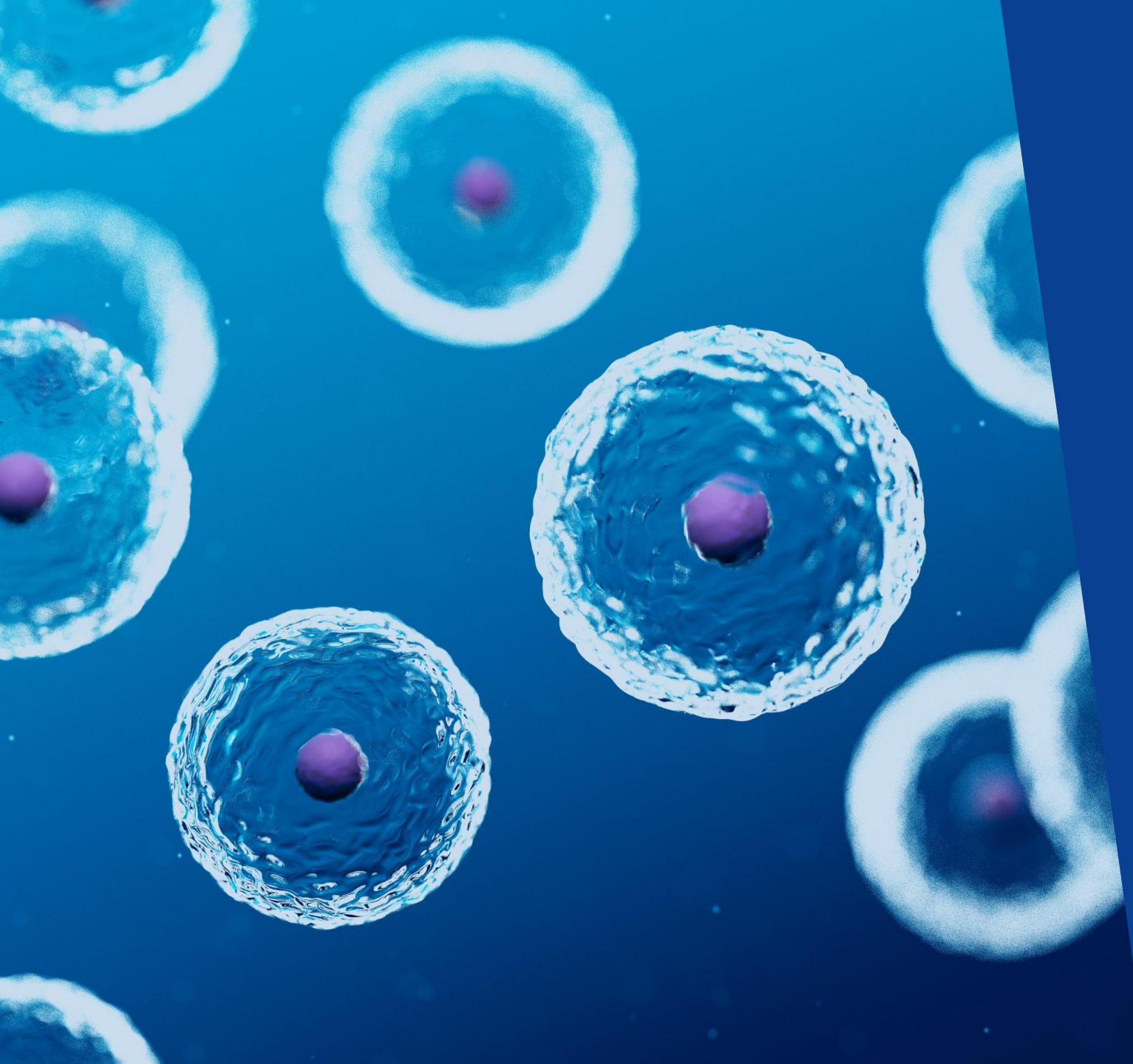

Analysis of a single-cell RNA-seq dataset

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December 2021

UCSF
University of California
San Francisco

GENERAL INFORMATION

- N = 28,700 cells from 4 murine mammary tumors.
- Information on the expression of > 20,000 genes per cell.
- Single cells were dissociated from mouse mammary gland tumors, immuno-stained and sorted on the CD45+ marker.
- In 2 samples, single cells were dissociated with enzymatic digestion and in 2 samples with the SimpleFlowTM System.
- Single cell RNA-seq was performed with 10×Genomics.

THE GOALS

Prepare a pipeline to analyze single-cell RNA-seq data for current and future use in our lab

Test whether there are differences between samples dissociated with distinct methodologies

(enzymatic digestion versus SimpleFlow)

INDEX

01

Quality control & cell filtering

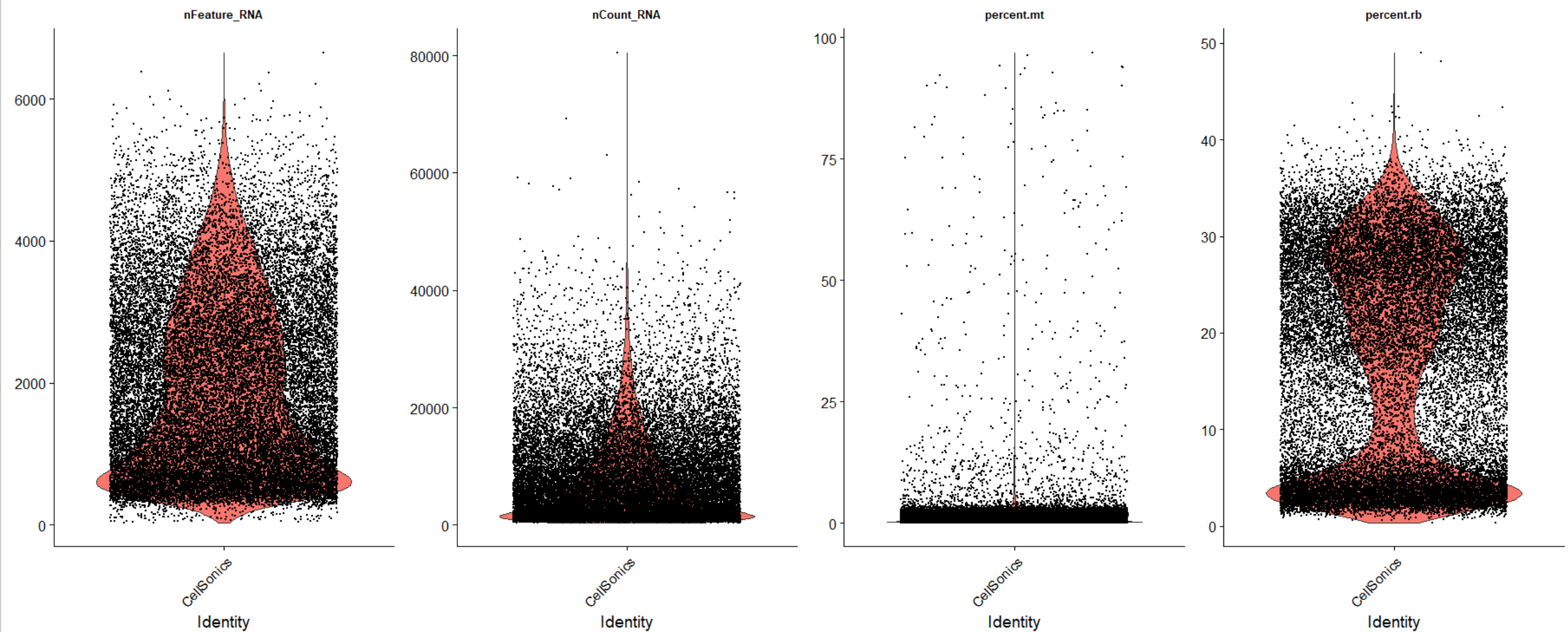
1. Import to R the output of 10X cell ranger (filtered_feature_bc_matrix)

2. Create a Seurat object

3. Calculate quality control metrics:
   - nCount RNA (UMI reads/cell)
   - nFeature RNA (number of detected genes/cell)
   - % mitochondrial genes
   - % ribosomal genes

(# detected genes/cell)

(UMI reads/cell)



4. Based on that, filter cells to remove dead cells / empty droplets / doublets

```
> table(srat[['QC']]) #Most cells (26.0007) pass the quality control.

             High_MT High_MT,Low_nFeature                   Pass
                  78                2615                  26007
```

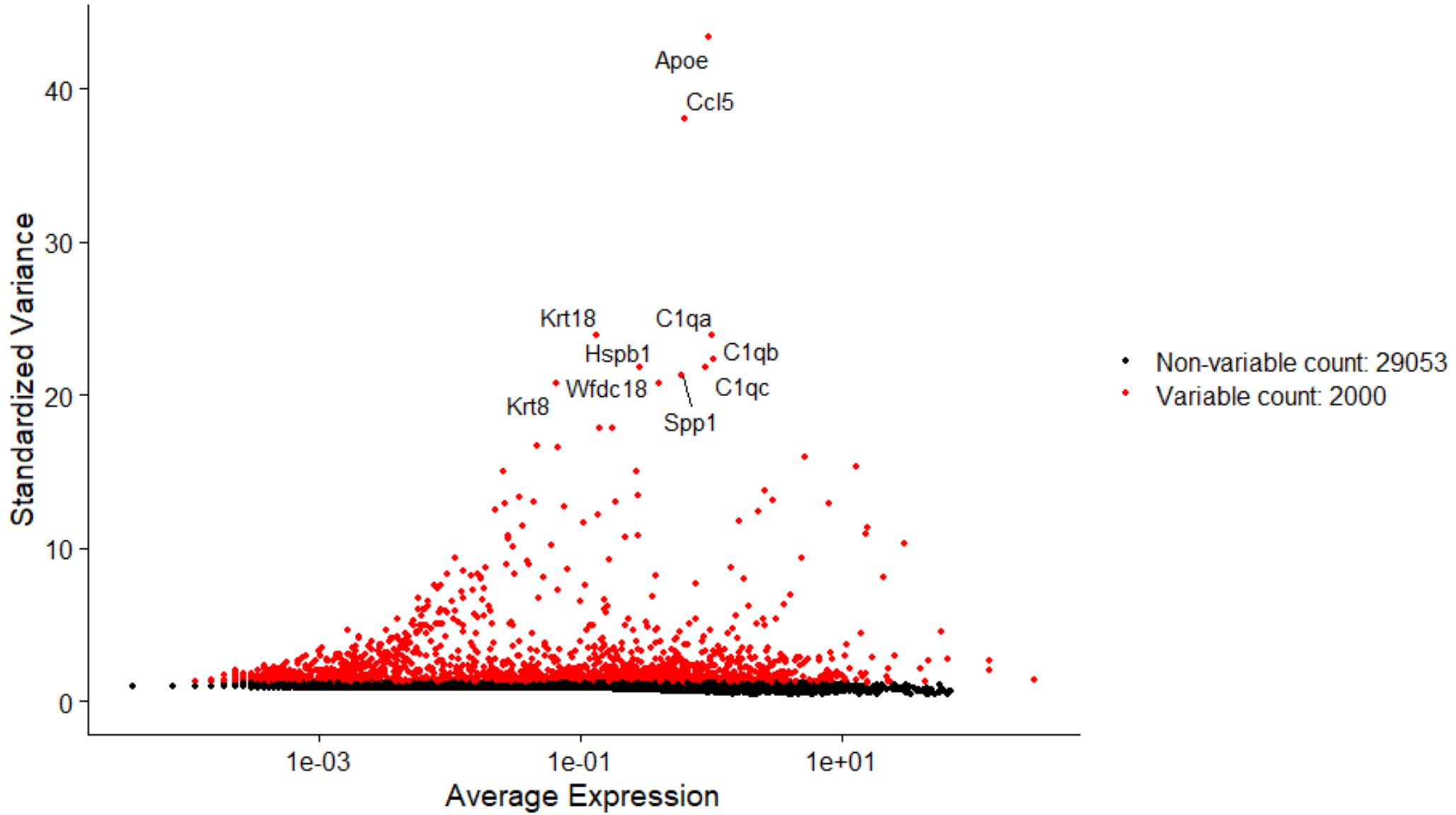
02

Normalization & dimensionality reduction

5. Normalize the data to account for sequencing depth using the NormalizeData() R function

   - Method: Feature counts for each cell are divided by the total counts for that cell and multiplied by the scale.factor (10.000 default). This is then log transformed.

