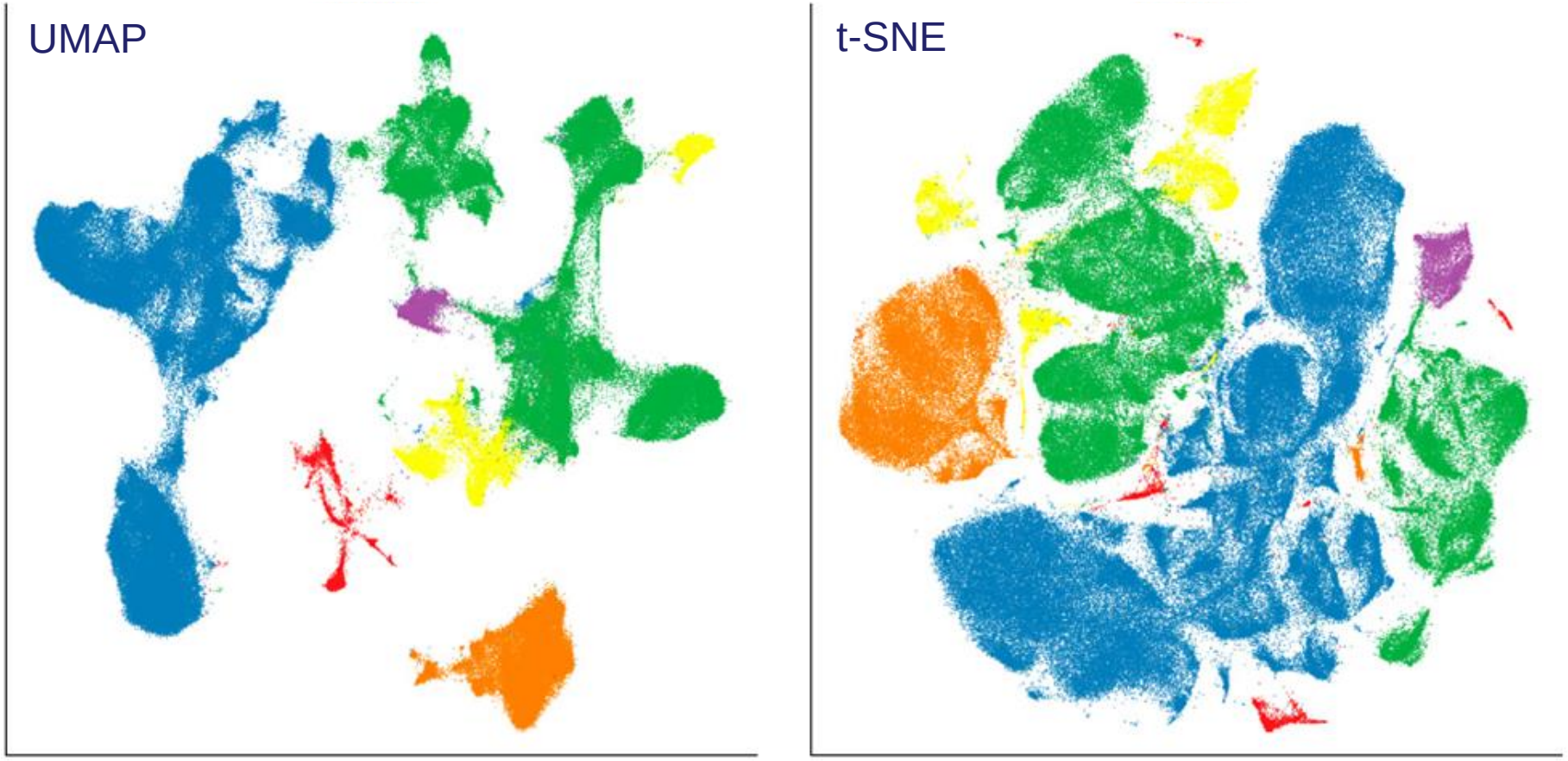
Single Cell Biology & Data Science Methods



Becht et al. 2018

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Web Applications
Research Assistant

Code & Examples for Today:

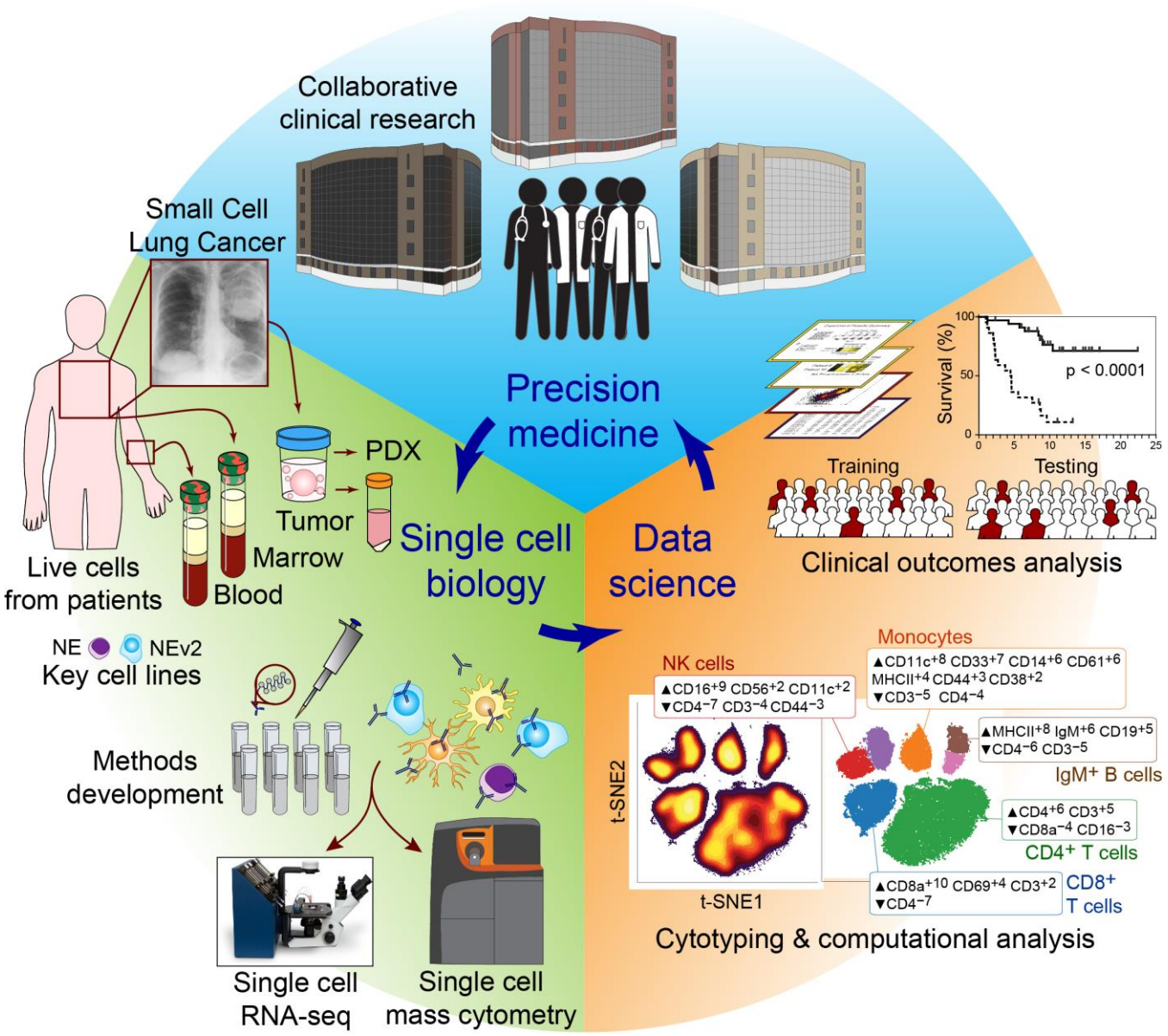
https://cytolab.shinyapps.io/cGVHD/

https://github.com/cytolab/irish-data-science

https://github.com/cytolab/mem

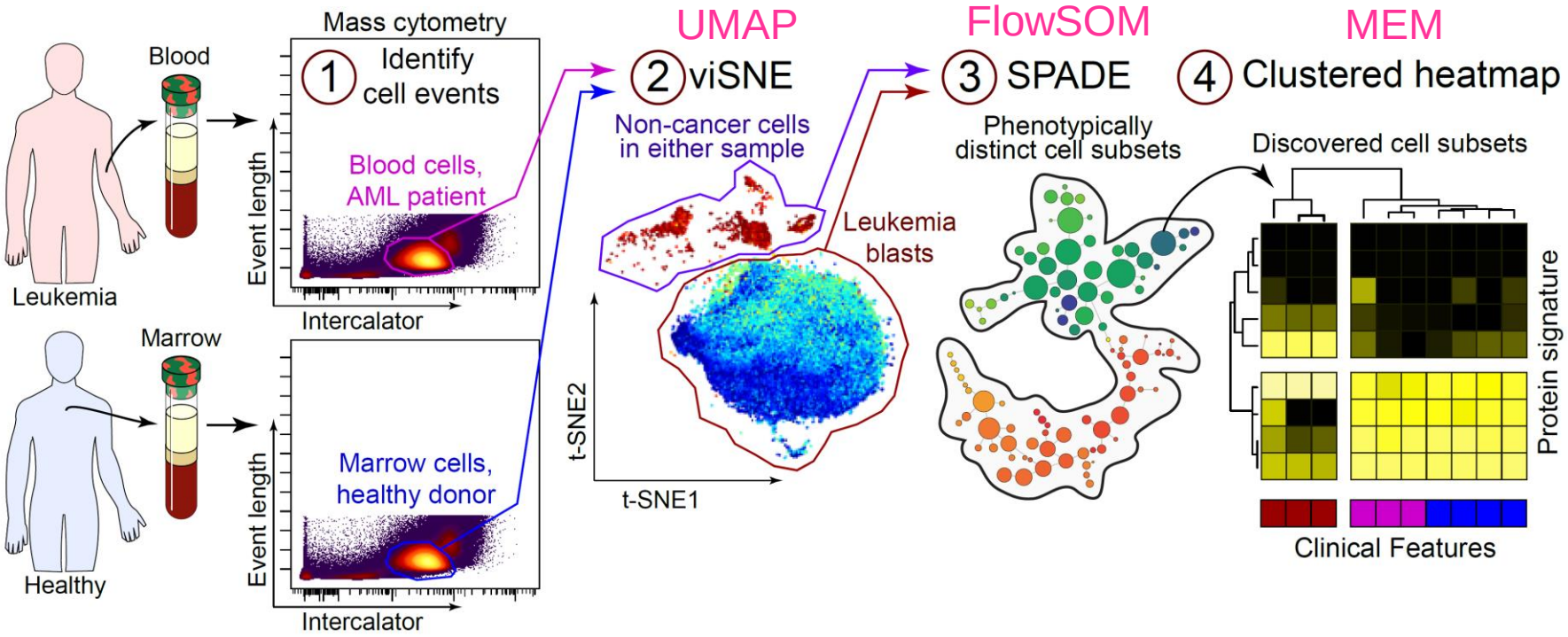
Part 1: Introduction to Data Science

Goal: Systematically Dissect Cellular Mechanisms Across Time, Treatments, Tissues, & Tumor Types



Irish & Diggins

Machine Learning Is a Key Skillset for Biologists (And the Tools Are Rapidly Evolving)



Protocol: Diggins et al., *Current Protocols in Cytometry* 2017
Data files: https://flowrepository.org/id/FR-FCM-ZZKZ
More great tools: Saeys et al, *Nature Reviews Immunology* 2016

Original research:
Diggins et al., *Methods* 2015
Diggins et al., *Nature Methods* 2017

Typical workflow and goal: learn & label cytotypes (cell identities), reveal and assess unexpected & abnormal cells

Need: human reference data (more examples) with annotations

Effective data analysis is critical in clinical research, & this now means working *with* computational tools that reveal and model patterns across data types

Tools from one area can be applied in others (economics, math, patients, cells, pixels, …)

Data science workshop can be self-taught:

https://github.com/cytolab/

Discussion Questions We Expect to Cover

1) What are key differences between tools (t-SNE/viSNE, SPADE, UMAP, FlowSOM, PCA, MEM, Citrus, etc.)? What is the difference between transforming, clustering, and modeling data? What type of modeling are we doing (if any)?

2) What do terms like linear or parametric analysis mean?
Does the data's scale matter (arcsinh5, arcsinh15, linear)?

3) What do all the settings do (e.g., t-SNE iterations, perplexity, SPADE downsampling & node #)? When should they be changed?

4) How does one compare new samples with a prior analysis?
How do we test tools with expert gating?

5) What are some "red flags" indicating problems? What does a good t-SNE, UMAP, FlowSOM, or other analysis run look like?

But first: what is data science?

Irish lab view of data science:

Systematically varying analytical elements
in order to test a hypothesis

(Varied analytical elements might be different data types, data sub-samples, different initial assumptions, contrasting analytical tools, input parameters, etc.)

It's relatively new that datasets are robust enough to enable mining & exploration.

Rumsfeldian Data Science

Known knowns: What do you know about your system?

Known unknowns: What do you know remains to be learned?

Unknown unknowns: What don’t you know you don’t know?

Donald Rumsfeld (Feb 12, 2002): Reports that say that something hasn't happened are always interesting to me, because as we know, there are known knowns; there are things we know we know. We also know there are known unknowns; that is to say we know there are some things we do not know. But there are also unknown unknowns – the ones we don't know we don't know. And if one looks throughout the history of our country and other free countries, it is the latter category that tend to be the difficult ones.

Socratic Data Science

Known knowns: What do you know about your system?

Known unknowns: What do you know remains to be learned?

Unknown unknowns: What don't you know you don't know?

Unknown knowns: What don't you know, but think you do?
i.e. Which 'priors' are incorrect?

If you fear incorrect priors, unsupervised analysis may be able to help.

Socrates according to Plato's *Apology*: I am wiser than this man, for neither of us appears to know anything great and good; but he fancies he knows something, although he knows nothing; whereas I, as I do not know anything, do not fancy I do. In this trifling particular, then, I appear to be wiser than he, because I do not fancy I know what I do not know.

Citrus & RAPID Connect Cell Clusters to Clinical Outcomes, RAPID is Designed for Unsupervised Analysis of Survival

	Citrus Bruggner, Tibshirani, et al., PNAS 2014	RAPID bioRxiv 2019
Finding cell clusters	Unsupervised (hierarchical clustering, cells may be in 2+ clusters)	Unsupervised (various: FlowSOM, KNN, t-SNE + FlowSOM)
Determining number of cell clusters to seek	Unsupervised (must be >5% of sample)	Unsupervised (seeks few clusters w/ low internal variation)
Modeling cluster features	Supervised, multivariate (lasso regularized logistic regression, nearest shrunken centroid)	Unsupervised, univariate (median or MEM, simply a statistical description of cluster)
Splitting patients into groups	Supervised, happens at start (expert knows cut points, assigns patients to groups)	Unsupervised, happens at end (cluster abundance as cut point, Cox model of hazard)

Defining Your System

1) Elements, the studied units of the system.

- Patients, cells, images, pixels, transcripts, genomes, peptides.
- We will envision elements as "rows" in a spreadsheet.

2) Features, the things measured for each element.

- Clinical outcomes, phospho-proteins, pixel density, nucleotides.
- We will envision features as "columns" in a spreadsheet.
- Feature selection may rely on hypotheses, rules, or prior knowledge.

3) Scales, the type & range of the measurements for each feature.

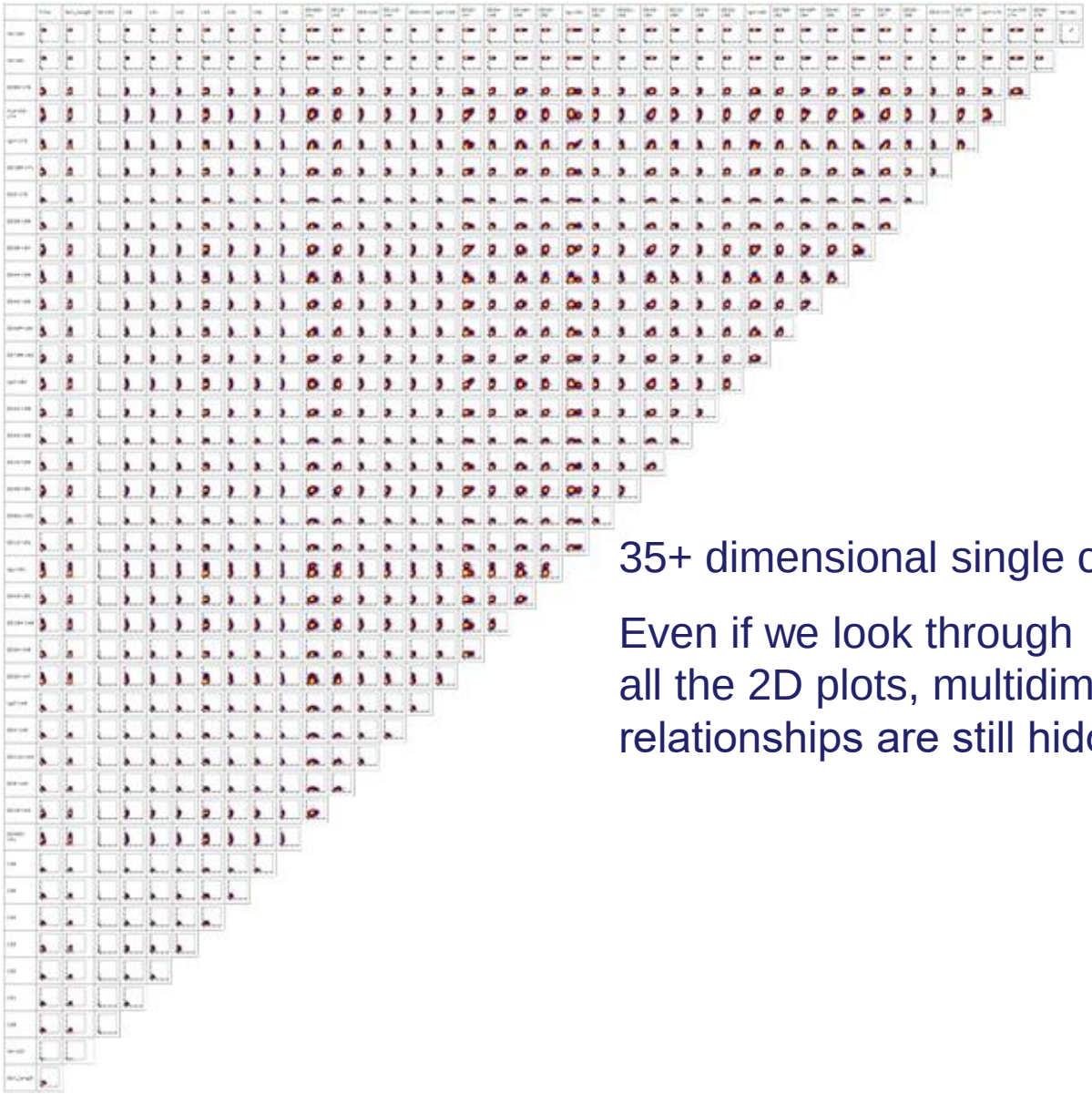
- Categorical, linear, log & base, arcsinh & cofactor.
- -150 to 262,144; 1 to 10,000; 0 to 50; 1 to 100; 0 to 1; NR, PR, CR.
- Will largely explore the data without units until we create reports.

4) Prior knowledge, the things assumed to be known for the system.

- Organization of elements (groups, order, etc.), feature relationships.
- Supervised analysis explicitly uses prior knowledge.
- Unsupervised analysis looks for patterns without prior knowledge.

~~Part 2: Quantifying Cell Biology & Cytometry Tools~~

We Now Make Billions of Multi-D Single Cell Measurements => Need for Machine Learning Tools & Human Readable Views



35+ dimensional single cell data:

Even if we look through
all the 2D plots, multidimensional
relationships are still hidden…

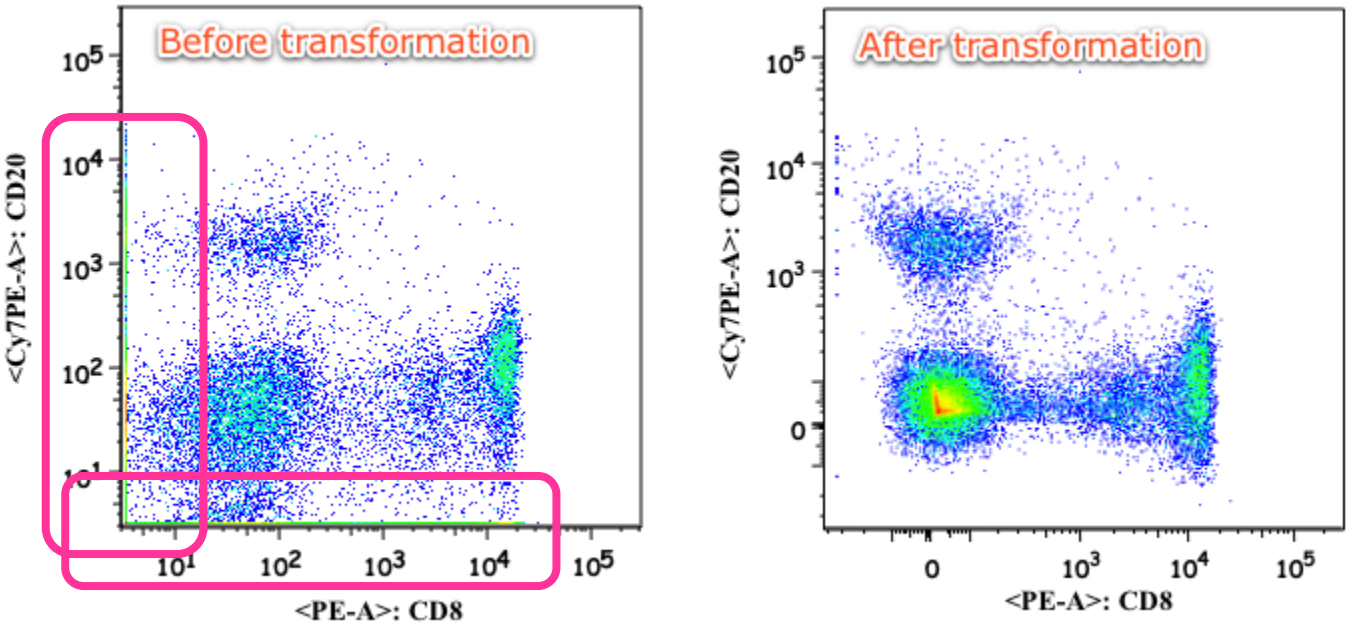
Part 3: The Importance of Scales

Before we get to ‘desert’, some math ‘veggies’:

Scales matter: poorly or variably scaled data can destroy an analysis, most issues arise near zero

(pre-processing & normalization can also be critical)

Have you ever noticed two peaks within the cells that are biologically 100% negative for a marker?

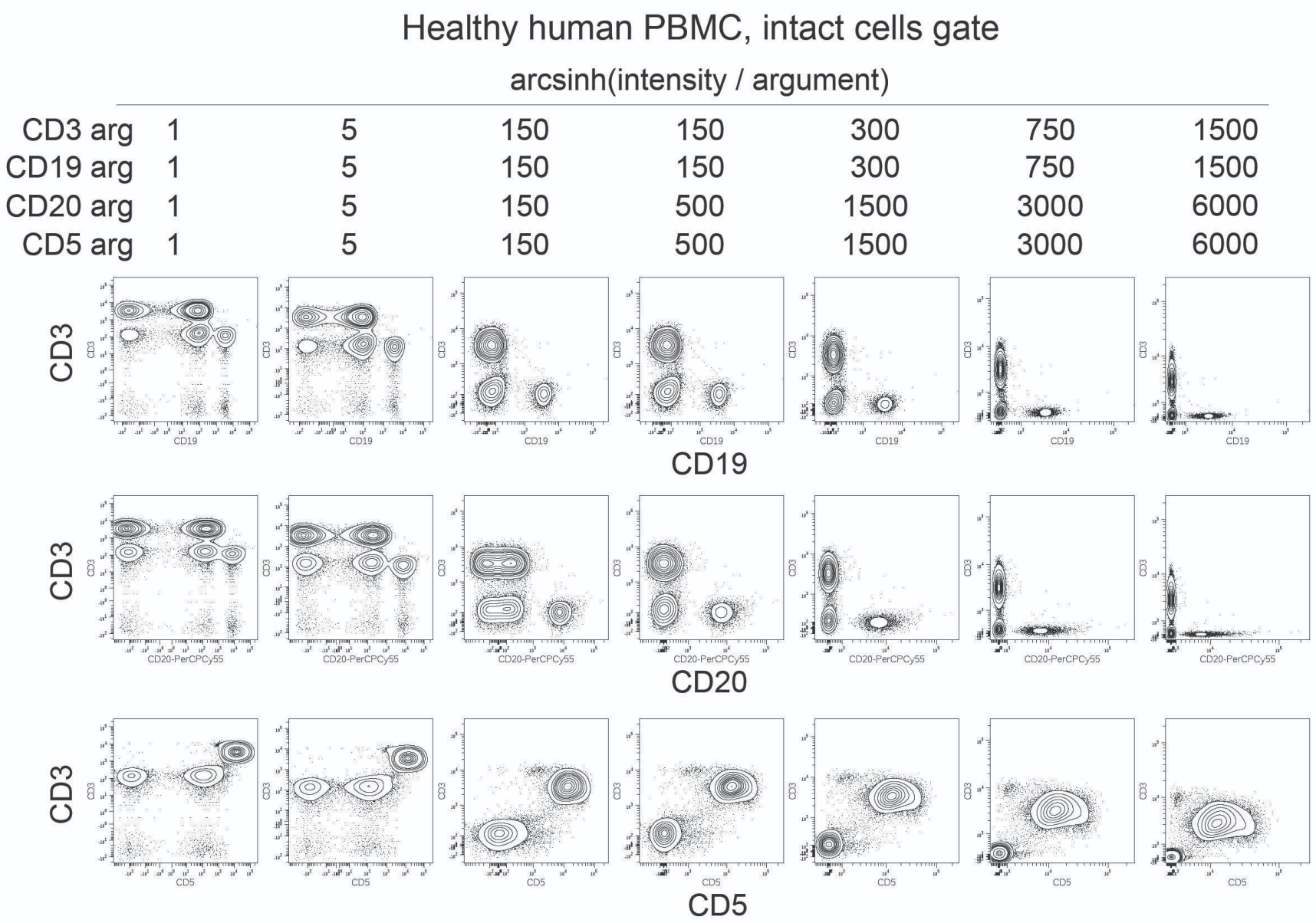


http://www.flowjo.com/v76/en/displaytransformwhy.html

Results from bad scaling (poor transformation)
and it can be an issue for computational analysis.

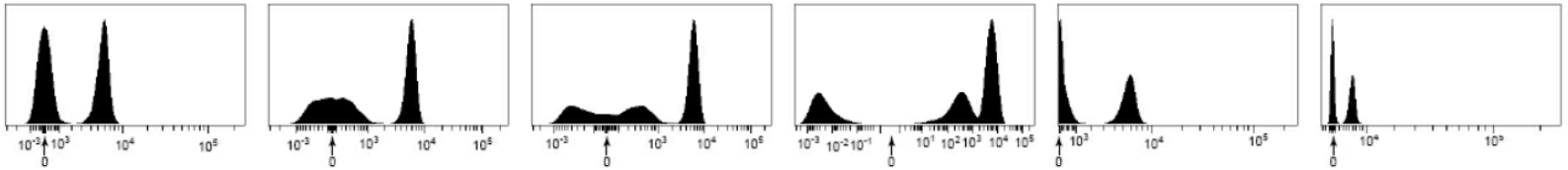
Scaling is important in both mass and fluorescence cytometry.

Scaling Matters for Measuring Distance (Fluorescence Flow)



Scaling Matters for Measuring Distance (Compensation Beads)

A 50:50 mix of + and - events stained only for PerCP-Cy5.5 is shown using different scales.



	A	B	C	D	E	F
Min:	-3000	-3000	-3000	-3000	1	-3000
Cofactor:	2500	500	150	1	2500	10,000
Result:	Good	Poor	Bad	Very Bad	Bad	Poor
Issue:		Under-transformed			Off scale	Over-transformed

arcsinh(x) with cofactor c = $\ln\left(\frac{x}{c} + \sqrt{1 + \left(\frac{x}{c}\right)^2}\right)$

For fluorescent flow cytometry data a biexponential or arcsinh transformation corrects the scale near zero.

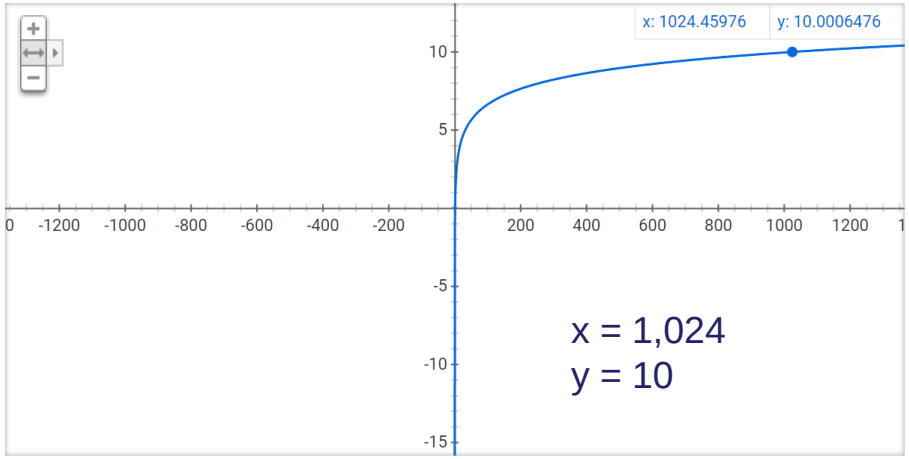
Since computational analysis techniques compare distance similar to what a person does when looking at a plot, these techniques can identify artificial populations near zero (see C and D) if data are not appropriately transformed prior to analysis.

More information: https://my.vanderbilt.edu/irishlab/protocols/scales-and-transformation/
http://www.flowjo.com/v76/en/displaytransformwhy.html

Examples of Four Common Data Scales

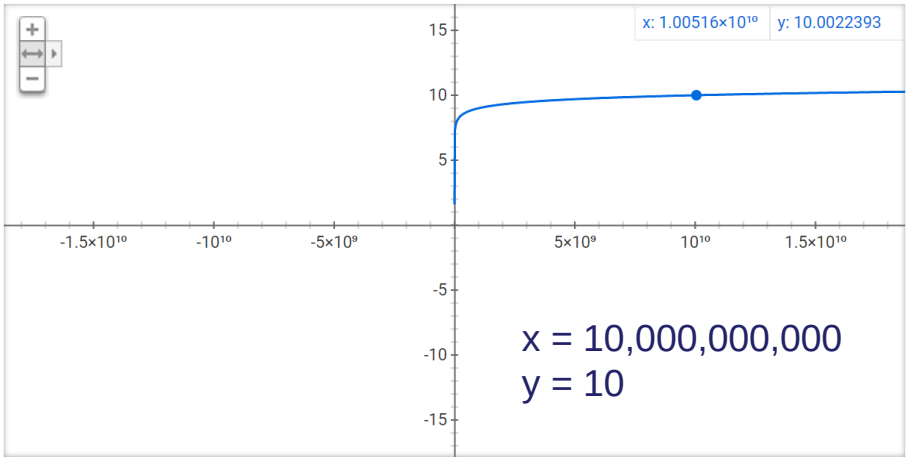
$\log_2(x)$ $\log_{10}(x)$ arcsinh(x/5) arcsinh(x/150)

Graph for ln(x)/ln(2)



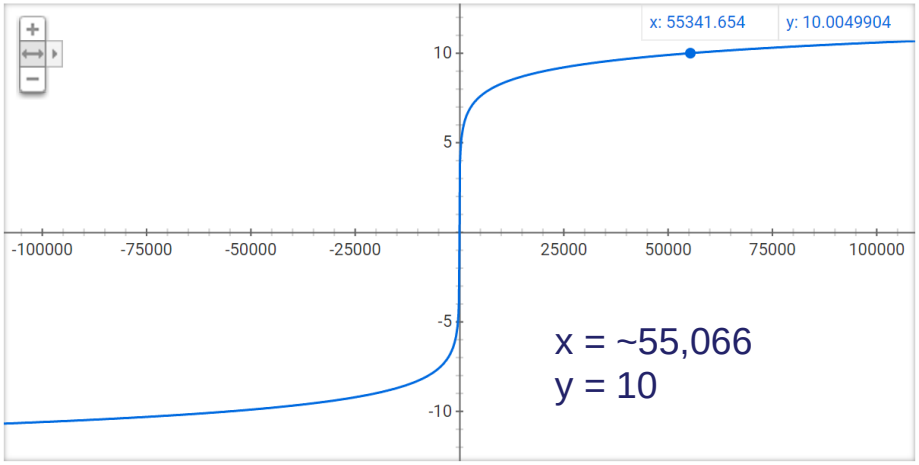
x = 1,024
y = 10

Graph for ln(x)/ln(10)



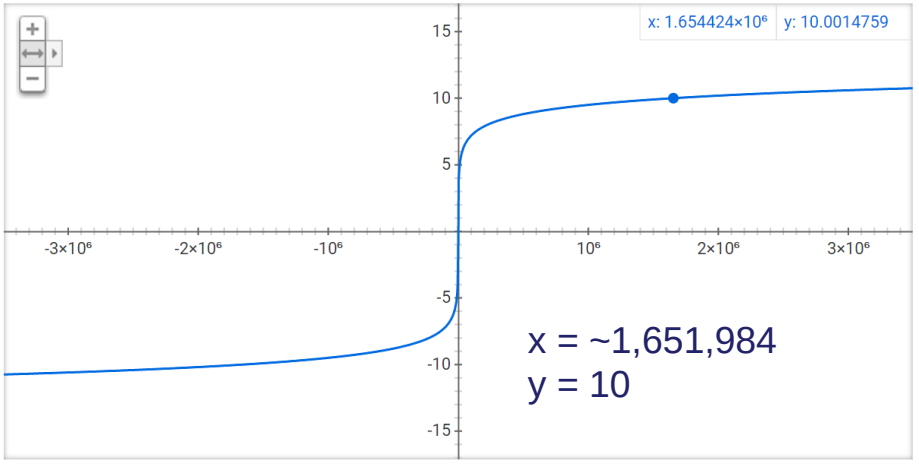
x = 10,000,000,000
y = 10

Graph for ln(x/5+sqrt(1+(x/5)^2)) = arcsinh(x/5)



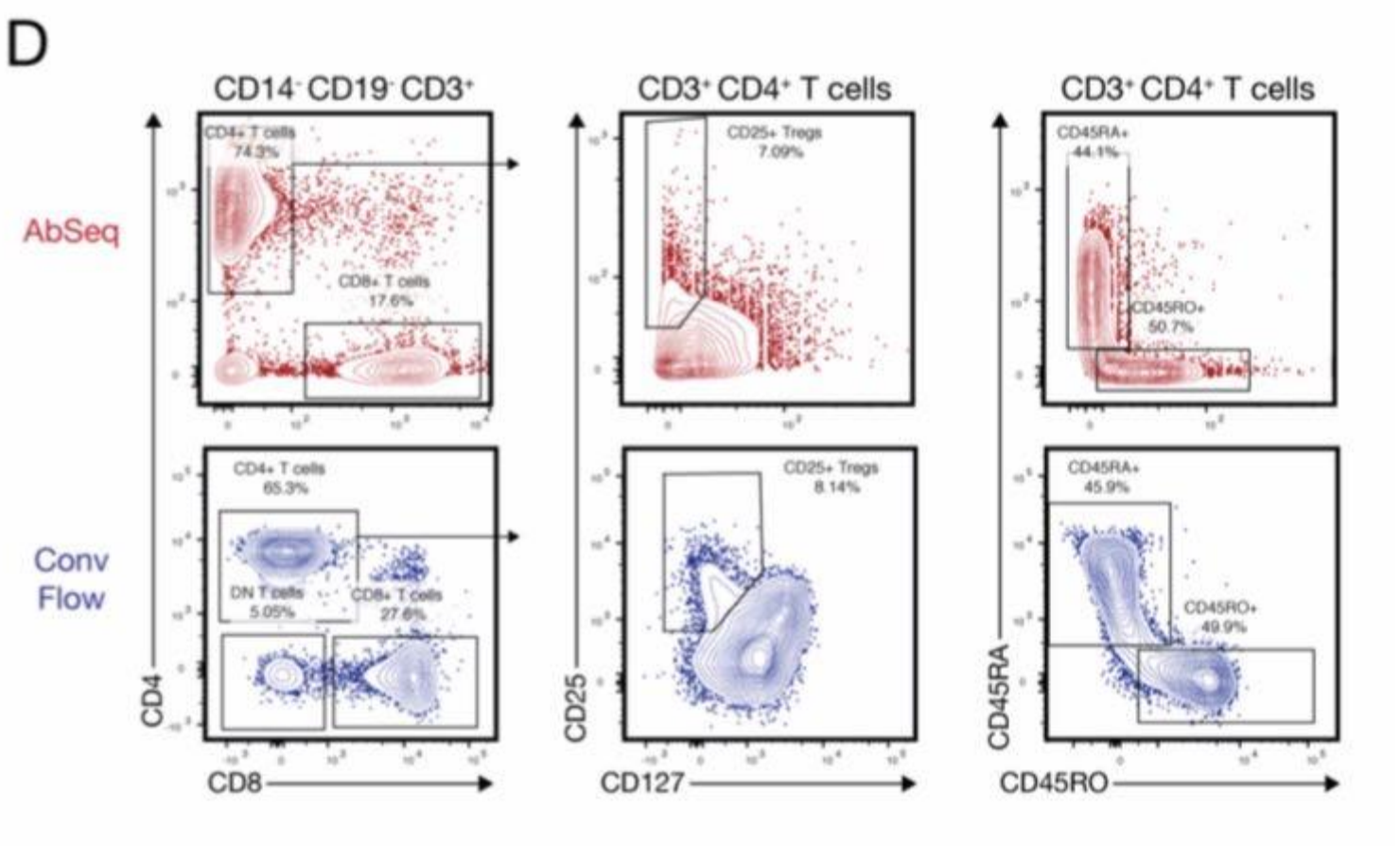
x = ~55,066
y = 10

Graph for ln(x/150+sqrt(1+(x/150)^2)) = arcsinh(x/150)



x = ~1,651,984
y = 10

Scaling Matters for Measuring Distance (RNA seq vs. Fluorescence Flow)



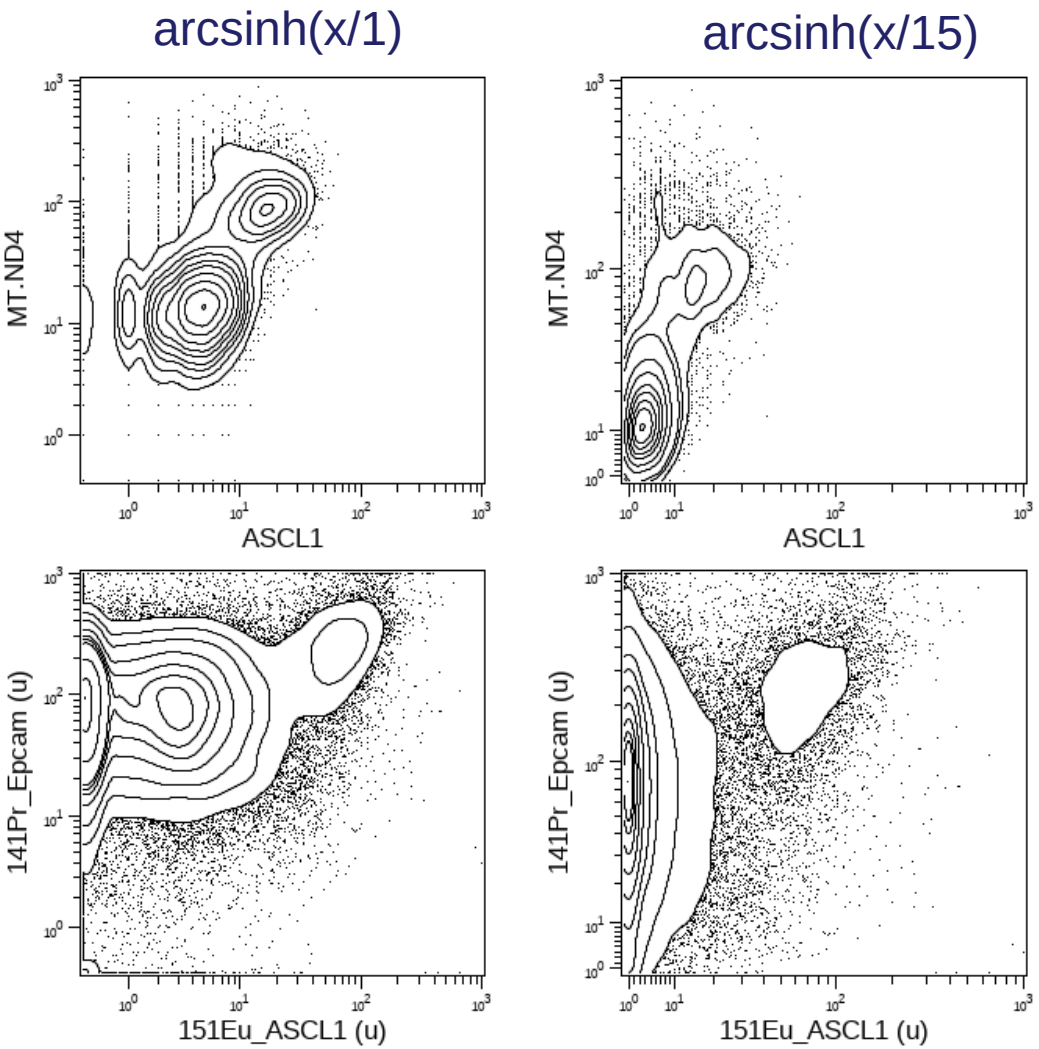
Example Challenge, Scaling to Compare scRNA-seq vs. CyTOF

(Here with one example cell line, DMS454, and ASCL1 on the x-axis)

DMS454
SCLC cell line

scRNA-seq

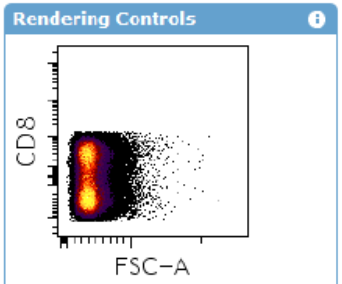
CyTOF

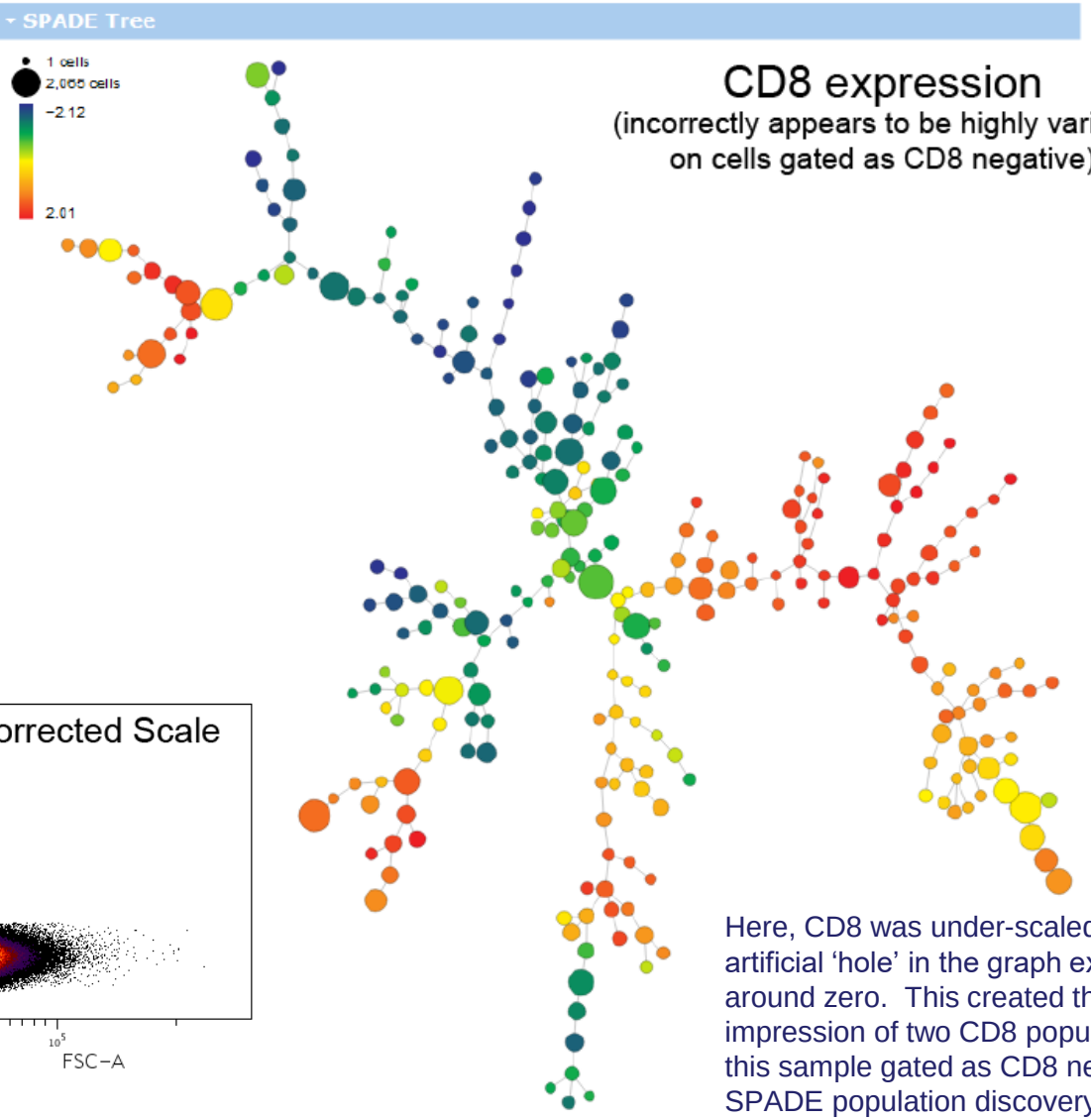


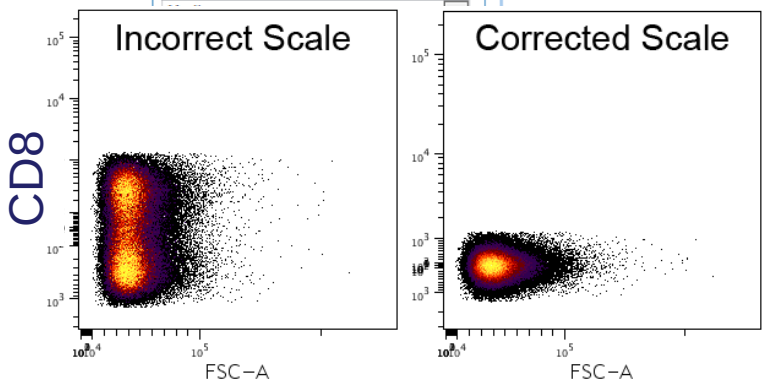
Qualitative: Is there a subset of cells that is ASCL1^{+}?

Quantitative: How much ASCL1 is in each cell? How much does ASCL1 change after Tx?

Inappropriate Scaling Can Lead to False Population Discovery





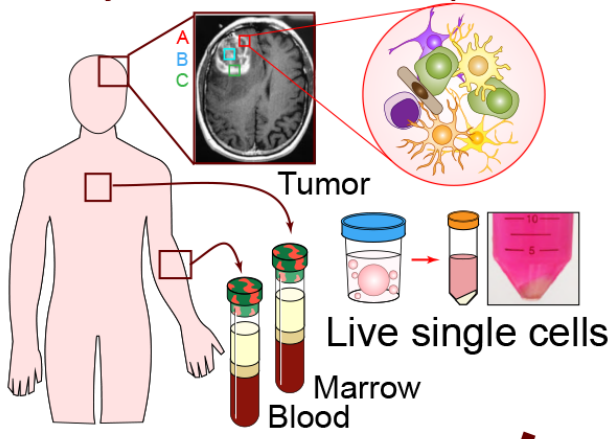


Here, CD8 was under-scaled so that an artificial 'hole' in the graph existed around zero. This created the false impression of two CD8 populations in this sample gated as CD8 negative. SPADE population discovery treated this as significant.

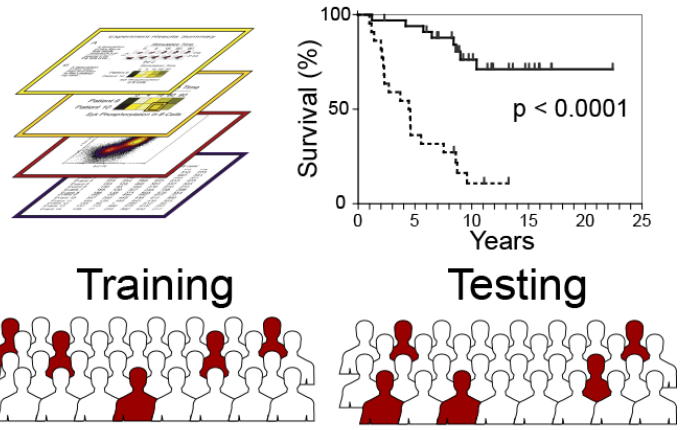
Part 4: Dimensionality Reduction Tools: t-SNE & UMAP Change Everything

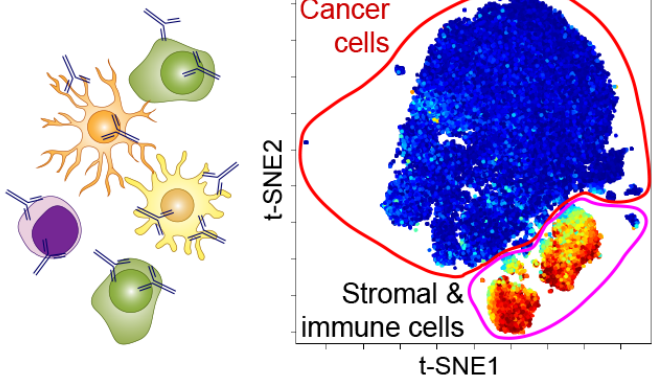
Elements of Translational Data Science

Sample patients over time
at key clinical decision points

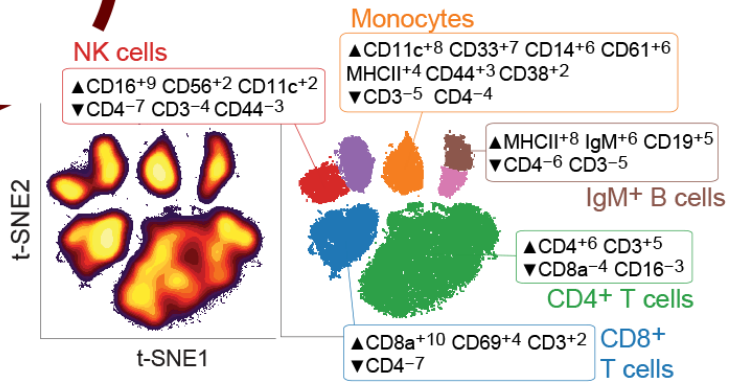


Integrate systems biology data
& clinical outcomes to guide treatment





Measure cell identity, signaling,
biomarkers, and functional responses



Apply machine learning tools
to reveal patterns & identify groups

Diggins et al., *in review*

Key Topic Areas, Terms, and Cytometry Workflows

1: Field Changes: Data Science & Latest Tools

2: History: Non-linear, PCA, Trajectories, Supervised

3: Dimensionality Reduction: t-SNE & UMAP

4: Clustering: SPADE (k-means), KNN, FlowSOM, Citrus

5: Enriched Features: MEM, ΔMEM, RMSD

6: Cytometry:
- 2004: Expert => Expert => Heatmap (Irish/Nolan)
- 2011: Expert => SPADE => Heatmap (Bendall/Qiu)
- 2013: t-SNE => Expert (viSNE/Pe'er, Van Der Maaten)
- 2014: t-SNE => DensVM => Heatmap (Newell)
- 2015: t-SNE => SPADE => Heatmap (Diggins/Irish)
- 2015: "KNN" (=> t-SNE) => Heatmap (Phenograph)
- 2015: FlowSOM (Van Gassen/Saeys)
- 2017: t-SNE => SPADE => MEM (Diggins/Irish)
- 2018: UMAP => Expert (Newell, McInnes)
- 2019: UMAP => FlowSOM => MEM (Barone/Irish)

Unsupervised Analysis: Not Using Prior Knowledge To Guide the Analysis

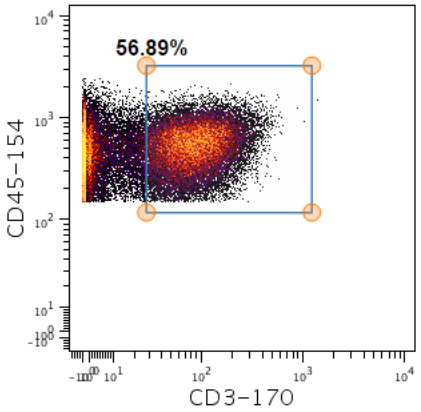
Prior knowledge examples: Stem cells express CD34,
these samples were from patients that responded to drug

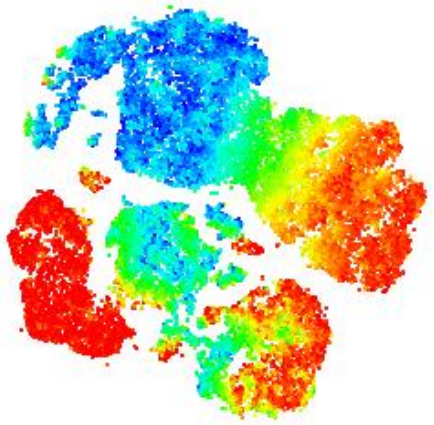
Supervised Approaches

- Expert gating
- Citrus
- CellCNN (neural network)
- Wanderlust

Unsupervised Approaches

- Most heatmap clustering
- SPADE, FlowSOM
- t-SNE / viSNE, UMAP
- Phenograph



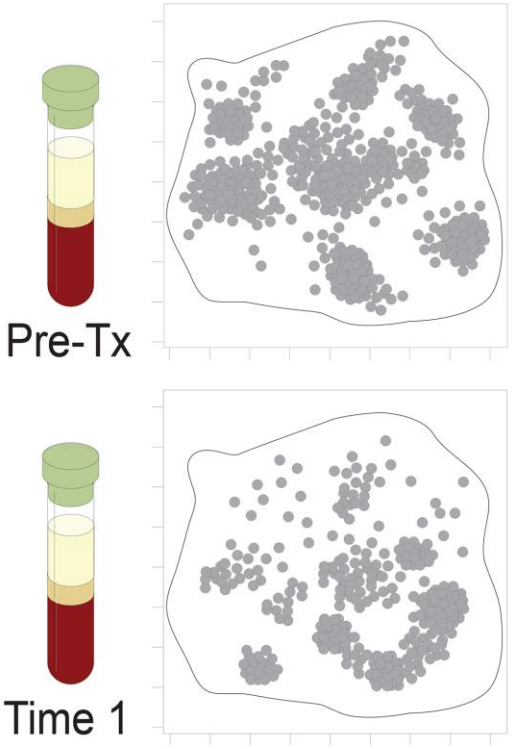


See Table 1 of Diggins et al., *Methods* 2015 for list of unsupervised tools

Clinical Trial Monitoring: What Do We Need to Know? Automate Four Key Readouts vs. Clinical Outcomes

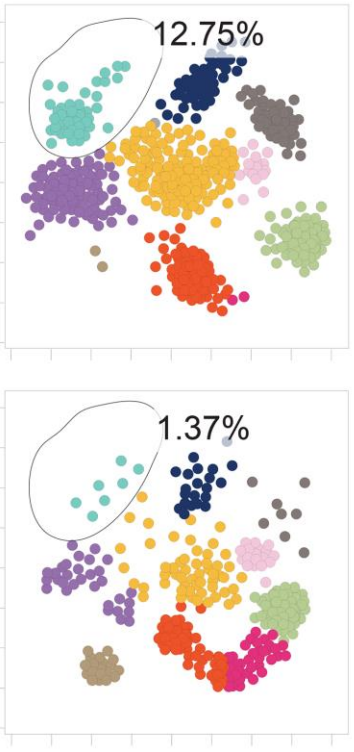
Features of Dynamic Populations

1 Systems Plasticity



Earth Mover's Distance on t-SNE or UMAP

2 Population abundance



Traditional gating or cluster frequency

3 Signature features

Pre-therapy

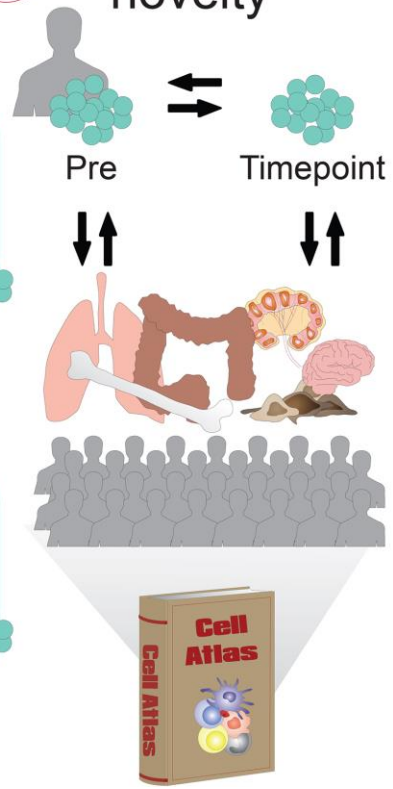
▲HLADR^{+2} CCR5^{+1} CD38^{+1} CD33^{+1}
▼CD8^{-8} CD45RO^{-6} CD3^{-6} CD4^{-4} CD45^{-2} CCR4^{-1} CCR7^{-1} CD28^{-1} CD27^{-1}

Time point 1

▲HLADR^{+2} CD38^{+1} CD45RA^{+1}
▼CD8^{-8} CD4^{-6} CD3^{-6} CD45RO^{-5} CCR5^{-2} CD45^{-2} CD28^{-2} CD20^{-1} CCR4^{-1} CD27^{-1}

Marker Enrichment Modeling (MEM)

4 Population novelty

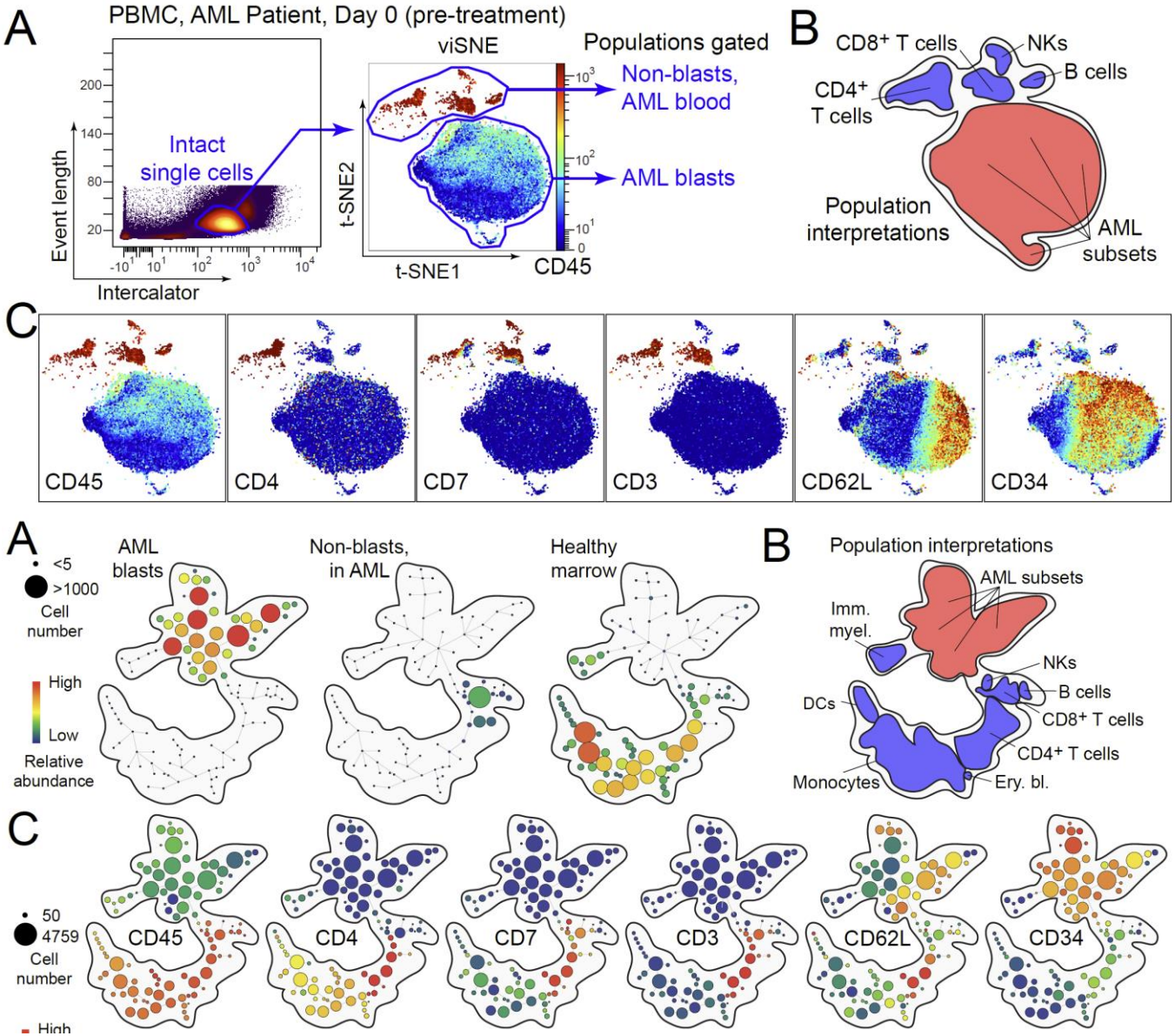


ΔMEM vs. Timepoint or Cell Atlas

How we quantified

Greenplate et al., *Cancer Immunology Research 2019*

Key Analysis Concepts: Dimensionality Reduction, Transformation, Clustering, Modeling, Visualization, & Integration



viSNE

Amir et al.

Nature biotech 2013

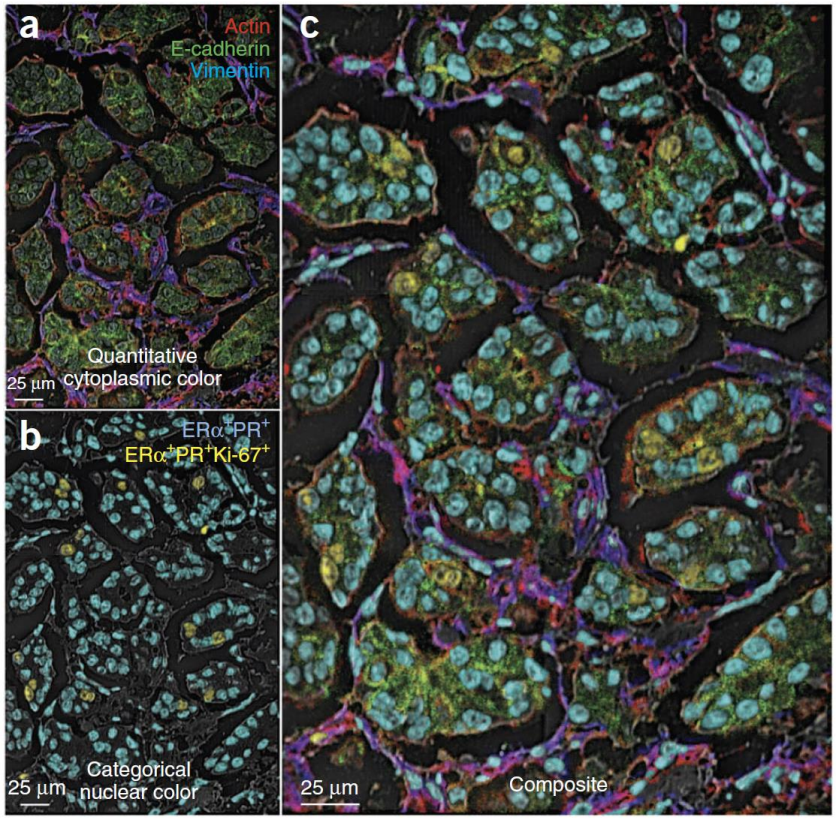
SPADE

Qiu et al.

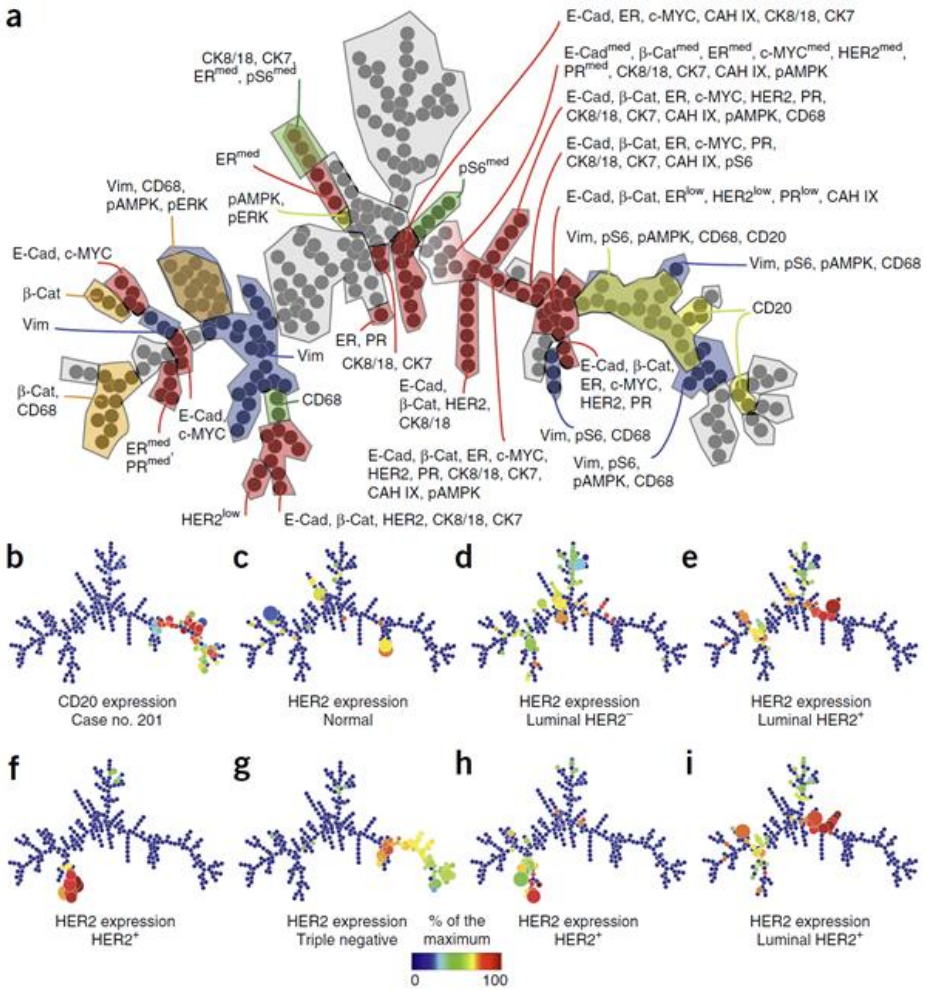
Nature biotech 2011

Diggins et al., *Methods* 2015

Cytomics (Cell Identity): Powered by Multiple Platforms: Imaging Mass Cytometry of Breast Cancer



Example MIBI breast cancer histology
Angelo et al., *Nature Medicine* 2014



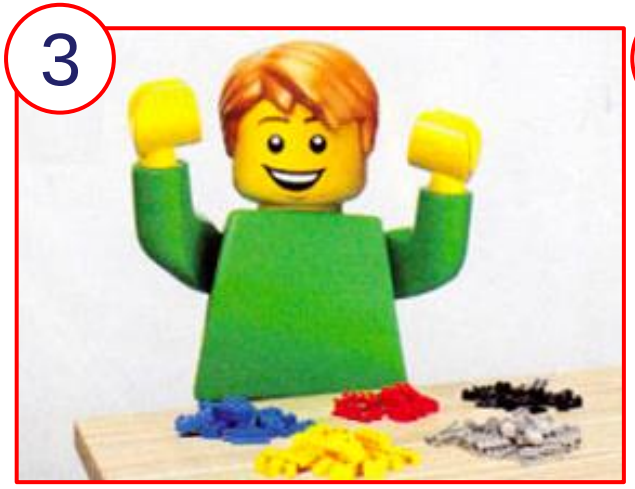
Analysis of IMC from 20+ breast cancer using SPADE
Giesen et al., *Nature Methods* 2014

t-SNE was a game changer for single cell

Teaching Computers To Spot Useful Patterns : Grouping Cells by Selected Features (e.g. Protein Expression)

HD cytometry!!

Woah, that's a lot of data…

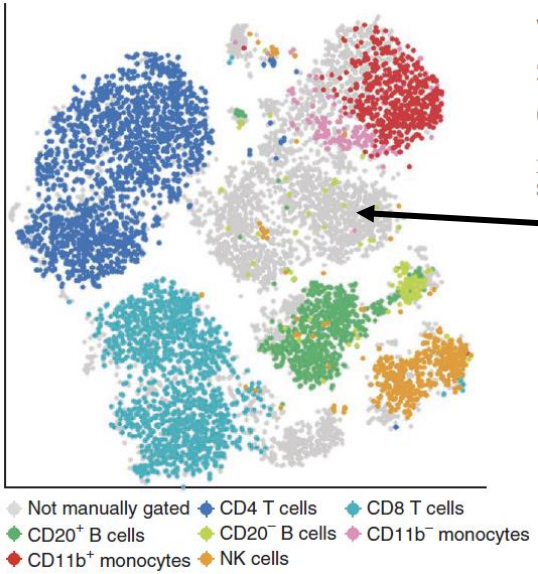


Computational tools

Biological knowledge

Adapted from LEGO 70803 Cloud Cuckoo Palace instructions 2014

Traditional Gating Overlooks Many Cells in Primary Samples



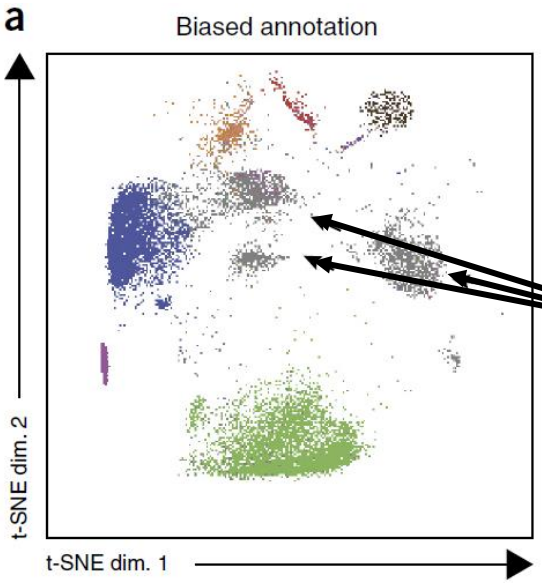
nature biotechnology

2013

viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia

El-ad David Amir[1], Kara L Davis[2,3], Michelle D Tadmor[1,3], Erin F Simonds[2,3], Jacob H Levine[1,3], Sean C Bendall[2,3], Daniel K Shenfeld[1,3], Smita Krishnaswamy[1], Garry P Nolan[2,4] & Dana Pe'er[1,4]

In all cases, the viSNE gate included cells that were not classified by the expert manually gated biaxial plots; these cells are labeled in gray in the viSNE map. Examination of the marker expression of these cells reveals that they are typically just beyond the threshold of one marker, but the viSNE classification is strongly supported based on the expression of all other markers. For example, in **Figure 1d**, wherein cells are colored for CD11b marker expression, the cells in the gated region express the canonical monocyte marker CD33 (**Supplementary Fig. 1b**). However, only 47% of these cells were classified as monocytes by the manual gating (**Fig. 1b**).



nature immunology

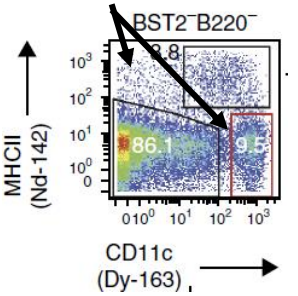
2014

High-dimensional analysis of the murine myeloid cell system

Burkhard Becher[1,4,5], Andreas Schlitzer[1,5], Jinmiao Chen[1,5], Florian Mair[2], Hermi R Sumatoh[1], Karen Wei Weng Teng[1], Donovan Low[1], Christiane Ruedl[3], Paola Riccardi-Castagnoli[1], Michael Poidinger[1], Melanie Greter[2], Florent Ginhoux[1] & Evan W Newell[1]

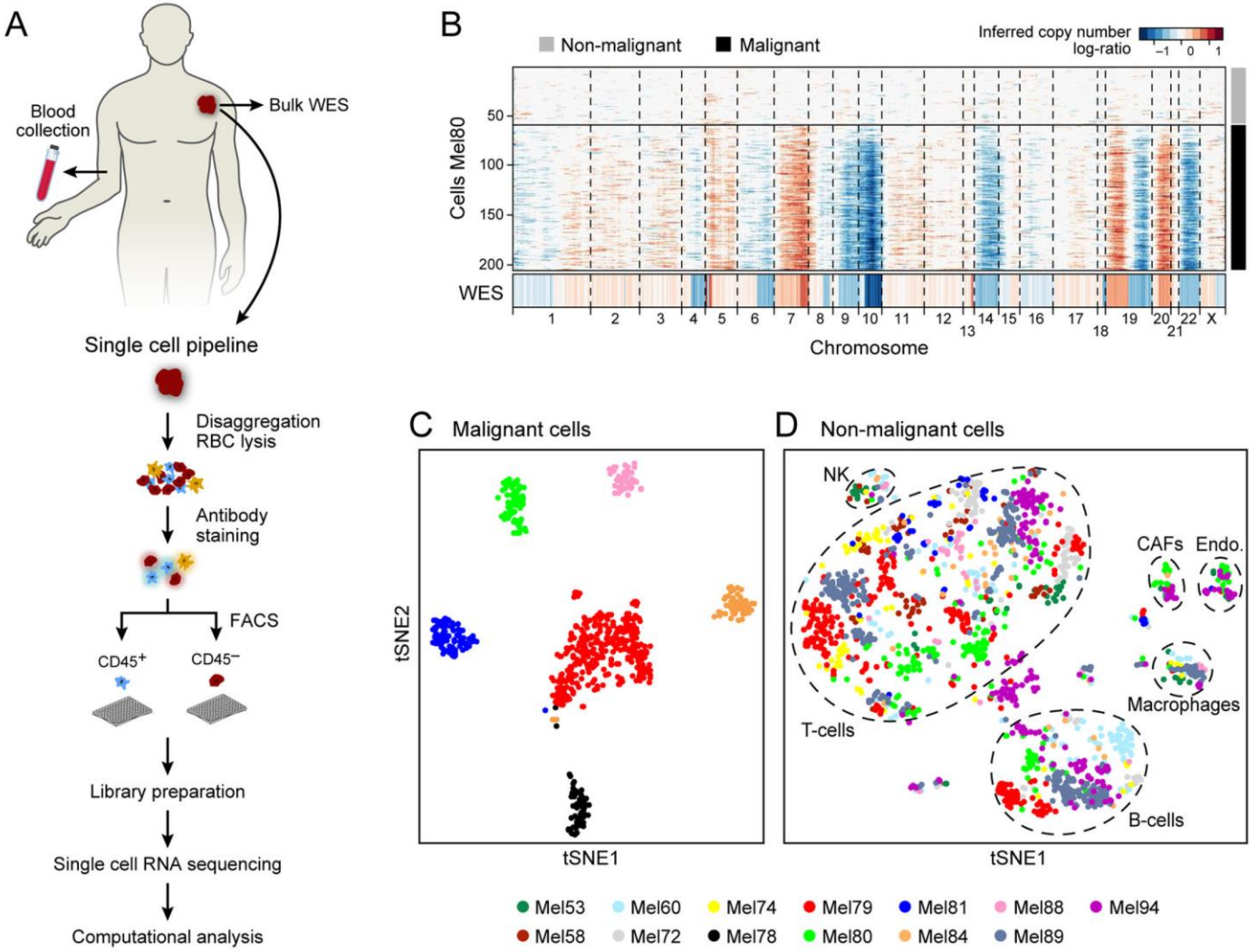
Notably, whereas traditional biased gating strategies allowed for identification of only 54.7 ± 2.6% (mean ± s.e.m., n = 3 mice) of lung myeloid cells (different DC subsets, macrophages, monocytes, neutrophils), the automatic, computational approach identified nearly 100% of the cells (96.6 ± 1.0% (mean ± s.e.m., n = 3 mice) accounted for by 14 predominant clusters).

