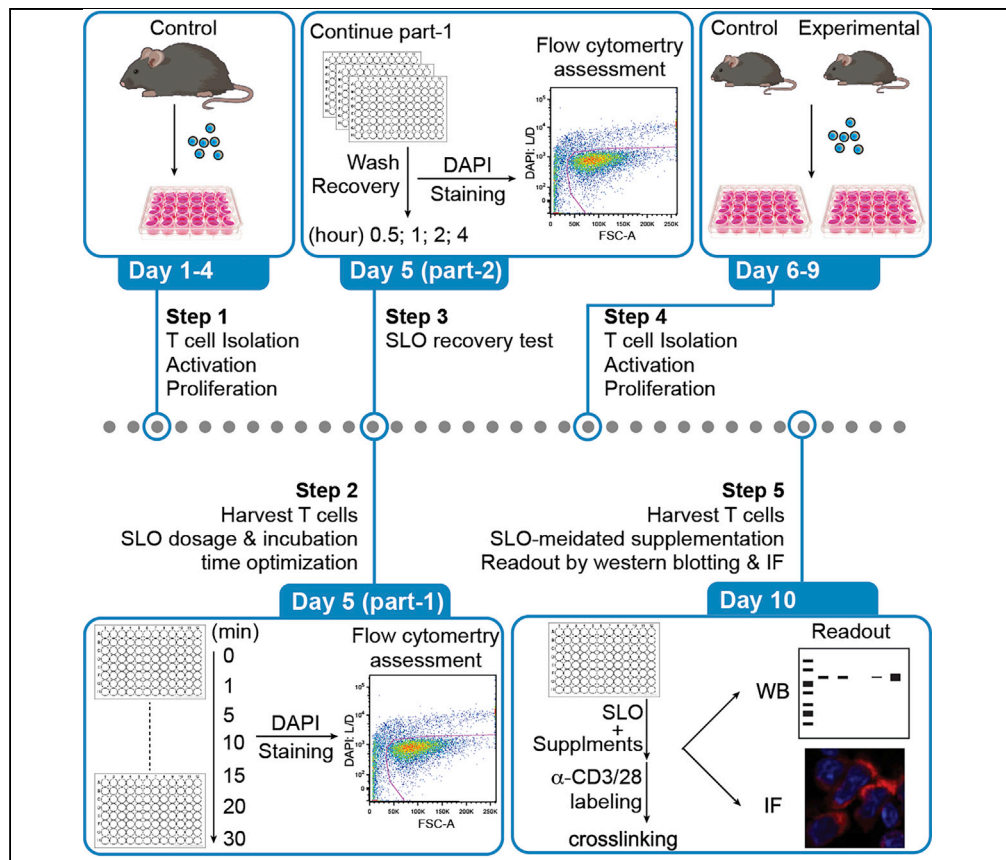


## Protocol

# Delivery of membrane impermeable molecules to primary mouse T lymphocytes



The pore-forming toxin streptolysin-O (SLO) enables intracellular delivery of molecules up to 100 kDa and has been used for short-term delivery of membrane-impermeable substances to assess their effects on cellular activities. A limitation of this technique is the loss of intracellular components and the potential unpredicted alterations of cellular metabolism and signaling. This protocol, optimized for primary mouse T lymphocytes, describes steps for SLO-mediated cell membrane permeabilization and substance supplementation, followed by immunoblotting and immunofluorescent microscopy for assessing cellular effects.

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### Highlights

Isolation, activation, and differentiation of mouse primary T cells

SLO-mediated cell membrane permeabilization protocol

SLO-mediated intracellular small molecule delivery protocol

## Protocol

## Delivery of membrane impermeable molecules to primary mouse T lymphocytes

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## SUMMARY

The pore-forming toxin streptolysin-O (SLO) enables intracellular delivery of molecules up to 100 kDa and has been used for short-term delivery of membrane-impermeable substances to assess their effects on cellular activities. A limitation of this technique is the loss of intracellular components and the potential unpredicted alterations of cellular metabolism and signaling. This protocol, optimized for primary mouse T lymphocytes, describes steps for SLO-mediated cell membrane permeabilization and substance supplementation, followed by immunoblotting and immunofluorescent microscopy for assessing cellular effects. For complete details on the use and execution of this protocol, please refer to Xu et al. (2021a and 2021b).

## BEFORE YOU BEGIN

## Isolation and activation of mouse naïve T cells

⌚ Timing: 4 days (including 1 day for plates coating with anti-CD3 Abs)

1. A day before the primary T cell isolation, coat a 24-well plate with a desired dosage of activating anti-CD3 $\epsilon$  antibodies (Clone 2C11) in 500  $\mu$ L of sterile PBS for each well. Seal the plate with parafilm and incubate 12–16 h (overnight) at 4°C.
  - a. 5  $\mu$ g/mL anti-CD3 can be used for CD8 T cell culture, and 1  $\mu$ g/mL anti-CD3 can be used for CD4 Th1, Th2, and Th17 cell culture.
  - b. For antibody titration, please check the [troubleshooting](#) section.
2. On the day of T cell isolation, prepare a six-well plate filled with 6 mL sterile HBSS in each well, and place a 70  $\mu$ m strainer (Fisherbrand™ Cell Strainers) in each well. Use one well for one mouse.
3. In a biosafety cabinet, sacrifice a mouse (8–12 weeks old, male or female) by CO<sub>2</sub>, and remove peripheral lymph nodes and spleen.
  - a. 2 inguinal lymph nodes, 2 axillary lymph nodes, 2 brachial lymph nodes, 4 superficial cervical lymph nodes can be harvested from one mouse.
4. Place lymph nodes and spleen from each mouse in a 70  $\mu$ m strainer and gently grind the lymph nodes and spleen with the flat end of a syringe plunger until the majority of the tissues become a single cell suspension.
5. Transfer the single cell suspension into a sterile 15 mL conical tube and spin down cells at 500 $\times$  g for 3 min at 20°C–22°C (room temperature).
6. Remove supernatant and lyse the cell pellet with 1 mL sterile red blood cells (RBC) lysis buffer for 3 min at 20°C–22°C (room temperature).



