Bioinformatics figures for publication

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11 August, 2022

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Chapter 1

Preface

One of the many questions Biologists ask Bioinformaticians is "How can I best represent these results in the form of a figure for my publication?". This workshop seeks to address this question by focusing on the figures that can be developed to address a biological question and provide explanations of how to interpret such figures. This is aimed at improving understanding of these figures generated in bioinformatics analysis and helping Biologists present their discoveries/observations in the best possible way. This workshop will be very helpful for Biologists to prepare bioinformatics figures for their publications and also for looking into their data in a better way. This will help improve the communications between them and Bioinformaticians as well.

Here we will go through some of the most commonly used figures in the bioinformatics analysis of bulk and single-cell RNA-seq data.

Chapter 2

Prerequisites

Before getting started with the rest of the analysis, it is important that we have the necessary data and software that will be used in this analysis.

2.1 Data

2.1.1 Bulk RNA-seq data

An RNA-seq dataset generated in a published study (Delconte., et al. Nature Immunology 2016) is used as an example dataset in this protocol.

Two samples (wild-type and Cish-/- natural killer cells) are included in this dataset and each sample has two biological replicates. These data were already deposited in the Gene Expression Omnibus database (GSE79409). However for the convenience of this analysis, processed counts from the Bulk RNA-seq analysis workshop and available here are used.

2.1.2 scRNA-seq data

A subset of the scRNA-seq dataset that was generated in a published study (Chen., et al. Nature Biotech 2020) is used as an example dataset. The data consists of two well-characterized cellular reference samples (human breast cancer cell line (HCC1395, sample A) and the matched normal B lymphocyte line (HCC1395BL, sample B)) that were captured using the 10X platform. The data is available in the SRA repository under accession code no. PRJNA504037. However, for the convenience of this analysis, processed counts from the scRNA-seq analysis workshop and available here are also used.

2.2 SOFTWARE

The following software tools should be installed on your computer:

- R [R Core Team, 2022]
- Rstudio [Allaire et al., 2022]
- Rsubread [Shi et al., 2022]
- limma [Smyth et al., 2022]
- edgeR
- org.Hs.eg.db
- statmod
- Seurat
- SingleR
- monocle

Consult the R Project website for the installation of R (https://www.r-project.org/) [R Core Team, 2022]. Make sure the latest release version of R is downloaded and installed. After R is installed, launch R and type the following commands to install *Rsubread*, *limma*, *edgeR*, *org.Hs.eg.db*, *statmod*, *Seurat*, *SingleR* and *Monocle*:

```
if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
if (!requireNamespace("devtools", quietly = TRUE))
  install.packages("devtools")
BiocManager::install(
  c(
    'Rsubread',
    "org.Hs.eg.db",
    "SingleR",
    'BiocGenerics',
    'DelayedArray',
    'DelayedMatrixStats',
    'S4Vectors',
    'SingleCellExperiment',
    'limma',
    'edgeR',
    'SummarizedExperiment',
    'batchelor',
    'Matrix.utils',
    'celldex',
    'lme4',
    'HDF5Array',
    'terra',
    'monocle',
```

```
    'ggrastr'
  ),
  update = T
)

if (!requireNamespace("statmod", quietly = TRUE))
  install.packages("statmod")
if (!requireNamespace("Seurat", quietly = TRUE))
  install.packages("Seurat")
if(requireNamespace("ggplot2"))
  install.packages(c("ggplot2","forcats"))
```

Chapter 3

Bulk RNA-seq data analysis

3.1 Background

Previous results from the Bulk RNA-seq analysis workshop were saved in the "Workshop_RNAseq" directory on All staff share as an R-object "Counts2.RData" or can also be downloaded from here. The R object will be imported into R in order to run the rest of the analysis.

```
#load the required package
library(limma)
load("Counts2.RData") # loads the counts dataset
```

There are a number of ways in which you can visualize the results from RNA-seq analysis, while in this chapter we attempt to cover the most common ways of visualizing the data, they are by no means exhaustive.

3.2 Multi-Dimensional scaling plot (MDS) or PCA

The Multi-Dimensional scaling (MDS) plot is a means of visualizing the level of similarity of individual samples in a dataset. In the plot, the distance between each pair of samples is the root-mean-square deviation (Euclidean distance) for the top genes. Distances on the plot can be interpreted as leading log2-fold-change, meaning the typical (root-mean-square) log2-fold-change between the samples for the genes that distinguish those samples.

```
col<-c("orange","purple")[factor(targets$CellType)]
plotMDS(
  y,
  cex = 0.8,
  col=col
)
```

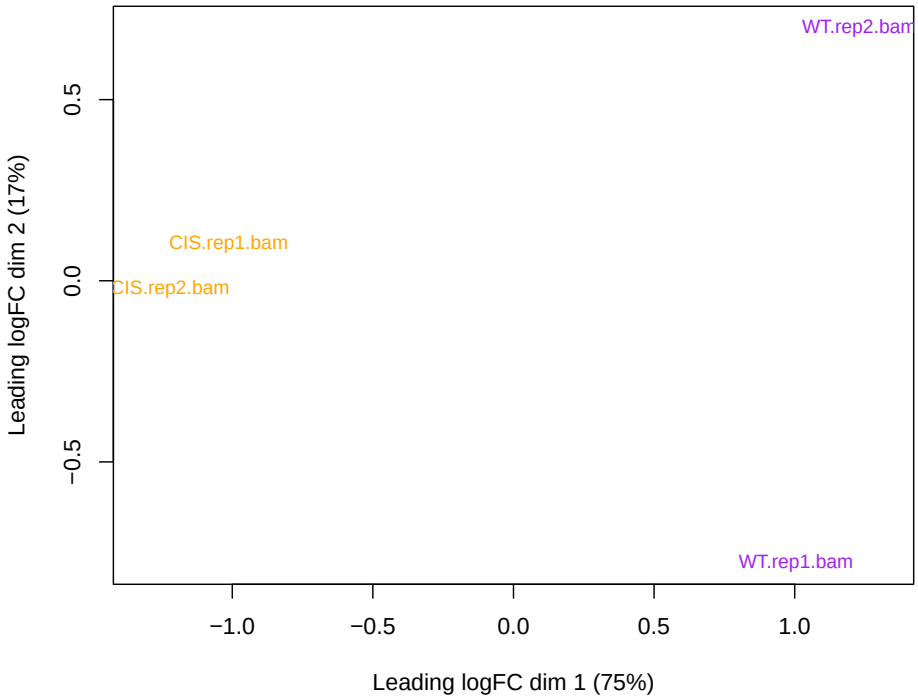


Figure 3.1: MDS plot showing speration and similarity of samples

We can also generate a PCA plot using the same function *plotMDS* by setting *gene.selection = "common"* which results in constructing a PCA plot from the top genes with the most considerable standard deviations across the samples.

```
col<-c("orange","purple")[factor(targets$CellType)]
plotMDS(
  y,
  cex = 0.8,
  gene.selection = "common",
  col=col
)
```

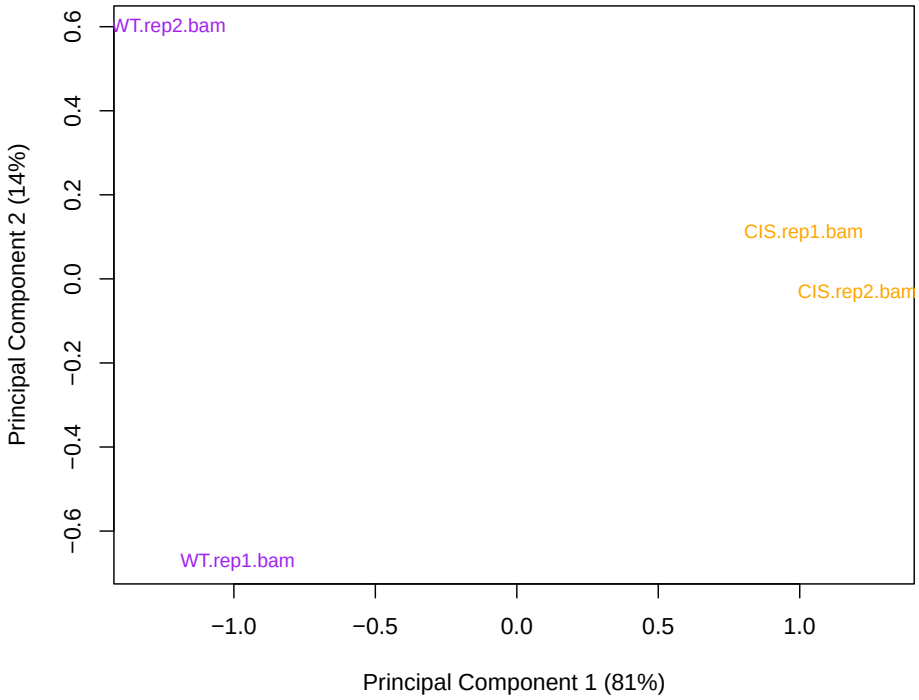


Figure 3.2: PCA plot showing speration and similarity of samples

3.3 Venn diagram

If differential expression analysis is performed on more than 1 comparison, a Venn diagram can be used to quickly compare the number of differentially expressed genes that are either uniquely DE in one comparison or DE in other comparisons. This can be easily done using the **vennDiagram** function in **limma**.

