TDS Facility

Analysis of flow cytometry data with R
Training for life scientists

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Translational Data Science – Facility

SIB Swiss Institute of Bioinformatics

Outline & Schedule

Day 3

01 **Importing data from Excel and arranging plots in a grid**

02 **Differential state analysis using a paired design**

03 **Normalization / batch correction**

04 **Working with gated data in R (Basics and Manual gating)**

Outline & Schedule

Day 4

04 **Working with gated data in R (Automated Gating)**

05 **Generate HTML or PDF reports**

Examples and exercises are integrated in the chapters

01

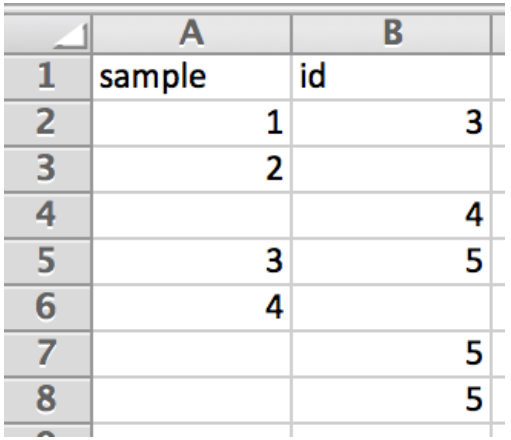
A. Importing data from Excel
B. Arranging plots in grids

Importing data from an Excel file

Package xlsx

https://cran.r-project.org/web/packages/xlsx/index.html

```
> data <- read.xlsx2(file = "excelfile.xlsx",
                     sheetName = "Sheet1")
```



	A	B
1	sample	id
2	1	3
3	2	
4		4
5	3	5
6	4	
7		5
8		5

The function r*ead.xlsx2* considers the empty cells as "" character.

```
> str(data)
'data.frame':   7 obs. of  2 variables:
 $ sample: chr  "1" "2" "" "3" ...
 $ id    : chr  "3" "" "4" "5" ...
```

The function *read.xlsx* considers the empty cells as NA

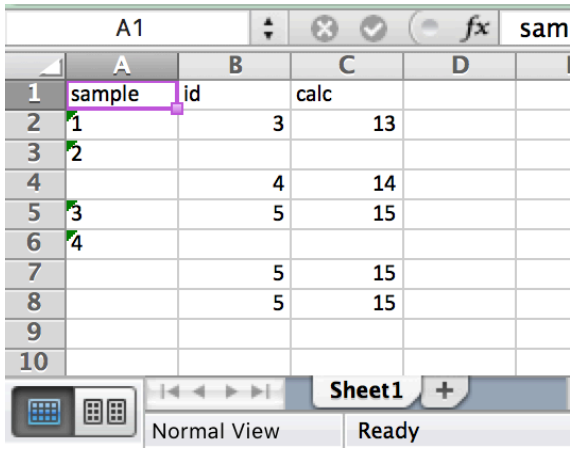
```
> str(data)
'data.frame':   7 obs. of  2 variables:
 $ sample: num  1 2 NA 3 4 NA NA
 $ id    : num  3 NA 4 5 NA 5 5
```

Exporting data to an Excel file

Advantage: no risk of gene name conversion to date like with csv files

Package *xlsx* can be used to export a data.frame after manipulating it within R:

```
> data$id<-as.numeric(data$id)
> data$calc<-data$id+10
> write.xlsx2(x=data,
                  file = "excelfile2.xlsx",
                  row.names = FALSE,
                 SheetName = "Sheet1")
```



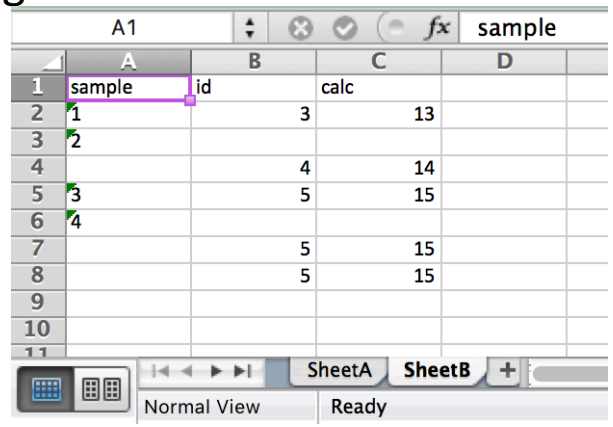
Another package: *openxlsx*

https://cran.r-project.org/web/packages/openxlsx/index.html

Allows to generate several separate sheets providing a named list of data.frame objects:

```
> openxlsx::write.xlsx(x = list(SheetA=data[ , c(1,2)],
                               SheetB=data),
                     file = "excelfile3.xlsx")
```

Doesn't export rownames by default.



Arranging ggplot2 plots in a grid

Can be useful to compare distribution of values before and after transformation, or to compare a UMAP with cells colored according to different markers.

Package *cowplot*

https://cran.r-project.org/web/packages/cowplot/index.html

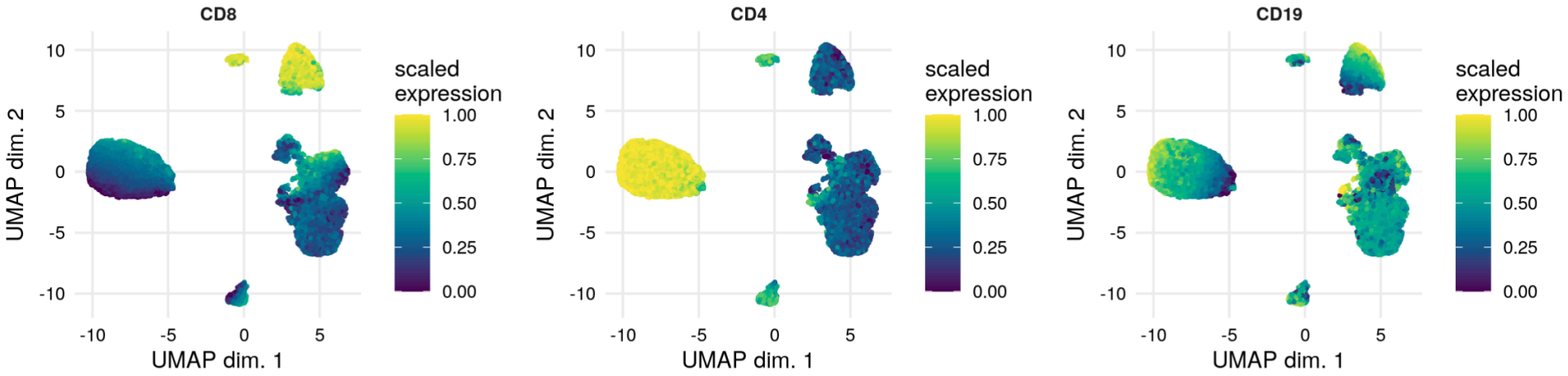
First save each ggplot2 plot to an object:

```
> p1 <- plotDR(sce,  dr =  "UMAP",
        assay = "exprs",  color_by="CD8" )
> p2 <- plotDR(sce,   dr =  "UMAP",
        assay = "exprs",   color_by="CD4")
> p3<-plotDR(sce,   dr =  "UMAP",
        assay = "exprs",   color_by="CD19")
```

Arranging ggplot2 plots in a grid

Use the *plot_grid* function to arrange them in a grid, e.g. all on the same row:

```
> plot_grid(p1, p2, p3, nrow = 1)
```



02

Differential state analysis with a paired design

Differential state (DS) analysis with paired design

Differential expression of cell state markers within clusters

```
> res_DS <- diffcyt(sce_PBMC,
                    clustering_to_use = ”final_annotation",
                    analysis_type = "DS",
                    method_DS = "diffcyt-DS-limma",
                    design = design,
                    contrast = contrast,
                    block_id = patient_id)
```

Methods for DS: uses the limma package

Vector of patient IDs

How to create a vector of patient IDs from the fcs file names?

```
> ei(sce)$sample_id
 # [1] 0BF51C_0.fcs  0BF51C_14.fcs 0BF51C_7.fcs  0E1F8E_0.fcs  0E1F8E_14.fcs
 # [6] 0E1F8E_7.fcs  180E1A_0.fcs  180E1A_14.fcs 180E1A_7.fcs  1A9B20_0.fcs
# [11] 1A9B20_14.fcs 1A9B20_7.fcs  61BBAD_0.fcs  61BBAD_14.fcs 61BBAD_7.fcs
```

Using gsub() :
This function replaces all characters having a same pattern with other characters.
We can replace the *_0.fcs*, *_14.fcs* and *_7.fcs* extensions by an empty character:

Create a vector of patient IDs for block design:

```
> patient_id <- ei(sce)$sample_id
```

Use gsub to replace the extensions for the matching elements within the vector:

```
> patient_id <- gsub("_0.fcs", "", patient_id)
> patient_id <- gsub("_14.fcs", "", patient_id)
> patient_id <- gsub("_7.fcs", "", patient_id)
```

```
> patient_id
[1] "0BF51C" "0BF51C" "0BF51C" "0E1F8E" "0E1F8E" "0E1F8E" "180E1A" "180E1A"
 [9] "180E1A" "1A9B20" "1A9B20" "1A9B20" "61BBAD" "61BBAD" "61BBAD"
```

Differential state analysis

Extract results table - same method as for unpaired design:

```
> tbl_DS <- rowData(res_DS$res)
> tbl_DS
```

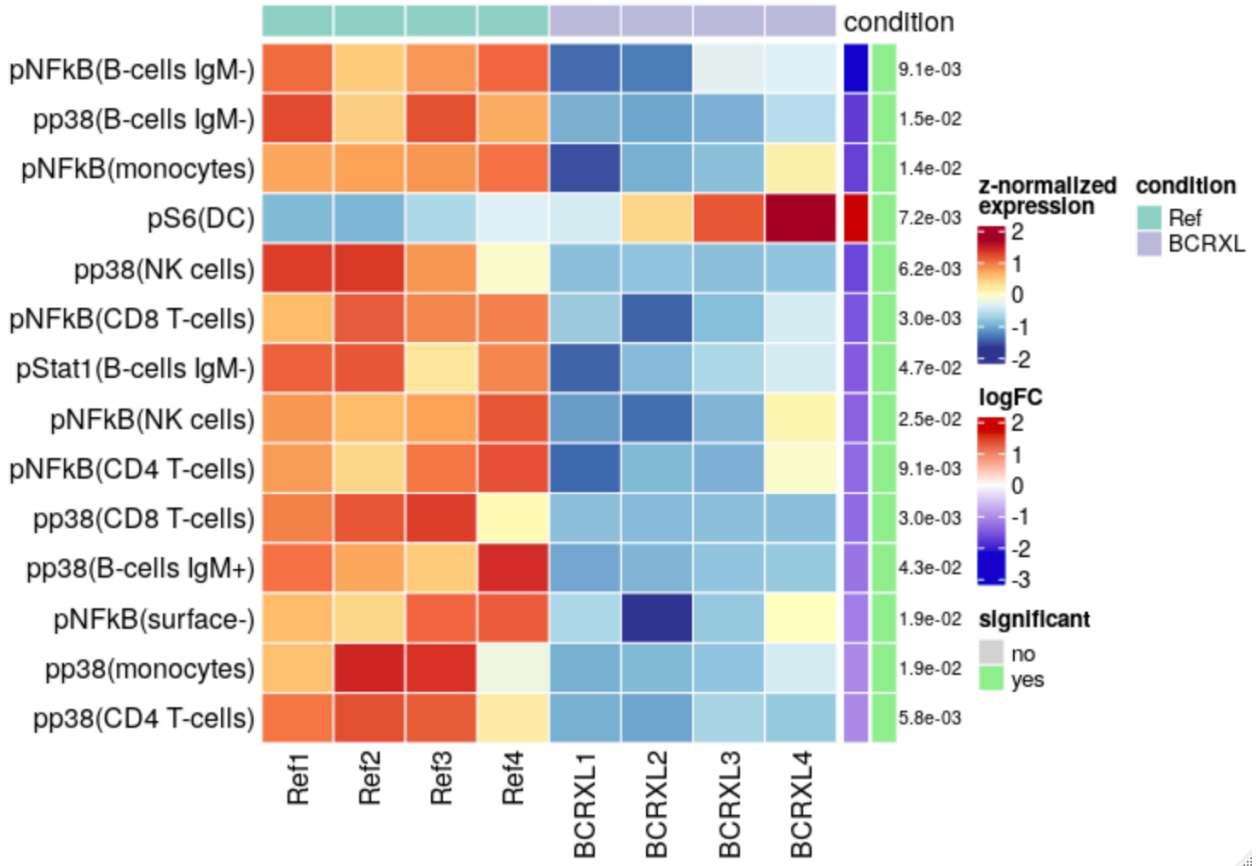
```
DataFrame with 20 rows and 4 columns
             cluster_id marker_id       p_val     p_adj
               <factor>  <factor>   <numeric> <numeric>
Other       Other          CD38   0.000883343  0.128085
Other       Other          TCR gd 0.006609759  0.479208
CD4 T cells CD4 T cells    CD141  0.027918113  0.481051
CD8 T cells CD8 T cells    CCR5   0.026749683  0.481051
Monocytes   Monocytes      CCR5   0.027850828  0.481051
...                  ...       ...         ...       ...
CD4 T cells CD4 T cells     CD16    0.0703599  0.534035
CD8 T cells CD8 T cells     CD16    0.0629339  0.534035
Other       Other           CD16    0.0688253  0.534035
CD8 T cells CD8 T cells     IgD     0.0704875  0.534035
Other       Other           CD161   0.0684019  0.534035
```

Differential state analysis

Plot results for all markers

Sorts results by absolute value of logFoldChange

```
> plotDiffHeatmap(sce_PBMC, tbl_DS, all=T , sort_by = "lfc", col_anno ="condition")
```



Let’s practice – 7 bis

In this exercise we will test if markers were differentially expressed between two time points (D14 compared to D0), **using a paired design**

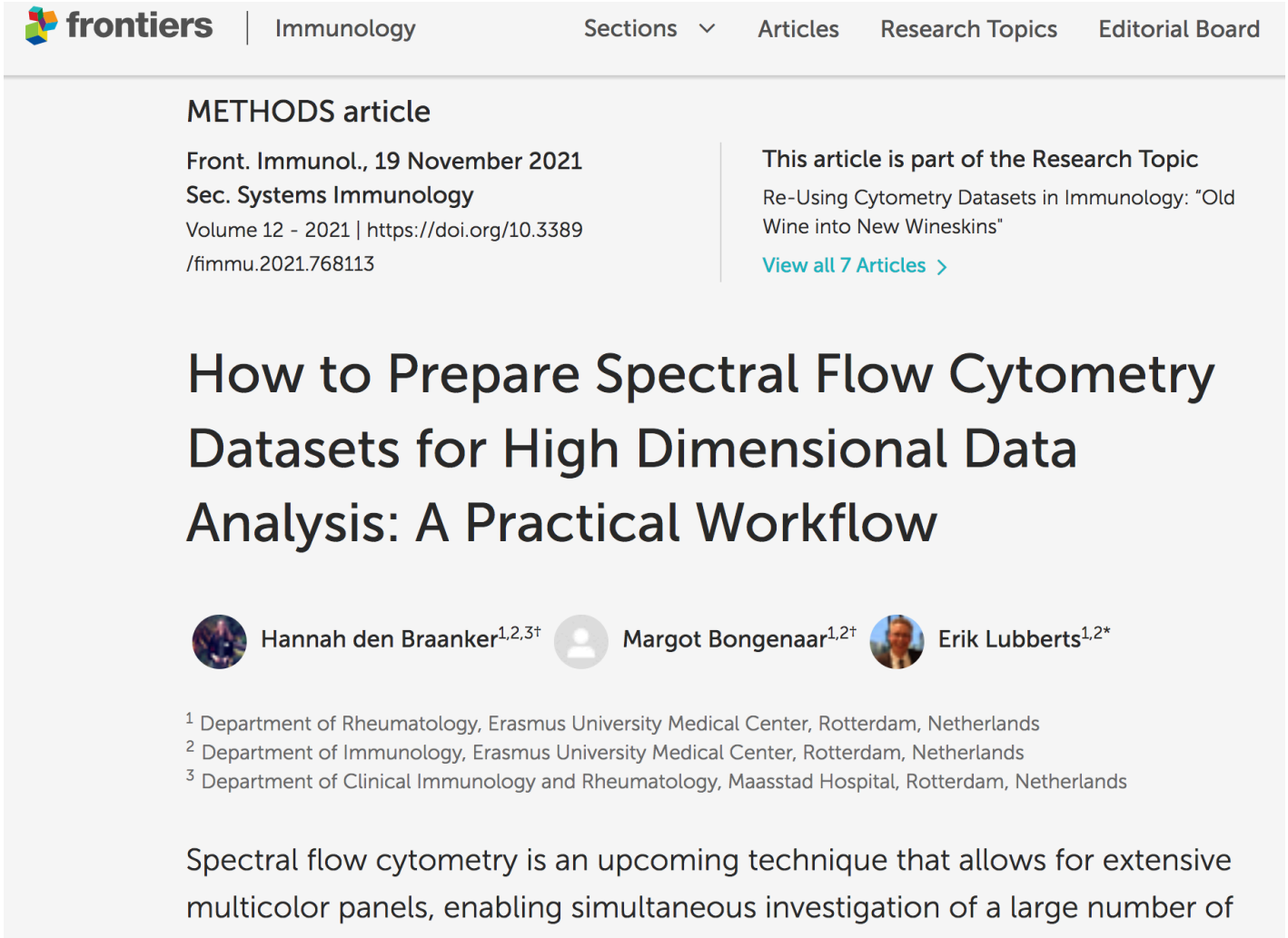
Create a new script in which you will

1) Load the sce object from exercise number 6 ("sce_annotated.RData").
2) Create a vector of patient IDs from the fcs file names using gsub()
3) Set up the design and contrast matrices.
4) Test for differences in marker expression between D14 and D0, including argument block_id
5) View table of results

03 Normalization / batch correction

Spectral flow cytometry analysis workflow

- Workflow based on



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METHODS article

Front. Immunol., 19 November 2021
Sec. Systems Immunology
Volume 12 - 2021 | https://doi.org/10.3389/fimmu.2021.768113

This article is part of the Research Topic
Re-Using Cytometry Datasets in Immunology: "Old Wine into New Wineskins"
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How to Prepare Spectral Flow Cytometry Datasets for High Dimensional Data Analysis: A Practical Workflow

