UNIVERSITY of MARYLAND
SCHOOL OF MEDICINE

KAMUZU UNIVERSITY
OF HEALTH SCIENCES

A semi-supervised pipeline for a comprehensive and scalable analysis of immune heterogeneity in human samples

David Rach, Nginache Nampota-Nkomba, Godfrey Mvula, Felix A. Mkandawire, Osward M. Nyirenda, Winter A. Okoth, Daniela Franco, Andrea G. Buchwald, Franklin R. Toapanta, Marcelo B. Sztein, Miriam K. Laufer, **Kirsten E. Lyke**, **Cristiana Cairo**.

University of Maryland School of Medicine

June 9, 2026

Slides

Spectral Flow Cytometry is amazing!!!

- ❑ Increasing number of fluorophores
- ❑ We can acquire millions of cells in a few minutes
- ❖ Characterization of rare subsets
- ❖ Expand our knowledge about human immune system heterogeneity
- ❖ Invaluable for studies with limited biospecimen

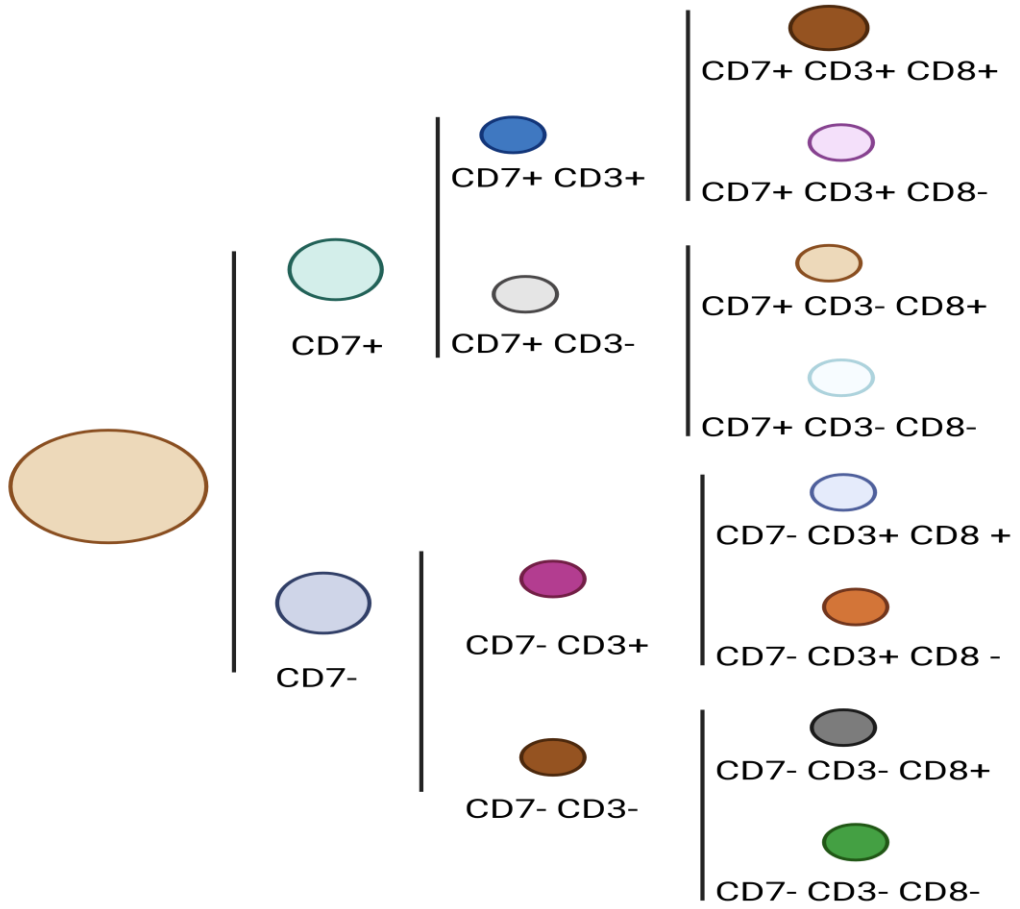
Increasing analytical complexity

- If you are interested only in a **single population** of cells, the 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.

Complex Datasets, Combinatorially Exponential Clusters

- If every marker in your panel split into **positive** and **negative** populations
- And you repeated this splitting process for **every marker**
- And **grouped cells** that shared the **same expressed markers**
- $2^{\text{NumberOfMarkers}}$



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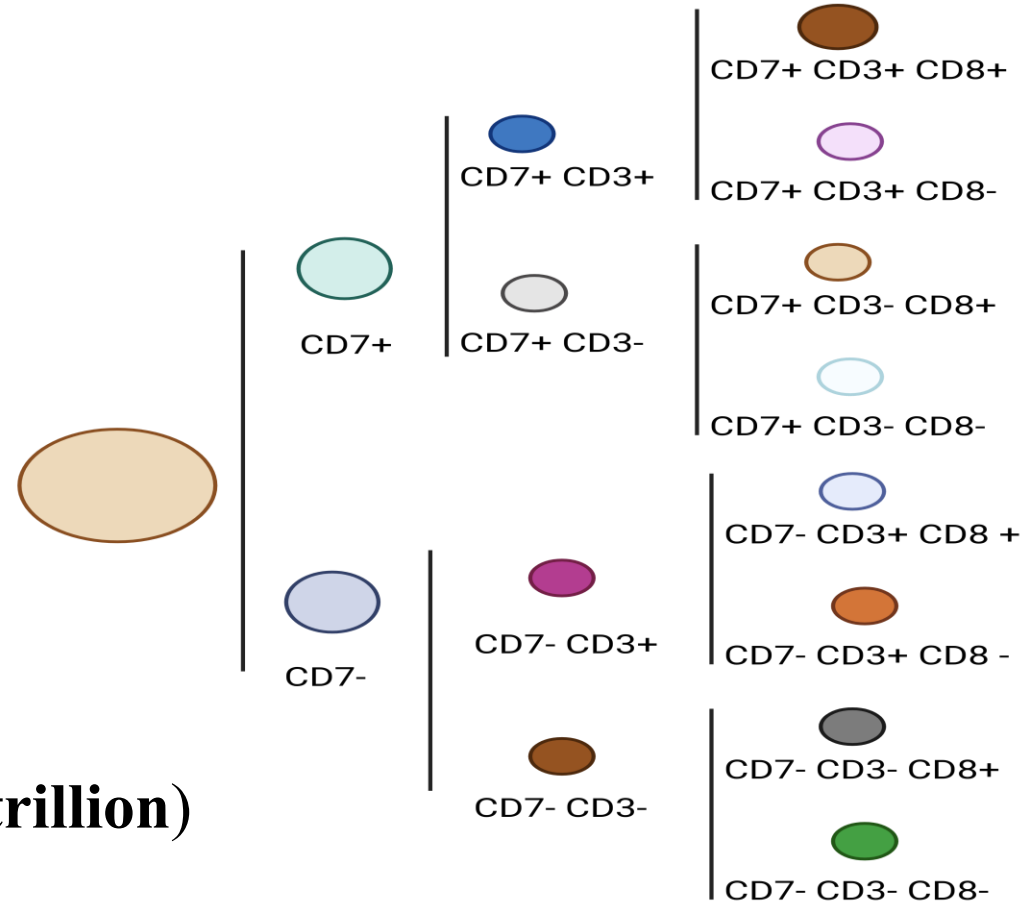
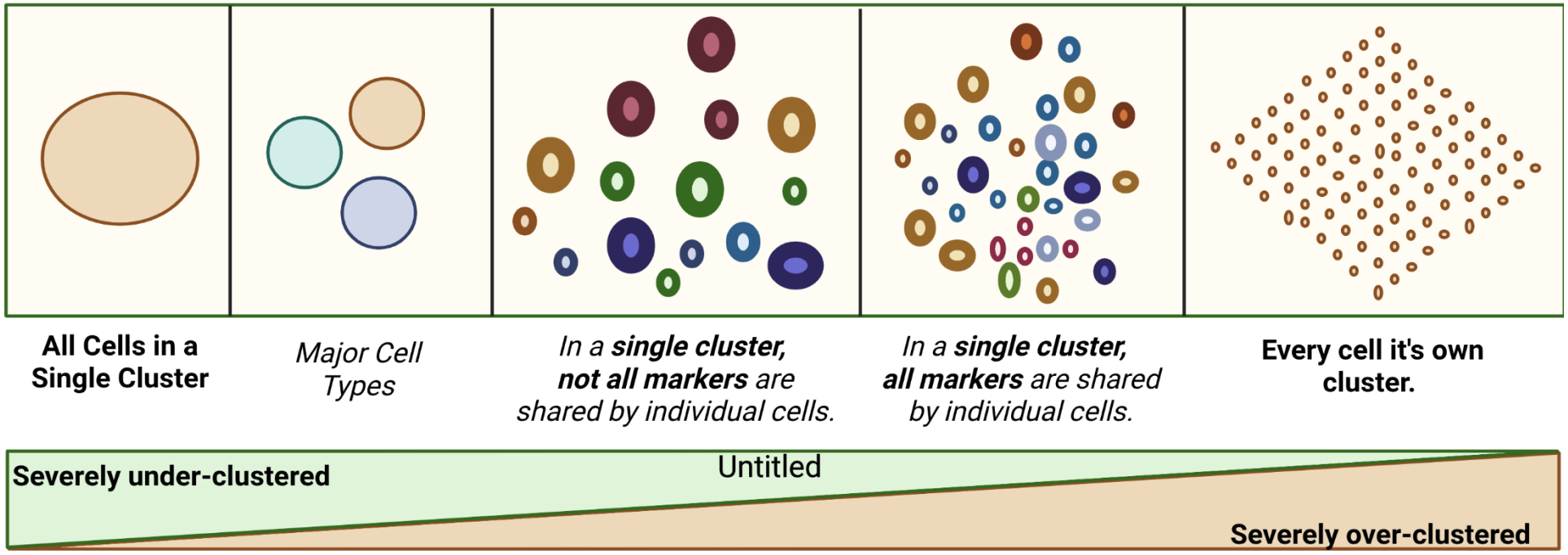
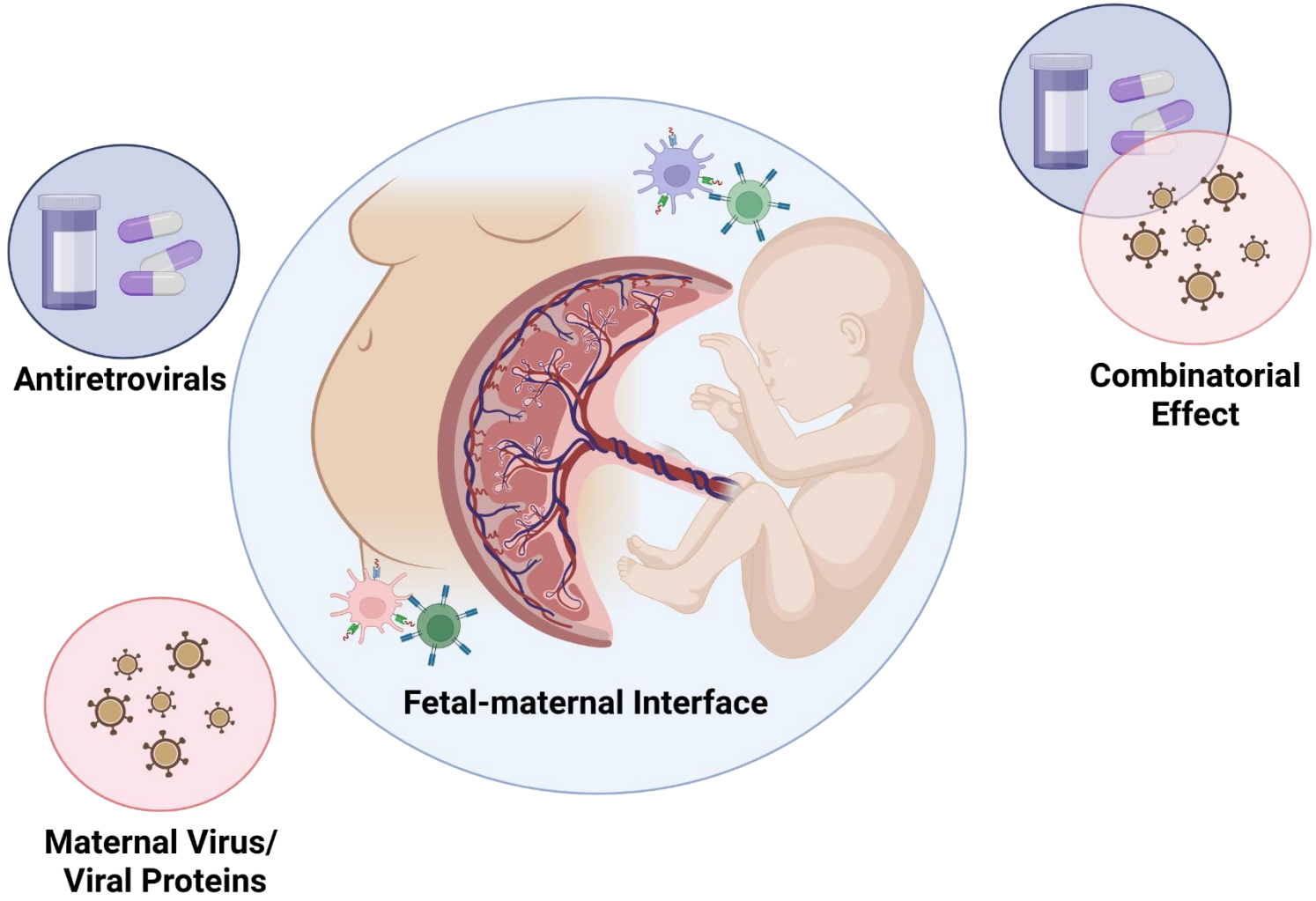


