QCN

Contents

Quality control and normalization

```
input_dataPath <- "../scClustViz_files/neurons_900_filtered_gene_bc_matrices/filtered_gene_bc_matrices/m
##  Path to 10X output directory (containing .mtx file)
##  i.e. http://cf.10xgenomics.com/samples/cell-exp/2.1.0/neurons_900/neurons_900_filtered_gene_bc_matri
dataPath <- "../scClustViz_files/demo_10Xneurons900/"
##  Path to analysis output directory
##  Will be created if it doesn't already exist
dataSpecies <- "mouse"
##  Set species ("mouse"/"human" or add your own - see below)
dataName <- "10Xneurons"
##  Name your analysis results


library(Matrix)
library(scales)
library(viridis)
```

```
## Loading required package: viridisLite
```

```
##
## Attaching package: 'viridis'
```

```
## The following object is masked from 'package:scales':
##
##     viridis_pal
```

```
library(RColorBrewer)
library(biomaRt)      # from Bioconductor
library(scran)        # from Bioconductor
```

```
## Loading required package: BiocParallel
```

```
## Loading required package: SingleCellExperiment
```

```
## Loading required package: SummarizedExperiment
```

```
## Loading required package: GenomicRanges

## Loading required package: stats4

## Loading required package: BiocGenerics

## Loading required package: parallel

##
## Attaching package: 'BiocGenerics'

## The following objects are masked from 'package:parallel':
##
##     clusterApply, clusterApplyLB, clusterCall, clusterEvalQ,
##     clusterExport, clusterMap, parApply, parCapply, parLapply,
##     parLapplyLB, parRapply, parSapply, parSapplyLB

## The following objects are masked from 'package:Matrix':
##
##     colMeans, colSums, rowMeans, rowSums, which

## The following objects are masked from 'package:stats':
##
##     IQR, mad, sd, var, xtabs

## The following objects are masked from 'package:base':
##
##     anyDuplicated, append, as.data.frame, basename, cbind,
##     colMeans, colnames, colSums, dirname, do.call, duplicated,
##     eval, evalq, Filter, Find, get, grep, grepl, intersect,
##     is.unsorted, lapply, lengths, Map, mapply, match, mget, order,
##     paste, pmax, pmax.int, pmin, pmin.int, Position, rank, rbind,
##     Reduce, rowMeans, rownames, rowSums, sapply, setdiff, sort,
##     table, tapply, union, unique, unsplit, which, which.max,
##     which.min

## Loading required package: S4Vectors

##
## Attaching package: 'S4Vectors'

## The following object is masked from 'package:Matrix':
##
##     expand

## The following object is masked from 'package:base':
##
##     expand.grid

## Loading required package: IRanges

##
## Attaching package: 'IRanges'

## The following object is masked from 'package:grDevices':
##
##     windows

## Loading required package: GenomeInfoDb

## Loading required package: Biobase
```

```
## Welcome to Bioconductor
##
##     Vignettes contain introductory material; view with
##     'browseVignettes()'. To cite Bioconductor, see
##     'citation("Biobase")', and for packages 'citation("pkgname")'.

## Loading required package: DelayedArray

## Loading required package: matrixStats

##
## Attaching package: 'matrixStats'

## The following objects are masked from 'package:Biobase':
##
##     anyMissing, rowMedians

##
## Attaching package: 'DelayedArray'

## The following objects are masked from 'package:matrixStats':
##
##     colMaxs, colMins, colRanges, rowMaxs, rowMins, rowRanges

## The following objects are masked from 'package:base':
##
##     aperm, apply
```

```
library(DropletUtils) # from Bioconductor


if (Sys.info()["sysname"] == "Windows") { sys <- "D:/" } else { sys <- "~/" }
if (dataSpecies == "mouse") {
  mart <- useMart("ensembl","mmusculus_gene_ensembl")
  speciesSymbol <- "mgi_symbol"
  speciesMito <- "^mt-"
  cycloneSpeciesMarkers <- "mouse_cycle_markers.rds"
} else if (dataSpecies == "human") {
  mart <- useMart("ensembl","hsapiens_gene_ensembl")
  speciesSymbol <- "hgnc_symbol"
  speciesMito <- "^MT-"
  cycloneSpeciesMarkers <- "human_cycle_markers.rds"
} else { print("Set species please!") }
dir.create(dataPath,recursive=T,showWarnings=F)

plotHistHoriz <- function(input,col="grey80",add=F) {
  tempH <- hist(input,breaks=50,plot=F)
  if (!add) {
    plot(x=NULL,y=NULL,xlim=range(tempH$counts),ylim=range(input),
         bty="n",ylab=NA,yaxt="n",xaxt="n",xlab=NA)
  }
  rect(xleft=rep(0,length(tempH$counts)),ybottom=tempH$breaks[-length(tempH$breaks)],
       xright=tempH$counts,ytop=tempH$breaks[-1],col=col)
}

rainbow2 <- function(n,a=1) {
  require(scales)
  hues = seq(15, 375, length = n + 1)
```

```
  alpha(hcl(h = hues, l = 60, c = 100)[1:n],a)
}
```