3.4 Mean-difference plot

A mean-difference plot or MD plot is a plot that can be used to show the fold-change differences against the average expression values of all genes used in the analysis. This can be generated easily using the *plotMD* function within the **limma** package.

```
plotMD(fit.contr, column = 1,status = dt,cex=0.8)
```

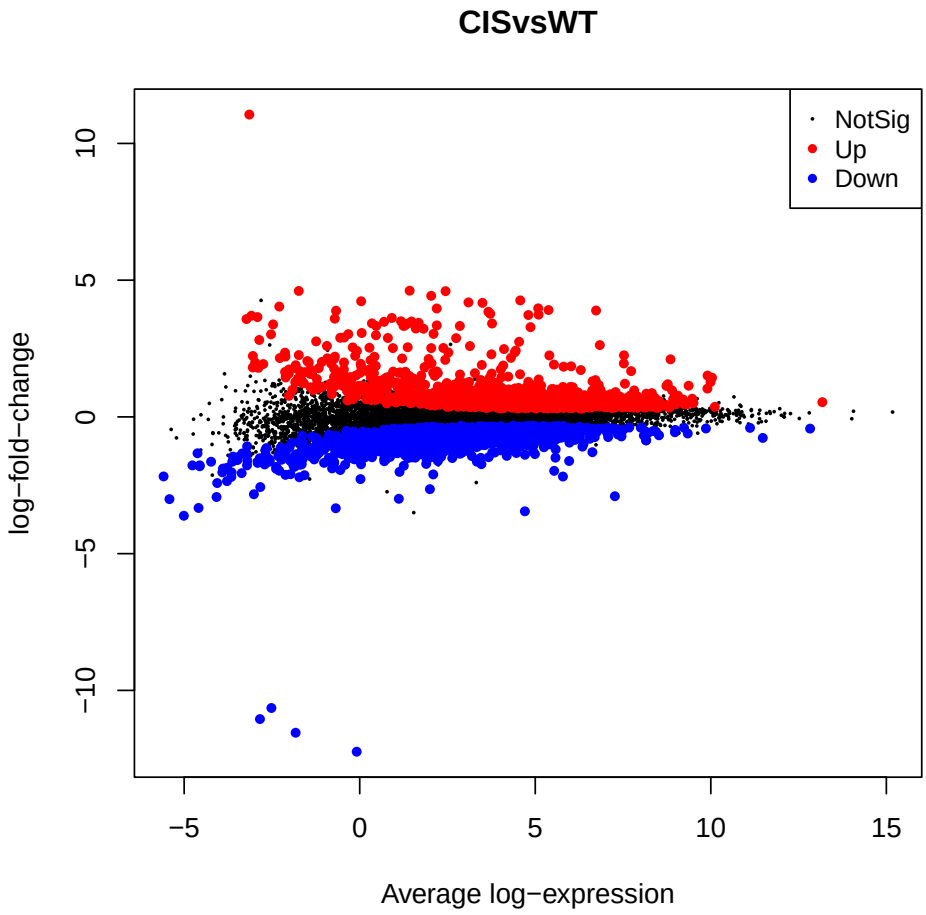


Figure 3.3: Mean-Difference plot. Represents relationship between mean expression and fold-changes

3.5 Volcano plot

A volcano plot shows the relationship between the log fold-change on the x-axis and a measure of statistical significance on the y-axis. The measure of significance can be -log(p-value) or the B-statistics. Here, we will use the *volcanoplot* function within the **limma** package.In the volcano plot below, differentially expressed genes are highlighted with red for up-regulated and blue for down-regulated genes.

```
#highlight de genes
col<-c("blue","black","red")[factor(dt[,"CISvsWT"])]
volcanoplot(fit.contr,col=col)
```

Alternatively, an interactive mean-difference plot can be generated using *glMDPlot* in **Glimma** which outputs an interactive html page with results displayed in the left panel and the expression values for the selected gene. A gene within the dataset can be searched through a search bar.

```
if(!require(Glimma))
{
  BiocManager::install("Glimma")
  library(Glimma)
}
```

```
## Loading required package: Glimma
```

```
glMDPlot(fit.contr, coef=1, status=dt, main=colnames(fit.contr)[1],
         side.main="ENTREZID", counts=y$E, groups=colnames(contr),
         launch = F)
```

Click here for interactive version

Hover over points to see sample-wise expression

- Click on column names to sort by column
- Click rows on tables to highlight gene

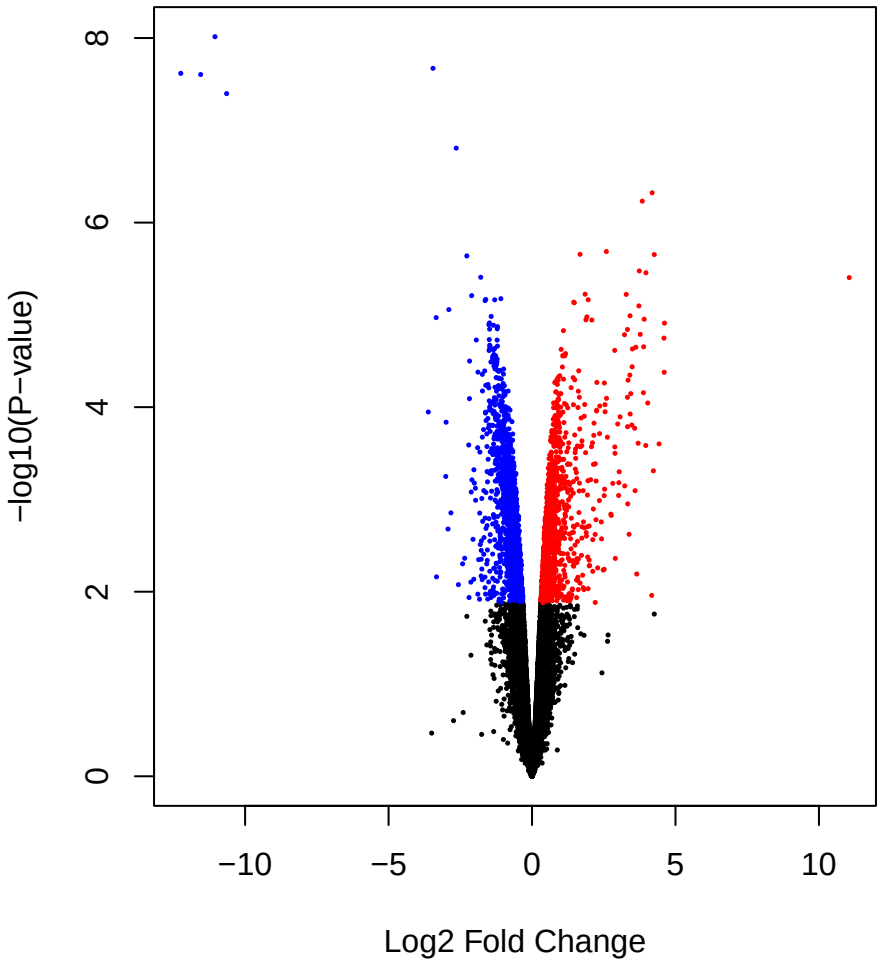


Figure 3.4: Volcano plot. Shows the relationship between fold-change and significance of change

3.6 Heatmaps

Heatmaps are a great way of demonstrating the differences in the expression patterns between different conditions in a dataset. *Heatmaps are commonly used in both bulk and single-cell RNAseq data presentation.*

The *coolmap* function within the **limma** package is an extension of the *heatmap.2* function within the **gplots** package is used to generate the heatmap. The heatmap below shows the top 100 differentially expressed genes' expression patterns between CIS and WT.

```
heat.data <- y$E[head(rownames(de.genes), n = 100), ]
colnames(heat.data) <- targets$Sample
rownames(heat.data) <- head(de.genes$Symbol, n = 100)
coolmap(
  heat.data,
  margin = c(5, 4),
  cexCol = 0.8,
  lhei = c(0.8, 4),
  cexRow = 0.6,
  adjRow=0
)
```

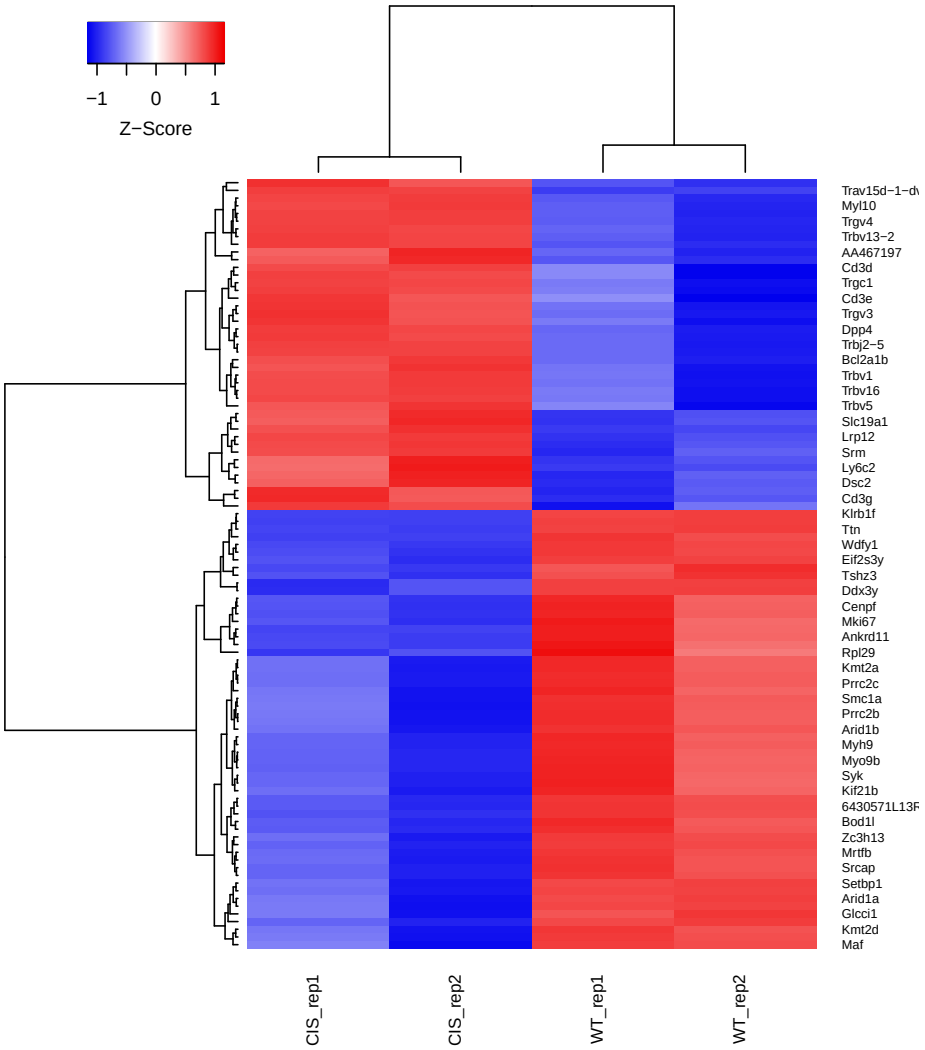


Figure 3.5: Heatmap plot of differentially expressed genes in CIS vs WT

3.7 Dendrogram

Similarly, we can also use a cluster dendrogram to show the relationship between samples.

```
d<-dist(t(heat.data))
hclst<-hclust(d,method = "ward.D2")
```

```
plot(
    hclst,
    cex.main = 1,
    cex.lab = 0.9,
    xlab ="",
    cex.axis = 0.8)
```

