Standardized voltage settings

For better resolution and experimental reproducibility

Research Flow Cytometry Core

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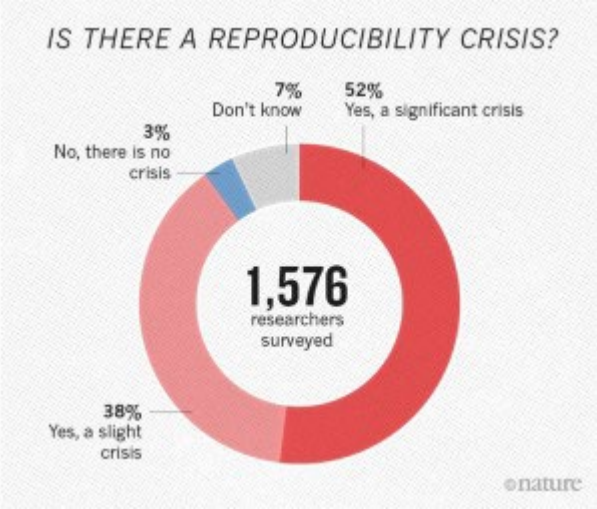
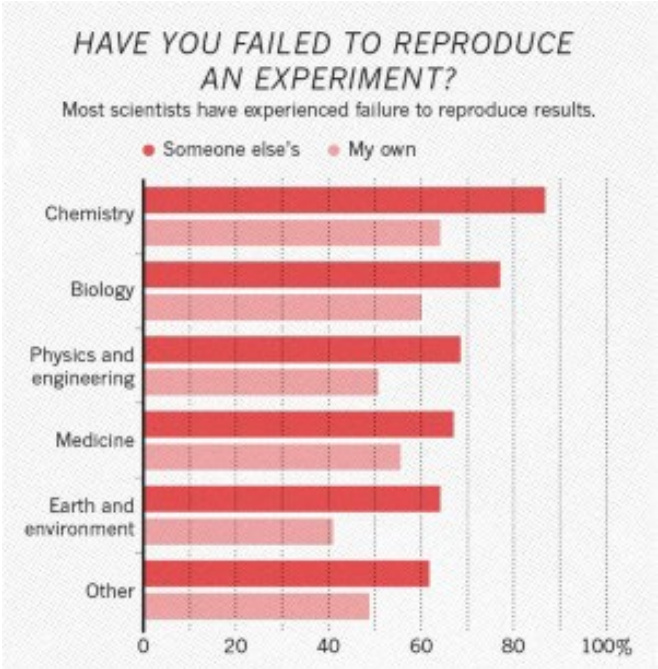
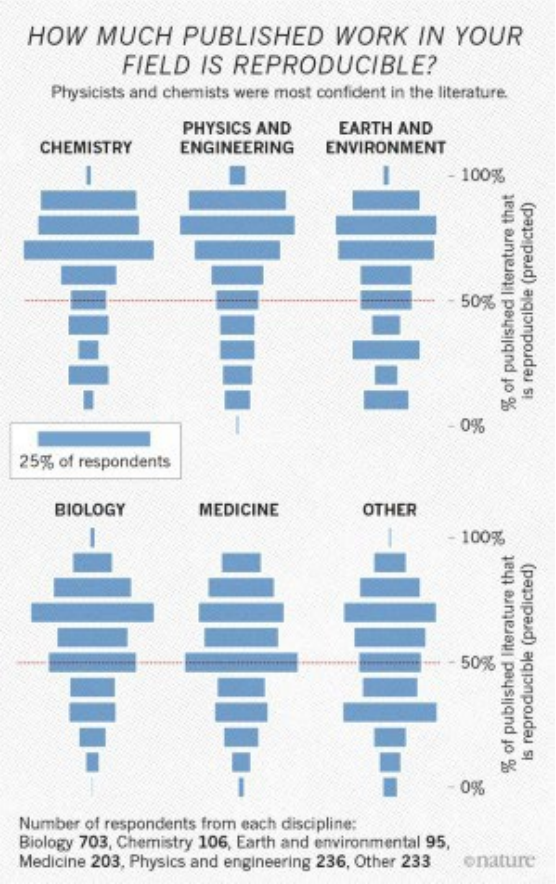
Why standardized settings are needed?

Lack of reproducibility in science







Baker, M. 1,500 scientists lift the lid on reproducibility. *Nature* **533**, 452–454 (2016).

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What is responsible for irreproducible findings in cytometry experiments?

