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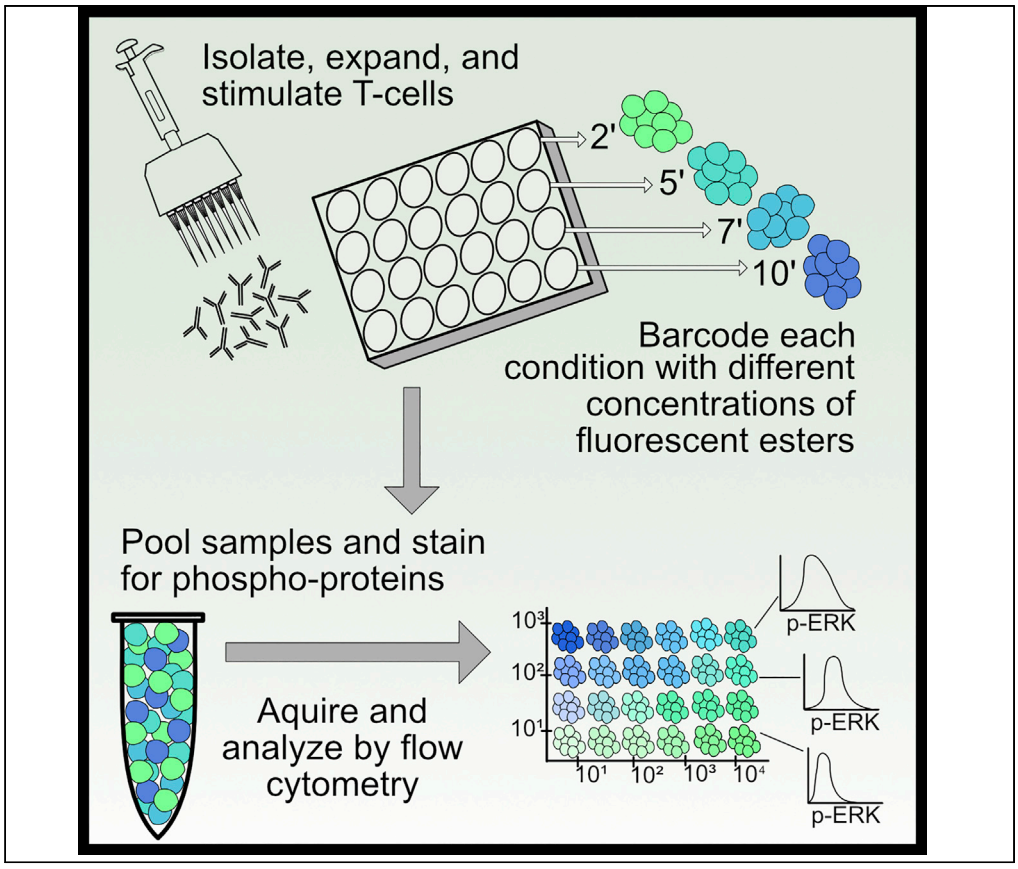
Protocol for Barcoding T Cells Combined with Timed Stimulations



Stimulation of naive T lymphocytes via the T cell receptor (TCR) induces distinct phosphorylation patterns that can be used to explore various signaling pathways within the cell. This protocol can be used to characterize different perturbations to the signaling pathways and the variations in time of stimulation. Here, we provide amethod of barcoding and consolidating amaximum of 24 different sample conditions using two florescent dyes. This single sample for phospho-staining and flow cytometry saves time and reagents.

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HIGHLIGHTS

Quantitative signaling analysis in primary T cells

Barcoding protocol for batch-wise processing

Minimizing experimental variation through batch experiments

A phospho-flow method amendable to other cell types

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SUMMARY

Stimulation of naive T lymphocytes via the T cell receptor (TCR) induces distinct phosphorylation patterns that can be used to explore various signaling pathways within the cell. This protocol can be used to characterize different perturbations to the signaling pathways and the variations in time of stimulation. Here, we provide a method of barcoding and consolidating a maximum of 24 different sample conditions using two florescent dyes. This single sample for phospho-staining and flow cytometry saves time and reagents.

For complete details on the use and execution of this protocol, please refer to Krutzik and Nolan (2006), Krutzik et al. (2012), Vercoulen et al. (2017), Ksionda et al. (2018), and Myers et al. (2019).

BEFORE YOU BEGIN

Coat Plate with Antibody

Timing: 15 min

1. On the day before the start of the experiment (day 0), coat a tissue culture-treated 24-well plate with α-CD3 antibody.
   a. Add 500 μL to each well at a concentration of 10 μg/mL, diluted in PBS.
2. Wrap plate in parafilm to prevent evaporation and place at 4°C for 12–24 h.

Note: Plate can be incubated for 2 h at 37°C for same-day experiment.

Prepare Reagents

Timing: 30 min

3. Prepare FACS buffer, MACS buffer, and T cell media according to MATERIALS AND EQUIPMENT, and store at 4°C.
4. Prepare a new stock of 8% paraformaldehyde (PFA) in PBS, and store at 25°C in the dark.

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Note: Fresh preparation of PFA is critical for its proper use.

5. Prepare a 90% methanol solution in ddH_2O, and store at −20°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD4 (fluorophore-conjugated, anti-mouse)	Tonbo Biosciences	Clone RM4-5
CD11b (fluorophore-conjugated, anti-mouse)	BD Biosciences	Clone M1/70
CD25 (fluorophore-conjugated, anti-mouse)	Tonbo Biosciences	Clone PC61
CD44 (fluorophore-conjugated, anti-mouse)	Tonbo Biosciences	Clone IM7
CD62L (fluorophore-conjugated, anti-mouse)	BD Biosciences	Clone Mel-14
B220 (fluorophore-conjugated, anti-mouse)	BD Biosciences	Clone RA3-6B2
CD3 (purified, anti-mouse)	UCSF Antibody Core	Clone 145-2C11
CD28 (purified, anti-mouse)	UCSF Antibody Core	Clone 37.51
Goat anti-Armenian Hamster IgG (H+L) secondary	Jackson Immunoresearch	Cat# 127-005-099; RRID: AB_2338971
pERK (P-44/42 MAPK) (unconjugated, anti-mouse)	Cell Signaling Technologies	Cat#4377; RRID: AB_331775
Donkey anti-Rabbit IgG secondary (APC conjugated)	Jackson Immunoresearch	Cat# 711-136-152; RRID: AB_2340601
Chemicals, Peptides, and Recombinant Proteins		
Recombinant murine IL-2	Peprotech	Cat# 212-12
Alexa Fluor488 Succinimidyl Ester	Thermo Fisher Scientific	Cat# A20000
Pacific Blue Succinimidyl Ester	Thermo Fisher Scientific	Cat# P10163
Critical Commercial Assays		
Naive CD4 T cell Isolation Kit (mouse)	Miltenyi Biotec	Cat# 130-104-453

