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**

Outline & Schedule

Day 4

05 **Generate html or PDF reports**

Examples and exercises are integrated in the chapters

01

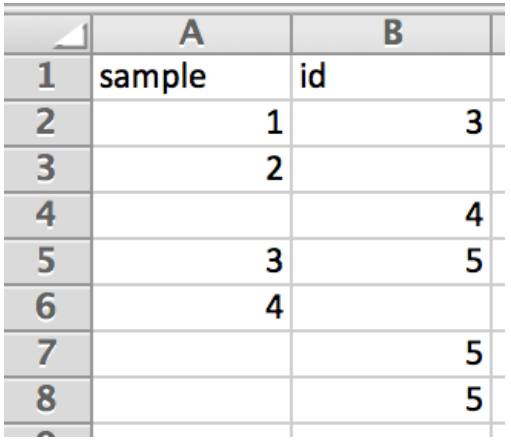
A. Importing data from Excel
B. Arranging plots in grids

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

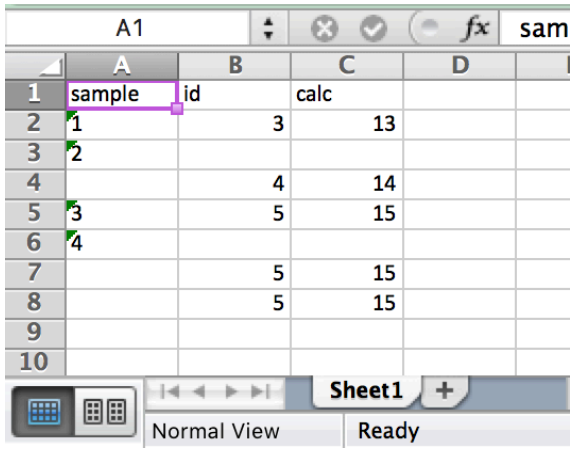
```
> str(data)
'data.frame':   7 obs. of  2 variables:
 $ sample: num  1 2 NA 3 4 NA NA
 $ id    : num  3 NA 4 5 NA 5 5
```

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")
```



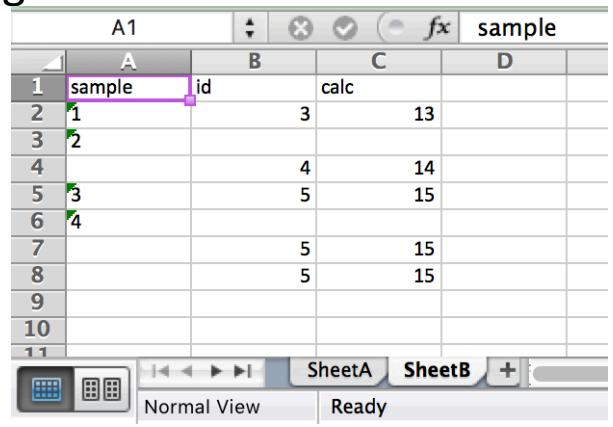
Another package: *openxlsx*

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

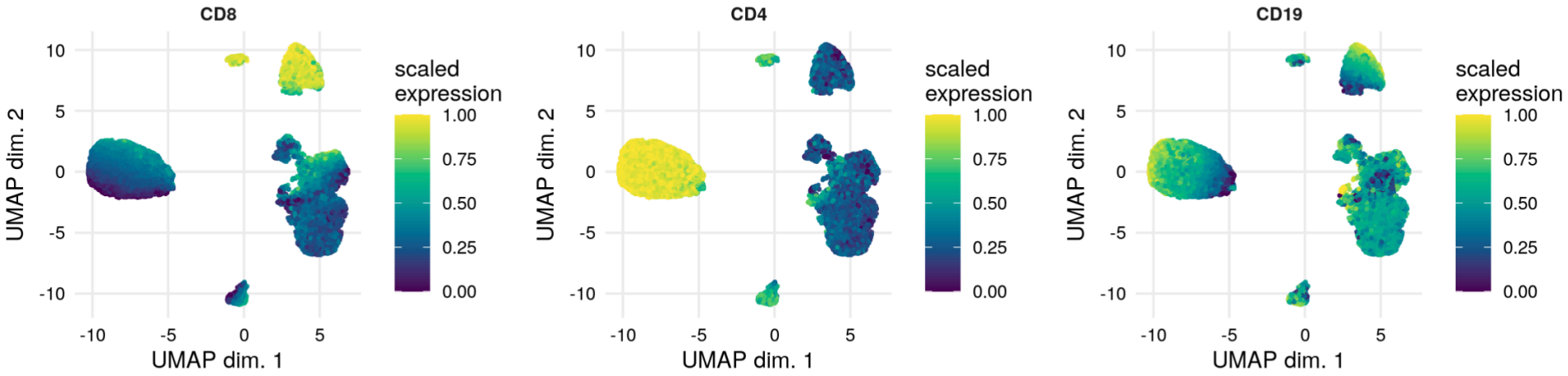
First save each ggplot2 plot to an object:

```
> 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
```

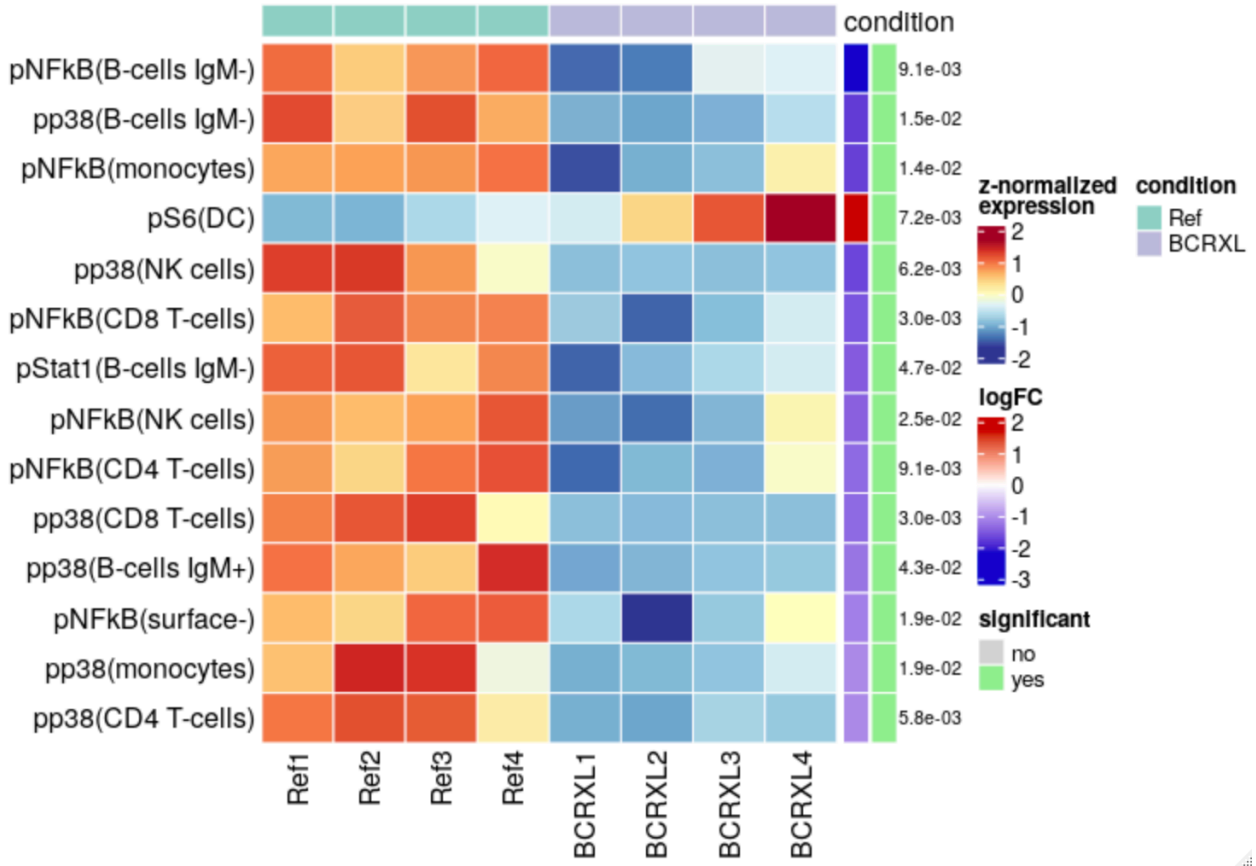
```
DataFrame with 20 rows and 4 columns
             cluster_id marker_id       p_val     p_adj
               <factor>  <factor>   <numeric> <numeric>
Other       Other          CD38   0.000883343  0.128085
Other       Other          TCR gd 0.006609759  0.479208
CD4 T cells CD4 T cells    CD141  0.027918113  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**

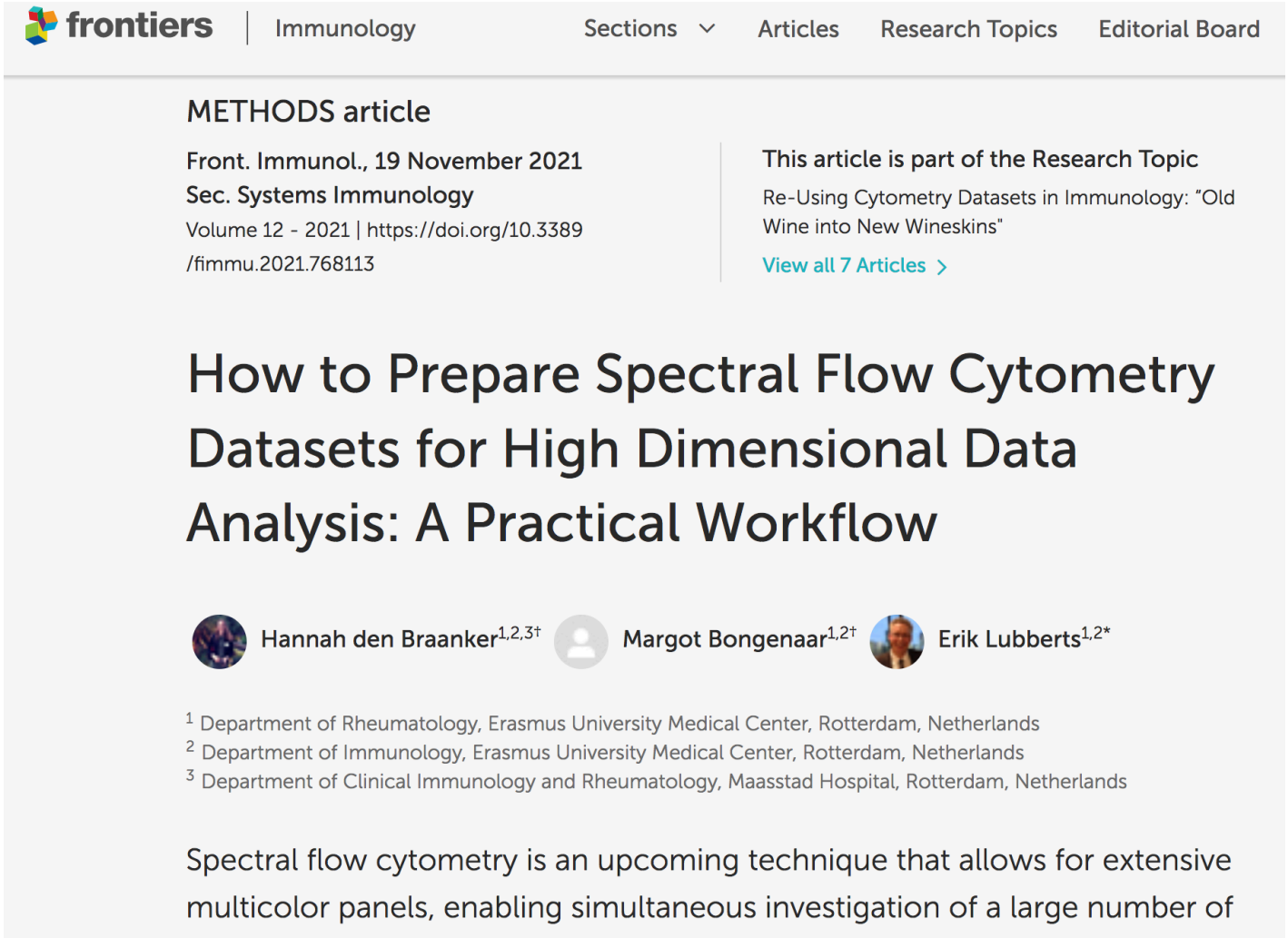
Create a new script in which you will

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

Hannah den Braanker[1,2,3†] Margot Bongenaar[1,2†] Erik Lubberts[1,2*]

