

SCnorm: robust normalization of single-cell RNA-seq data

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Contents

1	Introduction	1
2	Run SCnorm	2
2.1	Required inputs	2
2.2	SCnorm: Check count-depth relationship	5
2.3	SCnorm: Normalization	7
2.4	Evaluate choice of K	9
3	SCnorm: Multiple Conditions	10
4	SCnorm: UMI data	11
5	Spike-ins	12
6	Within-sample normalization	12
7	Session info	14
8	Frequently Asked Questions	15

1 Introduction

Normalization is an important first step in analyzing RNA-seq expression data to allow for accurate comparisons of a gene's expression across samples. Typically, normalization methods estimate a scale factor per sample to adjust for differences in the amount of sequencing each sample receives. This is because increases in sequencing typically lead to proportional increases in gene counts. However, in scRNA-seq data sequencing depth does not affect gene counts equally. SCnorm (as detailed in Bacher* and Chu* *et al.*, 2017) is a normalization approach we developed for single-cell RNA-seq data (scRNA-seq). SCnorm groups genes based on their count-depth relationship and within each group applies a quantile regression to estimate scaling factors to remove the effect of sequencing depth from the counts.

If you use SCnorm in published research, please cite:

SCnorm: robust normalization of single-cell RNA-seq data

Bacher R, Chu LF, Leng N, Gasch AP, Thomson J, Stewart R, Newton M, Kendziorski C. SCnorm: robust normalization of single-cell RNA-seq data. *Nature Methods*. 14 (6), 584-586 (<http://www.nature.com/nmeth/journal/v14/n6/full/nmeth.4263.html>)

If after reading through this vignette and the FAQ section you have questions or problems using SCnorm, please post them to <https://support.bioconductor.org> and tag "SCnorm". This will notify the package maintainers and benefit other users.

2 Run SCnorm

Before analysis can proceed, the SCnorm package must be installed.

The easiest way to install SCnorm is through Bioconductor (<https://bioconductor.org/packages/SCnorm>):

```
if (!requireNamespace("BiocManager", quietly=TRUE))
  install.packages("BiocManager")
BiocManager::install("SCnorm")
```

There are two additional ways to download SCnorm.

The first option is most useful if using a previous version of SCnorm (which can be found at <https://github.com/rhondabacher/SCnorm/releases>).

The second option is to download the most recent development version of SCnorm. This version may be used with versions of R below 3.4. This version includes use of summarized-Experiment, but parallel computation is maintained using the parallel package.

```
install.packages("SCnorm_x.x.x.tar.gz", repos=NULL, type="source")
#OR
library(devtools)
devtools::install_github("rhondabacher/SCnorm", ref="devel")
```

After successful installation, the package must be loaded into the working space:

```
library(SCnorm)
```

2.1 Required inputs

Data: Input to SCnorm may be either of class SummarizedExperiment or a matrix. The expression matrix should be a $G \times b \times S$ matrix containing the expression values for each gene and each cell, where G is the number of genes and S is the number of cells/samples. Gene names should be assigned as rownames and not a column in the matrix. Estimates of gene expression are typically obtained using RSEM, HTSeq, Cufflinks, Salmon or similar approaches.

The object `ExampleSimSCData` is a simulated single-cell data matrix containing 5,000 rows of genes and 90 columns of cells.

```
data(ExampleSimSCData)
ExampleSimSCData[1:5,1:5]

##           Cell_1    Cell_2    Cell_3    Cell_4    Cell_5
```

SCnorm: robust normalization of single-cell RNA-seq data

```
## Gene_1 3.809447 0.5891684 6.505612 33.052416 0.000000
## Gene_2 21.703998 3.9118515 0.000000 0.000000 5.651665
## Gene_3 2.128070 0.0000000 11.587132 11.352172 5.402953
## Gene_4 22.681505 0.0000000 27.495737 2.571482 8.248906
## Gene_5 0.000000 23.9983434 38.810055 1.244605 12.693561

str(ExampleSimSCData)

## num [1:5000, 1:90] 3.81 21.7 2.13 22.68 0 ...
## - attr(*, "dimnames")=List of 2
## ..$ : chr [1:5000] "Gene_1" "Gene_2" "Gene_3" "Gene_4" ...
## ..$ : chr [1:90] "Cell_1" "Cell_2" "Cell_3" "Cell_4" ...
```

