

Flow Cytometry Laboratory Considerations

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Introduction

- MTN
 - Rectal microbicide studies
 - Vaginal microbicide studies
- Non-MTN
 - HIV Prevention
 - Inflammatory Bowel Disease

Recent Studies

- MTN

- MTN006
- MTN007
- MTN008
- MTN011
- MTN017
- MTN024
- **MTN027**

- Non-MTN

- CHARM 01
- **CHARM 03**
- **MWRI-01**
- **A5330**
- **DIPY**
- Project Gel
- Merck IBD
- **HPTN069**
- HVTN-MIG
- **A5330**

MTN Studies Involving Flow Cytometry

STUDY	PBMC	MMC	CYTOBRUSH
MTN007		379	
MTN008	67		
MTN011			95
MTN017		77	
MTN024			112
MTN027			11
TOTAL	67	456	218

Non-MTN Studies Involving Flow Cytometry

STUDY	PBMC	MMC	CYTOBRUSH
A5330		15	
CHARM01		50	
CHARM03		29	
DIPY		70	
GADOH	26	26	
HPTN069		246	
HVTN-MIG	20	20	
PROJECT GEL		72	
GRAND TOTAL	113	984	218

Biological Samples for Flow Cytometry

- Peripheral Blood Mononuclear Cells (PBMC)
 - Density Gradient (exception MTN008)

Cell Type	Average Viability	Enumeration
PBMC	95-99%	~5-10 million/tube of whole blood

Biological Samples for Flow Cytometry

Size does matter!

- Mucosal Mononuclear Cells (MMC)
 - Mechanical and enzymatic dissociation

Cell Type	Average Viability	Enumeration
MMC	80-85%	~1 x 10 ⁶ /biopsy

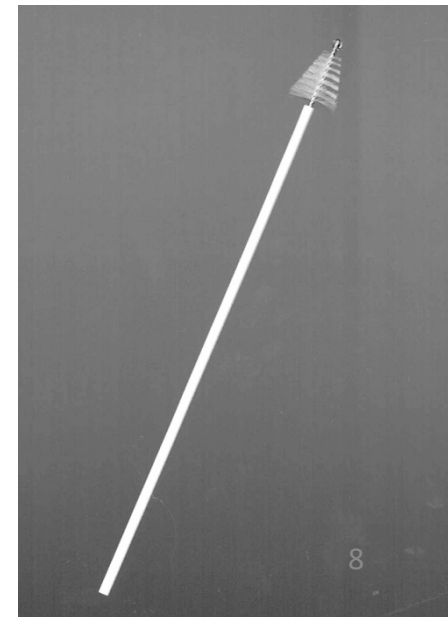


Weight (mg)
15.69
15.45
12.20
10.87
11.20
18.40
13.30
11.10
21.60
13.50
18.50
9.10
13.30
4.00
16.40
16.10

Biological Samples for Flow Cytometry

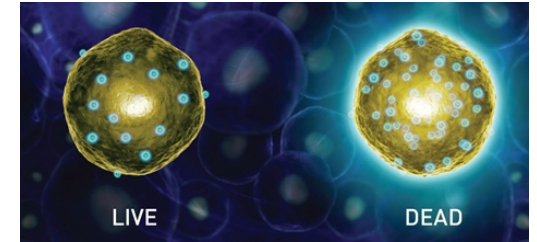
- Cervical Mononuclear Cells (CMC)
 - Mechanical dissociation

Cell Type	Average Viability	Enumeration
CMC	80%	50,000 – 1 million CMCs/cytobrush



