BENAROYA RESEARCH INSTITUTE VIRGINIA MASON

Getting started on the CyTOF: learning by living

Alice Long and BRI HIP core team

2013

BRI Timeline

Installation

Practice & Panel Development

Nov Dec Jan Feb Mar April May

Preparation

Training

Open for business

$

Preparation

- Funding decisions
  - External funding: Murdock foundation
  - Internal BRI funds to support HIP core CyTOF development
    - Pro's:
      - Not dependent on immediate grant funding
      - time to produce preliminary data for grants
    - Con's:
      - No solid commitment from staff to use the machine
- Gaining experience
  - Visited others facilities
    - OHSU – Mandy Boyd
    - FHCRC – Andrew Berger
    - Lessons learned
      - Instrument set-up: location of CyTOF in lab, gas and vacuum flow
      - Re-iteration of instrument operation and troubleshooting: common disposables
- Education
  - Potential of technology
    - BRI-wide and Faculty presentations
  - Comparison of flow vs CyTOF

Flow Cytometry vs. Mass Cytometry

Technology	Fluorescence flow cytometry	Mass Cytometry
Measurement	Fluorescent probes	Stable mass isotope probes
General Capabilities		
	20 parameters, 18 fluorescent (difficult)	37 ….and counting (easy*)
	-	Novel metals (i.e. barium, gold, iodine)
	Fluorescent barcoding	Metal tag barcoding
	FSC and SSC	-
	Ca^{2+} flux	-
	Mitochondrial Assessment	?
	Cell division (CFSE)	-
	Live cell sorting	-

Edited from Bendall S, et.al., Trends Immunol 2012

Flow Cytometry vs. Mass Cytometry

Technology	Fluorescence flow cytometry	Mass Cytometry
Sensitivity	0.1-10	1-2
Quantitative	Yes	Yes
Throughput		
Cells/s	25,000	1000 (500)
Cells/hr	25-60 million	1 million
Efficiency	> 95%	< 30%
Reagent Cost		
Per probe per test	$2.00-$8.00	$1.50-$3.00
Maintenance		
Daily	15 minutes	60 minutes
Weekly	30 minutes	4 hours

Installation and training

- Hands-on experience!
- Use DVS support!

Practice and Panel Development

- Initial practice and troubleshooting
- Surface panel development
  - Ab selection
  - Ab conjugation
  - Sample testing
- Current development projects
  - Class II tetramer
  - Phospho and ICS panels built of current surface panel
  - Mouse surface panel

Initial practice and troubleshooting

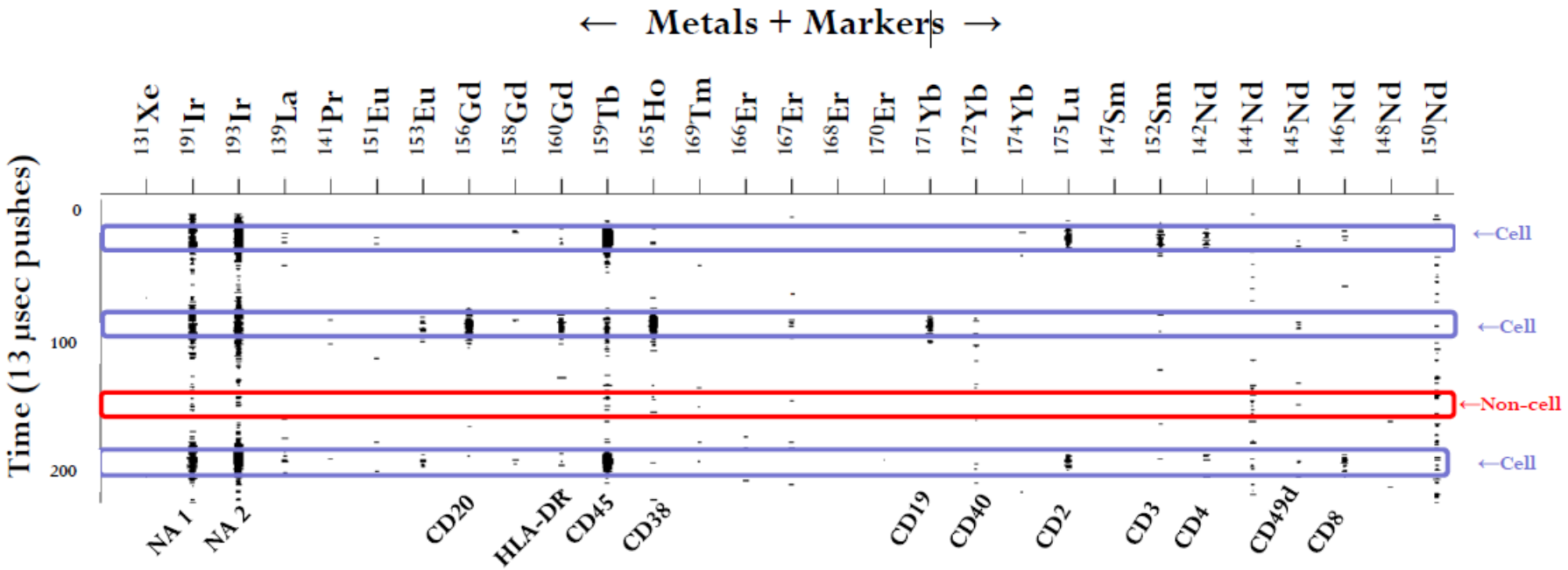
- Practice
  - Start the CyTOF daily for a few weeks, sometimes running basic samples to become comfortable with maintenance and troubleshooting
  - Become familiar with software and manuals

