Single Cell Analysis Tutorial

Samuele Soraggi

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This tutorial will give you the extensive basic commands and explanations for the single cell analysis of your own dataset.

- The **first part of the tutorial** (Section 2) is focused on **preprocessing** the data, which means primarily filtering and normalizing it.
- The **second part of the tutorial** (Section 3) is focused on **integrating** all sixteen datasets produced from the lab sessions (you will perform this integration analysis in groups), identifying cell types and find a population of cells expressing the HAR1 gene to analyze different conditions of mutant VS wild type Lotus japonicus.
- The **third part of the tutorial** (Section 4) applies tipycal gene analysis to detect genes conserved and differentially expressed between conditions
- The **fourth part of the tutorial** (Section 5) pivots around the study of groups of genes co-expressed in the data and in specific clusters and conditions

The first two parts follow the phylosophy of the best practices explained in Luecken and Theis (2019) and Heumos et al. (2023). The third part applies standard statistical tests on the average gene expressions in subsets of the data. The last part is based pulling cells transcripts together with different granularities to improve the statistical power of calculations based on their gene expression (as in Morabito et al. (2023)).

The tutorial is based on four samples of Lotus Japonicus (two rhizobia-infected and two wild types) from Frank et al. (2023). The last section follows some of the tutorials from hdWGCNA.

Learning outcomes

At the end of this tutorial **you will be able to use `R` to**

- **Filter** your data selecting specific criteria
- **Preprocess** your data for advanced analysis
- **Merge and integrate** datasets and perform **cross-data analysis**
- **Identify potential cell types** by markers and exploiting other datasets
- Perform and elaborate **differential and conserved** gene expression

- Infer **gene modules** from your data and isolate significant ones related to a cell type
- **Visualize gene modules** and extract their **gene ontology** to draw biological conclusions

0.1 A (rather very) short biological background

Lotus Japonicus is a legume characterized by the legume-rhizobium symbiotic interaction (rhizobia are soil microorganisms that can interact with leguminous plants to form root nodules within which conditions are favourable for bacterial nitrogen fixation. Legumes allow the development of very large rhizobial populations in the vicinity of their roots).

Rhizobial invasion of legumes is primarily mediated by a plant-made tubular invagination called an infection thread (IT). Research has shown that various genes are involved in some of the processes of the legume-rhizobia interaction.

- **RINRK1** (Rhizobial Infection Receptor-like Kinase1), that is induced by Nod factors (NFs) and is involved in IT formation but not nodule organogenesis. A paralog, RINRK2, plays a relatively minor role in infection. RINRK1 is required for full induction of early infection genes, including Nodule Inception (NIN), encoding an essential nodulation transcription factor. See Li et al. (2019).
- **HAR1** mediates nitrate inhibition and autoregulation of nodulation. Autoregulation of nodulation involves root-to-shoot-to-root long-distance communication, and HAR1 functions in shoots. HAR1 is critical for the inhibition of nodulation at 10 mM nitrate. The nitrate-induced CLE-RS2 glycopeptide binds directly to the HAR1 receptor, this result suggests that CLE-RS2/HAR1 long-distance signaling plays an important role in the both nitrate inhibition and the autoregulation of nodulation. See Okamoto and Kawaguchi (2015).
- **SYMRKL1**, encodes a protein with an ectodomain predicted to be nearly identical to that of SYMRK and is required for normal infection thread formation. See Frank et al. (2023).

1 UMI-based single cell data from microdroplets

The dataset is based on a **microdroplet-based method from 10X chromium**. We remember that a microdroplet single cell sequencing protocol works as follow:

- each cell is isolated together with a barcode bead in a gel/oil droplet
- each transcript in the cell is captured via the bead and assigned a cell barcode and a transcript unique molecular identifier (UMI)

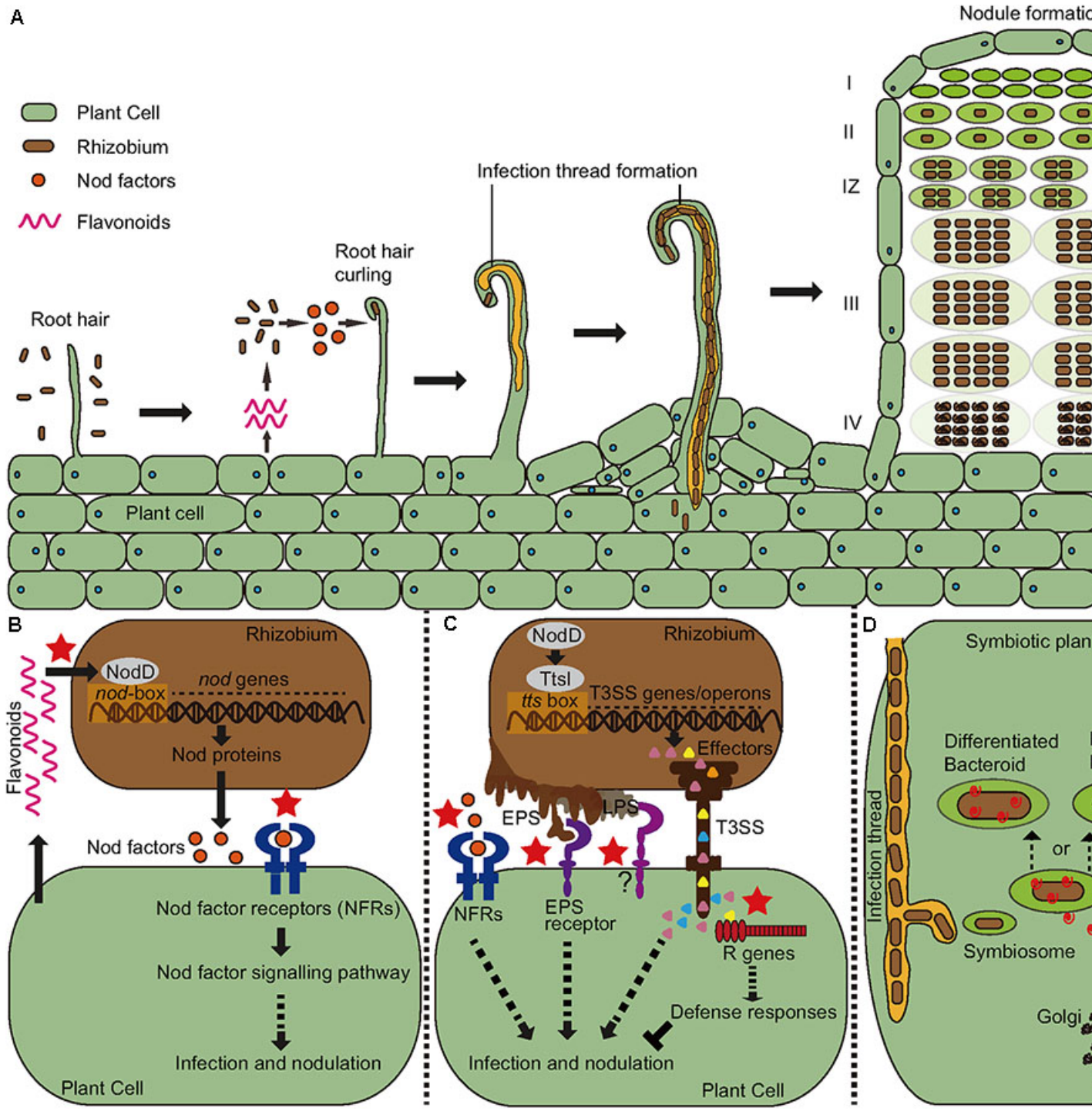


Figure 1: Figure and text from Wang, Liu, and Zhu (2018). Symbiosis signaling and plant immunity involved in recognition specificity in the legume-rhizobial interactions (indicated by the red stars). **A** The process of infection and nodule development. A mature indeterminate nodule contains a meristem zone (I), an infection zone (II), an interzone (IZ), a nitrogen fixing zone (III), and a senescent zone (IV). **B** The host secretes flavonoids to induce the expression of bacterial nodulation (nod) gene through the activation of NodD proteins. The enzymes encoded by the nod genes lead to the synthesis of Nod factors (NF) that are recognized by host Nod factor receptors (NFRs). Recognition specificity occurs both between Flavonoids and NodDs and between NF and NFRs. **C** In addition to NF signaling, bacteria also produce extracellular polysaccharides (EPS) and type III effectors to facilitate their infection in compatible interactions, but these molecules may also induce immune responses causing resistance to infection in incompatible interactions. **D** Certain legumes such

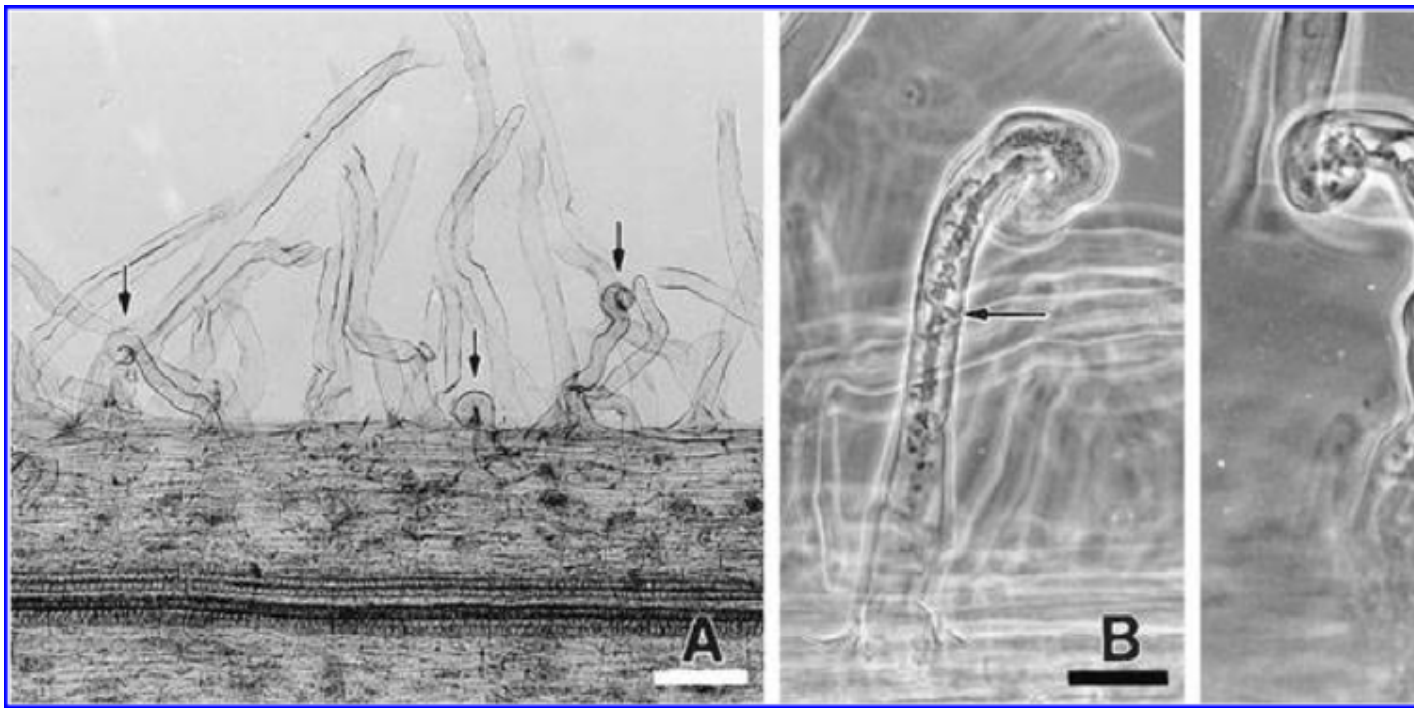


Figure 2: Figure and text from Szczyglowski et al. (1998). Primary infection of Lotus japonicus roots inoculated with Mesorhizobium loti strain NZP2235. **A**, Brightfield micrograph of root hair curlings resembling shepherd's-crook structures (arrows). **B**–**C**, Phase contrast micrographs of the intracellular infection threads within curled root hairs. Arrows point to infection threads with papilla-like structures on their outer surface. Bar, 70 µm **A**, 25 µm **B**, and 20 µm **C**.

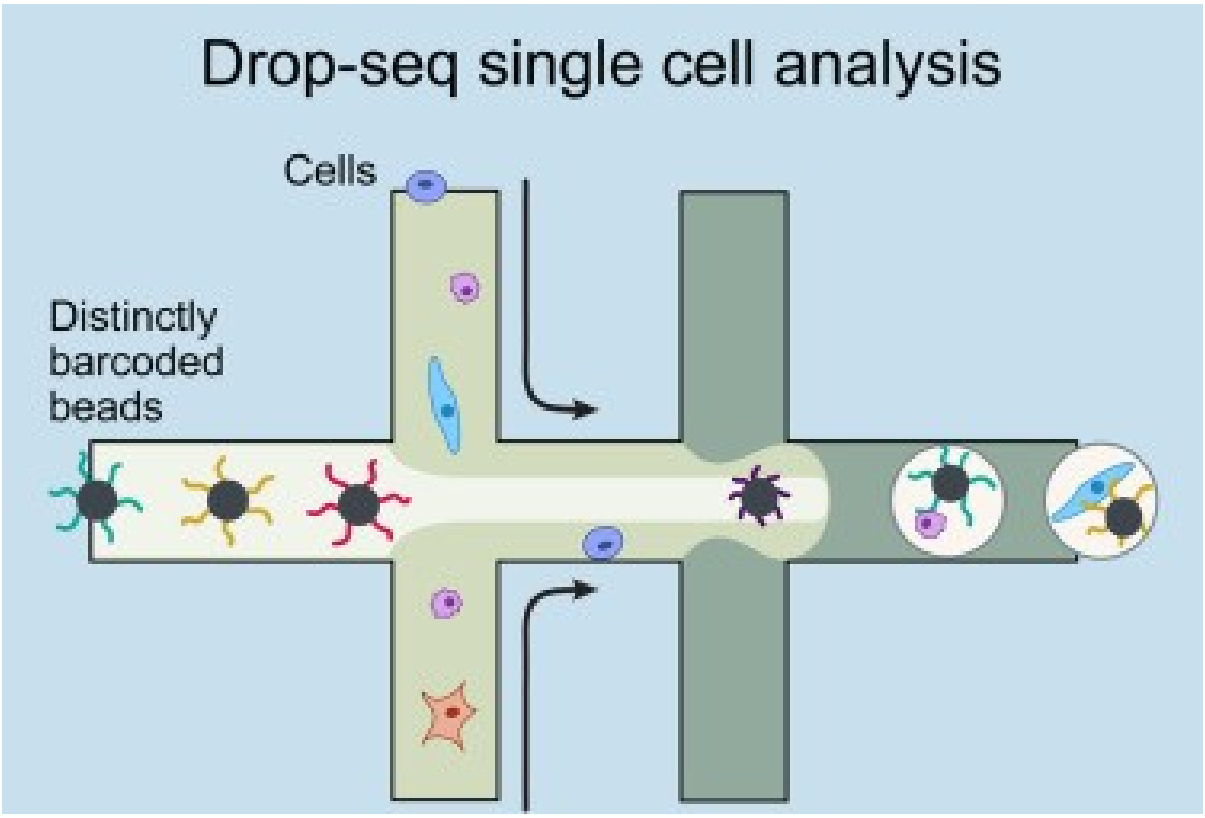


Figure 3: Isolation of cells and beads into microdroplets.

- 3' reverse transcription of mRNA into cDNA is then performed in preparation to the PCR amplification
- the cDNA is amplified through PCR cycles

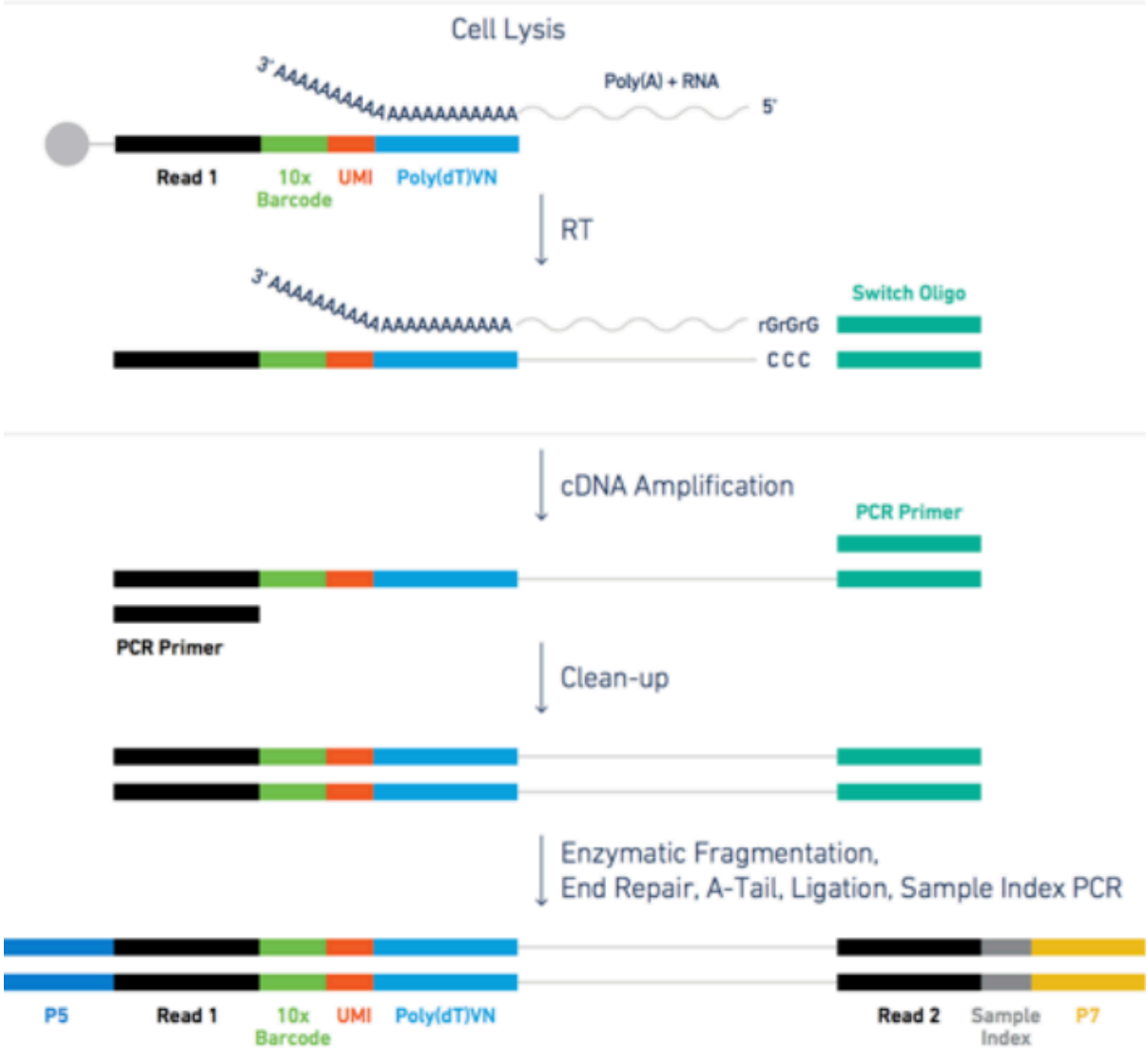


Figure 4: steps for the microdroplet-based single cell RNA sequencing after isolation.

1.1 The raw data in practice

Let's look at a specific read and its UMI and cell barcode. The data is organized in paired-end reads (written on `fastq` files), where the first `fastq` file contains reads in the following

format

```
@SRR8363305.1 1 length=26
NTGAAGTGTTAAGACAAGCGTGAACT
+SRR8363305.1 1 length=26
#AAFFJJJJJJJJJJJJJJJFJJJJ
```

Here, the first 16 characters `NTGAAGTGTTAAGACA` represent the cell barcode, while the last 10 characters `AGCGTGAACT` are the transcript UMI tag. The last line represents the quality scores of the 26 characters of barcode+UMI.

The associated second `fastq` file contains reads of 98nt as the following

```
@SRR8363305.1 1 length=98
NCTAAAGATCACACTAAGGCAACTCATGGAGGGGTCTTCAAAGA
    CCTTGCAAGAAGTACTAACTATGGAGTATCGGCTAAGTCAANCN
    TGTATGAGAT
+SRR8363305.1 1 length=98
#A<77AFJJFAAAJJJ7-7-<7FJ-7----<77--7FAAA--
    <JFFF-7--7<<-F77---FF---7-7A-777777A-<
    -7---#-#A-7-7--7--
```

The 98nt-long string of characters in the second line is a partial sequence of the cDNA transcript. Specifically, the 10X chromium protocol used for sequencing the data is biased towards the 3' end, because the sequencing is oriented from the 3' to the 5' end of the transcripts. The last line contains the corresponding quality scores.

1.2 Alignment and expression matrix

The data is aligned with `cellranger`, a completely automatized pipeline implemented by 10X for 10X-genomics data.

Apart from the data, the output contains an interactive document reporting the quality of the data and a small preliminary UMAP plot and clustering. In this report it is especially instructive to look at the **knee plot**.

The knee plot is created by plotting the number of unique molecular identifiers (UMIs) or reads against the number of cells sequenced, sorted in descending order. The UMIs or reads are a measure of the amount of RNA captured for each cell, and thus a measure of the quality of the data. The plot typically shows a **steep slope at the beginning, followed by a plateau, and then a gradual decrese into a second slope and a final plateau**.

- The steep slope represents the initial cells that are of **high quality** and have the highest number of UMIs or reads.
- The first plateau represents the cells that have **lower quality data**, and the gradual decrease represents the addition of droplets with even lower quality data.
- Usually, beyond the first slope, you have droplets that are **either empty or of so poor quality**, that they are not worth keeping for analysis.
- The height of the last plateau gives you an idea of the **presence of ambient RNA** inside droplets. If the last plateau is located high up, then the corresponding amount of UMIs consist of background ambient RNA which likely pollutes all cells in your data.

Below, the knee plot from the `control 1` sample used in this analysis. You can see that around 10,000 cells with above ~1000 UMIs seems to be coinsidered of decent quality by `cellranger` (the part of line coloured in blue). Note that the last plateau is located at a very low amount of UMIs, meaning there is not really any relevant contamination from ambient RNA.

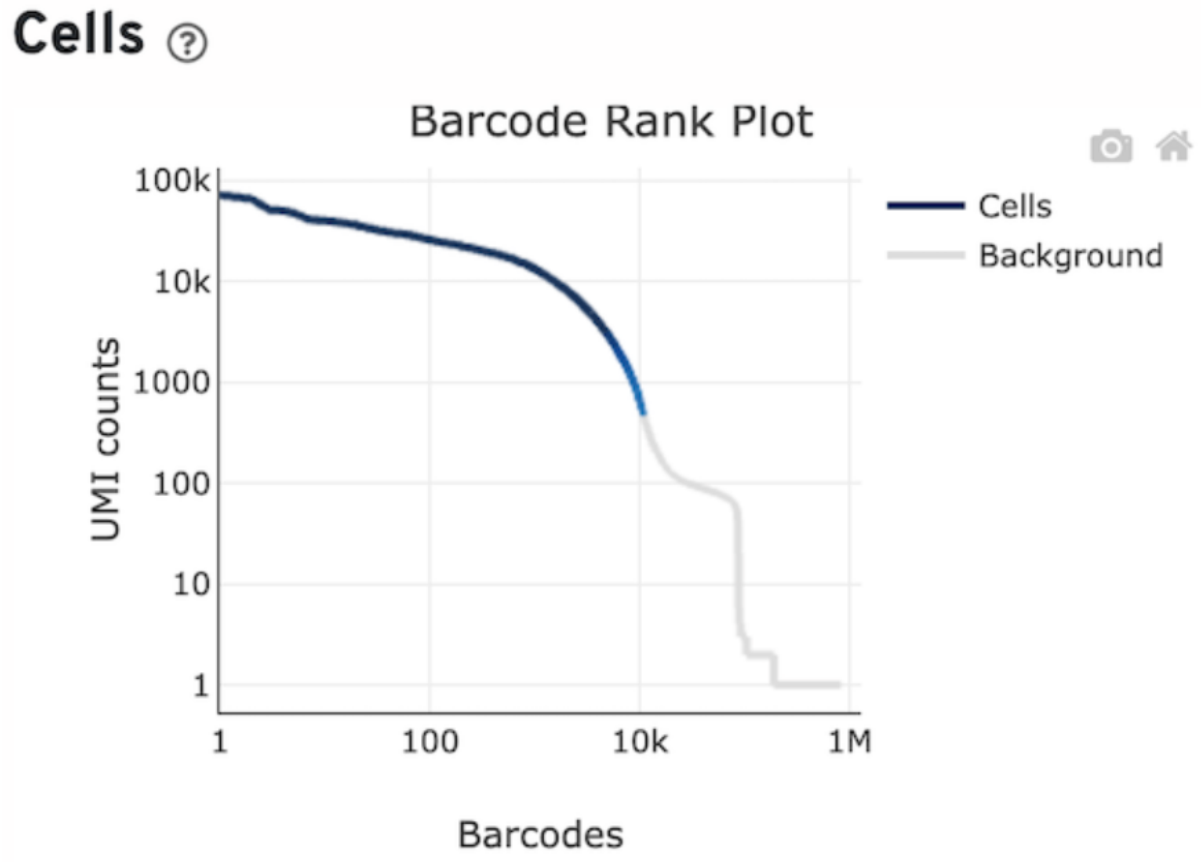


Figure 5: Knee plot of the Control 1 sample of the tutorial. Note the lower plateau of ambient RNA

Exercise

In this same folder you have the document `web_summary.html` that shows you the quality report of your own dataset. **Take some time to look at it and explore what it contains** (Click on `Trust HTML` on the top menu if the html remains blank after opening it). Get an idea of how many UMIs there are in cells of decent quality. We will work more on filtering out cells based on their quality in this tutorial.

Something more about knee plots

The background RNA (sequenced together with the transcript coming from the cell of interest) makes up the *ambient plateau*: the same background RNA is contained in empty droplets. If your dataset has extremely few UMI counts in empty droplets, then there is not much background RNA present - This is the best situation in which you can find yourself. See Exhibit A in Figure 6.
If you have a dataset where you can identify an *empty droplet plateau* by eye (Exhibit B in Figure 6), and these empty droplets have 50 or 100 or several hundred counts, then it can be advisable to use a specific software to remove the background transcripts (e.g. `CellBender` (Fleming et al. (2023)), `SoupX` (Young and Behjati (2020))).
If you have a dataset with so much background RNA that you cannot identify the *empty droplet plateau* yourself by eye (Exhibit C in Figure 6), then any software to remove background transcripts will also likely have a difficult time. Such the algorithms might be worth a try, but you should **strongly consider re-running the experiment, as the knee plot points to a real QC failure**

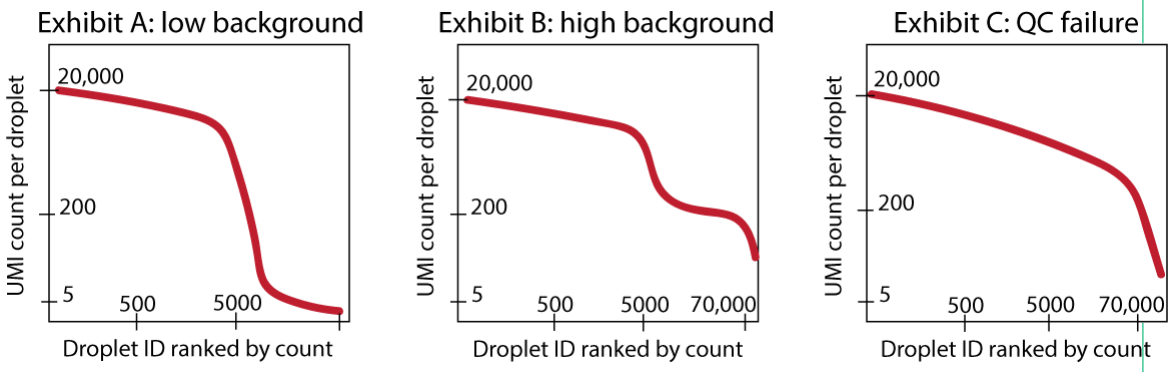


Figure 6: Various cases of knee plot you can encounter from sequenced data. Figure from the webpage of Cellbender.

2 Preprocessing

> **Learning outcome**
>
> We will answer to the following questions:
>
> - How can I **import single-cell data** into R?
> - How are different types of data/information (e.g. cell information, gene information, etc.) **stored and manipulated**?
> - How can I obtain basic **summary metrics** for cells and genes and **filter the data** accordingly?
> - How can I **visually explore** these metrics?

We start by loading all the packages necessary for the analysis and setup a few things

```
suppressPackageStartupMessages({
library(SeuratDisk);
library(Seurat);
library(DoubletFinder);
library(parallel);
library(multtest);
library(metap);
library(purrr);
library(dplyr);
library(stringr);
library(tibble);
library(ggplot2);
library(MAST);
library(WGCNA);
library(hdWGCNA);
library(patchwork);
library(doFuture);})
```

```
source("../Scripts/script.R")
```

```
options(future.seed=TRUE)
```

```
plan("multicore", workers = 8)
options(future.globals.maxSize = 8 * 1024^3)
```

2.1 Download data

We check if the data exists, otherwise a script will download the missing data files in the appropriate folder, which should be `../Data`.

```
downloadData()
```

2.2 Import data

We import the data reading the matrix files aligned by 10X. Those are usually contained in a folder with a name of the type `aligned_dataset/outs/filtered_bc_matrix`, that 10X Cellranger creates automatically after the alignment. You will use such a folder when your own data is aligned. In this tutorial, the aligned data is in the folder used below. The command for reading the data is simply `Read10X`.

