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Flow cytometer instrument set up

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HIV VACCINE TRIALS NETWORK

Instrument Set-up and Standardization

- Step 1: Ensure instrument alignment
- Step 2: Set PMT voltages
- Step 3: Collect standardization particles (used for trend analysis over time)
- Step 4: Begin sample collection - include unstained cells and compensation controls

BD Cytometer Setup and Tracking (CS&T) Beads

- We use a hybrid standardization procedure that uses CS&T and rainbow beads
- CS&T beads perform a number of functions:
  - They establish target MFI settings in each detector and set PMT voltages to match these targets.
  - They assess alignment and report any issues
  - They set area scaling factors and laser delay settings
- CS&T beads are run each day, but we over-ride the PMT settings and use target settings based on rainbow beads

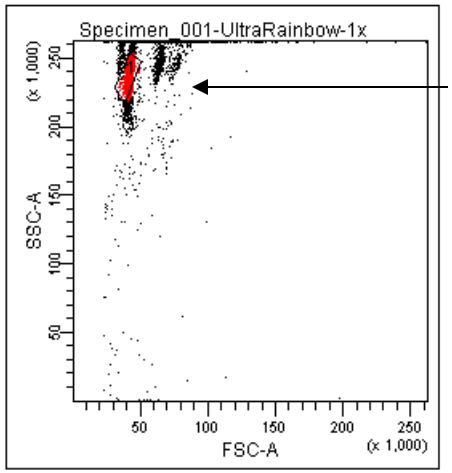
Instrument Alignment

- Even though the FACS facility checks this each day, you should check this yourself before every experiment
- It is a simple procedure to check alignment:
  - Use fluorescent particles (beads), e.g. “rainbow” beads. These fluoresce in most channels.
  - Run beads at low flow rate and determine CV for every channel of interest (after gating on “singlets”)
  - It is useful to have a collection template showing histograms for each channel along with median fluorescence and CV
  - Acceptable upper limit of CV differs for different channels

Alignment Template

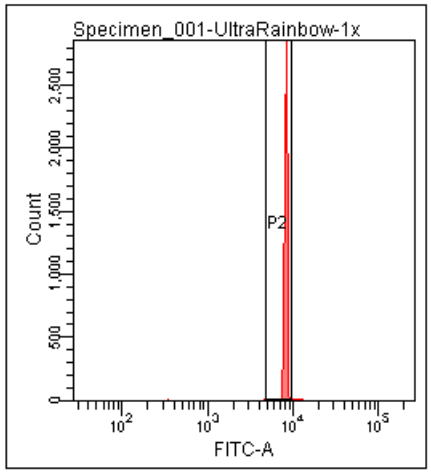
UltraRainbow-1x

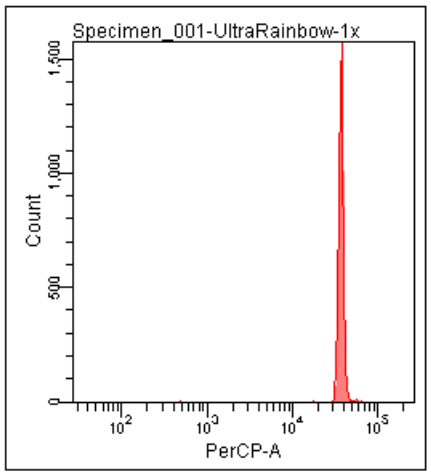
Tabs for each laser



Singlet gate on beads

Collection template created in DiVa software for BD LSRII





Tube Name: UltraRainbow-1x

Population	#Events	%Parent	FITC-A Median	FITC-A CV	PerCP-A Median	PerCP-A CV
P1	6,531	65.3	8,258	4.7	37,553	7.0
P2	6,485	99.3	8,257	2.9	37,533	5.9

CV for FITC
(P2 is secondary gate to remove doublets)

