Phospho-flow Course Overview, Example Results, and Detailed Manual

This manual is originally from the 2008 EMBO Advanced Flow Cytometry and Cell Sorting course organized by Andreas Thiel in Berlin (http://cwp.embo.org/pc08-15/programme.html). The phospho-flow workshop was taught by Jonathan Irish and June Myklebust. This manual describes three starter signaling experiments using primary human blood and is intended for students already familiar with flow cytometry. For more information, please contact:

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Experiment Overview

Overall Goal: Cytokine stimulate a mixture of cell types in primary human peripheral blood mononuclear cells (PBMCs) and quantify signaling by measuring protein phosphorylation in cell subsets using flow cytometry.

Experiment Outline (see next page for reagents and manual for detailed protocols for each step)

1. There are three different experiment options: a signaling timecourse, a titration of the input stimuli, and a mini-profile. Each has a specific experimental goal, but four tubes overlap in all three groups for global comparisons. Take a look at the experiment overview and decide which variation your group will do.
2. **Obtain cells** – Human peripheral blood cells have been prepared for you and are waiting in the 37 °C incubator. Take a flask of PBMC cells from the incubator (concentration: 1 million / mL; total volume 10 mL).
3. **Aliquot cells into tubes** – Arrange and label tubes to get familiar with your group's experiment. Listed below is each tube, the volume of cells to put into the tube, and the label for that tube's stimulation condition.

Group 1 (Signaling Timecourse)
1. 0.5 mL cells, Unstimulated
2. 0.5 mL cells, 1' IL-4
3. 0.5 mL cells, 2' IL-4
4. 0.5 mL cells, 4' IL-4
5. 0.5 mL cells, 8' IL-4
6. 0.5 mL cells, 12' IL-4
7. 0.5 mL cells, 16' IL-4
8. 0.5 mL cells, Unstimulated
9. 0.5 mL cells, 1' IL-7
10. 0.5 mL cells, 2' IL-7
11. 0.5 mL cells, 4' IL-7
12. 0.5 mL cells, 8' IL-7
13. 0.5 mL cells, 12' IL-7
14. 0.5 mL cells, 16' IL-7
15. 0.5 mL cells, 16' IL-4 + IL-7
16. 0.5 mL cells, 16' IFN-α
17. 0.5 mL cells, Unstimulated

Group 2 (Titration of Stimuli)
1. 0.5 mL cells, Unstimulated
2. 0.5 mL cells, 1/3125X IL-4
3. 0.5 mL cells, 1/625X IL-4
4. 0.5 mL cells, 1/125X IL-4
5. 0.5 mL cells, 1/25X IL-4
6. 0.5 mL cells, 1/5X IL-4
7. 0.5 mL cells, 1X IL-4
8. 0.5 mL cells, Unstimulated
9. 0.5 mL cells, 1/3125X IL-7
10. 0.5 mL cells, 1/625X IL-7
11. 0.5 mL cells, 1/125X IL-7
12. 0.5 mL cells, 1/25X IL-7
13. 0.5 mL cells, 1/5X IL-7
14. 0.5 mL cells, 1X IL-7
15. 0.5 mL cells, 1X IL-4 + 1X IL-7
16. 0.5 mL cells, 1X IFN-α
17. 0.5 mL cells, Unstimulated

Group 3 (Mini-Profile)
1. 1 mL cells, Unstimulated
2. 1 mL cells, IL-4
3. 1 mL cells, IL-7
4. 1 mL cells, PMA
5. 1 mL cells, PMA + ionomycin
6. 1 mL cells, IL-4 + IL-7
7. 1 mL cells, IFN-α
8. 1 mL cells, IL-4 + IL-7 + IFN-α + PMA + ionomycin
9. 0.5 mL cells, Unstimulated

Note: Group 3 will later divide tubes 1-8 in half and stain them with two different antibody staining panels. Groups 1 and 2 are using just one staining panel. This is why Group 3 is using twice the cells during stimulation as the other groups.

4. **Stimulate cells** – Once you have the cells aliquoted into tubes, put the cells back in the incubator and prepare the stimuli. The working stocks of each stimulus each contain 200 µL (enough for each the groups). Below is the summary of what you need for stimulation. Refer to Part 4 in the course manual for more details.

Group 1 (Signaling Timecourse)
1. One tube 1 µg/mL (20X) IL-4
2. One tube 1 µg/mL (20X) IL-7
3. One tube 0.02 µg/mL (20X) IFN- α
4. One tube of 2 mL 16% (10X) PFA

Add 25 µL of each stim to the 500 µL cells for the 16' timepoints. Wait 4 minutes, then add the 12' timpoints.

At time zero, fix each tube by adding 50 µL 16% PFA.

Group 2 (Titration of Stimuli)
1. One tube, 1 µg/mL (20X) IL-4
2. One tube, 1 µg/mL (20X) IL-7
3. One tube, 0.02 µg/mL (20X) IFN- α
4. One tube of 2 mL 16% (10X) PFA

Starting with the 20X stock solutions, prepare four 5-fold serial dilutions. Dilute 10 uL stock into 40 uL of PBS (=1/5X). Dilute 10 uL 1/5X into 40 uL of PBS (=1/25X).

Add 25 µL of each stim to 500 µL cells.

Fix each tube by adding 50 µL 16% PFA at 16 minutes after adding the stimuli.

Group 3 (Mini-Profile)
1. One tube 1 µg/mL (20X) IL-4
2. One tube 1 µg/mL (20X) IL-7
3. One tube 0.02 µg/mL (20X) IFN- α
4. One tube 100 µg/mL (20X) PMA
5. One tube 100 µg/mL (20X) iono.
6. One tube of 2 mL 16% (10X) PFA

Add 50 µL of each stim to 1 mL cells.

Fix each tube by adding 100 µL 16% PFA at 16 minutes after adding the stimuli.

5. **Fix and permeabilize cells** – At the end of the stimulation, fix with para-formaldehyde. Allow cells to fix for 5 minutes at room temperature. Then wash the cells by adding 2 mL PBS to each tube, spin at 800 rcf (2000 RPM) for 5 minutes, check for a pellet, and discard all but ~75 uL of the supernatant (see manual for details). Vortex the pellet to resuspend cells and then add 2 mL of very cold methanol (stored at -20 °C or lower) to permeabilize the cells and permeabilize on ice or in a -20 °C freezer for 10 minutes.

6. **Rehydrate cells** – After pemeabilization, the goal is to get the cells into an appropriate buffer for flow cytometry staining.
   a. Wash the cells by adding 2 mL PBS to each tube, spin at 800 rcf (2000 RPM) for 5 minutes, check for a pellet, and discard the supernatant.
   b. Then wash a second time by adding 2 mL flow cytometry staining media (PBS + 1% BSA) to each tube, spin at 800 rcf (2000 RPM) for 5 minutes, check for a pellet, and discard the supernatant.
   c. While washing, prepare antibody staining panels for the next step.
   d. **Group 3**: After washing, you need to split tubes 1-8 for two antibody staining panels. Label a fresh set of 16 tubes with the same labels as tubes 1-8. Then add the label "P1" to half and "P2" to the other half (indicating Staining Panels 1 and 2). After the last wash, resuspend cells in 100 uL PBS + 1% BSA and then split out
7. **Stain cells for flow cytometry** – Prepare enough 'master mix' of antibody staining panels for your samples. Below is the master mix recipe for each group. Once the antibody staining panel master mix is prepared, add 50 uL of master mix to each tube of 100 uL of cells.

Group 1 (Signaling Timecourse)

Staining Panel 1, stain tubes 1 – 16:
1. 900 µL PBS + 1% BSA
2. 80 µL α-CD5-PE
3. 80 µL α-CD20c-PerCPCy5.5
4. 160 µL α-p-Stat6-Ax488
5. 160 µL α-p-Stat5-Ax647

All Groups: *Don't forget to set aside tube 17, your unstained control!*

Group 2 (Titration of Stimuli)

Staining Panel 1, stain tubes 1 – 16:
1. 900 µL PBS + 1% BSA
2. 80 µL α-CD5-PE
3. 80 µL α-CD20c-PerCPCy5.5
4. 160 µL α-p-Stat6-Ax488
5. 160 µL α-p-Stat5-Ax647

Group 3 (Mini-Profile)

Staining Panel 1, stain tubes 1 - 8:
1. 450 µL PBS + 1% BSA
2. 40 µL α-CD5-PE
3. 40 µL α-CD20c-PerCPCy5.5
4. 80 µL α-p-Stat6-Ax488
5. 80 µL α-p-Stat5-Ax647

Staining Panel 2, stain tubes 9 - 16:
1. 450 µL PBS + 1% BSA
2. 40 µL α-CD5-PE
3. 40 µL α-CD20c-PerCPCy5.5
4. 80 µL α-p-NFkB-Ax488
5. 80 µL α-p-Erk1/2-PECy7
6. 80 µL α-p-Stat1-Ax647

8. **Wash samples and prepare compensation tubes** – Allow staining to proceed for 15 minutes at room temperature in the dark. Then wash the samples by adding 2 mL flow cytometry staining media (PBS + 1% BSA) to each tube, spin at 800 rcf (2000 RPM) for 5 minutes, check for a pellet, and discard the supernatant
9. **Collect samples and upload to the Public Cytobank** – Add 250 uL PBS to the cell pellet and collect. When done, export the FCS files and then upload them to Cytobank (http://cytobank.stanford.edu/public/). See full manual for details. When at the cytometer, you will be collecting 17 tubes with these sample names:

Group 1 (Signaling Timecourse)
1. Unstimulated #1 / 0' IL-4
2. 1' IL-4
3. 2' IL-4
4. 4' IL-4
5. 8' IL-4
6. 12' IL-4
7. 16' IL-4
8. Unstimulated #2 / 0' IL-7
9. 1' IL-7
10. 2' IL-7
11. 4' IL-7
12. 8' IL-7
13. 12' IL-7
14. 16' IL-7
15. IL-4 + IL-7 (16')
16. IFN-α (16')
17. Unstained

All: 1X & Staining Panel 1 (P1)

Group 2 (Titration of Stimuli)
1. Unstimulated #1 / 0 ng/mL IL-4
2. 0.016 ng/mL IL-4 (1/3125X)
3. 0.080 ng/mL IL-4 (1/625X)
4. 0.40 ng/mL IL-4 (1/125X)
5. 2.0 ng/mL IL-4 (1/25X)
6. 10 ng/mL IL-4 (1/5X)
7. 50 ng/mL IL-4 (1X)
8. Unstimulated 21 / 0 ng/mL IL-7
9. 0.016 ng/mL IL-7 (1/3125X)
10. 0.080 ng/mL IL-7 (1/625X)
11. 0.40 ng/mL IL-7(1/125X)
12. 2.0 ng/mL IL-7 (1/25X)
13. 10 ng/mL IL-7 (1/5X)
14. 50 ng/mL IL-7 (1X)
15. IL-4 + IL-7 (1X each)
16. IFN-α (1X)
17. Unstained

All: 16' & Staining Panel 1 (P1)

Group 3 (Mini-Profile)
1. Unstimulated x P1
2. IL-4 x P1
3. IL-7 x P1
4. PMA x P1
5. PMA+ionomycin x P1
6. IL-4 + IL-7 x P1
7. IFN-α x P1
8. IL-4+IL-7+IFN-α+PMA+iono. x P1
9. Unstimulated x P2
10. IL-4 x P2
11. IL-7 x P2
12. PMA x P2
13. PMA+ionomycin x P2
14. IL-4 + IL-7 x P2
15. IFN-α x P2
16. IL-4+IL-7+IFN-α+PMA+iono. x P2
17. Unstained

All: 16' & 1X

Reagent List

Items Needed			*Amount Needed*	*Cat # / Source*
Cells	Human PBMC		18 x [0.5 x 10^6 cells / 500 µL RPMI+10%FBS] = 9 million PBMCs in 9 mL for 18 stims	Buffy coat Ficoll prep
Stimuli	IL-4		50 ng/mL per stim of 1 µg/mL (20X); see Protocol	14-8049-62 / eBiosci.
	IL-7		50 ng/mL per stim of 1 µg/mL (20X); see Protocol	14-8079-62 / eBiosci.
	IFN Universal Type I (IFN-α)		1 ng/mL per stim of 0.02 µg/mL (20X); *Mini-Profile*	11200-1 / PBL Interferon Source
	phorbol 12-myristate 13-acetate (PMA)		5 µg/mL per stim of 100 µg/mL (20X); *Mini-Profile*	P1585-1MG / Sigma
	Ionomycin		5 µg/mL per stim of 100 µg/mL (20X); *Mini-Profile*	I0634-1MG / Sigma
Fix, perm & buffers	para-formaldehyde (PFA)		16 x [10 µL of 16% (10X) PFA], room temp.	15710 / Electron Microscopy Sciences
	cold methanol (-20 °C)		500 mL, kept at -20 °C	
	phosphate buffered saline (PBS)		500 mL, kept at 4 °C	Lab stock
	PBS + 1% BSA for staining		500 mL, kept at 4 °C [other staining media OK]	Lab stock
Lineage antibodies	α-CD20c-PerCPCy5.5		16 x [5 µL/stain]	558021 / BD
	α-CD5-PE		16 x [5 µL/stain]	347307 / BD
Phospho-antibodies	P1	α-p-Stat6(Y641)-Ax488	10 µL per stain, see Protocol	612600 / BD
		α-p-Stat5(Y694)-Ax647	10 µL per stain, see Protocol	612599 / BD
	P2	α-p-NFκBp65(S529)-Ax488	10 µL per stain, see Protocol; *Mini-Profile only*	558421 / BD
		α-p-Erk1/2(T202/Y204)-PECy7	10 µL per stain, see Protocol; *Mini-Profile only*	560116 / BD
		α-p-Stat1(Y701)-Ax647	10 µL per stain, see Protocol; *Mini-Profile only*	612597 / BD
Comp Beads	(+) α-mouse-κ		5 x [10 µL beads per compensation tube]	51-BP80212-01 / BD
	(-) FBS		5 x [10 µL beads per compensation tube]	51-BP80212-02 / BD

