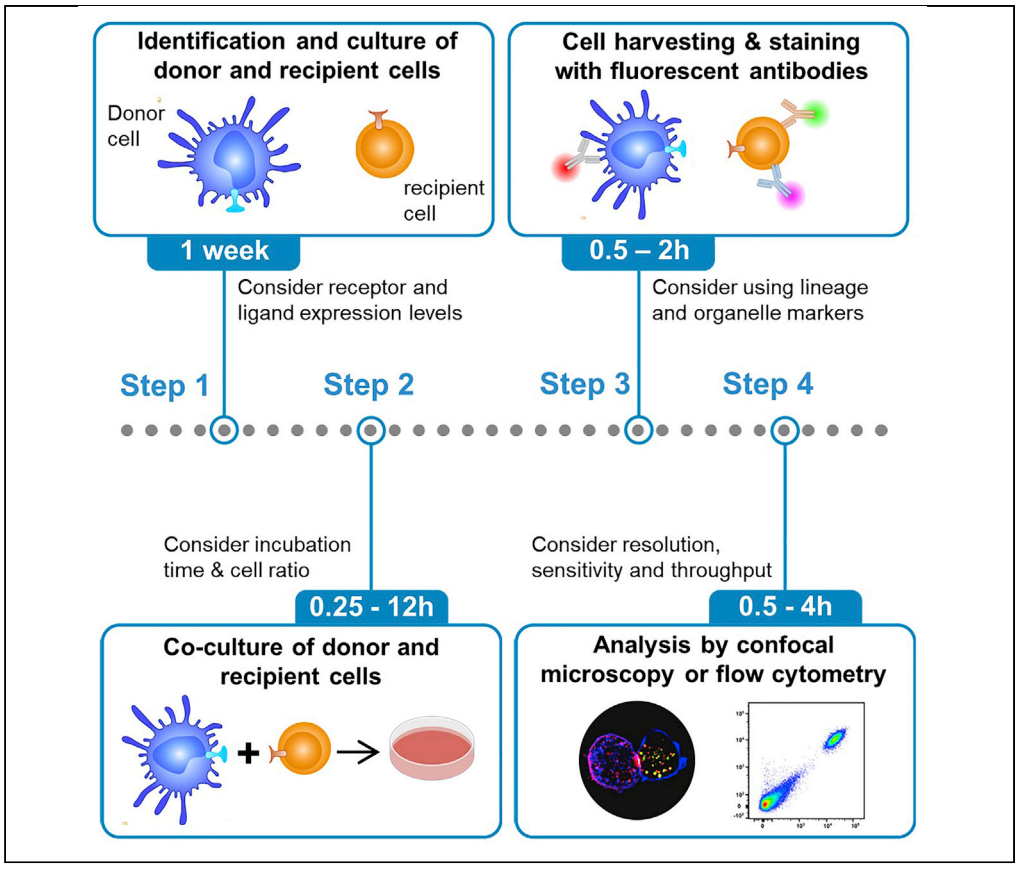
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Analyzing trogocytosis of T lymphocytes by flow cytometry and confocal microscopy



Here, we present a protocol to examine the mechanisms underlying the intercellular transfer of transmembrane molecules, termed trogocytosis, and the fate of transferred molecules. We describe the steps needed from T lymphocyte isolation, via co-culture with cells expressing the ligand of interest, to cell harvest and subsequent staining for flow cytometry and confocal microscopy. Furthermore, we showcase critical parameters and pitfalls, which allow easy adaptation of the protocol to investigate trogocytosis of various cell surface receptors in different cell types.

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

Alicia Zink, Simon Zenke, Teresa Wiese, Niklas Beyersdorf, Tim Lämmermann, Jan C. Rohr

alicia.zink@uniklinik-freiburg.de (A.Z.)
jan.rohr@uniklinik-freiburg.de (J.C.R.)

Highlights

Detailed protocol for studying T cell trogocytosis by flow cytometry and microscopy

Provision of strategies to distinguish between donor and recipient cells

Comprehensive overview on principles and pitfalls for assay optimization

Easy adaptability of assay to study trogocytosis by other cell types

Zink et al., STAR Protocols 4, 102013
March 17, 2023
https://doi.org/10.1016/j.xpro.2022.102013

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Analyzing trogocytosis of T lymphocytes by flow cytometry and confocal microscopy

Alicia Zink,[1,6,*] Simon Zenke,[1,4] Teresa Wiese,[2] Niklas Beyersdorf,[2] Tim Lämmermann,[3] and Jan C. Rohr[1,5,6,7,*]

[1]Institute of Immunodeficiency, Medical Center and Faculty of Medicine, Albert-Ludwigs-University, Freiburg, Germany

[2]Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany

[3]Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany

[4]Present address: Matterhorn Biosciences GmbH, Basel, Switzerland

[5]Present address: Novartis Institutes for Biomedical Research (NIBR), Novartis Pharma AG, Basel, Switzerland

[6]Technical contact

[7]Lead contact

*Correspondence: alicia.zink@uniklinik-freiburg.de (A.Z.), jan.rohr@uniklinik-freiburg.de (J.C.R.)
https://doi.org/10.1016/j.xpro.2022.102013

SUMMARY

Here, we present a protocol to examine the mechanisms underlying the intercellular transfer of transmembrane molecules, termed trogocytosis, and the fate of transferred molecules. We describe the steps needed from T lymphocyte isolation, via co-culture with cells expressing the ligand of interest, to cell harvest and subsequent staining for flow cytometry and confocal microscopy. Furthermore, we showcase critical parameters and pitfalls, which allow easy adaptation of the protocol to investigate trogocytosis of various cell surface receptors in different cell types.

For complete details on the use and execution of this protocol, please refer to Zink and Rohr.[1]

BEFORE YOU BEGIN

Trogocytosis denotes the intercellular transfer of transmembrane molecules between neighboring cells, which has been mainly studied in lymphocytes, but also occurs in other cell types.[2] In immune cells, trogocytosis is believed to regulate processes like antigen-presentation and co-stimulation.[3] Mechanistically, trogocytosis requires interaction between a receptor expressed on the surface of one cell and its cognate ligand expressed on the surface of another.[4] Usually receptor-expressing recipient cells acquire ligands from donor cells, although also bidirectional transfer has been described.[5,6] Some receptors have been shown to endocytose ligands upon trogocytosis ("trans-endocytosis").[7]

Below we list important parameters to consider upon studying trogocytosis. In the "troubleshooting" section we then outline strategies to optimize assays in this regard.

Trogocytosis is generally studied by co-culturing donor cells expressing a ligand of interest with recipient cells expressing the corresponding receptor. Ideally, ligand and donor expression are dichotomic, i.e., confined to donor and recipient cells, respectively. If this is not the case, receptor-ligand interactions on one cell in cis may limit trogocytosis. Furthermore, if recipient cells synthesize receptor and ligand, it may be impossible to distinguish between these and those acquired by trogocytosis.

The performance of trogocytosis assays depends on the efficiency of intercellular molecule transfer, which itself is influenced by many factors. Among those are the affinity of the receptor-ligand

interaction, as well as cell surface expression levels of receptor and ligand. In general, the higher the affinity and the expression levels of receptors and ligands are, the more molecules are transferred, and, the better is the signal-to-background ratio.