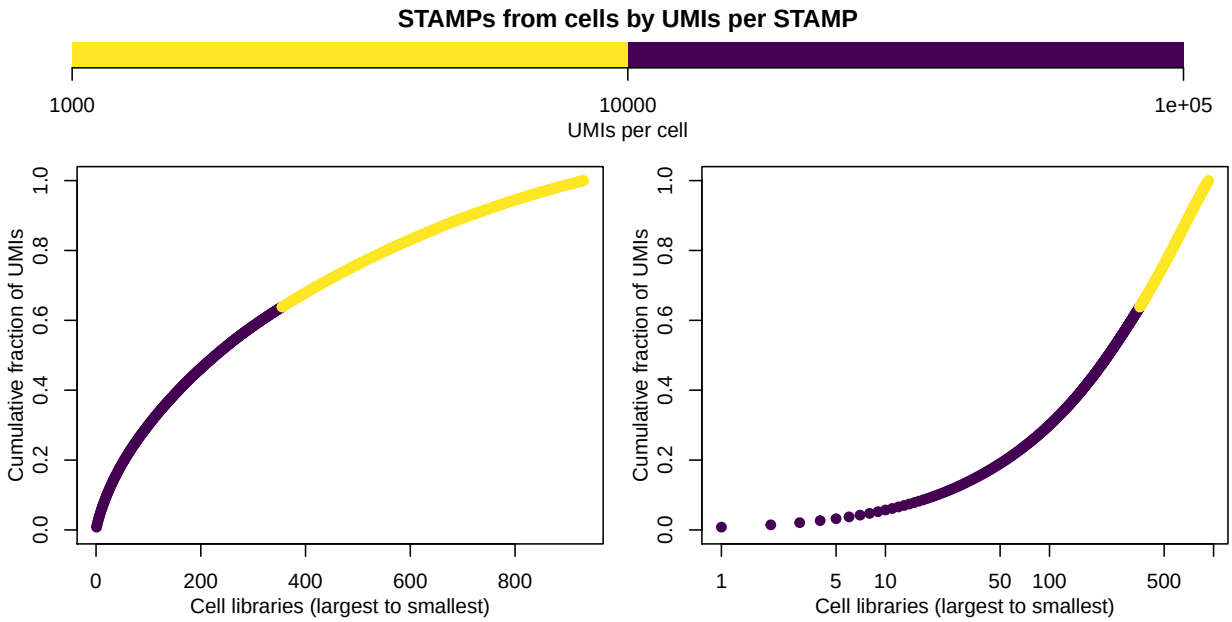
Cell filtering

```
ebRaw <- counts(read10xCounts(input_dataPath,col.names=T))
ebRaw <- ebRaw[,Matrix::colSums(ebRaw) > 0]
ebRaw <- ebRaw[Matrix::rowSums(ebRaw) > 0,]

##  Check rownames at this point.  This workflow expects MGI or HGNC gene symbols as the
##  rownames.  If the data was aligned to a reference genome that used ensembl gene IDs or
##  another annotation you will have to either convert them to gene symbols, or identify
##  which ensembl IDs correspond to the mitochondrial genome for the mitoFilt step.

geneRowNames <- "ensembl_gene_id" # Set if rownames are ensemble gene IDs
#geneRowNames <- speciesSymbol   # Set if rownames are gene symbols (no conversion).

####

if (geneRowNames != speciesSymbol) {
  e2g <- getBM(attributes=c(geneRowNames,speciesSymbol),
               mart=mart,filters=geneRowNames,
               values=rownames(ebRaw))
  if (nrow(e2g) < 10) {
    stop("Check row names and select appropriate rowname identifier!")
  }
  ##  Removing unmapped gene symbols from conversion table
  e2g <- e2g[e2g[,speciesSymbol] != "",]
  print(paste(sum(duplicated(e2g[,geneRowNames])),
              geneRowNames,"mapped to multiple",speciesSymbol))
  ##  Arbitrarily picking one mapping for the above duplicates,
  ##  since these generally map to predicted genes anyway.
  e2g <- e2g[!duplicated(e2g[,geneRowNames]),]
  rownames(e2g) <- e2g[,geneRowNames]
  ebRaw <- ebRaw[e2g[,geneRowNames],] # removing unmapped genes from data
  temp_repName <- unique(e2g[,speciesSymbol][duplicated(e2g[,speciesSymbol])])
  print(paste(length(temp_repName),speciesSymbol,"mapped to multiple",geneRowNames))
  ##  Going to collapse these by summing UMI counts between duplicated rows.
  temp_repRow <- ebRaw[e2g[,speciesSymbol] %in% temp_repName,]
  ebRaw <- ebRaw[!e2g[,speciesSymbol] %in% temp_repName,]
  ##  Removed duplicated rows from data, saved as separate object
  rownames(ebRaw) <- e2g[rownames(ebRaw),speciesSymbol] # renamed rows in data as symbols
  temp_repRow <- Matrix(t(sapply(temp_repName,function(X)
    Matrix::colSums(temp_repRow[e2g[,geneRowNames][e2g[,speciesSymbol] == X],]))),sparse=T)
  ##  Collapsed by summing each duplicated gene symbol's row
  ebRaw <- rbind(ebRaw,temp_repRow)  # added those data back to matrix
}
```

```
## [1] "0 ensembl_gene_id mapped to multiple mgi_symbol"
## [1] "12 mgi_symbol mapped to multiple ensembl_gene_id"
```

```
##  Consolidating duplicated gene names
##  (if genes are in under their MGI and HGNC symbols, or some other annotation mixup)
if (any(duplicated(toupper(rownames(ebRaw))))) {
  temp_repName <- unique(toupper(rownames(ebRaw)[duplicated(toupper(rownames(ebRaw)))]))
  print(paste(dataName,"-",length(temp_repName),"duplicated gene names."))
  ##  Going to collapse these by summing UMI counts between duplicated rows.
  temp_repRow <- ebRaw[toupper(rownames(ebRaw)) %in% temp_repName,]
  ebRaw <- ebRaw[!toupper(rownames(ebRaw)) %in% temp_repName,]
  if (!exists("e2g")) {
    e2g <- getBM(attributes=speciesSymbol,
                 mart=mart,filters=speciesSymbol,
                 values=rownames(temp_repRow))
  }
  rownames(e2g) <- toupper(e2g$mgi_symbol)
  if (any(!temp_repName %in% rownames(e2g))) {
    warning(paste("Some of your duplicated rownames aren't",
                  speciesSymbol,"and are being removed."))
    temp_repName <- temp_repName[temp_repName %in% rownames(e2g)]
  }
  temp_repRow <- Matrix(t(sapply(temp_repName,function(X)
    Matrix::colSums(temp_repRow[toupper(rownames(temp_repRow)) == X,]))),sparse=T)
  rownames(temp_repRow) <- e2g[rownames(temp_repRow),"mgi_symbol"]
  ebRaw <- rbind(ebRaw,temp_repRow)  # added those data back to matrix
  rm(temp_repName,temp_repRow)
}
rm(list=ls()[grepl("temp",ls())])
```

```
libSize <- Matrix::colSums(ebRaw)
cumCounts <- cumsum(libSize[order(libSize,decreasing=T)])
maxCount <- 10^ceiling(log10(max(libSize)))
countCols <- cut(log10(libSize[order(libSize,decreasing=T)]),
                 breaks=floor(log10(min(libSize))):ceiling(log10(max(libSize))),
                 labels=F,include.lowest=T)
layout(matrix(c(3,3,1:2),nrow=2,byrow=T),heights=c(1,3.2))
par(mar=c(3,3,1,1),mgp=2:0)
plot(seq_along(cumCounts),cumCounts/max(cumCounts),pch=19,ylim=c(0,1),
     col=viridis(max(countCols),d=-1)[countCols],
     xlab="Cell libraries (largest to smallest)",ylab="Cumulative fraction of UMIs")
plot(seq_along(cumCounts),cumCounts/max(cumCounts),pch=19,log="x",ylim=c(0,1),
     col=viridis(max(countCols),d=-1)[countCols],
     xlab="Cell libraries (largest to smallest)",ylab="Cumulative fraction of UMIs")
par(mar=c(3,1,2,1))
barplot(rep(1,max(countCols)),col=viridis(max(countCols),d=-1),
        space=0,border=NA,axes=F,xlab="UMIs per cell")
axis(side=1,at=0:max(countCols),
     labels=sapply(floor(log10(min(libSize))):ceiling(log10(max(libSize))),
                   function(X) 10^X))
title(main="STAMPs from cells by UMIs per STAMP")
```



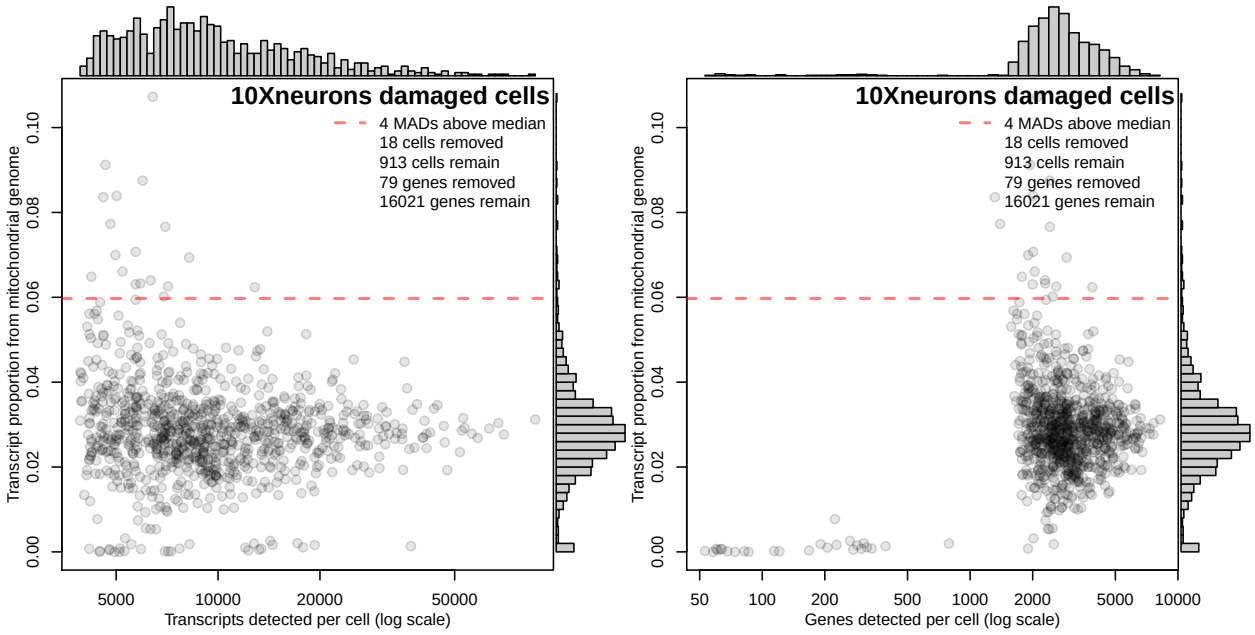
This is to check the cell filtering done in CellRanger. If you have cells with small library sizes, or a fairly horizontal line in the knee plot, you might consider revisiting the filtering for "cells" vs empty droplets.