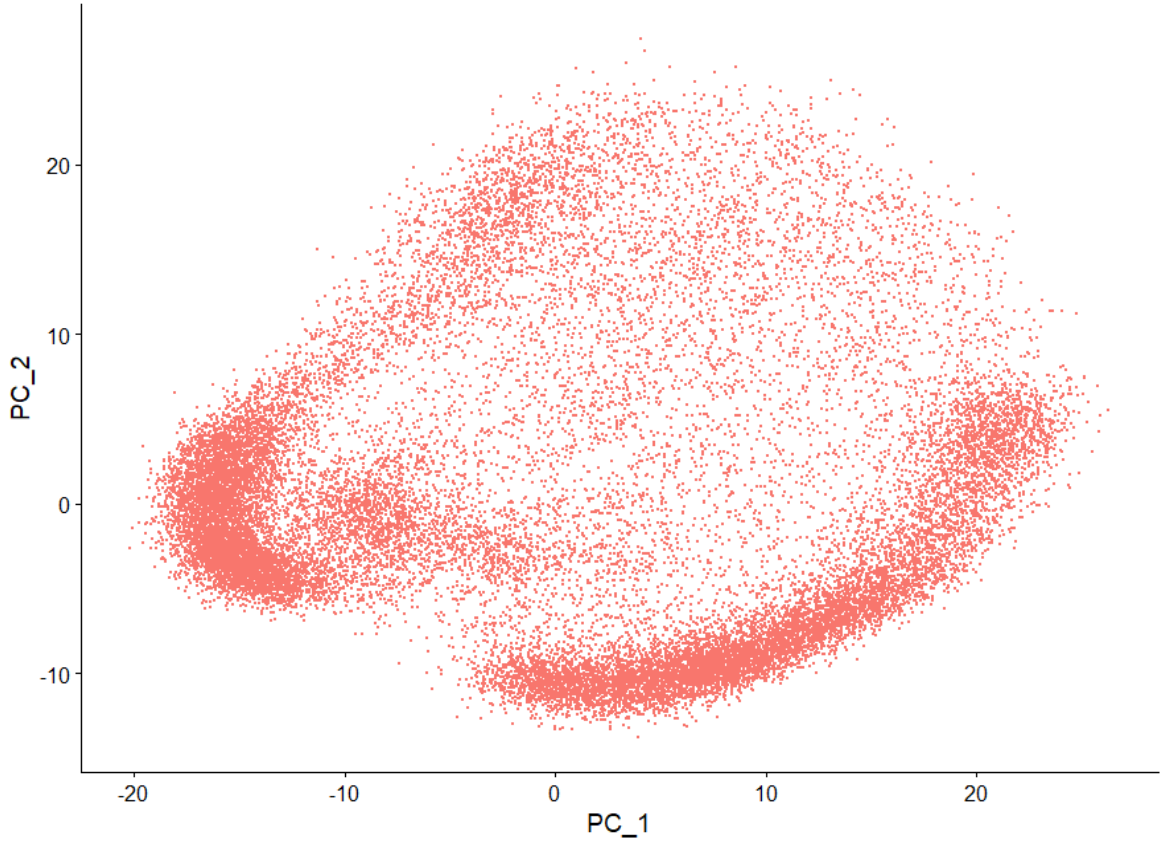
6. Identify the 2.000 genes with the highest variance between samples using the FindVariableFeatures() R function

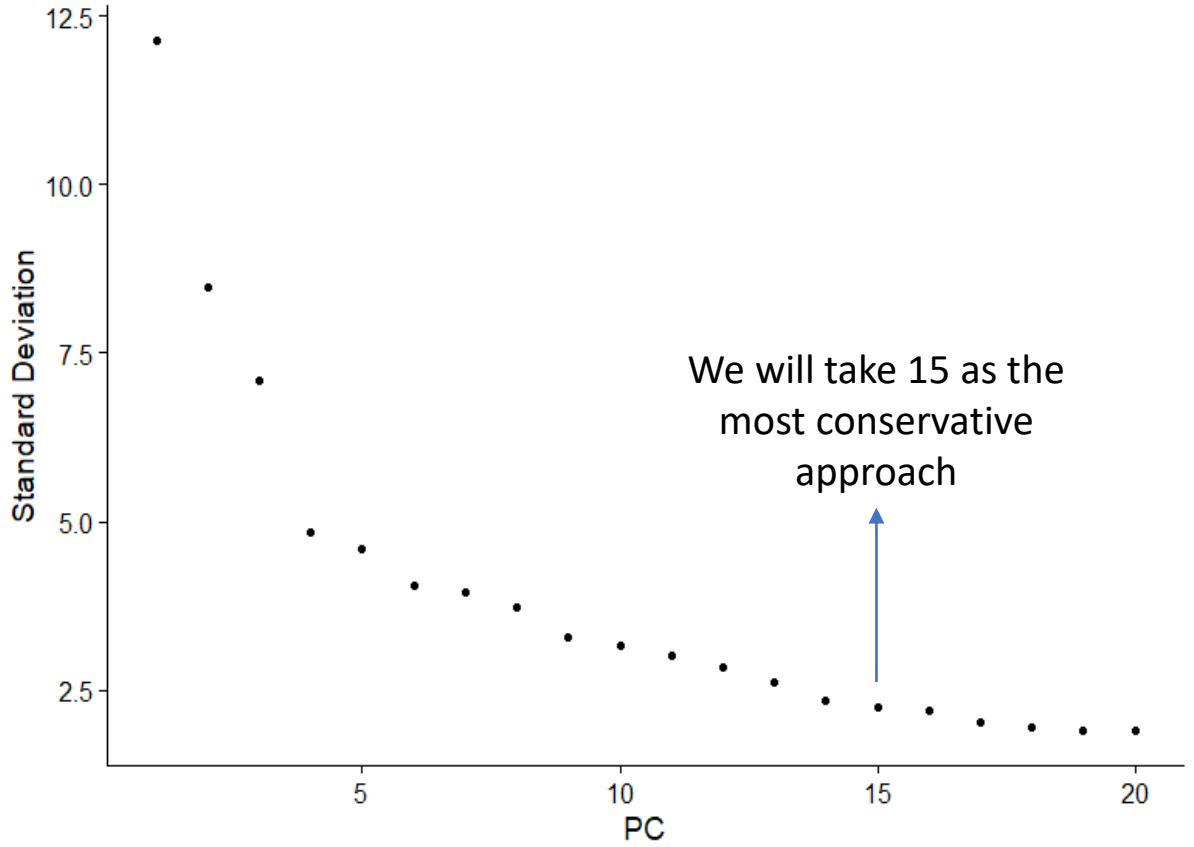

Apoe
Ccl5
Krt18
C1qa
Hspb1
C1qb
Wfdc18
C1qc
Krt8
Spp1
Standardized Variance
40
30
20
10
0
1e-03
1e-01
1e+01
Average Expression
Non-variable count: 29053
Variable count: 2000

7. Scale the data by Z-score transforming the gene expression

8. Perform a Principal Component Analysis (PCA) dimensionality reduction using the 2.000 genes with the highest variance

9. Identify how many Principal Components (PCs) we should use in downstream analyses by ranking the % of variance explained by each of them using the ElbowPlot() R function


PC_2
20
10
0
-10
-20
-10
0
10
20
PC_1

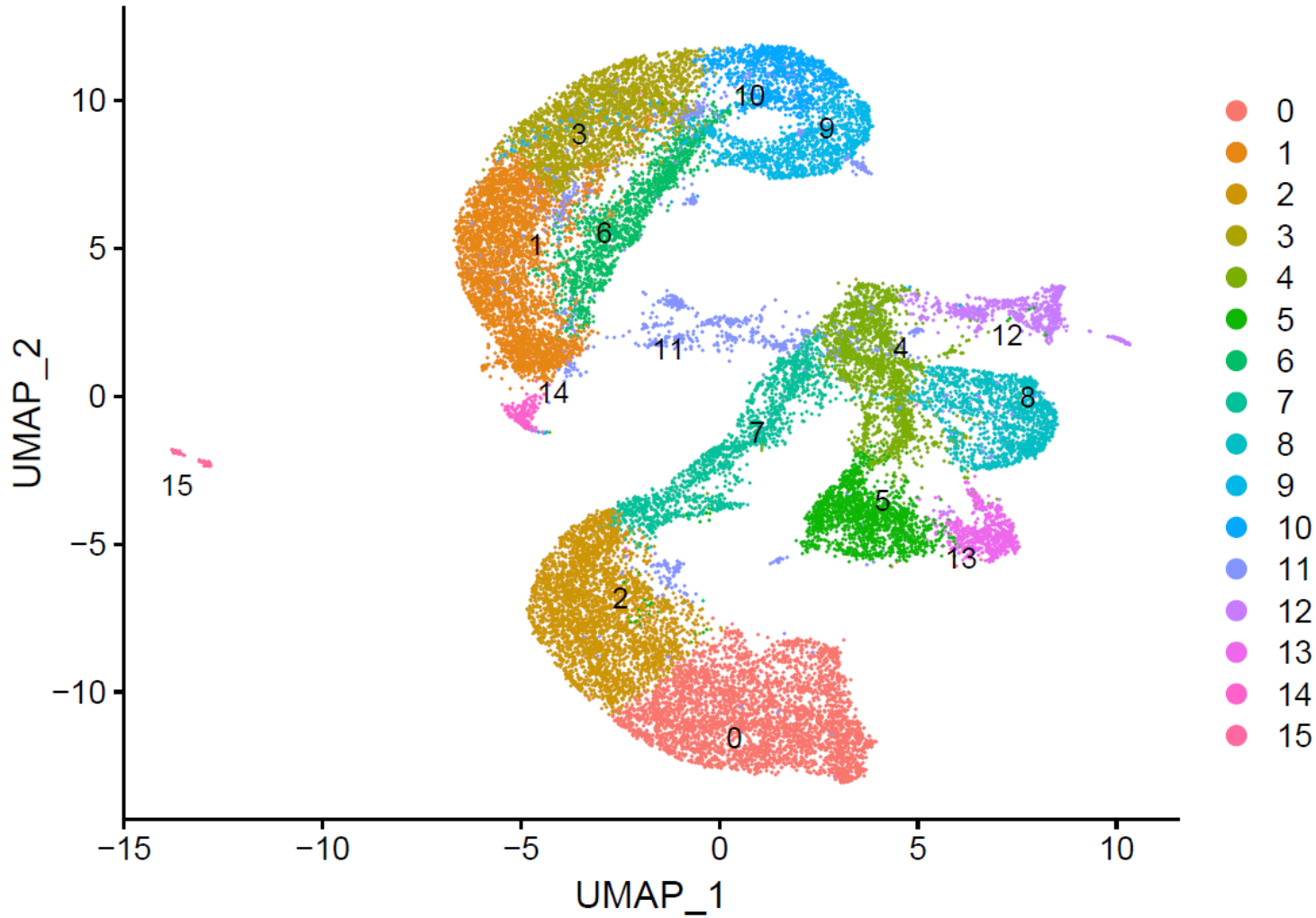

Standard Deviation
12.5
10.0
7.5
5.0
2.5
5
10
15
20
PC
We will take 15 as the most conservative approach

