Bioconductor workflow for single-cell RNA-seq data analysis: dimensionality reduction, clustering, and pseudotime ordering

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Abstract Abstracts should be up to 300 words and provide a succinct summary of the article. Although the abstract should explain why the article might be interesting, care should be taken not to inappropriately over-emphasise the importance of the work described in the article. Citations should not be used in the abstract, and the use of abbreviations should be minimized.

Keywords
single-cell, workflow, single-cell-genomics, pipeline, rna-seq, gene-expression, statistical-models, r

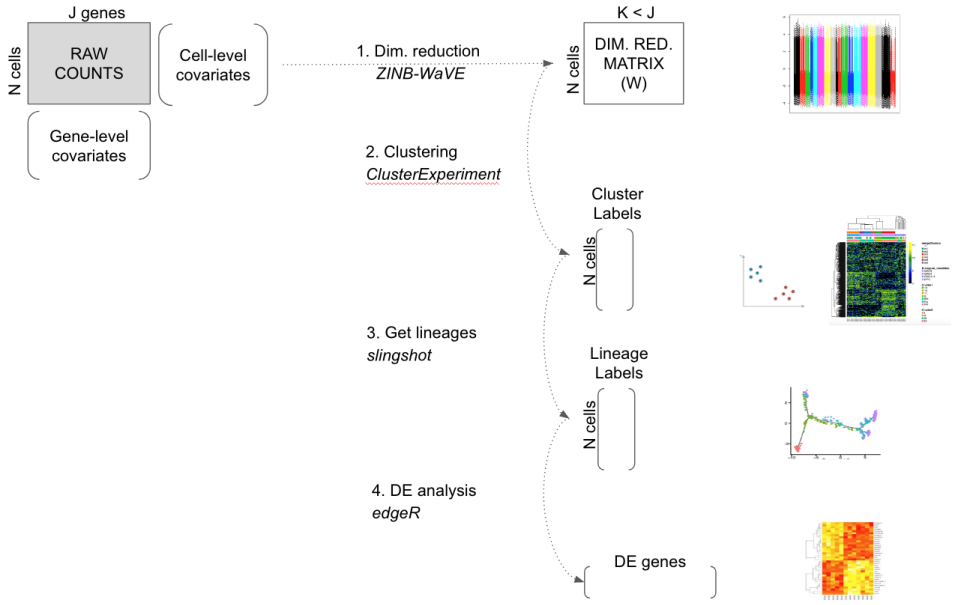


Figure 1. Workflow for analyzing single-cell RNA-seq datasets. On the right side, major plots generated by the workflow.

EAP: small request. Can everyone put a line between the beginning of a r chunk and text? It makes it nicely formated for my text editor.

Introduction

- growing interest in single-cell RNA-seq data. New statistical tools need to be developped compared to bulk RNA-seq because bias: drop-outs, amplification bias. There is also increasing size of datasets (10X-genomics, 1.3M cells) -> need of integrated workflows of new statistical tools

- previously developped : F1000 Research bioc (A. T. Lun, McCarthy, and Marioni 2016) and scater (McCarthy et al. 2017) are pipelines containing several useful methods for quality control, visualisation and pre-processing of data. In these pipelines, single-cell expression data are organized in objects of the SCESet class which allows integrated workflows. However, they are used mostly to prepare the data for further downstream analysis and do not focus on steps like clustering, pseudotime ordering, and DE analysis.

- we propose an integrated workflow with dowstream analysis: dimensionality reduction adjusting for gene and cell-level covariates, robust and stable clustering using resumpling, pseudotime ordering, and DE analysis for the clusters found. We use a summarizedExperiment object to have an integrated pipeline. We also propose useful functions for visualization.

Analysis of olfactory stem cell lineages using single cell RNA-seq data

Overview

The workflow is illustrated using data from a scRNA-seq study of stem cell differentiation in the mouse olfactory epithelium (Fletcher et al. 2017). The olfactory epithelium contains mature olfactory sensory neurons that are continuously renewed in the epithelium via neurogenesis through differentiation of globose basal cells (GBCs) which are the actively proliferating cells in the epithelium. When a severe injury to the entire tissue happens, the olfactory epithelium can regenerate from normally quiescent stem cells called horizontal basal cells (HBCs) which become activated to differentiate and reconstitute all major cell types in the epithelium.
The dataset we work with in this workflow was generated to study the differentitation from the HBCs stem cells to different cell types present in the epithelium. To map the developmental trajectories of the multiple cell lineages arising from the olfactory epithelium's HBC stem cell, a single-cell RNA-seq analysis was performed on FACS-purified cells using the Fluidigm C1 microfluidics cell capture platform followed by Illumina sequencing. Then, the expression of each gene was quantified by counting the total number of reads mapped to its cell to get a cell by gene matrix of raw counts. Different lineages were then assigned to each cell in the dataset using a set of statistical tools and results were confirmed experimentally using in vivo lineage tracing. Details on the data generation and steps of the analysis are available in (Fletcher et al. 2017).
It was found that the first major bifurcation in the HBC lineage trajectory occurs prior to cell division, producing either mature sustentacular (mSUS) cells or GBCs. Then, the GBC lineage, in turn, branches to give rise to mature olfactory sensory neurons (mOSN), microvillous (MV) cells, and cells of the Bowman's gland. See

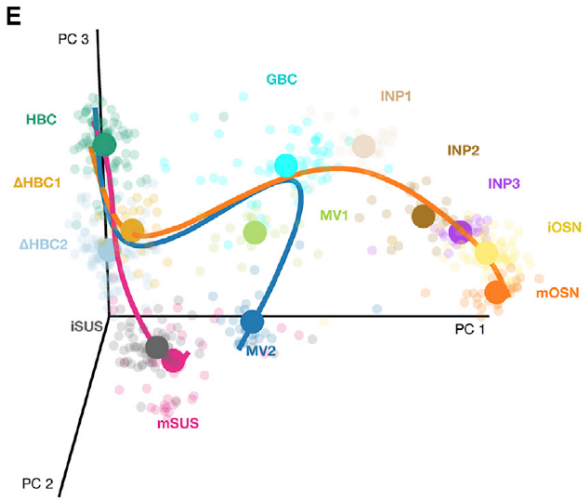


Figure 2. Stem cell differentiation in the mouse olfactory epithelium. This figure has been reproduced with kind permission from [@Fletcher2017].

Figure 2. In this study, we show possible steps to recover the lineages found in the original study from the matrix of raw counts publicly available.
FP: ask Russell if we can use the Figure 2 here.

Pre-processing

Counts for all genes in each cell were obtained from NCBI Gene Expression Omnibus (GEO) with accession number GSE95601. Before filtering, the dataset has 849 cells and 28361 detected genes.

```
load("../data/GSE95601_oeHBCdiff_Cufflinks_eSet.Rda")

## count matrix
E <- assayData(Cufflinks_eSet)$counts_table

# Remove undetected genes
E <- na.omit(E)
E <- E[rowSums(E)>0,]
dim(E)
```

```
## [1] 28361   849
```

We removed the genes corresponding to ERCC spike-ins and the CreER gene as it corresponds to a marker gene for the HBCs cells. Davide, is that correct?

```
# remove ERCC and CreER genes
cre <- E["CreER",]
ercc <- E[grep("^ERCC-", rownames(E)),]
E <- E[grep("^ERCC-", rownames(E), invert = TRUE), ]
E <- E[-which(rownames(E)=="CreER"), ]
dim(E)
```

```
## [1] 28284   849
```

Along the workflow, we use a SummarizedExperiement object to keep the counts and the metadata in the same object. We dispose of quality control measures as well as information on the batches in which the cells were sequenced and lineages found in the original publication (Fletcher et al. 2017). Cells with a lineage of -2 are cells not assigned to a lineage in the orginal publication.

