Differential gene expression

Material

https://youtu.be/6Y5uCQWRRbg?si=mcV9qJUap1qvFjy3

- More information on pseudobulk analysis
- Muscat for pseudobulk DGE.
- Paper on the robustness of different differential expression analysis methods

Exercises

Find all markers for each cluster

Load the seu dataset you have created yesterday:

And load the following packages (install them if they are missing):

Code starts here:

```
library(Seurat)
library(edgeR) # BiocManager::install("edgeR")
library(limma)
library(dplyr)
library(scuttle)
```

Code ends here

The function FindAllMarkers performs a Wilcoxon plot to determine the genes differentially expressed between each cluster and the rest of the cells. Other types of tests than the Wilcoxon test are available. Check it out by running ?Seurat::FindAllMarkers.

Now run analysis:

Code starts here:

```
de_genes <- Seurat::FindAllMarkers(seu,  min.pct = 0.25,
                                   only.pos = TRUE)
```

Code ends here

Subset the table to only keep the significant genes, and you can save it as a csv file if you wish to explore it further. Then extract the top 3 markers per cluster:

Code starts here:

```
de_genes <- subset(de_genes, de_genes$p_val_adj < 0.05)
write.csv(de_genes,
          "day3/de_genes_FindAllMarkers.csv",
          row.names = F, quote = F)

top_specific_markers <- de_genes %>%
  group_by(cluster) %>%
  top_n(3, avg_log2FC)
```

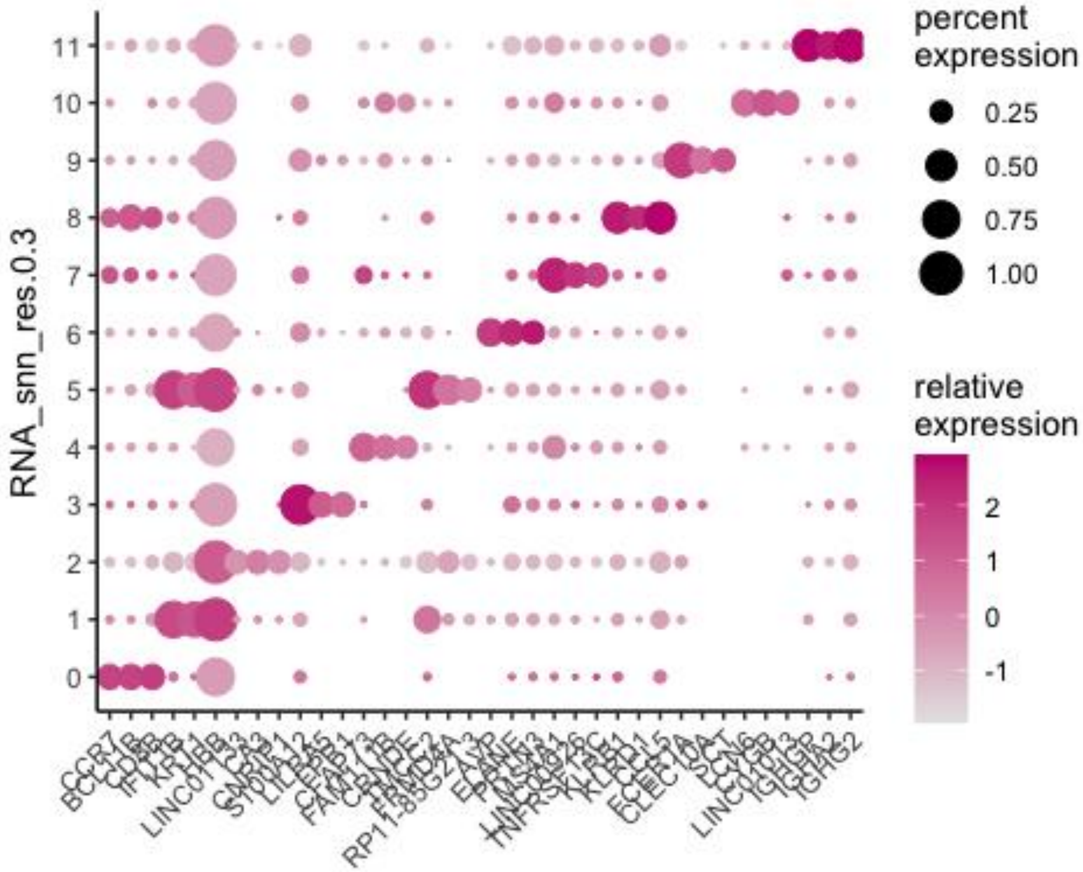
Code ends here

And generate e.g. a dotplot:

Code starts here:

```
dittoSeq::dittoDotPlot(seu,
                       vars = unique(top_specific_markers$gene),
                       group.by = "RNA_snn_res.0.3")
```

Code ends here



Exercise

What are significant marker genes in cluster 0 and 8? Are the T cell genes in there?

You can re-load the vector with immune genes with:

Code starts here:

```
tcell_genes <- c("IL7R", "LTB", "TRAC", "CD3D")
```

Answer

Code starts here:

```
de_genes[de_genes$gene %in% tcell_genes,] |> knitr::kable()
```

Code ends here

	p_val	avg_log2FC	pct.1	pct.2	p_val_adj	cluster	gene
CD3D	0	2.9119600	0.772	0.226	0	0	CD3D
TRAC	0	2.5821466	0.628	0.202	0	0	TRAC
LTB	0	1.8792775	0.761	0.394	0	0	LTB
IL7R	0	2.8925743	0.437	0.114	0	0	IL7R
LTB1	0	1.3839002	0.676	0.465	0	7	LTB
TRAC1	0	2.3960975	0.749	0.274	0	8	TRAC
CD3D1	0	2.1124053	0.795	0.326	0	8	CD3D
LTB2	0	1.8691213	0.757	0.462	0	8	LTB
IL7R1	0	1.9115516	0.468	0.172	0	8	IL7R
LTB3	0	0.9057526	0.743	0.466	0	10	LTB

So, yes, the T-cell genes are highly significant markers for cluster 0 and 8.

Differential expression between groups of cells

The `FindMarkers` function allows to test for differential gene expression analysis specifically between 2 groups of cells, i.e. perform pairwise comparisons, eg between cells of cluster 0 vs cluster 2, or between cells annotated as T-cells and B-cells.

