

Automated Flow Cytometry Analysis

R. Burke Squires

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SOURCES

Ryan Brinkman, PhD
Bioinformatics.ca

Outline

MORNING

Flow Cytometry Review

Flow Cytometry Standards and Analysis
(FlowCAP)

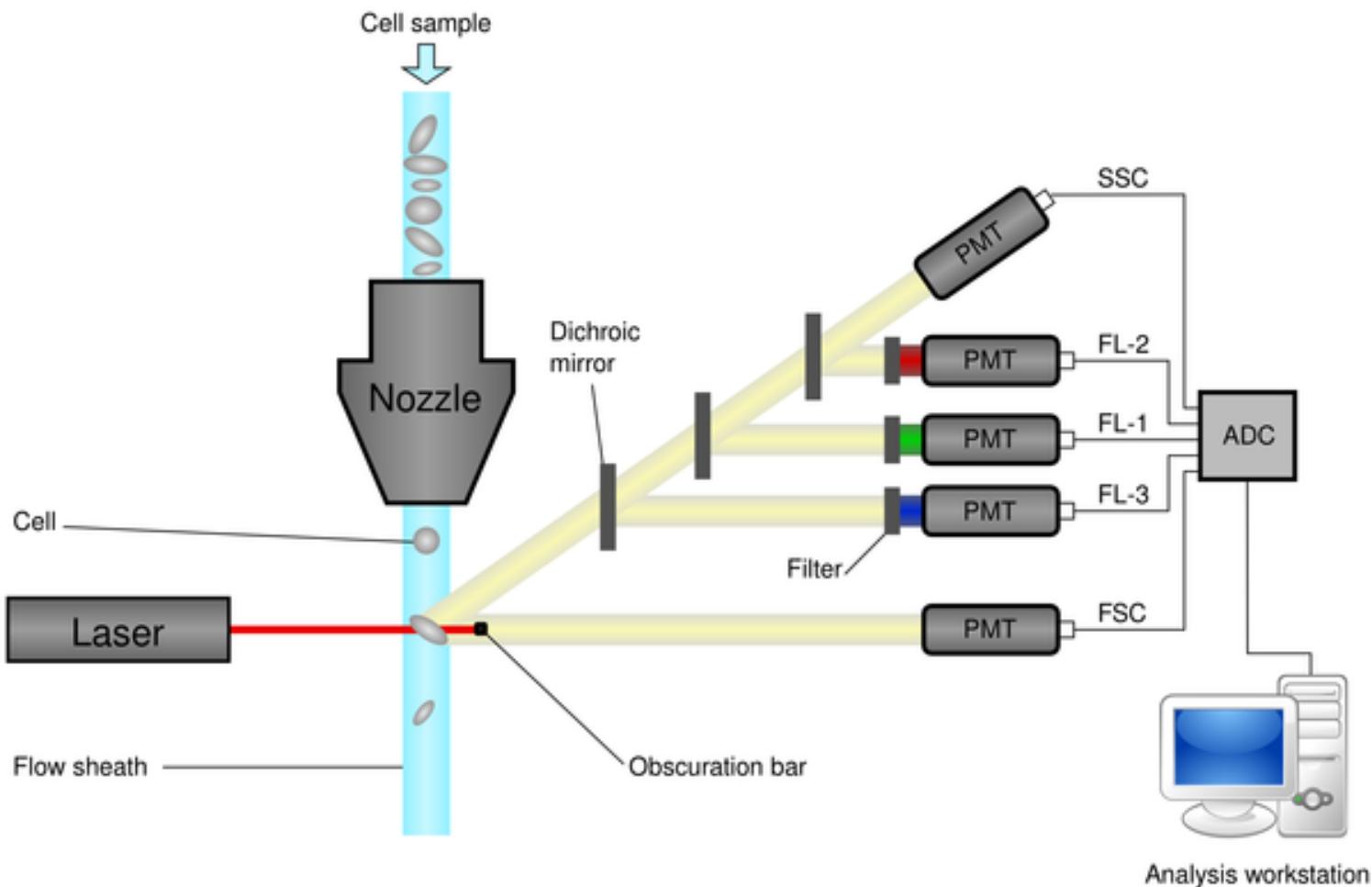
AFTERNOON

Automated Flow Cytometry Analysis



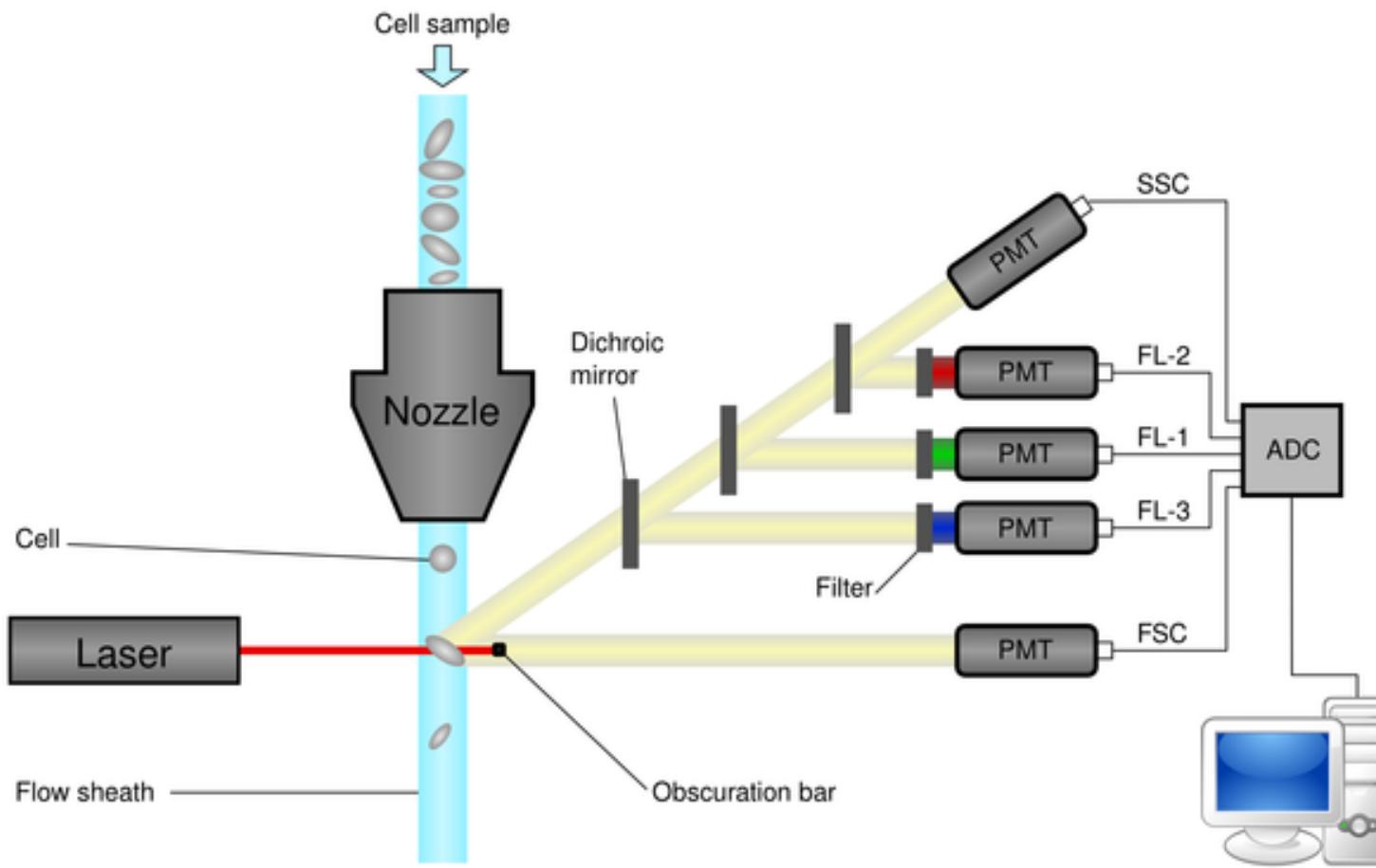
Flow Cytometry Review

Schematic Diagram Of A Flow Cytometer



O'Neill K, Aghaeepour N, Špidlen J, Brinkman R (2013) Flow Cytometry Bioinformatics. PLOS Computational Biology 9(12): e1003365. doi:10.1371/journal.pcbi.1003365
<http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1003365>

Schematic Diagram Of A Flow Cytometer

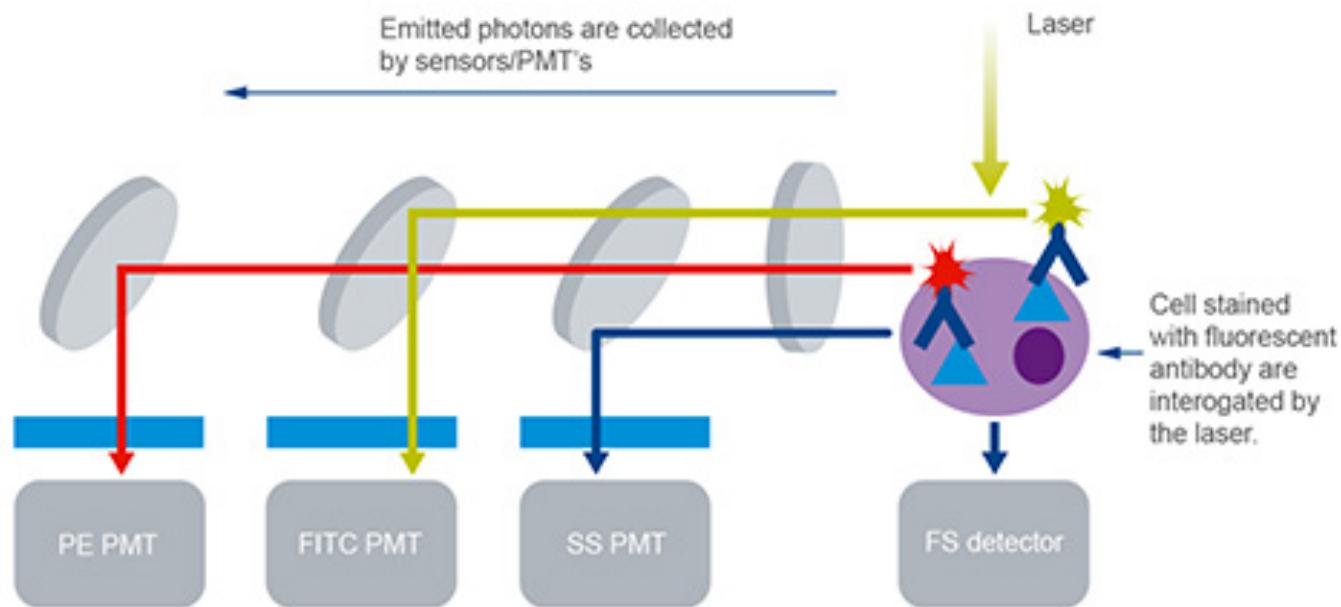


O'Neill K, Aghaeepour N, Špidlen J, Brinkman R (2013) Flow Cytometry Bioinformatics. PLOS Computational Biology 9(12): e1003365
doi:10.1371/journal.pcbi.1003365
<http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1003365>