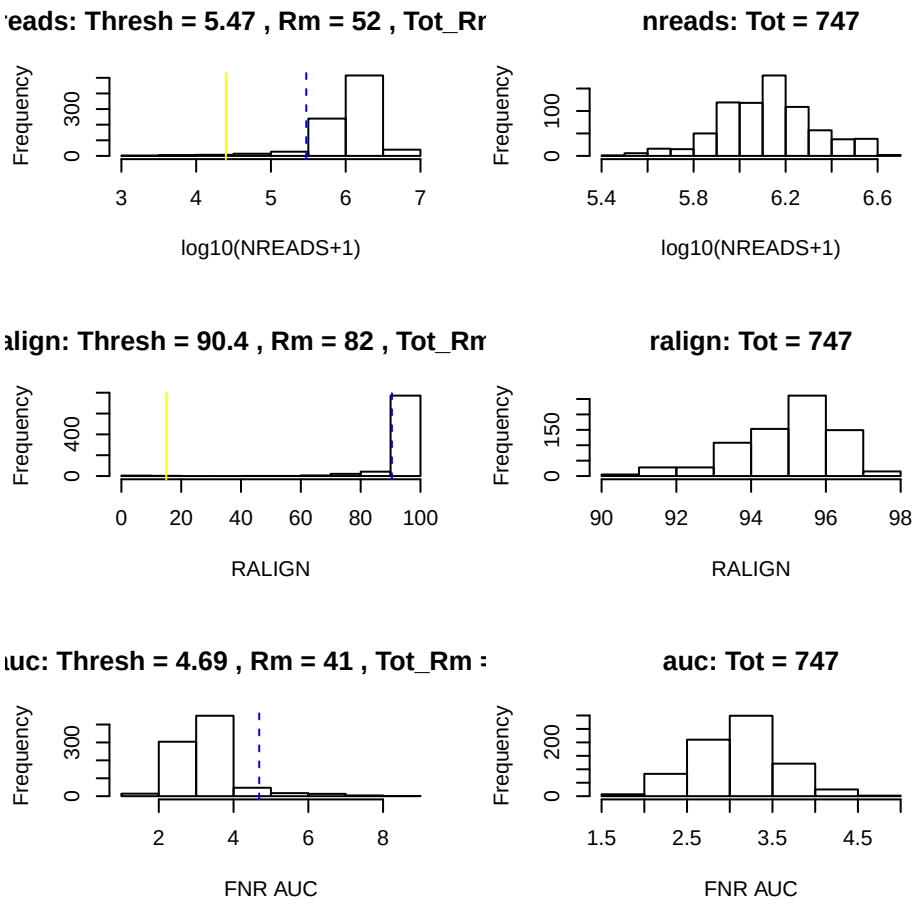
```
# Extract QC metrics
qc <- as.matrix(protocolData(Cufflinks_eSet)@data)[,c(1:5, 10:18)]
qc <- cbind(qc, CreER = cre, ERCC_reads = colSums(ercc))

# Extract metadata
```

```
batch <- droplevels(pData(Cufflinks_eSet)$MD_c1_run_id)
bio <- droplevels(pData(Cufflinks_eSet)$MD_expt_condition)
clusterLabels <- read.table("../data/oeHBCdiff_clusterLabels.txt",
                            sep = "\t", stringsAsFactors = FALSE)
m <- match(colnames(E), clusterLabels[, 1])

# Create metadata data.frame
metadata <- data.frame("Experiment" = bio,
                       "Batch" = batch,
                       "clusterLabels" = clusterLabels[m,2],
                       qc)

# symbol for cell not assigned to a lineage in original data
metadata$clusterLabels[is.na(metadata$clusterLabels)] <- -2

se <- SummarizedExperiment(assays = list(counts = E),
                           colData = metadata)
se
```

```
## class: SummarizedExperiment
## dim: 28284 849
## metadata(0):
## assays(1): counts
## rownames(28284): Xkr4 LOC102640625 ... Ggcx.1 eGFP
## rowData names(0):
## colnames(849): OEP01_N706_S501 OEP01_N701_S501 ... OEL23_N704_S503
##   OEL23_N703_S502
## colData names(19): Experiment Batch ... CreER ERCC_reads
```

Using bioconductor package scone, we removed low quality cells that did not pass our quality control rule, that is ... See Figure **??**. Davide, would you like to describe your choices for scone here?

```
# Metric-based Filtering sample-filtering
data("housekeeping")
hk = rownames(se)[toupper(rownames(se)) %in% housekeeping$V1]

mfilt <- metric_sample_filter(assay(se), nreads = colData(se)$NREADS,
                              ralign = colData(se)$RALIGN,
                              pos_controls = rownames(se) %in% hk,
                              zcut = 3, mixture = FALSE,
                              plot = TRUE)
```




```
# Simplify to a single logical
mfilt <- !apply(simplify2array(mfilt[!is.na(mfilt)]), 1, any)
se <- se[, mfilt]
dim(se)
```

```
## [1] 28284   747
```

Finally, to speed up the computations, we kept only the 1000 most variable genes. We recommend not to be so stringent in your analysis and perform the analysis on more genes.

```
# filtering to top 1000 most variable
vars <- rowVars(log1p(assay(se)))
names(vars) <- rownames(se)
vars <- sort(vars, decreasing = TRUE)
core <- se[names(vars)[1:1000],]
save(core, file="../data/oe_se_1000Var.rda")
```

Dataset structure

Overall, after the preprocessing steps, our dataset has 1000 genes and 747 cells

```
core
```

```
## class: SummarizedExperiment
## dim: 1000 747
## metadata(0):
## assays(1): counts
## rownames(1000): Cbr2 Cyp2f2 ... Rnf13 Atp7b
## rowData names(0):
## colnames(747): OEP01_N706_S501 OEP01_N701_S501 ... OEL23_N704_S503
##   OEL23_N703_S502
## colData names(19): Experiment Batch ... CreER ERCC_reads
```

Metadata for the cells is stored in the object colData from the SummarizedExperiment object. Cells have been processed in 18 different batches

```
batch <- colData(core)$Batch
col_batch = rep(brewer.pal(9, "Set1"), 2)
names(col_batch) = unique(batch)
table(batch)
```

```
## batch
## GBC08A GBC08B GBC09A GBC09B    P01    P02   P03A   P03B    P04    P05
##     39     40     35     22     31     48     51     40     20     23
##    P06    P10    P11    P12    P13    P14    Y01    Y04
##     51     40     50     50     60     47     58     42
```

In original work (Fletcher et al. 2017), cells have been clustered into 14 different clusters where 151 cells were not assigned to a lineage

```
clus.labels <- colData(core)[, "clusterLabels"]
col_clus <- c("transparent", brewer.pal(12, "Set3"), brewer.pal(8, "Set2"))
col_clus <- col_clus[1:length(unique(clus.labels))]
names(col_clus) <- sort(unique(clus.labels))
table(clus.labels)
```

```
## clus.labels
##  -2   1   2   3   4   5   7   8   9  10  11  12  14  15
## 151  90  25  54  35  93  58  27  74  26  21  35  26  32
```