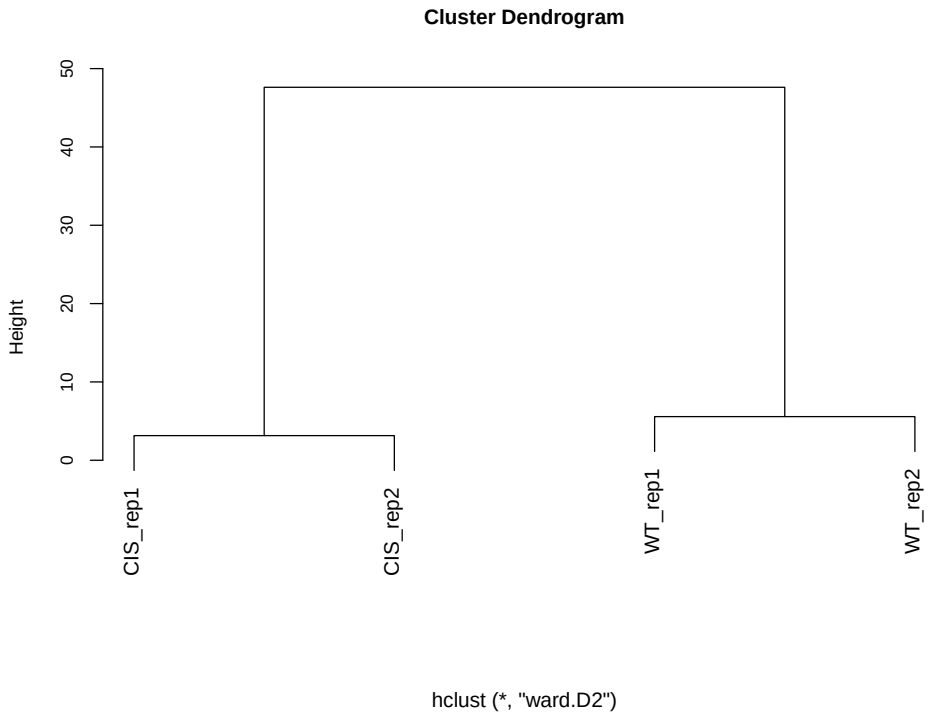


Figure 3.6: Dendrogram showing clustering of samples

3.8 Pathway Analysis

From the differential expression analysis, there were some 3181 differentially expressed genes. This is quite a long list of genes to interpret and nearly impossible to go through a gene at a time to gain any meaningful biological understanding. A common downstream analysis step is to understand pathways/gene networks in that the genes are implicated.

```
enrich.pvalue<-0.00001
```

3.8.1 Gene Ontology enrichment

Here, we use the *goana* function within the **limma** package to test for enrichment of differentially expressed genes between the CIS vs WT. Note that the *p-values* returned by *goana* are **unadjusted for multiple testing**. It is therefore, advisable that if the results are to be published, only terms with very small p-values should be included. For instance, in the example below, only terms with a p-value $< 10^{-5}$ are retained.

We use the output from the differential expression analysis and test for over representation for the Up and Down-regulated genes separately.

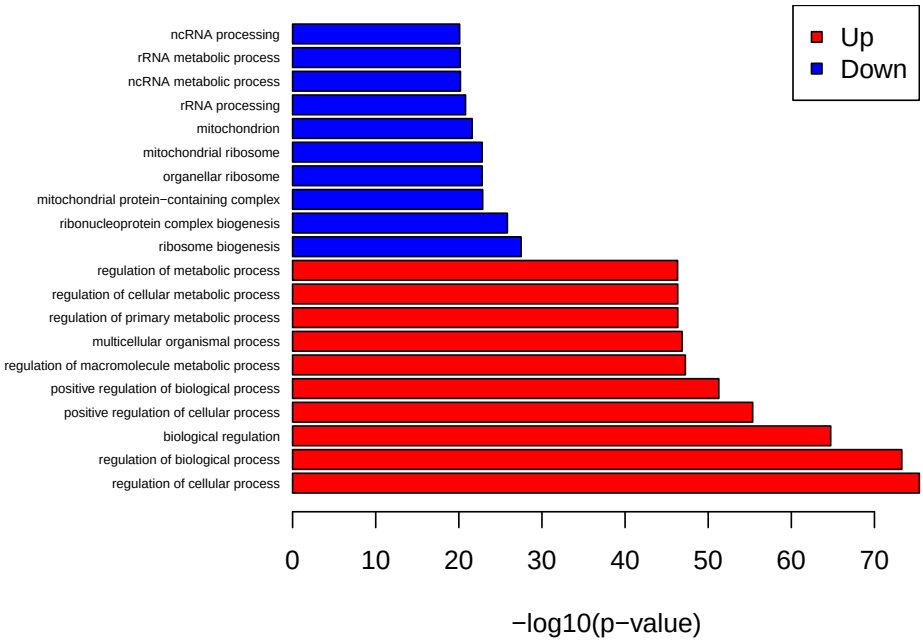
```
if(!requireNamespace("GO.db"))
  BiocManager::install("GO.db")
library(GO.db)
go.rst<-goana(fit.contr,species="Mm")
#exclude terms not meeting our cut-off
go.rst<-go.rst[rowSums(go.rst[,c("P.Up","P.Down")]<enrich.pvalue)==1,]
```

A simple way to visualize the enrichment results is through a bar plot as shown below which shows the top 10 enriched terms in each direction.

```
top.up <- head(go.rst[order(go.rst$P.Up, decreasing = F), ], n = 10)
top.down <-
  head(go.rst[order(go.rst$P.Down, decreasing = F), ], n = 10)
bar.data <-
  rbind(
    data.frame(
      Term = top.up$Term,
      P.value = top.up$P.Up,
      Dir = "up"
    ),
    data.frame(
      Term = top.down$Term,
```

```
      P.value = top.down$P.Down,
      Dir = "down"
    )
  )

bar.data <- bar.data[order(bar.data$P.value, decreasing = F), ]
par(mai = c(0.8, 2, 0.5, 0.5))
bb <- barplot(
  -log10(bar.data$P.value),
  horiz = T,
  xlab = " -log10(p-value)",
  cex.names = 0.5,
  col = c("red", "blue")[factor(bar.data$Dir)]
)
axis(
  2,
  line = -0.8,
  at = bb,
  labels = bar.data$Term,
  tick = F,
  las = 2,
  cex.axis = 0.5
)
legend(
  "topright",
  legend = c("Up", "Down"),
  pch = 22,
  pt.bg = c("red", "blue")
)
```


Up
Down
ncRNA processing
rRNA metabolic process
ncRNA metabolic process
rRNA processing
mitochondrion
mitochondrial ribosome
organellar ribosome
mitochondrial protein−containing complex
ribonucleoprotein complex biogenesis
ribosome biogenesis
regulation of metabolic process
regulation of cellular metabolic process
regulation of primary metabolic process
multicellular organismal process
regulation of macromolecule metabolic process
positive regulation of biological process
positive regulation of cellular process
biological regulation
regulation of biological process
regulation of cellular process
0
10
20
30
40
50
60
70
−log10(p−value)

An alternative popular choice is the use of bubble plots to represent the enrichment results. One can use the **ggplot2** to generate a simple bubble plot using the results from **goana** as follows. Enriched terms have been ordered by the Ontology term. The size of the bubbles is the proportion of genes that were up-regulated in that pathway.

```
library(ggplot2)
library(forcats)
#Get pathways enriched in the CIS condition when compared to the WT condition
go.rst_up <- go.rst[go.rst$P.Down > go.rst$P.Up,
                    c("Term", "Ont", "N", "Up", "P.Up")]
ggplot(go.rst_up, aes(
  y = reorder(Term, as.numeric(factor(Ont))),
  x = -log10(P.Up),
  size = Up / N
)) + geom_point(aes(color = -log10(P.Up)), alpha = 1.0) +
  geom_tile(aes(width = Inf, fill = Ont), alpha = 0.1) +
  scale_fill_manual(values = c("green", "red", "blue"))
```

Add gene information (optional)

Sometimes you may want to know what genes are enriched in each of the terms, this section allows you to add gene details.

First, we extract the genes that are associated with the catalytic activity GO terms. This results in a list mapping the GO term and the associated genes.

```
if(!require(GO.db))
  BiocManager::install("GO.db")
if(!require(org.Mm.eg.db))
  BiocManager::install("org.Mm.eg.db")
```

```
## Loading required package: org.Mm.eg.db
```

```
go.EntrezID <- as.list(org.Mm.egGO2ALLEGS)
go.rst <- tibble::rownames_to_column(go.rst, var = "GO.ID")
```

```
#Add gene details
go.rst$Genes.Up <- unlist(lapply(go.rst$GO.ID, function(x) {
  xx <- go.EntrezID[[x]]
  if (is.null(xx) |
      with(go.rst[go.rst$GO.ID == x,], Up == 0 |
           P.Up > enrich.pvalue))
    return("-")
  x <- intersect(with(de.genes,EntrezID[logFC>0]),xx)
```

Figure 3.7: Bubble plot showing GO terms that were up-regulated in CIS vs WT

```
  x <-
    paste0(with(fit.contr$genes, Symbol[EntrezID %in% xx]), collapse = "|")
  return(x)
}))
go.rst$Genes.Down <- unlist(lapply(go.rst$GO.ID, function(x) {
  xx <- go.EntrezID[[x]]
  if (is.null(xx) |
      with(go.rst[go.rst$GO.ID == x,], Down == 0 |
           P.Down > enrich.pvalue))
    return("-")
  x <- intersect(with(de.genes,EntrezID[logFC<0]),xx)
  x <-
    paste0(with(fit.contr$genes, Symbol[EntrezID %in% xx]), collapse = "|")
  return(x)
}))
```

