

Characterization of 786-O and 786-O/VHL Cell-Derived Extracellular Vesicle Subpopulations

PART I - CD24 MEASUREMENT USING FLOW CYTOMETRY

Supplementary Information: Data analysis and annotated source code

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Abstract

In this analysis, we suggest a “best practices” method using open-source software (R/bioconductor) for analyzing small-particle flow cytometry data in respect to the potential biases attributed to the “swarm effect”. A special experimental design is required for analysis of the swarm effect in samples containing small particles, an example of which is described here.

A deconvolution technique is applied for the first time to the analysis of swarm effect on population counts using maximum-likelihood (ML) expectation-maximization (EM) estimates for mixed distribution modeling. Constraints derived from experimental design allows for simplification of distribution parameters. We implement a noise-reduction technique to minimize noise-related artefacts across sample dilutions. We suggest a simple statistical method for interpreting population count differences across samples and across sample dilutions.

The results of significance testing for our data appear to indicate a statistically significant difference between 786-O and VHL cell-derived extracellular vesicles for the absolute count of CD24mid events, as well as for the overall proportion of CD24+ events and the overall proportion of CD24mid events. While multiple comparisons statistical testing reveals no significant differences in CD24hi population counts, fingerprinting reveals that most of the overall between-sample variation occurs in the CD24hi region. Lack of significance is perhaps owing to the difficulty of detecting rare events in very dilute samples. These data also provide with a better understanding of below-threshold particle size differences between samples: in particular, 786-O cells are found to generate significantly more CD24hi EVs smaller than the size detection threshold of the instrument compared to 786-O/VHL EVs.

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1 Production environment

1.1 Required libraries

In order to install all of the required libraries listed below, you will first need to source from **bioconductor**. For more information about **bioconductor**, please visit <https://www.bioconductor.com/>.

When troubleshooting any of the following code, please note that the order of library imports matters, as certain packages may interfere with each other in unpredictable ways.

```
library("Biobase")
library("gridExtra")
library("reshape2")
library("HH")
library("mixtools")
library("diptest")
library("ggplot2")
library("dplyr")
library("tidyR")
library("purrr")
library("car")

# Flow cytometry specific packages
library("flowCore")
library("flowWorkspace")
library("flowUtils")
library("flowStats")
library("flowFP")
library("MetaCyto")
library("gcyto")

# Used to generate this annotated PDF report
# https://haozhu233.github.io/kableExtra/awesome_table_in_pdf.pdf
library("kableExtra")
library("pander")
library("knitr")
```

1.2 SessionInfo()

This may be useful for troubleshooting and is included for the sake of reproducibility.

```
pander::pander(utils::sessionInfo(), locale=TRUE, compact=TRUE)
```

R version 4.0.2 (2020-06-22)

Platform: x86_64-apple-darwin17.0 (64-bit)

locale: en_US.UTF-8||en_US.UTF-8||en_US.UTF-8||C||en_US.UTF-8||en_US.UTF-8

attached base packages: *grid, parallel, stats, graphics, grDevices, utils, datasets, methods and base*

other attached packages: *knitr(v.1.30), pander(v.0.6.3), kableExtra(v.1.3.1), ggcryo(v.1.18.0), ncdfFlow(v.2.36.0), BH(v.1.72.0-3), RcppArmadillo(v.0.10.1.0.0), MetaCyto(v.1.12.0), flowFP(v.1.48.0), flowViz(v.1.54.0), flowStats(v.4.2.0), flowUtils(v.1.54.0), flowWorkspace(v.4.2.0), flowCore(v.2.2.0), car(v.3.0-10), carData(v.3.0-4), purrr(v.0.3.4), tidyR(v.1.1.2), dplyr(v.1.0.2), ggplot2(v.3.3.2), diptest(v.0.75-7), mixtools(v.1.2.0), HH(v.3.1-42), multcomp(v.1.4-14), TH.data(v.1.0-10), MASS(v.7.3-53), survival(v.3.2-7), mvtnorm(v.1.1-1), latticeExtra(v.0.6-29), lattice(v.0.20-41), reshape2(v.1.4.4), gridExtra(v.2.3), Biobase(v.2.50.0) and BiocGenerics(v.0.36.0)*

loaded via a namespace (and not attached): *readxl(v.1.3.1), backports(v.1.2.0), Hmisc(v.4.4-1), wrapr(v.2.0.4), plyr(v.1.8.6), igraph(v.1.2.6), ConsensusClusterPlus(v.1.54.0), splines(v.4.0.2), gmp(v.0.6-1), fda(v.5.1.5.1), digest(v.0.6.27), htmltools(v.0.5.0), fansi(v.0.4.1), magrittr(v.1.5), checkmate(v.2.0.0), CytoML(v.2.2.1), cluster(v.2.1.0), ks(v.1.11.7), aws.signature(v.0.6.0), openxlsx(v.4.2.3), fastcluster(v.1.1.25), RcppParallel(v.5.0.2), matrixStats(v.0.57.0), sandwich(v.3.0-0), cytolib(v.2.2.0), jpeg(v.0.1-8.1), colorspace(v.1.4-1), rvest(v.0.3.6), rrcov(v.1.5-5), haven(v.2.3.1), xfun(v.0.19), crayon(v.1.3.4), jsonlite(v.1.7.1), hexbin(v.1.28.1), graph(v.1.68.0), zoo(v.1.8-8), glue(v.1.4.2), gtable(v.0.3.0), zlibbioc(v.1.36.0), webshot(v.0.5.2), kernlab(v.0.9-29), IDPmisc(v.1.1.20), Rgraphviz(v.2.34.0), Rmpfr(v.0.8-1), DEoptimR(v.1.0-8), abind(v.1.4-5), scales(v.1.1.1), Rcpp(v.1.0.5), viridisLite(v.0.3.0), xtable(v.1.8-4), htmlTable(v.2.1.0), foreign(v.0.8-80), mclust(v.5.4.6), FlowSOM(v.1.22.0), Formula(v.1.2-4), stats4(v.4.0.2), tsne(v.0.1-3), vcd(v.1.4-8), htmlwidgets(v.1.5.2), httr(v.1.4.2), RColorBrewer(v.1.1-2), ellipsis(v.0.3.1), farver(v.2.0.3), pkgconfig(v.2.0.3), XML(v.3.99-0.5), nnet(v.7.3-14), labeling(v.0.4.2), tidyselect(v.1.1.0), rlang(v.0.4.8), later(v.1.1.0.1), munsell(v.0.5.0), cellranger(v.1.1.0), tools(v.4.0.2), cli(v.2.1.0), generics(v.0.1.0), aws.s3(v.0.3.21), evaluate(v.0.14), stringr(v.1.4.0), fastmap(v.1.0.1), yaml(v.2.2.1), zip(v.2.1.1), robustbase(v.0.93-6), nlme(v.3.1-150), RBGL(v.1.66.0), mime(v.0.9), leaps(v.3.1), xml2(v.1.3.2), compiler(v.4.0.2), rstudioapi(v.0.11), curl(v.4.3), png(v.0.1-7), tibble(v.3.0.4), pcaPP(v.1.9-73), stringi(v.1.5.3),forcats(v.0.5.0), Matrix(v.1.2-18), vctrs(v.0.3.4), pillar(v.1.4.6), lifecycle(v.0.2.0), RUnit(v.0.4.32), lmtest(v.0.9-38), data.table(v.1.13.2), corpcor(v.1.6.9), httpuv(v.1.5.4), R6(v.2.5.0), promises(v.1.1.1), KernSmooth(v.2.23-18), RProtoBufLib(v.2.2.0), rio(v.0.5.16), codetools(v.0.2-18), assertthat(v.0.2.1), withr(v.2.3.0), metafor(v.2.4-0), S4Vectors(v.0.28.0), hms(v.0.5.3), rpart(v.4.1-15), rmarkdown(v.2.5), segmented(v.1.3-0), shiny(v.1.5.0), base64enc(v.0.1-3) and tinytex(v.0.27)*

1.3 Global variables and convenience functions

```
# This is where all my data files reside on my local machine
setwd("/Users/gp/Documents/Maitrise/Results/N3L2/CD24 quantification/")

# The following colours are used consistently enough in this document
colorPalette <- list(
  "red"   = "#E87D72",
  "blue"  = "#54BCC2",
  "green" = "#00AA00"
)

# Output dataframe in PDF-friendly format
printDataFrame <- function(dataframe, caption, fontsize=7) {
  knitr::kable(
    dataframe,
    format="latex",
    booktabs=TRUE,
    longtable=TRUE,
    caption=caption
  ) %>%
  kableExtra::kable_styling(
    font_size=fontsize,
    latex_options=c(
      "hold_position",
      "repeat_header",
      "striped"
    )
  )
}

# kmeans() returns an error when one of the clusters turns out empty. Since in
# context we expect this to happen, we can define more useful behaviour.
safeClustering <- function(
  data,
  centers=matrix(data=c(0, 0, 0, 0), ncol=2),
  principal=1
) {
  # Linter bindings
  cluster <- NULL

  attemptClustering <- try(
    expr={
      km <- stats::kmeans(data, centers=centers)$cluster
    },
    silent=TRUE
  )

  if (class(attemptClustering) == "try-error") {
    km <- principal
  }

  km
```

```
}

# This function takes an anova object, the return value of aov(), and returns
# the p-Value of row 1 (ie.: [[1]])
getANOVApValue <- function(aov) {
  summary(aov)[["Error: Within"]][[1]][["Pr(>F)"]][1]
}
```

2 Data pre-processing

2.1 Experiment meta-data

The Biobase package provides a standard class called `AnnotatedDataFrame` to store and manipulate the experiment metadata.

References:

- <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4509590/>

Technical documentation:

- <https://bioconductor.org/packages/devel/bioc/vignettes/Biobase/inst/doc/ExpressionSetIntroduction.pdf>
- <https://bioconductor.org/packages/devel/bioc/manuals/Biobase/man/Biobase.pdf>

Experiment metadata is stored in a tab-delimited `.txt` file, which can be read using `read.table()`. In this case, as the FC500 instrument does not support integration with any of the `bioconductor` tools, the `phenoData.txt` file was created by hand in Microsoft Excel. Alternatively, any other plain text editor may be used. Let's take a look at the contents of this file:

```
pData <- utils::read.table(  
  file="phenoData.txt",  
  header=TRUE,  
  row.names=1,  
  sep="\t",  
  as.is=TRUE  
)  
  
printDataFrame(  
  pData,  
  "phenoData.txt",  
  fontsize=7  
)
```

Table 1: `phenoData.txt`

	id	cellLine	n	a23187	dilution	facetRow	facetCol	temps
data/786 n1/786n1_Ca1uM4h_samp_20x 00016119 613.LMD	1	786-O	1	1	20	r1	c1	4
data/786 n1/786n1_Ca1uM4h_samp_60x 00016122 616.LMD	2	786-O	1	1	60	r1	c2	4
data/786 n1/786n1_Ca1uM4h_samp_100x 00016125 619.LMD	3	786-O	1	1	100	r1	c3	4
data/786 n1/786n1_Ca1uM4h_samp_140x 00016128 622.LMD	4	786-O	1	1	140	r1	c4	4
data/786 n1/786n1_Ca1uM4h_samp_180x 00016131 625.LMD	5	786-O	1	1	180	r1	c5	4
data/786 n1/786n1_Ca1uM4h_samp_220x 00016134 628.LMD	6	786-O	1	1	220	r1	c6	4
data/786 n2/786n2_Ca1uM4h_samp_20x 00016158 632.LMD	7	786-O	2	1	20	r2	c1	4
data/786 n2/786n2_Ca1uM4h_samp_60x 00016161 635.LMD	8	786-O	2	1	60	r2	c2	4
data/786 n2/786n2_Ca1uM4h_samp_100x 00016164 638.LMD	9	786-O	2	1	100	r2	c3	4
data/786 n2/786n2_Ca1uM4h_samp_140x 00016167 641.LMD	10	786-O	2	1	140	r2	c4	4
data/786 n2/786n2_Ca1uM4h_samp_180x 00016170 644.LMD	11	786-O	2	1	180	r2	c5	4
data/786 n2/786n2_Ca1uM4h_samp_220x 00016173 647.LMD	12	786-O	2	1	220	r2	c6	4
data/786 n3/786n3_Ca1uM4h_samp_20x 00016176 650.LMD	13	786-O	3	1	20	r3	c1	4
data/786 n3/786n3_Ca1uM4h_samp_60x 00016179 653.LMD	14	786-O	3	1	60	r3	c2	4
data/786 n3/786n3_Ca1uM4h_samp_100x 00016182 656.LMD	15	786-O	3	1	100	r3	c3	4
data/786 n3/786n3_Ca1uM4h_samp_140x 00016185 659.LMD	16	786-O	3	1	140	r3	c4	4
data/786 n3/786n3_Ca1uM4h_samp_180x 00016188 662.LMD	17	786-O	3	1	180	r3	c5	4
data/786 n3/786n3_Ca1uM4h_samp_220x 00016191 665.LMD	18	786-O	3	1	220	r3	c6	4
data/VHL n1/VHLLn1_Ca1uM4h_samp_20x 00016196 670.LMD	19	VHL	1	1	20	r4	c1	4
data/VHL n1/VHLLn1_Ca1uM4h_samp_60x 00016199 673.LMD	20	VHL	1	1	60	r4	c2	4
data/VHL n1/VHLLn1_Ca1uM4h_samp_100x 00016202 676.LMD	21	VHL	1	1	100	r4	c3	4
data/VHL n1/VHLLn1_Ca1uM4h_samp_140x 00016205 679.LMD	22	VHL	1	1	140	r4	c4	4
data/VHL n1/VHLLn1_Ca1uM4h_samp_180x 00016208 682.LMD	23	VHL	1	1	180	r4	c5	4
data/VHL n1/VHLLn1_Ca1uM4h_samp_220x 00016211 685.LMD	24	VHL	1	1	220	r4	c6	4

Table 1: phenoData.txt (*continued*)

	id	cellLine	n	a23187	dilution	facetRow	facetCol	temps
data/VHL n2/VHLn2_Ca1uM4h_samp_20x 00016215 689.LMD	25	VHL	2	1	20	r5	c1	4
data/VHL n2/VHLn2_Ca1uM4h_samp_60x 00016218 692.LMD	26	VHL	2	1	60	r5	c2	4
data/VHL n2/VHLn2_Ca1uM4h_samp_100x 00016221 695.LMD	27	VHL	2	1	100	r5	c3	4
data/VHL n2/VHLn2_Ca1uM4h_samp_140x 00016224 698.LMD	28	VHL	2	1	140	r5	c4	4
data/VHL n2/VHLn2_Ca1uM4h_samp_180x 00016227 701.LMD	29	VHL	2	1	180	r5	c5	4
data/VHL n2/VHLn2_Ca1uM4h_samp_220x 00016230 704.LMD	30	VHL	2	1	220	r5	c6	4
data/VHL n3/VHLn3_Ca1uM4h_samp_20x 00016234 708.LMD	31	VHL	3	1	20	r6	c1	4
data/VHL n3/VHLn3_Ca1uM4h_samp_60x 00016237 711.LMD	32	VHL	3	1	60	r6	c2	4
data/VHL n3/VHLn3_Ca1uM4h_samp_100x 00016240 714.LMD	33	VHL	3	1	100	r6	c3	4
data/VHL n3/VHLn3_Ca1uM4h_samp_140x 00016243 717.LMD	34	VHL	3	1	140	r6	c4	4
data/VHL n3/VHLn3_Ca1uM4h_samp_180x 00016246 720.LMD	35	VHL	3	1	180	r6	c5	4
data/VHL n3/VHLn3_Ca1uM4h_samp_220x 00016249 723.LMD	36	VHL	3	1	220	r6	c6	4

The row labels correspond to the path of all the sample data files (relative to `getwd()`, as defined previously with `setwd()`). There are two cell lines in this experiment: the ATCC 786-O renal cell carcinoma cell line, as well as a 786-O/VHL line with functional VHL gene. We can see that this experiment features biological triplicates, and that each sample is measured in serial dilutions of 1:20, 1:60, 1:100, 1:140, 1:180 and 1:220. The experimental conditions corresponding to each sample file are otherwise identical (adherent cells are incubated with 1 uM A23187 in serum-starved medium for 4 hours before conditioned medium is collected, processed and analyzed).

The variables `facetRow` and `facetCol` have been added for convenience, as these data will be organized in an ordered grid, much like a plate. Let's label our experiment columns and rows while we're at it: these labels will be used for plotting later on.

```
# To make the faceted graph easier to interpret we can label columns and rows
facetLabels <- c(
  "c1"      = "1:20",
  "20"       = "1:20",
  "c2"       = "1:60",
  "60"       = "1:60",
  "c3"       = "1:100",
  "100"      = "1:100",
  "c4"       = "1:140",
  "140"      = "1:140",
  "c5"       = "1:180",
  "180"      = "1:180",
  "c6"       = "1:220",
  "220"      = "1:220",
  "r1"       = "786 n1",
  "r2"       = "786 n2",
  "r3"       = "786 n3",
  "786-0"    = "786-0 (n=3)",
  "r4"       = "VHL n1",
  "r5"       = "VHL n2",
  "r6"       = "VHL n3",
  "VHL"      = "786-0/VHL (n=3"
)
```

The AnnotatedDataFrame is then created directly from `pData`, an object of type `DataFrame`.

```
# Otherwise flowCore::read.flowSet() doesn't work
pData$filename <- row.names(pData)

phenoData <- new(
```

```
"AnnotatedDataFrame",
  data=pData,
  varMetadata=data.frame(
    labelDescription=BiocGenerics::colnames(pData),
    row.names=BiocGenerics::colnames(pData)
  )
)

phenoData

## An object of class 'AnnotatedDataFrame'
##   rowNames: data/786 n1/786n1_Ca1uM4h_samp_20x 00016119 613.LMD
##   data/786 n1/786n1_Ca1uM4h_samp_60x 00016122 616.LMD ... data/VHL
##   n3/VHLn3_Ca1uM4h_samp_220x 00016249 723.LMD (36 total)
##   varLabels: id cellLine ... filename (9 total)
##   varMetadata: labelDescription
```

2.2 Basic data structure

2.2.1 Creating a FlowSet

At this stage, we use our `AnnotatedDataFrame` to create a `FlowSet` for our experiment. A `FlowSet` is simply a collection of related `FlowFrame` objects which store individual measurements. Each row in `pData` contains the file path of each individual measurement that comprises a `FlowFrame` within the `FlowSet`. As we will see later, a `FlowFrame` object can be plotted or manipulated on its own, or a `FlowSet` object can be used to plot or manipulate a collection of `FlowFrames` in a consistent fashion. In other words, in `flowCore` parlance a `FlowFrame` is a flow cytometry measurement (which corresponds to a single row in the `pData`) whereas a `FlowSet` could be an entire experiment made up of many samples (which corresponds to the collection of all rows in the `pData`). See the `flowCore` package for more in-depth explanations.

```
# Load all samples into memory as a flowSet object
flowset <- flowCore::read.flowSet(
  path=getwd(),
  dataset=1,
  transformation=FALSE,
  alter.names=TRUE,
  phenoData=phenoData
)

flowset

## A flowSet with 36 experiments.
##
## column names(15): FS.Lin SS.Lin ... FL5.Lin TIME
```

There are other ways to access flow data in R, however I have found this method to be the most elegant. Loading `FlowSet` data using an `AnnotatedDataFrame` has the following advantages:

- **Clearer experimental design:** the experiment metadata explicitly defined in the `AnnotatedDataFrame` should clarify the experimental design.
- **Better code separation:** the file paths are all located in an external tab-delimited `.txt` file rather than being defined at any given location within the source code itself. File handling can otherwise take up a significant number of lines of code.
- **Cleaner code:** with this method, an entire experiment is set up within a `FlowSet` in one command, without the need for repetitive code for file handling or cumbersome operations on individual `FlowFrame` objects.

2.2.2 Constructing a minimal GatingSet

While the `FlowSet` contains the actual data for the experiment, we need a means to construct and manipulate a gating tree. This is where the `GatingSet` object provided by the `flowWorkspace` package comes in. A `GatingSet` object contains a reference to a `FlowSet` experiment, as well as additional objects such as transformations (eg.: linear, log, biexponential, logicle, etc), compensation matrices, gates, as well as population statistics in the gating tree. The `flowWorkspace` tools allow for non-destructive manipulation of flow cytometry data and integrate very well with `flowJo` (which is of little help to me, but is still noteworthy).

The ideal workflow as of writing this appears to be creating an XML workspace with the `flowJo` commercial software in order to import directly into a fully constructed `GatingSet` object. Unfortunately, `flowWorkspace` does not yet appear to support importing of standard GatingML-compliant files, although as we will see, some limited support for this is offered by `flowUtils`.

Technical documentation:

- <https://www.bioconductor.org/packages/release/bioc/html/flowWorkspace.html>

We start by constructing a minimal `GatingSet` for the experiment.

```

gatingset <- flowWorkspace::GatingSet(flowset)
gatingset

```

A GatingSet with 36 samples

This GatingSet is currently quite useless, but we will be making good use of it shortly.

2.2.3 Accessing instrument settings

While this section may at first glance appear unimportant or to be full of gibberish and utter nonsense, there are at least two good reasons for diving into the instrument settings included in .FCS or .LMD files:

- **Low-level data access:** TODO: Include explanation of all the things you can find in there
- MIFlowCyt: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2773297/>

As per MIFlowCyt, the following list of instrument and acquisition parameters is included for informational purposes.

```

# Extract experiment's instrument metadata
keywords <- flowCore::keyword(gatingset[[1]])

# Format
keywordsDataFrame <- data.frame(BiocGenerics::t(data.frame(keywords)))
keywordsDataFrame$key <- BiocGenerics::rownames(keywordsDataFrame)
keywordsDataFrame <- keywordsDataFrame[c(2, 1)] # Reorder columns

BiocGenerics::colnames(keywordsDataFrame) <- c("key", "value")
BiocGenerics::rownames(keywordsDataFrame) <- c()

# Print
printDataFrame(
  keywordsDataFrame,
  "Instrument settings",
  fontsize=6
)

```

Table 2: Instrument settings

key	value
FCVersion	3
X.ABSCALFACTOR	NOT SET
X.Acquisition.Protocol.Offset	0000565308
X.AUX_SIGNAL	N/A
X.BARCODE	NoRead
X.BASELINEOFFSET	OFF
X.BUILDNUMBER	3707
X.CAROUSEL	1
X.COMPENSATIONMODE	Advanced
X.CYTOMETERID	AG10041
X.DISCRIMINATOR	FS,1
X.FILEGUID	9DF5EB9B1AA39E49A66D67F4D2E0DDC7
X.HENELASER	ENABLED
X.LOCATION	
X.PI0ADDRESS	14
X.PI0C	ARITHMETIC
X.PI0GAIN	1.000000
X.PI0Q	FL1 Lin
X.PI0U	
X.PI0X	0.0, 0.0
X.PI0Z	ON
X.PI1ADDRESS	18
X.PI1C	ARITHMETIC
X.PI1GAIN	1.000000
X.PI1Q	FL2 Lin

Table 2: Instrument settings (*continued*)

key	value
X.P11U	
X.P11X	0.0, 0.0
X.P11Z	ON
X.P12ADDRESS	22
X.P12C	ARITHMETIC
X.P12GAIN	1.000000
X.P12Q	FL3 Lin
X.P12U	
X.P12X	0.0, 0.0
X.P12Z	ON
X.P13ADDRESS	26
X.P13C	ARITHMETIC
X.P13GAIN	1.000000
X.P13Q	FL4 Lin
X.P13U	
X.P13X	0.0, 0.0
X.P13Z	ON
X.P14ADDRESS	32
X.P14C	ARITHMETIC
X.P14GAIN	1.000000
X.P14Q	FL5 Lin
X.P14U	
X.P14X	0.0, 0.0
X.P14Z	ON
X.P15ADDRESS	4
X.P15C	ARITHMETIC
X.P15Q	TIME
X.P15U	
X.P15X	1023,0,500.0
X.P15Z	ON
X.PIADDRESS	6
X.PIC	ARITHMETIC
X.PIGAIN	2.000000
X.PIQ	FS Lin
X.PIU	
X.PIX	0.0, 0.0
X.PIZ	ON
X.P2ADDRESS	8
X.P2C	ARITHMETIC
X.P2GAIN	5.000000
X.P2Q	SS Lin
X.P2U	
X.P2X	0.0, 0.0
X.P2Z	ON
X.P3ADDRESS	15
X.P3C	GEOMETRIC
X.P3GAIN	1.000000
X.P3Q	FL1 Log
X.P3U	LOG Channels
X.P3Z	ON
X.P4ADDRESS	19
X.P4C	GEOMETRIC
X.P4GAIN	1.000000
X.P4Q	FL2 Log
X.P4U	LOGChannels
X.P4Z	ON
X.P5ADDRESS	23
X.P5C	GEOMETRIC
X.P5GAIN	1.000000
X.P5Q	FL3 Log
X.P5U	LOG Channels
X.P5Z	ON
X.P6ADDRESS	27
X.P6C	GEOMETRIC
X.P6GAIN	1.000000
X.P6Q	FL4 Log
X.P6U	
X.P6Z	ON

Table 2: Instrument settings (*continued*)

key	value
X.P7ADDRESS	33
X.P7C	GEOMETRIC
X.P7GAIN	1.000000
X.P7Q	FL5 Log
X.P7U	
X.P7Z	ON
X.P8ADDRESS	7
X.P8C	GEOMETRIC
X.P8GAIN	2.000000
X.P8Q	FS Log
X.P8U	
X.P8Z	ON
X.P9ADDRESS	9
X.P9C	GEOMETRIC
X.P9GAIN	5.000000
X.P9Q	SS Log
X.P9U	
X.P9Z	ON
X.PANEL	MitoSafe with CD44 (FL1 FITC)
X.RATIO_DENOMINATOR	N/A
X.RATIO_NUMERATOR	N/A
X.RATIODENOMINATORMUX	8
X.RATIONUMERATORMUX	6
X.RESAVEDFILE	RUNTIME PROTOCOL
X.SAMPLEID1	786n1_Ca1uM4h_samp_20x
X.SAMPLEID2	
X.SAMPLEID3	
X.SAMPLEID4	
X.SETTINGSFILE	MPs - MTDRFM et CD44 (FITC).PRO
X.SETTINGSFILEDATETIME	16-May-2018, 15:24:45
X.STOPREASON	PROTOCOL
X.TARGETLASERPOWER	20 mW
X.TUBENO	1
X.Y2KDATE	20180516
X.BEGINANALYSIS	0
X.BEGINDATA	4325
X.BEGINSTEXT	0
X.BTIM	16:53:04
X.BYTEORD	4,3,2,1
X.CELLS	
X.CYT	Cytomics FC 500
X.DATATYPE	F
X.DATE	16-May-18
X.DFC1TO2	3.00
X.DFC1TO3	5.20
X.DFC1TO4	0.00
X.DFC1TO5	0.00
X.DFC2TO1	0.00
X.DFC2TO3	0.00
X.DFC2TO4	0.00
X.DFC2TO5	0.00
X.DFC3TO1	0.00
X.DFC3TO2	0.00
X.DFC3TO4	0.00
X.DFC3TO5	0.00
X.DFC4TO1	1.60
X.DFC4TO2	0.00
X.DFC4TO3	2.60
X.DFC4TO5	25.00
X.DFC5TO1	0.00
X.DFC5TO2	0.00
X.DFC5TO3	0.00
X.DFC5TO4	0.00
X.ENDANALYSIS	0
X.ENDDATA	1118044
X.ENDSTEXT	0
X.ETIM	17:01:24
X.EXP	

Table 2: Instrument settings (*continued*)

key	value
X.FIL	786n1_Ca1uM4h_samp_20x 00016119 613.LMD
X.INST	Universite de Moncton
X.INSTADDRESS	
X.MODE	L
X.NEXTDATA	0
X.OP	El Guigui
X.P10B	32
X.P10E	0,0
X.P10G	1.000000
X.P10N	FL1.Lin
X.P10R	1024
X.P10S	FL1 Lin
X.P10V	440
X.P11B	32
X.P11E	0,0
X.P11G	1.000000
X.P11N	FL2.Lin
X.P11R	1024
X.P11S	FL2 Lin
X.P11V	555
X.P12B	32
X.P12E	0,0
X.P12G	1.000000
X.P12N	FL3.Lin
X.P12R	1024
X.P12S	FL3 Lin
X.P12V	250
X.P13B	32
X.P13E	0,0
X.P13G	1.000000
X.P13N	FL4.Lin
X.P13R	1024
X.P13S	FL4 Lin
X.P13V	306
X.P14B	32
X.P14E	0,0
X.P14G	1.000000
X.P14N	FL5.Lin
X.P14R	1024
X.P14S	FL5 Lin
X.P14V	250
X.P15B	32
X.P15E	0,0
X.P15N	TIME
X.P15R	1024
X.P15S	TIME
X.P15V	0
X.P1B	32
X.P1E	0,0
X.P1G	2.000000
X.P1N	FS.Lin
X.P1R	1024
X.P1S	FS Lin
X.P1V	503
X.P2B	32
X.P2E	0,0
X.P2G	5.000000
X.P2N	SS.Lin
X.P2R	1024
X.P2S	SS Lin
X.P2V	508
X.P3B	32
X.P3E	0,0
X.P3G	1.000000
X.P3N	FL1.Log
X.P3R	1024
X.P3S	FL1 Log
X.P3V	440
X.P4B	32

Table 2: Instrument settings (*continued*)

key	value
X.P4E	0,0
X.P4G	1.000000
X.P4N	FL2.Log
X.P4R	1024
X.P4S	FL2 Log
X.P4V	555
X.P5B	32
X.P5E	0,0
X.P5G	1.000000
X.P5N	FL3.Log
X.P5R	1024
X.P5S	FL3 Log
X.P5V	250
X.P6B	32
X.P6E	0,0
X.P6G	1.000000
X.P6N	FL4.Log
X.P6R	1024
X.P6S	FL4 Log
X.P6V	306
X.P7B	32
X.P7E	0,0
X.P7G	1.000000
X.P7N	FL5.Log
X.P7R	1024
X.P7S	FL5 Log
X.P7V	250
X.P8B	32
X.P8E	0,0
X.P8G	2.000000
X.P8N	FS.Log
X.P8R	1024
X.P8S	FS Log
X.P8V	503
X.P9B	32
X.P9E	0,0
X.P9G	5.000000
X.P9N	SS.Log
X.P9R	1024
X.P9S	SS Log
X.P9V	508
X.PAR	15
X.PROJ	
X.RUNNUMBER	00016119
X.SMNO	00016119
X.SRC	
X.SYS	CXP
X.TOT	18562
ACQTIME	500.0
FILENAME	/private/var/folders/23/z5mf0z_x2sj5xw7m95mdhgjh0000gn/T/RtmpqrUtTBD/file1797e6e1b4adf/data_786 n1_786n1_Calum4h_samp_20x 00016119 613.LMD
GUID	data_786 n1_786n1_Calum4h_samp_20x 00016119 613.LMD
GUID.original	data_786 n1_786n1_Calum4h_samp_20x 00016119 613.LMD
ORIGINALGUID	data_786 n1_786n1_Calum4h_samp_20x 00016119 613.LMD
TESTFILE	MPs - MTDRFM et CD44 (FITC)
TESTNAME	MPs - MTDRFM et CD44 (FITC)
X.CYTOLIB_VERSION	2.2.0
transformation	applied
flowCore_-P1Rmax	1024
flowCore_-P1Rmin	0
flowCore_-P2Rmax	1024
flowCore_-P2Rmin	0
flowCore_-P3Rmax	1024
flowCore_-P3Rmin	0
flowCore_-P4Rmax	1024
flowCore_-P4Rmin	0
flowCore_-P5Rmax	1024
flowCore_-P5Rmin	0
flowCore_-P6Rmax	1024

Table 2: Instrument settings (*continued*)

key	value
flowCore_P6Rmin	0
flowCore_P7Rmax	1024
flowCore_P7Rmin	0
flowCore_P8Rmax	1024
flowCore_P8Rmin	0
flowCore_P9Rmax	1024
flowCore_P9Rmin	0
flowCore_P10Rmax	1024
flowCore_P10Rmin	0
flowCore_P11Rmax	1024
flowCore_P11Rmin	0
flowCore_P12Rmax	1024
flowCore_P12Rmin	0
flowCore_P13Rmax	1024
flowCore_P13Rmin	0
flowCore_P14Rmax	1024
flowCore_P14Rmin	0
flowCore_P15Rmax	1024
flowCore_P15Rmin	0

2.3 GatingML

```
flowEnv <- new.env()  
flowUtils::read.gatingML("GatingML.xml", flowEnv)
```

2.3.1 Applying the gating tree onto the GatingSet

In theory, with an appropriate GatingML-compliant file this should be incredibly straightforward. However, support for standard GatingML-compliant files in `flowWorkspace` is nonexistent. From what I have been able to figure out, support for this in `flowUtils` is either broken, incomplete or improperly documented. As a result, the following code is not as elegant a demonstration as it could be, but this should not detract from the fact that the R/bioconductor open source flow cytometry tools are extremely powerful.