Selected issues encountered	solutions
Takes a few times to start	Make sure nebulizer is completely gassed-out
Pulse count, dual count, Tb TOF low (not pass QC)	SUPER clean copper contacts with EtOH
Sampler orifice damage?	Installed replacement
No fcs file after run	Stopped early, but data retrievable
Mass calibration off	Leslie from DVS fixed remotely
Oxidation high	Optimize make-up and nebulizer gas flows

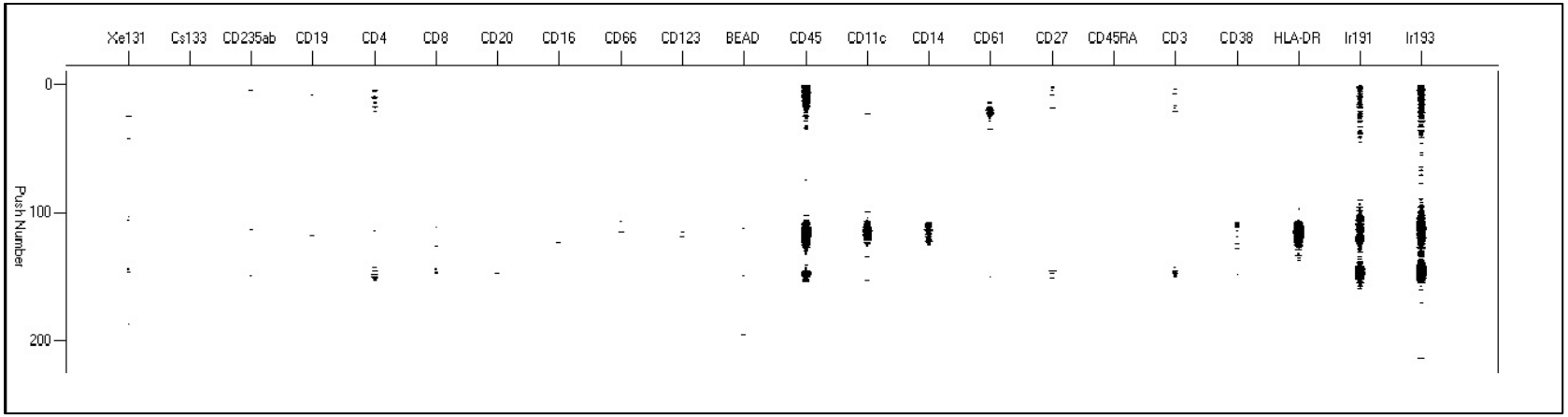
Other lessons learned

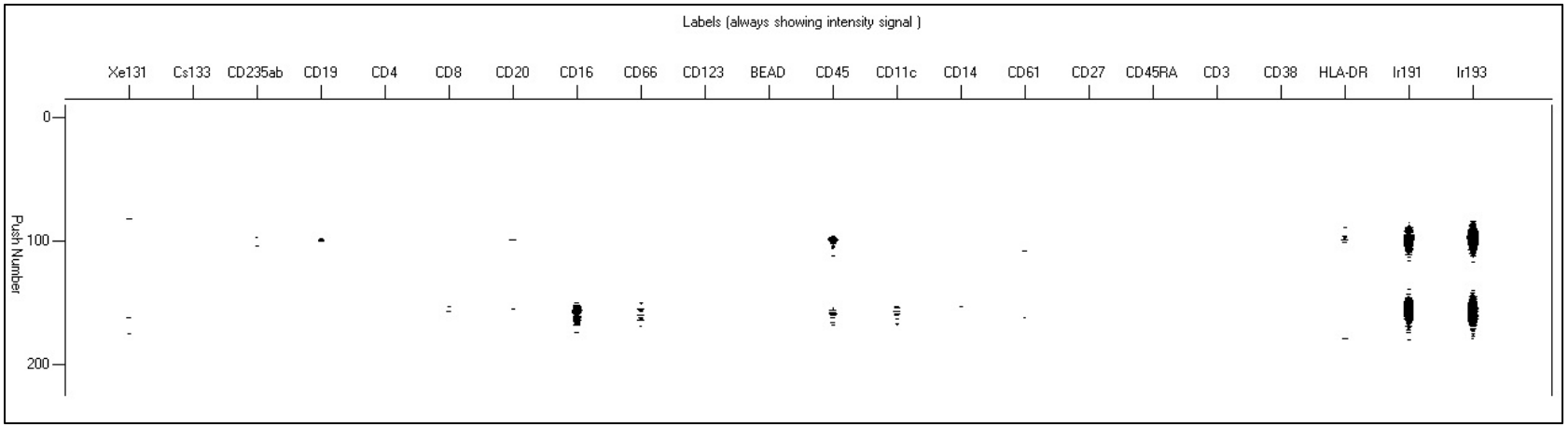
- Running samples
  - Importance of counting cells and flow rate
  - Optimizing protocols
    - Reduce washes
    - Least amount of time in water
    - Stability of stained cells
    - Need for permebilization and DNA labeling?
- Ask questions!
  - Thanks for help from DVS and Stanford team

DVS Raw Cytof Data



Really Raw Cytof Data





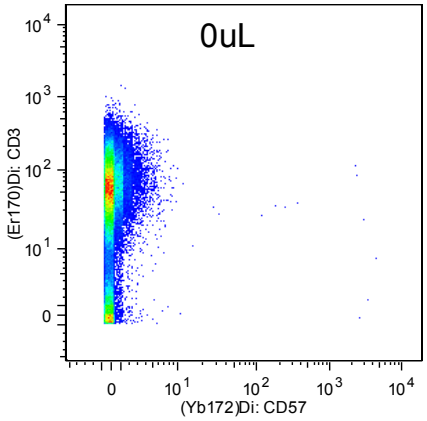
Development of a 28 Marker Mass Cytometry Panel

