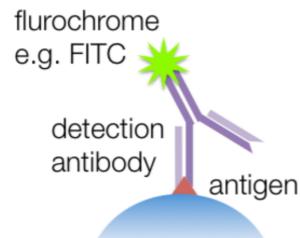


# Flow Cytometry Bioinformatics

Julián Candia

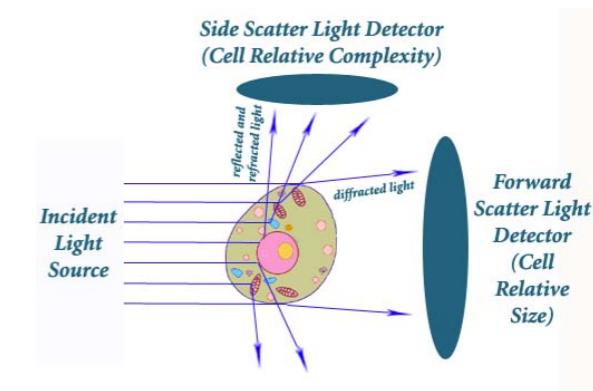
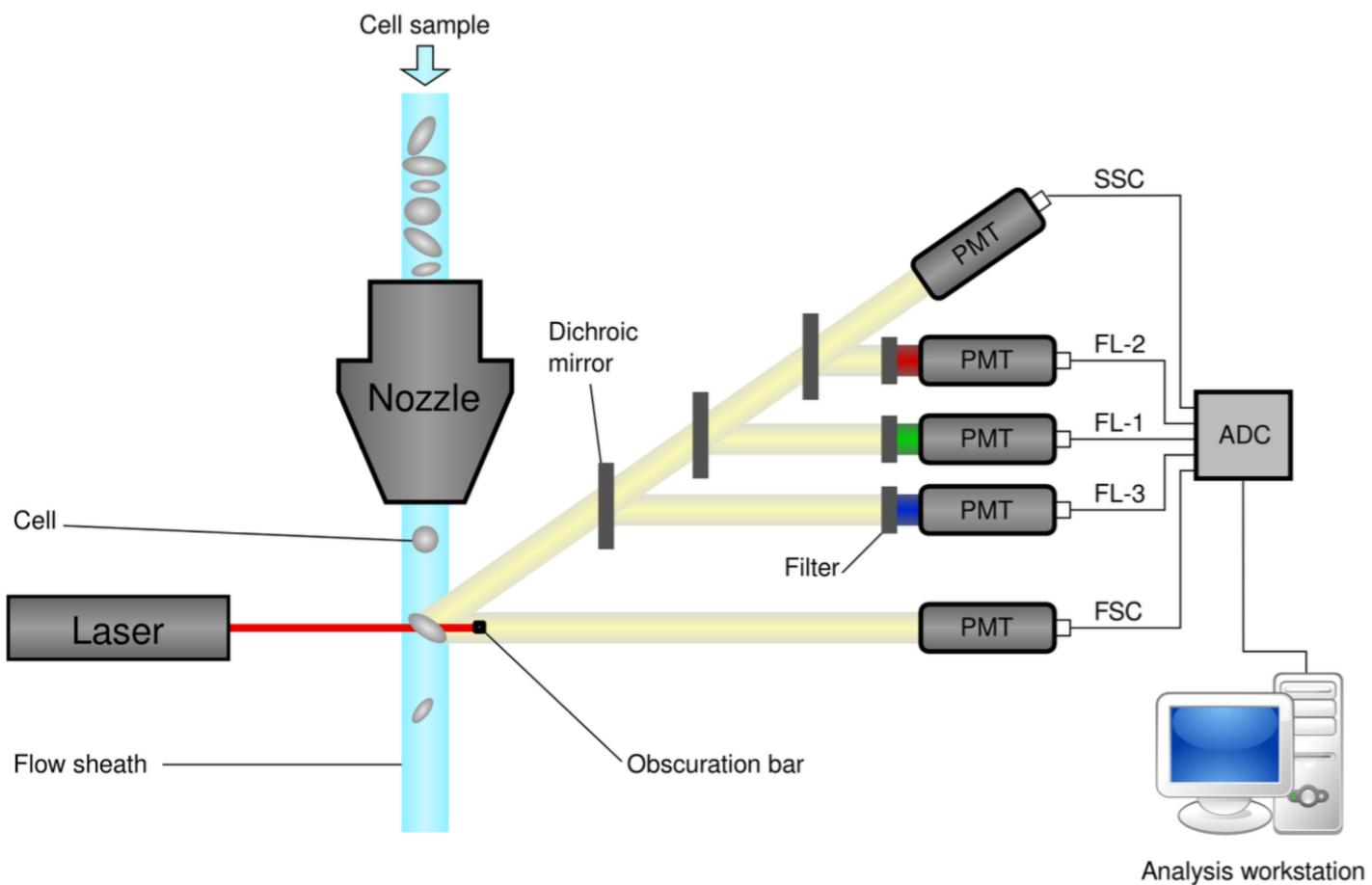


# Flow cytometry in a Nutshell

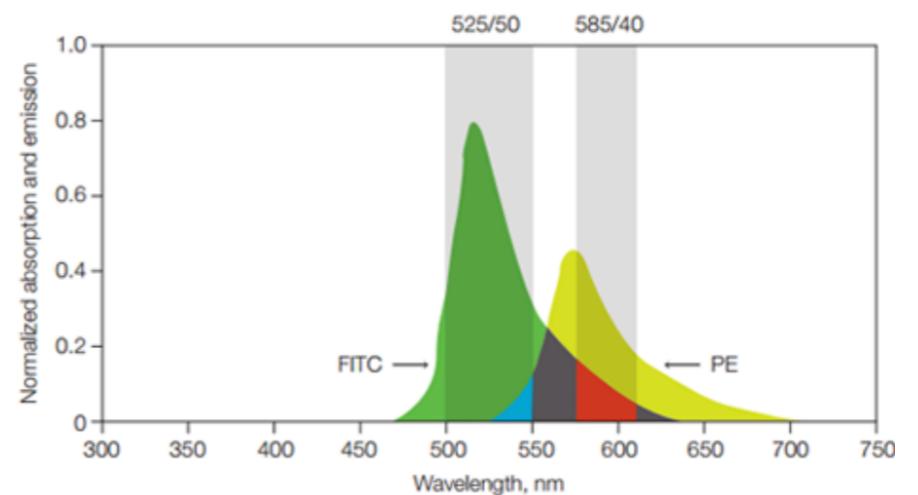


Fluorochrome-conjugated antibodies  
are used to target surface or  
intracellular proteins

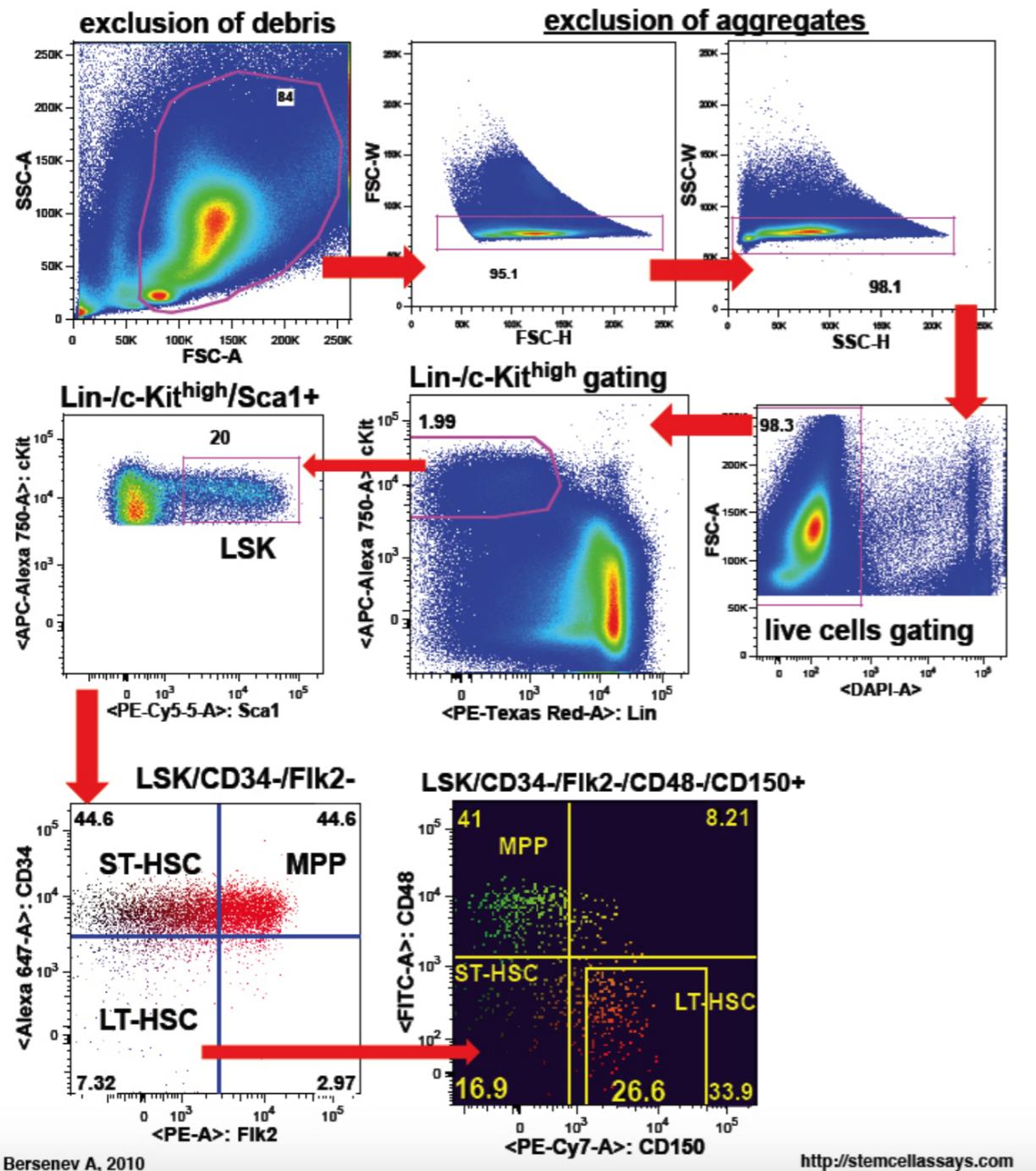
Forward- and side-scattered light provide information  
on the cell's relative size, shape, and complexity



Optical filters are set near the fluorochrome's emission peak. Due to spillover, data needs to be compensated and the number of simultaneous measurements is limited to ~15



# Flow cytometry (manual) data analysis



A sequence of boundaries (gates) is applied to 1D (histograms) or 2D (density scatterplots) marker selections to zoom into cell phenotypes of interest (of known marker signature).

The final outcome is usually reported as a fraction of the parent cell population.

FlowJo is a popular licensed software to perform manual data analysis.