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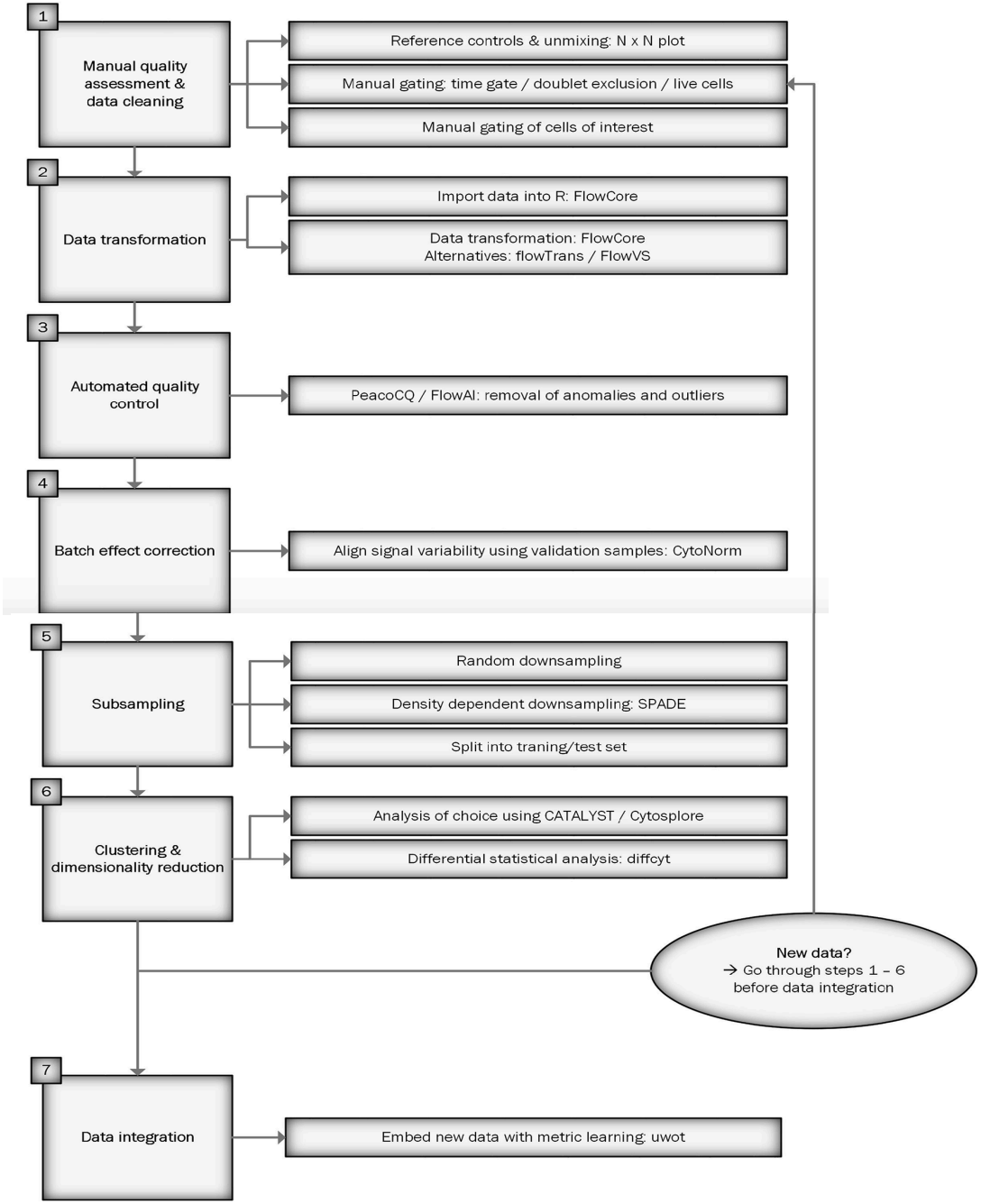
Spectral flow cytometry is an upcoming technique that allows for extensive multicolor panels, enabling simultaneous investigation of a large number of

Provide R code to perform the proposed workflow

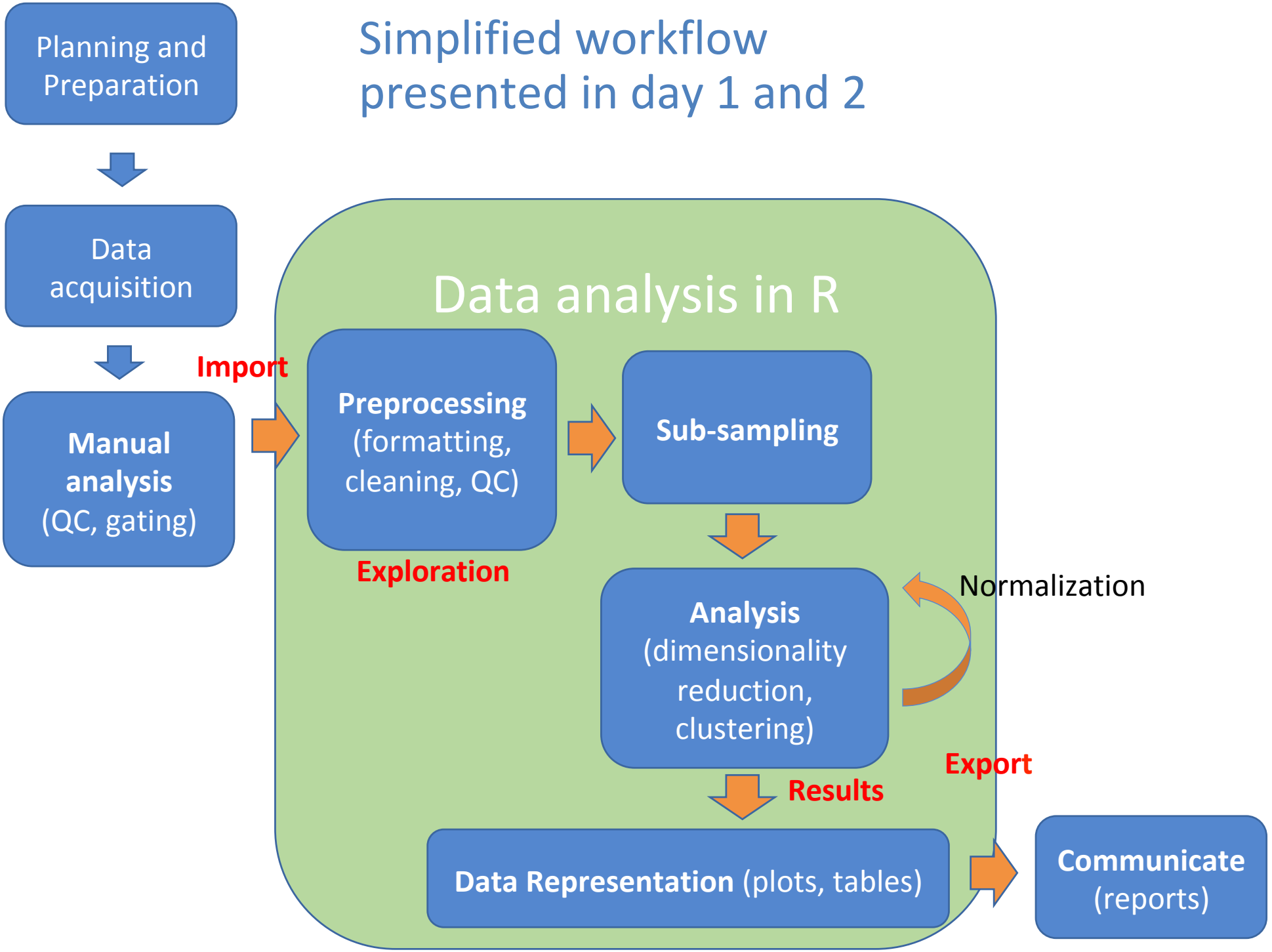
https://doi.org/10.3389/fimmu.2021.768113

Suggested workflow: Figure 1, den Braanker et al



https://doi.org/10.3389/fimmu.2021.768113

Simplified workflow presented in day 1 and 2



CytoNorm

Available on github: https://github.com/saeyslab/CytoNorm
van Gassen et al, 2020 https://onlinelibrary.wiley.com/doi/epdf/10.1002/cyto.a.23904

Install using devtools package:

```
> library(devtools)
> install_github('saeyslab/CytoNorm')
```

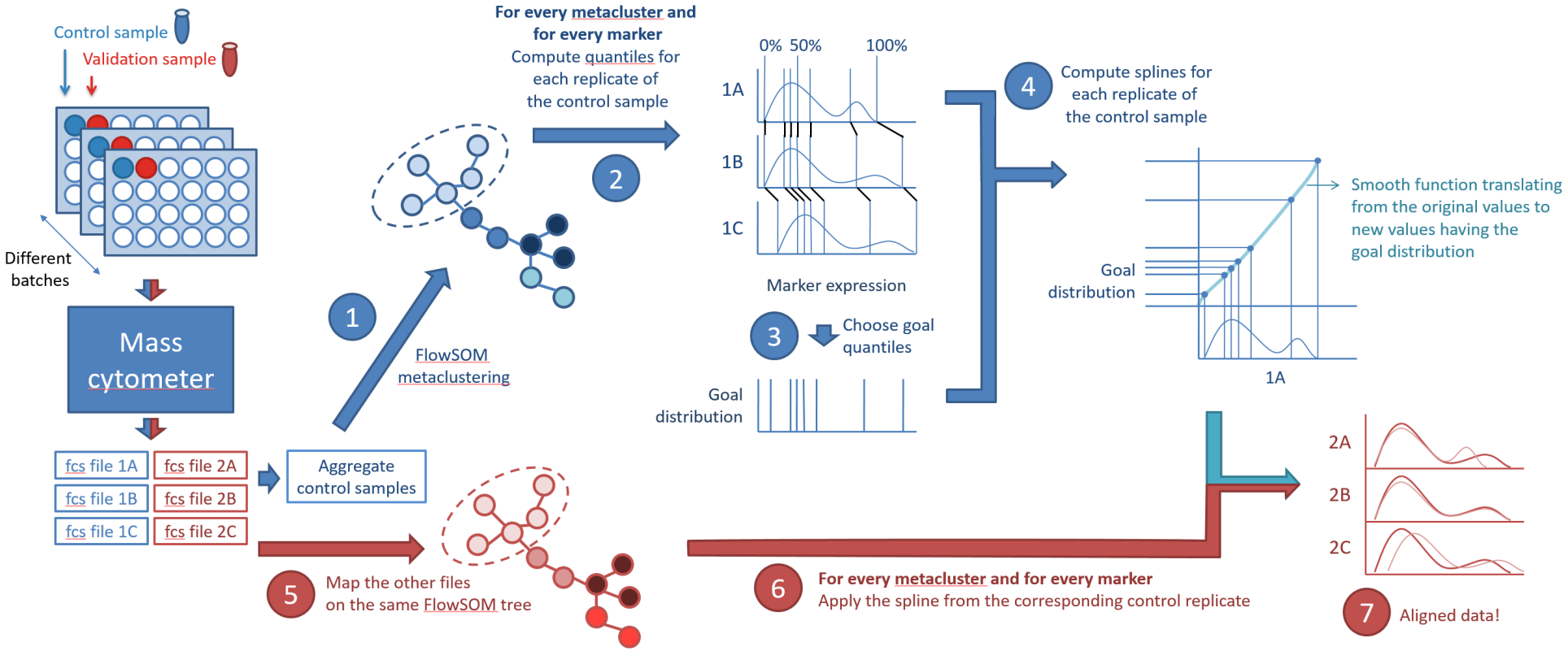
Why do we need to normalize?

Shifts that occur because of batch can have effects on DR for example.

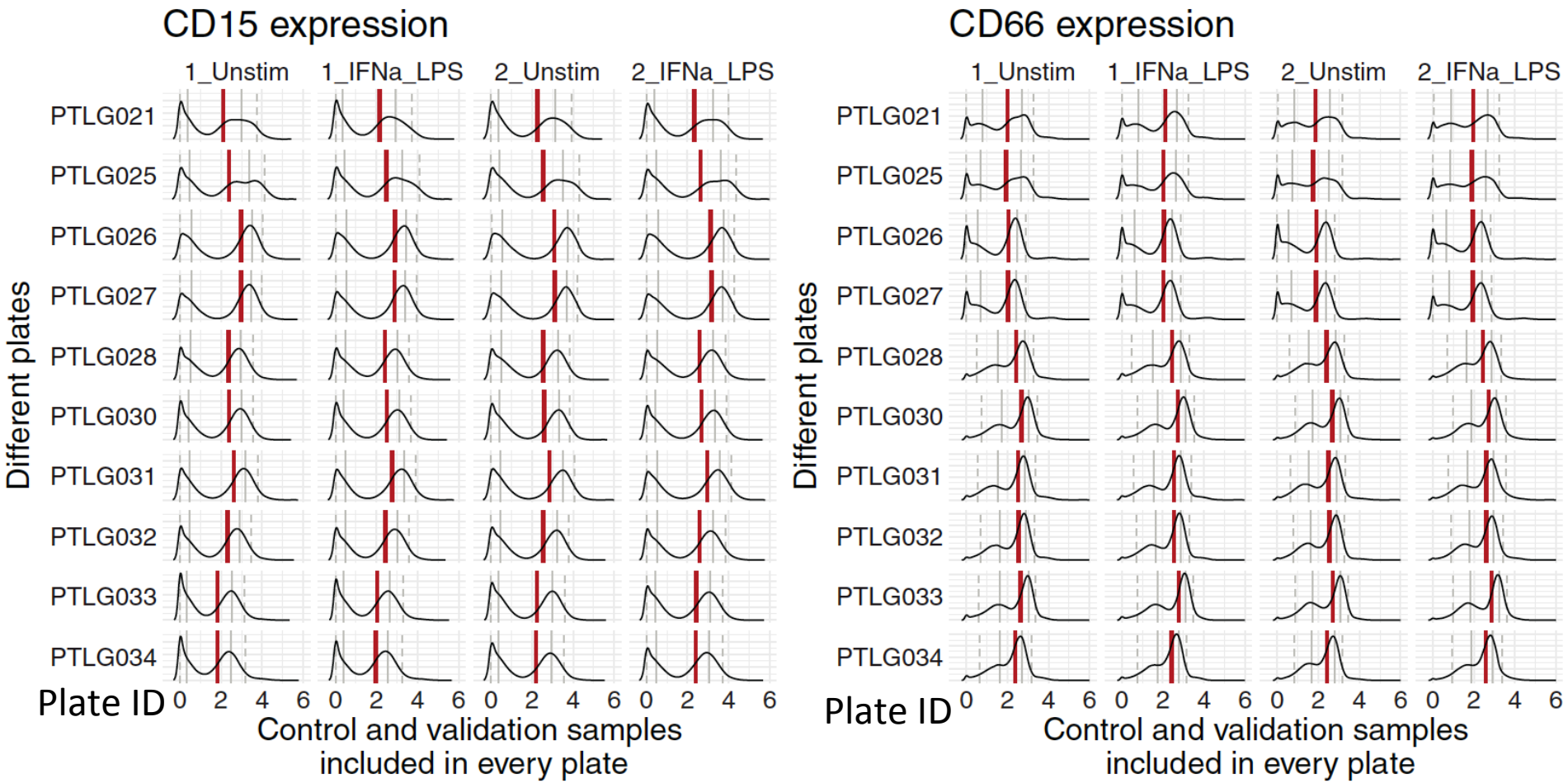
Computational methods can align the distribution of markers across samples.

Normalization methods without a reference sample can hide biologically relevant differences in cell subsets. Different cell types are impacted differently by batch effects (Finak et al, 2014, Cytom. Part A)

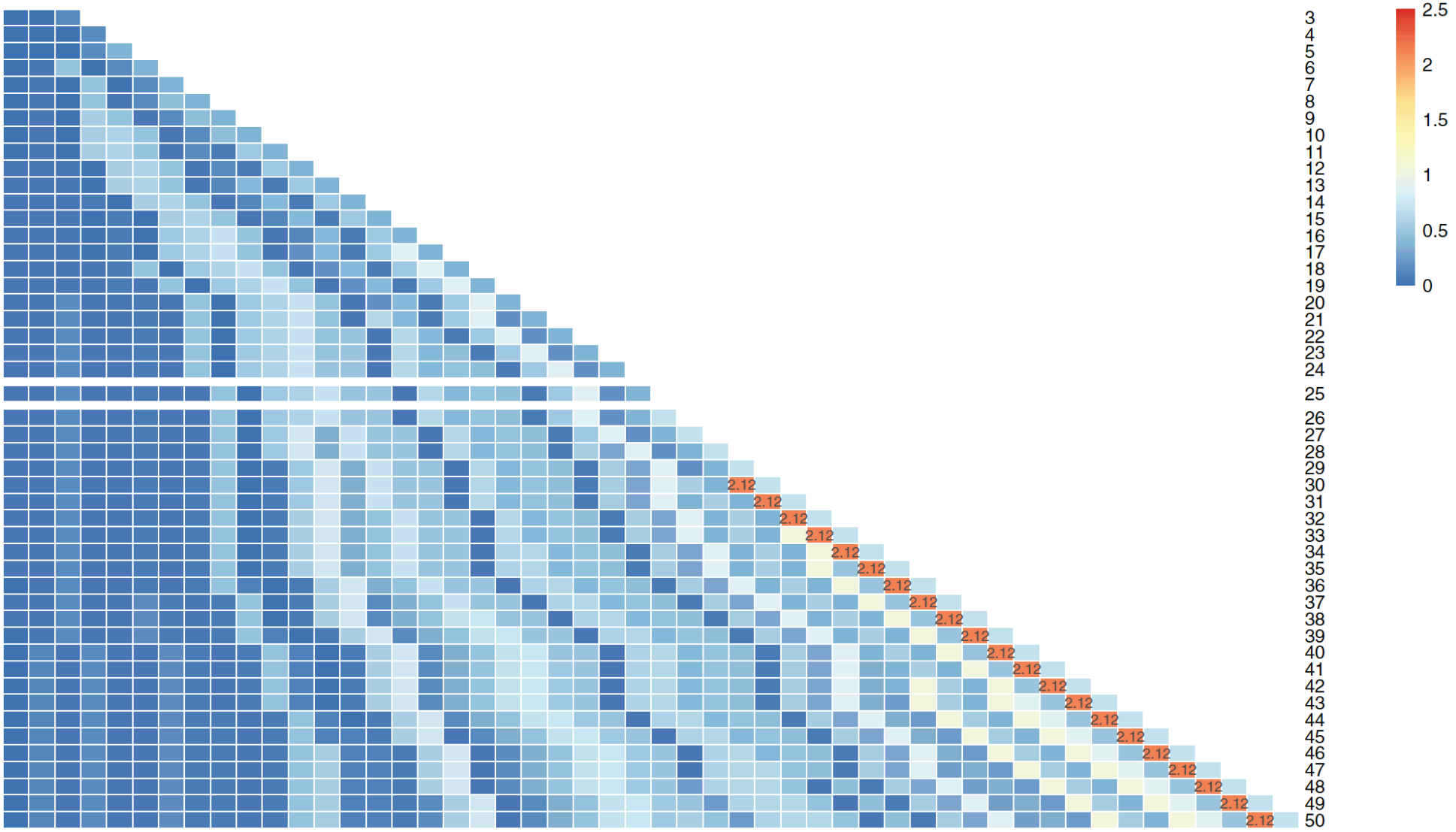
CytoNorm – algorithm overview



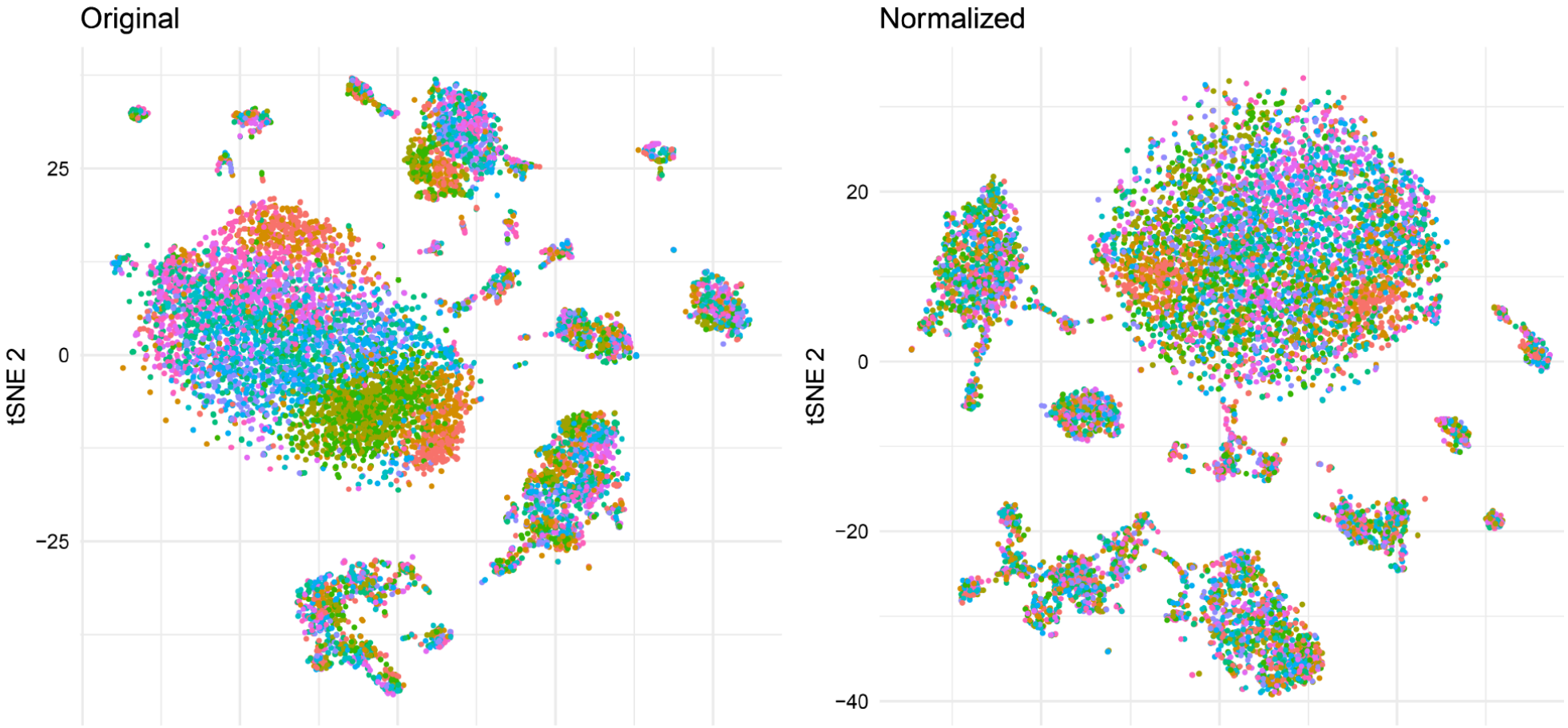
An example of shifts across plates



Assumption: clusters are not affected by batch



Normalization enhances DR plot



1,000 randomly sampled cells for each of 10 samples

Functions of CytoNorm

Functions use flowSet objects containing transformed data (eg arcsinh with fixed cofactor).