Cell Yields and Viability

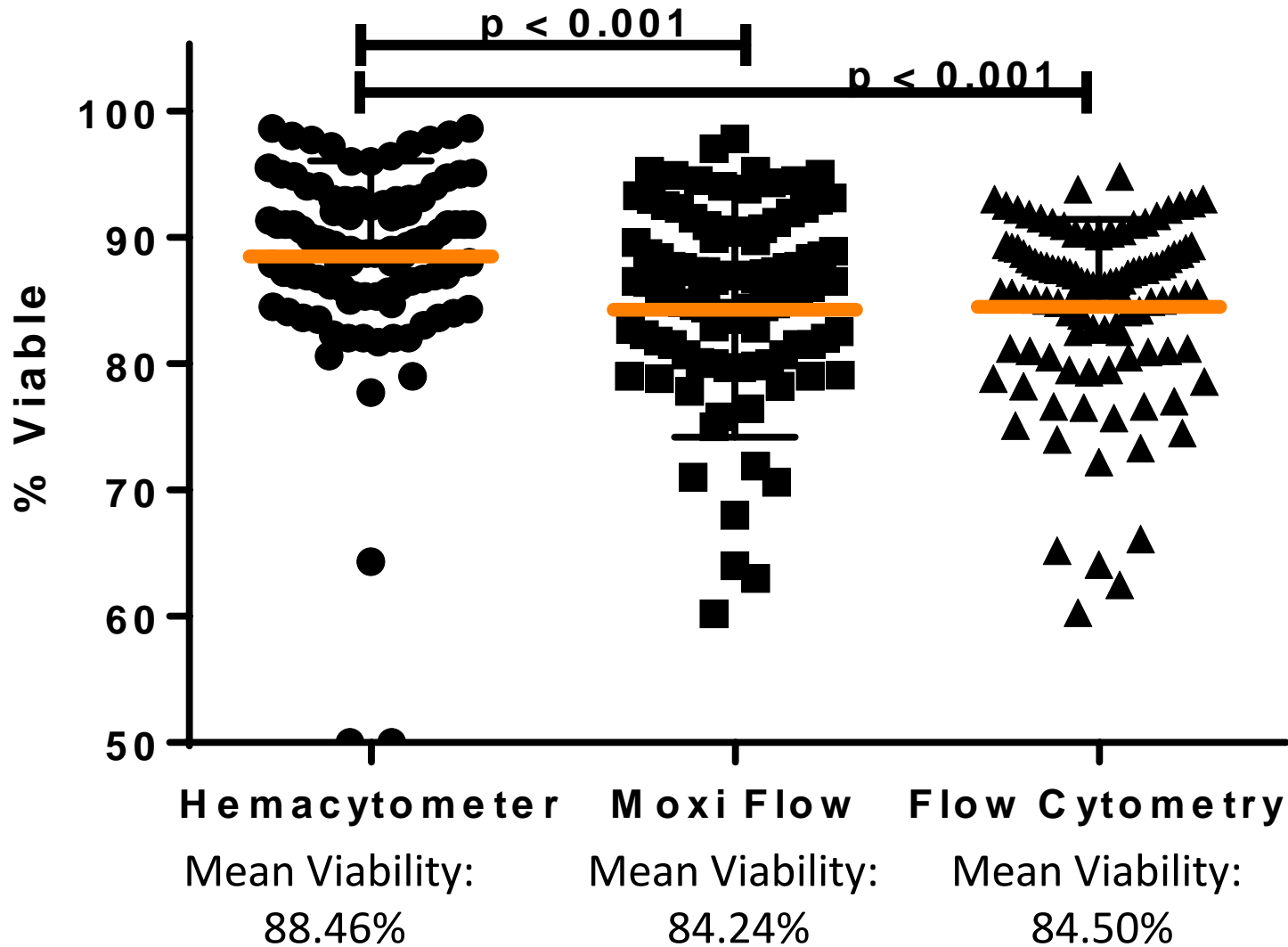
- Approach:
 - Trypan Blue Dye Exclusion
 - Moxi Flow™
 - Flow Cytometry



Cell Type	Average Viability	Enumeration
PBMC	95-99%	~5-10 million /tube of whole blood
MMC	80-85%	~1 x 10 ⁶ MMCs/biopsy
CMC	80%	50,000- 1 million CMCs/cytobrush

MMC Viability

N=104



External Assessment

- Quality Assurance/Quality Assessment Proficiency Testing for assay validation
 - Immunology Quality Assessment Center (IQA)
 - External Quality Assurance Oversight Laboratory (EQAPOL)

Immunology Quality Assessment Center (IQA)

- Quarterly Proficiency Testing of PBMCs
 - Isolation
 - Enumeration
 - Cryopreservation
 - Recovery



External Quality Assurance Program Oversight Laboratory (EQAPOL)



- 8 Color ICS Panel
 - Cryopreserved PBMCs
 - Overnight rest/Stimulation
 - Counting/Staining/Fixation
 - Proficiency in instrument set up
 - Data Analysis