MATERIALS AND EQUIPMENT

FACS Buffer

Reagent	Final Concentration	Add to 500 mL FACS Buffer
Dulbecco's Phosphate Buffered Saline (DPBS), Calcium-, Magnesium-free	n/a	483.5 mL
Fetal Bovine Serum (FBS)	2% (v/v)	10 mL
EDTA (0.5 M)	2 mM	2 mL
Sodium Azide (10%) (v/v)	0.09% (v/v)	4.5 mL

Store at 4°C.

MACS Buffer

Reagent	Final Concentration	Add to 500 mL MACS Buffer
Dulbecco's Phosphate Buffered Saline (DPBS), Calcium-, Magnesium-free	n/a	498 mL
Bovine Serum Antigen (BSA)	0.5% (w/v)	2.5 g
EDTA (0.5 M)	2 mM	2 mL

Filter sterilize the MACS Buffer (0.2 μm filter or smaller). Store at 4°C.

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Primary T Cell Media

Reagent	Final Concentration	Add to 500 mL RPMI
RPMI 1640 Media	n/a	500 mL
Fetal Bovine Serum (FBS)	10% (v/v)	50 mL
MEM Non-Essential Amino Acids (100×)	1% (v/v)	5 mL
Sodium Pyruvate (11 mg/mL)	0.11 mg/mL	5 mL
HEPES (1 M)	2.5 mM	5 mL
Penicillin-Streptomycin-Glutamine (100×)	1% (v/v)	5 mL
Beta-mercaptoethanol (55mM)	55 μM	0.5 mL

Store at 4°C.

STEP-BY-STEP METHOD DETAILS

Harvest and Plate Mouse CD4$^+$ T Cells

Timing: 2 h bench time, 2.5 days incubation time

T cells are harvested, purified, and then activated to allow proliferation for 48 h via α-CD3/ α-CD28 stimulation.

Note: We use four mice in this protocol, which could represent replicates and/or different genotypes. This sample size can be adjusted, keeping in mind that the total barcoding pool is optimized for 24 samples, which includes six time points of stimulation for each of these four mice (6 × 4 = 24).

1. Euthanize four mice with CO_2 according to animal facility guidelines.
2. Isolate and pool cervical, brachial, axillary, and inguinal lymph nodes (LN) in one well per mouse (Figure 1A).
   a. Add LN to a 70 μm filter in a tissue culture-treated 6-well plate, with 6 mL sterile MACS Buffer (Figure 1B).

Note: Keep reagents and samples on ice as much as possible.

Note: Mice can be dissected outside of sterility, but all future steps should be performed in a laminar flow hood with aseptic technique.

3. Use the plunger from a 1 mL syringe to mash LN through each 70 μm filter per mouse.
4. Collect buffer in separate 15 mL falcon tubes per mouse and centrifuge for 5 min at 500 × *g*.

Note: Centrifugation at 4°C is ideal, however spins at 25°C are sufficient.

5. Resuspend pellets in 5 mL MACS Buffer and count cells.
   a. Add 1 mL first with p1000 pipette to break up the pellet before adding additional 4 mL.
      i Use p20 pipette to remove any fatty clumps in suspension before counting cells.

Optional: Reserve a small aliquot of cells before MACS purification for a purity check of the T cell isolation. Keep on ice.

Note: The following protocol is adapted from the Miltenyi Biotec MACS Naive CD4$^+$ T Cell Isolation Kit mouse protocol, with few modifications. Cell numbers are adjustable, with a maximum of 2 × 10^9 cells per column.

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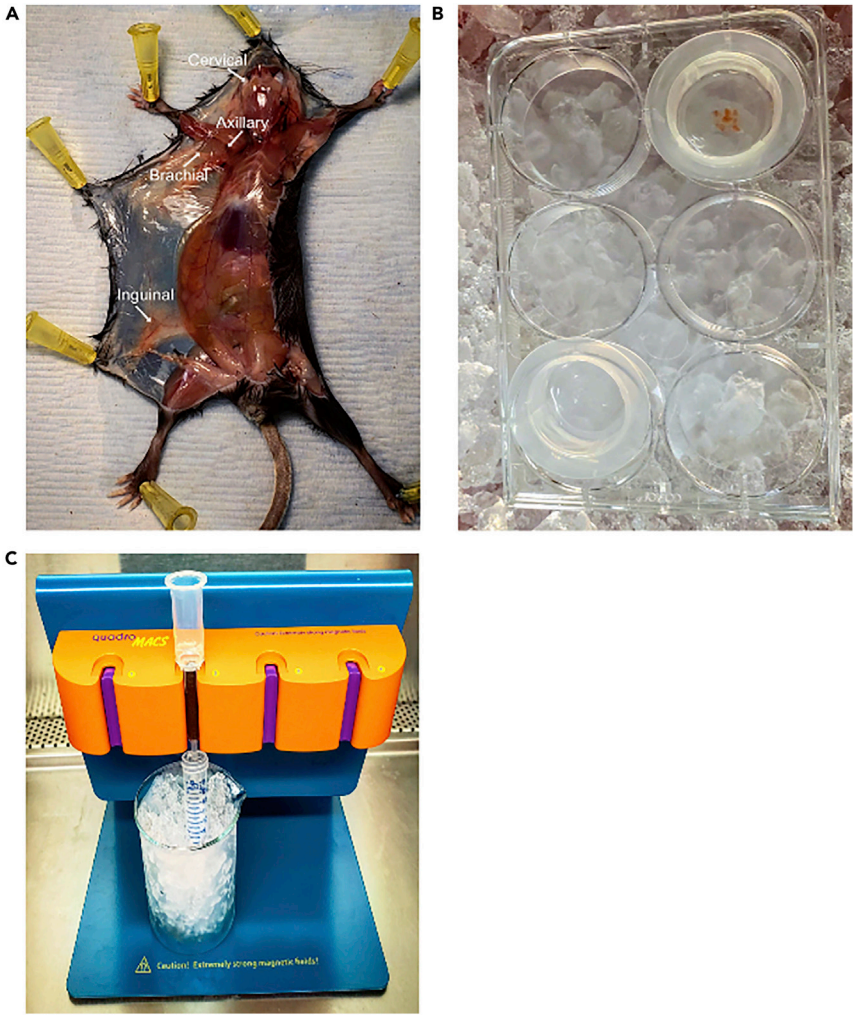

Figure 1. Isolating T Cells from Mouse Lymph Nodes

(A) Anatomical location of cervical, axillary, brachial, and inguinal lymph nodes.

