

High Parameter Meeting

February 15, 2023

Insight into the Standardization of Instrument Setup Across Cytek[®] Systems; presented by Cytek

- Maria Jaimes, PhD
- www.cytekbio.com
Resources>Videos>Webinars

Goal of Instrument Standardization

Same results regardless of instrument used (same results on Aurora and Maleficent, etc.)

1. Populations fall within same gates (can use same template across instruments).
2. Population percentages are the same.
3. Positive population MFIs are the same.
4. Resolution of dim markers is the same.



How can we achieve instrument standardization?

Three levels of instrument standardization, achieving different objectives

Standardization of
instrument setup
with hard-dye
beads

Standardization of
instrument setup
with fluorescent
labeled particles

Standardization of
instrument
performance





How does Cytek standardize instrument setup using hard-dye beads?

The Cytek Northern Lights™, Aurora, and Aurora CS come with setup standardization using SpectroFlo® QC beads and Daily QC

What are hard-dye beads?

- Beads that fluoresce across all detectors/channels
- Uses surrogate dyes, not using traditional fluorochromes
- SpectroFlo QC beads

Why use hard-dye beads?

- Highly stable over time and across temperatures
- Reproducible across instruments, across sites
- Widely available
- Small CVs, which is important for accuracy

What does Daily QC do?

- For each detector, set gain so that bead's positive population is at target MFI
- CytekAssaySettings (CAS) are adjusted based on Daily QC so that MFIs remain constant when the same QC particles are run



Why use CytekAssaySettings (CAS)?

❖ *Overall goal*

- To provide users with a starting point to run their assays
- CAS are NOT meant to be universal / optimal for all applications

❖ *Criteria used to establish settings*

- Based on biological samples performance
- Preserve spectral characteristics of each dye
- Optimize resolution of cells stained with anti-human CD4
- Provide enough range to accommodate bright signals (anti-human CD8)
- Minimize spread (critical for multicolor applications)

❖ *Software implementation around CAS*

- CAS are saved as MFI target for every channel using hard dyed beads
- Gains for CAS are automatically updated upon running QC
- **Same functionality is applied to any user defined instrument settings**



QC beads: a practical tool for setup standardization

Pros

- Hard-dye beads are highly stable, robust, and low cost
- Hard-dye beads emit in every detector for every laser onboard the Aurora/NL
- Automated way to achieve setup standardization to a good level of precision for immunophenotyping assays

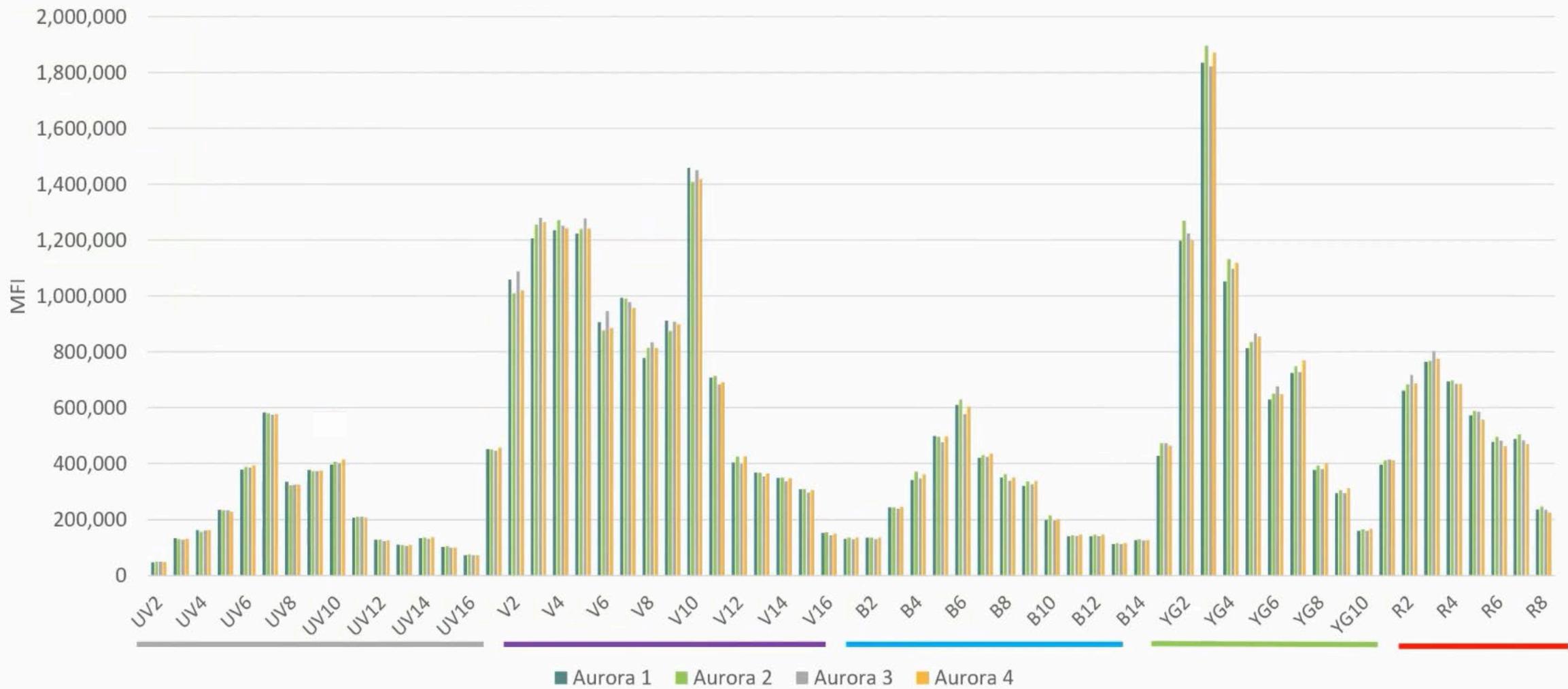
Cons

- Hard-dye beads are not spectrally matched with fluorochrome-labeled particles
- Can expect average of 10-15% variation when using hard-dye beads to set MFI, then moving to actual fluorochromes



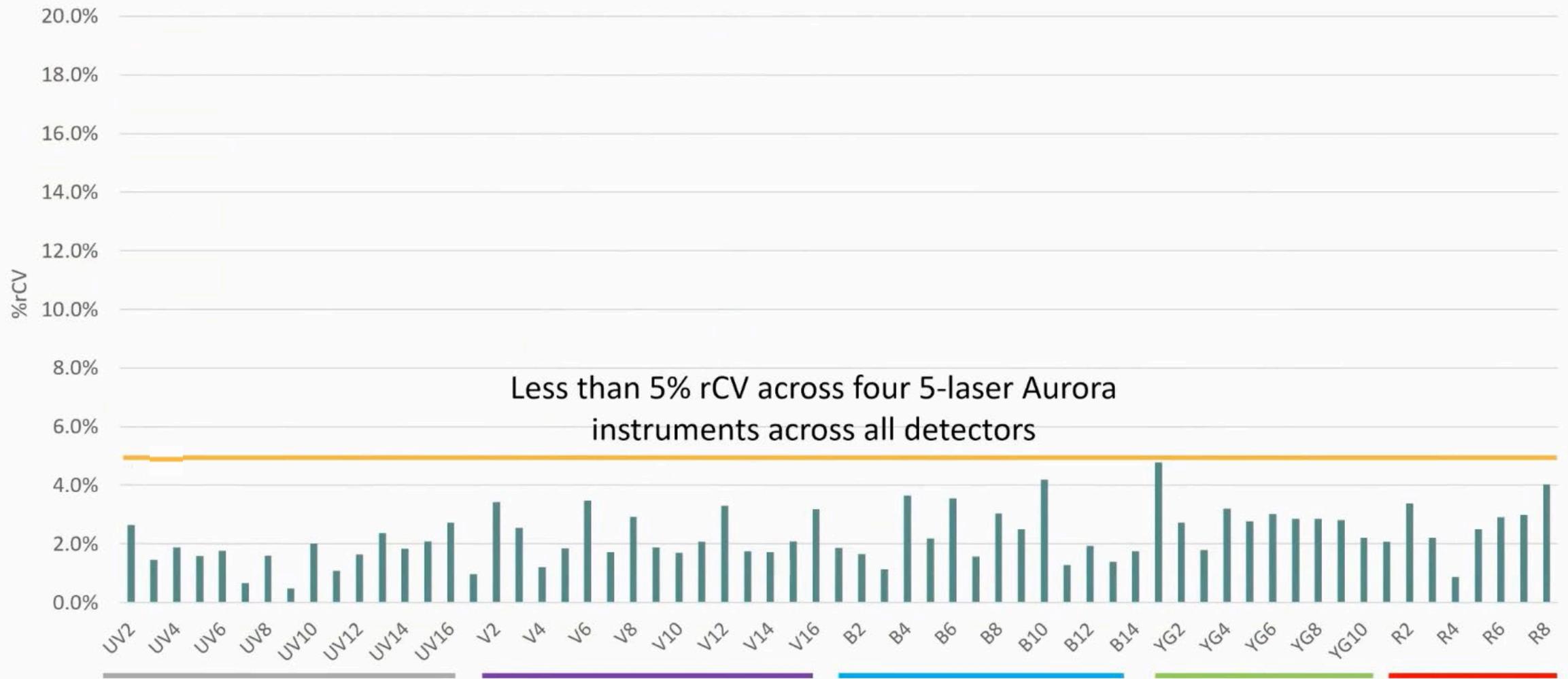
High consistency of hard-dye bead MFI (1)

MFI of QC beads are highly consistent in all detectors across four 5-laser Cytex Aurora systems, standardized using hard-dye beads



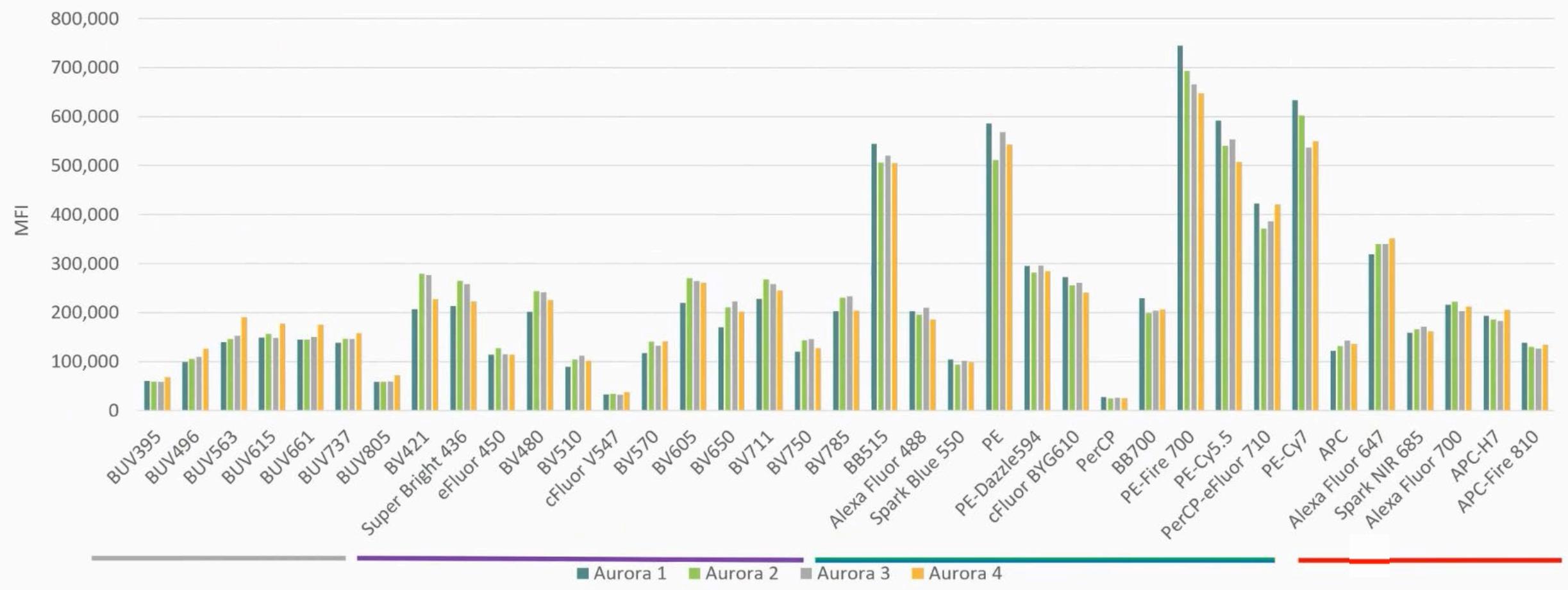


High consistency of hard-dye bead MFI (2): CVs



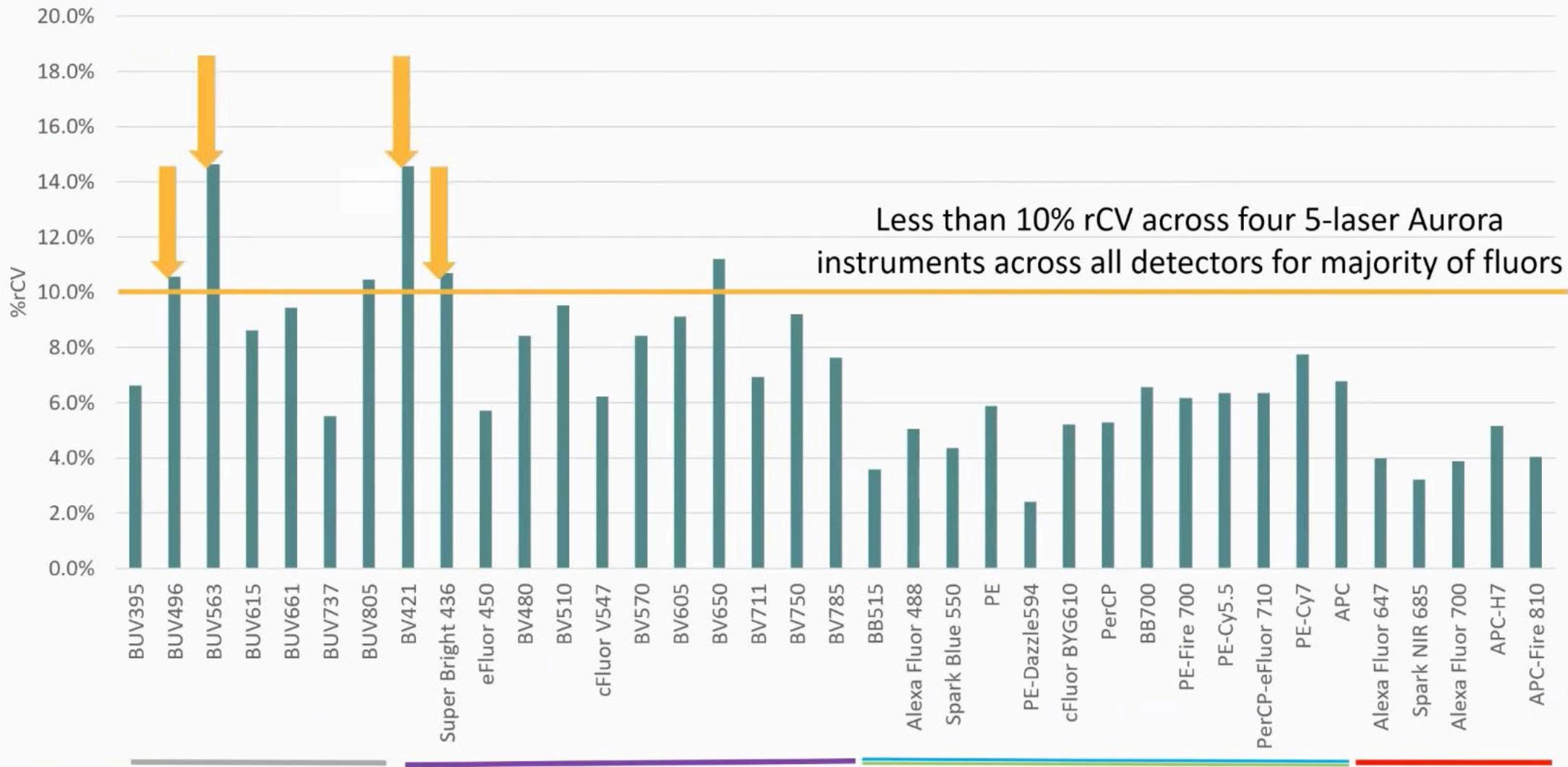


Higher variation of fluorochrome-stained bead MFI (1)





