to culture cells with flasks placed horizontally.
- Passage cells into new T75 flasks every 2–3 days and maintain a cell density of between 0.2 to  $1 \times 10^6$  cells/mL. At each split, cells are counted, and viability measured.

△ **CRITICAL:** Do not exceed a cell density  $> 1 \times 10^6$  cells/mL as cells will die. Replace media every 2–3 days. Do not culture for longer than 3 months or passage 20 (whichever condition is reached sooner).

**Note:** After seeding, NK-92 cells usually require a “recovery period” (1.5–2 weeks) but this time can vary.

**Note:** NK cells tend to grow in suspension as aggregates which is also an indication that they are healthy. Many aggregates sink to the bottom of the flask.

**Note:** For the first two weeks after seeding, NK cells can be fragile and/or have poor viability. Gentle media aspiration leaving about 2 mL (T25 flask) or 5 mL (T75 flask) media is therefore recommended. Once cultures are established transfer cells to a conical tube (15 or 50 mL), centrifuge 300g, 5 min, 20°C–26°C, resuspend in RPMI-1640 + IL-2 and transfer to a new flask. It is critical when adding media to follow the seeding density indicated above (step 5.a. e. f.).

### Isolating peripheral blood mononuclear cells (PBMCs)

⌚ **Timing:** 1.5 h

Specific steps for isolating PBMCs from human whole blood drawn from healthy donors by density gradient centrifugation with Ficoll-Paque™ PLUS are given below. The protocol includes some modifications we have made to the manufacturer’s instructions.

6. Collecting blood.
  - a. Obtain IRB approval.
  - b. Draw blood (up to 10 mL) into a BD Vacutainer™ plastic blood collection tube with sodium heparin.
  - c. Invert tube gently several times to mix and keep at 20°C–26°C.

**Note:** Blood is kept at 20°C–26°C throughout transfer to the lab for processing.

7. Isolating PBMCs using Ficoll.
  - a. Transfer blood from collection tube into a 50 mL conical tube and dilute 1:1 with sterile PBS. Subsequent steps are for 10 mL blood.
  - b. Invert Ficoll-Paque™ PLUS bottle several times to ensure thorough mixing.
  - c. Prepare a SepMate™-50 tube for Ficoll separation by adding 14 mL of Ficoll to the opening of the SepMate™ insert.
  - d. Carefully layer diluted blood from a. (20 mL) over Ficoll in the SepMate™-50 tube.
  - e. Centrifuge, 800g, 22 min, 20°C–26°C with the centrifuge brake OFF.

**Note:** Keeping the brake off maintains the mononuclear cells as a discrete band and minimizes intermixing with the upper and lower layers.

**Optional:** For Ficoll separation, a 50 mL conical tube can be used as an alternative to a SepMate™-50 tube.

- f. Gently aspirate the upper layer (plasma and platelets), taking care not to disturb the mononuclear cell layer (buffy coat) which forms as a narrow band above the Ficoll (containing a mix of red blood cells and granulocytes).
- g. Using a 10 mL disposable polystyrene serological pipette, carefully transfer the mononuclear cell layer into a new 50 mL conical tube.

- h. Add sterile PBS to conical tube to a final volume of 30 mL. Centrifuge, 300g, 10 min, 20°C–26°C.
  - i. Remove supernatant and resuspend cells in 1 mL of PBS.
  - j. Add an additional 9 mL of PBS and mix well.
  - k. Count cells and determine viability (step 4).
  - l. Centrifuge, 300g, 10 min, 20°C–26°C.
  - m. At this stage, cells are ready for downstream applications, or they can be frozen and stored for later use (step 8).
8. Freezing PBMCs.
- a. Prepare freezing media (FBS with 10% DMSO).
  - b. Resuspend cells in freezing media and use a minimum cell concentration of  $10 \times 10^6$  cells/mL.
  - c. Prepare 1 mL aliquots of the PBMC suspension in cryotubes and place in CoolCell LX cell freezing container ([key resources table](#)) at –80°C for 24 h before transferring to liquid nitrogen.

**Note:** Blood samples should be processed as soon as possible after collection to ensure optimal viability of immune cells which is especially important for functional studies. Delays in processing result in loss of cell viability, lower recovery and increased contamination with granulocytes and erythrocytes. Time from blood draw to isolation of PBMCs should not exceed 6 h.

### Preparing PBMCs for coculture

⌚ **Timing:** Thawing 30 min with resting 16–18 h

9. Preparing thawing media.
- a. Add benzonase (final concentration, 25 U/mL) to supplemented RPMI-1640 (step 2.b.) immediately before use.

**Note:** Benzonase is a nuclease added to the thawing media to prevent cell clumping due to the release of “sticky” DNA from dying cells.

10. Preparing PBMCs from frozen stock for coculture.
- a. Rapidly thaw a vial of frozen PBMCs from a healthy donor by gentle agitation in a 37°C water bath. We typically store PBMCs at  $20 \times 10^6$  cells/mL freezing media (FBS + 10% DMSO) in liquid nitrogen.
  - b. After thawing, wipe outside of vial with 70% ethanol.
  - c. Immediately transfer cells dropwise into a 15 mL conical tube containing 9 mL pre-warmed thawing media. Centrifuge, 300g, 5 min, 20°C–26°C.
  - d. Discard supernatant and wash cells once more with thawing media.
  - e. Resuspend cells in 5 mL of supplemented RPMI-1640.

**Note:** To minimize toxic effects of DMSO, cells are thawed and diluted quickly into thawing media.

**Note:** There are many variations of PBMC thawing protocols in the literature and across laboratories ([Higdon et al., 2016](#); [Ramachandran et al., 2012](#)). Here we present the thawing protocol established in our lab that is optimal for our coculture assays.

- f. Rest PBMCs from 1 h to overnight (16–18 h) in a humidified cell culture incubator, 37°C.

- g. After resting, count cells and determine viability (step 4). Filter out any cell clumps with a 70  $\mu\text{m}$  cell strainer. If viability >90% proceed.
- h. Resuspend PBMCs at  $0.8 \times 10^6$  cells/mL in supplemented RPMI-1640 + IL-2. PBMCs are ready for coculture with target cells of interest.

**Note:** Cell viability of 90% is critical for meaningful functional data. In our hands, resting PBMCs from 1 h to overnight (16–18 h) does not adversely affect viability.

### Culturing HGSC cell lines

⌚ Timing: 1 week

The protocol below describes specific steps for culturing OVCAR4, Kuramochi, and TYK-nu HGSC cell lines (Domcke et al., 2013; Gonzalez et al., 2021, 2022).

11. Preparing supplemented culture media, as specified for each HGSC cell line.
  - a. OVCAR4: M199 media supplemented with 5% FBS, 1% L-glutamine, and 1% PS.
  - b. Kuramochi: supplemented RPMI-1640 (step 2.a.).
  - c. TYK-nu: EMEM media supplemented with 10% FBS, 1% L-glutamine, and 1% PS.
12. Seeding and maintaining HGSC cell lines in 10 cm plates.
  - a. Thaw vial of frozen HGSC cell line by gentle agitation in a 37°C water bath.
  - b. After thawing, wipe outside of vial with 70% ethanol.
  - c. Immediately transfer cells dropwise into a 15 mL conical tube containing 9 mL pre-warmed supplemented culture media. Centrifuge, 300g, 5 min, 20°C–26°C.
  - d. Discard supernatant and resuspend in 1 mL of unsupplemented RPMI-1640.
  - e. Count cells and determine viability as described above (step 4). If > 90% proceed.
  - f. Seed  $0.25\text{--}0.5 \times 10^6$  cells into 10 mL media (specified above) in a 10 cm plate.
  - g. Change media 2–3 times a week.
  - h. Passage cell lines at confluence of 70%–80% according to recommended split ratios below.
    - i. OVCAR4: 1:3 to 1:6.
    - ii. Kuramochi: 1:3 to 1:4.
    - iii. TYK-nu: 1:10 to 1:20.

**Note:** Cocultures are sensitive to cell passage numbers. Although we do not have an explanation, at high passage numbers the efficiency of trogocytosis declines and replicates are variable. For HGSC cell lines we used passages between 2 and 20. However, researchers should determine optimal passage numbers according to the cells of interest.

### Coculturing NK cells (cell lines or primary cells) with ovarian tumor cell lines

⌚ Timing: 1–1.5 h for aliquoting cells; 6 h for incubating cocultures

In this protocol, cocultures are set up between target HGSC cell lines (OVCAR4, Kuramochi, TYK-nu) and NK effector cells (NK-92, NKL or primary NK cells from PBMCs). Selected HGSC cell lines were molecularly and genetically comparable to primary newly diagnosed HGSC tumors, as reported by the TCGA (Cancer Genome Atlas Research Network, 2011; Domcke et al., 2013). As a brief rationale for the design of this coculture system, it is based on data from our recent CyTOF study in which we identified CD9-expressing decidual-like (dl)-NK cells in newly diagnosed HGSC tumors (Gonzalez et al., 2021). Given that decidual NK cells are highly immune suppressive we used cocultures as a model system to enhance our understanding of the function of intra-tumoral dl-NK cells. After cocultures between HGSC cell lines and NK cells the latter acquired CD9 from the HGSC cells. The methods described below were used to establish that CD9 acquisition by NK cells *in vitro* occurred by trogocytosis. The same procedures are applicable to determine whether trogocytosis by NK cells

occurs with non-HGSC cell lines (e.g., HCT116, A431, MCF7 or CaCo). As well as *in vitro* studies (Gary et al., 2012; Gonzalez et al., 2021; Reed and Wetzel, 2019; Tilburgs et al., 2015), trogocytosis can also be investigated *in vivo* (Hamieh et al., 2019).

The number of cells to be used in coculture will need to be established from study to study. Here, unless otherwise stated, the total number of cells per well (96-well plate) was 200,000/250  $\mu$ L media. Analyses were always performed on cells harvested from 10 such wells.

13. Harvesting and seeding target HGSC cell lines for coculturing with NK cells.
  - a. For a 10 cm plate with HGSC cells at ~80% confluence (cell numbers indicated below), aspirate media.
    - i. OVCAR4:  $1\text{--}2.5 \times 10^6$  cells per plate.
    - ii. Kuramochi:  $1\text{--}2 \times 10^6$  cells per plate.
    - iii. TYK-nu:  $2.5\text{--}4 \times 10^6$  cells per plate.
  - b. Add 4 mL TrypLE and incubate 5 min, 37°C.
  - c. As cells detach from the plate, gently pipette up and down with a 10 mL disposable polystyrene serological pipette to break up clumps creating a uniform cell suspension.
  - d. Add 4 mL of appropriate complete media to plate and mix with cells by gentle pipetting.

**Note:** Trypsinization times vary depending on cell line.

- e. Aliquot cells into 15 mL conical tube.
- f. Centrifuge cells, 300g, 5 min, 20°C–26°C.
- g. Discard supernatant and resuspend cells in 1 mL of unsupplemented RPMI-1640.
- h. Count cells and determine viability (step 4). If >90% proceed.
- i. Centrifuge harvested HGSC cell line from step 13.g. and resuspend cell pellet in supplemented RPMI-1640 adjusting volume to  $0.8 \times 10^6$  cells/mL.
- j. Aliquot 125  $\mu$ L ( $1 \times 10^5$  cells) into each well of a U-bottom 96-well plate seeding a row of 10 wells for each tumor cell line.
- k. Avoid using outermost wells due to “edge effects” but adding PBS to these wells to ensure that evaporation is uniform across the plate.

**Note:** Cocultures are in 96 wells as the small surface area is optimal to ensure that NK cells are in contact with target cells.

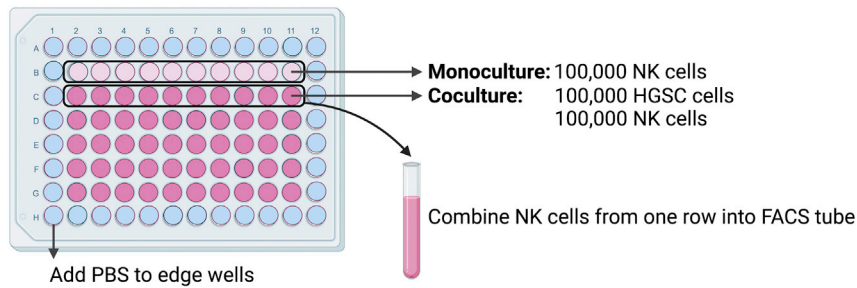
14. Harvesting and seeding effector NK cells for coculture with HGSC cell lines.
  - a. Collect NK cells (NK-92, NKL or PBMCs thawed from frozen stocks) in 15 mL conical tubes.
  - b. Centrifuge cells, 300g, 5 min, 20°C–26°C.
  - c. Discard supernatant and resuspend cells in 1 mL of unsupplemented RPMI-1640.
  - d. Count cells and determine viability as described above (step 4). If >90% proceed.
  - e. Centrifuge cells from step 14.c. and resuspend cell pellet in supplemented RPMI + IL-2 (400 IU/ mL) adjusting volume with media to  $0.8 \times 10^6$  cells/mL.

**Note:** As the NK cell suspension will get diluted when combined with HGSC cells we add IL-2 at 400 IU/mL (2 $\times$ ).

- f. Aliquot 125  $\mu$ L ( $1 \times 10^5$  cells) NK effector cells into wells containing target HGSC cells.
- g. Centrifuge 96-well plate, 250g, 4 min, 20°C–26°C to consolidate target and effector cells into bottom of wells.
- h. Incubate 96-well coculture plate in a humidified cell culture incubator, 6 h, 37°C.