References:

- <https://onlinelibrary.wiley.com/doi/epdf/10.1002/cyto.a.20637>

Technical documentation:

- <http://flowcyt.sourceforge.net/gating/latest.pdf>
- <http://bioconductor.riken.jp/packages/3.7/bioc/manuals/flowUtils/man/flowUtils.pdf>

Let's begin this mess by defining two convenience functions (which I hope to get rid of in the near future). We can then construct a gating tree from the gates defined in the GatingML file.

```
getGate <- function(gateName) {  
  flowEnv[[gateName]]@filters[[1]]  
}  
  
getGateParent <- function(gateName) {  
  flowEnv[[gateName]]@filters[[2]]@name  
}  
  
flowWorkspace::gs_pop_add(  
  gs=gatingset,  
  gate=getGate("boundary"),  
  parent=getGateParent("boundary"))  
)  
  
flowWorkspace::gs_pop_add(  
  gs=gatingset,  
  gate=getGate("nonNoise"),  
  parent=getGateParent("nonNoise"))  
)  
  
flowWorkspace::gs_pop_add(  
  gs=gatingset,  
  gate=getGate("beadsA"),  
  parent=getGateParent("beadsA"))  
)  
  
flowWorkspace::gs_pop_add(  
  gs=gatingset,  
  gate=getGate("beadsB"),  
  parent=getGateParent("beadsB"))  
)
```

```

flowWorkspace::gs_pop_add(
  gs=gatingset,
  gate=flowWorkspace::booleanFilter(`beadsA & beadsB`),
  name="beads",
  parent="nonNoise"
)

flowWorkspace::gs_pop_add(
  gs=gatingset,
  gate=flowWorkspace::booleanFilter(`nonNoise & !beads`),
  name="events",
  parent="nonNoise"
)

flowWorkspace::gs_pop_add(
  gs=gatingset,
  gate=getGate("cd24pos"),
  parent=getGateParent("cd24pos")
)

flowWorkspace::gs_pop_add(
  gs=gatingset,
  gate=getGate("cd24neg"),
  parent=getGateParent("cd24neg")
)

flowWorkspace::gs_pop_add(
  gs=gatingset,
  gate=getGate("cd24mid"),
  parent=getGateParent("cd24mid")
)

flowWorkspace::gs_pop_add(
  gs=gatingset,
  gate=getGate("cd24hi"),
  parent=getGateParent("cd24hi")
)

```

Because individually they are completely irrelevant to our analysis, we can hide the “helper” bead count nodes from our gating tree, instead focusing on the semantically more useful beads boolean gate. We can then apply the gating tree to the GatingSet: this will also compute population statistics down the tree.

```

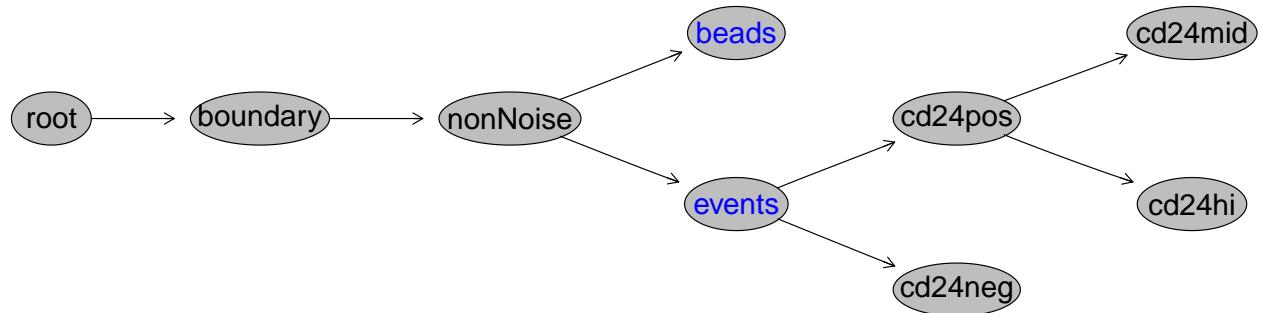
invisible(flowWorkspace::gs_pop_set_visibility(gatingset, "beadsA", FALSE))
invisible(flowWorkspace::gs_pop_set_visibility(gatingset, "beadsB", FALSE))

# Apply the gating tree to the underlying data
flowWorkspace::recompute(gatingset)

```

We can then plot the gating tree in order to visualize the general gating strategy for this experiment.

```
plot(gatingset, bool=TRUE)
```



2.4 Data normalization

There is some purely instrumental variability: beads sometimes appear at slightly different locations, so we normalize for this here.

References:

- <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3648208/>

Technical documentation:

- <https://bioconductor.org/packages/release/bioc/manuals/flowStats/man/flowStats.pdf>

```

## Estimating landmarks for channel FS.Log ...Estimating landmarks for channel SS.Log ...
## Registering curves for parameter FS.Log ...
## Registering curves for parameter SS.Log ...
## Estimating landmarks for channel FS.Log ...Estimating landmarks for channel SS.Log ...
## Registering curves for parameter FS.Log ...
## Registering curves for parameter SS.Log ...

```

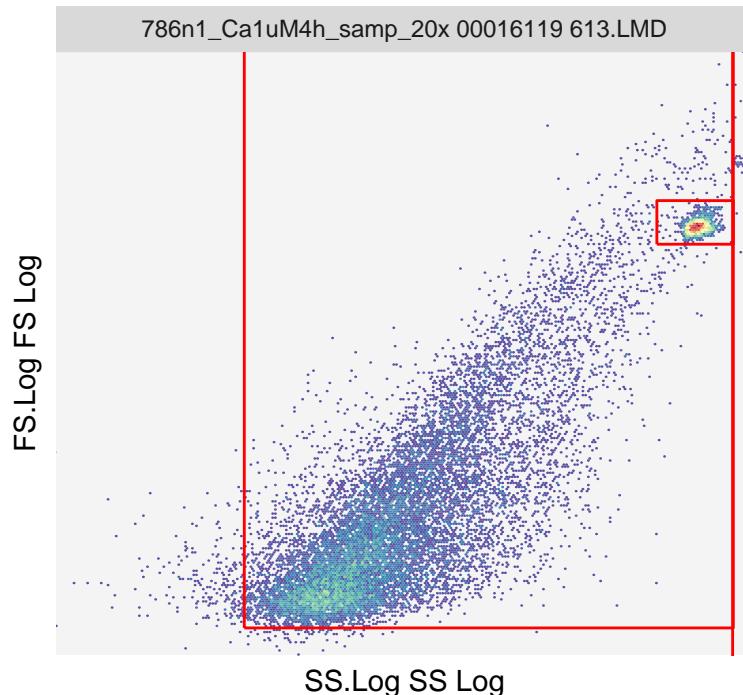
3 Data visualization

3.1 Single sample morphology plot

Gated cytometry data can be plotted with ggCyto.

```
ggcyto::ggcyto(
  data=gatingset[[1]],
  mapping=ggplot2::aes(
    x=SS.Log,
    y=FS.Log),
  subset="root"
) +
  ggplot2::geom_hex(bins=256) +
  ggplot2::scale_x_continuous(expand=c(0, 0)) +
  ggplot2::scale_y_continuous(expand=c(0, 0)) +
  ggcyto::geom_gate("boundary") +
  ggcyto::geom_gate("nonNoise") +
  ggcyto::geom_gate("beadsA") +
  ggplot2::theme(
    legend.position="none",
    panel.background=ggplot2::element_rect(fill="#F4F4F4"),
    panel.grid.major.x=ggplot2::element_blank(),
    panel.grid.minor.x=ggplot2::element_blank(),
    panel.grid.major.y=ggplot2::element_blank(),
    panel.grid.minor.y=ggplot2::element_blank(),
    panel.spacing=grid::unit(0.1, units="lines"),
    axis.text.x=ggplot2::element_blank(),
    axis.text.y=ggplot2::element_blank(),
    axis.ticks=ggplot2::element_blank()
```

root



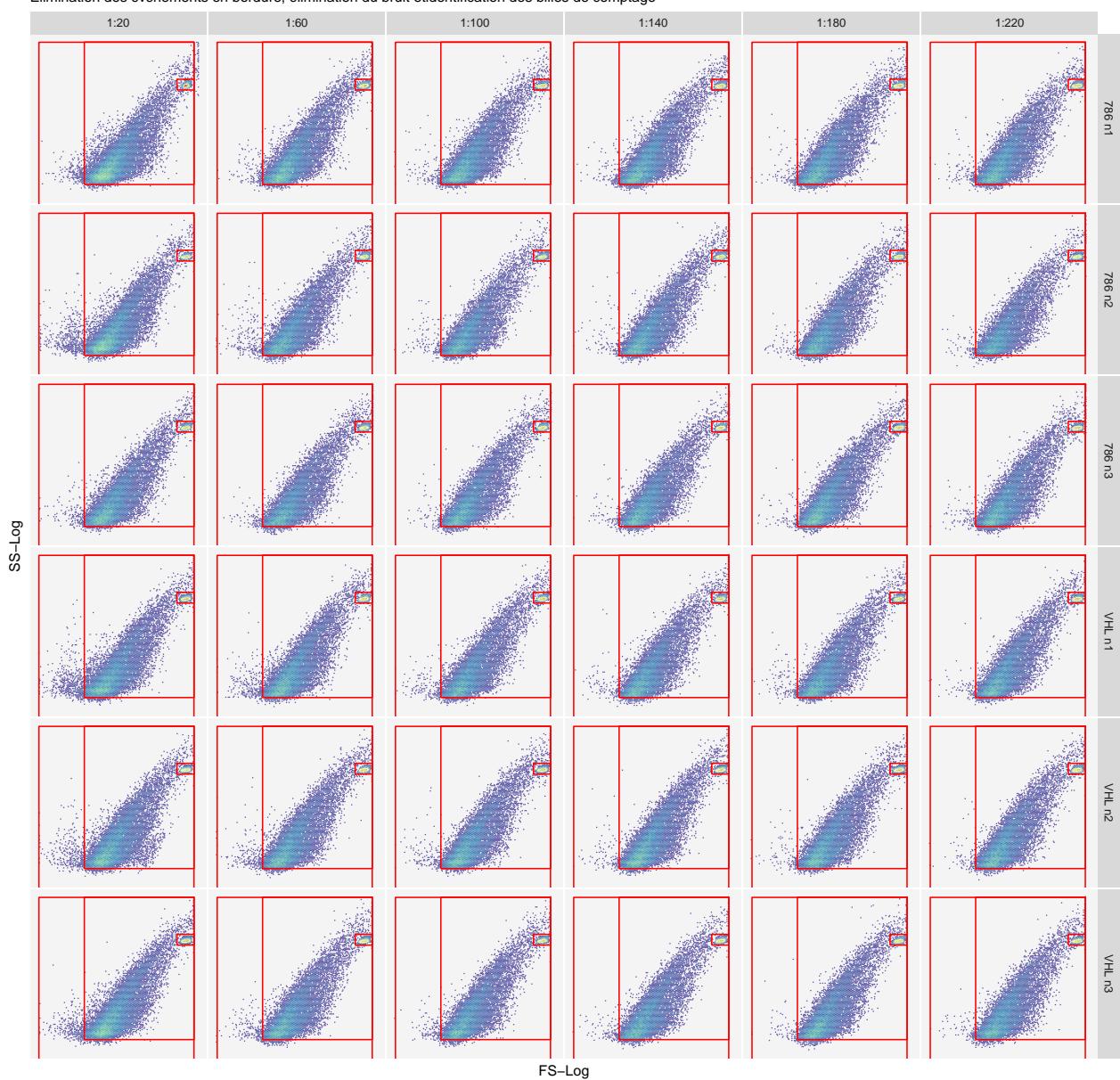
3.2 Whole experiment morphology plot

Let's plot the entire Gatingset so we can visualize the morphology plane on all samples simultaneously!

```
ggcyto::ggcyto(
  data=gatingset,
  mapping=ggplot2::aes(
    x=SS.Log,
    y=FS.Log),
  subset="root"
) +
  ggplot2::labs(
    title="Stratégie de gating sur les paramètres de morphologie",
    subtitle=paste0(
      "Élimination des évènements en bordure, élimination du bruit et",
      "identification des billes de comptage"),
    x="FS-Log",
    y="SS-Log") +
  ggplot2::geom_hex(bins=128) +
  ggcyto::geom_gate("boundary") +
  ggcyto::geom_gate("nonNoise") +
  ggcyto::geom_gate("beadsA") +
  ggplot2::facet_grid(
    rows=dplyr::vars(facetRow),
    cols=dplyr::vars(facetCol),
    labeller=ggplot2::as_labeller(facetLabels)) +
  ggplot2::theme(
    legend.position="none",
    panel.background=ggplot2::element_rect(fill="#F4F4F4"),
    panel.grid.major.x=ggplot2::element_blank(),
    panel.grid.minor.x=ggplot2::element_blank(),
    panel.grid.major.y=ggplot2::element_blank(),
    panel.grid.minor.y=ggplot2::element_blank(),
    panel.spacing=grid::unit(0.1, units="lines"),
    axis.text.x=ggplot2::element_blank(),
    axis.text.y=ggplot2::element_blank(),
    axis.ticks=ggplot2::element_blank())
```

Stratégie de gating sur les paramètres de morphologie

Élimination des événements en bordure, élimination du bruit et identification des billes de comptage



3.3 Auxiliary bead gate visualization

Visualizing the auxiliary bead gates:

```
gatingset <- flowStats::normalize(  
  data=gatingset,  
  populations=c("beadsB"),  
  dims=c("SS.Lin"),  
  minCountThreshold=100  
)  
  
## cloning the gatingSet...  
## Normalize beadsB  
  
## Estimating landmarks for channel SS.Lin ...  
## Registering curves for parameter SS.Lin ...  
  
## normalizing sample 1  
## normalizing sample 2  
## normalizing sample 3  
## normalizing sample 4  
## normalizing sample 5  
## normalizing sample 6  
## normalizing sample 7  
## normalizing sample 8  
## normalizing sample 9  
## normalizing sample 11  
## normalizing sample 12  
## normalizing sample 13  
## normalizing sample 14  
## normalizing sample 15  
## normalizing sample 16  
## normalizing sample 17  
## normalizing sample 18  
## normalizing sample 19  
## normalizing sample 20  
## normalizing sample 21  
## normalizing sample 22  
## normalizing sample 23  
## normalizing sample 24  
## normalizing sample 25  
## normalizing sample 26  
## normalizing sample 27
```

```

## normalizing sample 28
## normalizing sample 29
## normalizing sample 30
## normalizing sample 31
## normalizing sample 32
## normalizing sample 33
## normalizing sample 34
## normalizing sample 35
## normalizing sample 36
## normalizing sample 37

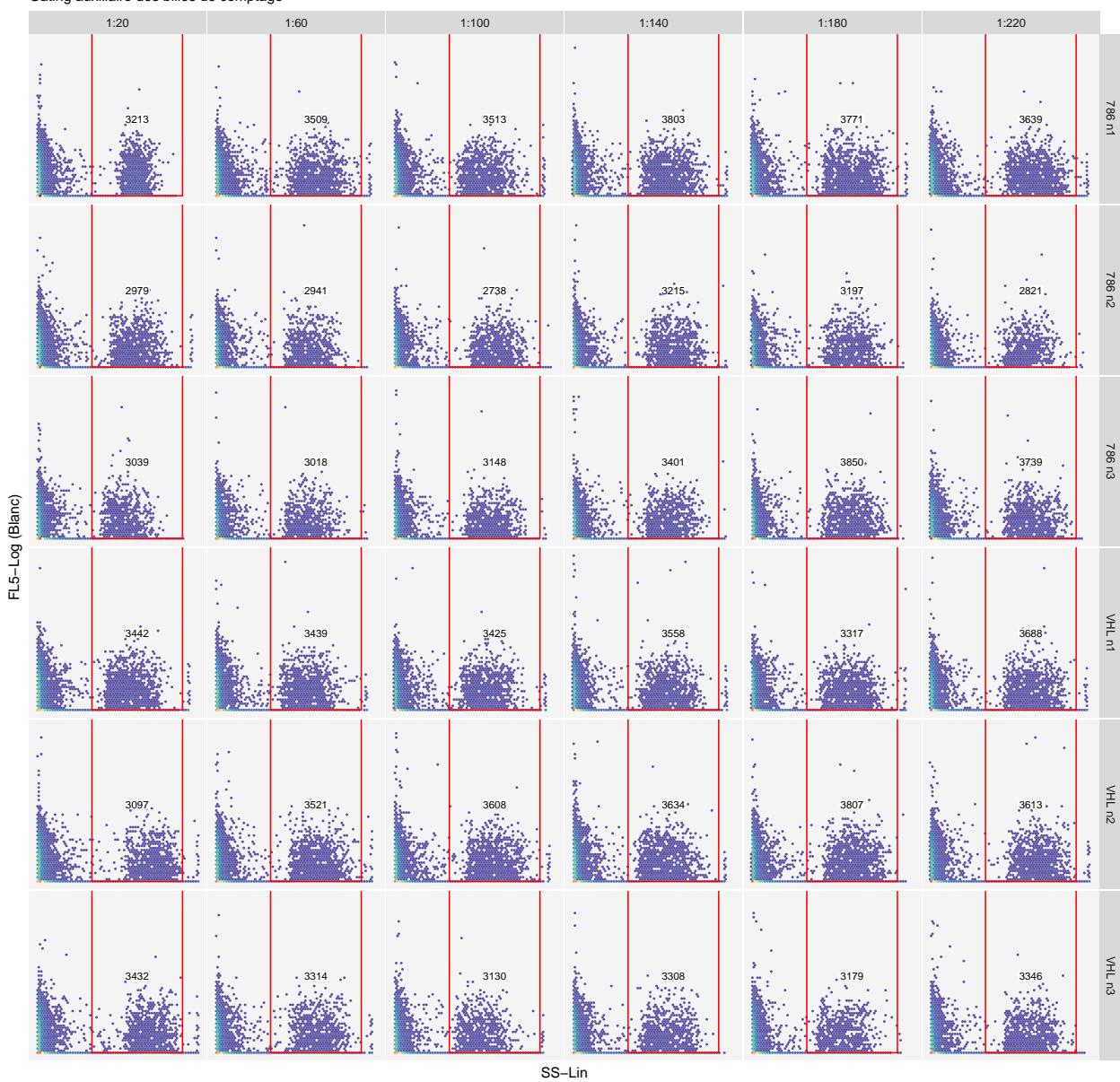
## done!

ggcyto::ggcyto(
  data=gatingset,
  mapping=ggplot2::aes(
    x=SS.Lin,
    y=FL5.Log),
  subset="nonNoise"
) +
  ggplot2::labs(
    title="Stratégie de gating pour l'élimination des beads",
    subtitle="Gating auxiliaire des billes de comptage",
    x="SS-Lin",
    y="FL5-Log (Blanc)") +
  ggplot2::geom_hex(bins=64) +
  ggcyto::geom_gate("beadsB") +
  ggcyto::geom_stats(
    size=2.7,
    type="count") +
  ggplot2::facet_grid(
    rows=dplyr::vars(facetRow),
    cols=dplyr::vars(facetCol),
    labeller=ggplot2::as_labeller(facetLabels)) +
  ggplot2::theme(
    legend.position="none",
    panel.background=ggplot2::element_rect(fill="#F4F4F4"),
    panel.grid.major.x=ggplot2::element_blank(),
    panel.grid.minor.x=ggplot2::element_blank(),
    panel.grid.major.y=ggplot2::element_blank(),
    panel.grid.minor.y=ggplot2::element_blank(),
    panel.spacing=grid::unit(0.1, units="lines"),
    axis.text.x=ggplot2::element_blank(),
    axis.text.y=ggplot2::element_blank(),
    axis.ticks=ggplot2::element_blank())

```

Stratégie de gating pour l'élimination des beads

Gating auxiliaire des billes de comptage



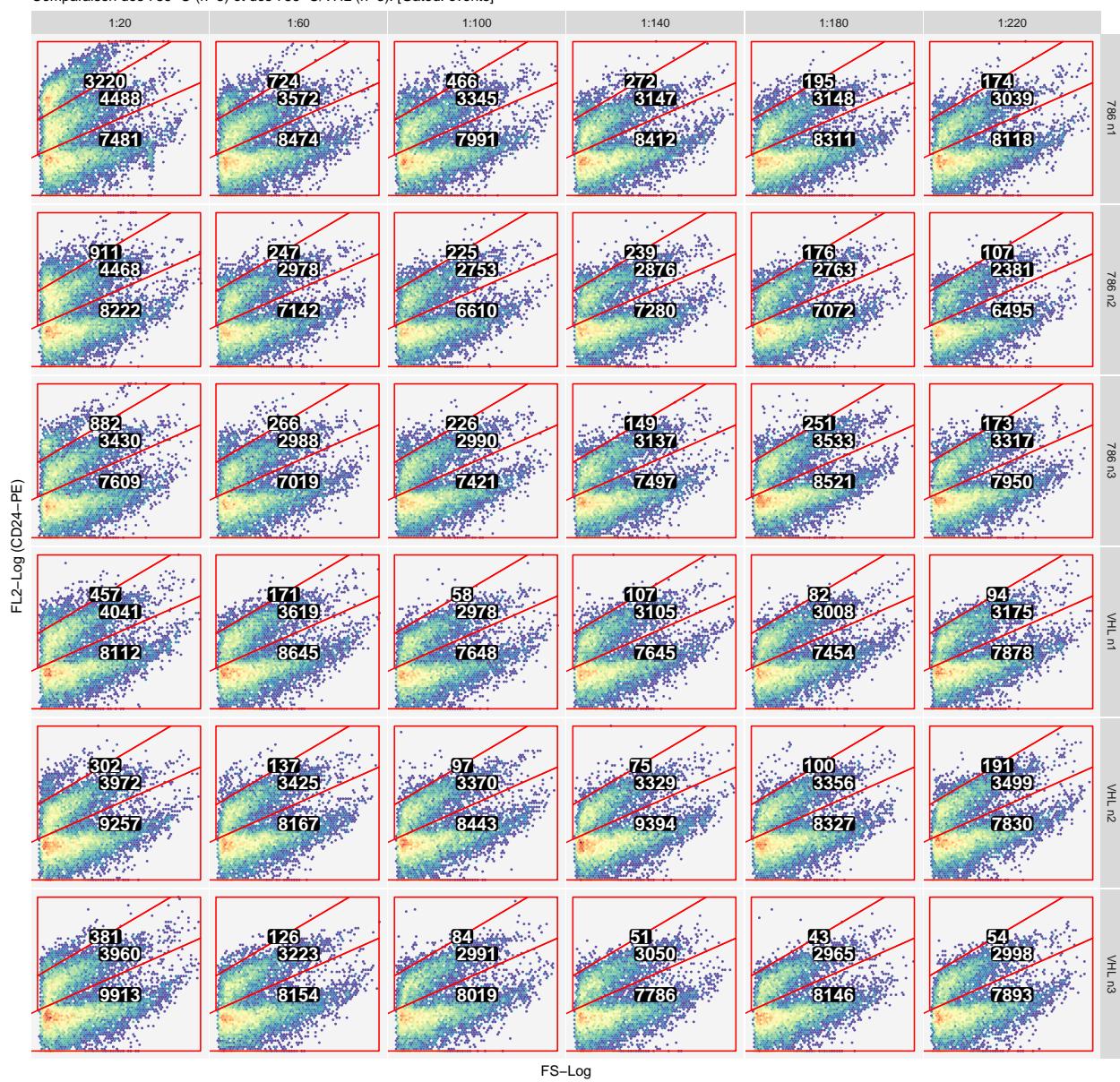
3.4 Whole experiment fluorescence with event counts

And finally, let's visualize the non-bead events on the fluorescence channel (anti-CD24 antibodies)

```
ggcyto::ggcyto(
  data=gatingset,
  mapping=ggplot2::aes(
    x=FS.Log,
    y=FL2.Log),
  subset="events"
) +
  ggplot2::labs(
    title="Quantification des microparticules marquées au CD24-PE",
    subtitle=paste0(
      "Comparaison des 786-0 (n=3) et des 786-0/VHL (n=3). ",
      "[Gated: events]"
    ),
    x="FS-Log",
    y="FL2-Log (CD24-PE)") +
  ggplot2::geom_hex(bins=64) +
  ggcyto::geom_gate(c(
    "cd24hi",
    "cd24mid",
    "cd24neg")) +
  ggcyto::geom_stats(
    color="#FFFFFF",
    fill="#000000",
    size=5,
    fontface="bold",
    type="count") +
  ggplot2::facet_grid(
    rows=dplyr::vars(facetRow),
    cols=dplyr::vars(facetCol),
    labeller=ggplot2::as_labeller(facetLabels)) +
  ggplot2::theme(
    legend.position="none",
    panel.background=ggplot2::element_rect(fill="#F4F4F4"),
    panel.grid.major.x=ggplot2::element_blank(),
    panel.grid.minor.x=ggplot2::element_blank(),
    panel.grid.major.y=ggplot2::element_blank(),
    panel.grid.minor.y=ggplot2::element_blank(),
    panel.spacing=grid::unit(0.1, units="lines"),
    axis.text.x=ggplot2::element_blank(),
    axis.text.y=ggplot2::element_blank(),
    axis.ticks=ggplot2::element_blank())
```

Quantification des microparticules marquées au CD24-PE

Comparaison des 786-O (n=3) et des 786-O/VHL (n=3). [Gated: events]



4 Analysis

4.1 Data-driven methods

4.1.1 Fingerprinting

Fingerprinting is a quick and easy way to observe population-agnostic, data-driven differences between a large number of samples.

References:

- <https://www.ncbi.nlm.nih.gov/pubmed/19956416>

Technical documentation:

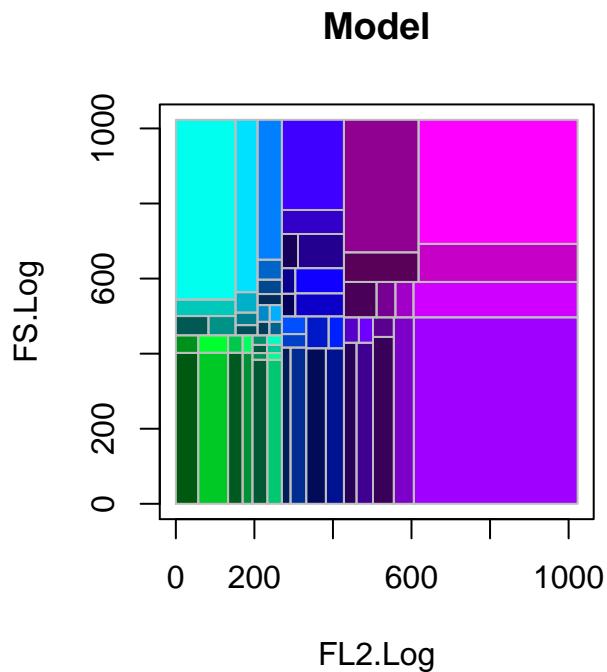
*https://bioconductor.org/packages/release/bioc/vignettes/flowFP/inst/doc/flowFP_HowTo.pdf

```
# Remove all irrelevant populations, such as beads and noise
fingerprintingData <- flowWorkspace::gs_pop_get_data(gatingset, "events")
fingerprintingData <- flowWorkspace::cytoset_to_flowSet(fingerprintingData)

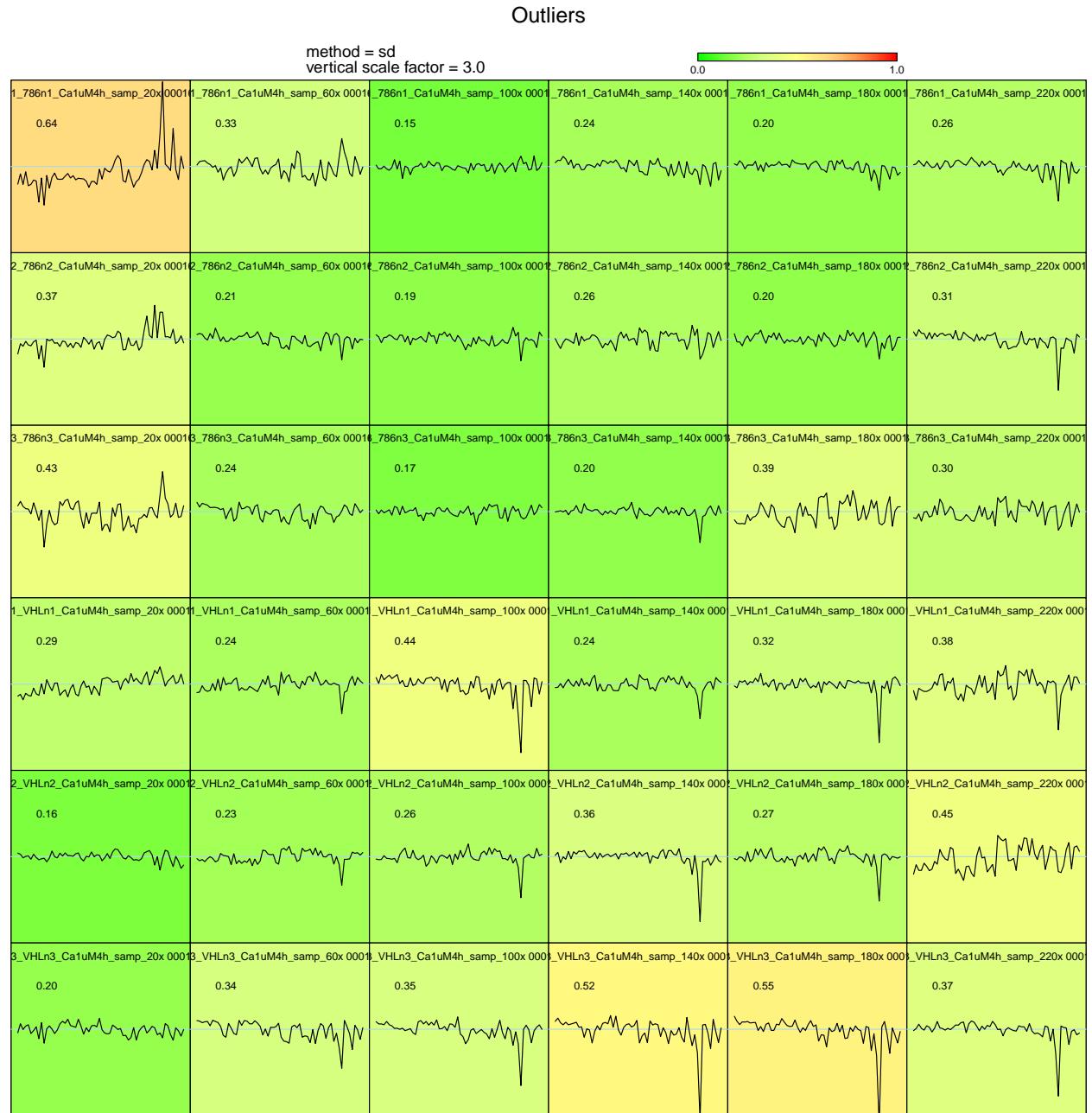
# Generate a fingerprinting model with the data in the "events"-gated FlowSet
fingerprintingModel <- flowFP::flowFPMModel(
  fcs=fingerprintingData,
  name="CD24/FS.Log Model",
  parameters=c("FS.Log", "FL2.Log"),
  nRecursions=6
)

# Generate fingerprints for all samples
fingerprints <- flowFP::flowFP(
  fcs=fingerprintingData,
  model=fingerprintingModel
)

# Visualize model
plot(fingerprintingModel)
```



```
# Detect outliers
plot(fingerprints, type="qc", main="Outliers")
```

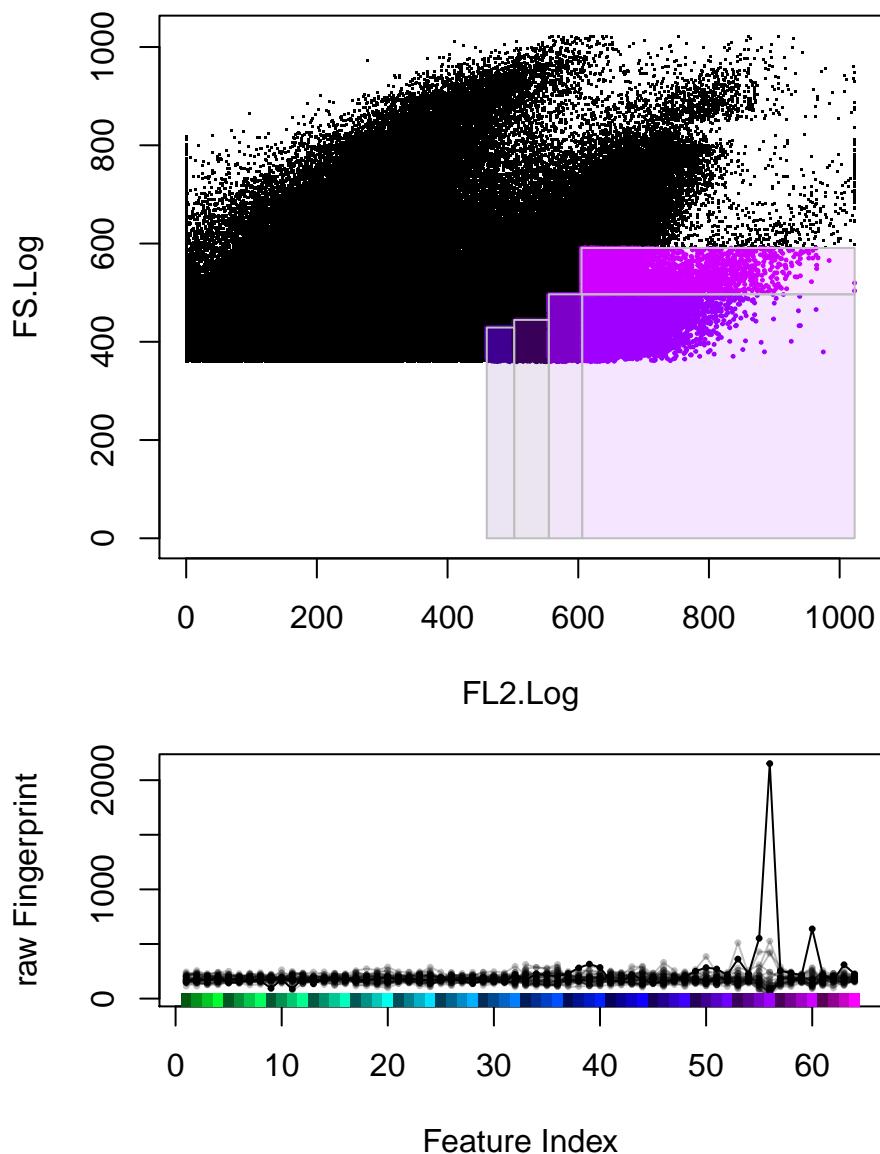


```

# Visualize a specific sample and its outlier bins
plot(
  fingerprints,
  fingerprintingData,
  hi=1,
  showbins=c(50, 53, 55, 56, 60),
  pch=20,
  cex=0.3,
  main="Visualization of the outlier bins"
)

```

Visualization of the outlier bins



4.2 Swarm deconvolution

This is a novel application of a deconvolution strategy that has been validated in entirely different contexts.