Generate pre-clustering with flowSOM:

```
> fsom <- prepareFlowSOM(train_files,
                         colsToUse = markerstotransf,
                         transformList = NULL,
                         FlowSOM.params = list(xdim=10,
                                               ydim=10,
                                               nClus=20, scale=FALSE))
```

flowSet with technical replicates of a single sample

Markers to use for clustering (eg. "type" marker_class)

FlowSOM parameters: number of grids and of metaclusters

Test for coefficient of variation within clusters:

```
> cvs <- CytoNorm::testCV(fsom, cluster_values = c(5,10,15,20), plot=TRUE)
> range(cvs$cvs$`20`) # 0.05758965 1.43114512
```

If the clusters are impacted by batch effects, CV values of >1.5 - 2 will occur, then you can choose to put FlowSOM.params to NULL and skip clustering.

Functions of CytoNorm

Train the model, i.e. evaluate quantiles from technical replicates:

```
model <- CytoNorm.train(files = train_files,
                        labels = labels_train,
                        channels = markerstotransf,
                        transformList = NULL,
                        FlowSOM.params = list(nCells = 6000,
                                              xdim = 10,
                                              ydim = 10,
                                              nClus = 5,
                                              scale = FALSE),
                        normMethod.train = QuantileNorm.train,
                        normParams = list(nQ = 101,
                                          goal = "mean"),
                        seed = 1,
                        verbose = TRUE)
```

flowSet with technical replicates of a single sample

Vector of batch ID labels for each technical replicate within the train flowSet

Markers to use for clustering (eg. "type" marker_class)

FlowSOM parameters: number of grids and of metaclusters

Compute quantiles to describe the distribution of the data, and infer spline functions to equalize these distributions over the files.

Functions of CytoNorm

Normalize the rest of the samples:

```
> CytoNorm.normalize(model = model,
                     files = validation_files,
                     labels = label_norm,
                     transformList = NULL,
                     transformList.reverse = NULL,
                     normMethod.normalize = QuantileNorm.normalize,
                     outputDir = "course_datasets/FR_FCM_Z4KT/Normalized",
                     prefix = "Norm_",
                     clean = TRUE,
                     verbose = TRUE)
```

Model with quantiles obtained using CytoNorm.train()

flowSet with the rest of the samples

Vector of batch ID labels for each sample of the samples to be normalized

Compute quantiles and infer spline functions to equalize these distributions over the files.

Output folder where new fcs files will be created with prefix "Norm_

Before the exercise: the grep() function

It allows to search for a pattern of characters within a vector:

```
> grep("pattern", myvector)
```

Will return numbers of elements within the vector that correspond to that pattern:

```
> myvector <- c("abc", "xyz", "abcd", "abxyz", "cdy")
> grep("abc", myvector)
# [1] 1 3
```

Use it on flowSet sampleNames to split a flowSet based on sample ID:

```
> train_files <- fcs_transform[grep("REU271", sampleNames(fcs_transform))]

> validation_files <- fcs_transform[-c(grep("REU271", sampleNames(fcs_transform)))]
```

Let's practice – 8

In this exercise we will perform normalization (i.e. batch correction) for fcs files provided in accession **FR_FCM_Z4KT** of the FlowRepository.

Create a new script in which you will:

1) Create a flowSet called fcs_data of all samples within the */course_dataset/FR_FCM_Z4KT* folder
2) Generate a panel data.frame using colnames(fcs_data) antigen names extracted with pData(parameters(fcs_data[[1]]))$desc. Create a new column called marker_class that will contain the type of markers: all that are not NA should be labeled as "type", except PD-1 which should be labeled as "state". Make sure that the antigen "Zombie UV" is labeled as "none" and not as "type". Save the panel to an Excel file using write.xlsx2().
3) Transform the data: extract a vector from the panel data.frame which are the channels to be transformed, which are not labeled with "none". Perform asinh transformation with a cofactor of 3000 for all channels to be transformed, using transFlowVS() from the flowVS package.
4) Split the flowSet resulting from transformation into a *training* flowSet containing all flowFrames from the sample "REU271", and a flowSet with the rest of the flowFrames not corresponding to sample "REU271". Use the grep() function on the sampleNames of the flowSet.

Let’s practice – 8 - continued

5) Perform pre-clustering with flowSOM with function prepareFlowSOM(), providing the flowSet with the training flowFrames, the vector of channels used for transformation (i.e not equal to "none" in marker class), and FlowSOM.params = list(xdim=10, ydim=10, nClus=20, scale=FALSE)

6) Test the coefficient of variation within clusters with the testCV() function.

7) Import the metadata with the batch label of each sample contained in the excel file md.xlsx, using read.xlsx2(). Create 2 vectors using the column "batch" in the md.xlsx file. One vector contains the batch labels of the samples that correspond to sample "REU271", and another vector contains the batch labels of the other samples (i.e. not "REU271").

8) Estimate quantiles from the training flowSet using CytoNorm.train(). Use FlowSOM.params = list(nCells = 6000, xdim = 10, ydim = 10, nClus = 5, scale = FALSE)

9) Normalize the rest of the samples using CytoNorm.normalize(), and using outputDir = "course_datasets/FR_FCM_Z4KT/Normalized" ; Make sure this is a new folder.

10) Choosing one channel, create a ridge plot of its distribution within samples before normalization (without the training samples), and one for the normalized samples. For this, you need to create a new flowSet with the created Norm_ fcs files. Use the densityplot() function for each flowSet, storing the output in 2 objects, then use the cowplot plot_grid() function to plot one above the other.

Working with gated data in R

Basics and Manual Gating

Gating data in R

- The *GatingSet* class of objects (*flowWorkspace*) for working with gating data in R
- Import a *FlowJo* or *Cytobank* workspace (xml file) with gating data into R
- Manual gating from scratch
  - Using functions from the *flowWorkspace* package
  - Using a graphic-based, interactive tool (*flowGate* package)
- Automated gating methods
  - *flowClust* package
  - *OpenCyto* package

flowWorkspace

https://bioconductor.org/packages/release/bioc/html/flowWorkspace.html

- Provides the *GatingSet* class of objects as an efficient data structure to store, query and visualize gated flow data
- A *GatingSet* (**gs**) stores multiple *GatingHierarchy* (**gh**) objects associated with individual samples

GatingSet ~ *flowSet*

GatingHierarchy ~ *flowFrame*

- Unlike *flowSet*s, functions that operate on a *GatingSet* have the potential side-effect of modifying the object (all the modifications are made to the *external pointer*, rather than the R object itself)

flowWorkspaceData

https://bioconductor.org/packages/release/bioc/html/flowWorkspace.html

- Contains FCS data files, XML workspaces and GatingSets for testing the flowWorkspace and openCyto packages
- Data from whole blood

Installation

To install this package, start R (version "4.3") and enter:

```
if (!require("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("flowWorkspaceData")
```

Create a *gatingSet*

Ways to generate a *gatingSet:*

- Imported from workspace XML files from FlowJo, CytoBank or other software using *CytoML* package
- Built from scratch within R (**manual gating**)
- Generated by **automated gating** methods (e.g. *openCyto* package)

Import a workspace using *CytoML*

https://bioconductor.org/packages/release/bioc/html/CytoML.html

```
if (!require("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("CytoML")
```

- Uses platform-specific implementations of the GatingML2.0 standard to exchange gated cytometry data

HHS Public Access

Author manuscript

Cytometry A. Author manuscript; available in PMC 2016 July 01.

Published in final edited form as:

Cytometry A. 2015 July ; 87(7): 683–687. doi:10.1002/cyto.a.22690.

ISAC's Gating-ML 2.0 data exchange standard for gating description

Josef Spidlen[1], **Wayne Moore**[2], **ISAC Data Standards Task Force**[3], and **Ryan R. Brinkman**[†,1,4]

[1]Terry Fox Laboratory, BC Cancer Agency, Vancouver, British Columbia, Canada

[2]Genetics Department, Stanford University School of Medicine, Stanford, California, United States of America

[3]Full lists of members and affiliations appear at the end of the paper

[4]Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada

```
# import workspace from FlowJo
> ws <- open_flowjo_xml("course_datasets/flowWorspaceData/manual.xml")
> gs <- flowjo_to_gatingset(ws, name = "T-cell")
```

flowWorkspace: basics on *GatingSet* objects

 # List the samples stored in the GatingSet

```
> sampleNames(gs)
[1] "CytoTrol_CytoTrol_1.fcs_119531" "CytoTrol_CytoTrol_2.fcs_115728"
```

 # Access metadata

```
> pData(gs)
                                                                name condition
CytoTrol_CytoTrol_1.fcs_119531 CytoTrol_CytoTrol_1.fcs treatment
CytoTrol_CytoTrol_2.fcs_115728 CytoTrol_CytoTrol_2.fcs   control
```

 # Add metadata

```
pData(gs)$condition <- c("treatment","control")
```

 # Subset a GatingSet by metadata column

```
> subset(gs, subset = treatment == "control")
A GatingSet with 1 samples
```

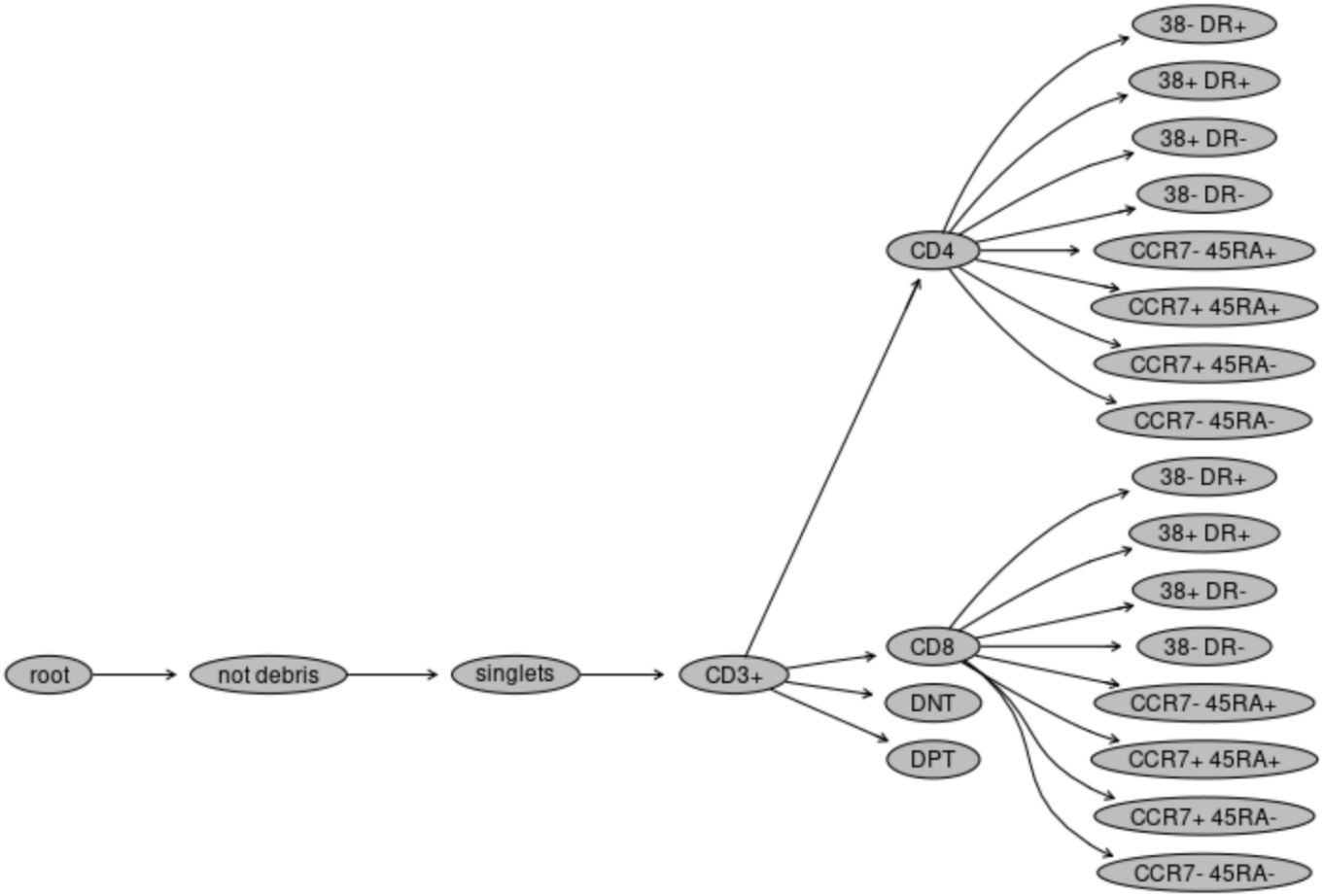
 # Retrieve a GatingHierarchical (one sample)

```
> gh <- gs[[1]]
> gh
Sample:  CytoTrol_CytoTrol_1.fcs_119531
GatingHierarchy with  24  gates
```

flowWorkspace: basics on *GatingSet* objects

 # plot the gating hierarchy (tree)

> plot(gs)

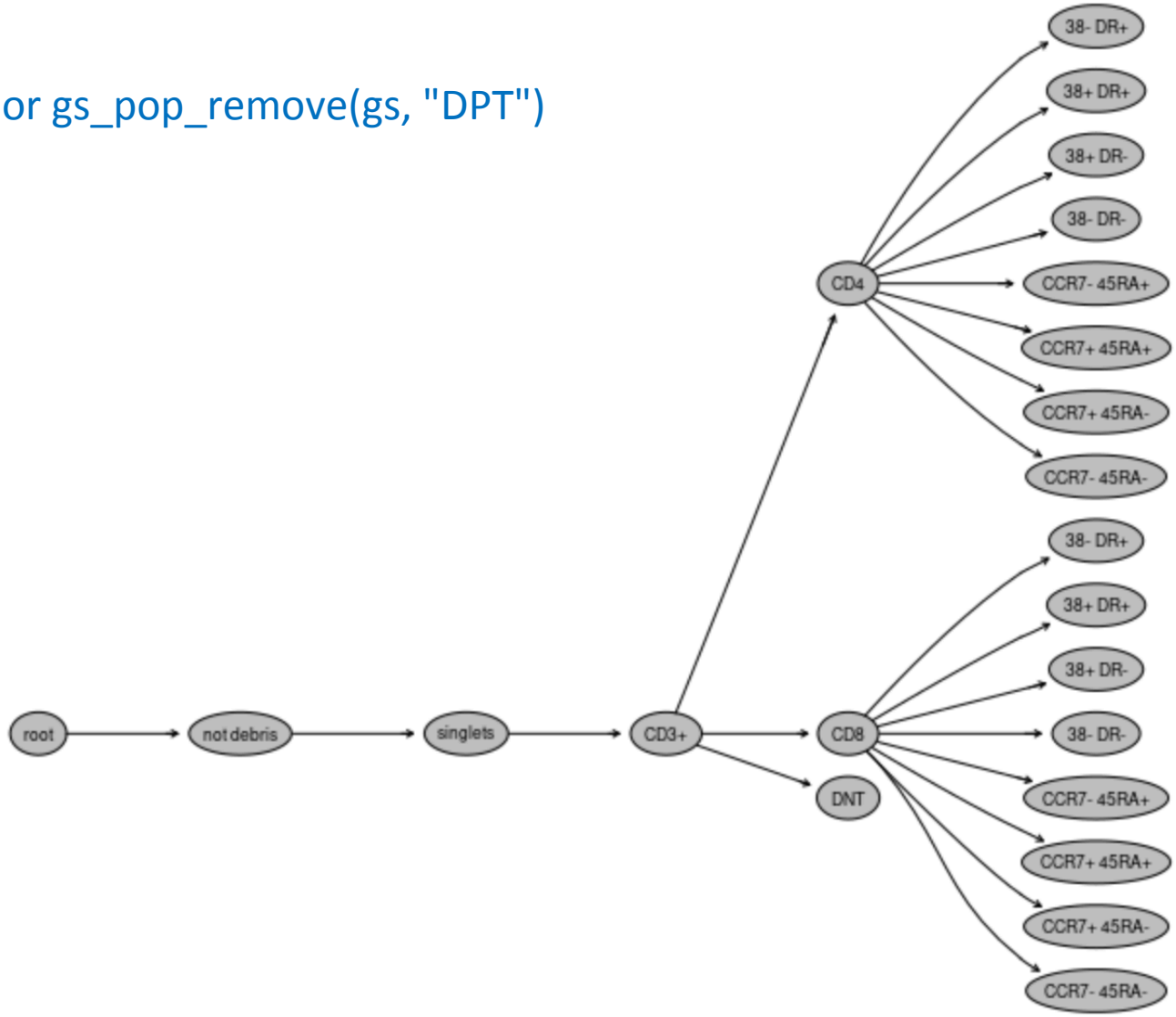


flowWorkspace: basics on *GatingSet* objects

 # Delete a gate

> Rm(gs, "DPT") or gs_pop_remove(gs, "DPT")

> plot(gs)



flowWorkspace: basics on *GatingSet* objects

 # list nodes (cell populations)

```
> gs_get_pop_paths(gs, path = 2)
 [1] "root"                "not debris"          "not debris/singlets" "singlets/CD3+"       "CD3+/CD4"
 [6] "CD4/38- DR+"         "CD4/38+ DR+"         "CD4/38+ DR-"         "CD4/38- DR-"         "CD4/CCR7- 45RA+"
[11] "CD4/CCR7+ 45RA+"     "CD4/CCR7+ 45RA-"     "CD4/CCR7- 45RA-"     "CD3+/CD8"            "CD8/38- DR+"
[16] "CD8/38+ DR+"         "CD8/38+ DR-"         "CD8/38- DR-"         "CD8/CCR7- 45RA+"     "CD8/CCR7+ 45RA+"
[21] "CD8/CCR7+ 45RA-"     "CD8/CCR7- 45RA-"     "CD3+/DNT"            "CD3+/DPT"
```

```
> gs_get_pop_paths(gs, path = "full")
 [1] "root"                                      "/not debris"
 [3] "/not debris/singlets"                      "/not debris/singlets/CD3+"
 [5] "/not debris/singlets/CD3+/CD4"             "/not debris/singlets/CD3+/CD4/38- DR+"
 [7] "/not debris/singlets/CD3+/CD4/38+ DR+"     "/not debris/singlets/CD3+/CD4/38+ DR-"
 [9] "/not debris/singlets/CD3+/CD4/38- DR-"     "/not debris/singlets/CD3+/CD4/CCR7- 45RA+"
[11] "/not debris/singlets/CD3+/CD4/CCR7+ 45RA+" "/not debris/singlets/CD3+/CD4/CCR7+ 45RA-"
[13] "/not debris/singlets/CD3+/CD4/CCR7- 45RA-" "/not debris/singlets/CD3+/CD8"
[15] "/not debris/singlets/CD3+/CD8/38- DR+"     "/not debris/singlets/CD3+/CD8/38+ DR+"
[17] "/not debris/singlets/CD3+/CD8/38+ DR-"     "/not debris/singlets/CD3+/CD8/38- DR-"
[19] "/not debris/singlets/CD3+/CD8/CCR7- 45RA+" "/not debris/singlets/CD3+/CD8/CCR7+ 45RA+"
[21] "/not debris/singlets/CD3+/CD8/CCR7+ 45RA-" "/not debris/singlets/CD3+/CD8/CCR7- 45RA-"
[23] "/not debris/singlets/CD3+/DNT"             "/not debris/singlets/CD3+/DPT"
```

```
> gs_get_pop_paths(gs, path = "auto")
 [1] "root"            "not debris"      "singlets"        "CD3+"            "CD4"             "CD4/38- DR+"     "CD4/38+ DR+"
 [8] "CD4/38+ DR-"     "CD4/38- DR-"     "CD4/CCR7- 45RA+" "CD4/CCR7+ 45RA+" "CD4/CCR7+ 45RA-" "CD4/CCR7- 45RA-" "CD8"
[15] "CD8/38- DR+"     "CD8/38+ DR+"     "CD8/38+ DR-"     "CD8/38- DR-"     "CD8/CCR7- 45RA+" "CD8/CCR7+ 45RA+" "CD8/CCR7+ 45RA-"
[22] "CD8/CCR7- 45RA-" "DNT"             "DPT"
```

