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Standards and Future Directions for Quantitative Flow Cytometry Assays

Joshua Welsh, Advanced Technologies

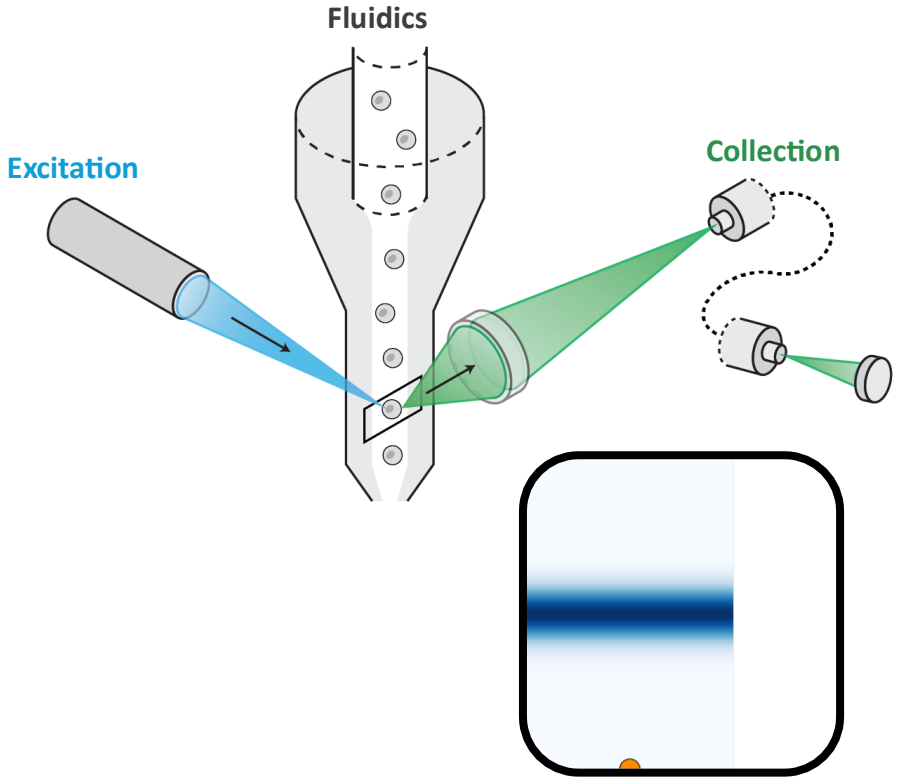
Disclosures

- **Sr. Staff Scientist**
  Advanced Technology Group,
  Waters Biosciences
- Director & Owner
  The Measuring Stick, Ltd
- **Associate Editor**
  Cytometry Part A
- **Councilor**
  International Society for
  Advancement of Cytometry

What is a flow cytometer?

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1. Samples are labelled with fluorescent reagents, typically fluorophore-conjugated antibodies.
2. Particles are suspended in a stream that passes through lasers sequentially.
3. As the particle passes through the lasers fluorescence and scattered light is collected and directed to a detector.
4. The detected signal (‘pulse’) from an event/particle are summarized as single statistics.
5. Event statistics are plotted on an arbitrary unit scale based on the electronic bit depth and a user’s detector gain settings



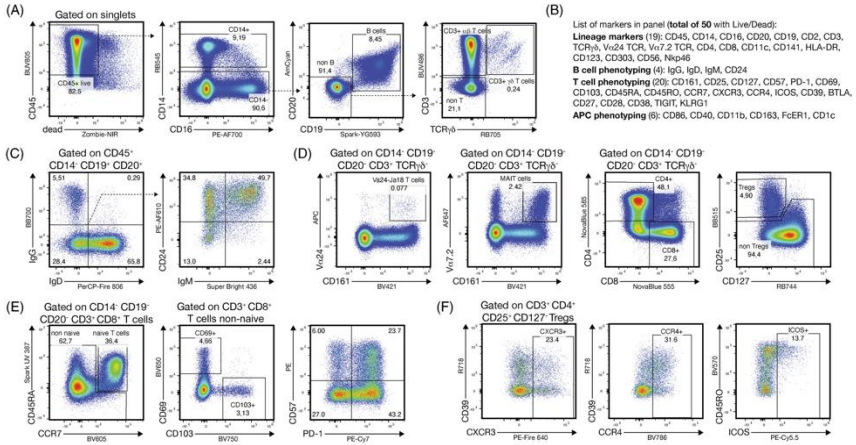
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Why use flow cytometry?

- Flow cytometers can measure tens of thousands of cells per second from heterogenous sample sources
- Spectral flow cytometers have demonstrated phenotyping across 50+ parameters.
- Flow cytometers clinical use spans:
  - Functional diagnostics
  - Cancer diagnoses
  - Disease monitoring
  - Immune profiling
  - Transplantation
  - Cell therapy

OMIP-102: 50-color phenotyping of the human immune system with in-depth assessment of T cells and dendritic cells

