Methods for Discovery & Characterization of Cell Subsets

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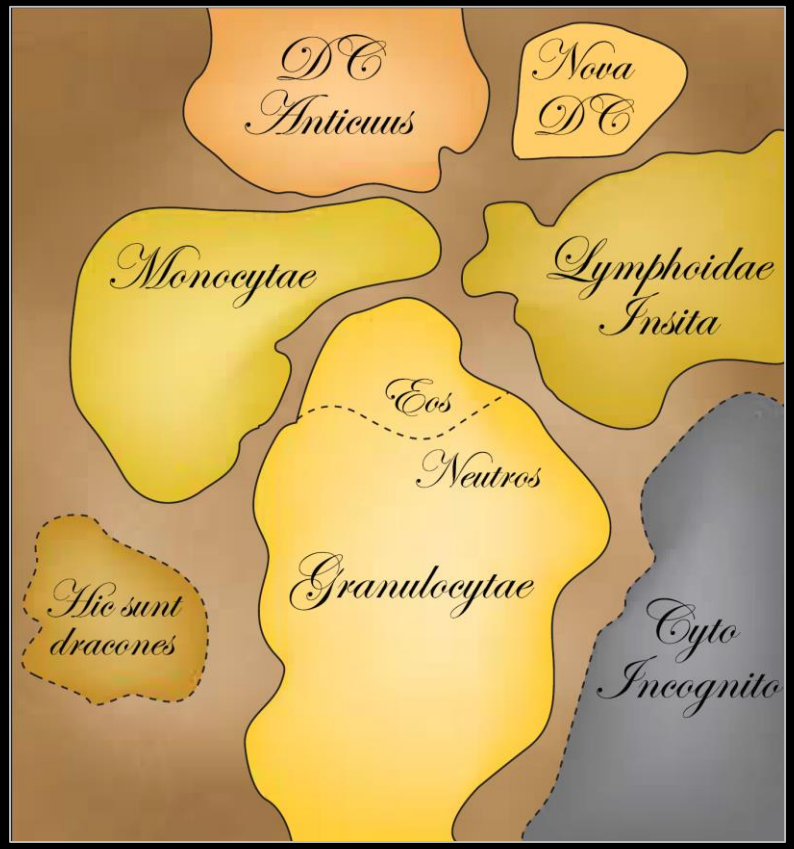
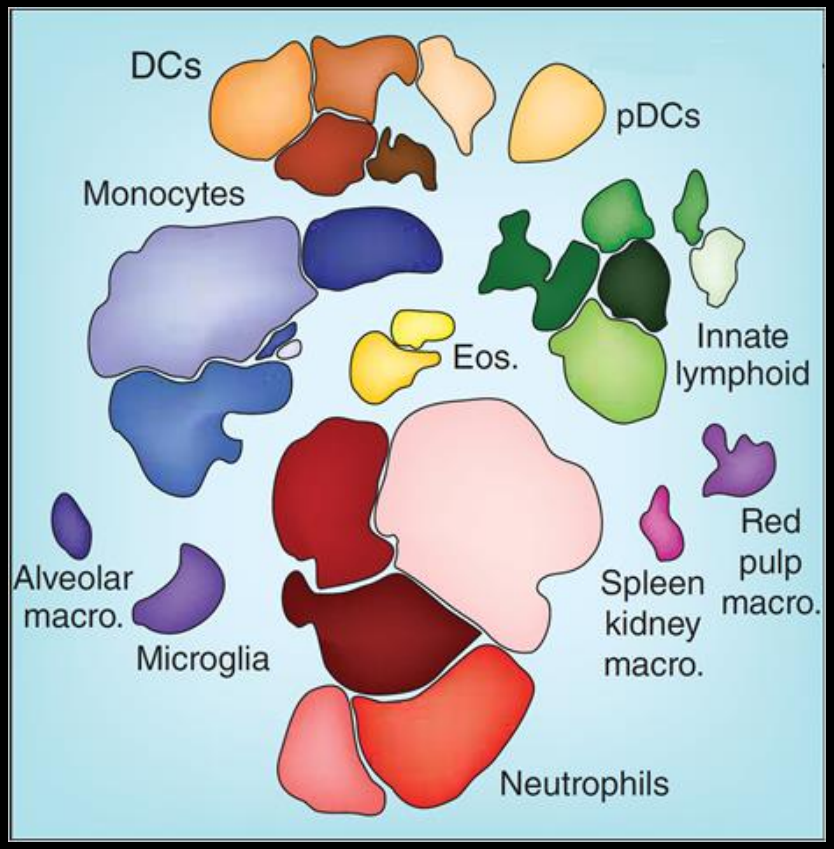
Australasian Cytometry Society

10 October 2015

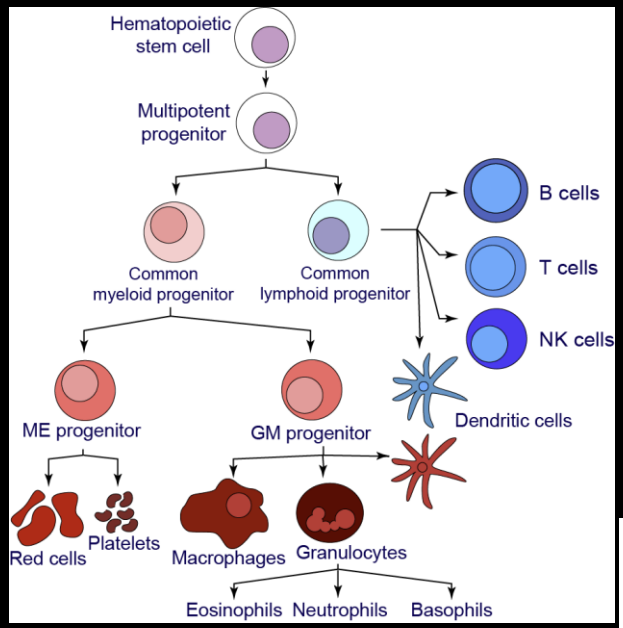


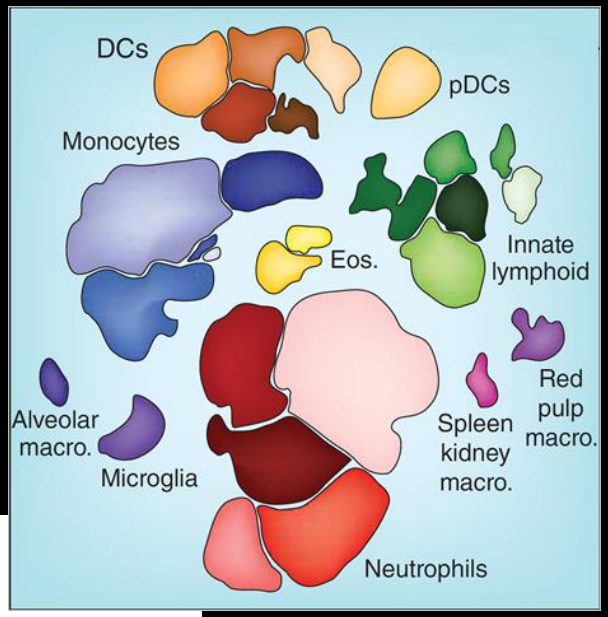


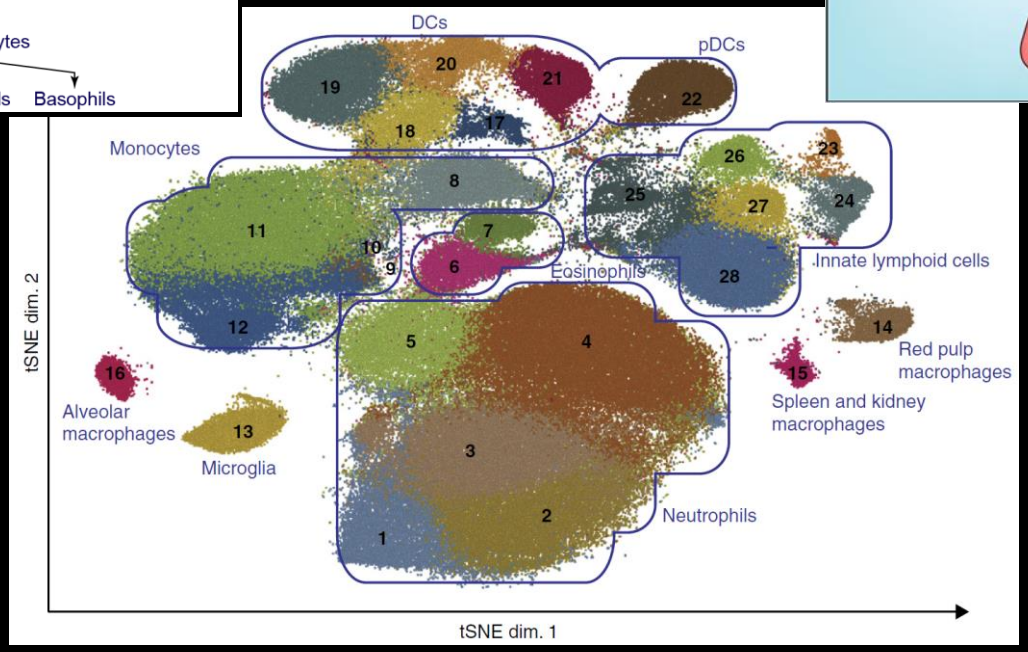
Disclosures for Jonathan Irish, Vanderbilt University

Co-founder & board	Cytobank
Clinical research	Incyte, Karyopharm
Speaking honorarium	Novartis
Invited speaker	Fluidigm

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



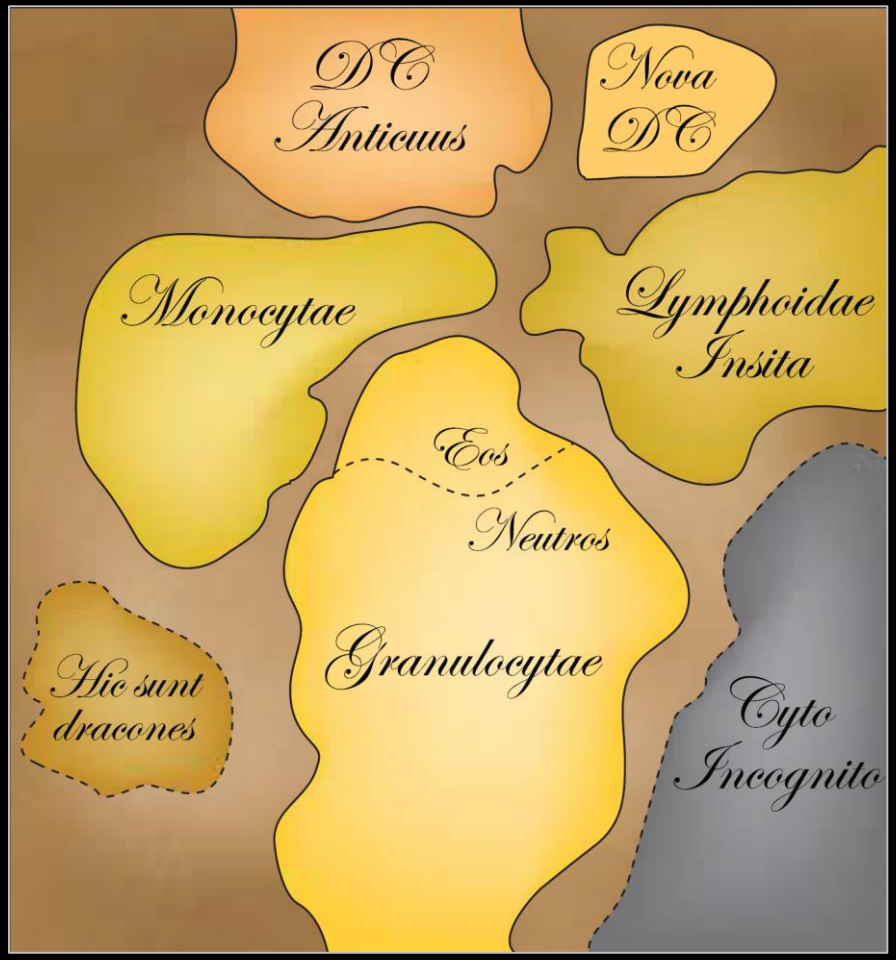




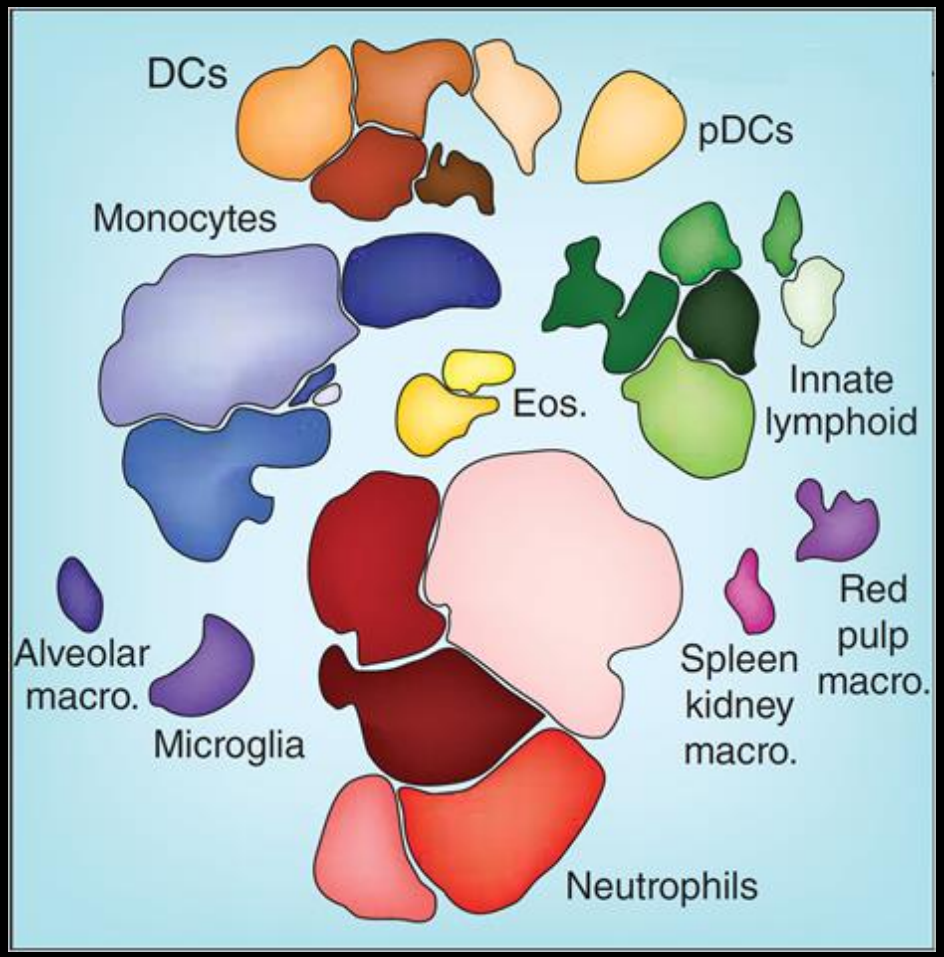
Irish, *Nature Immunology* 2014
Based on Becher et al., *Nature Immunology* 2014

Tools from Machine Learning + High Content Data: Comprehensive, Automatic Mapping of Cell Types

Classical map of the 'myeloid cell system'



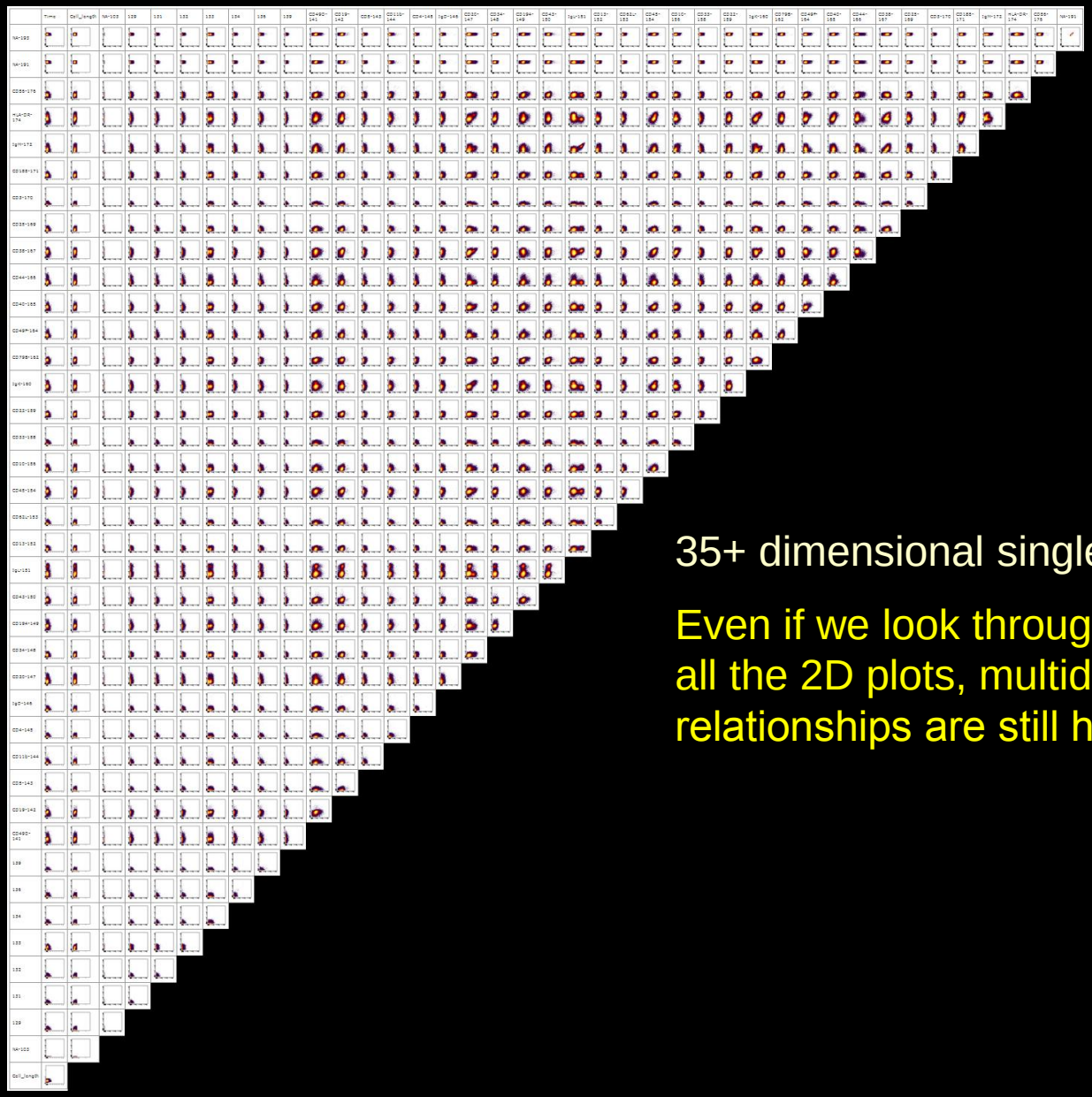
Modern map, computationally generated



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

Effective data analysis is critical to successful cytometry

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…

Unsupervised Analysis: Not Using Prior Knowledge To Guide the Analysis

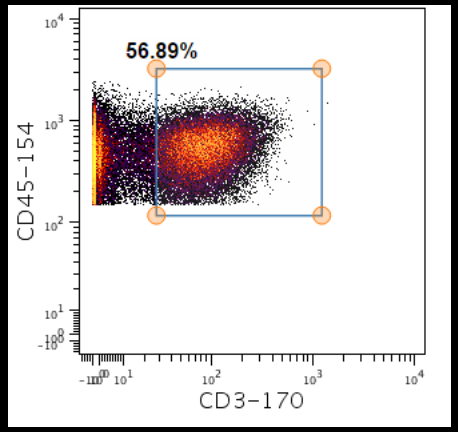
Prior knowledge examples: Stem cells express CD34, AML cells express CD45

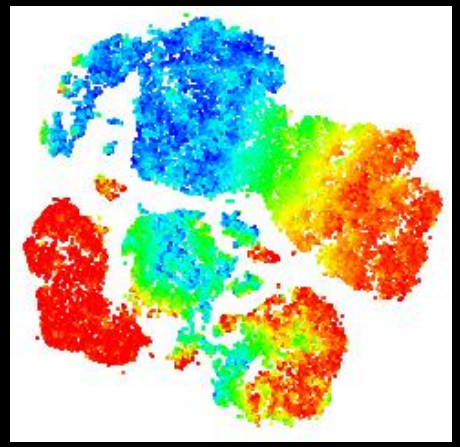
Supervised Approaches

- Expert gating
- Gemstone
- Wanderlust
- Citrus

Unsupervised Approaches

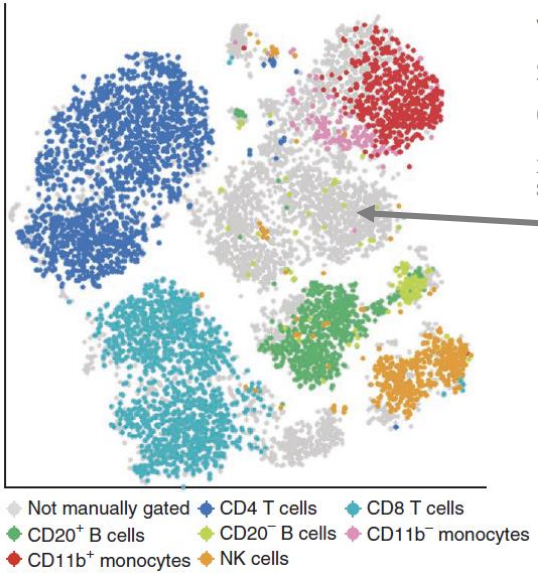
- Heatmap clustering
- SPADE
- viSNE
- Phenograph





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

Traditional Gating Overlooks Many Cells in Primary Samples

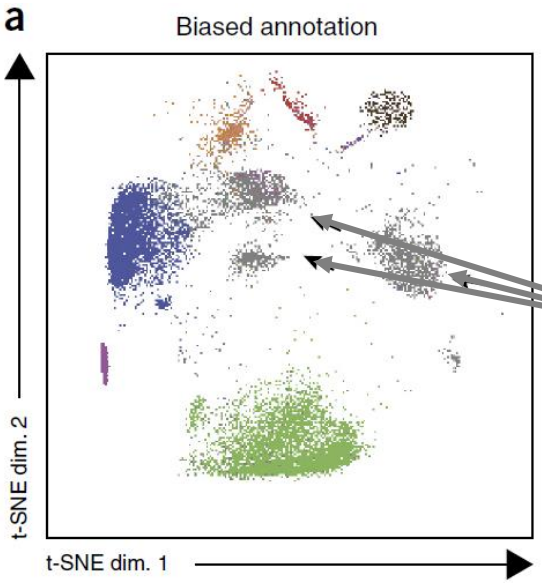


viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia

nature biotechnology
2013

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**).



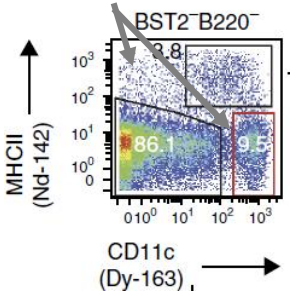
High-dimensional analysis of the murine myeloid cell system

nature immunology
2014

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

Major Steps in Most Single Cell Biology Workflows

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 gating
Revealing cell subsets	7) Feature selection 8) Dimensionality reduction 9) Identify cell clusters 10) Cluster refinement
Characterizing cell subsets	11) Feature comparison 12) Model populations 13) Learn cell identity 14) Statistical testing

How much can be automated?

Where do computers outperform humans?

How do we select tools and use them well?

Diggins et al., *Methods* 2015

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

Many Great Tools Exist, But Key Gaps Remain

Table 1 – A modular machine learning workflow for unsupervised high-dimensional single cell data analysis

Analysis step		Traditional	Additional methods§	Method here
Data collection	1) Panel design	Human expert	-	-
	2) Data collection	Human expert	-	-
Data processing	3) Cell event parsing	Instrument software	Bead normalization and event parsing [31]	-
	4) Scale transformation	Human expert	Logicle [36]	-
Distinguishing initial populations	5) Live single cell gating 6) Focal population gating	Biaxial gating + human expert	No event restriction, AutoGate [48]	viSNE + human expert (Figure 1)†
Revealing cell subsets	7) Select features	Human expert	Statistical threshold [40]	Human expert†
	8) Reduce dimensions or transform data	N/A	Heat plots [49], SPADE [12], t-SNE [50], viSNE [9], ISOMAP [23], LLE [25], PCA in R/flowCore [51]	SPADE†, viSNE
	9) Identify clusters of cells	Human expert	SPADE, k-medians, R/flowCore, flowSOM [52], Misty Mountain [13], JCM [26], Citrus [14], ACCSENSE [53], DensVM [24], AutoGate	SPADE (Figure 2)†, viSNE + human expert (Figure 1)
	10) Cluster refinement	Human expert	Citrus, DensVM, R/flowCore	-
Characterizing cell subsets	11) Feature comparison	Select biaxial single cell views	viSNE, SPADE, Heatmaps [34, 40], Histogram overlays [34, 40], Violin or box and whiskers plots [51]	Heatmaps (Figure 3A)†, viSNE (Figure 1C), SPADE (Figure 2C)
	12) Model populations	N/A	JCM, PCA	-
	13) Learn cell identity	Human expert	-	Human expert† (Figure 1B, Figure 2B, and Figure 3B)
	14) Statistical testing	Prism, Excel	R/flowCore	-

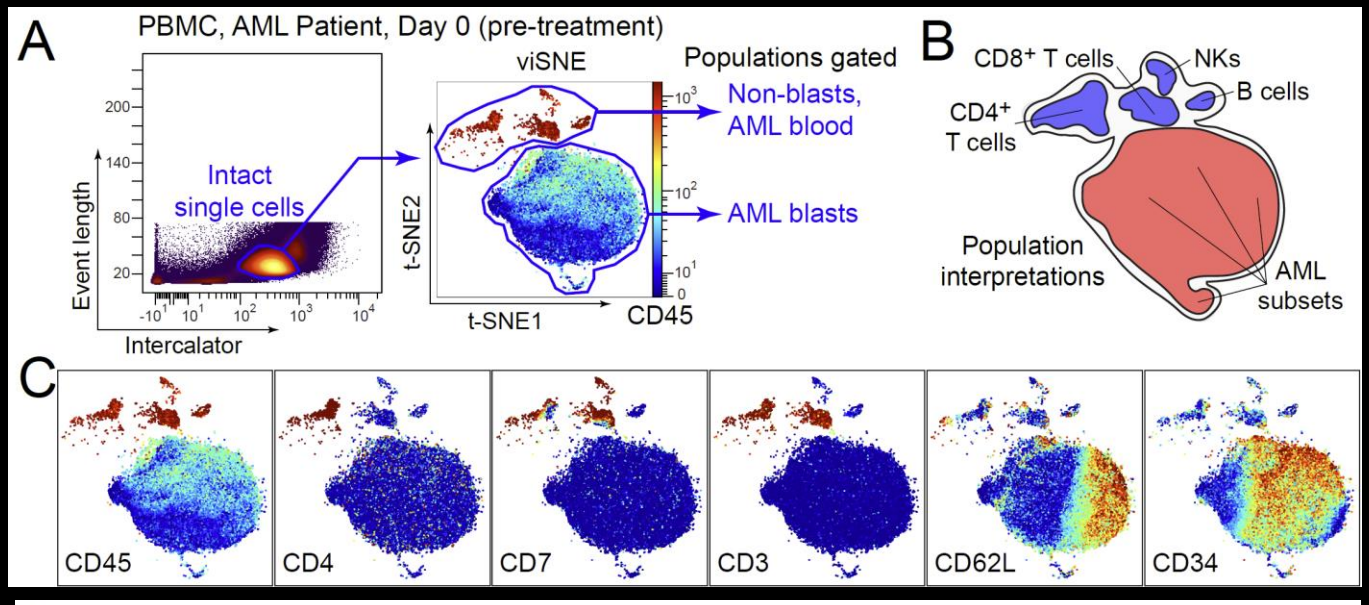
§Methods with broad application (e.g. R/flowCore) are listed minimally at select steps based on particular strengths or published applications.

†Denotes the primary approach used at each step in the sequential analysis workflow shown here.

A major gap in the field is in true <u>learning</u> of cell identity

Diggins et al., *Methods* 2015

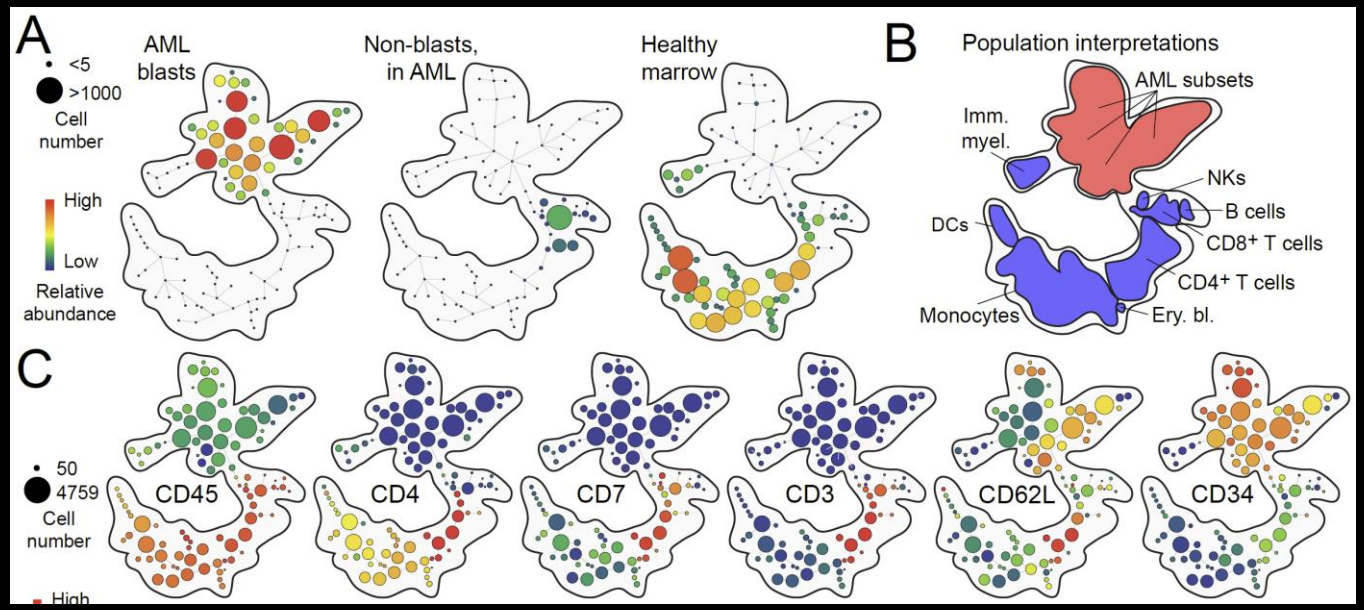
Key Analysis Concepts: Dimensionality Reduction, Transformation, Clustering, Modeling, Visualization, & Integration



viSNE

Amir et al.

Nature biotech 2013



SPADE

Qiu et al.

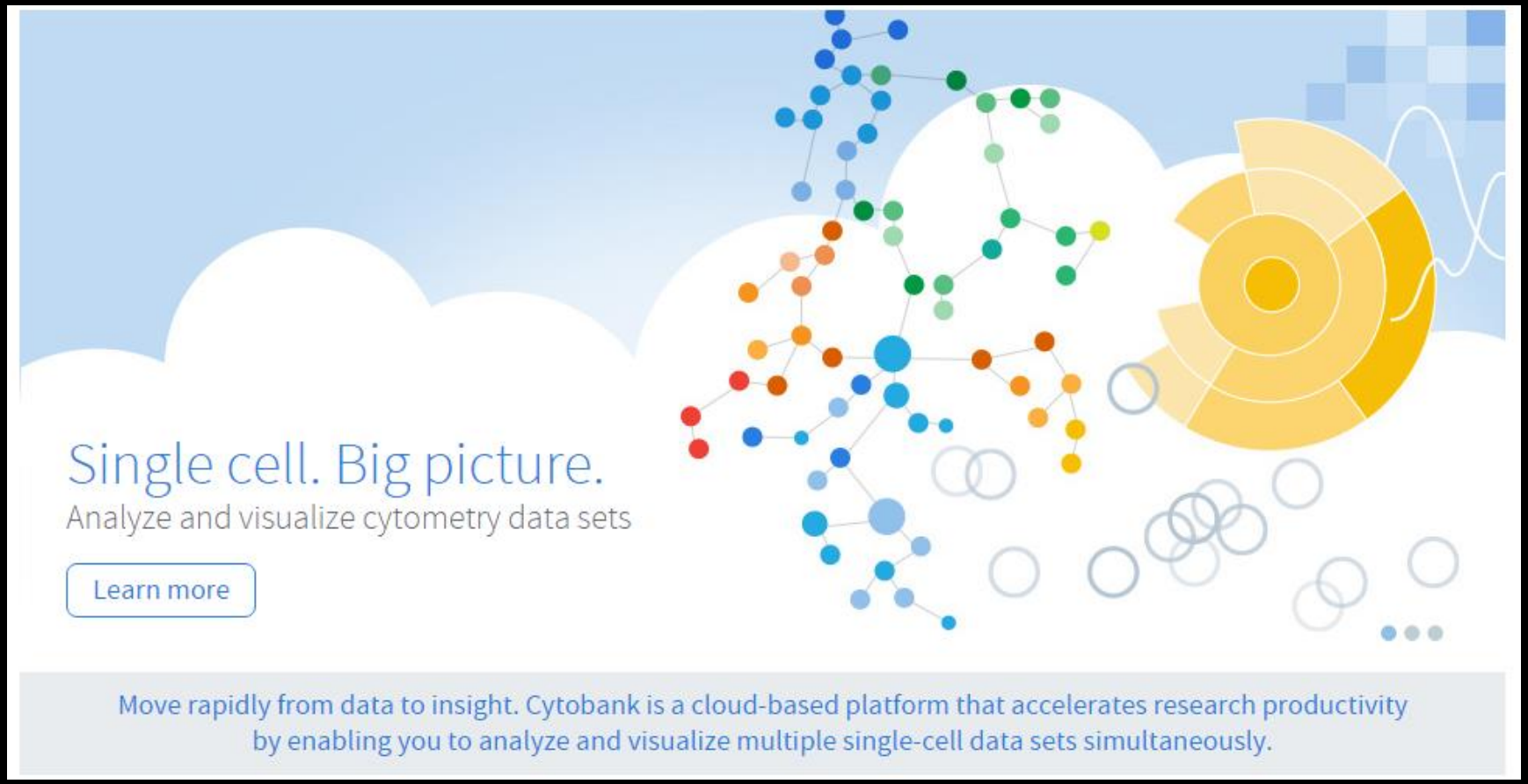
Nature biotech 2011

Diggins et al., *Methods* 2015

We Will Use Cytobank Software for viSNE & SPADE

Cytobank (www.cytobank.org) is a commercial tool
for web-based data storage, annotation, analysis, and visualization

30-day free trial with viSNE & SPADE: https://premier.cytobank.org/signup



Discussion Questions Covered in Today's Course

1) What are key differences between tools (viSNE, SPADE, PCA)? What is the difference between transforming, clustering, and modeling data? What type of modeling are we doing (if any)?

2) What does non-linear vs. linear analysis mean? Does the data's scale matter for analysis (arcsinh5, arcsinh15, linear)?