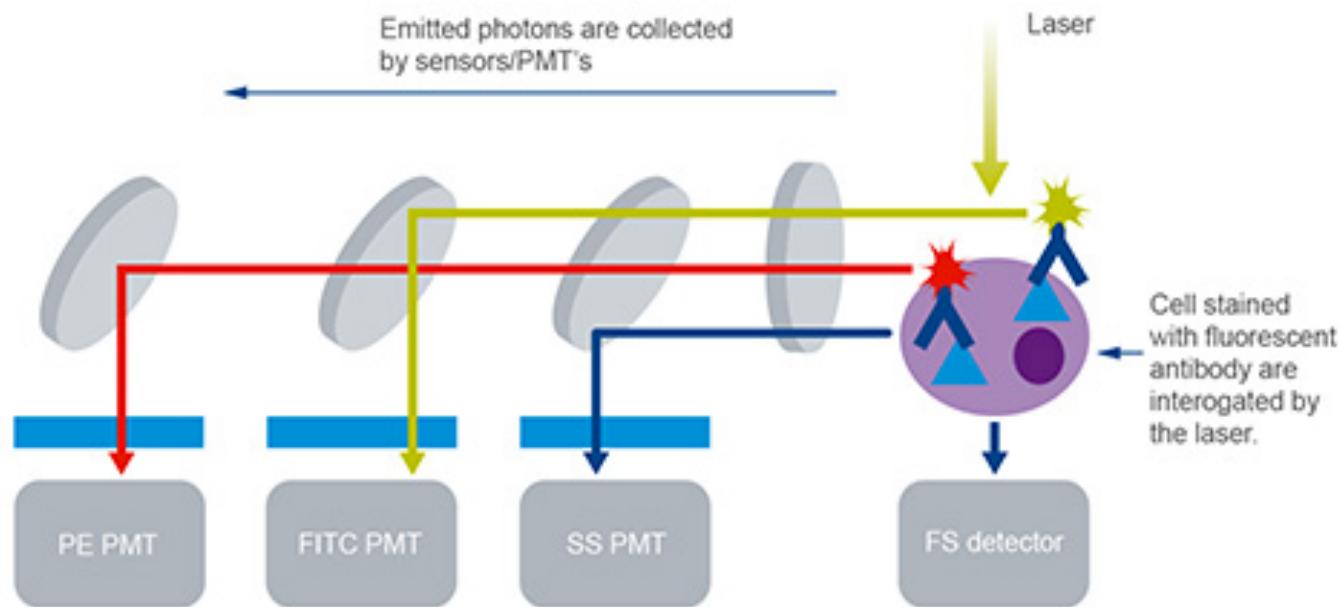


COMPUTATIONAL
BIOLOGY

Fluorescent Light Is Filtered So That Each PMT Detects A Specific Wavelength

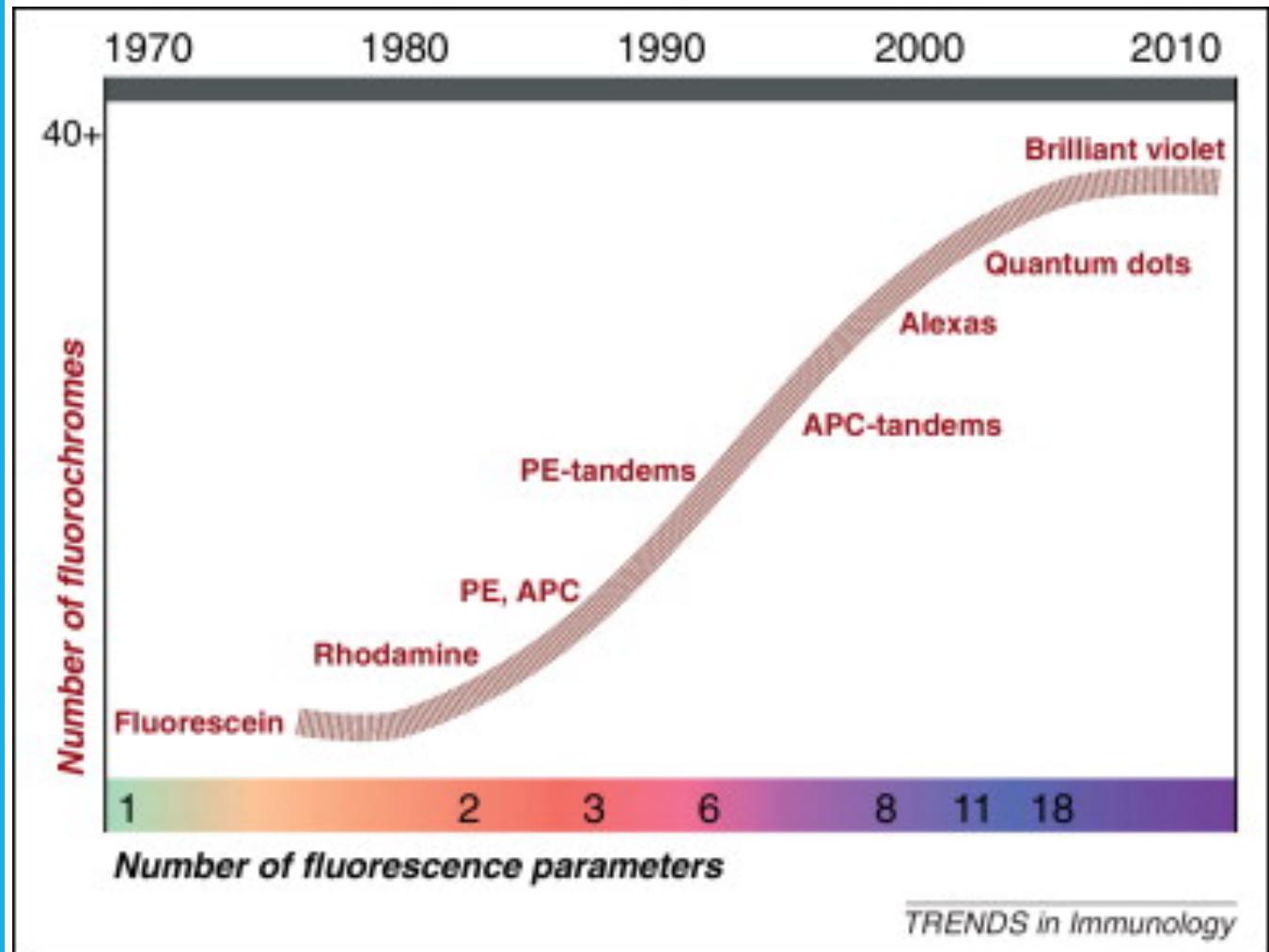


Fluorescent Light Is Filtered So That Each PMT Detects A Specific Wavelength



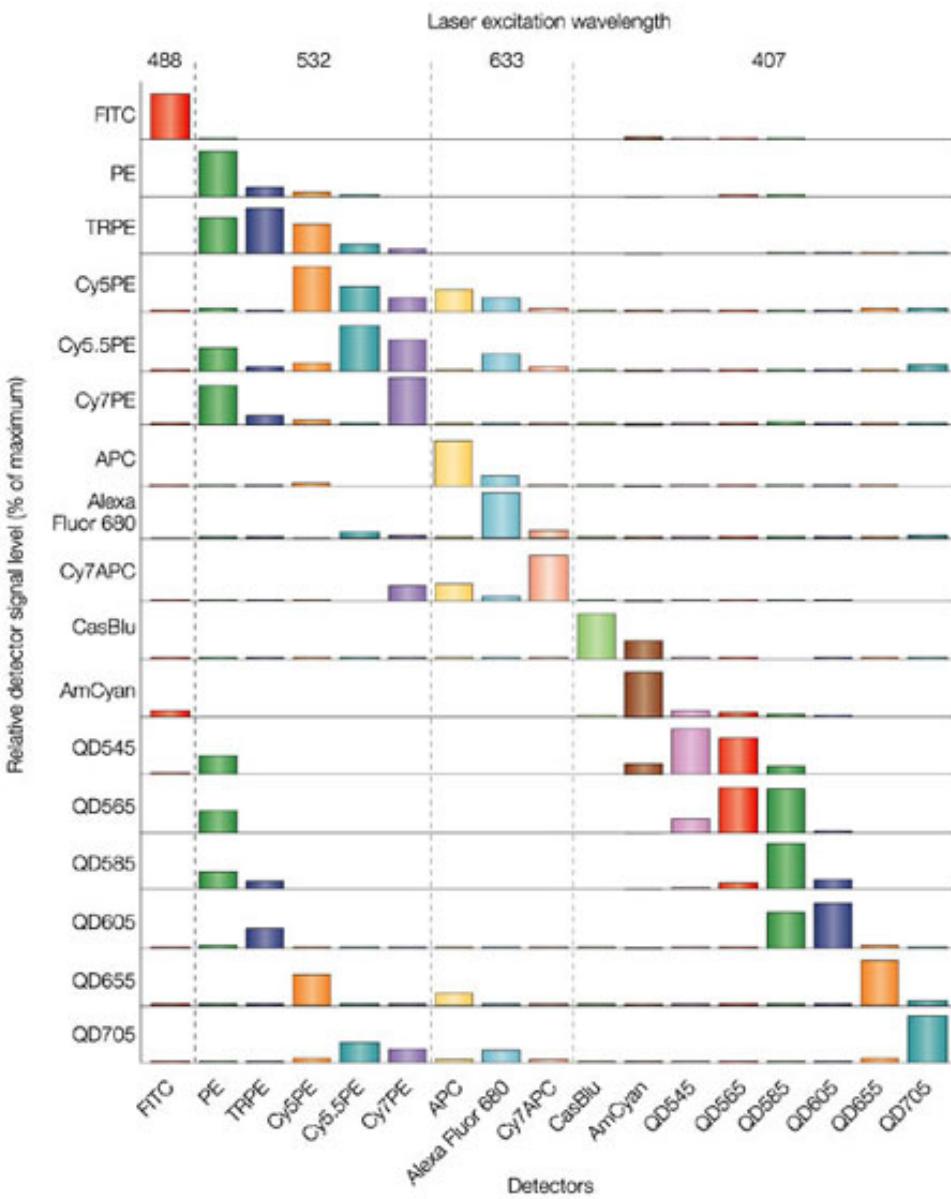
Fluorochromes

History

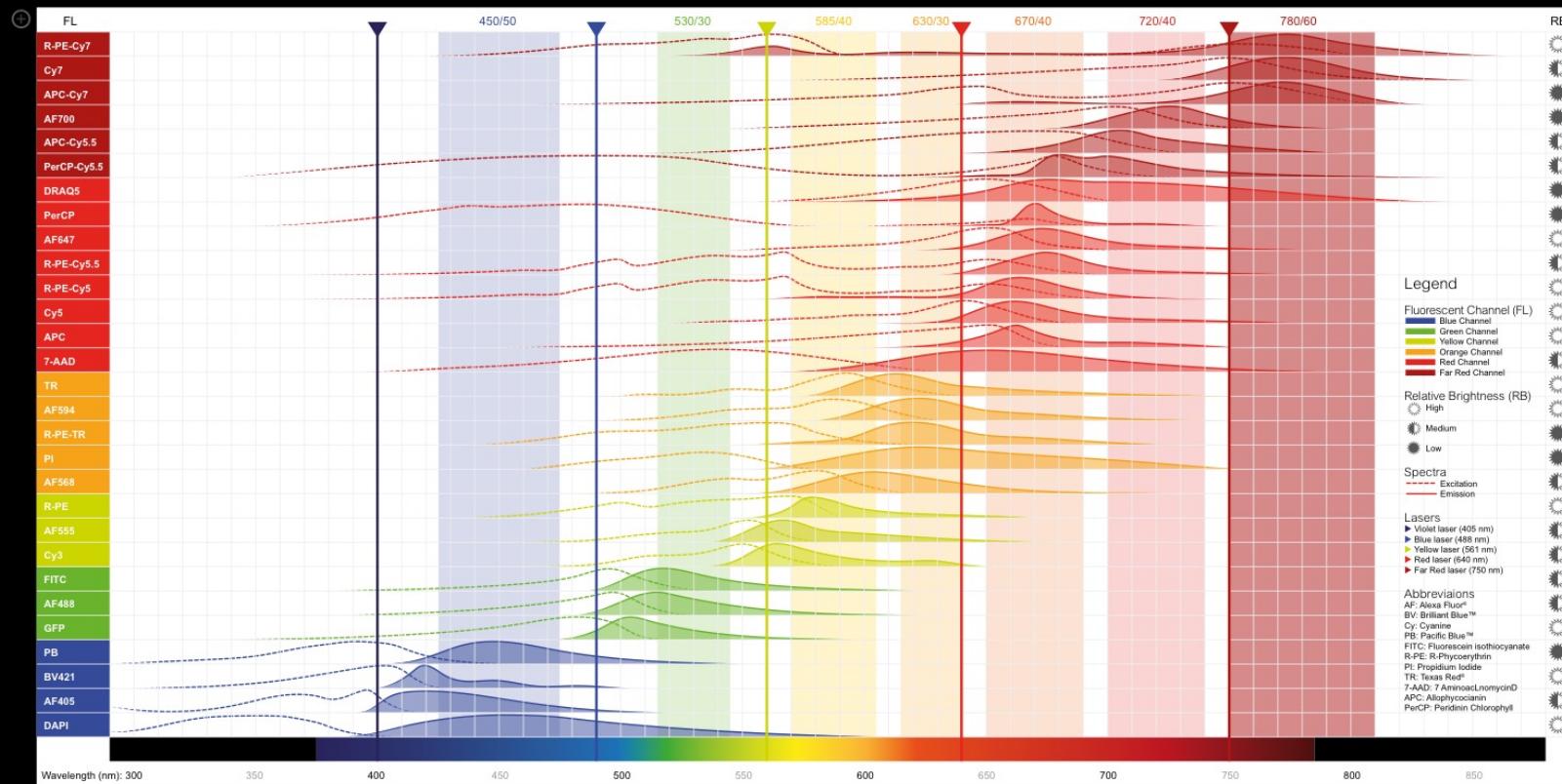


Bendall, S. C., Nolan, G. P., Roederer, M. & Chattopadhyay, P. K. A Deep Profiler's Guide to Cytometry. *Trends in Immunology* **33**, 323–332 (2012).

Fluorochromes



Fluorochrome chart



How to use this chart

- Check your instrument:
Type, number of lasers, filters and detectors dictate the fluorochromes that can be used.
- Try to choose a fluorochrome for each laser excitation range
- Select bright dyes:
It is possible to rank available dyes according to their brightness on a particular instrument.
