



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-Nkombamba, Godfrey Mvula, Felix A. Mkandawire, Oswald 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.

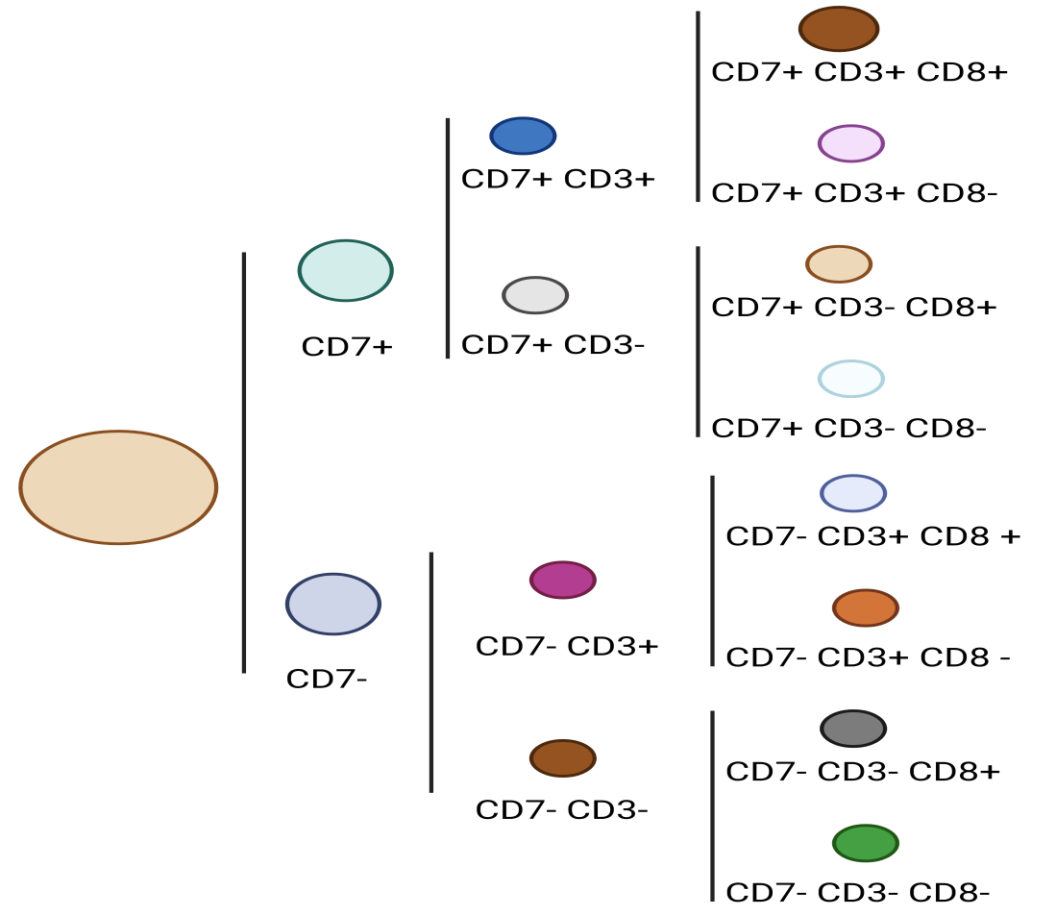


- If your goal is to characterize **all cell populations** that may be present, the breadth and depth complicate the analysis.

JAKE-CLARK.TUMBLR

Complex Datasets, Combinatorially Exponential Clusters

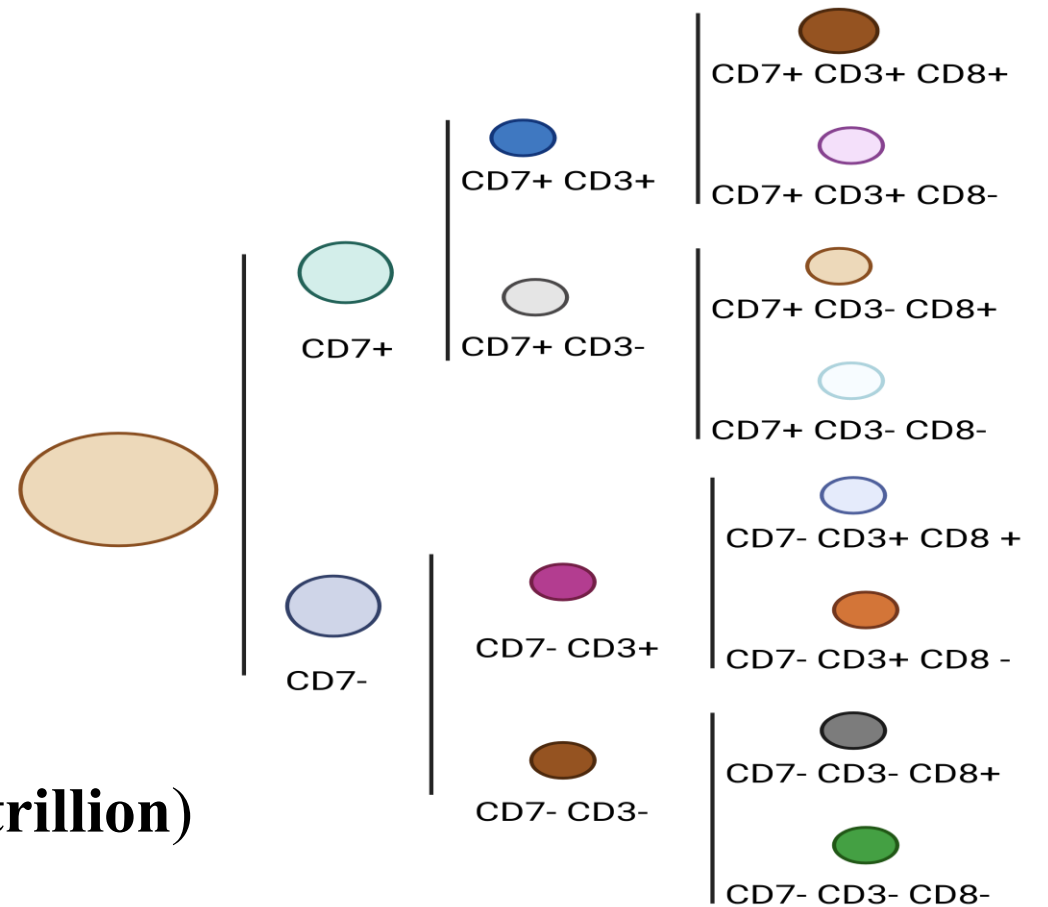
- If every marker in your panel split into **positive** and **negative** populations
- And you repeated this splitting process for **every marker**
- And **grouped cells** that shared the **same expressed markers**
- $2^{\text{NumberOfMarkers}}$



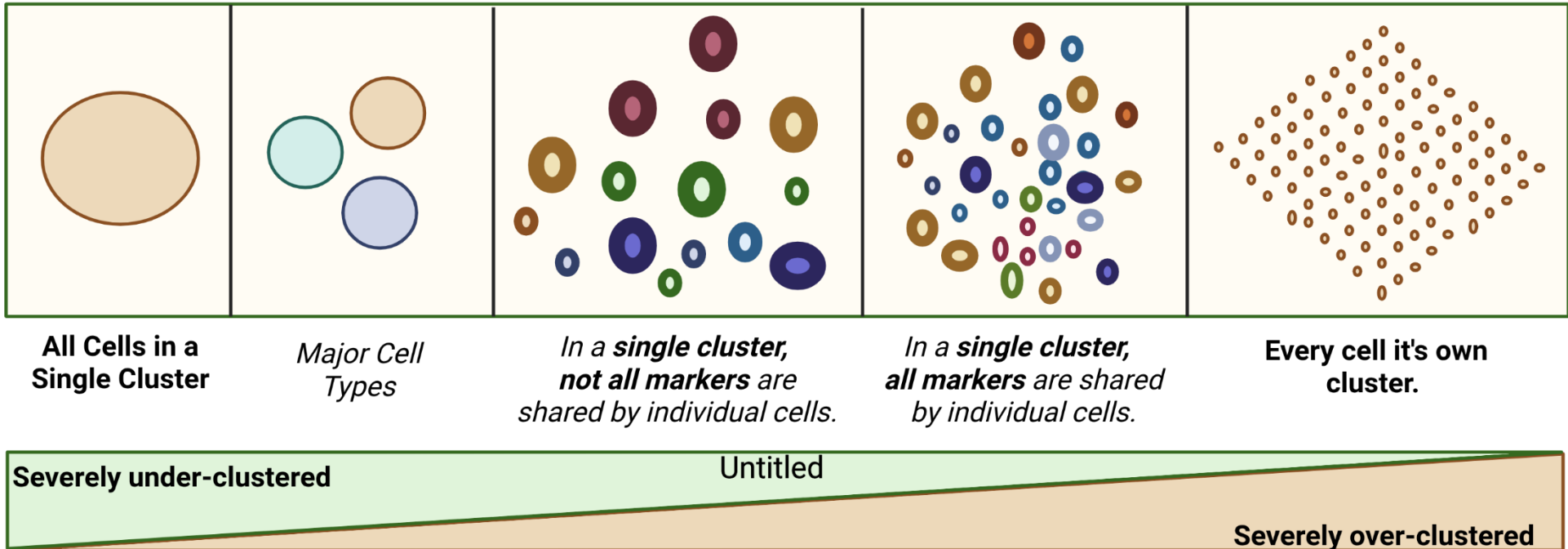
Complex Datasets, Combinatorially Exponential Clusters