7. Add 10 mL sterile HBSS to the cell suspension in the RBC lysis buffer to wash the cells and spin down at  $500\times g$  for 3 min at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  (room temperature).
8. Resuspend the cell pellet with a sterile single cell suspension buffer (MACS buffer) (refer to the [materials and equipment](#) section) and filter through a  $70\ \mu\text{m}$  mesh to remove aggregated cell debris. Prepare the single cell suspension in MACS buffer at a concentration of  $1\times 10^8$  cell/mL.
9. Proceed with the naïve CD4 or naïve CD8 T cell isolation protocol provided by the manufacturer of the specific isolation kit (the STEMCELL mouse naïve CD4 or CD8 isolation kits are recommended, while other brands such as Miltenyi and Biolegend isolation kit can be used as equivalents).
10. Prepare T cell culture medium (TCM) with a desired dosage of CD28 antibodies (Clone 37.51) and the cytokine cocktails (refer to the [materials and equipment](#) section) for CD4 or CD8 T cell activation and differentiation (refer to reagent tables for T cell differentiation conditions).
  - a.  $2\ \mu\text{g}/\text{mL}$  anti-CD28 can be used for CD8 T cell culture, and  $1\ \mu\text{g}/\text{mL}$  anti-CD28 can be used for CD4 Th1, Th2, and Th17 cell culture.
  - b. For antibody titration, please check the [troubleshooting](#) section.
11. Wash the 24-well plate coated with anti-CD3 (see step 1) with  $500\ \mu\text{L}$  sterile PBS twice.
12. Resuspend isolated naïve T cells at the concentration of  $2\times 10^5$ – $3\times 10^5$  cell/mL in TCM (see step 10) and transfer 1 mL of the cell suspension to each well of the coated 24-well plate.
13. To activate T cells, place the plate in a tissue culture incubator at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 3 days.
14. After 3 days of culturing, harvest T cells and proceed to the downstream assay.

### Streptolysin-O (SLO) optimization for cell membrane permeabilization

⌚ Timing: 2–3 h

15. Prepare SLO solution from stock powder:
    - a. The SLO used in the referenced articles was purchased from Sigma (Cat. # S5265-25KU).
    - b. Reconstitute SLO stock powder with sterile PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ .
    - c. Make the stock SLO solution at concentration of 10,000 Unit/mL and aliquot  $10\ \mu\text{L}$  SLO into sterile 1.5 mL Eppendorf tubes ( $100\ \text{Unit}/10\ \mu\text{L}$ ).
    - d. Store the SLO solutions in a  $-80^{\circ}\text{C}$  freezer and it should be used within one year.
    - e. The SLO cannot be reused after freeze and thaw.
- ⚠ CRITICAL: Using  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free PBS to reconstitute SLO is important because  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  are critical components for promoting cell membrane resealing.**
16. Harvest activated mouse T cells (see step 14 in the previous section) from the 24-well plates:
    - a. Use a P-1000 pipette to resuspend semi-adherent mouse T cells from a 24-well plate.
    - b. Transfer all cultured cells from the same mouse/genotype into one 15 mL conical tube and spin down at  $500\times g$  for 3 min at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  (room temperature).
  17. Resuspend the cell pellet with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS and spin down the cell pellet with the same setup as above.
  18. Repeat step 17 twice for a total of three times to wash cells thoroughly.
    - a. Remove  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  contained in the culture medium to avoid membrane resealing during the SLO-mediated pore forming step.
  19. After cell washing, resuspend the cell pellet with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS in a small volume.
    - a. 1 or 2 mL of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS is recommended to resuspend the cell pellet.
  20. Count the cell number in the single cell suspension.
    - a. Cell counting can be performed with an automated cell counter or hemocytometer.
    - b. Dead cells can be excluded by using trypan blue dye.
  21. Depending on the experimental setup, transfer 1 million live cells from different genotypes or 1 million live cells from the same sample to a 96-well plate.
  22. Spin down the plate at  $500\times g$  for 3 min at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  (room temperature), and resuspend cells in  $100\ \mu\text{L}$   $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS.

23. Titrate SLO to cell samples: (Starting point of SLO optimization)
  - a. All titration is done with  $10^6$  live cells/sample in a volume of 100  $\mu\text{L}$   $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS.
  - b. Suggested SLO titration dosages: 1 Unit/  $10^6$  cells, 5 Unit/  $10^6$  cells, 10 Unit/  $10^6$  cells, 20 Unit/  $10^6$  cells, 30 Unit/  $10^6$  cells, and 50 Unit/  $10^6$  cells.
  - c. The SLO stock solution (see step 15) is diluted in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS in a total volume of 100  $\mu\text{L}$ .
  - d. Seed cells in 3 replicates for each SLO titration dose and each incubation time point.
24. Incubate samples in a tissue culture incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  for different time lengths.
  - a. The incubation time is also a critical factor for membrane permeabilization.
  - b. Suggested SLO permeabilization time: 1 min, 5 min, 10 min, 15 min, 20 min, and 30 min.
25. At each designated incubation time point, take out 30–40  $\mu\text{L}$  of cell samples and wash with HBSS containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  twice by centrifugation at  $500\times g$  for 3 min at  $20^\circ\text{C}$ – $22^\circ\text{C}$  (room temperature).
26. After washing, resuspend the cell sample with 300  $\mu\text{L}$  HBSS or RPMI 1640 in a 96-well plate.
  - a. The HBSS or RPMI 1640 must contain  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  to promote cell membrane resealing.
27. Take out 40  $\mu\text{L}$  of cell samples and assess membrane permeability by DAPI staining.
  - a. Make DAPI (Invitrogen) stock solution with deionized  $\text{H}_2\text{O}$ : 5 mg/mL (14.3 mM).
  - b. For 1 million cell samples, resuspend with 200  $\mu\text{L}$  FACS staining solution.
  - c. Add DAPI to the staining solution to a final concentration of 3  $\mu\text{M}$  and mix well.
  - d. The DAPI staining is very fast, and can be finished in a minute.
  - e. Use flow cytometry (BD Biosciences) to assess DAPI staining.
28. Check the efficiency of SLO-mediated cell permeabilization by flow cytometry.
  - a. DAPI positive cells can be seen as SLO permeabilized.
  - b. A typical DAPI staining will result in two distinct populations with positive and negative DAPI signals, which can be used to calculate the membrane permeabilization efficiency, and  $> 85\%$  DAPI positivity is sufficient for downstream assays.

$$\text{SLO lysis efficiency} = \frac{\text{Cell\#} \times \text{DAPI}^+ \%( \text{after SLO} ) - \text{Cell\#} \times \text{DAPI}^+ \%( \text{before SLO} )}{\text{Cell\#} \times \text{DAPI}^- \%( \text{before SLO} )} \times 100\%$$

### Streptolysin-O titration for cell membrane recovery efficiency

© Timing: 2 h

29. After sampling cells for DAPI analysis (see steps 27 to 28), incubate the rest of the SLO-permeabilized cells in 300  $\mu\text{L}$   $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -containing HBSS or RPMI 1640 in a tissue culture incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  for different time lengths.
  - a. This step is to check cell viability after SLO-mediated membrane permeabilization.
  - b. Suggested time points to check membrane resealing: 30 min, 1 h, 2 h, and 4 h.
30. At each designated recovery time point, take 40  $\mu\text{L}$  of cell samples and wash with PBS twice by centrifugation at  $500\times g$  for 3 min at  $20^\circ\text{C}$ – $22^\circ\text{C}$  (room temperature).
31. After washing, resuspend the cell sample with 100  $\mu\text{L}$  PBS and add DAPI (see steps 27 and 28 in the previous section).
32. Check the efficiency of cell membrane resealing by flow cytometry.
  - a. DAPI negative cells can be seen with the intact membrane structure and are live.
  - b. It is highly recommended to assess the cell protein loss by western blotting.
  - c. Calculate the percentage of cells that were able to recover after the SLO treatment.

$$\text{Recovery efficiency} = \frac{\text{Cell\#} \times \text{DAPI}^- \%( \text{Recovery phase at time point} )}{\text{Cell\#} \times \text{DAPI}^+ \%( \text{After SLO} ) - \text{Cell\#} \times \text{DAPI}^+ \%( \text{Before SLO} )} \times 100\%$$

- d.  $> 85\%$  of recovery rate is acceptable.