- Brightest fluorochromes for dim antibodies and vice versa
- Minimize spillover:
The amount of spectral overlap will determine whether compensation is required.
- Sacrifice brightness to avoid spillover
- Avoid spillover from bright cell populations into detectors requiring high sensitivity for those populations

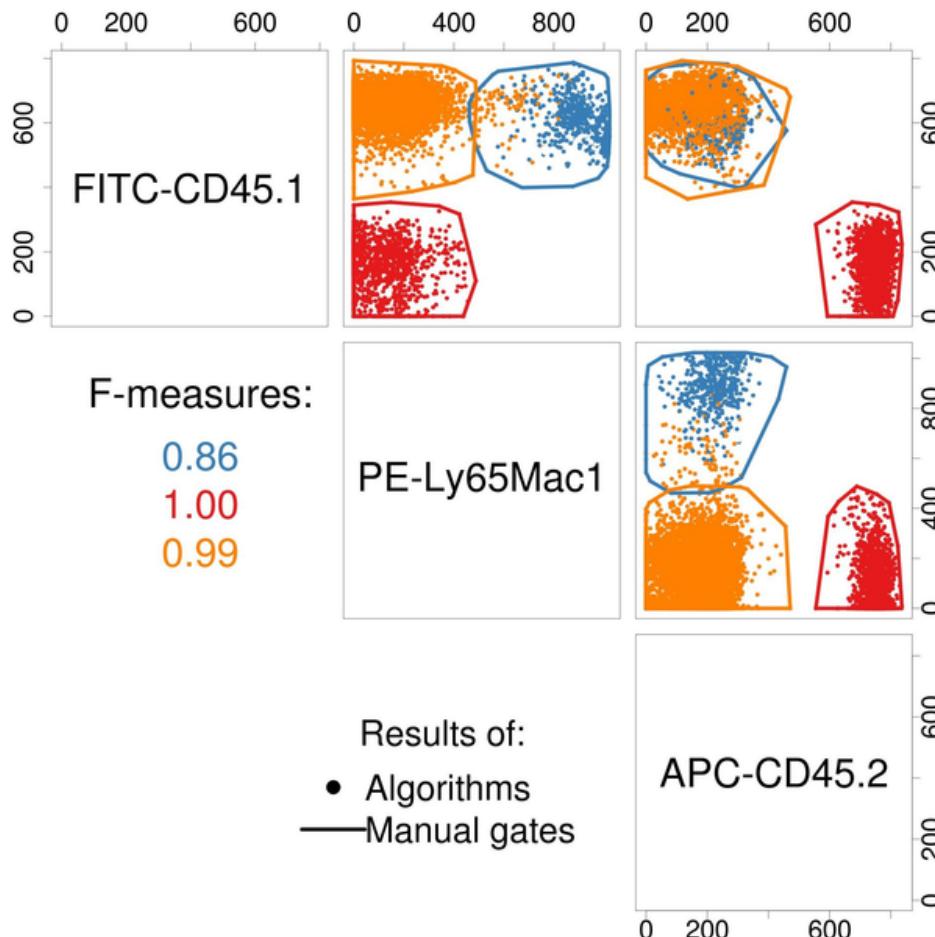
Examples

Fluorochrome	Target Expression	Lasers	Channels	Brightness	Compensation	Combination
FITC APC	High Low	Blue Red	Green Red	Medium High	Mild	Good
FITC PE	High Low	Blue Yellow	Green Yellow	Medium High	Moderate	Medium
PerCP 7-AAD	High Low	Blue Blue	Red Red	Low Medium	Severe	Poor (not recommended)

Discover more at abcam.com/fluorochrome-chart

Alexa Fluor® is a registered trademark of Life Technologies. Alexa Fluor® dye conjugates contain(s) technology licensed to Abcam by Life Technologies.