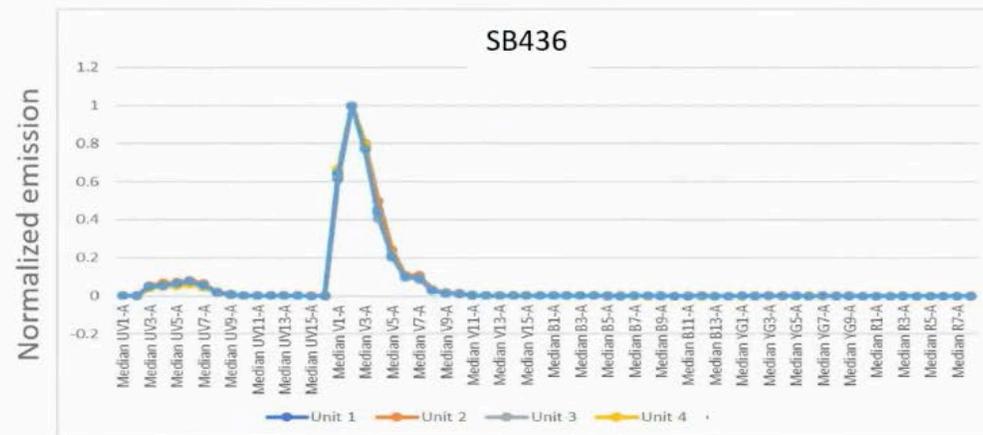
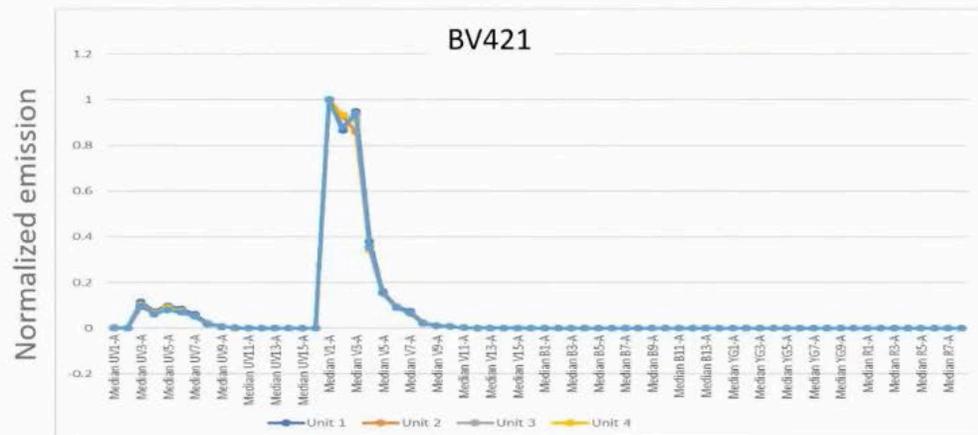
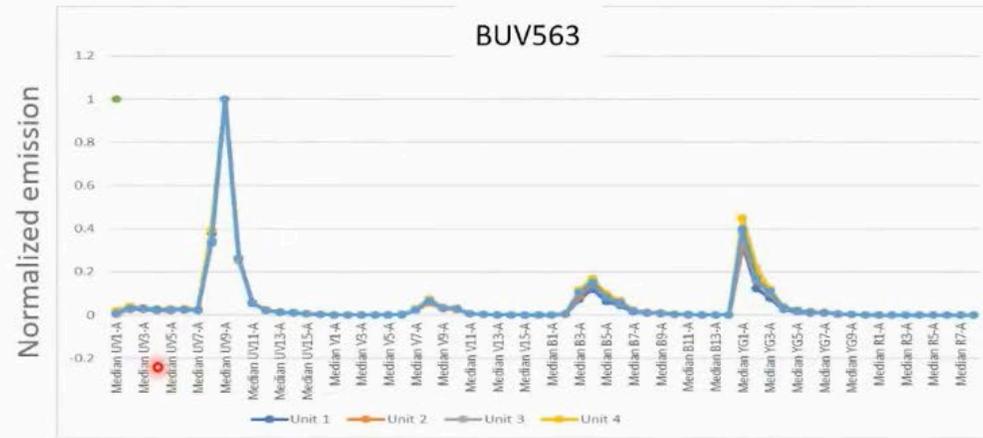
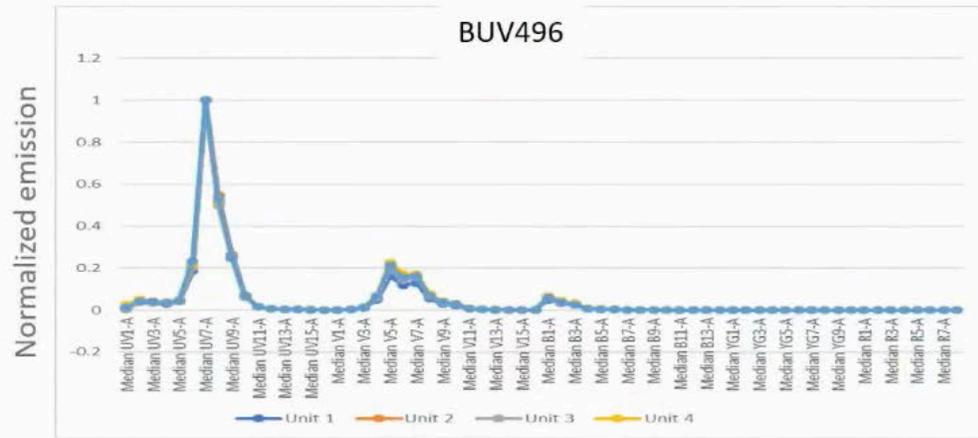
Higher variation of fluorochrome-stained bead MFI (2)





Consistency of spectral signatures at Cytek Assay Settings

High consistency in spectral signatures across all five 5-laser Cytek Aurora systems and across fluorochromes



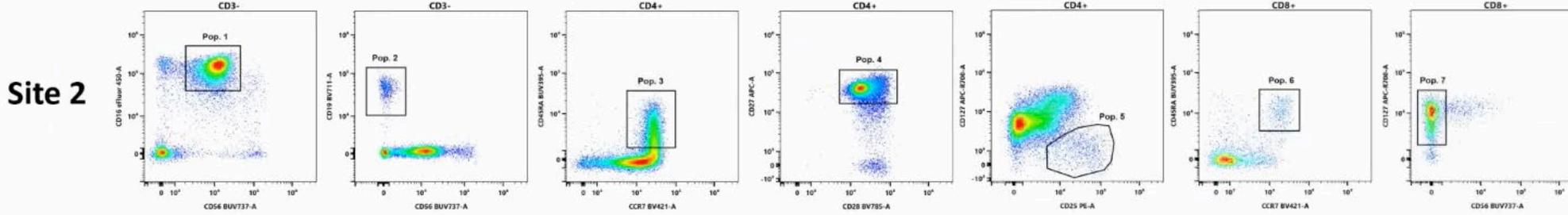
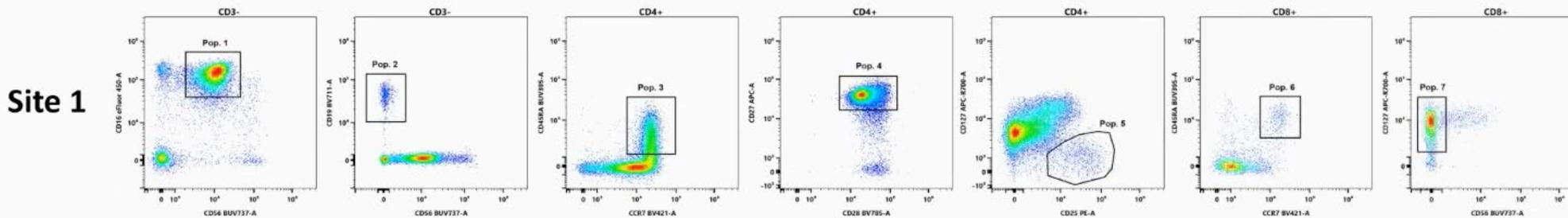
Cross-Site Study Using Hard-Dye Beads Standardization

Three data sets using pre-stained lyophilized 18-color immunophenotyping kit:

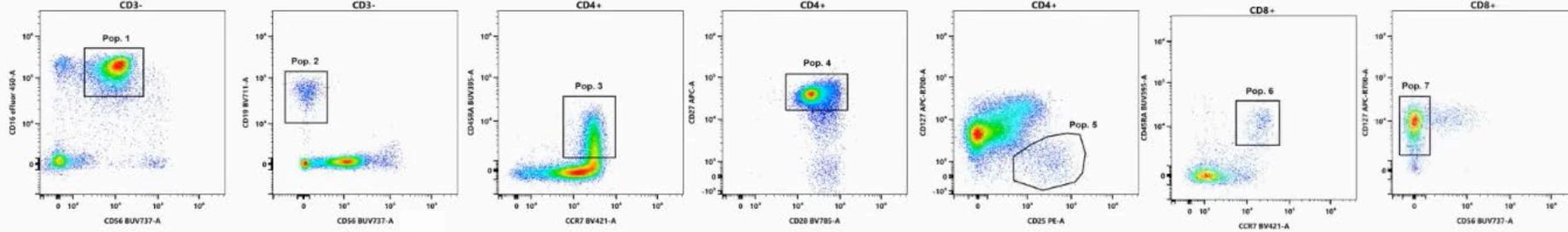
1. Gold Standard: independent runs from a single kit lot, on a single instrument, by a single operator
2. Cross-Site Standardized Sample Resuspension: runs from 9 different sites, from a single kit lot, following a standardized sample reconstitution and acquisition protocol
3. Cross-Site Non-Standardized Sample Resuspension: runs from 15 different sites, from a single kit lot, following non-standardized sample reconstitution and acquisition protocol



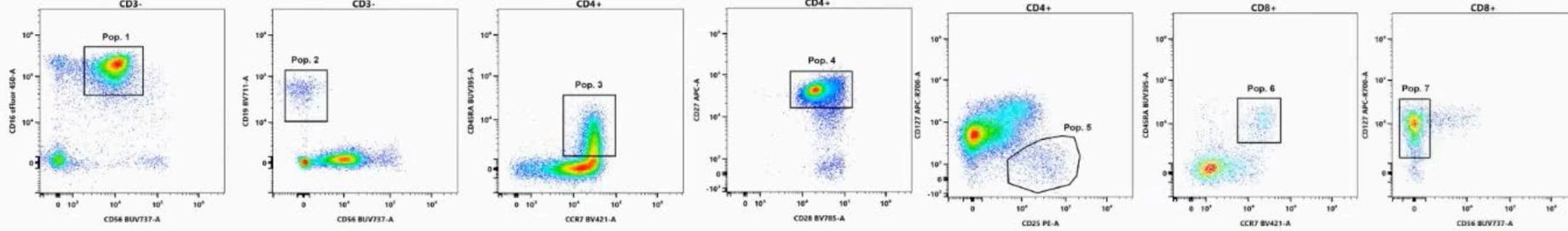
High consistency in visual data and manual gating