03

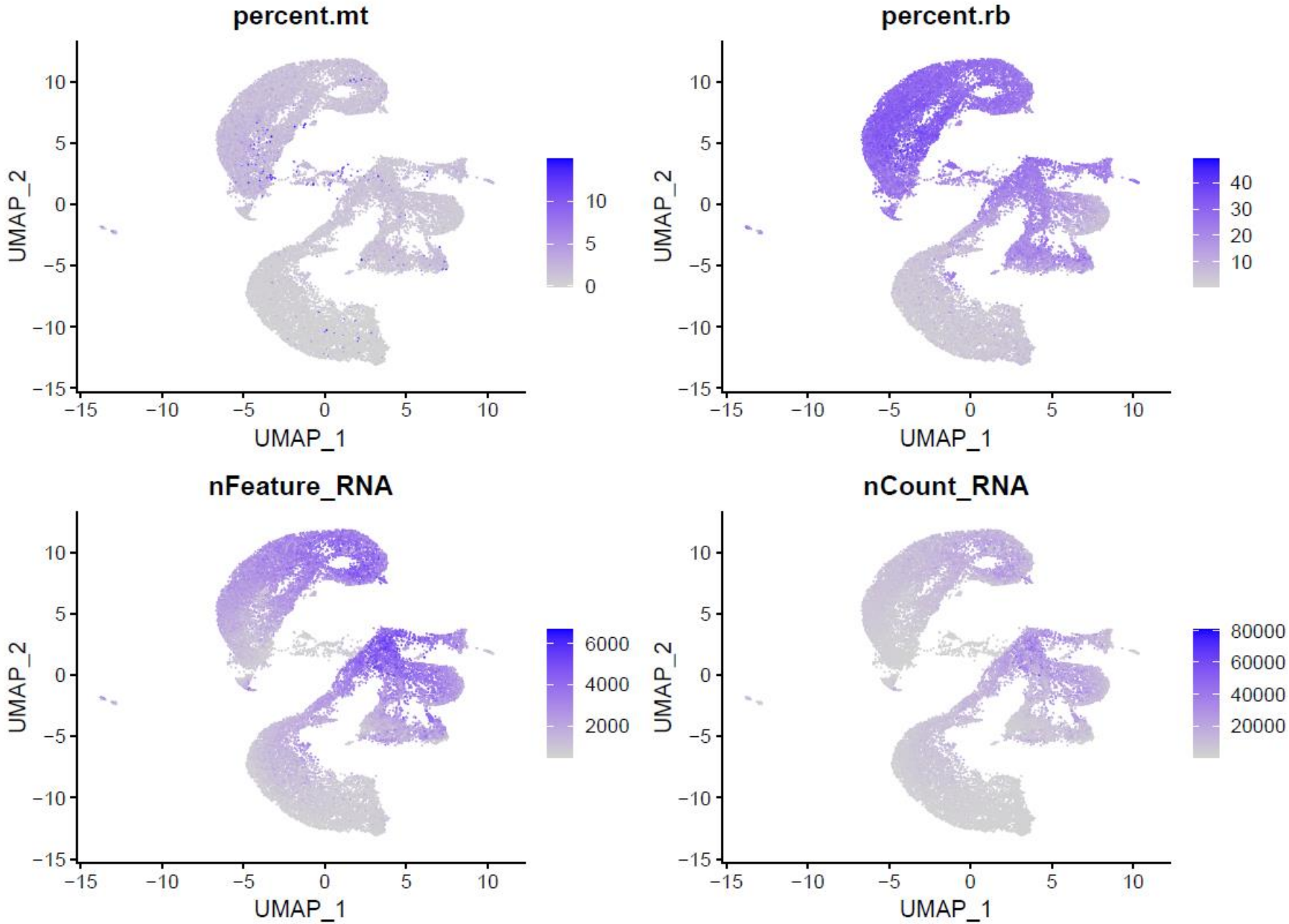
Clustering & artifact control

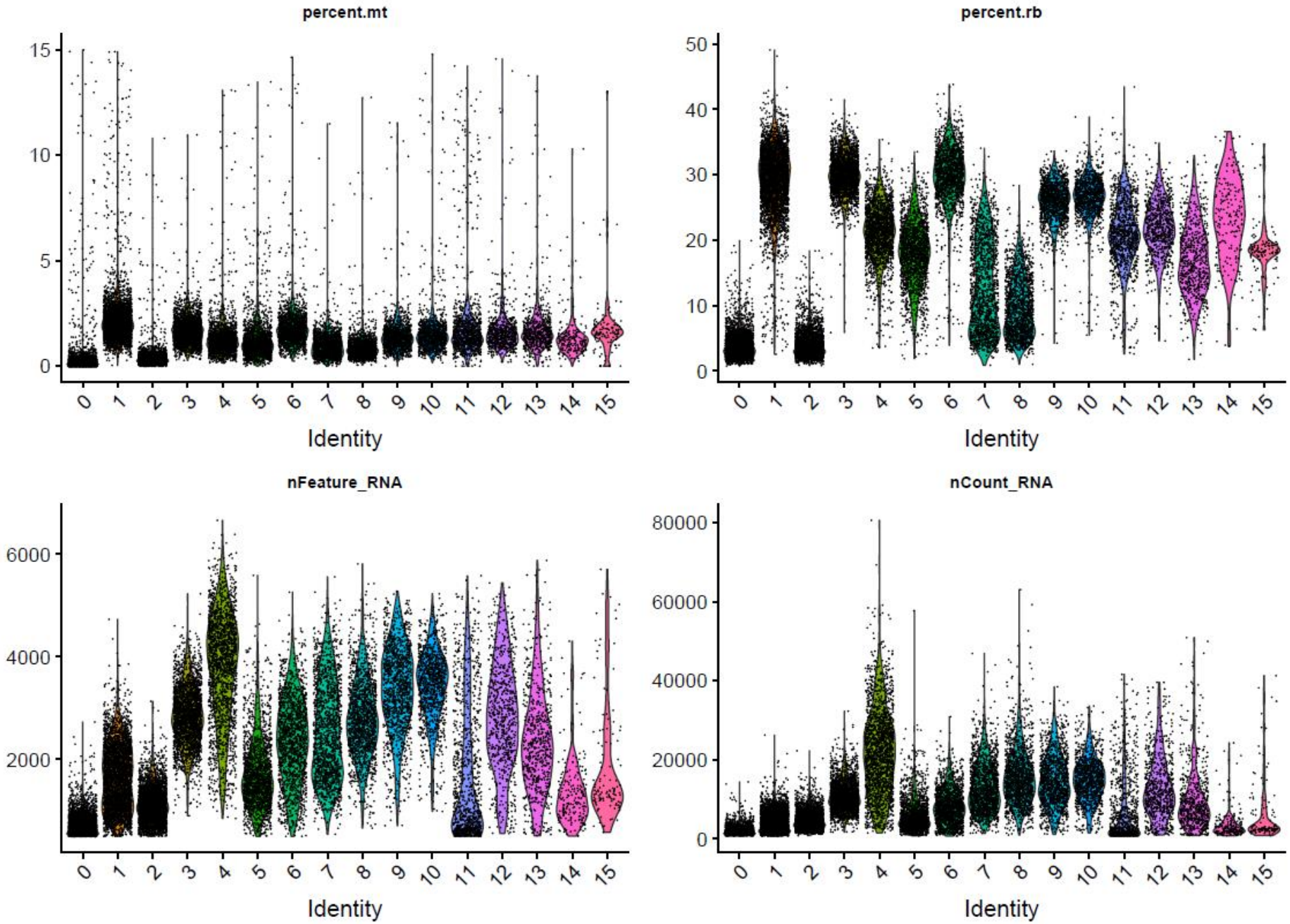
10. Using these number of PCs, perform clustering of the cells using the K-Nearest Neighbors (KNN) algorithm based on the euclidean distance in PCA space with the FindNeighbors() and FindClusters() R functions

11. For visualization purposes, generate UMAP reduced dimensionality representation of the results


UMAP_2
UMAP_1
10
5
0
−5
−10
−15
−10
−5
0
5
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

12. In order to control for possible artifacts, compare the distribution within clusters of the quality control (QC) metrics (nCount RNA, nFeature RNA, % mitochondrial genes, % ribosomal genes, +/- cell cycle score)


percent.mt
UMAP_2
UMAP_1
10
5
0
−5
−10
−15
percent.rb
40
30
20
nFeature_RNA
6000
4000
2000
nCount_RNA
80000
60000
40000
20000


percent.mt
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Identity
percent.rb
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Identity
nFeature_RNA
6000
4000
2000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Identity
nCount_RNA
80000
60000
40000
20000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Identity

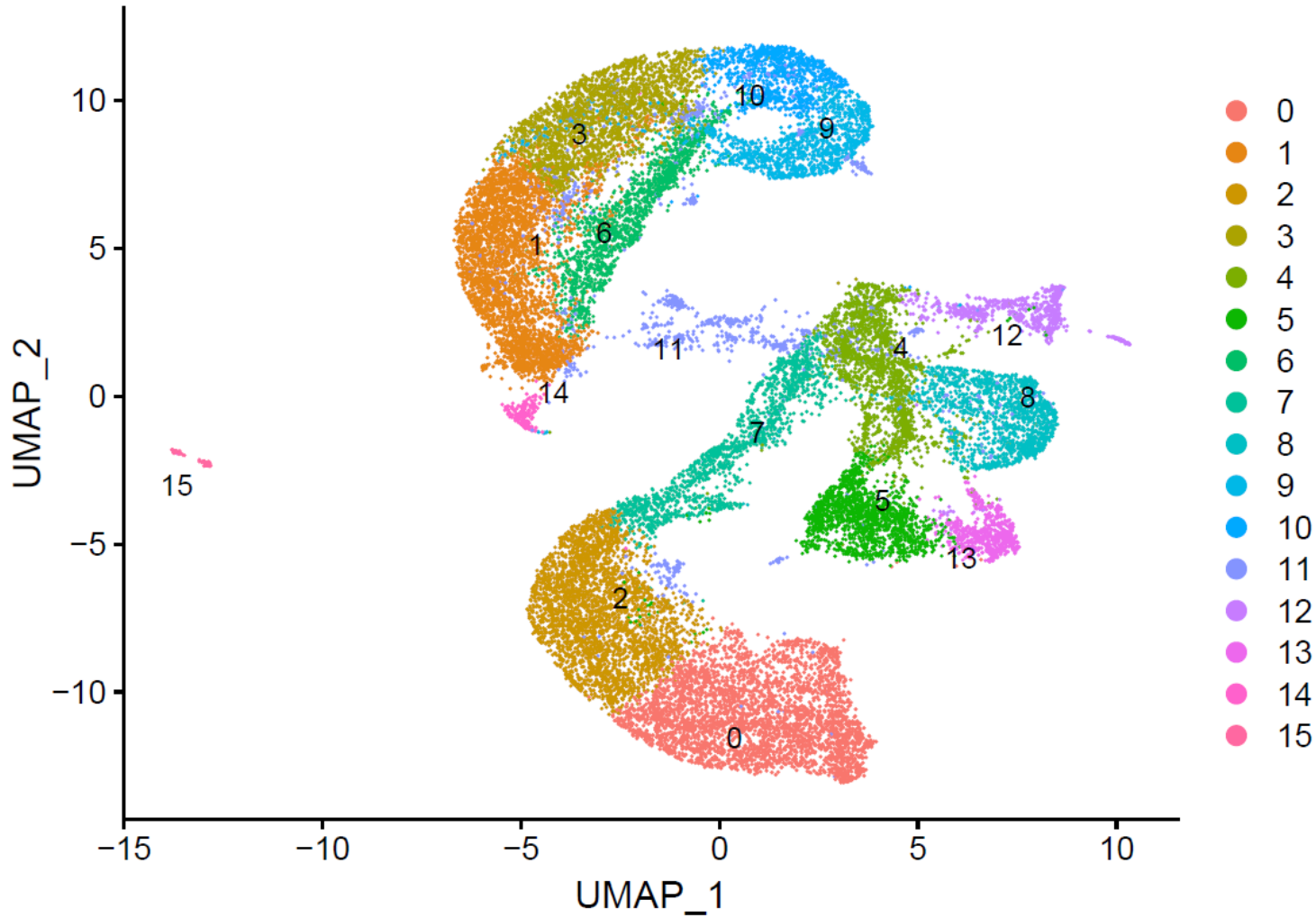
13. Correct for the identified confounding factors that change dramatically between clusters using using vars.to.regres from the SCTransform() R function

    In this case, however, the distribution of none of the metrics seems to be an artifact after consultation with the experts (Ye lab from UCSF), so no correction is needed

04

Marker selection

14. Find markers for every cluster by comparing it to all remaining cells using the Wilcoxon Rank Sum test with the FindAllMarkers() R function


UMAP_2
UMAP_1
10
5
0
−5
−10
−15
−10
−5
0
5
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Top 5 markers of each cluster

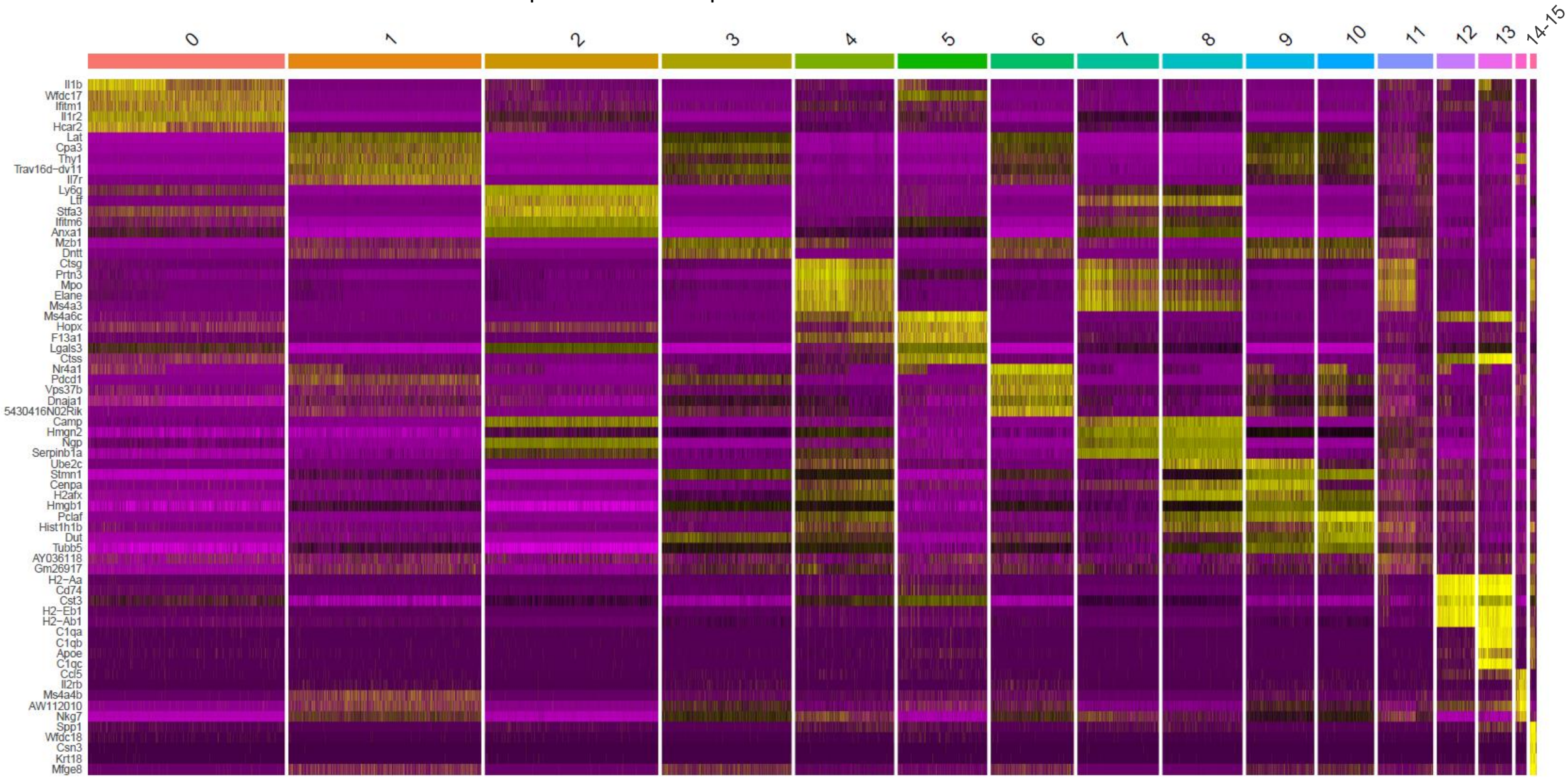
cluster	gene
0	Il1b
0	Wfdc17
0	Ifitm1
0	Il1r2
0	Hcar2
1	Lat
1	Cpa3
1	Thy1
1	Trav16d-dv11
1	Il7r
2	Ly6g
2	Ltf
2	Stfa3
2	Ifitm6
2	Anxa1
3	Lgals1
3	Cpa3
3	Mzb1
3	Dntt
3	Pebp1

cluster	gene
4	Ctsg
4	Prtn3
4	Mpo
4	Elane
4	Ms4a3
5	Ms4a6c
5	Hopx
5	F13a1
5	Lgals3
5	Ctss
6	Nr4a1
6	Pdcd1
6	Vps37b
6	Dnaja1
6	5430416N02Rik
7	Elane
7	Mpo
7	Prtn3
7	Camp
7	Hmgn2