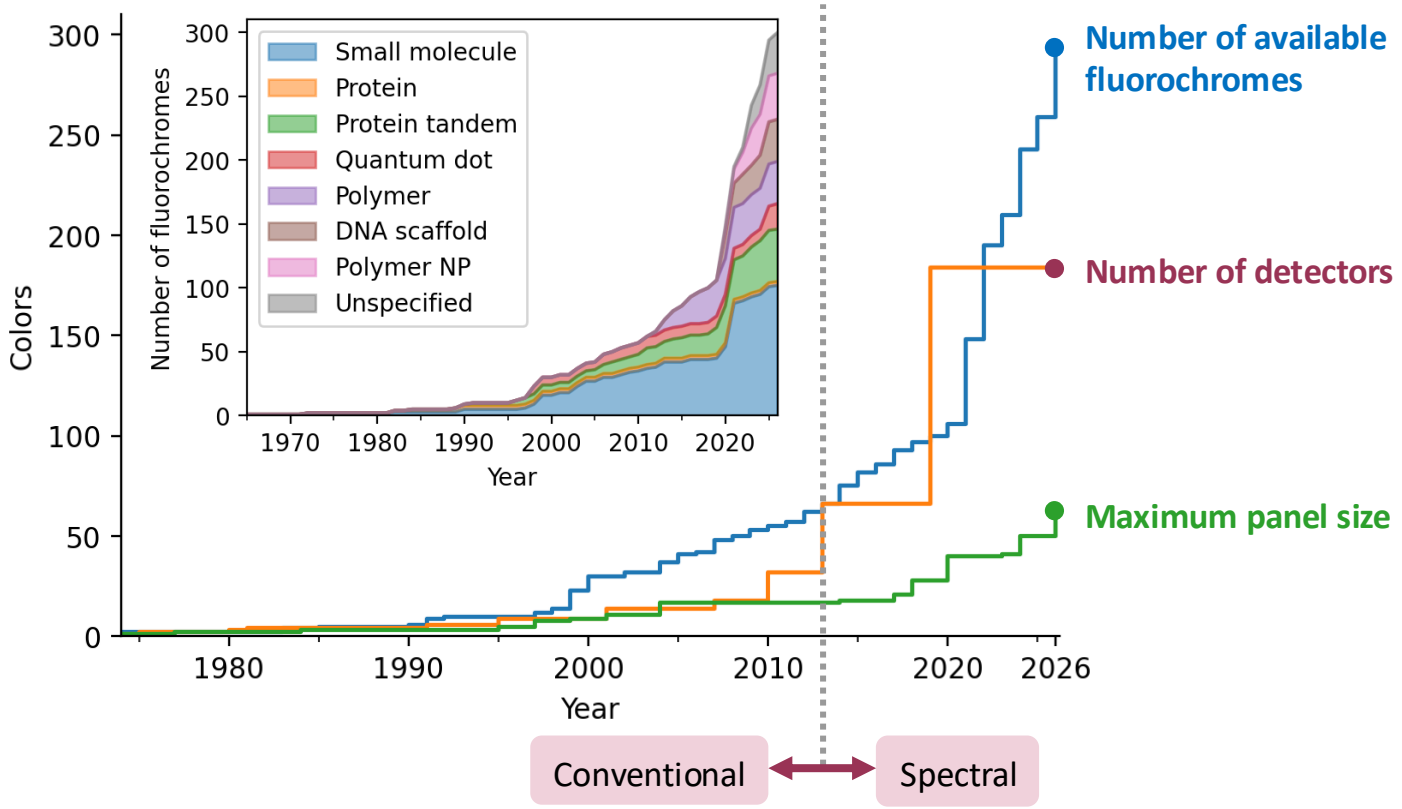
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The evolution in flow cytometry assay diversity

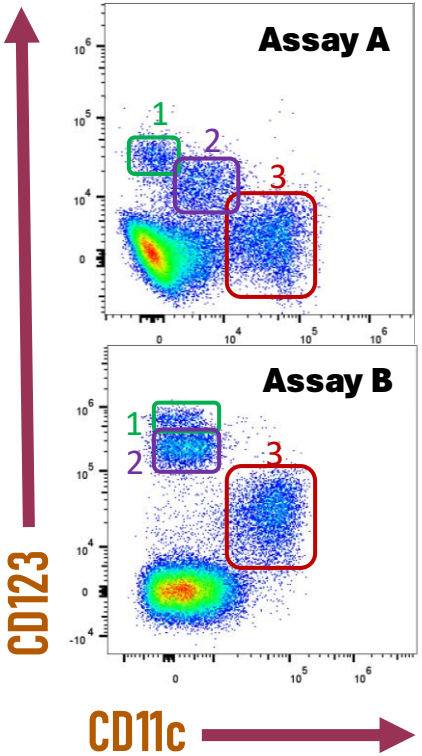
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Flow cytometry data currently semi-quantitative

Interpretation is heavily influenced by assay and instrument variation



Sample: Cryopreserved human PBMC
Markers: CD123/CD11c
Fluorophores: SB436/eFluor 450, PE-Cy5/BV510
FCM Technologies: Spectral
Experiment Sites: USA, Europe
Arbitrary Units: MFI

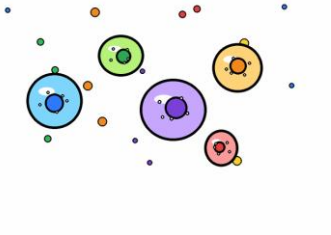
Population	OMIP 69	OMIP 102	Same in both?
1	CD123 Low CD11c Negative	CD123 High CD11c Negative	No Yes
2	CD123 Low CD11c Low	CD123 Medium CD11c Negative	No No
3	CD123 Negative CD11c Medium	CD123 Low CD11c Medium	No Yes

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“The same cell should not tell a different story”