Reference Site Day 1



Reference Site Day 10



Populations

1

2

3

4

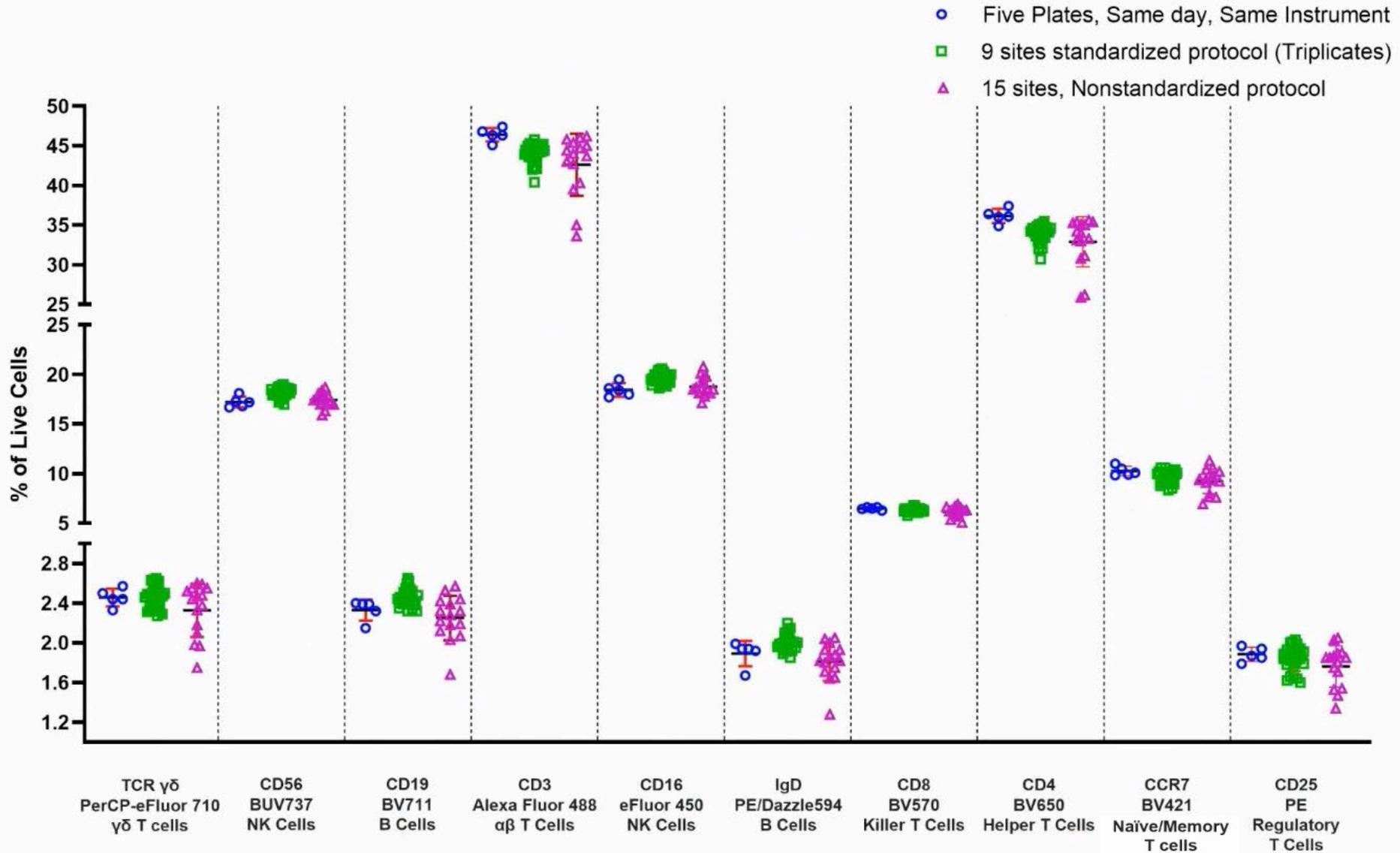
5

6

7



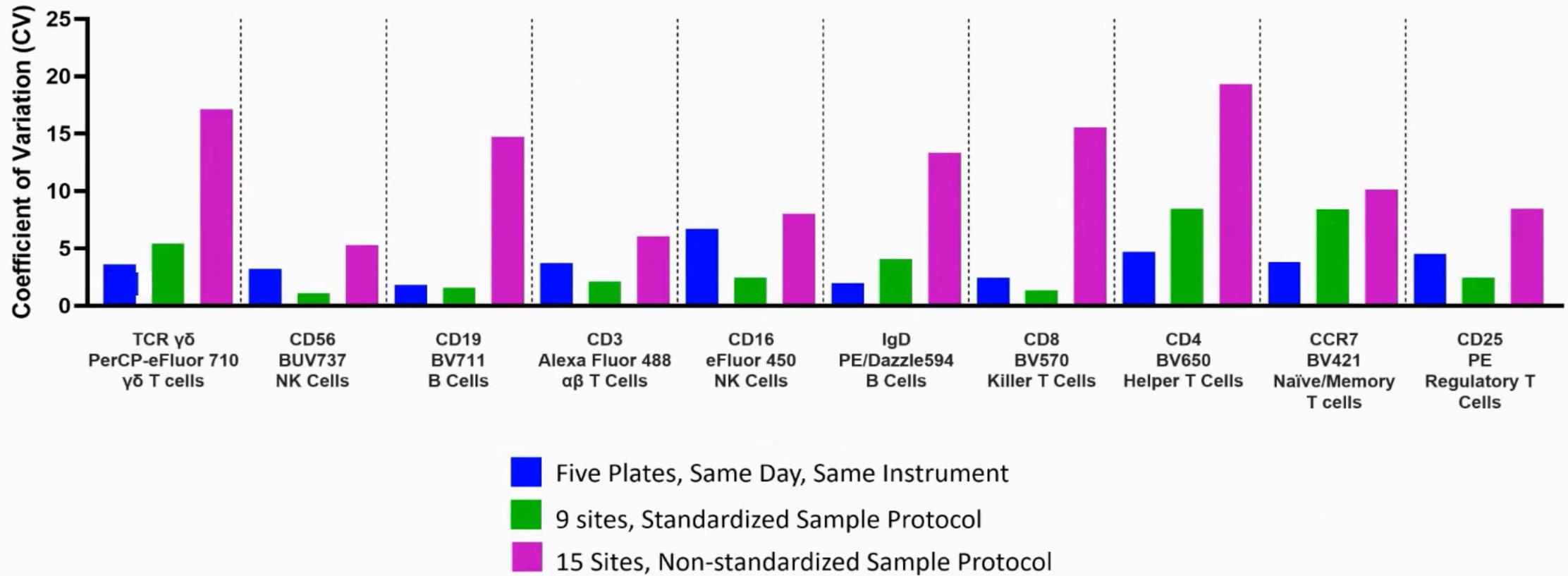
High consistency in population frequency across sites (1)





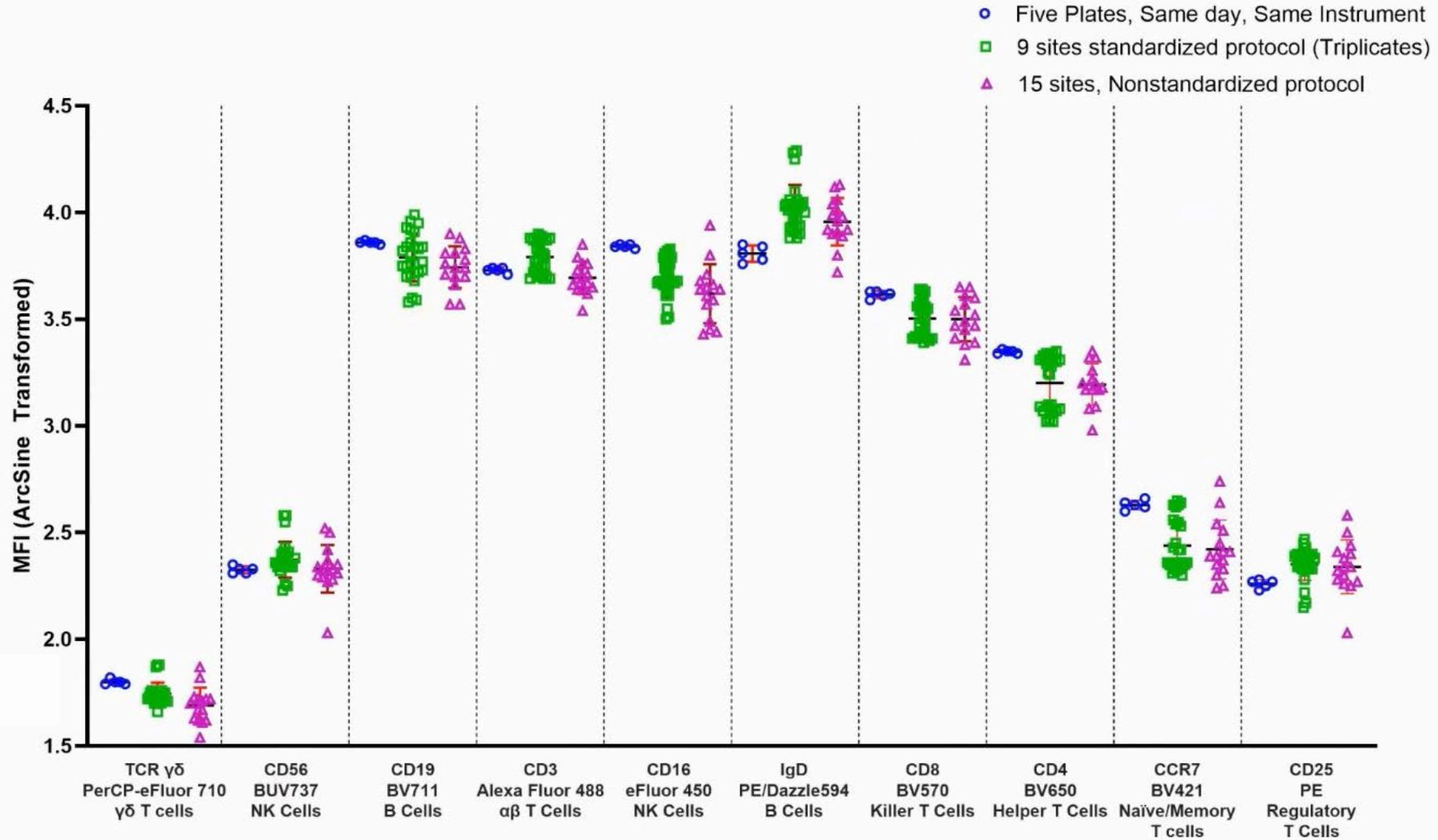
High consistency in population frequency across sites (2)

% Coefficient of Variation of Abundance of Populations of Interest Under Different Conditions





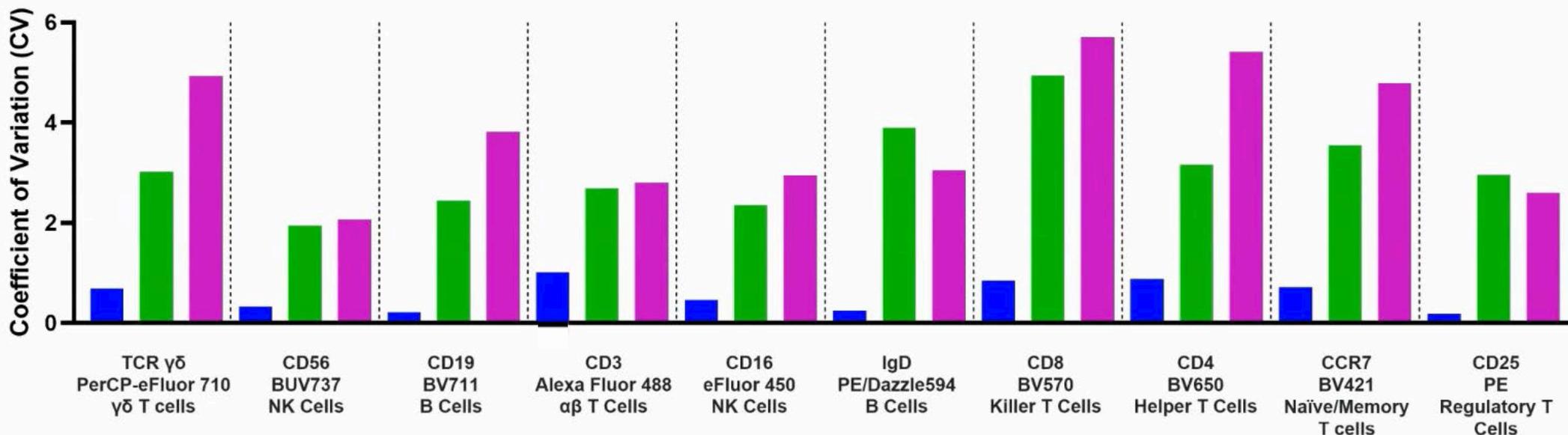
High consistency in fluorescence intensity (1)





High consistency in fluorescence intensity (2)

% Coefficient of Variation of MFI of Populations of Interest Under Different Conditions

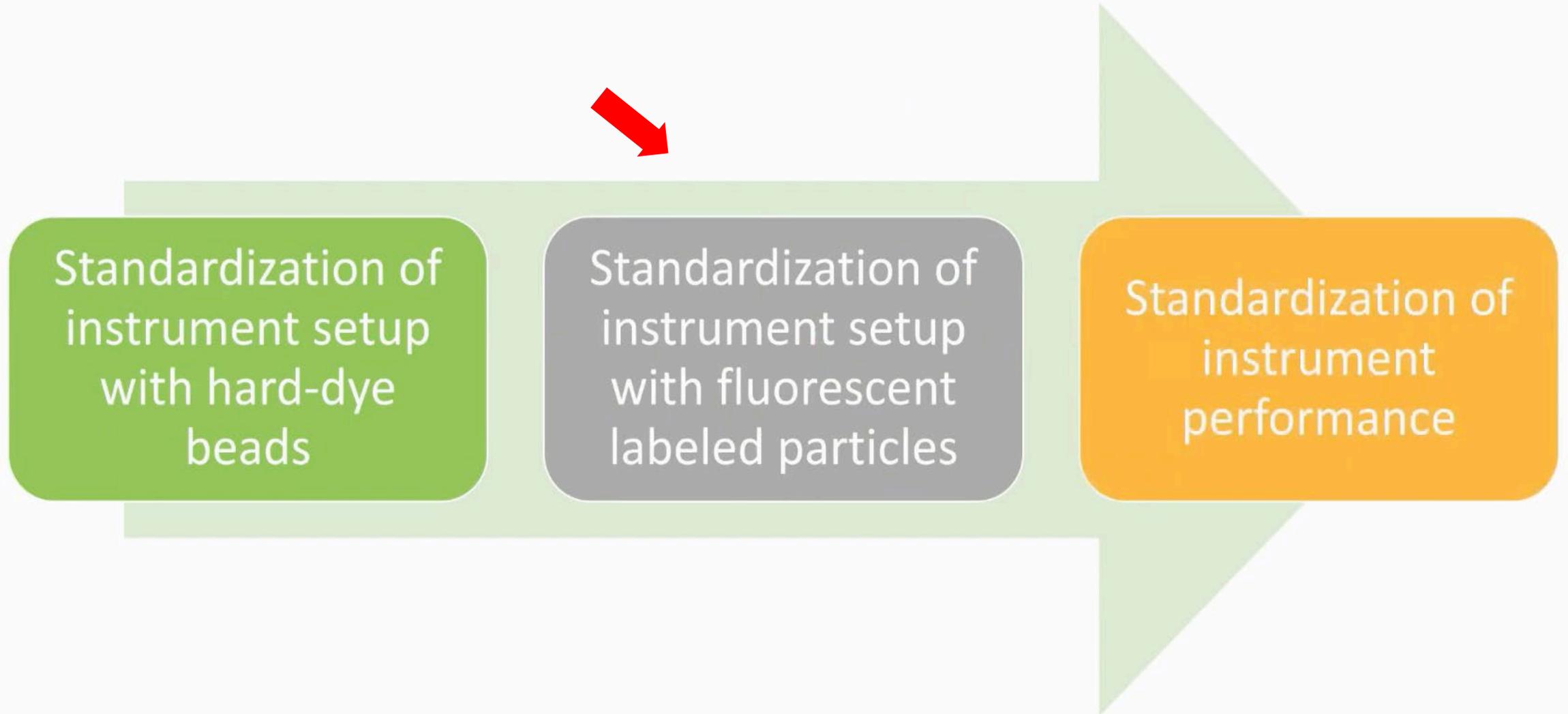


- Five Plates, Same Day, Same Instrument**
- 9 sites, Standardized Sample Protocol**
- 15 Sites, Non-standardized Sample Protocol**



How can we achieve instrument standardization?

Three levels of instrument standardization, achieving different objectives





Why use fluorochrome labeled beads to standardize instrument setup?

Pros

- To increase standardization accuracy by having exact match to fluors used in specific assay
- Allow to compared changes in MFI for a given marker across sites

Cons

- Difficult to find stable fluorochrome labeled particles
- Difficult to find stable particles for ALL needed fluorochromes
- Expensive
- Difficult to reproduce over time (lot to lot variation on calibrator)
- More labor intensive



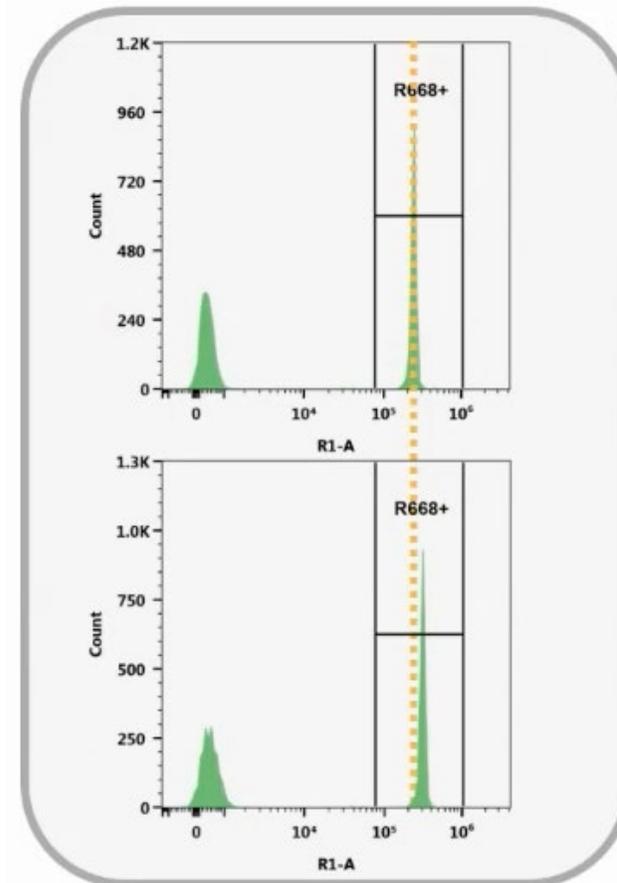
Standardization with Fluorochrome Labeled Particles

1. Create single-color reference controls using capture beads stained with antibodies labeled with experiment specific fluorochromes.