Overlooked cells 'between' traditional gates add up to ~half the sample



Amir et al., *Nature Biotechnology* 2013; Becher et al., *Nature Immunology* 2014

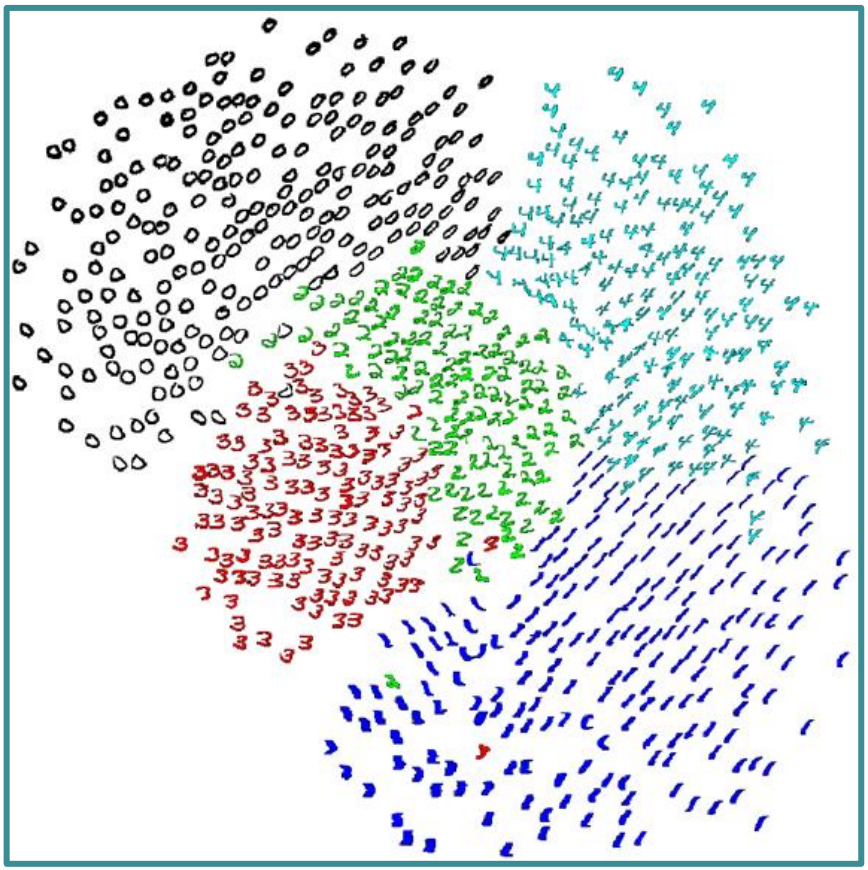
Cytomics (Cell Identity): Powered by Multiple Platforms
Melanoma Cell Diversity Based on scRNA-seq Data



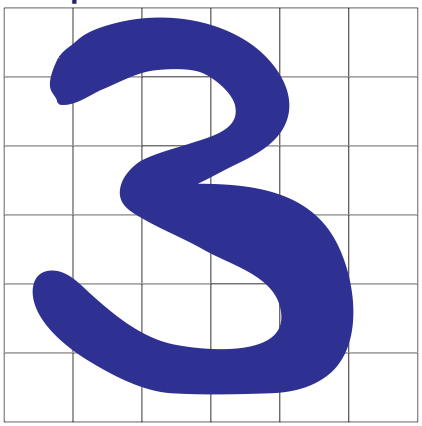
Tirosh et al., *Science* 2016

Stochastic Neighbor Embedding (SNE)

- SNE used for image recognition
- 60,000 handwritten greyscale images
- 28x28 pixels each



Example: 6x6 Pixel Image

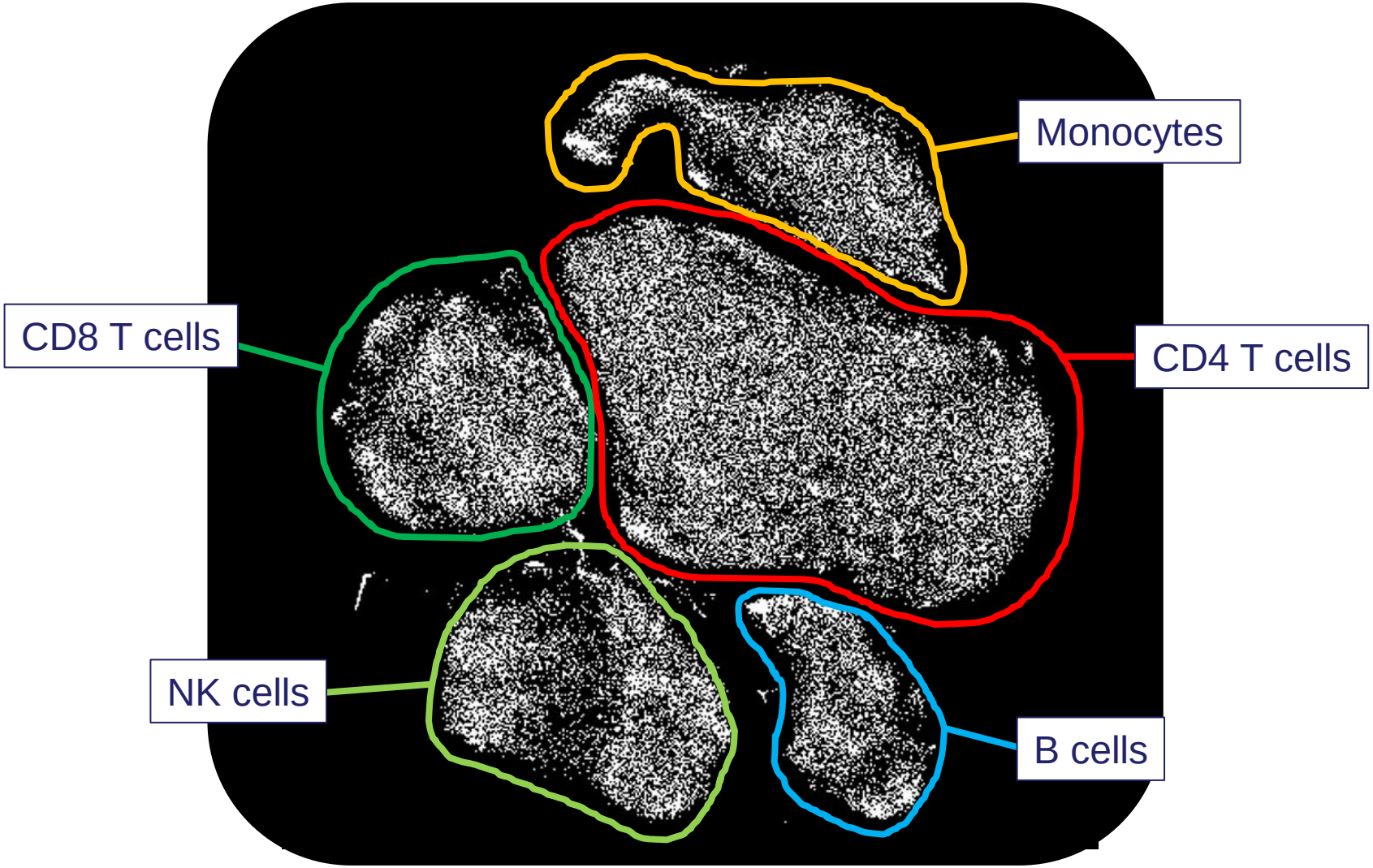


Vectorize (1x36)

tSNE on all pixels

Hinton et al., "*Advances in neural information processing systems*." 2002.

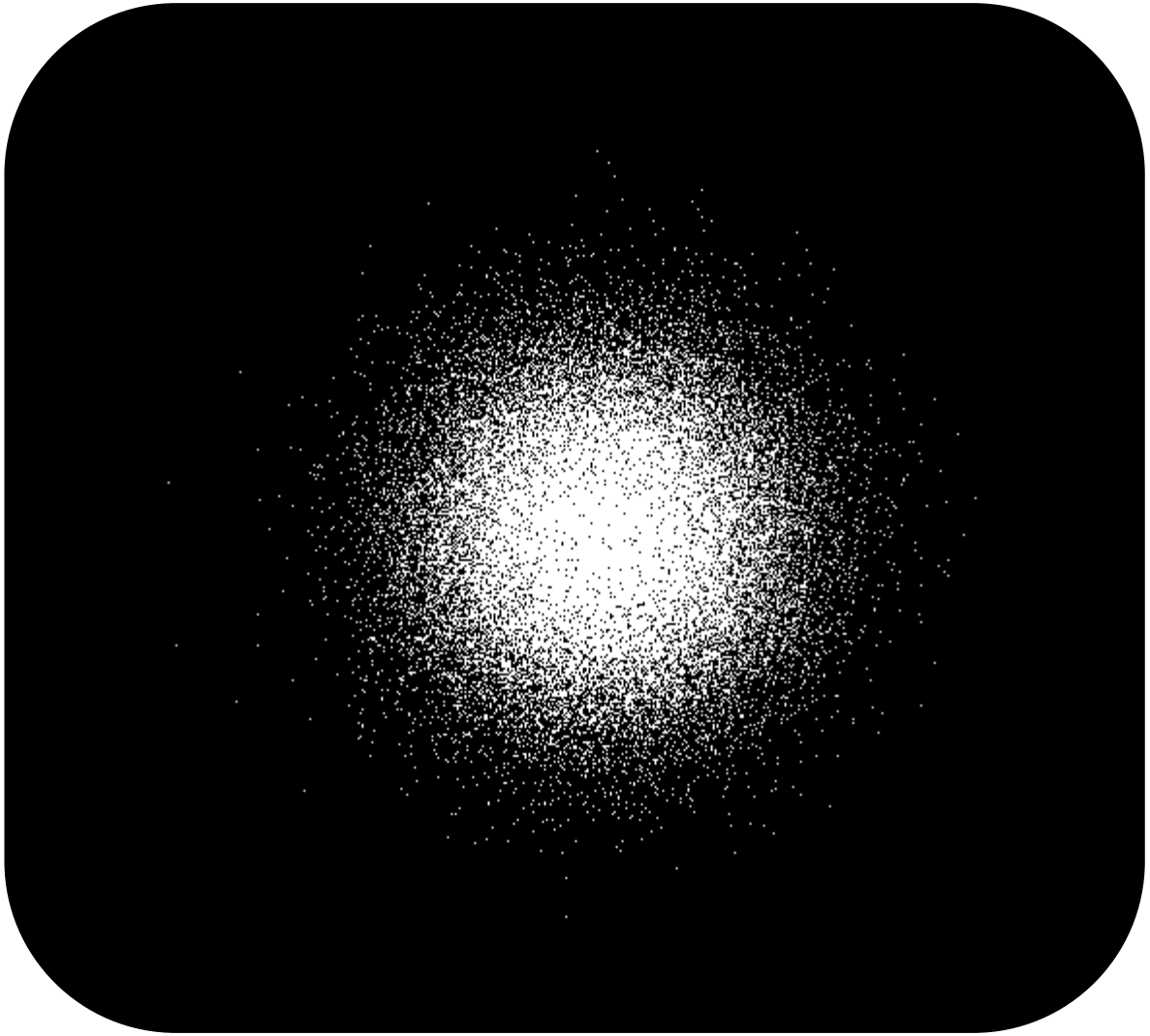
viSNE / t-SNE Arranges Cells in 2D by Multi-D Similarity



Healthy human blood, mass cytometry,
26 markers measured, viSNE analysis tool

Animation created by Cytobank team from iterations of viSNE / t-SNE using PBMC (26 features)

viSNE / t-SNE Arranges Cells in 2D by Multi-D Similarity



Healthy human blood, mass cytometry,
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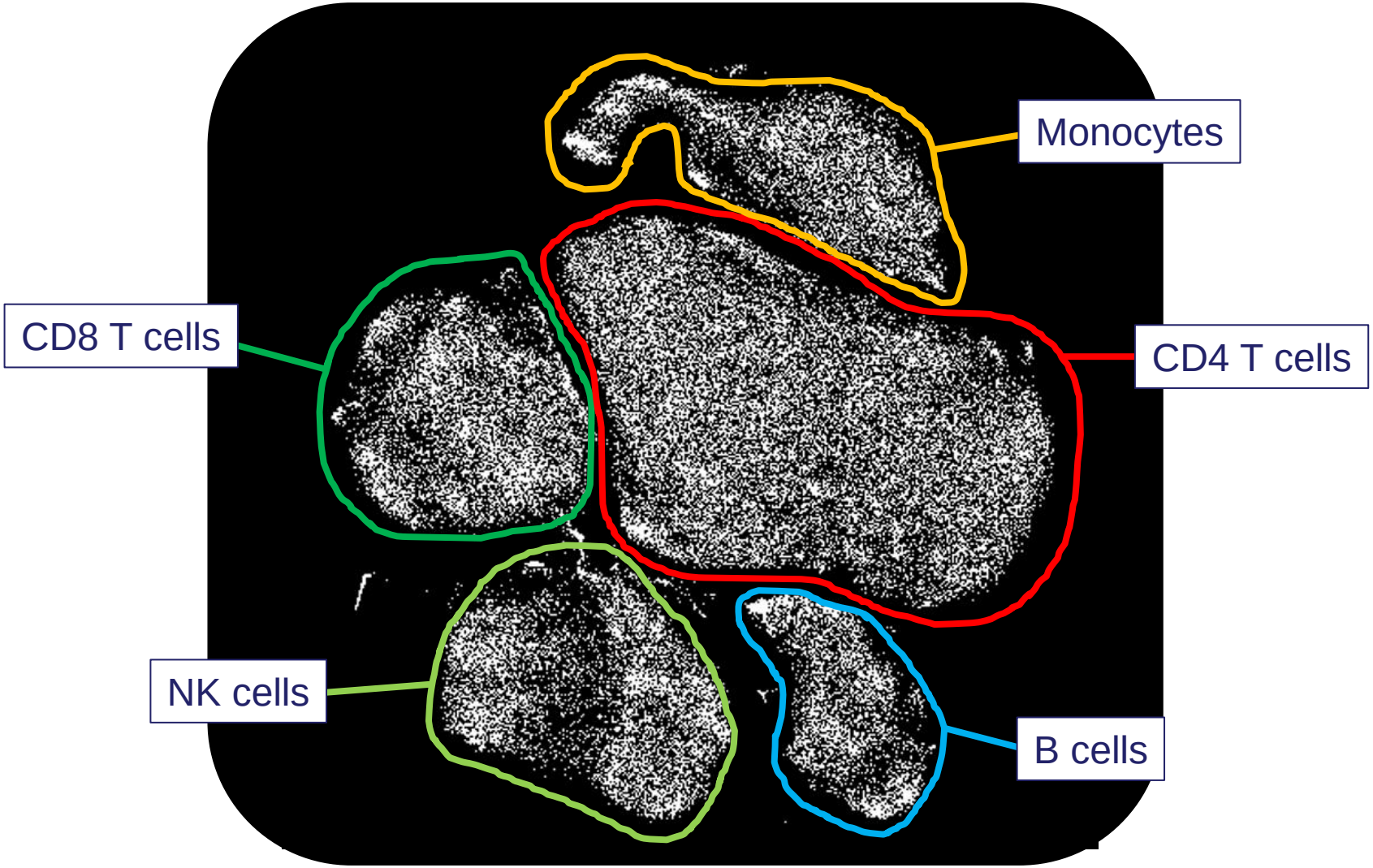
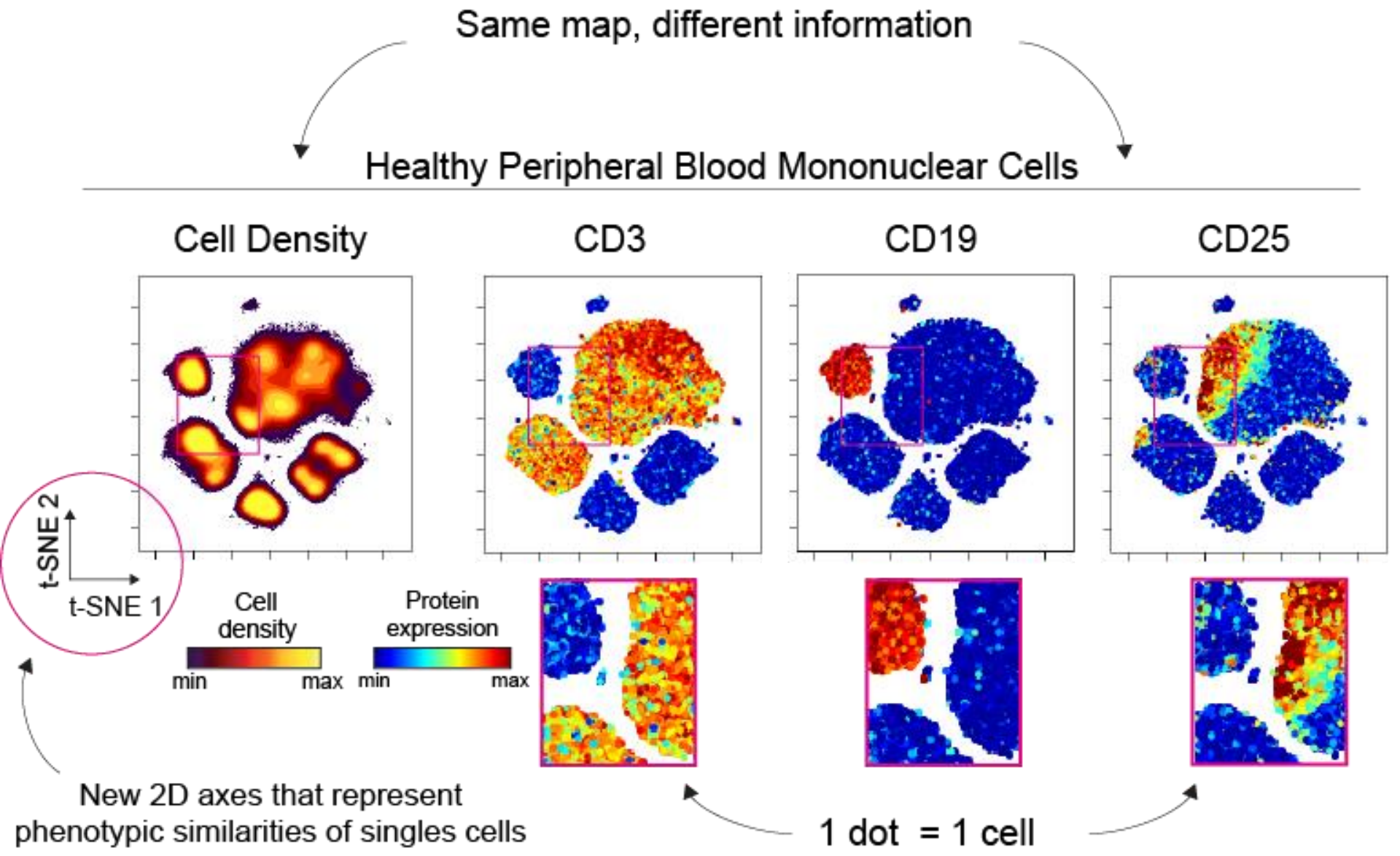
viSNE / t-SNE Arranges Cells in 2D by Multi-D Similarity



Healthy human blood, mass cytometry,
26 markers measured, viSNE analysis tool

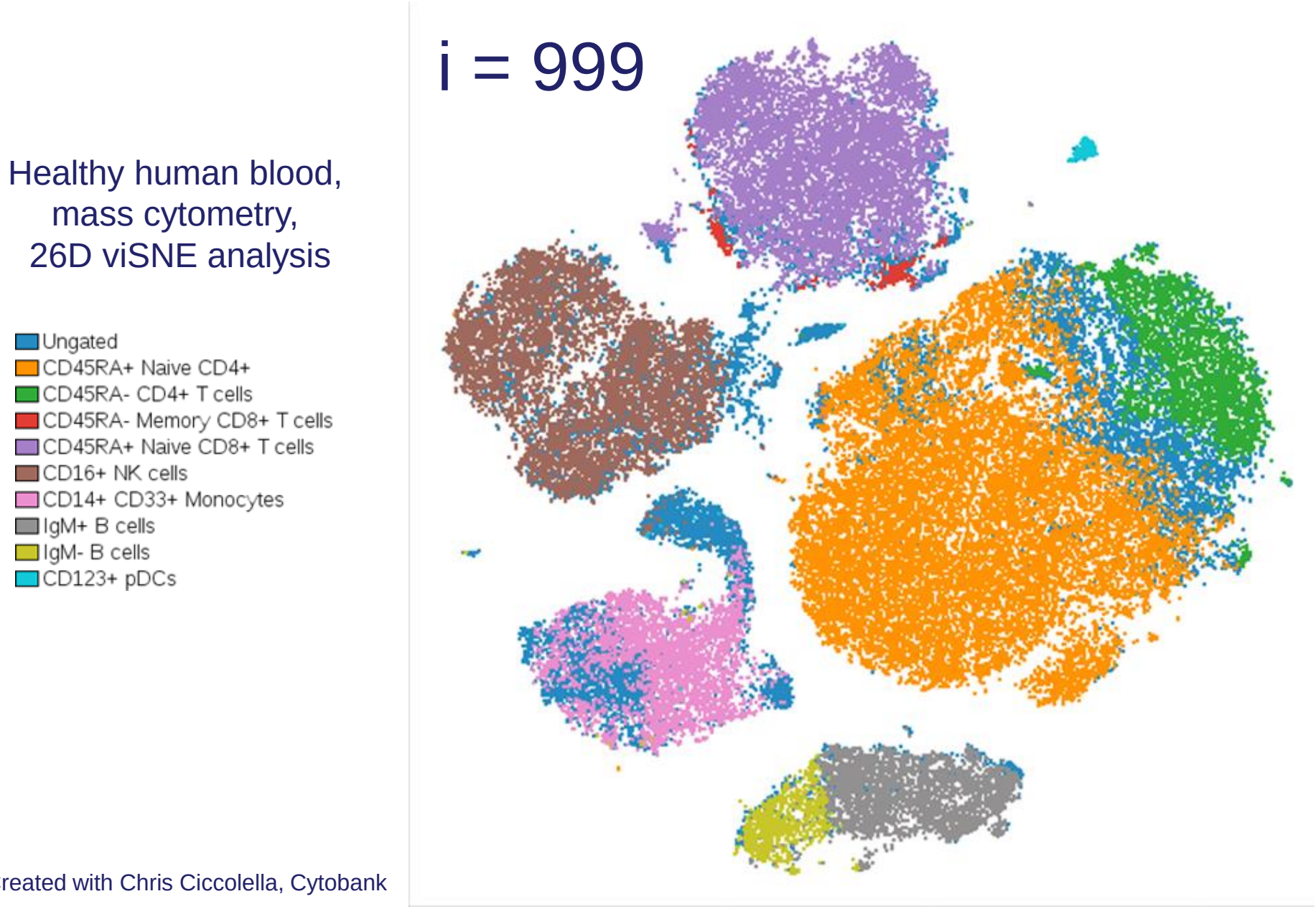
Animation created by Cytobank team from iterations of viSNE / t-SNE using PBMC (26 features)

t-SNE Analysis Allows 2D Visualization of High Dimensional Single Cell Data



Viewing Expert Gates with viSNE Reveals Cyto Incognito

Healthy human blood,
mass cytometry,
26D viSNE analysis

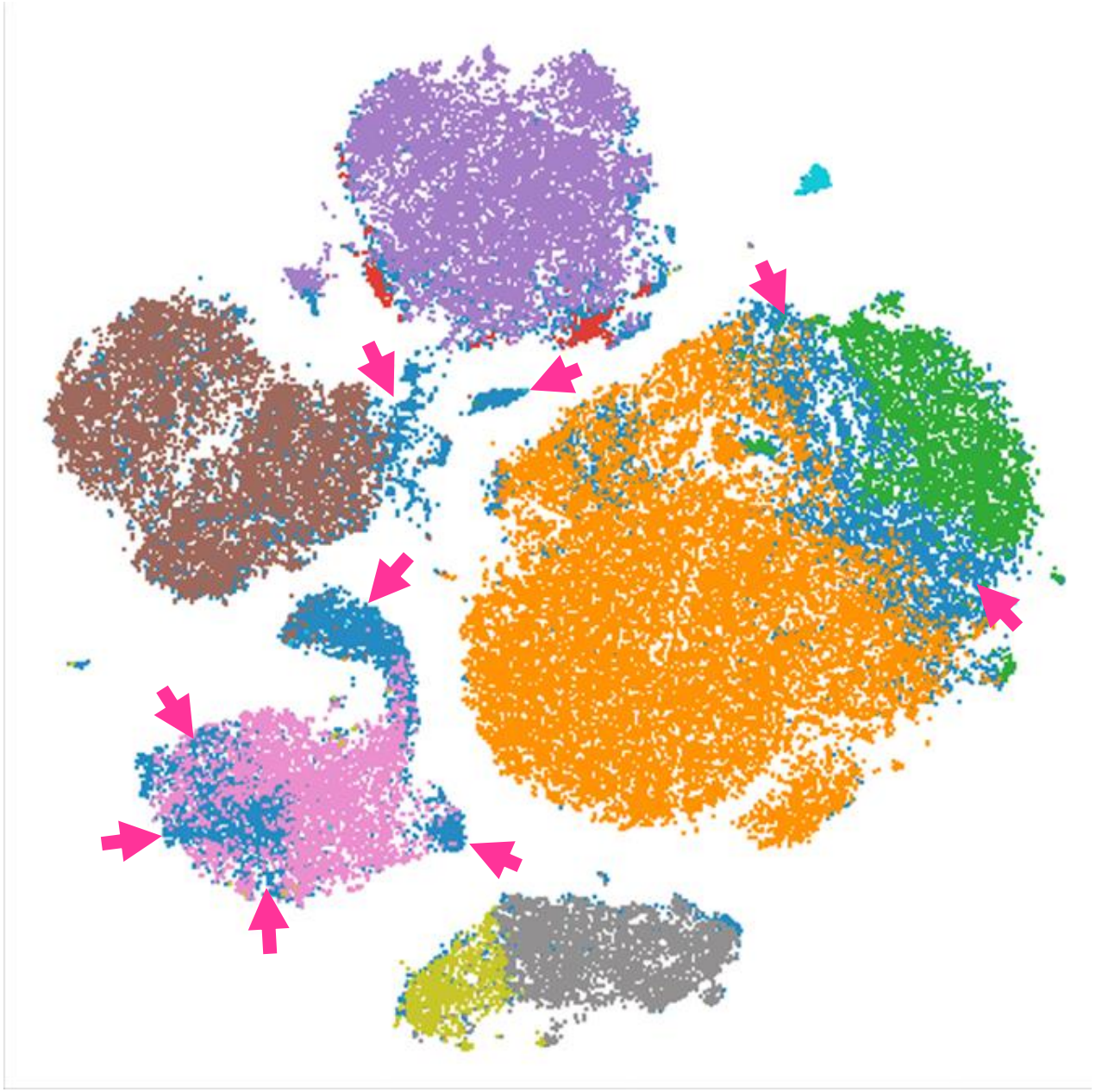


Created with Chris Ciccolella, Cytobank

Viewing Expert Gates with viSNE Reveals Cyto Incognito

Healthy human blood,
mass cytometry,
26D viSNE analysis

- Ungated
- CD45RA+ Naive CD4+
- CD45RA- CD4+ T cells
- CD45RA- Memory CD8+ T cells
- CD45RA+ Naive CD8+ T cells
- CD16+ NK cells
- CD14+ CD33+ Monocytes
- IgM+ B cells
- IgM- B cells
- CD123+ pDCs

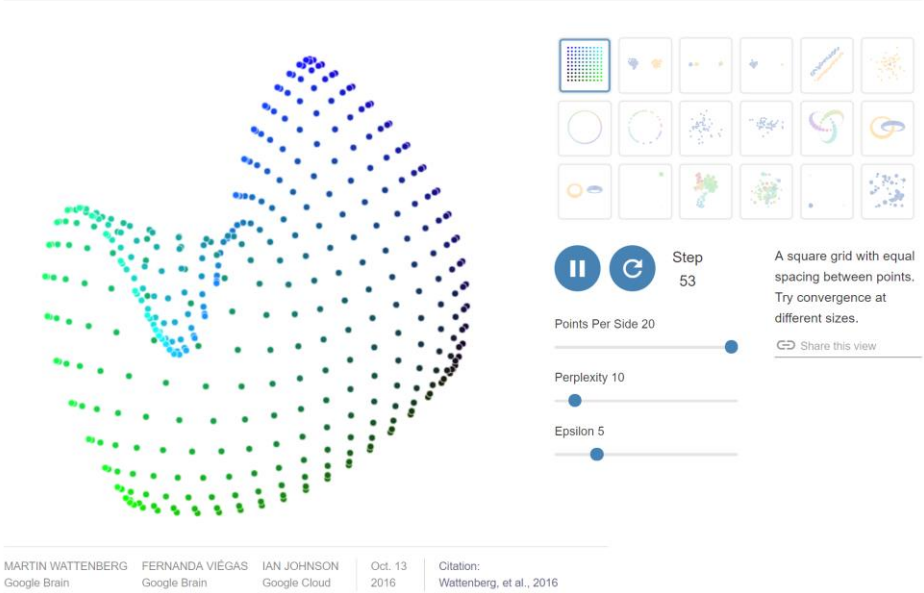


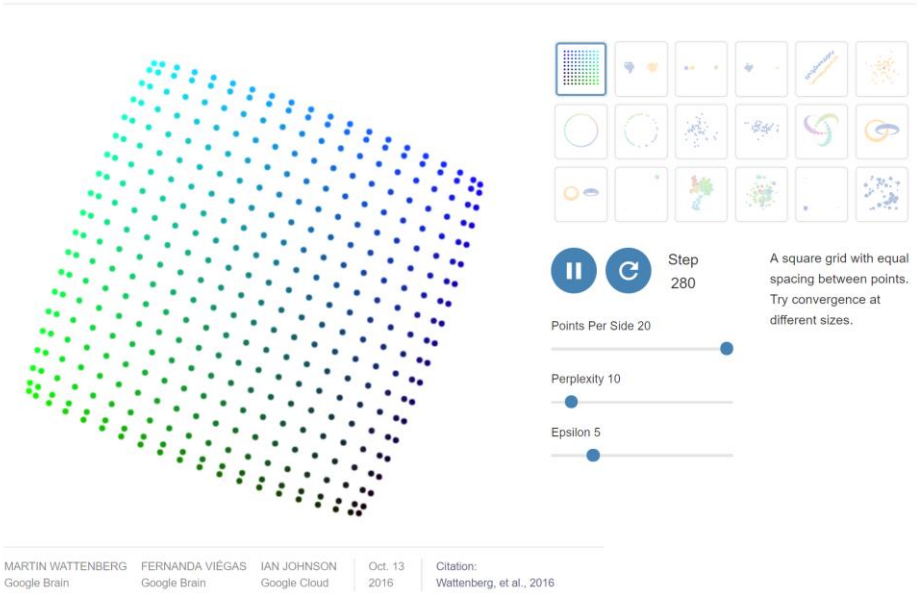
→ Cyto incognito
(Cells overlooked or
hidden in expert gating)

Created with Chris Ciccolella, Cytobank

t-SNE 2D Examples with Animations and Settings

http://distill.pub/2016/misread-tsne/





opt-SNE Provides Automated Optimization of t-SNE Parameters and PCA Initialization (Fast, Reliable)

nature communications

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Article | Open Access | Published: 28 November 2019

Automated optimized parameters for T-distributed stochastic neighbor embedding improve visualization and analysis of large datasets

Anna C. Belkina, Christopher O. Ciccolella, Rina Anno, Richard Halpert, Josef Spidlen & Jennifer E. Snyder-Cappione

Nature Communications **10**, Article number: 5415 (2019) | Cite this article

12k Accesses | **53** Citations | **67** Altmetric | Metrics

Abstract

Accurate and comprehensive extraction of information from high-dimensional single cell datasets necessitates faithful visualizations to assess biological populations. A state-of-the-art algorithm for non-linear dimension reduction, t-SNE, requires multiple heuristics and fails to produce clear representations of datasets when millions of cells are projected. We develop opt-SNE, an automated toolkit for t-SNE parameter selection that utilizes Kullback-Leibler divergence evaluation in real time to tailor the early exaggeration and overall number of gradient descent iterations in a dataset-specific manner. The precise calibration of early exaggeration together with opt-SNE adjustment of gradient descent learning rate dramatically improves computation time and enables high-quality visualization of large cytometry and transcriptomics datasets, overcoming limitations of analysis tools with hard-coded parameters that often produce poorly resolved or misleading maps of fluorescent and mass cytometry data. In summary, opt-SNE enables superior data resolution in t-SNE space and thereby more accurate data interpretation.

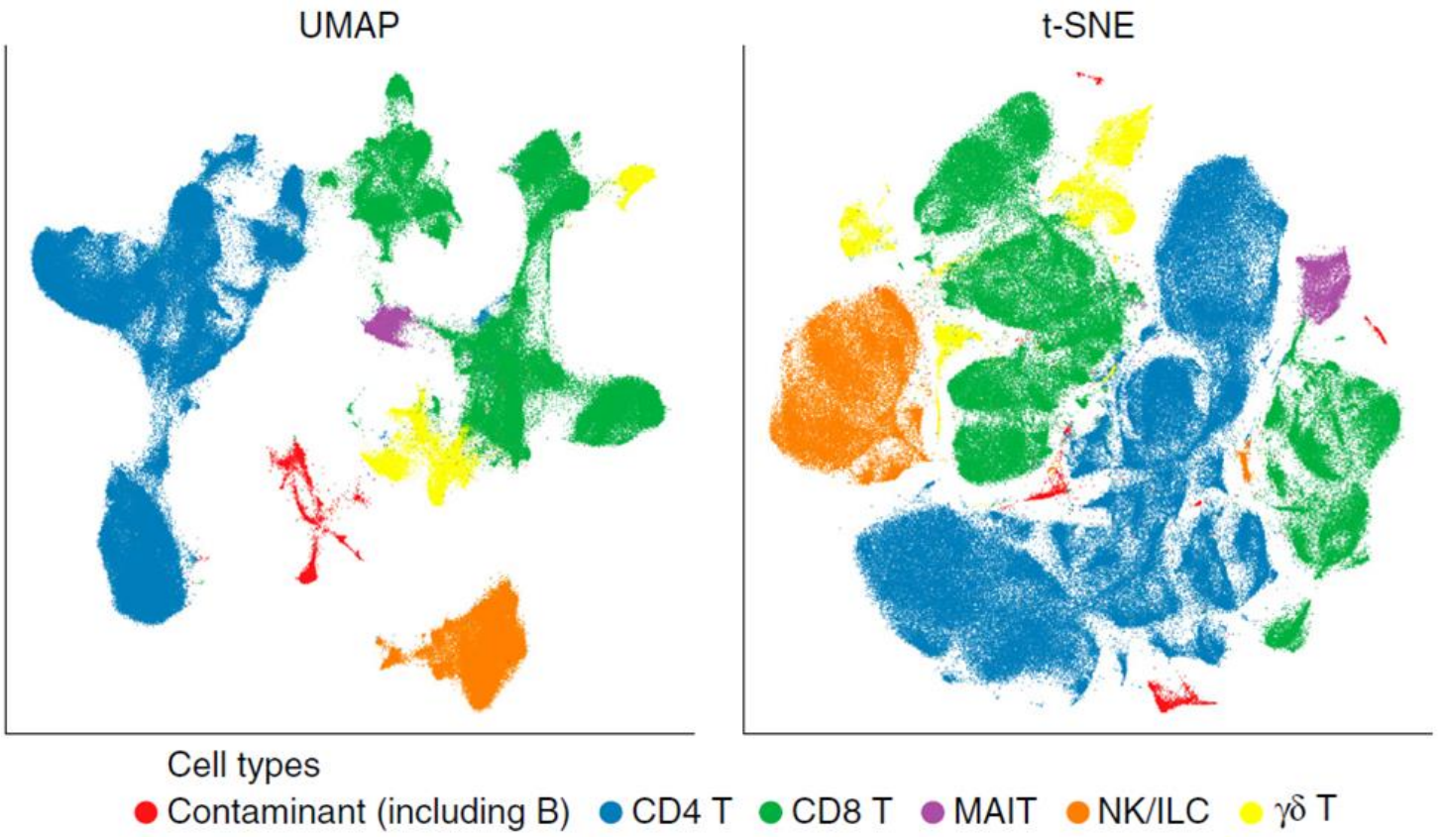
Belkina et al., *Nature Communications* 2019

Check out Dr. Anna Belkina's opt-SNE Webinar on ISAC's CYTO U Learning Portal

CYTO U Webinar: https://learning.isac-net.org/products/dont-leave-home-without-a-map-powerful-dimensionality-reduction-methods-for-cytometry-data-visualization

Becht et al., UMAP Preserves Local and Global Structure (Analysis of Tissue T Cells; Color = Expert Knowledge / Source)

(a) UMAP better split CD8 T cells, γδ T cells, and contaminating cells

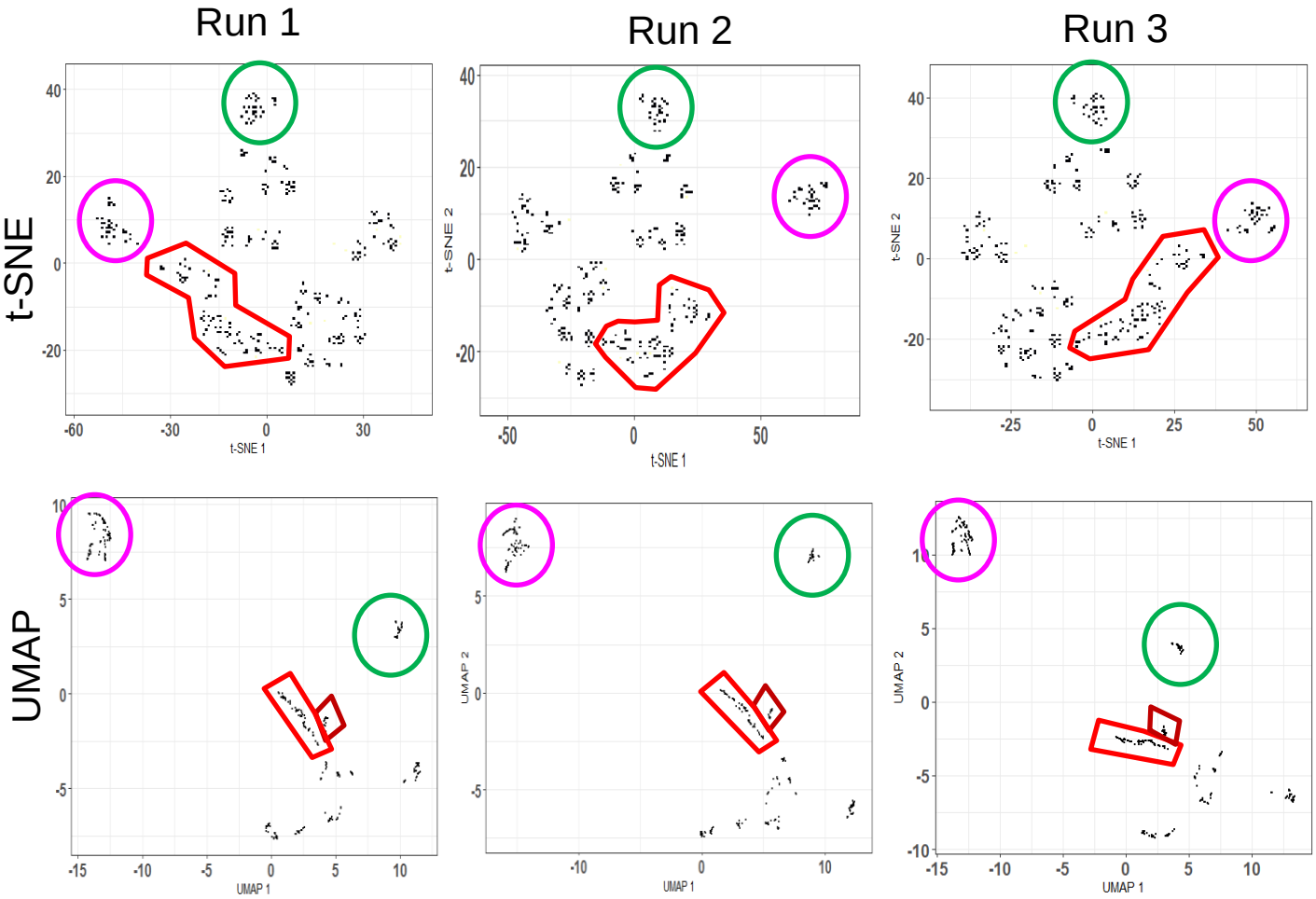


Dataset covering 35 samples originating from 8 distinct human tissues enriched for T and natural killer (NK) cells, of more than >300,000 cell events with 39 protein targets (Wong et al. dataset).

Becht et al., *Nature Biotechnology* 2018

Multiple Runs of t-SNE vs. UMAP on a Patient Dataset (n = 339)

Gandelman et al., cGVHD Patient Dataset

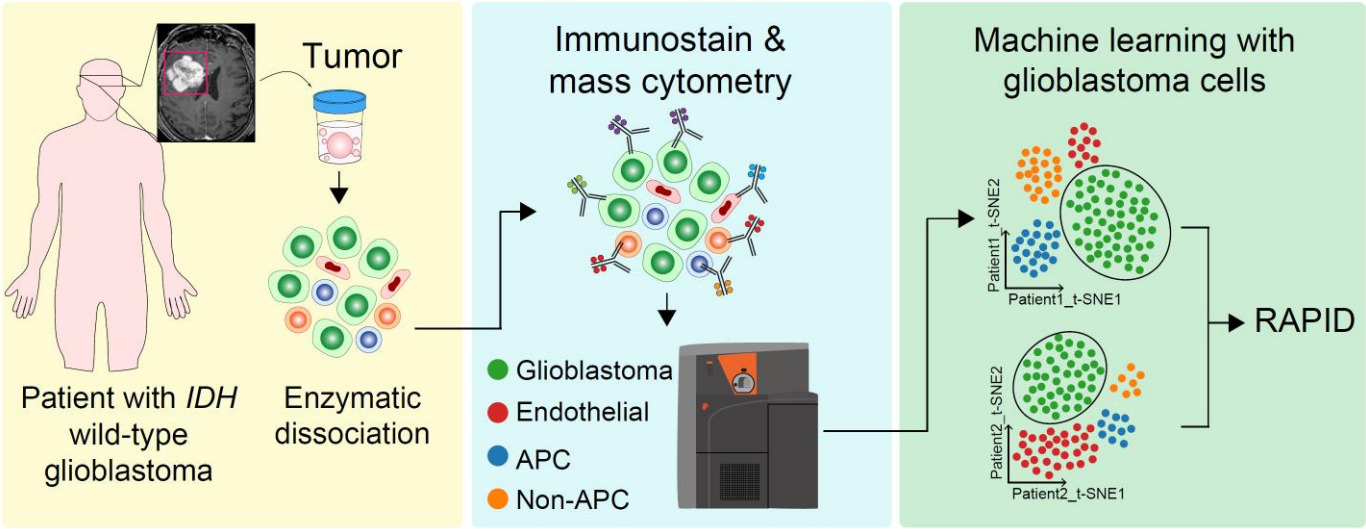


In the t-SNE plots, the relative relationship of the major islands ("global structure") alters between runs; t-SNE focuses on local structure

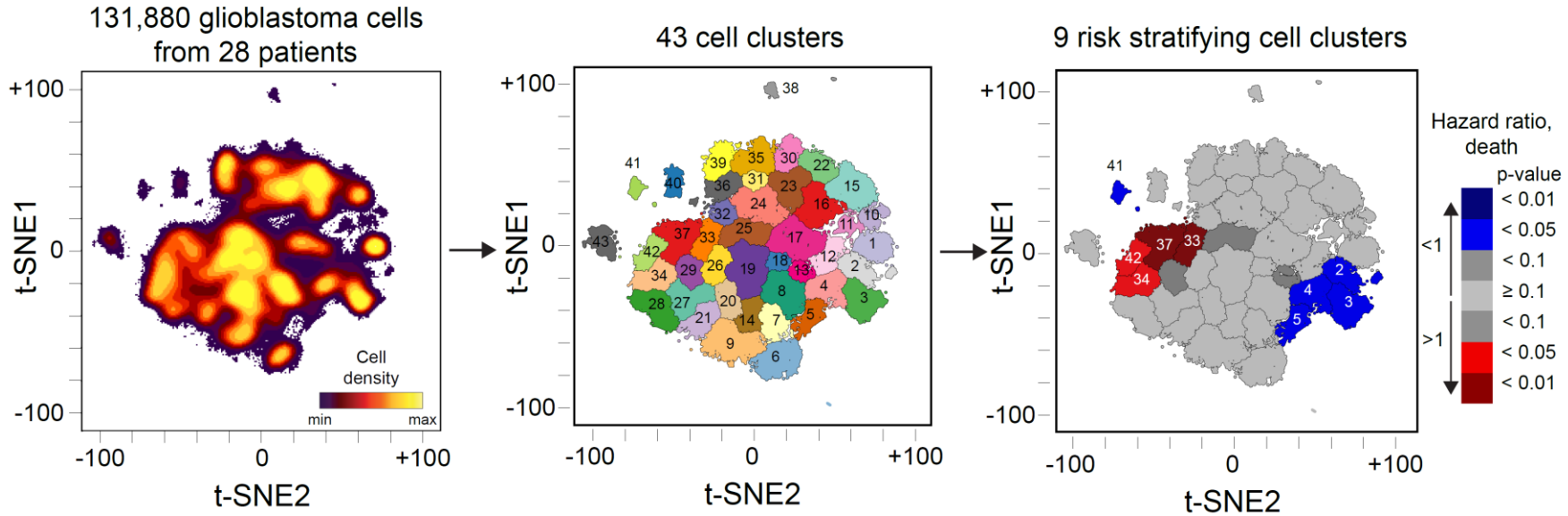
Relative island position ("global structure") is more stable & reflects original measurements in UMAP

Principle Component Analysis (PCA) is linear and deterministic, meaning that it strictly preserves global structure (and can overlook significant local structures / paths / trajectories)

RAPID Maps Clinical Outcomes Onto Clusters (in t-SNE, UMAP, 2D image, original features, PCA, etc.)

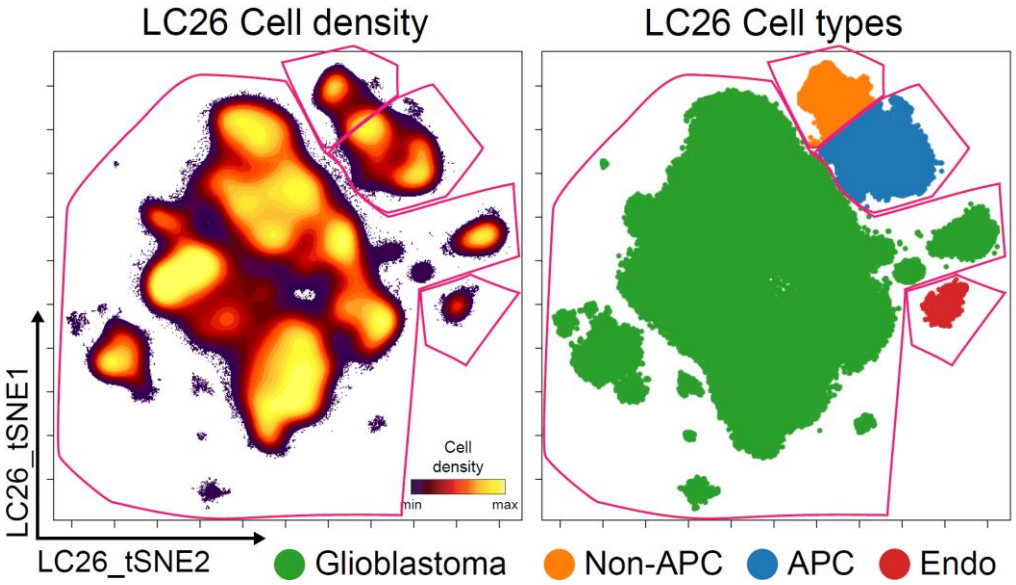


Risk Assessment Population IDentification (RAPID) Maps Outcome onto t-SNE



Leelatian & Sinnaeve et al., eLife 2020

t-SNE Can Help Computationally Isolate Cancer Cells from Endothelial and Leukocyte Cell Subsets

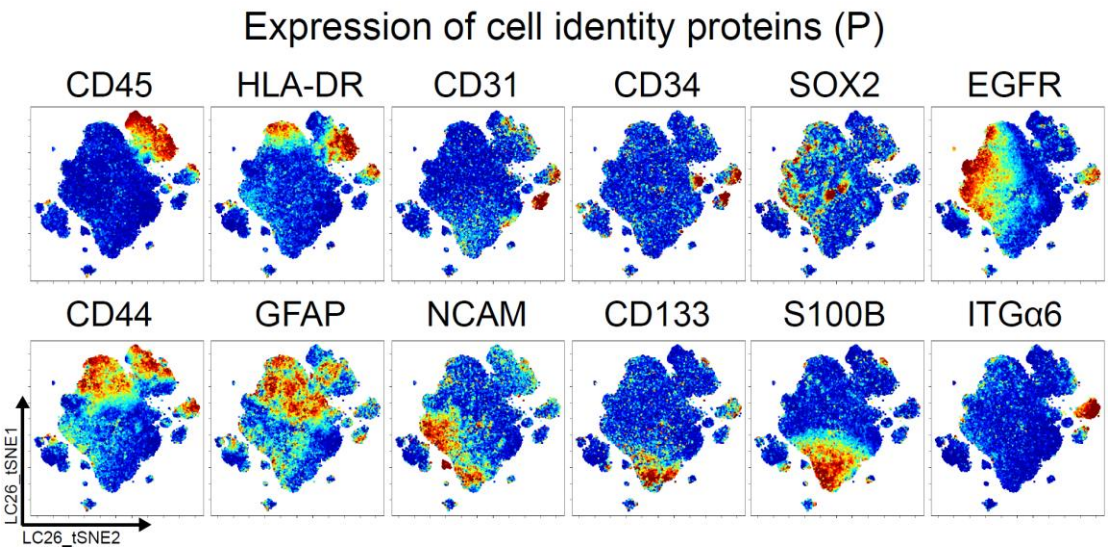


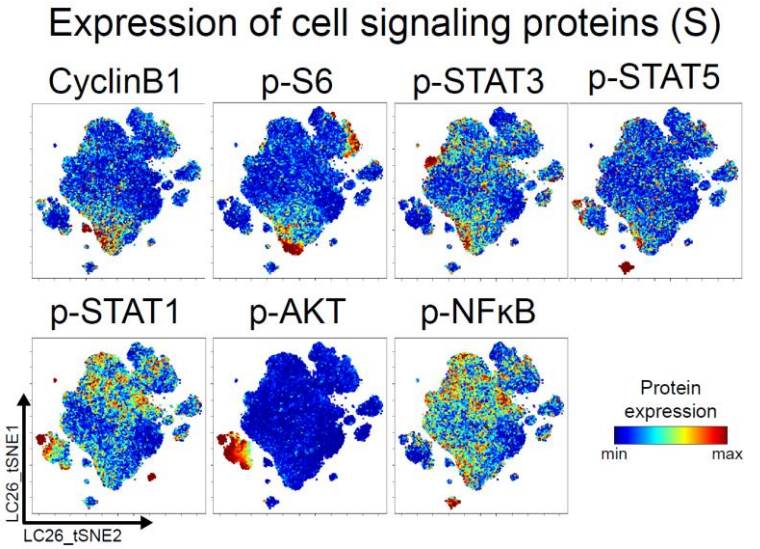
▲$CD45^{+7}$ $CD44^{+4}$
▼$EGFR^{-7}$ $S100B^{-3}$ $SOX2^{-2}$ — Non-APC

▲$CD45^{+6}$ $HLADR^{+4}$
▼$EGFR^{-5}$ $S100B^{-4}$ — APC

▲$CD31^{+5}$ $CD34^{+3}$ $PDGFRA^{+2}$ $CD49F^{+2}$
▼$EGFR^{-5}$ $CD44^{-4}$ $NCAM^{-3}$ $HLA\text{-}DR^{-2}$ $S100B^{-2}$ — Endothelial

▲$EGFR^{+2}$
▼$CD45^{-10}$ $HLADR^{-6}$ $CD31^{-3}$ $CD44^{-2}$ — Glioblastoma





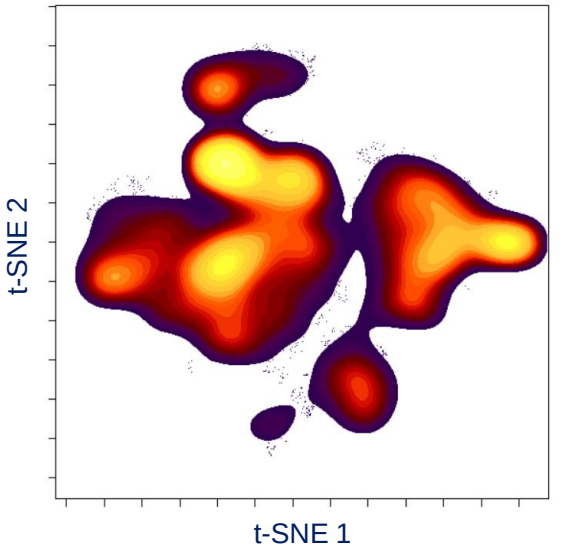
Leelatian & Sinnaeve et al., *eLife* 2020

UMAP (Uniform Manifold Approximation and Projection) is Another Dimensionality Reduction Tool

Superior run times

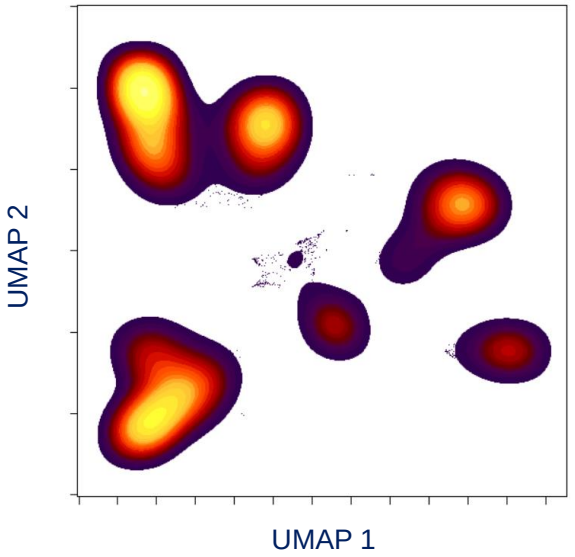
Emphasis on both global and local structure in the data

t-SNE



VS.

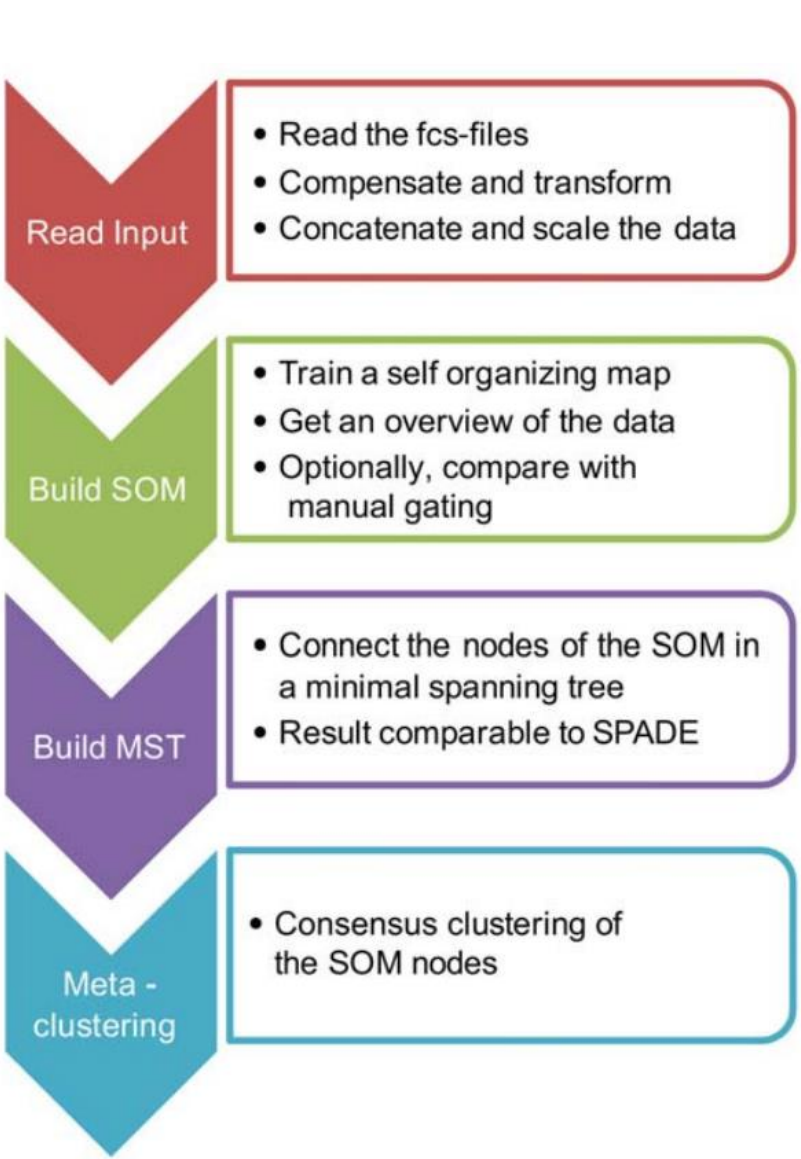
UMAP

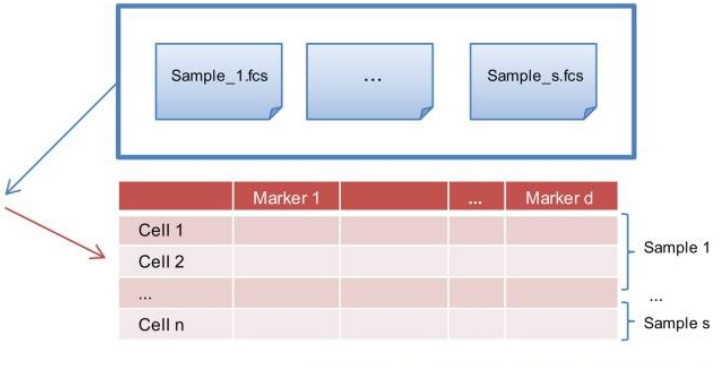


UMAP: McInnes et al., *arXiv* 2018; Becht et al., *Nature Biotech* 2019

Data used for t-SNE and UMAP: human PBMC

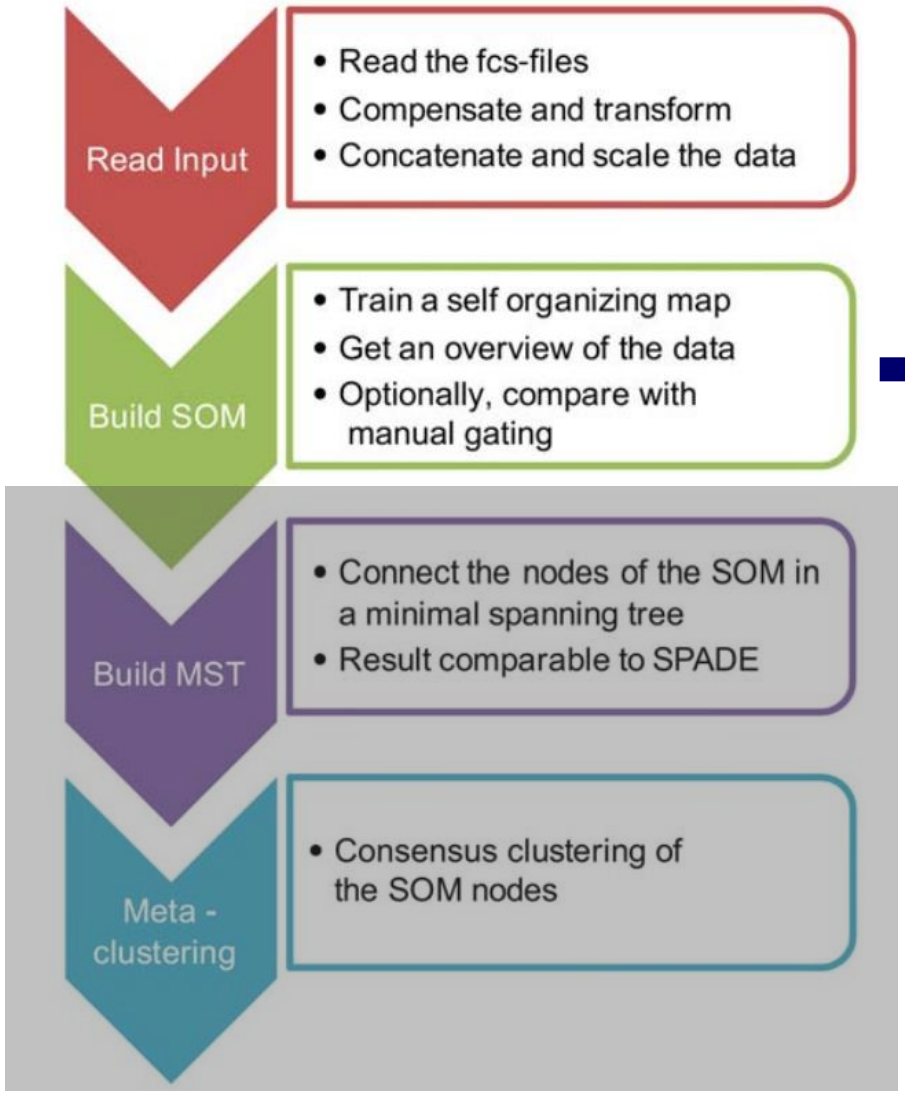
Clustering with FlowSOM: Self-organizing Maps





Van Gassen et al., *Cytometry A* 2015
See also, FlowSOM does well in a comparison of clustering tools: Weber & Robinson, *Cytometry A* 2017

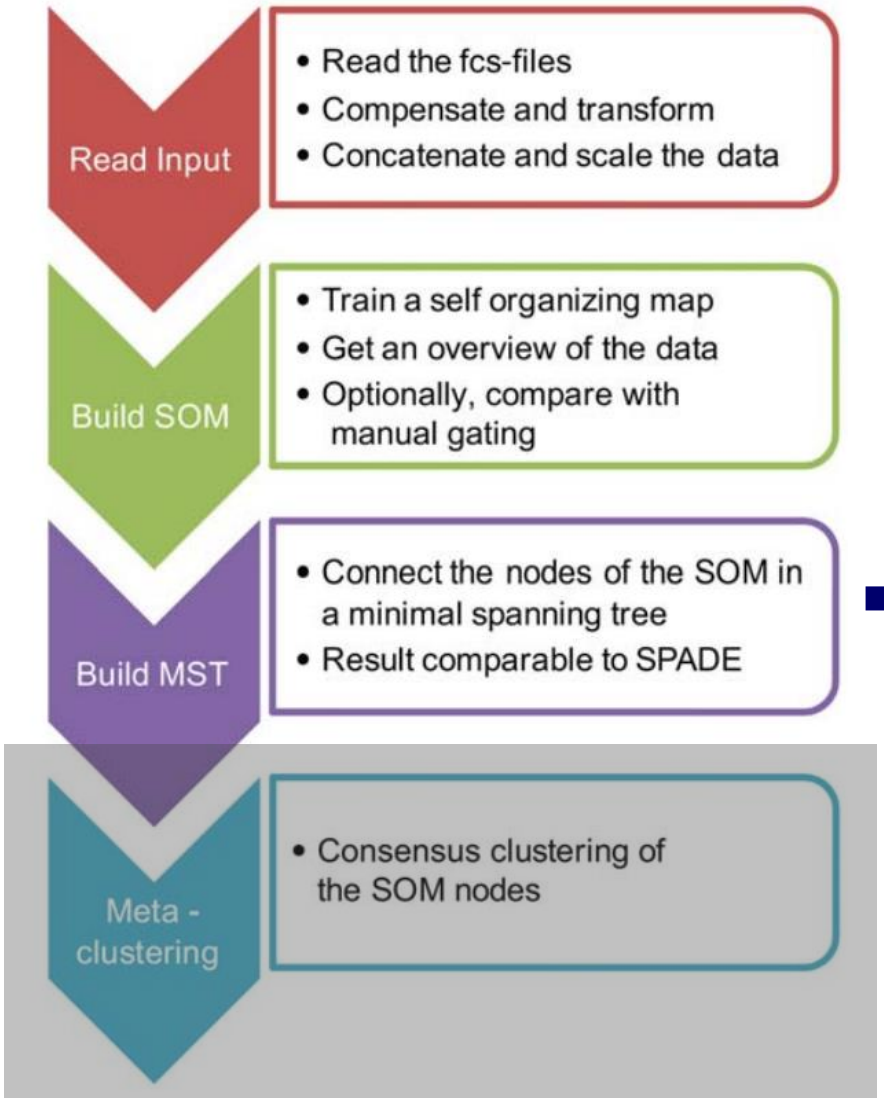
Clustering with FlowSOM: Self-organizing Maps



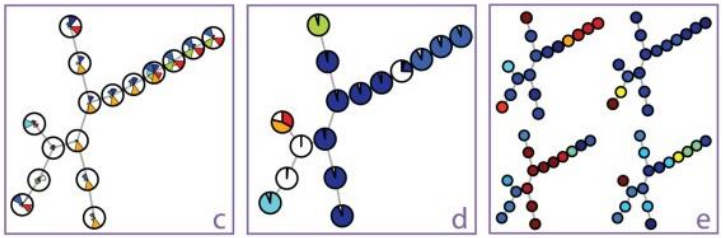
A SOM is created by assigning data points/cells to nodes based on their multidimensional phenotypes, updating this map repeatedly until each cell is assigned to a node with the most similar cells

Van Gassen et al., *Cytometry A* 2015
See also, FlowSOM does well in a comparison of clustering tools: Weber & Robinson, *Cytometry A* 2017

Clustering with FlowSOM: Self-organizing Maps



The next step is to arrange the nodes along a minimal spanning tree (MST), so that nodes that are most similar are closest on the tree
not used in our visualization



Van Gassen et al., *Cytometry A* 2015
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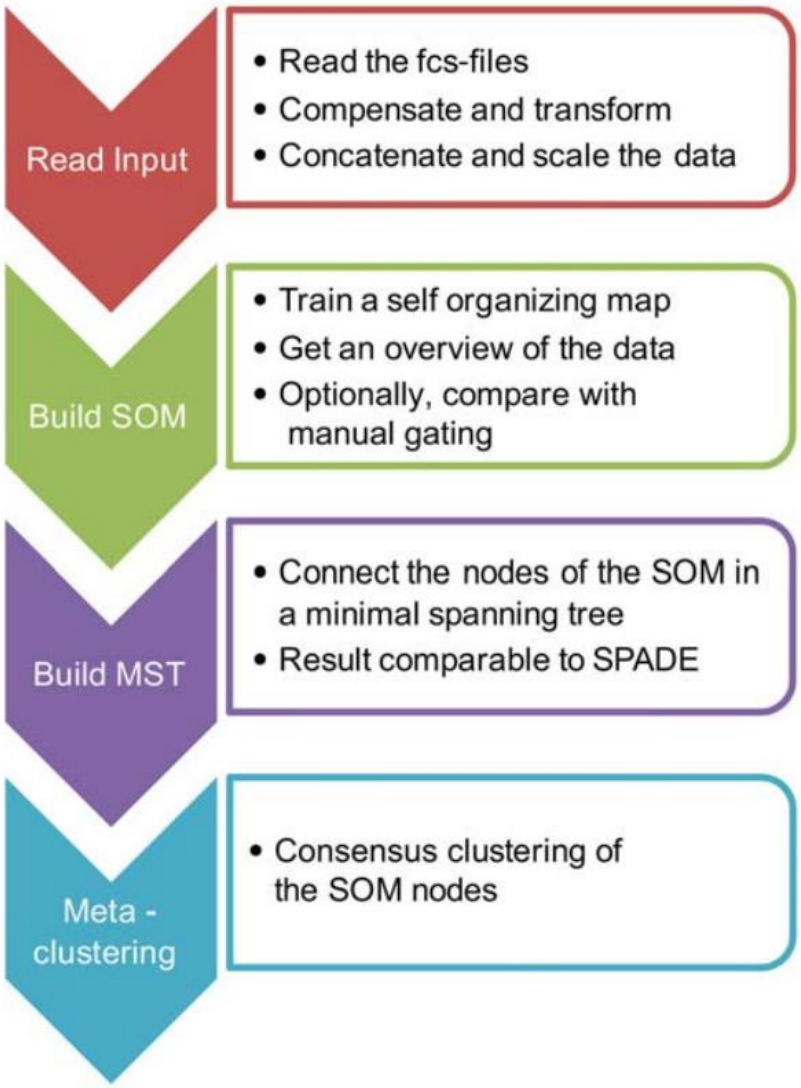
Clustering with FlowSOM: Self-organizing Maps

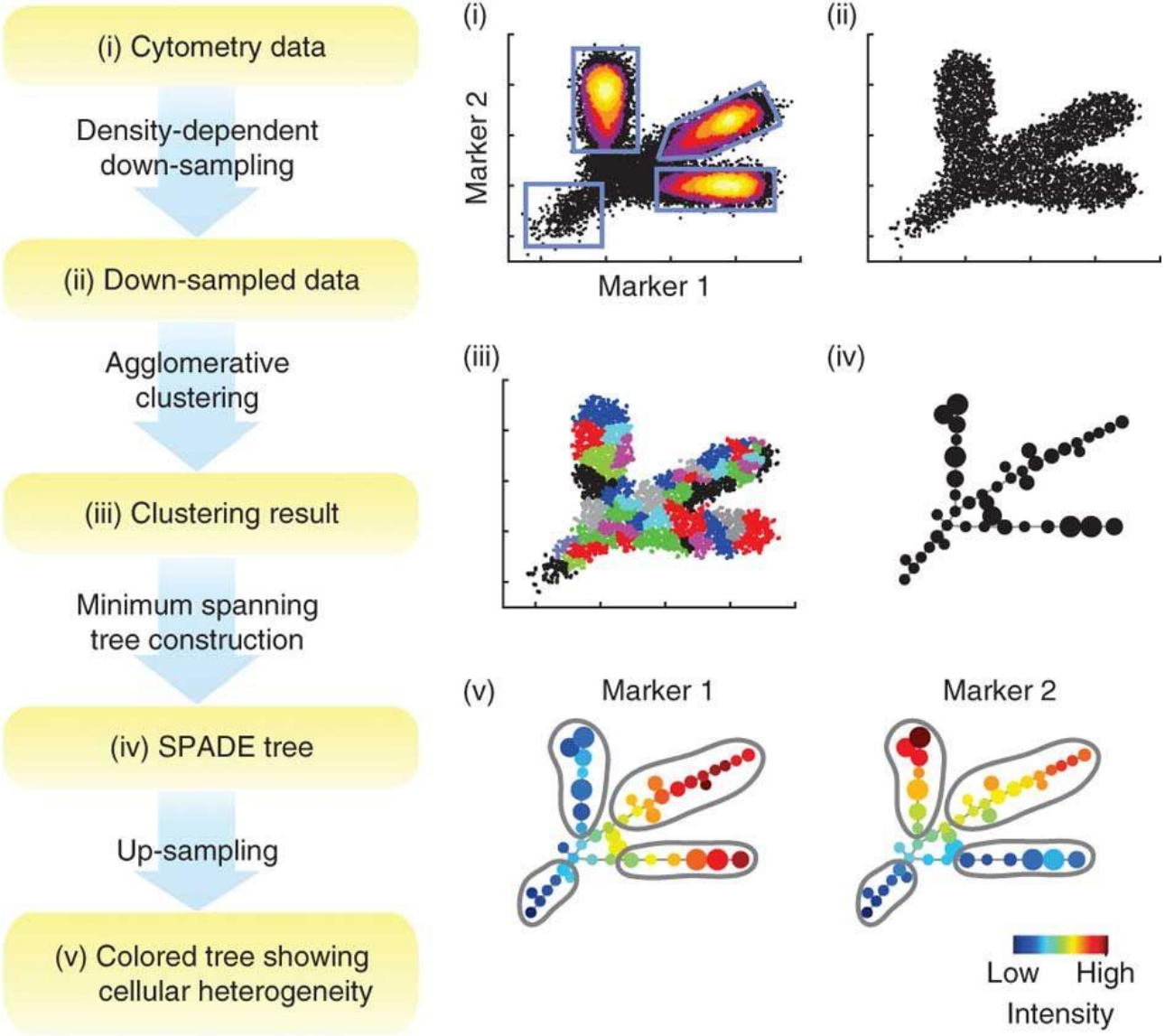


Finally, similar nodes are combined based on the number of desired clusters defined by the user. This desired number can be based on prior knowledge or a specific goal (i.e. minimizing intracluster variance)

Van Gassen et al., *Cytometry A* 2015

See also, FlowSOM does well in a comparison of clustering tools: Weber & Robinson, *Cytometry A* 2017

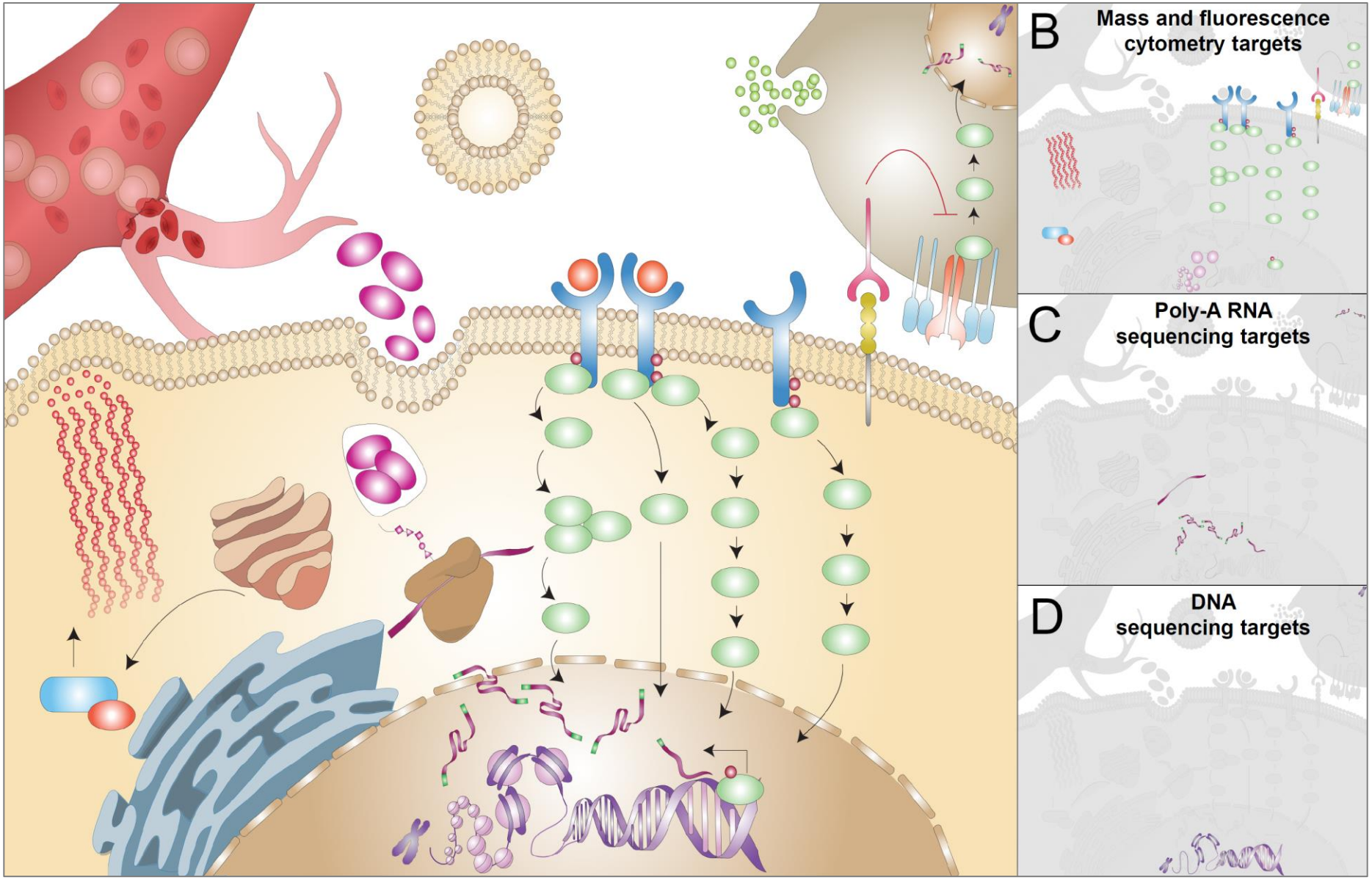
Spanning-Tree Progression Analysis of Density-Normalized Events (SPADE) is an Alternative Clustering Tool



Qui et al., *Nature Biotechnology* 2011

Part 5: What Is This Cluster?
MEM Labels & Identifies Group of Cells

Challenge to the Field: Multiplex Across Cell Functions

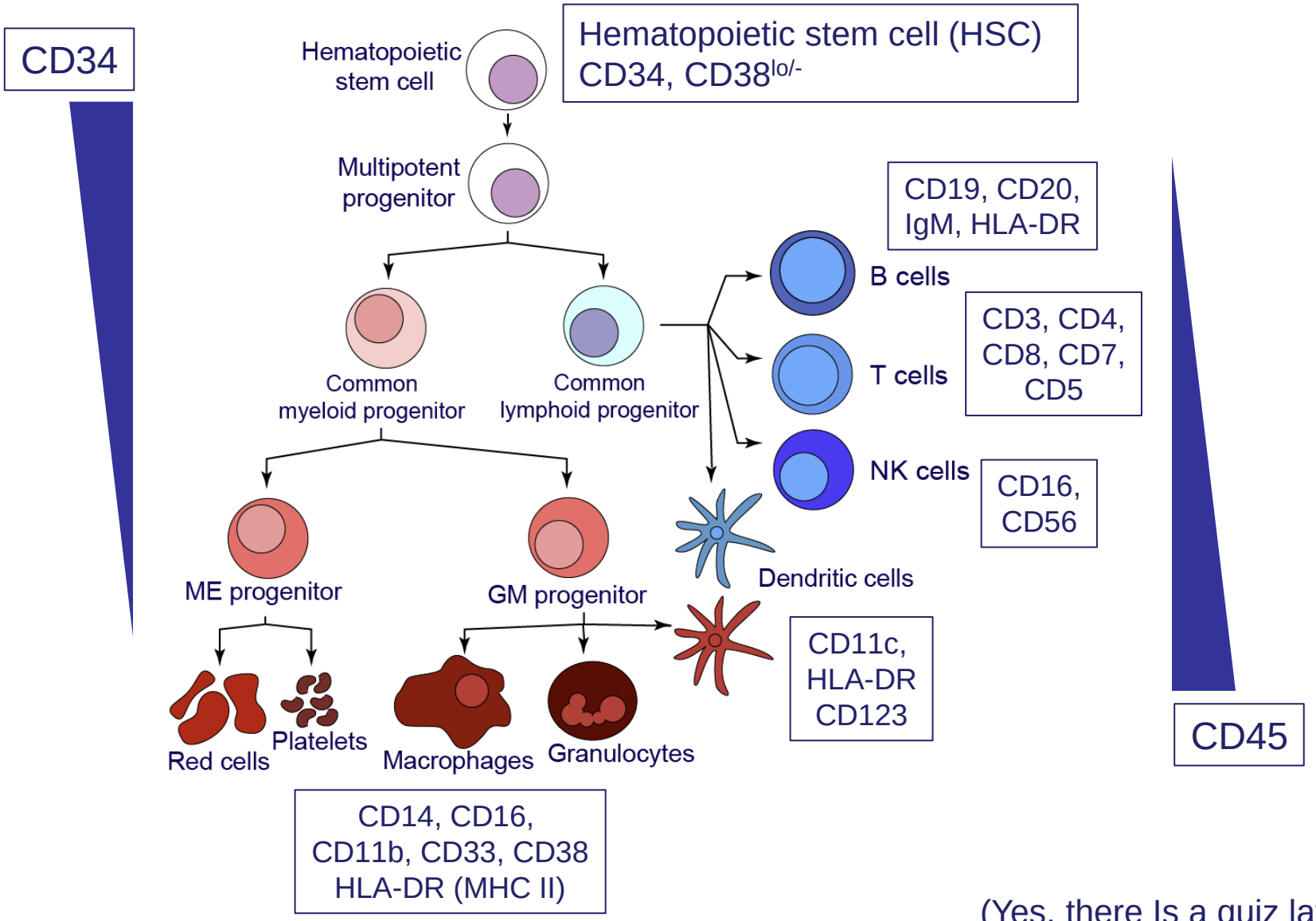


Beyond the Message: Advantages of Snapshot Proteomics with Single-Cell Mass Cytometry in Solid Tumors
Mistry et al., *FEBS Journal* 2018

MEM summarizes a population's special features
and is used in workflows "at the end"
(in place of box and whisker plots or heatmaps)

[So MEM complements tools from other steps, including
t-SNE, SPADE, Citrus, FlowSOM, SCAFFOLD, Phenograph]

Human Bone Marrow Hematopoiesis & “Famous” Cell Identity Markers



(Yes, there Is a quiz later)

Despite advances, no computational tools learn & label cell identity,
a human must “stare and compare” using expert knowledge

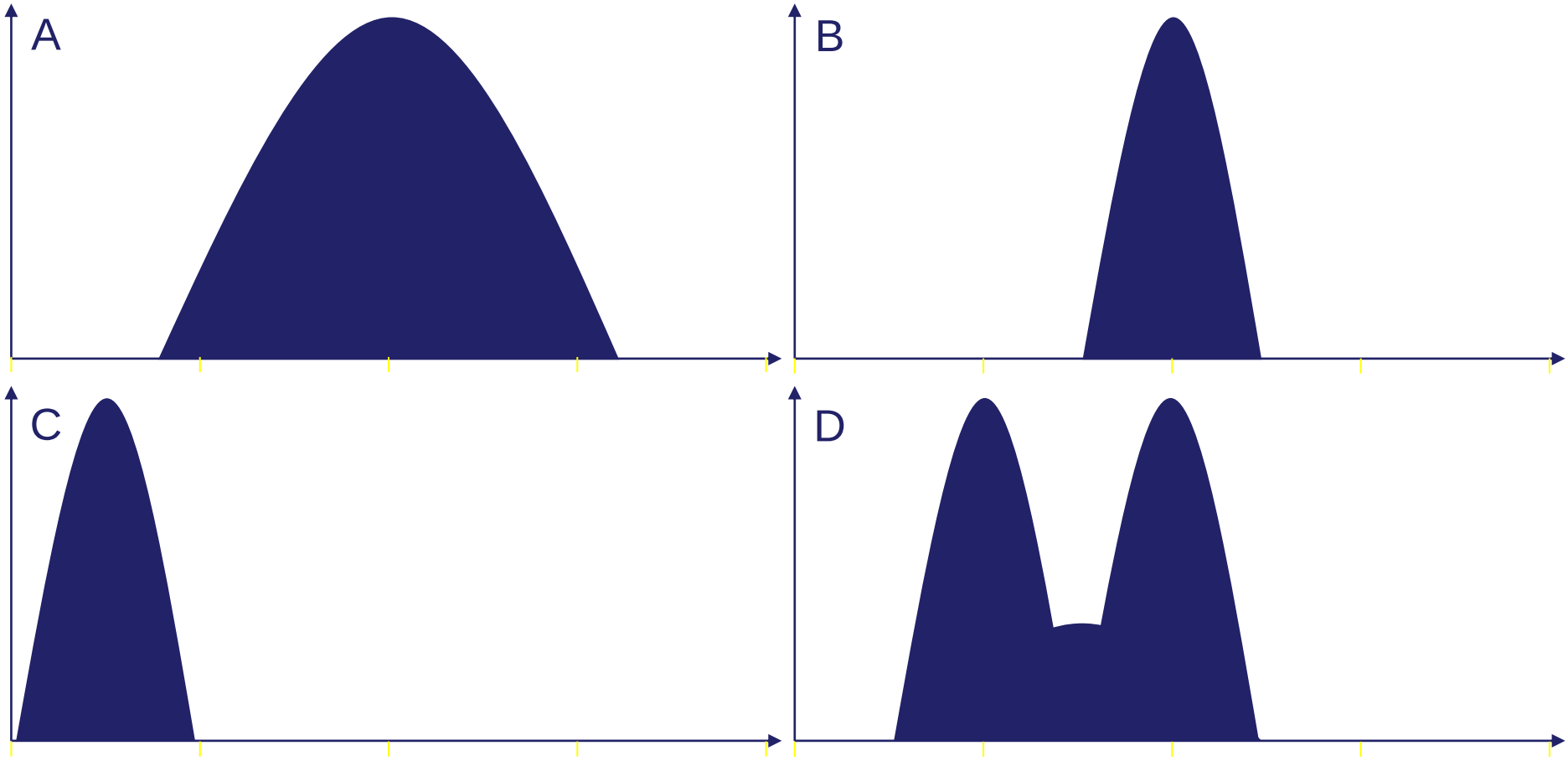
Diggins et al., *Methods* 2015

Populations are often labeled by metaphors of function
(“cancer stem cells”, “central memory T cells”)
or incomplete labels based on a few features (e.g. “PD-1+ CD8 T cells”).