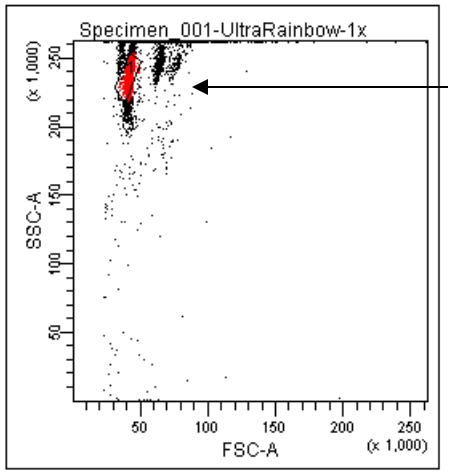
Instrument Standardization

- Ensure data collected on different days are comparable
- A method to set PMT voltages:
  - Use fluorescent particles (beads), e.g. “rainbow” beads. These fluoresce in most channels.
  - At the beginning of a study determine the optimal target values for median fluorescence intensity (MFI) for the beads in each channel
  - Each time the instrument is used for that study, set the PMT voltages so that the MFI matches the targets (+/-10%)
  - Note: using the same PMT voltages for all experiments is not appropriate standardization, although PMT voltages across experiments should be similar

Alignment Template

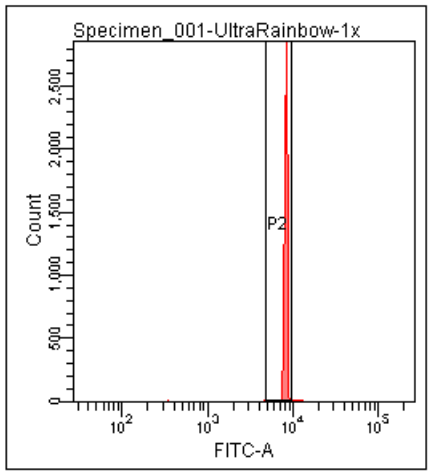
UltraRainbow-1x

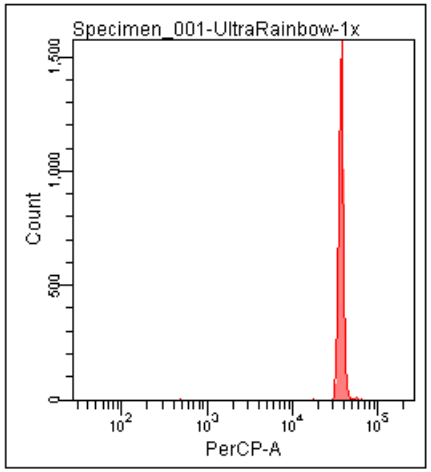
Tabs for each laser



Singlet gate on beads

Same template used to set PMT voltages





Tube Name: UltraRainbow-1x

Population	#Events	%Parent	FITC-A Median	FITC-A CV	PerCP-A Median	PerCP-A CV
P1	6,531	65.3	8,258	4.7	37,553	7.0
P2	6,485	99.3	8,257	2.9	37,533	5.9

