November 25th , 2022

Invitrogen Nuclear FoxP3 Staining vs BD Fix Perm

Specimen	Status	Location	Conc	Date	Tasks	Volume	Ly	Ly+Mon	Total	2E+6	.3E+6
ND006	Adult wt 1 2nd vax		15E+6	03-04-21		2	5.35	6.71	10.7 13.42		
ND006	Adult 2nd vax		15E+6	03-04-21		2	6.13	7.59	12.26 15.18		
									23.0m		
						4	5.74	7.15		Ø.348µl	
						5	4.60	5.72		Ø.434µl	65µl

Thaw Span @ 8:39 am 1200rpm 10min
count stain @ 8:59 am → 9:06
count done @ 9:20 am
Incubation @ 37°C start @ 9:52pm → 3:52pm
Ab prep @ 2:24 pm
Done @ 3:19 pm
3:56 pm cells out.
1:29 aliquoted 2 in 1300/8spin
[16:46 pm → cells into fridge
not ideal, but lot sc's to deal with]
4:49 sc start → 5:19
(CCR6 FoxP3) secondary, need to take ~50µl from unstained, hindsight should've taken from VδI Fitc.
Also realized intracellular has Fitc FoxP3 so yeah. Planning to spike in 0.5µl for the ~~not intra~~ markers just in case they do matter...
Hot sc's @ 5:28 pm → 5:58pm
Cold sc's @ 5:29 pm → 5:59pm
Samples & LD sc @ 5:32pm → 5:47pm
Spin @ 5:48pm
[sc's out @ 5:51 pm]
53/54 56 58
59 00 63 64
Samples hidden @ 6:08
Run at FBS!

For stop, Ebioscience mix @ 1µl ~ 500µl
but instead of PMA → substitute SEB (5µg/ml)
for the activation condition.
Go for 6 hours
FoxP3 Cr ctrl 9am sc
Ebio FoxP3 ctrl SEB 9am sc
(for scs)
(act SEB + FoxP3
- Fix Perm stain
- FoxP3 stain)
Just a → make 2 sets of sc's ←
unstained
act FoxP3 Vδ1
act FoxP3
3µl ~ 500?
6µl/ml?
CD107A sc
24 ~ 26 million cells range

29 colors
× 2
58 scs
× .3
17.4

23.0M
17.4M
5.60 M

6 × .3 = 2M SEB scs

52
× .3
156 → (16M)

2M/500µl?

CD107A F.Perm
CD107A FoxP3
SEB Vδ1
SEB FoxP3
unst F.Perm
unst FoxP3
SEBd

434
× 2
868

66 R10
+ ?
132 R10

434µl + ~~residual~~ 66µl R10 = 500µl
2.5µl SEB + 1µl stop

FBS: 68mls
24mls
~~92~~ 92
~~132~~ 68
+8mls
Nuclear Perm wash 136mls 76
+16
152 mls

2 tubes w/ CD107A samples
2 tubes activated SEB sc's + unstained
8 tubes ctrl tubes?

34
× 24
136
~~68~~ ~~136~~

2 + 2 unstained samples
× 4
8mls
-300

6mls 5% FBS-PBS
8mls Wash (4)
1ml Fix Nuclear

800
× 4
3200µl
+ Aqua

72
× 3
216 → 25 mls RBC lysis

68
÷ 5
→ 3.4 mls PFA-PBS

10:40
36 Nuclear Fix

5:53 6:07

6:16 pm scs in FBS wash post RBC lyse

Samples into 37° stain @ 6:20 pm → 6:50 pm

6:35 streptavidin scs ⇒ 6:50 pm

6:37 Fix - 6:40 → 50 → 7:00

6:43 → 53 → 03 → 13

scs → stained ✓

Lysed → ✓

(streptavidin ✓)

→ 4°C @ 7:01 → 7:31

→ Fixed/Permed @ 7-7:10 7:20 7:30

Perm Wash spin 1 7:07 pm → 7:13 pm

2nd wash @ _____

Nuc wash 2nd @ _____

7:31 pm

Rbc lysed @ 7:37

last colci needs it's final nuclear wash

samples need streptavidin.