We need an unbiased way to label & identify cells
(regardless of how they are found)

Enrichment Tracks Feature Exclusivity In a Subset

A, B, C, and D are 4 subsets where Protein X was measured.
In which subset is Protein X most distinct? (Which would be easiest to gate?)



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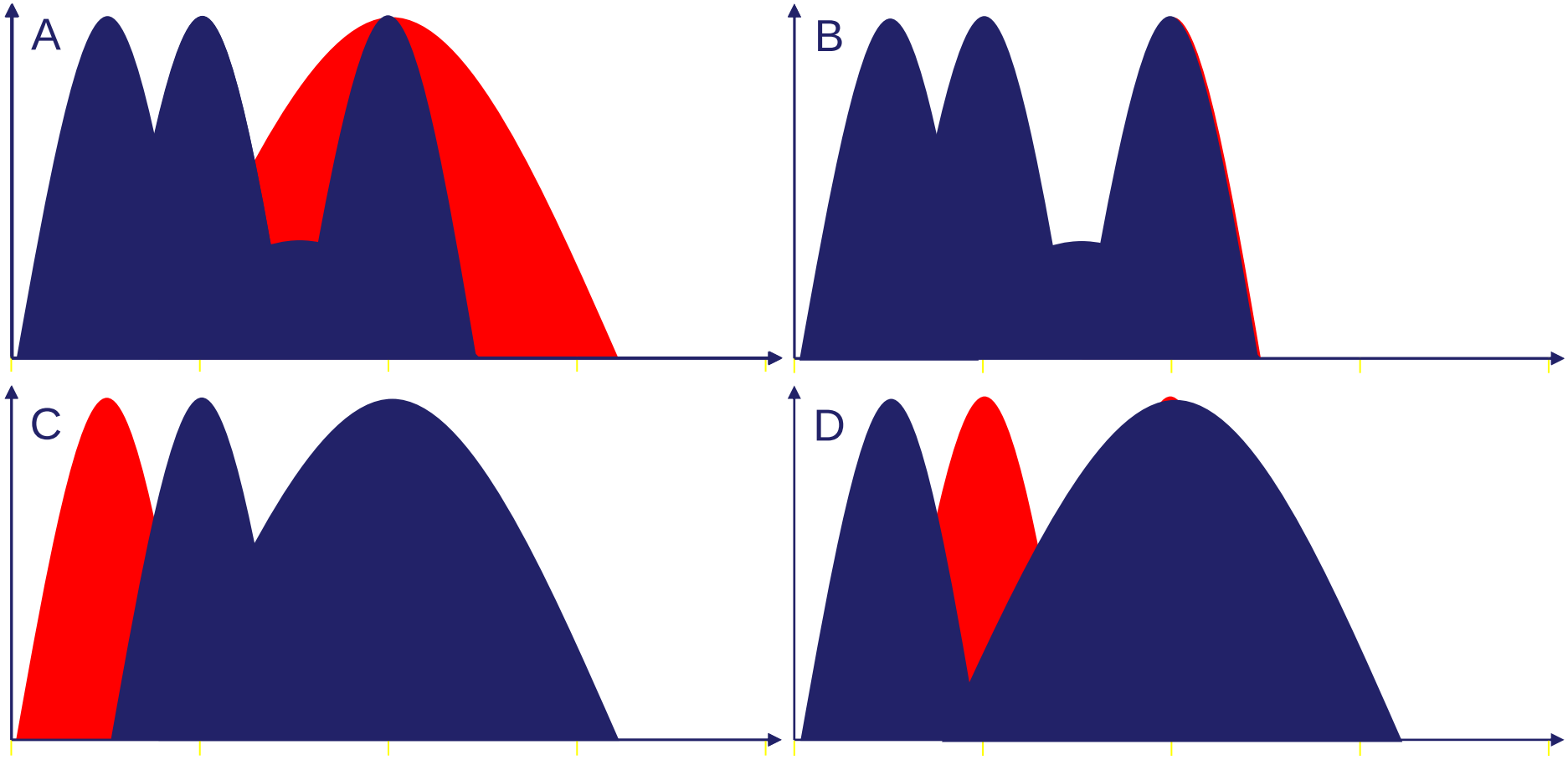
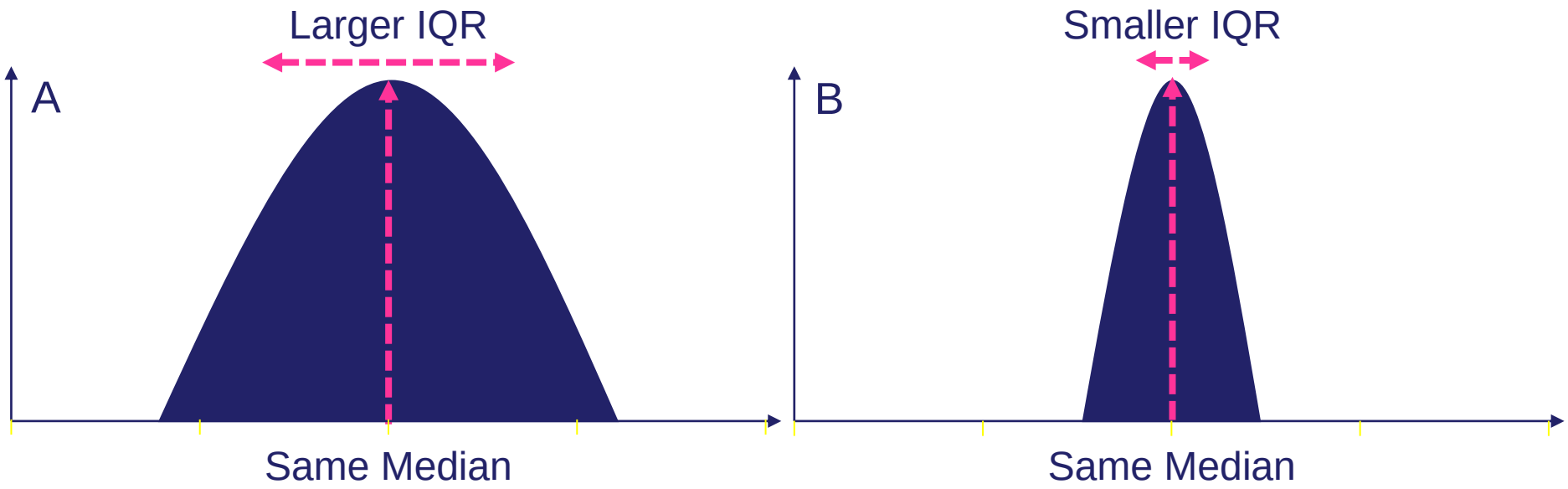


Median (50%) and Interquartile Range (25%-75%) Represent Key Features of Distributions



Core idea in MEM: given two protein distributions with equal medians, a smaller interquartile range (IQR) indicates greater enrichment

Not captured by median & IQR are other elements of shape (skewness, symmetry, # peaks, outliers, etc.)

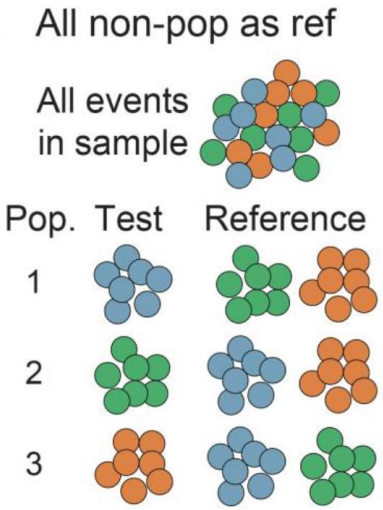
Diggins et al., *Nature Methods* 2017

MEM Quantifies Relative Enrichment By Combining Magnitude & Interquartile Range

$$MEM = |MAG_{test} - MAG_{ref}| + \frac{IQR_{ref}}{IQR_{test}} - 1$$

Linear transformation to -10 to +10
(d20 scale, cause that's how we roll)

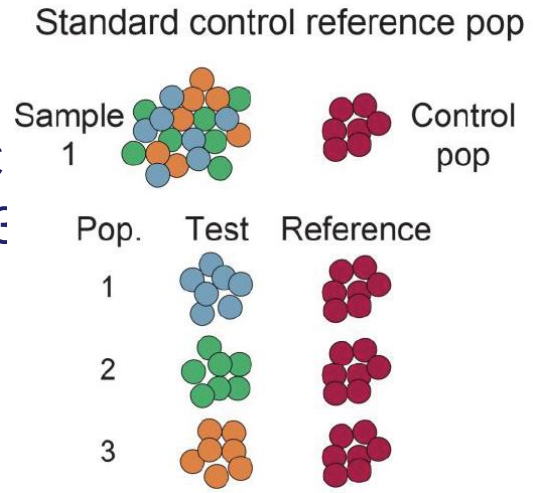
If $MAG_{test} - MAG_{ref} < 0$, MEM = -MEM



MEM label (CD19^{+} cells)

▲HLADR^{+10} CD20^{+9} CD19^{+7} IgM^{+5} C
CD45RA^{+3} CXCR4^{+2} CD47^{+2} CD33

▼CD7^{-2}



Diggins et al., *Nature Methods* 2017

Quiz Time: What Are These Cell Subsets & What Is This Tissue?

Stem cells (HSCs)

▲$CD34^{+6}$ $CD33^{+4}$ $CD15^{+3}$ $CD38^{+3}$
$MHCII^{+3}$ $CXCR4^{+2}$
▼$CD44^{-5}$ $CD45^{-5}$ $CD7^{-3}$ 0.07%

Natural killer cells

▲$CD16^{+9}$ $CD7^{+6}$ $CD38^{+5}$ $CD56^{+4}$ $CD161^{+4}$
$CD45RA^{+3}$ $CD8^{+2}$ $CD11b^{+2}$ $CD47^{+2}$ 5.27%

Progenitors

▲$MHCII^{+10}$ $CD33^{+7}$ $CD38^{+5}$ $CD123^{+3}$
$CD117^{+3}$ $CD19^{+2}$ $CD34^{+2}$ $CD13^{+2}$
$CD14^{+2}$ $CXCR4^{+2}$
▼$CD45^{-3}$ $CD15^{-2}$ 0.002%

$CD8^+$ T cells

▲$CD8^{+8}$ $CD7^{+5}$ $CD3^{+3}$
$CD45RA^{+3}$ $CXCR4^{+2}$ 9.25%

Early myeloid cells

▲$MHCII^{+9}$ $CD33^{+8}$ $CD38^{+5}$
$CD4^{+3}$ $CD15^{+2}$ $CD14^{+2}$
▼ $CD45^{-2}$ $CD7^{-2}$ 0.02%

$CD4^+$ T cells

▲$CD4^{+7}$ $CD7^{+5}$ $CD3^{+5}$
$CD47^{+2}$ $CD45RA^{+2}$ 8.12%

Monocytes

▲$CD33^{+10}$ $CD14^{+8}$ $CD11b^{+7}$
$MHCII^{+5}$ $CD4^{+4}$ $CD11c^{+4}$
$CD38^{+4}$ $CD13^{+3}$
▼ $CXCR4^{-2}$ $CD47^{-2}$ 10.57%

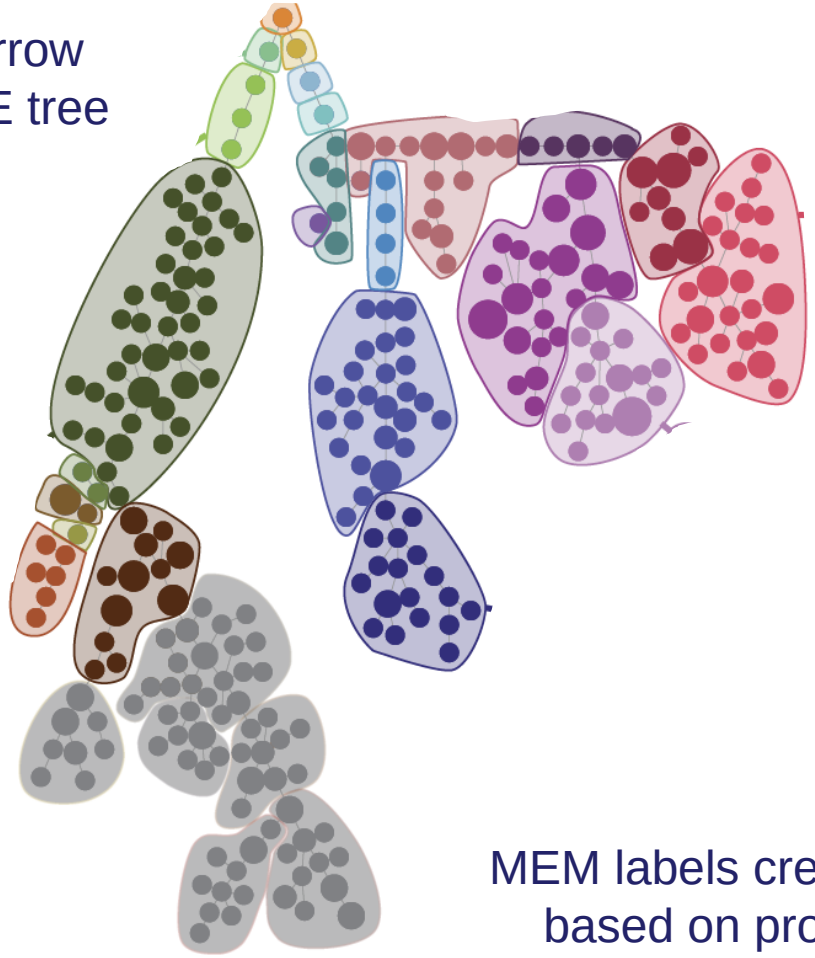
B cells

▲$MHCII^{+10}$ $CD20^{+9}$ $CD19^{+7}$ IgM^{+5} $CD34^{+3}$
$CD45RA^{+3}$ $CXCR4^{+2}$ $CD47^{+2}$ $CD33^{+2}$
▼$CD7^{-2}$ 2.44%

Data from healthy human bone marrow, Bendall et al., *Science* 2011
Diggins et al., *Nature Methods* 2017

Marker Enrichment Modeling Automatically Labels Cell Types in Human Bone Marrow Using -10 to +10 Enrichment Values

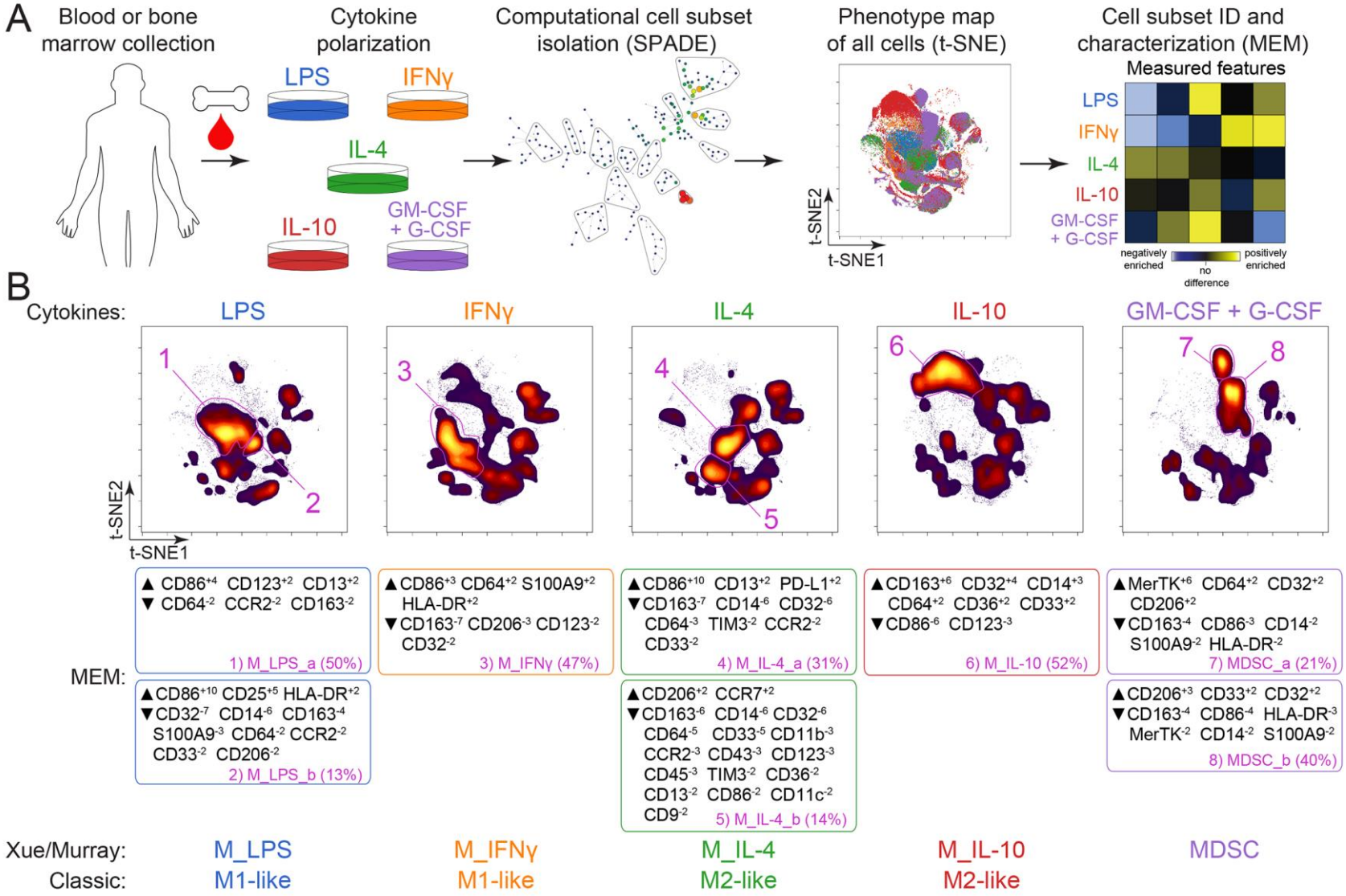
Cells from bone marrow grouped in a SPADE tree



MEM labels created automatically based on protein enrichment

Diggins et al., *Nature Methods* 2017
Data from Bendall et al., *Science* 2011

Functional Profiling: Polarization Capacity of Monocytes

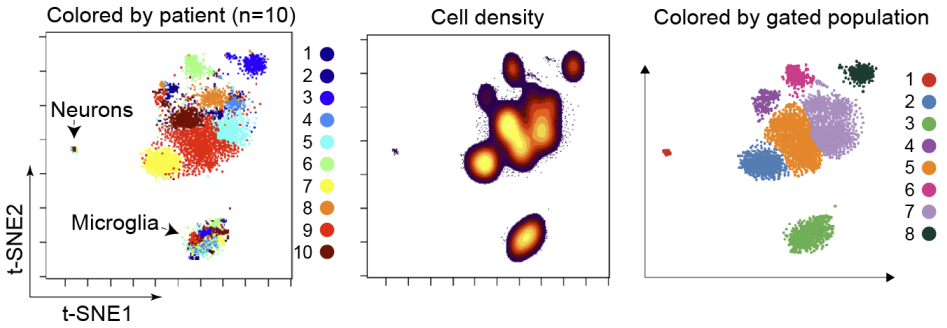


http://flowrepository.org/id/FR-FCM-Z2Z8

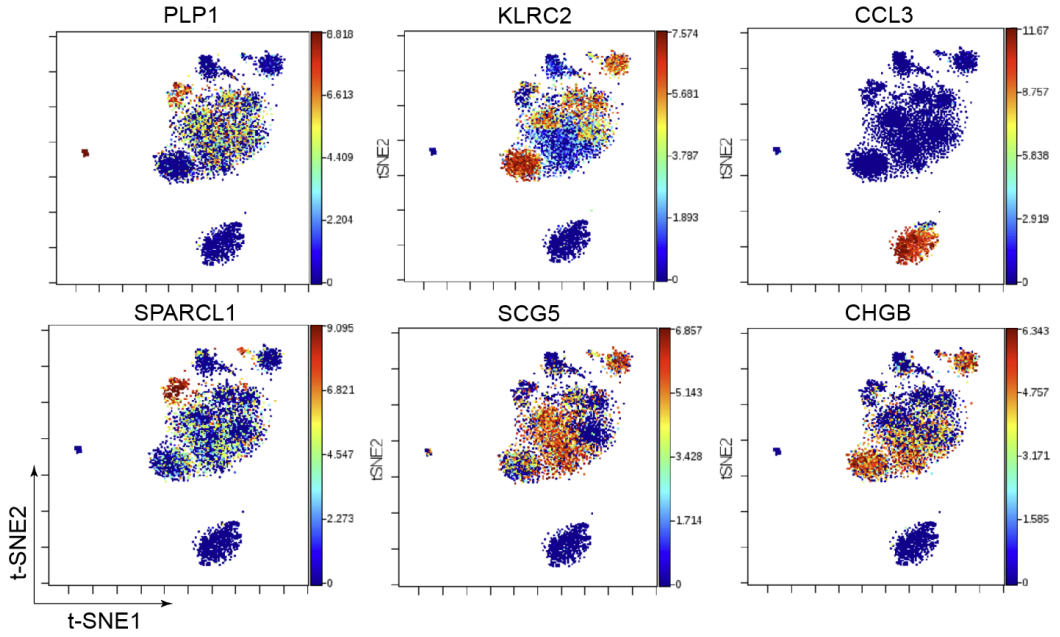
Roussel et al., *Methods in Molecular Biology* 2019, *in press*
Original work: Roussel et al., *Journal of Leukocyte Biology 2017*

MEM Classification of Cell Subsets in IDH-A Brain Tumors Based on scRNA-seq Transcript Expression

IDH-A brain tumor cells from 10 patients
Gating for 8 cell types: t-SNE using 500 most variable transcripts, gating on local density



Per-cell transcript expression, top 6 most positively enriched in gated populations



MEM Label, Population 3:

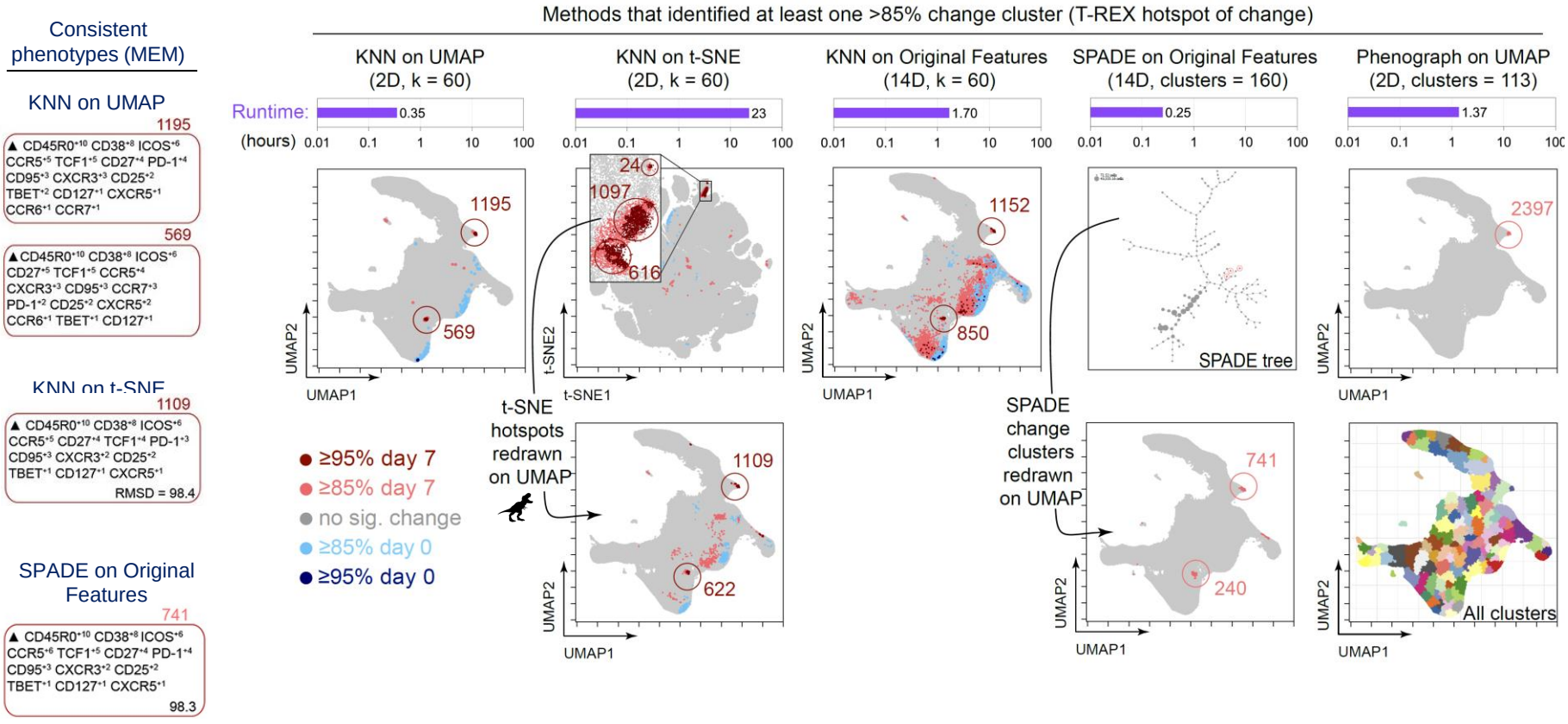
▲SAT1^{+6} CCL3^{+5} CCL4^{+5} CD74^{+5} HLA-DRA^{+5} RGS1^{+5} SPP1^{+5}

▼GFAP^{-8} OMG^{-8} APOD^{-7} KLRC2^{-7} SCG5^{-7} UCHL1^{-7}

(ID'd as microglia)

Data from leukemia patient blood, Venteicher et al., *Science* 2017
MEM for scRNA-seq: Diggins et al., *in preparation*

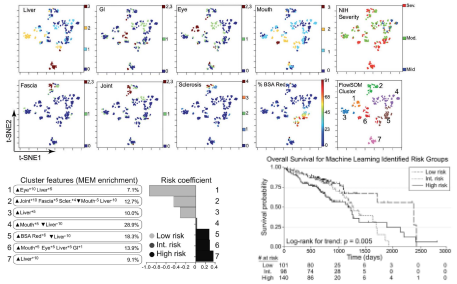
T-REX Worked with Other Algorithms to Identify Comparable Cells, But KNN on UMAP or t-SNE Outperformed KNN on Original Features



T-REX: Barone, Paul, Muehling et al., *eLife* 2021

Part 6: Example Data with cGVHD

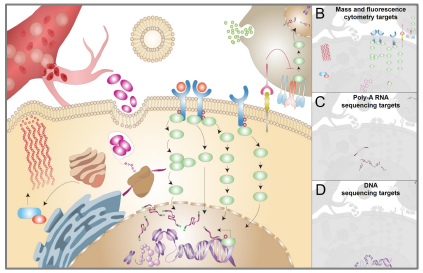
Adapting Advances in Machine Learning & Single Cell Biology for Immuno-Oncology



Haematologica 2018

1. Gandelman et al., Machine Learning Reveals Chronic Graft-Versus-Host Disease Phenotypes and Stratifies Survival After Stem Cell Transplant for Hematologic Malignancies. ***Haematologica*** 2018 PMC6312024.

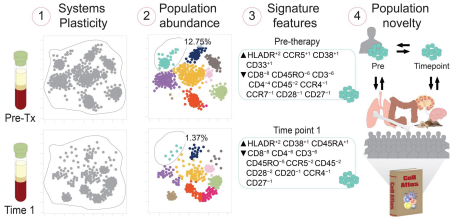
   Machine learning for patient medical phenotypes including t-SNE, FlowSOM, and MEM. Workflow inspired RAPID algorithm shown here for solid tumor cells.



FEBS J 2018

2. Mistry et al., Beyond the Message: Advantages of Snapshot Proteomics with Single-Cell Mass Cytometry in Solid Tumors. ***FEBS J*** 2018 Dec 13. PMID: 30549207 DOI: 10.1111/febs.14730.

   Reviews single cell mass cytometry with an emphasis on solid tumor and tissue research. Provides a detailed comparison with complementary single cell technologies, including single cell RNA-sequencing and quantitative multiplex imaging.



Cancer Immunology Research 2019

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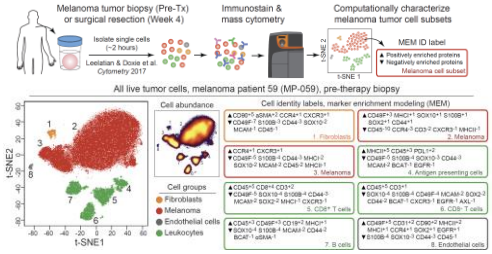
   New computational workflow for longitudinal single cell tumor immunology. Revealed abnormal immune cells present in multiple tumor types. Will be applied to solid tumors to track changes in immune microenvironment.

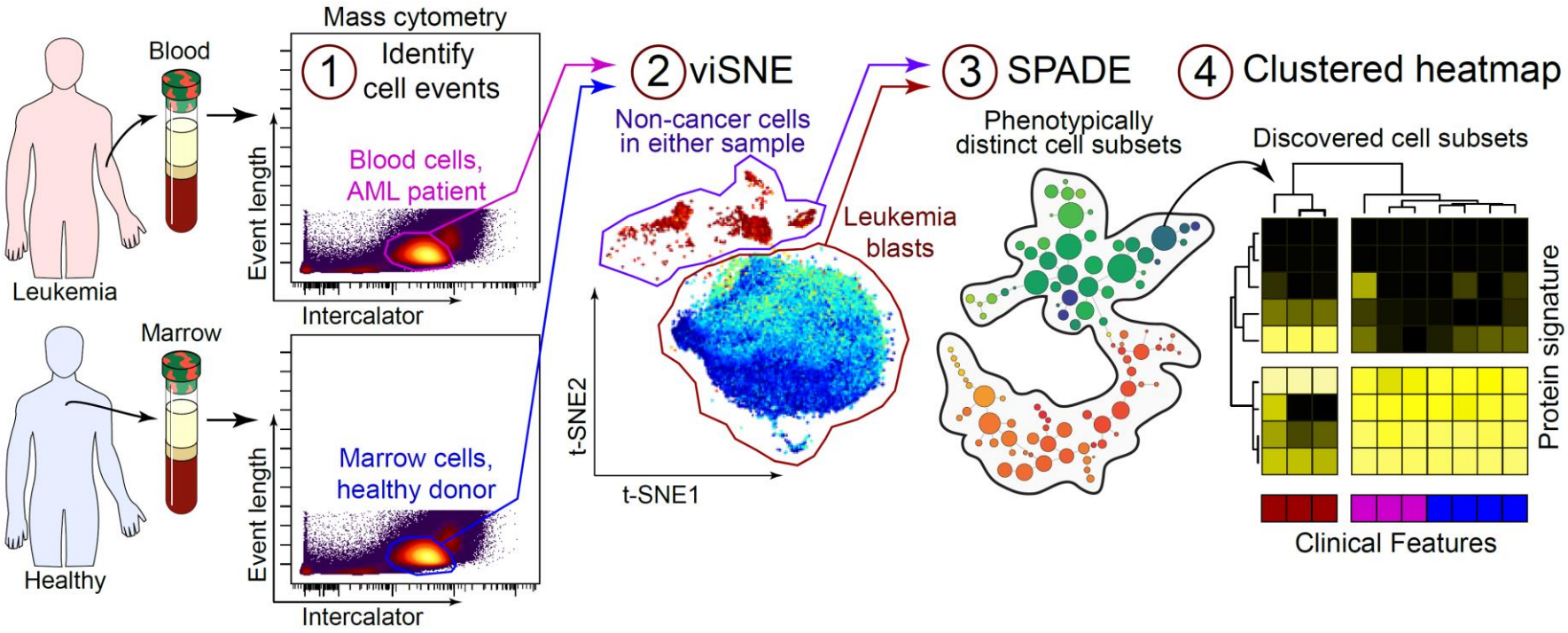


Pigment Cell & Melanoma Research 2018

4. Doxie et al., BRAF and MEK inhibitor therapy eliminates Nestin-expressing melanoma cells in human tumors. ***Pigment Cell Melanoma Research***. 2018 Jun 28. PubMed PMID: 29778085; PubMed Central PMCID: PMC6188784.

   Compared biopsies over time of melanoma patients on treatment with mass cytometry to reveal *in vivo* cellular changes on treatment following targeted therapy.

Machine Learning Tools Can Automate Sub-Population (Cluster / Subtype) Characterization

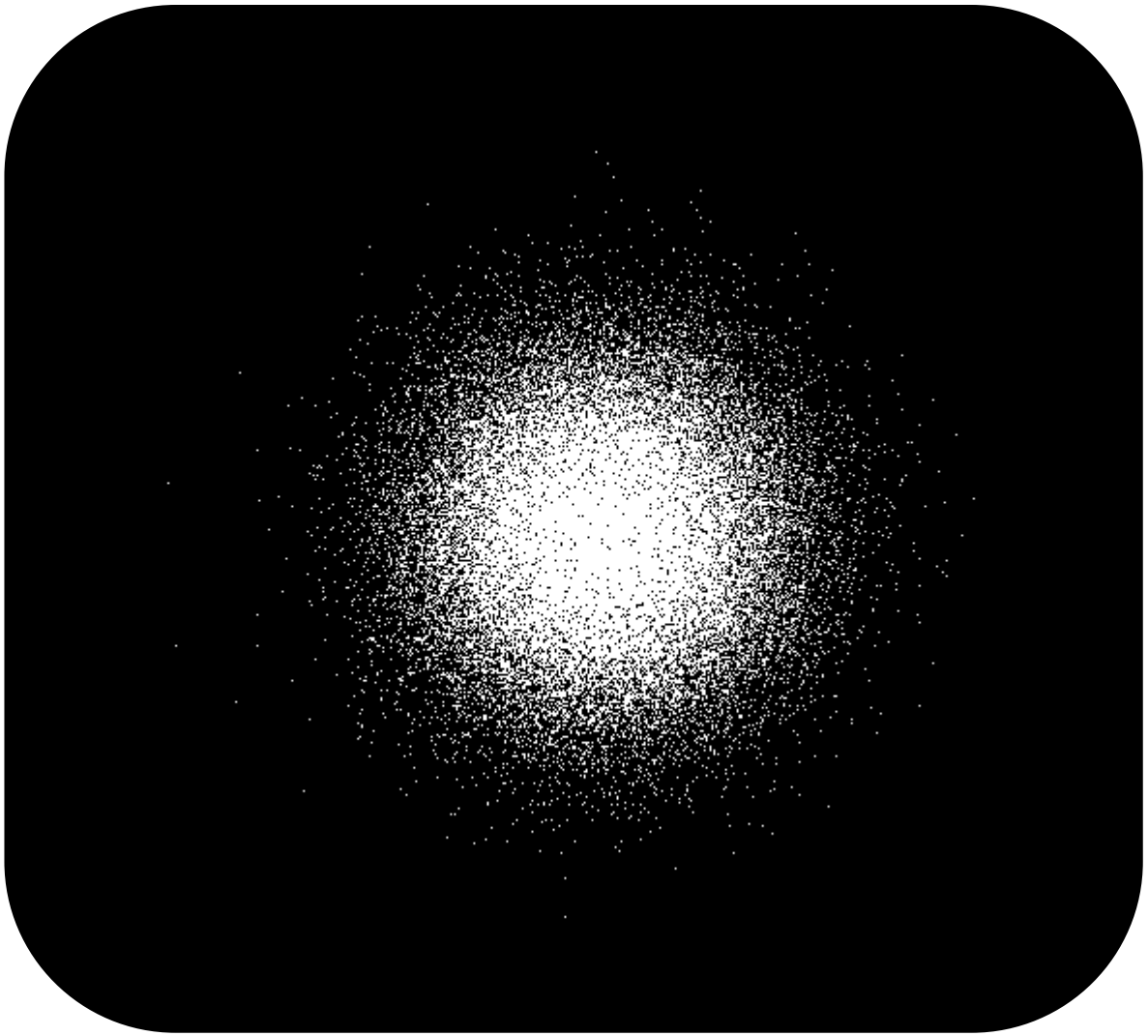


Protocol: Diggins et al., *Current Protocols in Cytometry* 2017
Data files: https://flowrepository.org/id/FR-FCM-ZZKZ
More great tools: Saeys et al, *Nature Reviews Immunology* 2016

Workflow: Diggins et al., *Methods* 2015
viSNE/t-SNE: Amir et al., *Nat Biotech* 2013
SPADE: Qiu et al., *Science* 2011

For sub-populations: quantify the known, reveal the unexpected, characterize the abnormal, & evaluate any associated risk

Key Algorithm Concept: t-SNE Visualizes (in 2D) Close Phenotypic Relationships Using Many Markers



Healthy human blood, mass cytometry, 26 markers measured, viSNE / t-SNE analysis tool
(Animation by Cytobank: each movie frame shows one iteration of t-SNE algorithm)

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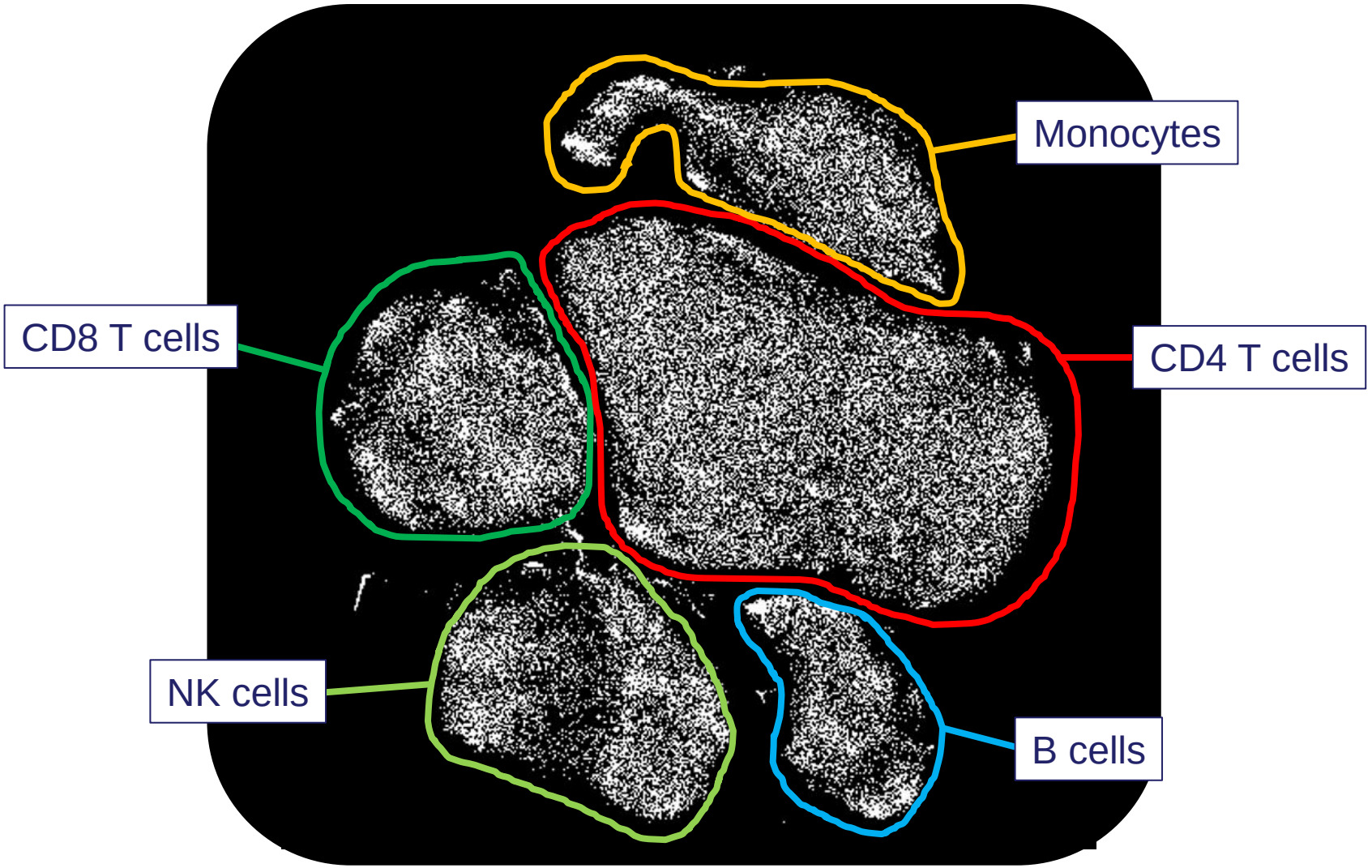

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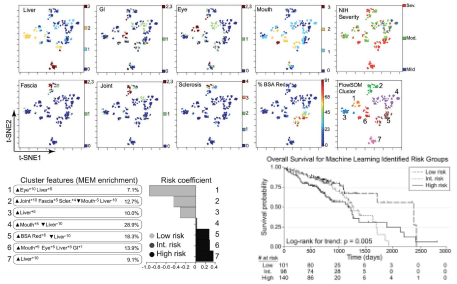

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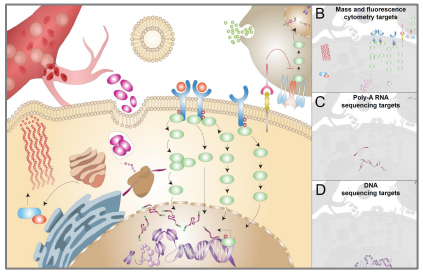

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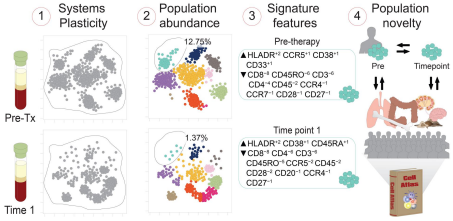

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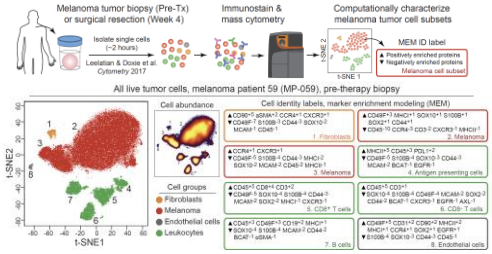

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   Compared biopsies over time of melanoma patients on treatment with mass cytometry to reveal *in vivo* cellular changes on treatment following targeted therapy.

cGVHD Arises from Immune Dysregulation, Is Clinically Heterogeneous, and Is Scored "Multidimensionally"

Areas of Potential Organ Involvement ("Dimensions"):

1. Liver
2. Gastrointestinal (GI)
3. Eye
4. Mouth
5. Fascia
6. Joint
7. Skin sclerosis
8. Skin redness
9. Lung

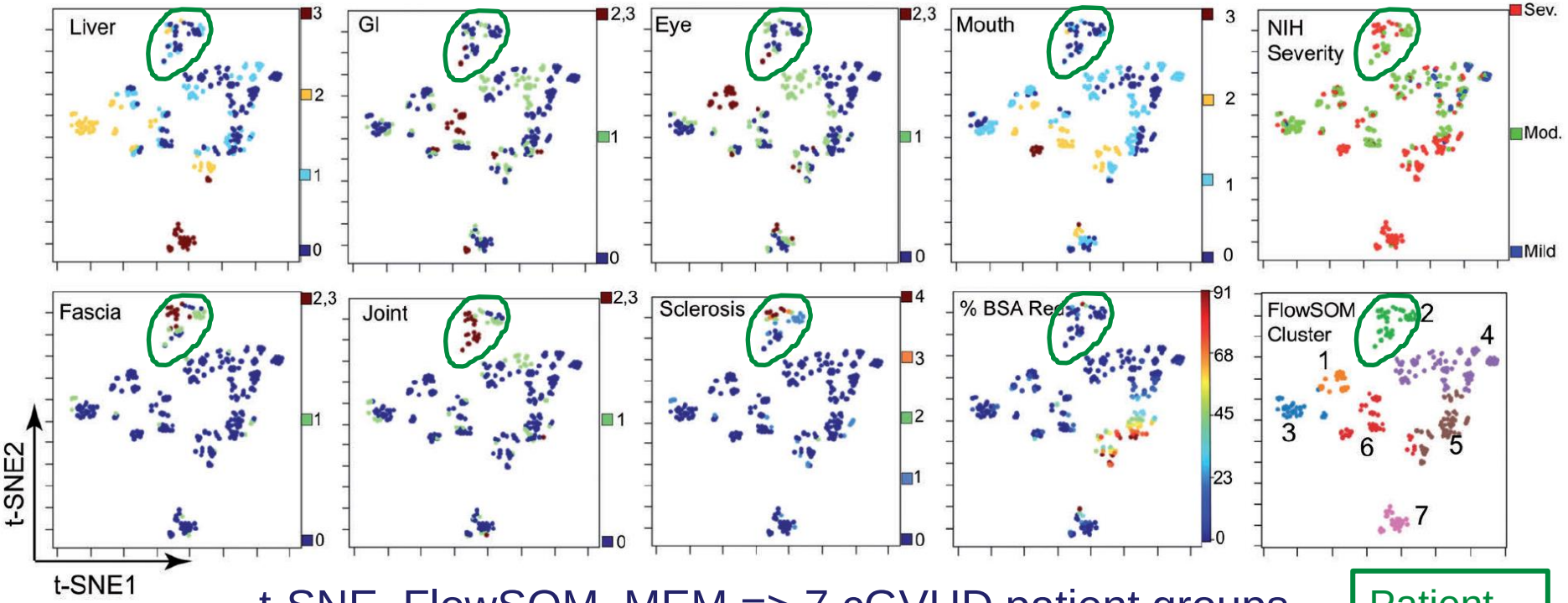
Each is scored (e.g. 0 to 3)

Sum of scores = NIH severity

Gandelman et al., *Haematologica* 2018

Data Science Strategies from Single Cell Analysis Reveal cGVHD Patient Groups Using Medical Phenotypes

Dots = 339 cGVHD patients
t-SNE = 8 dimensional medical phenotype
FlowSOM = identifies 7 cGVHD patient groups



t-SNE, FlowSOM, MEM => 7 cGVHD patient groups

Patient Cluster 2

Chronic graft-*versus*-host disease (cGVHD)
t-SNE on 8 organ domain scores (e.g., liver involvement from 0 to 3)

Gandelman et al., *Haematologica* 2018

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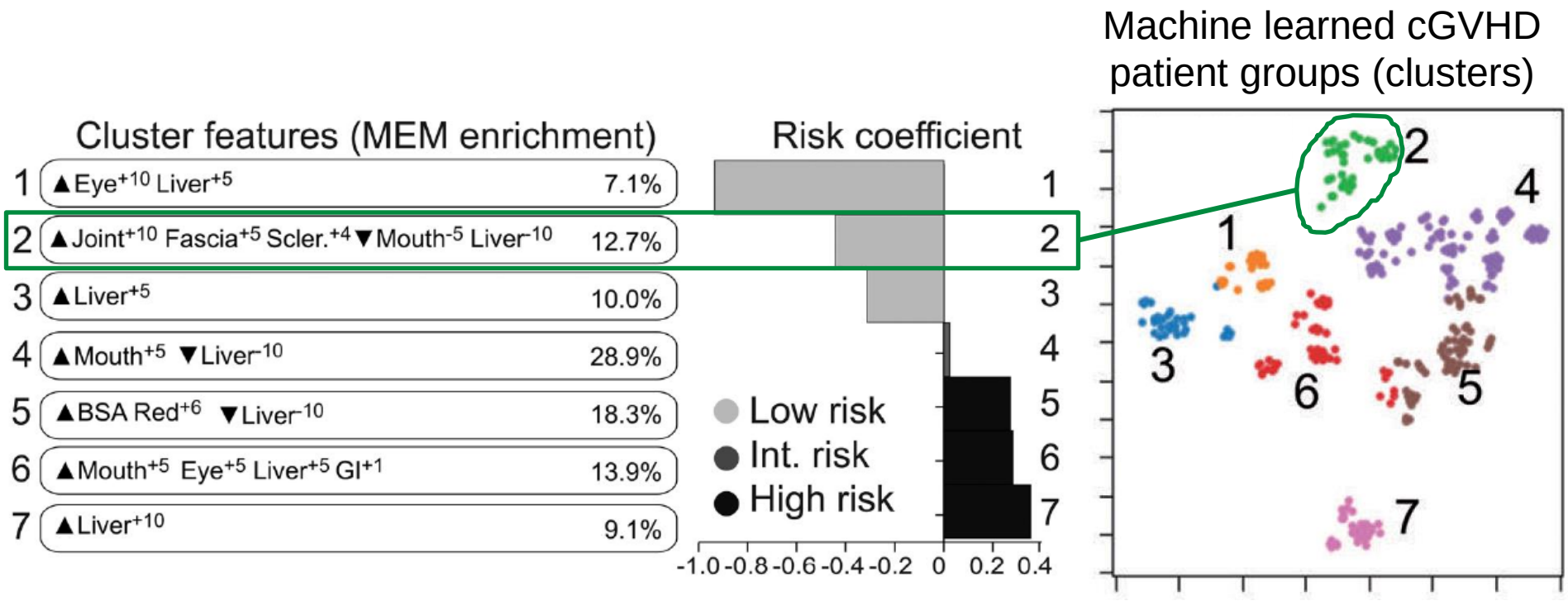
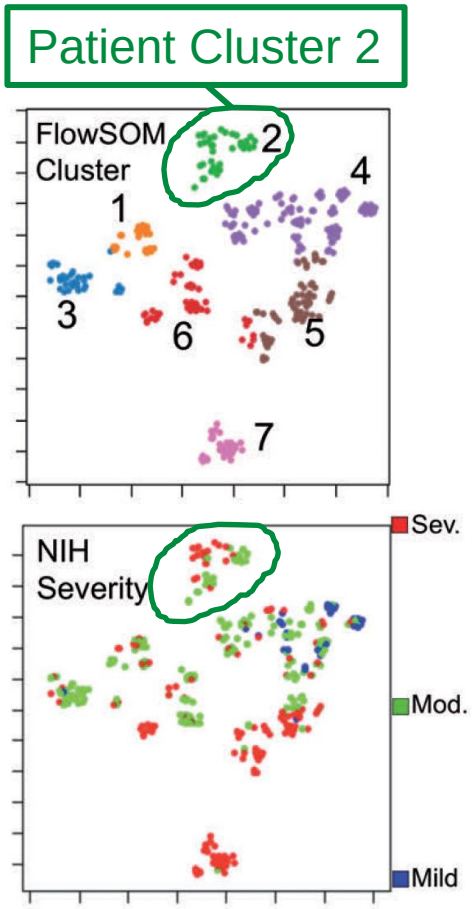
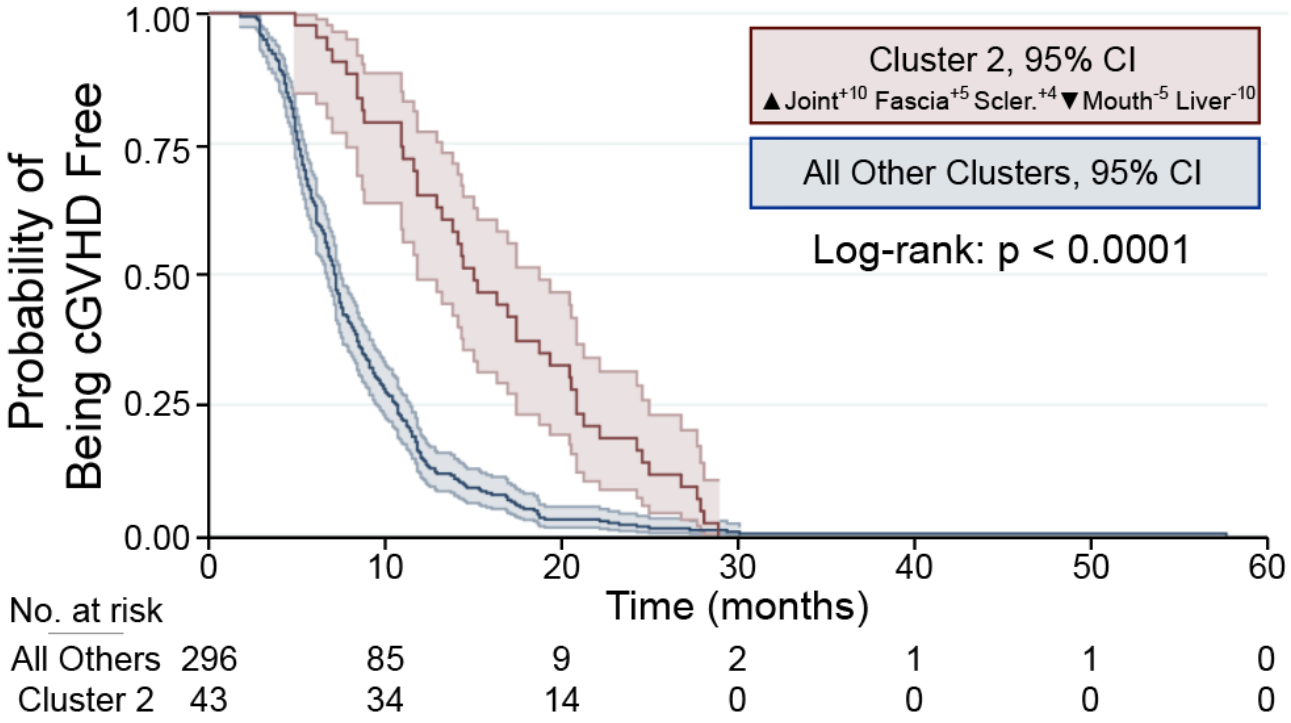


Chronic graft-*versus*-host disease (cGVHD)
t-SNE on 8 organ domain scores (e.g., liver involvement from 0 to 3)

Gandelman et al., *Haematologica* 2018

Cluster 2 Was a Distinct Subtype of cGVHD Patients with Longer Time from Stem Cell Transplant to cGVHD





Phenotype of cGVHD Patient Cluster 2 ("MEM label": +10 / -10)

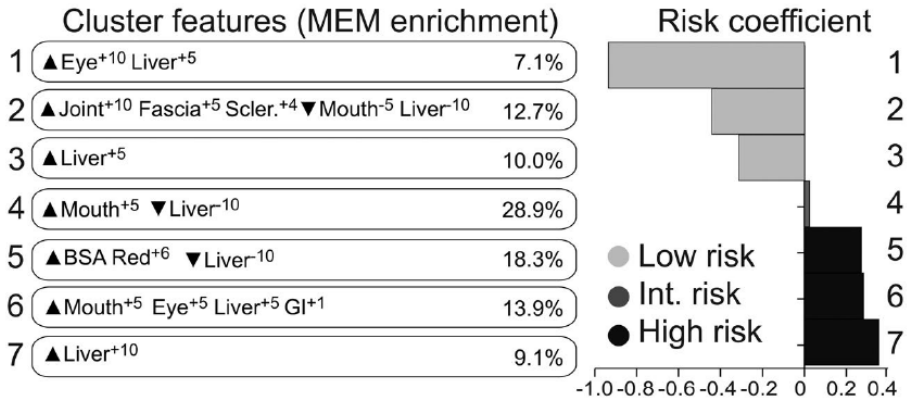
2 ▲Joint^{+10} Fascia^{+5} Scler.$^{+4}$ ▼Mouth^{-5} Liver^{-10} 12.7%

cGVHD patient data: http://flowrepository.org/id/FR-FCM-ZYSU

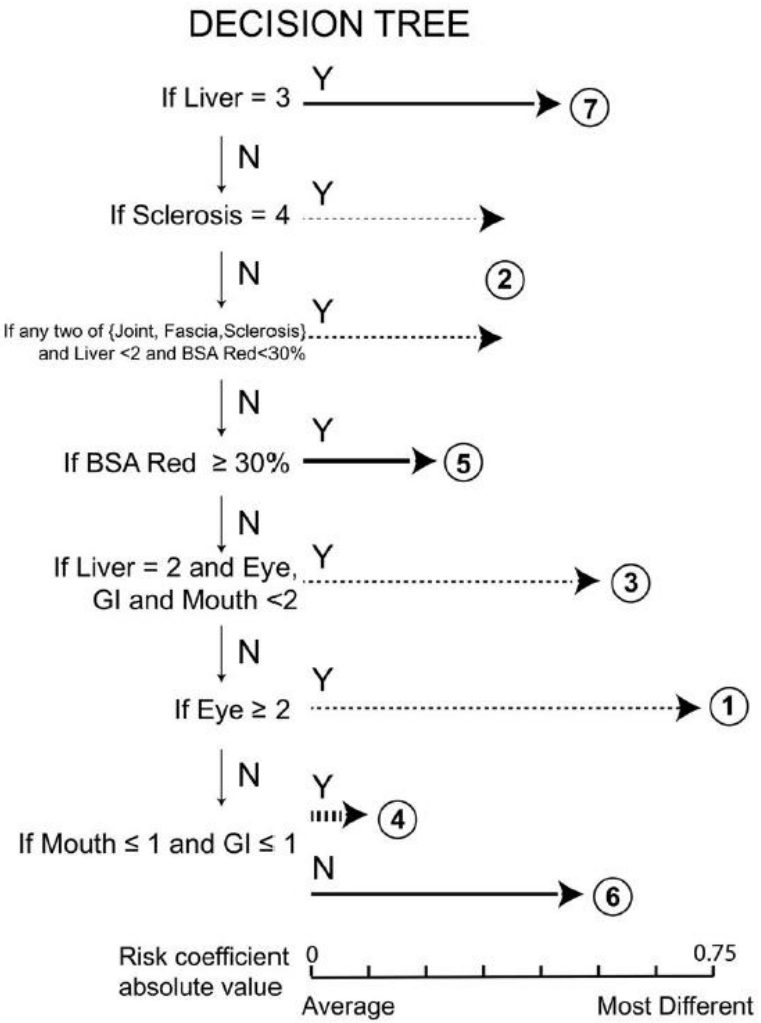
Gandelman et al., *Haematologica* 2018

Once Groups Are Revealed by Machine Learning, Clinically Accessible Strategies May Detect Them

7 Machine-Identified cGVHD patient groups



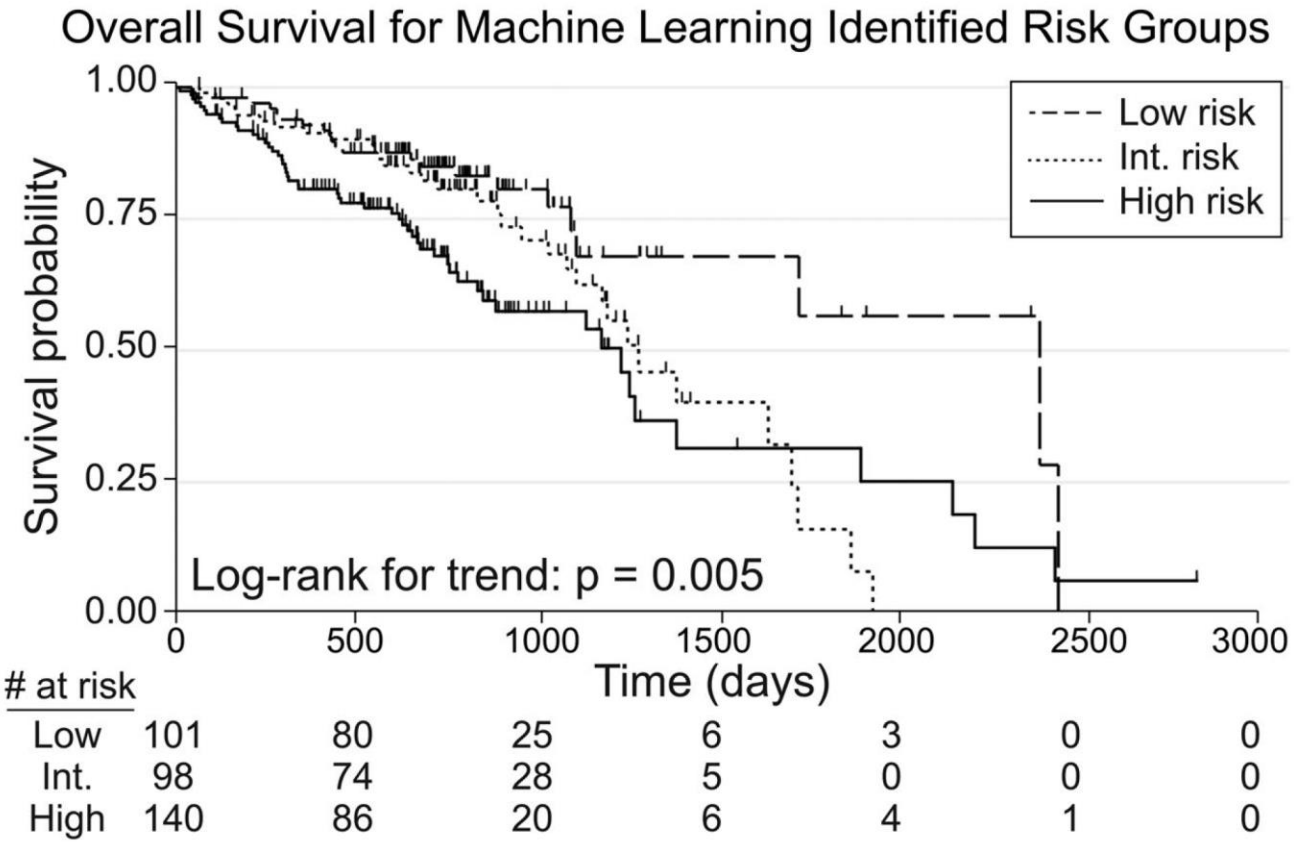
7 Comparable Patient Groups
Identified by Simple Yes/No Question Series



cGVHD patient data: http://flowrepository.org/id/FR-FCM-ZYSU

Gandelman et al., *Haematologica* 2018

Decision Tree cGVHD Patient Groups Are Also Risk Stratified for Overall Survival



Low risk: HR = 2.79,
95% CI: 1.58-4.91; p<0.001

High risk: HR = 2.65,
95% CI: 1.42-4.94; p<0.0001

Decision tree cGVHD patient groups (clusters)

	Risk	Freq.	
1	-0.76	8.6%	Low
2	-0.34	10.0%	
3	-0.53	8.6%	
4	-0.06	33.6%	Int.
5	+0.21	17.4%	High
6	+0.49	11.8%	
7	+0.46	10.0%	

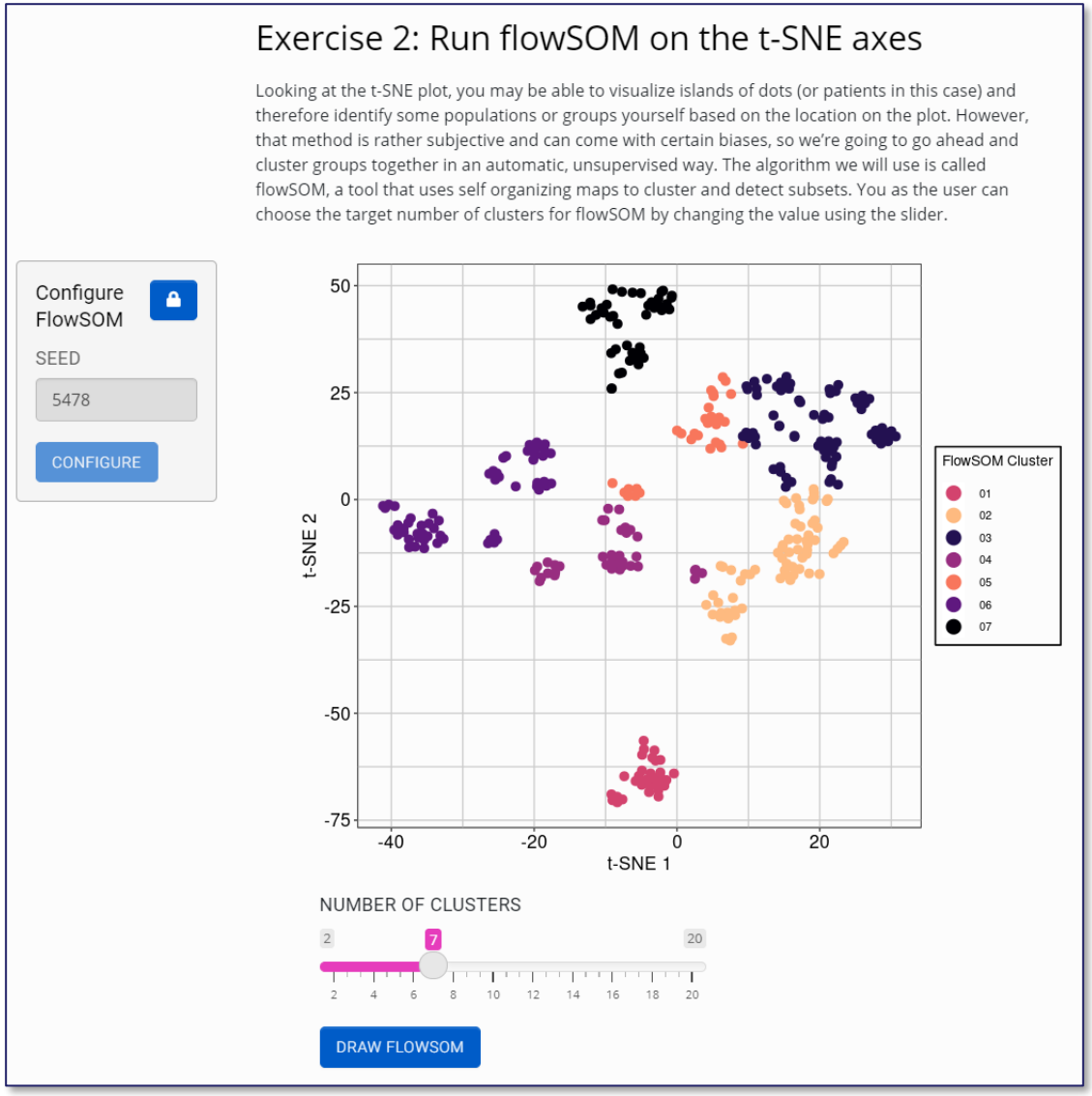
Chronic graft-*versus*-host disease (cGVHD)
t-SNE on 8 organ domain scores (e.g., liver involvement from 0 to 3)

Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/

cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/



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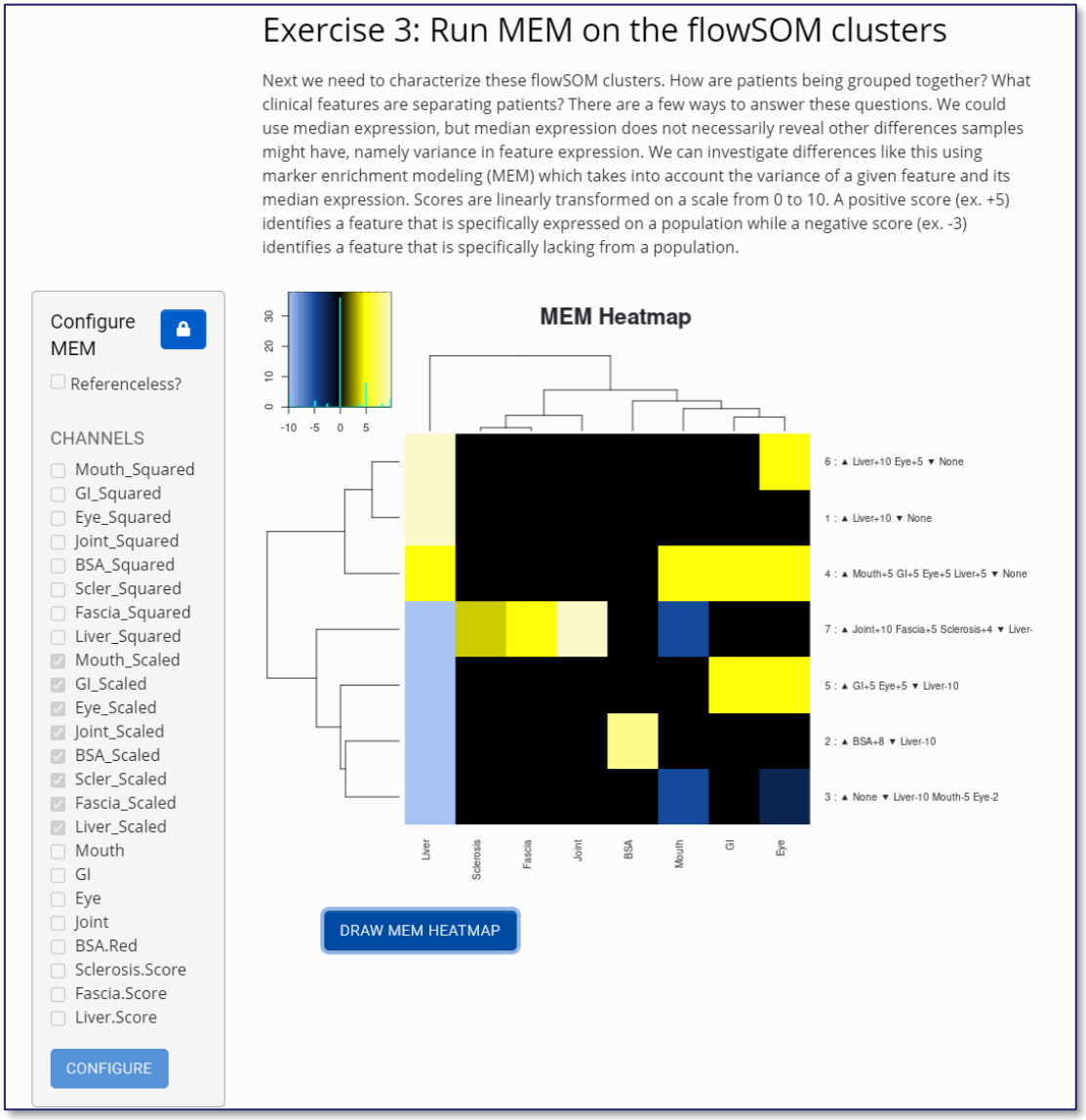

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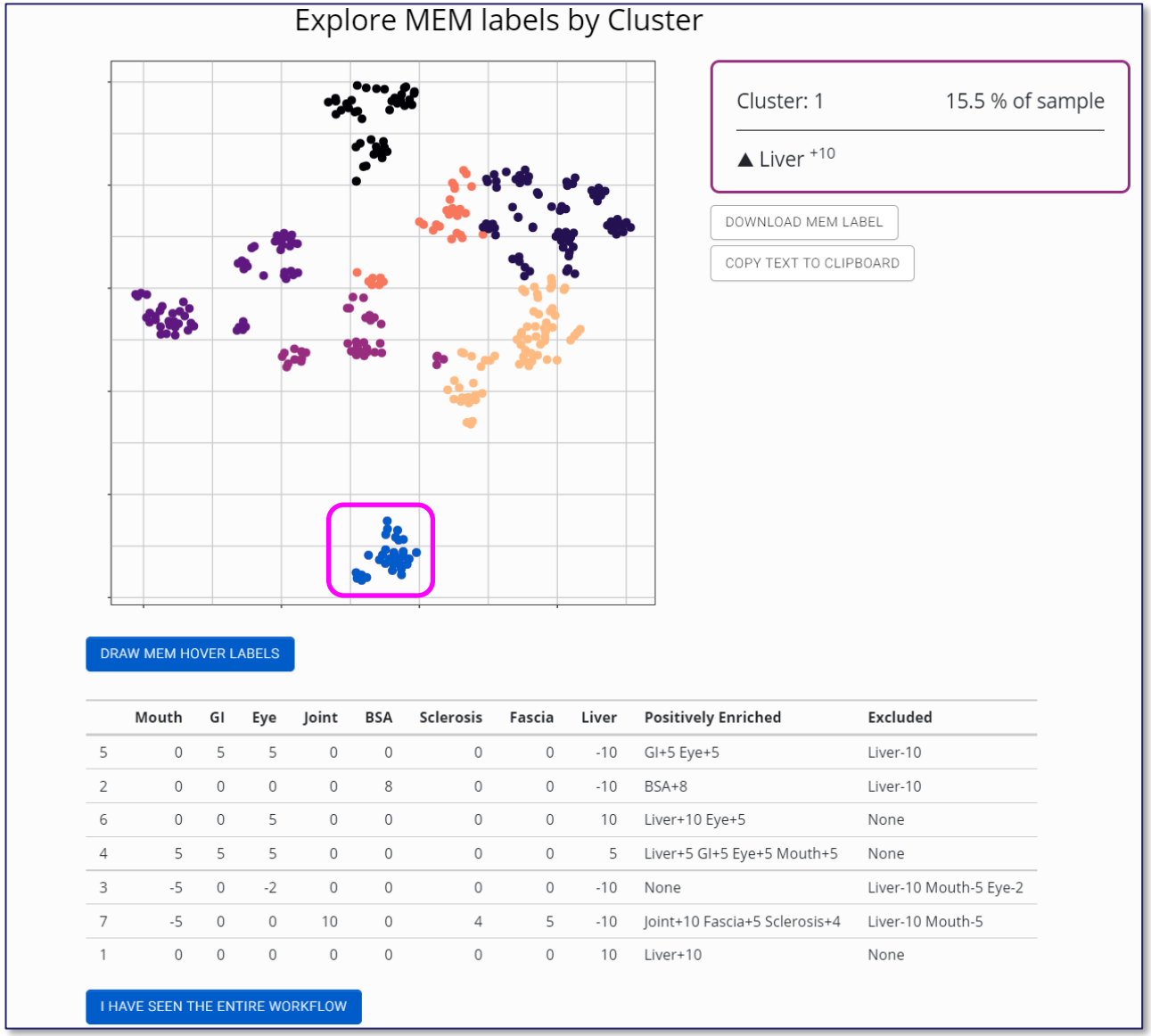
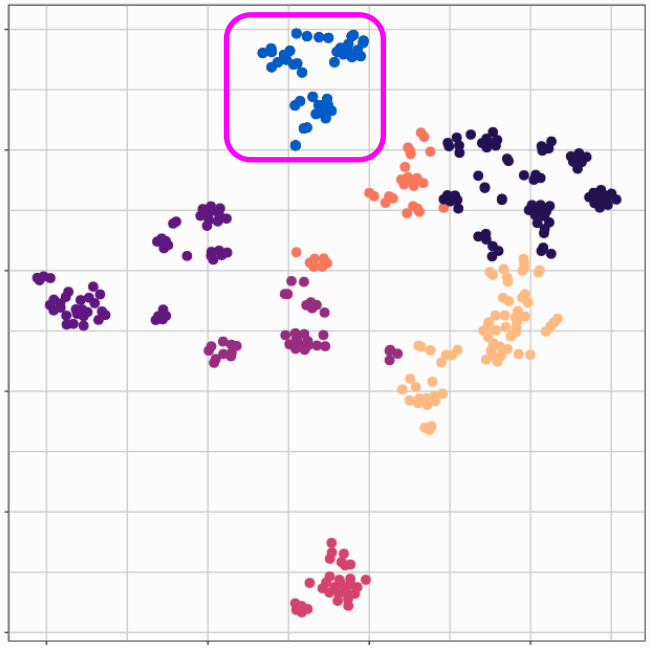