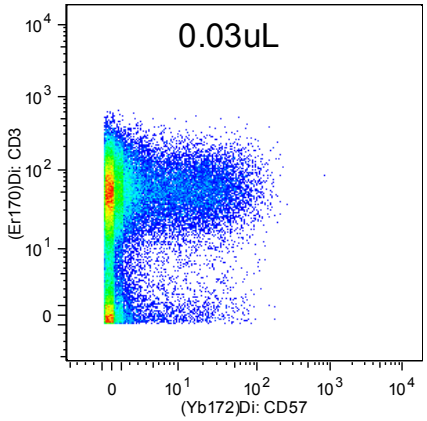
- Developed Metal Panel
  - Ordered 21 pre-conjugated human Antibodies from DVS Sciences as closely matching Stanford panels as possible
  - Conjugated 7 with MAXPAR® kits
  - Titrated all conjugates in the same tube on fresh and frozen PBMC
- Compared Panel to traditional Flow Cytometry
  - 3 human subjects, Ficoll separated
  - Assayed 4 fluorescent panels
  - Assayed 1 metal panel
  - Collected on LSRII and CyTOF

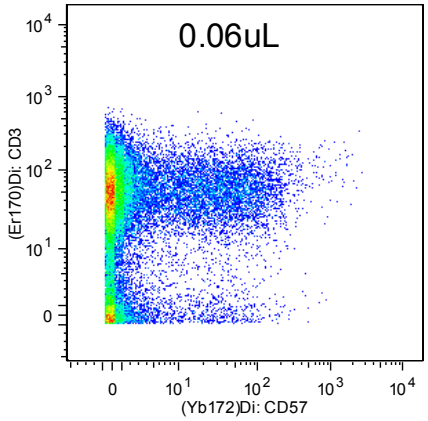
PBMC Surface Phenotyping Panel

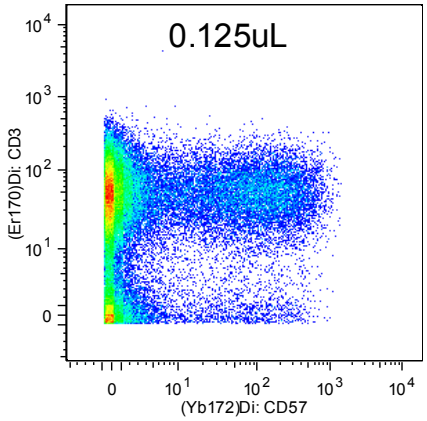
Ab	label	Ab	label	Ab	label
CCR4	149Sm	CD161	159Tb	CD45RO	152Sm
CCR6	141Pr	CD19	142Nd	CD56	176Yb
CCR7	150Nd	CD24	169Tm	CD8	168Er
CD10	156Gd	CD25	143Nd	CRTh2	175Lu
CD11c	162Dy	CD27	144Nd	CXCR3	158Gd
CD123	151Eu	CD3	170Er	HLA DR	174Yb
CD127	171Yb	CD38	167Er	IgD	146Nd
CD14	160Gd	CD4	145Nd	IgM	172Yb
CD15	164Dy	CD45	154Sm		
CD16	165Ho	CD45RA	153Eu		

Typical titration

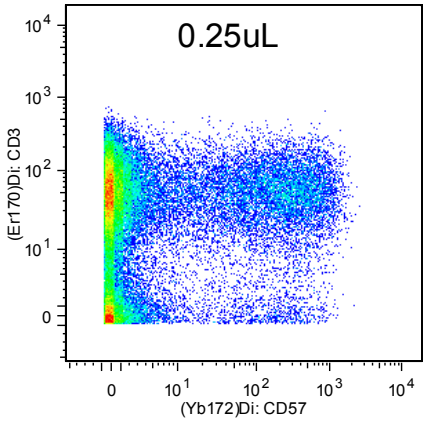


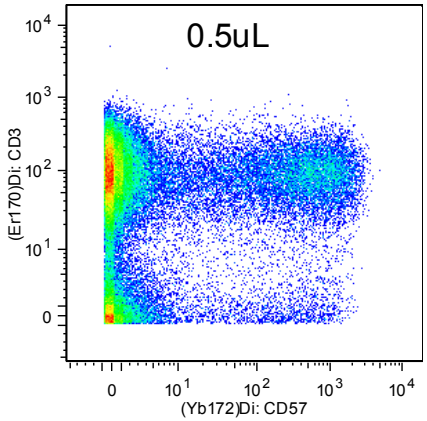


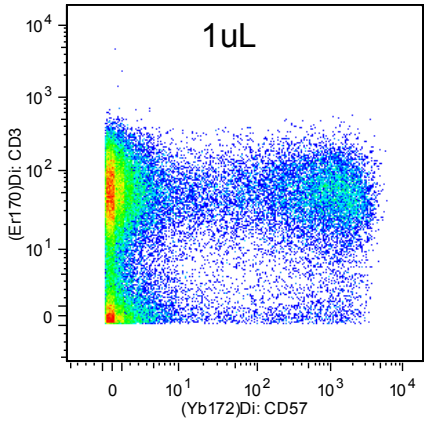


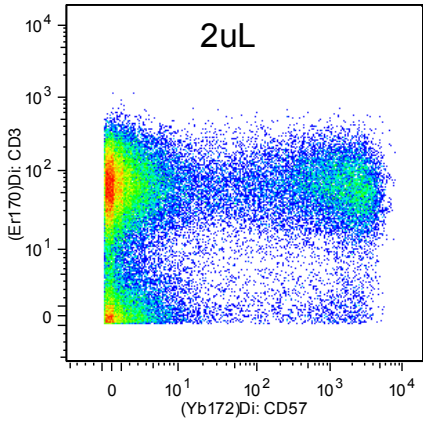


Use of isotype controls?