(B) 70 μm filter within a 6-well plate on ice to pool and mash lymph nodes.

(C) MACS separation magnet with a beaker of ice for cold collection.

6. Spin down suspension for 5 min at 500 × *g*.
7. Resuspend cell pellet in 40 μL MACS Buffer per 10^7 total cells.
8. Add 10 μL Biotin-Antibody Cocktail per 10^7 total cells.
9. Mix well by pipetting or flicking tube and incubate for 5 min in a 4°C refrigerator.

   Note: Increased incubation times may lead to nonspecific binding and working on ice may increase incubation times.

10. Add 30 μL MACS Buffer per 10^7 total cells.
11. Add 20 μL α-Biotin Microbeads per 10^7 total cells.
12. Mix well by pipetting or flicking tube and incubate for 10 min in a 4°C refrigerator.
13. During final 10-min incubation, prepare the LS columns by placing on the magnet and adding 3 mL MACS Buffer (Figure 1C).
    a. Discard the flow through.

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Note: Minimum volume for magnetic separation is 500 μL, so bring up the cell suspension volume with MACS Buffer, if necessary.

14. Apply the cell suspension to the column and let it flow by gravity.
    a. Collect the flow-through in 15 mL falcon tubes and keep on ice (Figure 1C).
        i. This contains the unlabeled $CD4^+$ T cells.
15. Wash the column by applying 3 mL of MACS Buffer and collecting the flow-through in the same collection tube.
16. Count the $CD4^+$ T cell flow-through.
17. Add up to 15 mL of cold T cell media and wash enriched cells by centrifuging for 5 min at 500 × *g*.

Optional: Take a small aliquot of cells after MACS purification for a purity check of the T cell isolation. Stain these cells along with the aliquot of cells from the pre-purification with fluorophore-conjugated CD4, CD11b, CD25, CD44, CD62L, and B220 antibodies. Acquire these samples on the flow cytometer to ensure a high purity of naive $CD4^+$ T cells post-T cell enrichment.

18. Resuspend cells at 4×10^6 cells/mL.
19. Add soluble α-CD28 antibody at a concentration of 5 μg/mL to the $CD4^+$ T cells.
20. Using the pre-coated 24-well plate from BEFORE YOU BEGIN steps 1 and 2, wash once with PBS.

Note: Vacuum out liquid from coated plate and add PBS to the walls of the wells to wash. Vacuum out PBS again, right before adding cells, without allowing the wells to dry.

21. Transfer 500 μL of cells to each pre-coated well (2×10^6 cells/well).

Note: Use multiple wells per mouse as number of T cells recovered per mouse should exceed 2×10^6.

22. Let cells activate and proliferate for 48 h in cell incubator (37°C, 5% CO_2).
23. 48 h post-stimulation collect cells from wells with p1000 pipette in 15 mL falcon tubes and wash cells with T cell media to remove leftover antibodies.
    a. Add 10 mL T cell media to cells and centrifuge for 5 min at 500 × *g* to wash.

Note: Cells will attach to plate. Pipet up and down for maximal cell recovery.

Note: Can pool wells from the same mouse that were previously split in step 21.

24. Resuspend cells at 1×10^6 cells/mL and add mIL-2 (100 U/mL).
    a. Resuspend a maximum of 5×10^6 cells in 5 mL of media in a well of a tissue culture-treated 6-well plate.
25. Incubate cells at 37°C for 12–16 h in T cell media supplemented with mIL-2.

Timed T Cell Receptor Stimulations

Timing: 2 h

Expanded $CD4^+$ T cells are rested in plain RPMI, then their T cell receptor (TCR) is stimulated with an α-CD3 antibody and crosslinked with an IgG antibody for varying amounts of time before the reaction is stopped and the cells are fixed with paraformaldehyde (PFA) and cell membranes are permeabilized with methanol.

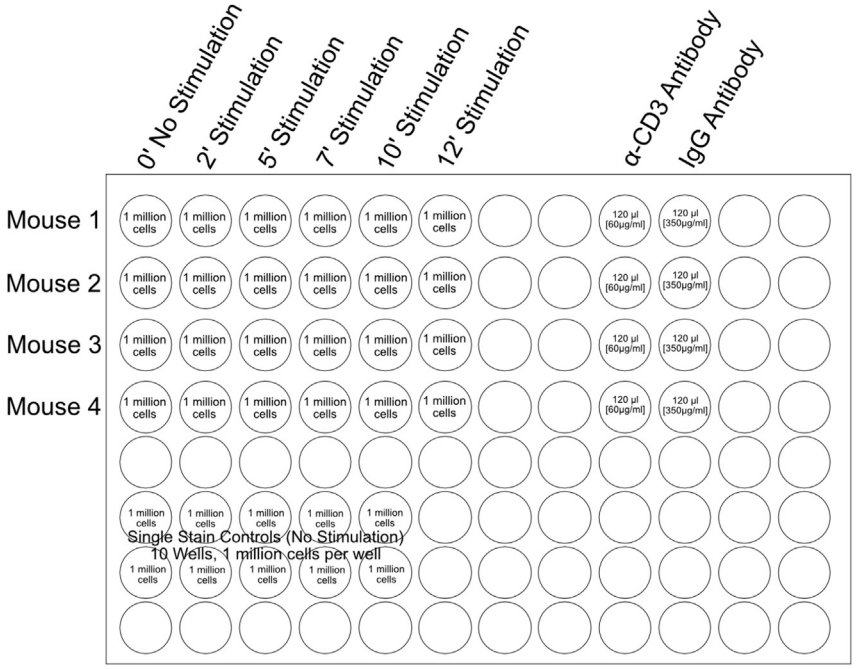

Figure 2. Plate Set-up for T Cell Stimulation

Example layout for acute TCR crosslinking of 24 test samples in a 96-well plate. Locations of the test samples and single stain controls used for flow cytometry setup are outlined. Additional wells are reserved for the stimulatory (a-CD3) and crosslinking (IgG) antibodies, which are added to the test-sample wells during the procedure.