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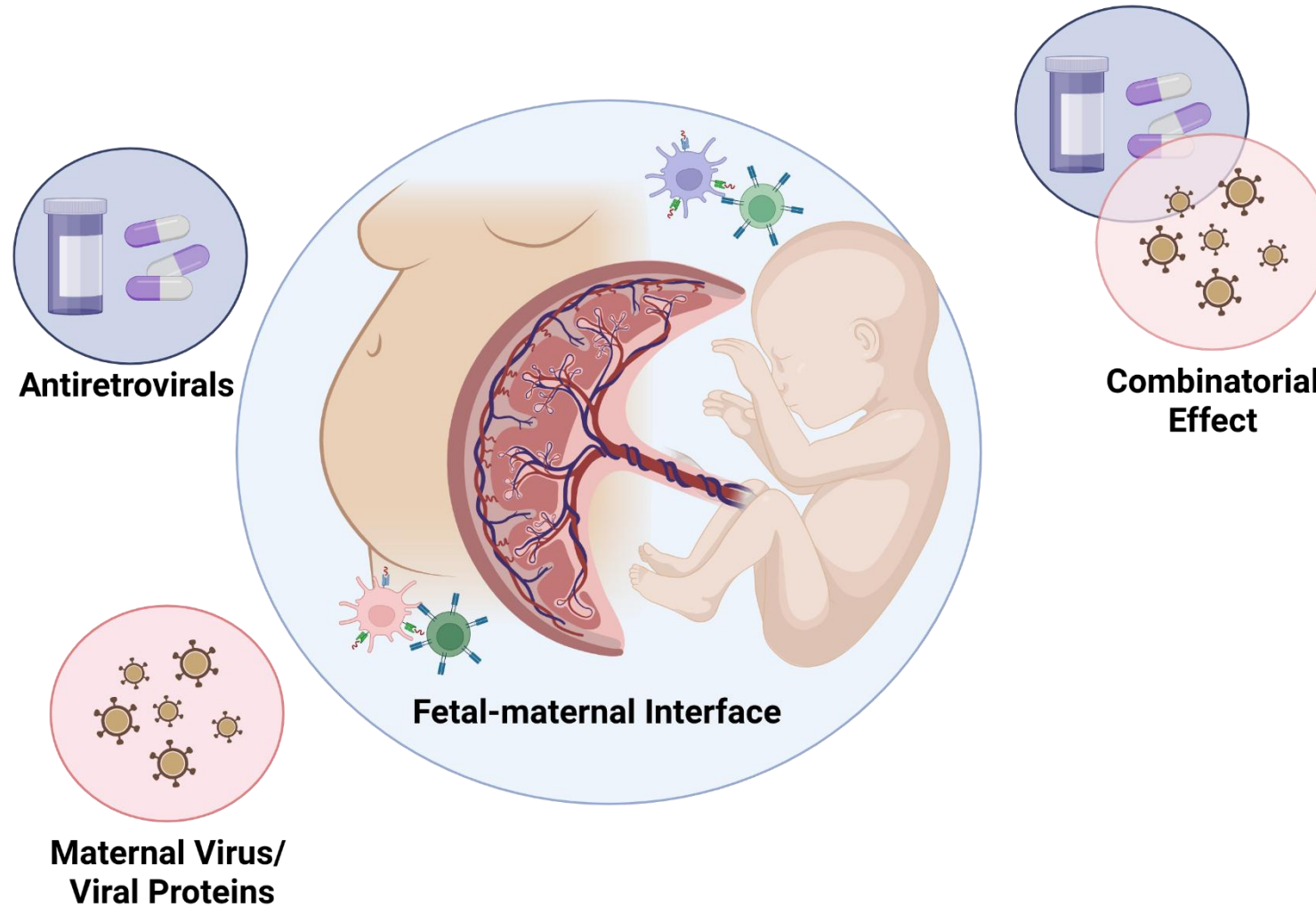
- **9-color** CFC panel: **512** groups at most
- **40-color** SFC panel: **1.1e+12** clusters at most (**trillion**)
- **60-color** SFC panel: **1.2e+18** clusters at most (**quintillion**)



The challenge for unsupervised approaches



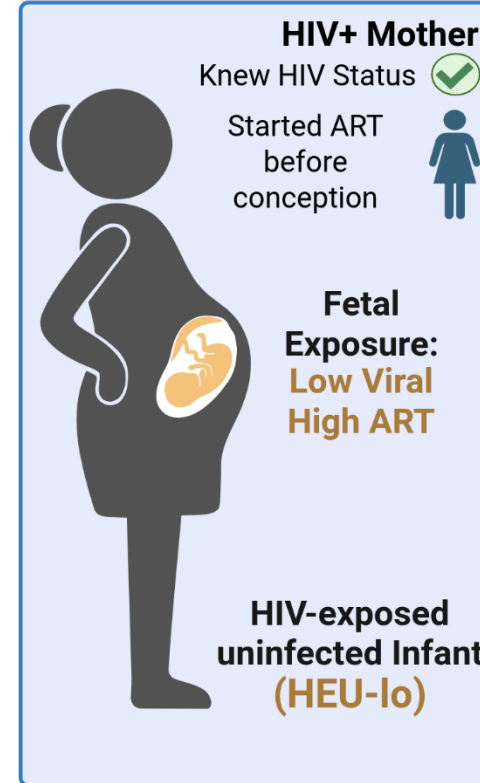
My PhD's focus: HIV-exposed Uninfected Infants



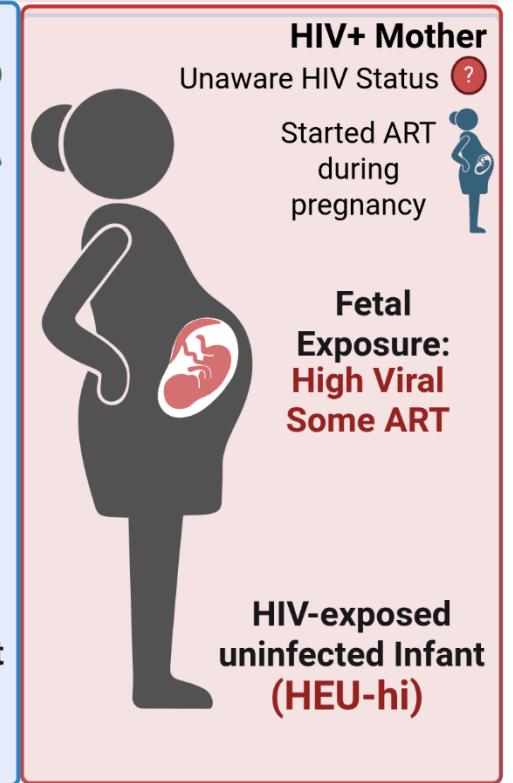
Our Malawian Cohort



Maternal Viral Load: Undetectable



Maternal Viral Load: Very High

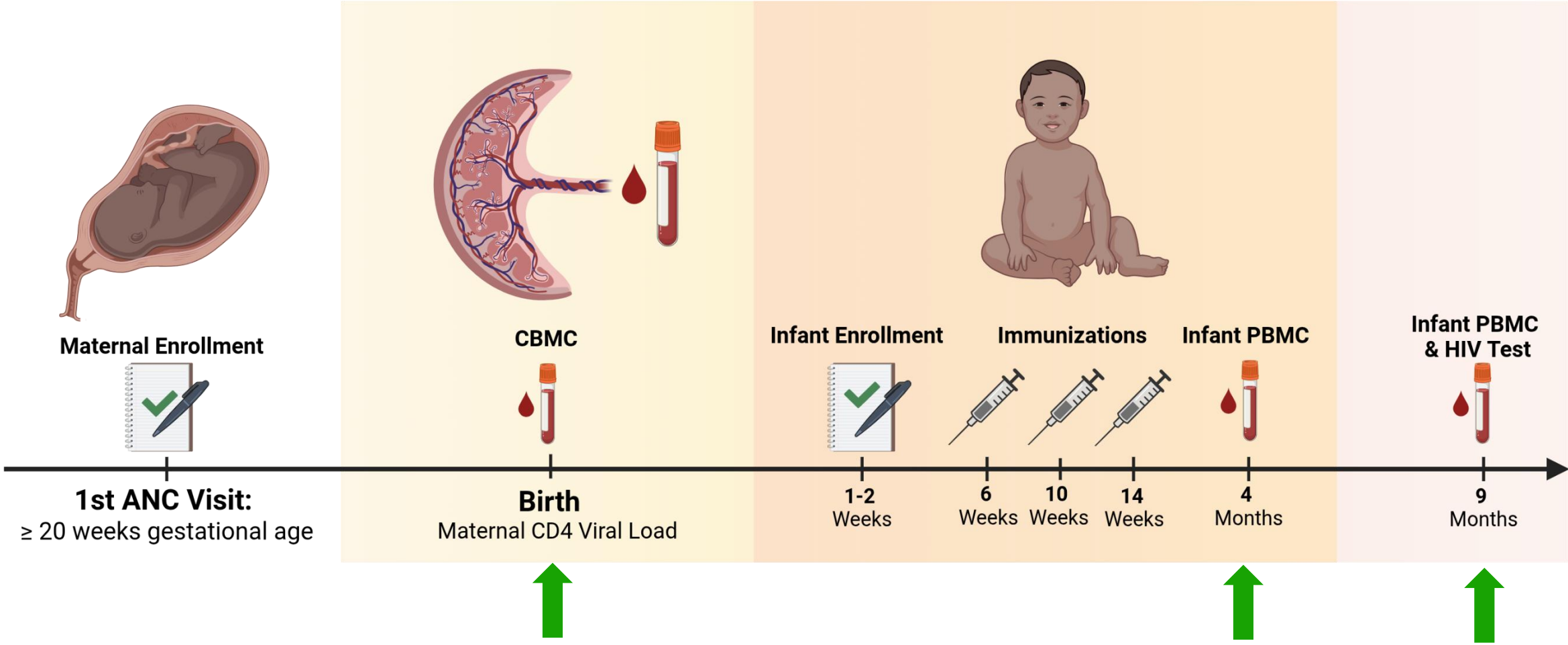


Fetal Antiretroviral Exposure: Extended

Fetal Antiretroviral Exposure: Limited



Study Timeline



32-Fluorophore Spectral Flow Cytometry Panel

UV1	Spark UV 387		
UV2	BUV395	CD62L	(SK11)
UV6	cFluor UV440		
UV7	BUIV496		
UV9	BUV563	CD69	(FN50)
UV10	BUV615	CCR4	(1G1)
UV11	BUV661	V82	(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	ID NIR 786		

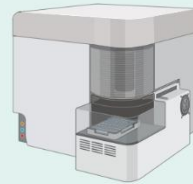
B2	Alexa Fluor 488	FoxP3	(236A/E7)
B3	Spark Blue 550	CD3	(SK7)
B4	Spark Blue 574	CD45	(2D1)
B6	RB613	PD1	(EH12.1)
B7	NovaFluorBlue660		
B8	SBB675		
B9	NovaFluorBlue690		
B10	RB705	CD26	(M-A261)
B12	RB744/		
B13	NovaFluorBlue760		
B14	RB780	CXCR5	(RF8B2)
YG1	PE	ICOS	(ISA-3)
YG2	Spark YG 593		
YG3	PE-Dazzle 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

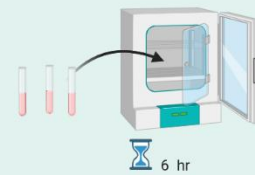
Thaw & Rest



1) Thaw



2) Count

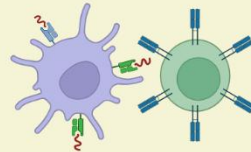


3) Rest
6 hr

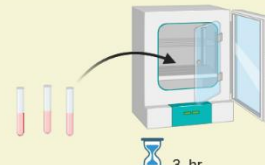
Antigen Incubation



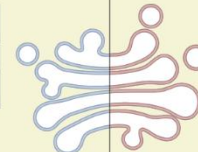
4) Aliquot by condition



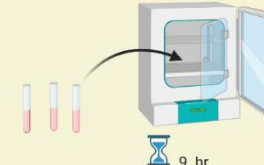
5) Add antigens
(PPD, SEB, Ctrl)