[1] Department of Rheumatology, Erasmus University Medical Center, Rotterdam, Netherlands
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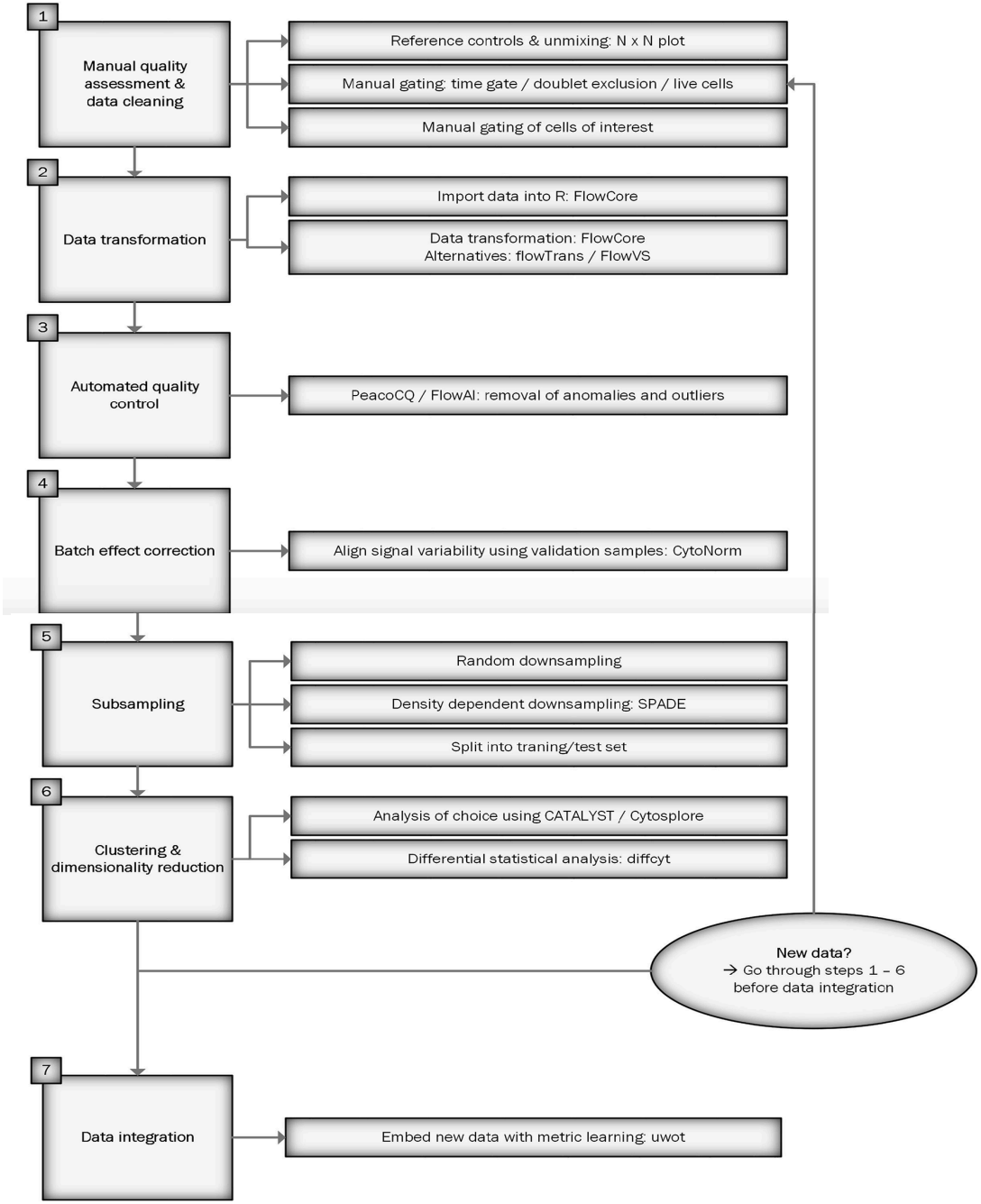
[3] Department of Clinical Immunology and Rheumatology, Maasstad Hospital, Rotterdam, Netherlands

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

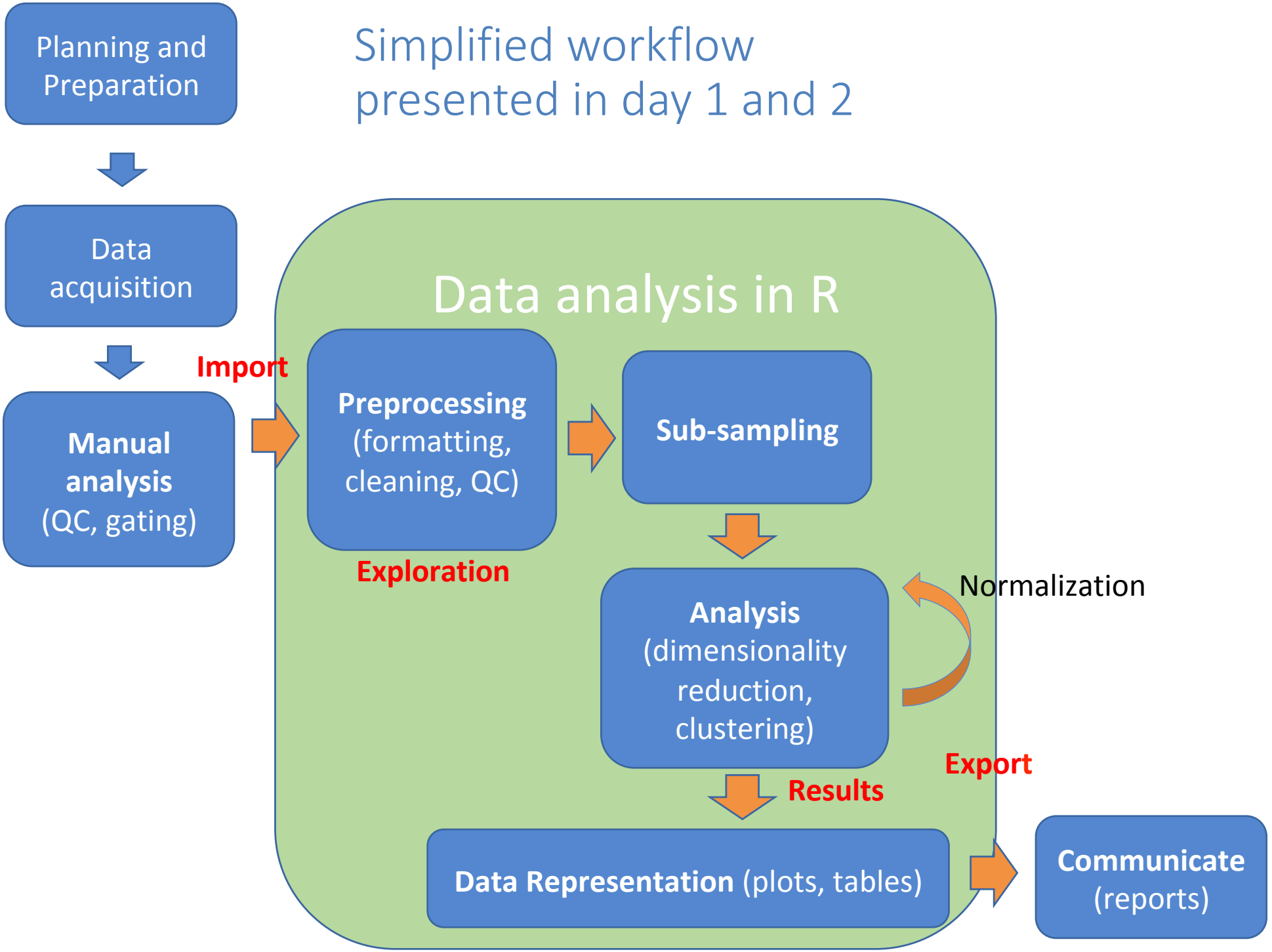
Provide R code to perform the proposed workflow

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

Suggested workflow: Figure 1, den Braanker et al



Simplified workflow presented in day 1 and 2



Spectral flow cytometry analysis workflow

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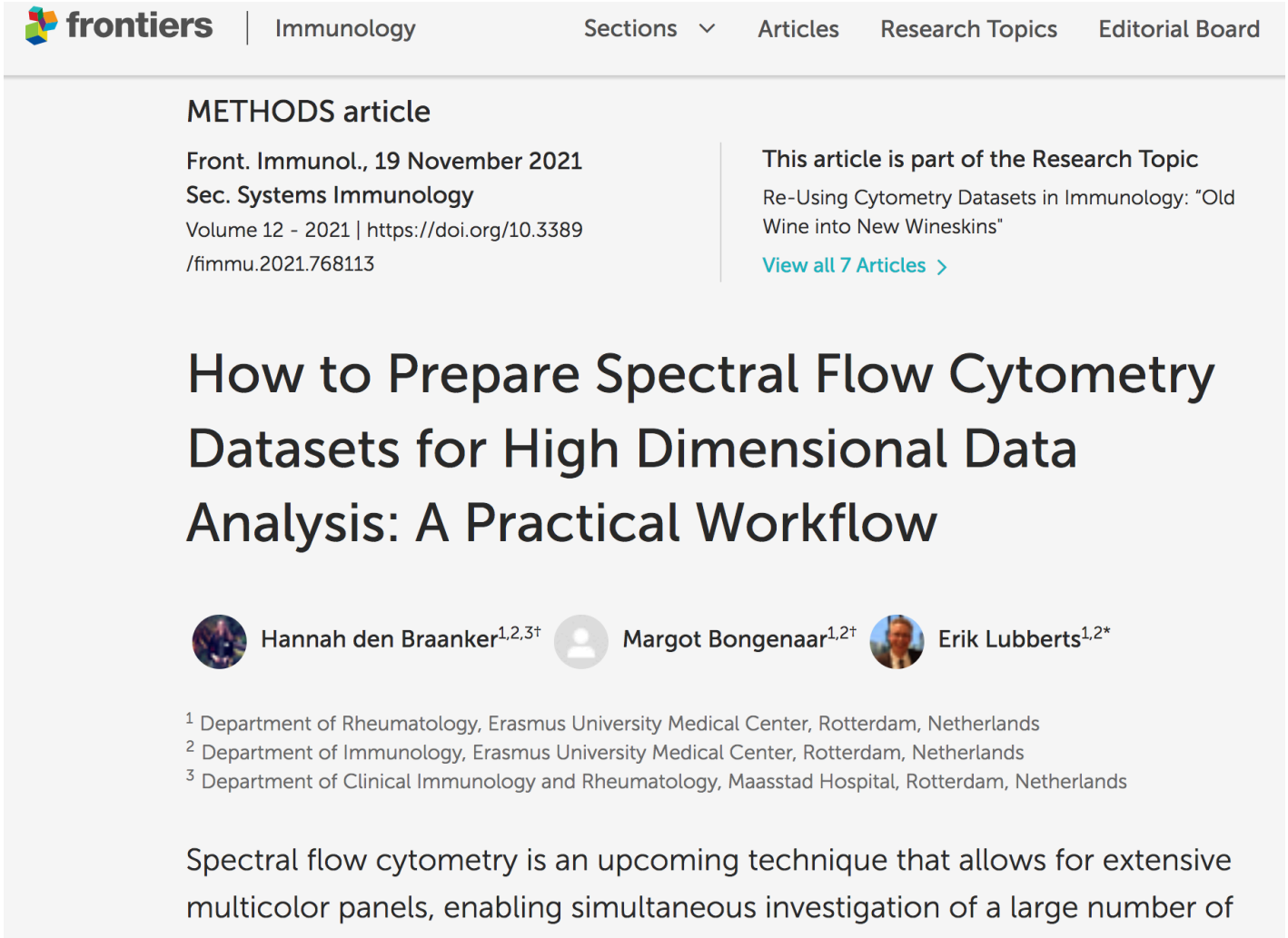

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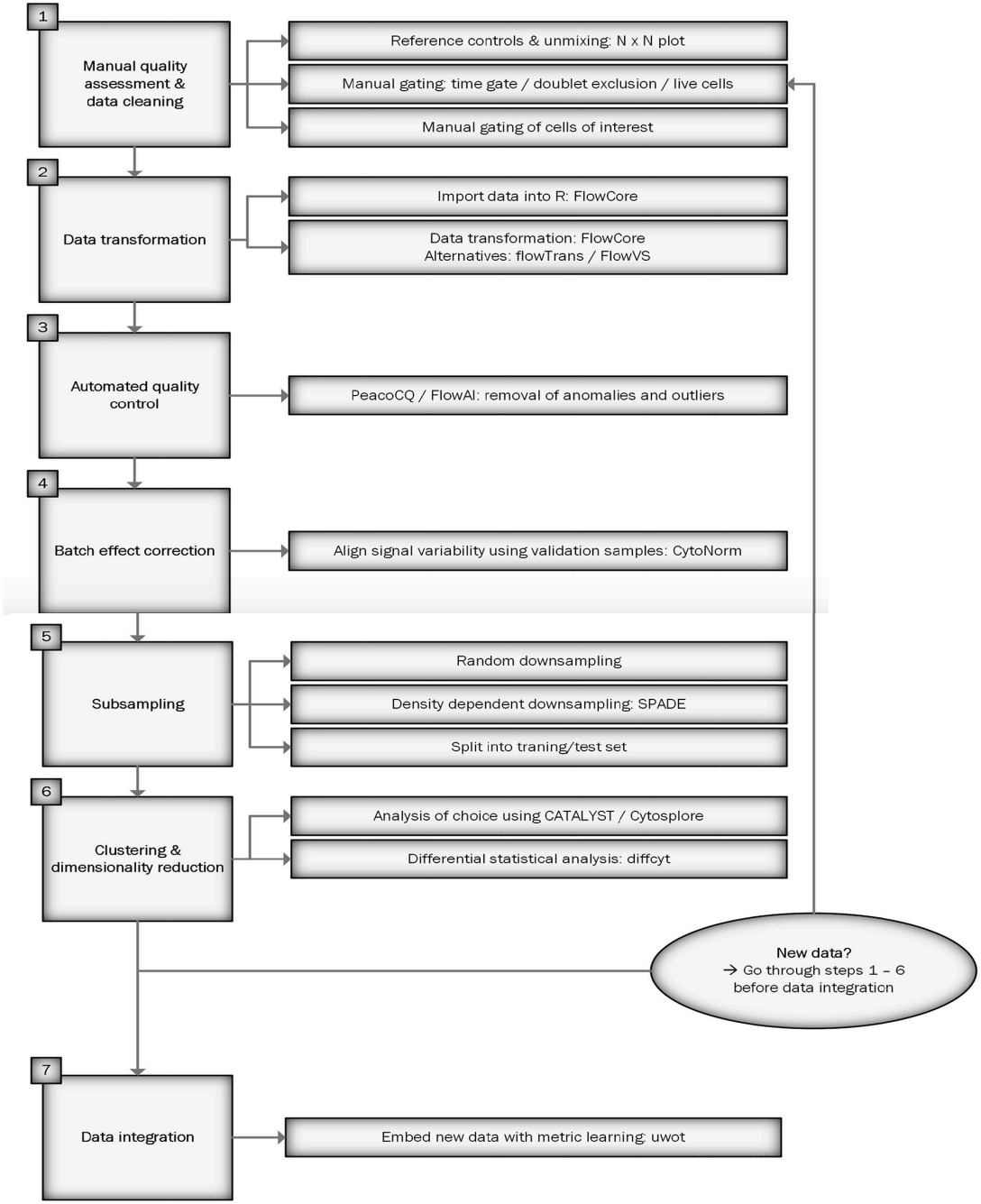