# Challenges of manual data analysis

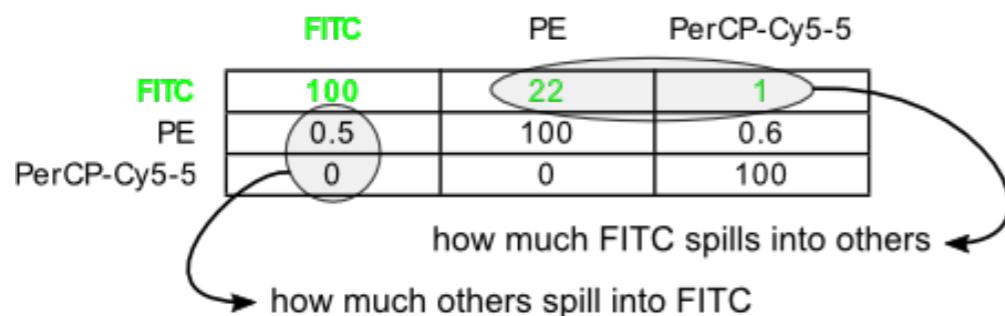
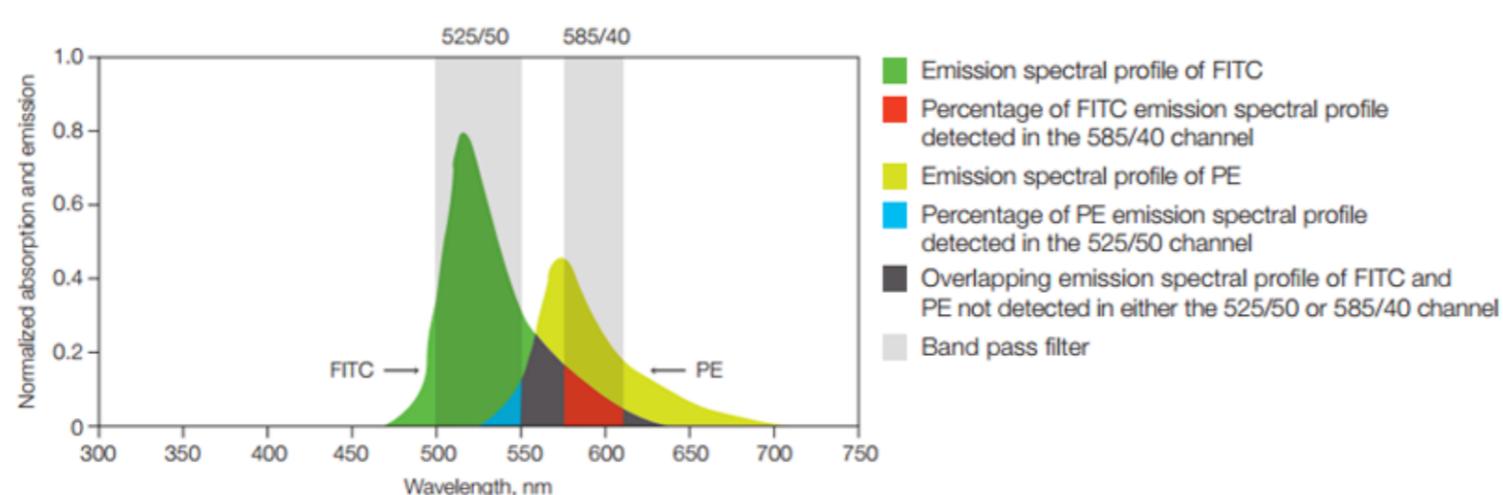
- Poor reproducibility:  
High variability arising from manual compensation and manual gating procedures
- Biased:  
Cell phenotypes restricted to known marker combinations
- Impractical when many markers are used:  
Number of marker combinations grows exponentially with the number of colors used (~ thousands for +/- combinations based on 10 colors)
- Not scalable:  
Analysis time grows linearly with the number of samples.

“Flow Bioinformatics”:

An emergent interdisciplinary field to create tools based on a variety of approaches for the unbiased, automated analysis of flow cytometry data.

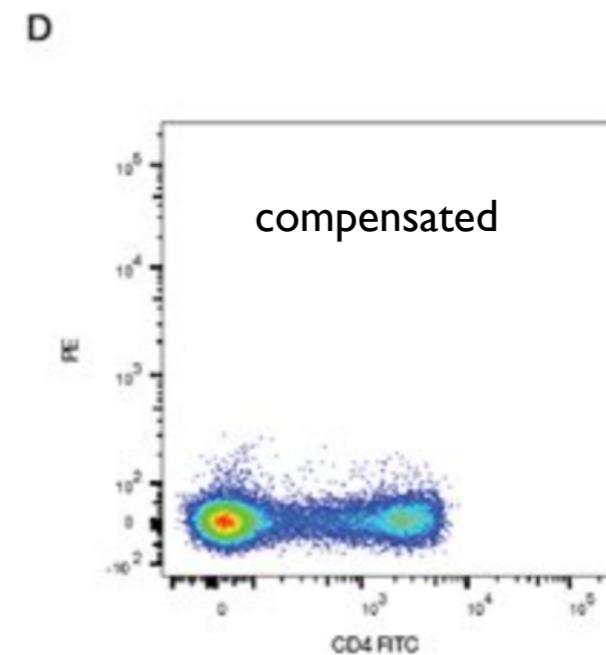
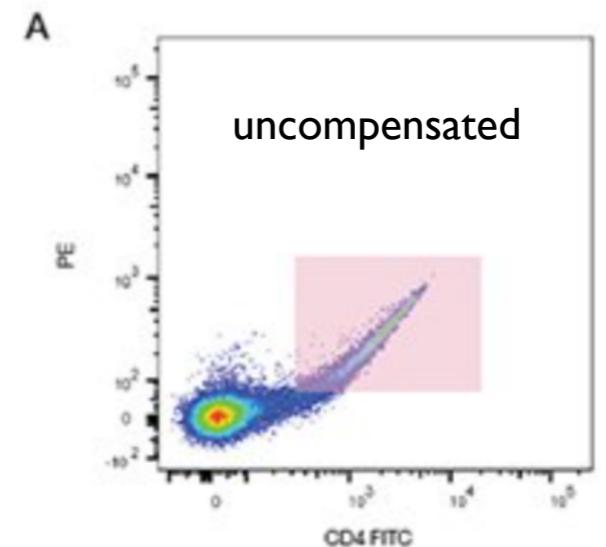
# Challenges in automated flow cytometry analysis:

## I. Data compensation



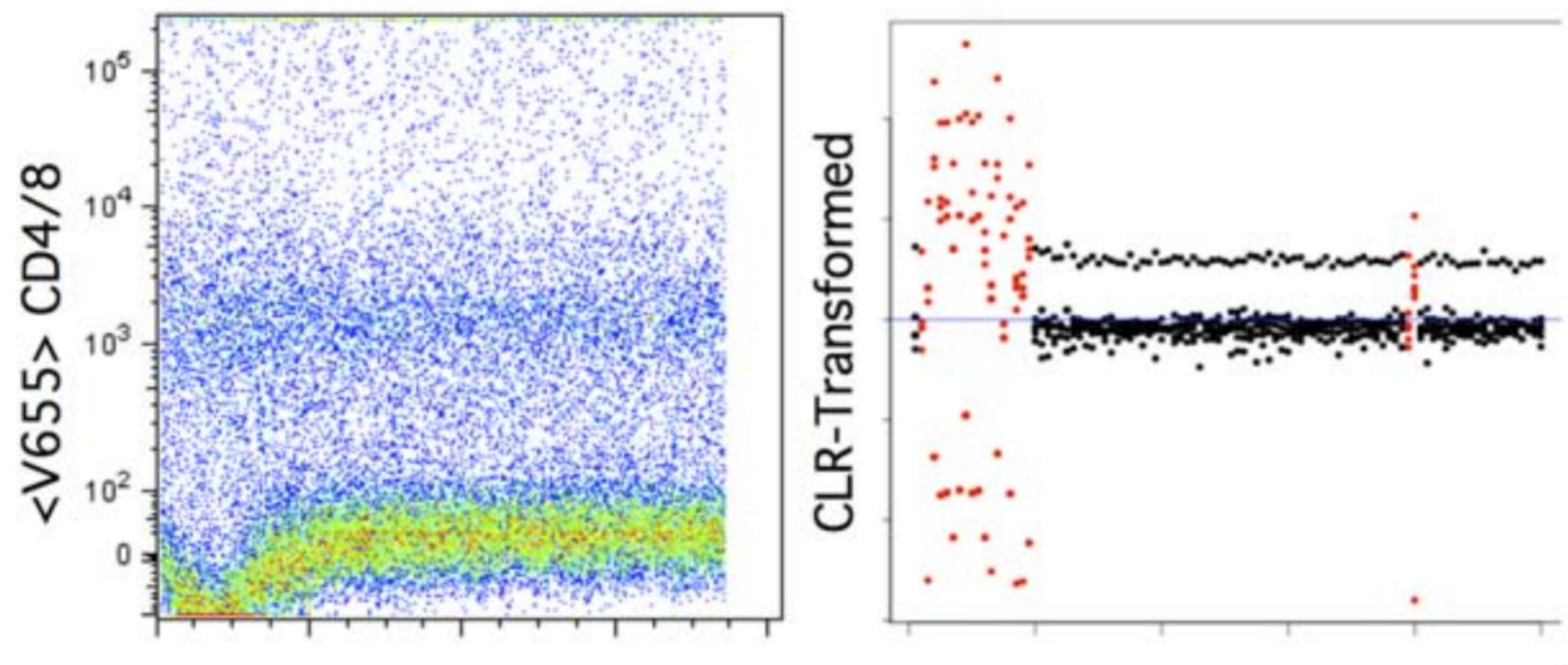
The spillover/compensation matrix subtracts the contribution of one signal to the other channels; the matrix needs to be adjusted to batch effects and other confounders.

With multicolor flow, the problem becomes complex.  
Open software available: `flowCore`, `flowUtils`.



# Challenges in automated flow cytometry analysis:

## 2. Fluid instabilities and other fluorescence anomalies



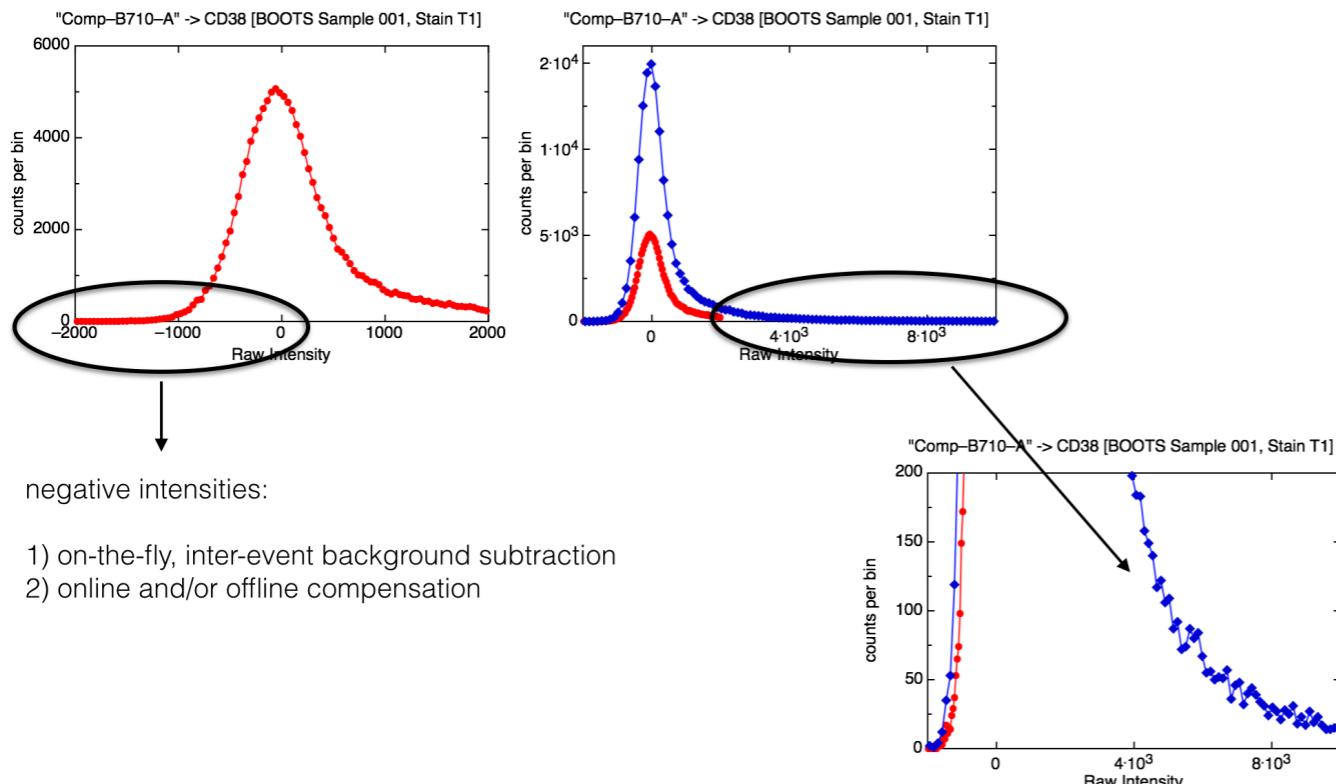
By tracking subset frequency changes within a sample during acquisition, time periods with fluorescence perturbations leading to the emergence of false populations are flagged.