Flow Cytometer

BD LSRFortessa™

Blue Laser

488nm

Violet Laser

405nm

Red Laser

640nm

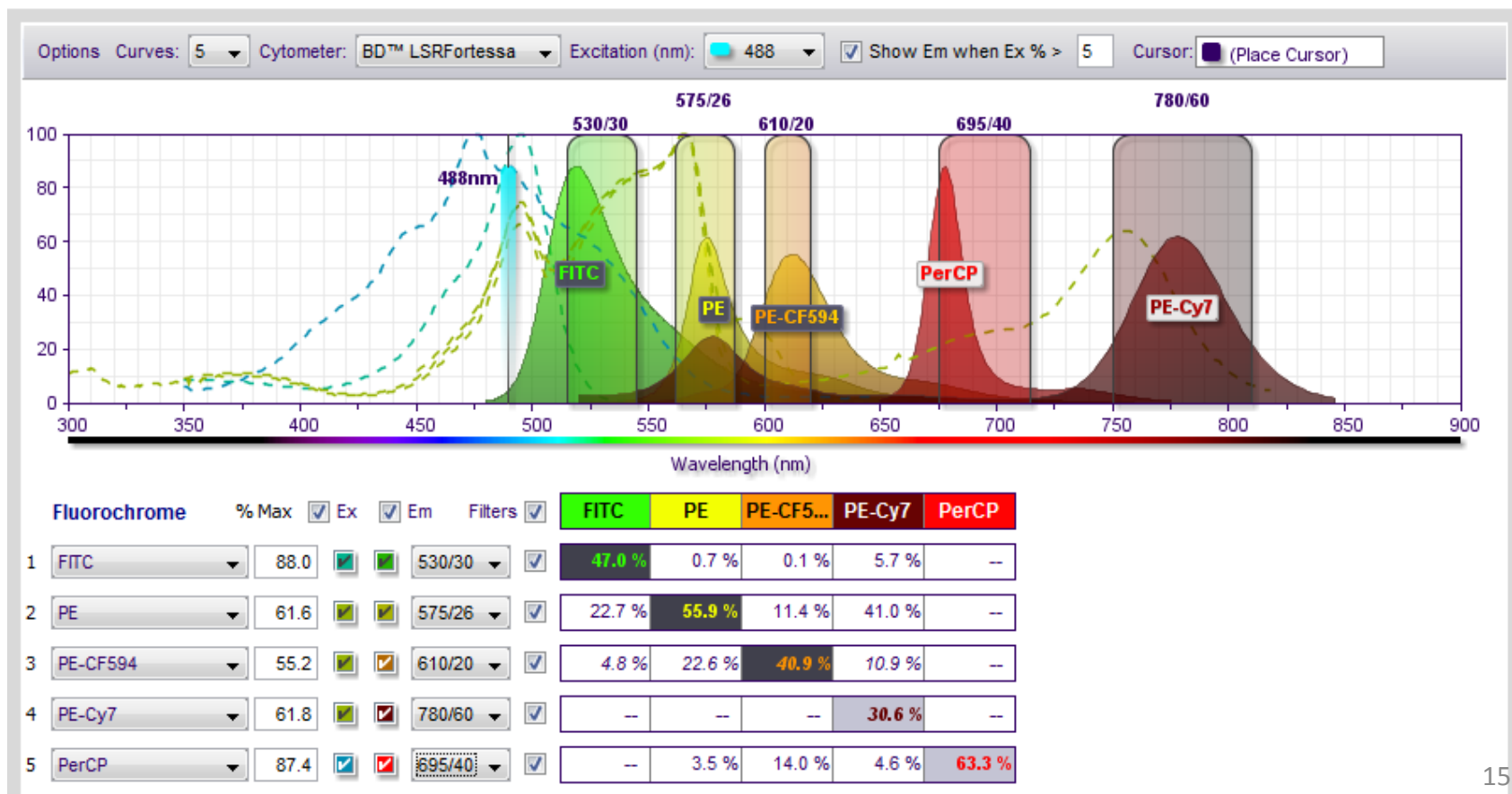
Ultra Violet Laser

355nm



Integrating Flow into Studies

- Panel Selection
 - BD Multi-Color Panel Design
 - BD Spectrum Viewer



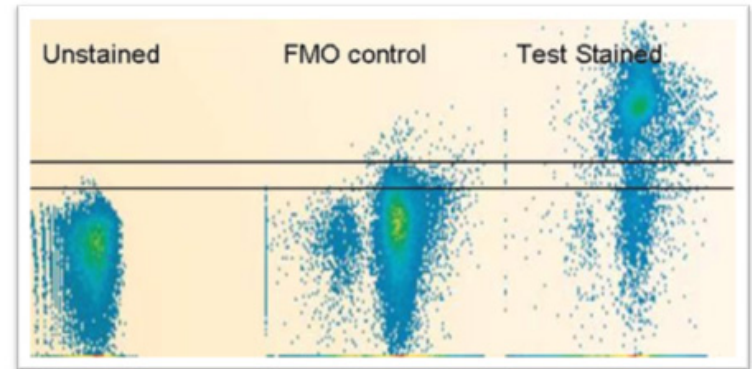
Preliminary Work

- Panel Selection: Things to Remember
 - Brightest fluorochrome for the lowest expressing antigens
 - Dimmest fluorochrome for your highest expressing antigen
 - Choose fluorochromes with emissions having the least spectral overlap