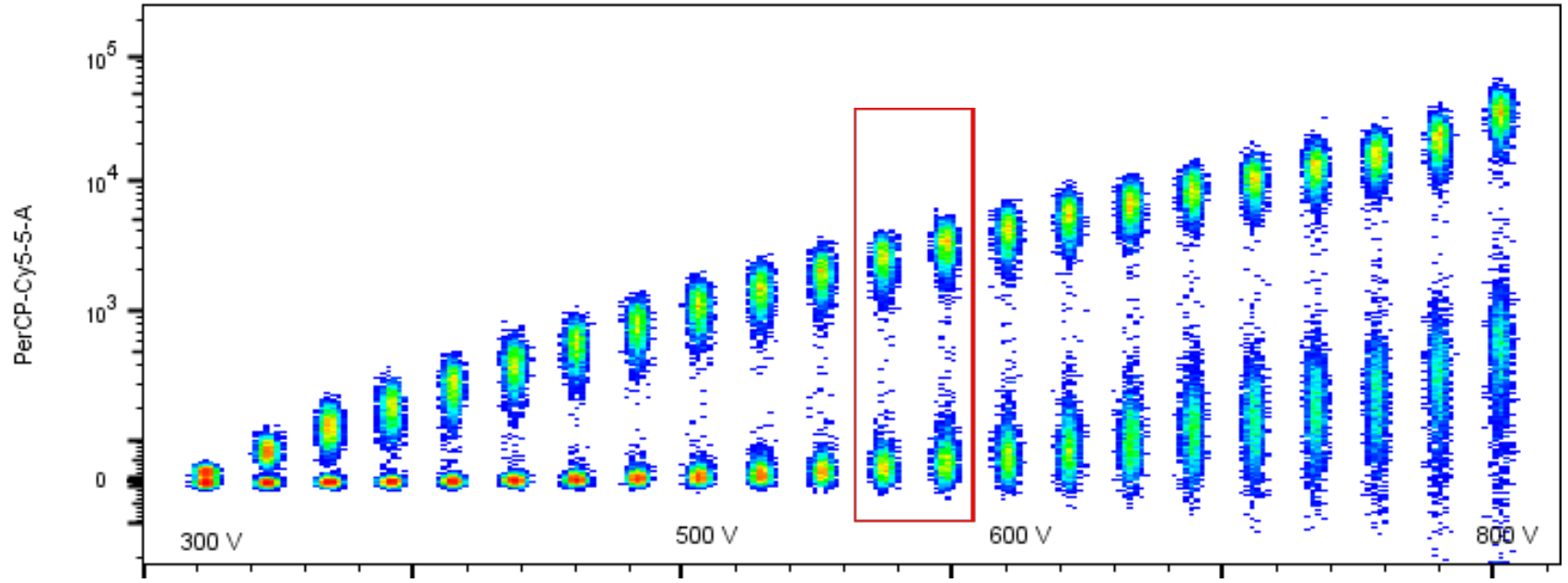
Statistics	Sample size, significance cutoffs
Reagents	Lot-to-lot variability, titration, specificity, reagent interactions
Instruments	Detector settings, optical filters, compensation, fluidic instability
Humans	Operator-to-operator inconsistency
Analysis	Data cleanup, manual gating vs computational approach

Different approaches to setting voltages

	By eye	QC	Copy from template	Matching target MFIs
Fast?	-	+++	+++	++
Reproducible?	-	+++	+	+++
Best separation?	?	+	?	+++

How standardized settings are determined

For each fluorescence detector: record cells stained with an appropriate anti-CD4 conjugate over range of PMT voltages