Fletez-Brant et al, Cytometry Part A 89A:461-471 (2016)  
Open software available: flowClean

# Challenges in automated flow cytometry analysis:

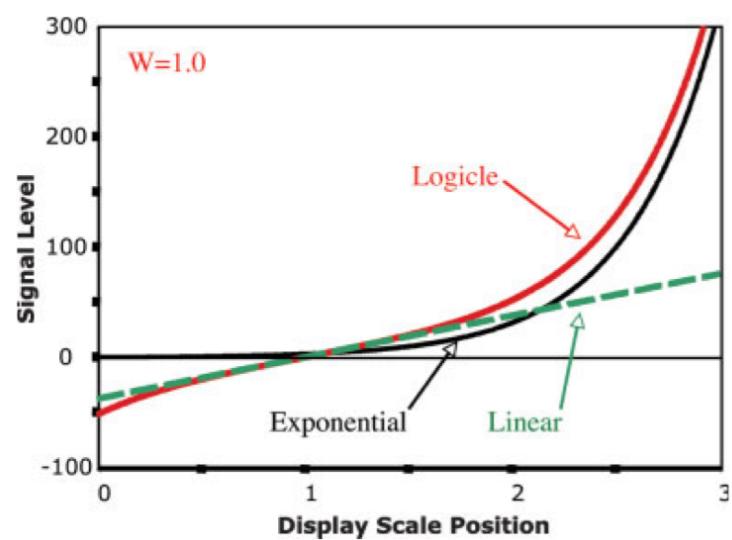
## 3. Data transformation

Fluorescence intensities from flow cytometry experiments have distributions with log-normal high-intensity events but also a sizable region of negative intensity values due to background subtraction and online/offline compensation



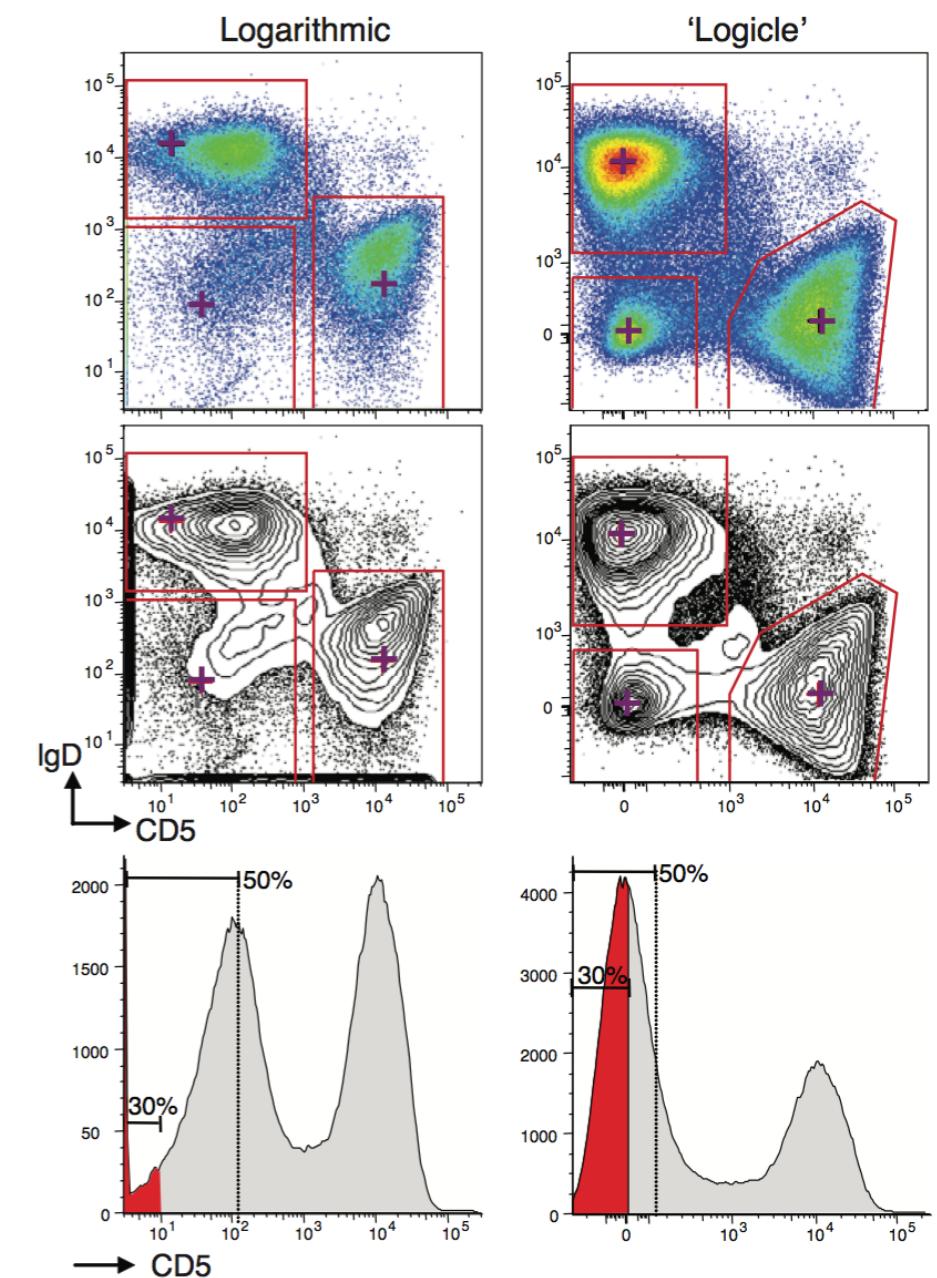
negative intensities:

- 1) on-the-fly, inter-event background subtraction
- 2) online and/or offline compensation



“Logicle”-transformation: linear around the negative/small-intensity region, logarithmic for high intensities. Depends on several transformation parameters.

Parks, Roederer & Moore, Cytometry Part A (2006).



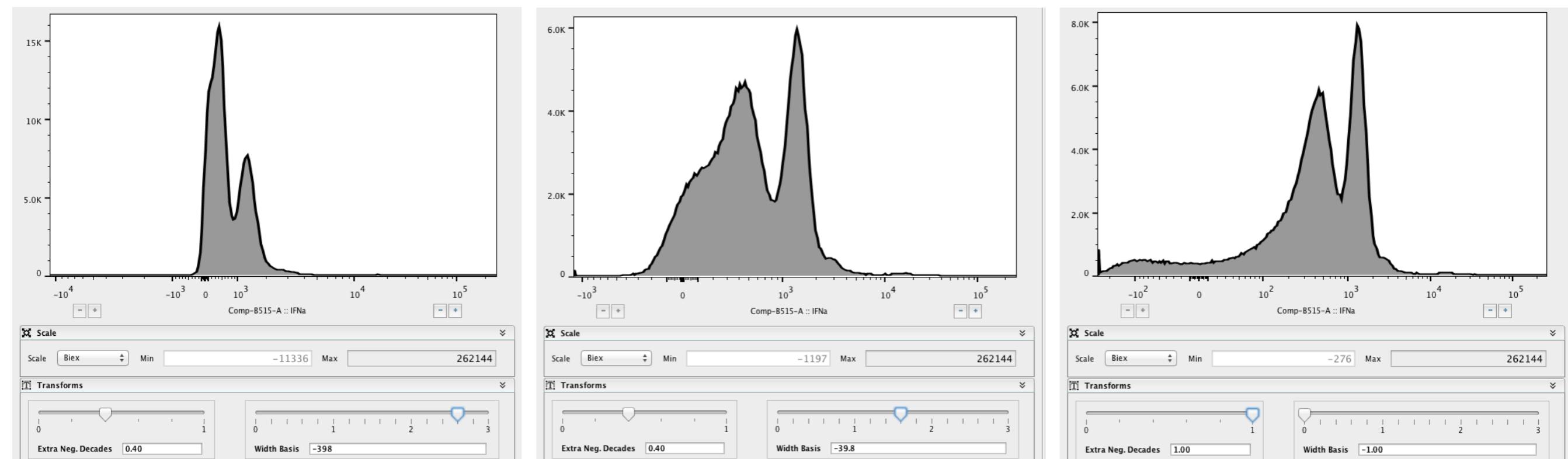
Herzenberg et al, Nat Immunology (2006)

# Challenges in automated flow cytometry analysis:

## 3. Data transformation (cont'd)

Logicle transformations depend on the choice of several transformation parameters, which usually occurs in FlowJo behind the scenes.

Changing those transformation parameters can dramatically affect the results.



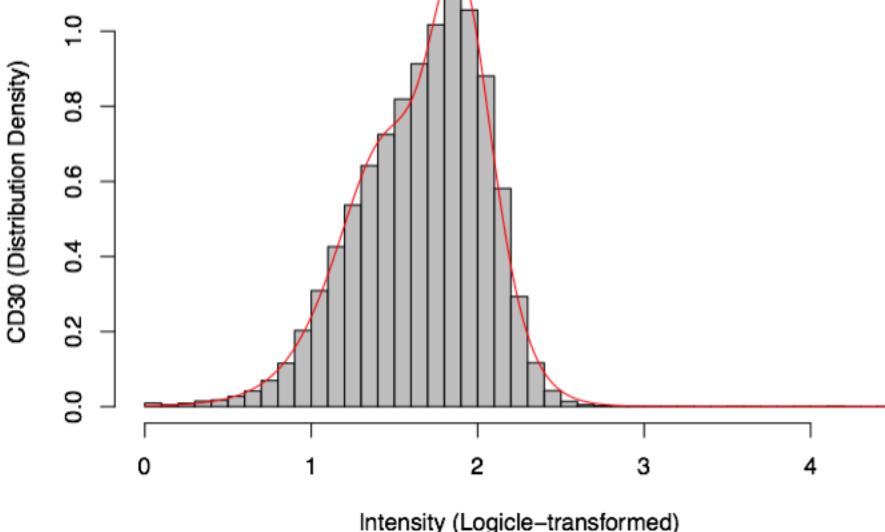
# Challenges in automated flow cytometry analysis:

## 4. Resolution of overlapping cell subpopulations

In these CD30 distributions (from the same patient at different time points), two peaks appear poorly resolved.