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Biological Assay

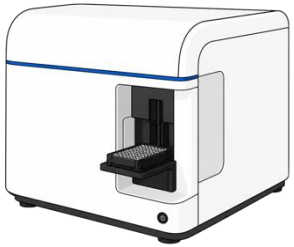




Samples

Reagents

Flow cytometers



Biological insights coupled to hardware

Biological Insights

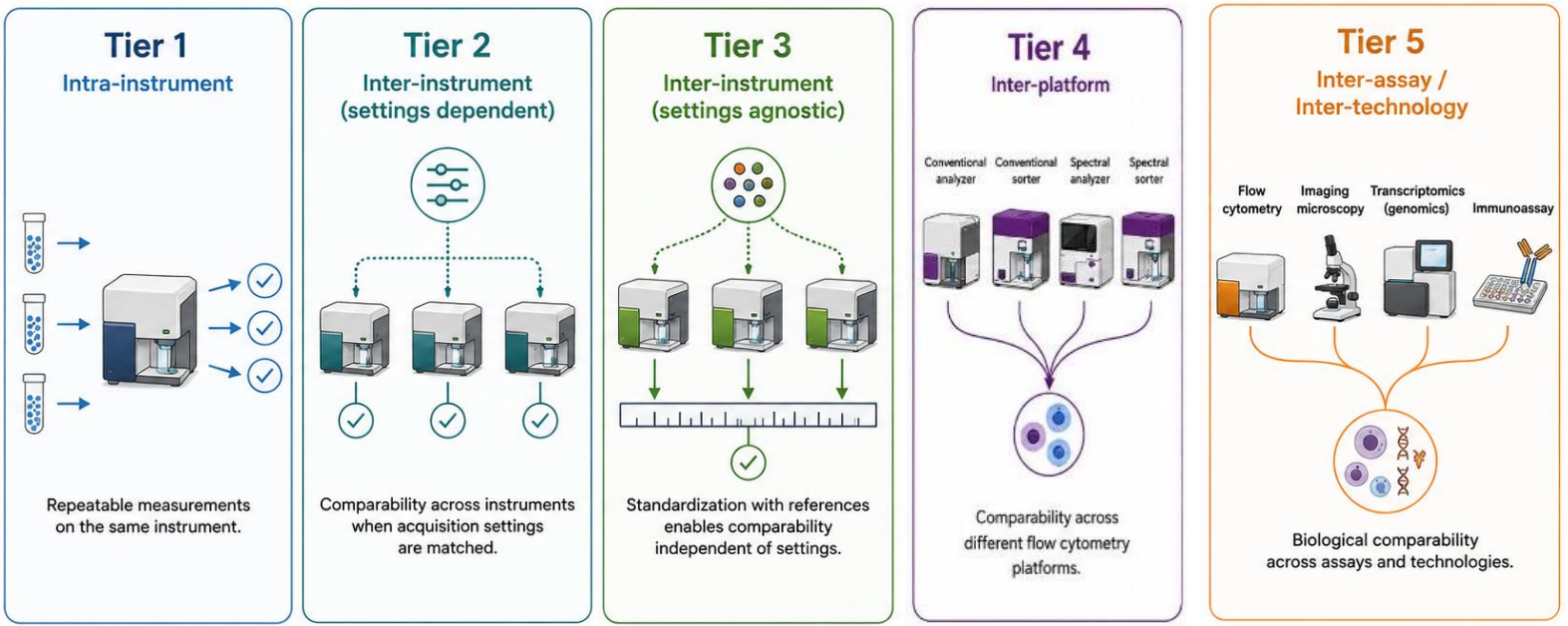


Current approaches to flow cytometry standardization can **mask biological insights** due to their hardware dependence

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Tiers of flow cytometry standardization

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From single-instrument reproducibility to cross-technology biological comparability.

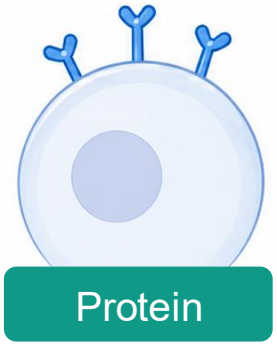
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Where flow cytometer assay variability arise?

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Pre-analytical variables

e.g. serum vs. plasma, separation methods, buffers, etc



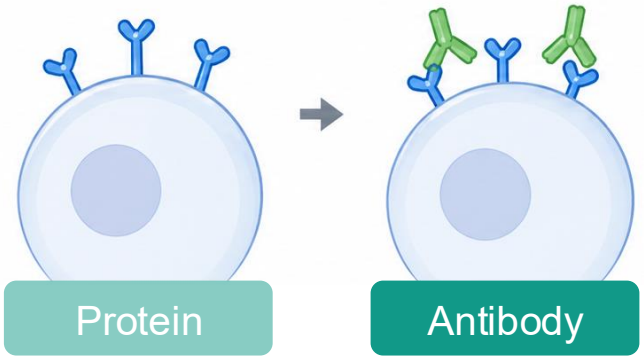
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Where flow cytometer assay variability arise?

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Different antibodies can bind to different protein domains

e.g. CD4 has 4 domains, SK3 is D1 specific, OKT-4 is D3 specific



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Where flow cytometer assay variability arise?