Manual Gating (polygons) Vs. Automated Gating (colored dots)



O'Neill K, Aghaeepour N, Špidlen J, Brinkman R (2013) Flow Cytometry Bioinformatics. PLOS Computational Biology 9(12): e1003365. doi:10.1371/journal.pcbi.1003365
<http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1003365>

Automated Flow Cytometry Analysis



Automated Flow Cytometry Analysis

“Automated algorithms for flow cytometry data analysis have reached a level of maturity that enables them to match and in many cases exceed the results produced by human experts.”¹

Supervised gating (diagnosis): OpenCyto, flowDensity²

Unsupervised biomarker discovery: FlowReMi, flowType/RchyOptimyx³

¹Aghaeepour et al., Nature Methods, 2013

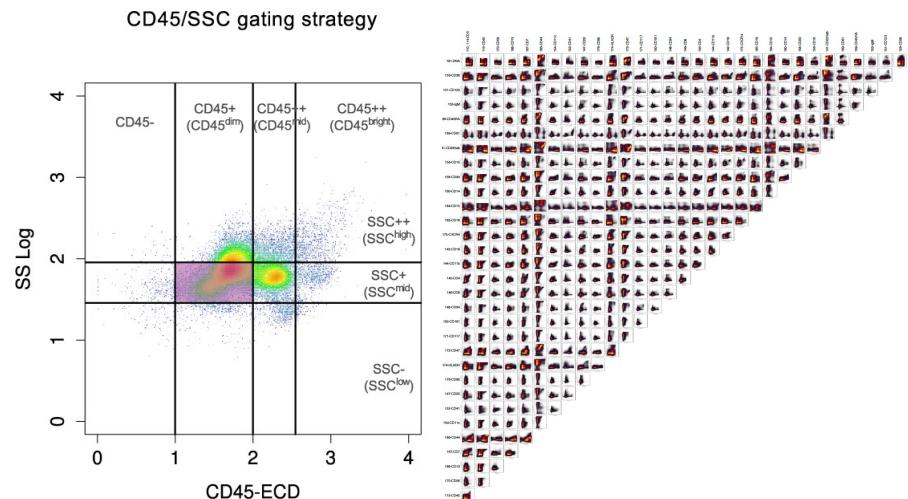
²Malek et al., Bioinformatics, 2014

³O’Neill et al., Bioinformatics, 2014

Big Flow Cytometry Data

AUTOMATED ANALYSIS OF FLOW CYTOMETRY DATA IN R/BIOCONDUCTOR

	1985 ¹	2012 ²	2016+ ³
Samples	1	466	77,000
Dimensions	5	13	50
Cells	50,000	400,000	1,000,000
Datasets	2.5^5	2.5^9	4^{11}



¹ Murphy *Cytometry* (1985)

² Aghaeepour *et al. Bioinformatics* (2012)

³ International Mouse Phenotyping Consortium (2015)

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Can Manual Analysis of “High-Dimensional” Data Be Improved Upon?

Time consuming, especially for “discovery”

Analysis guided by history with limited, intuitive exploration

Rarely (ever?) examine entire multidimensional dataset

Significant cross-individual variability (>10%)

No appropriate statistical basis to assess relative significance

Not fun (?)

“Unfortunately, the use of three or more independent fluorescent parameters complicates the analysis of the resulting data significantly.” Murphy Cytometry (1985)

“Despite the technological advances in acquiring [30] parameters per single cell, methods for analyzing multidimensional single-cell data remain inadequate.” Qiu et al. Nature Biotechnology (2011)

>50 Peer-reviewed, Free, Open Source Software Tools

45 R/BioConductor for data analysis

- A scripted approach to high throughput data analysis
- Non-interactive, self-documented, reproducible
- Breaks problem into smaller pieces (packages)
- Modules can plug-in & swap-out
- Collaborative, cross-platform development environment

9 additional software tools

- Java, Python, Matlab, C++
- Stand alone (single problem/solution)

Immunity
Letter

CellPress

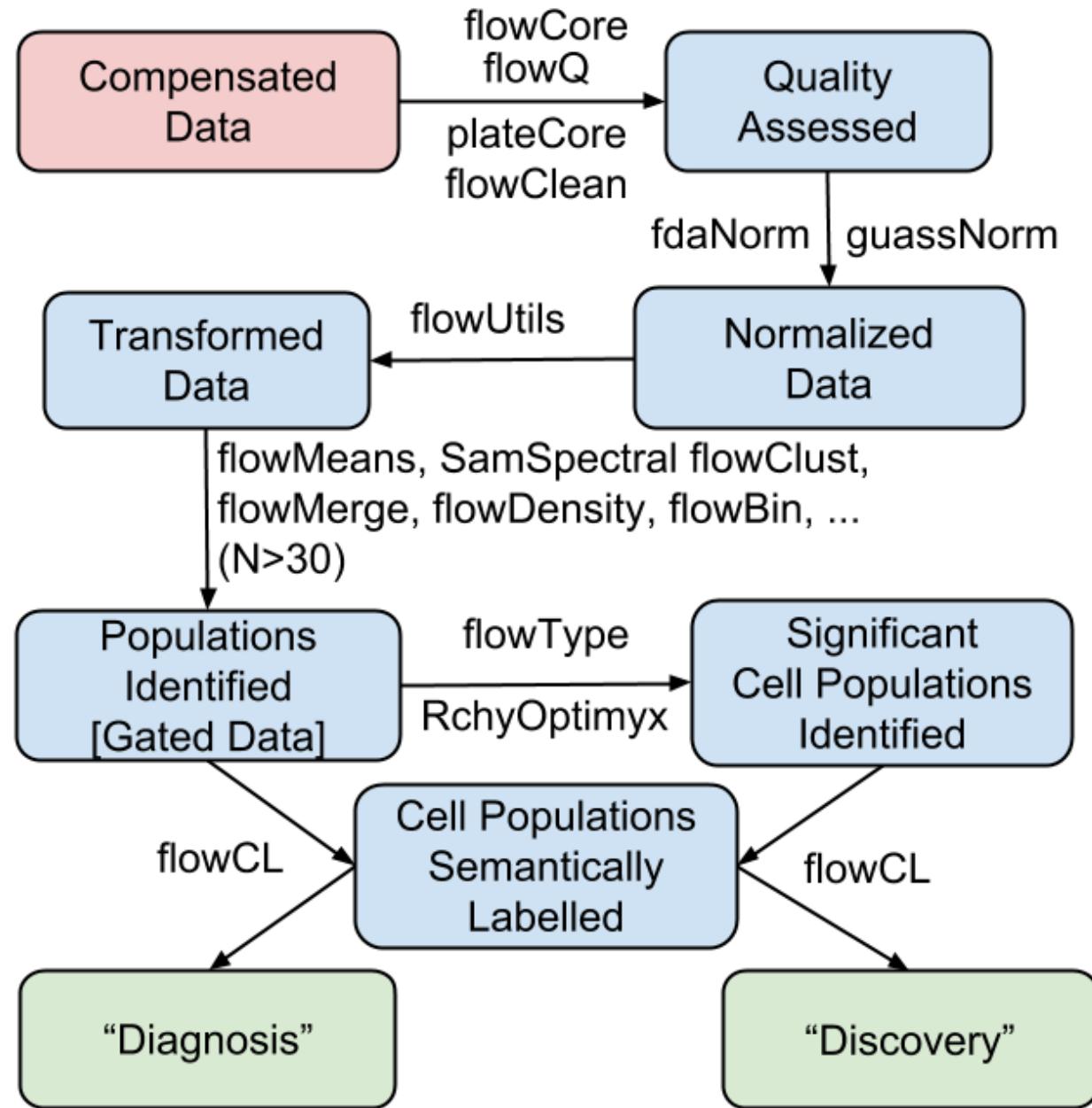
Thinking Outside the Gate: Single-Cell Assessments in Multiple Dimensions

Pia Kvistborg,^{1,19} Cécile Gouttefangeas,^{2,19} Nima Aghaeepour,³ Angelica Cazaly,⁴ Pratip K. Chattopadhyay,⁵ Cliburn Chan,⁶ Judith Eickl,⁷ Greg Finak,⁸ Sine Reker Hadrup,⁹ Holden T. Maecker,¹⁰ Dominik Maurer,¹¹ Tim Mosmann,¹² Peng Qiu,¹³ Richard H. Scheuermann,^{14,15} Marij J.P. Welters,¹⁶ Guido Ferrari,¹⁷ Ryan R. Brinkman,^{3,20,*} and Cedrik M. Britten^{18,20,21,*}

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BioConductor's Open, Extensible Infrastructure Packages are Interoperable & Interchangeable

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Which Automated Analysis Methods to Use?

Cytometry

Journal of the
International Society
for
Advancement of Cytometry

Rapid Cell Population Identification in Flow Cytometry Data

flowPeaks: a fast unsupervised clustering for flow cytometry data via K-means and density peak finding

Yongchao Ge¹* and Stuart C Sealfon¹

¹Department of Neurology and Center of Translational System Biology, Mount Sinai School of Medicine, New York, NY, 10029, USA



Automated high-dimensional flow cytometric data analysis

Research Article

Merging Mixture Components for Cell Population Identification in Flow Cytometry

Understanding GPU Programming for Statistical Computation: Studies in Massively Parallel Massive Mixtures

Zare et al. BMC Bioinformatics 2010, 11:403
<http://www.biomedcentral.com/1471-2105/11/403>

METHODOLOGY ARTICLE

BMC
Bioinformatics

Open Access

Data reduction for spectral clustering to analyze high throughput flow cytometry data

BMC
Bioinform

Open A

METHODOLOGY ARTICLE

Misty Mountain clustering: application to fast unsupervised flow cytometry gating

Cytometry Part B (Clinical Cytometry) 78B (Suppl. 1):S69–S82 (2010)

Elucidation of Seventeen Human Peripheral Blood B-Cell Subsets and Quantification of the Tetanus Response Using a Density-Based Method for the Automated Identification of Cell Populations in Multidimensional Flow Cytometry Data

Cytometry

Journal of the
International Society for
Advancement of Cytometry

Automated Gating of Flow Cytometry Data via Robust Model-Based Clustering

Naumann et al. BMC Bioinformatics 2010, 11:44
<http://www.biomedcentral.com/1471-2105/11/44>

METHODOLOGY ARTICLE

BMC
Bioinformatics

Open Access

The curvHDR method for gating flow cytometry samples

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Flow Cytometry Standards and Analysis (FlowCAP)