33. Choose the optimal combination of the SLO dosage and the incubation time for the specific immune cells type and use this setup to perform future experiments.
  - a. Choose the lowest SLO dosage that can permeabilize > 85% of  $10^6$  live cells between 15 to 30 min, with the majority (>85%) of permeabilized cells resealing their membrane completely within 4 h.
  - b. Choose the SLO dosage and the incubation time that efficiently supplement molecules without losing much of the protein of interest.
  - c. Cytosolic protein such as GAPDH can be used as a marker protein to assess protein loss after SLO treatment.
  - d. Usually, live cells can reseal their membrane in about 4 h.

△ **CRITICAL:** The selection of a SLO dosage and optimization of the incubation time are important parameters for downstream supplementation experiments. The sensitivity of distinct cell types to the SLO-mediated membrane permeabilization is different in part due to different levels of membrane cholesterol.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
IF & FC: Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647 (IF & FC: 1:400)	Invitrogen	Cat. # A32733; RRID: AB_2633282
WB & FC: Monoclonal Rabbit anti-pT308 Akt (clone D25E6) (WB & FC: 1:1000)	Cell Signaling Technology	Cat. # 13038S; RRID: AB_2629447
WB & FC: Polyclonal Rabbit anti-pS256 Foxo1 (WB: 1:1000 & FC: 1:200)	Cell Signaling Technology	Cat. # 9461S; RRID: AB_329831
WB & FC: Polyclonal Rabbit anti-pT24 Foxo1/pT32 Foxo3a (WB: 1:1000 & FC: 1:200)	Cell Signaling Technology	Cat. # 9464S; RRID: AB_329842
WB: Monoclonal Rabbit anti-PAN Akt (clone C67E7) (WB: 1:1000 & FC: 1:400)	Cell Signaling Technology	Cat. # 4691L; RRID: AB_915783
WB: Monoclonal Rabbit anti-Foxo1 (clone C29H4) (WB: 1:1000 & FC: 1:200)	Cell Signaling Technology	Cat. # 2880S; RRID: AB_2106495
WB: Polyclonal Rabbit anti-LDHA (WB: 1:1000)	Cell Signaling Technology	Cat. # 2012S; RRID: AB_2137173
WB: Monoclonal Mouse anti-β Actin (clone 8H10D10) (WB: 1:1000)	Cell Signaling Technology	Cat. # 3700S; RRID: AB_2242334
IF: Monoclonal anti-Phosphatidylinositol 3,4,5-trisphosphate (clone RC6F8) (IF: 1:100)	Invitrogen	Cat. # A-21328; RRID: AB_2535837
IF: DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen	Cat. # D1306; RRID: AB_2629482
<i>In vitro</i> : Monoclonal anti-mouse CD28-Biotin (clone 37.51)	Invitrogen	Cat. # 13-0281-81; RRID: AB_466410
<i>In vitro</i> : Monoclonal anti-mouse CD3ε-Biotin (clone 145-2C11; Functional Grade)	Invitrogen	Cat. # 36-0031-85; RRID: AB_469747
<i>In vitro</i> : Monoclonal anti-mouse CD28 (clone 37.51)	BioXcell	Cat. # BE0015-1; RRID: AB_1107624
<i>In vitro</i> : Monoclonal anti-mouse CD3ε (clone 145-2C11)	BioXcell	Cat. # BE0001-1; RRID: AB_1107634
<i>In vitro</i> : Monoclonal anti-mouse IFN-γ (clone XMG1.2)	BioXcell	Cat. #BE0055; RRID: AB_1107694
<i>In vitro</i> : Monoclonal anti-mouse IL-2 (clone S4B6)	BioXcell	Cat. #BE0043-1; RRID: AB_1107705
<i>In vitro</i> : Monoclonal anti-mouse IL-4 (clone 11B11)	BioXcell	Cat. #BE0045; RRID: AB_1107707
<b>Chemicals, peptides, and recombinant proteins</b>		
β-Mercaptoethanol	Gibco	Cat. # 31350010
RPMI 1640	Gibco	Cat. # 22400097
HBSS (Ca <sup>2+</sup> and Mg <sup>2+</sup> free)	Gibco	Cat. # 14170120
HBSS (with Ca <sup>2+</sup> and Mg <sup>2+</sup> )	Gibco	Cat. # 14025092
DPBS (Ca <sup>2+</sup> and Mg <sup>2+</sup> free)	Gibco	Cat. # 14190144
DPBS (with Ca <sup>2+</sup> and Mg <sup>2+</sup> )	Gibco	Cat. # 14040133
Adenosine 5'-triphosphate disodium salt hydrate	Sigma-Aldrich	Cat. # A2383-5G

