Introduction to single cell RNA-seq

Xabier Bujanda Cundin
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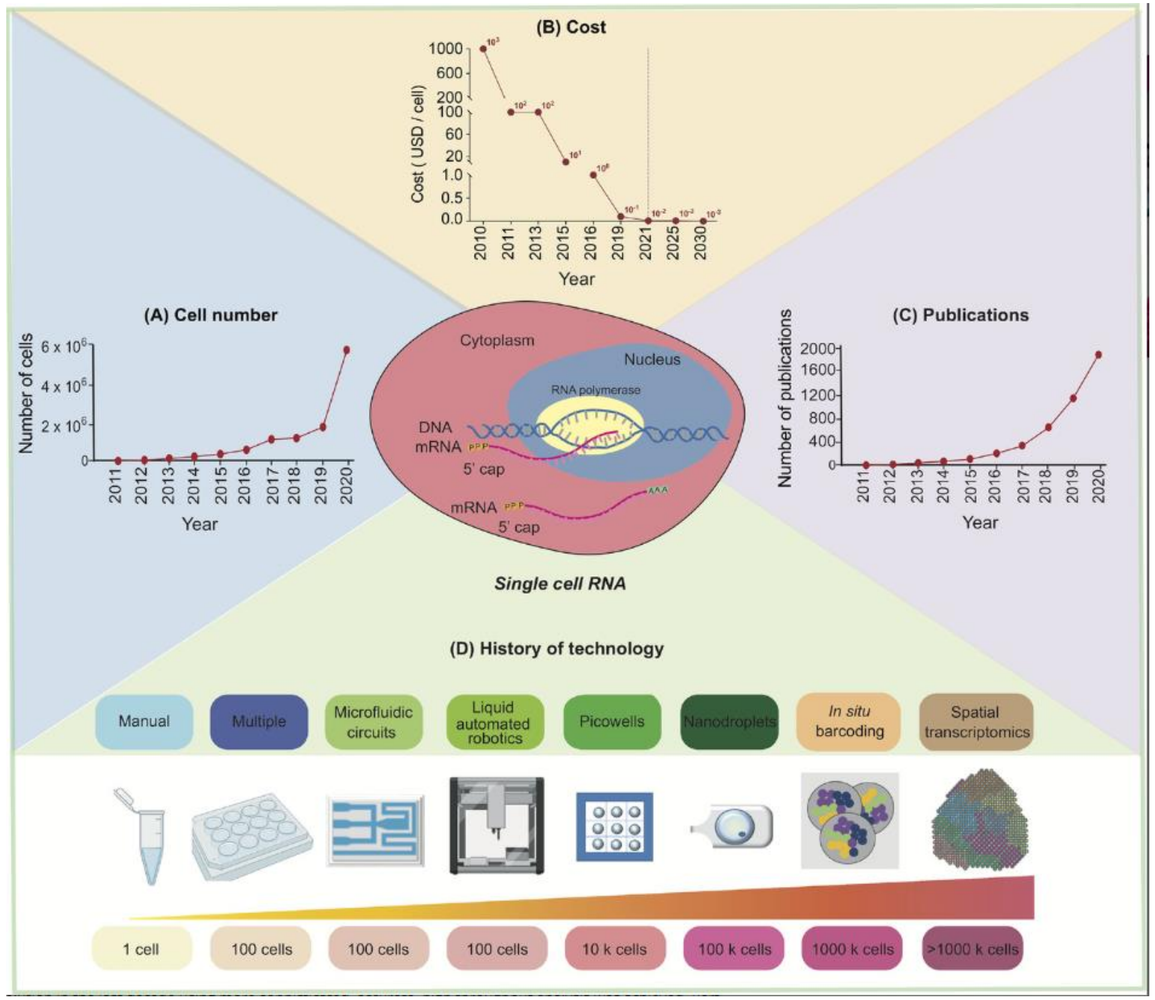
Why single cell?

- **Cell**: the smallest unit that can live on its own and that makes up all living organisms and the tissues of the body
- Identification of cell types and state in a heterogeneous sample
- Assess differences between and within cell types

Challenges:

- Huge amount of data to handle
- Low read depth
- High variability between cells/samples
- Data processing, analysis, presentation and interpretation



Jovic, D, Liang, X, Zeng, H, Lin, L, Xu, F, Luo, Y Single-cell RNA sequencing technologies and applications: A brief overview. *Clin Transl Med*. 2022; 12:e694. https://doi.org/10.1002/ctm2.694

scRNA-seq data can answer multiple biological questions

- Cell populations
- Expression differences cell clusters and conditions
- Time-series gene expression development

But…

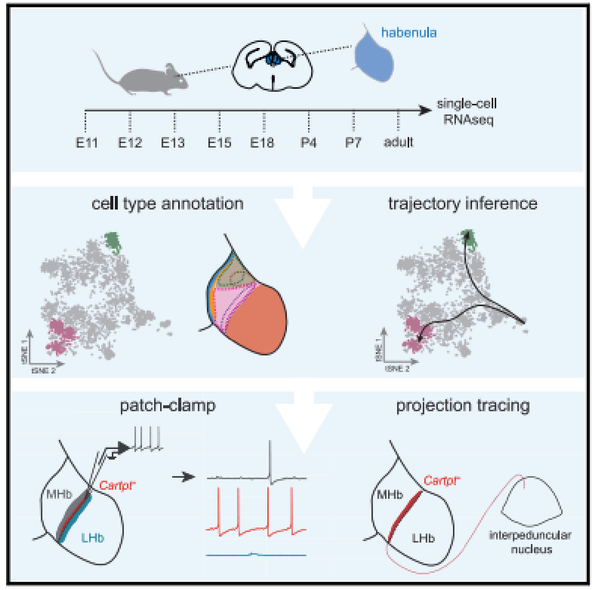
- Low number of transcripts can be detected
- Some cell types may not be compatible for some scRNA-seq techniques
- Low expressed transcript may not be detected

Article

Cell Reports

Molecular signatures and cellular diversity during mouse habenula development

Graphical abstract



Authors

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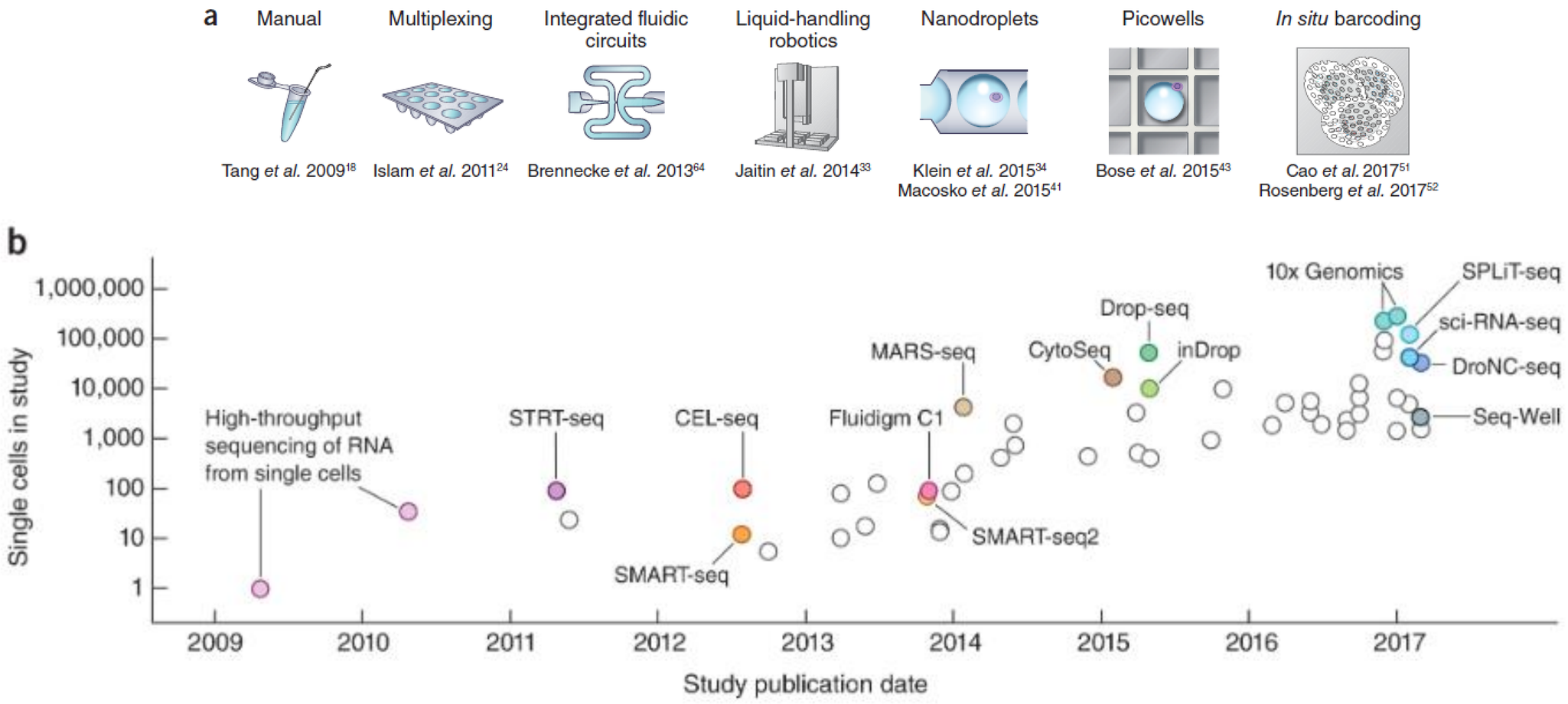
In brief

van de Haar et al. characterize cell populations of the developing mouse habenula using single-cell RNA sequencing. They reconstruct the developmental trajectories of molecularly distinct neuronal subsets and establish physiological and connectivity properties of *Cartpt* neurons. Their analysis indicates a possible link between developing habenula neurons and human psychiatric disorders.

Highlights

- scRNA-seq reveals cellular heterogeneity of the developing mouse habenula
- Trajectory inference reconstructs developmental paths of habenula cell types
- *Cartpt* marks physiologically distinct neurons that innervate the dorsal IPN
- Developing habenular cell types align to risk loci of human psychiatric disorders

Single cell RNA-seq technologies



Svensson, V., Vento-Tormo, R. & Teichmann, S. Exponential scaling of single-cell RNA-seq in the past decade. *Nat Protoc* **13**, 599–604 (2018). https://doi.org/10.1038/nprot.2017.149