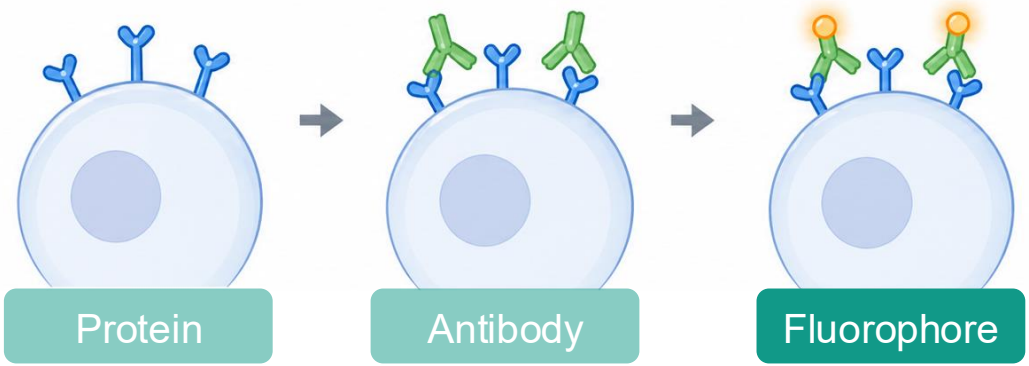
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Fluorophores can be different for the same targets

e.g. anti-CD4-FITC vs anti-CD4-PE antibodies

Antibody conjugates can change between lots

e.g. FITC average F/P 3-5, PE-Cy7 average 4-8 Cy7 per PE



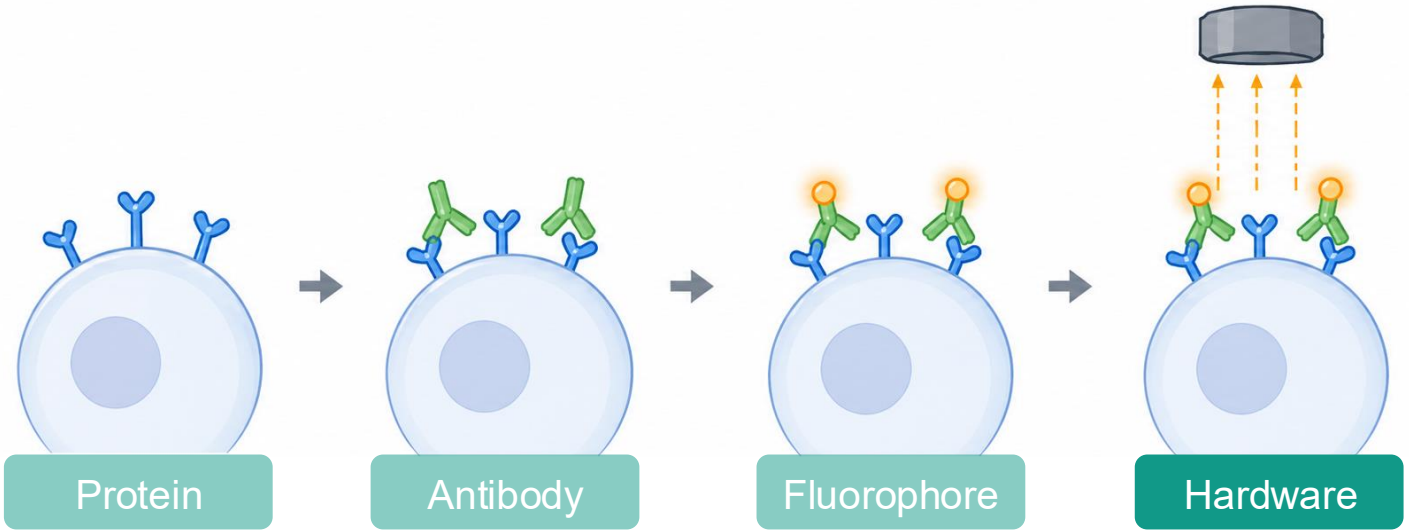
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Where flow cytometer assay variability arise?

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Fluorophore brightness depends on hardware and tolerances

e.g. FACSLyric vs FACSDiscover A8, Hardware tolerance: laser beam, drift, fluidics, etc



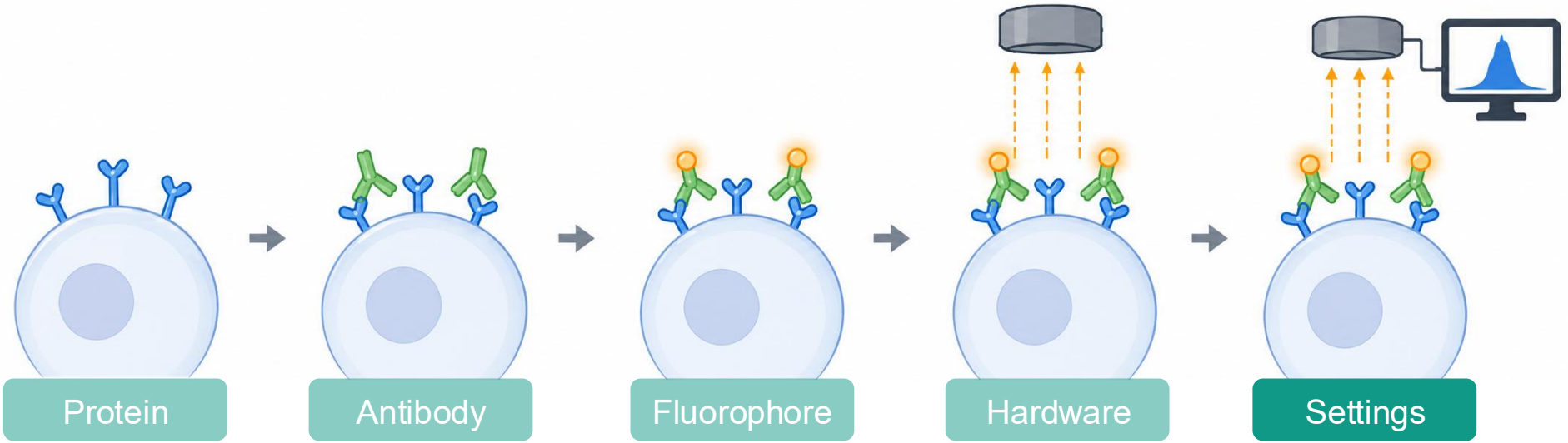
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Where flow cytometer assay variability arise?

