ABRF 2026 ANNUAL MEETING

INNOVATING AT THE INTERSECTION OF SCIENCE, TECHNOLOGY, AND COLLABORATION

PITTSBURGH, PA • MARCH 28-31, 2026

UNIVERSITY of MARYLAND
MARLENE AND STEWART GREENEBAUM
COMPREHENSIVE CANCER CENTER

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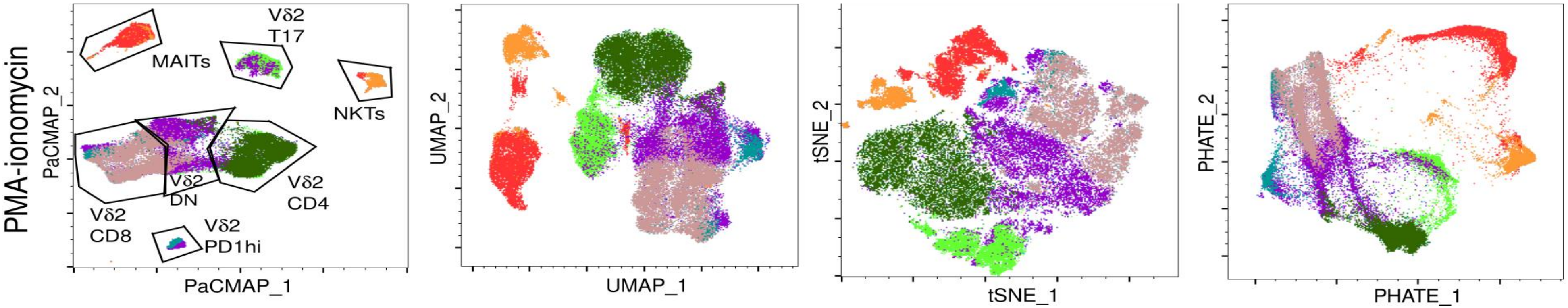
Shared Resource Laboratory



No Cells Left Behind: Wrangling the Full Biological Discovery Potential out of Spectral Flow Cytometry (SFC) datasets

by David Rach
UMGCCC Flow Cytometry Shared Resource

March 30, 2026

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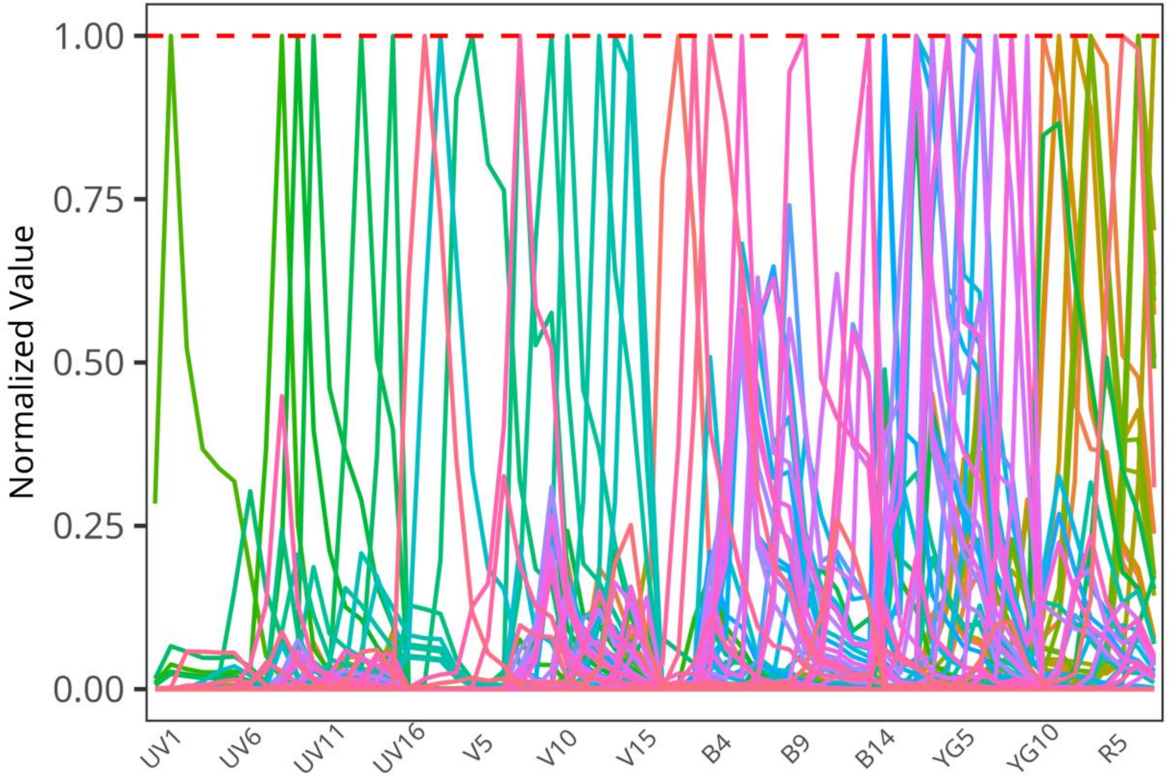
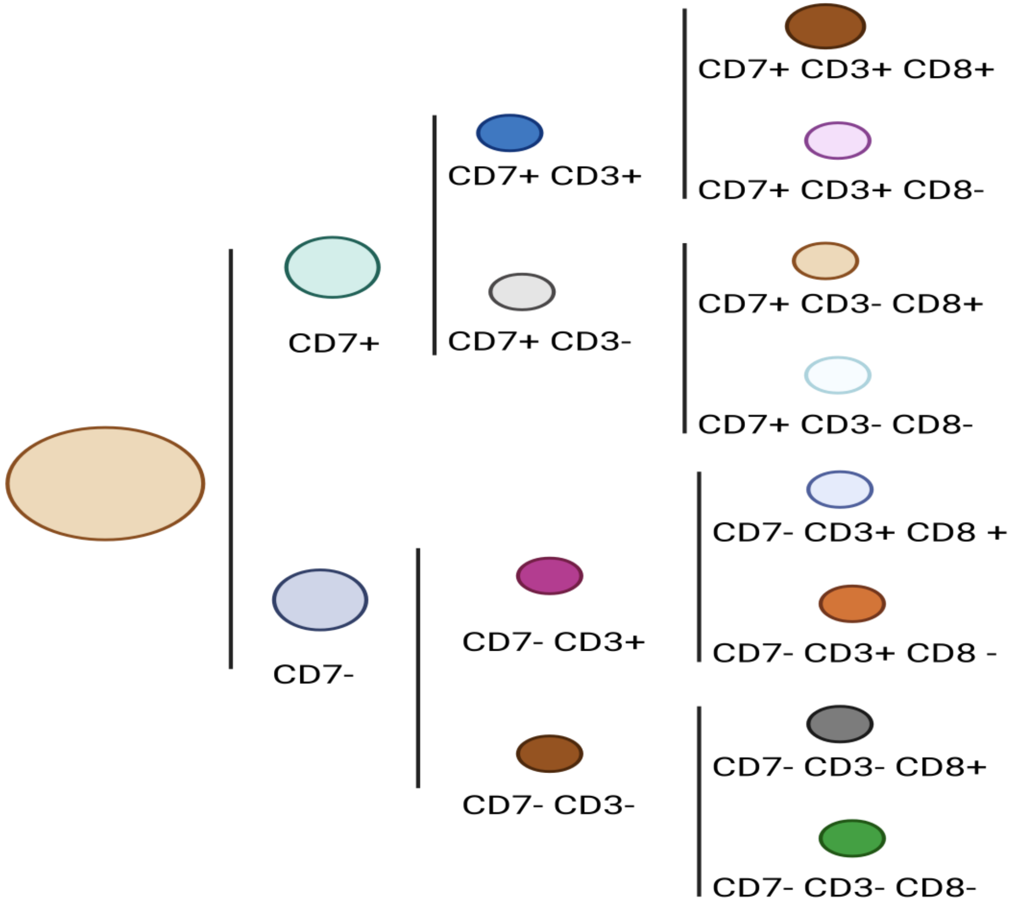
Spectral Flow Cytometry (SFC) is awesome!