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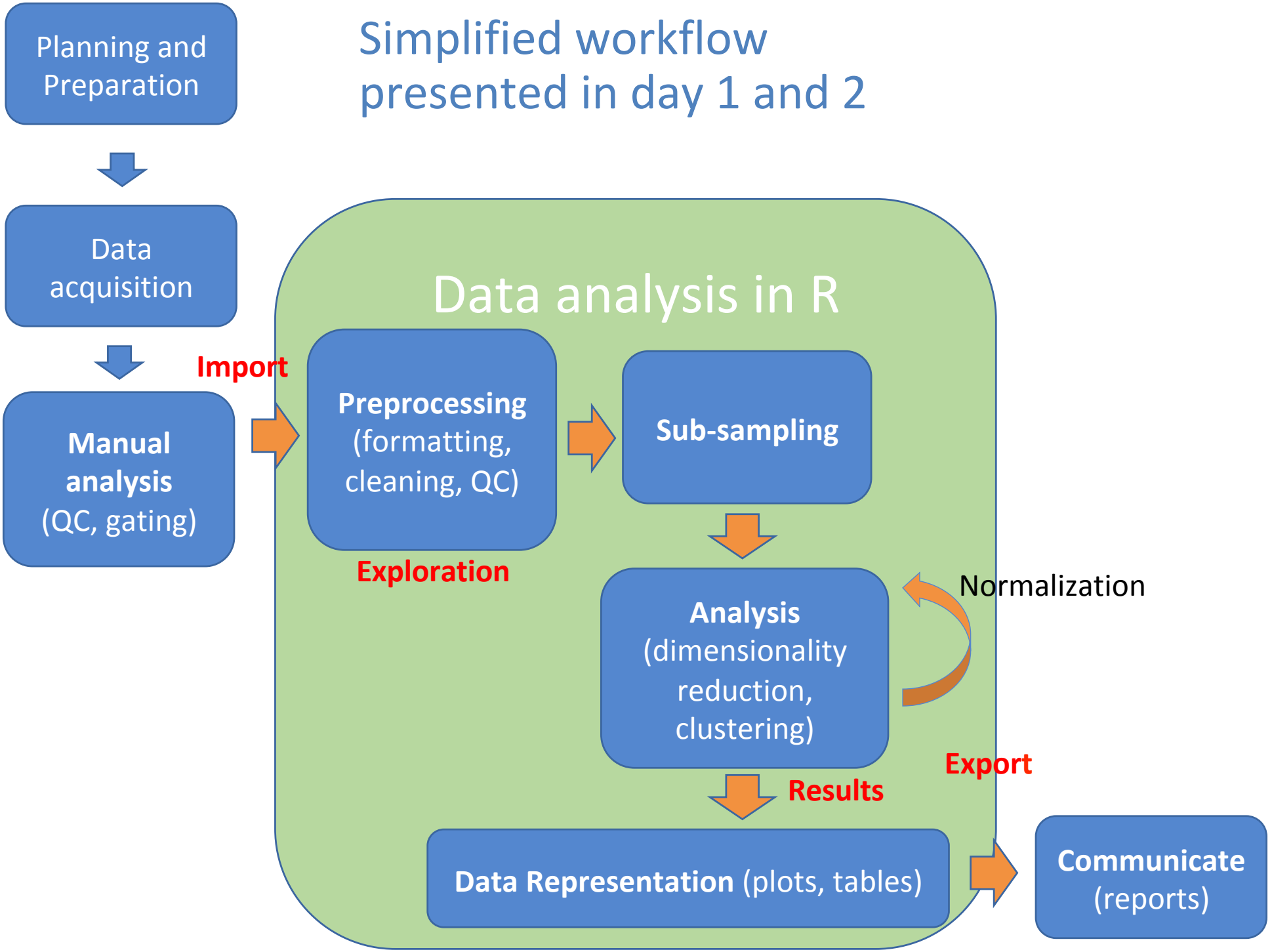


CytoNorm

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')
```

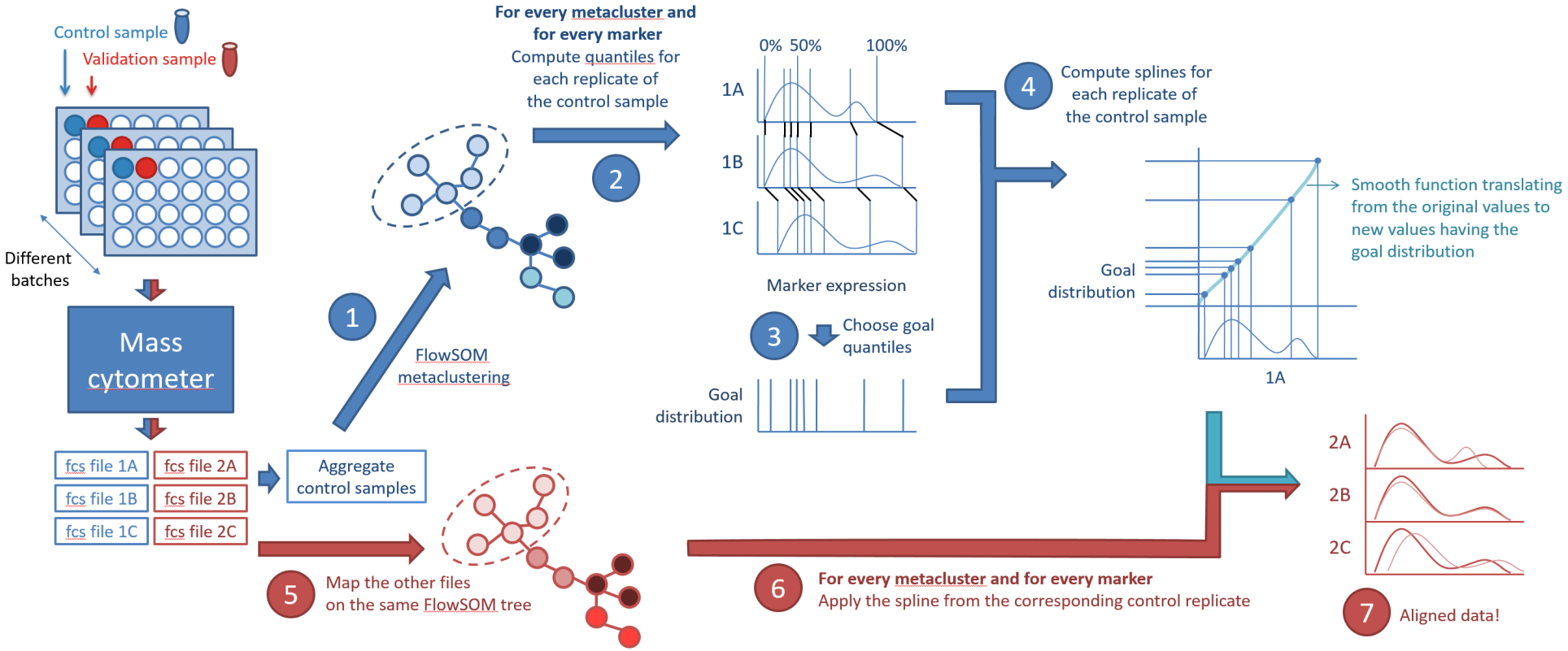
Why do we need to normalize?

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

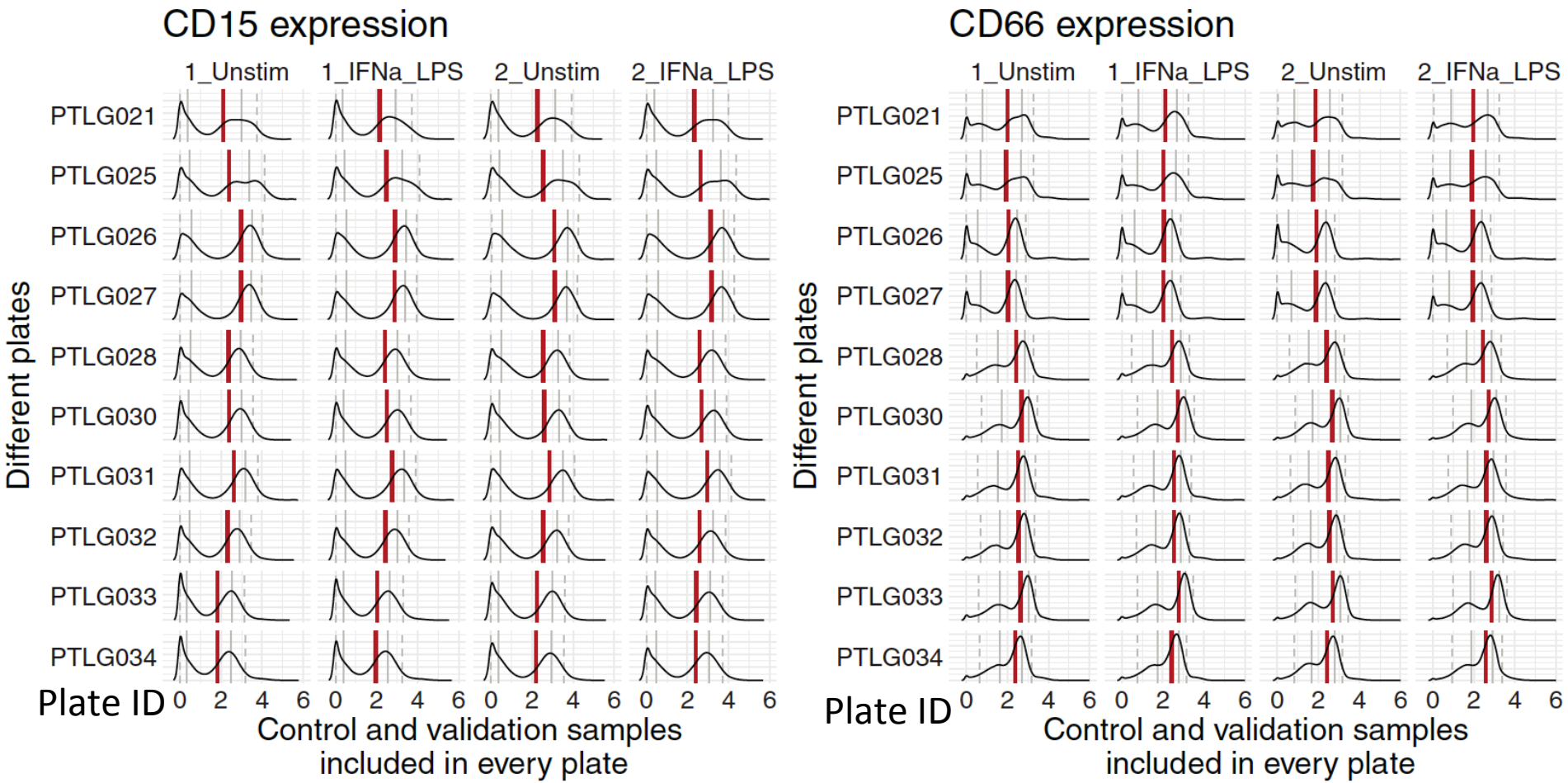
Computational methods can align the distribution of markers across samples.

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)

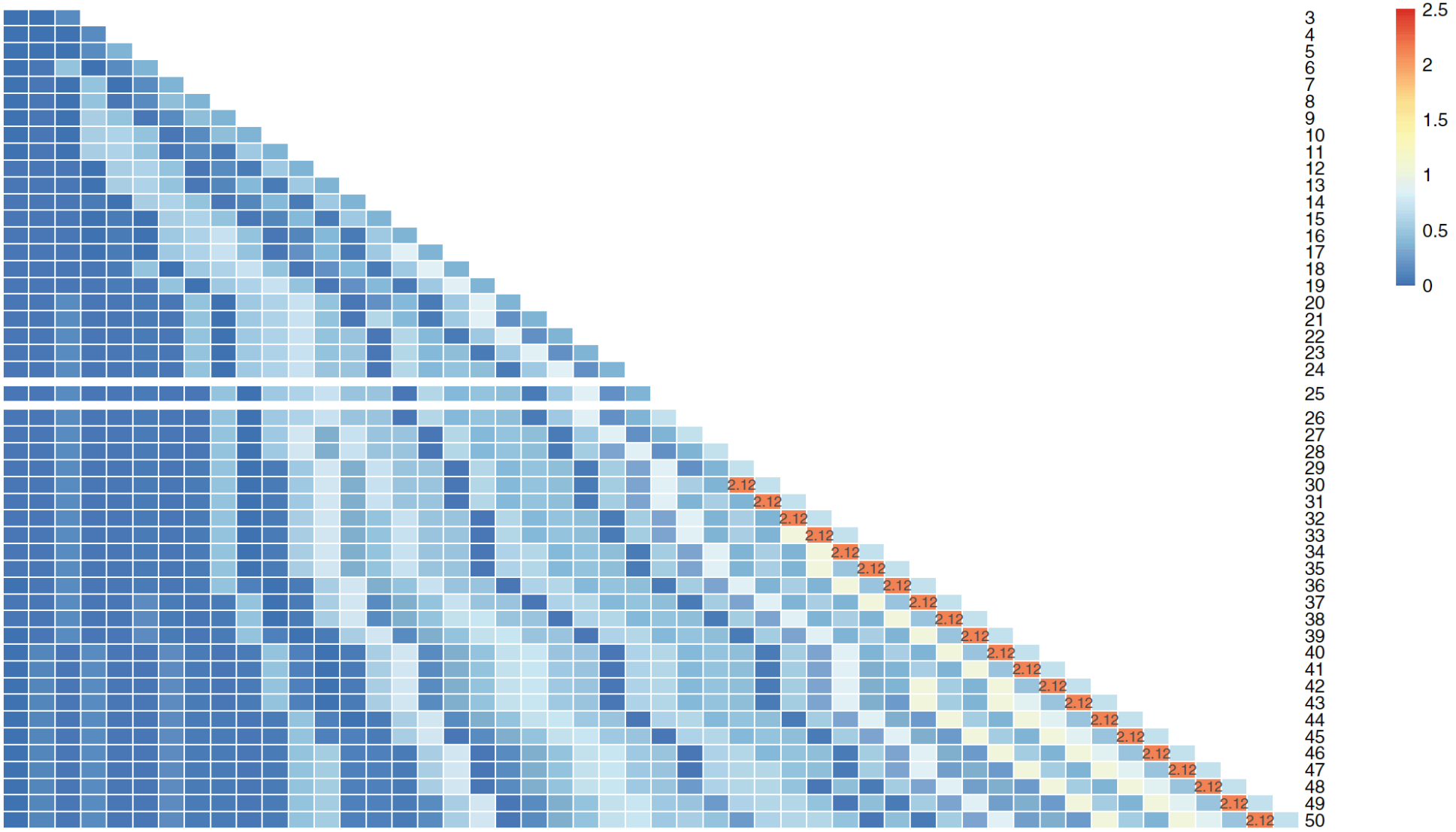
CytoNorm – algorithm overview



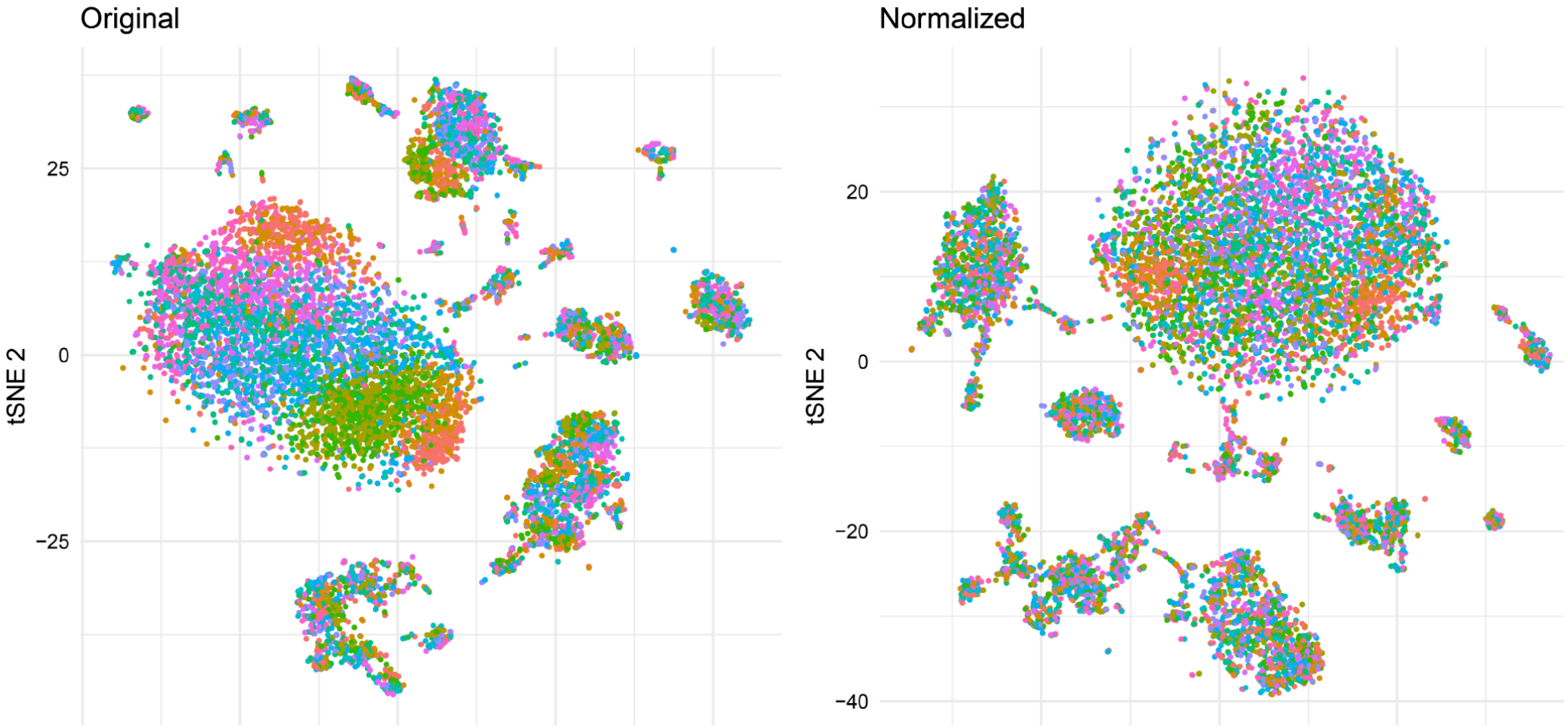
An example of shifts across plates



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".

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:

5) Perform pre-clustering with flowSOM with function prepareFlowSOM(), providing the flowSet with the training flowFrames, the vector of channels to transform, 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.

04 Working with gated data in R

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")
```