3.8.2 KEGG pathway enrichment

Pathway enrichment can also be performed against the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/) database. The KEGG database contains curated molecular pathways and disease signatures. To perform this analysis we use the *kegga* function within the **limma** which is similar to the *goana* function we used above.

```
kegg.rst<-kegga(fit.contr,species="Mm")
topKEGG(kegg.rst)
```

```
##                                                Pathway    N  Up Down
## path:mmu03008       Ribosome biogenesis in eukaryotes   75  37    4
## path:mmu01100                     Metabolic pathways 1038 206   75
## path:mmu04810        Regulation of actin cytoskeleton  143   6   45
## path:mmu05206                    MicroRNAs in cancer  121   6   40
## path:mmu04919       Thyroid hormone signaling pathway   93   3   32
## path:mmu03010                               Ribosome  137  39    1
## path:mmu01240              Biosynthesis of cofactors  111  32    2
## path:mmu04330                Notch signaling pathway   43   2   18
## path:mmu00310                     Lysine degradation   52   1   20
## path:mmu03020                         RNA polymerase   32  14    1
## path:mmu00190              Oxidative phosphorylation  118  32    0
## path:mmu04062            Chemokine signaling pathway  119   5   34
## path:mmu04611                    Platelet activation   77   0   25
## path:mmu05200                     Pathways in cancer  323  26   71
## path:mmu05205                  Proteoglycans in cancer  126   8   34
## path:mmu04658        Th1 and Th2 cell differentiation   69   6   22
## path:mmu05224                           Breast cancer   81   8   24
## path:mmu05202 Transcriptional misregulation in cancer  121  16   32
## path:mmu03050                             Proteasome   44  15    0
## path:mmu01232                  Nucleotide metabolism   65  19    0
##                       P.Up       P.Down
## path:mmu03008 1.182585e-14 9.951210e-01
## path:mmu01100 5.674601e-12 1.000000e+00
## path:mmu04810 9.998630e-01 4.679254e-08
## path:mmu05206 9.987434e-01 5.746746e-08
## path:mmu04919 9.996663e-01 4.319019e-07
## path:mmu03010 6.123733e-07 1.000000e+00
## path:mmu01240 4.561006e-06 9.999989e-01
## path:mmu04330 9.789811e-01 6.418221e-06
## path:mmu00310 9.991527e-01 9.325271e-06
## path:mmu03020 1.341355e-05 9.916924e-01
## path:mmu00190 1.832128e-05 1.000000e+00
## path:mmu04062 9.995796e-01 2.081620e-05
```

```
## path:mmu04611 1.000000e+00 2.435669e-05
## path:mmu05200 9.971966e-01 4.089375e-05
## path:mmu05205 9.933378e-01 7.494761e-05
## path:mmu04658 8.861202e-01 1.000116e-04
## path:mmu05224 8.230146e-01 1.784375e-04
## path:mmu05202 4.708773e-01 1.811724e-04
## path:mmu03050 2.086057e-04 1.000000e+00
## path:mmu01232 3.146027e-04 1.000000e+00
```

Exercise Use similar steps as above to generate a barplot and bubble plot to visualise the enrichment of KEGG pathways in CIS vs WT.

3.8.3 Gene Set Enrichment Analysis

GO and KEGG enrichment analysis uses the list of differentially expressed genes to test for over-representation of these genes in GO terms and KEGG pathways respectively. As such the results typically depend on the cut-offs used to get the differentially expressed genes.

Gene Set Enrichment Analysis is performed using *roast*, a gene set enrichment function within the **limma** package. Unlike above where we focused on a subset of genes(differentially genes), here all the genes in the set are used to test for gene expression signatures or pathways. A test is performed against the GO terms for demonstrative purposes, alternatively, if you wish to test against a specific gene set, this can quickly be done by importing that list.

We then use *roast* to test for enrichment between CIS and WT. The design matrix 'design', the expression values 'y' and the contrast matrix 'contr' from the imported results are used to perform gene set test and the GO term enrichment.

The gene set enrichment figures generated here for bulk RNA-seq data can also be used for scRNA-seq data.

```
go.all <- go.EntrezID[go.rst$GO.ID]
names(go.all) <- go.rst$Term
roast.GSEA <- roast(y,
                    index = go.all,
                    design = design,
                    contrast = contr)
head(roast.GSEA)
```

```
##                                    NGenes   PropDown    PropUp Direction
## 90S preribosome                        28 0.07142857 0.7142857        Up
## RNA processing                        809 0.26205192 0.4066749        Up
## preribosome                            79 0.06329114 0.6708861        Up
## meiotic chromosome segregation         63 0.55555556 0.0952381      Down
```

```
## mitotic sister chromatid segregation    150 0.52000000 0.1666667      Down
## regulation of chromosome separation      69 0.47826087 0.1884058      Down
##                                       PValue       FDR PValue.Mixed  FDR.Mixed
## 90S preribosome                       0.0005 0.1000000       0.0060 0.06227709
## RNA processing                        0.0005 0.1000000       0.0555 0.06227709
## preribosome                           0.0020 0.1253906       0.0130 0.06227709
## meiotic chromosome segregation        0.0035 0.1253906       0.0210 0.06227709
## mitotic sister chromatid segregation 0.0035 0.1253906       0.0135 0.06227709
## regulation of chromosome separation  0.0035 0.1253906       0.0160 0.06227709
```

```
term.test<-rownames(roast.GSEA)[1]
```

Next, we can visualize the gene set enrichment results using a barcode plot. The barcode plot is used to plot the positions of genes within a set in a ranked list of statistics. The statistics are ranked from left to right i.e. from smallest to largest.

To do so, the gene identified for the term should first be mapped to the index position of each gene in the expression matrix;

```
index <- rownames(fit.contr) %in% go.all[[rownames(roast.GSEA)[1]]]
barcodeplot(
  fit.contr$coefficients[, "CISvsWT"],
  index = index,
  labels = c("CS", "WT"),
  main = rownames(roast.GSEA)[1]
)
```

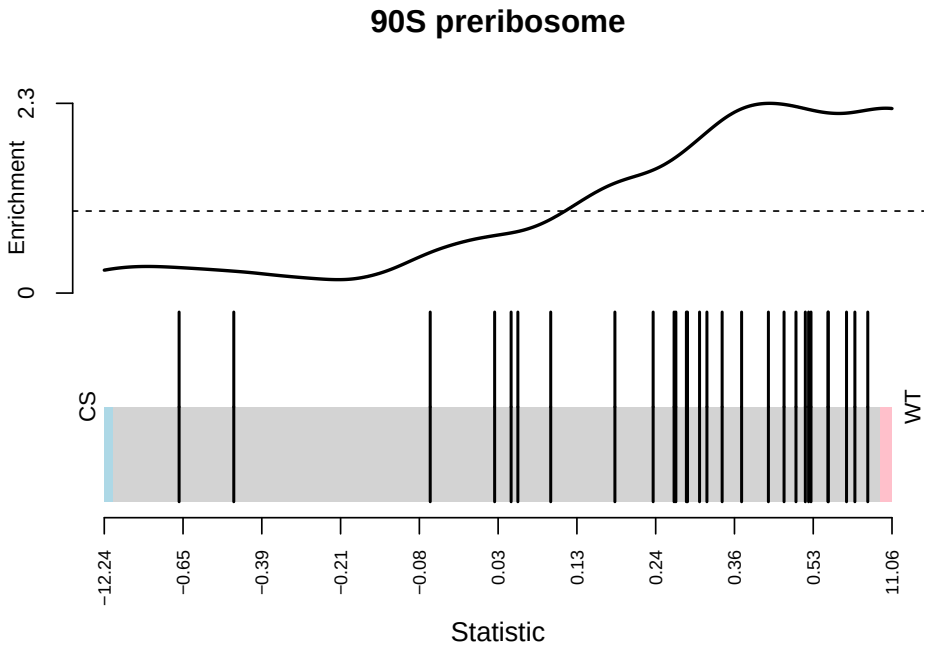


Figure 3.8: Barcode plot showing the enrichment of 90S preribosome in CS vs WT

Chapter 4

scRNA-seq data analysis

4.1 Background

As discussed previously in section 2.1.2, processed counts from the scRNA-seq workshop are used here to demonstrate the different types of figures that can be generated for publications from scRNA-seq. These can be downloaded from here or from the "All Staff Share/Workshop_scRNAseq"

4.2 tSNE plots

t-distributed stochastic neighbor embedding (t-SNE) is a statistical method for visualizing high-dimensional data by giving each data point a location in a two or three-dimensional map. It is a dimensionality reduction technique which is a way to graphically simplify very large datasets.

```
library(Seurat)
```

```
## Attaching SeuratObject
```

```
## Attaching sp
```

```
library(ggplot2)
load("Counts_scRNA-norm.RData")
```

```
DimPlot(
  counts_st,
```

```
  reduction = "tsne",
  label = T,
  size = 0.5,
  repel = T,
  cols = DiscretePalette(length(levels(Idents(
    counts_st
  ))))
)
```

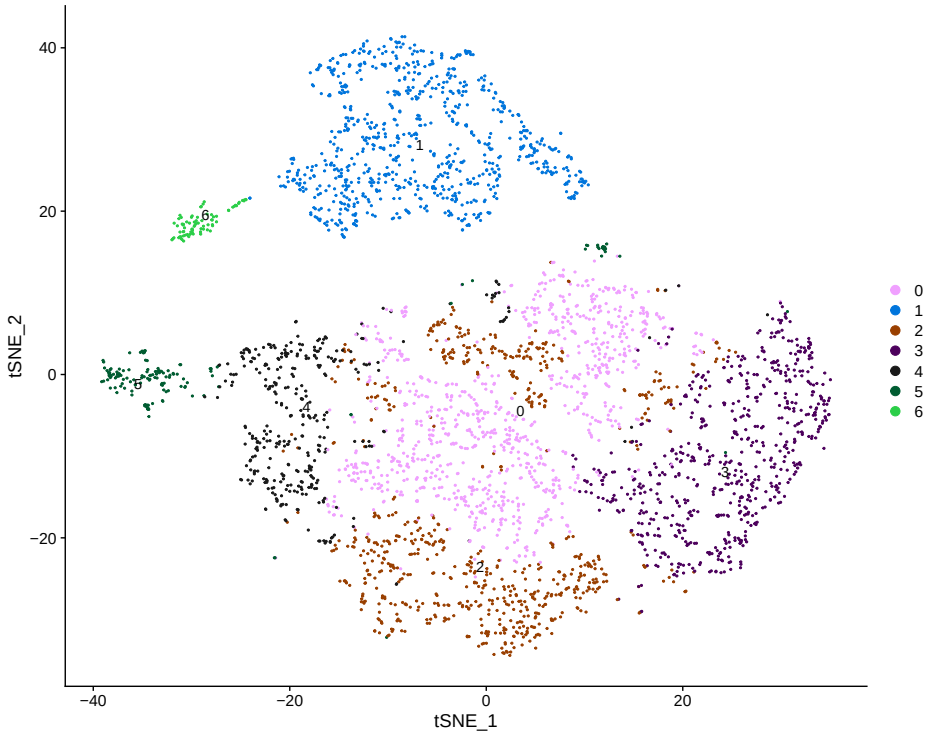


Figure 4.1: tSNE plot showing the clustering of cells

```
DimPlot(
  counts_st,
  reduction = "tsne",
  label = T,
  group.by = "Sample",
  size = 0.5,
  repel = T
)+ggtitle("")
```