**Note:** From our kinetic studies ([step-by-step method details](#), “[determining the kinetics of CD9 uptake by NK-92 cells](#)”, step 4) CD9 uptake by NK cells was maximal at 6 h and did not





**Figure 1. Cocultures in 96-well plate**

HGSC target cells seeded at 100,000 cells/125  $\mu$ L supplemented RPMI + IL-2 per well and followed immediately with addition of effector NK cells at 100,000 cells/125  $\mu$ L supplemented RPMI + IL-2 are cocultured for 6 h at 37°C. For each condition 10 wells are combined into one FACS tube, and cells are mixed with gentle pipetting.

increase significantly up to 48 h. For reasons of convenience, and unless otherwise specified, the duration of coculture for many of our experiments was 6 h. However, it is advisable to optimize the duration of coculture for the system under study.

**Note:** For PBMCs the cell number ( $1 \times 10^5$ ) represents a heterogeneous immune cell population and necessarily includes non-NK cell phenotypes. This permits measurements of CD9 uptake by primary NK cells within this cell population.

15. Processing cocultured cells for live/dead cell staining and fixation.
  - a. Collect cells from wells by gently pipetting up and down.
  - b. For each condition pool 10 wells of cells into a 5 mL FACS tube (Figure 1).

**Note:** The majority of cells in the supernatant are NK cells and gentle pipetting up and down with a P1000 pipette releases any NK cells that may have attached to the tumor cells.

**Note:** For cocultures with PBMCs we include antibodies against surface markers for the major immune cell types (T, B, myeloid) allowing us to gate them out and analyze the NK cell populations (CD16+ CD56<sup>dim</sup> and CD56<sup>bright</sup> CD16-) (key resources table).

- c. Centrifuge cells, 300g, 5 min, 20°C–26°C and discard supernatant.
- d. Wash cells with 4 mL unsupplemented RPMI-1640, centrifuge 300  $\times$  g, 5 min, 20°C–26°C and discard supernatant.
- e. Resuspend cell pellet in 1 mL unsupplemented RPMI-1640 and proceed to steps for live/dead cell determination for either fluorescence or mass cytometry.

**Note:** Cocultures are always performed in media used for culturing NK-92 cells (steps 1 and 2) as NK cells in general are sensitive to culture conditions.

**Note:** For most of our cocultures we used effector: target ratios of 1 : 1. In certain cases these ratios are changed (See [step-by-step method details](#), “Visualizing transferred plasma membrane fragments with embedded CD9 on NK-92 cells” and “Detecting trogocytosis by fluorescence microscopy”).

### Processing samples for CyTOF and fluorescence-based flow cytometry

© Timing: Live/dead cell staining for CyTOF 30 min, for fluorescence 45 min, for fixing and freezing 45 min

For both cytometry platforms there are key steps that must be performed to generate interpretable data. In this next section we provide the steps for live/dead cell staining, cell fixation and freezing, antibody titrations and sample processing before introduction into the instrument. These steps are the same for cocultures and monocultures. For additional details, we refer the researcher to many published protocols that detail sample processing for CyTOF and fluorescence-based flow cytometry (Gonzalez et al., 2018, 2022; Han et al., 2018; Hartmann et al., 2019; Sahaf et al., 2020; Thrash et al., 2020).

### 16. Live/dead cell staining for CyTOF.

- a. Wash cells from step 15.e. once with 4 mL unsupplemented RPMI-1640, centrifuge 300g, 5 min, 20°C–26°C and discard supernatant.
- b. Resuspend the cells in 1 mL unsupplemented RPMI-1640.
- c. Add 1 mL 50  $\mu$ M cisplatin in unsupplemented RPMI-1640 (final cisplatin concentration 25  $\mu$ M) to cells for 1 min exactly with rocking at 20°C–26°C.
- d. Quench reaction by adding equal volume (1 mL) of supplemented RPMI-1640.
- e. Centrifuge cells, 300g, 5 min, 20°C–26°C and discard supernatant.
- f. Wash once with 4 mL unsupplemented RPMI-1640 (centrifuge 300g, 5 min, 20°C–26°C) and discard supernatant.
- g. Resuspend cell pellet in 900  $\mu$ L unsupplemented RPMI-1640. Remove a small aliquot to count cells (step 4).
- h. At this stage cells can be fixed and frozen (step 18) or fixed and processed further for antibody staining and introduction into mass cytometer.

**△ CRITICAL:** CyTOF is very sensitive to metal contaminants. This necessitates use of high-grade analytical reagents (key resources table).

**Optional:** For a live/dead cell stain alternative to cisplatin, incubate cells with Cell-ID Interpolator 103-Rhodium (key resources table), final concentration 1  $\mu$ M, 15 min, 37°C.

### 17. Live/dead cell staining for fluorescence-based flow cytometry.

- a. For fluorescence-based flow cytometry, wash cells from step 15.e. once with 4 mL PBS, centrifuge 300g, 5 min, 20°C–26°C and discard supernatant.
- b. Resuspend the cells in 1 mL PBS in 5 mL FACS tube.
- c. Add 1  $\mu$ L LIVE/DEAD<sup>TM</sup> Fixable Aqua Stain to cells and incubate protected from light, 15 min, 37°C.
- d. Quench reaction by adding 1 mL of supplemented RPMI-1640.
- e. Centrifuge cells, 300g, 5 min, 20°C–26°C and discard supernatant.
- f. Wash once with 4 mL unsupplemented RPMI-1640 (centrifuge 300g, 5 min, 20°C–26°C) and discard supernatant.
- g. Resuspend cell pellet in 900  $\mu$ L unsupplemented RPMI-1640 and remove a small aliquot (10–20  $\mu$ L) to count cells (step 4).
- h. At this stage cells can be fixed and frozen (step 18) or fixed and processed further for antibody staining and introduction into flow cytometer.

**Note:** The steps above for live/dead staining are compatible with fixed cells but can also be used for cells without fixation. In either case cells need to be washed in carrier-free-buffers which can lead to cell loss.

**Optional:** LIVE/DEAD<sup>TM</sup> Fixable Near-IR Stain (0.5  $\mu$ L/mL) is an alternative.

**Optional:** For analyzing live cells, 4',6-diamidino-2-phenylindole (DAPI, a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA) (0.1  $\mu$ g/mL) is preferred as it does not need to be removed prior to analysis.

18. Fixing and freezing cells.

- a. This protocol can be used for further analysis by both mass (from step 16.g.) and fluorescence-based flow cytometry (from step 17.g.).
- b. Add 100  $\mu\text{L}$  filtered 16% paraformaldehyde (PFA) to cells in 900  $\mu\text{L}$  unsupplemented RPMI-1640 (final concentration 1.6%).

**△ CRITICAL:** Trace metal contaminants must be removed from PFA with a Millex Syringe filter (0.1  $\mu\text{m}$ ). The filtered solution can be stored in a 15 mL polypropylene conical tube in the dark at 20°C–26°C up to two weeks. PFA is an irritant. Avoid inhalation and contact with skin and eyes.

- c. Incubate exactly 10 min at 20°C–26°C.

**Note:** Do not incubate cells in PFA for longer than 10 min (over-fixing) as this may mask epitopes resulting in diminished antibody staining.

- d. Add 4 mL cell staining media (CSM, [key resources table](#)) directly to cells in PFA, centrifuge 500g, 5 min, 20°C–26°C and discard supernatant. Wash twice more.
- e. Resuspend cell pellet in 45  $\mu\text{L}$  CSM.
- f. At this point cells can be processed further for CyTOF or fluorescence-based cytometry or stored frozen for analysis at a later time.
- g. For storage resuspend cells in CSM and aliquot  $1\text{--}6 \times 10^6$  cells /100–200  $\mu\text{L}$  per freezing tube.
- h. Snap freeze on dry ice and store at  $-80^\circ\text{C}$ .

**Note:** Freezing aliquots of  $6 \times 10^6$  cells/100–200  $\mu\text{L}$  is a convenient concentration for 6-point antibody titrations (steps 19–27).

**△ CRITICAL:** Removing all residual PFA before freezing is critical to avoid damaging cells when they are thawed for later use.

### Titrating antibodies for CyTOF and fluorescence-based flow cytometry

⌚ **Timing:** 3–4 h (titration) and 1–2 h (data acquisition by CyTOF or fluorescence-based flow cytometry)

We routinely titrate antibodies for both CyTOF (conjugated to metal-chelating polymers) and fluorescence-based flow cytometry (conjugated to fluorochromes) to determine optimal antibody concentration to achieve maximal signal-to-noise ratio. Many antibodies suitable for CyTOF are now commercially available but if not, or they prove unsuitable, we perform in-house conjugations. We titrate antibodies with a 6-point two-fold serial dilution typically ranging from 8 to 0.5  $\mu\text{g}$  antibody/mL, using  $1 \times 10^6$  cells/100  $\mu\text{L}$  CSM. It is critical for titrations to be performed with cells both positive and negative (or negligible expression) for the epitope of interest. Determining background signal is especially important to assess autofluorescence and to this end we also include a sample without added antibody. Selection of optimal positive and negative cells for a given antibody is mostly based on prior experience or documentation in the literature. However, when a negative control is not available, a knock-down for the protein of interest becomes necessary to validate a given antibody. In some cases, e. g., PBMCs, titrations may be performed in the presence of counterstains to enable gating of the population of interest. For more details about titrating antibodies for CyTOF, the reader is referred to ([Gonzalez et al., 2022](#); [Han et al., 2018](#); [Sahaf et al., 2020](#); [Thrash et al., 2020](#)).

Here we present the steps for titrating antibodies, for both fluorescence and mass cytometry read-outs, against exemplar surface proteins (CD45 and CD9) as well as an intracellular protein (cleaved (c-) poly (ADP-ribose) polymerase (c-PARP), a marker for apoptosis).

Datasets from CyTOF and fluorescence-based flow cytometry are gated and analyzed using flow cytometry software packages, e.g., CellEngine and Cytobank ([key resources table](#)). A detailed description of methods for analyzing CyTOF data is beyond the scope of this manuscript. Here we provide the general measures that need to be taken into account. And refer the researcher to our earlier publications for the precise computational approaches used ([Gonzalez et al., 2021, 2022](#)).

19. Preparing cells for CyTOF antibody titrations.

- a. Aliquot PFA-fixed cells into FACS tubes ( $6 \times 10^6$  cells each for positive and negative controls).
- b. If frozen (step 16), thaw at  $20^{\circ}\text{C}$ – $26^{\circ}\text{C}$ .
- c. Wash once with 4 mL CSM, centrifuge 500g, 5 min,  $4^{\circ}\text{C}$ .
- d. Discard supernatant and resuspend the pellet in 270  $\mu\text{L}$  CSM.
- e. Aliquot 45  $\mu\text{L}$  cell suspension ( $1 \times 10^6$  cells) into each cluster tube.
- f. Add 5  $\mu\text{L}$  Fc Block to cells, mix and incubate 10 min,  $20^{\circ}\text{C}$ – $26^{\circ}\text{C}$ .

**Note:** Fc Block is an important control that blocks the non-specific binding of an antibody's Fc domain to Fc cell receptors.

20. Titrating antibodies against surface proteins ([Figure 2A](#)).

- a. Incubate cells with 50  $\mu\text{L}$  of  $2\times$  antibody solution (serial dilution from 16 to 1  $\mu\text{g}$  antibody/mL CSM) against CD45 or CD9 on shaker, 45 min,  $20^{\circ}\text{C}$ – $26^{\circ}\text{C}$ .
- b. Wash cells twice with 1 mL CSM, centrifuge 600g, 5 min,  $4^{\circ}\text{C}$  and discard supernatants.
- c. Permeabilize cells with 1 mL 100% ice-cold methanol, 20 min,  $4^{\circ}\text{C}$ .
- d. Centrifuge, 600g, 5 min,  $4^{\circ}\text{C}$  and discard supernatant.
- e. Wash cells twice with 1 mL CSM, centrifuge 600g, 5 min,  $4^{\circ}\text{C}$  and discard supernatants.
- f. Permeabilize cells with 1 mL ice-cold 100% methanol, 20 min,  $4^{\circ}\text{C}$ .
- g. Wash twice with 1 mL CSM, centrifuge 600g, 5 min,  $4^{\circ}\text{C}$ .
- h. At this step, samples can be incubated with DNA-iridium intercalator (detects nucleated cells, step 22 below) or used to titrate intracellular antibodies.

21. Titrating antibodies against intracellular proteins ([Figure 2B](#)).

- a. Add 50  $\mu\text{L}$  CSM.
- b. Incubate with 50  $\mu\text{L}$   $2\times$  antibody solution (serial dilution from 16 to 1  $\mu\text{g}$  antibody/mL CSM) against c-PARP on shaker, 1 h,  $20^{\circ}\text{C}$ – $26^{\circ}\text{C}$ .
- c. Wash twice with 1 mL CSM, centrifuge 600g, 5 min,  $4^{\circ}\text{C}$  and discard supernatant.