FlowCAP (Critical Assessment of Automated Analysis Methods)

Community-based evaluation of flow bioinformatics tools

FlowCAP-I (2010): Matching manual gating (1st gen tools)

FlowCAP-II (2011): (Too simple) sample classification

FlowCAP-III (2012): 2nd generation automated gating tools

FlowCAP-IV (2014): (Hard) Biomarker discovery

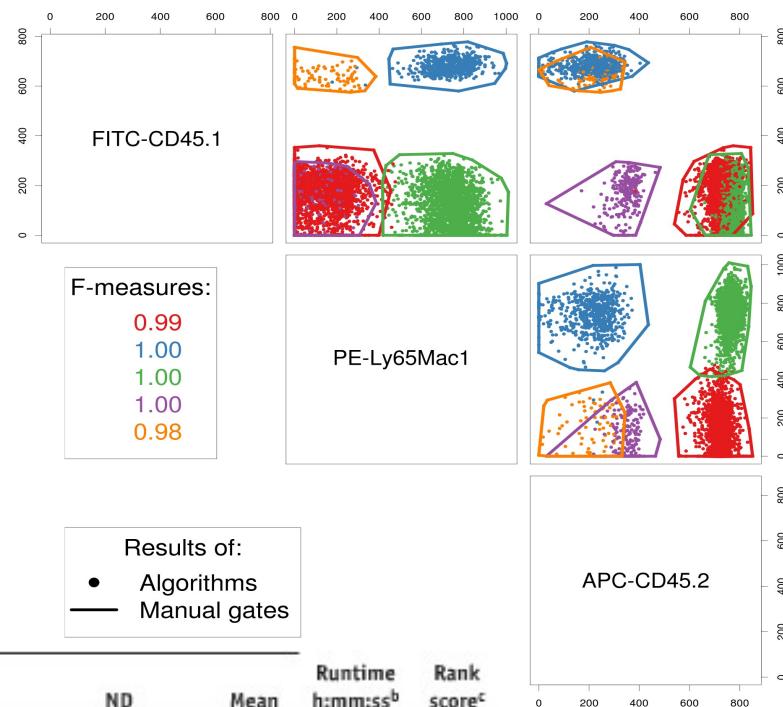
<http://flowcap.flowsite.org>

FlowCAP-I: Unsupervised gating (discovery) = humans'

Individual performance can vary on specific cell populations (might not matter)

- *Aghaeepour et al., Nature Methods (2013)
- <http://flowcap.flowsite.org>

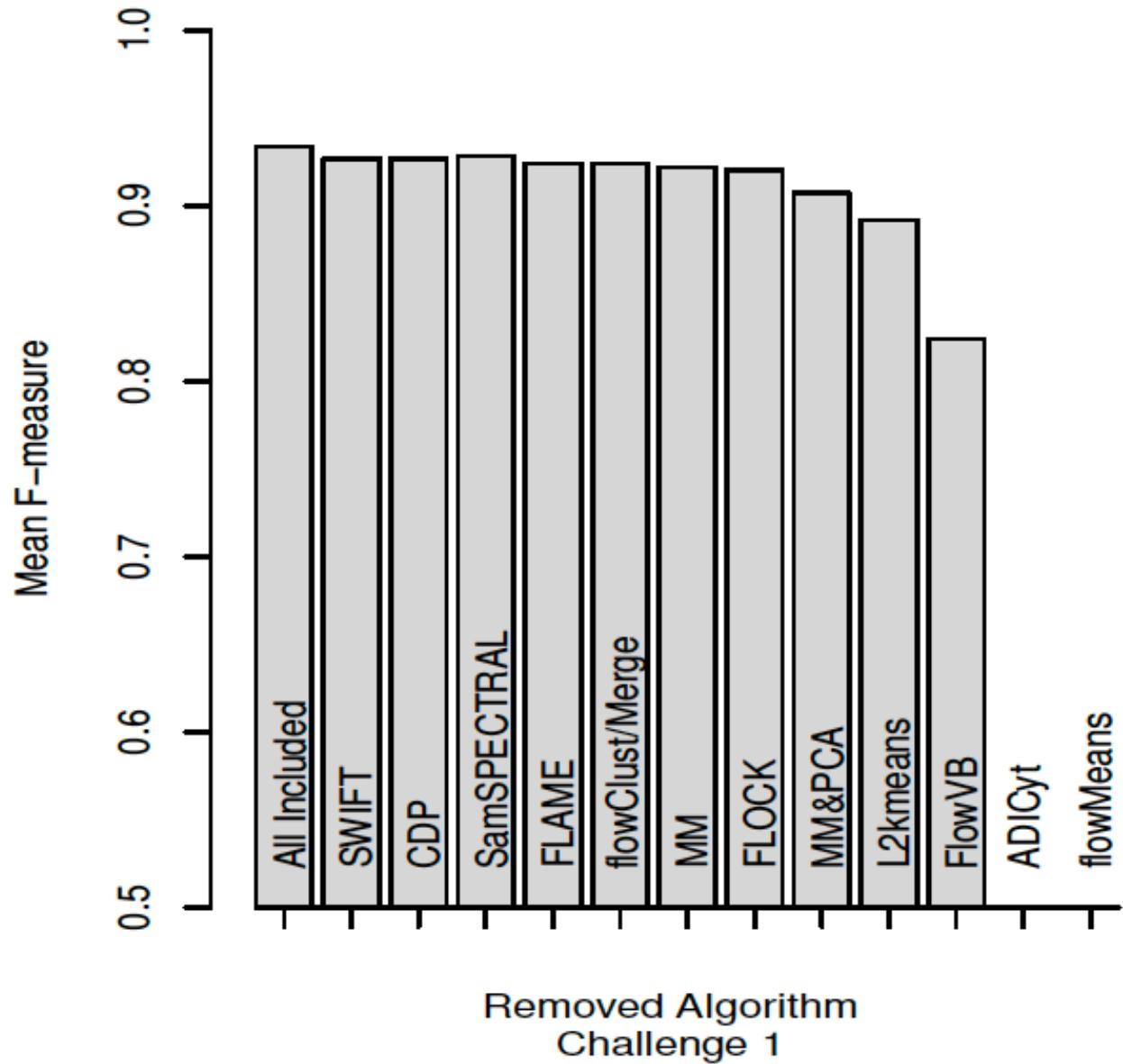
15 different tools on 5 datasets



	GvHD	DLBCL	HSCT	WNV	ND	Mean	Runtime h:mm:ss ^b	Rank score ^c
Challenge 1: completely automated								
ADICyt	0.81 (0.72, 0.88)	0.93 (0.91, 0.95)	0.93 (0.90, 0.96)	0.86 (0.84, 0.87)	0.92 (0.92, 0.93)	0.89	4:50:37	52
flowMeans	0.88 (0.82, 0.93)	0.92 (0.89, 0.95)	0.92 (0.90, 0.94)	0.88 (0.86, 0.90)	0.85 (0.76, 0.92)	0.89	0:02:18	49

There is No Single Best Gating Solution

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FlowCAP-II: Tools for Clinical Classification

Several algorithms performed perfectly

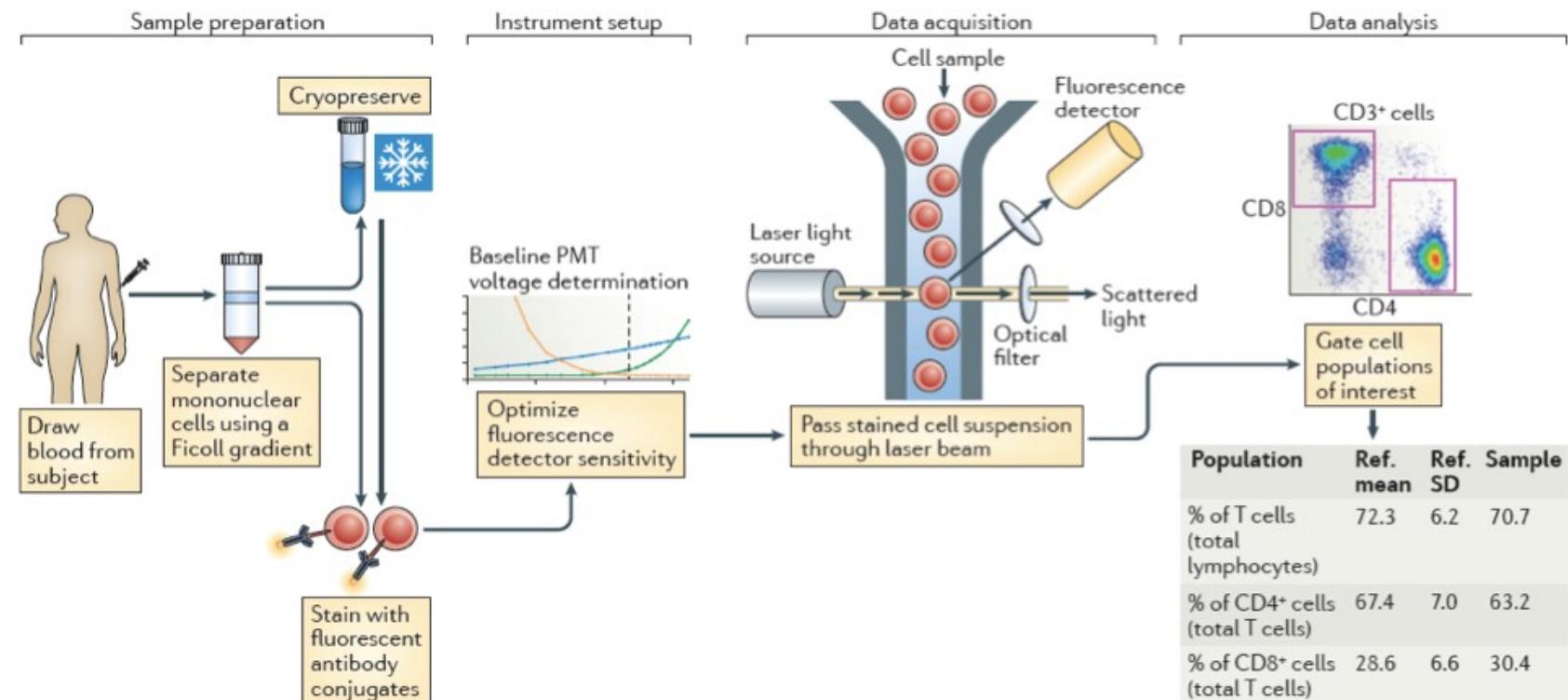
There were misclassifications

	Sensitivity	Specificity	Accuracy
flowType-FeaLect	1.00	1.00	1.00
flowPeaks	1.00	1.00	1.00
SPADE	1.00	1.00	1.00
...