- Capacity to resolve spectrally similar fluorophores: greater number markers per panel than Conventional Flow Cytometry (CFC)
- Faster acquisition speed: typically far more cells per .fcs file than Mass Cytometry (MC)
- Analytical breadth and depth permits characterization rare cell populations (and potentially millions of other non-target cells)
- Invaluable for human clinical samples with limited biospecimen

Complex Datasets, Combinatorially Exponential Clusters

- If you are interested in a single population of cells, analysis process remains essentially unchanged.
- If your goal is to characterize all cell populations that may be present, the breadth and depth complicate the analysis.



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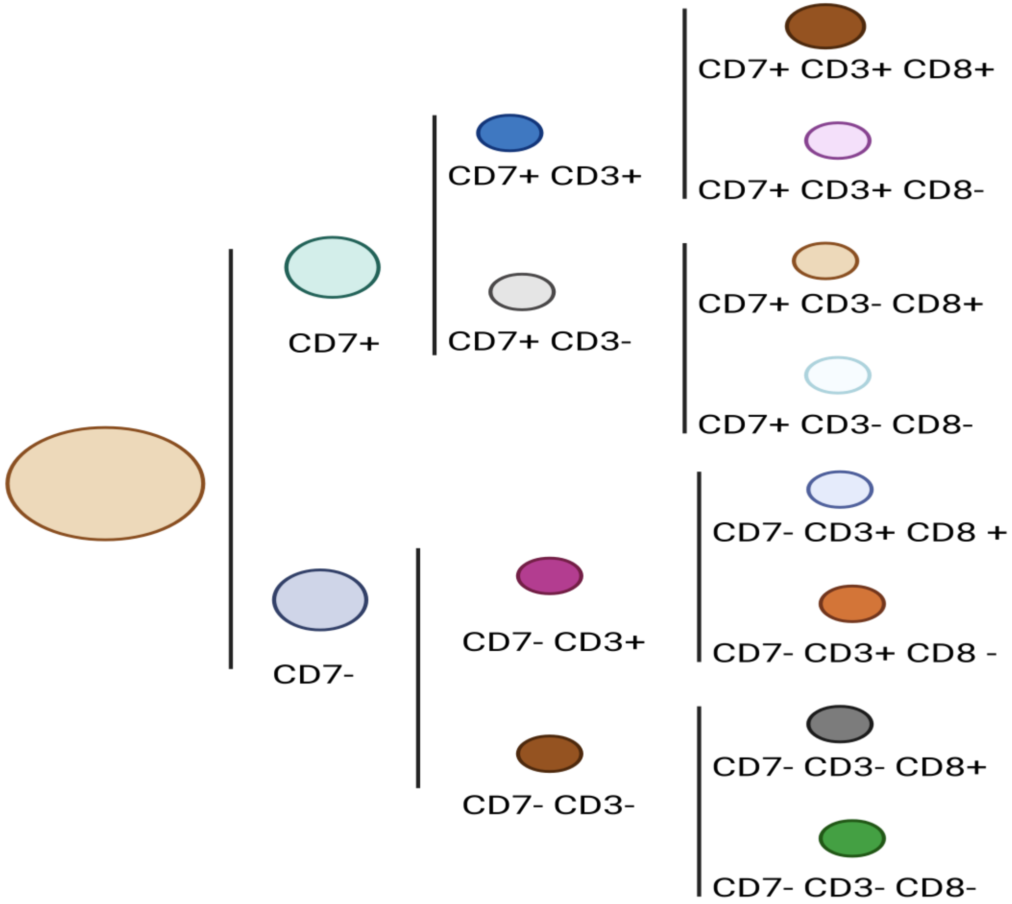
If we step back, and consider for a given marker:

- We split cells positive and negative populations
- Repeat the splitting process for every marker
- Group cells that have shared expression of every single marker
- Theoretical upper number of clusters would be 2^n



CD7+
CD7-

CD7+ CD3+
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CD7+ CD3+ CD8+
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CD7- CD3+ CD8 +
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CD7- CD3- CD8+
CD7- CD3- CD8-

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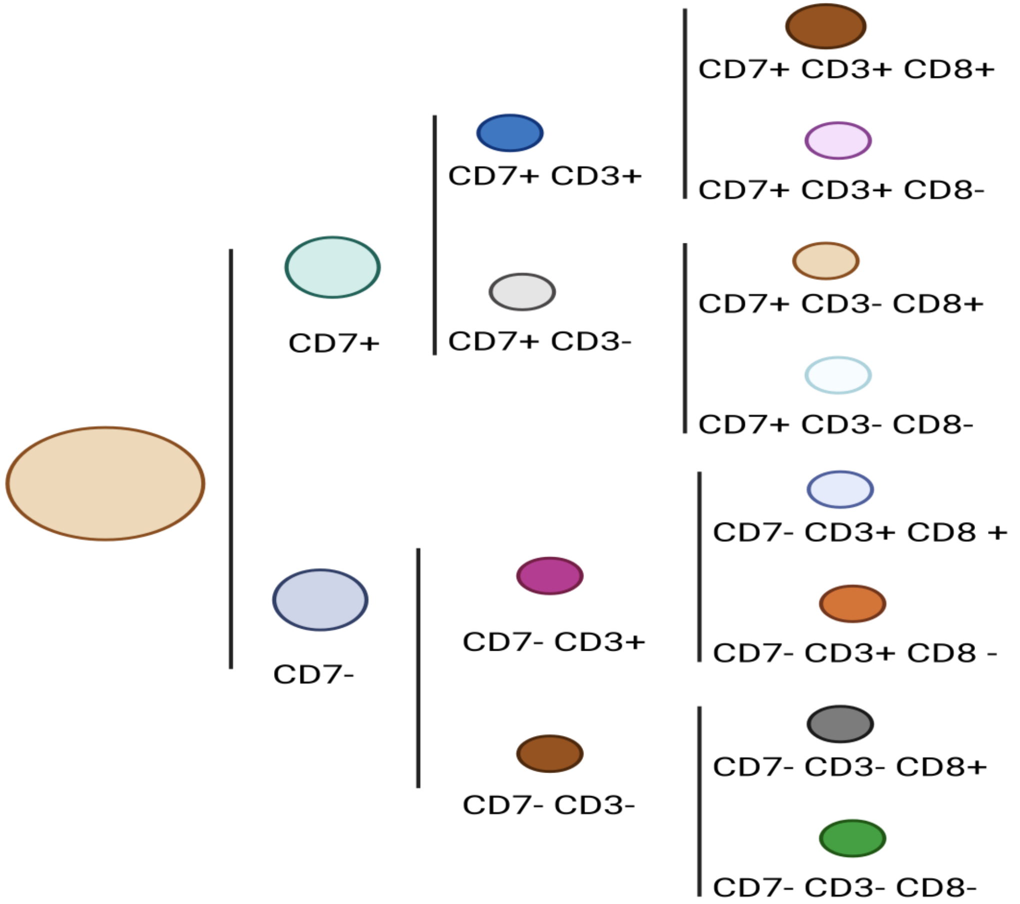
- **9-color** CFC panel: **512** clusters at most
- **40-color** SFC panel: **1.09e+12** clusters at most



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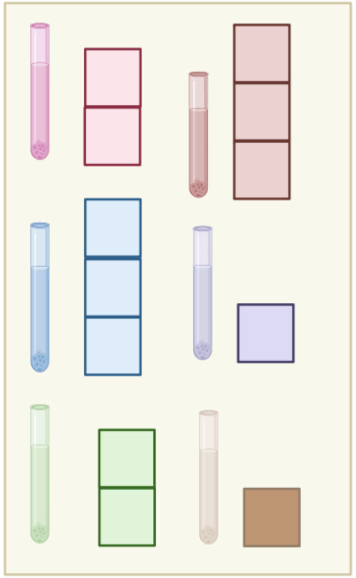
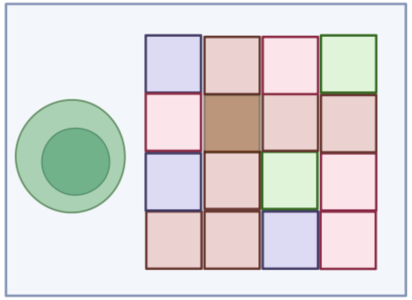
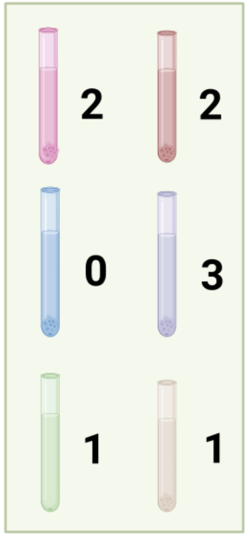
A crossroads deal with the Spectral Signature Devil