22. Detecting nucleated cells with DNA-iridium (Ir) intercalator solution.

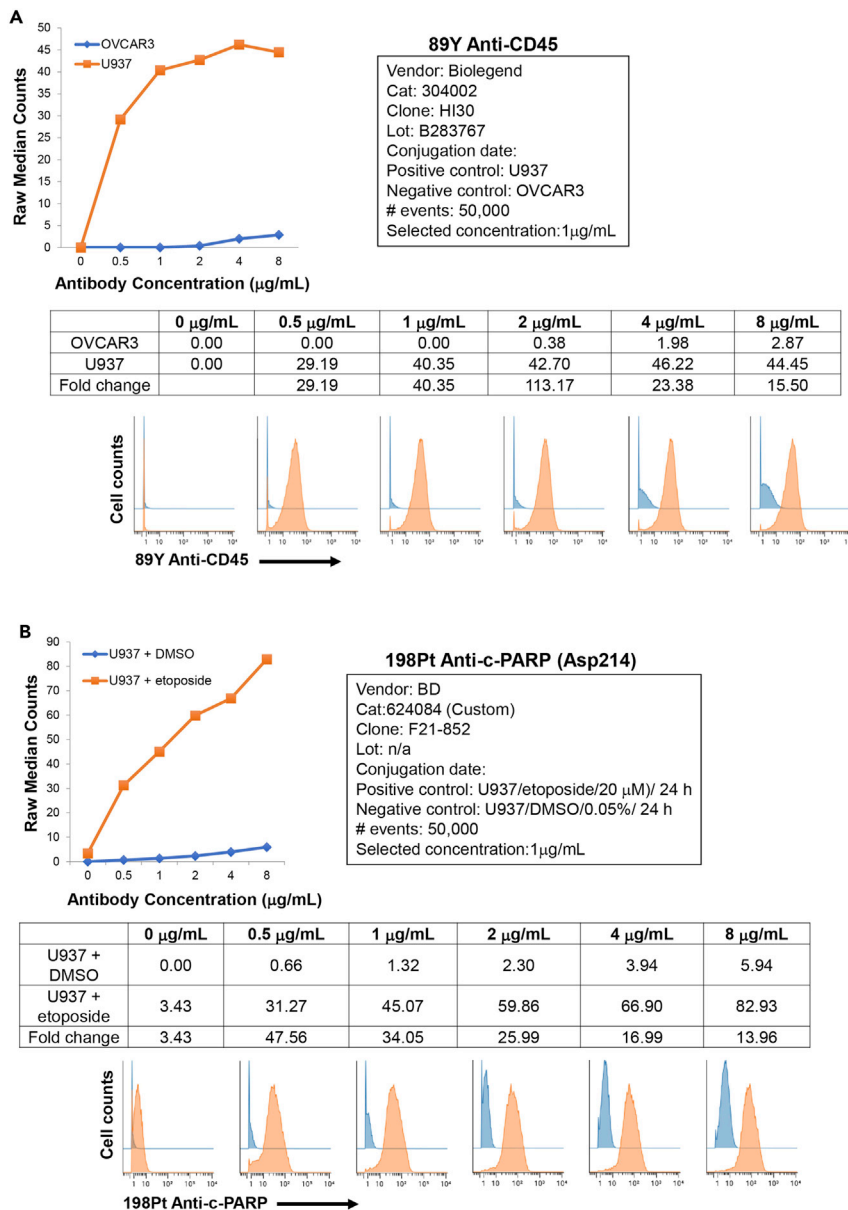
- a. Prepare 1 mL 1.6% PFA in PBS using filtered PFA and PBS (1 mL for each sample).
- b. Prepare working DNA-iridium intercalator solution in PFA/PBS solution (1:5,000 dilution, 0.1  $\mu\text{M}$ , final concentration).
- c. Add 1 mL DNA-iridium intercalator solution to each sample (from step 20.h.).
- d. Incubate for 1 h at  $20^{\circ}\text{C}$ – $26^{\circ}\text{C}$  or store for no longer than 48 h,  $4^{\circ}\text{C}$ .

**Note:** Some researchers leave samples in intercalator for up to a week at  $4^{\circ}\text{C}$ , but in our experience, we do not incubate for longer than 48 h to avoid loss of signal.

**Note:** Iridium is a composite of two naturally occurring stable isotopes,  $^{191}\text{Ir}$  and  $^{193}\text{Ir}$  and both channels can be used for gating nucleated single cells (step 28).

23. Introducing samples into mass cytometer.

- a. Centrifuge cells from step 22.d, 600g, 5 min,  $4^{\circ}\text{C}$  and discard supernatant.



**Figure 2. Antibody titrations for a surface and intracellular antibody using CyTOF**

For the examples shown, antibody conjugations to metal chelating polymers were performed in-house. Select the antibody concentration based on the optimal signal-to-noise ratio from the channel medians of the positive and negative controls.

(A) Titration for 89Y anti-CD45, a surface antibody, shows high signal-to-noise ratios over most of the antibody concentration range measured. In this case, take cost-saving into account and select concentration of 1 µg/mL.

(B) Titration for 198Pt anti-c-PARP, an intracellular antibody, shows high signal-to-noise ratios over most of the antibody concentration range measured. Record antibody characteristics, experimental conditions, and results on template. (See [key resources table](#) for further details about these antibodies).

- Wash once with 1 mL CSM, centrifuge 600g, 5 min, 4°C and discard supernatant.
- Wash twice with 1 mL CyTOF filtered water ([key resources table](#)), centrifuge 600g, 5 min, 4°C and discard supernatant.
- Resuspend each cell pellet at a concentration of  $1-2 \times 10^6$  cells/mL in CyTOF water containing a  $0.1 \times$  EQ4 calibration bead solution.

- e. Samples are loaded into the CyTOF instrument using an autosampler or manually using a 1 mL syringe without a needle. Adjust flow rate to 300–400 cells/s.

**Note:** In some cases, samples for CyTOF are barcoded with palladium isotopes. The steps for debarcoding occur after the normalization step (Matlab or R – Single Cell Debarcoder) (Gonzalez et al., 2022; Zunder et al., 2015).

24. Many steps of the antibody titrations are the same for CyTOF and fluorescence-based flow cytometry but there are some key differences. For convenience we provide a continuous protocol for titrating antibodies for fluorescence-based flow cytometry.
25. Titrating antibodies for fluorescence-based flow cytometry.
  - a. Thaw fixed frozen cells at 20°C–26°C and prepare for titrations (step 19).
26. Staining with fluorescent antibodies against surface proteins.
  - a. Incubate with 50  $\mu$ L of 2 $\times$  antibody solution (serial dilution from 16 to 1  $\mu$ g antibody/mL CSM, final staining reaction 100  $\mu$ L) against surface markers CD45 and CD9 on shaker protected from light, 30 min, 20°C–26°C.
  - b. Wash twice with 1 mL CSM, centrifuge 600g, 5 min, 4°C and discard supernatant.
  - c. Resuspend in 500  $\mu$ L CSM.
  - d. At this point cells may be introduced into a flow cytometer or stained with antibodies against intracellular proteins (step 27).
27. Staining with fluorescent antibodies against intracellular proteins.
  - a. Permeabilize cells with 1 mL 100% ice-cold methanol, 20 min, 4°C.
  - b. Centrifuge, 600g, 5 min, 4°C and discard supernatant.
  - c. Wash twice with 1 mL CSM, centrifuge 600g, 5 min, 4°C and discard supernatant.
  - d. Incubate with 50  $\mu$ L of 2 $\times$  antibody solution (serial dilution from 16 to 1  $\mu$ g antibody/mL CSM antibody against c-PARP), on shaker, protected from light, 30 min, 20°C–26°C.
  - e. Wash twice with 1 mL CSM, centrifuge 600g, 5 min, 4°C and discard supernatant.
  - f. Resuspend in 500  $\mu$ L CSM and introduce samples into flow cytometer, e.g., LSRII.

**Note:** The c-PARP antibody provides the option of excluding or including apoptotic cells in subsequent analyses. Apoptotic cells are distinguished from dead cells as they have an intact plasma membrane. Their inclusion in single cell data can confound the results. However, the researcher has the option to include them if needed.

28. Determining CyTOF data quality and gating viable cell population before analysis.
  - a. Normalize data from bead standards using Matlab or R – Normalizer ([key resources table](#)).
  - b. Gate the following populations sequentially: (i) nucleated cells DNA1 (191-Ir) vs DNA2 (193-Ir), (ii) singlets (DNA vs event length), (iii) live cells (cisplatin-negative), (iv) viable cells (c-PARP-negative).
29. Determining data quality and gating viable cell population from fluorescence-based cytometry.
  - a. Gate the following populations sequentially: (i) singlets (FSC-A vs FSC-H), (ii) cells (FSC-A vs SSC-A), (iii) live cells (DAPI-negative or Aqua-negative) (iv) viable cells (c-PARP-negative).

**Note:** If data is of high quality, the fourth gate will comprise a high frequency of viable cells. The resultant FSC files are analyzed further with the cell populations of interest.

### Titration of PKH67 and PKH26 fluorescent membrane dyes

⌚ Timing: 3.5–4 h

This section describes the steps to titrate PKH67 and PKH26, two lipophilic membrane dyes. OVCAR4 cells were stained with PKH67 (green fluorescent dye) and NK-92 cells with PKH26 (red fluorescent dye) using the appropriate cell linker kit. The titration steps have general applicability

to determine optimal dye concentrations for cell lines. The effects of each dye on cell viability can be determined after labeling by staining an aliquot of cells with LIVE/DEAD™ Fixable Near-IR Stain. Media for washes will vary depending on cell lines being used. This protocol uses  $1 \times 10^6$  cells for each dye concentration.

30. Preparing membrane dye solutions.

- a. Prepare 1 mL of a fresh 40  $\mu\text{M}$  solution for PKH26 and PKH67 in diluent C (as provided by vendor and listed in [key resources table](#)).
- b. Prepare two-fold serial dilutions of each membrane dye for six concentrations plus one vehicle control from 40  $\mu\text{M}$ , to 1.25  $\mu\text{M}$  in diluent C. Final PKH67 dye solutions are 20, 10, 5, 2.5, 1.25, and 0.625  $\mu\text{M}$  each in a volume of 500  $\mu\text{L}$ .

**△ CRITICAL: Protect tubes from light.**

31. Labeling cell lines with dyes.

- a. Harvest  $7 \times 10^6$  of each cell line into a 15 mL conical tube (steps 13 and 14).
- b. Centrifuge cells, 300g, 5 min, 20°C–26°C, and discard supernatant.
- c. Resuspend cells in 7 mL unsupplemented media ( $1 \times 10^6$  cells/mL) and transfer 1 mL to each of seven FACS tubes.
- d. Centrifuge cells, 300g, 5 min, 20°C–26°C, and discard supernatant.
- e. Resuspend each cell pellet in 500  $\mu\text{L}$  diluent C.
- f. Add 500  $\mu\text{L}$  of one of the six serial dilutions of dye and mix with gentle pipetting (final concentration  $1 \times 10^6$  cells/mL). To control for background fluorescence with no dye, add 500  $\mu\text{L}$  diluent C or ethanol.
- g. Incubate protected from light, 5 min, 20°C–26°C.
- h. Quench by adding 1 mL filtered FBS and incubate protected from light, 1 min, 20°C–26°C.
- i. Centrifuge cells, 300g, 5 min, 4°C, and discard supernatant.
- j. Resuspend cell pellet in 2 mL supplemented RPMI-1640 and wash three times with this media, centrifuge 300g, 5 min, 4°C, and discard supernatant.
- k. Wash cells with 2 mL PBS, centrifuge 300g, 5 min, 4°C, and discard supernatant.
- l. Resuspend labeled cell pellets in 1 mL PBS and determine viability.

32. Live/dead cell staining.

- a. Prepare 10 mL LIVE/DEAD™ Fixable Near-IR Dead Cell stain in PBS (0.5  $\mu\text{L}/\text{mL}$  working solution) according to [manufacturer's instructions](#).

**Note:** Other viability dyes can be used ([key resources table](#)).

- b. Centrifuge cells 300g, 5 min, 4°C and discard supernatant.
- c. Resuspend cell pellet in 1 mL LIVE/DEAD™ Fixable Near-IR Dead Cell Stain.
- d. Incubate protected from light, 30 min, 20°C–26°C.
- e. Quench by adding 3 mL of supplemented RPMI-1640.
- f. Centrifuge cells, 300g, 5 min, 4°C and discard supernatant.
- g. Wash cell pellet with 2 mL unsupplemented media, centrifuge 300g, 5 min, 4°C, and discard supernatant.
- h. Wash cell pellet twice with 2 mL PBS, centrifuge 300g, 5 min, 4°C, and discard supernatant.

33. Preparing cells for fluorescence-based flow cytometry.

- a. Fix cells by adding 1 mL 1.6% PFA/PBS solution, 10 min, 20°C–26°C (step 18).
- b. Add 1 mL of CSM, centrifuge cells, 500g, 5 min, 4°C and discard supernatant.
- c. Wash cells twice with 2 mL CSM, centrifuge 500g, 5 min, 4°C and discard supernatant.
- d. Resuspend cells in 500  $\mu\text{L}$  of CSM and introduce into flow cytometer, e.g., LSRII.

▮▮ **Pause point:** Fixed cells can be stored at 4°C for 3 days.