Mitochondrial gene content

Filtering cells based on the proportion of mitochondrial gene transcripts per cell. A high proportion of mitochondrial gene transcripts are indicative of poor quality cells, probably due to compromised cell membranes. Removal of these cells should not decrease the complexity of the overall dataName (measured by number of genes detected), while removing a source of noise.

```
### Parameters you could edit ###
drop_mitoMads <- 4
##  ^ Median absolute deviations from the median to use as
##  threshold for mitochondrial transcript proprotion.
hard_mitoCut <- 0.4
# ^ Hard threshold for mitochondrial transcript proportion

### Calculations and filtering ###
temp_geneDetectFx <- function(ebRaw) {
  if (is.null(slotNames(ebRaw))) {
    apply(ebRaw,2,function(X) sum(X>0))
  } else if ("j" %in% slotNames(ebRaw)) {
    as.vector(table(ebRaw@j))
  } else {
    as.vector(table(rep(seq_along(diff(ebRaw@p)),diff(ebRaw@p))))
  }
}
cellStats <- data.frame(
  libSize=Matrix::colSums(ebRaw),
  geneDetect=temp_geneDetectFx(ebRaw),
  mitoPct=Matrix::colSums(ebRaw[grepl(speciesMito,rownames(ebRaw)),]) /
    Matrix::colSums(ebRaw)
)
```

```
drop_mitoCut <- median(cellStats$mitoPct) + mad(cellStats$mitoPct) * drop_mitoMads
if (drop_mitoCut > hard_mitoCut) { drop_mitoCut <- hard_mitoCut }
drop_mito <- cellStats$mitoPct > drop_mitoCut

ebRawF <- ebRaw[,!drop_mito]
ebRawF <- ebRawF[Matrix::rowSums(ebRawF) > 0,]
cellStatsF <- cellStats[!drop_mito,]

### Plotting ###
layout(matrix(c(2,1,0,3,5,4,0,6),2),c(3.7,.5,3.7,.5),c(0.5,3.7))
par(mar=c(3,3,0,0),mgp=2:0)
plot(mitoPct~libSize,data=cellStats,log="x",
     pch=21,cex=1.2,col=alpha("black",0.2),bg=alpha("black",0.1),
     xlab="Transcripts detected per cell (log scale)",
     ylab="Transcript proportion from mitochondrial genome")
abline(h=drop_mitoCut,lwd=2,lty=2,col=alpha("red",0.5))
mtext(paste(dataName,"damaged cells"),side=3,adj=0.98,line=-1.5,font=2,cex=1)
legend("topright",bty="n",inset=c(0,.05),
       lty=c(2,NA,NA,NA,NA),lwd=c(2,NA,NA,NA,NA),col=alpha(c("red",NA,NA,NA,NA),0.5),
       legend=c(paste(drop_mitoMads,"MADs above median"),
                paste(sum(drop_mito),"cells removed"),
                paste(ncol(ebRawF),"cells remain"),
                paste(nrow(ebRaw)-nrow(ebRawF),"genes removed"),
                paste(nrow(ebRawF),"genes remain")))
par(mar=c(0,3,0,0))
hist(log10(cellStats$libSize),freq=F,breaks=50,col="grey80",
     main=NULL,xlab=NA,ylab=NA,xaxt="n",yaxt="n")
par(mar=c(3,0,0,0))
plotHistHoriz(cellStats$mitoPct)

par(mar=c(3,3,0,0))
plot(mitoPct~geneDetect,data=cellStats,log="x",
     pch=21,cex=1.2,col=alpha("black",0.2),bg=alpha("black",0.1),
     xlab="Genes detected per cell (log scale)",
     ylab="Transcript proportion from mitochondrial genome")
abline(h=drop_mitoCut,lwd=2,lty=2,col=alpha("red",0.5))
mtext(paste(dataName,"damaged cells"),side=3,adj=0.98,line=-1.5,font=2,cex=1)
legend("topright",bty="n",inset=c(0,.05),
       lty=c(2,NA,NA,NA,NA),lwd=c(2,NA,NA,NA,NA),col=alpha(c("red",NA,NA,NA,NA),0.5),
       legend=c(paste(drop_mitoMads,"MADs above median"),
                paste(sum(drop_mito),"cells removed"),
                paste(ncol(ebRawF),"cells remain"),
                paste(nrow(ebRaw)-nrow(ebRawF),"genes removed"),
                paste(nrow(ebRawF),"genes remain")))
par(mar=c(0,3,0,0))
hist(log10(cellStats$geneDetect),freq=F,breaks=50,col="grey80",
     main=NULL,xlab=NA,ylab=NA,xaxt="n",yaxt="n")
par(mar=c(3,0,0,0))
plotHistHoriz(cellStats$mitoPct)
```