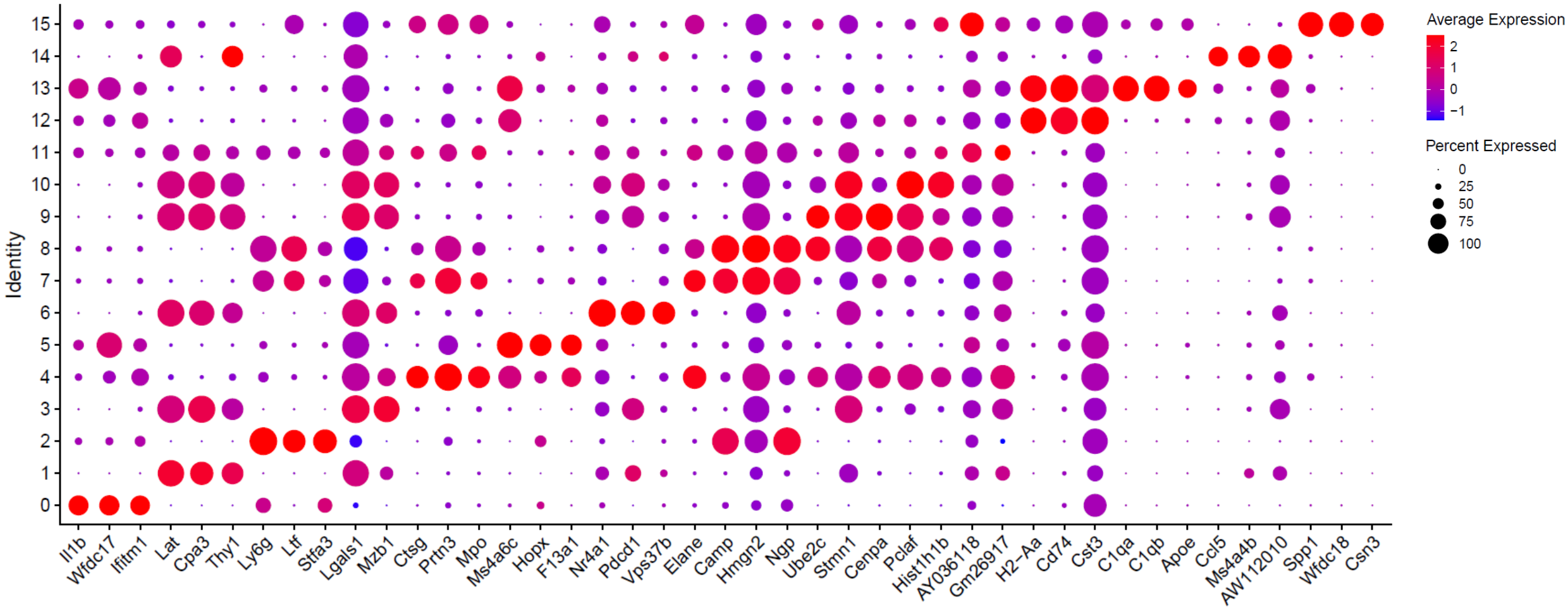
cluster	gene
8	Camp
8	Hmgn2
8	Ngp
8	Serpinb1a
8	Ube2c
9	Ube2c
9	Stmn1
9	Cenpa
9	H2afx
9	Hmgb1
10	Pclaf
10	Stmn1
10	Hist1h1b
10	Dut
10	Tubb5
11	mt-Cytb
11	Elane
11	Prtn3
11	AY036118
11	Gm26917

cluster	gene
12	H2-Aa
12	Cd74
12	Cst3
12	H2-Eb1
12	H2-Ab1
13	C1qa
13	C1qb
13	Apoe
13	C1qc
13	Cd74
14	Ccl5
14	Il2rb
14	Ms4a4b
14	AW112010
14	Nkg7
15	Spp1
15	Wfdc18
15	Csn3
15	Krt18
15	Mfge8

Gene expression heatmap of the main markers across clusters



Gene expression bubble chart of the main markers across clusters



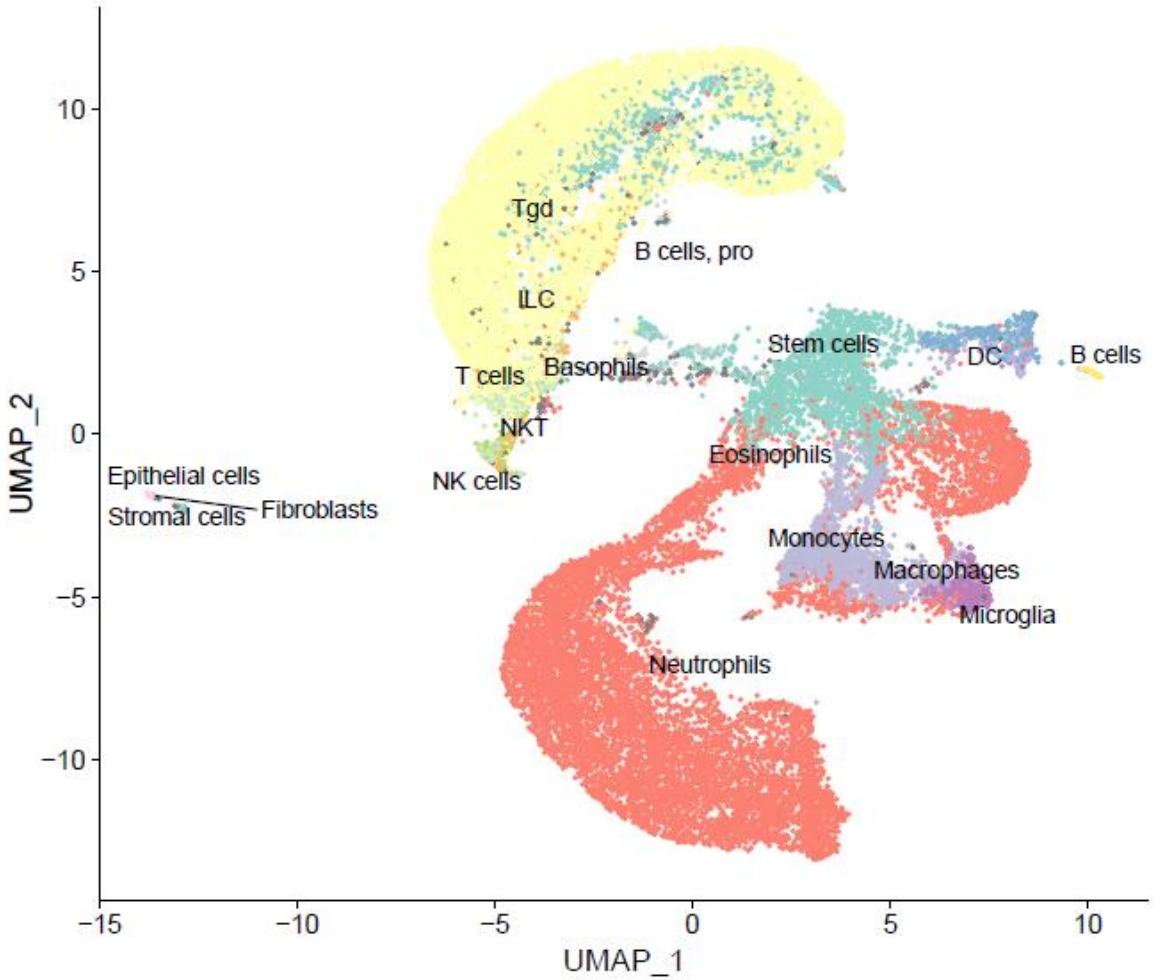
05

Cell type annotation

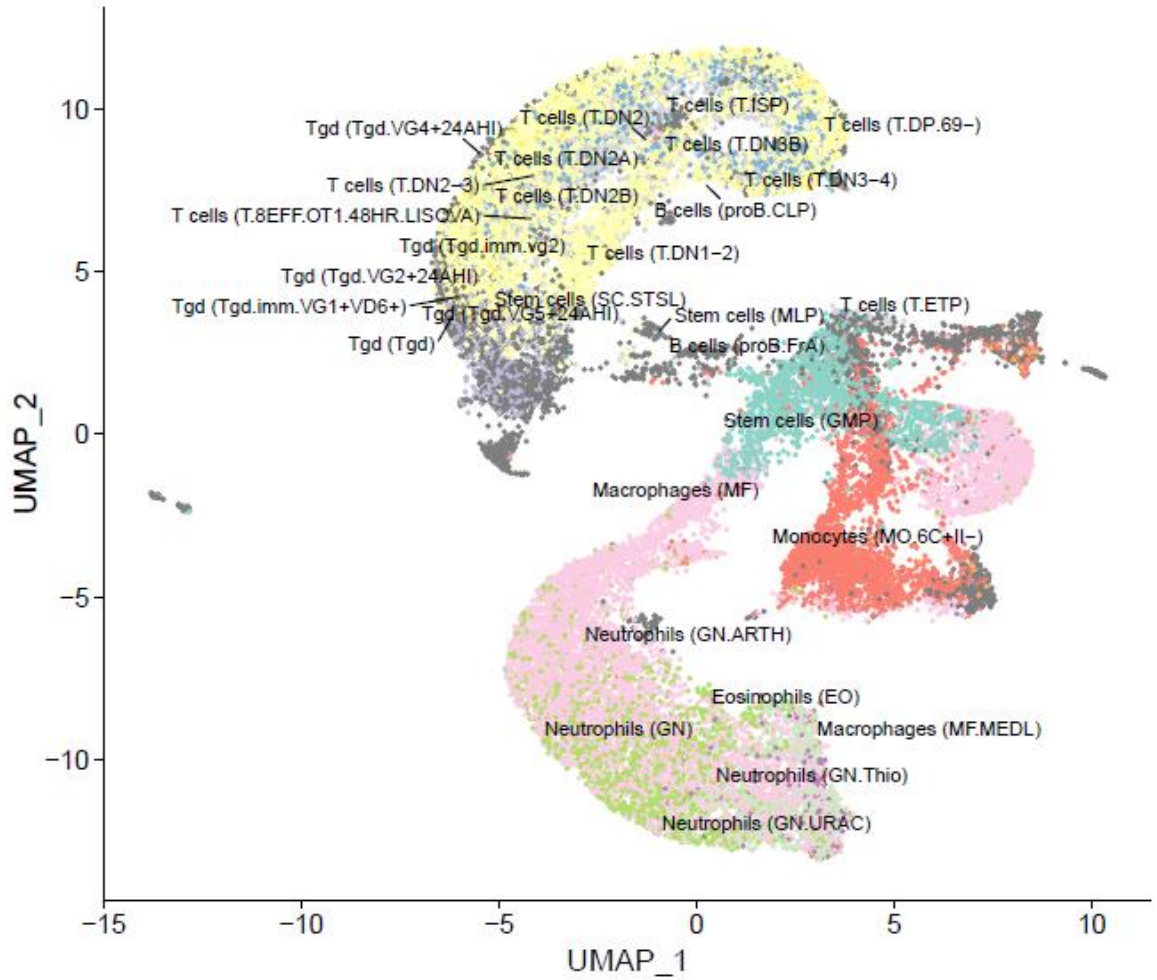
15. Assign cell types to the clusters using the ImmGen database as reference

- Convert the Seurat object into a single cell experiment object
- Get the ImmGen reference database from celldex package
- Assign cell types to the clusters using SingleR

Cell types: ImmGen (main labels)



Cell types: ImmGen (fine labels)