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Experimental Results

On the follow pages are the results from the Tuesday afternoon phospho-flow tutorial. These experiments can be viewed on the Public Cytobank and the associated files downloaded, here:

https://cytobank.stanford.edu/public/

Experimental Details: Files Collected for Each Group

Signaling Timecourse
1. Unstimulated #1 / 0' IL-4
2. 1' IL-4
3. 2' IL-4
4. 4' IL-4
5. 8' IL-4
6. 12' IL-4
7. 16' IL-4
8. Unstimulated #2 / 0' IL-7
9. 1' IL-7
10. 2' IL-7
11. 4' IL-7
12. 8' IL-7
13. 12' IL-7
14. 16' IL-7
15. IL-4 + IL-7 (16')
16. IFN-α (16')
17. Unstained

All: 1X & Staining Panel 1 (P1)

Titration of Stimuli
1. Unstimulated #1 / 0 ng/mL IL-4
2. 0.016 ng/mL IL-4 (1/3125X)
3. 0.080 ng/mL IL-4 (1/625X)
4. 0.40 ng/mL IL-4 (1/125X)
5. 2.0 ng/mL IL-4 (1/25X)
6. 10 ng/mL IL-4 (1/5X)
7. 50 ng/mL IL-4 (1X)
8. Unstimulated 21 / 0 ng/mL IL-7
9. 0.016 ng/mL IL-7 (1/3125X)
10. 0.080 ng/mL IL-7 (1/625X)
11. 0.40 ng/mL IL-7(1/125X)
12. 2.0 ng/mL IL-7 (1/25X)
13. 10 ng/mL IL-7 (1/5X)
14. 50 ng/mL IL-7 (1X)
15. IL-4 + IL-7 (1X each)
16. IFN-α (1X)
17. Unstained

All: 16' & Staining Panel 1 (P1)

Mini-Profile
1. Unstimulated x P1
2. IL-4 x P1
3. IL-7 x P1
4. PMA x P1
5. PMA+ionomycin x P1
6. IL-4 + IL-7 x P1
7. IFN-α x P1
8. IL-4+IL-7+IFN-α+PMA+iono. x P1
9. Unstimulated x P2
10. IL-4 x P2
11. IL-7 x P2
12. PMA x P2
13. PMA+ionomycin x P2
14. IL-4 + IL-7 x P2
15. IFN-α x P2
16. IL-4+IL-7+IFN-α+PMA+iono. x P2
17. Unstained

All: 16' & 1X

Experimental Details: Staining Panels

Antibody			***Amount Needed***	***Cat # / Source***
Lineage antibodies		α-CD20c-PerCPCy5.5	5 µL/stain	558021 / BD
		α-CD5-PE	5 µL/stain	347307 / BD
Phospho-antibodies	P1	α-p-Stat6(Y641)-Ax488	10 µL per stain, see Protocol	612600 / BD
		α-p-Stat5(Y694)-Ax647	10 µL per stain, see Protocol	612599 / BD
	P2	α-p-NFκBp65(S529)-Ax488	10 µL per stain, see Protocol	558421 / BD
		α-p-Erk1/2(T202/Y204)-PECy7	10 µL per stain, see Protocol	560116 / BD
		α-p-Stat1(Y701)-Ax647	10 µL per stain, see Protocol	612597 / BD

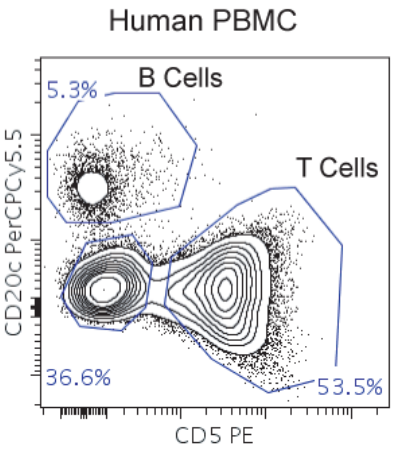
Experiment Results – Titration

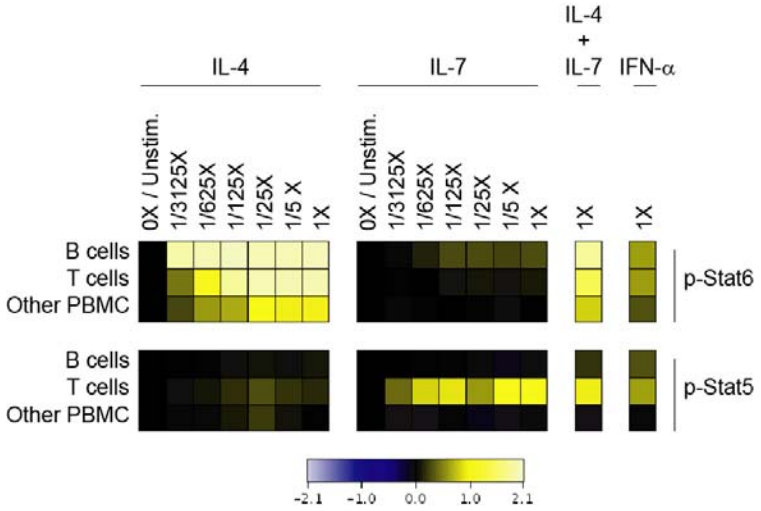
Collected by the second group to use the 'left side' upstairs LSR II
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=112

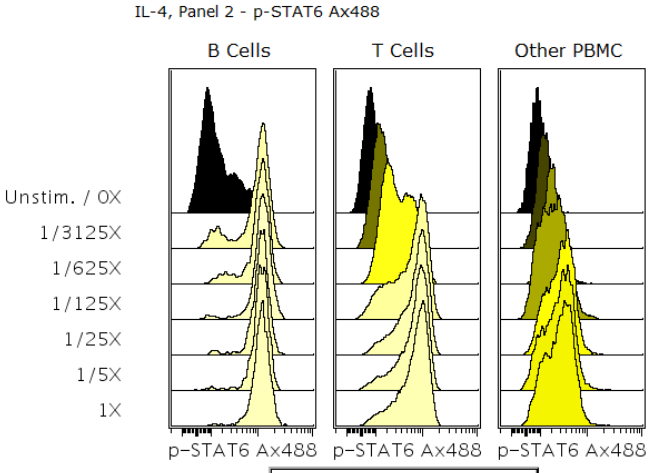
Summary: The majority of PBMC B cells were highly sensitive to IL-4 signaling, followed by T cells and 'other PBMC'. IL-7 signaling was cell type and phospho-protein specific. This experiment suggests that the dose of cytokines used for the course (50 ng/mL) is far above the saturation point for PBMC B and T cells.

IL-4 ► p-Stat6 in B cells, fold change: 2.04 (excellent!)

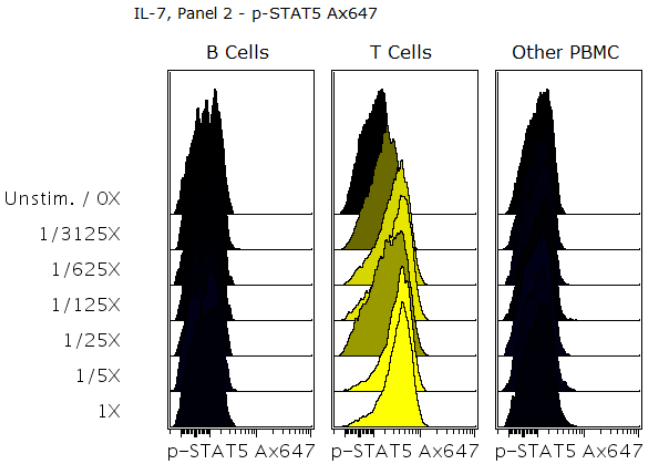
Issues: One of the 17 samples (Tube #1, Unstimulated #1) was unstained, so it was good that there was a backup unstimulated sample! 1/25X IL-7 was a minor outlier – perhaps the stimulus was not completely added? Otherwise, great work!







	B Cells	T Cells	Other PBMC
Unstim. / 0X	0	0	0
1/3125X	1.94	0.49	0.29
1/625X	2	1.16	0.63
1/125X	2.07	1.87	0.7
1/25X	2.01	2	1.05
1/5X	2	1.99	1
1X	2.03	1.99	1.01



	B Cells	T Cells	Other PBMC
Unstim. / 0X	0	0	0
1/3125X	-0	0.44	-0.05
1/625X	-0.01	0.88	-0.06
1/125X	-0.01	0.95	-0.02
1/25X	-0.03	0.63	-0.09
1/5X	-0.09	1.06	-0.07
1X	-0.04	1.07	-0.03

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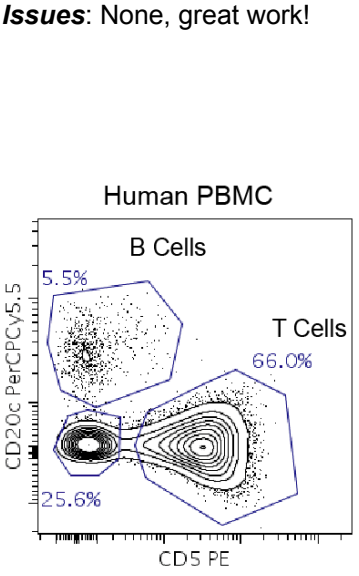
Experiment Results – Timecourse I

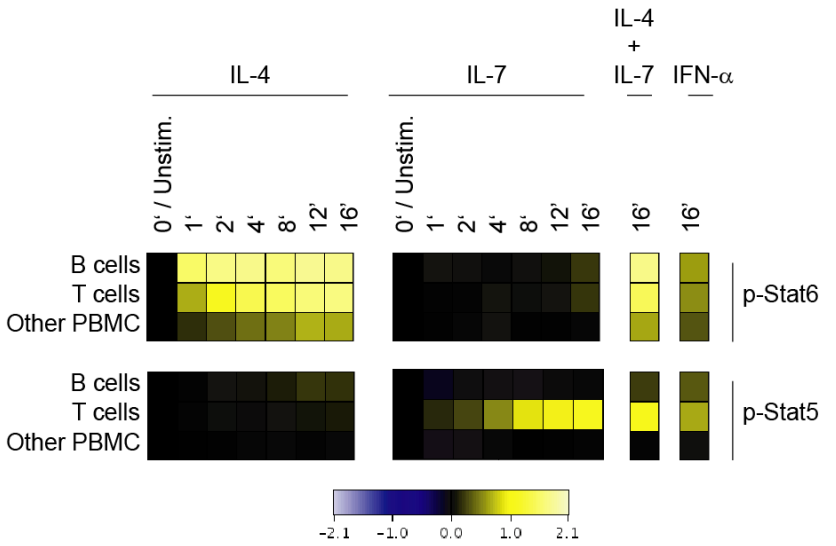
Collected by the first group to use the "left side" upstairs LSR II
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=116

Summary: Very nice timecourse of signaling. Different IL-4 signaling kinetics were observed in PBMC subsets.

Best IL-4 ► p-Stat6 in B cell fold change: 1.77 (great)

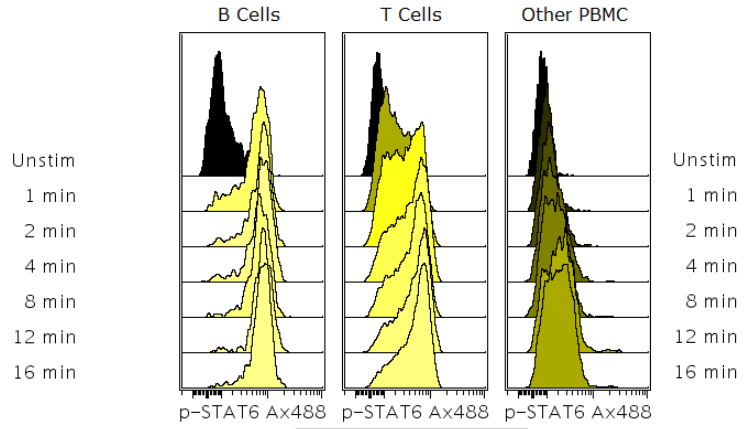
Issues: None, great work!