Another important parameter is the specificity of the receptor-ligand interaction: if receptor or ligand have different interaction partners, which are co-expressed on donor or recipient cells, this can impact the receptor ligand interaction studied and limit assay performance.

Furthermore, care should be taken to clearly discriminate between donor and recipient cells, as inadvertant inclusion of donor cells in the analysis will compromise the validity of the results.

Both flow cytometry and fluorescence microscopy are frequently used as readout systems for trogocytosis assays. The high-throughput nature of flow cytometry makes it well suited for analyzing trogocytosis in many samples. This can be useful for time-course experiments or drug-screenings. In contrast, the advantage of microscopy imaging is the spatial information it provides. Specifically, microscopy enables researchers to determine and quantify the location of trogocytosed ligands within recipient cells. Such considerations should be taken into account upon choosing the readout system for a given research question.

Below we provide a detailed description of a trogocytosis assay workflow using Chinese hamster ovary (CHO) cells transduced to express fluorescently tagged CD80 as donor cells and CTLA4-expressing T cell lymphoma lines and primary human T cells as recipient cells.

Institutional permissions

The use of primary human and murine blood cells or tissue requires approval by an Institutional Review Board. All human samples specified in this protocol were obtained and used following institutional guidelines and were approved by the local Ethics Committee of the Faculty of Medicine of the University of Würzburg.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-rat CD90/mouse CD90.1 (Thy-1.1) AF 700 (used at 1:1000 dilution)	BioLegend	202528 (RRID: AB_1626244)
Anti-mouse CD8a (53-6.7) APC (used at 1:100 dilution)	Life Technologies	17008182 (RRID: AB_469335)
Anti-human CD4 (RPA-T4) FITC (used at 1:200 dilution)	BioLegend	300506 (RRID: AB_314074)
Anti-human CD3 (HIT3a) (used at 1:10.000 dilution)	BioLegend	300301 (RRID: AB_314037)
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit	Thermo Fisher Scientific	L10119
Chemicals, peptides, and recombinant proteins		
Poly-L-ornithine hydrobromide	Sigma	P3655
Formaldehyde solution	MERCK	252549
Wheat germ agglutinin (WGA), CF™405S conjugate, blue (404/431 nm)	Biotium	29027
Iscove's Modified Dulbecco's Medium (IMDM)	Life Technologies	21980065
Phosphate buffered saline (PBS)	Thermo Fisher Scientific	70011044
Trypsin-EDTA, 0.05%	Life Technologies	25300054
Hanks' Balanced Salt Solution (HBSS)	Life Technologies	14025050
Penicillin/Streptomycin	Sigma-Aldrich	P4333
β-Mercaptoethanol	Life Technologies	31350010
Sodium azide (NaN_3)	Sigma-Aldrich	26628-22-8
Fetal calf serum (FCS)	PAN Biotech GmbH	P30-1502

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Histopaque-107	Sigma-Aldrich	10771
Sodium pyruvate (100 mM)	Thermo Fisher Scientific	11360-039
Minimum essential medium (MEM) non-essential amino acids (NEAA) (100×)	Thermo Fisher Scientific	11140035
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (1 M)	Thermo Fisher Scientific	15630-049
Human AB serum	Sigma-Aldrich	H4522-100ML
Bovine serum albumin (BSA)	Carl Roth	8076.3
KCl	Carl Roth	6781.1
KH_2PO_4	Carl Roth	3904.1
$Na_2HPO_4 \cdot 2\ H_2O$	Carl Roth	4984.2
NaCl	Carl Roth	3957.2
$C_{10}H_{14}N_2Na_2O_8 \cdot 2\ H_2O$	Carl Roth	8043.1
$CaCl_2 \cdot 4\ H_2O$	VWR (Merck)	1.02384.0100
$MgCl_2 \cdot 6\ H_2O$	Carl Roth	2189.1
$MgSO_4 \cdot 7\ H_2O$	Merck	1.05886.0500
$C_6H_{12}O_6 \cdot H_2O$ (α-D(+)Glucose)	Carl Roth	6887.1
Phenol red	VWR (Merck)	1.07241.0025
Roswell Park Memorial Institute (RPMI) 1640 medium	Gibco	21875034
Experimental models: Cell lines		
Murine 58 αβ T cell lymphoma cell line	Groettrup et al.[8]	PMID: 1385777
Chinese hamster ovary (CHO) cells	Puck et al.[9]	PMID: 13598821
Mouse embryonic fibroblasts (MEFs)	ATCC	CRL-2907
Human embryonic kidney (HEK) 293T cells	ATCC	CRL-3216
Human Jurkat T cell lymphoma cell line	ATCC	TIB-152
Recombinant DNA		
pMx-mCD80-mScarlet	This manuscript	N/A
pMx-mCD80-mTagRFP	This manuscript	N/A
pMX-mCTLA4-IRES-GFP	This manuscript	N/A
Software and algorithms		
Prism8 (v8.1.0)	GraphPad Software, Inc.	https://www.graphpad.com/
FlowJo X (v10.0.7r2 and v10.2)	BD Bioscience	https://www.flowjo.com/
Inkscape (v0.92)	The Inkscape project	https://inkscape.org
Other		
48 well plate tissue culture treated	Greiner Bio-One GmbH	677180
96 well plate tissue culture treated	Falcon	353072
96 well plate u bottom	Corning	3795
96 well plate v bottom	Corning	3896
µ-dish 35 mm glass bottom	Ibidi GmbH	81158
Partec Celltrics™ 100 µm filter size	Wolflabs	04-004-232
Humidified 37°C and 5% CO2 incubator	HERA	Cell240
Laminar flow cell culture cabinet	EHRET	SafeFAST Classic
Cell culture microscope	Leica	WILD MPS52
Benchtop centrifuge	Thermo Fisher Scientific	Heraeus Multifuge 3SR+
Flow cytometer	Becton Dickinson	Fortessa
Confocal fluorescence microscope	Zeiss	Cell Observer SD

MATERIALS AND EQUIPMENT

- Donor cells: Chinese hamster ovary cells expressing CD80-mScarlet.

  Note: Other cell types we have successfully used as donor cells:

  - Human embryonic kidney (HEK) 293T cells.
  - Murine embryonic fibroblasts (MEFs)s.

- Primary murine dendritic cells.

- Recipient cells: human peripheral blood mononuclear cells (PBMCs) / T lymphocytes or murine 58 αβ T cell lymphoma cell line transduced to express CTLA4.

Note: Other cell types we have successfully used as recipient cells:

- Primary murine T- and B-lymphocytes.
- Human Jurkat T cell lymphoma cell line.

Cell culture medium

Reagent	Final concentration	Amount
IMDM	N/A	500 mL
FCS	10%	50 mL
Penicillin/Streptomycin	1%	5 mL
50 μM β-Mercaptoethanol	0.01%	50 μL
Total	**N/A**	**555 mL**

Store at 4°C, use within 2 weeks.