REMOVE THIS SLIDE?

OR JUST mention we are using the testing data from this package. I will copy the files to the posit, but you can also access the data through this package (AND THEN ADD THE CODE SHOWING HOW ?)

Create a *gatingSet*

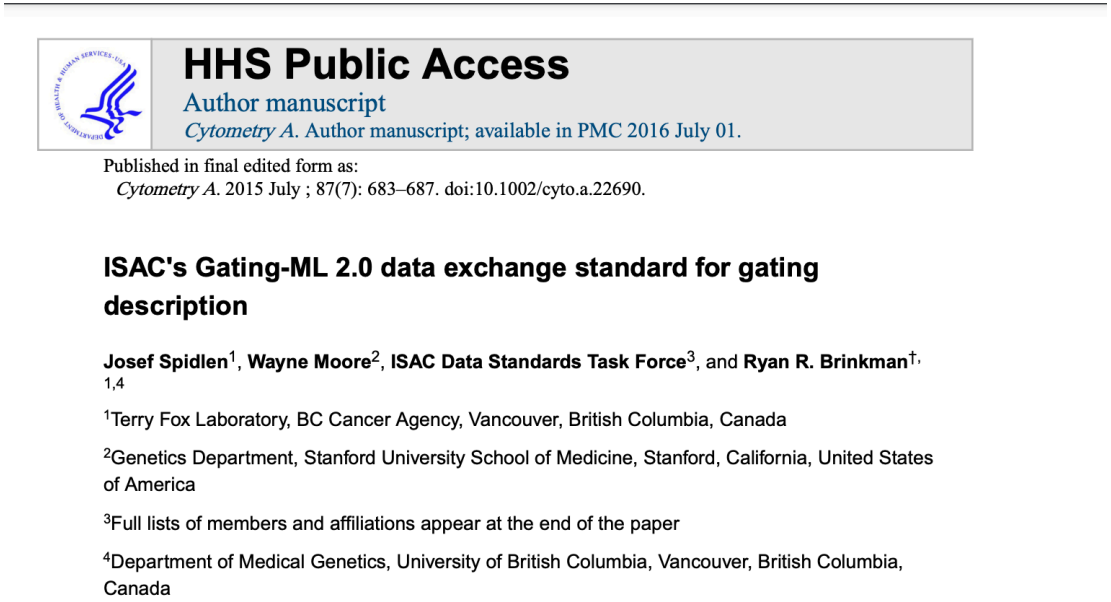
Ways to generate a *gatingSet:*

- Imported from workspace XML files from FlowJo, CytoBank or other software 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

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



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")
```

Check the name of the function for cytobank

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
```