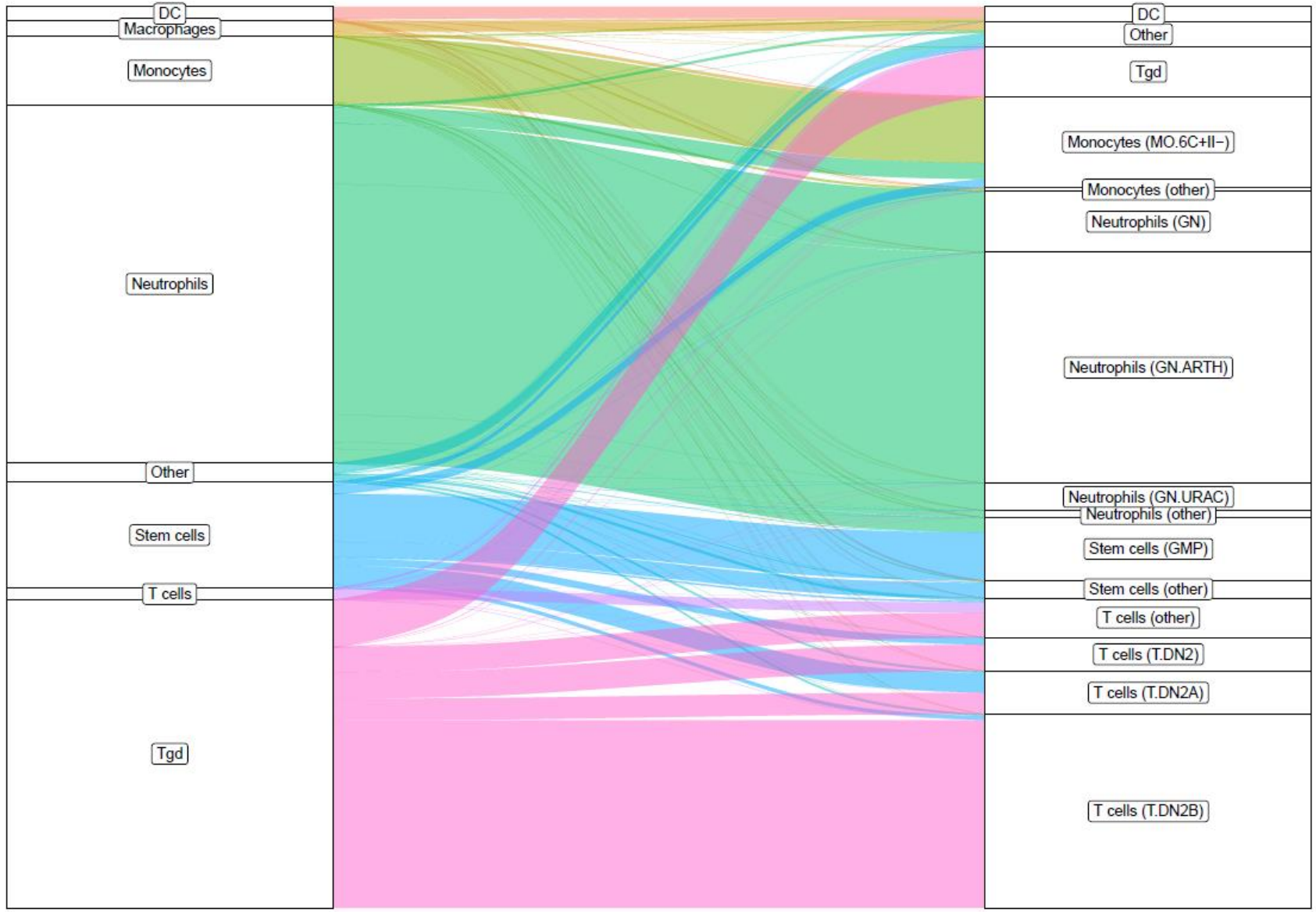


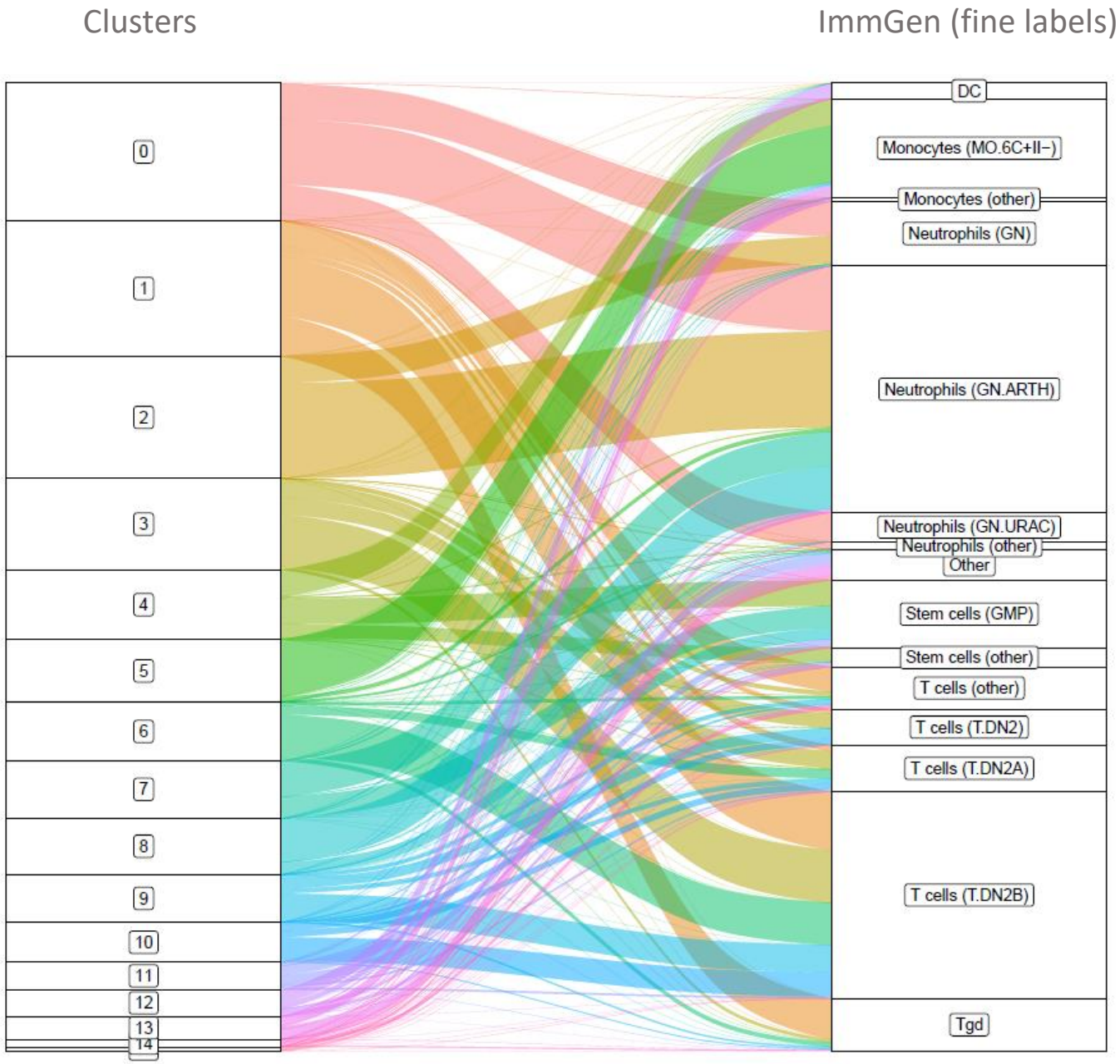
16. Check whether the cell types assigned with the main labels (general) and the fine labels (detailed subgroups) match well

17. Check what are the cell types from each cluster

ImmGen (main labels)

ImmGen (fine labels)



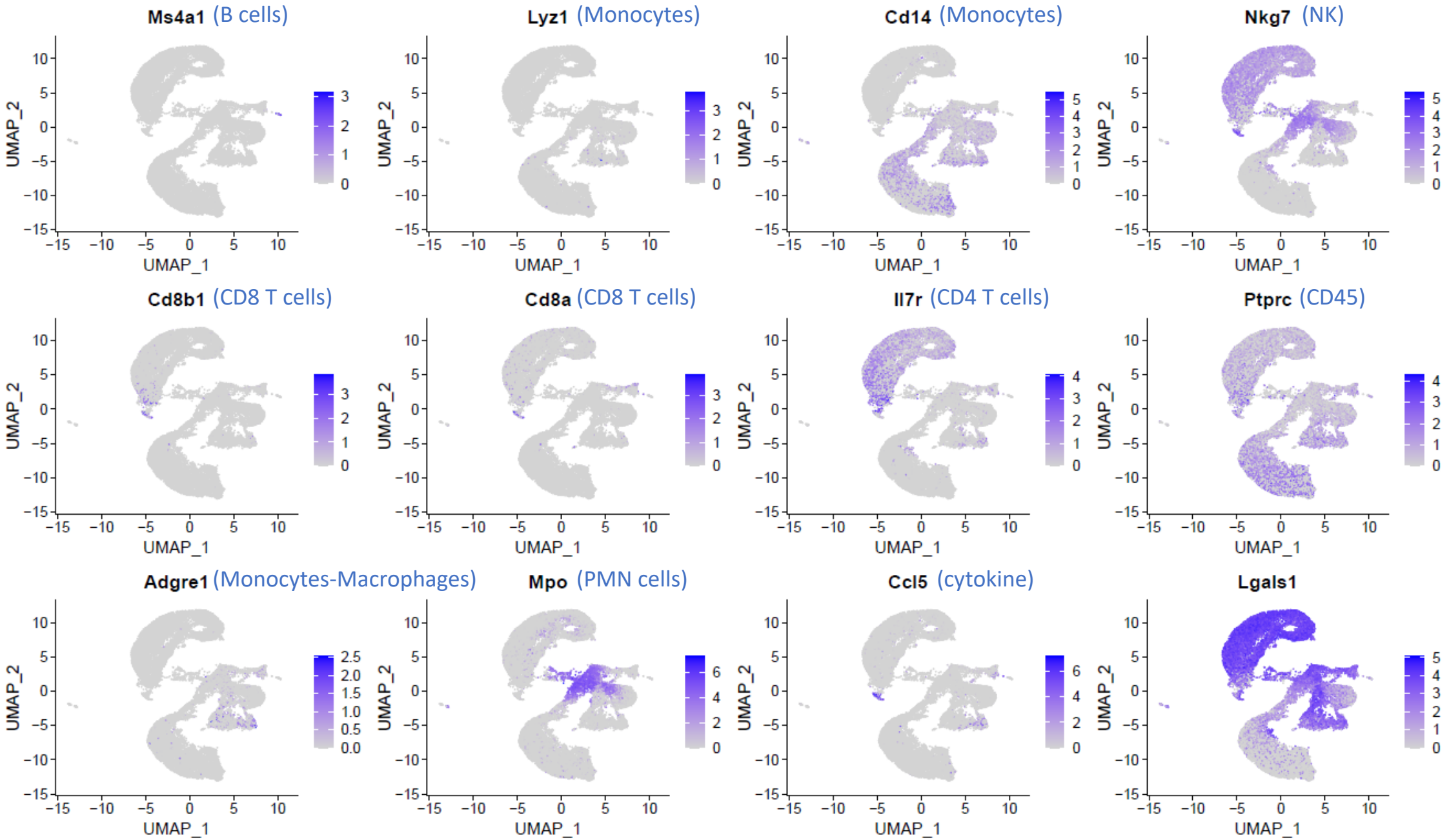

Clusters
ImmGen (fine labels)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
DC
Monocytes (MO.6C+II−)
Monocytes (other)
Neutrophils (GN)
Neutrophils (GN.ARTH)
Neutrophils (GN.URAC)
Neutrophils (other)
Other
Stem cells (GMP)
Stem cells (other)
T cells (other)
T cells (T.DN2)
T cells (T.DN2A)
T cells (T.DN2B)
Tgd