Note that some of the batches are confounded with the lineages found in the original work which could be a problem when we correct for batch effect in the dimensionality reduction step.

```
table(data.frame(batch = as.vector(batch),
                 cluster = clus.labels))
```

```
##         cluster
## batch    -2  1  2  3  4  5  7  8  9 10 11 12 14 15
##   GBC08A  3  0  2 12  9  0  0  0  0  0  2  0  2  9
##   GBC08B  8  0  7  5  3  0  0  0  1  2  3  0  5  6
##   GBC09A  6  0  1  5  8  0  0  0  1  1  0  0  6  7
##   GBC09B 12  0  2  1  3  0  0  0  1  0  0  0  3  0
##   P01     7  0  2  4  3 15  0  0  0  0  0  0  0  0
##   P02     5  2  0  9  3 15  3  3  2  3  0  2  1  0
##   P03A   15  3  0  2  0 12  2  9  4  2  0  2  0  0
##   P03B    9  1  2  1  1 11  1  2  8  1  1  2  0  0
##   P04     8  0  0  0  0  9  1  0  1  1  0  0  0  0
##   P05     3  0  0  0  1 11  3  0  1  0  2  2  0  0
##   P06    12  1  2  3  0  8  2  4  8  4  1  2  2  2
##   P10     7  3  1  4  0  3  5  8  1  0  2  5  0  1
##   P11     6  2  1  1  0  1  5  1 22  3  1  6  0  1
##   P12    10  0  2  0  0  4 10  0  8  2  3  6  4  1
##   P13    13  1  2  4  0  4 15  0  4  5  6  1  3  2
##   P14     9  0  0  1  2  0 11  0 12  2  0  7  0  3
##   Y01     8 46  1  1  2  0  0  0  0  0  0  0  0  0
##   Y04    10 31  0  1  0  0  0  0  0  0  0  0  0  0
```

Dimensionality reduction adjusting for batches

In single-cell RNA-seq analysis, dimensionality reduction is often used as a preliminary step prior to downstream analysis where clustering, pseudotime ordering, or differential expression analysis are performed. It allows the data to become more tractable, both from a statistical (cf. curse of dimensionality) and computational point of view. Additionally, technical noise can be reduced while preserving the often intrinsically low dimensional signal of interest (Dijk et al. 2017).

Here, we perform dimensionality reduction using bioconductor package zinbwave which allows to fit a zero-inflated negative binomial model (ZINB-WaVE) to get a low-dimensional representation of the data while accounting for zero inflation (dropouts), over-dispersion, and the count nature of the data. The model can include a sample-level intercept, which serves as a global-scaling normalization factor. The user can also include both gene-level and sample-level covariates. The inclusion of observed and unobserved sample-level covariates enables normalization for complex, non-linear effects (often referred to as batch effects), while gene-level covariates may be used to adjust for sequence composition effects, such as gene length and GC-content effects. See more details in (Risso et al. 2017).

As with most of the dimensionality reduction methods, the user needs to specify the number of dimensions of the new low dimensional space. Here, we use 50 dimensions and adjusted for the batches.

```
fn <- '../data/zinb_batch.rda'
if (runZinb & !file.exists(fn)){
  print(system.time(se <- zinbDimRed(core, K = 50, X = '~ Batch',
                                     residuals = TRUE,
                                     normalizedValues = TRUE)))
  save(se, file = fn)
}else{
  load(fn)
}
```

DR: the chunk above and the similar one for clusterExperiment are fine for now, but in the published workflow, we should just rely on the markdown cache system (the code above doesn't check for changes to the file or code – just that a version of the file exists).

Normalized values

When function `zinbDimRed` is called, normalized values of the counts can be computed and used for visualization (e.g. in heatmaps). The normalized values are the residuals of the fit of the ZINB-WaVE model where the gene and cell covariates were specified by the user when calling function `zinbDimRed` but no low dimensional matrix is computed. In that case, the residuals represents the counts adjusted for the gene and cell covariates and should capture the biological signal of interest. To compute the residuals, the deviance is used.

```
norm <- assays(se)$normalizedValues
if (sum(is.infinite(norm))>0){
  maxNorm = max(norm[!is.infinite(norm)])
  assays(se)$normalizedValues[is.infinite(norm)] <- maxNorm
  norm <- assays(se)$normalizedValues
}
norm[1:3,1:3]
```

```
##         OEP01_N706_S501 OEP01_N701_S501 OEP01_N707_S507
## Cbr2           4.557371        4.375069       -4.142697
## Cyp2f2         4.321644        4.283266        4.090283
## Gstm1          4.796498        4.663366        4.416324
```

As expected, the normalized values are uniformly distributed for the different batches. See Figure 3.

```
norm_order <- norm[, order(as.numeric(batch))]
col_order <- as.numeric(batch)[order(as.numeric(batch))]
boxplot(norm_order, col = col_order, staplewex = 0, outline = 0,
        border = col_order, xaxt = 'n')
abline(h=0)
```

The principal component analysis (PCA) on the normalized values where colors are for batches on the left and original clusters on the right shows that, as expected, cells do not cluster by batch but by orginal published lineage. See Figure 4. Overall, looking at the normalized values, it seems that the batch effect has been removed.

```
pca <- prcomp(t(norm))
par(mfrow = c(1,2))
plot(pca$x, col = col_batch[batch], pch = 20, main = '')
plot(pca$x, col = col_clus[as.character(clus.labels)], pch = 20, main = '')
```

Dimensionality reduction

Calling function zinbDimRed, we performed dimensionality reduction with `K = 50` dimensions in the low dimensional space. We can visualize the low dimensional matrix (with `K = 50` dimensions) by performing multidimensional scaling (MDS) in two dimensions and euclidian distance. To make sure that the low dimensional matrix in `K = 50` dimensions captured the biological signal of interest, we plotted the MDS with colors corresponding to the original published lineages. See Figure 5.

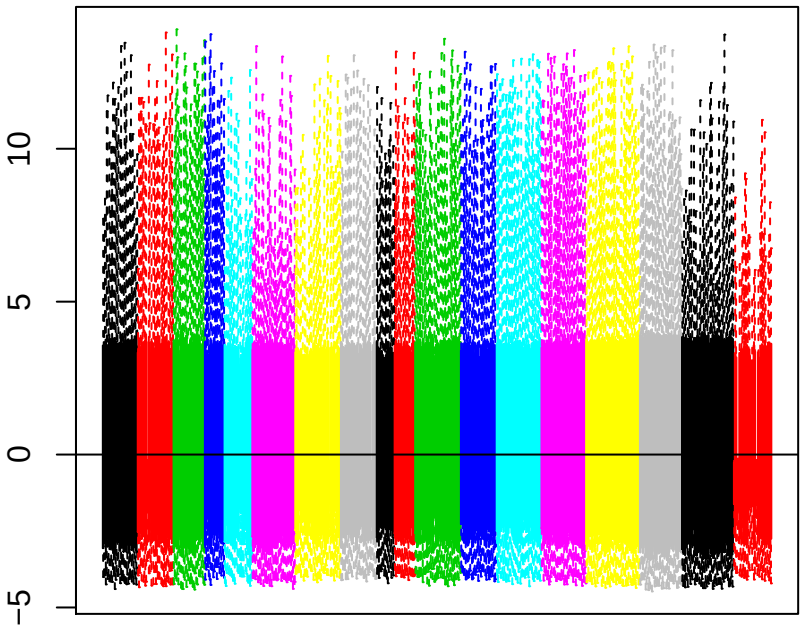


Figure 3. Boxplot of normalized values. Normalized values are uniformly distributed for the different batches