	Mouth	GI	Eye	Joint	BSA	Sclerosis	Fascia	Liver	Positively Enriched	Excluded
5	0	5	5	0	0	0	0	-10	GI+5 Eye+5	Liver-10
2	0	0	0	0	8	0	0	-10	BSA+8	Liver-10
6	0	0	5	0	0	0	0	10	Liver+10 Eye+5	None
4	5	5	5	0	0	0	0	5	Liver+5 GI+5 Eye+5 Mouth+5	None
3	-5	0	-2	0	0	0	0	-10	None	Liver-10 Mouth-5 Eye-2
7	-5	0	0	10	0	4	5	-10	Joint+10 Fascia+5 Sclerosis+4	Liver-10 Mouth-5
1	0	0	0	0	0	0	0	10	Liver+10	None

cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/

Explore MEM labels by Cluster



Cluster: 7 — 21.5 % of sample

▲ Joint $^{+10}$ Fascia $^{+5}$ Sclerosis $^{+4}$

▼ Liver $^{-10}$ Mouth $^{-5}$

DOWNLOAD MEM LABEL

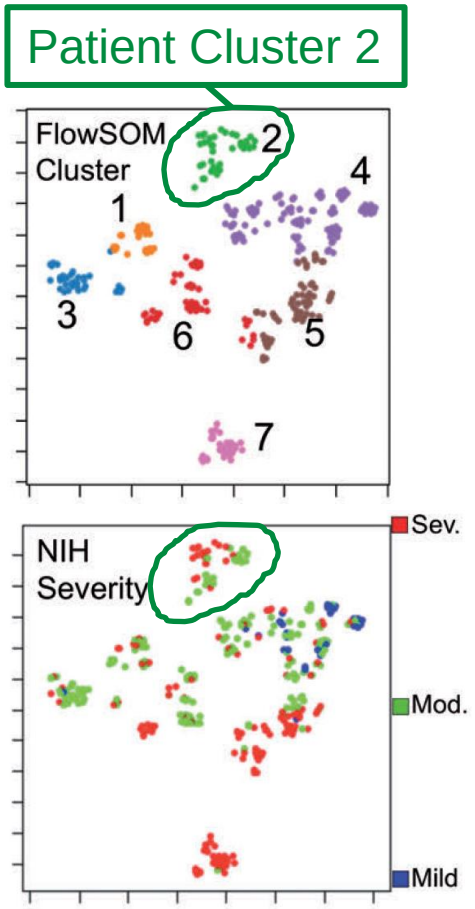
COPY TEXT TO CLIPBOARD

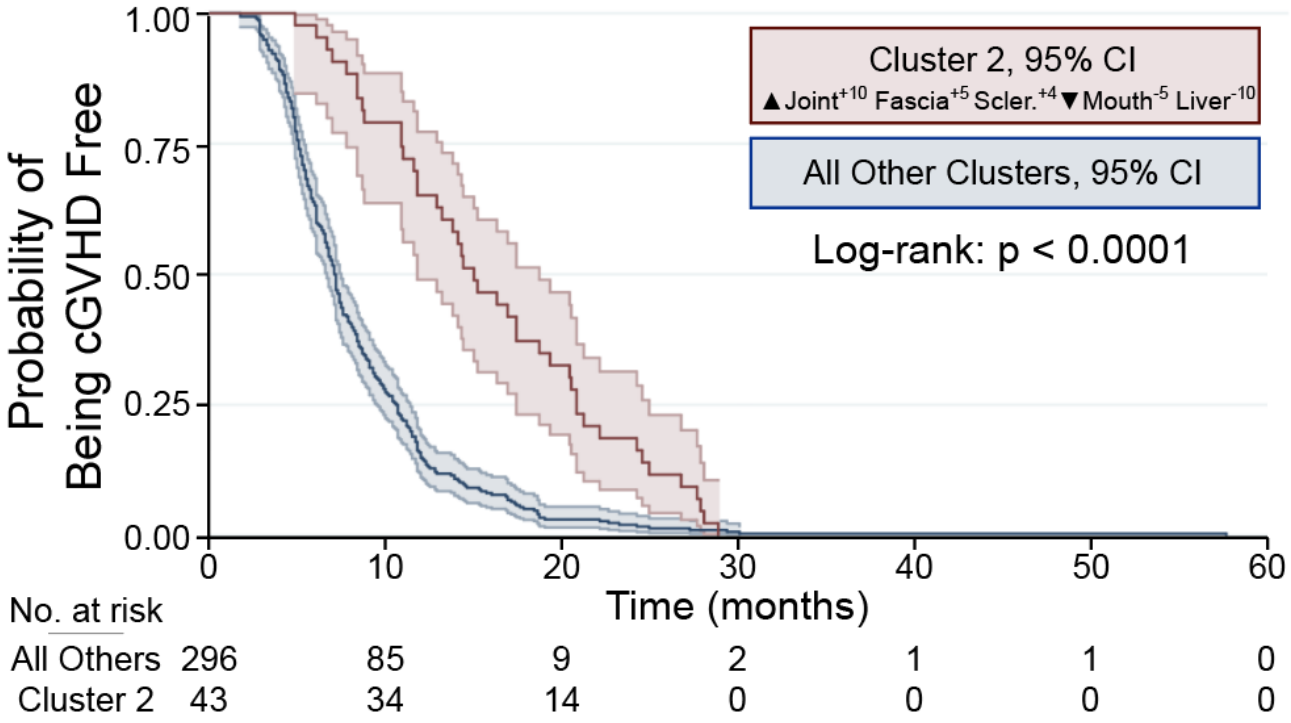
DRAW MEM HOVER LABELS

	Mouth	GI	Eye	Joint	BSA	Sclerosis	Fascia	Liver	Positively Enriched	Excluded
5	0	5	5	0	0	0	0	-10	GI+5 Eye+5	Liver-10
2	0	0	0	0	8	0	0	-10	BSA+8	Liver-10
6	0	0	5	0	0	0	0	10	Liver+10 Eye+5	None
4	5	5	5	0	0	0	0	5	Liver+5 GI+5 Eye+5 Mouth+5	None
3	-5	0	-2	0	0	0	0	-10	None	Liver-10 Mouth-5 Eye-2
7	-5	0	0	10	0	4	5	-10	Joint+10 Fascia+5 Sclerosis+4	Liver-10 Mouth-5
1	0	0	0	0	0	0	0	10	Liver+10	None

cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

Cluster 2 Was a Distinct Subtype of cGVHD Patients with Longer Time from Stem Cell Transplant to cGVHD





Phenotype of cGVHD Patient Cluster 2 ("MEM label": +10 / -10)

2 ▲Joint^{+10} Fascia^{+5} Scler.$^{+4}$ ▼Mouth^{-5} Liver^{-10} 12.7%

cGVHD patient data: http://flowrepository.org/id/FR-FCM-ZYSU

Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/

	Mouth	GI	Eye	Joint	BSA	Sclerosis	Fascia	Liver	Positively Enriched	Excluded
5	0	5	5	0	0	0	0	-10	GI+5 Eye+5	Liver-10
2	0	0	0	0	8	0	0	-10	BSA+8	Liver-10
6	0	0	5	0	0	0	0	10	Liver+10 Eye+5	None
4	5	5	5	0	0	0	0	5	Liver+5 GI+5 Eye+5 Mouth+5	None
3	-5	0	-2	0	0	0	0	-10	None	Liver-10 Mouth-5 Eye-2
7	-5	0	0	10	0	4	5	-10	Joint+10 Fascia+5 Sclerosis+4	Liver-10 Mouth-5
1	0	0	0	0	0	0	0	10	Liver+10	None

cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/

cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

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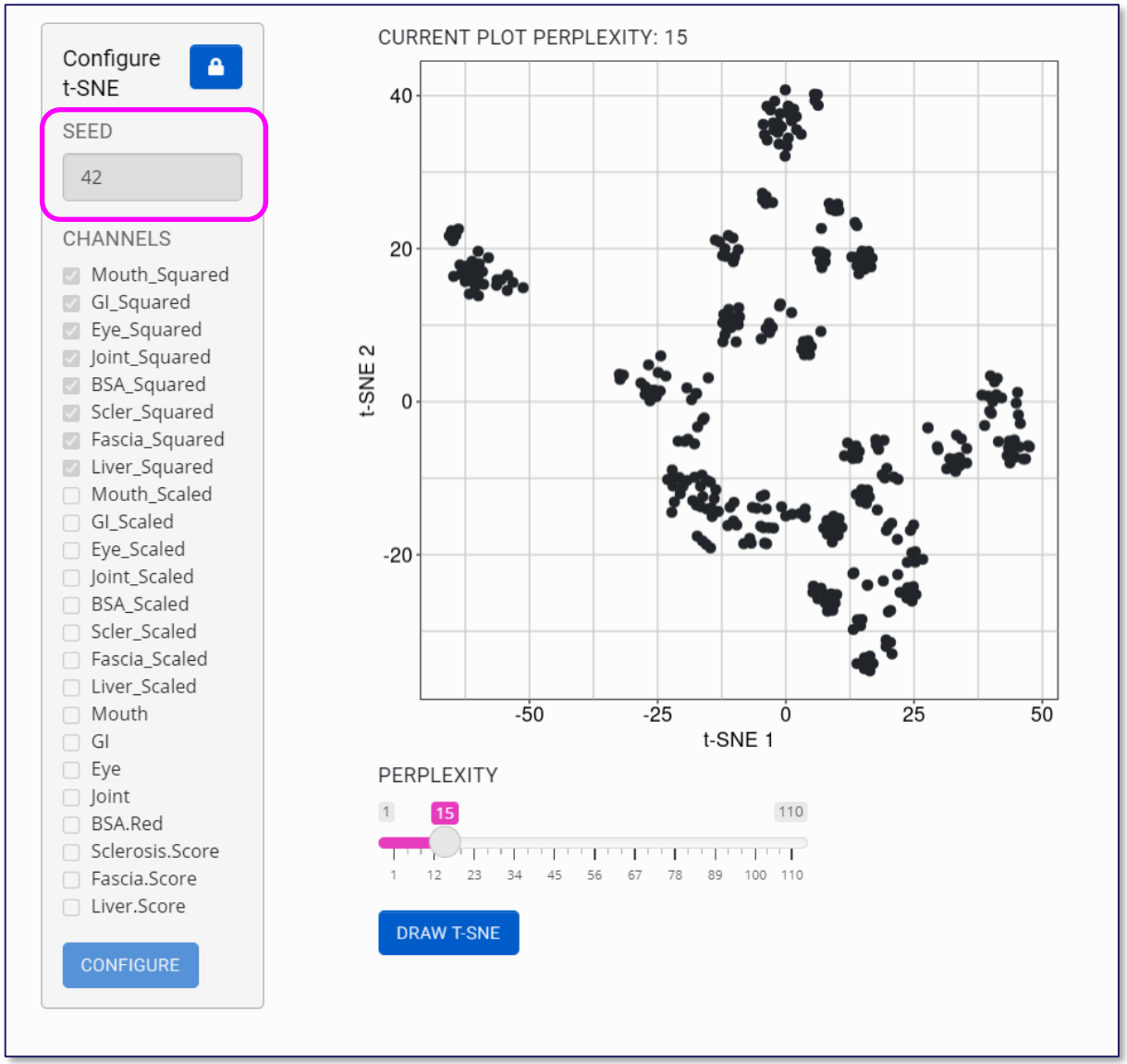
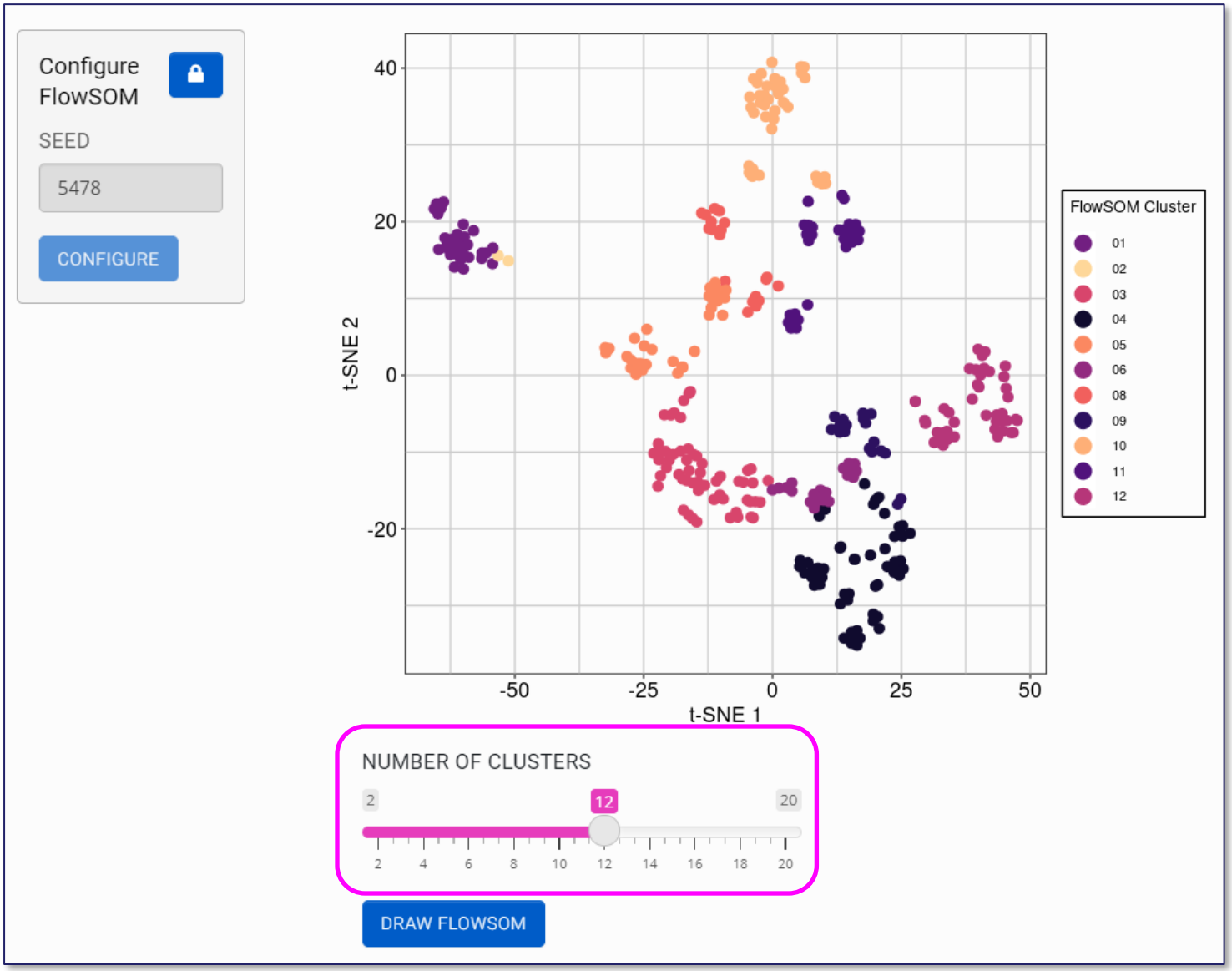


cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/

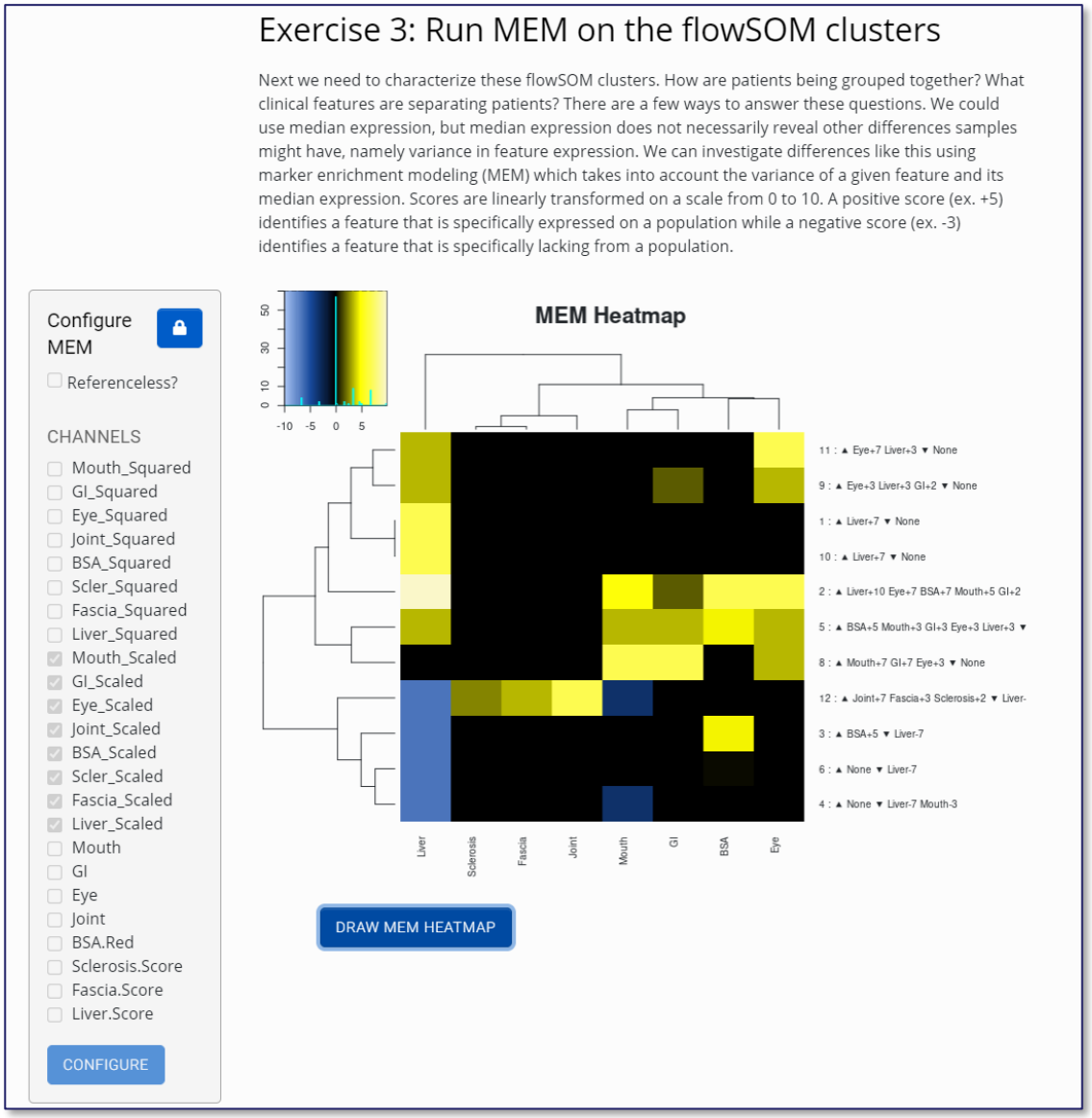
cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/



cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/



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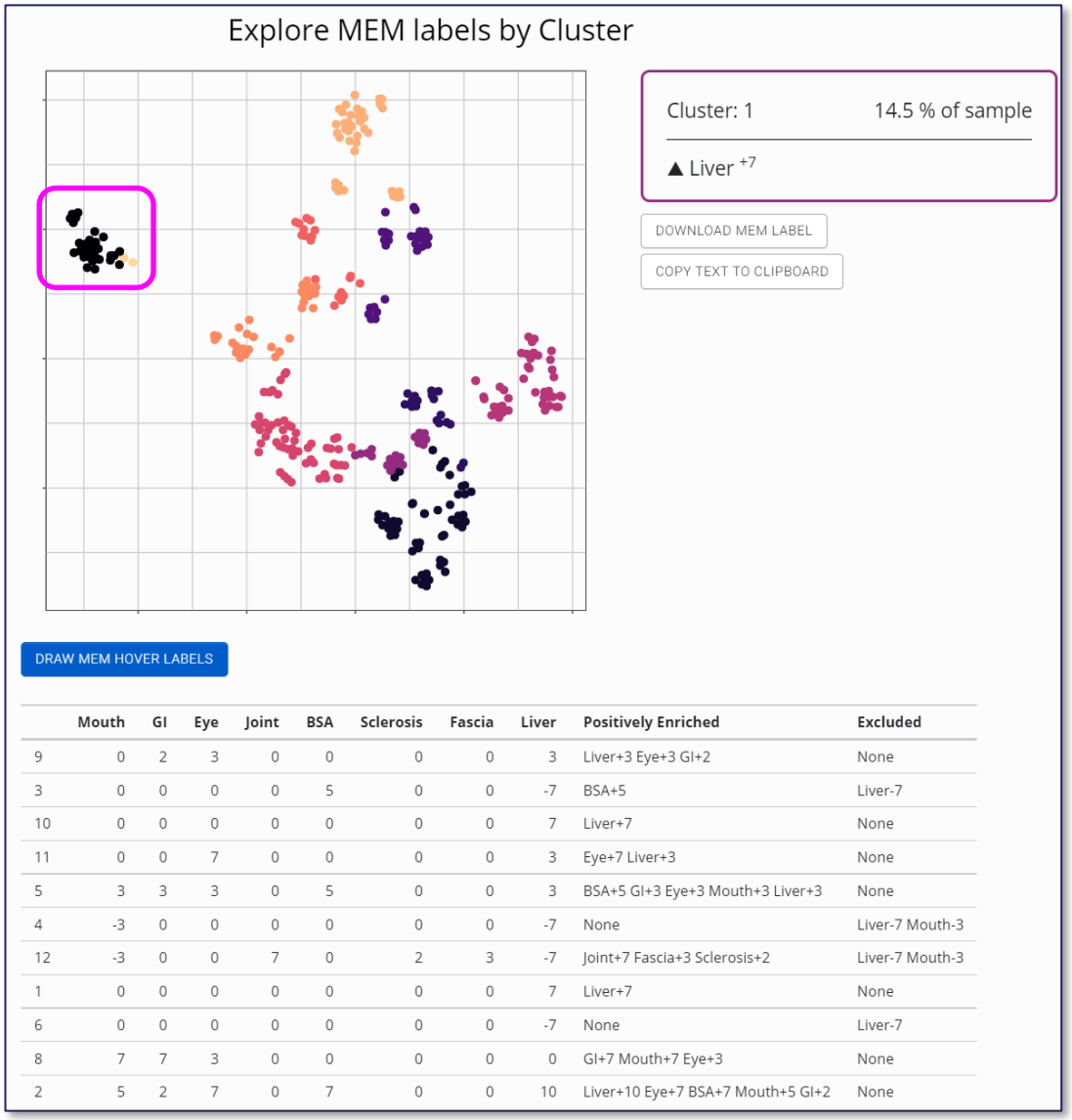
based on Gandelman et al., *Haematologica* 2018

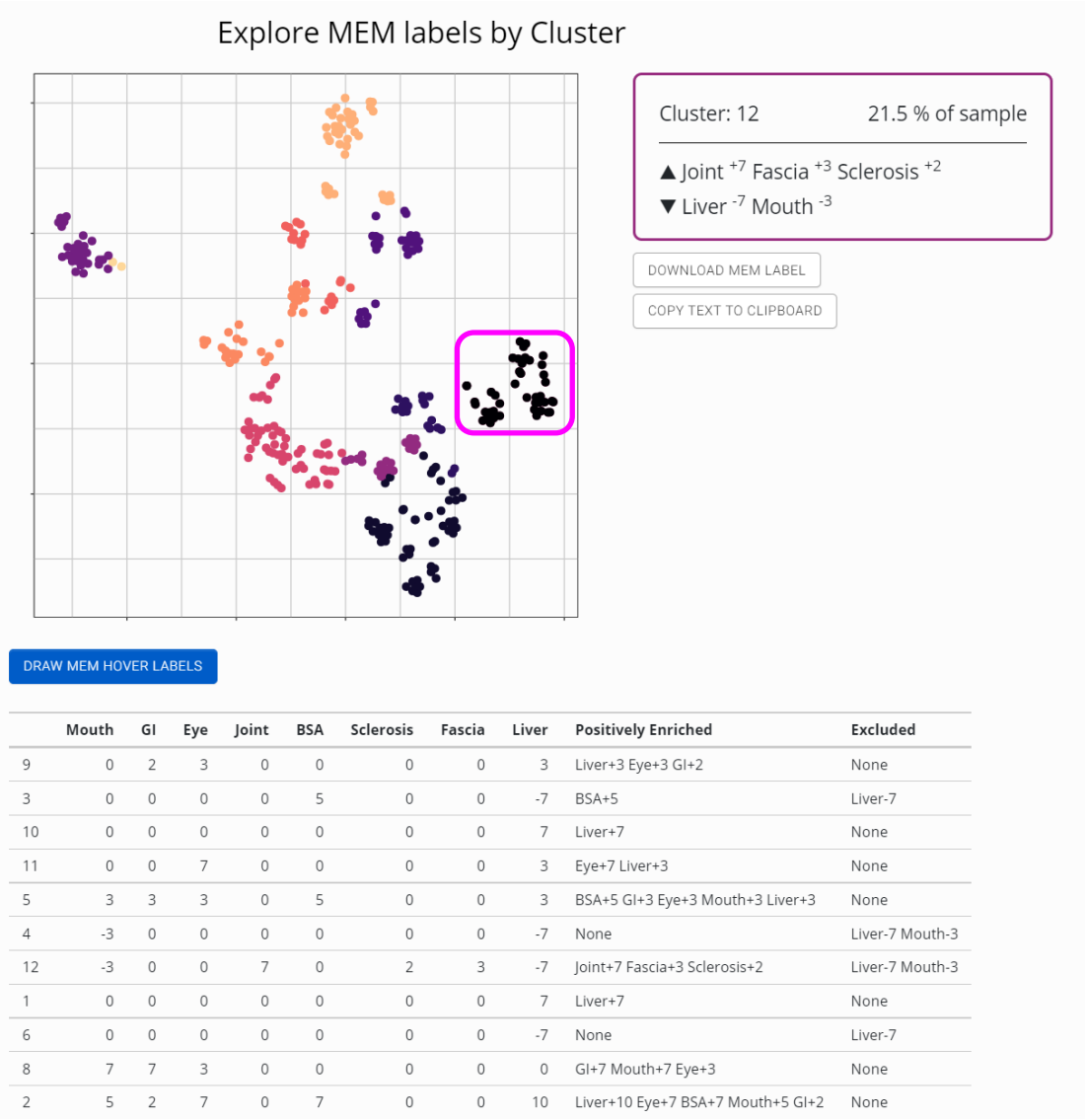
https://cytolab.shinyapps.io/cGVHD/



	Mouth	GI	Eye	Joint	BSA	Sclerosis	Fascia	Liver	Positively Enriched	Excluded
9	0	2	3	0	0	0	0	3	Liver+3 Eye+3 GI+2	None
3	0	0	0	0	5	0	0	-7	BSA+5	Liver-7
10	0	0	0	0	0	0	0	7	Liver+7	None
11	0	0	7	0	0	0	0	3	Eye+7 Liver+3	None
5	3	3	3	0	5	0	0	3	BSA+5 GI+3 Eye+3 Mouth+3 Liver+3	None
4	-3	0	0	0	0	0	0	-7	None	Liver-7 Mouth-3
12	-3	0	0	7	0	2	3	-7	Joint+7 Fascia+3 Sclerosis+2	Liver-7 Mouth-3
1	0	0	0	0	0	0	0	7	Liver+7	None
6	0	0	0	0	0	0	0	-7	None	Liver-7
8	7	7	3	0	0	0	0	0	GI+7 Mouth+7 Eye+3	None
2	5	2	7	0	7	0	0	10	Liver+10 Eye+7 BSA+7 Mouth+5 GI+2	None

cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

https://cytolab.shinyapps.io/cGVHD/



	Mouth	GI	Eye	Joint	BSA	Sclerosis	Fascia	Liver	Positively Enriched	Excluded
9	0	2	3	0	0	0	0	3	Liver+3 Eye+3 GI+2	None
3	0	0	0	0	5	0	0	-7	BSA+5	Liver-7
10	0	0	0	0	0	0	0	7	Liver+7	None
11	0	0	7	0	0	0	0	3	Eye+7 Liver+3	None
5	3	3	3	0	5	0	0	3	BSA+5 GI+3 Eye+3 Mouth+3 Liver+3	None
4	-3	0	0	0	0	0	0	-7	None	Liver-7 Mouth-3
12	-3	0	0	7	0	2	3	-7	Joint+7 Fascia+3 Sclerosis+2	Liver-7 Mouth-3
1	0	0	0	0	0	0	0	7	Liver+7	None
6	0	0	0	0	0	0	0	-7	None	Liver-7
8	7	7	3	0	0	0	0	0	GI+7 Mouth+7 Eye+3	None
2	5	2	7	0	7	0	0	10	Liver+10 Eye+7 BSA+7 Mouth+5 GI+2	None

cGVHD web app by Mayeda et al.
based on Gandelman et al., *Haematologica* 2018

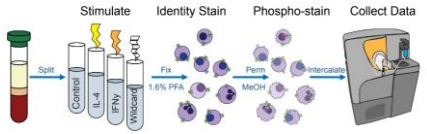
Part A: Installing Tools in R

https://github.com/cytolab

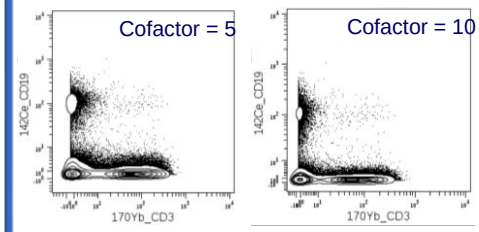
Irish lab examples, scripts, & walkthroughs
(MEM, RAPID, T-REX, & single cell courses)

Single Cell Biology Workflow

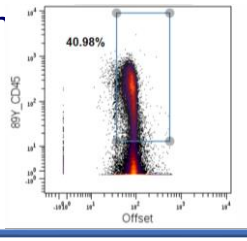
Data collection
1) Panel design
2) Data collection



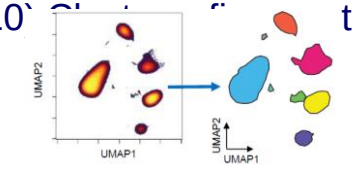
Data processing
3) Cell event parsing
4) Scale transformation



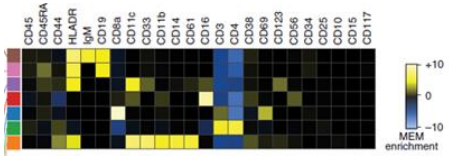
Distinguishing initial populations
5) Live single cell gating
6) Focal population gatir



Revealing cell subsets
7) Feature selection
8) Dimensionality reduction
9) Identify cell clusters
10) Cluster confirmation



Characterizing cell subsets
11) Feature comparison
12) Model populations
13) Learn cell identity
14) Statistical testing



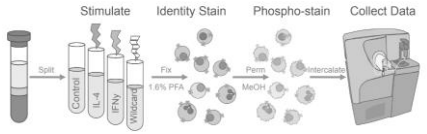
How much can be automated?

How do we select tools

Workflow Steps Adapted from Diggins et al., *Methods* 2015

Single Cell Biology Workflow

Data collection
1) Panel design
2) Data collection



Data processing
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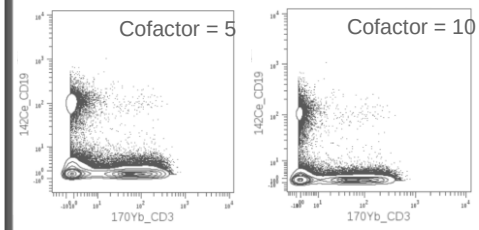
4) Scale transformation



Distinguishing initial populations
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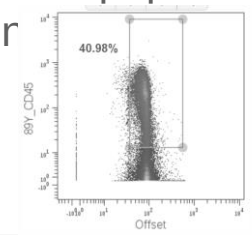

Revealing cell subsets
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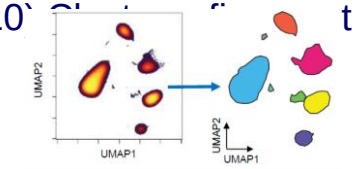

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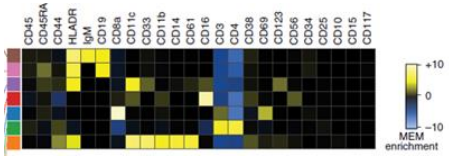


How much can be automated?

How do we select tools

Workflow Steps Adapted from Diggins et al., *Methods* 2015

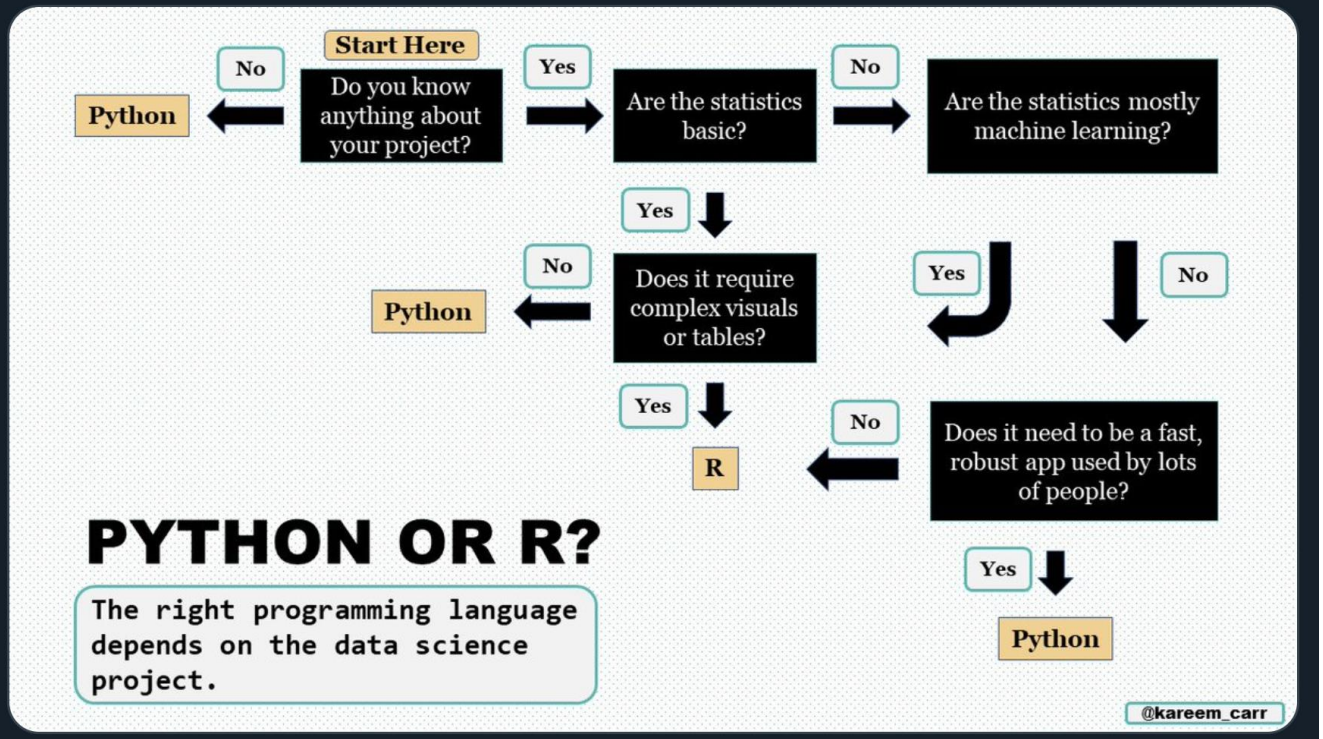
We will use R for the course

Tweet

You Retweeted

Kareem🔥data science thirst trap🔥Carr
@kareem_carr

People often ask me whether to learn R or Python for data science so I made a flowchart of my thought process.



3:30 PM · Sep 9, 2019 · TweetDeck

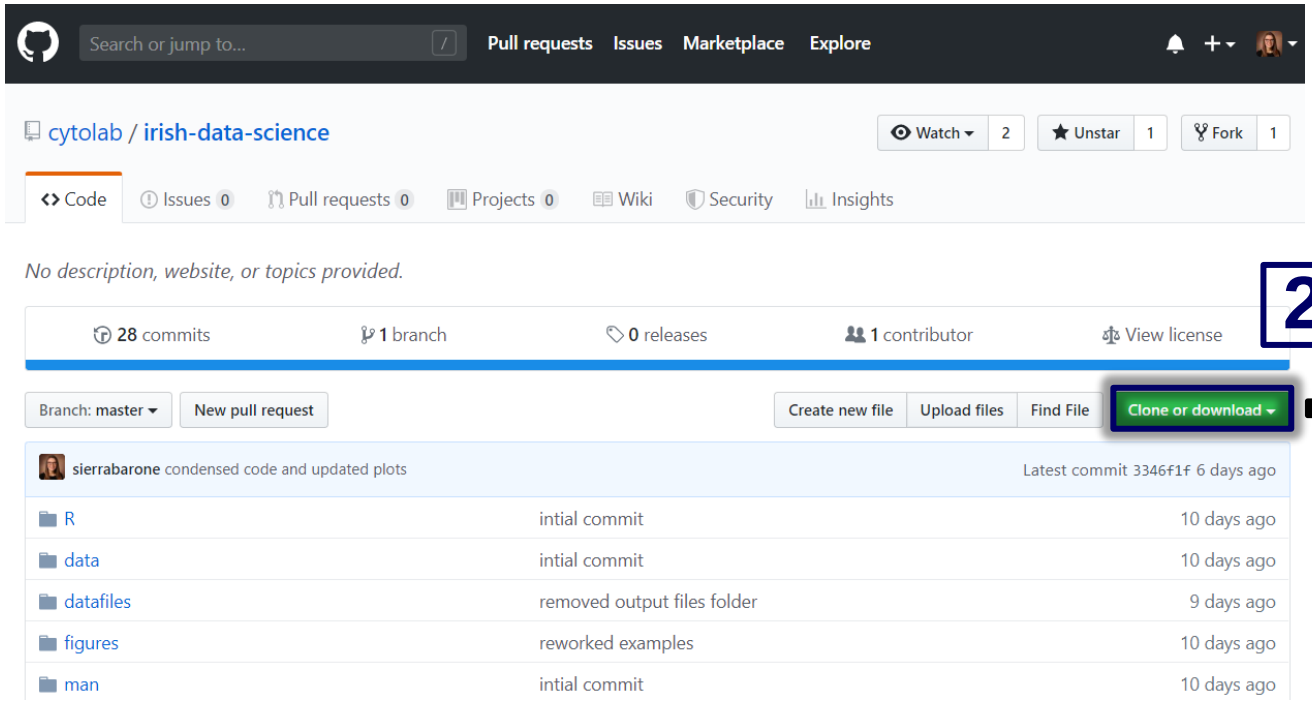
https://twitter.com/kareem_carr/status/1171068239087992833

Installation and Intro to Working in R

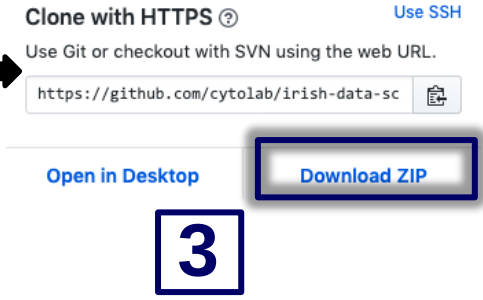
Download scripts, data, and R packages from GitHub

1 Go to link below and download repository:

https://github.com/JonathanIrish/datasci_london



2



3

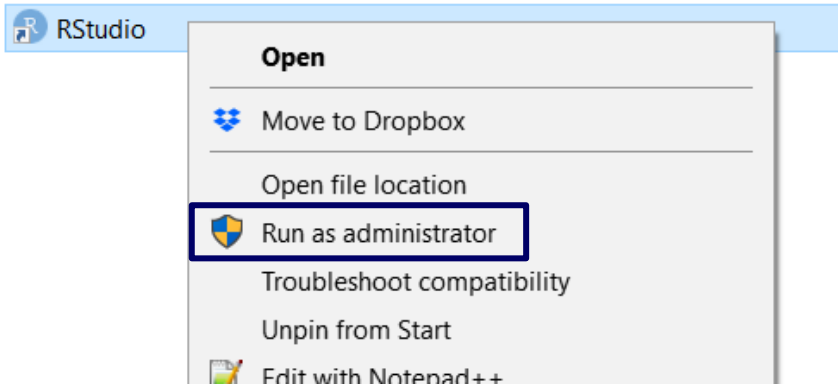
Github repository contents

0) installation script (R markdown, .rmd)
1) example analysis scripts (.rmd)
2) Data files (.fcs)
3) MEM package (.r, .rproj, etc.)
4) Documentation files (.rd, .md)
5) Other misc. files (.txt, .pdf, .rdata, etc.)

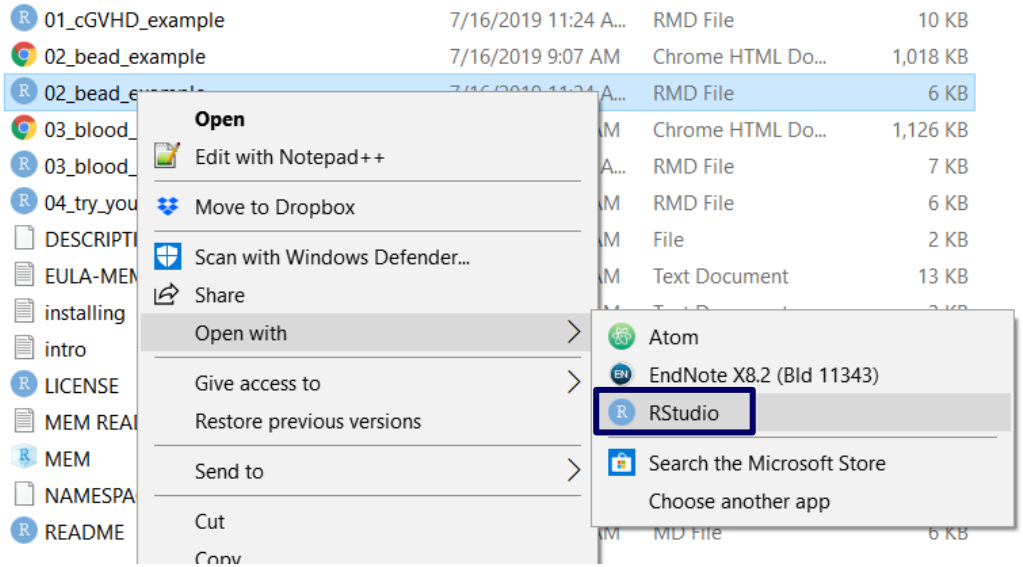
*make sure you unzip downloaded folder

We Will Open RStudio (Not R) For Interacting with Scripts

For PC users, run RStudio as administrator



For all, open .Rmd files with RStudio



*make sure you unzip downloaded folder

Scripts:

00_install_tools.rmd (run only once)

01_cGVHD_example.Rmd

02_bead_example.Rmd

03_blood_cell_PBMC_example.html

04_try_your_data.Rmd

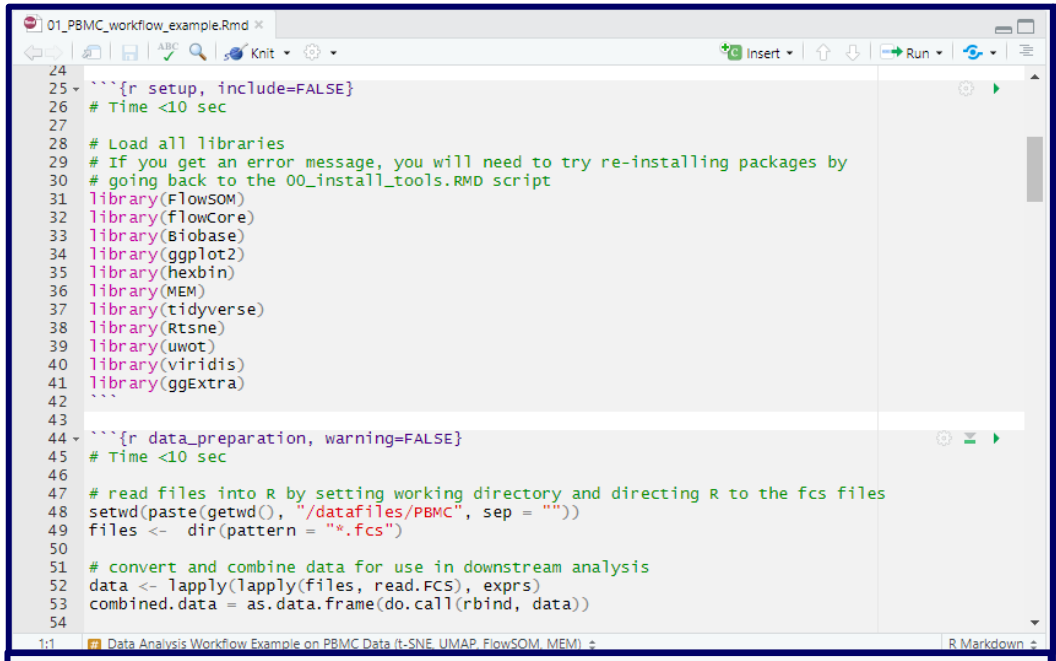
05_scRNA-seq_example.Rmd

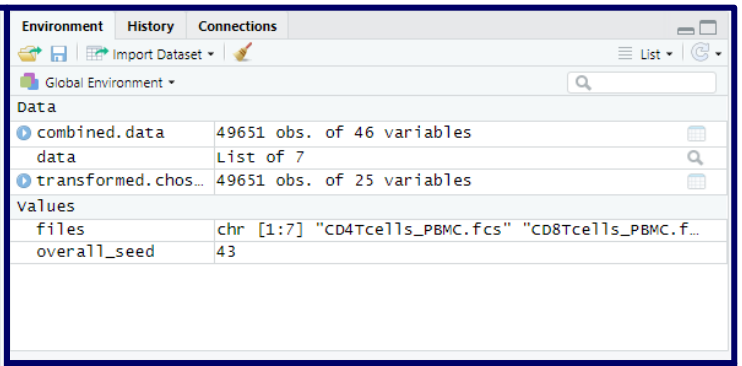
06_duncan_tsne_analysis.Rmd

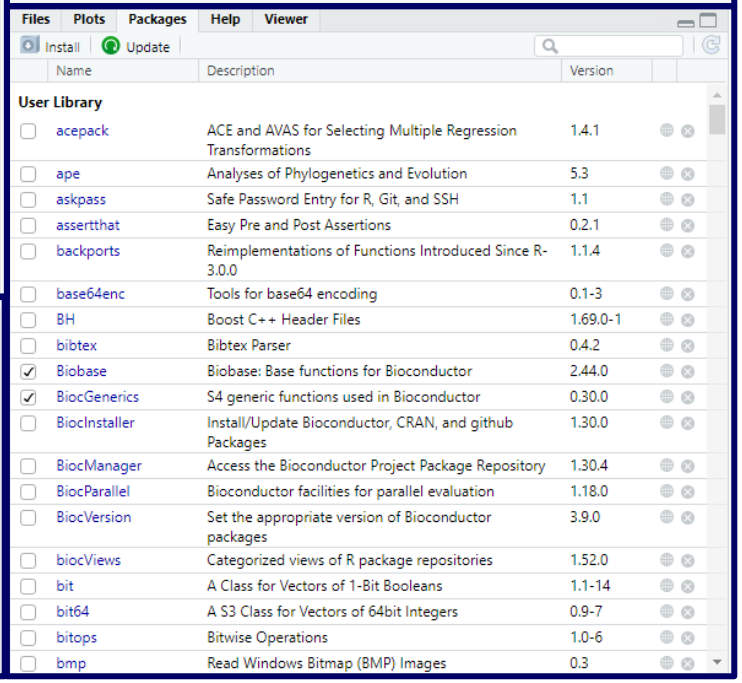
*make sure console is open

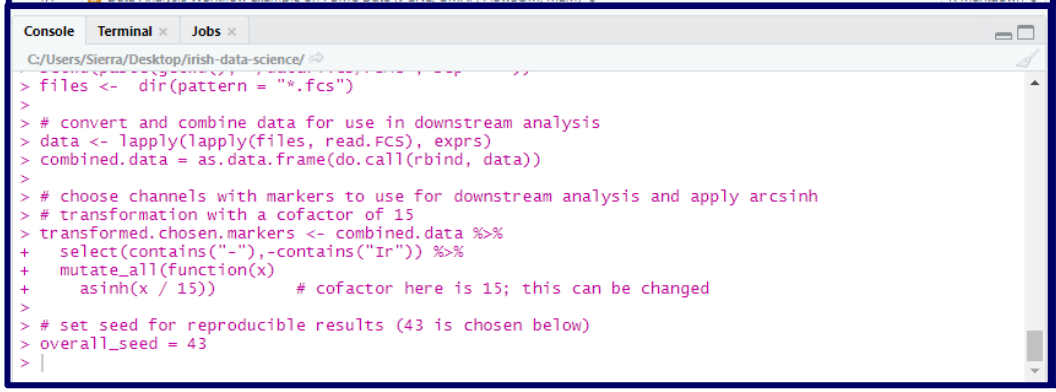
Working Script and Code

Environment







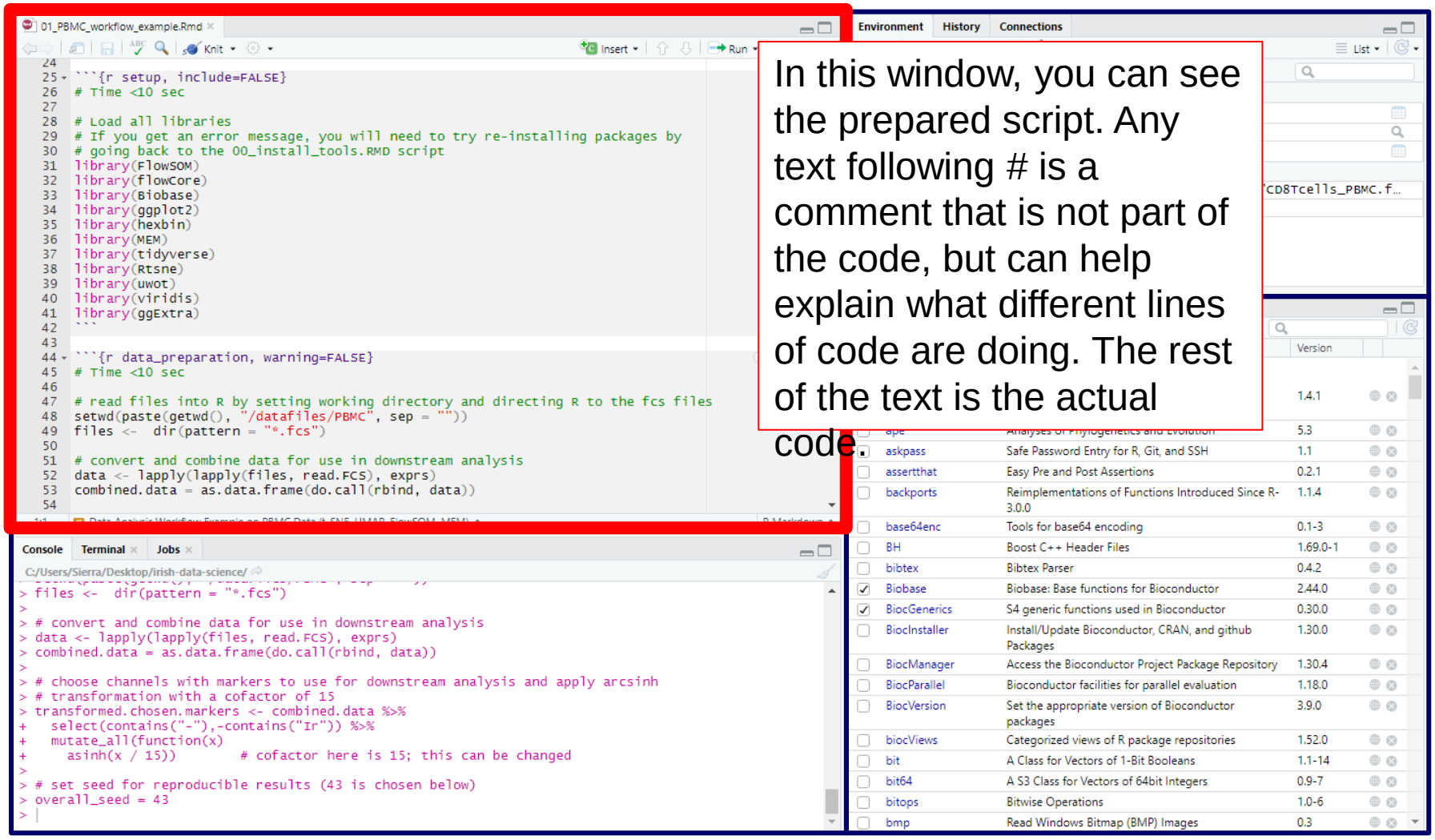


Console

Plots, Files, Help, etc.

Working Script and Code

Environment



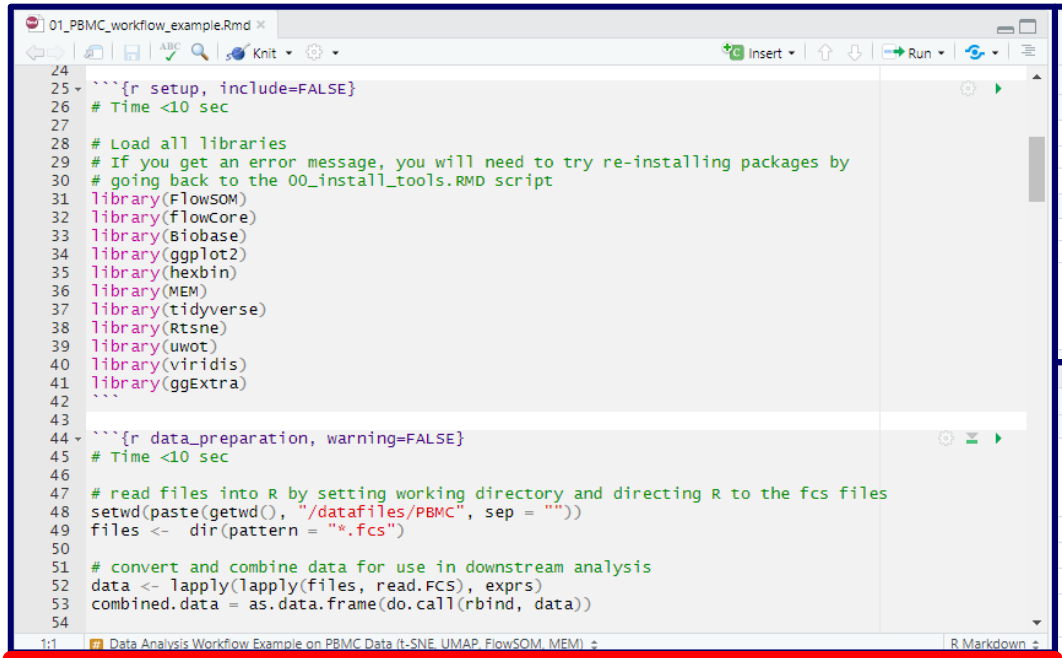
In this window, you can see the prepared script. Any text following # is a comment that is not part of the code, but can help explain what different lines of code are doing. The rest of the text is the actual code.

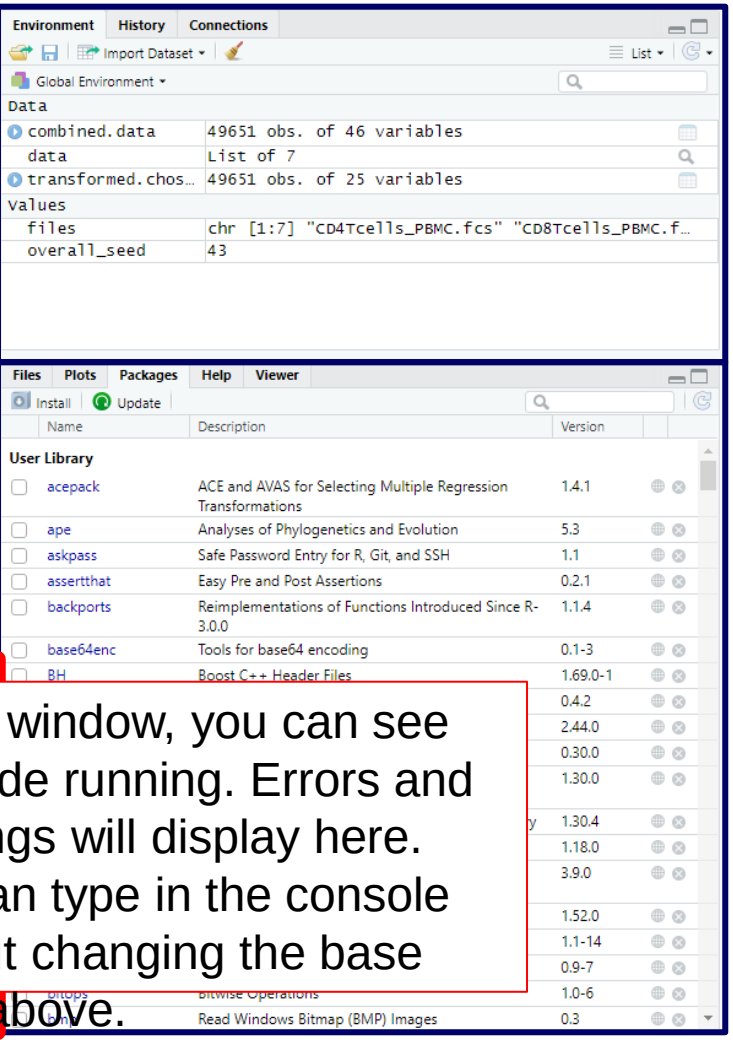
Console

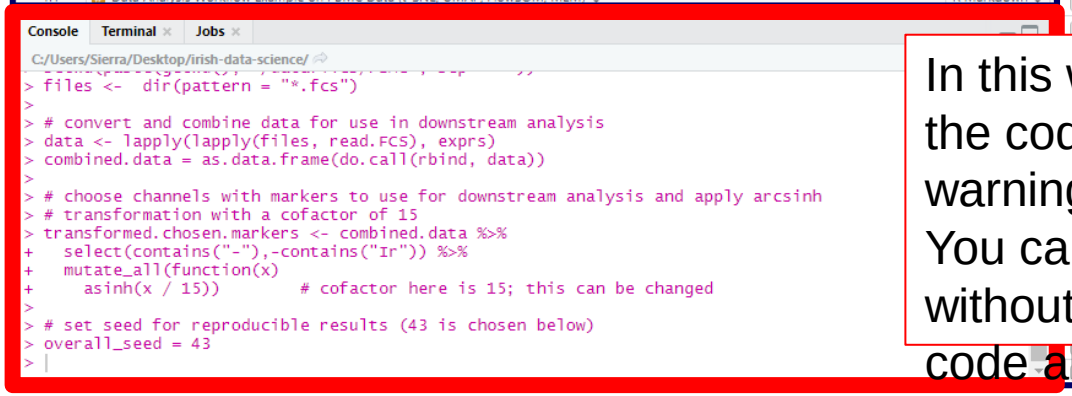
Plots, Files, Help, etc.

Working Script and Code

Environment







In this window, you can see the code running. Errors and warnings will display here. You can type in the console without changing the base code above.

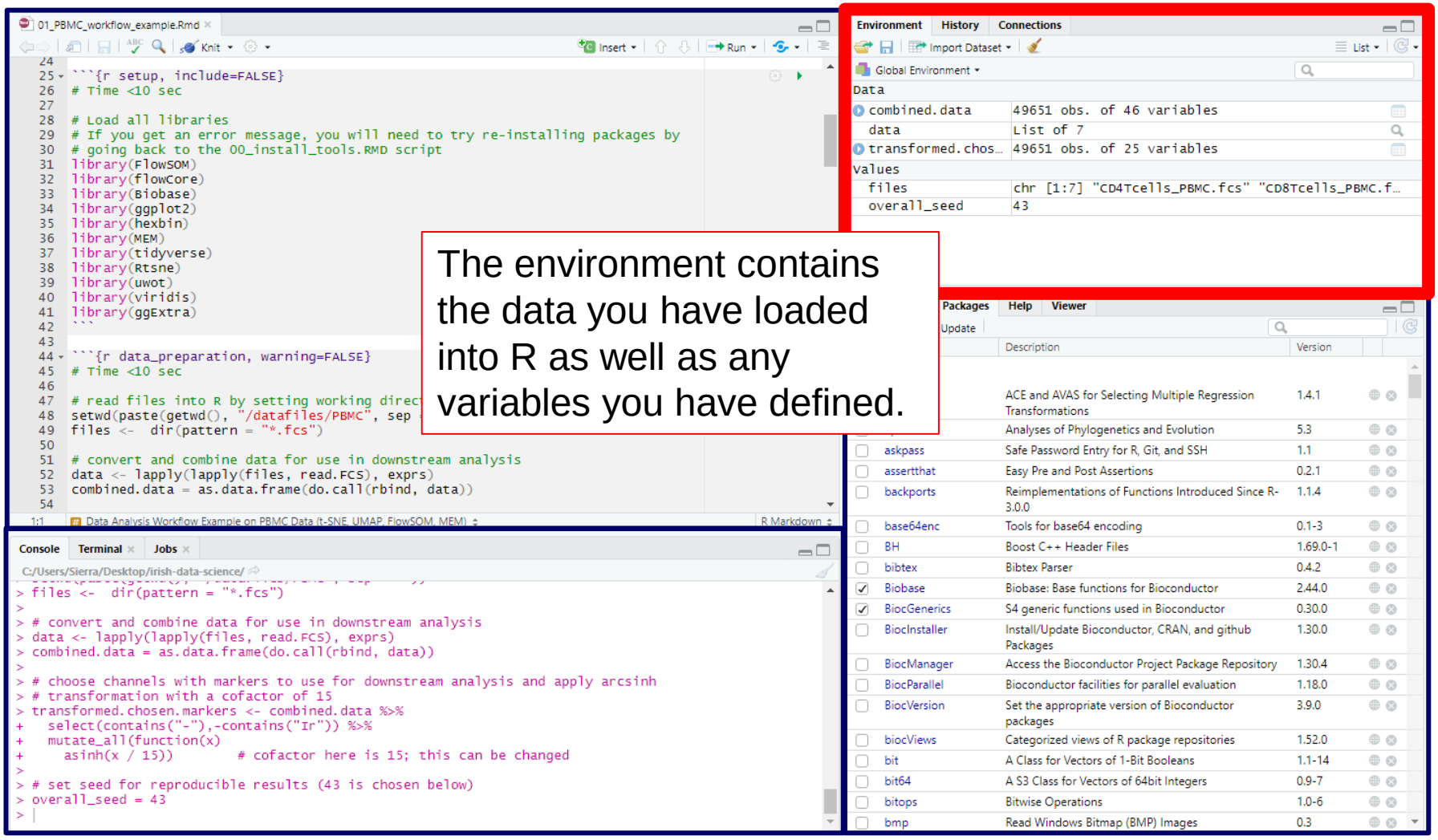
Console

Plots, Files, Help, etc.

*make sure console is open

Working Script and Code

Environment

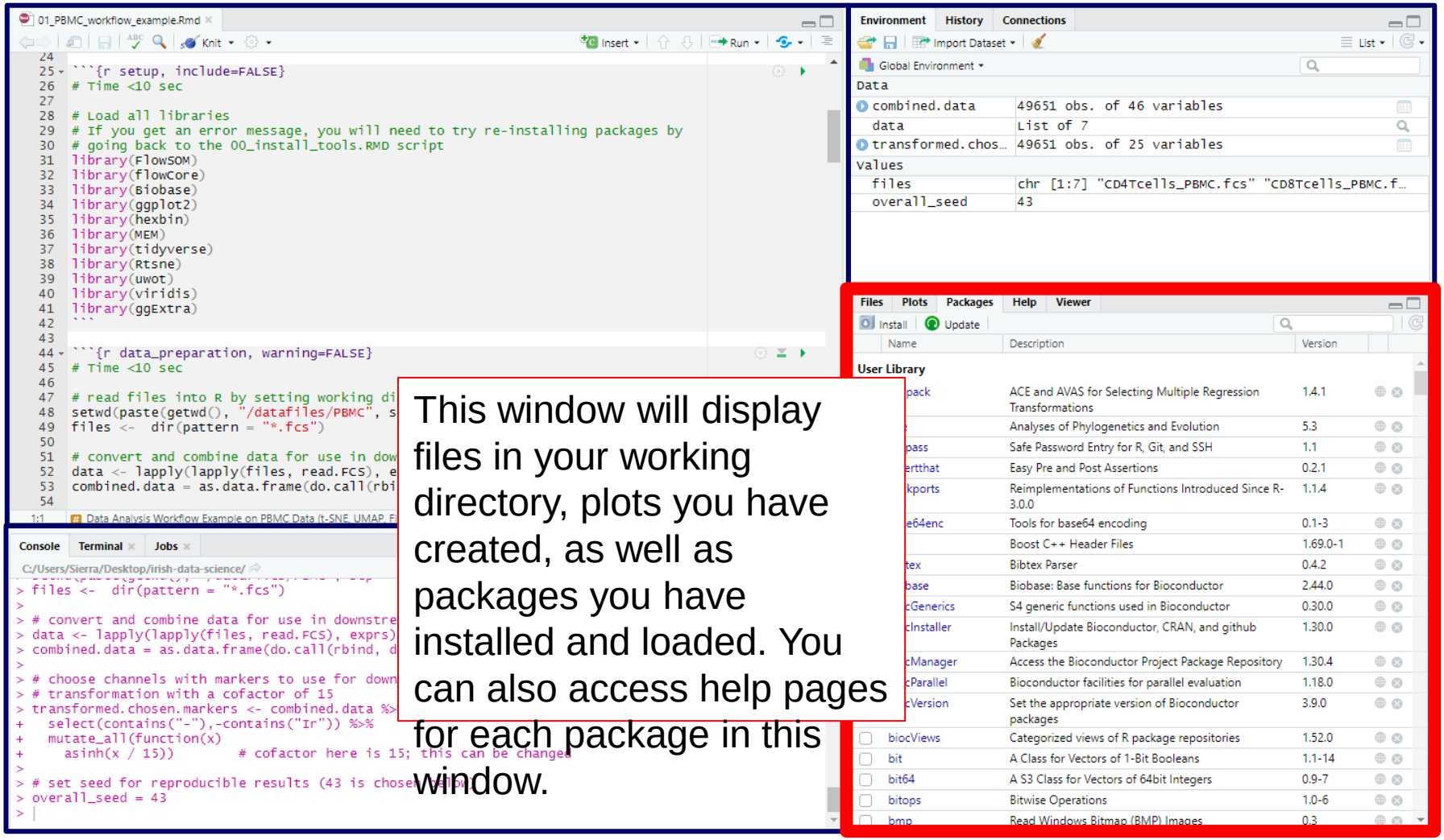


Console

Plots, Files, Help, etc.

Working Script and Code

Environment



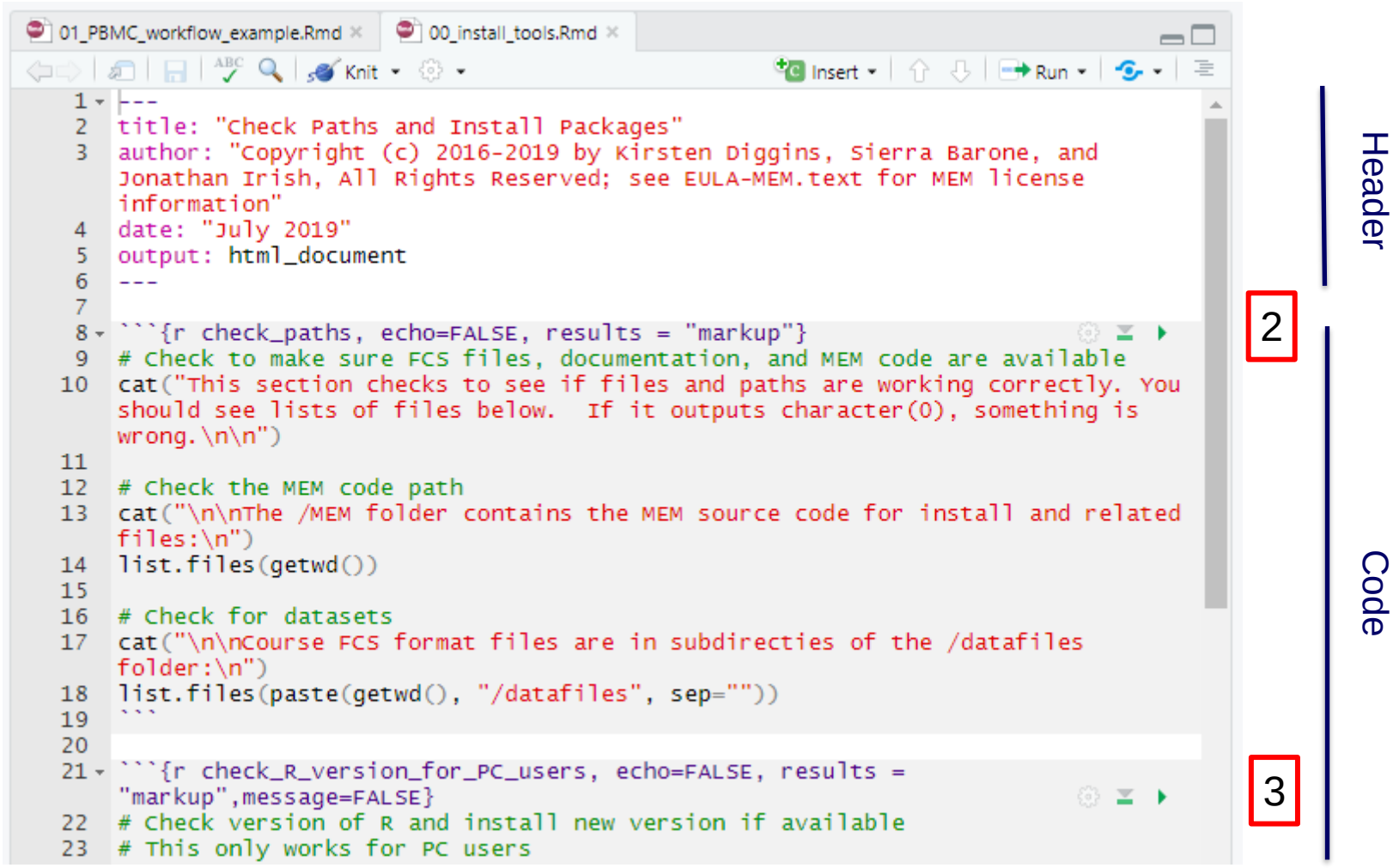
This window will display files in your working directory, plots you have created, as well as packages you have installed and loaded. You can also access help pages for each package in this window.

Console

Plots, Files, Help, etc.

Open **00_install_tools.rmd**

1



Header

2

Code

3

Open **00_install_tools.rmd** and begin installing required packages

CNTL-ENTER or COMMAND-RETURN to run a single line of code

OR

Press play to run entire section of code

Code Section Title

```
```{r check_paths, echo=FALSE, results = "markup"}
# Check to make sure FCS files, documentation, and MEM code are available
cat("This section checks to see if files and paths are working correctly. You
should see lists of files below.  If it outputs character(0), something is
wrong.\n\n")

# Check the MEM code path
cat("\n\nThe /MEM folder contains the MEM source code for install and related
files:\n")
list.files(getwd())

# Check for datasets
cat("\n\nCourse FCS format files are in subdirecties of the /datafiles
folder:\n")
list.files(paste(getwd(), "/datafiles", sep=""))
```
```

This section checks that the files we will need are accessible in our working directory

```
```{r installation_notes, echo=FALSE, results = "markdown"}
# Print the contents a help file that explains installing packages
writeLines(readLines(paste(getwd(), "installing.txt", sep="/")))
```
```

This section prints installation text

Open **00_install_tools.rmd** and begin installing required packages

```{r install_bioconductor_packages, echo=FALSE, results = "hide"}
# install bioconductor and flow cytometry tools for R
cat("If this works, you should see 4 sets of messages about downloading files
that end in a message saying something like package 'BiocManager' successfully
unpacked and MD5 sums checked.  You should see this for BioCManager, Biobase,
flowCore, and FlowSOM.\n\n")
install.packages("BiocManager", repos = "http://cran.us.r-project.org")

if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")
BiocManager::install("flowCore")
BiocManager::install("FlowSOM")
```

This section downloads Bioconductor and flow cytometry tools we will need

```{r test_flow_installs, echo=FALSE, results = "markdown"}
# Load and test whether bioconductor and flow packages are installed
cat("If this works, you may see Attaching Package messages or no message at
all; that's good.  If you get a warning, go back to the last CHUNK.\n\n")
library(FlowSOM)
library(flowCore)
library(Biobase)
```

This section tests to make sure Bioconductor and flow cytometry tools are installed

```{r install_ggplots, echo=FALSE, results = "markup"}
# install plotting packages
cat("If this works, you will see text about packages being downloaded.\n\n")
install.packages("gplots", repos = "http://cran.us.r-project.org")
install.packages("ggplot2", repos = "http://cran.us.r-project.org")
install.packages("hexbin", repos = "http://cran.us.r-project.org")
install.packages("viridis", repos = "http://cran.us.r-project.org")
install.packages("ggExtra", repos = "http://cran.us.r-project.org")
```

```{r load_gplots, echo=FALSE, results = "markup"}
# Load and test whether gplots and ggplot2 packages are installed
cat("If this works, you may see Attaching Package messages or no message at
all; that's good.  If you get a warning, go back to the last CHUNK.\n\n")
library(gplots)
library(ggplot2)
library(hexbin)
library(viridis)
library(ggExtra)
```

The next sections install and load the tools to make plots

Open **00_install_tools.rmd** and begin installing required packages

```
```{r install_MEM, echo=FALSE, results = "markup"}
# install MEM, load it, and test if it is all set
cat("If this works, you should see several lines about installing files, then
DONE (MEM) near the end.  The MEM help page will also open in the Help menu in
RStudio.\n\n")

# If you have previously installed MEM, you may get an error message. If this
is the case, try restarting your RStudio session
install.packages(getwd(), type="source", repos=NULL)
library(MEM)
?MEM

# OR
# install.packages("devtools", repos = "http://cran.us.r-project.org")
# devtools::install_github("cytolab/mem")
```
```

This section installs and loads the marker enrichment modeling tool

```
```{r install_last_packages, echo=FALSE, results = "markup"}
# install the last packages for UMAP, t-SNE and other tools
print("You may see a bunch of messages, this is OK as long as they are not
errors.\n\n")
install.packages("tidyverse", repos = "http://cran.us.r-project.org")
install.packages("Rtsne", repos = "http://cran.us.r-project.org")
install.packages("uwot", repos = "http://cran.us.r-project.org")
install.packages("RColorBrewer", repos = "http://cran.us.r-project.org")
```
```

```
```{r load_last_packages, echo=FALSE, results = "markup"}
# Load and test the last libraries
library(tidyverse)
library(Rtsne)
library(uwot)
library(RColorBrewer)
```
```

These sections install and load the other tools we will use for analysis

Part B: Example with Healthy Blood

Open **03_blood_cell_PBMC_example.html** and work through the example

03_blood_cell_PBMC_example.html

01_PBMC_workflow_example.Rmd

Knit | Insert | Run

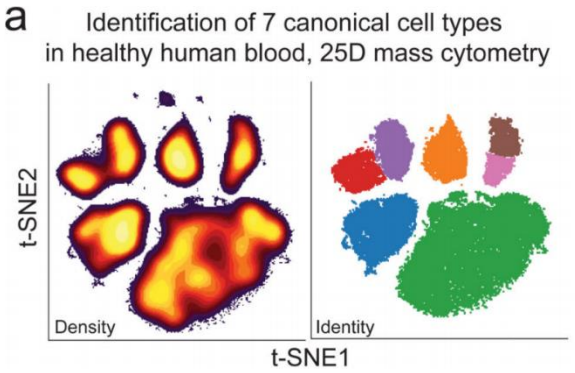
```
---
title: "Data Analysis Workflow Example on PBMC Data (t-SNE, UMAP, FlowSOM, MEM)"
author: "Copyright (c) 2016-2019 by Kirsten Diggins, Sierra Barone, and Jonathan Irish, All Rights Reserved; see EULA-MEM.text for MEM license information"
date: "July 2019"
output:
  pdf_document:
    latex_engine: xelatex
  html_document:
    df_print: paged
editor_options:
  chunk_output_type: inline
---

This data set contains 7 FCS (flow cytometry standard) files. Each FCS file contains single cell data for one cell subset that is a well-established, phenotypically distinct population. This is mass cytometry data for healthy human PBMC (peripheral blood mononuclear cells). The populations were expert gated following a t-SNE analysis. The first section of the code will run two dimensionality reduction tools, UMAP and t-SNE, on the data set. Next, you will run FlowSOM on the UMAP axes to cluster the islands of cell populations. Finally, you will run MEM to see enrichment scores for each of the FlowSOM clusters. The goal of this exercise is to run several computational tools on a single cell data set to get a feel for the workflow used in the Irish lab.