6) Incubate
3 hr



7) Add Golgi
Transport
Inhibitors



8) Incubate
9 hr

Flow Cytometry Staining



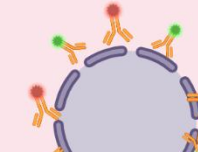
9) Pellet



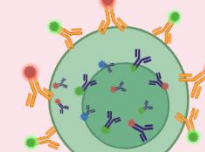
10) Viability
stain



11) Surface
antibodies



12) Fix &
permeabilize



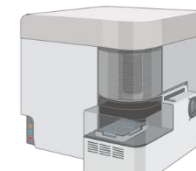
13) Intracellular
antibodies



14) Resuspend
in 0.4% PFA



15) Refrigerate
at 4°C



16) Acquisition
Cytek Aurora 5L

Personal Goal: No cell left behind



Repeated 3 AM thoughts

- ❑ 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

 frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 22 August 2025
DOI 10.3389/fimmu.2025.1628145

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

David Rach¹, Hao-Ting Hsu², Ngina Nampota-Nkomba^{3,4}, Godfrey Mvula³, Felix A. Mkandawire³, Oswald M. Nyirenda³, Bernadette Hritz¹, Francesca Boldrin⁵, Giulia Degiacomi⁵, Laura Cioetto Mazzabò⁵, Riccardo Manganelli⁵, Andrea G. Buchwald⁶, Franklin R. Toapanta⁶, Marcelo B. Sztein⁶, Miriam K. Laufer⁶, Kirsten E. Lyke^{6*} and Cristiana Cairo^{2,7*}

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 Check for updates

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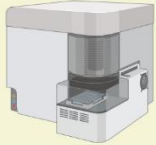
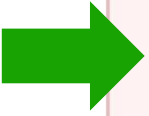
Paper

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



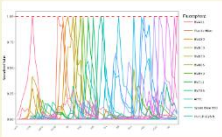
Pipeline: Acquisition



1) Acquisition
(Cytex Aurora 5L)



2) Unmixing Controls
(Lucifera)



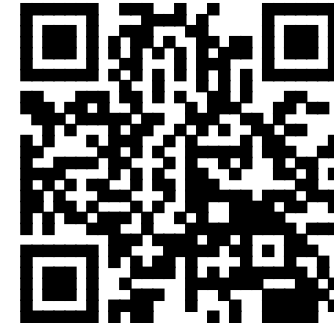
3) Unmixing
(OLS, TRU-OLS, AutoSpectral)



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

Iterating by Experiment

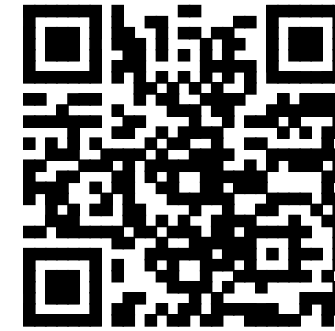
- Acquired day of staining on a 5-Laser Cytex Aurora
- Instrument flushed with 10% Contrad and Water for 20 minutes pre-use
- Instrument passed by both the UMGCCC FCSR and Cytex's QC criteria immediately before acquisition



UMGCCC FCSR
InstrumentQC



Plots for our
5L Aurora



Historical Data
5L Aurora



Dashboard
How-To-Set-Up



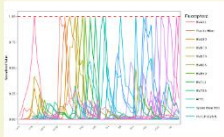
Pipeline: Evaluating Unmixing Controls



1) Acquisition
(Cytex Aurora 5L)



2) Unmixing Controls
(Luciernaga)



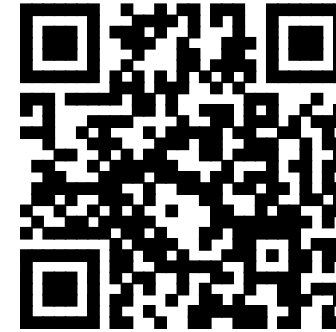
3) Unmixing
(OLS, TRU-OLS, AutoSpectral)



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

Iterating by Experiment

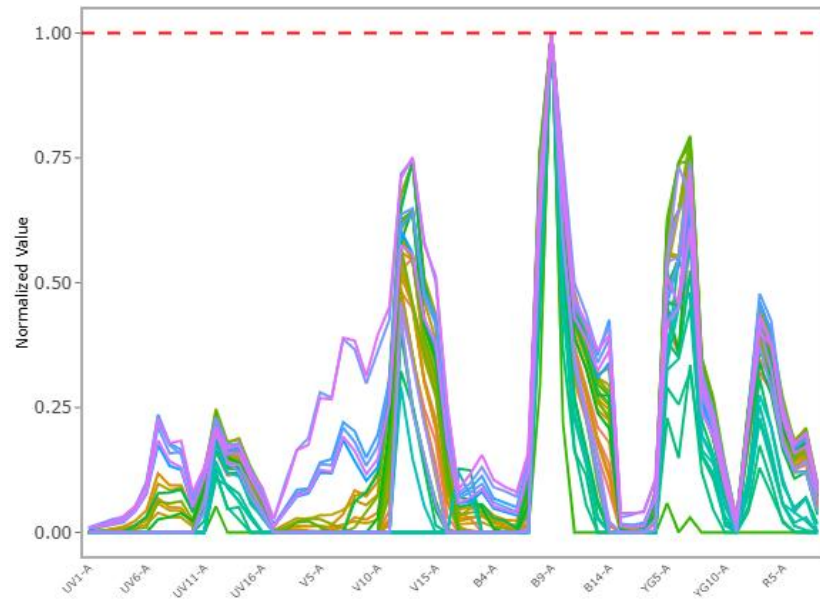
- ❖ New unmixing controls acquired with each experiment.
- ❖ Extra specimen cells acquired as additional unstained controls
- Unmixing Controls are screened for suitability for unmixing
- Single-colors: Tandem degradation, and autofluorescence contamination
- Unstained(s): Presence of multiple-autofluorescences in lymphocyte gate
- Flagged unmixing controls are replaced with equivalents from adjacent experimental date



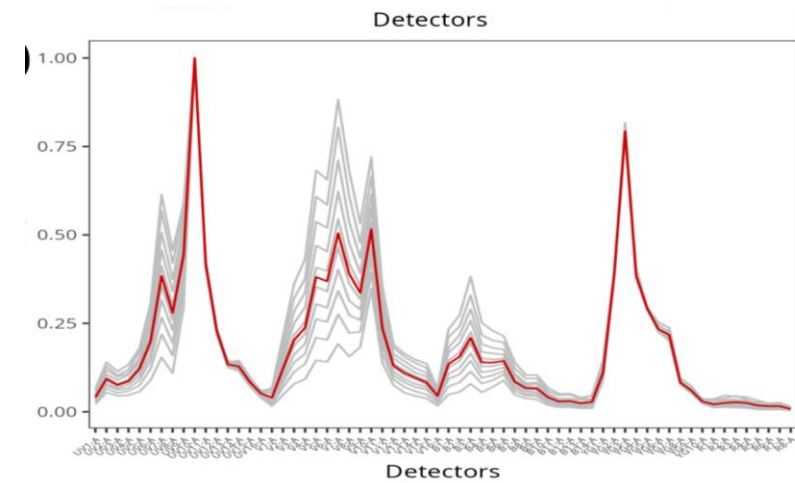
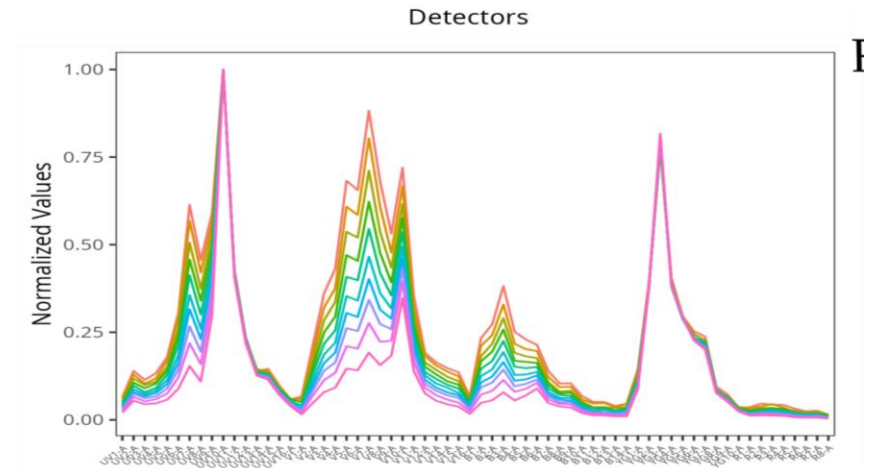
Luciernaga



Luciernaga
Vignette

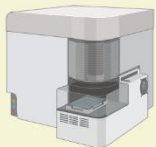


❖ Degrading PerCP-Cy5.5 antibody vial



❖ Leftover autofluorescence contribution to signature

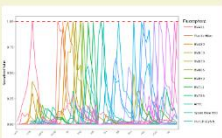
Pipeline: Unmixing



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

- ❖ 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



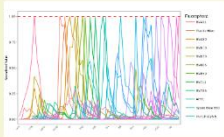
Pipeline: File Checks



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

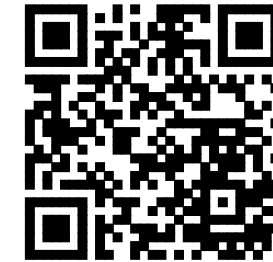
- ❑ Checking for fluidic or laser-related issues in the unmixed files
- R: PeacoQC or FlowAI
- Rust: PeacoQC (thanks James!)