> **!** Important
>
> When using your own data, you need to use the correct folder name below according to the `10X` folder structure `aligned_dataset/outs/filtered_bc_matrix`.

```
Control1 <- Read10X("../Data/control_1/")
```

Note that we are loading only one dataset (`control_1`, one of the two control replicates). Another control dataset, and two infected datasets, have already been preprocessed and will be used later - so **we will now focus on the preprocessing of a single dataset**. In general, when you have multiple datasets, you must preprocess them one at a time before integrating them together.

What we obtain in the command above is an expression matrix. Look at the first 10 rows and columns of the matrix (whose rows represent genes and columns droplets/cells) - the dots are zeros (they are not stored in the data, which has a compressed format called `dgCMatrix`), and **are the majority of the expression values obtained in scRNA data!**

```
Control1[1:10,1:10]
```

```
  [[ suppressing 10 column names 'AAACCCAAGGGCAGTT-1', 'AAACCCAAGTCAGCGA-1', 'AAACCCACACTAAC
```

```
10 x 10 sparse Matrix of class "dgCMatrix"

LotjaGi0g1v0000100    . . . . . . . . . .
LotjaGi0g1v0000200    . . . . . . . . . .
```

```
LotjaGi0g1v0000300    . . . . . .
LotjaGi0g1v0000400    . . . . . 1 1 . .
LotjaGi0g1v0000500    . . . . . .
LotjaGi0g1v0000600    . . . . . .
LotjaGi0g1v0000700    . . . . . .
LotjaGi0g1v0000800    . . . . 1 . . . . .
LotjaGi0g1v0000900_LC . . . . . .
LotjaGi0g1v0001000_LC . . . . . .
```

What is the percentage of zeros in this matrix? You can see it for yourself below - it is a lot, but quite surprisingly we can get a lot of information from the data!

```
cat("Number of zeros: ")
zeros <-  sum(Control1==0)
cat( zeros )
```

```
Number of zeros: 307060673
```

```
cat("Number of expression entries: ")
total <- dim(Control1)[1] * dim(Control1)[2]
cat( total )
```

```
Number of expression entries: 329798055
```

```
cat("Percentage of zeros: ")
cat( zeros / total * 100 )
```

```
Percentage of zeros: 93.10567
```

2.3 Create a single cell object in Seurat

We use our count matrix to create a Seurat object. A Seurat object allows you to **store the count matrix** and future modifications of it (for example its normalized version), together with **information regarding cells and genes** (such as clusters of cell types) and their **projections** (such as PCA and tSNE). We will go through these elements, but first we create the object with `CreateSeuratObject`:

```
Control1_seurat <- CreateSeuratObject(counts = Control1,
                                      project = "Control1_seurat",
                                      min.cells = 3,
                                      min.features = 200)
```

```
Warning message:
"Feature names cannot have underscores ('_'), replacing with dashes ('-')"
Warning message:
"Feature names cannot have underscores ('_'), replacing with dashes ('-')"
```

The arguments of the command are * `counts`: the count matrix * `project`: a project name * `min.cells`: a minimum requirement for genes, in our case saying they must be expressed in at least 3 cells. If not, they are filtered out already now when creating the object. * `min.features`: a minimum requirement for cells. Cells having less than 200 expressed genes are removed from the beginning from the data.

Values for the minimum requirements chosen above are standard checks when running the analysis. Droplets not satisfying those requirements are of extremely bad quality and not worth carrying on during the analysis (remember the knee plot).

How many genes and cells have been filtered out?

```
cat("Starting Genes and Cells:\n")
cat( dim(Control1) )

cat("\nFiltered Genes and Cells:\n")
cat( dim(Control1) - dim(Control1_seurat) )

cat("\nRemaining Genes and Cells:\n")
cat( dim(Control1_seurat) )
```

```
Starting Genes and Cells:
30585 10783
Filtered Genes and Cells:
6747 11
Remaining Genes and Cells:
23838 10772
```

We want to use this data later in the analysis with other `Control` and `Infected` datasets. Therefore we add a `Condition` to the metadata table, and for this dataset we establish that each cell is `Control`.

> **!** Important
>
> When using your own data, do not forget to **write the correct condition label**.

```
Control1_seurat <- AddMetaData(object = Control1_seurat,
                                        metadata = "Control",
                                        col.name = "Condition")
```

2.3.1 Content of a Seurat Object

What is contained in the Seurat object? We can use the command `str` to list the various *slots* of the object.

```
str(Control1_seurat, max.level = 2)
```

```
Formal class 'Seurat' [package "SeuratObject"] with 13 slots
  ..@ assays      :List of 1
  ..@ meta.data   :'data.frame':    10772 obs. of  4 variables:
  ..@ active.assay: chr "RNA"
  ..@ active.ident: Factor w/ 1 level "Control1_seurat": 1 1 1 1 1 1 1 1 1 1 ...
  .. ..- attr(*, "names")= chr [1:10772] "AAACCCAAGGGCAGTT-1" "AAACCCAAGTCAGCGA-1" "AAACCCAC
  ..@ graphs      : list()
  ..@ neighbors   : list()
  ..@ reductions  : list()
  ..@ images      : list()
  ..@ project.name: chr "Control1_seurat"
  ..@ misc        : list()
  ..@ version     :Classes 'package_version', 'numeric_version'  hidden list of 1
  ..@ commands    : list()
  ..@ tools       : list()
```

The first slot is called `assays`, and it contains all the count matrices we have collected during our analysis when, for example, normalizing data or doing other transformations of it. Right now we only have the `RNA` assay with the raw counts:

```
Control1_seurat@assays
```

```
$RNA
Assay data with 23838 features for 10772 cells
First 10 features:
 LotjaGi0g1v0000100, LotjaGi0g1v0000200, LotjaGi0g1v0000300,
LotjaGi0g1v0000400, LotjaGi0g1v0000500, LotjaGi0g1v0000700,
LotjaGi0g1v0000800, LotjaGi0g1v0001100, LotjaGi0g1v0001200,
LotjaGi0g1v0001400
```

You can always select which matrix is currently in use for the analysis by assigning it to `DefaultAssay()`. The default assay is often changed automatically by Seurat, for example the normalized assay is used as default after normalization is performed.

```
DefaultAssay(object = Control1_seurat) <- "RNA"
```

```
cat("Your default assay is ")
cat(DefaultAssay(object = Control1_seurat))
```

```
Your default assay is RNA
```

The second slot is the one that contains the metadata for each cell. It is easily visualized as a table (the command `head` shows only the first 6 rows of the table):

```
head( Control1_seurat@meta.data )
```

A data.frame: 6 × 4

	orig.ident <fct>	nCount_RNA <dbl>	nFeature_RNA <int>	Condition <chr>
AAACCCAAGGGCAGTT-1	Control1_seurat	3567	1919	Control
AAACCCAAGTCAGCGA-1	Control1_seurat	7015	2751	Control
AAACCCACACTAACGA-1	Control1_seurat	1484	828	Control
AAACCCACATGATCGT-1	Control1_seurat	20942	4711	Control
AAACCCAGTAGCTTGT-1	Control1_seurat	29105	5157	Control
AAACCCAGTCTCGTAG-1	Control1_seurat	6115	2124	Control

The table contains a name for the dataset (`orig.ident`, useful to distinguish multiple datasets merged together), how many RNA transcripts are contained in each cell (`nCount_RNA`), the number of expressed genes in each cell (`nFeature_RNA`), and the `Condition` (added by us previously). More metadata can be added along the analysis, and some is added automatically by Seurat when running specific commands.

The `assays` and `meta.data` slots are the most relevant and useful to know - the other ones are mostly for internal use by Seurat and we do not go into detail with those.

2.4 Finding filtering criteria

We want to look in depth at which droplets do not contain good quality data, so that we can filter them out. The standard approach - which works quite well - is to study the **distribution of various quality measures and remove doublets** (droplets containing more than one cell) which can confound the analysis results. We will look at some plots and decide some threshold, then we will apply them at the end after looking at all the histograms.

2.4.1 Quality measure distributions

A first step is to calculate the percentage of mitochondrial and chloroplastic genes. A high percentage indicates the presence of spilled material from broken cells. We use the command `PercentageFeatureSet` and provide the pattern of the gene ID which corresponds to mitochondrial and ribosomal genes. The percentages are saved into the metadata simply by using the double squared brackets `[[`.

```
Control1_seurat[["percent.mt"]] <- PercentageFeatureSet(Control1_seurat,
                                                        pattern = "LotjaGiM1v")
Control1_seurat[["percent.chloroplast"]] <- PercentageFeatureSet(Control1_seurat,
                                                                 pattern = "LotjaGi
```

You can see the new metadata is now added for each cell

```
head( Control1_seurat@meta.data )
```

A data.frame: 6 × 6

	orig.ident <fct>	nCount_RNA <dbl>	nFeature_RNA <int>	Condition <chr>	percent.mt <dbl>	percent.chloroplast <dbl>
AAACCCAAGCGCCATT-1	Control1_seurat	3567	1919	Control	4.6818054	0.02803476
AAACCCAAGTTCACGC-1	Control1_seurat	7015	2751	Control	0.7127584	0.04276550
AAACCCACAGTTACCA-1	Control1_seurat	1484	828	Control	6.6037736	0.06738544
AAACCCACATCGATTC-1	Control1_seurat	20942	4711	Control	0.4775093	0.12415242
AAACCCAGTAGCTTGT-1	Control1_seurat	29105	5157	Control	0.2954819	0.05497337
AAACCCAGTCTTCACG-1	Control1_seurat	6415	2124	Control	1.6026165	0.01635323

2.4.1.1 Number of transcripts per cell

We plot a histogram of the number of transcripts per cell in Figure 7 below. On the right, we zoom into the histogram. We want to **filter out the cells with the lowest number of transcripts** - often there is a peak we can identify with a group of low-quality cells. Here we can choose to remove cells with less than ~700 transcripts (some people prefere to do a lighter filtering, and would for example set a threshold to a lower value). We remove also **cells with too many transcripts** that might contain some weird transcripts - which is also helpful for normalization because it removes some outlying values. For those we can set a limit to 30000, where there is a very thin tail in the histogram.

```
options(repr.plot.width=14, repr.plot.height=5)

plot1 <- ggplot(Control1_seurat@meta.data, aes(x=nCount_RNA)) +
     geom_histogram(fill="#69b3a2", color="#e9ecef", alpha=0.9)

plot2 <- ggplot(Control1_seurat@meta.data, aes(x=nCount_RNA)) +
     geom_histogram(fill="#69b3a2", color="#e9ecef", alpha=0.9) +
     xlim(0,2000)

plot1 + plot2
```

```
`stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
`stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
Warning message:
"Removed 7668 rows containing non-finite values (`stat_bin()`)."
Warning message:
"Removed 2 rows containing missing values (`geom_bar()`)."
```

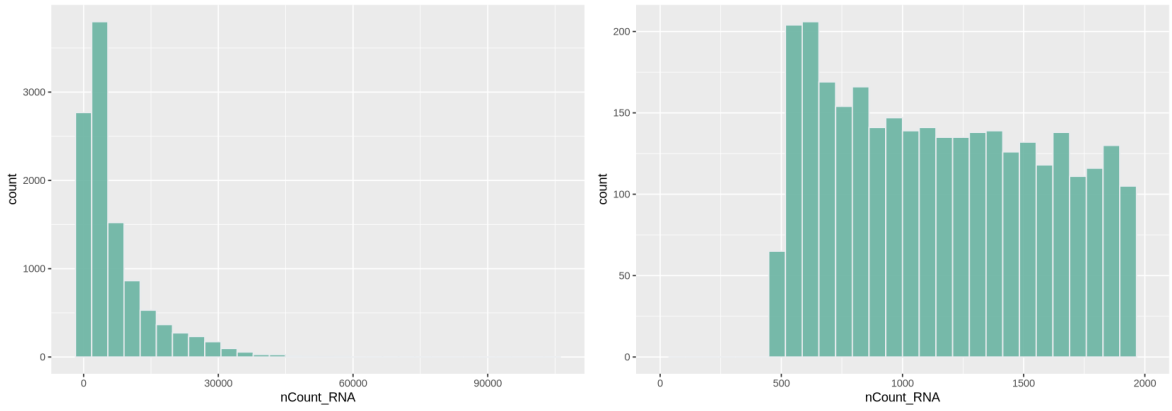


Figure 7: Histogram of transcripts per cell (left) and a zoom onto the histogram (right)

2.4.1.2 Number of detected genes per cell

Here we work similarly to filter out cells based on how many genes are detected (Figure 8). The right-side plot is a zoom into the histogram. It seems easy to set the thresholds at ~400 and ~7000 detected genes.

```
options(repr.plot.width=14, repr.plot.height=5)

plot1 <- ggplot(Control1_seurat@meta.data, aes(x=nFeature_RNA)) +
     geom_histogram(fill="#69b3a2", color="#e9ecef", alpha=0.9)

plot2 <- ggplot(Control1_seurat@meta.data, aes(x=nFeature_RNA)) +
     geom_histogram(fill="#69b3a2", color="#e9ecef", alpha=0.9) +
     xlim(0,1000)


plot1 + plot2
```

```
`stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
`stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
Warning message:
"Removed 7793 rows containing non-finite values (`stat_bin()`)."
Warning message:
"Removed 2 rows containing missing values (`geom_bar()`)."
```

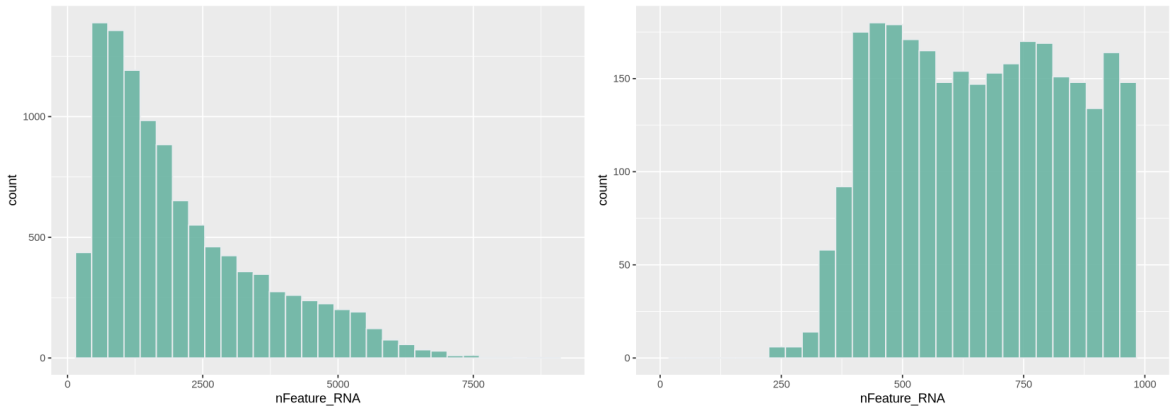


Figure 8: Histogram of detected counts per cell (left) and a zoom onto the histogram (right)

2.4.1.3 Mitochondrial and Chloroplast percentages

The percentages of mitochondrial and chloroplastic transcripts tells us the data is of good quality, since most cells have low values of those (Figure 9). Thresholds are usually set between 5% and 20% in single cell data analysis. In the paper, thresholds were for example set at 20%.

```
options(repr.plot.width=14, repr.plot.height=5)

plot1 <- ggplot(Control1_seurat@meta.data, aes(x=percent.mt)) +
     geom_histogram(fill="#69b3a2", color="#e9ecef", alpha=0.9)

plot2 <- ggplot(Control1_seurat@meta.data, aes(x=percent.chloroplast)) +
     geom_histogram(fill="#69b3a2", color="#e9ecef", alpha=0.9)

plot1 + plot2
```

```
`stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
`stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```

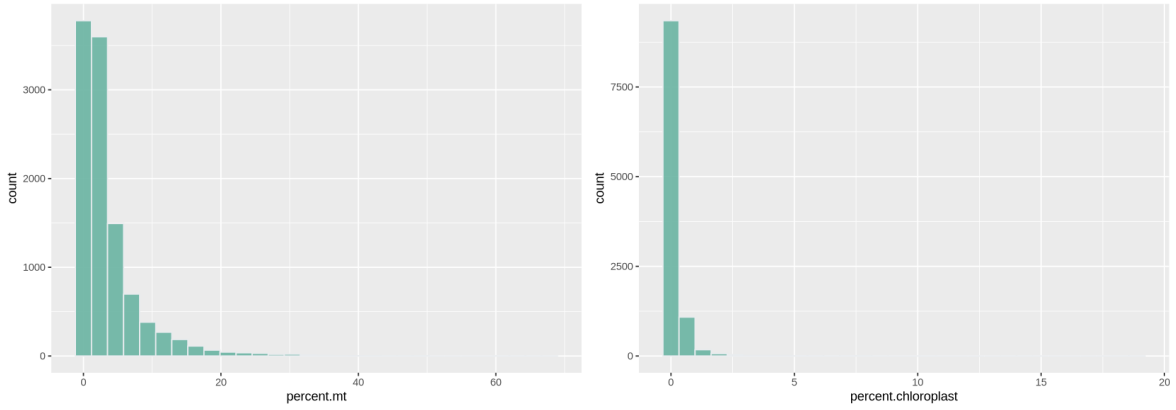


Figure 9: Histogram of mitochondrial (left) and chloroplastic (right) percentage of transcripts in each cell

2.4.1.4 Counts-Features relationship

In Figure 10 below, we look at the plot of the number of transcripts per cell vs the number of detected genes per cell. Usually, those two measure grow simultaneously. At lower counts the relationship is quite linear, then becomes a curve, typically bending in favour of the number of transcripts per cell. You can see below that each dot (representing a droplet) is coloured by percentage of mitochondria. Droplets with a high percentage of mitochondrial genes also have very low amount of transcripts and detected genes, **confirming that high mitochondrial content is a measure of low quality**.

```
options(repr.plot.width=14, repr.plot.height=5)

meta <- Control1_seurat@meta.data %>% arrange(percent.mt)

plot1 <- ggplot( meta, aes(x=nCount_RNA, y=nFeature_RNA, colour=percent.mt)) +
         geom_point(alpha=0.75, size=5)+
         geom_smooth(se=TRUE, method="loess")

plot1
```

```
`geom_smooth()` using formula = 'y ~ x'
Warning message:
"The following aesthetics were dropped during statistical transformation: colour
 This can happen when ggplot fails to infer the correct grouping structure in
  the data.
 Did you forget to specify a `group` aesthetic or to convert a numerical
  variable into a factor?"
```

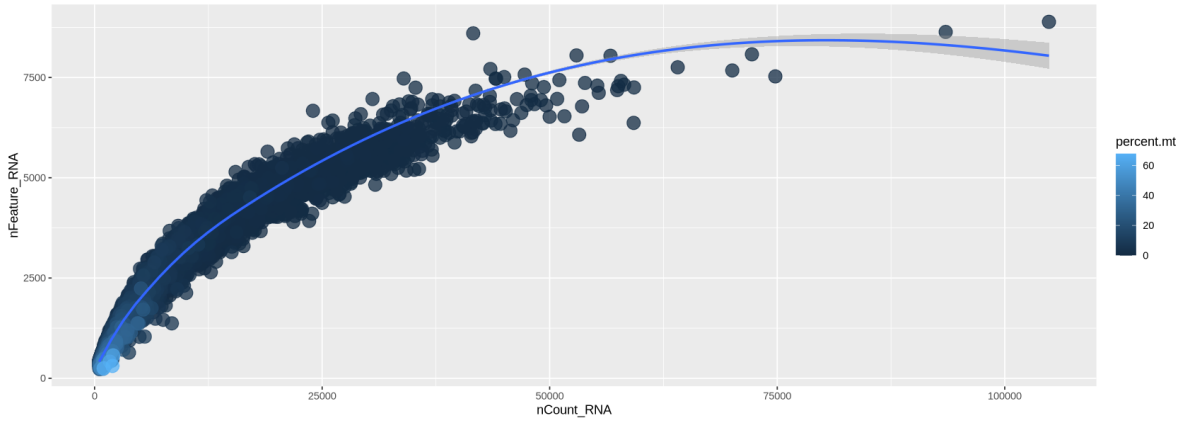


Figure 10: Histogram of the relationship between detected genes and transcripts per cell, coloured by mitochondrial content.

In a similar way the chloroplastic genes confirm the pattern of low quality droplets.

```
options(repr.plot.width=14, repr.plot.height=5)

meta <- Control1_seurat@meta.data %>% arrange(percent.chloroplast)

plot1 <- ggplot( meta, aes(x=nCount_RNA, y=nFeature_RNA, colour=percent.chloroplast)) +
```

```
          geom_point(alpha=0.75, size=5)+
          geom_smooth(se=TRUE, method="loess")

plot1
```

```
`geom_smooth()` using formula = 'y ~ x'
Warning message:
"The following aesthetics were dropped during statistical transformation: colour
  This can happen when ggplot fails to infer the correct grouping structure in
  the data.
  Did you forget to specify a `group` aesthetic or to convert a numerical
  variable into a factor?"
```

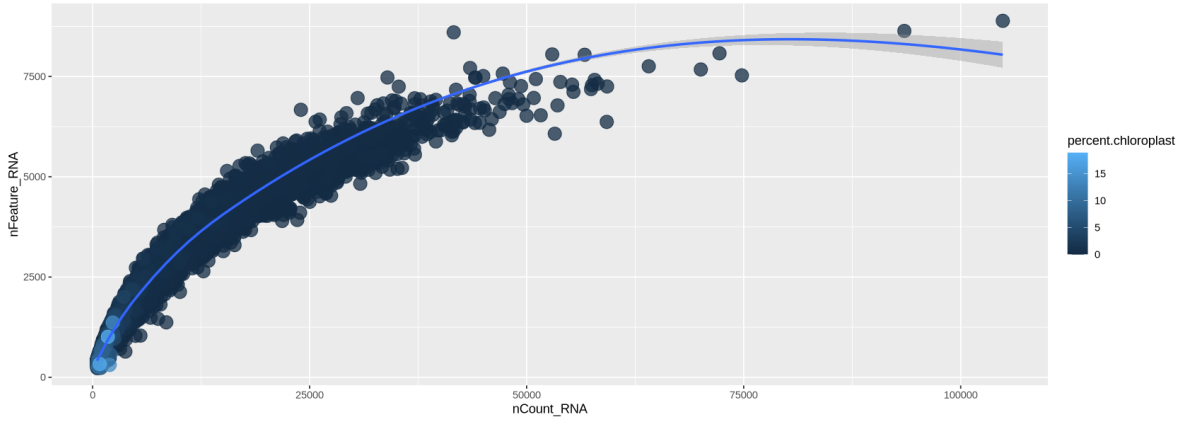


Figure 11: Histogram of the relationship between detected genes and transcripts per cell, coloured by chloroplastic content.

2.4.2 Filtering with the chosen criteria

Here we use the command **subset** and impose the criteria we chose above looking at the histograms. We set each criteria for keeping cells of good quality using the names of the features in metadata. We print those names to remember them.

```
cat("Meta data names:\n")
cat( names(Control1_seurat@meta.data), sep='; ' )
```

```
Meta data names:
orig.ident; nCount_RNA; nFeature_RNA; Condition; percent.mt; percent.chloroplast
```

The filtered object is called `Control1_seurat_filt`

```
Control1_seurat_filt <- subset(x = Control1_seurat,
                                        subset = nCount_RNA > 700 &
                                                 nCount_RNA < 35000 &
                                                 nFeature_RNA > 400 &
                                                 nFeature_RNA < 7000 &
                                                 percent.mt < 5 &
                                                 percent.chloroplast < 5)

cat("Filtered Genes and Cells: ")
cat( dim(Control1_seurat) - dim(Control1_seurat_filt) )
cat("\nRemaining Genes and Cells: ")
cat( dim(Control1_seurat_filt) )
```

```
Filtered Genes and Cells: 0 2762
Remaining Genes and Cells: 23838 8010
```

Now the transcripts vs genes can be seen in Figure 12. The relationship is much more linear than previously after the removal of extreme values for transcripts and detected genes.

```
options(repr.plot.width=14, repr.plot.height=5)

meta <- Control1_seurat_filt@meta.data %>% arrange(percent.mt)

plot1 <- ggplot( meta, aes(x=nCount_RNA, y=nFeature_RNA, colour=percent.mt)) +
         geom_point(alpha=0.75, size=5)+
         geom_smooth(se=TRUE, method="loess")

plot1
```

```
`geom_smooth()` using formula = 'y ~ x'
Warning message:
"The following aesthetics were dropped during statistical transformation: colour
 This can happen when ggplot fails to infer the correct grouping structure in
  the data.
 Did you forget to specify a `group` aesthetic or to convert a numerical
  variable into a factor?"
```

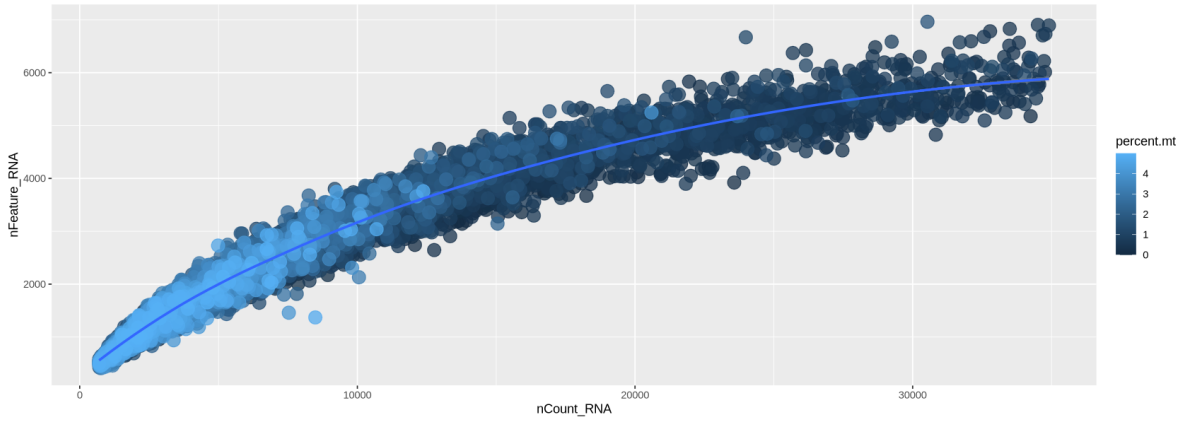


Figure 12: Count vs features after filtering with the chosen criteria

2.5 Normalization

scRNA-seq data is **affected by highly variable RNA quantities and qualities** across different cells. Furthermore, it is often subject to **batch effects, sequencing depth differences, and other technical biases** that can confound downstream analyses.

Normalization methods are used to **adjust for these technical variations so that true biological differences between cells can be accurately identified**.

Some commonly used normalization methods in scRNA-seq data include the following:

- **Total count normalization**: Normalizing the read counts to the total number of transcripts in each sample
- **TPM (transcripts per million)** normalization: Normalizing the read counts to the total number of transcripts in each sample, scaled to a million
- **Library size normalization:** Normalizing the read counts to the total number of reads or transcripts in each sample, adjusted for sequencing depth

All the above suffer from distorting some gene expressions, especially if the data varies a lot in term of sequencing depth. A new and more advanced method, at the moment the state-of-the-art, is `SCTransform` (Hafemeister and Satija (2019)), a software package that can **correct for technical sources of variation and remove batch effects**.

2.5.1 Finding technical sources of variation

Before normalizing we want to check for technical sources of variation in the data. One of those is the total number of transcripts: two similar cells might be sequenced at different depth.

This influences of course normalization. The influence of the number of transcripts per cell is however always removed by `SCtransform`.

We want to look into other possible sources of variation. Those are usually quantities we calculate for each cell, for example the percentage of mitochondrial and chloroplastic genes.

To see if those quantities actually influence our data a lot, we check how much is their highest correlation with the first 10 components of the PCA of the dataset. In short, **we see if any technical variation is such that it explains much of the variability of the data, covering possibly biological signal**.

We now use the function `plotCorrelations` to plot the highest correlation of three quantities with the PCA: number of transcripts, percent of mitochondrial genes and percent of chloroplastic genes. You will see in Figure 14 how **there is little correlation for the two percentages**, for which we do not need to worry about, while **there is correlation with the total number of transcripts per cell** (this is always expected and, as mentioned before, is removed automatically by the normalization process). We created the function `plotCorrelations` specifically for the course, together with a few others, mostly for plotting or handling tables. You can find them in the file `script.R`.

2.5.2 Executing normalization

We run `SCtransform` normalization below. Here you can choose to subsample some cells to do the normalization (`ncells` option): this is **useful to avoid ending up waiting for a long time**. A few thousands cells is enough.

You can also choose how many genes to consider for normalization (`variable.features.n` option): in this case it is best to **use the genes that vary the most their expression across cells**. We look at a histogram (Figure 15) of the variance of each gene to choose a threshold to identify highly-variable genes.