SCnorm now implements the *SingleCellExperiment* class. The input data should be formatted as following, where *ExamplesSimSCData* is the expression matrix:

```
library(SingleCellExperiment)

## Loading required package: SummarizedExperiment
## Loading required package: MatrixGenerics
## Loading required package: matrixStats
##
## Attaching package: 'MatrixGenerics'
## The following objects are masked from 'package:matrixStats':
##
## colAlls, colAnyNAs, colAnys, colAvgPerRowSet, colCollapse,
## colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
## colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
## colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
## colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
## colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
## colWeightedMeans, colWeightedMedians, colWeightedSds,
## colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAvgPerColSet,
## rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
## rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
## rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
## rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
## rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs,
## rowVars, rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
## rowWeightedSds, rowWeightedVars

## Loading required package: GenomicRanges
## Loading required package: stats4
## Loading required package: BiocGenerics
## Loading required package: generics
##
## Attaching package: 'generics'
```

SCnorm: robust normalization of single-cell RNA-seq data

```
## The following objects are masked from 'package:base':  
##  
##   as.difftime, as.factor, as.ordered, intersect, is.element,  
##   setdiff, setequal, union  
  
##  
## Attaching package: 'BiocGenerics'  
  
## The following objects are masked from 'package:stats':  
##  
##   IQR, mad, sd, var, xtabs  
  
## The following objects are masked from 'package:base':  
##  
##   Filter, Find, Map, Position, Reduce, anyDuplicated, aperm,  
##   append, as.data.frame, basename, cbind, colnames, dirname,  
##   do.call, duplicated, eval, evalq, get, grep, grepl, is.unsorted,  
##   lapply, mapply, match, mget, order, paste, pmax, pmax.int, pmin,  
##   pmin.int, rank, rbind, rownames, sapply, saveRDS, table, tapply,  
##   unique, unsplit, which.max, which.min  
  
## Loading required package: S4Vectors  
  
##  
## Attaching package: 'S4Vectors'  
  
## The following object is masked from 'package:utils':  
##  
##   findMatches  
  
## The following objects are masked from 'package:base':  
##  
##   I, expand.grid, unname  
  
## Loading required package: IRanges  
  
## Loading required package: Seqinfo  
  
## 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)".  
  
##  
## Attaching package: 'Biobase'  
  
## The following object is masked from 'package:MatrixGenerics':  
##  
##   rowMedians  
  
## The following objects are masked from 'package:matrixStats':  
##  
##   anyMissing, rowMedians  
  
ExampleSimSCData <- SingleCellExperiment(assays = list('counts' = ExampleSimSCData))
```

SCnorm: robust normalization of single-cell RNA-seq data

If the data was originally formatted as a *SummarizedExperiment*, then it can be coerced using:

```
ExampleSimSCData <- as(ExampleSimSCData, "SingleCellExperiment")
```

Conditions: The object `Conditions` should be a vector of length S indicating which condition/group each cell belongs to. The order of this vector MUST match the order of the columns in the `Data` matrix.

```
Conditions = rep(c(1), each= 90)
head(Conditions)
## [1] 1 1 1 1 1 1
```

2.2 SCnorm: Check count-depth relationship

Before normalizing using SCnorm it is advised to check the relationship between expression counts and sequencing depth (referred to as the count-depth relationship) for your data. If all genes have a similar relationship then a global normalization strategy such as median-by-ratio in the DESeq package or TMM in edgeR will also be adequate. However, we find that when the count-depth relationship varies among genes using global scaling strategies leads to poor normalization. In these cases we strongly recommend proceeding with the normalization provided by SCnorm.

The `plotCountDepth` function will estimate the count-depth relationship for all genes. The genes are first divided into ten equally sized groups based on their non-zero median expression. The function will generate a plot of the distribution of slopes for each group. We also recommend checking a variety of filter options in case you find that only genes expressed in very few cells or very low expressors are the main concern.

The function `plotCountDepth` produces a plot as output which may be wrapped around a call to `pdf()` or `png()` to save it for future reference. The function also returns the information used for plotting which may be utilized if a user would like to generate a more customized figure. The returned object is a list with length equal to the number of conditions and each element of the list is a matrix containing each genes's assigned group (based on non-zero median expression) and its' estimated count-depth relationship (Slope).

```
pdf("check_exampleData_count-depth_evaluation.pdf", height=5, width=7)
countDeptEst <- plotCountDepth(Data = ExampleSimSCData, Conditions = Conditions,
                               FilterCellProportion = .1, NCores=3)

## Warning: Using 'size' aesthetic for lines was deprecated in ggplot2 3.4.0.
## i Please use 'linewidth' instead.
## i The deprecated feature was likely used in the SCnorm package.
## Please report the issue at
## <https://github.com/rhondabacher/SCnorm/issues>.
## This warning is displayed once per session.
## Call 'lifecycle::last_lifecycle_warnings()' to see where this warning was
## generated.

## Warning: The 'size' argument of 'element_rect()' is deprecated as of ggplot2
3.4.0.
## i Please use the 'linewidth' argument instead.
## i The deprecated feature was likely used in the SCnorm package.
```