FlowWorkspace: basics on *GatingSet*

```
# retrieve data from all nodes as a cytoset
 > cs <- gs_pop_get_data(gs)
 > class(cs)
[1] "cytoset"
attr(,"package")
[1] "flowWorkspace"
```

```
# convert the cytoset to a flowSet
 > fs <- cytoset_to_flowSet(cs)
```

```
# check the number of cells in the flowSet
 > fsApply(fs, nrow)
```

```
                                                  [,1]
CytoTrol_CytoTrol_1.fcs_119531 119531
CytoTrol_CytoTrol_2.fcs_115728 115728
```

```
# retrieve data associated to one node (gate)
 > cs <- gs_pop_get_data(gs, "CD4")
 > fs <- cytoset_to_flowSet(cs)
 > fsApply(fs, nrow)
```

```
                                                  [,1]
CytoTrol_CytoTrol_1.fcs_119531 34032
CytoTrol_CytoTrol_2.fcs_115728 33751
```

FlowWorkspace: basics on *GatingSet* object

 # Get membership indices with respect to a gate

```
> gh_pop_get_indices(gs[[1]], "CD4")
```

```
  [1]  TRUE FALSE FALSE FALSE FALSE  TRUE FA
 [16]  TRUE FALSE  TRUE  TRUE  TRUE FALSE FA
 [31] FALSE FALSE FALSE FALSE  TRUE FALSE FA
```

```
> table(gh_pop_get_indices(gs[[1]], "CD4"))
```

```
FALSE  TRUE
85499 34032
```

 # save / load a GatingSet

```
> save_gs(gs, path = "course_datasets/flowWorspaceData/gs")
> gs2 <- load_gs("course_datasets/flowWorspaceData/gs")
```

The regular R assignement (<-) or save() routine doesn't work for *GatingSet* objects

Build a *GatingSet* from scratch

Start from a *flowSet* (*flowCore*)

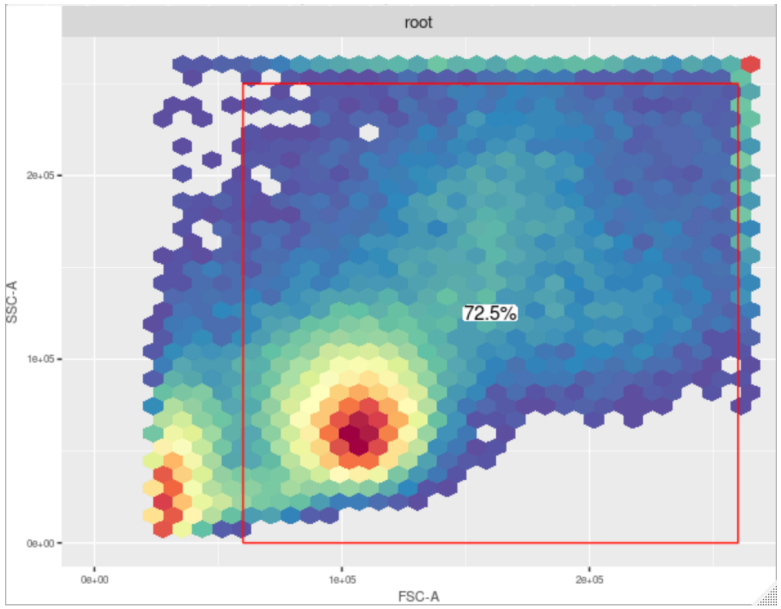
```
# Read the FCS files
> fs <- read.flowSet(path="course_datasets/flowWorspaceData/", pattern = "*.fcs")

# Arcsinh transform with flowVS
> panel <- pData(parameters(fs[[1]]))
> markerstotransf <- as.character(panel$name[!is.na(panel$desc)])
> fcs_transform <- transFlowVS(fs, channels = markerstotransf,
                               cofactors = rep(3000,length(markerstotransf)))
# Convert to a GatingSet
> gs <- GatingSet(fcs_transform) # convert the flowSet to GatingSet
```

Start from a single cell experiment object (*CATALYST*)

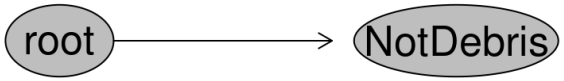
```
> load("course_datasets/FR_FCM_Z4KT/DA_example_sce_PBMC.RData") # load the sce
> fs <- sce2fcs(sce_PBMC, assay = "exprs") # convert sce to flowSet (CATALYST)
> gs <- GatingSet(fs) # convert flowSet to GatingSet
```

Manual gating : rectangle gate

```
# Create a rectangle gate
> rg1  <- rectangleGate("FSC-A"=c(60000,260000),
                        "SSC-A"=c(1, 250000),
                        filterId="NotDebris")
```




```
# Add the gate to the GatingSet object
> gs_pop_add(gs, rg1, parent = "root")

# Apply the gate to the data
> recompute(gs)

# Plot the gate
> autoplot(gs[[1]], gate = "NotDebris")
```



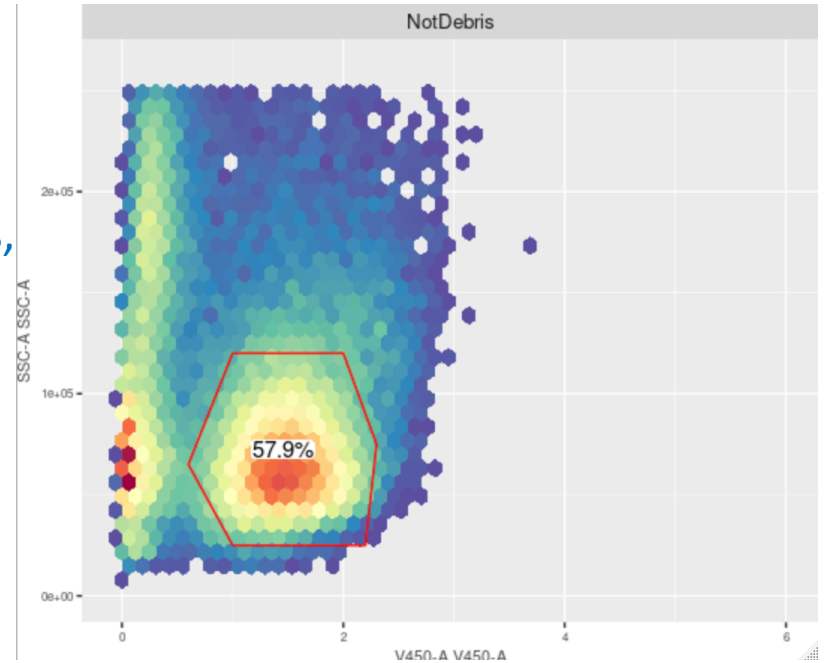

```
# Check gating hierarchy
> plot(gs)

# Get statistics
> gs_pop_get_stats(gs, "NonDebris") # counts
> gs_pop_get_stats(gs, "NonDebris", type = "percent") # proportions
```

```
                          sample       pop count
1: CytoTrol_CytoTrol_1.fcs NotDebris 86699
2: CytoTrol_CytoTrol_2.fcs NotDebris 86689
```

```
                          sample       pop   percent
1: CytoTrol_CytoTrol_1.fcs NotDebris 0.7253265
2: CytoTrol_CytoTrol_2.fcs NotDebris 0.7490754
```

Manual gating: polygon gate

```
# Define the vertices of the polygon
> my_vertices <-matrix(c(1,0.6,1,2,2.3,2.2,
                         25000,65000,120000,120000,75000,25000),
                         ncol=2,nrow=6)
colnames(my_vertices) <- c("V450-A","SSC-A")

# Create polygon gate "singlets"
> rg2  <- polygonGate( boundaries= my_vertices,
                  filterId="CD3")

# Add the gate to the GatingSet
> gs_pop_add(gs, rg2, parent = "NotDebris")

# Recompute the GatingSet
> recompute(gs)

# Check
> autoplot(gs[[1]], gate = "CD3")
> plot(gs)
```



Manual gating: quadrant gate

 # Create quadrant gate "CD4 CD8"

```
> rg3  <- quadGate("B710-A"= 1.5,
                   "R780-A"= 3,
                   filterId = "CD4 CD8")
```

 # Add the gate to the GatingSet

```
> gs_pop_add(gs, rg3, parent = "CD3")
```

 # Recompute the GatingSet

```
> recompute(gs)
```

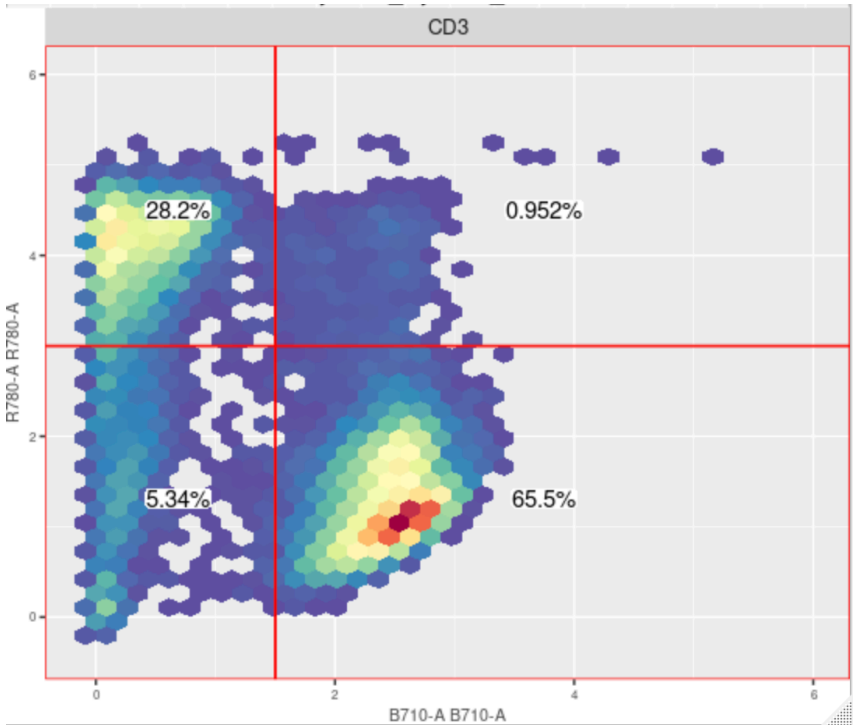
 # Check

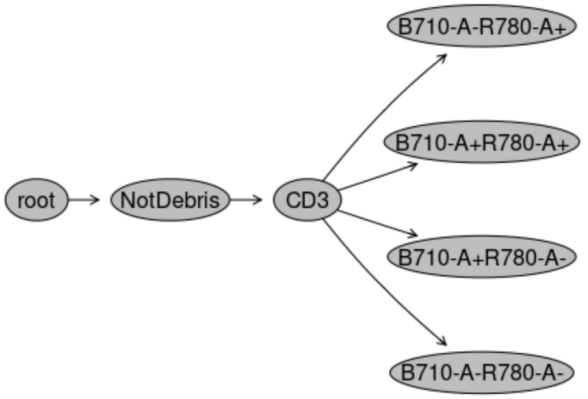
```
> gs_get_pop_paths(gs)
```

```
[1] "root"                          "/NotDebris"
[3] "/NotDebris/CD3"                "/NotDebris/CD3/B710-A-R780-A+"
[5] "/NotDebris/CD3/B710-A+R780-A+" "/NotDebris/CD3/B710-A+R780-A-"
[7] "/NotDebris/CD3/B710-A-R780-A-"
```

 # Plot

```
> autoplot(gs[[1]], gate = gs_get_pop_paths(gs)[4:7])
> plot(gs)
```

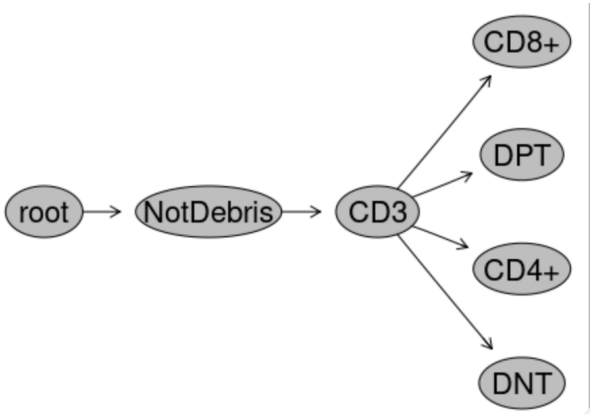




FlowWorkspace: other utilities

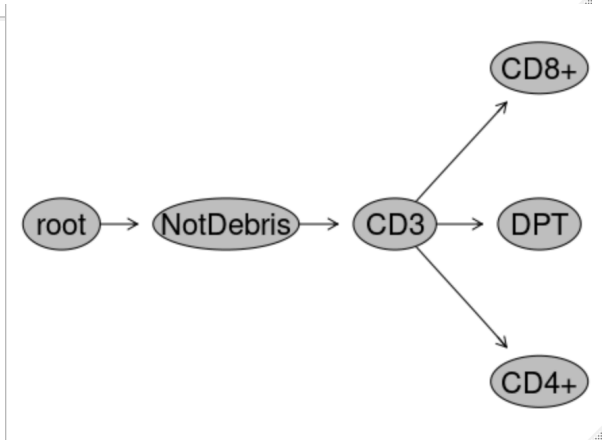
 # Rename nodes (gates)

```
> gs_pop_set_name(gs,"B710-A-R780-A+","CD8+")
> gs_pop_set_name(gs,"B710-A+R780-A-","CD4+")
> gs_pop_set_name(gs,"B710-A-R780-A-","DNT")
> gs_pop_set_name(gs,"B710-A+R780-A+","DPT")
> plot(gs)
```



 # Remove a node

```
> gs_pop_remove(gs, "DNT")
> plot(gs)
```



 # Retrieve the flow data for a node

```
> fs_CD8 <- gs_pop_get_data( gs, "CD8+")
```

Interactive gating with *flowGate*

https://bioconductor.org/packages/release/bioc/html/flowGate.html

```
if (!require("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("flowGate")
```

- Interactive cytometry gating in R
- Based on a shiny app (web application using R)
- Especially geared toward wet-lab cytometerists looking to take advantage of R without having a lot of experience
- Uses GatingSet objects
- You can use transformed data, but flowGate was designed to apply desired transformations at the plotting level only

Interactive gating with *FlowGate*

 # Load FCS files and preprocess

```
> fs <- read.flowSet(path="course_datasets/flowWorspaceData/", pattern = "*.fcs")
 > panel <- pData(parameters(fs[[1]]))
 > markerstotransf <- as.character(panel$name[!is.na(panel$desc)])
 > fs <- transFlowVS(fs, channels = markerstotransf,
                          cofactors = rep(3000,length(markerstotransf)))
```

 # Convert to GatingSet

```
> gs <- GatingSet(fs)
```

1st: chose type of gate

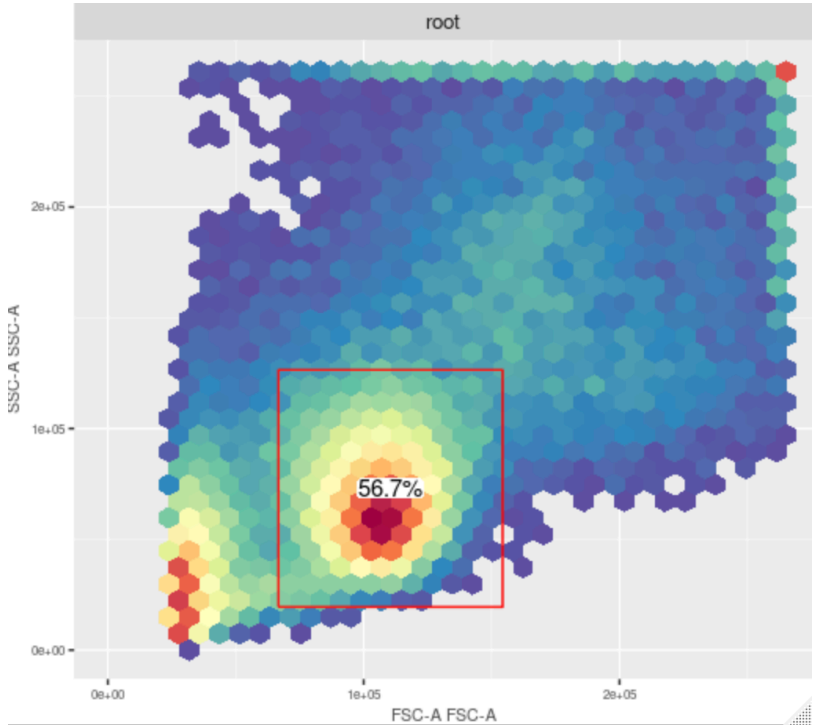
 # Start the interactive gating

```
 > gs_gate_interactive(gs,
              filterId = "NotDebris",
              dims = list("FSC-A", "SSC-A"))
```

Interactive gating with *FlowGate*

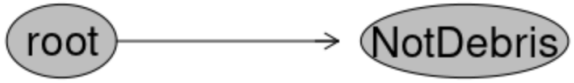
 # Plot the data with the new gate

```
> autoplot(gs[[1]], gate = "NotDebris")
```



 # Plot hierarchy

```
> plot(gs)
```



Interactive gating with *FlowGate*

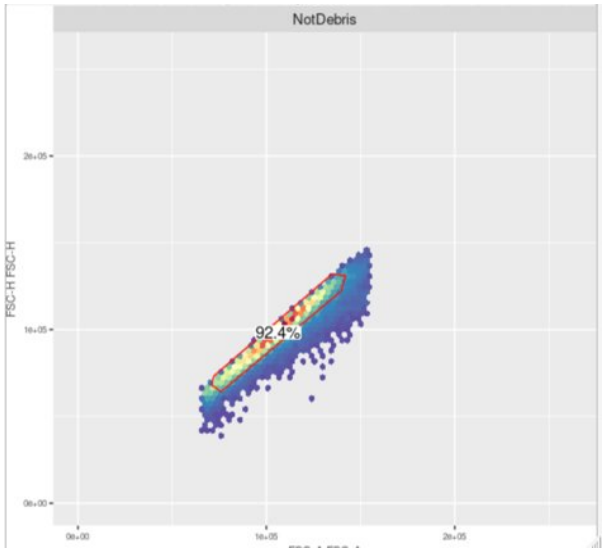
Create a 2-D *polygon* gate

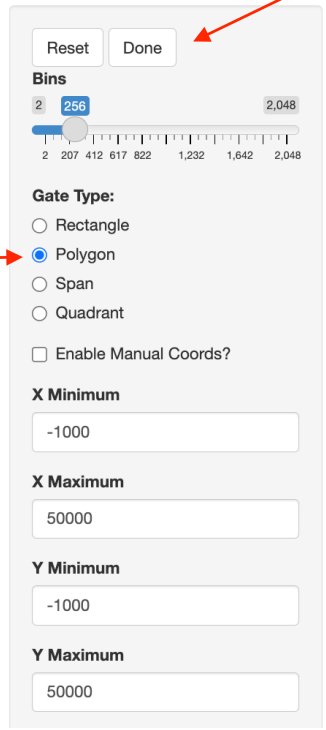
 # Create a polygon gate

```
> gs_gate_interactive(gs,
                      filterId = "singlets",
                      dims = list("FSC-A", "FSC-H"),
                      subset = "NotDebris")
```