06

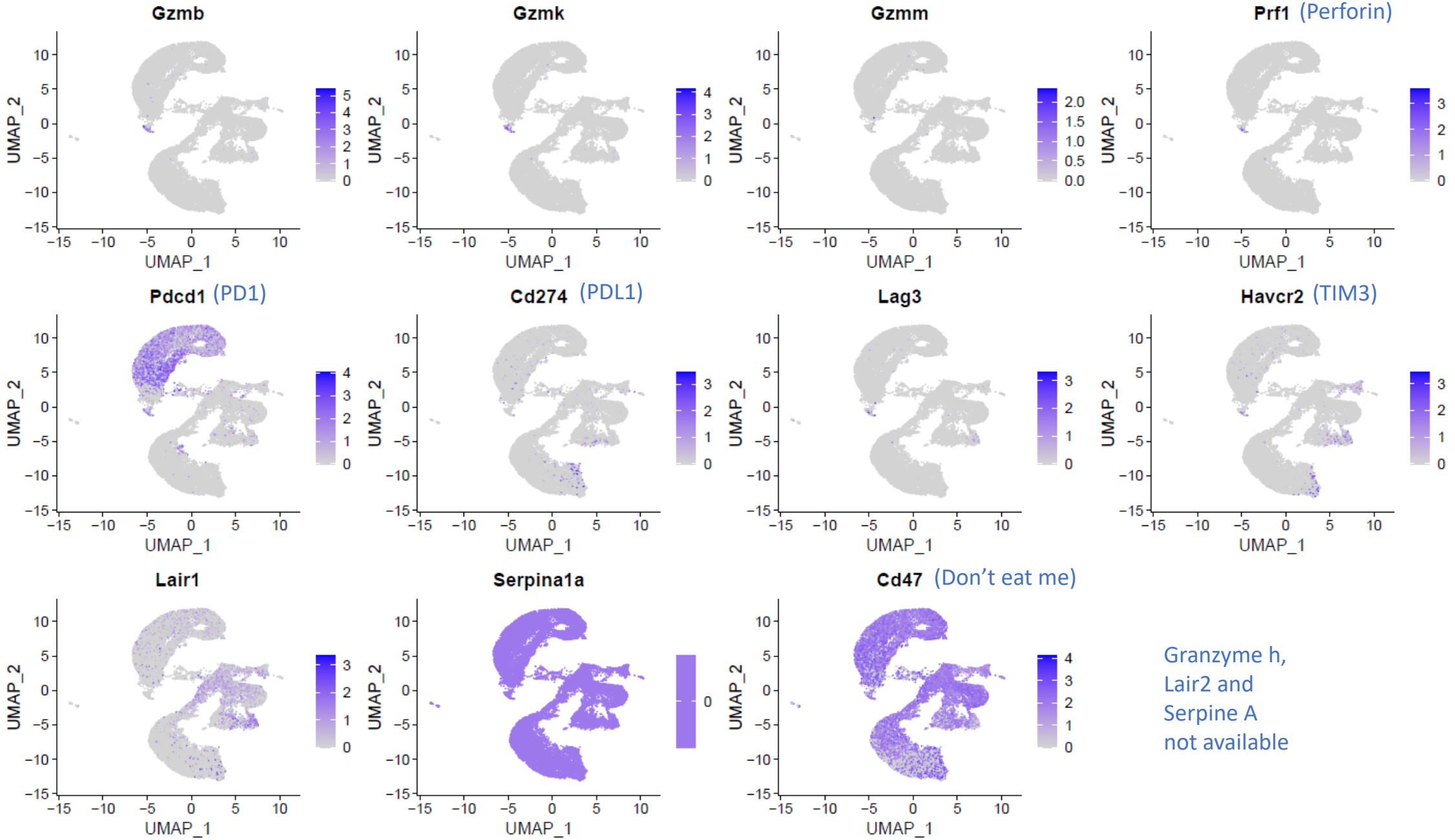
Coloring by genes

18. Visualize the distribution of the expression of some interesting genes across cells

Distribution of some famous markers

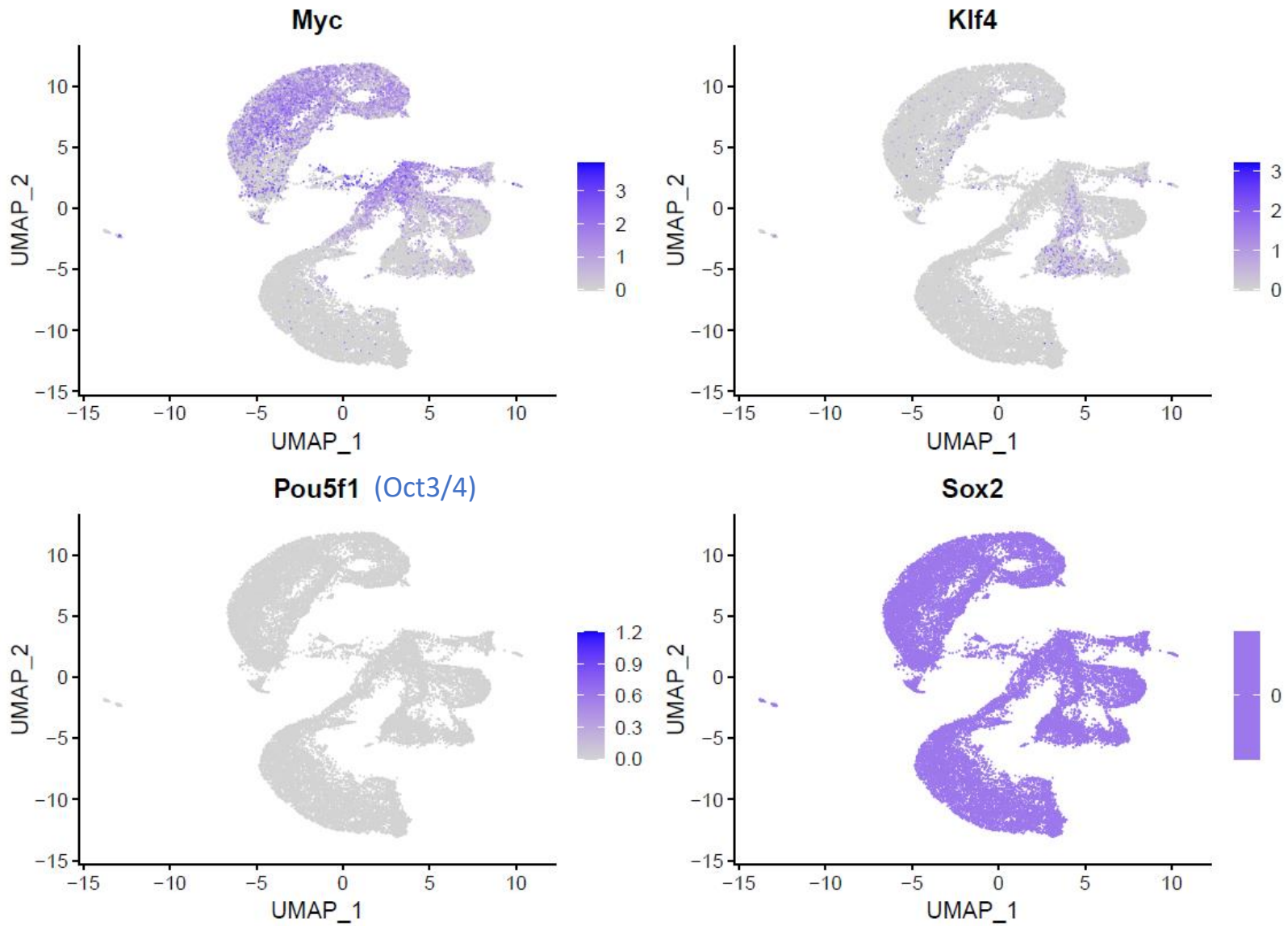


Distribution of some markers we were curious about



Granzyme h,
Lair2 and
Serpine A
not available

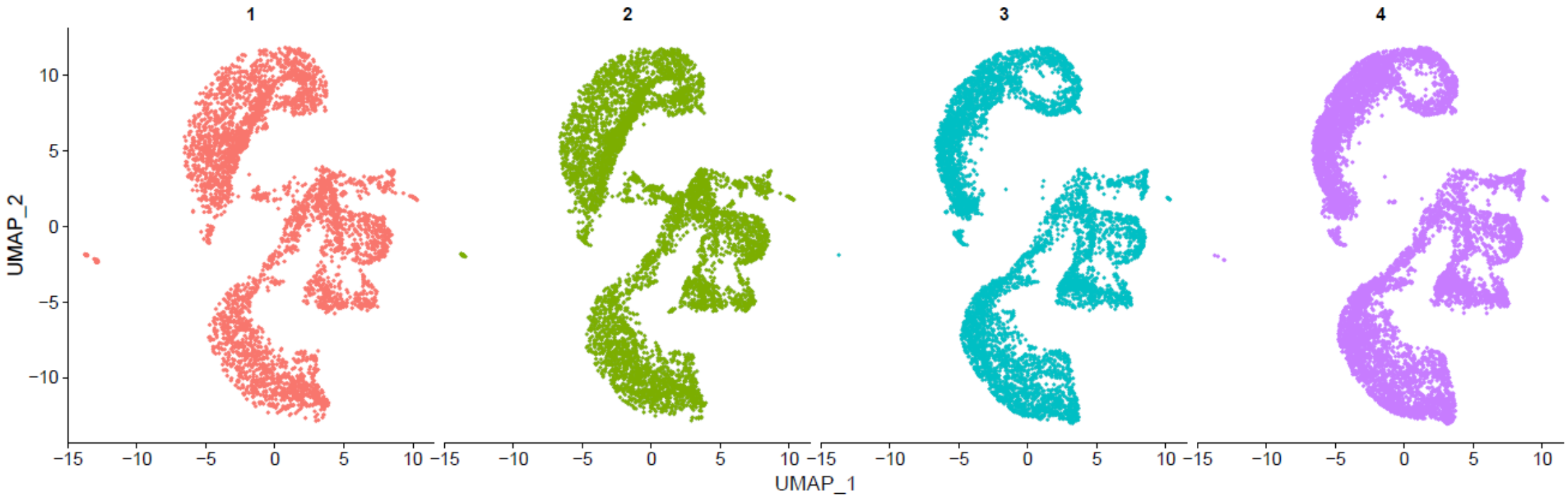
Distribution of the Yamanaka factors



07

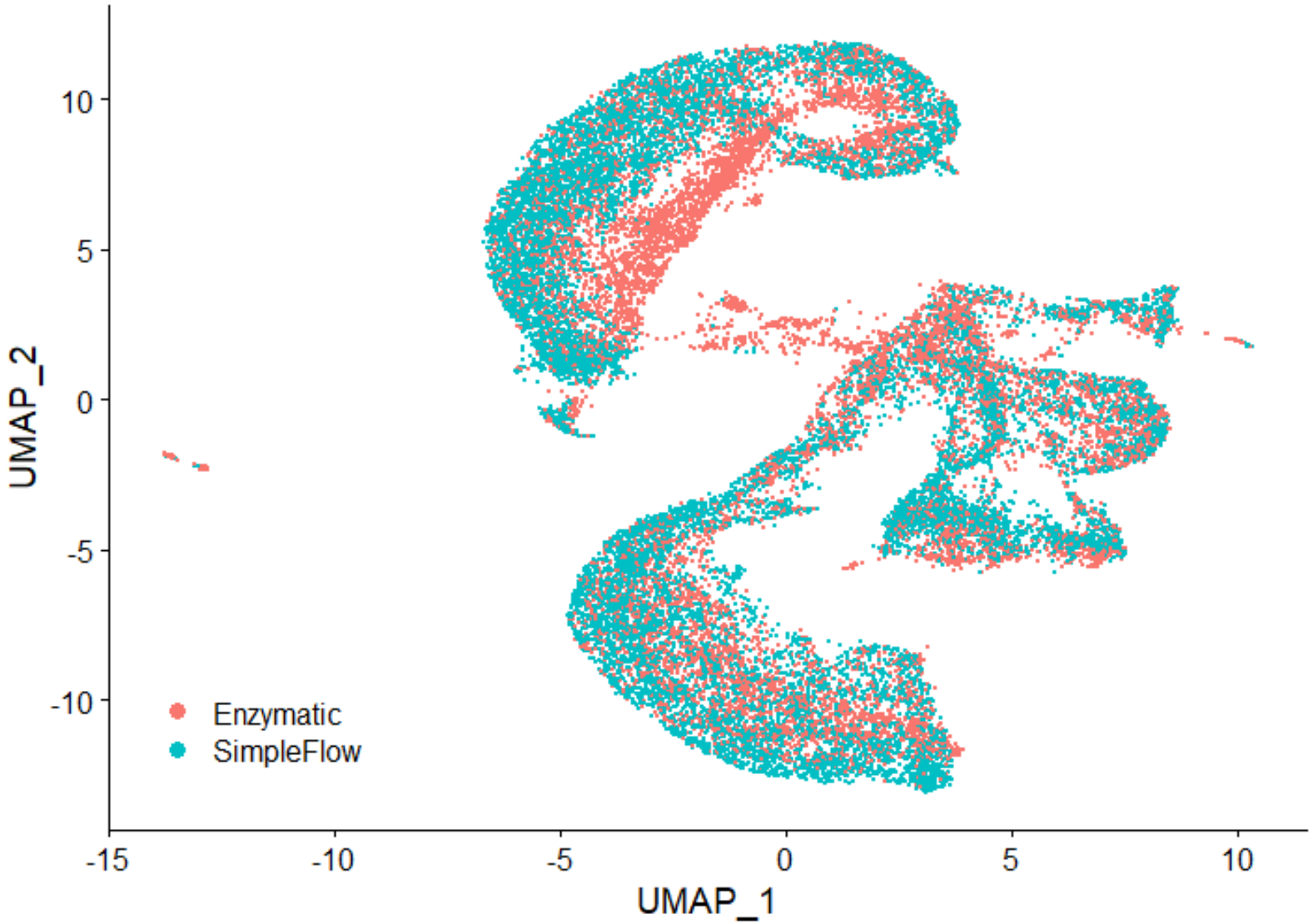
Comparison of the dissociation methods

1. Visualize the distribution of cells from each murine mammary tumor and from the two dissociation groups



Samples 1 & 2: Single cells were dissociated with Enzymatic digestion

Samples 3 & 4: Single cells were dissociated with SimpleFlowTM System


10
5
0
-5
-10
UMAP_2
-15
-10
-5
0
5
10
UMAP_1
Enzymatic
SimpleFlow

2. Perform a differential gene expression (DGE) analysis between the two dissociation groups to identify the expression of what genes changes for cells of the same type under different dissociation techniques