```{r setup, include=FALSE}
# Time <10 sec

# Load all libraries
# If you get an error message, you will need to try re-installing packages by
# going back to the 00_install_tools.RMD script
library(FlowSOM)
library(flowCore)
library(Biobase)
library(ggplot2)
library(hexbin)
library(MEM)
library(tidyverse)
library(Rtsne)
library(uwot)
library(viridis)
library(ggExtra)
```
```

A description of the code and its purpose



This section loads the necessary libraries

Diggins et al., *Nature Methods* 2017

03_blood_cell_PBMC_example.html

Data Preparation

````
```{r data_preparation, warning=FALSE}
# Time <10 sec

# read files into R by setting working directory and directing R to
files
setwd(paste(getwd(), "/datafiles/PBMC", sep = ""))
files <-  dir(pattern = "*.fcs")

# convert and combine data for use in downstream analysis
data <- lapply(lapply(files, read.FCS), exprs)
combined.data = as.data.frame(do.call(rbind, data))

# choose channels with markers to use for downstream analysis and apply arcsinh
# transformation with a cofactor of 15
transformed.chosen.markers <- combined.data %>%
  select(contains("-"),-contains("Ir")) %>%
  mutate_all(function(x)
    asinh(x / 15))         # cofactor here is 15; this can be change

# set seed for reproducible results (43 is chosen below)
overall_seed = 43
```
````

Read the data files into R and format for analysis

Select channels and scale the data

Choose parameters

03_blood_cell_PBMC_example.html

Run t-SNE

```
66 ```{r run_t-SNE}
67 # Time ~5 min
68
69 set.seed(overall_seed)
70
71 # the line below will run t-SNE on the scaled surface markers (to see help page
   for t-SNE, type "?Rtsne -- enter" in console)
72 # you can view t-SNE progress by opening up the console below
73 mytSNE = Rtsne(
74   transformed.chosen.markers,                          # input scaled data
75   dims = 2,                                            # number of final
   dimensions
76   initial_dims = length(transformed.chosen.markers), # number of initial
   dimensions
77   perplexity = 30,          # perplexity (similar to # of nearest neighbors,
78                             # will scale with data sets, cannot be greater t
79                             # the number of events minus 1 divided by 3)
80   check_duplicates = FALSE,
81   max_iter = 1000,          # number of iterations
82   verbose = TRUE
83 )
84 tsne.data = as.data.frame(mytSNE$Y)
85 ```
86
87 ```{r plot_t-SNE}
88 # Time <10 sec
89
90 # setting aspect ratio for plots
91 range <- apply(apply(tsne.data, 2, range), 2, diff)
92 graphical.ratio <- (range[1] / range[2])
93
94 # t-SNE flat dot plot and density dot plot (1 dot = 1 cell)
95 tsne.plot <- data.frame(x = tsne.data[, 1], y = tsne.data[, 2])
```

This section will run a t-SNE analysis on the PBMC data with set parameters

You can choose the resulting numbers of dimensions, the perplexity, and the iterations

This section will plot the t-SNE results. Two plots will appear, a “flat” dot plot and a density plot

Go to t-SNE talk while this runs

03_blood_cell_PBMC_example.html

Run UMAP

```
```{r run_UMAP}
# Time ~1 min

# Run UMAP on all scaled surface markers
set.seed(overall_seed)

# the line below will run UMAP on the data set (to see help page for UMA
"UMAP -- enter" in console)
# you can view UMAP progress by opening up the console below
myumap <-
  umap(transformed.chosen.markers,  # input scaled data
       n_neighbors = 15,            # number of nearest neighbors to look at,
scales with data set
       n_threads = 1,               # this argument makes UMAP reproducible
       verbose = TRUE)
umap.data = as.data.frame(myumap)
```

```{r plot_UMAP}
# Time <10 sec

# setting aspect ratio for plots
range <- apply(apply(umap.data, 2, range), 2, diff)
graphical.ratio <- (range[1] / range[2])

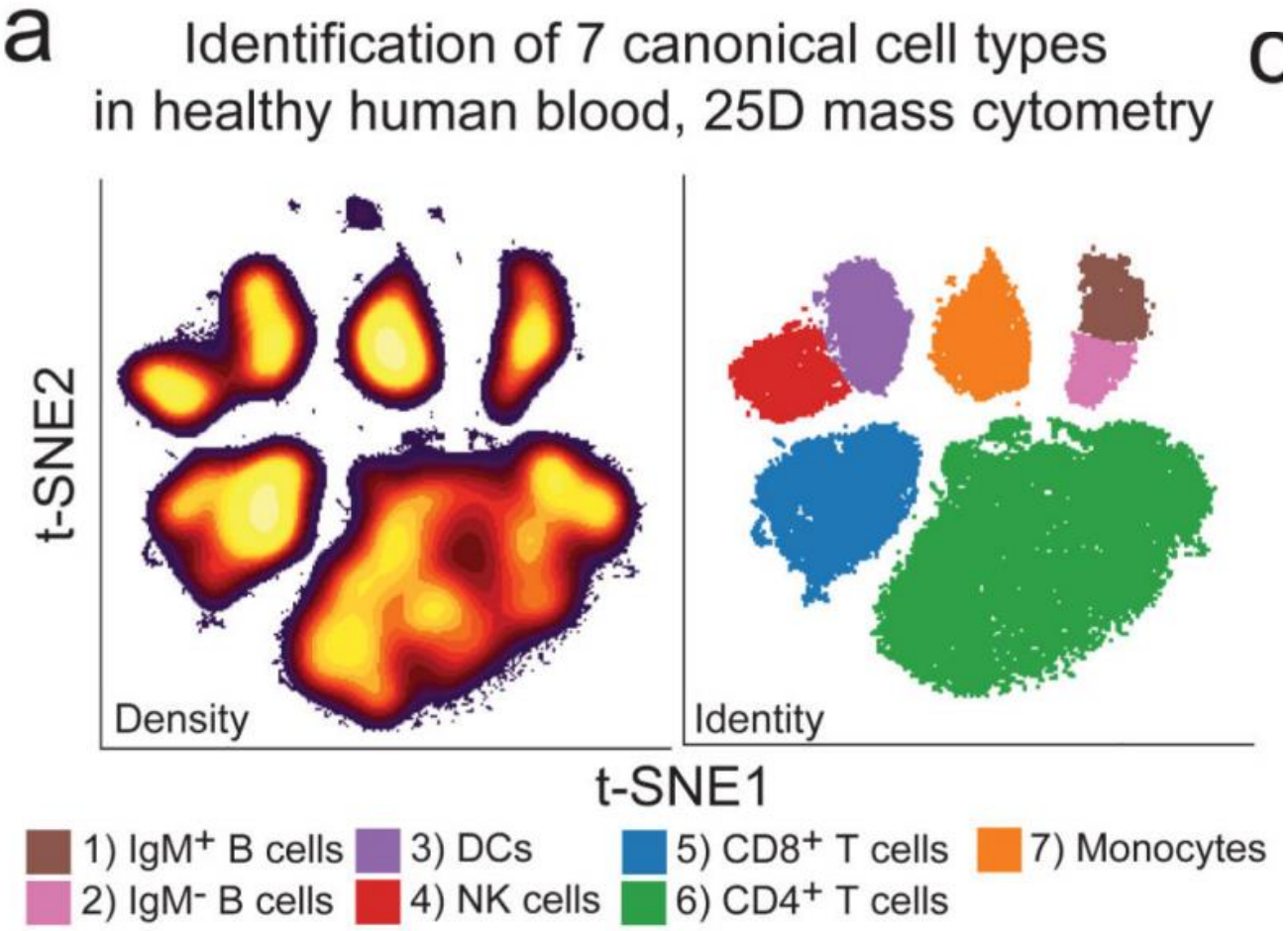
# UMAP flat dot plot and density dot plot (1 dot = 1 cell)
UMAP.plot <- data.frame(x = umap.data[, 1], y = umap.data[, 2])

# dot plot
ggplot(UMAP.plot) + coord_fixed(ratio = graphical.ratio) +
  geom_point(aes(x = x, y = y), cex = 0.5) + labs(x = "UMAP 1", y = "UMAP 2",
                                                  title = "PBMC Data on UMAP
Axes") +
  theme_bw() +
```

This section will run a UMAP analysis on the PBMC data using set parameters

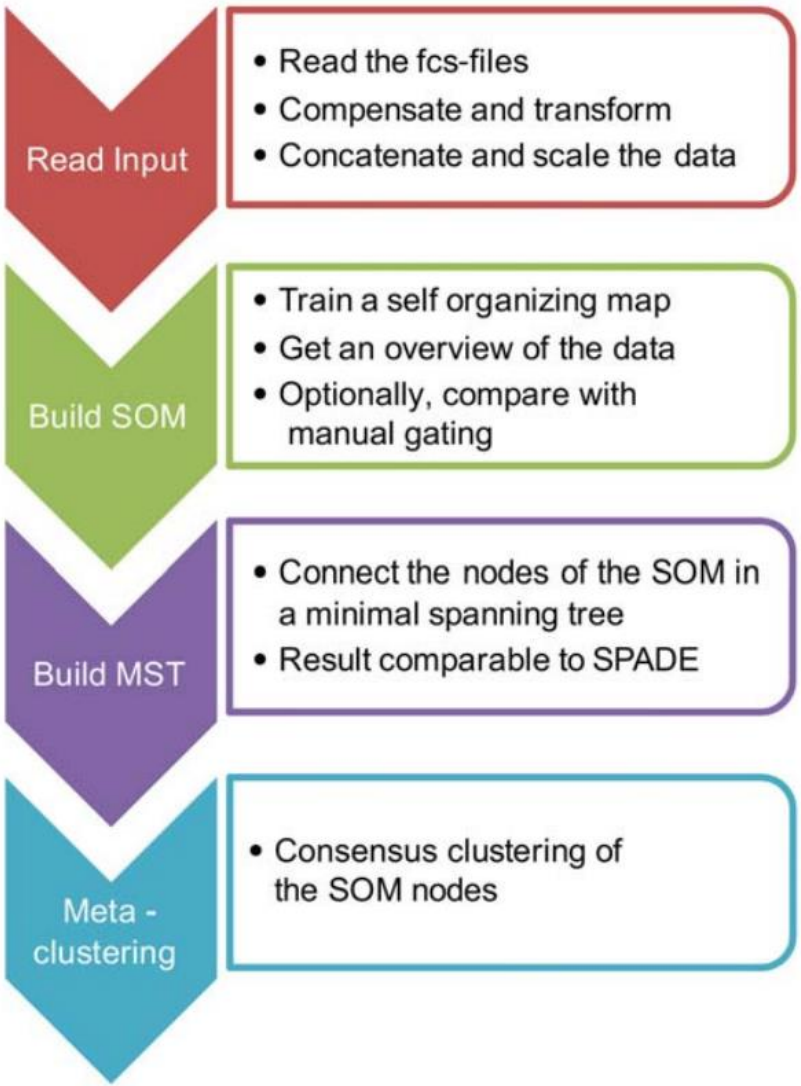
This section will plot the UMAP results. Two plots will appear, a “flat” dot plot and a density plot
```

Clusters can be Identified Based on Dimensionality Reduction Results



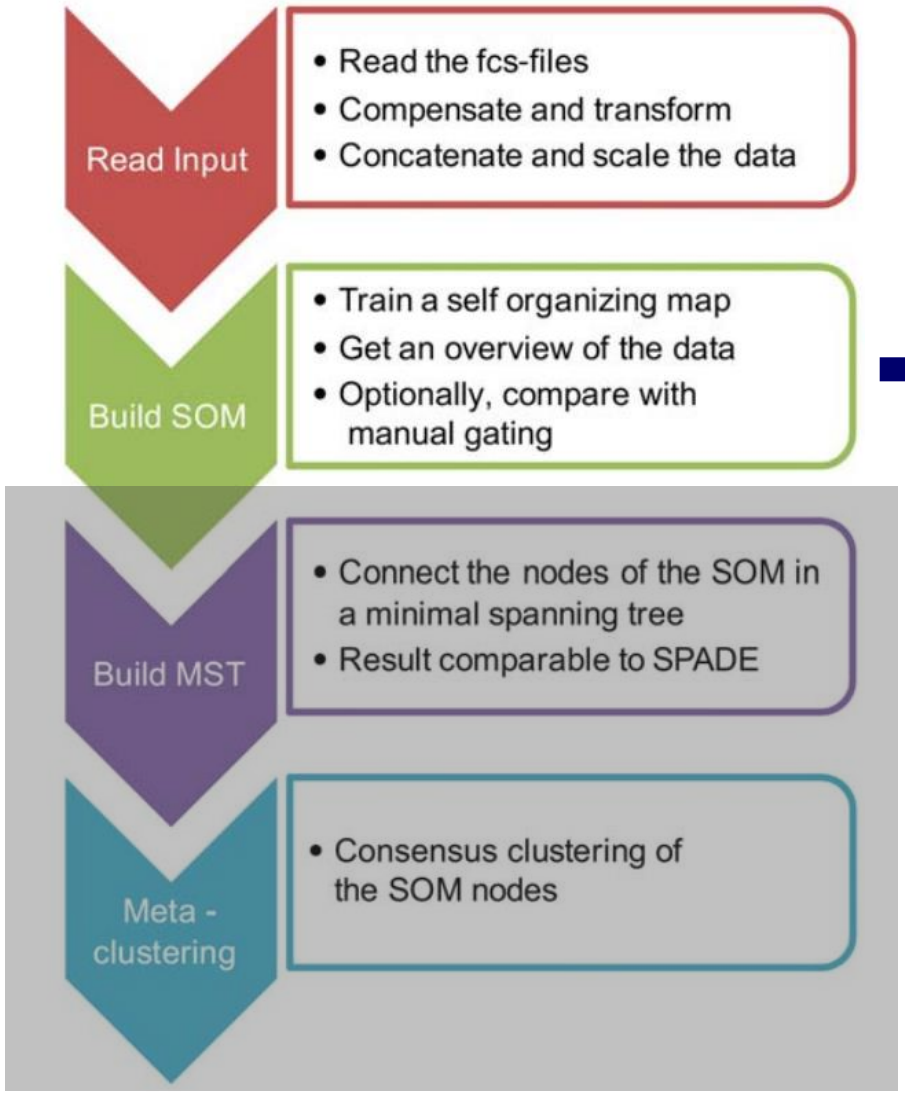
Diggins et al., *Nature Methods* 2017

Clustering with FlowSOM: Self-organizing Maps



Van Gassen et al., *Cytometry A* 2015
See also, FlowSOM does well in a comparison of clustering tools: Weber & Robinson, *Cytometry A* 2017

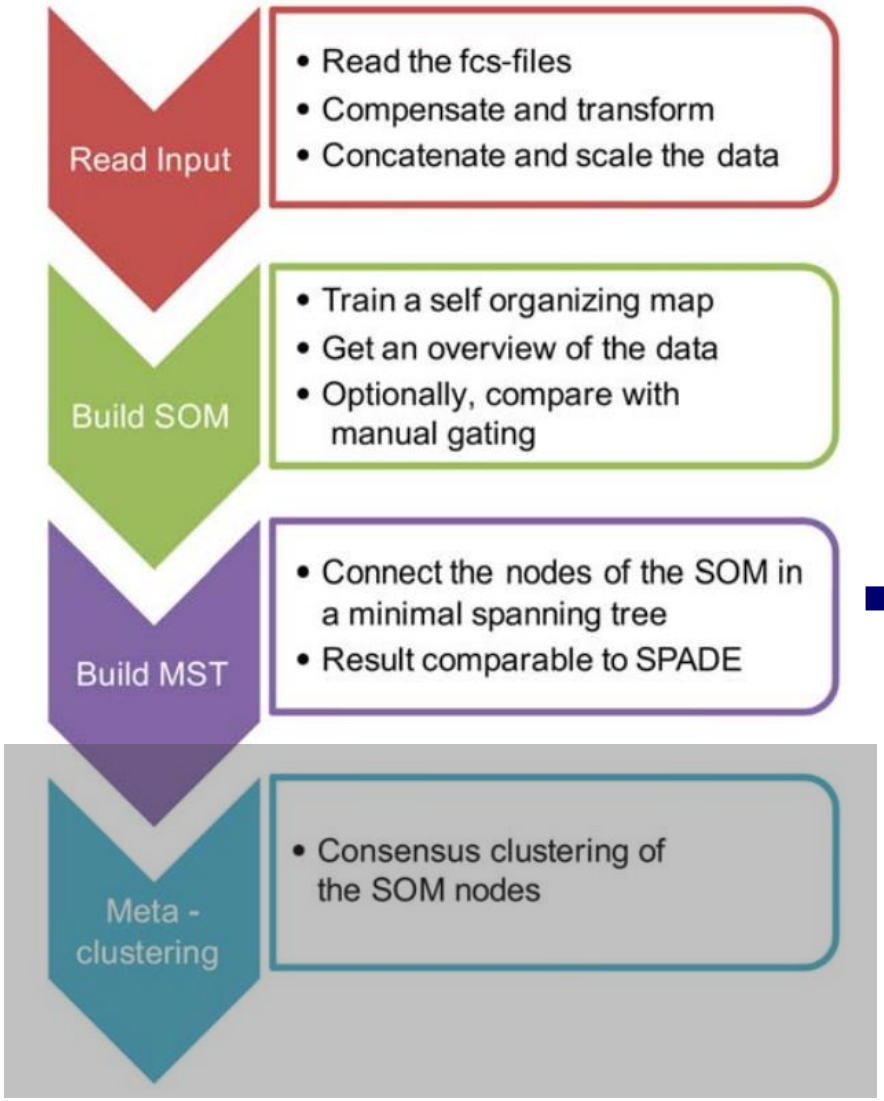
Clustering with FlowSOM: Self-organizing Maps



A SOM is created by assigning data points/cells to nodes based on their multidimensional phenotypes, updating this map repeatedly until each cell is assigned to a node with the most similar cells

Van Gassen et al., *Cytometry A* 2015
See also, FlowSOM does well in a comparison of clustering tools: Weber & Robinson, *Cytometry A* 2017

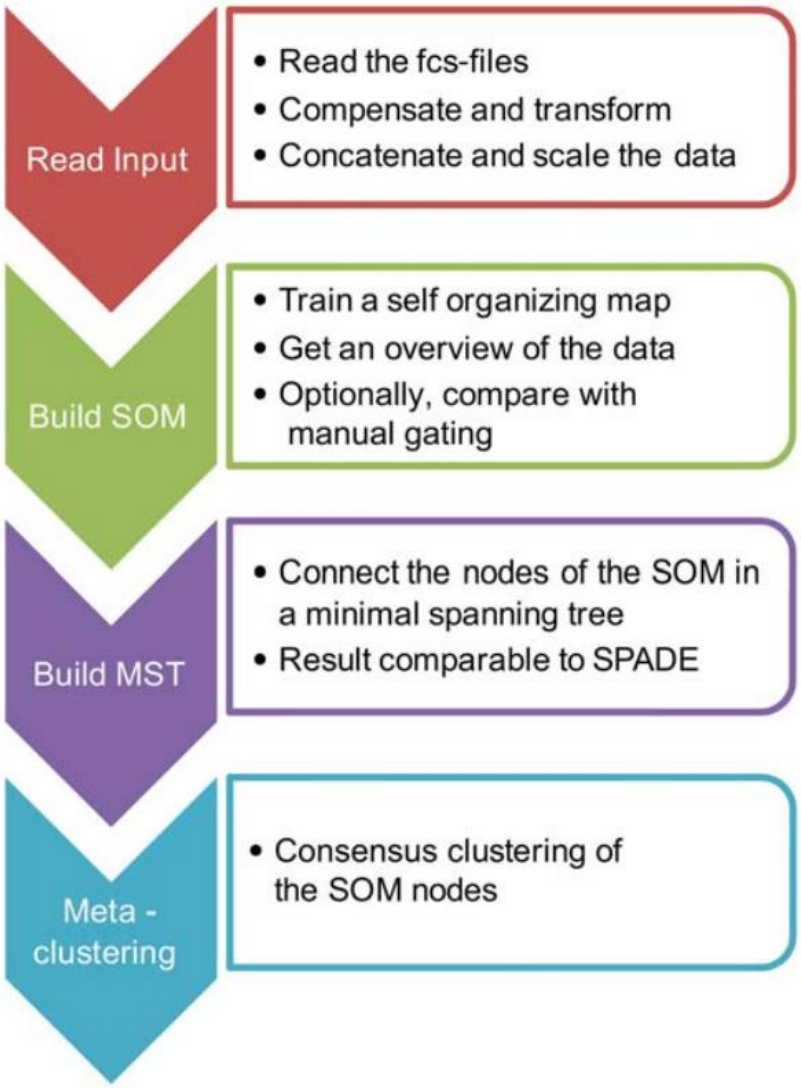
Clustering with FlowSOM: Self-organizing Maps



The next step is to arrange the nodes along a minimal spanning tree (MST), so that nodes that are most similar are closest on the tree
not used in our visualization

Van Gassen et al., *Cytometry A* 2015
See also, FlowSOM does well in a comparison of clustering tools: Weber & Robinson, *Cytometry A* 2017

Clustering with FlowSOM: Self-organizing Maps

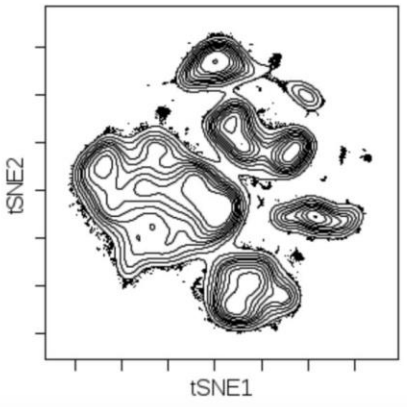


Finally, similar nodes are combined based on the number of desired clusters defined by the user. This desired number can be based on prior knowledge or a specific goal (i.e. minimizing intracluster variance)

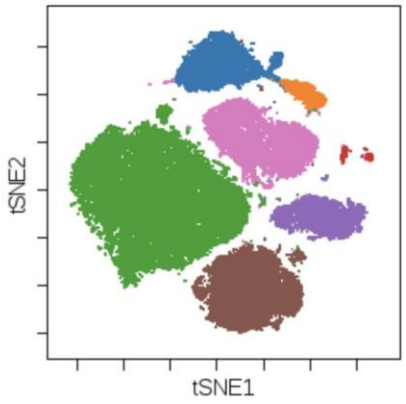
Van Gassen et al., *Cytometry A* 2015
See also, FlowSOM does well in a comparison of clustering tools: Weber & Robinson, *Cytometry A* 2017

Clustering with FlowSOM: Self-organizing Maps

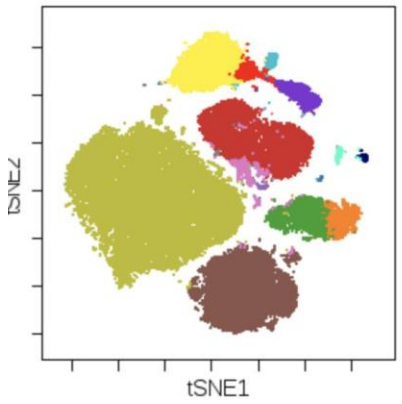
Contour plot of viSNE map



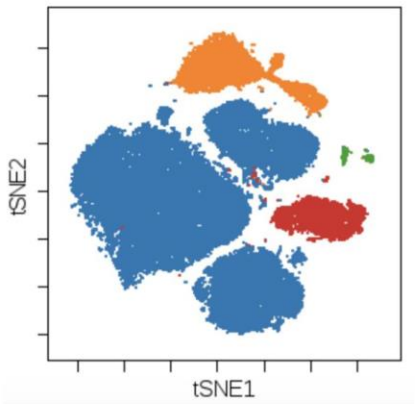
FlowSOM metaclusters overlaid on viSNE map



 # metaclusters = 7



 # metaclusters = 15



 # metaclusters = 4

03_blood_cell_PBMC_example.html

Run FlowSOM

````
```{r run_FlowSOM}
# Time <10 sec

# create flowFrame for FlowSOM input (using umap axes as input)
matrix <- as.matrix(umap.data)
metadata <-
  data.frame(name = dimnames(matrix)[[2]],
             desc = paste('UMAP', dimnames(matrix)[[2]]))
metadata$range <- apply(apply(matrix, 2, range), 2, diff)
metadata$minRange <- apply(matrix, 2, min)
metadata$maxRange <- apply(matrix, 2, max)
flowframe <- new("flowFrame",
                     exprs = matrix,
                     parameters = AnnotatedDataFrame(metadata))

# implement the FlowSOM on the data by running the line below (to see help for FlowSOM, type "FlowSOM -- enter" in console)
fsom <-
  FlowSOM(
    flowframe,      # input flowframe
    colsToUse = c(1:2),  # columns to use
    nClus = 10,          # target number of clusters (this can be changed)
    seed = overall_seed  # set seed for reproducibility
  )
FlowSOM.clusters <-
  as.matrix(fsom[[2]][fsom[[1]]$map$mapping[, 1]])
```

```{r plot_clusters}
# Time <10 sec

# plot FlowSOM clusters on UMAP axes
ggplot(UMAP.plot) + coord_fixed(ratio=graphical.ratio) +
  geom_point(aes(x=x, y=y, color=FlowSOM.clusters), cex = 0.5) +
  labs(x = "UMAP 1", y = "UMAP 2",title = "FlowSOM Clustering on UMAP Axes
       color = "Cluster") + theme_bw() +
  guides(colour = guide_legend(override.aes = list(size=4)))+
  labs(caption = "Data from Diggins et al., Nat Methods 2017, 14: 275-278 \nFlow Repository: FR-FCM-ZY63") +
  theme(panel.grid.major = element_blank(),
                panel.grid.minor = element_blank())
```
````

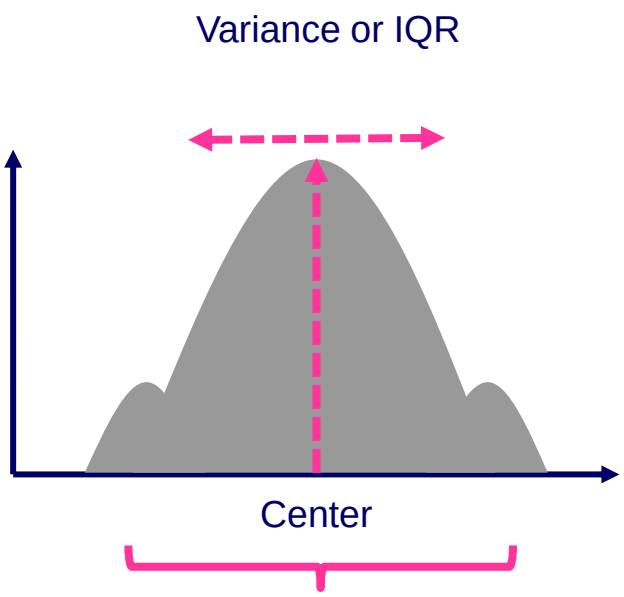
This section performs FlowSOM clustering on the UMAP results

You can choose the parameters the clustering is performed on (UMAP axes vs. measured markers) as well as a seed and desired number of clusters

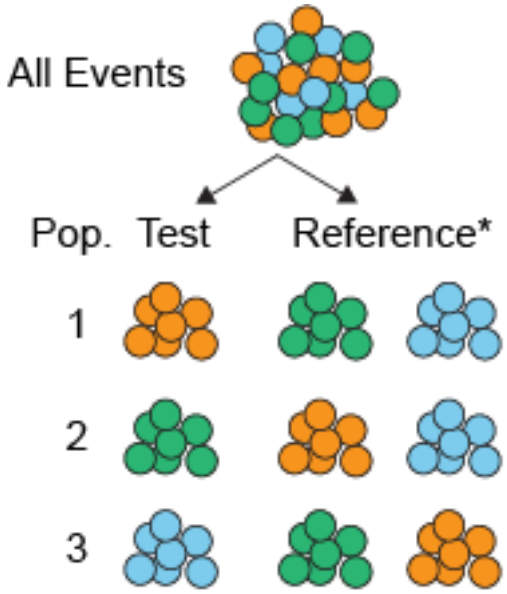
This section plots the identified clusters back onto the UMAP axes and generates a plot (a colored version of the UMAP plot from before)

Marker Enrichment Modeling Analysis Identifies Markers that are Specifically Expressed or Lacking on Populations

MEM accounts for variance and median of markers to identify enriched features on subsets of cells



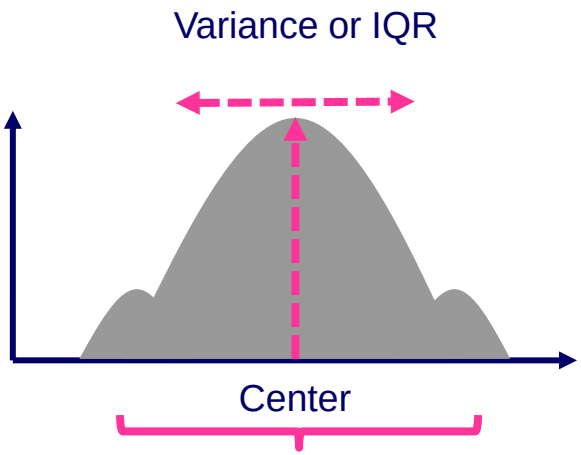
Shape (skewness, symmetry
peaks, outliers, etc.)



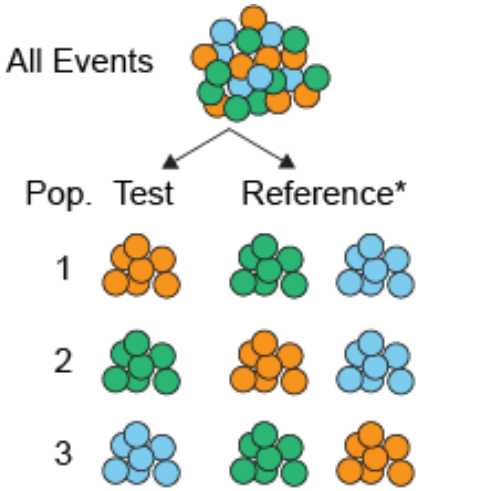
*All non-population as reference

Adapted from Kirsten Diggins

MEM Quantifies Relative Enrichment by Combining Magnitude and Interquartile Range



Shape (skewness, symmetry
peaks, outliers, etc.)



*All non-population as reference

MEM label

▲HLADR^{+10} CD20^{+9} CD19^{+7} IgM^{+5} CD34^{+3} CD45RA^{+3} CXCR4^{+2} CD47^{+2} CD33^{+2}
▼CD7^{-2}

Linear transformation to -10 to +10

If $MAG_{test} - MAG_{ref} < 0$, MEM = -MEM

Adapted from Kirsten Diggins

03_blood_cell_PBMC_example.html

Run MEM

```
```{r run_MEM}
# Time ~30 sec

# Run MEM on the FlowSOM clusters found from using UMAP axes
cluster = as.numeric(as.vector((FlowSOM.clusters)))
MEM.data = cbind(transformed.chosen.markers, cluster)

MEM.values = MEM(
  MEM.data,              # input data (last column must contain cluster values)
  transform = FALSE,     # data is already scaled in this case
  cofactor = 1,
  choose.markers = FALSE,
  markers = "all",       # use all transformed, chosen markers from previous selection
  choose.ref = FALSE,    # reference will be all other cells
  zero.ref = FALSE,
  rename.markers = FALSE,
  new.marker.names =
"CD19,CD117,CD11b,CD4,CD8,CD20,CD34,CD61,CD123,CD45RA,CD45,CD10,CD33,C
,CD69,CD15,CD16,CD44,CD38,CD25,CD3,IgM,HLA-DR,CD56", # rename channels labels
  file.is.clust = FALSE,
  add.fileID = FALSE,
  IQR.thresh = NULL
)

# build MEM heatmap and output enrichment scores
build.heatmaps(
  MEM.values,                  # input MEM values
  cluster.MEM = "both",        # dendrogram for columns and rows
  display.thresh = 3,          # display threshold for MEM scores
  newWindow.heatmaps = FALSE,
  output.files = TRUE,         # makes txt and PDF files for heatmap and MEM scores
  labels = TRUE,               # include labels in heatmap
  only.MEMheatmap = FALSE
)
```
```

This section performs MEM analysis on the identified FlowSOM clusters

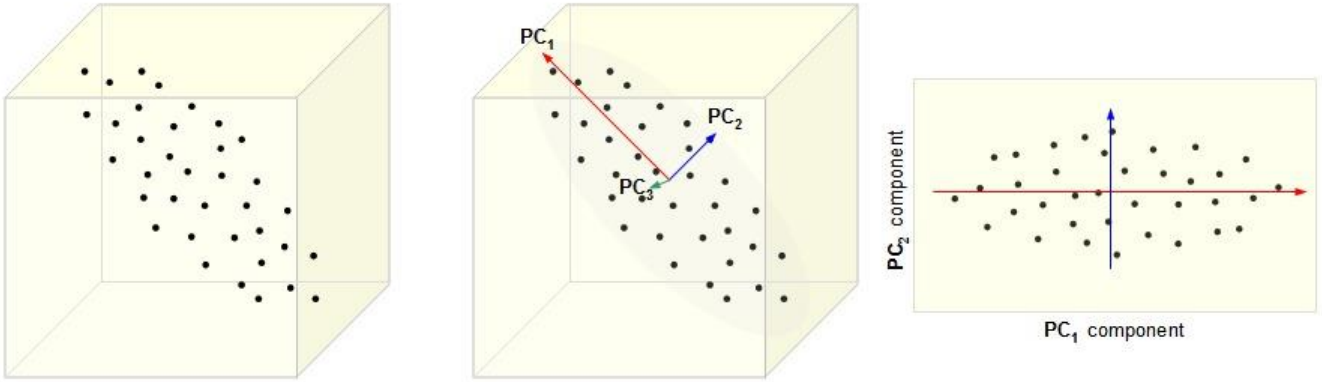
You can choose the markers for the MEM analysis as well as their names and the reference population

This section produces heatmaps and MEM (enrichment) scores

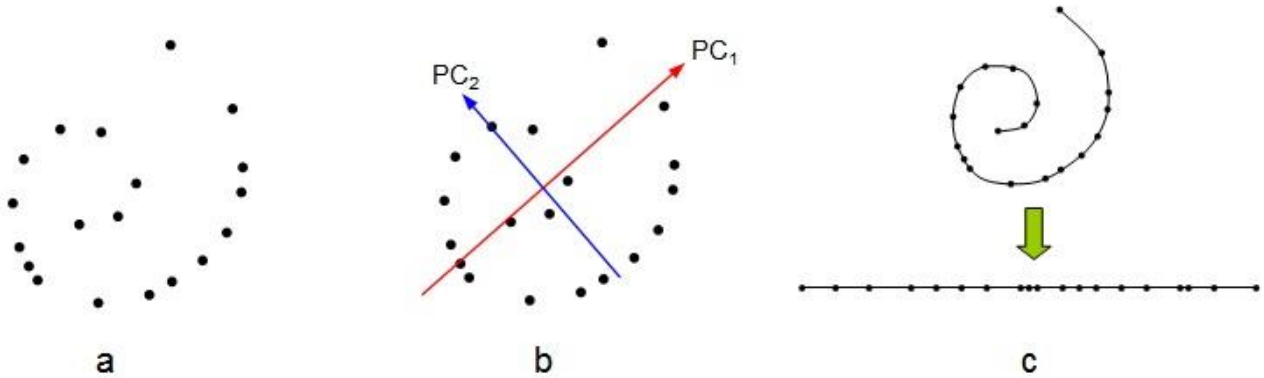
Part 7: Many More Tools!

PCA, Citrus, Trajectories

PCA is a Linear Dimensionality Reduction Tool

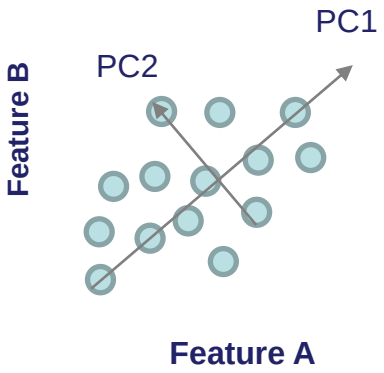


An illustration of PCA. **a)** A data set given as 3-dimensional points. **b)** The three orthogonal Principal Components (PCs) for the data, ordered by variance. **c)** The projection of the data set into the first two PCs, discarding the third one.



Effects of dimensionality reduction on an inherently non-linear data set. **a)** The original data given as a two-dimensional set. **b)**PCA identifies two PCs as contributing significantly to explain the data variance. **c)** However, the inherent topology (connectivity) of the data helps identify the set as being one-dimensional, but non-linear.

Principal Component Analysis

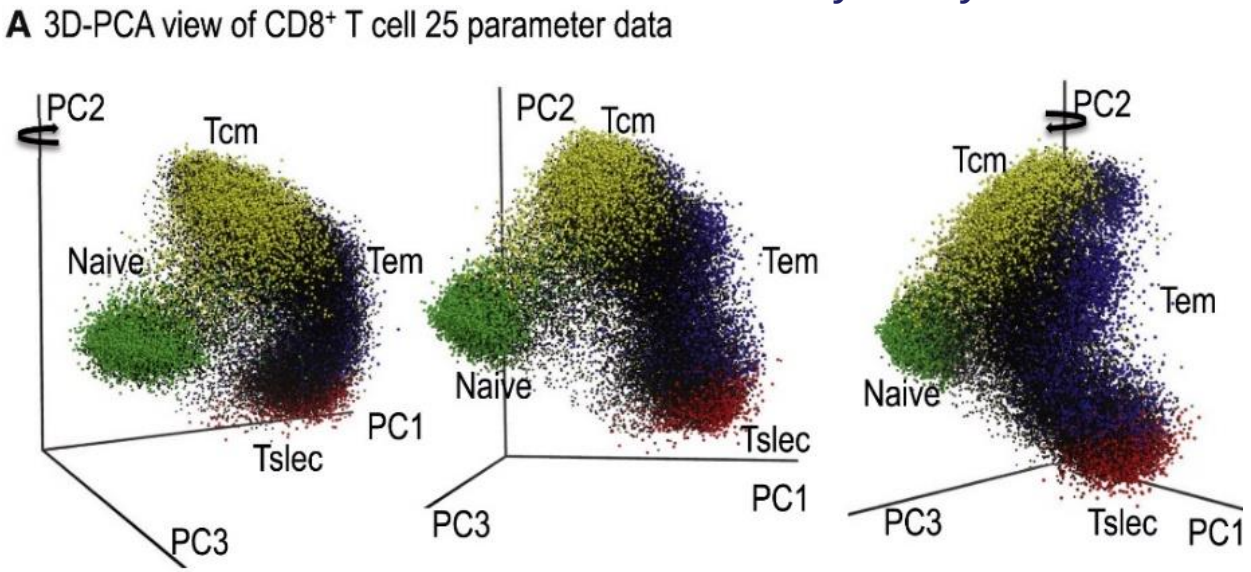


$PC1 = X_{A1}A + X_{B1}B + X_{C1}C$

$PC2 = X_{A2}A + X_{B2}B + X_{C2}C$

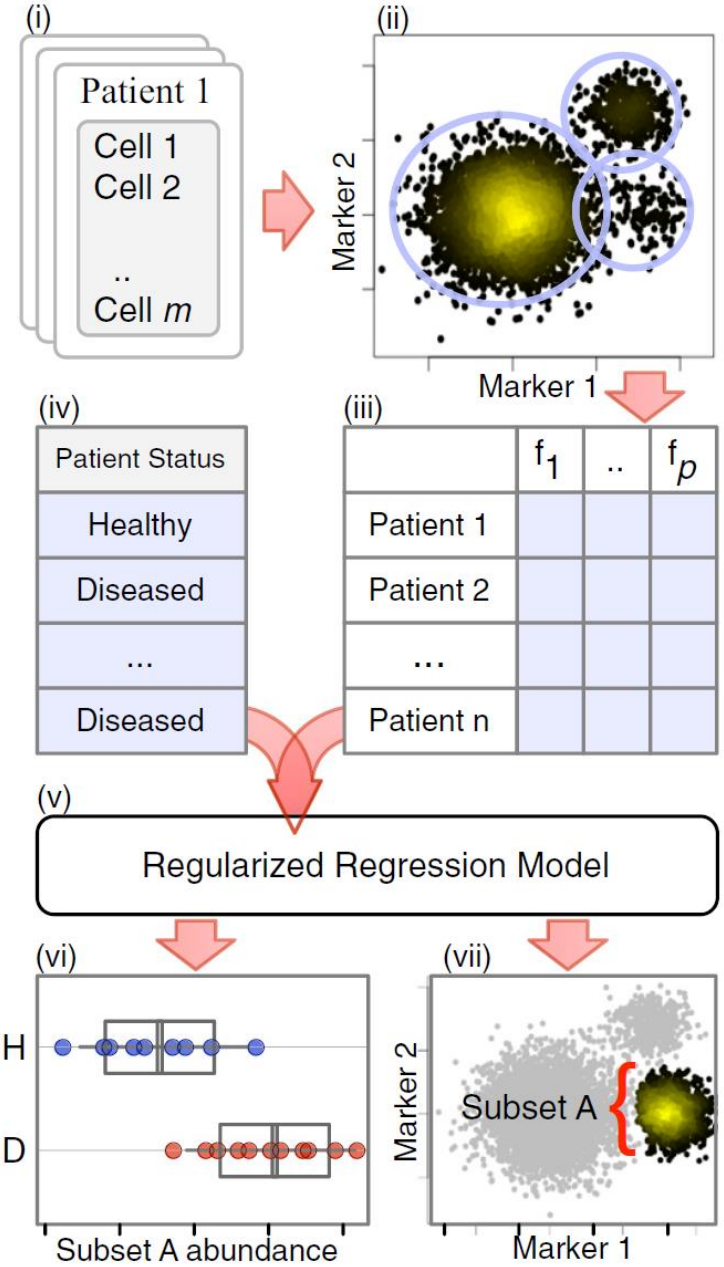
$PC3 = X_{A3}A + X_{B3}B + X_{C3}C$

PCA used to Reduce Dimensionality of CyTOF Data



Newell et al 2012, *Immunity*

Citrus: Supervised Population Finding

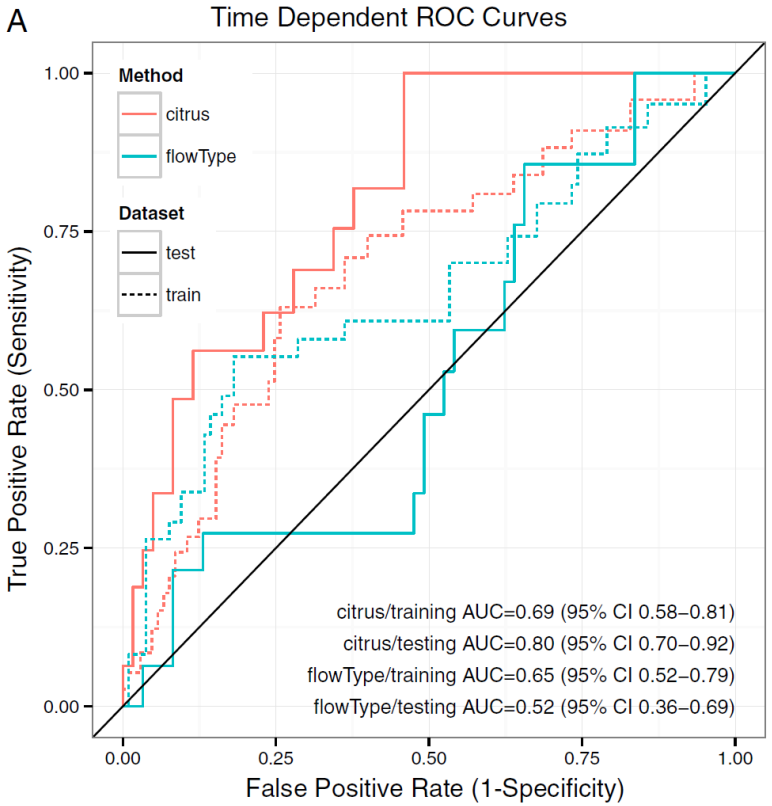


Automated identification of stratifying signatures in cellular subpopulations

Robert V. Bruggner[a,b], Bernd Bodenmiller[c], David L. Dill[d], Robert J. Tibshirani[e,f,1], and Garry P. Nolan[b,1]

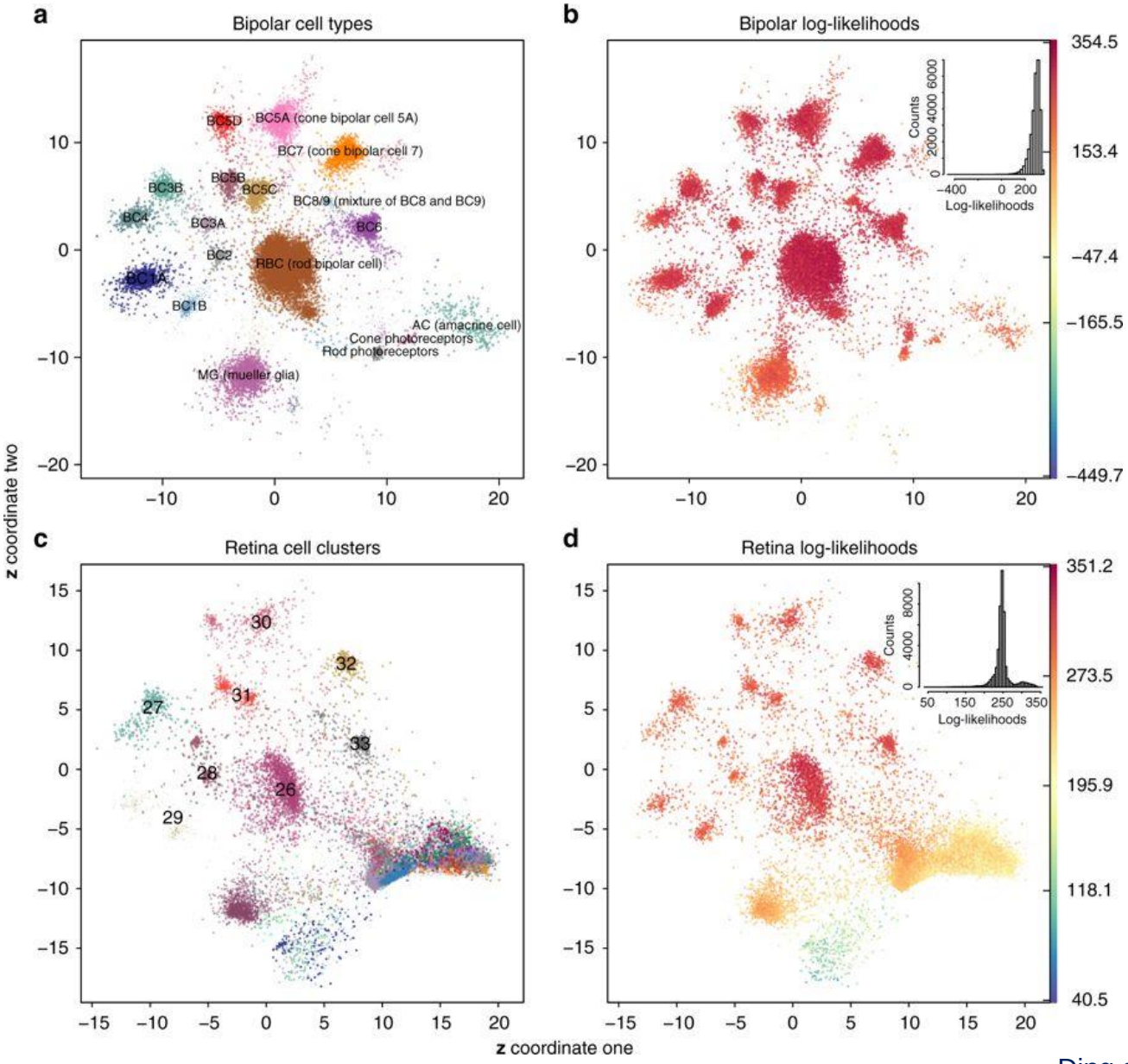
[a]Biomedical Informatics Training Program, Stanford University Medical School, Stanford, CA 94305; [b]Baxter Laboratory for Stem Cell Biology, Department of Microbiology and Immunology, and Departments of [d]Computer Science, [e]Health Research and Policy, and [f]Statistics, Stanford University, Stanford, CA 94305; and [c]Institute of Molecular Life Sciences, University of Zurich, CH-8057 Zurich, Switzerland

Contributed by Robert J. Tibshirani, May 14, 2014 (sent for review February 12, 2014)



Bruggner et al., *PNAS* 2014

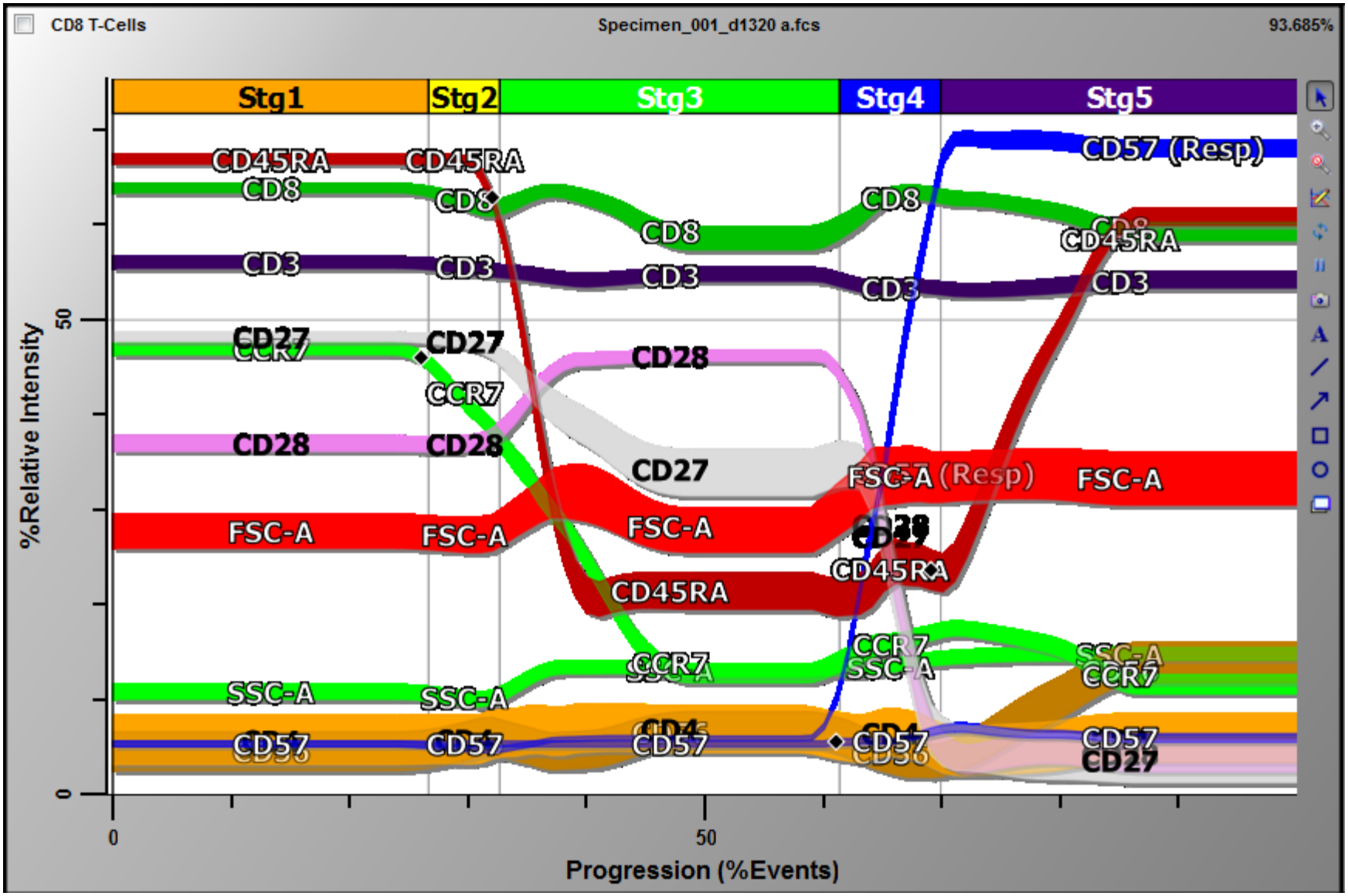
In 2018, Ding et al. Used scvis & *Probability* to Characterize / Identify Cells (scRNA-seq)



Learning a probabilistic mapping function from the bipolar data and applying the function to the independently generated mouse retina dataset. **a** scvis learned two-dimensional representations of the bipolar dataset, **b** coloring each point by the estimated log-likelihood, **c** the whole mouse retina dataset was directly projected to a two-dimensional space by the probabilistic mapping function learned from the bipolar data, and **d** coloring each point from the retina dataset by the estimated log-likelihood

Ding et al., *Nature Communications* 2018

Gemstone Uses Supervised Analysis to Identify Progressions

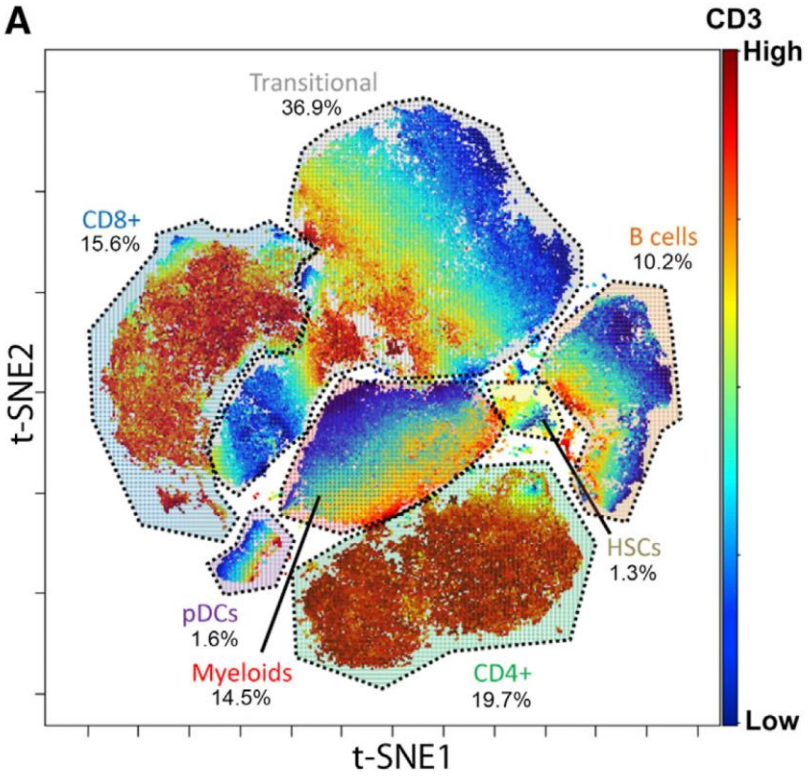


Verity Software House (www.vsh.com)

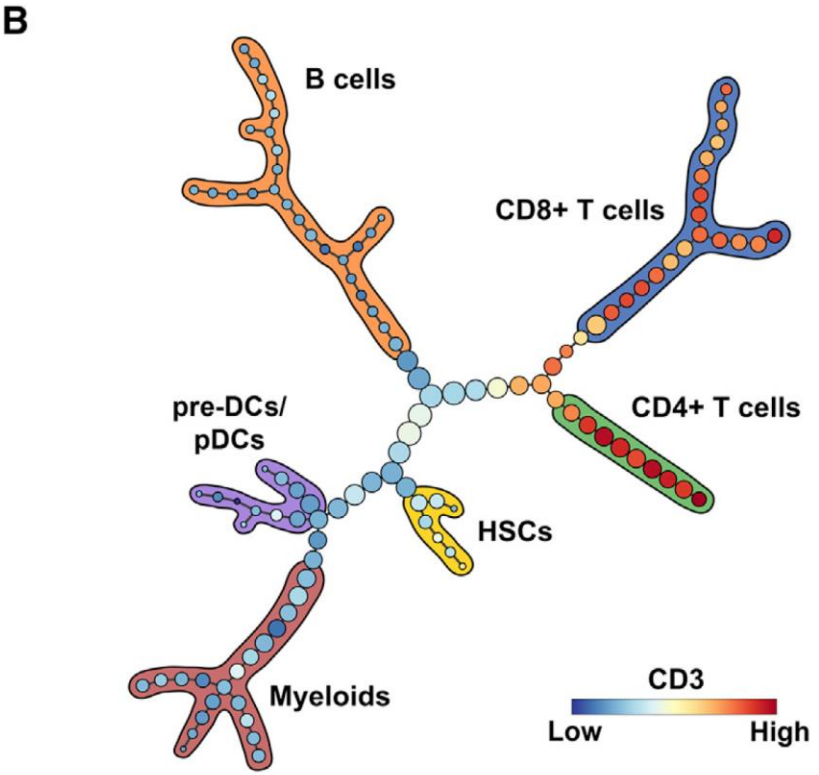
Inokuma, *JIM* 2013

In 2018, Ken Lau's Group Developed p-Creode to Infer Continua in Single Cell Data (e.g., human bone marrow, CyTOF)

t-SNE

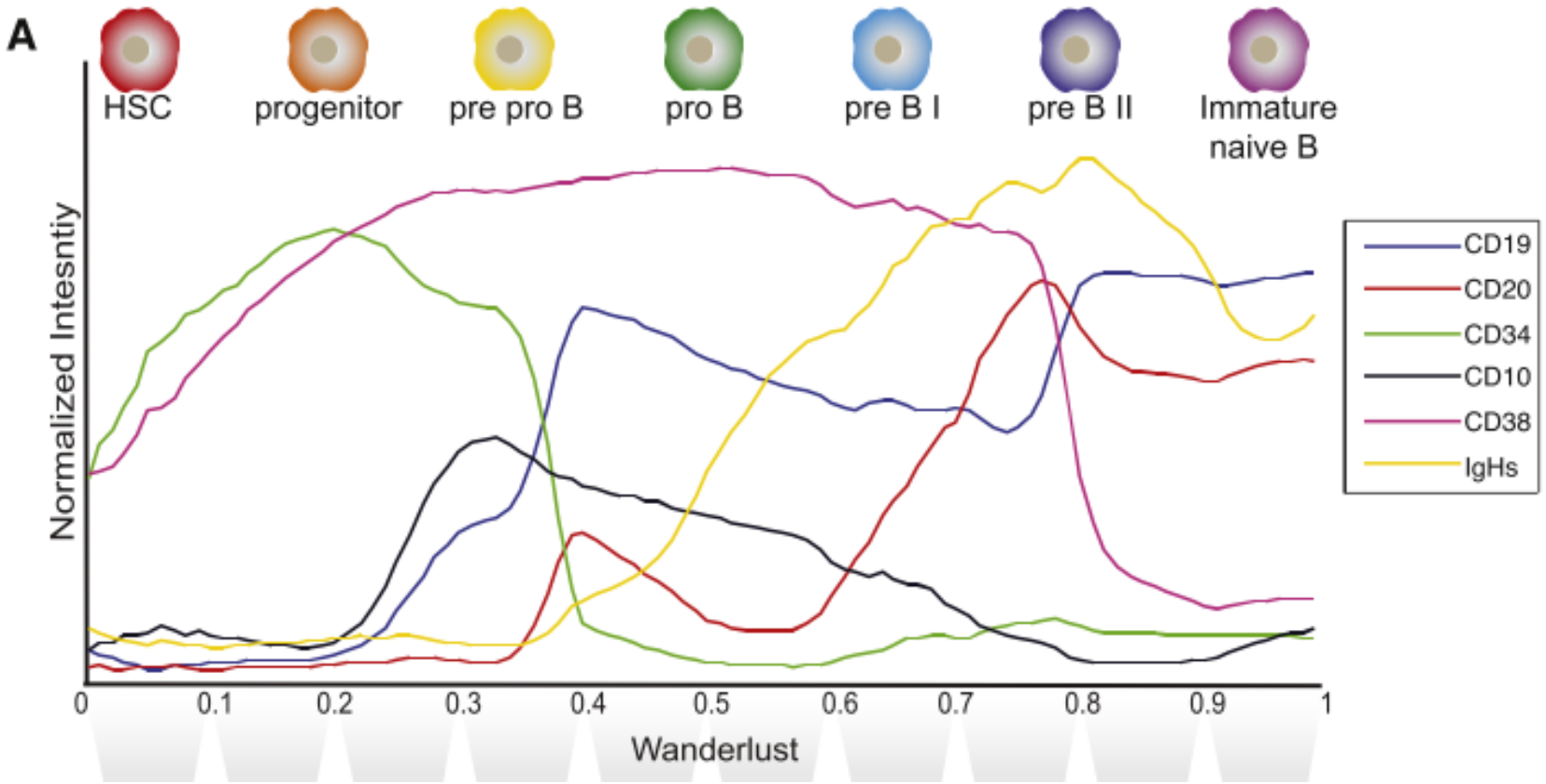


p-Creode



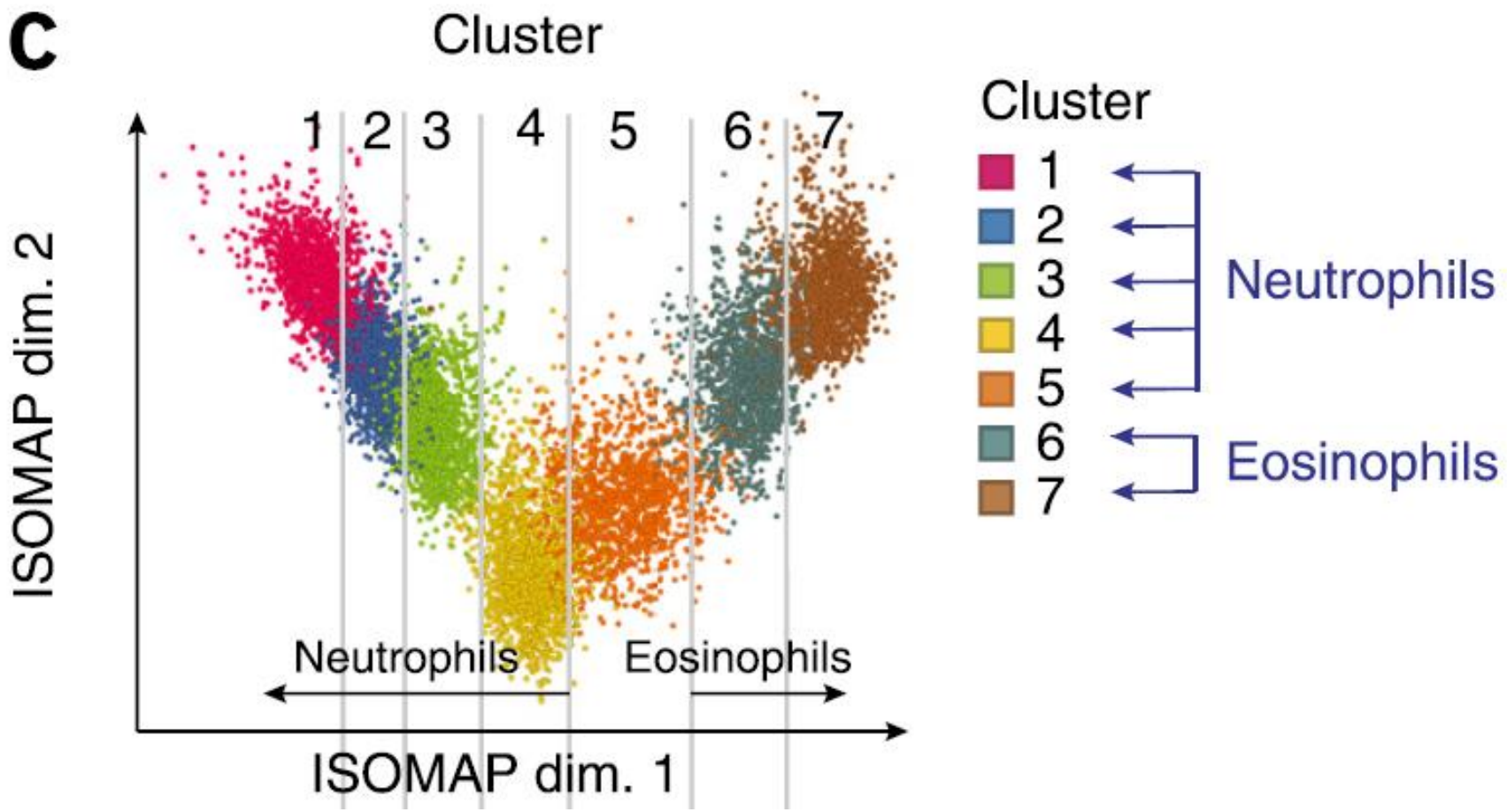
Herring et al., *Cell Systems* 2018

Wanderlust Identifies Phenotypic Progression



Bendall, *Cell* 2014

ISOMAP guided analysis

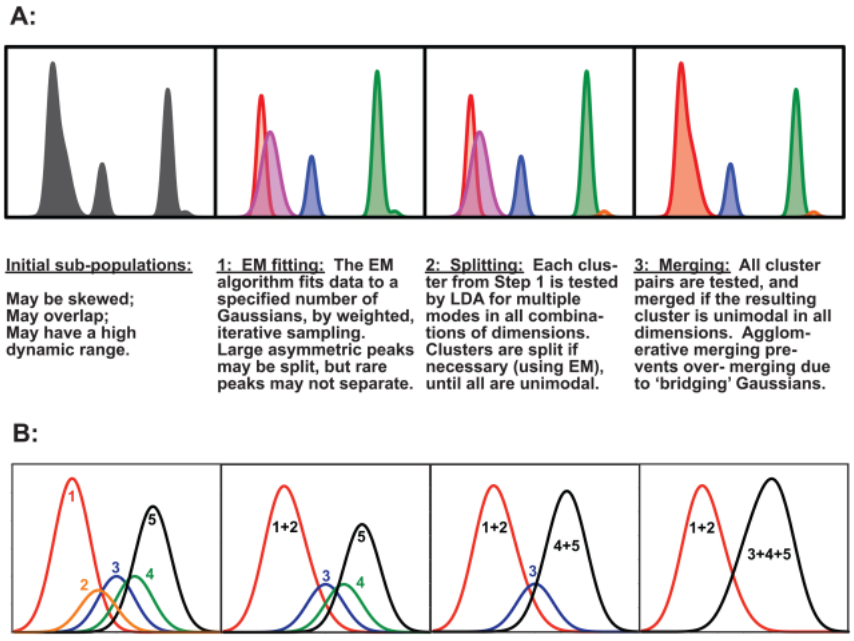


(**c**) ISOMAP dimensionality reduction to compare overall phenotypic relatedness of populations of neutrophil-like and eosinophil-like cells[31]. Top, cells color-coded by DensVM cluster number are plotted by their scores for ISOMAP dimensions 1 and 2. Binned median expression of defining markers (middle) and the tissue composition (percentage of each cluster as a fraction of total granulocytes from each tissue, bottom) of cells along this phenotypic progression defined by ISOMAP dimension 1 and DensVM clusters 1–7 are plotted.

Becher et al., *Nature Immunology* 2014

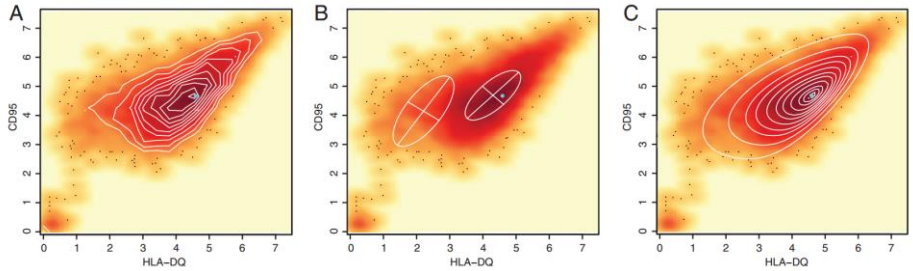
Mixture Modeling

SWIFT



Mosmann et al, 2014 *Cytometry A*

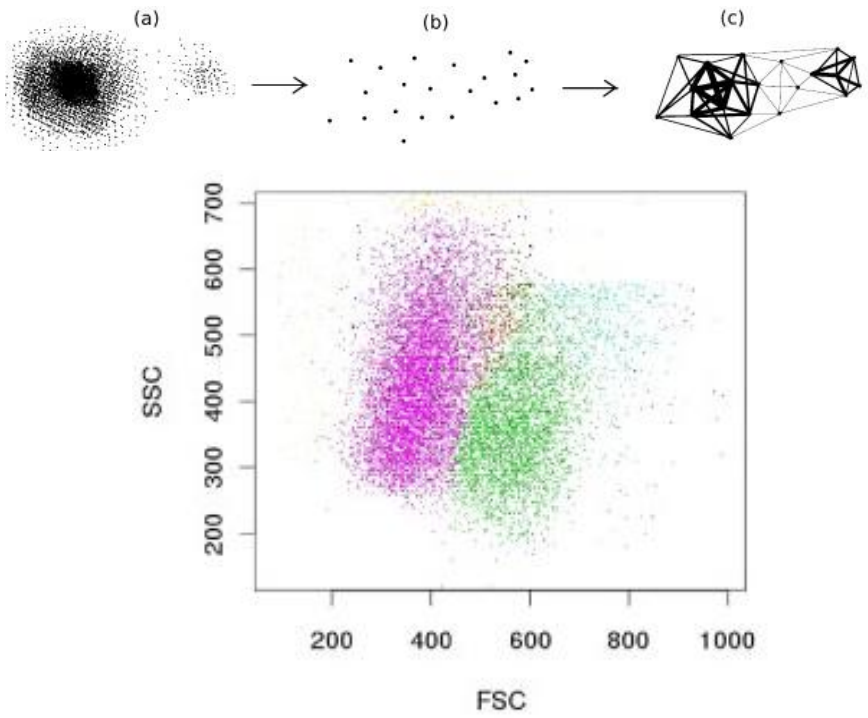
FLAME



Pyne et al, 2009 *PNA*

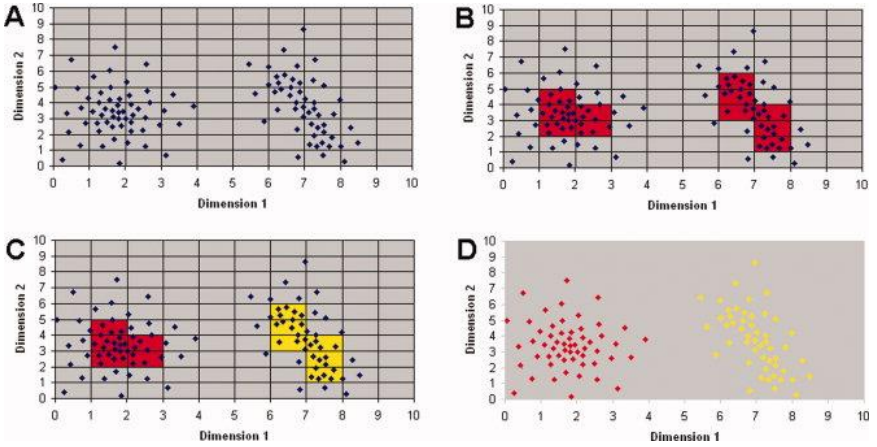
Automated Clustering and Population Identification Methods Based on Density

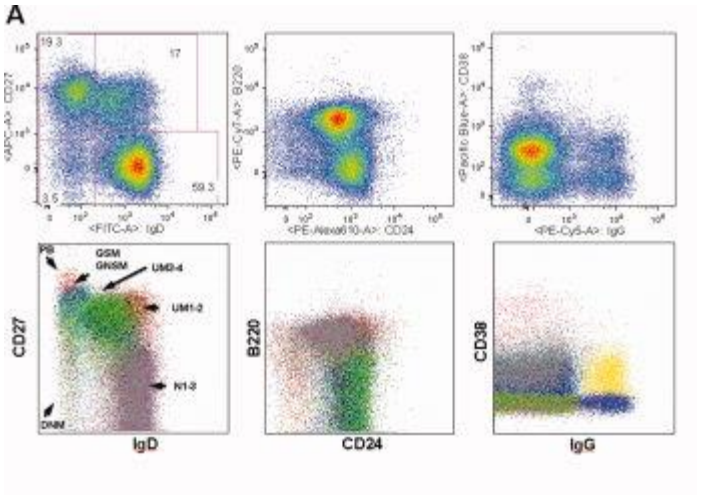
SamSpectral



Zare et al, 2010 *BMC Bioinformatics*

FLOCK

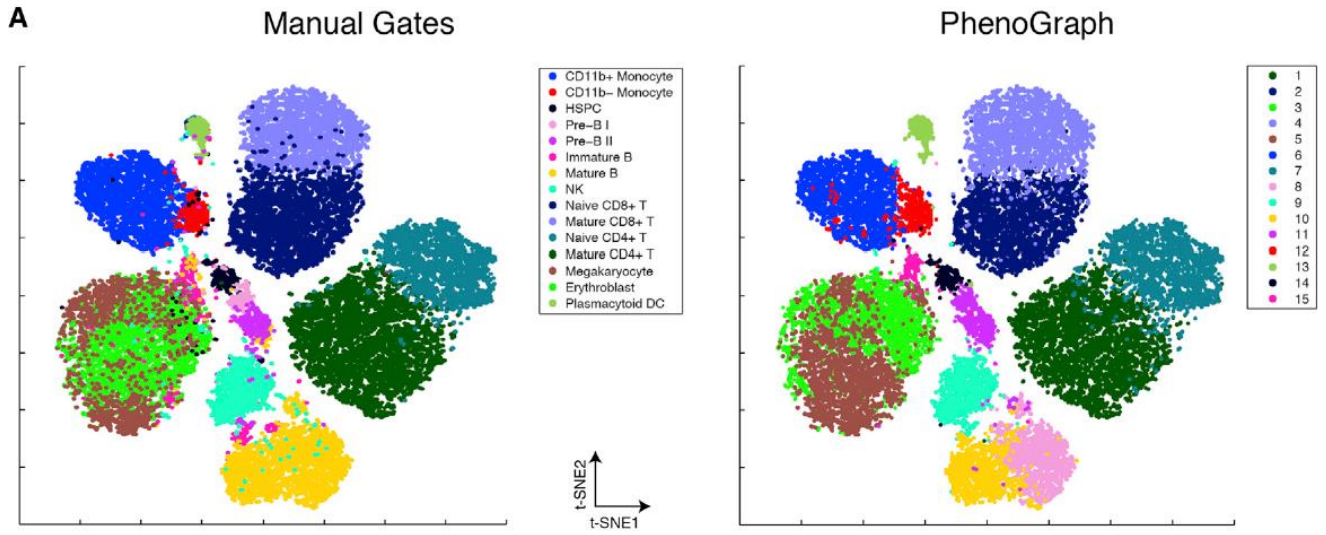


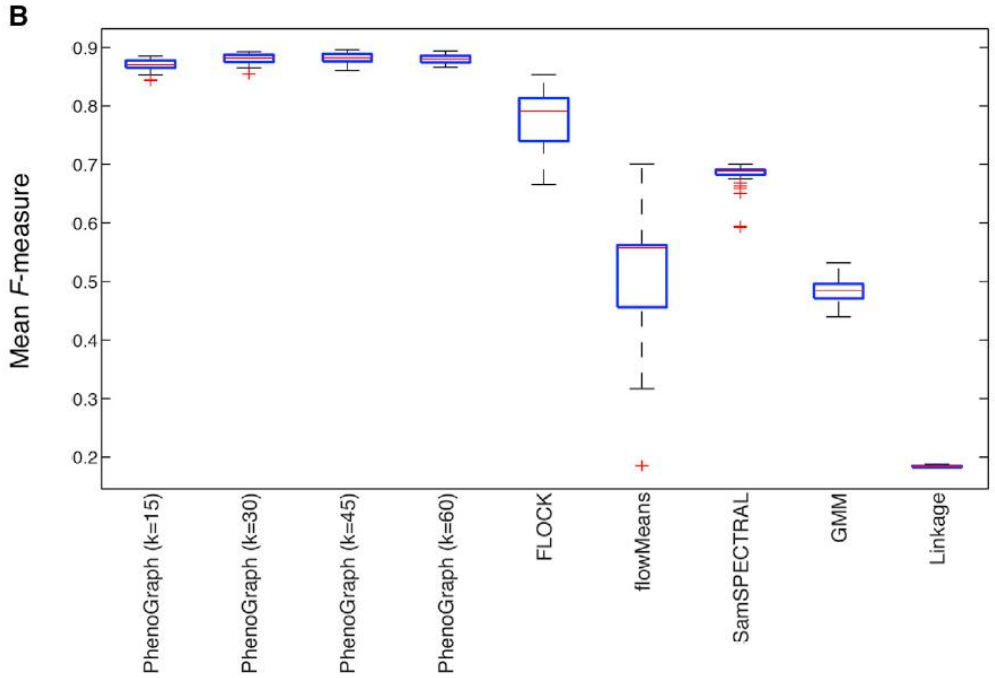


Qian et al, 2010 *Cytometry B Clin Cytom*

Phenograph

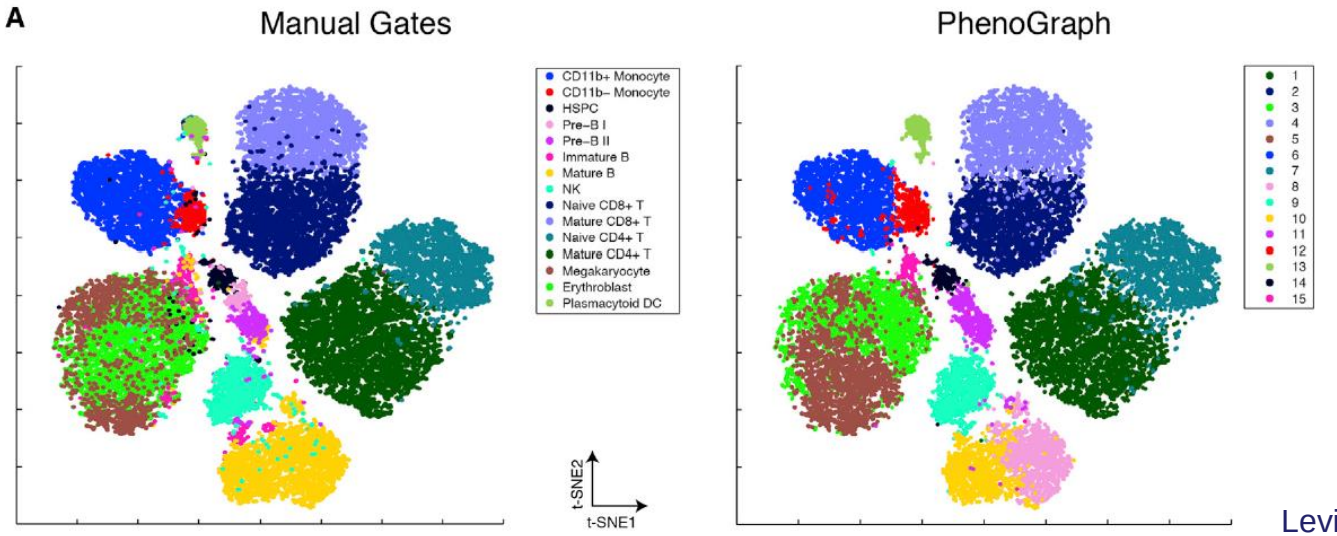
Phenograph Adds Fast Clustering & Meta-Analysis to viSNE





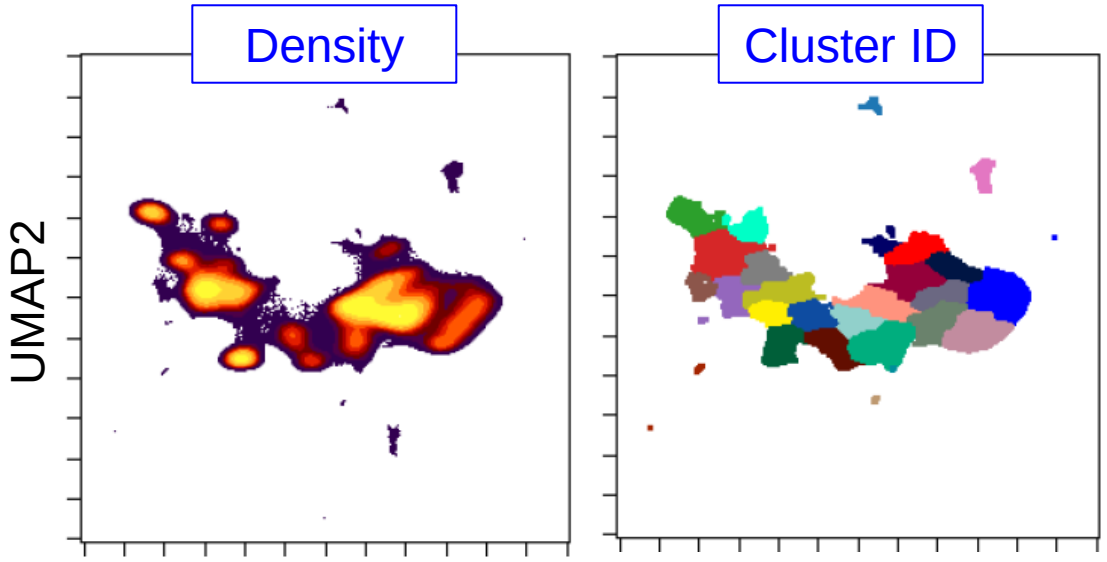
Levine et al., *Cell* 2015

Phenograph: Clustering 35 Features => t-SNE (Not the Reverse)



Levine et al., *Cell* 2015

Diggins: t-SNE or UMAP on Features => Clustering on 2 axes



Diggins et al., *Methods* 2015

UMAP

ANALYSIS

nature biotechnology

Dimensionality reduction for visualizing single-cell data using UMAP

Etienne Becht[1], Leland McInnes[2], John Healy[2], Charles-Antoine Dutertre[1], Immanuel W H Kwok[1], Lai Guan Ng[1], Florent Ginhoux[1] & Evan W Newell[1,3]

Advances in single-cell technologies have enabled high-resolution dissection of tissue composition. Several tools for dimensionality reduction are available to analyze the large number of parameters generated in single-cell studies. Recently, a nonlinear dimensionality-reduction technique, uniform manifold approximation and projection (UMAP), was developed for the analysis of any type of high-dimensional data. Here we apply it to biological data, using three well-characterized mass cytometry and single-cell RNA sequencing datasets. Comparing the performance of UMAP with five other tools, we find that UMAP provides the fastest run times, highest reproducibility and the most meaningful organization of cell clusters. The work highlights the use of UMAP for improved visualization and interpretation of single-cell data.

https://arxiv.org/abs/1802.03426

UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction

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December 7, 2018

Abstract

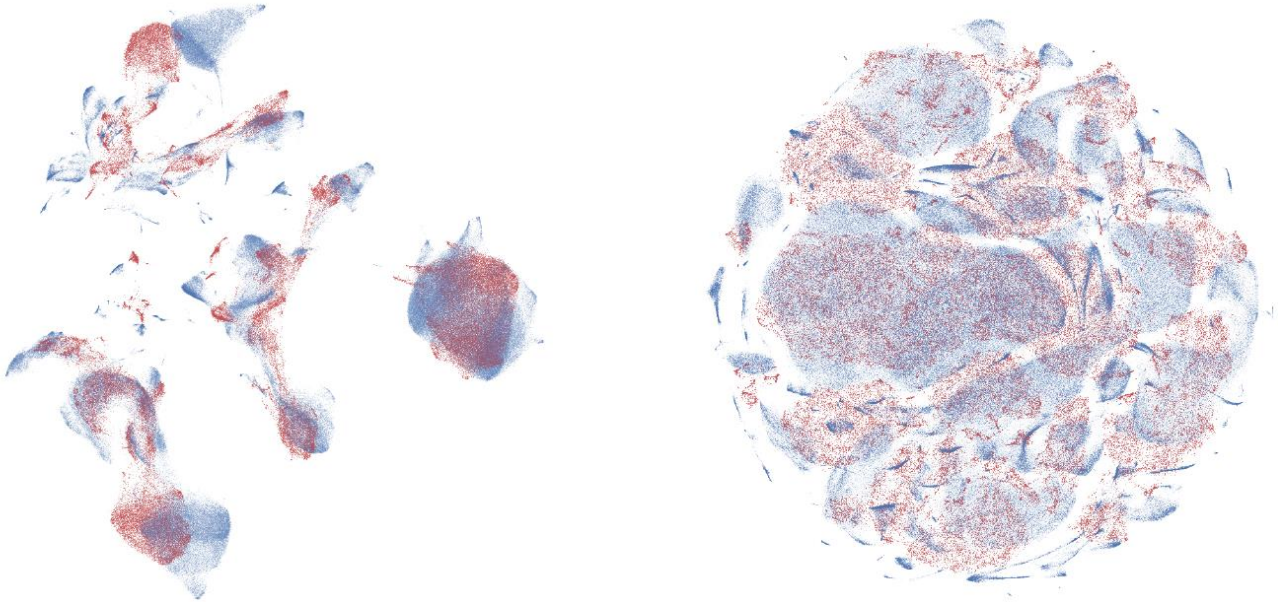
UMAP (Uniform Manifold Approximation and Projection) is a novel manifold learning technique for dimension reduction. UMAP is constructed from a theoretical framework based in Riemannian geometry and algebraic topology. The result is a practical scalable algorithm that applies to real world data. The UMAP algorithm is competitive with t-SNE for visualization quality, and arguably preserves more of the global structure with superior run time performance. Furthermore, UMAP has no computational restrictions on embedding dimension, making it viable as a general purpose dimension reduction technique for machine learning.