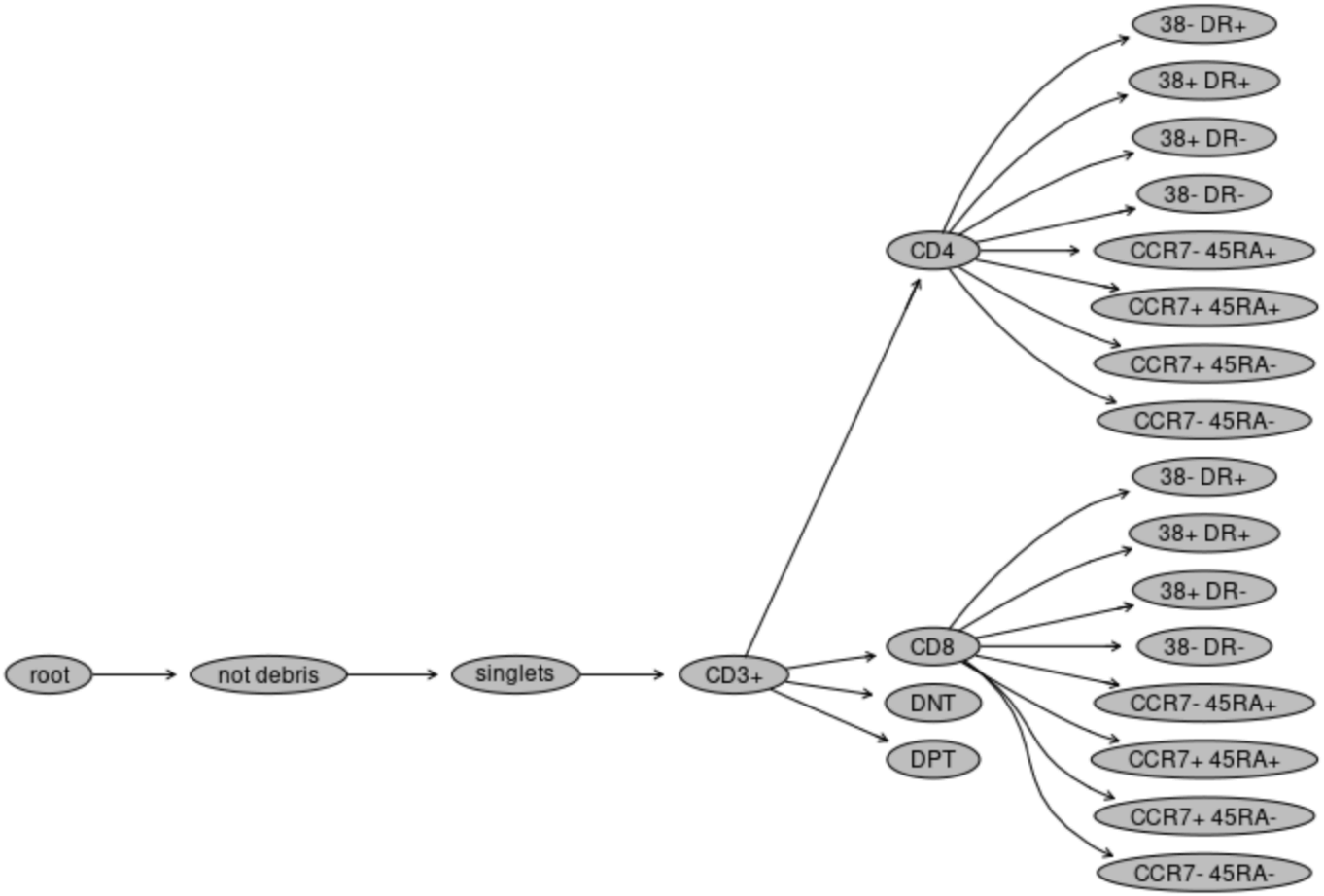
 # Retrieve a GatingHierarchical (one sample)

```
> 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)

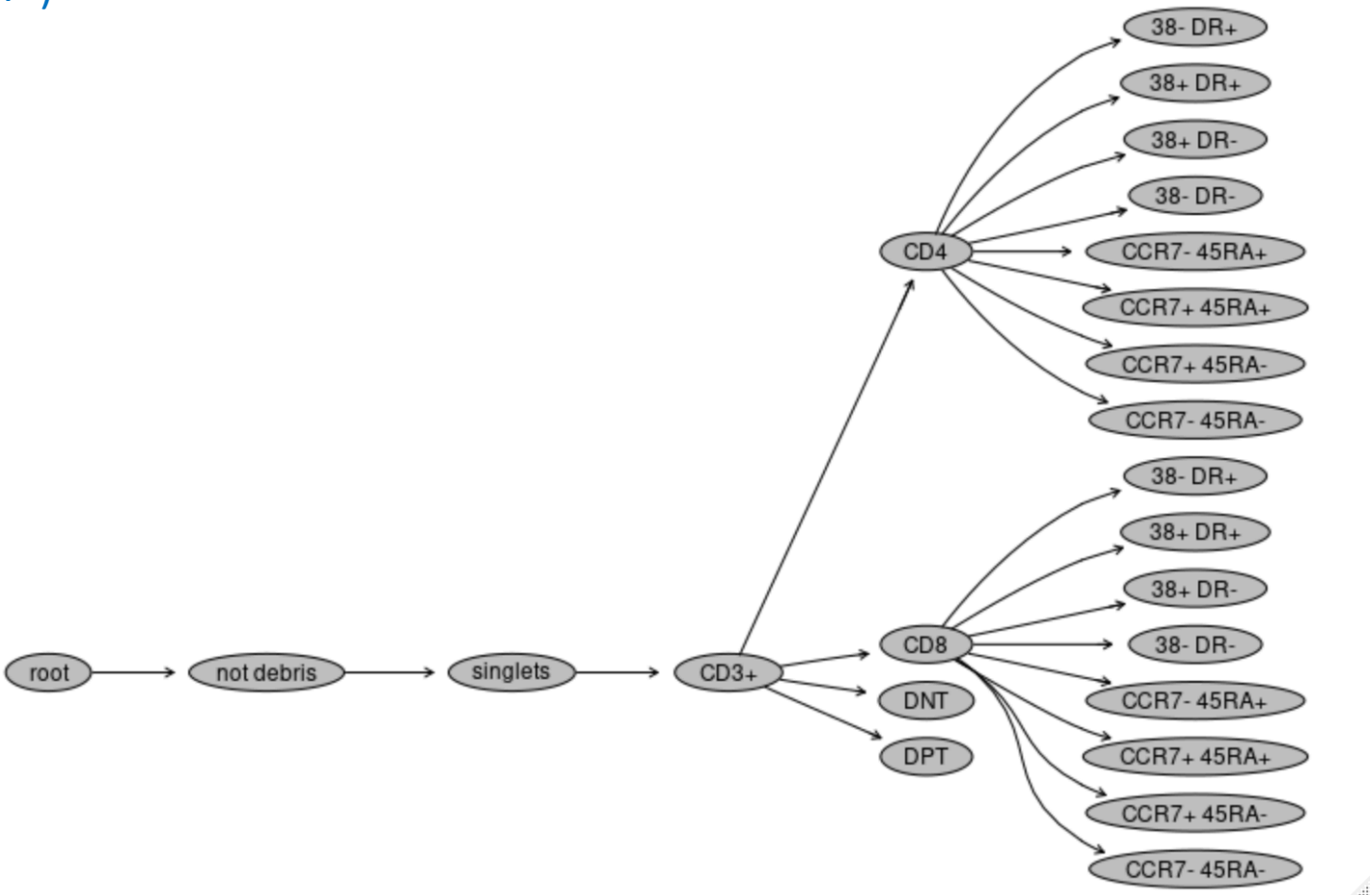


flowWorkspace: basics on *GatingSet* objects

 # Delete a gate

> Rm(gs, "DPT")

> 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

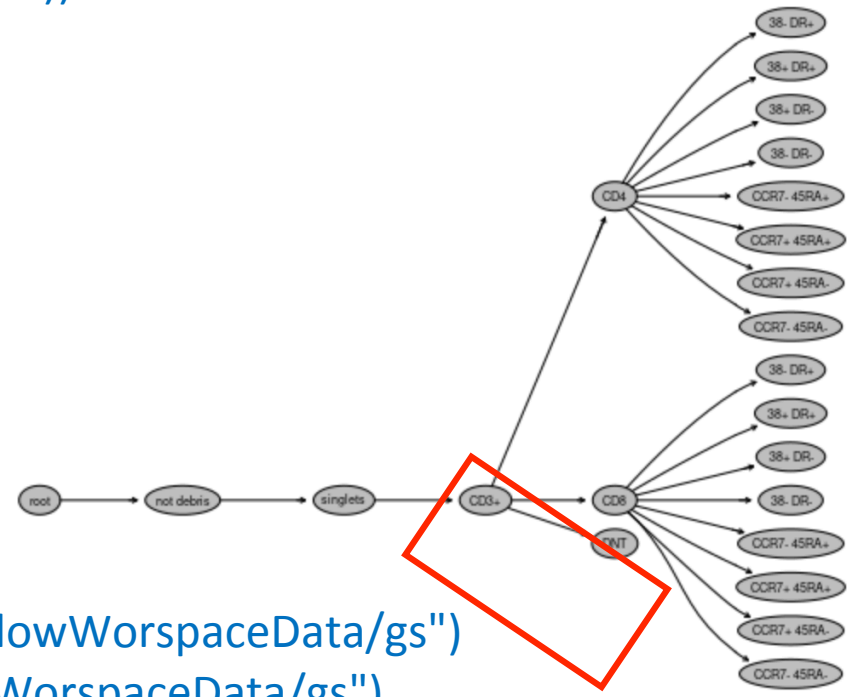
```
> gh_pop_get_indices(gs[[1]], "CD4")
> table(gh_pop_get_indices(gs[[1]], "CD4"))
```

```
FALSE  TRUE
85499 34032
```

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

 # Delete a gate

```
> Rm('DPT', gs)
> plot(gs)
```



 # 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

FlowWorkspace: build the *GatingSet* from scratch

Start from a *flowSet* (*flowCore*)

```
> load("course_datasets/FR_FCM_Z4KT/fcs_transform.RData") # load the flowSet
> 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
```

flowWorkspace: preprocessing functions

Possibility of transforming data (same as with a *flowSet*)

```
# Logicle transformation
 > tf <- estimateLogicle(gs[[1]], channels)
 > gs <- transform(gs, tf)
```

```
# Arcsinh transformation
 > trans.obj <- asinhGml2_trans()
 > channels <- c("","")
 > transList <- transformerList(channels, trans.obj )
 > gs <- transform(gs, tf)
 > autoplot(...)
```

Other built in functions: flowjo_biexp_trans, flowjo_fasinh_trans and logicle_trans

Will test these

FlowWorkspace: transformations

Retrieve the transformations

```
> trans <- gh_get_transformations(gs[[1]])
> trans[[1]]
```

Could we also apply QC methods ?

FlowWorkspace: basic 1-D gating

THIS PART COMES FROM THE FLOWCORE VIGNETTE

Create a basic gate and add to the gs

```
> rg1  <- rectangleGate("FSC-A"=c(50), filterID="NonDebris")
> gs_pop_add(gs, rg1, parent = "root")
```

Show the nodes beforec

Check the nodes (populations)

```
> gs_get_pop_paths(gs)
```

Replace this with real code

Show the nodes afterwards

Gate the data and plot the gate

```
> recompute(gs)
> autoplot(gs, "NonDebris")
```

Add plots

Get population statistics

```
> gs_pop_get_stats(gs[[]1], "NonDebris") # counts
> gs_pop_get_stats(gs[[]1], "NonDebris", type = "percent") # proportions
```

Move this to the end

FlowWorkspace: 2-D gating with polygon

Create a matrix with polygon coordinates

```
> mat  <- matrix(c(...), nrow=5)
> colnames(mat) <- c("FSC-A","FSC-H")
```

Create the gate and add to the gs

```
> pg <- polygonGate(mat)
> gs_pop_add(gs, pq, parent = "NonDebris", name = "Singlets")
```

Gate the data

```
> recompute(gs)
> autoplot(gs, "Singlets")
```

Add results

FlowWorkspace: 2-D gating with quadrant gates

A *quadrantGate* results in four sub-populations

```
> qg  <- quadGate("B710-A" = 2000, "R780-A" = 3000)
>  gs_pop_add(gs, qg, parent = "CD3", name = c("CD8","DPT","CD4","DNT"))
```

Check the gating hierarchy

```
> gs_pop_get_children(gs[[1]], "CD3")
```

Gate the data

```
> recompute(gs)
```

I skiped one gating here, but it would be nice to have a slide before. See the flowCore vignette

Add results

FlowWorkspace: Boolean gates

A *booleanFilter* selects several quadrants

```
> bg  <- booleanFilter("CD8-CD4+|CD8-CD4+")
>  gs_pop_add(gs, bg, parent = "CD3")
```

Check the gating hierarchy

```
> gs_pop_get_children(gs[[1]], "CD3")
```

Gate the data

```
> recompute(gs)
```

Plot the hierarchy

```
> plot(gs)
> plot(gs, bool=TRUE) # with boolean gate
```

Plot the gates

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

I skiped one gating here, but it would be nice to have a slide before. See the flowCore vignette

Add results

THIS WILL HAVE TO BE SPLIT INTO SEVERAL SLIDES

FlowWorkspace: other utilities

Remove nodes from a gating set object

```
> Rm('CD3', gs)
> gs_get_pop_paths(gs)
```

SHOULD THIS BE HERE OR WITH THE BASICS ?

FlowWorkspace: retrieve flow Data and stats

Retrieve the underlying flow data for a gated population

```
> fs_nonDebris <- getData( gs, "NonDebris")
```

THIS IS DUPLICATED

Get statistics

```
> gs_pop_get_count_fast(gs)
```

Add results

EXERCISE ON GATINGSET

- Import a xml workspace from FlowJo
- Plot the gating hierarchy
- Plot the gates
- Add a gate
- Statistics
- Export flow data

flowGate

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

- 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 gatingSets
- You can use transformed data, but flowGate was designed to apply desired transformations at the plotting level only

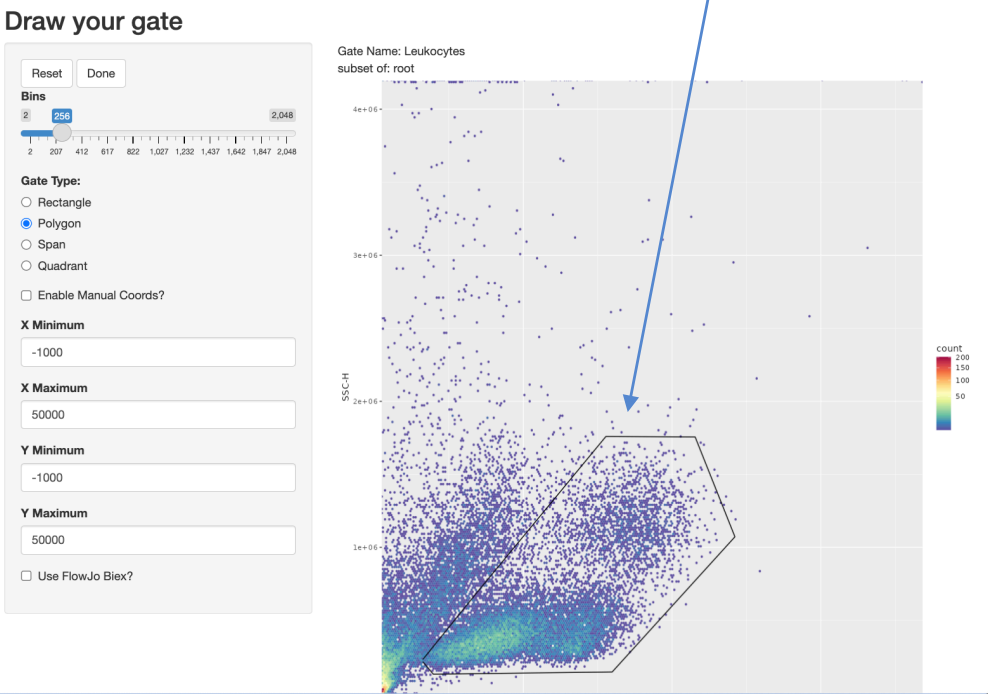
Interactive gating with *FlowGate*

Create a 2-D gate