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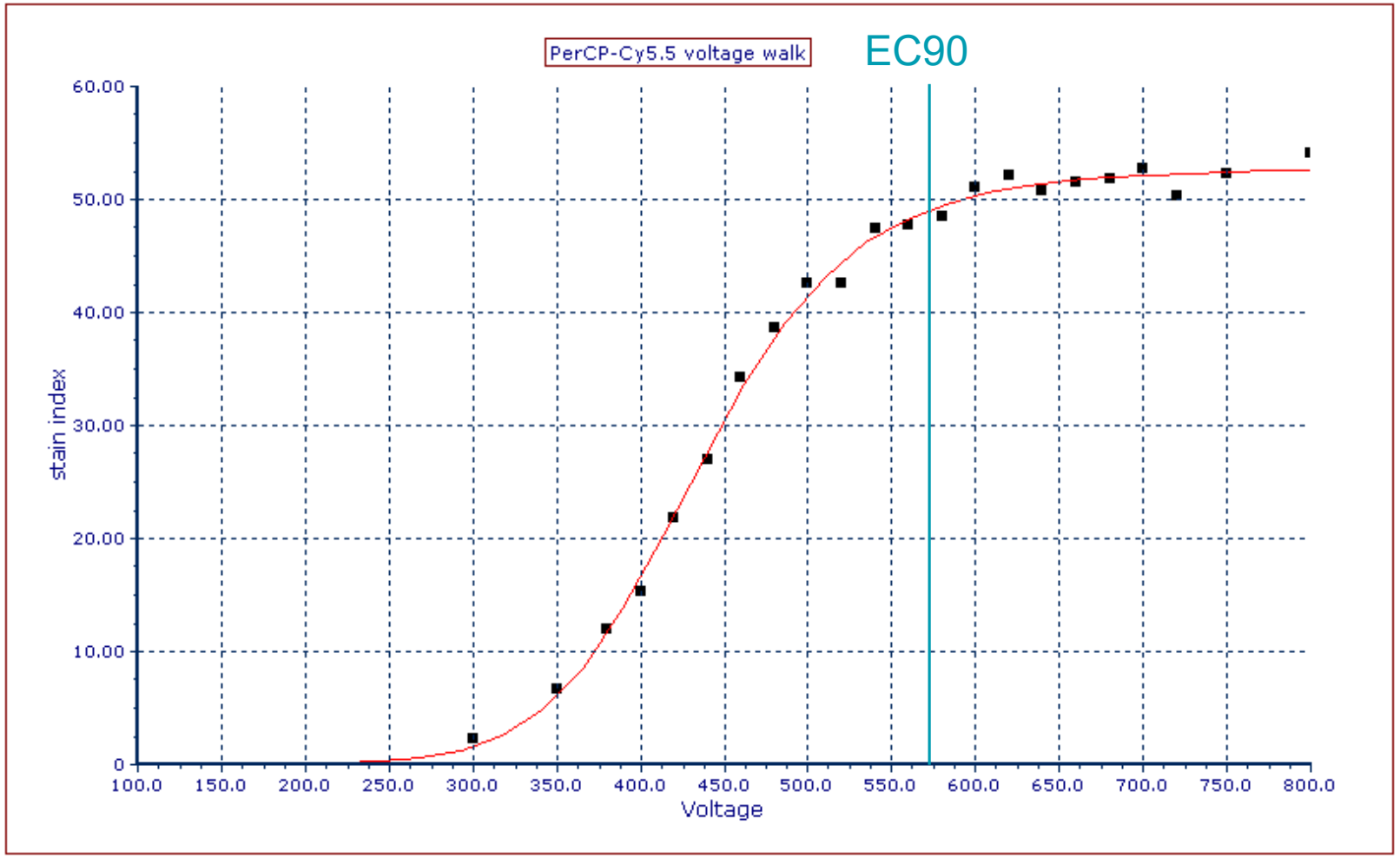
How standardized settings are determined

Calculate stain index and plot SI vs voltage. Determine EC90 voltage.



Regression Model Options

Regression Type

FourParmLogistic

Name	Fixed	Last Used
Imin	0	0
Imax		52.754
IC50		435.09
HillSlope		9.4433
r2		0.996476798829

Number of regression dots

30

Standardized settings don’t depend on the cell type or the actual fluorochrome used