Reference:

- <http://tinyheero.github.io/2016/01/03/gmm-em.html>

DECONVOLUTION TOOLS:

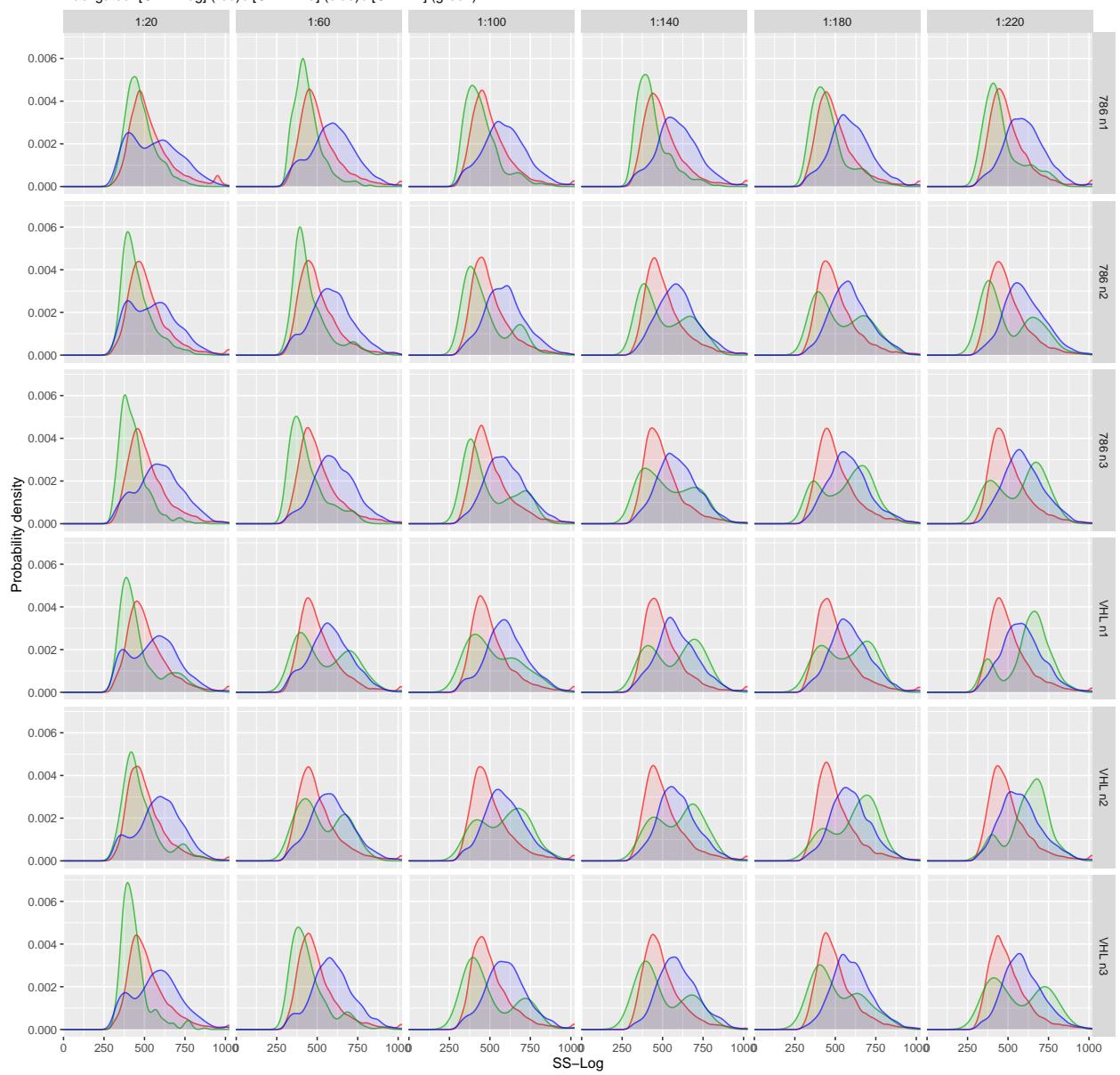
- <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0067620>
- <https://bioconductor.org/packages/release/bioc/vignettes/flowFit/inst/doc/HowTo-flowFit.pdf>

4.2.1 Visualizing the swarm effect

We can back-gate any of our fluorescent populations into a morphology axis:

```
# Morphology characteristics of fluorescence-gated data can be visualized to
# gain quantitative insight into the magnitude of per-subpopulation swarm effect
ggplot2::ggplot(
  data=flowWorkspace::gs_pop_get_data(gatingset, "events"),
  mapping=ggplot2::aes(x=SS.Log)
) +
  ggplot2::labs(
    title="Morphology of CD24 subpopulations by fluorescence intensity",
    subtitle="Backgated: [CD24neg] (red) / [CD24mid] (blue) / [CD24hi] (green)",
    x="SS-Log",
    y="Probability density") +
  ggplot2::scale_x_continuous(
    limits=c(0, 1023),
    expand=c(0, 0)) +
  ggplot2::geom_density(
    data=flowWorkspace::gs_pop_get_data(gatingset, "cd24neg"),
    color="#FF0000AA",
    fill="red",
    alpha=0.1) +
  ggplot2::geom_density(
    data=flowWorkspace::gs_pop_get_data(gatingset, "cd24hi"),
    color="#00AA00AA",
    fill="#00AA00",
    alpha=0.1) +
  ggplot2::geom_density(
    data=flowWorkspace::gs_pop_get_data(gatingset, "cd24mid"),
    color="#0000FFAA",
    fill="blue",
    alpha=0.1) +
  ggplot2::facet_grid(
    rows=dplyr::vars(facetRow),
    cols=dplyr::vars(facetCol),
    labeller=ggplot2::as_labeller(facetLabels))
```

Morphology of CD24 subpopulations by fluorescence intensity
Backgated: [CD24neg] (red) / [CD24mid] (blue) / [CD24hi] (green)



It might be easier to visualize this by combining data from all triplicates:

```
cellLineOrdering <- c("786-0", "VHL")
dilutionOrdering <- c("20", "60", "100", "140", "180", "220")

eventsData <- flowWorkspace::gs_pop_get_data(gatingset, "events")
eventsData <- flowWorkspace::cytoset_to_flowSet(eventsData)
cd24negData <- flowWorkspace::gs_pop_get_data(gatingset, "cd24neg")
cd24negData <- flowWorkspace::cytoset_to_flowSet(cd24negData)
cd24midData <- flowWorkspace::gs_pop_get_data(gatingset, "cd24mid")
cd24midData <- flowWorkspace::cytoset_to_flowSet(cd24midData)
cd24hiData <- flowWorkspace::gs_pop_get_data(gatingset, "cd24hi")
cd24hiData <- flowWorkspace::cytoset_to_flowSet(cd24hiData)

eventsData@phenoData@data$dilution <- factor(
  eventsData@phenoData@data$dilution,
  levels=dilutionOrdering)
cd24negData@phenoData@data$dilution <- factor(
  cd24negData@phenoData@data$dilution,
  levels=dilutionOrdering)
cd24midData@phenoData@data$dilution <- factor(
  cd24midData@phenoData@data$dilution,
  levels=dilutionOrdering)
cd24hiData@phenoData@data$dilution <- factor(
  cd24hiData@phenoData@data$dilution,
  levels=dilutionOrdering)

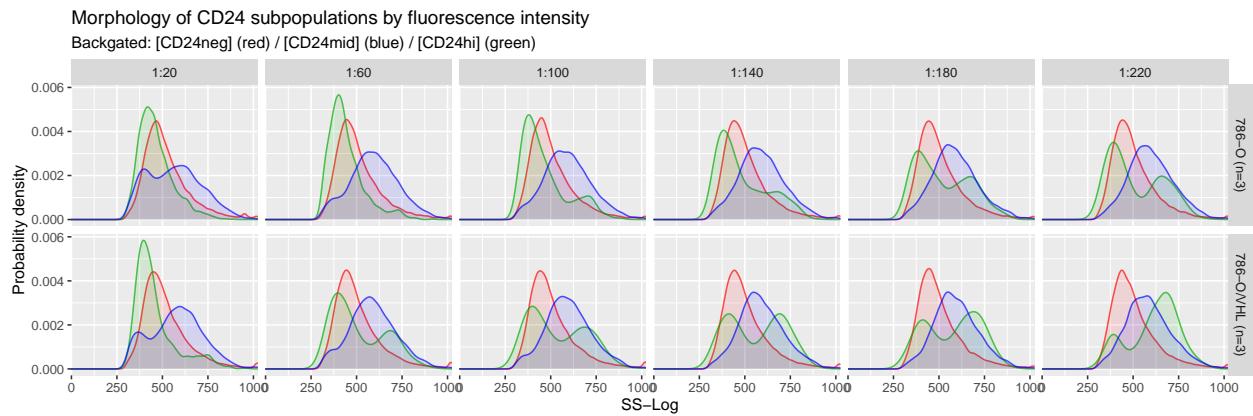
eventsData@phenoData@data$cellLine <- factor(
  eventsData@phenoData@data$cellLine,
  levels=cellLineOrdering)
cd24negData@phenoData@data$cellLine <- factor(
  cd24negData@phenoData@data$cellLine,
  levels=cellLineOrdering)
cd24midData@phenoData@data$cellLine <- factor(
  cd24midData@phenoData@data$cellLine,
  levels=cellLineOrdering)
cd24hiData@phenoData@data$cellLine <- factor(
  cd24hiData@phenoData@data$cellLine,
  levels=cellLineOrdering)

ggplot2::ggplot(
  data=eventsData,
  mapping=ggplot2::aes(x=SS.Log)
) +
  ggplot2::labs(
    title="Morphology of CD24 subpopulations by fluorescence intensity",
    subtitle="Backgated: [CD24neg] (red) / [CD24mid] (blue) / [CD24hi] (green)",
    x="SS-Log",
    y="Probability density") +
  ggplot2::scale_x_continuous(
    limits=c(0, 1023),
    expand=c(0, 0)) +
  ggplot2::geom_density(
    data=cd24negData,
```

```

color="#FF0000AA",
fill="red",
alpha=0.1) +
ggplot2::geom_density(
  data=cd24hiData,
  color="#00AA00AA",
  fill="#00AA00", # Dark green
  alpha=0.1) +
ggplot2::geom_density(
  data=cd24midData,
  color="#0000FFAA",
  fill="blue",
  alpha=0.1) +
ggplot2::facet_grid(
  rows=dplyr::vars(cellLine),
  cols=dplyr::vars(dilution),
  labeller=ggplot2::as_labeller(facetLabels))

```



4.2.2 A step-by-step example: CD24mid swarm deconvolution

4.2.2.1 Mixed distribution modeling

Let's compute the ML estimate of the mixed distribution using an expectation-maximization algorithm provided by mixtools. This will serve as an initial estimation of subpopulation characteristics before we proceed with model optimization.

Technical documentation:

- <https://cran.r-project.org/web/packages/mixtools/mixtools.pdf>
- <https://cran.r-project.org/web/packages/mixtools/vignettes/mixtools.pdf>

```
estimate <- list(
  "mu"     = c(373, 650),
  "sigma"  = c(35.6, 120)
)

generateSwarmModel <- function(
  gs,
  axis="SS.Log",
  gate="cd24pos",
  mu=c(0, 0),
  sigma=c(1, 1),
  maxIterations=10000
) {
  # Linter bindings
  pNeg <-
  pPos <-
  facetRow <-
  facetCol <- NULL

  numberOffFlowframes <- length(flowWorkspace::sampleNames(gs))
  swarmModel <- list()

  for (i in seq_len(numberOffFlowframes)) {
    y <- flowWorkspace::gs_pop_get_data(gs, gate)
    y <- flowWorkspace::cytoseq_to_flowSet(y)
    y <- y[[i]]@exprs[, axis]

    mixEM <- mixtools::normalmixEM(y, mu=mu, sigma=sigma, maxit=maxIterations)

    # Extract important results obtained from normalmixEM()
    distribution1 <- c(
      "mu"       = mixEM$mu[1],
      "sigma"    = mixEM$sigma[1],
      "proportion" = mixEM$lambda[1]
    )

    distribution2 <- c(
      "mu"       = mixEM$mu[2],
      "sigma"    = mixEM$sigma[2],
      "proportion" = mixEM$lambda[2]
    )
  }
}
```

```

# Ensure we correctly identify the true positive distribution
if (distribution1[["mu"]] < distribution2[["mu"]]) {
  negDistribution <- distribution1
  posDistribution <- distribution2
} else {
  negDistribution <- distribution2
  posDistribution <- distribution1
}

hartigansDipTestStatistic <-
  diptest::dip.test(y, B=2000, simulate.p.value=FALSE)$p.value

# Assign values to the appropriate columns
swarmModel[[i]] <- c(
  "id"      = i,
  "muNeg"   = negDistribution[["mu"]],
  "sigmaNeg" = negDistribution[["sigma"]],
  "pNeg"    = negDistribution[["proportion"]],
  "muPos"   = posDistribution[["mu"]],
  "sigmaPos" = posDistribution[["sigma"]],
  "pPos"    = posDistribution[["proportion"]],
  "hartigans" = hartigansDipTestStatistic
)
}

# Create the dataframe from all generated values
swarmModel <- BiocGenerics::as.data.frame(
  BiocGenerics::do.call("rbind", swarmModel)
)

# Compute number of events in each distribution
swarmModel$nNeg <- round(length(y) * swarmModel$pNeg, digits=0)
swarmModel$nPos <- round(length(y) * swarmModel$pPos, digits=0)

# Population statistics
swarmModel$welchTTest <-
  abs(swarmModel$muNeg - swarmModel$muPos) /
  sqrt(
    ((swarmModel$sigmaNeg^4)/swarmModel$nNeg) +
    ((swarmModel$sigmaPos^4)/swarmModel$nPos))

# Assign faceting values for plotting convenience
swarmModel$facetRow <- pData$facetRow
swarmModel$facetCol <- pData$facetCol

swarmModel
}

# TODO: mu and sigma may need to be tweaked recursively on a per-experiment
# basis
cd24midSwarmModel <- generateSwarmModel(
  gs=gatingset,
  axis="SS.Log",

```

```
    gate="cd24mid",
    mu=estimate$mu,
    sigma=estimate$sigma,
    maxIterations=10000
)

## number of iterations= 58
## number of iterations= 58
## number of iterations= 85
## number of iterations= 72
## number of iterations= 405
## number of iterations= 1909
## number of iterations= 65
## number of iterations= 51
## number of iterations= 2352
## number of iterations= 1540
## number of iterations= 1481
## number of iterations= 1598
## number of iterations= 54
## number of iterations= 234
## number of iterations= 1301
## number of iterations= 1092
## number of iterations= 1860
## number of iterations= 2904
## number of iterations= 51
## number of iterations= 67
## number of iterations= 57
## number of iterations= 1420
## number of iterations= 1067
## number of iterations= 1128
## number of iterations= 53
## number of iterations= 1713
## number of iterations= 1858
## number of iterations= 1743
## number of iterations= 4735
## number of iterations= 744
## number of iterations= 46
## number of iterations= 271
## number of iterations= 2196
## number of iterations= 2416
## number of iterations= 4254
## number of iterations= 1368
```

Let's check out how our model data looks so far.

```
printDataFrame(
  dataframe=cd24midSwarmModel,
  caption=
    "Swarm deconvolution model: initial ML estimate on a per-sample basis",
  fontsize=6
)
```

Table 3: Swarm deconvolution model: initial ML estimate on a per-sample basis

id	muNeg	sigmaNeg	pNeg	muPos	sigmaPos	pPos	hartigans	nNeg	nPos	welchTTest	facetRow	facetCol
1	396.2502	45.47151	0.2786944	627.9247	130.73896	0.7213056	0.0016110	836	2162	0.6186288	r1	c1
2	365.6965	31.41805	0.0695375	611.6603	126.74412	0.9304625	0.8431423	208	2790	0.7890223	r1	c2
3	342.1689	18.63243	0.0237496	597.3301	130.42876	0.9762504	0.9904091	71	2927	0.8046048	r1	c3
4	338.2743	20.14274	0.0195229	597.2074	125.27364	0.9804771	0.8238249	59	2939	0.8799455	r1	c4
5	532.5384	40.17127	0.1032401	599.3679	131.19264	0.8967599	0.9452544	310	2688	0.1940496	r1	c5
6	568.3445	105.04887	0.7503599	697.4892	129.81054	0.2496401	0.9157378	2250	748	0.1960944	r1	c6
7	390.0803	41.11500	0.2338327	600.0275	124.48321	0.7661673	0.0000530	701	2297	0.6370339	r2	c1
8	350.2707	22.82875	0.0349921	597.7028	125.29142	0.9650079	0.9639120	105	2893	0.8352025	r2	c2
9	564.5228	104.37870	0.8642001	775.7460	95.03780	0.1357999	0.8424461	2591	407	0.4256464	r2	c3
10	548.2789	95.97593	0.6961261	697.7414	120.09558	0.3038739	0.7689191	2087	911	0.2881748	r2	c4
11	556.6123	94.94854	0.8173546	758.1528	90.27656	0.1826454	0.9804892	2450	548	0.5129438	r2	c5
12	541.0980	88.72532	0.5796585	684.1912	117.44337	0.4203415	0.9791798	1738	1260	0.3312157	r2	c6
13	375.6986	36.05993	0.0924091	612.5806	127.51756	0.9075909	0.8959220	277	2721	0.7371015	r3	c1
14	538.8935	46.07314	0.0894822	612.4264	130.85571	0.9105178	0.8371142	268	2730	0.2086391	r3	c2
15	535.8466	91.58676	0.5656349	677.7136	119.41410	0.4343651	0.9258856	1696	1302	0.3190953	r3	c3
16	527.7733	88.34556	0.5245285	665.5280	116.14627	0.4754715	0.9131216	1573	1425	0.3376672	r3	c4
17	559.5073	103.39002	0.8999176	803.2029	92.73420	0.1000824	0.8848766	2698	300	0.4534202	r3	c5
18	546.1494	90.07902	0.6949464	711.8545	102.68036	0.3050536	0.9078441	2083	915	0.4235016	r3	c6
19	358.6581	31.61328	0.1609291	598.2758	127.41530	0.8390709	0.0018243	482	2516	0.7331242	r4	c1
20	348.7908	23.21432	0.0367609	591.9374	125.03770	0.9632391	0.8692413	110	2888	0.8230283	r4	c2
21	337.4829	18.00975	0.0184092	607.3286	124.23067	0.9815908	0.9922233	55	2943	0.9375203	r4	c3
22	538.1248	97.80425	0.6452774	678.6644	124.46504	0.3547226	0.9746102	1935	1063	0.2689524	r4	c4
23	542.9171	94.69992	0.6254976	673.4692	124.63938	0.3745024	0.9901248	1875	1123	0.2571255	r4	c5
24	537.4444	96.47954	0.6087726	665.4303	122.83263	0.3912274	0.6369635	1825	1173	0.2604129	r4	c6
25	351.5245	27.66856	0.0842573	613.2109	119.25198	0.9157427	0.6555798	253	2745	0.9492897	r5	c1
26	540.1524	101.75438	0.6448455	677.8514	125.51206	0.3551545	0.9656689	1933	1065	0.2563736	r5	c2
27	543.7559	96.76154	0.6440770	678.0266	121.22791	0.3559230	0.9897836	1931	1067	0.2697235	r5	c3
28	545.2358	90.79942	0.6670083	685.6656	116.44953	0.3329917	0.9171138	2000	998	0.3006010	r5	c4
29	557.0062	94.89092	0.7557905	714.6018	105.31833	0.2442095	0.9388683	2266	732	0.3490460	r5	c5
30	525.7306	90.28834	0.6552078	680.7267	123.23781	0.3447922	0.7701094	1964	1034	0.3057926	r5	c6
31	365.2591	32.92286	0.1306669	608.7118	129.57235	0.8693331	0.0496624	392	2606	0.7301999	r6	c1
32	557.6619	56.22707	0.1555773	603.2374	137.08106	0.8444227	0.9569248	466	2532	0.1136172	r6	c2
33	585.0356	113.56672	0.9384696	833.1352	86.17794	0.0615304	0.9763759	2814	184	0.4141510	r6	c3
34	562.3668	97.19094	0.8285732	758.5040	92.87097	0.1714268	0.7710601	2484	514	0.4614665	r6	c4
35	562.0383	95.08174	0.7643518	722.4078	109.97641	0.2356482	0.7564614	2292	706	0.3254188	r6	c5
36	543.5745	89.65853	0.6475734	684.1402	119.42589	0.3524266	0.9707170	1941	1057	0.2958508	r6	c6

Let's visualize this.

```
clusterModel <- function(
  model,
  centers=matrix(c(0, 0, 0, 0), ncol=2)
) {
  numberOfClusters <- BiocGenerics::nrow(centers)

  model$negCluster <- factor(
    safeClustering(
      data=model[, c("muNeg", "sigmaNeg")],
      centers=centers,
      principal=1
    ),
    levels=seq_len(numberOfClusters)
  )

  model$posCluster <- factor(
    safeClustering(
      data=model[, c("muPos", "sigmaPos")],
      centers=centers,
      principal=2
    ),
    levels=seq_len(numberOfClusters)
  )
}

model
}

plotModelClusters <- function(model, ylim=c(0, 1023), xlim=c(0, 1023)) {
  plot1 <- ggplot2::ggplot(
    data=model,
    mapping=ggplot2::aes(
      x=muNeg,
      y=sigmaNeg,
      color=negCluster
    )
  ) +
    ggplot2::labs(
      title="Estimated as negative",
      x="Estimate for mu",
      y="Estimate for sigma") +
    ggplot2::coord_cartesian(
      ylim=ylim,
      xlim=xlim) +
    ggplot2::scale_x_continuous(expand=c(0, 0)) +
    ggplot2::scale_y_continuous(expand=c(0, 0)) +
    ggplot2::theme(legend.position="none") +
    ggplot2::geom_point(size=2) +
    ggplot2::scale_color_manual(
      values=c(
        "1" = colorPalette[["red"]],
        "2" = colorPalette[["blue"]]
      ),
      guide=FALSE
    )
}
```

```

drop=FALSE)

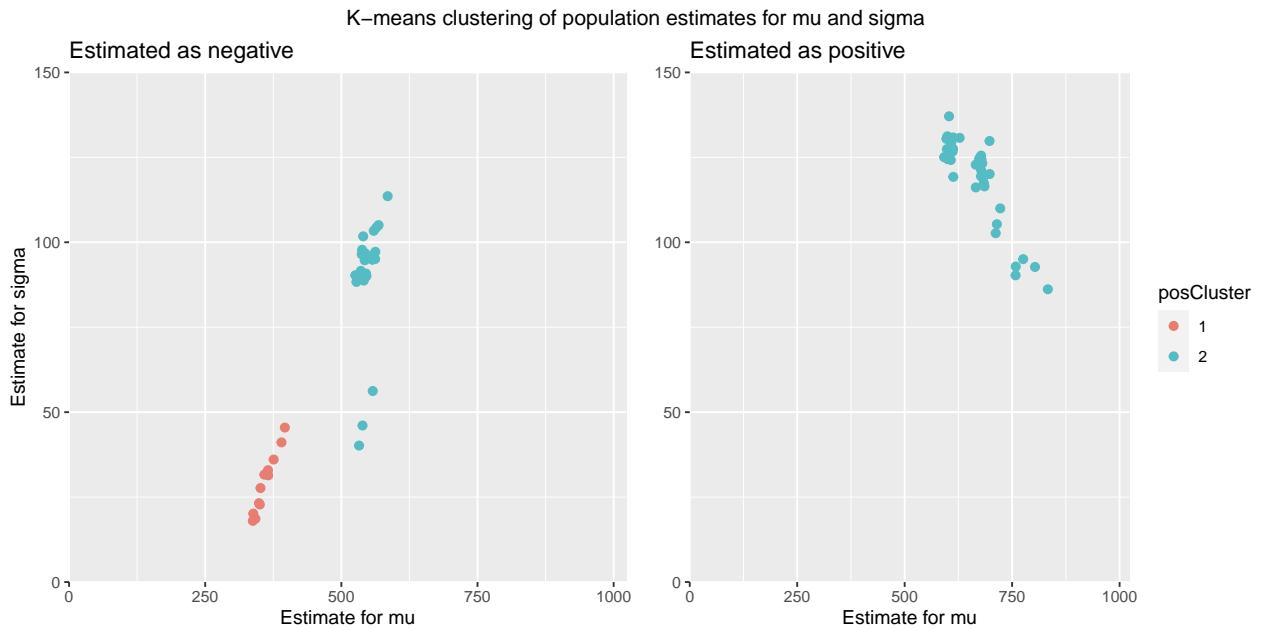
plot2 <- ggplot2::ggplot(
  data=model,
  mapping=ggplot2::aes(
    x=muPos,
    y=sigmaPos,
    color=posCluster
  )
) +
  ggplot2::labs(
    title="Estimated as positive",
    x="Estimate for mu",
    y=ggplot2::element_blank()) +
  ggplot2::coord_cartesian(
    ylim=ylim,
    xlim=xlim) +
  ggplot2::scale_x_continuous(expand=c(0, 0)) +
  ggplot2::scale_y_continuous(expand=c(0, 0)) +
  ggplot2::geom_point(size=2) +
  ggplot2::scale_color_manual(
    values=c(
      "1" = colorPalette[["red"]],
      "2" = colorPalette[["blue"]]),
    drop=FALSE)

gridExtra::grid.arrange(
  plot1, plot2,
  layout_matrix=cbind(c(1), c(2)),
  top="K-means clustering of population estimates for mu and sigma"
)
}

cd24midSwarmModel <- clusterModel(
  model=cd24midSwarmModel,
  centers=matrix(c(estimate$mu, estimate$sigma), ncol=2)
)

plotModelClusters(
  model=cd24midSwarmModel,
  ylim=c(0, 150),
  xlim=c(0, 1023)
)

```



```
# Get an idea of the distribution of the calculated averages
# Good results would be unimodal for each single parameter

# Bimodal: looks like ~375 would be a better starting parameter for negative pop
# Bimodal: based on values of mu, ~30 would be a good estimator of the negative
#           pop stddev
# Unimodal: looks like 650 is a good starting parameter for positive pop
# Unimodal: looks like 120 is a good starting parameter for positive pop stddev
```

4.2.2.2 Parameter optimization

Now that we've generated a mixed model for every sample, we can compute best-fit parameters for the whole experiment globally.

```
estimateGlobalParameters <- function(model) {
  # Calculate means weighted in favour of multimodality:
  # result hovers around 373
  # Calculate stddev weighted in favour of multimodality:
  # result hovers around 36
  negStats <- model %>%
    dplyr::filter(negCluster == 1, posCluster == 2) %>%
    dplyr::summarise(
      mu      = stats::weighted.mean(muNeg,   w=1-hartigans),
      sigma   = stats::weighted.mean(sigmaNeg, w=1-hartigans),
      .groups = "drop"
    )

  posStats <- model %>%
    dplyr::filter(negCluster == 1, posCluster == 2) %>%
    dplyr::summarise(
      mu      = stats::weighted.mean(muPos,   w=1-hartigans),
      sigma   = stats::weighted.mean(sigmaPos, w=1-hartigans),
      .groups = "drop"
    )

  params <- rbind("neg" = negStats, "pos" = posStats)
  params
}

estimatedParameters <- estimateGlobalParameters(model=cd24midSwarmModel)

printDataFrame(
  dataframe=estimatedParameters,
  caption="Estimated parameters"
)
```

Table 4: Estimated parameters

	mu	sigma
neg	372.9355	35.68217
pos	608.2555	127.16479

```
applyGlobalParameters <- function(model, params) {
  # Update our model with our best global parameter estimates
  model$muNeg    <- params["neg", "mu"]
  model$sigmaNeg <- params["neg", "sigma"]
  model$muPos    <- params["pos", "mu"]
  model$sigmaPos <- params["pos", "sigma"]
  model
}

cd24midSwarmModel <- applyGlobalParameters(
  model=cd24midSwarmModel,
```

```

    params=estimatedParameters
)

printDataFrame(
  datafram=cd24midSwarmModel,
  caption="Swarm deconvolution model: best-fit mu and sigma global parameters",
  fontsize=5
)