```
variance_genes <- apply( as.matrix(Control1_seurat_filt[['RNA']]@counts), 1, var)

options(repr.plot.width=14, repr.plot.height=5)

plot1 <- ggplot(data.frame(variance_genes), aes(variance_genes)) +
     geom_histogram(fill="#69b3a2", color="#e9ecef", alpha=0.9) + xlim(0,1)

plot1
```

```
Warning message in asMethod(object):
"sparse->dense coercion: allocating vector of size 1.4 GiB"
`stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```

```
plotCorrelations( object=Control1_seurat, measures=c('nCount_RNA', 'percent.mt', 'percent.chl
```

```
`geom_smooth()` using formula = 'y ~ x'
`geom_smooth()` using formula = 'y ~ x'
`geom_smooth()` using formula = 'y ~ x'
```

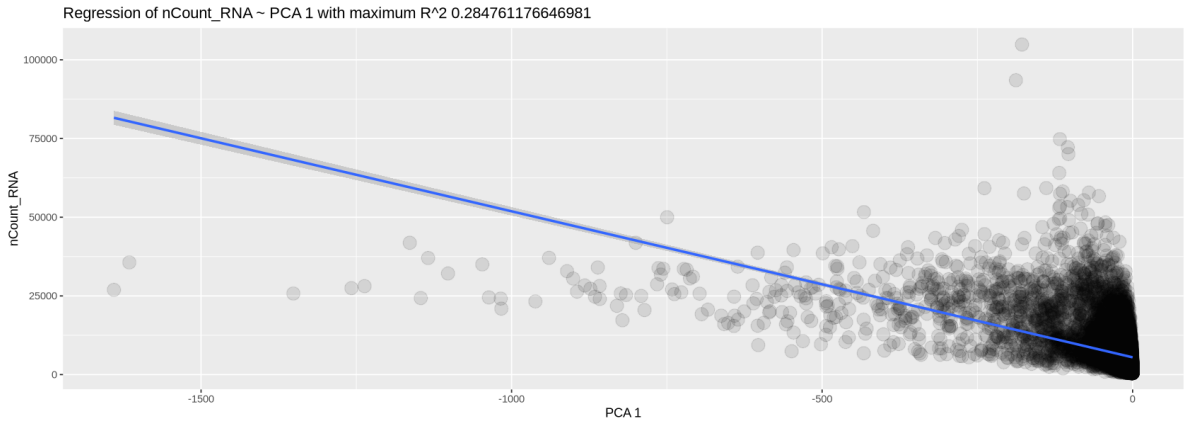
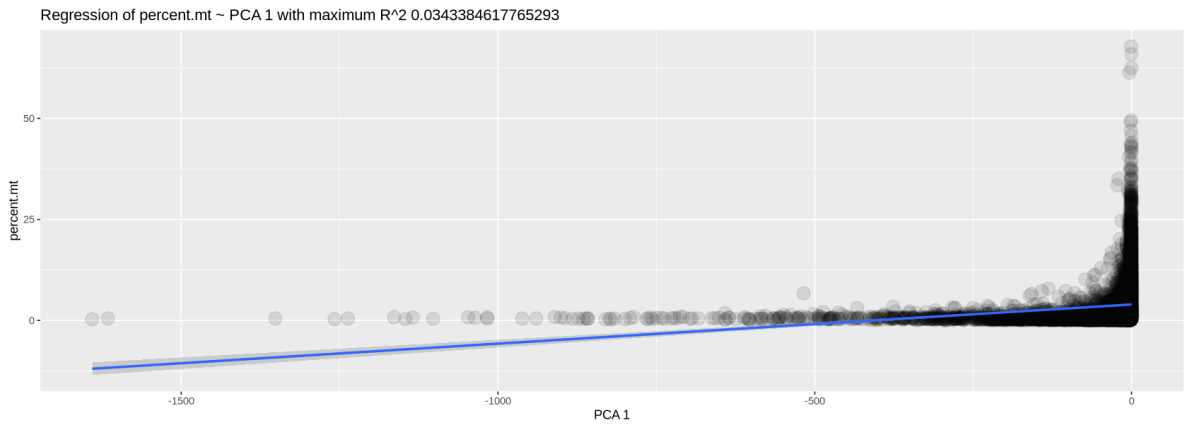


Figure 13: Relationships of maximal correlation between cell quantities and principal components



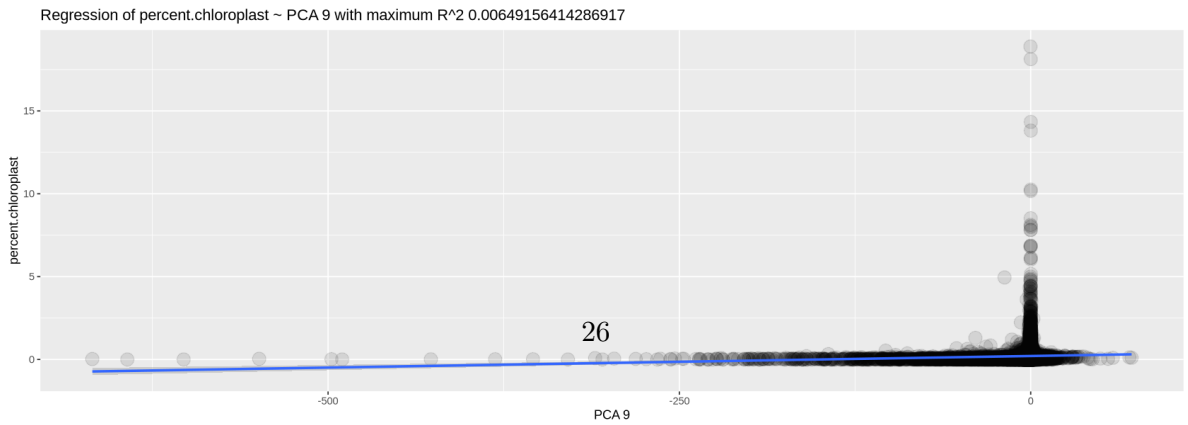


Figure 14: **?(caption)**

```
Warning message:
"Removed 3289 rows containing non-finite values (`stat_bin()`)."
Warning message:
"Removed 2 rows containing missing values (`geom_bar()`)."
```

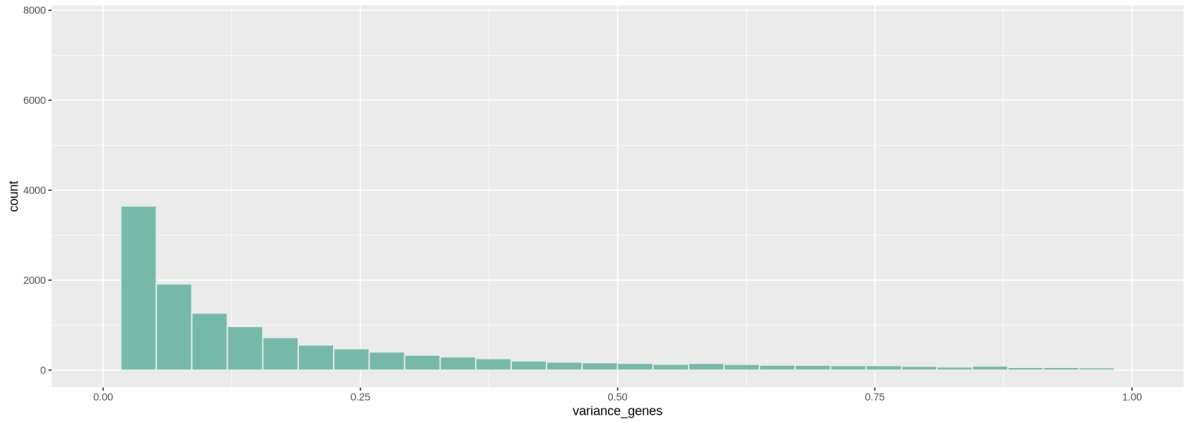


Figure 15: Histogram of genes variance. We choose the threshold 0.1 to identify highly variable genes.

```
hvighly_var_genes <- variance_genes > .1
cat("The total number of highly variable genes selected is: ")
cat(sum( hvighly_var_genes ))
```

```
The total number of highly variable genes selected is: 9955
```

```
Control1_seurat_norm <- SCTransform(Control1_seurat_filt,
                                    return.only.var.genes = FALSE,
                                    ncells = 3000,
                                    variable.features.n = sum( hvighly_var_genes ),
                                    verbose = FALSE)
```

Normalized data is now in the object `Control1_seurat_norm`, in a new assay called `SCT`. This assay is now the default used for data analysis: you can verify it very easily below:

```
cat("Your default assay is ")
cat(DefaultAssay(object = Control1_seurat_norm))
```

```
Your default assay is SCT
```

2.5.3 Visualizing the result

Now we plot the UMAP plot of the data to have a first impression of how the data is structured (presence of clusters, how many, etc.). First of all, we create a PCA plot, which tells us how many PCA components are of relevance with the elbow plot of Figure 16. In the elbow plot, we see the variability of each component in descending order. Note how, after a few rapidly descending components, there is an elbow. We schoose a threshold just after the elbow (for example at 15), which means those components will be used to calculate some other things of relevance in the data, such as distance between cells and the UMAP projection of Figure 17: specific commands using PCA allow to choose the components, and we will set 10 with the option `dims = 1:15`.

```
Control1_seurat_norm <- FindVariableFeatures(Control1_seurat_norm,
                                             nfeatures = sum( hvighly_var_genes ))
```

```
Control1_seurat_norm <- RunPCA(object = Control1_seurat_norm,
                               verbose = FALSE, seed.use = 123)
```

```
ElbowPlot(Control1_seurat_norm, ndims = 30)
```

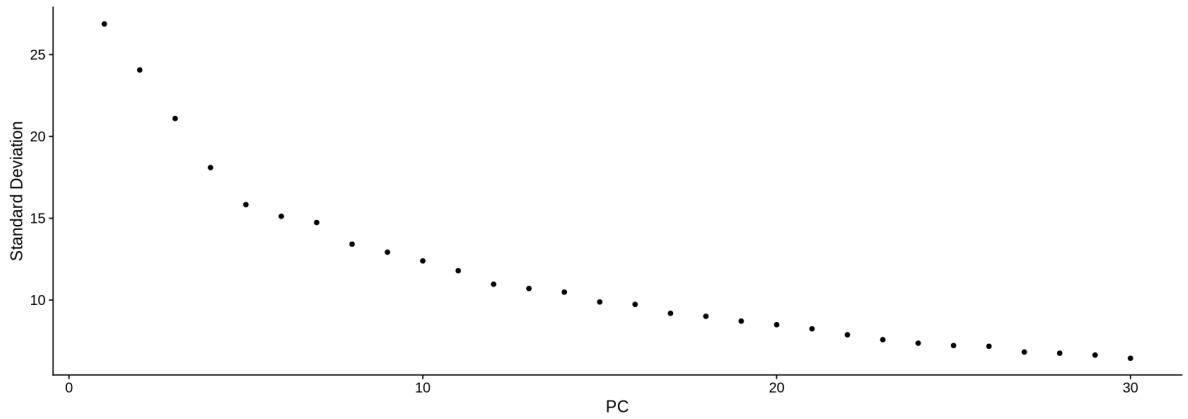


Figure 16: Elbow plot of the first 30 principal components calculated from the data

We calculate the projection using the UMAP algorithm (McInnes et al. (2018), Becht et al. (2019)). The parameters `a` and `b` will change how stretched or sparsed the data looks like. When you do your own UMAP projection, you can avoid setting `a` and `b`, and those will be chosen automatically by the command.

```
Control1_seurat_norm <- RunUMAP(object = Control1_seurat_norm,
                                a = .8, b=1,
                                dims = 1:15,
                                verbose = FALSE,
                                seed.use = 123)
```

```
Warning message:
"The default method for RunUMAP has changed from calling Python UMAP via reticulate to the R-
To use Python UMAP via reticulate, set umap.method to 'umap-learn' and metric to 'correlatio
This message will be shown once per session"
Found more than one class "dist" in cache; using the first, from namespace 'spam'

Also defined by ‘BiocGenerics’

Found more than one class "dist" in cache; using the first, from namespace 'spam'

Also defined by ‘BiocGenerics’
```

In Figure 17 we can see the resulting projection. The result looks pretty neat and structured (we can clearly see there are various clusters).

```
options(repr.plot.width=10, repr.plot.height=8)
UMAPPlot(object = Control1_seurat_norm)
```

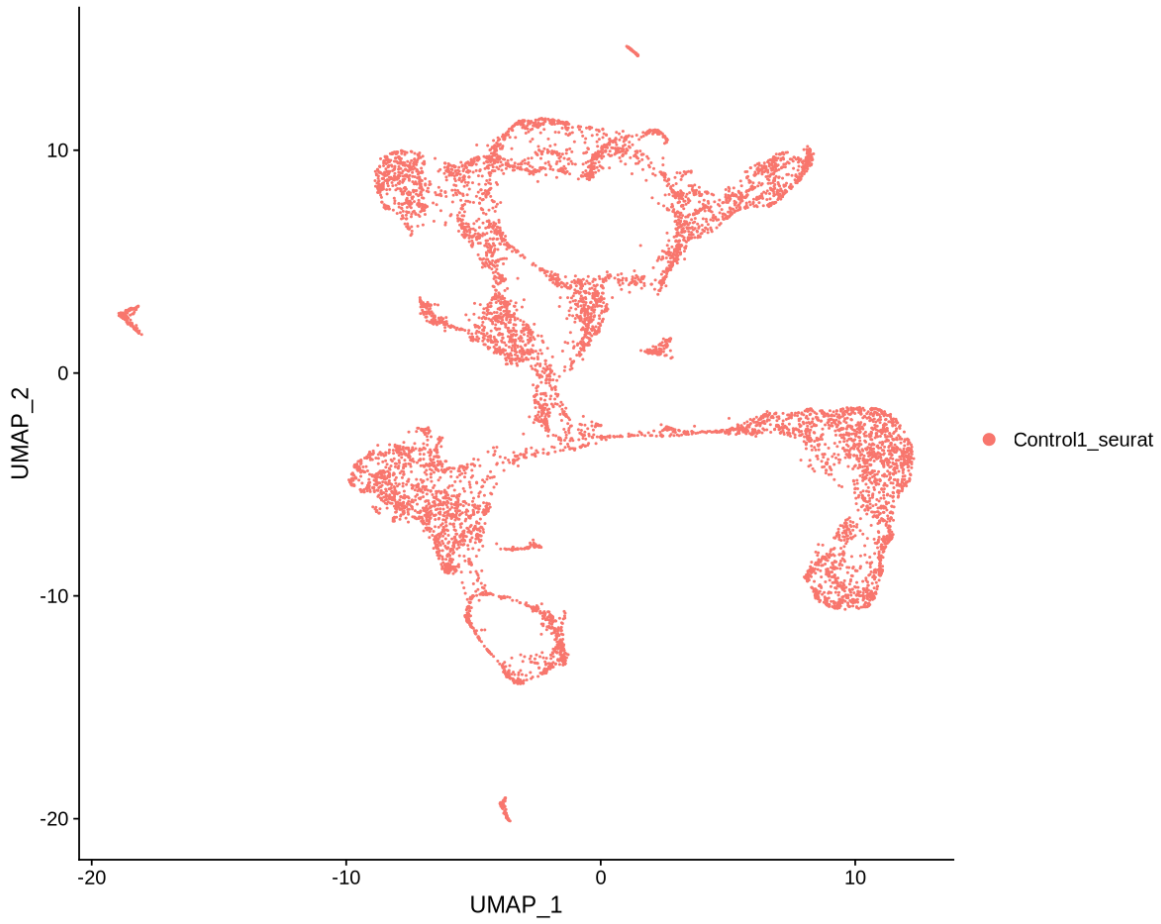


Figure 17: UMAP projection of the data

2.6 Removing doublets

Doublets removal is part of filtering, but it needs normalized data to work. This is why we do it after using `SCtransform`.

Doublets (and the very rare multiplets) refer to droplets that **contain the transcriptional profiles of two or more distinct cells**. Doublets can occur during the cell dissociation process or when two or more cells are captured in the same droplet during the library preparation step.

It is quite obvious that a doublet transcriptional profile can confound downstream analyses, such as cell clustering and differential gene expression analysis. Most doublet detectors, like `DoubletFinder` (McGinnis, Murrow, and Gartner (2019)) which we will use, **simulates doublets and then finds cells in the data which are similar to the simulated doublets**.

Most such packages need an idea of the number/proportion of expected doublets in the dataset. **As indicated from the Chromium user guide, expected doublet rates are about as follows:**

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~870	~500
~0.8%	~1700	~1000
~1.6%	~3500	~2000
~2.3%	~5300	~3000
~3.1%	~7000	~4000
~3.9%	~8700	~5000
~4.6%	~10500	~6000
~5.4%	~12200	~7000
~6.1%	~14000	~8000
~6.9%	~15700	~9000
~7.6%	~17400	~10000

Figure 18: Table of expected doublet rates based on the number of cells.

The data we are using contained about 10000 cells per sample (as in the knee plot at the beginning), hence we can assume that it originates from around 18000 loaded cells and should have a doublet rate at about 7.6%.

> **Note**
>
> Doublet prediction, like the rest of the filtering, **should be run on each sample separately**.

Here, we apply `DoubletFinder` to predict doublet cells. Most parameters are quite standard, we mostly need to choose `nExp` (expected number of doublets), `PCs` (number of principal components to use), `sct` (use the normalized data). The last three option are not part of the package, but have been added by creating a slightly modified version (here) - they allow to use multiple cores and a subset of cells for calculations for a considerable speedup. However, the code takes some time to run, so be patient.

```
nExp <- round(ncol(Control1_seurat_norm) * 0.076)  # expected doublet rate
```

```
Control1_seurat_norm <- doubletFinder_v3(Control1_seurat_norm,
                                         pN = 0.25, #proportion of doublets to simu
                                         pK = 0.09,
                                         nExp = nExp,
```

```
                                                    PCs = 1:15,
                                                    sct=TRUE,
                                                    workers=8,
                                                    future.globals.maxSize = 8*1024^13,
                                                    seurat.ncells=3000)
```

```
Loading required package: fields

Loading required package: spam

Spam version 2.10-0 (2023-10-23) is loaded.
Type 'help( Spam)' or 'demo( spam)' for a short introduction
and overview of this package.
Help for individual functions is also obtained by adding the
suffix '.spam' to the function name, e.g. 'help( chol.spam)'.


Attaching package: ‘spam’


The following object is masked from ‘package:stats4’:

    mle


The following objects are masked from ‘package:base’:

    backsolve, forwardsolve


Loading required package: viridisLite


Try help(fields) to get started.

Loading required package: KernSmooth

KernSmooth 2.23 loaded
Copyright M. P. Wand 1997-2009

Loading required package: future
```

```
Loading required package: sctransform

Calculating cell attributes from input UMI matrix: log_umi

Variance stabilizing transformation of count matrix of size 23388 by 10680

Model formula is y ~ log_umi

Get Negative Binomial regression parameters per gene

Using 2000 genes, 3000 cells

Found 151 outliers - those will be ignored in fitting/regularization step


Second step: Get residuals using fitted parameters for 23388 genes

Computing corrected count matrix for 23388 genes

Calculating gene attributes

Wall clock passed: Time difference of 1.655047 mins

Determine variable features

Place corrected count matrix in counts slot

Centering data matrix

Set default assay to SCT

PC_ 1
Positive:  LotjaGi2g1v0360900, LotjaGi5g1v0359500, LotjaGi6g1v0155900, LotjaGi6g1v0155800, Lo
       LotjaGi3g1v0009600, LotjaGi1g1v0539300, LotjaGi3g1v0450900, LotjaGi2g1v0269100, LotjaG
       LotjaGi1g1v0014300, LotjaGi5g1v0359400, LotjaGi6g1v0071000, LotjaGi3g1v0012400, LotjaG
Negative:  LotjaGi6g1v0254300, LotjaGi3g1v0068000, LotjaGi6g1v0286800-LC, LotjaGi1g1v0080000
       LotjaGi3g1v0358300, LotjaGi3g1v0329100, LotjaGi3g1v0445300, LotjaGi1g1v0646500-LC, Lot
       LotjaGi1g1v0577100, LotjaGi3g1v0395900-LC, LotjaGi5g1v0031100, LotjaGi5g1v0288600, Lot
PC_ 2
Positive:  LotjaGi3g1v0358300, LotjaGi6g1v0254300, LotjaGi1g1v0646500-LC, LotjaGi3g1v0038800
       LotjaGi3g1v0329100, LotjaGi6g1v0043900, LotjaGi1g1v0261700, LotjaGi4g1v0313900, LotjaG
       LotjaGi1g1v0690000, LotjaGi6g1v0155800, LotjaGi4g1v0293000-LC, LotjaGi2g1v0360900, Lot
Negative:  LotjaGi5g1v0248500, LotjaGi1g1v0405300, LotjaGi1g1v0074900, LotjaGi1g1v0683300, Lo
```

```
       LotjaGi2g1v0019900, LotjaGi4g1v0217400, LotjaGi5g1v0248600, LotjaGi4g1v0256800, LotjaG
       LotjaGi1g1v0109000, LotjaGi3g1v0493400-LC, LotjaGi5g1v0159400, LotjaGi3g1v0086100-LC,
PC_ 3
Positive:  LotjaGi3g1v0445300, LotjaGi3g1v0505900, LotjaGi1g1v0594900, LotjaGi1g1v0502700, Lo
       LotjaGi6g1v0028000-LC, LotjaGi4g1v0207600, LotjaGi3g1v0115600, LotjaGi1g1v0475000-LC,
       LotjaGi5g1v0266100, LotjaGi2g1v0402200, LotjaGi3g1v0359600, LotjaGi3g1v0506700, LotjaG
Negative:  LotjaGi1g1v0405300, LotjaGi5g1v0248500, LotjaGi4g1v0018700-LC, LotjaGi1g1v0683300
       LotjaGi4g1v0217400, LotjaGi4g1v0256800, LotjaGi2g1v0160200, LotjaGi3g1v0204100, LotjaG
       LotjaGi6g1v0315500, LotjaGi3g1v0086100-LC, LotjaGi3g1v0358300, LotjaGi6g1v0246700, Lot
PC_ 4
Positive:  LotjaGi5g1v0005800, LotjaGi1g1v0558200, LotjaGi5g1v0288600, LotjaGi3g1v0174100, Lo
       LotjaGi4g1v0121800, LotjaGi3g1v0395900-LC, LotjaGi2g1v0126700, LotjaGi5g1v0099800, Lot
       LotjaGi2g1v0368200, LotjaGi1g1v0393600, LotjaGi1g1v0114400, LotjaGi4g1v0064700, LotjaG
Negative:  LotjaGi5g1v0269800-LC, LotjaGi5g1v0120700, LotjaGi3g1v0420400, LotjaGi2g1v0157900
       LotjaGi1g1v0022100, LotjaGi2g1v0303000, LotjaGi3g1v0055400, LotjaGi2g1v0176500-LC, Lot
       LotjaGi3g1v0115400, LotjaGi3g1v0505900, LotjaGi1g1v0723600-LC, LotjaGi1g1v0690000, Lot
PC_ 5
Positive:  LotjaGi3g1v0328800, LotjaGi3g1v0222100, LotjaGi4g1v0417500, LotjaGi3g1v0328900, Lo
       LotjaGi3g1v0395900-LC, LotjaGi6g1v0069200, LotjaGi6g1v0155800, LotjaGi6g1v0286800-LC,
       LotjaGi1g1v0601700, LotjaGi3g1v0174100, LotjaGi1g1v0594900, LotjaGi5g1v0266100, LotjaG
Negative:  LotjaGi4g1v0121800, LotjaGi3g1v0068000, LotjaGi5g1v0099800, LotjaGi3g1v0178400, Lo
       LotjaGi3g1v0192300, LotjaGi2g1v0301500, LotjaGi4g1v0300800-LC, LotjaGi3g1v0162600, Lot
       LotjaGi1g1v0137600, LotjaGi3g1v0001800, LotjaGi1g1v0760600, LotjaGi5g1v0094100, LotjaG


[1] "Creating 2670 artificial doublets..."
[1] "Creating Seurat object..."
[1] "Running SCTransform..."
  |======================================================================| 100%
  |======================================================================| 100%
  |======================================================================| 100%
[1] "Running PCA..."
[1] "Calculating PC distance matrix..."
[1] "Computing pANN..."
[1] "Classifying doublets.."
```

We visualize the UMAP plot and which cells are estimated doublets in Figure 19. Fortunately, there are only a few to discard.

```
options(repr.plot.width=10, repr.plot.height=10)

DF.name = colnames(Control1_seurat_norm@meta.data)[grepl("DF.classification", colnames(Contro
```

```
DimPlot(Control1_seurat_norm, group.by = DF.name, pt.size = 2,
        split.by = DF.name)
```

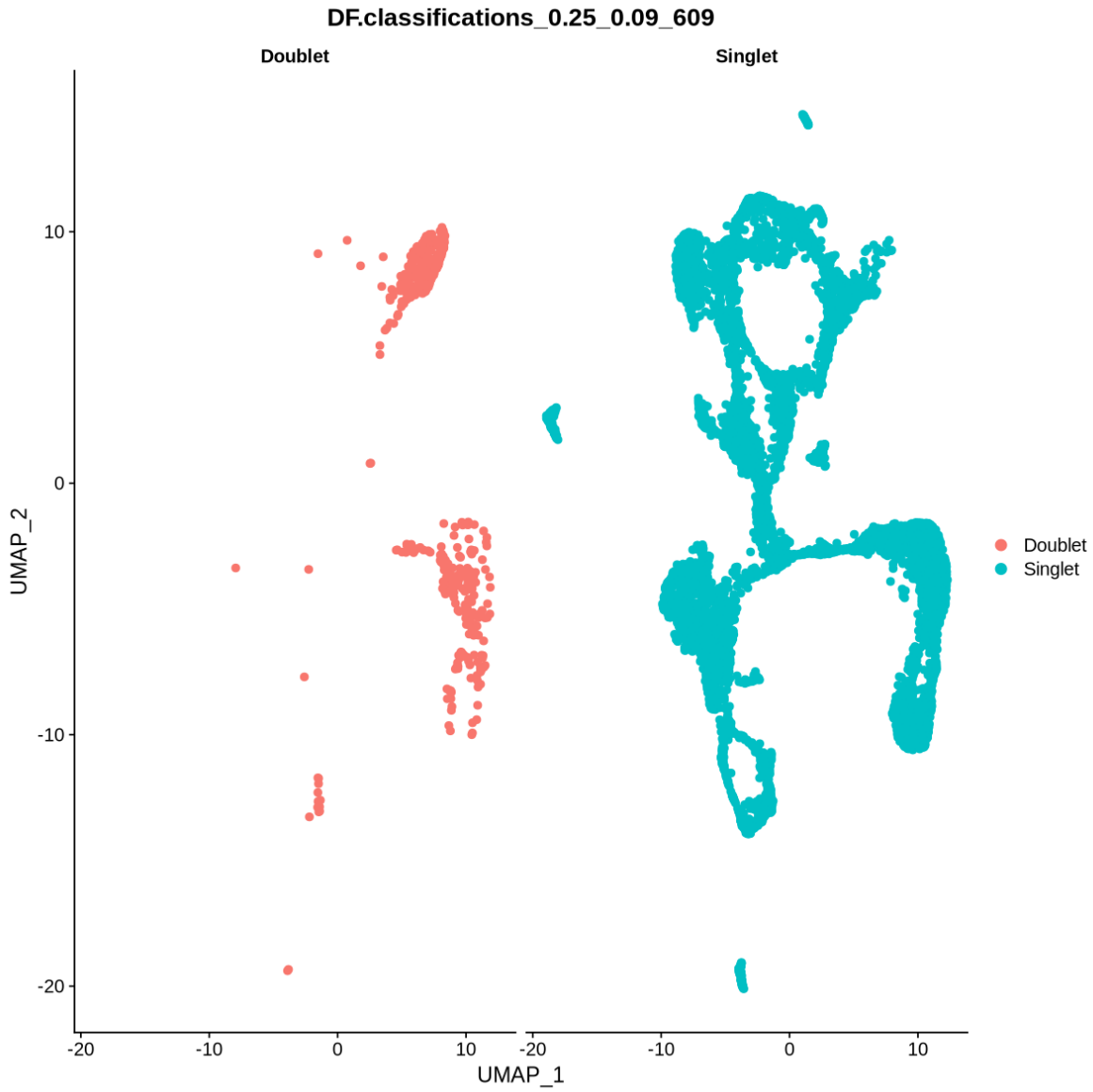
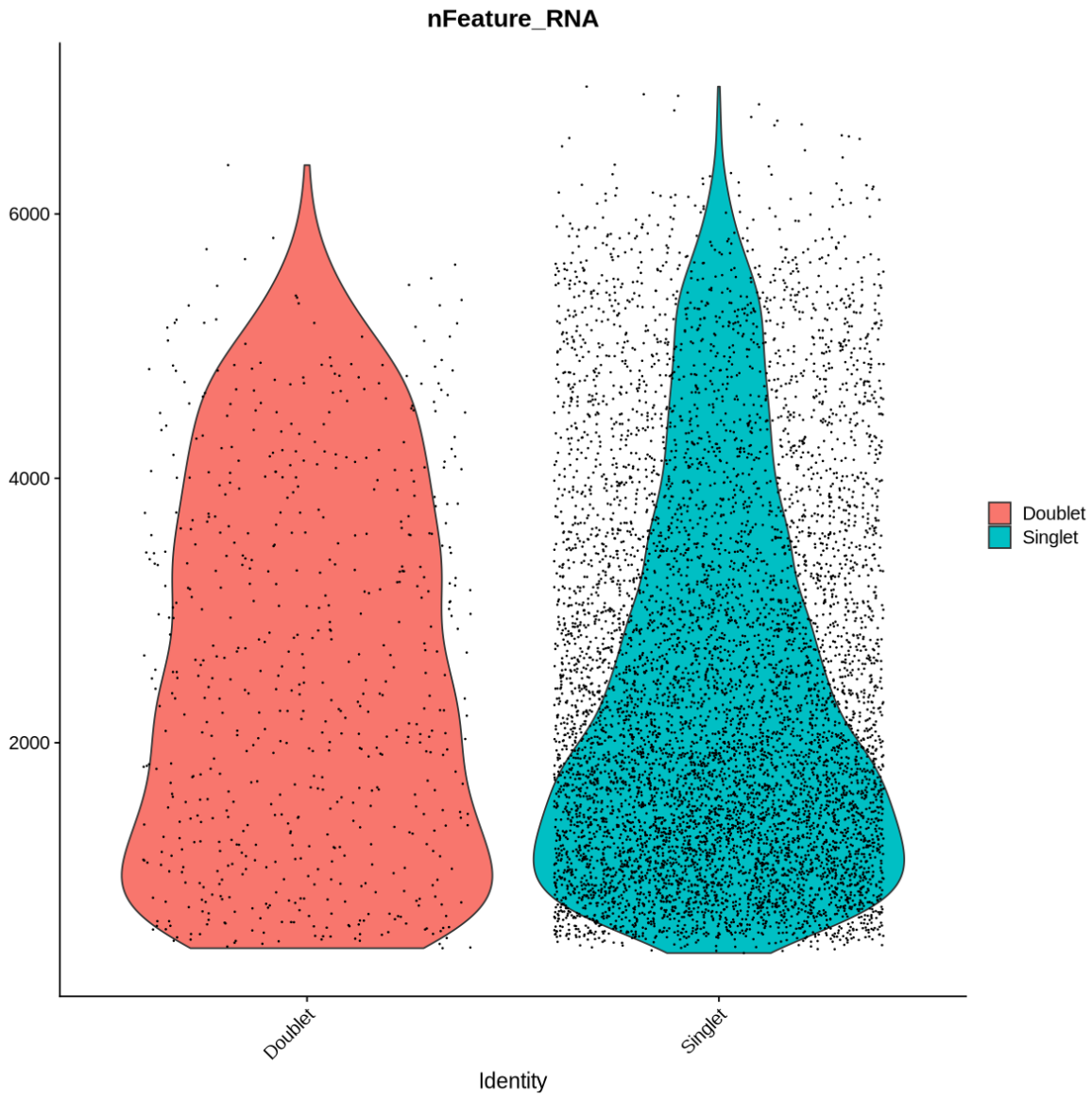


Figure 19: UMAP projection of the data coloured by doublet or singlet label

Sometimes doublets have more detected genes than a single cell. In our case, some of the droplets have higher number of genes than the average (the red violin is large also above 3000 detected genes), so there is aclear sign of the presence of some doublets. Of course, as with

any filtering, we might remove some actual cells. To be more effective in our filtering, we can select doublets with more than 2000 detected genes when we filter.

```
VlnPlot(Control1_seurat_norm, features = "nFeature_RNA", group.by = DF.name, pt.size = 0.1)
```



Here we keep only singlets:

```
Control1_seurat_norm = Control1_seurat_norm[, (Control1_seurat_norm@meta.data[, DF.name] == '
```

We save our data after all the filtering work!

```
SaveH5Seurat(object = Control1_seurat_norm,
             filename = "control1.normalized.h5Seurat",
             overwrite = TRUE,
             verbose = FALSE)
```

```
Warning message:
"Overwriting previous file control1.normalized.h5Seurat"
Creating h5Seurat file for version 3.1.5.9900
```

3 Integration

Integration of scRNA-seq data is useful to combine datasets from different experimental conditions (in our case the Control vs Infected) and sequencing runs, to gain a broader understanding of cellular processes. Integration is challenging due to technical variations and biological differences between the datasets (where we want to remove the formers to study correctly the latters).