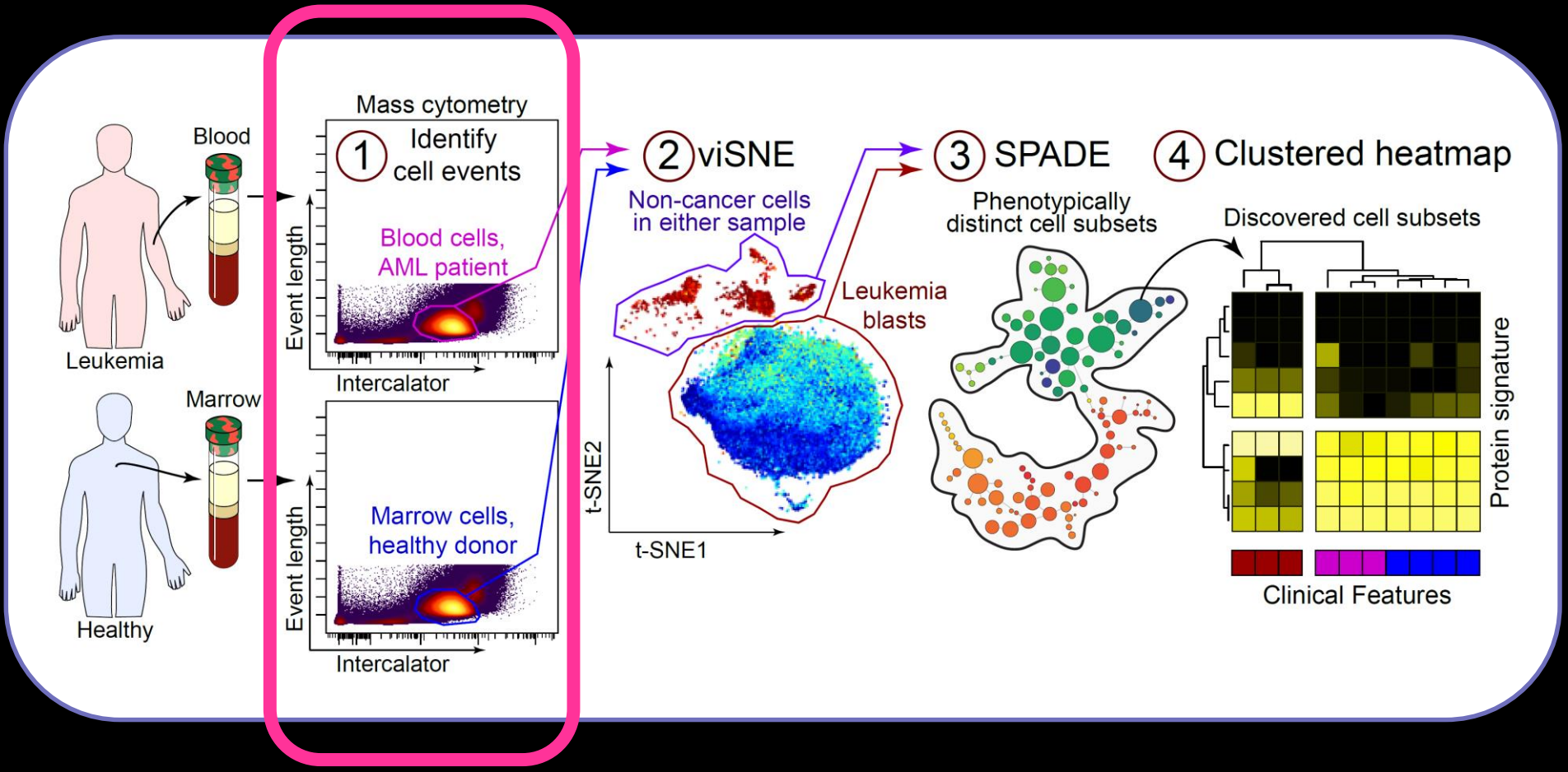
3) What do viSNE and SPADE settings do (viSNE iterations, SPADE downsampling & node #)? When should they be changed?

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

6) What are some "red flags" indicating problems? What does a good viSNE or SPADE analysis run look like?

Onward, to the analysis!

Discovery and Characterization of Cell Subsets: Towards Machine Learning Cell Identity



Diggins et al., *Methods* 2015

Single Cell Biology Workflow

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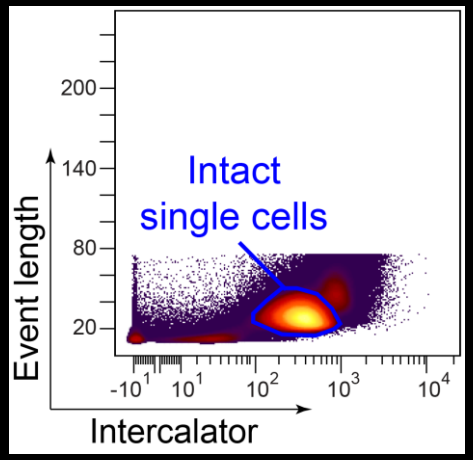
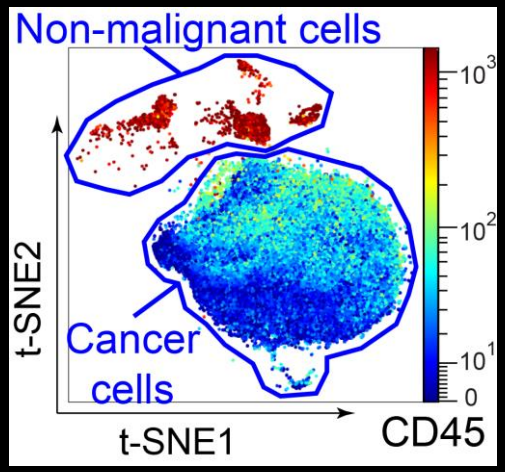
Expert gating



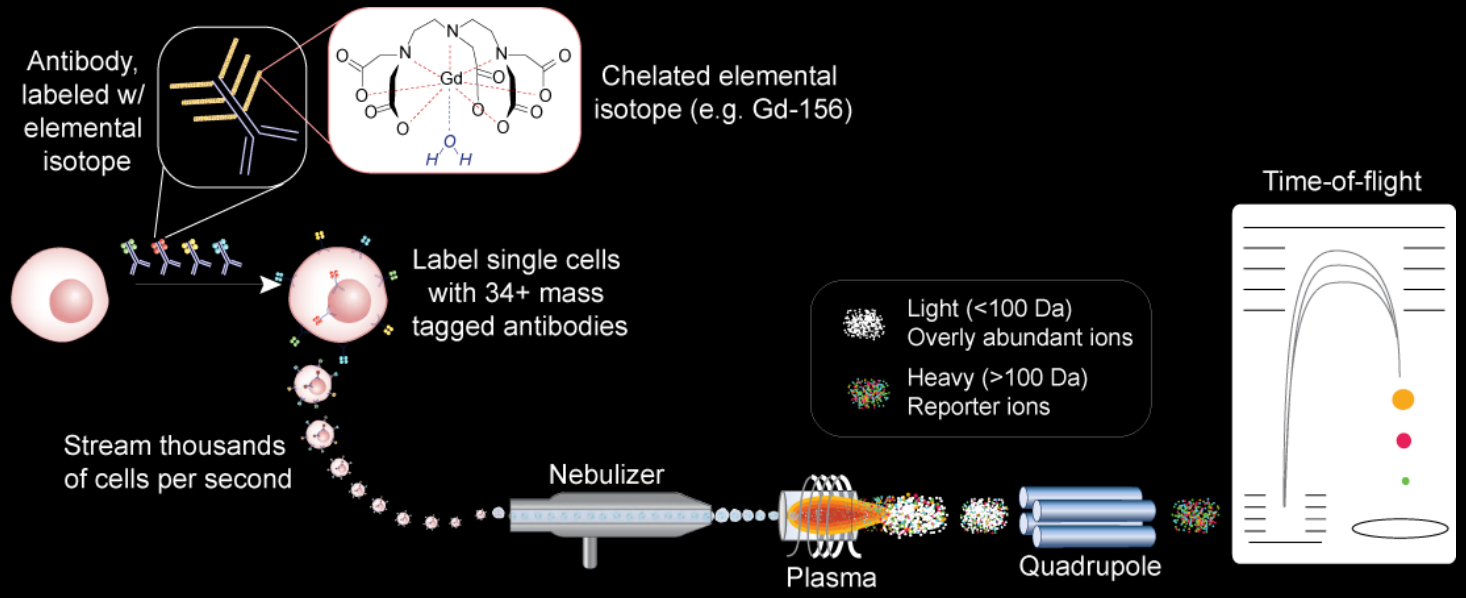
viSNE + expert gating



Diggins et al., *Methods* 2015

Mass Cytometry: 35+ Dimensional Analytical Cytometry

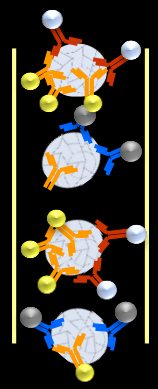
Vanderbilt Mass Cytometer



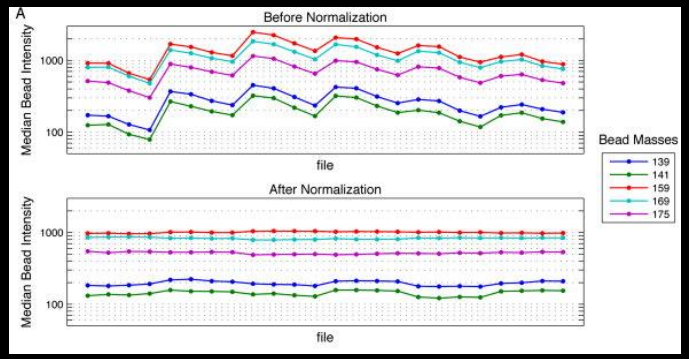
Adapted from Bendall et al., *Science* 2011, Cytobank, & DVS Sciences

Mass Cytometry Data Pre-Processing

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



FCS File 1
+
FCS File 2
+
FCS File 3



arcsinh(x/15)

Fink et al, *Cytometry Part A* 2013

Resources:

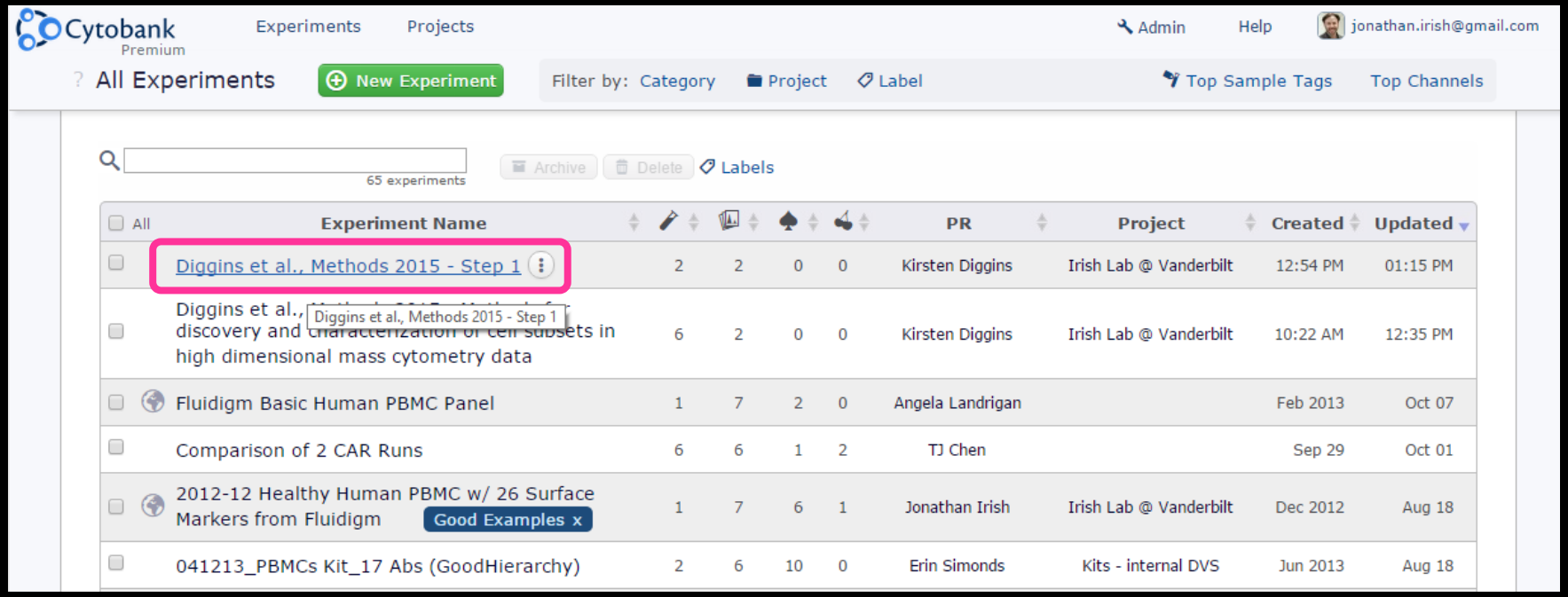
- Concatenation: downloadable tool from Cytobank (http://support.cytobank.org/help/kb/cytobank-utilities/concatenating-fcs-files)
- Normalization: Cytometry Part A Volume 83A, Issue 5, pages 483-494, 19 MAR 2013 DOI: 10.1002/cyto.a.22271 http://onlinelibrary.wiley.com/doi/10.1002/cyto.a.22271/full#fig6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

Steps 1-3: Getting Started & Preparing for viSNE

Workflow summary:

1) Clone experiment “Diggins et al., Methods 2015 – Step 1”
2) Set the Scales so that the scale argument is 15 for all channels.
3) Gate for “Intact cells”

- Navigate to Diggins et al., Methods 2015 – Step 1
- Click the experiment name to load it



https://premium.cytobank.org/cytobank/experiments/44410/

Steps 1-3: Getting Started & Preparing for viSNE

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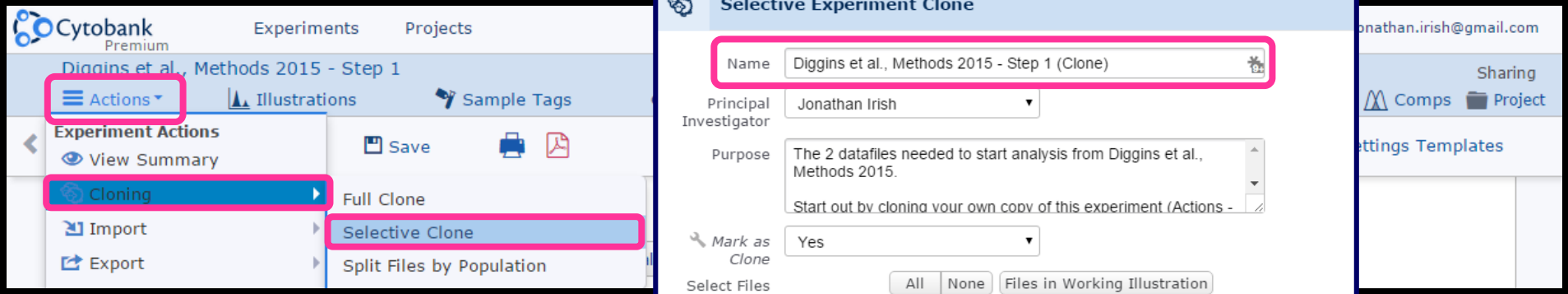
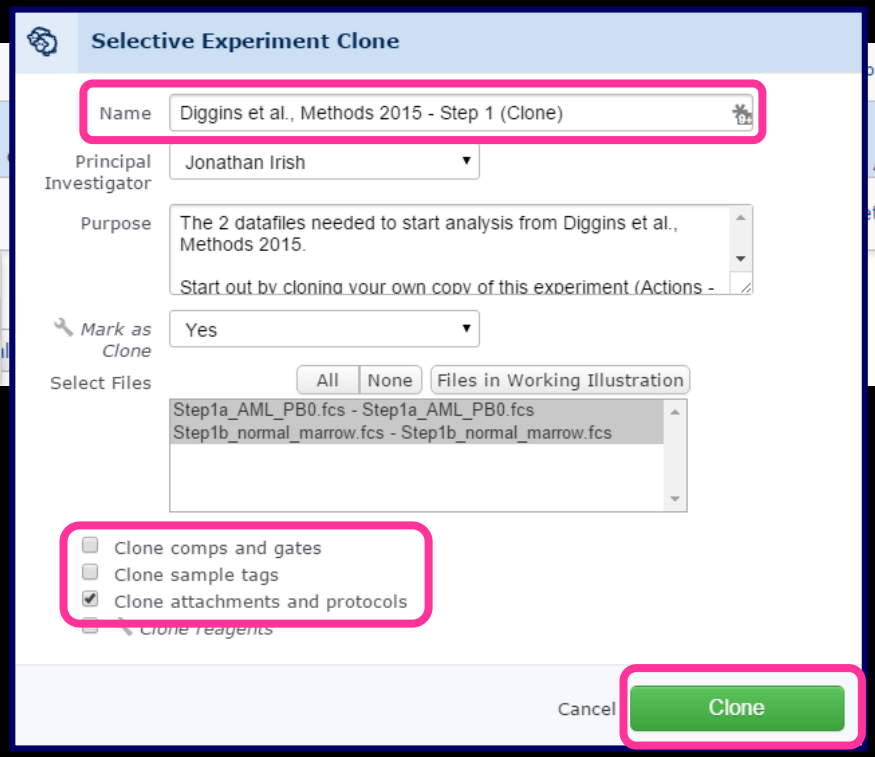
3) Gate for “Intact cells”

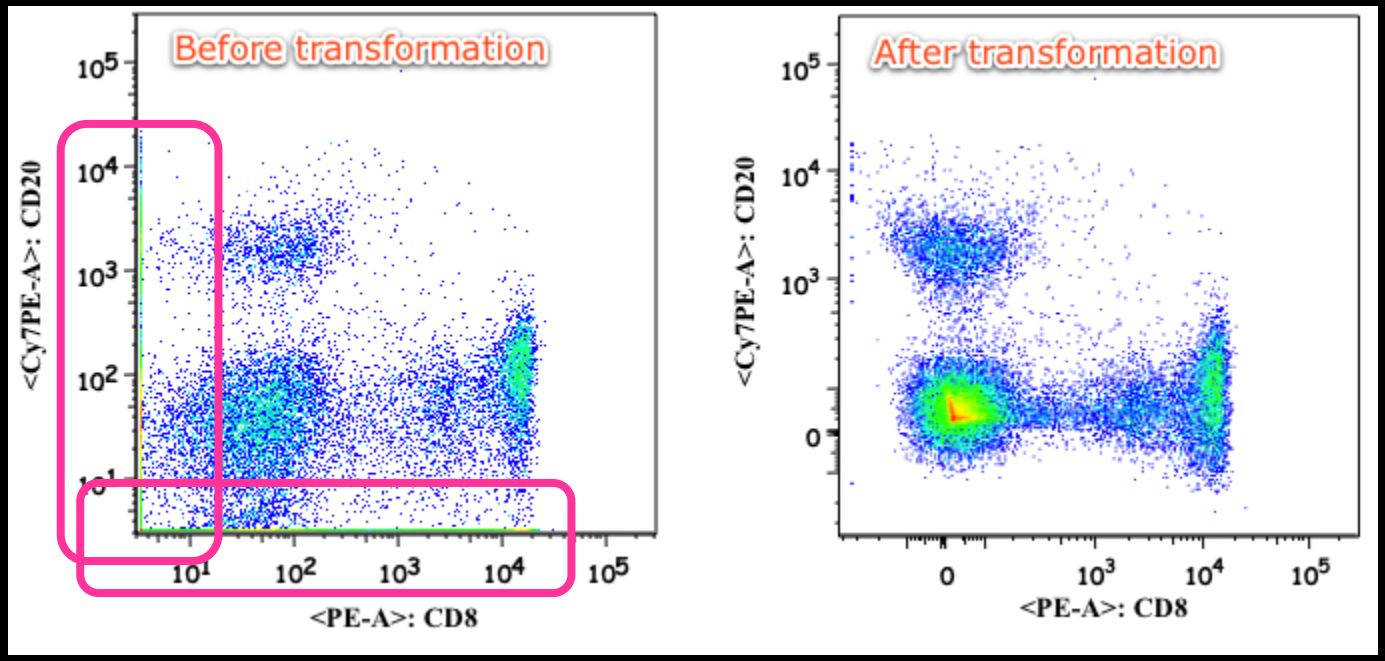
- Make a clone using Actions => Cloning => Selective Clone
- Edit the name, uncheck all except “clone attachments and protocols”, press Clone button.





https://premium.cytobank.org/cytobank/experiments/44410/

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



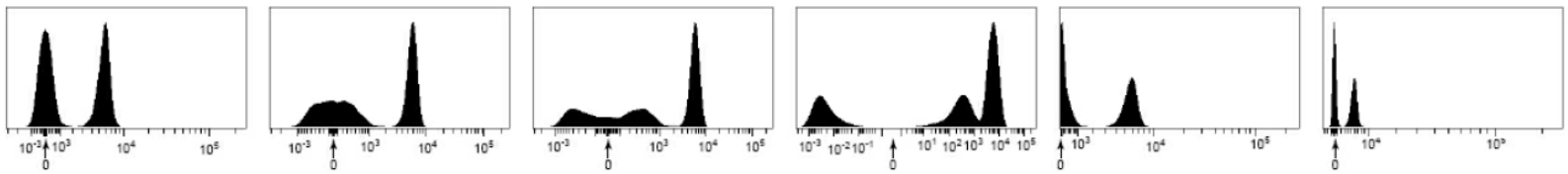
http://www.flowjo.com/v76/en/displaytransformwhy.html

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

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

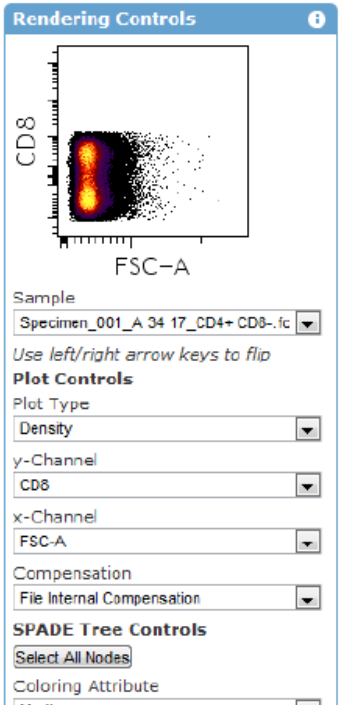
$$\text{arcsinh(x) with cofactor c} = \ln\left(\frac{x}{c} + \sqrt{1 + \left(\frac{x}{c}\right)^2}\right)$$

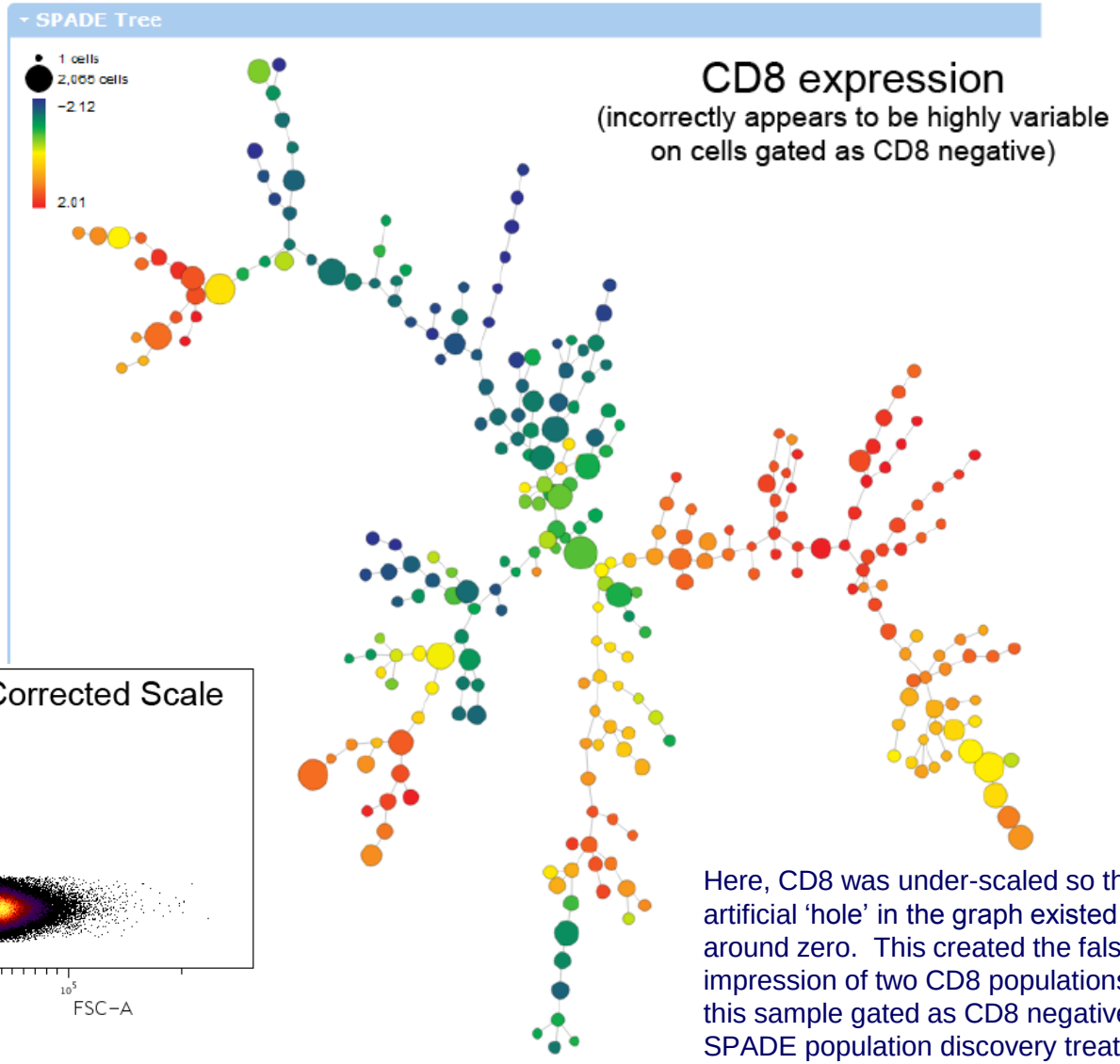
For fluorescent flow cytometry data a biexponential or arcsinh transformation corrects the scale near zero.

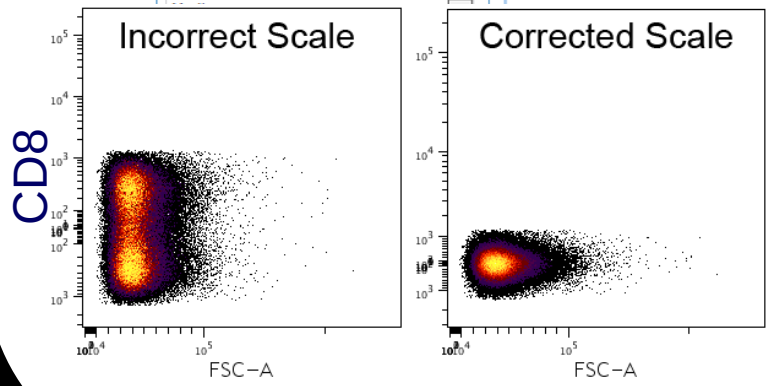
Since computational analysis techniques compare distance similar to what a person does when looking at a plot, these techniques can identify artificial populations near zero (see C and D) if data are not appropriately transformed prior to analysis.

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

Inappropriate Scaling Can Lead to False Population Discovery







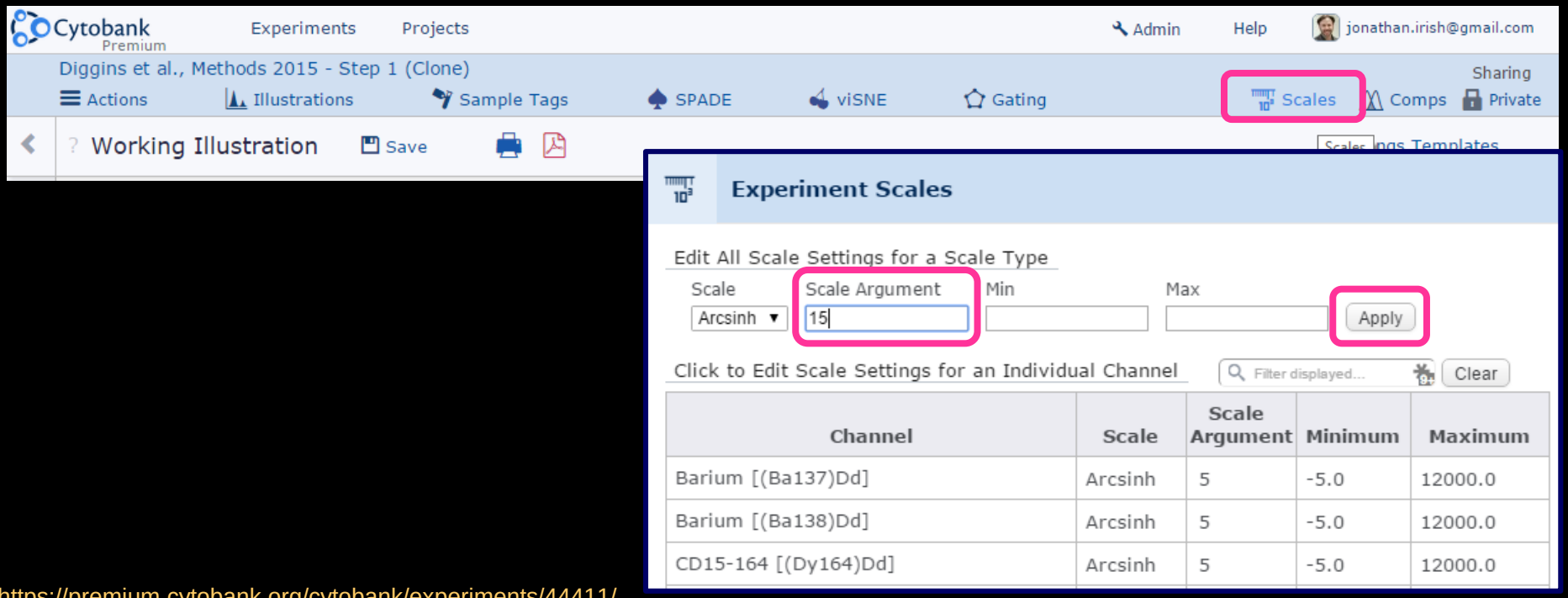
Here, CD8 was under-scaled so that an artificial 'hole' in the graph existed around zero. This created the false impression of two CD8 populations in this sample gated as CD8 negative. SPADE population discovery treated this as significant.

Steps 1-3: Getting Started & Preparing for viSNE

Workflow summary:

1) Clone experiment “Diggins et al., Methods 2015 – Step 1”
2) Set the Scales so that the scale argument is 15 for all channels.
3) Gate for “Intact cells”

- Open the Scales and set the scale argument to 15 for all channels, then press Apply.
- Choose “OK” when the popup asks if you really want to do it.



https://premium.cytobank.org/cytobank/experiments/44411/

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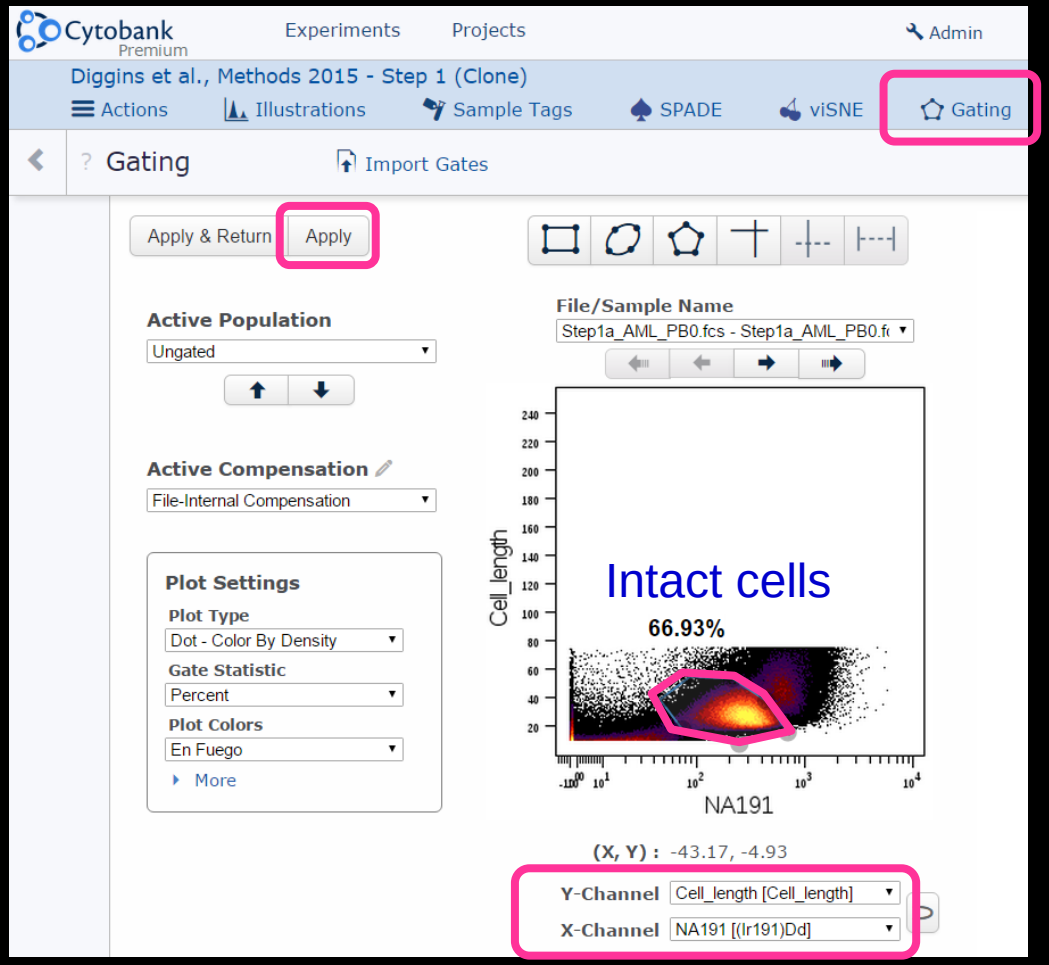
- Go into Gating, change the y-axis to “Cell Length” (Event Length) and the x-axis to NA191 (Intercalator).
- Draw a polygon gate like the one below and call it “Intact cells”.
- Apply the gate.



https://premium.cytobank.org/cytobank/experiments/44411/

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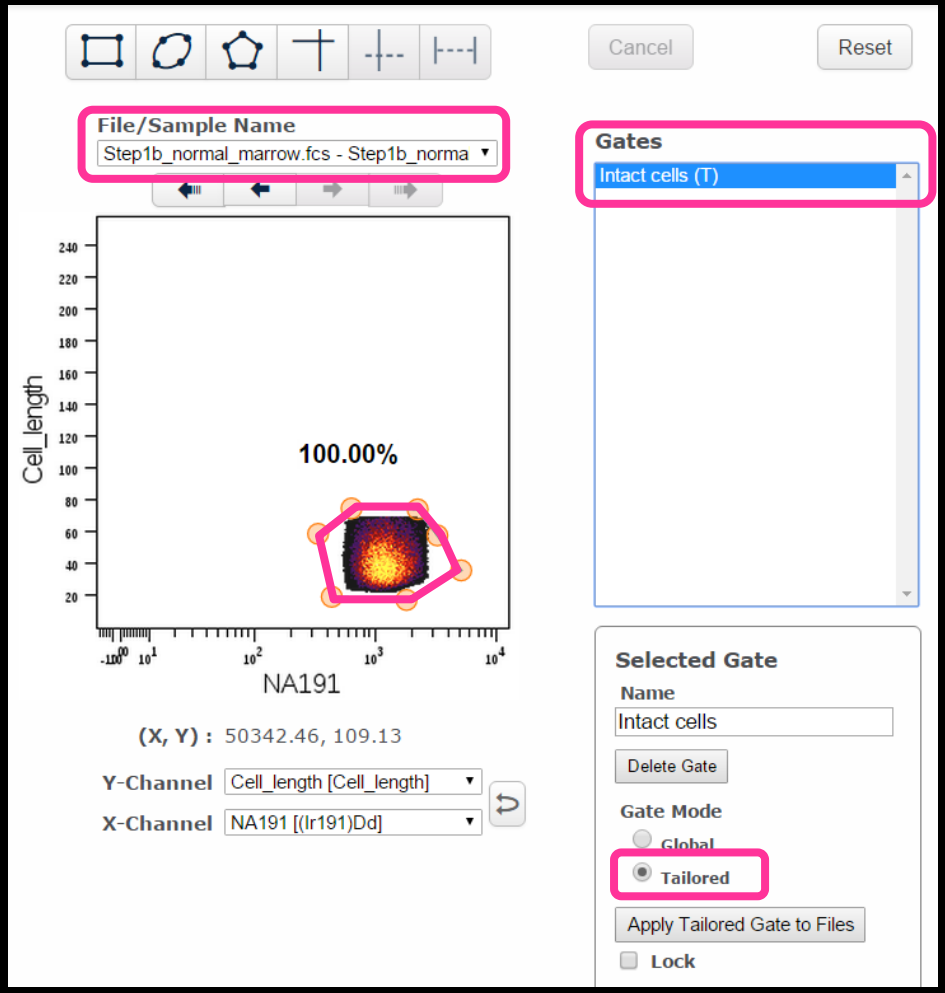
3) Gate for "Intact cells"

- Click on the "Intact cells" gate in the list of gates to the right.
- Make the gate a tailored gate by clicking the "Tailored" radio circle.
- Select the other file (Step1b_normal_marrow)
- Move the gate's vertices to include 100% of normal marrow events.
- Make sure to to Apply the gate. Optional: "Check gate".

Note: we're doing this because intercalator levels tend to vary from run to run (in contrast with antibody staining).



