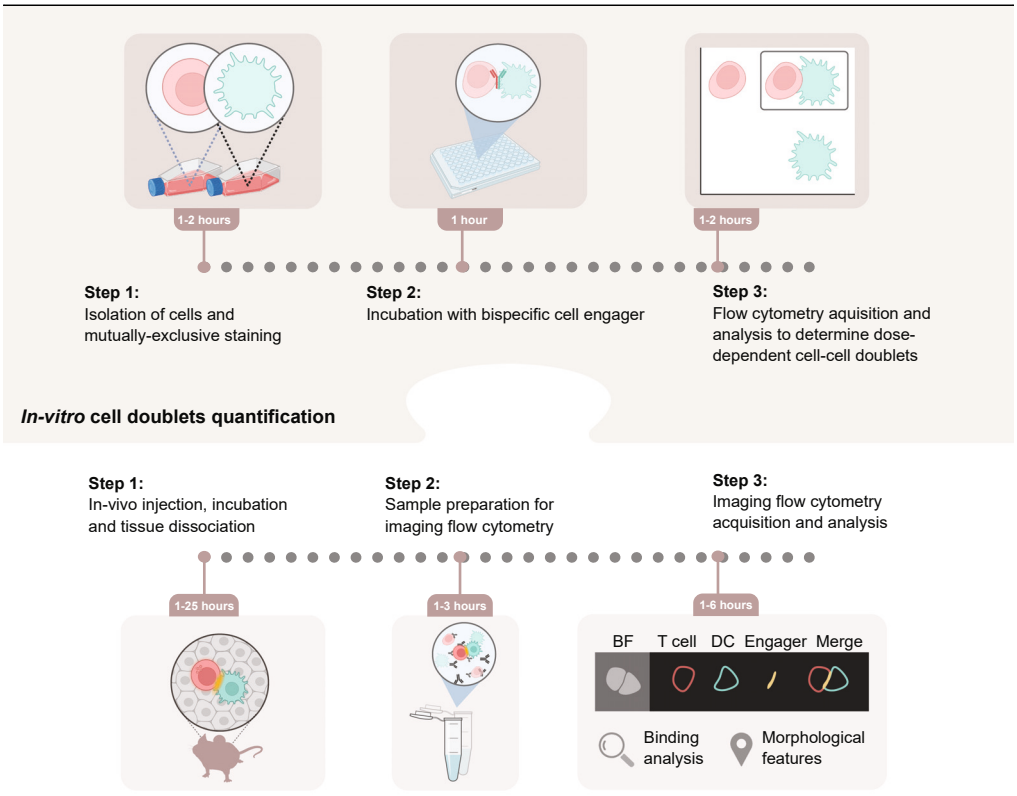
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for assessing murine cell doublet engagement and subsequent effects using flow cytometry and imaging flow cytometry



Physical interactions between two immune cells or between immune and cancer cells play a major role in shaping the immune response in the tumor microenvironment, making them prime therapeutic targets for bispecific engagers. Here, we present a protocol for assessing murine cell doublet engagement and subsequent effects using flow cytometry and imaging flow cytometry. We describe steps for identifying bispecific cell engager antibodies at the cell-cell interface, doublet quantification, and characterizing cellular protein morphology and processes within the doublet.

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

Yuval Shapir Itai, Ziv Porat, Rony Dahan

yuval.shapir@weizmann.ac.il (Y.S.I.)
rony.dahan@weizmann.ac.il (R.D.)

Highlights

Two approaches for determining physical interactions between two cells

In vitro system and flow cytometry protocol for cell binding analysis

Imaging flow cytometry protocol for engager localization at the cell-cell interface

Applications to assess cell activation, internalization, synapse formation, or death

Shapir Itai et al., STAR Protocols 5, 103152 December 20, 2024
https://doi.org/10.1016/j.xpro.2024.103152

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for assessing murine cell doublet engagement and subsequent effects using flow cytometry and imaging flow cytometry

Yuval Shapir Itai,[1,3,*] Ziv Porat,[2] and Rony Dahan[1,4,*]

[1]Department of Systems Immunology, Weizmann Institute of Science, Rehovot 7610001, Israel

[2]Flow Cytometry Unit, Life Science Core Facility, Weizmann Institute of Science, Rehovot 7610001, Israel

[3]Technical contact

[4]Lead contact

*Correspondence: yuval.shapir@weizmann.ac.il (Y.S.I.), rony.dahan@weizmann.ac.il (R.D.)
https://doi.org/10.1016/j.xpro.2024.103152

SUMMARY

Physical interactions between two immune cells or between immune and cancer cells play a major role in shaping the immune response in the tumor microenvironment, making them prime therapeutic targets for bispecific engagers. Here, we present a protocol for assessing murine cell doublet engagement and subsequent effects using flow cytometry and imaging flow cytometry. We describe steps for identifying bispecific cell engager antibodies at the cell-cell interface, doublet quantification, and characterizing cellular protein morphology and processes within the doublet.

For complete details on the use and execution of this protocol, please refer to Shapir Itai et al.[1]

BEFORE YOU BEGIN

Immune cell communication is governed by cell-cell interactions, which may result in the modulation of the immune cells within the interaction or with their surrounding cells and stroma. Bispecific engagers are a family of antibodies designed to modulate the interactions of the immune cells with each other, or with cancer cells *in vivo*. This protocol describes two approaches to evaluate dual-cell engagement using flow-based methods which were used previously in our laboratory and are described in Shapir Itai et al. Each approach can be done at a separate time point, and there is no necessity to execute both methods. The read-out from each protocol is different, and their combination will allow a more thorough investigation of cell-cell engagement. This protocol is optimized for HEK293 cells, lymphocytes, and myeloid cells from mouse lymph nodes. Nevertheless, it can be further optimized for a variety of cell types. Results using a bispecific T cell/dendritic cell (DC) immune cell engager (BiCE) are shown, however this protocol can be used using other cell engagers or to measure various markers and cell states within cell-cell doublets in principle.

A list of all reagents required for this protocol is described in the key resources table section. All reagents and buffers required should be prepared in advance.

Institutional permissions

The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Weizmann Institute of Science. Researchers interested in performing this protocol must obtain prior permission from their relevant institution.

Sample preparation for in-vitro cell doublets assessment by flow cytometry assay

Timing: Timing may vary, depending on the availability of the cell lines

The in-vitro cell doublets assessment flow cytometry assay is able to quantify the dual cell-binding of two cell types to each other by an increasing dose of bispecific antibody engager. The assay is based on flow cytometry gating using mutually exclusive markers. To execute this protocol, first prepare single-cell suspensions of both cell types.