   For that, we will use the FindAllMarkers() R function that uses the Wilcoxon Rank Sum test

   For simplicity, we will focus on the largest cell types

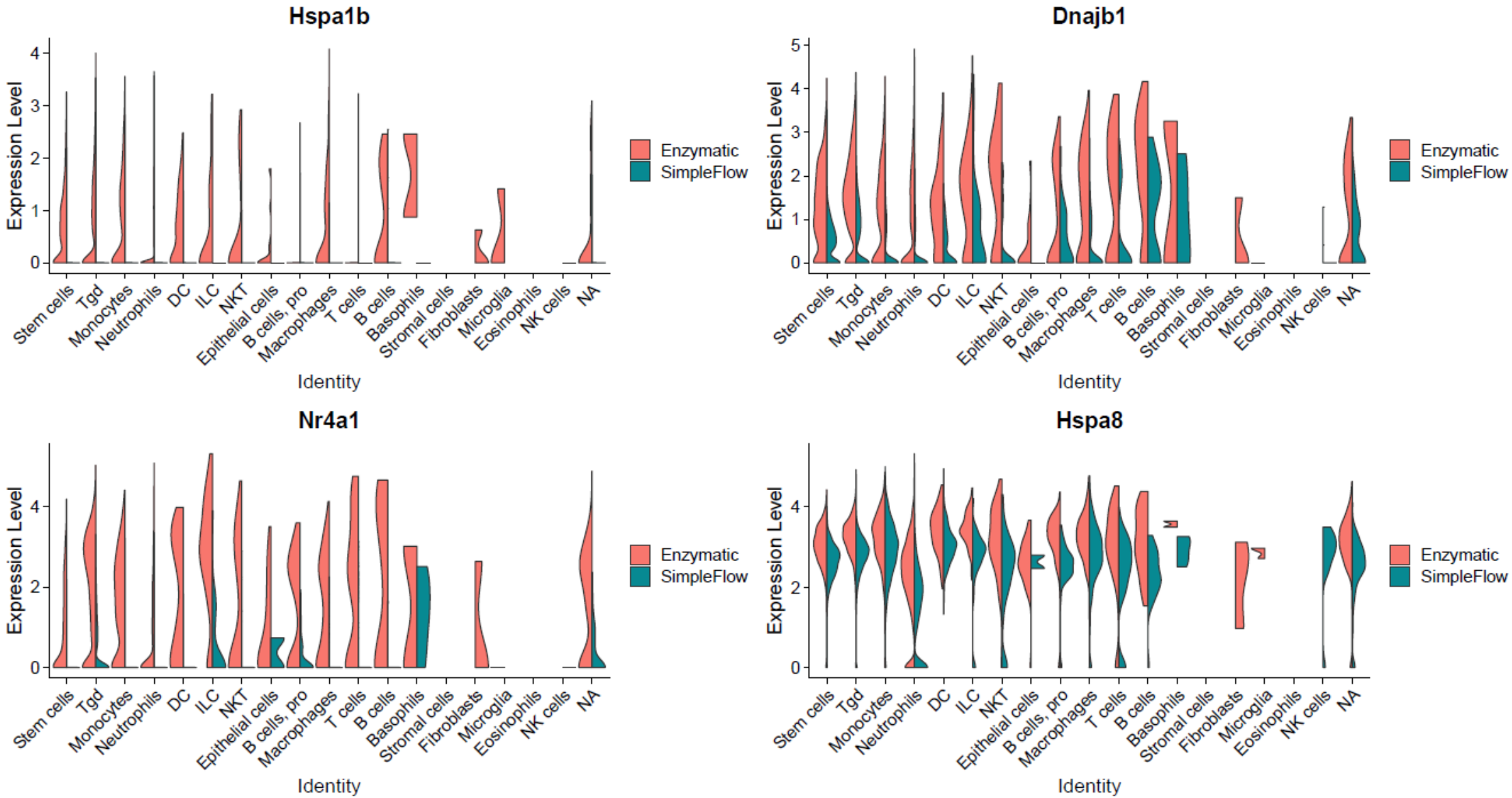
Top 15 differentially expressed genes between dissociation methods by cell type

```
> head(Monocytes, n = 15)
                 p_val avg_log2FC pct.1 pct.2     p_val_adj
Hspa1b   1.040215e-155  1.5454332 0.538 0.031 3.230179e-151
Nr4a1    5.232288e-130  2.4541140 0.743 0.241 1.624782e-125
Hspa1a   4.230262e-110  1.5763296 0.497 0.068 1.313623e-105
Dnajb1    1.186188e-75  1.6965480 0.659 0.305  3.683470e-71
Atf3      9.978361e-59  2.0161393 0.790 0.593  3.098580e-54
Ccl4      4.264070e-51  2.0004175 0.622 0.319  1.324122e-46
Hsp90aa1  5.711892e-46  1.0097817 0.786 0.552  1.773714e-41
Cdkn1a    7.336598e-39  1.0691153 0.688 0.447  2.278234e-34
H3f3b     1.700543e-37  0.5771917 1.000 0.999  5.280697e-33
Btg2      4.847031e-35  0.8355704 0.874 0.785  1.505149e-30
Srgn      8.298998e-34  0.8085828 0.903 0.772  2.577088e-29
Osm       4.413170e-32  1.0151987 0.428 0.199  1.370422e-27
Ier3      1.714669e-31  1.1314937 0.852 0.734  5.324563e-27
Ccl3      2.459093e-31  0.8946007 0.772 0.589  7.636220e-27
Il1b      1.501253e-30  2.0214160 0.450 0.237  4.661841e-26
```

```
> head(Neutrophils, n = 15)
                 p_val avg_log2FC pct.1 pct.2     p_val_adj
G0s2      0.000000e+00  2.2647916 0.762 0.531  0.000000e+00
Hspa8     0.000000e+00  1.1134147 0.879 0.711  0.000000e+00
Nr4a1     0.000000e+00  1.9639366 0.435 0.071  0.000000e+00
Hspa1b    0.000000e+00  0.7527038 0.256 0.011  0.000000e+00
Csrnp1   1.446207e-240  1.0648900 0.448 0.153 4.490907e-236
Dnajb1   2.659682e-220  1.1938296 0.607 0.334 8.259110e-216
Klf6     1.515432e-217  0.8794987 0.892 0.740 4.705870e-213
Trem1    6.158587e-211  1.0356728 0.686 0.403 1.912426e-206
Plaur    1.202167e-208  1.0528873 0.825 0.656 3.733089e-204
Hsp90aa1 1.559493e-204  0.9325669 0.580 0.320 4.842694e-200
Fosl1    1.995955e-200  0.8110221 0.302 0.076 6.198039e-196
Nfkbid   6.757802e-194  0.8075579 0.298 0.078 2.098500e-189
Tnfaip6  5.856903e-184  0.6785843 0.175 0.016 1.818744e-179
Hspa1a   1.105735e-183  0.5400613 0.163 0.011 3.433638e-179
Hsp90ab1 2.126125e-175  0.8851453 0.778 0.622 6.602257e-171
```

```
> head(Tgd, n = 15)
                 p_val avg_log2FC pct.1 pct.2     p_val_adj
Dnajb1    0.000000e+00  1.4599982 0.854 0.613  0.000000e+00
Hspa8     0.000000e+00  0.6472637 0.996 0.985  0.000000e+00
Nr4a1     0.000000e+00  2.6383861 0.785 0.420  0.000000e+00
Hspa1b    0.000000e+00  1.5541028 0.560 0.016  0.000000e+00
Hspa1a    0.000000e+00  1.6776488 0.446 0.014  0.000000e+00
Hsp90aa1 2.583884e-275  0.6212419 0.969 0.909 8.023735e-271
Hsp90ab1 3.865601e-230  0.3527013 0.999 0.997 1.200385e-225
Cdkn1a   2.824298e-223  1.1446326 0.448 0.169 8.770294e-219
Ppp1r15a 3.299994e-209  0.8235387 0.765 0.499 1.024747e-204
Plaur    9.011602e-207  1.0946360 0.645 0.361 2.798373e-202
Atf3     4.832174e-199  0.9064369 0.207 0.015 1.500535e-194
Tnfsf11  2.023240e-198  0.6370298 0.195 0.011 6.282768e-194
Csrnp1   5.380092e-192  0.8110122 0.475 0.203 1.670680e-187
Nr4a2    5.067422e-191  0.8392308 0.403 0.147 1.573587e-186
Jun      1.030369e-186  0.8721499 0.897 0.748 3.199606e-182
```

```
> head(Stem.cells, n = 15)
                 p_val avg_log2FC pct.1 pct.2     p_val_adj
Hspa1b   2.711773e-247  1.2752226 0.660 0.047 8.420869e-243
Hspa1a   1.161467e-155  1.2118726 0.489 0.044 3.606703e-151
Dnajb1   7.792747e-132  1.5208896 0.839 0.710 2.419882e-127
Nr4a1    3.926794e-123  1.9038705 0.587 0.220 1.219387e-118
Hspa8    2.028165e-112  0.6117943 0.987 0.995 6.298060e-108
Hsp90aa1  2.567353e-91  0.5895800 0.962 0.991  7.972401e-87
Jun       7.146602e-67  0.8336549 0.907 0.888  2.219234e-62
Hsph1     7.508380e-61  0.4787178 0.433 0.167  2.331577e-56
Hnrnpab   8.344769e-60 -0.4266460 0.874 0.980  2.591301e-55
Rps18     2.037699e-56  0.3285766 0.948 0.988  6.327668e-52
Bag3      2.962035e-56  0.5430598 0.403 0.160  9.198009e-52
Srsf2     4.995195e-56 -0.3655303 0.882 0.973  1.551158e-51
Gnai2     9.259230e-50 -0.4248351 0.819 0.947  2.875269e-45
Ppp1cc    1.249587e-49 -0.3403015 0.826 0.946  3.880342e-45
Gm10260   1.705010e-48 -0.2793919 0.980 1.000  5.294567e-44
```


Hspa1b
Dnajb1
Nr4a1
Hspa8
Expression Level
Identity
Enzymatic
SimpleFlow
Stem cells
Tgd
Monocytes
Neutrophils
DC
ILC
NKT
Epithelial cells
B cells, pro
Macrophages
T cells
B cells
Basophils
Stromal cells
Fibroblasts
Microglia
Eosinophils
NK cells
NA

3. Perform pathway analysis of the top 15 differentially expressed genes per cell type between dissociation groups using the DAVID platform (link)

Main differentially expressed pathways between dissociation methods by cell type

Sublist	Category	Term	RT	Genes	Count	%	P-Value	Fold Enrichment	Benjamini
	UP_KEYWORDS	Stress response	RT		6	40.0	6.8E-10	121.0	3.0E-8
	UP_KEYWORDS	Chaperone	RT		6	40.0	7.6E-8	47.5	1.7E-6
	GOTERM_MF_DIRECT	unfolded protein binding	RT		5	33.3	2.2E-7	83.1	1.6E-5
	KEGG_PATHWAY	Protein processing in endoplasmic reticulum	RT		6	40.0	2.5E-7	30.5	5.2E-6
	KEGG_PATHWAY	Antigen processing and presentation	RT		5	33.3	8.1E-7	52.1	8.5E-6

Neutrophils (n=10,202):

1) Stress response

Sublist	Category	Term	RT	Genes	Count	%	P-Value	Fold Enrichment	Benjamini
	UP_KEYWORDS	Stress response	RT		7	46.7	3.1E-12	141.1	1.6E-10
	UP_KEYWORDS	Chaperone	RT		6	40.0	7.6E-8	47.5	1.9E-6
	KEGG_PATHWAY	Protein processing in endoplasmic reticulum	RT		7	46.7	1.5E-7	22.9	9.2E-6
	GOTERM_MF_DIRECT	unfolded protein binding	RT		5	33.3	3.1E-7	77.5	2.9E-5
	KEGG_PATHWAY	Estrogen signaling pathway	RT		6	40.0	3.6E-7	33.6	1.1E-5

Tgd (n=9,706):

1) Stress response

Sublist	Category	Term	RT	Genes	Count	%	P-Value	Fold Enrichment	Benjamini
	UP_KEYWORDS	Stress response	RT		6	40.0	6.8E-10	121.0	3.8E-8
	INTERPRO	Heat shock protein 70 family	RT		4	26.7	7.1E-8	422.4	1.4E-6
	INTERPRO	Heat shock protein 70, conserved site	RT		4	26.7	7.1E-8	422.4	1.4E-6
	UP_KEYWORDS	Chaperone	RT		6	40.0	7.6E-8	47.5	1.5E-6
	UP_KEYWORDS	Acetylation	RT		12	80.0	8.1E-8	5.8	1.5E-6

Stem cells (n=3,109):