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Users can change detector settings

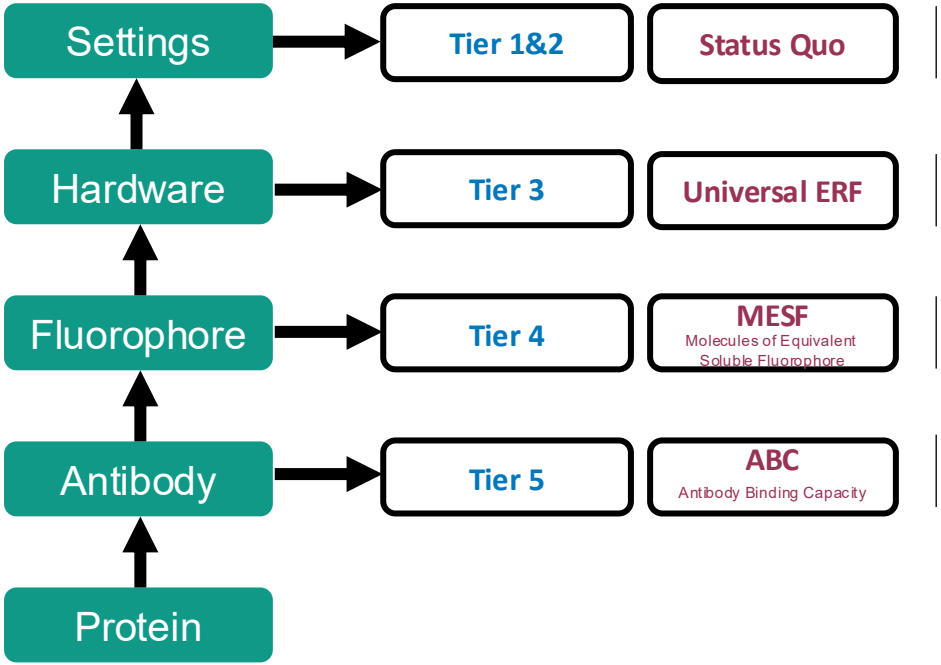
e.g. if user deviates from previous experiment settings, data is no longer directly comparable



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Relating standardization tiers to assay variability

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Provides consistent data on an instrument for a given set of settings

Accounts for detector setting variation on a given platform (current NIST effort)

Accounts for hardware variation for a given fluorophore

Accounts for conjugate variation and lot-lot variation

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“The same cell should not tell a different story”

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Human PBMC

10 COLOR PANEL	
CD3	BV605
CD4	BUV496
CD8	APCH7
CD14	BV510
CD16	BUV563
CD19	RY610
CD28	BUV737
CD45	PerCP
CD56	BV650
CD57	FITC



Fresh PBMCs



BD FACSDiscover™ A8

BD FACSymphony™ A5SE

- ***10 fluorophores***,
- ***10 markers***,
- ***2 platforms*** ,
- ***3 distinct optical modes****

*Optical modes refers to differences in core stream velocity, detector types, collection filters, beam geometry, & laser power

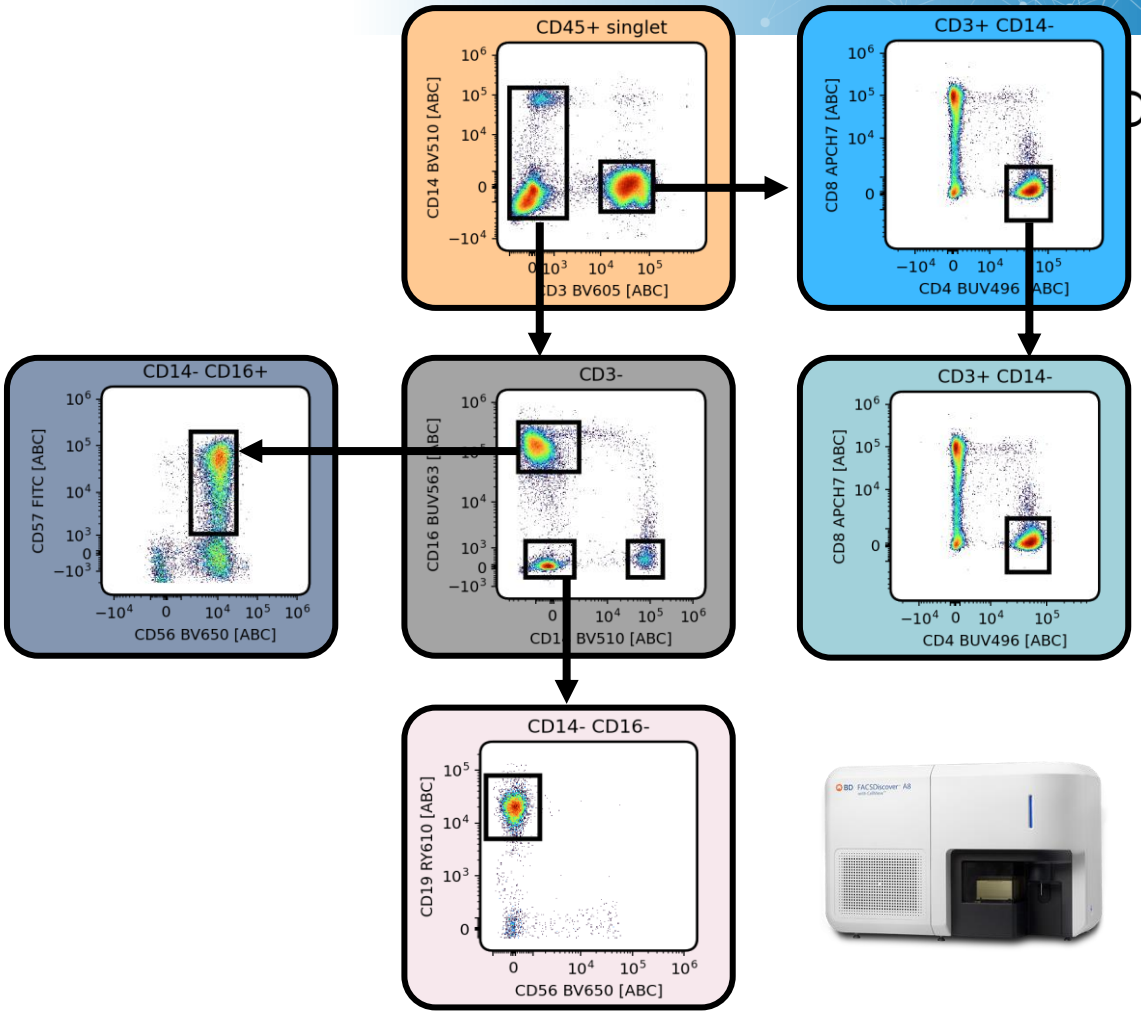
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Gating hierarchy