Standardization with Fluorochrome Labeled Particles

2. Run controls on Instrument A, set target MFIs, and note the MFI values.
3. Run Controls on Instrument B with Cytek Assay Settings and note MFI values and gain settings.



Standardization with Fluorochrome Labeled Particles

4. Calculate gain change. Will need to do for all fluorochromes in experiment.
5. Save as standardization settings.

$$233,680/299,906 \times 220 = 171$$

Instrument A:

cFluorR668+ Target MFI =
233,680

Instrument B:

cFluorR668+ MFI = 299,906
R1 Gain = 220

**Standardization settings R1
gain Instrument B**

$$\frac{\text{Target MFI A}}{\text{Observed MFI B}} \times \text{R1 B gain}$$

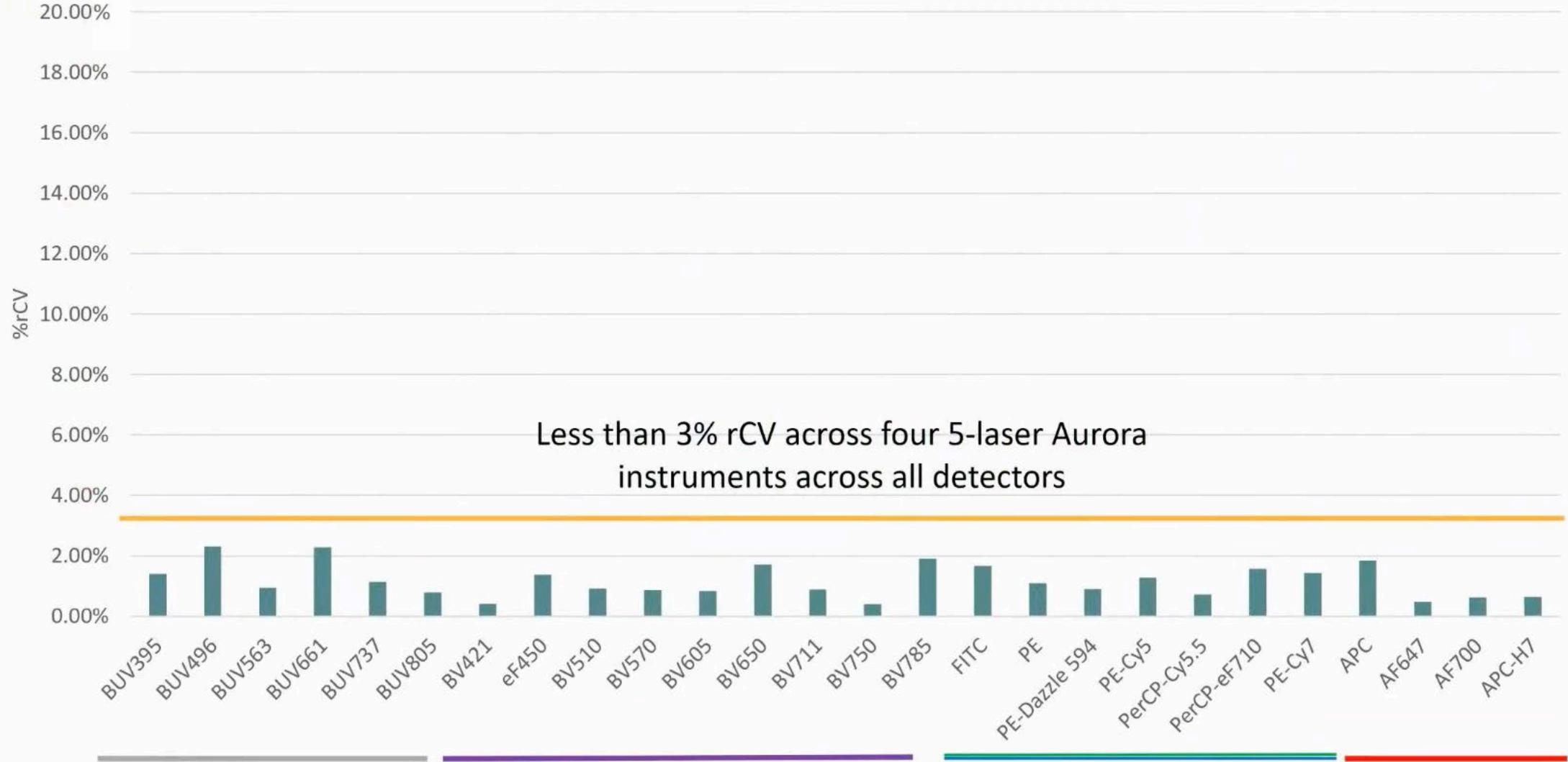


Is the MFI of fluorochrome stained beads more consistent?





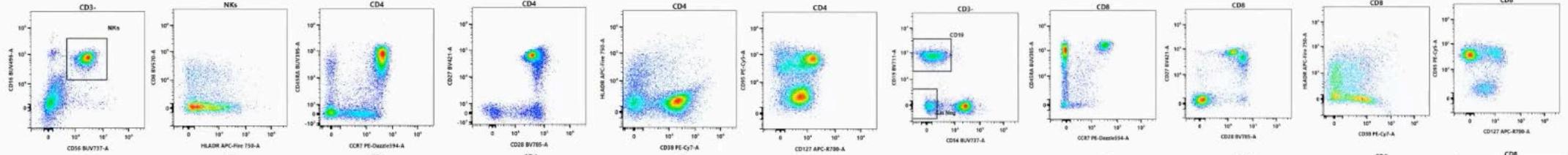
Does instrument setup standardization improve when using fluorescent-stained particles?



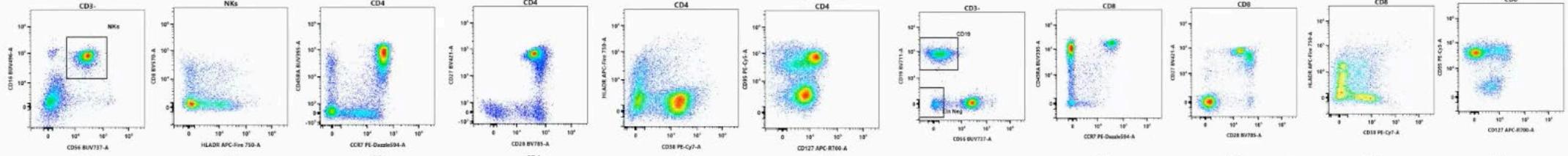


Assay performance at fluorochrome standardized settings

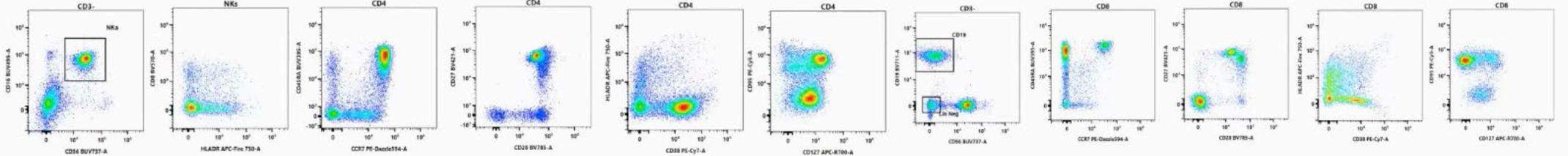
Unit 1



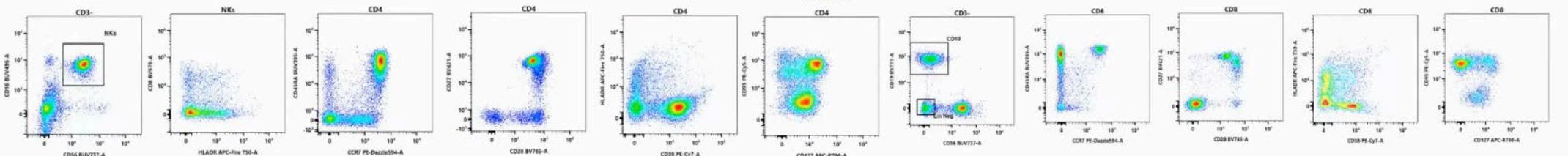
Unit 2



Unit 3



Unit 4



Unit/MFI	CD14 BV605	CD3 BUV661	CD4 BV750	CD8 BV570	CD56 BUV737	CD19 BV711
Unit 1	80502	115189	121386	181301	24924	73631
Unit 2	75606	122312	123863	181623	25811	78221
Unit 3	80533	116272	126003	179946	25038	75171
Unit 4	72398	110489	125604	175005	25159	76772
MFI CV%	5%	4%	2%	2%	2%	3%



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Standardization of
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beads

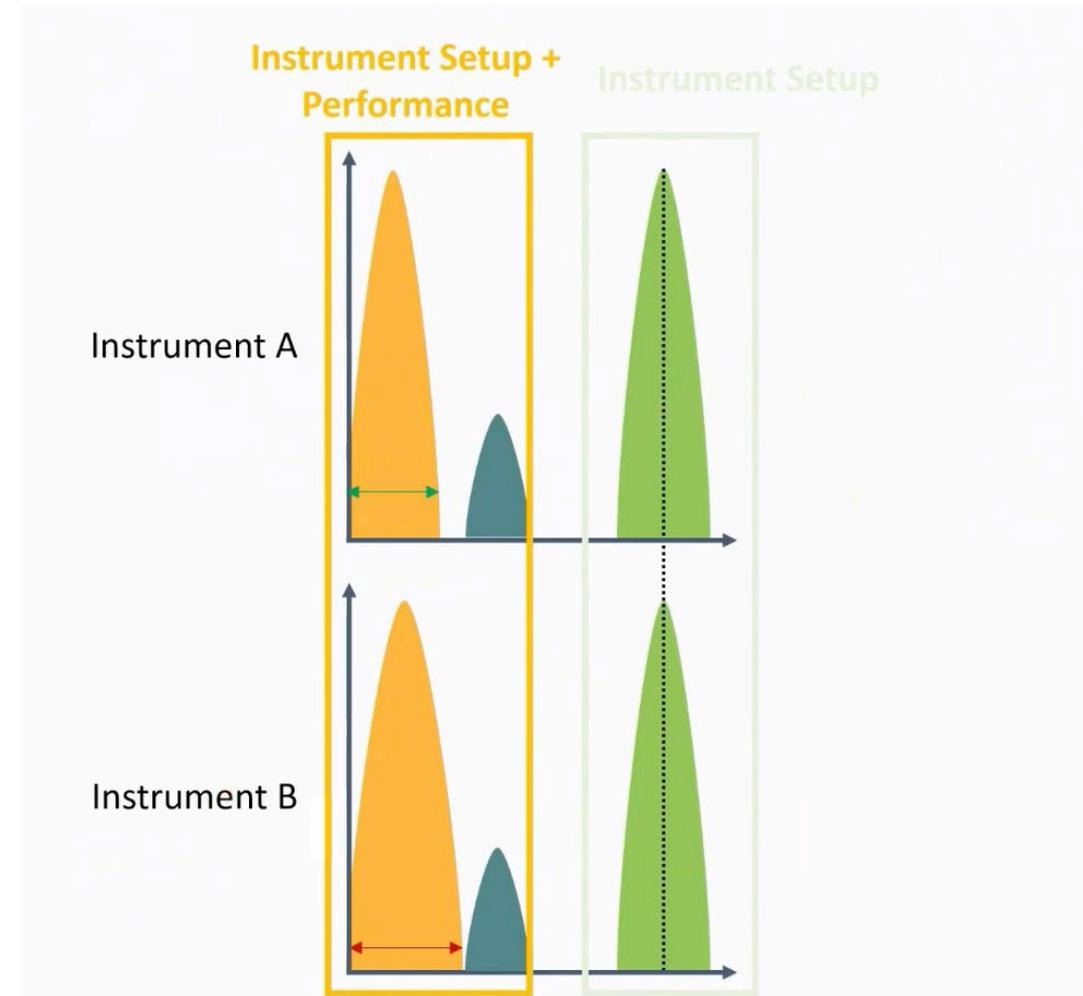
Standardization of
instrument setup
with fluorescent
labeled particles

Standardization of
instrument
performance



Standardization of Instrument Performance

1. Populations fall within same gates.
2. Population percentages are the same.
3. Positive population MFIs are the same.
4. Resolution of dim markers is the same.





Factors affecting instrument sensitivity/resolution

Positive Signal

Excitation Optics

- Laser power
- Laser wavelength
- Laser alignment

Collection Optics

- Optical detector
- Optical filters

Negative rSD

Optical Background

Electronic Noise

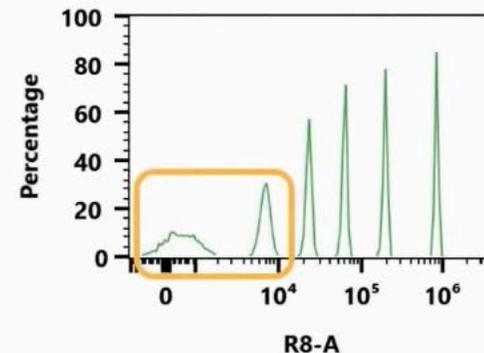
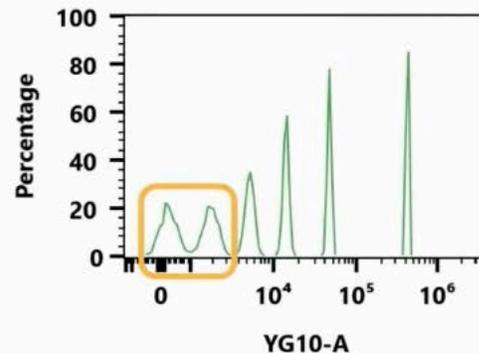
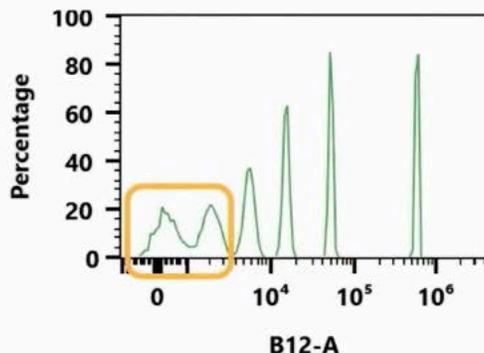
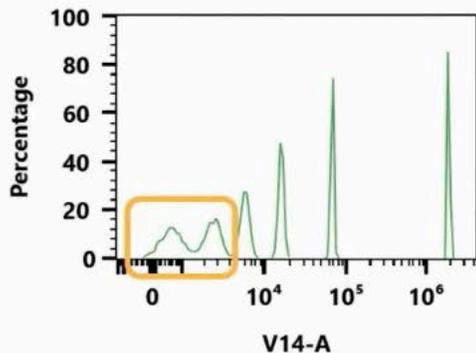
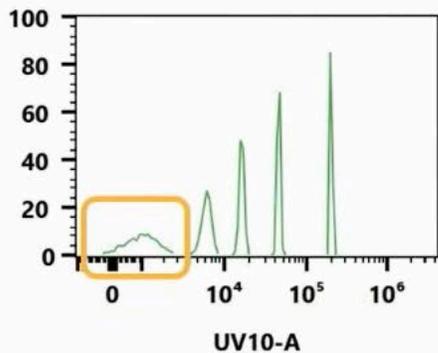
Cytek Manufacturing Specifications to Standardize Instrument Performance