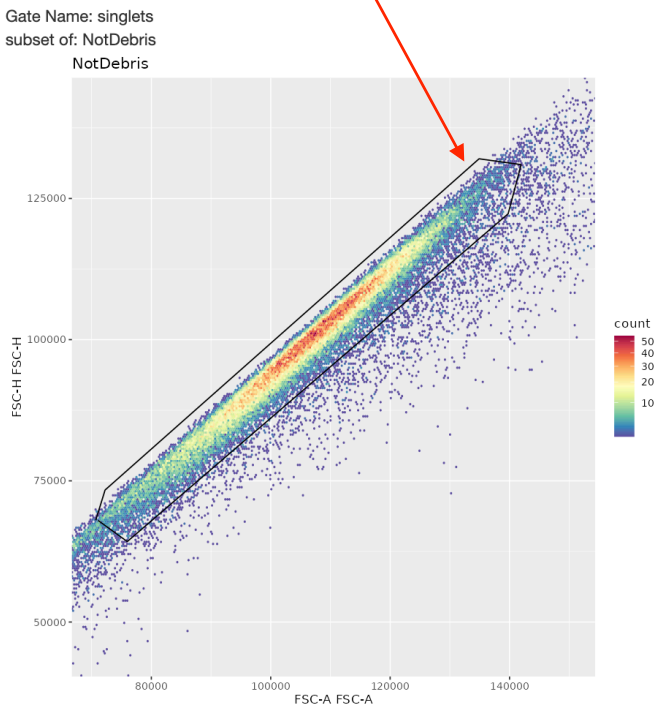
 # Check

```
> autoplot(gs[[1]], gate = "singlets")
```





Click on the vertices



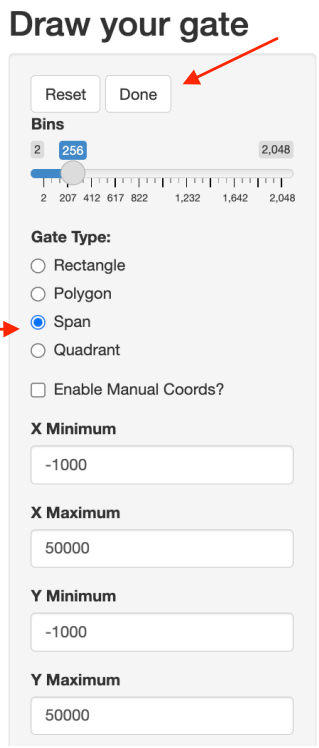

```
> plot(gs)
```

Interactive gating with *FlowGate*

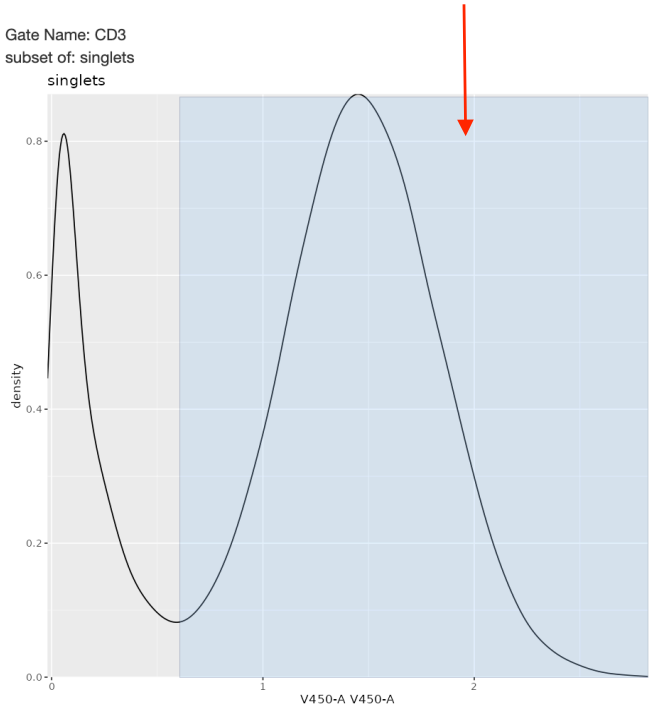
Create a 1-D *span* gate

 # Create a span gate

```
> gs_gate_interactive(gs,
                      filterId = "CD3",
                      dims = "V450-A",
                      subset = "singlets")
```

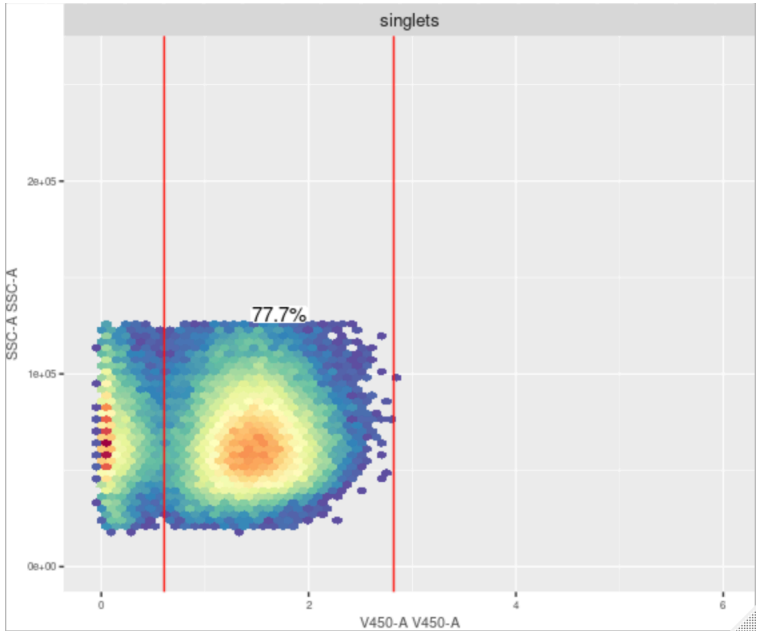


Drag a window

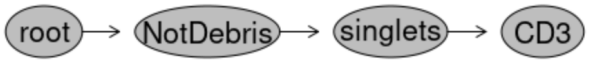


 # Check the gate

```
> autoplot(gs[[1]], gate = "CD3")
```




```
> plot(gs)
```



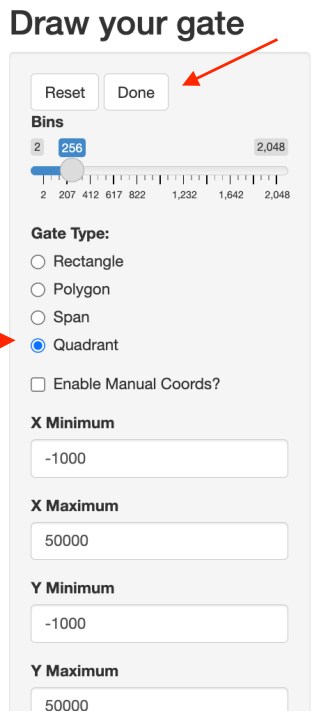
Interactive gating with *FlowGate*

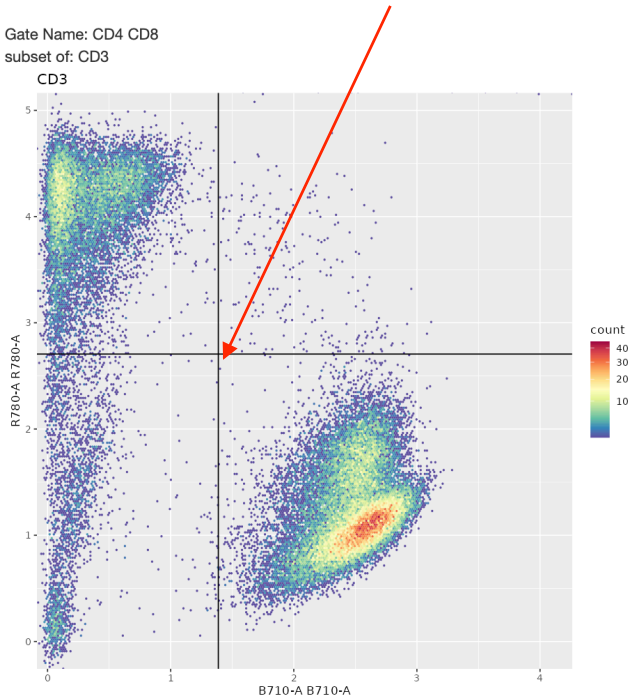
Create a 2-D quadrant gate

Select the origin of the quadrant

 # Create a quadrant gate

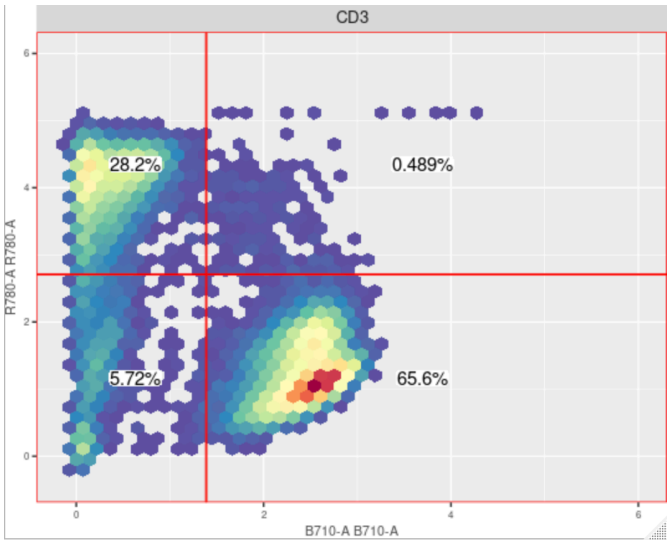
```
> gs_gate_interactive(gs,
                      filterId = "CD4 CD8",
                      dims = c("B710-A",
                               "R780-A"),
                      subset = "CD3")
```



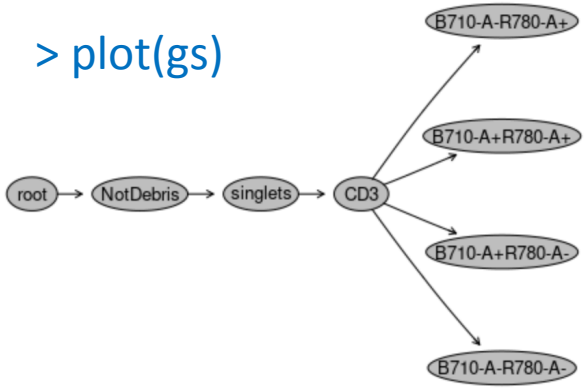


 # Check

```
my_nodes <- gs_pop_get_children(gs,"CD3")
autoplot(gs[[1]], my_nodes)
```




```
> plot(gs)
```



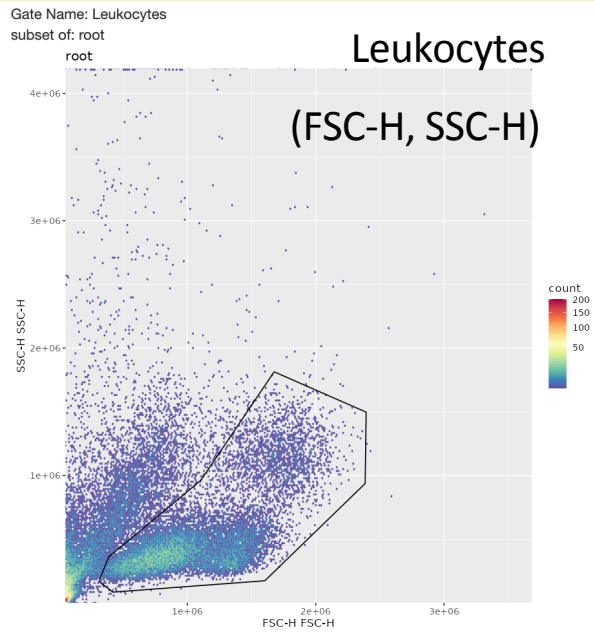
Let's practice – 9

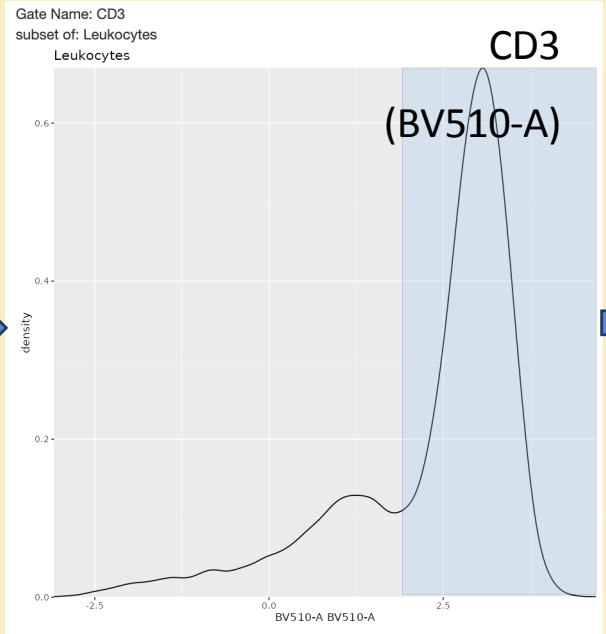
In this exercise we will do some manual gating using *flowGate* and data from the **FR_FCM_Z3WR** dataset of the FlowRepository.

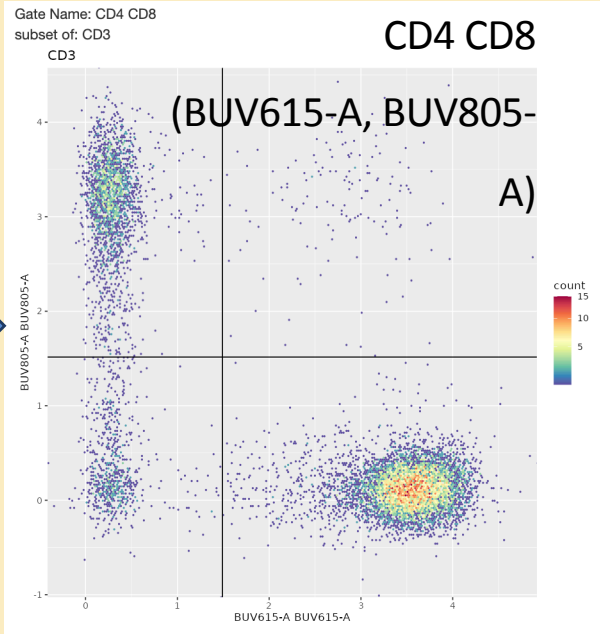
Create a new script in which you will:

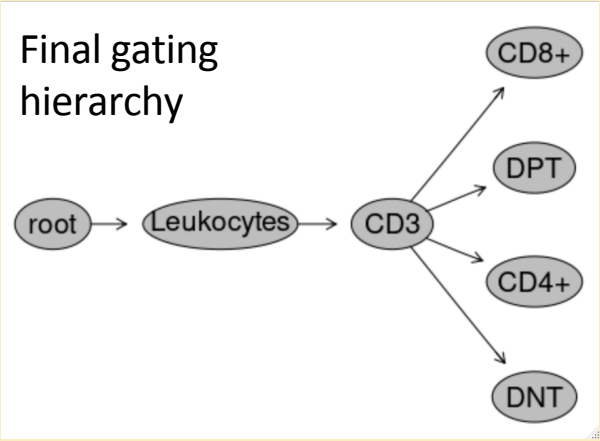
1) Create a *flowSet* of all samples within the /course_dataset/FR_FCM_Z3WR folder
2) Perform asinh transformation with a cofactor of 3000 for all channels not labeled with "none", using transFlowVS() from the flowVS package. You can use the csv file with the panel previously created (/course_datasets/FR_FCM_Z3WR/panel_with_marker_classes.csv)
3) Convert the *flowSet* to a *GatingSet*
4) Using *flowGate*, create a gating hierarchy according to the scheme depicted in the following slide. Don't forget to check your gating with scatter or density plots.
5) Perform the necessary adjustements so that your gating hierarchy looks like the one depicted in the next slide
6) What is the percentage of CD8+ T cells among T cells ("CD3")

Let’s practice – 9 : Gates









04 Working with gated data in R

Automated Gating

Automated gating with *flowClust*

https://www.bioconductor.org/packages/release/bioc/html/flowClust.html

```
if (!require("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("flowClust")
```

- Robust Model-based Clustering of Flow Cytometry Data (Lo et al. 2008)
- Identify cell populations in flow cytometry data
- Based on a multivariate t mixture model with Box-Cox transformation
- Options for estimating the number of clusters when it is unknown
- Input are *flowFrames*

Automated gating with *flowClust*

https://onlinelibrary.wiley.com/doi/10.1002/cyto.a.20531

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

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• **Abstract**

The capability of flow cytometry to offer rapid quantification of multidimensional characteristics for millions of cells has made this technology indispensable for health research, medical diagnosis, and treatment. However, the lack of statistical and bioinformatics tools to parallel recent high-throughput technological advancements has hindered this technology from reaching its full potential. We propose a flexible statistical model-based clustering approach for identifying cell populations in flow cytometry data based on *t*-mixture models with a Box–Cox transformation. This approach generalizes the popular Gaussian mixture models to account for outliers and allow for nonelliptical clusters. We describe an Expectation-Maximization (EM) algorithm to simultaneously handle parameter estimation and transformation selection. Using two publicly available datasets, we demonstrate that our proposed methodology provides enough flexibility and robustness to mimic manual gating results performed by an expert researcher. In addition, we present results from a simulation study, which show that this new clustering framework gives better results in terms of robustness to model misspecification and estimation of the number of clusters, compared to the popular mixture models. The proposed clustering methodology is well adapted to automated analysis of flow cytometry data. It tends to give more reproducible results, and helps reduce the significant subjectivity and human time cost encountered in manual gating analysis.

