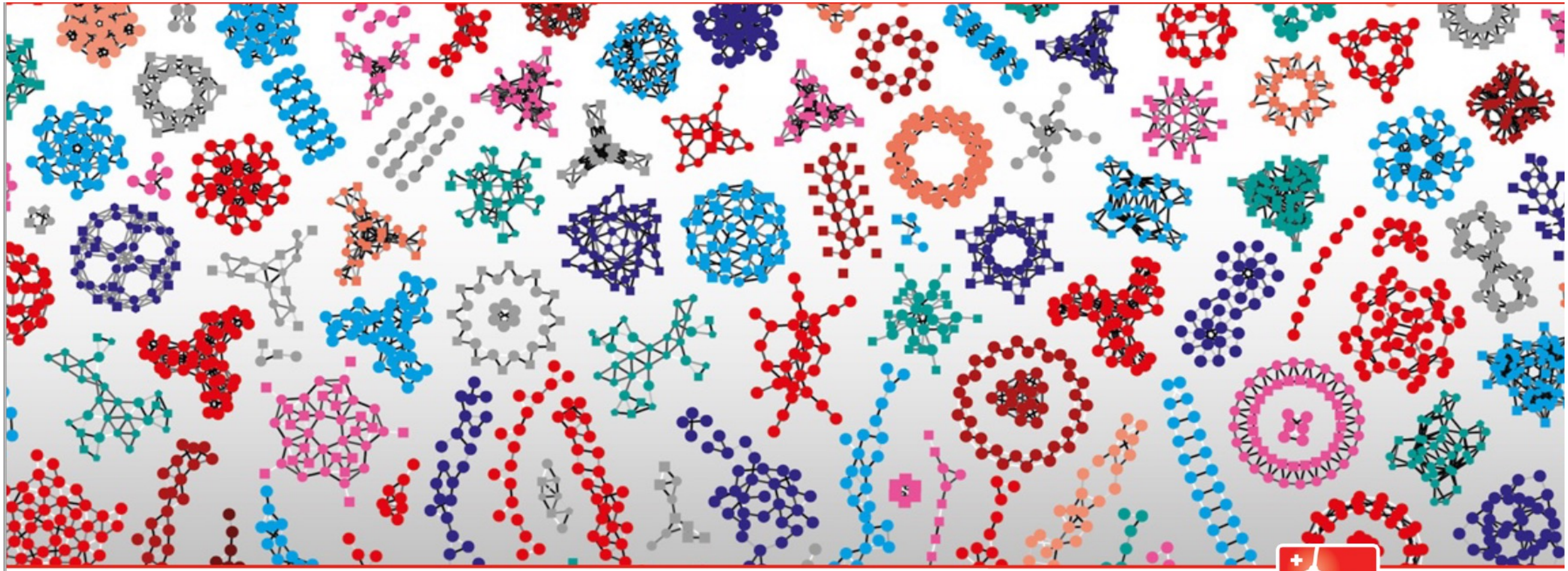


Swiss Institute of Bioinformatics

Single cell transcriptomics data analysis

Marker gene identification
&
Differential gene expression analysis

Two types of gene expression analysis:

- **Marker gene identification**:

  genes overexpressed by each cell type, cell cluster, ..., within the dataset => can *help with cell type annotation*

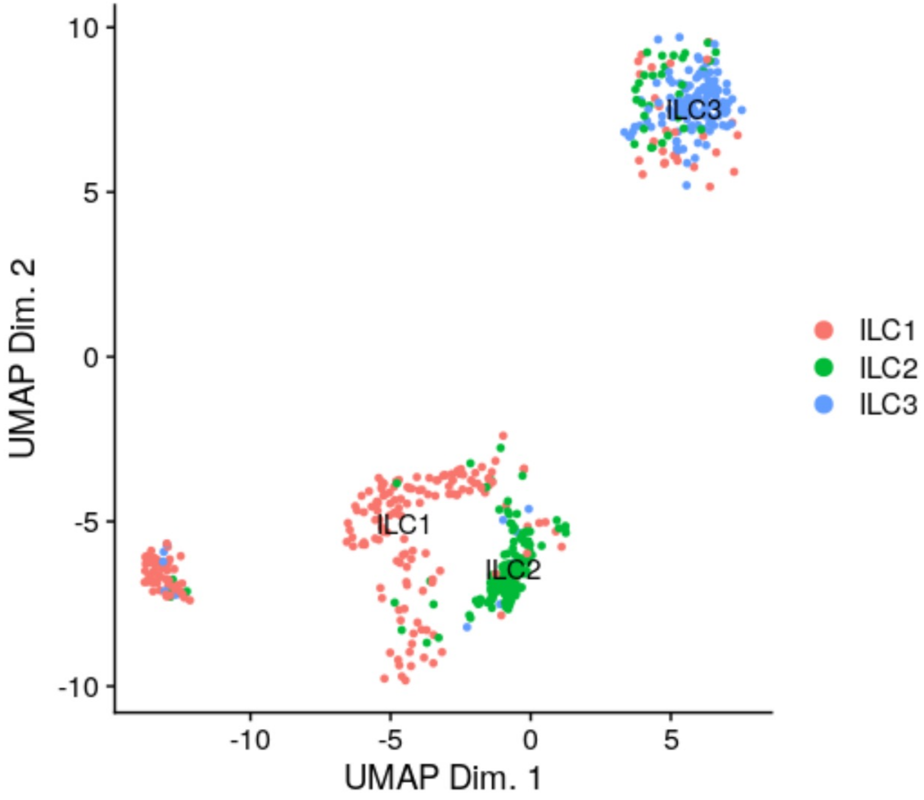
- **Differential gene expression analysis**:

  genes impacted by experimental conditions within a cell type, cell cluster, ..., etc

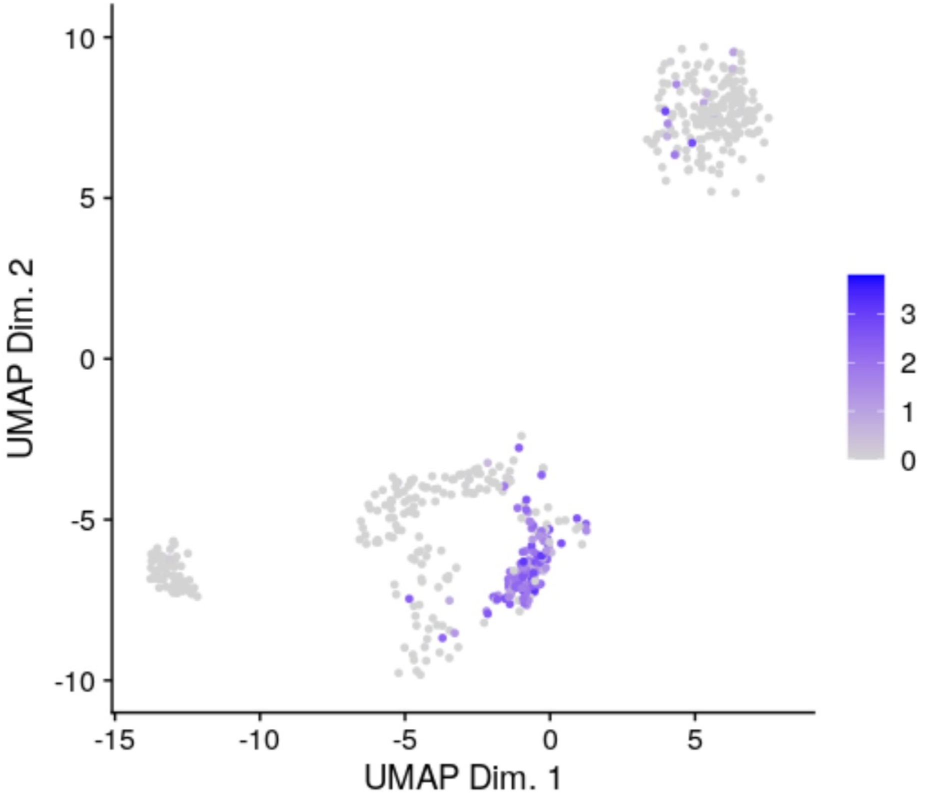
Marker gene identification

Which genes are more (or less) expressed in one cell type than in the others?