```

Table 5: Swarm deconvolution model: best-fit mu and sigma global parameters

id	muNeg	sigmaNeg	pNeg	muPos	sigmaPos	pPos	hartigans	nNeg	nPos	welchTTest	facetRow	facetCol	negCluster	posCluster
1	372.9355	35.68217	0.2786944	608.2555	127.1648	0.7213056	0.0016110	836	2162	0.6186288	r1	c1	1	2
2	372.9355	35.68217	0.0695375	608.2555	127.1648	0.9304625	0.8431423	208	2790	0.7890223	r1	c2	1	2
3	372.9355	35.68217	0.0237496	608.2555	127.1648	0.9762504	0.9904091	71	2927	0.8046048	r1	c3	1	2
4	372.9355	35.68217	0.0195229	608.2555	127.1648	0.9804771	0.8238249	59	2939	0.8799455	r1	c4	1	2
5	372.9355	35.68217	0.1032401	608.2555	127.1648	0.8967599	0.9452544	310	2688	0.1940496	r1	c5	2	2
6	372.9355	35.68217	0.7503599	608.2555	127.1648	0.2496401	0.9157378	2250	748	0.1960944	r1	c6	2	2
7	372.9355	35.68217	0.2338327	608.2555	127.1648	0.7661673	0.0000530	701	2297	0.6370339	r2	c1	1	2
8	372.9355	35.68217	0.0349921	608.2555	127.1648	0.9650079	0.9639120	105	2893	0.8352025	r2	c2	1	2
9	372.9355	35.68217	0.8642001	608.2555	127.1648	0.1357999	0.8424461	2591	407	0.4256464	r2	c3	2	2
10	372.9355	35.68217	0.6961261	608.2555	127.1648	0.3038739	0.7689191	2087	911	0.2881748	r2	c4	2	2
11	372.9355	35.68217	0.8173546	608.2555	127.1648	0.1826454	0.9804892	2450	548	0.5129438	r2	c5	2	2
12	372.9355	35.68217	0.5796585	608.2555	127.1648	0.4203415	0.9791798	1738	1260	0.3312157	r2	c6	2	2
13	372.9355	35.68217	0.0924091	608.2555	127.1648	0.9075909	0.8959220	277	2721	0.7371015	r3	c1	1	2
14	372.9355	35.68217	0.0894822	608.2555	127.1648	0.9105178	0.8371142	268	2730	0.2086391	r3	c2	2	2
15	372.9355	35.68217	0.5656349	608.2555	127.1648	0.4343651	0.9258856	1696	1302	0.3190953	r3	c3	2	2
16	372.9355	35.68217	0.5245285	608.2555	127.1648	0.4754715	0.9131216	1573	1425	0.3376672	r3	c4	2	2
17	372.9355	35.68217	0.8999176	608.2555	127.1648	0.1000824	0.8848766	2698	300	0.4534202	r3	c5	2	2
18	372.9355	35.68217	0.6949464	608.2555	127.1648	0.3050536	0.9078441	2083	915	0.4235016	r3	c6	2	2
19	372.9355	35.68217	0.1609291	608.2555	127.1648	0.8390709	0.0018243	482	2516	0.7331242	r4	c1	1	2
20	372.9355	35.68217	0.0367609	608.2555	127.1648	0.9632391	0.8692413	110	2888	0.8230283	r4	c2	1	2
21	372.9355	35.68217	0.0184092	608.2555	127.1648	0.9815908	0.9922233	55	2943	0.9375203	r4	c3	1	2
22	372.9355	35.68217	0.6452774	608.2555	127.1648	0.3547226	0.9746102	1935	1063	0.2689524	r4	c4	2	2
23	372.9355	35.68217	0.6254976	608.2555	127.1648	0.3745024	0.9901248	1875	1123	0.2571255	r4	c5	2	2
24	372.9355	35.68217	0.6087726	608.2555	127.1648	0.3912274	0.6369635	1825	1173	0.2604129	r4	c6	2	2
25	372.9355	35.68217	0.0842573	608.2555	127.1648	0.9157427	0.6555798	253	2745	0.9492897	r5	c1	1	2
26	372.9355	35.68217	0.6448455	608.2555	127.1648	0.3551545	0.9656689	1933	1065	0.2563736	r5	c2	2	2
27	372.9355	35.68217	0.6440770	608.2555	127.1648	0.3559230	0.9897836	1931	1067	0.2697235	r5	c3	2	2
28	372.9355	35.68217	0.6670083	608.2555	127.1648	0.3329917	0.9171138	2000	998	0.3006010	r5	c4	2	2
29	372.9355	35.68217	0.7557905	608.2555	127.1648	0.2442095	0.9388683	2266	732	0.3490460	r5	c5	2	2
30	372.9355	35.68217	0.6552078	608.2555	127.1648	0.3447922	0.7701094	1964	1034	0.3057926	r5	c6	2	2
31	372.9355	35.68217	0.1306669	608.2555	127.1648	0.8693331	0.0496624	392	2606	0.7301999	r6	c1	1	2
32	372.9355	35.68217	0.1555773	608.2555	127.1648	0.8444227	0.9569248	466	2532	0.1136172	r6	c2	2	2
33	372.9355	35.68217	0.9384696	608.2555	127.1648	0.0615304	0.9763759	2814	184	0.4141510	r6	c3	2	2
34	372.9355	35.68217	0.8285732	608.2555	127.1648	0.1714268	0.7710601	2484	514	0.4614665	r6	c4	2	2
35	372.9355	35.68217	0.7643518	608.2555	127.1648	0.2356482	0.7564614	2292	706	0.3254188	r6	c5	2	2
36	372.9355	35.68217	0.6475734	608.2555	127.1648	0.3524266	0.9707170	1941	1057	0.2958508	r6	c6	2	2

4.2.2.3 Resolving the distributions using an EM algorithm

Reference: * <http://tinyheero.github.io/2016/01/03/gmm-em.html>

This first function is the expectation step of the EM algorithm:

```
# Expectation Step of the EM Algorithm
eStep <- function(x, muVector, sdVector, alphaVector) {
  negDistributionProbabilities <-
    stats::dnorm(x, mean=muVector[1], sd=sdVector[1]) * alphaVector[1]
  posDistributionProbabilities <-
    stats::dnorm(x, mean=muVector[2], sd=sdVector[2]) * alphaVector[2]

  sumOfProbabilities <-
    negDistributionProbabilities + posDistributionProbabilities

  negPosteriorDistribution <-
    negDistributionProbabilities / sumOfProbabilities
  posPosteriorDistribution <-
    posDistributionProbabilities / sumOfProbabilities

  sumOfProbabilitiesLn <- log(sumOfProbabilities, base=exp(1))
  logLikelihood <- sum(sumOfProbabilitiesLn)

  list(
    "logLikelihood" = logLikelihood,
    "posteriorDf" = BiocGenerics::cbind(
      negPosteriorDistribution,
      posPosteriorDistribution
    )
  )
}
```

And this is for the maximization step of the EM algorithm:

```
# Maximization Step of the EM Algorithm
mStep <- function(x, posteriorDf) {
  negProportion <- sum(posteriorDf[, 1])
  posProportion <- sum(posteriorDf[, 2])

  negMu <- (1/negProportion) * sum(posteriorDf[, 1] * x)
  posMu <- (1/posProportion) * sum(posteriorDf[, 2] * x)

  negVar <- sum(posteriorDf[, 1] * (x - negMu)^2) * (1/negProportion)
  posVar <- sum(posteriorDf[, 2] * (x - posMu)^2) * (1/posProportion)

  negAlpha <- negProportion / length(x)
  posAlpha <- posProportion / length(x)

  list(
    "mu"     = c(negMu, posMu),
    "var"    = c(negVar, posVar),
    "alpha"   = c(negAlpha, posAlpha)
  )
}
```

And we can now iterate over both steps up to 50 times for each sample:

```
solveSwarmProportions <- function(gs, gate, axis, model) {  
  # Linter bindings  
  muNeg <-  
  muPos <-  
  sigmaNeg <-  
  sigmaPos <-  
  pNeg <-  
  pPos <-  
  nNeg <-  
  nPos <-  
  nTot <-  
  alpha <- NULL  
  
  populationsMu <- c(model$muNeg[1], model$muPos[1])  
  populationsSd <- c(model$sigmaNeg[1], model$sigmaPos[1])  
  populationsProportions <- c(model$pNeg[1], model$pPos[1])  
  
  numberOfWorkspace <- length(flowWorkspace::sampleNames(gs))  
  
  for (i in seq_len(numberOfWorkspace)) {  
    y <- flowWorkspace::gs_pop_get_data(gs, gate)  
    y <- flowWorkspace::cytoset_to_flowSet(y)  
    y <- y[[i]]@exprs[, axis]  
  
    eStepResultForAlpha <- function(alpha) {  
      eStep(  
        y,  
        muVector=populationsMu,  
        sdVector=populationsSd,  
        alphaVector=alpha  
      )  
    }  
  
    mStepResultForPosterior <- function(posterior) {  
      mStep(y, posteriorDf=posterior)  
    }  
  
    for (j in 1:50) {  
      if (j == 1) {  
        eStepResult <- eStepResultForAlpha(populationsProportions)  
        mStepResult <- mStepResultForPosterior(eStepResult[["posteriorDf"]])  
        currentLogLikelihood <- eStepResult[["logLikelihood"]]  
        logLikelihoodVector <- eStepResult[["logLikelihood"]]  
      } else {  
        eStepResult <- eStepResultForAlpha(mStepResult[["alpha"]])  
        mStepResult <- mStepResultForPosterior(eStepResult[["posteriorDf"]])  
        logLikelihoodVector <-  
          c(logLikelihoodVector, eStepResult[["logLikelihood"]])  
        logLikelihoodDiff <-  
          abs((currentLogLikelihood - eStepResult[["logLikelihood"]]))  
  
        if (logLikelihoodDiff < 1e-6) {  
          break  
        }  
      }  
    }  
  }  
}
```

```

        break
    } else {
        currentLogLikelihood <- eStepResult[["logLikelihood"]]
    }
}

model$pNeg[i] <- mStepResult$alpha[1]
model$pPos[i] <- mStepResult$alpha[2]
}
}

model$nTot <- model$nNeg + model$nPos
model$nNeg <- round(model$nTot * model$pNeg, digits=0)
model$nPos <- round(model$nTot * model$pPos, digits=0)
model
}

cd24midSwarmModel <- solveSwarmProportions(
  gs=gatingset,
  gate="cd24mid",
  axis="SS.Log",
  model=cd24midSwarmModel
)

```

Let's check out how our model data looks so far.

```

printDataFrame(
  dataframe=cd24midSwarmModel,
  caption="Swarm deconvolution final model for [CD24mid]",
  fontsize=5
)

```

Table 6: Swarm deconvolution final model for [CD24mid]

id	muNeg	sigmaNeg	pNeg	muPos	sigmaPos	pPos	hartigans	nNeg	nPos	welchTTest	facetRow	facetCol	negCluster	posCluster	nTot
1	372.9355	35.68217	0.2094181	608.2555	127.1648	0.7905819	0.0016110	628	2370	0.6186288	r1	c1	1	2	2998
2	372.9355	35.68217	0.0706969	608.2555	127.1648	0.293031	0.8431423	212	2786	0.7890223	r1	c2	1	2	2998
3	372.9355	35.68217	0.0486096	608.2555	127.1648	0.9513904	0.9090491	146	2852	0.8046048	r1	c3	1	2	2998
4	372.9355	35.68217	0.0311993	608.2555	127.1648	0.9688007	0.8238249	94	2904	0.8799455	r1	c4	1	2	2998
5	372.9355	35.68217	0.0218967	608.2555	127.1648	0.9781033	0.9452544	66	2932	0.1940496	r1	c5	2	2	2998
6	372.9355	35.68217	0.0065449	608.2555	127.1648	0.9934551	0.9157378	20	2978	0.1960944	r1	c6	2	2	2998
7	372.9355	35.68217	0.2073226	608.2555	127.1648	0.7926774	0.0000530	622	2376	0.6370339	r2	c1	1	2	2998
8	372.9355	35.68217	0.0489161	608.2555	127.1648	0.9510839	0.9639120	147	2851	0.8352025	r2	c2	1	2	2998
9	372.9355	35.68217	0.0199490	608.2555	127.1648	0.9800510	0.8424461	60	2938	0.4256464	r2	c3	2	2	2998
10	372.9355	35.68217	0.0104065	608.2555	127.1648	0.9895935	0.7689191	31	2967	0.2881748	r2	c4	2	2	2998
11	372.9355	35.68217	0.0021640	608.2555	127.1648	0.9978360	0.9804892	6	2992	0.5129438	r2	c5	2	2	2998
12	372.9355	35.68217	0.0003180	608.2555	127.1648	0.9996820	0.9791798	1	2997	0.3312157	r2	c6	2	2	2998
13	372.9355	35.68217	0.0864478	608.2555	127.1648	0.9135522	0.8959220	259	2739	0.7371015	r3	c1	1	2	2998
14	372.9355	35.68217	0.0082127	608.2555	127.1648	0.9917873	0.8371142	25	2973	0.2086391	r3	c2	2	2	2998
15	372.9355	35.68217	0.0046754	608.2555	127.1648	0.9953246	0.9258856	14	2984	0.3190953	r3	c3	2	2	2998
16	372.9355	35.68217	0.0104424	608.2555	127.1648	0.9895576	0.9131216	31	2967	0.3376672	r3	c4	2	2	2998
17	372.9355	35.68217	0.0281939	608.2555	127.1648	0.9718061	0.8848766	85	2913	0.4534202	r3	c5	2	2	2998
18	372.9355	35.68217	0.0011684	608.2555	127.1648	0.9988316	0.9078441	4	2994	0.4235016	r3	c6	2	2	2998
19	372.9355	35.68217	0.1866447	608.2555	127.1648	0.8133553	0.0018243	560	2438	0.7331242	r4	c1	1	2	2998
20	372.9355	35.68217	0.0597391	608.2555	127.1648	0.9402609	0.8692413	179	2819	0.8230283	r4	c2	1	2	2998
21	372.9355	35.68217	0.0164383	608.2555	127.1648	0.9835617	0.9922233	49	2949	0.9375203	r4	c3	1	2	2998
22	372.9355	35.68217	0.0289232	608.2555	127.1648	0.9710768	0.9746102	87	2911	0.2689524	r4	c4	2	2	2998
23	372.9355	35.68217	0.0114944	608.2555	127.1648	0.9885056	0.9901248	34	2964	0.2571255	r4	c5	2	2	2998
24	372.9355	35.68217	0.0263442	608.2555	127.1648	0.9736558	0.6369635	79	2919	0.2604129	r4	c6	2	2	2998
25	372.9355	35.68217	0.0822313	608.2555	127.1648	0.9177687	0.6555798	247	2751	0.9492897	r5	c1	1	2	2998
26	372.9355	35.68217	0.0321891	608.2555	127.1648	0.9678109	0.9656689	97	2901	0.2563736	r5	c2	2	2	2998
27	372.9355	35.68217	0.0122120	608.2555	127.1648	0.9877880	0.9897836	37	2961	0.2697235	r5	c3	2	2	2998
28	372.9355	35.68217	0.0023415	608.2555	127.1648	0.9976585	0.9171138	7	2991	0.3006010	r5	c4	2	2	2998
29	372.9355	35.68217	0.0002050	608.2555	127.1648	0.9997950	0.9388683	1	2997	0.3490460	r5	c5	2	2	2998
30	372.9355	35.68217	0.0298702	608.2555	127.1648	0.9701298	0.7701094	90	2908	0.3057926	r5	c6	2	2	2998
31	372.9355	35.68217	0.1405790	608.2555	127.1648	0.8594210	0.0496624	421	2577	0.7301999	r6	c1	1	2	2998

Table 6: Swarm deconvolution final model for [CD24mid] (*continued*)

id	muNeg	sigmaNeg	pNeg	muPos	sigmaPos	pPos	hartigans	nNeg	nPos	welchITest	facetRow	facetCol	negCluster	posCluster	nTot
32	372.9355	35.68217	0.0268358	608.2555	127.1648	0.9731642	0.9569248	80	2918	0.1136172	r6	c2	2	2	2998
33	372.9355	35.68217	0.0151463	608.2555	127.1648	0.9848537	0.9763759	45	2953	0.4141510	r6	c3	2	2	2998
34	372.9355	35.68217	0.0000994	608.2555	127.1648	0.9999006	0.7710601	0	2998	0.4614665	r6	c4	2	2	2998
35	372.9355	35.68217	0.0000017	608.2555	127.1648	0.9999983	0.7564614	0	2998	0.3254188	r6	c5	2	2	2998
36	372.9355	35.68217	0.0004802	608.2555	127.1648	0.9995198	0.9707170	1	2997	0.2958508	r6	c6	2	2	2998

4.2.2.4 Plotting the solutions

Now that we have obtained the optimal solution for our data set, we can create model distributions for use in plotting:

```
genDistributionsForPlotting <- function(model) {
  numberOfflowframes <- length(row.names(model))
  mixedModelDistributions <- list()

  for (i in seq_len(numberOfflowframes)) {
    x <- seq(0, 1023)

    negDistribution <-
      stats::dnorm(x, mean=model$muNeg[i], sd=model$sigmaNeg[i]) * model$pNeg[i]

    posDistribution <-
      stats::dnorm(x, mean=model$muPos[i], sd=model$sigmaPos[i]) * model$pPos[i]

    facetRow <- paste0("r", ((i-1) %% 6)+1)
    facetCol <- paste0("c", ((i-1) %% 6)+1)

    tempData <- data.frame(
      "distributionXAxis" = x,
      "distributionYAxis" = c(negDistribution, posDistribution),
      "population" = rep(c("neg", "pos"), each=1024),
      "facetRow" = rep(facetRow, times=2048),
      "facetCol" = rep(facetCol, times=2048)
    )

    mixedModelDistributions <-
      BiocGenerics::rbind(mixedModelDistributions, tempData)
  }

  mixedModelDistributions
}

plotDeconvolutedExperiment <- function(gs, model, gate, axis) {
  mixedModelDistributions <- genDistributionsForPlotting(model)

  ggplot2::ggplot(
    data=flowWorkspace::gs_pop_get_data(gs, gate),
    mapping=ggplot2::aes_string(x=axis)
  ) +
    ggplot2::scale_x_continuous(
      limits=c(0, 1024),
      expand=c(0, 0)) +
    ggplot2::geom_histogram(
      mapping=ggplot2::aes_string(
        x=axis,
        y=".density."),
      binwidth=40,
      colour="#AAAAAA",
      fill="#E6E6E6",
      size=0.1) +
    ggplot2::geom_line()
```

```

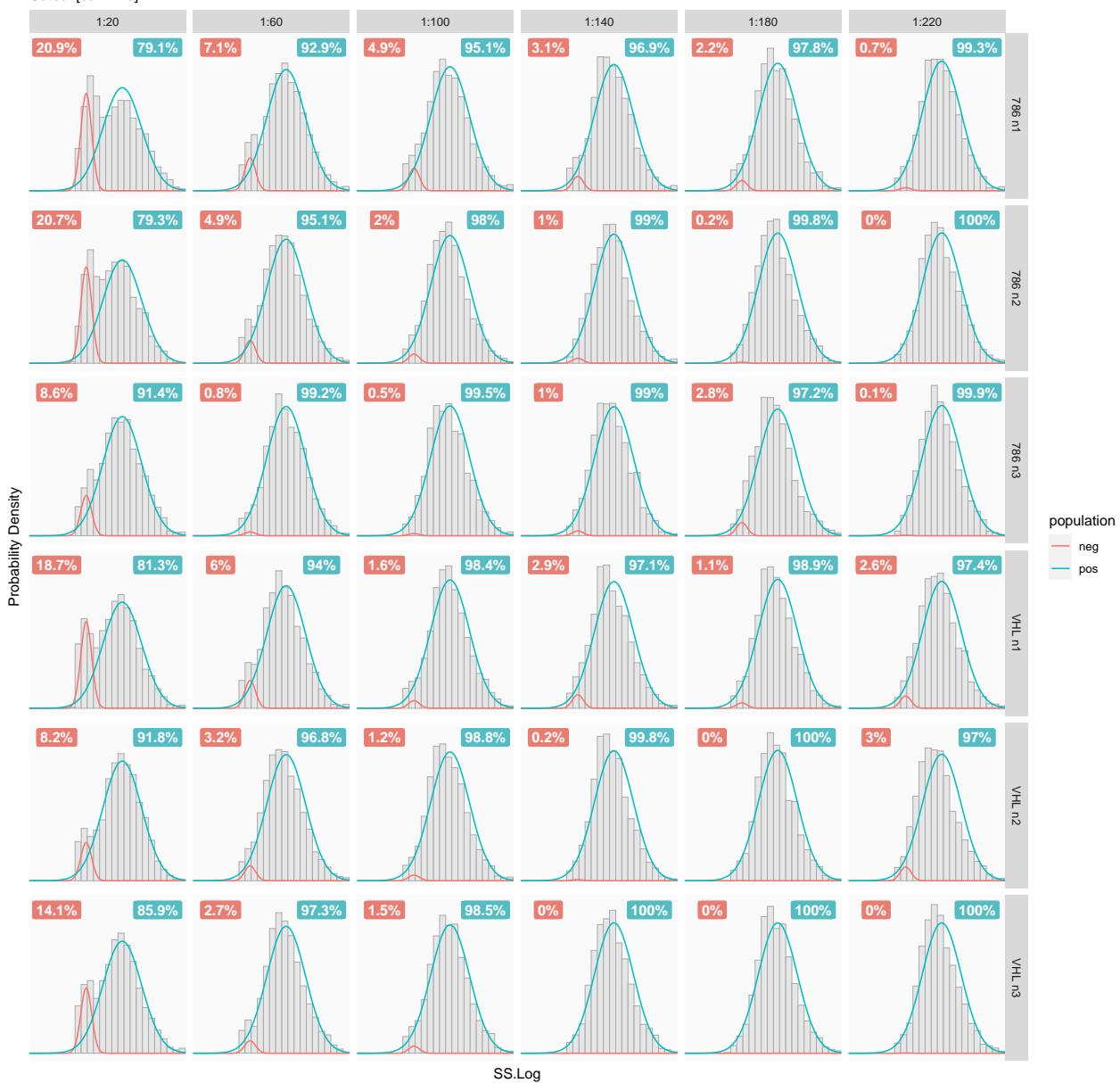
data=mixedModelDistributions,
mapping=ggplot2::aes(
  x=distributionXAxis,
  y=distributionYAxis,
  colour=population)) +
ggplot2::labs(
  title="Swarm deconvolution with a mixed gaussian model",
  subtitle=paste0("Gated: [", gate, "]"),
  x=axis,
  y="Probability Density") +
ggplot2::theme(
  panel.grid.major=ggplot2::element_blank(),
  panel.grid.minor=ggplot2::element_blank(),
  panel.background=ggplot2::element_rect(fill="#FAFAFA"),
  axis.text.x=ggplot2::element_blank(),
  axis.text.y=ggplot2::element_blank(),
  axis.ticks.x=ggplot2::element_blank(),
  axis.ticks.y=ggplot2::element_blank()) +
ggplot2::geom_label(
  data=model,
  x=180,
  y=0.00345,
  mapping=ggplot2::aes(label=paste0(round(pNeg*100, 1), "%")),
  fill="#E87D72",
  color="#FFFFFF",
  fontface="bold",
  size=4) +
ggplot2::geom_label(
  data=model,
  x=840,
  y=0.00345,
  mapping=ggplot2::aes(label=paste0(round(pPos*100, 1), "%")),
  fill="#54BCC2",
  color="#FFFFFF",
  fontface="bold",
  size=4) +
ggplot2::facet_grid(
  rows=dplyr::vars(facetRow),
  cols=dplyr::vars(facetCol),
  labeller=ggplot2::as_labeller(facetLabels))
}

plotDeconvolutedExperiment(
  gs=gatingset,
  model=cd24midSwarmModel,
  gate="cd24mid",
  axis="SS.Log"
)
## Warning: Removed 72 rows containing missing values (geom_bar).

```

Swarm deconvolution with a mixed gaussian model

Gated: [cd24mid]



4.2.3 Putting it all together: CD24hi swarm deconvolution

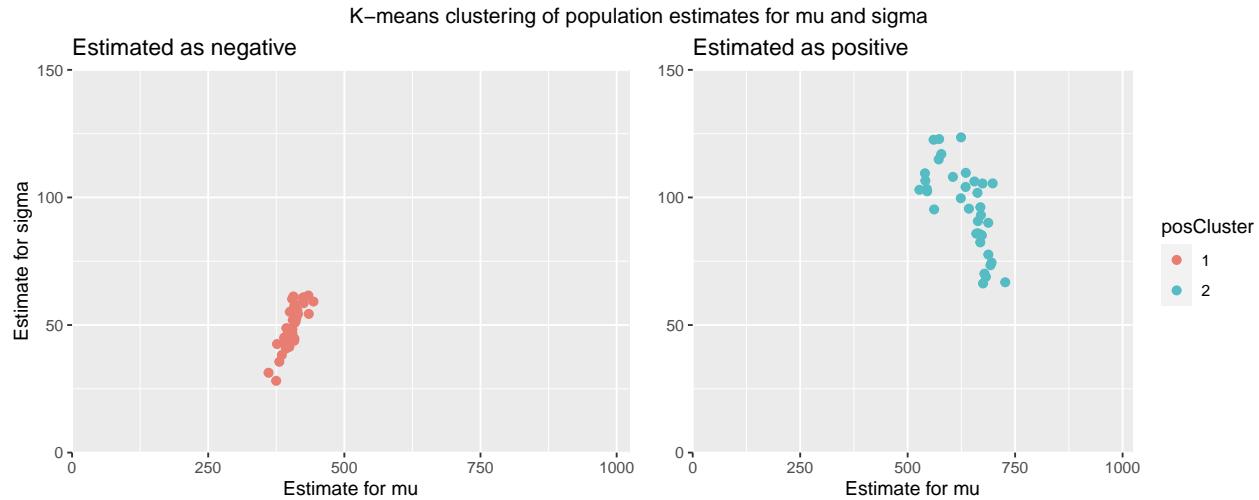
```
estimate <- list(  
  "mu"    = c(373, 650),  
  "sigma" = c(80, 80)  
)  
  
cd24hiSwarmModel <- generateSwarmModel(  
  gs=gatingset,  
  axis="SS.Log",  
  gate="cd24hi",  
  mu=estimate[["mu"]],  
  sigma=estimate[["sigma"]],  
  maxIterations=10000  
)  
  
## number of iterations= 80  
## number of iterations= 95  
## number of iterations= 104  
## number of iterations= 53  
## number of iterations= 64  
## number of iterations= 56  
## number of iterations= 90  
## number of iterations= 77  
## number of iterations= 48  
## number of iterations= 41  
## number of iterations= 61  
## number of iterations= 19  
## number of iterations= 80  
## number of iterations= 73  
## number of iterations= 144  
## number of iterations= 72  
## number of iterations= 33  
## number of iterations= 22  
## number of iterations= 45  
## number of iterations= 30  
## number of iterations= 52  
## number of iterations= 17  
## number of iterations= 32  
## number of iterations= 12  
## number of iterations= 252  
## number of iterations= 37  
## number of iterations= 35  
## number of iterations= 35  
## number of iterations= 34  
## number of iterations= 16  
## number of iterations= 35  
## number of iterations= 52  
## number of iterations= 65  
## number of iterations= 6  
## number of iterations= 30  
## number of iterations= 36  
  
cd24hiSwarmModel <- clusterModel(  
  model=cd24hiSwarmModel,
```

```

  centers=matrix(c(estimate[["mu"]], estimate[["sigma"]]), ncol=2)
)

plotModelClusters(
  model=cd24hiSwarmModel,
  ylim=c(0, 150),
  xlim=c(0, 1023)
)

```



```

cd24hiSwarmModel <- applyGlobalParameters(
  model=cd24hiSwarmModel,
  params=estimateGlobalParameters(cd24hiSwarmModel)
)

cd24hiSwarmModel <- solveSwarmProportions(
  gs=gatingset,
  gate="cd24hi",
  axis="SS.Log",
  model=cd24hiSwarmModel
)

printDataFrame(
  dataframe=cd24hiSwarmModel,
  caption="Swarm deconvolution final model for [CD24hi]",
  fontsize=5
)

```

Table 7: Swarm deconvolution final model for [CD24hi]

id	muNeg	sigmaNeg	pNeg	muPos	sigmaPos	pPos	hartigans	nNeg	nPos	welchITest	facetRow	facetCol	negCluster	posCluster	nTot
1	403.037	49.24012	0.7273018	666.7118	87.27702	0.2726982	0.4342748	39	15	0.0577825	r1	c1	1	2	54
2	403.037	49.24012	0.8693629	666.7118	87.27702	0.1306371	0.9191252	47	7	0.0418459	r1	c2	1	2	54
3	403.037	49.24012	0.8123504	666.7118	87.27702	0.1876496	0.9924966	44	10	0.0441530	r1	c3	1	2	54
4	403.037	49.24012	0.8267901	666.7118	87.27702	0.1732099	0.9821544	45	9	0.0546781	r1	c4	1	2	54
5	403.037	49.24012	0.7940708	666.7118	87.27702	0.2059292	0.9923969	43	11	0.0585287	r1	c5	1	2	54
6	403.037	49.24012	0.7457569	666.7118	87.27702	0.2542431	0.9932503	40	14	0.0871625	r1	c6	1	2	54
7	403.037	49.24012	0.8000080	666.7118	87.27702	0.1999920	0.9105907	43	11	0.0551437	r2	c1	1	2	54
8	403.037	49.24012	0.8026214	666.7118	87.27702	0.1973786	0.9945522	43	11	0.0466224	r2	c2	1	2	54
9	403.037	49.24012	0.7170202	666.7118	87.27702	0.2829798	0.0899201	39	15	0.1323929	r2	c3	1	2	54
10	403.037	49.24012	0.5486561	666.7118	87.27702	0.4513439	0.1515512	30	24	0.1416747	r2	c4	1	2	54
11	403.037	49.24012	0.5088555	666.7118	87.27702	0.4911445	0.1195755	27	27	0.1218253	r2	c5	1	2	54
12	403.037	49.24012	0.5918482	666.7118	87.27702	0.4081518	0.0023661	32	22	0.1873881	r2	c6	1	2	54
13	403.037	49.24012	0.8887473	666.7118	87.27702	0.1121527	0.8378481	48	6	0.0433730	r3	c1	1	2	54
14	403.037	49.24012	0.8144597	666.7118	87.27702	0.1855403	0.9901901	44	10	0.0607364	r3	c2	1	2	54

Table 7: Swarm deconvolution final model for [CD24hi] (continued)

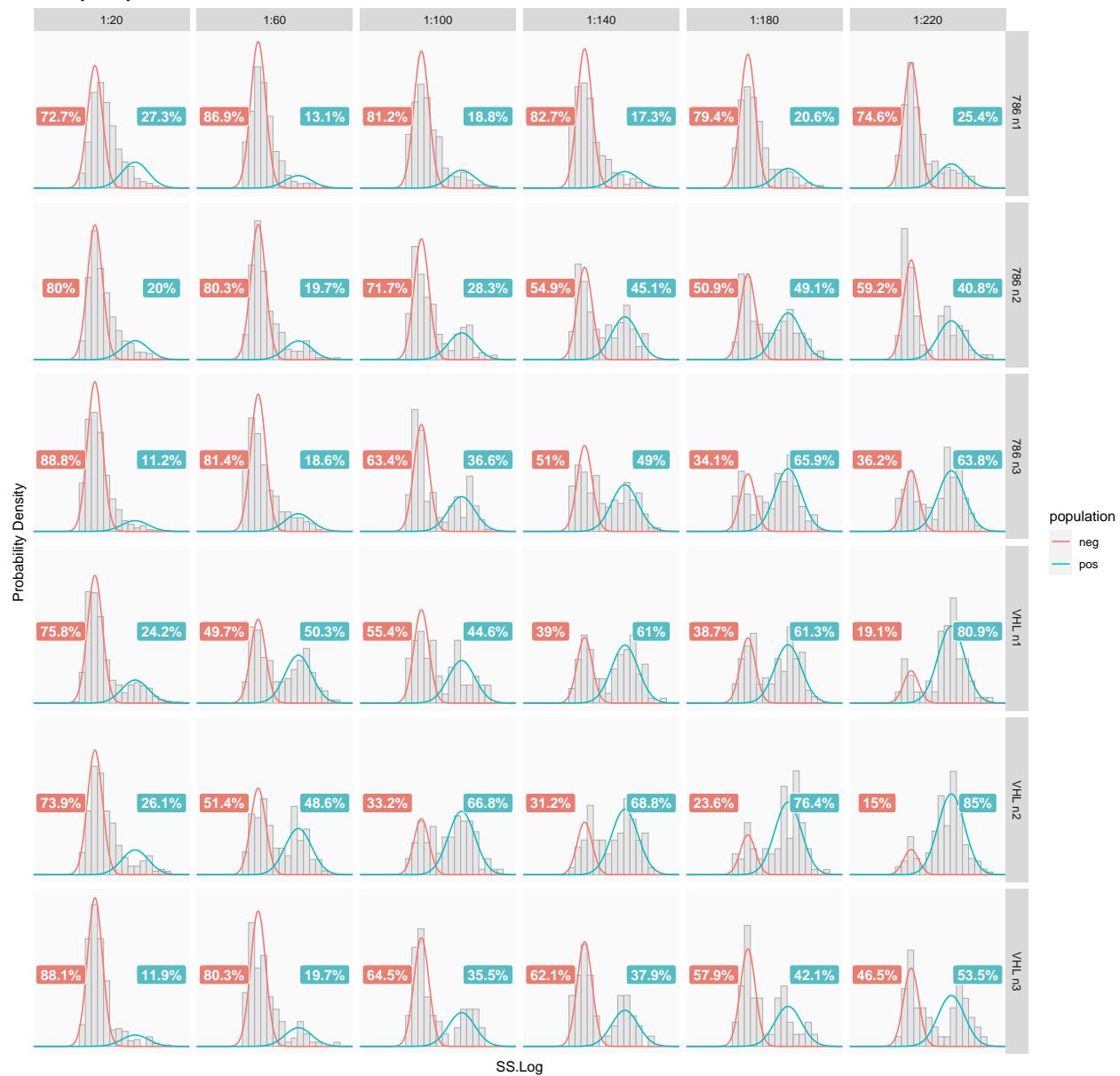
id	muNeg	sigmaNeg	pNeg	muPos	sigmaPos	pPos	hartigans	nNeg	nPos	welchTTest	facetRow	facetCol	negCluster	posCluster	nTot
15	403.037	49.24012	0.6344637	666.7118	87.27702	0.3655363	0.0236009	34	20	0.1033085	r3	c3	1	2	54
16	403.037	49.24012	0.5095351	666.7118	87.27702	0.4904649	0.5332946	28	26	0.1414295	r3	c4	1	2	54
17	403.037	49.24012	0.3407171	666.7118	87.27702	0.6592829	0.0006891	18	36	0.1579796	r3	c5	1	2	54
18	403.037	49.24012	0.3621810	666.7118	87.27702	0.6378190	0.0051107	20	34	0.2355596	r3	c6	1	2	54
19	403.037	49.24012	0.7580404	666.7118	87.27702	0.2419596	0.9549641	41	13	0.0609301	r4	c1	1	2	54
20	403.037	49.24012	0.4967604	666.7118	87.27702	0.5032396	0.0243285	27	27	0.1689630	r4	c2	1	2	54
21	403.037	49.24012	0.5537852	666.7118	87.27702	0.4462148	0.6175997	30	24	0.1157849	r4	c3	1	2	54
22	403.037	49.24012	0.3902391	666.7118	87.27702	0.6097609	0.0080804	21	33	0.2393098	r4	c4	1	2	54
23	403.037	49.24012	0.3867298	666.7118	87.27702	0.6132702	0.0056401	21	33	0.1965290	r4	c5	1	2	54
24	403.037	49.24012	0.1914878	666.7118	87.27702	0.8085122	0.3538832	10	44	0.2603653	r4	c6	1	2	54
25	403.037	49.24012	0.7394497	666.7118	87.27702	0.2605503	0.9925139	40	14	0.0674253	r5	c1	1	2	54
26	403.037	49.24012	0.5137004	666.7118	87.27702	0.4862996	0.0061349	28	26	0.2209991	r5	c2	1	2	54
27	403.037	49.24012	0.3321736	666.7118	87.27702	0.6678264	0.2398218	18	36	0.1556184	r5	c3	1	2	54
28	403.037	49.24012	0.3119157	666.7118	87.27702	0.6880843	0.2860918	17	37	0.2059403	r5	c4	1	2	54
29	403.037	49.24012	0.2362620	666.7118	87.27702	0.7637380	0.2530825	13	41	0.1958968	r5	c5	1	2	54
30	403.037	49.24012	0.1497174	666.7118	87.27702	0.8502826	0.7357624	8	46	0.2186312	r5	c6	1	2	54
31	403.037	49.24012	0.8810589	666.7118	87.27702	0.1189411	0.9939827	48	6	0.0392644	r6	c1	1	2	54
32	403.037	49.24012	0.8033030	666.7118	87.27702	0.1966970	0.9546455	43	11	0.0487754	r6	c2	1	2	54
33	403.037	49.24012	0.6448596	666.7118	87.27702	0.3551404	0.0632619	35	19	0.1160994	r6	c3	1	2	54
34	403.037	49.24012	0.6206470	666.7118	87.27702	0.3793530	0.1258550	34	20	0.2565239	r6	c4	1	2	54
35	403.037	49.24012	0.5794288	666.7118	87.27702	0.4205712	0.3785653	31	23	0.1469762	r6	c5	1	2	54
36	403.037	49.24012	0.4649789	666.7118	87.27702	0.5350211	0.0095742	25	29	0.2754260	r6	c6	1	2	54

```

plotDeconvolutedExperiment(
  gs=gatingset,
  model=cd24hiSwarmModel,
  gate="cd24hi",
  axis="SS.Log"
)
## Warning: Removed 72 rows containing missing values (geom_bar).