REAGENT	CLONE	FILTER	STAIN INDEX
PE	RPA-T4	585/40	356.3
Alexa Fluor 647	RPA-T4	660/20	313.1
APC	RPA-T4	660/20	279.2
PE-Cy7	RPA-T4	780/60	278.5
PE-Cy5	RPA-T4	695/40	222.1

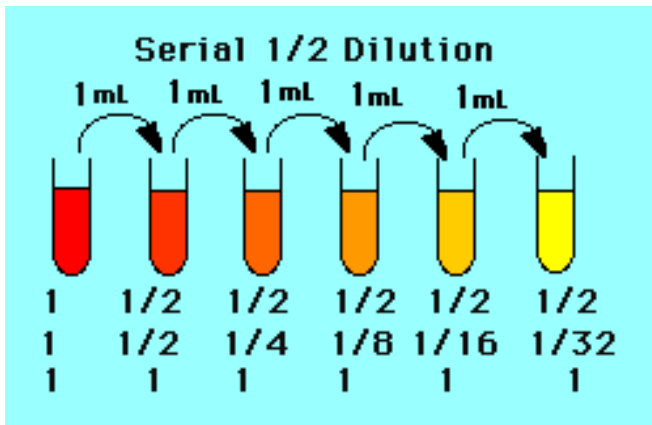
Preliminary Work

- Antibody Titrations
- Application settings
- FMO's
- Test it out!



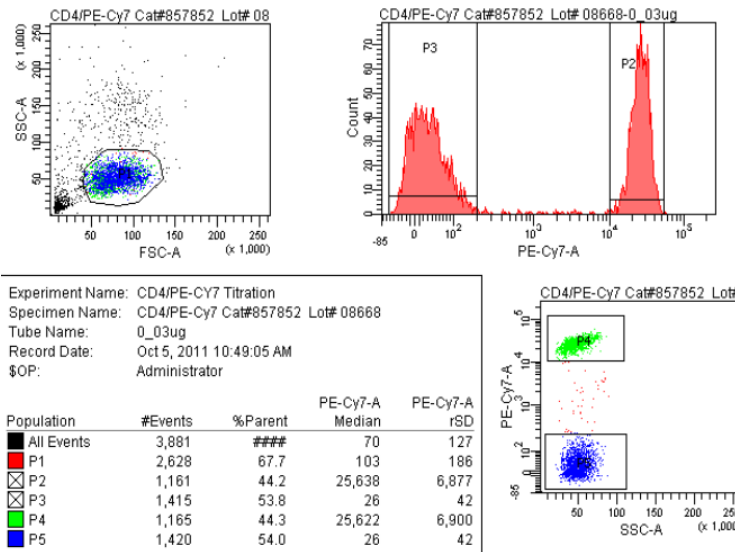
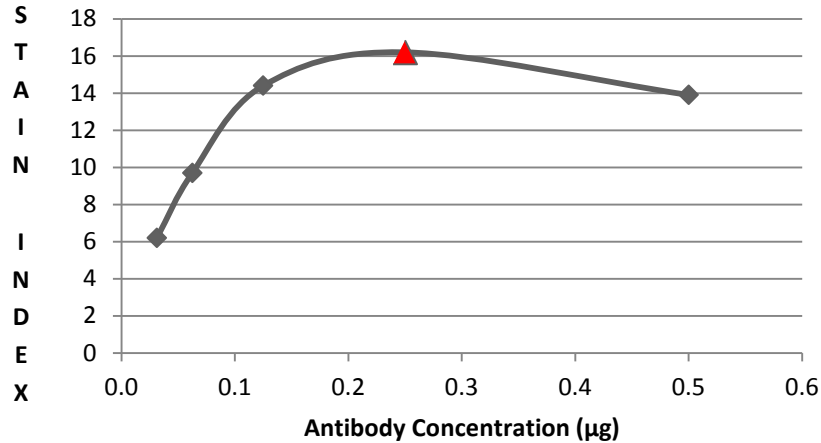
PITT/LSRFortessa (Baseline Report)