```
> fs <- read.flowSet(path="course_datasets/FR_FCM_Z3WR/",
                     pattern = "*.fcs",
                     transformation = FALSE,
                     truncate_max_range = FALSE)
> gs <- GatingSet(fs)
> gs_gate_interactive(gs,
            filterId = "Leukocytes",
            dims = list("FSC-H", "SSC-H"))
```

gate

type of gate



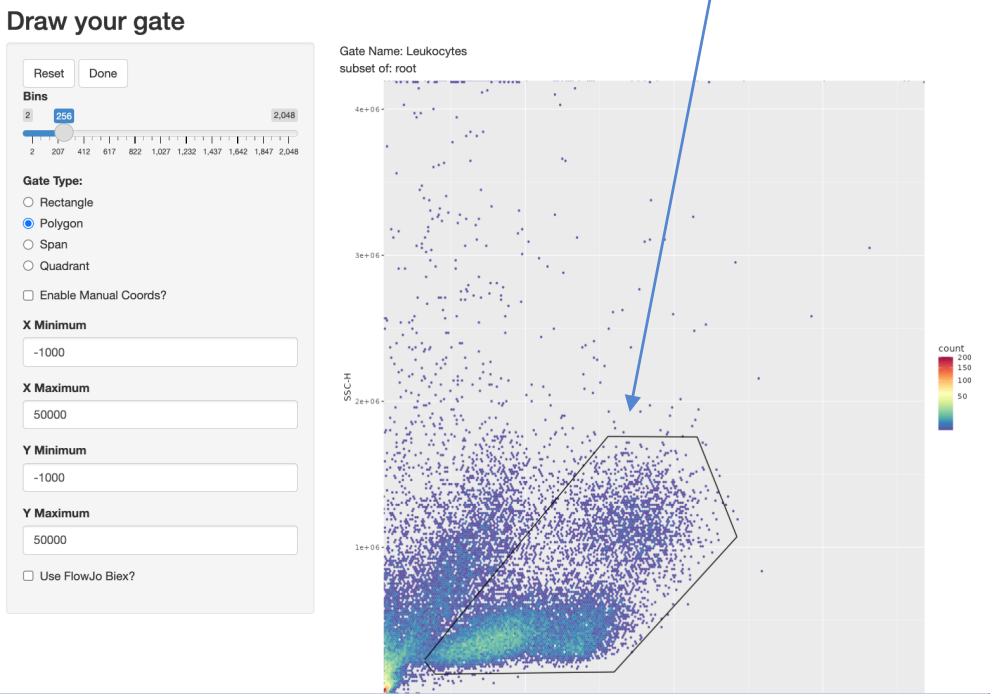
Interactive gating with *FlowGate*

Create a 2-D *polygon* gate

```
> fs <- read.flowSet(path="course_datasets/FR_FCM_Z3WR/",
                     pattern = "*.fcs",
                     transformation = FALSE,
                     truncate_max_range = FALSE)
> gs <- GatingSet(fs)
> gs_gate_interactive(gs,
            filterId = "Leukocytes",
            dims = list("FSC-H", "SSC-H"))
```

gate

type of gate



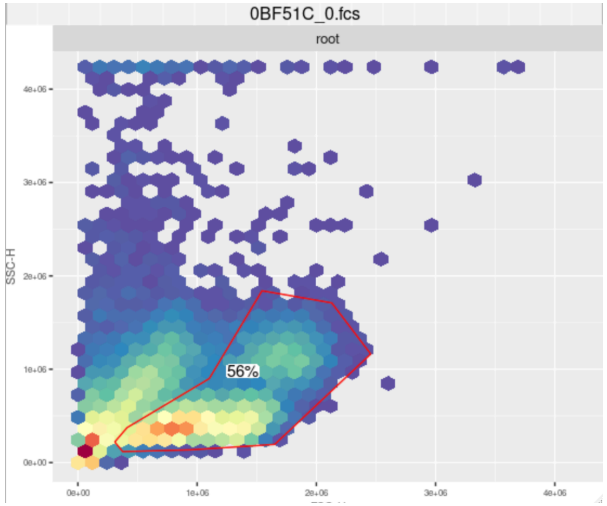
Output message

```
Listening on http://127.0.0.1:5217
done!
$Gate
Polygonal gate 'Leukocytes' with 10 vertices in dimensions FSC-H and SSC-H

$Bins
[1] 256

$Scaling
[1] "unused"
```

Interactive gating with *FlowGate*

Plot the gate

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



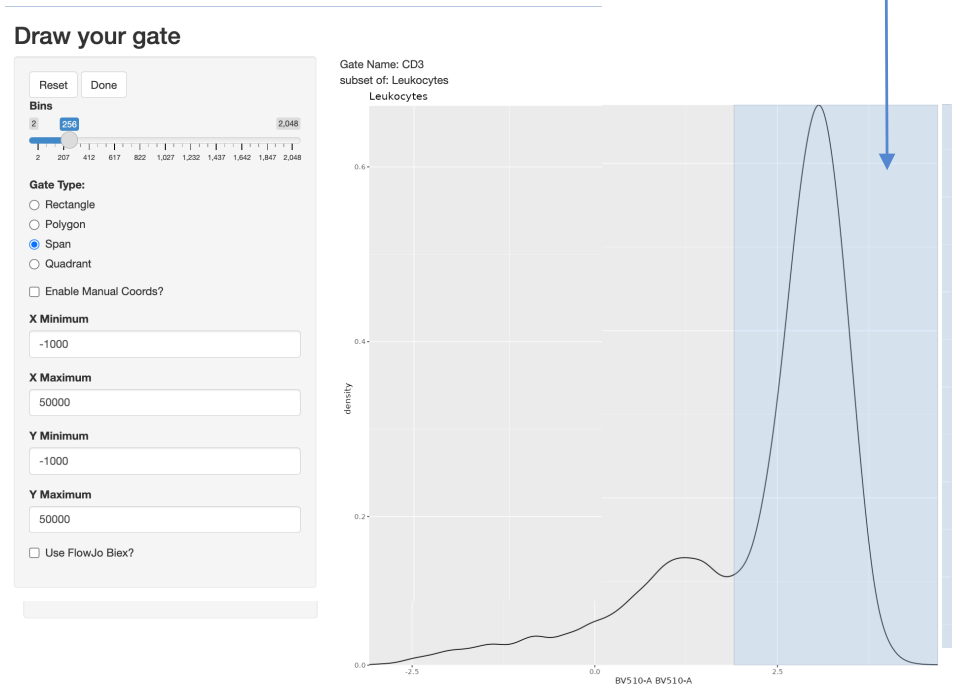
Interactive gating with *FlowGate*

Create a 1-D *span* gate

```
> gs_gate_interactive(gs,
                      filterId = "CD3",
                      dims = "BV510-A",
                      subset = "Leukocytes")
```

gate

type of gate



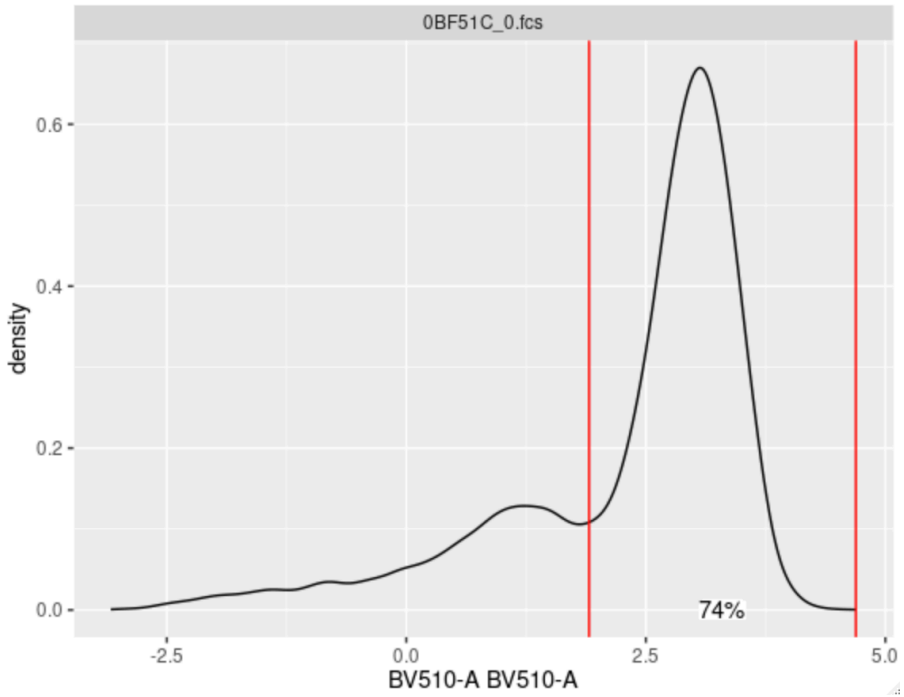
Output message

```
Listening on http://127.0.0.1:6815
done!
$Gate
Rectangular gate 'CD3' with dimensions:
  BV510-A: (1.9068187688965,4.6925115585313)

$Bins
[1] 256

$Scaling
[1] "unused"
```

Interactive gating with *FlowGate*

Plot the 1-D gate

```
> ggcyto(gs[[1]], aes("BV510-A")) +
      geom_density() +
      geom_gate("CD3") +
      geom_stats()
```



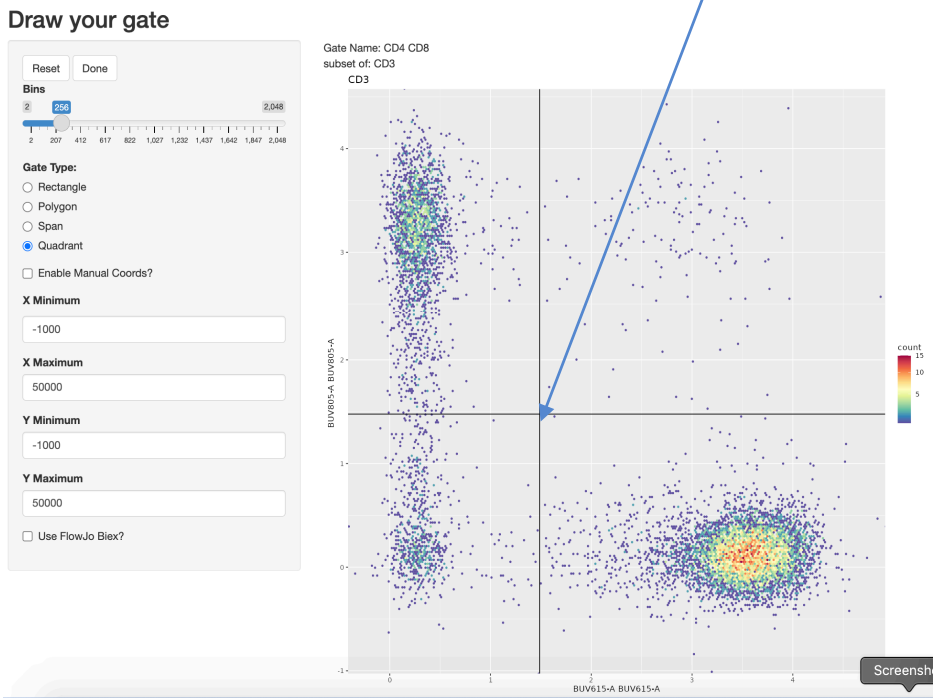
Interactive gating with *FlowGate*

Create a 2-D quadrant gate

```
> gs_gate_interactive(gs,
                      filterId = "CD4 CD8",
                      dims = c("BV510-A",),
                      subset = "Leukocytes")
```

quadrant

type of gate



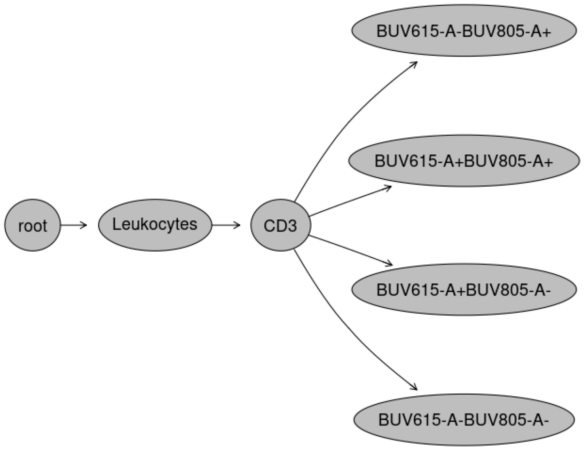
Output message

```
Listening on http://127.0.0.1:6815
Coordinate system already present. Adding new coordinate system, which will
replace the existing one.
done!
$Gate
Quadrant gate 'CD4 CD8' with dimensions:
  BUV615-A: 1.487559
  BUV805-A: 1.476832