Unmixing Controls (Single Color and Unstained)



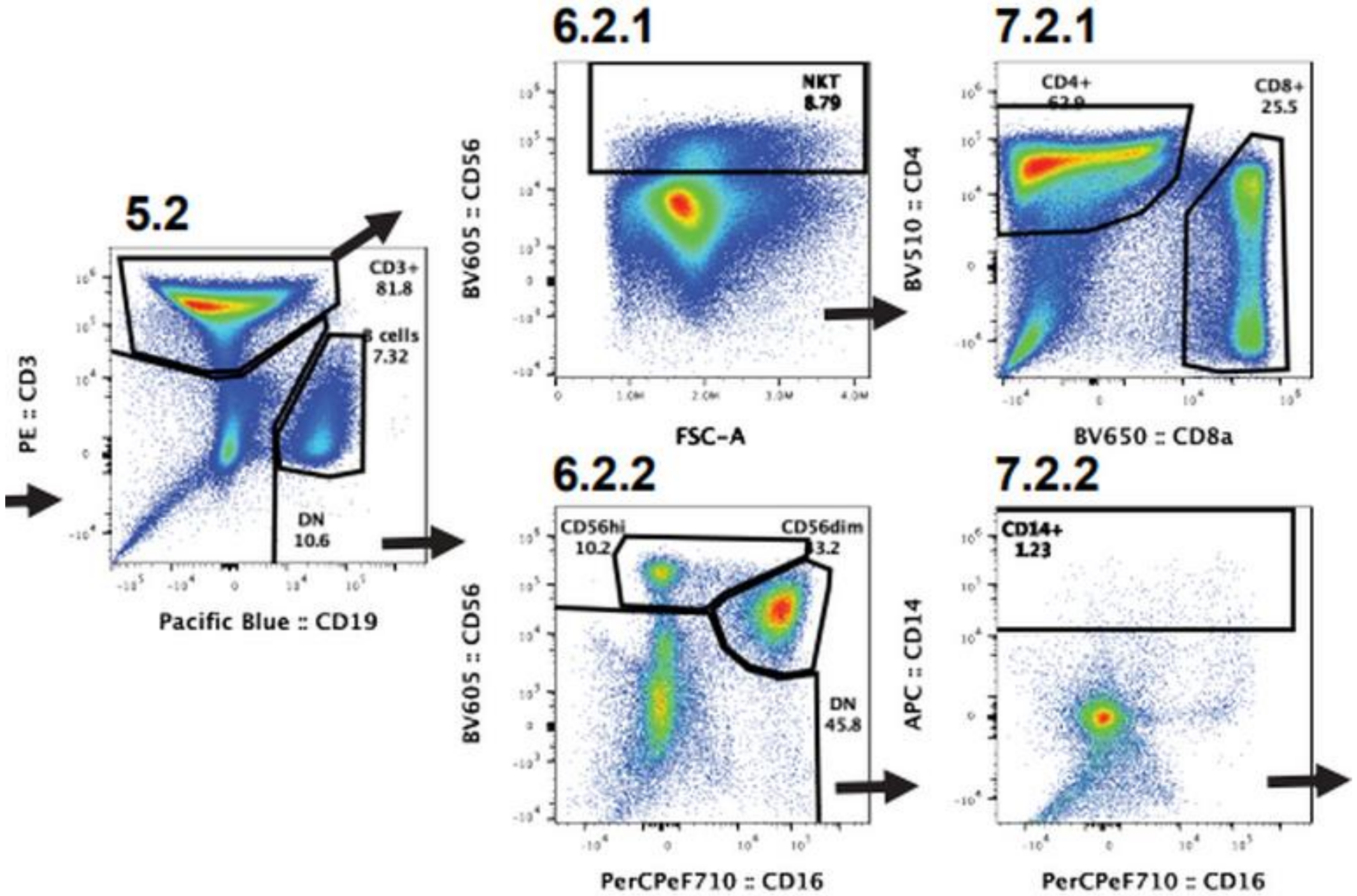
Single cell from full-stained sample



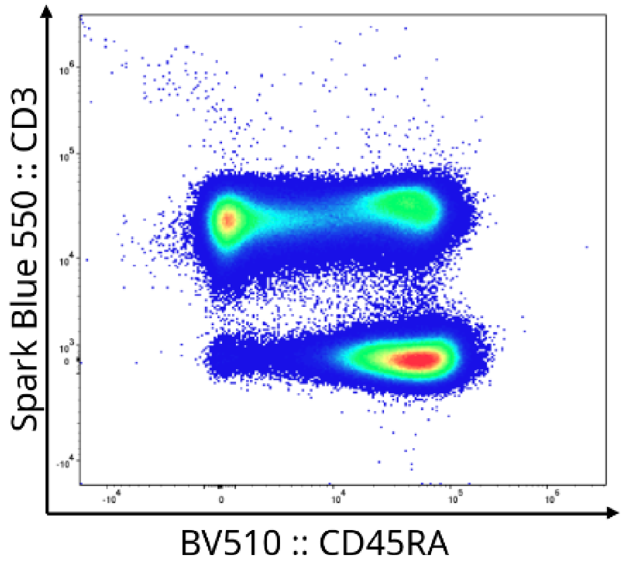
What fluorophores are present, and roughly how much

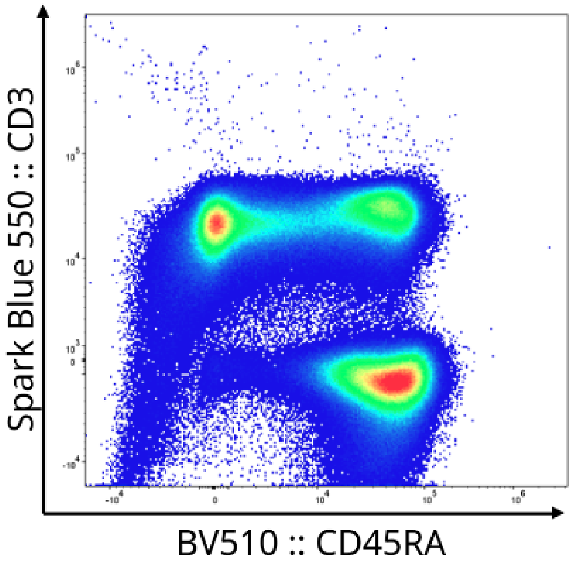
- Consequently, there is a need for semi-supervised and unsupervised algorithmic analytical approaches
- Immense potential for biological discovery, but at the cost of a major trade-off
- Quality of our final data depends on our ability to infer the fluorophores present on individual fully-stained cells
- How closely the fluorescent signatures of our unmixing controls (single-color and unstained) match the spectral signatures as present on the full-stained cells is critical to this final data quality.

“We discovered this new dendritic cell cluster….”