First we can set the default cell identity to the cell types defined by `SingleR`:

Code starts here:

```
seu <- Seurat::SetIdent(seu, value = "SingleR_annot")
```

Code ends here

Run the differential gene expression analysis and subset the table to keep the significant genes:

Code starts here:

```
deg_cd8_cd4 <- Seurat::FindMarkers(seu,
                                   ident.1 = "CD8+ T cells",
                                   ident.2 = "CD4+ T cells",
                                   group.by = seu$SingleR_annot,
                                   test.use = "wilcox")
deg_cd8_cd4 <- subset(deg_cd8_cd4, deg_cd8_cd4$p_val_adj<0.05)
```

Code ends here

Exercise

Are CD8A, CD8B and CD4 in there? What does the sign (i.e. positive or negative) mean in the log fold change values? Are they according to the CD8+ and CD4+ annotations? Check your answer by generating a violin plot of a top differentially expressed gene.

Answer

You can check out the results with:

Code starts here:

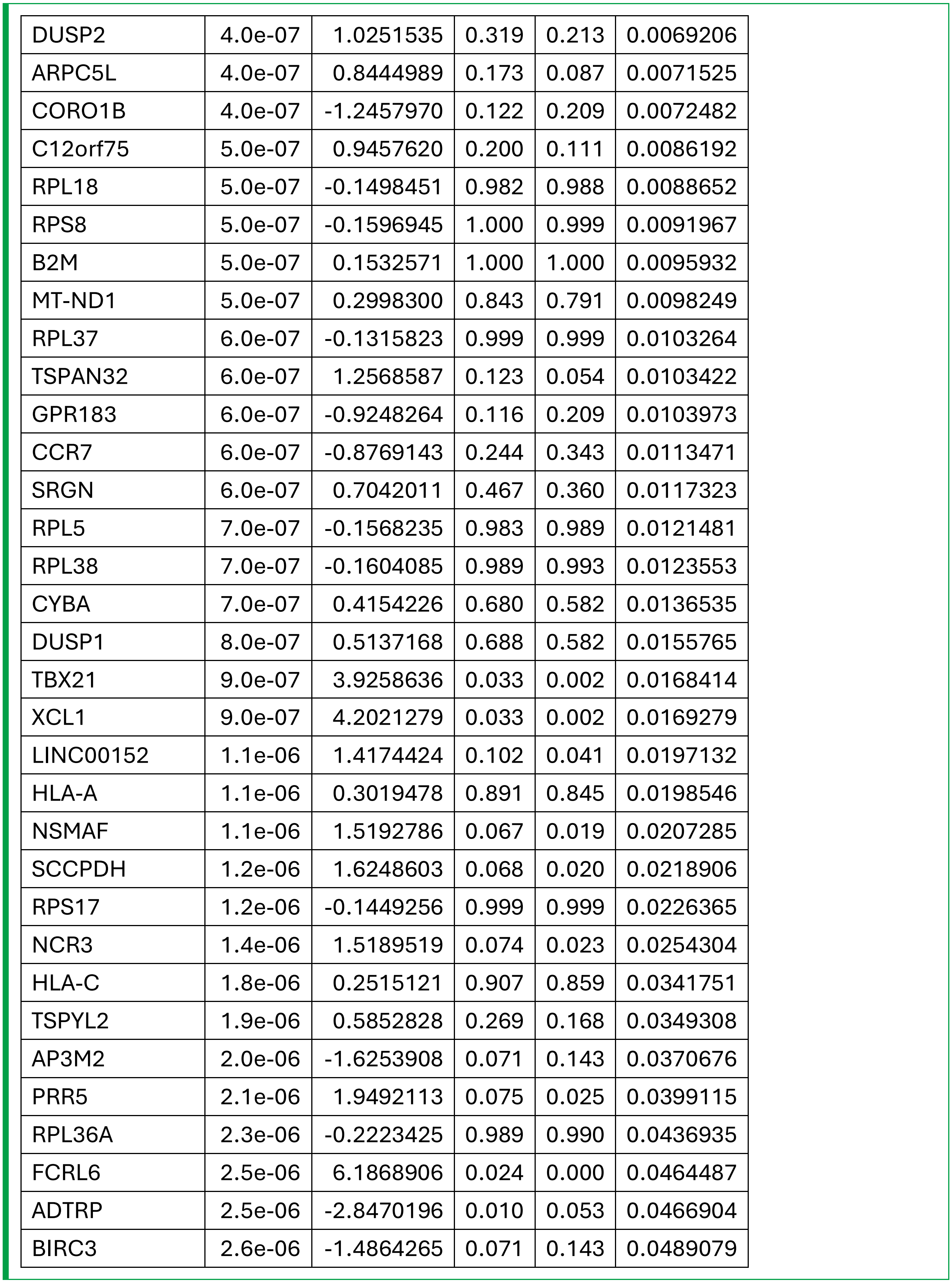
```
View(deg_cd8_cd4)
```

Code ends here

	p_val	avg_log2FC	pct.1	pct.2	p_val_adj
CD8A	0.0e+00	5.8036617	0.336	0.008	0.0000000
CTSW	0.0e+00	3.4118773	0.276	0.030	0.0000000
CCL5	0.0e+00	4.2922622	0.285	0.062	0.0000000
CD8B	0.0e+00	1.3123538	0.470	0.178	0.0000000
NKG7	0.0e+00	4.5376500	0.225	0.037	0.0000000
CST7	0.0e+00	4.4123574	0.143	0.012	0.0000000
GZMA	0.0e+00	3.8501654	0.169	0.025	0.0000000
TRGC2	0.0e+00	3.4931638	0.144	0.018	0.0000000
RPS27	0.0e+00	-0.1820605	1.000	1.000	0.0000000
KLRD1	0.0e+00	4.3589947	0.108	0.008	0.0000000
ID2	0.0e+00	0.8632969	0.565	0.353	0.0000000
GZMK	0.0e+00	3.0113231	0.130	0.022	0.0000000
HCST	0.0e+00	0.7949700	0.681	0.495	0.0000000
MT-CO1	0.0e+00	0.3243268	0.989	0.979	0.0000000
TRGC1	0.0e+00	3.6853476	0.110	0.013	0.0000000
FHIT	0.0e+00	-1.6706990	0.110	0.273	0.0000000
RP11-291B21.2	0.0e+00	1.3507107	0.222	0.077	0.0000000
MT-ND4	0.0e+00	0.3226206	0.963	0.932	0.0000000
CD4	0.0e+00	-3.3247772	0.011	0.105	0.0000000
MT-CO2	0.0e+00	0.2401784	0.993	0.995	0.0000000
TRDC	0.0e+00	2.6894608	0.105	0.019	0.0000000
CRTAM	0.0e+00	3.7124154	0.068	0.003	0.0000000
PECAM1	0.0e+00	2.6701767	0.096	0.015	0.0000000
LYAR	0.0e+00	1.6580580	0.198	0.076	0.0000000
GZMH	0.0e+00	4.8543018	0.067	0.003	0.0000000
PRF1	0.0e+00	2.8596139	0.099	0.018	0.0000000
ACTB	0.0e+00	0.3093718	0.965	0.925	0.0000000