- **9-color** CFC panel: **512** groups at most
- **40-color** SFC panel: **1.1e+12** clusters at most (**trillion**)
- **60-color** SFC panel: **1.2e+18** clusters at most (**quintillion**)

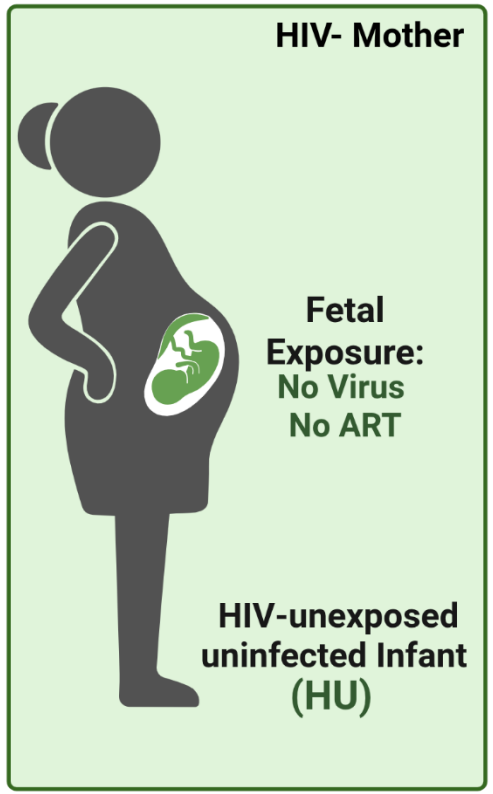
The challenge for unsupervised approaches

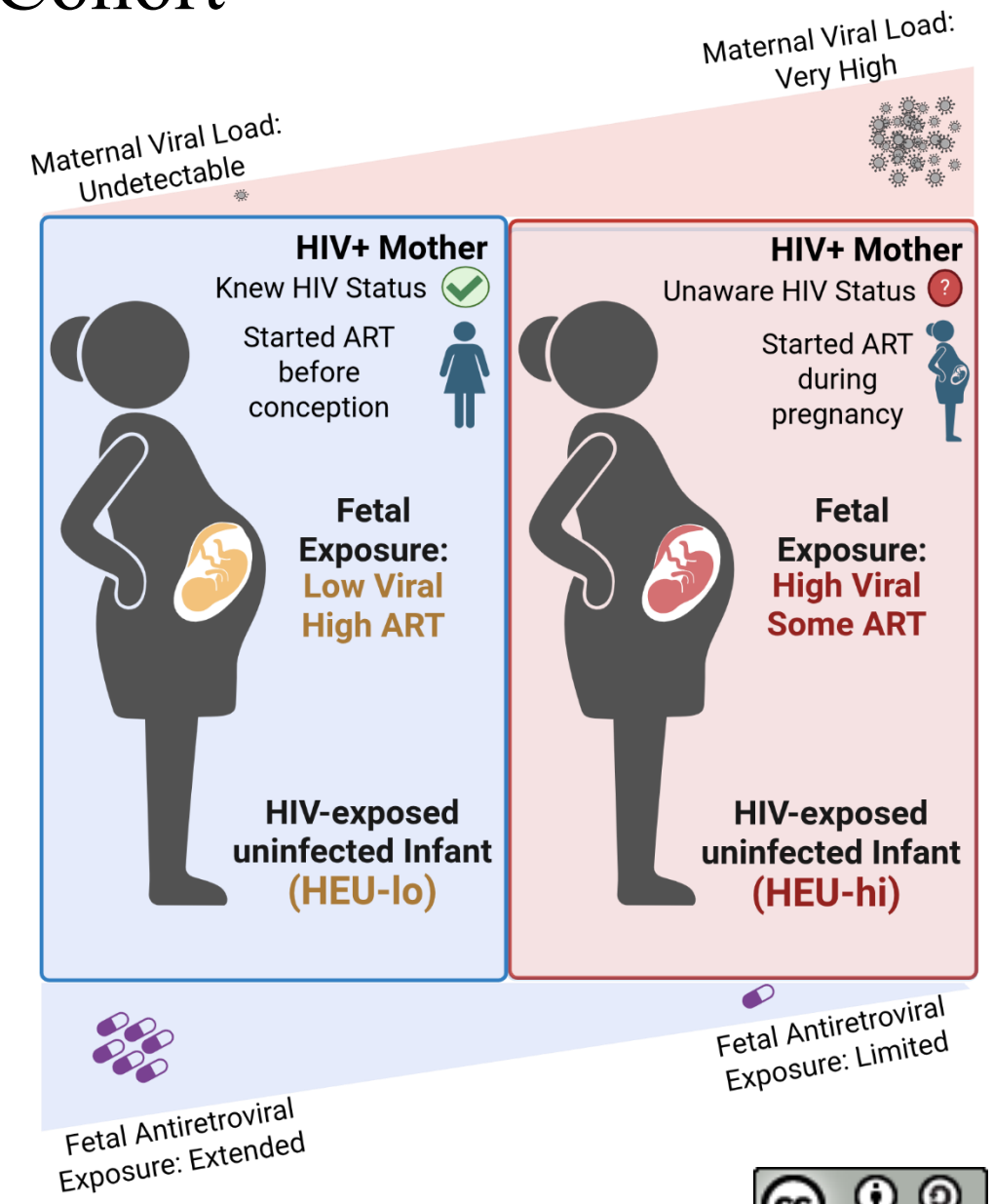


My PhD's focus: HIV-exposed Uninfected Infants

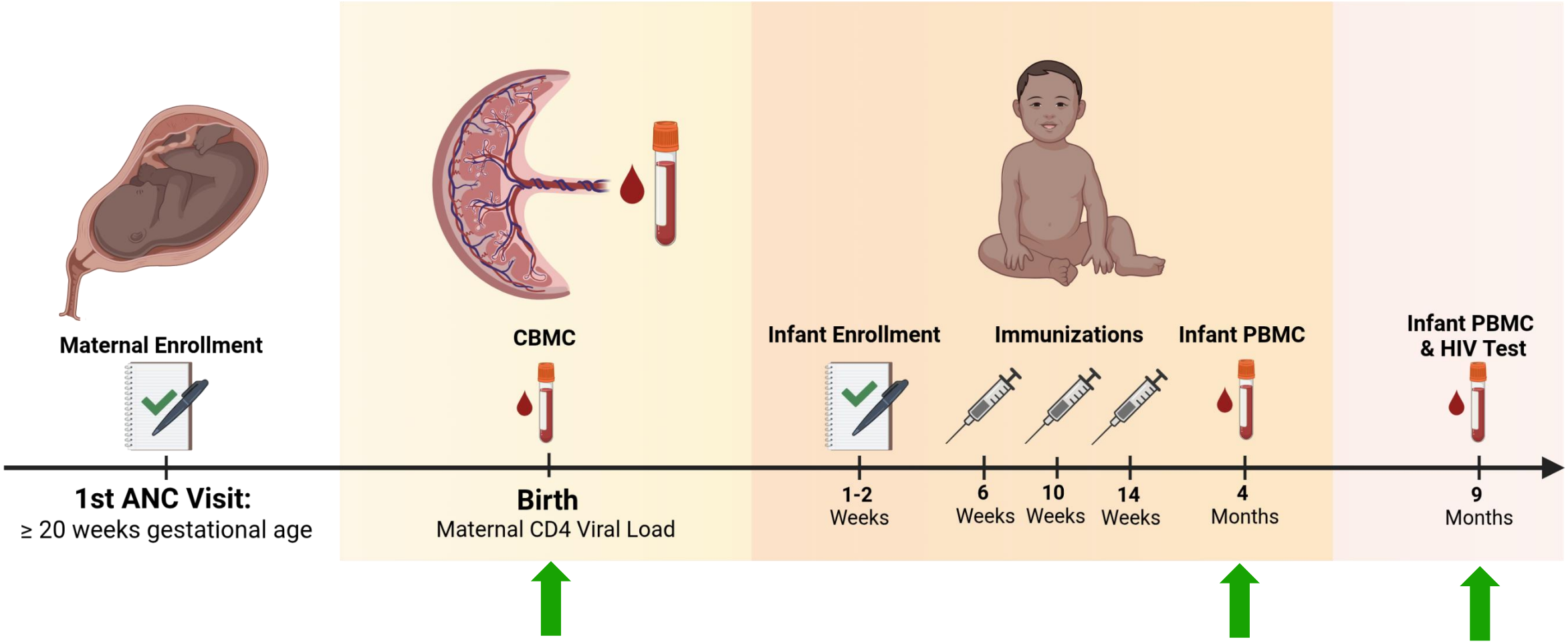


Our Malawian Cohort





Study Timeline



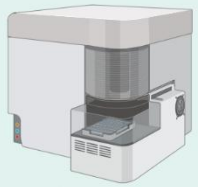
32-Fluorophore Spectral Flow Cytometry Panel

UV1	Spark UV 387		
UV2	BUV395	CD62L	(SK11)
UV6	cFluor UV440		
UV7	BUV496		
UV9	BUV563	CD69	(FN50)
UV10	BUV615	CCR4	(1G1)
UV11	BUV661	Vδ2	(B6)
UV14	BUV737	CD38	(HB7)
UV16	BUV805	CD4	(SK3)
V1	BV421/		
V2	Spark Violet 423		
V3	Pacific Blue	CD14	(M5E2)
V3	Pacific Blue	CD19	(SJ25C)
V4	SYTOX Blue		
V5	BV480	CD161	(HP-3G10)
V6	SBV515		
V7	BV510		
V8	BV570	CD16	(3G8)
V9	Qdot 585		
V10	BV605	CD45RA	(HI100)
V11	BV650	CD8	(RPA-T8)
V13	BV711	Va7.2	(3C10)
V14	BV750	IFNγ	(B27)
V15	BV786	CCR6	(11A9)
V16	LD NIR 786		
B2	Alexa Fluor 488	FoxP3	(236A/E7)
B3	Spark Blue 550	CD3	(SK7)
B4	Spark Blue 574	CD45	(2D1)
B6	RB613	PD1	(EH12.1)
B7	NovaFluorBlue660		
B8	SBB675		
B9	NovaFluorBlue690		
B10	RB705	CD26	(M-A261)
B12	RB744/		
B13	NovaFluorBlue760		
B14	RB780	CXCR5	(RF8B2)
YG1	PE	ICOS	(ISA-3)
YG2	Spark YG 593		
YG3	PE-Dazzle 594	TNFα	(MAB11)
YG4	PE-Fire 640/		
YG5	PE-Cy5	CXCR3	(G025H7)
YG6	NovaFluorYellow690		
YG7	PE-Fire 700	CD127	(A019D5)
YG8	PE-Fire 744	CD25	(M-A251)
YG9	PE-Vio 770	HLA-DR	(REA805)
YG10	PE-Fire810		
R1	APC	CD39	(A1)
R2	Alexa Fluor 647	IL2	(MQ1-
R3	cFluor R685		
R4	APC-R700	CD107a	(H4A3)
R5	NovaFluorRed725		
R6	Zombie NIR	L/D	
R7	APC-Fire 750	CD27	(O323)
R8	APC-Fire 810	CCR7	(G043H7)