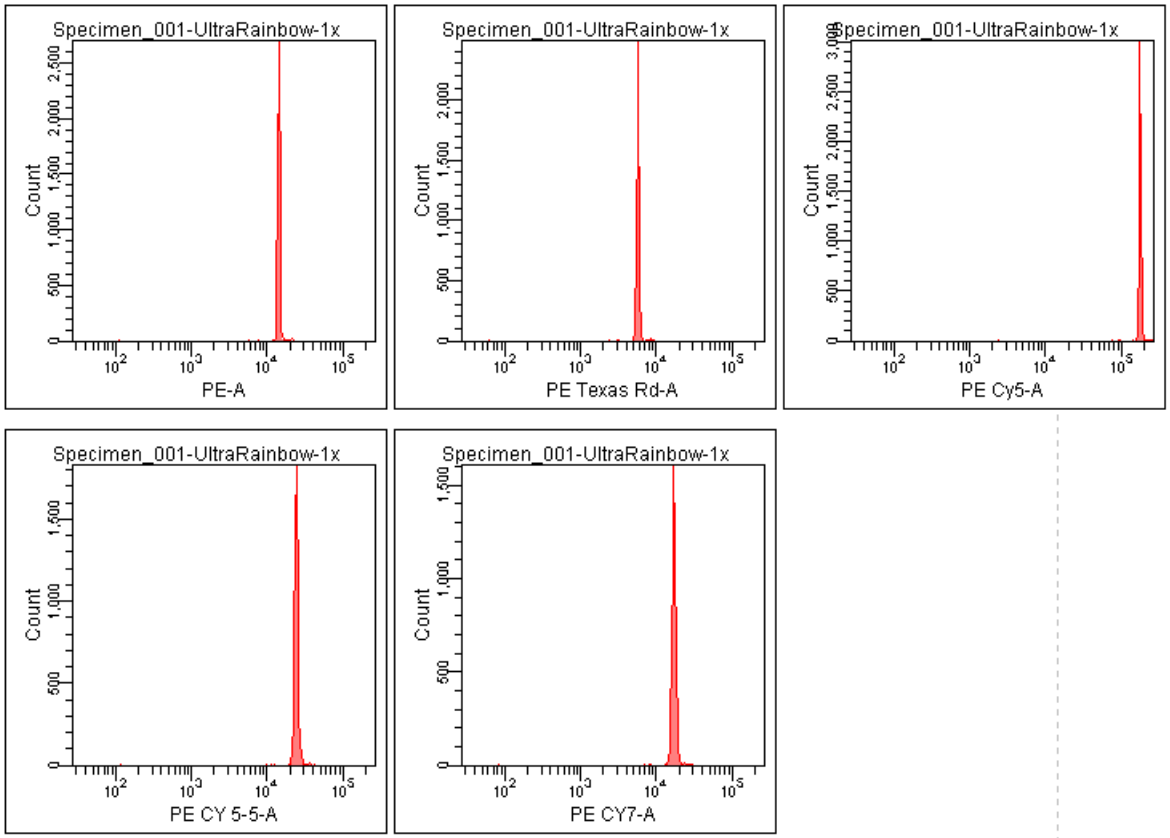
PMT for FITC is adjusted so that MFI matches target