Comments: Reference implementation available at this http URL
Subjects: **Machine Learning (stat.ML)**; Computational Geometry (cs.CG); Machine Learning (cs.LG)
Cite as: **arXiv:1802.03426 [stat.ML]**
(or **arXiv:1802.03426v2 [stat.ML]** for this version)

Submission history

From: Leland McInnes [view email]
[v1] Fri, 9 Feb 2018 19:39:33 UTC (958 KB)
[v2] Thu, 6 Dec 2018 18:54:07 UTC (7,966 KB)

Flow Cytometry Data That Looks Like a Blob on a t-SNE Plot Appears to Have Structure on a UMAP Plot



(a) UMAP (b) t-SNE

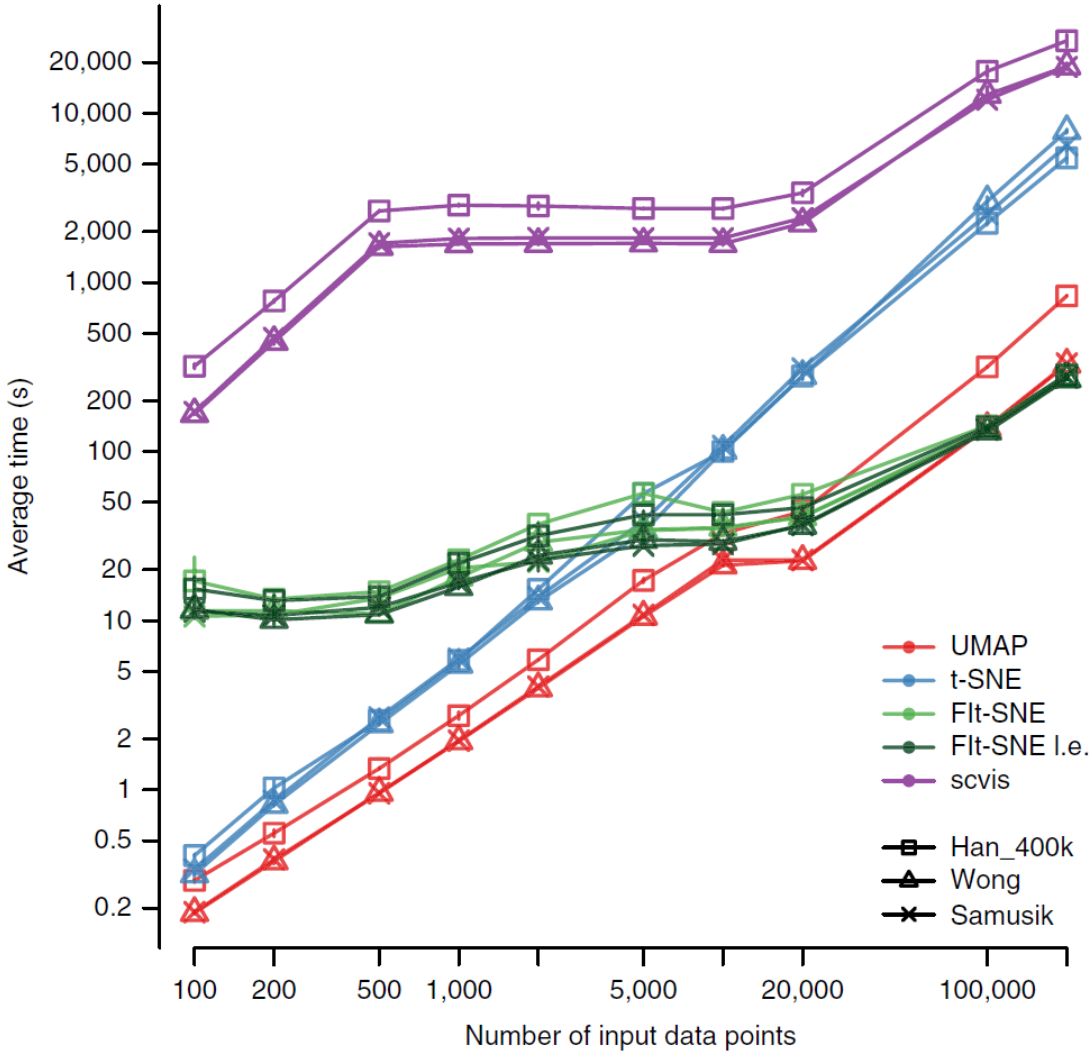
Figure 3: Procrustes based alignment of a 10% subsample (red) against the full dataset (blue) for the flow cytometry dataset for both UMAP and t-SNE.

In Greek mythology, **Procrustes** (Ancient Greek: Προκρούστης *Prokroustes*) or "the stretcher [who hammers out the metal]", also known as **Prokoptas** or **Damastes**(Δαμαστής, "subduer"), was a rogue smith and bandit from Attica who attacked people by stretching them or cutting off their legs, so as to force them to fit the size of an iron bed.

The word "Procrustean" is thus used to describe situations **where different lengths or sizes or properties are fitted to an arbitrary standard**.

McInnes et al., *Rxiv* 2018

UMAP & Fit-SNE are Much Faster than Traditional t-SNE



20,000 seconds = 5.5 hours

5,000 seconds = ~1.4 hours
(similar to our run times)

500 seconds = 8.3 minutes

CyTOF datasets are ~100,000 to 50,000,000 cell data points

[scRNA-seq is 1,000 to 100,000]

Figure 3 Run times of five dimensionality reduction methods for inputs of varying sizes. The average run time of three random subsamples is represented, with vertical bars representing s.d. after log-transforming the run times.

Becht et al., *Nature Biotechnology* 2018

Now, McInnes et al., UMAP Preserves Local and Global Structure

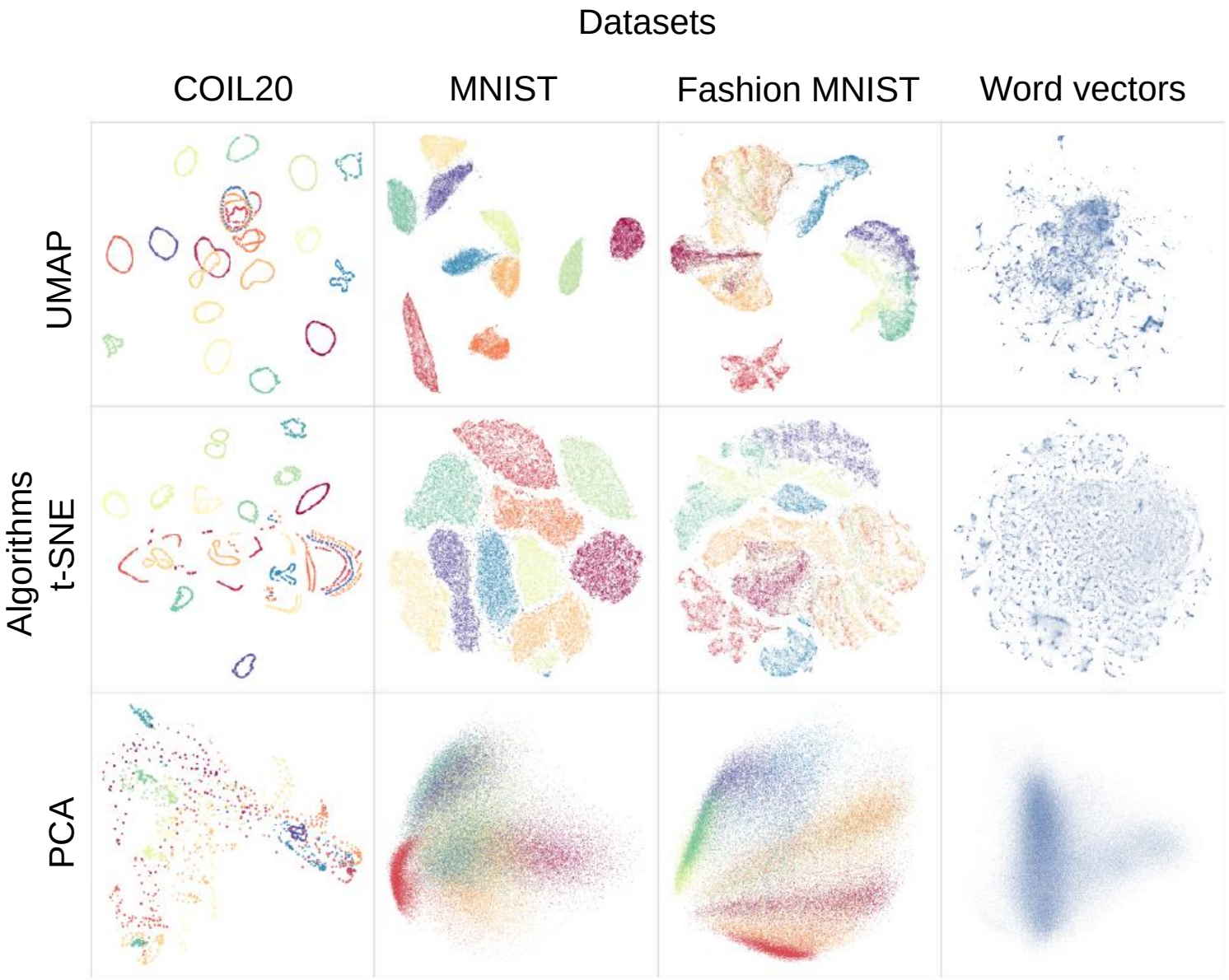


Figure 2: A comparison of several dimension reduction algorithms. We note that UMAP successfully reflects much of the large scale global structure that is well represented by Laplacian Eigenmaps and PCA (particularly for MNIST and Fashion-MNIST), while also preserving the local fine structure similar to t-SNE and LargeVis.

McInnes et al., *Rxiv* 2018

Becht et al., UMAP Preserves Local and Global Structure (Analysis of Tissue T Cells; Color = Expert Knowledge / Source)

(a) UMAP better split CD8 T cells, γδ T cells, and contaminating cells

(b) color-coding the tissues of origin; t-SNE separated cell populations according to their tissue of origin more often than UMAP. UMAP instead ordered events according to their origin within each major cluster, roughly from 1) cord blood and peripheral blood mononuclear cells, to liver and spleen, and to tonsils on the one end to skin, gut and lung on the other. **Continua not apparent in t-SNE.**

Dataset covering 35 samples originating from 8 distinct human tissues enriched for T and natural killer (NK) cells, of more than >300,000 cell events with 39 protein targets (the Wong dataset).

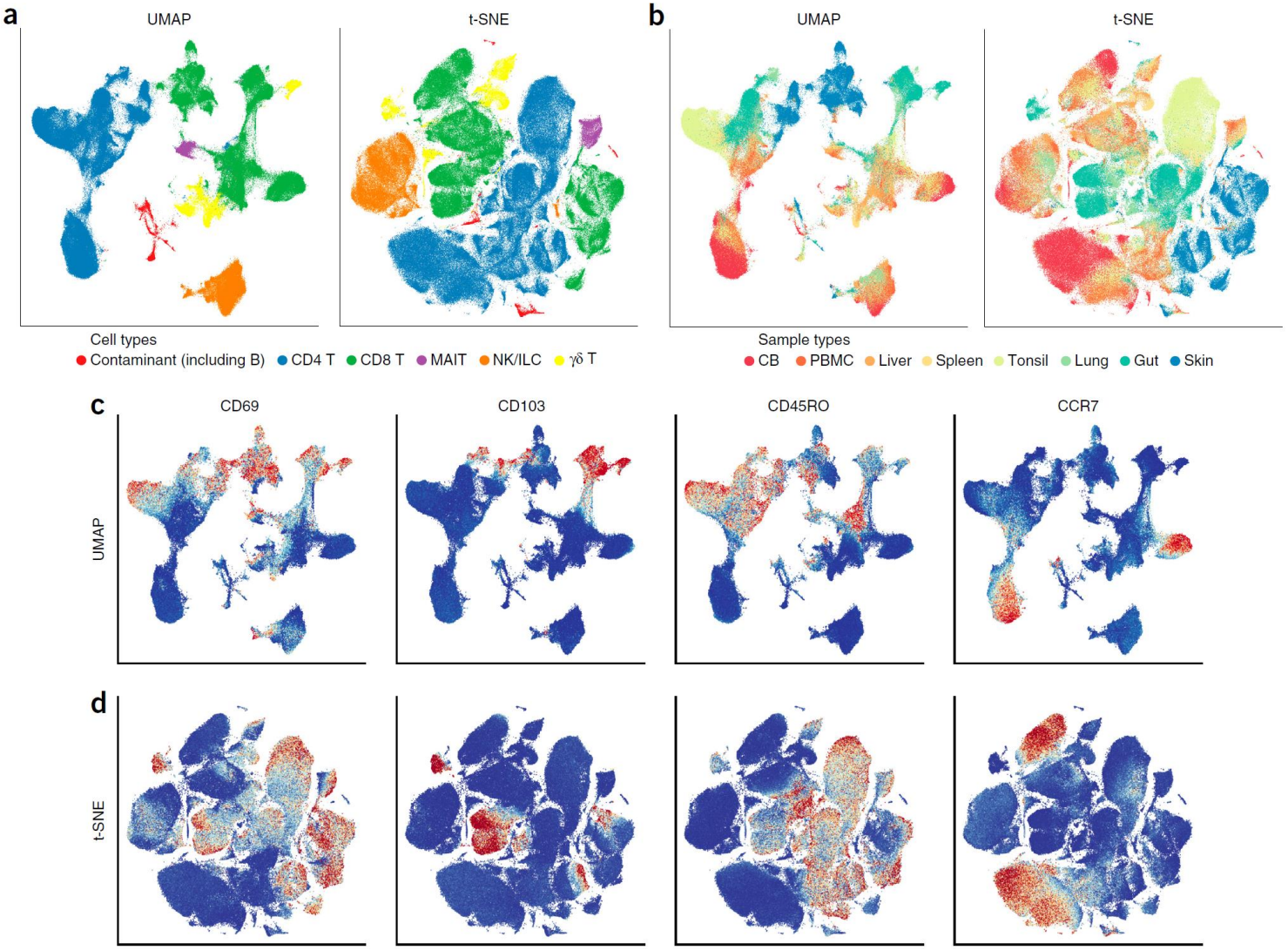


Figure 1 UMAP embeds local and large-scale structure of the data. UMAP and t-SNE projections of the Wong *et al.* dataset colored according to (**a**) broad cell lineages, (**b**) tissue of origin, and for (**c**) UMAP and (**d**) t-SNE, the expression of CD69, CD103, CD45RO and CCR7. For **c** and **d**, blue denotes minimal expression, beige intermediate and red high. MAIT, mucosal-associated invariant T cell; ILC, innate lymphoid cell; CB, cord blood; PBMC, peripheral blood mononuclear cell.

Becht et al., *Nature Biotechnology* 2018

Becht et al., UMAP Captures Developmental Trajectories

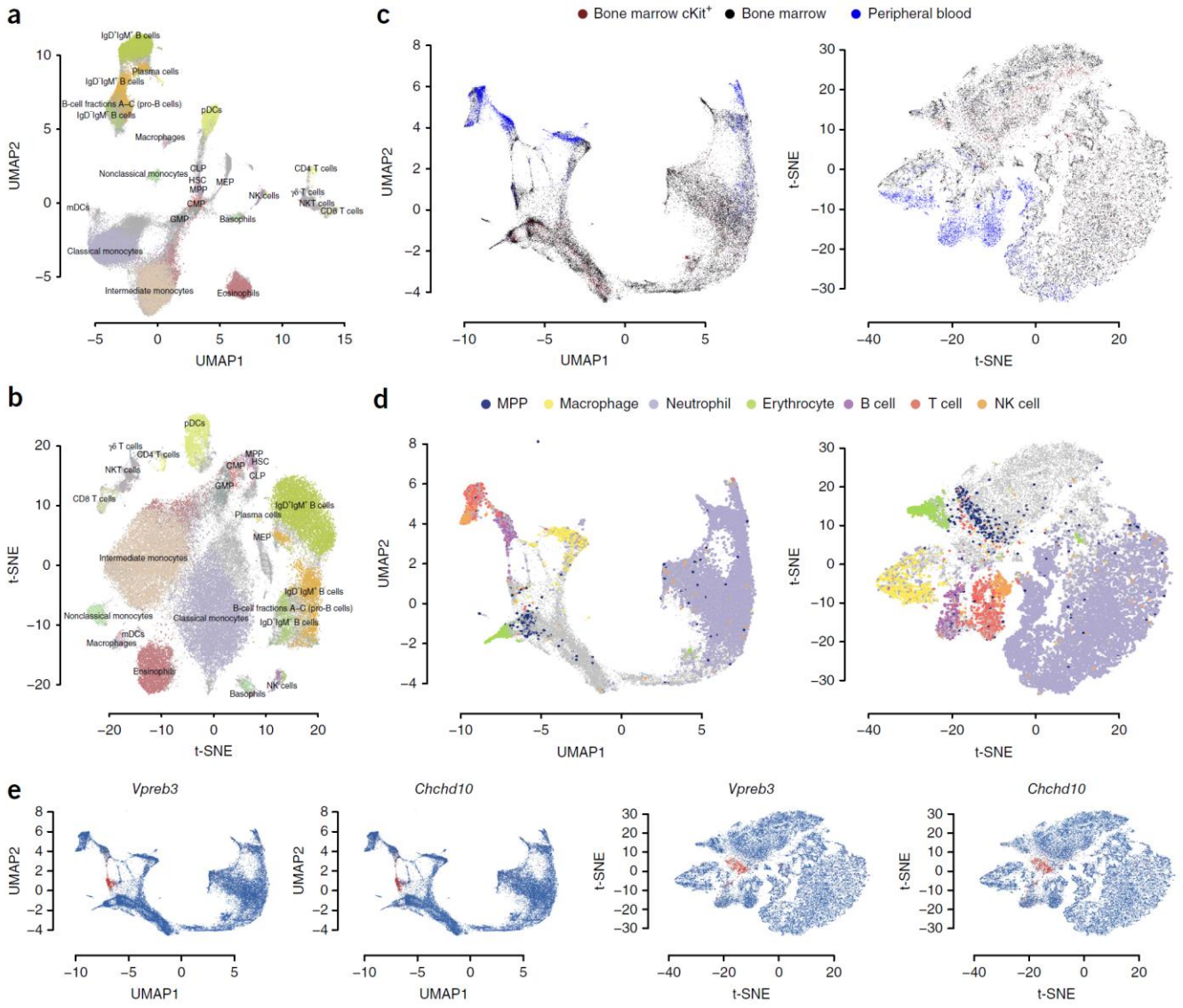


Figure 2 UMAP embeddings of bone marrow and blood samples recapitulate hematopoiesis. (**a**) UMAP and (**b**) t-SNE projection of the Samusik_01 dataset. Events are color-coded according to manual gates provided by the authors of the dataset. (**c**,**d**) UMAP and t-SNE projections of the Han dataset, color-coded by (**c**) tissue of origin or (**d**) cell populations. (**e**) Expression of the V-set pre-B cell surrogate light chain 3 (*Vpreb3*) and *Chchd10* genes on the UMAP and t-SNE projections of the Han dataset. Blue denotes minimal expression, beige intermediate and red high. pDC, plasmacytoid dendritic cell; mDC, myeloid dendritic cell; NKT, natural killer T.

Becht et al., *Nature Biotechnology* 2018

Speed is Nothing without Accuracy; UMAP is Also Accurate

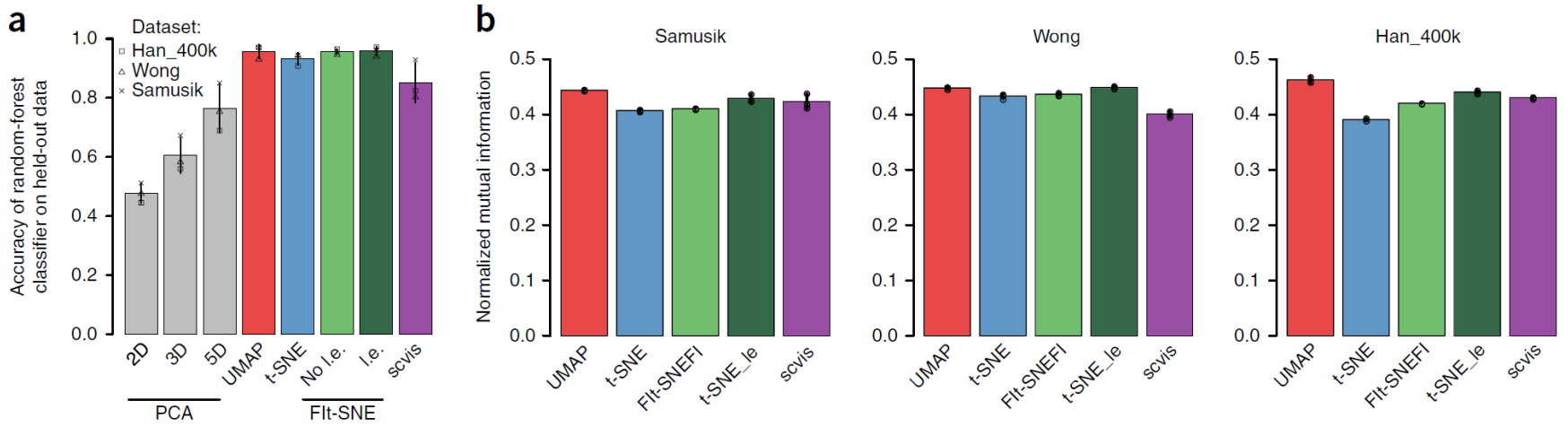
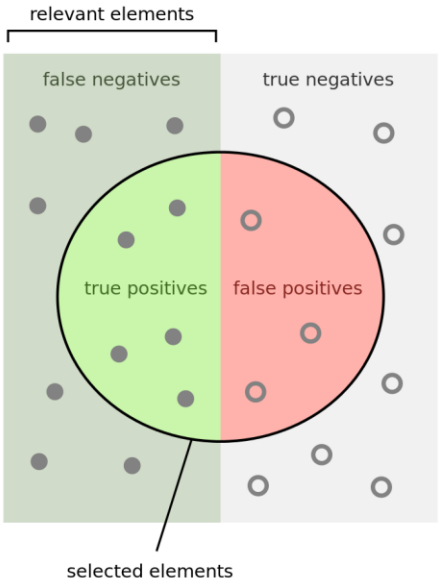


Figure 4 Analysis of local data structure in embeddings produced by each algorithm. (**a**) Accurate classification rate on held-out data of random-forest classifiers predicting Phenograph cluster labels using embedded coordinates as input. The average across the three datasets is shown, with vertical bars representing s.d. (**b**) Average normalized mutual information of *k*-means clustering ($k = 100$) performed on the embeddings of data subsamples and *k*-means clustering ($k = 100$) performed on total datasets. The average across the three random subsamples of size 200,000 is shown, with vertical bars representing s.d.



How many selected items are relevant?

$$\text{Precision} = \frac{\text{true positives}}{\text{true positives} + \text{false positives}}$$

How many relevant items are selected?

$$\text{Recall} = \frac{\text{true positives}}{\text{true positives} + \text{false negatives}}$$

F_1 score = accuracy

$$2 \cdot \frac{\text{precision} \cdot \text{recall}}{\text{precision} + \text{recall}}$$

Becht et al., *Nature Biotechnology* 2018

UMAP Preserves "Large-Scale Structure" That t-SNE Ignores (Large = Position of Islands; Fine = Position of Cells in an Island)

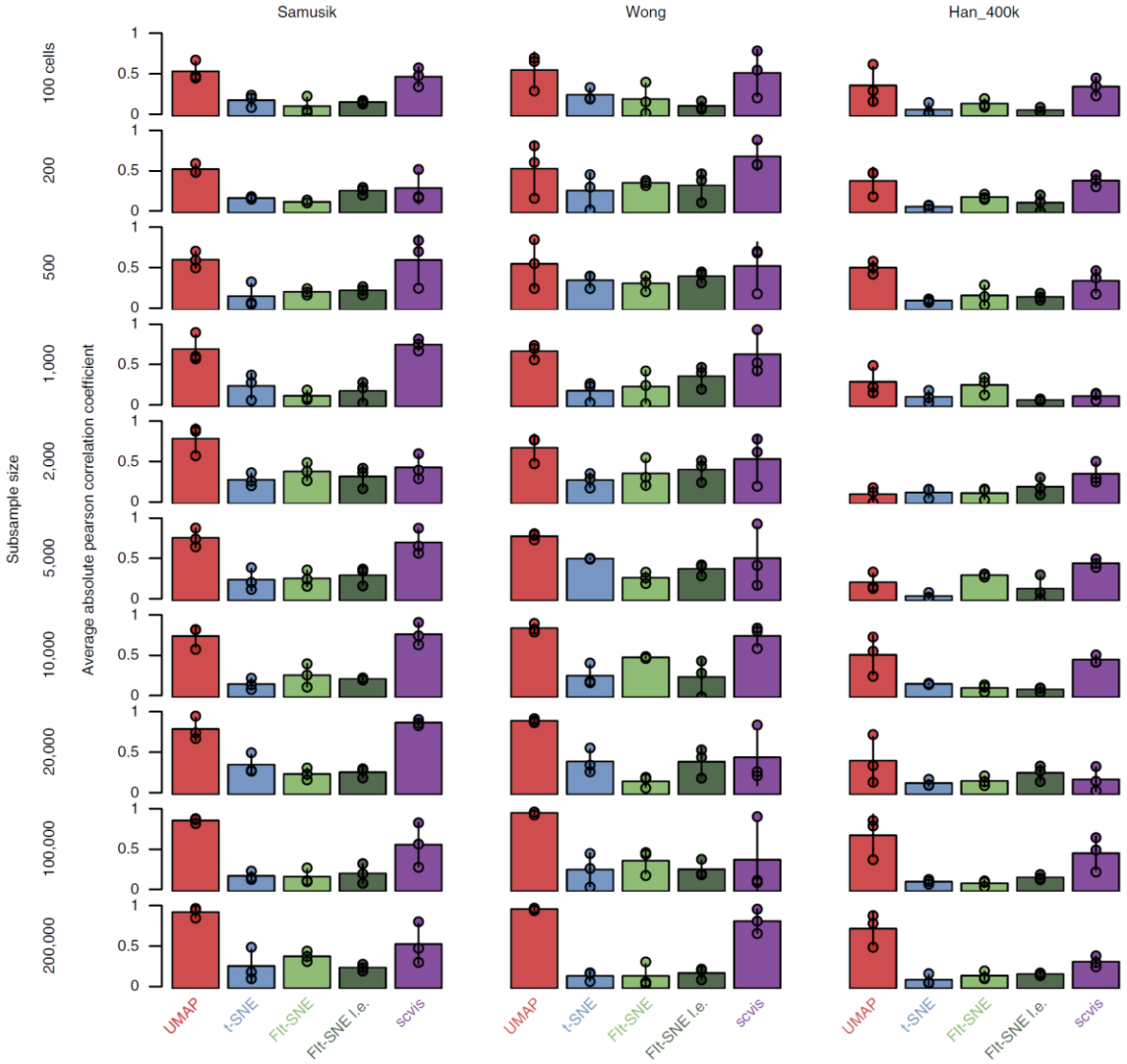
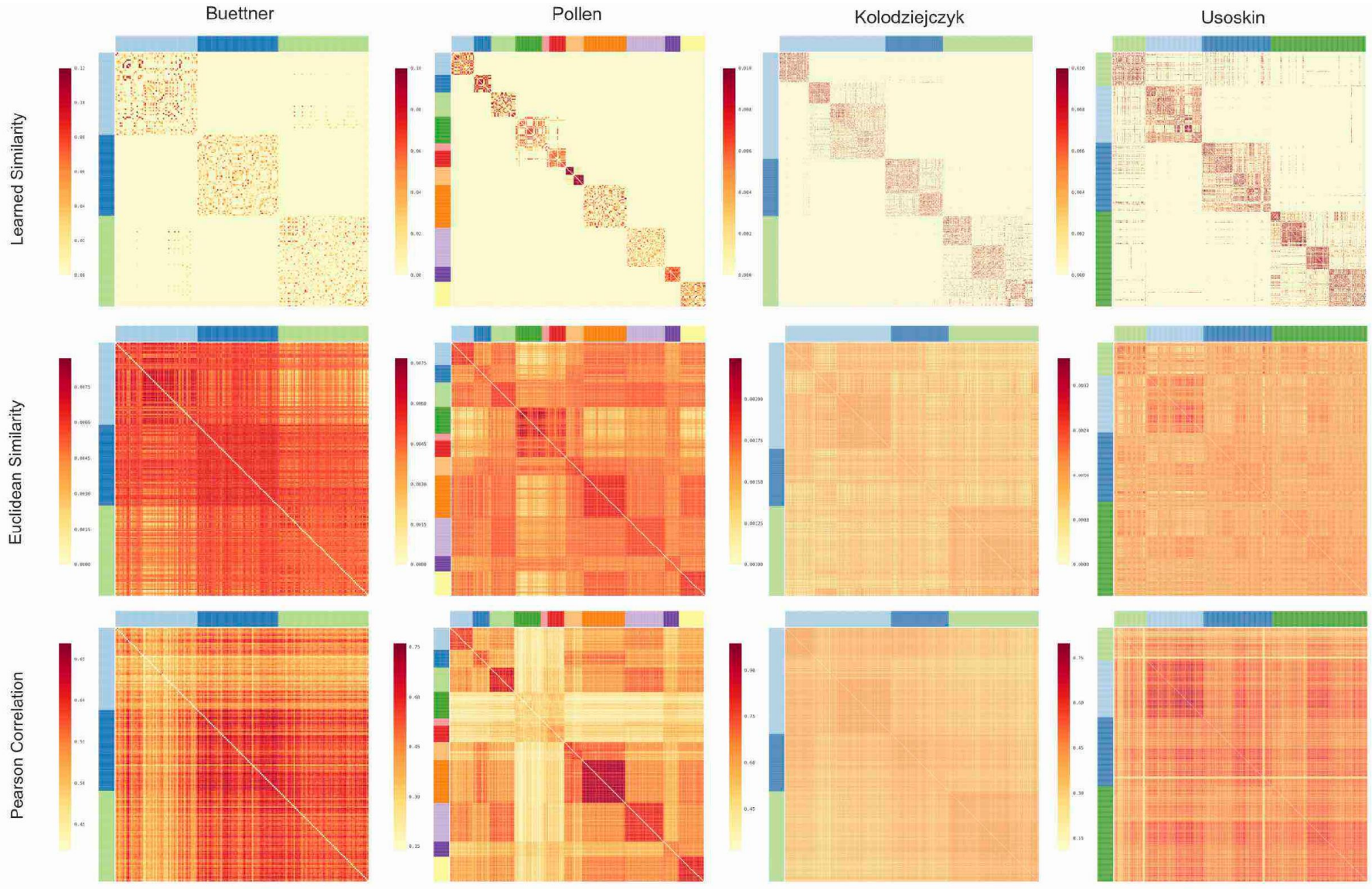


Figure 6 Reproducibility of large-scale structures in embeddings. Bar plots represent the average unsigned Pearson correlation coefficient of the points' coordinates in the embedding of subsamples versus in the embedding of the full dataset, thus measuring the correlation of coordinates in subsamples versus in the embedding of the full dataset, up to symmetries along the graph axes. Bar heights represent the average across three replicates and vertical bars the corresponding s.d.

Becht et al., *Nature Biotechnology* 2018

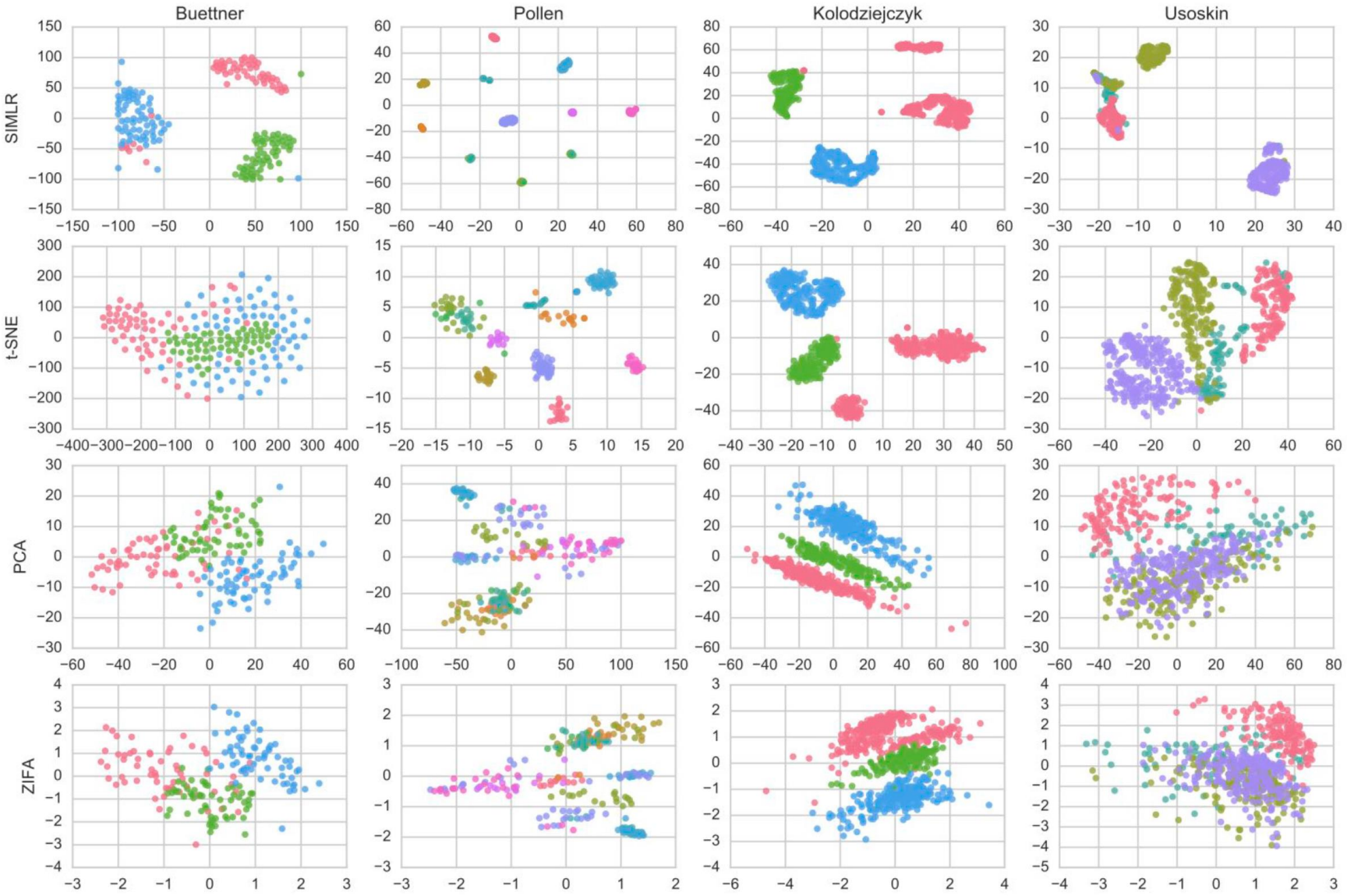
SIMLR

Visualization and analysis of single-cell RNA-seq data by kernel-based similarity learning (SIMLR)



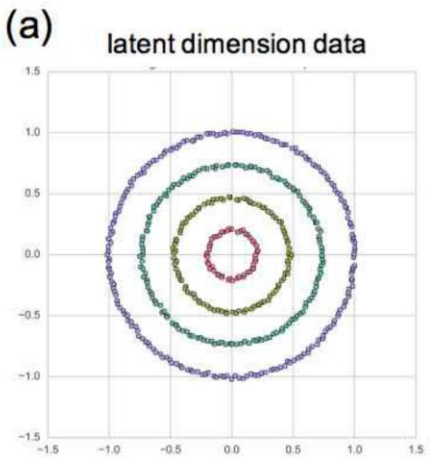
Wang et al., *Nature Methods* 2017

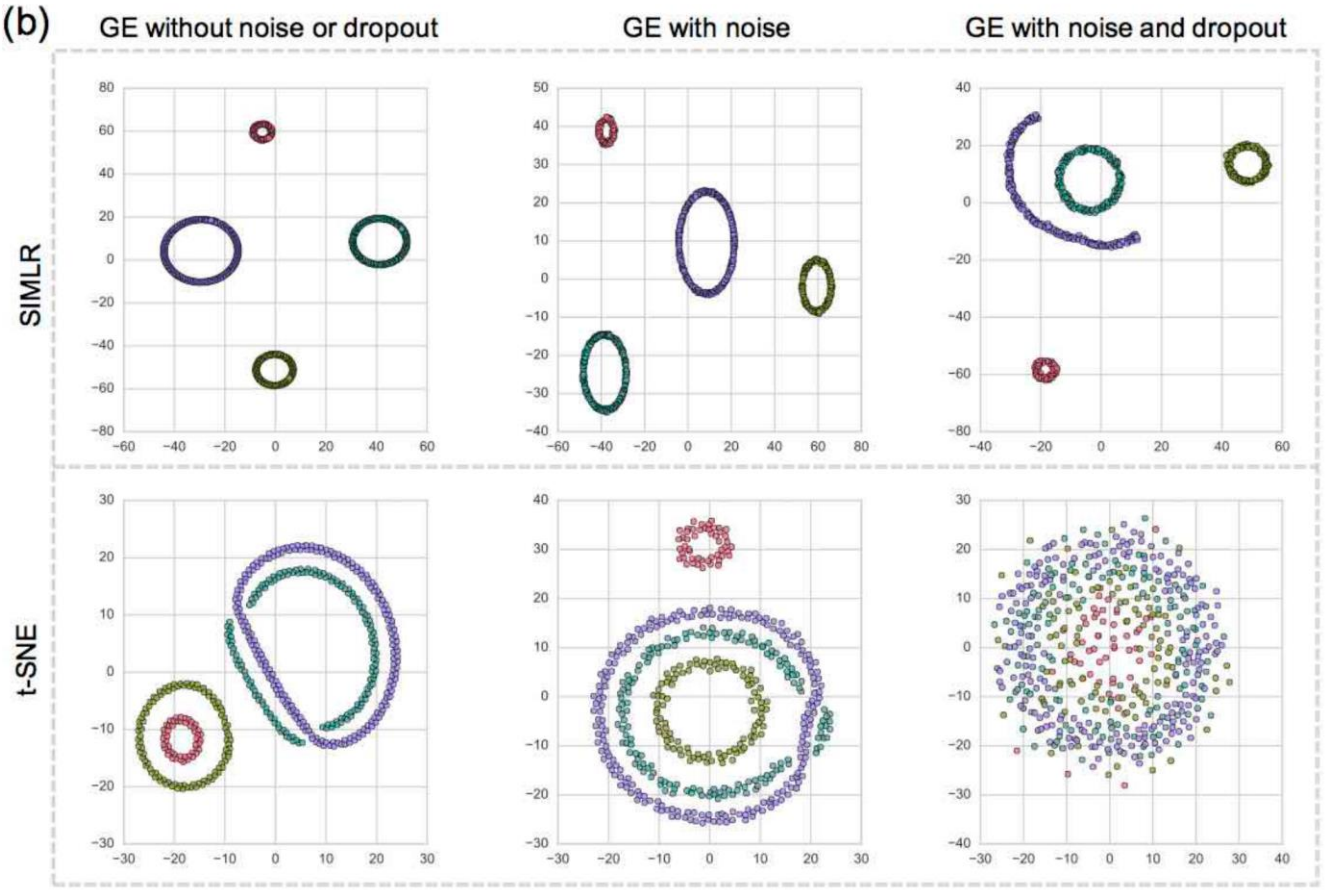
SIMLR vs. t-SNE vs. PCA on Four scRNA-seq Datasets



Wang et al., *Nature Methods* 2017

Analysis of “Toy” Gene Expression Data +/- Noise and Data Dropout



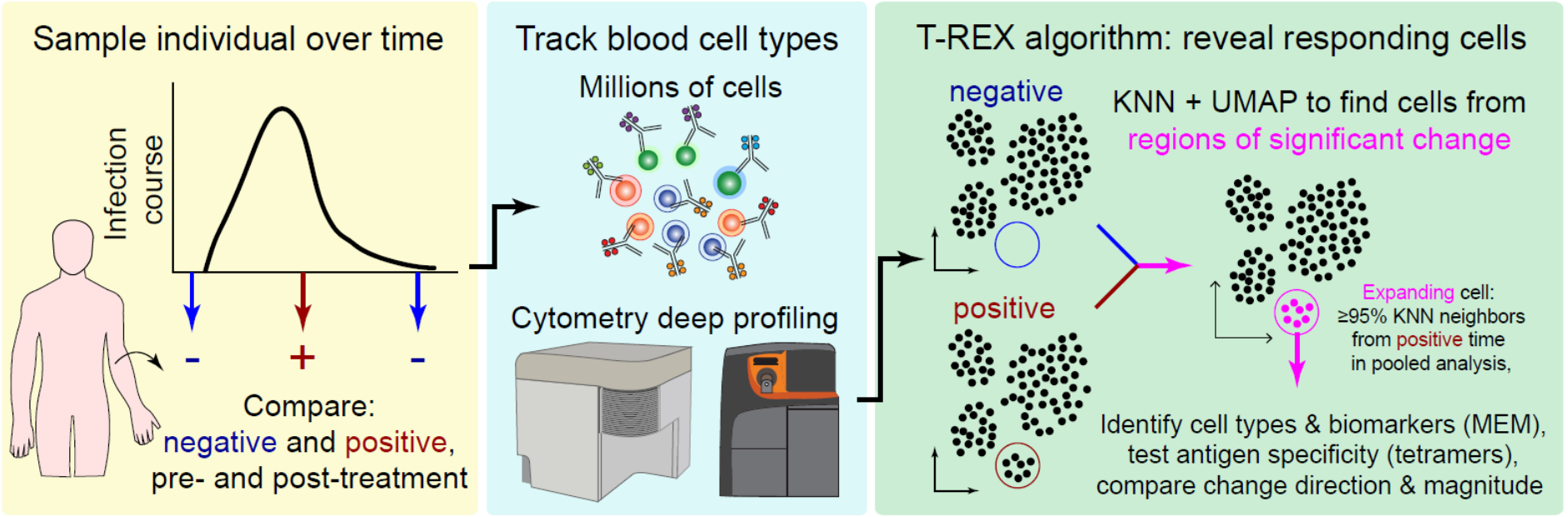


Wang et al., *Nature Methods* 2017

Part 8: T-REX!

Pinpointing Ultra-Rare Cells (e.g., virus-specific T cells)

T-REX: Machine Learning Reveals Virus-Specific Cells in Longitudinal Immune Monitoring Cytometry

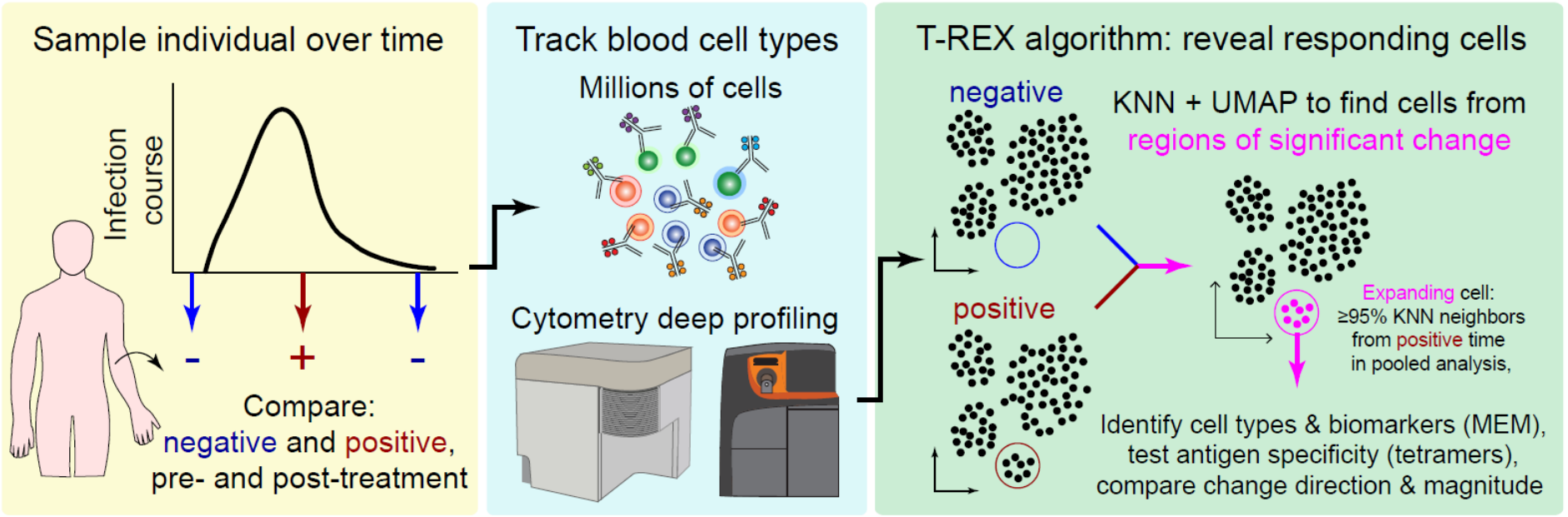


New algorithm: T-REX (Tracking Responders EXpanding)

Code: https://github.com/cytolab/t-rex
Manuscript: https://elifesciences.org/articles/64653

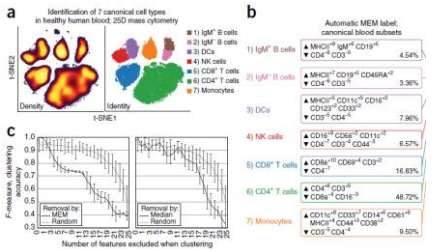
T-REX: Barone, Paul, Muehling et al., *eLife* 2021 | SARS-CoV-2 vaccine response: Kramer, Wilfong, Voss et al., *bioRxiv* 2021
No disclosures / conflicts, will show immune cell data from individuals receiving BNT162b2 SARS-CoV-2 vaccine

Key Ideas & Findings in Today's Talk



- Idea 1: T-REX automatically reveals virus-specific T cells in rhinovirus & SARS-CoV-2 vaccine response (without the need for tetramers, sorting, or sequencing)
- Idea 2: Approach focuses on extreme change & can summarize disease, therapy, or perturbation response (direction & magnitude of change; rhinovirus, COVID-19, cancer therapy, compound screening)
- Finding: Mass cytometry + T-REX characterized SARS-CoV-2 vaccine-induced memory CD4 and CD8 T cells (phenotype: CD38++ ICOS++ CD45R0+ PD-1+ Ki-67+ CXCR5-)
- Finding : This phenotype of SARS-CoV-2 vaccine responding T cells closely matched rhinovirus-specific T cells

T-REX Builds on Cytometry Data Science Tools



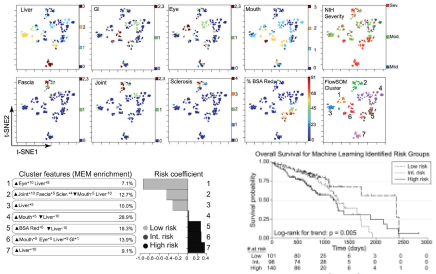
Nature Methods 2017

MEM

Diggins et al., PMC5330853

- MEM: machine labeling & identification of cell type clusters
- Enabled comparison across single cell platforms
- E.g., memory CD4+ T cells, 10-point enrichment scale:

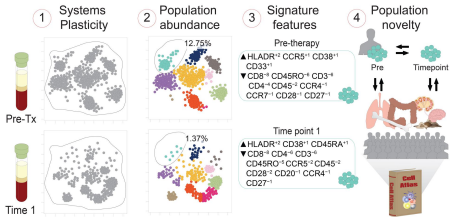
  $ICOS^{+8}$ $CD38^{+8}$ $CD4^{+7}$ $CD45R0^{+6}$ $CD3^{+5}$ $Ki\text{-}67^{+4}$



Hematologica 2018

Gandelman et al., PMC6312024

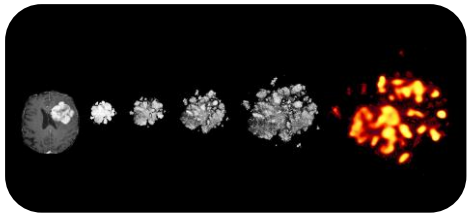
- Updated Diggins et al., 2015, precursor for RAPID
- Risk stratified cGVHD patients based on 8 organ scores
- Workflow: t-SNE => FlowSOM on embedding => MEM



Cancer Immunology Research 2019

Greenplate et al., PMC6318034

- Set of tools for longitudinal single cell tumor immunology.
- Revealed abnormal immune cells in multiple tumor types.
- Includes datasets (AML, melanoma) used by T-REX
- See Greenplate et al.'s outstanding COVID-19 work (*Science* 2020)



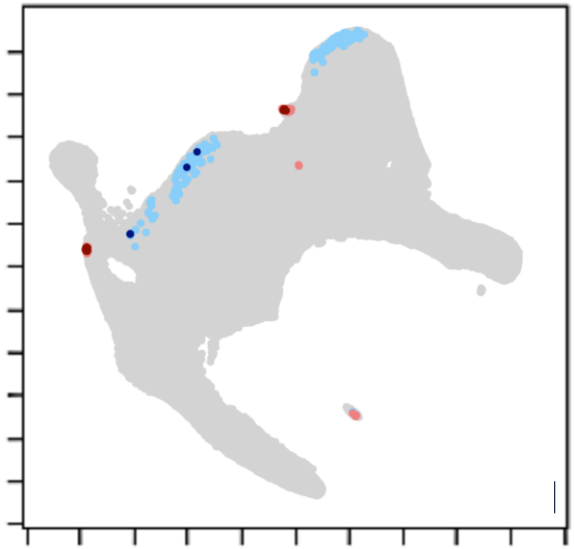
eLife 2020

RAPID

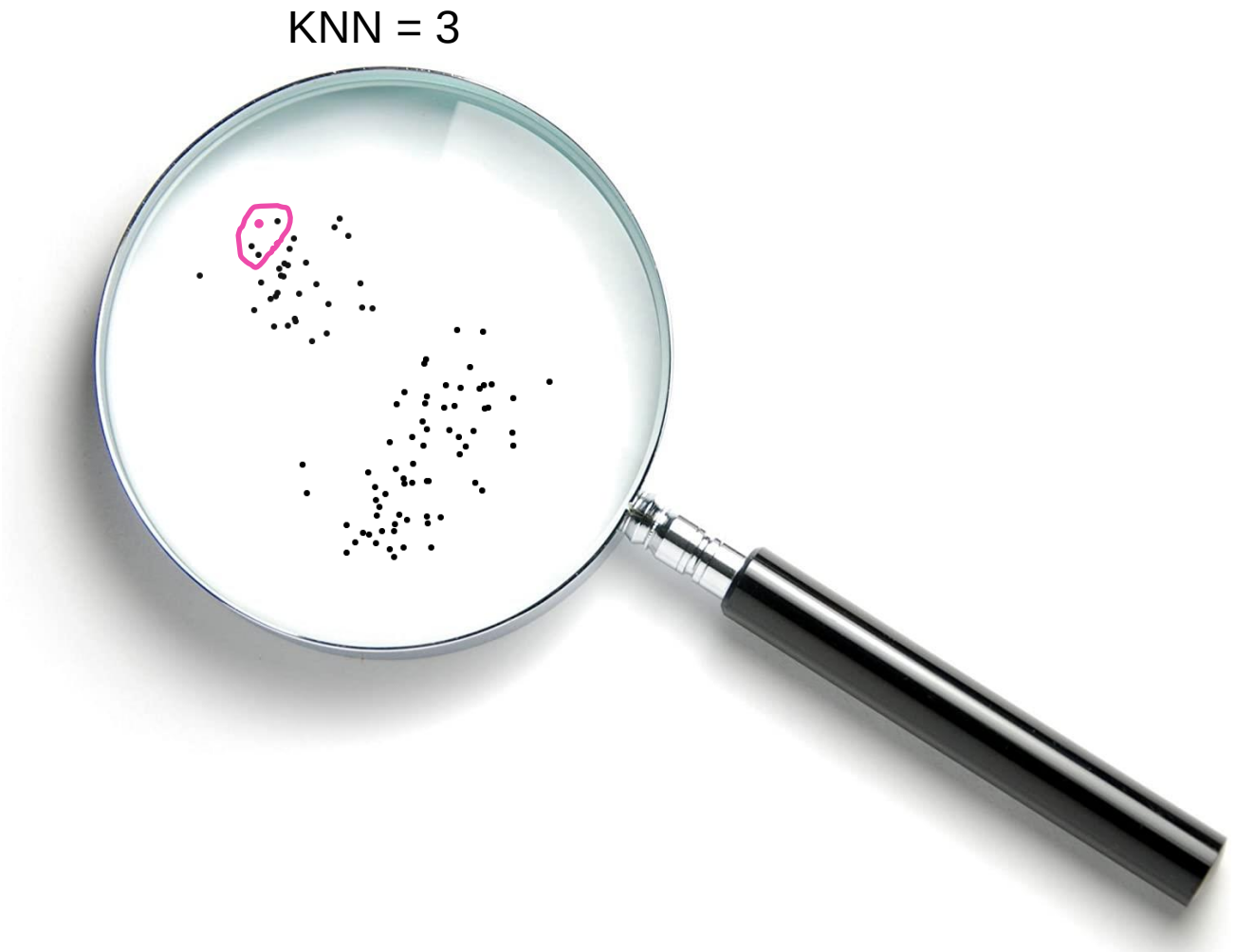
Leelatian and Sinnaeve et al., PMC7340505

- RAPID: maps probabilistic clinical outcomes on t-SNE & UMAP
- Reveals associations with extreme clinical outcomes
- Revealed JAK + AKT cooperation in glioblastoma cells

T-REX Draws on Sconify's K-Nearest Neighbors (KNN) Approach To Make Each Cell A Phenotypic Neighborhood



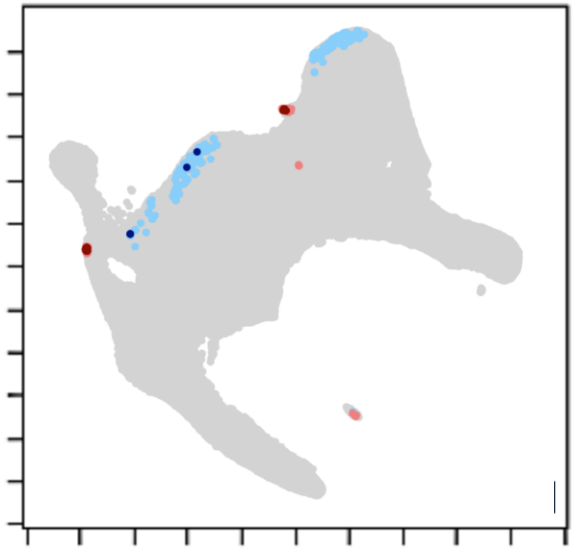
T-REX will use k = 60* for cell neighborhoods



(*shout out to ISAC's CYTO meeting, where questions & poster discussions in 2019 helped inspire T-REX)

Sconify: Tyler Burns, Garry Nolan, and Nikolay Samusik, bioRxiv 2018; R/bioconductor 2020

T-REX Draws on Sconify's K-Nearest Neighbors (KNN) Approach To Make Each Cell A Phenotypic Neighborhood



T-REX will use k = 60* for cell neighborhoods

KNN = 3

(*shout out to ISAC's CYTO meeting, where questions & poster discussions in 2019 helped inspire T-REX)

Sconify: Tyler Burns, Garry Nolan, and Nikolay Samusik, bioRxiv 2018; R/bioconductor 2020

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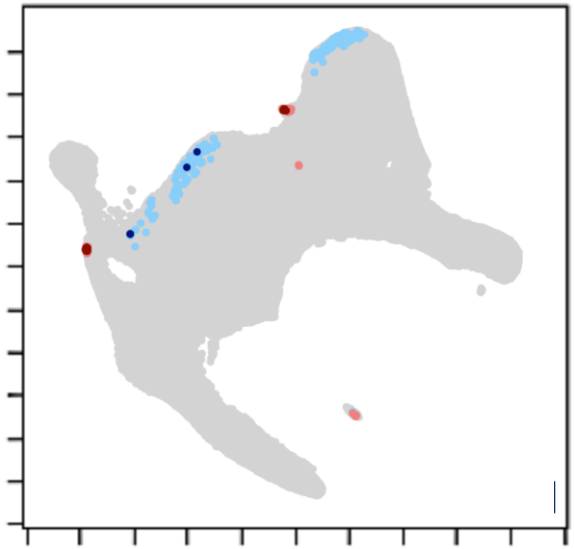
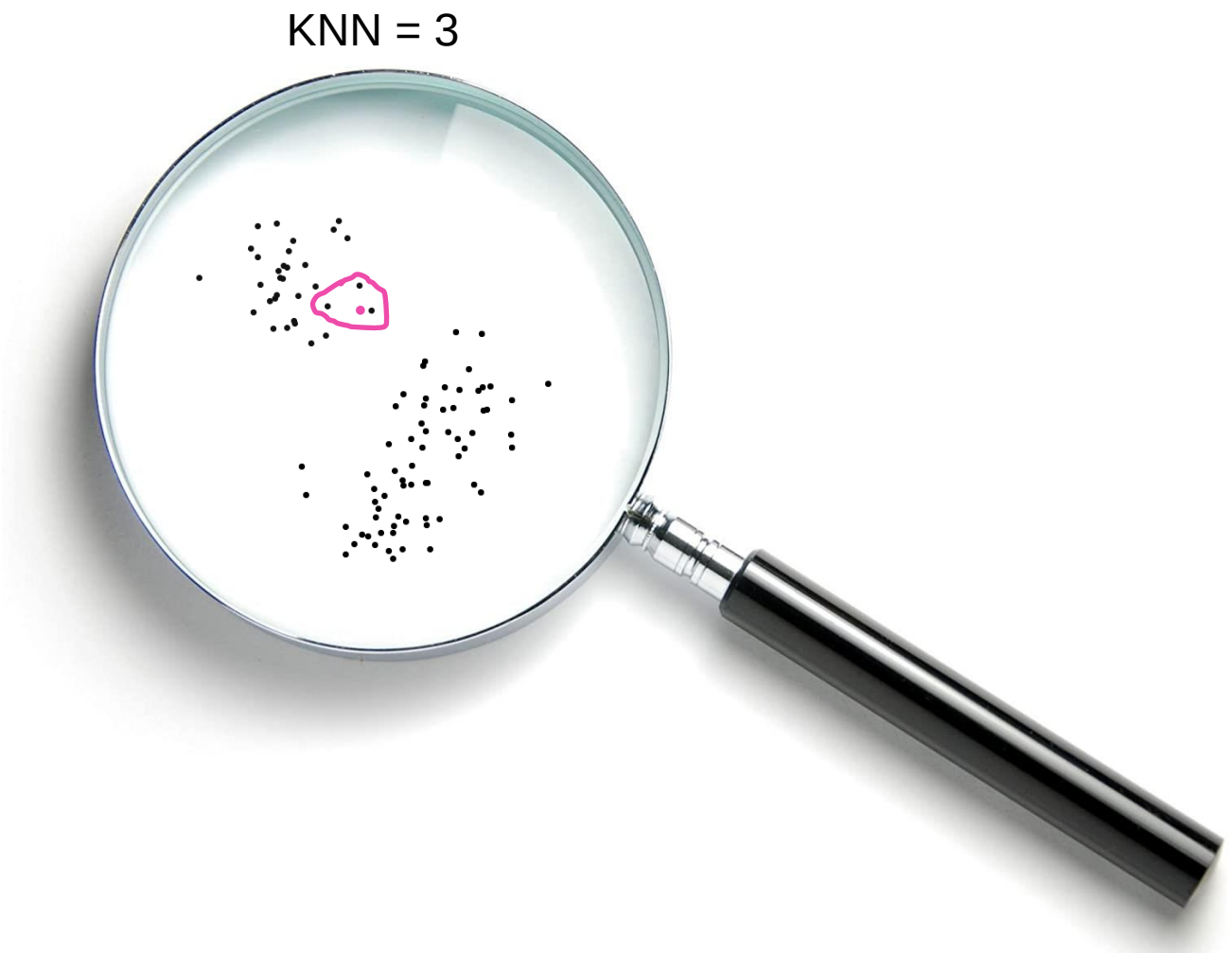


T-REX will use k = 60* for cell neighborhoods

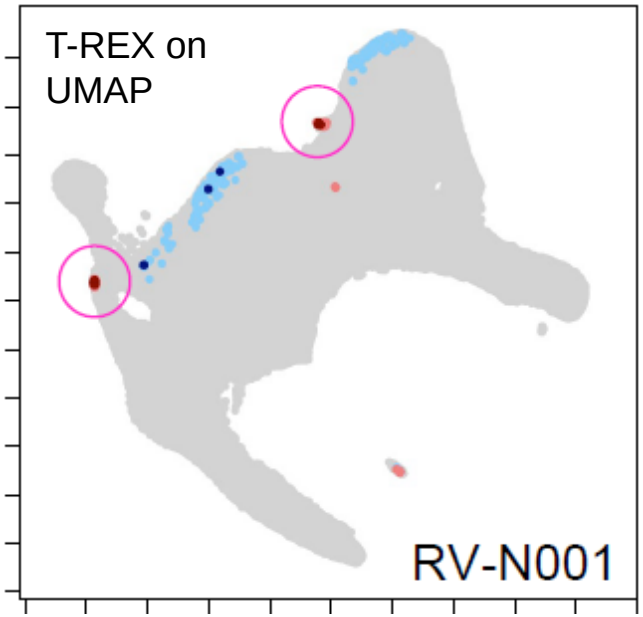


(*shout out to ISAC's CYTO meeting, where questions & poster discussions in 2019 helped inspire T-REX)

Sconify: Tyler Burns, Garry Nolan, and Nikolay Samusik, bioRxiv 2018; R/bioconductor 2020

T-REX: Tracking Responders EXpanding, Every Cell Is Characterized in a Search for Hotspots of Change

Live CD4+ T cells



>95% from d0
>75% from d0
>95% from d7
>75% from d7
>5% of neighbors tetramer+

CD4 T cells, Day 0 vs. Day 7,
individual infected with rhinovirus (RV-N001)
no cell enrichment, Aurora data, ~3 x 10^6 cells

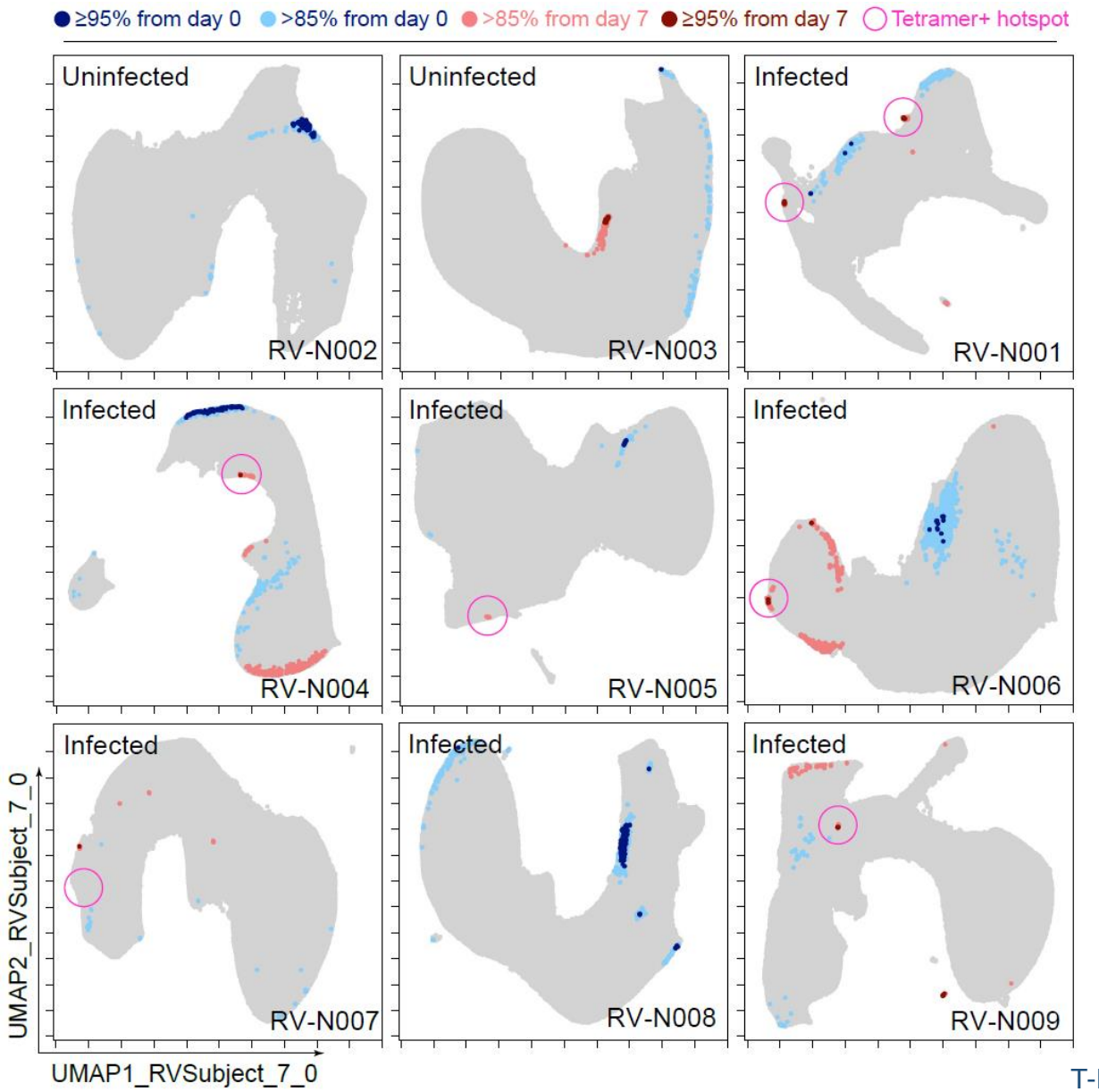
Color: cells in that phenotypic neighborhood
are mostly from one sample

Dark red = cells mostly from day 7 (expanding)

MHCII tetramers marking rhinovirus specific CD4 T cells
were not used to make the UMAP, instead used to show:
Change hotspots were enriched for virus-specific T cells

T-REX: Barone, Paul, Muehling et al., *eLife* 2021

In Analysis of a Rhinovirus Challenge Cohort, T-REX Revealed Virus-Specific Cell Phenotypes



CD4 T cells, Day 0 vs. Day 7,
individuals infected with rhinovirus
no cell enrichment, Cytek Aurora data

In 5 of 7 infected individuals, expansion hotspots were enriched for virus-specific cells

The phenotype of rhinovirus-specific memory CD4+ T cells included:
CCR5+ ICOS+ CD38+ PD-1+ CXCR5-

Gating on MEM enriched proteins =>
identified tetramer+ cells
(without gating on tetramers),
suggesting: FACS sort based on MEM label

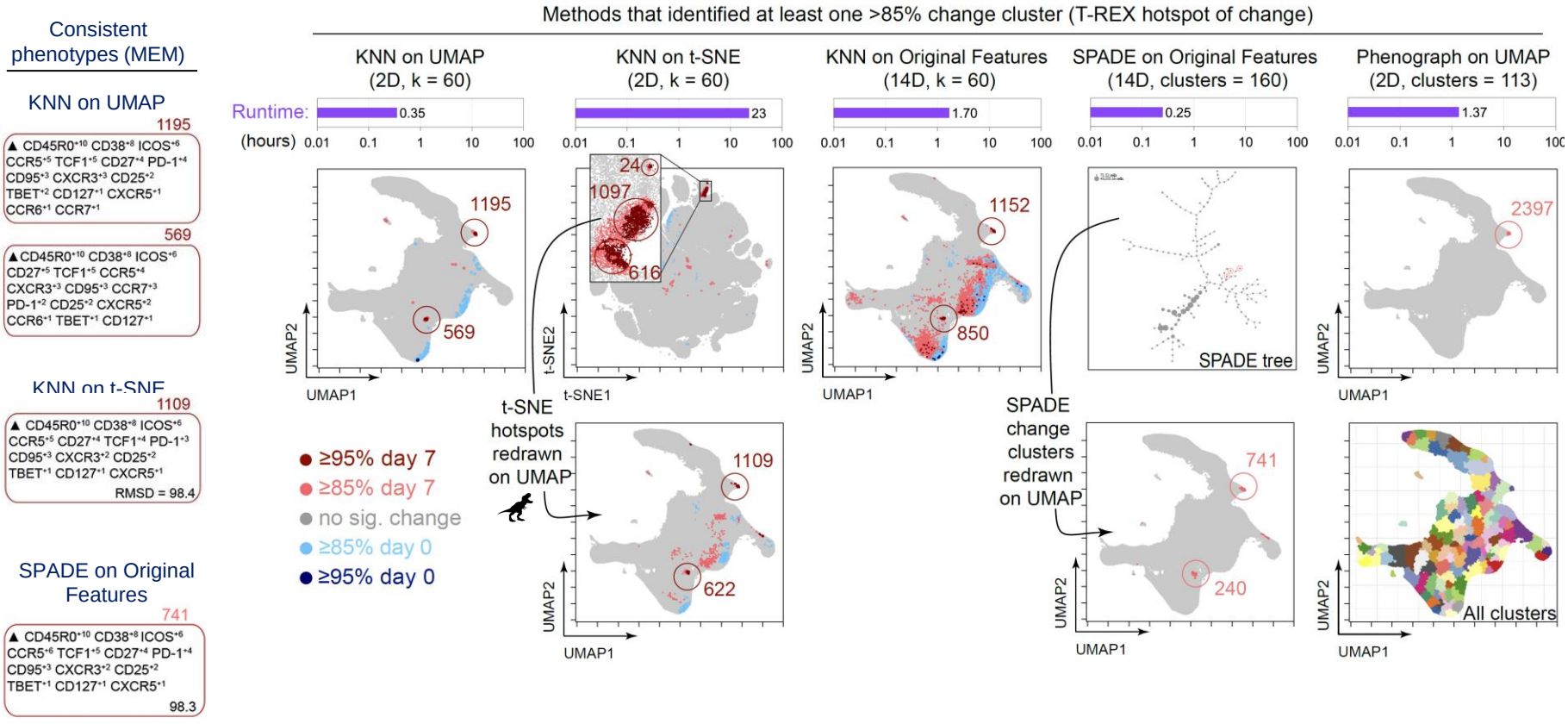
T-REX: Barone, Paul, Muehling et al., *eLife* 2021

T-REX revealed virus-specific T cells without tetramers

Would this approach work with other clustering algorithms?
Is it ‘OK’ to do KNN on UMAP axes as parameters?
(Perhaps: all embeddings are wrong, but some are useful…)

T-REX: Barone, Paul, Muehling et al., *eLife* 2021

T-REX Worked with Other Algorithms to Identify Comparable Cells, But KNN on UMAP or t-SNE Outperformed KNN on Original Features



T-REX: Barone, Paul, Muehling et al., *eLife* 2021

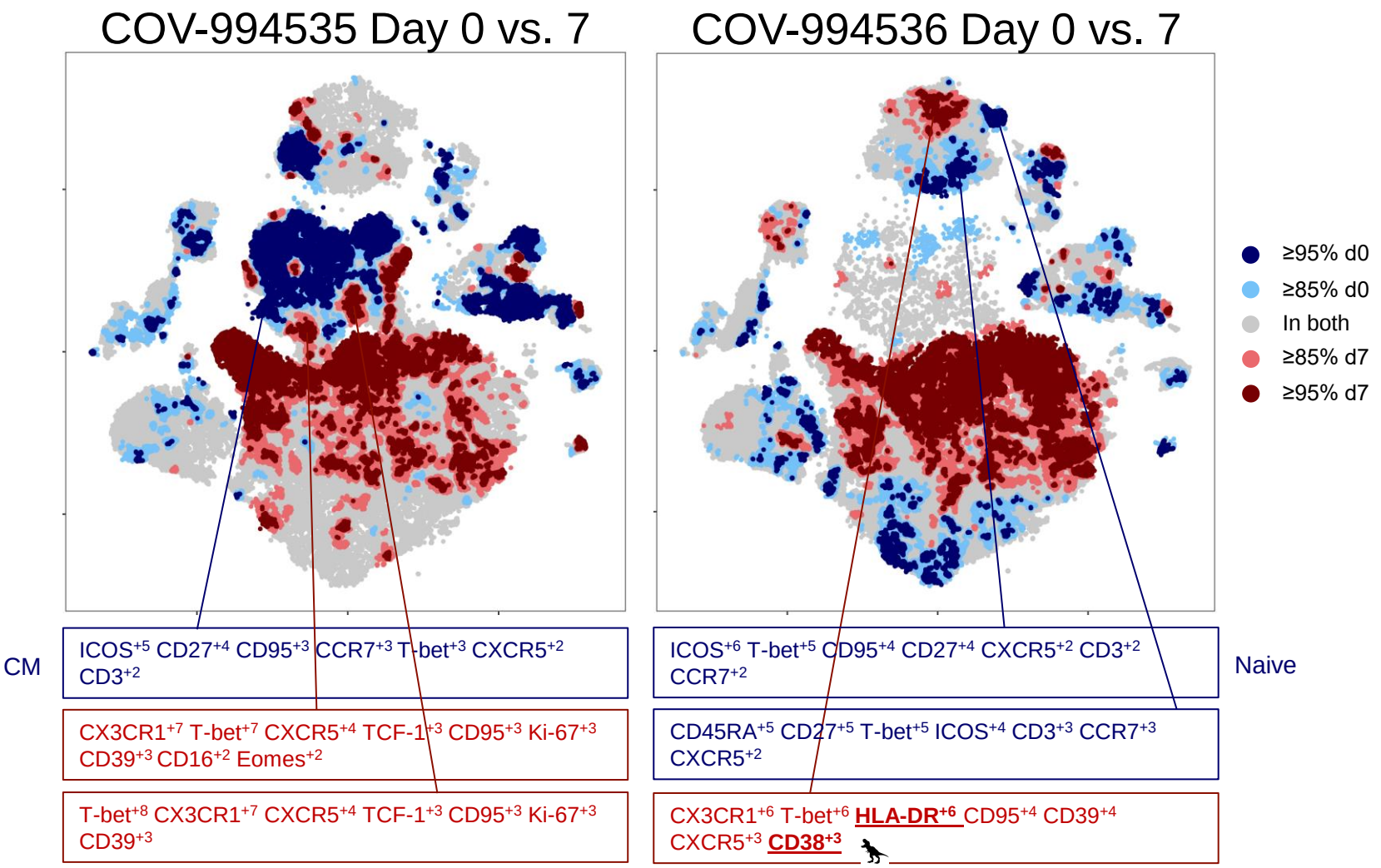
T-REX revealed virus-specific T cells without tetramers

Also found to work for:

- a range of k-values (k = 60 was optimal)
- post-infection as the comparison point to day 7
- data from a range of cytometers, studies, and labs
- COVID-19, melanoma immunotherapy response, AML

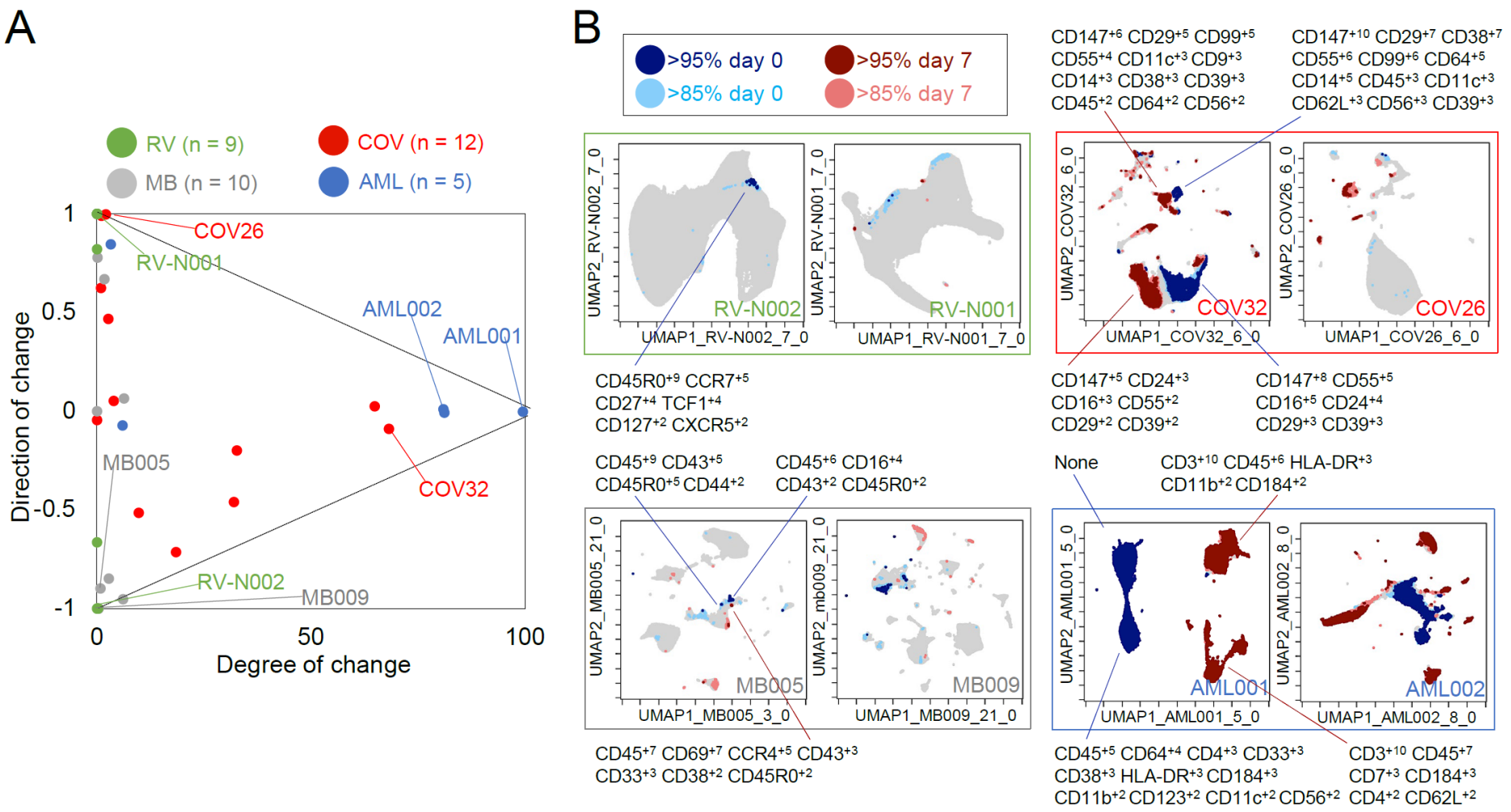
(see the manuscript for this & more!)