```

Swarm deconvolution with a mixed gaussian model
Gated: [cd24hi]



```
distForAggregatePlotting <- function(model) {
  number_of_flowframes <- length(row.names(model))
  mixedModelDistributions <- list()

  for (i in seq_len(number_of_flowframes)) {
    x <- seq(0, 1023)

    negDistribution <- stats::dnorm(
      x,
      mean=model$muNeg[i],
      sd=model$sigmaNeg[i]
    ) * model$pNeg[i]

    posDistribution <- stats::dnorm(
      x,
      mean=model$muPos[i],
      sd=model$sigmaPos[i]
    ) * model$pPos[i]

    mixedModelDistributions[[i]] <- negDistribution + posDistribution
  }
}
```

```

    x,
    mean=model$muPos[i],
    sd=model$sigmaPos[i]
) * model$pPos[i]

cellLine <- model$cellLine[i]
dilution <- model$dilution[i]

tempData <- data.frame(
  "distributionXAxis" = x,
  "distributionYAxis" = c(negDistribution, posDistribution),
  "population"        = rep(c("neg", "pos"), each=1024),
  "cellLine"          = rep(cellLine, times=2048),
  "dilution"          = rep(dilution, times=2048)
)

mixedModelDistributions <-
  BiocGenerics::rbind(mixedModelDistributions, tempData)
}

mixedModelDistributions
}

plotDeconvAggregateExperiment <- function(gs, model, gate, axis) {
  # Linter bindings
  cellLine <-
  dilution <- NULL

  mapFacetRowToCellLine <- wrapr::qc(
    "r1" = "786-0",
    "r2" = "786-0",
    "r3" = "786-0",
    "r4" = "VHL",
    "r5" = "VHL",
    "r6" = "VHL"
  )

  mapFacetColToDilution <- wrapr::qc(
    "c1" = "20",
    "c2" = "60",
    "c3" = "100",
    "c4" = "140",
    "c5" = "180",
    "c6" = "220"
  )

  model <- model %>%
    dplyr::mutate(., cellLine=mapFacetRowToCellLine[facetRow])

  model <- model %>%
    dplyr::mutate(., dilution=mapFacetColToDilution[facetCol])

  model <- model %>%
    group_by(cellLine, dilution) %>%

```

```

    summarize(
      muNeg     = mean(muNeg),
      sigmaNeg = mean(sigmaNeg),
      muPos     = mean(muPos),
      sigmaPos = mean(sigmaPos),
      pNeg      = sum(nNeg) / sum(nTot),
      pPos      = sum(nPos) / sum(nTot),
      nNeg      = sum(nNeg),
      nPos      = sum(nPos),
      nTot      = sum(nTot),
      .groups   = "drop"
    )

mixedModelDistributions <- distForAggregatePlotting(model)

cellLineOrdering <- c("786-0", "VHL")
dilutionOrdering <- c("20", "60", "100", "140", "180", "220")

model$cellLine <- factor(model$cellLine, levels=cellLineOrdering)
model$dilution <- factor(model$dilution, levels=dilutionOrdering)

mixedModelDistributions$cellLine <- factor(
  mixedModelDistributions$cellLine,
  levels=cellLineOrdering
)

mixedModelDistributions$dilution <- factor(
  mixedModelDistributions$dilution,
  levels=c(dilutionOrdering)
)

data <- flowWorkspace::gs_pop_get_data(gs, gate)
data <- flowWorkspace::cytoseq_to_flowSet(data)

data@phenoData@data$dilution <- factor(
  data@phenoData@data$dilution,
  levels=dilutionOrdering
)

data@phenoData@data$cellLine <- factor(
  data@phenoData@data$cellLine,
  levels=cellLineOrdering
)

ggplot2::ggplot(
  data=data,
  mapping=aes_string(x=axis)
) +
  ggplot2::scale_x_continuous(
    limits=c(0, 1024),
    expand=c(0, 0)) +
  ggplot2::geom_histogram(
    mapping=ggplot2::aes_string(

```

```

    x=axis,
    y="..density.."),
binwidth=40,
colour="#AAAAAA",
fill="#E6E6E6",
size=0.1) +
ggplot2::geom_line(
  data=mixedModelDistributions,
  mapping=ggplot2::aes(
    x=distributionXAxis,
    y=distributionYAxis,
    colour=population)) +
ggplot2::labs(
  title="Swarm deconvolution with a mixed gaussian model",
  subtitle=paste0("Gated: [", gate, "]"),
  x=axis,
  y="Probability Density") +
ggplot2::theme(
  panel.grid.major=ggplot2::element_blank(),
  panel.grid.minor=ggplot2::element_blank(),
  panel.background=ggplot2::element_rect(fill="#FAFAFA"),
  axis.text.x=ggplot2::element_blank(),
  axis.text.y=ggplot2::element_blank(),
  axis.ticks.x=ggplot2::element_blank(),
  axis.ticks.y=ggplot2::element_blank()) +
ggplot2::geom_label(
  data=model,
  x=180,
  y=0.00345,
  mapping=ggplot2::aes(label=paste0(round(pNeg*100, 1), "%")),
  fill="#E87D72",
  color="#FFFFFF",
  fontface="bold",
  size=4) +
ggplot2::geom_label(
  data=model,
  x=840,
  y=0.00345,
  mapping=ggplot2::aes(label=paste0(round(pPos*100, 1), "%")),
  fill="#54BCC2",
  color="#FFFFFF",
  fontface="bold",
  size=4) +
ggplot2::facet_grid(
  rows=dplyr::vars(cellLine),
  cols=dplyr::vars(dilution),
  labeller=ggplot2::as_labeller(facetLabels))
}

plotDeconvAggregateExperiment(
  gs=gatingset,
  model=cd24midSwarmModel,
  gate="cd24mid",

```

```

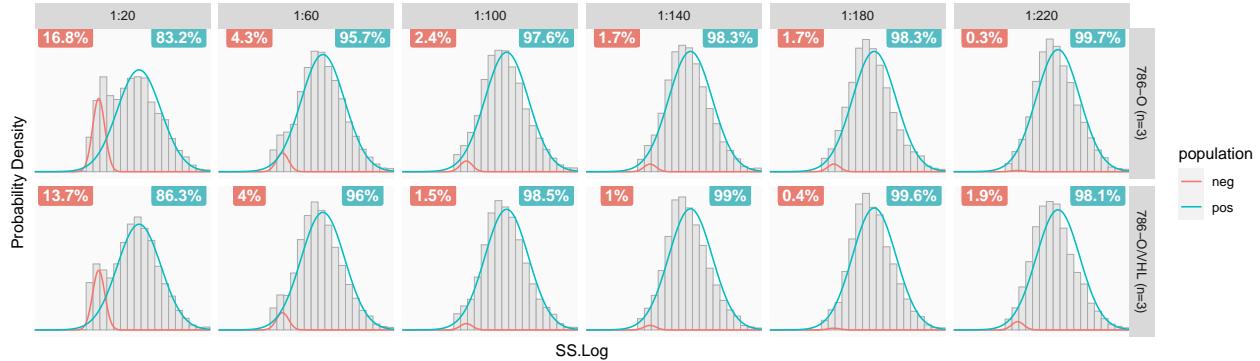
    axis="SS.Log"
)

```

Warning: Removed 24 rows containing missing values (geom_bar).

Swarm deconvolution with a mixed gaussian model

Gated: [cd24mid]



```
plotDeconvAggregateExperiment(

```

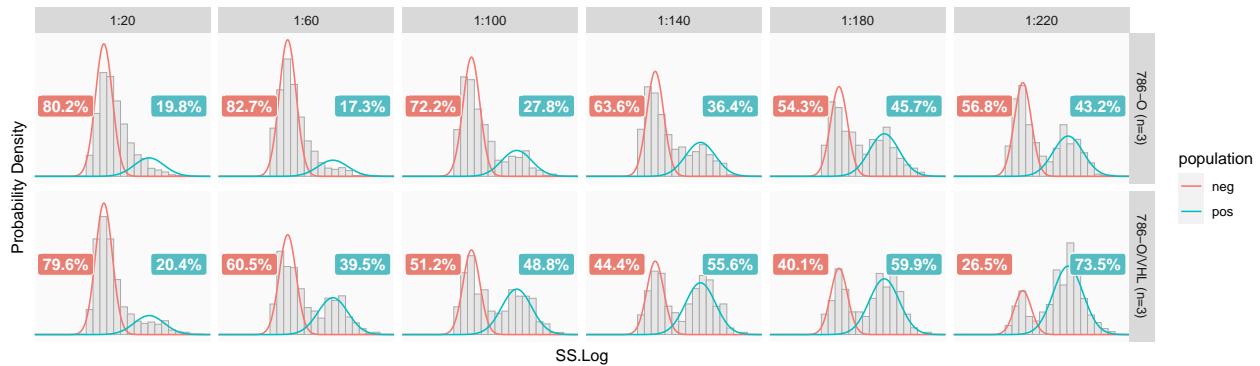
```

  gs=gatingset,
  model=cd24hiSwarmModel,
  gate="cd24hi",
  axis="SS.Log"
)
```

Warning: Removed 24 rows containing missing values (geom_bar).

Swarm deconvolution with a mixed gaussian model

Gated: [cd24hi]



4.3 Small particle quantification

4.3.1 Building our datasets

```
# Create a dataframe to hold all the experimental data obtained
experiment <- pData

# Get a list of all relevant populations
populations <- flowWorkspace::gs_get_pop_paths(gatingset, path="auto")
populations <- populations[!(populations %in% c("root", "boundary", "nonNoise"))]
]

populations

## [1] "beads"    "events"    "cd24pos"   "cd24neg"   "cd24mid"   "cd24hi"

# Get a list of all samples
sampleNames <- row.names(experiment)

# Get all population statistics
populationStatistics <- flowWorkspace::gs_pop_get_count_fast(gatingset)

# Change to camelCase
populationStatistics <- populationStatistics %>%
  dplyr::rename(
    "population"="Population",
    "parent"="Parent",
    "count"="Count",
    "parentCount"="ParentCount"
  )

# Populate the experimental data with all the relevant gate population counts,
# in "tidy" format
#
# References:
# https://www.jstatsoft.org/article/view/v059i10

for (sampleName in sampleNames) {
  # https://github.com/RGLab/flowWorkspace/issues/341#issuecomment-691742173
  escapedSampleName <- gsub("[/:\\\\\\]", "_", sampleName)
  sampleStatistic <- populationStatistics %>%
    dplyr::filter(populationStatistics$name == escapedSampleName)

  sampleStatistic$population <- sampleStatistic$population %>%
    purrr::map(function(x) {
      tail(strsplit(x, "/")[[1]], n=1)
    })

  for (populationName in populations) {
    samplePopulationStatistic <- sampleStatistic %>%
      dplyr::filter(sampleStatistic$population == populationName)

    experiment[sampleName, populationName] <- samplePopulationStatistic$count
  }
}
```

```

}

orderOfCellLines <- c("786-0", "VHL")

experiment$temp <- as.factor(experiment$temp)
experiment$a23187 <- as.factor(experiment$a23187)
experiment$n <- as.factor(experiment$n)
experiment$cellLine <- factor(experiment$cellLine, levels=orderOfCellLines)

# Integrate swarm deconvolution data. The true-positive statistic for the CD24+
# population can be derived from cd24mid and cd24hi
experiment$cd24midTp <- cd24midSwarmModel$pPos
experiment$cd24hiTp <- cd24hiSwarmModel$pPos
experiment$cd24posTp <- (
  (experiment$cd24mid * experiment$cd24midTp) +
  (experiment$cd24hi * experiment$cd24hiTp)) / experiment$cd24pos

# Let's not go too crazy with significant digits
experiment$cd24midTp <- round(experiment$cd24midTp, 3)
experiment$cd24hiTp <- round(experiment$cd24hiTp, 3)
experiment$cd24posTp <- round(experiment$cd24posTp, 3)

```

```

interestingColumns <- c(
  "beads",
  "events",
  "cd24pos",
  "cd24neg",
  "cd24mid",
  "cd24hi",
  "cd24posTp",
  "cd24midTp",
  "cd24hiTp"
)

printDataFrame(
  dataframe=experiment[, interestingColumns],
  caption="Gated event counts",
  fontsize=5
)

```

Table 8: Gated event counts

	beads	events	cd24pos	cd24neg	cd24mid	cd24hi	cd24posTp	cd24midTp	cd24hiTp
data/786 n1/786n1_Ca1uM4h_samp_20x 00016119 613.LMD	2938	15217	7708	7481	4488	3220	0.574	0.791	0.273
data/786 n1/786n1_Ca1uM4h_samp_60x 00016122 616.LMD	3382	12773	4296	8474	3572	724	0.795	0.929	0.131
data/786 n1/786n1_Ca1uM4h_samp_100x 00016125 619.LMD	3421	11805	3811	7991	3345	466	0.858	0.951	0.188
data/786 n1/786n1_Ca1uM4h_samp_140x 00016128 622.LMD	3684	11832	3419	8412	3147	272	0.906	0.969	0.173
data/786 n1/786n1_Ca1uM4h_samp_180x 00016131 625.LMD	3666	11654	3343	8311	3148	195	0.933	0.978	0.206
data/786 n1/786n2_Ca1uM4h_samp_220x 00016134 628.LMD	3542	11332	3213	8118	3039	174	0.953	0.993	0.254
data/786 n2/786n2_Ca1uM4h_samp_20x 00016158 632.LMD	2886	13606	5379	8222	4468	911	0.692	0.793	0.200
data/786 n2/786n2_Ca1uM4h_samp_60x 00016161 635.LMD	2848	10367	3225	7142	2978	247	0.893	0.951	0.197
data/786 n2/786n2_Ca1uM4h_samp_100x 00016164 638.LMD	2646	9588	2978	6610	2753	225	0.927	0.980	0.283
data/786 n2/786n2_Ca1uM4h_samp_140x 00016167 641.LMD	3101	10395	3115	7280	2876	239	0.948	0.990	0.451
data/786 n2/786n2_Ca1uM4h_samp_180x 00016170 644.LMD	3112	10011	2939	7072	2763	176	0.967	0.998	0.491
data/786 n2/786n2_Ca1uM4h_samp_220x 00016173 647.LMD	2751	8983	2488	6495	2381	107	0.974	1.000	0.408
data/786 n3/786n3_Ca1uM4h_samp_20x 00016176 650.LMD	2938	11926	4312	7609	3430	882	0.750	0.914	0.112
data/786 n3/786n3_Ca1uM4h_samp_60x 00016179 653.LMD	2919	10275	3254	7019	2988	266	0.926	0.992	0.186
data/786 n3/786n3_Ca1uM4h_samp_100x 00016182 656.LMD	3051	10637	3216	7421	2990	226	0.951	0.995	0.366
data/786 n3/786n3_Ca1uM4h_samp_140x 00016185 659.LMD	3319	10783	3286	7497	3137	149	0.967	0.990	0.490
data/786 n3/786n3_Ca1uM4h_samp_180x 00016188 662.LMD	3723	12305	3784	8521	3533	251	0.951	0.972	0.659
data/786 n3/786n3_Ca1uM4h_samp_220x 00016191 665.LMD	3652	11440	3490	7950	3317	173	0.981	0.999	0.638
data/VHL n1/VHLn1_Ca1uM4h_samp_20x 00016196 670.LMD	3341	12611	4498	8112	4041	457	0.755	0.813	0.242
data/VHL n1/VHLn1_Ca1uM4h_samp_60x 00016199 673.LMD	3340	12436	3790	8645	3619	171	0.921	0.940	0.503
data/VHL n1/VHLn2_Ca1uM4h_samp_100x 00016202 676.LMD	3319	10684	3036	7648	2978	58	0.973	0.984	0.446
data/VHL n1/VHLn2_Ca1uM4h_samp_140x 00016205 679.LMD	3466	10858	3212	7645	3105	107	0.959	0.971	0.610
data/VHL n1/VHLn2_Ca1uM4h_samp_180x 00016208 682.LMD	3219	10544	3090	7454	3008	82	0.979	0.989	0.613
data/VHL n1/VHLn2_Ca1uM4h_samp_220x 00016211 685.LMD	3590	11147	3269	7878	3175	94	0.969	0.974	0.809
data/VHL n2/VHLn2_Ca1uM4h_samp_20x 00016215 689.LMD	2988	13532	4274	9257	3972	302	0.871	0.918	0.261
data/VHL n2/VHLn2_Ca1uM4h_samp_60x 00016218 692.LMD	3391	11729	3562	8167	3425	137	0.949	0.968	0.486
data/VHL n2/VHLn2_Ca1uM4h_samp_100x 00016221 695.LMD	3494	11910	3467	8443	3370	97	0.979	0.988	0.668
data/VHL n2/VHLn2_Ca1uM4h_samp_140x 00016224 698.LMD	3522	12799	3404	9394	3329	75	0.991	0.998	0.688
data/VHL n2/VHLn2_Ca1uM4h_samp_180x 00016227 701.LMD	3709	11784	3456	8327	3356	100	0.993	1.000	0.764
data/VHL n2/VHLn2_Ca1uM4h_samp_220x 00016230 704.LMD	3494	11520	3690	7830	3499	191	0.964	0.970	0.850
data/VHL n3/VHLn3_Ca1uM4h_samp_20x 00016234 708.LMD	3273	14254	4341	9913	3960	381	0.794	0.859	0.119
data/VHL n3/VHLn3_Ca1uM4h_samp_60x 00016237 711.LMD	3191	11503	3349	8154	3223	126	0.944	0.973	0.197
data/VHL n3/VHLn3_Ca1uM4h_samp_100x 00016240 714.LMD	3050	11094	3075	8019	2991	84	0.968	0.985	0.355
data/VHL n3/VHLn3_Ca1uM4h_samp_140x 00016243 717.LMD	3236	10887	3101	7786	3050	51	0.990	1.000	0.379
data/VHL n3/VHLn3_Ca1uM4h_samp_180x 00016246 720.LMD	3095	11154	3008	8146	2965	43	0.992	1.000	0.421
data/VHL n3/VHLn3_Ca1uM4h_samp_220x 00016249 723.LMD	3229	10945	3052	7893	2998	54	0.991	1.000	0.535

```

# Constants for bead quantification procedure
normalizedToXBeads <- 5000

normalizedExperiment <- experiment

normalizedExperiment$events <-
  (experiment$events / experiment$beads) *
  normalizedToXBeads * experiment$dilution * 10

normalizedExperiment$cd24pos <-
  (experiment$cd24pos / experiment$beads) *
  normalizedToXBeads * experiment$dilution * 10

normalizedExperiment$cd24neg <-
  (experiment$cd24neg / experiment$beads) *
  normalizedToXBeads * experiment$dilution * 10

normalizedExperiment$cd24mid <-
  (experiment$cd24mid / experiment$beads) *
  normalizedToXBeads * experiment$dilution * 10

normalizedExperiment$cd24hi <-
  (experiment$cd24hi / experiment$beads) *
  normalizedToXBeads * experiment$dilution * 10

# To be or not to be, that is the question.
normalizedExperiment$events <- round(normalizedExperiment$events, digits=0)
normalizedExperiment$cd24pos <- round(normalizedExperiment$cd24pos, digits=0)
normalizedExperiment$cd24neg <- round(normalizedExperiment$cd24neg, digits=0)
normalizedExperiment$cd24mid <- round(normalizedExperiment$cd24mid, digits=0)
normalizedExperiment$cd24hi <- round(normalizedExperiment$cd24hi, digits=0)

# For normalized data, bead counts lose their significance...
# but we may be interested in the events' corresponding concentration
# in undiluted conditioned-medium (CM)
normalizedExperiment$beads <- NULL
normalizedExperiment$cmVolume <- 40 # TEMPORARY, FIX THIS

```

```

interestingColumns <- c(
  "cmVolume",
  "events",
  "cd24pos",
  "cd24neg",
  "cd24mid",
  "cd24hi",
  "cd24posTp",
  "cd24midTp",
  "cd24hiTp"
)

printDataFrame(
  dataframe=normalizedExperiment[, interestingColumns],
  caption="Gated event counts",
  fontsize=5
)

```

Table 9: Gated event counts

	cmVolume	events	cd24pos	cd24neg	cd24mid	cd24hi	cd24posTp	cd24midTp	cd24hiTp
data/786 n1/786n1_CaluM4h_samp_20x 00016119 613.LMD	40	5179374	2623553	2546290	1527570	1095984	0.574	0.791	0.273
data/786 n1/786n1_CaluM4h_samp_60x 00016122 616.LMD	40	11330278	3810763	7516854	3168539	642224	0.795	0.929	0.131
data/786 n1/786n1_CaluM4h_samp_100x 00016125 619.LMD	40	17253727	5570009	11679334	4888921	681087	0.858	0.951	0.188
data/786 n1/786n1_CaluM4h_samp_140x 00016128 622.LMD	40	22482085	6496471	15983713	5979642	516830	0.906	0.969	0.173
data/786 n1/786n1_CaluM4h_samp_180x 00016131 625.LMD	40	28610475	8207038	20403437	7728314	478723	0.933	0.978	0.206
data/786 n1/786n1_CaluM4h_samp_220x 00016134 628.LMD	40	35192547	9978261	25211180	9437888	540373	0.953	0.993	0.254
data/786 n2/786n2_CaluM4h_samp_20x 00016158 632.LMD	40	4714484	1863825	2848926	1548164	315662	0.692	0.793	0.200
data/786 n2/786n2_CaluM4h_samp_60x 00016161 635.LMD	40	10920295	3397121	7523174	3136938	260183	0.893	0.951	0.197
data/786 n2/786n2_CaluM4h_samp_100x 00016164 638.LMD	40	18117914	5627362	12490552	5202192	425170	0.927	0.980	0.283
data/786 n2/786n2_CaluM4h_samp_140x 00016167 641.LMD	40	23465011	7031603	16433409	6492099	539503	0.948	0.990	0.451
data/786 n2/786n2_CaluM4h_samp_180x 00016170 644.LMD	40	28952121	8499679	20452447	7990681	508997	0.967	0.998	0.491
data/786 n2/786n2_CaluM4h_samp_220x 00016173 647.LMD	40	35918939	9948382	25970556	9520538	427844	0.974	1.000	0.408
data/786 n3/786n3_CaluM4h_samp_20x 00016176 650.LMD	40	4059224	1467665	2589857	1167461	300204	0.750	0.914	0.112
data/786 n3/786n3_CaluM4h_samp_60x 00016179 653.LMD	40	10560783	3344296	7213772	3070915	273381	0.926	0.992	0.186
data/786 n3/786n3_CaluM4h_samp_100x 00016182 656.LMD	40	17431990	5270403	12161586	4900033	370370	0.951	0.995	0.366
data/786 n3/786n3_CaluM4h_samp_140x 00016185 659.LMD	40	22742091	6930401	15811690	6616149	314251	0.967	0.990	0.490
data/786 n3/786n3_CaluM4h_samp_180x 00016188 662.LMD	40	29746172	9147462	20598711	8540693	606769	0.951	0.972	0.659
data/786 n3/786n3_CaluM4h_samp_220x 00016191 665.LMD	40	34457831	10512048	23945783	9990964	521084	0.981	0.999	0.638
data/VHL n1/VHLn1_CaluM4h_samp_20x 00016196 670.LMD	40	3774618	1346304	2428016	1209518	136785	0.755	0.813	0.242
data/VHL n1/VHLn1_CaluM4h_samp_60x 00016199 673.LMD	40	11170060	3404192	7764970	3250599	153593	0.921	0.940	0.503
data/VHL n1/VHLn1_CaluM4h_samp_100x 00016202 676.LMD	40	16095209	4573667	11521543	4486291	87376	0.973	0.984	0.446
data/VHL n1/VHLn1_CaluM4h_samp_140x 00016205 679.LMD	40	21929025	6487017	15439988	6270917	216099	0.959	0.971	0.610
data/VHL n1/VHLn1_CaluM4h_samp_180x 00016208 682.LMD	40	29479963	8639329	20840634	8410065	229264	0.979	0.989	0.613
data/VHL n1/VHLn1_CaluM4h_samp_220x 00016211 685.LMD	40	34155153	10016435	24138719	9728412	288022	0.969	0.974	0.809
data/VHL n2/VHLn2_CaluM4h_samp_20x 00016215 689.LMD	40	4528782	1430388	3098059	1329317	101071	0.871	0.918	0.261
data/VHL n2/VHLn2_CaluM4h_samp_60x 00016218 692.LMD	40	10376585	3151283	7225302	3030080	121203	0.949	0.968	0.486
data/VHL n2/VHLn2_CaluM4h_samp_100x 00016221 695.LMD	40	17043503	4961362	12082141	4822553	138809	0.979	0.988	0.668
data/VHL n2/VHLn2_CaluM4h_samp_140x 00016224 698.LMD	40	25438103	6765474	18670642	6616411	149063	0.991	0.998	0.688
data/VHL n2/VHLn2_CaluM4h_samp_180x 00016227 701.LMD	40	28594230	8386088	20205716	8143435	242653	0.993	1.000	0.764
data/VHL n2/VHLn2_CaluM4h_samp_220x 00016230 704.LMD	40	36267888	11617058	24650830	11015741	601317	0.964	0.970	0.850
data/VHL n3/VHLn3_CaluM4h_samp_20x 00016234 708.LMD	40	4355026	1326306	3028720	1209899	116407	0.794	0.859	0.119
data/VHL n3/VHLn3_CaluM4h_samp_60x 00016237 711.LMD	40	10814478	3148543	7665935	3030085	118458	0.944	0.973	0.197
data/VHL n3/VHLn3_CaluM4h_samp_100x 00016240 714.LMD	40	18186885	5040984	13145902	4903279	137705	0.968	0.985	0.355
data/VHL n3/VHLn3_CaluM4h_samp_140x 00016243 717.LMD	40	23550371	6707973	16842398	6597651	110321	0.990	1.000	0.379
data/VHL n3/VHLn3_CaluM4h_samp_180x 00016246 720.LMD	40	32434895	8747011	23687884	8621971	125040	0.992	1.000	0.421
data/VHL n3/VHLn3_CaluM4h_samp_220x 00016249 723.LMD	40	37285537	10397027	26888510	10213069	183958	0.991	1.000	0.535

4.3.1.1 Dataset 1 - Raw gate counts

```

dataset1 <- normalizedExperiment

dataset1$cd24posTp <- NULL
dataset1$cd24midTp <- NULL
dataset1$cd24hiTp <- NULL

dataset1$cd24posProp <- dataset1$cd24pos / dataset1$events
dataset1$cd24midProp <- dataset1$cd24mid / dataset1$events
dataset1$cd24hiProp <- dataset1$cd24hi / dataset1$events

interestingColumns <- c(
  "cmVolume",
  "events",
  "cd24pos",
  "cd24neg",
  "cd24mid",
  "cd24hi",
  "cd24posProp",
  "cd24midProp",
  "cd24hiProp"
)

printDataFrame(
  datafram=dataset1[, interestingColumns],
  caption="Raw gate event counts, exhibiting swarm effect",
  fontsize=5
)