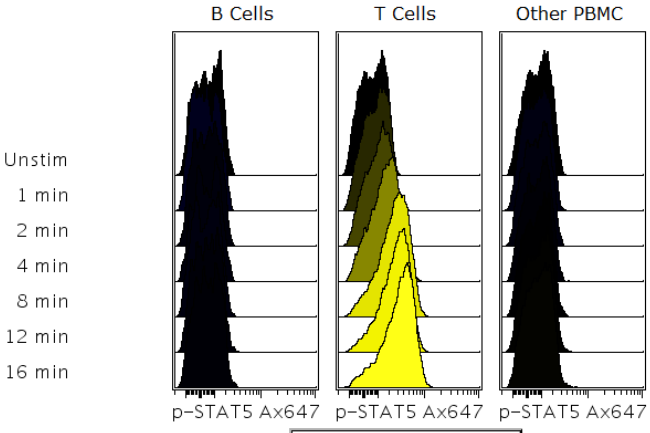


Panel 2 - p-STAT6 Ax488, IL-4

Panel 2 - p-STAT5 Ax647, IL-7



	B Cells	T Cells	Other PBMC
Unstim	0	0	0
1 min	1.59	0.71	0.19
2 min	1.75	1.17	0.32
4 min	1.78	1.47	0.46
8 min	1.7	1.55	0.53
12 min	1.81	1.68	0.73
16 min	1.77	1.72	0.7



	B Cells	T Cells	Other PBMC
Unstim	0	0	0
1 min	-0.12	0.16	-0.07
2 min	-0.04	0.29	-0.05
4 min	-0.05	0.56	-0.02
8 min	-0.06	0.94	-0
12 min	-0.03	1	0.01
16 min	-0.02	1.17	0.02

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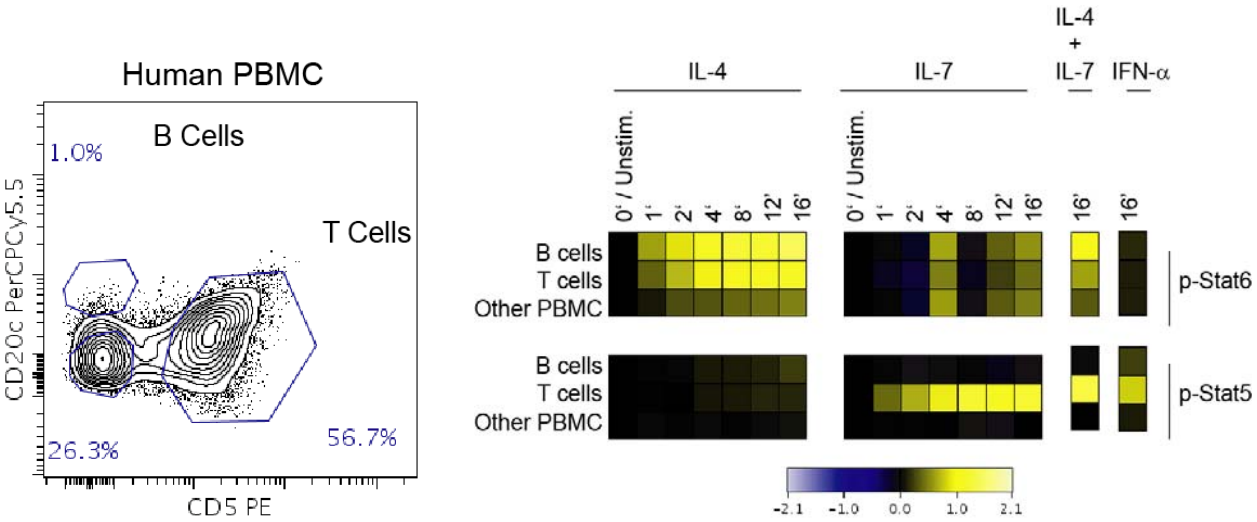
Experiment Results – Timecourse II

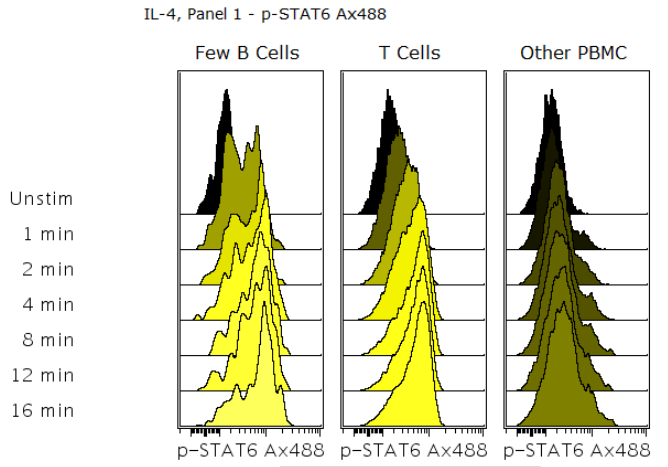
Collected by the first group to use the downstairs LSR II.
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=116

Summary: Good timecourse of signaling. Different IL-4 signaling kinetics were observed in PBMC subsets.

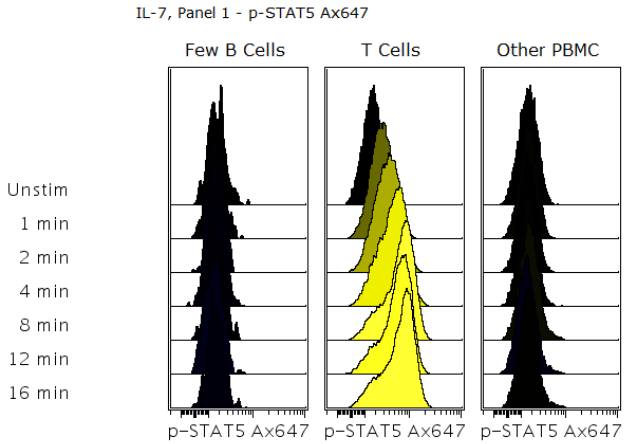
Best IL-4 ► p-Stat6 in B cell fold change: 1.53 (good, but could be better)

Issues: IL-7 / p-Stat6 panel was unexpectedly positive in the 4', 12', and 16' timepoints (but not the 8' timepoint). Lower fold changes than those observed in other groups. CD20 PerCP-Cy5.5 staining was very low or B cells were lost, but a B-cell specific signal was still detectable.





	Few B Cells	T Cells	Other PBMC
Unstim	0	0	0
1 min	0.66	0.4	0.1
2 min	0.94	0.76	0.32
4 min	1.22	1	0.34
8 min	1.34	1.18	0.41
12 min	1.32	1.19	0.46
16 min	1.53	1.22	0.53



	Few B Cells	T Cells	Other PBMC
Unstim	0	0	0
1 min	-0.02	0.43	0.01
2 min	-0.06	0.71	-0.01
4 min	-0.04	0.99	0.01
8 min	-0.02	1.32	0.05
12 min	-0.1	1.21	-0.07
16 min	-0.05	1.33	0

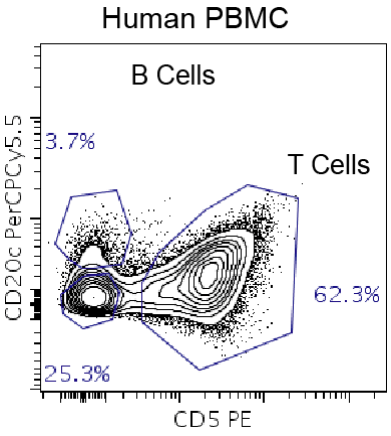
Experiment Results – Mini-Profile III

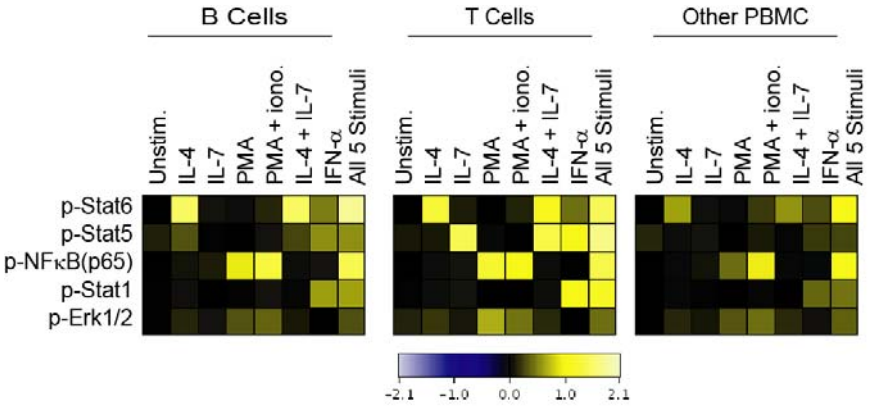
Collected by the second group to use the downstairs LSR II.
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=117

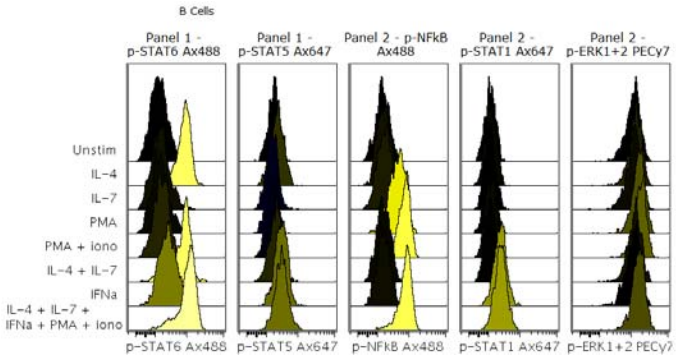
Summary: In general, the expected signaling profile for PBMC was observed. Staining consistency was excellent (there was no problem with 'lower than basal signal'). Different signaling profiles of PBMC subsets were apparent.

Best IL-4 ► p-Stat6 in B cell fold change: 1.56 (good, but could be better)

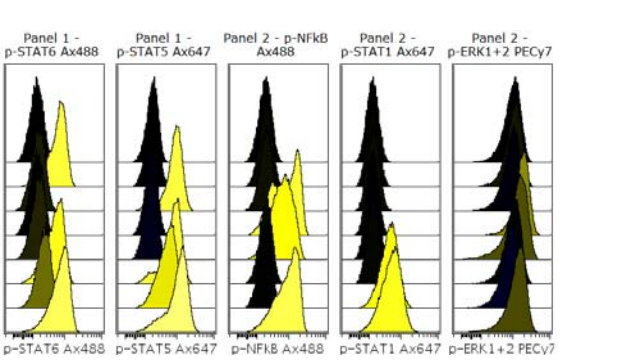
Issues: p-Erk1/2 responses were very weak due to high basal Erk1/2 phosphorylation. Smaller fold change values than other mini-profiles were observed. CD20 PerCP-Cy5.5 staining was very low or B cells were lost, but a B-cell specific signal was still detectable.