More unmixing errors, more downstream batch effects





- Despite the critical importance unmixing controls play, few tools permit us to determine quality of the individual control before unmixing occurs
- Existing quality control algorithms (PeacoQC, FlowAI, etc.) primarily focus on detecting laser and fluidic issues in already acquired .fcs files
- Unmixing errors can be introduced by tandem degradation, additional autofluorescences, undiagnosed instrumental issues, etc.
- Unmixing errors contribute to batch effects, and require complicated troubleshooting to be able to proceed with the downstream analysis

Open-source tool sets specifically for Spectral Flow Cytometry's quirks

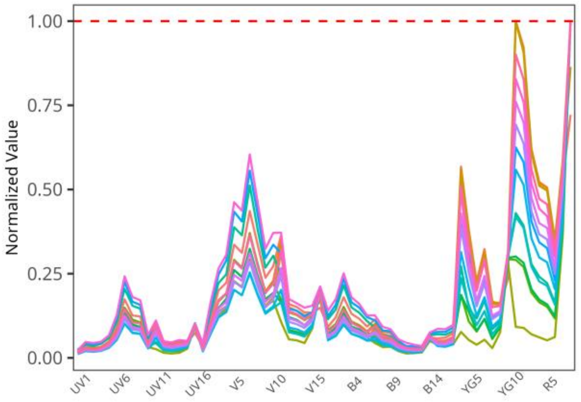
Spectral Signature Characterization and Isolation

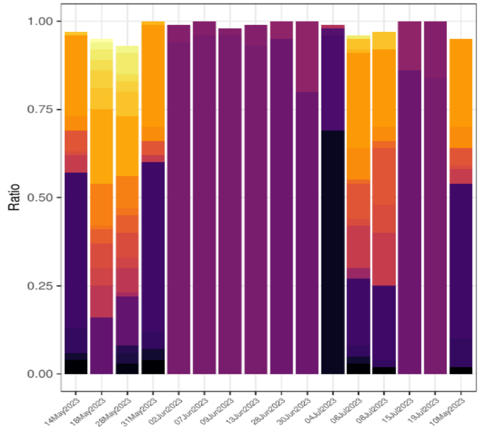
Automated Instrument Quality-Control Dashboards

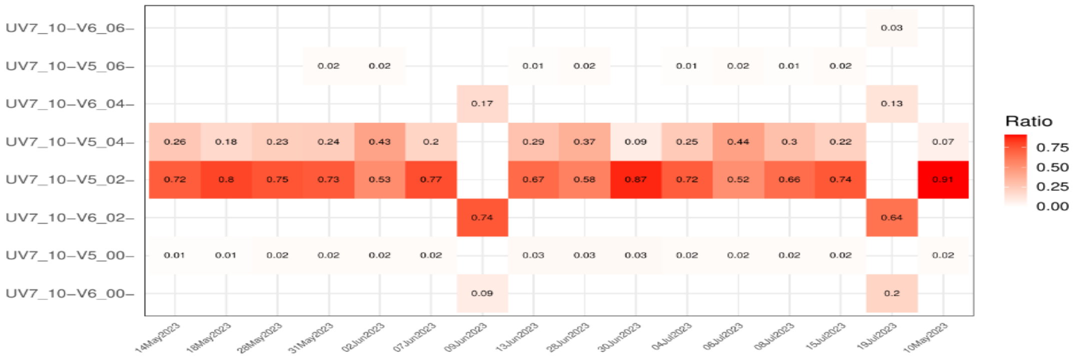
Semi-supervised analysis via incorporation of manually-annotated gating information

Spectral Signature Characterization and Clustering

- For individual cells, it characterizes the cell's normalized fluorescent signature
- Cells that share normalized spectral signature peaks at individual detectors are then clustered
- **Fluorophore vials in good condition**: we retrieve few normalized signatures from the raw .fcs file for each single-color unmixing control
- When the **tandem fluorophore decouples or degrades**, noticeable increase in variant signatures, flag the issue
- Setting up automation to retrieve and process reference controls for individual users experiments during off-hours







	14May2023	18May2023	28May2023	31May2023	02Jun2023	07Jun2023	09Jun2023	13Jun2023	28Jun2023	30Jun2023	04Jul2023	06Jul2023	08Jul2023	15Jul2023	19Jul2023	10May2023
UV7_10–V6_06															0.03	
UV7_10–V5_06				0.02	0.02			0.01	0.02		0.01	0.02	0.01	0.02		
UV7_10–V6_04							0.17								0.13	
UV7_10–V5_04	0.26	0.18	0.23	0.24	0.43	0.2		0.29	0.37	0.09	0.25	0.44	0.3	0.22		0.07
UV7_10–V5_02	0.72	0.8	0.75	0.73	0.53	0.77		0.67	0.58	0.87	0.72	0.52	0.66	0.74		0.91
UV7_10–V6_02							0.74								0.64	
UV7_10–V5_00	0.01	0.01	0.02	0.02	0.02	0.02		0.03	0.03	0.03	0.02	0.02	0.02	0.02		0.02
UV7_10–V6_00							0.09								0.2	