SCnorm: robust normalization of single-cell RNA-seq data

```
## Please report the issue at
## <https://github.com/rhondabacher/SCnorm/issues>.
## This warning is displayed once per session.
## Call 'lifecycle::last_lifecycle_warnings()' to see where this warning was
## generated.

dev.off()

str(countDeptEst)

head(countDeptEst[[1]])
```

Since gene expression increases proportionally with sequencing depth, we expect to find the estimated count-depth relationships near 1 for all genes. This is typically true for bulk RNA-seq datasets. However, it does not hold in most single-cell RNA-seq datasets. In this example data, the relationship is quite variable across genes.

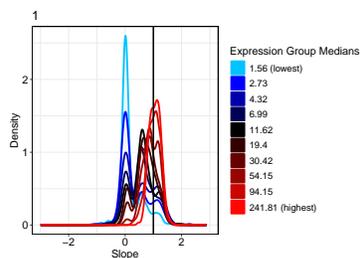


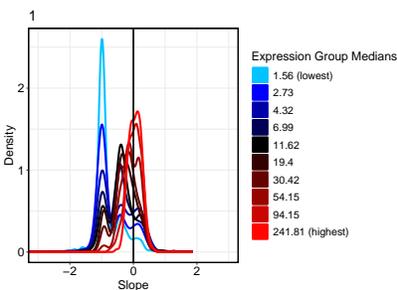
Figure 1: Evaluation of count-depth relationship in un-normalized data.

After normalization the count-depth relationship can be estimated on the normalized data, where slopes near zero indicate successful normalization. We can use the function `plotCountDepth` to evaluate normalized data:

```
ExampleSimSCData = SingleCellExperiment::counts(ExampleSimSCData)
# Total Count normalization, Counts Per Million, CPM.
ExampleSimSCData.CPM <- t((t(ExampleSimSCData) / colSums(ExampleSimSCData)) *
  mean(colSums(ExampleSimSCData)))
```

SCnorm: robust normalization of single-cell RNA-seq data

```
countDeptEst.CPM <- plotCountDepth(Data = ExampleSimSCData,  
                                   NormalizedData = ExampleSimSCData.CPM,  
                                   Conditions = Conditions,  
                                   FilterCellProportion = .1, NCores=3)
```



```
str(countDeptEst.CPM)  
  
## List of 1  
## $ 1:'data.frame':5000 obs. of 3 variables:  
## ..$ Gene : chr [1:5000] "Gene_1" "Gene_10" "Gene_100" "Gene_1000" ...  
## ..$ Group: int [1:5000] 4 1 3 3 3 4 1 1 2 2 ...  
## ..$ Slope: num [1:5000] -0.923 -1.005 -0.962 -1.017 -1.042 ...  
  
head(countDeptEst.CPM[[1]])  
  
##      Gene Group      Slope  
## 1   Gene_1      4 -0.9231680  
## 2   Gene_10     1 -1.0046535  
## 3   Gene_100    3 -0.9619995  
## 4 Gene_1000    3 -1.0172538  
## 5 Gene_1001    3 -1.0419565  
## 6 Gene_1002    4 -1.6735488
```

If normalization is successful the estimated count-depth relationship should be near zero for all genes. Here the highly expressed genes appear well normalized, while moderate and low expressors have been over-normalized and have negative slopes.

2.3 SCnorm: Normalization

SCnorm will normalize across cells to remove the effect of sequencing depth on the counts. The function `SCnorm` returns: the normalized expression counts, a list of genes which were not considered in the normalization due to filter options, and optionally a matrix of scale factors (if `reportSF = TRUE`).

The default filter for SCnorm only considers genes having at least 10 non-zero expression values. The user may wish to adjust the filter and may do so by changing the value of `FilterCellNum`. The user may also wish to not normalize very low expressed genes. The parameter `FilterExpression` can be used to exclude genes which have non-zero medians lower than a threshold specified by `FilterExpression` (`FilterExpression=0` is default).

SCnorm starts at $K = 1$, which normalizes the data assuming all genes should be normalized in one group. The sufficiency of $K = 1$ is evaluated by estimating the normalized count-depth relationships. This is done by splitting genes into 10 groups based on their non-zero unnormalized median expression (equally sized groups) and estimating the mode for each

SCnorm: robust normalization of single-cell RNA-seq data

group. If all 10 modes are within .1 of zero, then $K = 1$ is sufficient. If any mode is large than .1 or less than -.1, SCnorm will attempt to normalize assuming $K = 2$ and repeat the group normalizations and evaluation. This continues until all modes are within .1 of zero. (Simulation studies have shown .1 generally works well and is default, but may be adjusted using the parameter `Thresh` in the `SCnorm` function). If `PrintProgressPlots = TRUE` is specified, the progress plots of SCnorm will be created for each value of K tried (default is `FALSE`).