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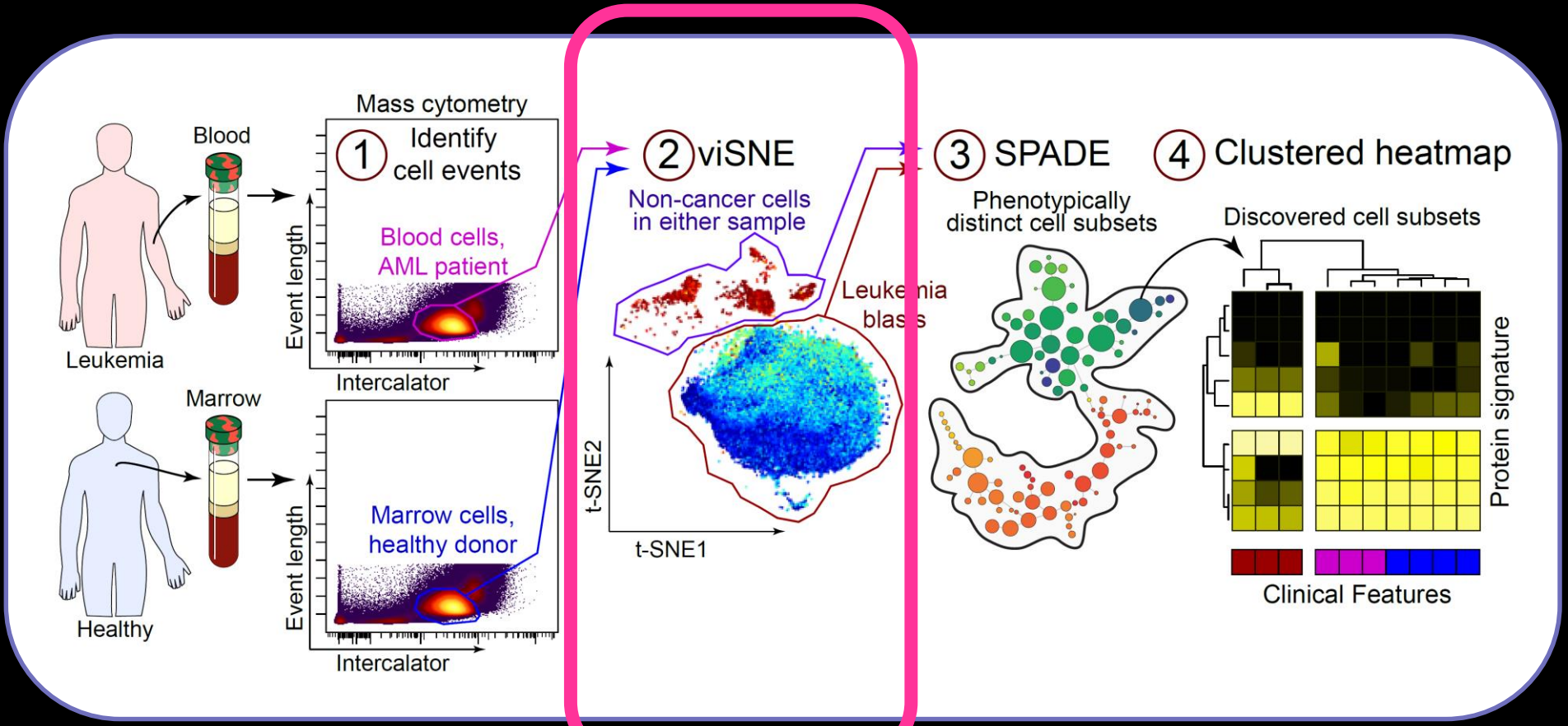
Discovery and Characterization of Cell Subsets: Towards Machine Learning Cell Identity



Diggins et al., *Methods* 2015

Single Cell Biology Workflow

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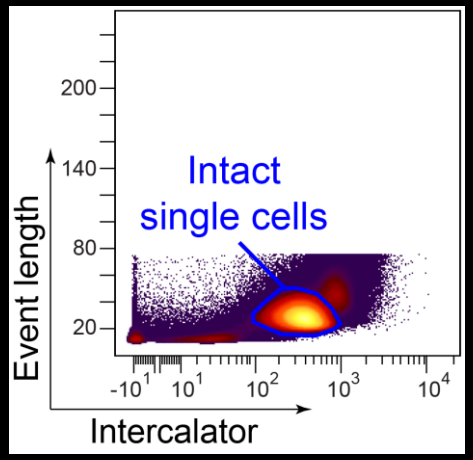
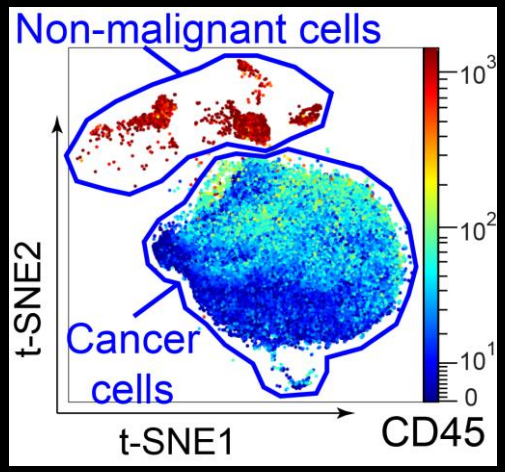
Data processing	3) Cell event parsing 4) Scale transformation
Distinguishing initial populations	5) Live single cell gating 6) Focal population gating
Revealing cell subsets	7) Feature selection 8) Dimensionality reduction 9) Identify cell clusters 10) Cluster refinement
Characterizing cell subsets	11) Feature comparison 12) Model populations 13) Learn cell identity 14) Statistical testing

Expert gating

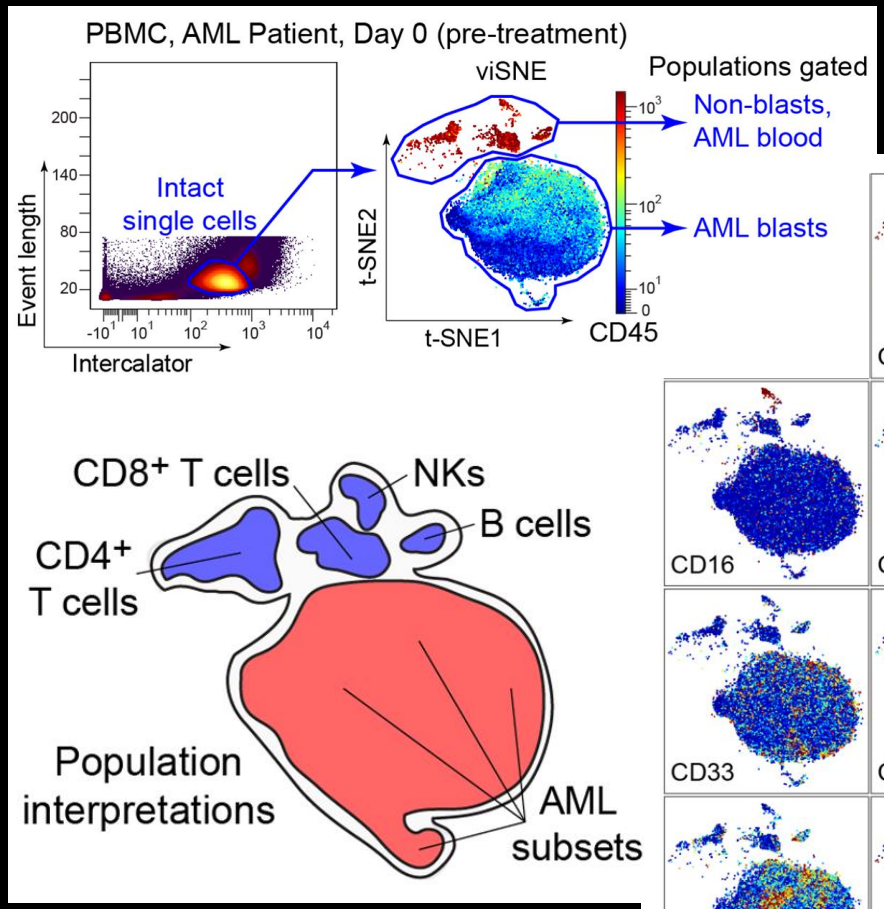


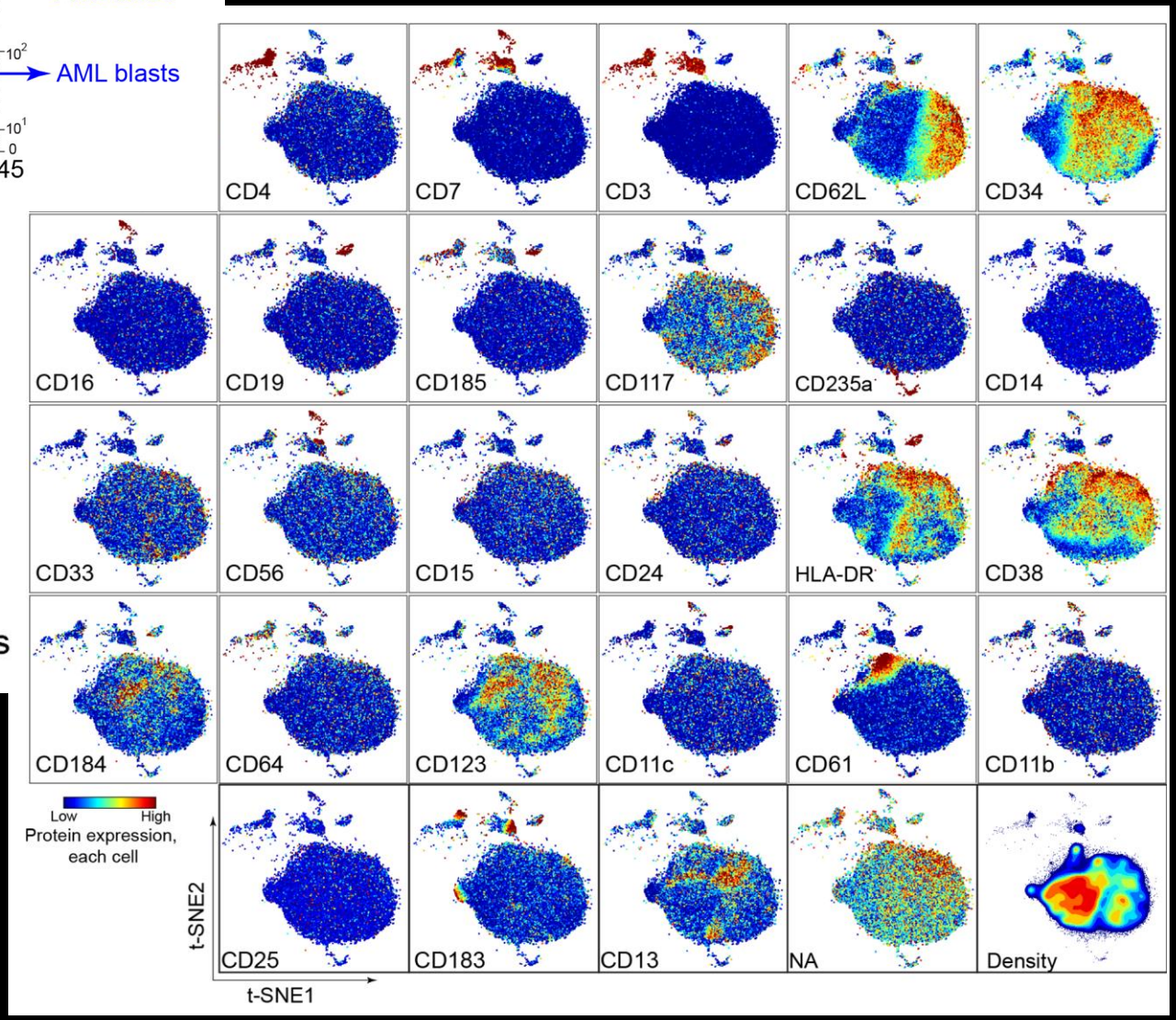
viSNE + expert gating



Diggins et al., *Methods* 2015

Current Goal: Get to Figure 1 from Diggins et al. with viSNE

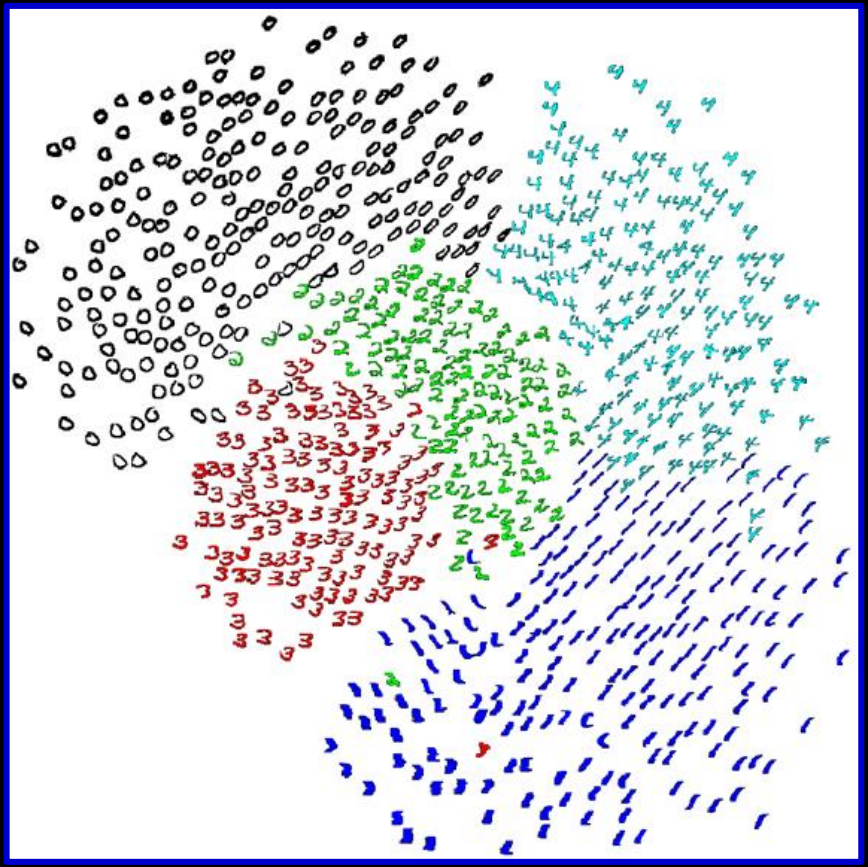




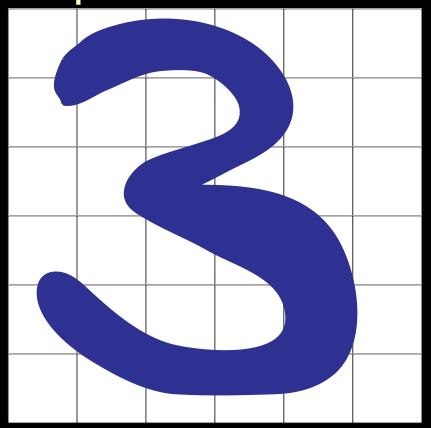
Diggins, et al *Methods* 2015

Stochastic Neighbor Embedding (SNE)

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



Example: 6x6 Pixel Image

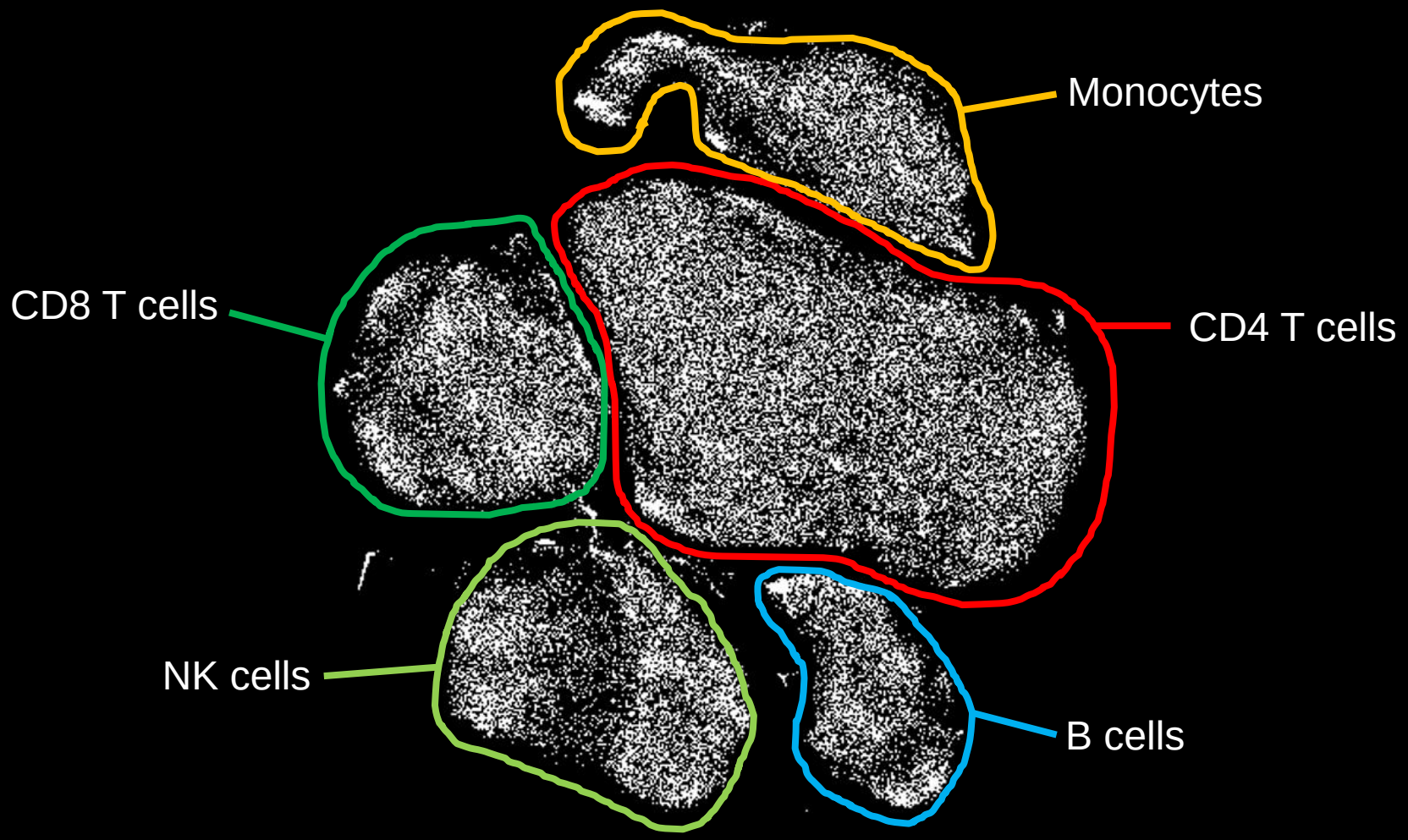


Vectorize (1x36)

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

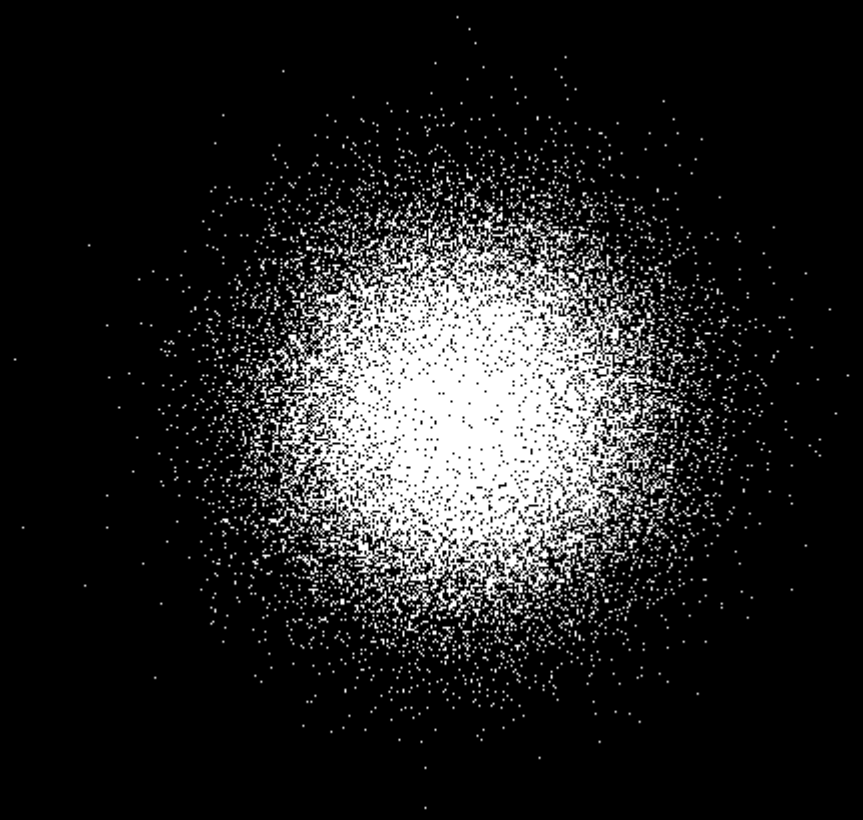


Healthy human blood, mass cytometry,
26 markers measured, viSNE analysis tool

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, 26D viSNE analysis

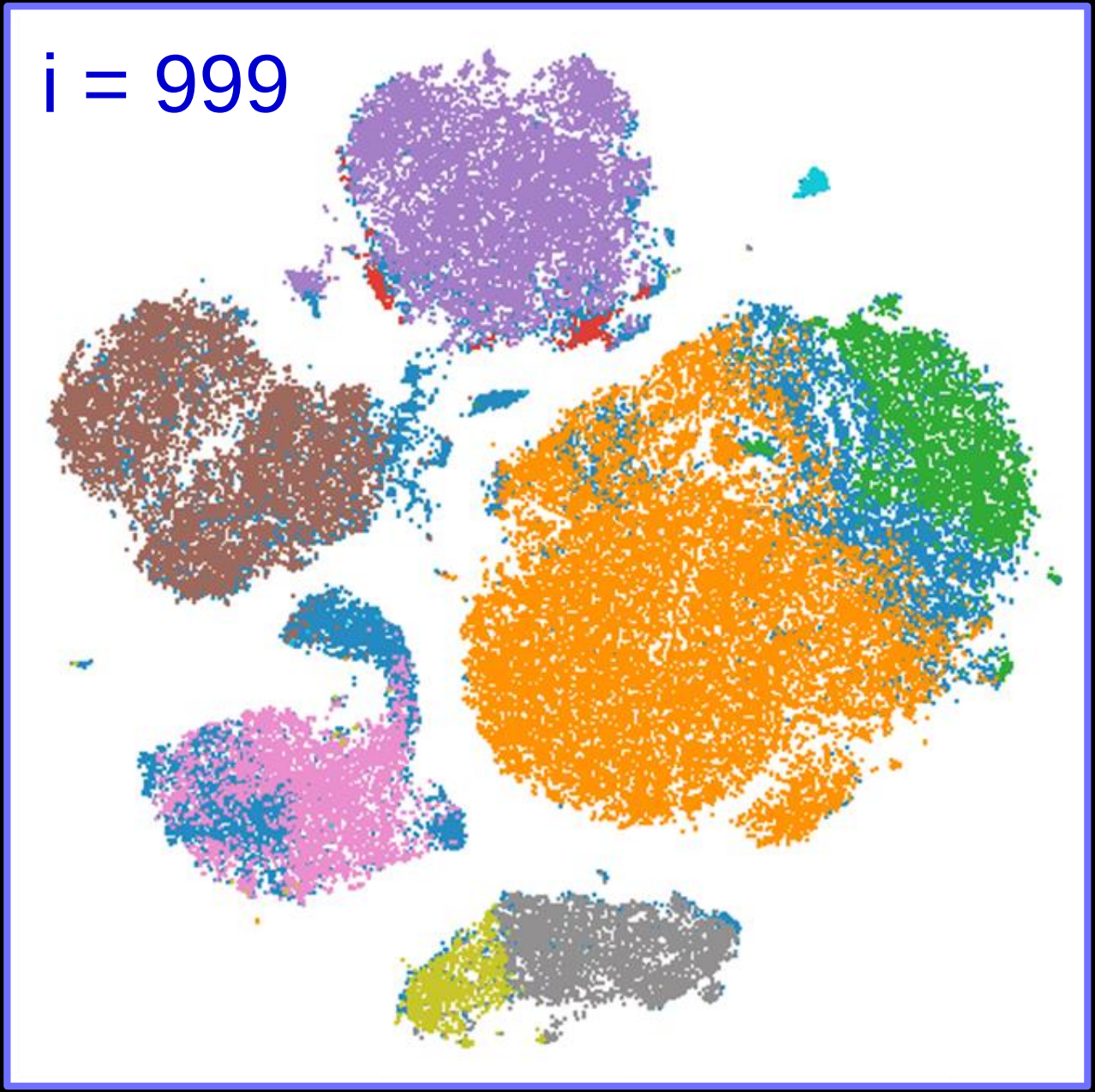


Animation created by Cytobank team from iterations of viSNE / t-SNE using PBMC (26 features)

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



Created with Chris Ciccolella, Cytobank

Viewing Expert Gates with viSNE Reveals *Cyto Incognito*

Healthy human blood,
mass cytometry,
26D viSNE analysis

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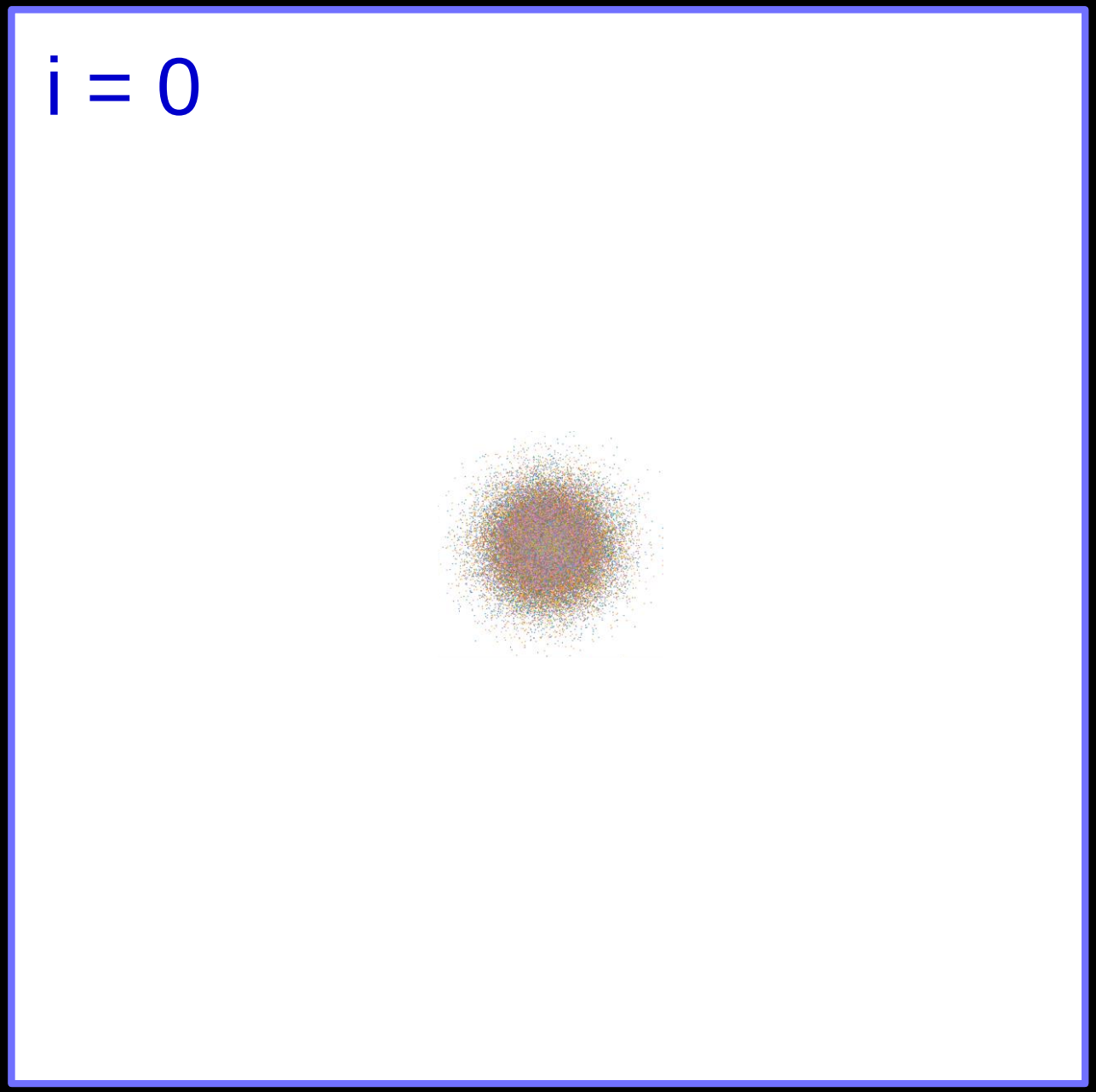

Created with Chris Ciccolella, Cytobank

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Healthy human blood,
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- IgM- B cells
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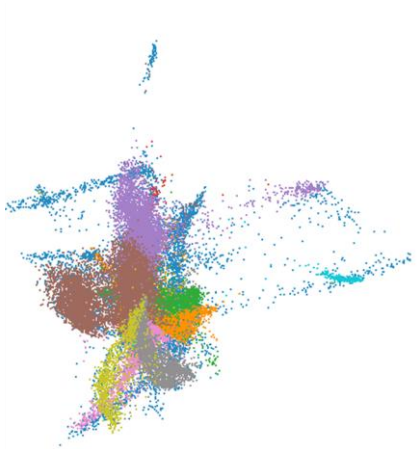
i = 45



Created with Chris Ciccolella, Cytobank

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Healthy human blood,
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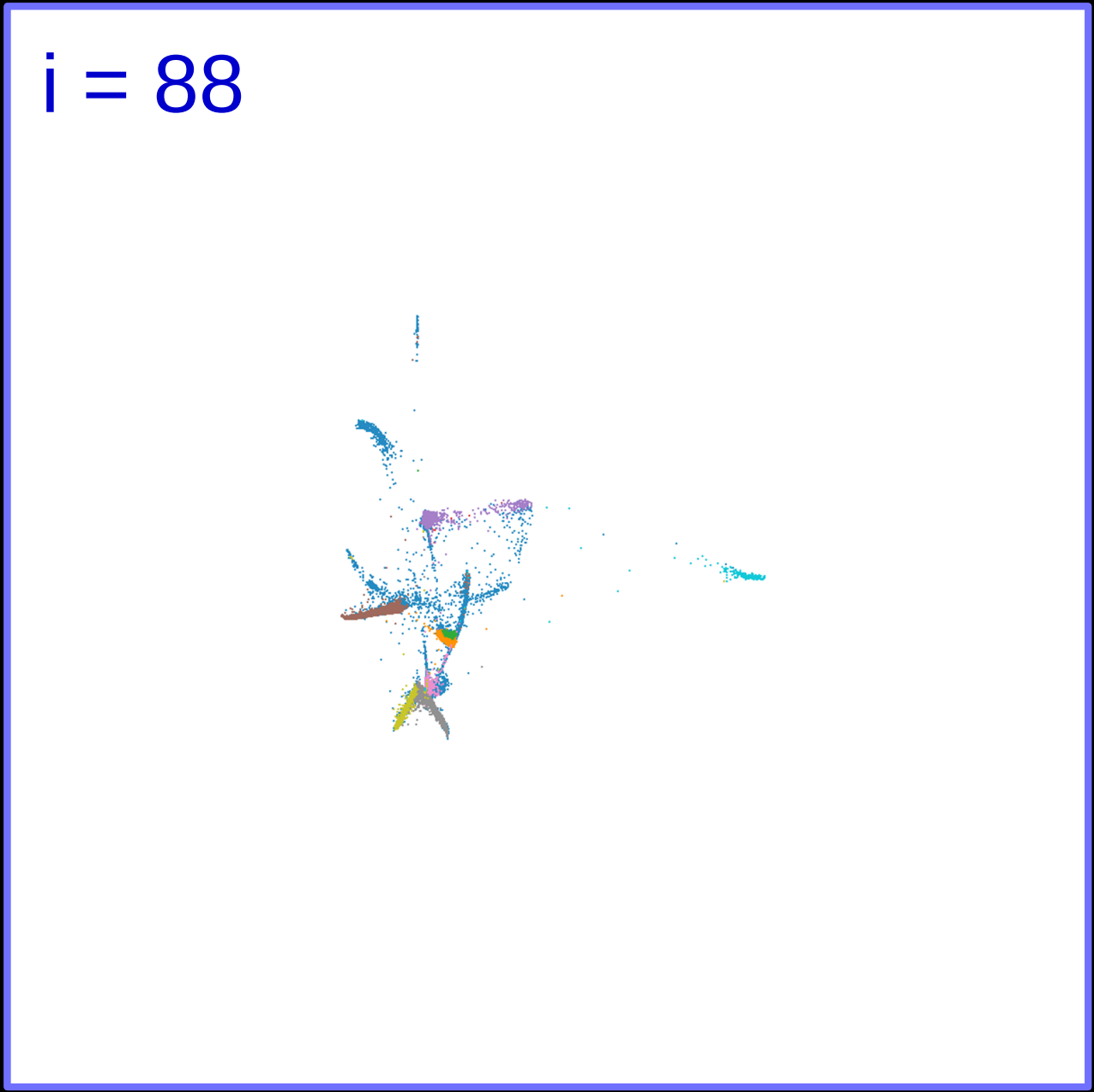

Created with Chris Ciccolella, Cytobank

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Healthy human blood,
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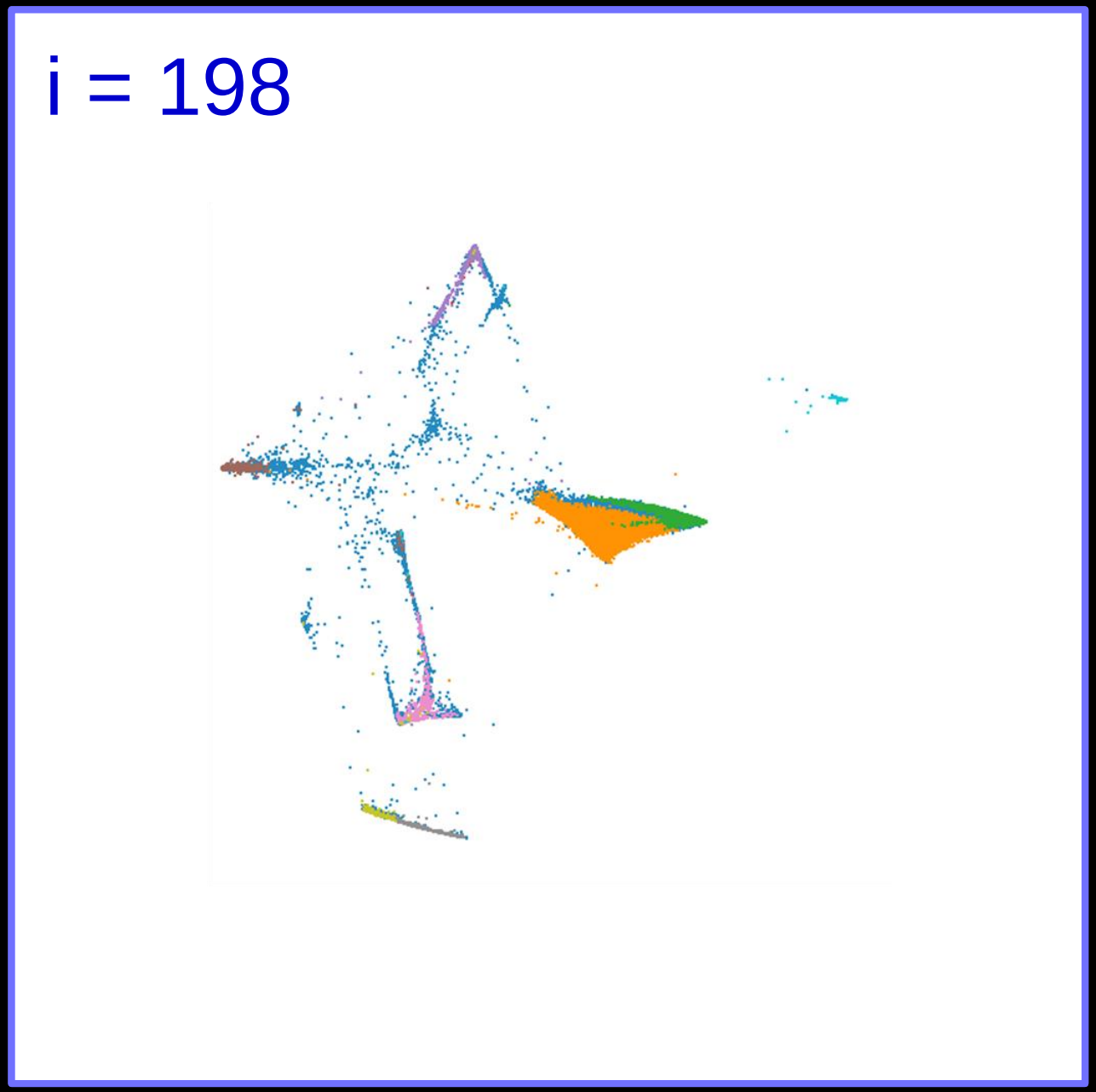