26. Pre-warm plain RPMI to 37°C for use during this section of the protocol.
27. Collect cells from plate and wash by adding up to 15 mL plain RPMI and centrifuging at 25°C for 5 min at 500 × *g*.
    a. Repeat wash two times.

⚠ CRITICAL: Use plain RPMI throughout this section, without FBS and without supplements, to allow the cells to fully rest at their basal state.

28. Determine the time points for stimulation, i.e. −12 min (12 min), 10 min, 7 min, 5 min, 2 min, and 0 min (no stimulation).

Note: The number of time points that can be used depends on the number of mice chosen previously. As this protocol is optimized for 24 samples, the number of time points (6) is replicated for each mouse (4) and has to be at or below 24.

29. Resuspend cells in plain RPMI at 10^7 cells/mL.
30. Plate cells in a tissue culture-treated 96-well round-bottom plate by adding 100 µL per well (10^6 cells/well) for each of the six stimulation time points (Figure 2).
    a. Plate the cells across a row (1–6) to allow for the use of a multichannel pipette.
    b. Plate the cells from each mouse one right below the other (Rows A, B, C, and D for Mouse 1, 2, 3, and 4, respectively).

31. Plate ten extra wells for use as single stain dye controls.

Optional: In the case of low cellularity, it is sufficient to make just two single stain control wells for the strongest dilutions of the dyes.

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Note: These single stain control wells will be unstimulated and will receive the same treatment as the 0-min timepoint wells in step 43.

32. Incubate the cells in the plate at 37°C for 1 h to allow them to rest in plain RPMI and return to basal signaling.
33. While cells are resting, prepare 20 μL of murine α-CD3 antibody per well (480 μL total) for stimulation at a final concentration of 10 μg/mL.
    a. The working concentration for the murine α-CD3 antibody on the cells is 10 μg/mL, so make a 6× stock solution of α-CD3 at 60 μg/mL.
34. Prepare 20 μL of the crosslinking IgG antibody per well (480 μL total) for stimulation at a final concentration of 50 μg/mL.
    a. The working concentration for the crosslinking IgG antibody on the cells is 50 μg/mL, so make a 7× stock solution of IgG at 350 μg/mL.

Note: Each well will receive 20 μL of each antibody, except the 0 min timepoint (no stimulation=no antibody, so the extra well preparation will account for any evaporation or pipetting error).

35. Plate the α-CD3 antibody in preparation for the stimulation, in wells separate from the cells (Figure 2).
    a. Plate 120 μL of the antibody in each of four wells down a column to match the number of mice replicates present.
36. Plate the IgG antibody in wells directly next to the α-CD3.
    a. Plate 120 μL of the antibody in each of four wells down a column to match the number of mice replicates present.

Note: Antibodies can be plated on the same 96-well plate as the resting cells, to reduce waste of an additional plate. Be sure to leave empty columns between the resting cells and the antibodies to prevent splash into timepoint wells.

37. After the cells have rested for 1 h at 37°C, remove from incubator and set-up a timer to count down, starting at the longest timepoint: 12-min stimulation.
    a. Set the timer to 12 min 30 s.

Note: Because of the secondary crosslinking antibody (IgG), the stimulation will be performed by first adding the α-CD3 antibody and 30 s later, adding the IgG.

38. Using a multichannel pipette, add 20 μL of the primary α-CD3 antibody to the column of 12-min timepoints for each of the four mice (Column 6).
    a. Pipet up and down twice to mix.
39. Start the 12 min 30 s countdown timer.
40. When the timer reaches 12 min (30 s after the addition of the α-CD3 antibody), add 20 μL of the IgG to the same column of 12-min timepoints.
    a. Pipet up and down twice to mix.
41. Repeat steps 38–40 to continue with timepoints 10-min to 2-min, adding the primary α-CD3 antibody to the wells 30 s before the actual timepoint is reached.

Note: It is important to add all antibodies to the same column at the same time and mix consistently to reduce variations in the stimulation.

Note: Place the stimulation plate back in the 37°C incubator during wait times to maintain cells at physiological temperature as much as possible.

42. After the countdown reaches 0, immediately add 50 μL of 8% PFA to wells column-wise which have been stimulated to stop the reactions, taking care to add the PFA to all the wells of a single column at the same time.
    a. Always stop the shorter timepoints first to avoid larger variations – so 2-min time point first (Column 2), then 5 min, 7 min, 10 min, and lastly, the 12-min timepoint (Column 6).
    b. Pipet up and down twice to mix.

Note: 8% PFA stock should not be placed in the 96-well plate with the cells. Use a multichannel pipet basin to hold the PFA immediately before stopping the reactions.

43. Finally, add 40 μL of plain RPMI to the 0-min timepoint (Column 1) and then immediately 50 μL of 8% PFA.

Note: The 0-min time point receives no stimulation, so add cell media in place of antibody, and then immediately add 8% PFA.

Note: Address the 0 min column last (Column 1), after the addition of PFA into the wells that had been stimulated, as the timing is not critical for these wells given that they did not receive stimulation.

44. Repeat step 43 for the ten single stain control wells.
45. Incubate plate at 25°C for 20 min.
46. Centrifuge the plate at 1,100 × *g* for 2 min and discard the supernatant by quickly dumping it upside down, flicking the liquid into a waste container, and blotting the plate once on a paper towel to dry out the edges and avoid spillover between wells.

Note: Step 46 requires the proper disposal of PFA into a designated hazardous waste container. If needed, supernatant can be aspirated out for the proper disposal of the PFA.

47. Add 200 μL of PBS to all the wells for two washes, referring to step 46.
48. Gently tap on the sides of the plate to loosen the pellets.
49. Add 200 μL of ice-cold 90% methanol drop-wise to each well.