Normalized data can be accessed using the `results()` function.

```
Conditions = rep(c(1), each= 90)
pdf("MyNormalizedData_k_evaluation.pdf")
par(mfrow=c(2,2))
DataNorm <- SCnorm(Data = ExampleSimSCData,
                  Conditions = Conditions,
                  PrintProgressPlots = TRUE,
                  FilterCellNum = 10, K = 1,
                  NCores=3, reportSF = TRUE)

## Setting up parallel computation using 3 cores

## SCnorm will normalize assuming 1 is the optimal number of groups. It is not
## advised to set this.

## Gene filter is applied within each condition.

## 0 genes in condition 1 will not be included in the normalization due to
## the specified filter criteria.

## A list of these genes can be accessed in output,
## see vignette for example.

## Done!

dev.off()

## pdf
## 2

DataNorm

## class: SingleCellExperiment
## dim: 5000 90
## metadata(2): ScaleFactors GenesFilteredOut
## assays(2): counts normcounts
## rownames(5000): Gene_1 Gene_2 ... Gene_4999 Gene_5000
## rowData names(0):
## colnames(90): Cell_1 Cell_2 ... Cell_89 Cell_90
## colData names(0):
## reducedDimNames(0):
## mainExpName: NULL
## altExpNames(0):

NormalizedData <- SingleCellExperiment::normcounts(DataNorm)
NormalizedData[1:5,1:5]

##           Cell_1      Cell_2      Cell_3      Cell_4      Cell_5
## Gene_1  4.927704  0.4627998  5.239220  26.2840393  0.0000000
```

SCnorm: robust normalization of single-cell RNA-seq data

```
## Gene_2 28.075171 3.0728125 0.000000 0.000000 5.782488
## Gene_3 2.752762 0.000000 9.331563 9.0275072 5.528019
## Gene_4 29.339624 0.000000 22.143373 2.0449014 8.439849
## Gene_5 0.000000 18.8510249 31.255229 0.9897386 12.987387
```

The list of genes not normalized according to the filter specifications may be obtained by:

```
GenesNotNormalized <- results(DataNorm, type="GenesFilteredOut")
str(GenesNotNormalized)

## List of 1
## $ GenesFilteredOutGroup1: chr(0)
```

If scale factors should be returned, then they can be accessed using:

```
ScaleFactors <- results(DataNorm, type="ScaleFactors")
str(ScaleFactors)

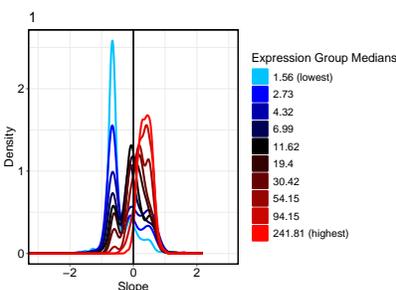
## num [1:5000, 1:90] 0.773 0.773 0.773 0.773 0.773 ...
## - attr(*, "dimnames")=List of 2
## ..$ : chr [1:5000] "Gene_1" "Gene_2" "Gene_3" "Gene_4" ...
## ..$ : chr [1:90] "Cell_1" "Cell_2" "Cell_3" "Cell_4" ...
```

2.4 Evaluate choice of K

SCnorm first fits the model for $K = 1$, and sequentially increases K until a satisfactory stopping point is reached. In the example run above, $K = 4$ is chosen, once all 10 slope densities have an absolute slope mode $< .1$.

We can also use the function `plotCountDepth` to make the final plot of the normalized count-depth relationship:

```
countDeptEst.SCNORM <- plotCountDepth(Data = ExampleSimSCData,
                                     NormalizedData = NormalizedData,
                                     Conditions = Conditions,
                                     FilterCellProportion = .1, Ncores=3)
```



```
str(countDeptEst.SCNORM)

## List of 1
## $ 1:'data.frame':5000 obs. of 3 variables:
## ..$ Gene : chr [1:5000] "Gene_1" "Gene_10" "Gene_100" "Gene_1000" ...
## ..$ Group: int [1:5000] 4 1 3 3 3 4 1 1 2 2 ...
```

SCnorm: robust normalization of single-cell RNA-seq data

```
## ..$ Slope: num [1:5000] -0.593 -0.675 -0.597 -0.692 -0.684 ...  
head(countDeptEst.SCNorm[[1]])  
##      Gene Group      Slope  
## 1   Gene_1      4 -0.5927619  
## 2   Gene_10     1 -0.6748594  
## 3   Gene_100    3 -0.5970708  
## 4 Gene_1000    3 -0.6915538  
## 5 Gene_1001    3 -0.6835126  
## 6 Gene_1002    4 -1.3863016
```

3 SCnorm: Multiple Conditions

When more than one condition is present SCnorm will first normalize each condition independently then apply a scaling procedure between the conditions. In this step the assumption is that most genes are not differentially expressed (DE) between conditions and that any systematic differences in expression across the majority of genes is due to technical biases and should be removed.