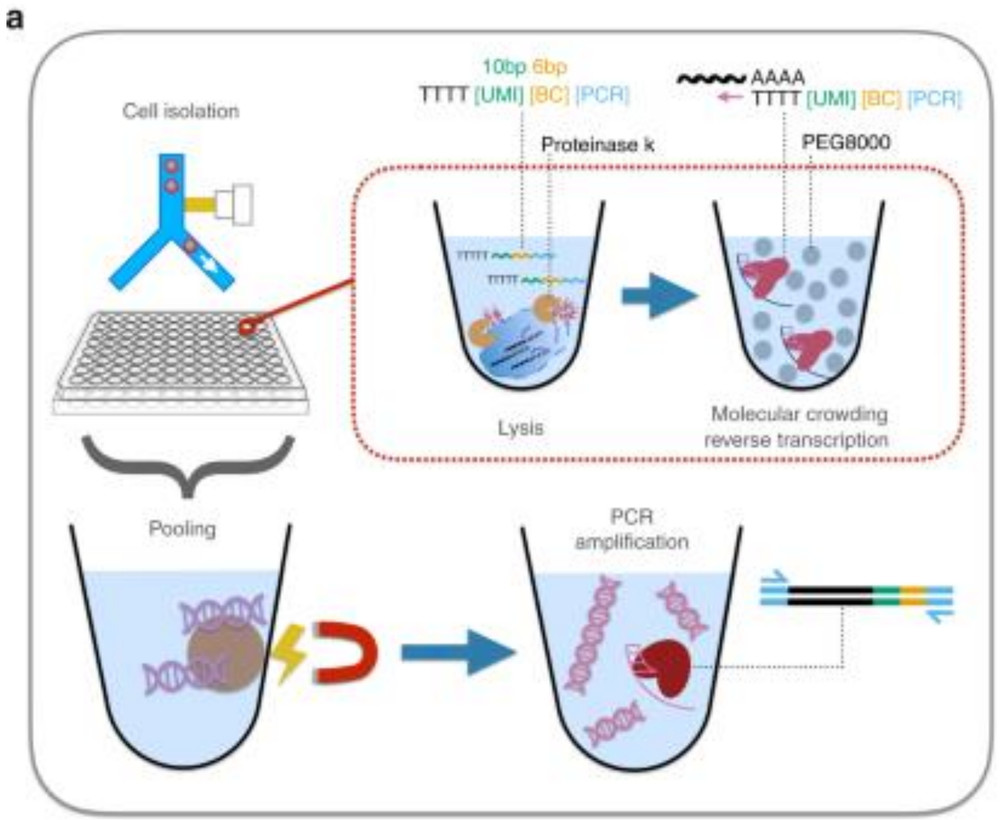
Single cell RNA-seq technologies

TABLE 1 Comparison of different single-cell RNA sequencing (scRNA-seq) technology and experimental protocols

Platforms	Isolation strategies	Tissue	Cell numbers	Targets	UMI	Amplification methods	Region	Published year
Smart-seq	FACS	Dissociated cell	Hundreds of cells	/	×	PCR	Full-length	2012
Smart-seq2	FACS	Dissociated cell	Hundreds of cells	/	×	PCR	Full-length	2013
Fluidigm C1	Micro-fluidic	Dissociated cell	Hundreds of cells	No poly(A) minus RNA detection	×	PCR	Full-length	2013
Drop-seq	Microdroplets	Dissociated cell	Large number of cell	No poly(A) minus RNA detection	√	PCR	3′ end	2015
10x Genomics	Microdroplets	Dissociated cell	Large number of cells	No poly(A) minus RNA detection	√	PCR	3′ end	2016
MATQ-seq	FACS	Dissociated cell	Hundreds of cells	No poly(A) minus RNA detection	√	PCR	Full-length	2017
Seq-Well	Micro-fluidic	Dissociated cell	Large number of cells	No poly(A) minus RNA detection	√	PCR	3′ end	2017
CEL-seq	FACS	Dissociated cell	Hundreds of cells	No poly(A) minus RNA detection	√	IVT	3′ end	2012
MARS-seq	FACS	Dissociated cell	Hundreds of cells	No poly(A) minus RNA detection	√	IVT	3′ end	2014
inDrop-seq	Microdroplets	Dissociated cell	Large number of cell	No poly(A) minus RNA detection	√	IVT	3′ end	2015
DNBelab C4	Microdroplets	Dissociated cell	Large number of cells	No poly(A) minus RNA detection	√	PCR	3′ end	2019

SMART-seq technology

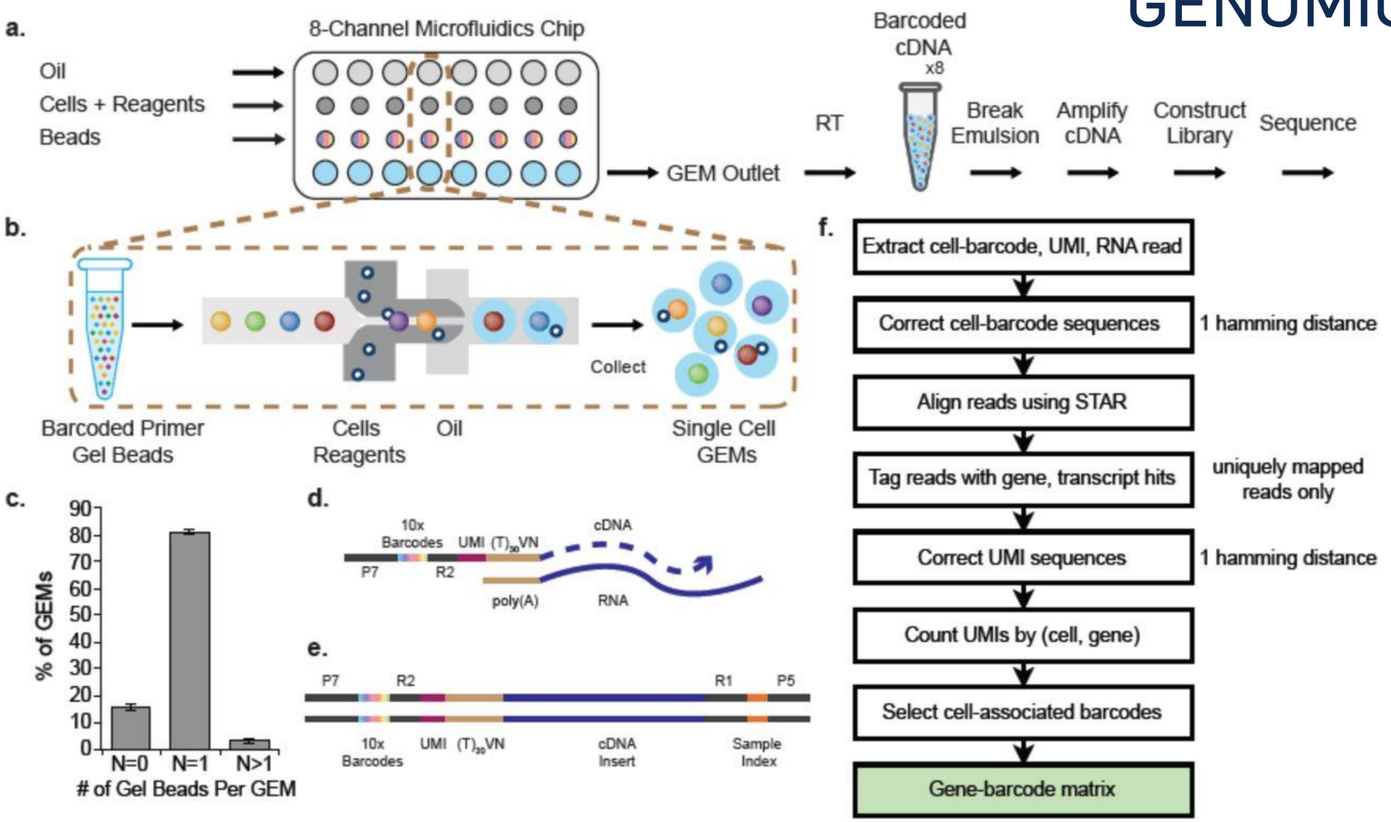
- Cell separation through FACS
- Library preparation by cell
- Poor depth
- Whole gene sequencing (isoforms identification)



Bagnoli, J.W., Ziegenhain, C., Janjic, A. *et al.* Sensitive and powerful single-cell RNA sequencing using mcSCRB-seq. *Nat Commun* **9**, 2937 (2018).

10X Genomics technology

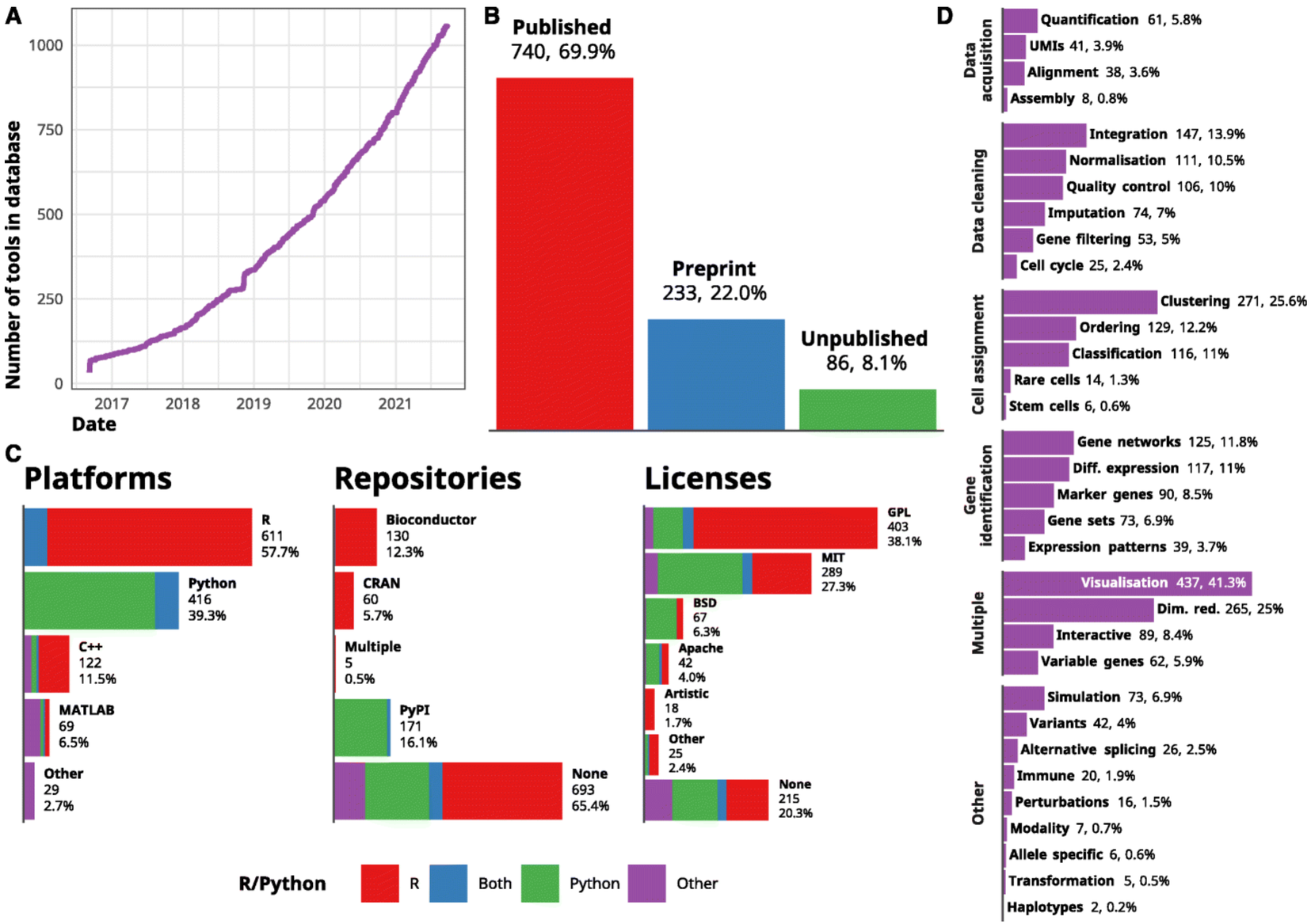
- High Sensitivity
- Lower technical noise
- High price
- Simpler data processing steps



Zheng, G., Terry, J., Belgrader, P. *et al.* Massively parallel digital transcriptional profiling of single cells. *Nat Commun* **8**, 14049 (2017). https://doi.org/10.1038/ncomms14049

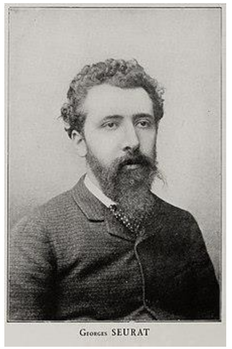
Platforms:

- Seurat (R)
- Scanpy (Python)
- Galaxy (webtool)
- Scater + scran (R, Bioconductor)
- Monocle3 and psupertime (R)

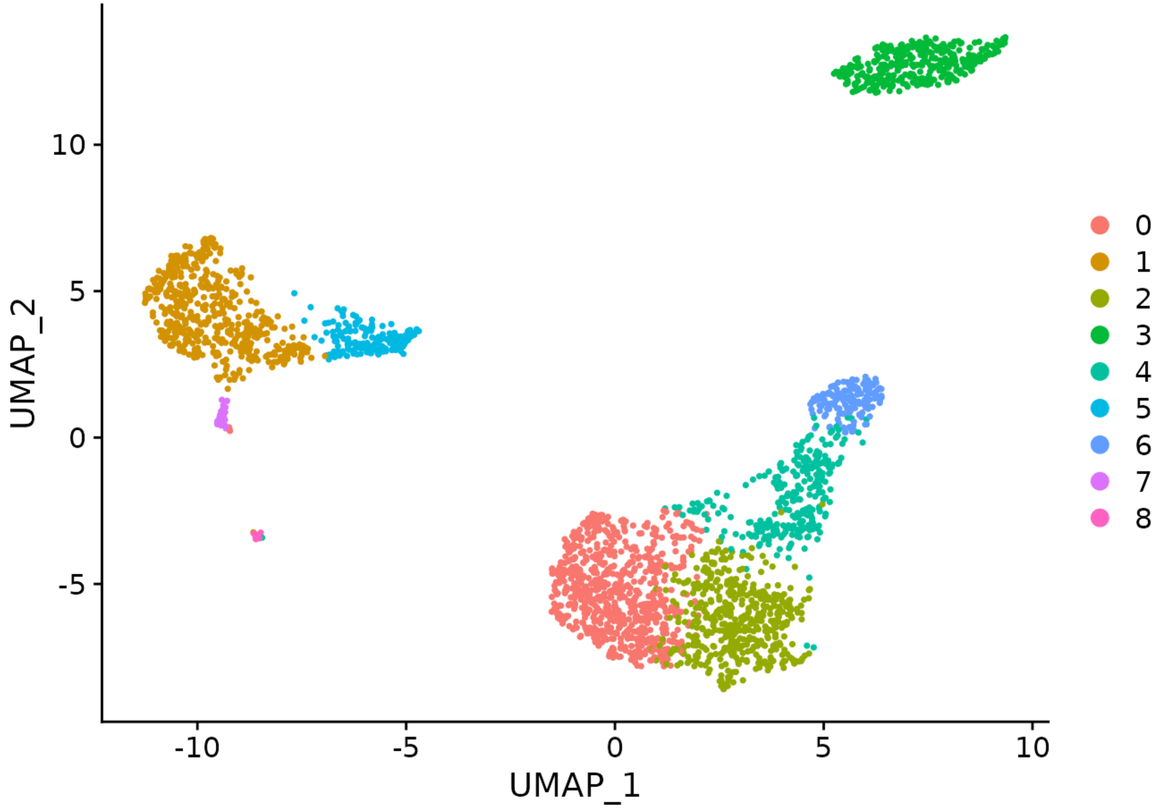


Zappia, L., Theis, F.J. Over 1000 tools reveal trends in the single-cell RNA-seq analysis landscape. *Genome Biol* **22**, 301 (2021). https://doi.org/10.1186/s13059-021-02519-4

3. Seurat



Georges SEURAT



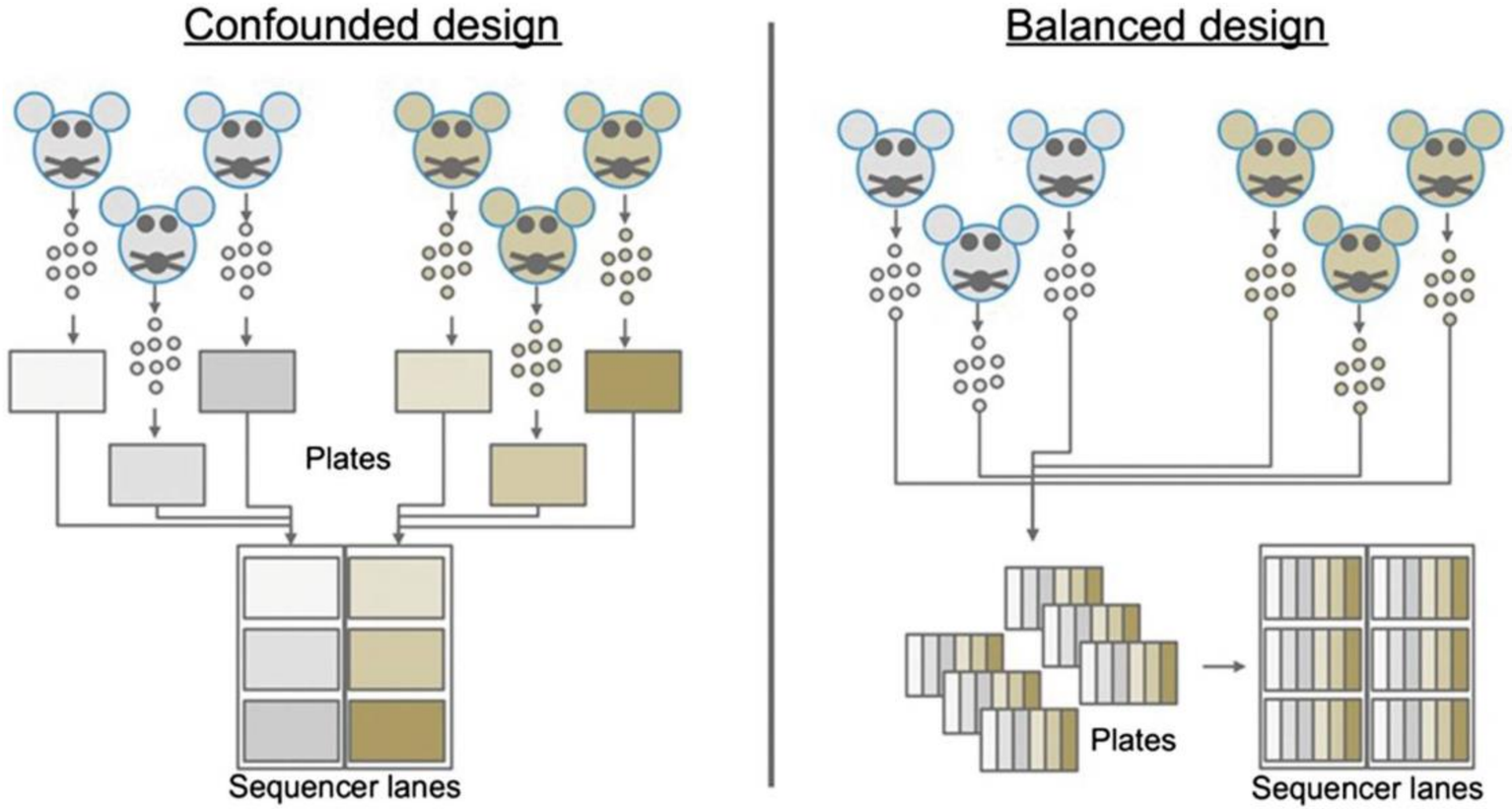
Why Seurat?

- Easy to use
- Very popular, supported and updated
- Deep documentation and good data model
- Several built-in functionalities
- Easy to extend with your own functionality
- Seurat objects extend to other packages

Adapted from https://satijalab.org/seurat/articles/pbmc3k_tutorial.html

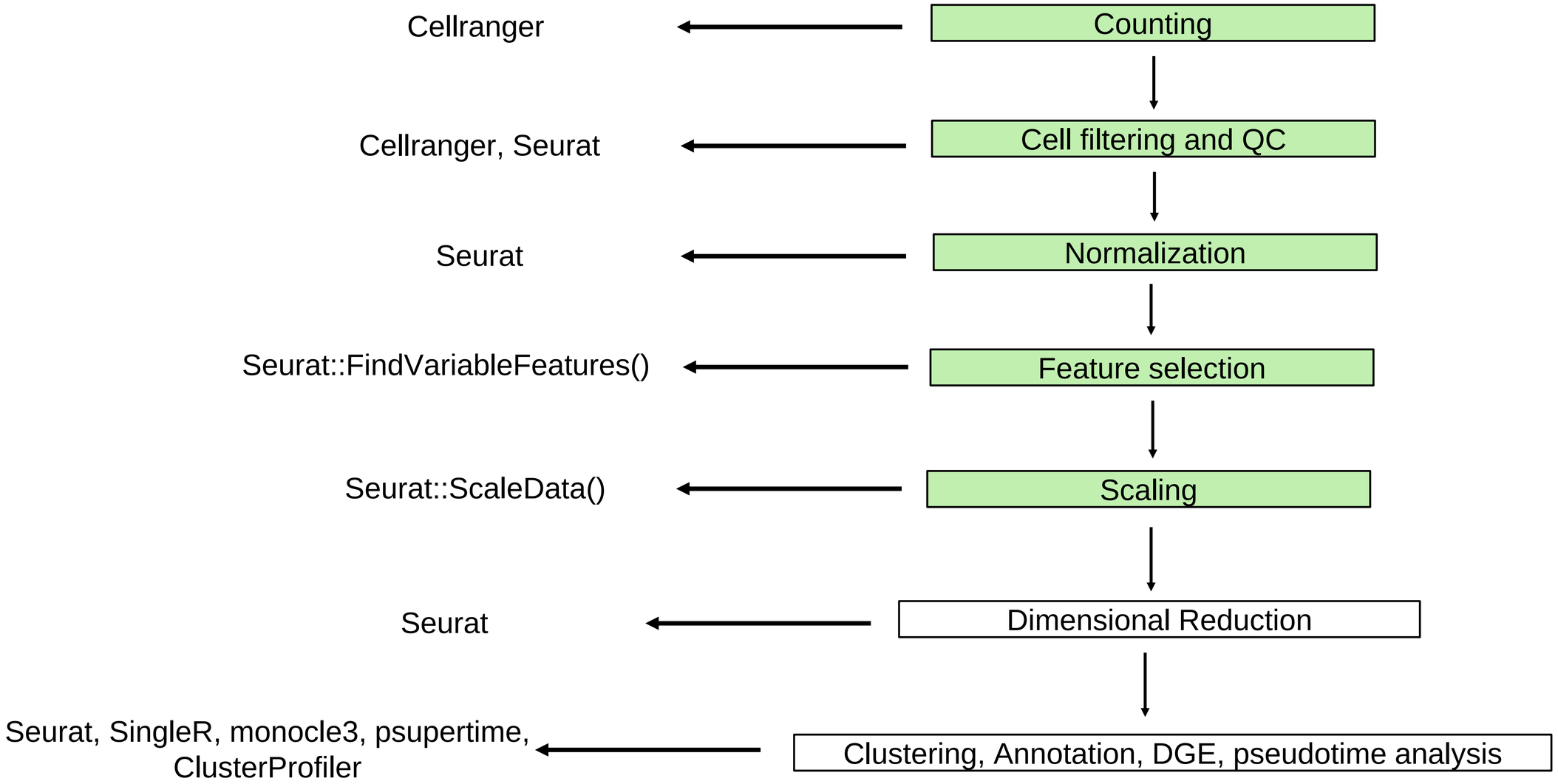
Experimental Design

- Replication and randomization
- Be aware of potential biases that can affect the results
- Record any factor for downstream correction



Baran-Gale J, Chandra T, Kirschner K. Experimental design for single-cell RNA sequencing. Brief Funct Genomics. 2018 Jul 1;17(4):233-239. doi: 10.1093/bfgp/elx035.