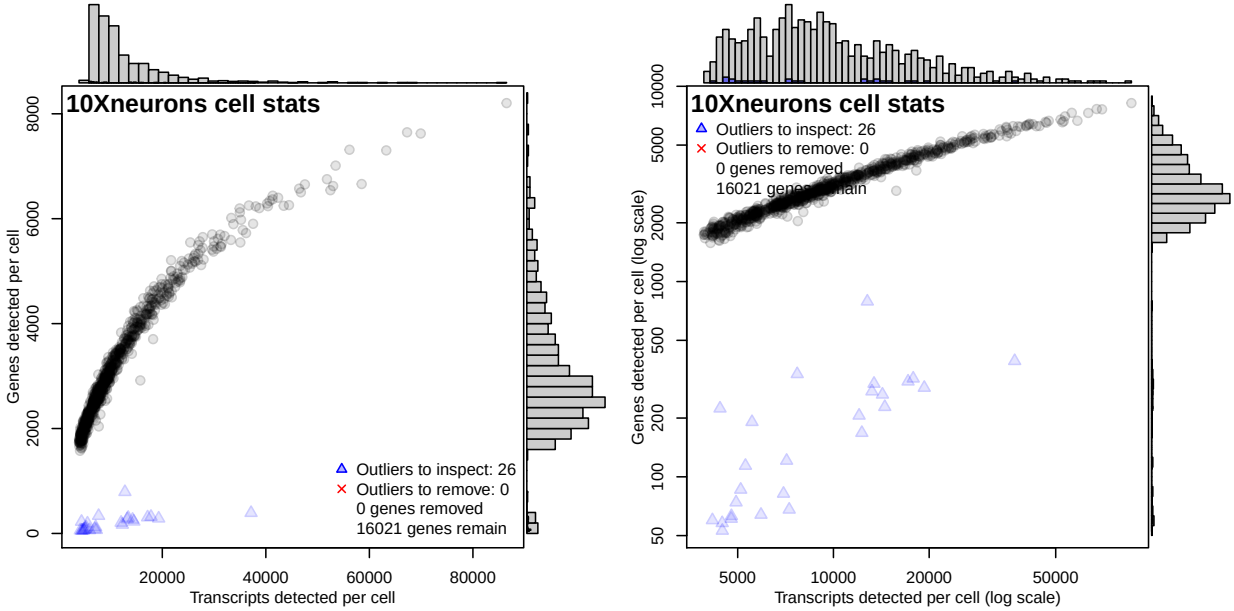
Outlier filtering

Doublet rate is best controlled experimentally, by reducing the concentration of the cells in the input suspension. Filtering for doublets by library size doesn't really work, since the dispersion of library sizes means that doublets containing two "small" cells will still have a smaller library size than one "large" cell. However, it is important to manually inspect the relationship between library size and gene detection rates per cell to identify obvious outliers. Outliers can be identified systematically using a sufficiently extreme number of median absolute deviations from the median, assuming a moderately normal distribution. Since library sizes tend to be log-normal, we use log-transformed library size to identify outliers.

```
### Parameters you could edit ###
#outToInspect <- rep(F,nrow(cellStatsF)) # None
outToInspect <- with(cellStatsF,geneDetect < (libSize * 0.08 + 50))
##  Defining a line below which the curious cells lie

# outToRemove <- rep(F,nrow(cellStatsF)) # None
numMADs <- 4 # Median absolute deviations from the median to consider as an outlier.
outToRemoveHi <- log10(cellStatsF$libSize) > median(log10(cellStatsF$libSize)) +
  mad(log10(cellStats$libSize)) * numMADs
outToRemoveLo <- log10(cellStatsF$libSize) < median(log10(cellStatsF$libSize)) -
  mad(log10(cellStats$libSize)) * numMADs
outToRemove <- outToRemoveHi | outToRemoveLo
##  Assuming an approximately log-normal distribution of library sizes, this removes
##  obvious outliers. (4 MADs from the median of the log-transformed library size)


### Calculations and filtering ###
ebRawF2 <- ebRawF[,!outToRemove]
ebRawF2 <- ebRawF2[Matrix::rowSums(ebRawF2) > 0,]

### Plotting ###
layout(cbind(matrix(c(2,1,0,3),2),matrix(c(5,4,0,6),2)),
       widths=c(3.5,.7,3.5,.7),heights=c(.7,3.5))
```

```
par(mar=c(3,3,0,0),mgp=2:0)
plot(geneDetect~libSize,data=cellStatsF[!outToInspect,],
     xlim=range(cellStatsF$libSize),ylim=range(cellStatsF$geneDetect),
     pch=21,col=alpha("black",0.2),bg=alpha("black",0.1),cex=1.2,
     xlab="Transcripts detected per cell",ylab="Genes detected per cell")
points(geneDetect~libSize,data=cellStatsF[outToInspect,],
       pch=24,col=alpha("blue",0.2),bg=alpha("blue",0.1),cex=1.2)
points(geneDetect~libSize,data=cellStatsF[outToRemove,],
       pch=4,cex=1.2,col="red")
mtext(paste(dataName,"cell stats"),side=3,adj=0.02,line=-1.5,font=2,cex=1)
legend("bottomright",bty="n",pch=c(24,4,NA,NA),
       col=c("blue","red",NA,NA),pt.bg=alpha(c("blue",NA,NA,NA),0.3),
       legend=c(paste("Outliers to inspect:",sum(outToInspect)),
                paste("Outliers to remove:",sum(outToRemove)),
                paste(nrow(ebRawF)-nrow(ebRawF2),"genes removed"),
                paste(nrow(ebRawF2),"genes remain")))
par(mar=c(0,3,1,0))
hist(cellStatsF$libSize[!outToInspect | !outToRemove],
     freq=T,breaks=50,col="grey80",main=NULL,xlab=NA,ylab=NA,xaxt="n",yaxt="n")
if (any(outToInspect)) {
  hist(cellStatsF$libSize[outToInspect],add=T,
       freq=T,breaks=50,col=alpha("blue",.5),main=NULL,xlab=NA,ylab=NA,xaxt="n",yaxt="n")
}
if (any(outToRemove)) {
  hist(cellStatsF$libSize[outToRemove],add=T,
       freq=T,breaks=50,col=alpha("red",.5),main=NULL,xlab=NA,ylab=NA,xaxt="n",yaxt="n")
}
par(mar=c(3,0,0,1))
plotHistHoriz(cellStatsF$geneDetect[!outToInspect | !outToRemove])
if (any(outToInspect)) {
  plotHistHoriz(cellStatsF$geneDetect[outToInspect],col=alpha("blue",.5),add=T)
}
if (any(outToRemove)) {
  plotHistHoriz(cellStatsF$geneDetect[outToRemove],col=alpha("red",.5),add=T)
}

par(mar=c(3,3,0,0),mgp=2:0)
plot(geneDetect~libSize,data=cellStatsF[!outToInspect,],log="xy",
     xlim=range(cellStatsF$libSize),ylim=range(cellStatsF$geneDetect),
     pch=21,col=alpha("black",0.2),bg=alpha("black",0.1),cex=1.2,
     xlab="Transcripts detected per cell (log scale)",
     ylab="Genes detected per cell (log scale)")
points(geneDetect~libSize,data=cellStatsF[outToInspect,],
       pch=24,col=alpha("blue",0.2),bg=alpha("blue",0.1),cex=1.2)
points(geneDetect~libSize,data=cellStatsF[outToRemove,],
       pch=4,cex=1.2,col="red")
mtext(paste(dataName,"cell stats"),side=3,adj=0.02,line=-1.5,font=2,cex=1)
legend("topleft",bty="n",inset=c(0,.05),pch=c(24,4,NA,NA),
       col=c("blue","red",NA,NA),pt.bg=alpha(c("blue",NA,NA,NA),0.3),
       legend=c(paste("Outliers to inspect:",sum(outToInspect)),
                paste("Outliers to remove:",sum(outToRemove)),
                paste(nrow(ebRawF)-nrow(ebRawF2),"genes removed"),
                paste(nrow(ebRawF2),"genes remain")))
```

```
par(mar=c(0,3,1,0))
hist(log10(cellStatsF$libSize[!outToInspect | !outToRemove]),
     freq=T,breaks=50,col="grey80",main=NULL,xlab=NA,ylab=NA,xaxt="n",yaxt="n")
if (any(outToInspect)) {
  hist(log10(cellStatsF$libSize[outToInspect]),add=T,
       freq=T,breaks=50,col=alpha("blue",.5),main=NULL,xlab=NA,ylab=NA,xaxt="n",yaxt="n")
}
if (any(outToRemove)) {
  hist(log10(cellStatsF$libSize[outToRemove]),add=T,
       freq=T,breaks=50,col=alpha("red",.5),main=NULL,xlab=NA,ylab=NA,xaxt="n",yaxt="n")
}
par(mar=c(3,0,0,1))
plotHistHoriz(log10(cellStatsF$geneDetect[!outToInspect | !outToRemove]))
if (any(outToInspect)) {
  plotHistHoriz(log10(cellStatsF$geneDetect[outToInspect]),col=alpha("blue",.5),add=T)
}
if (any(outToRemove)) {
  plotHistHoriz(log10(cellStatsF$geneDetect[outToRemove]),col=alpha("red",.5),add=T)
}
```



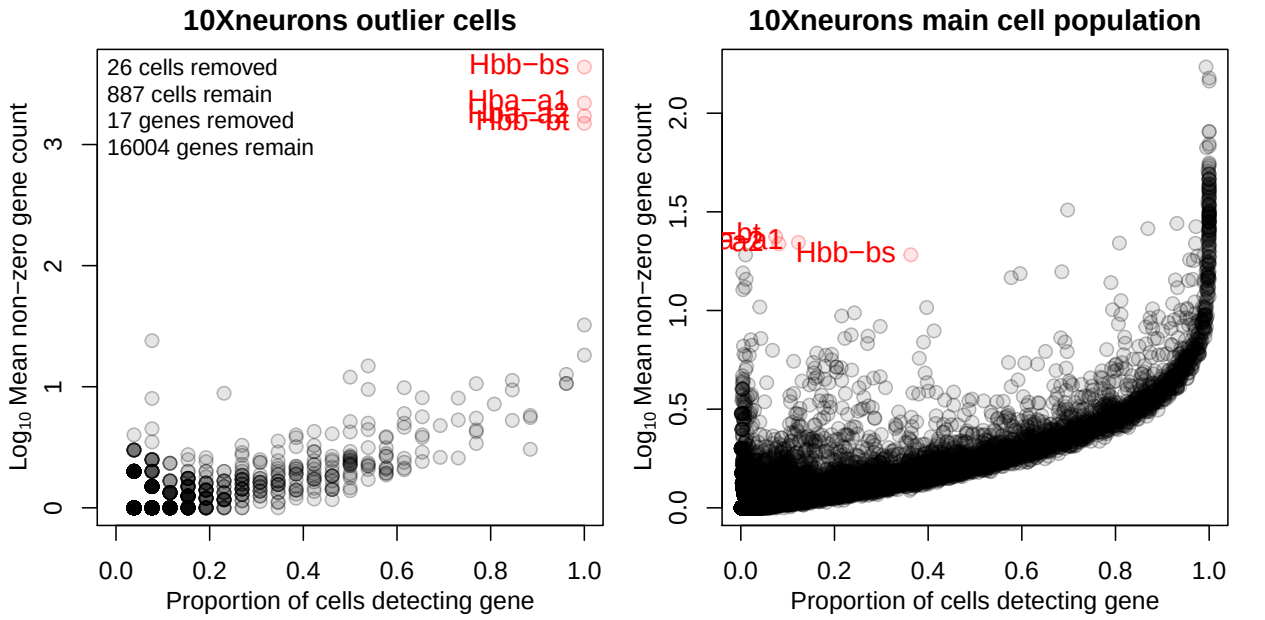
It's never a bad idea to inspect libraries before removing them, or at least ensuring that by removing them you are not losing genes from the analysis (which might imply that those cells were a unique population/cell-type).