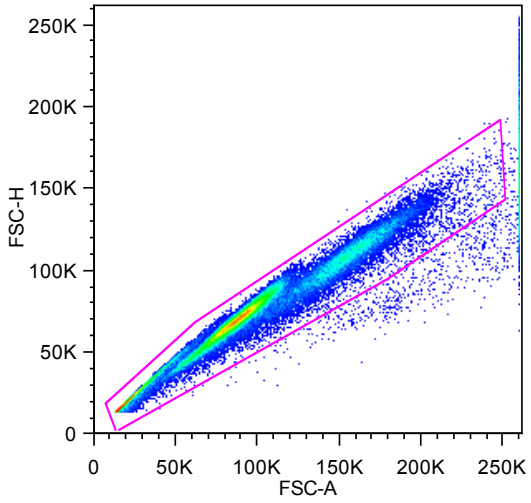


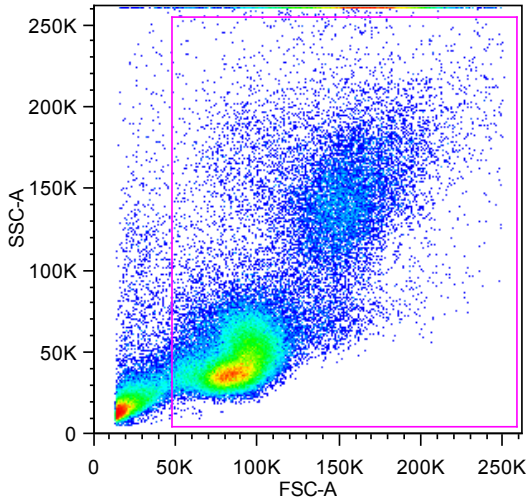


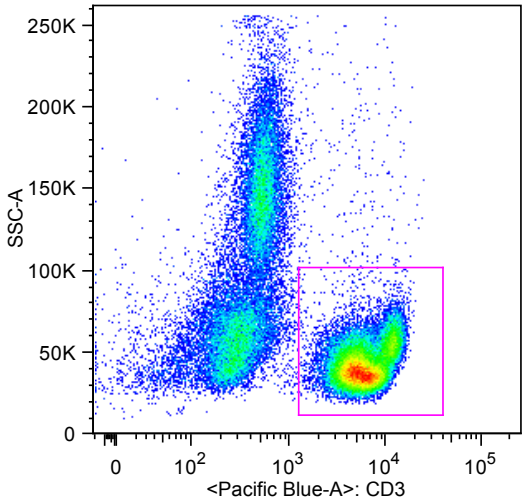


50 µL total volume

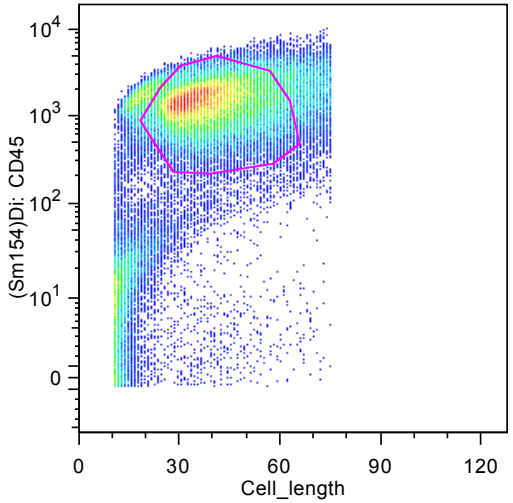
Gating for traditional Flow Cytometry

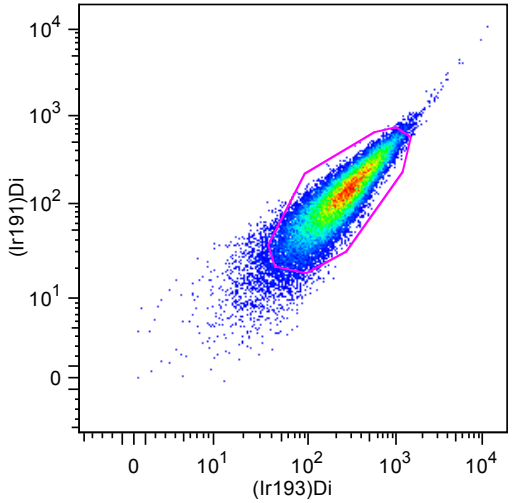