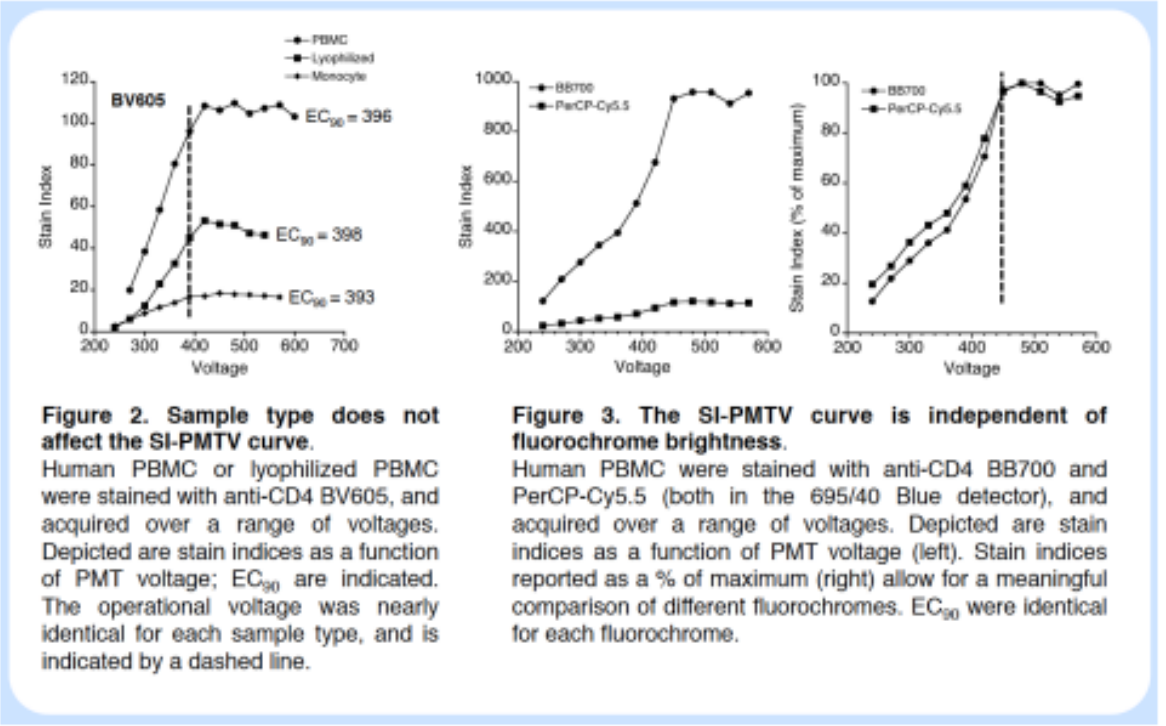


Figure 2. Sample type does not affect the SI-PMTV curve.
Human PBMC or lyophilized PBMC were stained with anti-CD4 BV605, and acquired over a range of voltages. Depicted are stain indices as a function of PMT voltage; EC_{90} are indicated. The operational voltage was nearly identical for each sample type, and is indicated by a dashed line.

Figure 3. The SI-PMTV curve is independent of fluorochrome brightness.
Human PBMC were stained with anti-CD4 BB700 and PerCP-Cy5.5 (both in the 695/40 Blue detector), and acquired over a range of voltages. Depicted are stain indices as a function of PMT voltage (left). Stain indices reported as a % of maximum (right) allow for a meaningful comparison of different fluorochromes. EC_{90} were identical for each fluorochrome.

Jones, DD, et. al., U Penn Flow Cytometry & Cell Sorting Resource Laboratory

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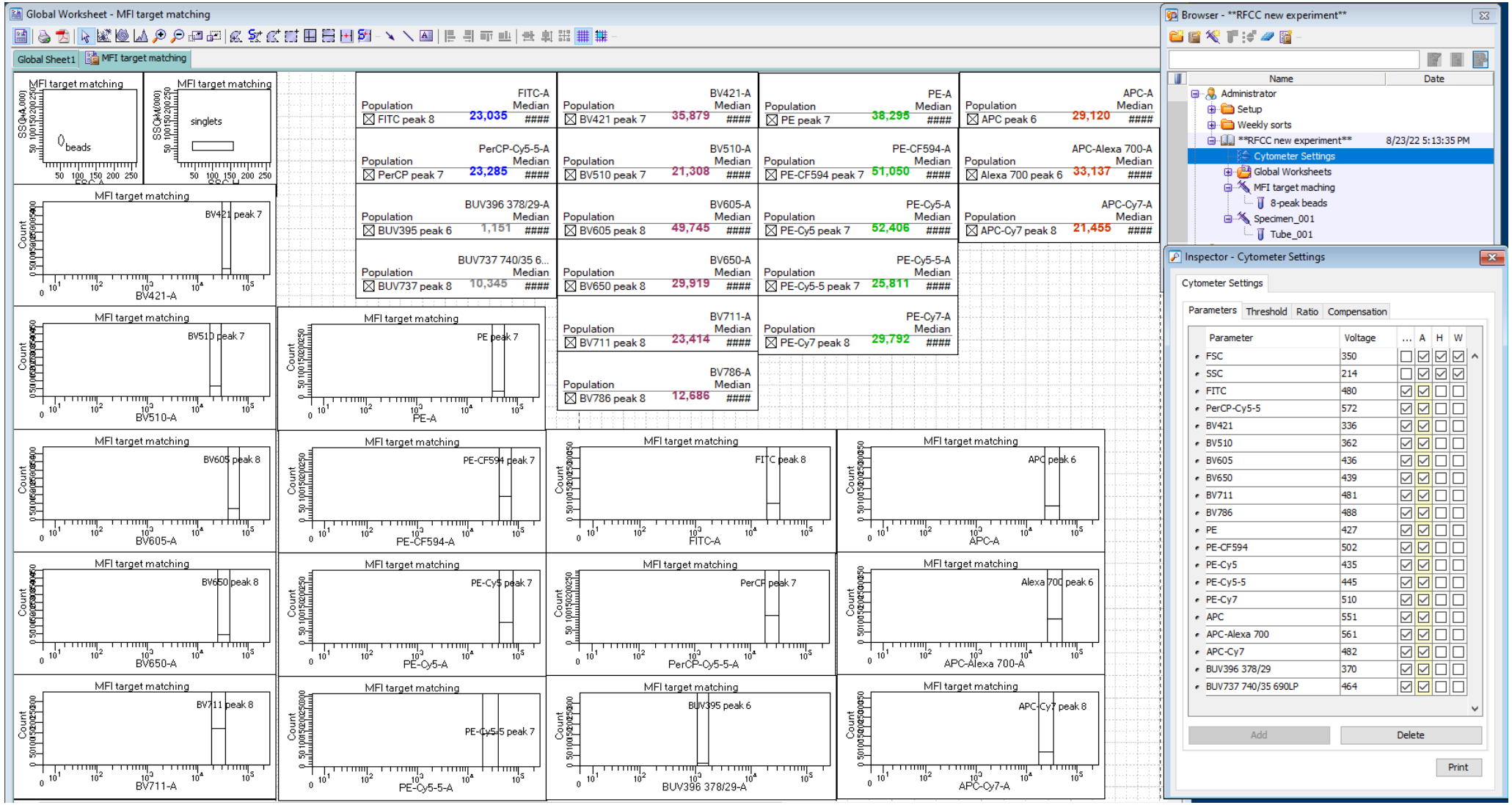
MFI values should stay consistent, not voltages