Outlier inspection

```
if (any(outToInspect)) {
  ### Parameters you could edit ###
  topNum <- 4
  ##  Number of highly expressed genes in outlier population to highlight
  removeOutlierPopulation <- T
  ##  Do you want to remove this outlier population from the analysis?
```

```
### Calculations ###
gsOut <- data.frame(
  DR=apply(ebRawF2[,outToInspect[!outToRemove]],1,function(X) sum(X > 0)/length(X)),
  MDTC=apply(ebRawF2[,outToInspect[!outToRemove]],1,function(X) mean(X[X>0])),
  MTC=Matrix::rowMeans(ebRawF2[,outToInspect[!outToRemove]])
)
rownames(gsOut) <- rownames(ebRawF2)
gsIn <- data.frame(
  DR=apply(ebRawF2[,!outToInspect[!outToRemove]],1,function(X) sum(X > 0)/length(X)),
  MDTC=apply(ebRawF2[,!outToInspect[!outToRemove]],1,function(X) mean(X[X>0])),
  MTC=Matrix::rowMeans(ebRawF2[,!outToInspect[!outToRemove]])
)
rownames(gsIn) <- rownames(ebRawF2)
if (removeOutlierPopulation) {
  ebRawF3 <- ebRawF2[,!outToInspect[!outToRemove]]
  ebRawF3 <- ebRawF3[Matrix::rowSums(ebRawF3) > 0,]
} else { ebRawF3 <- ebRawF2 }
topHits <- 1:nrow(gsOut) %in% head(order(gsOut$MTC,decreasing=T),topNum)

### Plotting ###
par(mfrow=c(1,2),mar=c(3,3,2,1),mgp=2:0)
plot(log10(MDTC)~DR,data=gsOut,
     pch=21,cex=1.2,xlab="Proportion of cells detecting gene",
     ylab=expression(Log[10]~"Mean non-zero gene count"),
     col=alpha(c("black","red"),0.3)[topHits+1],
     bg=alpha(c("black","red"),0.1)[topHits+1],
     main=paste(dataName,"outlier cells"))
text(log10(MDTC)~DR,data=gsOut[topHits,],
     labels=rownames(gsOut)[topHits],pos=2,col="red",cex=1.2)
if (removeOutlierPopulation) {
  legend("topleft",inset=c(-.06,-.02),bty="n",cex=0.9,
         legend=c(paste(ncol(ebRawF2)-ncol(ebRawF3),"cells removed"),
              paste(ncol(ebRawF3),"cells remain"),
              paste(nrow(ebRawF2)-nrow(ebRawF3),"genes removed"),
              paste(nrow(ebRawF3),"genes remain")))
} else {
  legend("topleft",inset=c(-.06,-.02),bty="n",cex=0.9,
         legend=c(paste(sum(outToInspect[!outToRemove]),"outlier cells"),
              paste(sum(!outToInspect[!outToRemove]),"cells in main population"),
              paste(nrow(ebRawF3) -
                      sum(Matrix::rowSums(ebRawF3[,!outToInspect[!outToRemove]]) > 0),
                    "genes unique to outliers"),
              paste(sum(Matrix::rowSums(ebRawF2[,!outToInspect[!outToRemove]]) > 0),
                    "genes in main population")))
}

plot(log10(MDTC)~DR,data=gsIn,
     pch=21,cex=1.2,xlab="Proportion of cells detecting gene",
     ylab=expression(Log[10]~"Mean non-zero gene count"),
     col=alpha(c("black","red"),0.3)[topHits+1],
     bg=alpha(c("black","red"),0.1)[topHits+1],
     main=paste(dataName,"main cell population"))
text(log10(MDTC)~DR,data=gsIn[topHits,],
```

```
      labels=rownames(gsIn)[topHits],pos=2,col="red",cex=1.2)
} else { ebRawF3 <- ebRawF2 }
```



This outlier population looks to be a red blood cell or progenitor (based on the very high expression of haemoglobin, and low genetic complexity), which seems to be a common contaminant in brain single-cell data. We removed this population from downstream analysis.

```
cellStatsF3 <- cellStats[colnames(ebRawF3),]
rm(list=ls()[!ls() %in% c("ebRawF3","cellStatsF3","dataName","dataPath","plotHistHoriz",
                          "rainbow2","mart","speciesSymbol","cycloneSpeciesMarkers",
                          "geneLengthPath")])
gc()
```

```
##            used  (Mb) gc trigger  (Mb) max used  (Mb)
## Ncells  5496340 293.6    8449315 451.3  8449315 451.3
## Vcells 13882150 106.0   65669397 501.1 82086461 626.3
```

Data processing will now be performed based on a workflow published by the Marioni group (Lun et al., F1000Research 2016; http://dx.doi.org/10.12688/f1000research.9501.2).

```
ebRawS <- SingleCellExperiment(list(counts=ebRawF3))
ebRawS <- scater::calculateQCMetrics(ebRawS)
```

```
## Note that the names of some metrics have changed, see 'Renamed metrics' in ?calculateQCMetrics.
## Old names are currently maintained for back-compatibility, but may be removed in future releases.
```

```
colData(ebRawS)$mitoPct <- cellStatsF3$mitoPct
rm(ebRawF3,cellStatsF3)
gc()
```

```
##            used  (Mb) gc trigger  (Mb) max used  (Mb)
## Ncells  5502659 293.9    8449315 451.3  8449315 451.3
## Vcells 14005612 106.9   52535517 400.9 82086461 626.3
```

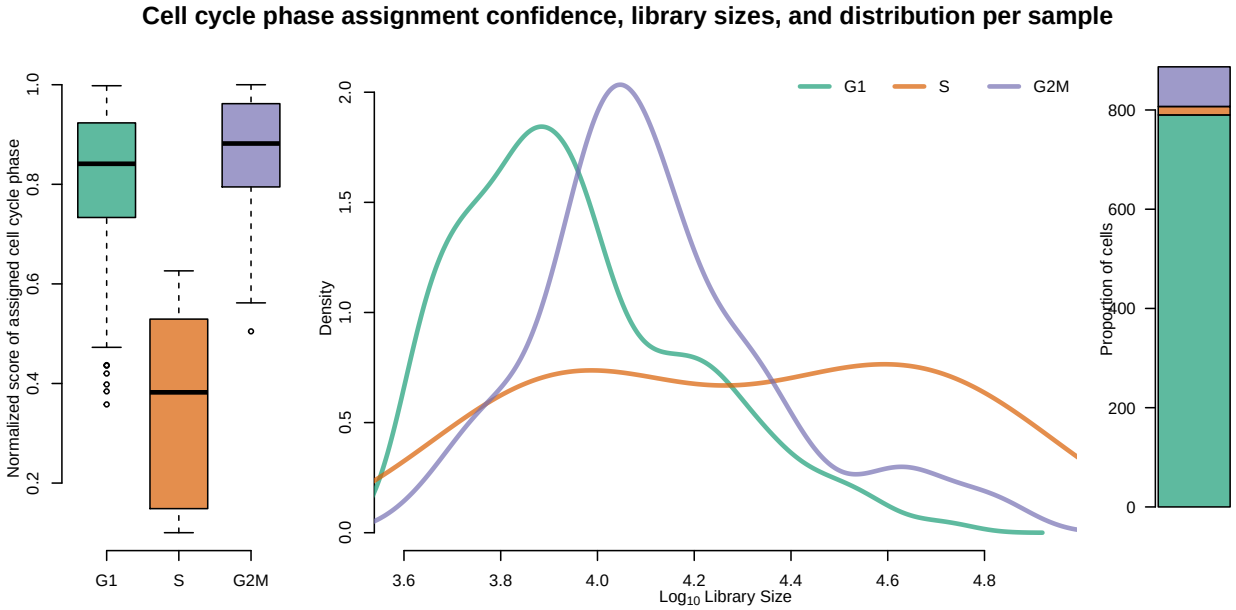
Cell cycle prediction using *Cyclone*

```
if (!file.exists(paste0(dataPath,"ebRawS.RData"))) {
  g2e <- getBM(attributes=c(speciesSymbol,"ensembl_gene_id"),
               mart=mart,filters=speciesSymbol,
               values=rownames(ebRawS))
  cycPairs <- readRDS(system.file("exdata",cycloneSpeciesMarkers,package="scran"))
  g2e <- g2e[g2e$ensembl_gene_id %in% unique((unlist(cycPairs))),]
  tempCyc <- cyclone(ebRawS[g2e$mgi_symbol,],
                     gene.names=g2e$ensembl_gene_id,pairs=cycPairs)
  tempCyc$confidence <- sapply(seq_along(tempCyc$phases),function(i)
    tempCyc$normalized.scores[i,tempCyc$phases[i]])
  colnames(tempCyc$scores) <- paste0("cycScore.",colnames(tempCyc$scores))
  colnames(tempCyc$normalized.scores) <- paste0("cycScoreNorm.",
                                                colnames(tempCyc$normalized.scores))
  colData(ebRawS) <- cbind(colData(ebRawS),tempCyc$scores,tempCyc$normalized.scores)
  colData(ebRawS)$cycPhase <- factor(tempCyc$phases,levels=c("G1","S","G2M"))
  colData(ebRawS)$cycConfidence <- tempCyc$confidence

  save(ebRawS,file=paste0(dataPath,"ebRawS.RData"))
} else {
  load(paste0(dataPath,"ebRawS.RData"))
  print(paste("Data loaded from",paste0(dataPath,"ebRawS.RData")))
}
```

```
## [1] "Data loaded from ../scClustViz_files/demo_10Xneurons900/ebRawS.RData"
```

```
layout(matrix(c(1,2,1,3,1,4),2),widths=c(2,5,1),heights=c(1,9))
par(mar=rep(0,4),mgp=2:0)
plot.new()
title("Cell cycle phase assignment confidence, library sizes, and distribution per sample",
      line=-2,cex.main=1.5)

par(mar=c(3,3,1,1),bty="n")
boxplot(tapply(colData(ebRawS)$cycConfidence,colData(ebRawS)$cycPhase,c),
        col=alpha(brewer.pal(3,"Dark2"),0.7),
        ylab="Normalized score of assigned cell cycle phase")

par(mar=c(3,3,1,1))
cycDlibSize <- tapply(log10(colData(ebRawS)$total_counts),colData(ebRawS)$cycPhase,
                      function(X) density(X))
plot(x=NULL,y=NULL,ylab="Density",xlab=expression(Log[10]~"Library Size"),
     xlim=range(log10(colData(ebRawS)$total_counts)),
     ylim=c(min(sapply(cycDlibSize,function(X) min(X$y))),
            max(sapply(cycDlibSize,function(X) max(X$y)))))
for (x in 1:length(cycDlibSize)) {
  lines(cycDlibSize[[x]],col=alpha(brewer.pal(3,"Dark2"),0.7)[x],lwd=3)
}
legend("topright",bty="n",horiz=T,lwd=rep(3,3),
       col=alpha(brewer.pal(3,"Dark2"),0.7),legend=levels(colData(ebRawS)$cycPhase))

par(mar=c(5,3,1,1))
barplot(cbind(table(colData(ebRawS)$cycPhase)),
        col=alpha(brewer.pal(3,"Dark2"),0.7),
```

```
        ylab="Proportion of cells",las=2)
```