An example of such is shown in Shapir Itai et al., using HEK293 cells overexpressed with PD-1 or CLEC9A, incubated with a PD-1/CLEC9A bispecific engager.[1]

1. If the creation of new cell lines is needed, transfect two populations of HEK293 cells with expression plasmids containing the targets of interest using the manufacturer's protocol. Otherwise, obtain two cell lines, each expressing one target protein of interest.
   a. Culture the cells separately to obtain roughly 10 million cells of each cell line.
   b. Remove medium from culture dish by gentle aspiration.
   c. Wash cells twice with 1× PBS.
   d. Add enough cell dissociation buffer to completely cover the cells (see here for recommended volumes).
   e. Incubate at 37°C for 5 min or until cells have completely detached.
   f. Add medium to stop the dissociation reaction, at 4× volume of the dissociation.
   g. Centrifuge at 300 × *g* for 5 min at 20°C.
   h. Aspirate and resuspend in 5 mL of 1× PBS.
   i. Pass through 70 μm strainer to obtain single-cell suspensions.
   j. Determine the concentration of viable cells and keep the cells on ice.

⚠ CRITICAL: It is recommended to dissociate the cells using cell dissociation buffer or similar, as standard trypsin may alter cell surface antigens before flow cytometry.

Note: The cell doublets flow cytometry assay can also be extended to isolated primary cells. In this case, each cell population must exclusively express only one of the targets.

Sample preparation for in-vivo imaging flow cytometry assay

Timing: Timing may vary, depending on the cell types, location of engagement, and antibody characteristics

The in-vivo imaging flow cytometry assay is able to quantify the dual-binding of two cell types to each other by the injection of a bispecific engager in-vivo at a single dose and assess the morphology and localization of the bispecific antibody and additional target proteins and cellular states within the cell-cell doublet. The cell types must be identifiable using a mutually exclusive gating strategy. Assessing the cell-cell interactions using imaging flow cytometry allows a high-throughput method to identify doublets of rare cell types and assess their morphological features. To execute this protocol, first label and inject the bispecific antibody to the desired mouse model.

2. If a bispecific cell engager is to be injected, first label the antibody using the SAIVI Rapid Antibody Labeling Kit (Invitrogen) using the manufacturer's protocol (linked here).
   a. If performing cell-engagement in-vivo, inject the labeled cell engager to the animal.

⚠ CRITICAL: The SAIVI Rapid Antibody Labeling Kit may be substituted with other antibody labeling kits. If intending to inject the labeled engager in-vivo, it is essential to use a kit suitable for animal injection, such as this one.

Note: This step may also be performed ex-vivo by applying the antibody to a single-cell suspension of the target cell mix and incubating in-vitro (as described here in steps 2–4 in step-by-step method details).

b. Incubation times may vary depending on the characteristics of the engager. In our example, 500 μg of the bispecific engager was injected intraperitoneally to tumor-bearing mice, and draining lymph nodes were collected after 24 h.[1]

3. Following in-vivo incubation, harvest the target organ using standard animal care procedures, and carefully dissociate the cells.

Note: Dissociation may require careful calibration to attain a single-cell suspension without disrupting cell-cell doublets (see troubleshooting).

4. Strain using a 70 μm cell strainer and keep on ice.

⚠ CRITICAL: Do not vortex cell doublets throughout the protocol. If necessary, pipette gently.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies (will vary depending on cell populations of interest)		
Anti-mouse PD-1/CLEC9A BiCE (RMP1-14/10B4)	Shapir Itai et al.[1]	N/A
Anti-mouse BV510-conjugated MHCII (clone M5/11415.2) (diluted 1:200)	BioLegend	Cat#107635; RRID: AB_2561397
Anti-mouse PE/Cy7-conjugated CD3 (clone 17A2) (diluted 1:200)	BioLegend	Cat#100219; RRID: AB_1732068
TruStain FcX (anti-mouse CD16/32) (clone 93) (diluted 1:25)	BioLegend	Cat#101319; RRID: AB_1574973
PE AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG (diluted 1:200)	Jackson ImmunoResearch	Cat#109-116-170; RRID: AB_2337681
Chemicals, peptides, enzymes, and recombinant proteins		
PBS (phosphate-buffered saline)	Sigma-Aldrich	Cat#D8537
Ethylenediaminetetraacetic acid disodium salt solution (EDTA)	Sigma-Aldrich	Cat#03690
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A3294
Cell dissociation buffer, enzyme-free, PBS	Gibco	Cat#13151014
DMEM (Dulbecco's modified Eagle's medium)	Gibco	Cat#41965-039
Fetal bovine serum, certified, South America (FBS)	Gibco	Cat#12657-029
Penicillin-Streptomycin solution	Sartorius	Cat#03-031-1B
CellTrace Violet Cell Proliferation Kit	Invitrogen	Cat#C34557
CFSE Cell Division Tracker Kit	BioLegend	Cat#423801
7-AAD viability staining solution	BioLegend	Cat#420403
LIVE/DEAD Fixable Violet Dead Cell Stain Kit	Invitrogen	Cat#L34955
Critical commercial assays		
SAIVI Alexa Fluor 647 Antibody Labeling Kit	Invitrogen	Cat#S30044
Experimental models: Organisms/strains		
Mouse: C57BL/6JOlaHsd (8–12 weeks mice of both sexes may be used)	Envigo	N/A
Experimental models: Cell lines		
Cancer cell line: B16F10	ATCC	Cat# CRL-6475
Software and algorithms		
GraphPad Prism version 9	Prism	RRID: SCR_002798
FlowJo Software version 10.6.2	FlowJo, LLC	RRID: SCR_008520
Acquisition software – INSPIRE version 200.1.681.0	Amnis/part of Luminex	https://www.luminexcorp.com/imagestreamx-mk-ii/#software
Analysis software – IDEAS version 6.3	Amnis/part of Luminex	https://www.luminexcorp.com/imagestreamx-mk-ii/#software

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
CytoFLEX flow cytometer	Beckman Coulter	N/A
ImageStreamX markII	Amnis/part of Luminex	N/A

Alternatives: All reagents can be substituted by similar alternatives from other companies. The antibodies detailed are those that have been used in our example but can be adapted to fit one's specific needs. The protocol is specifically tailored to use by the ImageStreamX markII and its acquisition and analysis software.

MATERIALS AND EQUIPMENT

DMEM Medium		
Reagent	Final concentration	Amount
DMEM	N/A	445 mL
Heat-inactivated FBS	10%	50 mL
Pen/Strep solution (10,000 units/mL)	1%	5 mL
Total	**N/A**	**500 mL**
Prepare sterile and filter through 0.45 μm membrane. Store at 4°C for up to 1 month.		