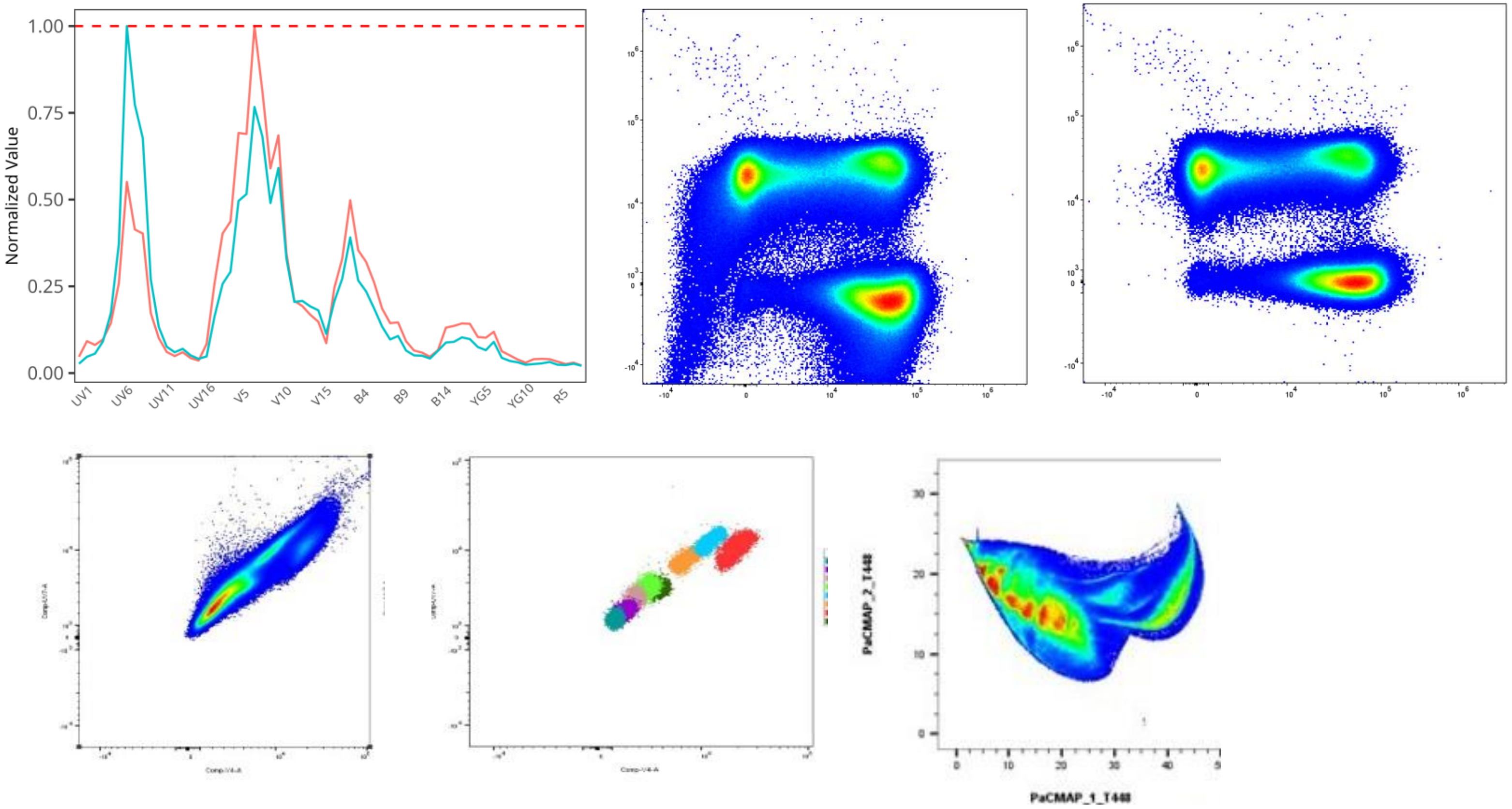
Luciérnaga

Automated isolation of autofluorescence signatures

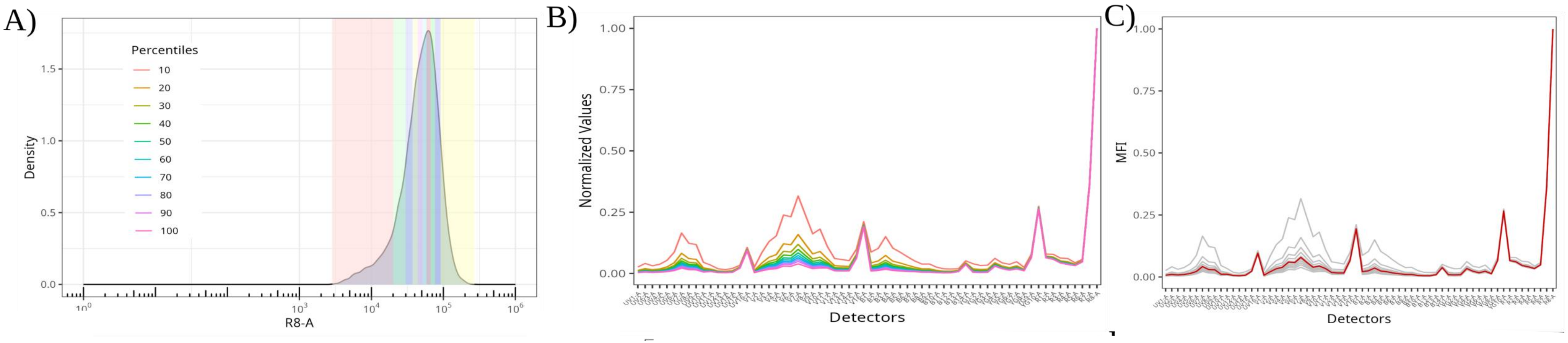
- Adapt this appproach to isolate out
- normalized autofluorescence signatures
- from unstained controls

- Create .fcs files with purified autofloresence signatures, to use for unmixing with commercial software.

- Isolated cells share same normalized signature, only differing in brightness, unlike the current Autoflorescence Explorer or gating a dimensionality-visualized island style approaches to autofluorescence isolation

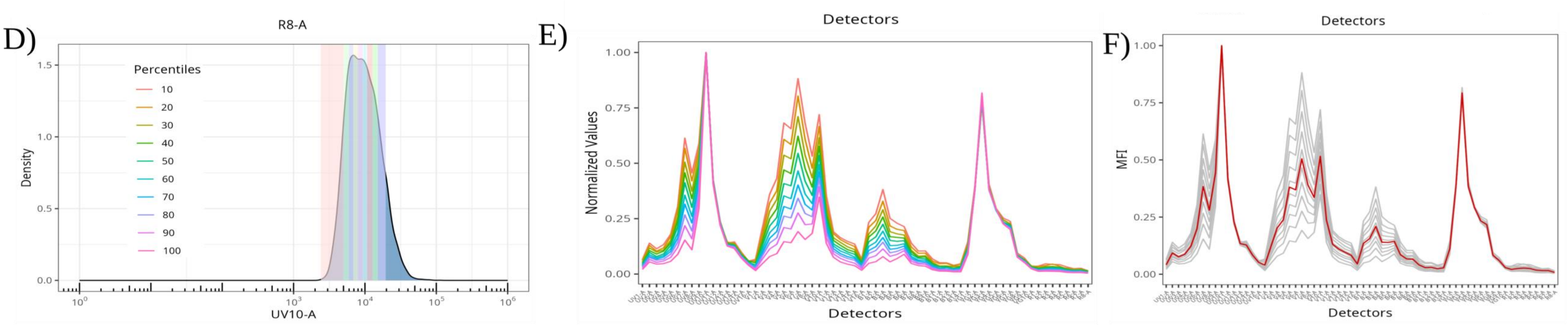


Residual Autofluorescence peaks in Single-Color Signatures



For cell controls with a **moderate density antigen** on **bright fluorophores**, we see that any residual autofluorescence contributes minimally to the overall isolated single-color signature when gated according to best practices

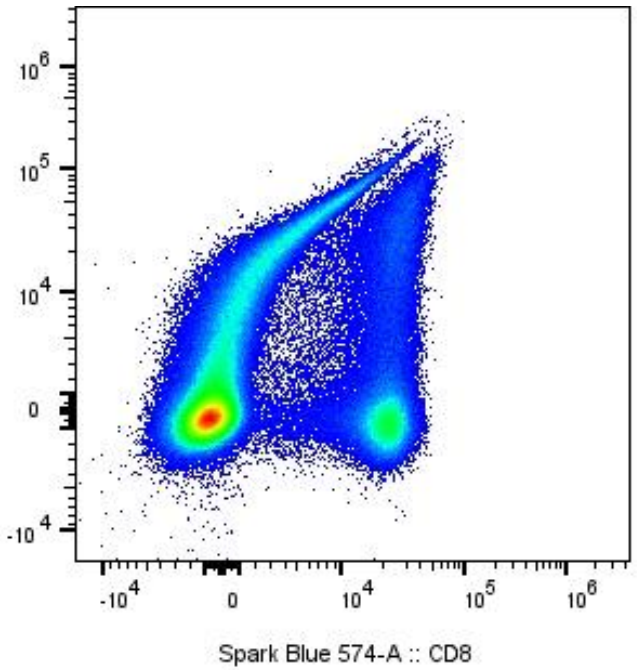
Residual Autofluorescence peaks in Single-Color Signatures



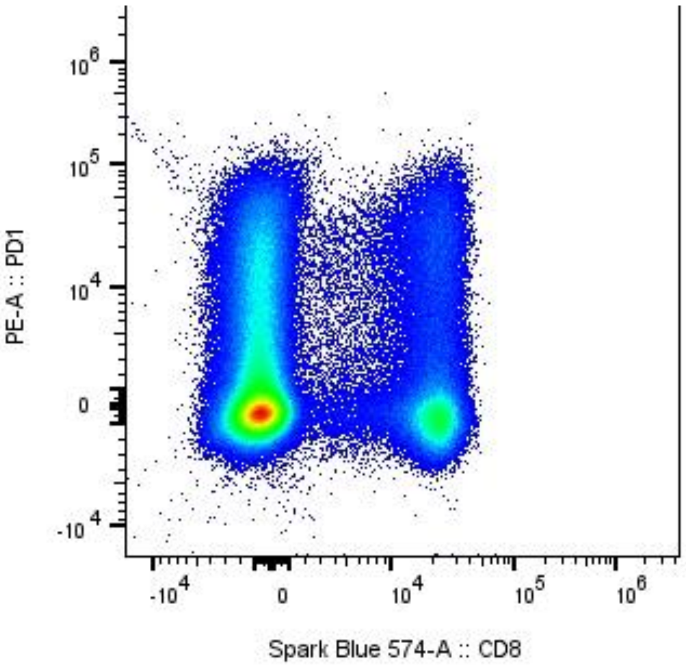
For cell controls with a **moderate density antigen** on **dim fluorophores,** we see substantial residual autofluorescence contributions to the isolated single-color normalized signatures even when gated according to best practices