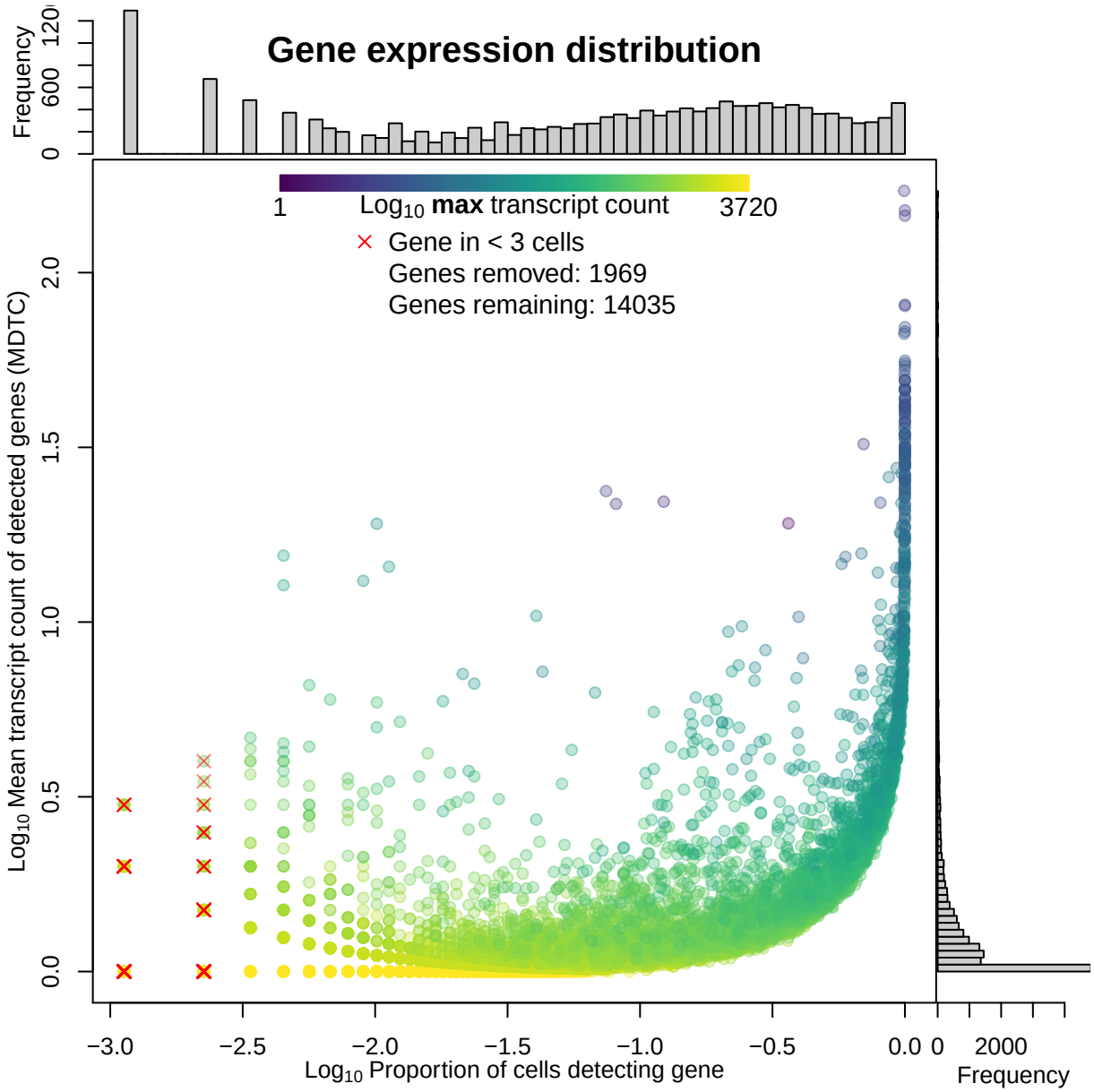
Cyclone generates individual scores for each cell cycle phase. G1 and G2/M are assigned based on these scores, and any cells not strongly scoring for either phase are assigned to S phase.
Later in the pipeline we will also attempt to predict cell cycle using the method from *Seurat*.

Filter out low abundance genes

Noisy genes must be removed to prevent them from skewing normalization. The filtering method in *Seurat* removes only genes detected in very few cells, which is sufficient for normalization while removing as few genes as possible.

```
geneStats <- data.frame(DR=apply(counts(ebRawS),1,function(X) sum(X > 0)/length(X)),
                        MDTC=apply(counts(ebRawS),1,function(X) mean(X[X > 0])),
                        cellMax=apply(counts(ebRawS),1,max))
drop_g <- geneStats$DR < 3/ncol(ebRawS)
ebRawSF <- ebRawS[!drop_g,]

layout(matrix(c(2,1,0,3),2),widths=c(6,1),heights=c(1,6))
par(mar=c(3,3,0,0),mgp=2:0)
temp_H <- cut(log10(geneStats[order(geneStats$cellMax,decreasing=F),"cellMax"]),
              breaks=100,labels=F)
plot(log10(MDTC)~log10(DR),data=geneStats[order(geneStats$cellMax,decreasing=F),],
     pch=21,col=viridis(100,0.5,1,0)[temp_H],bg=viridis(100,0.3,1,0)[temp_H],
     xlab=expression(Log[10]~"Proportion of cells detecting gene"),
     ylab=expression(Log[10]~"Mean transcript count of detected genes (MDTC)"))
points(log10(MDTC)~log10(DR),data=geneStats[drop_g,],
       pch=4,col=alpha("red",0.5),cex=1.2)
legend("top",bty="n",pch=c(4,NA,NA),col=c("red",NA,NA),cex=1.1,inset=c(0,.06),
       legend=c("Gene in < 3 cells",
                paste("Genes removed:",sum(drop_g)),
                paste("Genes remaining:",nrow(ebRawSF))))
segments(x0=seq(quantile(range(log10(geneStats$DR)),.2),
```

```
                quantile(range(log10(geneStats$DR)),.8),length.out=1000),
         y0=rep(max(log10(geneStats$MDTC)) * 1.02),
         y1=rep(max(log10(geneStats$MDTC))),col=viridis(1000))
text(x=c(quantile(range(log10(geneStats$DR)),.2),
         median(range(log10(geneStats$DR))),
         quantile(range(log10(geneStats$DR)),.8)),
     y=rep(max(log10(geneStats$MDTC)) * .98,3),
     labels=c(min(geneStats$cellMax),
              expression(Log[10]~bold(max)~transcript~count),
              max(geneStats$cellMax)),cex=1.1)

par(mar=c(0,3,0,0))
hist(log10(geneStats$DR),freq=T,breaks=100,col="grey80",main=NULL,xaxt="n")
title("Gene expression distribution",line=-2,cex.main=1.5)
par(mar=c(3,0,0,0))
barplot(hist(log10(geneStats$MDTC),breaks=100,plot=F)$counts,
        horiz=T,space=0,col="grey80",main=NULL,xlab="Frequency")
```




```
rm(list=ls()[!ls() %in% c("ebRawSF","dataName","dataPath","plotHistHoriz","rainbow2")])
gc()
```

```
##            used  (Mb) gc trigger  (Mb) max used  (Mb)
## Ncells  5529584 295.4    8449315 451.3  8449315 451.3
## Vcells 14027163 107.1   60661715 462.9 82086461 626.3
```

Normalization

Next step is normalization. Marioni proposed a normalization technique that attempts to generate cell-specific size factors that are robust to differential expression between genes in a heterogenous sample, unlike simple library-size normalization (https://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-0947-7). This method correlates strongly with library size normalization for homogenous samples, but solves a series of linear equations to deconvolute cell-specific size factors for normalization. In order to better

handle heterogenous data, they suggest separating the data by simple heirarchical clustering of a Spearman correlation-based distance metric so that they can normalize the separate subpopulations separately to prevent the suppression of true differential expression during normalization.