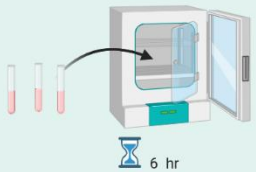
Flow Cytometry

Thaw & Rest

1) Thaw



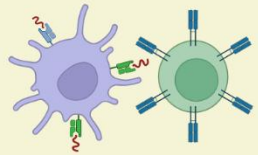
2) Count



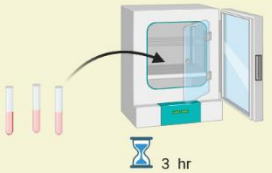
3) Rest

Antigen Incubation

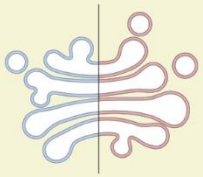
4) Aliquot by condition



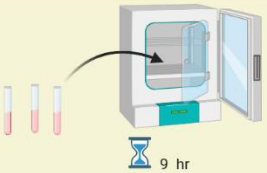
5) Add antigens (PPD, SEB, Ctrl)



6) Incubate



7) Add Golgi Transport Inhibitors

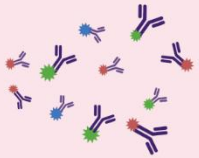


8) Incubate

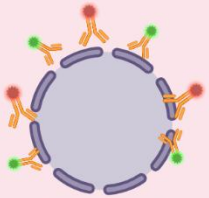
Flow Cytometry Staining

9) Pellet

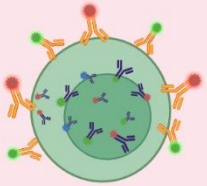
10) Viability stain



11) Surface antibodies



12) Fix & permeabilize



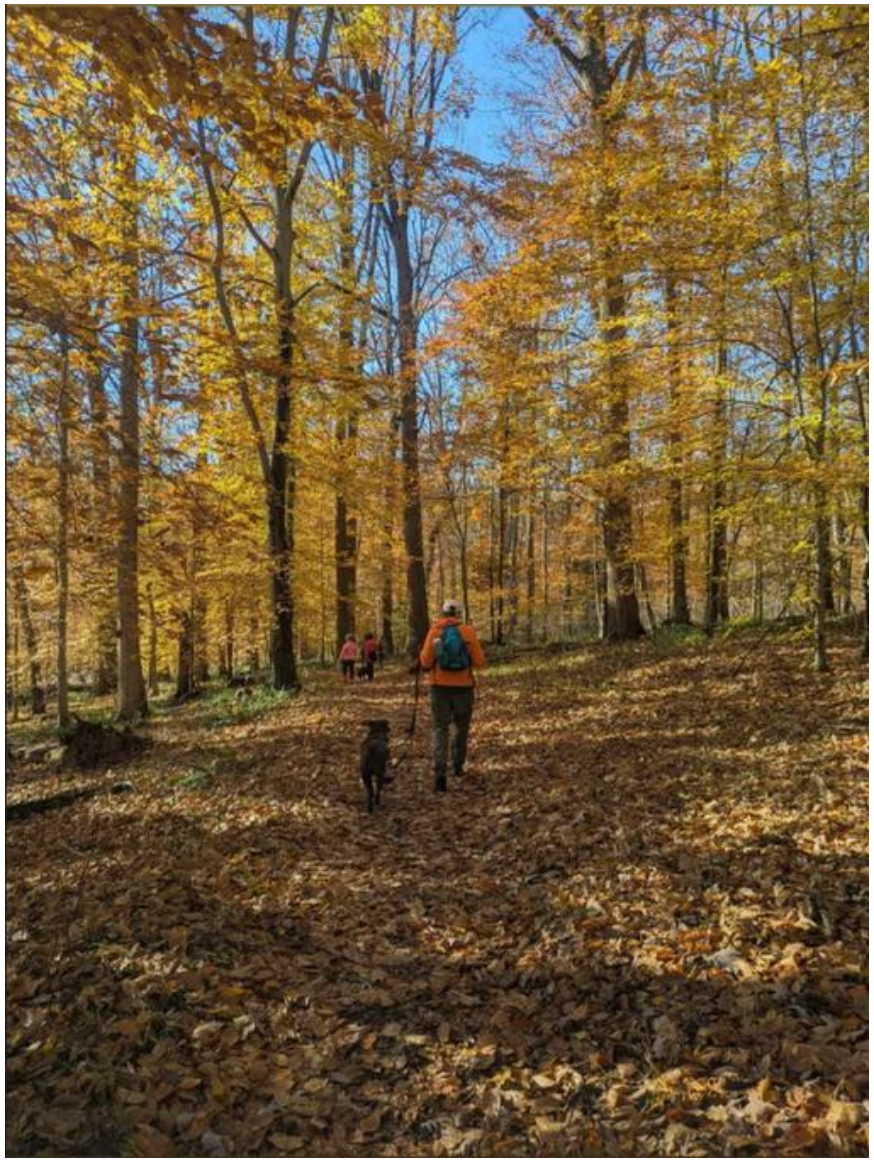
13) Intracellular antibodies

14) Resuspend in 0.4% PFA

15) Refrigerate at 4*C

16) Acquisition Cytek Aurora 5L

Personal Goal: No cell left behind

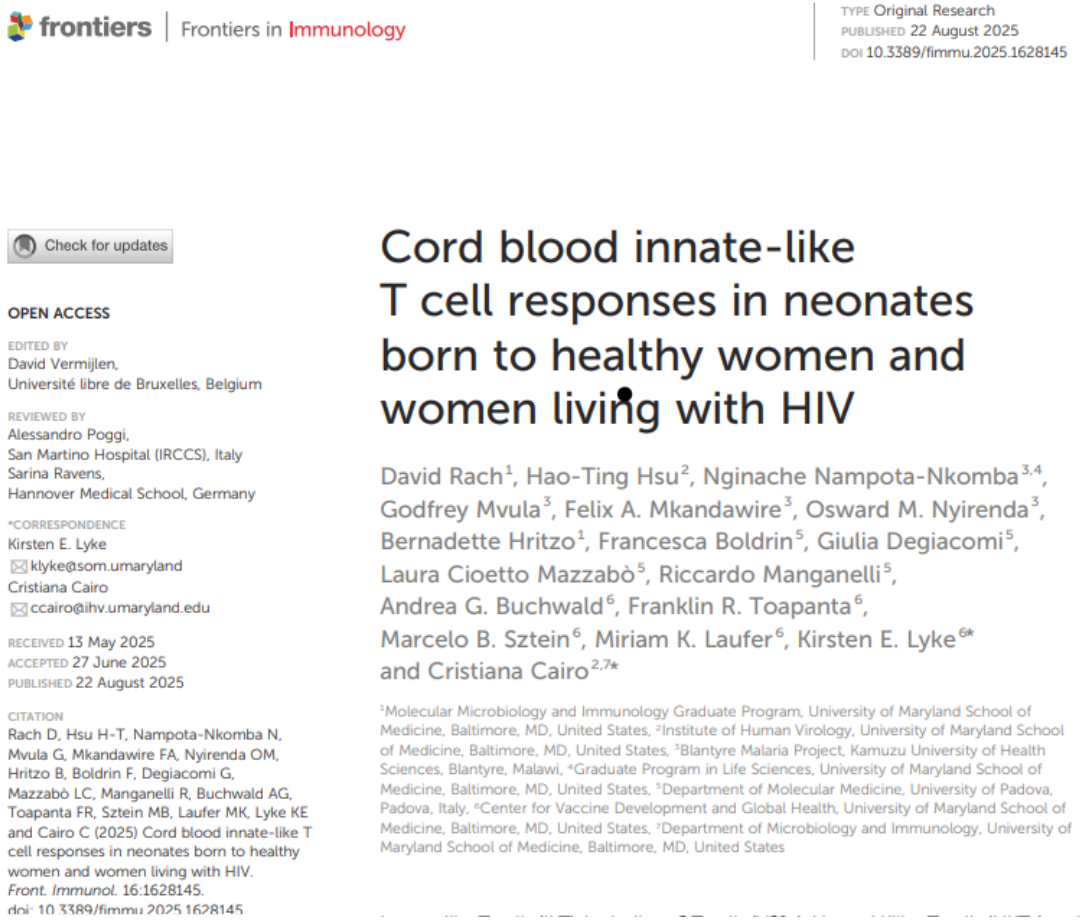


<u>Repeated 3 AM thoughts</u>

- ❑ What makes spectral flow cytometry data amazing is both the depth and the breadth
- ❑ How to fully profile the entirety of the acquired dataset, not just single cell population of interest

Previously looked at unconventional T cells

Paper



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EDITED BY
David Vermijlen,
Université libre de Bruxelles, Belgium

REVIEWED BY
Alessandro Poggi,
San Martino Hospital (IRCCS), Italy
Sarina Ravens,
Hannover Medical School, Germany

*CORRESPONDENCE
Kirsten E. Lyke
klyke@som.umaryland
Cristiana Cairo
ccairo@ihv.umaryland.edu

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Cord blood innate-like T cell responses in neonates born to healthy women and women living with HIV

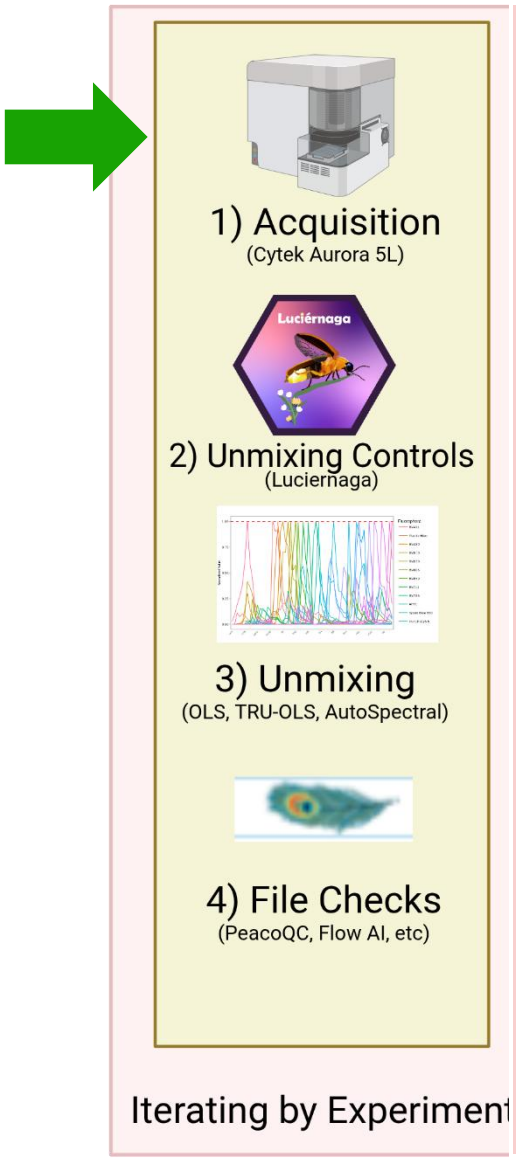
David Rach[1], Hao-Ting Hsu[2], Nginache Nampota-Nkomba[3,4], Godfrey Mvula[3], Felix A. Mkandawire[3], Osward M. Nyirenda[3], Bernadette Hritzo[1], Francesca Boldrin[5], Giulia Degiacomi[5], Laura Cioetto Mazzabò[5], Riccardo Manganelli[5], Andrea G. Buchwald[6], Franklin R. Toapanta[6], Marcelo B. Sztein[6], Miriam K. Laufer[6], Kirsten E. Lyke[6*] and Cristiana Cairo[2,7*]