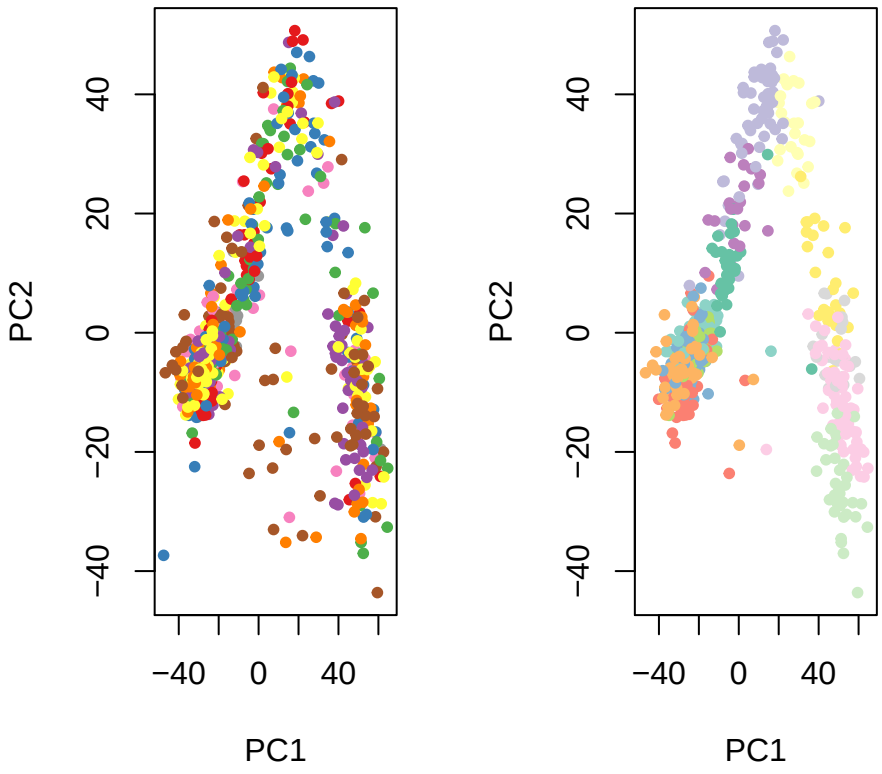


Figure 4. PCA of normalized values. In the left panel, colors correspond to the different batches. In the right panel, colors correspond to the original published lineages.

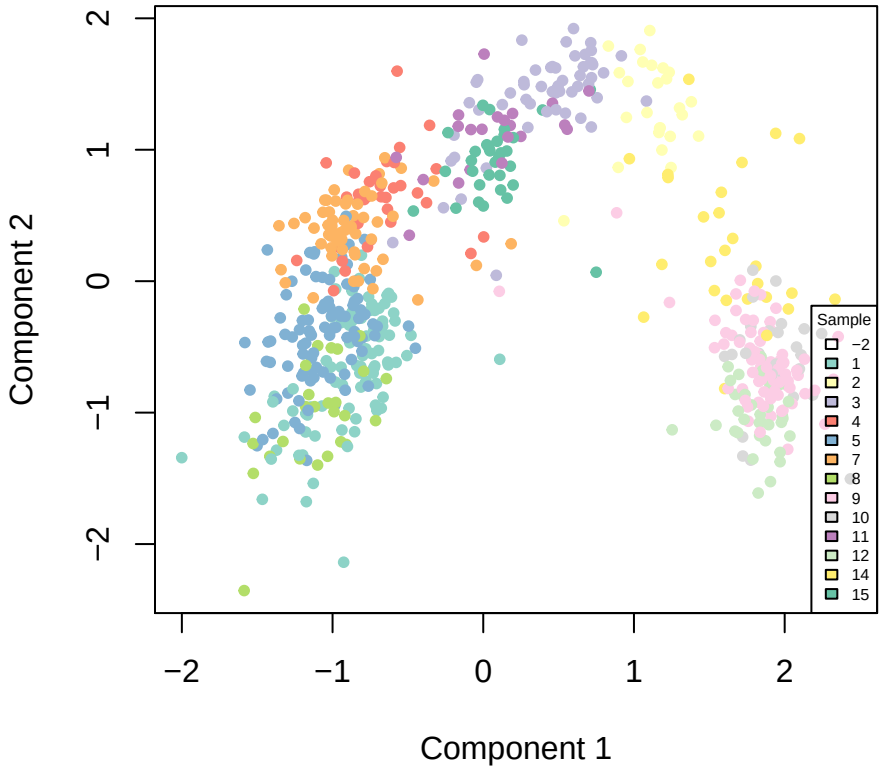


Figure 5. MDS of the low dimensional matrix with K = 50 dimensions. Colors correspond to the original published lineages.

```
W <- colData(se)[, grepl('^W', colnames(colData(se)))]
W <- as.matrix(W)
d <- dist(W)
fit <- cmdscale(d, eig = TRUE, k = 2)
plot(fit$points, col = col_clus[as.character(clus.labels)], main = '',
     pch = 20, xlab = 'Component 1', ylab = 'Component 2')
legend(x = 'bottomright', legend = unique(names(col_clus)), cex = .5,
       fill = unique(col_clus), title = 'Sample')
```

Clustering of the cells

The new step of the workflow is to cluster the cells according to the low dimensional matrix computed in the previous step. We used function RSEC from bioconductor package clusterExperiment. Elizabeth, would you like to explain the rational and arguments of the different functions?

```
fn <- '../data/RSEC_W_combineMinSize10.rda'
if (runClus & !file.exists(fn)){
  #symbol for samples missing from original clustering
  seObj <- SummarizedExperiment(t(W), colData = colData(core))
  print(system.time(ceObj <- RSEC(seObj, k0s = 4:15, alphas = c(0.1),
                                  betas = 0.8, dimReduce="none",
                clusterFunction = "hierarchical01", minSizes=1,
                ncores = NCORES, isCount=FALSE,
                subsampleArgs = list(resamp.num=100,
                                     clusterFunction="kmeans",
                                     clusterArgs=list(nstart=10)),
                seqArgs = list(k.min=3, top.can=5), verbose=TRUE,
                combineProportion = 0.7,
                mergeMethod = "none", random.seed=424242,
                combineMinSize = 10)))
  save(ceObj, file = fn)
}else{
  load(fn)
}
```

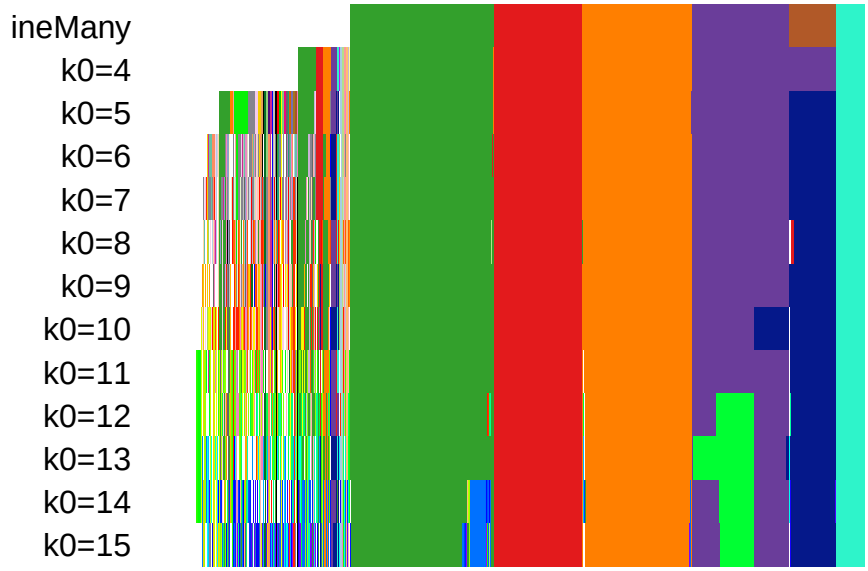


Figure 6. Clusters found using function RSEC from clusterExperiment package. Cells are in the columns, and different clusterings on the rows. Each sample is color coded based on its clustering for that row, where the colors have been chosen to try to match up clusters across different clusterings that show large overlap.