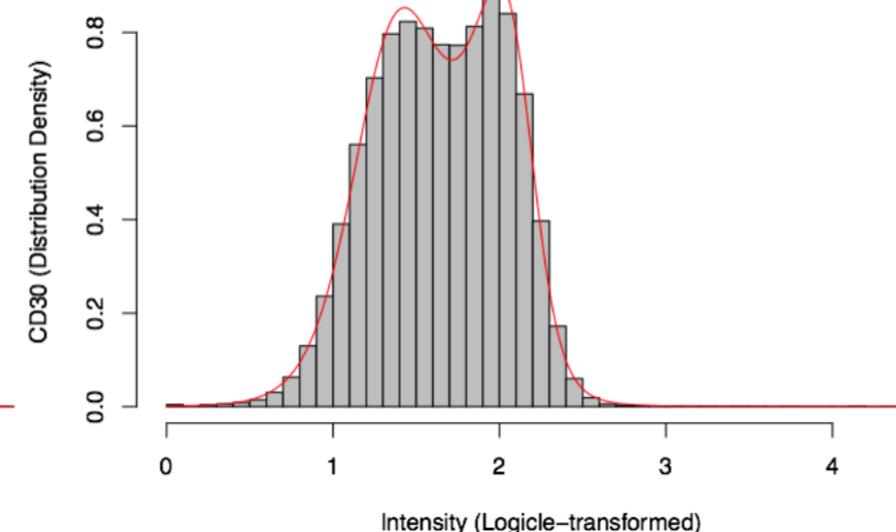
sample#4: Uveitis-015 w0

**Histogram of Skew.t fit**



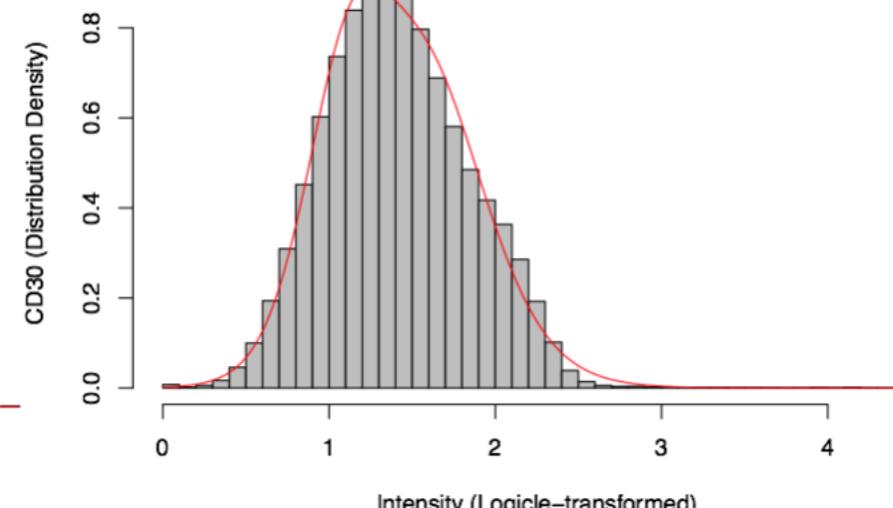
sample#5: Uveitis-015 w24

**Histogram of Skew.t fit**



sample#6: Uveitis-015 w52

**Histogram of Skew.t fit**



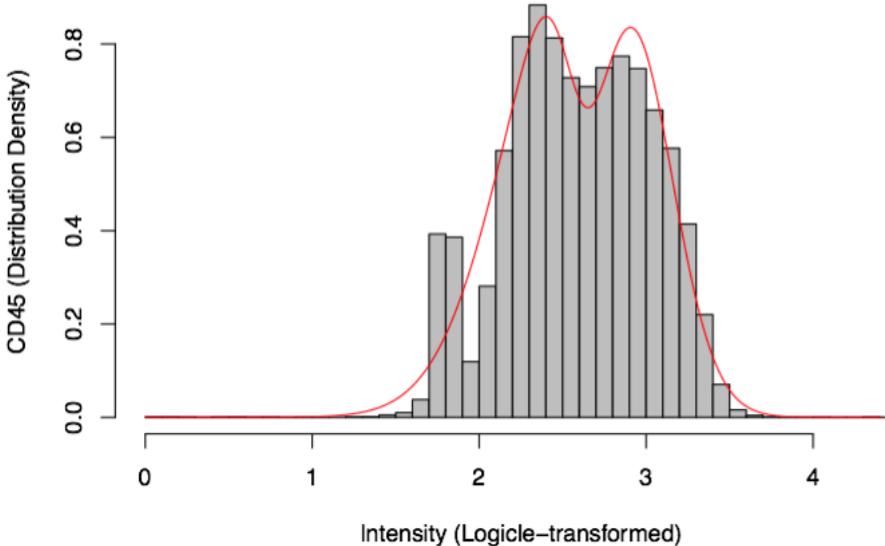
# Challenges in automated flow cytometry analysis:

## 5. Consistency of features across the cohort

The shape of CD45 distributions appears inconsistent across samples

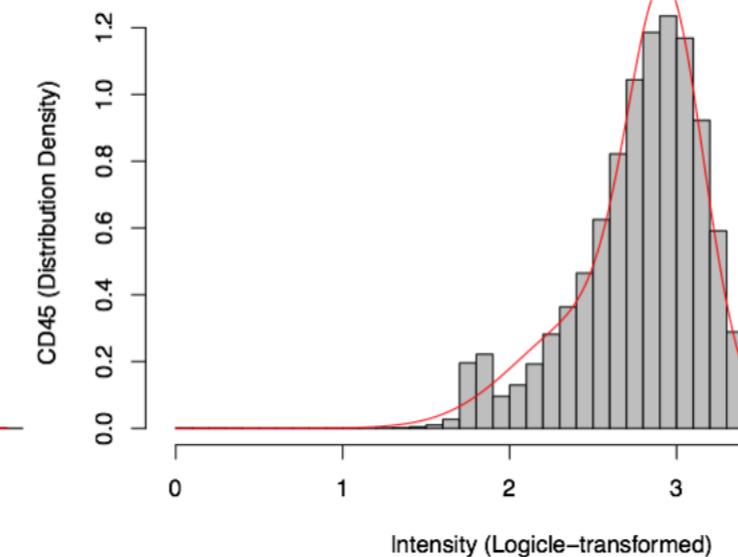
sample#37: Uveitis-020 w0

**Histogram of Skew.t fit**



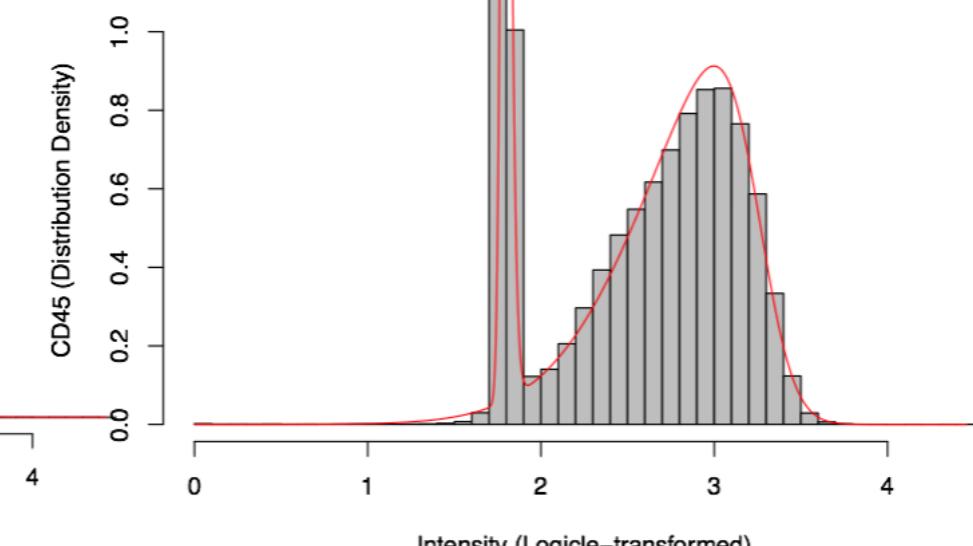
sample#40: Uveitis-023 w0

**Histogram of Skew.t fit**



sample#43: Uveitis-002 w0

**Histogram of Skew.t fit**

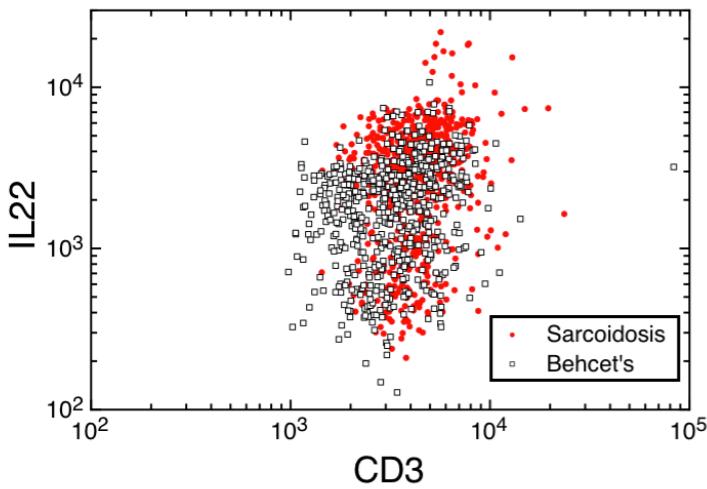


# Methods for automated gating... many proposed so far!

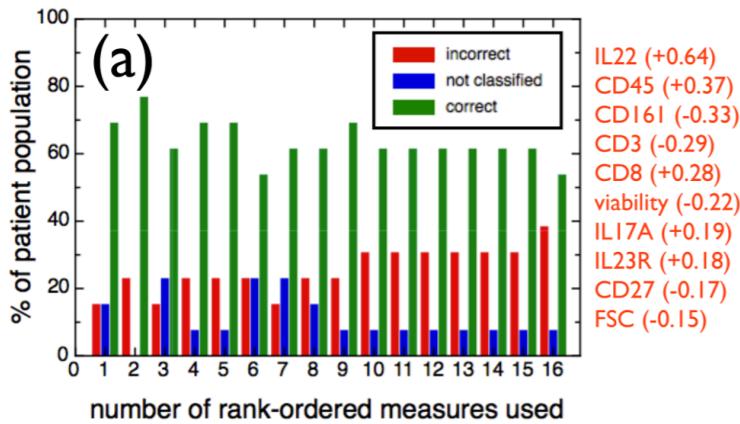
There is no single best method: choice depends on the structure of the data and the goals of the analysis.

## I. “Supercell” method

Due to cell heterogeneity and phenotype similarity, cells from Behçet’s and Sarcoidosis patients are highly overlapping in marker space.

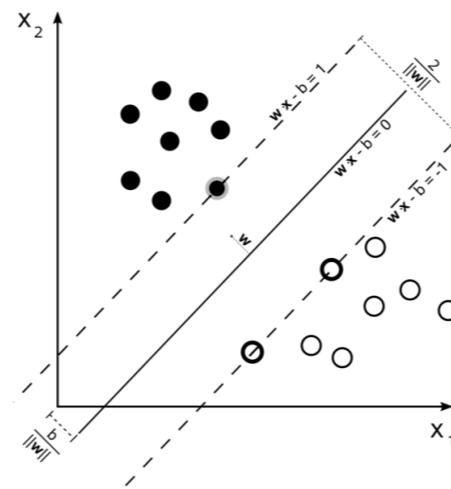


all cells

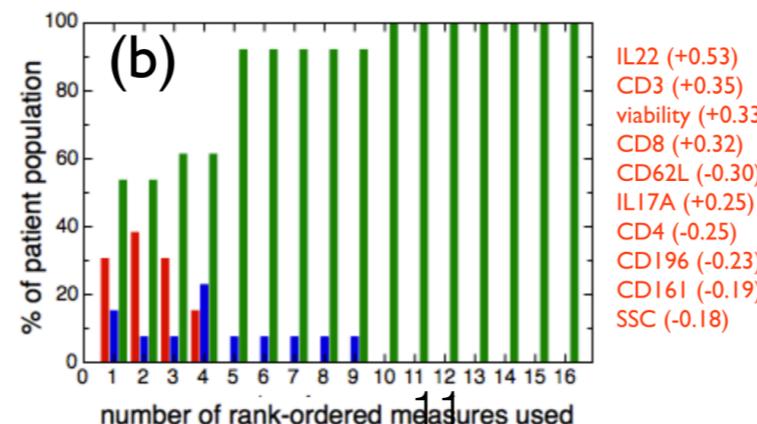


Candia et al, Plos Comp Biol (2013); J Phys: Condens Matt (2014)

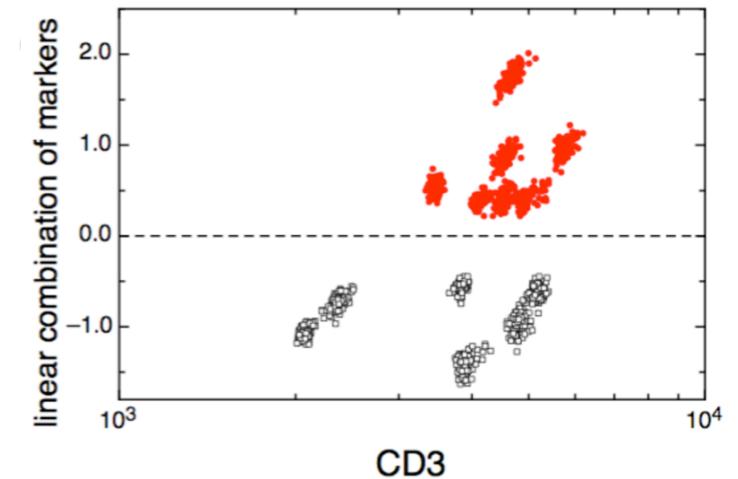
We propose “supercells” as a cell-averaging procedure to reduce variability in combination with a machine-learning approach such as SVMs