Zeros are not included in the estimations of scaling factors across conditions by default, this will make the means across conditions equal when zeros are not considered (which can then be used for downstream differential expression analyses with *MAST*, for example). However, if the proportion of zeros is very unequal between conditions and the user plans to use a downstream tool which includes zeros in the model (such as *DESeq* or *edgeR*), then the scaling should be estimated with zeros included in this calculation by using the `useZerosToScale=TRUE` option:

```
Conditions = rep(c(1, 2), each= 90)  
DataNorm <- SCnorm(Data = MultiCondData,  
                  Conditions = Conditions,  
                  PrintProgressPlots = TRUE,  
                  FilterCellNum = 10,  
                  NCores=3,  
                  useZerosToScale=TRUE)
```

Generally the definition of condition will be obvious given the experimental setup. If the data are very heterogeneous within an experimental setup it may be beneficial to first cluster more similar cells into groups and define these as conditions in SCnorm.

For very large datasets, it might be more reasonable to normalize each condition separately. To do this, first normalize each Condition separately:

```
DataNorm1 <- SCnorm(Data = ExampleSimSCData[,1:45],  
                  Conditions = rep("1", 45),  
                  PrintProgressPlots = TRUE,  
                  FilterCellNum = 10,  
                  NCores=3, reportSF = TRUE)  
  
DataNorm2 <- SCnorm(Data = ExampleSimSCData[,46:90],  
                  Conditions = rep("2", 45),
```

SCnorm: robust normalization of single-cell RNA-seq data

```
PrintProgressPlots = TRUE,  
FilterCellNum = 10,  
NCores=3, reportSF = TRUE)
```

Then the final scaling step can be performed as follows:

```
normalizedDataSet1 <- results(DataNorm1, type = "NormalizedData")  
normalizedDataSet2 <- results(DataNorm2, type = "NormalizedData")  
  
NormList <- list(list(NormData = normalizedDataSet1),  
               list(NormData = normalizedDataSet2))  
OrigData <- ExampleSimSCData  
Genes <- rownames(ExampleSimSCData)  
useSpikes = FALSE  
useZerosToScale = FALSE  
  
DataNorm <- scaleNormMultCont(NormData = NormList,  
                              OrigData = OrigData,  
                              Genes = Genes, useSpikes = useSpikes,  
                              useZerosToScale = useZerosToScale)  
  
str(DataNorm$ScaledData)  
myNormalizedData <- DataNorm$ScaledData
```

4 SCnorm: UMI data

If the single-cell expression matrix is very sparse (having a lot of zeros) then SCnorm may not be appropriate. Currently, SCnorm will issue a warning if at least one cell/sample has less than 100 non-zero values or total counts below 10,000. SCnorm will fail if the filtering criteria or quality of data leaves less than 100 genes for normalization.

If the data have many tied count values consider setting the option `ditherCounts=TRUE` (default is `FALSE`) which will help in estimating the count-depth relationships. This introduces some randomness but results will not change if the command is rerun.

It is highly recommended to check the count-depth relationship before and after normalization. In some cases, it might be desired to adjust the threshold used to decide K . Lowering the threshold may improve results for some datasets (default `Thresh = .1`).

For larger datasets, it may also be desired to increase the speed. One way to do this is to change the parameter `PropToUse`. `PropToUse` controls the proportion of genes nearest to the overall group mode to use for the group fitting (default is `.25`).

```
checkCountDepth(Data = umiData, Conditions = Conditions,  
                FilterCellProportion = .1, FilterExpression = 2)  
  
DataNorm <- SCnorm(Data = umiData, Conditions= Conditions,  
                  PrintProgressPlots = TRUE,  
                  FilterCellNum = 10,  
                  PropToUse = .1,
```

SCnorm: robust normalization of single-cell RNA-seq data

```
Thresh = .1,  
ditherCounts = TRUE)
```