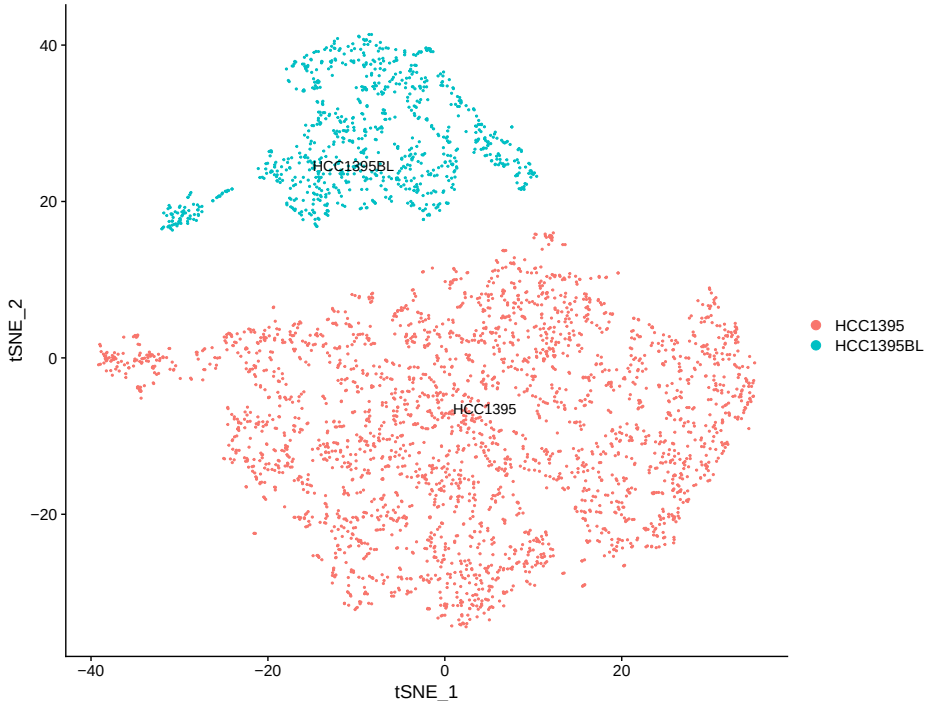


Figure 4.2: tSNE plot overlaid with sample information

4.3 UMAP

Optionally, we can also perform dimension reductionality reduction using Uniform Manifold Approximation and Projection (UMAP). UMAP is a dimension reduction technique that can be used for visualization similarly to t-SNE, but also for general non-linear dimension reduction.

```
DimPlot(
  counts_st,
  reduction = "umap",
  label = T,
  size = 0.5,
  repel = T,
  cols = DiscretePalette(length(levels(Idents(
    counts_st
  ))))
)
```

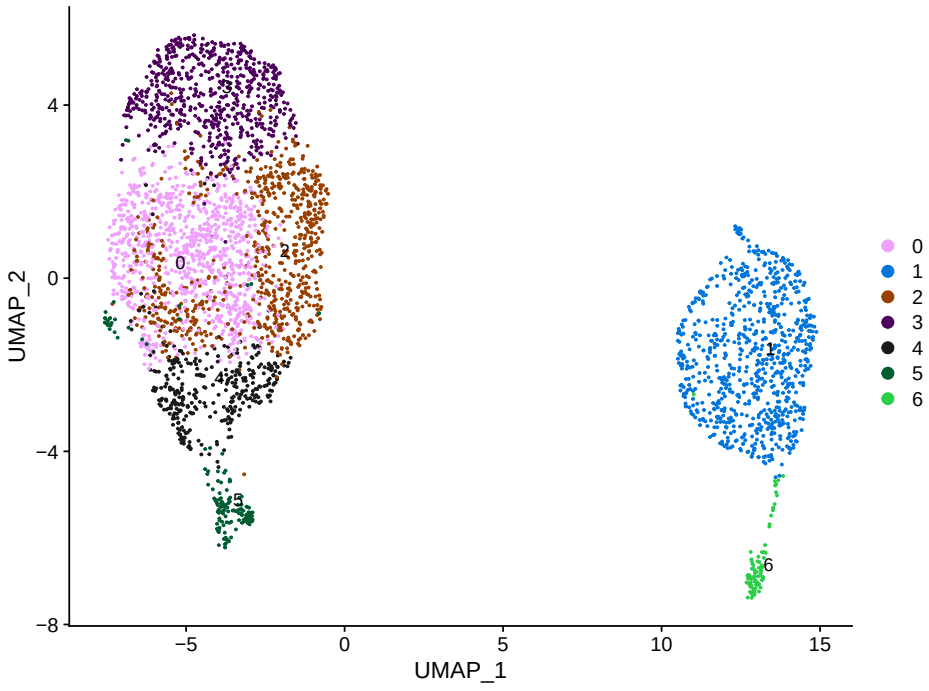


Figure 4.3: UMAP plot showing the clustering of cells

```
DimPlot(
  counts_st,
  reduction = "umap",
  label = T,
  group.by = "Sample",
  size = 0.5,
  repel = T
)+ggtitle("")
```

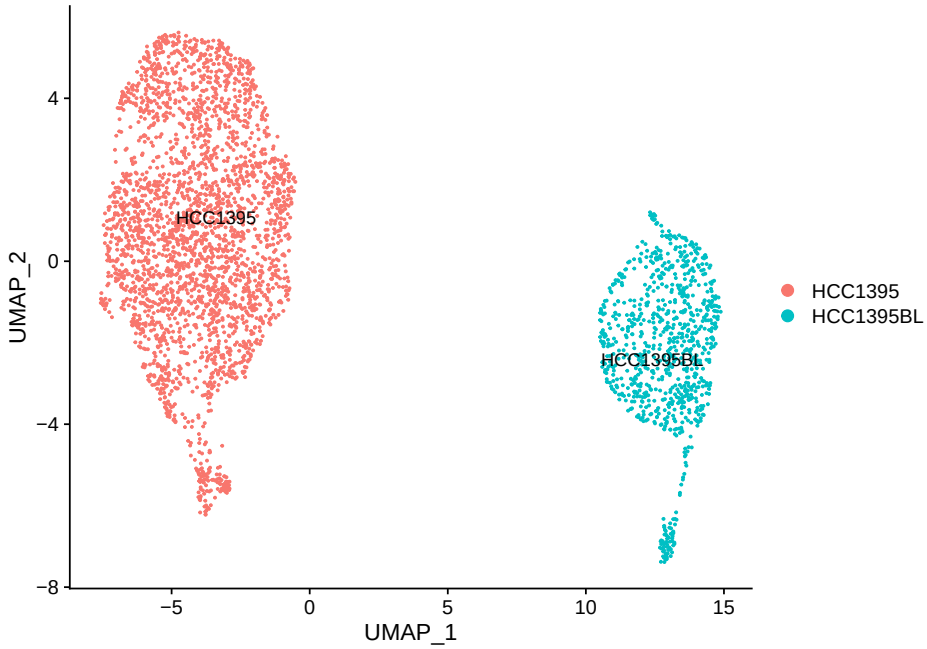


Figure 4.4: UMAP plot overlaid with sample information

To identify differentially expressed genes between clusters we can use the *FindMarker* function in Seurat. As an example, DE genes between clusters 0 and 1 are computed below.

```
DE.cluster0_1 <- FindMarkers(counts_st,
                             ident.1 = 0,
                             ident.2 = 1,
                             verbose = F)
head(DE.cluster0_1[order(abs(DE.cluster0_1$avg_log2FC),
                         decreasing = T), ])
```

```
##                p_val avg_log2FC pct.1 pct.2     p_val_adj
## CD74    0.000000e+00  -5.891577 0.008 1.000  0.000000e+00
## IGHM    0.000000e+00  -5.729445 0.000 0.998  0.000000e+00
## S100A6  0.000000e+00   5.598281 1.000 0.047  0.000000e+00
## TMSB4X 1.657565e-278  -4.911549 0.970 1.000 2.935713e-274
## CCL3   6.580286e-308  -4.861987 0.000 0.945 1.165434e-303
## KRT81  4.273972e-296   4.593045 0.962 0.000 7.569632e-292
```

4.4 Heatmaps

Marker genes which can be used to uniquely identify each of the clusters are identified using the *FindAllMarkers* function.

```
library(dplyr)
all.markers %>%
  group_by(cluster) %>%
  slice_max(n = 5, order_by = avg_log2FC)
```

```
## # A tibble: 35 x 7
## # Groups:   cluster [7]
##        p_val avg_log2FC pct.1 pct.2 p_val_adj cluster gene
##        <dbl>      <dbl> <dbl> <dbl>     <dbl> <fct>   <chr>
##  1 1.61e-155      1.23  0.94  0.525 2.86e-151 0       IGFBP3
##  2 4.07e- 65      1.17  0.417 0.149 7.21e- 61 0       IGFBP5
##  3 2.12e-147      1.06  0.998 0.714 3.75e-143 0       TPM1
##  4 2.31e-202      0.938 0.994 0.869 4.09e-198 0       CSNK2B
##  5 1.78e-131      0.901 0.998 0.665 3.15e-127 0       TPM2
##  6 0              4.79  0.998 0.021 0         1       IGHM
##  7 0              4.63  1     0.037 0         1       CD74
##  8 0              4.22  0.945 0.015 0         1       CCL3
##  9 0              4.08  1     0.959 0         1       TMSB4X
## 10 0              3.48  0.999 0.033 0         1       HLA-DRA
## # ... with 25 more rows
## # i Use `print(n = ...)` to see more rows
```

```
top10.markers<-all.markers %>%
    group_by(cluster) %>%
    slice_max(n = 10, order_by = avg_log2FC)
```

```
DoHeatmap(counts_st, features = top10.markers$gene)
```