[1]Molecular Microbiology and Immunology Graduate Program, University of Maryland School of Medicine, Baltimore, MD, United States, [2]Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD, United States, [3]Blantyre Malaria Project, Kamuzu University of Health Sciences, Blantyre, Malawi, [4]Graduate Program in Life Sciences, University of Maryland School of Medicine, Baltimore, MD, United States, [5]Department of Molecular Medicine, University of Padova, Padova, Italy, [6]Center for Vaccine Development and Global Health, University of Maryland School of Medicine, Baltimore, MD, United States, [7]Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, United States

Main differences this time around:

- ❑ Shifting from 50%-50% FlowJo – R to increasingly more R
- ❑ Targeted re-writes in Rust to speed up original R code
- ❑ Building around reproducibility from the start (not as an after thought)
- ❑ Improved Bioconductor interoperability

Pipeline: Acquisition



- ❑ Acquired day of staining on a 5-Laser Cytek Aurora
- ❑ Instrument flushed with 10% Contrad and Water for 20 minutes pre-use
- ❑ Instrument passed by both the UMGCCC FCSR and Cytek's QC criteria immediately before acquisition

UMGCCC FCSR InstrumentQC

Plots for our 5L Aurora

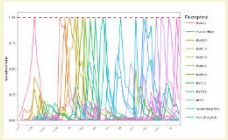
Historical Data 5L Aurora

Dashboard How-To-Set-Up

Pipeline: Evaluating Unmixing Controls

1) Acquisition
(Cytek Aurora 5L)

2) Unmixing Controls
(Luciernaga)



3) Unmixing
(OLS, TRU-OLS, AutoSpectral)

4) File Checks
(PeacoQC, Flow AI, etc)

Iterating by Experiment

- ❖ New unmixing controls acquired with each experiment.
- ❖ Extra specimen cells acquired as additional unstained controls

- ❑ Unmixing Controls are screened for suitability for unmixing
- ❑ Single-colors: Tandem degradation, and autofluorescence contamination
- ❑ Unstained(s): Presence of multiple-autofluorescences in lymphocyte gate

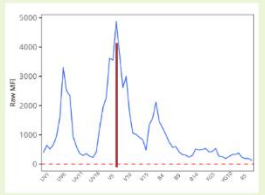
- ❑ Flagged unmixing controls are replaced with equivalents from adjacent experimental date

Luciernaga

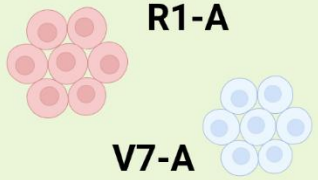
Luciernaga Vignette

Luciernaga: Individual Cell Normalized Signatures

Code

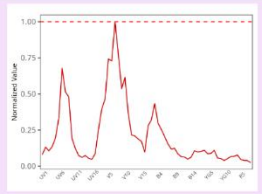


Identify detector with brightest MFI for each cell

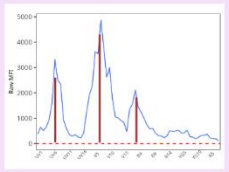


Group cells with shared peak detectors

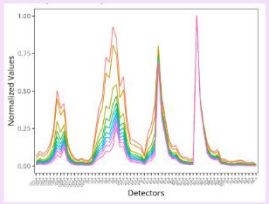
Subtract Out Autofluorescence



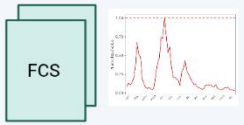
Normalize individual cell fluorescent signatures



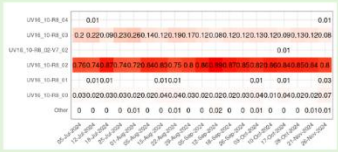
Identify peaks using local maxima



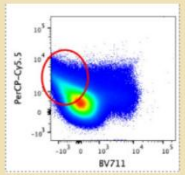
Group cells with shared peaks & heights



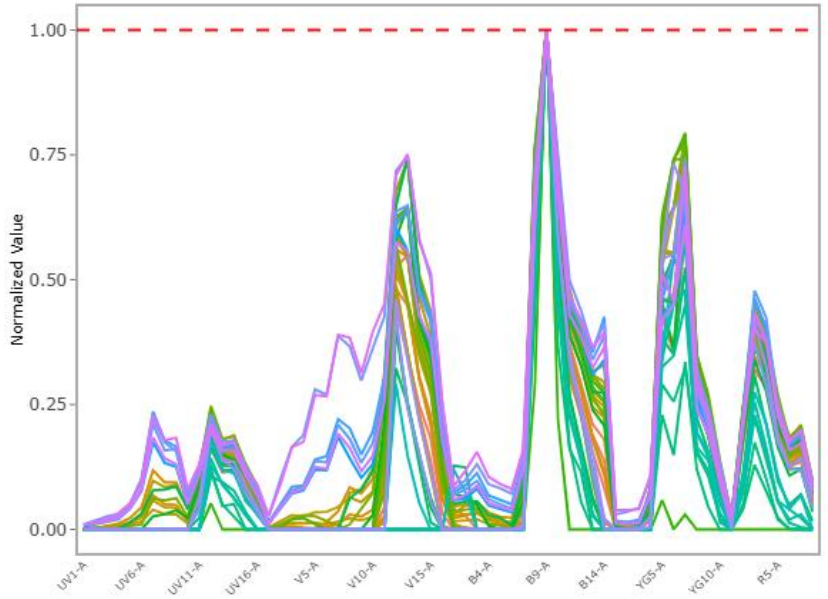
Enumerate and export out cells that share signature as .fcs files



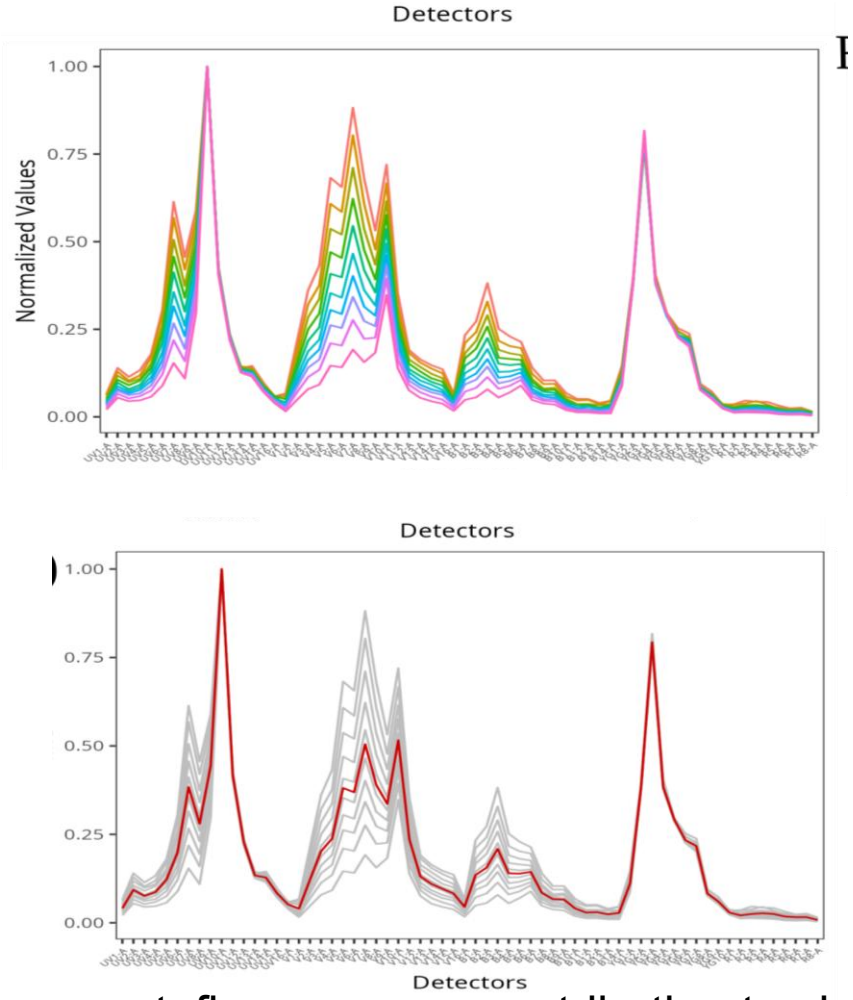
Checks for tandem degradation



Autofluorescence Isolation

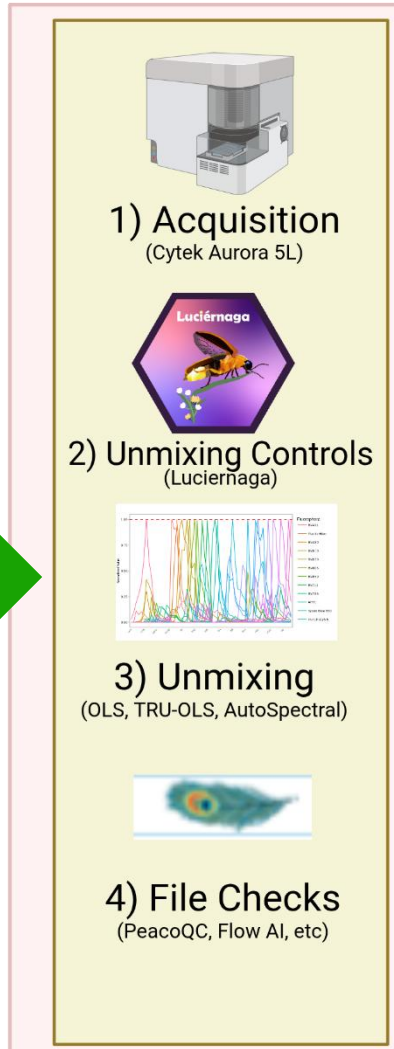


❖ Degrading PerCP-Cy5.5 antibody vial



❖ Leftover autofluorescence contribution to signature

Pipeline: Unmixing



- ❖ Unmixing Method Agnostic
- ❑ Unmixing in SpectroFlo (with modified single-color and unstained inputs via Luciernaga)
- ❑ Unmixing in R
  - OLS (Luciernaga using Rust)
  - TRU-OLS (via Julia wrapper)
  - AutoSpectral

TRU-OLS

TRU-OLS Walkthrough

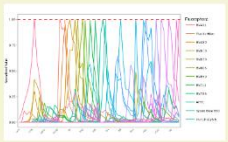
AutoSpectral

AutoSpectral Walk-through

Pipeline: File Checks

1) Acquisition
(Cytek Aurora 5L)

2) Unmixing Controls
(Luciernaga)



3) Unmixing
(OLS, TRU-OLS, AutoSpectral)

4) File Checks
(PeacoQC, Flow AI, etc)

Iterating by Experiment

- ❑ Checking for fluidic or laser-related issues in the unmixed files

- ➢ R: PeacoQC or FlowAI
- ➢ Rust: PeacoQC (thanks James!)