PBMC culture medium

Reagent	Final concentration	Amount
RPMI 1640	N/A	500 mL
Sodium pyruvate 100 mM (100×)	1%	5 mL
MEM NEAA (100×)	1%	5 mL
HEPES buffer solution 1 M	1%	5 mL
Penicillin/Streptomycin	1%	5 mL
50 μM β-Mercaptoethanol	0.1%	500 μL
Human AB serum	10%	50 mL
Total	**N/A**	**570 mL**

Store at 4°C, use within 2 weeks.

Versene buffer

Reagent	Concentration [mM]	Amount
KCl	13.4	1 g
KH_2PO_4	7.3	1 g
$Na_2HPO_4 \cdot 2\ H_2O$	16.1	2.86 g
NaCl	701.6	41 g
Na_2EDTA	2.7	1 g
ddH_20	N/A	5 L
Total	**N/A**	**5 L**

Store at 4°C, use within 4 weeks.

BSS/BSA buffer

Reagent	Concentration [mM]	Amount
$CaCl_2 \cdot 2\ H_2O$	11.2	2.046 g
KCl	59.0	4.4 g
NaCl	1505.8	88 g
$MgCl_2 \cdot 6\ H_2O$	10.8	2.2 g
$MgSO_4 \cdot 7\ H_2O$	8.9	2.2 g
$C_6H_{12}O_6 \cdot H_2O$	55.5	11 g
KH_2PO_4	4.8	0.66 g
$Na_2HPO_4 \cdot 2\ H_2O$	14.7	2.618 g

(*Continued on next page*)

Continued		
Reagent	Concentration [mM]	Amount
Phenole red	0.3	0.11 g
BSA	0.3	20 g
ddH_20	N/A	10 L
Total	**N/A**	**10 L**

Store at 4°C, use within 4 weeks.

Flow cytometry (FACS) buffer		
Reagent	Final concentration	Amount
PBS	N/A	500 mL
FCS	2%	10 mL
NaN_3	0.01%	50 µL
Total	**N/A**	**510 mL**

Store at 4°C, use within 2 weeks.

STEP-BY-STEP METHOD DETAILS

⚠ CRITICAL: To minimize the risk of microbial contamination all cell culture work should be performed in a laminar flow cabinet.

Note: This protocol describes the workflow for CHO and MEF as donor cells and 58αβ T cells or primary human PBMCs as recipient cells. As different cell types may prefer different culture conditions (e.g., cell culture media, cell densities, etc.), please adjust the protocol to the cells being used.

Cell culture

Timing: 5–15 min hands-on time for cell splitting + 1 week incubation

Note: All media, buffers and other solutions used for cell culture handling can be used at normal lab temperatures (19°C–23°C), unless stated otherwise.

1. Thaw cryo-preserved donor (CHO, MEF) and recipient (58 αβ) cell lines.
   a. transfer cells into 15 mL Falcon tube.
   b. add PBS to a final volume of 10 mL.
   c. centrifuge at 400 × *g* for 5 min.
   d. Aspirate and discard supernatant.
   e. resuspend cell pellet in 1 mL of cell culture medium.
   f. transfer each cell line into a separate 25 cm^2 (or T25) cell culture flask and add 4 mL of culture medium.
2. Store cell culture flasks in a humidified 37°C, 5% CO_2 incubator.
3. Once the **adherent donor cells** reach 80%–90% confluence (by visual inspection),
   a. Aspirate medium and rinse donor cells once with 5 mL PBS, then aspirate PBS.
   b. Add 0.5–1 mL of 0.05% trypsin and incubate for 2–5 min at 37°C until cells detach from cell culture flask.
   c. Stop trypsinization by adding 4 mL of cell culture medium.

Note: For larger or smaller sized cell culture flasks scale the amount of Trypsin-EDTA and of cell culture medium accordingly.

d. Detach the cells by slowly pipetting up and down, discard 4.5 mL of cell suspension and keep the remaining 0.5 mL.
e. Add 4.5 mL of fresh cell culture medium to split cells 1:10.

4. Split non-adherent recipient cells when the originally red cell culture medium starts to turn yellowish:
   a. Slowly pipet up and down, discard 4.5 mL of cell suspension and keep the remaining 0.5 mL.
   b. Add 4.5 mL of fresh cell culture medium to split cells 1:10.
5. Store the cell culture flasks in a humidified 37°C, 5% CO_2 incubator for at least a week and split cells as indicated.

Day 0

Poly-L-ornithine coating of cell culture dishes for microscopy

Timing: 15 min + 1–18 h incubation + 1 h drying

Note: For flow cytometry-based experiments this step can be omitted.

Note: There are many different types of coating glass surfaces to facilitate cellular adhesion. While in our experimental setup poly-L-ornithine coating worked well, other coatings may work better for other cell types.

6. Dilute poly-L-ornithine stock in sterile H_2O to a final concentration of 0.01% (w/v 0.1 mg/mL).
7. Add 500 µL 0.01% poly-L-ornithine coating solution to 35 mm glass bottom µ-dishes to coat it. Make sure to cover the entire bottom of the dish.
8. Incubate for 1 h at approximately 20°C or for 16–18 h at 4°C.
9. Aspirate coating solution without scratching the coated surface and dry dish at normal lab temperatures (19°C–23°C) for 30–60 min.

Day 1

Counting and plating of donor cells

Timing: 30 min + 16–18 h incubation

10. Aspirate medium from donor cells and rinse them once with 5 mL PBS, then aspirate PBS.
11. add of 0.5–1 mL 0.05% Trypsin-EDTA and incubate for 2–5 min at 37°C until cells detach from cell culture flask.
12. Stop trypsinization by adding 4 mL of cell culture medium.

Note: For larger or smaller sized cell culture flasks scale amount of trypsin and cell culture medium accordingly.

13. Harvest cells into 15 mL Falcon tube, pipet up and down several times in order to singulate cells.

Note: In case singulation of cells turns out to be difficult by trypsinization alone, consider adding a filtering step using a cell filter appropriately sized for the cells used (e.g., Partec Celltrics™ 100 µm filter size).

14. Count cells using a Neubauer counting chamber or other suitable counting device.
15. Adjust the concentration of cells by dilution with cell culture medium as needed.

Note: Seeding 1–3.5 × 10^4 donor cells per well in a 48- or 96-well flat bottom plate often yields a confluent monolayer on the following day, which we found to be optimal for

trogocytosis assays measured by flow cytometry. These numbers may differ between cell lines and culture conditions and should thus be optimized.

⚠ CRITICAL: Optimizing the number of donor cells to achieve a confluent monolayer is important for achieving reproducible results across different experiments. This is because the trogocytosis rate of the recipient cells depends on the total amount of ligand molecules available, which scales with the number of donor cells present.

In contrast, for microscopic analyses a confluent monolayer may be less desired to allow for visualization of interactions between individual donor and recipient cells. In this case, lower cell densities may be beneficial. In our systems, seeding 0.5–1 × 10^5 donor cells in a 35 mm glass bottom μ-dish allowed for imaging of individual cells.

16. Plate donor cells in 96- or 48-well flat bottom cell culture plate, or - for microscopic analyses – in a suitable, coated glass-bottom plate or dish.
17. Incubate for 16–18 h in a humidified 37°C 5% CO_2 incubator.