3 cell types (clusters)



PTGDR2



Mazzurana et al, Cell Research, 2021

Marker gene identification

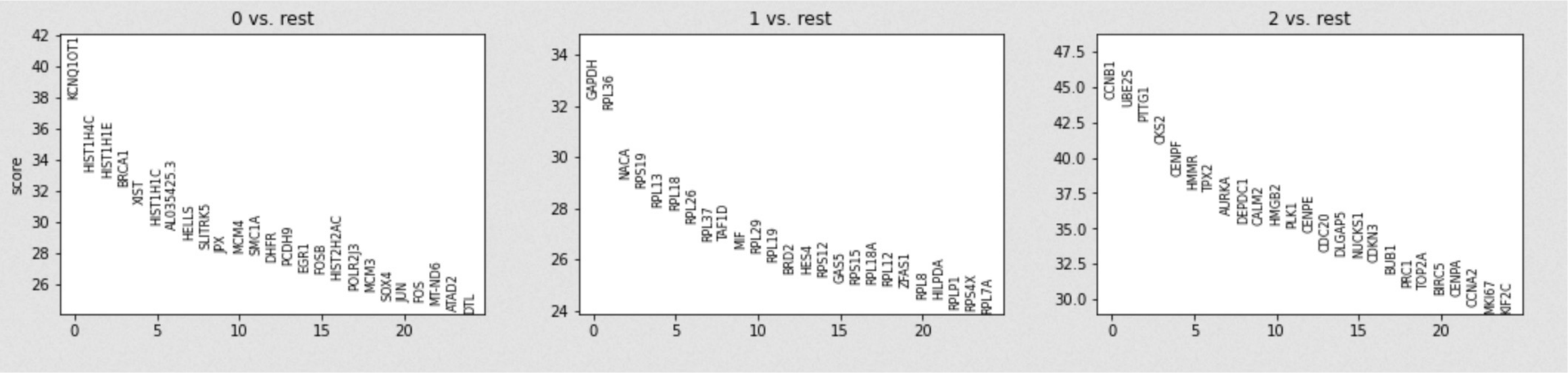
- Compare each cluster of cells against all other cells = 2 group-comparison
- **Wilcoxon Rank Sum test** (Mann-Whitney U test)

```
sc.tl.rank_genes_groups(adata,
groupby="leiden", method="wilcoxon)
```

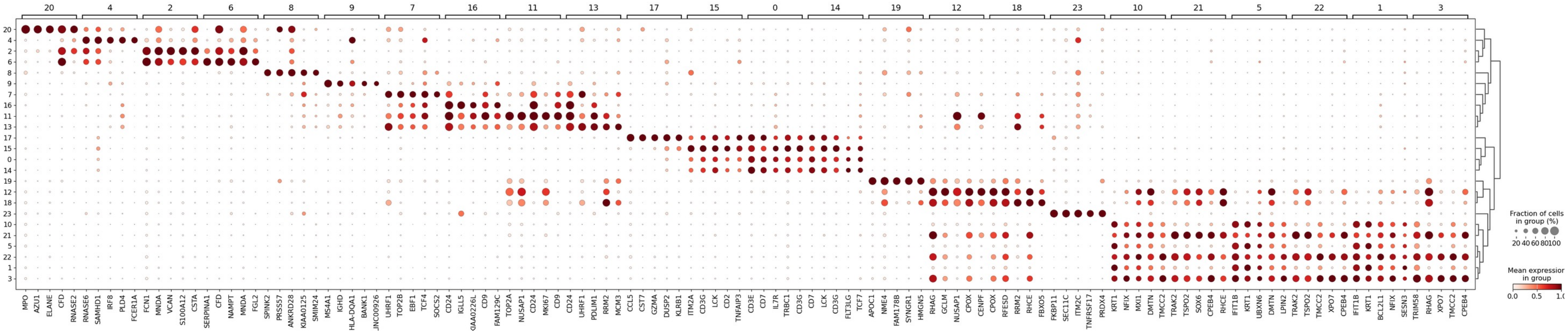
-> Returns an AnnData

- Plot the MW U statistic - the higher, the more significant:

```
sc.pl.rank_genes_groups(adata, n_genes=25)
```



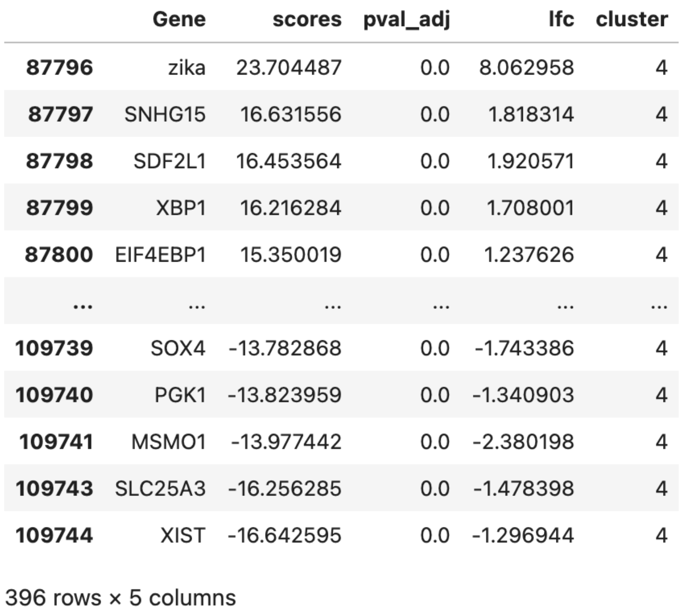
Marker gene identification



```
sc.pl.rank_genes_groups_dotplot(adata, groupby="leiden", n_genes=5)
```

Table of marker genes in each cluster versus all other cells - obtain $\log_2$(fold change), p-value and adjusted p-value