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FlowCAP-III: Reducing Variability in Translational Immunology

Sample prep (BD lyoplates), Instrumentation, Acquisition, Automated Analysis



Maecker et al. *Nature Reviews Immunology*, 2012

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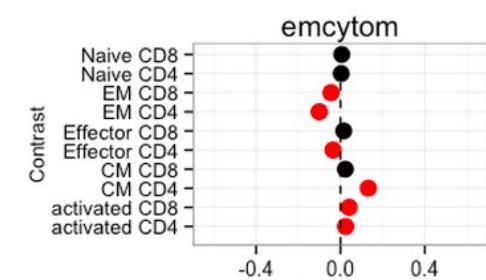
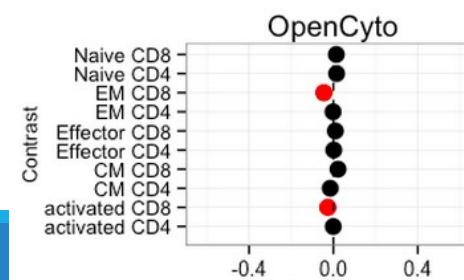
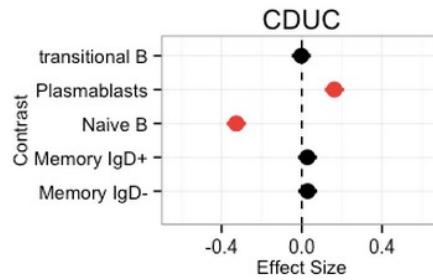
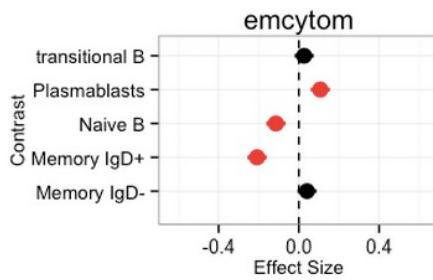
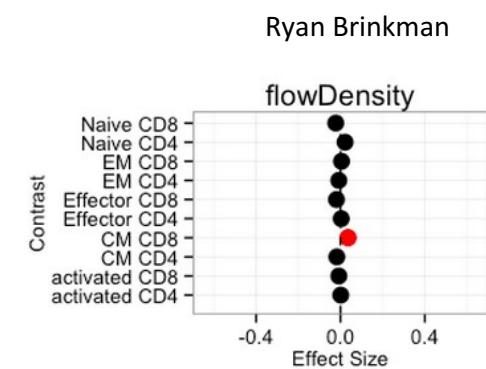
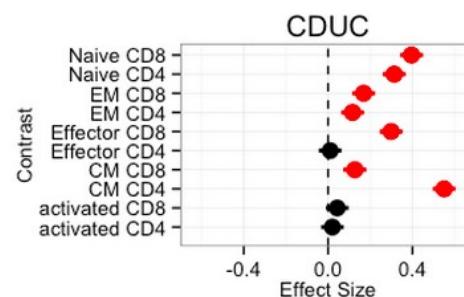
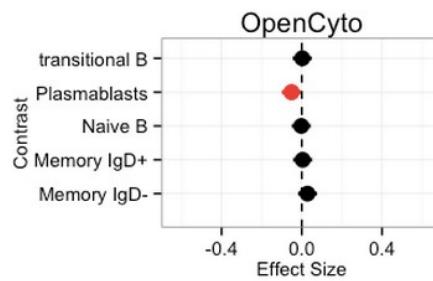
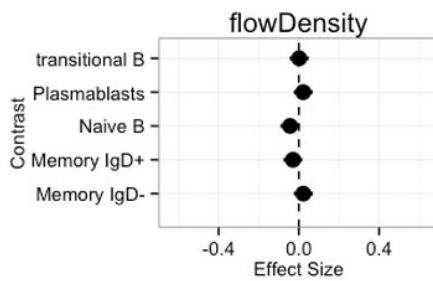
FlowCAP-III: Supervised Analysis for “Diagnostics”

2 automated tools can match human gating

9 clinical sites, 4 replicates of cryopreserved cells per site.

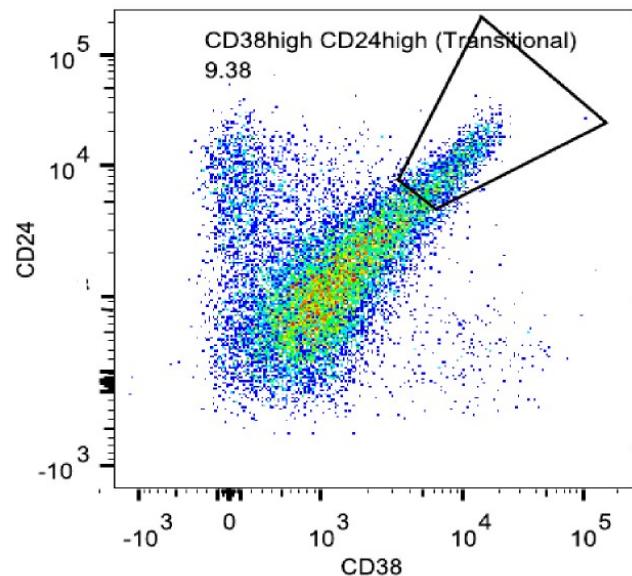
Centralized gating of data based on a consensus best approach.

Automated algorithms vs. centralized gating.

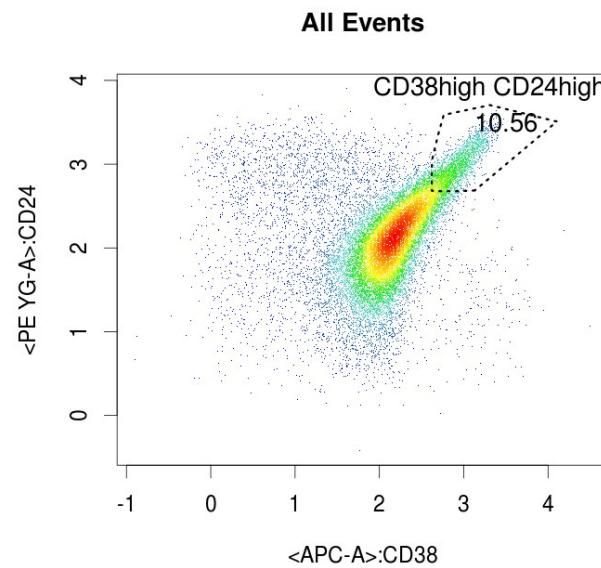


Gating Transitionals From Lymphocytes

MANUAL: 9.38



AUTO: 10.56



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FlowCAP-III Conclusion: Stop Manually Gating

Supervised gating can match manual analysis when it is data-driven

Automated gating is unbiased relative to manual gating

Variability is as low or lower than manual gating

Even when biased, the bias is associated with populations that have low cell counts and CV is lower than manual gating

Not following SOPs can result in large variability

*Finak *et al.*, *Nature Scientific Reports* (2016)

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FlowCAP-IV: Unsupervised Clustering and Classification

Biomarker discovery

388 patients (split training and test)

14 parameter data

Predict survival time (onset of HIV) & identify biomarkers

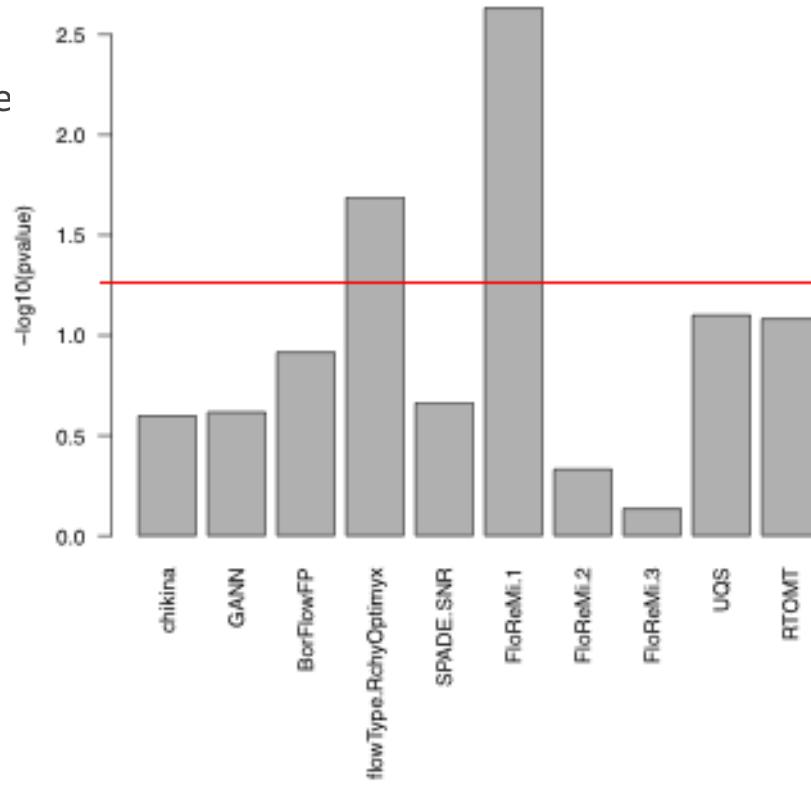
Thorough manual analysis (NIH/VRC) had failed to identify any biomarker

*Aghaeepour *et al.*, *Cytometry A* (2016)

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FlowCAP-IV: Unsupervised Clustering and Classification

Two similar methods (included flowDensity/flowType) had significant re on test data



*p-value not adjusted for testing multiple cell populations, but not algorithms

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Automated Analysis For Discovery And Diagnosis In Big Flow Cytometry Data

FLOWDENSITY: PIPELINE FOR DIAGNOSIS

- Finds what you want to find, how you want find it
- Based on density estimation techniques
- Seconds per FCS file
- Identical to the manual practice of 2D gating

FLOWTYPE / RCHYOPTIMYX: PIPELINE FOR DISCOVERY

- You split FCS files into groups
- Pipeline finds best cell populations that correlate with that split
- One graph summary of very large datasets
- Can be used as input to large multi-group studies

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What You Can Do To Get Started?



