

Ariel Phospho FACS Notes:

Before beginning, these are some VERY IMPORTANT NOTES:

- Do not use Saponin for permeabilization of cells. Use -20C Methanol. (Ethanol is ok as a substitute)
- Do not fix with Paraformaldehyde, only use formaldehyde for fixation. Paraformaldehyde seems to have an impact on total fluorescence (makes overall fluorescence much less)
- Perform staining incubation at room temperature and not 4C. Incubation at 4C also significantly decreases total staining, so leave cells/solution at room temperature during this step.
- Place Methanol at -20C before starting the experiment so it's cold and ready when your to that step.

Starts with 100,000 cells/100ul in cells normal medium (in this case, for Ariel's cells RPMI w/ 10%FCS)

Setup plate with Wt and Ko (control vs. Experimental) across from each other. This is very important so that the stimulation timepoints correspond to each other.

Here is the plate setup Ariel used today:

		<u>Stimulation Timepoints</u>					<u>Stimulation Timepoints</u>								
		0	5	15	30	60	Empty	0	5	15	30	60	Empty		
Wt Mouse #1	pERK	○	○	○	○	2	X	○	○	○	○	1	X	pERK	Ko Mouse #1
	pJNK	○	○	○	○	4	X	○	○	○	○	3	X	pJNK	
	p#3	○	○	○	○	6	X	○	○	○	○	5	X	p#3	
	p#4	○	○	○	○	8	X	○	○	○	○	7	X	p#4	
Wt Mouse #2	pERK	○	○	○	○	10	X	○	○	○	○	9	X	pERK	Ko Mouse #2
	pJNK	○	○	○	○	12	X	○	○	○	○	11	X	pJNK	
	p#3	○	○	○	○	14	X	○	○	○	○	13	X	p#3	
	p#4	○	○	○	○	16	X	○	○	○	○	15	X	p#4	

Incubate cells at 37C for 10 minutes

Heat activator/s at 37C as well so there is no change in solution temp when activator is added.

Ariel adds his activator at 1/10 to his cell solutions. Thus 100ul cell solutions receive 11ul of activator (e.coli in this experiment) and then are immediately added back to the incubator.

IMPORTANT: When adding the activator you want to do so from right to left (or visa-versa) and NOT up and down your columns. So complete each row before moving to the next. This is so each Wt and Ko are done at almost (~1 to 2 sec delay) at the exact same time. Even if repeats for the same group are a bit off each other, they are matched to a point of the opposing group (control or experimental group) which should make your data equivalent. For this experiment Ariel did four mice for each group total, thus he had two plates. It took him around 50 seconds to add the activator to all 32 wells needed. For which well to add when, see the plate above (in numerical order). Adding the activator should go in order 1,2,3, etc to 16. One half an hour later, perform same step for your 30 minute activations so the data can come off at the same time.

Make up your formaldehyde solution. If using the Fisher 37% stock, dilute 1 part formaldehyde to 9.25 parts diluent to give 4% formaldehyde.

Also during the incubation time, you can adjust your pipette tips such that is ready for adding solutions to your plate w/ you multichannel (if you have columns missing you can remove those tips before hand to make it faster/more efficient).

When it's time to stop the activation, pipette 111 of 4% formaldehyde into the wells. Do this quickly and just add to each well don't bother mixing each yet.

Once every well has received the formaldehyde, go ahead and go back and mix each well to ensure cells will not get stuck to the plate (not unlikely given that your fixing them as well at this point so don't forget)

Let cells incubate in 2% formaldehyde/media solution for 10 minutes at room temperature.

Spin down cells at 3,500 rpm for 2 minutes. (You can spin this fast b/c cells are fixed and are less likely to burst under pressure of faster spin).

After spin, dump off supernatants (formaldehyde/media) and add 50-60ul of -20C methanol. Once all cells have received methanol go back and mix cells by pipetting up and down.

(If you need to stop during this experiment this is the place to do it. Store cells in solution at 4C. Check on methanol periodically for evaporation. This is the best part to stop, even better than stopping after staining. Ariel says he's left samples here for weeks and the still run well at this point. Others claim as much as a month is fine)

If performing all in one day (likely case), let cells permabilize in methanol for 15 minutes on ice in plate.

Ariel's FACS buffer is as follows: HBSS w/ .1% BSA +.02% Sodium Azide.

While incubating, pipette out your master mixes for your stain such that each well only requires adding sample once. The final volume added to each well should be 100ul. For the phospho antibodies Ariel adds 5ul of each, 5ul for the F4/80 antibody and 1ul of the CD11b antibody and dilutes to 100 (per sample) with FACS buffer.

-for any antibody Ariel usually starts w/ half of what the product sheet recommends then adjusts.

Antibodies Used in this experiment:

Ariel says that the 647 has a stronger signal than 488 so your best bet is to put your weaker antibodies on 647.

From Cell Signaling:

Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Mouse mAb Alexa Fluor 647 conjugate 500ul @ 5ug/ml #4552.

Phospho-NF-KappaB p65 (ser536) (93H1) Rabbit mAb Alexa Fluor 647 conjugate 500ul @ 5ug/ml #4887

Phospho-SapK/JNK (Thr183/Tyr 185)(G9) mouse mAb Alexa Fluor 647 conjugate 500ul @ 20ug/ml #9257

Phospho-p44/42 MAPK (Erk 1/2) (Thr202/Tyr204)(E10) mouse mAb Alexa Fluor 647 500ul @ 7.5ug/ml #4375

-AlexaFluor 647 on FL4

From Biolegend:

Anti-phosphotyrosine (PY20) Alexa Fluor 488 200ul @ .5mg/ml Cat#309306

-AlexaFluor 488 on FL1

From AbD Serotec:

Rat Anti-Mouse F4/80 Antigen RPE Cat#MCA497PE

-PE on FL2

From BD Pharmingen:

Rat anti-mouse CD11b (M1/70) PE-Cy7 @ .2mg/ml Cat #552850

-PE-Cy7 runs on FL3

Once the 15 minute methanol incubation is complete add 140-150ul of FACS buffer (final volume 200ul) and then spin down the plate again and dump off supernatant when done spinning.

Ariel tries to minimize the number of spins to reduce the number of cells lost since starting amount is relatively low.

Add master mixes to samples, then after all master mixes are added, go back and mix well by pipetting up and down with a multi-channel pipette.

Let cells incubate in staining master mixes at room temperature for 30 minutes.

After incubation is complete add 100ul FACS buffer and spin and dump off supernatants.

Resuspend cells in 200ul of FACS buffer in FACS tubes.

Cells are ready for FACS analysis.