PeacoQC



PeacoQC
Vignette



FlowAI



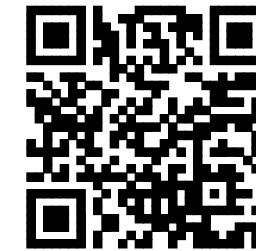
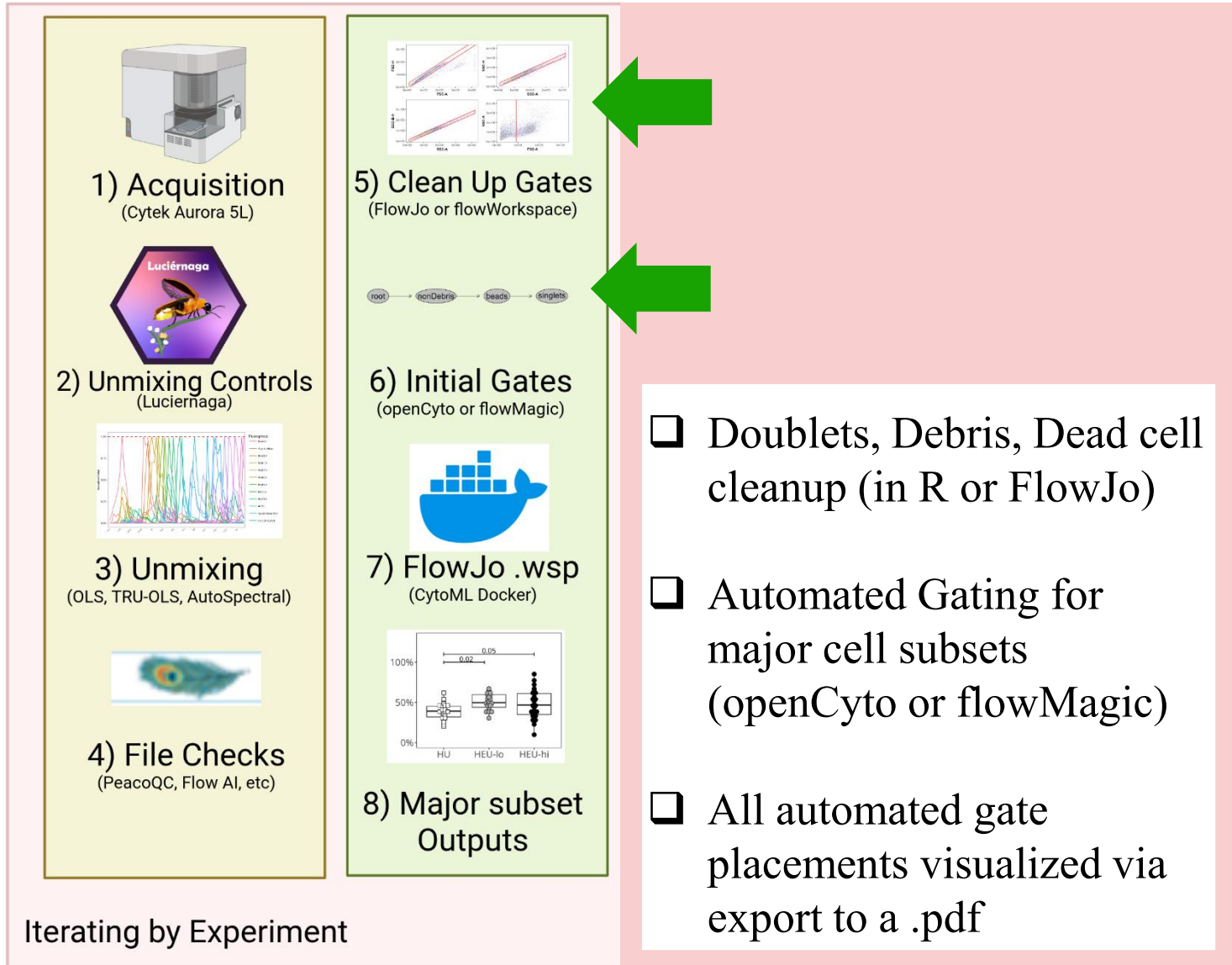
FlowAI
Vignette



PeacoQC
in Rust



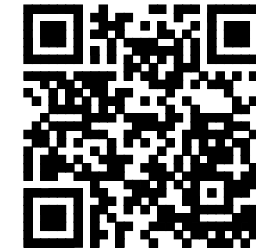
Pipeline: Manual and Automated Gating



flowGate



flowGate
Walkthrough



openCyto



openCyto
Walk-through



flowMagic



flowMagic



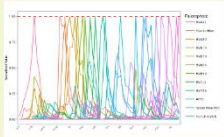
Pipeline: Save to FlowJo v10 .wsp



1) Acquisition
(Cytek Aurora 5L)



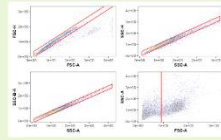
2) Unmixing Controls
(Lucifera)



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



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



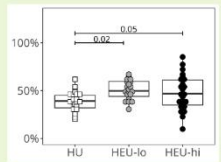
5) Clean Up Gates
(FlowJo or flowWorkspace)



6) Initial Gates
(openCyto or flowMagic)

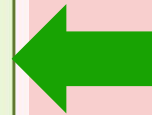


7) FlowJo .wsp
(CytoML Docker)

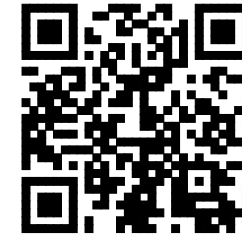
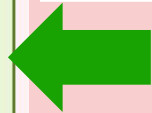


8) Major subset
Outputs

- 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

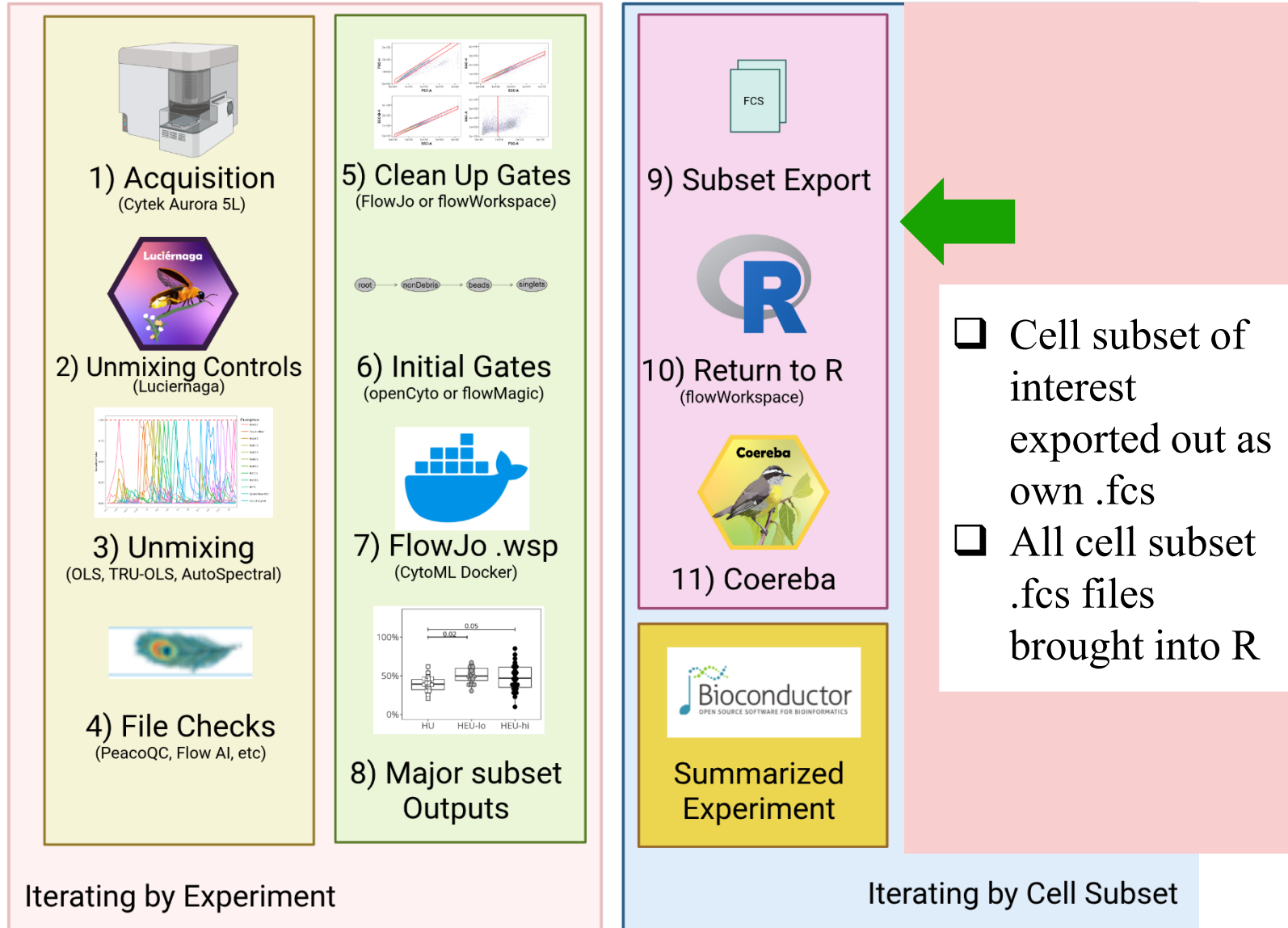


Data for Statistics
Walkthrough

Iterating by Experiment



Pipeline: Iterating through cell subsets



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

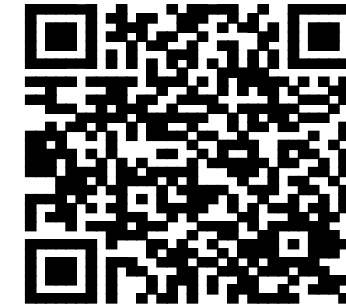
10) Return to R
(flowWorkspace)

11) Coereba

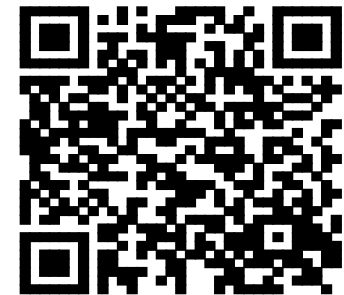
Summarized Experiment

Coereba

Iterating by Cell Subset



Subset Export Walkthrough



GatingSets Walkthrough



Coereba




Coereba Vignette



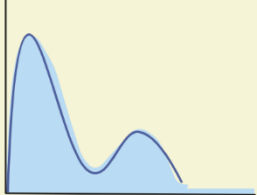
Coereba



Code

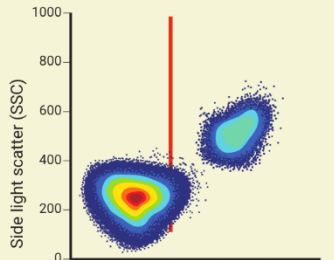


1) Load into R
(flowWorkspace)

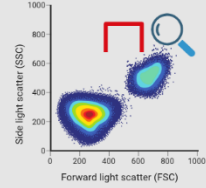


Fluorophore

2) Calculate Splitpoints



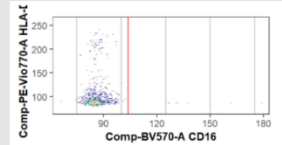
3) Visualize



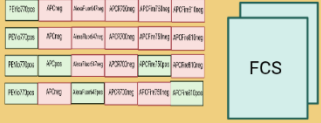
4) Gate Constraints



5) Shiny App adjustments



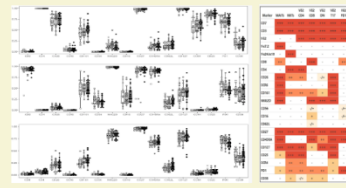
6) Splitpoints updated



7) CoerebaID



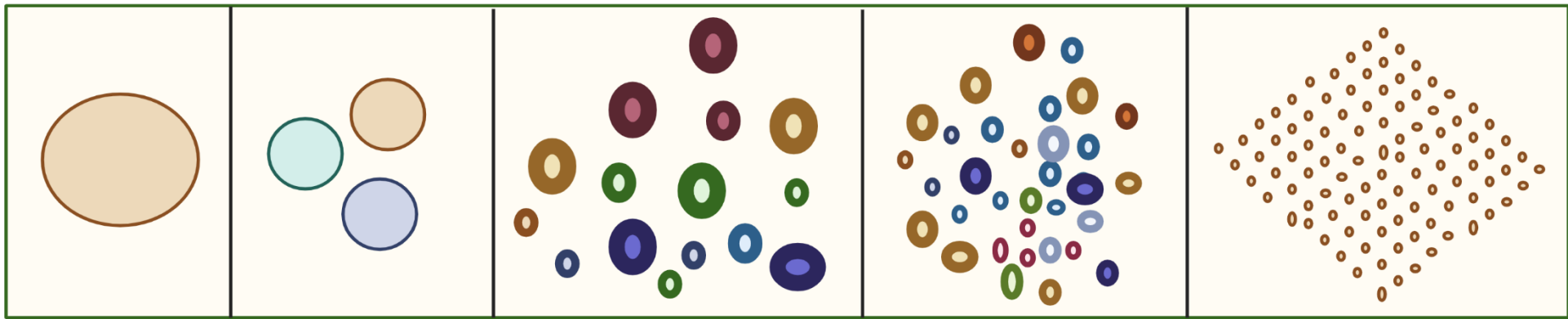
8) Summarized Experiment



9) Visualize



Grouping cells that share every marker



All Cells in a Single Cluster

Major Cell Types

In a *single cluster*, **not all markers** are shared by individual cells.