Note: Do not pipet methanol up and down, as this will shear the cell membranes.

50. Store plate at −20°C for 12–16 h, until barcoding the following day.

Pause Point: Plate can be stored at −20°C for a week before barcoding.

Preparing Barcoding Dyes and Color Combinations

Timing: 30 min

Follow Table 1 to make 50× dilutions of two staining dyes (AlexaFluor488 and Pacific Blue) to create ten different dye dilutions which will be used to make 24 distinct two-color combinations for the respective number of samples.

Optional: Different combinations and concentrations of various dyes can also be used, with empirically designed titrations.

Optional: Make the 50× dyes in PCR tube strips, as it makes it possible to use a multichannel pipette in the later steps.

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Table 1. Dye Dilutions

AlexaFluor488 Tubes (Highest to Lowest Concentration)	DMSO Volume (µL)	Add from Previous Tube (µL)	50× Concentration (µg/mL)	Dilution Factor (from Previous Tube)
A1	24.8		15	1.33
A2	53.2	26.8	5	3
A3	58.4	21.2	1.3	3.8
A4	61.2	18.8	0.3	4.3
A5	60.0	20.0	0.075	4
A6	44.8	14.0	0.018	4.2
Pacific Blue Tubes (Highest to Lowest Concentration)	DMSO Volume (µL)	Add from previous tube (µL)	50× Concentration (µg/mL)	Dilution Factor (from previous tube)
P1	20.0		2000	1.25
P2	83.6	16.4	325	6.15
P3	90.8	9.2	30	10.83
P4	70.0	10.0	3.75	8

Table detailing the set-up and serial dilutions of the Alexa Fluor488 and Pacific Blue stock dyes.

51. Begin by adding the appropriate volume of dimethyl sulfoxide (DMSO) into each of 10 PCR tubes, according to Table 1 "DMSO Volume".
52. Label the tubes based on the different dyes used as well as the concentrations of each dye.
    a. The labeling strategy here is A1, A2, …, A6 and P1, P2, P3, P4 representing the six Alexa Fluor488 (AF488) tubes and the four Pacific Blue (PacBlue) tubes, respectively.
53. To prepare A1, add 75.2 µL of the commercially available AF488 to 24.8 µL of DMSO into the first PCR tube (A1) as a 1.33 times dilution (Figure 3A).
    a. Pipet up and down twice to mix.

54. To prepare A2, take 26.8 µL from A1, and add it to A2, with the 53.2 µL of DMSO as a 3 times dilution from the previous tube (A1).
    a. Pipet up and down twice to mix.
55. Continue with these serial dilutions according to Table 1 for a total of six tubes.
56. To prepare P1, add 80 µL of the commercially available PacBlue to 20 µL DMSO into the first PacBlue PCR tube (P1) as a 1.25 times dilution.
    a. Pipet up and down twice to mix.
57. Similar to the AF488 dilutions, follow Table 1 and Figure 3A to finish the PacBlue dilutions.
58. To prepare the dye combinations, set up 24 PCR tubes with six tubes across and four tubes down, referring to Figure 3B.
59. Add 88 µL of cold 90% methanol to all tubes.

Note: Methanol will evaporate, so perform these steps right before staining the cells.

60. Assign each dye combination to a specific sample from the experiment.

Optional: Assign dye combination based on Table 2. From the first mouse in the timed stimulation, the 0-min timepoint (Mouse 1, 0 min) will be assigned the strongest dye combination (A1 with P1) and this will represent Staining Tube 1.

61. Arrange the AF488 dilutions (A1-A6) in a row, six across (Figure 3B).
62. Arrange the PacBlue dilutions (P1-P4) in a column, four down.