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ProLong™ Gold Antifade Mountant	Invitrogen	Cat. # P36930
Cell Lysis Buffer (10×)	Cell Signaling Technology	Cat. # 9803
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche	Cat. # 4693132001
Fetal Bovine Serum (heat inactivated)	Sigma-Aldrich	Cat. # F4135-500ML
Foxp3 / Transcription Factor Fix/Perm Concentrate (4×)	Tonbo	Cat. # TNB-1020-L050
Foxp3 / Transcription Factor Fix/Perm Diluent (1×)	Tonbo	Cat. # TNB-1022-L160
Flow Cytometry Perm Buffer (10×)	Tonbo	Cat. # TNB-1213-L150
GlutaMAX™ Supplement	Gibco	Cat. # 35050061
Hoechst 33342	Invitrogen	Cat. # H1399
Lactate Dehydrogenase A Inhibitor, FX11	Millipore	Cat. # 427218 -10MG
32% Paraformaldehyde Solution (EM Grade)	Electron Microscopy Sciences	Cat. # 15714-S
100× Penicillin/Streptomycin Solution	Gemini Bio-Products	Cat. # 400-109
PhosSTOP (20 tablets in EASYpacks)	Roche	Cat. # 4906837001
Poly-D-lysine	Gibco	Cat. # A3890401
Recombinant mouse interleukin-1β	BioLegend	Cat. # 575102
Recombinant mouse IL-12 P70 (carrier-free)	BioLegend	Cat. # 577002
Recombinant human IL-2	NIH	N.A.
Recombinant mouse interleukin-23	BioLegend	Cat. # 589002
Recombinant mouse interleukin-4 (carrier-free)	BioLegend	Cat. # 574302
Recombinant mouse interleukin-6	BioLegend	Cat. # 575706
Recombinant human TGF-β1	R&D Systems	Cat. # 240-B-002
Sodium azide	Sigma-Aldrich	Cat. # 71289-5G
Streptavidin	ProZyme	Cat. # SA10
Streptolysin O from <i>Streptococcus pyogenes</i> (SLO)	Sigma-Aldrich	Cat. # 55265-25KU
<b>Critical commercial assays</b>		
EasyStep™ Mouse Naïve CD4 <sup>+</sup> T Cell Isolation Kit	STEMCELL	Cat. # 19765
EasyStep™ Mouse Naïve CD8 <sup>+</sup> T Cell Isolation Kit	STEMCELL	Cat. # 19858
<b>Experimental models: Organisms/strains</b>		
B6 CD45.2: C57BL/6J	Jackson Laboratory	Cat. # 000664
<b>Other</b>		
BD LSR II Flow Cytometer	BD Biosciences	N/A
CytoOne 24-well TC plate, clear	USA Scientific	Cat. # CC7682-7524
Falcon™ tissue culture plates, 96-well; standard tissue culture; U-bottom	Fisher Scientific	Cat. # 353077
Fisherbrand™ Cell Strainers - 70µm	Fisher Scientific	Cat. # 22-363-548
Fisherbrand™ Gel-Loading Tips, 1–200µL - Volume: 1–200µL; Length: 2.75	Fisher Scientific	Cat. # 02-707-138
MilliporeSigma™ Steritop™ Sterile Vacuum Bottle-Top Filters	Millipore	Cat. # SCGPS05RE
Inverted Zeiss LSM880 laser scanning confocal microscope	Zeiss	N/A
<b>Software and algorithms</b>		
FlowJo V10.0.7	FlowJo	<a href="https://www.flowjo.com">https://www.flowjo.com</a>
ImageJ/Fiji V1.52P	Fiji	<a href="https://fiji.sc">https://fiji.sc</a>
Prism V7.0 d	GraphPad	<a href="https://www.graphpad.com">https://www.graphpad.com</a>

## MATERIALS AND EQUIPMENT

### T cell culture medium (TCM)

Reagent	Final concentration	Amount
RPMI 1640	n/a	440 mL
FBS (Heat-inactivated)	10% (v/v)	50 mL
Penicillin/Streptomycin 100×	1% (v/v)	5 mL
Gluta-MAX 100×	1% (v/v)	5 mL
β-Mercaptoethanol	50 µM	0.5 mL
<b>Total</b>	<b>n/a</b>	<b>500. 5 mL</b>

The prepared TCM is recommended to store at 4°C and use within 1 month.

**CD4 Th1 cell differentiation cytokine cocktail (Recommend making 1000× cytokine or antibody stocks)**

Reagent	Final concentration	Amount
IL-2	100 Unit/mL Stock: 100,000 U/mL	Depending on total volume
IL-12	10 ng/mL Stock: 10 µg/mL	Depending on total volume
Anti-IL-4	10 µg/mL Stock: 10 mg/mL	Depending on total volume
<b>Total</b>	<b>n/a</b>	Depending on total volume

The prepared cytokine and antibody stocks are recommended to store at –80°C and use within 1 year.

**CD4 Th2 cell differentiation cytokine cocktail**

Reagent	Final concentration	Amount
IL-2	100 Unit/mL Stock: 100,000 U/mL	Depending on total volume
IL-4	20 ng/mL Stock: 20 µg/mL	Depending on total volume
Anti-IFN $\gamma$	10 µg/mL Stock: 10 mg/mL	Depending on total volume
<b>Total</b>	<b>n/a</b>	Depending on total volume

The prepared cytokine and antibody stocks are recommended to store at –80°C and use within 1 year.

**CD4 Th17 cell differentiation cytokine cocktail**

Reagent	Final concentration	Amount
TGF- $\beta$ 1	10 ng/mL Stock: 10 µg/mL	Depending on total volume
IL-6	10 ng/mL Stock: 10 µg/mL	Depending on total volume
IL-1 $\beta$	10 ng/mL Stock: 10 µg/mL	Depending on total volume
IL-23	10 ng/mL Stock: 10 µg/mL	Depending on total volume
Anti-IL-4	10 µg/mL Stock: 10 mg/mL	Depending on total volume
Anti-IL-2	10 µg/mL Stock: 10 mg/mL	Depending on total volume
Anti-IFN $\gamma$	10 µg/mL Stock: 10 mg/mL	Depending on total volume
<b>Total</b>	<b>n/a</b>	Depending on total volume

The prepared cytokine and antibody stocks are recommended to store at 80°C and use within 1 year.

**CD8 cell differentiation cytokine cocktail**

Reagent	Final concentration	Amount
IL-2	100 Unit/mL Stock: 100,000 U/mL	Depending on total volume
<b>Total</b>	<b>n/a</b>	Depending on total volume

The prepared cytokine and antibody stocks are recommended to store at –80°C and use within 1 year.

$\Delta$  **CRITICAL:** All antibody and cytokine stock solutions should be aliquoted and stored at –80°C. Avoid freeze and thaw more than 3 times. Generally, antibodies are more stable and can be stored in 4°C for 1 month after thaw.

### RBC lysis buffer

Reagent	Final concentration	Amount
NH <sub>4</sub> Cl	8.02 g/L	8.02 g
NaHCO <sub>3</sub>	0.84 g/L	0.84 g
EDTA Di-Sodium	0.37 g/L	0.37 g
ddH <sub>2</sub> O	n/a	Fill up to 1000 mL
<b>Total</b>	<b>n/a</b>	<b>1000 mL</b>

The prepared RBC buffer is recommended to store at 20°C–22°C and use within 6 months. The RBC lysis buffer has to be filtered by a 0.22 µm filter.

### MACS buffer

Reagent	Final concentration	Amount
BSA	1% (w/v)	5 g
EDTA Di-Sodium	2 mM	336.2 mg
PBS	n/a	500 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

The prepared MACS buffer is recommended to store at 4°C and use within 6 months. The MACS buffer has to be filtered by a 0.22 µm filter.

### FACS staining buffer

Reagent	Final concentration	Amount
BSA	1% (w/v)	5 g
EDTA Di-Sodium	2 mM	336.2 mg
Sodium Azide	0.1% (v/v)	2.5 mL from 20% stock solution
PBS	n/a	500 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

The prepared FACS buffer is recommended to store at 4°C and use within 6 months. The FACS buffer has to be filtered by a 0.22 µm filter.

### 4% PFA fixation buffer

Reagent	Final concentration	Amount
32% Paraformaldehyde Solution (EM Grade)	4% (w/v)	10 mL
PBS	n/a	70 mL
<b>Total</b>	<b>n/a</b>	<b>80 mL</b>

It is recommended to prepare fresh PFA buffer on the same day of the experiment and use fresh buffer every time for best outcome.

## STEP-BY-STEP METHOD DETAILS

### Harvest and rest the activated T cells

⌚ Timing: 3.5 h

Prepare activated mouse T cells for resting without TCR or cytokine stimulation.