5 Spike-ins

SCnorm does not require spike-ins, however if high quality spike-ins are available then they may be used to perform the between condition scaling step. If `useSpikes=TRUE` then only the spike-ins will be used to estimate the scaling factors. If the spike-ins do not span the full range of expression, SCnorm will issue a warning and will use all genes to scale. SCnorm also assumes the spike-ins are named with the prefix "ERCC-".

```
ExampleSimSCData.SCE <- SingleCellExperiment::SingleCellExperiment(assays = list('counts' = ExampleSimSCData  
  
## Assuming the spikes are ERCC, otherwise specify which features are spike-ins manually using the splitAlt  
myspikes <- grepl("^ERCC-", rownames(ExampleSimSCData.SCE))  
ExampleSimSCData.SCE <- SingleCellExperiment::splitAltExps(ExampleSimSCData.SCE, ifelse(myspikes, "ERCC", "g  
  
DataNorm <- SCnorm(Data = ExampleSimSCData.SCE, Conditions = Conditions,  
PrintProgressPlots = TRUE,  
FilterCellNum = 10, useSpikes=TRUE)
```

6 Within-sample normalization

SCnorm allows correction of gene-specific features prior to the between-sample normalization. We implement the regression based procedure from

Risso et al., 2011 in *EDASeq*. To use this feature you must set `withinSample` equal to a vector of gene-specific features, one per gene. This could be anything, but is often GC-content or gene length.

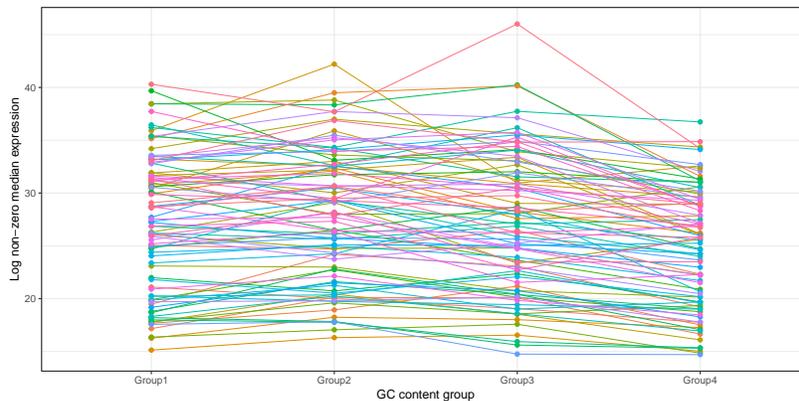
A function to evaluate the extent of bias in expression related to the gene-specific feature is `plotWithinFactor()`. Genes are split into 4 (`NumExpressionGroups = 4` is default) equally sized groups based on the gene-specific factor provided. For each cell the median expression of genes in each group is estimated and plotted. The function `plotWithinFactor` returns the information used for plotting as a matrix of the expression medians for each group for all cells.

In this example I generate a random vector of gene specific features, this may be the proportion of GC content for example. Each cell receives a different color

```
#Colors each sample:  
exampleGC <- runif(dim(ExampleSimSCData)[1], 0, 1)  
names(exampleGC) <- rownames(ExampleSimSCData)  
withinFactorMatrix <- plotWithinFactor(Data = ExampleSimSCData, withinSample = exampleGC)  
  
## Warning: 'aes_()' was deprecated in ggplot2 3.0.0.  
## i Please use tidy evaluation idioms with 'aes()'  
## i The deprecated feature was likely used in the SCnorm package.  
## Please report the issue at  
## <https://github.com/rhondabacher/SCnorm/issues>.
```

SCnorm: robust normalization of single-cell RNA-seq data

```
## This warning is displayed once per session.  
## Call 'lifecycle::last_lifecycle_warnings()' to see where this warning was  
## generated.
```



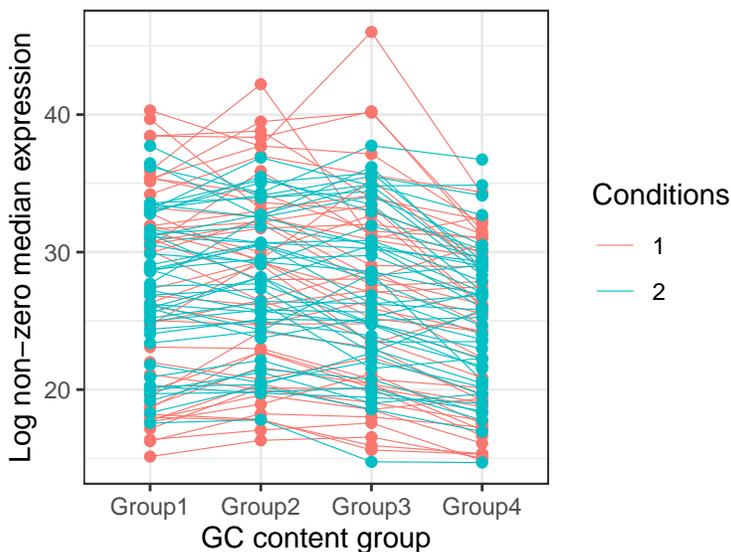
```
head(withinFactorMatrix)
```

```
##   Sample Condition  Group1  Group2  Group3  Group4  
##   <char>   <fctr>   <num>   <num>   <num>   <num>  
## 1: Cell_1       1 21.09794 19.99439 19.05631 17.75502  
## 2: Cell_2       1 35.90814 42.20586 30.94471 32.57404  
## 3: Cell_3       1 35.42286 33.60837 33.04583 29.90441  
## 4: Cell_4       1 38.44794 38.34422 40.23223 31.14260  
## 5: Cell_5       1 24.69504 29.38946 25.88768 24.24165  
## 6: Cell_6       1 27.30387 29.31169 25.18426 24.23136
```