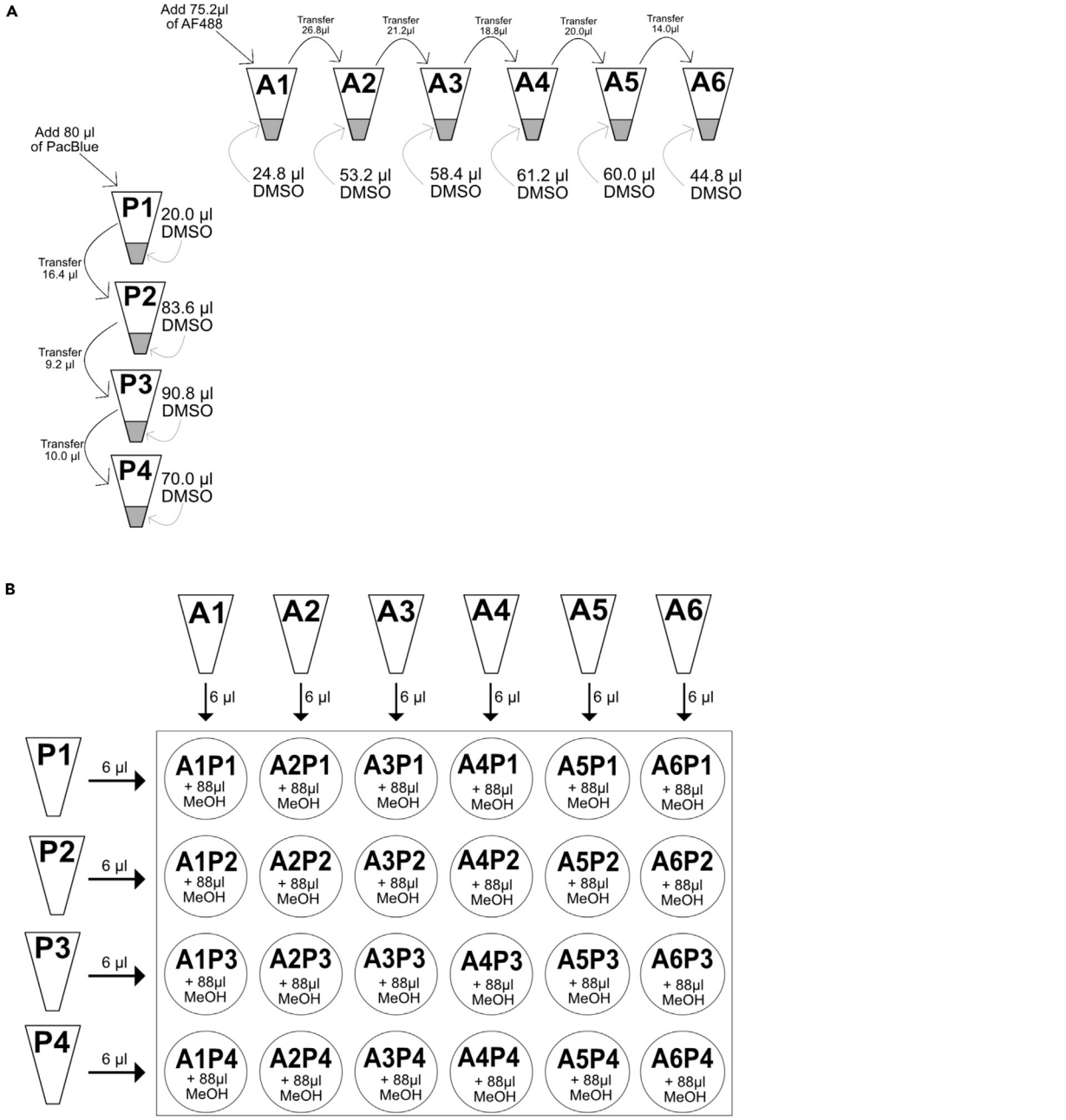

Figure 3. Creating Barcoding Dilutions and Combinations

(A) Cartoon of serial dilutions to prepare six and four stock concentrations of Alexa Fluor488 and Pacific Blue, respectively.

(B) Well set-up to prepare the 24 unique dye combinations for barcoding samples. MeOH: Methanol

63. Using a multichannel pipette, take 6 µL from each of the six stock AF488 tubes (A1-A6) and add them to the first row horizontally of the 24 PCR tubes with methanol (Methods Video S1).
    a. Continue by adding 6 µL of the stock AF488 dilutions to the second, third, and fourth rows of the 24 PCR tubes.

64. Add 6 µL of the four PacBlue stock dilutions (P1-P4) to the first column vertically of the 24 PCR tubes with methanol.

Table 2. Dye to Sample Assignments

Staining Tube	Dye Combination	Sample ID
1	A1P1	Mouse 1 0 min
2	A2P1	Mouse 1 2 min
3	A3P1	Mouse 1 5 min
4	A4P1	Mouse 1 7 min
5	A5P1	Mouse 1 10 min
6	A6P1	Mouse 1 12 min
7	A1P2	Mouse 2 0 min
8	A2P2	Mouse 2 2 min
9	A3P2	Mouse 2 5 min
10	A4P2	Mouse 2 7 min
11	A5P2	Mouse 2 10 min
12	A6P2	Mouse 2 12 min
13	A1P3	Mouse 3 0 min
14	A2P3	Mouse 3 2 min
15	A3P3	Mouse 3 5 min
16	A4P3	Mouse 3 7 min
17	A5P3	Mouse 3 10 min
18	A6P3	Mouse 3 12 min
19	A1P4	Mouse 4 0 min
20	A2P4	Mouse 4 2 min
21	A3P4	Mouse 4 5 min
22	A4P4	Mouse 4 7 min
23	A5P4	Mouse 4 10 min
24	A6P4	Mouse 4 12 min

Table assigning each of the unique dye combinations to individual samples to be analyzed.

a. Continue by adding 6 μL of the stock PacBlue dilutions to the second through sixth columns of the 24 PCR tubes.

Note: This will create the barcoding Staining Tubes 1–24, as well as dilute the stocks from a 50× to a 3× solution for addition to the cells.

Note: It is important when creating these barcoding dyes to pay attention to the dyes added horizontally versus vertically, as well as accurate labeling of the tubes, as this is needed to deconvolve the samples during analysis after flow cytometry.

Optional: If there are less than 24 samples, only create the number of dye combinations appropriate for the number of samples present, starting with the strongest dilutions first.

65. Create 10 single stain controls by adding 6 μL of each of the stock dilutions (A1-A6 and P1-P4) into separate PCR tubes in 94 μL of 90% methanol.

Optional: In case of low cellularity, it is sufficient to make just barcoding dye two single stain controls for flow cytometry compensation, with the strongest dilution of each dye (A1 and P1).

Note: In further steps, there will be two additional controls for flow cytometry compensation: unstained control and the phospho- control.

Barcoding the Samples

Timing: 2.5 h

Permeabilized cells are labeled with the 24 distinct dye combinations and the samples are pooled for labeling of intracellular markers of interest and then prepared for flow cytometry.

66. Retrieve cells stored at −20°C after permeabilization in methanol.
67. Add 50 μL of the corresponding Staining Tubes to each of the 24 wells of permeabilized cells, referring to Table 2 for sample assignment.

Note: Add the dyes drop-wise to cells to mix to avoid shearing cells (Methods Video S2). DO NOT pipet to mix.

Note: Cells are in 100 μL methanol, and do not require a wash step, as 50 μL of the barcoding dyes prepared as a 3× stock in methanol will be diluted appropriately to 1× when added to the existing 100 μL of cells.

68. Add 50 μL of the appropriate single stain controls (A1-A6 and P1-P4) to the unstimulated single stain control wells.
69. Incubate the cells with dyes for 15 min at 25°C and protected from light.
70. Centrifuge the plate at 1,100 × *g* for 2 min and discard the supernatant by quickly dumping it upside down and blotting the plate once on a paper towel to dry out the edges and avoid spilling between wells.
71. Tap on the sides of the plate to loosen the pellets.
72. Add 200 μL of FACS buffer to all the wells for two washes, without pipetting up and down, referring to steps 70 and 71.
73. After final wash, add 200 μL of FACS buffer to the first well *only*.
74. Pipet up and down twice to mix and transfer the whole 200 μL to the next well to pool the cells.
    a. Pipet up and down twice to mix.
75. Continue this process in step 74 to pool all 24 test wells (Methods Video S3).
76. For the single stain controls, pool each color individually by first adding 200 μL of FACS buffer only to the AF488 first single stain control well (A1).
77. Pipet up and down twice to mix and transfer the whole 200 μL to the next well (A2) to pool the cells.
    a. Pipet up and down twice to mix.
78. Continue this process in step 77 to pool all six AF488 single stain controls.
79. Repeat steps 76–78 to pool the four PacBlue single stain controls.