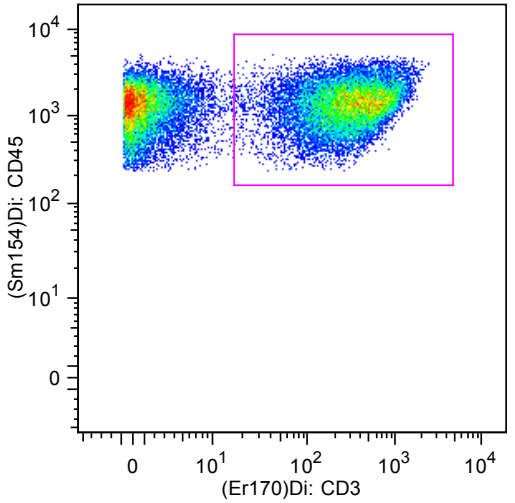




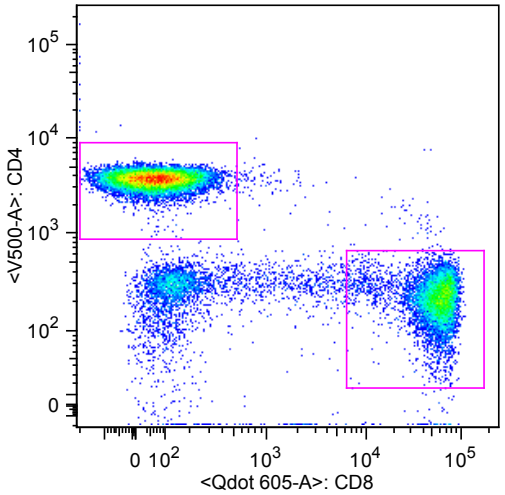
Gating for Mass Cytometry

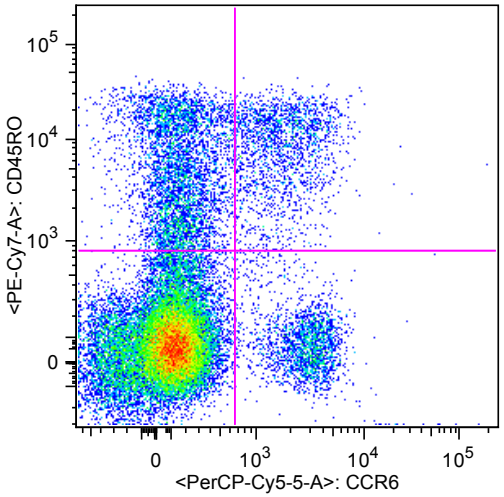


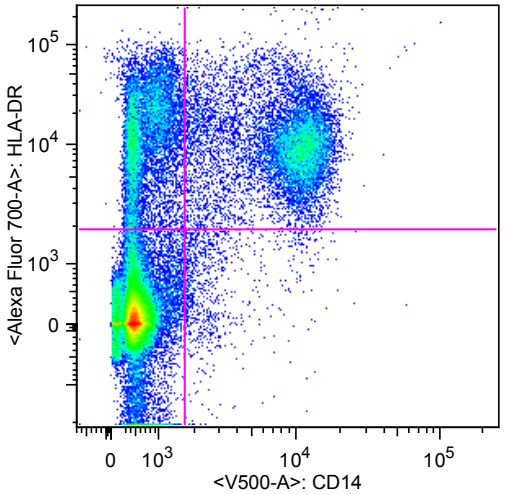




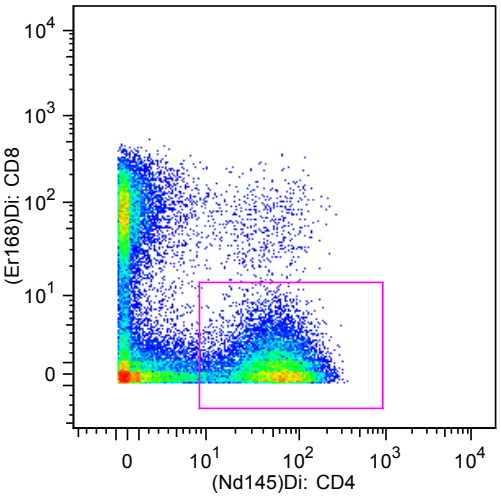
Gating for traditional Flow Cytometry