Fluorochrome	Lin Max	rSDEN	Multiplier		Cellular Target Value Range	
FITC	248317	17.0	2.5	3.0	42.5	51.0
PE	236569	14.5	2.5	3.0	36.3	43.5
PE-Texas Red	130840	43.9	2.5	3.0	109.8	131.7
PerCP	239607	35.2	2.5	3.0	88.0	105.6
PE-Cy7	231709	14.4	2.5	3.0	36.0	43.2
APC	235442	16.0	2.5	3.0	40.0	48.0
Alexa Fluor 700	237247	15.4	2.5	3.0	38.5	46.2
APC-H7	240983	18.1	2.5	3.0	45.3	54.3
Pacific Blue	232983	17.1	2.5	3.0	42.8	51.3
UV395			2.5	3.0		
UV737			2.5	3.0		
Aqua Blue	231607	11.1	2.5	3.0	27.8	33.3



Antibody Titrations

HLA-DR/Alexa Fluor 700

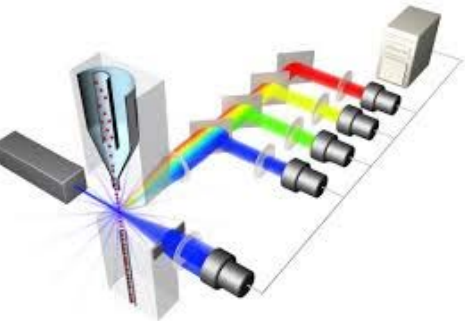


Stain Index = Median pos - Median neg / 2rSD

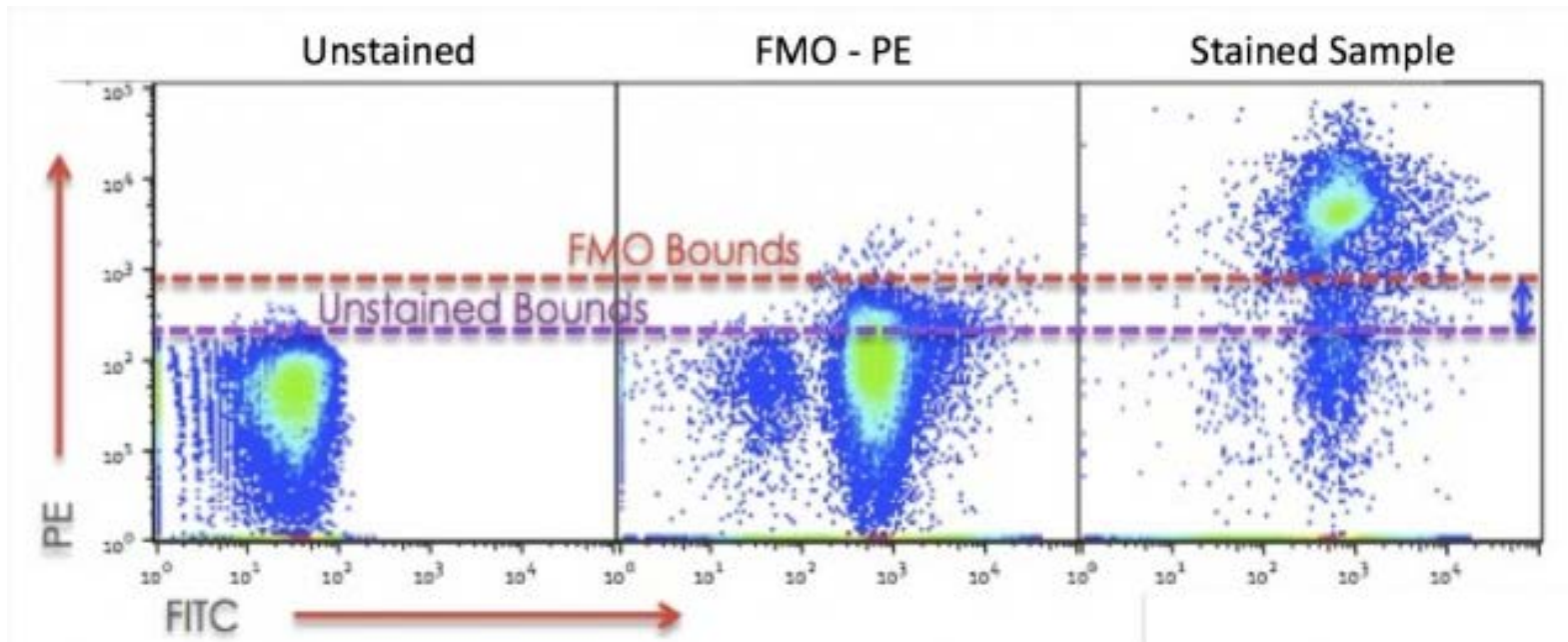