	Panel 1 - p-STAT6 Ax488	Panel 1 - p-STAT5 Ax647	Panel 2 - p-NFkB Ax488	Panel 2 - p-STAT1 Ax647	Panel 2 - p-ERK1+2 PECy7
Unstim	0	0	0	0	0
IL-4	1.56	0.21	0.08	0.04	0.14
IL-7	0.07	-0.12	0.11	-0.01	0.05
PMA	0.04	-0.13	0.97	0.01	0.33
PMA + iono	0.15	-0.08	1.39	0.05	0.4
IL-4 + IL-7	1.56	0.15	0.04	0.01	0.09
IFNa	0.51	0.45	0.05	0.63	-0.01
IL-4 + IL-7 + IFNa + PMA + iono	1.85	0.46	1.47	0.65	0.31



	Panel 1 - p-STAT6 Ax488	Panel 1 - p-STAT5 Ax647	Panel 2 - p-NFkB Ax488	Panel 2 - p-STAT1 Ax647	Panel 2 - p-ERK1+2 PECy7
Unstim	0	0	0	0	0
IL-4	1.36	0	0.04	0.02	0.09
IL-7	0.11	1.39	0.06	0.05	-0.05
PMA	-0	-0.07	1.32	-0.01	0.58
PMA + iono	0.14	-0.09	1.06	-0.01	0.33
IL-4 + IL-7	1.16	1.34	0.04	0.03	0.02
IFNa	0.45	0.96	0.01	1.06	-0.14
IL-4 + IL-7 + IFNa + PMA + iono	1.52	1.66	1.46	1.21	0.31

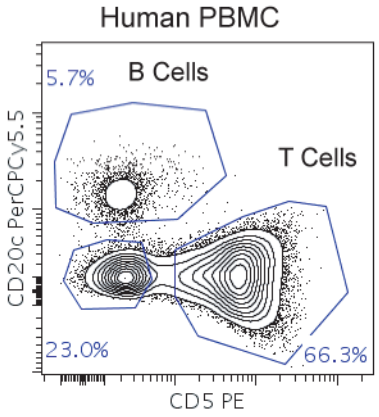
Experiment Results – Mini-Profile I

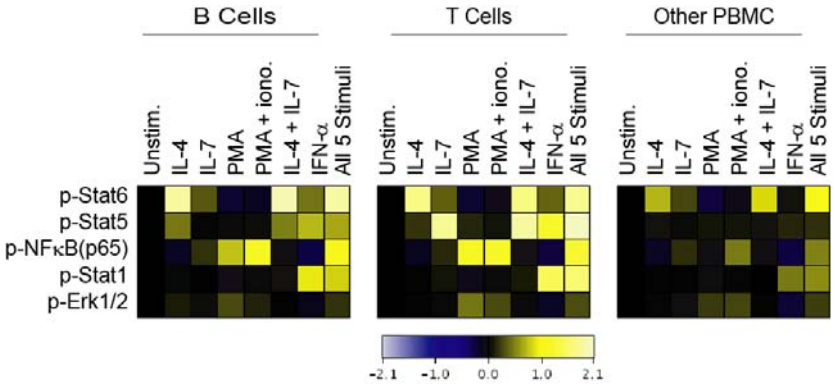
Collected by the first group to use the 'right side' upstairs LSR II
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=113

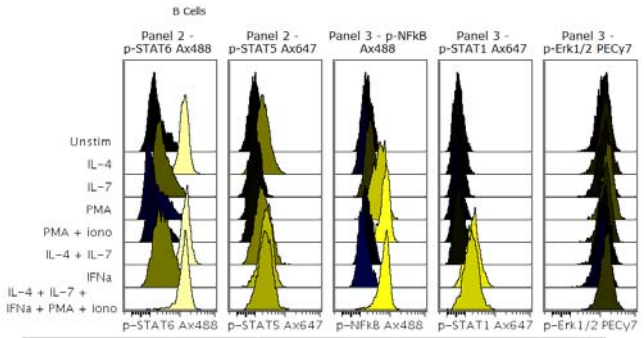
Summary: In general, the expected signaling profile for PBMC was observed. Different signaling profiles of PBMC subsets were apparent.

IL-4 ► p-Stat6 in B cells, fold change: 1.88 (excellent)

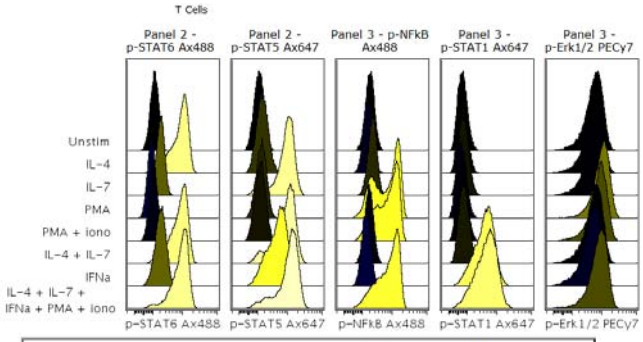
Issues: p-Erk1/2 responses were very weak due to high basal Erk1/2 phosphorylation. Some signals appeared to be lower after stimulation than in the unstimulated sample.







	Panel 2 - p-STAT6 Ax488	Panel 2 - p-STAT5 Ax647	Panel 3 - p-NFkB Ax488	Panel 3 - p-STAT1 Ax647	Panel 3 - p-Erk1/2 PECy7
Unstim	0	0	0	0	0
IL-4	1.88	0.49	-0.14	0.03	0.11
IL-7	0.36	0.02	0.2	0.01	0.04
PMA	-0.2	0.03	0.8	-0.08	0.3
PMA + iono	-0.13	0.04	1.2	-0.03	0.14
IL-4 + IL-7	1.99	0.52	-0.04	0.05	0.02
IFNa	0.47	0.76	-0.27	0.97	-0.11
IL-4 + IL-7 + IFNa + PMA + iono	1.91	0.69	1.16	0.86	0.21



	Panel 2 - p-STAT6 Ax488	Panel 2 - p-STAT5 Ax647	Panel 3 - p-NFkB Ax488	Panel 3 - p-STAT1 Ax647	Panel 3 - p-Erk1/2 PECy7
Unstim	0	0	0	0	0
IL-4	1.75	0.21	-0.13	0.01	-0.02
IL-7	0.39	1.82	0.17	0.08	-0.03
PMA	-0.21	0.15	1.23	-0.08	0.49
PMA + iono	-0.08	0.08	1.26	-0.03	0.3
IL-4 + IL-7	1.72	1.9	-0.06	0.1	-0.03
IFNa	0.41	1.27	-0.25	1.52	-0.16
IL-4 + IL-7 + IFNa + PMA + iono	1.8	2.07	1.33	1.64	0.31

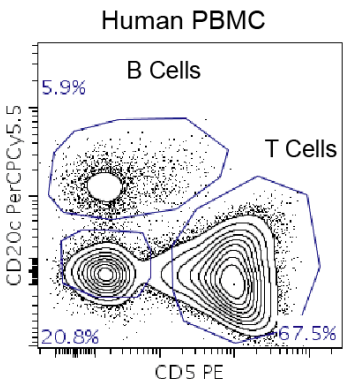
Experiment Results – Mini-Profile II

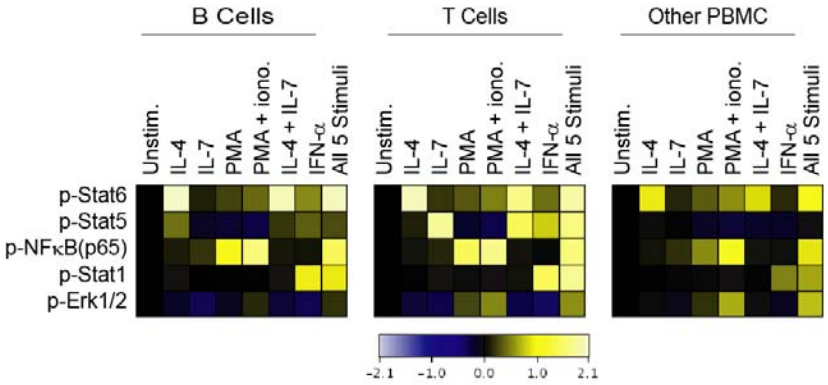
Collected by the second group to use the 'right side' upstairs LSR II
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=114

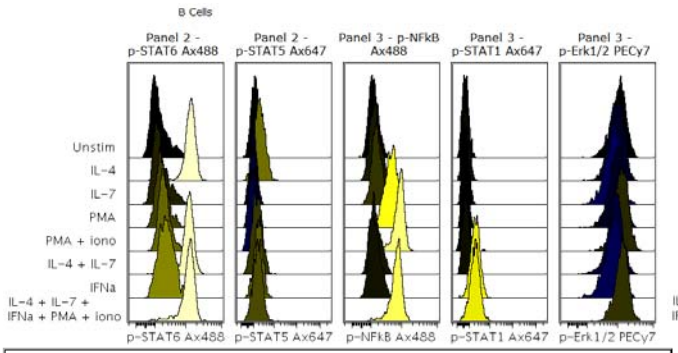
Summary: In general, the expected signaling profile for PBMC was observed. Different signaling profiles of PBMC subsets were apparent.

Best IL-4 ► p-Stat6 in B cell fold change: 2.13 (outstanding!)

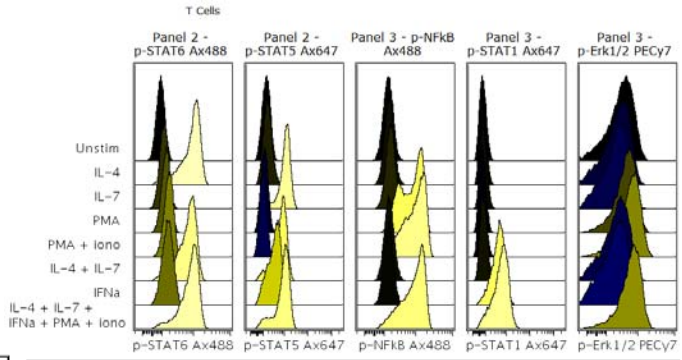
Issues: p-Erk1/2 responses were very weak due to high basal Erk1/2 phosphorylation. Some signals appeared to be higher and lower after stimulation than in the unstimulated sample.







	Panel 2 - p-STAT6 Ax488	Panel 2 - p-STAT5 Ax647	Panel 3 - p-NFkB Ax488	Panel 3 - p-STAT1 Ax647	Panel 3 - p-Erk1/2 PECy7
Unstim	0	0	0	0	0
IL-4	2.13	0.46	0.11	0.05	-0.16
IL-7	0.13	-0.14	0.21	-0	-0.34
PMA	0.28	-0.2	1.14	-0.01	-0.13
PMA + iono	0.43	-0.27	1.69	-0.01	0.16
IL-4 + IL-7	2.02	0.23	0.09	0.06	-0.23
IFNa	0.56	0.38	0.08	1	-0.34
IL-4 + IL-7 + IFNa + PMA + iono	2.05	0.31	1.52	0.97	0.21



	Panel 2 - p-STAT6 Ax488	Panel 2 - p-STAT5 Ax647	Panel 3 - p-NFkB Ax488	Panel 3 - p-STAT1 Ax647	Panel 3 - p-Erk1/2 PECy7
Unstim	0	0	0	0	0
IL-4	2.03	0.13	0.07	0.01	-0.22
IL-7	0.21	1.86	0.15	0.05	-0.31
PMA	0.35	-0.17	1.51	-0.03	0.28
PMA + iono	0.52	-0.31	1.77	-0.05	0.56
IL-4 + IL-7	1.75	1.51	0.06	0.07	-0.29
IFNa	0.45	0.85	0.03	1.54	-0.41
IL-4 + IL-7 + IFNa + PMA + iono	1.92	1.72	1.69	1.82	0.59

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Part 1 – Obtain cells (for the course, 1 and 2 have been done, start at Part 3)

Primary cells: Thaw a cryopreserved aliquot of primary peripheral blood mononuclear cells (PBMC). Once thawed, use a low speed spin to remove DMSO and dead cells, resuspend the cells at high concentration, count the cells, and then dilute the cells to the concentration used for stimulation.

1. Warm to 25 - 37 °C enough medium for Parts 1 and 2 (e.g. ~50 mL medium).

2. Prepare a 15 mL tube (e.g. a Falcon tube from BD) containing at least 5 mL of warmed medium (ideally 10 mL). Cap the tube and invert it, then vortex it, in order to coat the inside walls of the tube with warmed medium.

3. Take an aliquot of cryopreserved cells out of frozen storage and warm it in your hands until the suspended cells thaw to a small piece of ice in mostly liquid. The aliquot might contain anywhere from 5 – 500 x 10^6 cells, although >20 x 10^6 cells per aliquot is recommended when starting. For standard PBMC aliquots, we usually freeze ~50 x 10^6 cells per vial and recover ~75-85% as viable cells. In the beginning, you may want to plan for only recovering ~50% of the amount of cells reported to be frozen in an aliquot.

4. Once the sample thaws, carefully pour the cells into the pre-warmed medium. It is best if you can directly touch the lip of the cryotube to the surface of the warmed medium, which will quickly transfer the thawed cells into the medium. Pipetting with regular size tips can injure the cells and should be avoided. You can use a 'wide bore' P1000 tip to transfer the cells (cut a standard size tip with a razor to make an opening ~½ cm in diameter).

5. Wash out any remaining cells from the cryotube very gently with warmed medium, minimizing pipetting.

6. Spin the cells at a low speed for 5 minutes to gently pellet them (170 RCF, which is 900 RPM on common rotors). Carefully pipette off the dead cell supernatant and discard it. Resuspend the cell pellet in ~1 mL of warmed medium.

7. Count the cells and resuspend them at the appropriate concentration for stimulation (see Part 2). The concentration of cells used at stimulation is higher for primary cells, such as healthy PBMC (1 – 10 x 10^6 cells / mL), than it is for cell lines (see below). For plate based stimulation, we use ~200 uL of 10 x 10^6 PBMC / mL.

    a. For the course, we are using a large volume of dilute PBMC in cytometry tubes: 1 x 10^6 PBMC / mL in 0.5 mL, per stimulation.

8. Try to complete steps 1-7 in less than 15 minutes. If you are thawing a lot of samples, you should get people to help (you want all samples to have been out of the freezer for the same amount of time before stimulation).

Cell lines: For suspension cell lines like Ramos, Jurkat, or U937, you can do the stimulation directly on cells in log phase growth (0.5 - 1 x 10^6 cells / mL). If you have them growing, great! Just make sure they are healthy and not overgrown. If they overgrow, use the low speed spin to 'clean them up' (see below).

1. Do not concentrate cell lines to greater than 1 x 10^6 cells / mL.

2. Split the cells no later than the night before the experiment (16hr prior to the experiment). It has been suggested that adding fresh medium containing serum the morning of the experiment can alter signaling. It is not necessary to 'starve' cell lines in low serum. Starving cells changes basal signaling and signaling responses (starving does not always 'remove basal signaling'; e.g. stress response pathways are activated).

