Normalization and scaling

Learning outcomes

After having completed this chapter you will be able to:

- Describe and perform standard procedures for normalization and scaling with the package `Seurat`
- Select the most variable genes from a `Seurat` object for downstream analyses

Material

Material

https://youtu.be/QS6ZH_41b4s?si=fvaNGkqjsIcxTEFn

- Seurat vignette

Exercises

Code starts here:

```
library(Seurat)
seu <- readRDS("day1/seu_day1-2.rds")
```

Code ends here

Normalization

After removing unwanted cells from the dataset, the next step is to normalize the data. By default, Seurat employs a global-scaling normalization method `"LogNormalize"` that normalizes the feature expression measurements for each cell by the total expression, multiplies this by a scale factor (10,000 by default), and log-transforms the result. Normalized values are stored in the “RNA” assay (as item of the `@assay` slot) of the seu object.

This is how you can call the function (don’t run it yet! Read the exercise first):

Code starts here:

```
# Don't run it yet! Read the exercise first
seu <- Seurat::NormalizeData(seu,
                     normalization.method = "LogNormalize",
                     scale.factor = 10000)
```

```
Normalizing layer: counts
```

Code ends here

Exercise

Have a look at the assay data before and after running `NormalizeData()`. Did it change?

Tip

You can extract assay data with the function `Seurat::GetAssayData`. By default, the slot `data` is used (inside the slot `assay`), containing normalized counts. Use `Seurat::GetAssayData(seu, slot = "counts")` to get the raw counts.

Answer

You can check out some assay data with:

Code starts here:

```
Seurat::GetAssayData(seu)[1:10,1:10]
```

Code ends here

Returning:

Before normalization

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

  [[ suppressing 10 column names 'PBMMC-1_AAACCTGCAGACGCAA-1', 'PBMMC-1_AA
ACCTGTCATCACCC-1', 'PBMMC-1_AAAGATGCATAAAGGT-1' ... ]]


RP11-34P13.7  . . . . . . . . . .
FO538757.3    . . . . . . . . . .
FO538757.2    1 . . . . . 2 . . .
AP006222.2    . . . . . . . . . .
RP4-669L17.10 . . . . . . . . . .
RP5-857K21.4  . . . . . . . . . .
RP11-206L10.9 . . . . . . . . . .
LINC00115     . . . . . . . . . .
FAM41C        . . . . . . . . . .
RP11-54O7.1   . . . . . . . . . .
```

After normalization

```
Normalizing layer: counts

10 x 10 sparse Matrix of class "dgCMatrix"

  [[ suppressing 10 column names 'PBMMC-1_AAACCTGCAGACGCAA-1', 'PBMMC-1_AA
ACCTGTCATCACCC-1', 'PBMMC-1_AAAGATGCATAAAGGT-1' ... ]]


RP11-34P13.7  .        . . . . . .        . . .
FO538757.3    .        . . . . . .        . . .
FO538757.2    1.641892 . . . . . 1.381104 . . .
AP006222.2    .        . . . . . .        . . .
RP4-669L17.10 .        . . . . . .        . . .
RP5-857K21.4  .        . . . . . .        . . .
RP11-206L10.9 .        . . . . . .        . . .
LINC00115     .        . . . . . .        . . .
FAM41C        .        . . . . . .        . . .
RP11-54O7.1   .        . . . . . .        . . .
```

Updating `seu`

As you might have noticed, this function takes the object `seu` as input, and it returns it to an object named `seu`. We can do this because the output of such calculations are added to the object, without loosing information.

Variable features

We next calculate a subset of features that exhibit high cell-to-cell variation in the dataset (i.e, they are highly expressed in some cells, and lowly expressed in others). Focusing on these genes in downstream analysis helps to highlight biological signal in single-cell datasets. The procedure in Seurat models the mean-variance relationship inherent in single-cell data, and is implemented in the `FindVariableFeatures()` function. By default, 2,000 genes (features) per dataset are returned and these will be used in downstream analysis, like PCA.

Code starts here:

```
seu <- Seurat::FindVariableFeatures(seu,
                            selection.method = "vst",
                            nfeatures = 2000)

Finding variable features for layer counts
```

Code ends here

Let's have a look at the 10 most variable genes:

Code starts here:

```
# Identify the 10 most highly variable genes
top10 <- head(Seurat::VariableFeatures(seu), 10)
top10