AC092580.4	0.0e+00	1.3359936	0.173	0.061	0.0000000
CCL4	0.0e+00	3.7724347	0.118	0.031	0.0000001
RPS27A	0.0e+00	-0.1605123	1.000	1.000	0.0000001
RPL11	0.0e+00	-0.1685948	1.000	1.000	0.0000002
KLRC1	0.0e+00	8.3089065	0.048	0.000	0.0000004
MT-CO3	0.0e+00	0.2753652	0.966	0.954	0.0000005
TPST2	0.0e+00	1.8352805	0.137	0.045	0.0000009
RUNX3	0.0e+00	1.0535174	0.180	0.071	0.0000009
HLA-B	0.0e+00	0.3203112	0.970	0.934	0.0000012
RPL21	0.0e+00	-0.1713527	1.000	1.000	0.0000012
MT-ND2	0.0e+00	0.2971960	0.958	0.918	0.0000013
RPS29	0.0e+00	-0.1172011	1.000	1.000	0.0000032
GNLY	0.0e+00	3.8610459	0.132	0.046	0.0000038
IL32	0.0e+00	0.5401192	0.739	0.624	0.0000060
FAM173A	0.0e+00	1.1506784	0.194	0.087	0.0000076
NR4A2	0.0e+00	0.8415276	0.341	0.203	0.0000094
IL2RB	0.0e+00	2.2000968	0.078	0.015	0.0000148
HOPX	0.0e+00	2.3701004	0.089	0.022	0.0000197
CXCR3	0.0e+00	2.4269612	0.072	0.013	0.0000234
RPL30	0.0e+00	-0.1749260	0.992	0.998	0.0000259
PLEK	0.0e+00	2.7963063	0.059	0.008	0.0000327
CBLB	0.0e+00	1.2237121	0.127	0.045	0.0000494
RPS25	0.0e+00	-0.1727553	1.000	1.000	0.0000532
BZW1	0.0e+00	0.7778875	0.354	0.223	0.0000597
RPL34	0.0e+00	-0.1552375	1.000	1.000	0.0000684
MT-ND3	0.0e+00	0.2539291	0.955	0.930	0.0000920
ACTG1	0.0e+00	0.4120757	0.744	0.622	0.0001017
RPL31	0.0e+00	-0.1426997	1.000	0.998	0.0001340
CD160	0.0e+00	4.6296720	0.042	0.002	0.0001448
NT5E	0.0e+00	4.1692640	0.042	0.002	0.0001452
TRAT1	0.0e+00	-1.2378085	0.135	0.242	0.0001694
RPL35A	0.0e+00	-0.1498209	0.999	0.999	0.0001911
RPL32	0.0e+00	-0.1349986	1.000	1.000	0.0001959

MAL	0.0e+00	-1.0983742	0.149	0.257	0.0001977
MATK	0.0e+00	1.5714195	0.092	0.026	0.0002149
CD40LG	0.0e+00	-1.9356543	0.020	0.087	0.0002240
TGFBR3	0.0e+00	3.2149127	0.047	0.004	0.0003054
CLIC3	0.0e+00	2.7202513	0.054	0.008	0.0004215
JUN	0.0e+00	0.5742398	0.681	0.588	0.0004373
KLRC4	0.0e+00	3.3549690	0.048	0.005	0.0005192
RPS23	0.0e+00	-0.1485187	1.000	1.000	0.0005639
TMSB10	0.0e+00	-0.1783588	0.996	0.999	0.0006274
IFRD1	0.0e+00	0.7876254	0.303	0.182	0.0006301
ICOS	1.0e-07	-1.8295856	0.035	0.107	0.0009510
MT-ATP6	1.0e-07	0.2791020	0.932	0.902	0.0009630
XCL2	1.0e-07	5.1473347	0.035	0.001	0.0009734
LITAF	1.0e-07	0.6455639	0.407	0.277	0.0010564
KLRC2	1.0e-07	4.5769235	0.038	0.002	0.0011382
LAG3	1.0e-07	2.6152771	0.054	0.009	0.0012129
KLRG1	1.0e-07	1.8532056	0.103	0.037	0.0013752
IFNG	1.0e-07	3.4727589	0.051	0.008	0.0013816
RPS15A	1.0e-07	-0.1390132	1.000	1.000	0.0014859
MT1F	1.0e-07	1.8145433	0.086	0.026	0.0015767
S100B	1.0e-07	2.0010809	0.105	0.038	0.0018264
LCP1	1.0e-07	0.7192442	0.312	0.198	0.0021165
MT-CYB	1.0e-07	0.2155921	0.959	0.958	0.0021248
GZMB	1.0e-07	4.7551476	0.037	0.002	0.0021433
HLA-DPB1	2.0e-07	0.9458630	0.210	0.113	0.0028339
MAP3K8	2.0e-07	2.3308513	0.058	0.012	0.0037843
STK17A	3.0e-07	0.6044699	0.426	0.306	0.0049752
ZFP36	3.0e-07	0.6981065	0.394	0.276	0.0052431
A1BG	3.0e-07	0.9879067	0.147	0.068	0.0052530
ACTN4	3.0e-07	1.6884121	0.074	0.021	0.0057168
GZMM	3.0e-07	0.5590226	0.354	0.231	0.0057598
RPL13A	3.0e-07	-0.1027637	1.000	1.000	0.0062383
ABCB1	4.0e-07	2.0901339	0.057	0.012	0.0067877



DUSP2	4.0e-07	1.0251535	0.319	0.213	0.0069206
ARPC5L	4.0e-07	0.8444989	0.173	0.087	0.0071525
CORO1B	4.0e-07	-1.2457970	0.122	0.209	0.0072482
C12orf75	5.0e-07	0.9457620	0.200	0.111	0.0086192
RPL18	5.0e-07	-0.1498451	0.982	0.988	0.0088652
RPS8	5.0e-07	-0.1596945	1.000	0.999	0.0091967
B2M	5.0e-07	0.1532571	1.000	1.000	0.0095932
MT-ND1	5.0e-07	0.2998300	0.843	0.791	0.0098249
RPL37	6.0e-07	-0.1315823	0.999	0.999	0.0103264
TSPAN32	6.0e-07	1.2568587	0.123	0.054	0.0103422
GPR183	6.0e-07	-0.9248264	0.116	0.209	0.0103973
CCR7	6.0e-07	-0.8769143	0.244	0.343	0.0113471
SRGN	6.0e-07	0.7042011	0.467	0.360	0.0117323
RPL5	7.0e-07	-0.1568235	0.983	0.989	0.0121481
RPL38	7.0e-07	-0.1604085	0.989	0.993	0.0123553
CYBA	7.0e-07	0.4154226	0.680	0.582	0.0136535
DUSP1	8.0e-07	0.5137168	0.688	0.582	0.0155765
TBX21	9.0e-07	3.9258636	0.033	0.002	0.0168414
XCL1	9.0e-07	4.2021279	0.033	0.002	0.0169279
LINC00152	1.1e-06	1.4174424	0.102	0.041	0.0197132
HLA-A	1.1e-06	0.3019478	0.891	0.845	0.0198546
NSMAF	1.1e-06	1.5192786	0.067	0.019	0.0207285
SCCPDH	1.2e-06	1.6248603	0.068	0.020	0.0218906
RPS17	1.2e-06	-0.1449256	0.999	0.999	0.0226365
NCR3	1.4e-06	1.5189519	0.074	0.023	0.0254304
HLA-C	1.8e-06	0.2515121	0.907	0.859	0.0341751
TSPYL2	1.9e-06	0.5852828	0.269	0.168	0.0349308
AP3M2	2.0e-06	-1.6253908	0.071	0.143	0.0370676
PRR5	2.1e-06	1.9492113	0.075	0.025	0.0399115
RPL36A	2.3e-06	-0.2223425	0.989	0.990	0.0436935
FCRL6	2.5e-06	6.1868906	0.024	0.000	0.0464487
ADTRP	2.5e-06	-2.8470196	0.010	0.053	0.0466904
BIRC3	2.6e-06	-1.4864265	0.071	0.143	0.0489079