3. If you want to 'clean up' suspension cell lines, do so the night before the experiment. Spin the cells at a low speed for 5 minutes to gently pellet them (170 RCF, which is 900 RPM on common rotors). Resuspend the cells in 1 mL of warmed medium. Count the cells, and dilute to a concentration that, given a night of growth, will be approximately 0.75 x 10^6 cells / mL the day of the experiment.

4. The concentration of cells used at stimulation is much lower for cell lines (0.5 – 1.0 x 10^6 cells / mL) than that used for primary cells (see above).

Part 2 - Dilute and equilibrate cells

Primary cells: The goal for this step is to get the cells suspended at the right concentration, let them rest and equilibrate briefly between thawing and stimulation, and remove any large clumps of cells sticking together (see below).

Cell lines: This step is mostly skipped for cell lines, since they are growing in culture and you use them as-is.

The first thing you need to do is determine what concentration and amount of cells you need so that you can dilute the cells into the proper amount of media. To do this, you need to go through your experiment design and determine several things:

1. Decide the volume in which you would like to stimulate the cells. A volume between 100 uL and 750 uL usually provides a good balance between conserving reagents and getting enough cells in each staining tube. For the course, we will use flow cytometry tubes (vs. plates) and larger, less conservative volumes.

    a. **Groups 1 and 2**: Since you are doing 1 staining panel per tube, use a stimulation volume of 0.5 mL.

    b. **Group 3**: Since you are doing 2 staining panels per tube, use a stimulation volume of 1.0 mL.

2. Determine how many cells you need total, based on the experiment design. Around 0.5 - 2.0 x 10^6 cells per staining tube is typical. This number then gets multiplied by timepoints, staining panels and other experiment variables to give the total number of cells.

    a. For the course, all groups will use 0.5 x 10^6 cells per staining panel. Since each group will have 17 tubes, each group needs 8.5 million cells total.

    b. **Group 1**: 1 staining panel x [(7 timepoints x 2 stimuli) + 2 control stimuli] + 1 unstained sample
    = 17 staining tubes x 0.5 million cells in 0.5 mL = 8.5 million total cells

    Staining Panel 1 (x 16 samples):

α-CD20c-PerCPCy5.5	(B cells)
α-CD5-PE	(T cells and rare B1 B cells)
α-p-Stat6-Ax488	(IL-4 signaling, all cells)
α-p-Stat5-Ax647	(IL-7 signaling, T cells; IFN-α signaling; all cells)

    c. **Group 2**: 1 staining panel x [(7 titrations x 2 stimuli) + 2 control stimuli] + 1 unstained sample
    = 17 staining tubes x 0.5 million cells in 0.5 mL = 8.5 million total cells

    Staining Panel 1 (x 16 samples):

α-CD20c-PerCPCy5.5	(B cells)
α-CD5-PE	(T cells and rare B1 B cells)
α-p-Stat6-Ax488	(IL-4 signaling, all cells)
α-p-Stat5-Ax647	(IL-7 signaling, T cells; IFN-α signaling; all cells)

    d. **Group 3**: 2 staining panels x [panel of 8 stimulation conditions] + 1 unstained sample
    = 17 staining tubes x 0.5 million cells in 0.5 mL = 8.5 million total cells

    Staining Panel 1 (x 8 samples):

α-CD20c-PerCPCy5.5	(B cells)
α-CD5-PE	(T cells and rare B1 B cells)
α-p-Stat6-Ax488	(IL-4 signaling, all cells)
α-p-Stat5-Ax647	(IL-7 signaling, T cells; IFN-α signaling; all cells)

    Staining Panel 2 (x 8 samples):

α-CD20c-PerCPCy5.5	(B cells)
α-CD5-PE	(T cells and rare B1 B cells)
α-p-NFκBp65(S529)-Ax488	(PMA+ionomycin signaling; all cells)
α-p-Erk1/2(T202/Y204)-PECy7	(PMA+ionomycin; all cells)
α-p-Stat1(Y701)-Ax657	(IFN-α signaling, all cells)

3. Once you know your stimulation volume and the total number of cells, you can determine the concentration of cells you need during stimulation and the total volume of cells in media you need.

    a. For the course, the density of cells during stimulation will be 1 x 10^6 cells / mL for all groups.

    b. For the course, the 8.5 million cells will be at 1 x 10^6 cells / mL, so you will need 8.5 mL of cells total.

4. Continuing from Part 1, you have a cell pellet after the low speed spin. Resuspend the cells in a small volume of pre-warmed medium and note the time. This is considered the start of the resting period.

5. Count the cells, and add any remaining medium needed to achieve the concentration for stimulation.

6. Once the cells are resuspended in medium, allow them to rest for a total of 30 minutes at 37 °C.

7. Check for clumping and disperse or remove cell clumps.

    a. With primary cells, it is common to find layers of adherent cells or clumps of dying cells and/or platelets. If you see clumps, disperse them using vigorous pipetting with a P1000 or P200 tip. If you get a particularly large clump, after attempting to disperse it, remove it using the pipette tip.

    b. The higher the quality of the cell prep that is frozen, the fewer clumps are observed. In some cases you may not have control over the samples before they are frozen, but if you do, it is ideal to remove all non-PBMC material from the sample (e.g. platelets, neutrophils, dead cells, red blood cells).

8. This completes the first equilibration period, which should be ~30 minutes.

Part 3 - Aliquot cells into tubes / wells

Primary cells: Primary cells will be aliquoted into tubes and then rested again briefly before stimulation.

Cell lines: Split the cell line into different tubes and then go directly to Step 4 – no resting needed.

After the equilibration period, aliquot the cells into your favorite flow cytometry tubes (e.g. Falcon 2052 from BD). From this point on the whole protocol can be done in these tubes (or wells of a plate, with smaller volumes).

1. Split the sample into tubes for stimulation.
    a. **Groups 1 and 2**: make 17 tubes of cells for stimulation with 0.5 mL of 1 x 10^6 / mL cells in each.
    b. **Group 3**: make 8 tubes of cells for stimulation with 1.0 mL of 1 x 10^6 / mL cells in each and 1 additional tube of 0.5 mL of 1 x 10^6 / mL cells for the unstained control.
    c. It is important to try and keep the flask of cells evenly suspended while aliquoting. I frequently pipet up and down with a 10 mL pipettor if the volume is large. This helps to ensure even numbers of cells are distributed into each tube (which will even out stimulation and staining).
    d. For primary samples in a small volume, I usually use a 1 mL electronic multi-pipettor for this step as I feel it is rapid, accurate, and cuts down on carpal tunel.
2. For primary cells, allow the cells to rest for an additional 45 to 90 minutes at 37 ^{o}C before stimulation.
    a. Ideally, your longest timepoint plus your total rest time should be ~2 hours. For example, if you are doing a timecourse including 0', 15', 30', and 45', your total rest time should be 2h – 45 min (the longest timepoint) = 1 hour 15 minutes total. Longer rest times are not necessarily bad, but signaling can begin to change as the cells produce cytokines and adapt (or die) in culture.
    b. This is a good time to label the stimulation tubes, if you are not using plates.

Part 4 – Stimulate the cells

For stimulation of signaling experiments it's good to have the workplace, stimulation reagents, and fixative all arranged before you begin (especially the fix – you don't want to get to the end of a timecourse and realize you don't have enough formaldehyde). Be careful not to overload yourself with so many tubes if you have close timepoints.

1. **Group 1**: For a time course, add stimuli in reverse time points:
    a. For a 16 minute time course, the first stimulation could be considered "Time minus 16". Add stimuli to tubes, vortex briefly, and return the tubes to 37 ^{o}C.
        i. For the course, all of the stimuli are "20X", so you will be adding 25 uL of stim to each 0.5 mL tube of cells. In addition to the timecourse, you have two 16 minute 'positive control' stimuli that all groups will be doing: IL-4 + IL-7 (add 25 uL of each) and IFN-α.
    b. For the 12 minute time point, repeat the addition of stimuli at Time -12 (4 minutes after you did Time -16), and so on. Once "Time 0" is reached, quickly add the fixative directly to all the tubes and vortex (see below for fix).
2. **Group 2**: For a titration, prepare serial dilutions of stimuli:
    a. You will need ~125 uL of 1 µg/mL stock solution of IL-4 and IL-7 to make all the titration points.
    b. Starting with the 1 µg/mL stock solutions, prepare four 5-fold serial dilutions. You need at least 25 uL of each concentration, so dilute 10 uL stock into 40 uL of PBS, and repeat. You should end with five tubes with 40 uL each of 200 ng/mL, 40 ng/mL, 8.0 ng/mL, 1.6 ng/mL, and 0.32 ng/mL for both IL-4 and IL-7. In addition, you should have your starting "20X" tube containing 115 uL of 1 µg/mL stock.
    c. Add 25 uL of each diluted stimulus to the 0.5 mL of cells.
    d. In addition to the titration, you have two 'positive control' stimuli that all groups will be doing: IL-4 + IL-7 (add 25 uL of each) and IFN-α.
    e. After 16 minutes of stimulation, quickly add the fixative directly to all the tubes and vortex (see Step 5).
3. **Group 3**: For the mini-profile, you will be stimulating twice the volume / number of cells as the other groups, since you will be doing two antibody staining panels.
    a. Add 50 uL of each "20x" stimulus to the 1 mL tube of cells in medium.
    b. For combination stimuli (e.g. IL-7 + IL-4), add 50 uL of each.
    c. After 16 minutes of stimulation, quickly add the fixative directly to all the tubes and vortex (see Step 5).
4. End stimulation by fixing all tubes in unison. Add para-formaldehyde directly to each tube for a final concentration of ~1.6% PFA (Source: Electron Microscopy Services). Both 16% and 32% PFA solutions are available and work well.
    a. **Groups 1 and 2**: For a volume of 0.5 mL of cells, add 50 uL of 16% PFA.
    b. **Group 3**: For a volume of 1.0 mL of cells, add 100 uL of 16% PFA
    c. After vortexing, you should see the medium turn yellow when the PFA is mixed in well.

5. Notes on the conditions being used in the course:

   a. **IL-4**
   50 ng/mL IL-4 stimulates B, T and monocyte lineage cells. Phosphorylation of Stat6 should be apparent at 15 minutes following IL-4 in U937 and Ramos. Lower concentrations and earlier time point are also effective (typical profiling concentration is 20 ng/mL for 15 minutes). Source: eBioscience.

   b. **IL-7**
   50 ng/mL IL-7 specifically stimulates Stat5 phosphorylation in T cells. Lower concentrations and earlier time point are also effective (typical profiling concentration is 20 ng/mL for 15 minutes). Source: eBioscience.

   c. **IFN Type 1 / IFN-α**
   1 ng/mL of Type I interferon alpha (Universal Type I IFN [Recombinant Human IFN-alpha A/D) stimulates B, T and myeloid lineage cells. Phosphorylation of Stat5 and/or Stat1 should be apparent at 15 minutes. Souce: Cat# 11200-1, 100000U (100,000 U = ~0.22 ug), from R&D, made by PBL Bioscience Laboratories, now called PBL Interferon Source. This can be used at lower concentrations (0.2 ng/mL) and still trigger significant signaling in cell lines.

   d. **PMA + ionomycin**
   5 ug/mL PMA and 5 ug/mL ionomycin stimulates B, T and monocyte lineage cells. Phosphorylation of Erk1/2, NFkB, and p38 should be apparent at 15 minutes following PMA/iono in U937 and Ramos. Lower concentrations and earlier time point are also effective (typical profiling concentration is 1 ug/mL each for 15 minutes). Source: Sigma.