Before integrating scRNA-seq datasets, we have applyed **quality control and normalization to each sample** to ensure consistency and accuracy of the data. Integration can happen using various methods (Adossa et al. (2021)). Seurat uses **canonical correlation analysis (CCA)** (Stuart et al. (2019), Xinming (2022)) to integrate scRNA-seq datasets from different experimental conditions. CCA identifies shared variation between two datasets while accounting for technical differences, such as batch effects.

The shared covariance patterns **can represent biological signals that are common across the datasets**, such as cell types or signaling pathways.

We load another control and two infected datasets. Those have been previously preprocessed, so you will not need to. Remember again: each dataset must be preprocessed separately before integration.

```
Control1_seurat_norm <- LoadH5Seurat("control1.normalized.h5Seurat", verbose = FALSE)
```

```
Validating h5Seurat file
```

```
Control2_seurat_norm <- LoadH5Seurat("../Data/control2.normalized.h5Seurat", verbose = FALSE)
Infected1_seurat_norm <- LoadH5Seurat("../Data/infected1.normalized.h5Seurat", verbose = FALS
Infected2_seurat_norm <- LoadH5Seurat("../Data/infected2.normalized.h5Seurat", verbose = FALS
```

```
Validating h5Seurat file
```

```
Validating h5Seurat file
```

```
Validating h5Seurat file
```

To integrate the datasets, we need to start creating a list with all datasets.

```
Gifu.list <- list(Control1_seurat_norm,
                  Control2_seurat_norm,
                  Infected1_seurat_norm,
                  Infected2_seurat_norm)
```

We then start by normalizing each dataset of the list with `SCtransform`. Here we also have a commented command (with the symbol `#`) that is not executed, to show how you add technical variations to be removed with the option `vars.to.regress`, if necessary (this is not the case of the tutorial).

```
Gifu.list <- lapply(X = Gifu.list, FUN = function(x) {
  message("Normalizing\n")
  #x <- SCTransform(x, vars.to.regress = c("percent.mt",  "percent.chloroplast"), variable.f
  x <- SCTransform(x, ncells=3000, variable.features.n = 10000, return.only.var.genes = FALSE
})
```

```
Normalizing
```

```
Normalizing
```

```
Normalizing
```

```
Normalizing
```

Now we apply the CCA (Canonical Correlation Analysis) to put datasets together according to their similarities, while removing differences. The number of genes to use during integration is expressed below as `nfeatures`. We choose a reasonable number of features, for example 10000, which is similar to what we used in the normalization steps along the tutorial.

```
Gifu.features <- SelectIntegrationFeatures(object.list = Gifu.list, nfeatures = 10000)

Gifu.list <- PrepSCTIntegration(object.list = Gifu.list, anchor.features = Gifu.features)
```

```
Gifu.anchors <- FindIntegrationAnchors(object.list = Gifu.list, normalization.method = "SCT"
                                       anchor.features = Gifu.features, reference = c(1,2))

seurat.integrated <- IntegrateData(anchorset = Gifu.anchors, normalization.method = "SCT")
```

```
Warning message in CheckDuplicateCellNames(object.list = object.list):
"Some cell names are duplicated across objects provided. Renaming to enforce unique cell name
Finding anchors between all query and reference datasets

Running CCA

Merging objects

Finding neighborhoods

Finding anchors

    Found 8777 anchors

Filtering anchors

    Retained 8517 anchors

Running CCA

Merging objects

Finding neighborhoods

Finding anchors

    Found 8962 anchors
```

```
Filtering anchors
    Retained 8046 anchors
Running CCA
Merging objects
Finding neighborhoods
Finding anchors
    Found 10128 anchors
Filtering anchors
    Retained 9486 anchors
Running CCA
Merging objects
Finding neighborhoods
Finding anchors
    Found 7768 anchors
Filtering anchors
    Retained 7424 anchors
Running CCA
Merging objects
Finding neighborhoods
Finding anchors
    Found 8694 anchors
Filtering anchors
```

```
    Retained 8493 anchors

Building integrated reference

Merging dataset 1 into 2

Extracting anchors for merged samples

Finding integration vectors

Finding integration vector weights

Integrating data


Integrating dataset 3 with reference dataset

Finding integration vectors

Finding integration vector weights

Integrating data

Warning message:
"UNRELIABLE VALUE: One of the ‘future.apply’ iterations (‘future_lapply-1’) unexpectedly gene

Integrating dataset 4 with reference dataset

Finding integration vectors

Finding integration vector weights

Integrating data

Warning message:
"UNRELIABLE VALUE: One of the ‘future.apply’ iterations (‘future_lapply-2’) unexpectedly gene
```

Now the default assay used for analysis has changed into `integrated`:

```
cat("The default assay of the data is now called: ")
cat(DefaultAssay(seurat.integrated))
```

```
The default assay of the data is now called: integrated
```

We need to recalculate PCA and UMAP to look at all datasets integrated together. We choose again 10 principal components from Figure 20. The newUMAP is in Figure 21.

```
seurat.integrated <- RunPCA(object = seurat.integrated, verbose = FALSE)
```

```
ElbowPlot(seurat.integrated, ndims = 30)
```

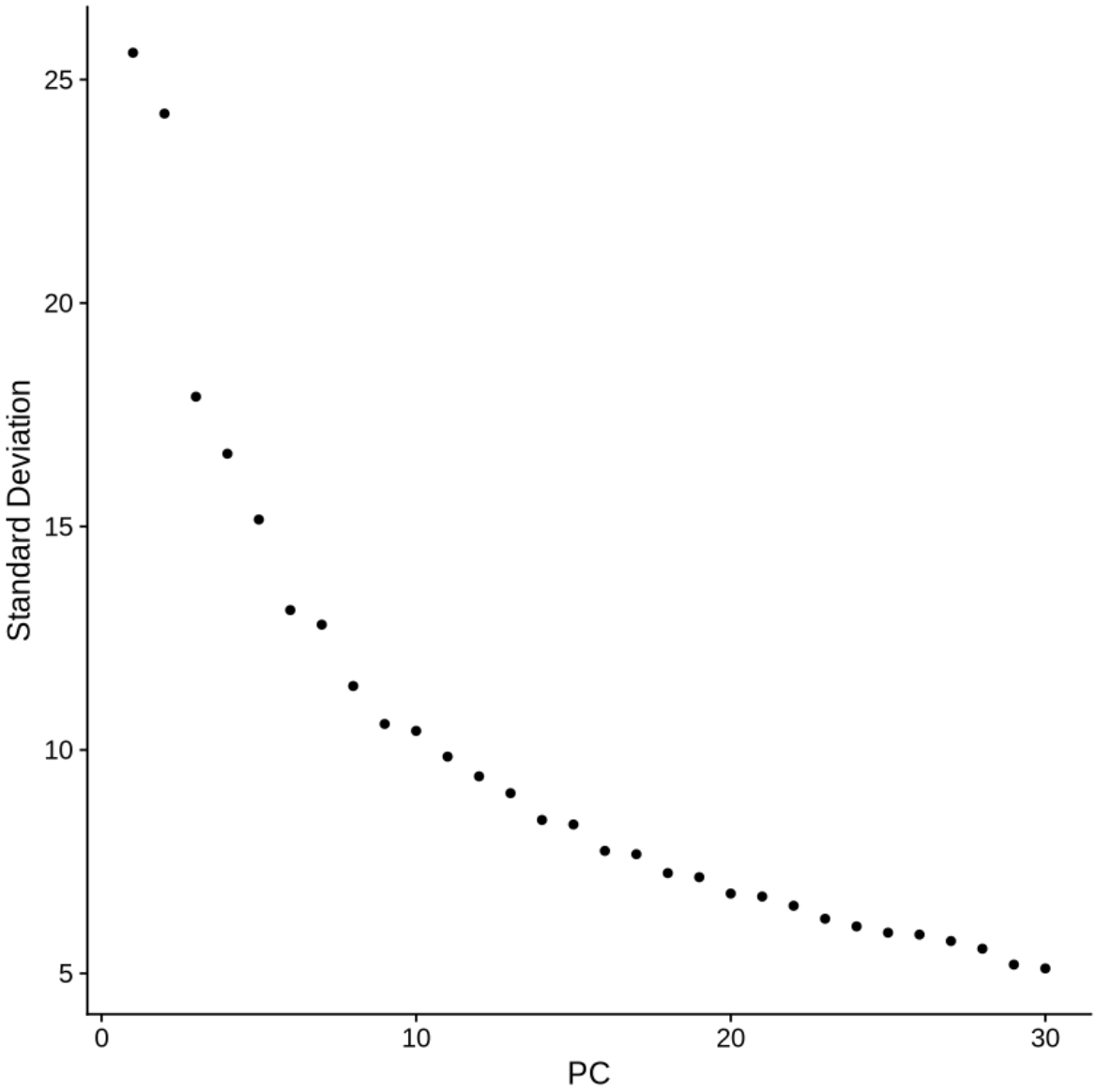


Figure 20: Elbow plot of integrated datasets

```
seurat.integrated <- FindNeighbors(object = seurat.integrated, dims = 1:20, k.param = 5)
```

```
Computing nearest neighbor graph
```

```
Computing SNN
```

```r
seurat.integrated <- RunUMAP(object = seurat.integrated, dims = 1:20)
```

```
Warning message:
"The default method for RunUMAP has changed from calling Python UMAP via reticulate to the R-
To use Python UMAP via reticulate, set umap.method to 'umap-learn' and metric to 'correlatio
This message will be shown once per session"
10:31:42 UMAP embedding parameters a = 0.9922 b = 1.112

Found more than one class "dist" in cache; using the first, from namespace 'spam'

Also defined by ‘BiocGenerics’

10:31:42 Read 17721 rows and found 20 numeric columns

10:31:42 Using Annoy for neighbor search, n_neighbors = 30

Found more than one class "dist" in cache; using the first, from namespace 'spam'

Also defined by ‘BiocGenerics’

10:31:42 Building Annoy index with metric = cosine, n_trees = 50

0%   10   20   30   40   50   60   70   80   90   100%

[----|----|----|----|----|----|----|----|----|----|

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10:31:44 Writing NN index file to temp file /tmp/Rtmp4PPgeq/file580bd59165

10:31:44 Searching Annoy index using 8 threads, search_k = 3000

10:31:45 Annoy recall = 100%

10:31:47 Commencing smooth kNN distance calibration using 8 threads
```

```
 with target n_neighbors = 30

10:31:49 Initializing from normalized Laplacian + noise (using irlba)

10:31:57 Commencing optimization for 200 epochs, with 723462 positive edges

10:32:06 Optimization finished
```

```
seurat.integrated <- SetIdent(seurat.integrated, value = "orig.ident")
```

```
options(repr.plot.width=10, repr.plot.height=8)
DimPlot(object = seurat.integrated, reduction = "umap",  label = T, repel = TRUE, pt.size = (
```

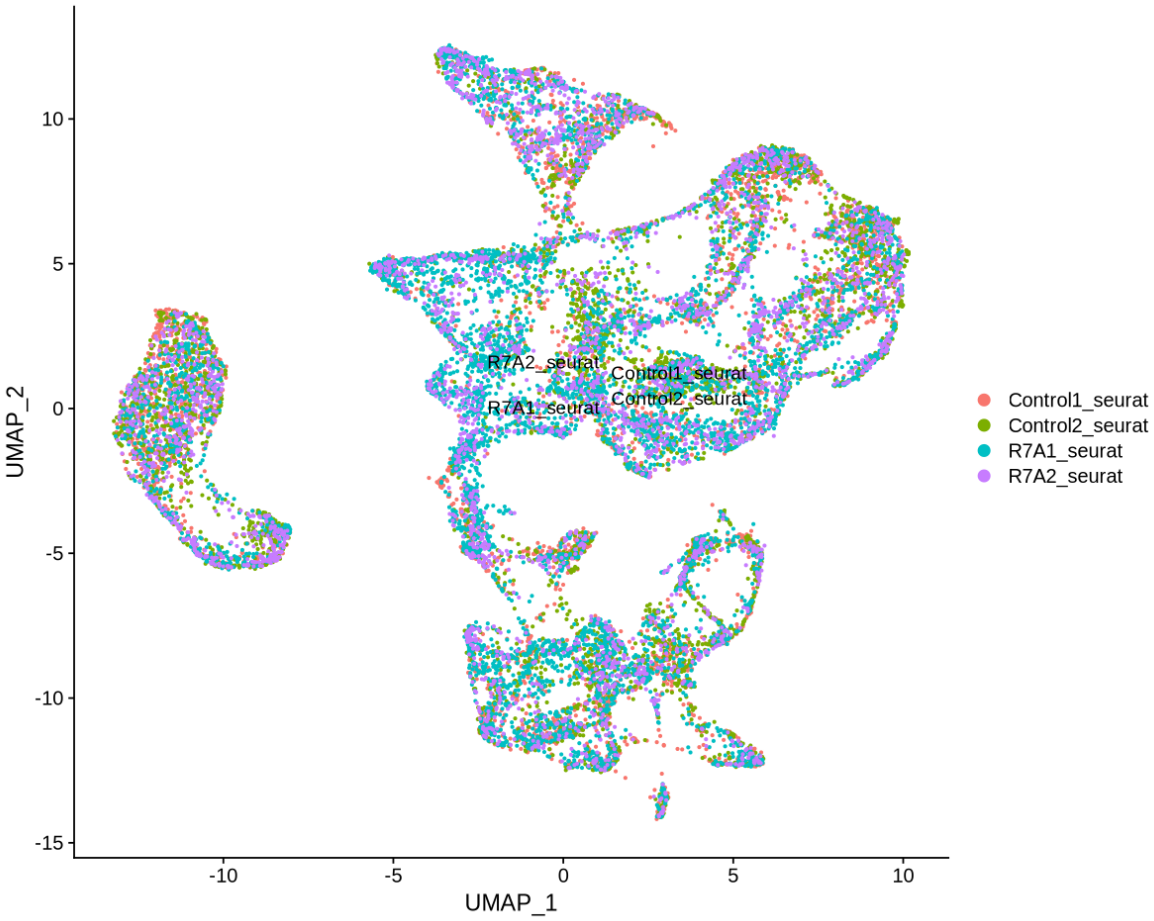


Figure 21: UMAP of the integrated datasets.

We save our integrated data

```
SaveH5Seurat(object = seurat.integrated, filename = "seurat.integrated.h5Seurat", overwrite=T
```

```
Warning message:
"Overwriting previous file seurat.integrated.h5Seurat"
Creating h5Seurat file for version 3.1.5.9900

Adding counts for SCT

Adding data for SCT

Adding scale.data for SCT

No variable features found for SCT

No feature-level metadata found for SCT

Writing out SCTModel.list for SCT

Adding counts for RNA

Adding data for RNA

No variable features found for RNA

No feature-level metadata found for RNA

Adding data for integrated

Adding scale.data for integrated

Adding variable features for integrated

No feature-level metadata found for integrated

Writing out SCTModel.list for integrated

Adding cell embeddings for pca

Adding loadings for pca
```

```
No projected loadings for pca

Adding standard deviations for pca

No JackStraw data for pca

Adding cell embeddings for umap

No loadings for umap

No projected loadings for umap

No standard deviations for umap

No JackStraw data for umap
```

3.1 Clustering and cell type assignment

We perform clustering on the data using the leiden algorithm (blondel_fast_2008, Traag, Waltman, and Van Eck (2019)). Then, we look at a typical strategy of **naming clusters by visualizing known markers**. Since this is very subjective and biased, we then resort to naming cell types **using a reference annotated dataset**. An overview of cell type assignment procedures can be found at Cheng et al. (2023).

```
seurat.integrated <-  LoadH5Seurat("seurat.integrated.h5Seurat", verbose=FALSE)
```

```
Validating h5Seurat file

Warning message:
"Adding a command log without an assay associated with it"
```

Clustering function `FindClusters`. The resolution is used to change the number of clusters. We do not need many, so we set on to 0.25. Usual values range between 0.1 and 1.

```
seurat.integrated <- FindClusters(object = seurat.integrated, resolution = .25)
```

```
Modularity Optimizer version 1.3.0 by Ludo Waltman and Nees Jan van Eck

Number of nodes: 17721
Number of edges: 165857
```

```
Running Louvain algorithm...
Maximum modularity in 10 random starts: 0.9631
Number of communities: 21
Elapsed time: 0 seconds

Warning message:
"UNRELIABLE VALUE: One of the ‘future.apply’ iterations (‘future_lapply-1’) unexpectedly gen
```

The clusters are saved in the meta data table as `integrated_snn_res.0.25`. **Note that the name changes with the resolution**. Also observe how much metadata we have: many columns come from tools we have applied, such as doubletfinder (DF) and nearest neighbor distances (snn).

```
head( seurat.integrated@meta.data )
```

A data.frame: 6 × 18

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	<dbl>	<int>	<dbl>	<int>	<chr>	<chr>	<dbl>	<dbl>	<dbl>	<chr>	<dbl>	<chr>	<dbl>	<chr>	<dbl>	<chr>	<fct>	<fct>
[illegible] 1_1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	NA	NA	NA	NA	NA	NA	4	4
[illegible] 1_1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	NA	NA	NA	NA	NA	NA	9	9
[illegible] 1_1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	NA	NA	NA	NA	NA	NA	0	0
[illegible] 1_1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	NA	NA	NA	NA	NA	NA	16	16
[illegible] 1_1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	NA	NA	NA	NA	NA	NA	17	17
[illegible] 1_1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	NA	NA	NA	NA	NA	NA	2	2

We can plot the clusters in the UMAP plot

```
options(repr.plot.width=10, repr.plot.height=8)
DimPlot(object = seurat.integrated, reduction = "umap",  label = T, repel = TRUE, pt.size = 
```

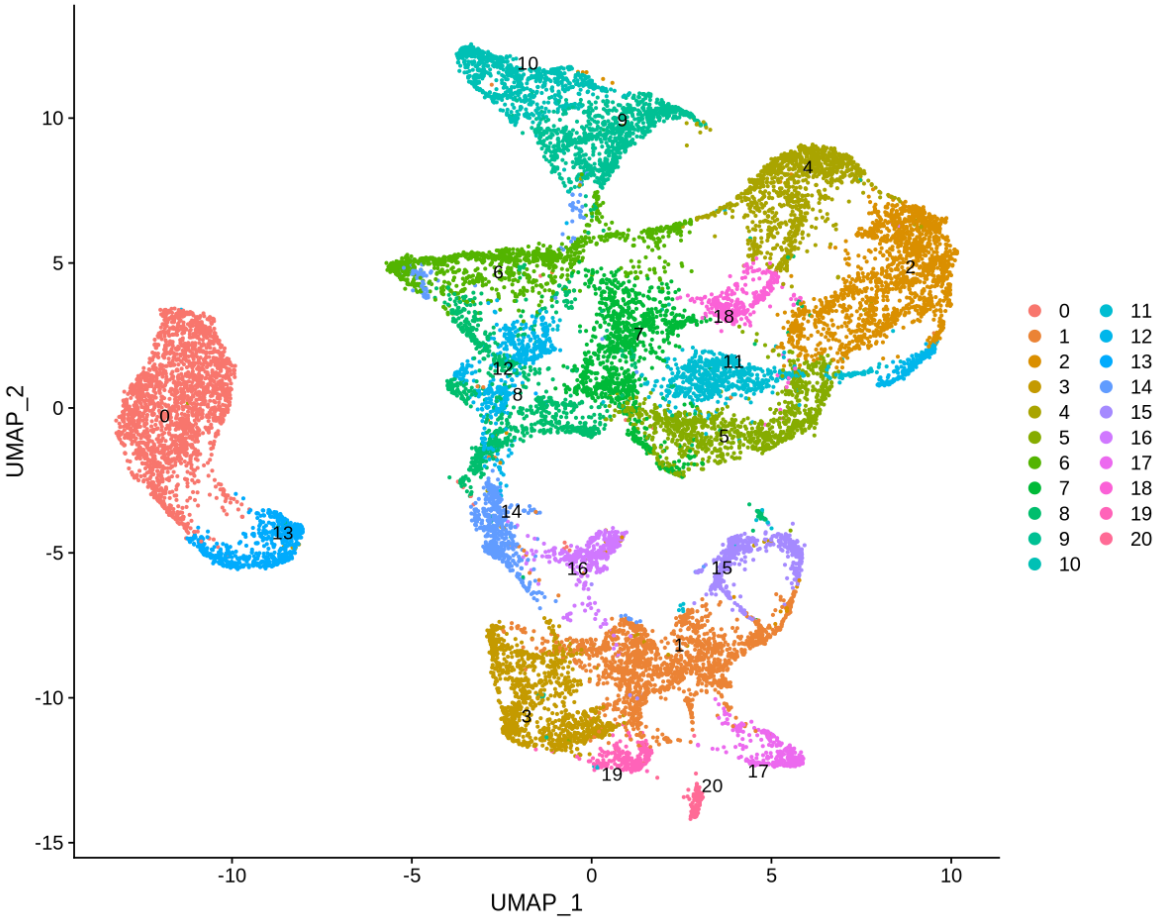


Figure 22: UMAP of the integrated datasets with clusters (still unassigned to cell types).

3.1.1 Cluster assignment from visualized marker scores

Here, we look at how to assign names based on known markers. In this procedure, biological knowledge of the cell types is needed. Below, there is a list of known markers for each cell type, extracted from the supplementary data of Frank et al. (2023).

```
features_list <- list(
    'Cortex_scoring' = c("LotjaGi1g1v0006200",
                "LotjaGi1g1v0022100",
                "LotjaGi1g1v0261700",
                "LotjaGi1g1v0348000",
                "LotjaGi2g1v0303000",
                "LotjaGi3g1v0505900"),
```

```
    'Epidermis_scoring' = c("LotjaGi1g1v0080000",
                    "LotjaGi1g1v0377600",
                    "LotjaGi1g1v0613100",
                    "LotjaGi3g1v0070500"),
    'Endodermis_scoring' = c("LotjaGi1g1v0114400",
                     "LotjaGi1g1v0221300",
                     "LotjaGi1g1v0240900-LC",
                     "LotjaGi1g1v0707500"),
    'RootCap_scoring' = c("LotjaGi1g1v0020900",
                   "LotjaGi1g1v0039700-LC",
                   "LotjaGi1g1v0040300",
                   "LotjaGi1g1v0147500"),
    'Meristem_scoring'= c("LotjaGi4g1v0300900",
                          "LotjaGi6g1v0056500",
                          "LotjaGi1g1v0594200"),
    'Phloem_scoring'= c("LotjaGi1g1v0028800",
                "LotjaGi1g1v0085900",
                "LotjaGi1g1v0119300",
                "LotjaGi1g1v0149100"),
    'QuiescentCenter_scoring' = c("LotjaGi1g1v0004300",
                            "LotjaGi1g1v0021400",
                            "LotjaGi1g1v0052700",
                            "LotjaGi1g1v0084000"),
    'RootHair_scoring'= c("LotjaGi1g1v0014300",
                   "LotjaGi1g1v0109000",
                   "LotjaGi1g1v0109100",
                   "LLotjaGi1g1v0143900"),
    'Pericycle_scoring'= c("LotjaGi3g1v0222100",
                   "LotjaGi3g1v0395900-LC",
                   "LotjaGi5g1v0166000-LC",
                   "LotjaGi3g1v0395500-LC",
                   "LotjaGi1g1v0783700-LC",
                   "LotjaGi2g1v0333200",
                   "LotjaGi4g1v0293000-LC"),
    'Stele_scoring' = c("LotjaGi2g1v0126700",
                "LotjaGi1g1v0558200",
                "LotjaGi4g1v0215500",
                "LotjaGi3g1v0174100",
                "LotjaGi5g1v0288600",
                "LotjaGi3g1v0129700"),
    'Xylem_scoring' = c("LotjaGi1g1v0623100",
                "LotjaGi1g1v0569300",
```

```
                "LotjaGi1g1v0443000",
                "LotjaGi1g1v0428800")
    )
```

Here, we need a function calculating the scores for each cell type. This is the average expression of the markers in the list, from which we remove the average expression of some control genes, which are supposed not to be specific for the cell type of interest. The cells matching the desired type should retain a high score.

```
seurat.clustered <- AddModuleScore(
  object = seurat.integrated,
  features = features_list,
  ctrl = 5,
  name = 'LJ_scores'
)
```

```
Warning message:
"The following features are not present in the object: LLotjaGi1g1v0143900, not searching fo
```

We also apply a function (from `script.R`) to rename the scores in the metadata. Their names are not intuitive by default, they are all called with the name chosen above and a number after it:

```
names(seurat.clustered@meta.data)
```

1. 'nCount_RNA'
2. 'nFeature_RNA'
3. 'nCount_SCT'
4. 'nFeature_SCT'
5. 'orig.ident'
6. 'Condition'
7. 'percent.mt'
8. 'percent.chloroplast'
9. 'pANN_0.25_0.09_609'
10. 'DF.classifications_0.25_0.09_609'
11. 'pANN_0.25_0.09_329'
12. 'DF.classifications_0.25_0.09_329'
13. 'pANN_0.25_0.09_309'
14. 'DF.classifications_0.25_0.09_309'
15. 'pANN_0.25_0.09_110'
16. 'DF.classifications_0.25_0.09_110'

17. 'integrated_snn_res.0.25'
18. 'seurat_clusters'
19. 'LJ_scores1'
20. 'LJ_scores2'
21. 'LJ_scores3'
22. 'LJ_scores4'
23. 'LJ_scores5'
24. 'LJ_scores6'
25. 'LJ_scores7'
26. 'LJ_scores8'
27. 'LJ_scores9'
28. 'LJ_scores10'
29. 'LJ_scores11'

```
seurat.clustered <- renameScores(markers_list = features_list, seurat_data = seurat.clustered
```

```
Scores renamed FROM
```

```
TO
```

```
LJ_scores1
LJ_scores2
LJ_scores3
LJ_scores4
LJ_scores5
LJ_scores6
LJ_scores7
LJ_scores8
LJ_scores9
LJ_scores10
LJ_scores11
Cortex_scoring
Epidermis_scoring
Endodermis_scoring
RootCap_scoring
Meristem_scoring
```

```
Phloem_scoring
QuiescentCenter_scoring
RootHair_scoring
Pericycle_scoring
Stele_scoring
Xylem_scoring
```

Now we run the function `plotScoresUMAP` (from the file `script.R`). In Figure 23 we can see that some clusters are easy to classify (phloem and xylem), but many others are not. This is mainly due to the fact that the change of many cell types is a continuum, and this manual annotation is very subjective.

```
plotScoresUMAP(markers_list = features_list, seurat_data = seurat.clustered)
```

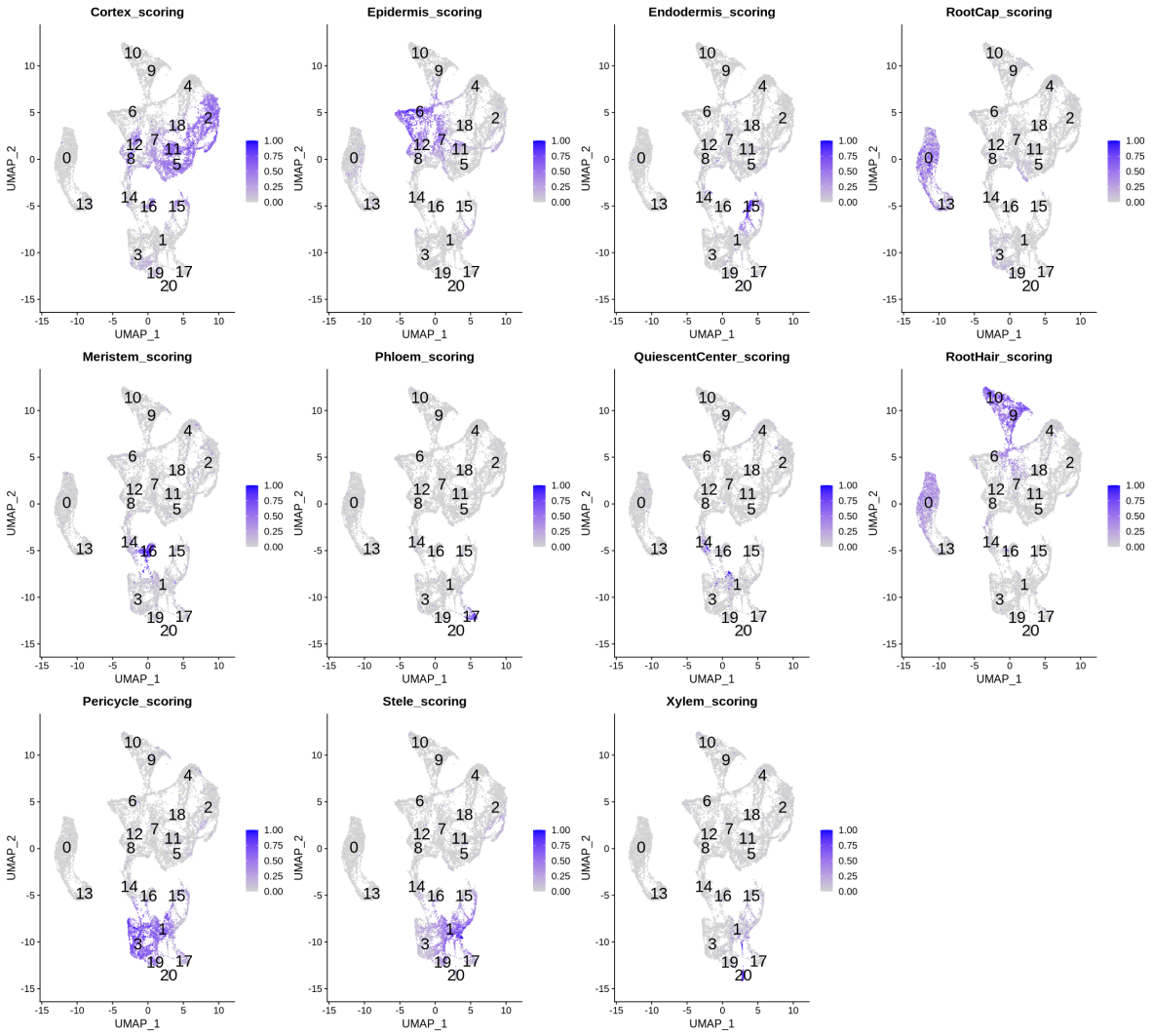


Figure 23: Scores of the list of markers for the Lotus Japonicus clusters. Those should help identifying cell types by plotting the average of markers subtracted the average of other random genes in the data.