Human PBMC

10 COLOR PANEL	
CD3	BV605
CD4	BUV496
CD8	APCH7
CD14	BV510
CD16	BUV563
CD19	RY610
CD28	BUV737
CD45	PerCP
CD56	BV650
CD57	FITC

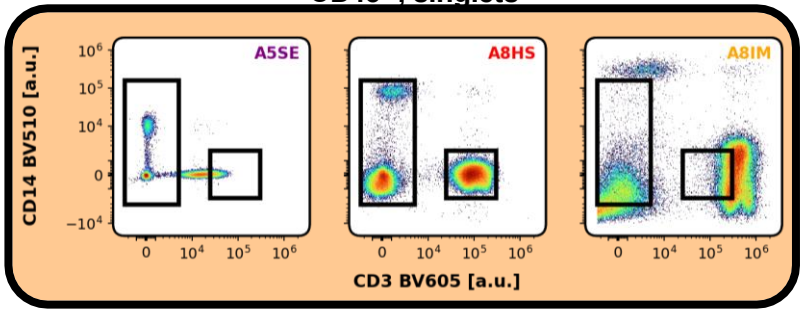
- ***10 fluorophores***,
- ***10 markers***,
- ***2 platforms*** ,
- ***3 distinct optical modes****



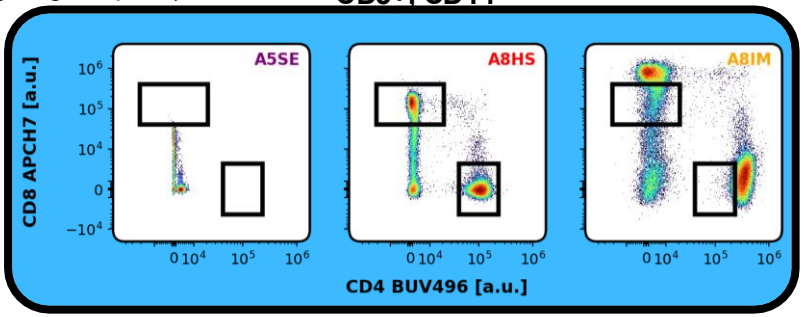
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Uncalibrated (A8HS gating template)