Day 2

Isolation of primary recipient cells from human blood

Timing: 40 min

Note: When using T cell lines like murine 58 αβ or human Jurkat as recipient cells steps 19–26 can be omitted. For studying trogocytosis of human primary T cells, we used leukocyte-enriched blood samples from healthy donors (provided by the Department of Transfusion Medicine of the University Hospital Würzburg). However, fresh blood samples collected in EDTA- or heparin-coated tubes should work as well. Alternatively, primary T cells can be prepared from murine spleens or lymph nodes by immunomagnetic isolation, using commercially available kits.

18. Transfer blood sample to a 50 mL Falcon tube.
19. Add 25 mL of Versene buffer to the blood sample.
20. For density-gradient centrifugation, prepare another 50 mL tube filled with 15 mL Histopaque-1077.
21. Carefully layer the Versene-diluted blood sample onto the Histopaque using a 25 mL pipette.

Note: To avoid mixing of blood with Histopaque it can be helpful to hold the tube at a 20–45 degree angle. Then slowly dispense the blood onto the inside of the tube letting it run down the tube wall onto the Histopaque to get two clearly separated layers (Figure 1).

22. Centrifuge at 1,160 × *g* for 15 min at normal lab temperatures (19°C–23°C) (no brake).
23. Carefully collect the layer of PBMCs, which can be identified as a white band located between the Histopaque and serum layers (Figure 1).
24. Wash PBMC twice with BSS/BSA buffer. Centrifuge settings: 430 × *g* for 3 min at 4°C.
25. Resuspend in 10 mL PBMC culture medium.

Note: In our experience non-activated human T cells display only little trogocytic activity, and this can be considerably improved by T cell activation. For this, one may add Anti-human CD3 antibody (clone: HIT3a, final concentration 0.1 μg/mL at recipient cell concentration of 1.5 × 10^5 cells/mL) to the recipient cells just before co-culturing them with the donor cells. In contrast, we have found murine primary T cells as well as different T cell lines (58αβ, Jurkat) to also efficiently trogocytose ligands without concomitant activation.

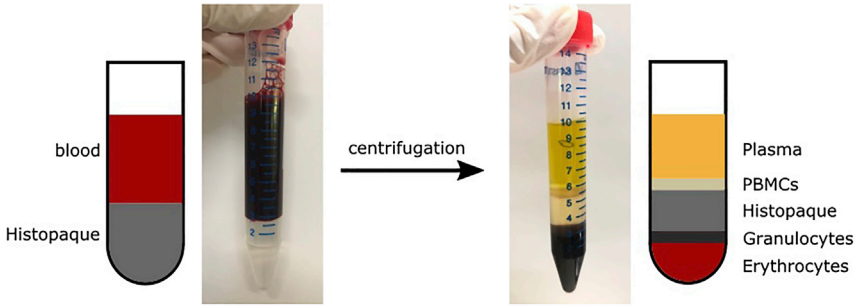


Figure 1. PBMC preparation with Histopaque before (left) and after (right) centrifugation

Setting up co-culture of donor and recipient cells

Timing: 30 min

26. Adjust concentration of recipient cells to 1.5×10^5 cells/mL.

Note: Optimal cell concentrations can vary across different experimental systems and should be optimized accordingly.

27. Carefully remove medium from donor cell culture.
    a. For experiments using flow cytometry as readout add 3×10^4 recipient cells (=200 µL) to donor cells cultured in 96-well plates.
    b. For fluorescence microscopy-based experiments add 1×10^5 recipient cells (=660 µL) to donor cells cultured in 35 mm glass bottom µ-dish.

Note: For microscopy-based experiments involving subsequent fixation and staining of cells prior to microscopic analysis, serum-free medium can be used as it promotes adhesion of lymphocytes to the coated glass-surface. After fixation this will prevent loss of cells in subsequent sample processing steps.

28. Incubate the co-cultured donor and recipient cells in a humidified 37°C 5% CO_2 incubator for the desired amount of time. Alternatively, place the cells in the live-imaging compartment of a confocal microscope.

Note: Whereas trogocytosis can be very rapid, i.e., being detectable after a few minutes, the speed of the process can differ substantially depending on the receptor-ligand pair investigated as well as the type and mobility of the cells used. It is advisable to perform a kinetic analysis comparing different incubation periods when establishing the assay. For T lymphocytes we usually employ co-culture periods of 0.25–12 h (rule of thumb: shorter incubation times can be used when studying the actual molecule transfer, longer incubation times should be used if the focus is on the subsequent fate of acquired molecules).

Note: It takes a few minutes until recipient cells have sunken down onto the donor cells by gravity. A brief centrifugation step (e.g., 200 × *g*, 30 s, 4°C) can accelerate this. Particularly for short experiments, the centrifugation step promotes homogenous trogocytosis rates among recipient cells within a well by aligning starting times of the process.

Optional: In case the effect of pharmacological agents on trogocytosis shall be studied, consider that trogocytosis can occur very rapidly, whereas pharmacological agents may take a while to exert their functions. Hence, it may be necessary to expose donor and recipient cells to such agents for a while before co-culture to assess their effects on trogocytosis.

29. Depending on whether flow cytometry or fluorescence microscopy shall be used as readout either proceed with paragraph "harvest of recipient cells and staining for flow cytometric analysis" (step 31) or "preparation of cells for fluorescent microscopy" (step 41).

Harvest of recipient cells and staining for flow cytometric analysis

Timing: 90 min

Note: From now on keep cells cooled at all times (on ice or in fridge)!

30. Carefully collect and discard 100 μL supernatant from of each well without removing the cells at the bottom.
31. Per well add 100 μL ice-cold FACS Buffer (aids in detaching the cells from one another).
32. Carefully pipet up and down several times.

Note: Aim the flow generated by pipetting to all areas of the well, but without touching the bottom of the plate, to detach the recipient cells from the donor cells. While the goal here is to detach the recipient but not the donor cells, it is usually unavoidable that also some donor cells are inadvertently harvested during this process. Therefore, implementing strategies to unambiguously distinguish between donor and recipient cells is highly recommended to avoid the unintended inclusion of donor cells in the analysis of recipient cells. This can, for example, be accomplished by antibody-based detection of molecules differentially expressed between donor and recipient cells (e.g., so called "lineage-markers"). Please also see problem 1 in the troubleshooting section for further details.

33. For subsequent staining, transfer 200 μL of cell suspension to a new 96-well u- or v-shaped plate.
34. Pellet the cells by centrifugation (400 × *g* for 5 min at 4°C).
    a. During the centrifugation, prepare a staining mix of antibodies in FACS buffer.

Note: For primary human T cells (PBMCs) and the 58αβ T cell lymphoma cell line Anti-human CD4-FITC antibody (1:200 dilution) and Anti-mouse CD8α-APC antibody (1:100 dilution) were used, respectively. Other antibodies can be used to identify cells or interest and discriminate donor and recipient cells. Also a viability dye may be included in the staining mix.

