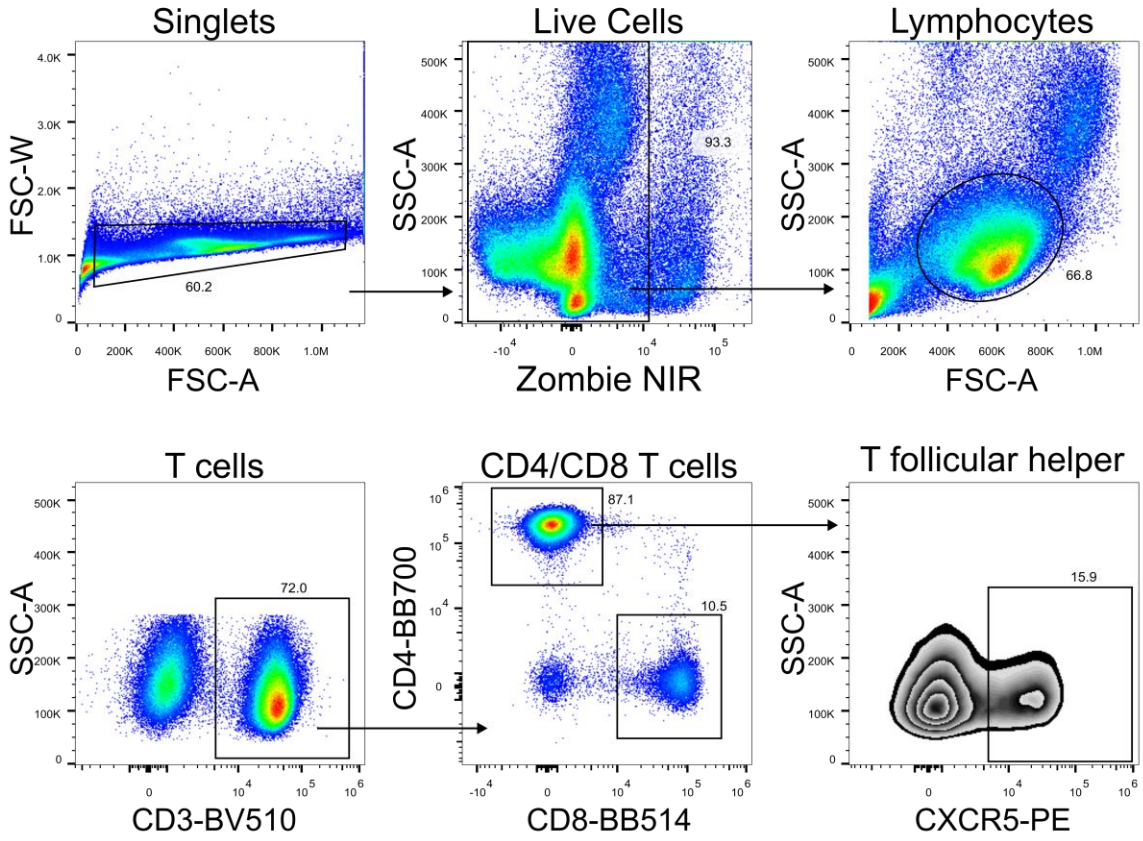
T follicular helper cell stain

1. Thaw frozen PBMCs in water bath.
2. Add to 8 mL pre-warmed cRPMI in a 15 mL conical tube (can put the cRPMI in the water bath ahead of time to warm it up).
3. Spin down PBMCs at 1500 rpm for 6 mins, 4°C. Decant and resuspend in 5ml of cRPMI.
4. Count PBMC live cell number.
5. Spin down, remove supernatant, then resuspend to 5 million cells/mL (500,000 cells per 100ul) in PBS (no protein, not FACS Wash).
6. Use a 96 well u-bottom plate for staining
7. Add 100ul of cells per well (500,000 cells per well). This stain does not require any stimulation. 3 wells per sample- unstained (US), Isotype, full stain. So, need 1.5 million cells each
8. Add 100ul PBS per well, mix, spin down, remove supernatant
9. Prepare ZNIR at 1:10,000 in PBS, add 100ul per well. US wells get 100ul PBS, not 100ul ZNIR.
10. Incubate for 30 mins in the dark at room temperature.
11. Add 100ul more of PBS to each well, spin down, remove supernatant.
12. Wash again with 200ul FACS wash, spin down, remove supernatant
13. Prepare antibody cocktails. PD-1 and ICOS need isotypes, so you have two master mixes- one for full stain, one for isotype.
14. Add 50ul of surface antibody cocktail to each corresponding well (stained or isotype). US well gets 50ul FACS Wash
15. Incubate 30 mins in the dark at room temp
16. Add 150ul facs wash to each well, spin down and remove supernatant
17. Resuspend the cells in 120ul FACS buffer.
    a. Or you could resuspend in 240ul if want it to run more slowly, just make sure your cytometer settings match your volume for acquisition, and consistently use the same volume for your experiments.
18. Filter the cells, don't add beads

   a. beads for PBMCs don't give us great count information because it doesn't relate back to like per mL of blood or per gram of tissue or something
   b. You could add count beads if it's useful for your panel and sample type. Do not filter the count beads, you add them after filtering, directly into the wells (don't want the filter to catch some of the beads and mess up the count)
      i. If you add count beads, make sure to record the count bead concentration from the vial, the volume you added, and your well volume so you can calculate numbers afterward.