- Harvest the day 3-activated mouse T cells (see section [isolation and activation of mouse naïve T cells](#)) from 24-well plates:
  - Use a P-1000 pipette to resuspend the semi-adherent mouse T cells from a 24-well plate.
  - Transfer all the cultured cells from the same mouse/genotype into one 15 mL conical tube and spin down with 500× g for 3 min at 20°C–22°C (room temperature).



2. After spin down, resuspend the cell pellet with 10 mL of RPMI 1640 and spin down with the same setup again.
  - a. The RPMI 1640 used here contains no other supplements.
3. Repeat step 2 twice for a total of three times to wash cells thoroughly.
4. After cell washing, resuspend cell pellets in a small volume of RPMI 1640.
  - a. 1 or 2 mL of RPMI 1640 is recommended to resuspend the cell pellet.
5. Count the cell number in the single cell suspension.
  - a. Cell counting can be performed with an automated cell counter or hemocytometer.
  - b. Dead cells must be excluded by using the trypan blue dye.
  - c. All experimental samples described in the protocol are based on using  $10^6$  live cells per sample.
6. Depending on the experimental setup, transfer 1 million live cells from different genotypes or 1 million live cells from the same sample to a 96-well plate.
  - a. Fill up each well containing cell samples with RPMI 1640 to the final volume of 300  $\mu$ L.
7. Rest the cell samples in a tissue culture incubator at 37°C with 5% CO<sub>2</sub> for 3 h.
  - a. The RPMI 1640 should not contain any supplement and make sure the cells receive minimal growth factor signals or cytokine signals.
  - b. The resting step is to lower the signaling event to a basal level and is necessary for conducting assays that assess signaling events after acute receptor-ligand re-stimulation.
  - c. Skip this step if the readout of the downstream assay is not influenced by previous culture conditions.

### **SLO-mediated T-cell membrane permeabilization and substance (e.g., ATP) supplementation and their impact on acute TCR signaling as assessed by immunoblotting**

⌚ Timing: 1–1.5 h (depending on the optimal condition determined previously)

8. After resting cells or directly following the cell harvest procedure, spin down the cells in the 96-well plate at 500 $\times$  g for 3 min at 20°C–22°C (room temperature).
9. Resuspend and wash the cell pellets with 300  $\mu$ L Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS solution and spin down at 500 $\times$  g for 3 min at 20°C–22°C (room temperature).
10. Repeat the wash with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS 3 times.

**⚠ CRITICAL: Using Ca<sup>2+</sup> and Mg<sup>2+</sup>-free HBSS to wash the cells is critical to promote membrane resealing and improve the efficiency of SLO-mediated membrane permeabilization.**

11. While washing the cells, dilute the SLO stock solution to the optimal concentration determined previously (see section [streptolysin-O optimization for cell membrane permeabilization](#)).
  - a. Use Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS solution as the base solution.
  - b. The final volume is 100  $\mu$ L/10<sup>6</sup> live cells.
12. Add the substance to be delivered intracellularly to the SLO-containing solution.
  - a. The concentration of the substance should be higher than that of the intracellular compartment because the supplementation is dependent on a passive diffusion and concentration gradient.
  - b. Titration of the concentration of the substance is also recommended before conducting the experiment.
  - c. In the articles published by [Xu et al. \(2021a and 2021b\)](#), ATP is supplemented in a concentration of 10 mM.
  - d. The final volume is 100  $\mu$ L/10<sup>6</sup> live cells.
13. Warm up the SLO and the supplement solution to 37°C in a water bath.
14. When cell washing is completed, resuspend the cell samples in the SLO solution with or without the supplement substance and incubate in a tissue culture incubator at 37°C with 5% CO<sub>2</sub> for the time length determined previously (see section [streptolysin-O optimization for cell membrane permeabilization](#)).
  - a. The final volume is 100  $\mu$ L/10<sup>6</sup> live cells.

15. While cells are undergoing permeabilization and supplementation, prepare a solution that contains biotinylated CD3 and CD28 antibodies with or without the substance to be delivered intracellularly.
  - a. The base solution can be  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -containing HBSS or RPMI 1640.
  - b. The dosage of anti-CD3 and anti-CD28 is dependent on the user's need.
  - c. For a typical acute anti-CD3 and anti-CD28 crosslinking experiment, we recommend 5  $\mu\text{g}/\text{mL}$  and 2  $\mu\text{g}/\text{mL}$ , respectively.
  - d. The final volume is 100  $\mu\text{L}/10^6$  live cells.
16. After incubation, take out the cell samples and wash the cells with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -containing HBSS or RPMI 1640 2–3 times with 500 $\times$  g centrifugation for 3 min at 20°C–22°C (room temperature).
  - a.  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  can promote cell membrane resealing even at 4°C.
17. After washing, resuspend the samples with 100  $\mu\text{L}$  solutions that contain anti-CD3/anti-CD28 with or without the supplementary substance, and incubate the samples in a 4°C fridge for 30–40 min.
  - a. This step is to coat T cells with activating antibodies.
  - b. It is important to keep supplementing the substance with the same concentration after the removal of SLO.
  - c. Incubating T cells with antibodies is recommended to be done at 4°C or on ice to minimize endocytosis.

△ **CRITICAL:** If the substance is not supplied after the removal of SLO, the concentration gradient will cause a depletion of that substance while the plasma membrane is healed, and will possibly result in the opposite phenotype.

18. While incubating, prepare a 20 $\times$  streptavidin solution (Prozyme) in either  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -containing HBSS or RPMI 1640 for biotin-streptavidin-induced antibody crosslinking and keep it in a 37°C incubator.
19. After antibody coating, warm up the bottom of the 96-well plate in a water bath for 2 min.
  - a. Be careful not to contaminate the samples with water.
20. Quickly add 20 $\times$  streptavidin solution (5  $\mu\text{L}$  into 100  $\mu\text{L}$  per sample) and mix well. Keep the plate either in a 37°C water bath or an incubator for 5 min to allow crosslinking.
  - a. The time length of crosslinking can vary depending on the signaling kinetics of the specific pathway.
  - b. 5 min is recommended for assessing the proximal TCR and the downstream pathways including Akt-Foxo1 and mTORC.
21. After crosslinking, quickly spin down the cells and immediately add 100  $\mu\text{L}$  of 1 $\times$  cell lysis buffer (Cell Signaling Technology, with phosphatase and protease inhibitors) for every  $10^6$  cells to prepare immunoblotting samples.
  - a. The 10 $\times$  lysis buffer stock solution should be warmed up to allow proteases and phosphatases to be completely dissolved (see below).
  - b. Dilute the 10 $\times$  buffer with  $\text{ddH}_2\text{O}$ , and add phosphatase (Roche) and protease inhibitor (Roche) cocktails before use.