FACS Buffer		
Reagent	Final concentration	Amount
PBS 1×	N/A	498 mL
EDTA 0.5 M	2 mM	2 mL
BSA	0.5%	2.5 g
Total	**N/A**	**500 mL**
Prepare sterile and filter through 0.45 μm membrane. Store at 4°C for up to 3 months.		

STEP-BY-STEP METHOD DETAILS

In-vitro cell doublets assay for flow cytometry

In this section, we describe the protocol for the preparation, dual-binding incubation, and analysis of cell-cell doublets using conventional flow cytometry (following the appropriate sample preparation detailed earlier). This protocol can be used to assess the dual cell-binding ability of a bispecific antibody or similar reagent, titered at different antibody concentrations (Figure 1A).

In-vitro cell doublets assay for flow cytometry

Timing: 2–3 h

1. Stain each HEK293 cell population in a unique color for simple discrimination via flow cytometry:
   a. Centrifuge cell solutions from each cell population at 300 × *g* at 4°C for 3 min.
   b. Resuspend cell pellet up to 10^7 cells in each staining solution:
      i. **CellTrace Violet** (CTV) – Population A: Dilute 1 μL CellTrace in 5 mL 1× PBS in a 15 mL tube to obtain a 1:5,000 dilution. Resuspend cell pellet in entire volume.
      ii. **CFSE** – Population B: Dilute 1 μL CFSE in 5 mL 1× PBS in a 15 mL tube to obtain a 1:5,000 dilution. Resuspend cell pellet in entire volume.

Note: To lower CFSE staining intensity, we first diluted the CFSE stock solution 1:5 in DMSO, resulting in a final 1:25,000 dilution. Staining intensity/flow cytometer laser voltage may need to be tested and adapted prior to the experiment to obtain optimal acquisition of the cells.

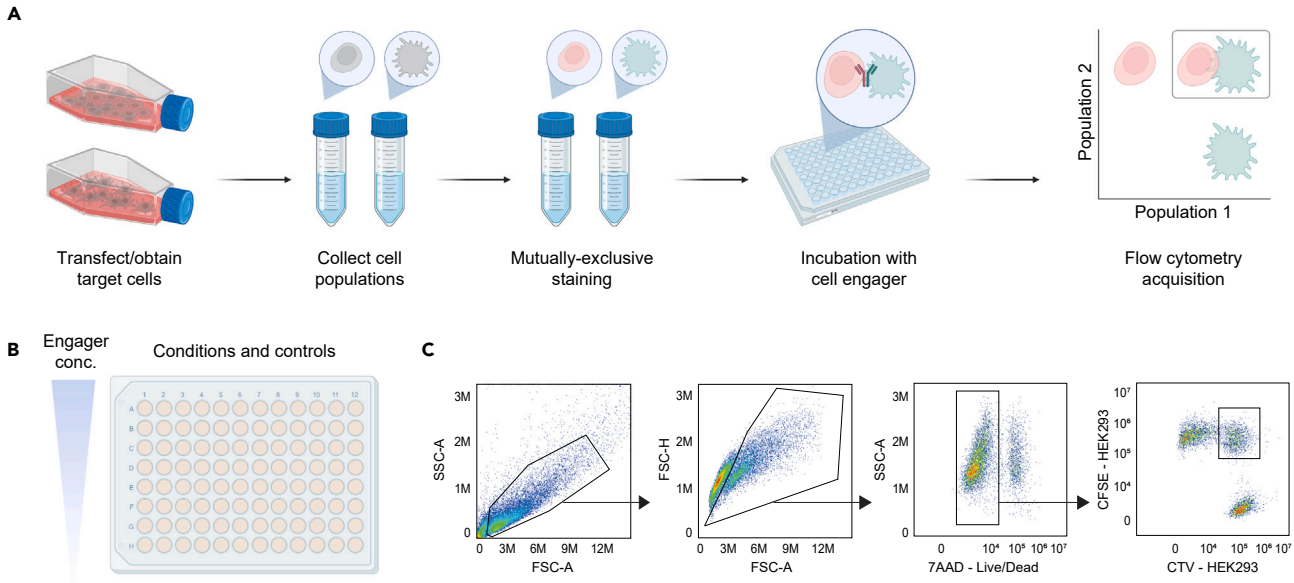


Figure 1. In-vitro dual cell binding assessment for flow cytometry

(A) Illustration depicting the main stages of the expected workflow.

(B) Suggestion for 96-well flow cytometry plate layout, with bispecific cell engager titration.

(C) Gating strategy for dual-cell engagement using mutually-exclusive cell staining strategy, adapted with permission from Shapir Itai et al.

c. Incubate at 37°C for 20 min in the dark.
d. Centrifuge cell solutions at 300 × *g* at 4°C for 3 min.
e. Wash twice in 15 mL of DMEM medium using the same parameters as above, waiting 5 min between washes.
f. Wash once more in 15 mL FACS buffer, resuspend in 5 mL FACS buffer, and count the cells from each cell population. Keep on ice protected from light.

Note: The CellTrace/CFSE labeling protocol has been calibrated for up to 10 million cells from each cell type. If more cells are desired, it is recommended to scale up the staining quantities.

Optional: Instead of labeling the cells using CellTrace/CFSE labeling at this stage, cells can be labeled prior to flow cytometry acquisition by traditional FACS staining using mutually exclusive markers.

2. Plate cells from both populations at a 1:1 ratio in a 96-well plate (Thermo Scientific Nunc U-shaped bottom 96-well plate or similar), using approximately 150,000 cells of each type in 150 µL FACS buffer.

Note: We used a 96-well plate for this assay, and a flow cytometer with a plate reader. The protocol can be modified for FACS tubes by scaling up all volumes appropriately.

Optional: If using cells dissociated ex-vivo (such as cells from mouse lymph nodes, spleens, etc.), we recommend adapting cell quantities to reach an approximate 1:1 ratio, either by separating your population of interest using a commercial kit prior to this step, or by altering cell quantities according to the relative quantity of the population of interest within the cell mix. If the cell population is rare within the mix, cell quantities may be increased.

3. Dual cell binding:
   a. Add bispecific antibody in a 50 μL solution in FACS buffer using a multipipette and gently resuspend the cells. In our example, we titered a 3 μg/mL antibody solution using 1:2 serial dilutions spanning 8 concentrations (Figure 1B).
   b. Incubate for 1 h on ice in the dark.