PeacoQC

PeacoQC Vignette

FlowAI

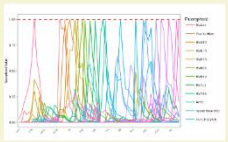
FlowAI Vignette

PeacoQC in Rust

Pipeline: Manual and Automated Gating

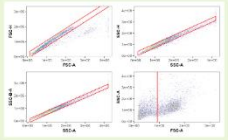
1) Acquisition
(Cytek Aurora 5L)

2) Unmixing Controls
(Luciernaga)



3) Unmixing
(OLS, TRU-OLS, AutoSpectral)

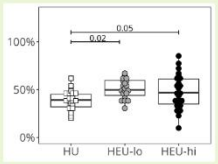
4) File Checks
(PeacoQC, Flow AI, etc)



5) Clean Up Gates
(FlowJo or flowWorkspace)

6) Initial Gates
(openCyto or flowMagic)

7) FlowJo .wsp
(CytoML Docker)



8) Major subset Outputs

Iterating by Experiment

- ❑ Doublets, Debris, Dead cell cleanup (in R or FlowJo)
- ❑ Automated Gating for major cell subsets (openCyto or flowMagic)
- ❑ All automated gate placements visualized via export to a .pdf

flowGate

flowGate Walkthrough

openCyto

openCyto Walk-through

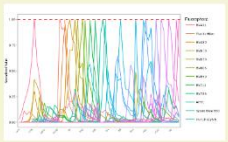
flowMagic

flowMagic

Pipeline: Save to FlowJo v10 .wsp

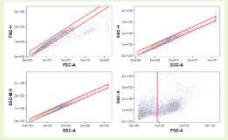
1) Acquisition
(Cytek Aurora 5L)

2) Unmixing Controls
(Luciernaga)



3) Unmixing
(OLS, TRU-OLS, AutoSpectral)

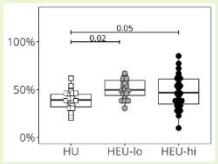
4) File Checks
(PeacoQC, Flow AI, etc)



5) Clean Up Gates
(FlowJo or flowWorkspace)

6) Initial Gates
(openCyto or flowMagic)

7) FlowJo .wsp
(CytoML Docker)



8) Major subset Outputs

Iterating by Experiment

- ❑ Everything is saved to a FlowJo v10 .wsp, via CytoML
- ❑ Any automated gates that need adjustment get corrected
- ❑ Major cell subset statistics exported and analyzed

flowWorkspace

CytoML

FlowJo to R

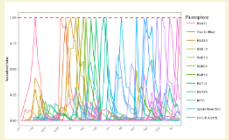
R to FlowJo

Data for Statistics Walkthrough

Pipeline: Iterating through cell subsets

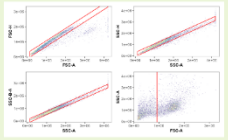
1) Acquisition
(Cytek Aurora 5L)

2) Unmixing Controls
(Luciernaga)

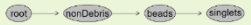


3) Unmixing
(OLS, TRU-OLS, AutoSpectral)

4) File Checks
(PeacoQC, Flow AI, etc)

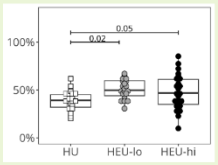


5) Clean Up Gates
(FlowJo or flowWorkspace)



6) Initial Gates
(openCyto or flowMagic)

7) FlowJo .wsp
(CytoML Docker)



8) Major subset Outputs

Iterating by Experiment

9) Subset Export

10) Return to R
(flowWorkspace)

11) Coereba

Summarized Experiment

Iterating by Cell Subset

- ❑ Cell subset of interest exported out as own .fcs
- ❑ All cell subset .fcs files brought into R

Subset Export Walkthrough

GatingSets Walkthrough

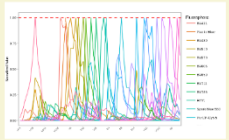
Coereba

Coereba Vignette

Pipeline: Coereba

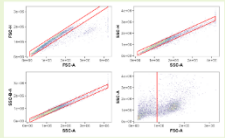
1) Acquisition
(Cytek Aurora 5L)

2) Unmixing Controls
(Luciernaga)



3) Unmixing
(OLS, TRU-OLS, AutoSpectral)

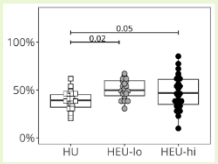
4) File Checks
(PeacoQC, Flow AI, etc)



5) Clean Up Gates
(FlowJo or flowWorkspace)

6) Initial Gates
(openCyto or flowMagic)

7) FlowJo .wsp
(CytoML Docker)



8) Major subset Outputs

Iterating by Experiment

9) Subset Export

10) Return to R
(flowWorkspace)

11) Coereba

Summarized Experiment

Iterating by Cell Subset

❑ Coereba

Subset Export Walkthrough

GatingSets Walkthrough

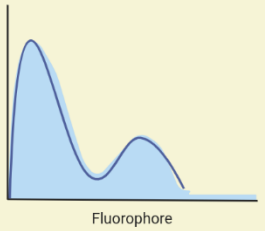
Coereba

Coereba Vignette

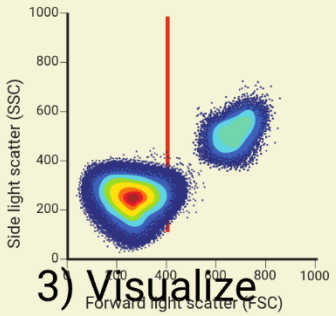
Coereba

Code

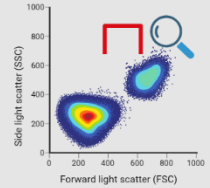
1) Load into R
(flowWorkspace)