34. Data analysis.
  - a. Use flow cytometry software packages (CellEngine or Cytobank) to gate population of interest out of the viable cell population.
  - b. Plot titration data (e.g., Prism, [key resources table](#)).
  - c. The optimal concentration for staining cells (20 μM for both dyes) is based on maximal uptake of dye and minimal cell death.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
PE anti-human CD9 (clone M-L13), 1:20 dilution	BD Biosciences	Cat#555372; RRID: AB_395774
Anti-PE-165Ho (clone PE001), 1:100 dilution	Fluidigm	Cat#3165015B; RRID: AB_2714168
Anti-human CD9 (clone MEM-61), 1:100 dilution	Abcam	Cat#ab2215; RRID: AB_302894
Anti-human CD45 (clone HI30)-89Y, 1:200 dilution	Fluidigm	Cat#3089003B RRID: AB_2661851
APC anti-human CD45 (clone HI30), 1:20 dilution	BioLegend	Cat#304037; RRID: AB_2562049
BV421 anti-human CD9 (clone M-L13), 1:20 dilution	BD Biosciences	Cat#743047; RRID: AB_2741243
Anti-human CD9 (clone M-L13) in-house conjugated to 156Gd, 1 μg/mL	BD Biosciences/Fantl Lab, Stanford	Cat#555370
Anti-cleaved (c)-PARP (Asp214) (clone F21-852) in-house conjugated to 171Yb, 1 μg/mL	BD Biosciences	Cat#552597; RRID: AB_394438
Anti-human CD11c (clone 3.9) in-house conjugated to 157Gd, 4 μg/mL	BioLegend	Cat#301601; RRID: AB_314171
Anti-human CD3 (clone UCHT1)-170Er, 1:200 dilution	Fluidigm	Cat#3170001B; RRID: AB_2661807
Anti-human CD19 (clone HIB19)-169Tm, 1:100 dilution	Fluidigm	Cat#3169011B; RRID: AB_2893034
Anti-human CD14 (clone M5E2)-160Gd, 1:50 dilution	Fluidigm	Cat#3160001B; RRID: AB_2687634
Anti-human CD7 (clone M-T701) in-house conjugated to 144Nd, 3 μg/mL	BD Pharmingen	Cat#555359; RRID: AB_395762
Anti-human CD16 (clone 3G8)-165Ho, 1:100 dilution	Fluidigm	Cat#3165001B; RRID: AB_2802109
Anti-human CD56 (clone NCAM16.2)-176Yb, 1:100 dilution	Fluidigm	Cat#3176008B; RRID: AB_2661813
<b>Biological samples</b>		
Peripheral blood mononuclear cells (PBMCs) from healthy donors	Stanford Blood Center	<a href="https://stanfordbloodcenter.org/">https://stanfordbloodcenter.org/</a>
<b>Chemicals, peptides, and recombinant proteins</b>		
RPMI-1640 medium	Life Technologies	Cat#21870-092
Gibco™ Medium 199 (M199)	Thermo Fisher Scientific	Cat#11-150-059
Eagle's Minimum Essential Medium (EMEM)	ATCC	Cat#30-2003
HyClone™ Fetal Bovine Serum (U.S.), defined (FBS)	Thermo Fisher Scientific	Cat#SH3007003
Penicillin-Streptomycin (10,000 U/mL)	Life Technologies	Cat#15140-122
L-Glutamine (200 mM)	Life Technologies	Cat#25030-081
TrypLE™ express enzyme (1 ×)	Life Technologies	Cat#12605-036
Recombinant Human IL-2 protein	R&D Systems	Cat#202-IL-050
Protease-free Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat#A3059
Sodium azide	Sigma-Aldrich	Cat#71289
Benzonase	Sigma-Aldrich	Cat#E8263-25KU
Ficoll-Paque™ PLUS	Thermo Fisher Scientific	Cat#45-001-750
Cisplatin	Sigma-Aldrich	Cat#P4394
Cytochalasin D	Sigma-Aldrich	Cat#C2618
EDTA	Teknova	Cat#E0308
Nocodazole	Sigma-Aldrich	Cat#M1404-10MG
Concanamycin A	Sigma-Aldrich	Cat#C9705-25UG
LY294002	Selleck Chemicals	Cat#1105
Etoposide	Sigma-Aldrich	Cat# E1383
Cell-ID intercalator-Ir	Fluidigm	Cat#201192B

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell-ID intercalator-Rh	Fluidigm	Cat#201103A
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D2650
EQ4 Element calibration beads	Fluidigm	SKU 201078
2-Mercaptoethanol	Sigma-Aldrich	Cat#63689
NaCl	Thermo Fisher Scientific	Cat#S271-10
KCl	Thermo Fisher Scientific	Cat#P330-500
Na <sub>2</sub> HPO <sub>4</sub>	Sigma-Aldrich	Cat#S9390-500
KH <sub>2</sub> PO <sub>4</sub>	Sigma-Aldrich	Cat#P-0662
DAPI solution	BD Biosciences	Cat#564907
NaOH pellets	Sigma-Aldrich	Cat#S5881
Trypan Blue solution, 0.4%	Sigma-Aldrich	Cat#T8154-100mL
PKH67 Green fluorescent cell linker mini kit for general membrane labeling	Sigma-Aldrich	MINI67-1KT
PKH26 Red fluorescent cell linker mini kit for general membrane labeling	Sigma-Aldrich	MINI26-1KT
LIVE/DEAD™ Fixable Aqua dead cell stain kit	Invitrogen	Cat#L34957
LIVE/DEAD™ Fixable near-IR dead cell stain kit, for 633/ 635 nm excitation	Invitrogen	Cat#L10119
16% Paraformaldehyde aqueous solution (PFA)	Electron Microscopy Sciences	Cat#15711
Human TruStain FcX (FC-Receptor blocking solution)	BioLegend	Cat#422302
Fluoromount mounting medium	Thermo Fisher Scientific	Cat#00-4958-02
<b>Critical commercial assays</b>		
MaxPar X8 Antibody Labeling kit	Fluidigm	N/A
RNeasy kit	QIAGEN	Cat#74004
High-Capacity cDNA reverse transcription kit	Applied Biosystems, Thermo Fisher Scientific	Cat#4368814
TaqMan gene expression master mix	Applied Biosystems, Thermo Fisher Scientific	Cat#4370048
TaqMan gene expression assay: Hs00170423_m1 (CDH1-FAM)	Applied Biosystems, Thermo Fisher Scientific	Cat#4453320
TaqMan gene expression assay: Hs00894716_m1 (PTPRC-FAM)	Applied Biosystems, Thermo Fisher Scientific	Cat#4448892
TaqMan gene expression assay: Hs01124022_m1 (CD9-FAM)	Applied Biosystems, Thermo Fisher Scientific	Cat#4453320
TaqMan gene expression assay: Hs02758991_g1 (GADPH-VIC)	Applied Biosystems, Thermo Fisher Scientific	Cat#4448489
<b>Experimental models: Cell lines</b>		
OVCAR4 (Ovarian)	Fox Chase Cancer Center	N/A
NK-92 – isolated from a male with non-Hodgkins lymphoma	ATCC	CRL-2407
NKL – isolated from a male with natural killer cell lymphoblastic leukemia	Dr. Lewis Lanier (UCSF)	RRID: CVCL_0466
Kuramochi (Ovarian)	JCRB Cell Bank	JCRB0098
TYK-nu (Ovarian)	JCRB Cell Bank	JCRB0234.0
<b>Software and algorithms</b>		
CellEngine™ analysis software	CellCarta	<a href="https://cellengine.com">https://cellengine.com</a>
Prism	GraphPad Software	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a> , Version 9
Matlab or R – Normalizer	(Finck et al., 2013)	<a href="https://github.com/nolanlab/bead-normalization/wiki/Normalizing-FCS-Files">https://github.com/nolanlab/bead-normalization/wiki/Normalizing-FCS-Files</a> <a href="https://github.com.laneproxy.stanford.edu/ParkerICI/premessa—R">https://github.com.laneproxy.stanford.edu/ParkerICI/premessa—R</a>
Matlab or R – Single Cell Debarcoder	(Zunder et al., 2015)	<a href="https://github.com/nolanlab/single-cell-debarcoder">https://github.com/nolanlab/single-cell-debarcoder</a> <a href="https://github.com.laneproxy.stanford.edu/ParkerICI/premessa—R">https://github.com.laneproxy.stanford.edu/ParkerICI/premessa—R</a>
Cytobank	(Kotecha et al., 2010)	<a href="https://cytobank.org/">https://cytobank.org/</a>
Microsoft Excel	Microsoft	<a href="https://www.microsoft.com/en-us/microsoft-365/excel">https://www.microsoft.com/en-us/microsoft-365/excel</a>

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Other</b>		
Corning™ TC-treated culture dishes, 10 cm	Thermo Fisher Scientific	Cat# 08-772-22
Corning™ T-25 flasks	Thermo Fisher Scientific	Cat#10-126-28
Corning™ T-75 flasks	Thermo Fisher Scientific	Cat#07-202-000
Corning™ 96 well TC-treated microplates	Thermo Fisher Scientific	Cat#3799
Corning™ HTS Transwell™ -96 permeable support system	Thermo Fisher Scientific	Cat#09-761-80
Corning™ 2 mL cryogenic vials	Thermo Fisher Scientific	Cat#430659
Eppendorf™ snap-cap microcentrifuge safe-lock™ tubes for freezing	Thermo Fisher Scientific	Cat#05-402-25
Corning™ Stripette™ All-Plastic Wrapped, Polystyrene Serological Pipettes, Sterile	Thermo Fisher Scientific	Cat#07-200-12
Corning cluster tube	Thermo Fisher Scientific	Cat#07-200-317
BD Vacutainer™ Plastic Blood Collection Tubes with Sodium Heparin	Thermo Fisher Scientific	Cat#02-689-6
SepMate™-50 (IVD)	STEMCELL Tech	Cat#85460
Corning CoolCell LX, Alcohol-free Cell Freezing Container	VWR Scientific	Cat#75779-716
Falcon 15 mL conical centrifuge tubes	Thermo Fisher Scientific	Cat#14-959-53A
Falcon round-bottom polystyrene test tubes (5 mL FACS tube, no lid)	Thermo Fisher Scientific	Cat#14-959-6
Falcon round-bottom polystyrene tubes with cell strainer (35 µm)	Thermo Fisher Scientific	Cat#0877123
Falcon 70 µm cell strainer	Thermo Fisher Scientific	Cat#087712
PCR 384-well plate	Applied Biosystems, Thermo Fisher Scientific	Cat#AB1384
Axygen™ microplate sealing film	Thermo Fisher Scientific	Cat#14-222-342
Steriflip-GP sterile centrifuge tube top filter unit, 0.22 µm pore size	Milipore	Cat#SCGP00525
Millex-VV Syringe filter unit, 0.1 µm	Milipore	Cat#SLVV033RS
Nalgene Rapid-Flow™ sterile vacuum filter units	Thermo Fisher Scientific	Cat#156-4020
Hemocytometer	N/A	N/A
Cell counting slides	Bio-Rad	Cat#145-0011
Microscope glass slides	Ted Pella	Cat#260386
Benchtop refrigerated centrifuge	N/A	N/A
CyTOF2 mass cytometer	Fluidigm	N/A
Sony SH800 cell sorter	Sony Biotechnology	N/A
7900HT Fast Real-Time PCR System	Applied Biosystems	N/A
BD LSRII Flow Cytometer	BD Biosciences	N/A
Keyence BZ-X800	KEYENCE	N/A
TC10™ Automated cell counter	Bio-Rad	N/A
Nanodrop 2000 Spectrophotometer	ThermoFisher Scientific	N/A

**Note:** Fluidigm has been renamed Standard BioTools.

**MATERIALS AND EQUIPMENT**

**Supplemented RPMI-1640 media for NK-92, NKL cell lines and Kuramochi cell lines**

Reagent	Final concentration	Amount
RPMI-1640 media	n/a	500 mL
FBS	10%	50 mL
Penicillin-Streptomycin (10,000 U/mL)	1%	5 mL
L-Glutamine (200 mM)	1%	5 mL
<b>Total</b>		<b>560 mL</b>

Store for up to 2 months at 4°C. IL-2 added fresh each time media is used.

**Complete M199 media for OVCAR4 cells**

Reagent	Final concentration	Amount
M199 media	n/a	500 mL

(Continued on next page)

**Continued**

Reagent	Final concentration	Amount
FBS	5%	25 mL
Penicillin-Streptomycin (10,000 U/mL)	1%	5 mL
<b>Total</b>		<b>530 mL</b>

Store for up to 2 months at 4°C.

**Complete EMEM media for TYK-nu cells**

Reagent	Final concentration	Amount
EMEM media	n/a	500 mL
FBS	10%	50 mL
Penicillin-Streptomycin (10,000 U/mL)	1%	5 mL
<b>Total</b>		<b>565 mL</b>

Store for up to 2 months at 4°C.

**Thawing media for frozen PBMCs**

Reagent	Final concentration	Amount
Supplemented RPMI-1640 media	n/a	50 mL
Benzonase	25 U/mL	5 µL
<b>Total</b>		<b>50 mL</b>

Add benzonase to pre-warmed supplemented RPMI-1640 media before use.

**10× CyTOF PBS (pH 7.4)**

Reagent	Final concentration	Amount
NaCl	1.37 M	320 g
KCl	0.027 M	8 g
Na <sub>2</sub> HPO <sub>4</sub>	0.043 M	46 g
KH <sub>2</sub> PO <sub>4</sub>	0.015 M	8 g
ddH <sub>2</sub> O (Milli-Q)	n/a	4 L
NaOH	n/a	n/a
<b>Total</b>		<b>500 mL</b>

Store up to 1 year at 20°C–26°C.

NaOH pellets are added to adjust pH to 7.4.

**CyTOF water**

Reagent	Final concentration	Amount
Double-distilled (Milli-Q) purified water	n/a	500 mL
Nalgene Rapid-Flow™ sterile vacuum filter units	n/a	n/a
<b>Total</b>		<b>500 mL</b>

Store up to 6 months at 20°C–26°C.

**Cell staining media (CSM) for CyTOF**

Reagent	Final concentration	Amount
Sterile-filtered 1× PBS	n/a	500 mL
BSA	2%	2.5 g
Sodium azide	0.5%	100 mg
<b>Total</b>		<b>500 mL</b>

Store up to 6 months at 4°C.