- Lasers
 - Power (+/- 10% expected power output)
 - Wavelength (+/- 2nm)
- Optical Filters
 - Center Wavelength (+/- 2nm)
 - Each filter is scanned for transmission and center wavelength measurement
- APD module performance and laser alignment
- Electronic noise

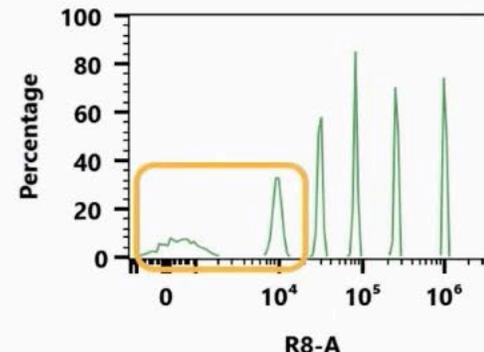
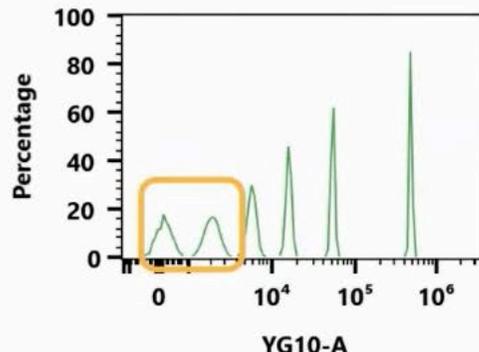
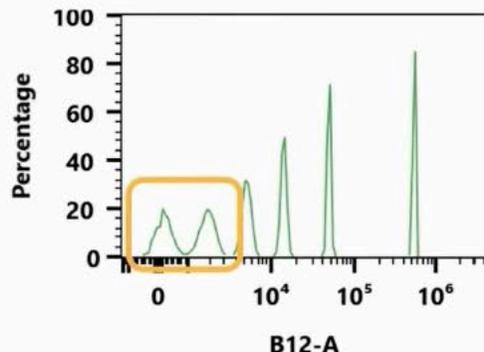
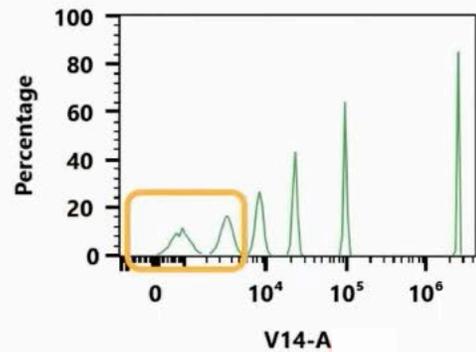
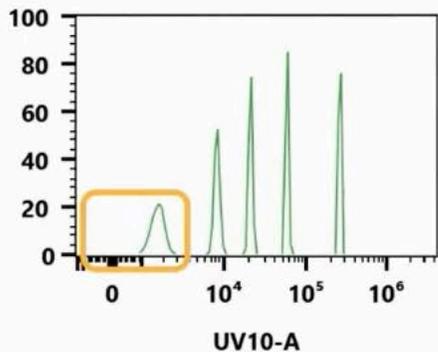


Instrument sensitivity /resolution: multipeak beads

Aurora A



Aurora B



UV- 610nm

V- 750nm

B- 760nm

YG- 810nm

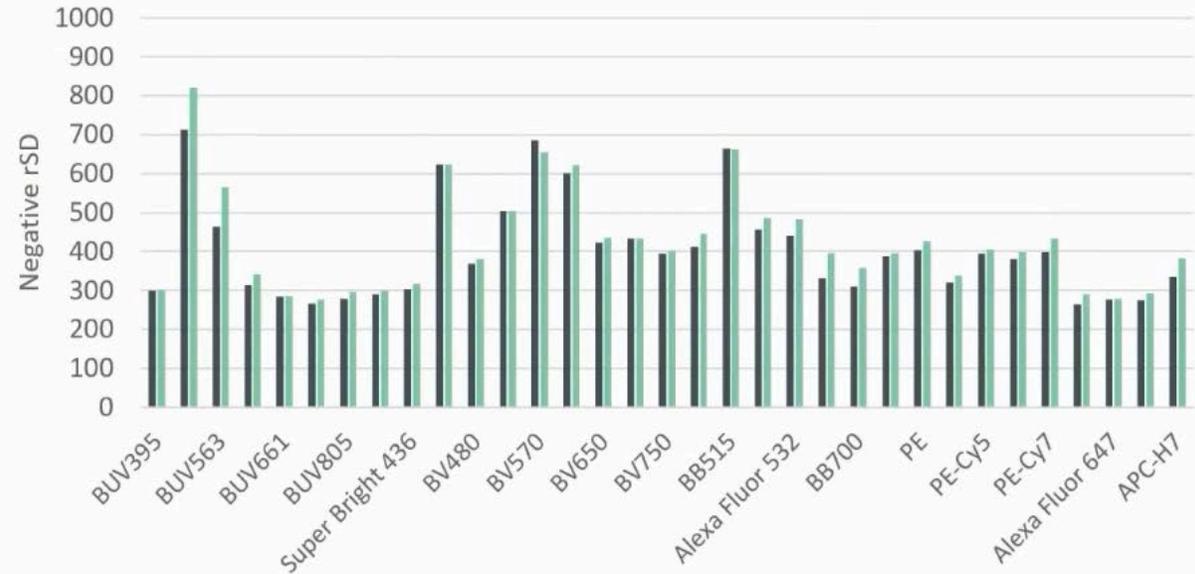
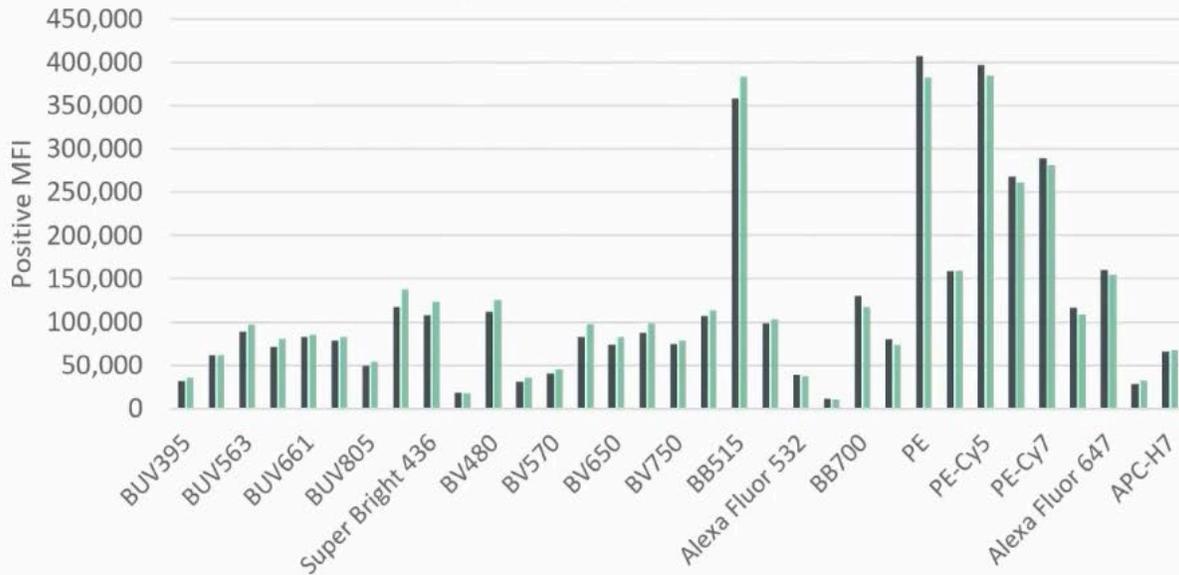
R- 810nm



Instrument sensitivity /resolution: Stain Index



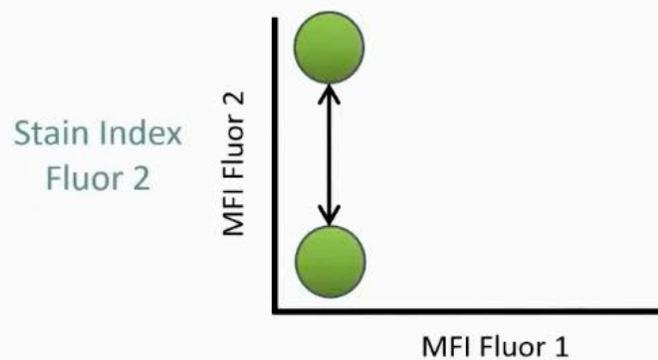
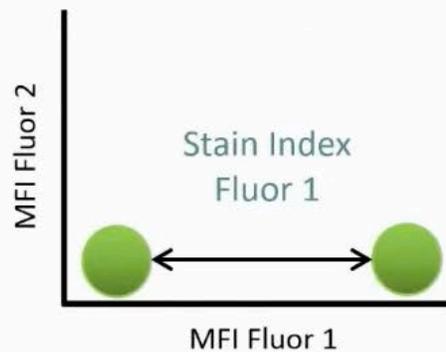
$$SI = \frac{\text{Median Pos.} - \text{Median Neg.}}{2 (\text{rSD Neg.})}$$





Instrument sensitivity /resolution: SPREAD

Single Color Scenario



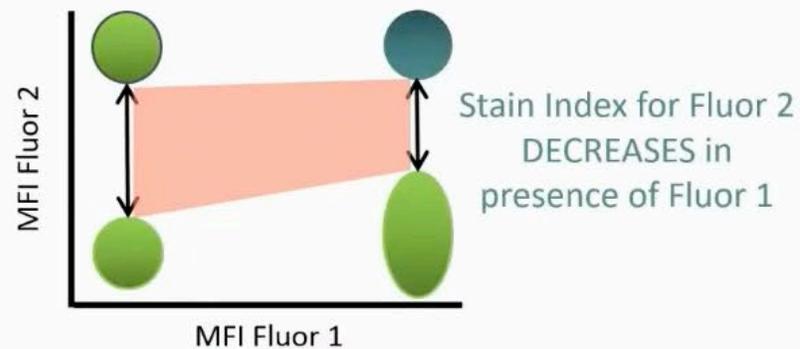
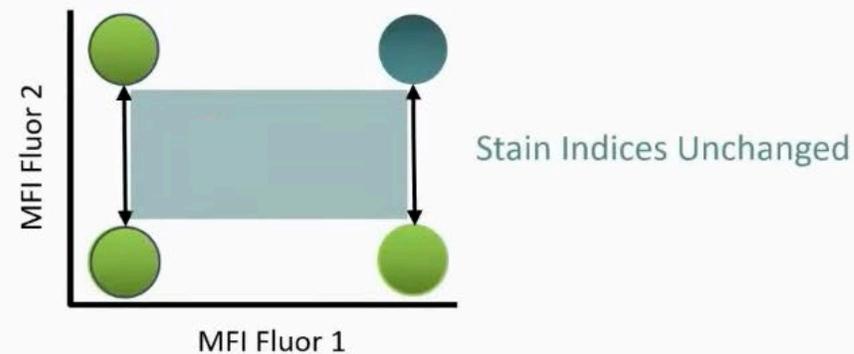
Fluor 1 DOES NOT spread into Fluor 2

Fluor 2 DOES NOT spread into Fluor 1

Fluor 1 DOES spread into Fluor 2

Fluor 2 DOES NOT spread into Fluor 1

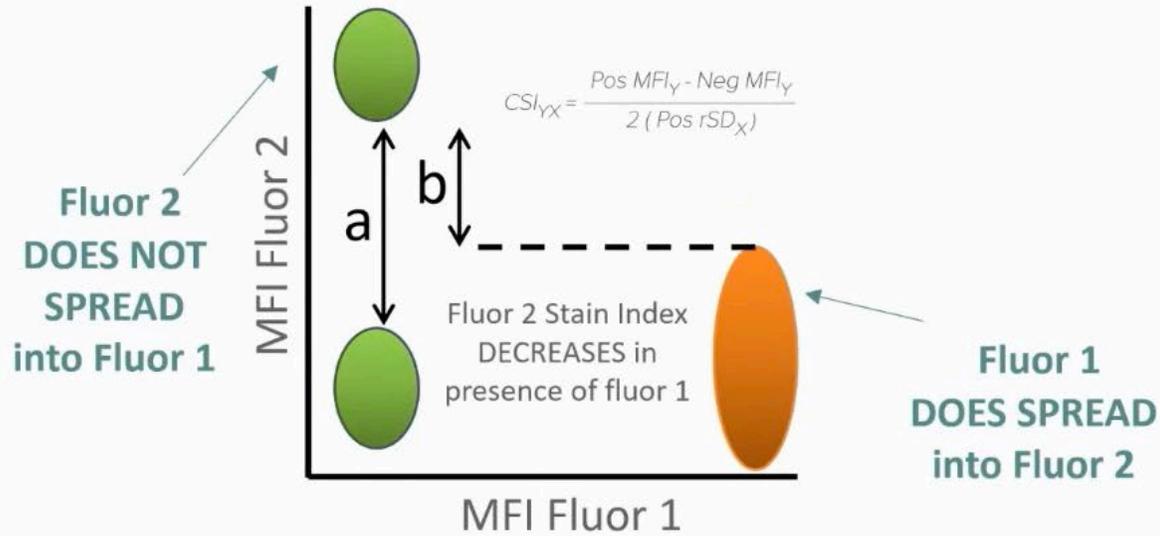
Multicolor Scenario





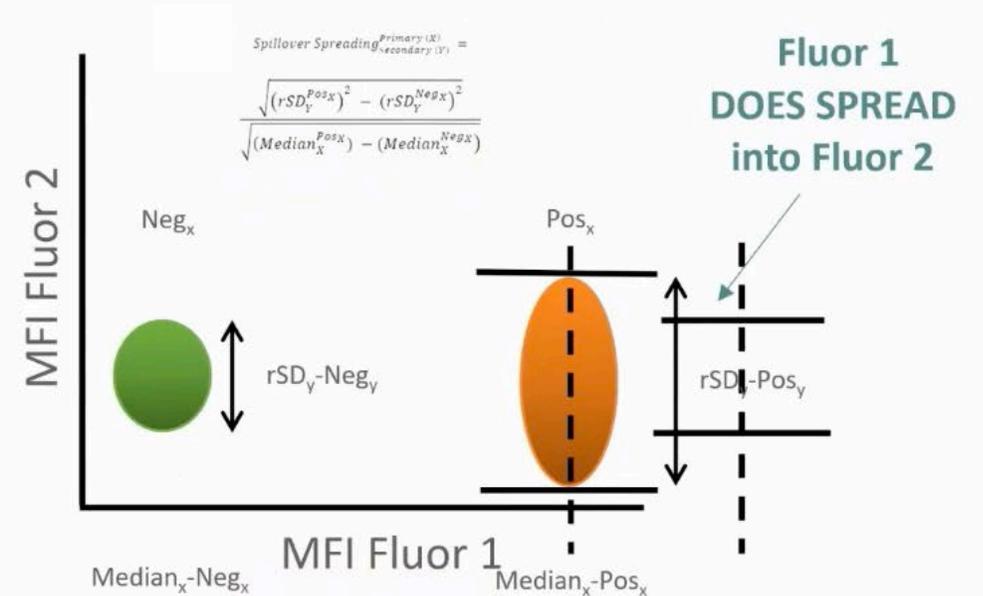
Quantification of spread

Cross Stain Index (CSI)



- CSI value represents loss of resolution of fluor 2 due to spread contributed by fluor 1.
- CSI value depends on the expression level of the antigen detected using fluor 1

Spillover Spread Value (SSV)



- SS value represents the spread increase of the fluor 1 positives into fluor 2, when compared to negatives in the same channel.
- SSV is not dependent on the expression level of the antigen detected by fluor 2.