Note: There should be two separate pools for the single stain controls. One containing the six AF488 stains and the other containing the four PacBlue stains. These wells will not receive any additional staining and should be left in 200 μL FACS buffer at 25°C and protected from light until flow cytometry.

80. Divide the pooled sample containing the 24 barcoded wells into two new wells, one for an intracellular phospho-ERK stain and the other well as a secondary-only antibody control.
81. Stain the test well with p-ERK primary antibody by diluting 1 μL of the antibody in 100 μL of FACS buffer and then adding it to an equivalent volume in the test well for a final antibody dilution of 1:200.
82. Add 100 μL of FACS buffer to the secondary-only antibody control well.
83. Incubate wells for 1 h at 25°C, protected from light.
84. Centrifuge the plate at 1,100 × *g* for 2 min and discard the supernatant by quickly dumping it upside down and blotting the plate once on a paper towel to dry out the edges and avoid spilling between wells.

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85. Tap on the sides of the plate to loosen the pellets.
86. Add 200 μL of FACS buffer to the wells for two washes, according to steps 84 and 85.
87. After the final wash, discard the supernatant and tap on the sides of the plate to loosen pellets.
88. Stain both wells with APC-conjugated donkey α-rabbit secondary antibody by diluting 2 μL of the antibody (at 0.5 mg/mL) in 400 μL of FACS buffer and then adding 200 μL of this stain to each well for a 1:200 antibody dilution.
89. Incubate wells for 30 min at 25°C, protected from light.

Optional: Direct APC-conjugated intracellular phospho-ERK antibody can be used in lieu of primary and secondary antibody staining. With this, an isotype control antibody conjugated to the same fluorophore should be used as a negative control for staining and added to the cells at the same concentration as the p-ERK antibody.

Optional: Other fluorescent antibodies can be used instead of APC as long they do not interfere with the barcoding dyes.

90. Centrifuge the plate at 1,100 × *g* for 2 min and discard the supernatant by quickly dumping it upside down and blotting the plate once on a paper towel to dry out the edges and avoid spilling between wells.
91. Tap on the sides of the plate to loosen the pellets.
92. Add 200 μL of FACS buffer to the wells for two final washes, according to steps 90 and 91.
93. After final wash, resuspend both wells in 300 μL FACS buffer and transfer to FACS tubes, along with the two wells of single stain controls.
94. Acquire samples on flow cytometer.
    a. Acquire the single stain controls to enable compensation and define parameters for the barcoded cells, post-acquisition (Figure 4).
    b. Be sure to acquire the APC secondary antibody only control well to account for nonspecific antibody binding.

EXPECTED OUTCOMES

A successful barcoding experiment will yield a flow cytometry plot with 24 distinguishable populations. These populations can be gated independently to deconvolve samples and assess phosphorylated ERK levels. An example of the gating strategies, flow cytometry plots, and phospho-ERK histograms are shown in Figure 5. Figure 5B can be used as a de-barcoding key to identify and characterize the 24 barcoded samples. The most concentrated dye combination (A1P1) stained the cell population that is found in the upper right corner of the flow plot displaying the two barcoding dyes. And the most dilute dye combination (A6P4) is found in the lower left corner of the same plot.

LIMITATIONS

This specific protocol should not be adapted for use with more than 24 samples barcoded together. Changes in the dilutions of the dyes to accommodate larger samples sizes may yield undistinguishable sample groupings within the flow cytometry plot. If additional staining of proteins of interest are desired (i.e. – S6), fluorescent antibodies too close in emission to the barcoding dyes should not be used due to the spectral overlap.

TROUBLESHOOTING

Problem

Purity of $CD4^+$ T cells post-MACS isolation is lower than 95% (from Optional Purity Check, after step 17).

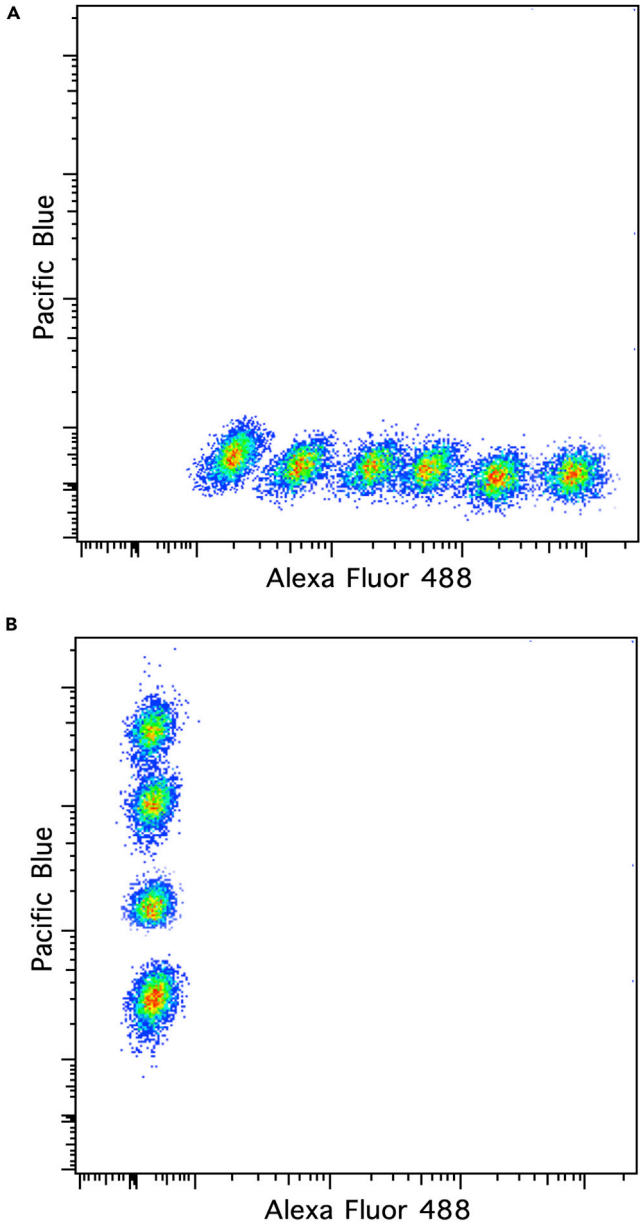

Figure 4. Example Flow Plots of Single Stain Controls

(A) Representative flow cytometry plot of the six Alexa Fluor488 and (B) four Pacific Blue single stain controls optimally spaced for distinguishing between populations.