For an explanation of the log fold change have a look at `?Seurat::FindMarkers`. At **Value** it says:

> `avg_logFC`: log fold-chage of the average expression between the two groups. Positive values indicate that the gene is more highly expressed in the first group

To view CD8A, CD8B and CD4:

Code ends here

```
deg_cd8_cd4[c("CD4", "CD8A", "CD8B"),]

            p_val avg_log2FC pct.1 pct.2    p_val_adj
CD4  2.290800e-14  -3.324777 0.011 0.105 4.277611e-10
CD8A 2.889582e-74   5.803662 0.336 0.008 5.395717e-70
CD8B 3.756143e-34   1.312354 0.470 0.178 7.013846e-30
```

Code ends here

Indeed, because we compared ident.1 = “CD8+ T cells” to ident.2 = “CD4+ T cells”, a negative log2FC for the CD4 gene indicates a lower expression in CD8+ T-cells than in CD4+ T-cells, while a positive log2FC for the CD8A and CD8B genes indicates a higher expression in CD8+ T-cells.

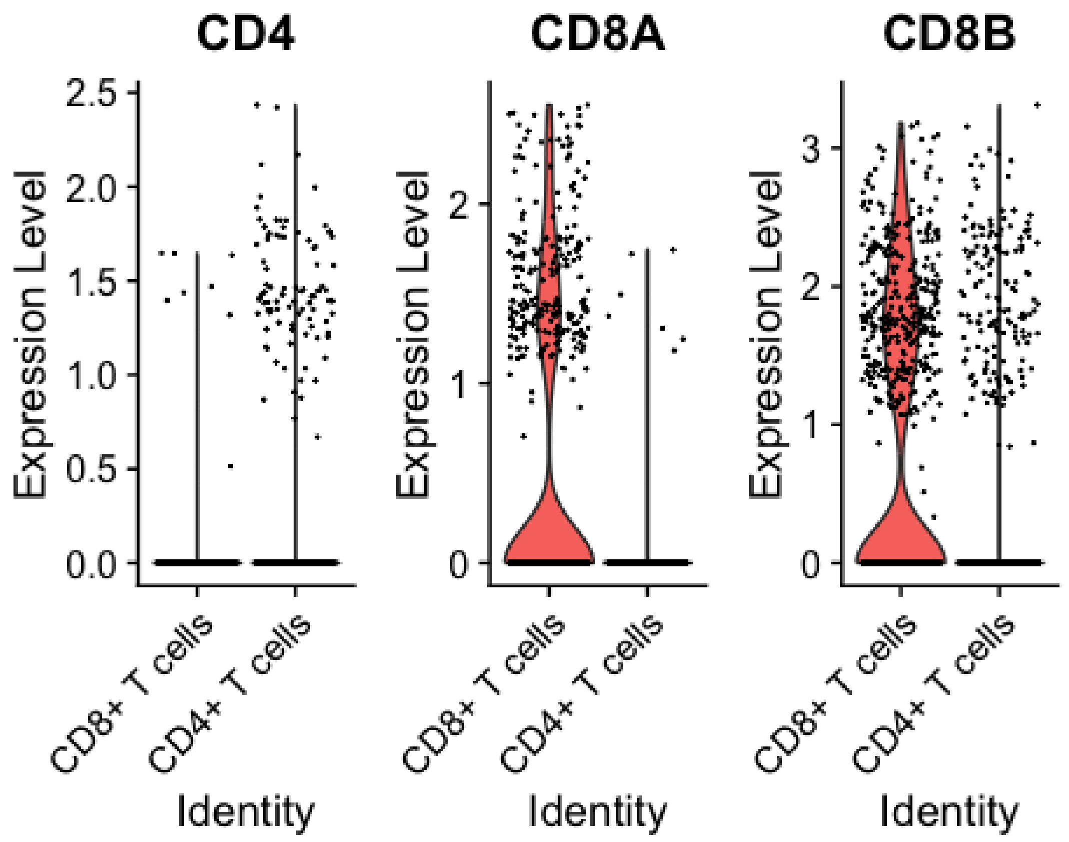
Plotting the genes in these two T-cell groups only:

Code ends here

```
Seurat::VlnPlot(seu,
            features = c("CD4", "CD8A", "CD8B"),
            idents = c("CD8+ T cells", "CD4+ T cells"))

Warning: The `slot` argument of `FetchData()` is deprecated as of SeuratOb
ject 5.0.0.
ℹ Please use the `layer` argument instead.
ℹ The deprecated feature was likely used in the Seurat package.
  Please report the issue at <https://github.com/satijalab/seurat/issues>.
```

Code ends here



Differential expression using `limma`

The Wilcoxon test implemented in `FindMarkers` does not allow you to test for complex design (eg factorial experiments) or to include batch as a covariate. It doesn't allow you to run paired-sample T tests for example.

For more complex designs, we can use `edgeR` or `limma` which are designed for microarray or bulk RNA seq data and provide a design matrix that includes covariates for example, or sample IDs for paired analyses.

We will load an object containing only pro B cells, both from healthy tissues (PBMMC), and malignant tissues (ETV6-RUNX1).

Warning

> Please NOTE that in the original design of this data set, the healthy and malignant tissues were not patient-matched, i.e. the real design was not the one of paired healthy and malignant tissues. However, for demonstration purposes, we will show you how to run a paired analysis, and do as if the PBMMC-1 and ETV6-RUNX1-1 samples both came from the same patient 1, the PBMMC-2 and ETV6-RUNX1-2 samples both came from the same patient 2, etc...