Green Laser Alignment Template

Worksheet

Blue Laser | Green Laser | Red Laser | Violet Laser

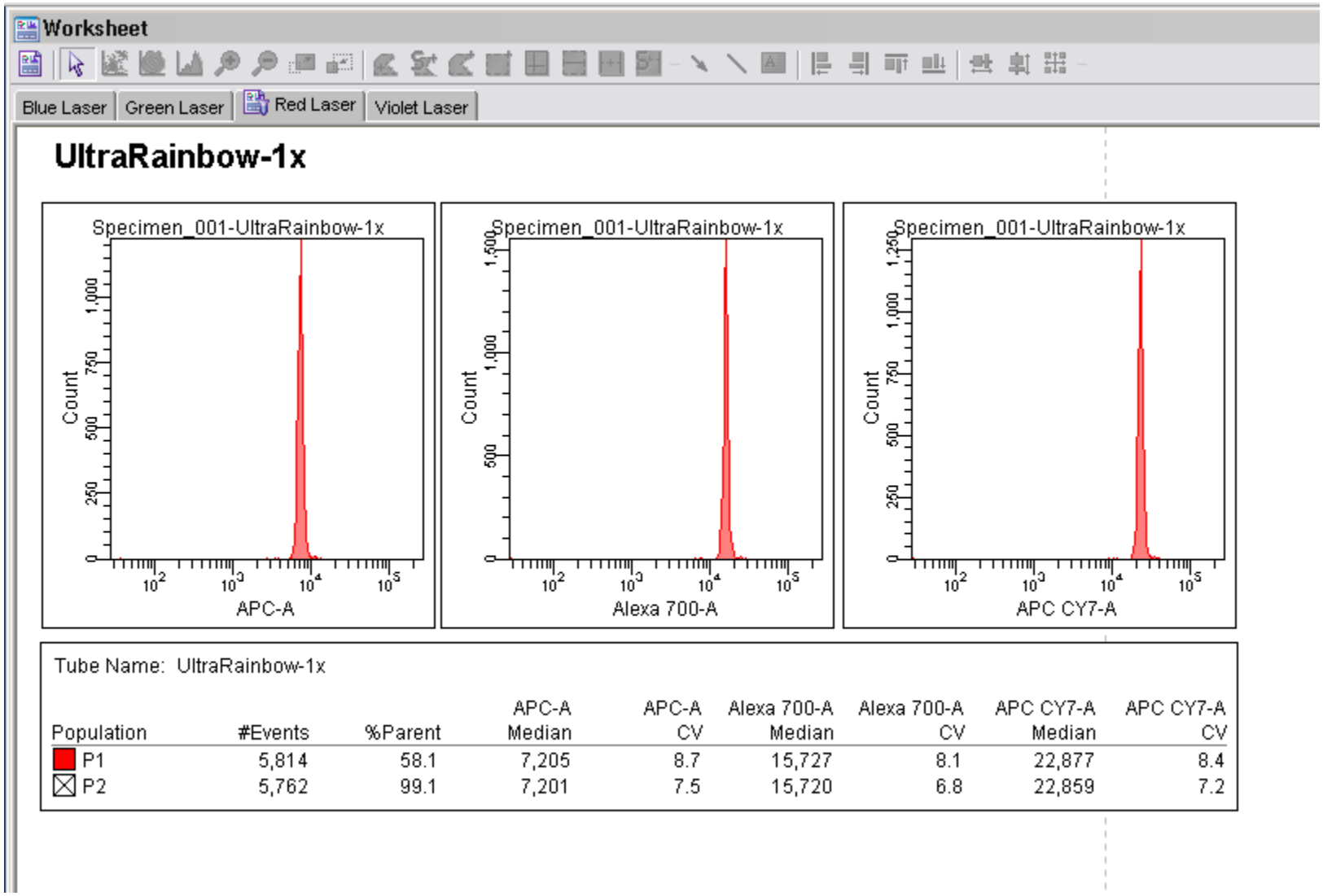
UltraRainbow-1x



Tube Name: UltraRainbow-1x

Population	#Events	%Parent	PE-A Median	PE-A CV	PE Texas R... Median	PE Texas R... CV	PE Cy5-A Median	PE Cy5-A CV	PE CY 5-5-A Median	PE CY 5-5-A CV	PE CY7-A Median	PE CY7-A CV
P1	5,814	58.1	14,279	5.3	5,706	5.2	180,430	4.7	24,351	6.7	17,155	7.4
P2	5,762	99.1	14,275	3.2	5,704	3.2	180,395	2.6	24,345	5.1	17,147	5.9