Created with Chris Ciccolella, Cytobank

Viewing Expert Gates with viSNE Reveals *Cyto Incognito*

Healthy human blood,
mass cytometry,
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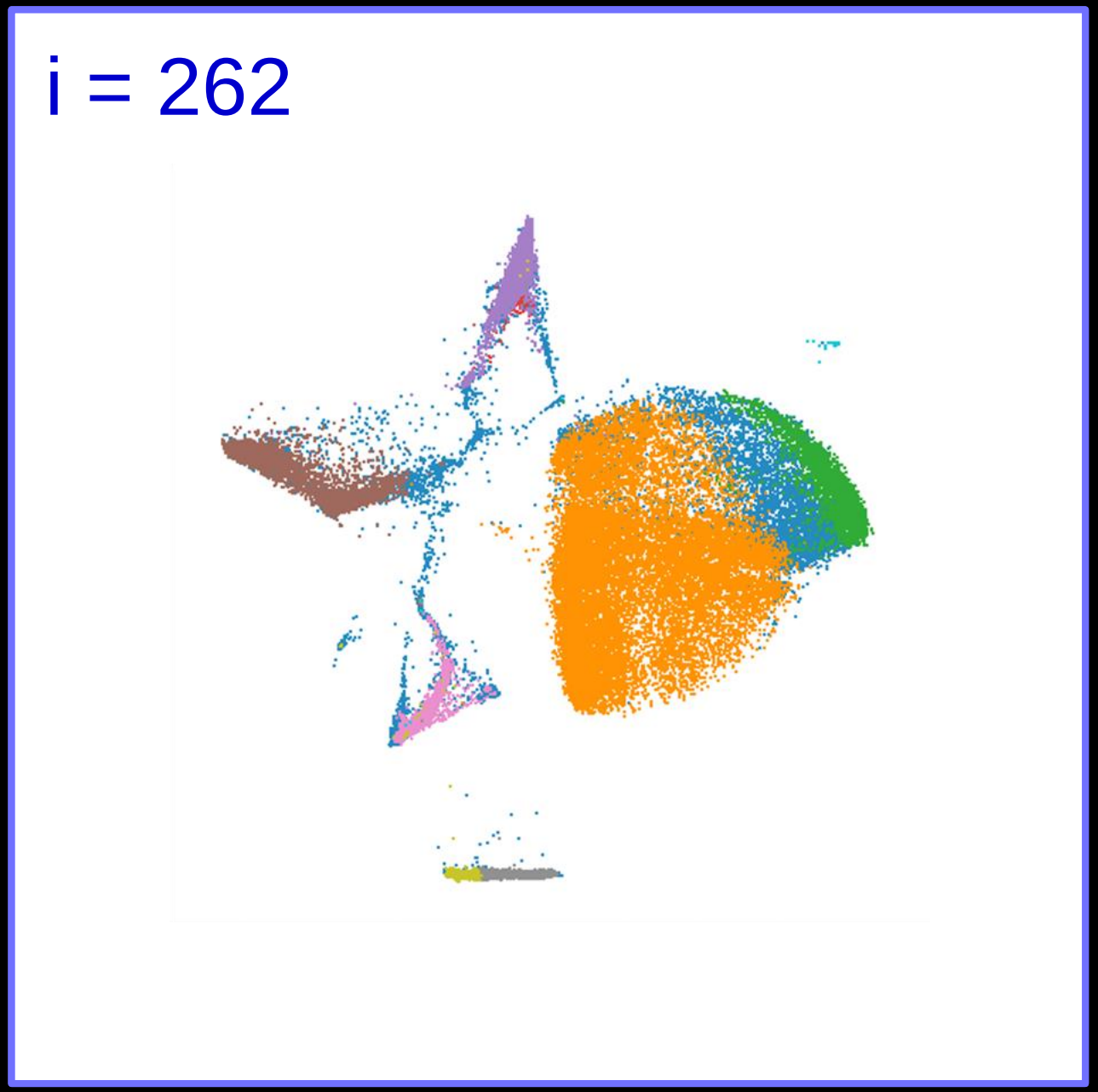

Created with Chris Ciccolella, Cytobank

Viewing Expert Gates with viSNE Reveals *Cyto Incognito*

Healthy human blood,
mass cytometry,
26D viSNE analysis

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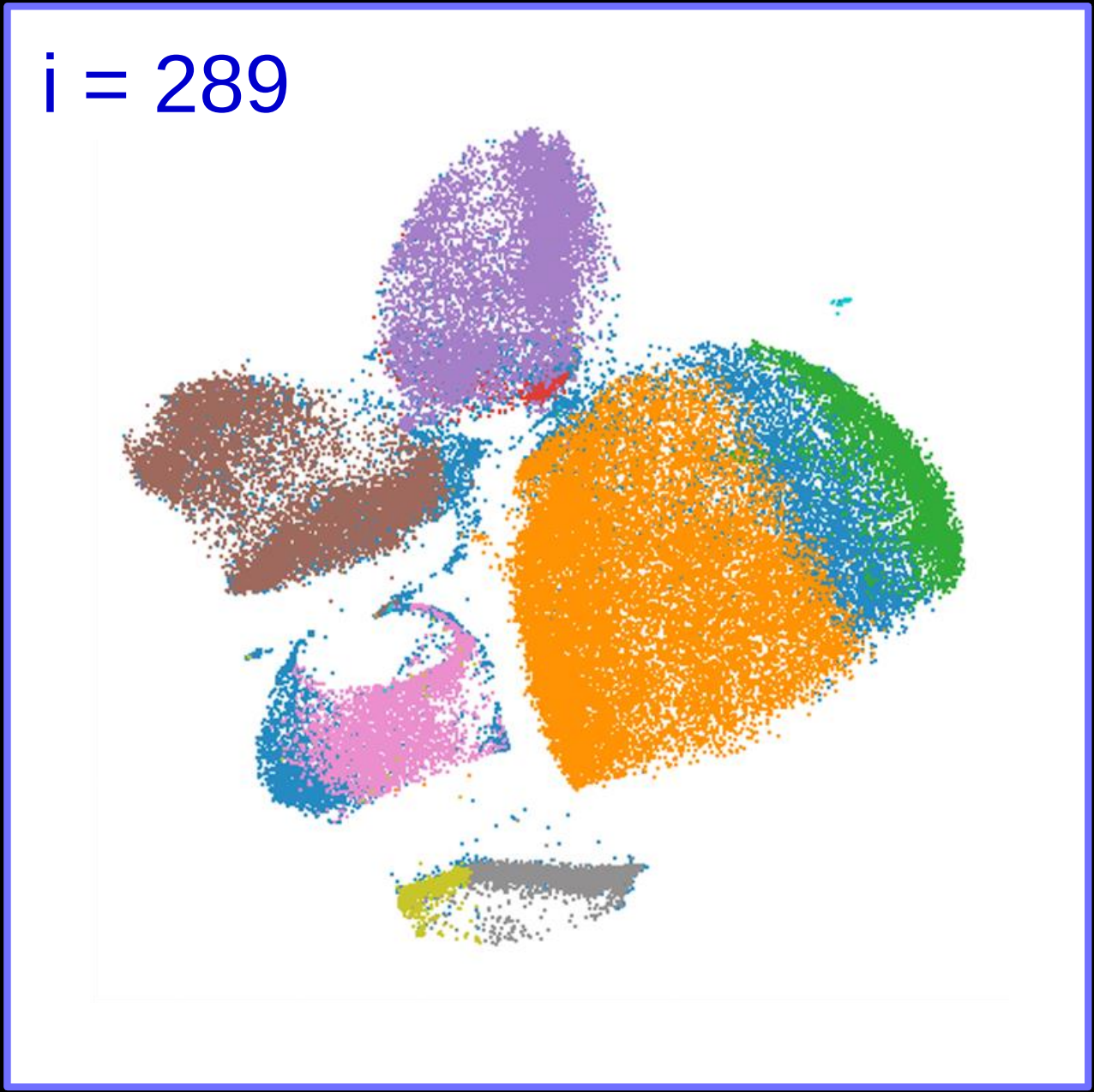

Created with Chris Ciccolella, Cytobank

Viewing Expert Gates with viSNE Reveals *Cyto Incognito*

Healthy human blood,
mass cytometry,
26D viSNE analysis

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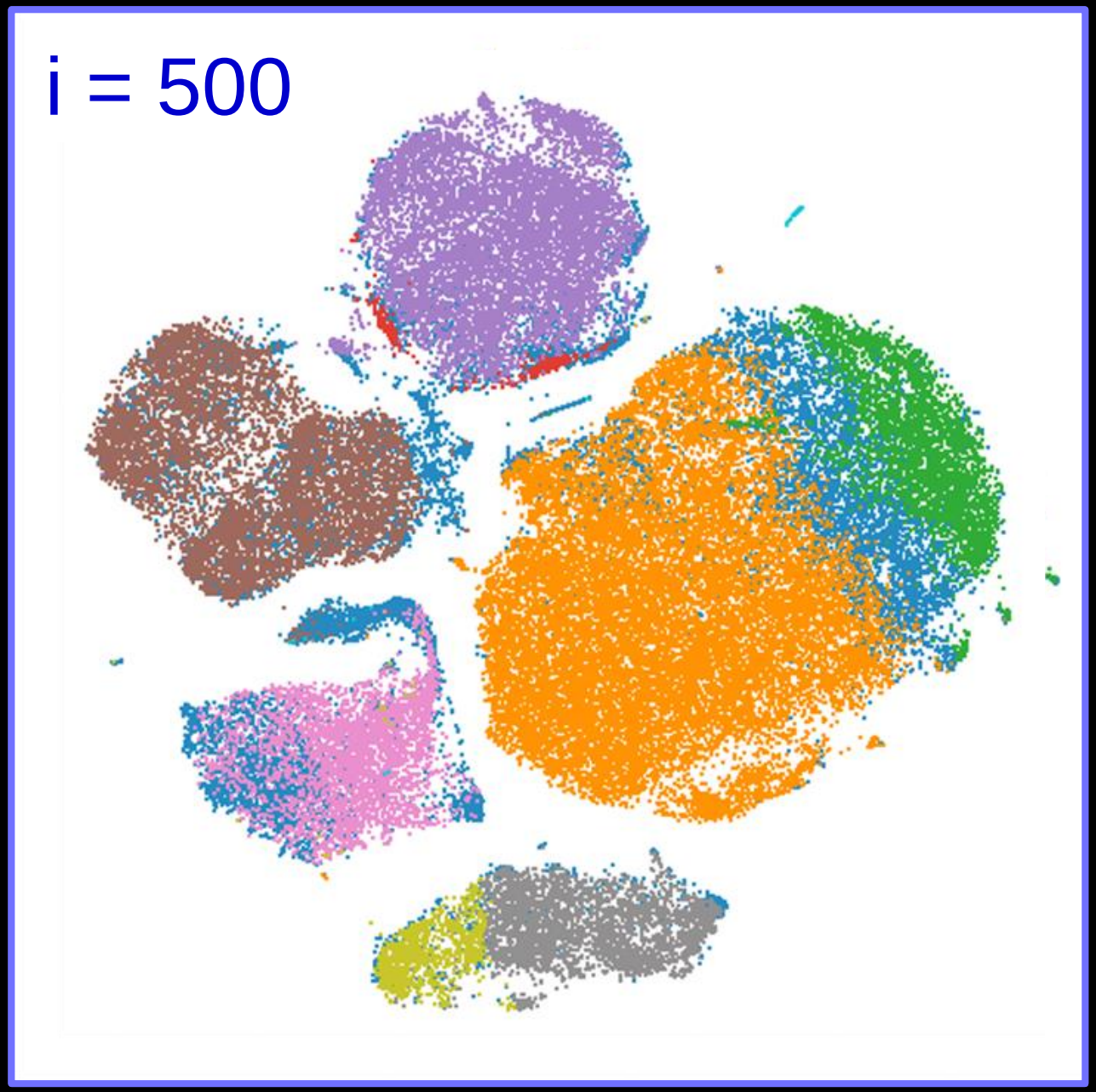

Created with Chris Ciccolella, Cytobank

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Healthy human blood,
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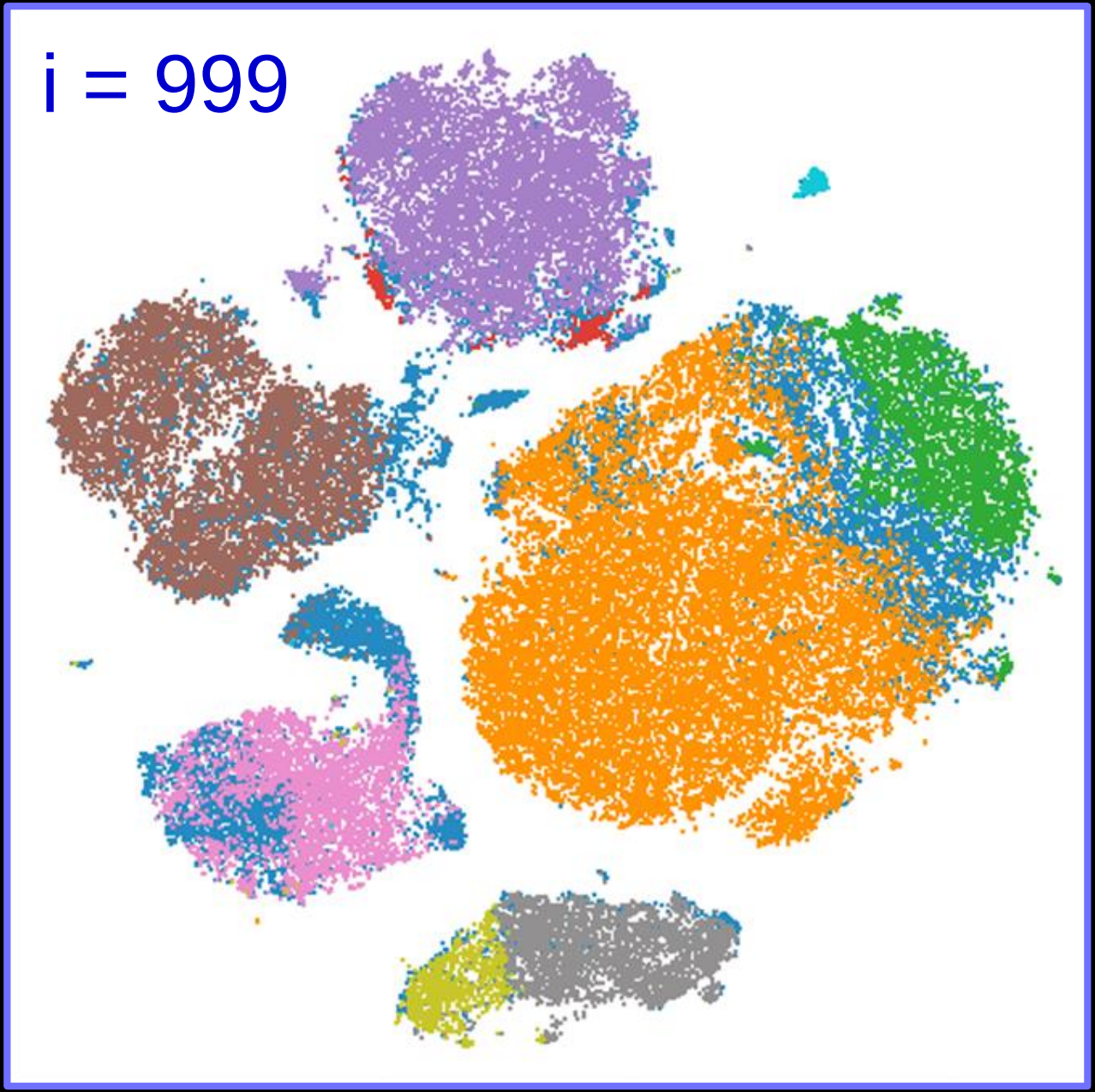

Created with Chris Ciccolella, Cytobank

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Healthy human blood,
mass cytometry,
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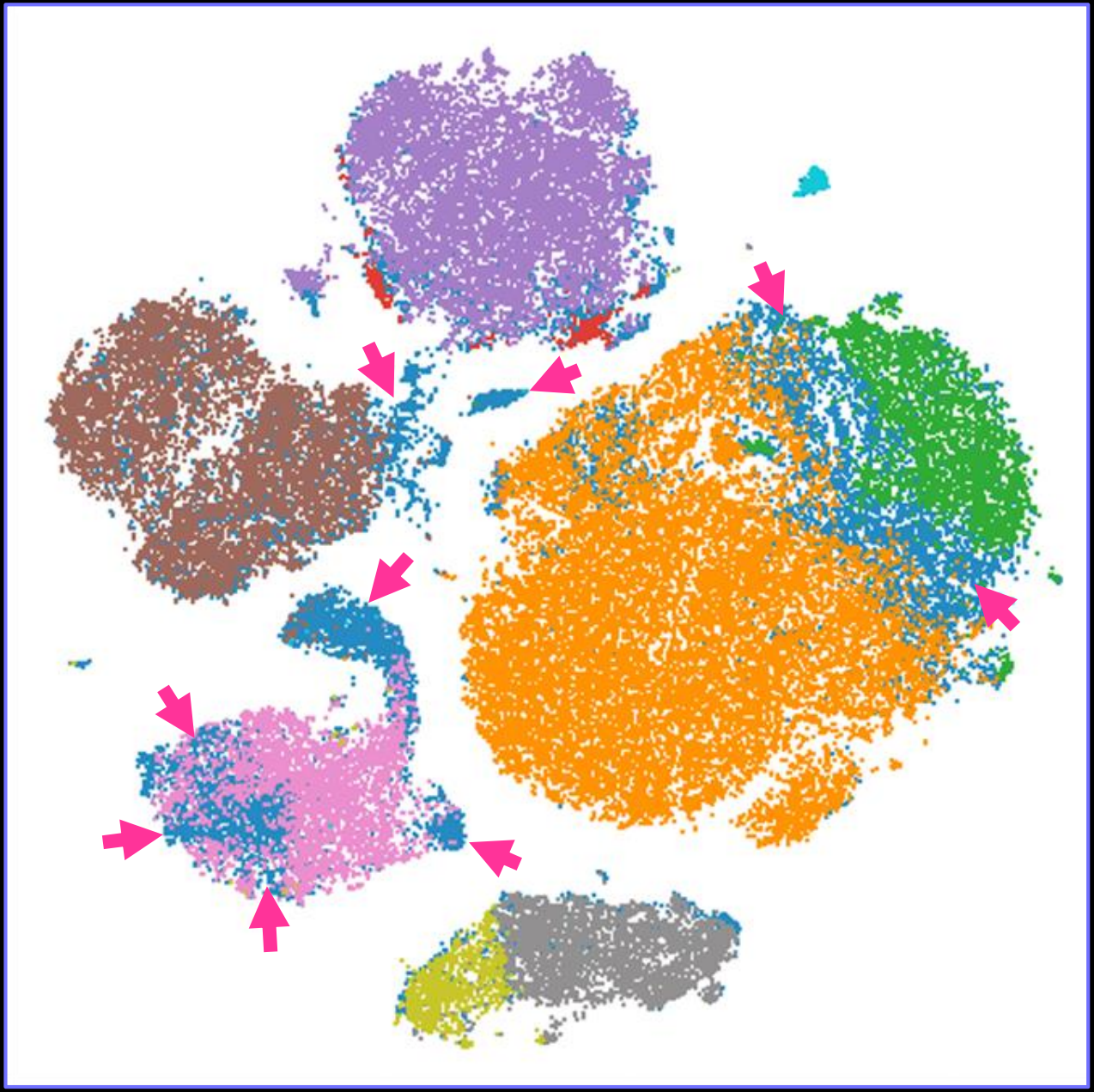
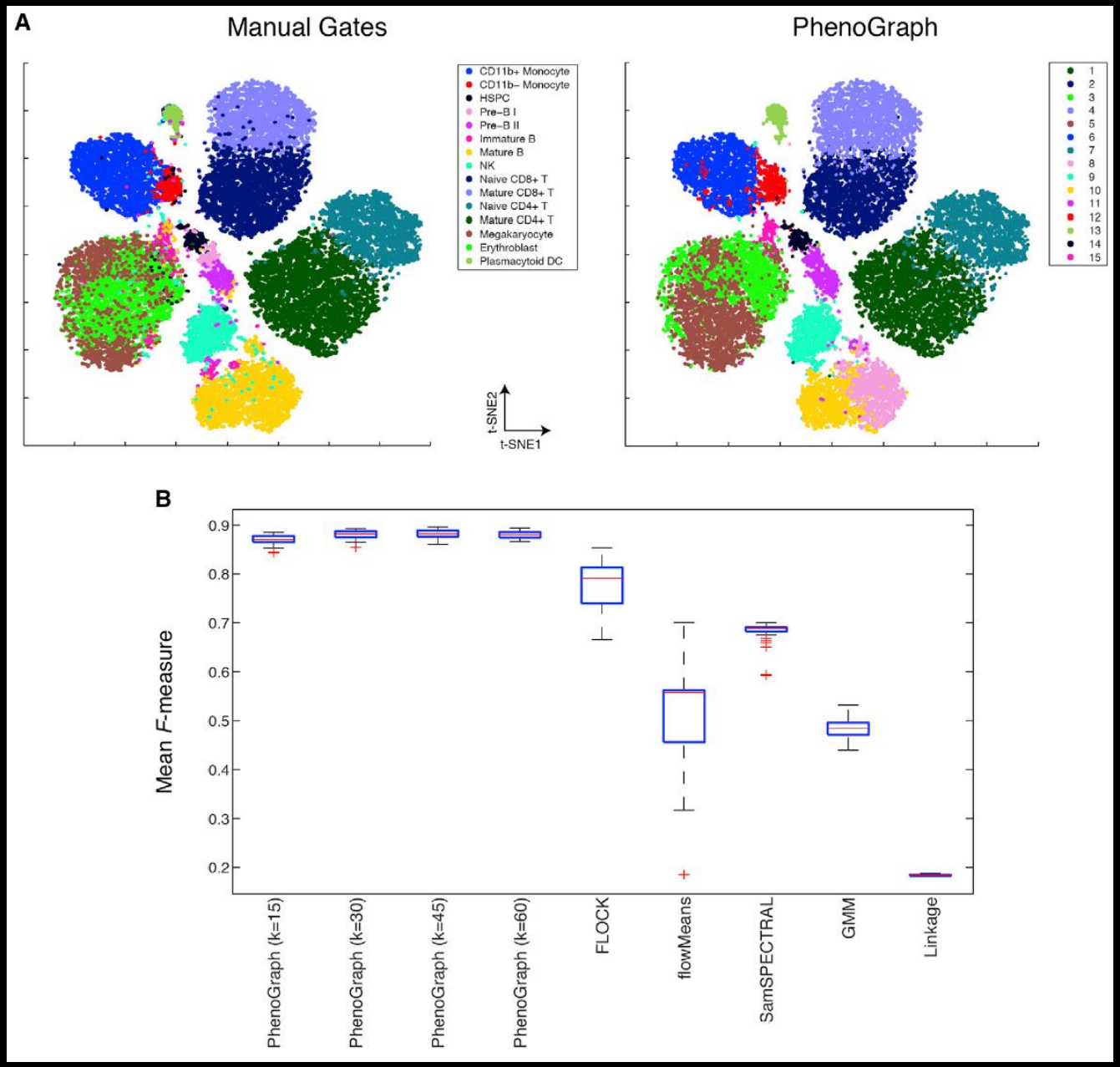
- CD16+ NK cells
- CD14+ CD33+ Monocytes
- IgM+ B cells
- IgM- B cells
- CD123+ pDCs

Cyto incognito
(Cells overlooked or
hidden in expert gating)



Created with Chris Ciccolella, Cytobank

Phenograph Adds Fast Clustering & Meta-Analysis to viSNE



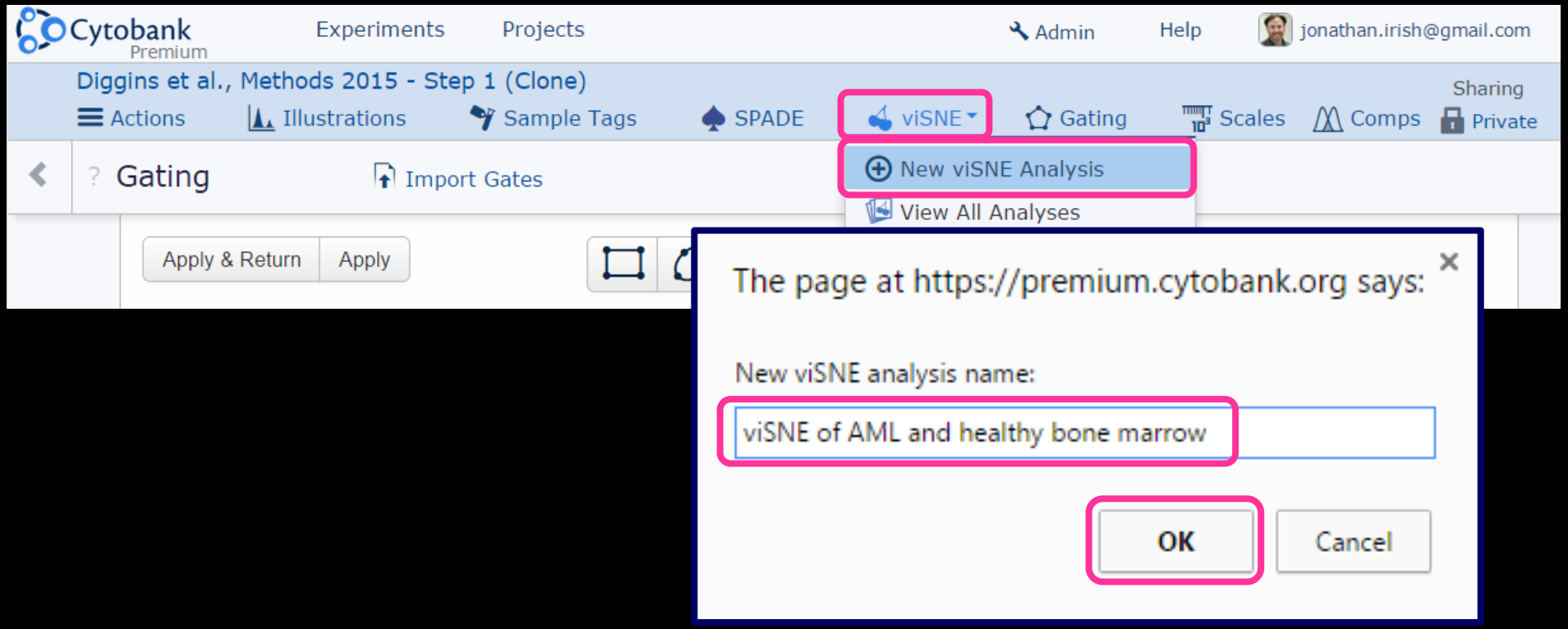
Levine et al., *Cell* 2015

Step 4: Run viSNE on Intact Cells from Both Samples

Workflow summary:

4) Run viSNE.

- Pick viSNE => New viSNE Analysis
- Name it “viSNE of AML and healthy marrow”



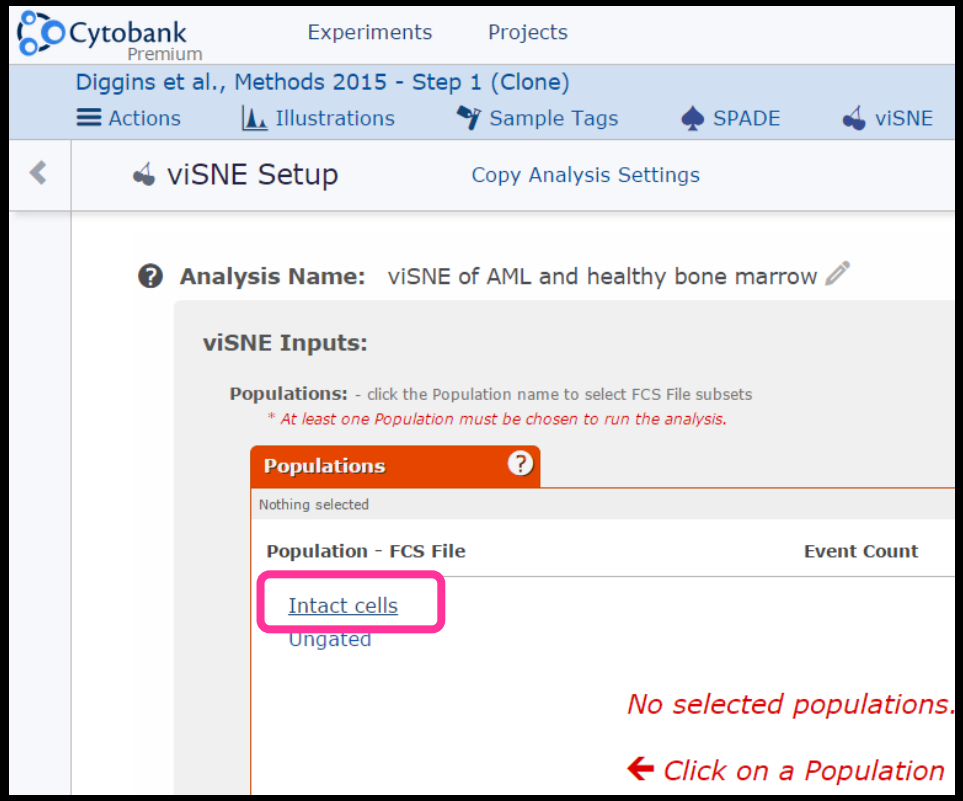
https://premium.cytobank.org/cytobank/experiments/44411/

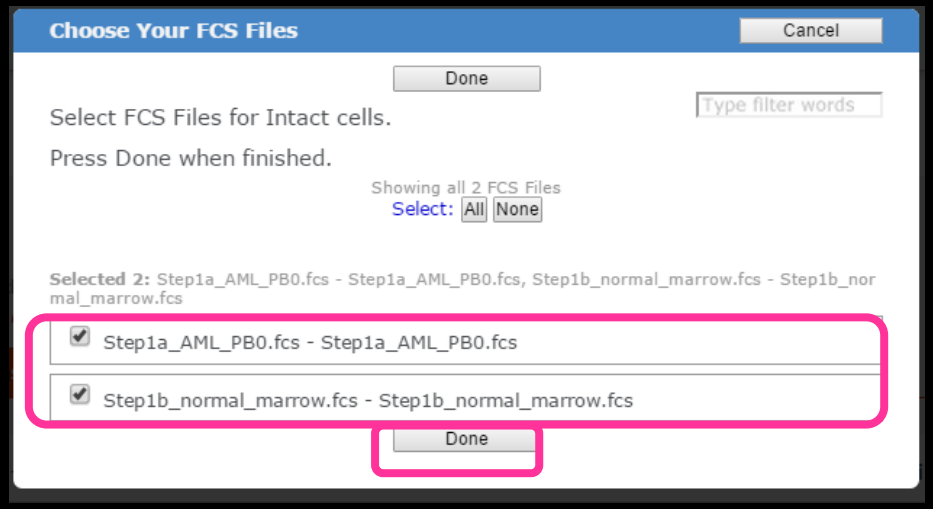
Step 4: Run viSNE on Intact Cells from Both Samples

Workflow summary:

4) Run viSNE.

- Pick Intact Cells from the Population selection area.
- Select both files to include in the viSNE analysis.





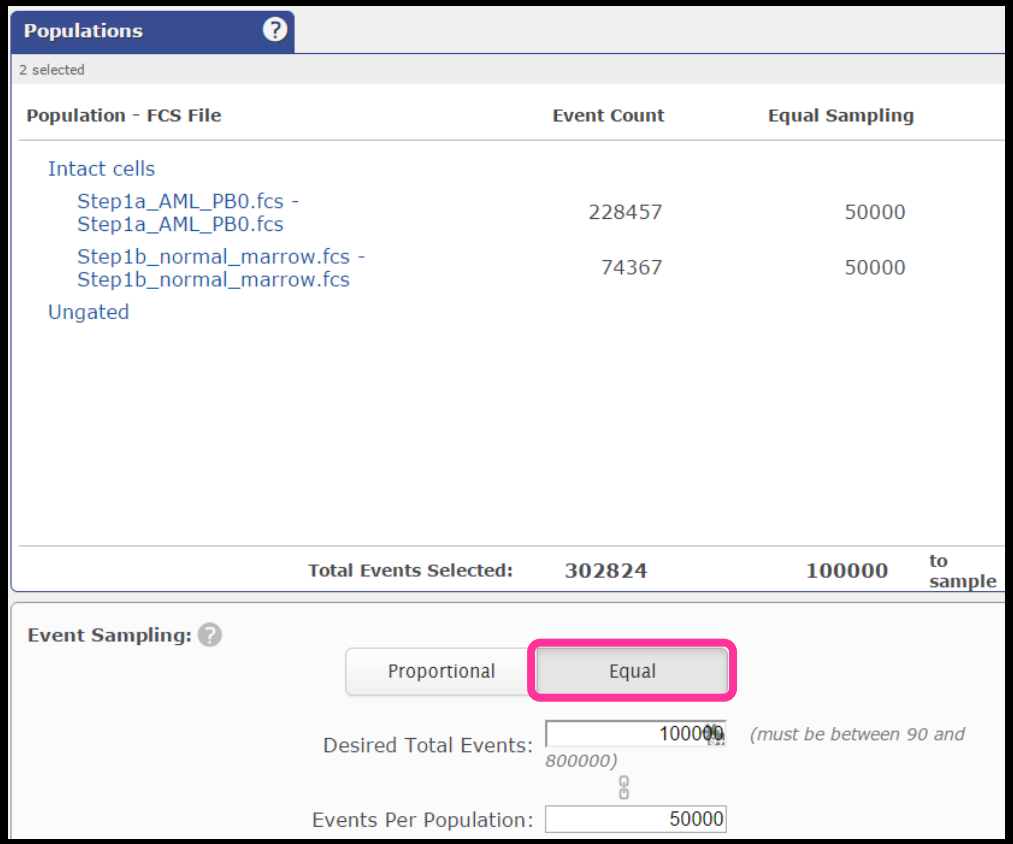
https://premium.cytobank.org/cytobank/experiments/44411/

Step 4: Run viSNE on Intact Cells from Both Samples

Workflow summary:

4) Run viSNE.

- Choose Equal subsampling and leave the “events per population” at the default of 50,000 (so 100,000 total events).



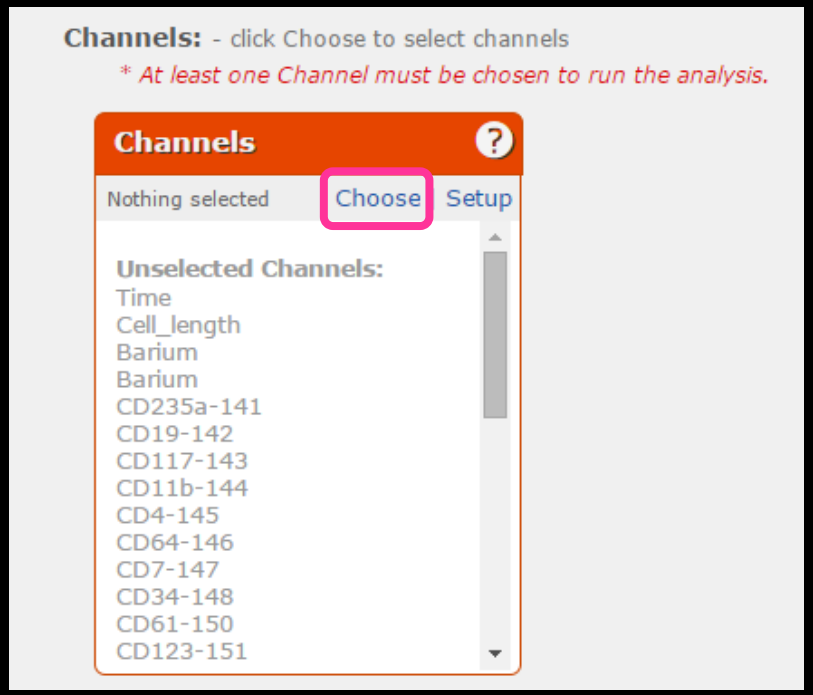
https://premium.cytobank.org/cytobank/experiments/44411/

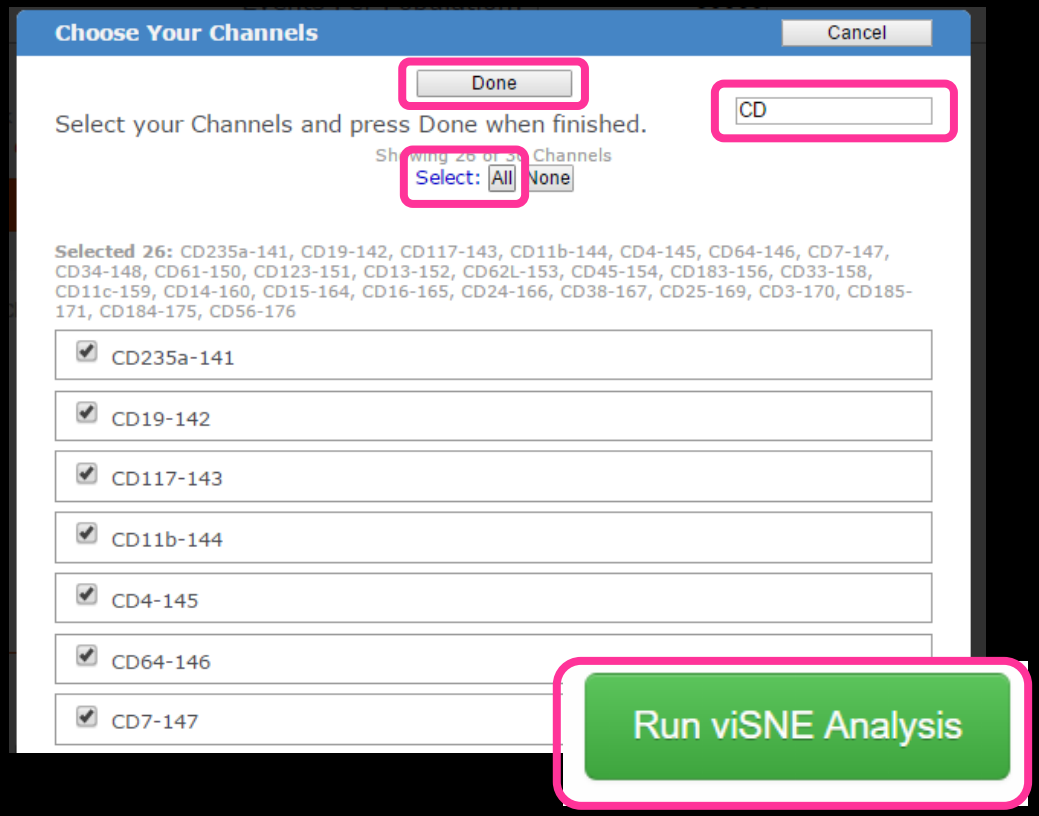
Step 4: Run viSNE on Intact Cells from Both Samples

Workflow summary:

4) Run viSNE.