Data Analysis Overview

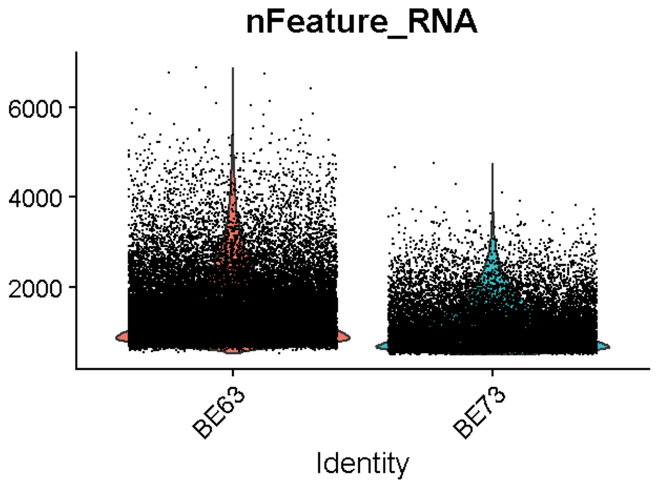


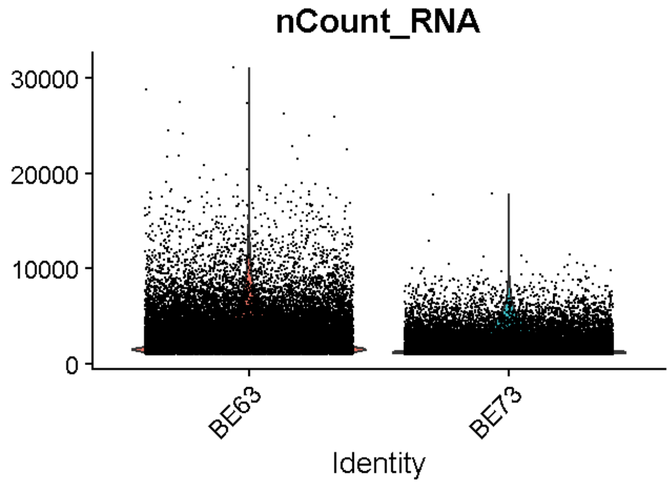
Counting

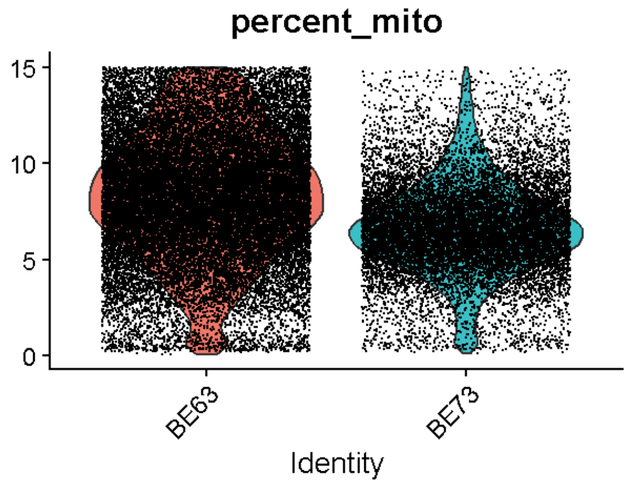
```
#Export path to make cellranger 7.1.0  avaliable
export PATH=$PATH:/home/tigem/software/cellranger-7.1.0

# Define the path to the main directory containing the subfolders with input files
main_dir="/home/tigem/x.bujandac/Polishchuk_scRNAseq/MAN2-HRP_TC_sc"

# Loop through each subfolder
for subfolder in "$main_dir"/*; do

        echo "Processing files in $subfolder..."

        # Run cellranger count on the files in the subfolder
        cellranger count --id=$(basename "$subfolder") \
        --transcriptome=/home/tigem/x.bujandac/Polishchuk_scRNAseq/GRCh38 \
        --fastqs=$subfolder \
        --sample=$(basename "$subfolder") \
        --localcores 8 \
        --localmem 64

        echo "Finished processing files in $subfolder."
        echo
done
```

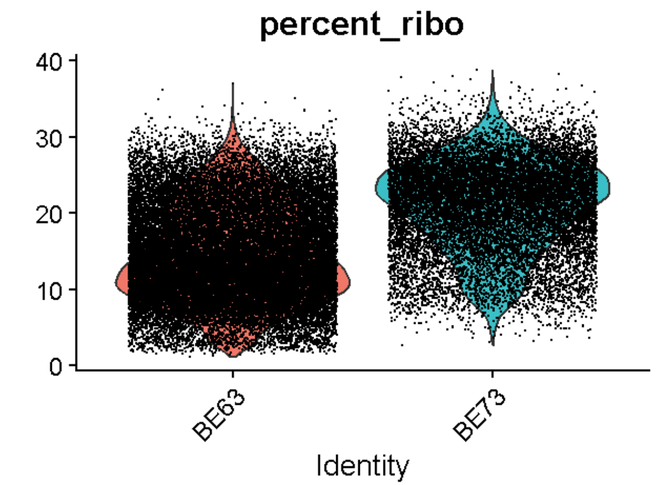
	Cell1	Cell2	...	CellN
Gene1	3	2	.	13
Gene2	2	3	.	1
Gene3	1	14	.	18
...	.	.	.	.
...	.	.	.	.
...	.	.	.	.
GeneM	25	0	.	0

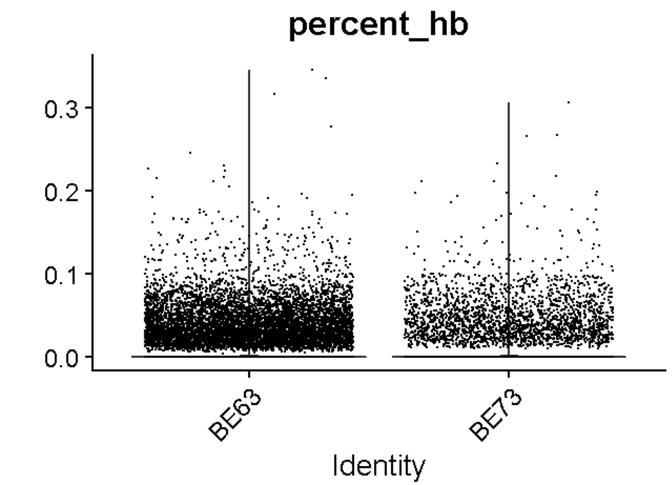
Quality Control











Differences among samples in:

- nFeatures and nCounts
- Eventual apoptotic cells
- Ceck for ribosomal genes and blood related genes

Quality Control

```r
# Create Seurat Object
counts <- CreateSeuratObject(counts = counts, meta.data = metadata, project = "scRNAseqBE")

#============= Quality Control ==============#

#calculate the % of mitocondrial genes for each cell
counts <- PercentageFeatureSet(counts, "^MT-", col.name = "percent_mito")

#calculate the % of ribosomal genes for each cell
counts <- PercentageFeatureSet(counts, "^RP[SL]", col.name = "percent_ribo")

# Percentage hemoglobin genes - includes all genes starting with HB except HBP.
counts <- PercentageFeatureSet(counts, "^HB[^(P)]", col.name = "percent_hb")

#Plot basic statistics
feats <- c("nFeature_RNA", "nCount_RNA", "percent_mito", "percent_ribo", "percent_hb")
VlnPlot(countsFiltered, group.by = "orig.ident", features = feats, pt.size = 0.1, ncol = 3) +
  NoLegend()
```

Data Normalization and Scaling

There are different ways to normalize data:

Logaritmic normalization:

$$x_i = \frac{The\ read\ count\ of\ gene\ X\ in\ cell\ i}{Total\ counts\ of\ cell\ i} \times 10^4 \ (Eq.1)$$

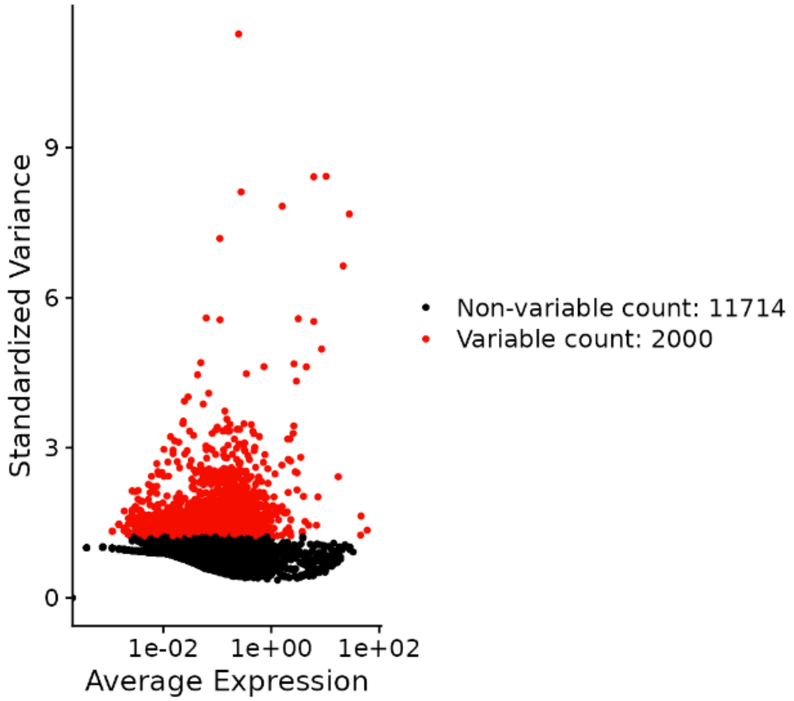
$$f(x_i) = ln(x_i + 1) \ (Eq.2)$$

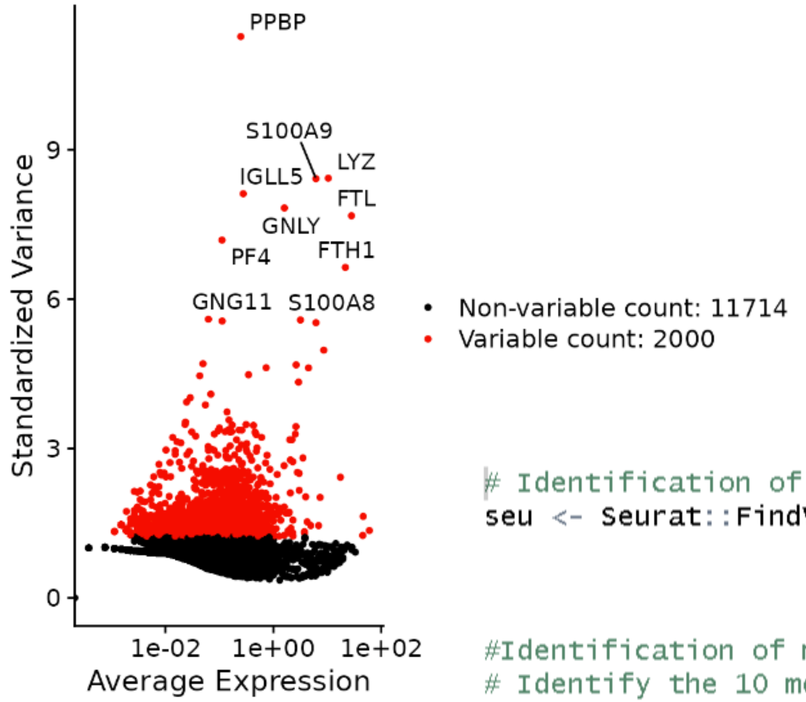
CPM, TPM, RPKM (old but gold)