△ **CRITICAL:** For CyTOF, to minimize metal contamination use new sterile plasticware (Nalgene polypropylene bottle) to store CSM. Prepare 1× PBS from 10× PBS using double distilled (Milli-Q) purified water.

△ **CRITICAL:** Sodium azide is highly toxic. Avoid contact with skin, eyes, and clothing. Fatal if swallowed.

### Trogocytosis inhibitors

Reagent	Final concentration	Amount
Concanamycin A (stock: 115 μM in DMSO)	100 nM	1.3 μL
Cytochalasin D (stock: 10 mM in DMSO)	10 μM	1.5 μL
EDTA (stock: 500 mM in ddH <sub>2</sub> O)	2 mM	6 μL
LY294002 (stock: 10 mM in DMSO)	10 μM	1.5 μL
Nocodazole (stock: 100 μM in DMSO)	100 nM	1.5 μL
Supplemented RPMI-1640 media plus IL-2 (200 IU/mL)	n/a	1.5 mL
<b>Total</b>		<b>n/a</b>

Prepare fresh in supplemented RPMI-1640 media + IL-2 at time of use.

### PKH67 working solution

Reagent	Final concentration	Amount
PKH67 dye	40 μM	40 μL
Diluent C	n/a	960 μL
<b>Total</b>		<b>1 mL</b>

Prepare immediately prior to use and keep protected from light.

Do not store.

### PKH26 working solution

Reagent	Final concentration	Amount
PKH26 dye	40 μM	40 μL
Diluent C	n/a	960 μL
<b>Total</b>		<b>1 mL</b>

Prepare immediately prior to use and keep protected from light.

Do not store.

### Reverse Transcriptase (RT) PCR master mix (RT-PCR MM)

Reagent	Final concentration	Amount
10× RT buffer	1×	2.2 μL
25× dNTP mix (100 mM)	1×	0.88 μL
10× RT random primers	1×	2.2 μL
<b>Total</b>		<b>5.28 μL</b>

Prepare reagents fresh at 4°C. Do not store.

Volumes are calculated for 10% excess.

Discard unused reagents.

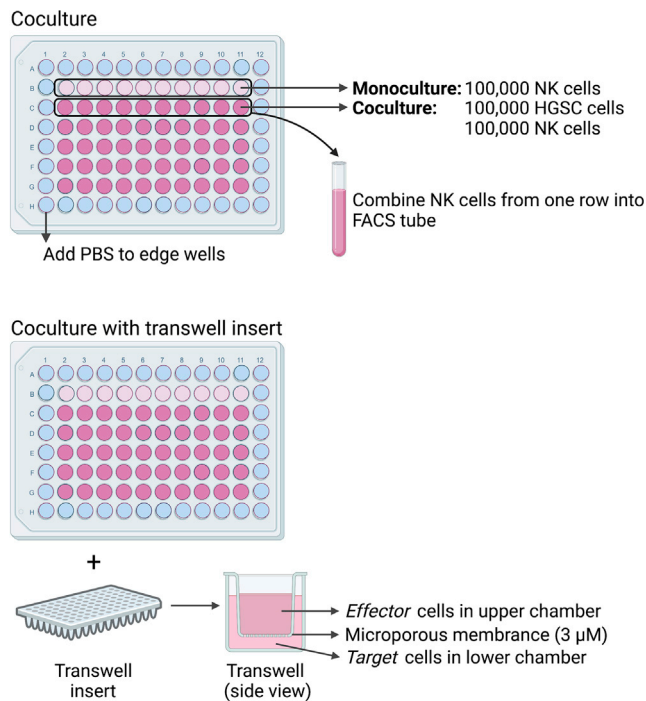
### TaqMan qPCR master mix

Reagent	Final concentration	Amount
2× TaqMan™ gene expression master mix	1×	128 μL
GADPH-VIC probe	n/a	19.8 μL
Gene of interest-FAM probe	n/a	19.8 μL
<b>Total</b>		<b>237.6 μL</b>

Prepare reagents fresh at 4°C. Do not store.

Volumes are calculated for 10% excess.

Discard unused reagents.



**Figure 3. Coculture with transwell insert**

HGSC target cells (100,000 cells/well) are seeded into a 96-well U-bottom plate. A transwell membrane insert, pre-equilibrated in NK cell growth media, is placed into the plate. NK effector cells (100,000 cells/well) are seeded into the upper chamber. After coculture, the transwell membrane insert is transferred into an empty 96-well plate. NK cells are released from the membrane, by gently pipetting up and down. Samples are pooled from 10 wells into a FACS tube for further processing.

## STEP-BY-STEP METHOD DETAILS

### Coculturing cells with a transwell barrier

⌚ Timing: 9–10 h

To confirm that the protein(s) of interest has been acquired by trogocytosis, cocultures separated by a membrane barrier (pore size 3  $\mu\text{m}$ ) should be set up with a transwell plate. The large 3  $\mu\text{m}$  pore size allows extracellular vesicles, such as exosomes (100 nm), to pass through but blocks cell – cell contact that is essential for trogocytosis.

- Pre-equilibrating Transwell™ -96 permeable support system.
  - Fill each of the 96 wells of the U-shaped bottom plate with 200  $\mu\text{L}$  supplemented RPMI-1640 (Figure 3).
  - Immerse the Transwell™ -96 permeable support (upper plate) into the wells of the bottom plate.
  - Equilibrate in a humidified cell culture incubator, 37°C for least 1 h before use.
- Coculturing OVCAR4 and NK-92 cells across a membrane barrier.
  - Aliquot 125  $\mu\text{L}$  OVCAR4 ( $1 \times 10^5$  cells) into wells of a U-shaped bottom plate.
  - Place equilibrated transwell membrane insert (from step 1.c.) into this plate.
  - Aliquot 125  $\mu\text{L}$  NK-92 ( $1 \times 10^5$  cells) into transwells.
  - Incubate the dual chamber 96-well plate (OVCAR4, bottom chamber and NK-92 cells, upper chamber) at 37°C for 6 h (see further details below for time course).
- Collecting NK-92 cells cocultured across a membrane barrier.

- a. Lift transwell insert and place in an empty sterile 96-well plate.
- b. Release NK-92 cells from membrane by gently pipetting up and down.
- c. Pool cells from 10 transwells into 5 mL FACS tubes as described above (Section “before you begin”, steps 15.b.–e., Figure 1).
- d. Samples can be processed for CyTOF or fluorescence-based flow cytometry (Section “before you begin”, “processing samples for CyTOF and fluorescence-based flow cytometry,” and “titrating antibodies for CyTOF and fluorescence-based flow cytometry”, steps 16–27).

## Determining the kinetics of CD9 uptake by NK-92 cells

⌚ Timing: 3.5–10 h

Trogocytosis occurs within minutes of cell-cell contact and involves the transfer of plasma membrane fragments from one cell to another (Miyake and Karasuyama, 2021; Reed et al., 2021). In a kinetic assay we detected CD9+ NK-92 cells within 15 min of starting the coculture, with a steady increase up to 360 min (Gonzalez et al., 2021) (Figure 5).

4. Coculturing NK-92 cells and OVCAR4 cells over time.
  - a. NK-92 and OVCAR4 cells (1:1 ratio) were cocultured for 15, 30, 60, 120 and 360 min in a humidified cell culture incubator, 37°C.
  - b. To measure frequencies of CD9+ NK-92 cells after coculture follow the steps described above (“before you begin”, steps 13 and 14).
  - c. For CyTOF and fluorescence-based flow cytometry follow Section “before you begin”, “processing samples for CyTOF and fluorescence-based flow cytometry”, “titrating antibodies for CyTOF and fluorescence-based flow cytometry” and “Determining data quality and gating viable cell population before analysis”, steps 16–29.
  - d. Analyze FCS files and plot data (Figure 5).

## Inhibiting trogocytosis

⌚ Timing: 7–9 h

Further evidence that trogocytosis is the mechanism of protein transfer from one cell to another, requires cocultures to be performed in the presence of cytoskeletal inhibitors (Aucher et al., 2008; Gary et al., 2012; Gonzalez et al., 2021; Reed and Wetzel, 2019; Vanherberghen et al., 2004). Available inhibitors include concanamycin A, latrunculin, cytochalasin A, cytochalasin D, EDTA, LY294002 and nocodazole, each capable of altering the cytoskeleton. In our study, of those tested, cytochalasin D was the most inhibitory (range 34%–53%) (Figure 6).

5. Pre-treating NK-92 cells with inhibitors of trogocytosis.
  - a. Pre-incubate NK-92 cells ( $1 \times 10^6$  cells/mL) with: concanamycin A (100 nM), cytochalasin D (10  $\mu$ M), EDTA (2 mM), LY294002 (10  $\mu$ M) or nocodazole (100 nM), with appropriate vehicle controls, 2 h, 37°C (See “materials and equipment: Trogocytosis inhibitors”).
  - b. During incubation period, prepare 500  $\mu$ L of  $0.5 \times 10^6$  target cells (HGSC or non-HGSC cell lines) in FACS tubes.
  - c. Add 500  $\mu$ L NK-92 cells pre-treated with inhibitor (a) to FACS tubes containing target cells (effector : target ratio of 1 : 1 at  $0.5 \times 10^6$  cells for each line).
  - d. Centrifuge cocultures to maximize cell-cell contact, 200g, 2 min, 20°C–26°C.
  - e. Incubate cocultures, 3 h, 37°C. There are no media changes, and the inhibitor added (step 5.a.) is present for duration of coculture.

**Note:** Given that trogocytosis inhibitors block proteins within the cytoskeleton, we adjusted co-culture incubation times to maximize CD9 uptake while minimizing overall cellular toxicity.

**Note:** Our and other studies have indicated that the block on trogocytosis may vary from inhibitor to inhibitor. It is therefore advisable to perform pilot experiments to find the most potent inhibitor for the cells being studied.

6. Processing inhibitor-treated cocultures for CyTOF or fluorescence cytometry (Section “before you begin,” “processing samples for CyTOF and fluorescence-based flow cytometry” and “titrating antibodies for CyTOF and fluorescence-based flow cytometry”, steps 16–27).
7. Analyze FCS files and plot data (Figure 6).
  - a. For CyTOF analysis, gate the following populations sequentially after bead normalization: (i) nucleated cells (DNA1 (191-Ir) vs DNA2 (193-Ir), (ii) singlets (DNA vs event length), (iii) live cells (cisplatin-negative), (iv) viable cells (c-PARP-negative), (v) NK-92 cells (CD45+).
  - b. For fluorescence-based flow cytometry analysis, gate the following populations sequentially: (i) singlets (FSC-A vs FSC-H), (ii) cells (FSC-A vs SSC-A), (iii) live cells (DAPI-negative or Aqua-negative), (iv) NK-92 cells (CD45+).

### Investigating potential intracellular pools of CD9

⌚ Timing: 4 h

To verify that the presence of CD9 on NK-92 cells is due to its transfer from OVCAR4 cells, and not due to trafficking from their own pre-existing intracellular pools induced by intercellular synapse formation (Figure 7).

8. Determining intracellular pools of CD9.
  - a. Process NK-92 and OVCAR4 cells grown in monoculture for live/dead cell staining with cisplatin and fix with PFA (Section “before you begin”, steps 16, 17, and 18).
  - b. Wash cells with 4 mL CSM, centrifuge 500g, 5 min, 4°C and discard supernatant.
  - c. Resuspend in 90 µL CSM, add 5 µL Fc-Block, incubate, 10 min, 20°C–26°C.
  - d. To detect surface CD9, add 5 µL of anti-CD9-phycoerythrin (PE), incubate on shaker 45 min, 20°C–26°C.
  - e. Wash once with 4 mL CSM, centrifuge 500g, 5 min, 4°C and discard supernatant.
  - f. Resuspend cells in 100 µL CSM.
  - g. Add 1 µL anti-PE-165Ho, incubate 30 min, 20°C–26°C.

**Optional:** Instead of two-step antibody staining, (steps 8.d.–g.) anti-CD9 conjugated directly to another metal can be used.

- h. Wash once with 4 mL CSM, centrifuge 500g, 5 min, 4°C and discard supernatant.
- i. Permeabilize cells by resuspending in 1 mL 100% ice-cold methanol and incubate on ice, 10 min.
- j. Wash twice with 4 mL CSM, centrifuge 500g, 5 min, 4°C and discard supernatant.
- k. To detect intracellular CD9, resuspend cells in 100 µL CSM with 0.5 µL anti-CD9-156Gd antibody and incubate 1 h, 20°C–26°C.
- l. Wash twice with 4 mL CSM, centrifuge 500g, 5 min, 4°C and discard supernatant.
- m. Add 1 mL freshly prepared DNA-iridium intercalator solution.
- n. Incubate for 1 h, 20°C–26°C or up to 48 h, 4°C.
- o. Introduce samples into mass cytometer (Section “before you begin”, step 23).
- p. Analyze FCS files and plot data (Section “before you begin”, step 28) (Figure 7).