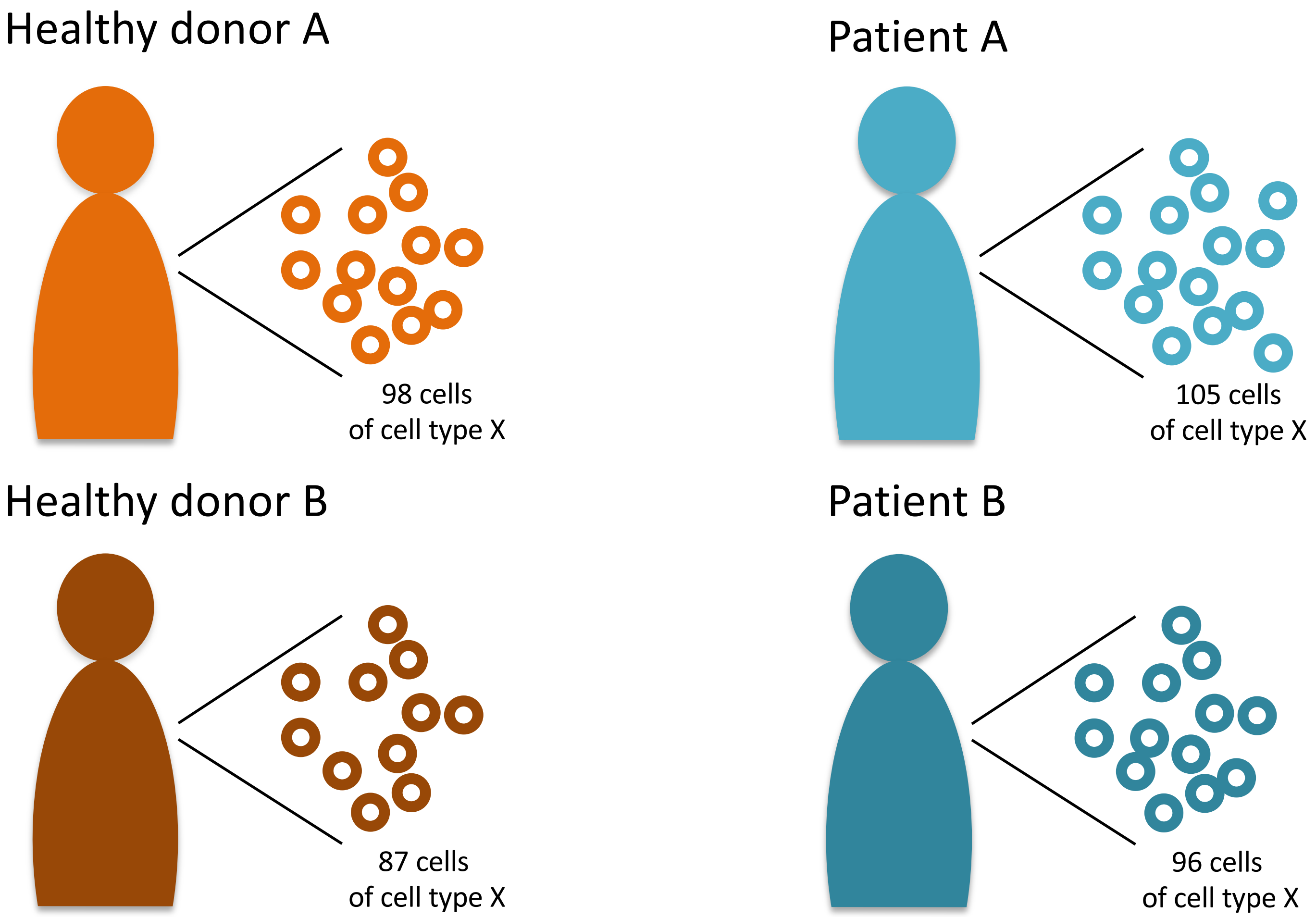
https://github.com/mousepixels/sanbomics_scripts/blob/main/Scanpy_intro_pp_clustering_markers.ipynb
https://www.youtube.com/watch?v=5HuOGZEu2HY&t=21s&ab_channel=Sanbomics



	Gene	scores	pval_adj	lfc	cluster
87796	zika	23.704487	0.0	8.062958	4
87797	SNHG15	16.631556	0.0	1.818314	4
87798	SDF2L1	16.453564	0.0	1.920571	4
87799	XBP1	16.216284	0.0	1.708001	4
87800	EIF4EBP1	15.350019	0.0	1.237626	4
...	...	...	...	...	...
109739	SOX4	-13.782868	0.0	-1.743386	4
109740	PGK1	-13.823959	0.0	-1.340903	4
109741	MSMO1	-13.977442	0.0	-2.380198	4
109743	SLC25A3	-16.256285	0.0	-1.478398	4
109744	XIST	-16.642595	0.0	-1.296944	4

396 rows × 5 columns

DGE analysis between 2 conditions :



Healthy donor A

98 cells
of cell type X

Patient A

105 cells
of cell type X

Healthy donor B

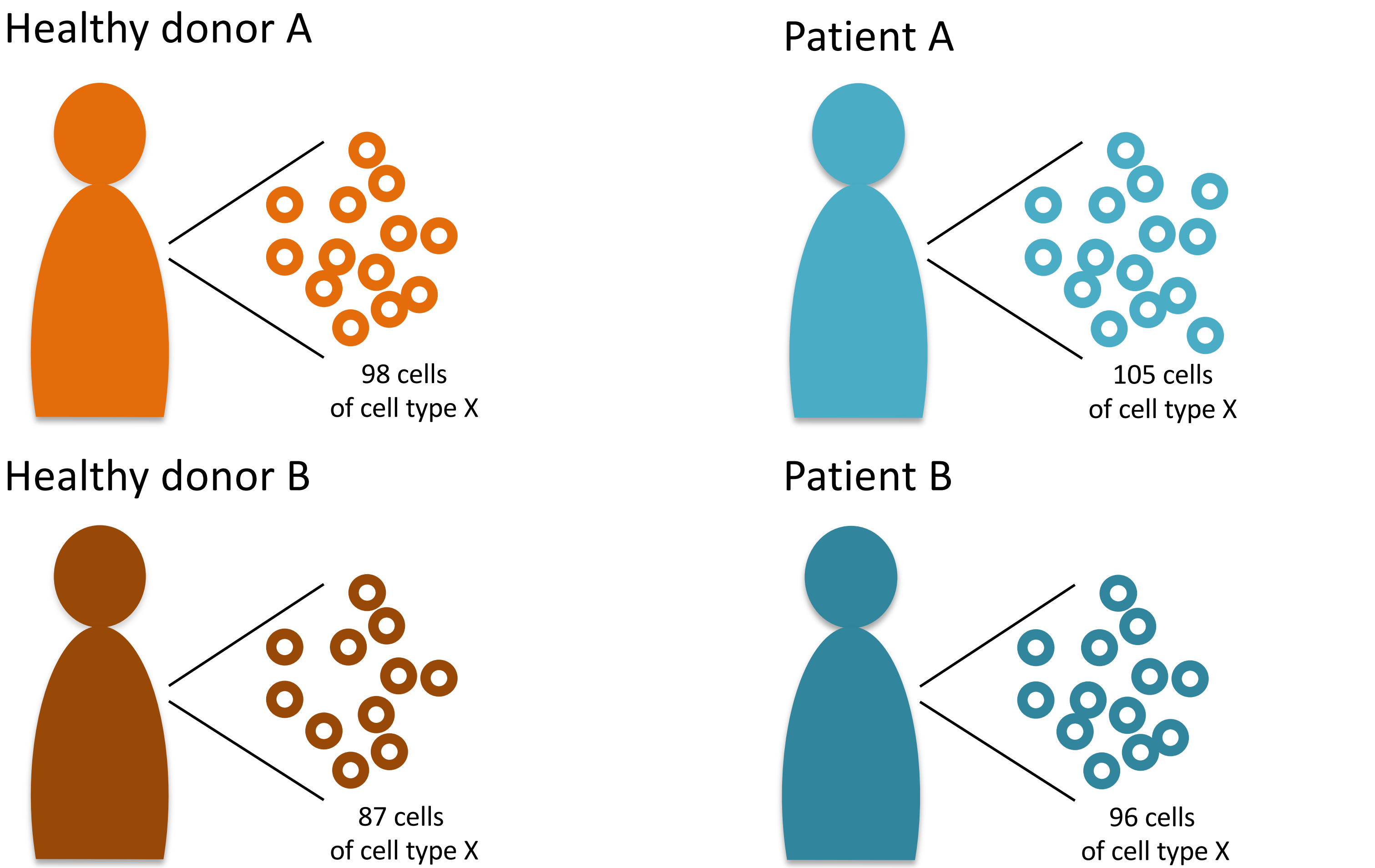
87 cells
of cell type X

Patient B

96 cells
of cell type X

DGE analysis between 2 conditions : Problem of pseudo-replication?