Spread between optimally performing units is similar but not identical

Aurora A	BUV395	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	Super Bright 436	eFluor 450	BV480	BV510	BV570	BV605	BV650	BV711	BV750	BV785	BB515	Alexa Fluor 488	Alexa Fluor 532	PerCP	BB700	PerCP-eFluor 710	PE	PE-Dazzle594	PE-Cy5	PE-Cy5.5	PE-Cy7	APC	Alexa Fluor 647	Alexa Fluor 700	APC-H7	
BUV395		2.32	0.60	0.47	0.40	0.26	0.25	0.99	0	0	1.53	0	1.19	0.22	0	0	0	0	0	0	0	0.23	0.52	0	0.54	0	0	0	0	0	0	0	0	0.16
BUV496	0.23		1.45	0.55	0.30	0.20	0.19	0	0	3.57	1.22	5.31	1.41	0.78	0	0	0	0	5.02	4.75	0.63	0	0	0	0.71	0.26	0.47	0.23	0	0.32	0.34	0	0	
BUV563	0.36	0.24		1.09	0.66	0.39	0.23	0	0	2.69	1.12	2.36	1.36	0.87	0	0	0	0	2.77	3.58	1.99	0.56	0.73	0.51	3.34	0.91	0.71	0.56	0.17	0.43	0.39	0	0.17	
BUV615	0	0	1.33		1.47	1.26	1.03	0	0.34	0	0.26	0	0	1.58	1.00	0.92	0.65	0.65	1.84	1.11	0.21	1.51	1.58	1.26	0.95	3.00	1.94	1.78	0.48	1.01	1.15	0.52	0.11	
BUV661	0	0	0.38	0.51		2.16	1.59	0.40	0.36	0	0.54	0	0	0.22	1.80	1.25	1.24	0.98	0	0	1.83	1.92	1.48	0.47	0.19	2.58	1.75	0.53	4.01	4.09	1.52	0.98		
BUV737	0.22	0	0.62	0	0.58		3.04	0	0.40	0	0	0	0	0	0.19	1.83	2.03	1.67	0	1.28	0	0.61	1.15	2.68	0.42	0	0.33	1.63	0.80	0.87	0.91	1.90	1.31	
BUV805	0.67	0	0	0	0	1.03		0	0	2.00	0	0	0.93	0	0	0	0	0.64	0	0	0.44	0	0	0.45	0	0	0	0.20	0.26	0.84	0.26	0	1.18	
BV421	0.54	1.40	0.91	0.31	0.26	0	0.26		4.58	3.54	1.99	4.78	1.33	0.26	0	0	0.12	0	0	0	0	0	0.34	0.16	0.41	0	0.16	0	0.12	0.38	0.41	0	0.18	
Super Bright 436	0.26	1.57	0.84	0	0.15	0	0	3.45		4.16	2.27	4.70	1.42	0.53	0	0	0	0	0	1.17	0.24	0	0	0	0.34	0	0	0	0	0.43	0.09	0	0	
eFluor 450	0	0	1.99	0.72	0.73	0	0	0	0		1.90	6.87	4.37	2.57	1.51	0	0	0.70	0	0	0	0.52	0.82	0	1.64	0	0	0	0	0	0.39	0.37	0.16	
BV480	0.11	3.71	1.64	0.56	0.25	0	0.11	0.60	0.23	8.36		9.45	3.55	1.63	0.75	0	0.46	0.59	5.32	5.09	1.27	0.24	0	0	1.27	0.39	0	0	0	0	0	0.18	0	
BV510	0	3.06	1.90	0.67	0.55	0.31	0.65	0.78	0.72	6.68	1.95		3.88	2.17	1.36	0.51	0.82	0.20	0	0	0.44	0	0	0.29	1.32	0.33	0.41	0.48	0.10	0	0.53	0	0.25	
BV570	0	0	1.56	0.73	0.55	0.42	0.23	0.51	1.51	0	0	0		2.25	1.19	0.80	0.68	0.92	0	0	0.62	0.72	0.50	0.68	2.14	0.93	1.01	0.66	0	0.58	0.72	0	0	
BV605	0	0	1.01	1.64	0.78	0.71	0.45	0.89	1.17	1.42	0.88	2.00	0.98		1.91	1.41	1.24	0.93	0	0	0	1.10	1.10	0.93	0.86	1.19	1.48	1.17	0.52	0.82	0.77	0.28	0.22	
BV650	0	0	0.43	0.44	1.45	0.86	0.72	0.96	1.07	1.31	0.66	1.43	0.27	0.80		1.86	1.74	1.40	2.49	2.58	0	1.19	1.20	1.10	0	0.42	1.57	0.99	0.38	1.68	1.62	0.64	0.45	
BV711	0	0	0	0.27	1.01	2.95	1.26	1.23	1.25	0	0	0	0.52	0	0.41		3.13	2.98	0.40	0.90	0	0.49	3.11	1.60	0.74	0.26	0.77	3.22	0.45	1.60	1.39	2.95	1.04	
BV750	0	0	0	0	0.23	1.90	1.57	0.92	1.03	0	0	0	0	0	0	1.21		3.66	0	1.01	0	0	0.69	0.61	0	0	0	0.40	0.39	0	0.11	0.73	0.85	
BV785	0	0	0	0	0	0.87	1.73	0.96	0.91	0	0	0	0.11	0	0	0.20	2.00		0.56	0	0	0.30	0.25	0.38	0	0.07	0	0	0.36	0	0	0.36	0.90	
BB515	0	0.75	0.58	0.20	0.20	0	0	0.07	0.38	3.43	0.41	2.68	1.44	0.42	0	0.16	0.22	0.05		17.64	3.60	0.15	0.20	0.13	0.73	0.56	0.18	0.21	0	0.05	0.12	0.07	0.07	
Alexa Fluor 488	0	0	0.40	0	0	0	0.07	0	0	3.24	0	1.70	1.44	0.61	0	0	0	0	16.79		3.70	0.25	0.37	0.42	0.68	0.52	0.27	0.37	0.13	0	0.39	0	0.13	
Alexa Fluor 532	0	0	0.52	0.46	0	0.15	0.52	0.54	0	3.58	0	3.32	2.59	1.23	0.71	0	0	0	7.42	9.17		0.65	1.04	0.94	1.43	0.92	0.61	0.52	0	0.65	0.91	0	0	
PerCP	0	0	0	0.39	0.95	1.46	1.18	0	0	0	0	0	0	0	0.67	1.92	1.94	1.34	0	0	1.29		3.12	1.98	0	0	1.46	2.29	0	1.08	1.89	0.95	0	
BB700	0	0	0.45	0.29	0.77	0.81	0.71	0.40	0.29	1.78	0.09	1.42	0.72	0.44	1.76	3.27	2.03	1.87	1.15	1.33	0.64	1.77		3.60	0.52	0.37	1.11	2.41	0.97	3.85	5.01	1.92	0.91	
PerCP-eFluor 710	0	0	0.21	0.16	0.70	1.28	1.10	0.79	0.63	0	0.30	0	0	0	1.04	4.37	2.67	2.58	0	0	0.57	1.64	5.14		0.73	0.21	0.82	4.17	1.67	1.87	2.28	2.36	1.02	
PE	0	0.70	0.79	0.47	0.29	0.18	0.15	0	0	1.96	0.50	2.40	2.52	1.01	0.50	0.33	0.25	0.28	3.46	3.87	1.93	0.75	0.80	0.64		1.24	0.94	0.69	0.20	0.45	0.48	0.10	0	
PE-Dazzle594	0	0	0.81	1.35	0.64	0.32	0.18	0	0	1.25	0.37	2.17	0.78	1.37	0.79	0.92	0.44	0	3.13	3.66	1.36	1.77	2.16	1.50	1.65		2.16	1.73	0.56	1.05	1.17	0.32	0.17	
PE-Cy5	0	0.06	0.36	0.75	0.63	0.68	0.41	0.25	0.26	0	0.18	0.17	0	0.32	1.57	2.52	0.86	0.77	2.21	2.49	0.34	3.55	5.16	2.82	0.85	0.56		3.14	1.33	2.20	3.28	0.82	0.52	
PE-Cy5.5	0	0	0.25	0.28	0.35	0.69	0.49	0.14	0	1.19	0.31	0.54	0.73	0.50	1.24	3.07	1.20	1.03	1.46	1.59	0.67	1.75	5.70	3.91	2.04	0.53	1.04		2.01	1.97	2.75	0.90	0.49	
PE-Cy7	0	0	0	0.14	0.11	0.51	0.85	0	0	0	0	0	0	0.09	0.05	1.50	0.77	1.51	0	0.48	0	0.29	0.53	1.76	0.37	0.25	0.30	1.75		0.21	0.36	0.39	1.57	
APC	0	0	0.26	0	0.68	0.68	0.46	0	0.48	0	0	0	0	0	1.70	0.87	0.92	0.89	0	0	0	1.68	1.53	1.21	0.14	0	2.71	1.43	0.53	3.33	1.28	0.93		
Alexa Fluor 647	0	0	0	0.18	0.42	0.39	0.21	0	0	0.61	0	0	0	0.29	0.83	0.37	0	0.26	0	0	0	1.10	1.02	0.87	0	0.17	1.39	0.98	0.33	3.19		1.42	1.10	
Alexa Fluor 700	0	0	0.56	0	0.13	0.95	0.92	0	0	0	0	0	0	0	0	1.20	1.21	1.04	0	0	0	0.57	1.66	1.33	0	0	0.72	1.06	0.61	1.87	1.86		1.58	
APC-H7	0	0	0	0	0.34	0.44	1.02	0	0	0	0	0	0	0	0.39	0	0.78	1.71	0	0.90	0	0.36	0.33	0.21	0.42	0	0	0.22	1.11	0.41	0	0.31		



Spread between optimally performing units is similar but not identical

B	BUV395	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	Super Bright 436	eFluor 450	BV480	BV510	BV570	BV605	BV650	BV711	BV750	BV785	BB515	Alexa Fluor 488	Alexa Fluor 532	PerCP	BB700	PerCP-eFluor 710	PE	PE-Dazzle594	PE-Cy5	PE-Cy5.5	PE-Cy7	APC	Alexa Fluor 647	Alexa Fluor 700	APC-H7
BUV395		1.62	0.62	0	0	0.23	0.18	0.73	1.04	0	1.35	0	0.27	0	0.49	0.22	0.27	0.45	0	0	0	0.64	0	0	0	0.23	0	0.11	0	0.50	0	0.43	
BUV496	0		1.53	0.52	0.39	0.45	0.32	0.24	0	4.63	1.48	5.40	1.50	0.35	0	0	0	0	4.39	3.96	0.77	0.33	0	0.37	0.77	0.36	0	0.40	0	0	0.13	0.09	
BUV563	0.31	0		1.16	0.62	0.39	0.41	0	0.42	2.75	0.82	2.09	1.13	0.87	0	0.30	0.29	0	0	1.95	0.97	0.59	0.33	0.18	2.99	1.06	0.57	0.34	0.07	0.42	0.17	0	0.16
BUV615	0.40	1.66	1.19		1.39	1.29	1.10	0.20	0	1.26	0.39	0.71	0.59	1.87	1.01	0.55	0.39	0.41	1.04	2.11	0.76	1.46	1.30	1.01	0.86	3.25	1.75	1.42	0.52	1.00	0.86	0.48	0
BUV661	0.26	0	0.25	0.46		2.32	1.91	0.57	0	1.14	0.77	0.21	0	0	2.18	1.30	1.38	1.14	0	0	0	1.90	1.63	1.33	0	0.34	2.43	1.55	0.67	3.54	3.59	1.51	1.08
BUV737	0.21	0.27	0.11	0.14	0.47		3.92	0.23	0	0.73	0	0	0.38	0	0.47	1.49	2.00	1.67	0	0	0	0.53	0.75	1.32	0	0.37	0	0.93	0.69	0.92	1.02	2.05	1.47
BUV805	0.96	0.84	0.67	0	0.29	1.04		0	0	0.67	0	0	0.47	0	0	0	0.79	1.18	0.27	1.01	0	0	0	0	0.19	0.31	0.40	0.32	0.76	0.63	0.43	1.27	
BV421	0	1.56	0.81	0	0	0	0		4.60	4.53	2.12	5.34	1.42	0.71	0.28	0.11	0.23	0.14	1.85	1.65	0	0.28	0	0	0.44	0	0.07	0	0	0.42	0.35	0	0.16
Super Bright 436	0	1.29	0.63	0	0	0	0	3.36		3.59	2.22	4.81	1.26	0.57	0	0	0	0.27	0	0	0	0.10	0	0.34	0.51	0.20	0	0	0.08	0	0.15	0	0
eFluor 450	0	1.67	1.23	0.77	0.56	0.63	0.59	0.58	0		0.92	5.44	4.39	2.34	1.22	0	0.23	0.87	0	0	0	0	0.62	0.86	1.56	0.28	0.45	0	0	0	0	0.85	0.61
BV480	0	3.23	1.61	0.48	0.30	0.12	0.07	0.56	0.49	8.52		10.15	3.63	1.72	0.66	0	0	0	4.23	4.21	0.95	0	0	0	1.14	0.41	0.20	0	0.15	0.51	0.45	0.06	0
BV510	0	2.31	1.56	0.73	0.41	0.44	0.52	0.68	0.86	7.41	1.77		4.00	2.29	1.16	0.73	0.38	0.58	1.01	2.29	0.22	0.19	0	0	1.45	0.53	0.25	0.24	0.19	0	0.24	0	0.10
BV570	0	0	1.29	0.47	0.36	0.40	0.45	1.58	1.78	0	1.08	0		2.33	1.18	0.93	0.80	0	0.78	2.25	0.64	0.49	0.85	0.35	2.25	0.86	0.91	0.80	0	0.49	0.78	0.33	0.25
BV605	0	0.80	0.88	1.20	0.75	0.63	0.52	1.05	1.03	2.17	0.70	2.44	1.28		1.83	1.29	1.23	1.02	0	0	0	1.02	0.95	0.76	0.78	1.18	1.27	0.85	0.37	0.64	0.81	0.36	0
BV650	0	0	0	0.44	1.12	0.79	0.78	0.82	1.13	0	0.60	0	0	1.02		1.94	1.72	1.55	0	0	0	1.09	1.04	0.85	0.35	0.28	1.35	0.96	0.46	1.28	1.31	0.69	0.42
BV711	0	0	0.42	0	0.47	1.33	1.52	1.05	1.20	1.57	0.60	2.07	0.58	0.21	0.54		3.21	2.90	0	0	0	0.43	1.16	1.07	0	0	0.30	0.86	0.51	1.17	1.14	1.64	1.06
BV750	0	0	0.11	0	0.21	1.68	1.83	0.36	0.61	0	0	0	0	0	0	1.03		3.85	0	0	0	0.32	0.43	0.27	0.39	0	0.26	0.40	0.45	0.55	0.25	0.73	0.83
BV785	0	0	0.32	0	0	0.86	1.99	1.11	0.93	0	0.74	0	0.29	0	0	0	2.08		0	0	0	0	0	0.17	0	0	0	0	0.38	0	0	0.37	0.89
BB515	0	0.76	0.43	0.24	0.13	0	0.09	0.07	0	2.74	0.19	2.03	1.19	0.41	0	0	0.16	0		15.86	2.94	0.14	0.19	0.17	0.64	0.45	0.18	0.20	0	0.31	0	0.13	0.13
Alexa Fluor 488	0	0	0.56	0	0.17	0	0.18	0.27	0.65	3.04	0.32	2.31	1.29	0.23	0	0.34	0	0.33		16.78	3.32	0	0.23	0.21	0.70	0.48	0.29	0.26	0	0.14	0.28	0	0
Alexa Fluor 532	0	0	1.02	0.36	0.39	0	0.21	0	0	5.49	0.83	2.62	2.82	0.97	0	0.59	0.27	0	0	8.20	9.32	0.73	0.86	0.61	1.60	0.33	0.60	0.66	0.31	0.74	0	0	0
PerCP	0	0	1.22	0	0.90	1.61	1.37	0	0	2.36	0	0	0.83	0	1.61	0	0.67	0	0	0	0	0	2.71	0	0	0	1.25	0	0	0.79	2.12	0.36	0
BB700	0	0	0.24	0.45	0.79	0.98	0.87	0.27	0.36	0.59	0	0	0.45	0.55	1.66	3.77	2.21	1.95	1.05	1.80	0.43	1.76		2.82	0.49	0.35	1.11	2.16	0.96	2.34	3.02	1.29	1.14
PerCP-eFluor 710	0	0	0.31	0	0.67	1.48	1.34	0	0	0	0.13	0	0	0	1.05	4.71	3.01	2.73	1.73	0.39	0.67	1.38	3.88		0.49	0	0.76	3.28	1.76	1.80	2.04	2.01	1.31
PE	0	0.23	1.58	0.37	0.23	0.13	0	0.37	0	2.02	0.59	2.24	4.51	1.37	0.44	0.37	0	0.14	2.52	2.95	1.40	0.67	0.68	0.55		1.02	0.81	0.59	0.22	0.44	0.49	0.11	0.04
PE-Dazzle594	0	0	0.58	0.80	0.48	0.34	0.29	0	0	1.78	0.49	1.66	0.48	2.19	0.87	0.93	0	0.34	1.29	1.85	0.69	1.67	1.53	1.35	1.33		1.94	1.56	0.57	0.81	1.15	0.29	0.16
PE-Cy5	0	0	0	0.45	1.02	0.60	0.38	0.27	0.24	1.00	0.20	0.46	0.08	0.47	2.89	1.72	0.87	0.84	1.43	1.56	0.39	2.94	3.73	2.40	0.72	0.35		3.15	1.30	2.27	2.71	0.96	0.67
PE-Cy5.5	0	0	0.10	0.30	0.48	0.76	0.62	0.38	0	0	0	0	0.34	0.19	1.22	2.76	1.05	1.04	0.86	1.05	0.41	1.63	4.15	3.19	1.64	0.44	1.05		2.25	1.38	1.97	0.82	0.52
PE-Cy7	0	0	0	0	0.16	0.61	1.00	0.21	0	0	0	0	0.30	0.22	0.19	1.25	0.79	2.42	0.71	0	0	0.29	0.54	1.19	0.55	0.11	0.28	1.08		0.12	0	0.40	0.98
APC	0	0	0.32	0.24	1.00	0.75	0.53	0.56	0.31	0	0	0	0.25	0.35	3.32	0.98	0.88	0.89	0	0	0.44	1.89	1.38	1.02	0	0	3.24	1.48	0.63		3.60	1.78	1.17
Alexa Fluor 647	0	0	0	0.09	0.48	0.41	0.17	0.43	0.23	0	0	0	0	0	0.87	0.19	0	0.27	0	0	0.25	1.18	1.11	0.77	0.21	0.14	1.56	0.93	0.32	3.08		1.73	1.42
Alexa Fluor 700	0	0	0.91	0.39	0.57	0.91	0.36	0.68	0.28	0	0	0	0	0	0	1.37	1.31	1.23	0	2.92	0	0.61	1.50	0.92	0.72	0.37	0.50	0.89	0.66	1.51	1.54		1.81
APC-H7	0	0	0	0	0.19	0.51	1.25	0	0	0	0.50	1.36	0	0.28	0	0	0.71	1.91	0	0	0	0.28	0	0	0	0.22	0	0	1.15	0.60	0.46	0.23	