   Note: The optimal antibody concentration and incubation time will depend on the characteristics of your chosen cell engager and tested cell populations. While these conditions were proven successful for a variety of ex-vivo lymphocyte populations and overexpressing HEK293 cells using bispecific human IgG1-based antibodies, they may need to be calibrated for your application of choice.

4. Gently wash the cells:
   a. Centrifuge the plate at 300 × *g* at 4°C for 3 min.
   b. Remove the supernatant by flipping the plate over the sink with a sharp hand movement.
   c. Resuspend gently in 150 μL FACS buffer.
   d. Repeat a-c, for 2 total washes.

   Optional: If performing antibody staining for each cell population, the bispecific antibody, or additional cell markers, add the following sub-steps:

   e. Add TruStain FcX (anti-mouse CD16/32) Antibody (Fc-block) to the cells 1:25 in 25 μL FACS buffer and gently resuspend. Incubate for 15 min in the dark at 20°C–25°C.
   f. Meanwhile, prepare the extracellular antibody staining solution. Dilute the antibodies at 2× of their desired concentration in 25 μL FACS buffer.

   Note: Remember to use mutually exclusive defining markers for each cell population in the doublets.

   g. Add 25 μL antibody staining mix on top of Fc-block incubated samples, to result in a 1× staining concentration in 50 μL of final volume.
   h. Incubate for 30 min in the dark, on ice.

   Note: If required, the bispecific antibody can also be stained at this step. We used Jackson ImmunoResearch's PE conjugated-aHuman IgG to bind the human IgG region of our bispecific antibody.[1]

   i. Following incubation with the antibody staining mix, repeat washing (step 4 a-d).
5. Resuspend in 150 μL of FACS buffer with 3 μL of 7AAD (or similar) for viability staining.
6. Store on ice protected from light until ready for flow cytometer acquisition.

Flow cytometry acquisition and analysis

Timing: 1–3 h

7. Turn on the flow cytometer and set up the relevant lasers.

   Note: It is recommended that you compensate your chosen panel beforehand if you are using multiple markers.

8. Using flow cytometry, run the samples at a steady flow rate, reading up to 135 μL per sample (to prevent loading air into the flow cell).

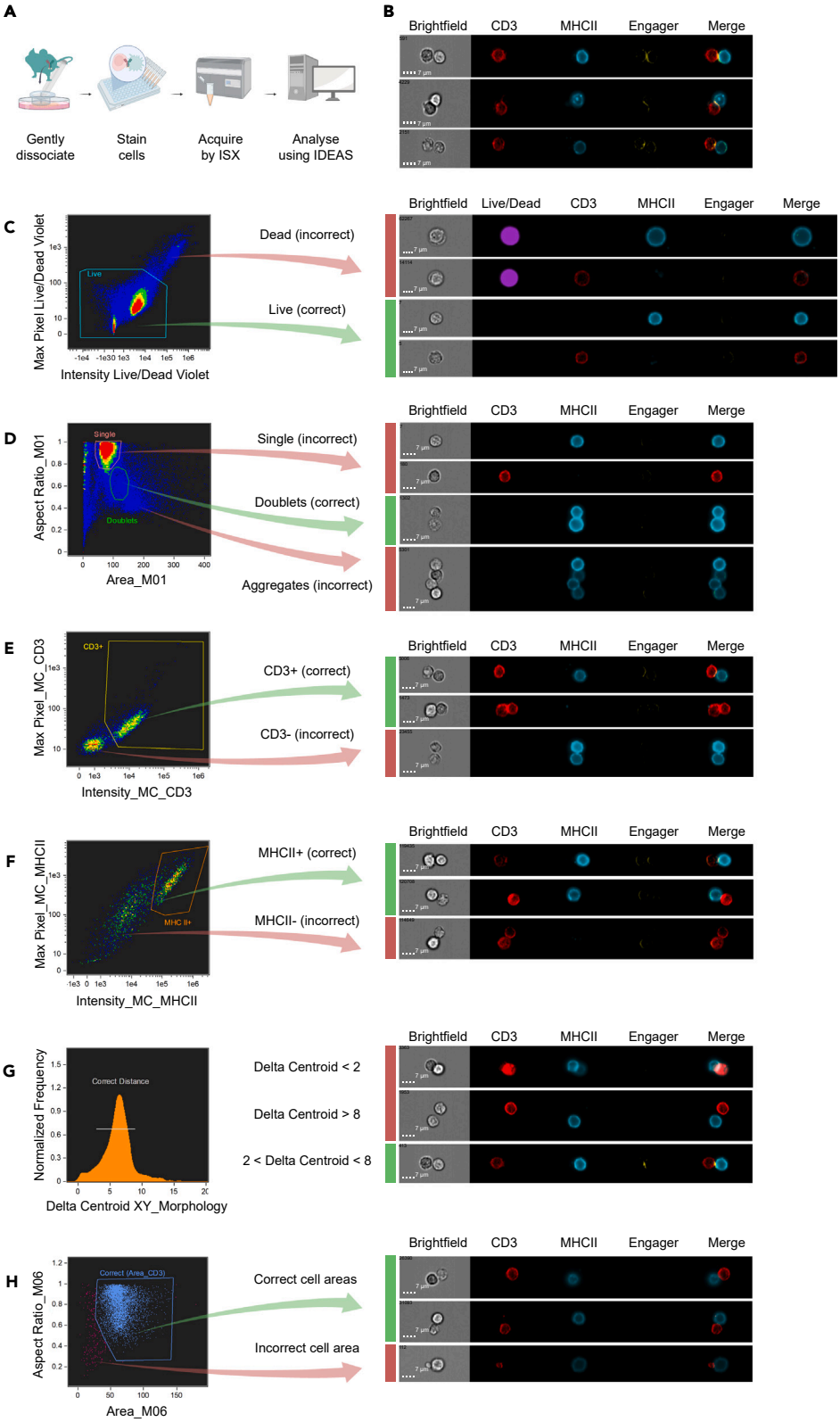

A
Gently dissociate
Stain cells
Acquire by ISX
Analyse using IDEAS
B
Brightfield
CD3
MHCII
Engager
Merge
C
Max Pixel Live/Dead Violet
Intensity Live/Dead Violet
Live
Dead (incorrect)
Live (correct)
Brightfield
Live/Dead
CD3
MHCII
Engager
Merge
D
Aspect Ratio_M01
Area_M01
Single
Doublets
Single (incorrect)
Doublets (correct)
Aggregates (incorrect)
E
Max Pixel_MC_CD3
Intensity_MC_CD3
CD3+
CD3+ (correct)
CD3- (incorrect)
F
Max Pixel_MC_MHCII
Intensity_MC_MHCII
MHC II+
MHCII+ (correct)
MHCII- (incorrect)
G
Normalized Frequency
Delta Centroid XY_Morphology
Correct Distance
Delta Centroid < 2
Delta Centroid > 8
2 < Delta Centroid < 8
H
Aspect Ratio_M06
Area_M06
Correct (Area_CD3)
Correct cell areas
Incorrect cell area

Figure 2. Defining cell-cell doublets by imaging flow cytometry using two target populations

(A) Illustration depicting the main stages of the expected workflow.