**Note:** This protocol also applies to staining cells for fluorescence-based flow cytometry.

**Fluorescence-activated cell sorting of CD9+ and CD9- NK-92 populations after coculture**

⌚ Timing: 2 days

Ruling out that a trogocytosed protein is not endogenously produced but that it is being transferred from an interacting cell is an important mechanistic validation (Caumartin et al., 2007; Gonzalez et al., 2021; Nakayama et al., 2011; Hasim et al., 2022). To this end we looked for the presence of CD9 transcripts using the reverse transcription polymerase chain reaction (RT-PCR). We describe the steps for isolating CD9+ and CD9- NK-92 cells from cocultures by fluorescence-activated cell sorting (FACS). For these assays the number and ratio of cells in coculture is study dependent. The goal is to optimize the number of cells of interest that can be sorted from the starting coculture population. In our studies with an effector : target ratio 1:1 we harvested  $1 \times 10^6$  NK cells from the coculture supernatant. Of these around 80% were viable cells. For practical reasons the duration of cocultures was 24 h with no adverse effects noted for cell viability.

9. Coculturing OVCAR4 and NK-92 cells 24 h, 37°C and transferring the cell culture supernatant to FACS tubes (Section “before you begin”, steps 13–15).
10. Staining cells with fluorochrome-conjugated antibodies for FACS.
  - a. Resuspend cells in 90  $\mu$ L of unsupplemented RPMI-1640.
  - b. Add 5  $\mu$ L anti-human CD9-PE (2.5  $\mu$ g/mL) and 5  $\mu$ L anti-human CD45-APC (0.45  $\mu$ g/mL).
  - c. Mix well and incubate protected from light, 30 min, on ice.
  - d. Add 2 mL unsupplemented RPMI-1640, centrifuge 300g, 5 min, 4°C and discard supernatant.
  - e. Wash cells twice with 2 mL unsupplemented RPMI-1640, centrifuge 300g, 5 min, 4°C, discard supernatant and stain cells with nuclear stain DAPI as a dead cell probe.
  - f. Dilute DAPI (1 mg/mL) in unsupplemented RPMI-1640 for a final concentration of 0.1  $\mu$ g/mL (1:10,000 v/v dilution).
  - g. Resuspend cells in 1 mL of a 0.1  $\mu$ g/mL DAPI solution (above).
  - h. Incubate protected from light, 10 min, 20°C–26°C.
  - i. Quench with 1 mL supplemented RPMI-1640, centrifuge 300g, 5 min, 4°C and discard supernatant.
  - j. Wash twice with 2 mL supplemented RPMI-1640, centrifuge 300g 5 min, 4°C and discard supernatant.
  - k. Resuspend cells in 1 mL supplemented RPMI-1640.
  - l. Remove aggregates with a 35  $\mu$ m cell strainer.
11. Sorting gated cell populations (Figure 8).
  - a. Introduce cells from step 10.l. into sorter, e.g., BD FACS Aria or Sony SH800.
  - b. Create gates for sorting using the relevant software e.g., BD FACSDiva™ or Sony SH800.
  - c. For singlets, gate on FSC-H vs FSC-A.
  - d. For live cells (DAPI-negative), gate singlets on DAPI vs FSC-A.
  - e. For CD45+ NK-92 cells, gate live cells on CD45-APC vs FSC-A.
  - f. For CD9+ and CD9- NK-92 cells, gate CD45+ NK-92 cells on CD9-PE vs CD45-APC.
  - g. Sort using the purity mode.
  - h. Collect sorted CD9+ and CD9- NK-92 cells in ice-cold FBS.
  - i. Centrifuge cell populations, 300g, 5 min, 4°C and discard supernatant.
  - j. Resuspend cells in 1 mL of unsupplemented RPMI-1640 and count.

**Note:** At this point, sorted NK-92 cells can be used for different purposes. In our case, we used them for quantitative reverse transcription PCR analysis (next section).

**Note:** The gating strategy shown in Figure 8 should be performed for all experiments when fluorescence-based flow cytometry is the readout. (See also step 7.b.).



△ **CRITICAL:** Include monocultures of both cell lines as controls for FACS sorting: i) OVCAR4 cells and NK-92 cells each stained with anti-human CD9-PE antibody, (ii) NK-92 cells unstained, (iii) NK-92 cells stained with DAPI.

△ **CRITICAL:** All centrifugations must be performed at 4°C to minimize cell death. Sorted CD9+ and CD9- NK-92 cells are collected into ice-cold FBS in FACS tubes kept on ice and protected from light.

### Performing quantitative reverse transcription PCR analysis (qRT-PCR)

⌚ **Timing:** 6–8 h

This procedure can detect low levels of mRNA transcripts. A negative result for CD9 mRNA in NK-92 cells would further validate CD9 trogocytosis by these cells. RNeasy micro kit (QIAGEN) was used to isolate RNA from cell lysates and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to convert RNA to cDNA. Preparation of cell lysates from monocultures of OVCAR4 and NK-92 cells as controls for the presence and absence of CD9 transcripts. E-cadherin and CD45 act as positive controls for transcripts from OVCAR4 and NK-92 cells respectively.

#### 12. Lysing cells for RNA isolation.

- a. Centrifuge cells (FACS-purified and control monocultures,  $1 \times 10^5$ – $1 \times 10^6$  cells) 300g, 5 min, 4°C and discard supernatant.
- b. Resuspend  $2$ – $5 \times 10^4$  cells in 350  $\mu$ L RLT buffer (component of RNeasy kit) containing  $\beta$ -mercaptoethanol (10  $\mu$ L/mL RLT buffer).
- c. Lyse and homogenize cells according to the manufacturer's protocol (RNeasy kit [protocol](#)) and freeze the lysate at  $-80^\circ\text{C}$ .

⏸ **Pause point:** Cell lysates can be stored at  $-80^\circ\text{C}$  until they are processed for RNA isolation.

#### 13. Isolating total RNA from cell lysates.

- a. Perform RNA extraction according to the manufacturer's protocol (RNeasy kit [protocol](#)).
- b. Quantify RNA using NanoDrop microvolume spectrophotometer.
- c. Dilute RNA samples with RNAase-free water to 5–10 ng/ $\mu$ L.

#### 14. Synthesizing cDNA with High-Capacity cDNA Reverse Transcription Kit.

- a. Thaw kit components on ice.
- b. Label PCR tubes and place on ice.
- c. Aliquot 12  $\mu$ L of each RNA sample into PCR tubes on ice.
- d. Prepare RT master mix scaling up for number of samples (4.8  $\mu$ L RT buffer, 2.2  $\mu$ L RNase free  $\text{H}_2\text{O}$ , 1  $\mu$ L MultiScribe™ (RTase)).
- e. Add 8  $\mu$ L master mix to each RNA sample (final reaction volume 20  $\mu$ L).
- f. RT-PCR samples (triplicates) to include mRNAs from: NK-92 cells in monoculture, FACS-purified CD9+ and CD9- NK-92 cells, OVCAR4 cells (positive CD9 control). Negative controls to include samples without RNA and without RTase respectively. Final reaction volumes to be made up to 20  $\mu$ L with RNase free  $\text{H}_2\text{O}$ .
- g. Keep samples on ice and gently pipette up and down to mix.
- h. Load PCR tubes into a thermal cycler and set conditions according to High-Capacity cDNA Reverse Transcription kit instructions ([Table 1](#)).

⏸ **Pause point:** cDNA can be stored at 4°C or  $-20^\circ\text{C}$ .

#### 15. Preparing reaction mixes for duplex qPCR.

- a. Dilute cDNA (20  $\mu$ L) by adding 60  $\mu$ L dd $\text{H}_2\text{O}$ .

**Table 1. RT-PCR conditions**

RT-PCR cycling conditions		
Steps	Temperature	Time
1	25°C	10 min
2	37°C	120 min
3	85°C	5 min
4	4°C	Forever

**Note:** Plan experiment for transcripts of interest and controls. For our study we prepared 54 PCR reactions (3 genes, 6 conditions in triplicate) (Figure 9).

- b. Label PCR tubes, place on ice and aliquot 12  $\mu$ L of TaqMan qPCR master mix containing a Victoria (VIC) labeled probe against GAPDH (control housekeeping gene) and a fluorescein amidite (FAM) labeled probe against one gene of interest (18 tubes per gene) (Figure 9).
  - i. E-cadherin: CDH1-FAM.
  - ii. CD45: PTPRC-FAM.
  - iii. CD9: CD9-FAM.
- c. Add 8  $\mu$ L cDNA to each PCR tube.
- d. Pipette up and down with P20 pipette to mix well and avoid creating bubbles.
- e. Transfer mix from PCR tubes to 384-well plate and seal it with aluminum sealing film.
- f. Centrifuge 384-well plate, 300g, 2 min, 20°C–26°C.
- g. Load 384-well plate onto a 7900HT Fast Real-Time PCR System and run program below (Table 2).
- h. Analyze data and plot transcript copy numbers (Figure 10A) and fold transcript expression compared to GAPDH control (Figure 10B).

### Visualizing transferred plasma membrane fragments with embedded CD9 on NK-92 cells

⌚ Timing: 2 days

During trogocytosis, the direction of membrane capture (i.e., which of the two cells donates or accepts plasma membrane fragments) can have important functional consequences. This method is designed to address the directionality of trogocytosis with cocultures prepared where either one or both cell lines have been pre-stained with a fluorescent lipophilic membrane dye. Here, as mentioned above, we are focusing on directionality from HGSC to NK cells. Effector : target ratios are titrated to quantify transferred plasma membrane fragments and to rule out that transfer is a random event. OVCAR4 cells are stained with PKH67 (green fluorescent) and NK-92 cells are unstained.

16. Prepare monocultures of OVCAR4 and NK-92 cells (Section “before you begin” steps 13 and 14 respectively).
  - a. Each cell line is prepared at a concentration of  $2.5 \times 10^6$  cells/mL specified media.
17. Labeling OVCAR4 cells with PKH67 dye.
  - a. Harvest OVCAR4 cells (Section “before you begin” step 13).

**Table 2. qPCR cycling conditions**

Temperature	Time	Cycles
50°C	2 min	–
95°C	10 min	–
95°C	15 s	40 cycles
60°C	1 min	

**Table 3. Cell dilutions for indicated effector : target ratios**

Effector : Target ratio	Volume NK cells	Volume OVCAR4 cells	Additional media
5 : 1	100 $\mu$ L	20 $\mu$ L	130 $\mu$ L
2.5 : 1	50 $\mu$ L	20 $\mu$ L	180 $\mu$ L
1 : 1	40 $\mu$ L	40 $\mu$ L	170 $\mu$ L
1 : 2.5	20 $\mu$ L	50 $\mu$ L	180 $\mu$ L
1 : 5	20 $\mu$ L	100 $\mu$ L	130 $\mu$ L

- b. Resuspend cells in unsupplemented RPMI-1640 and transfer  $7 \times 10^6$  OVCAR4 cells to a 15 mL conical tube, centrifuge 300g, 5 min, 4°C and discard supernatant.
  - c. Add volume of diluent C for cell suspension of  $1 \times 10^7$  cells/mL.
  - d. Add an equal volume of PKH67 working solution to the OVCAR4 cell suspension (final concentration of  $5 \times 10^6$  cells/mL) (20  $\mu$ M PKH67 was determined to be the optimal concentration (“before you begin” step 32)).
  - e. Mix well by gently pipetting up and down and incubate 5 min, 20°C–26°C.
  - f. Quench by adding an equal volume of filtered FBS (Steriflip-GP sterile centrifuge tube top filter unit, 0.22  $\mu$ m pore size) and incubate 1 min, 20°C–26°C.
  - g. Centrifuge cells, 300g, 5 min, 4°C and discard supernatant.
  - h. Wash cells with 10 mL of supplemented RPMI-1640 three times, centrifuge 300g, 5 min, 4°C.
  - i. Resuspend labeled OVCAR4 cells in supplemented RPMI-1640 + IL-2 ( $2.5 \times 10^6$  cells/mL).
  - j. Keep protected from light before setting up coculture.
18. Seeding PKH67 stained OVCAR4 cells with unstained NK-92 cells for coculture.
    - a. See “before you begin”, steps 13.e.–k. and 14.
    - b. Seed prepared cells at effector : target ratios indicated (Table 3) into 96-well U-bottom plate. 10 wells are seeded for each target effector ratio.
    - c. Centrifuge 96-well plate, 250g, 4 min, 20°C–26°C.
    - d. Incubate coculture in a humidified cell culture incubator, 24 h, 37°C.

**Note:** Our kinetic experiments (step 4 above and data not shown) indicated that CD9 expression on NK recipient cells was maintained for up to 48 h. This provides flexibility when designing experiments. In this experiment we chose a 24 h coculture time not only for convenience but to maximize our chances of measuring a CD9 signal on NK cells especially when cell ratios were not optimal for trogocytosis.

**Note:** To ensure a detectable signal, the minimum cell number for either of the cell lines was 50,000.

**Note:** Although the total cell numbers varied between ratios, the data demonstrated that levels of CD9 uptake by NK-92 cells correlated with the number of OVCAR4 cells, which was the goal of this experiment.

19. Harvesting cocultures.
  - a. Pipette cocultured cells up and down before transferring from wells to FACS tubes.
  - b. Centrifuge cells, 300g, 5 min, 4°C and discard supernatant.
  - c. Wash cells with PBS, centrifuge 300g, 5 min, 4°C and discard supernatant.
20. Live/dead cell staining.
  - a. “before you begin”, steps 32.a.–h.
21. Antibody staining.
  - a. “before you begin”, step 26.
22. Preparing cells for fluorescence-based flow cytometry.
  - a. “before you begin”, step 33.
23. Data analysis.

a. FCS files are gated for live cells (step 7.b.). Data are presented as histograms (Figure 11).