- Beads were recorded with the EC90 voltages to obtain “target” MFI values
- Before each experiment, the same beads will be run and voltage adjusted to obtain the same MFI value

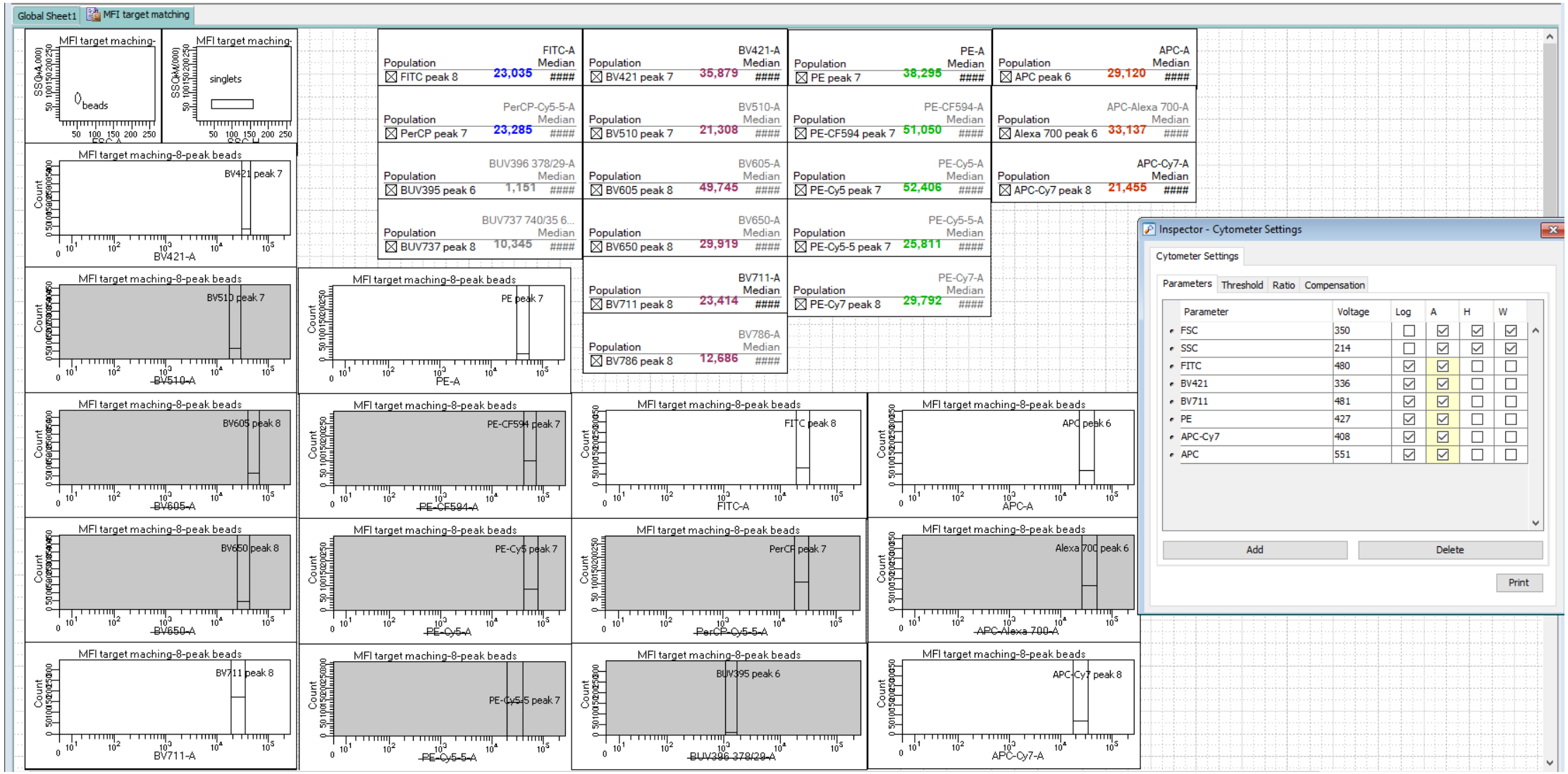
How to use standardized settings

1. Create new experiment using the “RFCC new experiment” template



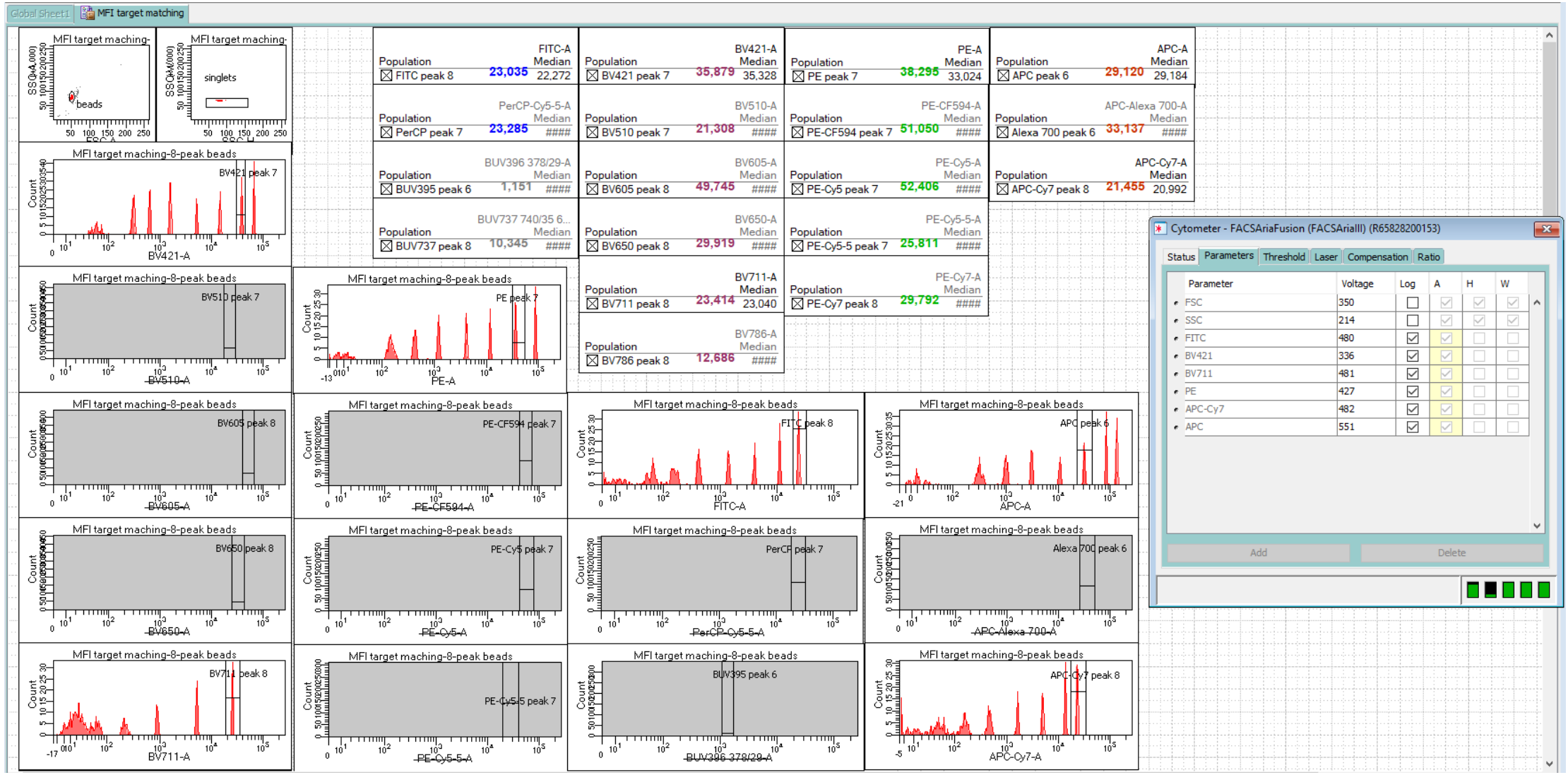
How to use standardized settings

2. Delete unwanted parameters



How to use standardized settings