How many independent replicates do we have, ~200 or 2 replicates per condition?



Healthy donor A

98 cells
of cell type X

Patient A

105 cells
of cell type X

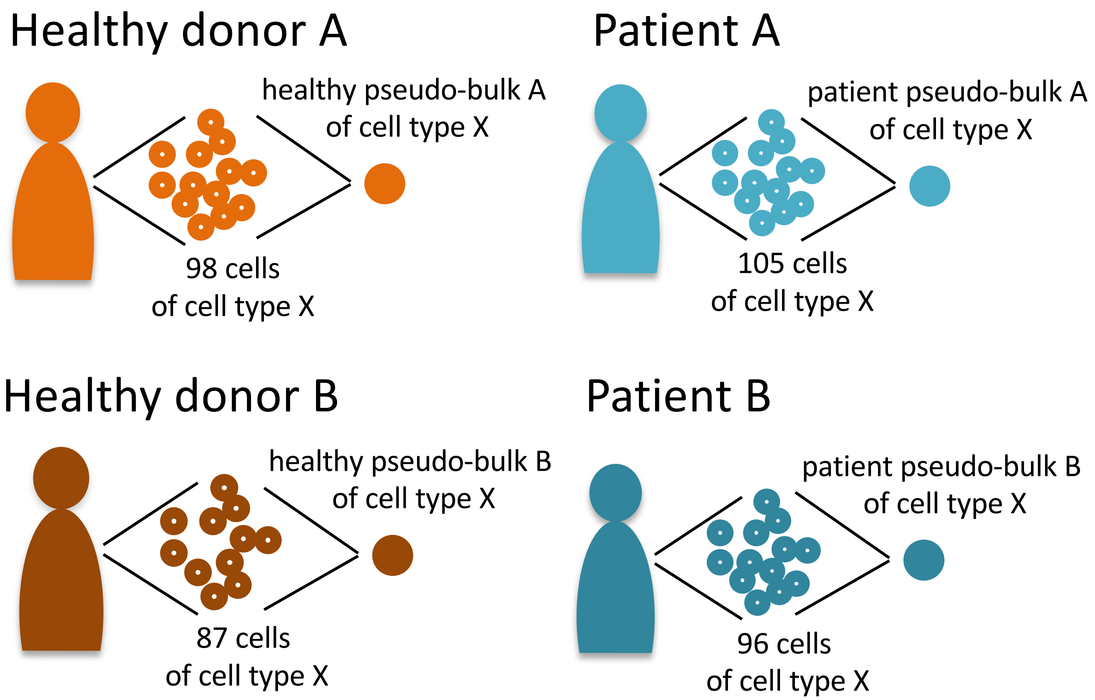
Healthy donor B

87 cells
of cell type X

Patient B

96 cells
of cell type X

Pseudo-bulk DE analysis


Healthy donor A
healthy pseudo-bulk A of cell type X
98 cells of cell type X
Patient A
patient pseudo-bulk A of cell type X
105 cells of cell type X
Healthy donor B
healthy pseudo-bulk B of cell type X
87 cells of cell type X
Patient B
patient pseudo-bulk B of cell type X
96 cells of cell type X

For cell type/cluster X:
From a matrix of
386 cells × 33694 genes

↓

To a matrix of
4 pseudo-bulk samples × 33694 genes

↓

Perform a DGE analysis of
patient vs healthy with n=2 per condition

↓

Repeat for every individual
cell type/cluster

PyDESeq2

https://pydeseq2.readthedocs.io/en/latest/auto_examples/index.html

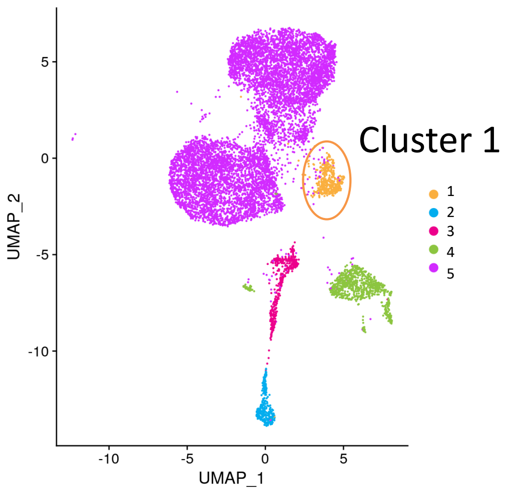
- Implementation of R’s DESeq2 package: Method designed for bulk RNA seq analysis

- **Wald test**: DESeq2 models count data using a negative binomial distribution. It fits a GLM to the counts of each gene. The Wald test is used to determine the significance of individual coefficients in the GLM fitted to each gene.

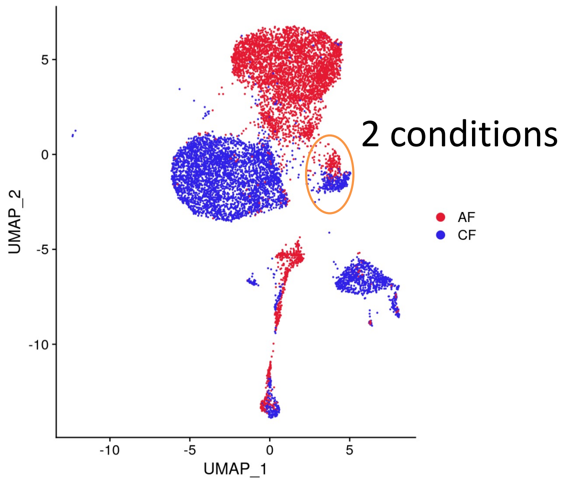
  $\text{counts}_i \sim \text{condition} + \text{other covariates}$

- It can incorporate factors such as experimental conditions (multi-factor, factorial, …), batch effects and other covariates.
- How to create design matrices and contrasts:
  https://doi.org/10.12688%2Ff1000research.27893.1

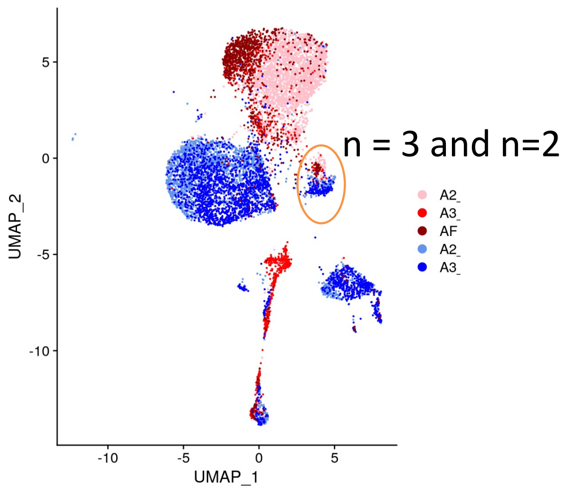
DGE analysis between 2 conditions :