SI = 25638 - 26 / 2 (42) = 304.9

Application Settings

- Consistency
 - Using cells of interest in the study
 - Study lot specific CS&T to set MFIs
 - Allows for transfer to another instrument allowing recreation of the application settings and standardization of other cytometers



Use of FMOs

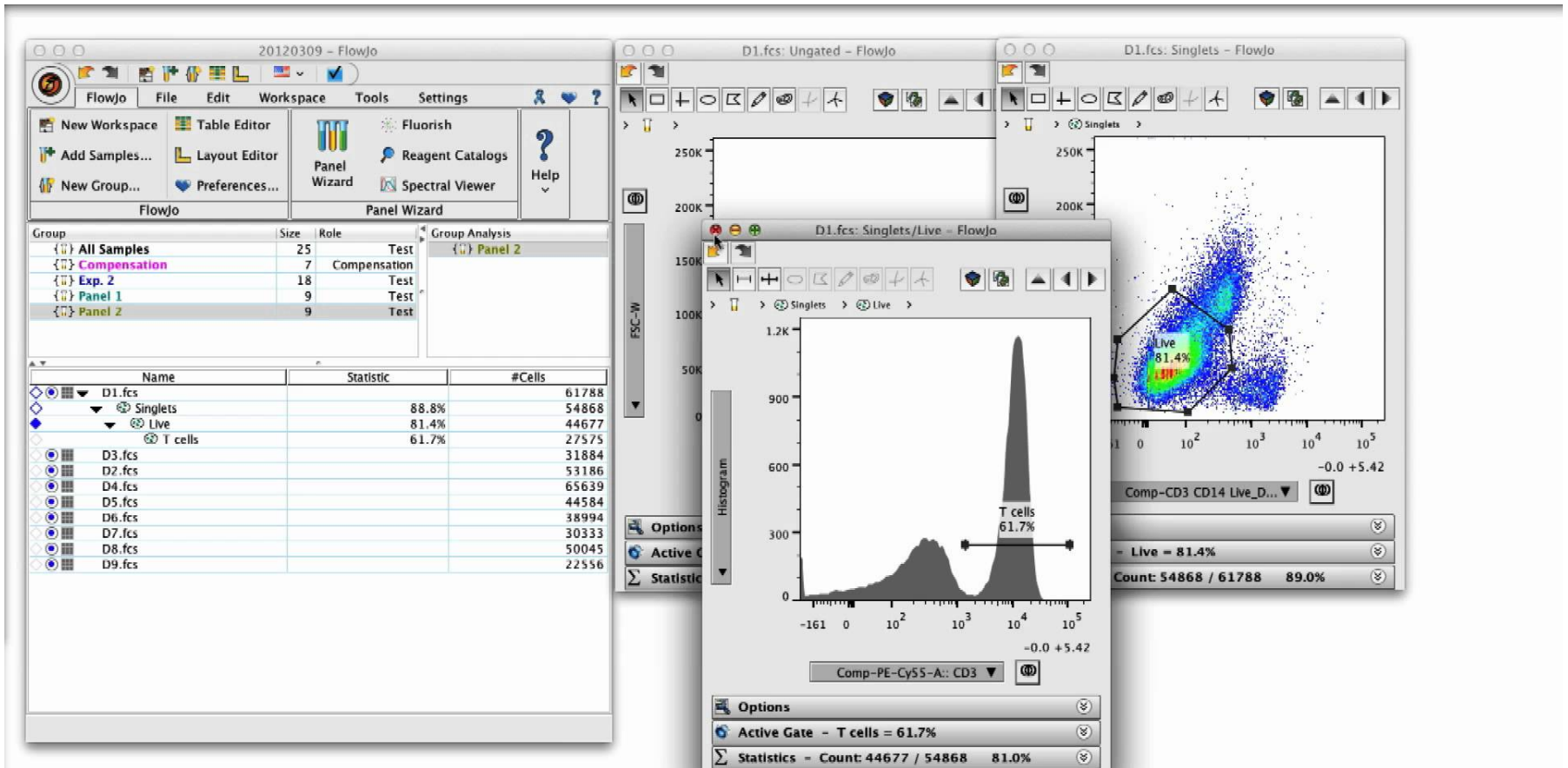


Next steps

- Once a panel has been designed, optimized, and tested on relevant biological samples...
- It is time to order study supplies
- Try and order sufficient volume to last for the entire study to avoid the need for re-titration of a new lot
- This can be challenging for slow enrolling studies (e.g. ACTG pathogenesis studies)