(B) Exemplary experiment of CD3/MHCII cell-cell doublets formed by a labeled bispecific engager, as shown in Shapir Itai et al.

(C–H) Gating on strategy for correct cell-cell doublets. Gates were validated by manual inspection: green indicates correct gating, while red depicts incorrect examples. Live cells were selected (C), and gated on doublets (D). Then, CD3+ positive cells were selected (E), that were bound to MHCII+ cells (F). (G) Interacting CD3/MHCII cells were selected by eliminating overlapping or distanced cell doublets. (H) From them, cell doublets will appropriate cell areas were selected, to remove additional debris.

⚠ CRITICAL: To prevent the detachment of doublets, aggressive 'mixing' by the flow cytometer between each well is not recommended.

9. Gate on the target cell population using the expected SSC-A and FSC-A values.

⚠ CRITICAL: Do not perform doublet exclusion by using an FSC-H and FSC-A gate.

10. Following acquisition, gate for cell-cell doublets as shown in Figure 1C.
    a. Gate on the target cell population using the expected SSC-A and FSC-A gate, removing debris, etc.
    b. Gate on the doublet population, using the FSC-H and FSC-A gate.
    c. Gate on live cells, using the 7AAD stain.
    d. From here, the percent of CTV/CFSE double positive conjugates can be calculated.

Optional: As noted before, the gating strategy can be modified for ex-vivo dual binding assays. An example of such is shown on mouse splenocytes in Shapir Itai et al., with a detailed gating strategy. Additionally, different expression markers can be added to the antibody staining panel to investigate their specific expression on cells within the doublet.[1]

11. Using this data, the formation of doublets as a function of bispecific antibody concentration can be graphed.

Imaging flow cytometry of in-vivo cell-cell doublets

In this section, we describe the protocol for the preparation, image acquisition, and analysis of cell-cell doublets using the Image-Stream X instrument, as shown in Figure 2A (following the appropriate sample preparation detailed earlier).

Sample preparation for imaging flow cytometry of in-vivo doublets

Timing: 1–3 h

12. Plate an adequate number of cells per sample in a 96-well plate. For our example, an entire dissociated draining lymph node/sample was plated in each well.

Note: Remember to plate additional samples for single-stain compensation controls at this step.

Note: This can also be done in Eppendorf tubes/FACS tubes by scaling up washing and staining volumes, appropriately.

13. Add viability staining in 50 μL staining volume to the samples according to the manufacturer's protocol.

Note: In our example, 50 μL of Live/Dead Fixable Violet (diluted 1:1000 in PBS) was added, and cells were incubated for 10 min in the dark at 20°C–25°C.

Note: Create a single-stain control for the viability staining at this stage. Additionally, save several wells of unstained samples for single-stain controls for extra/intracellular staining according to your desired panel in subsequent steps.

⚠ CRITICAL: From this step, all incubations should be done in the dark to prevent photobleaching.

14. Gently wash the cells:
    a. Centrifuge the plate at 300 × *g* at 4°C for 3 min.
    b. Remove the supernatant by flipping the plate over the sink with a sharp hand movement.
    c. Resuspend gently in 150 μL FACS buffer.
    d. Repeat a-c twice, for 3 total washes.
15. Add Fc-block to the cells 1:25 in 25 μL FACS buffer and gently resuspend. Incubate for 15 min in the dark at 20°C–25°C.
16. Meanwhile, prepare the extracellular antibody staining solution. Dilute the antibodies at 2× of their desired concentration in 25 μL FACS buffer.

⚠ CRITICAL: Use mutually exclusive defining markers for each cell population in the doublets.

Note: Additional markers can be added according to the optimal setup of the Image-Stream X (ISX) machine.

17. Add 25 μL antibody staining mix on top of Fc-block incubated samples, to result in a 1× staining concentration in 50 μL of final volume. Incubate for 30 min in the dark, on ice.

Note: Include staining of single-stain controls in this step.

18. Gently wash the cells, by repeating step 14.

Optional: If the staining of intracellular proteins or actin is desired, fix and permeabilize the cells and continue to intracellular staining according to your desired protocol. Remember to add a single-stain control. Following intracellular staining, gently wash the cells, by repeating step 14.

Note: BioLegend's True-Nuclear Transcription Factor Buffer Set (Cat#424401) used according to the manufacturer's protocol, or fixation with 1.5% PFA followed by permeabilization with 0.1% triton-X100[2] have worked equally well for our purposes, but different fixation strategies may affect antibody staining intensity (see here). If necessary, calibrate fixation according to your desired staining strategy prior to your experiment.

19. Resuspend the cells in a final volume of 30 μL of FACS buffer and transfer to Eppendorf tubes for ISX acquisition.

Image acquisition by image-stream X

Timing: 1–3 h

20. Turn on the ISX, server, and acquisition computer. Log in to the acquisition computer.

Note: For a detailed explanation of how to use the instrument and analysis software, please refer to the user manual.

21. Start the acquisition software, INSPIRE. Once loaded, click on 'STARTUP', and make sure that all the calibrations and tests have passed (marked green).
22. Check the excitation/emission data for your dyes and turn on the relevant lasers. Set the lasers to maximal power.

    Note: In the example we provide (Figure 2B), we used the 561 nm laser (200 mW) for the PE-Cy7 (CD3) dye and 405 nm laser (120 mW) for the Live/Dead Violet, BV510 (MHC II), and 642 nm laser (150 mW) for AF647 (bsAb).

23. Turn on the relevant acquisition channels for your dyes.

    Note: These should include the bright-field channels (#1 and #9 for a two-camera instrument) and side-scatter channel (#6 or #12, depending on your dye combination).

    Note: In the example we provide, PE-Cy7 was acquired on channel 6, and Live/Dead Violet was read on channel 7, MHC II on Channel 8 and the bsAb on Channel 11.

24. Load your first sample: start with the sample that contains all dyes, which is expected to produce the highest staining intensity.
25. Create a scatter plot of the Area vs. Aspect Ratio of the bright-field channel. Gate on both single cells and doublets by including also higher-area, low-aspect-ratio cells.

    Optional: Create a histogram of Gradient_RMS of the bright-field channel (usually channel 1). Draw a linear gate that includes values between 50 and 90, to collect only cells that are focused.