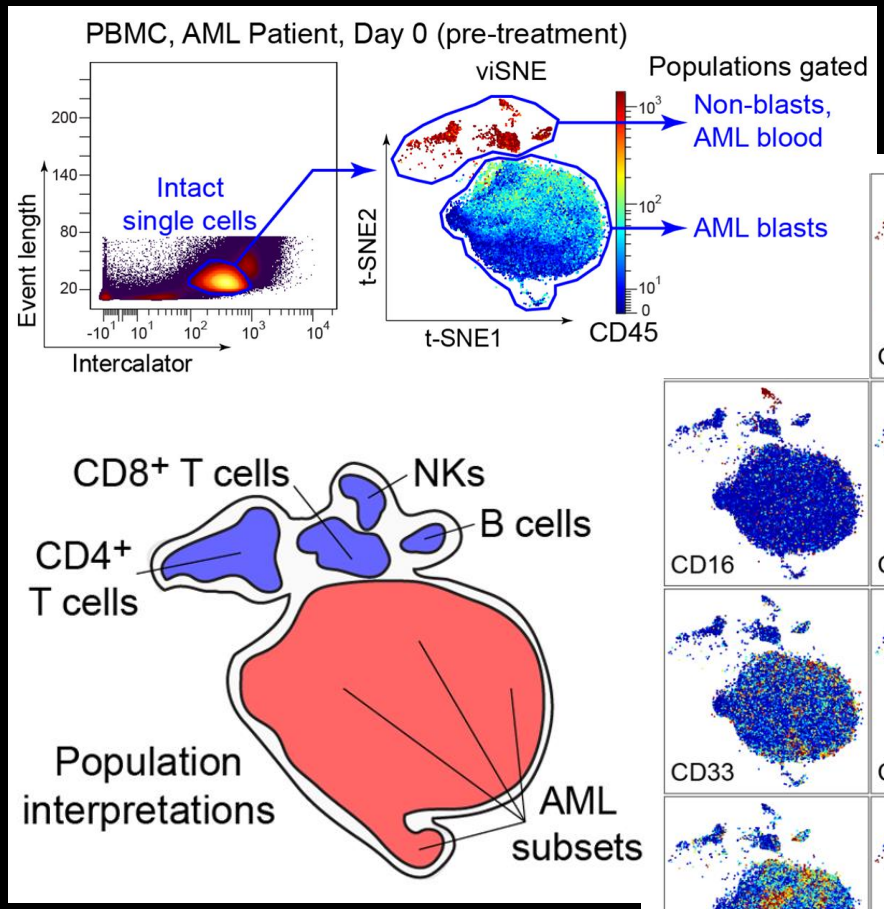
- Scroll down, and in the channel selector, pick the channels you want to be used in the comparison analysis (to “make the map”).
- Pick “all 27 markers”. Search on “CD” and “select all” (selects 26), then clear the selection and add HLA-DR to the list. Click “Done” and “Run viSNE Analysis”

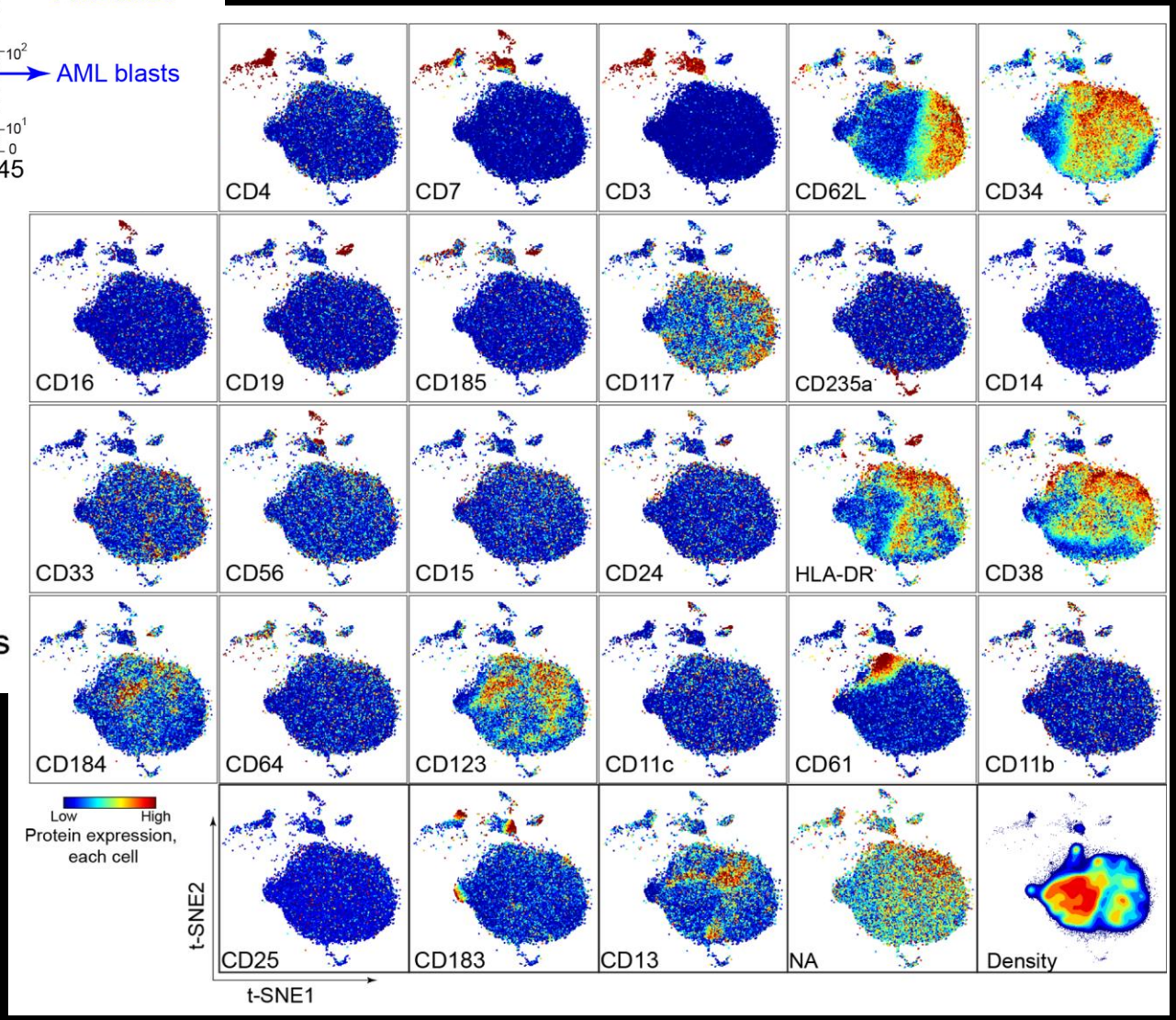




https://premium.cytobank.org/cytobank/experiments/44411/

Next: View All Channels to Make Figure 1 from Diggins et al.



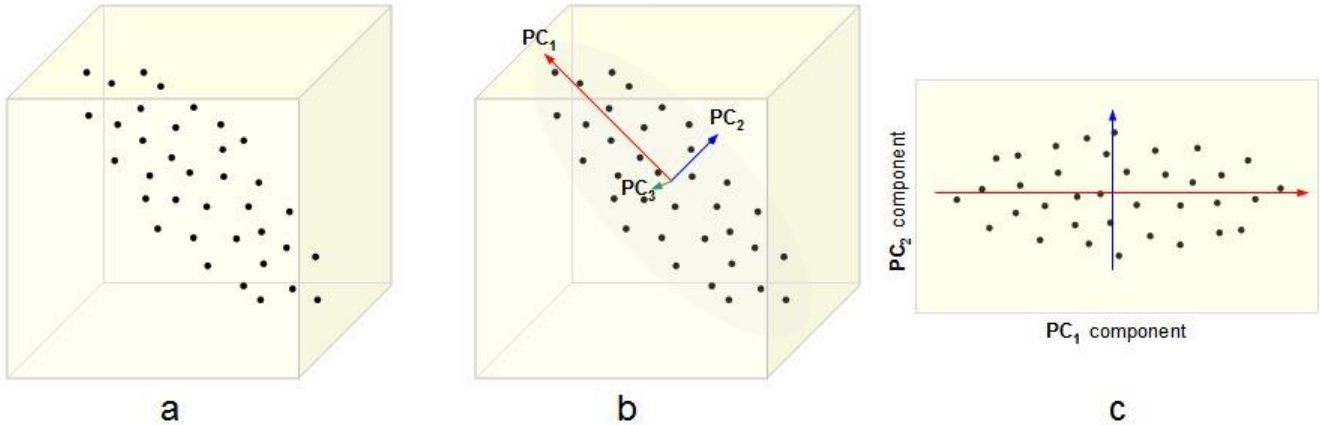


Diggins, et al *Methods* 2015

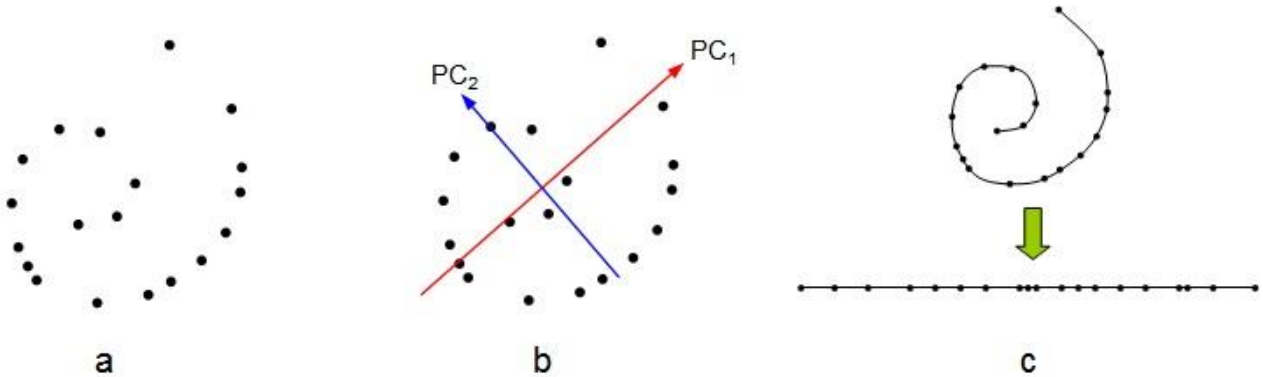
While viSNE runs…

A brief interlude about topology

Is There 'Inherent Topology' in the Data?

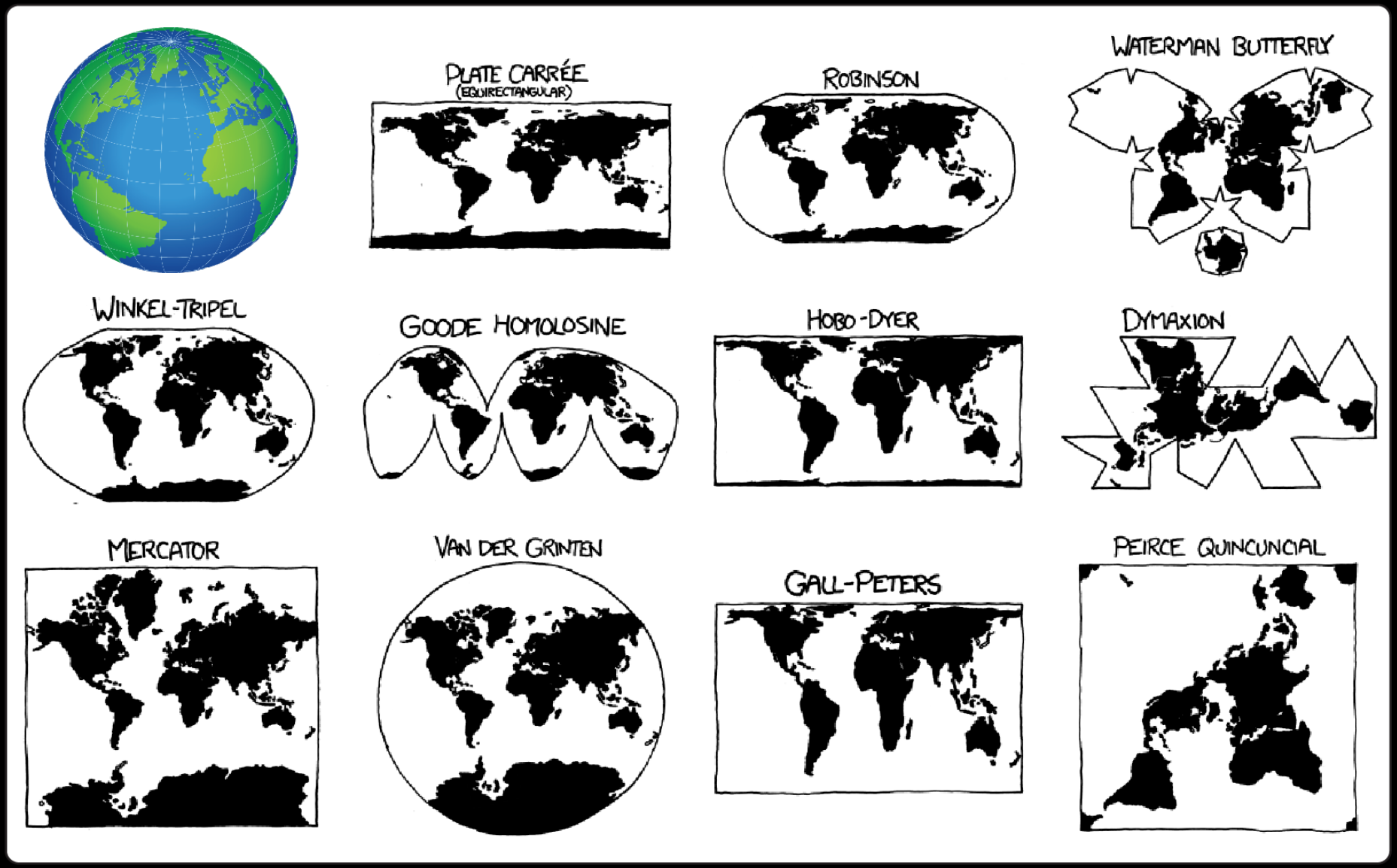


An illustration of PCA. **a)** A data set given as 3-dimensional points. **b)** The three orthogonal Principal Components (PCs) for the data, ordered by variance. **c)** The projection of the data set into the first two PCs, discarding the third one.



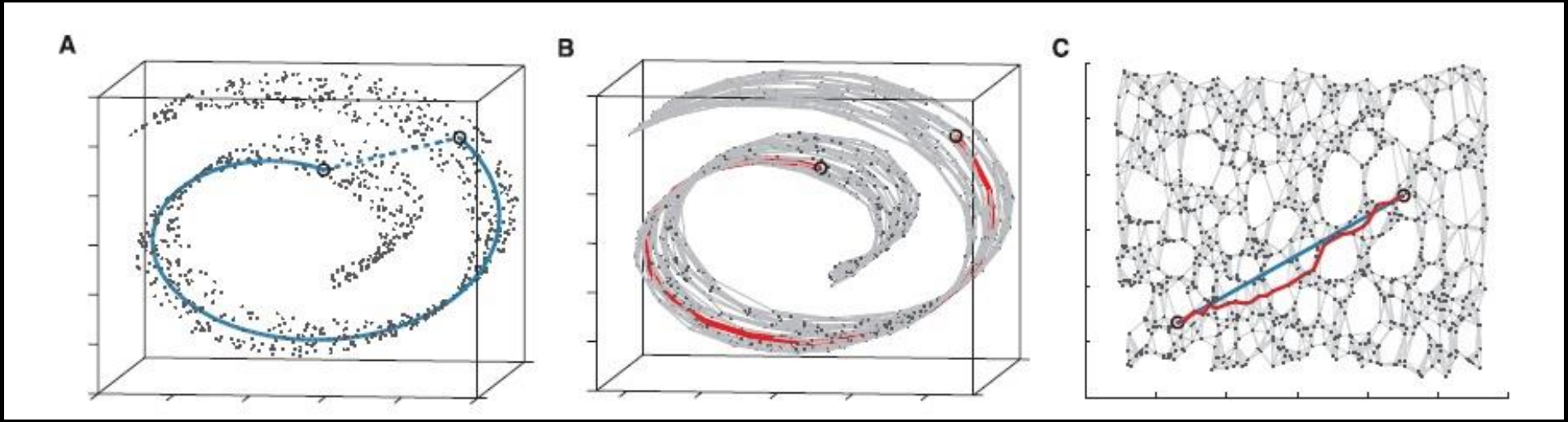
Effects of dimensionality reduction on an inherently non-linear data set. **a)** The original data given as a two-dimensional set. **b)**PCA identifies two PCs as contributing significantly to explain the data variance. **c)** However, the inherent topology (connectivity) of the data helps identify the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

Mapping Topology Matters When Considering “Travel”



Cartoon maps adapted from http://xkcd.com/977/

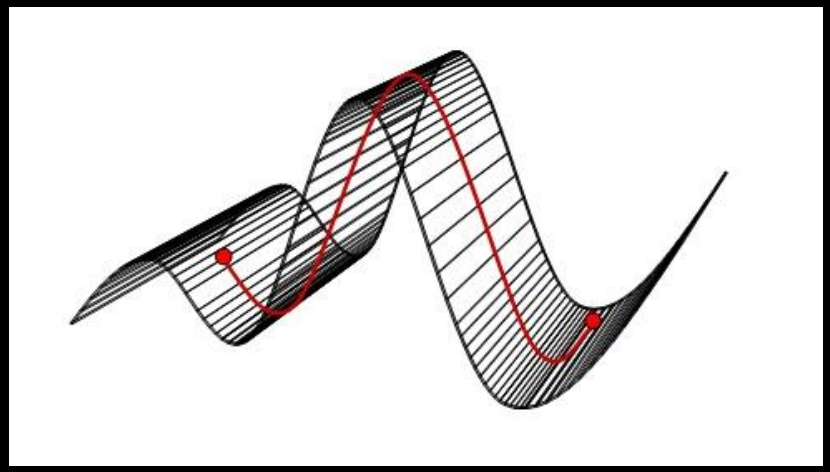
Multidimensional Scaling Can “Flatten” or “Unroll” Multidimensional Progressions / Topologies for Linear Analysis



Isomap at work on the "swiss roll" data set. **a)** The input data are given as three-dimensional, but **<u>are really two-dimensional in nature</u>**. **b)** Each point in the data set is connected to its neighbors to form a neighborhood graph, overlaid in light grey. Geodesic distances are approximated by computing **<u>shortest paths along the neighborhood graph</u>** (red). **c)** MDS applied to the geodesic distances has the effect of "unrolling" the swiss roll into its natural, two-dimensional parameterization. The neighborhood graph has been overlaid for comparison. Now, the Euclidean distances (in blue) approximate the original geodesic distances (in red).

Lydia E. Kavraki, *Geometric Methods in Structural Computational Biology*

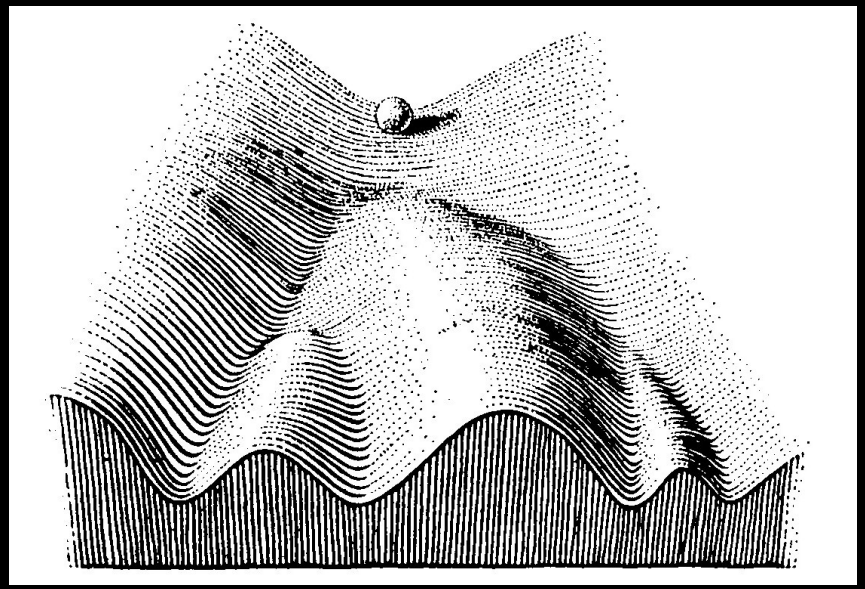
Geodesic Distance Measurements May Better Capture Developmental Progressions



Geodesic distance. The geodesic distance between the two red points is the length of the geodesic path, which is the shortest path between the points, **that lies on the surface**.

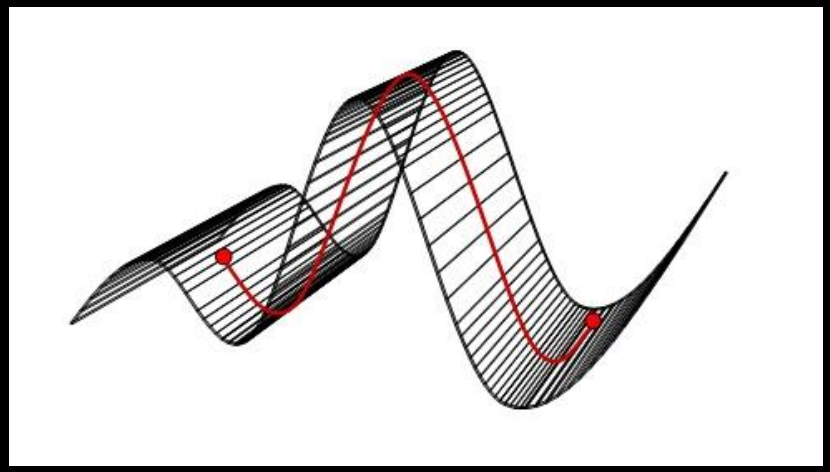
Lydia E. Kavraki, *Geometric Methods in Structural Computational Biology*

"**Creode** is a neologism coined by the biologist C.H. Waddington to represent the developmental pathway followed by a cell as it grows to form part of a specialized organ. Combining the Greek roots for "necessary" and "path," the term was inspired by the property of regulation."



http://en.wikipedia.org/wiki/Creode

Geodesic Distance Measurements May Better Capture Developmental Progressions



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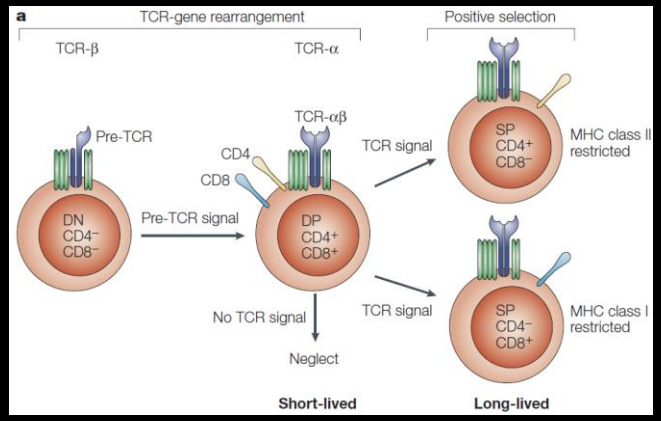
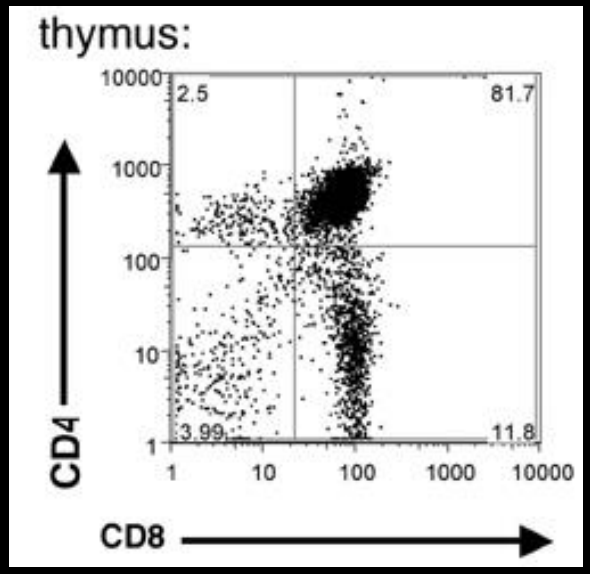
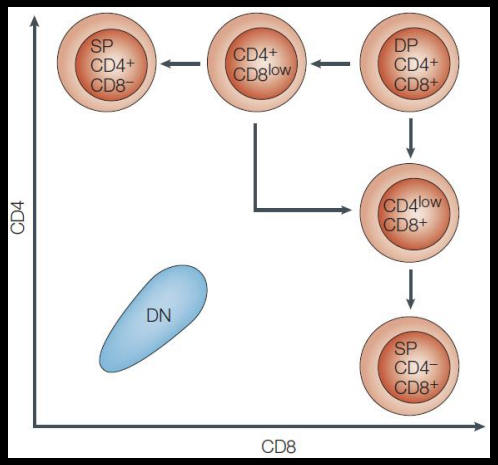






Bosslet, *Nature Rev. Immuno.* 2004

OK, back to work…

Steps 5-7: Gating on viSNE & Exporting for SPADE

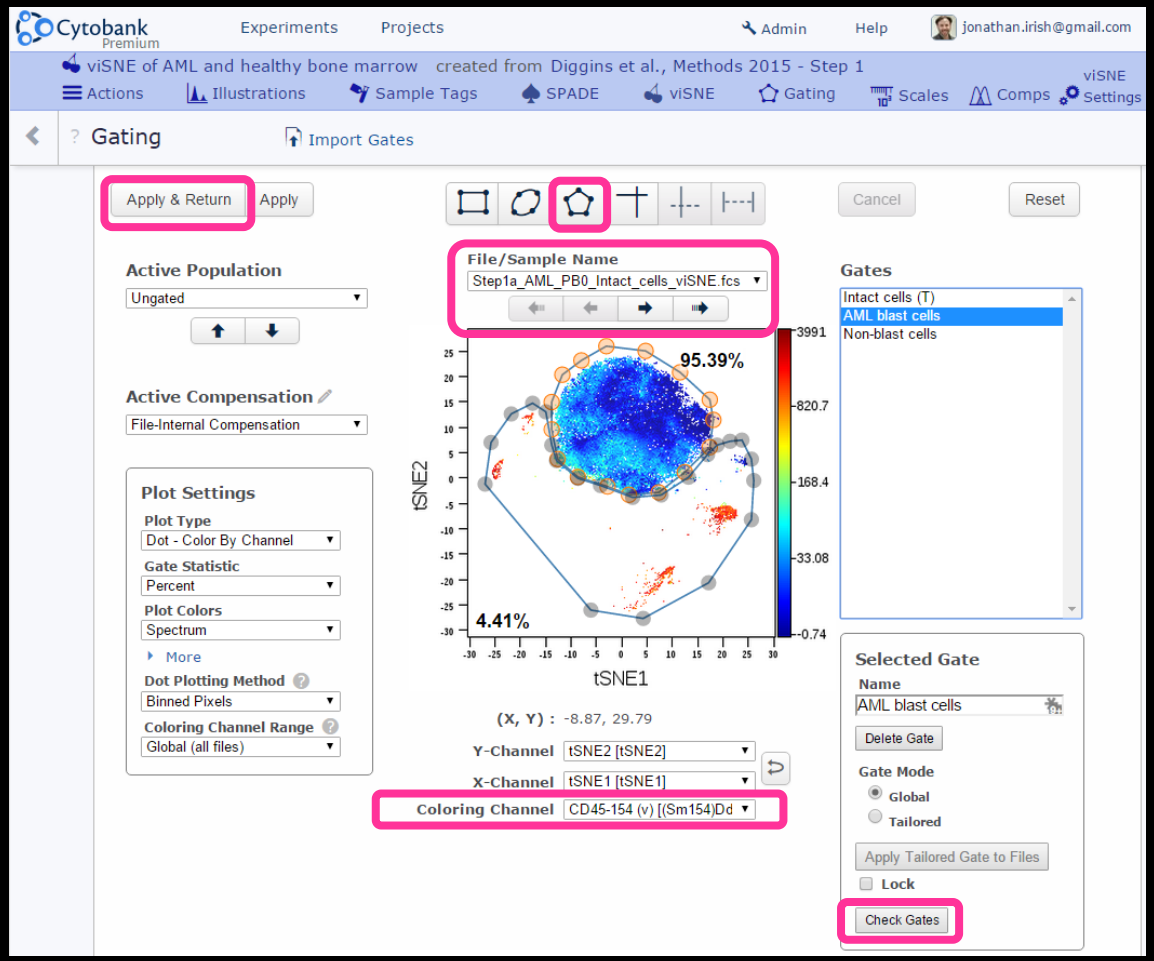
Workflow summary:

5) Gate on the t-SNE axes for “AML PB blasts” ($CD45^{lo}$) and “AML PB non-blasts” ($CD45^{hi}$)

6) Export gated populations as 4 new FCS files & discard the empty one.

7) Concatenate the 3 remaining files into a merged file.

- Change the z-axis coloring channel to CD45.
- Using the knowledge the AML blasts are $CD45^{lo}$, draw gates for “AML blast cells” and “non-blast cells”.
- Use “Check Gates” button to make sure gates work well on both files. Healthy marrow should have <3% cells in the AML blast gate.
- Apply & Return.



https://premium.cytobank.org/cytobank/experiments/44415/

Steps 5-7: Gating on viSNE & Exporting for SPADE

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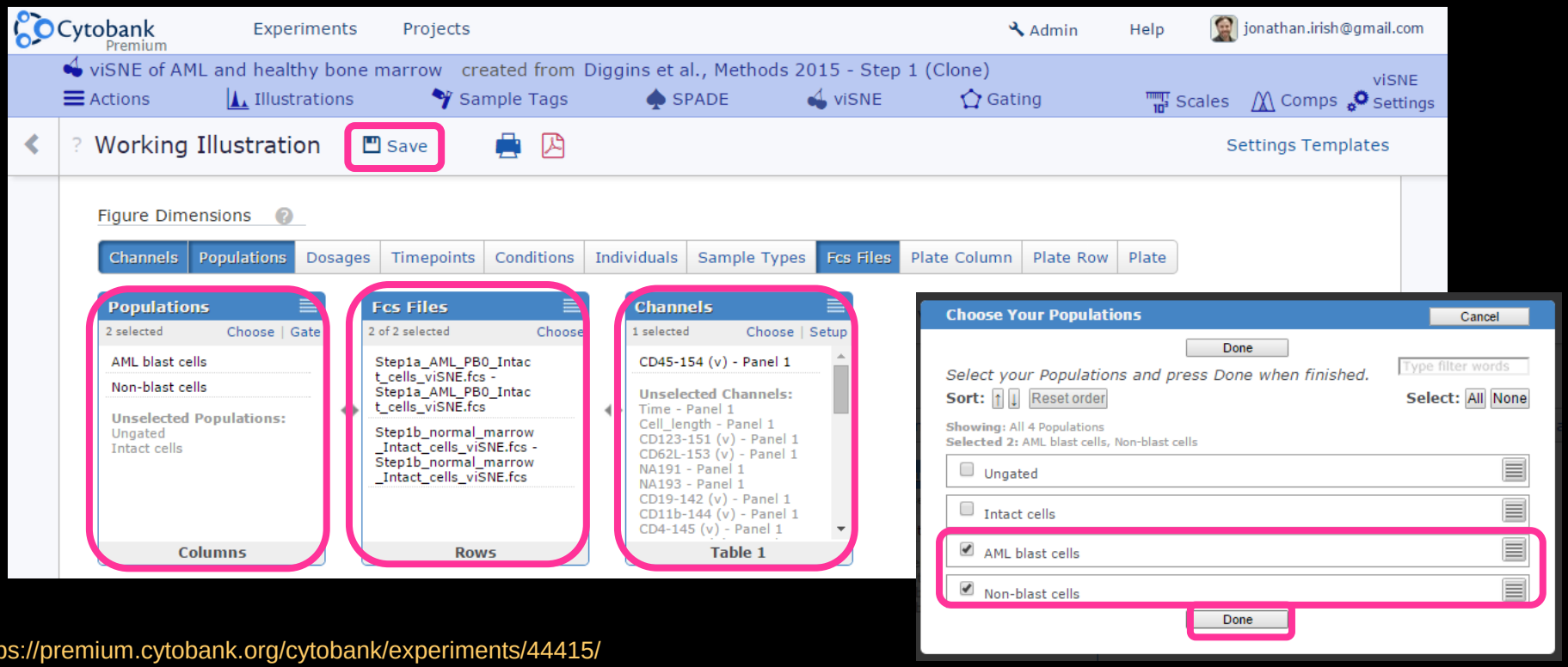
6) Export gated populations as 4 new FCS files & discard the empty one.

7) Concatenate the 3 remaining files into a merged file.

- Set up an illustration to export the viSNE gated data: Pick 2 Populations ("AML blast cells" & "Non-blast cells"). Pick one Channel (CD45). Activate "FCS files". Update & Save.



https://premium.cytobank.org/cytobank/experiments/44415/

Steps 5-7: Gating on viSNE & Exporting for SPADE

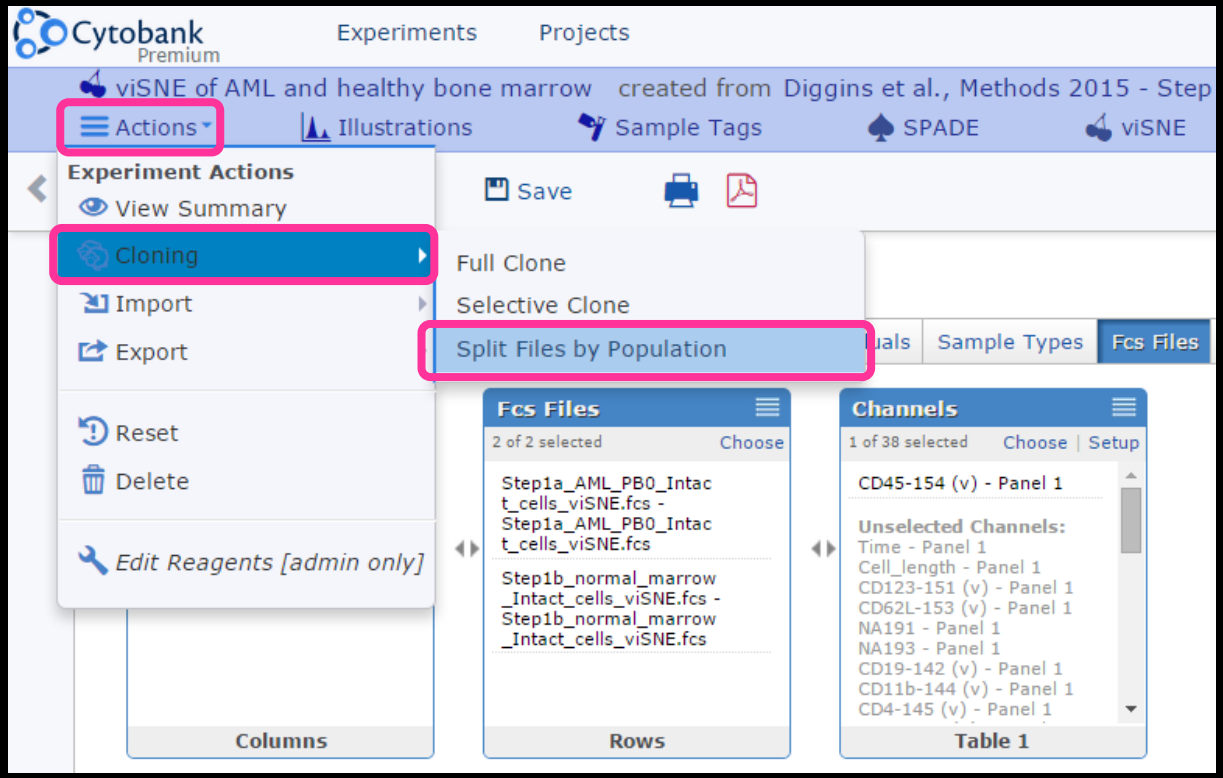
Workflow summary:

5) Gate on the t-SNE axes for "AML PB blasts" ($CD45^{lo}$) and "AML PB non-blasts" ($CD45^{hi}$)

6) Export gated populations as 4 new FCS files & discard the empty one.

7) Concatenate the 3 remaining files into a merged file.