6. Notes on BCR network stimulation (not part of the course, but may be interesting to some):

   a. **α-IgG, α-IgM F(ab')$_2$**
   F(ab')$_2$ fragments raised against IgG and IgM can be used to crosslink the BCR of most primary B cells. Primary human B cells tend to be ~5 to 10% of PBMC, ~80% of which are IgM and ~10% of which are IgG. A ~1:1 mixture of AHI1301 and AHI1601 from Biosource can be used to trigger signaling in most B cells. The polyclonal goat reagents from Biosource vary in concentration, but tend to be ~1 mg/mL. The goal is to make a stock solution of 0.5 mg / mL of each F(ab')$_2$. Optimal BCR crosslinking tends to occur at 5 - 10 ug / mL α-BCR, although there is a wide range of concentrations over which BCR crosslinking is equivalent. In primary cells, I use 6 uL of this mixture per 300 uL stimulation volume for a final concentration of ~10 ug / mL (or 4 uL in 200 uL volume, more recently).

      i. Full length antibody and crosslinked full length antibody were not significantly better than F(ab')$_2$ in stimulation of human primary and cell line B cells. Bead or plate-bound antibody is tough to use as a stim in flow experiments.

      ii. For 1 mL of Ramos, use 10 uL of 1.0 mg/mL α-IgM (not a mixture; saves antibody). Ramos cells are IgM heavy chain isotype and lambda light chain isotype.

      iii. Note that the kinetics of BCR (and TCR) signaling in primary cells are fairly rapid. A good timepoint for signaling is around 4 minutes following BCR crosslinking. By 15 minutes, many signals are decreasing, and by 30 minutes most signals have returned to basal.

   b. **H_2O_2**
   a dose of 3.3 mM H_2O_2 is useful for profiling signaling. This dose was identified as one that stimulates little signaling on its own in primary human B and T cells but effectively inactivates the catalytic cysteine of phosphatases so that subsequent (PTP-regulated) signals proceed unchecked. For an example titration in primary human PBMC B cells see Irish et al., J Immunol 2006, Fig 3.

      i. Caution! H_2O_2 can trigger low level ligand independent signaling in B and T cells on its own, and 3.3 mM is at a point in the activity curve where a small increase can stimulate significant signaling. 5 mM H_2O_2 is sufficient to stimulate dramatic signaling in both B and T cells. This sensitivity to a small change in concentration is unlike the other stimuli, where one generally uses a high dose near or at the activity plateau. T cells may be slightly more sensitive than B cells to H_2O_2 alone. See Reth M, Nature Immunology 2002.

      ii. In B cell lines, such as Ramos, H_2O_2 stimulates significant signaling, in contrast with healthy primary B cells.

      iii. A stock of 166.6 mM H_2O_2 ("50X") can be kept at 4 ^{o}C in an eppendorf and protected from light for several weeks. For a stimulation volume of 200 uL, use 4 uL of this 166.6 mM stock for a final concentration of 3.3 mM. Source: MP Biomedical.

   c. **α-IgG, α-IgM F(ab')$_2$ + H_2O_2**
   A combined stimulation of BCR crosslinking plus H_2O_2 delivers a powerful signal to B cells. This can be a useful control in cases where there appears to be tight regulation of B cell signaling. Add α-IgG/M followed by H_2O_2 within 15 seconds. H_2O_2 can also be added just before α-IgG/M and still be effective in amplification and extension of signaling, but it is critical to add H_2O_2 no earlier than a minute before BCR crosslinking or the 3.3 mM dose will be ineffective (see Irish et al., J Immunol 2006, last figure).

      i. The addition of H_2O_2 + BCR crosslinking increases the maximum level of per-cell phosphorylation and extends the time signaling is active. In primary healthy PBMC B cells, signaling tends to peak at 30 - 45 minutes following BCR + H_2O_2, and stays active for 90 – 120 minutes.

      ii. Caution! B and T cells can become exquisitely sensitive to input signals once exposed to H_2O_2. You may find background activation of signaling in T cells that depends on the source of crosslinking reagent and/or the dose of H_2O_2.

Part 5 – Fix and permeabilize (cells can be stored in methanol at -80 °C)

There are many variations on the fix/perm procedure that can be used to optimize staining for challenging epitopes. The protocol here is one that works well for detection of phospho-proteins in human cells, but a significant caveat is that the methanol step will destroy many native epitopes detected by 'classic' antibodies for human lineage markers.

1. Once signaling has been halted by PFA fixation, transfer the tubes to your bench and leave them at room temperature. Allow fixation to proceed for at least 5 minutes at room temperature and no more than 30 minutes. 5-10 minutes is ideal.

2. Following fixation, add 2 mL of PBS to each tube as a wash and pellet the cells by fairly high speed centrifugation (830 RCF, which is 2000 – 2500 RPM on common rotors) for 5 minutes. Since the cells are fixed, they can be spun at higher g-force (and this is required for an efficient pellet).

3. Check to make sure you have a cell pellet. You should be able to see it if you have 0.5 - 1x10^6 cells. Carefully dump out the supernatant of media, PFA, and PBS from the cell pellet.

    a. Technique A: When dumping, do not try to remove all liquid and leave a void volume of ~75 uL. Vortex the cells to resuspend them in this void volume.

    b. Technique B: When dumping, continue to hold the tube when upside down and blot the lip on a piece of paper towel. This will remove nearly all the liquid from the tube. Now, add back a small volume (~75 uL) of PBS to resuspend the cell pellet.

    c. Both techniques are OK. Technique B is ideal when the next step requires an accurate volume (e.g. when antibody staining or barcoding).

4. Add 2 mL of very cold (-20 or -80 °C) high quality methanol to a final concentration of >90% methanol. Source: Fisher Scientific Optima methanol.

    a. Varying amounts of methanol are OK, as long as the final amount is more than 90%.

    b. At the minimum, allow the cells to be permeabilized in methanol for 10 minutes at 4 °C before proceeding to rehydrate and stain.

    c. Fix/perm'd cells can be stored in methanol at -80 °C for ~1-2 months with very little loss of phospho-epitopes. See Krutzik and Nolan, Cytometry 2003 Figure 5: http://www3.interscience.wiley.com/cgi-bin/fulltext/105057021/HTMLSTART

    d. Keep the cells in methanol cold for optimum staining (do not let them warm up until staining). There are some epitopes thought to partially or fully degrade if the samples are warm (>= 4 °C; e.g. CD4, CD3).

5. Other permeabilization techniques:

    a. In some cases you may not be able to find an antibody clone that will work after cells have been permeabilized by methanol. Here is a multi-step protocol that you can use in these cases:

        i. Stimulate cells, then fix to stop signaling. Permeabilize first with a gentle technique (triton, saponin, or commercial fix/perm buffers).

        ii. Stain for methanol-sensitive epitopes. Make sure to use antibodies conjugated to small molecule fluorophores (e.g. Pacific Blue, Alexa dyes), since proteins (PE, APC) and especially tandem dyes are very sensitive to methanol. Alternatively, PE is less sensitive to ethanol (instead of methanol).

        iii. Pemeabilize with methanol and stain for phospho-proteins. Most phospho-specific antibodies seem to work much better following methanol permeabilization (e.g. p-STATs).

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Part 6 – Rehydrate cells

The goal of this step is to wash out the methanol and get the cells into a solution suitable for antibody staining, such as PBS + 1% BSA (the amount of BSA can vary and doesn't matter much, it's there for blocking).

1. Add 2 mL PBS to cells in methanol. Pellet the cells by a higher speed centrifugation (e.g. 2000 – 2500 RPM on most rotors) for 5 minutes.

    a. Adding PBS to this solution makes it easier for the cells to form a good pellet. It is really important to add PBS to dilute the methanol to <= 50% of the solution before pelleting the cells, otherwise it is likely you will have less efficient recovery of cells out of methanol.

    b. BSA in the PBS is optional at this step, so most use PBS alone.

2. Check to make sure you have a cell pellet (if your cell number is high enough for it to be visible). Carefully dump out the supernatant of methanol and PBS from the cell pellet.

    a. The methanol in the solution makes it easy to disrupt the cell pellet at this point, so be especially careful.

3. Resuspend the cell pellet in 2 mL PBS + 1% BSA to wash. Pellet the cells by a higher speed centrifugation (e.g. 2000 – 2500 RPM on most rotors) for 5 minutes.

4. Check to make sure you have a cell pellet (if your cell number is high enough for it to be visible). Carefully dump out the supernatant of PBS/BSA from the cell pellet.

    a. This time you want to make sure to evenly dump out the supernatant. I recommend getting rid of all of the supernatant (no void volume) using "Technique B" described in Step 5. This will make the next steps easier.

5. Resuspend the cells in 100 uL PBS + 1% BSA. If you used Technique B, you should have fairly close to 100 uL total volume in each tube. This makes it easy to do splits in the next step, if you have more than one panel.

    a. **Groups 1 and 2**: Since you have one staining panel, you can now go on to the next step (no split needed).

6. **Group 3**: Since you have more than one staining panel, split the cells from each stimulation tube into a set of new tubes for staining. The number of splits depends on the number of staining panels you are using (for the course we have two staining panels). Split each of the 8 stimulation tubes in half, resulting in 16 total tubes for staining (two sets of 8 staining tubes).

    a. For optimally even staining, you can move both splits of the cells into new tubes (discarding the original tubes). This is the best way to ensure even staining, but you will lose some material this way (some cells will remain behind in the original tubes).

    b. After splitting as two 50 uL aliquots, bring up the volume to 100 uL by adding 50 uL to each tube. This way the staining will be comparable to the other groups.

Part 7 - Stain for flow cytometry

In this step the cells are stained using primary-conjugated antibodies and analyzed by flow cytometry. This protocol is fairly similar to standard flow cytometry staining. You probably want to start here while rehydrating in Part 6.

1. **Group 3**: Since you have more than one staining panel, re-arrange the staining tubes so that all the tubes that will be stained with a given panel of antibodies are all in their own rack. In this case you have two racks of 8 staining tubes (and one unstained tube that you can set to the side on one rack). This is more useful when you have a lot of tubes, but it's a good habit to get into now.

2. Make antibody master mixes in PBS + 1% BSA while the cells are pelleting.

    c. For most phospho stains, we use 10 uL per tube of the commercial BD reagents (half of the recommended volume). This means that a tube of 1 mL of phospho-antibody has 100 tubes worth of stain in it. For lineage marker stains, you can use even less of the commercial antibodies to obtain a better signal / background staining ratio. It is ideal to titrate antibodies yourself to identify what works well for you, but typically around 0.5 to 5 uL of stain is used for lineage / surface markers.

    d. For each staining panel, prepare a starting tube with enough PBS + 1% BSA for all the staining tubes. This step is flexible, and it's a good idea to work out your own master mix and antibody titration conditions.

        i. **Groups 1 and 2**: You have 16 staining tubes for your staining panel. You need 50 uL of PBS + 1% BSA per tube, plus an extra 100 uL of buffer as 'slop' for error.

            1. Make a tube of 900 uL PBS + 1% BSA (50 uL x 16 staining tubes + 100 uL slop for error).

        ii. **Group 3**: You have 8 staining tubes per staining panel. You need 50 uL of PBS + 1% BSA per tube, plus an extra 50 uL of buffer as 'slop' for error.

            1. Make two tubes of of 450 uL PBS + 1% BSA, one for each staining panel (50 uL x 8 staining tubes + 50 uL slop for error).

        iii. Some people subtract the volume of the antibodies from the master mix. This is good if you will be using the antibodies in very different staining panels and want to keep the concentration identical.

    e. Add enough of each antibody for all the staining tubes to the tube of PBS + 1% BSA

        i. **Groups 1 and 2**, Panel 1:

            1. Add 16 x 5 uL each lineage antibody (80 uL): α-CD5-PE, α-CD20c-PerCPCy5.5

            2. Add 16 x 10 uL each phospho antibody (160 uL): α-p-Stat6-Ax488, α-p-Stat5-Ax657

        ii. **Group 3**, Panel 1:

            1. Add 8 x 5 uL each lineage antibody (40 uL): α-CD5-PE, α-CD20c-PerCPCy5.5

            2. Add 8 x 10 uL each phospho antibody (80 uL): α-p-Stat6-Ax488, α-p-Stat5-Ax657

        iii. **Group 3**, Panel 2:

            1. Add 8 x 5 uL each lineage antibody (40 uL): α-CD5-PE, α-CD20c-PerCPCy5.5

            2. Add 8 x 10 uL each phospho antibody (80 uL): α-p-NFkB-Ax488, α-p-Erk1/2-PECy7, α-p-Stat1-Ax657

3. Set aside your unstained control so that you don't accidentally stain it.

4. Split the master mix evenly among the tubes of cells.

    a. To make sure you don't run out of master mix, add 50 uL to each tube of 100 uL cells (from Step 6, part 5).

    b. You could check to make sure you have enough master mix and add more of it to each sample (e.g. 70 uL / tube), but this is not necessary. Electronic repeater pipettes can be useful, since you can take up enough master mix for all the samples (to make sure you have enough), and then aliquot it out one at a time.

5. Vortex, and allow the cells to stain in the dark at room temperature for ~30 minutes.

6. After staining, resuspend the stained cells in 2 mL PBS each to wash. Pellet the cells by a higher speed centrifugation (e.g. 2000 – 2500 RPM on most rotors) for 5 minutes.

7. Check to make sure you have a cell pellet (if your cell number is high enough for it to be visible). Carefully dump out the supernatant of PBS from the cell pellet.

8. Resuspend the cells in an appropriate volume of PBS for flow cytometry, collect and analyze within 10-16 h.

    a. For the course, resuspend samples in 250 uL PBS.

    b. We typically use 125 – 175 uL of PBS to resuspend the sample and collect without the 'sample draw sheath' on the cytometer. The sample draw sheath will suck up ~60 uL of your sample each time you place it on the machine, which is a problem at low volumes. However, the sheath prevents spillover between samples.

    c. Stained samples of cells can be stored in the dark at 4 ^{o}C overnight with little loss phospho epitopes, but should be analyzed as soon as possible. Some epitopes may be sensitive to this, so beware! Longer time periods can lead to significant loss of signal.

Data storage using the Public Cytobank

Cytobank is an open source, web-based tool for data storage, sharing and analysis. We'll use Cytobank to compare the results between the different groups, and you can use Cytobank to download your data from home after the course.