```

Table 10: Raw gate event counts, exhibiting swarm effect

	cmVolume	events	cd24pos	cd24neg	cd24mid	cd24hi	cd24posProp	cd24midProp	cd24hiProp
data/786 n1/786n1_Ca1uM4h_samp_20x 00016119 613.LMD	40	5179374	2623553	2546290	1527570	1095984	0.5065386	0.2949333	0.2116055
data/786 n1/786n1_Ca1uM4h_samp_60x 00016122 616.LMD	40	11330278	3810763	7516854	3168539	642224	0.3363345	0.2796524	0.0566821
data/786 n1/786n1_Ca1uM4h_samp_100x 00016125 619.LMD	40	17253727	5570009	11679334	4888921	681087	0.3228293	0.2833545	0.0394748
data/786 n1/786n1_Ca1uM4h_samp_140x 00016128 622.LMD	40	22482085	6496471	15983713	5979642	516830	0.2889621	0.2659736	0.0229885
data/786 n1/786n1_Ca1uM4h_samp_180x 00016131 625.LMD	40	28610475	8207038	20403437	7728314	478723	0.2868543	0.2701218	0.0167324
data/786 n1/786n1_Ca1uM4h_samp_220x 00016134 628.LMD	40	35192547	9978261	25211180	9437888	540373	0.2835334	0.2681786	0.0153548
data/786 n2/786n2_Ca1uM4h_samp_20x 00016158 632.LMD	40	4714484	1863825	2848926	1548164	315662	0.3953402	0.3283846	0.0669558
data/786 n2/786n2_Ca1uM4h_samp_60x 00016161 635.LMD	40	10920295	3397121	7523174	3136938	260183	0.3110833	0.2872576	0.0238256
data/786 n2/786n2_Ca1uM4h_samp_100x 00016164 638.LMD	40	18117914	5627362	12490552	5202192	425170	0.3105966	0.2871297	0.0234668
data/786 n2/786n2_Ca1uM4h_samp_140x 00016167 641.LMD	40	23465011	7031603	16433409	6492099	539503	0.2996633	0.2766715	0.0229918
data/786 n2/786n2_Ca1uM4h_samp_180x 00016170 644.LMD	40	28952121	8499679	20452442	7990681	508997	0.2935771	0.2759964	0.0175806
data/786 n2/786n2_Ca1uM4h_samp_220x 00016173 647.LMD	40	35918939	9948382	25970556	9520538	427844	0.2769676	0.2650562	0.0119114
data/786 n3/786n3_Ca1uM4h_samp_20x 00016176 650.LMD	40	4059224	1467665	2589857	1167461	300204	0.3615629	0.2876069	0.0739560
data/786 n3/786n3_Ca1uM4h_samp_60x 00016179 653.LMD	40	10560123	3344296	7213772	3070915	273381	0.3166910	0.2908030	0.0258881
data/786 n3/786n3_Ca1uM4h_samp_100x 00016182 656.LMD	40	17431990	5270403	12161586	4900033	370370	0.3023409	0.2810943	0.0212466
data/786 n3/786n3_Ca1uM4h_samp_140x 00016185 659.LMD	40	22742091	6930401	15811690	6616149	314251	0.3047390	0.2909209	0.0138180
data/786 n3/786n3_Ca1uM4h_samp_180x 00016188 662.LMD	40	29746172	9147462	2059871	8540693	606769	0.2075173	0.2871191	0.0203982
data/786 n3/786n3_Ca1uM4h_samp_220x 00016191 665.LMD	40	34457831	10512048	23945783	9990964	521084	0.3050699	0.2899476	0.0151224
data/VHL n1/VHLn1_Ca1uM4h_samp_20x 00016196 670.LMD	40	3774618	1346304	2428016	1209518	136785	0.3566729	0.3204345	0.0362381
data/VHL n1/VHLn1_Ca1uM4h_samp_60x 00016199 673.LMD	40	11170060	3404192	7764970	3250599	153593	0.3047604	0.2910100	0.0137504
data/VHL n1/VHLn1_Ca1uM4h_samp_100x 00016202 676.LMD	40	16095209	4573667	11521543	4486291	87376	0.2841633	0.2787346	0.0054287
data/VHL n1/VHLn1_Ca1uM4h_samp_140x 00016205 679.LMD	40	21929025	6487017	15439988	6270917	216099	0.2958188	0.2859642	0.0098545
data/VHL n1/VHLn1_Ca1uM4h_samp_180x 00016208 682.LMD	40	29479963	8639329	20840634	8410065	229264	0.2930577	0.2852807	0.0077769
data/VHL n1/VHLn1_Ca1uM4h_samp_220x 00016211 685.LMD	40	34155153	10016435	24138719	9728412	288022	0.2932628	0.2848300	0.0084328
data/VHL n2/VHLn2_Ca1uM4h_samp_20x 00016215 689.LMD	40	4528782	1430388	3098059	1329317	101071	0.3158439	0.2935264	0.0223175
data/VHL n2/VHLn2_Ca1uM4h_samp_60x 00016218 692.LMD	40	10376585	3151283	7225302	3030080	121203	0.3036917	0.2920113	0.0116804
data/VHL n2/VHLn2_Ca1uM4h_samp_100x 00016221 695.LMD	40	17043503	4961362	12082141	4822553	138809	0.2910999	0.2829555	0.0081444
data/VHL n2/VHLn2_Ca1uM4h_samp_140x 00016224 698.LMD	40	25438103	6765474	18670642	6616411	149063	0.2659583	0.2600984	0.0058598
data/VHL n2/VHLn2_Ca1uM4h_samp_180x 00016227 701.LMD	40	28594230	8386088	20205716	8143435	242653	0.2932790	0.2847929	0.0084861
data/VHL n2/VHLn2_Ca1uM4h_samp_220x 00016230 704.LMD	40	36267888	11617058	24650830	11015741	601317	0.3203125	0.3037326	0.0165799
data/VHL n3/VHLn3_Ca1uM4h_samp_20x 00016234 708.LMD	40	4355026	1326306	3028720	1209899	116407	0.3045461	0.2778167	0.0267293
data/VHL n3/VHLn3_Ca1uM4h_samp_60x 00016237 711.LMD	40	10814478	3148543	7665935	3030085	118458	0.2911415	0.2801878	0.0109536
data/VHL n3/VHLn3_Ca1uM4h_samp_100x 00016240 714.LMD	40	18186885	5040984	13145902	4903279	137705	0.2771769	0.2696052	0.0075717

Table 10: Raw gate event counts, exhibiting swarm effect (*continued*)

	cmVolume	events	cd24pos	cd24neg	cd24mid	cd24hi	cd24posProp	cd24midProp	cd24hiProp
data/VHL n3/VHLn3_Ca1uM4h_samp_140x 00016243 717.LMD	40	23550371	6707973	16842398	6597651	110321	0.2848351	0.2801506	0.0046845
data/VHL n3/VHLn3_Ca1uM4h_samp_180x 00016246 720.LMD	40	32434895	8747011	23687884	8621971	125040	0.2696790	0.2658239	0.0038551
data/VHL n3/VHLn3_Ca1uM4h_samp_220x 00016249 723.LMD	40	37285537	10397027	26888510	10213069	183958	0.2788488	0.2739150	0.0049338

4.3.1.2 Dataset 2 - Swarm-deconvoluted

```
dataset2 <- normalizedExperiment

dataset2$cd24mid <- dataset2$cd24mid * dataset2$cd24midTp
dataset2$cd24hi <- dataset2$cd24hi * dataset2$cd24hiTp
dataset2$cd24pos <- dataset2$cd24mid + dataset2$cd24hi

dataset2$cd24posProp <- dataset2$cd24pos / dataset2$events
dataset2$cd24midProp <- dataset2$cd24mid / dataset2$events
dataset2$cd24hiProp <- dataset2$cd24hi / dataset2$events

dataset2$cd24posTp <- NULL
dataset2$cd24midTp <- NULL
dataset2$cd24hiTp <- NULL

printDataFrame(
  dataframe=dataset2[, interestingColumns],
  caption="Swarm-deconvoluted event counts",
  fontsize=5
)
```

Table 11: Swarm-deconvoluted event counts

	cmVolume	events	cd24pos	cd24neg	cd24mid	cd24hi	cd24posProp	cd24midProp	cd24hiProp
data/786 n1/786n1_Ca1uM4h_samp_20x 00016119 613.LMD	40	5179374	1507512	2546290	1208307.9	299203.63	0.2910606	0.2332923	0.0577683
data/786 n1/786n1_Ca1uM4h_samp_60x 00016122 616.LMD	40	11330278	3027704	7516854	2943572.7	84131.34	0.2672224	0.2597970	0.0074254
data/786 n1/786n1_Ca1uM4h_samp_100x 00016125 619.LMD	40	17253727	4777408	11679334	4649363.9	128044.36	0.2768914	0.2694701	0.0074213
data/786 n1/786n1_Ca1uM4h_samp_140x 00016128 622.LMD	40	22482085	5883685	15983713	5794273.1	89411.59	0.2617055	0.2577285	0.0039770
data/786 n1/786n1_Ca1uM4h_samp_180x 00016131 625.LMD	40	28610475	7656908	20403437	7558291.1	98616.94	0.2676260	0.2641792	0.0034469
data/786 n1/786n1_Ca1uM4h_samp_220x 00016134 628.LMD	40	35192547	9509078	25211180	9371822.8	137254.74	0.2702015	0.2663014	0.0039001
data/786 n2/786n2_Ca1uM4h_samp_20x 00016158 632.LMD	40	4714484	1290826	2848926	1227694.1	63132.40	0.2738002	0.2604090	0.0133912
data/786 n2/786n2_Ca1uM4h_samp_60x 00016161 635.LMD	40	10920295	3034484	7523174	2983228.0	51256.05	0.2778757	0.2731820	0.0046937
data/786 n2/786n2_Ca1uM4h_samp_100x 00016164 638.LMD	40	18117914	5218471	12490552	5098148.2	120323.11	0.2880283	0.2813871	0.0066411
data/786 n2/786n2_Ca1uM4h_samp_140x 00016167 641.LMD	40	23465011	6670494	16433409	6427178.0	243315.85	0.2842741	0.2739048	0.0103693
data/786 n2/786n2_Ca1uM4h_samp_180x 00016170 644.LMD	40	28952121	8224617	20452442	7974699.6	249917.53	0.2840765	0.2754444	0.0086321
data/786 n2/786n2_Ca1uM4h_samp_220x 00016173 647.LMD	40	35918939	9695098	25970556	9520538.0	174560.35	0.2699161	0.2650562	0.0048598
data/786 n3/786n3_Ca1uM4h_samp_20x 00016176 650.LMD	40	4059224	1100682	2589857	1067059.4	33622.85	0.2711558	0.2628727	0.0082831
data/786 n3/786n3_Ca1uM4h_samp_60x 00016179 653.LMD	40	10560123	30791797	7213772	3046347.9	50848.87	0.2932917	0.2884765	0.0048152
data/786 n3/786n3_Ca1uM4h_samp_100x 00016182 656.LMD	40	17431990	5011088	12161586	4875532.8	135555.42	0.2874651	0.2796888	0.0077762
data/786 n3/786n3_Ca1uM4h_samp_140x 00016185 659.LMD	40	22742091	6703970	15811690	6549987.5	153982.99	0.2947825	0.2880117	0.0067708
data/786 n3/786n3_Ca1uM4h_samp_180x 00016188 662.LMD	40	29746172	8701448	20598711	8301553.6	399860.77	0.2925222	0.2790797	0.0134424
data/786 n3/786n3_Ca1uM4h_samp_220x 00016191 665.LMD	40	34457831	10313425	23945783	9980973.0	332451.59	0.293057	0.2896576	0.0096481
data/VHL n1/VHLn1_Ca1uM4h_samp_20x 00016196 670.LMD	40	3774618	1016440	2428016	983338.1	33101.97	0.2692829	0.2605133	0.0087696
data/VHL n1/VHLn1_Ca1uM4h_samp_60x 00016199 673.LMD	40	11170060	3132820	7764970	3055563.1	77257.28	0.2804658	0.2735494	0.0069165
data/VHL n1/VHLn1_Ca1uM4h_samp_100x 00016202 676.LMD	40	16095209	4453480	11521543	4414510.3	38969.70	0.2766960	0.2742748	0.0024212
data/VHL n1/VHLn1_Ca1uM4h_samp_140x 00016205 679.LMD	40	21929025	6220881	15439988	6089060.4	131820.39	0.2836825	0.2776713	0.0060112
data/VHL n1/VHLn1_Ca1uM4h_samp_180x 00016208 682.LMD	40	29479963	8458093	20840634	8317554.3	140538.83	0.2869099	0.2821426	0.0047673
data/VHL n1/VHLn1_Ca1uM4h_samp_220x 00016211 685.LMD	40	34155153	9708483	24138719	9475473.3	233009.80	0.2842465	0.2774244	0.0068221
data/VHL n2/VHLn2_Ca1uM4h_samp_20x 00016215 689.LMD	40	4528782	1246693	3098059	1220313.0	26379.53	0.2752821	0.2694572	0.0058249
data/VHL n2/VHLn2_Ca1uM4h_samp_60x 00016218 692.LMD	40	10376585	2992022	7225302	2933117.4	58904.66	0.2883436	0.2826669	0.0056767
data/VHL n2/VHLn2_Ca1uM4h_samp_100x 00016221 695.LMD	40	17043503	4857407	12082141	4764682.4	92724.41	0.2850005	0.2795600	0.0054405
data/VHL n2/VHLn2_Ca1uM4h_samp_140x 00016224 698.LMD	40	25438103	6705734	18670642	6603178.2	102555.34	0.2636098	0.2595782	0.0040316
data/VHL n2/VHLn2_Ca1uM4h_samp_180x 00016227 701.LMD	40	28594230	8328822	20205716	8143435.0	185386.89	0.2912763	0.2847929	0.0064834
data/VHL n2/VHLn2_Ca1uM4h_samp_220x 00016230 704.LMD	40	36267888	11196388	24650830	10685268.8	511119.45	0.3087135	0.2946207	0.0140299
data/VHL n3/VHLn3_Ca1uM4h_samp_20x 00016234 708.LMD	40	4355026	1053156	3028720	1039303.2	13852.43	0.2418253	0.2386446	0.0031808
data/VHL n3/VHLn3_Ca1uM4h_samp_60x 00016237 711.LMD	40	10814478	2971609	7665935	2948272.7	23336.23	0.2747806	0.2726227	0.0021579
data/VHL n3/VHLn3_Ca1uM4h_samp_100x 00016240 714.LMD	40	18186885	4878615	13145902	4829729.8	48885.28	0.2682491	0.2655611	0.0026879
data/VHL n3/VHLn3_Ca1uM4h_samp_140x 00016243 717.LMD	40	23550371	6639463	16842398	6597651.0	41811.66	0.2819260	0.2801506	0.0017754
data/VHL n3/VHLn3_Ca1uM4h_samp_180x 00016246 720.LMD	40	32434895	8674613	23687884	8621971.0	52641.84	0.2674469	0.2658239	0.0016230
data/VHL n3/VHLn3_Ca1uM4h_samp_220x 00016249 723.LMD	40	37285537	10311487	26888510	10213069.0	98417.53	0.2765546	0.2739150	0.0026396

4.3.1.3 Dataset 3 - Swarm-deconvoluted and normalized

```

# This optimization function will figure out how much noise is in the population
# (ie.: for blank subtraction)
subtractNoise <- function(noiseCeiling, events, tpRate, beads, dilution) {
  normalizedToXBeads <- 5000
  multiplier <- (normalizedToXBeads * dilution * 10) / beads
  BiocGenerics::var(((events * tpRate) - noiseCeiling) * multiplier)
}

cd24hiNoiseCeiling <- optimize(
  f=subtractNoise,
  interval=c(1, 5000),
  events=experiment$cd24hi,
  tpRate=experiment$cd24hiTp,
  beads=experiment$beads,
  dilution=experiment$dilution
)$minimum

cd24midNoiseCeiling <- optimize(
  f=subtractNoise,
  interval=c(100, 10000),
  events=experiment$cd24mid,
  tpRate=experiment$cd24midTp,
  beads=experiment$beads,
  dilution=experiment$dilution
)$minimum

cd24negNoiseCeiling <- optimize(
  f=subtractNoise,
  interval=c(100, 9000),
  events=experiment$cd24neg,
  tpRate=1,
  beads=experiment$beads,
  dilution=experiment$dilution
)$minimum

# Make sure the noiseCeiling is not set above the measured signal
if (any(experiment$cd24hi < cd24hiNoiseCeiling)) {
  cd24hiNoiseCeiling <- min(experiment$cd24hi)
}
if (any(experiment$cd24mid < cd24midNoiseCeiling)) {
  cd24midNoiseCeiling <- min(experiment$cd24mid)
}
if (any(experiment$cd24neg < cd24negNoiseCeiling)) {
  cd24negNoiseCeiling <- min(experiment$cd24neg)
}

list(
  "cd24hiNoiseCeiling: " = cd24hiNoiseCeiling,
  "cd24midNoiseCeiling: " = cd24midNoiseCeiling,
  "cd24negNoiseCeiling: " = cd24negNoiseCeiling
)

```

```
## $`cd24hiNoiseCeiling: `  
## [1] 43  
##  
## $`cd24midNoiseCeiling: `  
## [1] 2381  
##  
## $`cd24negNoiseCeiling: `  
## [1] 6495
```

```

# Let's make a separate dataframe for these data
dataset3 <- experiment

# Normalize with noise reduction
factor <- (experiment$dilution * normalizedToXBeads * 10) / experiment$beads

dataset3$cd24mid <- (
  (experiment$cd24mid * experiment$cd24midTp) - cd24midNoiseCeiling) * factor

dataset3$cd24hi <- (
  (experiment$cd24hi * experiment$cd24hiTp) - cd24hiNoiseCeiling) * factor

dataset3$cd24neg <- (experiment$cd24neg - cd24negNoiseCeiling) * factor

# Replace negative values by zero
dataset3$cd24neg[dataset3$cd24neg < 0] <- 0
dataset3$cd24mid[dataset3$cd24mid < 0] <- 0
dataset3$cd24hi[dataset3$cd24hi < 0] <- 0

# To be or not to be, that is the question
dataset3$cd24neg <- round(dataset3$cd24neg, digits=0)
dataset3$cd24mid <- round(dataset3$cd24mid, digits=0)
dataset3$cd24hi <- round(dataset3$cd24hi, digits=0)

# Compute remaining columns
dataset3$cd24pos <- dataset3$cd24mid + dataset3$cd24hi
dataset3$events <- dataset3$cd24neg + dataset3$cd24pos

dataset3$cd24posProp <- dataset3$cd24pos / dataset3$events
dataset3$cd24midProp <- dataset3$cd24mid / dataset3$events
dataset3$cd24hiProp <- dataset3$cd24hi / dataset3$events

# We don't need these columns any longer
dataset3$beads <- NULL
dataset3$cd24posTp <- NULL
dataset3$cd24midTp <- NULL
dataset3$cd24hiTp <- NULL
dataset3$cmVolume <- 40

printDataFrame(
  dataframe=dataset3[, interestingColumns],
  caption="Swarm-deconvoluted and denoised event counts",
  fontsize=5
)

```

Table 12: Swarm-deconvoluted and denoised event counts

	cmVolume	events	cd24pos	cd24neg	cd24mid	cd24hi	cd24posProp	cd24midProp	cd24hiProp
data/786 n1/786n1_Ca1uM4h_samp_20x 00016119 613.LMD	40	1018062	682460	335602	397892	284568	0.6703521	0.3908328	0.2795193
data/786 n1/786n1_Ca1uM4h_samp_60x 00016122 616.LMD	40	2632967	877497	1755470	831509	45988	0.3332731	0.3158068	0.0174662
data/786 n1/786n1_Ca1uM4h_samp_100x 00016125 619.LMD	40	3421080	1234585	2186495	1169388	65197	0.3608758	0.3418184	0.0190574
data/786 n1/786n1_Ca1uM4h_samp_140x 00016128 622.LMD	40	4920329	1277821	3642508	1270114	7707	0.2597023	0.2581360	0.0015664
data/786 n1/786n1_Ca1uM4h_samp_180x 00016131 625.LMD	40	6171221	1712956	4458265	1712956	0	0.2775717	0.2775717	0.0000000
data/786 n1/786n1_Ca1uM4h_samp_220x 00016134 628.LMD	40	7021500	1981127	5040373	1977413	3714	0.2821515	0.2816226	0.0005289
data/786 n2/786n2_Ca1uM4h_samp_20x 00016158 632.LMD	40	1049315	450909	598406	402676	48233	0.4297175	0.3837513	0.0459662
data/786 n2/786n2_Ca1uM4h_samp_60x 00016161 635.LMD	40	1162644	481113	681531	475152	5961	0.4138094	0.4086823	0.0051271
data/786 n2/786n2_Ca1uM4h_samp_100x 00016164 638.LMD	40	855281	637972	217309	598904	39068	0.7459209	0.7002424	0.0456786
data/786 n2/786n2_Ca1uM4h_samp_140x 00016167 641.LMD	40	2970720	1198711	1772009	1052460	146251	0.4035086	0.3542778	0.0492308

Table 12: Swarm-deconvoluted and denoised event counts (*continued*)

	cmVolume	events	cd24pos	cd24neg	cd24mid	cd24hi	cd24posProp	cd24midProp	cd24hiProp
data/786 n2/786n2_Ca1uM4h_samp_180x 00016170 644.LMD	40	2883036	1214334	1668702	1088774	125560	0.4211997	0.3776484	0.0435513
data/786 n2/786n2_Ca1uM4h_samp_220x 00016173 647.LMD	40	2623	2623	0	0	2623	1.0000000	0.0000000	1.0000000
data/786 n3/786n3_Ca1uM4h_samp_20x 00016176 650.LMD	40	654801	275631	379170	256644	18987	0.4209386	0.3919420	0.0289966
data/786 n3/786n3_Ca1uM4h_samp_60x 00016179 653.LMD	40	1144473	605932	538541	599276	6656	0.5294419	0.5236262	0.0058158
data/786 n3/786n3_Ca1uM4h_samp_100x 00016182 656.LMD	40	2556155	1038620	1517535	973533	65087	0.4063212	0.3808584	0.0254629
data/786 n3/786n3_Ca1uM4h_samp_140x 00016185 659.LMD	40	3704875	1591588	2113287	1528295	63293	0.4295929	0.4125092	0.0170837
data/786 n3/786n3_Ca1uM4h_samp_180x 00016188 662.LMD	40	7739287	2841624	4897663	2545712	295912	0.3671687	0.3289337	0.0382350
data/786 n3/786n3_Ca1uM4h_samp_220x 00016191 665.LMD	40	7394750	3012220	4382530	2809286	202934	0.4073458	0.3799028	0.0274430
data/VHL n1/VHLn1_Ca1uM4h_samp_20x 00016196 670.LMD	40	774896	290909	483987	270677	20232	0.3754168	0.3493075	0.0261093
data/VHL n1/VHLn1_Ca1uM4h_samp_60x 00016199 673.LMD	40	2886712	955574	1931138	916940	38634	0.3310251	0.3176417	0.0133834
data/VHL n1/VHLn1_Ca1uM4h_samp_100x 00016202 676.LMD	40	2564556	827587	1736969	827587	0	0.3227019	0.3227019	0.0000000
data/VHL n1/VHLn1_Ca1uM4h_samp_140x 00016205 679.LMD	40	3647887	1325325	2322562	1280348	44977	0.3633131	0.3509835	0.0123296
data/VHL n1/VHLn1_Ca1uM4h_samp_180x 00016208 682.LMD	40	4362100	1680833	2681267	1660518	20315	0.3853266	0.3806694	0.0046572
data/VHL n1/VHLn1_Ca1uM4h_samp_220x 00016211 685.LMD	40	6518789	2281185	4237604	2179930	101255	0.3499400	0.3344072	0.0155328
data/VHL n2/VHLn2_Ca1uM4h_samp_20x 00016215 689.LMD	40	1359812	435448	924364	423459	11989	0.3202266	0.3114100	0.0088167
data/VHL n2/VHLn2_Ca1uM4h_samp_60x 00016218 692.LMD	40	2326732	847522	1479210	826659	20863	0.3642542	0.3552876	0.0089667
data/VHL n2/VHLn2_Ca1uM4h_samp_100x 00016221 695.LMD	40	4176240	1388604	2787636	1357413	31191	0.3325010	0.3250323	0.0074687
data/VHL n2/VHLn2_Ca1uM4h_samp_140x 00016224 698.LMD	40	7649800	1888017	5761783	1870924	17093	0.2468061	0.2445716	0.0022344
data/VHL n2/VHLn2_Ca1uM4h_samp_180x 00016227 701.LMD	40	6892316	2446913	4445403	2365867	81046	0.3550204	0.3432615	0.0117589
data/VHL n2/VHLn2_Ca1uM4h_samp_220x 00016230 704.LMD	40	7767939	3565020	4202919	3189276	375744	0.4589403	0.4105691	0.0483711
data/VHL n3/VHLn3_Ca1uM4h_samp_20x 00016234 708.LMD	40	1356853	312551	1044302	311836	715	0.2303499	0.2298230	0.0005270
data/VHL n3/VHLn3_Ca1uM4h_samp_60x 00016237 711.LMD	40	2269488	709789	1559699	709789	0	0.3127529	0.3127529	0.0000000
data/VHL n3/VHLn3_Ca1uM4h_samp_100x 00016240 714.LMD	40	3424812	926451	2498361	926451	0	0.2705115	0.2705115	0.0000000
data/VHL n3/VHLn3_Ca1uM4h_samp_140x 00016243 717.LMD	40	4239802	1447157	2792645	1447157	0	0.3413266	0.3413266	0.0000000
data/VHL n3/VHLn3_Ca1uM4h_samp_180x 00016246 720.LMD	40	6499192	1698223	4800969	1698223	0	0.2612976	0.2612976	0.0000000
data/VHL n3/VHLn3_Ca1uM4h_samp_220x 00016249 723.LMD	40	6864354	2101889	4762465	2101889	0	0.3062035	0.3062035	0.0000000

4.3.2 Computing summary data

4.3.2.1 Dataset 1 - Raw gate counts

```
# Compute summary statistics for each biological triplicate
dataset1Summary <- dataset1 %>%
  dplyr::group_by(cellLine, dilution) %>%
  dplyr::summarise(
    n = dplyr::n(),
    eventsAvg = BiocGenerics::mean(events),
    eventsSem = BiocGenerics::sd(events) / dplyr::n(),
    cd24posAvg = BiocGenerics::mean(cd24pos),
    cd24posSem = BiocGenerics::sd(cd24pos) / dplyr::n(),
    cd24negAvg = BiocGenerics::mean(cd24neg),
    cd24negSem = BiocGenerics::sd(cd24neg) / dplyr::n(),
    cd24hiAvg = BiocGenerics::mean(cd24hi),
    cd24hiSem = BiocGenerics::sd(cd24hi) / dplyr::n(),
    cd24midAvg = BiocGenerics::mean(cd24mid),
    cd24midSem = BiocGenerics::sd(cd24mid) / dplyr::n(),
    cd24posPropAvg = BiocGenerics::mean(cd24pos/events),
    cd24posPropSem = BiocGenerics::sd(cd24pos/events) / dplyr::n(),
    cd24negPropAvg = BiocGenerics::mean(cd24neg/events),
    cd24negPropSem = BiocGenerics::sd(cd24neg/events) / dplyr::n(),
    cd24midPropAvg = BiocGenerics::mean(cd24mid/events),
    cd24midPropSem = BiocGenerics::sd(cd24mid/events) / dplyr::n(),
    cd24hiPropAvg = BiocGenerics::mean(cd24hi/events),
    cd24hiPropSem = BiocGenerics::sd(cd24hi/events) / dplyr::n(),
    .groups="drop"
  )
```

```

interestingColumns <- function(metric) {
  c(
    "cellLine",
    "dilution",
    "n",
    paste0("events", metric),
    paste0("cd24pos", metric),
    paste0("cd24neg", metric),
    paste0("cd24mid", metric),
    paste0("cd24hi", metric),
    paste0("cd24posProp", metric),
    paste0("cd24negProp", metric),
    paste0("cd24midProp", metric),
    paste0("cd24hiProp", metric)
  )
}

printDataFrame(
  dataframe=dataset1Summary[, interestingColumns("Avg")],
  caption="Normalized experiment summary: calculated means",
  fontsize=5
)

```

Table 13: Normalized experiment summary: calculated means

cellLine	dilution	n	eventsAvg	cd24posAvg	cd24negAvg	cd24midAvg	cd24hiAvg	cd24posPropAvg	cd24negPropAvg	cd24midPropAvg	cd24hiPropAvg
786-O	20	3	4651027	1985014	2661691	1414398	570616.7	0.4211473	0.5779771	0.3036416	0.1175058
786-O	60	3	10936899	3517393	7417933	3125464	391929.3	0.3213696	0.6784873	0.2859043	0.0354653
786-O	100	3	17601210	5489258	12110491	4997049	492209.0	0.3119223	0.6879930	0.2838595	0.0280627
786-O	140	3	22896396	6819492	16076271	6362630	456861.3	0.2977881	0.7021837	0.2778553	0.0199328
786-O	180	3	29102923	8618060	20484863	8086563	531496.3	0.2959829	0.7040171	0.2777458	0.0182371
786-O	220	3	35189772	10146230	25042506	9649797	496433.7	0.2885236	0.7114469	0.2743941	0.0141295
VHL	20	3	4219475	1367666	2851598	1249578	118087.7	0.3256876	0.6742614	0.2972592	0.0284283
VHL	60	3	10787041	3234673	7552069	3103588	131084.7	0.2998645	0.7001087	0.2877364	0.0121282
VHL	100	3	17108532	4858671	12249862	4737374	121296.7	0.2841467	0.7158534	0.2770984	0.0070483
VHL	140	3	23639166	6653488	16984343	6494993	158494.3	0.2822041	0.7177392	0.2754044	0.0067996
VHL	180	3	30169696	8590809	21578078	8391824	198985.7	0.2853386	0.7146331	0.2786325	0.0067060
VHL	220	3	35902859	10676840	25226020	10319074	357765.7	0.2974747	0.7025253	0.2874926	0.0099821

```

printDataFrame(
  dataframe=dataset1Summary[, interestingColumns("Sem")],
  caption="Normalized experiment summary: calculated SEMs",
  fontsize=5
)

```

Table 14: Normalized experiment summary: calculated SEMs

cellLine	dilution	n	eventsSem	cd24posSem	cd24negSem	cd24midSem	cd24hiSem	cd24posPropSem	cd24negPropSem	cd24midPropSem	cd24hiPropSem
786-O	20	3	187588.2	195798.77	54535.65	71367.25	151682.367	0.0252850	0.0255546	0.0072463	0.0271893
786-O	60	3	128448.6	85144.94	58945.71	16604.39	72287.322	0.0044199	0.0044527	0.0018991	0.0061344
786-O	100	3	152089.7	63897.03	136005.19	59248.73	55284.048	0.0034361	0.0034810	0.0010164	0.0033151
786-O	140	3	169768.9	94761.23	107009.25	112475.66	41341.124	0.0026846	0.0026692	0.0041719	0.0017652
786-O	180	3	194224.3	160419.52	33864.67	138196.67	22307.471	0.0035132	0.0035132	0.0028775	0.0006397
786-O	220	3	243519.3	105719.81	340956.91	99445.19	20059.421	0.0049003	0.0048953	0.0045199	0.0006415
VHL	20	3	131644.0	18410.50	122822.58	23018.75	5972.073	0.0091407	0.0091512	0.0071842	0.0023714
VHL	60	3	132482.8	48938.15	95762.72	42438.42	6513.683	0.0025244	0.0025331	0.0021855	0.0004837
VHL	100	3	349117.7	83336.92	275021.88	73719.67	9793.782	0.0023205	0.0023205	0.0022746	0.0004772
VHL	140	3	585407.8	49002.31	539999.23	64760.69	17838.656	0.0050344	0.0050342	0.0045235	0.0009034
VHL	180	3	670362.7	61762.96	618173.05	79929.65	21462.597	0.0045207	0.0045290	0.0036984	0.0008314
VHL	220	3	532265.6	278731.25	487450.28	216726.09	72414.906	0.0070168	0.0070168	0.0050287	0.0019919

4.3.2.2 Dataset 2 - Swarm-deconvoluted

```
# Do the same with swarm-deconvoluted data
dataset2Summary <- dataset2 %>%
  dplyr::group_by(cellLine, dilution) %>%
  dplyr::summarise(
    n = dplyr::n(),
    eventsAvg = BiocGenerics::mean(events),
    eventsSem = BiocGenerics::sd(events) / dplyr::n(),
    cd24posAvg = BiocGenerics::mean(cd24pos),
    cd24posSem = BiocGenerics::sd(cd24pos) / dplyr::n(),
    cd24negAvg = BiocGenerics::mean(cd24neg),
    cd24negSem = BiocGenerics::sd(cd24neg) / dplyr::n(),
    cd24hiAvg = BiocGenerics::mean(cd24hi),
    cd24hiSem = BiocGenerics::sd(cd24hi) / dplyr::n(),
    cd24midAvg = BiocGenerics::mean(cd24mid),
    cd24midSem = BiocGenerics::sd(cd24mid) / dplyr::n(),
    cd24posPropAvg = BiocGenerics::mean(cd24pos/events),
    cd24posPropSem = BiocGenerics::sd(cd24pos/events) / dplyr::n(),
    cd24negPropAvg = BiocGenerics::mean(cd24neg/events),
    cd24negPropSem = BiocGenerics::sd(cd24neg/events) / dplyr::n(),
    cd24midPropAvg = BiocGenerics::mean(cd24mid/events),
    cd24midPropSem = BiocGenerics::sd(cd24mid/events) / dplyr::n(),
    cd24hiPropAvg = BiocGenerics::mean(cd24hi/events),
    cd24hiPropSem = BiocGenerics::sd(cd24hi/events) / dplyr::n(),
    .groups="drop"
  )
```

```

printDataFrame(
  datafram=dataset2Summary[, interestingColumns("Avg")],
  caption="Normalized experiment summary (deconvoluted): calculated means",
  fontsize=5
)