### **SLO-mediated T-cell membrane permeabilization and substance (e.g., ATP) supplementation and their impact on acute TCR signaling as assessed by immunofluorescent microscopy**

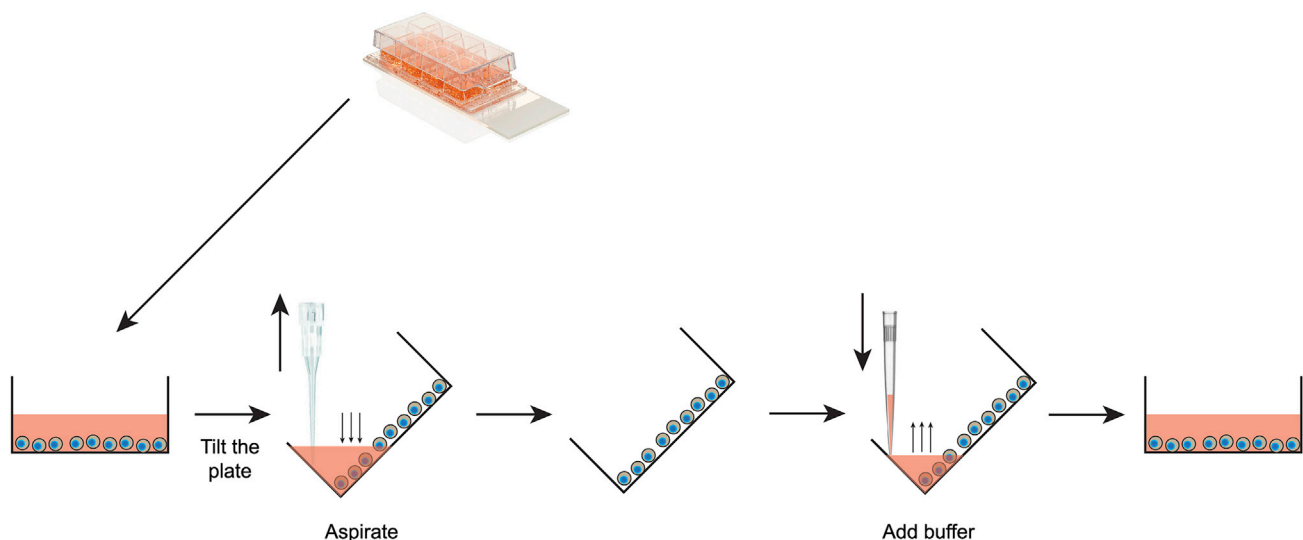
Ⓞ **Timing:** 1.5–2 h (depending on the optimal condition determined previously. Timing for cell fixation/permeabilization, antibody and hoechst staining etc. should be considered here as well)

22. Coat a chamber slide with poly-D-lysine or other cell adhesives a night before the assay.

- a. Dilute the poly-D-lysine solution (Gibco) in sterile PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , and prepare a working solution with 50  $\mu\text{g}/\text{mL}$  poly-D-lysine.
- b. Coat the chamber slide surface with 250–300  $\mu\text{L}$  of the working solution.
- c. Incubate the slides at 20°C–22°C (room temperature) for 1 h.
- d. Remove the solution and wash the slides 3 times with 300  $\mu\text{L}$  of distilled water.
- e. Make sure to rinse the chamber thoroughly because excess poly-D-lysine solution can be toxic to the cells.
- f. After washing, leave the coated slides uncovered in the laminar hood to dry.
- g. The dried plate can be used immediately or stored at 4°C sealed with parafilm.
23. After finishing step 17 described in the previous section, transfer the entire groups of cell samples to different wells of the chamber slide and spin down at 500 $\times$   $g$  for 5 min to let the cells adhere to the bottom of the slide.
24. Warm up the chamber slide in a water bath for 2 min.
  - a. Be careful not to contaminate the samples with water.
25. Gently take out half (50  $\mu\text{L}$ ) of the solution in the chamber slide and add 50  $\mu\text{L}$  of the 2 $\times$  streptavidin solution in each well of the chamber slide. Keep the chamber slide either in a 37°C water bath or an incubator for 5 min to allow crosslinking.
26. Quickly spin down the chamber slide and remove the supernatant. Immediately add 200  $\mu\text{L}$  of 4% PFA solution to fix the cells at 20°C–22°C (room temperature) for 10 min.

**△ CRITICAL:** This fixation step is critical for preserving phosphorylated proteins after TCR or cytokine stimulation because phospho signaling can be quickly diminished within minutes or even seconds. Therefore, it is important to fix the cells as quickly as possible after a quick spin down.

27. Aspirate the supernatant slowly and carefully without disturbing the cells. (Figure 1)
28. Wash the cells with 250  $\mu\text{L}$  of PBS once in the chamber slide. (Figure 1)
  - a. Do not directly add PBS to the cells, this will detach the cells.
  - b. Add 250  $\mu\text{L}$  of PBS by touching the pipette tip to the chamber wall and slowly discharge the washing buffer.



**Figure 1. Schematic demonstration of how to aspirate and add buffers to the chamber slides**

Activated T cells are semi-adhesive and easily detach from the slide. Therefore, it is highly recommended to manipulate the slides with extra care. In order to preserve the cells, it is recommended to tilt the slide and aspirate the liquid from the edge with a thin western loading tip (Fisherbrand™ Gel-Loading Tips) to minimize the flow and disturbance. When adding a buffer to the slide, it is also recommended to tilt the slide and add the liquid from the edge with a regular tip, and then slowly lay down the slide.

29. After washing, fix the cells again with 250  $\mu$ L of Tonbo nuclear protein fixation buffer at 20°C–22°C (room temperature) for 40–60 min.
30. After fixation, aspirate the supernatant and wash the cells once with 250  $\mu$ L of Tonbo permeabilization buffer slowly and carefully without disturbing the cells.
31. While washing, prepare the primary antibody or conjugated antibody cocktail.
  - a. Prepare 250  $\mu$ L antibody solution per sample.
  - b. Fluorophore-conjugated antibodies are recommended in order to minimize the wash steps to preserve the samples.
  - c. Use the Tonbo permeabilization buffer to make the cocktail solution.
32. Add 250  $\mu$ L of the antibody cocktail to the samples and incubate at 4°C 12–16 h (overnight).
  - a. The optimal amount of primary antibody needs to be titrated before staining or used as instructed in the manual.
33. On the next day, aspirate the primary antibodies slowly and carefully, and wash the sample twice with PBS at 20°C–22°C (room temperature).
34. Add secondary antibodies to the samples (250  $\mu$ L per sample) and incubate 1 h at 20°C–22°C (room temperature).
  - a. The optimal amount of secondary antibody needs to be titrated before staining or used as instructed in the manual.
35. After secondary antibodies staining, wash the cells with PBS twice at 20°C–22°C (room temperature).
36. Incubate cell samples with hoechst diluted in PBS for 10 min at 20°C–22°C (room temperature) and wash with PBS twice.
  - a. The optimal amount of hoechst needs to be titrated before staining or used as instructed in the manual.
  - b. Make hoechst stock solution at the concentration of 10 mg/mL and dilution in PBS with 1:500 to 1:2000 ratio.
37. Remove the chamber wall slowly and carefully.
38. Add 2–3 drops or enough of antifade mountant on the top of the samples slowly and carefully, and apply the cover slide carefully.
39. The images can be taken with immunofluorescent microscopy (ZEISS LSM 880) with a desired setup and magnification.