Cluster 1



2 conditions

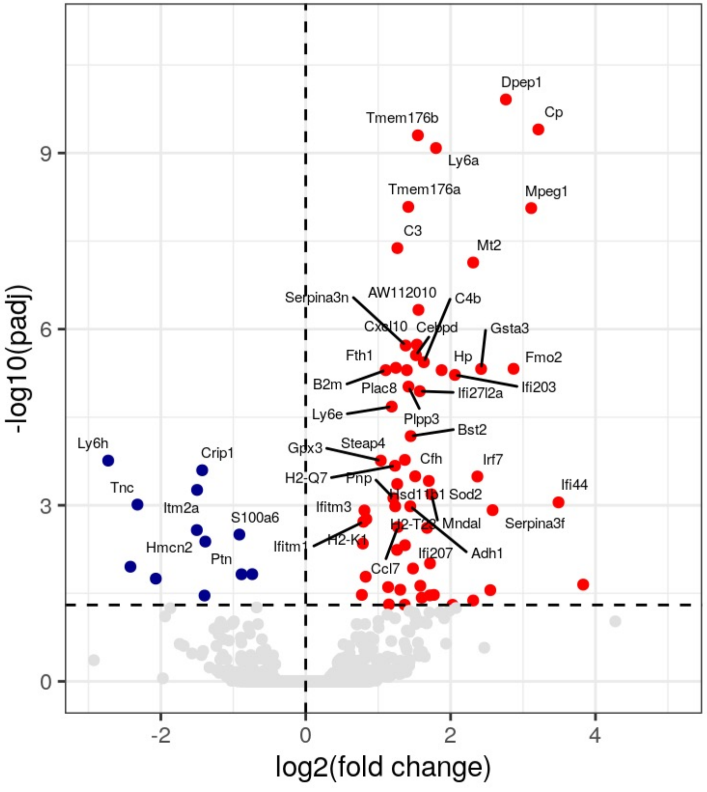


n = 3 and n=2

Sum counts of all cells of cluster 1 per sample:

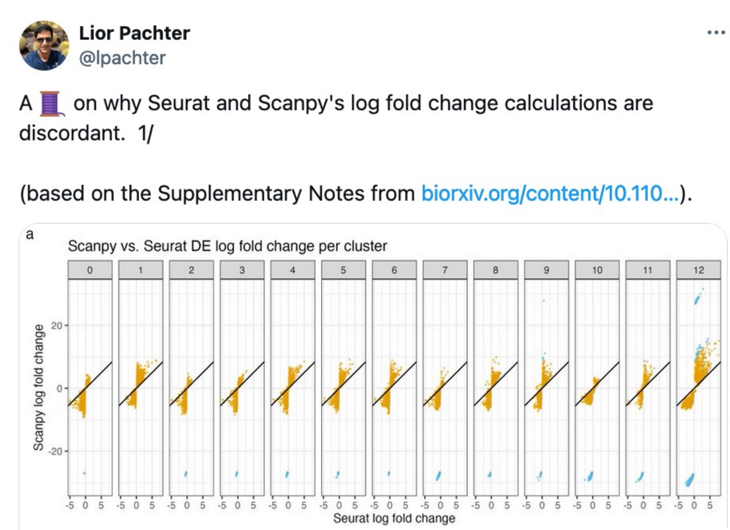
	Xkr4	Gm1992	Gm3738	Rp1	Sox17	Gm3732	..
A2_AF	18	1	240	0	4	68	..
A3_AF	25	0	489	0	5	45	..
AF	40	2	500	0	1	32	..
A2_CF	70	0	407	0	0	45	..
A3_CF	36	2	230	0	3	23	..

DGE with PyDESeq2



https://github.com/mousepixels/sanbomics_scripts/blob/main/pseudobulk_pyDeseq2.ipynb
https://www.youtube.com/watch?v=Ee0PQUwVH8Q&ab_channel=Sanbomics

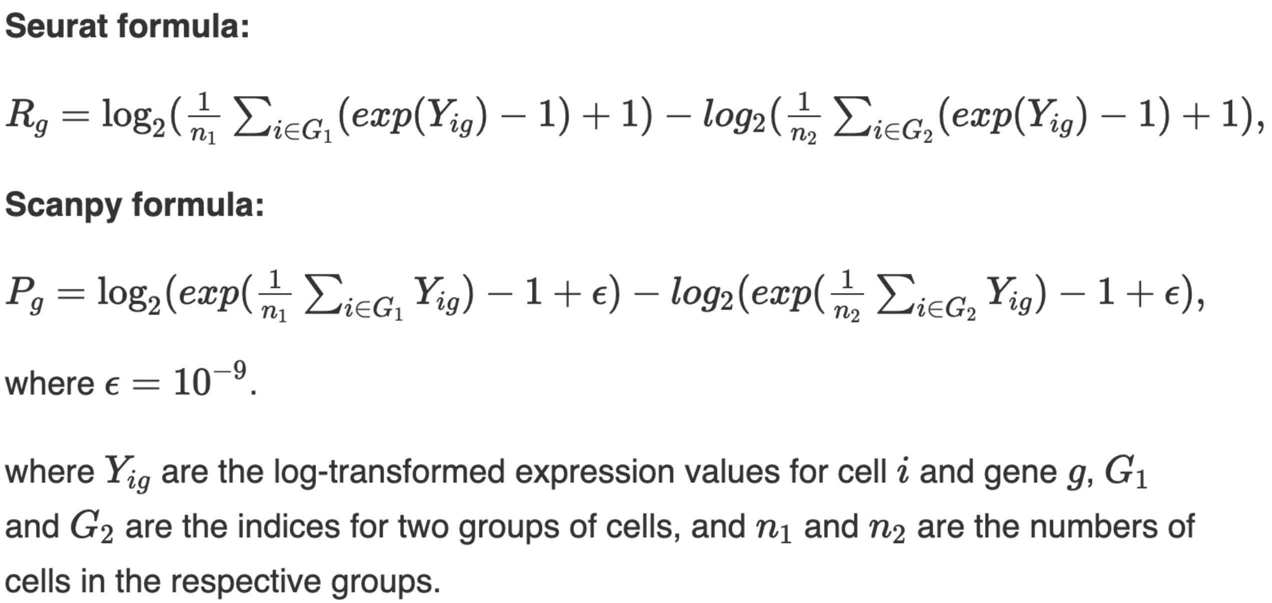
$\log_2$(fold change) discrepancy?



Lior Pachter
@lpachter

A 🧵 on why Seurat and Scanpy's log fold change calculations are discordant. 1/

(based on the Supplementary Notes from biorxiv.org/content/10.110...).