Specifying a Conditions vector will color each cell according to its associated condition:

```
#Colors samples by Condition:  
Conditions <- rep(c(1,2), each=45)  
withinFactorMatrix <- plotWithinFactor(ExampleSimSCData, withinSample = exampleGC,  
                                       Conditions=Conditions)
```

SCnorm: robust normalization of single-cell RNA-seq data



```
head(withinFactorMatrix)
```

```
##   Sample Condition  Group1  Group2  Group3  Group4
##   <char>   <fctr>   <num>   <num>   <num>   <num>
## 1: Cell_1       1 21.09794 19.99439 19.05631 17.75502
## 2: Cell_2       1 35.90814 42.20586 30.94471 32.57404
## 3: Cell_3       1 35.42286 33.60837 33.04583 29.90441
## 4: Cell_4       1 38.44794 38.34422 40.23223 31.14260
## 5: Cell_5       1 24.69504 29.38946 25.88768 24.24165
## 6: Cell_6       1 27.30387 29.31169 25.18426 24.23136
```

```
# To run correction use:
```

```
DataNorm <- SCnorm(ExampleSimSCData, Conditions,
  PrintProgressPlots = TRUE,
  FilterCellNum = 10, withinSample = exampleGC)
```

For additional evaluation on whether to correct for these features or other options for correction, see: Risso, D., Schwartz, K., Sherlock, G. & Dudoit, S. GC-content normalization for RNA-Seq data. BMC Bioinformatics 12, 480 (2011).

7 Session info

Here is the output of sessionInfo on the system on which this document was compiled:

```
print(sessionInfo())
## R version 4.5.2 (2025-10-31)
## Platform: x86_64-pc-linux-gnu
## Running under: Ubuntu 24.04.3 LTS
##
## Matrix products: default
## BLAS: /usr/lib/x86_64-linux-gnu/openblas-pthread/libblas.so.3
## LAPACK: /usr/lib/x86_64-linux-gnu/openblas-pthread/libopenblas-p-r0.3.26.so; LAPACK version 3.12.0
```

SCnorm: robust normalization of single-cell RNA-seq data

```
##
## locale:
## [1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C
## [3] LC_TIME=en_US.UTF-8        LC_COLLATE=C
## [5] LC_MONETARY=en_US.UTF-8    LC_MESSAGES=en_US.UTF-8
## [7] LC_PAPER=en_US.UTF-8      LC_NAME=C
## [9] LC_ADDRESS=C               LC_TELEPHONE=C
## [11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C
##
## time zone: Etc/UTC
## tzcode source: system (glibc)
##
## attached base packages:
## [1] stats4      stats      graphics  grDevices  utils      datasets  methods
## [8] base
##
## other attached packages:
## [1] SingleCellExperiment_1.33.0 SummarizedExperiment_1.41.0
## [3] Biobase_2.71.0             GenomicRanges_1.63.1
## [5] Seqinfo_1.1.0             IRanges_2.45.0
## [7] S4Vectors_0.49.0          BiocGenerics_0.57.0
## [9] generics_0.1.4            MatrixGenerics_1.23.0
## [11] matrixStats_1.5.0         SCnorm_1.33.0
##
## loaded via a namespace (and not attached):
## [1] SparseArray_1.11.10 lattice_0.22-7      magrittr_2.0.4
## [4] digest_0.6.39         evaluate_1.0.5     grid_4.5.2
## [7] RColorBrewer_1.1-3   fastmap_1.2.0     Matrix_1.7-4
## [10] tinytex_0.58          survival_3.8-6    BiocManager_1.30.27
## [13] scales_1.4.0          codetools_0.2-20  abind_1.4-8
## [16] cli_3.6.5             rlang_1.1.7       BiocStyle_2.39.0
## [19] XVector_0.51.0        splines_4.5.2     withr_3.0.2
## [22] DelayedArray_0.37.0  yaml_2.3.12       otel_0.2.0
## [25] S4Arrays_1.11.1      tools_4.5.2       parallel_4.5.2
## [28] MatrixModels_0.5-4   SparseM_1.84-2    BiocParallel_1.45.0
## [31] ggplot2_4.0.1        forcats_1.0.1     buildtools_1.0.0
## [34] vctrs_0.7.1          R6_2.6.1          lifecycle_1.0.5
## [37] MASS_7.3-65          cluster_2.1.8.1   pillar_1.11.1
## [40] gtable_0.3.6         glue_1.8.0        data.table_1.18.2.1
## [43] moments_0.14.1       xfun_0.56         highr_0.11
## [46] sys_3.4.3            knitr_1.51        farver_2.1.2
## [49] htmltools_0.5.9     labeling_0.4.3    rmarkdown_2.30
## [52] maketools_1.3.2     compiler_4.5.2    quantreg_6.1
## [55] S7_0.2.1
```