Normalization is carried out by assigning size factors per gene by the pooling and deconvolution method, then taking the log-ratio between each count and its size factor, and adding a pseudocount of one. Log-transforming the data stabilizes variance by reducing the impact of a few highly variable genes.

Following this, it is suggested to investigate sources of technical variance, but without spike-ins or any annotated possible sources of variation, this step is impossible with this data.

```
if (!file.exists(paste0(dataPath,"ebNorm.RData"))) {
  qClust <- quickCluster(ebRawSF,min.size=200)
  ##  Clustering of heterogenous data is suggested prior to normalization,
  ##  but if you have few cells or homogenous data, it may not be necessary.
  names(qClust) <- colnames(ebRawSF)
  ebRawSF <- computeSumFactors(ebRawSF,clusters=qClust)
  ebN <- ebRawSF[,!sizeFactors(ebRawSF) <= 0]
  ebN <- normalize(ebN)
  naCells <- apply(exprs(ebN),2,function(X) any(is.na(X)))
  if (any(naCells)) {
    exprs(ebN)[,naCells] <- min(apply(exprs(ebN),1,function(X) min(X,na.rm = T)))
  }
  save(qClust,ebN,file=paste0(dataPath,"ebNorm.RData"))
} else {
  load(paste0(dataPath,"ebNorm.RData"))
  print(paste("Data loaded from",paste0(dataPath,"ebNorm.RData")))
}
```

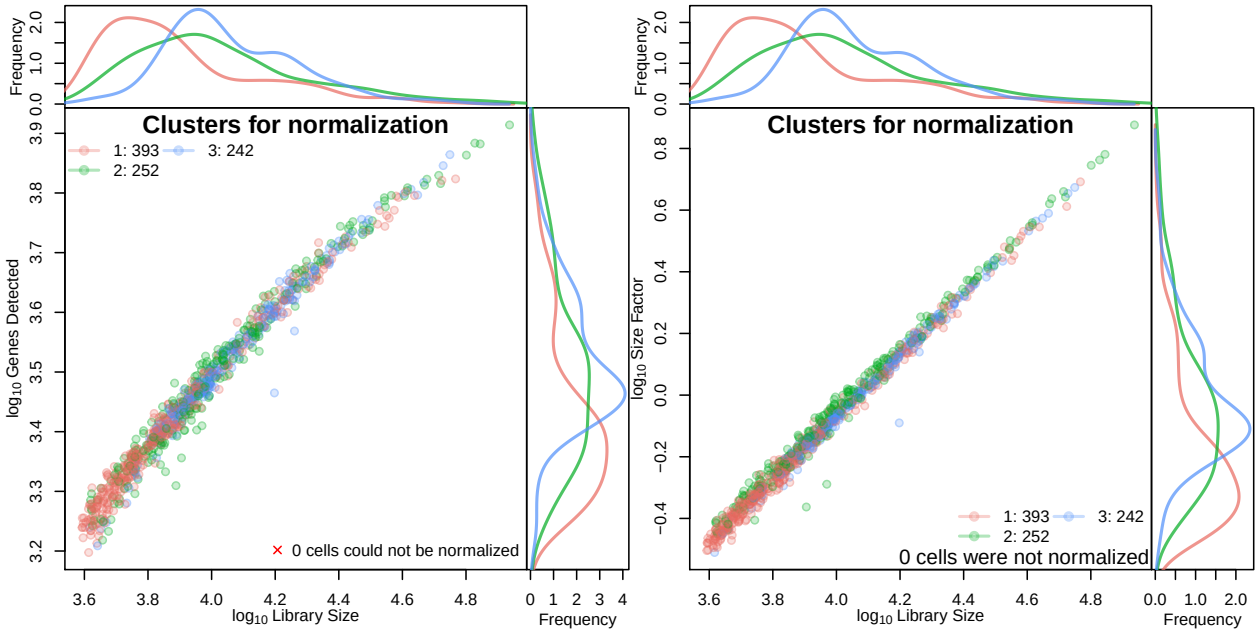
```
## [1] "Data loaded from ../scClustViz_files/demo_10Xneurons900/ebNorm.RData"
```

```
geneStatsN <- data.frame(
  DR=apply(exprs(ebN),1,function(X) sum(X > 0))/ncol(ebN),
  MDTC=apply(exprs(ebN),1,function(X) mean(X[X > 0])),
  MTC=Matrix::rowMeans(exprs(ebN)),
  sumTC=Matrix::rowSums(exprs(ebN)),
  cellMax=apply(exprs(ebN),1,max)
)

clustCols <- rainbow2(length(levels(qClust)))
temp_randcells <- sample.int(ncol(ebN))
```

```
temp_times <- as.factor(sub("_.+$","",colnames(ebRawSF)))
layout(matrix(c(2,1,0,3,5,4,0,6),2),
       widths=c(3.5,.7,3.5,.7),heights=c(.7,3.5))

## GeneDetect~LibSize by QuickCluster
par(mar=c(3,3,0,0),mgp=2:0)
plot(x=log10(colData(ebRawSF)$total_counts)[temp_randcells],
     y=log10(colData(ebRawSF)$total_features)[temp_randcells],
     pch=21,col=alpha(clustCols,0.4)[qClust][temp_randcells],
     bg=alpha(clustCols,0.2)[qClust][temp_randcells],
     xlab=expression(log[10]~"Library Size"),ylab=expression(log[10]~"Genes Detected"))
points(log10(colData(ebRawSF)$total_counts)[!colnames(ebRawSF) %in% colnames(ebN)],
       log10(colData(ebRawSF)$total_features)[!colnames(ebRawSF) %in% colnames(ebN)],
       pch=4,col="red")
```

```
mtext("Clusters for normalization",side=3,line=-1.5,font=2)
legend("topleft",bty="n",ncol=2,inset=c(0,.05),lwd=2,pch=21,
       col=alpha(clustCols[seq_along(levels(qClust))],0.3),
       pt.bg=alpha(clustCols[seq_along(levels(qClust))],0.1),
       legend=paste0(levels(qClust),": ",table(qClust)))
legend("bottomright",bty="n",pch=4,col="red",
       legend=paste(ncol(ebRawSF) - ncol(ebN),"cells could not be normalized"))

par(mar=c(0,3,.1,0))
temp_density <- tapply(log10(colData(ebRawSF)$total_counts),qClust,
                       function(X) density(X))
plot(x=NULL,y=NULL,xlim=range(log10(colData(ebRawSF)$total_counts)),
     ylim=range(unlist(lapply(temp_density,function(X) range(X$y)))),
     xlab=NULL,ylab="Frequency",xaxt="n")
for (x in seq_along(levels(qClust))) {
  lines(temp_density[[x]],lwd=2,col=alpha(clustCols,0.7)[x])
}

par(mar=c(3,0,0,.1))
temp_density <- tapply(log10(colData(ebRawSF)$total_features),qClust,
                       function(X) density(X))
plot(x=NULL,y=NULL,ylim=range(log10(colData(ebRawSF)$total_features)),
     xlim=range(unlist(lapply(temp_density,function(X) range(X$y)))),
     ylab=NULL,xlab="Frequency",yaxt="n")
for(x in seq_along(levels(qClust))) {
  lines(x=temp_density[[x]]$y,y=temp_density[[x]]$x,
        lwd=2,col=alpha(clustCols,0.7)[x])
}

## SizeFactor~LibSize by QuickCluster
qClustF <- qClust[colnames(ebN)]
par(mar=c(3,3,0,0),mgp=2:0)
plot(x=log10(colData(ebN)$total_counts)[temp_randcells],
     y=log10(sizeFactors(ebN))[temp_randcells],
     pch=21,col=alpha(clustCols,0.4)[qClustF][temp_randcells],
     bg=alpha(clustCols,0.2)[qClustF][temp_randcells],
     xlab=expression(log[10]~"Library Size"),ylab=expression(log[10]~"Size Factor"))
mtext("Clusters for normalization",side=3,line=-1.5,font=2)
legend("bottomright",bty="n",ncol=2,inset=c(0,.03),lwd=2,pch=21,
       col=alpha(clustCols[seq_along(levels(qClustF))],0.3),
       pt.bg=alpha(clustCols[seq_along(levels(qClustF))],0.1),
       legend=paste0(levels(qClustF),": ",table(qClustF)))
mtext(paste(ncol(ebRawSF) - ncol(ebN),"cells were not normalized"),
      side=1,line=-1.1,adj=.99,cex=.8)

par(mar=c(0,3,.1,0))
temp_density <- tapply(log10(colData(ebN)$total_counts),qClustF,
                       function(X) density(X))
plot(x=NULL,y=NULL,xlim=range(log10(colData(ebN)$total_counts)),
     ylim=range(unlist(lapply(temp_density,function(X) range(X$y)))),
     xlab=NULL,ylab="Frequency",xaxt="n")
for (x in seq_along(levels(qClustF))) {
  lines(temp_density[[x]],lwd=2,col=alpha(clustCols,0.7)[x])
```

```
}

par(mar=c(3,0,0,.1))
temp_density <- tapply(log10(sizeFactors(ebN)),qClustF,
                       function(X) density(X))
plot(x=NULL,y=NULL,ylim=range(log10(sizeFactors(ebN))),
     xlim=range(unlist(lapply(temp_density,function(X) range(X$y)))),
     ylab=NULL,xlab="Frequency",yaxt="n")
for(x in seq_along(levels(qClustF))) {
  lines(x=temp_density[[x]]$y,y=temp_density[[x]]$x,
        lwd=2,col=alpha(clustCols,0.7)[x])
}
```