- Export viSNE gated data: Actions => Cloning => Split Files by Population



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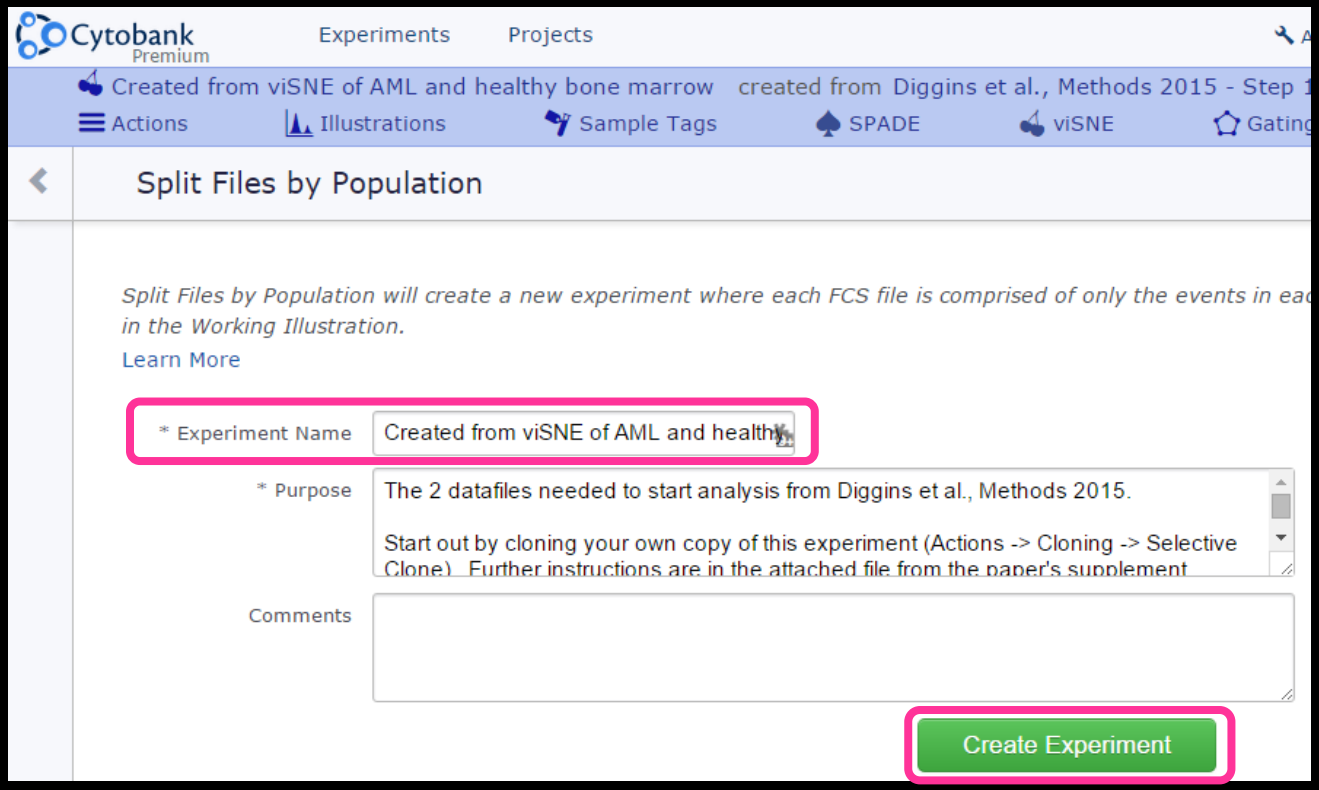
- Name the experiment “Diggins et al. - Export for SPADE” and click “Create Experiment”



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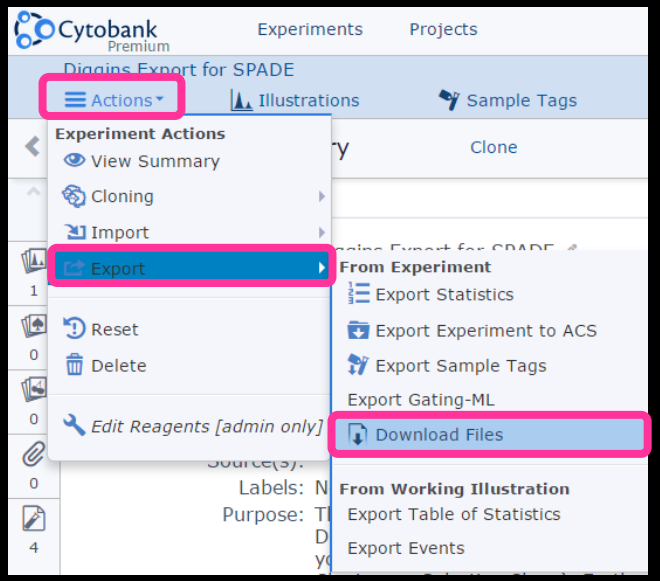
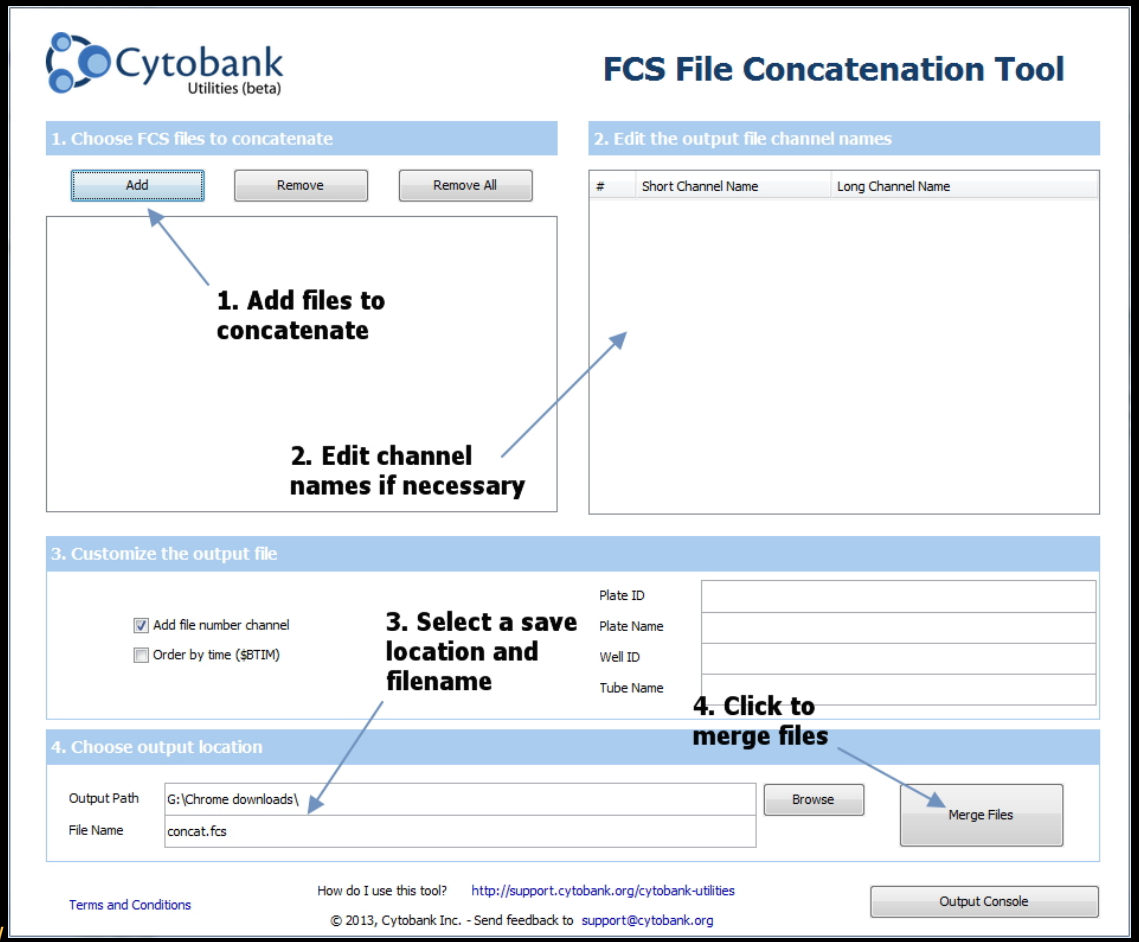
- Download the files and concatenate (merge) into 1 file.

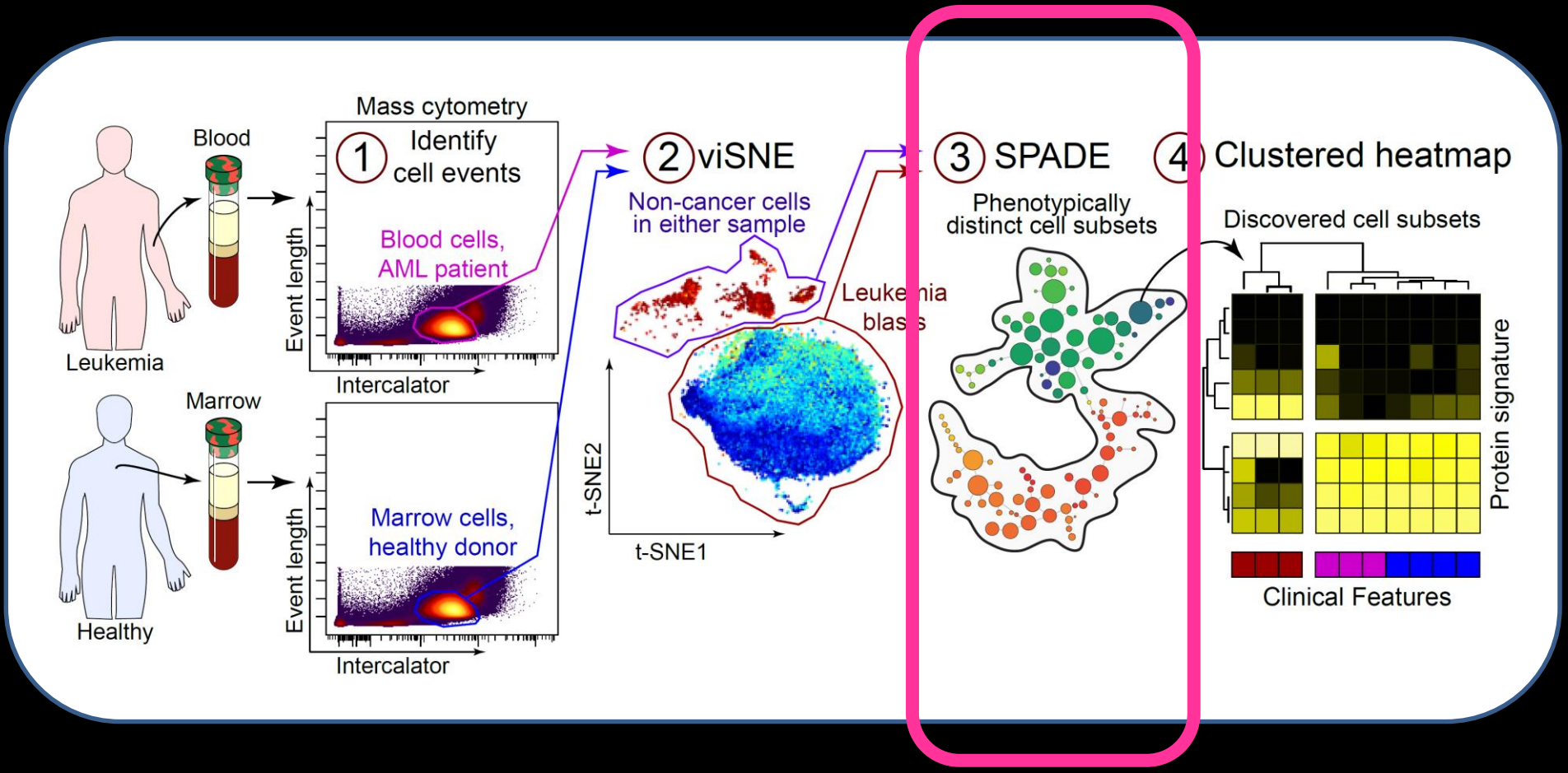
http://blog.cytobank.org/2013/10/29/new-fcs-file-concatenation-tool/





https://premium.cytobank.org/cytobank/experiments/44415/

Discovery and Characterization of Cell Subsets: Towards Machine Learning Cell Identity



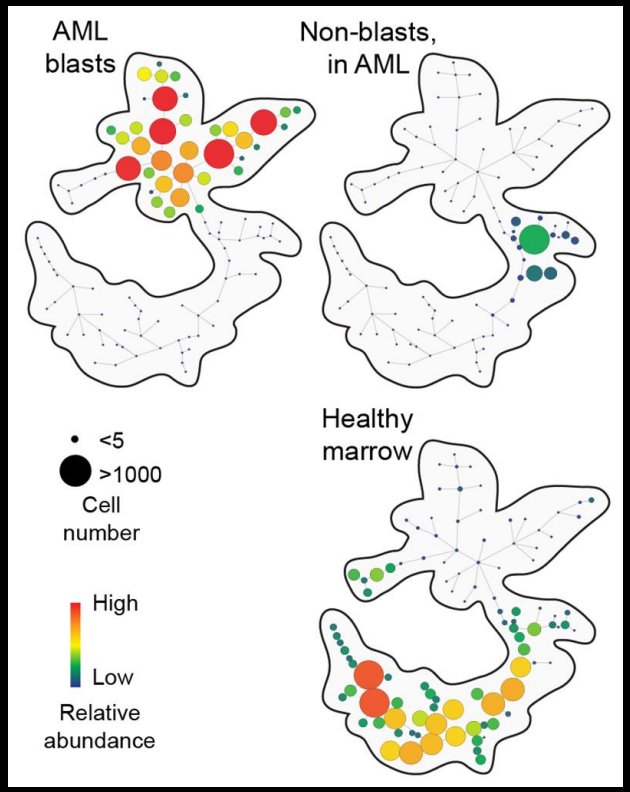
Diggins et al., *Methods* 2015

Single Cell Biology Data Analysis

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 gating
Revealing cell subsets	7) Feature selection 8) Dimensionality reduction 9) Identify cell clusters 10) Cluster refinement
Characterizing cell subsets	11) Feature comparison 12) Model populations 13) Learn cell identity 14) Statistical testing

Feature selection

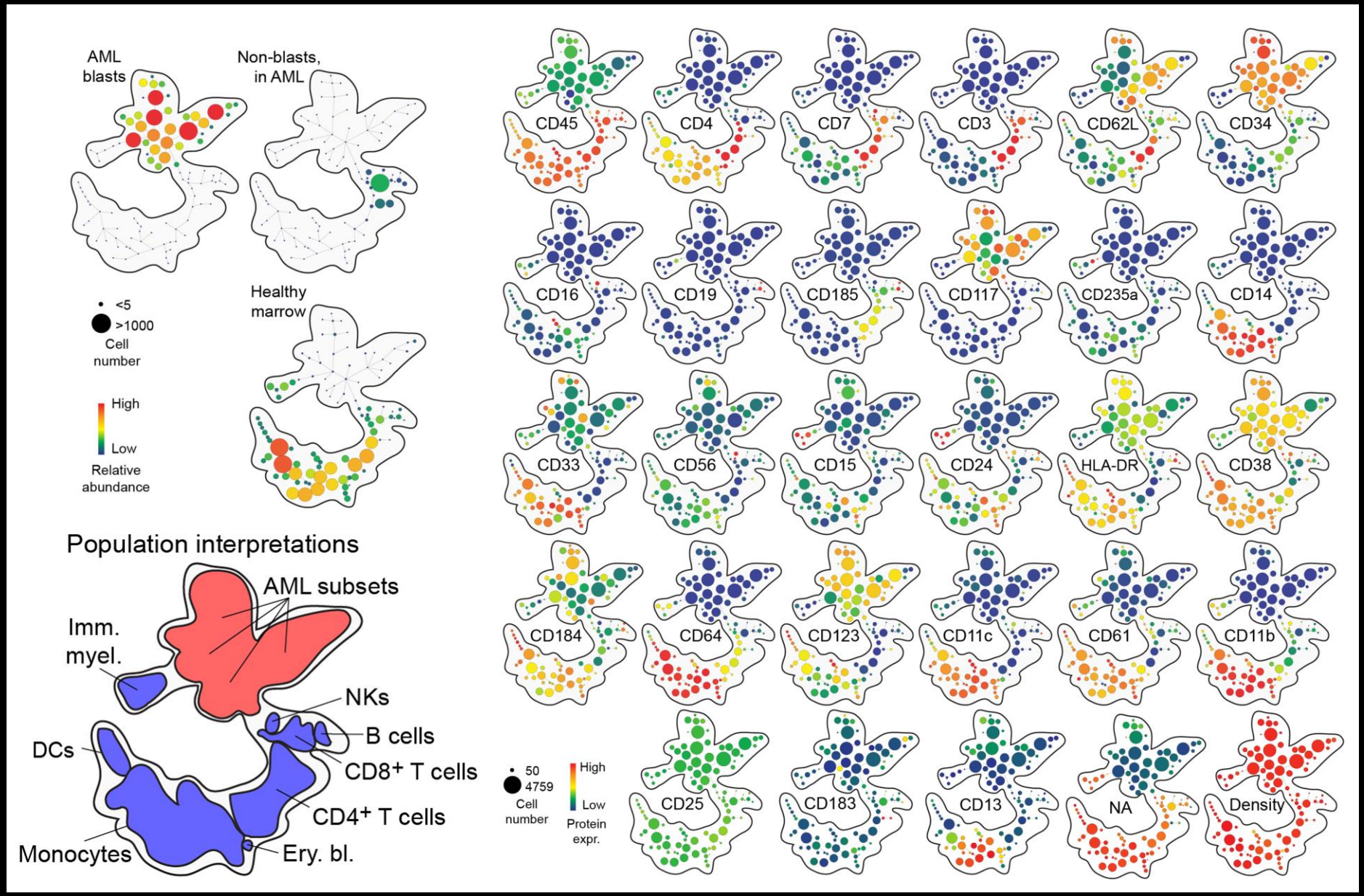
SPADE: cell subset clustering



Grouped populations of cells

Diggins et al., *Methods* 2015

Current Goal: Make Figure 2 from Diggins et al. with SPADE



Diggins, et al *Methods* 2015

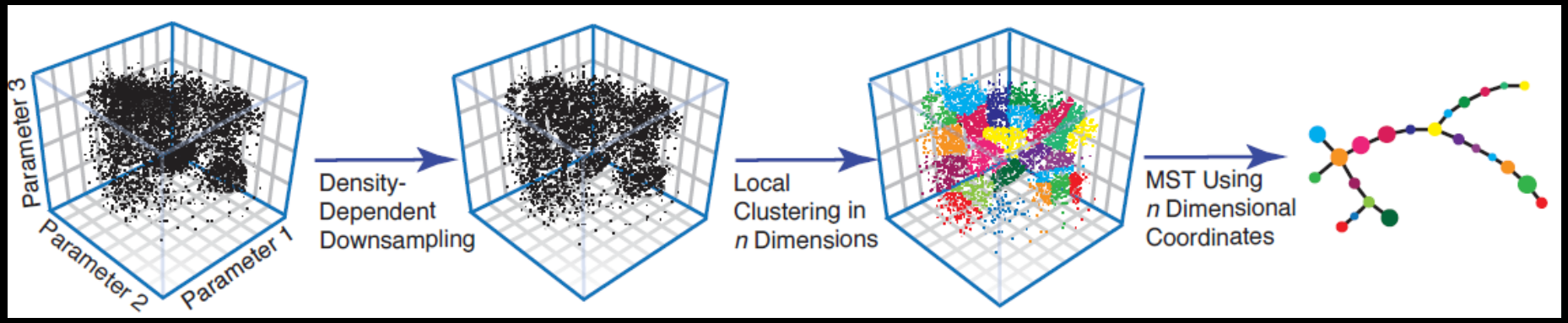
SPADE Extracts Population Hierarchies from Multi-D Space

Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE

Peng Qiu[1,2], Erin F Simonds[3], Sean C Bendall[3], Kenneth D Gibbs Jr[3], Robert V Bruggner[3], Michael D Linderman[4], Karen Sachs[3], Garry P Nolan[3] & Sylvia K Plevritis[1]

VOLUME 29 NUMBER 10 OCTOBER 2011 NATURE BIOTECHNOLOGY

SPADE method:



1) Get Data

2) Downsample
(preserves rare subsets)

3) Cluster
(group by similarity)

4) Project in 2D
(minimum spanning tree)

SPADE stands for 'Spanning-tree Progression Analysis of Density-normalized Events'. A graphical interface and updates for fluorescence datasets are implemented on Cytobank.

SPADE Trees Depict Multidimensional Similarity
(not necessarily developmental relationships)

SPADE trees can sometimes be reorganized so that closely related branches appear far apart. In order to remove loops from the SPADE tree, breakpoints may be added in unexpected places.

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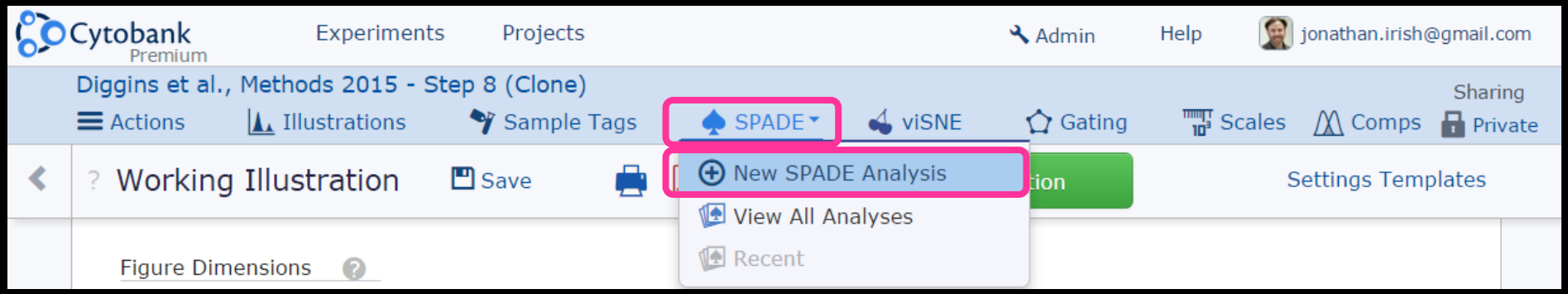
Steps 8 and 9: SPADE Clustering on t-SNE Channels

Workflow summary:

1) Clone experiment “Diggins et al., Methods 2015 – Step 8”
2) Set the Scales so that the scale argument is 15 for all channels.
3) Run SPADE.

- Navigate to Diggins et al., Methods 2015 – Step 8.
- Click the experiment name to load it.
- Clone the experiment, as before. Actions => Cloning => Selective Clone.
- Click Scales to edit the scales and set the argument to 15 for all channels. Click Apply and then close this window.
- The cells have already been gated, so no gating is needed.
- Choose SPADE => New SPADE Analysis & give it a name.



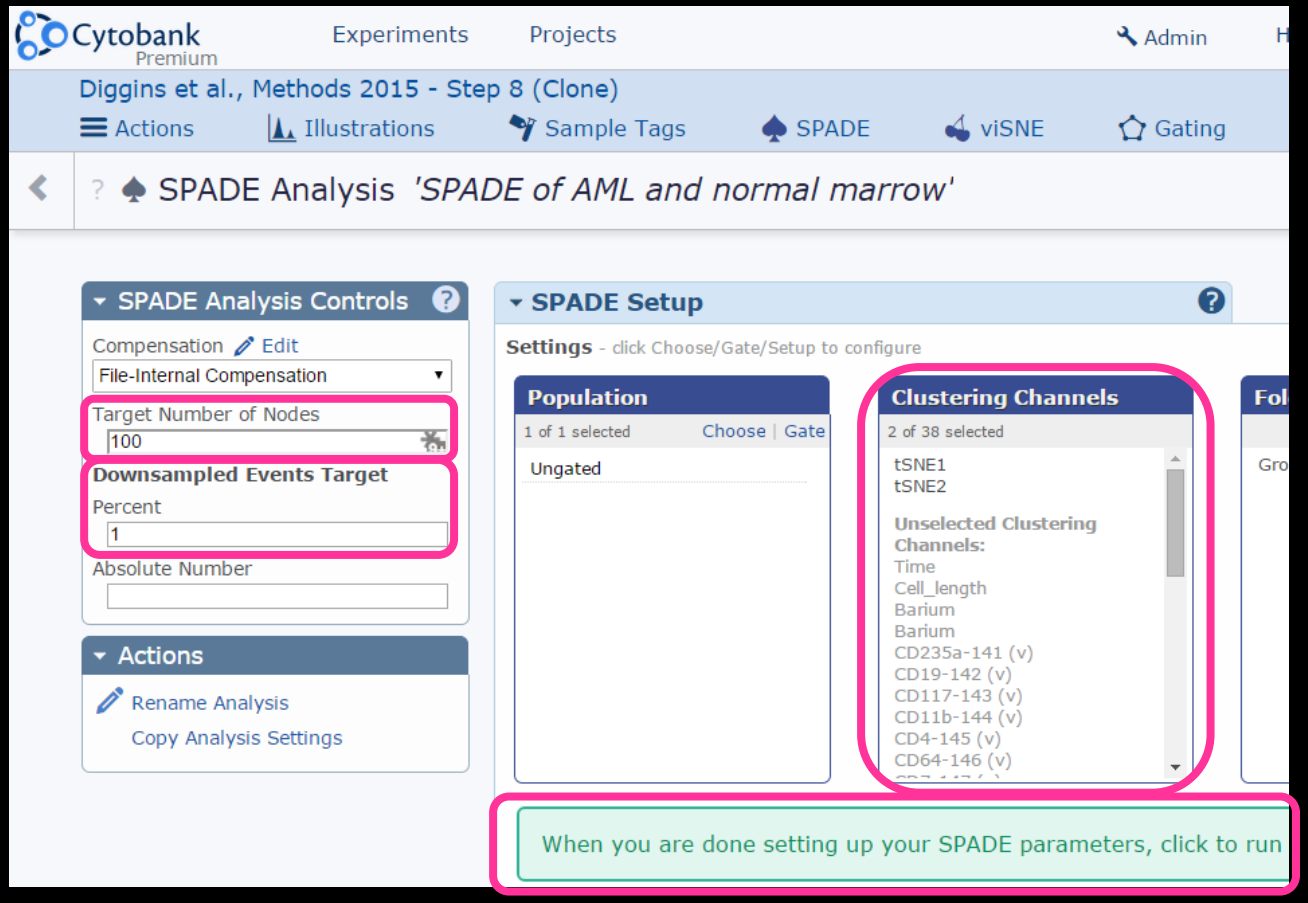
https://premium.cytobank.org/cytobank/experiments/44412

Steps 8 and 9: SPADE Clustering on t-SNE Channels

Workflow summary:

1) Clone experiment "Diggins et al., Methods 2015 – Step 8"
2) Set the Scales so that the scale argument is 15 for all channels.
3) Run SPADE.

- Set SPADE to use 100 nodes & 1% downsampling.
- Select just the 2 t-SNE channels.
- Click the green bar to run the analysis.



https://premium.cytobank.org/cytobank/experiments/44412

Steps 8 and 9: SPADE Clustering on t-SNE Channels

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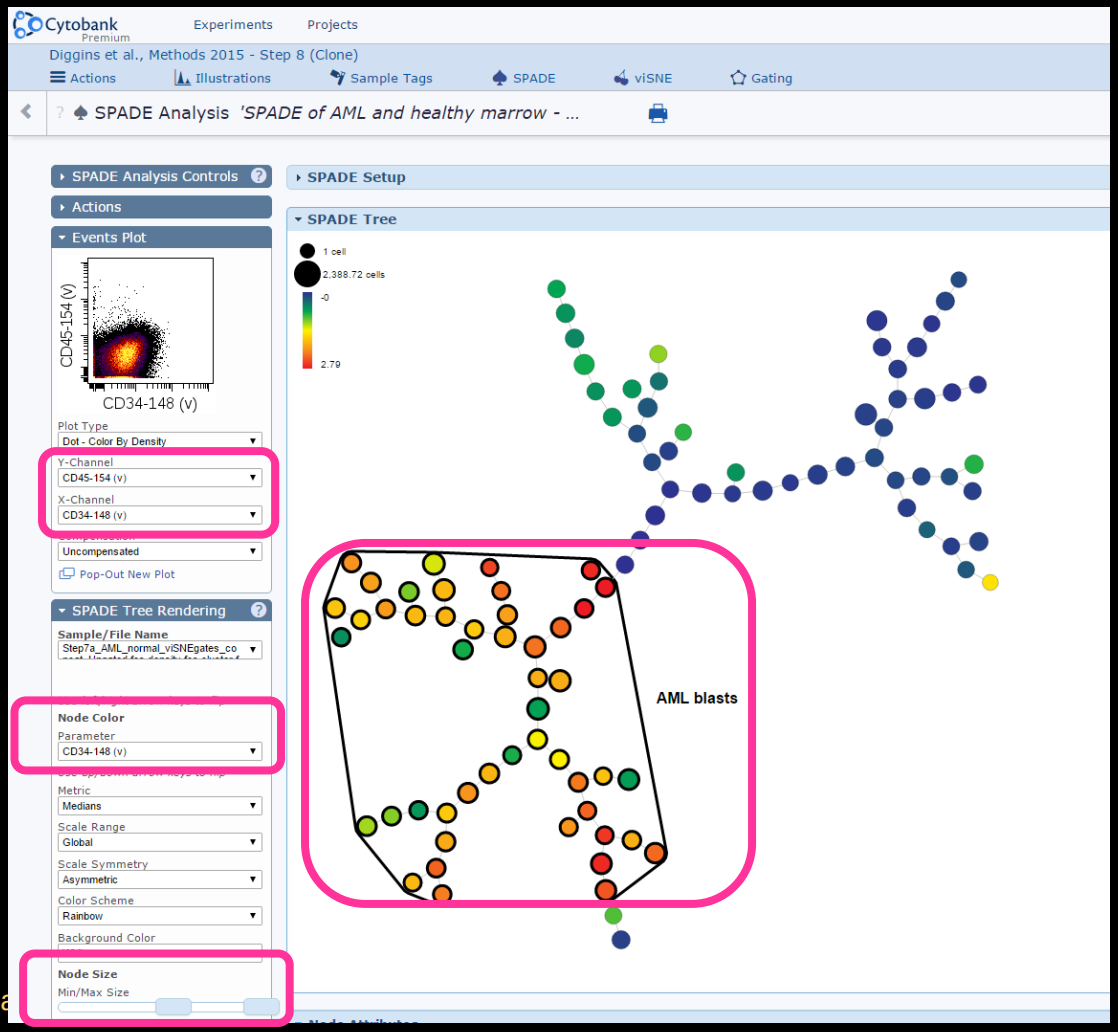
3) Run SPADE.

- Explore the data in the SPADE viewer.
- Make your node size larger. Set the x/y axes to CD45 and CD34. Set the Node Color Parameter to CD34.
- See if you can find the AML blasts and bubble them.
- View various Parameters.



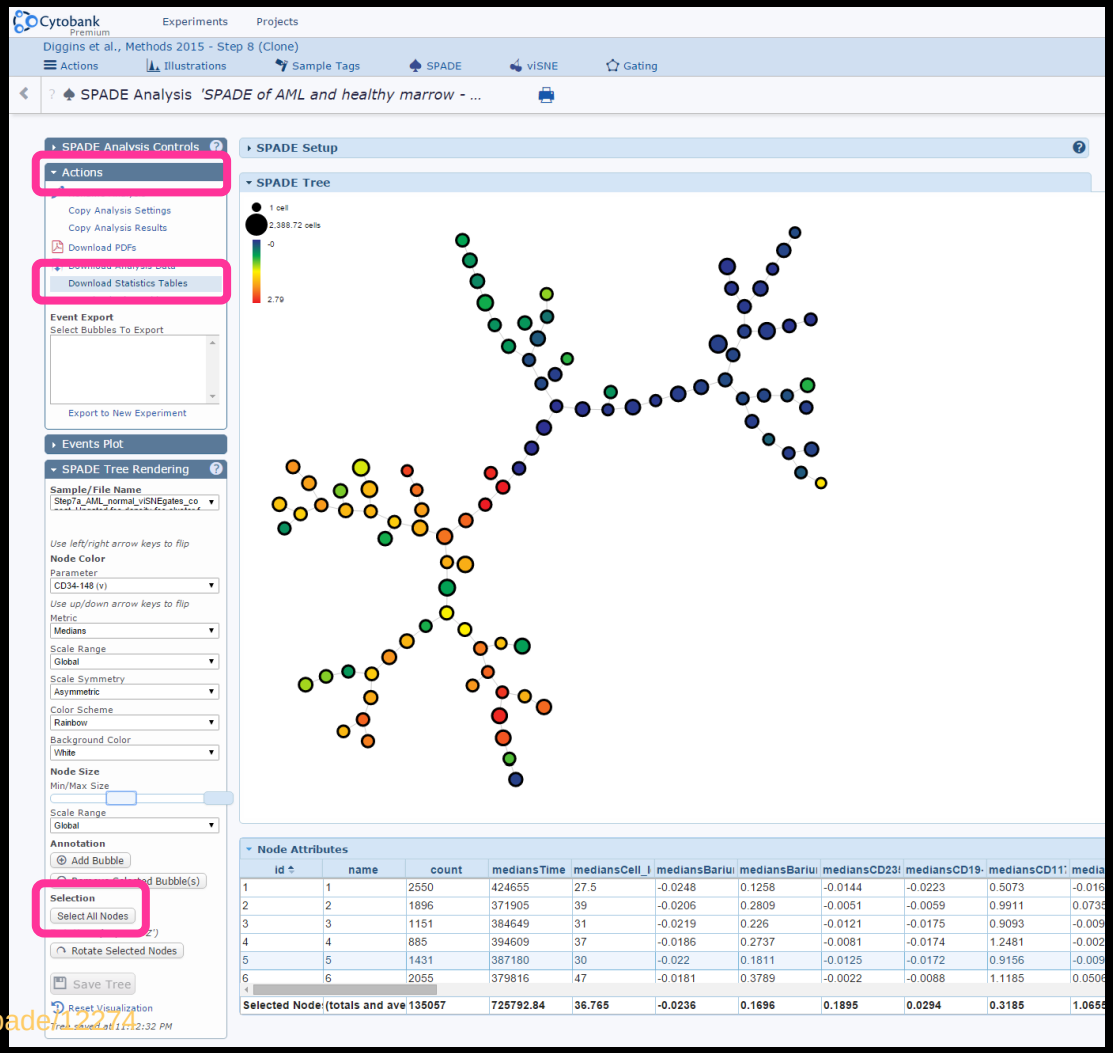
https://premium.cytobank.org/cytobank/experiments/44413/spa

Steps 8 and 9: SPADE Clustering on t-SNE Channels

Workflow summary:

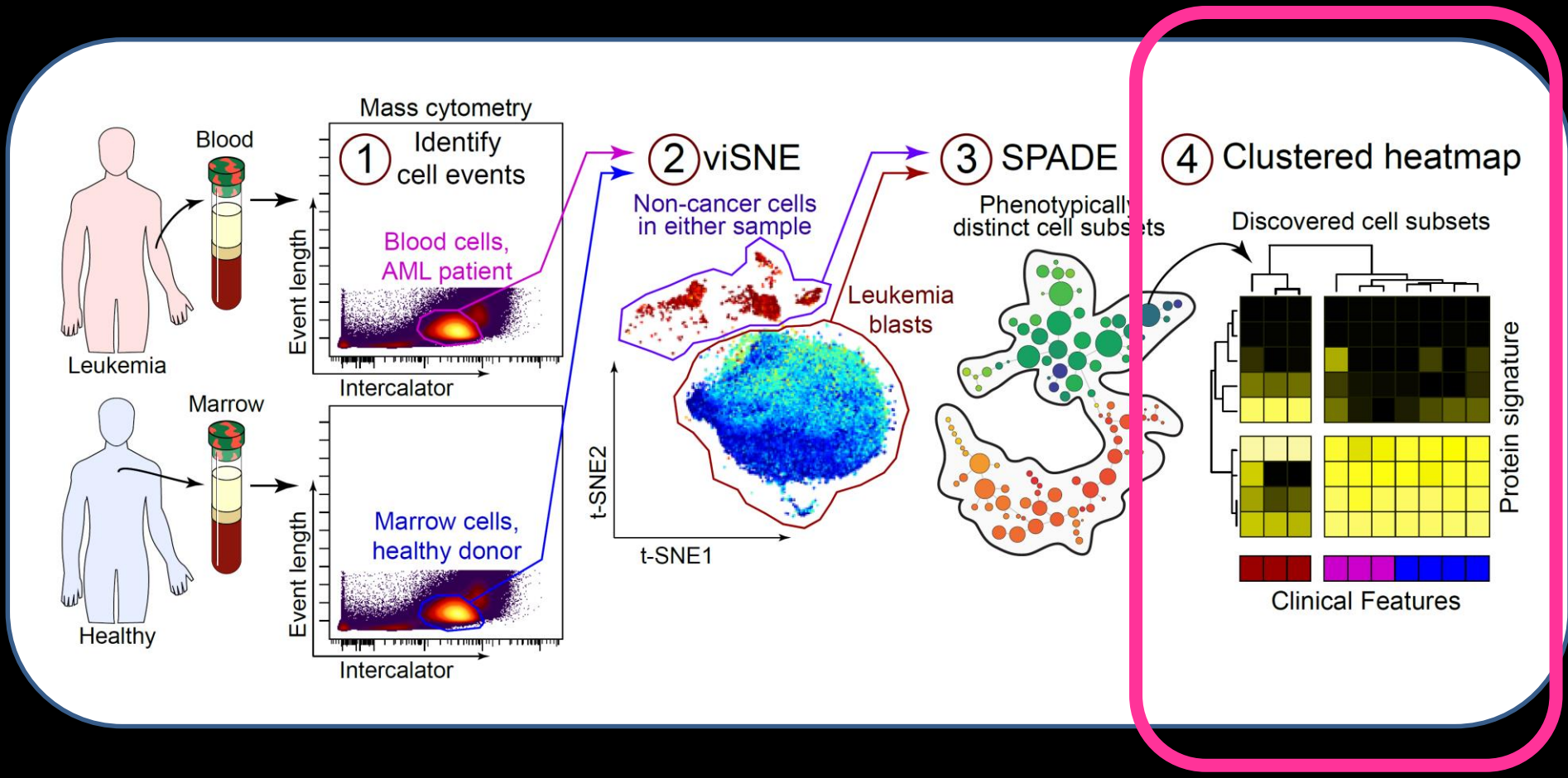
1) Clone experiment “Diggins et al., Methods 2015 – Step 8”
2) Set the Scales so that the scale argument is 15 for all channels.
3) Run SPADE.