Work well under the assumption that the amount of RNA is the same in all cells and a uniform scaling factor is applicable for all genes

Sctransform: Normalization and variation control in one step → Provides an excellent tool to not only normalize data, but also stabilize variance and regress unwanted variation

Feature selection





- Identify several genes that exhibit high variability between cells
- Simple mathematical model to detect the most variable genes
- These most variable genes represent the features to prioritize for the downstream analysis

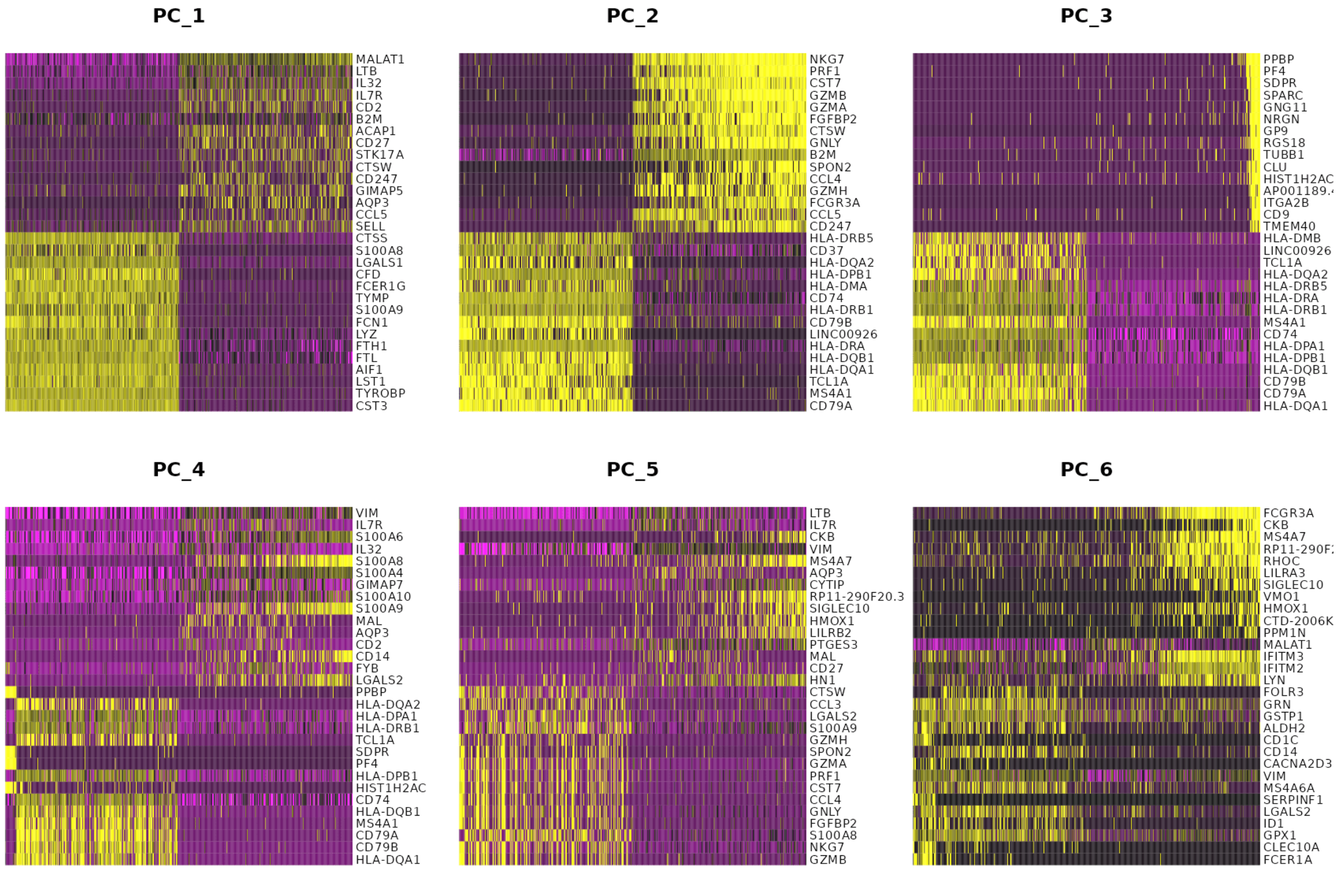
```
# Identification of highly variable features (feature selection)
seu <- Seurat::FindVariableFeatures(seu,
                                    selection.method = "vst",
                                    nfeatures = 2000)

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

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

Adapted from https://satijalab.org/seurat/articles/pbmc3k_tutorial.html

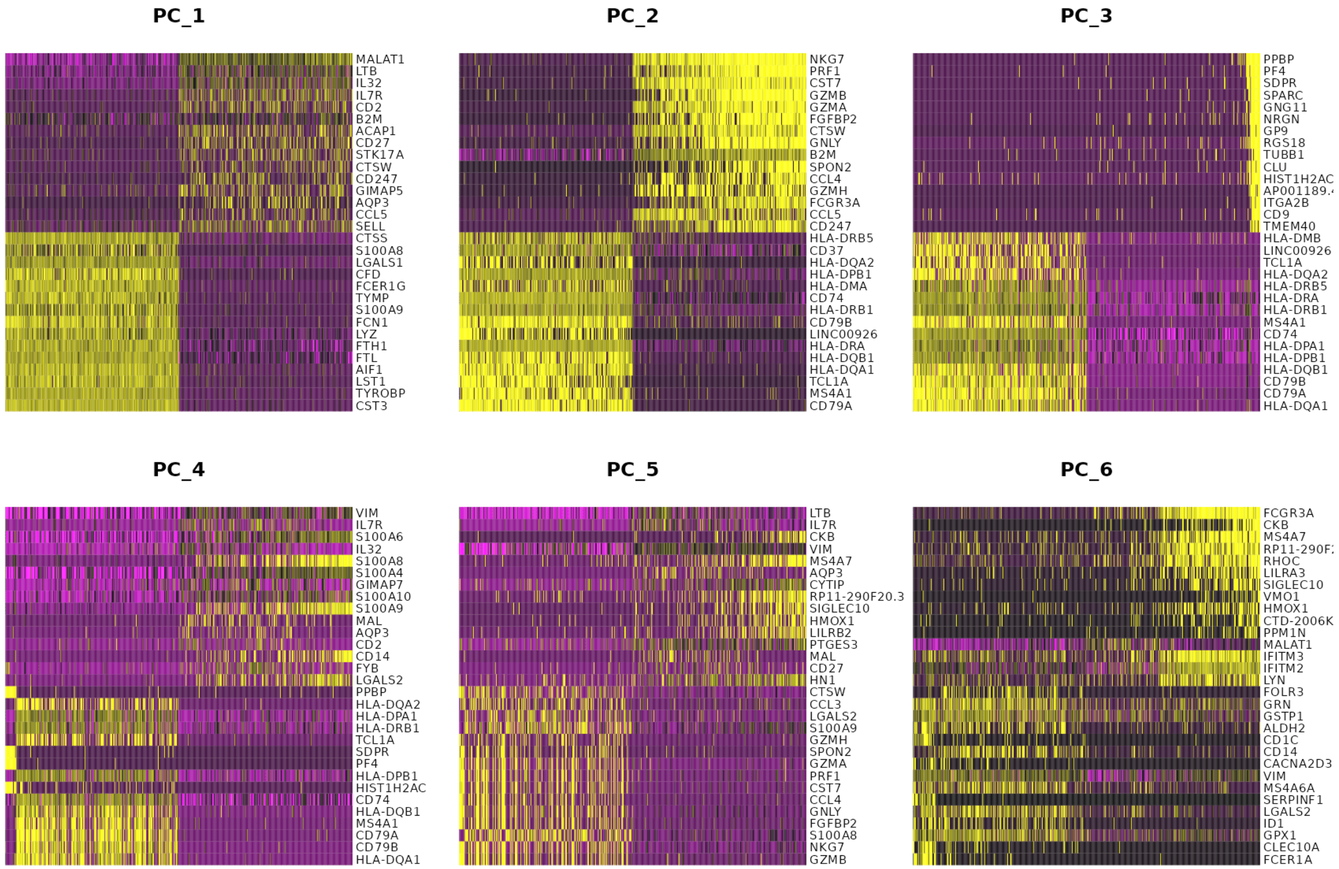
Linear Dimensional Reduction

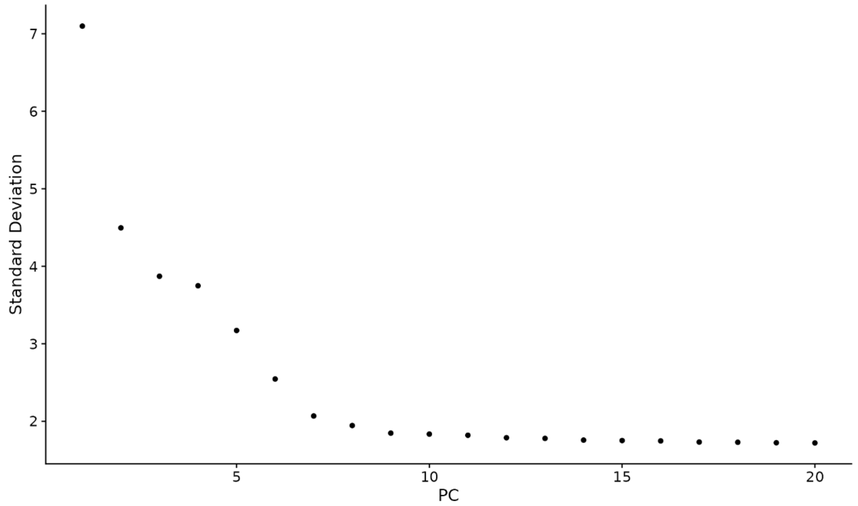


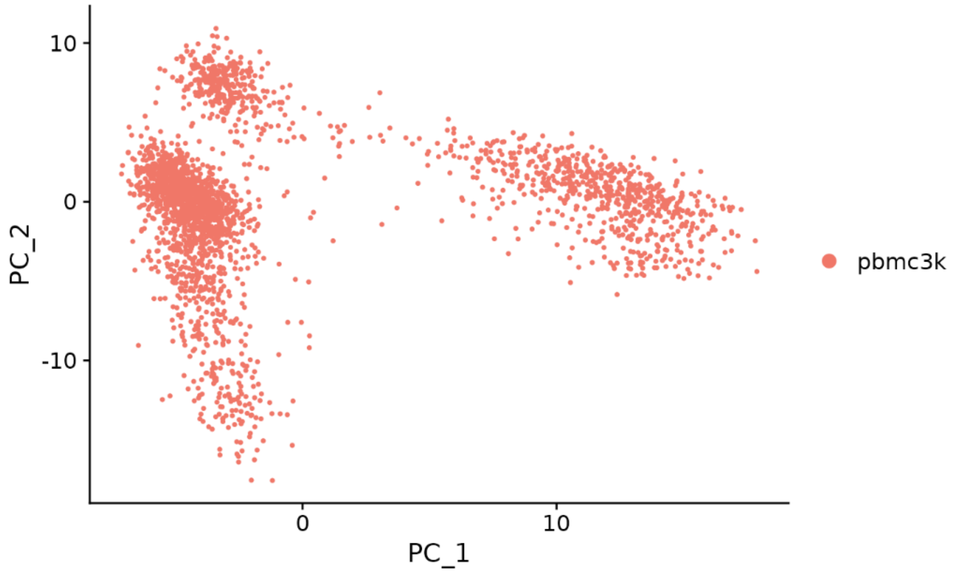
PCA is a statistical technique for reducing the dimensionality of the dataset by linearly transforming the data into a new coordinate system where most of the variation in the data can be described