Residual Autofluorescence peaks in Single-Color Signatures

PE signature with residual AF



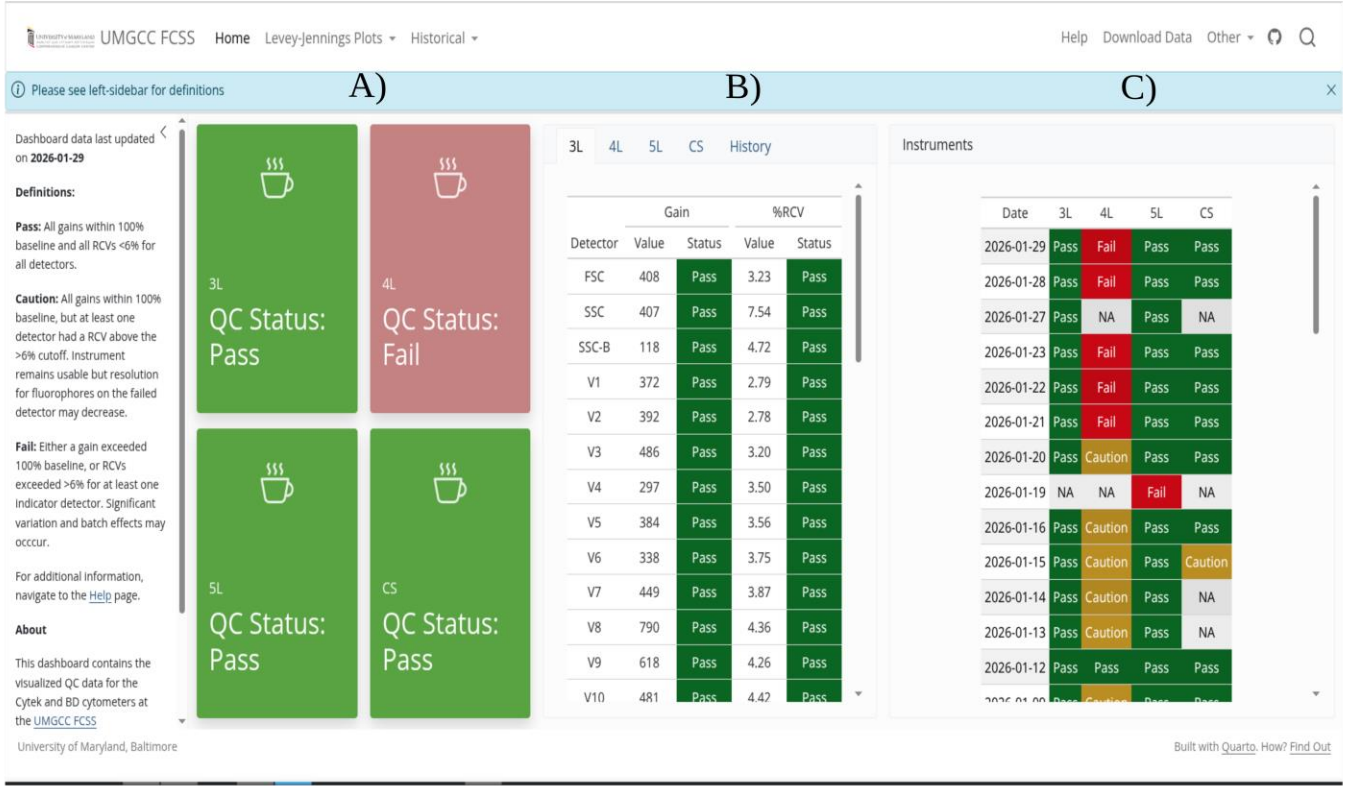
PE signature without residual AF



This residual autofluorescence contribution results in unmixing errors, and the panel unmixing significantly improved when switched out for bead controls

Instrument Quality-Control Dashboards

- Prior work assumes that the spectral flow cytometers are stably maintaining their quality control (QC) targets
- At our core: Daily acquire 5000 QC beads as .fcs files both before and after morning QC
- Automatically processed for MFI, Gain, and %rCV and displayed as Levey-Jennings plots
- Data for all instruments is deployed as interactive plots on a Quarto website hosted on GitHub



Please see left-sidebar for definitions

Dashboard data last updated on **2026-01-29**

Definitions:

Pass: All gains within 100% baseline and all RCVs <6% for all detectors.

Caution: All gains within 100% baseline, but at least one detector had a RCV above the >6% cutoff. Instrument remains usable but resolution for fluorophores on the failed detector may decrease.

Fail: Either a gain exceeded 100% baseline, or RCVs exceeded >6% for at least one indicator detector. Significant variation and batch effects may occcur.

For additional information, navigate to the Help page.

About

This dashboard contains the visualized QC data for the Cytek and BD cytometers at the UMGCC FCSS

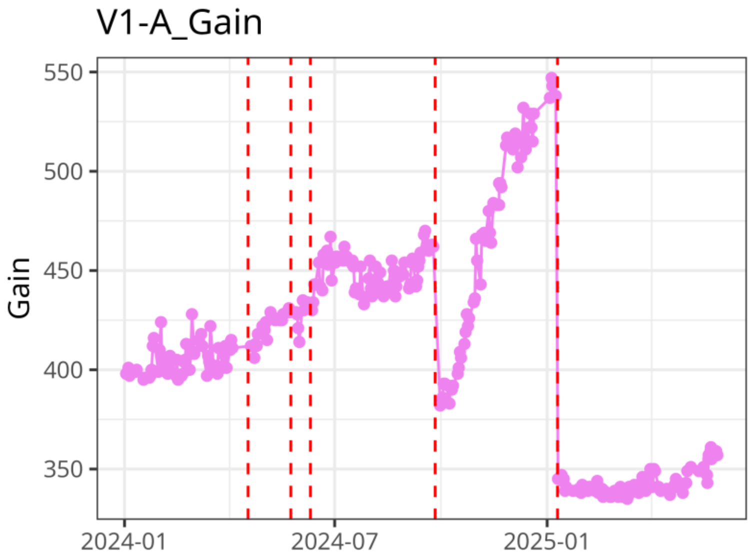
University of Maryland, Baltimore

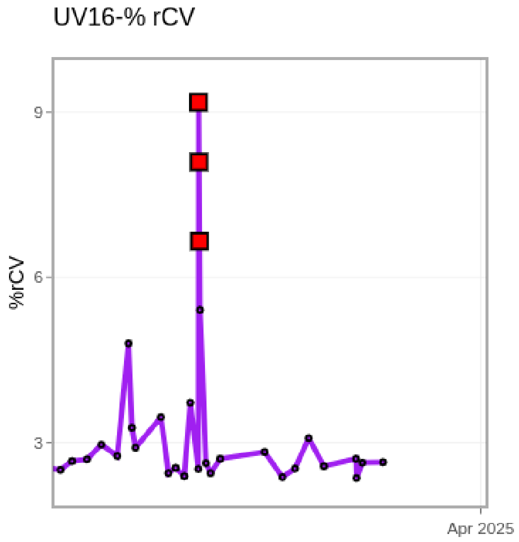
	Gain		%RCV	
Detector	Value	Status	Value	Status
FSC	408	Pass	3.23	Pass
SSC	407	Pass	7.54	Pass
SSC-B	118	Pass	4.72	Pass
V1	372	Pass	2.79	Pass
V2	392	Pass	2.78	Pass
V3	486	Pass	3.20	Pass
V4	297	Pass	3.50	Pass
V5	384	Pass	3.56	Pass
V6	338	Pass	3.75	Pass
V7	449	Pass	3.87	Pass
V8	790	Pass	4.36	Pass
V9	618	Pass	4.26	Pass
V10	481	Pass	4.42	Pass

Date	3L	4L	5L	CS
2026-01-29	Pass	Fail	Pass	Pass
2026-01-28	Pass	Fail	Pass	Pass
2026-01-27	Pass	NA	Pass	NA
2026-01-23	Pass	Fail	Pass	Pass
2026-01-22	Pass	Fail	Pass	Pass
2026-01-21	Pass	Fail	Pass	Pass
2026-01-20	Pass	Caution	Pass	Pass
2026-01-19	NA	NA	Fail	NA
2026-01-16	Pass	Caution	Pass	Pass
2026-01-15	Pass	Caution	Pass	Caution
2026-01-14	Pass	Caution	Pass	NA
2026-01-13	Pass	Caution	Pass	NA
2026-01-12	Pass	Pass	Pass	Pass

Built with Quarto. How? Find Out

Early detection of Instrumental Issues





- Permitted identification of laser issues a month before standard QC would have failed, allowing earlier scheduling of preventive maintenance visits
- By spotting the spike in %rCV values for last detector of each laser, we were able to link unmixing errors we were seeing to the Lot 2006 QC beads being bleach-sensitive