△ **CRITICAL:** Monocultures of OVCAR4 and NK-92 cells to be included as controls: i) OVCAR4 unstained, ii) OVCAR4 labeled with PKH67 dye, iii) OVCAR4 labeled with anti-human CD9-BV421 antibody, iv) NK-92 cells unstained, v) NK-92 cells stained with anti-human CD45-APC antibody, vi) NK-92 cells heat-killed for LIVE/DEAD™ Fixable Near-IR Dead Cell stain. Cover all tubes with foil to protect from light.

### Detecting trogocytosis by fluorescence microscopy

⌚ **Timing:** 2 days

OVCAR4 cells labeled with PKH67 (green fluorescence) are cocultured with NK-92 cells labeled with PKH26 (red fluorescence) as described above. Trogocytosed green membrane fragments with embedded CD9 (blue) can be visualized by microscopy. A total of  $3 \times 10^6$  each of OVCAR4 and NK-92 cells are required providing enough cells for cocultures and monoculture controls.

24. PKH67 labeling OVCAR4 cells and seeding for coculture.
  - a. Harvest monocultures of OVCAR4 (Section “before you begin” steps 13.a.–k.).
  - b. Label  $2 \times 10^6$  OVCAR4 cells with PKH67 as above (step 17).
  - c. Resuspend OVCAR4 cells in supplemented RPMI-1640 at a concentration of  $0.8 \times 10^6$  cells/mL.
  - d. Seed labeled OVCAR4 cells (125  $\mu$ L/well) in 96-well U-bottom plate for coculture with NK-92 cells (effector : target ratio 1 : 1). Seed a row of 10 wells per condition.
25. PKH26 labeling NK-92 cells and seeding for coculture.
  - a. Harvest NK-92 cell monocultures (Section “before you begin” steps 14.a.–d.).
  - b. Resuspend cells in unsupplemented RPMI-1640 and transfer  $2 \times 10^6$  NK-92 cells to a 15 mL conical tube, centrifuge 300g, 5 min, 4°C and discard supernatant.
  - c. Prepare a cell suspension of  $1 \times 10^7$  cells/mL in diluent C.
  - d. Label NK-92 cell suspension with an equal volume of 40  $\mu$ M PKH26 solution for a final concentration of  $5 \times 10^6$  cells/mL in PKH26 solution (20  $\mu$ M final concentration).
  - e. Mix well by pipetting up and down and incubate 5 min, 20°C–26°C.
  - f. Quench by adding an equal volume of filtered FBS and incubate 1 min, 20°C–26°C.
  - g. Centrifuge cells, 300g, 5 min, 4°C and discard supernatant.
  - h. Wash cells three times with 10 mL of supplemented RPMI-1640, centrifuge 300g, 5 min, 4°C.
  - i. Resuspend labeled NK-92 cells in supplemented RPMI-1640 with IL-2 (400 IU/mL, 2 $\times$ ) at a volume for  $0.8 \times 10^6$  cells/mL.
  - j. Add 125  $\mu$ L labeled NK-92 cells to 96-well U-bottom plate containing OVCAR4 target cells.
  - k. Centrifuge 96-well plate, 250g, 4 min, 20°C–26°C and incubate coculture in a humidified cell culture incubator, 3 h, 37°C.
26. Live/dead cell staining.
  - a. “before you begin”, step 32.a–h.
27. Antibody staining.
  - a. “before you begin”, step 26.
28. Preparing cells for fluorescence microscopy and imaging.
  - a. Resuspend cells in 1 mL of CSM.
  - b. Transfer 15  $\mu$ L onto a microscope slide and let it dry.
  - c. Add one drop of Fluoromount mounting medium to cells on microscope slide, place coverslip on top of slide, press gently and carefully remove excess mounting agent by blotting with a Kimwipe.
  - d. Dry for 5 min and image on microscope, e.g., Keyence BZ-X800 (Figure 12).

▮▮ **Pause point:** For optimal results image as soon as possible. However, mounted cells can be stored protected from light for 1 week at 4°C.

△ **CRITICAL:** Controls cells required: monocultures of OVCAR4 and NK-92 both unstained and stained with PKH67 and PKH26 respectively. Protect samples from light covering tubes with foil.

## EXPECTED OUTCOMES

The protocols described here enabled us to confirm that NK cells acquire CD9 from ovarian tumor cells by trogocytosis. Although most prevalent in immunology, trogocytosis has been described in a wide variety of biological settings e.g., fertilization, embryology, neuronal remodeling, and infectious diseases. Thus, the protocols described have wide applicability and will allow researchers to ascertain whether a cellular protein(s) of interest has been acquired by this mechanism. Trogocytosis requires cell - cell contact and occurs very rapidly, being initiated within minutes. If these two criteria are observed, then all (or a selection) of the additional methodologies can be used as confirmation for this mechanism of protein transfer.

We outline the steps to set up cocultures between NK cells and ovarian cancer cells, emphasizing that NK cells can be difficult to grow and passage number and cell density should be closely monitored. We recommend determining the kinetics of protein transfer in addition to the duration of its expression on the recipient cell to ensure subsequent experiments can be carried out at a time point when the transferred protein(s) are readily detectable. While trogocytosis occurs within minutes, its kinetics in different biological settings can vary considerably.

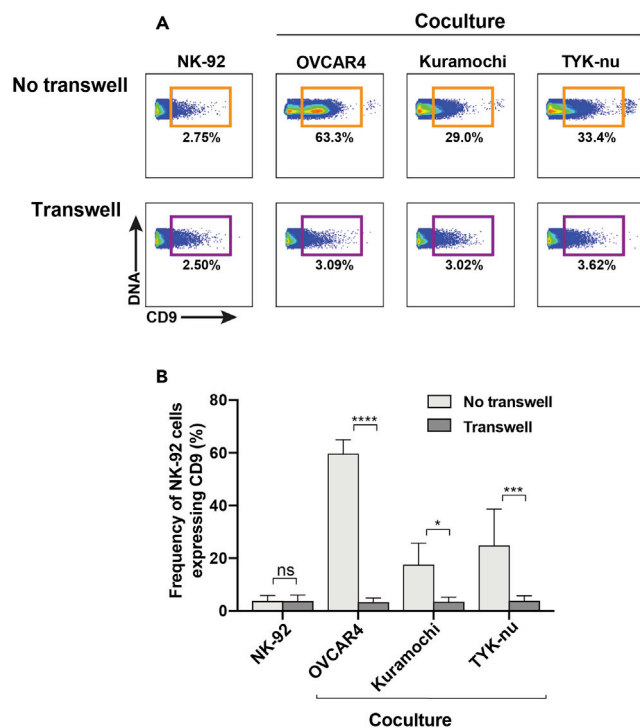
Since physical contact between cells is essential for trogocytosis, coculturing cells on either side of a membrane barrier with large pore size is key to confirming whether trogocytosis is the mechanism of protein transfer, rather than acquisition by smaller extracellular vesicles e.g., exosomes. If, in the presence of the membrane barrier, protein acquisition is reduced to background levels, this supports trogocytosis (Figure 4). Trogocytosis occurs within minutes of intercellular contact, with steady acquisition thereafter (Figure 5). In our system we detected the presence of CD9-expressing NK cells as early as 15 min, with maximal frequencies observed at around 6 h and persisting up to 48 h. At times greater than 48 h, cell density becomes greater resulting in cell death. Conversely a decrease in protein uptake is seen when cells are cocultured in the presence of cytochalasin D, an inhibitor of trogocytosis (Figure 6).

Further verification of trogocytosis requires the exclusion of pre-existing endogenous intracellular pools of the protein of interest in the effector NK cells (Figure 7). Likewise, the absence of RNA transcripts of the protein of interest (CD9) further supports the mechanism of trogocytosis. After coculture, CD9+ and CD9- NK-92 cells were FACS sorted (Figure 8) and CD9 transcripts were measured but not detected in either of these cell populations (Figure 10).

Trogocytosis involves the transfer of membrane patches in which the protein(s) of interest are embedded. This can be quantified by flow cytometry using cells labeled with fluorescently labeled lipophilic membrane dyes and antibodies. This also allows determination of whether trogocytosis is mono- or bi-directional. Furthermore, showing correlated increases and decreases in transfer of membrane patches when target:effector ratios are varied rules out that the process is random. (Figure 11). Finally, fluorescence microscopy is a gold-standard to visualize trogocytosis using cocultured cells stained with fluorescently labeled lipophilic membrane dyes and antibodies (Figure 12).

## QUANTIFICATION AND STATISTICAL ANALYSIS

All data and statistical analyses are implemented with Microsoft Excel, Matlab and GraphPad Prism. CyTOF datasets are analyzed with software available from Cytobank, CellEngine, and FlowJo. FACS-Sorter datasets are analyzed with FlowJo. Microscopy datasets are analyzed with ImageJ.



**Figure 4. CD9 uptake by NK-92 cells measured by CyTOF**

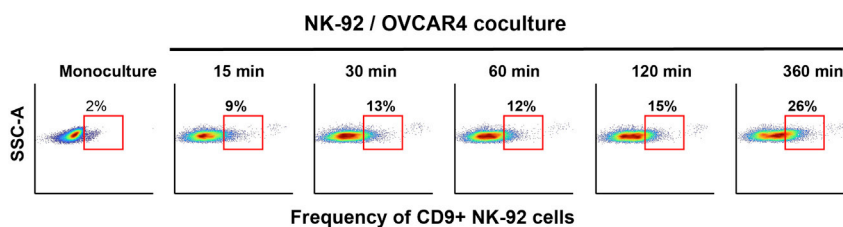
(A) 2D flow plots show increased frequency of CD9+ NK-92 cells after coculture with HGSC cell lines. When cocultured across a membrane barrier (transwells), frequencies of NK-92 cells expressing CD9 were reduced to background levels found for NK-92 cells grown in monoculture (lower row).

(B) Mean and standard deviations for CD9+ NK-92 cells after coculture in the absence and presence of a membrane barrier (transwell insert) (n = 4). Student's two-tailed t test, \*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001, \*\*\*\*p ≤ 0.0001.

## LIMITATIONS

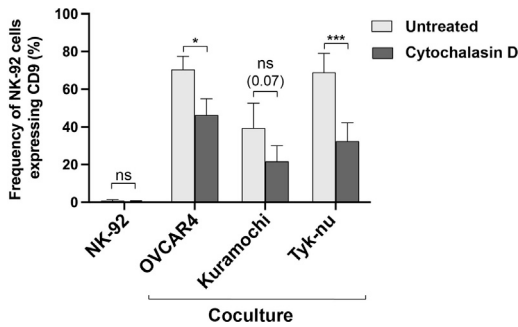
It is essential that each protocol described above is optimized for different coculture pairings selected for investigation. e.g., other cancer cell lines with either NK cell lines, T cell lines or with PBMCs. Pilot kinetic experiments should be performed to determine when transfer of the protein of interest is maximal. As different systems have varying responses to inhibitors, pilot experiments are recommended to determine which inhibitor(s) most effectively block uptake of the protein of interest in recipient cells.

If using NK cell lines, after thawing, their recovery time is unusually long (2 weeks) and this should be factored in to avoid experimental delays, especially when planning coculture assays.



**Figure 5. Kinetics of CD9 uptake by NK-92 cells from OVCAR4 cells analyzed by fluorescence cytometry**

One hallmark of trogocytosis is the rapid protein uptake by a recipient cell within minutes of cell – cell contact. In this example CD9 uptake was observed at 15 min and steadily increased over this 6 h time course.



**Figure 6. Cytochalasin D inhibition of CD9 uptake by NK-92 cells**

CD9 uptake was significantly reduced in the presence of cytochalasin D during coculture (effector : target ratio 1 : 1, 3 h, 37°C) as measured by fluorescence-based flow cytometry (n = 3). Student's two-tailed t test, \*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001, \*\*\*\*p ≤ 0.0001.

NK cells are difficult to maintain in culture and sensitive to passage number, cell density and IL-2 quality. All are crucial to maintain NK cell viability > 85% and to avoid a decrease in viability that will directly induce variability in coculture assays. (See below).

## TROUBLESHOOTING

### Problem 1

Low NK cell viability in Section “before you begin, culturing NK cell lines (step 5)”.

#### Potential solution

Passage cells as needed to prevent overgrowth i.e., > 1 × 10<sup>6</sup> cells/mL. All media changes for NK cells in culture should be with supplemented RPMI-1640 containing freshly thawed IL-2. If low viability persists, discard cells and thaw a new batch of NK cells.

### Problem 2

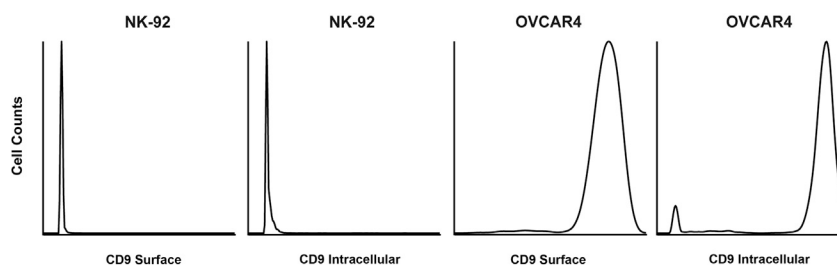
High variability in readouts from protocols that include coculture assays (all protocols in “step-by-step method details”).

#### Potential solution

Use cells with low passage numbers, < 20 (varies depending on cell line used) and viabilities > 85%. Biological and technical replicates are essential.