Just for the sake of the exercise, we set some names to the clusters below, at least for those ones that are well identifiable. Note that we use `idents` to define which is the clustering to be considered in use in the data.

```
Idents(seurat.clustered) <- 'integrated_snn_res.0.25'

seurat.clustered <- RenameIdents(object = seurat.clustered,
                                 "2"="Cortex", "5"="Cortex", "11"="Cortex",
```

```
                                    "6"="Epidermis", "12"="Epidermis", "7"="Epidermis",
                                    "15"="Endodermis",
                                    "0"="Root_Cap", "13"="Root_Cap",
                                    "16"="Meristem",
                                    "17"="Phloem",
                                    "10"="Root_Hair", "9"="Root_Hair",
                                    "1"="PericycleStele",
                                    "3"="Pericycle", "19"="Pericycle",
                                    "20"="Xylem")
```

we saved the renamed clusters in the metadata under `Cell_types`

```
seurat.clustered@meta.data$Cell_types <- Idents(seurat.clustered)
```

It seems from Figure 24 that we named most clusters. For some others we could not really be sure of the markers, and we will use the reference-based assignment.

```
options(repr.plot.width=10, repr.plot.height=10)
DimPlot(object = seurat.clustered, reduction = "umap", repel = TRUE, label=T, pt.size = 2, la
```

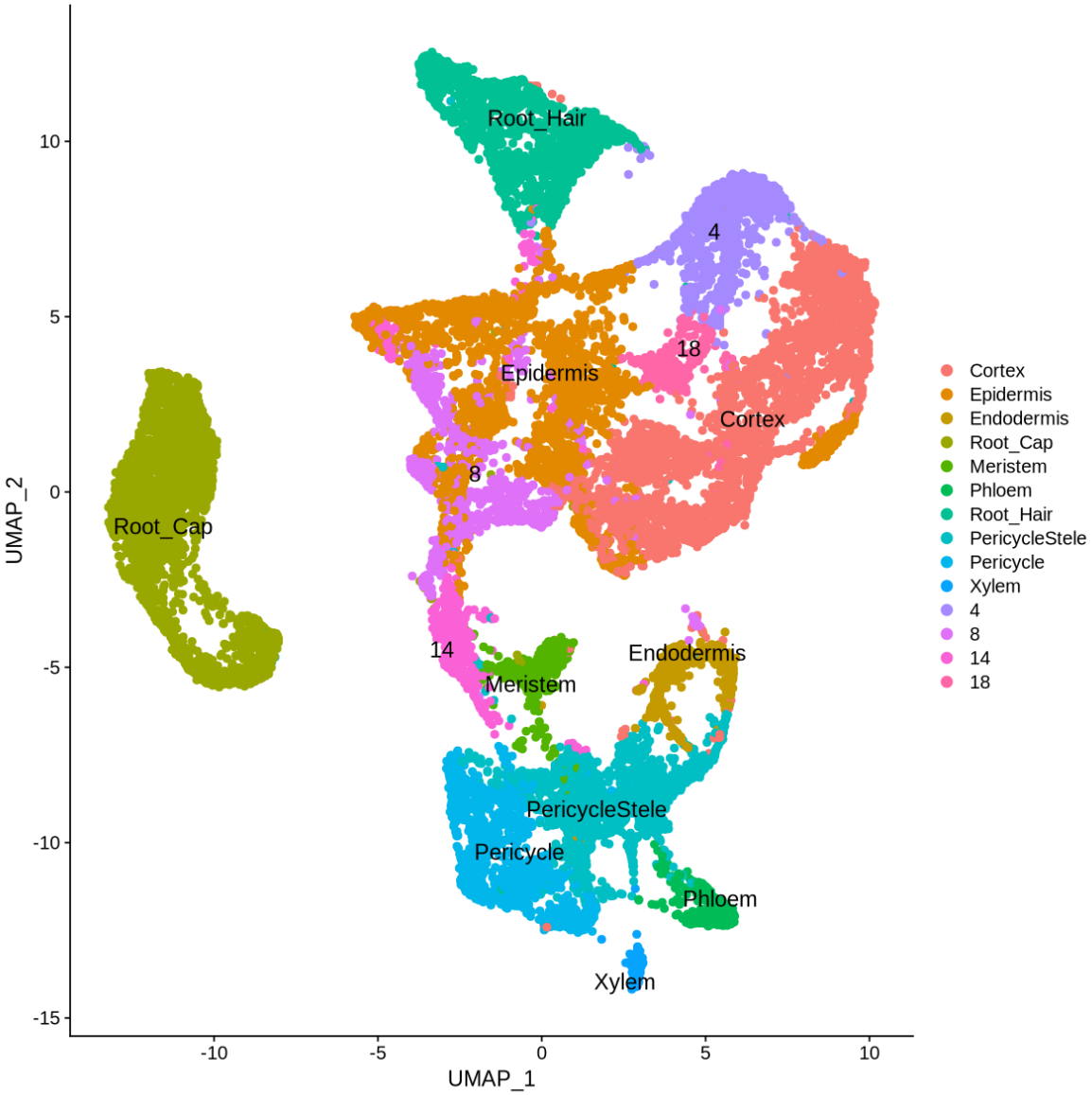


Figure 24: UMAP of the integrated datasets with clusters assigned by looking at markers scores. Note how we could not determine clearly some of the clusters, because of few cells with high scores (cluster 11, which might be epidermis; and cluster 16, which might be quiescent center) or cells that might be a mix of two types (like Pericycle and Stele).

3.1.1.1 Optional: assigning a mixed cluster

The cluster `Pericycle_Stele` is probably a mix of the Pericycle and Stele, because it shows

markers from both. We can assign at each cell of the cluster one of the cell types based on which marker score is highest. First we save the old clustering in the metadata to avoid losing it

```
seurat.clustered@meta.data$Cell_types_old <- Idents(seurat.clustered)
```

and then reassign the name to the cluster `Pericycle_Stele` checking the markers score of each cell.

```
peri <- seurat.clustered@meta.data$Pericycle_scoring[seurat.clustered@meta.data$Cell_types ==
stel <- seurat.clustered@meta.data$Stele_scoring[seurat.clustered@meta.data$Cell_types == 'P
peri_stele <- peri>=stel
finalcl = c()
for(i in peri_stele)
    finalcl = c(finalcl, ifelse(i, "Pericycle", "Stele"))

celltypes <- unfactor(seurat.clustered@meta.data$Cell_types)
celltypes[seurat.clustered@meta.data$Cell_types == 'PericycleStele'] <- finalcl
seurat.clustered@meta.data$Cell_types <- celltypes
Idents(seurat.clustered) <- seurat.clustered@meta.data$Cell_types
```

We managed to get a meaningful separation between the two clusters!

```
options(repr.plot.width=10, repr.plot.height=10)
DimPlot(object = seurat.clustered, reduction = "umap", repel = TRUE, label=T, pt.size = 2, la
```

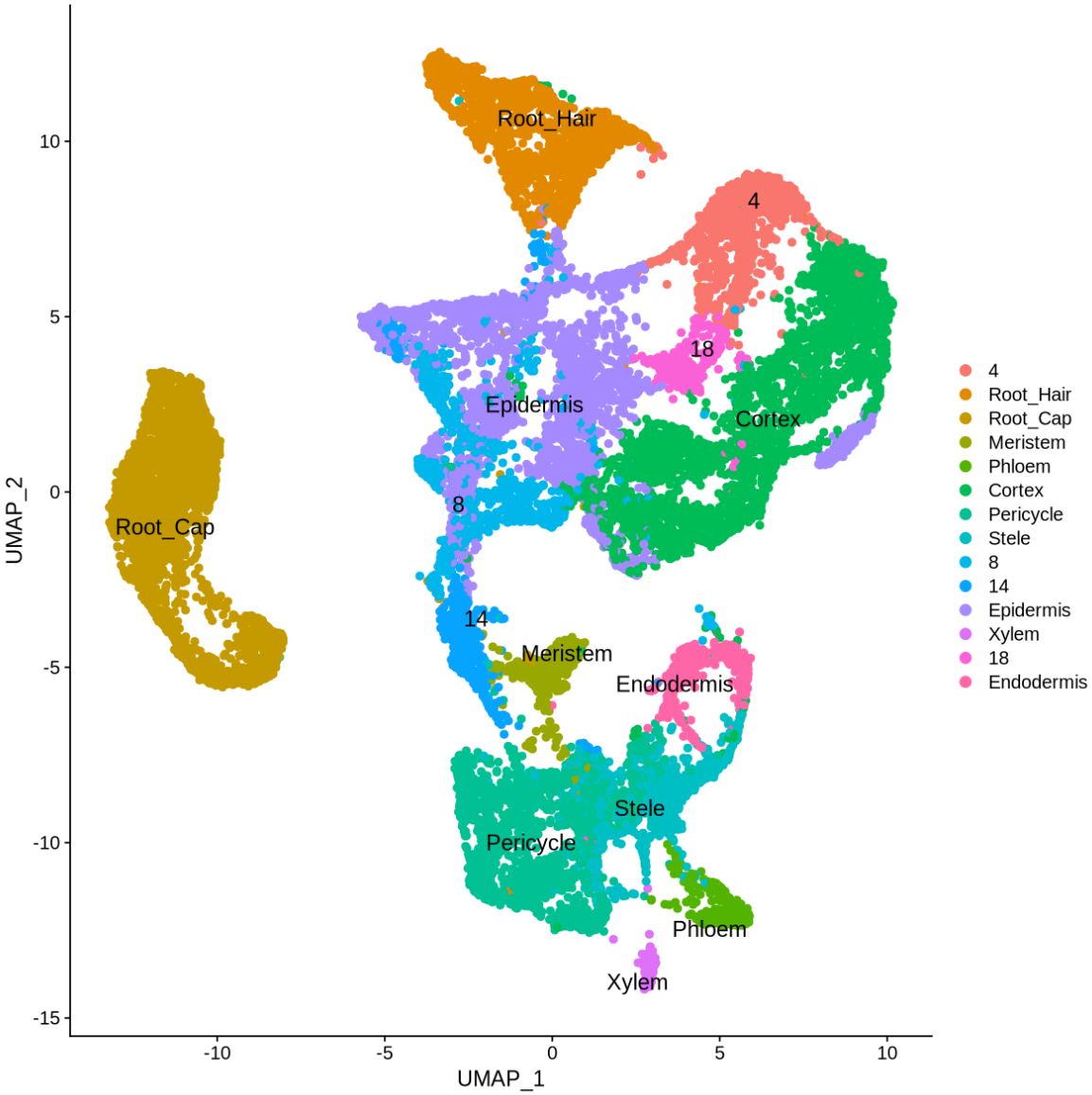


Figure 25: UMAP of the integrated datasets with clusters assigned by looking at markers scores and separated clusters of Peicycle and Stele. In this case, the subclustering has clearly been a success.

3.1.2 Cluster assignment from an annotated dataset

We now use the annotated data from Frank et al. (2023) (which is the same we are using in the tutorial) to transfer data labels to our own processed data. More about label transfer can

be read at Stuart et al. (2019). We load the data from the paper and define reference and query data.

```
seurat.reference <- readRDS("data_lavinia.RDS")
```

```
seurat.query <- seurat.clustered
```

We have to define the data integration between query and reference before we can transfer the cluster names. For the algorithm to work, we need to use the "RNA" assay, which contains raw expression values.

```
DefaultAssay(seurat.query) <- "RNA"
```

```
lotusjaponicus.anchors <- FindTransferAnchors(reference = seurat.reference,
                                              features = intersect( rownames(seurat.query),
                                                          rownames( seurat.reference[['SCT']]@scale
                                              query = seurat.query, dims = 1:20,
                                              reference.reduction = "pca",
                                              reference.assay='RNA')
```

```
Projecting cell embeddings
```

```
Finding neighborhoods
```

```
Finding anchors
```

```
    Found 25859 anchors
```

```
Filtering anchors
```

```
    Retained 13194 anchors
```

Calculating the integration of the labels from the reference takes time. So we save the calculated anchors for the integration. If you need to rerun the code, skip the command above and instead load the data with `readRDS` below.

```
saveRDS(lotusjaponicus.anchors, file = "anchors.RDS")
```

```
lotusjaponicus.anchors <- readRDS("anchors.RDS")
```

Now it is finally time to transfer the labels and add them to the metadata. The column in the metadata is called by default `predicted.id`.

```
predictions <- TransferData(anchorset = lotusjaponicus.anchors,
                            refdata = Idents(seurat.reference),
                            dims = 1:20)
```

```
Finding integration vectors

Finding integration vector weights

Predicting cell labels
```

```
seurat.clustered <- AddMetaData(seurat.clustered, metadata = predictions['predicted.id'])
```

Just as a reminder of what is in the metadata, we can quickly look at the column names. Those are ordered by when we added things along the analysis. If you read the names, you can recognize part of the analysis steps until now.

```
names( seurat.clustered@meta.data )
```

1. 'nCount_RNA'
2. 'nFeature_RNA'
3. 'nCount_SCT'
4. 'nFeature_SCT'
5. 'orig.ident'
6. 'Condition'
7. 'percent.mt'
8. 'percent.chloroplast'
9. 'pANN_0.25_0.09_609'
10. 'DF.classifications_0.25_0.09_609'
11. 'pANN_0.25_0.09_329'
12. 'DF.classifications_0.25_0.09_329'
13. 'pANN_0.25_0.09_309'
14. 'DF.classifications_0.25_0.09_309'
15. 'pANN_0.25_0.09_110'
16. 'DF.classifications_0.25_0.09_110'
17. 'integrated_snn_res.0.25'
18. 'seurat_clusters'

19. 'Cortex_scoring'
20. 'Epidermis_scoring'
21. 'Endodermis_scoring'
22. 'RootCap_scoring'
23. 'Meristem_scoring'
24. 'Phloem_scoring'
25. 'QuiescentCenter_scoring'
26. 'RootHair_scoring'
27. 'Pericycle_scoring'
28. 'Stele_scoring'
29. 'Xylem_scoring'
30. 'Cell_types'
31. 'Cell_types_old'
32. 'predicted.id'

Here we define as clustering for the data and the plots, the one transfered just before. We then have a look at Figure 26 to observe that the labels look fine.

```
Idents(seurat.clustered) <- 'predicted.id'
```

```
options(repr.plot.width=10, repr.plot.height=10)
DimPlot(object = seurat.clustered,
        reduction = "umap",
        repel = TRUE, label=T,
        pt.size = 0.5, label.size = 10, )
```

```
ERROR: Error in DimPlot(object = seurat.clustered, reduction = "umap", repel = TRUE, : could
```

Figure 26: **?(caption)**

We save the data

```
SaveH5Seurat(object = seurat.clustered,
             filename = "seurat.clustered.h5Seurat",
             overwrite = TRUE,
             verbose=FALSE)
```

```
Warning message:
"Overwriting previous file seurat.clustered.h5Seurat"
Creating h5Seurat file for version 3.1.5.9900
```

4 Gene Expression Analysis

In this section we explore the gene expression through

- inferring **conserved genes** across all treatments in the integrated dataset. Conserved genes are the ones preserving their expression across the grouping of interest.
- determining **differentially expressed genes** for the infected condition against the control. Differentially expressed genes are significantly more epressed in one of the two groups used for the comparison. Usually the wild type is used as query for the comparison, such that differentially expressed genes are referred to the perturbated condition (infection, knock-out, illness, ...)
- studying **coexpression modules** (a module is a group of gene similarly expressed across cells in the data) to find if
  - any of them contains the gene of interest,
  - they are significantly more expressed in specific cell groups (**Differential module expression**)
  - if there are specific known functions associated to some modules

We will also use gene ontology terms for understanding the function of groups of genes.

4.1 Conserved markers

We detect **conserved markers** across control and infected datasets. Below, we define a function that detect the conserved markers for each cell type, and calculates the average log-fold change of each conserved gene. The log-fold change is the magnitude of change of the gene expression in the cluster of interest against the rest of the data. This means that a marker with a high log-fold change and a significant p-value is also **differentially expressed in a specific cluster across the two conditions**.

```
seurat.clustered <- LoadH5Seurat("seurat.clustered.h5Seurat", verbose=FALSE)
```

```
Validating h5Seurat file

Warning message:
"Adding a command log without an assay associated with it"
```

```
get_conserved <- function(cluster){
  message(paste0("Conserved genes in ",cluster), appendLF=FALSE)
  FindConservedMarkers(seurat.clustered,
                       ident.1 = cluster,
                       grouping.var = "Condition",
```

```
                        only.pos = TRUE,
                        verbose=FALSE) %>%
    rownames_to_column(var = "gene") %>%
    cbind(cluster_id = cluster, .)
}

clusters <- levels(seurat.clustered@active.ident)
conserved_markers <- map_dfr(c(clusters), get_conserved)
conserved_markers$Average_log2FC <- (conserved_markers$Control_avg_log2FC + conserved_markers
conserved_markers$Max_p_val_adj <- apply(conserved_markers[, c('Control_p_val_adj', 'R7A_p_va
```

```
Conserved genes in Cortex
Conserved genes in Trichoblasts
Conserved genes in Root tip
Conserved genes in Meristem
Conserved genes in Phloem
Conserved genes in Pericycle
Conserved genes in Stele
Conserved genes in Atrichoblasts
Conserved genes in Xylem
Conserved genes in Endodermis
Conserved genes in Quiescent center
```

We can save conserved markers in a csv file. It can be downloaded and opened like a normal excel table to consult it, for example when writing an article or a report.

```
write.csv(conserved_markers, "conserved_markers.csv")
```

You can load the table in `R` with `read.csv`

```
conserved_markers <- read.csv("conserved_markers.csv")
```

The table contains, for each cluster, the genes that are conserved, the cluster they refer to, the mean log-fold change of the gene compared to the rest of the data, and the maximum p-value (adjusted). There are many other values, which are not of interest for interpreting results:

```
head(conserved_markers)
```

A data.frame: 6 × 17

	X	cluster	gene	Control_p_val	Control_avg_log2FC	Control_pct.1	Control_pct.2	Control_p_val_adj	STAT_p_val	STAT_avg_log2FC	STAT_pct.1	STAT_pct.2	STAT_p_val_adj	max_pval	minimump_adj	Average_log2FC	Max_p_val_adj
	<int>	<chr>	<chr>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>	<dbl>
1	1	Cortex	LotjaGi1g1v0022100	2.582286e-286	0.1860	0.000	1.085686e-282	0.0750	0.000000e+00	2.233984e+00	0.000	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	2.2339844	0.000000e+00
2	2	Cortex	LotjaGi1g1v0515200	7.531026e-286	0.2100	0.000	3.165031e-282	0.0921	1.525432e-281	1.521614e+00	0.324	6.411720e-277	6.411720e-277	1.525432e-281	1.525432e-281	1.5216140	1.525432e-281
3	3	Cortex	LotjaGi1g1v0588600	1.958688e-163	0.4520	0.000	8.230549e-160	0.2841	1.577754e-158	8.268144e-01	0.546	6.632464e-154	6.632464e-154	1.577754e-158	1.577754e-158	0.8268144	1.577754e-158
4	4	Cortex	LotjaGi3g1v0197600	8.912768e-121	0.5260	0.000	3.746136e-117	0.2678	8.310762e-117	1.196838e+00	0.362	6.979434e-113	6.979434e-113	8.310762e-117	8.310762e-117	0.1968384	8.310762e-117
5	5	Cortex	LotjaGi3g1v0443150	9.312598e-201	0.0820	0.021	3.913725e-197	0.0212	2.273017e-196	7.406281e-01	0.137	1.159590e-191	1.159590e-191	2.273017e-196	2.273017e-196	0.7406281	2.273017e-196
6	6	Cortex	LotjaGi3g1v0505900	2.074059e-01	0.1680	0.000	2.000000e+00	0.0780	0.000000e+00	2.532152e+00	0.000	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	2.5321521	0.000000e+00

We can easily look at the interesting columns:

```
head( conserved_markers[,c('cluster_id', 'gene', 'Average_log2FC', 'Max_p_val_adj')] )
```

A data.frame: 6 × 4

	cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>
1	Cortex	LotjaGi1g1v0022100	2.2339844	0.000000e+00
2	Cortex	LotjaGi1g1v0515200	1.5216140	1.525432e-281
3	Cortex	LotjaGi1g1v0588600	0.8268144	1.577754e-158
4	Cortex	LotjaGi3g1v0197600	0.1968384	8.310762e-117
5	Cortex	LotjaGi3g1v0443150	0.7406281	2.273017e-196
6	Cortex	LotjaGi3g1v0505900	2.5321521	0.000000e+00

So many of those columns are not significant in term of differential gene expression! This means that their expression is conserved across conditions, but they are **not specific to any cluster**. Rather, they are found with the same expression everywhere. It is definitely more interesting to look at the genes that are specific of some clusters. We define the table with only our columns of interest, and then we filter the non-significant genes. Also, we can filter by requiring that the log fold-change is above 1.5, so that we do not have too many genes to look at in each cluster. Remember we are using logarithms, so it means the expression is larger by a factor 2.83 (2 elevated at the power of 1.5).

```
conserved_filtered <- conserved_markers %>%
        select(cluster_id, gene, Average_log2FC, Max_p_val_adj) %>%
        filter(Max_p_val_adj < .001 & Average_log2FC>1.5 )
```

```
conserved_filtered
```

A data.frame: 643 × 4

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>
Cortex	LotjaGi1g1v0022100	2.233984	0.000000e+00
Cortex	LotjaGi1g1v0515200	1.521614	1.525432e-281
Cortex	LotjaGi3g1v0505900	2.532152	0.000000e+00
Cortex	LotjaGi4g1v0207600	1.838904	0.000000e+00
Cortex	LotjaGi4g1v0208100	1.776188	0.000000e+00
Cortex	LotjaGi4g1v0275500	1.839618	2.666024e-222
Cortex	LotjaGi5g1v0269800-LC	1.683960	2.442392e-295
Cortex	LotjaGi6g1v0028000-LC	1.655783	0.000000e+00
Cortex	LotjaGi6g1v0254700	2.159140	2.875239e-294
Cortex	LotjaGi2g1v0303000	1.988727	8.690812e-221
Cortex	LotjaGi6g1v0253800	2.043040	3.914669e-180
Cortex	LotjaGi3g1v0445300	1.869792	3.947225e-113
Cortex	LotjaGi2g1v0157900	1.589630	1.770177e-211
Cortex	LotjaGi1g1v0594900	2.092269	6.093304e-176
Trichoblasts	LotjaGi1g1v0074900	3.964291	0.000000e+00
Trichoblasts	LotjaGi1g1v0109000	3.138703	0.000000e+00
Trichoblasts	LotjaGi1g1v0171200-LC	1.837657	0.000000e+00
Trichoblasts	LotjaGi1g1v0268900	2.002680	0.000000e+00
Trichoblasts	LotjaGi1g1v0405300	3.843570	0.000000e+00
Trichoblasts	LotjaGi1g1v0413800	1.857181	0.000000e+00
Trichoblasts	LotjaGi1g1v0430800-LC	2.066899	0.000000e+00
Trichoblasts	LotjaGi1g1v0593900	1.844212	0.000000e+00
Trichoblasts	LotjaGi1g1v0636900	2.000571	0.000000e+00
Trichoblasts	LotjaGi1g1v0683300	3.703797	0.000000e+00
Trichoblasts	LotjaGi2g1v0160200	4.878893	0.000000e+00
Trichoblasts	LotjaGi2g1v0422000	2.102740	0.000000e+00
Trichoblasts	LotjaGi2g1v0426500	2.252654	0.000000e+00
Trichoblasts	LotjaGi3g1v0086100-LC	2.609181	0.000000e+00
Trichoblasts	LotjaGi3g1v0131300	2.006668	0.000000e+00
Trichoblasts	LotjaGi3g1v0204100	2.347129	0.000000e+00

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>
Quiescent center	LotjaGi3g1v0121000	2.947723	2.922592e-27
Quiescent center	LotjaGi1g1v0409900	3.073568	1.872145e-46
Quiescent center	LotjaGi5g1v0173900	1.651361	8.663520e-23
Quiescent center	LotjaGi1g1v0389100	1.656108	8.894005e-35
Quiescent center	LotjaGi5g1v0070400	1.779483	3.029826e-62
Quiescent center	LotjaGi5g1v0000600	2.562423	3.692719e-21
Quiescent center	LotjaGi5g1v0324200	1.837806	1.547016e-34
Quiescent center	LotjaGi1g1v0656500	2.788623	4.006635e-14
Quiescent center	LotjaGi6g1v0330700	3.329053	1.939902e-29
Quiescent center	LotjaGi3g1v0123200	2.347791	7.972779e-38
Quiescent center	LotjaGi3g1v0094400	3.077065	1.609653e-41
Quiescent center	LotjaGi3g1v0199700	1.784646	9.906537e-25
Quiescent center	LotjaGi4g1v0413300-LC	2.140779	7.802118e-36
Quiescent center	LotjaGi4g1v0070600	1.797423	2.355649e-41
Quiescent center	LotjaGi3g1v0255800	3.496842	2.199030e-12
Quiescent center	LotjaGi6g1v0213700	1.949240	3.477861e-33
Quiescent center	LotjaGi1g1v0512800	2.010809	2.086998e-21
Quiescent center	LotjaGi6g1v0266650	3.216128	3.940921e-16
Quiescent center	LotjaGi4g1v0336700	1.608371	5.043550e-17
Quiescent center	LotjaGi3g1v0153700-LC	2.145124	7.685686e-11
Quiescent center	LotjaGi6g1v0224300	1.650663	4.753793e-04
Quiescent center	LotjaGi2g1v0295300	1.650952	8.857559e-04
Quiescent center	LotjaGi1g1v0558200	2.721567	6.316628e-09
Quiescent center	LotjaGi1g1v0137100	1.726189	5.632273e-10
Quiescent center	LotjaGi3g1v0437000	2.766490	7.153966e-12
Quiescent center	LotjaGi2g1v0434500	2.513694	2.749486e-05
Quiescent center	LotjaGi4g1v0081300-LC	1.837138	7.581861e-04
Quiescent center	LotjaGi3g1v0217800	2.591970	9.094169e-04
Quiescent center	LotjaGi2g1v0371700	1.560628	7.675878e-13
Quiescent center	LotjaGi6g1v0040400	1.738781	1.123715e-04

We can use our list in combination witht he gene ontology table, so that we can find out the relevant go-terms for each cluster and the genes we have found.