19. Run on cytometer
   a. This panel was designed for use on the HITS Cytoflex LX, AIM configuration. If using another cytometer, check to make sure it can detect these colors.
   b. I imported compensation matrix and gain for my experiments, but if someone gets standardization up and running they may choose another option for gains instead.
20. I don't fix the samples for this stain, because LTC doesn't fix their PBMC AIM samples, and part of this stain is derived from the LTC AIM stain panel.



T follicular helper cell gating strategy. Debris and doublets were excluded, live cells gated on, and then lymphocytes were identified by scatter properties within the live cell gate. Within lymphocytes, $CD3^+$ events were identified as T cells, and these T cells were gated into $CD4^+$ and $CD8^+$ single positive cells. Within the $CD4^+$ T cell compartment, T follicular helper cells were identified as $CXCR5^+$ events.

This gating strategy is from my thesis. For simplicity there I just showed Tfh, but realistically I designed this panel to allow us to look at Tfh, Th1, Th2, Th17, and Tregs (CD4+CD39+). You could double check the literature to refine a strategy, but this would likely look like: within CD4+, gate CD39+ out as Tregs, take CD39- and gate for CXCR5. The CXCR5+ events are Tfh, the CD39-CXCR5- events move to be gated for Th1, Th2, and Th17 based on the existing gating strategies in our papers, using CXCR3, CCR4, CCR6. This stain does not use stimulated samples, as we didn't have

enough colors to be able to look at antigen-specific cells using AIM markers in addition to Tfh and Tfh phenotype, thus this tells us about overall Tfh, not antigen-specific Tfh.

PD-1 and ICOS are markers typically expressed on Tfh in SLOs, but some studies have suggested most circulating Tfh are negative for these markers[1]. Here are some references about what PD-1 and ICOS likely do, and how different phenotypes may be important[2–6]. I've included these markers to allow us to subtype/further examine the Tfh, and to cover our bases in case one day someone decides that their expression is indeed important for identifying true circulating Tfh.

Supplementary Table 1. Flow Cytometry Antibodies for Hybrid Immunity study

Specificity	Clone	Fluorophore	Manufacturer	Cat. Number	Dilution (1:X)
Live/Dead	NA	Zombie Near IR	BioLegend	423105	10,000
CD3	UCHT1	BV510	BD Biosciences	563109	50
CD4	SK3	BB700	BD Biosciences	566392	50
CD25	M-A251	PECy7	BD Biosciences	557741	50
CD8	RPA-T8	BB515	BD Biosciences	564526	50
CD39	A1	PEDazzle594	BioLegend	328224	100
CXCR3	G025H7	BV421	BioLegend	353716	25
CCR6	G034E3	BV785	BioLegend	353422	25
CCR4	L291H4	BV605	BioLegend	359418	25
CXCR5	J252D4	PE	BioLegend	356904	50
PD-1	EH12.2H7	APC	BioLegend	329908	25
ICOS	C398.4A	BV711	BioLegend	313548	25
mIgG1k (PD-1 isotype)	MOPC-21	APC	BioLegend	400120	*

Armenian Hamster IgG (ICOS isotype)	HTK888	BV711	BioLegend	400963	*

*Dilution for isotypes is lot dependent and should match final µg of corresponding antibody.

Surface Stain

Antibody	Fluorochrome	Ab uL per sample
CD3	BV510	1
CD4	BB700	1
CD25	PECy7	1
CD8	BB515	1
CD39	PEDazzle594	0.5
CXCR3	BV421	2
CCR6	BV785	2
CCR4	BV605	2
CXCR5	PE	1
PD-1	APC	2
ICOS	BV711	2
Brilliant Buffer	NA	10
PBS	NA	24.5

Surface Stain ISOTYPE

Antibody	Fluorochrome	Ab uL per sample
CD3	BV510	1
CD4	BB700	1
CD25	PECy7	1
CD8	BB515	1
CD39	PEDazzle594	0.5
CXCR3	BV421	2
CCR6	BV785	2
CCR4	BV605	2
CXCR5	PE	1
mIgG1k	APC	1
Armenian Hamster IgG	BV711	1
Brilliant Buffer	NA	10

PBS	NA	26.5

References

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