- Scroll way down and click “Select All Nodes”.
- In the “Actions” panel, Choose “Download Statistics Table”
- In the ZIP file, you will find a folder called “By Sample” that has spreadsheets of median expression by node for all samples. These can be sorted, filtered, and clustered.



https://premium.cytobank.org/cytobank/experiments/44413/spade/122774

Discovery and Characterization of Cell Subsets: Towards Machine Learning Cell Identity



Diggins et al., *Methods* 2015

Single Cell Biology Data Analysis

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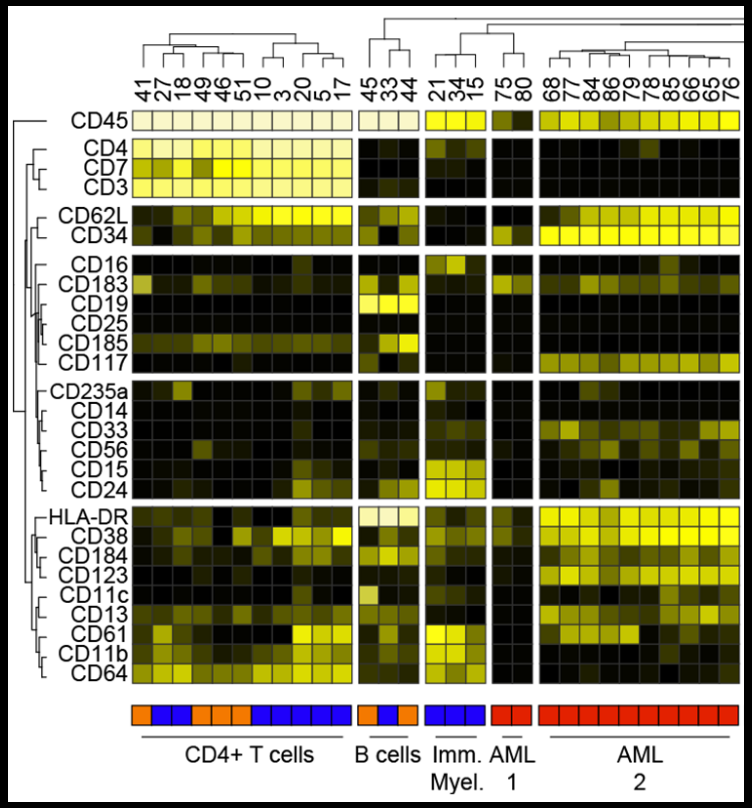
Grouped populations of cells

Heatmap: clustering populations

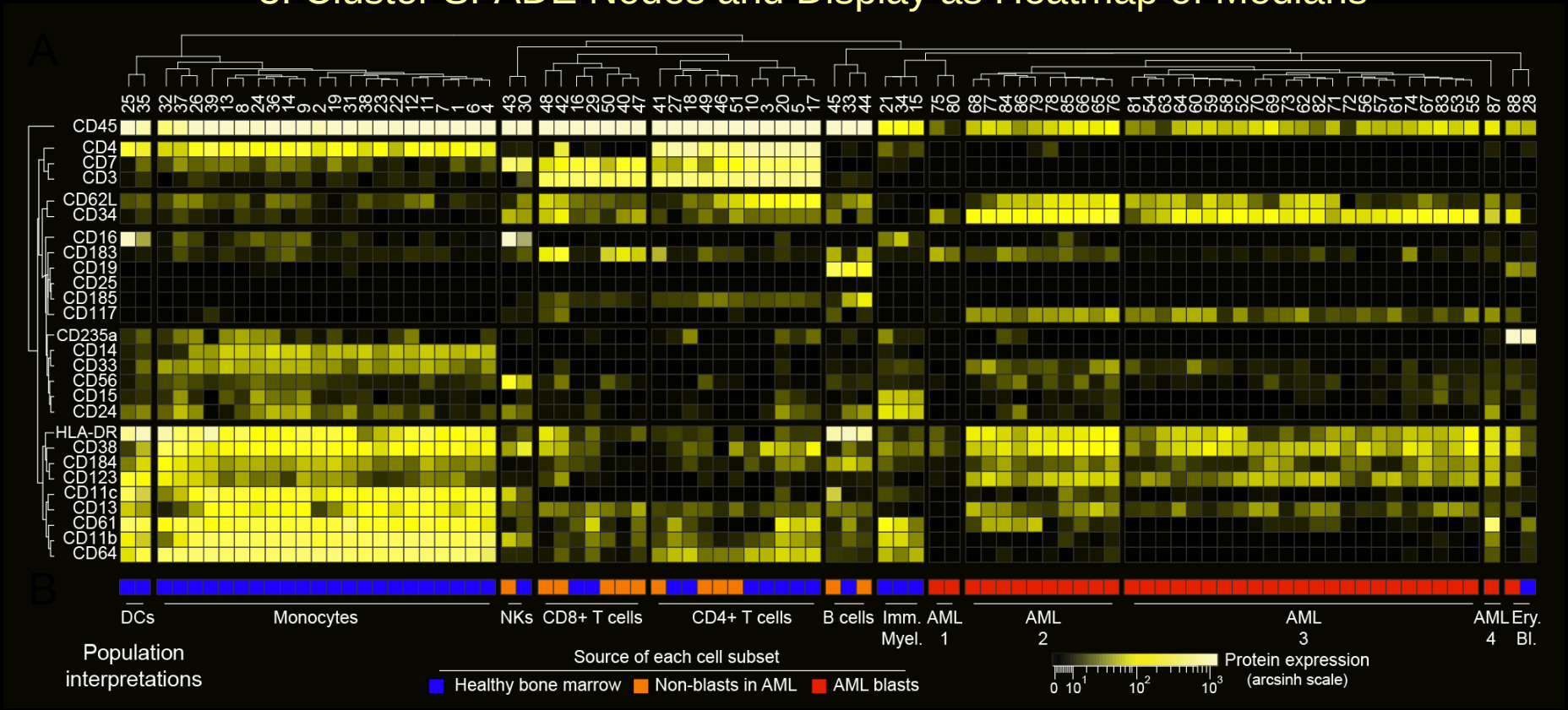


Compare and visualize populations across datasets

Diggins et al., *Methods* 2015

Finally, The Fun/Challenging Part: “Call” Subset Identity and Characterize Subset Features

3. Cluster SPADE Nodes and Display as Heatmap of Medians



Note: Heatmaps allow comparison of data from different viSNE and SPADE runs, different tools, different machines, etc.

Diggins, et al *Methods* 2015

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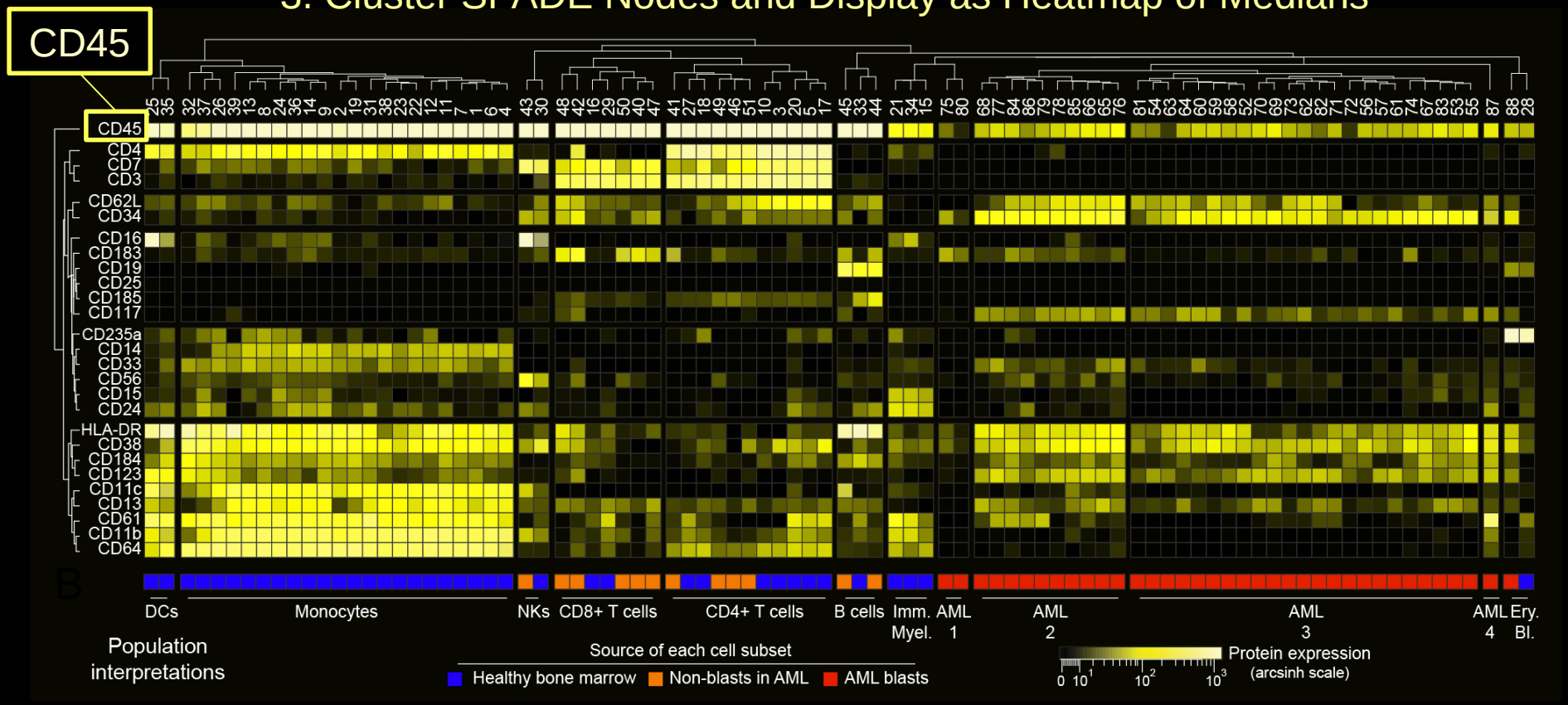

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Diggins, et al *Methods* 2015

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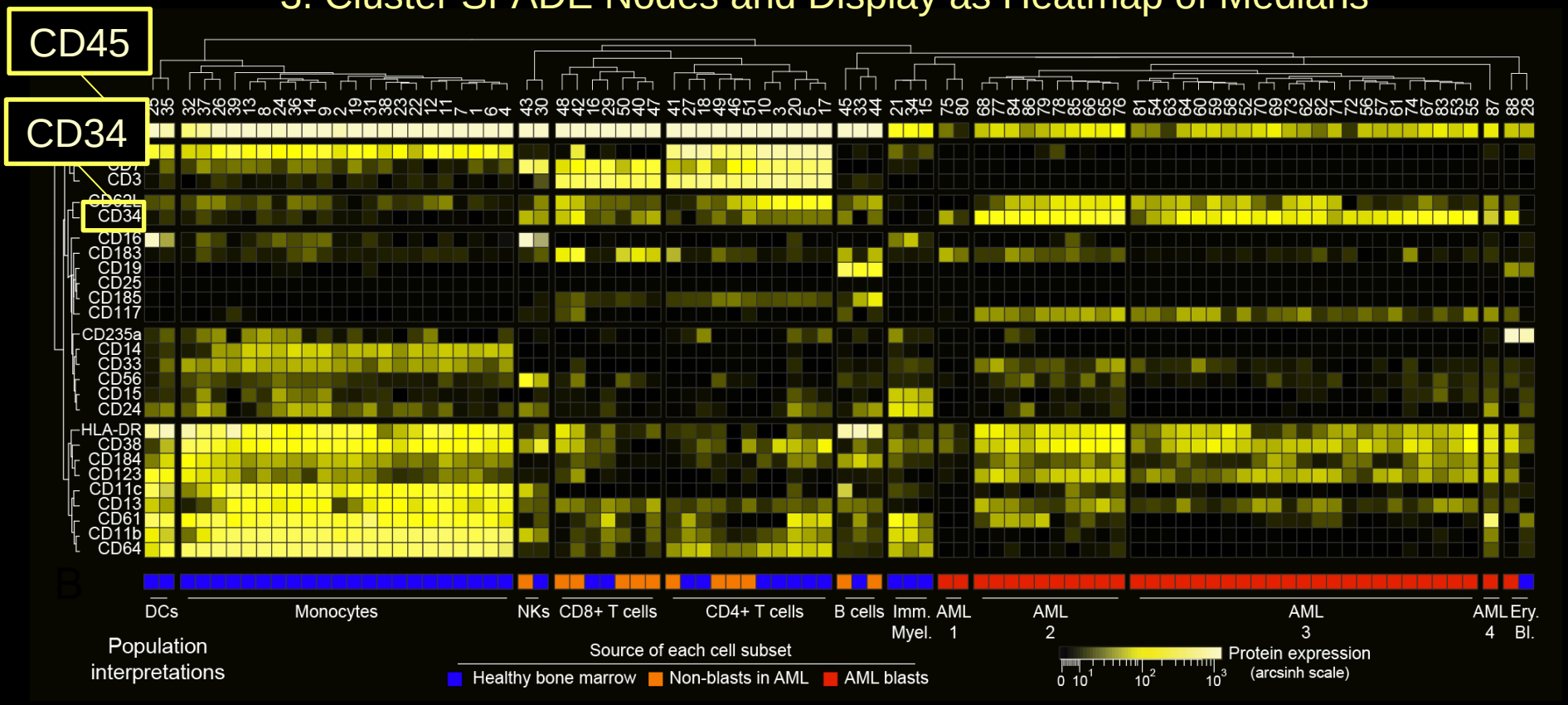

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Diggins, et al *Methods* 2015

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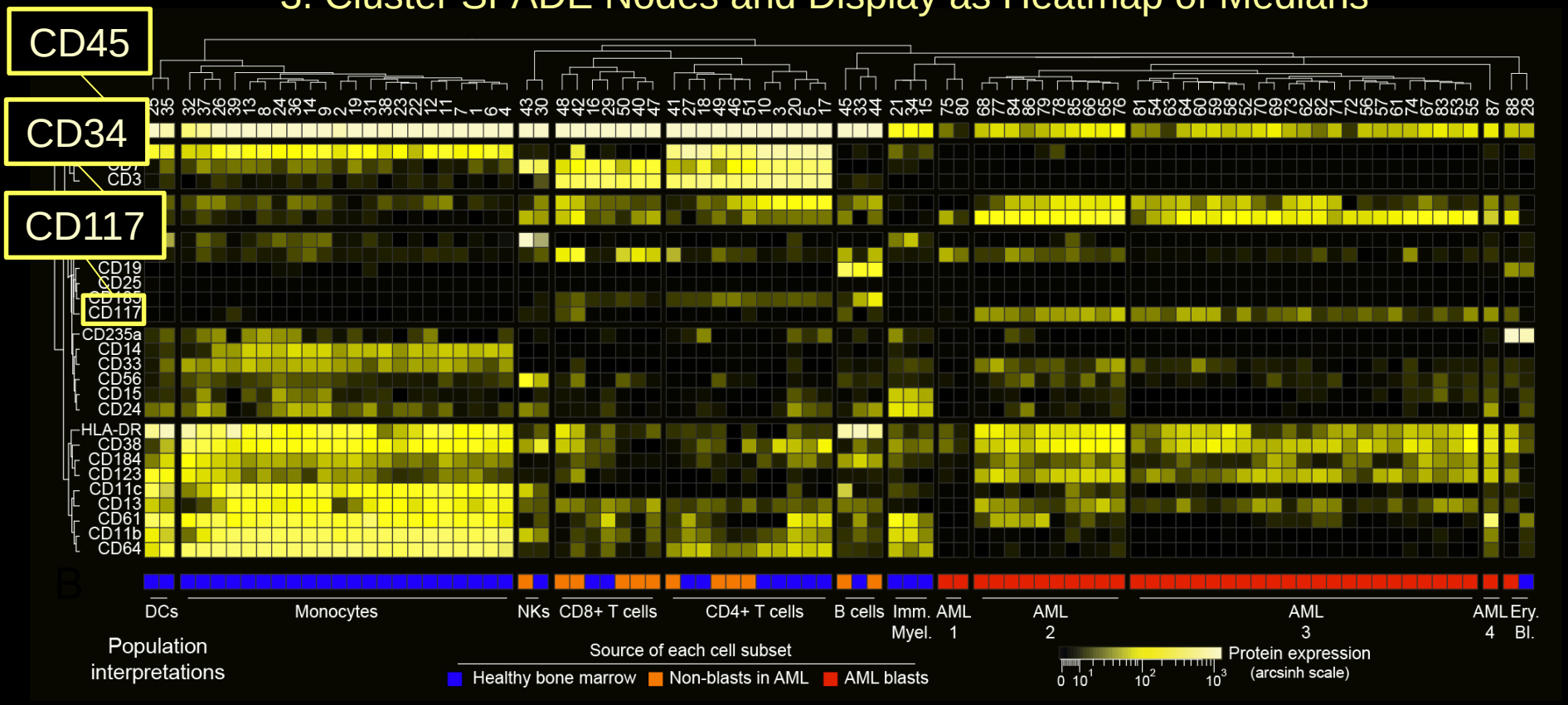
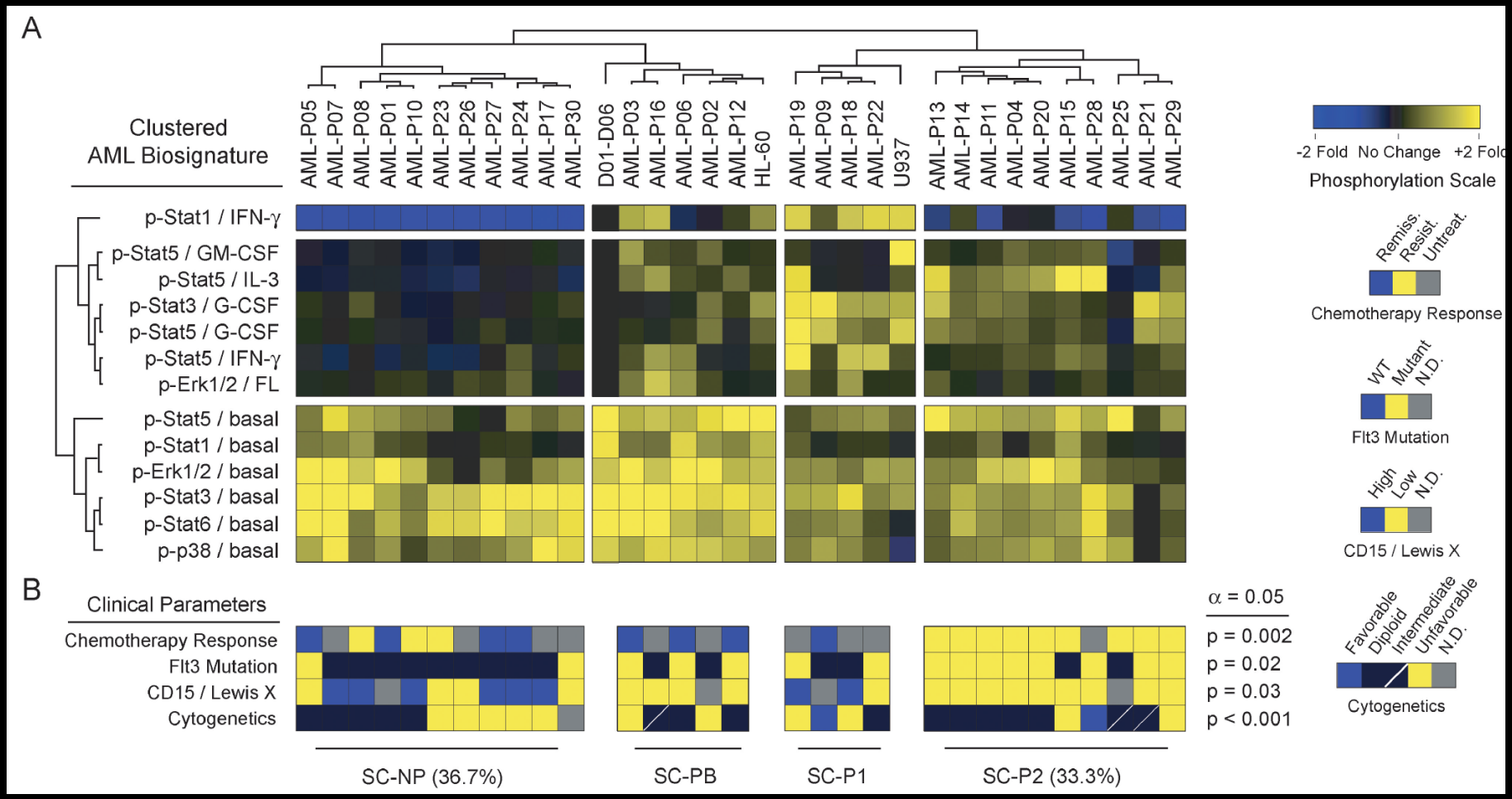


Note: Heatmaps allow comparison of data from different viSNE and SPADE runs, different tools, different machines, etc.

Diggins, et al *Methods* 2015

Heatmaps Also Visualize Other Data Types (e.g. Stratified Clinical Outcomes) and Compare Across Analysis Runs

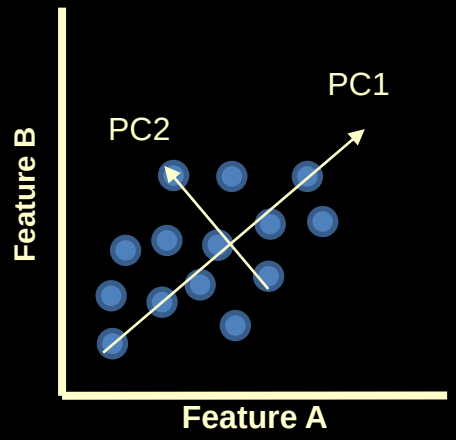


Heatmaps visualize across integrated data types,
e.g. clinical outcomes, cytogenetics, & signaling profiles

Irish, et al *Cell* 2004

Other analysis tools

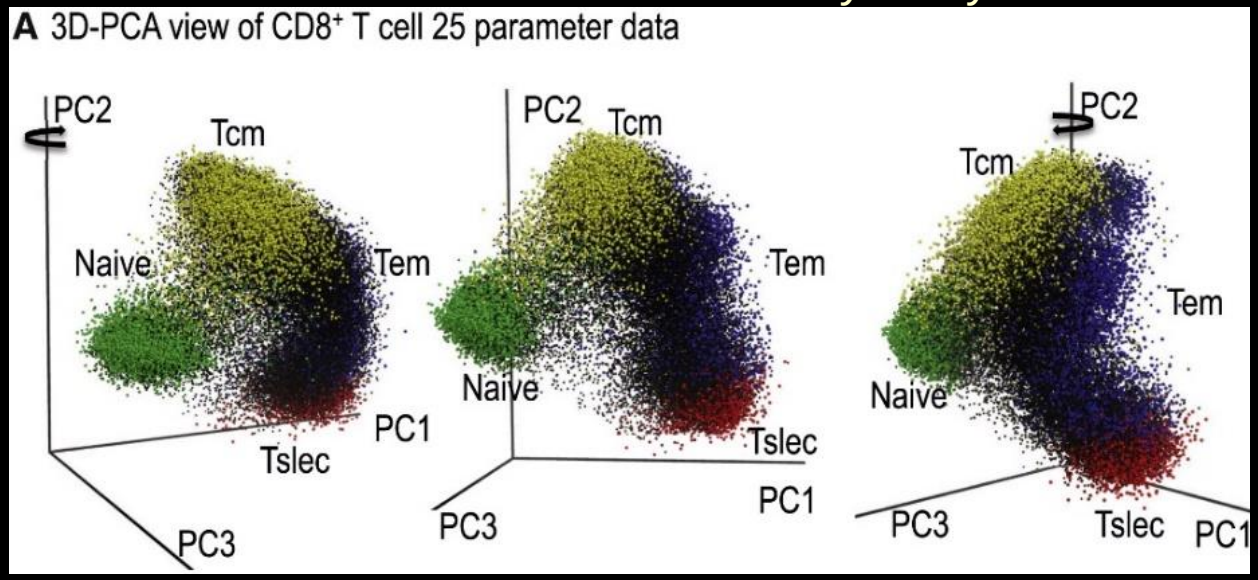
Principal Component Analysis



	Feature A	Feature B	Feature C
Principal Component 1	$X_{A1}A$	$X_{B1}B$	$X_{C1}C$
Principal Component 2	$X_{A2}A$	$X_{B2}B$	$X_{C2}C$
Principal Component 3	$X_{A3}A$	$X_{B3}B$	$X_{C3}C$

$$PC1 = X_{A1}A + X_{B1}B + X_{C1}C$$
$$PC2 = X_{A2}A + X_{B2}B + X_{C2}C$$
$$PC3 = X_{A3}A + X_{B3}B + X_{C3}C$$

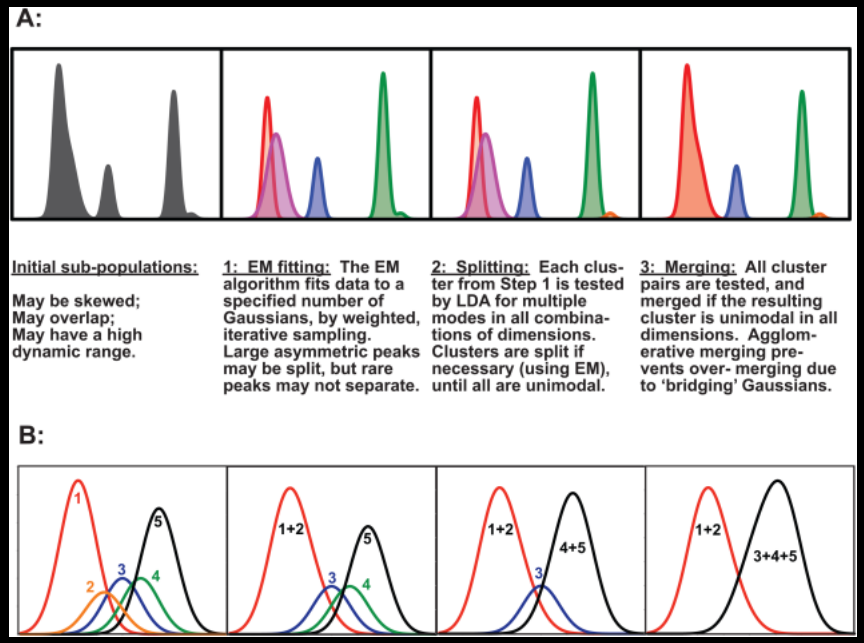
PCA used to Reduce Dimensionality of CyTOF Data



Newell et al 2012, *Immunity*

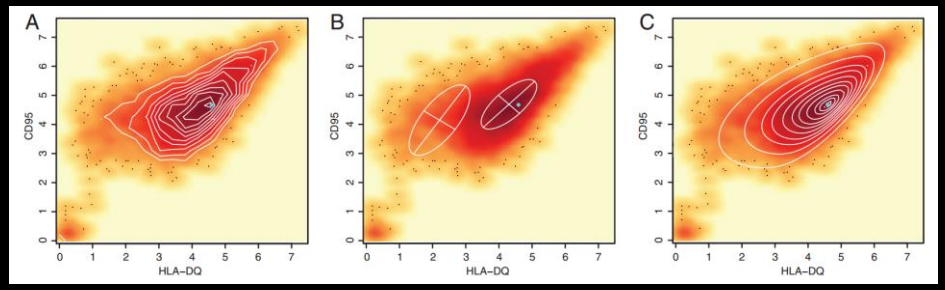
Mixture Modeling

SWIFT



Mosmann et al, 2014 *Cytometry A*

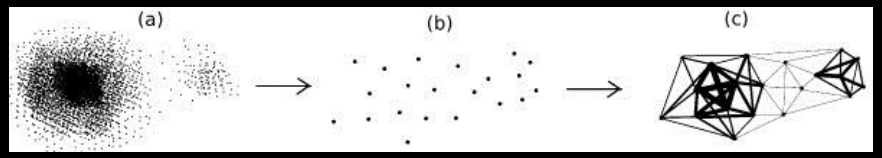
FLAME

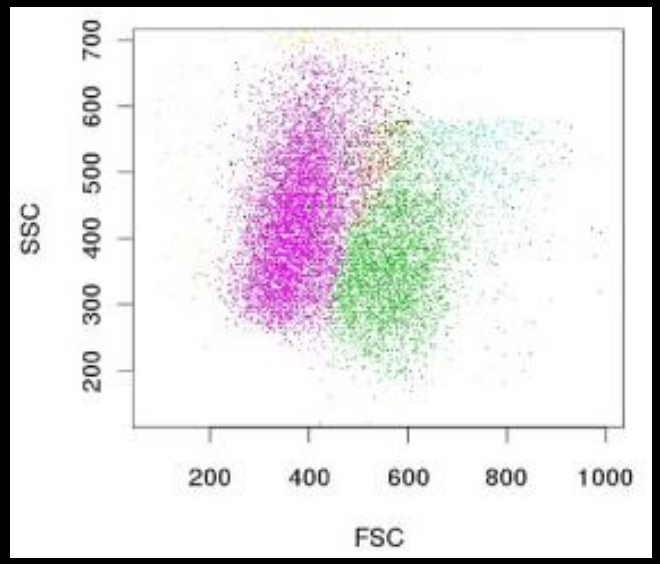


Pyne et al, 2009 *PNA*

Automated Clustering and Population Identification Methods Based on Density

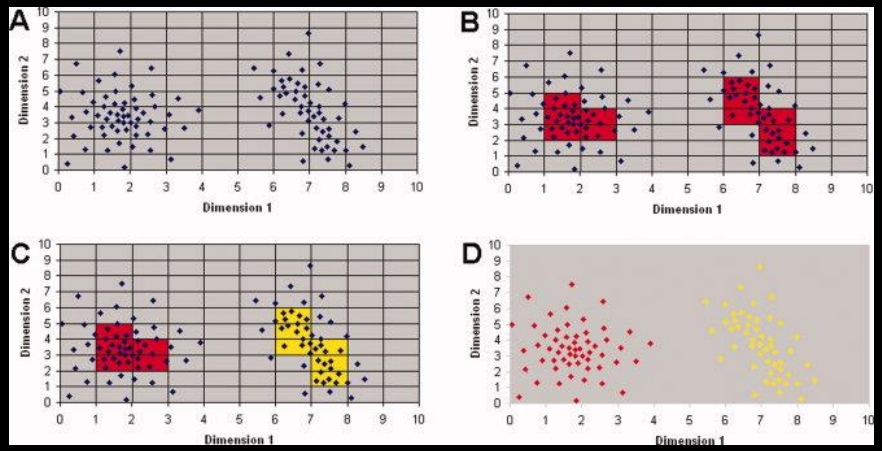
SamSpectral

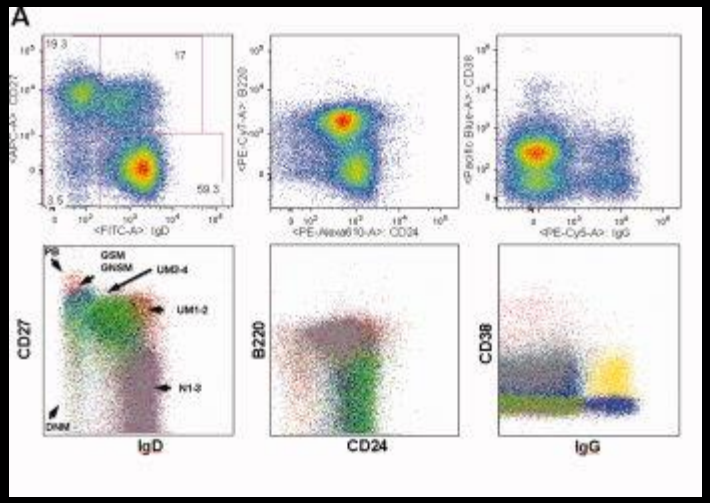




Zare et al, 2010 *BMC Bioinformatics*

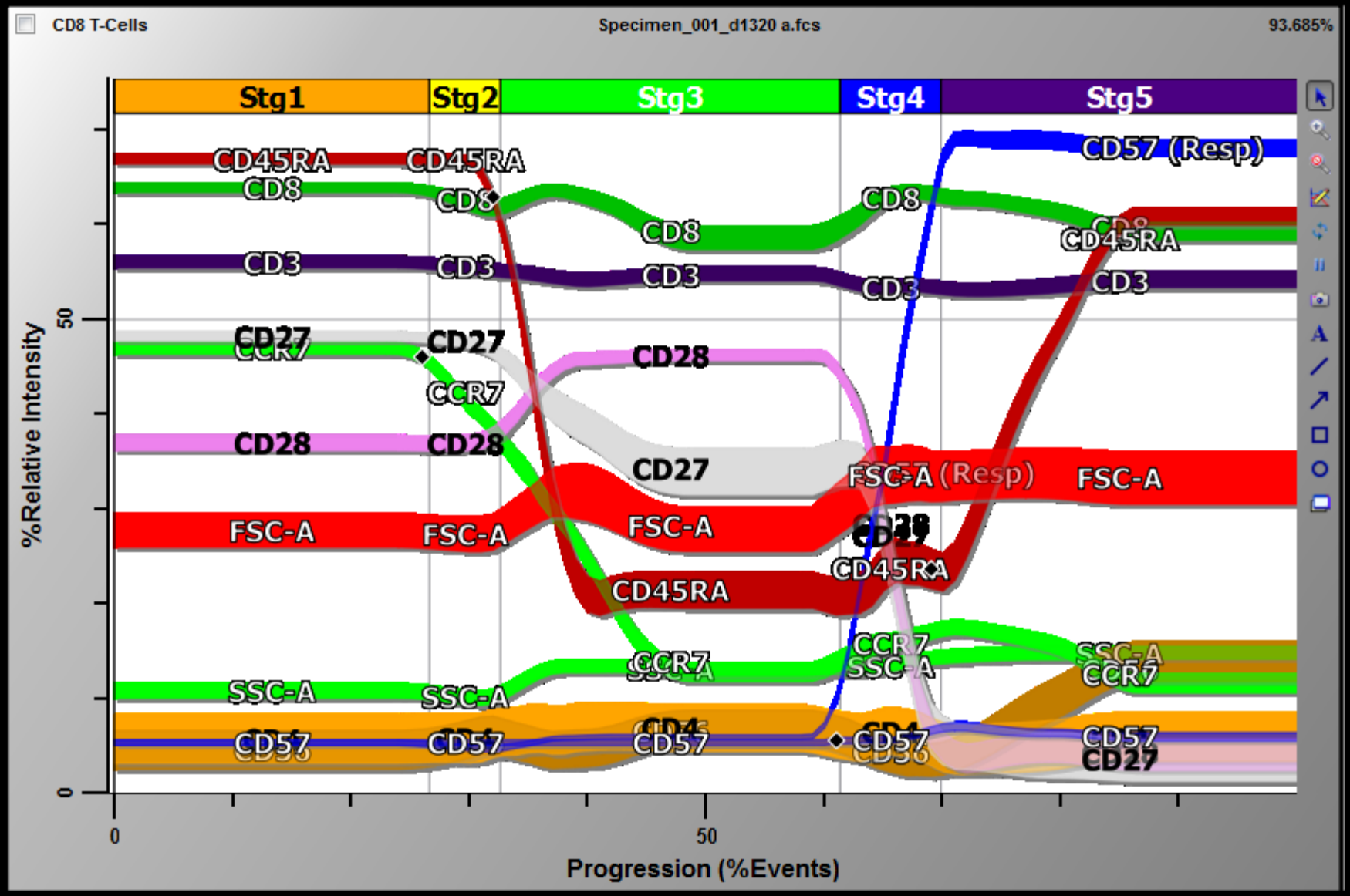
FLOCK





Qian et al, 2010 *Cytometry B Clin Cytom*

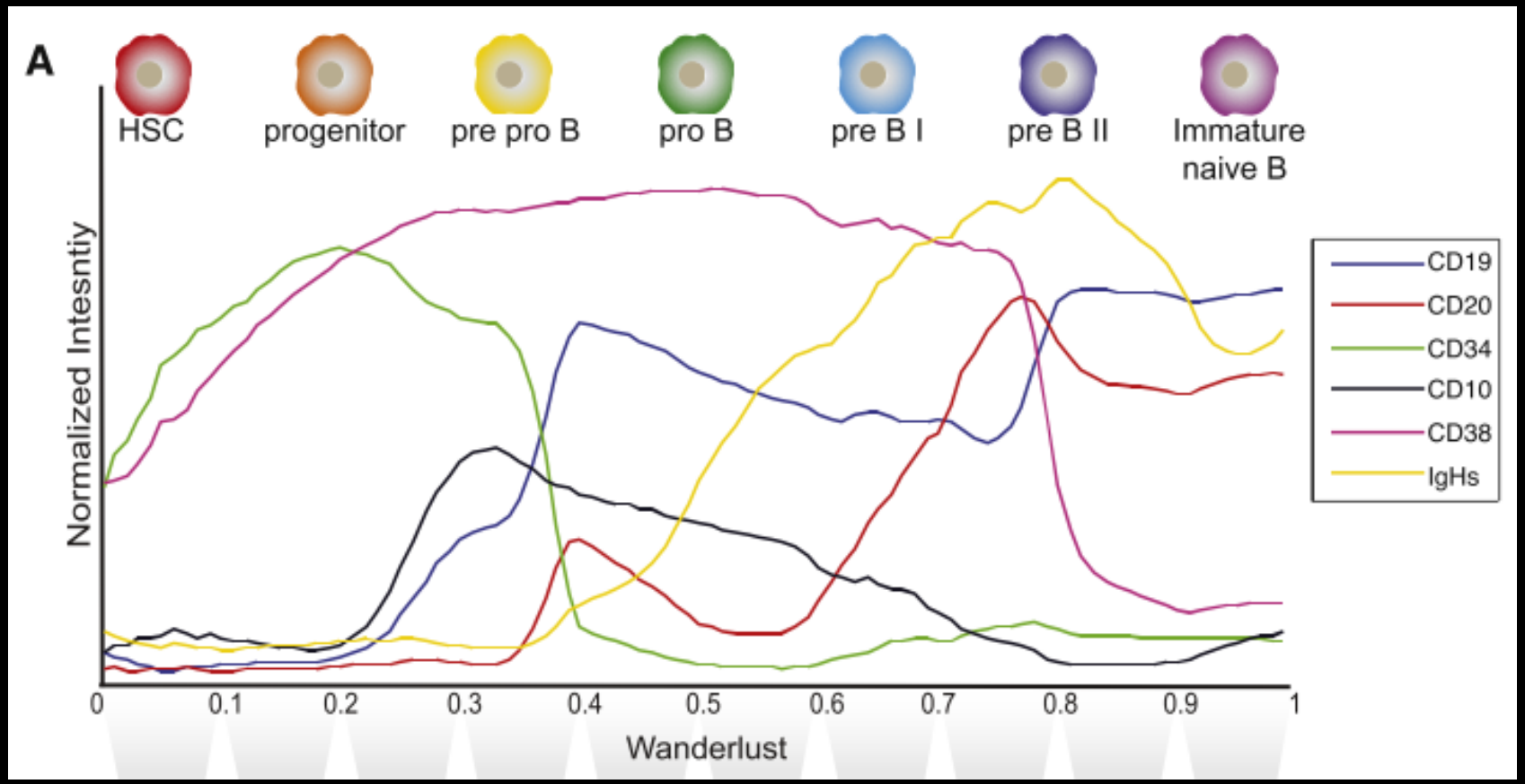
Gemstone Uses Supervised Analysis to Identify Progressions