CD8+ T cells



We build a marker signature that optimally separates cell subsets from the two disease cohorts

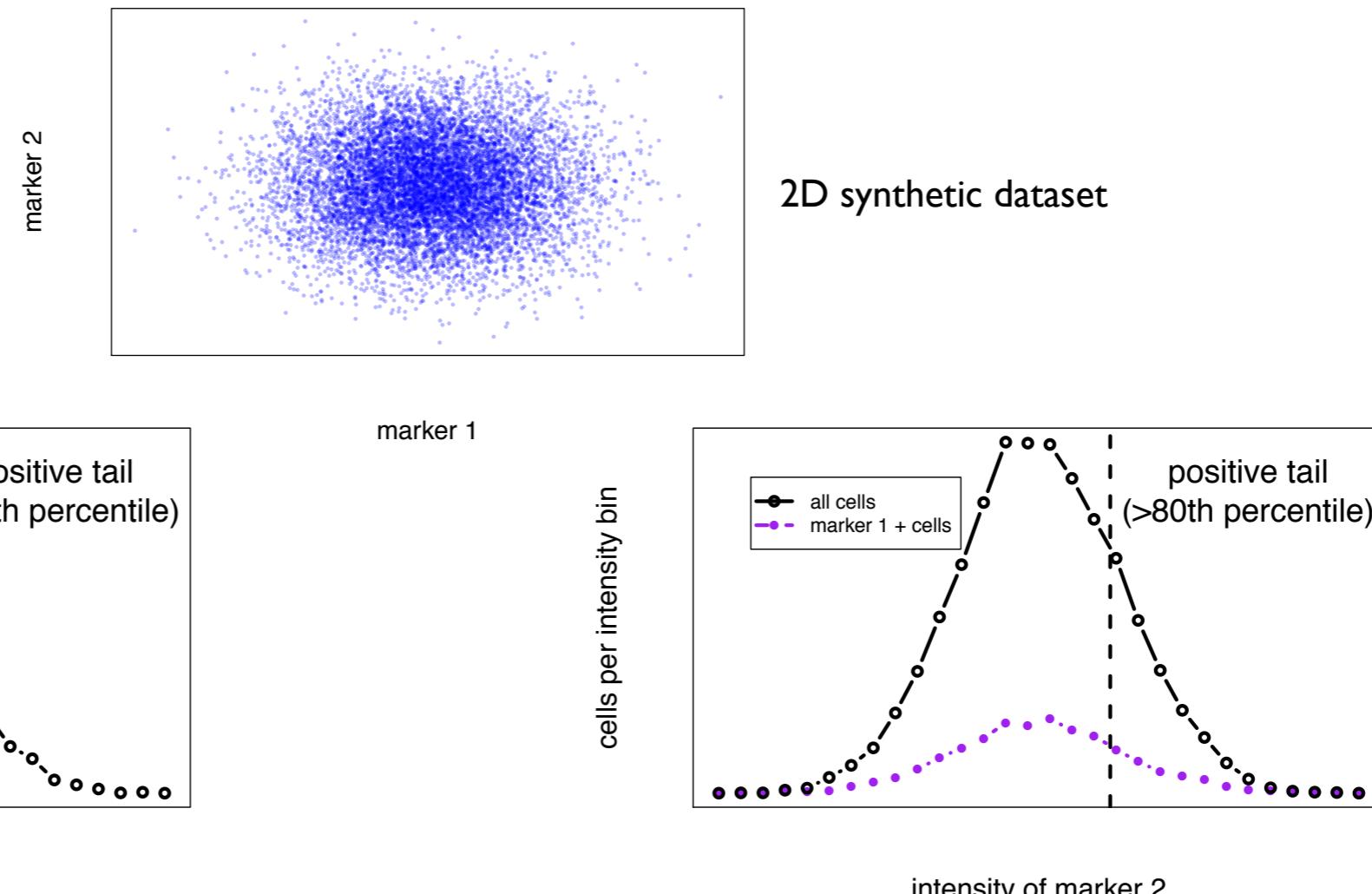


We find a signature of 5 markers (dependent on the normalized fluorescence intensity of CD8+ T cells) that is the most predictive of Sarcoidosis vs Behçet’s patients upon leave-one-out cross validation.

It works well as a “black box” classification machine, but it’s difficult to interpret back in term of the biology.

## II. QuantPheno: A non-parametric, quantile-based approach

(Candia et al, in preparation)



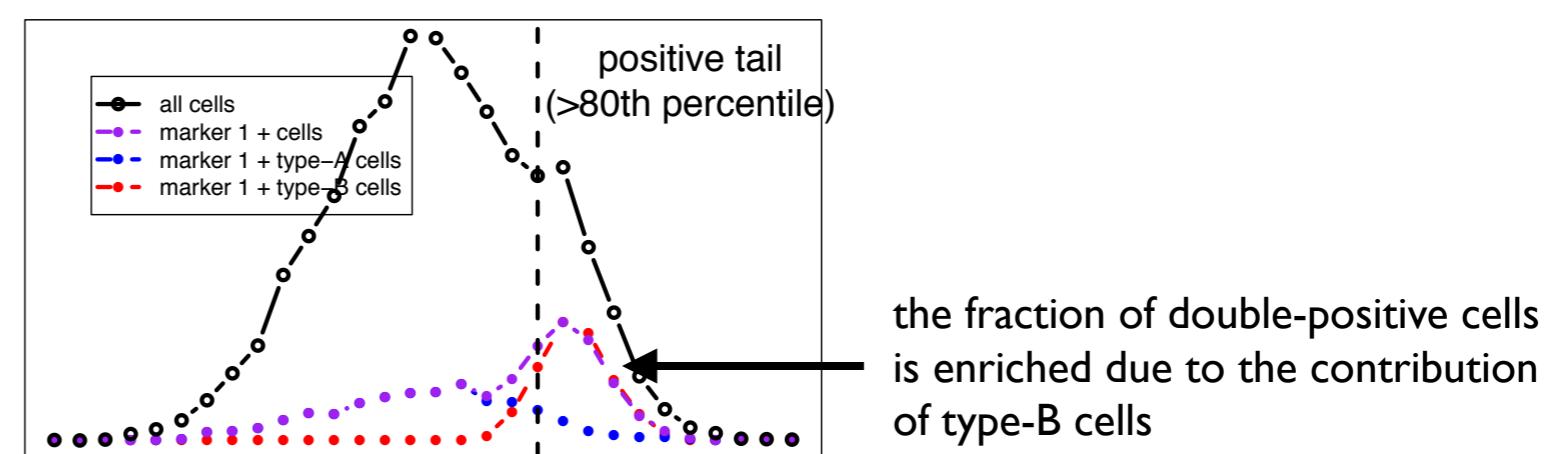
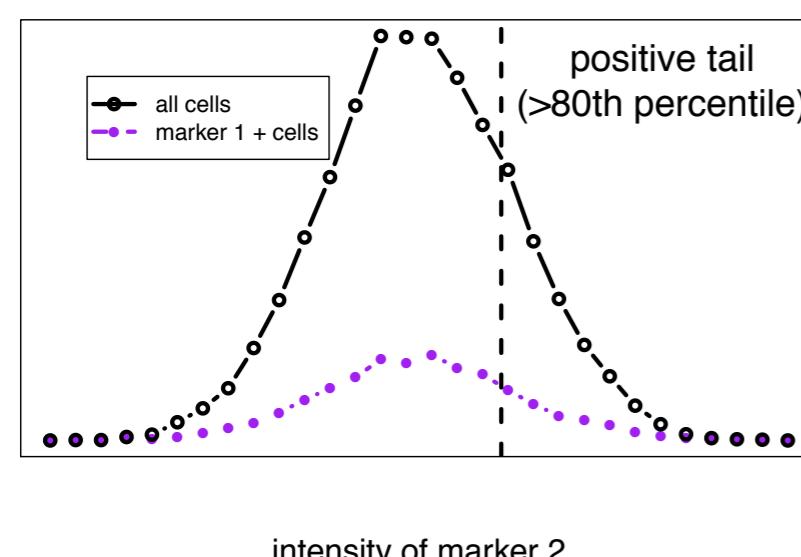
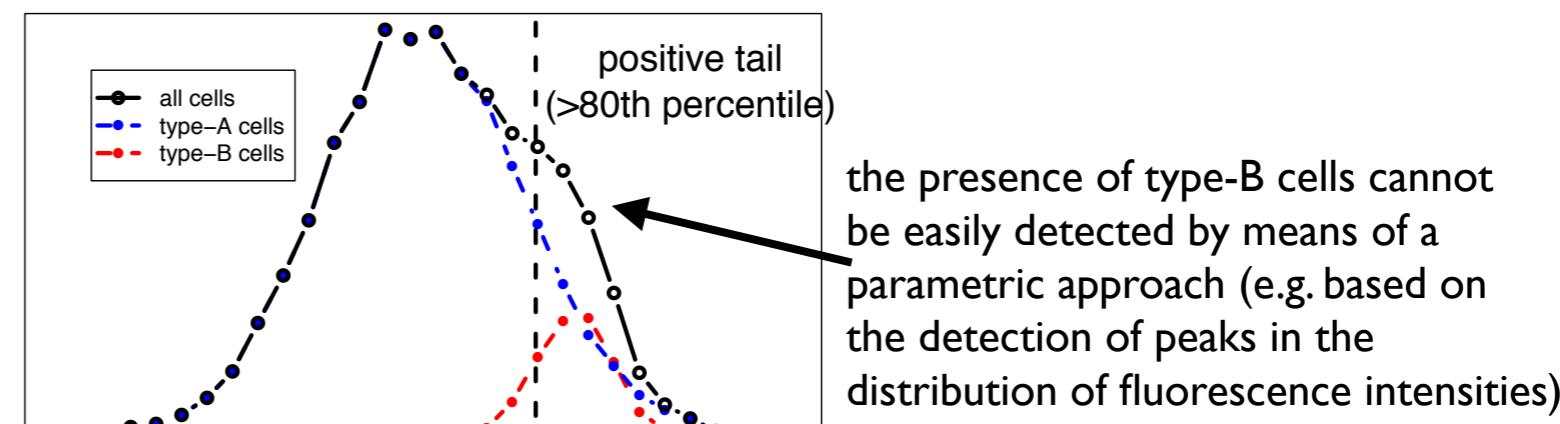
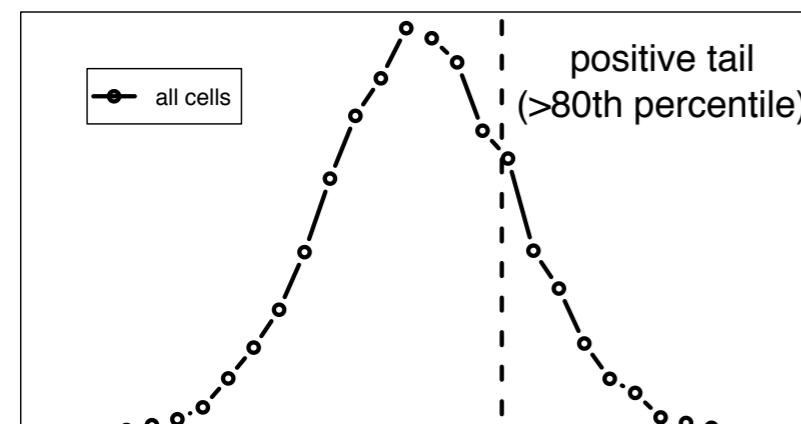
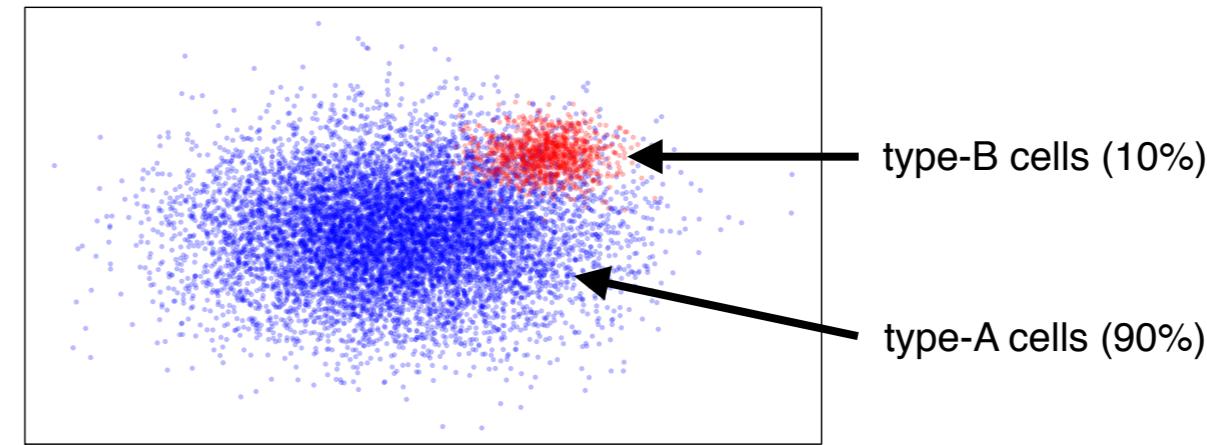
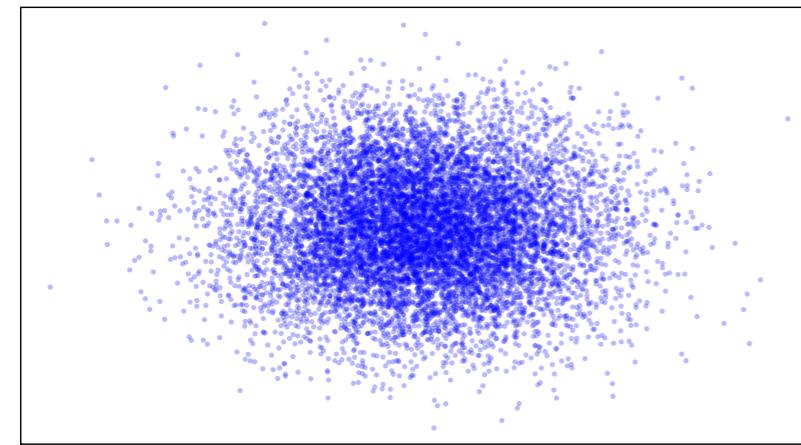
Based on marker 1, we label cells as negative (<20th percentile), intermediate, or positive (>80th percentile)