Massive Immune Change, Common Shifts in Expanding Cell Subsets Observed Between Day 0 and Day 7 in COVID-19



T-REX (*eLife* 2020) analysis of data from Mathew, Giles, Baxter, Oldridge, Greenplate, Wu, Alanio et al., *Science* 2020 (Wherry, Symphony)

Half of COVID-19 Patients Displayed Immune Changes Comparable to AML Patients with a Complete Response to Chemotherapy



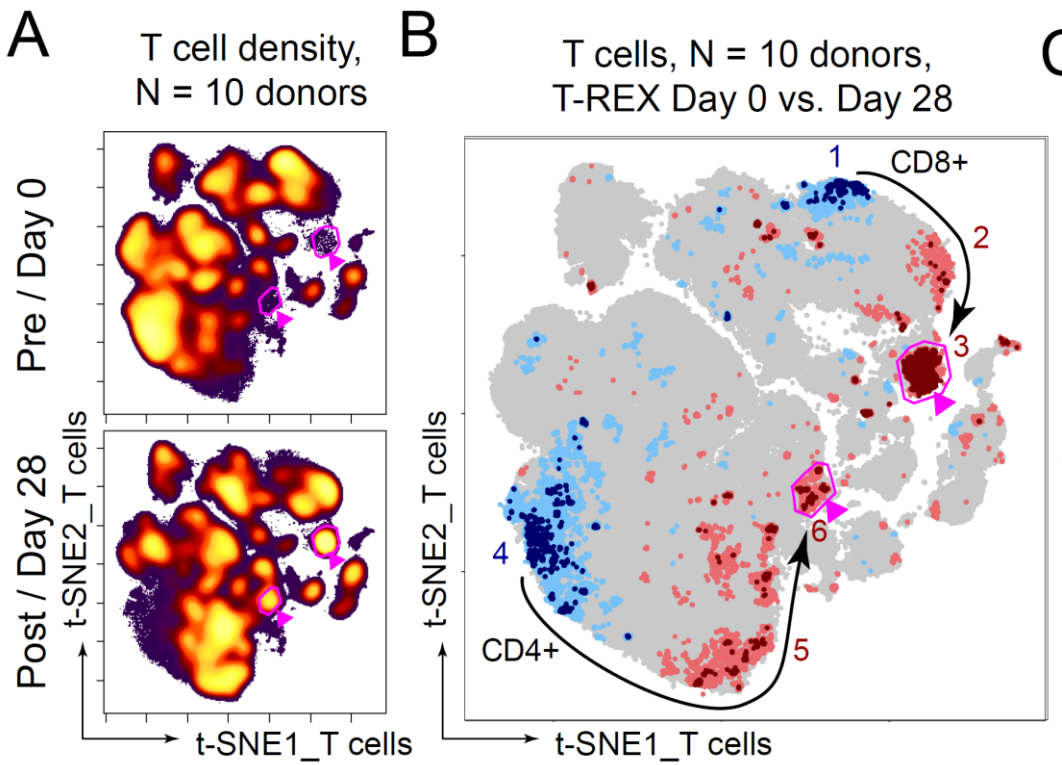
Barone et al., *eLife* 2021, T-REX analysis of data including COVID-19 from Rodriguez, Pekkarinen, Lakshmikanth, Tan, Consiglio et al., *Cell Reports Medicine* 2020 (Brodin, CyTOF)

T-REX revealed virus-specific T cells without tetramers
& characterized massive immune changes in COVID-19

Would it also work to characterize SARS-CoV-2 vaccine response?

T-REX: Barone, Paul, Muehling et al., *eLife* 2021

T-REX Reveals Memory CD4 & CD8 T Cell Phenotypes Expanding following BNT162b2 SARS-CoV-2 RNA Vaccine

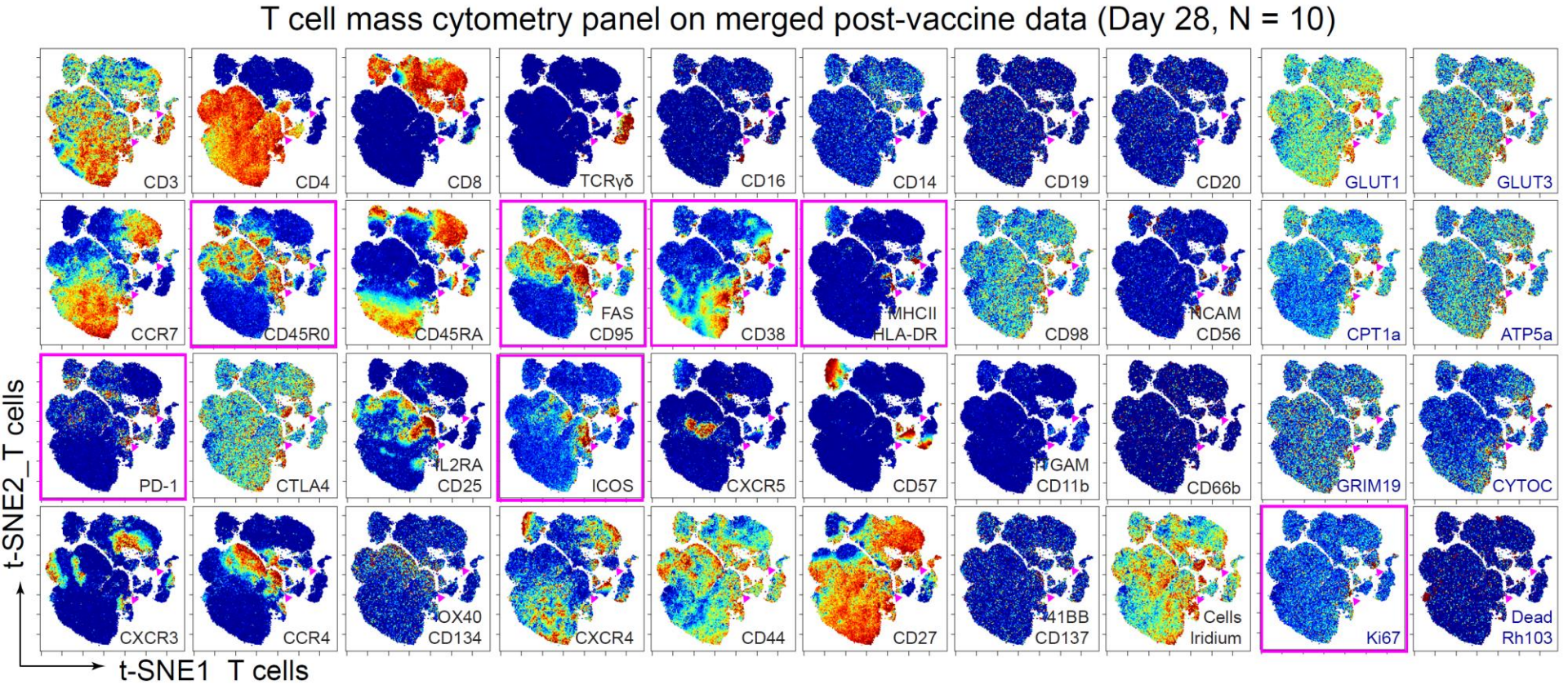


C

1	▲ CD45RA+9 CD8+6 CD27+6 CCR7+6 CD44+6 CD45+5 CPT1a+5 GLUT3+5 CD98+5 CTLA4+5 GLUT1+5 CD127+4 ATP5a+4 CXCR3+4 Ki-67+4 CD38+4 CXCR4+4	$CD38^{+}$ $ICOS^{-}$	Naive CD8+ T cells
2	▲ CD45RA+10 CD8+8 CD27+8 CCR7+8 CD44+8 CD45+7 CD38+7 GLUT1+6 CD3+5 CPT1a+5 CD127+5 CTLA4+5 CXCR4+5 ATP5a+4 ICOS+4 GLUT3+4 CD98+4	$CD38^{++}$ $ICOS^{+}$	
3	▲ CD44+8 CD38+8 CD8+7 ICOS+7 CD45+6 CD45R0+6 CD27+6 PD-1+6 CPT1a+5 CXCR3+5 GLUT1+5 CD95+5 HLA-DR+5 CD3+4 CD98+4 CTLA4+4 Ki-67+4 CD45RA+4	$CD38^{++}$ $ICOS^{++}$ $Ki67^{+}$ Memory CD8 T cells	
4	▲ CD45RA+8 CD44+7 CD45+6 CD4+6 CD27+6 CCR7+6 GLUT1+5 CD38+5 CPT1a+4 CD127+4 CTLA4+4 CXCR4+4	$CD38^{+}$ $ICOS^{-}$	Naive CD4+ T cells
5	▲ CD45RA+9 CCR7+8 CD44+8 CD38+8 CD45+7 CD4+7 CD27+7 CD3+6 CD127+5 ICOS+5 CTLA4+5 GLUT1+5 CXCR4+5 CPT1a+4 ATP5a+4 CD98+4 CD95+4	$CD38^{++}$ $ICOS^{+}$	
6	▲ ICOS+8 CD44+8 CD38+8 CD45+7 CD4+7 CD45R0+7 CD27+6 CD95+6 PD-1+6 CD3+5 CPT1a+5 CXCR3+5 CTLA4+5 GLUT1+5 CD127+4 ATP5a+4 GLUT3+4 CD98+4 Ki-67+4	$CD38^{++}$ $ICOS^{++}$ $Ki67^{+}$ Memory CD4 T cells	

Vaccine response: Kramer, Wilfong, Voss et al., *bioRxiv* 2021
T-REX: Barone, Paul, Muehling et al., *eLife* 2021

Mass Cytometry Phenotyping of ICOS+ CD38+ PD-1+ Ki-67+ CXCR5- Memory CD4 & CD8 T Cells following SARS-CoV-2 Vaccination



Vaccine response: Kramer, Wilfong, Voss et al., *bioRxiv* 2021
T-REX: Barone, Paul, Muehling et al., *eLife* 2021

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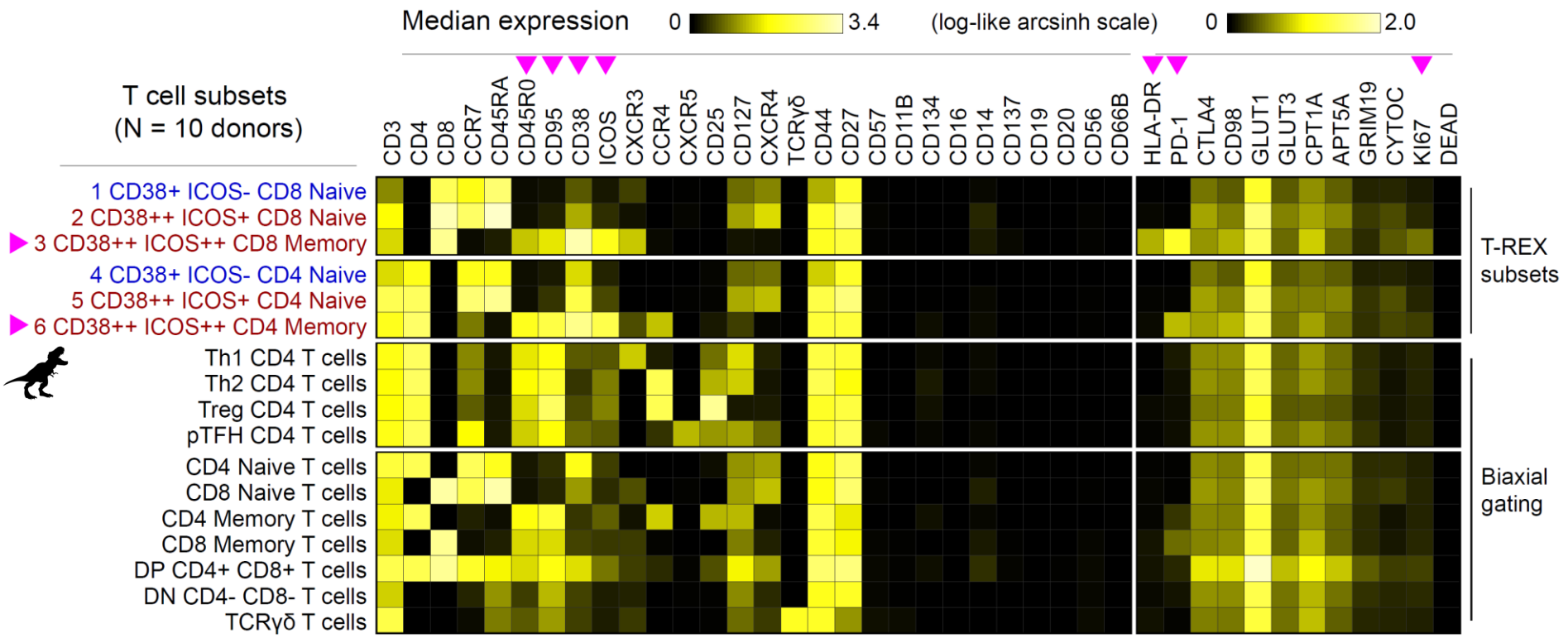
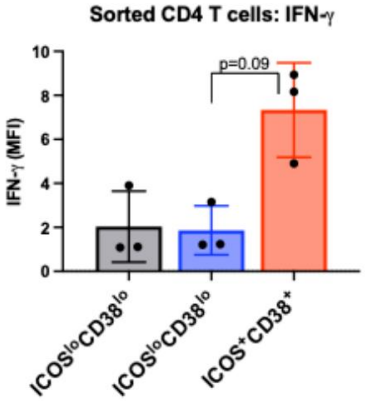
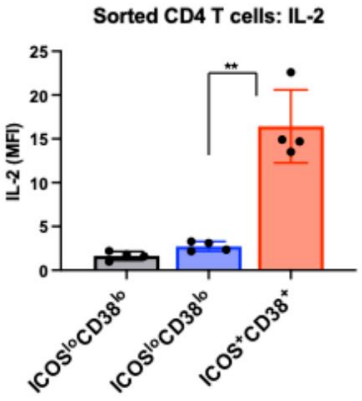
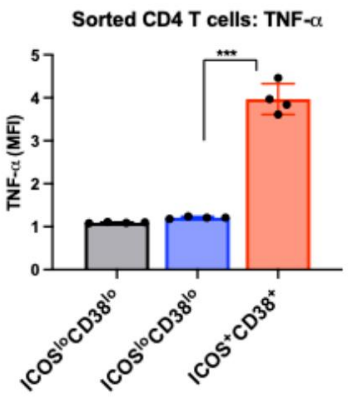


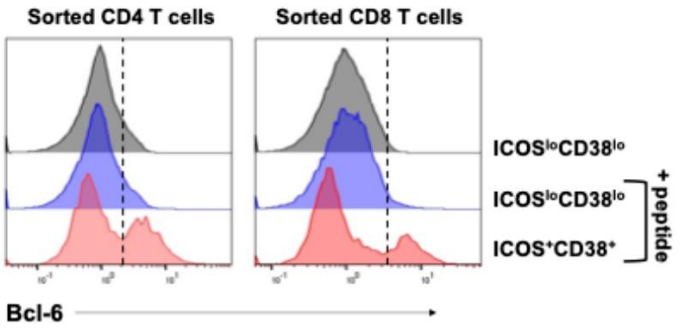
Vaccine response: Kramer, Wilfong, Voss et al., *bioRxiv* 2021
T-REX: Barone, Paul, Muehling et al., *eLife* 2021

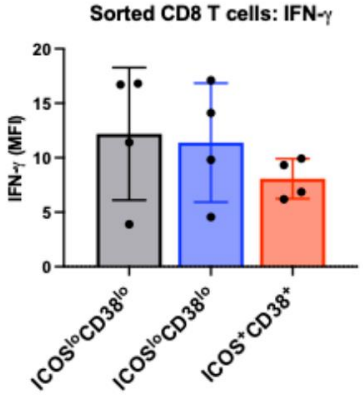
ICOS++ CD38++ T Cells from Day 28 Post-Vaccine Responded to SARS-CoV-2 Spike Peptide (Autologous PBMCs)

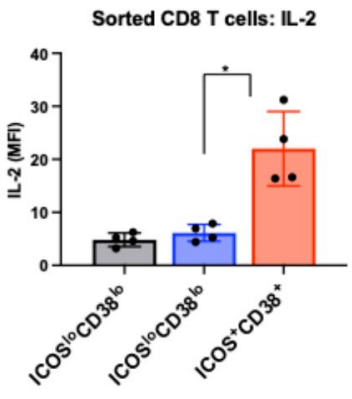


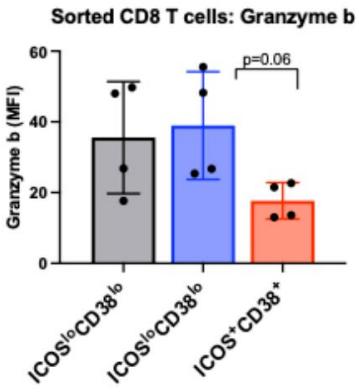


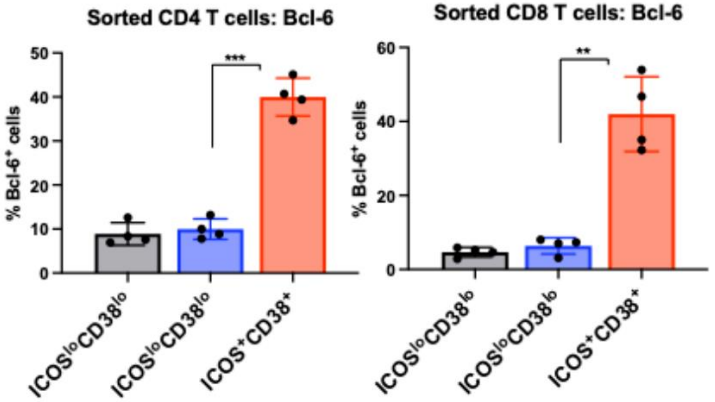












T_{FH}/T_{FC}? Only half of these cells were BCL-6+, and the cells from T-REX were CXCR5-

Vaccine response: Kramer, Wilfong, Voss et al., *bioRxiv* 2021
T-REX: Barone, Paul, Muehling et al., *eLife* 2021

T-REX revealed virus-specific T cells without tetramers,
characterized massive immune changes in COVID-19,
& identified a SARS-CoV-2 reactive non-canonical
memory T cell that expands by day 28 following RNA vaccination

Check out the pre-print for more, including plasmablasts, B cell LIBRA-seq, and a breakthrough case who did NOT generate the ICOS+ CD38+ T cells.

Vaccine response: Kramer, Wilfong, Voss et al., *bioRxiv* 2021
T-REX: Barone, Paul, Muehling et al., *eLife* 2021

T-REX & COVID-19 Acknowledgements

Irish Lab at Vanderbilt University
+ Cancer & Immunology Core

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VU Undergraduate

Sierra Barone Lima
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Program Coordinator

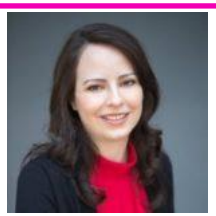
Todd Bartkowiak
PhD Postdoc
K00 Fellow

Caroline Roe
CIC/MCCE
Senior Research
Specialist

Madeline Hayes
Lab Development
Program
Coordinator

University of Virginia Collaborators
(Rhinovirus T-REX)

Alberta Paul



Lyndsey Muehling

Judith Woodfolk

Vanderbilt University Medical Center
(SARS-CoV-2 vaccine response)

Kevin Kramer

Erin Wilfong

Kelsey Voss

Rachel Bonami

Ivelin Georgiev

Jeff Rathmell

Thank you for the invitation!

Southeastern Brain Tumor Foundation (Ihrie & Irish), NIH/NCI : R00 CA143231 (Irish), R01 CA226833 (Bachmann & Irish), HIDI TIPS (Vanderbilt) U01 CA196405 (Massion), CSBC U54 CA217450 (Quaranta), U01 AI125056 (Woodfolk), R01 HL136664 (Rathmell), F31 CA199993 (Greenplate), T32 CA009592 & R25 GM062459 (Doxie), R25 CA136440 (Diggins), K12 CA090625 (Ferrell), P30 CA68485 (Vanderbilt-Ingram Cancer Center)

Part 2: Quantifying Cell Biology & Cytometry Tools

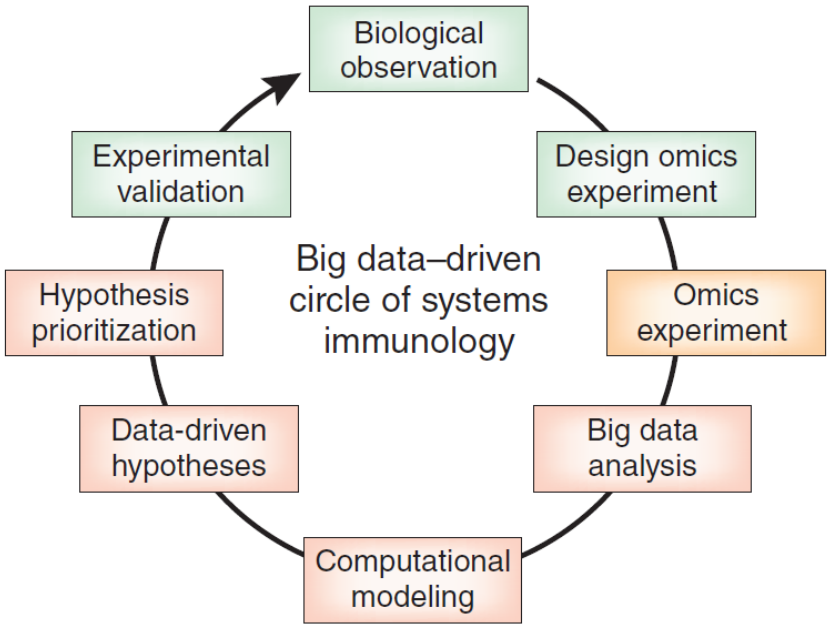
The data type for today: cytometry
(quantitative single cell measurements)

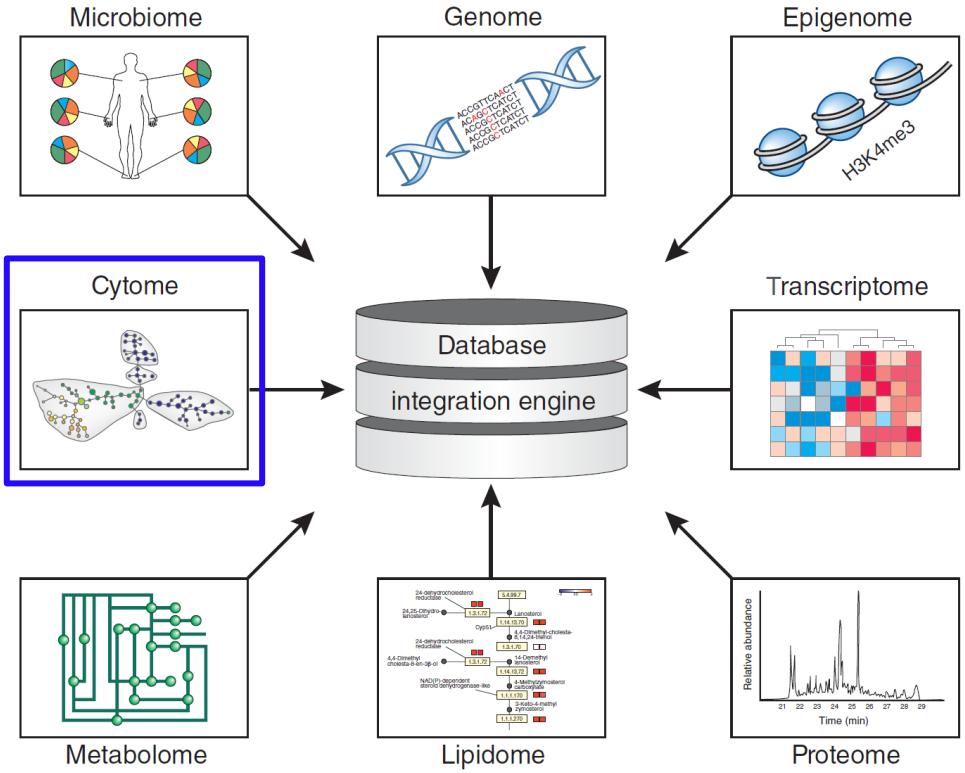
Cytomics: The 'Omics of Cells & Cell Identity

Teaching 'big data' analysis to young immunologists

NATURE IMMUNOLOGY
VOLUME 16 NUMBER 9 SEPTEMBER 2015

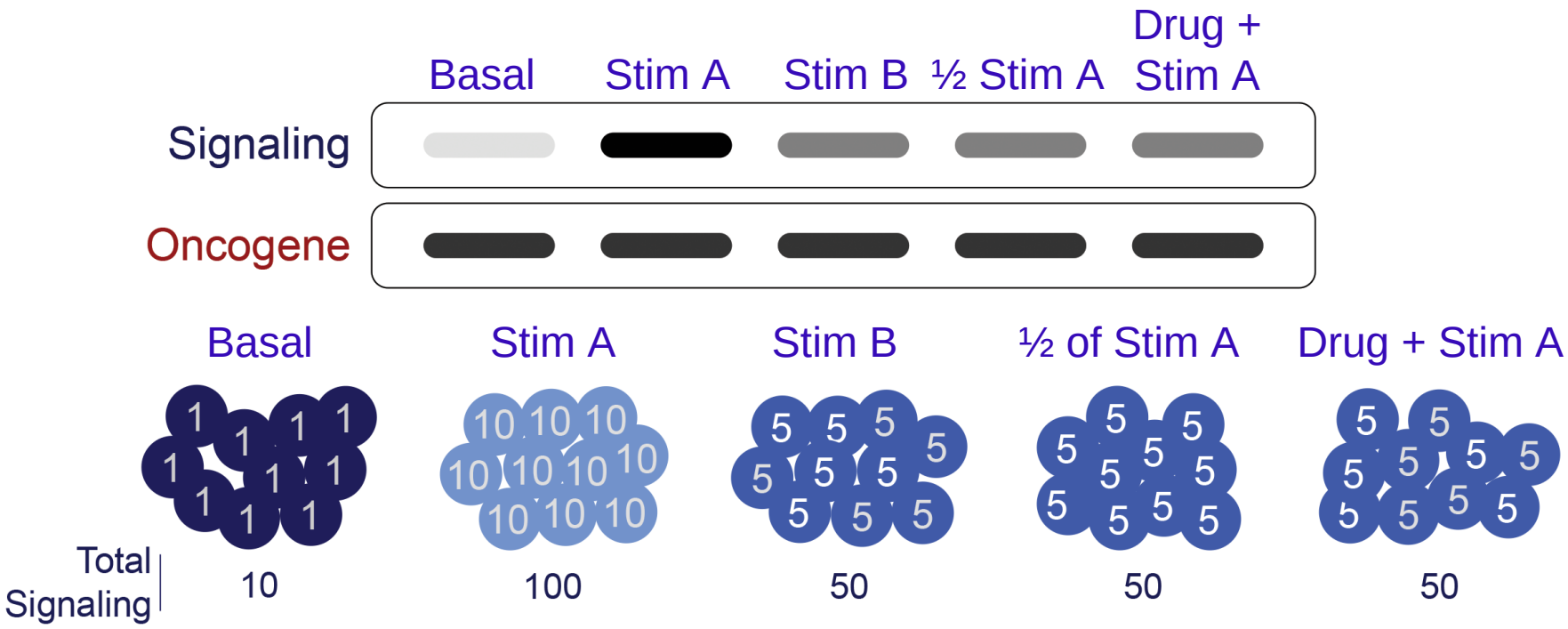
Joachim L Schultze





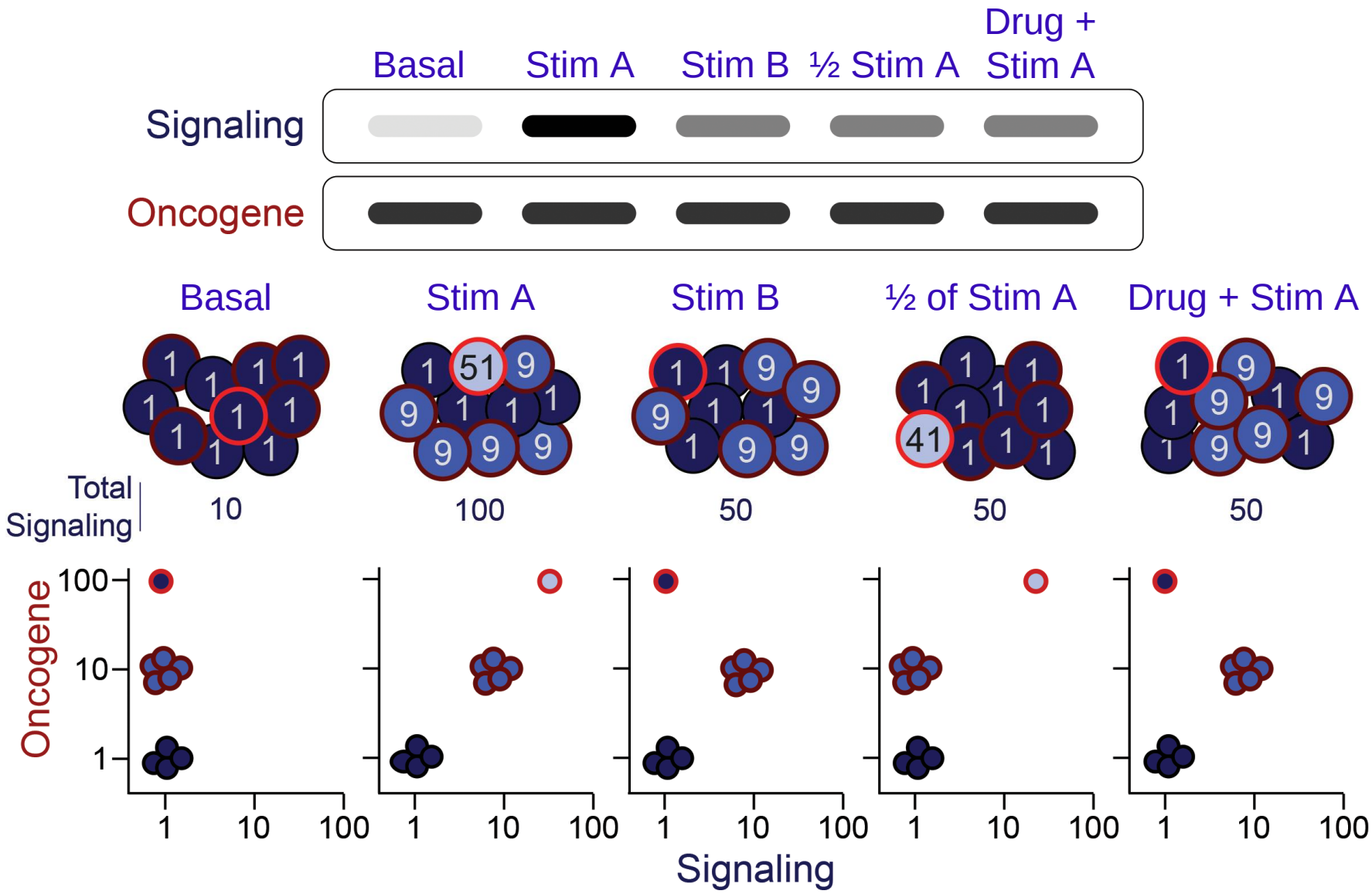
Schultze JL, *Nature Immunology* 2015

Single Cell Biology: Which Cell, How Much?



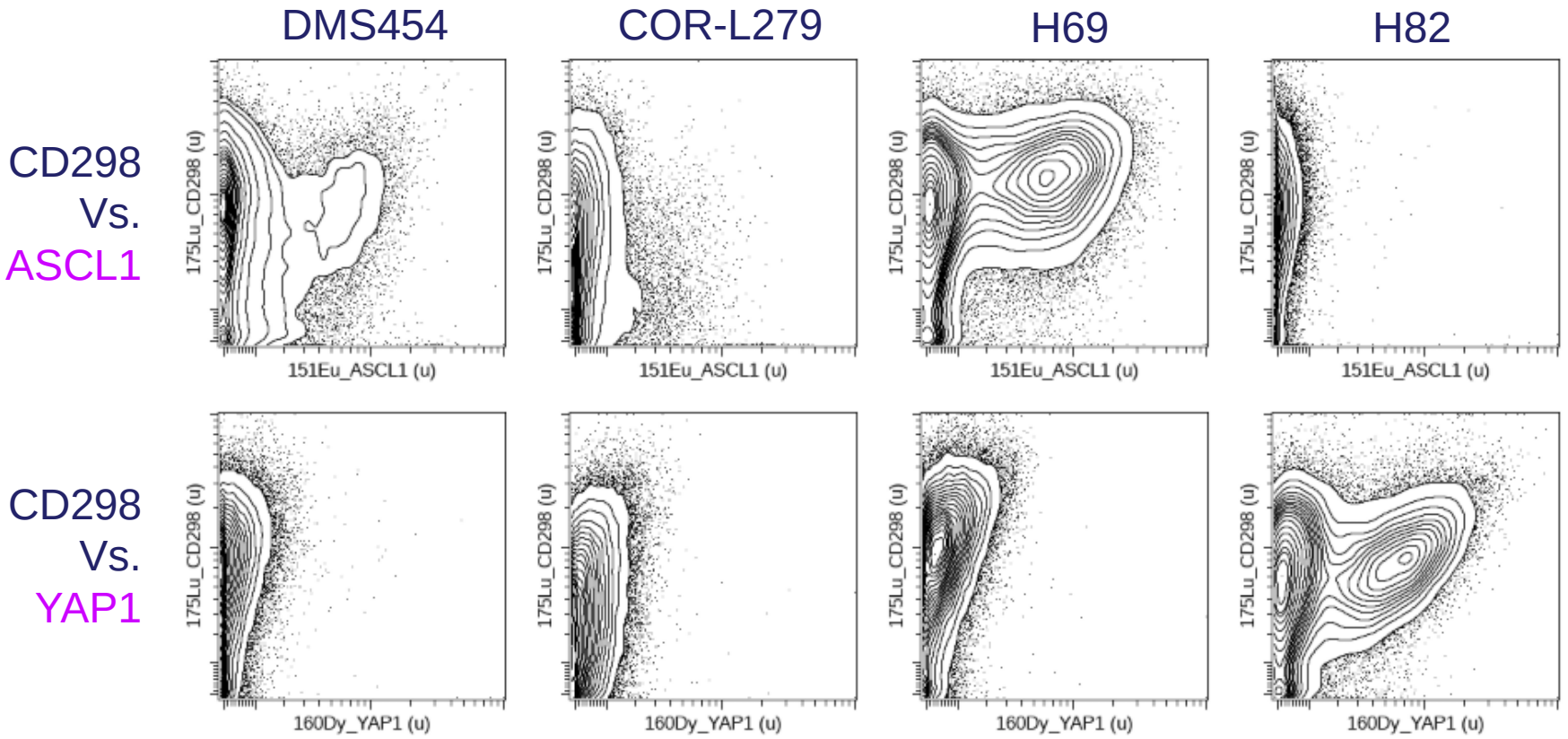
Doxie & Irish, *Current Topics in Microbiology & Immunology* 2014
& Krutzik et al., *Clinical Immunology* 2004

Single Cell Biology: Which Cell, How Much?



Doxie & Irish, *Current Topics in Microbiology & Immunology* 2014
& Krutzik et al., *Clinical Immunology* 2004

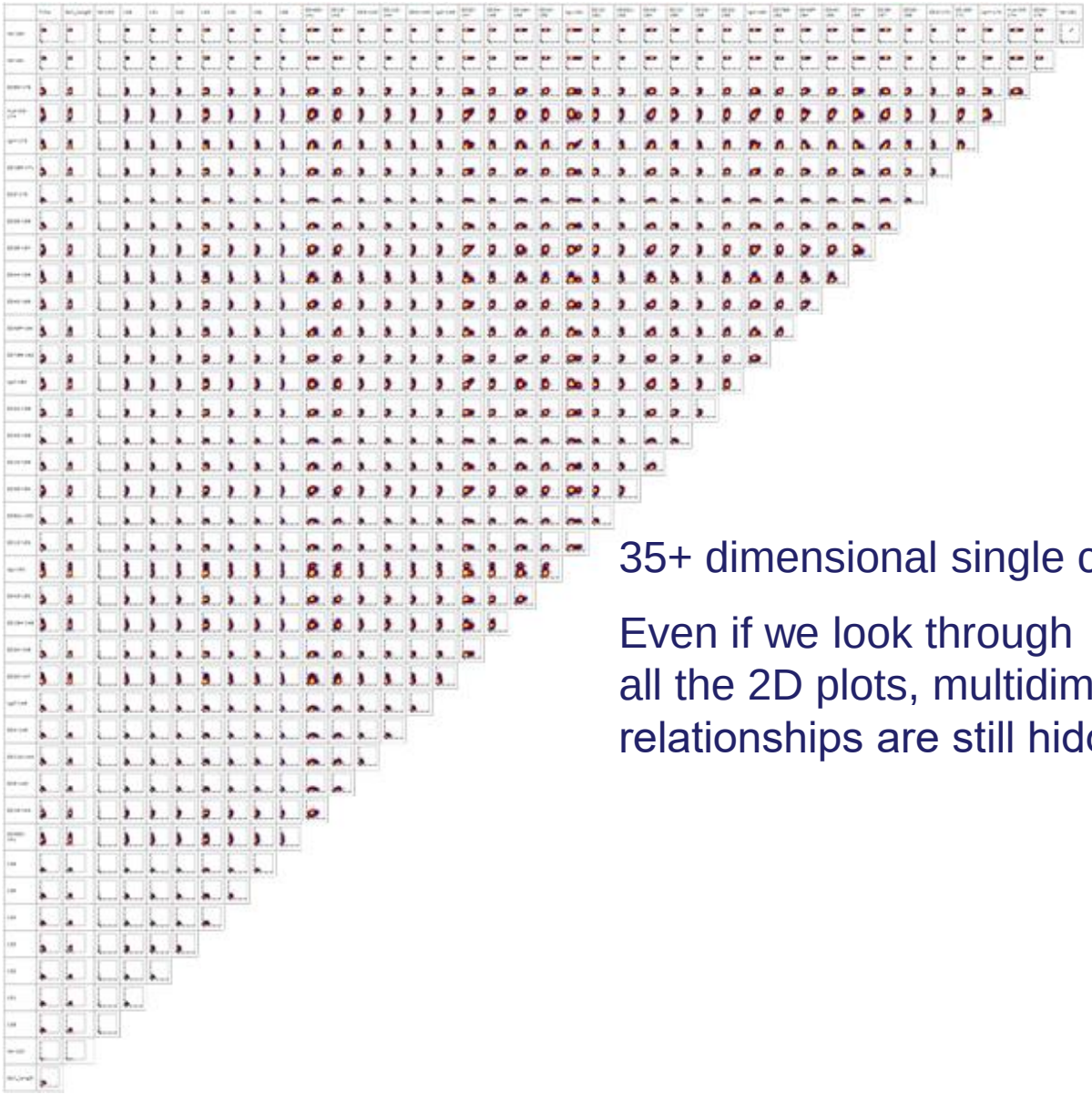
Cytometry Analysis Reveals Stable Subpopulations with Key Subtype-Defining Features Even in 'Clonal' Cell Lines (!)



CD298 (y-axis, Marker for All cells) vs. ASCL1 & YAP1 (x-axis, SCLC Subtypes), Staining on CyTOF (mass cytometry)

CD298-ATP1B3 Is a "Universal" Human Cell Marker Developed for Tracking PDXs (Lawson et al., *Nature* 2015 Werb) & Cellular Barcoding (Hartmann et al., *Scientific Reports* 2018)

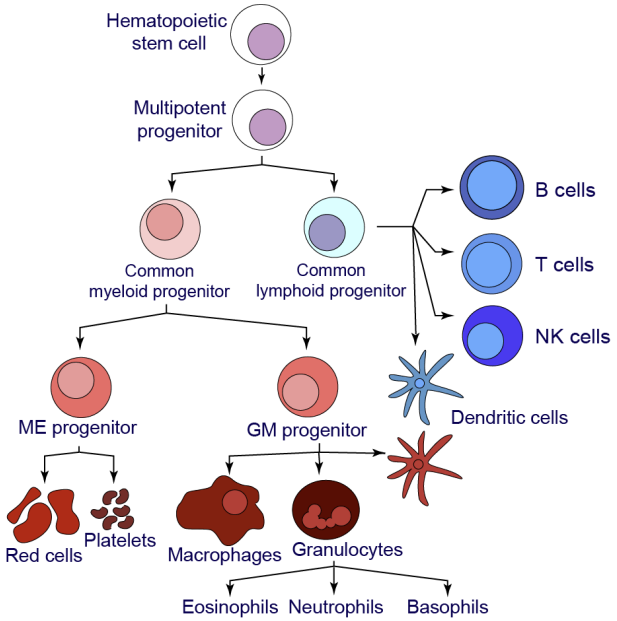
We Now Make Billions of Multi-D Single Cell Measurements => Need for Machine Learning Tools & Human Readable Views

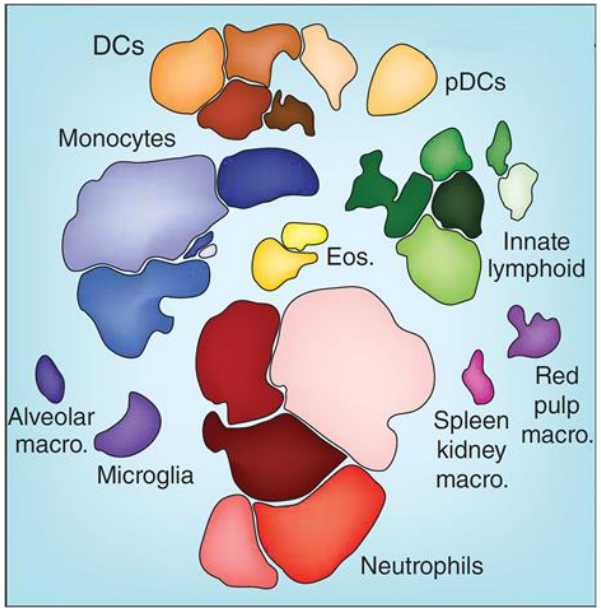


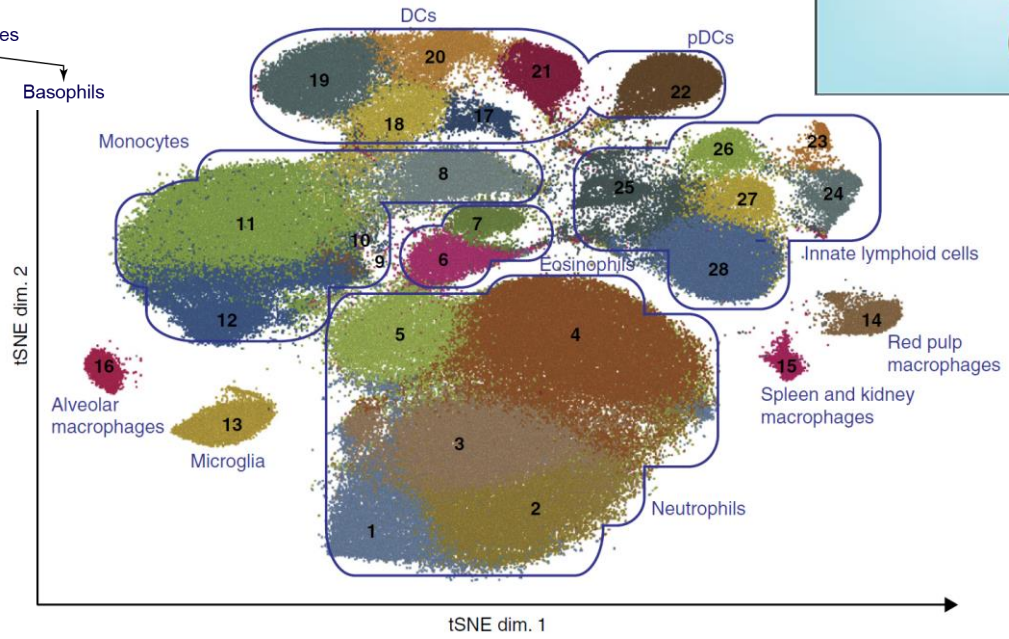
35+ dimensional single cell data:

Even if we look through
all the 2D plots, multidimensional
relationships are still hidden…

The Big Idea: Automatically Identify All Cell Types in Primary Tissues, Create Reference Models to Study Impact of Disease, Genetic Changes, etc.



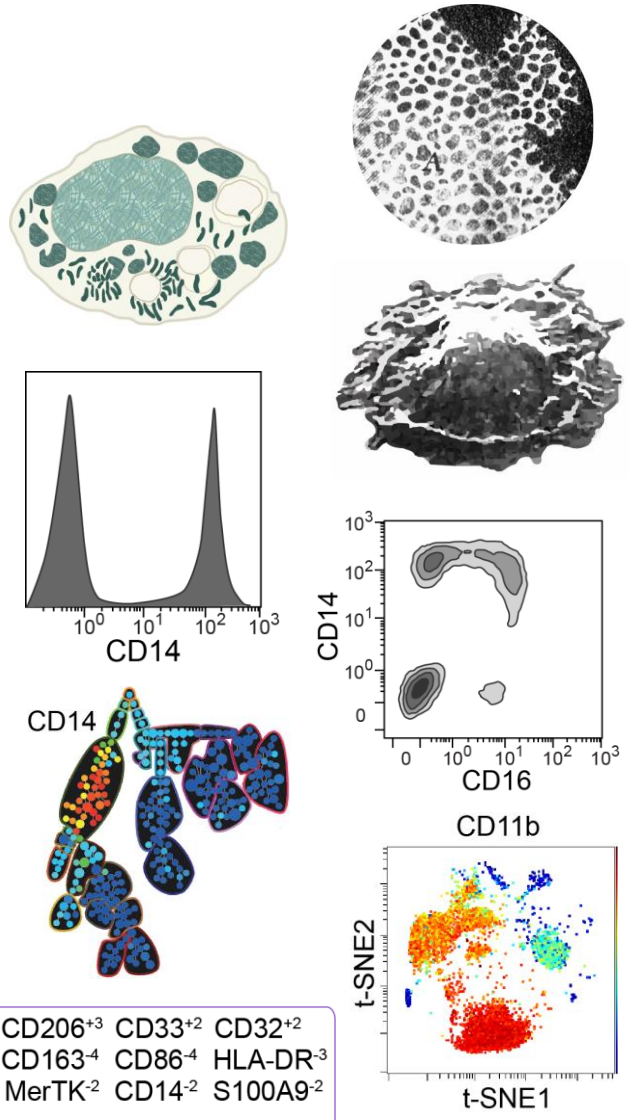




Irish, *Nature Immunology* 2014
Based on Becher et al., *Nature Immunology* 2014

New Technology Reveals & Characterizes New Cells

Date	Approach	Dimensions (D) Per Cell & Speed	
1665*	Light microscopy	Low	Low
1908**	Light microscopy	Low	Low
1946	Scanning EM	Low	Low
1989	Flow cytometry identification	Low	1K cells/s
2001	Flow cytometry subsetting	4D	2 – 50K cell/s
2011	Mass cytometry + SPADE	32D	500 cell/s
2014	Mass cytometry + t-SNE / viSNE	38D	500 cell/s
(now)	Flow or Imaging MC + UMAP, FlowSOM, MEM	38D	500 cell/s



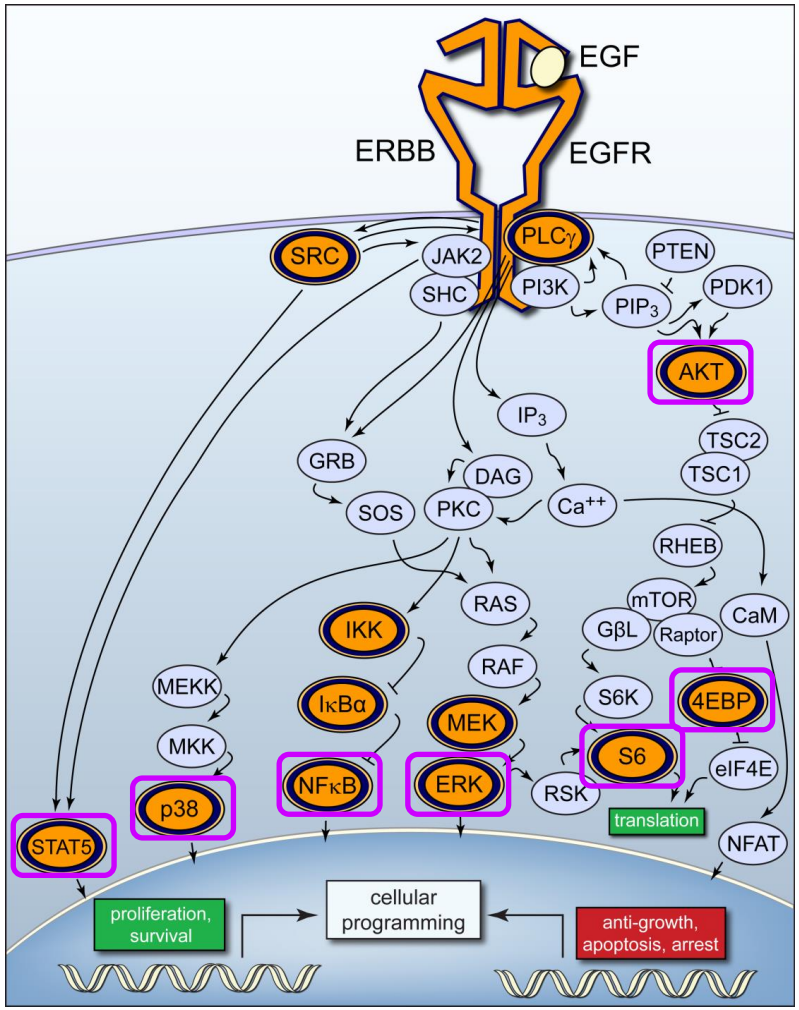
* Robert Hooke describes 'cells' in *Micrographia: or Some Physiological Descriptions of Miniature Bodies Made by Magnifying Glasses*

** Élie Metchnikoff characterizes mononuclear phagocytes: Lectures on the Comparative Pathology of Inflammation, Pasteur Institute in 1891, Nobel Prize in 1908 w/ Ehrlich.

Adapted from
Roussel et al., *Human Innate Immunity* 2016

An Updated Mass Cytometry Panel for Glioblastoma Cell Identity, Stemness, Function, & Signaling

Phospho-protein effectors of key mutant proteins

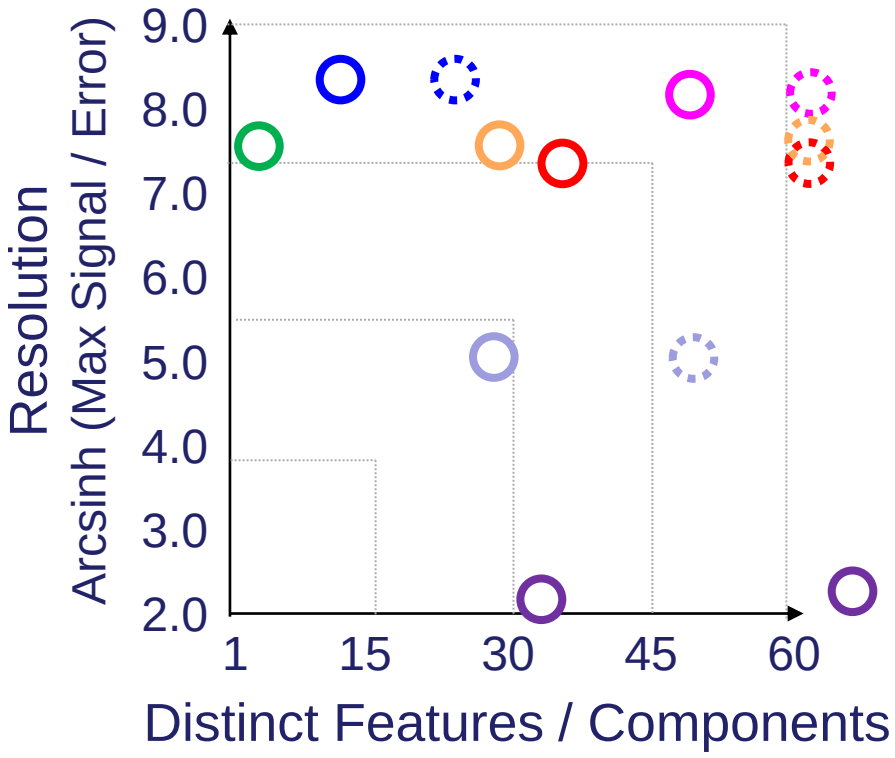


Phospho-flow on lymphoma tumors: Irish et al., *PNAS* 2010

Target	Mass	Clone	Signaling & proteins		Stain		
			Panel	t-SNE	Live	Sap	MeOH
Rhodium	103	-	●		✓		
Cyclin B1	139	GNS-1	●				✓
TUJ1	141	TUJ1	●	■			✓
cCasp3	142	5A1E	●				✓
CD117	143	104D2	●		✓		
S100B	144	19/S100B	●	■			✓
CD31	145	WM59	●	■*	✓		
γH2AX	147	JBW301	●				✓
CD34	148	581	●	■	✓		
p-4E-BP1 (T37/T46)	149	236B4	●				✓
p-STAT5 (Y694)	150	47	●	■			✓
BMX	151	40/BMX	●				✓
p-AKT (S473)	152	D9E	●	■			✓
p-STAT1 (Y701)	153	58D6	●	■			✓
CD45	154	HI30	●	■*	✓		
NCAM/CD56	155	HCD56	●	■	✓		
p-p38 (T180/Y182)	156	D3F9	●				✓
p-STAT3 (Y705)	158	4/P-STAT3	●	■			✓
ITGα6/CD49F	159	GoH3	●	■	✓		
CD133	160	AC133	●	■	✓		
PDGFRα	161	16A1	●	■	✓		
SOX2	163	O30-678	●	■		✓	
SSEA-1/CD15	164	W6D3	●	■	✓		
EGFR	165	AY13	●	■	✓		
p-NFκB p65 (S529)	166	K10-895.12.50	●	■			✓
L1CAM	167	5G3	●	■	✓		
Nestin	168	10C2	●	■			✓
CD44	169	BJ18	●	■	✓		
GFAP	170	1B4	●	■			✓
p-ERK1/2 (T202/Y204)	171	D13.14.4E	●				✓
p-S6 (S235/S236)	172	N7-548	●	■			✓
SOX10	173	A-2	●	■			✓
HLA-DR	174	L243	●	■	✓		
p-HH3	175	HTA28	●				✓
Histone H3	176	D1H2	●				✓

GBM panel: Leelatian & Sinnaeve et al., bioRxiv 2019
Tumor methods: Leelatian & Doxie et al., Cytometry B 2017

Cytometry Probes & Instruments Keep Improving (ca. 2019)



Symphony
27 probes (50)
262,144 / 3000
Res: 5.2

Aurora
40+ probes (60+)
4,194,300 / 6,000
Res: 7.2

scRNA-seq
30K genes
(35 PCAs?)
15 / 3
Res: 2.3

50 / 1
Res: 4.6

CyTOF 3.0
45 probes
(60+)
12,000 / 5
Res: 8.3

CyTOF 1.0
36 probes (50)
12,000 / 15
Res: 7.2

FACSCalibur
4 probes
10,000 / 10
Res: 7.6

LSR ii
14 probes (25)
262,144 /
150
Res: 8.2

Mistry et al., *FEBS J* 2018; "Resolution" calculated from typical published data; dashed lines indicate theoretical or unpublished values.

HD Cytometry Balances Signal, Throughput, & Cost

Mass cytometry: Imaging or flow cytometry method of multiplexed single cell analysis. Standard mass cytometry panels detect 37+ features per cell using pre-validated antibodies. The dynamic range is >10,000 intensity units per feature and a small flow-based mass cytometry dataset might include 1.2 million cells from 12 samples collected at a rate of 500 cells/second (~40 min instrument time) for a total cost of ~$4,500 ($0.004 per cell), including personnel time/effort.

Diggins et al., *Bench to Bedside to Bytes, in review*
Mass cytometry vs. other single cell technologies: Mistry et al., *FEBS J* 2018

Some of the literature from "big seq" is hilariously inaccurate when it comes to flow cytometry…

From: "Single cell RNA sequencing to explore immune cell heterogeneity", Nat Rev Immunology 2017

	FACS	CyTOF	qPCR	Plate-based protocols (STRT-seq, SMART-seq, SMART-seq2)	Fluidigm C1	Pooled approaches (CEL-seq, MARS-seq, SCRB-seq, CEL-seq2)	Massively parallel approaches (Drop-seq, InDrop)
Cell capture method	Laser	Mass cytometry	Micropipettes	FACS	Microfluidics	FACS	Microdroplets
Number of cells per experiment	Millions	Millions	300–1,000	50–500	48–96	500–2,000	5,000–10,000
Cost	$0.05 per cell	$35 per cell	$1 per cell	$3–6 per well	$35 per cell	$3–6 per well	$0.05 per cell
Sensitivity	Up to 17	Up to 40	10–30 genes	7,000–10,000 genes	6,000–9,00 genes for cell	7,000–10,000 genes per cell for cell lines; 2,000–6,000 genes per cell for primary cells	5,000 genes per cell for cell lines; 1,000–3,000 genes per cell for primary cells

Wait, so this review says each experiment costs… ?!
FACS: $50,000 ($0.05 x 1,000,000 cells)
CyTOF: $35,000,000 ($35.00 x 1,000,000 cells)

mass cytometry); FACS, fluorescence-activated cell sorting; CR, quantitative PCR; SCRB-seq, single-cell RNA barcoding and sequencing; STRT-seq, single-cell tagged reverse transcription sequencing.

HD Cytometry Dissects Cellular Mechanisms of Cancer Immune Response

Article

Cell

Systemic Immunity Is Required for Effective Cancer Immunotherapy

Spitzer et al., *Cell* 2017

Uses mass cytometry to characterize essential role of peripheral blood $CD4^+$ T cells in immunotherapy response

ARTICLE

doi:10.1038/nature22079

T-cell invigoration to tumour burden ratio associated with anti-PD-1 response

Huang et al., *Nature* 2017

Uses mass cytometry to reveal peripheral blood CD8 T cells associated with anti-PD-1 immunotherapy responses

Article

Cell

Distinct Cellular Mechanisms Underlie Anti-CTLA-4 and Anti-PD-1 Checkpoint Blockade

Wei et al., *Cell* 2017

Uses mass cytometry to characterize similar & distinct tumor-infiltrating immune cell subsets (mostly T cells) following immunotherapies

Referenced Comparisons of Single Cell Techniques

	Mass cytometry	Fluorescence cytometry	scRNA-seq
Detection method	Time-of-flight of ionized heavy elemental isotopes	Emitted light and light scatter	Nucleic acid sequence
Example probes	Metal-conjugated antibodies, metal-conjugated small molecules	Fluorochrome-conjugated antibodies, fluorescent molecules	Poly-A targeted oligonucleotides, antibody-conjugated oligonucleotides [144]
Example cellular features and targets measured	Proteins, phospho-proteins, chromatin modifications [124], RNA transcripts [131–133], platinum drug uptake [128,129], cell cycle status and DNA synthesis [130], cell size [125], apoptosis [48], and viability [27]	Proteins, phospho-proteins, chromatin modifications [145], RNA transcripts [146], fluorescent drug uptake [147], metabolism and redox state [148,149], cell cycle status and DNA synthesis [150], cell size and granularity, apoptosis [151] and viability [152]	Poly-adenylated RNA transcripts, CITE-seq probes [144]
Minimum cells per sample needed at start of protocol	50 000 cells	50 000 cells	200 cells [153][a]
Cell capture rate	30–60% [31,32]	> 95%	5–65% [154]
Target capture	> 95%	> 95%	10–40% [154–156]
Example of analyzed cell events per study	2 000 000 cells per study	20 000 000 cells per study	5000 cells per study [157]
Resolution, arcsinh (max/error)[b]	~ 6–9 e.g., arcsinh (12 000/5)	~ 5–9 e.g., arcsinh (262 144/150), arcsinh (4 194 300/6000)[c]	~ 2–3 e.g., arcsinh (10/1)
Cell throughput	Up to 10^4 cells·s^{-1} [33]	Up to 10^5 cells·s^{-1} [31]	Up to 10^4 cells·s^{-1} [155]
Features/available channels	50/200 channels	30/64 channels[c]	~ 30 000/N/A
Degree of crosstalk between parameters	3% (range: 0–8.6%) [55]	19.5% (4.9–51.1%) [56]	N/A
Accessibility	Major research institutions	> 20 features: major research institutions; ~ 4 color common	Major research institutions
Total cost	$0.004-$0.01/cell [31]	< $0.001/cell	$0.05-$3.00/cell [158]

Beyond the Message: Advantages of Snapshot Proteomics with Single-Cell Mass Cytometry in Solid Tumors
Mistry et al., *FEBS Journal* 2018

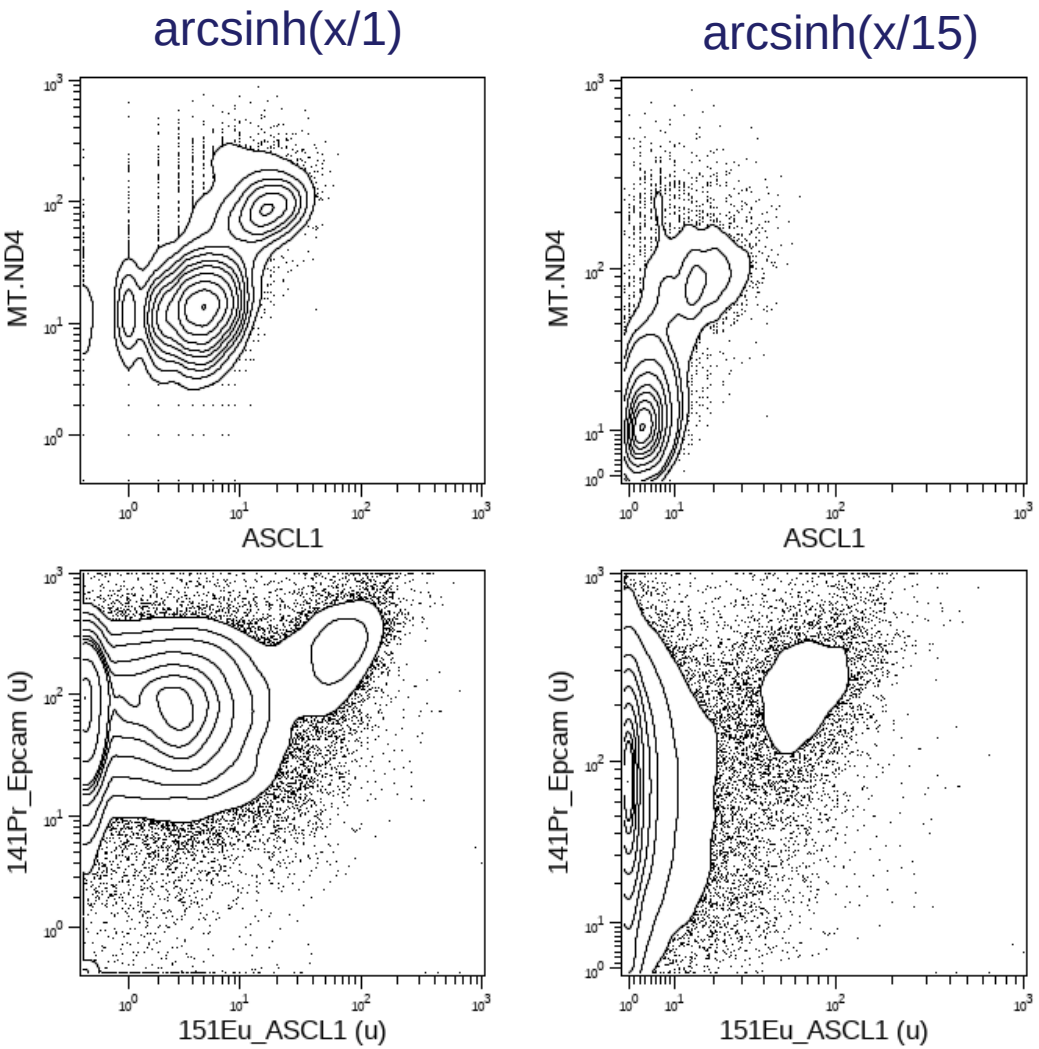
Example Challenge, Scaling to Compare scRNA-seq vs. CyTOF

(Here with one example cell line, DMS454, and ASCL1 on the x-axis)

DMS454
SCLC cell line

scRNA-seq

CyTOF



Qualitative: Is there a subset of cells that is ASCL1^{+}?

Quantitative: How much ASCL1 is in each cell? How much does ASCL1 change after Tx?

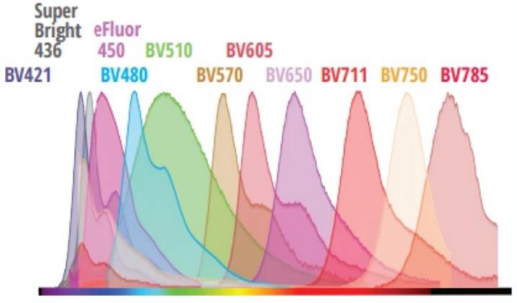
Spectral Flow Cytometry Can Separate ‘Overlapping’ Probes

More Choice, Greater Flexibility, Easier Setup

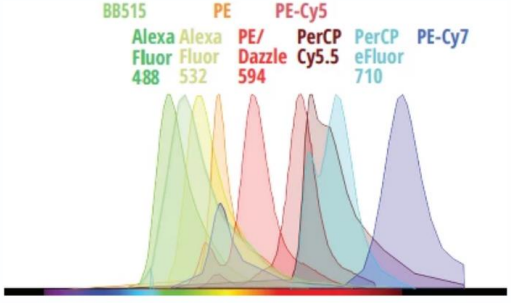
The optical design combined with the unmixing capability in SpectroFlo® software allows greater fluorochrome choice, panel flexibility, and easy setup without having to change filters. The three laser configuration provides outstanding multi-parametric data for a wide array of applications. Markers and fluorochromes in a 24-color panel designed for identification of circulating cell subsets in human peripheral blood are summarized in the table below:

SPECIFICITY	FLUOROCHROME	SPECIFICITY	FLUOROCHROME	SPECIFICITY	FLUOROCHROME
CCR7	Brilliant Violet 421™	CD11c	BD Horizon™ BB515	CD27	APC
CD19	Super Bright 436	CD45RA	Alexa Fluor® 488	CD123	Alexa Fluor® 647
CD16	eFluor® 450	CD3	Alexa Fluor® 532	CD127	BD Horizon™ APC R700
TCR γ/δ	BD Horizon™ BV480	CD25	PE	HLA DR	APC/Fire™ 750
CD14	Brilliant Violet 510™	IgD	PE/Dazzle™ 594		
CD8	Brilliant Violet 570™	CD95	PE-Cy™5		
CD1c	Brilliant Violet 605™	CD11b	PerCP-Cy™5.5		
PD-1	Brilliant Violet 650™	CD38	PerCP-eFluor® 710		
CD56	Brilliant Violet 711™	CD57	PE-Cy™7		
CD4	Brilliant Violet 750™				
CD28	Brilliant Violet 785™				

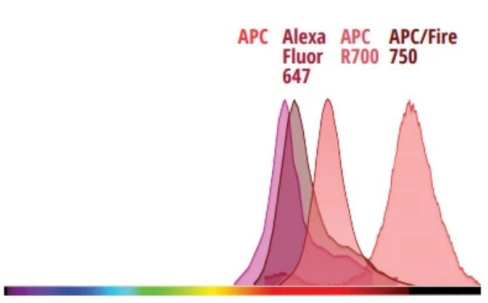
The 24-Color Panel Includes Many Highly Overlapping Dyes:



Violet Excited Dyes Emission Spectra

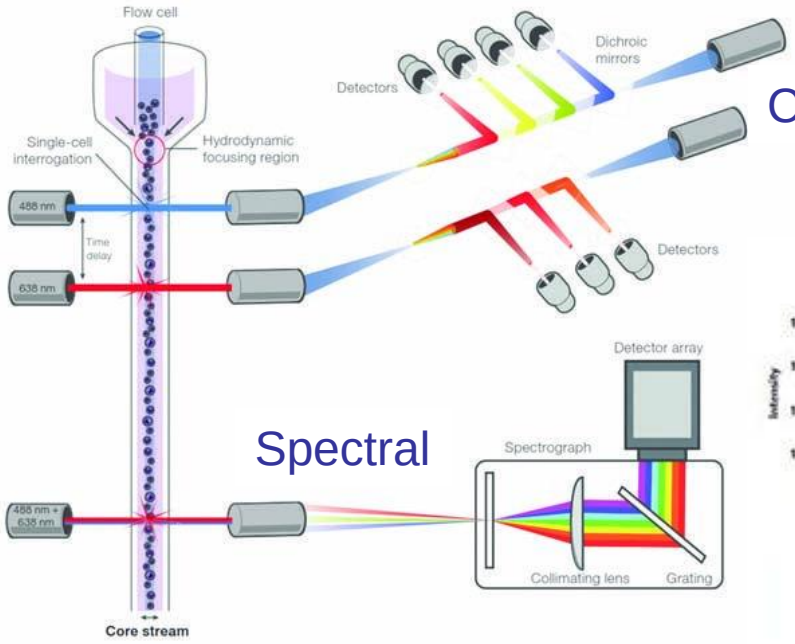


Blue Excited Dyes Emission Spectra

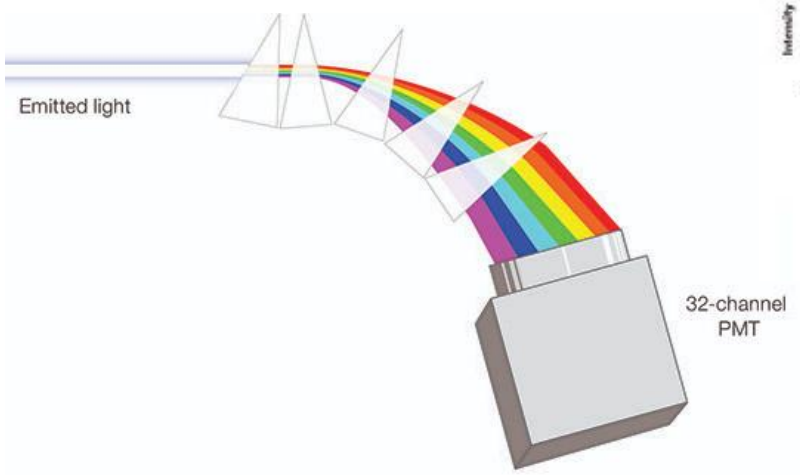


Red Excited Dyes Emission Spectra

By Detecting “Across the Spectrum” (vs. “Channels” of Light) Spectral Flow Distinguishes Probes with Overlapping Peaks

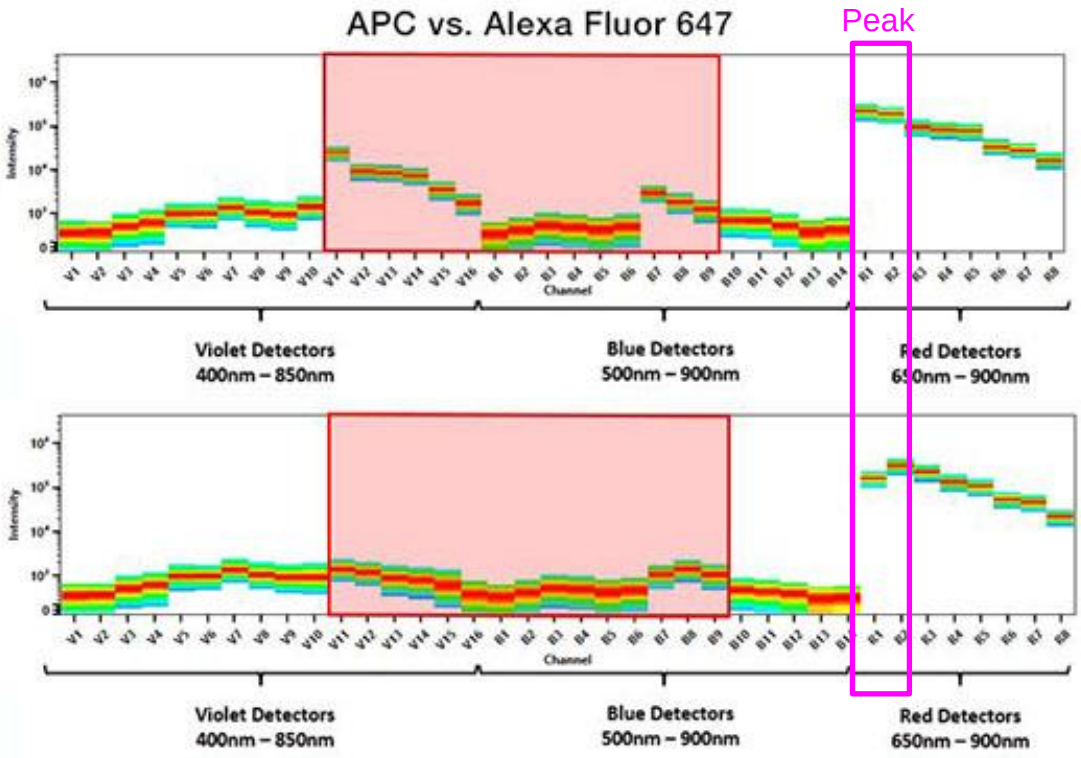


Themo Fisher, adapted from John Nolan et al. 2013



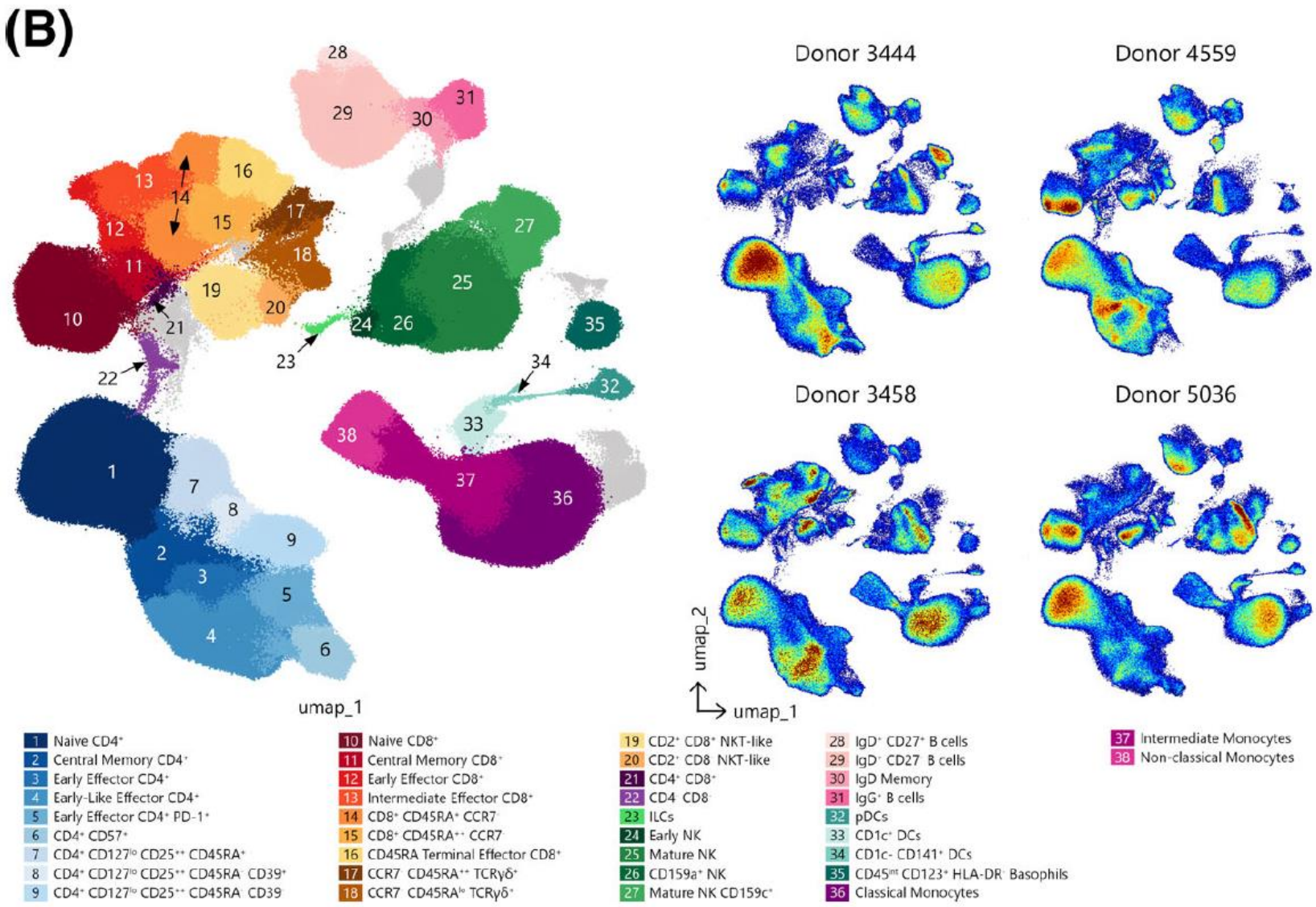
Conventional

Probes with similar “peaks” are resolved



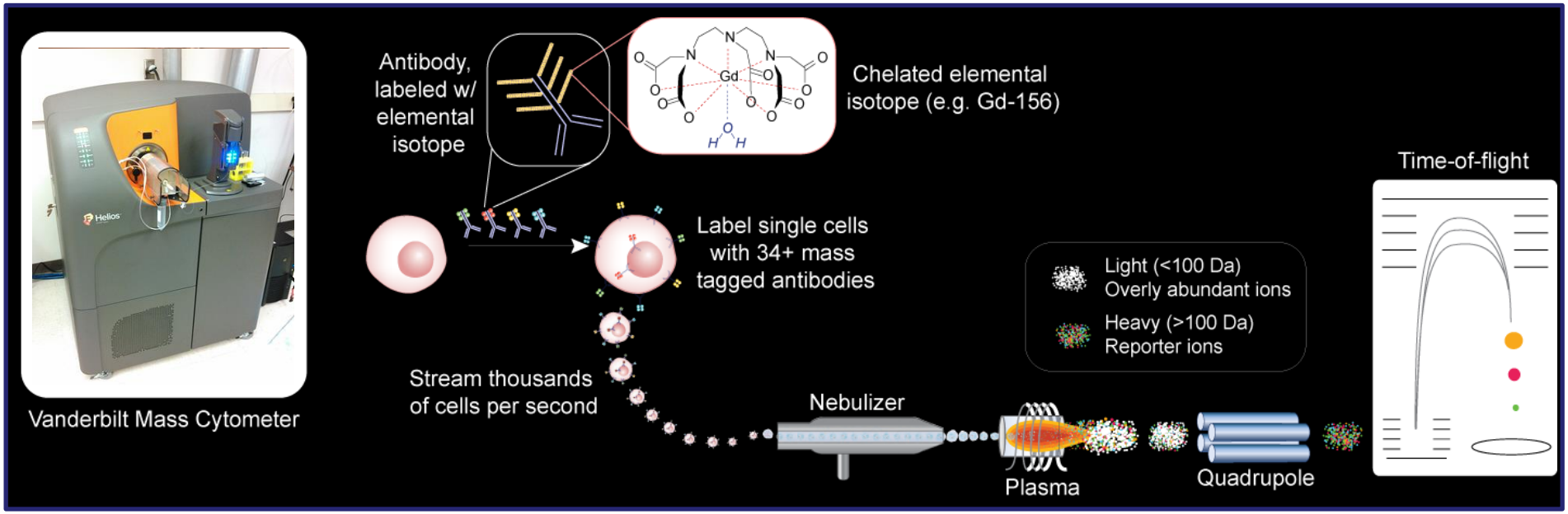
Conventional detector measures only peaks, uses dyes with distinct peaks

Spectral Flow Cytometry's State of the Art in 2020: Excellent Resolution of 40 Antibodies (Rivals CyTOF)

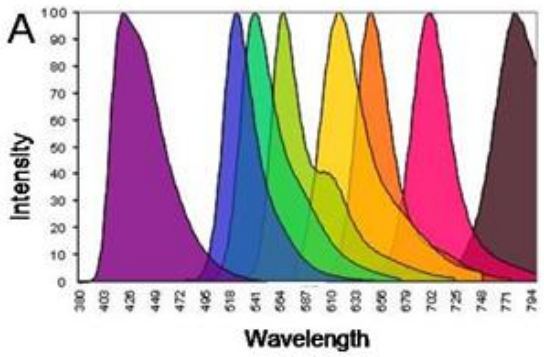


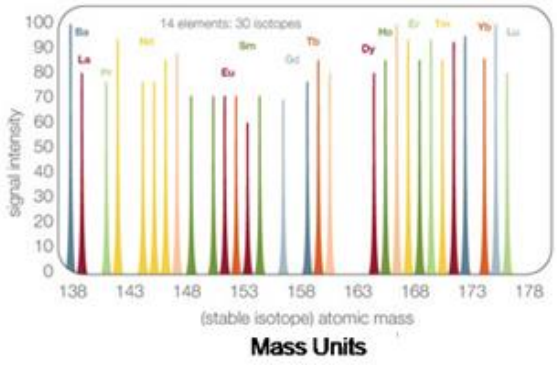
Park et al., *Cytometry* 2020; see Ferrer-Font et al., Cytometry 2020 for comparison of mass and spectral flow cytometry.