2) Calculate Splitpoints

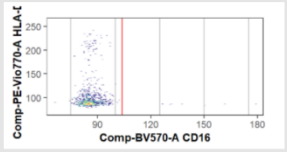


3) Visualize



4) Gate Constraints

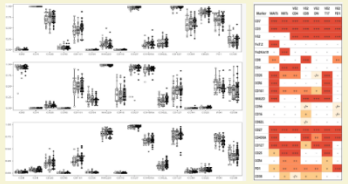
5) Shiny App adjustments



6) Splitpoints updated

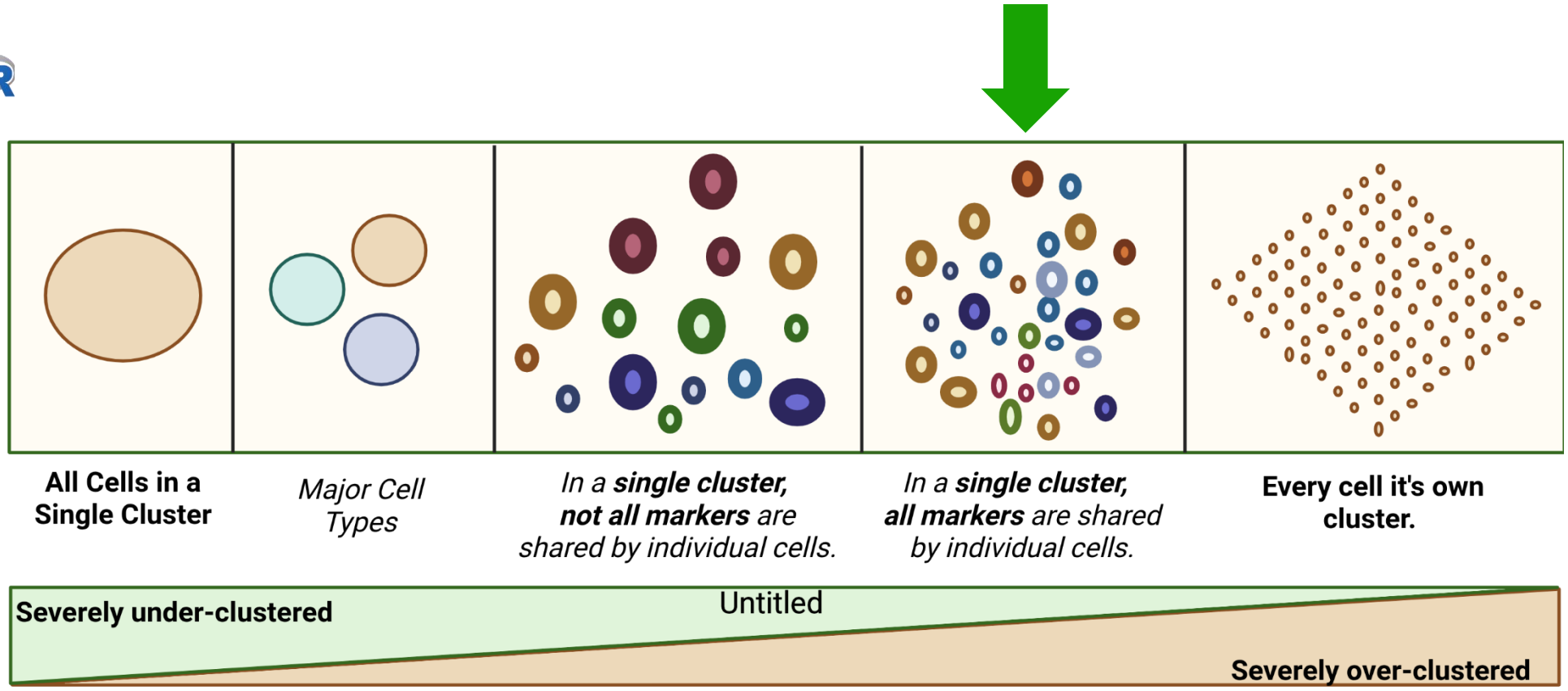
7) CoerebaID

8) Summarized Experiment

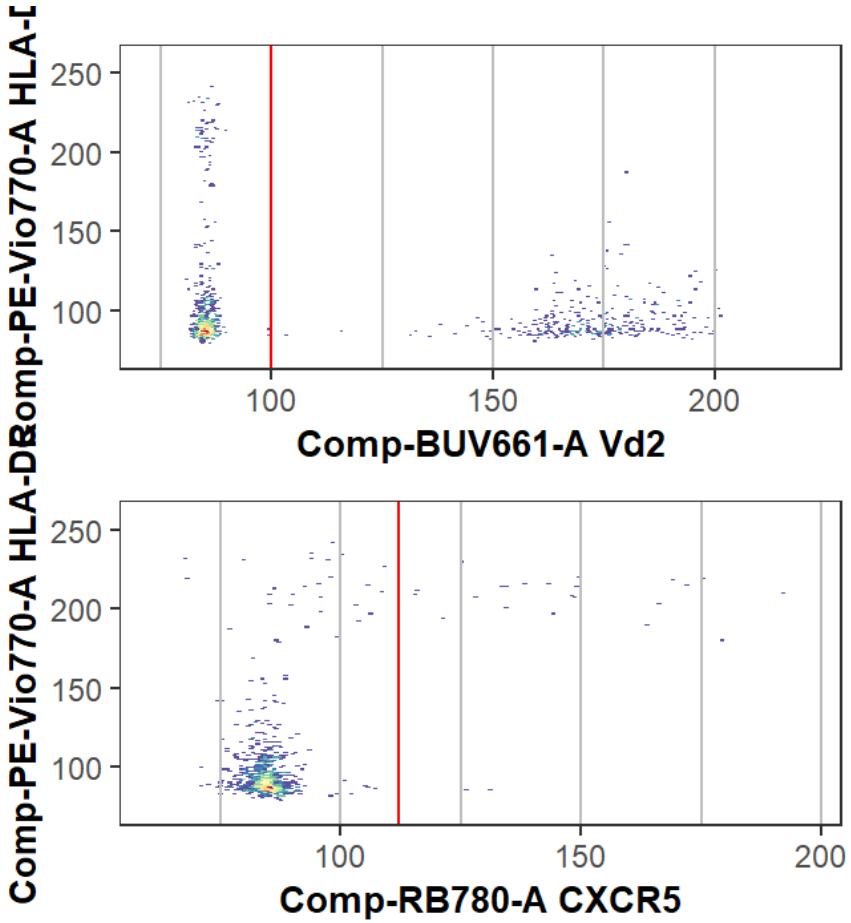


9) Visualize

Grouping cells that share every marker



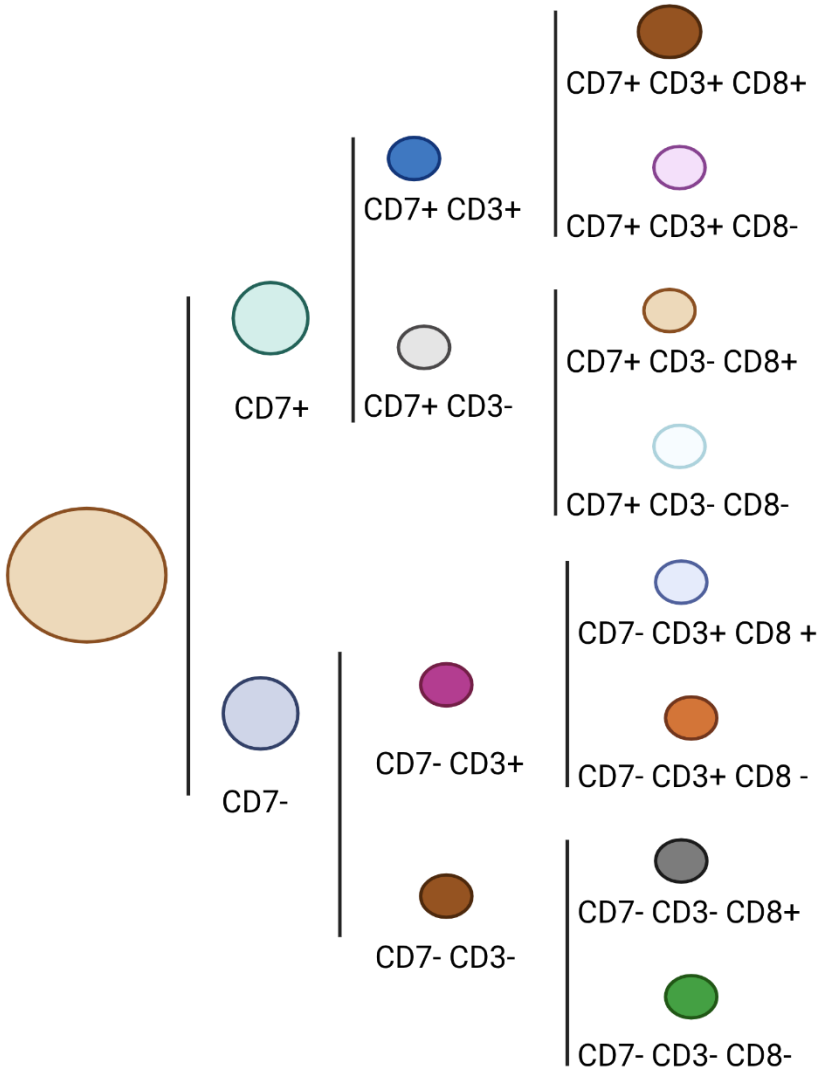
Initial automated gate, with ability to adjust



- Initial gates for designated markers calculated via openCyto (mindensity)
- Ability to update gate_range constraints
- Launch a Shiny app and designate interactively

CoerebaID column gets added to the .fcs file

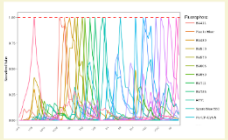
PEVio770pos	APCneg	AlexaFluor647neg	APCR700neg	APCFire750neg	APCFire810neg
PEVio770pos	APCneg	AlexaFluor647neg	APCR700neg	APCFire750neg	APCFire810neg
PEVio770pos	APCpos	AlexaFluor647neg	APCR700neg	APCFire750pos	APCFire810neg
PEVio770pos	APCneg	AlexaFluor647pos	APCR700neg	APCFire750neg	APCFire810pos



Pipeline: Summarized Experiment

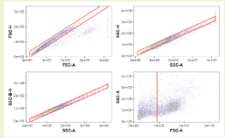
1) Acquisition
(Cytek Aurora 5L)

2) Unmixing Controls
(Luciernaga)

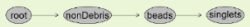


3) Unmixing
(OLS, TRU-OLS, AutoSpectral)

4) File Checks
(PeacoQC, Flow AI, etc)

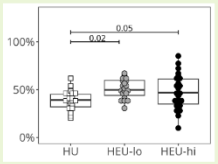


5) Clean Up Gates
(FlowJo or flowWorkspace)



6) Initial Gates
(openCyto or flowMagic)

7) FlowJo .wsp
(CytoML Docker)



8) Major subset Outputs

Iterating by Experiment

9) Subset Export

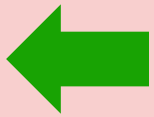
10) Return to R
(flowWorkspace)

11) Coereba

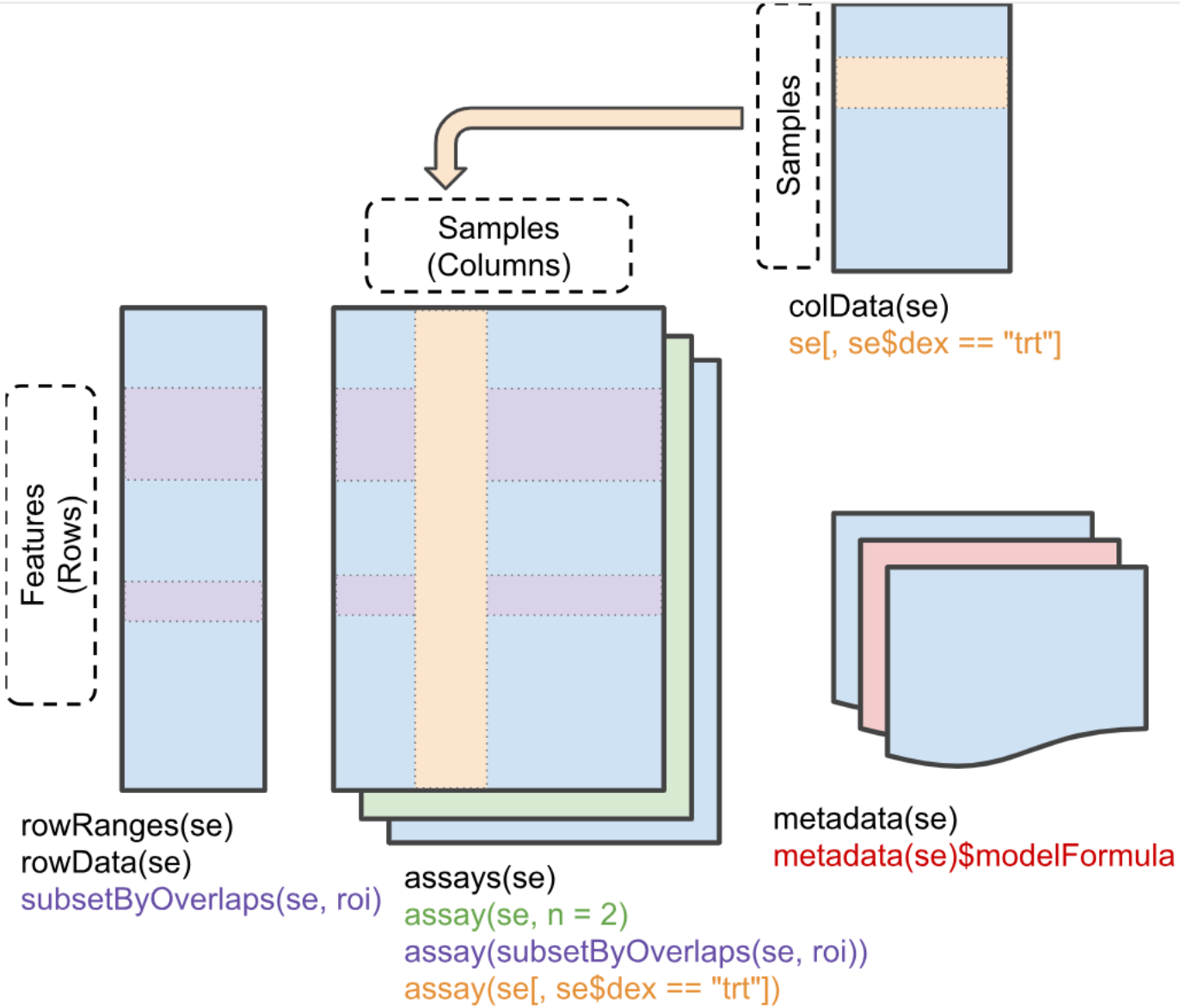
Summarized Experiment

Ite

- ❑ Data stored in a Summarized Experiment
- ❑ Interoperability with Bioconductor statistics packages