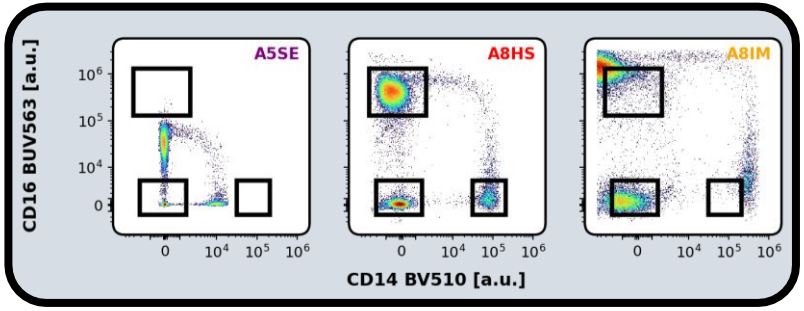
CD45+, singlets



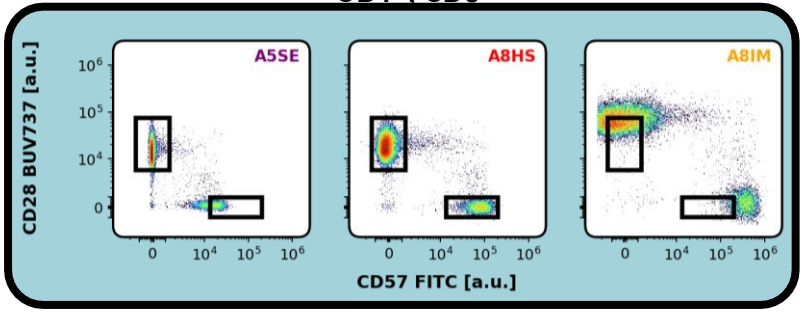
CD3+, CD14–



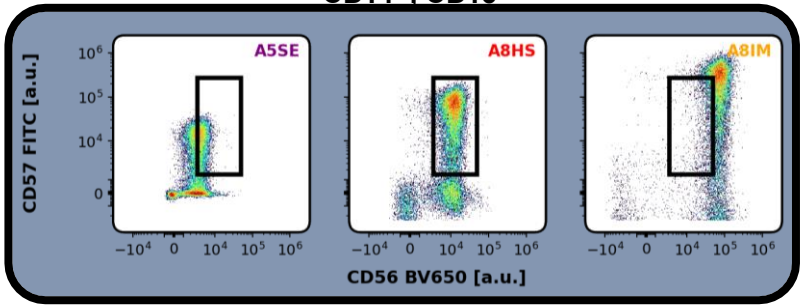
CD3–



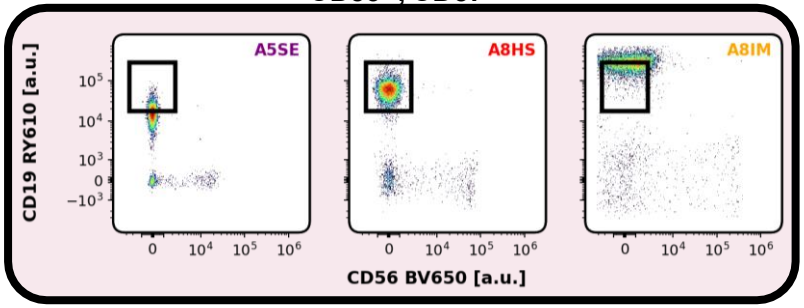
CD4+, CD8–



CD14–, CD16+

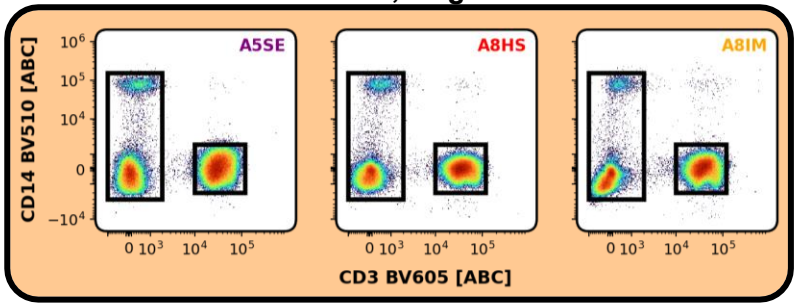


CD56+, CD57–

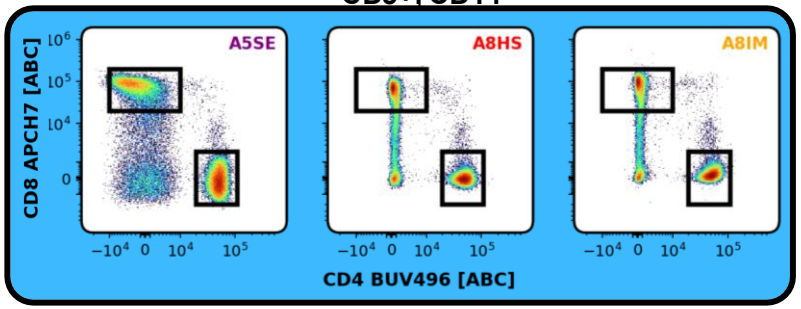


Calibrated (A8HS gating template)