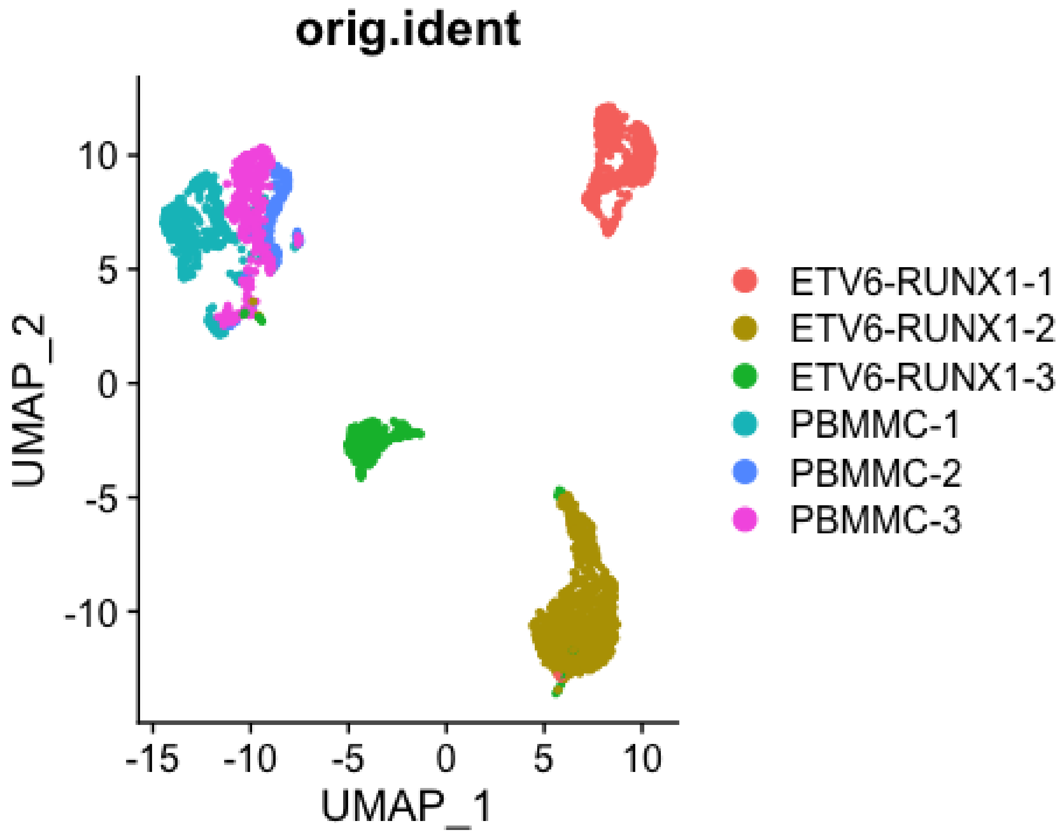
We can load the object and explore its UMAP and meta.data like this:

Code starts here:

```
proB <- readRDS("course_data/proB.rds")

Seurat::DimPlot(proB, group.by = "orig.ident")
```

Code ends here



Code starts here:

```
table(proB@meta.data$type)
```

```
ETV6-RUNX1      PBMMC
      2000       1021

head(proB@meta.data)

                           orig.ident nCount_RNA nFeature_RNA    SingleR_anno
t
PBMMC-1_AAATGCCAGACTGGGT-1    PBMMC-1       4886         1727 Pro-B_cell_CD34
+
PBMMC-1_AAATGCCTCCACTGGG-1    PBMMC-1       8397         2291 Pro-B_cell_CD34
+
PBMMC-1_AACACGTTCTTGACGA-1    PBMMC-1       3444         1204 Pro-B_cell_CD34
+
PBMMC-1_AACCATGAGAAGGTGA-1    PBMMC-1       8981         2437 Pro-B_cell_CD34
+
PBMMC-1_AACCGCGCATGGTCAT-1    PBMMC-1       3719         1368 Pro-B_cell_CD34
+
PBMMC-1_AAGCCGCCAGACGTAG-1    PBMMC-1       4573         1464 Pro-B_cell_CD34
+
                            type
PBMMC-1_AAATGCCAGACTGGGT-1 PBMMC
PBMMC-1_AAATGCCTCCACTGGG-1 PBMMC
PBMMC-1_AACACGTTCTTGACGA-1 PBMMC
PBMMC-1_AACCATGAGAAGGTGA-1 PBMMC
PBMMC-1_AACCGCGCATGGTCAT-1 PBMMC
PBMMC-1_AAGCCGCCAGACGTAG-1 PBMMC
```

Code ends here

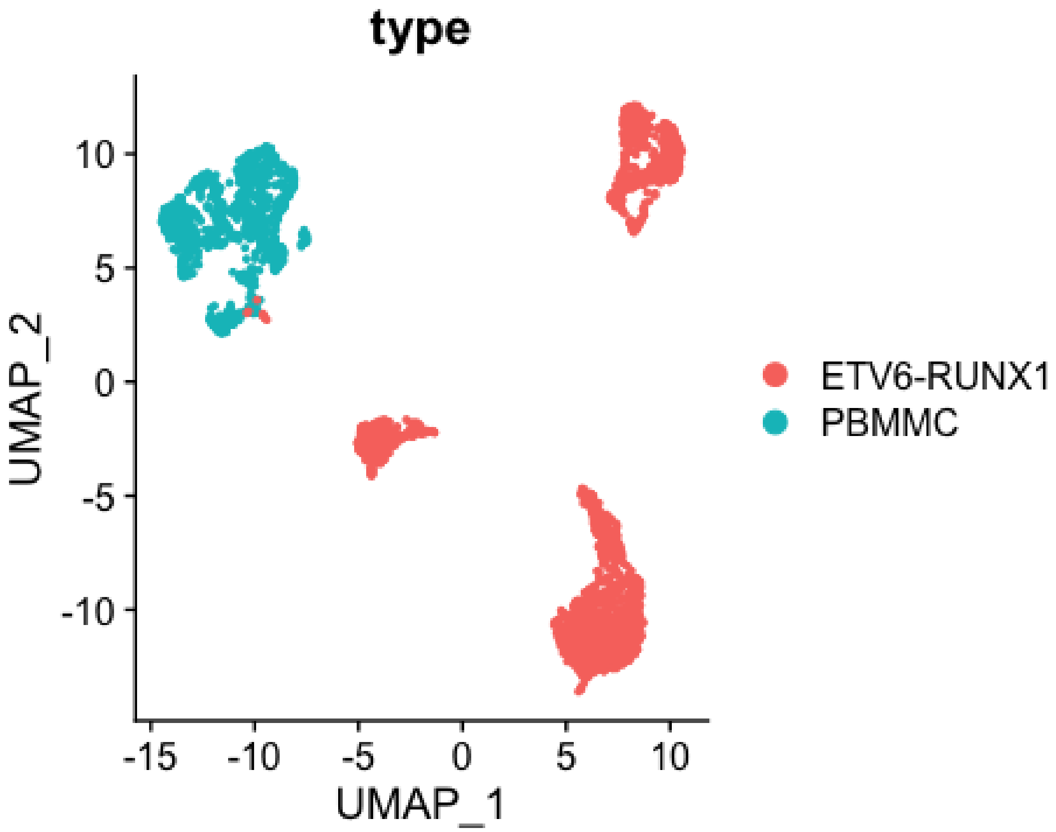
> Note
>
> If you want to know how this pro-B cell subset is generated, have a look at the script here.