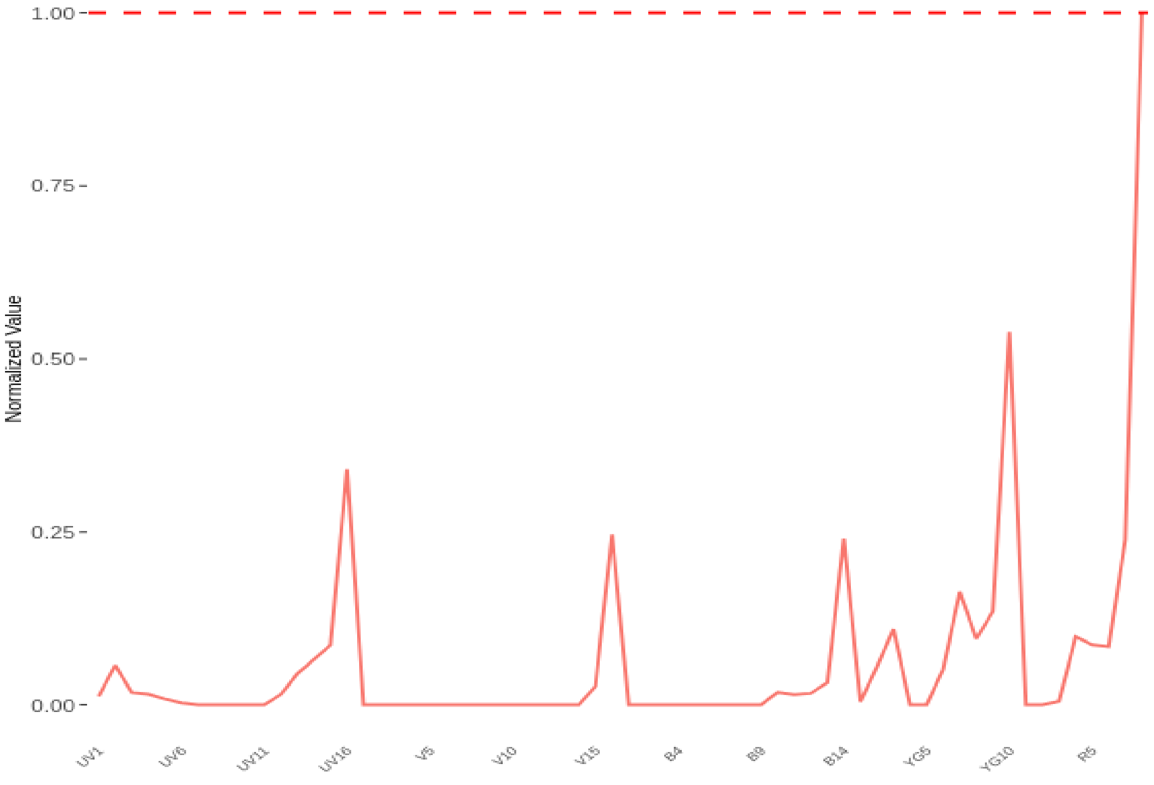
When automated QC goes awry

- Bleach stripped APC-Fire 810 like fluorophore from the
- QC beads

- MFI for the detectors where fluorophore signal are
- below the target value, gain is increased by automated QC

- Major unmixing issues result, because the instrument
- settings didn't need the gain increase

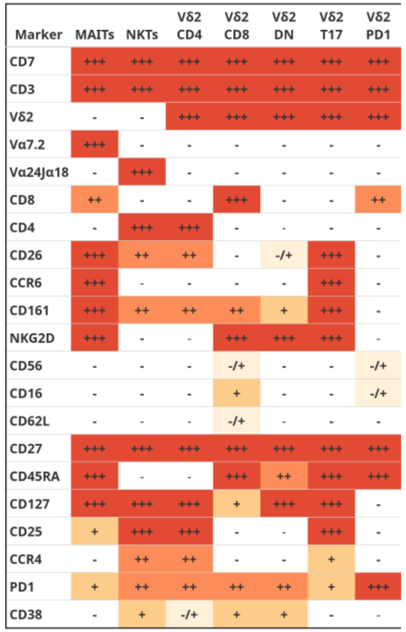
- Retrospective analysis suggest issue has been present
- for other bead lots, but was never flagged.



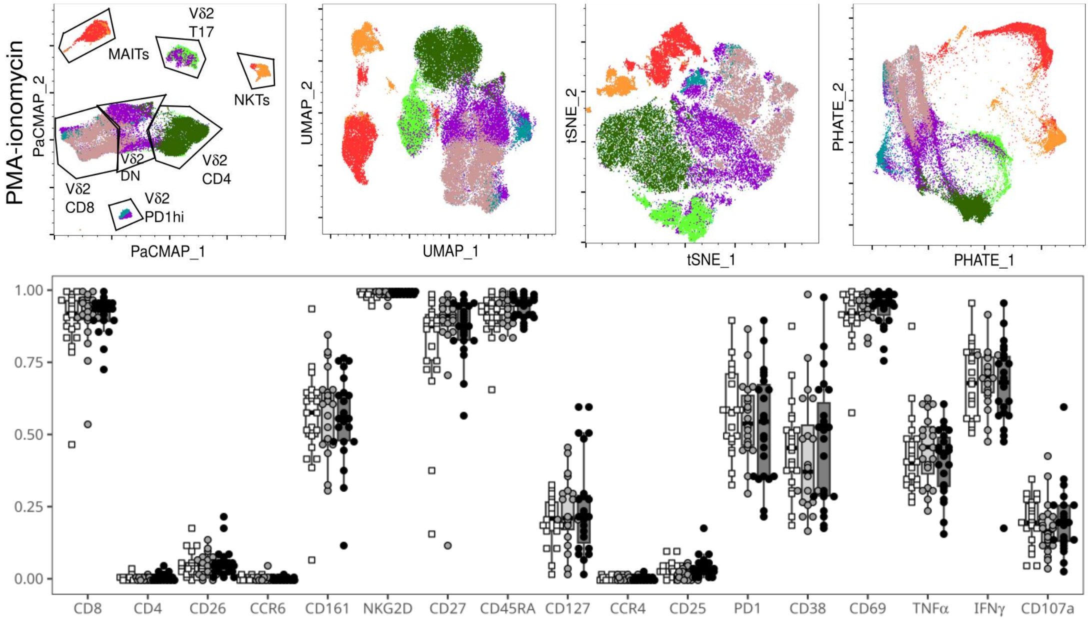
Coereba

Semi-supervised analysis by annotating in manual gates

- Comprehensive automated manual-gating information is appended to individual cells in an .fcs file
- Can be passed to unsupervised algorithms for quality control, normalization, clustering, dimensionality visualization
- Can retrieve underlying supervised gating information afterwards



Marker	MAITs	NKTs	Vδ2 CD4	Vδ2 CD8	Vδ2 DN	Vδ2 T17	Vδ2 PD1
CD7	+++	+++	+++	+++	+++	+++	+++
CD3	+++	+++	+++	+++	+++	+++	+++
Vδ2	-	-	+++	+++	+++	+++	+++
Vα7.2	+++	-	-	-	-	-	-
Vα24Jα18	-	+++	-	-	-	-	-
CD8	++	-	-	+++	-	-	++
CD4	-	+++	+++	-	-	-	-
CD26	+++	++	++	-	-/+	+++	-
CCR6	+++	-	-	-	-	+++	-
CD161	+++	++	++	++	+	+++	-
NKG2D	+++	-	-	+++	+++	+++	-
CD56	-	-	-	-/+	-	-	-/+
CD16	-	-	-	+	-	-	-/+
CD62L	-	-	-	-/+	-	-	-
CD27	+++	+++	+++	+++	+++	+++	+++
CD45RA	+++	-	-	+++	++	+++	+++
CD127	+++	+++	+++	+	+++	+++	-
CD25	+	+++	+++	-	-	+++	-
CCR4	-	++	++	-	-	+	-
PD1	+	++	++	++	++	+	+++
CD38	-	+	-/+	+	+	-	-



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- All packages are available via our GitHub
- Fixing bugs and updating documentation
- Submitting Bioconductor this year

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OPEN SOURCE SOFTWARE FOR BIOINFORMATICS