```

Table 15: Normalized experiment summary (deconvoluted): calculated means

cellLine	dilution	n	eventsAvg	cd24posAvg	cd24negAvg	cd24midAvg	cd24hiAvg	cd24posPropAvg	cd24negPropAvg	cd24midPropAvg	cd24hiPropAvg
786-O	20	3	4651027	1299673	2661691	1167687	131986.29	0.2786722	0.5779771	0.2521913	0.0264808
786-O	60	3	10936899	3053128	7417933	2991049	62078.75	0.2794633	0.6784873	0.2738185	0.0056447
786-O	100	3	17601210	5002323	12110491	4874348	127974.30	0.2841282	0.6879930	0.2768487	0.0072795
786-O	140	3	22896396	6419383	16076271	6257146	162236.81	0.2802540	0.7021837	0.2732150	0.0070391
786-O	180	3	29102923	8194313	20484863	7944848	249465.08	0.2814082	0.7040171	0.2729011	0.0085071
786-O	220	3	35189772	9839200	25042506	9624445	214755.56	0.2798077	0.7114469	0.2736717	0.0061360
VHL	20	3	4219475	1105429	2851598	1080985	24444.64	0.2621301	0.6742614	0.2562050	0.0059251
VHL	60	3	10787041	3032150	7552069	2978984	53166.05	0.2811967	0.7001087	0.2762797	0.0049170
VHL	100	3	17108532	4729834	12249862	4669641	60193.13	0.2766485	0.7158534	0.2731320	0.0035165
VHL	140	3	23639166	6522026	16984343	6429963	92062.46	0.2764061	0.7177392	0.2724667	0.0039394
VHL	180	3	30169696	8487176	21578078	8360987	126189.19	0.2818777	0.7146331	0.2775865	0.0042912
VHL	220	3	35902859	10405453	25226020	10124604	280848.93	0.2898382	0.7025253	0.2819867	0.0078515

```

printDataFrame(
  datafram=dataset2Summary[, interestingColumns("Sem")],
  caption="Normalized experiment summary (deconvoluted): calculated SEMs",
  fontsize=5
)

```

Table 16: Normalized experiment summary (deconvoluted): calculated SEMs

cellLine	dilution	n	eventsSem	cd24posSem	cd24negSem	cd24midSem	cd24hiSem	cd24posPropSem	cd24negPropSem	cd24midPropSem	cd24hiPropSem
786-O	20	3	187588.2	67852.96	54535.65	29227.86	48521.395	0.0036033	0.0255546	0.0054711	0.0090719
786-O	60	3	128448.6	12771.51	58945.71	17277.33	63663.396	0.0043690	0.0044527	0.0047834	0.0005144
786-O	100	3	152089.7	73554.05	136005.19	74798.16	2538.799	0.0020912	0.0034810	0.0021487	0.0001936
786-O	140	3	169768.9	154743.41	107009.25	135178.56	25761.135	0.0056337	0.0026692	0.0050511	0.0010682
786-O	180	3	194224.3	174304.05	33864.67	124176.45	50207.475	0.0042202	0.0035132	0.0025897	0.0016663
786-O	220	3	243519.3	140363.64	340956.91	105863.35	34540.144	0.0056288	0.0048953	0.0046194	0.0010264
VHL	20	3	131644.0	41235.71	122822.58	41287.99	3256.515	0.0059461	0.0091512	0.0052839	0.0009319
VHL	60	3	132482.8	29259.36	95762.72	22250.19	9138.251	0.0022703	0.0025331	0.0018503	0.0008229
VHL	100	3	349117.7	79854.78	275021.88	74443.47	9535.274	0.0027919	0.0023205	0.0023564	0.0005572
VHL	140	3	585407.8	87631.89	539999.23	98414.47	15304.205	0.0037056	0.0050342	0.0037435	0.0007065
VHL	180	3	670362.7	58240.12	618173.05	80735.48	22508.631	0.0042289	0.0045290	0.0034242	0.0008216
VHL	220	3	532265.6	249463.34	487450.28	203243.38	70156.298	0.0055976	0.0070168	0.0036937	0.0019319

4.3.2.3 Dataset 3 - Swarm-deconvoluted and normalized

```
# Compute summary statistics for each biological triplicate
dataset3Summary <- dataset3 %>%
  dplyr::group_by(cellLine, dilution) %>%
  dplyr::summarise(
    n           = dplyr::n(),
    eventsAvg   = BiocGenerics::mean(events),
    eventsSem   = BiocGenerics::sd(events) / dplyr::n(),
    cd24posAvg  = BiocGenerics::mean(cd24pos),
    cd24posSem  = BiocGenerics::sd(cd24pos) / dplyr::n(),
    cd24negAvg  = BiocGenerics::mean(cd24neg),
    cd24negSem  = BiocGenerics::sd(cd24neg) / dplyr::n(),
    cd24hiAvg   = BiocGenerics::mean(cd24hi),
    cd24hiSem   = BiocGenerics::sd(cd24hi) / dplyr::n(),
    cd24midAvg  = BiocGenerics::mean(cd24mid),
    cd24midSem  = BiocGenerics::sd(cd24mid) / dplyr::n(),
    cd24posPropAvg = BiocGenerics::mean(cd24pos/events),
    cd24posPropSem = BiocGenerics::sd(cd24pos/events) / dplyr::n(),
    cd24negPropAvg = BiocGenerics::mean(cd24neg/events),
    cd24negPropSem = BiocGenerics::sd(cd24neg/events) / dplyr::n(),
    cd24midPropAvg = BiocGenerics::mean(cd24mid/events),
    cd24midPropSem = BiocGenerics::sd(cd24mid/events) / dplyr::n(),
    cd24hiPropAvg = BiocGenerics::mean(cd24hi/events),
    cd24hiPropSem = BiocGenerics::sd(cd24hi/events) / dplyr::n(),
    .groups="drop"
  )
```

```

printDataFrame(
  datafram=dataset3Summary[, interestingColumns("Avg")],
  caption="Denoised and normalized experiment summary: calculated means",
  fontsize=5
)

```

Table 17: Denoised and normalized experiment summary: calculated means

cellLine	dilution	n	eventsAvg	cd24posAvg	cd24negAvg	cd24midAvg	cd24hiAvg	cd24posPropAvg	cd24negPropAvg	cd24midPropAvg	cd24hiPropAvg
786-O	20	3	907392.7	469666.7	437726.0	352404.0	117262.67	0.5070027	0.4929973	0.3888420	0.1181607
786-O	60	3	1646694.7	654847.3	991847.3	635312.3	19535.00	0.4255081	0.5744919	0.4160384	0.0094697
786-O	100	3	2277505.3	970392.3	1307113.0	913941.7	56450.67	0.5043726	0.4956274	0.4743064	0.0300663
786-O	140	3	3865308.0	1356040.0	2509268.0	1283623.0	72417.00	0.3642679	0.6357321	0.3416410	0.0226270
786-O	180	3	5597848.0	1922971.3	3674876.7	140490.67	0.3555134	0.6446866	0.3280512	0.0272621	
786-O	220	3	4806291.0	1665323.3	3140967.7	1595566.3	69757.00	0.5631658	0.4368342	0.2205085	0.3426573
VHL	20	3	1163853.7	346302.7	817551.0	335324.0	10978.67	0.3086645	0.6913355	0.2968468	0.0118176
VHL	60	3	2494310.7	837628.3	1656682.3	817796.0	19832.33	0.3360107	0.6639893	0.3285607	0.0074500
VHL	100	3	3388536.0	1047547.3	2340988.7	1037150.3	10397.00	0.3085715	0.6914285	0.3060819	0.0024896
VHL	140	3	5179163.0	1553499.7	3625663.3	1532809.7	20690.00	0.3171486	0.6828514	0.3122939	0.0048547
VHL	180	3	5917869.3	1941989.7	3975879.7	1908202.7	33787.00	0.3338815	0.6661185	0.3284095	0.0054720
VHL	220	3	7050360.7	2649364.7	4400996.0	2490365.0	158999.67	0.3716946	0.6283054	0.3503933	0.0213013

```

printDataFrame(
  datafram=dataset3Summary[, interestingColumns("Sem")],
  caption="Denoised and normalized experiment summary: calculated SEMs",
  fontsize=5
)

```

Table 18: Denoised and normalized experiment summary: calculated SEMs

cellLine	dilution	n	eventsSem	cd24posSem	cd24negSem	cd24midSem	cd24hiSem	cd24posPropSem	cd24negPropSem	cd24midPropSem	cd24hiPropSem
786-O	20	3	73102.74	68020.70	46949.25	27655.03	48542.236	0.0471776	0.0471776	0.0014811	0.0466660
786-O	60	3	284728.41	67556.23	221723.36	60296.99	7637.202	0.0328688	0.0328688	0.0347016	0.0023112
786-O	100	3	435132.85	101367.33	333771.62	96624.33	5017.977	0.0701391	0.0701391	0.0655459	0.0046316
786-O	140	3	328218.74	69263.38	332046.24	79401.66	23240.399	0.0304969	0.0304969	0.0259862	0.0081036
786-O	180	3	826126.23	277908.46	583744.64	243651.01	49506.646	0.0241814	0.0241814	0.0166814	0.0079196
786-O	220	3	1388094.16	509816.69	913324.07	481014.80	38445.318	0.1278178	0.1278178	0.0657290	0.1898115
VHL	20	3	112283.49	25985.60	98344.63	26350.94	3265.883	0.0244071	0.0244071	0.0203530	0.0043508
VHL	60	3	113677.58	41063.61	80356.19	34619.84	6445.870	0.0087034	0.0087034	0.0077583	0.0022731
VHL	100	3	268818.05	99823.88	180908.15	93908.73	6002.711	0.0111077	0.0111077	0.0102756	0.0014373
VHL	140	3	720002.02	98678.57	621601.87	101487.43	7567.742	0.0206341	0.0206341	0.0196159	0.0021897
VHL	180	3	453866.14	145787.62	378391.81	132265.68	14056.373	0.0215534	0.0215534	0.0203520	0.0019739
VHL	220	3	215004.60	266010.71	104507.12	202177.05	64804.598	0.0262193	0.0262193	0.0179960	0.0082320

4.3.3 Building sample graphs

4.3.3.1 Dataset 1 - Raw gate counts

```
# Let's generate plots to show the swarm effect

abcTemplate <- function(
  data,
  title,
  subtitle,
  measurement,
  scaleYLimits,
  scaleYLabels,
  legendPosition
) {
  measurement <- rlang::sym(measurement)

  ggplot2::ggplot(
    data=data,
    mapping=ggplot2::aes(
      x=dilution,
      y=!!measurement,
      colour=cellLine,
      group=interaction(cellLine, facetRow)
    )
  ) +
    ggplot2::labs(
      title=title,
      subtitle=subtitle,
      x=ggplot2::element_blank(),
      y=ggplot2::element_blank() +
        ggplot2::geom_point() +
        ggplot2::geom_line() +
        ggplot2::scale_x_continuous(
          limits=c(20, 220),
          breaks=c(0, 20, 60, 100, 140, 180, 220),
          minor_breaks=c(40, 80, 120, 160, 200)) +
        ggplot2::scale_y_continuous(
          limits=scaleYLimits,
          labels=scaleYLabels) +
        ggplot2::theme(
          legend.position=legendPosition,
          legend.justification=c(1, 1),
          legend.title=ggplot2::element_blank())
    )
}

plotA1 <- abcTemplate(
  data=dataset1,
  title="Proportion of CD24+ events",
  subtitle="Swarm effect",
  measurement="cd24posProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
```

```

)

plotA2 <- abcTemplate(
  data=dataset1,
  title="Proportion of CD24mid events",
  subtitle="Swarm effect",
  measurement="cd24midProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotA3 <- abcTemplate(
  data=dataset1,
  title="Proportion of CD24hi events",
  subtitle="Swarm effect",
  measurement="cd24hiProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition=c(1, 1)
)

plotA4 <- abcTemplate(
  data=dataset1,
  title="Absolute count of CD24+ events",
  subtitle="Swarm effect",
  measurement="cd24pos",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotA5 <- abcTemplate(
  data=dataset1,
  title="Absolute count of CD24mid events",
  subtitle="Swarm effect",
  measurement="cd24mid",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotA6 <- abcTemplate(
  data=dataset1,
  title="Absolute count of CD24hi events",
  subtitle="Swarm effect",
  measurement="cd24hi",
  scaleYLimits=c(0, 1.2e6),
  scaleYLabels=scales::scientific,
  legendPosition=c(1, 1)
)

```

4.3.3.2 Dataset 2 - Swarm-deconvoluted

```
# Let's generate plots for the swarm-deconvoluted data

plotB1 <- abcTemplate(
  data=dataset2,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24posProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotB2 <- abcTemplate(
  data=dataset2,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24midProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotB3 <- abcTemplate(
  data=dataset2,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24hiProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotB4 <- abcTemplate(
  data=dataset2,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24pos",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotB5 <- abcTemplate(
  data=dataset2,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24mid",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)
```

```

plotB6 <- abcTemplate(
  data=dataset2,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24hi",
  scaleYLimits=c(0, 1.2e6),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

```

4.3.3.3 Dataset 3 - Swarm-deconvoluted and normalized

```

# Let's generate plots to show the final result

plotC1 <- abcTemplate(
  data=dataset3,
  title="",
  subtitle="Swarm deconvoluted + Denoised",
  measurement="cd24posProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotC2 <- abcTemplate(
  data=dataset3,
  title="",
  subtitle="Swarm deconvoluted + Denoised",
  measurement="cd24midProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotC3 <- abcTemplate(
  data=dataset3,
  title="",
  subtitle="Swarm deconvoluted + Denoised",
  measurement="cd24hiProp",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotC4 <- abcTemplate(
  data=dataset3,
  title="",
  subtitle="Swarm deconvoluted + Denoised",
  measurement="cd24pos",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

```

```
)  
  
plotC5 <- abcTemplate(  
  data=dataset3,  
  title="",  
  subtitle="Swarm deconvoluted + Denoised",  
  measurement="cd24mid",  
  scaleYLimits=c(0, 1.2e7),  
  scaleYLabels=scales::scientific,  
  legendPosition="none"  
)  
  
plotC6 <- abcTemplate(  
  data=dataset3,  
  title="",  
  subtitle="Swarm deconvoluted + Denoised",  
  measurement="cd24hi",  
  scaleYLimits=c(0, 1.2e6),  
  scaleYLabels=scales::scientific,  
  legendPosition="none"  
)
```

4.3.4 Building Summary graphs

4.3.4.1 Dataset 1 - Raw gate counts

```
defTemplate <- function(  
  data,  
  title,  
  subtitle,  
  measurement,  
  sem,  
  scaleYLimits,  
  scaleYLabels,  
  legendPosition  
) {  
  measurement <- rlang::sym(measurement)  
  sem <- rlang::sym(sem)  
  
  ggplot2::ggplot(  
    data=data,  
    mapping=ggplot2::aes(  
      x=dilution,  
      y=!!measurement,  
      fill=cellLine  
    )  
  ) +  
  ggplot2::labs(  
    title=title,  
    subtitle=subtitle,  
    x=ggplot2::element_blank(),  
    y=ggplot2::element_blank() +  
  ggplot2::theme(  
    panel.background=ggplot2::element_rect(fill="#F4F4F4"),  
    panel.grid.major.x=ggplot2::element_blank(),  
    panel.grid.minor.x=ggplot2::element_blank(),  
    panel.grid.minor.y=ggplot2::element_line(colour="#EEEEEE"),  
    axis.ticks=ggplot2::element_blank(),  
    axis.title.x=ggplot2::element_text(vjust=-2),  
    axis.title.y=ggplot2::element_text(  
      angle=90,  
      vjust=2),  
    legend.position=legendPosition,  
    legend.justification=c(1, 1),  
    legend.title=ggplot2::element_blank()) +  
  ggplot2::geom_bar(  
    position=ggplot2::position_dodge(),  
    stat="identity") +  
  ggplot2::geom_errorbar(  
    mapping=ggplot2::aes(  
      ymin=!!measurement - !!sem,  
      ymax=!!measurement + !!sem,  
      color=cellLine),  
    width=15,  
    size=1,  
    position=ggplot2::position_dodge(35),
```

```

    show.legend=FALSE) +
  ggplot2::scale_fill_manual(
    name="Cell line",
    values=c(
      "#F3756E",
      "#1DBDC3")) +
  ggplot2::scale_color_manual(
    values=c(
      "#B8645B",
      "#43989C")) +
  ggplot2::scale_x_continuous(
    limits=c(0, 240),
    breaks=c(20, 60, 100, 140, 180, 220),
    minor_breaks=c(40, 80, 120, 160, 200)) +
  ggplot2::scale_y_continuous(
    limits=scaleYLimits,
    labels=scaleYLabels)
}

# Show the swarm effect

plotD1 <- defTemplate(
  data=dataset1Summary,
  title="Proportion of CD24+ events",
  subtitle="Swarm effect",
  measurement="cd24posPropAvg",
  sem="cd24posPropSem",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotD2 <- defTemplate(
  data=dataset1Summary,
  title="Proportion of CD24mid events",
  subtitle="Swarm effect",
  measurement="cd24midPropAvg",
  sem="cd24midPropSem",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotD3 <- defTemplate(
  data=dataset1Summary,
  title="Proportion of CD24hi events",
  subtitle="Swarm effect",
  measurement="cd24hiPropAvg",
  sem="cd24hiPropSem",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition=c(1, 1)
)

```

```

plotD4 <- defTemplate(
  data=dataset1Summary,
  title="Absolute CD24+ events count",
  subtitle="Swarm effect",
  measurement="cd24posAvg",
  sem="cd24posSem",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotD5 <- defTemplate(
  data=dataset1Summary,
  title="Absolute CD24mid events count",
  subtitle="Swarm effect",
  measurement="cd24midAvg",
  sem="cd24midSem",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotD6 <- defTemplate(
  data=dataset1Summary,
  title="Absolute CD24hi events count",
  subtitle="Swarm effect",
  measurement="cd24hiAvg",
  sem="cd24hiSem",
  scaleYLimits=c(0, 1.2e6),
  scaleYLabels=scales::scientific,
  legendPosition=c(1, 1)
)

```

4.3.4.2 Dataset 2 - Swarm-deconvoluted

```

# Swarm-deconvoluted data plots

plotE1 <- defTemplate(
  data=dataset2Summary,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24posPropAvg",
  sem="cd24posPropSem",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotE2 <- defTemplate(
  data=dataset2Summary,
  title="",
  subtitle="Swarm-deconvoluted",

```

```

measurement="cd24midPropAvg",
sem="cd24midPropSem",
scaleYLimits=c(0, 1),
scaleYLabels=scales::number,
legendPosition="none"
)

plotE3 <- defTemplate(
  data=dataset2Summary,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24hiPropAvg",
  sem="cd24hiPropSem",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotE4 <- defTemplate(
  data=dataset2Summary,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24posAvg",
  sem="cd24posSem",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotE5 <- defTemplate(
  data=dataset2Summary,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24midAvg",
  sem="cd24midSem",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotE6 <- defTemplate(
  data=dataset2Summary,
  title="",
  subtitle="Swarm-deconvoluted",
  measurement="cd24hiAvg",
  sem="cd24hiSem",
  scaleYLimits=c(0, 1.2e6),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

```

4.3.4.3 Dataset 3 - Swarm-deconvoluted and normalized

```

# Let's generate plots to show the final result

plotF1 <- defTemplate(
  data=dataset3Summary,
  title="",
  subtitle="Denoised and swarm-deconvoluted",
  measurement="cd24posPropAvg",
  sem="cd24posPropSem",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotF2 <- defTemplate(
  data=dataset3Summary,
  title="",
  subtitle="Denoised and swarm-deconvoluted",
  measurement="cd24midPropAvg",
  sem="cd24midPropSem",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotF3 <- defTemplate(
  data=dataset3Summary,
  title="",
  subtitle="Denoised and swarm-deconvoluted",
  measurement="cd24hiPropAvg",
  sem="cd24hiPropSem",
  scaleYLimits=c(0, 1),
  scaleYLabels=scales::number,
  legendPosition="none"
)

plotF4 <- defTemplate(
  data=dataset3Summary,
  title="",
  subtitle="Denoised and swarm-deconvoluted",
  measurement="cd24posAvg",
  sem="cd24posSem",
  scaleYLimits=c(0, 1.2e7),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotF5 <- defTemplate(
  data=dataset3Summary,
  title="",
  subtitle="Denoised and swarm-deconvoluted",
  measurement="cd24midAvg",
  sem="cd24midSem",
  scaleYLimits=c(0, 1.2e7),

```

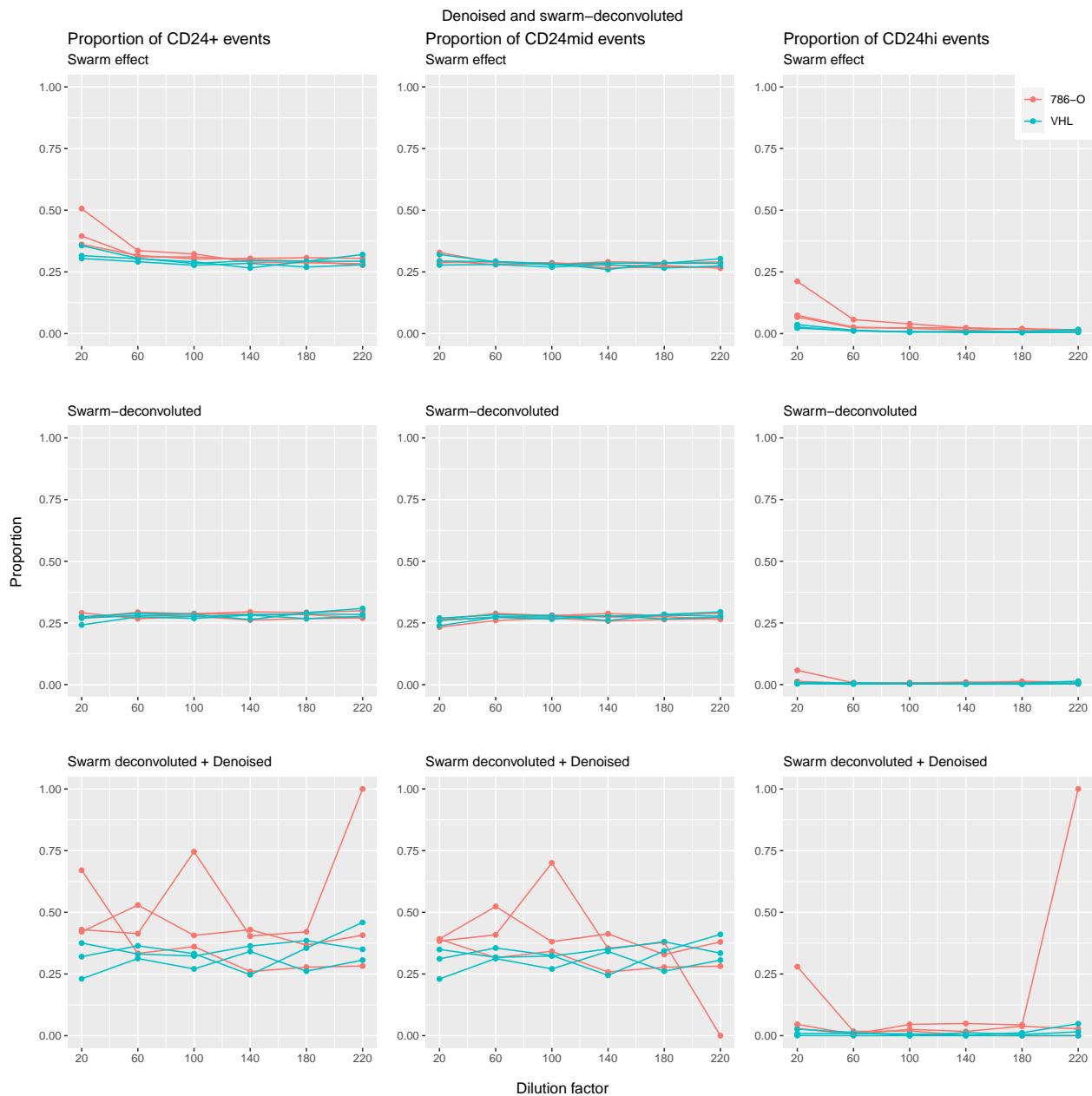
```
  scaleYLabels=scales::scientific,
  legendPosition="none"
)

plotF6 <- defTemplate(
  data=dataset3Summary,
  title="",
  subtitle="Denoised and swarm-deconvoluted",
  measurement="cd24hiAvg",
  sem="cd24hiSem",
  scaleYLimits=c(0, 1.2e6),
  scaleYLabels=scales::scientific,
  legendPosition="none"
)
```

4.3.5 Visualizing the process

4.3.5.1 Sample graphs

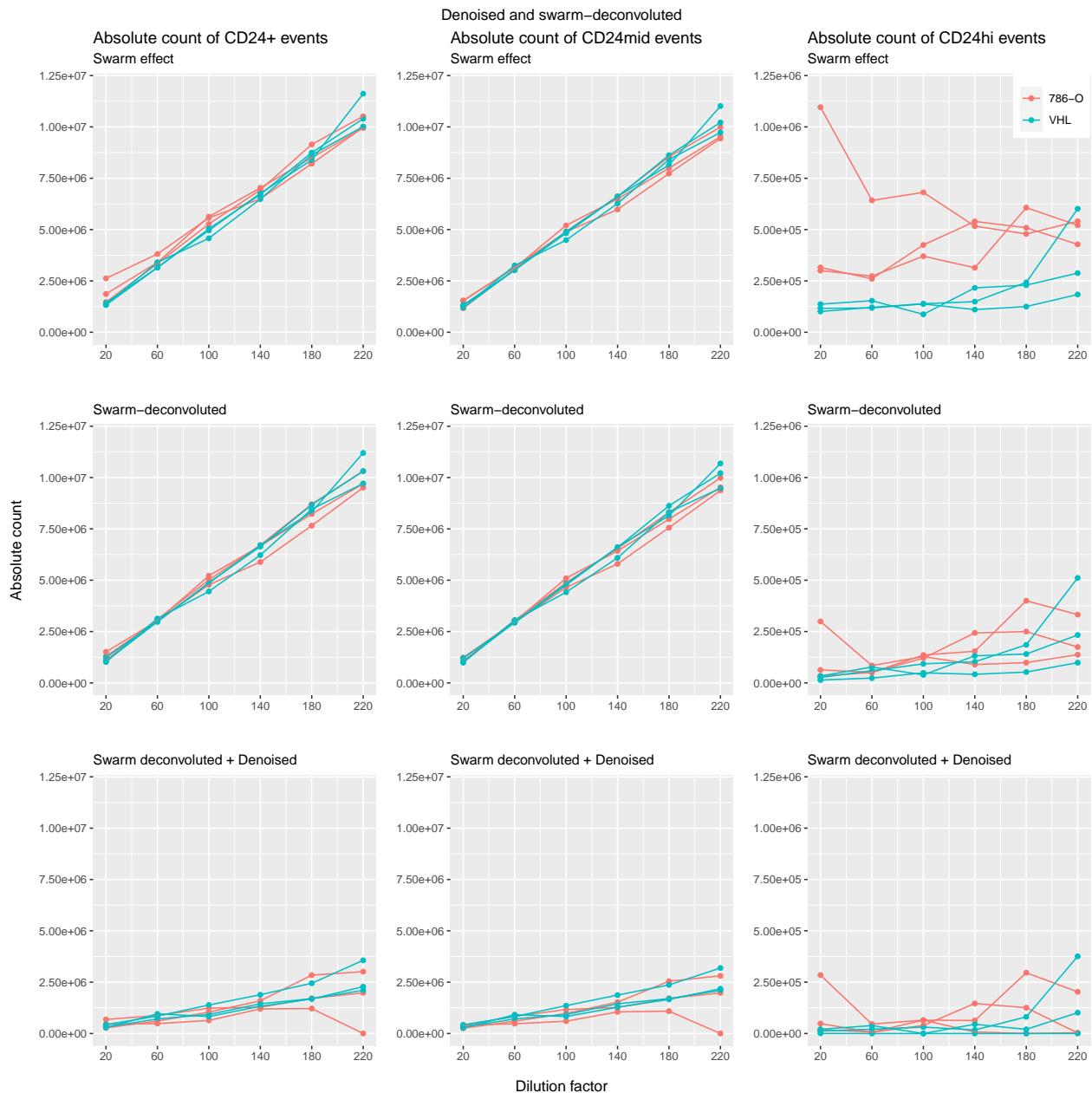
```
gridExtra::grid.arrange(
  plotA1, plotA2, plotA3,
  plotB1, plotB2, plotB3,
  plotC1, plotC2, plotC3,
  nrow=3,
  top="Denoised and swarm-deconvoluted",
  left="Proportion",
  bottom="Dilution factor"
)
```



```

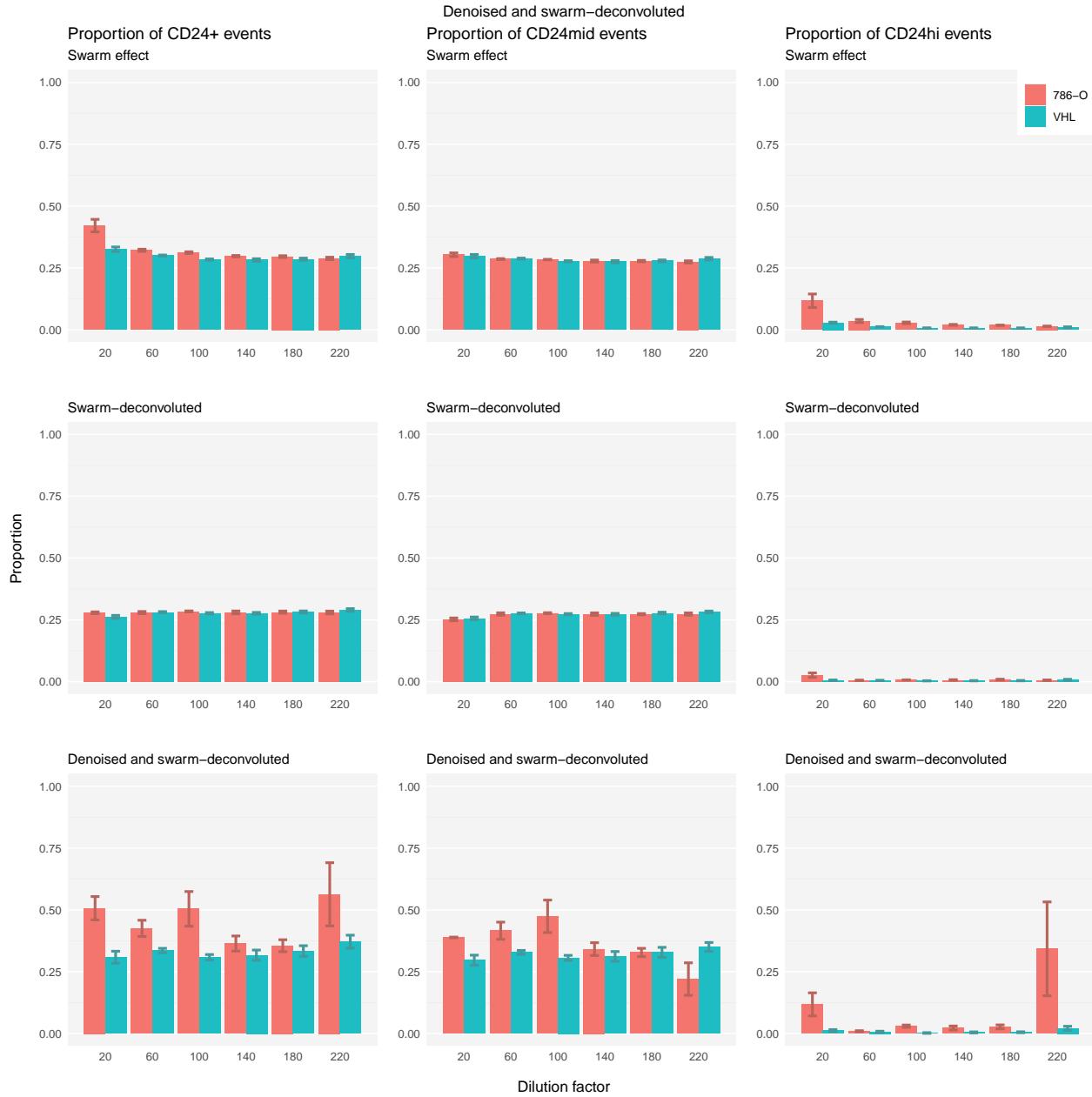
gridExtra::grid.arrange(
  plotA4, plotA5, plotA6,
  plotB4, plotB5, plotB6,
  plotC4, plotC5, plotC6,
  nrow=3,
  top="Denoised and swarm-deconvoluted",
  left="Absolute count",
  bottom="Dilution factor"
)