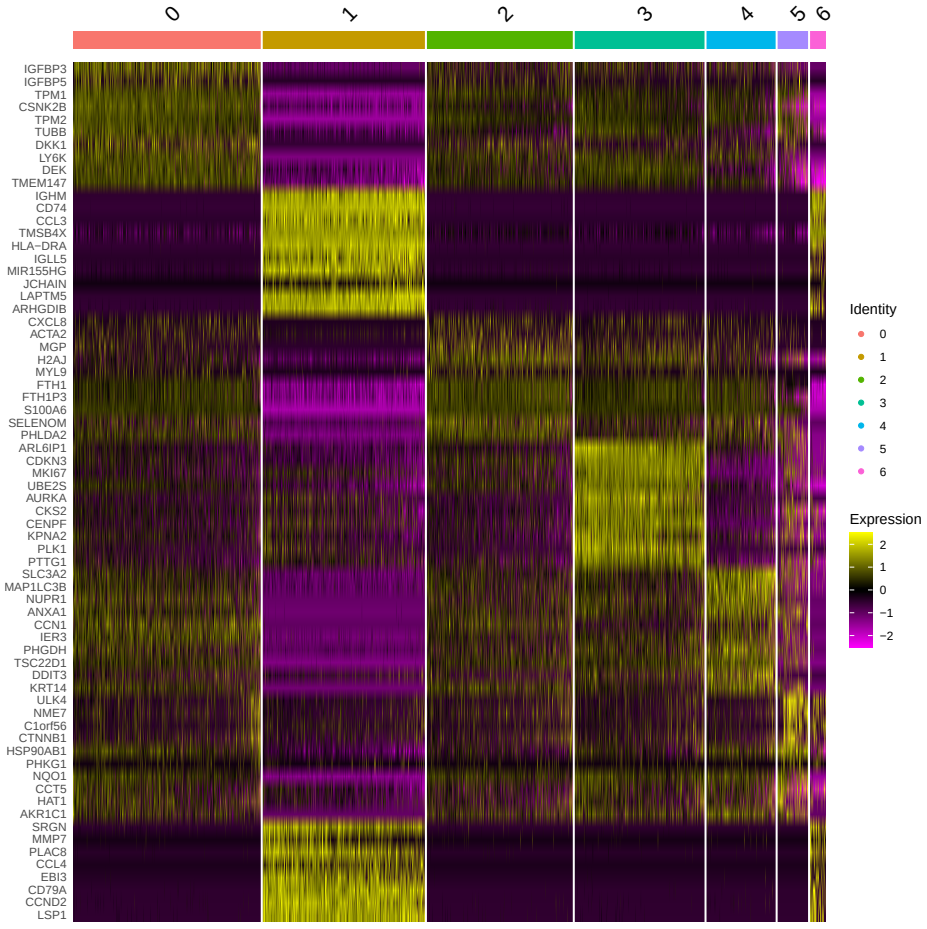


Figure 4.5: Heatmap of the top DE genes per cluster

4.5 Feature plots

We can also highlight the expression of genes of interest on the clusters by way of a tSNE plot.

```
#Get the top marker gene per cluster
top.markers<-all.markers %>%
    group_by(cluster) %>%
    slice_max(n = 1, order_by = avg_log2FC)
```

```
FeaturePlot(
  counts_st,
  features = top.markers$gene,
  ncol = 3,
  label = T,
  reduction = "tsne"
)
```

Alternatively, we can also use violin plots or boxplots to show the expression profile of genes of interest across cells by cluster or any other combination of cells

```
VlnPlot(counts_st,
        features = top.markers$gene,
        cols = DiscretePalette(length(unique(
          counts_st$seurat_clusters
        ))))
```

```
par(mfrow = c(3, 3))
for (n in top.markers$gene)
{
  n.data <- counts_st[n, ]
  boxplot(
    as.numeric(n.data@assays$RNA@data) ~ as.character(n.data$seurat_clusters),
    col = DiscretePalette(length(unique(
      counts_st$seurat_clusters
    ))),
    xlab = "Cluster",
    ylab = "Expression Level",
    main = n
  )
}
```