We open the gene ontology table

```
go_table <- read.table("./LJ_GO_terms.gaf", skip=6, sep='\t', fill=TRUE, quote = "\"")
```

This contains genes and ontology descriptions that can be useful for identifying functions of conserved markers.

```
go_table$V2[1]
```

'protein kinase family protein; TAIR: AT5G19450.1 calcium-dependent protein kinase 19; Swiss-Prot: sp|Q84SL0|CDPKK_ORYSJ Calcium-dependent protein kinase 20; TrEMBL-Plants: tr|A0A151TU37|A0A151TU37_CAJCA Calcium-dependent protein kinase 32; Found in the gene: LotjaGi0g1v0000400'

```
head( go_table )
```

A data.frame: 6 × 2

	V1 <chr>	V2 <chr>
1	LotjaGi0g1v0000400.1	protein kinase family protein; TAIR: AT5G19450.1 calcium-dependent protein kinase 19; Swiss-Prot: sp
2	LotjaGi0g1v0000400.1	protein kinase family protein; TAIR: AT5G19450.1 calcium-dependent protein kinase 19; Swiss-Prot: sp
3	LotjaGi0g1v0000400.1	protein kinase family protein; TAIR: AT5G19450.1 calcium-dependent protein kinase 19; Swiss-Prot: sp
4	LotjaGi0g1v0000400.1	protein kinase family protein; TAIR: AT5G19450.1 calcium-dependent protein kinase 19; Swiss-Prot: sp
5	LotjaGi0g1v0000400.2	protein kinase family protein; TAIR: AT3G57530.1 calcium-dependent protein kinase 32; Swiss-Prot: sp

	V1 <chr>	V2 <chr>
6	LotjaGi0g1v0000400.2	protein kinase family protein; TAIR: AT3G57530.1 calcium-dependent protein kinase 32; Swiss-Prot: sp

The code below adds the GO terms to our filtered table of conserved markers. We parse from the go table the terms from the TAIR (arabidopsis protein archive) and the reviewed `swiss-uniprotKB` protein archive for plants for the arabidopsis thaliana. It can take some time to run, but is usually fast because we parallelized the function (here we assign GO terms to 16 genes at a time).

```
conserved_filtered <- addGOterms(input_table = conserved_filtered,
                                 go_table = go_table,
                                 n.cores = 16)
```

You can choose any cluster and see its GO terms. We do not look at undefined GO terms.

```
conserved_filtered %>% filter(cluster_id=='Phloem' & GO!='Undefined')
```

A data.frame: 44 × 5

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi1g1v0119302	2.272687	0.000000e+00	Jacalin lectin family protein; TAIR: AT1G19715.1 Mannose-binding lectin superfamily protein; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi1g1v0544203	3.228466	0.000000e+00	Heavy-metal-associated domain-containing family protein; TAIR: AT5G17450.1 Heavy metal transport/detoxification superfamily protein; Swiss-Prot: sp
Phloem	LotjaGi1g1v0696901	0.811034	0.000000e+00	Heavy metal transport/detoxification superfamily protein; TAIR: AT2G37390.2 Chloroplast-targeted copper chaperone protein; Swiss-Prot: sp
Phloem	LotjaGi1g1v0768602	2.683491	0.000000e+00	Glutaredoxin family protein; TAIR: AT2G47880.1 Glutaredoxin family protein; Swiss-Prot: sp
Phloem	LotjaGi2g1v0102601	0.716500	0.000000e+00	Tetraspanin family protein; TAIR: AT3G12090.1 tetraspanin6; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi2g1v0309900	0.350798	0.000000e+00	Thioredoxin; TAIR: AT5G39950.1 thioredoxin 2; Swiss-Prot: sp
Phloem	LotjaGi2g1v0310000	0.772175	0.000000e+00	Thioredoxin; TAIR: AT5G39950.1 thioredoxin 2; Swiss-Prot: sp
Phloem	LotjaGi2g1v0400500	2.178100	0.000000e+00	Calcium binding protein; TAIR: AT2G15680.1 Calcium-binding EF-hand family protein; Swiss-Prot: sp
Phloem	LotjaGi3g1v0089200	0.687731	0.000000e+00	MLP-like protein; TAIR: AT5G28010.1 Polyketide cyclase/dehydrase and lipid transport superfamily protein; Swiss-Prot: sp
Phloem	LotjaGi3g1v0176800	0.563508	0.000000e+00	Myb family transcription factor APL; TAIR: AT3G04030.3 Homeodomain-like superfamily protein; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi4g1v0012200	1.799539	0.000000e+00	Aspartic proteinase; TAIR: AT1G62290.1 Saposin-like aspartyl protease family protein; Swiss-Prot: sp
Phloem	LotjaGi4g1v0267002	2.120445	0.000000e+00	GTP-binding nuclear protein; TAIR: AT5G20010.1 RAS-related nuclear protein-1; Swiss-Prot: sp
Phloem	LotjaGi5g1v0003001	1.588913	0.000000e+00	Calcium-dependent kinase; TAIR: AT1G76040.2 calcium-dependent protein kinase 29; Swiss-Prot: sp
Phloem	LotjaGi5g1v0084802	2.180794	2.328492e-297	Ras family protein; TAIR: AT5G03530.1 RAB GTPase homolog C2A; Swiss-Prot: sp
Phloem	LotjaGi5g1v0095500	1.741651	1.355926e-296	Carbonic anhydrase family protein; TAIR: AT3G52720.1 alpha carbonic anhydrase 1; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi2g1v0210200	0.943762	5.224239e-267	Heavy metal-associated protein; TAIR: AT3G25855.1 Copper transport protein family; Swiss-Prot: sp
Phloem	LotjaGi2g1v0061300	0.624828	3.013300e-260	MLP; TAIR: AT1G70890.1 MLP-like protein 43; Swiss-Prot: sp
Phloem	LotjaGi3g1v0532700	2.410816	1.369188e-219	Aquaporin-like protein; TAIR: AT3G54820.1 plasma membrane intrinsic protein 2;5; Swiss-Prot: sp
Phloem	LotjaGi4g1v0164700	2.218920	4.924690e-205	Spermidine synthase; TAIR: AT1G23820.1 spermidine synthase; Swiss-Prot: sp
Phloem	LotjaGi1g1v0028800	2.680094	1.462391e-188	Translation factor SUI1; TAIR: AT5G54940.1 Translation initiation factor SUI1 family protein; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi1g1v0560400	1.724744	1.920599e-182	Peptidyl-prolyl cis-trans isomerase; TAIR: AT2G16600.1 rotamase CYP 3; Swiss-Prot: sp
Phloem	LotjaGi5g1v0073002	2.147869	2.585078e-180	Copper transport protein family; TAIR: AT1G66240.1 homolog of anti-oxidant 1; Swiss-Prot: sp
Phloem	LotjaGi6g1v0289005	5.012208	2.064488e-166	1-aminocyclopropane-1-carboxylate oxidase; TAIR: AT1G05010.1 ethylene-forming enzyme; Swiss-Prot: sp
Phloem	LotjaGi3g1v0039400	1.740635	6.553991e-159	Trehalose-6-phosphate synthase; TAIR: AT1G78580.1 trehalose-6-phosphate synthase; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi1g1v0555003	3.082789	8.949235e-149	Bidirectional sugar transporter SWEET; TAIR: AT5G13170.1 senescence-associated gene 29; Swiss-Prot: sp
Phloem	LotjaGi2g1v0386602	2.779973	1.485060e-293	Peroxidase; TAIR: AT5G66390.1 Peroxidase superfamily protein; Swiss-Prot: sp
Phloem	LotjaGi1g1v0594302	2.821061	3.326878e-165	dCTP pyrophos-phatase 1; TAIR: AT3G25400.1 dCTP pyrophosphatase-like protein; Swiss-Prot: sp
Phloem	LotjaGi5g1v0184301	1.707773	1.469535e-76	Ethylene-responsive transcription factor; TAIR: AT3G23230.1 Integrase-type DNA-binding superfamily protein; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi3g1v0046502	0.641435	1.136575e-150	Fatty acid oxidation complex subunit alpha; TAIR: AT3G15290.1 3-hydroxyacyl-CoA dehydrogenase family protein; Swiss-Prot: sp
Phloem	LotjaGi3g1v0237700	0.647226	1.425559e-108	Early nodulin-like protein; TAIR: AT3G20570.1 early nodulin-like protein 9; Swiss-Prot: sp
Phloem	LotjaGi1g1v0393600	0.535070	8.655222e-170	Plasma membrane ATPase; TAIR: AT2G24520.2 plasma membrane H+-ATPase; Swiss-Prot: sp Plasma membrane ATPase; TAIR: AT2G24520.1 plasma membrane H+-ATPase; Swiss-Prot: sp Plasma membrane ATPase; TAIR: AT2G18960.1 H -ATPase 1; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi3g1v0149302	2.031048	2.521757e-55	Early nodulin-like protein; TAIR: AT3G20570.1 early nodulin-like protein 9; Swiss-Prot: sp
Phloem	LotjaGi1g1v0172502	2.372215	1.119822e-43	Glucosamine-6-phosphate deaminase; TAIR: AT5G24400.1 NagB/RpiA/CoA transferase-like superfamily protein; Swiss-Prot: sp
Phloem	LotjaGi3g1v0136902	1.535443	1.376879e-39	Ubiquitin, putative; TAIR: AT1G31340.1 related to ubiquitin 1; Swiss-Prot: sp
Phloem	LotjaGi4g1v0369602	1.832673	4.931855e-74	Xyloglucan endotransglucosylase/hydrolase; TAIR: AT3G23730.1 xyloglucan endotransglucosylase/hydrolase 16; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi2g1v0082500	0.661449	8.995617e-48	MYB-related transcription factor; TAIR: AT5G14340.1 myb domain protein 40; Swiss-Prot: sp Myb factor; TAIR: AT5G14340.1 myb domain protein 40; Swiss-Prot: sp
Phloem	LotjaGi1g1v0662000	0.515167	4.491462e-35	protein kinase family protein; TAIR: AT3G56760.1 Protein kinase superfamily protein; Swiss-Prot: sp protein kinase family protein; TAIR: AT2G41140.1 CDPK-related kinase 1; Swiss-Prot: sp
Phloem	LotjaGi3g1v0522700	0.611578	6.711625e-26	Cold shock protein; TAIR: AT2G21060.1 glycine-rich protein 2B; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi2g1v0286302	2.146353	6.768979e-53	S-adenosylmethionine decarboxylase proenzyme; TAIR: AT3G25570.1 Adenosylmethio-nine decarboxylase family protein; Swiss-Prot: sp
Phloem	LotjaGi3g1v0224503	3.186377	5.120955e-21	Metallothionein-like protein; TAIR: AT3G09390.1 metallothionein 2A; Swiss-Prot: sp
Phloem	LotjaGi1g1v0386601	0.785015	5.317097e-34	GTP-binding nuclear protein; TAIR: AT5G55190.1 RAN GTPase 3; Swiss-Prot: sp
Phloem	LotjaGi1g1v0386301	0.560275	2.666945e-24	GTP-binding nuclear protein; TAIR: AT5G55190.1 RAN GTPase 3; Swiss-Prot: sp
Phloem	LotjaGi6g1v0220901	0.759788	4.299561e-22	Sucrose synthase; TAIR: AT3G43190.1 sucrose synthase 4; Swiss-Prot: sp

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>
Phloem	LotjaGi6g1v0234000	0.621734	4.539221e-13	Calcium-transporting ATPase; TAIR: AT5G57110.1 autoinhibited Ca2+ -ATPase, isoform 8; Swiss-Prot: sp

As a last check, we can see if the gene RINRK1 is in the table. The gene ID we need for RINCR1 is `LotjaGi1g1v0732500`. Since this gene should be expressed only within the infected samples, we expect not to find it in the table of conserved genes:

```
RINRK1.id <- 'LotjaGi1g1v0182900'
```

```
conserved_filtered %>% filter(gene==RINRK1.id)
```

A data.frame: 0 × 5

cluster_id <chr>	gene <chr>	Average_log2FC <dbl>	Max_p_val_adj <dbl>	GO <chr>

Again, we saved the filtered table of conserved genes with the GO terms, so it can be downloaded and used outside the programming environment.

```
write.csv(conserved_filtered, "conserved_GO.csv")
```

4.2 Differential Gene Expression (DGE)

Here we test each cluster to see which are significantly more expressed genes in the infected samples compared to the wild-type samples. We also see if we find the gene RINRK1 as being significant. Again, the resulting genes can be useful to be integrated with the GO terms as we did before.

We first have a quick look to see how much the RINRK1 gene is expressed in the data. We use the `RNA` assay to plot the true expression values. The UMAP plot shows few cells expressing the genes, meaning its average expression is going to be very low, so it is likely we will not find the gene to be differentially expressed anywhere.

```r
RINRK1.id <- 'LotjaGi1g1v0182900'

DefaultAssay(seurat.clustered) <- "RNA"

FeaturePlot(seurat.clustered,
            reduction = "umap",
            features = c(RINRK1.id),
            order = TRUE,
            min.cutoff = 0,
            pt.size = 1,
            label = TRUE,
            label.size = 7) + theme(legend.position = "right")
```

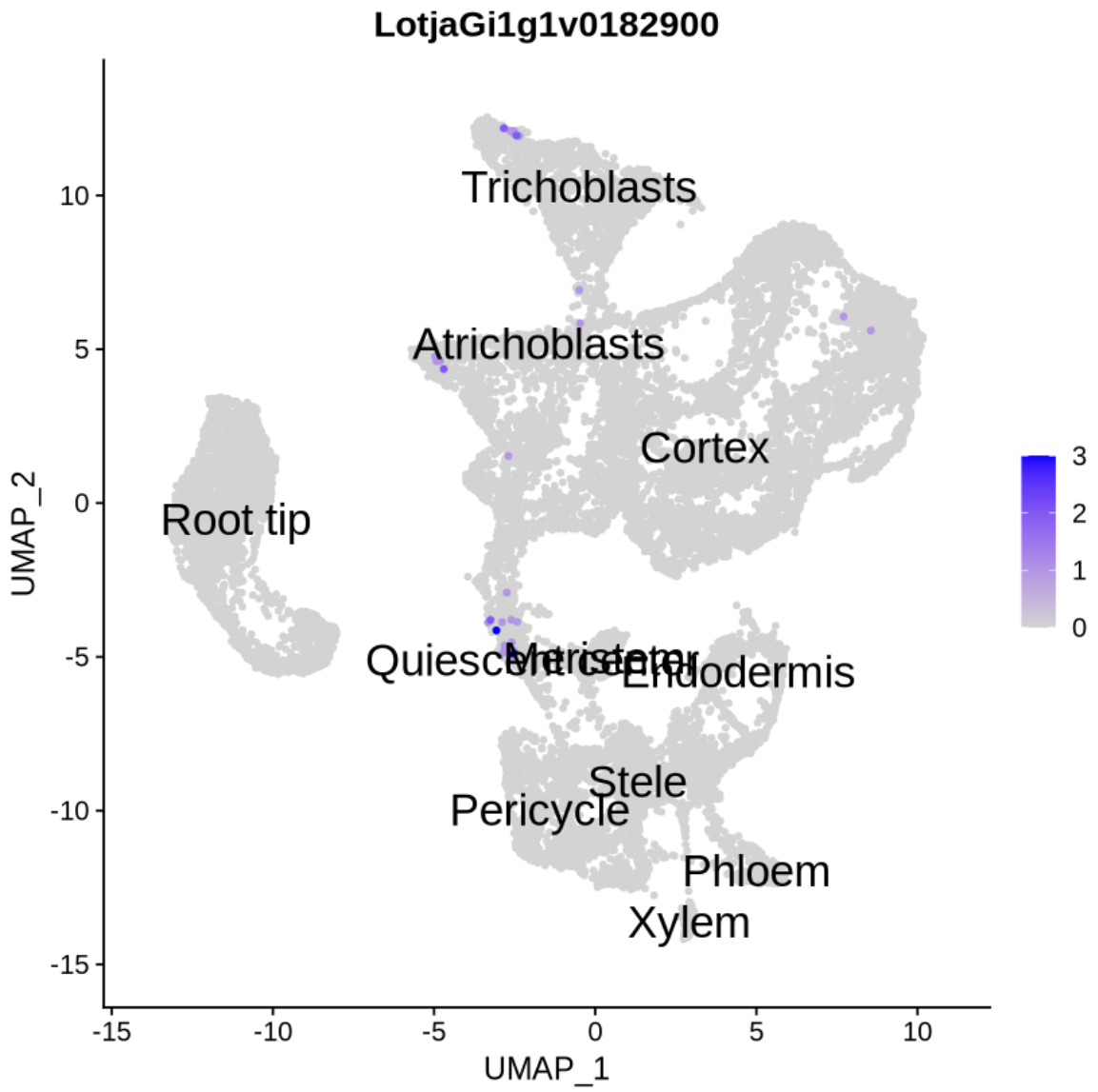


Figure 27: UMAP plot for the expression of gene RINRK1 in the data

From biological knowledge, we expect the gene mostly expressed in the cortex and trichoblasts upon inoculation with rhizobia, and that is what happens in our data as well. We can see it in the code and violin plot of Figure 28

```
cat("Cells in inoculated L.J. expressing", RINRK1.id, "\n")
```

```
cat( sum( as.numeric(GetAssayData(seurat.clustered[RINRK1.id,]))>0 &
     seurat.clustered@meta.data$Condition=="R7A" ) )

cat("\nCells in control L.J. expressing", RINRK1.id, "\n")

cat( sum( as.numeric(GetAssayData(seurat.clustered[RINRK1.id,]))>0 &
    seurat.clustered@meta.data$Condition=="Control" ) )
```

```
Cells in inoculated L.J. expressing LotjaGi1g1v0182900
32
Cells in control L.J. expressing LotjaGi1g1v0182900
0
```

```
VlnPlot(seurat.clustered,
        features = RINRK1.id)
```

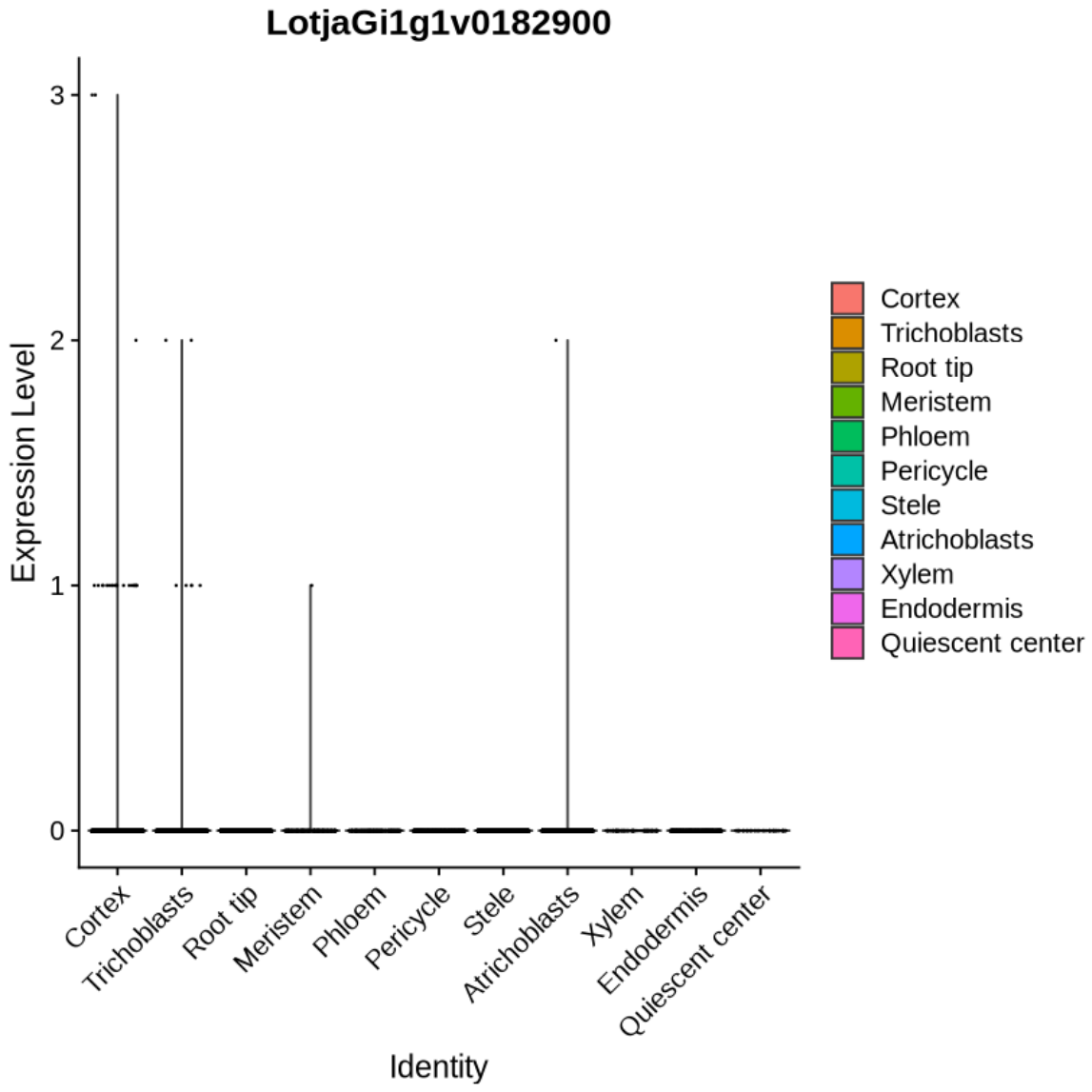


Figure 28: Violin plot for the expression of gene RINRK1 in various clusters

The code below uses `FindMarkers` to compare `R7A` condition against `Control` for each cluster, with a filter to remove non-singnificant genes (keeping p-value below 0.001 and log fold change > 1 and < -1). We keep also genes expressed 30% more than in the condition where they are underexpressed. We use only the 2000 most variable genes, since we are interested in very variable gene expressions across data.

```
seurat.clustered <- FindVariableFeatures(seurat.clustered, nfeatures = 2000, assay = "integra
```

```
Warning message:
"Not all features provided are in this Assay object, removing the following feature(s): Lotja
```

```
DEG_table <- FindMarkers(seurat.clustered,
                            assay='integrated',
                            ident.1 = "R7A",
                            ident.2 = "Control",
                            group.by = "Condition",
                            subset.ident="Cortex",
                            min.diff.pct = 0.3,
                            verbose = TRUE,
                            features = seurat.clustered@assays$integrated@var.features,
                            test.use = "wilcox") %>%
                            filter(p_val_adj <= 0.001 & abs(avg_log2FC)>1) %>%
                            select(-p_val)
```

```
DefaultAssay(seurat.clustered) <- "integrated" #return to the integrated data
DEG <- data.frame()
cluster.names <- unique(Idents(seurat.clustered))

for(CLUSTER in cluster.names){
    DEG_table <- FindMarkers(seurat.clustered,
                            assay='integrated',
                            ident.1 = "R7A",
                            ident.2 = "Control",
                            group.by = "Condition",
                            subset.ident=CLUSTER,
                            verbose = TRUE,
                            min.diff.pct = 0.3,
                            features = seurat.clustered@assays$integrated@var.features,
                            test.use = "wilcox") %>%
                            filter(p_val_adj <= 0.001 & abs(avg_log2FC)>1) %>%
                            select(-p_val)
    if(dim(DEG_table)[1]>0){
        DEG_table$'cluster' <- CLUSTER
        DEG <- rbind(DEG, DEG_table)}
    else{
        message(paste0("---> Warning: No DE genes in cluster ", CLUSTER), appendLF=FALSE)}
    message(paste0("Done with cluster ",CLUSTER), appendLF=FALSE)
```

```
    }

DEG <- as.data.frame(DEG)
DEG$gene <- rownames(DEG)
```

```
Done with cluster Cortex
Done with cluster Trichoblasts
Done with cluster Root tip
Done with cluster Meristem
Done with cluster Phloem
Done with cluster Pericycle
Done with cluster Stele
Done with cluster Atrichoblasts
Done with cluster Xylem
Done with cluster Endodermis
---> Warning: No DE genes in cluster Quiescent center
Done with cluster Quiescent center
```

The table looks like this. Columns represent:

- average logfoldchange between R7A and Control
- percentage of cells in R7A expressing the gene
- percentage of cells in Control expressing the gene
- adjusted p-value
- cluster
- gene

There are some genes in the table

```
dim(DEG)[1]
```

718

We now integrate GO terms

```
go_table <- read.table("./LJ_GO_terms.gaf", skip=6, sep='\t', fill=TRUE, quote = "\"")
```

```
DEG <- addGOterms(DEG, go_table, n.cores = 16)
```

So we can see which GO terms are relevant in each cluster for the infected samples against the control:

```
DEG %>% filter(cluster=="Cortex" & GO!="Undefined")
```

A data.frame: 2 × 7

	avg_log2FC <dbl>	pct.1 <dbl>	pct.2 <dbl>	p_val_adj <dbl>	cluster <chr>	gene <chr>	GO <chr>
LotjaGi1g1v0147200	0.747276	0.411	0.10	2.556740e-215	Cortex	LotjaGi1g1v0147200	0.0e-172 6-phosphate phosphatase; TAIR: AT5G10100.1 Haloacid dehalogenase-like hydrolase (HAD) superfamily protein; Swiss-Prot: sp
LotjaGi1g1v0154100	2.359368	0.422	0.12	2.637166e-97	Cortex	LotjaGi1g1v0154100	0.0e+00 flavonol synthase; TAIR: AT5G08640.1 flavonol synthase 1; Swiss-Prot: sp

Finally, we do not expect to find the RINRK1 gene as differentially expressed, because its expression is on average too low.

```
DEG %>% filter(gene==RINRK1.id)
```

A data.frame: 0 × 7

avg_log2FC <dbl>	pct.1 <dbl>	pct.2 <dbl>	p_val_adj <dbl>	cluster <chr>	gene <chr>	GO <chr>

We save the table:

```
write.csv(DEG_table, "DEG_table.csv")
```

5 Coexpression analysis

We use the package hdWGCNA to detect groups of cells expressed simultaneously, and we find which modules are differentially expressed in specific clusters. We look at the GO terms to gain biological insight in the data.

> **Note**
>
> Before running hdWGCNA, we first have to set up the Seurat object. Most of the information computed by hdWGCNA will be stored in the Seurat object's `@misc` slot.

Here we will set up the Seurat object using the `SetupForWGCNA` function, specifying the name of the `hdWGNCA` experiment. This function also selects the genes that will be used for WGCNA. The user can select genes using three different approaches using the posse gene_select parameter:

- variable: use the genes stored in the Seurat object's VariableFeatures.
- fraction: use genes that are expressed in a certain fraction of cells for in the whole dataset or in each group of cells, specified by `group.by`.
- custom: use genes that are specified in a custom list.

In this example, we will select genes that are expressed in at least 5% of cells in this dataset, and we will name our `hdWGCNA` experiment "tutorial".

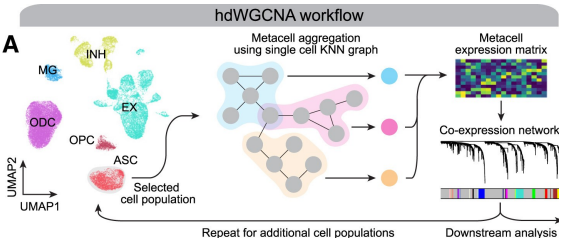
```
seurat.clustered <- LoadH5Seurat("seurat.clustered.h5Seurat", verbose=FALSE)
```

```
Validating h5Seurat file

Warning message:
"Adding a command log without an assay associated with it"
```

```r
seurat.clustered <- SetupForWGCNA(
  seurat.clustered,
  gene_select = "variable",
  #gene_select = "fraction", # the gene selection approach
  fraction = 0.05, # fraction of cells that a gene needs to be expressed in order to be incl
  wgcna_name = "tutorial" # the name of the hdWGCNA experiment
)
```

5.1 Construct metacells

After we have set up our Seurat object, the first step in running the hdWGCNA pipeline is to construct metacells from the single-cell dataset. Briefly, metacells are **aggregates of small groups of similar cells originating from the same biological sample of origin**.



{#fig-hdwgcna, width=800}

Something more about hdWGCNA

The k-Nearest Neighbors (KNN) algorithm is used to identify groups of similar cells to aggregate, and then the average or summed expression of these cells is computed, thus yielding a metacell gene expression matrix (**as if you had many bulk samples**). The **sparsity of the metacell expression matrix is considerably reduced** when compared to the original expression matrix, and therefore it is preferable to use. We were originally motivated to use metacells in place of the original single cells because correlation **network approaches such as WGCNA are sensitive to data sparsity**.
`hdWGCNA` includes a function `MetacellsByGroups` to construct metacell expression matrices given a single-cell dataset. This function constructs a new Seurat object for the metacell dataset which is stored internally in the `hdWGCNA` experiment. The `group.by` parameter determines which groups metacells will be constructed in. We only want to construct metacells from cells that came from the same biological sample of origin, so it is critical to pass that information to `hdWGCNA` via the `group.by` parameter. Additionally, we usually construct metacells for each cell type separately. Thus, in this example, we are **grouping by Sample and cell type** to achieve the desired result.
The number of cells to be aggregated `k` should be tuned based on the size of the input dataset, in general a lower number for `k` can be used for small datasets.

We generally use `k` values between 20 and 75. The dataset used for this tutorial has 21,369 cells, and here we use `k=20`. The amount of allowable overlap between metacells can be tuned using the `max_shared` argument. There should be a range of `k` values that are suitable for reducing the sparsity while retaining cellular heterogeneity for a given dataset, rather than a single optimal value.
Note: the authors of `hdWGCNA` have found that the metacell aggregation approach does not yield good results for extremely underrepresented cell types. For example, in this dataset, the `Meristem` and `Xylem` are the least represented, and will be excluded them from this analysis. `MetacellsByGroups` has a parameter `min_cells` to exclude groups that are smaller than a specified number of cells. **Errors are likely to arise if the selected value for `min_cells` is too low**.