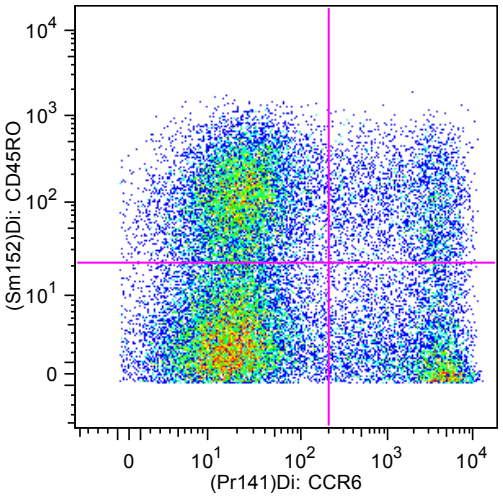


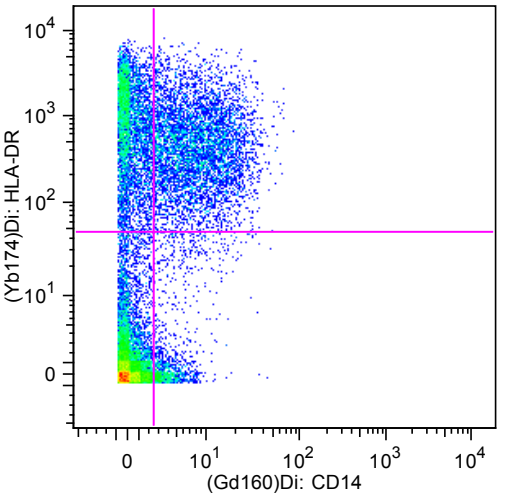




Gating for Mass Cytometry

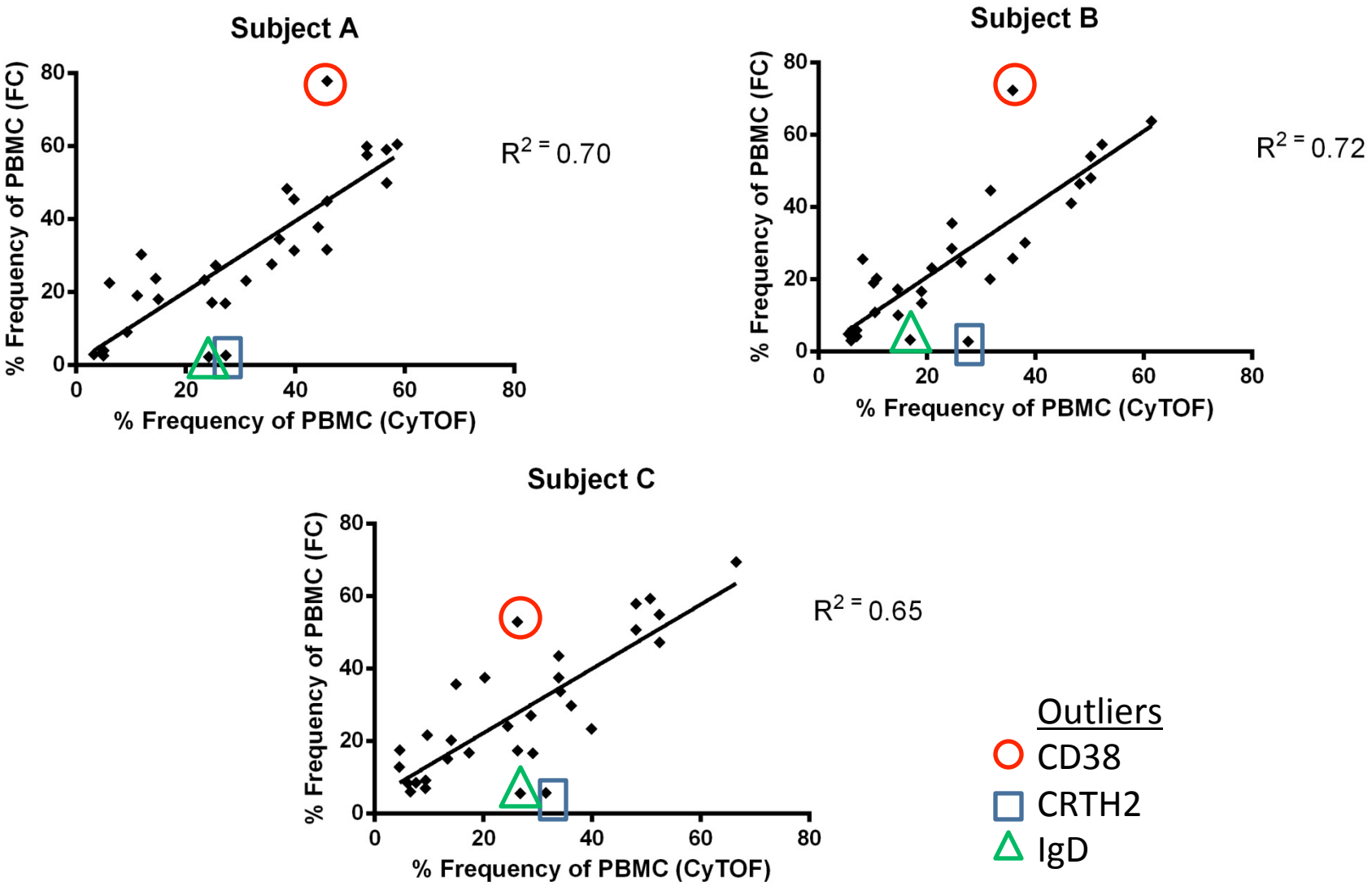






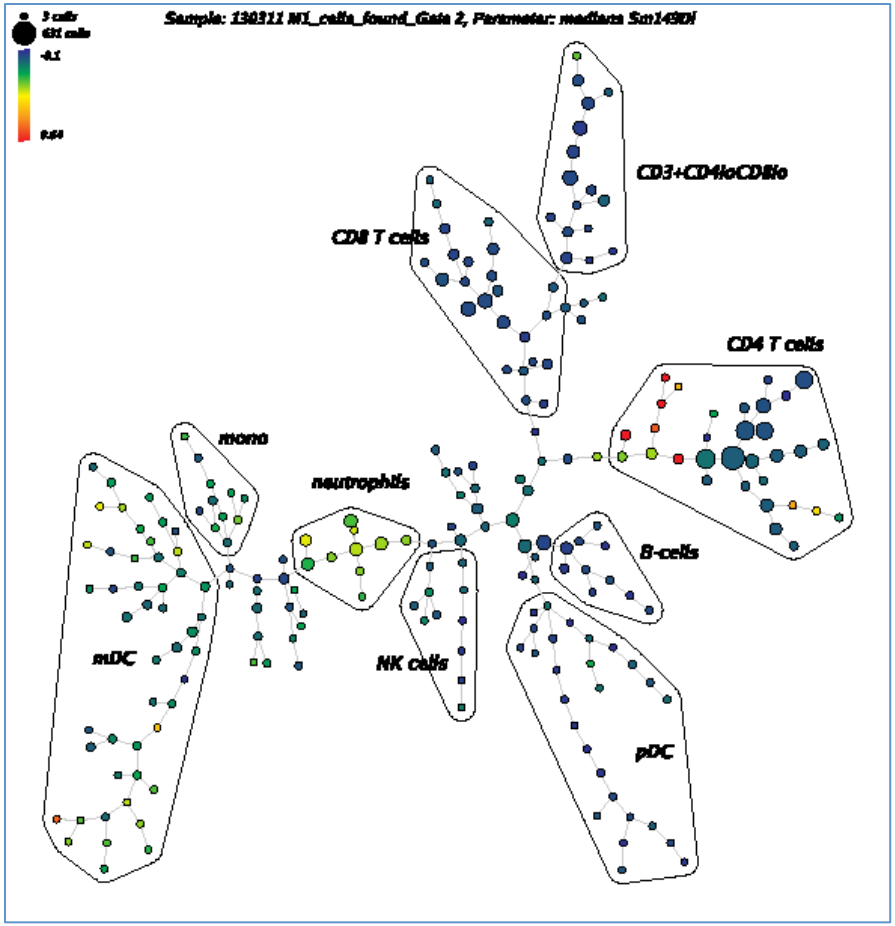
Differences in MFI and double positive populations?