Automated gating with *flowClust*

https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-10-145

BMC Bioinformatics

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flowClust: a Bioconductor package for automated gating of flow cytometry data

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Automated gating with *flowClust*

flowClust function

```
res2 <-
    flowClust(
        rituximab2,
        varNames = c("FL1.H", "FL3.H"),
        K = 1:6,
        B = 100
    )
```

flowFrame

markers to use

number of expected clusters

number of iterations

Select the best model based on the Bayesian Information Criterion (BIC)

```
criterion(res2, "BIC")
```

```
## [1] -34167.12 -33103.77 -33065.41 -32998.05 -32993.74 -32942.72
```

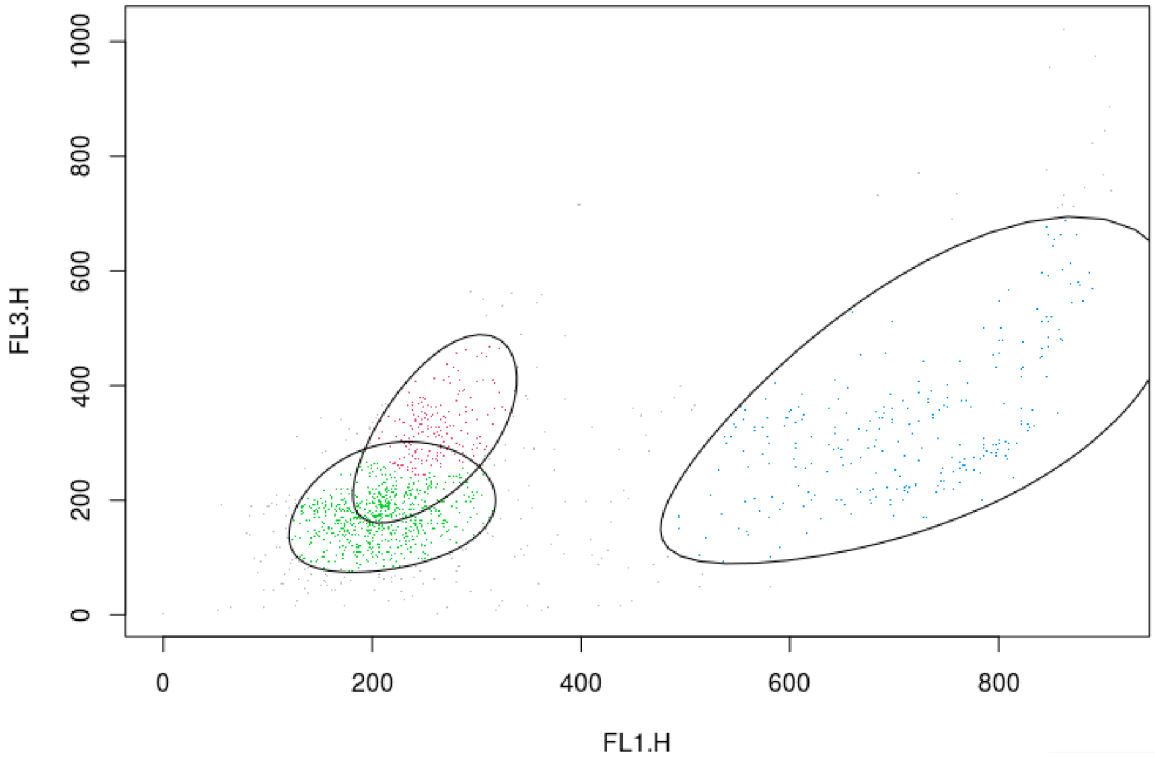
Automated gating with *flowClust*

flowClust function

```
plot(res2[[3]],
      data = rituximab2,
      level = 0.8,
      z.cutoff = 0)
```

Rule of identifying outliers:

- use a 80% quantile (level)
- assign an cellto a cluster only if the associated posterior probability is greater than z.cutoff



Automated gating with *openCyto*

https://bioconductor.org/packages/release/bioc/html/openCyto.html

```
if (!require("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("openCyto")
```

- Hierarchical gating pipeline for flow cytometry data
- Based on *GatingSet* objects
- Wide variety of methods, including *flowClust*
- Automated gating using a *gatingTemplate* (hierarchical gating scheme)
- Possibility of step-by-step gating without a template

Automated gating with *openCyto*

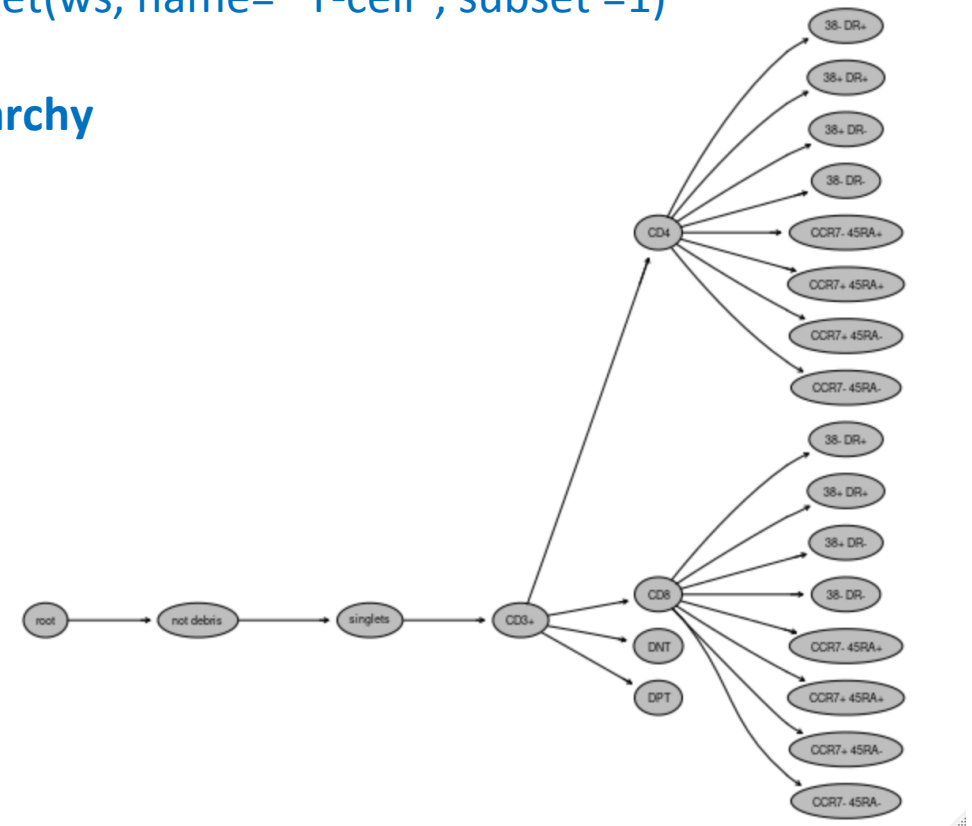
Example from *flowWorkspaceData* (gated data)

 # Load example gatingSet from FlowJo workspace (xml file)

```
> library(CytoML)
> ws <- open_flowjo_xml("course_datasets/flowWorspaceData/manual.xml")
> gs_final <- flowjo_to_gatingset(ws, name= "T-cell", subset =1)
```

 # Check complete gating hierarchy

```
> plot(gs_final[[1]])
```

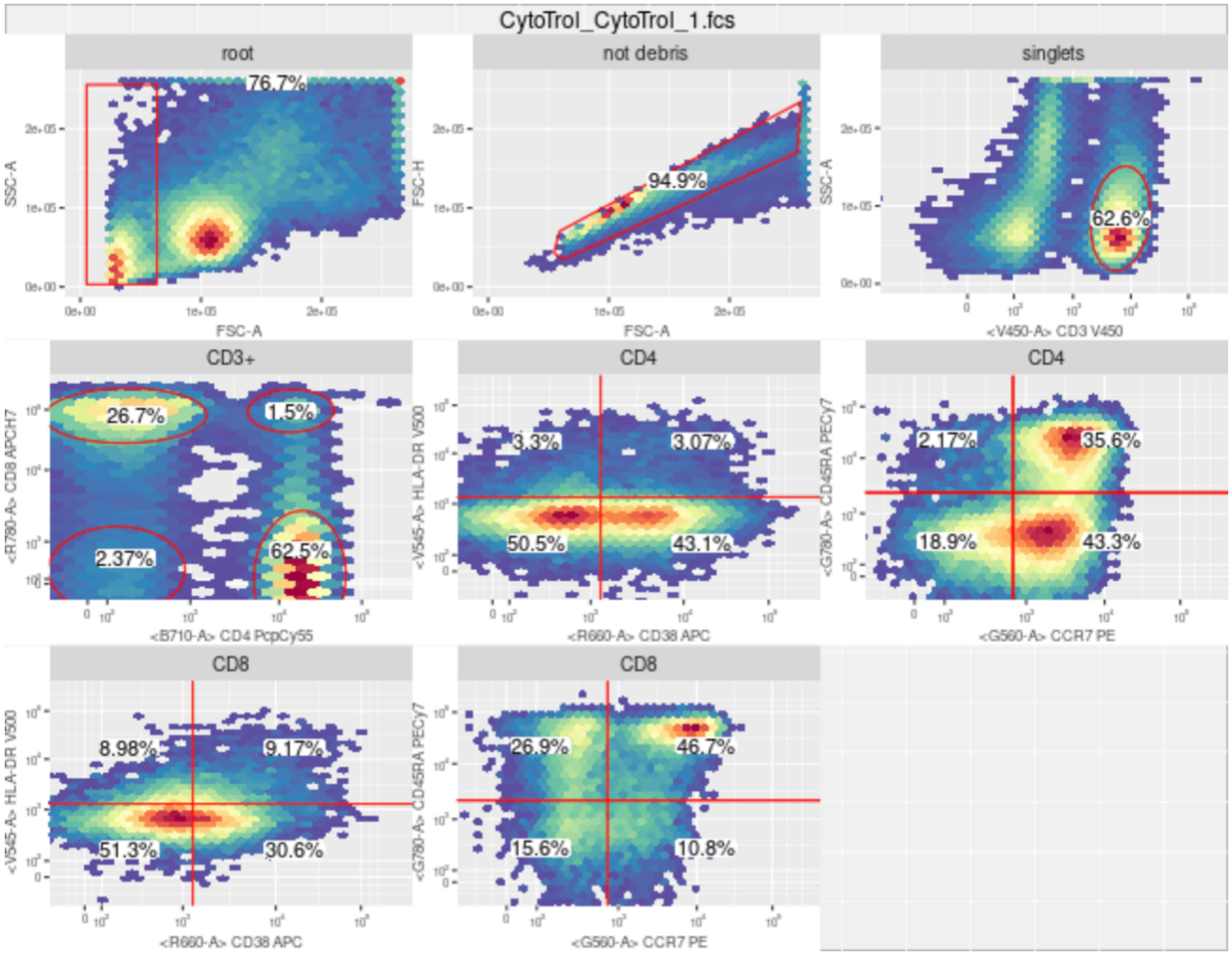


Automated gating with *openCyto*

Example from flowWorkspaceData (gated data)

 # Plot gates

> autoplot(gs_final[[1]])



gatingTemplate

```
# Check the structure of the gatingTemplate
> my_gt <- read.csv("course_datasets/flowWorkspaceData/tcell.csv")
> view(my_gt)
```

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

label of the cell population

parent poulation

method used for gating

collapse all samples into one, gate and then replicate across samples

should any preprocessing be applied

arguments for preprocessing

which side (1-D) or quadrant (2-D) to keep

dimensions used for gating

Arguments for the method

group every n samples

https://bioconductor.org/packages/release/bioc/vignettes/openCyto/inst/doc/HowToWriteCSVTemplate.html

gatingTemplate

Example of a 1-D scan gate

	alias	pop	parent	dims	gating_method	gating_args	collapseDataForGating	groupBy	preprocessing_method	preprocessing_args
1	nonDebris	+	root	FSC-A	gate_mindensity		*NA*	*NA*		*NA*

- The population name will be "nonDebris"
- The parent node is "root"
- The gating method is *mindensity*
- It will generate a scan gate on FSC-A and keep the only the positive cells

gatingTemplate

Example of a *singlet* gate

2	singlets	+	nonDebris	FSC-A,FSC-H	singletGate		NA	NA	NA

- The population name will be "singlets"
- The parent node is "nonDebris"
- It will use FSC-A and FSC-H
- The gating method is *singletGate*
- It will generate a polygon gate on FSC-A and FSC-H to keep only the singlets

gatingTemplate

Example of a *flowClust* gate

3	lymph	+	singlets	FSC-A,SSC-A	flowClust	K=2,target=c(1e5,5e4)	*NA*	*NA*	prior_flowClust	*NA*

- The population name will be "lymph"
- The parent node is "singlets"
- Use the method from *flowClust*
- It will generate an ellipsoid gate on FSC-A and SSC-A and split the cells in "peaks" (high density areas)
- There are parameters to be passed to flowClust: apply some preprocessing before gating; it should identify to populations, centered

gatingTemplate

Example of a quadrant gate

5	*	-/++/-	cd3	cd4,cd8	gate_mindensity	gate_range=c(1,3)	*NA*	*NA*		*NA*

- Quadrant gate
- Based on mindesity for determining the origin of the quadrant
- Specifies that population CD4+/-CD8+/- should be expanded into 6 cell populations
  - The first two gates are span gates on each channel (CD4 and CD8)
  - The other four gates are rectangle gates that correspond to the four quadrants in the 2-D projection

openCyto

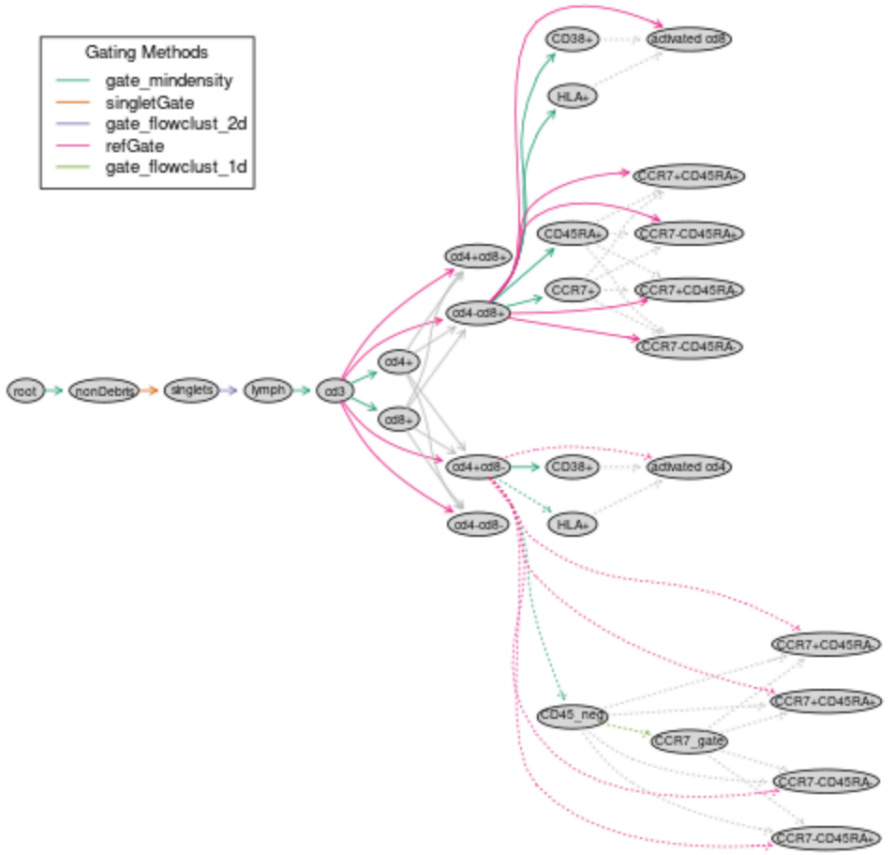
Create the gatingTemplate from the csv file

 # Create the gatingTemplate from a file

```
> gt_tcell <- gatingTemplate("course_datasets/flowWorkspaceData/tcell.csv")
```

Examine the gating scheme

```
> plot(gt_tcell)
```

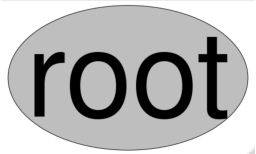


openCyto: run the gating pipeline

Load the raw data and convert to gatingSet

```
# Load the preprocessed but ungated data
# The code used to preprocess this data is avalable in
# /Code_slides/Code_preprocessing_data_openCyto.R
> gs <- load_gs("course_datasets/flowWorspaceData/gs_preprocessed")

# Check
> plot(gs)
```



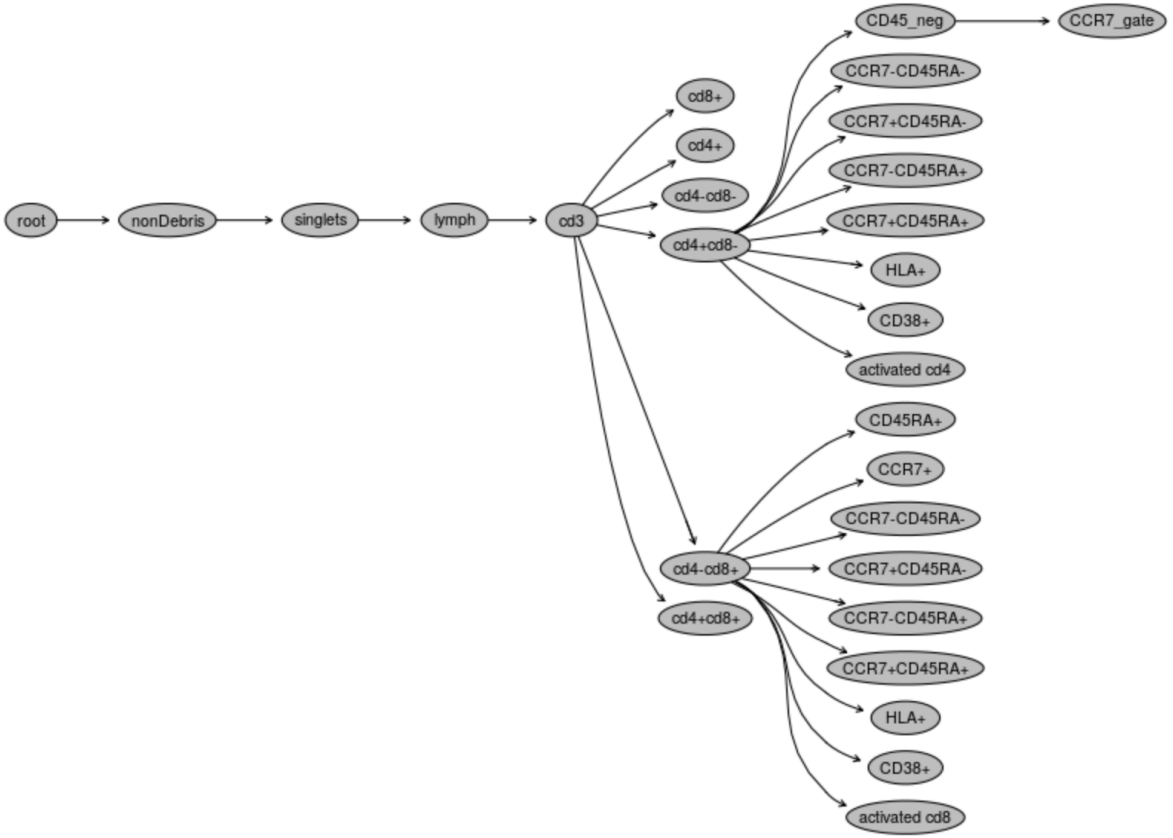
Apply the gating template

```
# Run the gating
 > gt_gating(gt_tcell, gs)
```

openCyto: run the gating pipeline

Check the gating

```
# Check the gating
 > plot(gs[[1]])
```



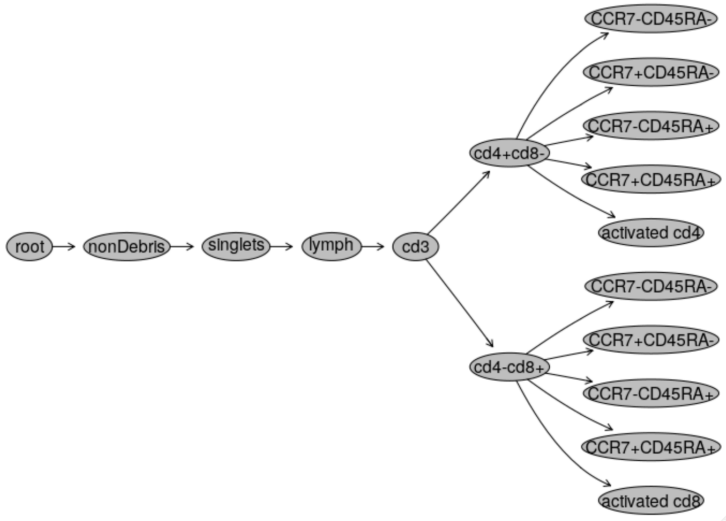
openCyto

Hide populations we are not interested in

```
# Define nodes to hide
 > nodesToHide  <- c("cd8+", "cd4+" , "cd4-cd8", "cd4+cd8+" , "cd4+cd8-/HLA+",
                     "cd4+cd8-/CD38+" , "cd4-cd8+/HLA+", "cd4-cd8+/CD38+" ,
                     "CD45_neg/CCR7_gate", "cd4+cd8-/CD45_neg" ,
                     "cd4-cd8+/CCR7+", "cd4-cd8+/CD45RA+" )

# Apply the gs_pop_set_visibility() function to the nodes to hide
 > lapply(nodesToHide, function(thisNode) gs_pop_set_visibility(gs, thisNode, FALSE))

# Check after hiding nodes
> plot(gs[[1]])
```