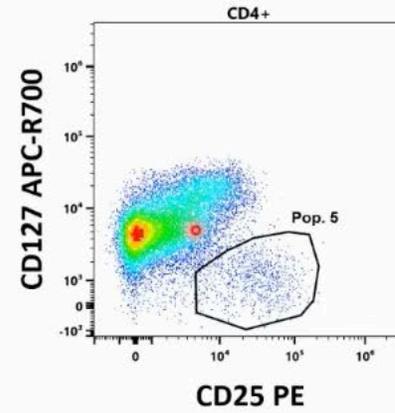
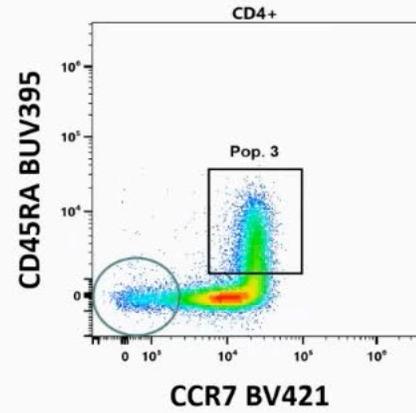
Spread between optimally performing units is very similar but not identical

Aurora B-Aurora A	BUV395	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	Super Bright 436	eFluor 450	BV480	BV510	BV570	BV605	BV650	BV711	BV750	BV785	BB515	Alexa Fluor 488	Alexa Fluor 532	PerCP	BB700	PerCP-eFluor 710	PE	PE-Dazzle594	PE-Cy5	PE-Cy5.5	PE-Cy7	APC	Alexa Fluor 647	Alexa Fluor 700	APC-H7
BUV395		-0.70	0.02	-0.47	-0.40	-0.03	-0.08	-0.26	1.04	0.00	-0.18	0.00	-0.92	-0.22	0.49	0.22	0.27	0.45	0.00	0.00	0.00	-0.23	0.12	0.00	-0.54	0.00	0.23	0.00	0.11	0.00	0.50	0.00	0.26
BUV496	-0.23		0.08	-0.03	0.09	0.25	0.14	0.24	0.00	1.06	0.26	0.09	0.08	-0.42	0.00	0.00	0.00	0.00	-0.62	-0.78	0.13	0.33	0.00	0.37	0.06	0.10	-0.47	0.17	0.00	-0.32	-0.34	0.13	0.09
BUV563	-0.05	-0.24		0.08	-0.04	0.00	0.18	0.00	0.42	0.07	-0.30	-0.28	-0.24	0.00	0.00	0.30	0.29	0.00	-2.77	-1.63	-1.01	0.04	-0.40	-0.33	-0.35	0.15	-0.14	-0.22	-0.10	-0.01	-0.22	0.00	-0.01
BUV615	0.40	1.66	-0.14		-0.08	0.03	0.07	0.20	-0.34	1.26	0.13	0.71	0.59	0.30	0.01	-0.38	-0.26	-0.25	-0.80	1.00	0.55	-0.05	-0.27	-0.25	-0.09	0.25	-0.19	-0.36	0.03	-0.02	-0.29	-0.04	-0.11
BUV661	0.26	0.00	-0.13	-0.05		0.15	0.32	0.17	-0.36	1.14	0.23	0.21	0.00	-0.22	0.38	0.05	0.14	0.16	0.00	0.00	0.00	0.07	-0.29	-0.14	-0.47	0.15	-0.15	-0.20	0.14	-0.47	-0.50	0.00	0.10
BUV737	-0.02	0.27	-0.51	0.14	-0.10		0.88	0.23	-0.40	0.73	0.00	0.00	0.38	0.00	0.28	-0.34	-0.02	0.00	0.00	-1.28	0.00	-0.09	-0.39	-1.36	-0.42	0.37	-0.33	-0.69	-0.10	0.05	0.11	0.15	0.16
BUV805	0.28	0.84	0.67	0.00	0.29	0.02		0.00	0.00	-1.33	0.00	0.00	-0.46	0.00	0.00	0.00	0.79	0.54	0.27	1.01	-0.44	0.00	0.00	-0.45	0.00	0.19	0.31	0.20	0.06	-0.09	0.36	0.43	0.09
BV421	-0.54	0.16	-0.10	-0.31	-0.26	0.00	-0.26		0.02	0.99	0.13	0.56	0.09	0.44	0.28	0.11	0.11	0.14	1.85	1.65	0.00	0.28	-0.34	-0.16	0.03	0.00	-0.09	0.00	-0.12	0.05	-0.06	0.00	-0.02
Super Bright 436	-0.26	-0.28	-0.22	0.00	-0.15	0.00	0.00	-0.09		-0.57	-0.05	0.11	-0.16	0.04	0.00	0.00	0.27	0.00	0.00	-1.17	-0.24	0.10	0.00	0.34	0.17	0.20	0.00	0.08	-0.43	0.06	0.00	0.00	
eFluor 450	0.00	1.67	-0.75	0.05	-0.17	0.63	0.59	0.58	0.00		-0.98	-1.43	0.02	-0.23	-0.29	0.00	0.23	0.17	0.00	0.00	0.00	-0.52	-0.20	0.86	-0.08	0.28	0.45	0.00	0.00	0.00	-0.39	0.48	0.46
BV480	-0.11	-0.48	-0.03	-0.08	0.05	0.12	-0.05	-0.04	0.26	0.16		0.69	0.08	0.09	-0.09	0.00	-0.46	-0.59	-1.09	-0.87	-0.32	-0.24	0.00	0.00	-0.13	0.02	0.20	0.00	0.15	0.51	0.45	-0.13	0.00
BV510	0.00	-0.75	-0.34	0.06	-0.14	0.13	-0.13	-0.10	0.14	0.73	-0.19		0.12	0.12	-0.20	0.22	-0.43	0.38	1.01	2.29	-0.23	0.19	0.00	-0.29	0.13	0.20	-0.15	-0.24	0.09	0.00	-0.29	0.00	-0.15
BV570	0.00	0.00	-0.28	-0.26	-0.18	-0.02	0.22	0.08	0.27	0.00	1.08	0.00		0.09	-0.01	0.12	0.12	-0.92	0.78	2.25	0.02	-0.23	0.35	-0.33	0.11	-0.07	-0.10	0.14	0.00	-0.09	0.07	0.33	0.25
BV605	0.00	0.80	-0.12	-0.44	-0.03	-0.08	0.07	0.16	-0.14	0.75	-0.18	0.44	0.30		-0.08	-0.13	-0.01	0.09	0.00	0.00	0.00	-0.09	-0.15	-0.17	-0.07	-0.01	-0.21	-0.32	-0.14	-0.18	0.03	0.08	-0.22
BV650	0.00	0.00	-0.43	0.00	-0.33	-0.07	0.06	-0.13	0.06	-1.31	-0.06	-1.43	-0.27	0.22		0.07	-0.02	0.14	-2.49	-2.58	0.00	-0.10	-0.16	-0.25	0.35	-0.14	-0.22	-0.03	0.09	-0.40	-0.31	0.05	-0.02
BV711	0.00	0.00	0.42	-0.27	-0.53	-1.62	0.25	-0.18	-0.05	1.57	0.60	2.07	0.06	0.21	0.13		0.08	-0.09	-0.40	-0.90	0.00	-0.05	-1.95	-0.53	-0.74	-0.26	-0.47	-2.36	0.06	-0.43	-0.25	-1.30	0.02
BV750	0.00	0.00	0.11	0.00	-0.02	-0.22	0.26	-0.56	-0.42	0.00	0.00	0.00	0.00	0.00	0.00	-0.18		0.19	0.00	-1.01	0.00	0.32	-0.27	-0.34	0.39	0.00	0.26	0.00	0.05	0.55	0.14	0.00	-0.02
BV785	0.00	0.00	0.32	0.00	0.00	-0.01	0.26	0.15	0.02	0.00	0.74	0.00	0.18	0.00	0.00	-0.20	0.08		-0.56	0.00	0.00	-0.30	-0.25	-0.21	0.00	-0.07	0.00	0.00	0.02	0.00	0.00	0.00	-0.01
BB515	0.00	0.00	-0.15	0.04	-0.07	0.00	0.09	0.00	-0.38	-0.69	-0.22	-0.65	-0.25	-0.01	0.00	-0.16	-0.05	-0.05		-1.77	-0.66	0.00	-0.01	0.04	-0.08	-0.11	0.00	-0.02	0.00	0.26	-0.12	0.05	0.06
Alexa Fluor 488	0.00	0.00	0.16	0.00	0.17	0.00	0.11	0.27	0.65	-0.19	0.32	0.61	-0.15	-0.39	0.00	0.34	0.00	0.33	-0.02		-0.38	-0.25	-0.14	-0.20	0.02	-0.04	0.02	-0.11	-0.13	0.14	-0.10	0.00	-0.13
Alexa Fluor 532	0.00	0.00	0.50	-0.10	0.39	-0.15	-0.31	-0.54	0.00	1.90	0.83	-0.71	0.24	-0.27	-0.71	0.59	0.27	0.00	0.78	0.15		0.08	-0.18	-0.33	0.17	-0.59	0.00	0.14	0.31	0.09	-0.91	0.00	0.00
PerCP	0.00	0.00	1.22	-0.39	-0.06	0.15	0.19	0.00	0.00	2.36	0.00	0.00	0.83	0.00	0.94	-1.92	-1.28	-1.34	0.00	0.00	-1.29		-0.41	-1.98	0.00	0.00	-0.21	-2.29	0.00	-0.29	0.23	-0.58	0.00
BB700	0.00	0.00	-0.21	0.16	0.02	0.17	0.16	-0.13	0.07	-1.19	-0.09	-1.42	-0.27	0.11	-0.10	0.50	0.18	0.08	-0.09	0.48	-0.21	-0.02		-0.77	-0.03	-0.03	-0.01	-0.26	-0.02	-1.51	-2.00	-0.63	0.23
PerCP-eFluor 710	0.00	0.00	0.11	-0.16	-0.03	0.21	0.24	-0.79	-0.63	0.00	-0.16	0.00	0.00	0.01	0.34	0.34	0.15	0.34	1.73	0.39	0.09	-0.25	-1.26		-0.24	-0.21	-0.06	-0.89	0.09	-0.07	-0.24	-0.35	0.29
PE	0.00	-0.47	0.79	-0.10	-0.06	-0.06	-0.15	0.37	0.00	0.06	0.09	-0.16	1.99	0.36	-0.06	0.04	-0.25	-0.14	-0.94	-0.93	-0.53	-0.08	-0.13	-0.09		-0.23	-0.13	-0.10	0.02	-0.01	0.00	0.00	0.04
PE-Dazzle594	0.00	0.00	-0.23	-0.55	-0.16	0.02	0.11	0.00	0.00	0.52	0.12	-0.51	-0.30	0.82	0.09	0.01	-0.44	0.34	-1.84	-1.80	-0.66	-0.10	-0.64	-0.15	-0.33		-0.22	-0.17	0.00	-0.24	-0.01	-0.03	0.00
PE-Cy5	0.00	-0.06	-0.36	-0.30	0.38	-0.07	-0.03	0.01	-0.02	1.00	0.02	0.30	0.08	0.15	1.32	-0.80	0.01	0.06	-0.78	-0.93	-0.35	-0.61	-1.44	-0.42	-0.13	-0.21		0.01	-0.02	0.07	-0.57	0.15	0.15
PE-Cy5.5	0.00	0.00	-0.14	0.02	0.13	0.07	0.13	0.24	0.00	-1.19	-0.31	-0.54	-0.39	-0.31	-0.03	-0.31	-0.16	0.01	-0.60	-0.54	-0.26	-0.12	-1.55	-0.72	-0.40	-0.09	0.00		0.24	-0.60	-0.78	-0.08	0.03
PE-Cy7	0.00	0.00	0.00	-0.14	0.05	0.10	0.15	0.21	0.00	0.00	0.00	0.00	0.30	0.13	0.14	-0.25	0.03	0.91	0.71	-0.48	0.00	0.00	0.00	-0.57	0.17	-0.14	-0.02	-0.67		-0.09	-0.36	0.01	-0.59
APC	0.00	0.00	0.07	0.24	0.32	0.07	0.07	0.56	-0.18	0.00	0.00	0.00	0.25	0.35	1.62	0.11	-0.04	0.00	0.00	0.00	0.44	0.21	-0.14	-0.19	-0.14	0.00	0.53	0.05	0.09		0.27	0.50	0.24
Alexa Fluor 647	0.00	0.00	0.00	-0.09	0.06	0.02	-0.04	0.43	0.23	-0.61	0.00	0.00	0.00	-0.29	0.03	-0.18	0.00	0.01	0.00	0.00	0.25	0.08	0.08	-0.10	0.21	-0.03	0.17	-0.05	-0.01	-0.11		0.31	0.32
Alexa Fluor 700	0.00	0.00	0.36	0.39	0.44	-0.04	-0.56	0.68	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.10	0.19	0.00	2.92	0.00	0.04	-0.17	-0.41	0.72	0.37	-0.22	-0.16	0.05	-0.36	-0.31		0.23
APC-H7	0.00	0.00	0.00	0.00	-0.15	0.07	0.23	0.00	0.00	0.00	0.50	1.36	0.00	0.28	-0.39	0.00	-0.07	0.20	0.00	-0.90	0.00	-0.09	-0.33	-0.21	-0.42	0.22	0.00	-0.22	0.04	0.19	0.46	-0.09	