Comparison of Mass Cytometry to Traditional Flow Cytometry (FC)

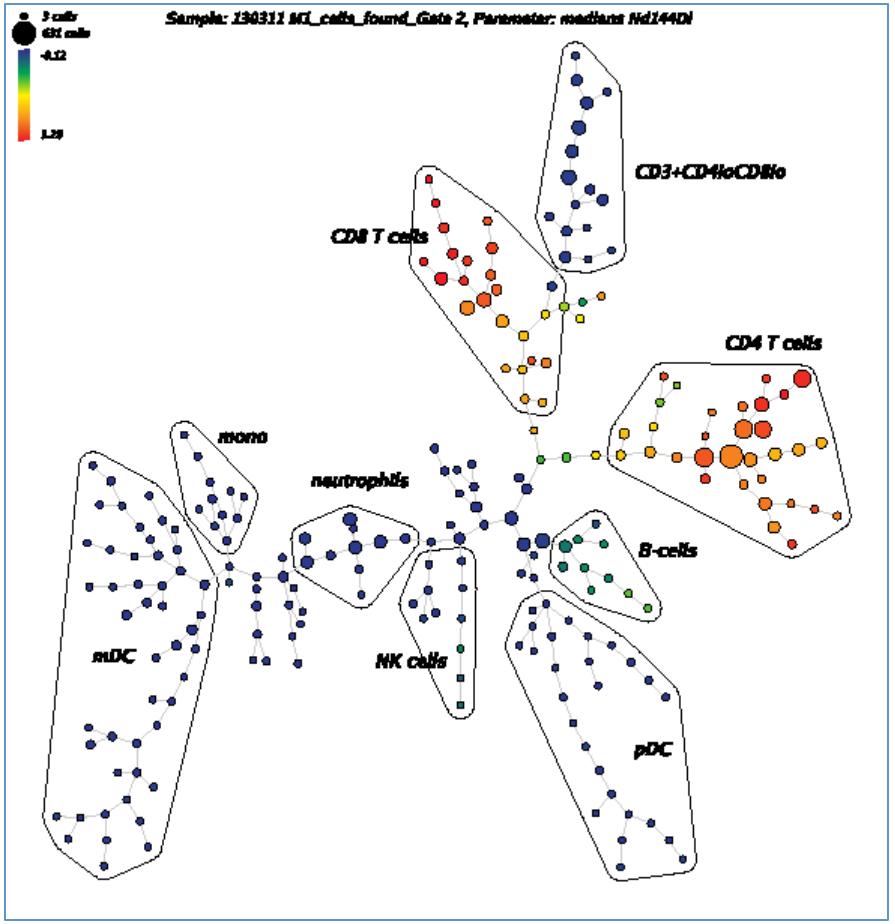


Examples of SPADE trees from whole blood samples

gate on live cells, cluster on CD19, CD4, CD8, CD3, CD123, CD56, CD11c



CCR4 density

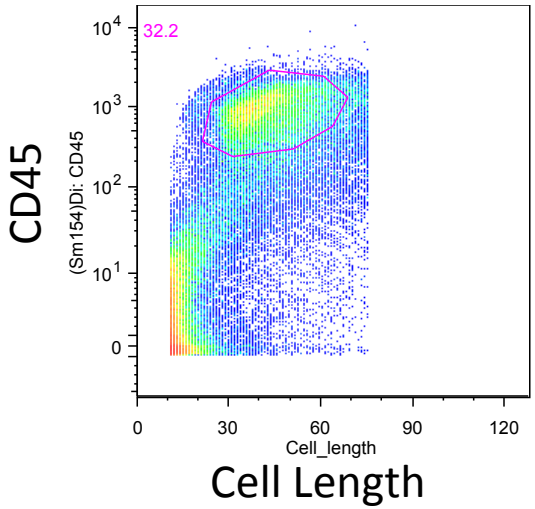


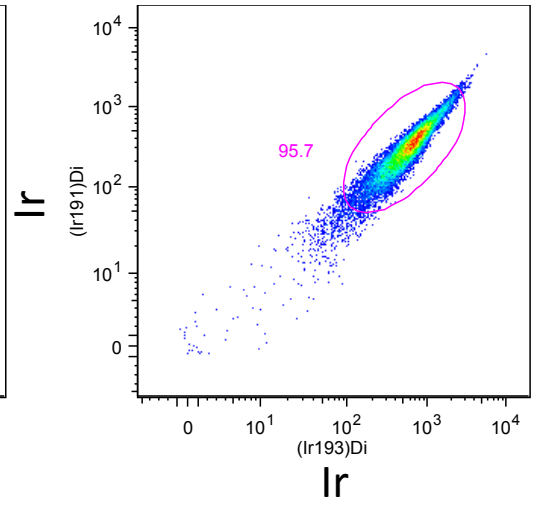
CD27 density

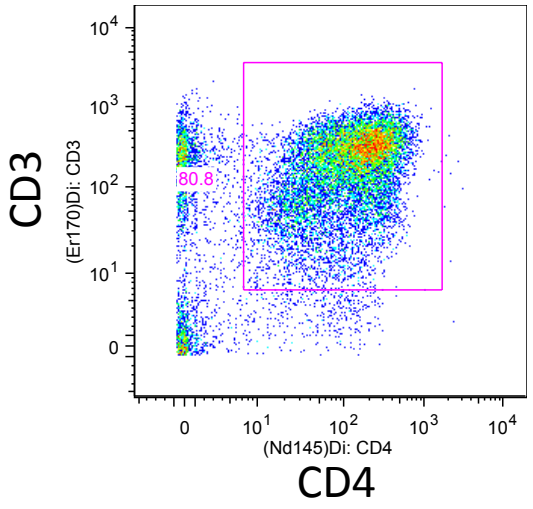
Antigen Specific Cell Phenotyping (Kwok Lab)