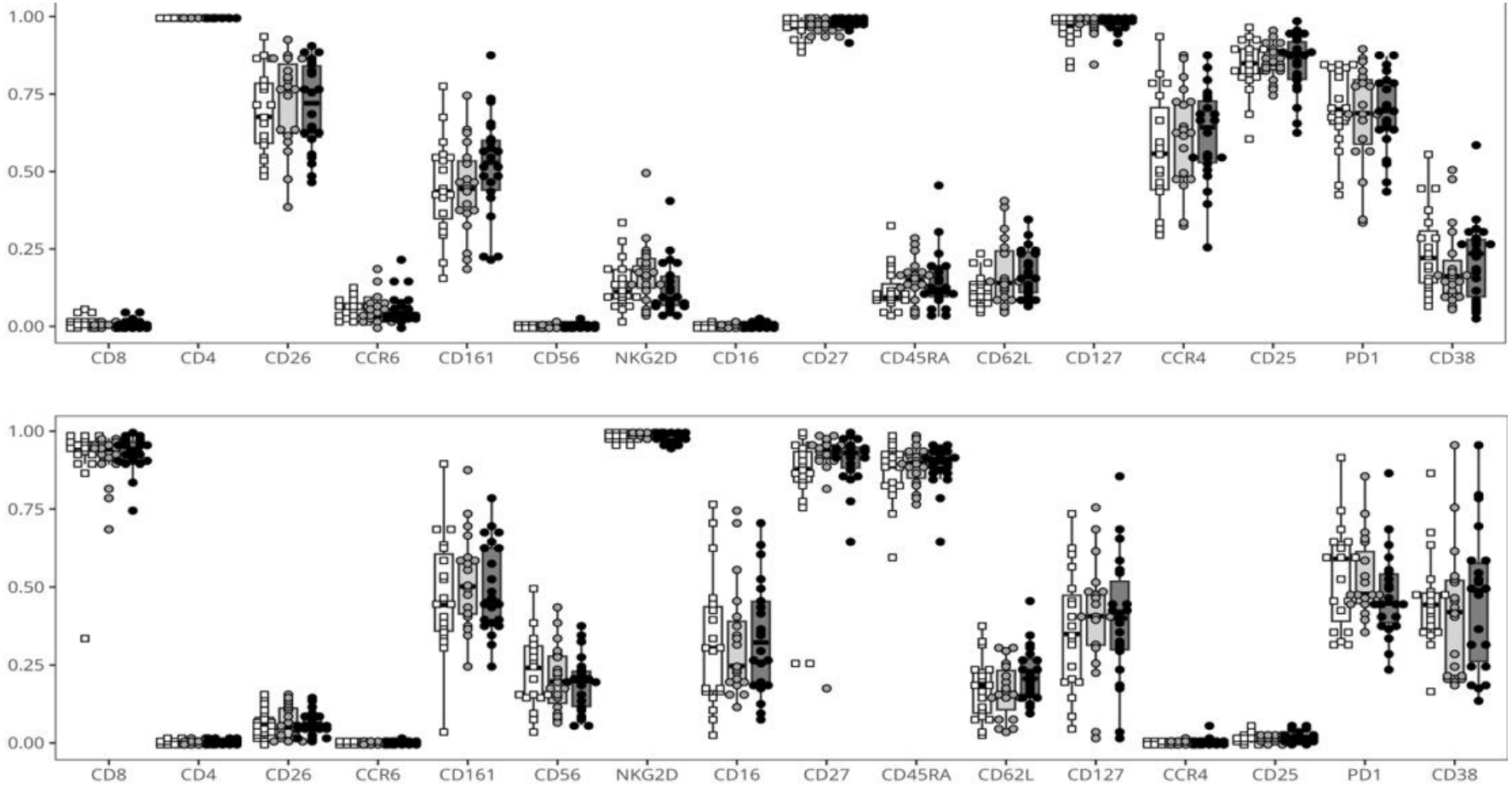


Summarized Experiment

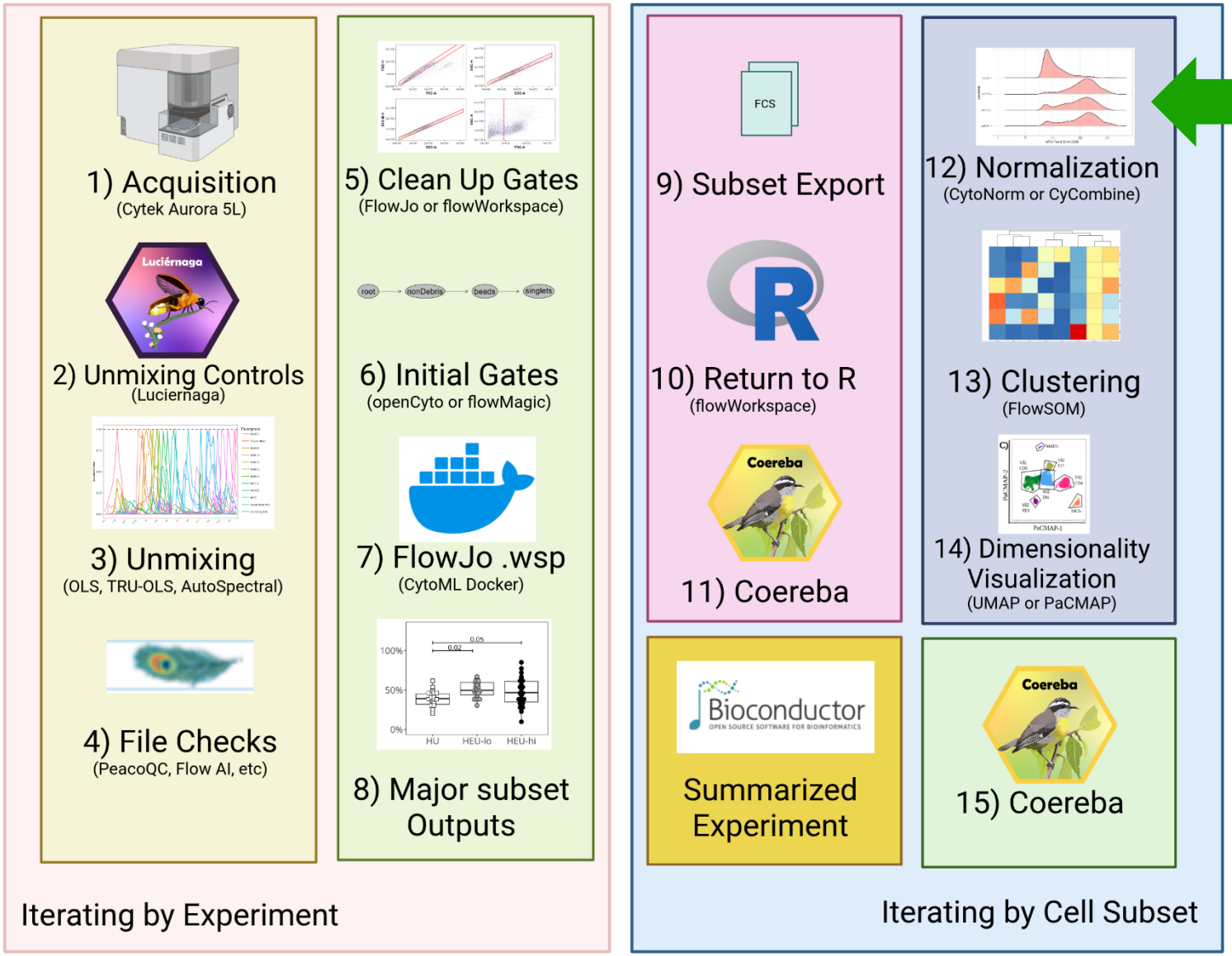


- Permits rapid re-aggregation of the terminal nodes for re-evaluation
- Ties in with downstream Bioconductor statistics packages
- Portable. Don't like how we aggregated groups of cells? File is small enough to store in the same data repository as .fcs files for subsequent re-analysis.
- Filterable by metadata or node.
  - Nodes only in HEU-hi?
  - Nodes unique to one individual?

Retrieval of marker expressions



Pipeline: Normalization



CytoNorm

CytoNorm Vignettes

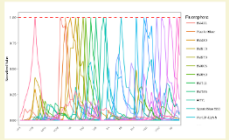
CyCombine

CyCombine Vignettes

Pipeline: Clustering

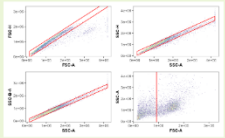
1) Acquisition
(Cytek Aurora 5L)

2) Unmixing Controls
(Luciernaga)

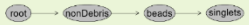


3) Unmixing
(OLS, TRU-OLS, AutoSpectral)

4) File Checks
(PeacoQC, Flow AI, etc)

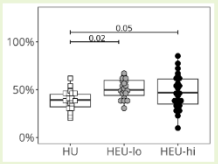


5) Clean Up Gates
(FlowJo or flowWorkspace)



6) Initial Gates
(openCyto or flowMagic)

7) FlowJo .wsp
(CytoML Docker)



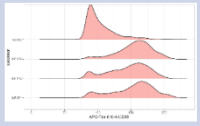
8) Major subset Outputs

Iterating by Experiment

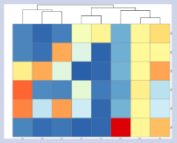
9) Subset Export

10) Return to R
(flowWorkspace)

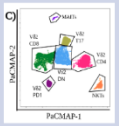
11) Coereba



12) Normalization
(CytoNorm or CyCombine)



13) Clustering
(FlowSOM)



14) Dimensionality Visualization
(UMAP or PaCMAP)