$Bins
[1] 256

$Scaling
[1] "unused"
```

Interactive gating with *FlowGate*

Plot the gating hierarchy

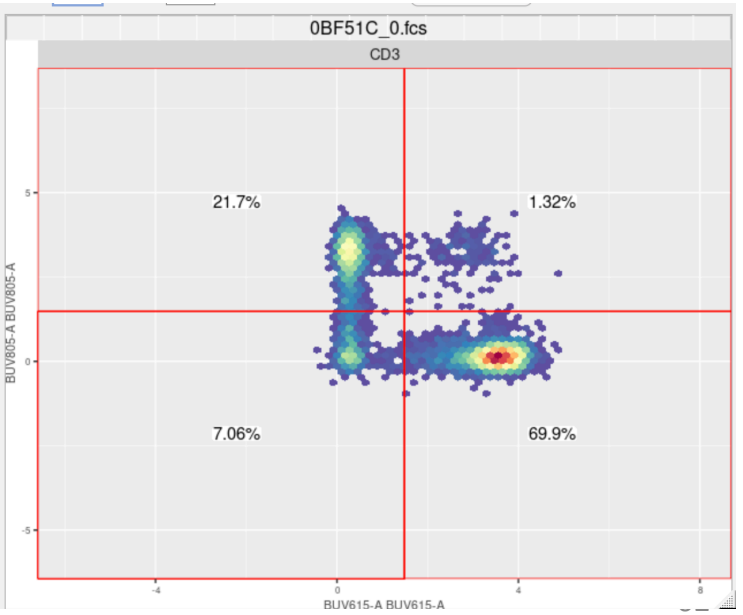
```
> plot(gs)
```



There is no node "CD4 CD8" !

Plot the 2-D quadrant gate

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



04 Automated Gating

flowClust

REMOVE
THIS PART

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

- 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
- ...
- Two options for **estimating the number of clusters when it is unknown**
- **Input are *flowFrames***

Check
paper !

Add some
figures
here !

openCyto

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

- Hierarchical gating pipeline for flow cytometry data
- Automated gating using a *gatingTemplate* (hierarchical gating scheme)

gatingTemplate

- Prepare a template in a spreadsheet (Excel)

	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

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

parent poulation

dimensions used for gating

method used for gating

Arguments for the method

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

group every n samples

should any preprocessing be applied

arguments for preprocessing

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

gatingTemplate

	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 is "nonDebris"
- The parent node is "root"
- Use *mindensity* :
- Generate a 1-D gate on FSC-A and keep the only the positive cells

gatingTemplate

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

- The population name is "singlets"
- The parent node is "nonDebris"
- Use *singletGate* :
- Generate a polygon gate on FSC-A and FSC-H keep the only the singlet cells (singlet+)

gatingTemplate

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

- The population name is "lymph"
- The parent node is "singlets"
- Use *flowClust* :
- Generate a 2-D gate on FSC-A and SSC-A and keep the only the positive cells
- Apply the preprocessing method from *flowClust* (before gating). This is actually an extra argument for *flowClust*

gatingTemplate

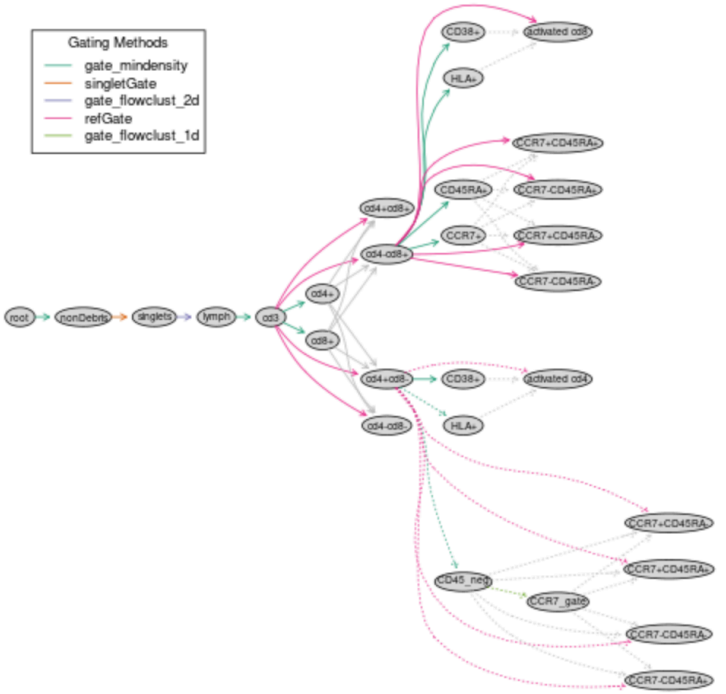
5	*	-/++/-	cd3	cd4,cd8	gate_mindensity	gate_range=c(1,3)	NA	NA		NA

- Specifies that population CD4+/-CD8+/- should be expanded into 6 rows
  - The first two rows are 1-D gates on each dimension
  - The other four rows are rectangle gates that correspond to the four quadrants in the 2-D projection

gatingTemplate

- Export the template in csv format and load it into R

```
# import the gatingTemplate
 > gtFile <- "course_datasets/gating/tcell_gating_template.csv"
 > gt_tcell <- gatingTemplate(gtFile)

# examine the gating template
 > plot(gt_tcell)
```



openCyto: run the gating pipeline

Load the data and convert to gatingSet

```
# Load the raw data
 > fcsFiles <- list.files(pattern = "CytoTrol", flowDataPath, full = TRUE)
 > fs <- read.flowSet(fcsFiles)


# Convert to a gatingSet
 > gs <- GatingSet(fs)
```

Preprocess the data using dedicated functions from flowWorkspace

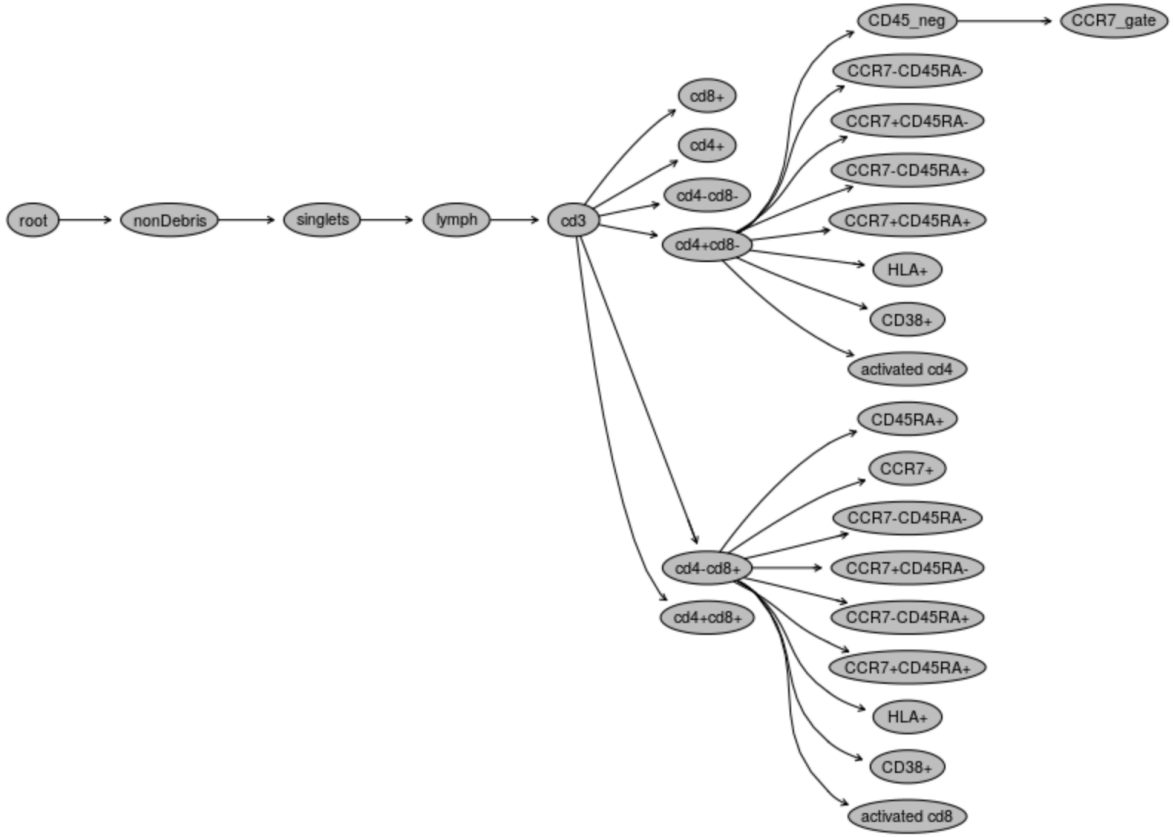
Apply the gating template