1) Stress response

2) Heat shock protein 70 family

Sublist	Category	Term	RT	Genes	Count	%	P-Value	Benjamini
	GOTERM_BP_DIRECT	response to heat	RT		4	26.7	1.1E-5	3.2E-3
	UP_KEYWORDS	Stress response	RT		4	26.7	1.2E-5	6.2E-4
	GOTERM_BP_DIRECT	negative regulation of cell proliferation	RT		5	33.3	1.7E-4	2.5E-2
	UP_KEYWORDS	Chaperone	RT		4	26.7	2.0E-4	3.8E-3
	UP_KEYWORDS	Cytokine	RT		4	26.7	2.3E-4	3.8E-3

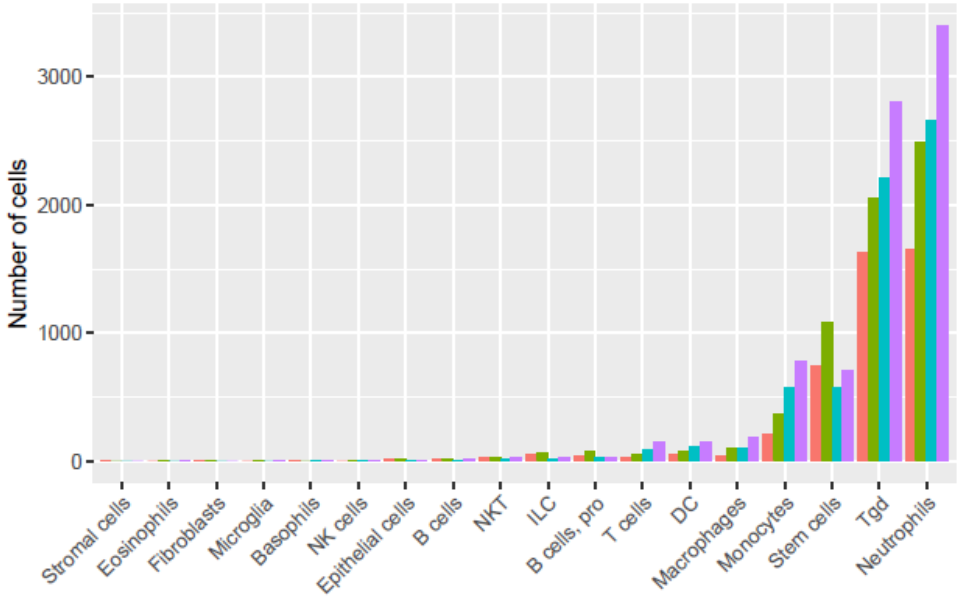
Monocytes (n=1,939):

1) Response to heat

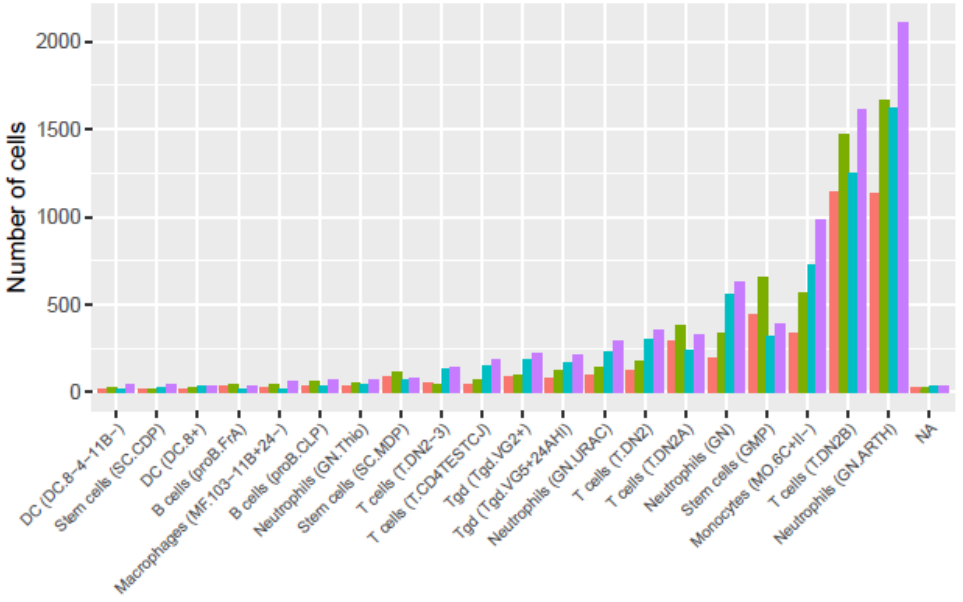
2) Stress response

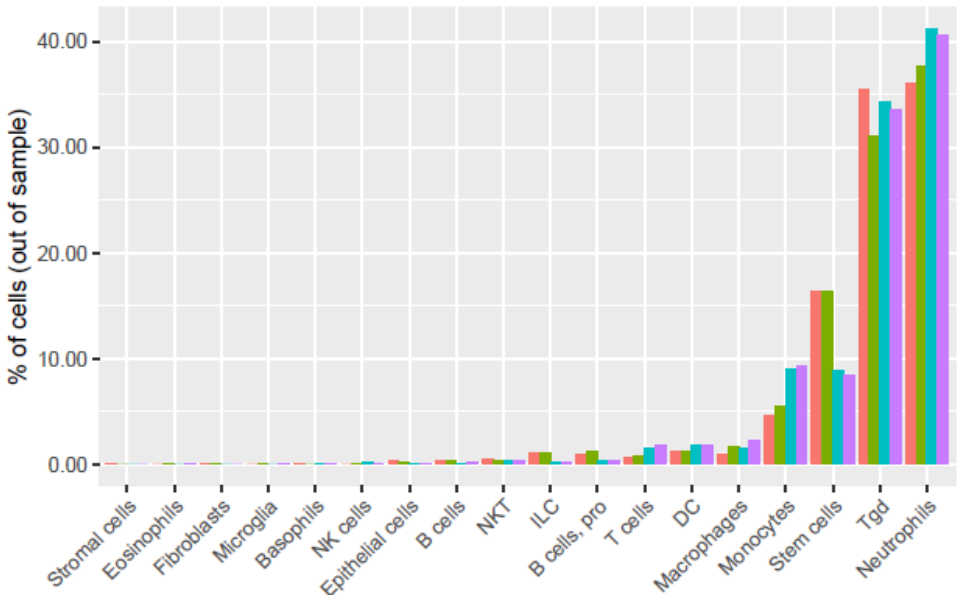
4. Compare the cell type composition of samples from the two dissociation groups

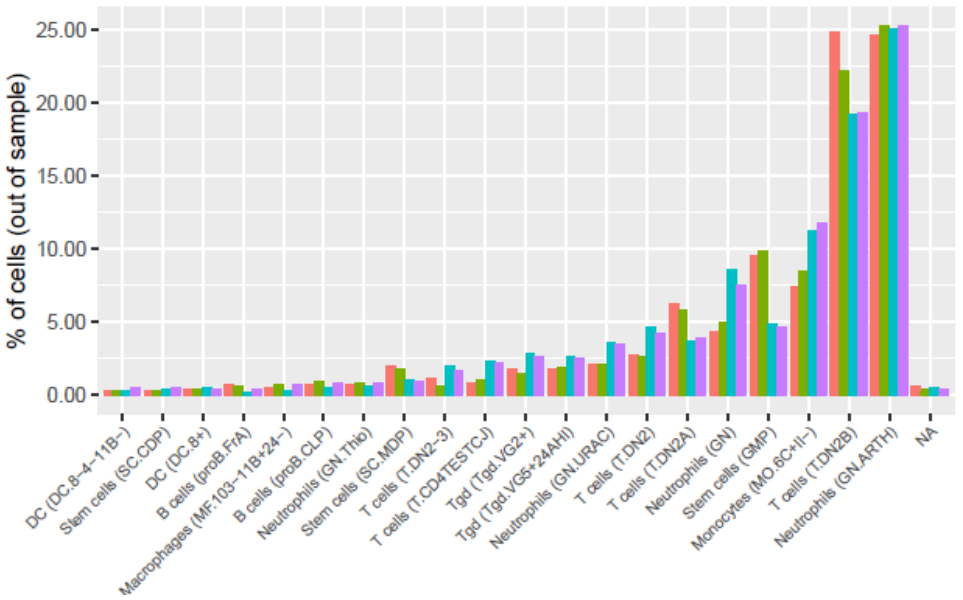
Cell types: ImmGen (main labels)



Cell types: ImmGen (fine labels)







Samples 1 & 2: Single cells were dissociated with enzymatic digestion

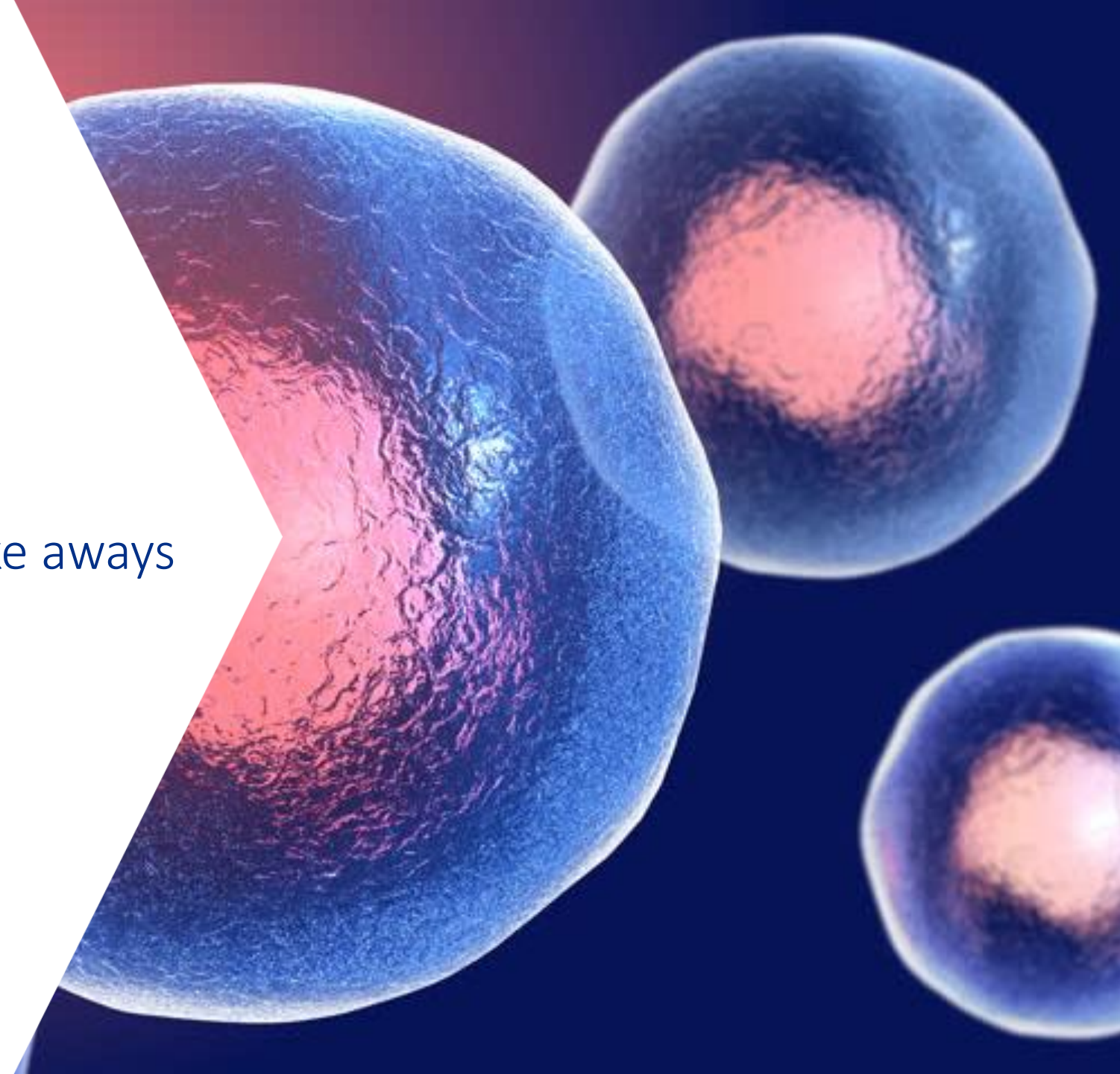
Samples 3 & 4: Single cells were dissociated with SimpleFlow TM

Total # cells per sample:

1	2	3	4
4596	6610	6458	8343

08

The take aways



Summary of key insights:

- The most frequent cell types in the samples are neutrophils (GN.ARTH), T cells (T.DN2B), and monocytes (MO.6C+II-).
- The cell type compositions of samples dissociated with enzymatic digestion and the SimpleFlowTM System are very similar, though samples dissociated with enzymatic digestion contain a slightly higher proportion of T cells (T.DN2B).
- Cells dissociated with enzymatic digestion have a substantial increase in expression of genes from the “stress response” and the “response to heat” pathways. Overexpression of these pathways is indicative of cellular stress conditions imposed by the circumstances of tissue digestion and the high temperature of enzymatic digestion (it is performed at 37º, whereas SimpleFlowTM is performed at 4º).
- Overall, the SimpleFlowTM system has demonstrated to be an excellent dissociation methodology of single cells because: 1) it generates similar results to those obtained with the conventional methodology of enzymatic digestion, and 2) reduces the cellular stress imposed by the procedures.

THANKS

Does anyone have any questions?