3. Run beads and adjust voltage to match target MFI



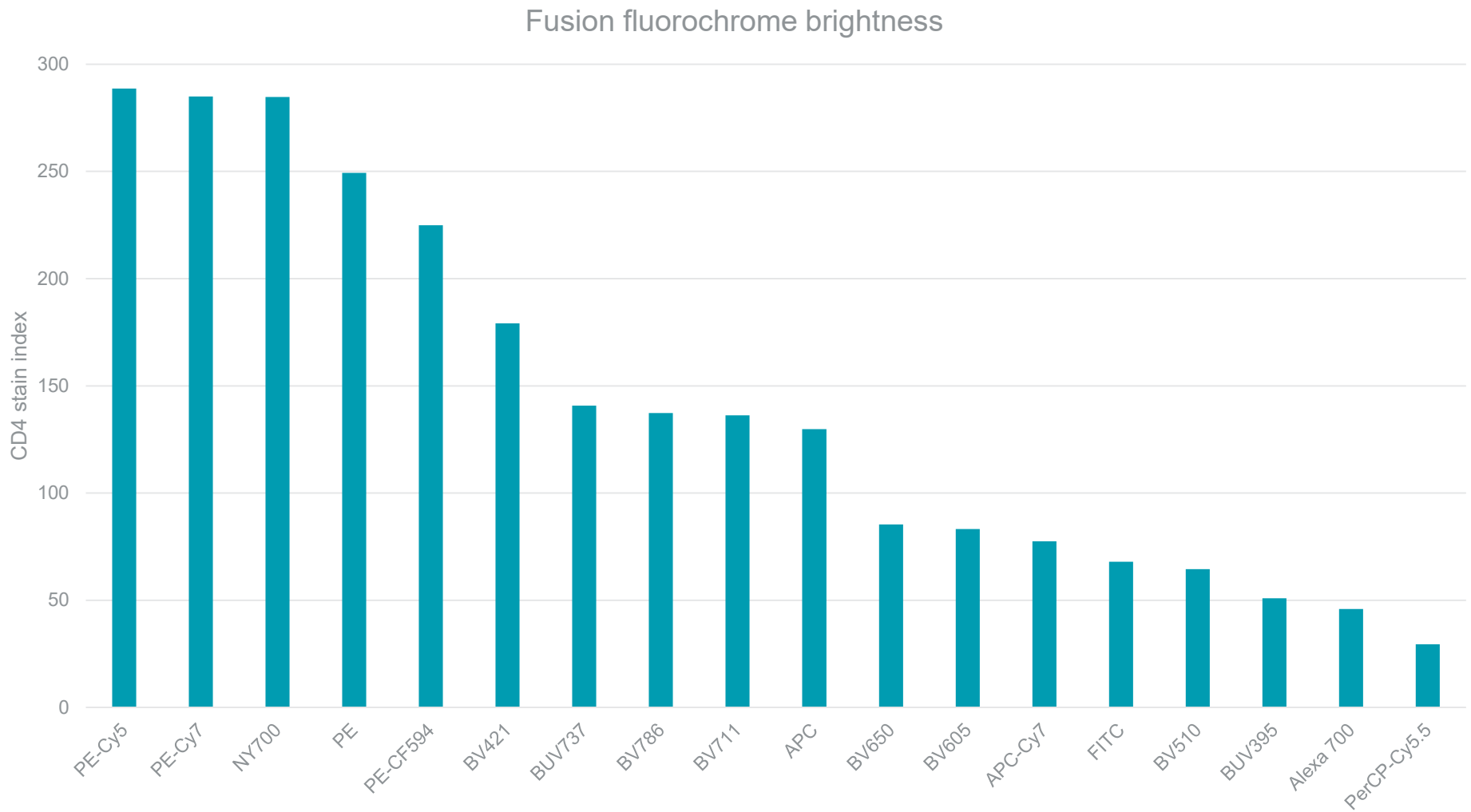
How to use standardized settings

4. Create compensation controls and look at full stain:
   - Adjust FSC and SSC voltage for your cells
   - Confirm all populations are on scale
   - ***Only*** adjust voltage to get positive events on scale!
5. Record controls and calculate compensation as usual
6. Load analysis template to apply plots and gates from previous experiment

Benefits to using standardized settings

- Best resolution
- Experimental rigor and reproducibility
  - Easier to compare data over time with fewer batch effects
  - Confidence that any change you see is biological
  - What if a reviewer asks how you set the voltages?
- Can quantify how Fortessa 2 is different from Fortessa 3
- Instrument specific information to aid in panel design
  - Fluorochrome brightness
  - Spread matrix