We can visualize the resulting clusterings using the plotClusters command. See Figure 6.

```
plotClusters(ceObj, colPalette = c(bigPalette, rainbow(199)))
```

We can also visualize the proportion of times these clusters were together across these clusterings. See Figure 7.

```
plotCoClustering(ceObj)
```

```
## Warning in .makeColors(clusters, colors = bigPalette): too many clusters to
## have unique color assignments
```

Overall, here is a summary of the number of cells clustered in each cluster

```
table(primaryClusterNamed(ceObj))
```

```
##
##  -1  c1  c2  c3  c4  c5  c6
## 172 161  98 123 108  52  33
```

```
sum(primaryCluster(ceObj) == -1)
```

```
## [1] 172
```

```
# set colors for cell clusterings
colData(ceObj)$clusterLabels <- as.factor(colData(ceObj)$clusterLabels)
origClusterColors<-bigPalette[1:nlevels(colData(ceObj)$clusterLabels)]
experimentColors<-bigPalette[1:nlevels(colData(ceObj)$Experiment)]
batchColors<-bigPalette[1:nlevels(colData(ceObj)$Batch)]
metaColors<-list("Experiment"=experimentColors,"Batch"=batchColors,"clusterLabels"=origClusterColors

plotHeatmap(ceObj, visualizeData = assays(se)$normalizedValues,
            whichClusters = "primary",clusterFeaturesData="all",
            clusterSamplesData = "dendrogramValue",
            sampleData=c("clusterLabels","Batch","Experiment"),
            clusterLegend=metaColors, annLegend=FALSE,
            main = '',
            breaks = 0.99)
```

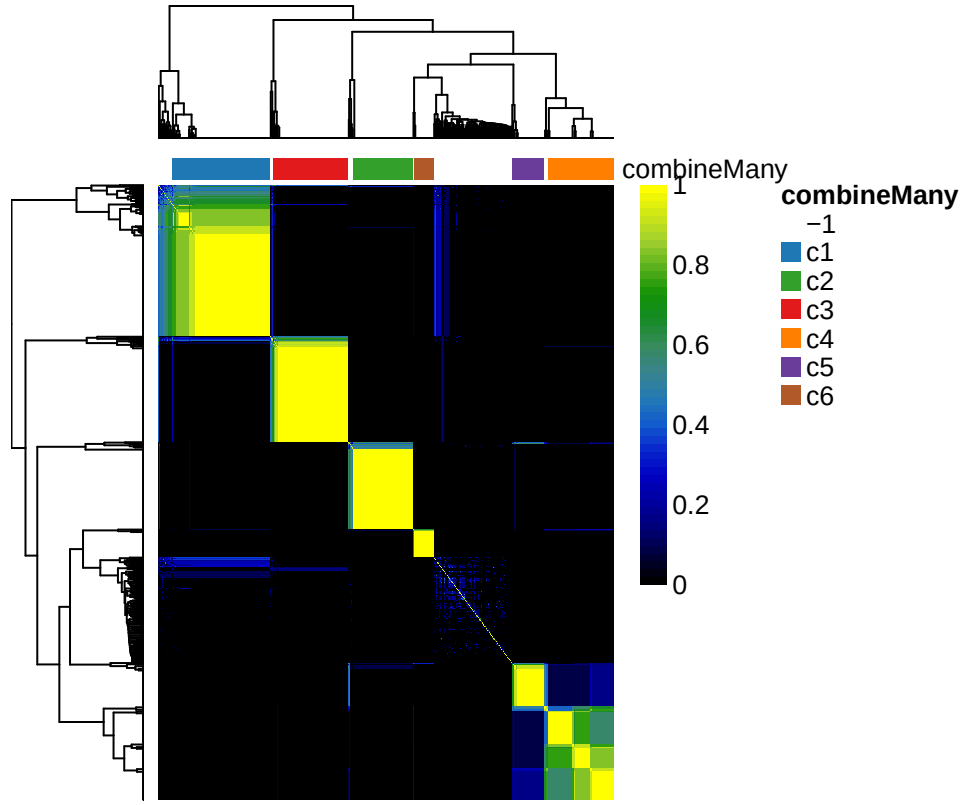


Figure 7. **Heatmap of the proportion of times the clusters were together across the different clusterings.**

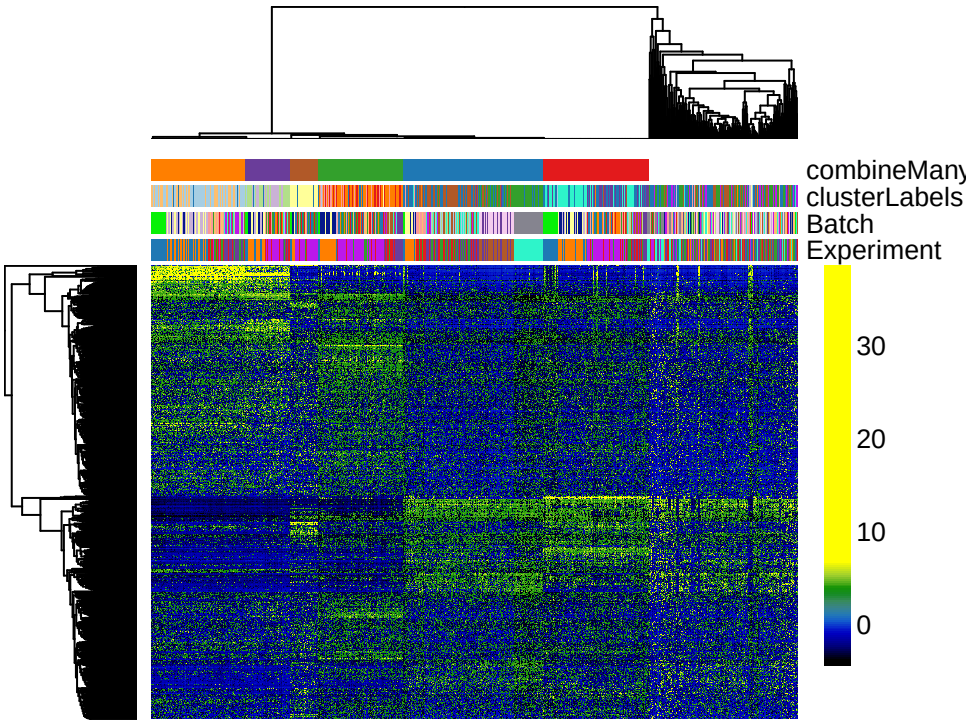


Figure 8. **Heatmap of the normalized values for the 1000 most variable genes (rows).**