Verity Software House (www.vsh.com)

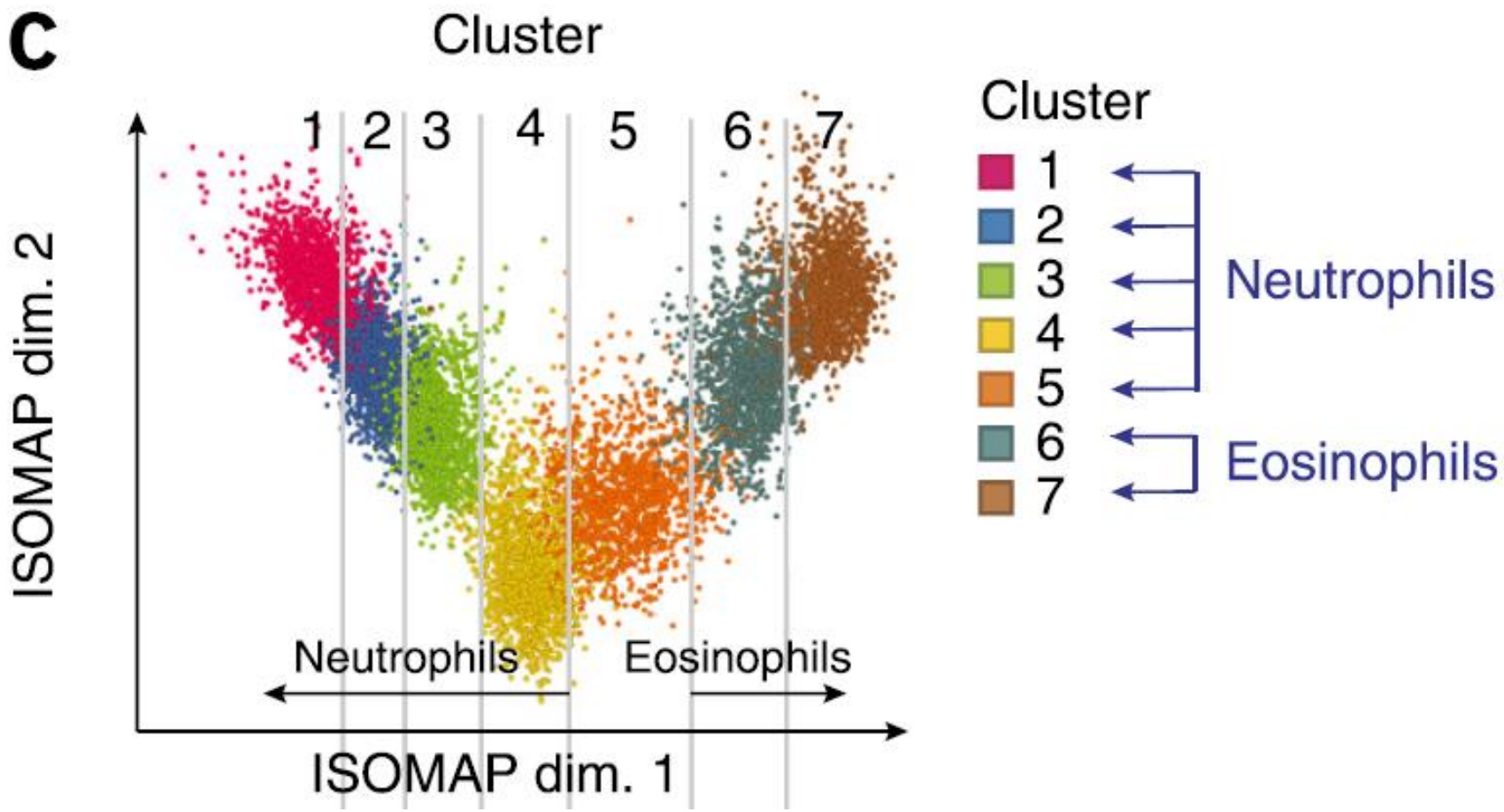
Inokuma, *JIM* 2013

Wanderlust Identifies Phenotypic Progression



Bendall, *Cell* 2014

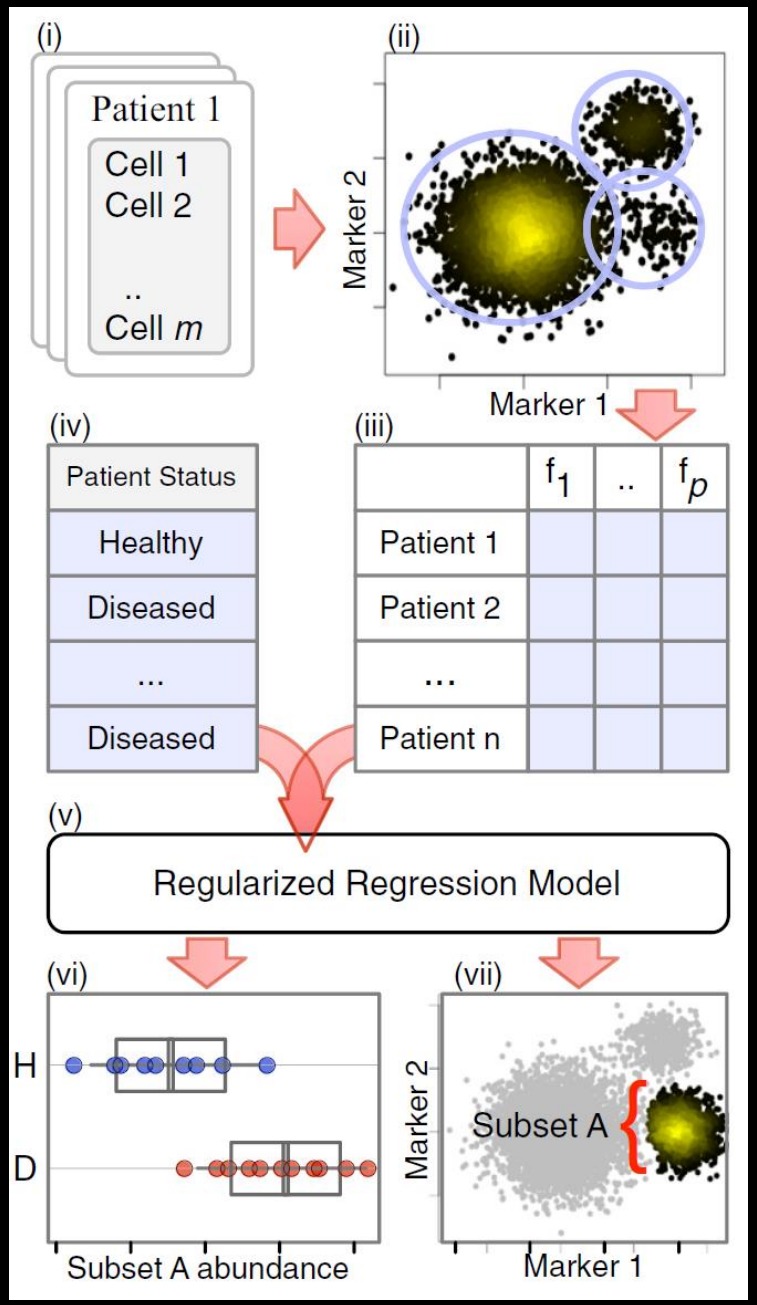
ISOMAP guided analysis



(**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

Citrus: Supervised Population Finding

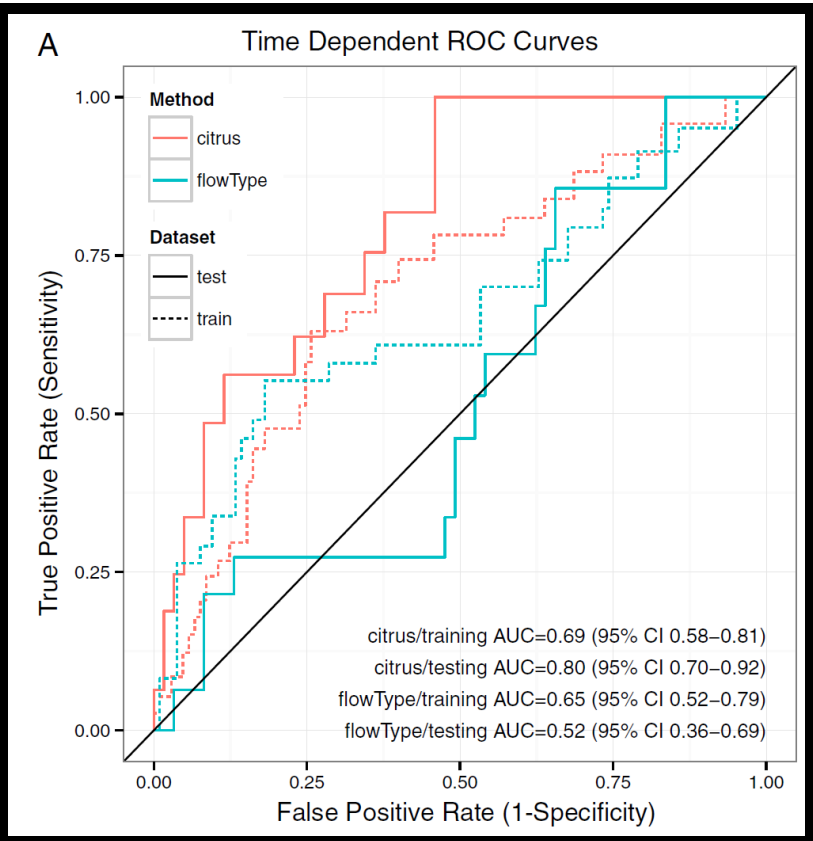


Automated identification of stratifying signatures in cellular subpopulations

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

Final notes & conclusions…

Computational Tools + HD Cytomics Together Are Powering A New Era in Clinical Oncology & Immunity

1) Need pre-treatment prognosis & prediction

If diagnostic scheme does not provide actionable information, fix it.
Who will benefit from expensive cell based therapies (~$500,000 ea.)?
In the absence of mutations, clinical response can be predicted by cell profiling.

2) Need to monitor treatment longitudinally

See early whether patient responded / adjust treatment, as needed.
Monitor whether treatment is still required.

3) Need to check multiple biomarkers with one test

As with genetic tests, multiplexing biomarkers will give more information per sample, catch the unexpected, and cost less than repeated testing

4) Need to monitor biomarkers on all cell types

PD-L1 is a great example – expressed by many cell types & can be activated.

5) Need to characterize all cell types to monitor cancer

Evolving cancer cells adopt unexpected phenotypes.

Conclusions: Data Analysis & Mapping Cell Identity

Workflow summary:

1) viSNE with minimal pre-gating
2) SPADE, works especially well on t-SNE axes
3) Heatmap to compare with other data, do statistical tests

1) A modular workflow allows comparison of different tools at each step. Synthetic channels are useful (e.g. t-SNEs).

2) Non-linear transformation may help; tool selection depends on ‘what works’, biology, & data shape.

3) Many outstanding tools are available (see Diggins et al. for reference list). Still need tools that learn.

Discussion Questions Covered in Today's Course

1) What are key differences between tools (viSNE, SPADE, PCA)? What is the difference between transforming, clustering, and modeling data? What type of modeling are we doing (if any)?

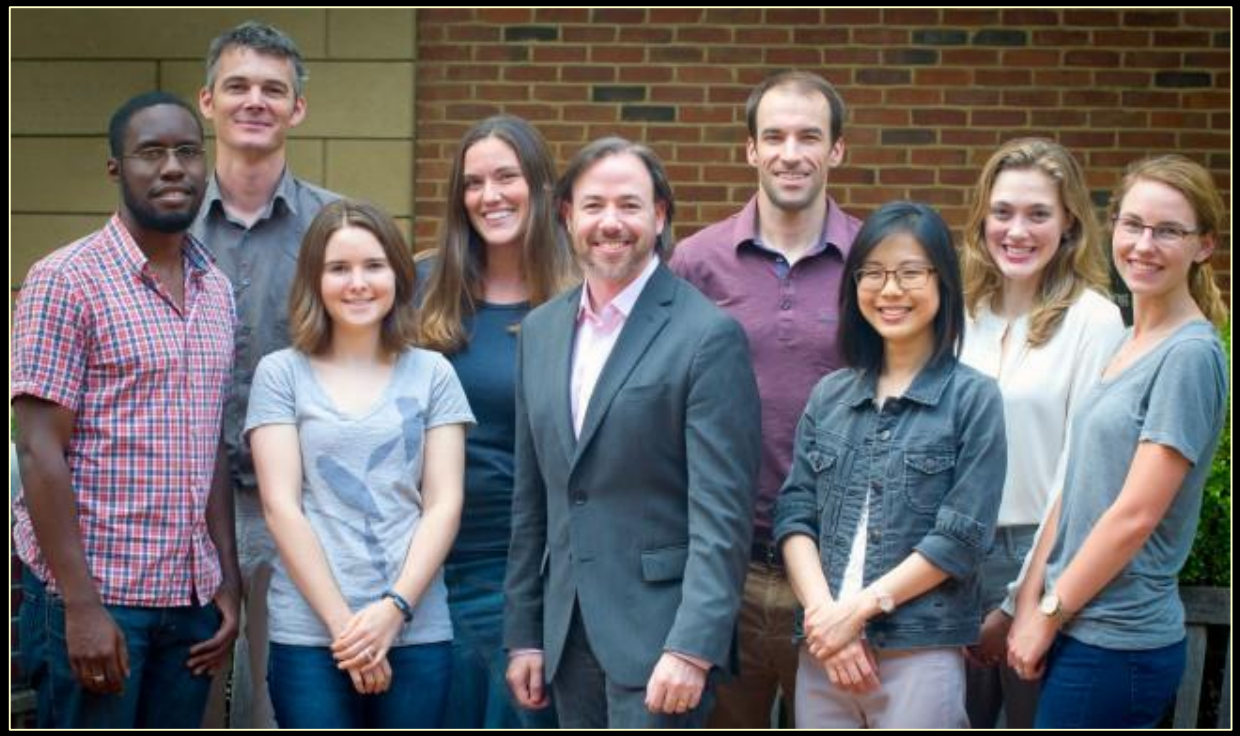
2) What does non-linear vs. linear analysis mean? Does the data's scale matter for analysis (arcsinh5, arcsinh15, linear)?

3) What do viSNE and SPADE settings do (viSNE iterations, SPADE downsampling & node #)? When should they be changed?

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

6) What are some "red flags" indicating problems? What does a good viSNE or SPADE analysis run look like?

Acknowledgements & Thank you!



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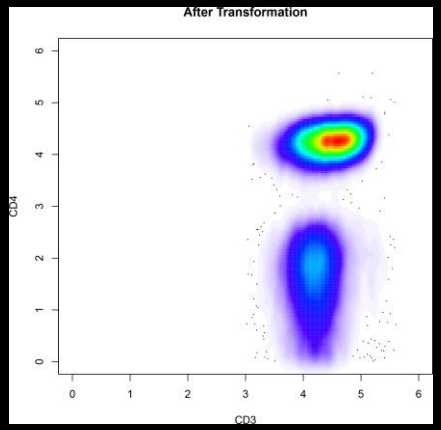
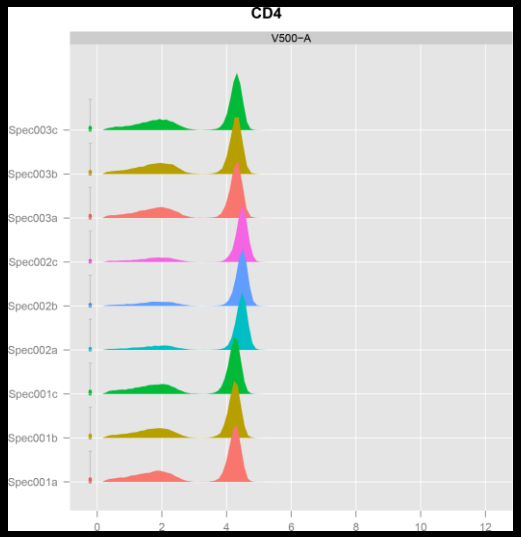
R/BIOCONDUCTOR

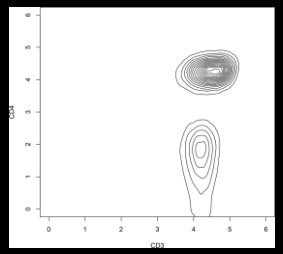
R Flow Cytometry Data Analysis

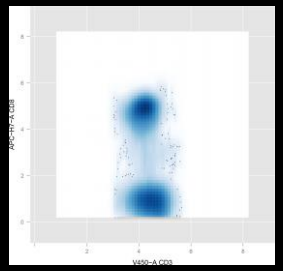
Arcsinh Transformation of Data



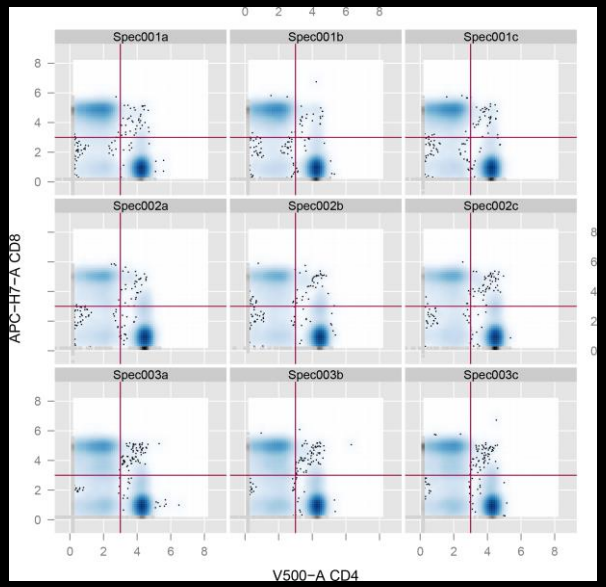
Visualization



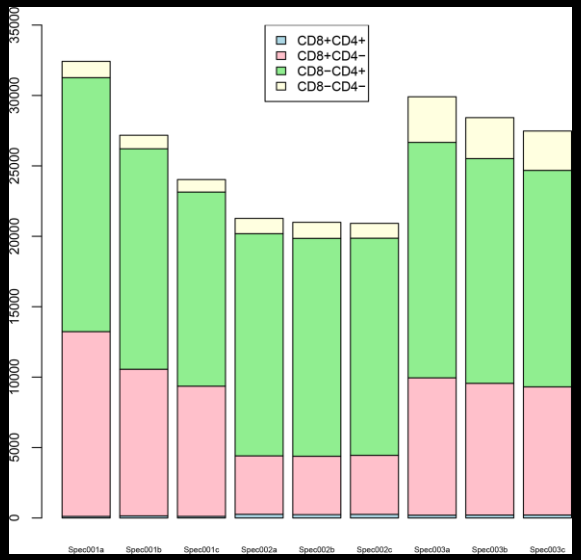




Gate T Cells on CD4 and CD8 Expression

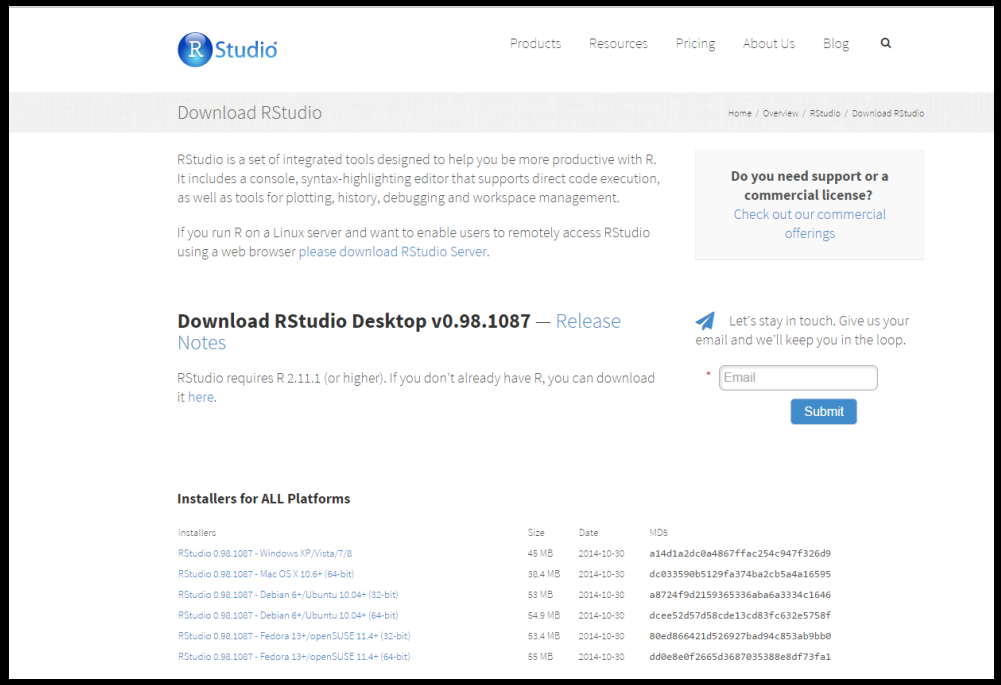


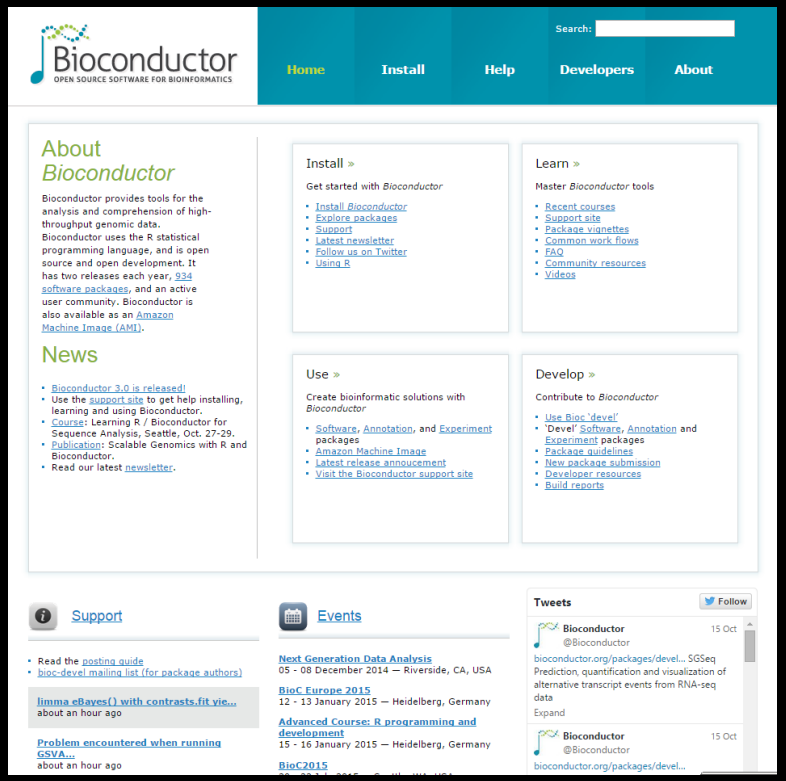
Compare T cell Subset Frequencies Between Individuals



What is R/Bioconductor?

- **R:** statistical and graphical programming language/environment
- **Bioconductor**: provides open source packages for bioinformatics analysis in R





Anatomy of an FCS File in R

FlowSet (set of FCS files)

FlowFrame1 (fcs file)	FlowFrame2 (fcs file)	FlowFrame3 (fcs file)	…

FlowFrame (fcs file)

Exprs

- Fluorescence intensity matrix

Cell	Parameter1	Parameter2
1	MFI_1-1	MFI_1-2
2	MFI_2-1	MFI_2-2

> exprs(FlowFrame)

Parameters

- Annotated data frame
- Info about parameters (stains)

rowNames	$P1,$P2,$P3…
varLabels	Name, desc…max range
varMetadata	labelDescription

> parameters(FlowFrame)

Description

-List of information from instrument

$FCSversion [1] “3”
$`$BEGINANALYSIS [1] “0”
$`$ENDANALYSIS [1] “0”
…

> description(FlowFrame)

Flow Analysis in R

- flowCore: access data in fcs files
- flowQ: quality control for flow data
- flowStats: statistical analysis of flow data
- flowViz: visualization of flow data
- flowClust: mixture-modeling to find clusters
- flowMerge: merge clusters from mixture modeling
- flowUtils: parse Gating-ML files
- flowFP: transforms high dimensional data using modeling to facilitate data analysis

flowCore

- R package flowCore
  - Read fcs files into R and perform basic analysis functions
  - View and access intensity matrix

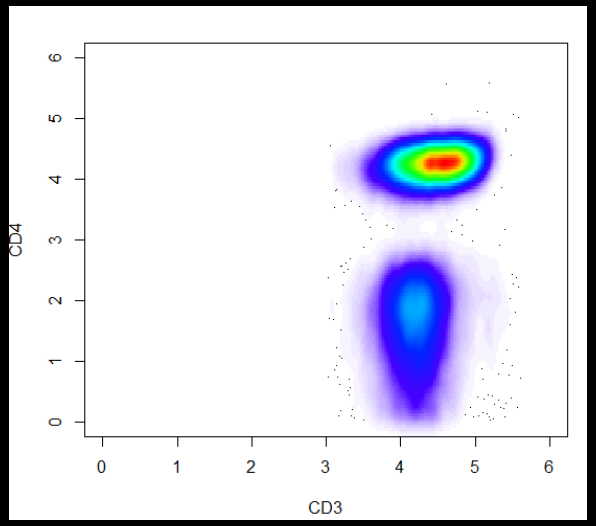
```
> head(exprs(TcellData$Spec001a))
         FSC-A FSC-H    FSC-W    SSC-A SSC-H     SSC-W FITC-A PerCP-Cy5-5-A  APC-A Alexa Fluor 700-A APC-H7-A  V450-A
[1,]  74313.46 64310 75730.17 26153.92 28082  61036.37 345.28       1647.36 1310.4              688.5    100.8 3174.22
[2,]  54602.26 45362 78885.72 25446.72 16436 101464.84 236.08       2462.72 2231.1             1161.9    846.9 8924.06
[3,]  67478.18 49500 89338.38 15140.32 17640  56249.21 143.52       2442.96 2241.0             1305.0    687.6 7780.16
[4,]  83260.04 64328 84823.56 31904.08 21015  99493.97 295.36       2546.96 2165.4             1104.3    538.2 8496.02
[5,]  54679.66 43581 82225.88 14768.00 15760  61410.89 260.00       1998.88 2108.7              945.9    346.5 9673.54
[6,] 107701.24 83691 84337.73 30740.32 23988  83983.55 101.92       5040.88 3944.7             2043.0   1160.1 5296.38
       V500-A    PE-A PE-Cy7-A Time
[1,] 6639.540   66.33   102.96  0.4
[2,] 7260.280 1548.36 10053.45  0.7
[3,] 7010.180 1472.13  9365.40  2.1
[4,] 7343.100 1545.39  5906.34  2.1
[5,] 9341.439 1074.15  3357.09  3.0
[6,] 1669.520 1062.27 16261.74  3.2
>
```

```
> print(TcellData$Spec001a)
flowFrame object 'Spec001a'
with 32423 cells and 16 observables:
                  name   desc  range minRange maxRange
$P1               FSC-A  FSC-A 262144        0   262144
$P2               FSC-H  FSC-H 262144        0   262144
$P3               FSC-W  FSC-W 262144        0   262144
$P4               SSC-A  SSC-A 262144        0   262144
$P5               SSC-H  SSC-H 262144        0   262144
$P6               SSC-W  SSC-W 262144        0   262144
$P7              FITC-A   CD57 262144     -111   262144
$P8       PerCP-Cy5-5-A   CD28 262144     -111   262144
$P9               APC-A   CD27 262144     -111   262144
$P10 Alexa Fluor 700-A   CD56 262144     -111   262144
$P11           APC-H7-A    CD8 262144     -111   262144
$P12             V450-A    CD3 262144        0   262144
$P13             V500-A    CD4 262144     -111   262144
$P14               PE-A   CCR7 262144     -111   262144
$P15           PE-Cy7-A CD45RA 262144     -111   262144
$P16               Time   Time 262144        0   262144
250 keywords are stored in the 'description' slot
```

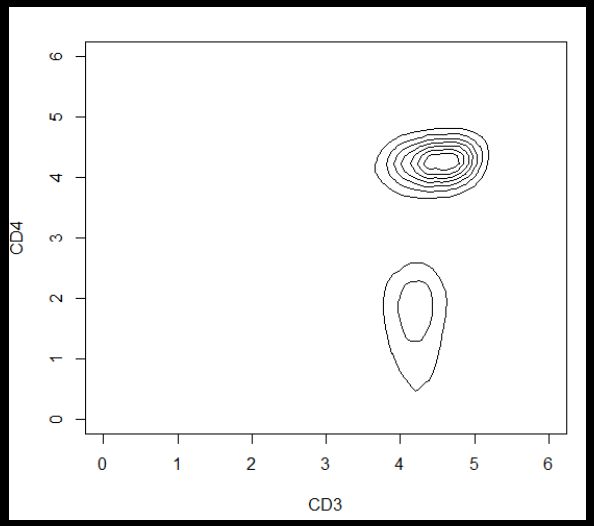
flowViz

- Contour plot, density plot, scatter plot, trellis plot, and histograms for flow data

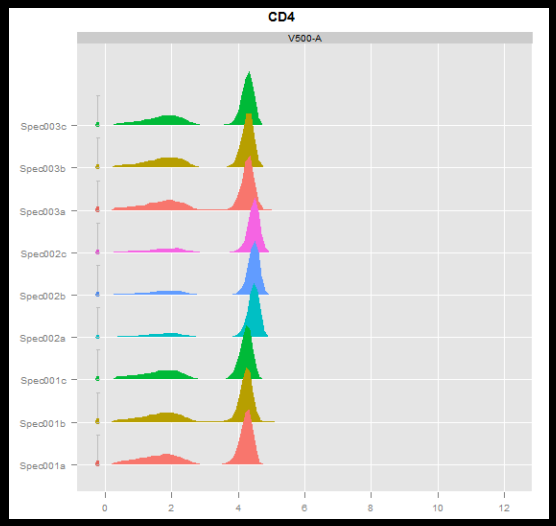
Density Dot (flowPlot(…))



Contour Plot (contour(…))



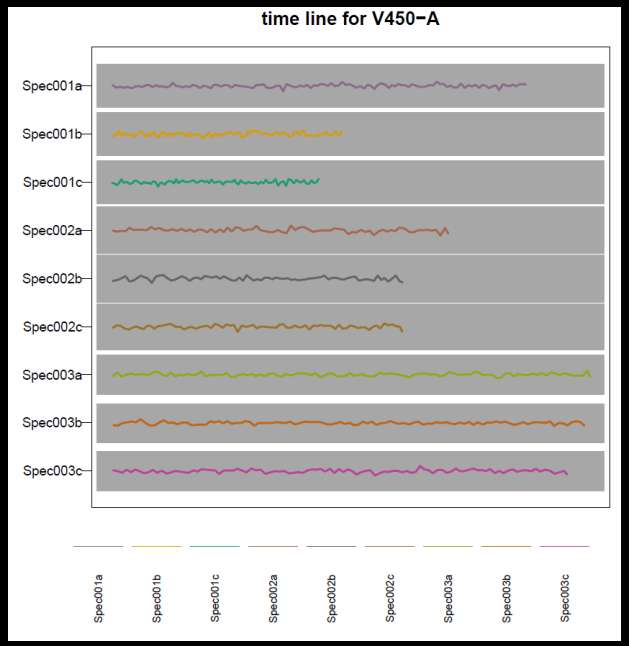
Histograms (densityplot(…))



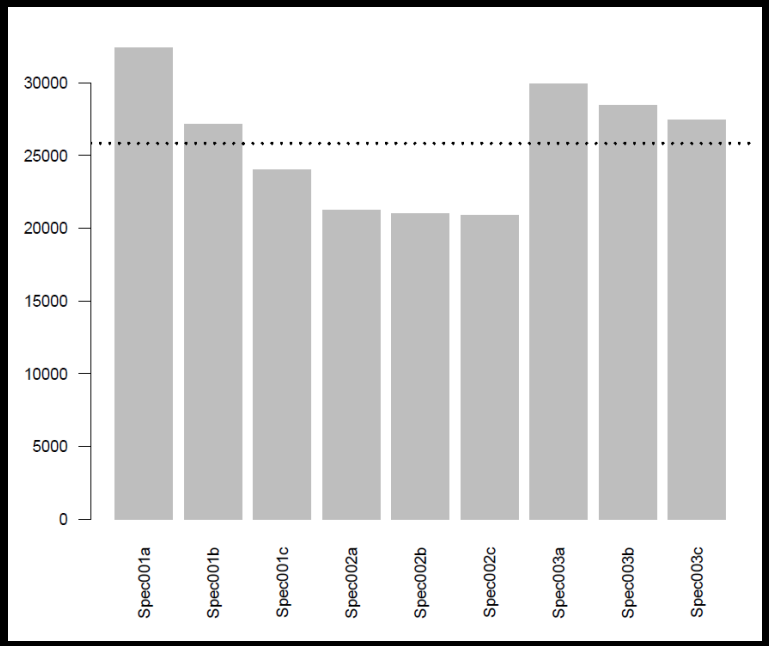
flowQ

- Compare number of cells between files
- Check for margin events
- Check for time anomalies
- Data normalization
- KL Divergence

Timeline



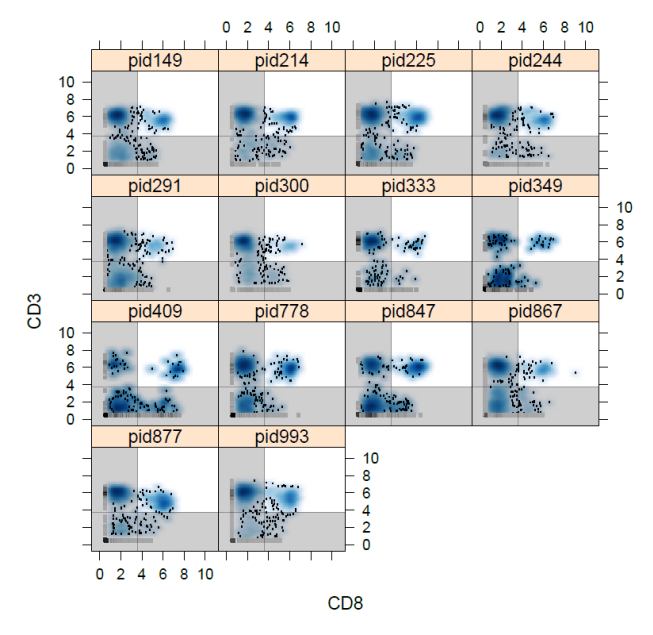
Cell Number



flowStats

- Functions for statistical analysis of flow data
  - Probability binning + Chi-squared test
  - Create filters for high density regions (auto-gating)
  - Quadrant gating
  - Methods for data normalization

Quadrant Gating



Normalization