1. Go to the public Cytobank: http://cytobank.stanford.edu/public/

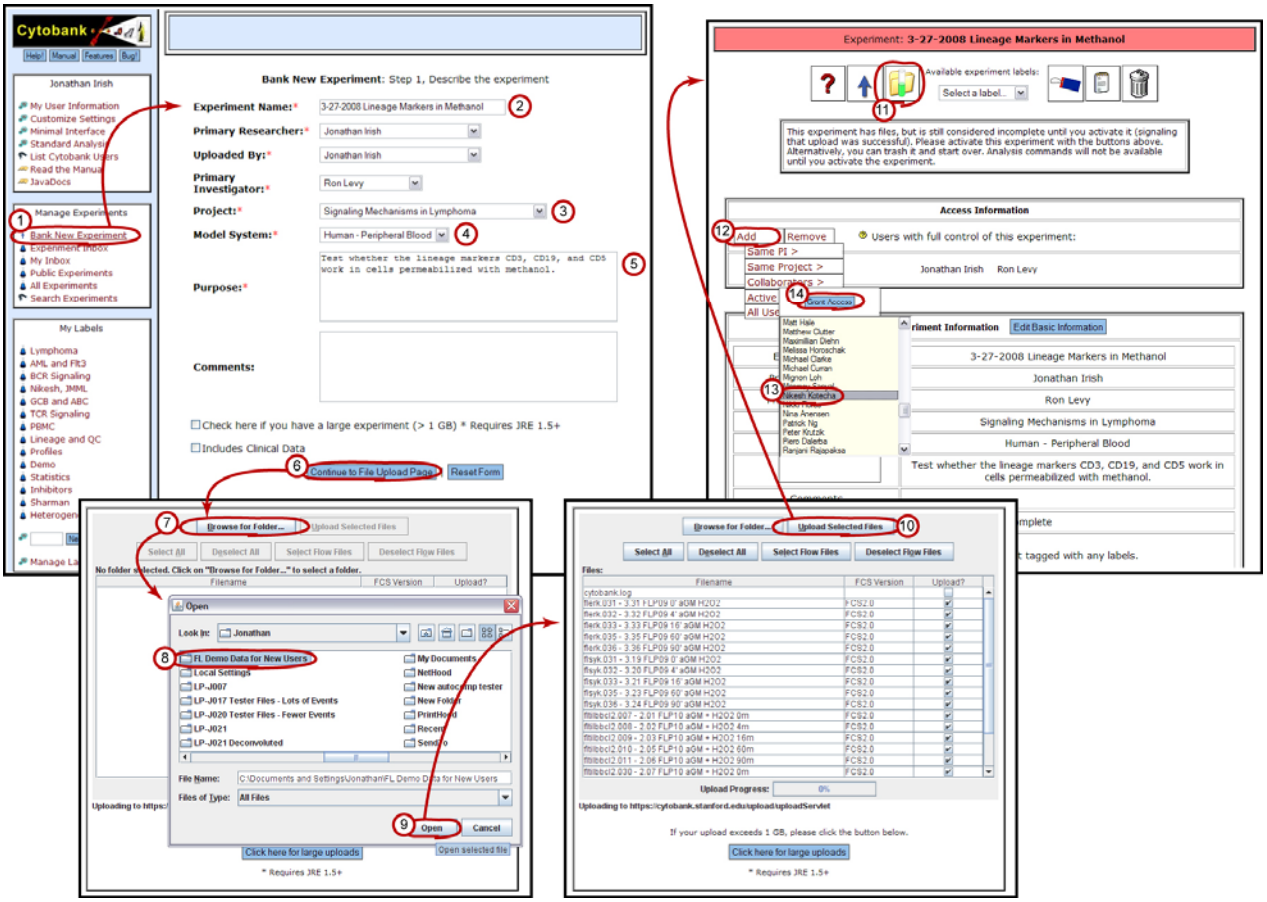
    a. On a PC, we recommend FireFox as the web browser. Internet Explorer works, but some formatting is not correct. On a Mac, we recommend FireFox or Safari as the web browser.

2. Choose "Enter with your Yahoo! ID", choose "Yahoo" as your OpenID source, and then click "Let Me In".

    a. Open IDs are being developed to allow single-sign-on access to different web tools. If you don't already have a Yahoo! ID, you can create one in just a couple minutes, and it's free.

3. Once you log in, you will see your "Experiment Inbox". If this is your first time using Cytobank, you will only see Public experiments with a green background, since you have not uploaded any data and no experiments have been shared with you.

4. Click the "Bank New Experiment" link on the left to begin the process to upload your data. Follow the instructions to upload your files (https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=usermanual#25). Once the files are uploaded, click the Folder / Tube button to "activate" the experiment (indicating upload was successful and stopping further upload of files).



Data analysis using the Public Cytobank

Cytobank creates interactive figures based on the design of your experiment. Instead of dragging and dropping files into a figure, with Cytobank you can say, "Show me a heatmap of timepoints in columns vs. cell subsets in rows." In order to do this, you must first outline the experiment design in Step 1 by tagging the experiment variables.

1. First off, share your experiment with your other group members who have logged into Cytobank. Click on the experiment title in your Inbox, or click on the "?" icon to see the basic details.
    a. Using the "Add" menu at the top of the page, find your group members' names and choose "Grant Access."
2. Next, go to Step 1 and annotate your experiment variables.
    a. Click "Compensation Controls" to identify your comp bead tubes. With Cytobank, you want to have a positive and negative population in each file, and you do not need a "universal unstained". (If you have only a + in each file and a universal unstained, ask for help and switch to advanced mode.)
    b. Click "Staining Panels" to identify what you stained on each channel. Since we entered this information into the acquisition software, you can just choose "Auto Detect Panels". (If you did not label the channels, or you mis-labeled something, you can enter names for the panels and hand-identify which files match them.)
        i. After pressing "Next", make sure it finds the right files for your staining panel(s).
        ii. Group 3 should see all the non-comp files in two panels. Groups 1 and 2 should see all the non-comp files checked in just one panel.
    c. Click "Conditions" and identify the different stimulation conditions
        i. **Groups 1 and 2**: Type in four conditions: IL-4, IL-7, IL-4 + IL-7, IFN-a
            1. Unstimulated will be 'timepoint zero' or 'dosage 0 ng/mL', so don't enter this tag yet
        ii. **Group 3**: Type in seven conditions: Unstim, IL-4, IL-7, PMA, PMA + ionomycin, IL-4 + IL-7, IFN-a, IL-4 + IL-7 + PMA + ionomycin + IFN-a
    d. Identify remaining variables:
        i. **Group 1**: Click "Timepoints" and enter timepoint search tags: 0', 1', 2', 4', 8' 12', 16'
            1. Make sure to tag unstimulated as 0'
        ii. **Group 2**: Click "Dosage Titrations" and enter titration search tags: 50, 10, 2.0, 0.40, 0.080, 0.016
            1. Make sure to tag unstimulated as 0 ng/mL
            2. Your tags might be different -- you may not have been able to tag files with "." in the name
3. Go to Step 2 and click "New Compensation", check to make sure it found the right files, and finish.
4. Go to Step 3 for gating.
    a. Select your compensation in the drop down menu to activate it. If you did the compensation on the cytometer, you can choose "Use This File's Compensation".

b. Draw your singlet, live cell, CD5+ CD20- T cell, CD5- CD20+ B cell, and CD5- CD20- "other cells" gates. All gates begin "global" and apply to all files.

5. Go to Step 4 to create "Figure Dimensions". Click "Auto Create". Then use this drop down menu to select the auto-created dimensions and make sure they are OK.

   a. You can re-organize and re-order the members of Figure Dimensions here.

   b. Most users just auto-create figure dimensions and move to Step 5. Step 4 is more useful once you're an advanced user.

6. Go to Step 5 to make Illustrations.

   a. Begin by dragging in your 'main' experiment variables, such as the timepoints, dosage titrations, or conditions (for Groups 1, 2, and 3, respectively).

   b. Add any remaining experiment variables into the figure, such as populations or "panels and channels" (which contains your phospho markers).

   c. As you add dimensions, you switch into 'placeholder mode' showing tubes. In this mode you can download files for each tube. It also gives a quick overview of the experiment.

   d. To switch out of placeholder mode, click the link above the illustration, or the "plot" icon in the upper left of the illustration controls.

   e. Filter to show the phosphos by Control-clicking just the phosphos and pressing "Update" in the "Panels and Channels" dimension.

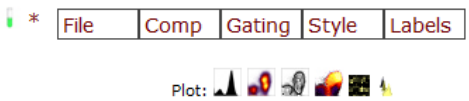
   f. Make sure to apply the live cell gate and software compensation.

   g. Try switching between types of illustrations, such as contour plots, heat maps, and histogram overlays.

   h. Note: If you delete Figure Dimensions in Step 4 or restart your annotations in Step 1, you should start a new Illustration in Step 5 or you will get a Cytobank error. Currently, your old Illustrations will be lost if you re-do the figure dimensions this way.

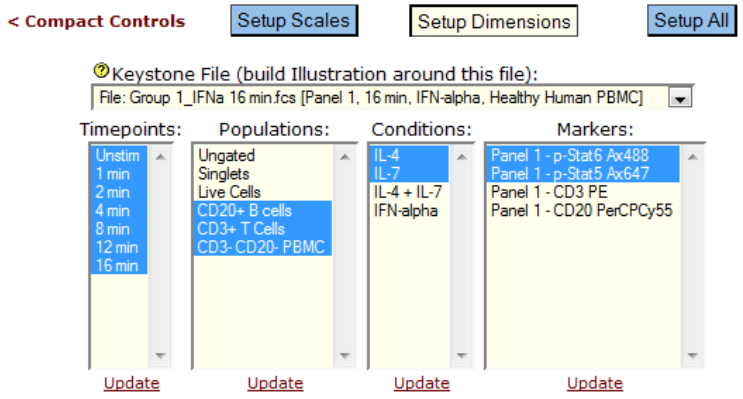
   i. Note: you can change your scales, but currently if you want to see a gate and the percentage of cells in the gate, the scales and compensation settings in the illustration must exactly match those used in Step 3 when you did the gating.

7. Example of the Illustration setup for Group 1 (Signaling Timecourse), illustrations shown on the following pages:



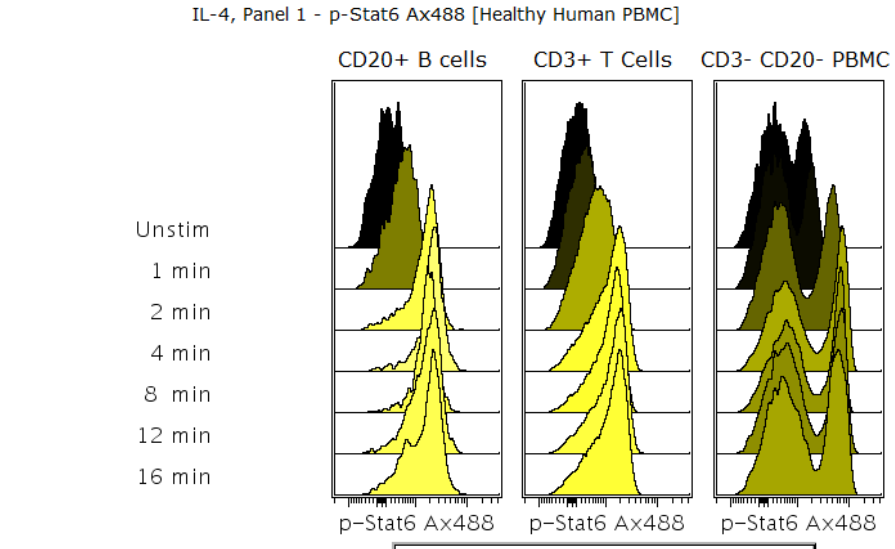
(X is variable)

Illustration Dimensions	
Include:	Arrange Active Dimensions:
Individuals	Timepoints (overlaid)
	Populations (in columns)
	Conditions (in tables)
	Markers (in tables)

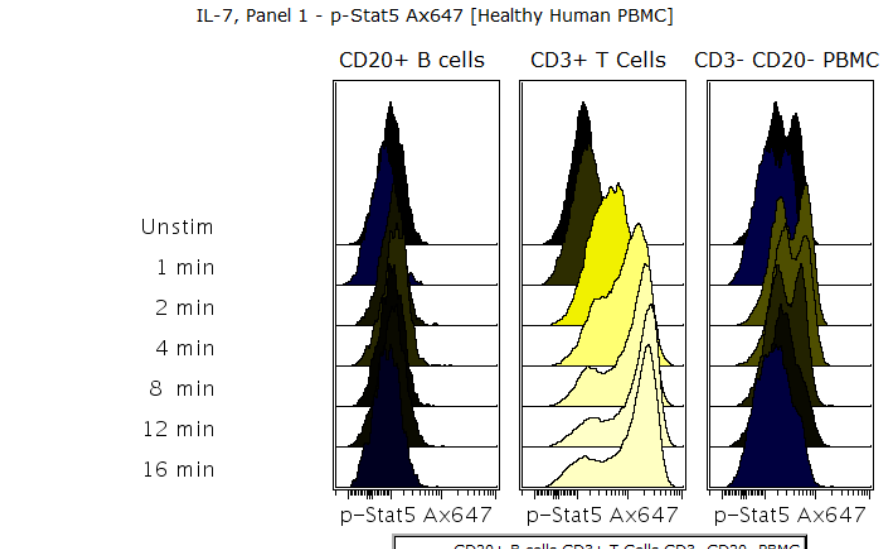


Example Group 1 Illustration – Signaling Kinetics – Histogram Overlays

Available on the Public Cytobank:
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=62