```
#========== Dimensional Reduction =========#
# PCA
countsFiltered <- RunPCA(countsFiltered, features = VariableFeatures(object = countsFiltered))
```

Linear Dimensional Reduction





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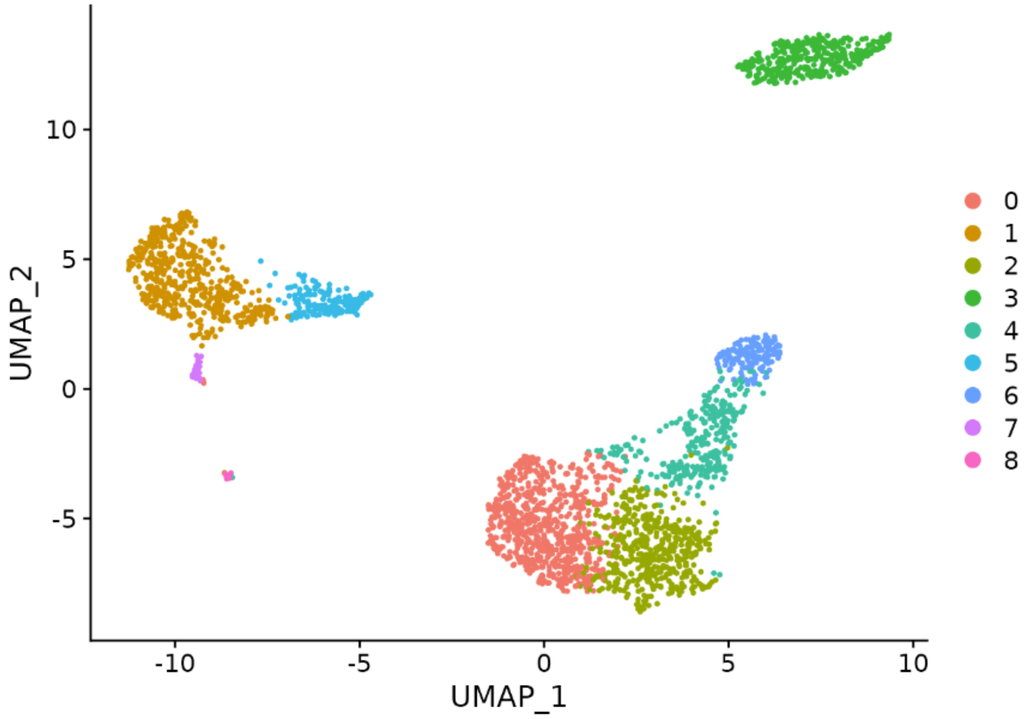
```

Non - Linear Dimensional Reduction




```
#============= Cluster the cells ==============#
countsFiltered <- FindNeighbors(countsFiltered, dims = 1:10)
countsFiltered <- FindClusters(countsFiltered, resolution = 0.5)
head(Idents(countsFiltered), 5)

#============= Non-linear dimensional reduction ==============#
#Run UMAP
countsFiltered <- RunUMAP(countsFiltered, dims = 1:6)

# individual clusters
DimPlot(countsFiltered, reduction = "umap",label = TRUE, repel = TRUE)
```

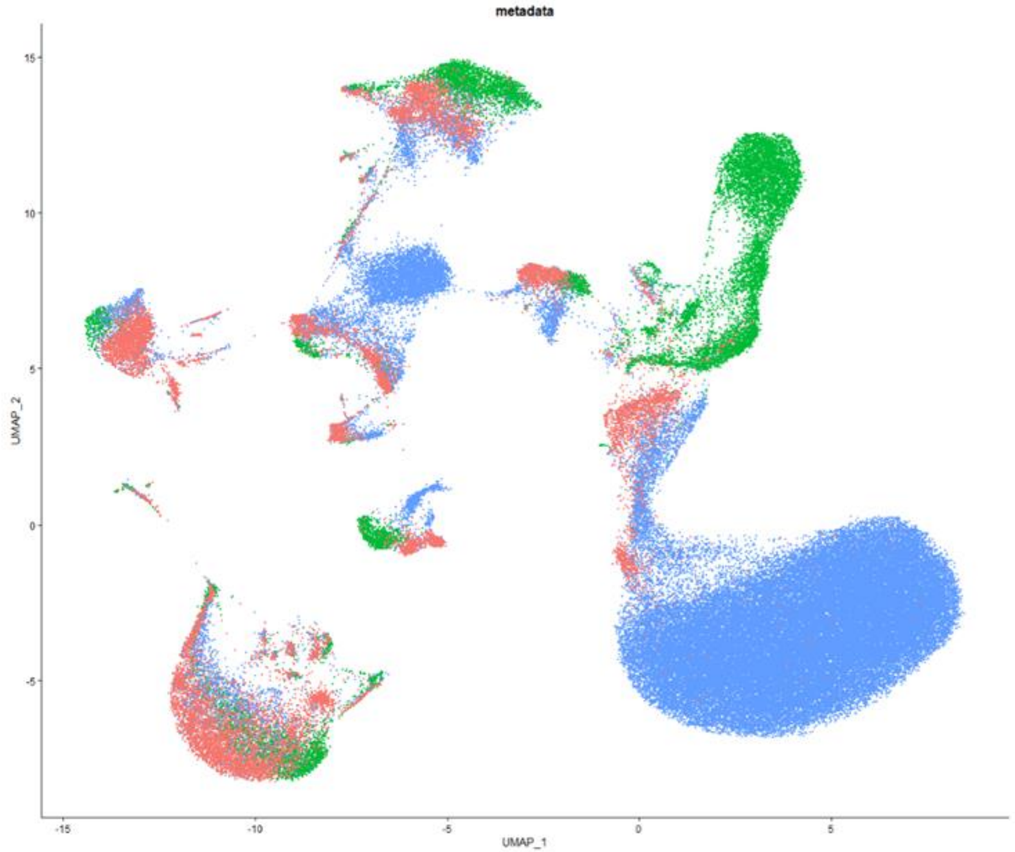
First we need to cluster cells!

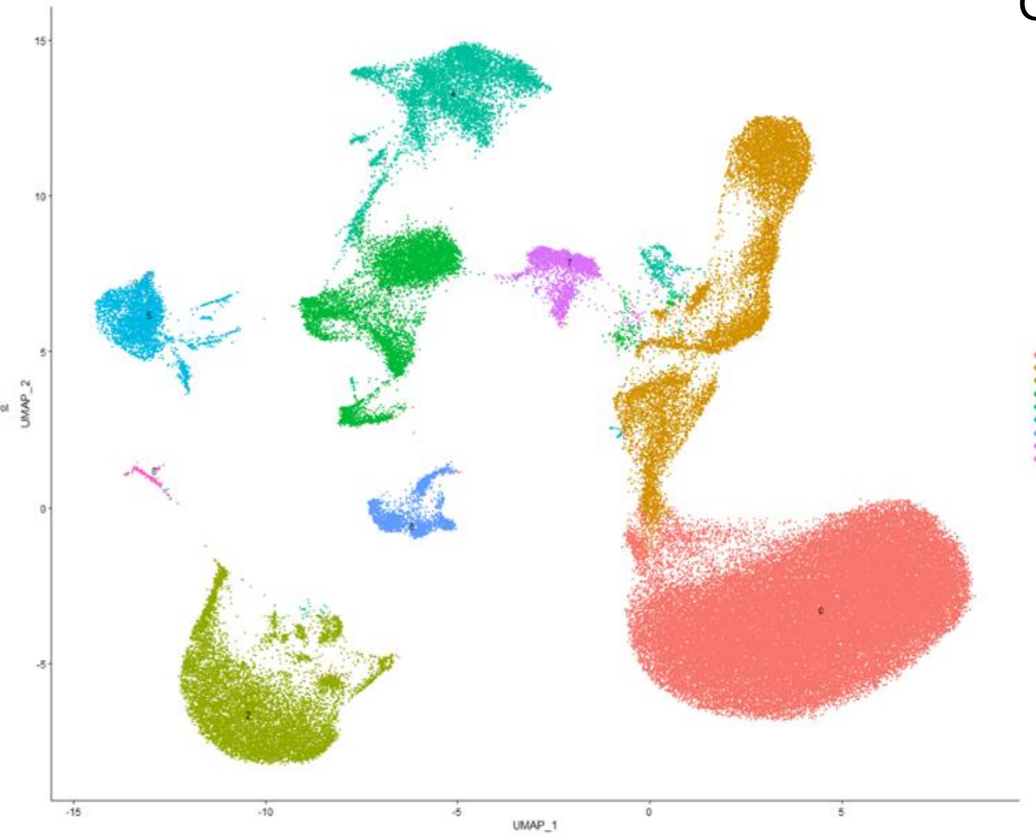
Clustering groups a set of objects in a way that objects in the same group (**cluster**) are more similar to each other than to those in other groups