Based on marker 2, we do the same. All cells have then a combined label based on both markers

Then, we compute cell frequencies associated with each possible “phenotype”, i.e. each combination of negative (1), positive (2) and neutral (0) values for each marker: “00”, “01”, “02”, “10”, “11”, “12”, “20”, “21”, “22”

Finally, we perform statistical tests (t-test, Wilcoxon) to determine each phenotype’s correlation with patient outcomes.

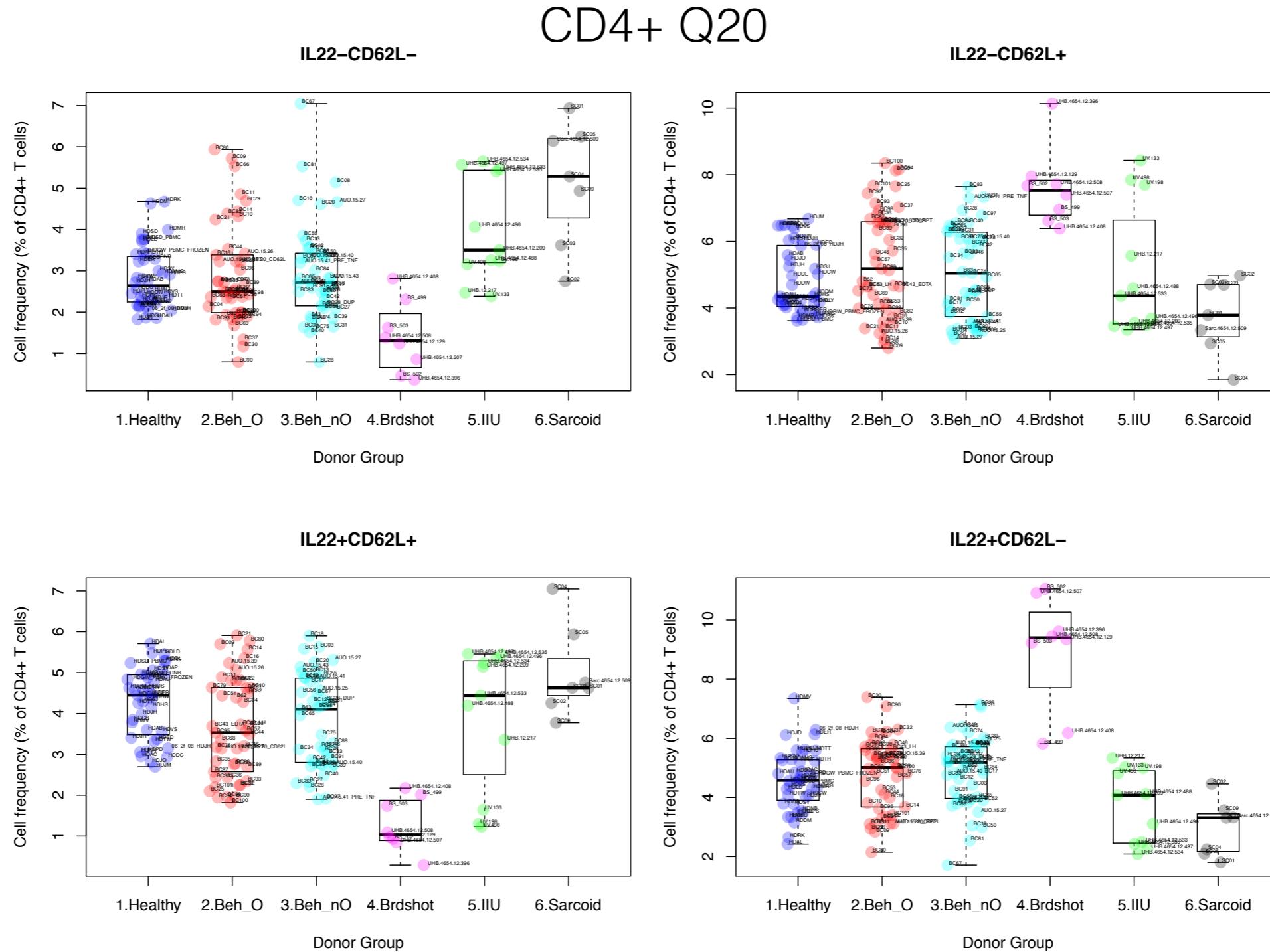
# Case vs control example based on synthetic data



# Comparison of autoimmune diseases with ocular manifestations

(Candia et al, in preparation)

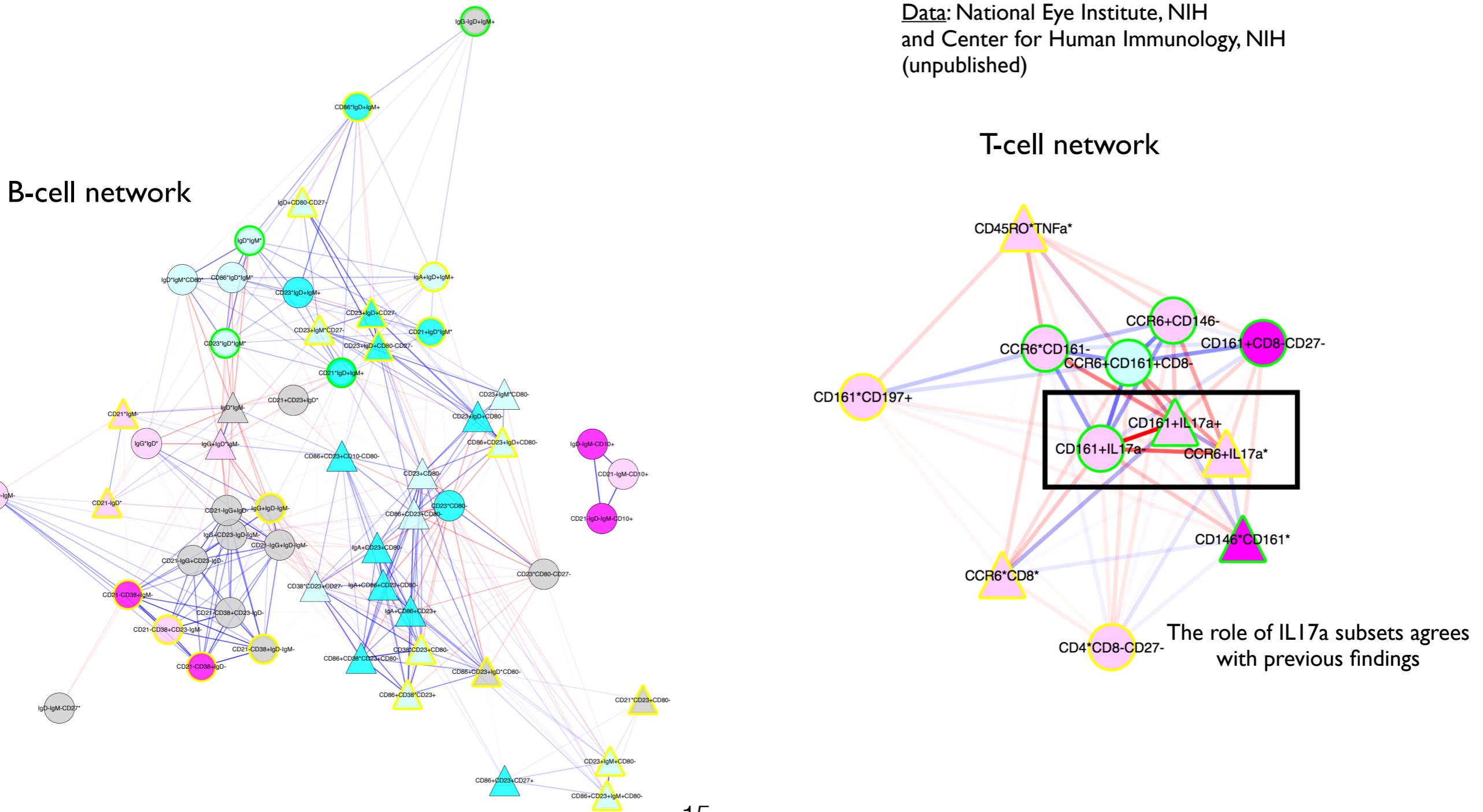
Data: Centre for Translational Inflammation Research,  
University of Birmingham, UK (unpublished)



# Cell Phenotype Networks:

## Based on correlations among cell subsets differentially expressed across cohorts

Data: National Eye Institute, NIH  
and Center for Human Immunology, NIH  
(unpublished)



# Features of a non-parametric, quantile-based approach

## 1) Data transformation:

This issue is avoided altogether, since quantile distributions are invariant under monotonic data transformations

## 2) Resolution of overlapping cell subpopulations:

This approach detects differences among highly overlapping cell populations (where methods such as clustering may be expected to fail)