    Note: Choosing focused cells could also be done in the analysis stage.

26. Create a 'Raw Max Pixel' histogram for each fluorescent channel, taken from the 'Single' gate.

    ⚠ CRITICAL: Make sure the values do not reach 4095, which indicates the camera is saturated. If they do, reduce the relevant laser intensity until the values are within the target range.

27. Collect at least 100,000 cells from each sample.

    Note: If the target population is rare, collect enough cells so that the final population contains at least 500 cells.

    Note: If the cell number is lower than expected, see here.

28. Maintain focus and cell position during the acquisition.

    Note: If the autofocus fails to maintain its focus, it could be disabled by going to Instrument → Advanced settings → Autofocus and disabling the 'Autofocus' option. Once the autofocus is disabled, the focus can be manually changed on the 'Focus' tab.

    Note: If the cell position in the flow is not centralized, it can be adjusted manually by moving its position on 'Centering'.

If additional troubleshooting is needed, see problem 4 in troubleshooting section.

29. After recording your samples, acquire single stained controls with the compensation wizard, using the same laser settings as those used for the experimental samples.

Note: If the compensation wizard fails, see problem 5 in troubleshooting section.

Image analysis by IDEAS

Timing: 1–3 h

30. Prepare the files for analysis.
    a. Start the IDEAS analysis software (preferably version 6.2 or higher).
    b. Open the first file and calculate the compensation using the single-stain files acquired, using the compensation wizard in IDEAS.
31. Gate on live, focused doublets of the desired cell populations.
    a. Draw a bivariate plot of the Intensity vs. Max Pixel of the Live/Dead Violet staining.
        i. Gate for viable cells negative for the staining (Figure 2C).
    b. Draw a bivariate plot of Area vs. Aspect Ratio of the bright-field image.
        i. Gate for single cells and for the doublet population (i.e., have higher area and lower aspect ratio).
        ii. Carefully adjust the gate by visual inspection according to the desired doublet population (Figure 2D).
    c. From the doublet population, draw a bivariate plot of Intensity vs. Max Pixel of the marker defining the first cell population (in this example, CD3).
        i. Gate on the positive population (Figure 2E).
    d. Based on this gated population, draw a bivariate plot of Intensity vs. Max Pixel of the marker defining the second cell population (in this example, MHC II).
        i. Gate on the positive population (Figure 2F).
    e. Create a morphology mask for each of the cell markers.
        i. Go to 'Analysis' → 'Masks' → 'New' → 'Function'.
        ii. Choose the 'Morphology' mask.
        iii. Select the relevant channels (In this example, CD3 in channel 6 and MHC II in channel 8).

Note: Utilizing the morphology mask allows a more accurate gating for true doublets.

    f. Further eliminate false doublets by determining the optimal range between the cells.
        i. Add the feature 'Delta Centroid XY' of the two markers.
        ii. Determine the optimal range by manual inspection, excluding single or overlapping cells (distance too short) and cells too far apart (distance too long).

Note: This feature calculates the distance between the two staining masks (in microns), so only two cells with the correct distance would be considered as doublets. In this example, CD3 and MHCII markers were used, and their optimal distance was between 2 and 8 (Figure 2G).

    g. Verify there is only one cell from each cell type.
        i. Draw a bivariate plot of the area vs. aspect ratio using the marker for each cell type.
        ii. Gate on single events (Figure 2H).
    h. Eliminate out-of-focus cells.
        i. Draw a bivariate plot using the Gradient RMS vs. Contrast features of the bright-field image.
        ii. Gate on focused cells.
        iii. Verify by visual inspection.

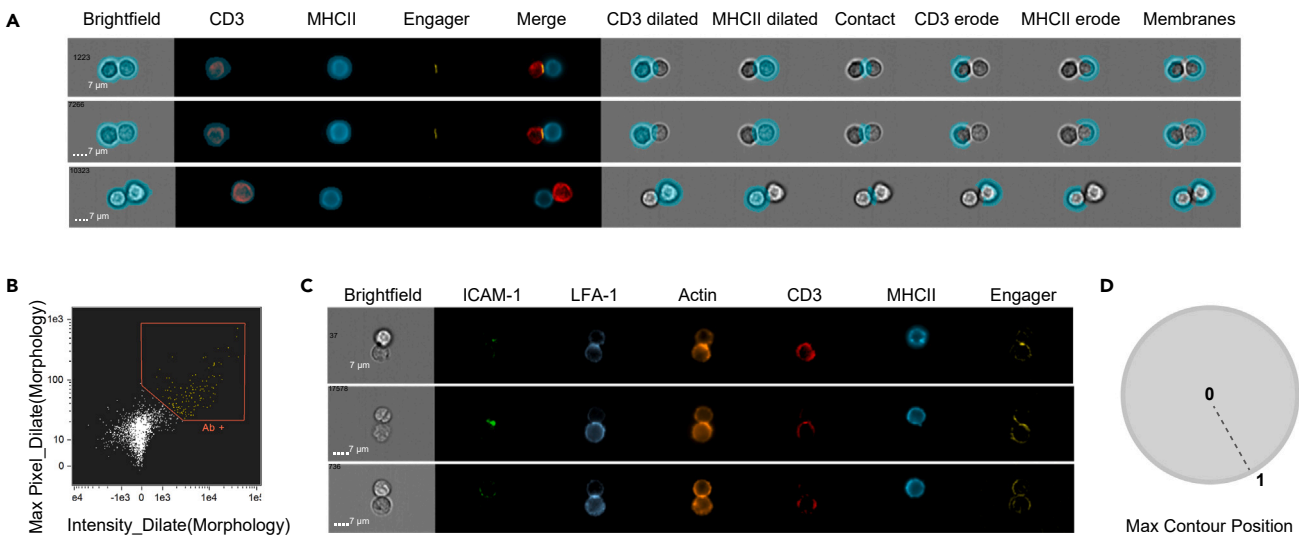


Figure 3. Analyzing morphological features within cell-cell doublets by imaging flow cytometry

(A) Analysis strategy to define the contract region between two cells within a doublet (middle) and their cell membranes (right).

(B) The intensity of the bispecific engager was calculated within the cell-cell contact region.

(C) Exemplary experiment with the addition of traditional immune synapse markers.

(D) The 'Max Contour Position' feature is demonstrated on a cell cartoon (gray).