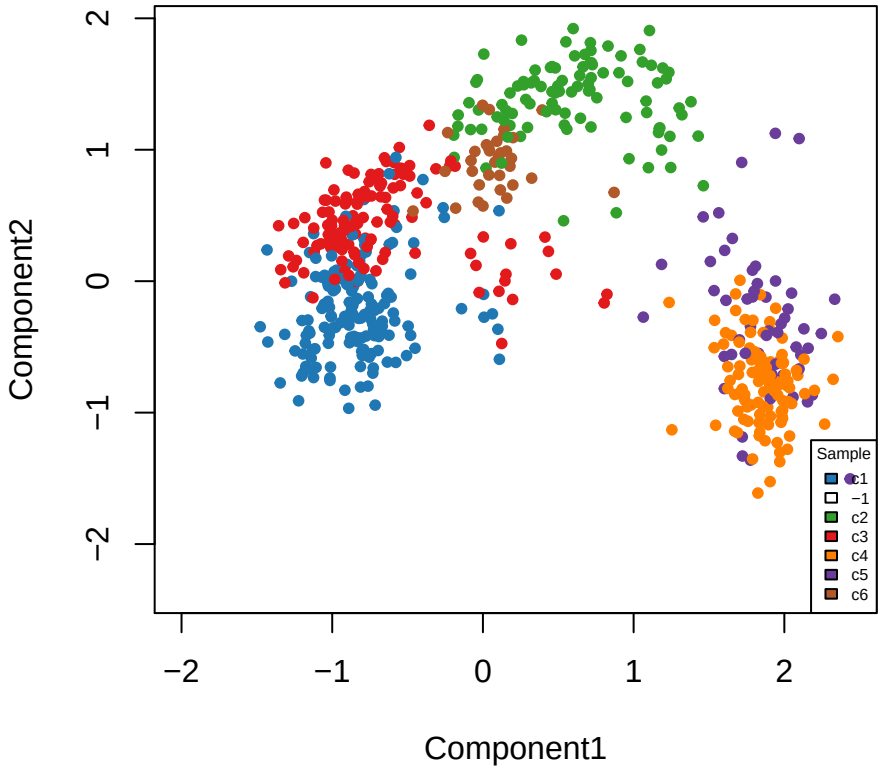


Figure 9. MDS of the low dimensional matrix with K = 50 dimensions. Colors correspond to the clusters found using our workflow.

We can now plot the MDS of the low dimensional matrix coloring the cells by their newly found clusters. See Figure 9 which is the same as Figure 5 but where colors for the cells are different.

```
palDF <- ceObj@clusterLegend[[1]]
pal <- palDF[, 'color']
names(pal) <- palDF[, 'name']
pal["-1"] = "transparent"
plot(fit$points, col = pal[primaryClusterNamed(ceObj)], main = '', pch = 20,
     xlab = 'Component1', ylab = 'Component2')
legend(x = 'bottomright', legend = names(pal), cex = .5,
       fill = pal, title = 'Sample')
```

Pseudotime ordering

We now want to order the clusters found using function RSEC from package clusterExperiment. From original published work, we know that the start clusters are 1 and 5 (HBC) and end original clusters are 15 (Microvillus), 9 and 12 (neuron), and 4, 7 (Sus). Additionally, we know that the GBC cluster should be a junction before the differentiation between Microvillus cells and Neurons. The correspondance with the original clusters is as follow

```
table(data.frame(original = clus.labels, ours = primaryClusterNamed(ceObj)))
```

```
##          ours
## original -1 c1 c2 c3 c4 c5 c6
##       -2 51 50  6 33  5  3  3
##       1  41 49  0  0  0  0  0
##       2   1  0 24  0  0  0  0
##       3   2  2 49  1  0  0  0
##       4   1  2  0 32  0  0  0
##       5  36 55  0  2  0  0  0
##       7   3  1  0 54  0  0  0
##       8  27  0  0  0  0  0  0
##       9   2  0  1  1 68  2  0
##       10  0  0  0  0  0 26  0
```

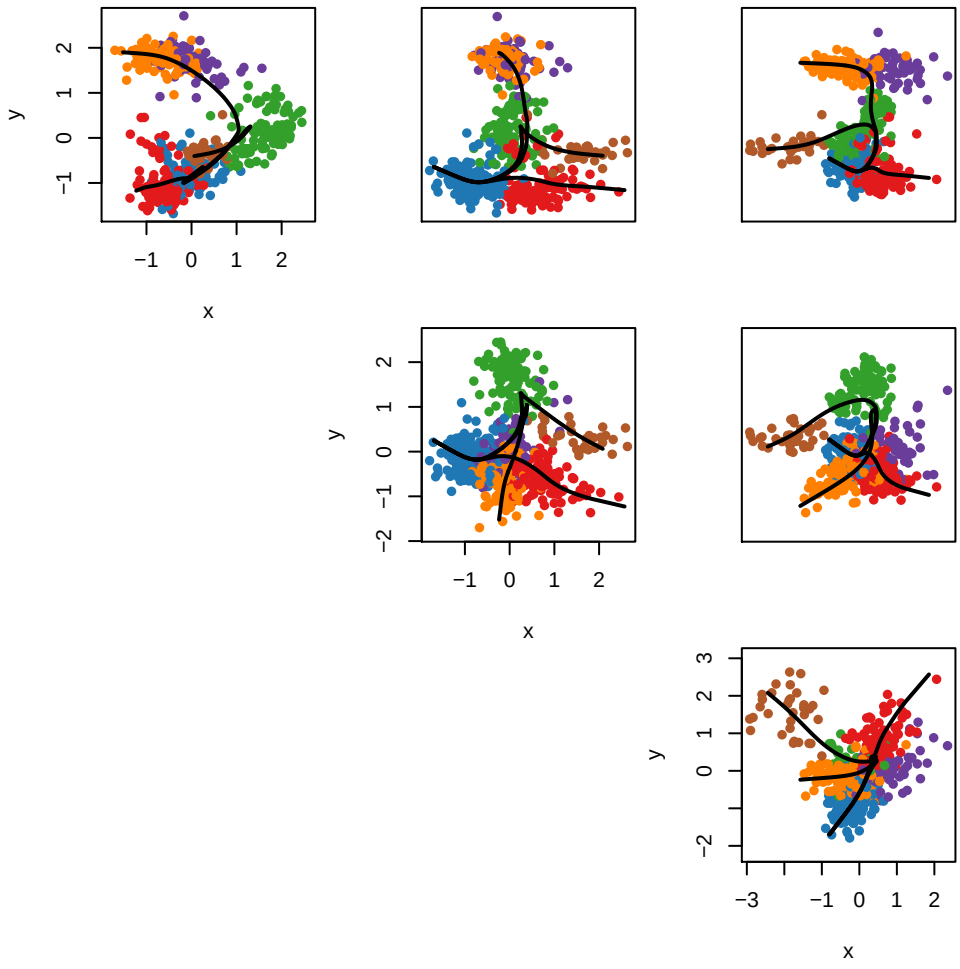
```
##         11  3  2 16  0  0  0  0
##         12  0  0  0  0 35  0  0
##         14  4  0  1  0  0 21  0
##         15  1  0  1  0  0  0 30
```

Cluster name	Description	Color	Correspondence
c1	HBC	blue	original 1, 5
c2	GBC	immature neurons	MV 1
c3	Sus	red	original 4, 7
c4	Neuron	orange	original 9, 12
c5	Immature Neuron	purple	original 10, 14
c6	Microvillus	cyan	original 15