Potential Solution

Decrease the incubation times during step 9 and step 12 to reduce the non-specific binding of primary and secondary antibodies.

Problem

Low cellularity or viability of expanded T cells (post-stimulation and expansion, step 29).

Potential Solution

Use new α-CD3 and α-CD28 antibodies and fresh mIL-2 for T cell expansion. Avoid freeze-thaw cycles of the mIL-2.

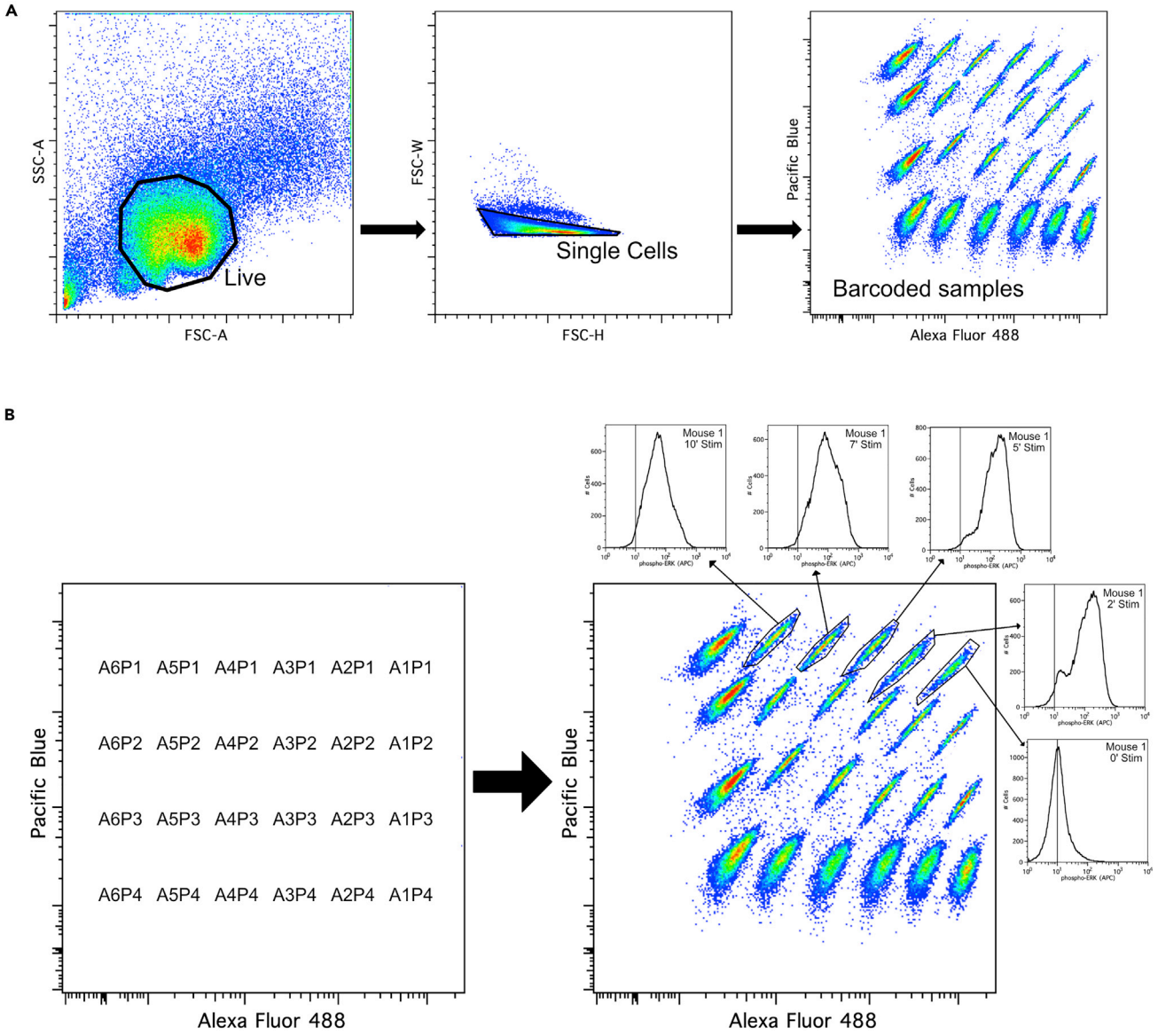

Figure 5. Example Outcome of Barcoding After Flow Cytometry Analysis

(A) Gating strategy identifying the live, single cell, barcoded populations.

(B) Further gating on individual samples using fluorescence intensity to deconvolve the barcoding and reach the targeted data.

Problem

High variation of T cell stimulation between replicates as observed in variations of phospho-ERK levels.

Potential Solution

Practice stimulating cells at precisely the same time with a multi-channel pipette (in steps 38–41) and stopping reactions by thoroughly pipetting to mix the PFA in the wells (step 42), leaving no time variations between wells of the same timepoint.

In addition, increase the time allowed for cells to rest in plain RPMI to ensure they are all at their basal state (step 32).

Problem

Inability to distinguish between some of the 24 barcoded samples on the flow cytometry plot.

Potential Solution

Keep barcoding dyes in the dark as much as possible, after diluting the dyes (steps 53–57), creating the color combinations (steps 63 and 64), and after adding the dyes to the cells (step 67). This will ensure that the fluorescent profiles in the color combinations are as close as possible to the empirically designed panel.

In addition, ensure voltages of lasers are accurately adjusted to create the maximal separation of samples within the plot.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeroen Roose (jeroen.roose@ucsf.edu).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate unique datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100067.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.R.M., O.K., and Y.V.; Writing – Original Draft, K.R.K.; Writing – Review & Editing, K.R.K., D.R.M., and D.R-M.; Funding Acquisition, J.P.R.; Supervision, J.P.R.

DECLARATION OF INTERESTS

J.P.R. is a co-founder and scientific advisor of Seal Biosciences Inc. and on the scientific advisory committee for the Mark Foundation for Cancer Research. All other authors declare no competing interests.

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