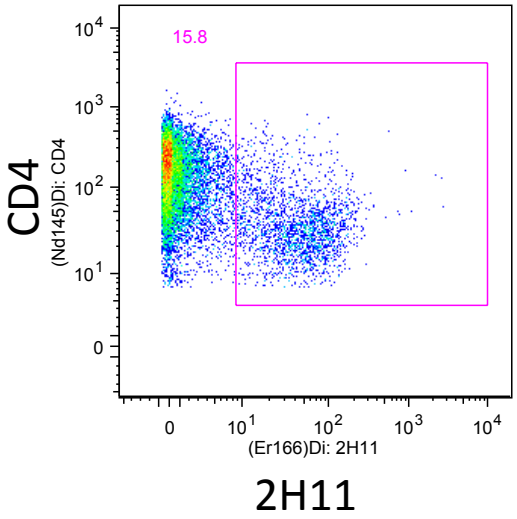
- HA Clonal Cells/PBMC spiked in.
- Cells surfaced labeled with Antibody (2H11) and metal-tagged tetramer (StAv metal)
- Washed and labeled with 2H11-166Er
- Fixed with 2% PFA
- Permeablized with Saponin
- Washed and labeled with Ir191,193
- Washed with MilliQ water
- Resuspended in MQ and Eu151,153

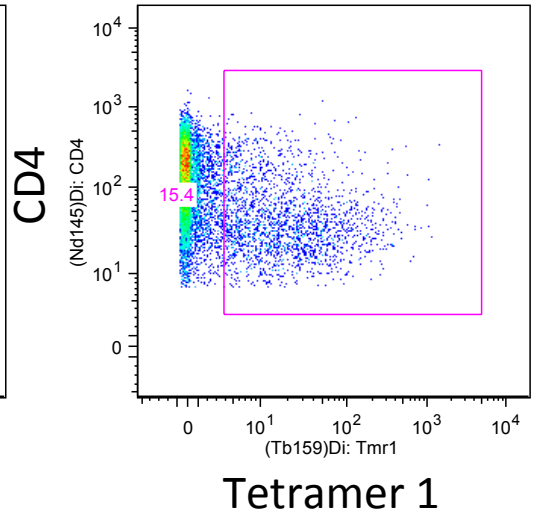
Class II - Flu Tetramer Positive

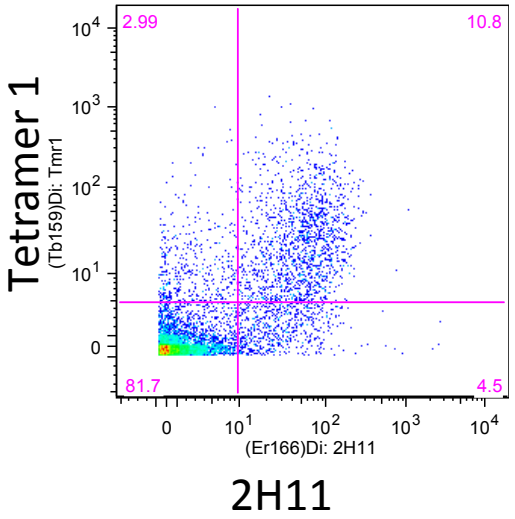












Collaboration with Kwok lab

Current troubleshooting with Class II Tmr

Issues	Possible solutions
Permeablization reduces Tmr binding, particularly for low affinity interactions	- Eliminate permeablization and identify cells by surface markers
Low frequency of Class II Tmr+ cells in PBMC	- Positively select Class II Tmr+ cells using anti-PE Miltenyi columns - Amplify signal using Strepavidin conjugated heavy metal or anti-biotin-metal Ab - Use surrogate marker of Tmr for initial tests of low frequency events
c-myc Class II Tmr production	- Trial and error

Current troubleshooting with ICS and phoshpo stains

Issues	Possible solutions
Pairing nuclear stains with ICS	- Testing BD transcription factor buffer by flow for multiple targets (flow vs CyTOF for nuclear stains) - Testing different FOXP3 antibodies
Selection of best heavy metal for bar-coding	- Trial and error
Best level of stimulation to detect subtle changes in autoimmune diseases	- Determining ½ maximal and maximal stim for phospho and ICS - Selection of positive control samples (PMA/I, cytokine capture techniques, lines and clones)

Open for Business

- BRI Resources
  - HIP core services
    - Maintenance of CyTOF
    - Scheduling CyTOF use
  - HIP core resources
    - Advice on experimental/panel design
    - Advice on protocols
      - Provide protocols and training
    - Metal conjugated Ab
    - Acquisition of data
    - FloJo QC and SPADE analysis of data

Cost structure

	Reagents	acquisition	FloJo analysis	SPADE analysis	BRI cost/ sample
Tier 1	HIP core	HIP core	HIP core		$240
Tier 1 (SPADE)	HIP core	HIP core	HIP core	HIP core	$300
Tier 2 (trained personnel)	BRI and/or HIP core ($300/vial) or $8/test	HIP core $200/hr	BRI or HIP core	$50/hr for analysis-training	
Tier 3 (specially trained personnel)	BRI and /or HIP core	BRI $100/hr	BRI		

SPADE is $1,200/year/user

CyTOF users to-date

- HIP core
  - Ian Frank
  - Jerill Thorpe
  - Katharine Schwedhelm
  - Alice Long
- Kwok lab for Tmr stains
- Jane Buckner and Alice Long 1st customers
  - Phospho and ICS stains on clinical samples

BENAROYA RESEARCH INSTITUTE

VIRGINIA MASON

Human Immunophenotyping Core (HIPc) at BRI

S. Alice Long

Katharine Schwedhelm

Jerill Thorpe

Ian Frank

BRI Investigators

Jerry Nepom

Bill Kwok

Erik Wambre

Jane Buckner

Funding Support

NIAID

NIAID

JDRF

JDRF IMPROVING LIVES. CURING TYPE 1 DIABETES.

Murdock Foundation