Let's have a look at the UMAP (again), coloured by celltype:

Code starts here:

```
Seurat::DimPlot(proB, group.by = "type")
```

Code ends here



Let’s say we are specifically interested to test for differential gene expression between the tumor and normal samples.

> Note
>
> Here we could also test for e.g. healthy versus diseased within a celltype/cluster.

Now we will run differential expression analysis between tumor and healthy cells using the patient ID as a covariate by using `limma`.

Prepare the pseudobulk count matrix:

Code starts here:

```
#taking the proB data
Seurat::DefaultAssay(proB) <- "RNA"
Seurat::Idents(proB) <- proB$orig.ident

## add the patient id also for paired DGE
proB$patient.id<-gsub("ETV6-RUNX1", "ETV6_RUNX1", proB$orig.ident)
proB$patient.id<-sapply(strsplit(proB$patient.id, "-"), '[', 2)
```

```
## Here we do perform pseudo-bulk:
##first a mandatory column of sample needs to be added to the meta data that
is the grouping factor, should be the samples
proB$sample <- factor(proB$orig.ident)

# aggergate the cells per sampple
bulk <- Seurat::AggregateExpression(proB, group.by = "sample",
                                    return.seurat = TRUE,
                                    assay = "RNA")

# create a metadata data frame based on the aggregated cells
meta_data <- unique(proB@meta.data[, c("orig.ident",
                                            "sample", "type",
                                            "patient.id")])
rownames(meta_data) <- meta_data$orig.ident
bulk@meta.data <- meta_data[colnames(bulk), ]

##have a look at the counts
counts <- Seurat::GetAssayData(bulk, layer = "counts") |> as.matrix()

head(counts)

              ETV6-RUNX1-1 ETV6-RUNX1-2 ETV6-RUNX1-3 PBMMC-1 PBMMC-2 PBMMC-3
RP11-34P13.7             0            0            0       2       0       0
FO538757.3               0            0            0       0       0       0
FO538757.2             138          275           74     129      40     112
AP006222.2              63           43           17      38      19      26
RP4-669L17.10            5           10            3       0       1       1
RP5-857K21.4             0            0            0       0       0       2

#have a look at the colData of our new object summed, can you see type and
#patient.id are there
head(bulk@meta.data)

               orig.ident       sample       type patient.id
ETV6-RUNX1-1 ETV6-RUNX1-1 ETV6-RUNX1-1 ETV6-RUNX1          1
ETV6-RUNX1-2 ETV6-RUNX1-2 ETV6-RUNX1-2 ETV6-RUNX1          2
ETV6-RUNX1-3 ETV6-RUNX1-3 ETV6-RUNX1-3 ETV6-RUNX1          3
PBMMC-1           PBMMC-1      PBMMC-1      PBMMC          1
PBMMC-2           PBMMC-2      PBMMC-2      PBMMC          2
PBMMC-3           PBMMC-3      PBMMC-3      PBMMC          3
```

Code ends here

Generate a `DGEList` object to use as input for `limma` and filter the genes to remove lowly expressed genes. How many are left?

Code starts here:

```
#As in the standard limma analysis generate a DGE object
```

```
y <- edgeR::DGEList(counts, samples = bulk@meta.data)

##filter lowly expressed (recommanded for limma)
keep <- edgeR::filterByExpr(y, group = bulk$type)
y <- y[keep,]

##see how many genes were kept
summary(keep)

   Mode   FALSE    TRUE
logical   11086   10017
```

Code ends here

Generate a design matrix, including patient ID to model for a paired analysis. If you need help to generate a design matrix, check out the very nice edgeR User Guide, sections 3.3 and 3.4. Extract the sample ID from the meta.data, then create the design matrix:

Code starts here:

```
## Create the design matrix and include the technology as a covariate:
design <- model.matrix(~0 + y$samples$type + y$samples$patient.id)

# Have a look
design

  y$samples$typeETV6-RUNX1 y$samples$typePBMMC y$samples$patient.id2
1                        1                   0                     0
2                        1                   0                     1
3                        1                   0                     0
4                        0                   1                     0
5                        0                   1                     1
6                        0                   1                     0
  y$samples$patient.id3
1                     0
2                     0
3                     1
4                     0
5                     0
6                     1
attr(,"assign")
[1] 1 1 2 2
attr(,"contrasts")
attr(,"contrasts")$`y$samples$type`
[1] "contr.treatment"

attr(,"contrasts")$`y$samples$patient.id`
[1] "contr.treatment"
```

```
# change column/rownames names to more simple group names:
colnames(design) <- make.names(c("ETV6-RUNX1", "PBMMC","patient2","patient3")
)
rownames(design) <- rownames(y$samples)
```

Code ends here

Specify which contrast to analyse:

Code starts here:

```
contrast.mat <- limma::makeContrasts(ETV6.RUNX1 - PBMMC,
                                     levels = design)
```