32. Define the contact region between the doublets and analyze the target intensity within this region (Figure 3A, middle).
    a. To quantify the antibody distribution, define the different parts of the immune contact region.
        i. To define the contact region, dilate the morphology mask (created in subitem 30e) by going to 'Analysis' → 'Masks' → 'New' → 'Function'.
        ii. Choose the previously created morphology mask, click on the down arrow to select it, and click on 'Function'.
        iii. Use the 'Dilate' mask to extend it (in this example by 4 pixels).
        iv. Do the same for the other cell marker.
    b. Define the contact area between the two cells as the overlap between the two dilated masks and calculate the intensity of the antibody in this region.
        i. Go to 'Analysis' → 'Masks' → 'New'.
        ii. Choose one dilated mask, click on the down arrow, then click on the 'And' operator and choose the other dilated mask.

    Note: Using this mask, one can calculate the intensity of any marker within the contact area between the two cells. In our example, we calculated the antibody intensity within this region. Adding new features is done by going to 'Analysis' → 'Features' → 'New'.

        iii. Choose the intensity feature and the mask from the previous item, and the channel of the antibody (channel 11 in this example).
    c. Draw a bivariate plot of Intensity vs. Max Pixel of the antibody and gate on the positive population. Define the gate according to the relevant control sample, e.g., samples in which the antibody does not bind (Figure 3B).
33. Calculate the distribution of the target proteins in the contact region relative to the cell membranes (Figure 3A, right).
    a. First define the cell membranes:
        i. Use the 'Adaptive Erode' mask on the morphology mask of each of the cell types (in this example, we used 70%).

ii. Merge these two masks using the 'Or' Boolean operator and subtract the contact area mask from the merged mask using the 'And Not' operator.

b. Calculate the intensity of the antibody on the cell membranes (excluding the contact area) using the new mask created in step 33 aii.

c. To calculate the interface/membrane ratio:
   i. Go to 'Analysis' → 'Features' → 'New' → 'Combined'.
   ii. Choose the intensity feature of the antibody within the contact area from subitem 32c.
   iii. Select on the down arrow, add the division marker, and choose the feature from subitem 33b.

Note: This ratio can be used to show the accumulation of the target antibody within the contact area and can be compared between different treatments.

Optional: Traditional markers of the immune synapse can include Actin, LFA1, ICAM, Lck3, and others.[2,3] Accumulation of these markers within the immune synapse can be quantified in a similar manner (Figure 3C).

34. Save the analysis template.
35. Open the files recorded from treated samples using this template.
36. Apply the gating on all the other experimental datasets.

Optional: If desired, calculate the localization of key target proteins within the cells in the doublets.

To quantify the localization of the fluorescent signal, the 'Max Contour Position' feature can be used. It calculates the location of the contour in the cell that has the highest intensity concentration, mapped between 0 and 1 (0 being the geometrical center of the cell and 1 being the periphery, Figure 3D). This could be done on each cell separately within the doublet by using the mask of the cell of interest and can be used to quantify the internalization or translocation of a protein marker within the cell (e.g., antibody internalization, as shown in Shapir Itai et al.[1]).

Optional: Additional morphological information can be gained on a cell of interest within a doublet, for example:

Transcription factor translocation to the nucleus can indicate cell activation resulting from cell-cell interactions. This can be quantified by labeling the nucleus and calculating the 'Similarity' feature (i.e., log-transformed Pearson's Correlation Coefficient, a measure of the degree to which two images are linearly correlated within a masked region) between the nuclear mask and the transcription factor labeling.[4,5] This should be done only on the mask of the cell of interest within the doublet.

Apoptosis of an individual cell population within the doublets can also be quantified. Apoptotic cells are characterized by condensed DNA staining and higher contrast of the bright field.

a. Add a threshold mask of the 30% top intensity pixels of the DNA staining channel.
b. Choose this mask and make a combined mask using the 'AND' Boolean operator with the mask of the cell of interest.
c. Add the 'Area' feature of this combined mask. This defines the area of the DNA staining.
d. Calculate the contrast feature of the bright-field channel using the mask of the target cell.
e. Plot a bivariate plot of the area of the top intensity pixels of the DNA staining and the contrast, and gate on the apoptotic cells with high contrast and low DNA area. This determines the percentage of doublets in which the target cell is apoptotic.
f. Alternatively, other markers for apoptosis can be used.[6]

Cytotoxic granules can also be quantified to evaluate the cell-killing potential within a doublet.

a. The granules can be defined using a combination of the 'Peak' and 'Threshold' mask on the granule staining channel. This should be explicitly calibrated according to the intensity and the background of the staining used.
b. Then, the number, intensity and size of the granule scan be quantified.[7]

Cell classification: As you can utilize up to 10 fluorescent channels, you can label additional markers to increase your resolution of specific population identification.

EXPECTED OUTCOMES

The complementary protocols allow for the characterization of mutual cell engagement, providing insights into the interactions between two cell types. This includes understanding the extent and nature of binding between two cell populations, both *in vitro* and *in vivo*. While the outcomes of these protocols will vary and depend greatly on both the intrinsic binding properties of the cell engager and the concentration of the engager, if used, the target cell populations, their locations, and the chosen method of incubation, when using these protocols one can expect to gain a comprehensive understanding of physical cell engagement, ranging from the initial binding events to downstream cellular responses, with potential applications in immunotherapy, cancer research, and beyond. The quantification of doublet formation determines the frequency of dual binding events, as well as potentially provides information on binding kinetics and affinity. Utilizing imaging flow cytometry, the protocol facilitates visualization of cell-cell interactions at a single-cell level, to allow for the observation of binding dynamics, spatial organization at the cell-cell interface, and potentially the formation of specialized structures such as synapses. Beyond binding events, the protocol allows for the investigation of cellular responses following dual-cell engagement. This may include activation, internalization of cell engagers or target antigens, synapse formation, or induction of cell death pathways. The protocol offers the flexibility to compare the outcomes of different cell engagers or to measure various markers and cell states within cell-cell doublets. This enables comparative studies to assess the efficacy and specificity of different therapeutic agents or to investigate the functional consequences of dual cell engagement under different conditions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry data (fcs files) were generated on the CytoFLEX LX (Beckman Coulter). Flow cytometry analysis was performed using FlowJo v.10.6.2. Images from imaging flow cytometry experiments were acquired by ImageStreamX mark II (Amnis, Part of Luminex, Au. TX) using a 60× lens (NA = 0.9), and analyzed using IDEAS 6.3 software (Amnis, Part of Luminex, Au. TX). Graphs and additional analysis of data from flow and imaging cytometry was plotted using GraphPad Prism (GraphPad Software).