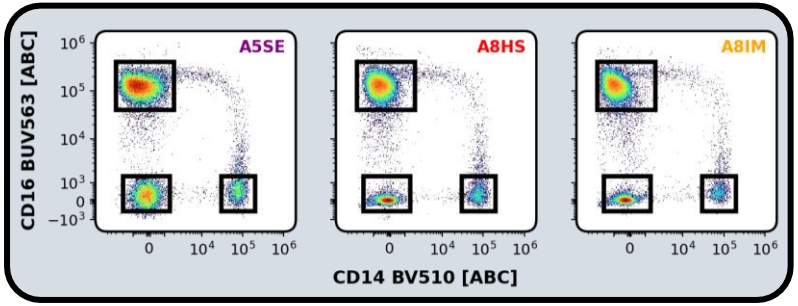
CD45+, singlets



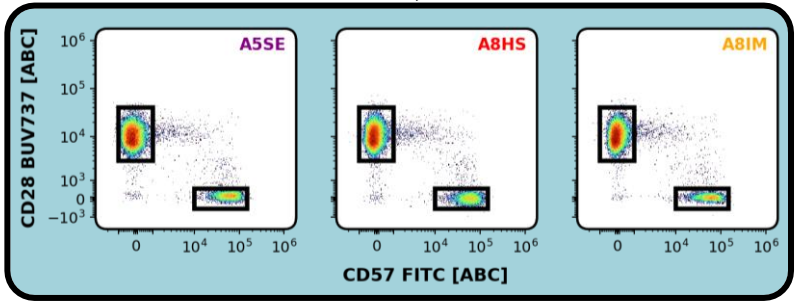
CD3+, CD14–



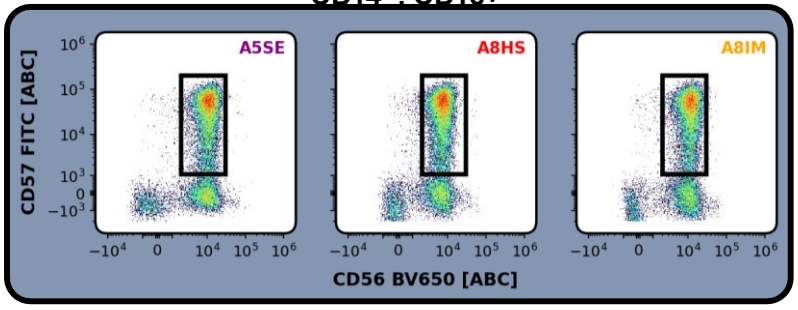
CD3–



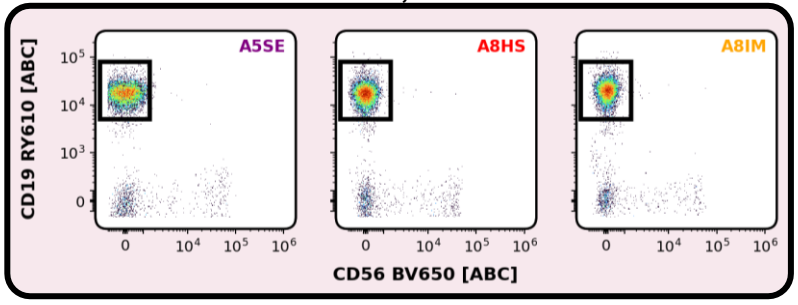
CD4+, CD8–



CD14–, CD16+

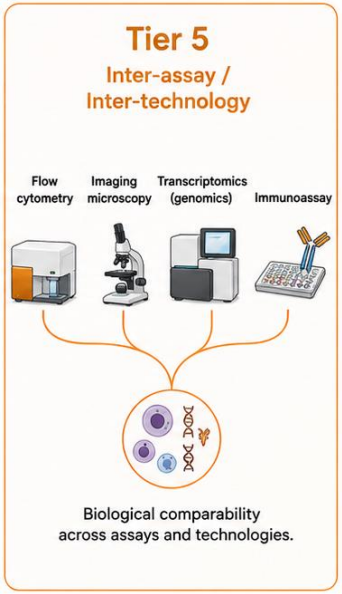


CD56+, CD57–



“The same cell should not tell a different story”

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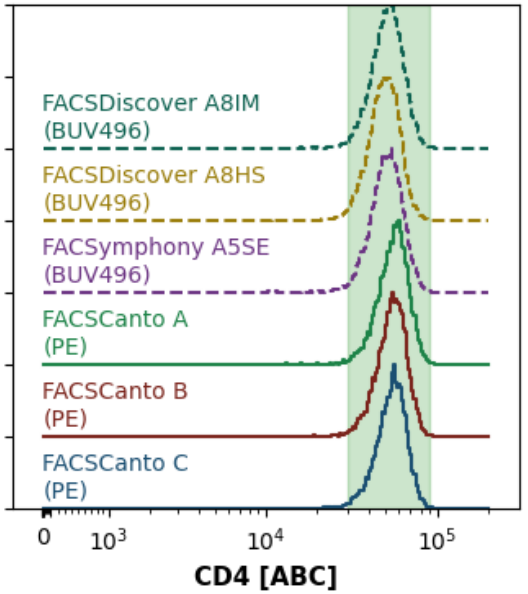


What’s different?

- ❑ **Assays** (10-color vs. 3-color)
- ❑ **CD4 Conjugates** (BUV496 vs. PE)
- ❑ **Platforms & Technologies** (Spectral vs. Conventional)
- ❑ **Days**
- ❑ **Donors**
- ❑ **Cross-calibrations**

What’s the same?

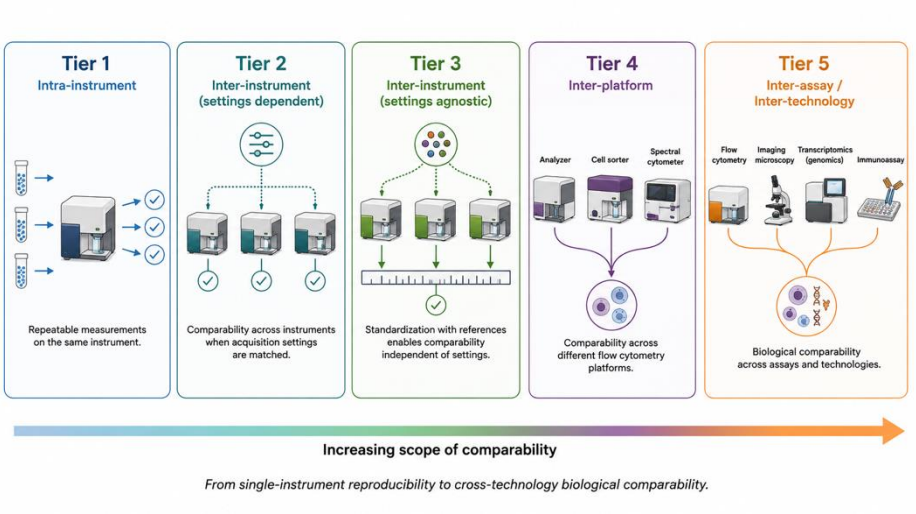
- ❑ **Antibody clone** (anti-CD4 SK3)
- ❑ **Staining** (to saturation)
- ❑ **Sample type/isolation protocol**



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Conclusions

- Fluorescence standardization can be broadly broken into 5 Tiers.
- Relative fluorescence of fluorophores is subjective and platform dependent
- Flow cytometry platforms natively standardize data to Tier 2
- Traceable standards do not exist beyond Tier 3
- Traceable standards catering to Tier 5 are needed



From single-instrument reproducibility to cross-technology biological comparability.

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