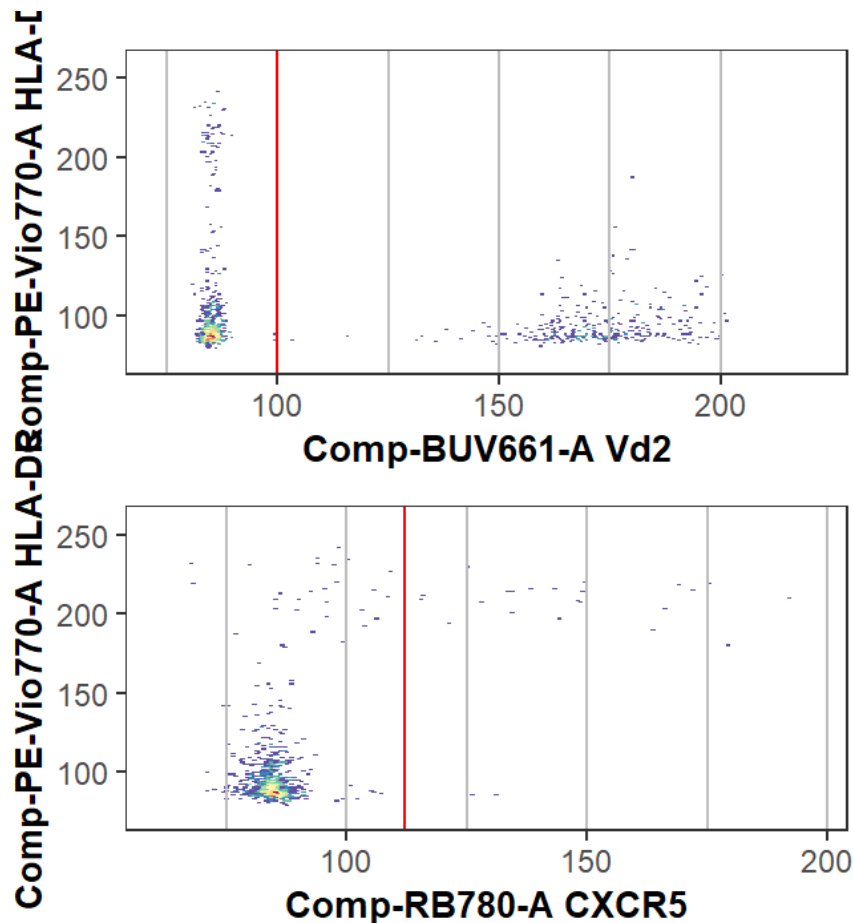
In a *single cluster*, **all markers** are shared by individual cells.

Every cell it's own cluster.





Initial automated gate, with ability to adjust

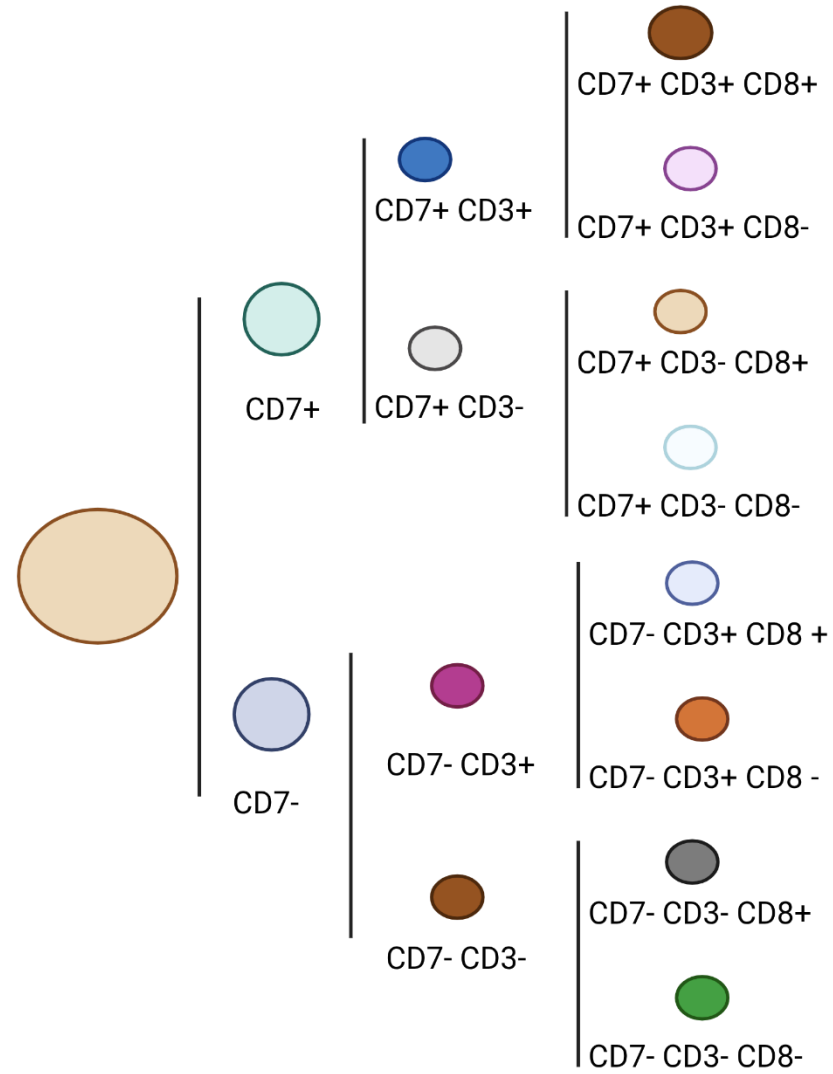


- Initial gates for designated markers calculated via openCyto (mindensity)
- Ability to update gate_range constraints
- Launch a Shiny app and designate interactively