LIMITATIONS

Imaging flow cytometry (IFC) combines the robust, uniform, informative data of microscopy with the rapid, high-throughput, unbiased quantification of both the quantity and morphological features of individual cells collected automatically by the ImageStream system. IFC can acquire up to 10 fluorescent markers, in addition to the bright-field and side-scatter (dark field image). It is also easier to image non-adherent cells, and due to the large number of cells acquired, rare populations are easier to identify. Despite the numerous advantages, there are a few limitations to this approach.

The number of cells acquired is limited relative to conventional flow cytometry, as practically not more than a few hundred thousand cells can be acquired, compared with up to 100 million in conventional flow cytometry. This can be challenging when investigating very rare cell populations within the doublets, and can be overcome by acquiring only a specific cell population, and by fixing the cells to prevent cell death within long acquisition times. Another limitation compared with

microscopy is that multiple steps are needed to prepare the samples, as tissue digestion, staining, washings etc. These steps may disrupt the integrity of the doublets, thus requiring optimizing the preparation protocol. In addition, separating the tissue to its components does not allow investigating the doubles in their spatial context, or their interaction with the surrounding tissue.

TROUBLESHOOTING

Problem 1

A low number of doublets was identified in flow cytometry or ISX.

Potential solution

A low number of doublets may be due to four main reasons. First, it is important to assess the biological context of the experiment. Incubation times, organs, or target cell populations may need to be calibrated to reach an optimal number of doublets formed. Secondly, the dissociation technique of the organ investigated may harm the doublets, though too little dissociation may cause cell aggregates and prevent access to the doublets of interest. In our example, we mechanically dissociated lymph nodes using gentle handling techniques to locate our populations of interest. Other organs or cell types may need careful dissociation calibration and may require the use of additional enzymes, such as collagenase and DNase. Similarly, we recommend gentle pippeting and aspiration of the dissociated cells so as not to break the doublets while preparing the samples. Finally, a suboptimal gating strategy and insufficient staining (see Problem 2) may affect the ability to gate on correct doublets. Remember to pick mutually exclusive staining markers to distinguish between the populations and compensate your panel for each experiment.

Problem 2

Insufficient flow cytometry or ISX staining following fixation.

Potential solution

Some antibodies may not yield sufficient staining in PFA or methanol fixation. Before performing the full experiment, it is recommended to test that the antibodies of choice are suitable for the chosen fixation.

Problem 3

A low number of cells is acquired by ISX (step 27).

Potential solution

Cells may have been lost during sample preparation while they were washed. To minimize such events, aspirate the samples carefully leaving a minimal volume of residual supernatant to avoid cell loss. In addition, the protein content of the washing buffer may be increased (e.g., 1% BSA).

Problem 4

The focus and location of cells in the ISX are not stable (step 28).

Potential solution

Return the sample, vortex it, and load again. If the cell concentration is too high, you can dilute the samples. In case of a bubble or a partial clog, use the 'Purge Bubbles' script (located under 'Instrument' in the main menu).

Problem 5

The ISX compensation wizard may fail if too few cells are acquired in a certain channel (step 29). This may occur when staining rare markers or cell types.

Potential solution

If necessary, use a highly concentrated single-stain cell sample. It is also possible to stain a more highly expressed target in the desired color or to use staining beads, to ease the compensation process.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rony Dahan (rony.dahan@weizmann.ac.il).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contact, Yuval Shapir Itai (yuval.shapir@weizmann.ac.il).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

This study did not generate new datasets or code.

ACKNOWLEDGMENTS

R.D. is an incumbent of the Rina Gudinski Career Development Chair, funded by the European Research Council, Israel Cancer Research Fund, Israel Science Foundation, Moross Integrated Cancer Center, Flight Attendant Medical Research Institute, Dwek Institute for Cancer Therapy Research, Teva Pharmaceuticals, Center for Immunotherapy at the Weizmann Institute of Science, David E. Stone and Sheri Hirschfield Stone 75th Anniversary Fund, Rising Tide Foundation, Miel de Botton, Garvan-Weizmann Partnership donors, and Elie Hirschfeld and Dr. Sarah Schlesinger. Y.S.I. is supported by the National Forum of Bioinnovators by Teva. We would like to extend our sincere gratitude to the Weizmann Institute of Science's Life Sciences Core Facility for their valuable contributions. Figures were created with BioRender and Adobe Illustrator.

AUTHOR CONTRIBUTIONS

Y.S.I. and Z.P. performed the experiments and wrote the manuscript. R.D. supervised and funded the project and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Shapir Itai, Y., Barboy, O., Salomon, R., Bercovich, A., Xie, K., Winter, E., Shami, T., Porat, Z., Erez, N., Tanay, A., et al. (2024). Bispecific dendritic-T cell engager potentiates anti-tumor immunity. Cell *187*, 375–389.e18. https://doi.org/10.1016/j.cell.2023.12.011.

2. Markey, K.A., Gartlan, K.H., Kuns, R.D., MacDonald, K.P.A., and Hill, G.R. (2015). Imaging the immunological synapse between dendritic cells and T cells. J. Immunol. Methods *423*, 40–44. https://doi.org/10.1016/j.jim.2015.04.029.

3. Huppa, J.B., and Davis, M.M. (2003). T-cell-antigen recognition and the immunological synapse. Nat. Rev. Immunol. *3*, 973–983. https://doi.org/10.1038/nri1245.

4. Yissachar, N., Sharar Fischler, T., Cohen, A.A., Reich-Zeliger, S., Russ, D., Shifrut, E., Porat, Z., and Friedman, N. (2013). Dynamic Response Diversity of NFAT Isoforms in Individual Living Cells. Mol. Cell *49*, 322–330. https://doi.org/10.1016/j.molcel.2012.11.003.

5. Maguire, O., Collins, C., O'Loughlin, K., Miecznikowski, J., and Minderman, H. (2011). Quantifying nuclear p65 as a parameter for NF-κB activation: Correlation between ImageStream cytometry, microscopy, and Western blot. Cytometry A. *79*, 461–469. https://doi.org/10.1002/cyto.a.21068.

6. Sevilya, Z., Chorin, E., Gal-Garber, O., Zelinger, E., Turner, D., Avidor, B., Berke, G., and Hassin, D. (2018). Killing of latently HIV-infected CD4 T cells by autologous CD8 T cells is modulated by Nef. Front. Immunol. *9*, 2068. https://doi.org/10.3389/fimmu.2018.02068.

7. Gropper, Y., Feferman, T., Shalit, T., Salame, T.M., Porat, Z., and Shakhar, G. (2017). Culturing CTLs under Hypoxic Conditions Enhances Their Cytolysis and Improves Their Anti-tumor Function. Cell Rep. *20*, 2547–2555. https://doi.org/10.1016/j.celrep.2017.08.071.