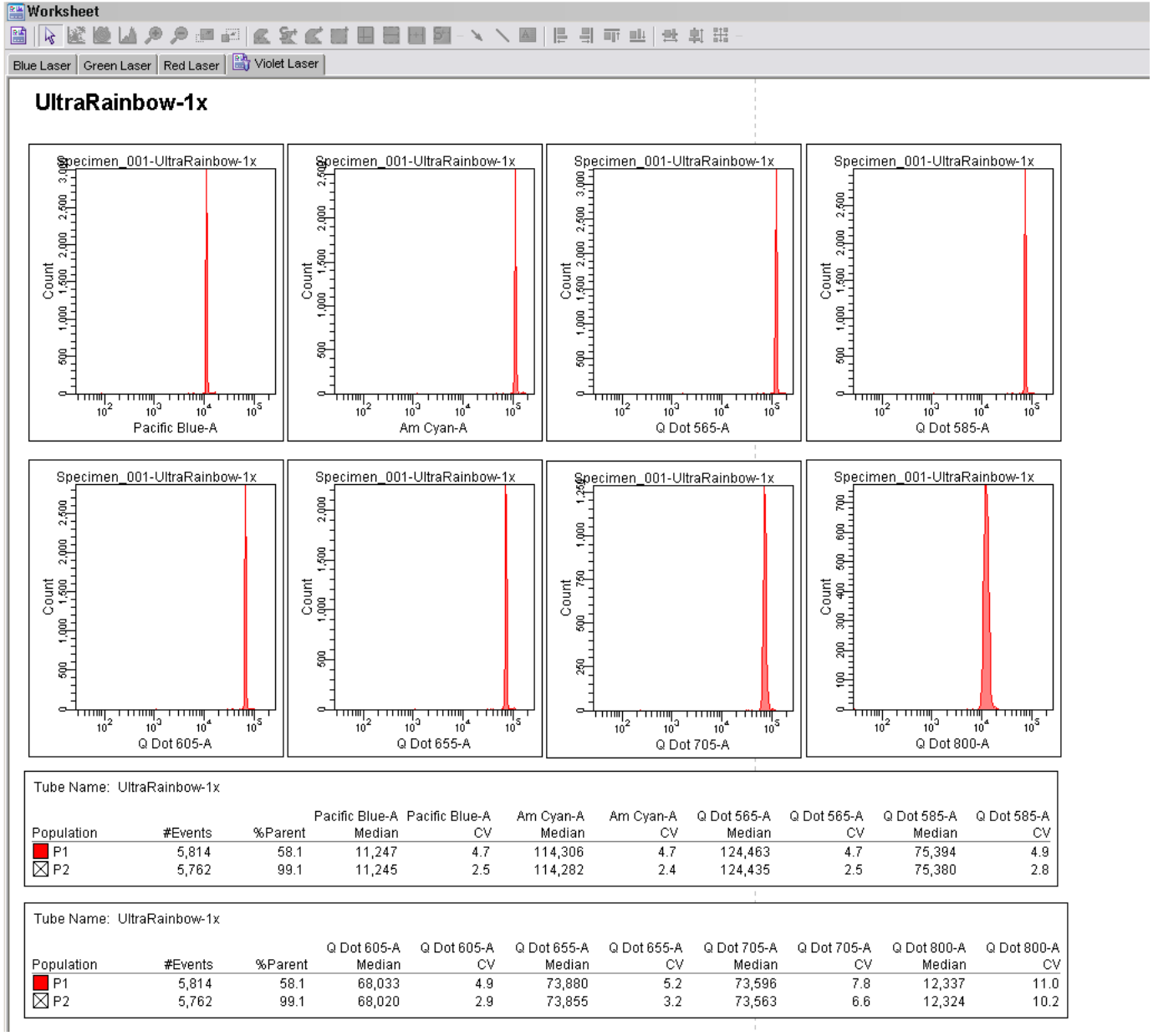
Red Laser Alignment Template



Tube Name: UltraRainbow-1x

Population	#Events	%Parent	APC-A Median	APC-A CV	Alexa 700-A Median	Alexa 700-A CV	APC CY7-A Median	APC CY7-A CV
P1	5,814	58.1	7,205	8.7	15,727	8.1	22,877	8.4
P2	5,762	99.1	7,201	7.5	15,720	6.8	22,859	7.2

Violet Laser Alignment Template



Tube Name: UltraRainbow-1x

Population	#Events	%Parent	Pacific Blue-A Median	Pacific Blue-A CV	Am Cyan-A Median	Am Cyan-A CV	Q Dot 565-A Median	Q Dot 565-A CV	Q Dot 585-A Median	Q Dot 585-A CV
P1	5,814	58.1	11,247	4.7	114,306	4.7	124,463	4.7	75,394	4.9
P2	5,762	99.1	11,245	2.5	114,282	2.4	124,435	2.5	75,380	2.8

Tube Name: UltraRainbow-1x

Population	#Events	%Parent	Q Dot 605-A Median	Q Dot 605-A CV	Q Dot 655-A Median	Q Dot 655-A CV	Q Dot 705-A Median	Q Dot 705-A CV	Q Dot 800-A Median	Q Dot 800-A CV
P1	5,814	58.1	68,033	4.9	73,880	5.2	73,596	7.8	12,337	11.0
P2	5,762	99.1	68,020	2.9	73,855	3.2	73,563	6.6	12,324	10.2