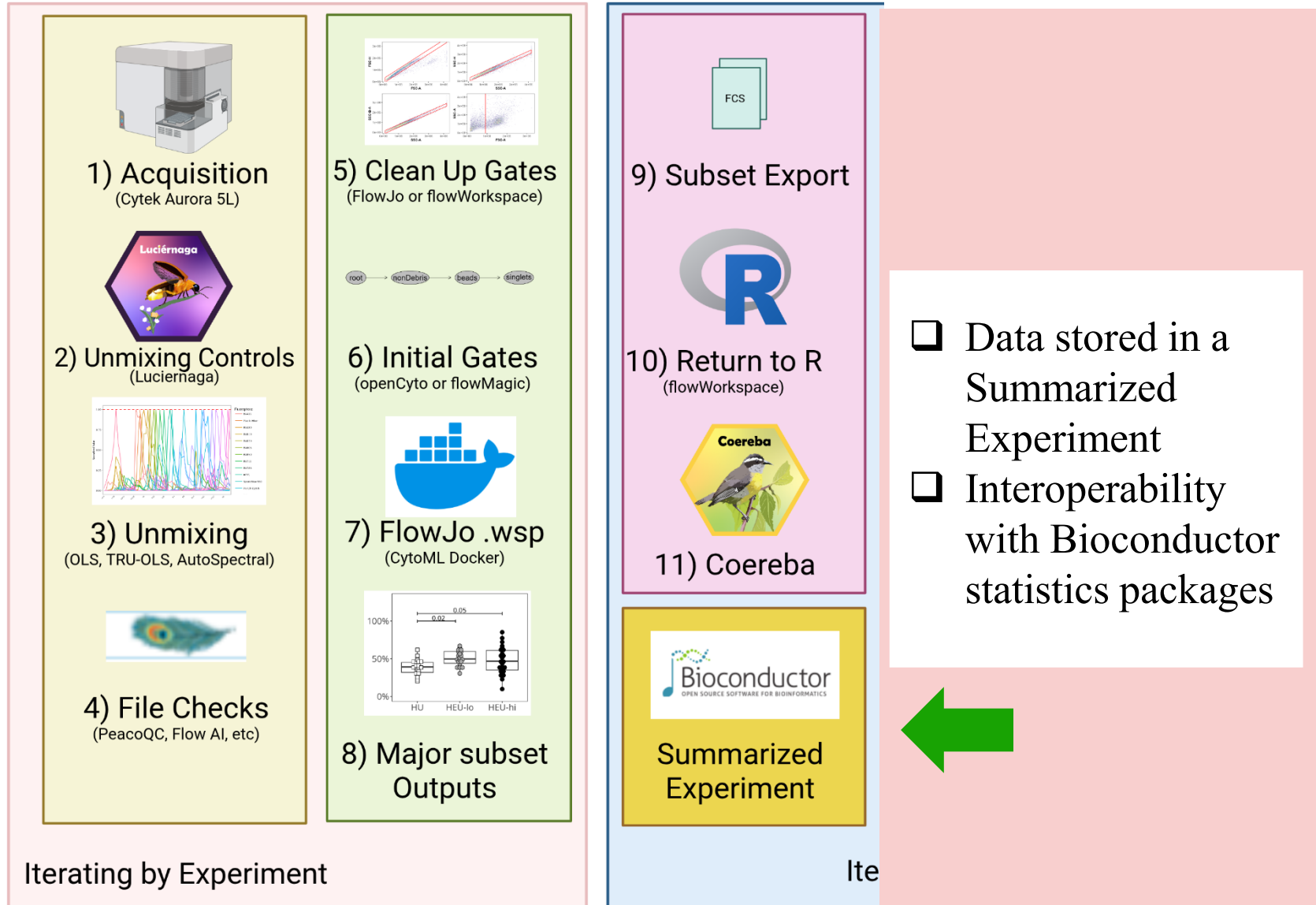


CoerebaID column gets added to the .fcs file

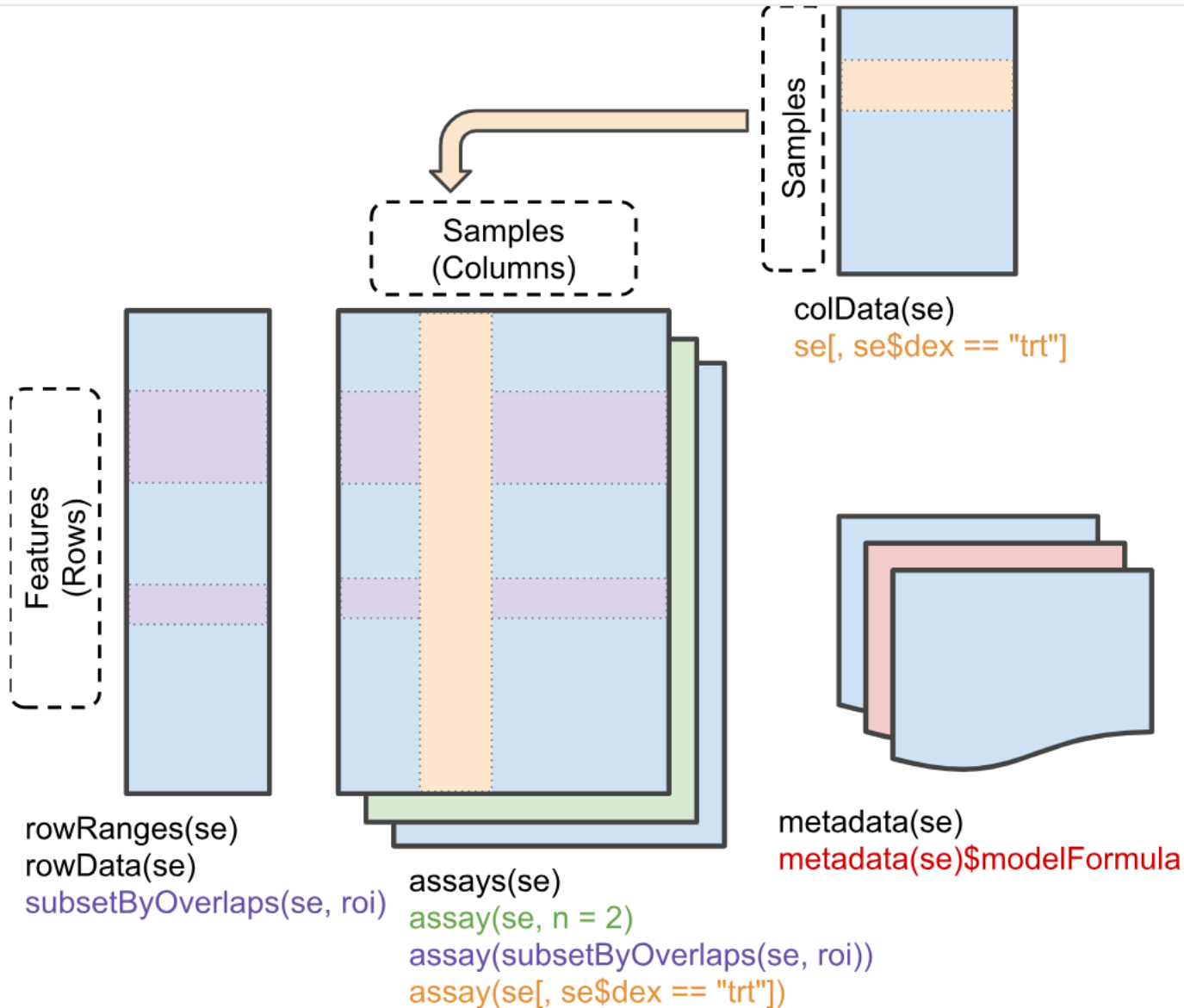
PEVio770pos	APCneg	AlexaFluor647neg	APCR700neg	APCFire750neg	APCFire810neg
PEVio770pos	APCneg	AlexaFluor647neg	APCR700neg	APCFire750neg	APCFire810neg
PEVio770pos	APCpos	AlexaFluor647neg	APCR700neg	APCFire750pos	APCFire810neg
PEVio770pos	APCneg	AlexaFluor647pos	APCR700neg	APCFire750neg	APCFire810pos



Pipeline: Summarized Experiment



Summarized Experiment

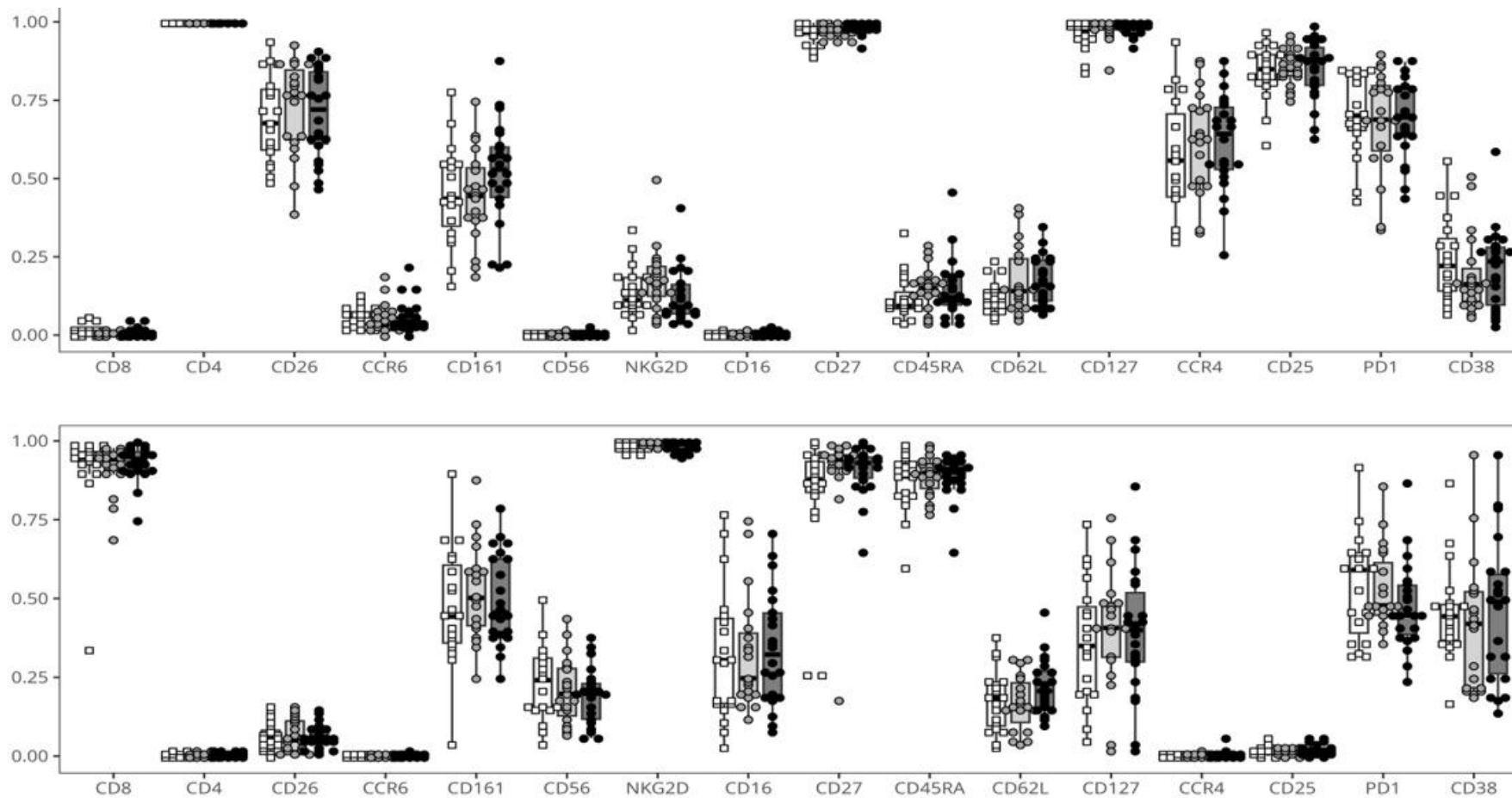


- ❑ Permits rapid re-aggregation of the terminal nodes for re-evaluation
- ❑ Ties in with downstream Bioconductor statistics packages
- ❑ Portable. Don't like how we aggregated groups of cells? File is small enough to store in the same data repository as .fcs files for subsequent re-analysis.
- ❑ Filterable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

Coereba



Retrieval of marker expressions



Pipeline: Normalization

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)