Seurat formula:

$$R_g = \log_2(\frac{1}{n_1}\sum_{i\in G_1}(exp(Y_{ig})-1)+1) - log_2(\frac{1}{n_2}\sum_{i\in G_2}(exp(Y_{ig})-1)+1),$$

Scanpy formula:

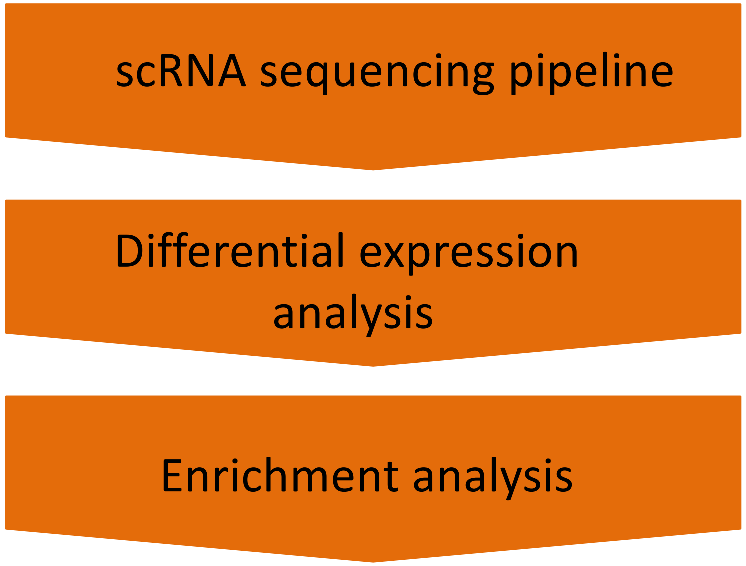
$$P_g = \log_2(exp(\frac{1}{n_1}\sum_{i\in G_1}Y_{ig})-1+\epsilon) - log_2(exp(\frac{1}{n_2}\sum_{i\in G_2}Y_{ig})-1+\epsilon),$$

where $\epsilon = 10^{-9}$.

where Y_{ig} are the log-transformed expression values for cell i and gene g, G_1 and G_2 are the indices for two groups of cells, and n_1 and n_2 are the numbers of cells in the respective groups.

https://twitter.com/lpachter/status/1694387749967847874
https://divingintogeneticsandgenomics.com/post/do-you-really-understand-log2fold-change-in-single-cell-rnaseq-data/

Once we have identified marker or DE genes, what do we do?



scRNA sequencing pipeline

Differential expression analysis

Enrichment analysis

Several methods available, *e.g.*:

- over-representation analysis (ORA)
- gene set enrichment analysis (GSEA)

Goal: to gain biologically-meaningful insights from long gene lists

- Pathways from a collection like KEGG or Gene Ontology?
- Transcription factor targets ?
- Custom gene list from a publication?
- Genes associated with a disease ?
- Etc…

Enrichment analysis

- GSEApy:

https://gseapy.readthedocs.io/en/latest/introduction.html

Tutorial:

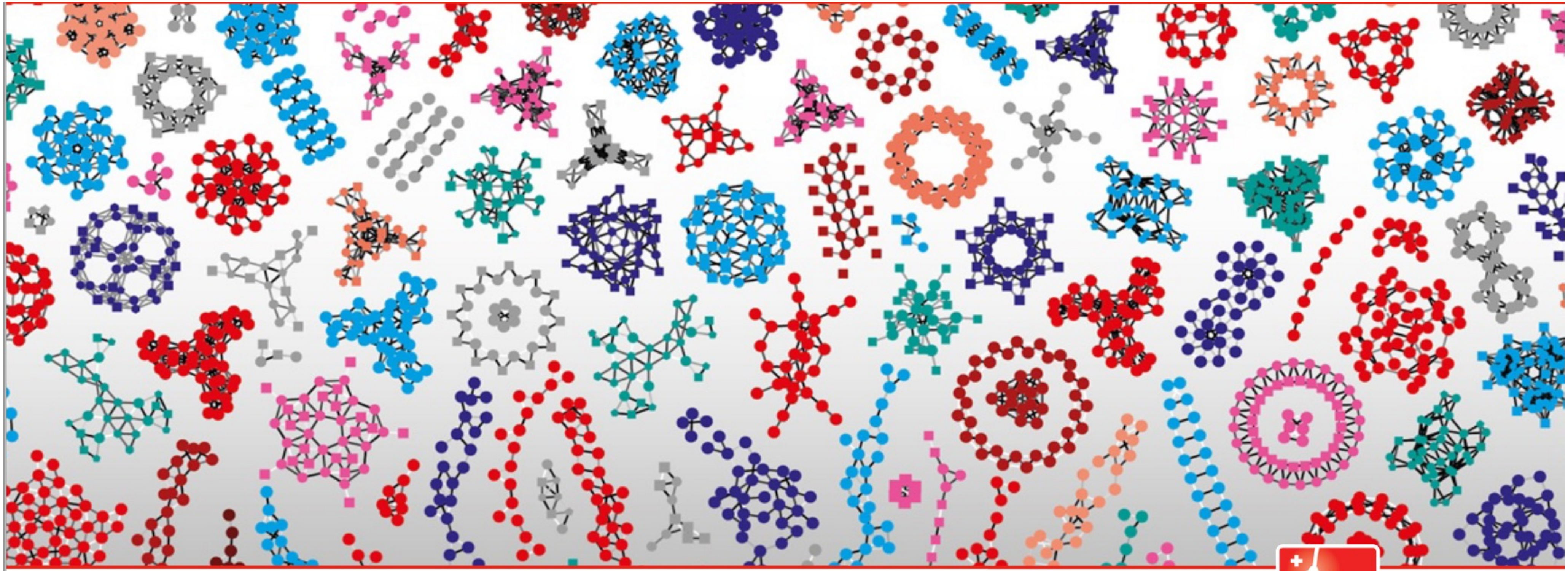
https://github.com/mousepixels/sanbomics_scripts/blob/main/GSEA_in_python.ipynb

https://www.youtube.com/watch?v=yOQcrUMCALw&t=302s&ab_channel=Sanbomics

https://nbisweden.github.io/workshop-scRNAseq/labs/scanpy/scanpy_05_dge.html#meta-dge_gsa_hyper

- (R: https://sib-swiss.github.io/enrichment-analysis-training/)

Question on marker gene/DGE analysis



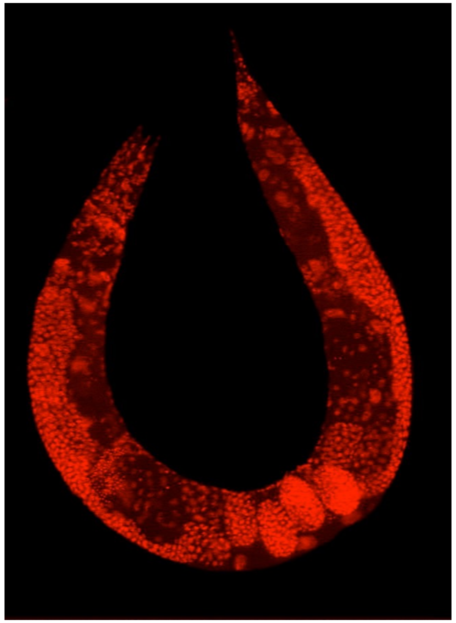
Swiss Institute of Bioinformatics

Single cell transcriptomics data analysis

Cell type annotation

What is a “cell type”?