Here **we construct metacells and normalize the resulting expression matrix** using the following code (read the additional text in the box above to understand the parameters). Note how the clusters Xylem and Quiescent center are removed, simply because they are too small and cannot be used. So from now on we do not consider them for the coexpression analysis.

```
# construct metacells  in each group
seurat.clustered <- MetacellsByGroups(
  seurat_obj = seurat.clustered,
  group.by = c("predicted.id", "Condition"), # specify metadata to split by cluster and cond
  reduction = 'pca', # select the dimensionality reduction to perform KNN on
  k = 30, # nearest-neighbors parameter
  max_shared = 10, # maximum number of shared cells between two metacells
  ident.group = 'predicted.id', # set the Idents of the metacell seurat object
  assay = "integrated",
  slot = "scale.data",
  min_cells = 100,
  wgcna_name = "tutorial",
  verbose=TRUE
)
```

```
Warning message in MetacellsByGroups(seurat_obj = seurat.clustered, group.by = c("predicted.:
"Removing the following groups that did not meet min_cells: Quiescent center#Control, Quiesc
Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 1.51461988304094
Median shared cells bin-bin: 0

Overlap QC metrics:
```

```
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 0.635690485005553
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 0.335219114968392
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 0.19742043684862
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 1.36752136752137
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 1.59090909090909
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 3.57575757575758
Median shared cells bin-bin: 2

Warning message in (function (seurat_obj, name = "agg", ident.group = "seurat_clusters", :
"On average, more than 10% of cells are shared between paired bins."
Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 9
Mean shared cells bin-bin: 4.66666666666667
Median shared cells bin-bin: 5
```

```
Warning message in (function (seurat_obj, name = "agg", ident.group = "seurat_clusters", :
"On average, more than 10% of cells are shared between paired bins."
Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 2.41578947368421
Median shared cells bin-bin: 0.5

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 1.13725490196078
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 4.05555555555556
Median shared cells bin-bin: 3

Warning message in (function (seurat_obj, name = "agg", ident.group = "seurat_clusters", :
"On average, more than 10% of cells are shared between paired bins."
Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 9
Mean shared cells bin-bin: 3.9
Median shared cells bin-bin: 3

Warning message in (function (seurat_obj, name = "agg", ident.group = "seurat_clusters", :
"On average, more than 10% of cells are shared between paired bins."
Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 0.690802348336595
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 0.659649122807018
Median shared cells bin-bin: 0

Overlap QC metrics:
```

```
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 1.33547632963179
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 0.9340592861464
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 1.6312292358804
Median shared cells bin-bin: 0

Overlap QC metrics:
Cells per bin: 30
Maximum shared cells bin-bin: 10
Mean shared cells bin-bin: 0.77909604519774
Median shared cells bin-bin: 0
```

```
#normalize metacell expression matrix:
seurat.clustered <- NormalizeMetacells(seurat.clustered)
```

```
Warning message:
"Cannot find a parent environment called Seurat"
```

Here we specify the expression matrix that we will use for network analysis. For example, we can choose the Cortex, which is important also because it expresses RINRK1 in infected samples.

```
seurat.clustered <- SetDatExpr(
  seurat.clustered,
  group_name = c('Pericycle','Cortex','Trichoblasts','Root tip',
                 'Phloem','Stele','Endodermis','Atrichoblasts',
                 'Meristem'), #all groups of interest in the data (we use all clusters)
  group.by='predicted.id', # the metadata column containing the cell type info. This same co
  assay = 'integrated', # using integrated assay
  slot = 'counts' # using count data
)
```

5.2 Select soft-power threshold

Next we will select the soft power threshold. This is an extremely important step in the pipeline. `hdWGCNA` constructs a gene-gene correlation adjacency matrix to infer co-expression relationships between genes. The correlations are **raised to a power to reduce the amount of noise present in the correlation matrix**, thereby **retaining the strong connections and removing the weak connections**. Therefore, it is critical to determine a proper value for the soft power threshold.

We use the function `TestSoftPowers` to perform a parameter sweep for different soft power thresholds. This function helps us to guide our choice in a soft power threshold for constructing the co-expression network by inspecting the resulting network topology for different power values. The following code performs the parameter sweep and outputs a summary figure.

```
# Test different soft powers:
seurat.clustered <- TestSoftPowers(powers = 1:100,
  seurat.clustered,
  networkType = 'signed'
)

# plot the results:
plot_list <- PlotSoftPowers(seurat.clustered)

# assemble with patchwork
wrap_plots(plot_list, ncol=2)
```

```
pickSoftThreshold: will use block size 1902.
 pickSoftThreshold: calculating connectivity for given powers...
   ..working on genes 1 through 1902 of 1902
    Power SFT.R.sq  slope truncated.R.sq  mean.k. median.k.   max.k.
1      1   0.7870  6.540         0.8230 1.14e+03  1.16e+03 1330.000
2      2   0.5050  2.320         0.7630 7.07e+02  7.28e+02  964.000
3      3   0.0603  0.528         0.6600 4.49e+02  4.62e+02  718.000
4      4   0.0241 -0.281         0.7740 2.91e+02  2.97e+02  548.000
5      5   0.2060 -0.780         0.9000 1.93e+02  1.93e+02  426.000
6      6   0.4090 -1.180         0.9510 1.30e+02  1.27e+02  338.000
7      7   0.5580 -1.460         0.9780 8.97e+01  8.48e+01  272.000
8      8   0.6540 -1.620         0.9910 6.27e+01  5.72e+01  222.000
9      9   0.7350 -1.740         0.9970 4.46e+01  3.87e+01  184.000
10    10   0.7830 -1.830         0.9810 3.22e+01  2.65e+01  153.000
11    11   0.8250 -1.890         0.9740 2.35e+01  1.81e+01  130.000
12    12   0.8580 -1.960         0.9810 1.74e+01  1.25e+01  110.000
13    13   0.8600 -2.020         0.9540 1.31e+01  8.80e+00   94.700
```

14	14	0.8390	-2.080	0.9060	9.96e+00	6.18e+00	81.800
15	15	0.8500	-2.070	0.9010	7.65e+00	4.37e+00	71.200
16	16	0.9030	-2.020	0.9560	5.94e+00	3.11e+00	62.400
17	17	0.8950	-2.030	0.9480	4.66e+00	2.24e+00	54.900
18	18	0.9090	-1.990	0.9560	3.69e+00	1.61e+00	48.600
19	19	0.9080	-1.970	0.9440	2.95e+00	1.16e+00	43.200
20	20	0.8770	-1.990	0.8950	2.37e+00	8.56e-01	38.500
21	21	0.8810	-1.960	0.8940	1.93e+00	6.31e-01	34.500
22	22	0.8930	-1.930	0.9030	1.57e+00	4.67e-01	31.100
23	23	0.9020	-1.900	0.9100	1.30e+00	3.41e-01	28.000
24	24	0.3400	-2.670	0.1990	1.07e+00	2.55e-01	25.400
25	25	0.3390	-2.620	0.1970	8.94e-01	1.89e-01	23.100
26	26	0.3420	-2.580	0.2000	7.50e-01	1.41e-01	21.000
27	27	0.3460	-2.540	0.2050	6.32e-01	1.05e-01	19.200
28	28	0.3480	-2.510	0.2080	5.35e-01	8.04e-02	17.600
29	29	0.3540	-2.490	0.2170	4.55e-01	6.06e-02	16.100
30	30	0.3530	-2.450	0.2170	3.89e-01	4.56e-02	14.800
31	31	0.3820	-2.500	0.3120	3.35e-01	3.53e-02	13.700
32	32	0.3200	-2.220	0.1280	2.89e-01	2.73e-02	12.600
33	33	0.3210	-2.190	0.1290	2.50e-01	2.08e-02	11.700
34	34	0.3220	-2.160	0.1310	2.17e-01	1.61e-02	10.900
35	35	0.3240	-2.140	0.1330	1.90e-01	1.25e-02	10.100
36	36	0.3260	-2.120	0.1350	1.66e-01	9.64e-03	9.400
37	37	0.3280	-2.090	0.1380	1.46e-01	7.42e-03	8.770
38	38	0.3280	-2.070	0.1390	1.29e-01	5.78e-03	8.200
39	39	0.3290	-2.040	0.1400	1.14e-01	4.42e-03	7.670
40	40	0.2780	-2.410	0.0721	1.01e-01	3.42e-03	7.190
41	41	0.2790	-2.380	0.0744	9.01e-02	2.64e-03	6.740
42	42	0.2790	-2.370	0.0737	8.05e-02	2.07e-03	6.340
43	43	0.2800	-2.340	0.0745	7.21e-02	1.62e-03	5.960
44	44	0.2810	-2.320	0.0757	6.47e-02	1.26e-03	5.620
45	45	0.2810	-2.310	0.0765	5.83e-02	1.00e-03	5.300
46	46	0.2830	-2.290	0.0784	5.26e-02	7.83e-04	5.010
47	47	0.3010	-2.320	0.1050	4.76e-02	6.10e-04	4.740
48	48	0.3000	-2.290	0.1040	4.32e-02	4.74e-04	4.480
49	49	0.2990	-2.270	0.1040	3.93e-02	3.75e-04	4.250
50	50	0.3030	-2.250	0.1090	3.58e-02	2.99e-04	4.030
51	51	0.3040	-2.230	0.1110	3.27e-02	2.39e-04	3.830
52	52	0.3050	-2.210	0.1120	2.99e-02	1.91e-04	3.640
53	53	0.3060	-2.200	0.1140	2.74e-02	1.51e-04	3.460
54	54	0.2920	-2.420	0.0894	2.52e-02	1.19e-04	3.300
55	55	0.2920	-2.400	0.0896	2.32e-02	9.27e-05	3.140
56	56	0.2930	-2.390	0.0906	2.14e-02	7.35e-05	3.000

57	57	0.2950	-2.370	0.0933	1.98e-02	5.97e-05	2.860
58	58	0.2960	-2.340	0.0947	1.83e-02	4.85e-05	2.730
59	59	0.2970	-2.320	0.0963	1.70e-02	3.86e-05	2.610
60	60	0.2980	-2.310	0.0981	1.58e-02	3.09e-05	2.500
61	61	0.2970	-2.290	0.0969	1.47e-02	2.46e-05	2.390
62	62	0.2990	-2.270	0.0994	1.37e-02	1.94e-05	2.290
63	63	0.2990	-2.250	0.0994	1.28e-02	1.54e-05	2.200
64	64	0.3010	-2.230	0.1020	1.19e-02	1.22e-05	2.110
65	65	0.3000	-2.220	0.1010	1.12e-02	9.72e-06	2.020
66	66	0.3010	-2.210	0.1020	1.05e-02	7.74e-06	1.940
67	67	0.3030	-2.200	0.1040	9.81e-03	6.17e-06	1.860
68	68	0.3040	-2.190	0.1060	9.22e-03	4.95e-06	1.790
69	69	0.3050	-2.180	0.1080	8.67e-03	3.99e-06	1.730
70	70	0.3190	-2.210	0.1310	8.16e-03	3.25e-06	1.660
71	71	0.3200	-2.200	0.1340	7.70e-03	2.58e-06	1.600
72	72	0.2990	-2.270	0.1080	7.26e-03	2.03e-06	1.550
73	73	0.2980	-2.260	0.1060	6.86e-03	1.62e-06	1.490
74	74	0.3000	-2.240	0.1080	6.49e-03	1.31e-06	1.440
75	75	0.3020	-2.230	0.1090	6.14e-03	1.08e-06	1.390
76	76	0.3040	-2.220	0.1110	5.82e-03	8.57e-07	1.350
77	77	0.3030	-2.210	0.1100	5.52e-03	6.87e-07	1.300
78	78	0.3050	-2.200	0.1110	5.24e-03	5.63e-07	1.260
79	79	0.3050	-2.180	0.1110	4.97e-03	4.58e-07	1.220
80	80	0.3040	-2.180	0.1100	4.73e-03	3.69e-07	1.180
81	81	0.3030	-2.190	0.1080	4.50e-03	2.97e-07	1.150
82	82	0.3030	-2.170	0.1080	4.28e-03	2.38e-07	1.110
83	83	0.3020	-2.150	0.1080	4.08e-03	1.90e-07	1.080
84	84	0.3040	-2.150	0.1090	3.89e-03	1.51e-07	1.040
85	85	0.3050	-2.140	0.1110	3.72e-03	1.20e-07	1.010
86	86	0.3070	-2.130	0.1120	3.55e-03	9.65e-08	0.982
87	87	0.3080	-2.120	0.1140	3.39e-03	7.71e-08	0.953
88	88	0.3090	-2.130	0.1150	3.24e-03	6.29e-08	0.926
89	89	0.3100	-2.140	0.1160	3.10e-03	5.03e-08	0.899
90	90	0.3110	-2.140	0.1170	2.97e-03	4.04e-08	0.874
91	91	0.3130	-2.130	0.1190	2.85e-03	3.23e-08	0.849
92	92	0.3140	-2.120	0.1200	2.73e-03	2.58e-08	0.826
93	93	0.3150	-2.130	0.1220	2.62e-03	2.08e-08	0.803
94	94	0.3270	-2.160	0.1350	2.51e-03	1.70e-08	0.781
95	95	0.3290	-2.150	0.1370	2.41e-03	1.40e-08	0.760
96	96	0.3300	-2.140	0.1390	2.32e-03	1.15e-08	0.740
97	97	0.3300	-2.130	0.1390	2.23e-03	9.37e-09	0.720
98	98	0.3320	-2.120	0.1410	2.14e-03	7.61e-09	0.701
99	99	0.3400	-2.130	0.1540	2.06e-03	6.14e-09	0.683

```
100   100   0.3410 -2.120         0.1550 1.98e-03  4.95e-09    0.666
  Power   SFT.R.sq      slope truncated.R.sq   mean.k. median.k.    max.k.
1     1 0.78715066  6.5419434      0.8226937 1142.8616 1164.8542 1330.4551
2     2 0.50547179  2.3222895      0.7627136  706.9821  727.8092  963.5889
3     3 0.06030832  0.5277599      0.6599972  448.5438  461.6084  717.8182
4     4 0.02406336 -0.2805631      0.7737510  291.1354  296.5913  547.5911
5     5 0.20640114 -0.7801742      0.9004039  192.9425  193.3074  426.3568
6     6 0.40937211 -1.1838404      0.9509733  130.3530  127.1297  337.9309

Warning message:
"executing %dopar% sequentially: no parallel backend registered"
```

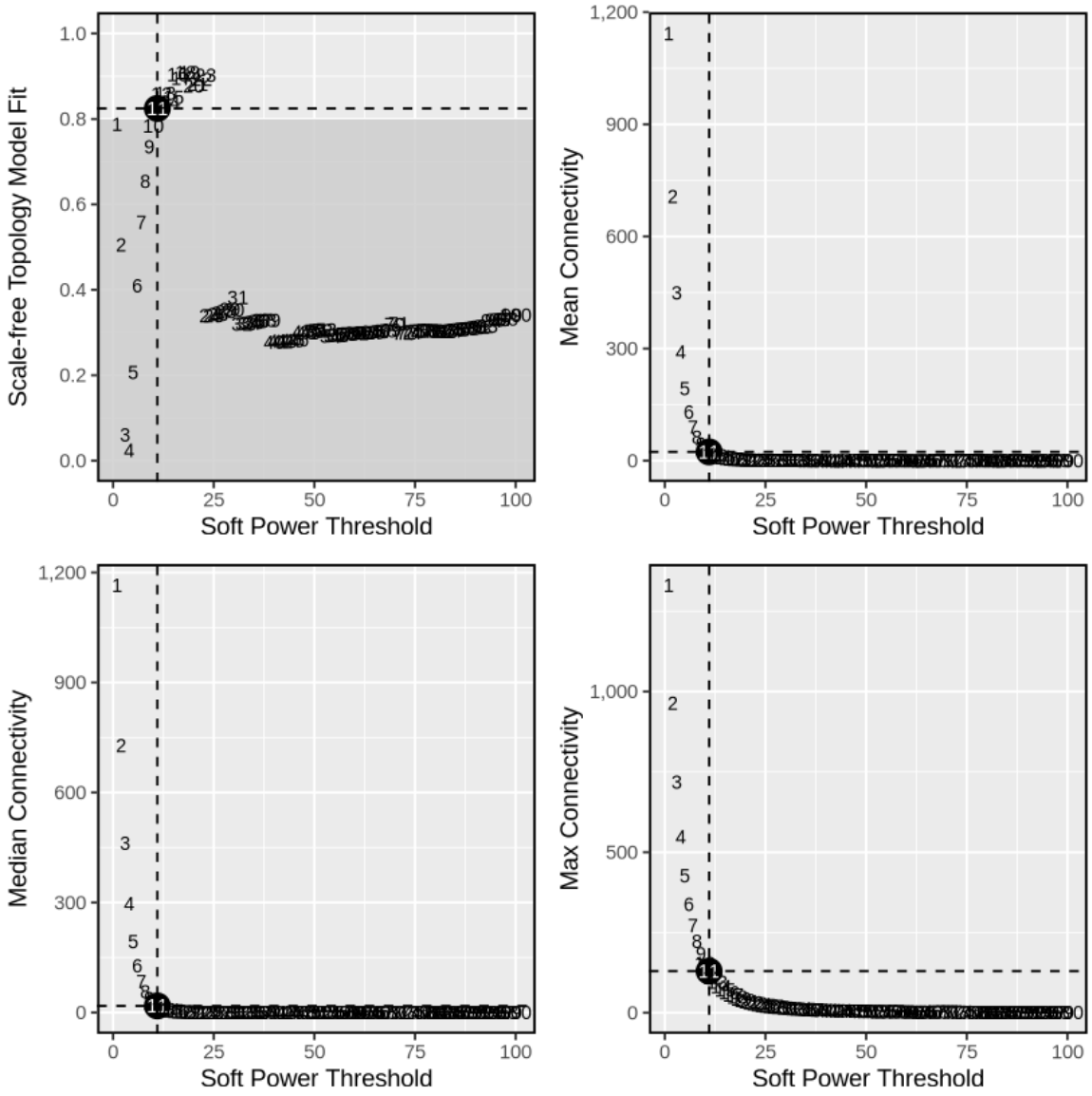


Figure 29: Parameter sweep to choose the soft power threshold

The general guidance for `hdWGCNA` is to pick the lowest soft power threshold that has Model Fit greater than or equal to 0.8, so in this case we would select our soft power threshold to be 11 following the illustration fo Figure 29. Later on, the `ConstructNetwork` command will automatically select the soft power threshold if the user does not provide one.

5.3 Construction of co-expression network

We now have everything that we need to construct our co-expression network. Here we use the `hdWGCNA` function `ConstructNetwork`. This function has quite a few parameters to play with if you are an advanced user (read this manual and the function `ConstructNetwork` is based on here), but we use default parameters that work well with many single-cell datasets.

The following code constructs the co-expression network using the soft power threshold selected before:

```
# construct co-expression network:
seurat.clustered <- ConstructNetwork(na.rm=TRUE,
  seurat.clustered,
  soft_power=11,
  setDatExpr=FALSE,
  tom_name = 'Cortex', # name of the topoligical overlap matrix written to disk
  overwrite_tom = TRUE
)
```

```
Warning message in ConstructNetwork(na.rm = TRUE, seurat.clustered, soft_power = 11, :
"Overwriting TOM TOM/Cortex_TOM.rda"
```

```
 Calculating consensus modules and module eigengenes block-wise from all genes
 Calculating topological overlaps block-wise from all genes
   Flagging genes and samples with too many missing values...
    ..step 1
    TOM calculation: adjacency..
    ..will not use multithreading.
     Fraction of slow calculations: 0.000000
    ..connectivity..
    ..matrix multiplication (system BLAS)..
    ..normalization..
    ..done.
 ..Working on block 1 .
 ..Working on block 1 .
 ..merging consensus modules that are too close..
```

We can plot the network dendrogram in Figure 30. Each leaf on the dendrogram represents a single gene, and the color at the bottom indicates the co-expression module assignment.

Note: the "gray" module consists of genes that were not grouped into any co-expression module. The gray module is to be ignored for all downstream analysis and interpretation.

```
PlotDendrogram(seurat.clustered, main='Phloem hdWGCNA Dendrogram')
```

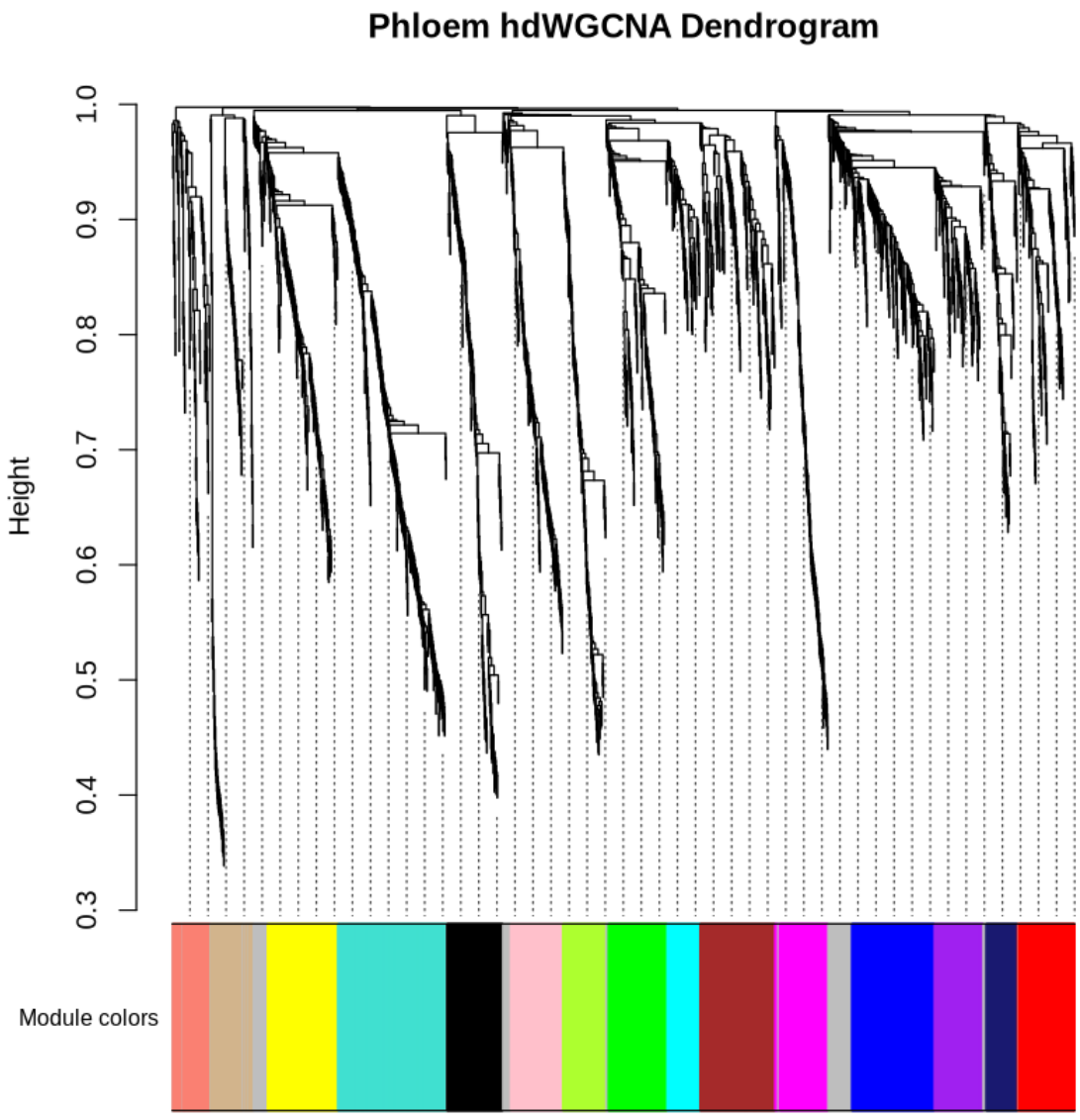


Figure 30: Dendrogram of the genes coexpression network. Leaves at the bottom are genes, and the colours represent a module of coexpression for the corresponding genes. Gray areas are for genes not included in any module.

5.3.1 Module Eigengenes (MEs)

We calculate harmonized module eigengenes. This is a way of doing PCA of the expression matrix of metacells including the genes of one coexpression module at a time. Thus we have a PCA of the data for each module. The first principal component of each PCA is called module eigengene: it is enough to distinguish one module from all the others and characterize the expression pattern of the module (Langfelder and Horvath (2007))!

Dimensionality reduction techniques are a very hot topic in single-cell genomics (Xiang et al. (2021)). It is well known that technical artifacts can muddy the analysis of single-cell datasets, and over the years there have been many methods that aim to reduce the effects of these artifacts. Therefore it stands to reason that MEs would be subject to these technical artifacts as well, and `hdWGCNA` seeks to alleviate these effects by using the integration software `Harmony` (Korsunsky et al. (2019)).

The following code performs the module eigengene computation harmonizing by the Sample of origin using the `group.by.vars` parameter.

```
# need to run ScaleData first or else harmony throws an error:
seurat.clustered <- ScaleData(seurat.clustered,
                              features=VariableFeatures(seurat.clustered),
                              verbose = FALSE)
```

```
# compute all MEs in the full single-cell dataset
seurat.clustered <- ModuleEigengenes(
 seurat.clustered,
 group.by.vars="orig.ident",
 verbose = FALSE
)
```

```
[1] "turquoise"
[1] "brown"
[1] "salmon"
[1] "black"
[1] "red"
[1] "midnightblue"
[1] "magenta"
[1] "yellow"
[1] "blue"
[1] "green"
[1] "cyan"
[1] "grey"
[1] "greenyellow"
```

```
[1] "tan"
[1] "pink"
[1] "purple"


Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Warning message:
"Key ‘harmony_’ taken, using ‘lsmpe_’ instead"
Centering and scaling data matrix

Warning message in irlba(A = t(x = object), nv = npcs, ...):
"You're computing too large a percentage of total singular values, use a standard svd instead
Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Warning message:
"Key ‘harmony_’ taken, using ‘nibyo_’ instead"
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Centering and scaling data matrix

Warning message in irlba(A = t(x = object), nv = npcs, ...):
"You're computing too large a percentage of total singular values, use a standard svd instead
Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Warning message:
"Key ‘harmony_’ taken, using ‘lkzye_’ instead"
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
```

```
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Warning message:
"Key ‘harmony_’ taken, using ‘ltnwf_’ instead"
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Warning message:
"Key ‘harmony_’ taken, using ‘xolrz_’ instead"
Centering and scaling data matrix

Warning message in irlba(A = t(x = object), nv = npcs, ...):
"You're computing too large a percentage of total singular values, use a standard svd instead
Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Warning message:
"Key ‘harmony_’ taken, using ‘ibzol_’ instead"
Centering and scaling data matrix

Warning message in irlba(A = t(x = object), nv = npcs, ...):
"You're computing too large a percentage of total singular values, use a standard svd instead
Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Centering and scaling data matrix

Warning message in irlba(A = t(x = object), nv = npcs, ...):
"You're computing too large a percentage of total singular values, use a standard svd instead
Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Warning message:
"Key ‘harmony_’ taken, using ‘pical_’ instead"
Centering and scaling data matrix
```

```
Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Centering and scaling data matrix

Warning message:
"Keys should be one or more alphanumeric characters followed by an underscore, setting key fi
Warning message:
"Key 'harmony_' taken, using 'vnltp_' instead"
```

5.3.2 Compute module connectivity

In co-expression network analysis, we often want to focus on the **hub genes, those which are highly connected within each module**. Therefore we wish to determine the eigengene-based connectivity, also known as kME, of each gene. The `ModuleConnectivity` function computes the kMEs in the single-cell dataset (rather than the metacell dataset). This function essentially computes pairwise correlations between genes and module eigengenes. kME can be computed for all cells in the dataset, but it is recommended computing kMEs in the cell type(s) or group(s) previously used to run `ConstructNetwork`.

```
# compute eigengene-based connectivity (kME):
seurat.clustered <- ModuleConnectivity(
  seurat.clustered,
  group.by = 'predicted.id',
  group_name = c('Pericycle','Cortex','Trichoblasts','Root tip',
                 'Phloem','Stele','Endodermis','Atrichoblasts',
                 'Meristem','Xylem'),
)
```

For convenience, we re-name the hdWGCNA modules to indicate that they are from the Cortex cluster.

```
# rename the modules
seurat.clustered <- ResetModuleNames(
  seurat.clustered,
  new_name = "Lotus-Mod"
)
```

We can visualize the genes in each module ranked by kME using the `PlotKMEs` function.

```
options(repr.plot.width=20, repr.plot.height=25)
p <- capture.output({PlotKMEs(seurat.clustered, ncol=3, text_size = 4);})
```

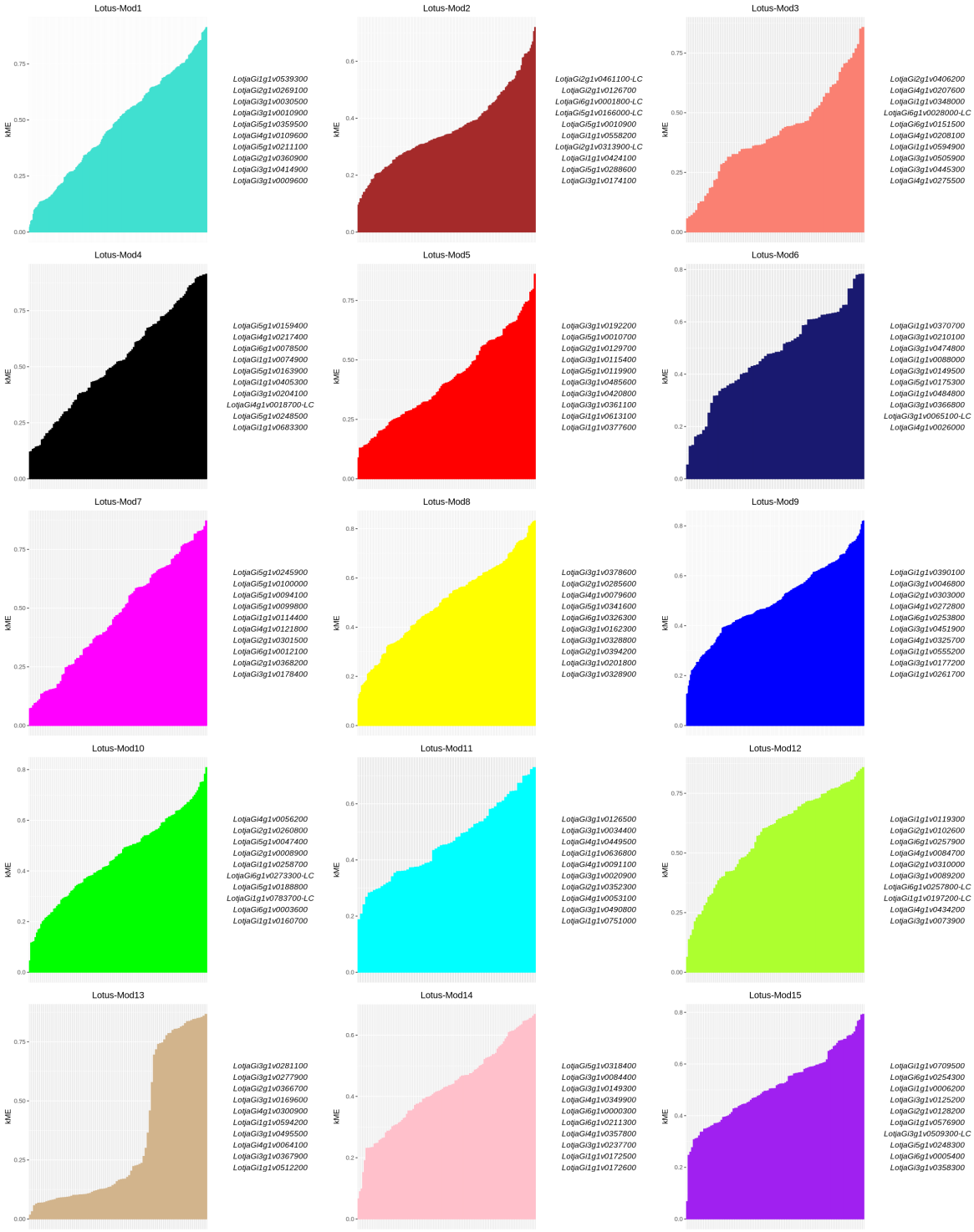


Figure 31: KMEs for each module, where the genes on the right of each plot gives the top genes based on the module's KMEs. Each plot is a histogram which has been rotated, so that bars starting on the left side of the y axis represent the counts of how many genes have the connectivity written on the right side of the y axis. The number of counts should be written on the x axis, but is not plotted by the function.