```
# Gating
 > gt_gating(gt_tcell, gs)
```

openCyto: run the gating pipeline

Check the gating

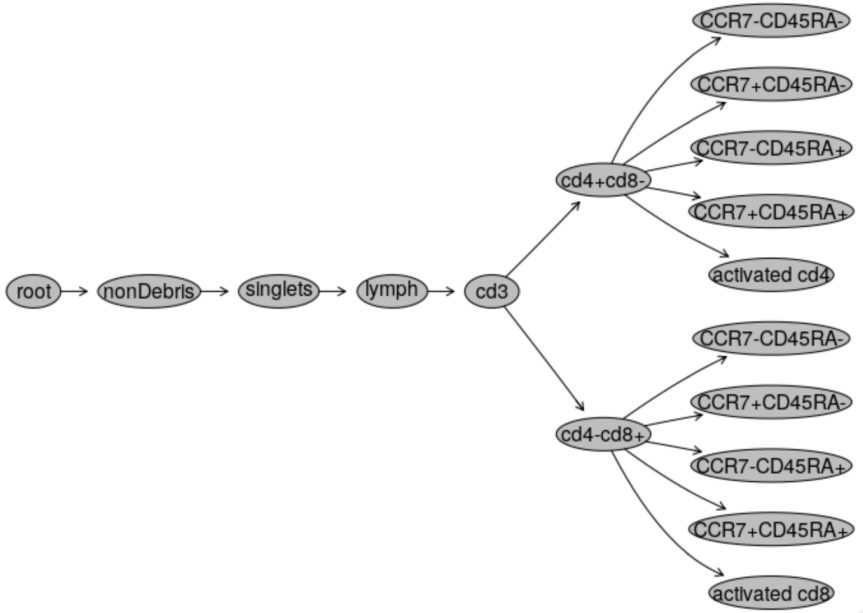
```
# Check the gating
 > plot(gs[[1]])
```



openCyto

Hide populations we are not interested in

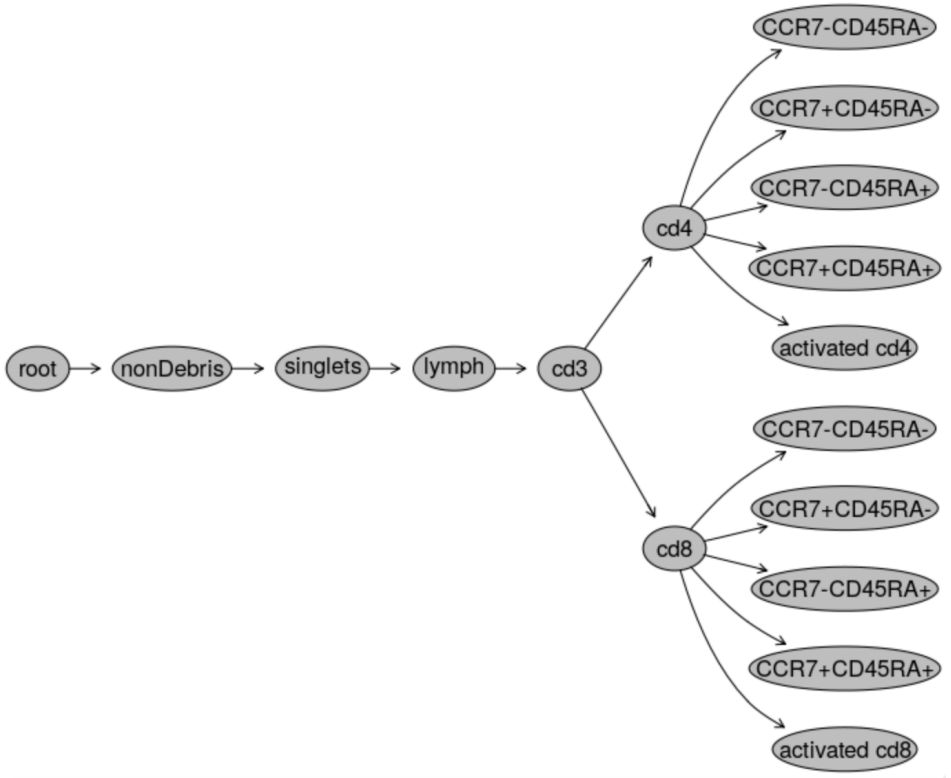
```
# Hide populations
 > nodesToHide  <- c("cd8+", "cd4+" , "cd4-cd8", "cd4+cd8+" , "cd4+cd8-/HLA+",
                     "cd4+cd8-/CD38+" , "cd4-cd8+/HLA+", "cd4-cd8+/CD38+" ,
                     "CD45_neg/CCR7_gate", "cd4+cd8-/CD45_neg" ,
                     "cd4-cd8+/CCR7+", "cd4-cd8+/CD45RA+" )
 > lapply(nodesToHide, function(thisNode) gs_pop_set_visibility(gs, thisNode, FALSE))
 > plot(gs[[1]])
```



openCyto

Rename populations

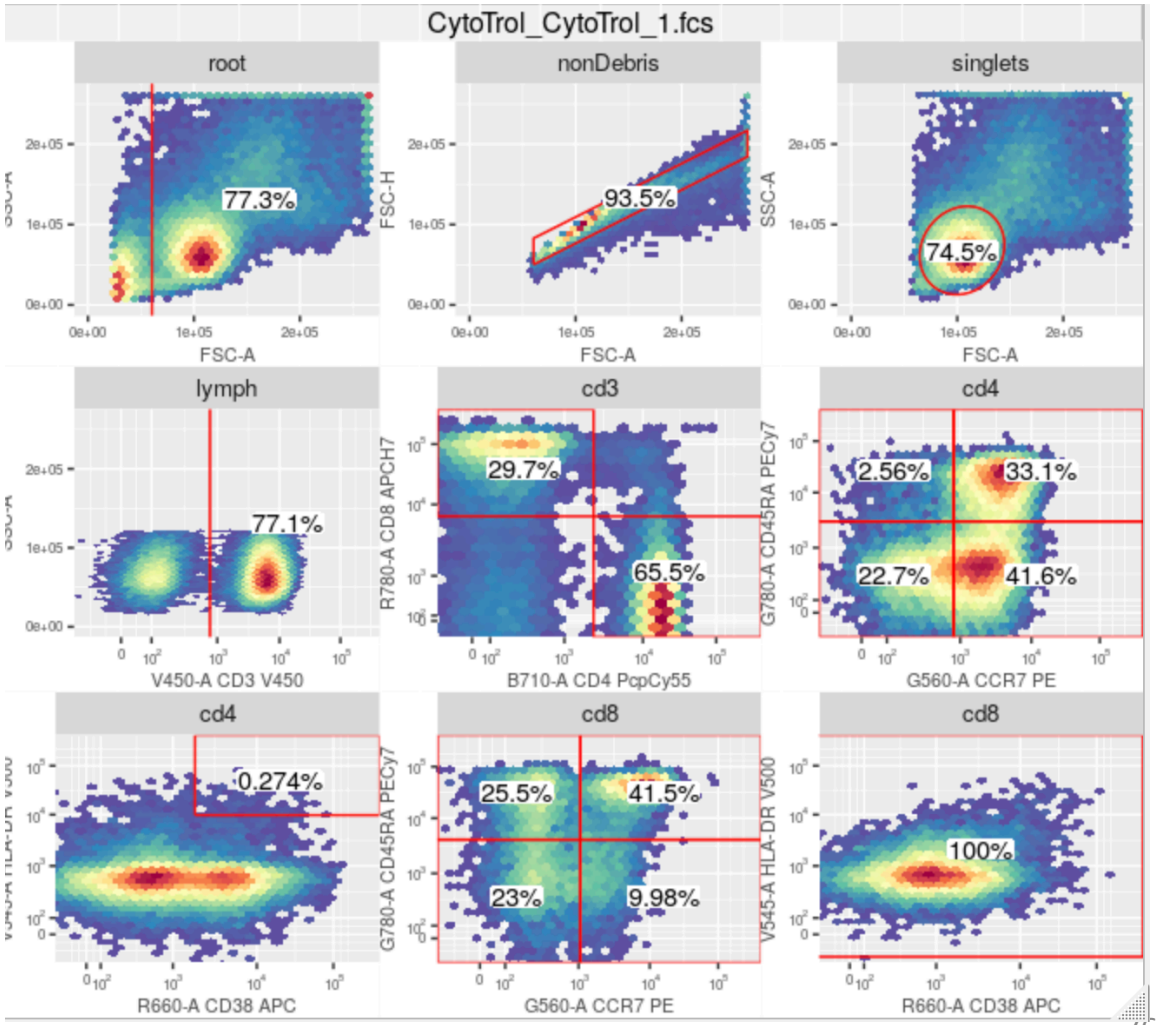
```
# Rename
 > gs_pop_set_name(gs,"cd4+cd8-","cd4")
 > gs_pop_set_name(gs,"cd4-cd8+","cd8")
 > plot(gs[[1]])
```



openCyto

Visualize the gates with *ggcyto*

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



openCyto: gating without csv template

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

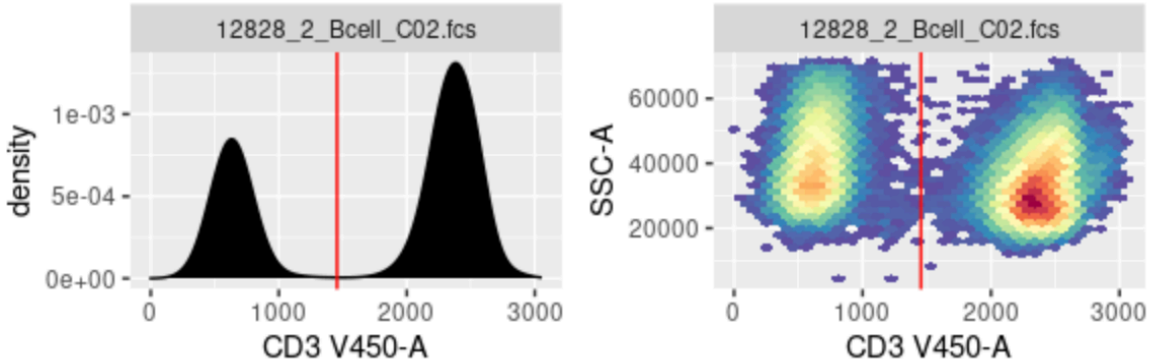
```
> gs_add_gating_method(gs,
                       alias = "non-activated cd4",
                       pop = "--",
                       parent = "cd4",
                       dims = "CD38,HLA",
                       gating_method = "tailgate")
> plot(gs[[1]])
```

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

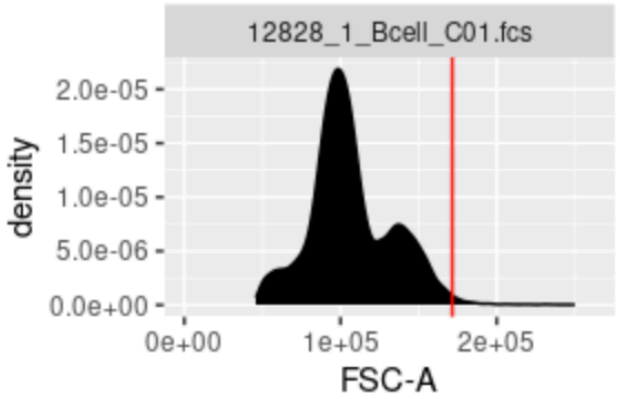
openCyto: overview of gating methods

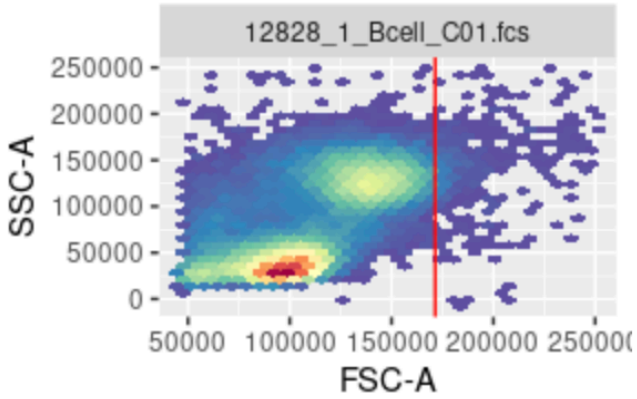
tailgate

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





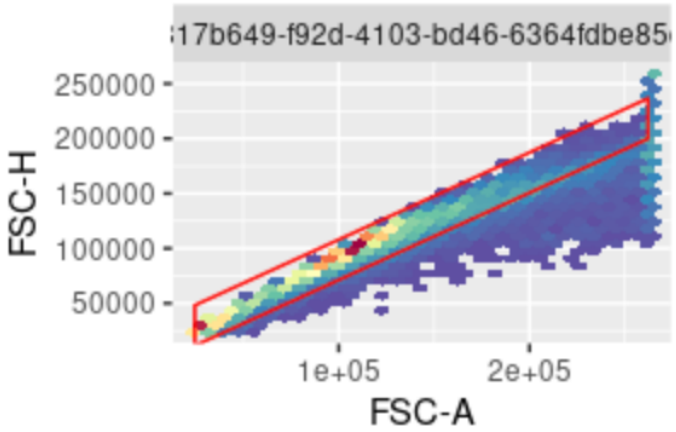
- More commonly used for rare populations (peak is not prominent enough)

openCyto: overview of gating methods

singletGate

Use the area vs height to gate

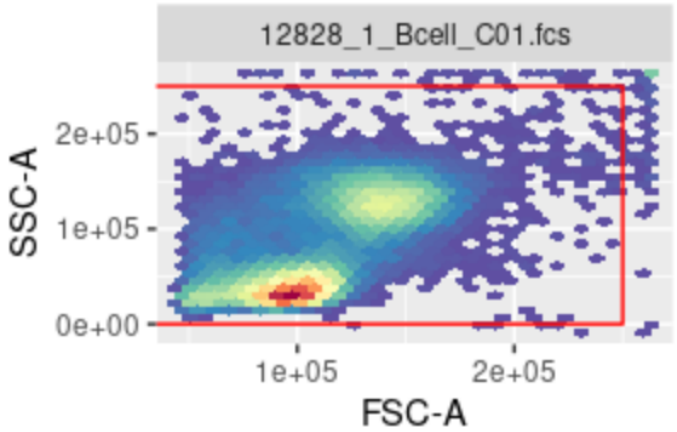
out the singlets



boundary

Constructs a rectangle gate

from input range (min and max)

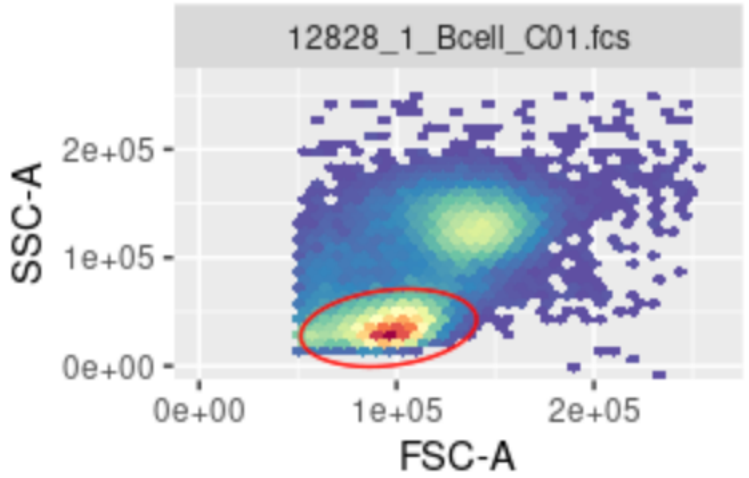


- Used for filtering out very extreme signals at the boundary

openCyto: overview of gating methods

flowClust

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

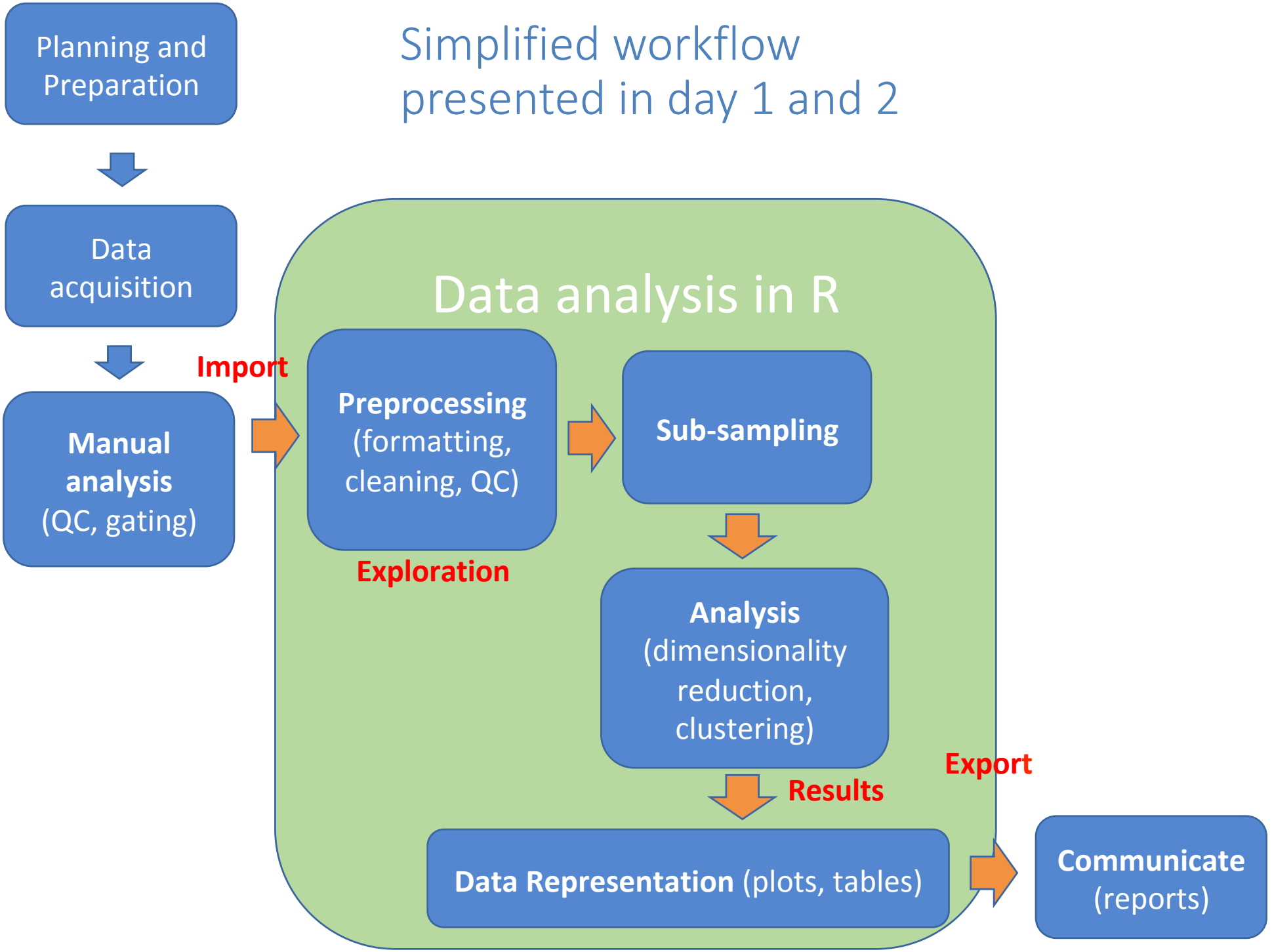


- *k*= how many cell populations are expected
- *target* = center of target population (by default the most prominent cluster)
- *quantile* = how large the ellipse should be

EXERCISE ON AUTOMATED GATING

Generate html or PDF reports

Simplified workflow presented in day 1 and 2



Rmarkdown

Thank you for your attention!

https://agora-cancer.ch/scientific-platforms/translational-data-science-facility/

Any questions? Contact us !

tds-facility@sib.swiss