Mass Cytometry Uses Isotopically Pure Metals As Probes



Mass cytometry: standard panels detect 35+ features per cell using pre-validated antibodies. The dynamic range is >10,000 intensity units per feature. A small dataset might include 1.2 million cells from 12 samples collected at a rate of 500 cells/second (~40 min instrument time) for a total cost of ~$4,500 ($0.004 per cell), including reagents & personnel.





Experiment Protocols: Leelatian et al., *Methods in Molecular Biology* 2015
Leelatian et al., *Current Protocols in Molecular Biology* 2017

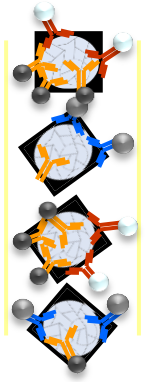
Analysis Protocols: Diggins et al., *Methods* 2015
Diggins et al., *Current Protocols in Cytometry* 2018

Reviewed in: Spitzer & Nolan, *Cell* 2016

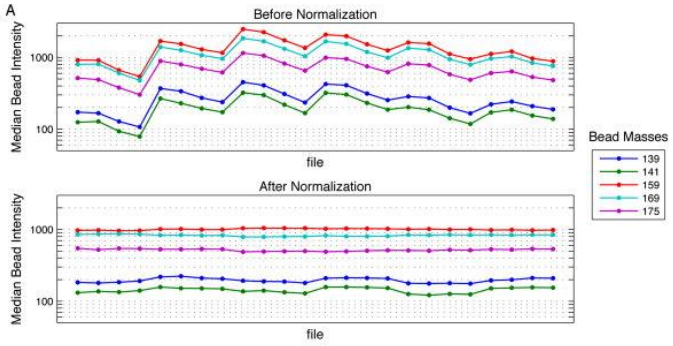
Adapted from Bendall et al., *Science* 2011

Modern Cytometry Includes Internal Batch Controls

Data Acquisition (IMD -> FCS) → Concatenation → Normalization → Debarcoding → Transformation → Analysis



FCS File 1
+
FCS File 2
+
FCS File 3



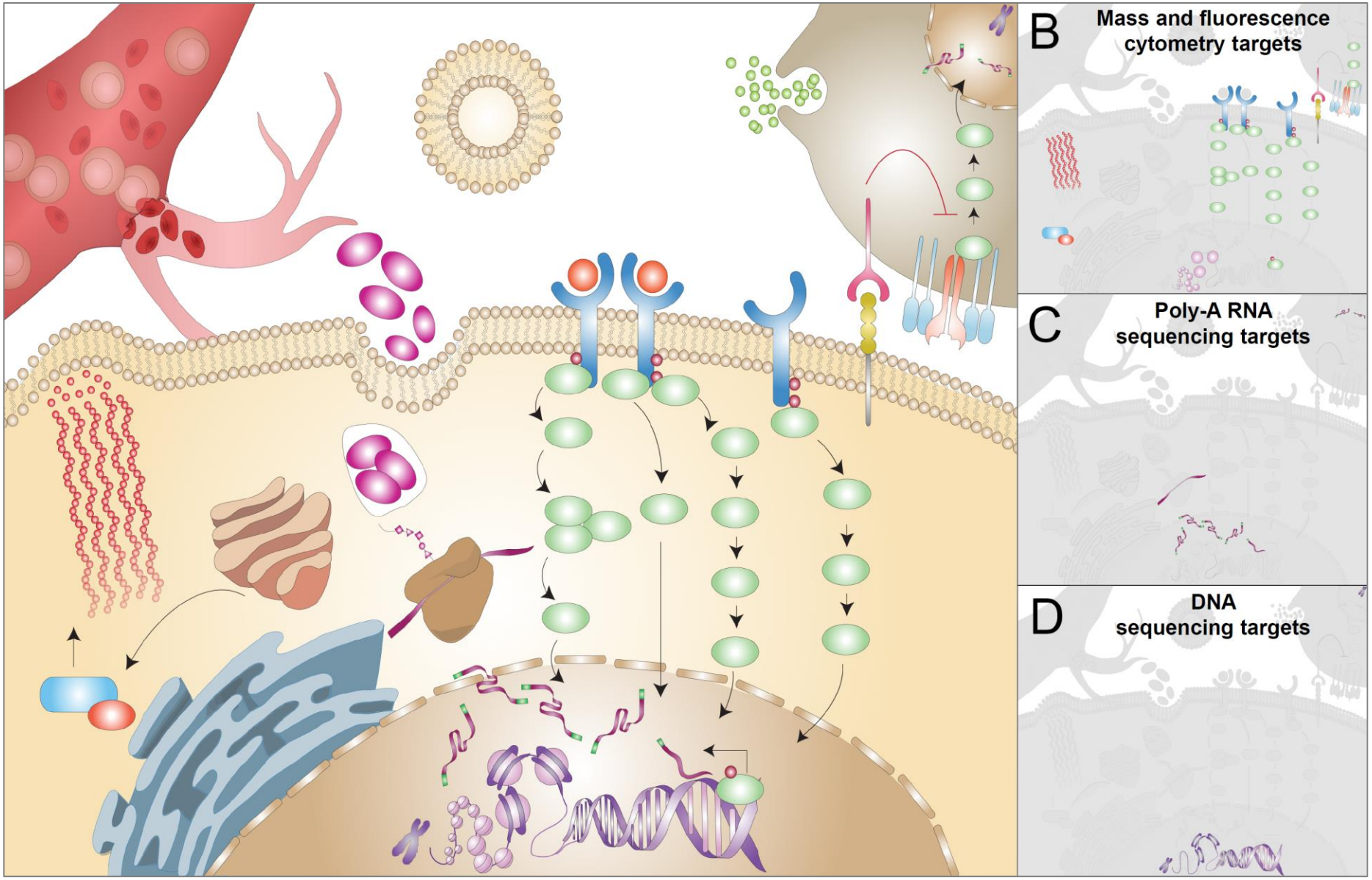
arcsinh(x/5)

Fink et al, *Cytometry Part A* 2013

Resources:

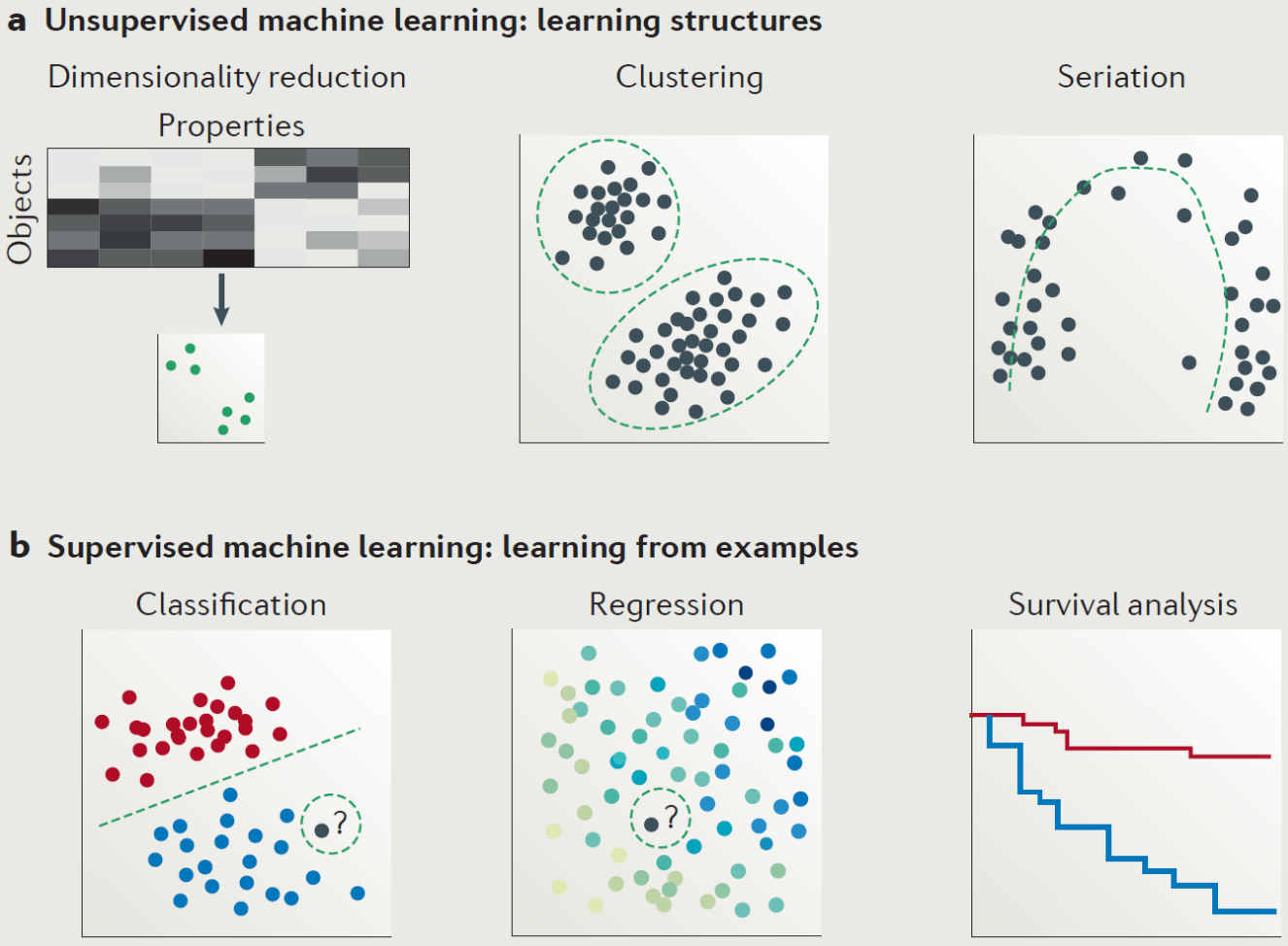
- Concatenation: downloadable tool from Cytobank (http://support.cytobank.org/help/kb/cytobank-utilities/concatenating-fcs-files)
- Normalization: Cytometry Part A Volume 83A, Issue 5, pages 483-494, 19 MAR 2013 DOI: 10.1002/cyto.a.22271 http://onlinelibrary.wiley.com/doi/10.1002/cyto.a.22271/full#fig6
- Barcoding: Bodenmiller et al, *Nature Biotechnology* 2012 (http://www.nature.com/nbt/journal/v30/n9/full/nbt.2317.html)

Challenge: Get a Full Picture of Cell Function (Multiple Data Types)



Beyond the Message: Advantages of Snapshot Proteomics with Single-Cell Mass Cytometry in Solid Tumors
Mistry et al., *FEBS Journal* 2018

Computational Flow Cytometry: There Is Help for Data Analysis

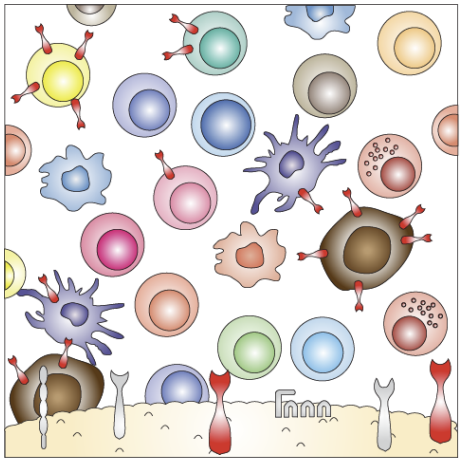


Saeys Y, Van Gassen S, and Lambrecht BN, *Nature Reviews Immunology* 2016

Part 9: Samples Over Time Reveal Immune System Dynamics

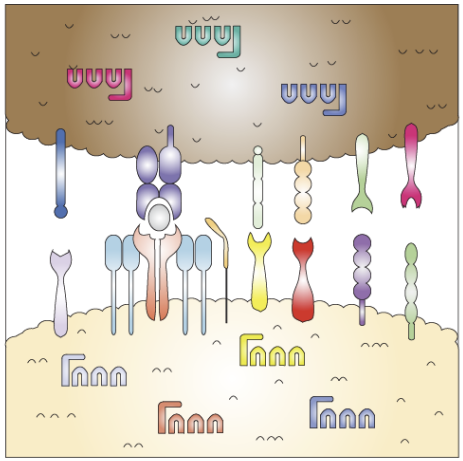
Applications of HD Cytometry in Cancer & Cell Biology

Microenvironment



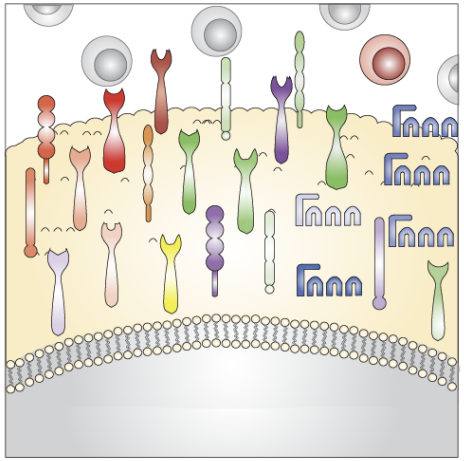
Track key biomarkers on all cell types; find cytokine producers

Cell:cell interactions



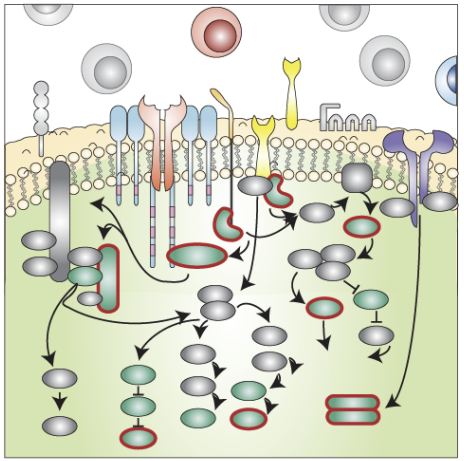
Monitor immune checkpoint proteins on T cells, APCs, & cancer cells

Immunophenotype



Measure differentiation; deep phenotype using fewer cells

Signaling & function

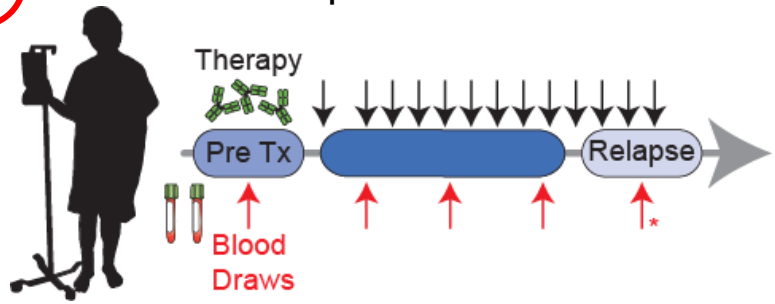


Dissect signaling changes; characterize mechanisms of treatment response

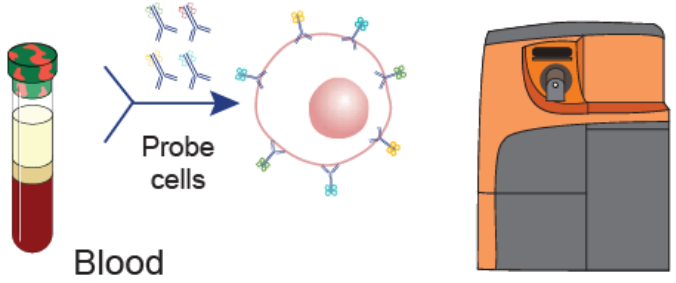
Greenplate et al., *Euro J Cancer* 2016

Human Immune Monitoring: T cell Focus

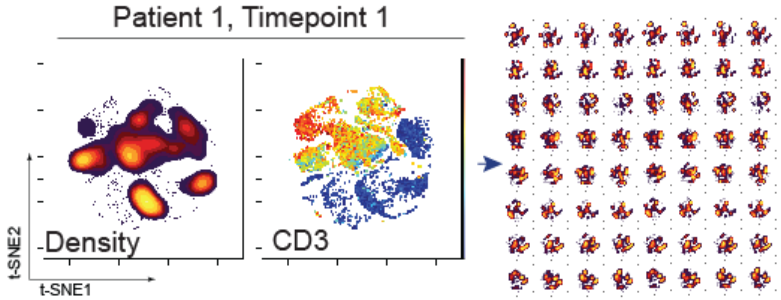
1 Live cells from patients



2 High dimensional, single cell measurements



3 Analyze systems immune changes



Tag Isotope #	Target
141 Pr	ICOS
142 Nd	CD19
143 Nd	TIM3
144 Nd	CCR5
145 Nd	CD4
146 Nd	CD64
147 Sm	CD20
148 Nd	CD38
149 Sm	CCR4
150 Ns	CD43
151 Eu	CD14
152 Sm	TCRgd
153 Eu	CD45RA
154 Sm	CD45
156 Gd	CXCR3
158 Gd	CD33
159 Tb	CCR7
160 Gd	CD28
161 Dy	CD32
162 Dy	CD69
163 Dy	HLA-DR
164 Dy	CD45RO
165 Ho	CD16
166 Er	CD44
167 Er	CD27
168 Er	CD8
169 Tm	CD25
170 Er	CD3
171 Yb	CXCR5
172 Yb	CD57
174 Yb	PD-1
175 Lu	PD-L1
176 Yb	CD56
191/193 Ir	Nucleic Acid
195 Pt	Viability

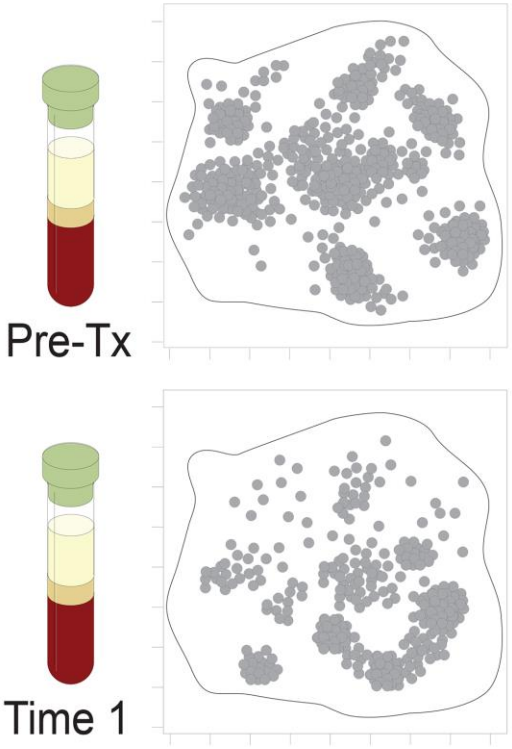
Custom conjugates
Commercially available

Greenplate et al., *Cancer Immunology Research* 2019

Clinical Trial Monitoring: What Do We Need to Know? Automate Four Key Readouts vs. Clinical Outcomes

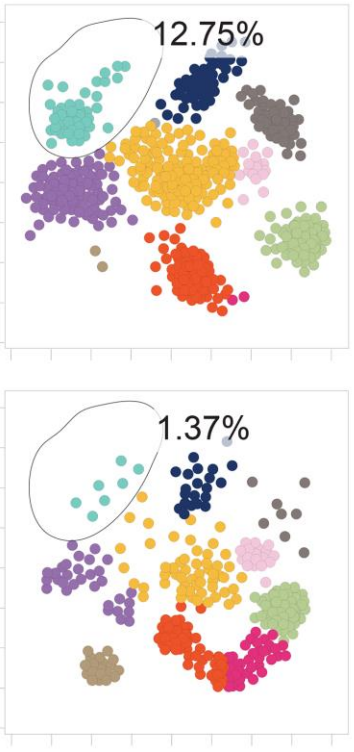
Features of Dynamic Populations

1 Systems Plasticity



Earth Mover's Distance on t-SNE or UMAP

2 Population abundance



Traditional gating or cluster frequency

3 Signature features

Pre-therapy

▲HLADR^{+2} CCR5^{+1} CD38^{+1} CD33^{+1}
▼CD8^{-8} CD45RO^{-6} CD3^{-6} CD4^{-4} CD45^{-2} CCR4^{-1} CCR7^{-1} CD28^{-1} CD27^{-1}

Time point 1

▲HLADR^{+2} CD38^{+1} CD45RA^{+1}
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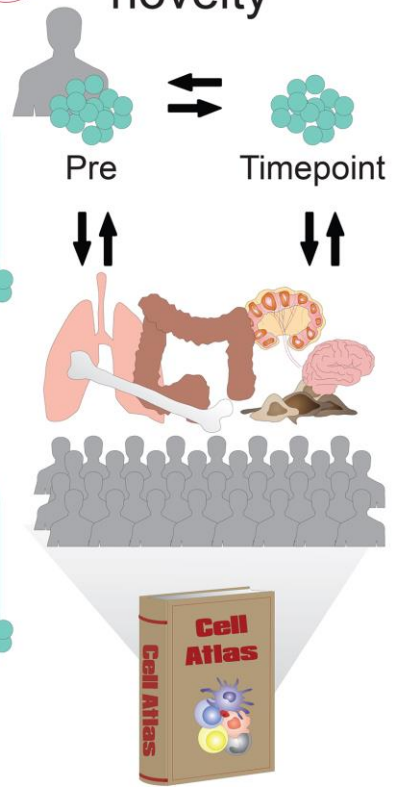
Marker Enrichment Modeling (MEM)

4 Population novelty



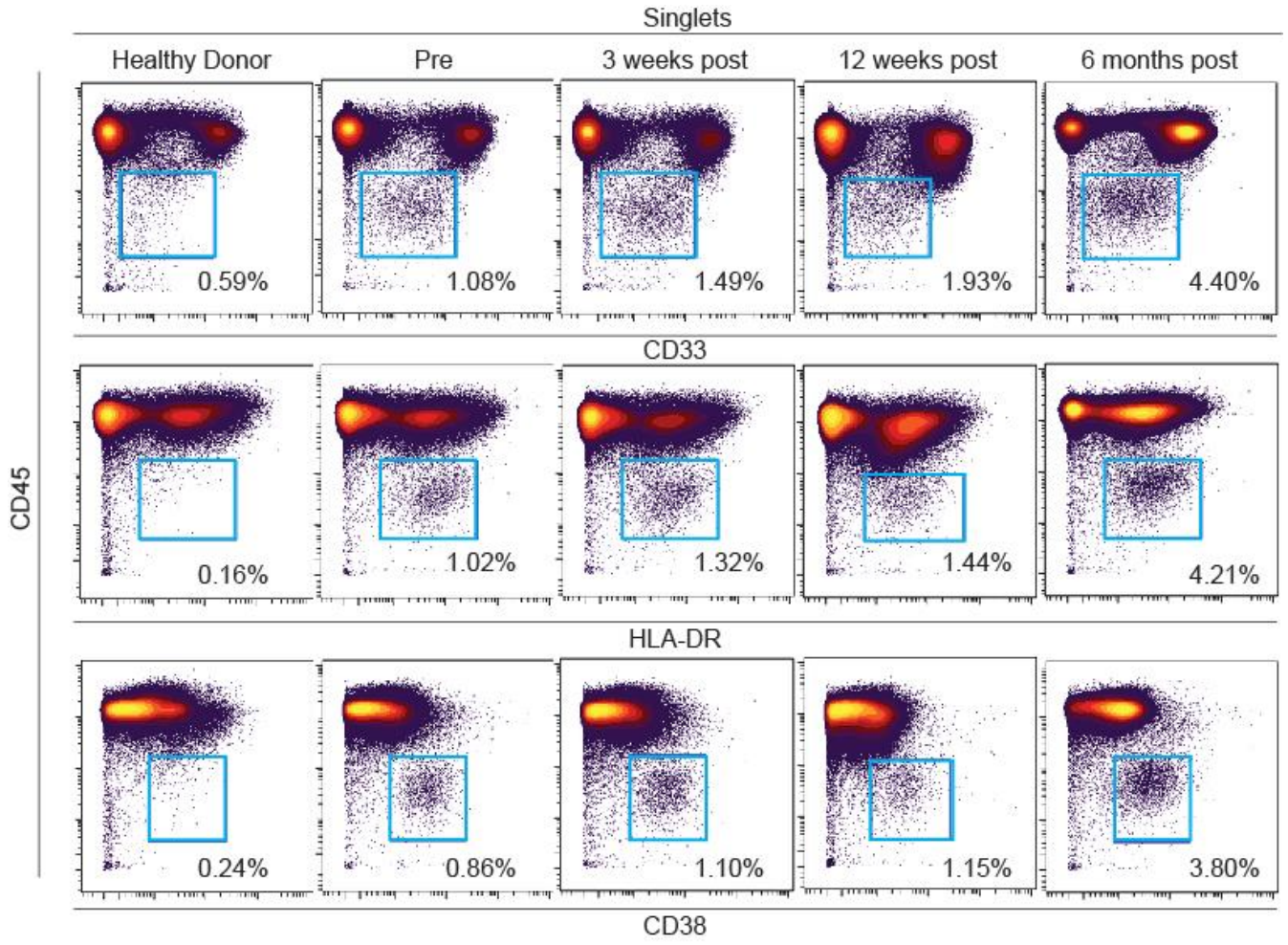
ΔMEM vs. Timepoint or Cell Atlas

How we quantified

Greenplate et al., *Cancer Immunology Research 2019*

A Case Study: Systems Immune Monitoring with Mass Cytometry Reveals A Clinically Significant Rare Cell Subset

MDS in Melanoma Patient Revealed During α-PD-1 Therapy



Mass cytometry data (CyTOF)

Healthy donor looks similar to melanoma in 2D views

At Pre-Tx, MDS blasts were not detected by standard CBC

High dimensional panel allowed review of PD-1 on MDS blasts w/ existing data

Melanoma data: https://flowrepository.org/id/FR-FCM-ZYDG

Greenplate et al., *Cancer Immunology Research* 2016

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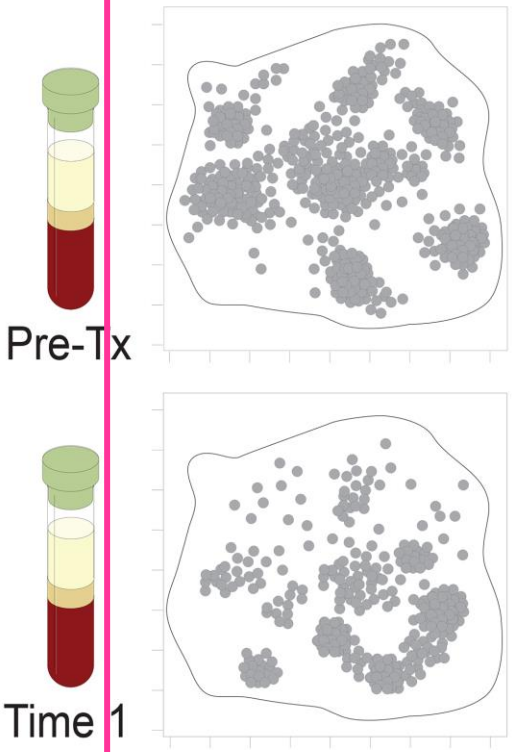
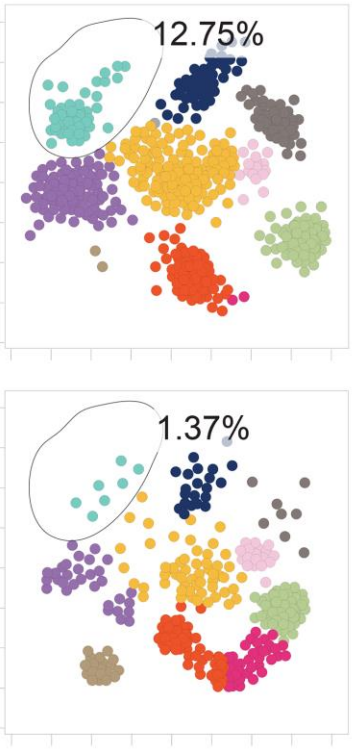


Earth Mover's Distance on t-SNE or UMAP

2 Population abundance



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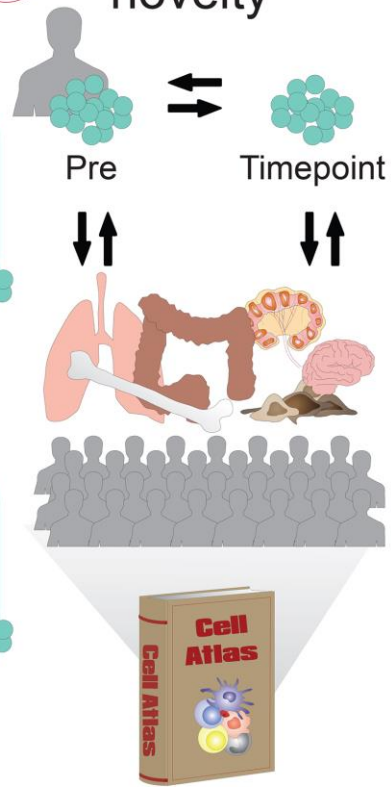


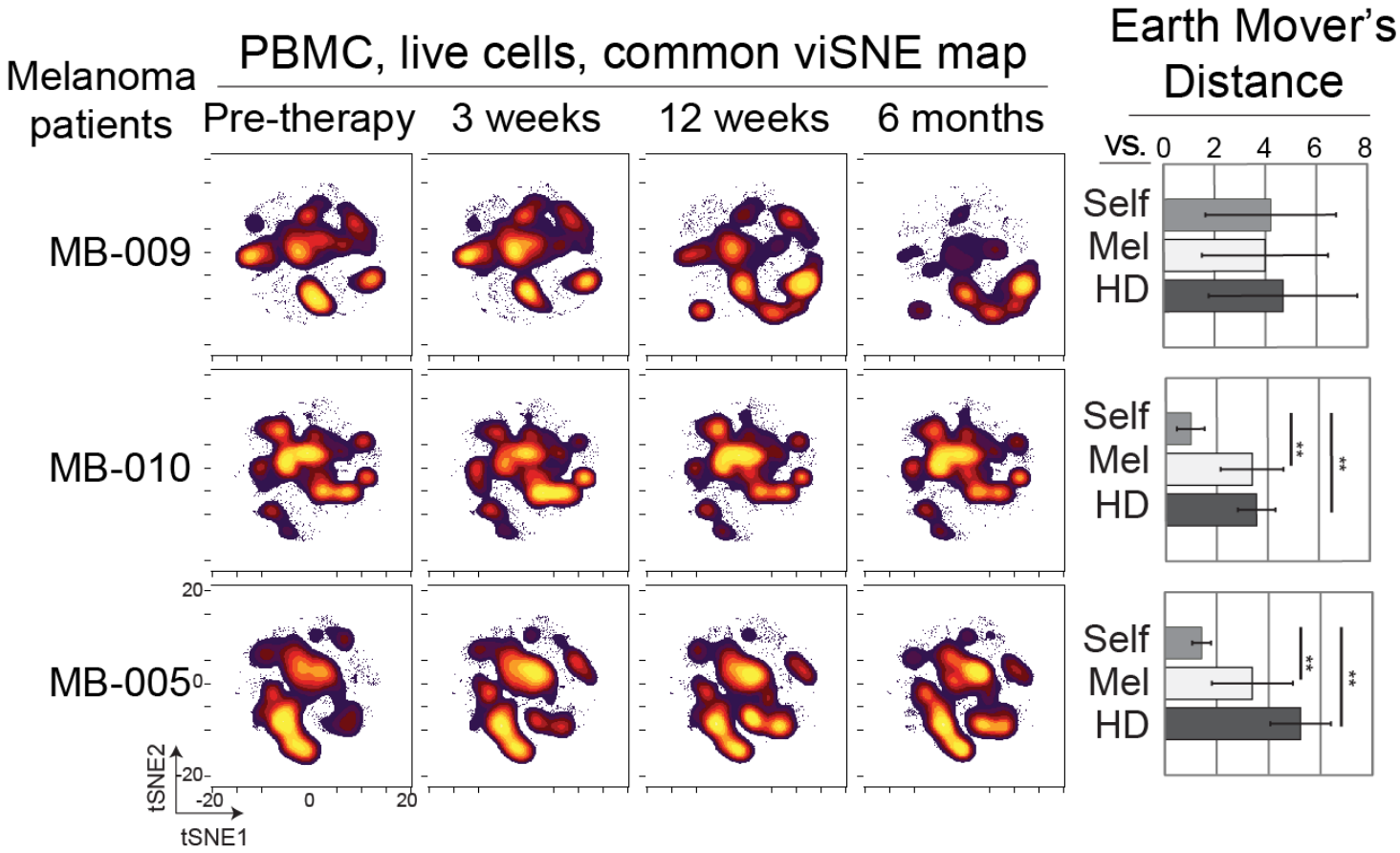
ΔMEM vs. Timepoint or Cell Atlas

How we quantified

Greenplate et al., *Cancer Immunology Research 2019*

Plasticity / Stability: Earth Mover's Distance Quantifies Change Over Time Within a t-SNE Analysis

Melanoma Patients Treated with α-PD-1 Therapy, Monitored by Mass Cytometry



Systems immune monitoring reveals an unexpected pattern in MB-009
Individuals can be their own significantly stable baseline

Melanoma data: https://flowrepository.org/id/FR-FCM-ZYDG

Greenplate et al., *Cancer Immunology Research* 2019
MB-009 Case Study from Greenplate et al., *CIR* 2016

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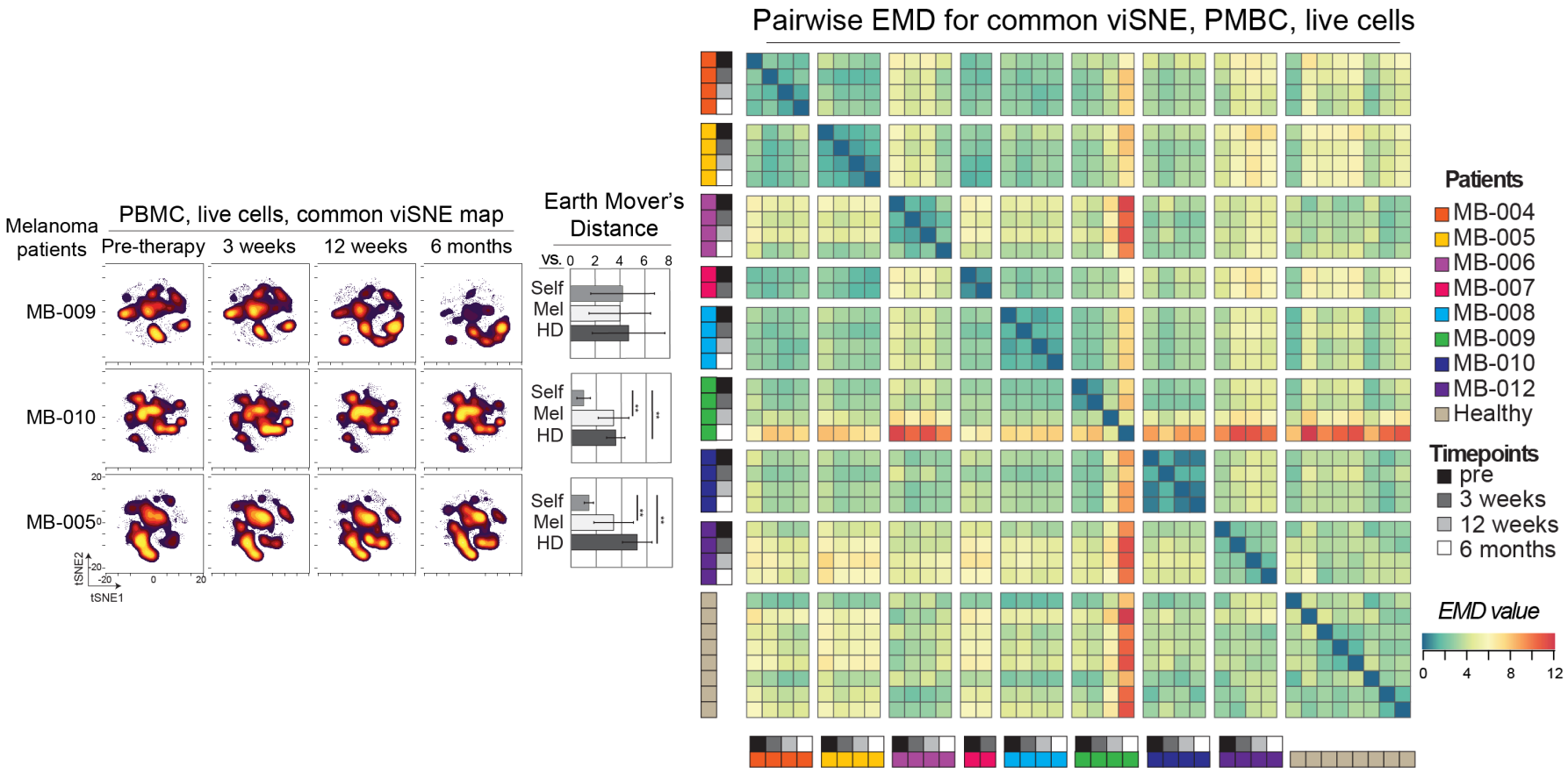

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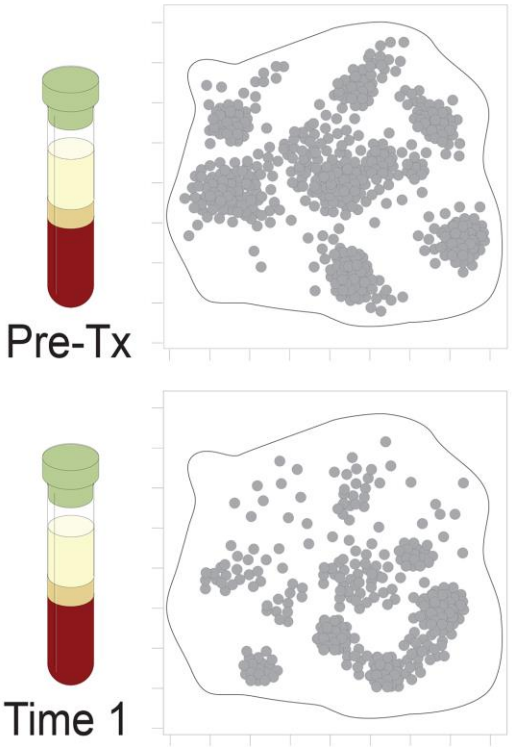

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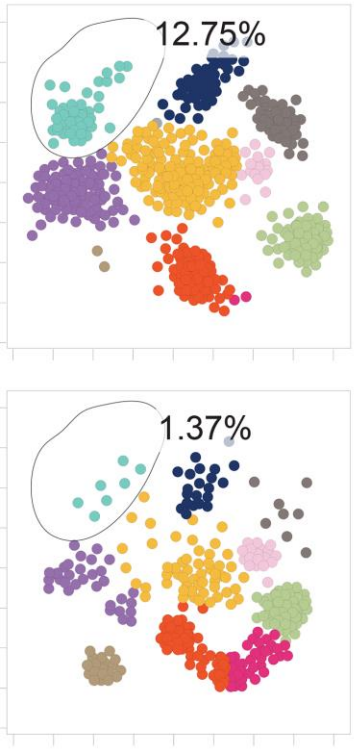

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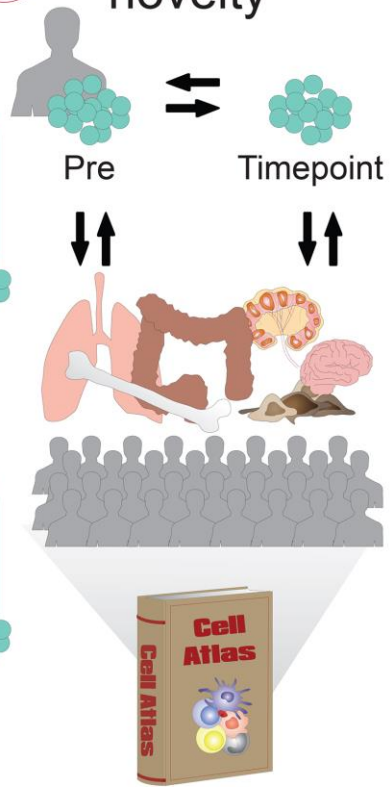

ΔMEM vs. Timepoint or Cell Atlas

How we quantified

Greenplate et al., *Cancer Immunology Research 2019*

Clinical Trial Monitoring: What Do We Need to Know? Automate Four Key Readouts vs. Clinical Outcomes

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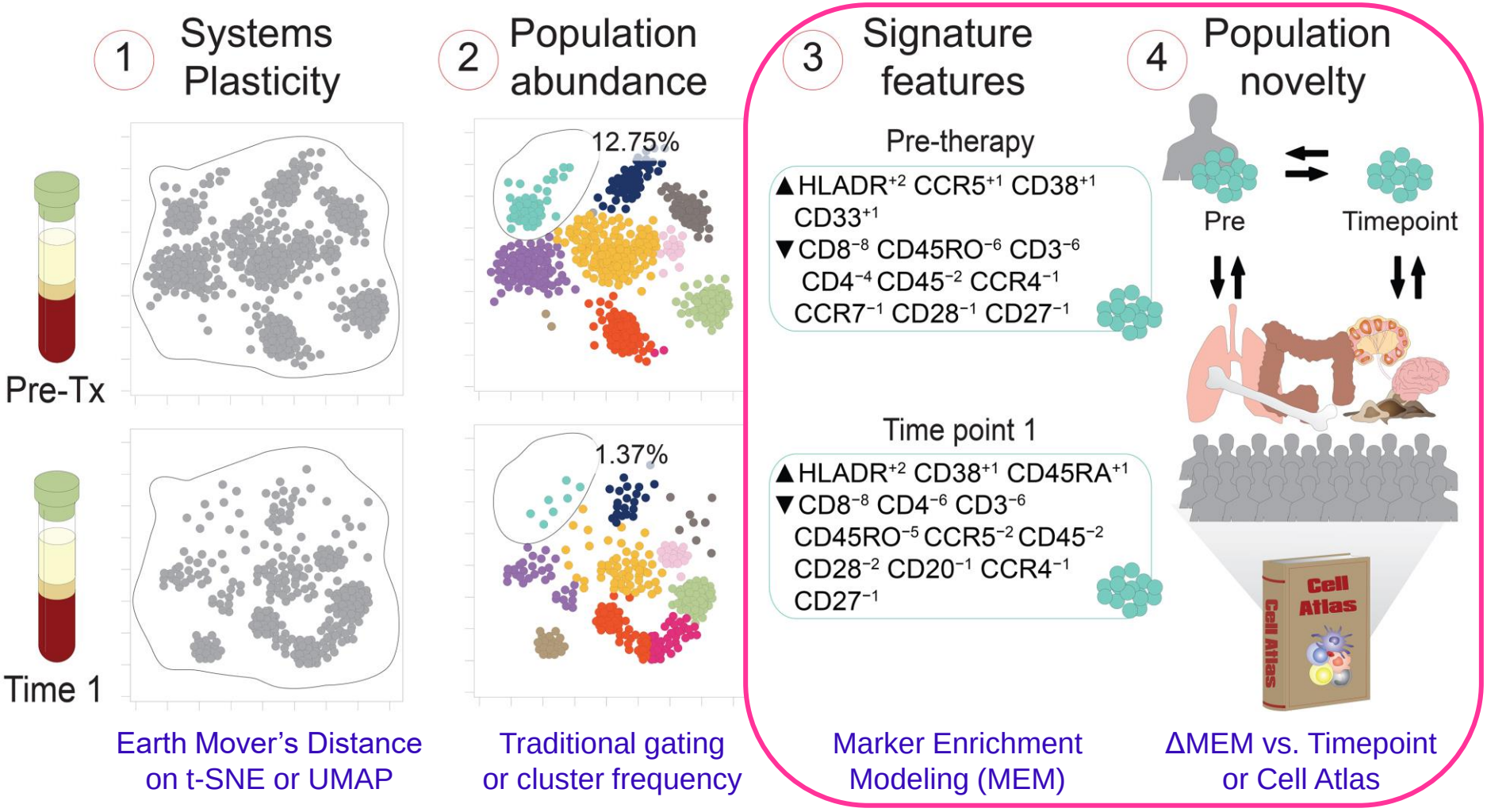
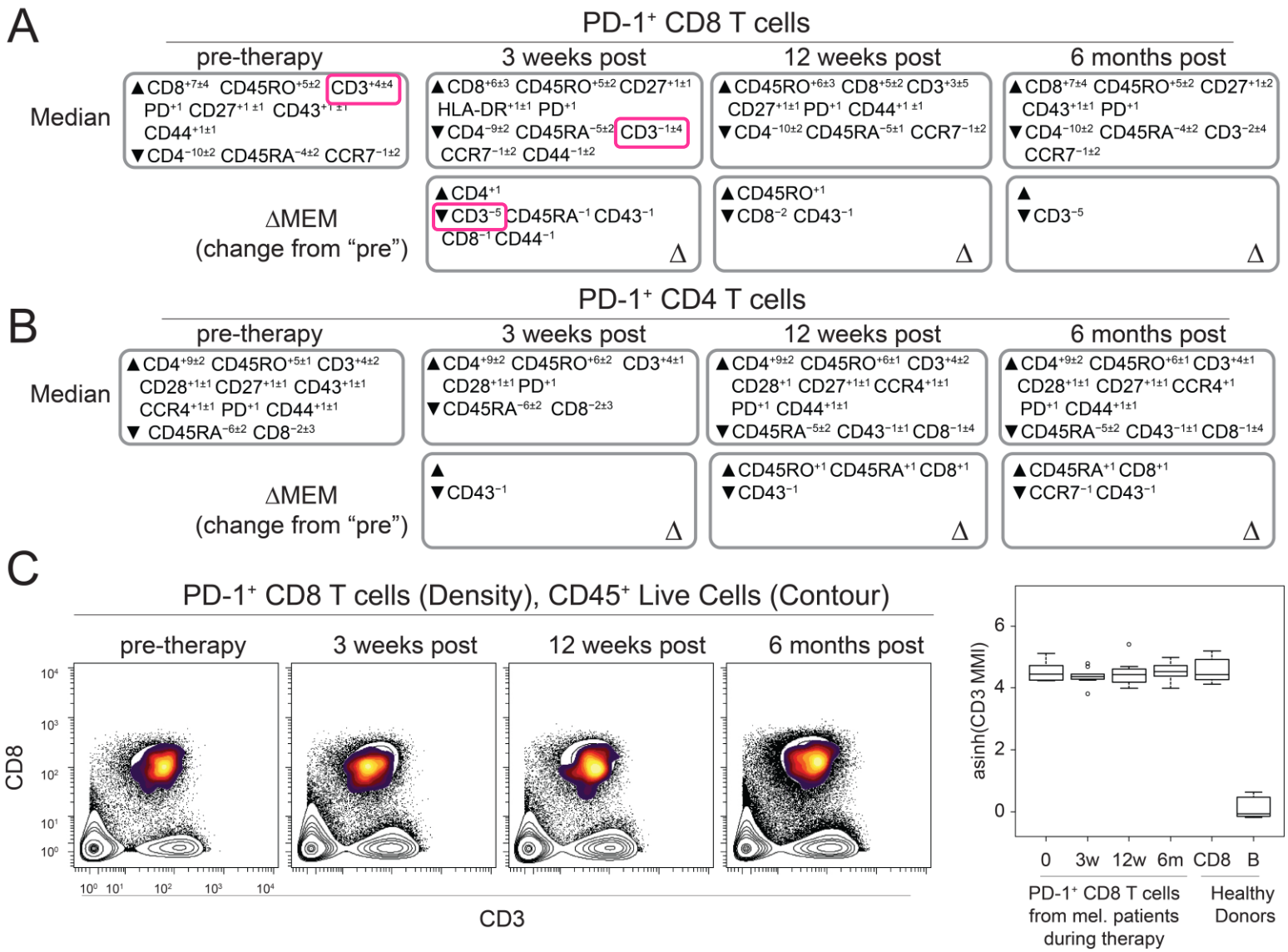


How we quantified

Greenplate et al., *Cancer Immunology Research 2019*

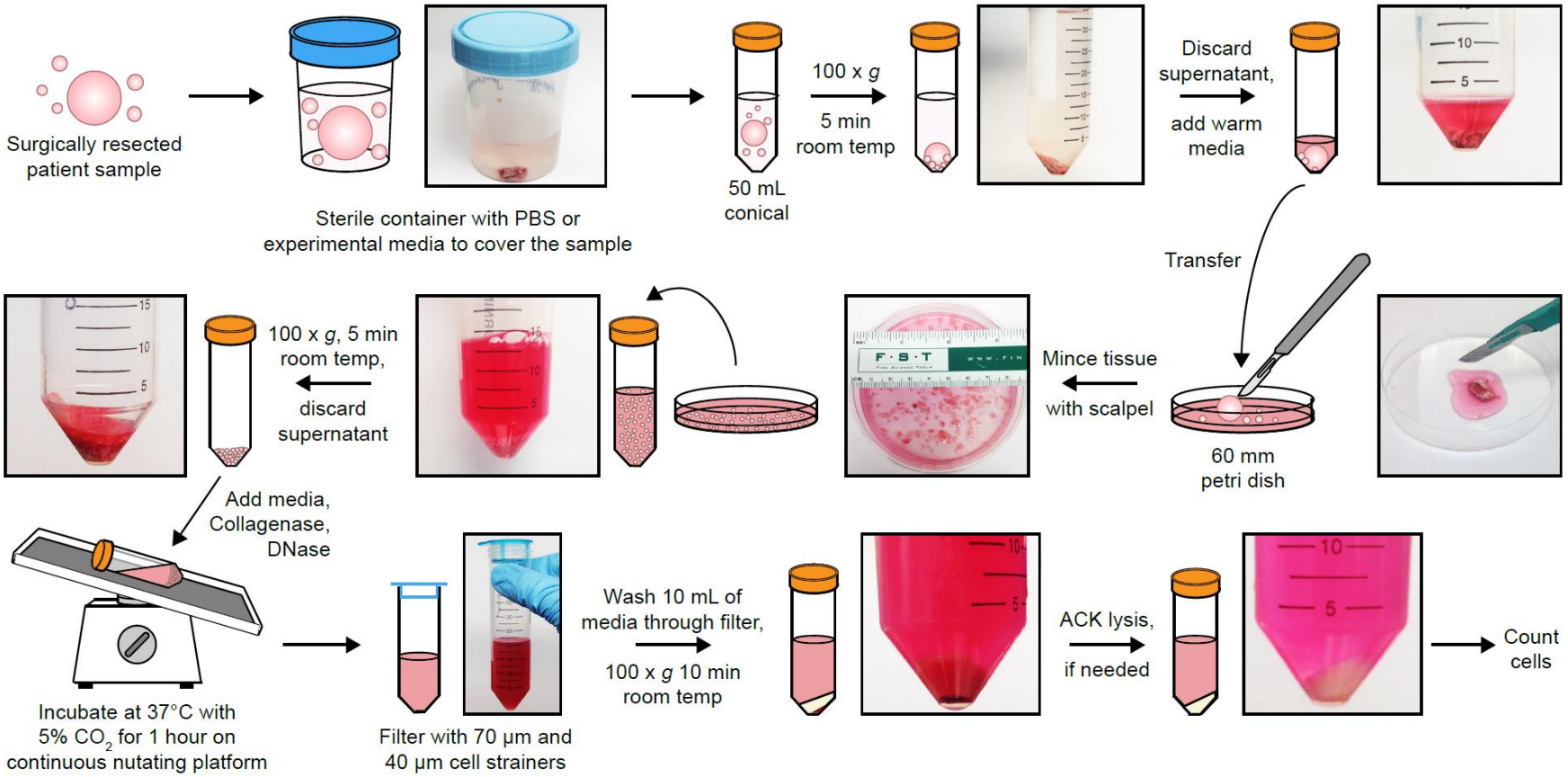
ΔMEM Reveals CD8⁺ Specific Decrease in Per-Cell CD3 in Melanoma Patient PBMC at 3 Weeks after α-PD-1 Therapy



Melanoma data: https://flowrepository.org/id/FR-FCM-ZYDG

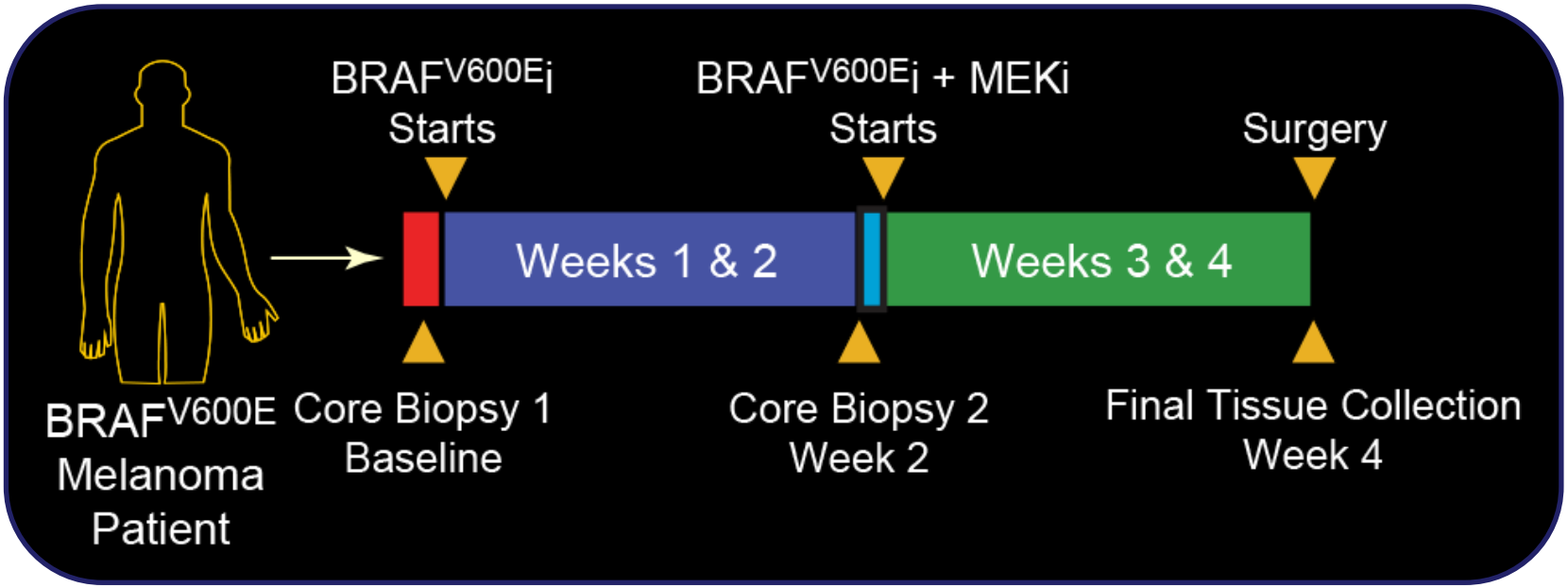
Greenplate et al., *Cancer Immunology Research* 2019

Tissue Biopsies Go from the Operating Room to the Lab and Viable Single Cells Isolated for Functional Studies



Protocol: Leelatian et al., *Current Protocols in Molecular Biology* 2017
Original research: Based on Leelatian and Doxie et al., *Cytometry B* 2017
CIC services: https://my.vanderbilt.edu/cancerimmunology/

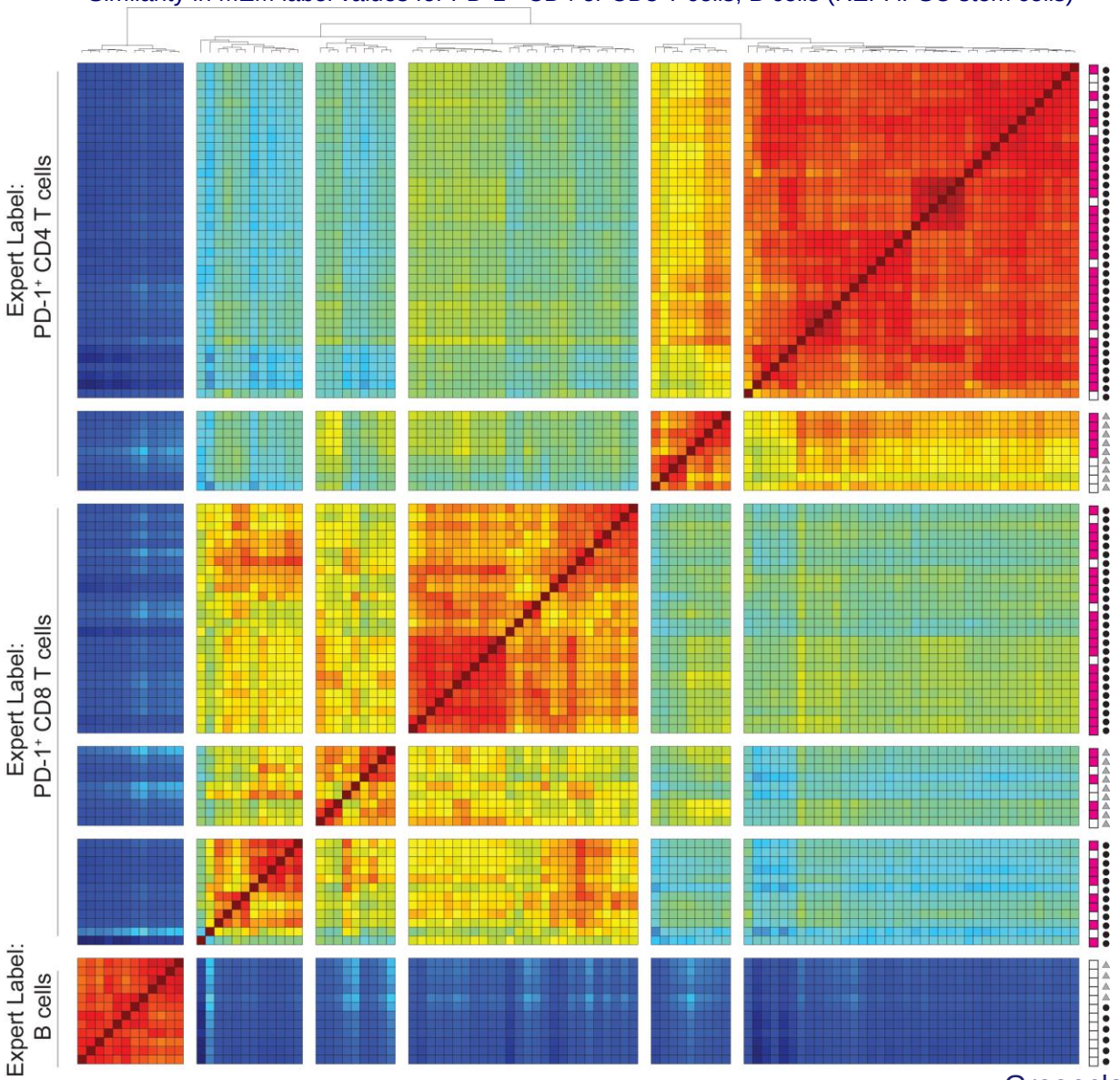
Identify and Characterize Tumor & Immune Cells That Regress or Persist During Clinical Trial Therapies

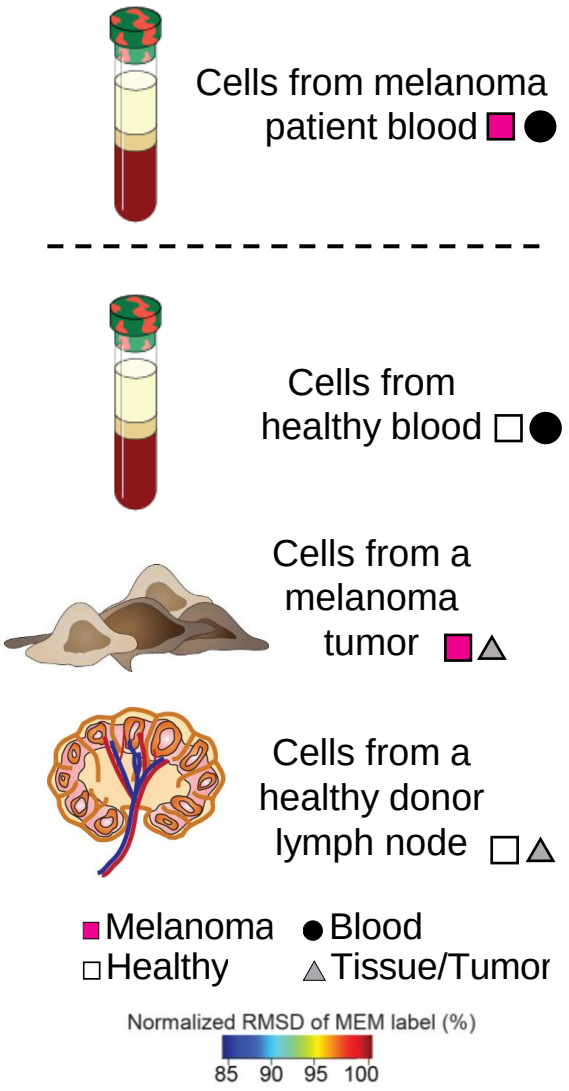


Melanoma patient tissues are brought directly from surgery to the lab for mass cytometry + machine learning single cell analysis

Doxie et al., *Pigment Cell & Melanoma Research* 2018
Greenplate et al., *Cancer Immunology Research* 2019

Distinct Phenotypes of PD-1^{+} CD8^{+} T cells in Melanoma Tumors Revealed by Quantitatively Comparing MEM Text Labels





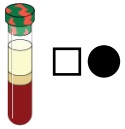
Greenplate et al., *Cancer Immunology Research* 2019

Data files: http://flowrepository.org/id/FR-FCM-ZYCC

Methods: Diggins et al., *Nature Methods* 2017; *Curr Prot Cyt* 2018

Blood vs. Tumor: CXCR3 Is Enriched on PD-1^{+} CD4 and CD8 Melanoma TIL; CD57 Is Gained on Tumor Infiltrating PD-1^{+} CD8

PD-1^{+} **CD8** T cells ΔMEM

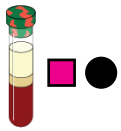


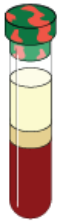
Melanoma tumor	Healthy blood
▲CD43^{+3} CD57^{+2} CCR4^{+2} CXCR3^{+2} CD27^{+1} CD44^{+1} ▼CD38^{-2} CD69^{-2} CD45RO^{-2} Δ	▲CCR4^{+2} CD45RO^{+1} ▼CD57^{-2} Δ

PD-1^{+} **CD4** T cells ΔMEM

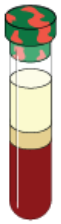
Melanoma tumor	Healthy blood
▲CD4^{+3} CD43^{+2} CXCR3^{+2} CCR5^{+1} CD44^{+1} ▼CD69^{-2} CD45RO^{-2} CD38^{-1} ICOS^{-1} Δ	No difference Δ

ΔMEM labels each subset vs. cognate in melanoma blood



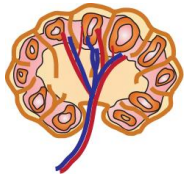


Cells from melanoma patient blood



Cells from healthy blood

Cells from a melanoma tumor



Cells from a healthy donor lymph node

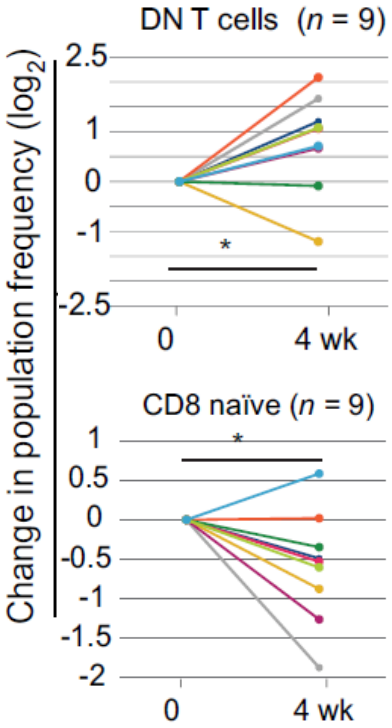
Melanoma ● Blood
□ Healthy △ Tissue/Tumor

Greenplate et al., *Cancer Immunology Research* 2019

Data files: http://flowrepository.org/id/FR-FCM-ZYCC

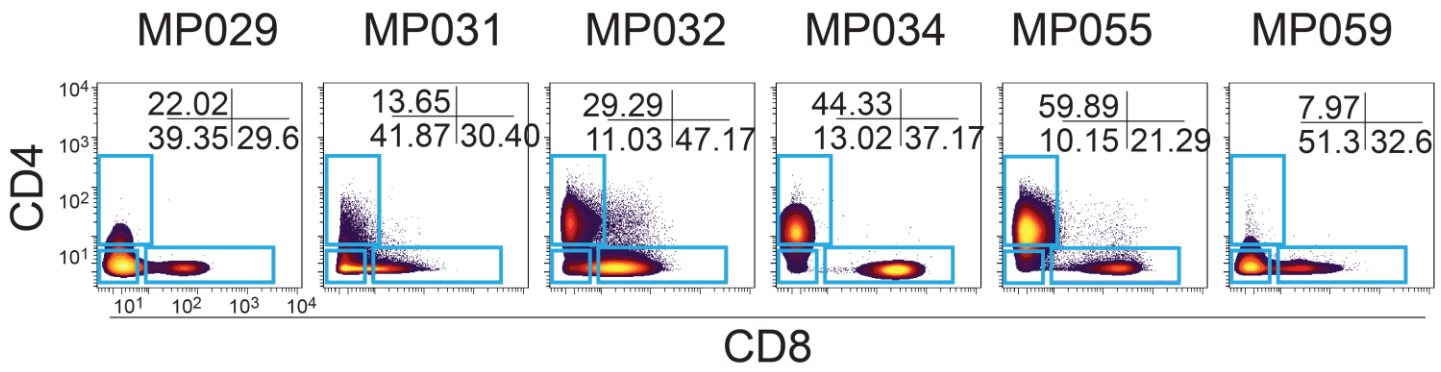
Methods: Diggins et al., *Nature Methods* 2017; *Curr Prot Cyt* 2018

Abnormal CD4^{-} CD8^{-} Double Negative (DN) T cells Are Enriched in Melanoma Tumors Following BRAFi + MEKi

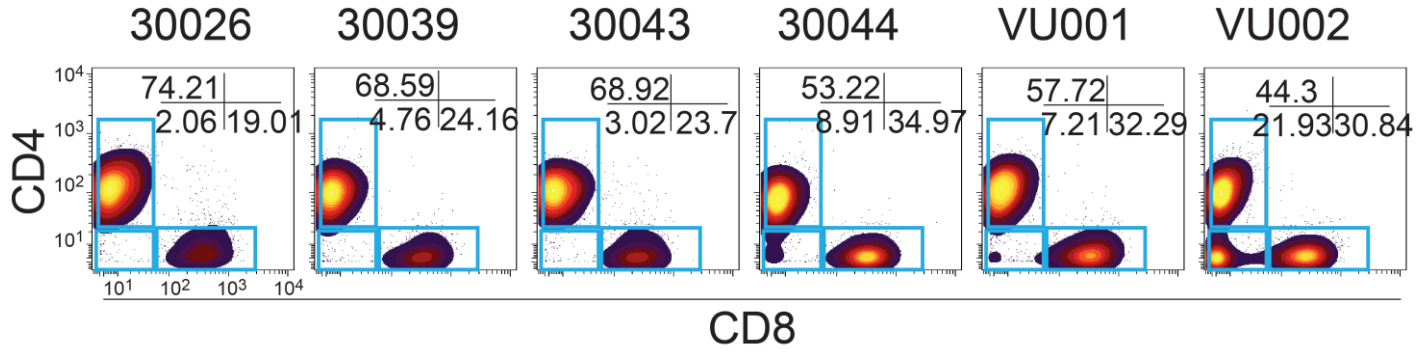


(DN T cells here were γδ negative)

Melanoma patient biopsy, all CD3+ tumor-infiltrating T cells (4 weeks after BRAF + MEK inhibitor therapy start)



Comparison point: activated T cells from healthy blood (Staphylococcal enterotoxin B stimulated)

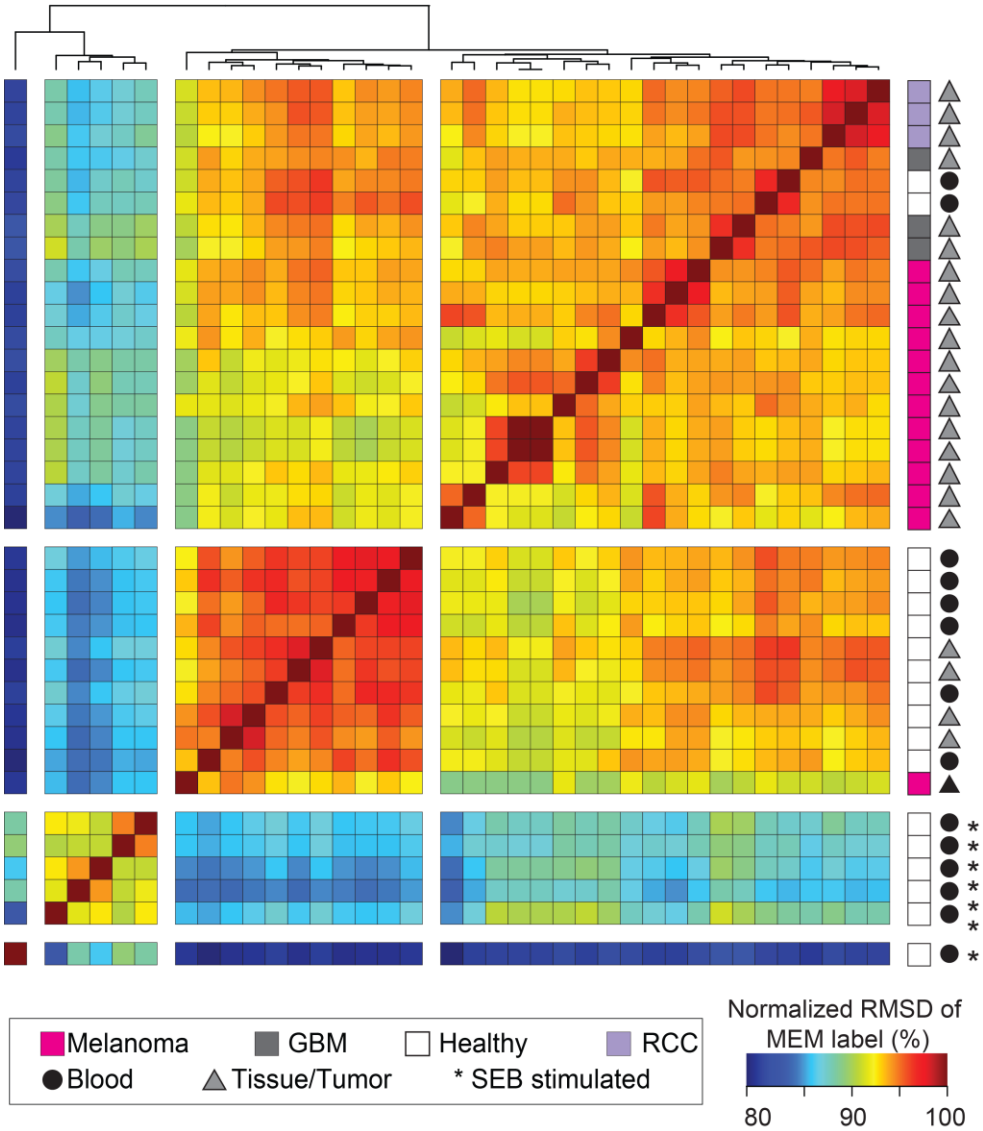


Data files: http://flowrepository.org/id/FR-FCM-ZYCC

Greenplate et al., *Cancer Immunology Research* 2019

Similar Abnormal Tumor-infiltrating $CD4^-$ $CD8^-$ DN T cells Are Observed Across Diverse Tumor Types

Comparing MEM labels for DN T cells across human tissues



Median MEM Labels iPCs as reference

RCC Tumor

▲$CD45^{+7\pm1}$ $CD45RO^{+2\pm0}$ $CD3^{+2\pm0}$ $CD44^{+2\pm1}$
▼$CD57^{-5\pm0}$ $CD56^{-3\pm0}$ $CCR7^{-1\pm0}$ $PD1^{-1\pm0}$
$CD45RA^{-1\pm1}$

GBM Tumor

▲$CD45^{+8\pm1}$ $CD45RO^{+3\pm1}$ $CD3^{+2\pm0}$ $CD69^{+2\pm1}$
$CD44^{+2\pm1}$
▼$CD57^{-5\pm2}$ $CD56^{-3\pm1}$ $PDL1^{-2\pm0}$ $CCR5^{-1\pm1}$

Melanoma Tumor

▲$CD45^{+7\pm3}$ $CD3^{+3\pm1}$ $CD45RO^{+2\pm3}$ $CD44^{+2\pm3}$
▼$CD57^{-6\pm2}$ $CCR4^{-3\pm1}$ $CD56^{-2\pm1}$ $PDL1^{-2\pm1}$

Non-malignant LN

▲$CD45^{+8\pm1}$ $CD3^{+4\pm0}$ $CD44^{+2\pm1}$ $CD45RA^{+1\pm2}$
▼$CD57^{-5\pm1}$ $CCR4^{-3\pm0}$ $CD56^{-3\pm0}$ $PDL1^{-2\pm0}$
$CCR7^{-1\pm0}$ $CXCR3^{-2\pm0}$

Healthy Donor PBMC

▲$CD45^{+10\pm1}$ $CD3^{+4\pm1}$ $CD44^{+2\pm1}$ $CD45RA^{+1\pm2}$
▼ $CD57^{-5\pm2}$ $CCR4^{-3\pm0}$ $CD56^{-3\pm0}$ $PDL1^{-2\pm0}$
$CCR7^{-1\pm0}$ $CD28^{-1\pm1}$ $CXCR3^{-1\pm1}$ $CCR5^{1\pm1}$

Activated T cells PBMC

▲$CD45^{+10\pm1}$ $CD45RO^{+6\pm2}$ $CD69^{+5\pm1}$ $CD4^{+4\pm2}$
$CD3^{+3\pm1}$ $CCR7^{+3\pm2}$ $HLADR^{+2\pm1}$ $CD8^{+2\pm1}$
$CD27^{+2\pm2}$ $CD25^{+2\pm3}$
▼$CD57^{-5\pm1}$ $CD16^{-1\pm0}$

Greenplate et al., *Cancer Immunology Research* 2019

Data files: http://flowrepository.org/id/FR-FCM-ZYCC

Methods: Diggins et al., *Nature Methods* 2017; *Curr Prot Cyt* 2018