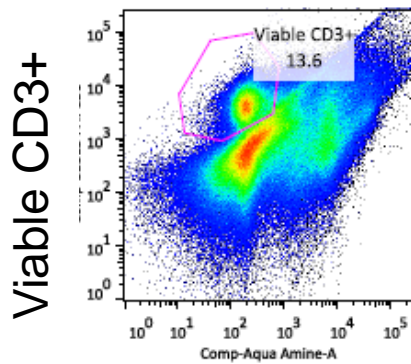
Flow Analysis

- Single Site Analysis



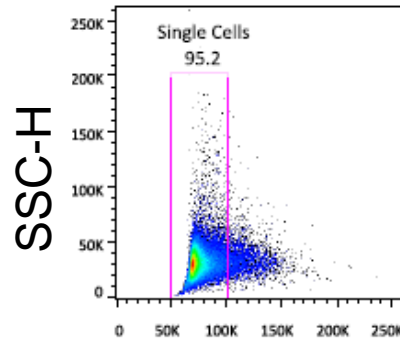
Panels

- Surface Staining
 - T Cell, Cytokine, & Activation Markers



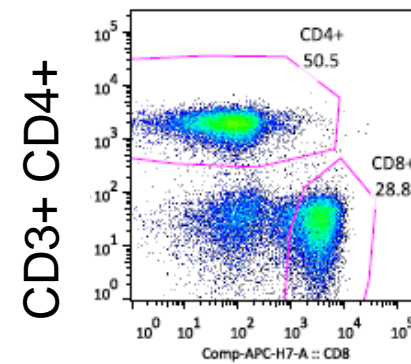
AVID

4.65E5



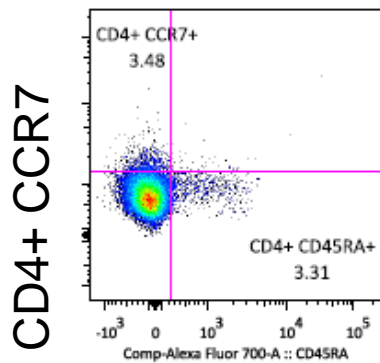
SSC-W

Viable CD3+
63246