Summarized Experiment

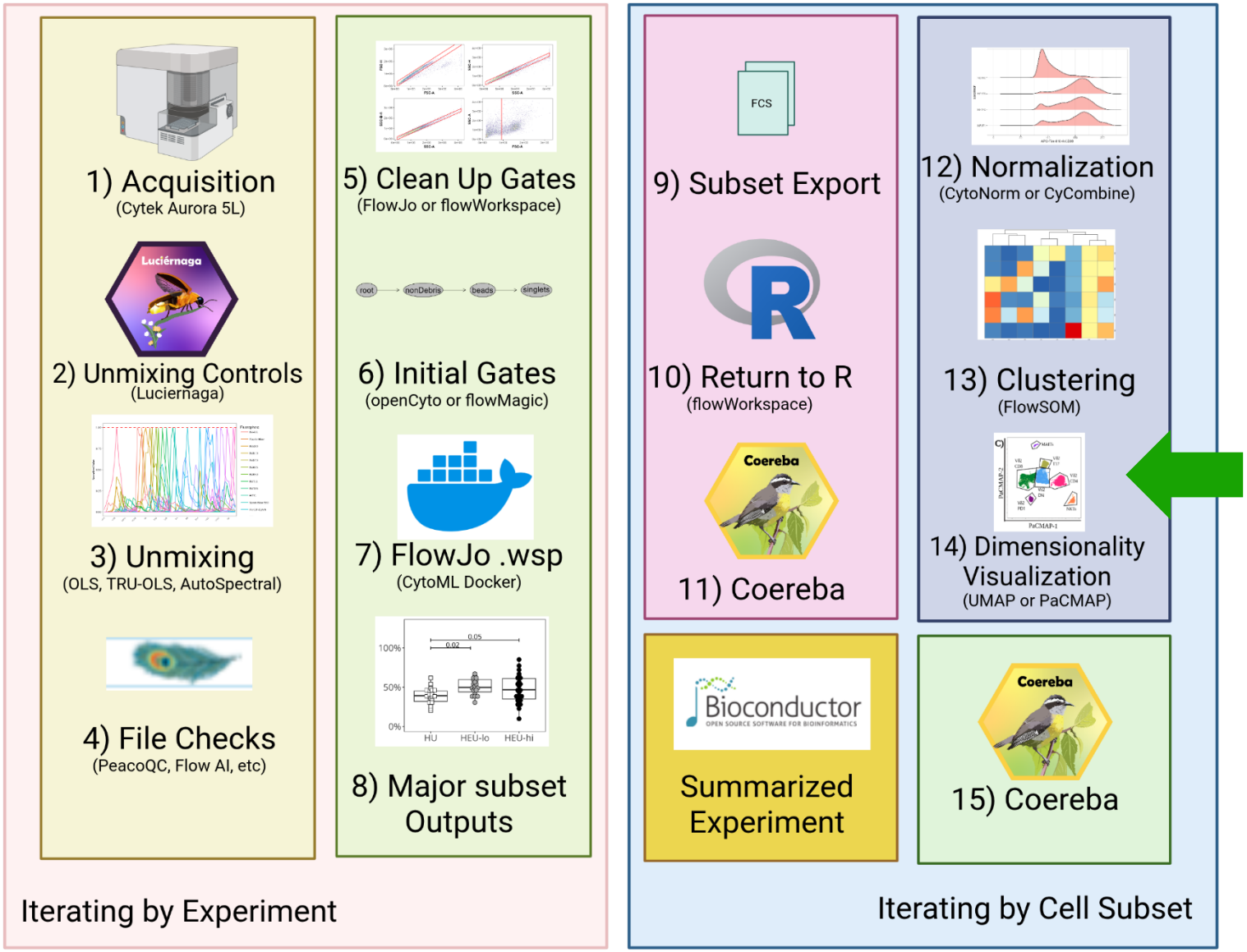
15) Coereba

Iterating by Cell Subset

FlowSOM

FlowSOM Vignettes

Pipeline: Dimensionality Visualization



UMAP

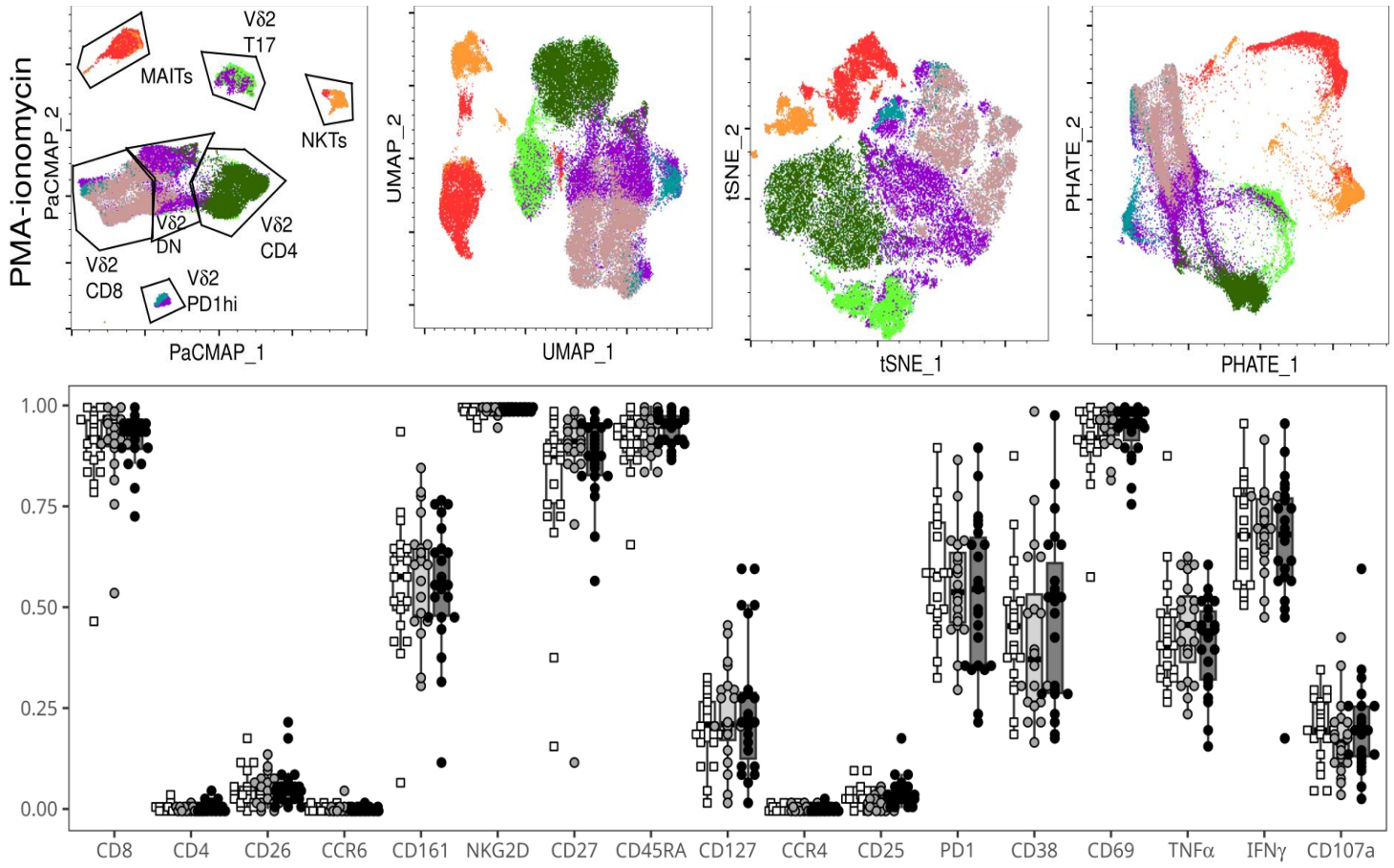
UMAP Wrapper

PaCMAP

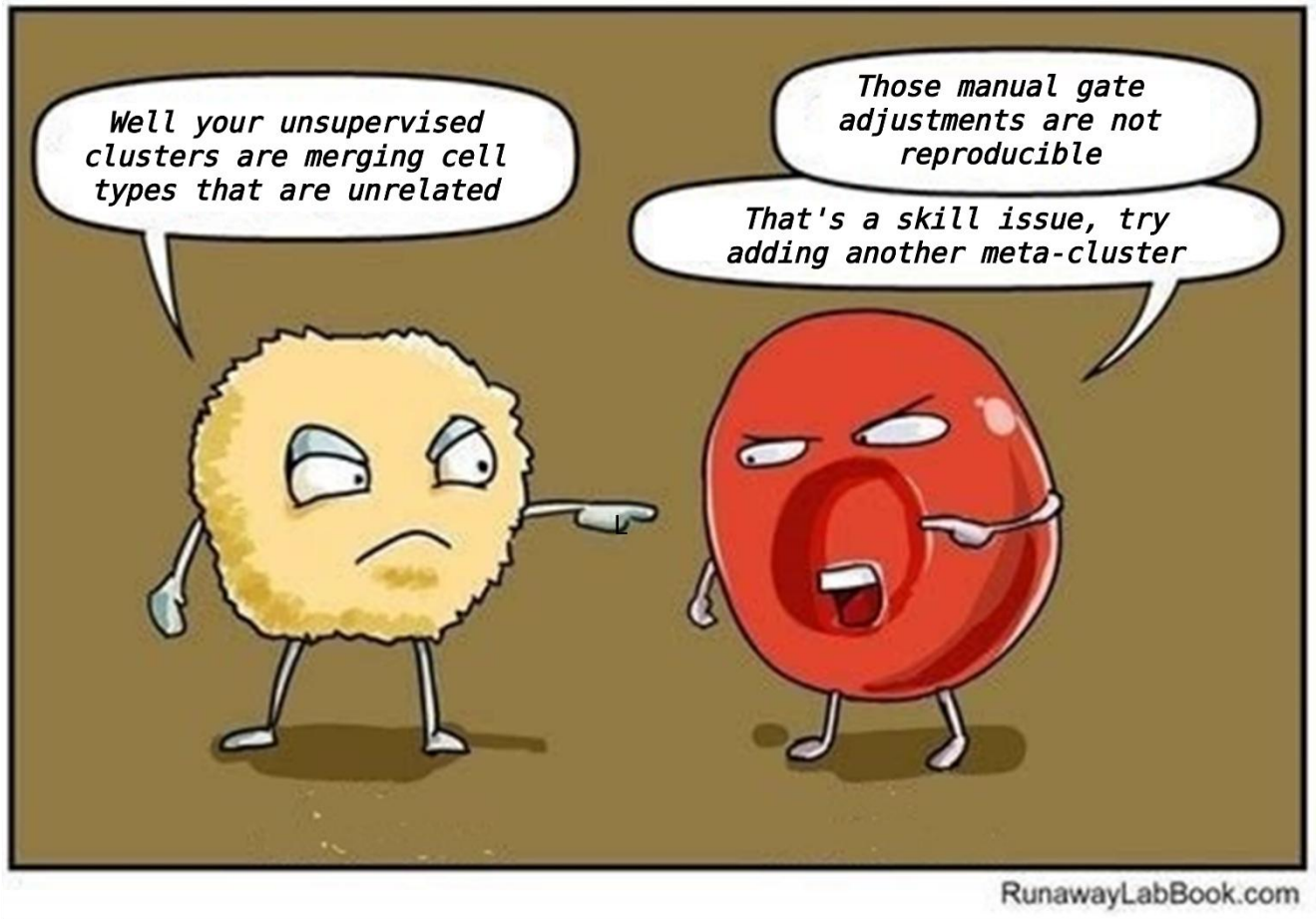
PaCMAP Wrapper

Retrieve manual annotated gating information following unsupervised steps

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	-	+	-/+	+	+	-	-



Functional and Iterative: plenty for everyone to dislike ☺



Fun off-label uses thus far:

- ❑ Contrasting where "manually-gated" cells end up vs. our unsupervised methods (normalization, clustering, etc.)
- ❑ Added benefit of looking-at-the-forest: Easy to identify badly cryopreserved specimens where cell populations were lost

Acknowledgements

All mothers and their infants, and their communities

UMGCCC FCSR

Dr. Xiaoxuan Fan
Dr. Natarajan Ayithan
Mikayla Trainor

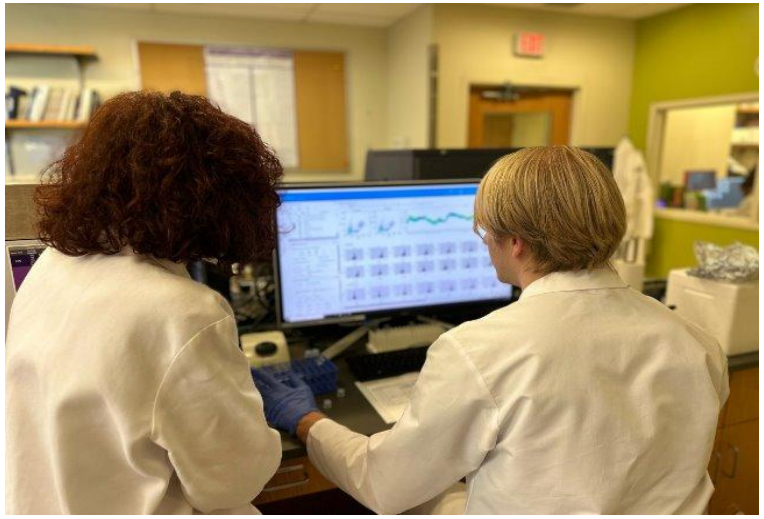
Bioconductor (S4 OOP help)

Hervé Pagès
Vince Carey

All Cytometry R package maintainers, past and present

https://github.com/DavidRach

@davidrach.bsky.social



U01 HD092308

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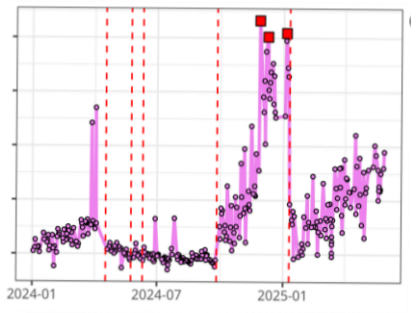
Molecular Microbiology Immunology Graduate Program

Dr. Eileen Barry
Dr. Brett Hassel
Dr. Heather Ezelle
Bess Tracey

Thesis Committee

Dr. Tonya Webb
Dr. Franklin Toapanta
Dr. Shannon Takala-Harrison
Dr. Yutaka Tagaya
Dr. Margaret Feeney

Questions?



UMGCCC FCSR
InstrumentQC dashboard

Luciernaga

Website - CytometryInR

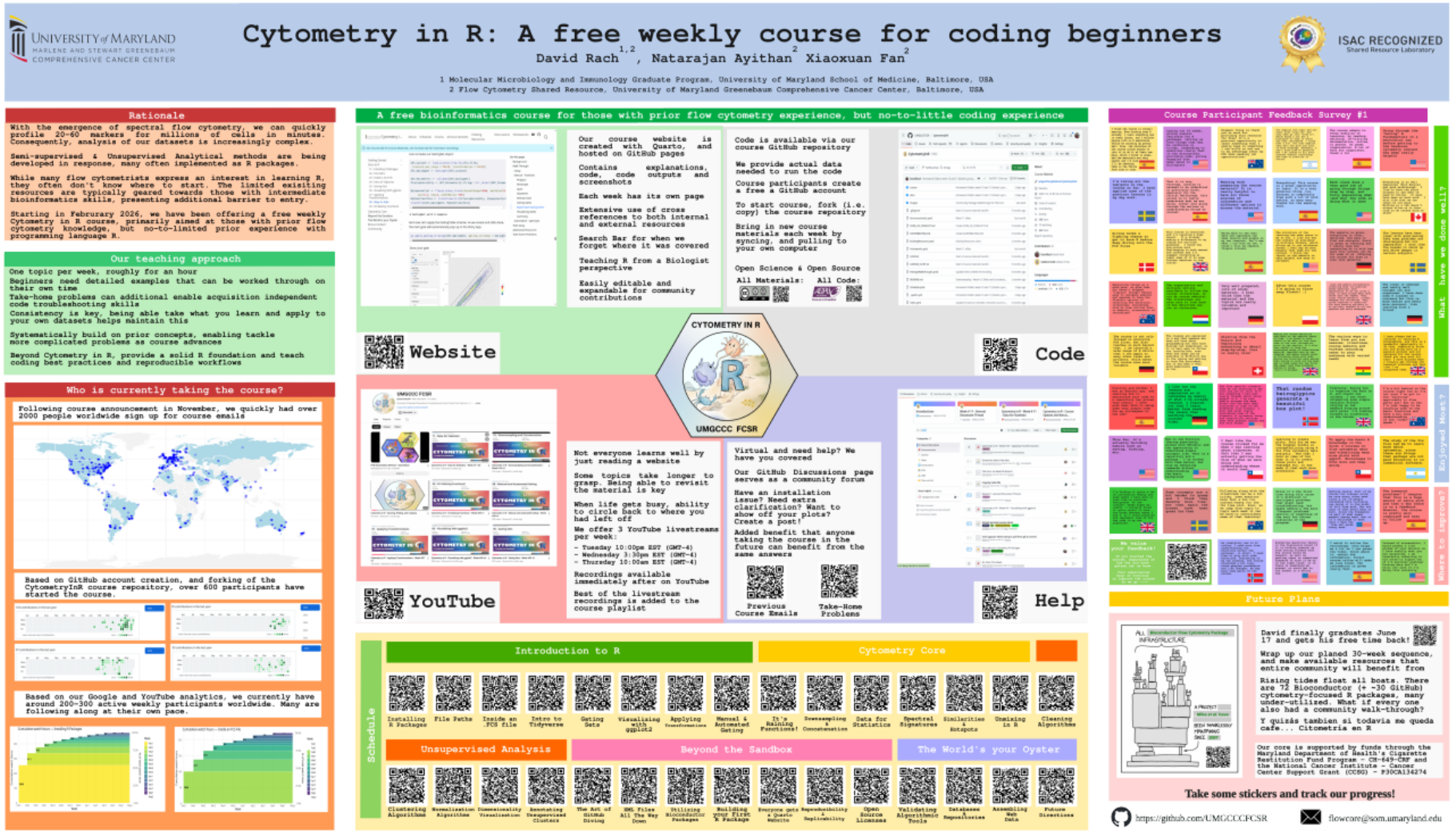
InstrumentQC How-To

Coereba

YouTube **@CytometryInR**

Learn R at your own pace

Website



- Graduate Next Week
- All coding side-projects will then resume post-defense