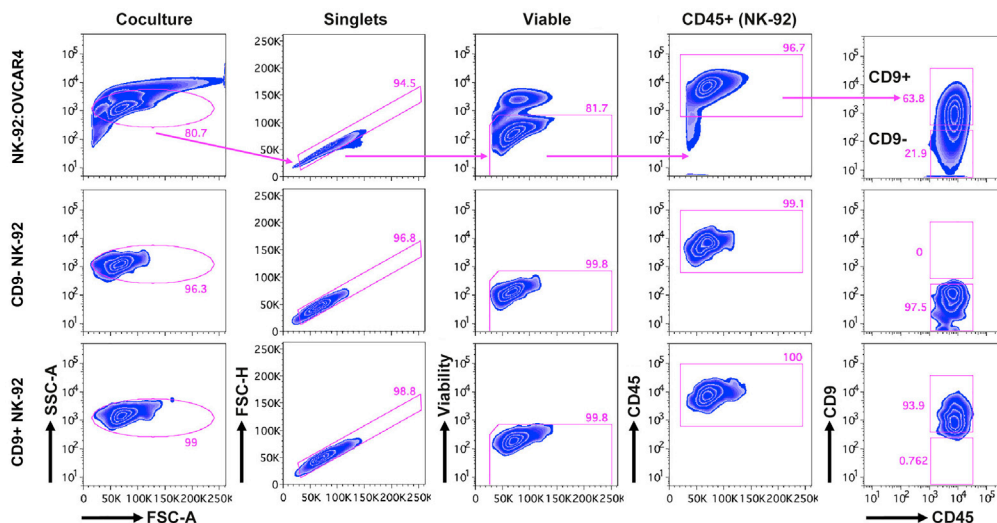
### Problem 3

Low, negative or variable antibody intensities seen for CyTOF readouts in Section “before you begin, titrating antibodies for CyTOF and fluorescence-based flow cytometry (step 23)”, and in Section “step-by-step method details”, protocols “coculturing cells with a transwell barrier (step 3)”, “determining the kinetics of CD9 uptake by NK-92 cells (step 4)”, “inhibiting trogocytosis (step 7)” and “investigating potential intracellular pools of CD9 (step 8)”.



**Figure 7. Measurement of cellular pools of CD9 protein by CyTOF**

The absence of CD9 protein in preexisting cellular pools within NK-92 cells. By contrast CD9 protein was readily detected within preexisting OVCAR4 cellular pools supporting them as the source of CD9.



**Figure 8. Gating strategy to sort CD9- and CD9+ NK-92 cells after coculture with OVCAR4 cells**

Cocultured cells were gated based on size and granularity (FSC and SSC respectively). Singlets were identified based on the linear correlation between FSC-A and FSC-H. Viable cells were identified based on absence of DAPI or Aqua dye. NK-92 cells were gated out of the viable cell population based on CD45 expression out of which CD9+ and CD9- NK-92 cells were identified and sorted. 2D flow plots show cells in coculture (upper row), sorted CD9- NK-92 cells (middle row), sorted CD9+ NK-92 cells (bottom row).

### Potential solution

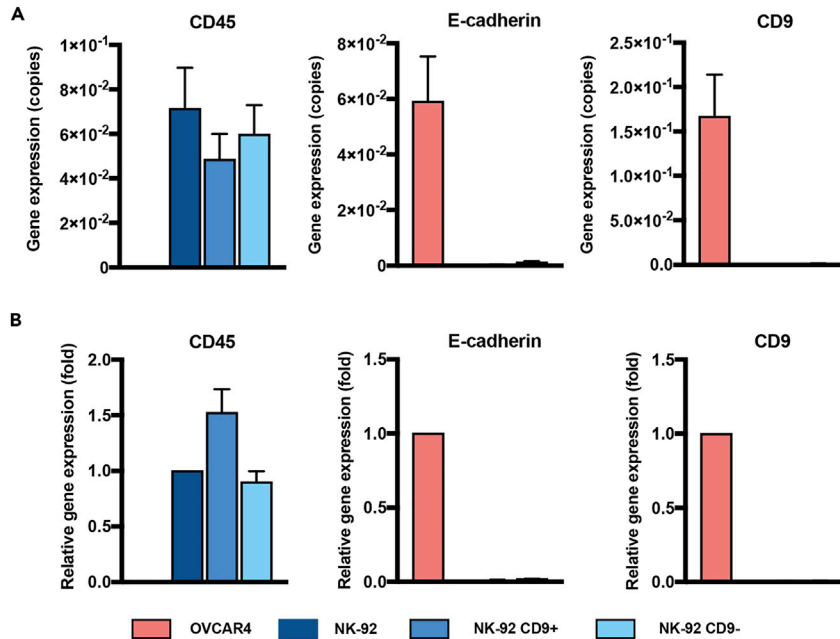
There are multiple factors that may account for low and/or variable CyTOF readouts. The most likely causes are: i) technical/human error, ii) low shelf life of a particular conjugated antibody and issues with the mass cytometer instrument. From our experience ii) is not a frequent occurrence but can be

CDH1 probe	PTPCR probe	CD9 probe
OVCAR4 	OVCAR4 	OVCAR4 
NK-92 	NK-92 	NK-92 
CD9+ NK-92 	CD9+ NK-92 	CD9+ NK-92 
CD9- NK-92 	CD9- NK-92 	CD9- NK-92 
No RTase 	No RTase 	No RTase 
No RNA 	No RNA 	No RNA 

**Figure 9. Template depicting qPCR reaction conditions**

Each circle represents a qPCR reaction condition. FAM-labeled probes for genes of interest are shown in columns: CDH1 (E-cadherin), orange, PTPCR (CD45), purple, and CD9, green. All reactions contain a VIC-labeled GAPDH probe. Cell lines from which RNA is extracted, and controls are shown in rows. For the control “No RTase”, RNA isolated from any of the cell lines can be used.



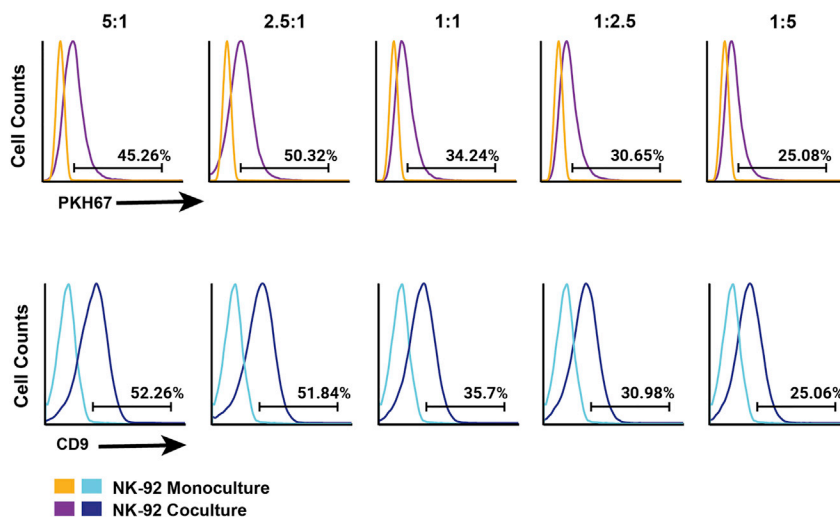


**Figure 10. qRT-PCR of FACS-purified CD9+ and CD9-NK-92 cells after coculture with OVCAR4 cells**

CD9 transcripts were not detected in CD9+ NK-92 cells after coculture but were readily detectable in OVCAR4 cells (far right upper and lower plots).

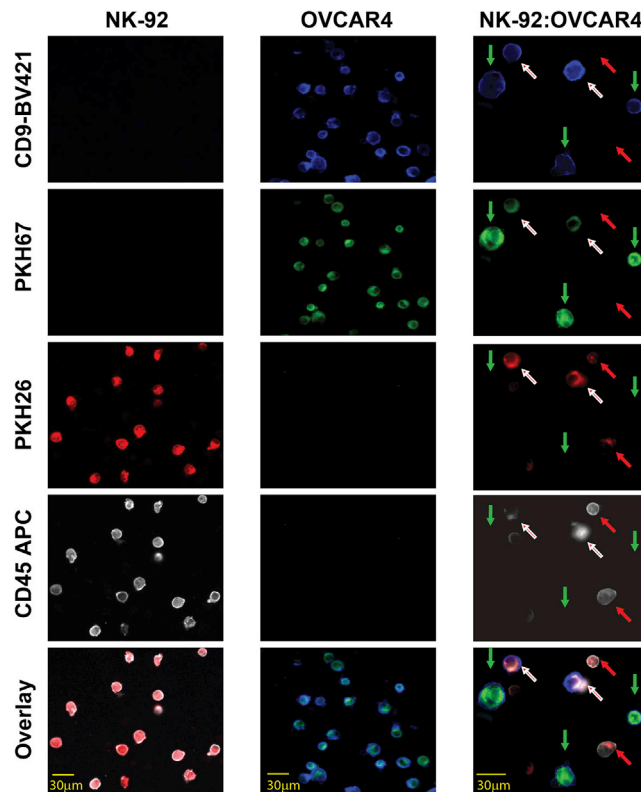
(A and B) (A) Transcript copy numbers (mean + SDs, n = 3) and (B) Gene expression levels relative to GAPDH (fold change). Controls: CD45 (positive for NK-92, negative for OVCAR4) and E-cadherin (negative for NK-92, positive for OVCAR4).

addressed immediately by performing a repeat antibody titration to confirm that the deterioration in performance is due to its shorter-than-expected half-life. To this end we keep reference cells. The problem can be readily addressed by antibody replacement (in-house conjugation or commercially available). Issues with the instrument could arise from incomplete tuning.



**Figure 11. Transfer of membrane fragments with embedded CD9 from OVCAR4 cells to NK-92 cells by fluorescence-based flow cytometry**

NK-92 cells cocultured with pre-stained OVCAR4 cells (green fluorescent membrane dye PKH67) at different target:effector ratios. Initial gating was performed to identify the viable CD45+NK-92 cell population (as Figure 7 above). Frequency of NK-92 cells that acquired PKH67 dye (upper histograms) and CD9 (lower histograms).



**Figure 12. CD9 uptake by NK-92 cells visualized by fluorescence microscopy**

NK-92 cells that acquired CD9 from OVCAR4 cells indicated with white arrows (far right column). Control monocultures: NK-92 cells stained with PKH67, green (left column), OVCAR4 cells stained with PKH26, red (center column), coculture with pre-stained NK-92 and OVCAR4 cell (far right column). Monocultures and cocultures were stained with CD45 and CD9 antibodies. Images were enhanced for brightness and contrast to optimize visualization on the printed image. Magnification; 20× monocultures, 60× cocultures including channel overlay.

#### Problem 4

Low, negative or variable antibody intensities seen for fluorescence readouts in Section “before you begin, titrating antibodies for CyTOF and fluorescence-based flow cytometry (step 27)”, and in Section “step-by-step method details”, protocols “coculturing cells with a transwell barrier (step 3)”, “determining the kinetics of CD9 uptake by NK-92 cells (step 4)”, “inhibiting trogocytosis (step 7)”, “investigating potential intracellular pools of CD9 (step 8)”, “fluorescence-activated cell sorting CD9+ and CD9- NK-92 populations after coculture (step 11)”, “fluorescence-based flow cytometry to visualize transferred plasma membrane fragments with embedded CD9 on NK-92 cells (step 23)”, and “detecting trogocytosis by fluorescence microscopy (step 28)”.

#### Potential solution

As with CyTOF the causative factors and solutions are largely comparable. For fluorescence-based reagents and treated cells protection from light is essential.

#### Problem 5

Low RNA yield and low or negative signals from qRT-PCR in Section “step-by-step method details, performing quantitative reverse transcription PCR analysis (qRT-PCR) (steps 13–15)”.

#### Potential solution

Higher number of cells provides higher RNA extraction yields. Always use ice and RNase free water to prevent RNA degradation. If PCR signal is low, use more RNA for the reverse transcription

reaction. If PCR is negative, including positive control samples, repeat the PCR using another thermocycler since they sometimes fail, or a fresh batch of reagents.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to Antonio Delgado Gonzalez ([adelgadogonzalez@stanford.edu](mailto:adelgadogonzalez@stanford.edu)) and Ying-Wen Huang ([ywhuang@stanford.edu](mailto:ywhuang@stanford.edu)) and will be fulfilled by the lead contact, Wendy J. Fantl ([wjfantl@stanford.edu](mailto:wjfantl@stanford.edu)).

### Materials availability

This study did not generate any unique reagents.

### Data and code availability

This study did not generate unique datasets or code.

## ACKNOWLEDGMENTS

This work was supported by funding from the Department of Defense (W81XWH-12-1-0591), NCI (1R01CA234553, R21CA231280), the 2019 Cancer Innovation Award, supported by the Stanford Cancer Institute, an NCI-designated Comprehensive Cancer Center; BRCA Foundation and the V Foundation for Cancer Research; a gift from the Gray Foundation; the Department of Urology at Stanford University; NHLBI (P01HL10879709); NIAID (U19AI057229); and a PICI Bedside to Bench grant. A.D.-G. thanks the Fundacion Alfonso Martin Escudero, Spain for his postdoctoral fellowship. We thank Shawn Hollahan for artwork ([paradigm\\_shiftin@mac.com](mailto:paradigm_shiftin@mac.com)).

## AUTHOR CONTRIBUTIONS

A.D.-G., Y.-W.H., and E.P. designed and performed experiments, analyzed data, and wrote the manuscript. K.D. designed, performed, and analyzed microscopy. V.D.G. designed and performed experiments and analyzed data. W.J.F. designed experiments, analyzed data, wrote the manuscript, and supervised the study.

## DECLARATION OF INTERESTS

The authors declare no competing interests. We have a published patent related to this study: WO2021/050200, PCT/US2020/046195.

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