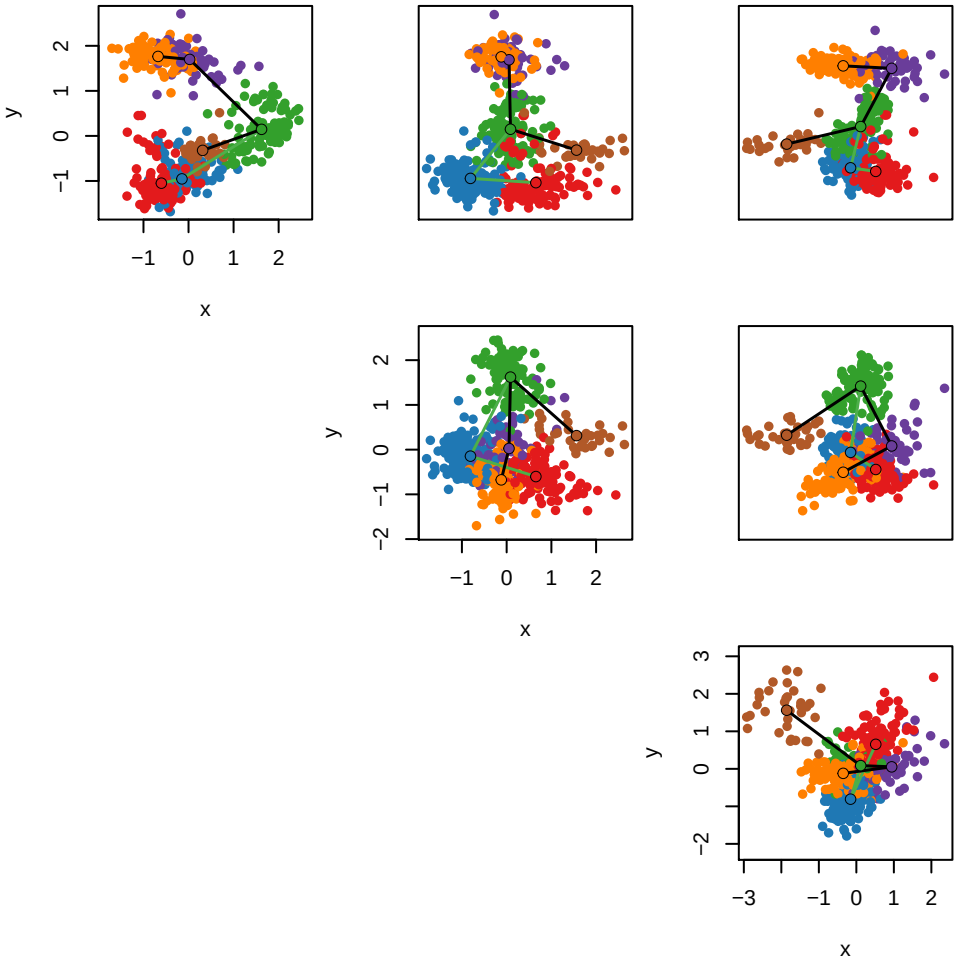
As the input for the speudotime ordering algorithm, we use the MDS of low dimensional matrix (K=50) where the number of dimensions for MDS is k=3. The algorithm is stable to the number of dimensions k in the MDS. We use the unsupervised version of slingshot where we only give the information of the start cluster but not the end clusters.
Kelly, would you like to give more information about slingshot?

```
our_cl <- primaryClusterNamed(ceObj)
cl = our_cl[our_cl != "-1"]
pal = pal[names(pal) != '-1']
```

```
X <- W[our_cl != "-1", ]
mds <- cmdscale(dist(X), eig = TRUE, k = 4)
X <- mds$points

lineages <- get_lineages(X, clus.labels = cl, start.clus = "c1")
curves <- get_curves(X, clus.labels = cl, lineages = lineages)
plot_curves(X, cl, curves, col.clus = pal)
```



```
plot_tree(X, cl, lineages, col.clus = pal)
```




```
print(lineages$lineage1)
```

```
## [1] "c1" "c2" "c5" "c4"
```

```
print(lineages$lineage2)
```

```
## [1] "c1" "c2" "c6"
```

```
print(lineages$lineage3)
```

```
## [1] "c1" "c3"
```

In this workflow, it is ok to use unsupervised. In some other cases, we have noticed that we need to give more knowledge to the algorithm to find the correct ordering. Here is the code to use slingshot in a supervised setting.

```
X <- W[our_cl != "-1", ]
mds <- cmdscale(dist(X), eig = TRUE, k = 4)
X <- mds$points

lineages <- get_lineages(X, clus.labels = cl, start.clus = "c1",
                         end.clus = c("c3", "c6"))
curves <- get_curves(X, clus.labels = cl, lineages = lineages)
plot_curves(X, cl, curves, col.clus = pal)
plot_tree(X, cl, lineages, col.clus = pal)

print(lineages$lineage1)
print(lineages$lineage2)
```

```
print(lineages$lineage3)
print(lineages$lineage4)
print(lineages$lineage5)
```

DE analysis

Kelly, would you like to complete this part? Here is the kind of plots we want to present

```
de <- read.csv('../data/oe_markers.txt', stringsAsFactors = F, header = F)
de <- de$V1
plotHeatmap(ceObj,
            visualizeData = assays(se)$normalizedValues[rownames(se) %in% de, ],
            clusterSamplesData = "dendrogramValue",
            whichClusters = "primary",
            main = 'Normalized values, 1000 most variable genes',
            breaks = 0.99)
```

Further developments

- DE analysis using slingshot
- DE analysis and imputation using zinbwave
- SingleCellExperiment class

Conclusion

This workflow provides guidance to perform downstream analysis of single-cell RNA-seq datasets in R. We propose a worflow in four steps: dimensionality reduction while adjusting for cell-level covariates, robust and stable clustering, pseudotime ordering, and DE analysis between the lineages. The workflow is general and flexible allowing the user to sustitute the statistical method used in each step of the worflow by a different method or R package and to input data that have been previously processed by different tools. We hope the workflow will ease the technical aspect of single-cell RNA-seq data analysis to help discovering new biological insights.

Software availability

This section will be generated by the Editorial Office before publication. Authors are asked to provide some initial information to assist the Editorial Office, as detailed below.

1. URL link to where the software can be downloaded from or used by a non-coder (AUTHOR TO PROVIDE; optional)
2. URL link to the author's version control system repository containing the source code (AUTHOR TO PROVIDE; required)
3. Link to source code as at time of publication (*F1000Research* TO GENERATE)
4. Link to archived source code as at time of publication (*F1000Research* TO GENERATE)
5. Software license (AUTHOR TO PROVIDE; required)