Samples streptavidin @ 7:47 pm → 8:02 pm

Sample streptavidin spin @ 8:04 pm

sc intracellular @ 8:18 pm +40 → 8:58 pm

Sample Fixes @ 8:21 pm → 31 → 41 (Fix Perm)

→ 51 (Nuclear)

- sc's 1st wash post stain @ 9:00

[last nuclear wash pre-intrac for samples]

Samples intra @ 9:09 → 9:49 pm

Final sc spin @ 9:15 pm

Sc's away @ 9:34 pm

1st wash @ 9:50 pm ✓

2nd wash @ 9:58 pm ✓

Done @ 10:51

November 25th, 2022 αβ T cell SFC panel

#	Filter	Single color (ul)	Ref ctrl type	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	2	L/D RT 15 min	RT for 10 min	37oC for 30min	2	Surface 4oC for 30min	2	4*C for 15 min	RBC Lyse then NuclearFix/Perm	Intra Mix RT for 40min	2
1	UV2				BUV395	CD62L	(DREG-56)								1.2	2.4				
					AF	AF-UV6														
2	UV7				BUV496	CD8	(RPA-T8)								0.7	1.4			0.25	0.5
3	UV9				BUV563	CD69	(FN50)								0.5	1			0.5	1
4	UV10				BUV615	CCR4	(1G1)						2.5	5					0.5	1
5	UV11				BUV661	Vd2	(B6)								0.7	1.4				
6	U14				BUV737	CXCR3	(1C6/CXCR3)						2.5	5					0.5	1
7	UV16				BUV805	CD4	(SK3)								1.5	3			0.25	0.5
8	V1				BV421	CD127	(A019D5)						1.5	3					0.5	1
9	V3				Pacific Blue	CD14	(M5E2)								2.0	4				
	V3				Pacific Blue	CD19	(HIB19)								2.0	4				
10	V5				BV480	CD161 - Biotin	(REA631)					1.5					Strep <0.8>		1	2
11	V7				BV510	CD45RA	(Hi100)								0.7	1.4				
					BV570															
12	V10				BV605	CXCR5	(J252D4)						2.5	5					0.5	1
13	V11				BV650	CCR7	(G043H7)						1.5	3		0			0.5	1
14	V13				BV711	Va7.2	(3C10)								1.2	2.4			0.25	0.5
15	V14				BV750	IFNg	(B27)												1.5	3
16	V15				BV786	CCR6	(11A9)						1.5	3						
17	B3				FITC	FOXP3/Vd1													4 3	8
18					Spark blue 550	CD3	(SK7)								1.2	2.4			0.25	0.5
19	B4				PE	PD-1	(EH12.2H7								1.2	2.4			0.25	0.5
20	B6				PE-CF594	CD26	(M-A261)						1.2	2.4						
21	B8				PE-Cy5	CD25	(M-A251)								1.2	2.4			0.25	0.5
22	B10				PerCP-Cy5.5	TNFa	(MAB11)												2.5	5
23	B13				PE-vio770	HLA-DR	(L243)								0.8	1.6				
24	R1				APC	CD39	(A1)						1.2	2.4						
25	R2				Alexa Fluor 647	IL2	(MQ1-												1.2	2.4
26	R4				APC-R700	CD107a	(H4A3)		<3>	######										
27	R6				Zombie NIR	L/D					<1:2500>									
28	R7				APC/Fire 750	CD27	(O323)						2	4						
29	R8				APC/Fire 810	CD38	(HIT2)						1.6	3.2						
						Antibody Total		0.0			Antibody Total		18	36	14.9	29.8			14.7	29.4
And UNSTAINED CONTROLS !!!						R10 Media		20.5			Brilliant Stain		50	100	50	100			50	100
						Pippette draw volume /sample		19.5			Pippette draw volume /sample		64		60.9				60.7	

Notes: Need to resolve the what gets 37*C, what gets 4*C and what gets Intracellular on Monday, can't be spiking two antibodies if avoidable for CD161....

Simplified Protocol

<Rest cells overnight in 6 well plate, 1E6/ml of R10>
Collect, count, aliquot cells 2.0E+6 Cells/ 5ml polystyrene tube
Add antigens, stim antibodies, **and during stain antibodies in 500 ul R10**
Cap and store incubator at 37*C for 2 two hours
Add golgi block, and **incubate for <6> hours**

Wash with 2 ml PBS, 1300rpm, 8min
800 ul Live/Dead 1:2500 @RT for 15min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

Add CD161 biotin antibody for 10 minutes at RT
Add hot Surface mix, incubate @37C for 30 min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

Add cold Surface mix, incubate @ 4C for 30min
Add 300-500ul 1x RBC Lysis for 3 minutes
Wash 2 ml 5% PBS-FBS 1500 rpm, 6min

1 ml Nuclear FixPerm, incubate @ 4C for 30min
(vortex every 10 minutes)
First PermWash: 2 ml NuclearWash 1500 rpm 6 min
Second Perm Wash: 2 ml NuclearWash 1500 rpm 6 min

Add Intracellular Stain, incubate @ RT for 40min
First PermWash: 2 ml NuclearWash 1500 rpm 6 min
Second Perm Wash: 2 ml NuclearWash 1500 rpm 6 min

Resuspend in 100 ul 0.4% PFA-PBS
Cap tubes, wrap rack in foil, store at 4*C

Not the LSR-II as reason ↓3µl for Human

New issues:

Biotin CD161 — Miltenyi Biolegend REA631 or HP-3610

CD62L — BD SK11 or BD Dreg56

Testing on single color ctrl (F.X)

∴ Supernatants in ctrl vs stim?