Instrument specific fluorochrome brightness



Use for critical tertiary antigens where resolution is most important

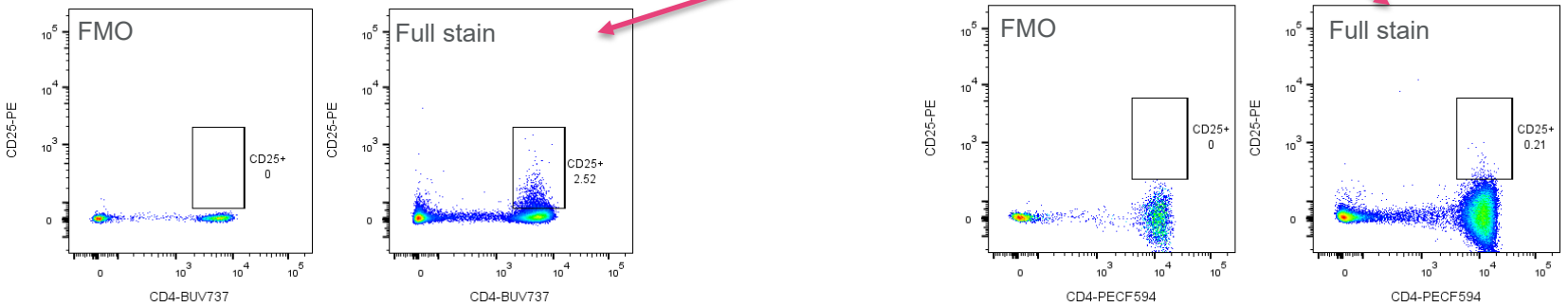
Use for primary antigens to decrease spread: CD45, CD4, CD8, etc

Instrument specific spread matrix

…will increase spread of this parameter

Staining with this dye…

	FITC 530/30	PerCP-Cy5-5 710/50	BV421 450/50	BV510 525/50	BV605 610/20	BV650 670/30	BV711 710/50	BV786 780/60	PE 586/15	PE-CF594 610/20	PE-Cy5 670/30	PE-Cy5-5 710/50	PE-Cy7 780/60	APC 670/30	APC-Alexa 700 710/50	APC-Cy7 780/60	BUV396 378/29	BUV737 740/35
FITC		34.8	16.3	17.4	14.7	14.1	16.8	17.9	11.9	11.9	26.6	25	13.6	18.5	35.9	13.6	21.2	11.4
PerCP-Cy5.5	14.1		16.8	16.3	14.7	27.7	45.6	41.3	11.4	11.9	47.8	48.9	31.5	53.8	113	25	21.7	23.4
BV421	15.2	29.9		20.7	16.8	14.7	16.8	17.9	11.9	13	27.2	25	14.1	19	36.9	14.1	22.3	11.4
BV510	15.8	34.2	17.9		46.2	39.7	35.3	32	20.1	22.8	27.2	25	13.6	28.2	58.2	14.1	21.2	17.4
BV605	14.7	72.3	18.5	17.4		77.2	71.2	56.5	66.3	76.7	88.1	52.2	31.5	52.7	106	14.7	21.2	28.2
BV650	15.2	68	19.6	17.4	48.4		94.7	70.7	22.3	29.9	84.8	50	28.8	108	180	26.6	22.3	33.7
BV711	15.2	144	21.7	16.3	15.7	33.7		137	11.9	11.9	45.1	61.4	49.4	62.5	365	64.7	22.3	63
BV786	14.7	35.9	25.5	17.4	15.2	16.8	30.4		11.9	11.9	27.7	25.5	33.1	22.8	62	38	21.7	28.2
PE	16.8	107	16.3	16.9	36.4	28.8	29.3	21.2		109	99.6	54.9	28.8	32	48.9	14.1	21.7	14.7
PE-CF594	14.7	201	16.8	16.3	45.6	39.7	45.1	27.2	79.4		177	99.6	59.8	47.8	69.6	15.2	21.7	18.5
PE-Cy5	15.2	694	20.1	16.9	23.4	76.7	131	51.1	33.1	26.6		237	137	267	321	52.7	22.8	42.4
NY700	15.2	286	16.8	16.9	15.7	33.1	60.3	23.9	50.5	37.5	212		89.2	212	802	86	21.7	22.8
PE-Cy7	15.2	58.7	17.9	15.8	14.1	14.7	20.1	82.7	28.8	20.6	33.7	35.8		20.6	44	38.6	21.7	22.8
APC	15.2	84.3	15.8	16.3	15.2	30.4	33.7	27.2	11.9	15.2	146	62	40.7		244	41.8	21.7	25
Alexa 700	15.2	42.9	16.8	16.3	13.6	13.6	27.2	28.2	11.4	11.9	32.6	41.3	33.7	31.5		41.8	21.2	22.3
APC-Cy7	14.7	35.9	16.8	16.3	13.6	14.7	18.5	45.6	11.4	11.9	42.9	29.3	90.3	53.2	77.2		21.2	18.5
BUV395	15.2	29.9	16.3	16.9	13.6	13	16.3	17.4	11.4	11.9	26.6	23.9	14.1	17.9	36.4	13.6		11.4
BUV737	15.2	166	17.9	16.9	14.1	22.8	49.4	60.9	11.9	11.9	31.5	52.2	50	33.1	238	68.5	22.8	



Future goals

- Measure CD4 single stains and generate target MFIs for the following RFCC instruments
  - Fusion (done) and AriaII
  - FACSymphony S6 (coming soon)
  - Bigfoot
  - MA900
  - All 3 Fortessas
- Publish instrument specific spread matrix and fluorochrome brightness ranking on RFCC website