8 Frequently Asked Questions

Does SCnorm work for all datasets?

SCnorm: robust normalization of single-cell RNA-seq data

No, while SCnorm has proven useful in normalizing many single-cell RNA-seq datasets, it will not be appropriate in every setting. It is important to evaluate normalized data for selected genes as well as overall (e.g. using `plotCountDepth`).

Can I use SCnorm on your 10X (or very sparse) dataset?

SCnorm is not intended for datasets with more than ~80% zero counts, often `K` will not converge in these situations. Setting the `FilterExpression` parameter to 1 or 2 may help, but is not a guarantee. It may also be helpful to use the `ditherCounts = TRUE` parameter for sparse UMI based data which may contain numerous tied counts (counts of 1 and 2 for example).

Can/Should I use SCnorm on my bulk RNA-seq data?

SCnorm can normalize bulk data. However, SCnorm should not be used to normalize data containing less than 10 cells/samples. Consideration must also be given to what downstream analysis methods will be used. Methods such as DESeq2 or edgeR typically expect a matrix of scaling factors to be supplied, these can be obtained in SCnorm by setting `reportSF=TRUE` in the SCnorm function.

The count-depth relationship of both unnormalized and normalized bulk RNA-seq data can be evaluated using the `plotCountDepth` function.

How can I speedup SCnorm if I have thousands of cells?

Utilizing multiple cores via the `NCores` parameter is the most effective way.

Splitting the data into smaller clusters may also improve speed. Here is one example on how to cluster and the appropriate way to run SCnorm: (This method of clustering single-cells was originally suggested by Lun et al., 2016 in [scrn](#).)

```
library(dynamicTreeCut)
distM <- as.dist( 1 - cor(BigData, method = 'spearman'))
htree <- hclust(distM, method='ward.D')
clusters <- factor(unnamed(cutreeDynamic(htree, minClusterSize = 50,
                                       method="tree", respectSmallClusters = FALSE)))
names(clusters) <- colnames(BigData)
Conditions = clusters

DataNorm <- SCnorm(Data = BigData,
                  Conditions = Conditions,
                  PrintProgressPlots = TRUE,
                  FilterCellNum = 10,
                  NCores=3, useZerosToScale=TRUE)
```

When should I set the parameter `useZerosToScale=TRUE` ?

This parameter is only used when specifying multiple conditions in SCnorm. The `useZerosToScale` parameter will determine whether zeros are used in estimating the condition based scaling factors.

Use the default, `useZerosToScale=FALSE`, when planning to use single-cell specific methods such as MAST that separately model continuous and discrete measurements.

If you plan on using an analysis method that explicitly uses zeros in the model like DESeq2, then `useZerosToScale=TRUE` should be used.

SCnorm was unable to converge.

SCnorm: robust normalization of single-cell RNA-seq data

SCnorm will fail if K exceeds 25 groups. It is unlikely that such a large number of groups is appropriate. This might happen if a large proportion of your genes have estimated count-depth relationships very close to zero. Typically this error can be resolved by removing genes with very small counts from the normalization using the parameter `FilterExpression`. Genes having non-zero median expression less than `FilterExpression` will not be scaled during normalization. An appropriate value for `FilterExpression` will be data dependent and decided based on exploring the data. One way that might help decide is to see if there are any natural cutoffs in the non-zero medians:

```
MedExpr <- apply(Data, 1, function(c) median(c[c != 0]))
plot(density(log(MedExpr), na.rm=T))
abline(v=log(c(1,2,3,4,5)))
# might set FilterExpression equal to one of these values.
```