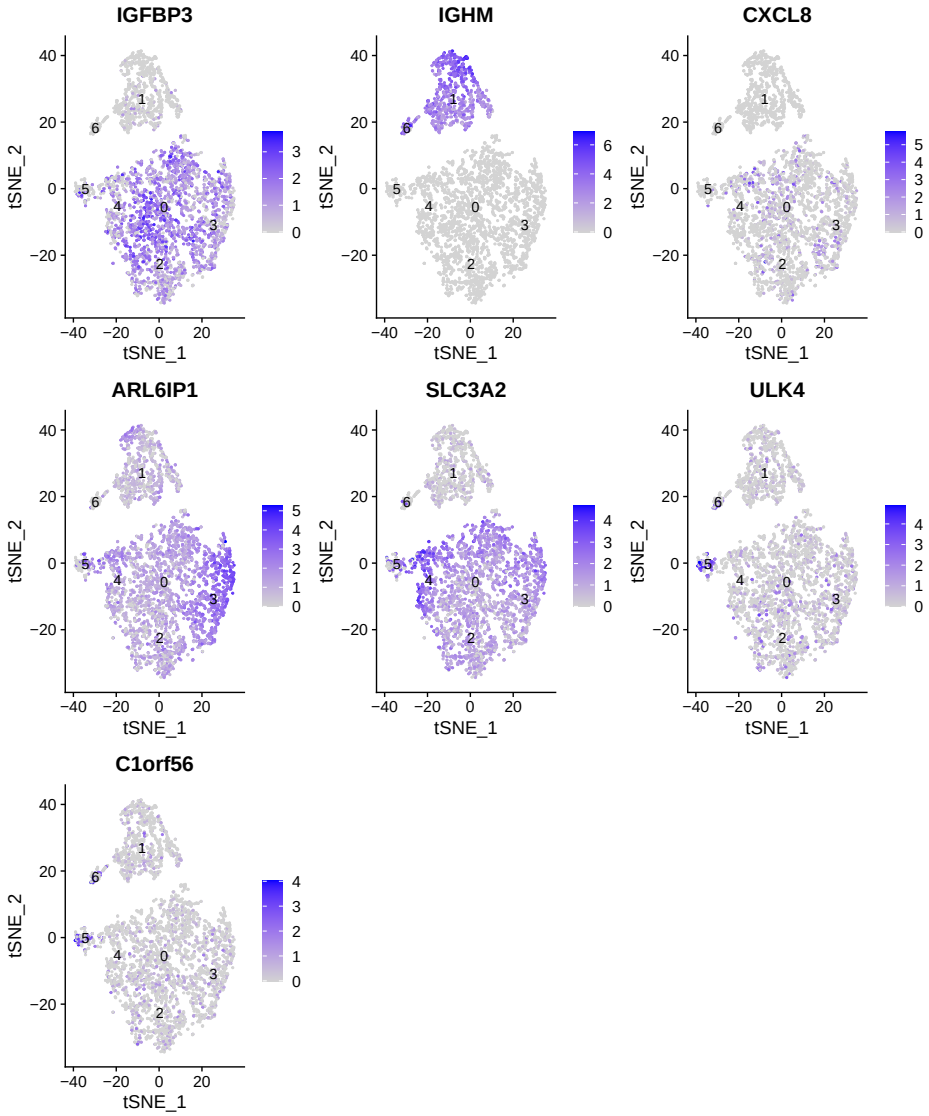


Figure 4.6: tSNE plots overlaid with the expression profile of genes

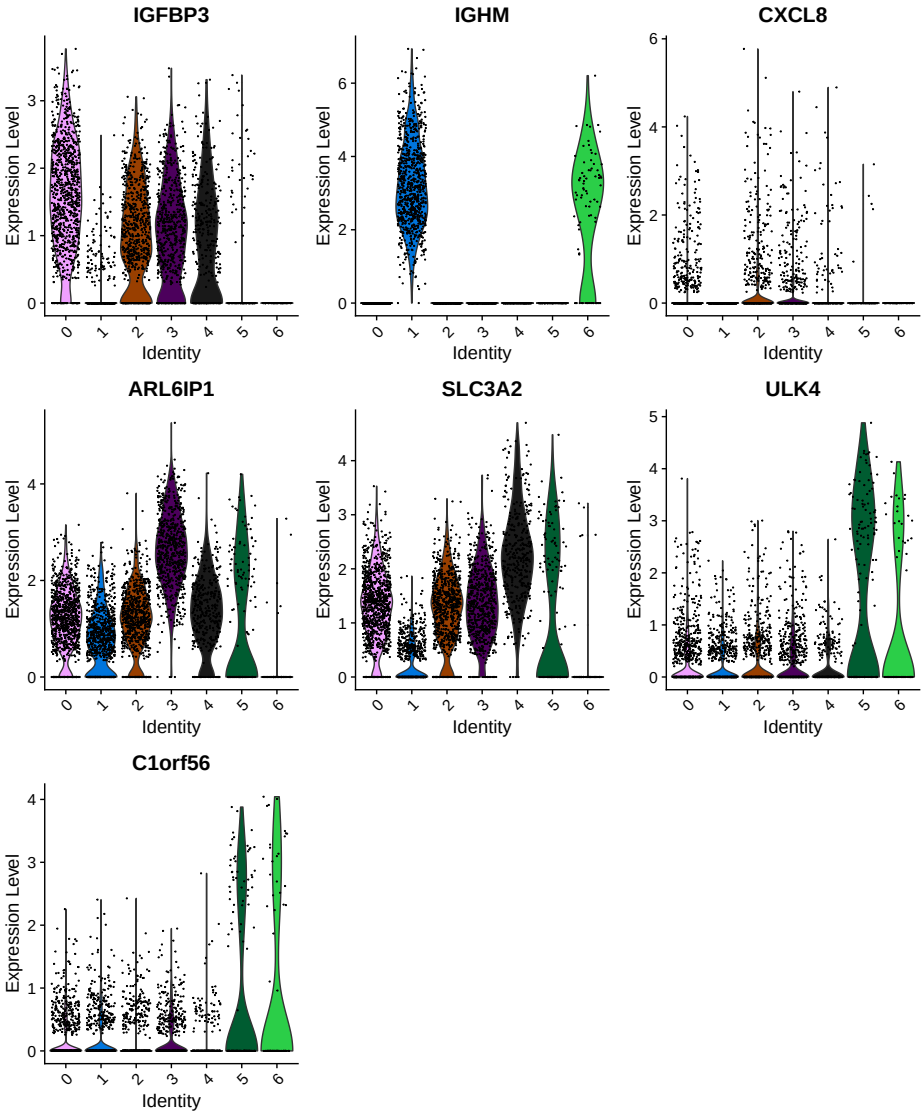


Figure 4.7: Violin plots showing the expression profile of some genes

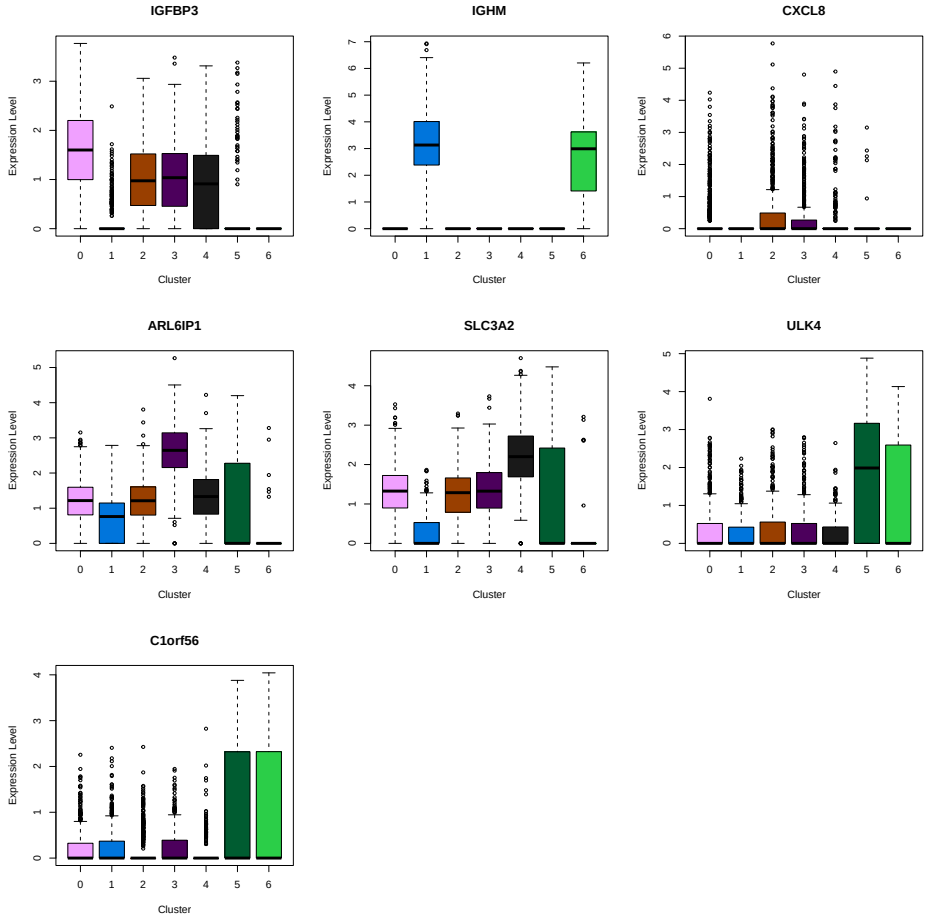


Figure 4.8: Boxplots showing the expression profile of some genes

4.6 Cell annotation

An unbiased cell type recognition is performed using **SingleR**. **celldex** has a range of annotations derived from Bulk RNA-seq data that can be used to annotate the identified clusters above. Here, we use the Human Primary Cell Atlas database as an example.

```
library(SingleR)
library(SingleCellExperiment)
```

```
cell.sce<- as.SingleCellExperiment(counts_st)
annot<-celldex::HumanPrimaryCellAtlasData()
cell.annots <- SingleR(
    test = cell.sce,
    ref = annot,
    clusters = cell.sce$seurat_clusters,
    labels = annot$label.main)

cell.annots.fine<-SingleR(
    test = cell.sce,
    ref = annot,
    clusters = cell.sce$seurat_clusters,
    labels = annot$label.fine)

save(cell.annots,cell.annots.fine,file = "Annotations.RData")
```

```
counts_st <-
  AddMetaData(counts_st, cell.annots[match(
    counts_st@meta.data$seurat_clusters,rownames(cell.annots)),
    "labels"], "Annot.main")
```

```
library(ggplot2)
```

```
DimPlot(
    counts_st,
    reduction = "tsne",
    group.by = "Annot.main",
    label = T,
    repel = T,
    pt.size = 0.1,
  )+ggtitle(label = "")
```

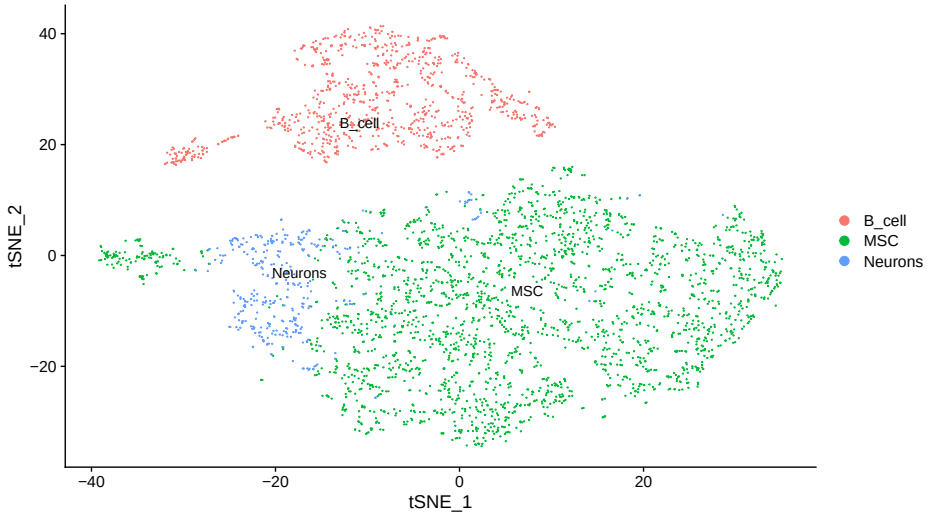


Figure 4.9: tSNE plot overlaid with cell annotation information

```
counts_st <-
  AddMetaData(counts_st, cell.annots.fine[match(
    counts_st@meta.data$seurat_clusters,
    rownames(cell.annots.fine))], "labels"], "Annot.fine")
```

```
DimPlot(
  counts_st,
  reduction = "tsne",
  group.by = "Annot.fine",
  label = T,
  repel = T,
  pt.size = 0.1
) + ggtitle(label = "")
```

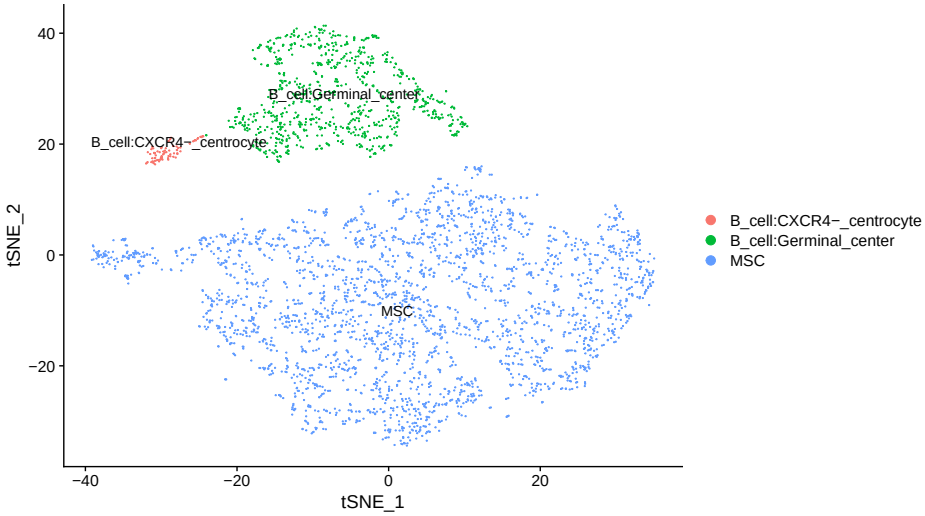


Figure 4.10: tSNE plots showing cell annotation information

4.7 Trajectory analysis

Pseudotime is a measure of how much progress an individual cell has made through a process such as cell differentiation.

Pseudotime analysis of the cells identified in the dataset was performed using **Monocle2**.

```
mono2.learn.traject <-
  function(X_counts,
           these.cell.types) {
    library(monocle) # It has to be Monocle 2.
    rds.fname <-"Trajectory-cds.rds"
    gsndf <- data.frame(gene_short_name = rownames(X_counts))
    csndf <- data.frame(cell.type = these.cell.types)
    rownames(gsndf) <- rownames(X_counts)
    rownames(csndf) <- colnames(X_counts)
    pd <- new("AnnotatedDataFrame", data = csndf)
    fd <- new("AnnotatedDataFrame", data = gsndf)
    cds <-
      newCellDataSet(
        X_counts,
        phenoData = pd,
        featureData = fd,
        expressionFamily = negbinomial.size()
      )
    return(cds)
    cds <- estimateSizeFactors(cds)
    cds <- estimateDispersions(cds)
    cds <- detectGenes(cds, min_expr = 0.1)
    disp_table <- dispersionTable(cds)
    ordering_genes <- subset(disp_table, mean_expression >= 0.1)
    cds <- setOrderingFilter(cds, ordering_genes)
    cds <- reduceDimension(cds)
    saveRDS(cds , rds.fname)
}
```

```
mono2.learn.traject(
  X_counts <- as.matrix(counts_st@assays$RNA@counts),
  these.cell.types <- counts_st[[]]$seurat_clusters
)
```

```
mono.rds <-readRDS("Trajectory-cds.rds")
mono.Tree <- t(mono.rds@reducedDimS)
```

```
do.Traj.lines <- function(traj) {
  for (i in 1:length(traj@minSpanningTree[[]])) {
    p1no <- unlist(traj@minSpanningTree[[i]])[1]
    p2no <- unlist(traj@minSpanningTree[[i]])[2]
    lines(
      c(traj@reducedDimK[1, p1no],
        traj@reducedDimK[1, p2no]),
      c(traj@reducedDimK[2, p1no],
        traj@reducedDimK[2, p2no]),
      lwd = 2
    )
  }
}
```

```
clusters <- counts_st[[]]$seurat_clusters
cols.traj <- c("red", "green", "blue","orange","purple","pink")
traj.cols <-  cols.traj[clusters]
plot(
    mono.Tree,
    cex = 0.7,
    pch = 16,
    bg = traj.cols,
    col = traj.cols,
    xlab = "Component 1",
    ylab = "Component 2",
    cex.axis = 0.8,
    cex.lab = 0.9
)
do.Traj.lines(mono.rds)
legend(
  "bottomright",
  legend = levels(clusters),
  pch = 16,
  col = cols.traj,
  bty = "n"
)
```