35. Carefully remove and discard the supernatant.
36. Resuspend pelleted cells in 30 μL staining mix and incubate at 4°C for 20–30 min in the dark.
37. Per well add 180 μL cold FACS buffer. Then spin down the cells by centrifugation at 400× *g* for 5 min and carefully discard the supernatant afterward.
38. Repeat step 38.
39. Resuspend cells in 200 μL of ice-cold FACS buffer and analyze them by flow cytometry. Alternatively, cells may now be permeabilized and stained for intracellular molecules (including trogocytosed ligands).

Preparation of cells for fluorescent microscopy

40. Fix cells by adding 500 μL of 4% formaldehyde solution to reach a final concentration of 2% formaldehyde and incubate for 15 min at normal lab temperatures (19°C–23°C) in the dark.

⚠ CRITICAL: Carefully add the fixative dropwise to prevent detachment of cells from the coated glass surface or disruption of cell-cell interactions.

41. Remove the fixative by carefully pouring it off the dish.

Note: After fixation cells should stick fairly strong to the coated surface, which facilitates subsequent washing and staining steps.

42. Wash the dish 3-times with 3 mL PBS.
    a. For this, add PBS to the dish without pipetting it directly onto the cell-coated area.
    b. Remove PBS by pouring it out of the dish.

Note: By tilting the dish at a 45° angle the residual liquid will form a drop which can be removed with a tissue or pipet.

Note: While cells can already be imaged at this stage, subsequent labeling of cells (e.g., cell membrane stain with WGA lectin) can help to determine the subcellular localization of trogocytosed molecules.

43. Remove all PBS from dish and add 200 µL of 5 µg/mL WGA-CF405S conjugate diluted in HBSS for cell membrane labeling.
44. Incubate for 10 min at RT.
45. Wash cells twice with HBSS as described in step 43.
46. Add 3 mL of fresh HBSS to dish and analyze cells using a fluorescence microscope. If you encounter difficulties to detect trogocytosis by microscopy, please also see problem 4 in the troubleshooting section.

EXPECTED OUTCOMES

Flow cytometry

For data analysis it is important to implement a strategy to reliably distinguish between donor and recipient cells. Inadvertent inclusion of donor cells into the analysis of recipient cells compromises the validity of results. For flow cytometry this can be achieved by gating on recipient cells (Figure 2). In the experimental system described here, donor and recipient cells differ in size and granularity, allowing their separation in a dot plot depicting forward and side scatter parameters. In a next step, we plot forward scatter-area versus -height to exclude cell doublets, i.e., cells sticking to each other. Further gating steps building on antibody-based staining of cellular antigens can be included as shown in Figure 2. Optionally, also a viability dye can be added to enable more rigorous exclusion of dead cells. After successfully gating out the donor cells, trogocytosed molecules can be displayed as dot plot or histogram or, in case comparisons across samples are preferred, as histogram overlay. Flow cytometric data can be quantified as change in mean fluorescence intensity (MFI) or as percentage of cells having trogocytosed ligand. Both of these can be calculated by subtracting a negative control sample (i.e., recipient cells only in the absence of trogocytosis) from the experimental sample.

Fluorescent microscopy

Also, for confocal microscopy it is indispensable to reliably distinguish between donor and recipient cells. In the images shown in Figure 3 this is achieved by using recipient cells expressing a green fluorescent CTLA4 receptor, which is absent from the donor cells. Upon co-culture with CD80-TagRFP expressing donor cells, recipient cells acquire the red fluorescent CD80 molecules. To determine whether acquired molecules are located at the cell surface or within the recipient cells, cell membranes can be stained (e.g., with blue fluorescent WGA-Lectin). Depending on the experimental question staining of other organelles may be used.

LIMITATIONS

Trogocytosed ligands may be shuttled to recipient cell lysosomes, where they become degraded and are then no longer detectable.[10] Hence, at any given point in time, the detectable fraction of trogocytosed molecules within recipient cells constitutes the net of all trogocytosed molecules

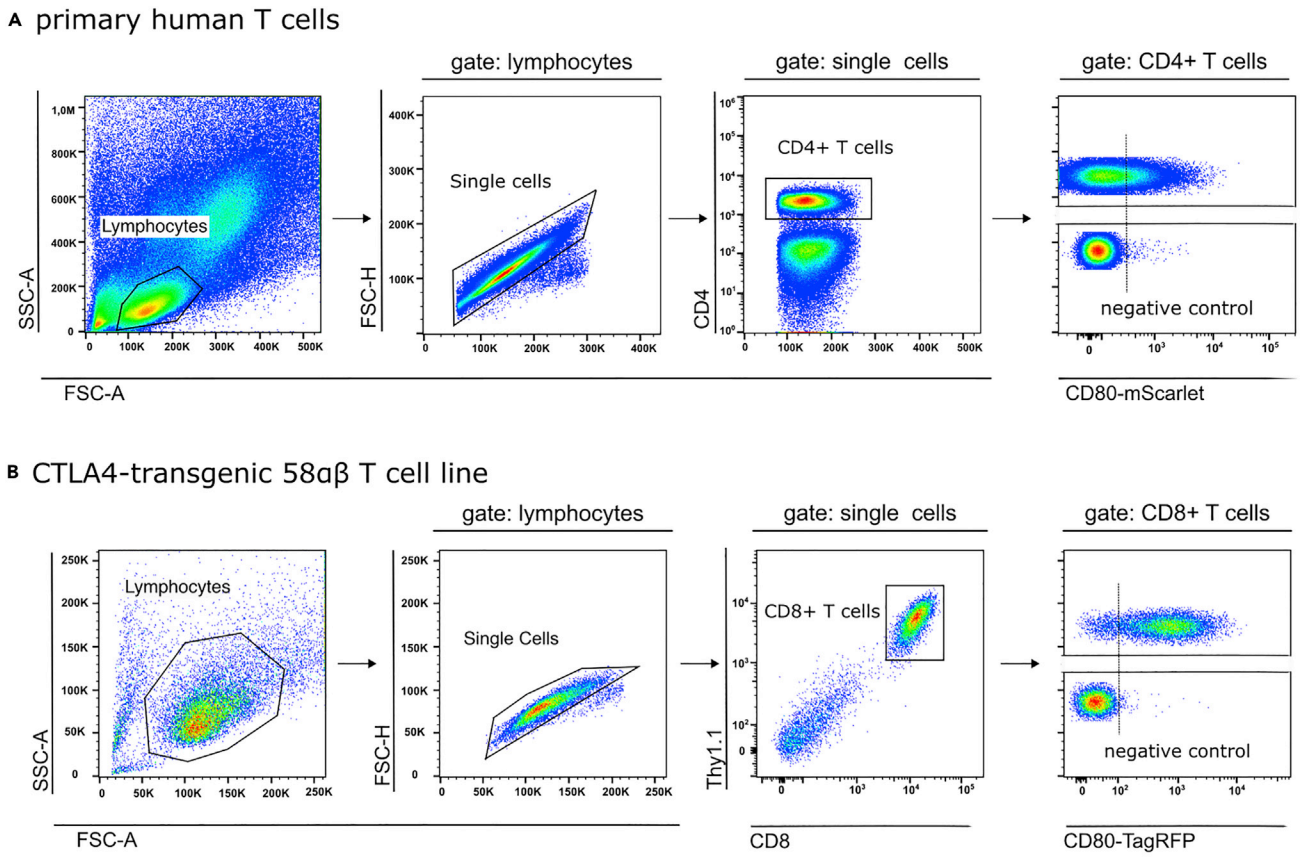


Figure 2. Exemplary gating strategies for primary human T cells and murine T cell line

(A) Trogocytosis by activated primary human $CD4^+$ T cells after 12 h co-culture of PBMC with CD80-mScarlet transgenic murine embryonic fibroblasts. Co-culture of PBMC with non-engineered MEFs was used as negative control.