```



4.3.5.2 Summary graphs

```
gridExtra::grid.arrange(
  plotD1, plotD2, plotD3,
  plotE1, plotE2, plotE3,
  plotF1, plotF2, plotF3,
  nrow=3,
  top="Denoised and swarm-deconvoluted",
  left="Proportion",
  bottom="Dilution factor"
)
```



```

gridExtra::grid.arrange(
  plotD4, plotD5, plotD6,
  plotE4, plotE5, plotE6,
  plotF4, plotF5, plotF6,
  nrow=3,
  top="Denoised and swarm-deconvoluted",
  left="Absolute count",
  bottom="Dilution factor"
)

```



4.3.6 Significance testing

First we must verify that test assumptions are met.

```
# Testing for univariate normality
significanceDfShapiro <- c(
  stats::shapiro.test(dataset3$cd24pos)$p.value,
  stats::shapiro.test(dataset3$cd24mid)$p.value,
  stats::shapiro.test(dataset3$cd24hi)$p.value,
  stats::shapiro.test(dataset3$cd24posProp)$p.value,
  stats::shapiro.test(dataset3$cd24midProp)$p.value,
  stats::shapiro.test(dataset3$cd24hiProp)$p.value
)

# Testing for homoscedasticity
significanceDfHov <- c(
  HH::hov(cd24pos ~ cellLine, data=dataset3)$p.value,
  HH::hov(cd24mid ~ cellLine, data=dataset3)$p.value,
  HH::hov(cd24hi ~ cellLine, data=dataset3)$p.value,
  HH::hov(cd24posProp ~ cellLine, data=dataset3)$p.value,
  HH::hov(cd24midProp ~ cellLine, data=dataset3)$p.value,
  HH::hov(cd24hiProp ~ cellLine, data=dataset3)$p.value
)

significanceDfBartlett <- c(
  stats::bartlett.test(cd24pos ~ cellLine, data=dataset3)$p.value,
  stats::bartlett.test(cd24mid ~ cellLine, data=dataset3)$p.value,
  stats::bartlett.test(cd24hi ~ cellLine, data=dataset3)$p.value,
  stats::bartlett.test(cd24posProp ~ cellLine, data=dataset3)$p.value,
  stats::bartlett.test(cd24midProp ~ cellLine, data=dataset3)$p.value,
  stats::bartlett.test(cd24hiProp ~ cellLine, data=dataset3)$p.value
)

significanceDfFligner <- c(
  stats::fligner.test(cd24pos ~ cellLine, data=dataset3)$p.value,
  stats::fligner.test(cd24mid ~ cellLine, data=dataset3)$p.value,
  stats::fligner.test(cd24hi ~ cellLine, data=dataset3)$p.value,
  stats::fligner.test(cd24posProp ~ cellLine, data=dataset3)$p.value,
  stats::fligner.test(cd24midProp ~ cellLine, data=dataset3)$p.value,
  stats::fligner.test(cd24hiProp ~ cellLine, data=dataset3)$p.value
)
```

```

# Display multiplot in 2 rows and 3 columns
graphics::par(mfrow=c(2, 3))

# Base plots for univariate normality test
stats::qqnorm(dataset3$cd24pos, main="CD24+ absolute count")
stats::qqline(dataset3$cd24pos, col=colorPalette[["green"]])

stats::qqnorm(dataset3$cd24mid, main="CD24mid absolute count")
stats::qqline(dataset3$cd24mid, col=colorPalette[["green"]])

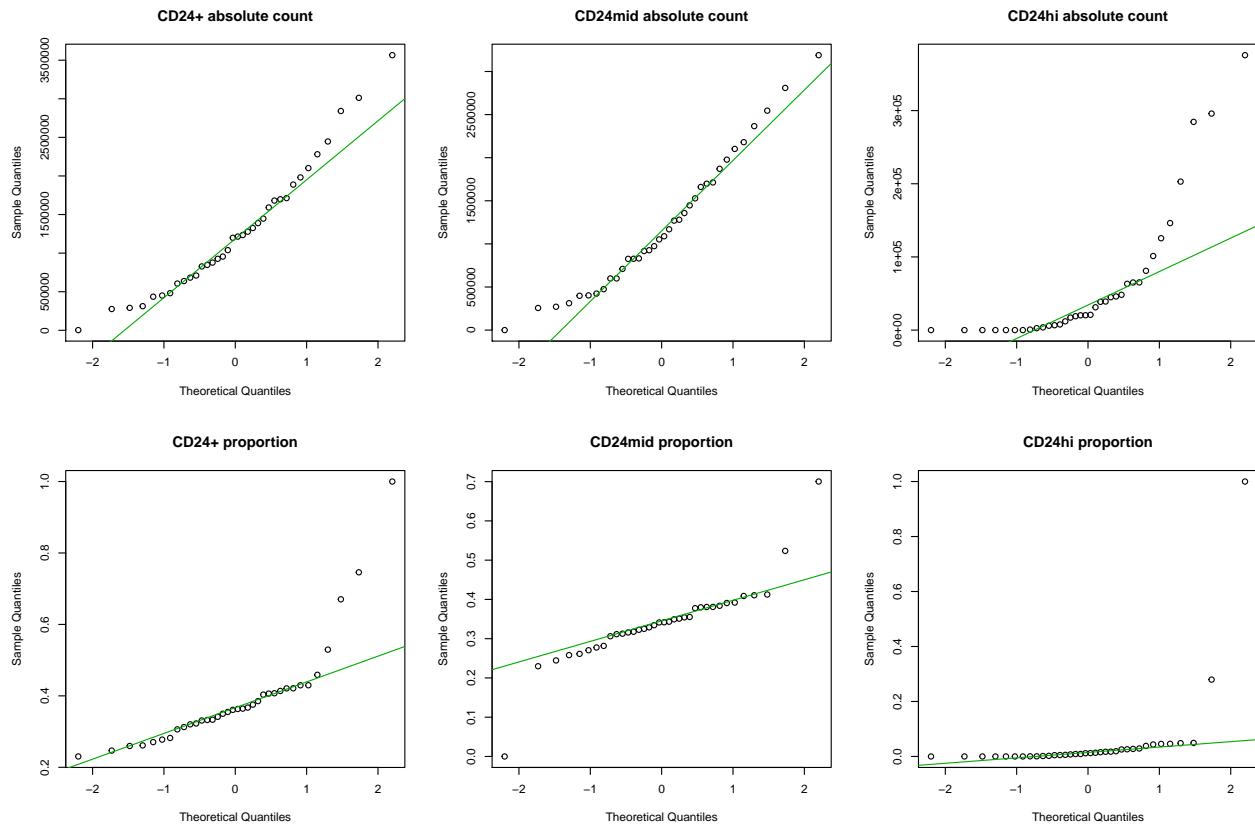
stats::qqnorm(dataset3$cd24hi, main="CD24hi absolute count")
stats::qqline(dataset3$cd24hi, col=colorPalette[["green"]])

stats::qqnorm(dataset3$cd24posProp, main="CD24+ proportion")
stats::qqline(dataset3$cd24posProp, col=colorPalette[["green"]])

stats::qqnorm(dataset3$cd24midProp, main="CD24mid proportion")
stats::qqline(dataset3$cd24midProp, col=colorPalette[["green"]])

stats::qqnorm(dataset3$cd24hiProp, main="CD24hi proportion")
stats::qqline(dataset3$cd24hiProp, col=colorPalette[["green"]])

```

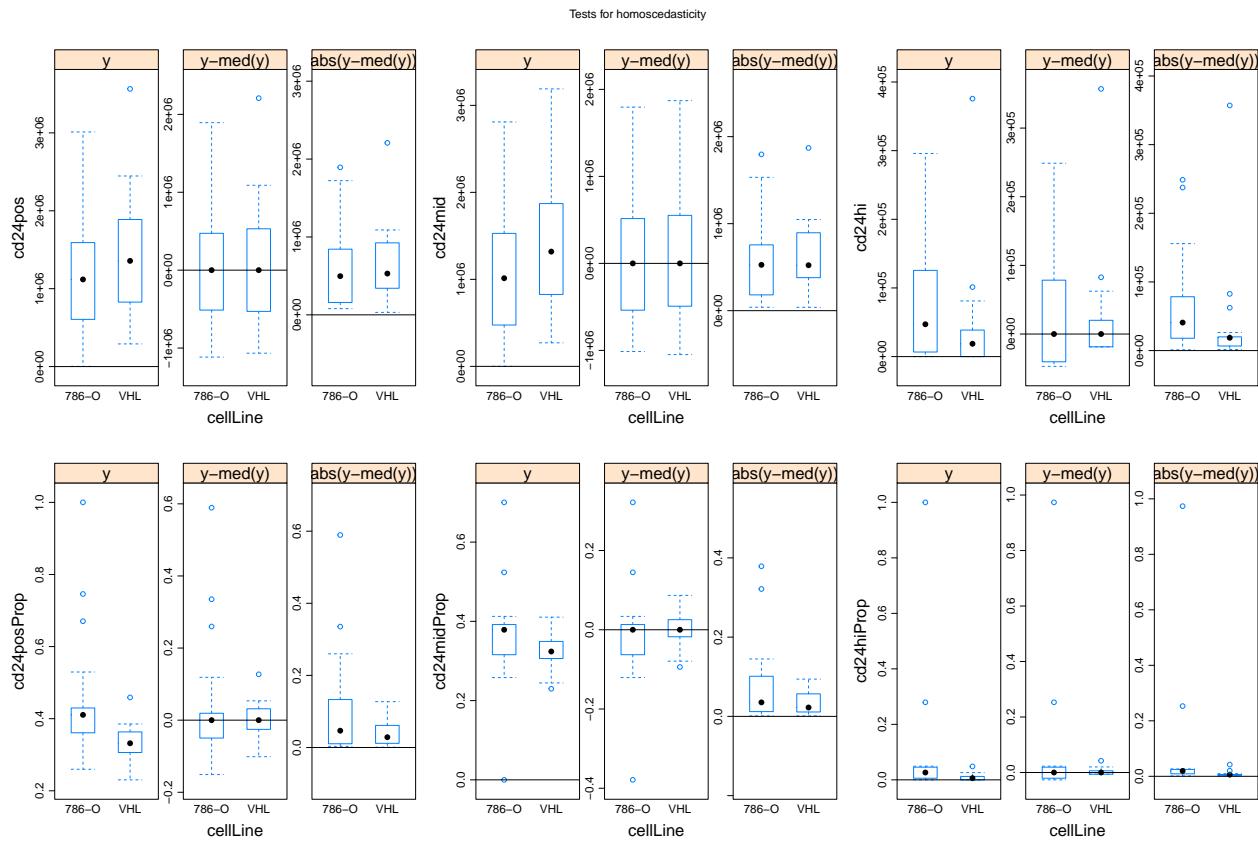


```

# Base plots for homoscedasticity test
cd24posHov      <- HH::hovPlot(cd24pos ~ cellLine, data=dataset3)
cd24midHov       <- HH::hovPlot(cd24mid ~ cellLine, data=dataset3)
cd24hiHov        <- HH::hovPlot(cd24hi ~ cellLine, data=dataset3)
cd24posPropHov   <- HH::hovPlot(cd24posProp ~ cellLine, data=dataset3)
cd24midPropHov   <- HH::hovPlot(cd24midProp ~ cellLine, data=dataset3)
cd24hiPropHov    <- HH::hovPlot(cd24hiProp ~ cellLine, data=dataset3)

gridExtra::grid.arrange(
  cd24posHov,      cd24midHov,      cd24hiHov,
  cd24posPropHov,  cd24midPropHov,  cd24hiPropHov,
  nrow=2,
  top="Tests for homoscedasticity"
)

```



The basic test we are interested in is whether there exists a difference in event counts between the two cell lines. In this case, event counts is the dependant variable, and the cell line is an independant variable. The basic notation for this is (DependantVariable ~ IndependantVariable).

In our experimental model, the independant variable `cellLine` is a fixed variable: it can either be '786-O' or 'VHL' with no possibility for error. Conversely, the dilution factor is not a fixed variable but is rather a random variable which must be taken into account: while a sample may be prepared to be diluted in a 1:120 ratio, there is inherent error or variability in instrument precision while making these measurements. This can be taken into account for the ANOVA with the notation `+ Error(RandomVariable/DependantVariable)`.

```
# Perform an anova on each parameter

cd24posAov <- stats::aov(
  cd24pos ~ cellLine + Error(dilution/cd24pos),
  data=dataset3
)

cd24posPropAov <- stats::aov(
  cd24posProp ~ cellLine + Error(dilution/cd24posProp),
  data=dataset3
)

cd24midAov <- stats::aov(
  cd24mid ~ cellLine + Error(dilution/cd24mid),
  data=dataset3
)

cd24midPropAov <- stats::aov(
  cd24midProp ~ cellLine + Error(dilution/cd24midProp),
  data=dataset3
)

cd24hiAov <- stats::aov(
  cd24hi ~ cellLine + Error(dilution/cd24hi),
  data=dataset3
)

cd24hiPropAov <- stats::aov(
  cd24hiProp ~ cellLine + Error(dilution/cd24hiProp),
  data=dataset3
)

# Get the p-Value for each ANOVA performed
significanceDfAnova <- c(
  getANOVApValue(cd24posAov),
  getANOVApValue(cd24posPropAov),
  getANOVApValue(cd24midAov),
  getANOVApValue(cd24midPropAov),
  getANOVApValue(cd24hiAov),
  getANOVApValue(cd24hiPropAov)
)

# Also perform a non-parametric equivalent of an ANOVA and retrieve the p-Values
significanceDfKruskal <- c(
  stats::kruskal.test(cd24pos ~ cellLine, data=dataset3)$p.value,
```

```

stats::kruskal.test(cd24mid ~ cellLine, data=dataset3)$p.value,
stats::kruskal.test(cd24hi ~ cellLine, data=dataset3)$p.value,
stats::kruskal.test(cd24posProp ~ cellLine, data=dataset3)$p.value,
stats::kruskal.test(cd24midProp ~ cellLine, data=dataset3)$p.value,
stats::kruskal.test(cd24hiProp ~ cellLine, data=dataset3)$p.value
)

# Create a big matrix with the p-Values for all the tests we did
significanceDf <- cbind(
  significanceDfShapiro,
  significanceDfHov,
  significanceDfBartlett,
  significanceDfFligner,
  significanceDfAnova,
  significanceDfKruskal
)

# Ensure rows and columns are named correctly
colnames(significanceDf) <- c(
  "Shapiro-Wilk",
  "Brown-Forsythe",
  "Bartlett",
  "Fligner-Killeen",
  "ANOVA",
  "Kruskal-Wallis"
)

significanceDfParameters <- c(
  "cd24pos",
  "cd24mid",
  "cd24hi",
  "cd24posProp",
  "cd24midProp",
  "cd24hiProp"
)

row.names(significanceDf) <- significanceDfParameters

# Convert the matrix into a dataframe
significanceDf <- data.frame(significanceDf)

# Let's see what we have
printDataFrame(
  dataframe=significanceDf,
  caption="Assumption testing and ANOVA significance testing summary",
  fontsize=7
)

```

Table 19: Assumption testing and ANOVA significance testing summary

	Shapiro.Wilk	Brown.Forsythe	Bartlett	Fligner.Killeen	ANOVA	Kruskal.Wallis
cd24pos	0.0748045	0.7869697	0.8767312	0.7393307	0.6300612	0.3588705
cd24mid	0.1565158	0.7899081	0.9471735	0.7638399	0.0118708	0.3266931
cd24hi	0.0000002	0.3343487	0.7653241	0.1245900	0.3347106	0.0610075
cd24posProp	0.0000010	0.0712290	0.0000082	0.1406199	0.0029515	0.0029392

Table 19: Assumption testing and ANOVA significance testing summary (*continued*)

	Shapiro.Wilk	Brown.Forsythe	Bartlett	Fligner.Killeen	ANOVA	Kruskal.Wallis
cd24midProp	0.0000742	0.0928031	0.0000599	0.1464645	0.0663233	0.0462361
cd24hiProp	0.0000000	0.1845365	0.0000000	0.0057232	0.1424608	0.0031465

```

# Transform this dataframe into a tidy format for plotting purposes
significanceDfMolten <- significanceDf
significanceDfMolten$parameter <- row.names(significanceDf)
significanceDfMolten <- reshape2::melt(
  data=significanceDfMolten,
  id.vars="parameter",
  value.name="p",
  variable.name="test"
)

# Break down p-Value into discrete intervals
significanceDfMolten$pDiscrete <- cut(
  significanceDfMolten$p,
  breaks=c(0.00, 0.01, 0.05, 0.10, 0.15, 0.20, 0.25, 1.00),
  labels=c(
    "p < 0.01",
    "p < 0.05",
    "p < 0.10",
    "p < 0.15",
    "p < 0.20",
    "p < 0.25",
    "p > 0.25"
  )
)

# Make sure the levels is plotted from top to bottom in order
significanceDfMolten$parameter <- factor(
  as.character(significanceDfMolten$parameter),
  levels=rev(significanceDfParameters)
)

```

```

# Let's see what we have
printDataframe(
  dataframe=significanceDfMolten,
  caption=
    "Assumption testing and ANOVA significance testing summary (longform)",
  fontsize=7
)

```

Table 20: Assumption testing and ANOVA significance testing summary (longform)

parameter	test	p	pDiscrete
cd24pos	Shapiro.Wilk	0.0748045	p < 0.10
cd24mid	Shapiro.Wilk	0.1565158	p < 0.20
cd24hi	Shapiro.Wilk	0.0000002	p < 0.01
cd24posProp	Shapiro.Wilk	0.0000010	p < 0.01
cd24midProp	Shapiro.Wilk	0.0000742	p < 0.01
cd24hiProp	Shapiro.Wilk	0.0000000	p < 0.01
cd24pos	Brown.Forsythe	0.7869697	p > 0.25
cd24mid	Brown.Forsythe	0.7899081	p > 0.25
cd24hi	Brown.Forsythe	0.3343487	p > 0.25
cd24posProp	Brown.Forsythe	0.0712290	p < 0.10
cd24midProp	Brown.Forsythe	0.0928031	p < 0.10
cd24hiProp	Brown.Forsythe	0.1845365	p < 0.20
cd24pos	Bartlett	0.8767312	p > 0.25
cd24mid	Bartlett	0.9471735	p > 0.25
cd24hi	Bartlett	0.7653241	p > 0.25
cd24posProp	Bartlett	0.0000082	p < 0.01
cd24midProp	Bartlett	0.0000599	p < 0.01
cd24hiProp	Bartlett	0.0000000	p < 0.01
cd24pos	Fligner.Killeen	0.7393307	p > 0.25
cd24mid	Fligner.Killeen	0.7638399	p > 0.25
cd24hi	Fligner.Killeen	0.1245900	p < 0.15
cd24posProp	Fligner.Killeen	0.1406199	p < 0.15
cd24midProp	Fligner.Killeen	0.1464645	p < 0.15
cd24hiProp	Fligner.Killeen	0.0057232	p < 0.01
cd24pos	ANOVA	0.6300612	p > 0.25
cd24mid	ANOVA	0.0118708	p < 0.05
cd24hi	ANOVA	0.3347106	p > 0.25
cd24posProp	ANOVA	0.0029515	p < 0.01
cd24midProp	ANOVA	0.0663233	p < 0.10
cd24hiProp	ANOVA	0.1424608	p < 0.15
cd24pos	Kruskal.Wallis	0.3588705	p > 0.25
cd24mid	Kruskal.Wallis	0.3266931	p > 0.25
cd24hi	Kruskal.Wallis	0.0610075	p < 0.10
cd24posProp	Kruskal.Wallis	0.0029392	p < 0.01
cd24midProp	Kruskal.Wallis	0.0462361	p < 0.05
cd24hiProp	Kruskal.Wallis	0.0031465	p < 0.01

```

# Create a heat map of p-Values for each parameter
heatmap <- ggplot2::ggplot(
  data=significanceDfMolten,
  mapping=ggplot2::aes(
    x=test,
    y=parameter)
) +
  ggplot2::geom_tile(
    mapping=ggplot2::aes(fill=pDiscrete),
    colour="white",
    size=1) +
  ggplot2::geom_text(
    mapping=ggplot2::aes(label=sprintf("%.2f", round(p, digits=2))),
    colour="white",
    fontface="bold",
    size=3) +
  ggplot2::labs(
    title="Assumption testing and ANOVA significance testing summary",
    subtitle="Swarm-deconvoluted and denoised event counts",
    x="",
    y="") +
  ggplot2::scale_y_discrete(expand=c(0, 0)) +
  ggplot2::scale_x_discrete(
    expand=c(0, 0),
    position="top") +
  ggplot2::scale_fill_manual(
    values=c(
      "#d53e4f",
      "#f46d43",
      "#fd9e61",
      "#fee08b",
      "#e6f598",
      "#abdda4",
      "#ddff1d"),
    na.value="grey90") +
  ggplot2::theme(
    plot.background=ggplot2::element_blank(),
    legend.title=ggplot2::element_blank(),
    axis.text.x=ggplot2::element_text(
      angle=90,
      hjust=0,
      vjust=0.5))

```

A Shapiro-Wilk test statistic > 0.05 indicates that the univariate normality condition is met. While normality is technically a test assumption, in practice the ANOVA is relatively robust to departures from normality.

A Brown-Forsythe test statistic > 0.05 indicates that the condition for homoscedasticity is met. This test is better suited to minor departures from normality.

A Bartlett test statistic > 0.05 indicates that the condition for homoscedasticity is met. This test is very sensitive to minor departures from normality.

A Fligner-Killeen test statistic > 0.05 indicates that the condition for homoscedasticity is met. This test is less sensitive to departures from normality or to the influence of outliers. Failing other tests but meeting the Fligner-Killeen test could justify the downstream use of nonparametric methods (Such as the Kruskal-Wallis rank sum test instead of the ANOVA).

```
# Visualization
```

```
heatmap
```

Assumption testing and ANOVA significance testii

Swarm-deconvoluted and denoised event counts



The results of significance testing for our data appear to indicate a statistically significant difference between 786-O and VHL cell-derived extracellular vesicles for the absolute count of cd24mid events, as well as for the overall proportion of CD24+ events and the overall proportion of cd24mid events.

```
datasetNormalizedForLm <- normalizedExperiment

datasetNormalizedForLm <- datasetNormalizedForLm %>%
  dplyr::mutate_at(
    c("events", "cd24pos", "cd24mid", "cd24hi", "dilution"),
    ~ (scale(.)) %>% as.vector
  )

lmFitBase <- stats::lm
```

```

cd24mid ~ cellLine,
  data=datasetNormalizedForLm
)

lmFitDilution <- stats::lm(
  cd24mid ~ cellLine + dilution,
  data=datasetNormalizedForLm
)

lmFitSwarmDilution <- stats::lm(
  cd24mid ~ cellLine + cd24midTp + dilution,
  data=datasetNormalizedForLm
)

afBase <- stats::anova(lmFitBase)
afBase$percentVariance <- (afBase$"Sum Sq" / sum(afBase$"Sum Sq")) * 100

afBase

## Analysis of Variance Table
##
## Response: cd24mid
##           Df Sum Sq Mean Sq F value Pr(>F) percentVariance
## cellLine    1  0.012  0.01217  0.0118  0.91405          0.035
## Residuals 34 34.988  1.02905                   99.965

afDilution <- stats::anova(lmFitDilution)
afDilution$percentVariance <-
  (afDilution$"Sum Sq" / sum(afDilution$"Sum Sq")) * 100

afDilution

## Analysis of Variance Table
##
## Response: cd24mid
##           Df Sum Sq Mean Sq   F value Pr(>F) percentVariance
## cellLine    1  0.012   0.012    1.1806  0.28511          0.035
## dilution    1 34.648  34.648 3361.8541  0.00000         98.994
## Residuals 33  0.340   0.010                   0.972

afSwarmDilution <- stats::anova(lmFitSwarmDilution)
afSwarmDilution$percentVariance <-
  (afSwarmDilution$"Sum Sq" / sum(afSwarmDilution$"Sum Sq")) * 100

afSwarmDilution

## Analysis of Variance Table
##
## Response: cd24mid
##           Df Sum Sq Mean Sq   F value Pr(>F) percentVariance
## cellLine    1  0.0122  0.0122   1.1527  0.29101          0.035
## cd24midTp  1 16.4313 16.4313 1556.7342  0.00000         46.947
## dilution    1 18.2188 18.2188 1726.0820  0.00000         52.054
## Residuals 32  0.3378  0.0106                   0.965

```

```

decreaseInVarianceFromDilution <- 1 - (
  afSwarmDilution[["dilution", "percentVariance"]]
  / afDilution[["dilution", "percentVariance"]])

decreaseInVarianceFromDilution

## [1] 0.4741714

datasetNormalizedForLm <- normalizedExperiment

datasetNormalizedForLm <- datasetNormalizedForLm %>%
  dplyr::mutate_at(
    c("events", "cd24pos", "cd24mid", "cd24hi", "dilution"),
    ~ (scale(.) %>% as.vector)
  )

lmFitBase <- stats::lm(
  cd24hi ~ cellLine,
  data=datasetNormalizedForLm
)

lmFitDilution <- stats::lm(
  cd24hi ~ cellLine + dilution,
  data=datasetNormalizedForLm
)

lmFitSwarmDilution <- stats::lm(
  cd24hi ~ cellLine + cd24hiTp + dilution,
  data=datasetNormalizedForLm
)

afBase <- stats::anova(lmFitBase)
afBase$percentVariance <- (afBase$"Sum Sq" / sum(afBase$"Sum Sq")) * 100

afBase

## Analysis of Variance Table
##
## Response: cd24hi
##           Df Sum Sq Mean Sq F value    Pr(>F) percentVariance
## cellLine     1 17.046 17.0461 32.281 2.2329e-06      48.703
## Residuals 34 17.954  0.5281                   51.297

afDilution <- stats::anova(lmFitDilution)
afDilution$percentVariance <-
  (afDilution$"Sum Sq" / sum(afDilution$"Sum Sq")) * 100

afDilution

## Analysis of Variance Table
##
## Response: cd24hi
##           Df Sum Sq Mean Sq F value    Pr(>F) percentVariance
## cellLine     1 17.046 17.0461 32.9770 0.000002      48.703
## dilution     1  0.896  0.8960  1.7334 0.197050      2.560
## Residuals 33 17.058  0.5169                   48.737

```

```

afSwarmDilution <- stats::anova(lmFitSwarmDilution)
afSwarmDilution$percentVariance <-
  (afSwarmDilution$"Sum Sq" / sum(afSwarmDilution$"Sum Sq")) * 100

afSwarmDilution

## Analysis of Variance Table
##
## Response: cd24hi
##           Df Sum Sq Mean Sq F value Pr(>F) percentVariance
## cellLine    1 17.0461 17.0461 33.8210 0.00000          48.703
## cd24hiTp   1  1.8256  1.8256  3.6222 0.06604          5.216
## dilution   1  0.0001  0.0001  0.0001 0.99081          0.000
## Residuals 32 16.1283  0.5040                      46.081

decreaseInVarianceFromDilution <- 1 - (
  afSwarmDilution["dilution", "percentVariance"]
  / afDilution["dilution", "percentVariance"])

decreaseInVarianceFromDilution

## [1] 0.9999242

datasetNormalizedForLm <- normalizedExperiment

datasetNormalizedForLm <- datasetNormalizedForLm %>%
  dplyr::mutate_at(
    c("events", "cd24pos", "cd24mid", "cd24hi", "dilution"),
    ~ (scale(.) %>% as.vector)
  )

lmFitBase <- stats::lm(
  cd24pos ~ cellLine,
  data=datasetNormalizedForLm
)

lmFitDilution <- stats::lm(
  cd24pos ~ cellLine + dilution,
  data=datasetNormalizedForLm
)

lmFitSwarmDilution <- stats::lm(
  cd24pos ~ cellLine + cd24posTp + dilution,
  data=datasetNormalizedForLm
)

afBase <- stats::anova(lmFitBase)
afBase$percentVariance <- (afBase$"Sum Sq" / sum(afBase$"Sum Sq")) * 100

afBase

## Analysis of Variance Table
##
## Response: cd24pos
##           Df Sum Sq Mean Sq F value Pr(>F) percentVariance
## cellLine    1  0.039  0.03852  0.0375 0.84768          0.11

```

```

## Residuals 34 34.961 1.02828                               99.89
afDilution <- stats::anova(lmFitDilution)
afDilution$percentVariance <-
  (afDilution$"Sum Sq" / sum(afDilution$"Sum Sq")) * 100

afDilution

## Analysis of Variance Table
##
## Response: cd24pos
##           Df Sum Sq Mean Sq   F value   Pr(>F) percentVariance
## cellLine    1  0.039  0.039    2.3744  0.13288          0.11
## dilution    1 34.426 34.426 2121.9408 0.00000          98.36
## Residuals 33  0.535  0.016                               1.53

afSwarmDilution <- stats::anova(lmFitSwarmDilution)
afSwarmDilution$percentVariance <-
  (afSwarmDilution$"Sum Sq" / sum(afSwarmDilution$"Sum Sq")) * 100

afSwarmDilution

## Analysis of Variance Table
##
## Response: cd24pos
##           Df Sum Sq Mean Sq   F value   Pr(>F) percentVariance
## cellLine    1  0.0385  0.0385   2.6202  0.11533          0.110
## cd24posTp  1 17.3953 17.3953 1183.2155 0.00000          49.701
## dilution    1 17.0957 17.0957 1162.8418 0.00000          48.845
## Residuals 32  0.4705  0.0147                               1.344

decreaseInVarianceFromDilution <- 1 - (
  afSwarmDilution["dilution", "percentVariance"]
  / afDilution["dilution", "percentVariance"])

decreaseInVarianceFromDilution

## [1] 0.5034072

type3Anova <- car::Anova(
  stats::lm(
    cd24mid ~ cellLine + cd24midTp + dilution,
    data=datasetNormalizedForLm,
  ),
  type=3
)

type3Anova$percentVariance <-
  (type3Anova$"Sum Sq" / sum(type3Anova$"Sum Sq")) * 100

type3Anova

## Anova Table (Type III tests)
##
## Response: cd24mid
##           Sum Sq Df   F value   Pr(>F) percentVariance
## (Intercept) 0.0019  1    0.1803  0.67393          0.010
## cellLine     0.0131  1    1.2429  0.27321          0.071

```

```
## cd24midTp  0.0023  1   0.2220 0.64073      0.013
## dilution  18.2188  1 1726.0820 0.00000      98.088
## Residuals  0.3378 32                           1.818
```

```

type3Anova <- car::Anova(
  stats::lm(
    cd24hi ~ cellLine + cd24hiTp + dilution,
    data=datasetNormalizedForLm,
  ),
  type=3
)

type3Anova$percentVariance <-
  (type3Anova$"Sum Sq" / sum(type3Anova$"Sum Sq")) * 100

type3Anova

## Anova Table (Type III tests)
##
## Response: cd24hi
##             Sum Sq Df F value Pr(>F) percentVariance
## (Intercept) 0.4395  1 0.8719 0.35741      1.324
## cellLine    15.6897  1 31.1299 0.00000     47.276
## cd24hiTp    0.9297  1  1.8446 0.18392      2.801
## dilution    0.0001  1  0.0001 0.99081      0.000
## Residuals   16.1283 32                      48.598

type3Anova <- car::Anova(
  stats::lm(
    cd24pos ~ cellLine + cd24posTp + dilution,
    data=datasetNormalizedForLm,
  ),
  type=3
)

type3Anova$percentVariance <-
  (type3Anova$"Sum Sq" / sum(type3Anova$"Sum Sq")) * 100

type3Anova

## Anova Table (Type III tests)
##
## Response: cd24pos
##             Sum Sq Df  F value Pr(>F) percentVariance
## (Intercept) 0.0713  1   4.8513 0.03495      0.403
## cellLine    0.0047  1   0.3208 0.57510      0.027
## cd24posTp   0.0649  1   4.4167 0.04355      0.367
## dilution    17.0957  1 1162.8418 0.00000     96.547
## Residuals   0.4705 32                      2.657

```

5 References