15) Coereba

Summarized Experiment

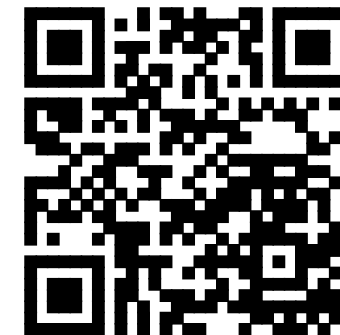
Iterating by Cell Subset



CytoNorm



CytoNorm Vignettes



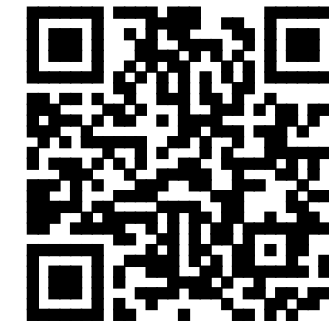
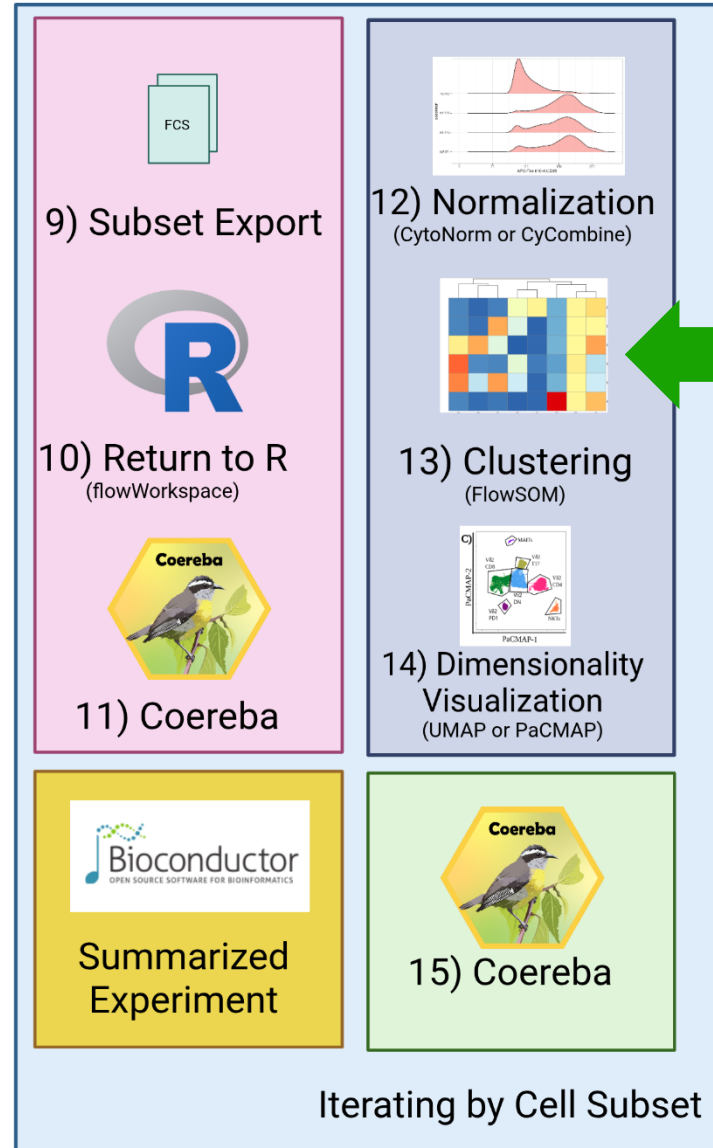
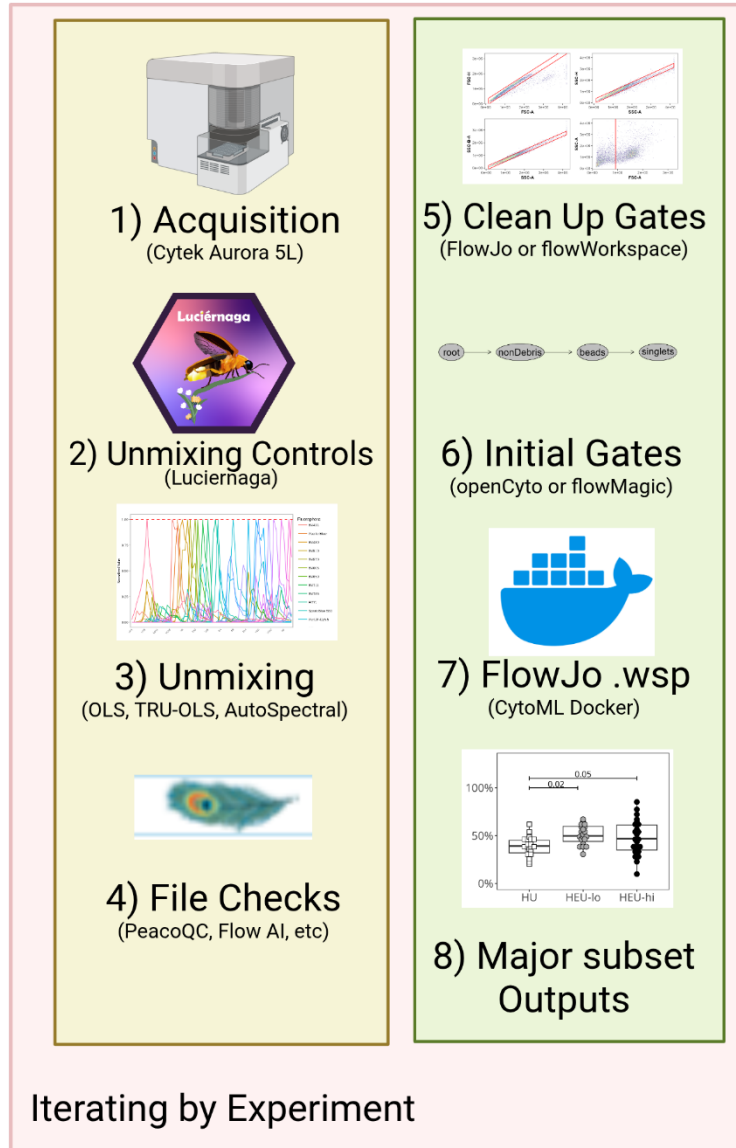
CyCombine



CyCombine Vignettes



Pipeline: Clustering



FlowSOM



FlowSOM
Vignettes

Pipeline: Dimensionality Visualization

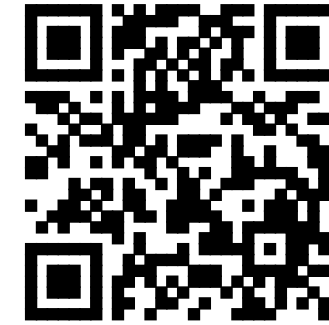
Iterating by Experiment

- 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 Cell Subset

- 9) Subset Export**
- 10) Return to R**
(flowWorkspace)
- 11) Coereba**
- 12) Normalization**
(CytoNorm or CyCombine)
- 13) Clustering**
(FlowSOM)
- 14) Dimensionality Visualization**
(UMAP or PaCMAP)
- 15) Coereba**

Summarized Experiment



UMAP



UMAP
Wrapper



PaCMAP

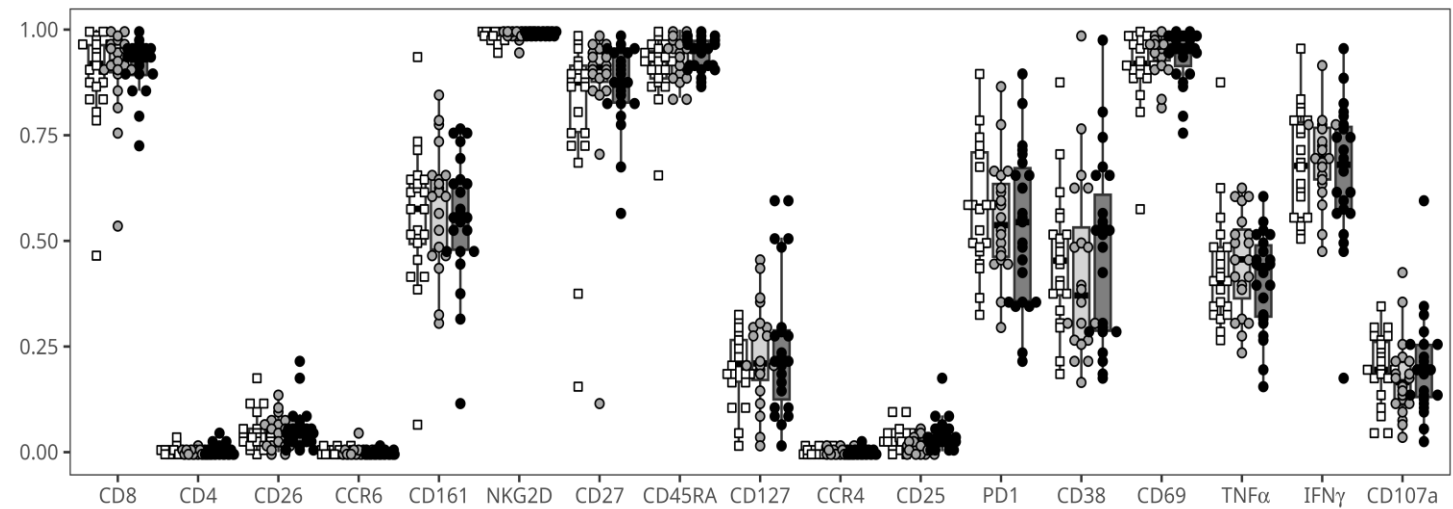
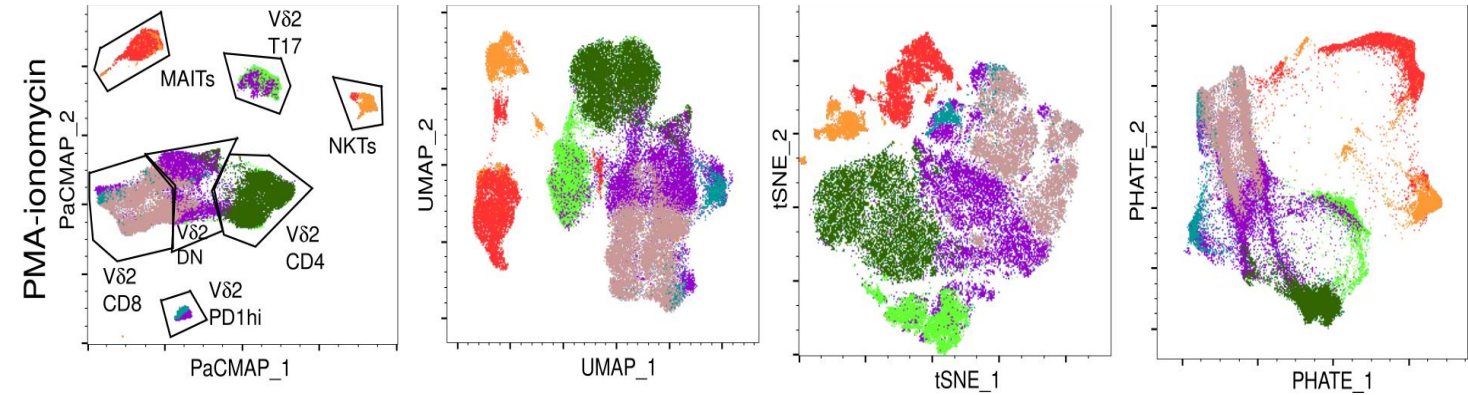