- Fundamental unit of life
- Originally defined in terms of function, location tissue type, cell morphology
- Later extended to
  - presence/absence of cell surface markers
  - gene expression (molecular profile)
- Currently very much less fixed
  - cell cycle phase
  - migration state
  - differentiation: cell state



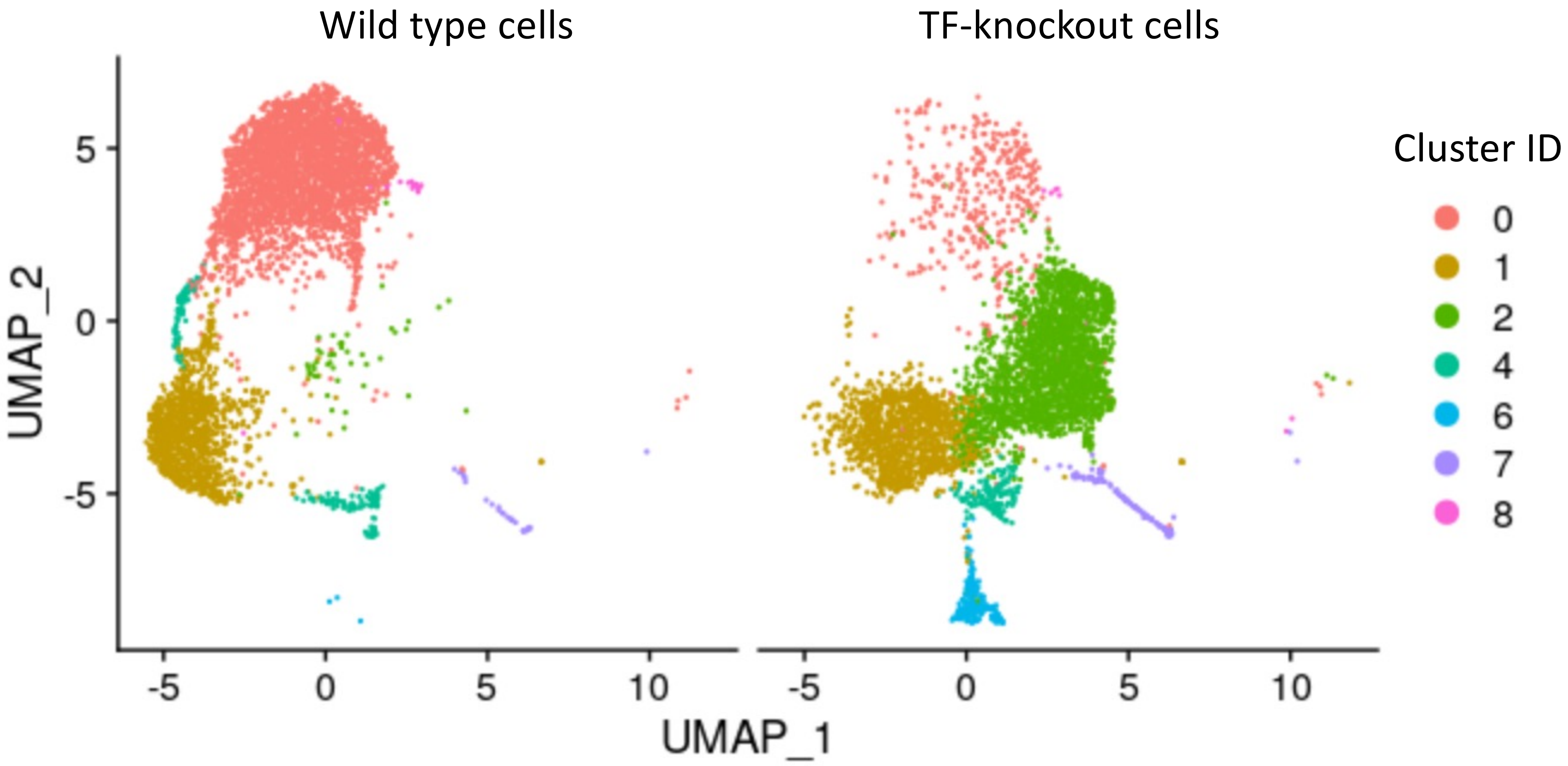
Wild-type *C. elegans* hermaphrodite stained to highlight the nuclei of all cells

Source article: (2005) The Evolution of Self-Fertile Hermaphroditism: The Fog Is Clearing. *PLoS Biol* **3**(1): e30
Creative Commons Attribution 2.5, Category:Media from PLOS journals

Why should we identify cell types?

- Samples are heterogeneous (in general)
- Tumor sample: how much do they differ from normal cell types?
- Find new cell types which have been missed by using "standard" surface markers
- Follow cell fate and determine cell differentiation mechanisms
- To determine which cell types might communicate with each other
- To compare the abundance of cell types in different conditions
- …

Change in cell type abundances: what are the new cells?



Mouse mesenteric lymphatic endothelial cells. From González-Loyola A et al 2021, DOI: 10.1126/sciadv.abf4335

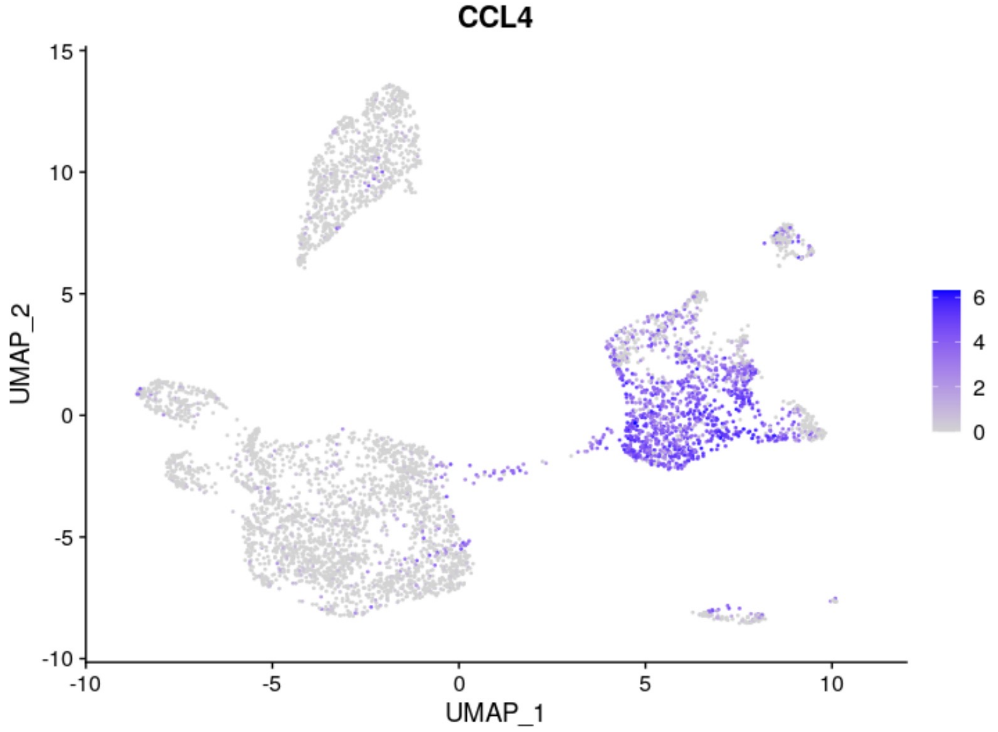
Manual *vs* automatic cell type annotation

- Manual: using marker genes
  - What most people do…
  - Time consuming
  - Requires expert knowledge
  - Sometimes subjective and inaccurate