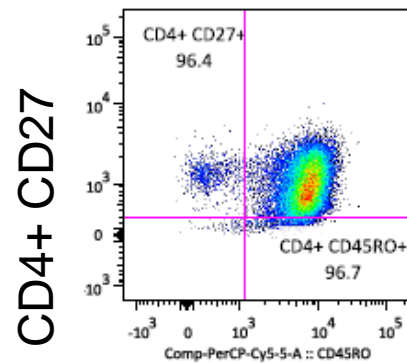


CD3+ CD8+

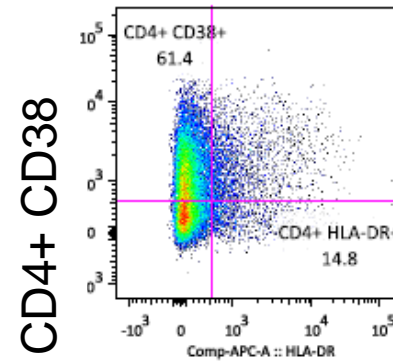
60228



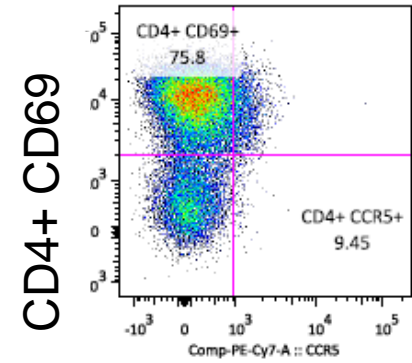
CD45RA



CD45RO

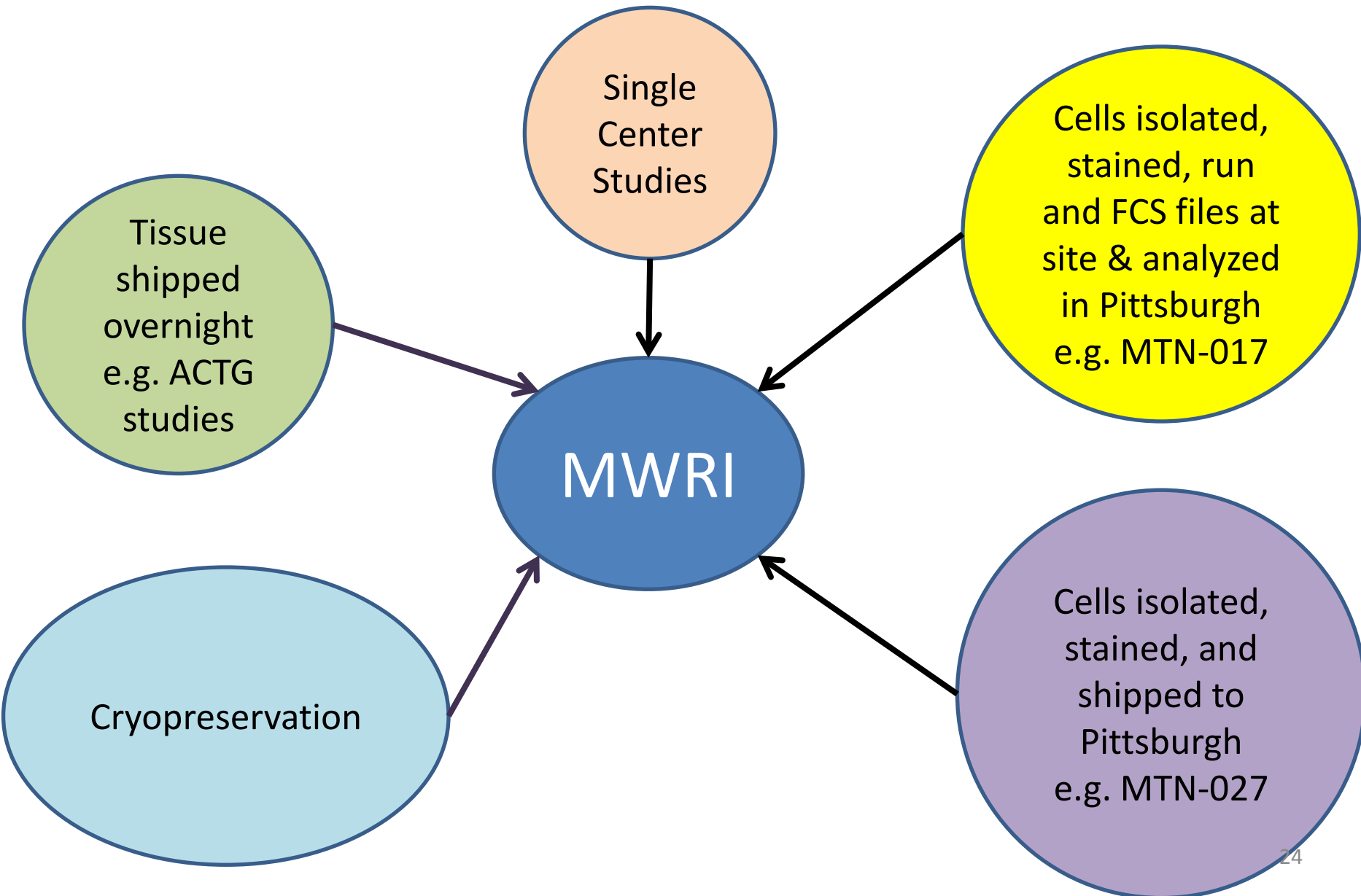


HLA-DR



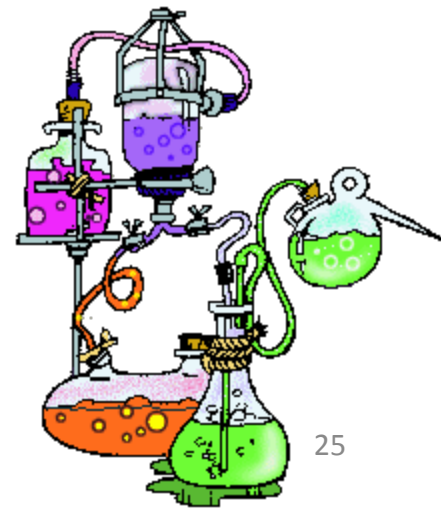
CCR5

Multi-Center Studies



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Questions ?