PaCMAP
Wrapper

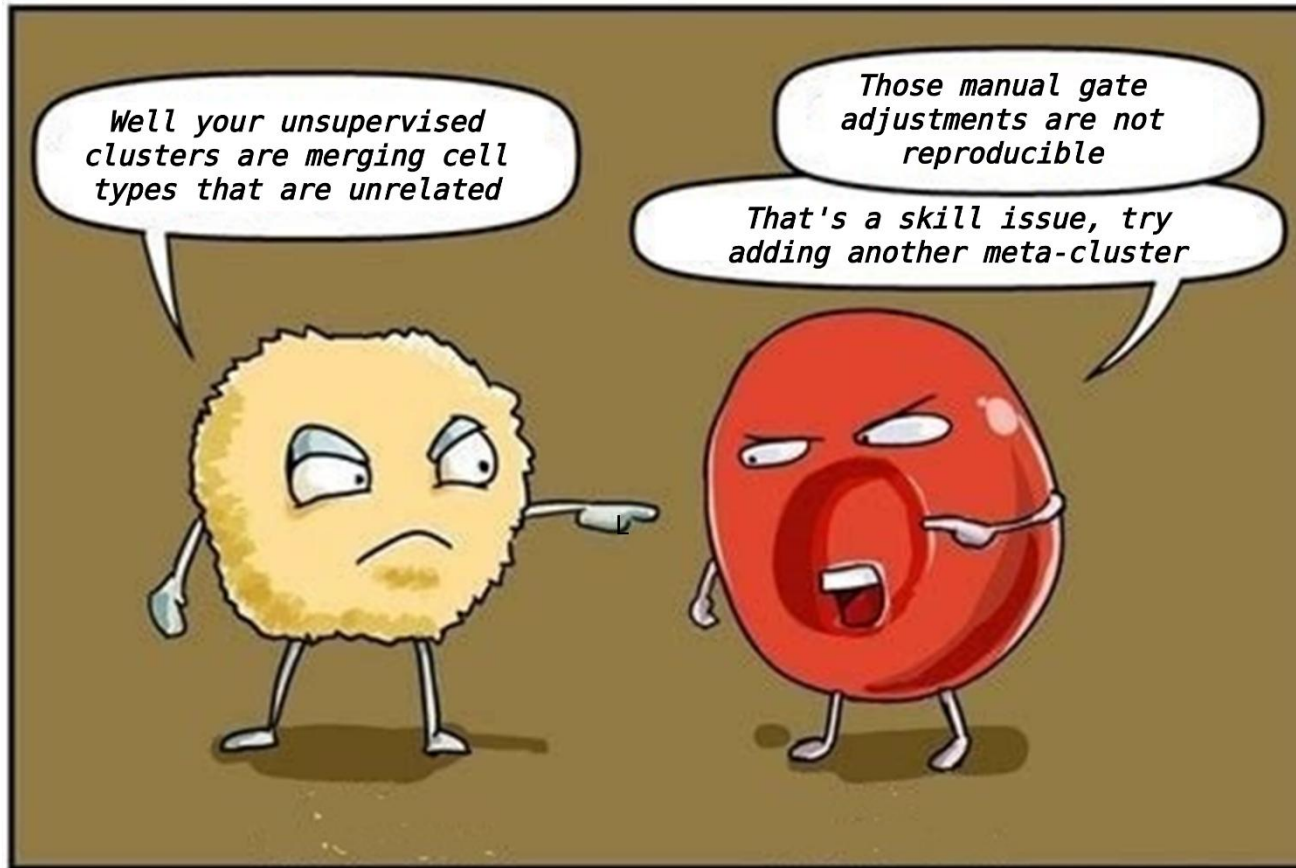


Retrieve manual annotated gating information following unsupervised steps

Marker	MAITs	NKTs	Vδ2 CD4	Vδ2 CD8	Vδ2 DN	Vδ2 T17	Vδ2 PD1
CD7	+++	+++	+++	+++	+++	+++	+++
CD3	+++	+++	+++	+++	+++	+++	+++
Vδ2	-	-	+++	+++	+++	+++	+++
Vα7.2	+++	-	-	-	-	-	-
Vα24Ja18	-	+++	-	-	-	-	-
CD8	++	-	-	+++	-	-	++
CD4	-	+++	+++	-	-	-	-
CD26	+++	++	++	-	-/+	+++	-
CCR6	+++	-	-	-	-	+++	-
CD161	+++	++	++	++	+	+++	-
NKG2D	+++	-	-	+++	+++	+++	-
CD56	-	-	-	-/+	-	-	-/+
CD16	-	-	-	+	-	-	-/+
CD62L	-	-	-	-/+	-	-	-
CD27	+++	+++	+++	+++	+++	+++	+++
CD45RA	+++	-	-	+++	++	+++	+++
CD127	+++	+++	+++	+	+++	+++	-
CD25	+	+++	+++	-	-	+++	-
CCR4	-	++	++	-	-	+	-
PD1	+	++	++	++	++	+	+++
CD38	-	+	-/+	+	+	-	-



Functional and Iterative: plenty for everyone to dislike 😊



RunawayLabBook.com

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



UNIVERSITY of MARYLAND
SCHOOL OF MEDICINE

Acknowledgements

All mothers and their infants, and their communities

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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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KAMUZU
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Molecular Microbiology Immunology Graduate Program

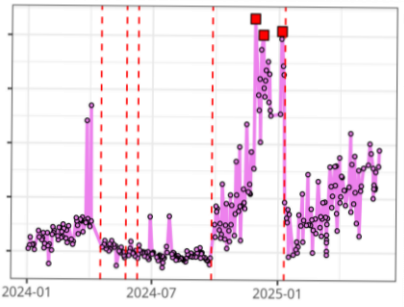
Dr. Eileen Barry
Dr. Brett Hassel
Dr. Heather Ezelle
Bess Tracey

Thesis Committee

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



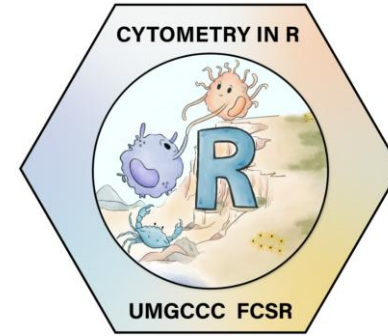
Questions?



UMGCCC FCSR
InstrumentQC dashboard



Luciernaga



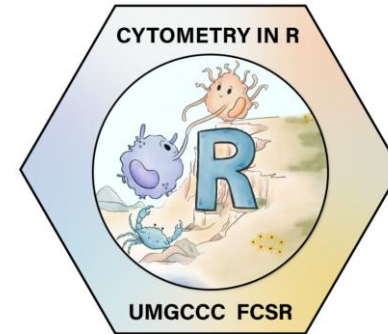
Website - CytometryInR



InstrumentQC How-To



Coereba



YouTube @CytometryInR

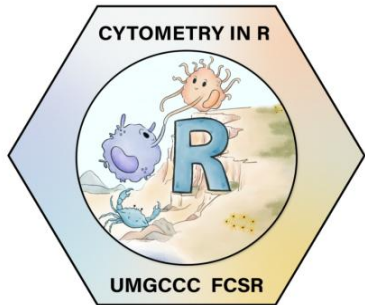
Learn R at your own pace



Cytometry in R: A free weekly course for coding beginners

David Rach^{1,2}, Natarajan Ayithan², Xiaoxuan Fan²

¹ Molecular Microbiology and Immunology Graduate Program, University of Maryland School of Medicine, Baltimore, USA
² Flow Cytometry Shared Resource, University of Maryland Greenebaum Comprehensive Cancer Center, Baltimore, USA



CYTOMETRY IN R



UMGCC FCSR



Website

Rationale

With the emergence of spectral flow cytometry, we can quickly profile 20+60 markers for millions of cells in minutes. Consequently, analysis of our datasets is increasingly complex. Semi-supervised & Unsupervised Analytical methods are being developed in response, many often implemented as R packages. While many flow cytometrists express an interest in learning R, they often don't know where to start. The limited existing resources are typically geared towards those with intermediate bioinformatics skills, presenting additional barriers to entry. Starting in February 2026, we have been offering a free weekly Cytometry in R course, primarily aimed at those with prior flow cytometry knowledge, but no-to-limited prior experience with programming language R.

Our teaching approach

One topic per week, roughly for an hour. Beginners need detailed examples that can be worked through on their own time. Take-home problems can additional enable acquisition independent code troubleshooting skills. Consistency is key, being able take what you learn and apply to your own datasets helps maintain this. Systematically build on prior concepts, enabling tackle more complicated problems as course advances. Beyond Cytometry in R, provide a solid R foundation and teach coding best practices and reproducible workflows.

Who is currently taking the course?

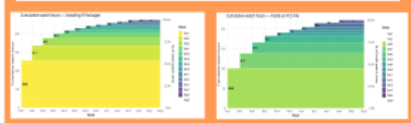
Following course announcement in November, we quickly had over 2000 people worldwide sign up for course emails.



Based on GitHub account creation, and forking of the CytometryinR course repository, over 600 participants have started the course.



Based on our Google and YouTube analytics, we currently have around 200-300 active weekly participants worldwide. Many are following along at their own pace.



A free bioinformatics course for those with prior flow cytometry experience, but no-to-little coding experience

Our course website is created with Quarto, and hosted on GitHub pages. Contains explanations, code, code outputs and screenshots. Each week has its own page. Extensive use of cross references to both internal and external resources. Search Bar for when we forget where it was covered. Teaching R from a Biologist perspective. Easily editable and expandable for community contributions.

Code is available via our course GitHub repository. We provide actual data needed to run the code. Course participants create a free a GitHub account. To start course, fork (i.e. copy) the course repository. Bring in new course materials each week by syncing, and pulling to your own computer. Open Science & Open Source. All Materials: All Code:

Website

Code

YouTube

Not everyone learns well by just reading a website. Some topics take longer to grasp. Being able to revisit the material is key. When life gets busy, ability to circle back to where you had left off. We offer 3 YouTube livestreams per week: - Tuesday 10:00am EST (GMT-4) - Wednesday 3:30pm EST (GMT-4) - Thursday 10:00am EST (GMT-4). Recordings available immediately after on YouTube. Best of the livestream recordings is added to the course playlist.

Virtual and need help? We have you covered. Our GitHub Discussions page serves as a community forum. Have an installation issue? Need extra clarification? Want to show off your plots? Create a post! Added benefit that anyone taking the course in the future can benefit from the same answers.

Schedule	Introduction to R										Cytometry Core																			
Schedule	Unsupervised Analysis										Beyond the Sandbox										The World's your Oyster									

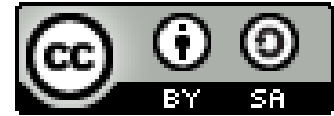
Course Participant Feedback Survey #1

Future Plans

David finally graduates June 17 and gets his free time back! Wrap up our planned 30-week sequence, and make available resources that entire community will benefit from. Rising tides float all boats. There are 12 Bioconductor (+ ~30 GitHub) cytometry-focused R packages, many under-utilized. What if every one also had a community walk-through? Y quizás también así todavía me queda café... Citometria en R. Our core is supported by funds through the Maryland Department of Health's Cigarette Restitution Fund Program - CR4F-CR and the National Cancer Institute - Cancer Center Support Grant (CCSG) - P30CA14746.

Take some stickers and track our progress!

<https://github.com/UMGCCFCSR> flowcore@som.umaryland.edu



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

IT'S BEEN A LONG TIME COMING

DOCTORAL DISSERTATION DEFENSE
DAVID THOMAS RACH

*"CHARACTERIZATION OF CORD BLOOD
INNATE-LIKE T CELL RESPONSES IN NEONATES BORN TO
HEALTHY WOMEN AND WOMEN LIVING WITH HIV"*

COMMITTEE CHAIRS
DRS. CRISTIANA CAIRO & KIRSTEN E. LYKE

WEDNESDAY, JUNE 17, 2026
12:00 PM — 1:00 PM EST (GMT-4)



<https://www.youtube.com/@CytometryInR>
<https://github.com/DavidRach>