### EXPECTED OUTCOMES

This protocol has been designed to deliver a cell membrane-impermeable substance into the cytosolic compartment of the target cells. Using this protocol, investigators can test whether a particular substance (nutrients, ATP, cofactors, ions, etc) can affect a particular cellular phenotype through readouts from immunoblotting or immunofluorescent imaging.

From one 8-12-week-old mouse, investigators can harvest about 5–8 million naïve CD4 or 4–5 million naïve CD8 T cells. After 3-day culture, investigators can recover over 20 million activated CD4 or 15 million activated CD8 T cells. Depending on the number of genotypes and tests, it is important to calculate the number of mice that need to be sacrificed. Usually, pooling more than 3 mice is not recommended due to inter-mice variability. If cell samples are harvested directly from peripheral non-lymphoid tissue, pooling several mice together is inevitable. We recommend using littermates in this case to minimize the inter-mice variation.

Assessment of the effect of ATP supplementation on the cellular signaling pathway by immunoblotting: Use ATP supplementation described in the published article (Xu et al., 2021a, 2021b) as examples. FX11 is a LDHA inhibitor that can effectively lower the intracellular ATP level so that phosphorylation of Akt and Foxo1 are compromised because the upstream PI3K's activity is sensitive to the level of intracellular ATP. Similarly, SLO alone can effectively deplete intracellular ATP due to the concentration gradient. When SLO and the high level of extracellular ATP are supplied, extracellular

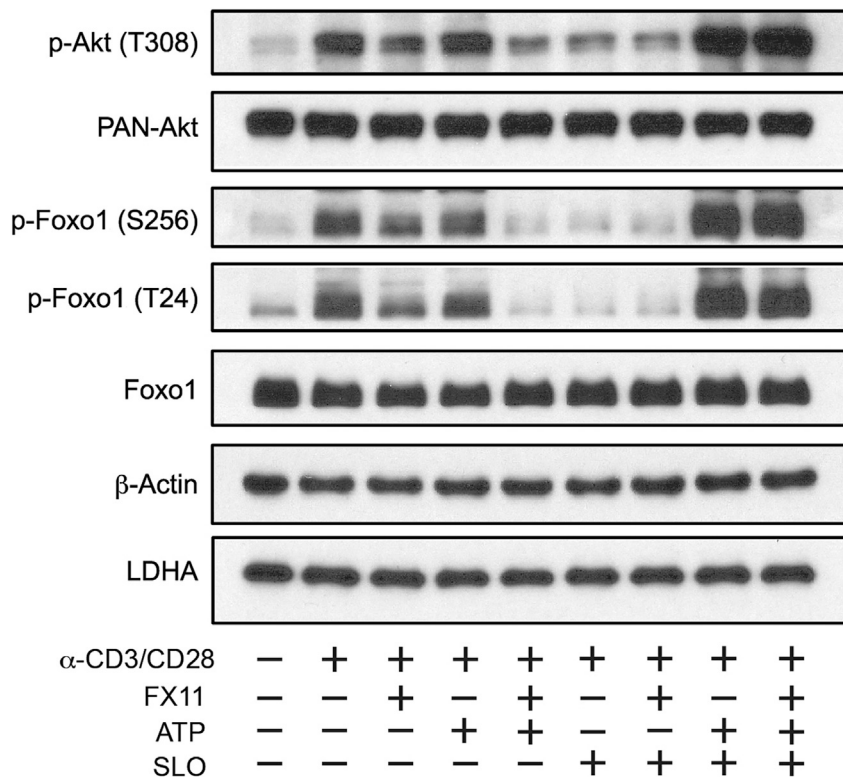
ATP can not only rescue the FX11- or SLO-induced diminished phosphorylation level of Akt and Foxo1, but also boost the phosphorylation of Akt and Foxo1 to higher levels (Figure 2).

Assessment of the effect of ATP supplementation on the kinase activity by immunofluorescent imaging: Use ATP supplementation described in the published article (Xu et al., 2021a) as an example. FX11 is a LDHA inhibitor that can effectively diminish the intracellular ATP level causing lower levels of PI3K-catalyzed production of PIP3. When SLO and the high level of extracellular ATP are supplied, extracellular ATP can not only rescue the FX11-induced diminished PIP3 production, but also boost the production of PIP3 to a significantly higher level (Figure 3).

### LIMITATIONS

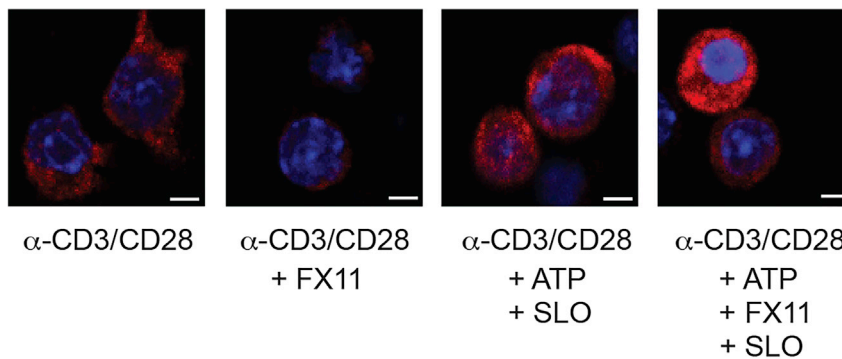
SLO-induced transient plasma membrane permeabilization has several limitations. First, this protocol utilizes a cholesterol-binding toxin and its pore-forming activity is dependent on the cholesterol content on the target cell membrane. The protocol described above is optimized for activated murine CD4 and CD8 T cells and human CD8 T cells, so it is critical to optimize a protocol for other immune or non-immune cells.

Second, the SLO-assisted delivery could not be performed in the long-term T cell culture, which prevented testing a hypothesis that requires long term observation. The delivery method is suitable for manipulating biochemical/enzymatic reactions (e.g., PIP3 production) and signaling transduction cascades (e.g., protein phosphorylation) that typically take place in seconds or minutes. It is



**Figure 2. Intracellular delivery of ATP and its effect on Akt-Foxo1 signaling assessed by western blotting**

Naïve wild type CD4<sup>+</sup> T cells were cultured under the Th17 differentiation conditions for 3 days, rested for 3 h in RPMI, and subsequently incubated in HBSS in the presence or absence of FX11, ATP, and SLO. T cells were then re-stimulated with anti-CD3 and anti-CD28 crosslinking for 5 min. Samples were fixed and lysed for immunoblotting. Cell lysates were collected and analyzed by immunoblotting for p-Akt (T308), Akt, p-Foxo1 (T24), p-Foxo1 (S256), Foxo1, LDHA, and β-actin.