(B) Trogocytosis of CTLA4-transgenic murine 58 αβ T cells after 2 h co-culture with CD80-TagRFP transgenic CHO cells. 58 αβ T cells lacking expression of CTLA4 and CD28 were used as negative control.

minus those that were degraded. The rate of ligand shuttling to lysosomal degradation may differ substantially for different receptors. Along this line, a receptor that rapidly shuttles acquired ligands to lysosomal degradation may be erroneously regarded as having a poor trogocytic capability.

Overexpression systems of ligand and/or receptor can be very helpful in studying trogocytosis because they frequently improve signal-to-background ratios of experimental systems. However, such overexpression systems may alter cellular behaviors, for example by saturating interaction partners required for molecule trafficking. By the same token, attaching fluorescent proteins to receptors or ligands may affect their folding, function and trafficking. It is thus important to keep in mind, that such engineered systems may not always adequately mirror physiological settings. Hence, we recommend to confirm observations made in overexpression systems by analogous assays using naturally expressed native receptors and ligands.

TROUBLESHOOTING

Problem 1 (Referring to protocol step 32)

It is difficult to tell apart recipient cells that have trogocytosed the molecule of interest from donor cells.

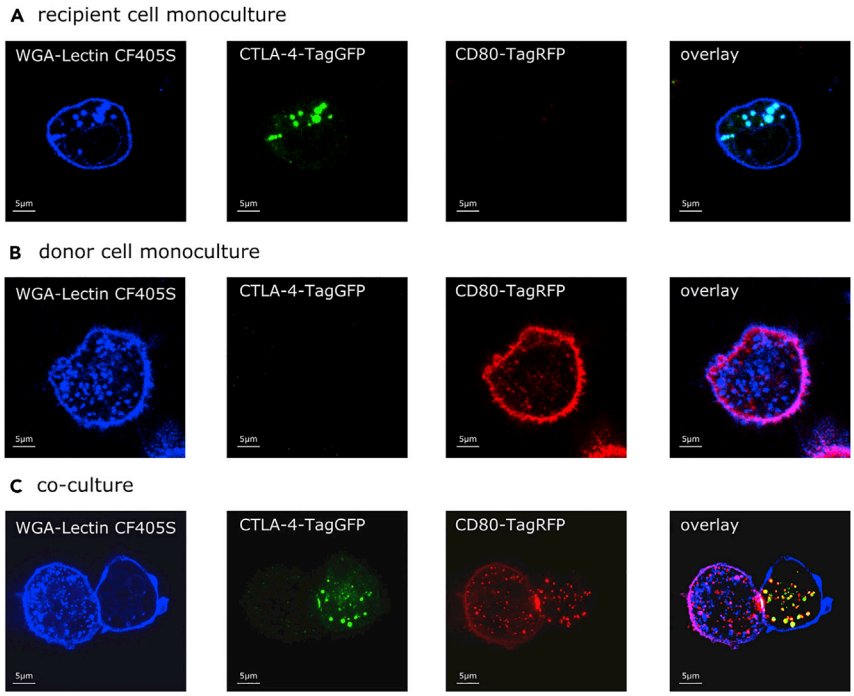


Figure 3. Exemplary microscopic images of separated and interacting donor and recipient cells

(A–C) Representative maximum-intensity projection of confocal images of (A) CTLA4-TagGFP-expressing T cells, (B) CD80-TagRFP-expressing CHO cells and (C) their interaction after 2 h co-culture. Cell membranes were stained with WGA-Lectin CF405S.

Possible solution

In order to reliably track molecule transfer between donor and recipient cells the ability to clearly distinguish between both cell types is absolutely crucial. Differences between donor and recipient cells in terms of size, morphology and adherence to the culture dish surface can be helpful in this regard, but are often not sufficient. One option is to stain one of the two cell types with dyes like Carboxyfluorescein succinimidyl ester (CFSE) or Cell trace violet (CTV) prior to co-culture. As these dyes are usually not transferred between donor and recipient cells, they facilitate the distinction of both cell types.

Another frequently used option to distinguish between donor and recipient cells is antibody-based staining of differentially expressed molecules (e.g., so called "lineage-markers"). Importantly, when using this approach, we do not recommend the use of trypsin or other proteases to detach cells upon harvesting. Such treatment can cleave off cell surface molecules and, hence, severely compromise subsequent staining by fluorescent antibodies. If detachment of cells is desired, one may try to use PBS without Ca^{2+}/Mg^{2+} supplemented with EDTA.

In general, combining several independent parameters to discriminate donor and recipient cells (e.g., size and lineage marker) improves distinction of donor and recipient cells.

Problem 2

The ligand of interest binds to different types of receptors on recipient cells.

Possible solution

Some ligands are able to bind to different types of receptors. In such situations, the expression levels and affinity of the competing receptors will determine their access to the shared ligand. If such competing receptors are co-expressed on recipient cells, this can make it difficult to study

trogocytosis by one of these receptors. Potential strategies to overcome such lack of ligand-exclusivity are antibody-mediated blockade or gene disruption of competing receptors (leaving all ligand to non-blocked / non-gene-disrupted receptors).

Problem 3

Recipient cells of interest express both receptor and corresponding ligand studied.

Possible solution

If in addition to expressing the receptor for the ligand of interest, recipient cells also synthesize the ligand themselves, this can compromise the validity of trogocytosis assays, as it makes it difficult to distinguish trogocytosed from endogenously synthesized ligands. To overcome this, one may disrupt the gene encoding the ligand in recipient cells (e.g., via Crispr/Cas9-based approaches). Alternatively, one may engineer donor cells to express ligands covalently tagged with fluorescent proteins at their cytoplasmic domain.[11] In such systems, the transfer of the fluorescent protein mirrors trogocytosis of the ligand. Both transient transfection and (retro-/lenti-)viral transduction of donor cells with fluorescently-tagged ligands can be used. Along this line, it can be very useful to generate transgenic donor cell lines stably expressing fluorescent ligands, because such lines improve the reproducibility of results across experiments (compared to the highly variable transgene expression levels observed upon transient plasmid transfection).

Problem 4 (referring to protocol step 46)

Trogocytosis is readily detectable by flow cytometry, but hard to observe by fluorescence microscopy.