Cells that fail to normalize are generally due to poor information content (small library size, weak gene expression relative to other cells).

```
rm(list=ls()[!ls() %in% c("ebN","dataPath")])
gc()
```

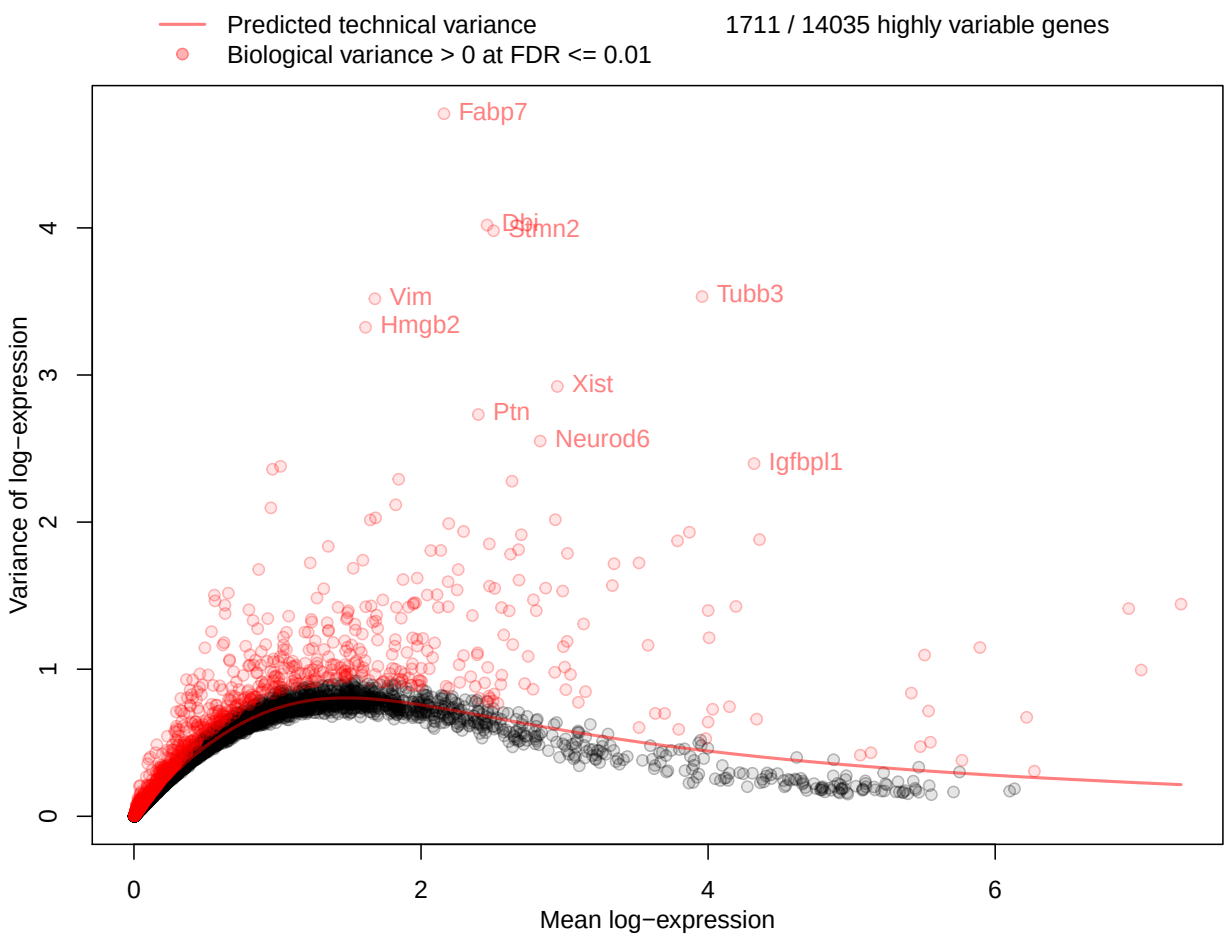
```
##            used  (Mb) gc trigger  (Mb) max used  (Mb)
## Ncells  5538850 295.9    8449315 451.3  8449315 451.3
## Vcells 18265603 139.4   58299246 444.8 82086461 626.3
```

Highly variable genes

Identification of highly variable genes is done by assuming most endogenous genes are not variably expressed, and fitting a curve to these genes when comparing variance to mean. This curve is presumed to represent technical variation, and thus highly variable genes are those with variance significantly greater than this curve. The method used here from *scran* isn't that different in logic to the *Seurat* method, but fitting a spline is just a little more refined way of going about it than Seurat's binning method.

```
var.fit <- trendVar(exprs(ebN),method="loess",parametric=T)
var.out <- decomposeVar(exprs(ebN),var.fit)
```

```
bioCut <- 0
bioCutFDR <- 1e-2
hvg <- var.out[which(var.out$FDR <= bioCutFDR & var.out$bio >= bioCut),]
hvg <- hvg[order(hvg$bio,decreasing=T),]

par(mar=c(3,3,3,1),mgp=2:0)
plot(total~mean, data=var.out[!rownames(var.out) %in% rownames(hvg),],
     ylim=range(var.out$total),xlim=range(var.out$mean),
     pch=21,col=alpha("black",0.3),bg=alpha("black",0.1),
     xlab="Mean log-expression",ylab="Variance of log-expression")
points(total~mean, data=var.out[rownames(var.out) %in% rownames(hvg),],
       pch=21,col=alpha("red",0.3),bg=alpha("red",0.1))
lines(var.out$mean[order(var.out$mean)],var.out$tech[order(var.out$mean)],
      col=alpha("red",0.5),lwd=2)
text(total~mean,data=var.out[rownames(hvg[1:10,]),],
     labels=rownames(hvg[1:10,]),pos=4,col=alpha("red",0.5))
legend("top",bty="n",inset=c(0,-.12),ncol=2,xpd=NA,lwd=c(2,NA,NA),pch=c(NA,21,NA),
       col=alpha(c("red","red",NA),0.5),pt.bg=alpha(c(NA,"red",NA),0.3),
       legend=c("Predicted technical variance",
                paste("Biological variance > 0 at FDR <=",bioCutFDR),
                paste(nrow(hvg),"/",nrow(ebN),"highly variable genes")))
```




```
ebNorm <- exprs(ebN)
pDat <- cbind(
```

```
  colData(ebN)[,c("total_features","total_counts")],
  colData(ebN)[,which(colnames(colData(ebN)) == "mitoPct"):ncol(colData(ebN))]
)
save(ebNorm,pDat,hvg,file=paste0(dataPath,"clustInputs.RData"))

##  Writing the normalized matrix as a .csv in case you want to use it for other things
##  (ie. loading in Python)
write.csv(as.matrix(exprs(ebN)),file=paste0(dataPath,"ebNorm.csv"),quote=F)
```