Adapted from https://satijalab.org/seurat/articles/pbmc3k_tutorial.html

Non - Linear Dimensional Reduction

We can take advantage of these methods to identify potential bath effects



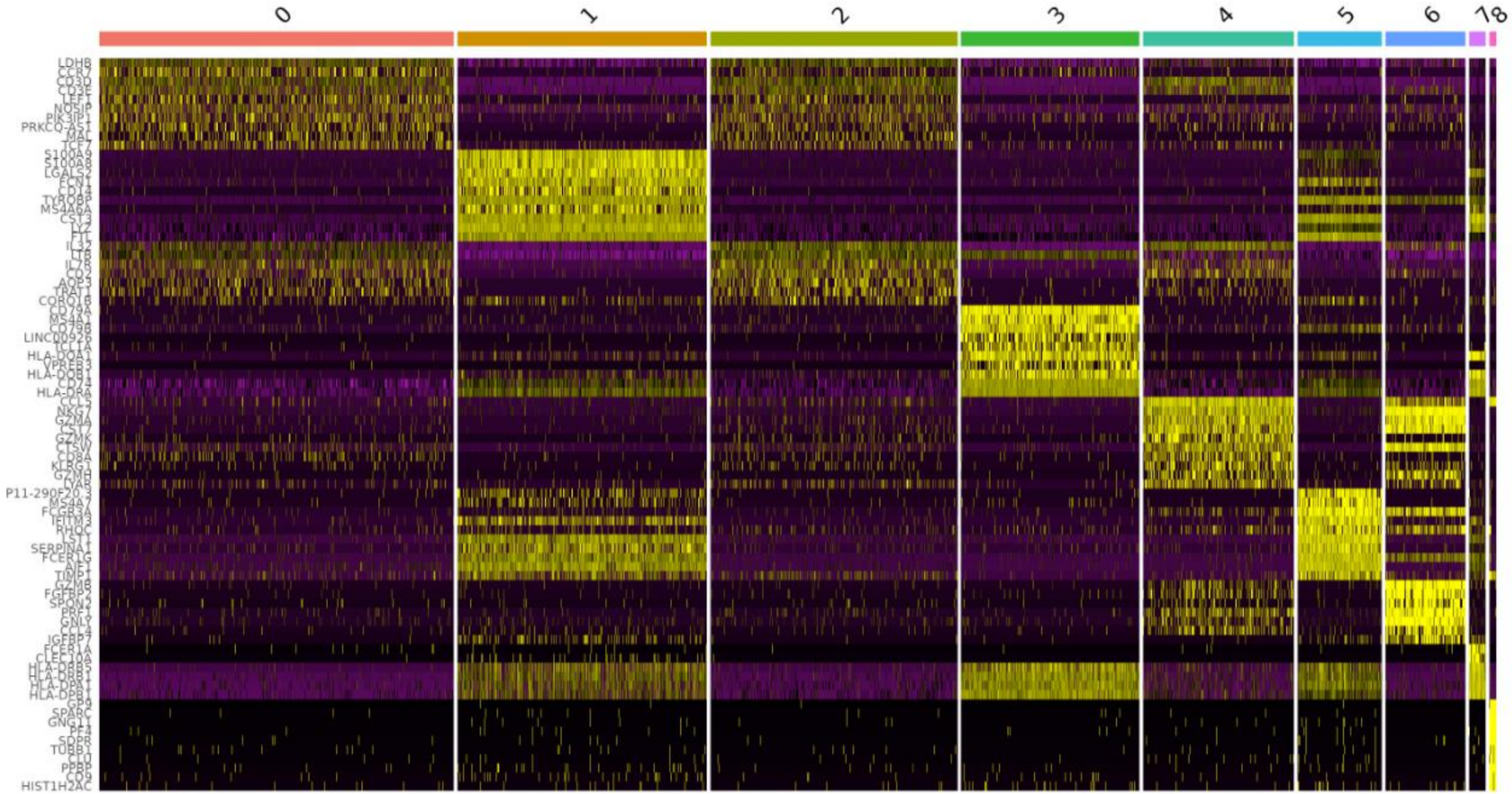


Clusters are driven by samples

↓

Batch correction

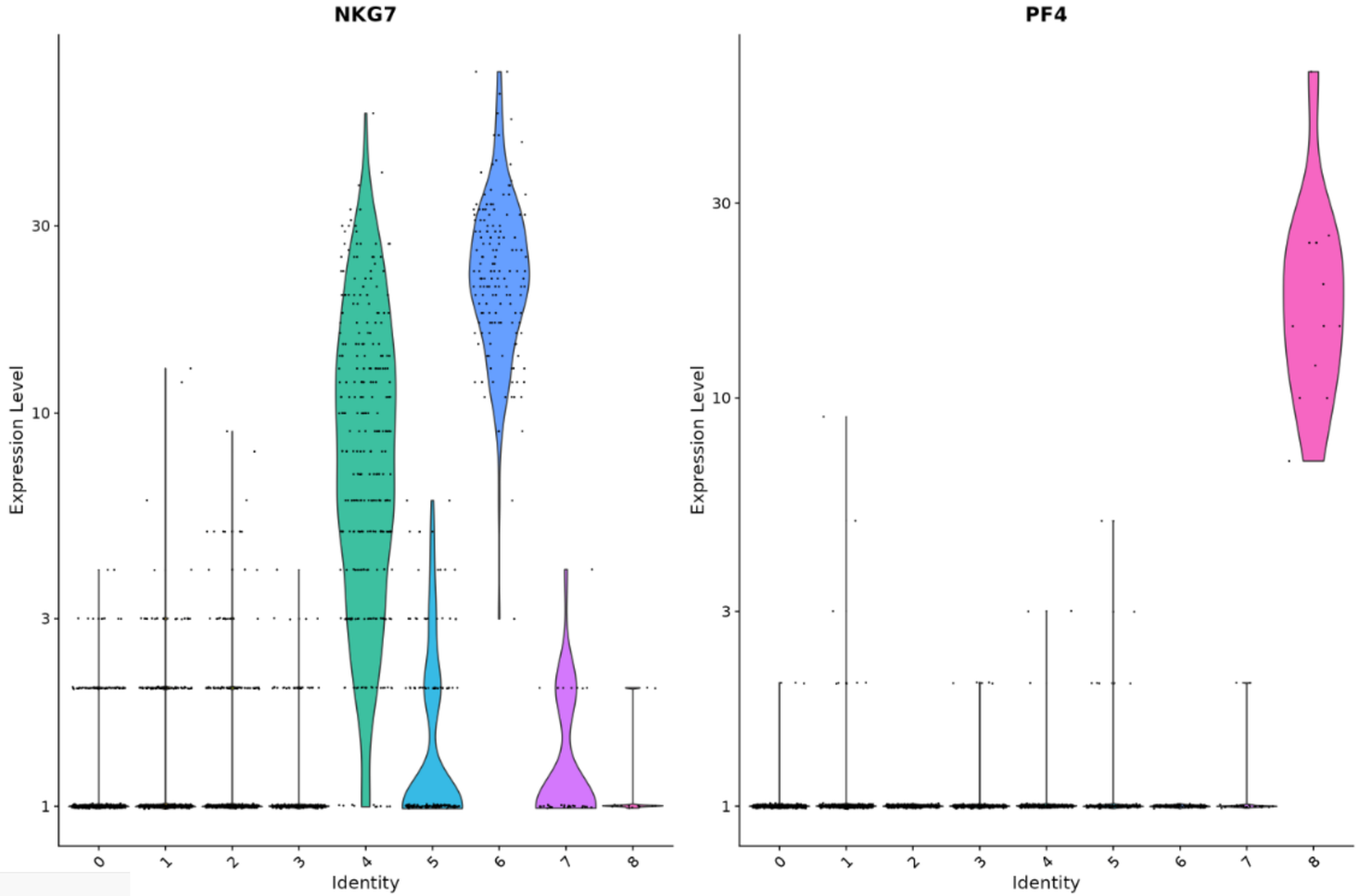
Finding Differentially Expressed Features




```
# find all markers of cluster 8
cluster8.markers <- FindMarkers(countsFiltered, ident.1 = 8, min.pct = 0.25)
```

Adapted from https://satijalab.org/seurat/articles/pbmc3k_tutorial.html

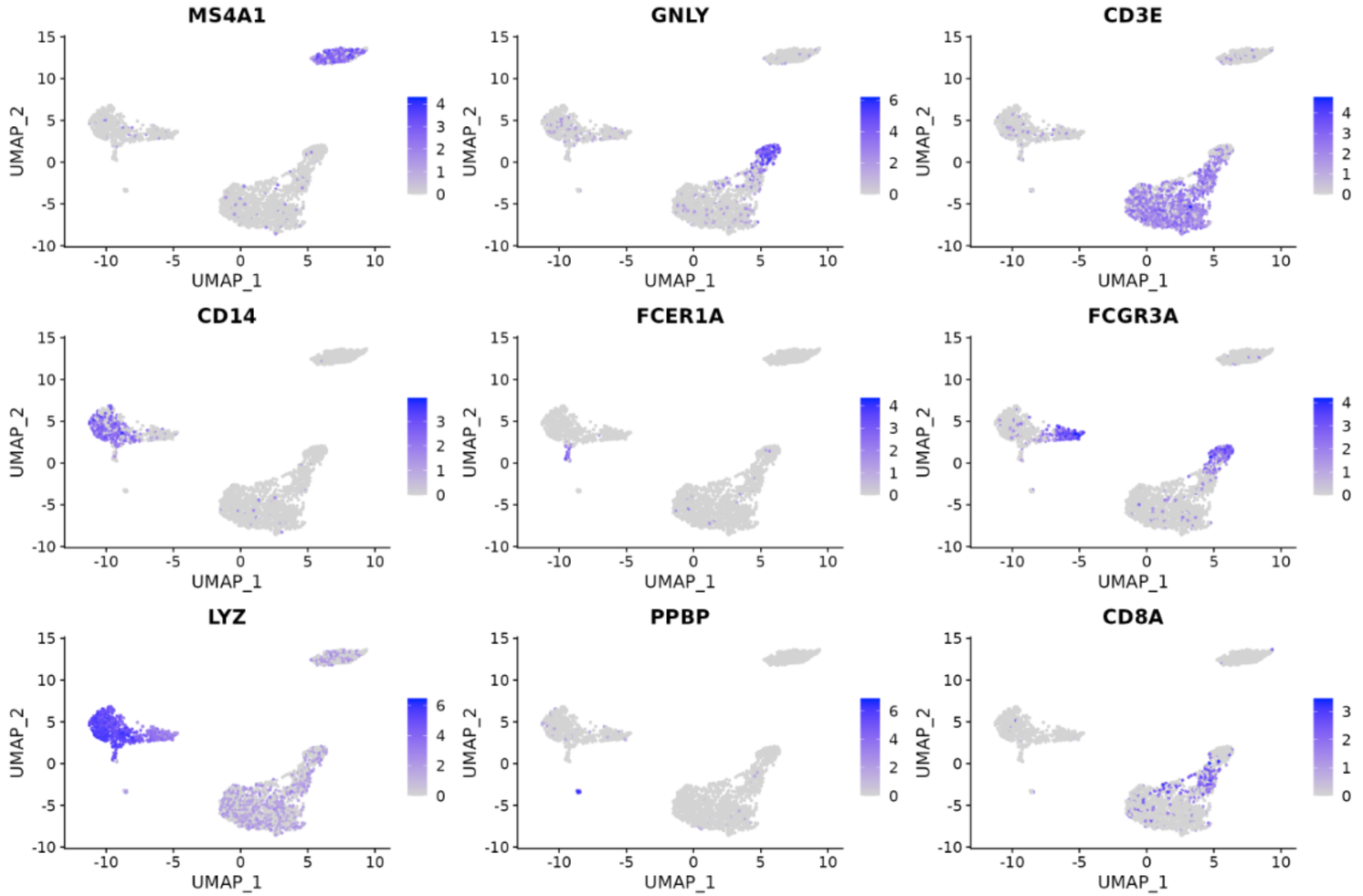
Finding Differentially Expressed Features




```
# you can plot raw counts as well
VlnPlot(pbmc, features = c("NKG7", "PF4"), slot = "counts", log = TRUE)
```

Adapted from https://satijalab.org/seurat/articles/pbmc3k_tutorial.html

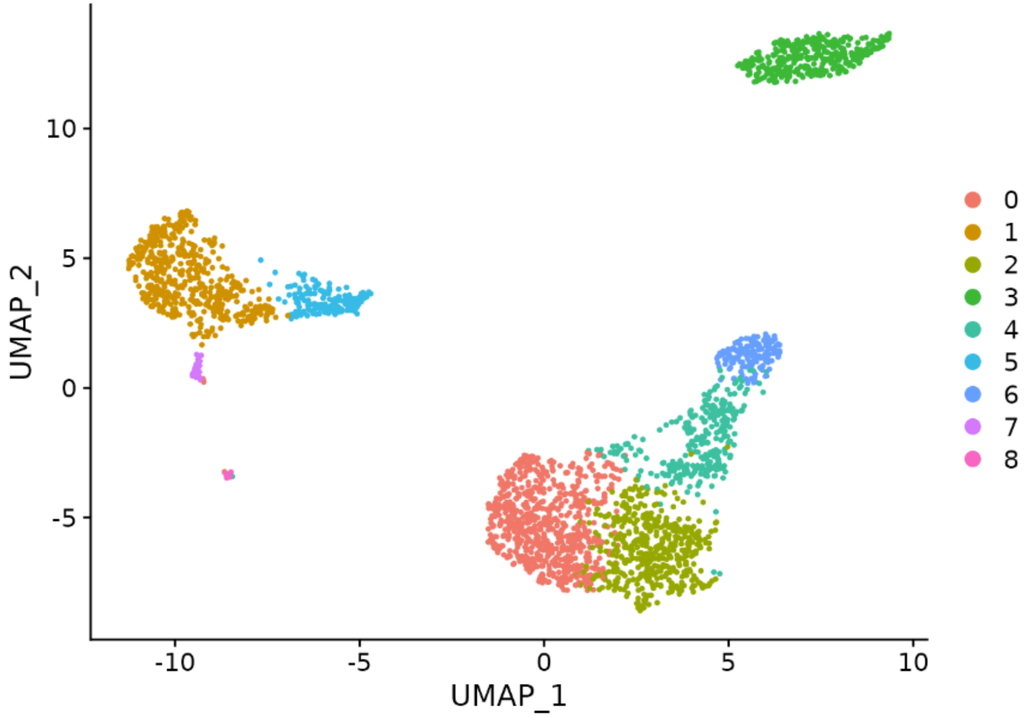
Finding Differentially Expressed Features




```
FeaturePlot(pbmc, features = c("MS4A1", "GNLY", "CD3E", "CD14", "FCER1A", "FCGR3A", "LYZ", "PPBP",
    "CD8A"))
```