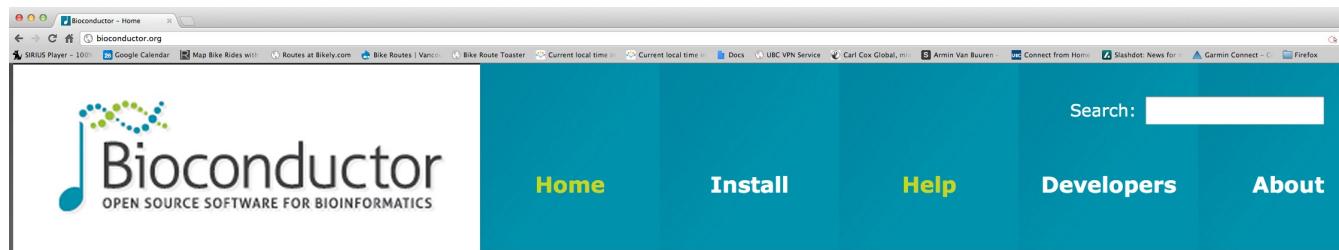
What You Can Do To Get Started

Practical Considerations for Automated Analysis

- Don't waste your time on 12 clinical samples
- Your study probably isn't sufficiently powered for unsupervised analysis
- Don't waste your time on automated discovery using 6 colors
 - Automated analysis will find everything you found by hand
- Good bioinformatics can't save bad data
- Discovery analysis is hypothesis generating
 - Finding cell populations that don't "make sense" will happen
- Its OK to ask for help (and to attend training! ☺)

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Learning R: BioConductor.org



The screenshot shows the Bioconductor.org homepage. The header features the Bioconductor logo with a stylized DNA helix icon and the text "Bioconductor OPEN SOURCE SOFTWARE FOR BIOINFORMATICS". Below the header is a navigation bar with links for "Home", "Install", "Help", "Developers", and "About". A search bar is also present. The main content area has a light gray background. On the left, there's a sidebar titled "About Bioconductor" with text about the software's purpose, its use of R, and its community resources. The main content area is titled "Use Bioconductor for..." and lists several applications:

- Microarrays**: Import Affymetrix, Illumina, Nimblegen, Agilent, and other platforms. Perform quality assessment, normalization, differential expression, clustering, classification, gene set enrichment, and other workflows.
- Variants**: Read and write VCF files. Identify structural location of variants and compute amino acid coding changes for non-synonymous variants. Use SIFT and PolyPhen database packages to predict consequence of amino acid coding changes.
- Sequence Data**: Import fasta, fastq, ELAND, MAQ, BWA, Bowtie, BAM, gff, bed, wig, and other sequence formats. Trim, transform, align and manipulate sequences. Perform quality assessment, ChIP-seq, differential expression, RNA-seq, and other workflows. Access the Sequence Read Archive.
- Annotation**: Use microarray probe, gene, pathway, gene ontology, homology and other annotations. Access GO, KEGG, NCBI, Biomart, UCSC, vendor, and other sources.
- High Throughput Assays**: Import, transform, edit, analyze and visualize flow cytometric, mass spec, HTqPCR, cell-based, and other assays.

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What next? BioC Courses & Mailing List



Analyzing flow cytometry data in Bioconductor

Instructors: Nishant Gopalakrishnan / Chao-Jen Wong

- [Brief Intro to R for Flow Packages Users](#)
- [Analyzing flow cytometry data in Bioconductor](#)
- [Flow cytometry analysis Lab](#)
- [Flow cytometry analysis Lab Solutions](#)

Install command:

```
source("http://bioconductor.org/course-packages/install-flowTrack.R")
```

Automated Gating and Metaclustering of High Content Flow Cytometry Data

Instructor: Greg Finak

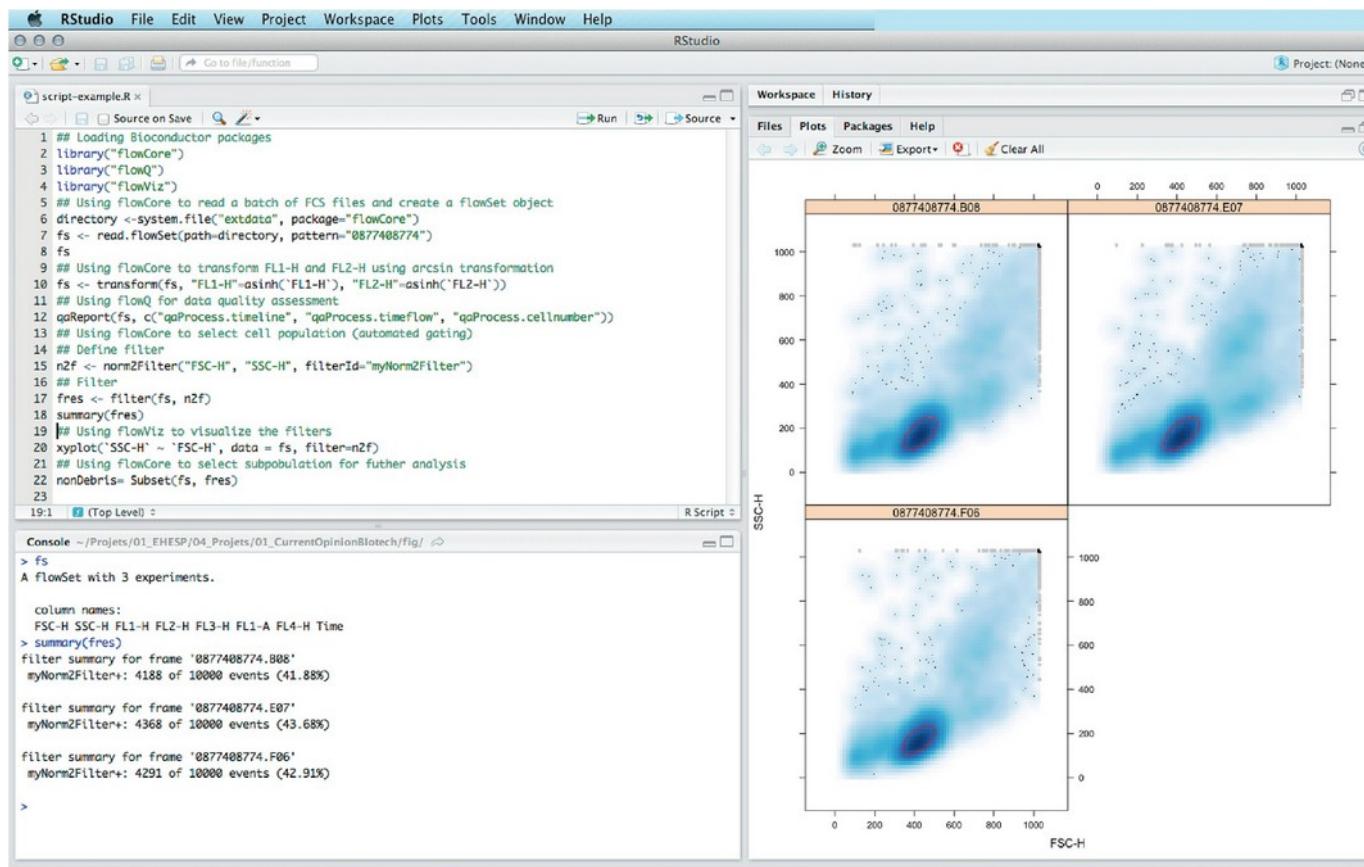
- [Automated Gating and Metaclustering for Flow Cytometry Data](#)
- [Automated Gating and Metaclustering for Flow Cytometry Data \(white paper\)](#)

Install command:

```
source("http://bioconductor.org/course-packages/install-flowTrack.R")
```