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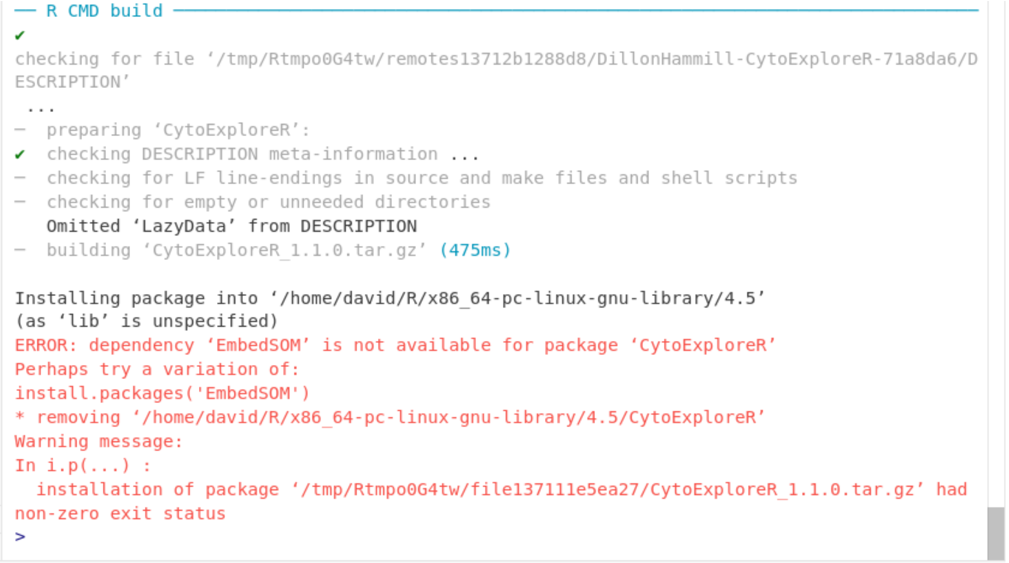

```
── R CMD build ─────────────────────────────────────────────────────────────────
✔
checking for file ‘/tmp/Rtmpo0G4tw/remotes13712b1288d8/DillonHammill-CytoExploreR-71a8da6/D
ESCRIPTION’
 ...
─  preparing ‘CytoExploreR’:
✔  checking DESCRIPTION meta-information ...
─  checking for LF line-endings in source and make files and shell scripts
─  checking for empty or unneeded directories
   Omitted ‘LazyData’ from DESCRIPTION
─  building ‘CytoExploreR_1.1.0.tar.gz’ (475ms)

Installing package into ‘/home/david/R/x86_64-pc-linux-gnu-library/4.5’
(as ‘lib’ is unspecified)
ERROR: dependency ‘EmbedSOM’ is not available for package ‘CytoExploreR’
Perhaps try a variation of:
install.packages('EmbedSOM')
* removing ‘/home/david/R/x86_64-pc-linux-gnu-library/4.5/CytoExploreR’
Warning message:
In i.p(...) :
  installation of package ‘/tmp/Rtmpo0G4tw/file137111e5ea27/CytoExploreR_1.1.0.tar.gz’ had
non-zero exit status
>
```

- What good is a tool that the community can't use?

CYTOMETRY IN R

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Flow Cytometry
Shared Resource

ISAC RECOGNIZED
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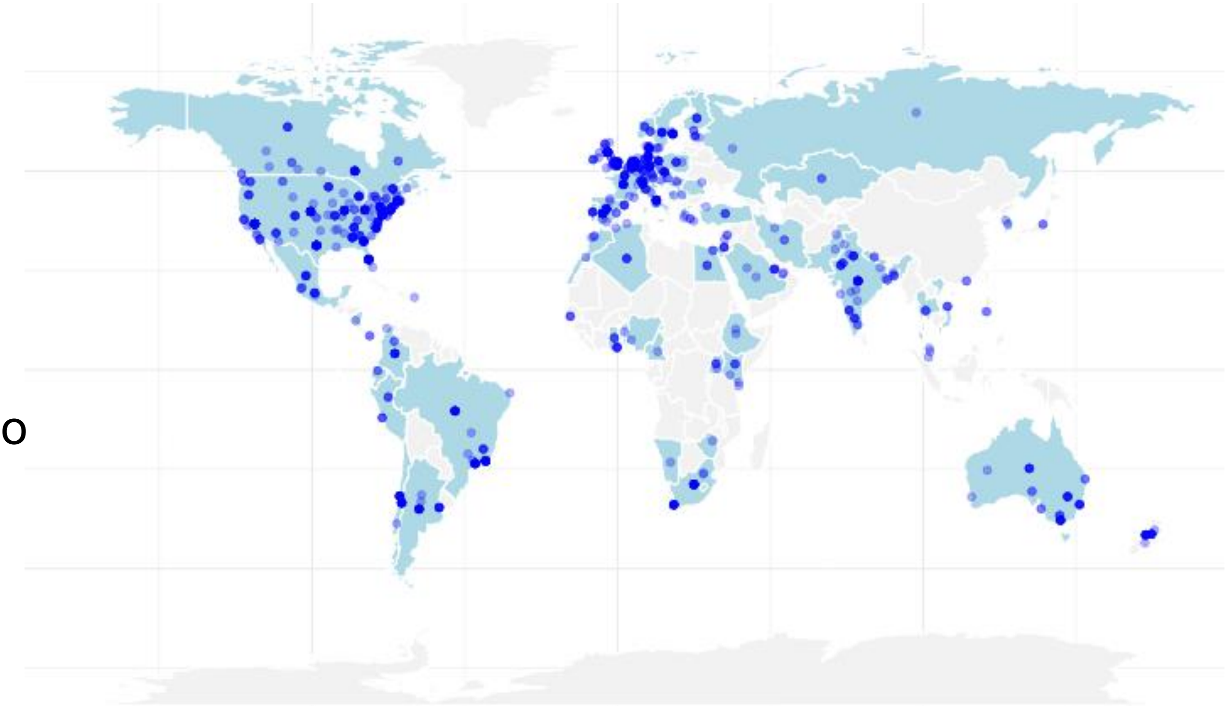
CYTOMETRY IN R

A COURSE FOR BEGINNERS

- Free weekly Cytometry in R course, offered both in-person and online via YouTube livestreams.
- Aimed at those with prior cytometry experience, but no-to-little coding experience
- Started in February, will be exploring from beginner to advanced topics for at least 30 weeks
- Around 2000 interest forms completed, between 300-500 weekly participants



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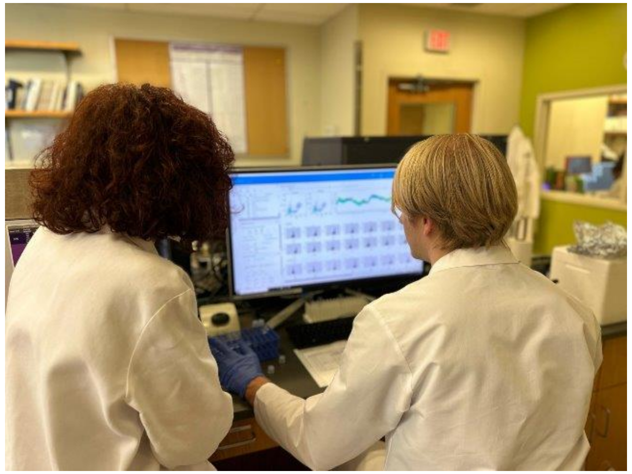


- **Molecular Microbiology Immunology Graduate Program**
- Dr. Eileen Barry
- Bess Tracey

Cytometry Discord

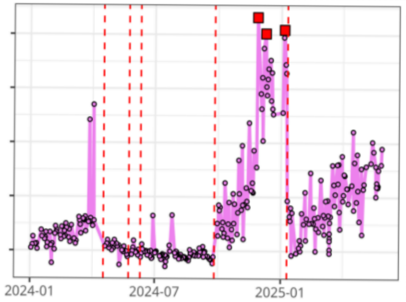
r/flowcytometry

Bioconductor Zulip

https://github.com/DavidRach

@davidrach.bsky.social

Questions?



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InstrumentQC dashboard

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Website - CytometryInR

InstrumentQC How-To

Coereba

YouTube **@CytometryInR**