The three packages zinbwave, clusterExperiment, and slingshot used in the workflow are bioconductor packages and are available on github at respectively `https://github.com/drisso/zinbwave`, `https://github.com/epurdom/clusterExperiment`, and `https://github.com/kstreet13/slingshot`. The source code for this package can be found at `https://github.com/fperraudeau/singlecellworkflow` under license XXX.

```
sessionInfo()
```

```
## R version 3.4.0 (2017-04-21)
## Platform: x86_64-apple-darwin15.6.0 (64-bit)
## Running under: macOS Sierra 10.12.5
##
## Matrix products: default
## BLAS: /Library/Frameworks/R.framework/Versions/3.4/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/3.4/Resources/lib/libRlapack.dylib
##
## locale:
```

```
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## attached base packages:
## [1] parallel  stats4    stats     graphics  grDevices utils     datasets
## [8] methods   base
##
## other attached packages:
##  [1] rARPACK_0.11-0             digest_0.6.12
##  [3] RColorBrewer_1.1-2         Rtsne_0.13
##  [5] magrittr_1.5               gplots_3.0.1
##  [7] ggplot2_2.2.1              doParallel_1.0.10
##  [9] iterators_1.0.8            foreach_1.4.3
## [11] slingshot_0.0.3-5          princurve_1.1-12
## [13] GEOquery_2.42.0            zinbwave_0.99.4.2
## [15] BiocParallel_1.10.1        clusterExperiment_1.3.1
## [17] scone_1.0.0                scRNAseq_1.2.0
## [19] SummarizedExperiment_1.6.3 DelayedArray_0.2.7
## [21] matrixStats_0.52.2         Biobase_2.36.2
## [23] GenomicRanges_1.28.3       GenomeInfoDb_1.12.2
## [25] IRanges_2.10.2             S4Vectors_0.14.3
## [27] BiocGenerics_0.22.0        knitr_1.16
## [29] BiocStyle_2.4.0
##
## loaded via a namespace (and not attached):
##   [1] shinydashboard_0.6.0     R.utils_2.5.0
##   [3] RSQLite_1.1-2            AnnotationDbi_1.38.1
##   [5] htmlwidgets_0.8          grid_3.4.0
##   [7] trimcluster_0.1-2        RNeXML_2.0.7
##   [9] DESeq_1.28.0             munsell_0.4.3
##  [11] codetools_0.2-15         statmod_1.4.29
##  [13] scran_1.4.4              DT_0.2
##  [15] miniUI_0.1.1             colorspace_1.3-2
##  [17] energy_1.7-0             uuid_0.1-2
##  [19] pspline_1.0-17           robustbase_0.92-7
##  [21] bayesm_3.0-2             NMF_0.20.6
##  [23] tximport_1.4.0           GenomeInfoDbData_0.99.0
##  [25] hwriter_1.3.2            rhdf5_2.20.0
##  [27] rprojroot_1.2            EDASeq_2.10.0
##  [29] diptest_0.75-7           R6_2.2.1
##  [31] ggbeeswarm_0.5.3         taxize_0.8.4
##  [33] locfit_1.5-9.1           flexmix_2.3-14
##  [35] bitops_1.0-6             reshape_0.8.6
##  [37] assertthat_0.2.0         scales_0.4.1
##  [39] nnet_7.3-12              beeswarm_0.2.3
##  [41] gtable_0.2.0             phylobase_0.8.4
##  [43] RUVSeq_1.10.0            bold_0.4.0
##  [45] rlang_0.1.1              genefilter_1.58.1
##  [47] splines_3.4.0            rtracklayer_1.36.3
##  [49] lazyeval_0.2.0           hexbin_1.27.1
##  [51] rgl_0.98.1               yaml_2.1.14
##  [53] reshape2_1.4.2           abind_1.4-5
##  [55] GenomicFeatures_1.28.3   backports_1.1.0
##  [57] httpuv_1.3.3             tensorA_0.36
##  [59] tools_3.4.0              bookdown_0.4
##  [61] gridBase_0.4-7           stabledist_0.7-1
##  [63] dynamicTreeCut_1.63-1    Rcpp_0.12.11
##  [65] plyr_1.8.4               progress_1.1.2
##  [67] visNetwork_1.0.3         zlibbioc_1.22.0
##  [69] purrr_0.2.2.2            RCurl_1.95-4.8
##  [71] prettyunits_1.0.2        viridis_0.4.0
##  [73] zoo_1.8-0                cluster_2.0.6
##  [75] data.table_1.10.4        RSpectra_0.12-0
##  [77] mvtnorm_1.0-6            whisker_0.3-2
##  [79] gsl_1.9-10.3             aroma.light_3.6.0
##  [81] mime_0.5                 evaluate_0.10
```

```
##  [83] xtable_1.8-2             XML_3.98-1.7
##  [85] mclust_5.3               gridExtra_2.2.1
##  [87] compiler_3.4.0           biomaRt_2.32.1
##  [89] scater_1.4.0             tibble_1.3.3
##  [91] KernSmooth_2.23-15       R.oo_1.21.0
##  [93] htmltools_0.3.6          pcaPP_1.9-61
##  [95] segmented_0.5-2.0        BiocWorkflowTools_1.2.0
##  [97] tidyr_0.6.3              geneplotter_1.54.0
##  [99] howmany_0.3-1            DBI_0.6-1
## [101] MASS_7.3-47              fpc_2.1-10
## [103] MAST_1.2.1               boot_1.3-19
## [105] compositions_1.40-1      ade4_1.7-6
## [107] ShortRead_1.34.0         Matrix_1.2-10
## [109] R.methodsS3_1.7.1        gdata_2.18.0
## [111] igraph_1.0.1             rncl_0.8.2
## [113] GenomicAlignments_1.12.1 registry_0.3
## [115] numDeriv_2016.8-1        locfdr_1.1-8
## [117] plotly_4.7.0             xml2_1.1.1
## [119] annotate_1.54.0          vipor_0.4.5
## [121] rngtools_1.2.4           pkgmaker_0.22
## [123] XVector_0.16.0           stringr_1.2.0
## [125] copula_0.999-16          ADGofTest_0.3
## [127] softImpute_1.4           Biostrings_2.44.1
## [129] rmarkdown_1.5            dendextend_1.5.2
## [131] edgeR_3.18.1             kernlab_0.9-25
## [133] shiny_1.0.3              Rsamtools_1.28.0
## [135] gtools_3.5.0             modeltools_0.2-21
## [137] rjson_0.2.15             nlme_3.1-131
## [139] jsonlite_1.5             viridisLite_0.2.0
## [141] limma_3.32.2             lattice_0.20-35
## [143] httr_1.2.1               DEoptimR_1.0-8
## [145] survival_2.41-3          glue_1.0.0
## [147] FNN_1.1                  prabclus_2.2-6
## [149] glmnet_2.0-10            class_7.3-14
## [151] stringi_1.1.5            mixtools_1.1.0
## [153] latticeExtra_0.6-28      caTools_1.17.1
## [155] memoise_1.1.0            dplyr_0.7.0
## [157] ape_4.1
```

Author contributions

In order to give appropriate credit to each author of an article, the individual contributions of each author to the manuscript should be detailed in this section. We recommend using author initials and then stating briefly how they contributed.

Competing interests

All financial, personal, or professional competing interests for any of the authors that could be construed to unduly influence the content of the article must be disclosed and will be displayed alongside the article. If there are no relevant competing interests to declare, please add the following: 'No competing interests were disclosed'.

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References

Dijk, David van, Juozas Nainys, Roshan Sharma, Pooja Kathail, Ambrose J Carr, Kevin R Moon, Linas Mazutis, Guy Wolf, Smita Krishnaswamy, and Dana Pe'er. 2017. "MAGIC: A diffusion-based imputation method reveals gene-gene interactions in single-cell RNA-sequencing data." *BioRxiv*. doi:10.1101/111591.

Fletcher, Russell B, Diya Das, Levi Gadye, Kelly N Street, Ariane Baudhuin, Allon Wagner, Michael B Cole, et al. 2017. "Deconstructing Olfactory Stem Cell Trajectories at Single-Cell Resolution." *Cell Stem Cell* 20 (6). Elsevier: 817–830.e8. doi:10.1016/j.stem.2017.04.003.

Lun, Aaron T.L., Davis J. McCarthy, and John C. Marioni. 2016. "A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Bioconductor." *F1000Research* 5 (October): 2122. doi:10.12688/f1000research.9501.2.

McCarthy, Davis J., Kieran R. Campbell, Aaron T. L. Lun, and Quin F. Wills. 2017. "Scater: pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R." *Bioinformatics*, January, btw777. doi:10.1093/bioinformatics/btw777.

Risso, Davide, Fanny Perraudeau, Svetlana Gribkova, Sandrine Dudoit, and Jean-Philippe Vert. 2017. "ZINB-WaVE: A general and flexible method for signal extraction from single-cell RNA-seq data." doi:10.1101/125112.