Adapted from https://satijalab.org/seurat/articles/pbmc3k_tutorial.html

Assigning Cell Identities to Clusters



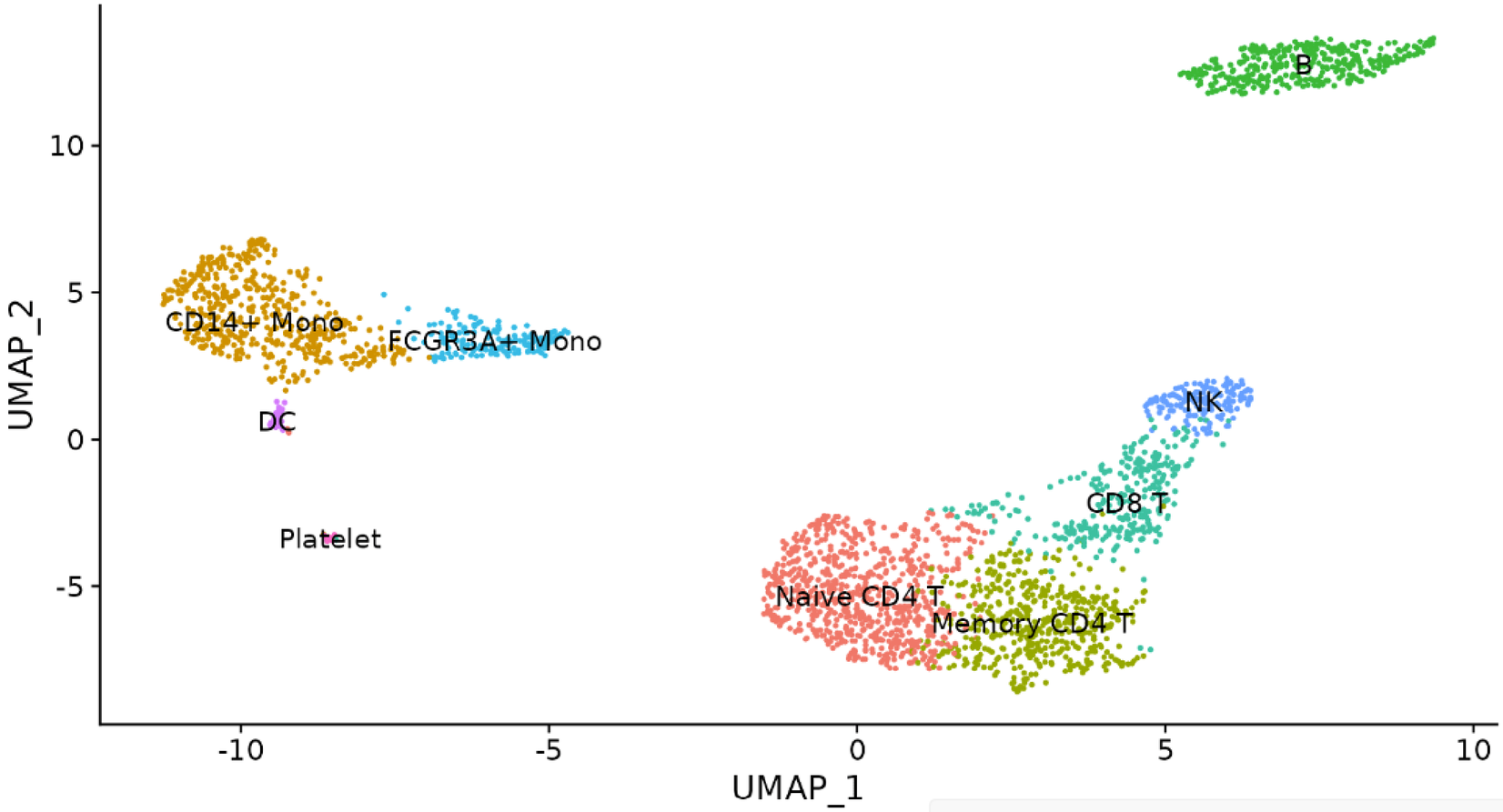
How to assign cell identities:

- Manually
- SingleR
- Using Machine Learning techniques

```
new.cluster.ids <- c("Naive CD4 T", "CD14+ Mono", "Memory CD4 T", "B", "CD8 T", "FCGR3A+ Mono",
    "NK", "DC", "Platelet")
names(new.cluster.ids) <- levels(pbmc)
pbmc <- RenameIdents(pbmc, new.cluster.ids)
DimPlot(pbmc, reduction = "umap", label = TRUE, pt.size = 0.5) + NoLegend()
```

Adapted from https://satijalab.org/seurat/articles/pbmc3k_tutorial.html

Assigning Cell Identities to Clusters



How to assign cell identities:

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```
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```

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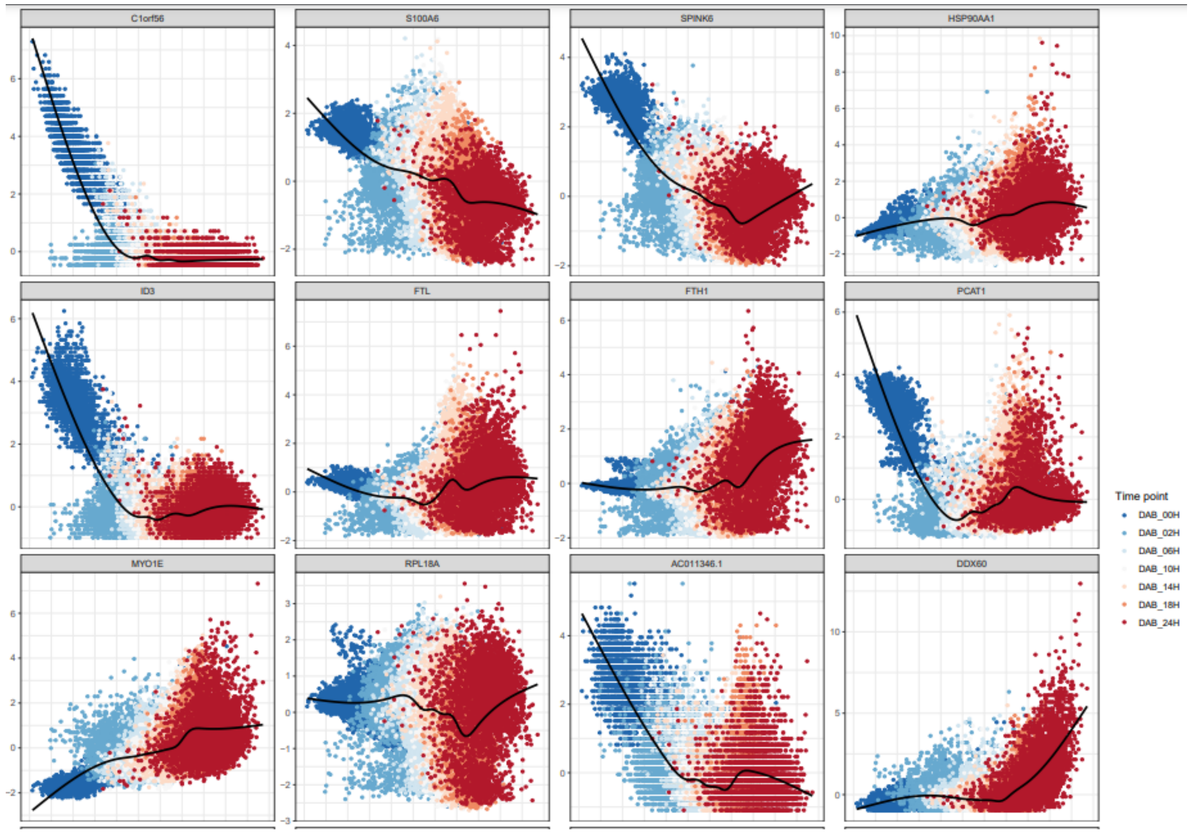
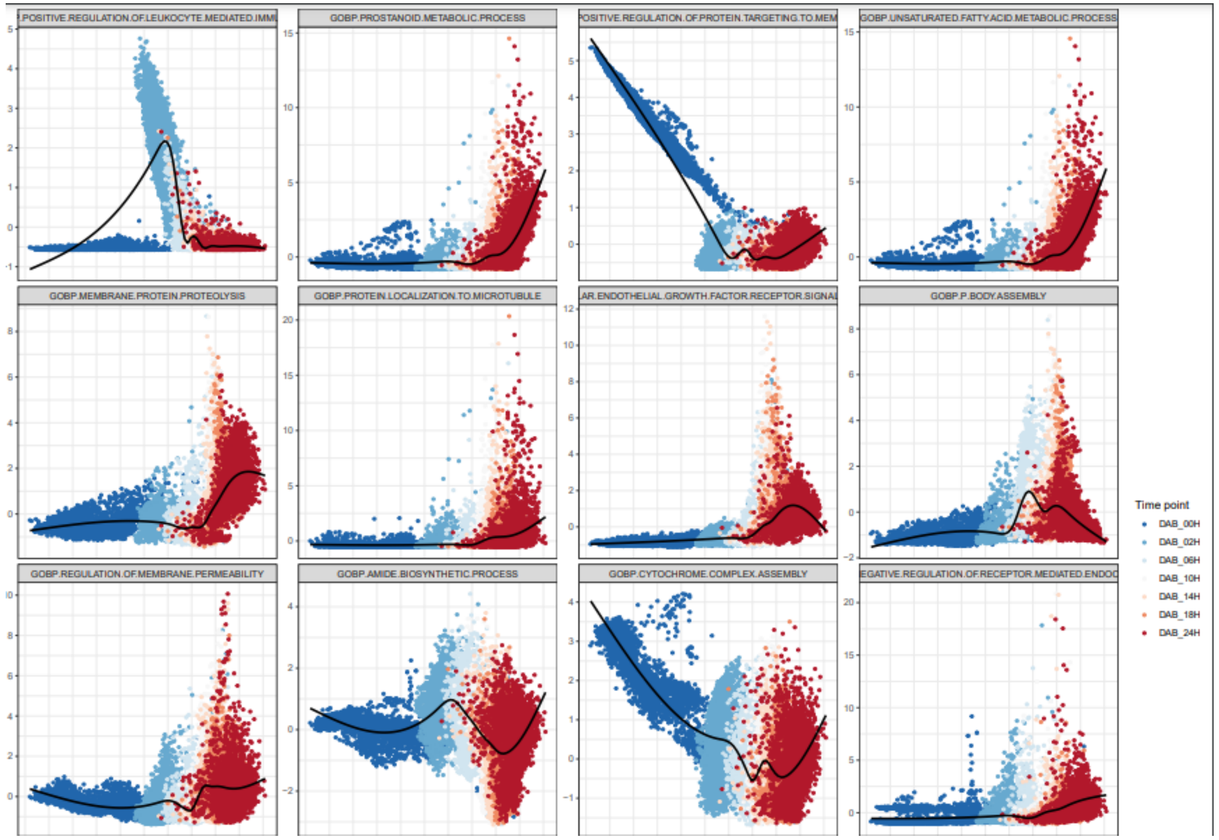
Pseudotime Analysis





Melania Franchini and others, Single-cell gene set enrichment analysis and transfer learning for functional annotation of scRNA-seq data, *NAR Genomics and Bioinformatics*, Volume 5, Issue 1, March 2023,

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