Code ends here

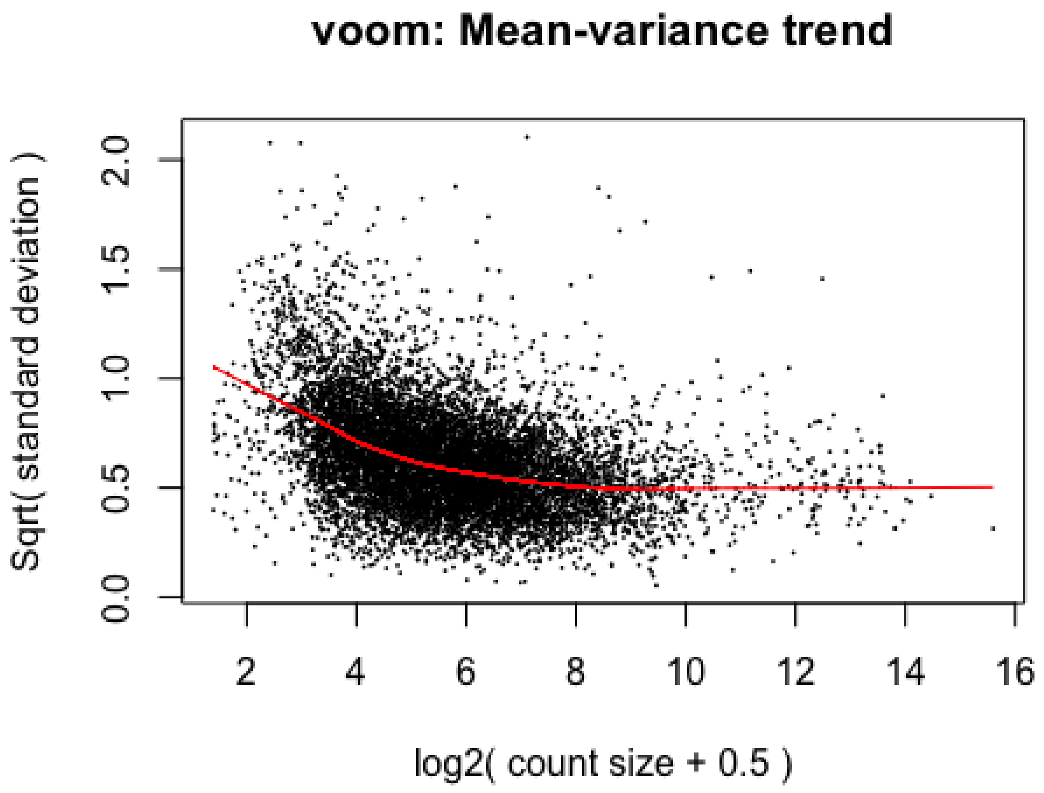
Firt, we perform TMM normalization using edgeR, and then `limma` can perform the transformation with `voom`, fit the model, compute the contrasts and compute test statistics with `eBayes`:

Code starts here:

```
dge <- edgeR::calcNormFactors(y)

#Do limma
vm <- limma::voom(dge, design = design, plot = TRUE)
```

Code ends here



Code starts here:

```
fit <- limma::lmFit(vm, design = design)
fit.contrasts <- limma::contrasts.fit(fit, contrast.mat)
fit.contrasts <- limma::eBayes(fit.contrasts)
```

Code ends here

We can use `topTable` to get the most significantly differentially expressed genes, and save the full DE results to an object. How many genes are significant? Are you suprised by this number?

Code starts here:

```
# Show the top differentially expressed genes:
limma::topTable(fit.contrasts, number = 10, sort.by = "P")

               logFC  AveExpr         t      P.Value    adj.P.Val        B
RPS4Y2      5.346800 6.347826  15.39674 3.361453e-08 0.0001152727 9.477129
SDC2        9.070465 2.708711  15.33434 3.493119e-08 0.0001152727 7.681010
IGLL1      -3.788160 9.287148 -15.19483 3.808426e-08 0.0001152727 9.465422
CTGF        4.368363 6.029640  14.89301 4.603081e-08 0.0001152727 9.141505
AP005530.2  8.770808 2.560369  14.27130 6.879238e-08 0.0001335825 7.294079
GNG11       3.500250 6.457777  13.70183 1.008304e-07 0.0001335825 8.495288
```

```
HLA-DQA1     2.982748 7.410939  13.39202 1.249067e-07 0.0001335825 8.287394
PTP4A3       3.734865 5.449894  13.23439 1.395246e-07 0.0001335825 8.126807
CD27         4.115561 5.843582  13.23011 1.399471e-07 0.0001335825 8.147433
ALOX5        4.142010 5.682900  13.16658 1.463814e-07 0.0001335825 8.098199
```

```
limma_de <- limma::topTable(fit.contrasts, number = Inf, sort.by = "P")
length(which(limma_de$adj.P.Val<0.05))
```

```
[1] 2738
```

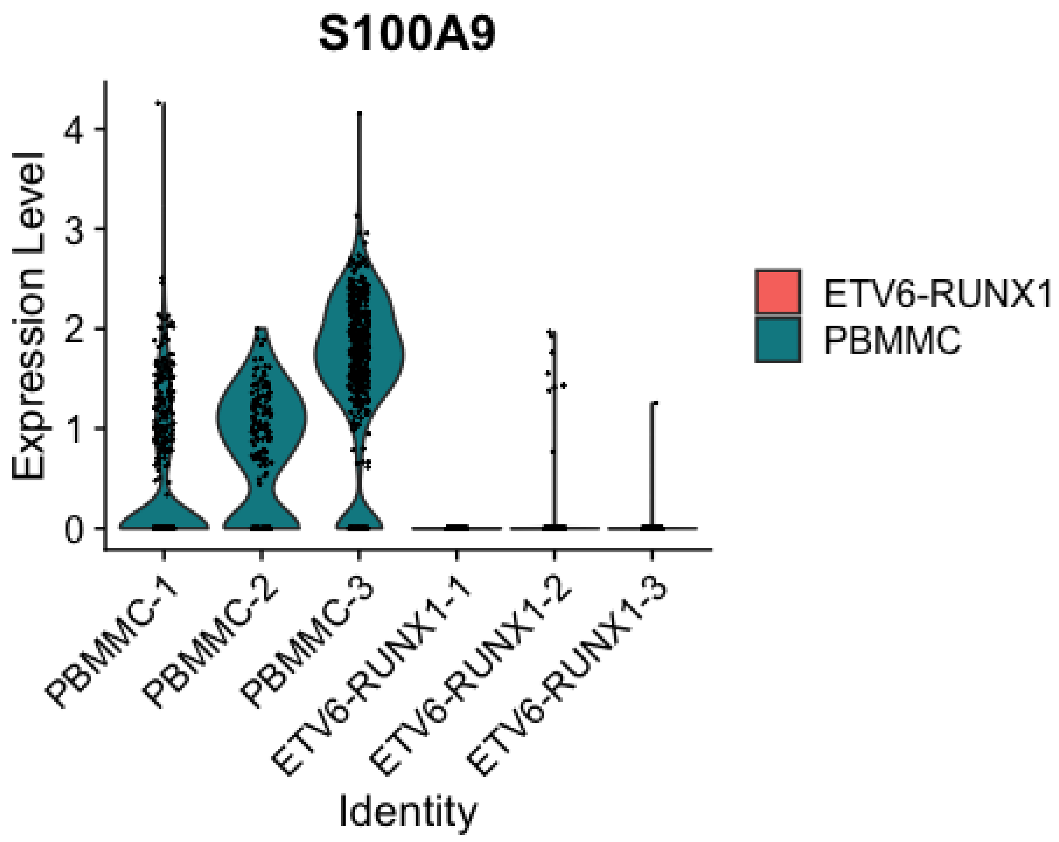
Code ends here

And we can check whether this corresponds to the counts by generating a violin plot, or a gene downregulated in tumor, or a gene upregulated in tumor:

Code starts here:

```
Seurat::VlnPlot(proB, "S100A9", split.by = "type")
```

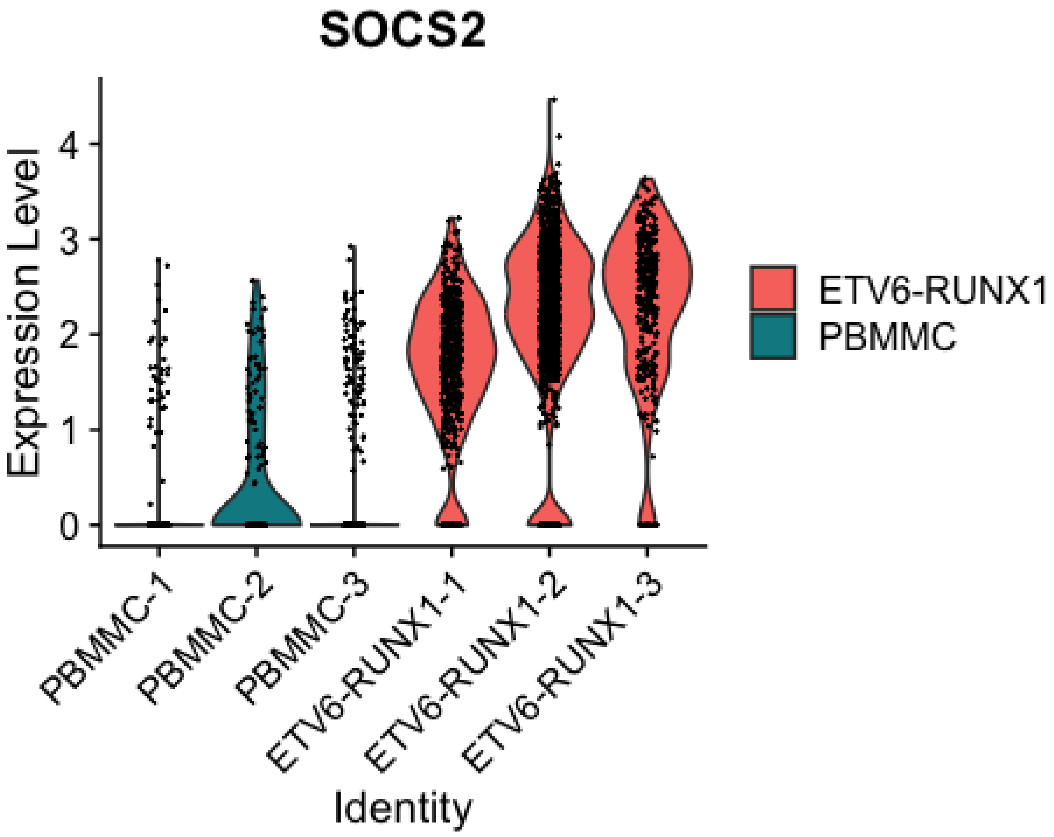
Code ends here



Code starts here:

```
Seurat::VlnPlot(proB, "SOCS2", split.by = "type")
```

Code ends here



We can run a similar analysis with `Seurat`, but this will not take into account the paired design. Run the code below.

Code starts here:

```
tum_vs_norm <- Seurat::FindMarkers(proB,
                                   ident.1 = "ETV6-RUNX1",
                                   ident.2 = "PBMMC",
                                   group.by = "type")
tum_vs_norm <- subset(tum_vs_norm, tum_vs_norm$p_val_adj<0.05)
```

Code ends here

Exercise (extra)

How many genes are significant? How does the fold change of these genes compare to the fold change of the top genes found by limma?

Answer

Code starts here:

```
dim(tum_vs_norm)

[1] 3820    5
```

Code ends here

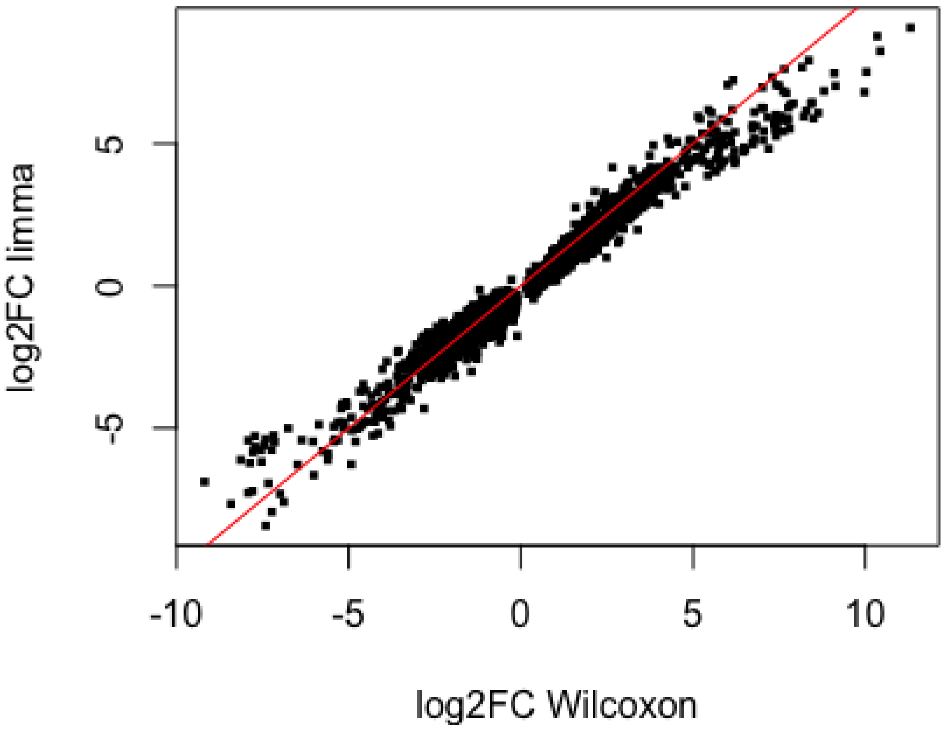
We find 3820 significant genes. If we merge the `FindMarkers` and the `limma` results, keep `limma`’s most significant genes and plot:

Code starts here:

```
merge_limma_FindMarkers <- merge(tum_vs_norm, limma_de, by="row.names",
                           all.x=T)


par(mar=c(4,4,4,4))
plot(merge_limma_FindMarkers$avg_log2FC,
    merge_limma_FindMarkers$logFC,
    xlab="log2FC Wilcoxon", ylab="log2FC limma",
    pch=15, cex=0.5)
abline(a=0, b=1, col="red")
```

Code ends here



Keep the object

Keep the `tum_vs_norm` and `limma_de` objects because we will use this output later for the enrichment analysis in the next section.