AB T cell SFC Panel

11/25/2022

Spectrum	UV				Violet				Blue				Red			
373	UV1															
388	UV2	BUV395	[2]	CD62L												
428	UV3				V1	BV421	[4]	CD127								
443	UV4				V2											
458	UV5				V3	PacBlue	[1]	CD14/19								
473	UV6				V4											
508					V5	BV480	[3]	CD161	B1							
514	UV7	BUV496	[2]	CD8												
525					V6				B2	FITC	[1.5]	Vd1/FoxP3				
542	UV8				V7	BV510	[1.5]	CD45RA	B3	SparkBlue 550	[1]	CD3				
582	UV9	BUV563	[3]	CD69	V8	BV570			B4	PE	[4]	PD1				
598					V9				B5							
613	UV10	BUV615	[3]	CCR4	V10	BV605	[3]	CXCR5	B6	PE-CF594	[4]	CD26				
664	UV11	BUV661	[3]	Vd2	V11	BV650	[3.5]	CCR7	B7				R1	APC	[3.5]	CD39
679									B8	PE-Cy5	[4.5]	CD25	R2	AF647	[3.5]	IL-2
697	UV12				V12				B9	PerCP-Cy5.5	[2]	TNFa	R3			
717	UV13				V13	BV711	[4]	Va7.2	B10				R4	APC-R700	[3]	CD107a
738									B11				R5			
750	UV14	BUV737	[3]	CXCR3	V14	BV750	[2.5]	IFNg								
760									B12				R6	Zombie NIR	[ ]	L/D
783	UV15				V15	BV786	[3]	CCR6	B13	Pe-Vio770	[3]	HLA-DR	R7	APC-Fire 750	[2]	CD27
812	UV16	BUV805	[1]	CD4	V16				B14				R8	APC-Fire 810	[3]	CD38

¿AF or Alexa? How stored?

November 25th , 2022

FoxP3 Staining

Universal Intracellular Mix

=Bioscience FoxP3/TF staining buffer set 00-5523 (Invitrogen/ThermoFisher)

FixPerm Concentrate 4x 1:3 diluent w/ H_2O

Buffer 10x

100µl PermBuffer, ~2 ml wash

1 ml FoxP3 fixPerm 30 minuts

2 ml 1x ~2 (400-600 g 5 min)

2ml ~2

Acquisition - Aurora on @ ~~10:07~~ 9:58 am ✓

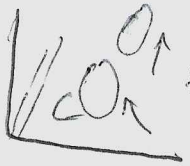
(moving my & Hastings files to backup disk (limited memory on Data :D)

Bailey is "using it for just an hour" sure, glances @ waste tank that I left full earlier that afternoon.

☆ Running 50µl H_2O @ 30 min mark, no cells in line. 60 x2min 120

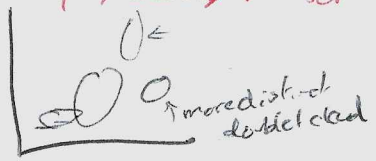
QC @ 10:36 am (Lot 2004) Important as keeps gains set ~ spot/daily.

FixPerm start @ 10:41 am. Unstimeds ← at whatever time frame they were at



(CD69 SEB)

collecting 50µl/sec → ✓ for this round. (or 100,000 events/



11:00 am (stop to fix sampleref names, swap in correct collection amounts for samples that got extra cells)

(will need to just duplicate w/o data to not need recreate again for FoxP3)

☆ (don't think we have enough Vd2's) ☆

... Did my SEB work? ... (did you vortex it?) ... IFNg no well ...

if any very little?

From CD45RA looks like we are having some major AF at least

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FoxP3 Staining

SEB that failed, or was it simultaneous addition of Stop? (did add stop ✓) (Remember)

Va 7.2 similar, lost in the ball?

TNFa also looks ≠, what happened SEB? did I add these abs? I think so.... culture condition same.... and the monitor tubes ✓ ... sigh...

↗ or stop @ start?

If brighter signal is on a Δ cell pop, does it matter if you focus exclusively on your own?

Lymphocytes are not no AF, they have it, just swamped vs monocyte levels.