**Figure 3. Intracellular delivery of ATP and its effect on PI3K-PIP3 signaling assessed by immunofluorescent imaging**  
Naïve CD4<sup>+</sup> T cells were cultured under Th17 cell differentiation conditions for 3 days, rested for 3 h in RPMI, and subsequently incubated in HBSS in the presence or absence of FX11, ATP, and SLO. T cells were then re-stimulated with anti-CD3 and anti-CD28 crosslinking for 5 min. Samples were fixed and stained for immunofluorescent imaging. Scale bar, 5 microns.

probably not suitable to test a hypothesis that requires readouts based on cell proliferation, epigenetic regulation, cytokine production that usually require hours or days to complete the actions.

Third, the robustness of the assay's readout is depending on the target protein's Michaelis Menten constants towards the supplemented substance ( $K_m$ -Suppl.). In theory, the higher the  $K_m$  that the target protein has, the more sensitive the protein's activity is towards the fluctuation of the intracellular concentration of the specific substance. The lower the  $K_m$  is, the less sensitive the protein activity will respond to the fluctuation of the intracellular concentration of the specific substance. This concept was demonstrated in the article (Xu et al., 2021a). PI3K $\delta$ 's ( $K_m$ -ATP  $\sim$ 118mM) activity is very sensitive towards the cytosolic concentration of ATP, but Zap70's ( $K_m$ -ATP  $\sim$ 3mM) activity is marginally affected either when ATP is depleted by SLO or supplied in excess.

Forth, the biochemical reactions and signal transduction take place in various subcellular compartments such as cytosol, mitochondria, and nucleus. SLO-mediated pore-forming activity is only explored in the context of the cell plasma membrane. Whether this protocol is suitable for delivering a substance into other subcellular compartments requires further investigation.

Lastly, SLO-induced cell membrane permeabilization is predicted to cause loss of intracellular components, including small molecule metabolites and cytosolic proteins with low molecular weight, which might affect cellular metabolism and signaling in an unpredicted manner.

## TROUBLESHOOTING

### Problem 1

RBCs are not completely lysed by RBC lysis buffer. This is an issue that is very often to encounter during mouse T cells isolation from spleen that contains large quantity of RBCs. (refer to step 7 of Isolation of mouse naïve T cells)

### Potential solution

Depending on the quantity of total isolated cells after single cell suspension, investigators can extend the RBC lysis time to an extra 1–2 min (do not exceed 5 min in total). The other solution is to add an extra 1–2 mL of RBC lysis buffer to the cells.

### Problem 2

Severe cell death after RBC lysis (step 8 in the section of [isolation and activation of mouse naïve T cells](#)).

### Potential solution

Using PBS to wash the isolated cells after RBC lysis often results in enormous cell death for an unknown reason. It is highly recommended to use  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -containing HBSS to complete the wash step after RBC lysis which results in a significantly reduced cell death level.

### Problem 3

Naïve T cell during 3-day culture do not generate sufficient T cells (step 14 in the section of [isolation and activation of mouse naïve T cells](#)).

### Potential solution

The density of naïve cells seeded onto the 24-well plate is an important factor that influence T cell activation and proliferation. It is highly recommended to seed 0.2–0.3 million naïve cells per well in a 24-well plate. If the seeding density is too low, T cell activation or proliferation will be dampened resulting in the lower yield of fully activated T cells in a 3-day culture experiment. If the cell number is low, culture for one extra day is recommended. If T cells are cultured for more than 4-day, large number of cell death could appear due to activation-induced cell death. In addition, the dosage of CD3 and CD28 antibodies can be titrated to induce optimal T cell activation and proliferation.

CD3 and CD28 antibody titration:

- Titration of antibody is recommended before conducting the actual experiment. For typical murine T cells, it is recommended to start coating the culture plate with 0.5  $\mu\text{g}/\text{mL}$  of anti-CD3 and gradually increase the dose to 1  $\mu\text{g}/\text{mL}$ , 2  $\mu\text{g}/\text{mL}$ , 5  $\mu\text{g}/\text{mL}$ , and 10  $\mu\text{g}/\text{mL}$ .
- With each different anti-CD3 concentration, investigators can vary the concentrations of anti-CD28. We recommend starting from 0.2  $\mu\text{g}/\text{mL}$  of anti-CD28 and gradually increasing the dose to 0.5  $\mu\text{g}/\text{mL}$ , 1  $\mu\text{g}/\text{mL}$ , 2  $\mu\text{g}/\text{mL}$ , 5  $\mu\text{g}/\text{mL}$ , and 10  $\mu\text{g}/\text{mL}$ .
- Choose the best combination of anti-CD3 and anti-CD28 doses that results in the most robust T cell proliferation based on the cell number.

### Problem 4

Cell viability is low after SLO-mediated cell membrane permeabilization (step 32 in the section of [streptolysin-O titration for cell membrane recovery efficiency](#)).

### Potential solution

It is critical to titrate the dosage of SLO and incubation time for the specific cell type before carrying the full-scale experiments. It is important to use the combination of the optimal SLO dosage and incubation time that produce the best membrane permeabilization efficiency and the most viable cells after recovery. This is also a critical step to make sure that cells will produce robust responses after SLO-induced membrane permeabilization.

### Problem 5

The background noise of the readouts is high such as a high basal level of protein phosphorylation (step 21 in the section of [SLO-mediated T-cell membrane permeabilization and substance \(e.g., ATP\) supplementation and their impact on acute TCR signaling as assessed by immunoblotting](#)).

### Potential solution

It is important to assess the phosphorylation dynamics of the particular protein before proceeding with the protocol. Determine the best time frame for detecting the strongest phospho-signal and the time range when phospho-signals start to decline. Usually, it is important to rest the cell samples in a non-stimulating environment for 3-h before re-stimulating the cells again. This step is to ensure that phospho-signals decline to the lowest level before re-stimulating again.

### Problem 6

Readouts detected by immunoblotting or immunofluorescent imaging are weak (step 21 in the section of SLO-mediated T-cell membrane permeabilization and substance (e.g., ATP) supplementation and their impact on acute TCR signaling as assessed by immunoblotting, or step 39 in the section of SLO-mediated T-cell membrane permeabilization and substance (e.g., ATP) supplementation and their impact on acute TCR signaling as assessed by immunofluorescent microscopy).

### Potential solution

The protocol is designed to assess acute biochemical reactions such as phosphorylation which is subjected to a negative feedback mechanism in a short time frame. Therefore, it is not only critical to determine the best time point to harvest the sample after re-stimulation, but also to preserve the samples with signal-fixation agents such as 4% PFA immediately after re-stimulation to minimize the loss of signals.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ming O. Li ([lim@mskcc.org](mailto:lim@mskcc.org)).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

The data that supports the findings of this study are available upon reasonable request.

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### AUTHOR CONTRIBUTIONS

K.X. and M.O.L. designed the study. K.X. carried out the experiments. K.X. prepared the figures. K.X. and M.O.L. wrote the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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