5.3.3 Getting the module assignment table

The plots of Figure 31 are a bit hard to read, though fancy to plot. `hdWGCNA` allows for easy access of the module assignment table using the `GetModules` function. This table consists of three columns: `gene_name` stores the gene's symbol or ID, module stores the gene's module assignment, and color stores a color mapping for each module (which is used in plotting steps). If `ModuleConnectivity` has been used on this hdWGCNA experiment, as it is our case, this table has additional columns with the connectivities plotted in Figure 31

```
# get the module assignment table:
modules <- GetModules(seurat.clustered)

# show the first 6 columns:
head(modules[,1:6])
```

A data.frame: 6 × 6

	gene_name <chr>	module <fct>	color <chr>	kME_Lotus-Mod1 <dbl>	kME_Lotus-Mod2 <dbl>	kME_Lotus-Mod3 <dbl>
LotjaGi3g1v0321700	LotjaGi3g1v0321700	Lotus-Mod1	turquoise	0.8397488	-0.1355572	-0.2505539
LotjaGi2g1v0360900	LotjaGi2g1v0360900	Lotus-Mod1	turquoise	0.9035133	-0.1649301	-0.2914684
LotjaGi3g1v0506700	LotjaGi3g1v0506700	Lotus-Mod1	turquoise	0.7470380	-0.1574194	-0.2274758
LotjaGi3g1v0228100	LotjaGi3g1v0228100	Lotus-Mod2	brown	-0.1650440	0.4451741	-0.1961163
LotjaGi3g1v0445300	LotjaGi3g1v0445300	Lotus-Mod3	salmon	-0.2402357	-0.1797755	0.8505632
LotjaGi6g1v0155800	LotjaGi6g1v0155800	Lotus-Mod1	turquoise	0.7323678	-0.1353630	-0.2581496

A table of the top N hub genes sorted by kME can be extracted using the `GetHubGenes` function. Here we choose the top 10 genes, but you can change the value if you want

```
# get hub genes
hub_df <- GetHubGenes(seurat.clustered, n_hubs = 10)

head( hub_df )
```

A data.frame: 6 × 3

	gene_name <chr>	module <fct>	kME <dbl>
1	LotjaGi1g1v0539300	Lotus-Mod1	0.8522461
2	LotjaGi2g1v0269100	Lotus-Mod1	0.8544151
3	LotjaGi3g1v0030500	Lotus-Mod1	0.8551061
4	LotjaGi3g1v0010900	Lotus-Mod1	0.8877517
5	LotjaGi5g1v0359500	Lotus-Mod1	0.8911171
6	LotjaGi4g1v0109600	Lotus-Mod1	0.8935191

Again, we can assign GO terms to better check associated functions

```
go_table <- read.table("./LJ_GO_terms.gaf", skip=6, sep='\t', fill=TRUE, quote = "\"")
```

```
hub_df <- addGOterms(input_table = hub_df,
                     go_table = go_table,
                     gene_column = 'gene_name',
                     n.cores = 16)
```

Now it is easy to look at each module and relevant GO terms.

```
hub_filtered <- hub_df %>% filter(module == "Lotus-Mod3")
```

```
hub_filtered
```

A data.frame: 10 × 4

gene_name <chr>	module <fct>	kME <dbl>	GO <chr>
LotjaGi2g1v0406200	Lotus-Mod3	0.6885007	Undefined
LotjaGi4g1v0207600	Lotus-Mod3	0.7048991	Undefined
LotjaGi1g1v0348000	Lotus-Mod3	0.7078898	Aspartic proteinase nepenthesin-1; TAIR: AT1G09750.1 Eukaryotic aspartyl protease family protein; Swiss-Prot: sp
LotjaGi6g1v0028000-LC	Lotus-Mod3	0.7213880	Undefined

gene_name <chr>	module <fct>	kME <dbl>	GO <chr>
LotjaGi6g1v0151500	Lotus-Mod3	0.7284848	Undefined
LotjaGi4g1v0208100	Lotus-Mod3	0.7491915	Undefined
LotjaGi1g1v0594900	Lotus-Mod3	0.7680713	Undefined
LotjaGi3g1v0505900	Lotus-Mod3	0.7862156	Undefined
LotjaGi3g1v0445300	Lotus-Mod3	0.8505632	Undefined
LotjaGi4g1v0275500	Lotus-Mod3	0.8577066	Undefined

We save the table for later use

```
write.csv(hub_df, "top_genes_networks.csv")
```

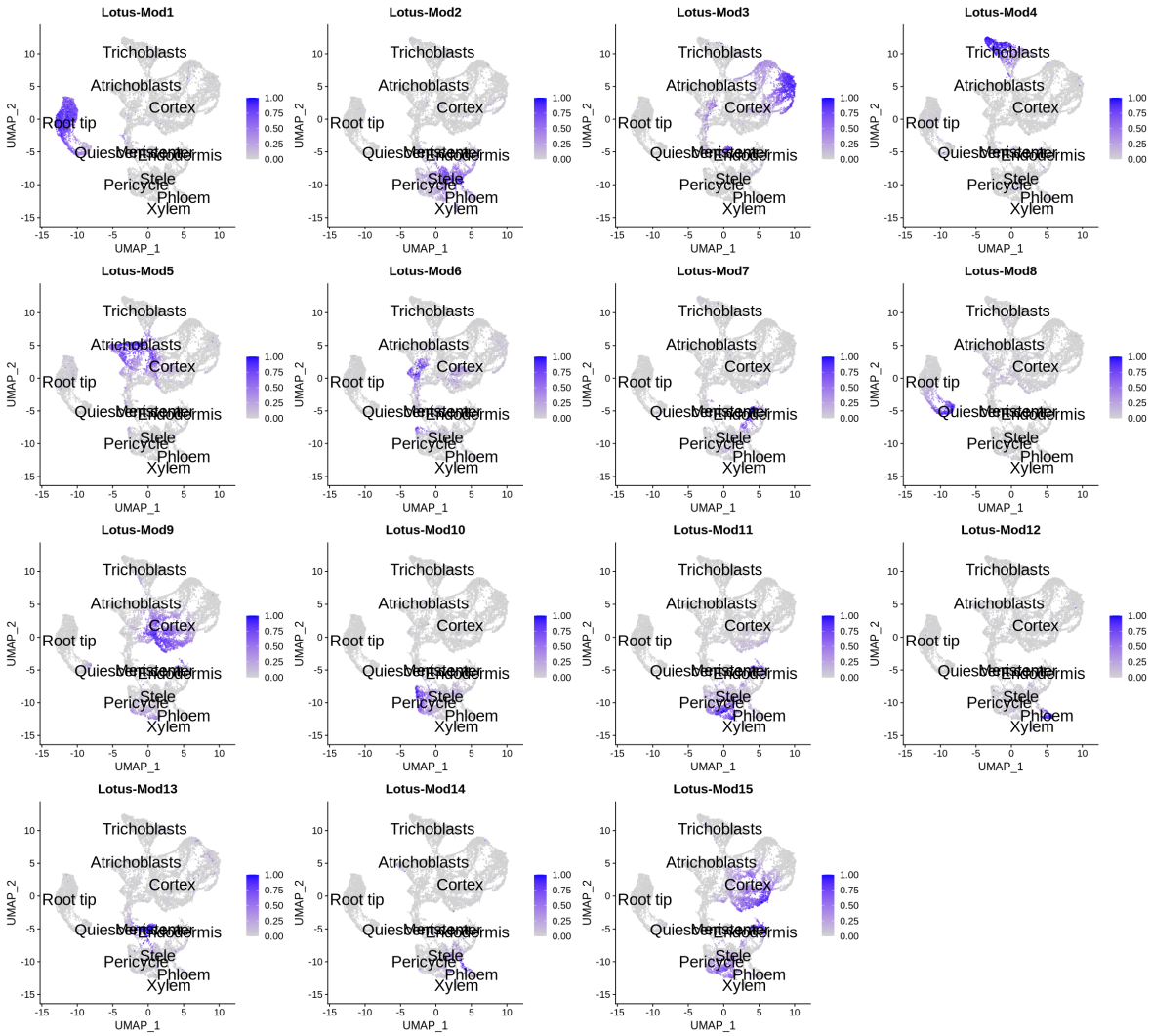
We can also plot the modules to see where they are most relevant on the UMAP plot. First we calculate the scores of each module.

```
features_list <- list()

for(MOD in hub_df$module){
    genes <- hub_df %>% filter(module == MOD)
    genes_mod <- genes$gene_name
    features_list[[MOD]] <- genes_mod
    }

seurat.clustered <- AddModuleScore(
  object = seurat.clustered,
  features = features_list,
  ctrl = 5,
)

seurat.clustered <- renameScores(markers_list = features_list, seurat_data = seurat.clustered
plotScoresUMAP(markers_list = features_list, seurat_data = seurat.clustered)
```

```
Scores renamed FROM
```

```
TO
```

Cluster1
Cluster2
Cluster3
Cluster4
Cluster5
Cluster6
Cluster7
Cluster8
Cluster9
Cluster10
Cluster11
Cluster12
Cluster13
Cluster14
Cluster15
Lotus-Mod1
Lotus-Mod2
Lotus-Mod3
Lotus-Mod4
Lotus-Mod5
Lotus-Mod6
Lotus-Mod7
Lotus-Mod8
Lotus-Mod9
Lotus-Mod10
Lotus-Mod11
Lotus-Mod12
Lotus-Mod13
Lotus-Mod14
Lotus-Mod15



We save the seurat object and the network (because for some inscrutable error, it cannot load together with the Seurat object when it is needed again).

```
SaveH5Seurat(seurat.clustered, 'seurat.network.h5Seurat', overwrite = TRUE, verbose=FALSE)
```

```
Warning message:
"Overwriting previous file seurat.network.h5Seurat"
Creating h5Seurat file for version 3.1.5.9900
```

```
saveRDS(seurat.clustered@misc, "network_lotus.RDS")
```

5.4 Differential module Expression (DME) analysis

Lastly, we can see which modules are most expressed in a specific cluster against the others, in a similar way to differential gene expression.

```
seurat.network <- LoadH5Seurat('seurat.network.h5Seurat', misc=FALSE, verbose=FALSE)
```

```
Validating h5Seurat file

Warning message:
"Adding a command log without an assay associated with it"

[1] "Couldn't delete        144115188075856517"
```

```
seurat.network@misc <- readRDS("network_lotus.RDS")
```

Here we discuss how to perform **DME testing between two different groups**. We use the `hdWGCNA` function `FindDMEs`, which is similar to the Seurat function FindMarkers. We use the Mann-Whitney U test, also known as the Wilcoxon test, to compare two groups, but other tests can be used at the user's discretion with the `test.use` parameter.

We are interested in defining our two groups both by condition and by choosing a cluster, for example `Cortex`. We filter DMEs by p-value and fold-change.

```
R7Agroup <- seurat.network@meta.data %>% subset(Condition == 'R7A' & predicted.id == 'Cortex
WTgroup <- seurat.network@meta.data %>% subset(Condition == 'Control' & predicted.id == 'Cor
```

```
DMEs <- FindDMEs(
  seurat.network,
  barcodes1 = R7Agroup,
  barcodes2 = WTgroup,
  test.use='wilcox',
  wgcna_name='tutorial'
) %>% filter(p_val_adj < 0.001 & abs(avg_log2FC)>1) %>% select(-p_val)
```

```
[1] 17721    15
 [1] "Lotus-Mod3"  "Lotus-Mod13" "Lotus-Mod5"  "Lotus-Mod6"  "Lotus-Mod2"
 [6] "Lotus-Mod12" "Lotus-Mod14" "Lotus-Mod7"  "Lotus-Mod10" "Lotus-Mod11"
[11] "Lotus-Mod9"  "Lotus-Mod15" "Lotus-Mod4"  "Lotus-Mod1"  "Lotus-Mod8"
```

The resulting table below is very similar to the one for differential gene expression. Now the p-values and fold changes are referred to the differential module expression in the inoculated cortex VS the control cortex.

```
DMEs
```

A data.frame: 4 × 5

	avg_log2FC <dbl>	pct.1 <dbl>	pct.2 <dbl>	p_val_adj <dbl>	module <chr>
Lotus-Mod11	1.321296	0.258	0.139	3.169398e-37	Lotus-Mod11
Lotus-Mod6	1.058396	0.445	0.329	2.570134e-32	Lotus-Mod6
Lotus-Mod1	-1.213882	0.022	0.042	1.141514e-05	Lotus-Mod1
Lotus-Mod4	-1.812261	0.063	0.087	8.976603e-04	Lotus-Mod4

We can also do a lollipop plot as in Figure 32, where the size of each circle is the p-value, and the x-axis is the log-fold change. A cross on a circle means the p-value is not significant. Module 4 and 11 are very interesting because of their over- and under-expression, though you should note the difference in percentages is pretty small. In our case we filtered out p-values that were too high, so we have only significant modules in the plot.

```
options(repr.plot.width=6, repr.plot.height=6)

PlotDMEsLollipop(
  seurat.network,
  DMEs,
  wgcna_name='tutorial',
  pvalue = "p_val_adj",
)
```

```
Loading required package: ggforestplot
```

```
[1] "Please be aware comparison group/groups are not provided, which may casue an ERROR. Plot
```

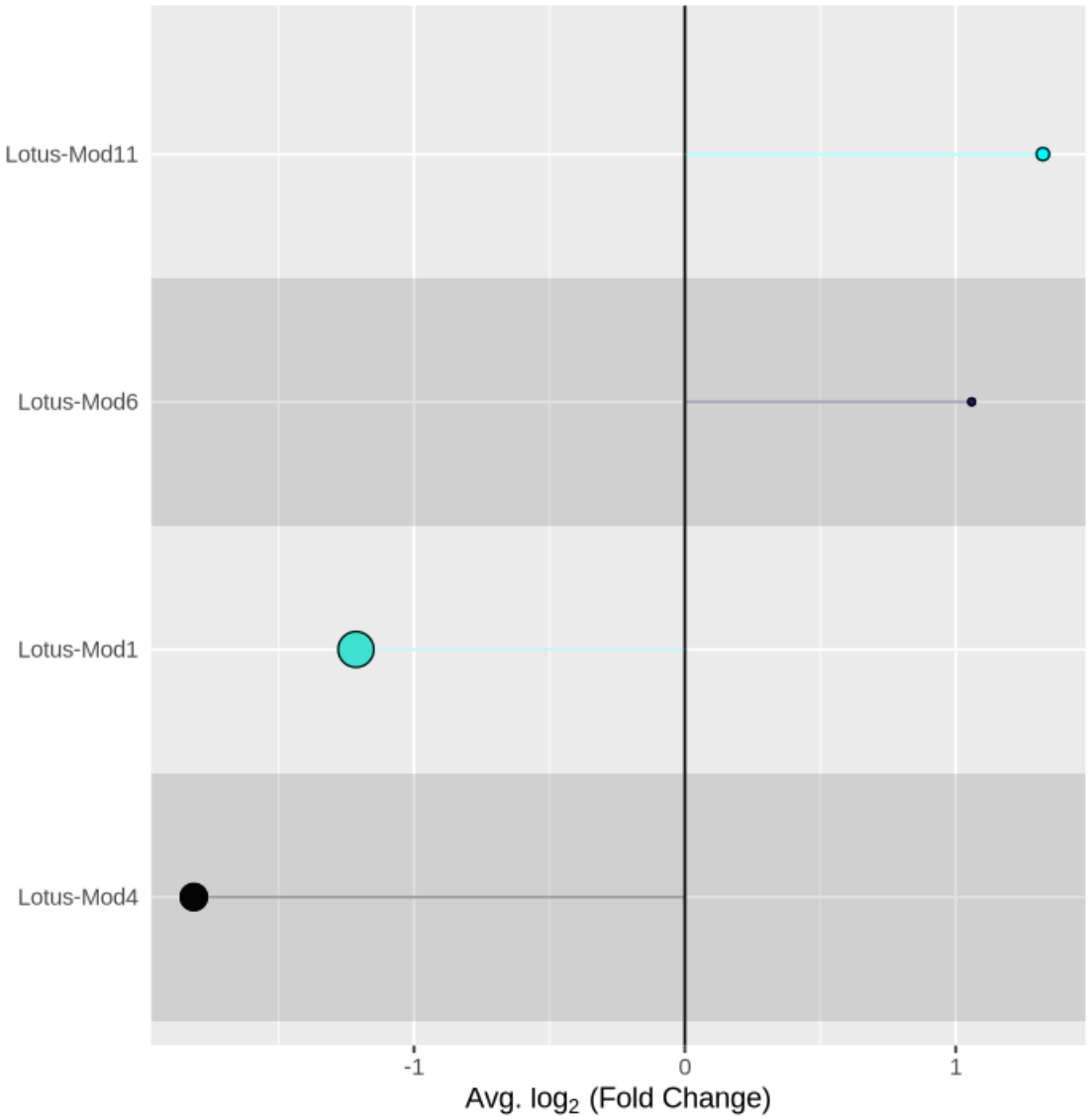


Figure 32: Lollipop plot that shovs the average log-fold change of each module and the p-value (size of each circle). Crossed circles, when present, have a non-significant p-value

5.4.1 One-versus-all DME analysis

This is another case of DME analysis, where each cluster is tested against the rest of the data to see which modules are differentially expressed. We can test by cell type (`predicted.id`) or by condition (`Condition`).

```
DMEs_all <- FindAllDMEs(
  seurat.network,
  group.by = 'predicted.id',
  wgcna_name = 'tutorial'
)
```

```
[1] "Cortex"
[1] "Trichoblasts"
[1] "Root tip"
[1] "Meristem"
[1] "Phloem"
[1] "Pericycle"
[1] "Stele"
[1] "Atrichoblasts"
[1] "Xylem"
[1] "Endodermis"
[1] "Quiescent center"
```

The table is usually big, but you can choose to filter by various parameters as below, and to look only at one cluster of interest

```
DMEs_cortex <- DMEs_all %>% filter(p_val_adj < .001 & abs(avg_log2FC)>1
                                   & is.finite(avg_log2FC)
                                   & group=='Cortex')
```

The DME analysis results in a lot of significant results for the cortex. But we can see that `pct.1` and `pct.2` vary a lot. Module 12 has more than 50% of the cells expressing the module, and 7% in the rest of the data, while Module 20 has percentages of 52% vs 22%. Here it is always best to report those percentages and log-fold changes starting from the highest ones.

```
DMEs_cortex
```

A data.frame: 14 × 7

	p_val <dbl>	avg_log2FC <dbl>	pct.1 <dbl>	pct.2 <dbl>	p_val_adj <dbl>	module <chr>	group <chr>
Cortex.Lotus-Mod3	0.000000e+00	2.0690457	0.518	0.149	0.000000e+00	Lotus-Mod3	Cortex
Cortex.Lotus-Mod6	0.000000e+00	2.0516551	0.397	0.137	0.000000e+00	Lotus-Mod6	Cortex

	p_val <dbl>	avg_log2FC <dbl>	pct.1 <dbl>	pct.2 <dbl>	p_val_adj <dbl>	module <chr>	group <chr>
Cortex.Lotus-Mod2	0.000000e+00	4.225120	0.075	0.348	0.000000e+00	Lotus-Mod2	Cortex
Cortex.Lotus-Mod12	0.000000e+00	4.736902	0.038	0.283	0.000000e+00	Lotus-Mod12	Cortex
Cortex.Lotus-Mod9	0.000000e+00	2.309667	0.522	0.249	0.000000e+00	Lotus-Mod9	Cortex
Cortex.Lotus-Mod1	0.000000e+00	5.372586	0.031	0.284	0.000000e+00	Lotus-Mod1	Cortex
Cortex.Lotus-Mod14	1.424592e-288	-3.210875	0.077	0.295	2.136888e-287	Lotus-Mod14	Cortex
Cortex.Lotus-Mod10	3.164844e-256	-4.539404	0.075	0.266	4.747266e-255	Lotus-Mod10	Cortex
Cortex.Lotus-Mod15	2.013474e-254	1.032737	0.476	0.218	3.020212e-253	Lotus-Mod15	Cortex
Cortex.Lotus-Mod4	8.327387e-179	-3.730921	0.073	0.224	1.249108e-177	Lotus-Mod4	Cortex
Cortex.Lotus-Mod11	1.287590e-108	-2.435203	0.209	0.325	1.931384e-107	Lotus-Mod11	Cortex
Cortex.Lotus-Mod7	1.020206e-39	-2.764012	0.122	0.188	1.530309e-38	Lotus-Mod7	Cortex
Cortex.Lotus-Mod8	2.830229e-24	-2.096240	0.246	0.291	4.245343e-23	Lotus-Mod8	Cortex
Cortex.Lotus-Mod13	3.424949e-07	-1.448706	0.172	0.202	5.137423e-06	Lotus-Mod13	Cortex

You can look at any other cluster. For example trichoblasts. Here you can see how module 4 pops up as being basically entirely expressed only in Trichoblasts.

```
DMEs_tricho <- DMEs_all %>% filter(p_val_adj < .001 & abs(avg_log2FC)>1
                                        & is.finite(avg_log2FC)
                                        & group=='Trichoblasts')
```

```
DMEs_tricho
```

A data.frame: 15 × 7

	p_val <dbl>	avg_log2FC <dbl>	pct.1 <dbl>	pct.2 <dbl>	p_val_adj <dbl>	module <chr>	group <chr>
Trichoblasts Lotus-Mod4	0.000000e+00	0.462799	0.998	0.078	0.000000e+00	Lotus-Mod4	Trichoblasts
Trichoblasts Lotus-Mod15	4.589976e-165	-6.220812	0.016	0.357	6.883613e-164	Lotus-Mod15	Trichoblasts
Trichoblasts Lotus-Mod11	2.472972e-134	-6.784385	0.011	0.303	3.709458e-133	Lotus-Mod11	Trichoblasts
Trichoblasts Lotus-Mod9	1.165473e-129	-3.592430	0.095	0.390	1.748210e-128	Lotus-Mod9	Trichoblasts
Trichoblasts Lotus-Mod3	6.823088e-111	-4.113294	0.065	0.327	1.023463e-109	Lotus-Mod3	Trichoblasts
Trichoblasts Lotus-Mod6	7.331605e-111	-5.105053	0.014	0.268	1.099741e-109	Lotus-Mod6	Trichoblasts
Trichoblasts Lotus-Mod2	1.103509e-101	-4.652608	0.016	0.255	1.655264e-100	Lotus-Mod2	Trichoblasts
Trichoblasts Lotus-Mod10	1.334983e-80	-6.542761	0.009	0.204	2.002475e-79	Lotus-Mod10	Trichoblasts
Trichoblasts Lotus-Mod12	5.703476e-72	-5.346382	0.017	0.197	8.555215e-71	Lotus-Mod12	Trichoblasts
Trichoblasts Lotus-Mod14	9.752613e-68	-3.932097	0.037	0.221	1.462892e-66	Lotus-Mod14	Trichoblasts
Trichoblasts Lotus-Mod5	1.462617e-64	-3.455537	0.089	0.271	2.193925e-63	Lotus-Mod5	Trichoblasts
Trichoblasts Lotus-Mod13	1.126768e-63	-2.689731	0.032	0.205	1.690151e-62	Lotus-Mod13	Trichoblasts
Trichoblasts Lotus-Mod1	6.001523e-29	-5.077260	0.090	0.187	9.002285e-28	Lotus-Mod1	Trichoblasts
Trichoblasts Lotus-Mod8	9.594551e-24	-2.423585	0.179	0.282	1.439183e-22	Lotus-Mod8	Trichoblasts
Trichoblasts Lotus-Mod7	6.536989e-21	-3.664154	0.083	0.168	9.805484e-20	Lotus-Mod7	Trichoblasts

For better visualization, you can always use the lolliplot plots using the correct table as an argument, so that you can plot multiple instances for the various clusters. In Figure 33 we plot boyh for cortex and trichoblasts from the tables determined in the code above.

```
options(repr.plot.width=12, repr.plot.height=6)

#lollipop with cortex table
p1 <- PlotDMEsLollipop(
```

```
  seurat.network,
  DMEs_cortex,
  wgcna_name='tutorial',
  pvalue = "p_val_adj",
)

#lollipop with trichoblasts table
p2 <- PlotDMEsLollipop(
  seurat.network,
  DMEs_tricho,
  wgcna_name='tutorial',
  pvalue = "p_val_adj",
)

#plot an add a title
p1 + ggtitle("DMEs of Cortex VS data") +
p2 + ggtitle("DMEs of Trichoblasts VS data")
```

```
[1] "Please be aware comparison group/groups are not provided, which may casue an ERROR. Plot
[1] "Please be aware comparison group/groups are not provided, which may casue an ERROR. Plot
```

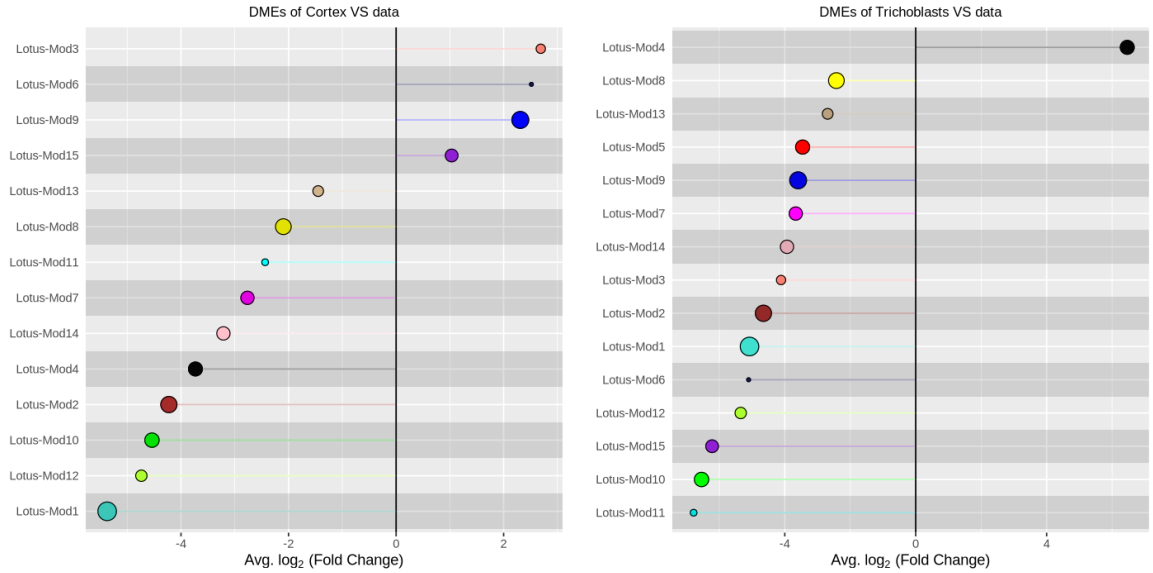


Figure 33: Lollipop plot that shovs the average log-fold change of each module and the p-value (size of each circle). Crossed circles, when present, have a non-significant p-value. Plotting lolliplot plots for various clusters can be useful to detect modules being simultaneously significant.

i Wrapping Up

This is the end of the tutorial. We have not shown much biological conclusion from the analysis - this is something that comes out by studying for example the GO terms of the various genes and modules identified. The scope of the tutorial was mainly to give all the means to perform your own analysis, from which you can then gain biological insight.
If you are interested in more resources to learn single cell analysis, you can find them at some of our courses. We have other single-cell analysis tutorials including different tools at

- **Introduction to NGS data analysis** (found in the `Genomics Sandbox` **at this link** - note that this is in the `python` language)
- **Introduction to scRNAseq analysis in R** (found in the `Transcriptomics Sandbox` **at this link**)

You can also find a lot of material in the **`Seurat` webpage**. If you create a new notebook in this environment, you are likely to have all the packages needed to try the `Seurat` tutorials.

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