openCyto

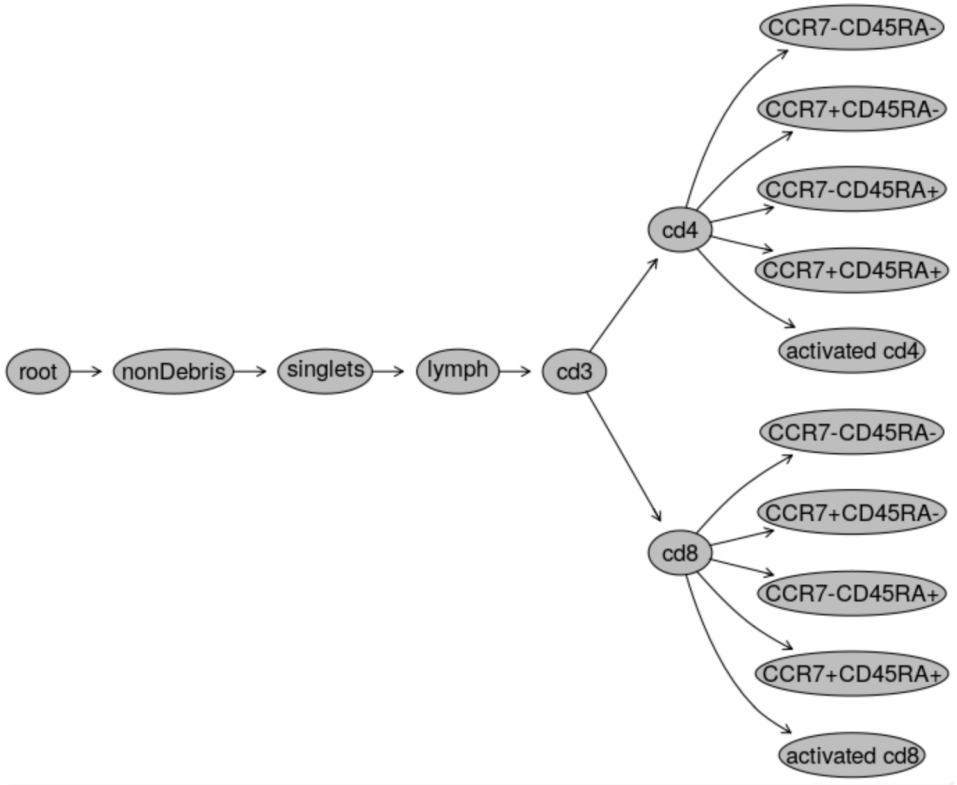
Rename some populations

 # Rename

```
> gs_pop_set_name(gs,"cd4+cd8-","cd4")
> gs_pop_set_name(gs,"cd4-cd8+","cd8")
```

 # Check

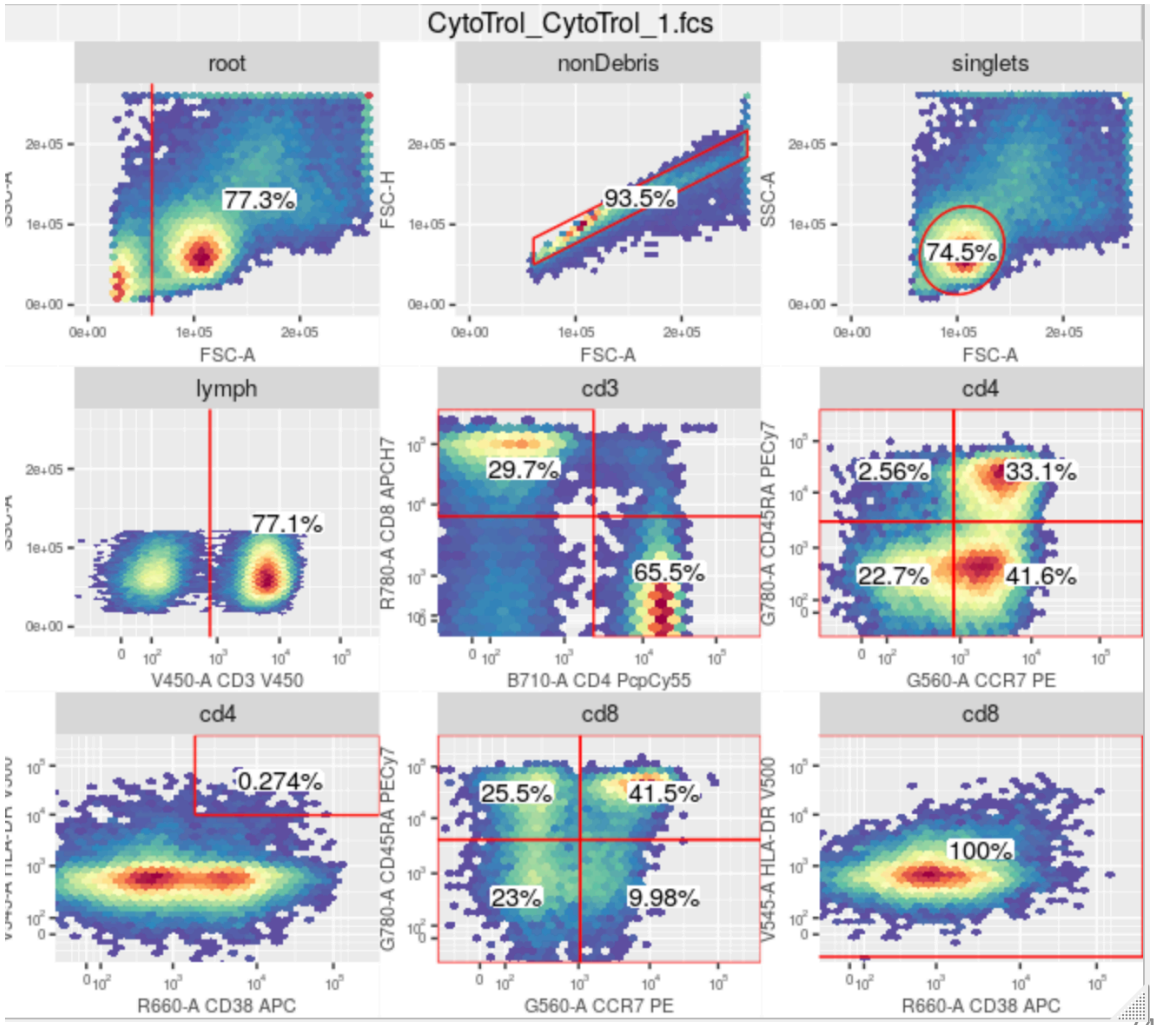
```
> plot(gs[[1]])
```



openCyto

Visualize the gates with *ggcyto*

```
> autoplot(gs[[1]])
```

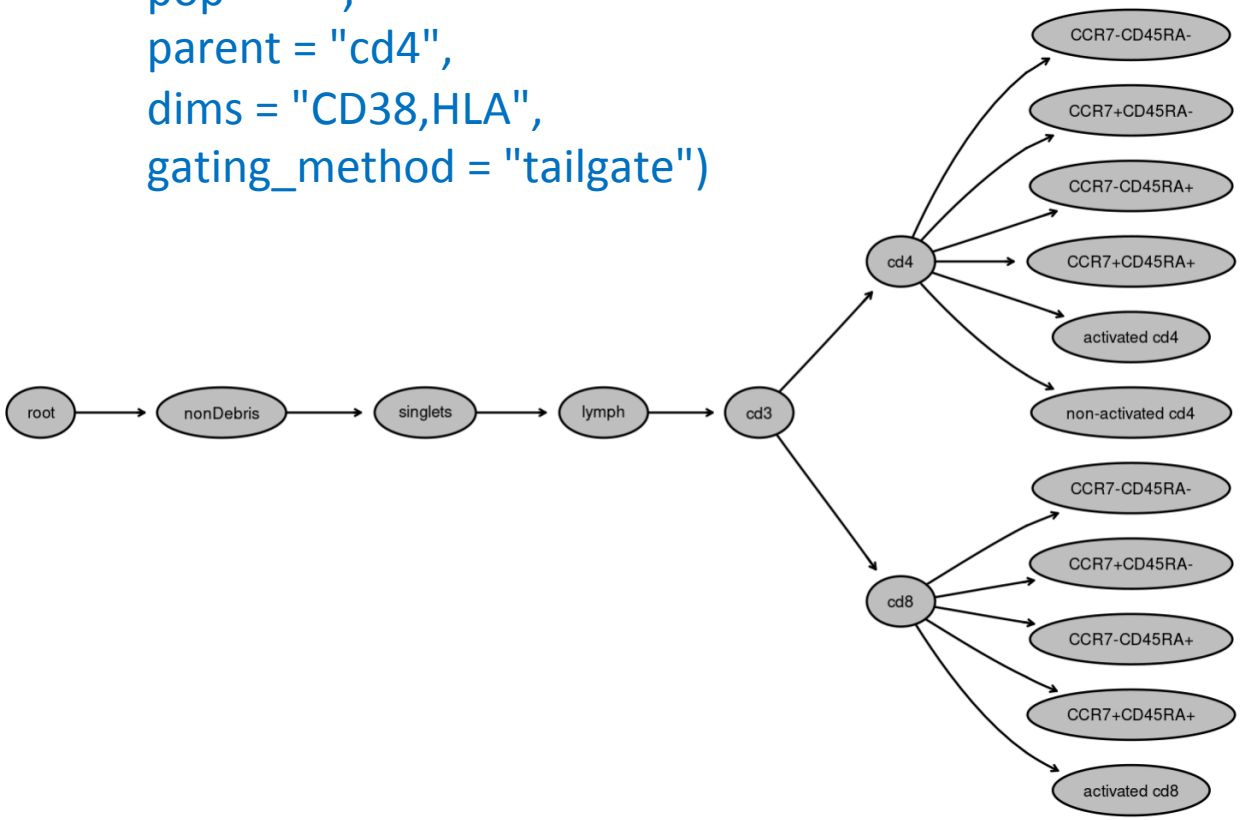


openCyto: gating without a template

We can apply each automated gating step using the same fields as in the template

```
> gs_add_gating_method(gs,
                       alias = "non-activated cd4",
                       pop = "--",
                       parent = "cd4",
                       dims = "CD38,HLA",
                       gating_method = "tailgate")
```

```
# check
> plot(gs[[1]])
```

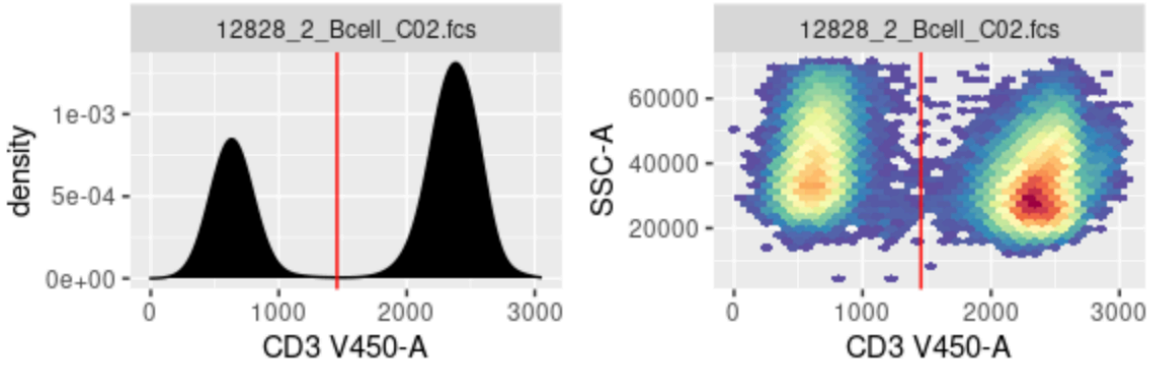


openCyto: overview of gating methods

https://bioconductor.org/packages/release/bioc/vignettes/openCyto/inst/doc/HowToAutoGating.html

mindensity

Finds the minimum as the cutpoint between positive and negative peaks in a 1-D density plot



- Fast, robust and easy to use
- For markers with a good separation between + and – peaks
- Needs more guidance when there are more than 2 peaks

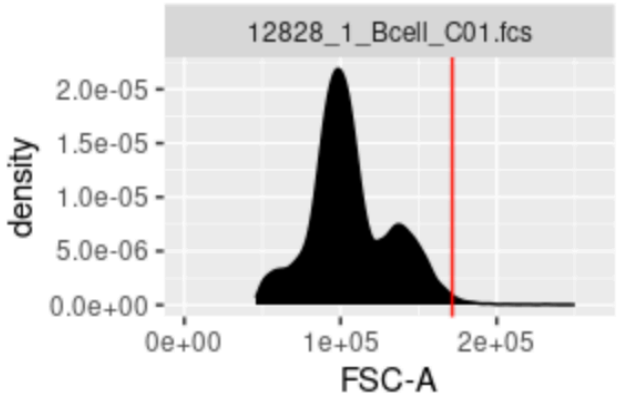
openCyto: overview of gating methods

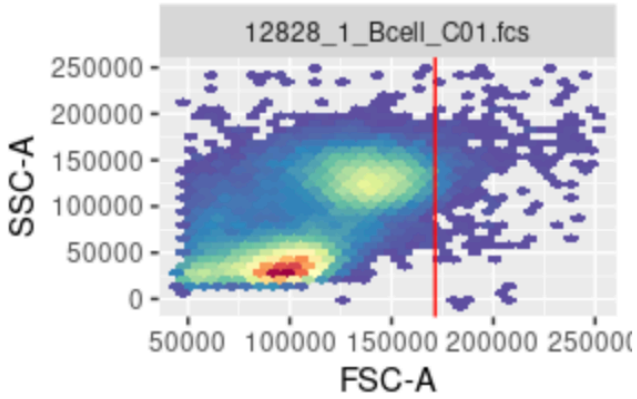
tailgate

Gates the right side or left side of the 1-D density based on a cutpoint (estimated) in the tail

quantileGate

Alternative to tailgate and it determines the cutpoint by the events quantile



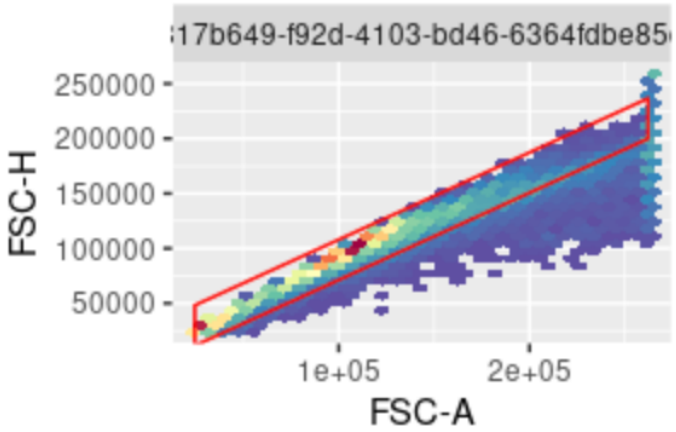


- More commonly used for rare populations (peak is not prominent enough)

openCyto: overview of gating methods

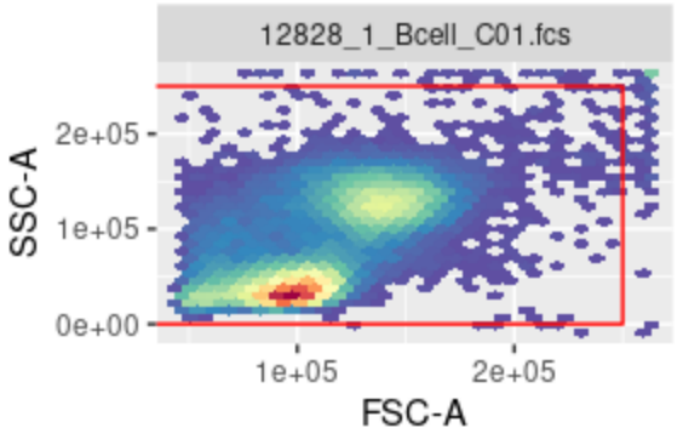
singletGate

Use the area vs height to gate out the singlets



boundary

Constructs a rectangle gate from input range (min and max)

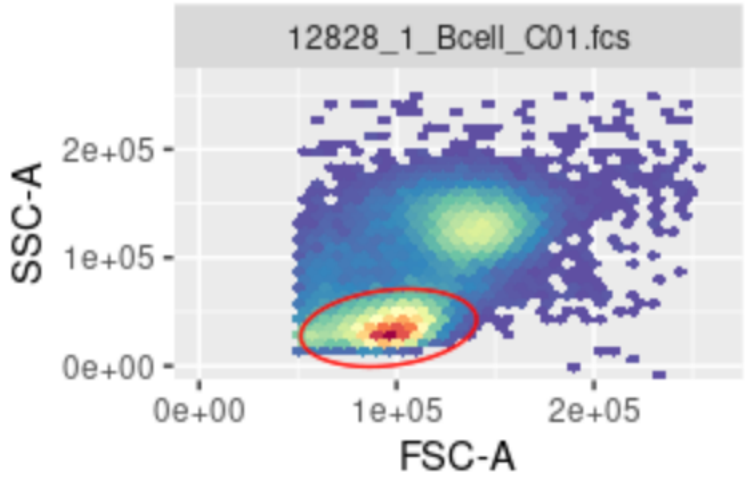


- Used for filtering out very extreme signals at the boundary

openCyto: overview of gating methods

flowClust

1-D or 2-D automated gating methods from flowClust (more details in ?flowClust)



- *k*= how many cell populations are expected
- *target* = center of target population (by default the most prominent cluster)
- *quantile* = how large the ellipse should be

Let’s practice – 10

In this exercise we will repeat the gating previously done with *flowGate* on the data from **FR_FCM_Z3WR** of the FlowRepository, but this time using automated gating with *openCyto*.

Create a new script in which you will:

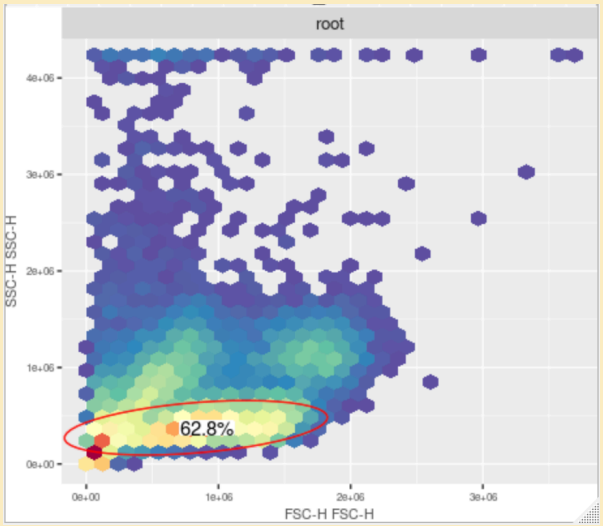
1) Repeat steps 1 to 4 from previous exercise (loading data from fcs files and preprocessing). You can also load the preprocessed GatingSet from /course_dataset/FR_FCM_Z3WR/gs_preprocessed/.
2) Using the *gs_add_gating_method()* function (i.e., without a template), create a gating hierarchy according to the scheme depicted in the following slide. Don't forget to check your gating with scatter or density plots.
3) Do necessary adjustements so that your gating hierarchy looks like the one depicted in the next slide (hide the "BUV805-A+" and "BUV615-A+" nodes from the tree, and rename the CD4, CD8, DNT and DPT nodes)
4) Create boxplots showing the percentage of CD8+ T cells among T cells ("CD3") as a function of time points

Let’s practice – 10 : Gates

Leukocytes

(FSC-H, SSC-H)

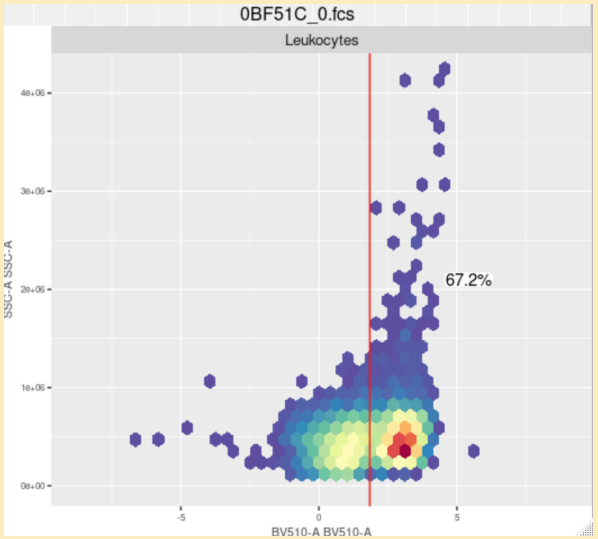
flowClust (K = 3)



CD3 +

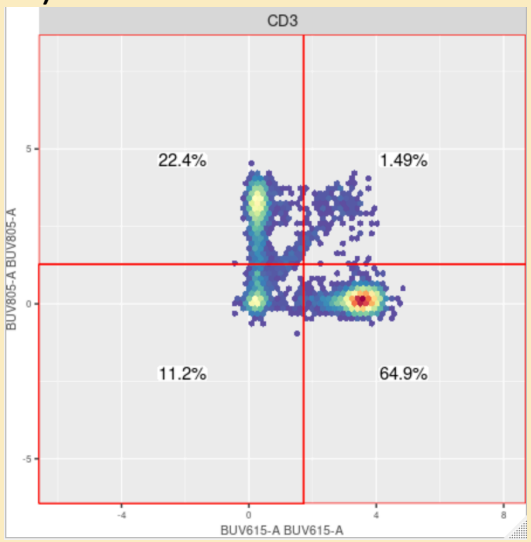
(BV510-A)

gate_mindensity

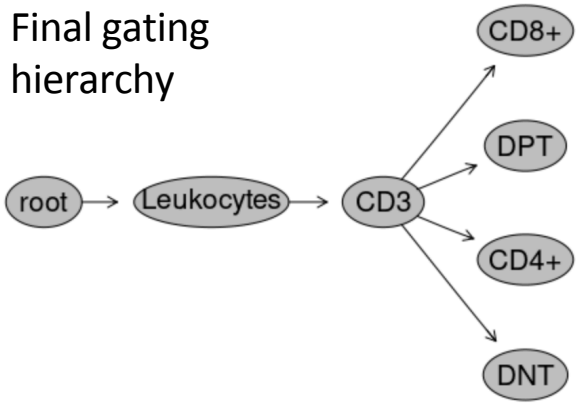


CD4 CD8

(BUV615-A, BUV805-A)