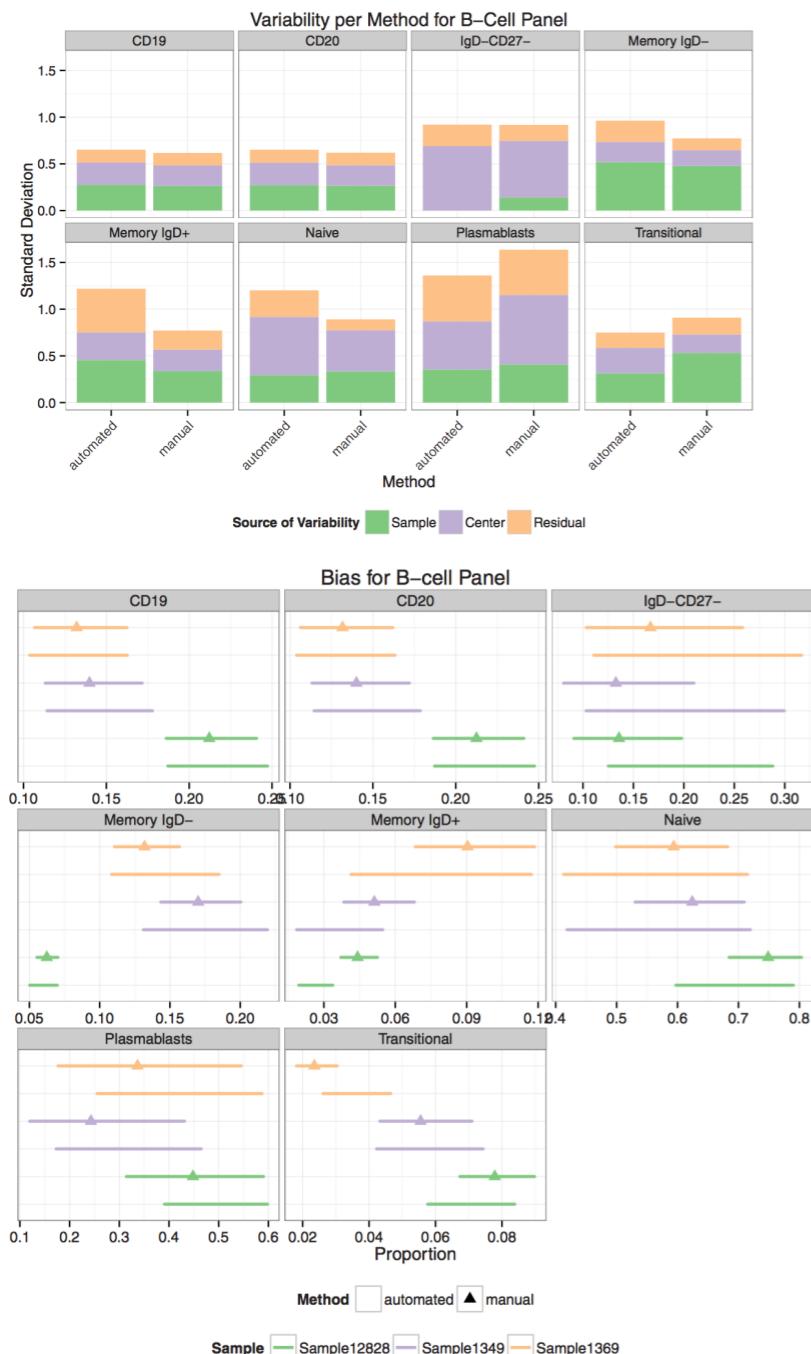
## 3) Consistency of features across the cohort:

This flexible, nonparametric approach does not rely on a fixed set of features that must be present across the cohort (as needed by parametric approaches such as Gaussian mixtures)

## 4) More intuitive interpretation in terms of marker combinations compared to unsupervised (e.g. clustering) and supervised (e.g. SVMs) machine-learning approaches. Scans exhaustively through all possibilities.

# Human ImmunoPhenotyping Consortium (HIPC): The importance of wet- and dry-lab standardization

Automated analysis shows bias and variance comparable to manual analysis  
(but only on select cell subsets and stain panels)



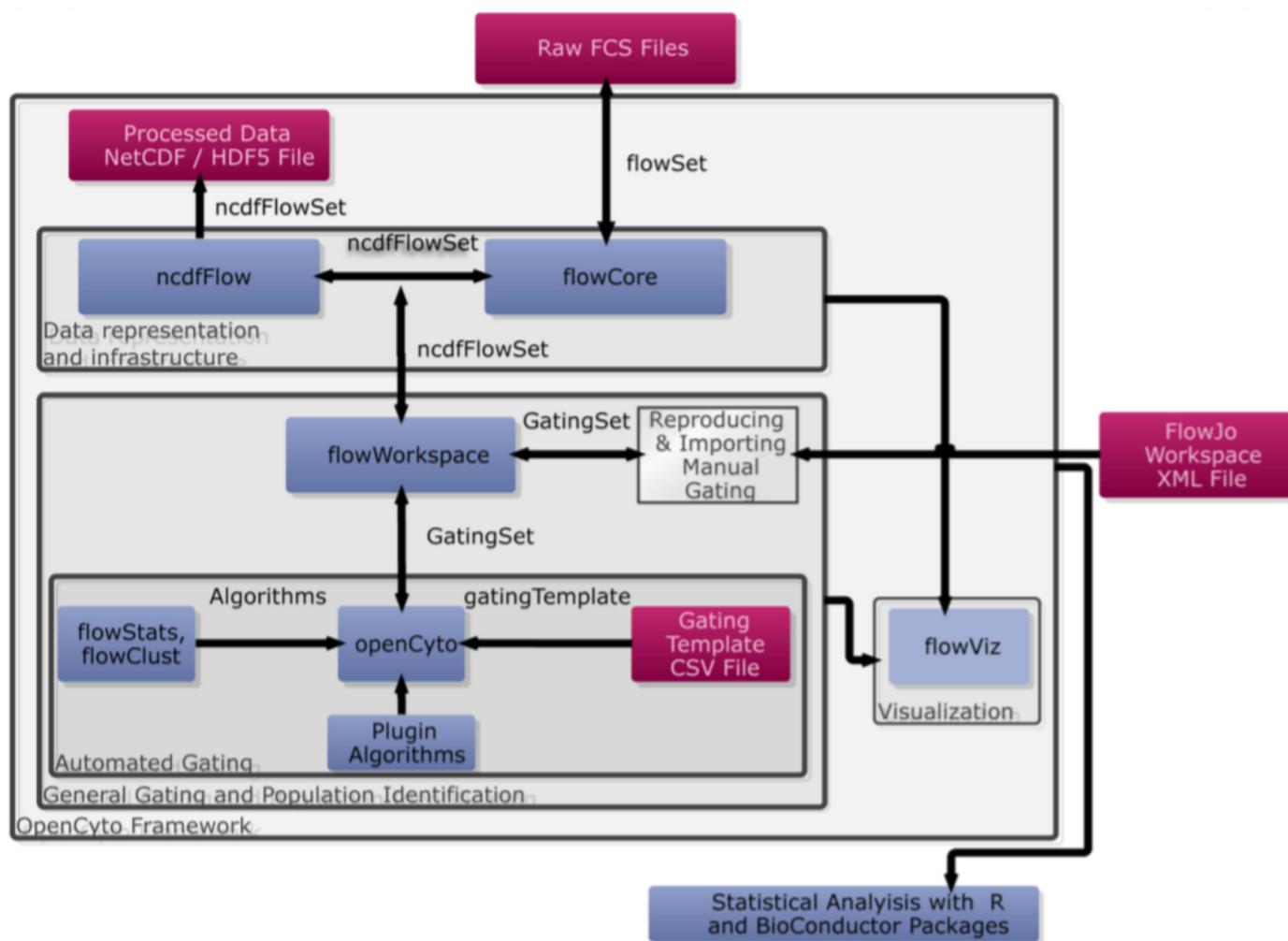
Finak et al, Scientific Reports (2016)

Panel	Population Name	Reliability	Corresponding Markers
T-cell	CD8 Activated	-	CD3+/CD8+/CD4-/CD38+/HLADR+
T-cell	CD4 Activated	+	CD3+/CD8-/CD4+/CD38+/HLADR+
T-cell	CD4 Central Memory	-	CD3+/CD8-/CD4+/CCR7-/CD45RA-
T-cell	CD8 Central Memory	-	CD3+/CD8+/CD4-/CCR7+/CD45RA-
T-cell	CD4 Effector	+	CD3+/CD8-/CD4+/CCR7-/CD45RA+
T-cell	CD8 Effector	+	CD3+/CD8+/CD4-/CCR7-/CD45RA+
T-cell	CD4 Effector Memory	+	CD3+/CD8-/CD4+/CCR7-/CD45RA-
T-cell	CD8 Effector Memory	-	CD3+/CD8+/CD4-/CCR7-/CD45RA-
T-cell	CD4 Naïve	+	CD3+/CD8-/CD4+/CCR7+/CD45RA+
T-cell	CD8 Naïve	+	CD3+/CD8+/CD4-/CCR7+/CD45RA+
B-cell	IgD-/CD27-	-	CD3-/CD19+/CD20+/IgD-/CD27-
B-cell	Transitional	+	CD3-/CD19+/CD20+
B-cell	Plasmablasts	-	CD3-/CD19+/CD20-/Cd24 <sup>high</sup> /CD38 <sup>high</sup>
B-cell	Naïve B	+	CD3-/CD19+/CD20+/CD27-/IgD+
B-cell	Memory IgD+	+	CD3-/CD19+/CD20+/IgD+/CD27+/IgD+
B-cell	CD19	+	CD3-/CD19+
B-cell	CD20	+	CD3-/CD20+
B-cell	Memory IgD-	+	CD3-/CD19+/CD20+/CD27-/IgD-
T-regulatory	Total T-regulatory	+	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4+ (as % of CD4)
T-regulatory	Memory T-regulatory	+	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4+/CD45RO+ (as % of total Treg)
T-regulatory	Naïve T-regulatory	+	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4+/CD45RO- (as % of total Treg)
T-regulatory	CCR4-/CD45RO-	-	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4-/CD45RO- (as % of parent)
T-regulatory	CCR4-CD45RO+	-	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4-CD45RO+ (as % of parent)
T-regulatory	CCR4-HLADR-	+	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4-HLADR- (as % of parent)
T-regulatory	CCR4-HLADR+	-	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4-/HLADR+ (as % of parent)
T-regulatory	CCR4+/CD45RO-	-	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4+/CD45RO- (as % of parent)
T-regulatory	CCR4+/HLADR+	+	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4+/HLADR+ (as % of parent)
T-regulatory	Total CD4	+	CD3+/CD4+/CD8- (as % of parent)
T-regulatory	LoCD127/HiCD25	+	CD3+/CD4+/CD8-/LoCD127/HiCD25 (as % of parent)
T-regulatory	Activated	+	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4+/HLADR+ (as % of total Treg)
DC/Mono/NK	CD11c-/CD123-	-	CD11c-/CD123-
DC/Mono/NK	CD11c-/CD123+	+	CD11c-/CD123+
DC/Mono/NK	CD11c+/CD123-	+	CD11c+/CD123-
DC/Mono/NK	CD11c+/CD123+	-	CD11c+/CD123+
DC/Mono/NK	CD14+/CD16+	-	CD14+/CD16+
DC/Mono/NK	CD16-/CD56+	+	CD16-/CD56+
DC/Mono/NK	CD16+/CD56-	-	CD16+/CD56-
DC/Mono/NK	CD16+/CD56+	+	CD16+/CD56+
DC/Mono/NK	HLADR+	-	HLADR+
DC/Mono/NK	Lin-CD14-	+	Lin-CD14-
DC/Mono/NK	Lin-/CD14+	+	Lin-/CD14+
DC/Mono/NK	CD16-/CD56-	-	CD16-/CD56-

# OpenCyto: a BioConductor framework for integrated analysis

Finak et al, PLoS Comp Biol (2014)

OpenCyto provides a flexible infrastructure to perform preprocessing, gating, cohort-level analysis and visualization