Possible solution

Obviously, laser lines and filter sets of the detection system should match the wavelengths of maximum excitation and emission of the fluorescent protein chosen. However, even when this is the case, it can be more challenging to detect trogocytosis by microscopy than by flow cytometry. This is because most flow cytometers have built-in photomultipliers, which are usually not present in microscopes. These photomultipliers enable flow cytometers to detect signals across very large dynamic ranges, which makes them considerably more sensitive than most fluorescence microscopes. Furthermore, the longer exposure times used for microscopy can result in considerable bleaching of fluorochromes (particularly but not only in live cell video microscopy experiments). Based on these considerations, for generating fluorescent protein-fusions of proteins of interests it is advisable to choose very bright and photostable monomeric fluorescent proteins that can be easily distinguished from autofluorescent background signals (e.g., mTFP1, mRuby3, Gamillus, mScarlet, etc). Further information on these and many other fluorescent proteins can be found in FPbase.[12] For antibody-based readouts it is worth to note, that some bright fluorophores that are frequently used for flow cytometry do not work equally well in immunofluorescence microscopy, because they are not very photostable (e.g., PE and APC).

Another aspect that is particularly important for time-lapse live cell imaging is phototoxicity due to prolonged illumination times, which can lead to death of the cells imaged. In general, light of lower wavelengths is more energetic and therefore phototoxic, than of higher wavelengths. Based on this, fluorescent proteins with higher wavelengths excitation maxima (i.e., those excitable by red rather than ultraviolet or blue laser lines) are generally better suited for such applications.

Furthermore, the efficiency of trogocytosis depends on the cell surface expression level of the receptor as well as its affinity to the ligand. For low affinity interactions or lowly expressed receptors, this can result in the transfer of only a small fraction of ligands expressed on donor cells. If in this case one adjusts microscope settings based on ligand expression level on donor cells, signals derived from trogocytosed ligands in recipient cells may fall below the detection limit and give the impression that no trogocytosis occurred. To circumvent this, it may be necessary to adjust microscopy settings in a way leading to overexposure of donor cell fluorescent signal. Alternatively, one may consider to physically separate donor and recipient cells prior to microscopic analysis (e.g., by flow cytometric cell sorting).

Problem 5

Within one experiment, the rate of trogocytosis appears to decrease over time.

Possible solution

Some receptors have been shown to endocytose ligands upon trogocytosis (Huang et al., 1999) and endocytosed molecules can be rapidly shuttled to lysosomes, where they are degraded. If ligands available for trogocytosis are more rapidly trogocytosed and degraded by the recipient cells than they are produced by the donor cells, in time-course experiments this will appear as a continuous decline in the amount of trogocytosed ligands in recipient cells. One possible solution is to decrease the recipient to donor cell ratio, as this increases ligand availability and at the same time decreases demand by recipient cells. Alternatively, inhibitors of lysosomal function (e.g., bafilomycin A) may be added to the co-culture of donor and recipient cells. A potential caveat of such treatments is that while they are often tolerated by cells if only applied for a few hours, longer blockade of lysosomal function can be detrimental to cells.

Furthermore, even if endocytosed ligands are not directly shuttled to lysosomes, the acidic pH present in most endosomes[13] can substantially quench signals derived from commonly used fluorescent protein like GFP. This will lead to an underestimation of the amount of trogocytosed molecules. To avoid signal loss due to fluorescence quenching in endosomes, it is advisable to use acid-stable monomeric fluorescent proteins, e.g., mTagRFP,[14] Gamillus[15] or mRuby3.[16]

Problem 6

Across multiple experiments, the rate of trogocytosis becomes consecutively lower and lower.

Potential solution

One cause for this can be a decline in the expression level of fluorescent ligand transgenes in donor cells over time. We have observed that overexpression of transgenes can confer a fitness cost to cells. Hence, particularly when using bulk populations of transfected or transduced donor cells, those with low or absent transgene expression may have a proliferative advantage, resulting in their outgrowth. Over time this will negatively impact the signal-to-background ratio of trogocytosis assays.

To avoid this, we recommend the derivation of a clonal donor cell population when working with transfected or transduced donor cells, e.g., from flow cytometrically sorted single cell clones. Alternatively, a selection marker (e.g., puromycin-resistance gene) may be co-transfected/-transduced, which allows to deplete cells that do not express the desired transgene by addition of puromycin to the culture medium. While both approaches are helpful in maintaining stable transgene expression, they do not guarantee this. Hence, we have chosen to take along a control sample of donor cells only (i.e., without recipient cells) in each experiment, in order to monitor the expression levels of the fluorescent ligand.

Problem 7

"Negative control cells" that do not express the receptor of interest appear to trogocytose ligands of this receptor.

Possible solution

There are several possible causes for such an effect, one being "lack of ligand exclusivity" (see problem 2). Another cause can be "bystander" trogocytosis, which describes the phenomenon of trogocytosis mediated by one specific receptor-ligand interaction dragging along other cell surface molecules located nearby. In this scenario presumed "negative control cells" lack expression of the receptor A interacting with ligand B (which are the molecules of interest), but they may express receptor C interacting with ligand D. If now receptor C trogocytoses ligand D, ligand B may be dragged along if it was located closely to D. Circumventing this problem does not necessarily require the identification of the receptors and ligands mediating such by-stander trogocytosis, as exemplified in Figure 4. In this experiment, the ability of two murine T cell lines, which either express CD28 (but not CTLA4) or lack expression of both

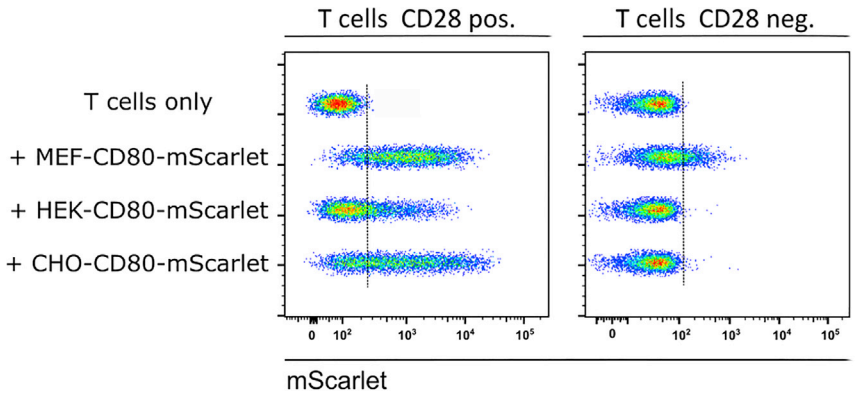


Figure 4. Concatenated flow cytometry plots of 58 αβ T cells either expressing CD28 or lacking expression of CD28 and CTLA-4 co-cultured for 2 h with donor cell lines expressing CD80-mScarlet

58 αβ T cells cultured without donor cells were used as a negative control.

CD28 and CTLA4 to trogocytose CD80 from three different cell lines expressing CD80-mScarlet was compared. As expected CD28-expressing T cells trogocytosed CD80 from all cell lines (albeit with variable efficacy). However, the presumed "negative control" T cells lacking CD28 and CTLA4 expression also trogocytosed CD80 from murine embryonic fibroblast (MEF) cells, but not from human embryonic kidney (HEK) and chinese hamster ovary (CHO) cells. This lack of "background" signal upon co-culture of murine T cells with HEK or CHO cells suggests that introducing a species barrier between donor and recipient cells may help to minimize undesired bystander trogocytosis.