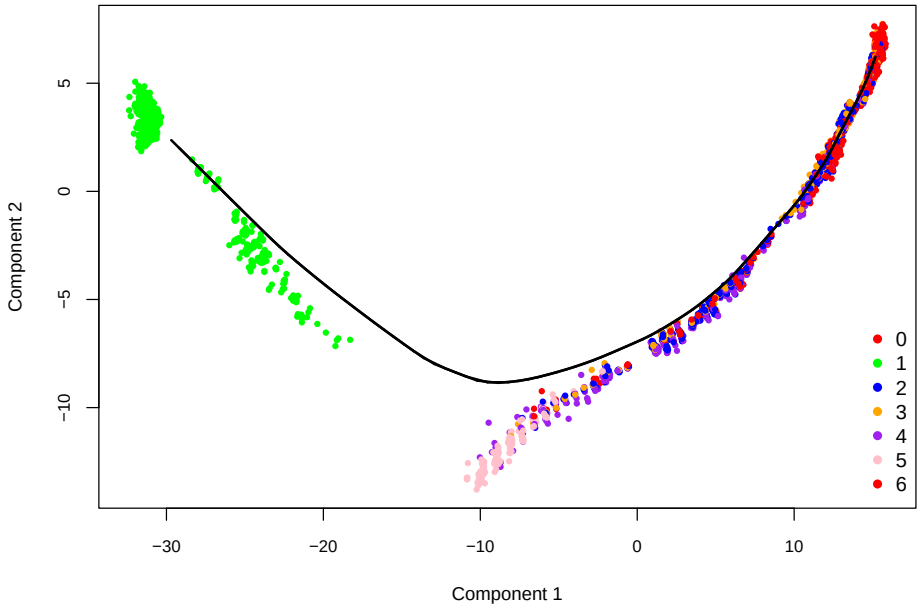


Figure 4.11: Pseudotime trajectory analysis

Session info

```
sessionInfo()
```

```
## R version 4.2.0 (2022-04-22)
## Platform: x86_64-apple-darwin17.0 (64-bit)
## Running under: macOS Big Sur/Monterey 10.16
##
## Matrix products: default
## BLAS:   /Library/Frameworks/R.framework/Versions/4.2/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/4.2/Resources/lib/libRlapack.dylib
##
## locale:
## [1] en_AU.UTF-8/en_AU.UTF-8/en_AU.UTF-8/C/en_AU.UTF-8/en_AU.UTF-8
##
## attached base packages:
## [1] stats4    stats     graphics  grDevices utils     datasets  methods
## [8] base
##
## other attached packages:
##  [1] SingleCellExperiment_1.18.0 SingleR_1.10.0
##  [3] SummarizedExperiment_1.26.1 GenomicRanges_1.48.0
##  [5] GenomeInfoDb_1.32.2         MatrixGenerics_1.8.1
##  [7] matrixStats_0.62.0          dplyr_1.0.9
##  [9] sp_1.5-0                    SeuratObject_4.1.0
## [11] Seurat_4.1.1                org.Mm.eg.db_3.15.0
## [13] forcats_0.5.1               ggplot2_3.3.6
## [15] GO.db_3.15.0                AnnotationDbi_1.58.0
## [17] IRanges_2.30.0              S4Vectors_0.34.0
## [19] Biobase_2.56.0              BiocGenerics_0.42.0
## [21] Glimma_2.6.0                limma_3.52.2
##
## loaded via a namespace (and not attached):
##   [1] utf8_1.2.2                reticulate_1.25
##   [3] tidyselect_1.1.2          RSQLite_2.2.15
##   [5] htmlwidgets_1.5.4         docopt_0.7.1
##   [7] combinat_0.0-8            grid_4.2.0
##   [9] BiocParallel_1.30.3       Rtsne_0.16
##  [11] munsell_0.5.0             ScaledMatrix_1.4.0
##  [13] codetools_0.2-18          ica_1.0-3
##  [15] future_1.27.0             miniUI_0.1.1.1
##  [17] withr_2.5.0               fastICA_1.2-3
##  [19] spatstat.random_2.2-0     colorspace_2.0-3
##  [21] progressr_0.10.1          highr_0.9
##  [23] knitr_1.39                rstudioapi_0.13
```

```
##  [25] ROCR_1.0-11               tensor_1.5
##  [27] listenv_0.8.0             labeling_0.4.2
##  [29] slam_0.1-50               GenomeInfoDbData_1.2.8
##  [31] polyclip_1.10-0           pheatmap_1.0.12
##  [33] bit64_4.0.5               farver_2.1.1
##  [35] parallelly_1.32.1         vctrs_0.4.1
##  [37] generics_0.1.3            xfun_0.31
##  [39] R6_2.5.1                  ggbeeswarm_0.6.0
##  [41] rsvd_1.0.5                VGAM_1.1-7
##  [43] locfit_1.5-9.6            bitops_1.0-7
##  [45] spatstat.utils_2.3-1      cachem_1.0.6
##  [47] DelayedArray_0.22.0       assertthat_0.2.1
##  [49] promises_1.2.0.1          scales_1.2.0
##  [51] rgeos_0.5-9               beeswarm_0.4.0
##  [53] gtable_0.3.0              beachmat_2.12.0
##  [55] globals_0.15.1            goftest_1.2-3
##  [57] rlang_1.0.4               genefilter_1.78.0
##  [59] splines_4.2.0             lazyeval_0.2.2
##  [61] spatstat.geom_2.4-0       yaml_2.3.5
##  [63] reshape2_1.4.4            abind_1.4-5
##  [65] httpuv_1.6.5              tools_4.2.0
##  [67] bookdown_0.27             ellipsis_0.3.2
##  [69] gplots_3.1.3              spatstat.core_2.4-4
##  [71] RColorBrewer_1.1-3        ggridges_0.5.3
##  [73] Rcpp_1.0.9                plyr_1.8.7
##  [75] sparseMatrixStats_1.8.0   zlibbioc_1.42.0
##  [77] purrr_0.3.4               RCurl_1.98-1.8
##  [79] rpart_4.1.16              deldir_1.0-6
##  [81] viridis_0.6.2             pbapply_1.5-0
##  [83] cowplot_1.1.1             zoo_1.8-10
##  [85] ggrepel_0.9.1             cluster_2.1.3
##  [87] magrittr_2.0.3            data.table_1.14.2
##  [89] scattermore_0.8           lmtest_0.9-40
##  [91] RANN_2.6.1                fitdistrplus_1.1-8
##  [93] patchwork_1.1.1           mime_0.12
##  [95] evaluate_0.15             xtable_1.8-4
##  [97] XML_3.99-0.10             sparsesvd_0.2
##  [99] gridExtra_2.3             HSMMSingleCell_1.16.0
## [101] compiler_4.2.0            tibble_3.1.8
## [103] KernSmooth_2.23-20        crayon_1.5.1
## [105] htmltools_0.5.3           mgcv_1.8-40
## [107] later_1.3.0               tidyr_1.2.0
## [109] geneplotter_1.74.0        DBI_1.1.3
## [111] MASS_7.3-58.1             leidenbase_0.1.11
## [113] Matrix_1.4-1              cli_3.3.0
## [115] parallel_4.2.0            igraph_1.3.4
```

```
## [117] pkgconfig_2.0.3          plotly_4.10.0
## [119] spatstat.sparse_2.1-1    annotate_1.74.0
## [121] vipor_0.4.5              XVector_0.36.0
## [123] stringr_1.4.0            digest_0.6.29
## [125] sctransform_0.3.3        RcppAnnoy_0.0.19
## [127] DDRTree_0.1.5            spatstat.data_2.2-0
## [129] Biostrings_2.64.0        rmarkdown_2.14
## [131] leiden_0.4.2             uwot_0.1.11
## [133] edgeR_3.38.2             DelayedMatrixStats_1.18.0
## [135] shiny_1.7.2              gtools_3.9.3
## [137] monocle_2.24.1           lifecycle_1.0.1
## [139] nlme_3.1-158             jsonlite_1.8.0
## [141] BiocNeighbors_1.14.0     viridisLite_0.4.0
## [143] fansi_1.0.3              pillar_1.8.0
## [145] lattice_0.20-45          ggrastr_1.0.1
## [147] KEGGREST_1.36.3          fastmap_1.1.0
## [149] httr_1.4.3               survival_3.3-1
## [151] glue_1.6.2               qlcMatrix_0.9.7
## [153] png_0.1-7                bit_4.0.4
## [155] stringi_1.7.8            blob_1.2.3
## [157] DESeq2_1.36.0            BiocSingular_1.12.0
## [159] caTools_1.18.2           memoise_2.0.1
## [161] irlba_2.3.5              future.apply_1.9.0
```

Bibliography

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Wei Shi, Yang Liao, and Gordon K Smyth with contributions from Jenny Dai. *Rsubread: Mapping, quantification and variant analysis of sequencing data*, 2022. URL http://bioconductor.org/packages/Rsubread. R package version 2.10.4.

Gordon Smyth, Yifang Hu, Matthew Ritchie, Jeremy Silver, James Wettenhall, Davis McCarthy, Di Wu, Wei Shi, Belinda Phipson, Aaron Lun, Natalie Thorne, Alicia Oshlack, Carolyn de Graaf, Yunshun Chen, Mette Langaas, Egil Ferkingstad, Marcus Davy, Francois Pepin, and Dongseok Choi. *limma: Linear Models for Microarray Data*, 2022. URL http://bioinf.wehi.edu.au/limma. R package version 3.52.2.