Example of a gating template to integrate domain-specific knowledge into the analysis pipeline

```
##           alias      pop   parent      dims gating_method
## 1:    nonDebris  nonDebris root     FSC-A mindensity
## 2:    singlets    singlets nonDebris FSC-A,FSC-H singletGate
## 3:     lymph     lymph  singlets FSC-A,SSC-A flowClust
## 4:      cd3      cd3   lymph    CD3 mindensity
## 5:      *       cd4-/+cd8+/- cd3   cd4,cd8 mindensity
## 6: activated cd4  CD38+HLA+ cd4+cd8- CD38,HLA tailgate
## 7: activated cd8  CD38+HLA+ cd4-cd8+ CD38,HLA tailgate
## 8:   CD45_neg   CD45RA- cd4+cd8- CD45RA mindensity
## 9: CCR7_gate    CCR7+  CD45_neg   CCR7 flowClust
## 10:    *       CCR7+/-CD45RA+/- cd4+cd8- CCR7,CD45RA refGate
## 11:    *       CCR7+/-CD45RA+/- cd4-cd8+ CCR7,CD45RA mindensity
##               gating_args collapseDataForGating groupBy
## 1:                               NA
## 2:                               NA
## 3: K=2,target=c(1e5,5e4)
## 4:                               TRUE
## 5:      gate_range=c(1,3)          NA
## 6:                               NA
## 7:      tol=0.08                NA
## 8:      gate_range=c(2,3)          NA
## 9:      neg=1,pos=1              NA
## 10:     CD45_neg:CCR7_gate      NA
## 11:      preprocessing_method preprocessing_args
## 1:                               NA
## 2:                               NA
## 3:      prior_flowClust        NA
## 4:                               NA
## 5:                               NA
## 6:      standardize_flowset    NA
## 7:      standardize_flowset    NA
## 8:                               NA
## 9:                               NA
## 10:                             NA
## 11:                             NA
```

# Public Repositories: lots of software and data!

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OPEN SOURCE SOFTWARE FOR BIOINFORMATICS

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  - SingleCell (5)
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- AnnotationData (939)
- ExperimentData (308)

Packages found under FlowCytometry:

Show All entries	Maintainer	Title
COMPASS	Greg Finak	Combinatorial Polyfunctionality Analysis of Single Cells
cytofkit	Jinmiao Chen, Hao Chen	cytofkit: an integrated mass cytometry data analysis pipeline
CytoML	Mike Jiang	GatingML interface for openCyto
flowAI	Gianni Monaco	Automatic and interactive quality control for flow cytometry data
flowBeads	Nikolas Pontikos	flowBeads: Analysis of flow bead data
flowBin	Kieran O'Neill	Combining multibyte flow cytometry data by binning
flowCHIC	Author: Joachim Schumann	Analyze flow cytometric data using histogram information
flowCL	Justin Meskas	Semantic labelling of flow cytometric
flowClean	Kipper Fletez-Brant	flowClean
flowClust	Greg Finak, Mike Jiang	Clustering for Flow Cytometry
flowCore	Mike Jiang	flowCore: Basic structures for flow cytometry
flowCyBar	Joachim Schumann	Analyze flow cytometric data using GatingML
flowDensity	Mehrnoush Malek	Sequential Flow Cytometry Data Gating
flowFit	Davide Rambaldi	Estimate proliferation in cell-tracking
flowFP	Herb Holyst	Fingerprinting for Flow Cytometry
flowMap	Chiawen Joyce Hsiao	Mapping cell populations in flow cytometry sample comparisons using the Friedmann algorithm
flowMatch	Ariful Azad	Matching and meta-clustering in flow cytometry
flowMeans	Nima Aghaeepour	Non-parametric Flow Cytometry Data Gating
flowMerge	Greg Finak	Cluster Merging for Flow Cytometry
flowPeaks	Yongchao Ge	An R package for flow data clustering
flowPloidy	Tyler Smith	Analyze flow cytometer data to determine cell ploidy
flowPlots	N. Hawkins	flowPlots: analysis plots and data visualization for flow cytometry data
flowQ	Mike Jiang	Quality control for flow cytometry

**FLOW Repository**

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**Help**

The following open access article describes how to upload and annotate flow cytometry data sets: Spidlen J, Breuer K and Brinkman R. Preparing a Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) Compliant Manuscript Using the International Society for Advancement of Cytometry (ISAC) FCS File Repository (FlowRepository.org). *Current Protocols in Cytometry*, UNIT 10.18, July 2012.

We also have a [Quick start guide](#) and a [FAQ section](#).

You may download [slides](#) from our Workshop at CYTO 2012: Publishing MIFlowCyt Compliant Data to ISAC's FlowRepository.org for Cytometry A and Other Journals

Additional links and help options are listed in our [support](#) page.

You can contact us by filling out a [support ticket](#).

**FlowRepository**

FlowRepository is a database of flow cytometry experiments where you can query and download data collected and annotated according to the [MIFlowCyt standard](#). It is primarily used as a data deposition place for experimental findings published in peer-reviewed journals in the flow cytometry field. Those datasets can be queried or browsed using the form and links below.

**Query FlowRepository**

Enter a term to search all publicly available experiments:

Query

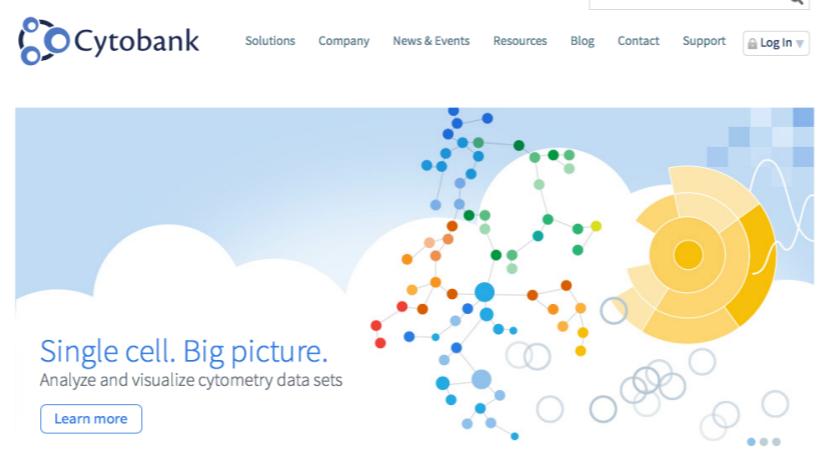
Show query fields

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-- Jan 10 -- **CisFileCreator v4 now available in pre-production**

We are also happy to offer the use of a pilot (beta) Grid of National Center for Genome Analysis Support (NCGAS) resources than we are able to provide through this Beta Grid to run many jobs, and/or compute intensive jobs, to obtain important information.

**Older Java Applet visualizers are no longer supported**

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**Getting Started**

**New! Web tours**

- Click here for a tour of [what's new in GenePattern](#)
- Click here for an [introductory tour of GenePattern](#)

**Analyzing genomic data in GenePattern**

- Select a [protocol](#) below for a step by step guide
- Click here for a [Quick Start tutorial](#) on how to use GenePattern

**Protocols for running common analyses**

	<b>Run an Analysis in GenePattern</b> Learn how to run an analysis in GenePattern
	<b>Differential Expression Analysis</b> Find genes that are significantly differentially expressed between two samples
	<b>Clustering</b> Group genes and/or samples by similarity
	<b>Prediction</b> Create a model, also referred to as classification
	<b>SNP Copy Number and Loss of Heterozygosity</b> Compute SNP copy number (CN) and loss of heterozygosity (LOH) for each sample

**AddFCSEventIndex** Adds indexes to events in a Flow Cytometry Standard (FCS) data file. [Flow Cytometry](#)

**AddFCSParameter** Add parameters and their values to a FCS data file. [Flow Cytometry](#)

**AddNoiseToFCS** Add noise to specified parameters in an FCS data file. [Flow Cytometry](#)

**ApplyGatingML** Apply a Gating-ML file on an FCS data file (gate and/or transform list mode data). [Flow Cytometry](#)

**CompensateFCS** Compensates an FCS data file. [Flow Cytometry](#)

**DeIdentifyFCS** DeIdentifyFCS an FCS data file; remove keywords from a list or matching a regular expression. [Flow Cytometry](#)

**ExtractFCSDataset** Extract one or more FCS datasets from an FCS data file. [Flow Cytometry](#)

**ExtractFCSKeywords** Extracts keyword(s) value(s) from a Flow Cytometry Standard (FCS) file. [Flow Cytometry](#)

**ExtractFCSParameters** Extract specified parameters (dimensions) from an FCS data file. [Flow Cytometry](#)

**FCMFeatureExtraction** Calculation of features from clustered flow cytometry data. [Flow Cytometry](#)

**FCMSinglePanelQC** Single panel flow cytometry data quality control. [Flow Cytometry](#)

# Conclusions

Flow Bioinformatics is an emerging field with a wealth of conceptual approaches and available implementations to perform data pre-processing, quality control, automated gating, data analysis, visualization and post-processing tasks.

There is no “right” or “best” tool - the method of choice and its performance will depend on the structure of the data and the goals of the analysis. As such, it is advisable to test a variety of approaches.

To build a robust, long-term, scalable flow cytometry platform, the design and implementation of careful wet- and dry-lab standardization procedures is of paramount importance. Exhaustive testing on sets of control samples will determine which stain panels, marker signatures and cell subsets are reliable. It is also key to establish fully quantitative metrics (coefficients of variation, bias, and statistical power).

# Suggested References

- 1) Summary of some of the bioinformatic tools available for flow cytometry data pre-processing, QC, gating, analysis and post-processing:

Kvistborg P, Gouttefangeas C, Aghaeepour N, Cazaly A, Chattopadhyay PK, Chan C, Eckl J, Finak G, Hadrup SR, Maecker HT, Maurer D, Mosmann T, Qiu P, Scheuermann RH, Welters MJ, Ferrari G, Brinkman RR, Britten CM. Thinking outside the gate: single-cell assessments in multiple dimensions. *Immunity*. 2015 Apr 21;42(4):591-2. doi: 10.1016/j.jimmuni.2015.04.006. PubMed PMID: 25902473; PubMed Central PMCID: PMC4824634.

- 2) Multi-center Manual vs Automated assessment using 8-color standardized lyo-plates:

Finak G, Langweiler M, Jaimes M, Malek M, Taghiyar J, Korin Y, Raddassi K, Devine L, Obermoser G, Pekalski ML, Pontikos N, Diaz A, Heck S, Villanova F, Terrazzini N, Kern F, Qian Y, Stanton R, Wang K, Brandes A, Ramey J, Aghaeepour N, Mosmann T, Scheuermann RH, Reed E, Palucka K, Pascual V, Blomberg BB, Nestle F, Nussenblatt RB, Brinkman RR, Gottardo R, Maecker H, McCoy JP. Standardizing Flow Cytometry Immunophenotyping Analysis from the Human ImmunoPhenotyping Consortium. *Sci Rep*. 2016 Feb 10;6:20686. doi: 10.1038/srep20686. PubMed PMID: 26861911; PubMed Central PMCID: PMC4748244.

- 3) Flexible platform for end-to-end flow data analysis:

Finak G, Frelinger J, Jiang W, Newell EW, Ramey J, Davis MM, Kalams SA, De Rosa SC, Gottardo R. OpenCyto: an open source infrastructure for scalable, robust, reproducible, and automated, end-to-end flow cytometry data analysis. *PLoS Comput Biol*. 2014 Aug 28;10(8):e1003806. doi:10.1371/journal.pcbi.1003806. PubMed PMID: 25167361; PubMed Central PMCID: PMC4148203.

- 4) Comparative assessment of different automated methods (FlowCAP challenges):

Aghaeepour N, Finak G; FlowCAP Consortium.; DREAM Consortium., Hoos H, Mosmann TR, Brinkman R, Gottardo R, Scheuermann RH. Critical assessment of automated flow cytometry data analysis techniques. *Nat Methods*. 2013 Mar;10(3):228-38. doi:10.1038/nmeth.2365. Erratum in: *Nat Methods*. 2013 May;10(5):445. PubMed PMID:23396282; PubMed Central PMCID: PMC3906045

- 5) (One of my papers) with exploratory techniques to find biomarker signatures:

Candia J, Maunu R, Driscoll M, Biancotto A, Dagur P, McCoy JP Jr, Sen HN, Wei L, Maritan A, Cao K, Nussenblatt RB, Banavar JR, Losert W. From cellular characteristics to disease diagnosis: uncovering phenotypes with supercells. *PLoS Comput Biol*. 2013;9(9):e1003215. doi: 10.1371/journal.pcbi.1003215. PubMed PMID:24039568; PubMed Central PMCID: PMC3763994.

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