How to determine target MFI's

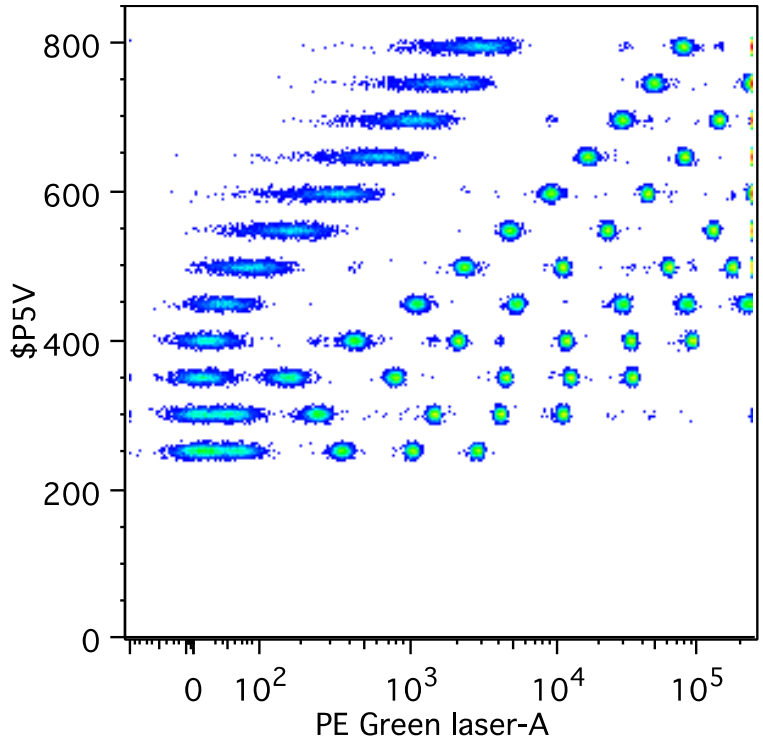
- Refer to Steve Perfetto's publication
  - Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay PK, Roederer M. Quality assurance for polychromatic flow cytometry using a suite of calibration beads. *Nature protocols*. Dec 2012;7(12):2067-2079
- As an alternate simplified procedure (not optimal) is to ensure that all positive cells are on-scale and all negative cells are well above the lower scale
- Typically, we prefer to have the upper edge of the negative cells at about 100

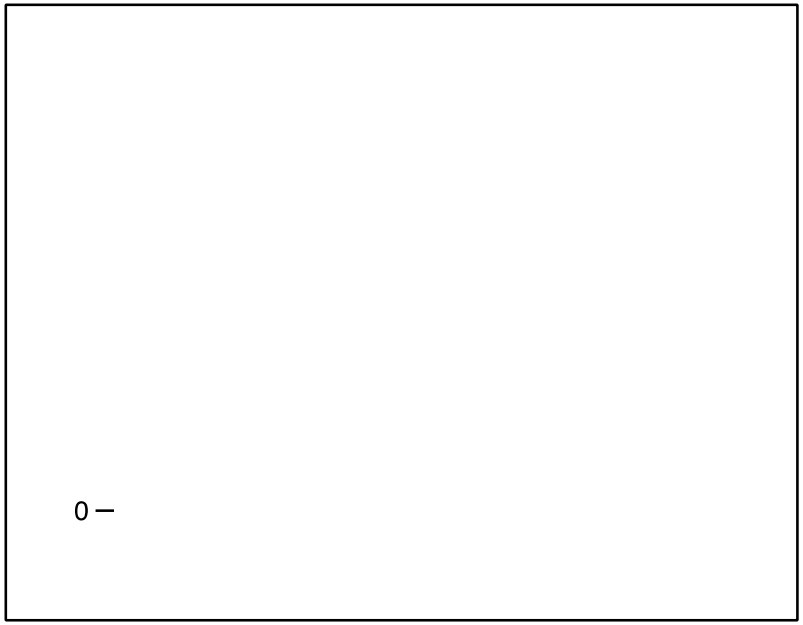
Calibrating and standardizing a flow cytometer

- First ensure instrument is optimized (alignment, laser delay, PMT efficiency, filters)
- Then, perform testing to identify range of PMT voltages to assure best sensitivity
- Choose a target PMT setting within this range to keeps cells on-scale and maintain compensation percentages <100%
- Beads to use for testing:
  - Cyto-Cal are hard-dyed and have signal in each detector
  - Quantum Simply Cellular Beads (QSCB) are antibody capture beads of 4 levels of intensity
  - Can also use single-stained cells

Cyto-Cal beads for calibration

- Adjust voltage in 50V increments
- Calculate stain index for separation of negative bead from first bead
- Identifies minimum voltage to achieve good sensitivity





Setting MFI target setting

- PMT voltage range determined using Cyto-Cal beads can be confirmed using single-stained cells
- Choose a voltage that is within the sensitive range and ensure positive cells are on-scale
- After PMT voltages are chosen for each detector, check if any compensation percentages are >100%. If so, increase PMT voltage for primary detector and decrease for secondary detector
- Once completed, collect single peak rainbow beads to determine the target MFI for these beads for each detector