Final gating hierarchy



04 Working with gated data in R

Phenotype discovery

Phenotype discovery and annotation with *FAUST*

https://github.com/RGLab/FAUST

- Full Annotation Using Shape-constrained Trees (FAUST)
- Machine-learning method
- Unsupervised discovery and annotation of phenotypes in single-cell data from flow and mass cytometry experiments
- Based on *GatingSet* data structures (*flowWorkspace*)

Phenotype discovery and annotation with *FAUST*

https://www.cell.com/patterns/pdfExtended/S2666-3899(21)00234-8

Patterns

CellPress
OPEN ACCESS

Article

New interpretable machine-learning method for single-cell data reveals correlates of clinical response to cancer immunotherapy

Evan Greene,[1,2,*] Greg Finak,[1,2] Leonard A. D'Amico,[1,4] Nina Bhardwaj,[7] Candice D. Church,[5] Chihiro Morishima,[5] Nirasha Ramchurren,[1,4] Janis M. Taube,[6] Paul T. Nghiem,[3,5] Martin A. Cheever,[3,4] Steven P. Fling,[1,4] and Raphael Gottardo[1,2,8,9,*]

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[8]Centre Hospitalier Universitaire Vaudois et Université de Lausanne, Lausanne, Switzerland
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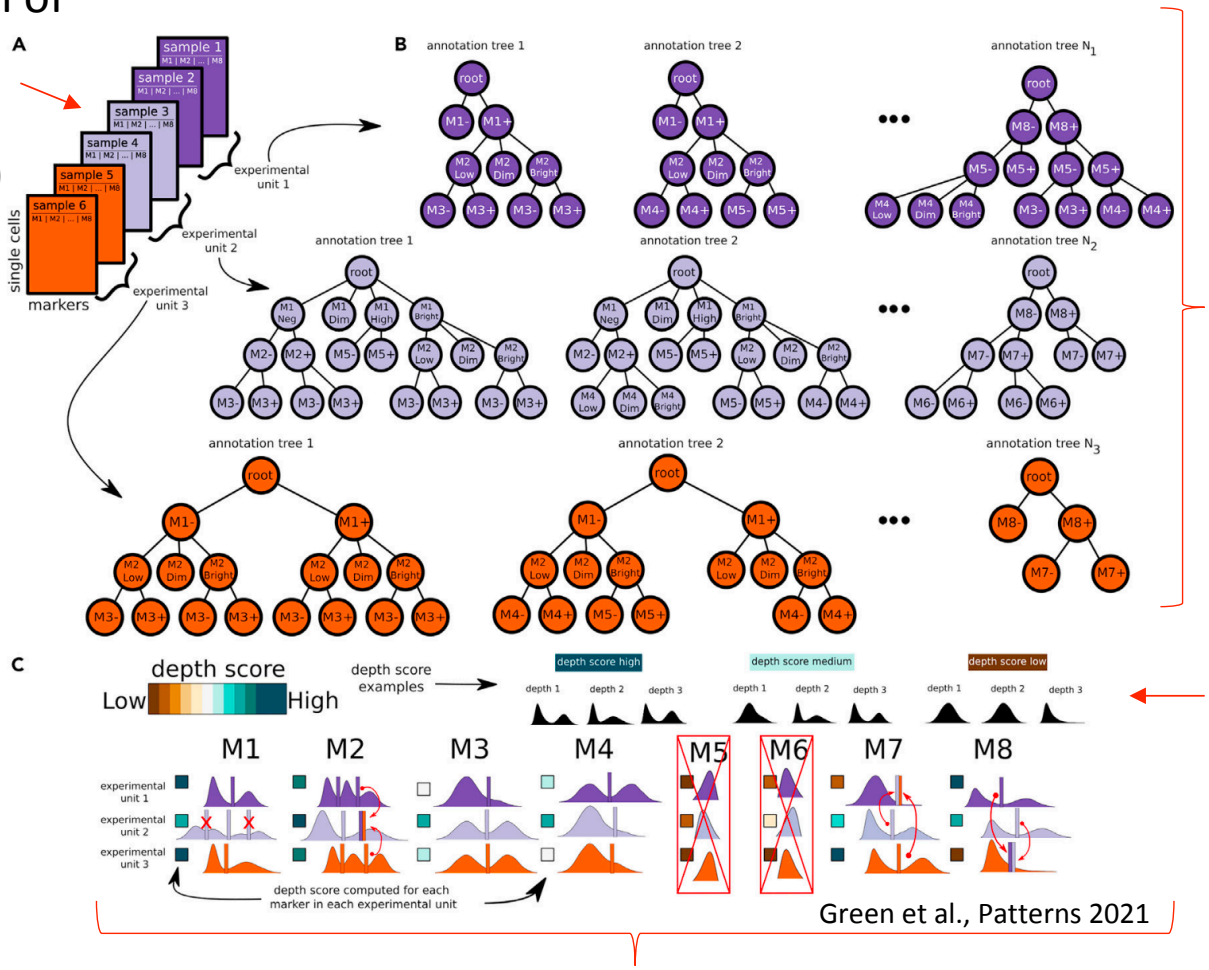
https://doi.org/10.1016/j.patter.2021.100372

Phenotype discovery and annotation with *FAUST*

Deriving a standardized set of thresholds for informative markers

Concatenation of samples by experimental unit (samples)



Annotation forest (exhaustive collection of all 3-marker gating strategies)

Depth score: how consistenly a marker separates into subpopulations

Select high scoring markers and standardize threshols

Phenotype discovery and annotation with *FAUST*

Deriving a standardized set of thresholds for informative markers

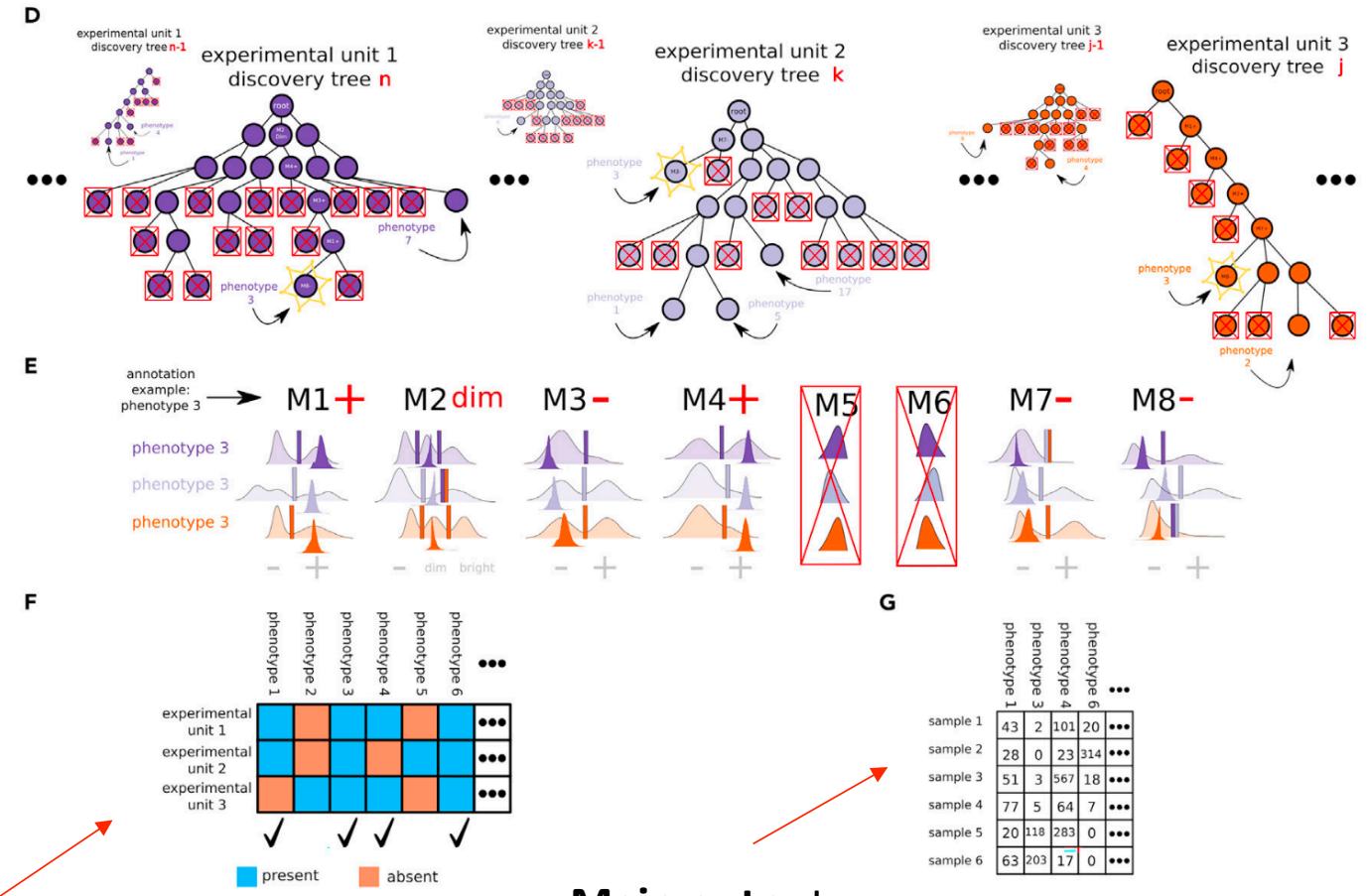
Multiple discovery forests for each experimental unit

+

Select a subset of high-scoring leaves (clusters) that jointly partition the experimental unit

+

Match clusters across experimental units



Main output:
Sample-by-phenotype cell count matrix

Phenotype discovery and annotation with *FAUST*

There are three vignettes in this packages.

- *faustIntro* is a quick introduction to the main faust function
- *faustTuning* has a discussion about how to tune different parameters available in the package
- *faustPFDA* provides an example of how to fit a PFDA (Phenotype and Functional Differential Abundance) model to the output count matrix

FAUST Workflow

A FAUST analysis has two phases :

- **1st Tuning the parameters**: marker scoring, selection, and threshold standardization (*generateAnnotationThresholds*)
- **2nd Phenotype discovery**: once the standardized thresholds have been approved by the user (*discoverPhenotypes)*

Tuning the parameters

```
> generateAnnotationThresholds(gatingSet = gs,
                               startingCellPop  = "root",
                               projectPath  = "course_datasets/FAUST/",
                               plottingDevice  = "png",
                               depthScoreThreshold = 0.85,
                               selectionQuantile   = 0.5,
                                activeChannels     = c("V1","V4","V5"))
```

Threshold to use for the depth score criterium

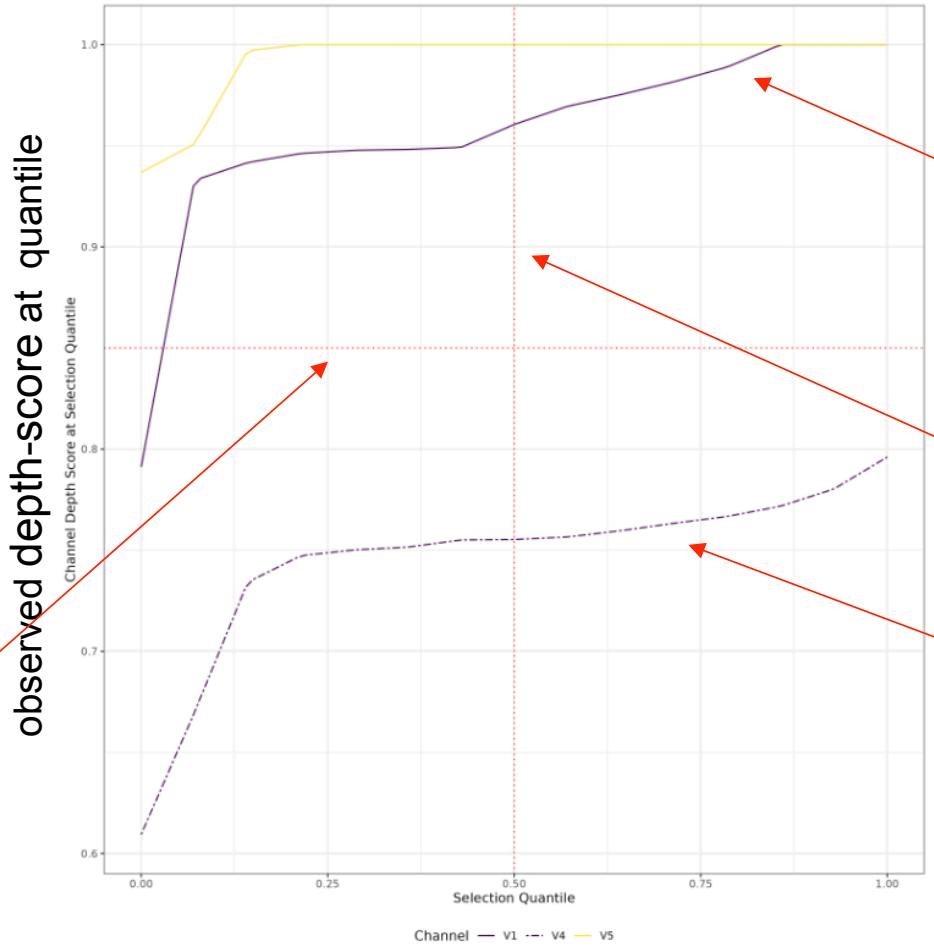
Threshold to use for the quantile criterium

markers to use (“type”)

Once complete, a *faustData* directory will be created inside the project path, with a sub-directory called *plotData*

Tuning the parameters - output

scoreLines.png



quantiles of the depth score

Solidly-colored markers = markers selected for analysis

threshold for the parameter *selectionQuantile*

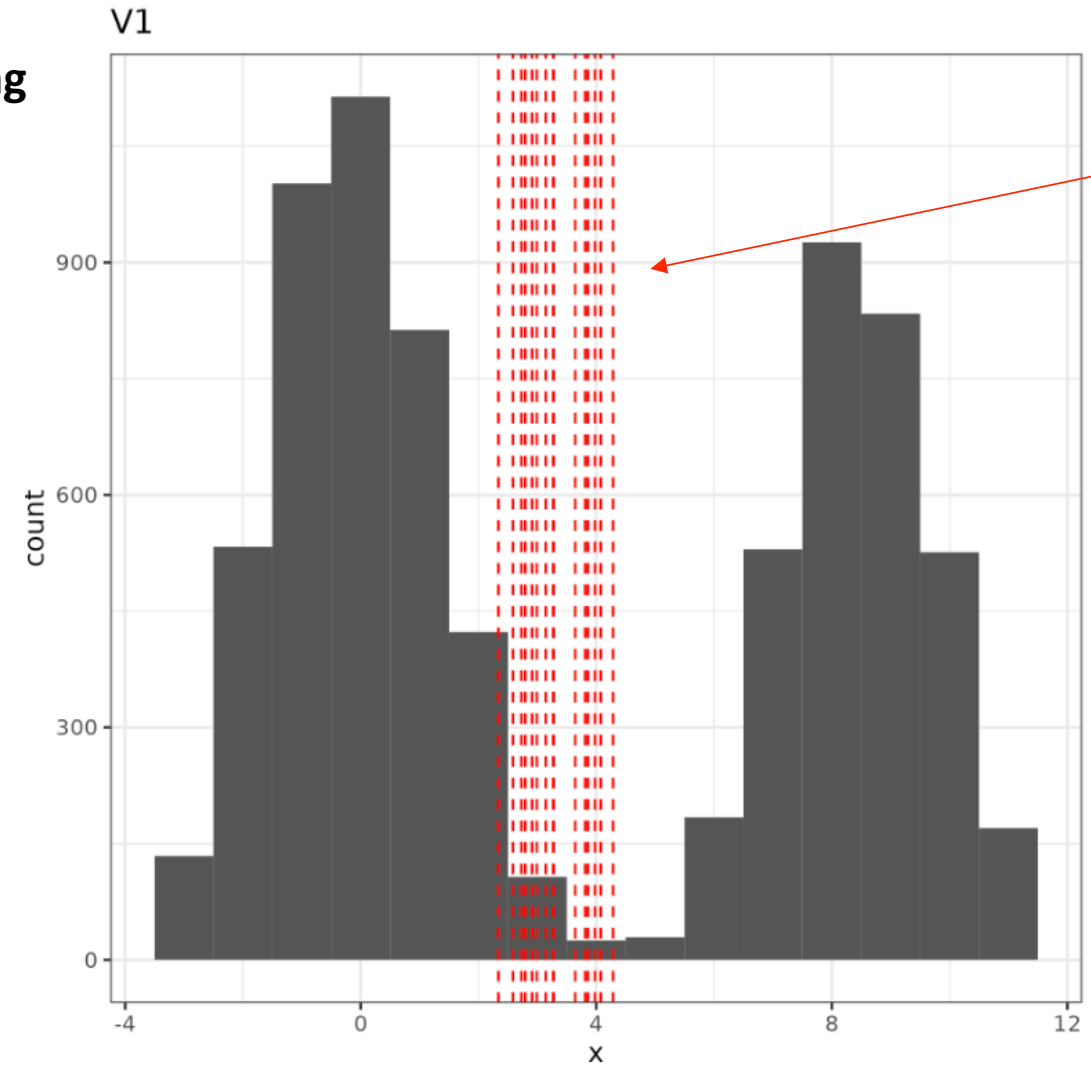
Dashed-colored markers = markers not selected for analysis

Threshold for the parameter *depthScore*

All markers with depth score above the *depthScore* threshold at the specified *selectionQuantile* will be included for phenotype discovery

Tuning the parameters - output

hist_V1_ab_1.png



annotated thresholds for each sample

These plots can be useful in checking for batch effects or for other technical effects that may affect a subset of samples in large experiments.

Discovering phenotypes

```
> discoverPhenotypes(gatingSet   = gs,
                     projectPath = "course_datasets/FAUST/",
                     threadNum   = 4)


# Alternative : run the faust function (wraper around the two previous functions)
> faust(gatingSet = gs,
        startingCellPop = "root",
        depthScoreThreshold = 0.85,
        selectionQuantile   = 0.5,
        projectPath = "course_datasets/FAUST/",
        activeChannels      = c("V1","V4","V5"),
        annotationsApproved = TRUE)
```

FALSE by default (encourage the user to review the proposed parameter values).
When set to TRUE, indicates the user wants to use the proposed parameters.

If you want to run the FAUST method totally unsupervised, set this parameter to true before running the faust function.

Examine output: annotated count matrix

```
# Annotated count matrix
> count_df <- readRDS("course_datasets/FAUST/faustData/faustCountMatrix.rds")
> count_df <- as.data.frame(count_df)
> count_df
```

discovered cell populations

samples

	V5-V1-	V5+V1-	V5+V1+	0_0_0_0_0
sample001	162	100	235	3
sample002	177	94	228	1
sample003	184	102	213	1
sample004	183	104	210	3
sample005	170	99	231	0
sample006	164	113	223	0
sample007	181	107	211	1
sample008	175	103	219	3
sample009	186	105	205	4
sample010	187	104	209	0
sample011	182	105	210	3
sample012	196	79	225	0
sample013	174	96	230	0
sample014	179	102	218	1
sample015	183	109	208	0

- The columns are annotated by a selected subset of markers used in conducting the experiment.
- The annotations define, in terms of these markers, the phenotypes of all cell populations discovered by the pipeline.

number of cells in a sample that belong to a discovered cell population

Examine output: UMAP with phenotypes

```
# Create DF with embedings (UMAP)
 > annoEmbed <-  makeAnnotationEmbedding( projectPath="course_datasets/FAUST/",
                                          sampleNameVec= pData(gs)$name)

# plot the UMAP
 > ggplot(annoEmbed,
            aes(x=umapX,
                y=umapY,
                color=faustLabels))+
     geom_point()+
     theme_classic()+
     xlab("Annotation Embedding X")+
     ylab("Annotation Embedding Y")+
     facet_grid(vars(sampleOfOrigin))+
     theme(legend.position="bottom")
```