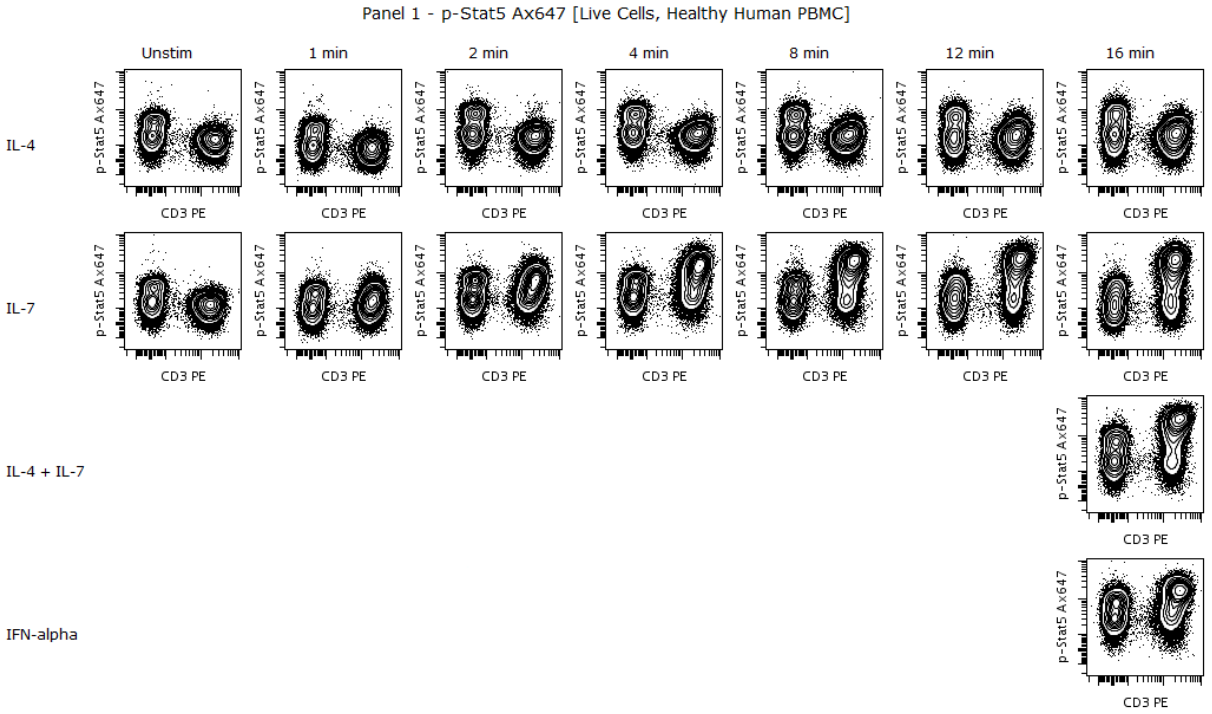
	CD20+ B cells	CD3+ T Cells	CD3- CD20- PBMC
Unstim	0	0	0
1 min	0.6	0.22	0.06
2 min	1.64	0.82	0.48
4 min	1.88	1.48	0.81
8 min	1.67	1.47	0.71
12 min	1.68	1.58	0.67
16 min	1.68	1.52	0.78

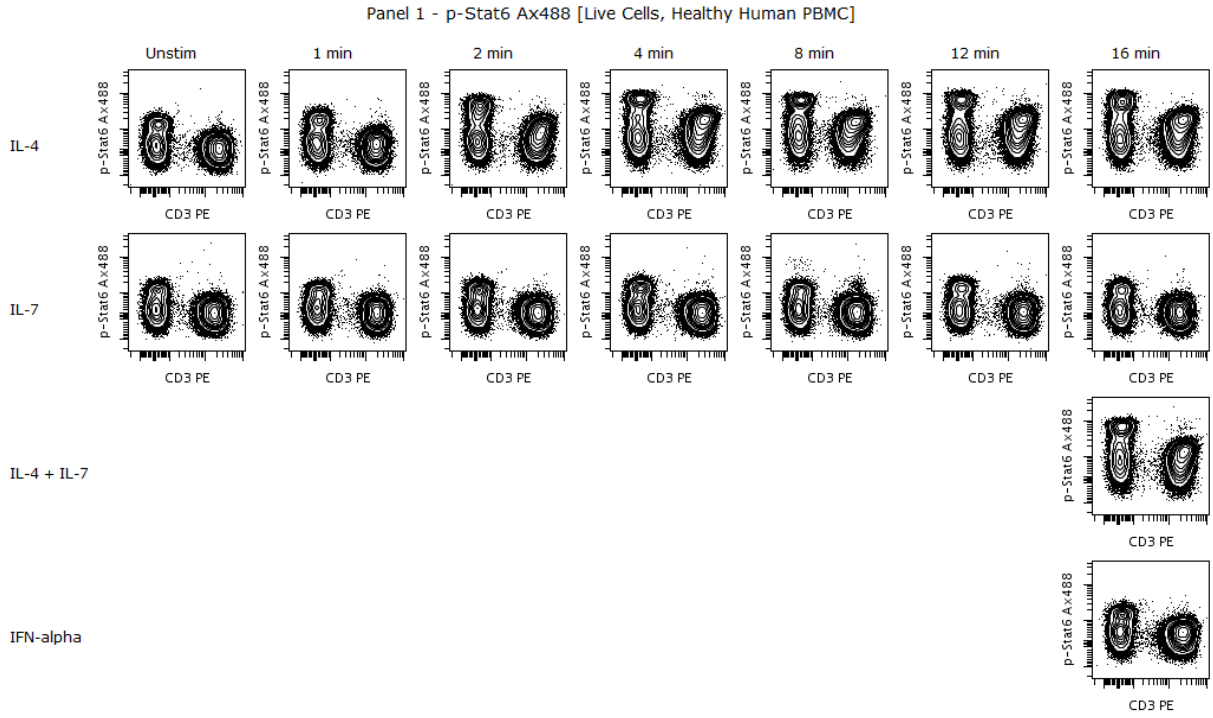


	CD20+ B cells	CD3+ T Cells	CD3- CD20- PBMC
Unstim	0	0	0
1 min	-0.29	0.22	-0.33
2 min	0.1	1.14	0.38
4 min	0.18	1.89	0.37
8 min	0.02	2.23	0.16
12 min	0.05	2.47	0.04
16 min	-0.15	2.36	-0.3

Example Group 1 Illustration – Signaling Kinetics – 2D Plots

Available on the Public Cytobank:
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=62

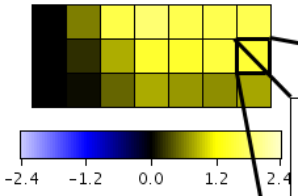




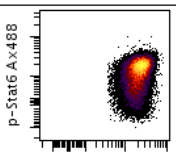
Example Group 1 Illustration – Signaling Kinetics – Heatmaps

Available on the Public Cytobank:
https://cytobank.stanford.edu/openidpublic/index.jsp?commandName=getIllustration&experimentID=62

IL-4, Panel 1 - p-Stat6 Ax488 [Healthy Human PBMC]



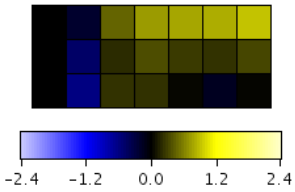
	Unstim	1 min	2 min	4 min	8 min
CD20+ B cells	0	0.6	1.64	1.88	1.67
CD3+ T Cells	0	0.22	0.82	1.48	1.47
CD3- CD20- PBMC	0	0.06	0.48	0.81	0.71



Row: CD3+ T Cells
Column: 16 min
Filename: Group 1_IL4 16 min.fcs

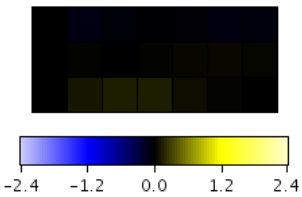
Click the tube icon to download the data file gated and compensated as shown.
Value: 1.52

IL-4, Panel 1 - p-Stat5 Ax647 [Healthy Hu



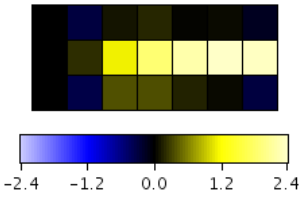
	Unstim	1 min	2 min	4 min	8 min	12 min	16 min
CD20+ B cells	0	-0.21	0.47	0.73	0.79	0.82	0.92
CD3+ T Cells	0	-0.48	0.2	0.36	0.28	0.24	0.33
CD3- CD20- PBMC	0	-0.6	0.24	0.23	0.03	-0.15	0.02

IL-7, Panel 1 - p-Stat6 Ax488 [Healthy Human PBMC]



	Unstim	1 min	2 min	4 min	8 min	12 min	16 min
CD20+ B cells	0	-0.08	-0.04	-0.01	-0.03	-0.07	-0.06
CD3+ T Cells	0	0.01	-0	0.02	0.04	0.04	0.03
CD3- CD20- PBMC	0	0.1	0.14	0.14	0.06	0.02	0

IL-7, Panel 1 - p-Stat5 Ax647 [Healthy Human PBMC]



	Unstim	1 min	2 min	4 min	8 min	12 min	16 min
CD20+ B cells	0	-0.29	0.1	0.18	0.02	0.05	-0.15
CD3+ T Cells	0	0.22	1.14	1.89	2.23	2.47	2.36
CD3- CD20- PBMC	0	-0.33	0.38	0.37	0.16	0.04	-0.3

Some published examples of signaling in primary human cells

The protocols listed in the following publications are fairly complete and the figures show examples of data from primary human cells:

- Bcl-2 and Kappa/Lambda subsetting of tumor and non-tumor B cells, B cell receptor crosslinking over a 2h timecourse (10 points) in primary follicular lymphoma samples, phosphatase inhibition by H_2O_2 (with titration), CD20 stain, p-Btk, p-Syk/Zap70, p-Erk1/2, p-p38, comparison of B cell subsets (IgG and IgM), tumor vs. non-tumor comparison:

    a. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=16835385&query_hl=1&itool=pubmed_docsum

    b. Irish JM, Czerwinski DK, Nolan GP, Levy R. *Altered B-cell receptor signaling kinetics distinguish human follicular lymphoma B cells from tumor-infiltrating nonmalignant B cells.* **Blood**. 2006 Nov 1;108(9):3135-42. Epub 2006 Jul 11.

- B cell receptor crosslinking over a 2h timecourse (10 points) in healthy primary human PBMC, phosphatase inhibition by H2O2 (with titration), CD20 stain, p-Btk, p-Syk/Zap70, p-Erk1/2, p-p38, comparison of B cell subsets (IgG and IgM):

    a. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=16849466&query_hl=1&itool=pubmed_docsum

    b. Irish JM, Czerwinski DK, Nolan GP, Levy R. *Kinetics of B cell receptor signaling in human B cell subsets mapped by phosphospecific flow cytometry*. **J Immunol**. 2006 Aug 1;177(3):1581-9.

- p53 phosphorylation and Flt3 mutational status in primary human acute myeloid leukemia blast cells, Bcl-2, p-p53-S15, p-p53-S20, p-p53-S37, p-p53-S47, p-p53-S392, 2D-PAGE of p53, idarubicin stimulation:

    a. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=17105820&query_hl=1&itool=pubmed_docsum

    b. Irish JM, Anensen N, Hovland R, Skavland J, Borresen-Dale AL, Bruserud O, Nolan GP, Gjertsen BT. *Flt3 Y591 duplication and Bcl-2 overexpression are detected in acute myeloid leukemia cells with high levels of phosphorylated wild-type p53.* **Blood**. Mar 15;109(6):2589-96. Epub 2006 Nov 14.

- Profiles of p-Stat1, p-Stat3, p-Stat5, p-Stat6, p-Erk1/2, p-p38 in response to GM-CSF, G-CSF, IL-3, IFNg, and Flt3 ligand in primary human acute myeloid leukemia cells, Flt3 mutational status and relationship to signaling in AML:

    a. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=15260991&query_hl=1&itool=pubmed_docsum

    b. Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud O, Gjertsen BT, Nolan GP. *Single cell profiling of potentiated phospho-protein networks in cancer cells* **Cell**. 2004 Jul 23;118(2):217-28.