 [1] "IGKC"   "HBG2"   "IGHG3"  "IGHG1"  "JCHAIN" "HBG1"   "IGHA1"  "IGHGP"
 [9] "IGLC2"  "IGLC3"
```

Code ends here

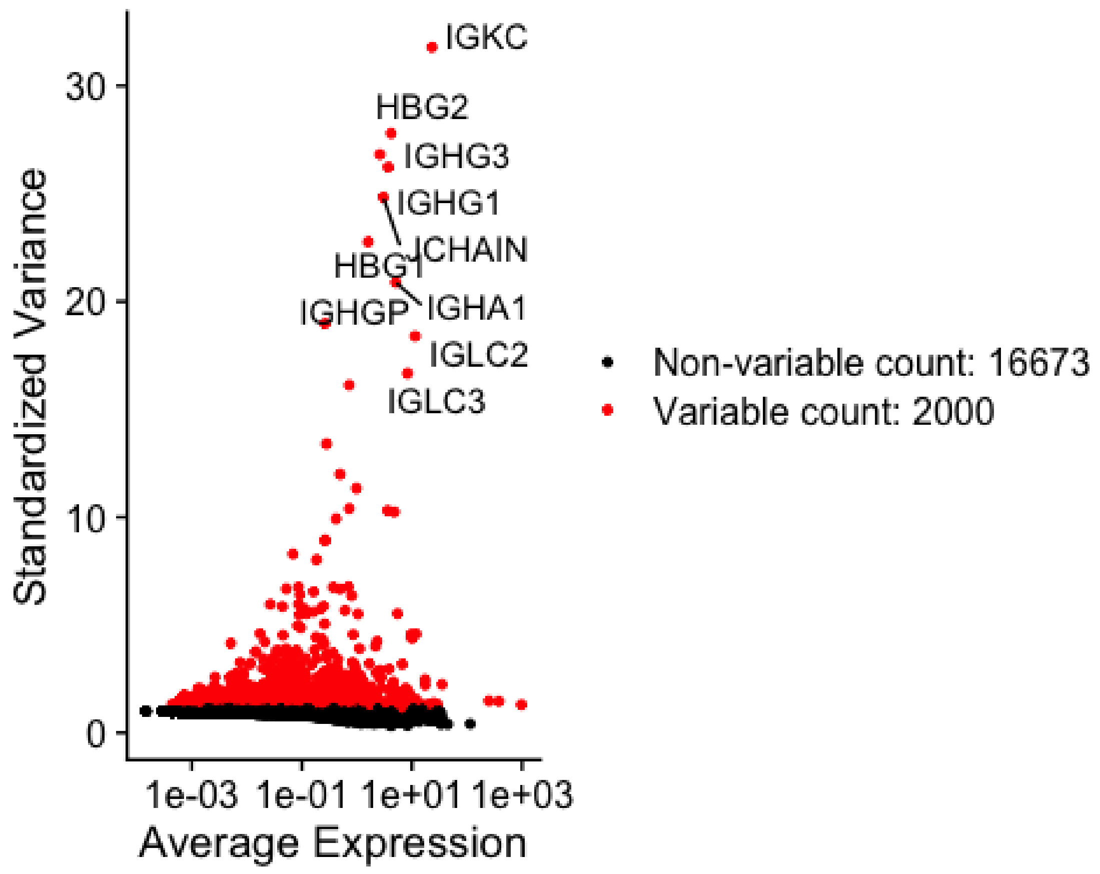
We can plot them in a nicely labeled scatterplot:

Code starts here:

```
vf_plot <- Seurat::VariableFeaturePlot(seu)
Seurat::LabelPoints(plot = vf_plot,
            points = top10, repel = TRUE)

When using repel, set xnudge and ynudge to 0 for optimal results
```

```
Code ends here
```



> Make sure the plotting window is large enough
>
> The function `LabelPoints` will throw an error if the plotting window is to small. If you get an error, increase plotting window size in RStudio and try again.

You can see that most of the highly variables are antibody subunits (starting with IGH, IGL). Not very surprising since we look at bone marrow tissue. We can have a look later in which cells they are expressed.

Scaling

Next, we apply scaling, a linear transformation that is a standard pre-processing step prior to dimensional reduction techniques like PCA. The `ScaleData()` function

1. shifts the expression of each gene, so that the mean expression across cells is 0
2. scales the expression of each gene, so that the variance across cells is 1

This step gives equal weight in downstream analyses, so that highly-expressed genes do not dominate. The results of this are stored in `seu$RNA@scale.data`

Code starts here:

```
seu <- Seurat::ScaleData(seu)

Centering and scaling data matrix
```

Code ends here

The use of `Seurat::SCTransform`

The functions `NormalizeData`, `VariableFeatures` and `ScaleData` can be replaced by the function `SCTransform`. The latter uses a more sophisticated way to perform the normalization and scaling, and is argued to perform better. However, it is slower, and a bit less transparent compared to using the three separate functions. Therefore, we chose not to use `SCTransform` for the exercises.

Bonus exercise

Run `SCTransform` on the `seu` object. Where is the output stored?

Answer

You can run it like so:

Code starts here:

```
seu <- Seurat::SCTransform(seu)
```

Code ends here

And it will add an extra assay to the object. `names(seu@assays)` returns:

```
[1] "RNA" "SCT"
```

Meaning that a whole new assay was added (including the sparse matrices with counts, normalized data and scaled data).

Warning

Running `SCTransform` will change `@active.assay` into `SCT`(in stead of `RNA`; check it with `DefaultAssay(seu)`). This assay is used as a default for following function calls. To change the active assay to `RNA` run:

Code starts here:

```
DefaultAssay(seu) <- "RNA"
```

Code ends here

Save the dataset and clear environment

Now, save the dataset so you can use it tomorrow:

Code starts here:

```
saveRDS(seu, "day2/seu_day2-1.rds")
```

Code ends here

Clear your environment:

Code starts here:

```
rm(list = ls())
gc()
.rs.restartR()
```

Code ends here