Problem 8

After co-culture of donor and recipient cells only very weak signals receptors and/or ligands mediating trogocytosis are detected by antibody-based staining.

Possible solution

While this can be due to lysosomal degradation of receptor and/or ligand (see problem 5), another cause can be sterical hindrance between the receptor-ligand interaction and antibody-binding. If the antibody-binding site overlaps with the receptor-ligand interaction site, then antibody-binding can be compromised resulting in weaker or even absent staining (as exemplified in Figure 5). This plot depicts an anti-CD80 antibody staining of CD80-expressing MEFs with or without pre-incubation of cells with CTLA4-Fc versus PBS. Note that the signal intensity of the fluorescent antibody staining is reduced by almost 1 log upon interaction of CD80 with CTLA4-Fc. While this may not be a big problem in flow cytometry (due to is high dynamic range), such reduced signal intensity

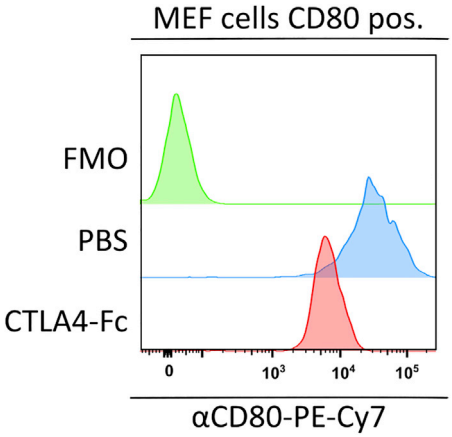


Figure 5. Competition between Anti-CD80 antibody staining and CTLA4-binding

Plot depicts representative histograms of anti-CD80 antibody surface staining of CD80-expressing MEF cells pre-incubated with PBS (control) or CTLA4-Fc. "FMO" denotes "fluorescence minus one" negative control sample.

can make a substantial difference in fluorescence microscopy. A solution for this kind of problem can be to generate recipient and donor cells expressing fluorescent protein fusions of receptor and corresponding ligand, respectively (as exemplified in Figure 3). Alternatively, one may use antibodies targeting a different domain of the protein of interest.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Jan C. Rohr (jan.rohr@uniklinik-freiburg.de) upon reasonable request.

Materials availability

Plasmids generated for this study will be deposited to a public repository.

Data and code availability

The authors declare that all data supporting the findings of this study are available within the article.

ACKNOWLEDGMENTS

We acknowledge Jan Bodinek for cell sorting and Hanna Hepp for the photographies of density gradient centrifugation samples. We are grateful to Peter Aichele and Stephan Ehl for discussions and support. Funding for this research was provided by German Research Foundation (DFG) grant no. RO 4120/2-1 and RO 4120/3-1 (to J.C.R.), FOR2123/P02 (to N.B.), and SFB1160 (to J.C.R.).

AUTHOR CONTRIBUTIONS

Conceptualization, A.Z., J.C.R.; Methodology, S.Z., A.Z., J.C.R., T.L.; Investigation, S.Z., A.Z., T.W., T.L.; Funding acquisition, J.C.R., N.B.; Supervision, J.C.R., N.B.; Writing – original draft, A.Z., S.Z., J.C.R.; Writing – review & editing, A.Z., J.C.R., T.L., N.B., T.W., S.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Zenke, S., Palm, M.M., Braun, J., Gavrilov, A., Meiser, P., Böttcher, J.P., et al. (2020). Quorum Regulation via Nested Antagonistic Feedback Circuits Mediated by the Receptors CD28 and CTLA-4 Confers Robustness to T Cell Population Dynamics. Immunity *52*, 313–327.e7.

2. Bettadapur, A., Miller, H.W., and Ralston, K.S. (2020). Biting off what can be chewed: trogocytosis in health, infection, and disease. Infect. Immun. *88*, 009300-19.

3. Dhainaut, M., and Moser, M. (2014). Regulation of immune reactivity by intercellular transfer. Front. Immunol. *5*, 112.

4. Joly, E., and Hudrisier, D. (2003). What is trogocytosis and what is its purpose? Nat. Immunol. *4*, 815.

5. Vanherberghen, B., Andersson, K., Carlin, L.M., Nolte-'t Hoen, E.N.M., Williams, G.S., Höglund, P., and Davis, D.M. (2004). Human and murine inhibitory natural killer cell receptors transfer from natural killer cells to target cells. Proc. Natl. Acad. Sci. USA *101*, 16873–16878.

6. Brown, K., Sacks, S.H., and Wong, W. (2008). Extensive and bidirectional transfer of major histocompatibility complex class II molecules between donor and recipient cells in vivo following solid organ transplantation. FASEB J. *22*, 3776–3784.

7. Huang, J.F., Yang, Y., Sepulveda, H., Shi, W., Hwang, I., Peterson, P.A., Jackson, M.R., Sprent, J., and Cai, Z. (1999). TCR-mediated internalization of peptide-MHC complexes acquired by T cells. Science *286*, 952–954.

8. Groettrup, M., Baron, A., Griffiths, G., Palacios, R., and von Boehmer, H. (1992). T cell receptor (TCR) beta chain homodimers on the surface of immature but not mature alpha, gamma, delta chain deficient T cell lines. EMBO J. *11*, 2735–2745.

9. Puck, T.T., Cieciura, S.J., and Robinson, A. (1958). Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. J. Exp. Med. *108*, 945–956.

10. Qureshi, O.S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E.M., Baker, J., Jeffery, L.E., Kaur, S., Briggs, Z., et al. (2011). Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science *332*, 600–603.

11. Crivat, G., and Taraska, J.W. (2012). Imaging proteins inside cells with fluorescent tags. Trends Biotechnol. *30*, 8–16.

12. Lambert, T.J. (2019). FPbase: a community-editable fluorescent protein database. Nat. Methods *16*, 277–278.

13. Maxfield, F.R., and Yamashiro, D.J. (1987). Endosome acidification and the pathways of receptor-mediated endocytosis. Adv. Exp. Med. Biol. *225*, 189–198.

14. Merzlyak, E.M., Goedhart, J., Shcherbo, D., Bulina, M.E., Shcheglov, A.S., Fradkov, A.F., Gaintzeva, A., Lukyanov, K.A., Lukyanov, S., Gadella, T.W.J., and Chudakov, D.M. (2007). Bright monomeric red fluorescent protein with an extended fluorescence lifetime. Nat. Methods *4*, 555–557.

15. Shinoda, H., Ma, Y., Nakashima, R., Sakurai, K., Matsuda, T., and Nagai, T. (2018). Acid-tolerant monomeric GFP from Olindias formosa. Cell Chem. Biol. *25*, 330–338.e7.

16. Bajar, B.T., Wang, E.S., Lam, A.J., Kim, B.B., Jacobs, C.L., Howe, E.S., Davidson, M.W., Lin, M.Z., and Chu, J. (2016). Improving brightness and photostability of green and red fluorescent proteins for live cell imaging and FRET reporting. Sci. Rep. *6*, 20889.