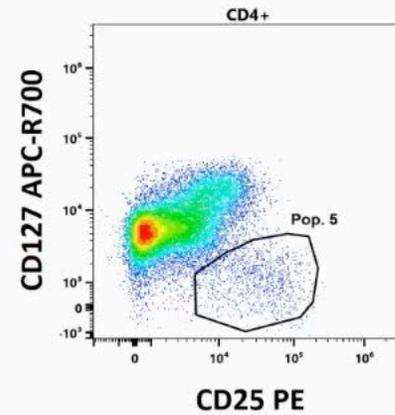
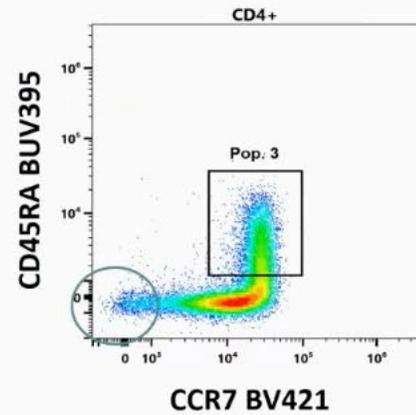


Resolution of dim markers in multicolor assay

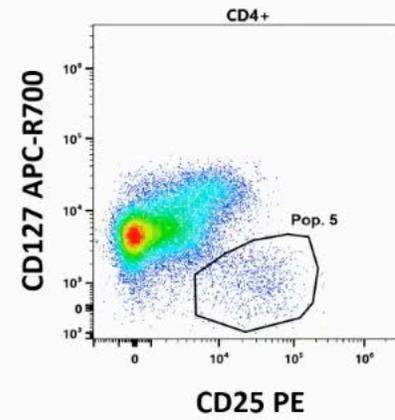
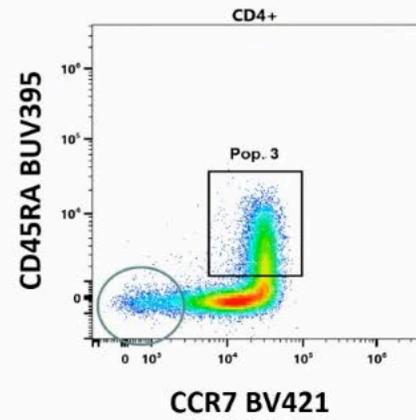
Site 1



Site 2



Site 3





Use of Aurora by CRO

With great power comes great responsibility: high-dimensional spectral flow cytometry to support clinical trials

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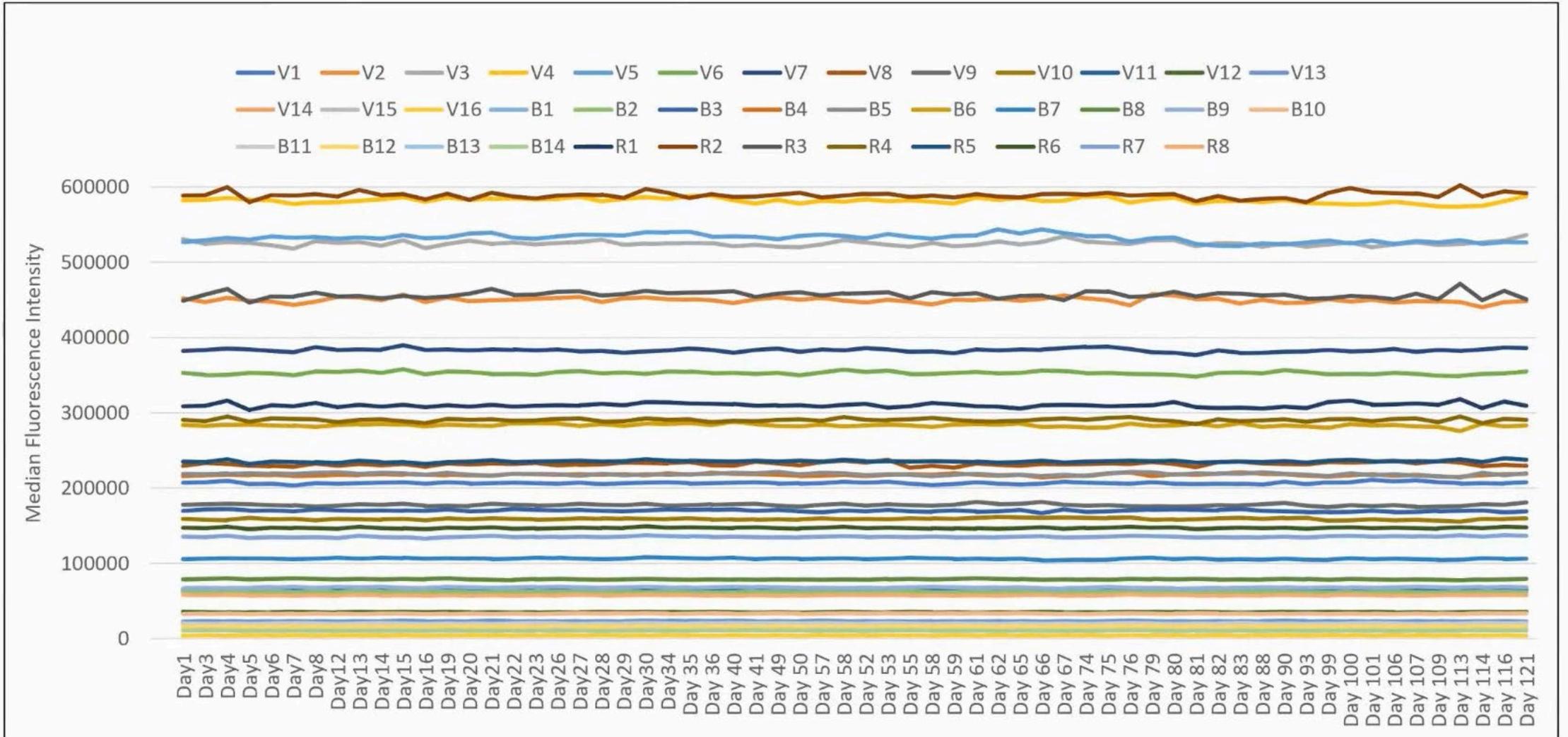
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User Settings stability over time





Stain index across 4 Auroras (Supplemental Figure)

Instrument / Fluorochrome	"A"	"B"	"C"	"D"
BUV395	101	115	89	102
BUV496	83	93	82	79
BUV563	266	234	213	214
BUV615	213	224	196	185
BUV661	238	252	221	207
BUV737	324	339	290	285
BUV805	180	183	160	159
BV421	158	179	152	132
Super Bright 436	92	109	89	77
eFluor 450	46	59	49	43
BV480	210	228	207	194
BV510	69	74	70	66
BV570	88	87	77	71
BV605	167	178	171	158
BV650	103	123	110	91
BV711	140	150	135	117
BV750	149	138	134	123
BV785	203	189	181	165
BB515	217	233	189	193
Alexa Fluor 488	21	23	18	18
Spark Blue 550	53	56	49	49
PerCP	7	7	7	7
PerCP-Cy5.5	17	19	16	16
PerCP-eFluor 710	111	129	111	109
PE	655	688	549	668
PE-Dazzle594	337	376	314	336
PE-Fire 640	101	124	113	118
PE-Cy5	187	190	186	182
PE-Cy5.5	343	329	314	329
PE-Cy7	798	799	751	789
APC	79	82	69	65
Alexa Fluor 647	82	84	74	69
Spark NIR 685	68	68	64	56
Alexa Fluor 700	77	79	74	71
APC-H7	137	142	126	127
APC-Fire 810	115	123	108	124

AVE	SD	CV
102	10.5	10.3
84	6.1	7.3
232	24.9	10.7
204	17.4	8.5
230	19.7	8.6
309	26.0	8.4
171	13.1	7.7
155	19.5	12.5
91	13.3	14.5
49	7.1	14.3
210	14.1	6.7
70	3.4	4.8
81	8.5	10.5
168	8.3	4.9
107	13.2	12.3
136	13.6	10.0
136	10.5	7.7
185	16.0	8.7
208	20.9	10.0
20	2.4	12.0
52	3.6	6.9
7	0.2	3.4
17	1.4	8.1
115	9.5	8.2
640	62.2	9.7
341	25.5	7.5
114	9.8	8.6
186	3.2	1.7
329	11.6	3.5
784	22.4	2.9
74	8.3	11.3
77	7.2	9.3
64	5.6	8.7
75	3.2	4.3
133	7.6	5.7
118	7.3	6.2



Spread assessment: cross-stain index comparison (figure 4)

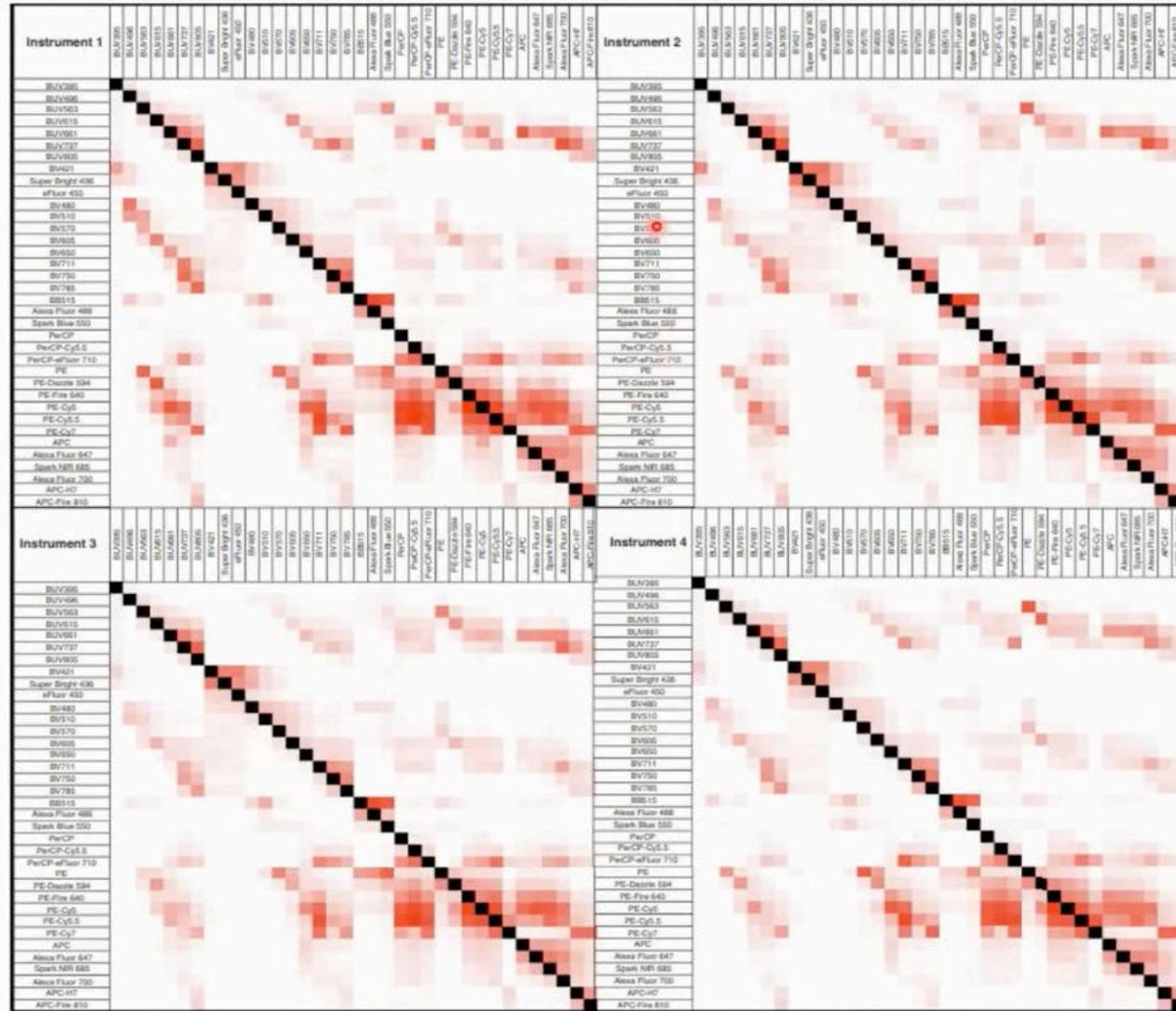


Figure 4. Instrument standardization. 36 color CSI heat maps from four optimally performing 5L (UV, V, B, Y/G, R) Cytek Auroras illustrating consistent spillover spreading across instruments. Fluorochromes in the columns spread into the detectors in the rows, with increased spread (e.g., reduced stain index/resolution) denoted by increased red intensity. The associated quantified cross-stain index values are found in Supplementary Table 1. CSI: Cross stain index.

Summary

Standardization of Instrument Setup

- Hard-dye beads standardization: good for immunophenotyping assays
 - Fast
 - Sufficient accuracy to obtain reproducible results
 - Can re-use worksheets without adjusting gates
- Fluorochrome based standardization: necessary for assays requiring precise measurement of changes in MFI

Standardization of Instrument Performance

- Single-Stained
 - Manufacturing process
 - Metrics for assessing: multipeak beads, stain index
- Multicolor
 - Evaluation of spread (CSI, SSV)
 - Visualize SSM differences for absolute values >5