↳ especially after culture? Y/N

¿Since log scale, how major is that split in pop. for monocytes @ uv detector

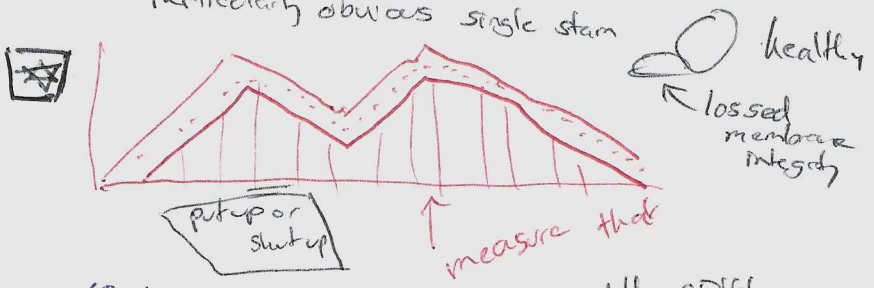
Effect freeze/thawed cells on baseline

~~Va7.2 similar~~

clear no cytokines or Δ between tubes

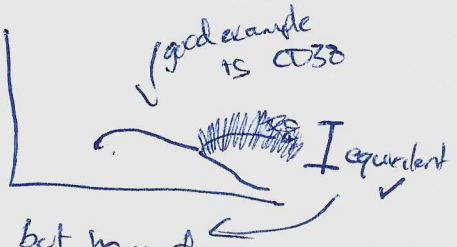
~some degranulation~

Particularly obvious single stain



¿whats it like to spend all your career and end up in an obsolete technology?

Talen codgers opinions on Celgene ~ ConvFlow vs spectral pops



but how does equivalent look compressed/spread over a log?

Not as easy as first glance

Half CD62L bits below margin events // (nope ~~poor quality~~) ditto CD16!

First set done @ 12:19 (1:40 min) 10:40

FoxP3 @ 12:25:

Wow.... vastly different. Almost on the threshold (250,000 // Lymphocyte average ~ 1M mark //

¿CD8 brighter w/ the FoxP3 ~ higher AF background?

[Set 5's at 50µl volume]

[distinct lack of creativity gets on my nerves] ... I am side-tracking though...

¿If you collect few events vs more events, effect on the final "color" signature sent to the matrix?

What does a decrease in FSC actually reflect?

// a decrease in internal cellular area // from Fix Perm? // loss cytosol

[¿Certain threshold % to be visualized on spectral line?]

They all pulled up CD107a stain from culture period, so yeah, analagous to Zombie NIR effectively

November 25th , 2022

FoxP3 Staining

If we do go nuclear stain route, we are going to need to change threshold, barely can gate on dead cells for its Zombie NIR SC.

(Swapping out mask before sample acquisition)

Sample acq @ 1:50 pm → Fixed 1.5 M FSC-A.

Manipulating FSC gain to have pop in same spot, but observing Δ in properties

¿Do you want to spend rest of your life counting deer year after year ~ want to be up all night doing flow running cells? ... no.

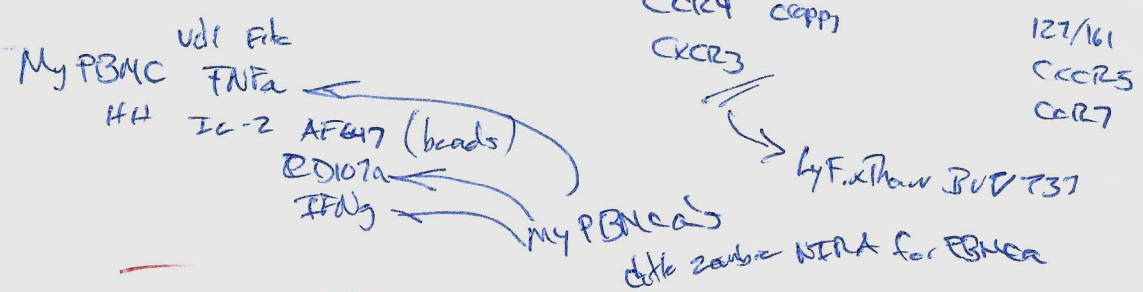
Leverage tech to find useful things.

Done @ 2:09 → cleaning up

Unmixing 11/28/2022

FoxP3 ⇒ used SC unstained for both activated unstained to start.

Last CD38 expression



No CXCR3 for FixPerm

127 intra

Matrix change of [0.05] as green cutoff.

¿Tag-on what?

48 62 6259.55
-17 -22 2203
31 40 40.29

0.05 - 0.10 green
0.10 - 0.15 yellow
0.15 - 0.20 orange
0.20 - 0.30 red
~~0.25 - 0.30~~
~~0.30 - 0.35~~
0.30 - 0.50 black

November 25th , 2022

FoxP3 Staining

Alexis meetings

Tuesday AM Wednesday AM Thursday AM

10:15 AM NYT → 2:15 AM
6:45
17:00
+6:45 → 9am

- Kirsten meeting -
- Miriam/Andrea -
- Alexis Gonzales -

Monday 5pm 6pm

Tuesday 5pm 6pm

Kirsten @ 2pm 10pm

Wednesday 5pm 6pm

Thursday 1pm

3-4pm