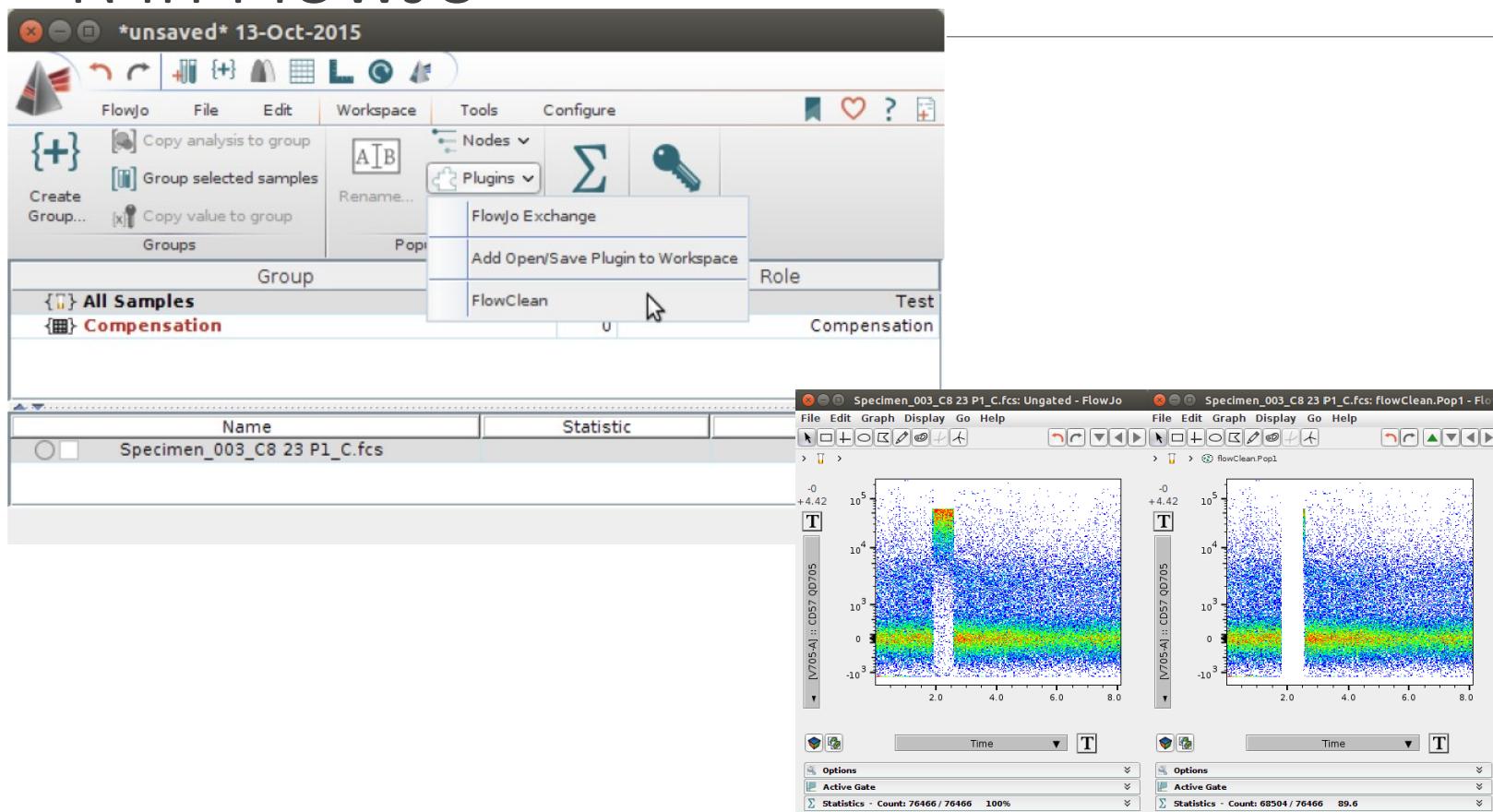
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RStudio



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R in FlowJo



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GenePattern

www.broadinstitute.org/cancer/software/genepattern/modules/flow_cytometry#Flow_Cytometry_Suite

SIRIUS Player - 100% Glotman•Simpson Come Ride With Us! Google Calendar Offliberty - evidence Live Sets: Fatboy Slim Map Bike Rides with

Gene Pattern
BROAD INSTITUTE

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Gene Expression Analysis
Proteomics
SNP Analysis
Data Format Conversion
RNA-seq Analysis
Flow Cytometry
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Cancer Program
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Back to top

GenePattern Flow Cytometry Suite

Quality Assessment

Flow cytometry [Quality Assessment](#) suite includes modules implementing various approaches to automatically assess the quality of flow cytometry data. Such an assessment represents an important part of any data analysis, and quality control tests should be included at the beginning of data analysis and often at other steps of an analytical pipeline to identify differences in samples originating from changes in conditions that are probably not biologically motivated. Generally, these methods establish a quality control criterion to give special consideration to abnormal samples or even exclude these from further analysis.

Data Preprocessing

Flow cytometry [Data Preprocessing](#) suite includes various utilities for [FCS](#) data preview and transformations, conversion between spreadsheets (i.e., CSV files) FCS files, merging and sub-sampling data, editing keywords in FCS files and other support tools.

Gating and Clustering

Flow cytometry [Gating and Clustering](#) suite includes modules for the application of manually created gates (saved in Gating-ML) on FCS data files and various clustering algorithms developed for the use with flow cytometry data.

Back to top

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Genepattern - Example

GenePattern - SamSPECTRALCluster - Mozilla Firefox

File Edit View History Bookmarks Tools Help

My Settings | Sign out jspidlen@bccr.ca

GenePattern

Modules & Pipelines Suites Job Results Resources Downloads Administration Help

SamSPECTRALCluster version 3

* required field

Show parameter descriptions

Run Reset properties | export | edit | help

Input FCS data file*: Browse... Specify File Path or URL Upload File Upload Multiple Files

The FCS file to be clustered.

Dimensions

A comma-separated list of dimensions (parameters/channels) that shall be used for clustering. Use either parameter names or indexes. All dimensions except for Time will be used if this parameter is not provided.

Transformation*: ASinh (Hyperbolic Arcus Sine), default

Which transformation to apply on the data prior clustering. Fluorescence channels are usually better visualized and clustered using a transformation: the ASinh transformation produces good results on most data.

Dimensions to transform

A comma-separated list of dimensions (parameters/channels) that shall be transformed as specified by previous parameter. This will be ignored if no transformation is specified above. If this parameter is not provided and transformation is specified above, the algorithm will use heuristics to identify parameters that shall be transformed. These heuristics are based on how parameters are stored in the FCS file, their resolution and their name. Again, you can use either parameter names or parameter indexes to specify dimensions to transform.

Sigma*: 250

A scaling parameter that determines the resolution in the spectral clustering stage. By increasing it, more spectral clusters are identified. This can be useful when small population are aimed. See documentation on more details how Sigma can be adjusted.

Separation factor*: 0.8

Threshold that controls to what extend clusters should be combined or kept separate. See documentation on more details how the separation factor can be adjusted.

Beta*: 4, default

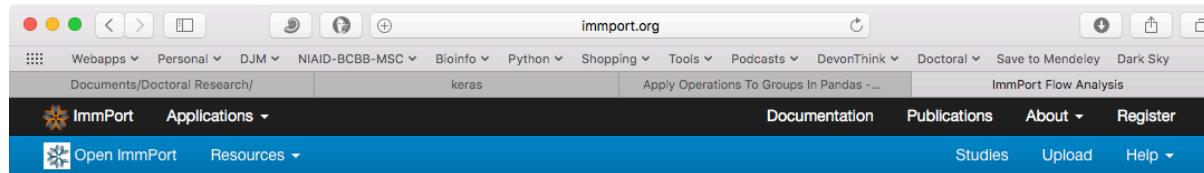
The size of neighborhood that is being searched during the conductance calculation step. The default value of 4 should not be changed unless the input data set contains measurements of more than 100,000. Setting beta to zero or a low value will reduce computation time.

Recent Jobs

- GBTDonorQuantification (1 849)Apr 19 02:09:47 PM GMBTDonorQuantification_execution_log.html
- GMBTPipelineManager workflow.csv
- gp_execution_log.txt
- GMBTAnnotateFlowset fs.annotated.RData
- gp_execution_log.txt
- GMBTPreprocessFlowset fs.preprocessed.RData
- gp_execution_log.txt
- GMBTExtractViableFlowset gateViable.images.csv
- fs.viable.RData
- stdout.txt
- gp_execution_log.txt
- GMBTEvaluateViableFlowset resAll.data.csv
- fs.donor.RData
- class.images.csv
- Results.csv
- stdout.txt
- gp_execution_log.txt
- GMBTPerformQA QA_Aggregator_Results.csv
- report.html
- Rplots.pdf
- stdout.txt
- gp_execution_log.txt
- SuggestNumberOfPopulations (1 848)Apr 13 05:03:27 PM ICLScorePlot.png
- suggestedNumberofPopulations.txt
- BICScorePlot.png
- stdout.txt
- gp_execution_log.txt
- StageFiles (1 847)Apr 13 05:02:18 PM staged_list.txt
- gp_execution_log.txt
- SetFCSKeywords (1 846)Apr 13 05:02:17 PM

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ImmPort - FLOCK



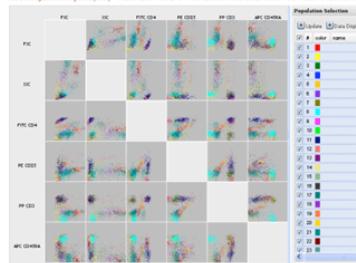
Flow Analysis ImmPort

Flow Cytometry Analysis Workflow

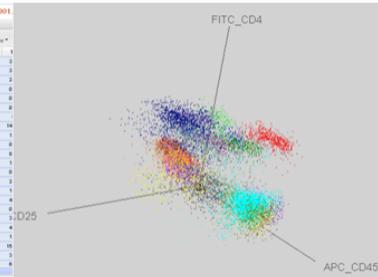


Two Dimensional Visualization

Result Adjustment System(RAS) - Task Name: v0-0-1312 base-rash-after-rash File Name: 04233281.001



Three Dimensional Visualization



The links below will direct you to the ImmPort Flow Cytometry Analysis tools which require a login to keep your data private.

ImmPort's flow cytometry analysis component includes:

- Data Management for Single File Upload, Multiple File Upload and Dataset Generation [+](#)
- Automated population identification using the FLOCK algorithm for individual sample or dataset [+](#)
- Automated mapping populations across sample for Cross-Sample Comparison [+](#)
- Result visualization and statistical analysis of population characteristics [+](#)

FLOCK Algorithm Overview

The FLOCK algorithm consists of four core steps: hyper-grid creation, identifying dense hyper-regions, merging neighboring dense hyper-regions, clustering based on centroids derived from the



Resources

Intro to Flow Cytometry

- <http://www.abcam.com/protocols/introduction-to-flow-cytometry>
- <https://www.bio-rad-antibodies.com/introduction-to-flow-cytometry.html>

Flow Cytometry GitHub Repository / Videos

- https://github.com/bioinformatics-ca/other_workshops/tree/master/flow_cytometry_2013
- http://bioinformatics-ca.github.io/flow_cytometry_2013/

R Programming

- <http://www.cyclismo.org/tutorial/R/>
- <https://cran.r-project.org/doc/contrib/Torfs+Brauer-Short-R-Intro.pdf>

Repository

- <https://flowrepository.org/>

Unix

- <http://www.ee.surrey.ac.uk/Teaching/Unix/>
- <http://www.rain.org/%7Emkummel/unix.html>

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