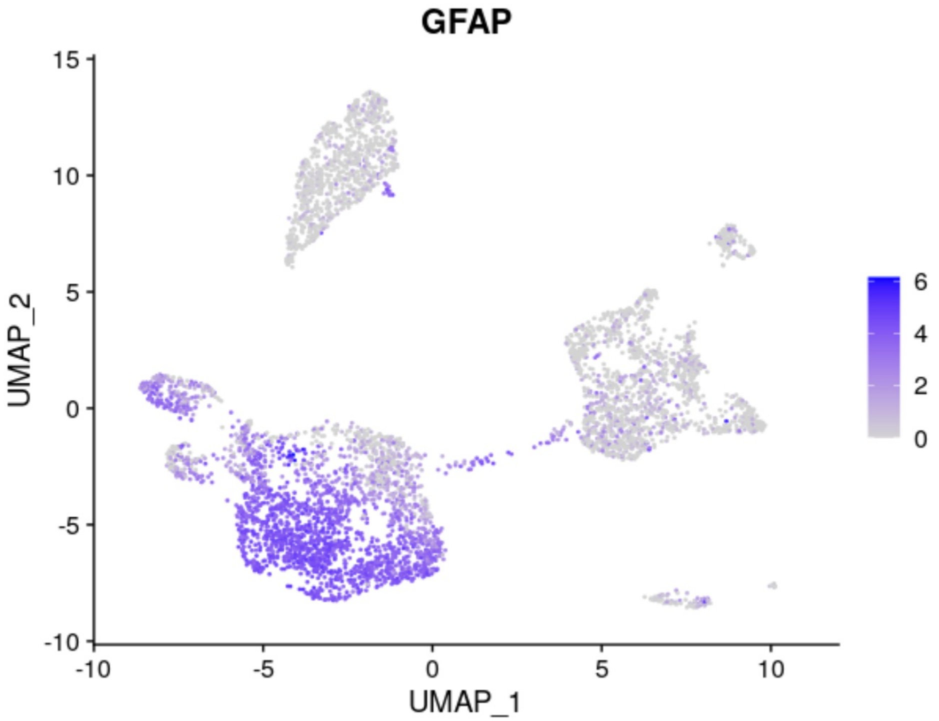
- Automatic: requires a reference
  - Use complete cell type-specific mRNA expression profiles based on bulk RNAseq from FACS-sorted 'pure' populations
  - OR: Use "a reference" of manually curated cells picked from scRNA-seq data sets
  - Can miss cell types if they are not included in the reference

Manual annotation using known marker genes

Microglia



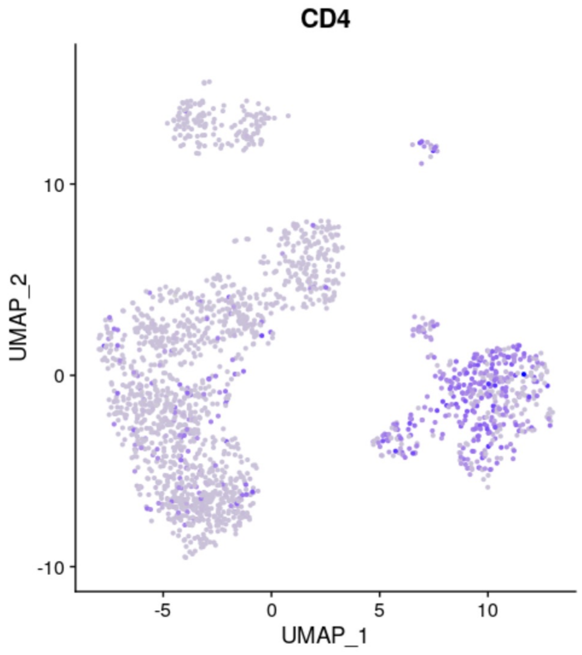
Astrocytes

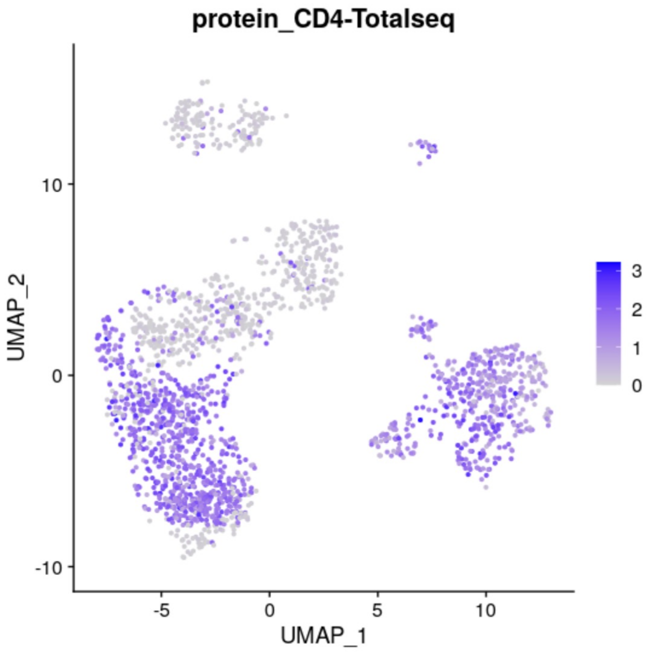


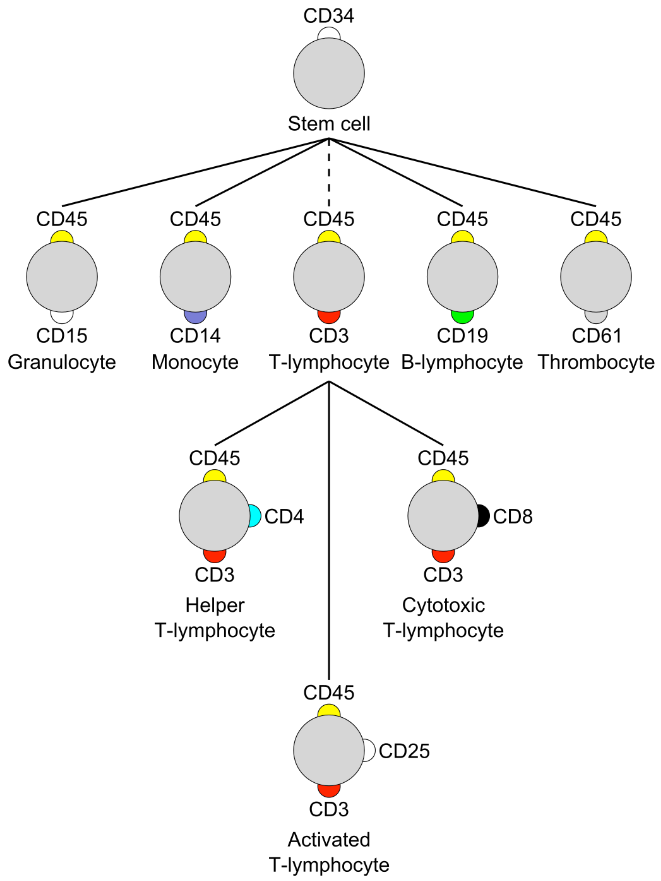
Human glioblastoma multiforme cells, 10x Genomics data (source of data to play with)
https://support.10xgenomics.com/single-cell-gene-expression/datasets/4.0.0/Parent_SC3v3_Human_Glioblastoma

Cell surface markers

- Often considered the gold standard esp. in immunology
- mRNA of cell surface markers sometimes lowly expressed or absent
- Use a combination of such marker genes, and also other genes like marker genes among clusters (eg secreted proteins or transcription factors)







By Lokal_Profil, CC BY-SA 2.5, https://commons.wikimedia.org/w/index.php?curid=8797123

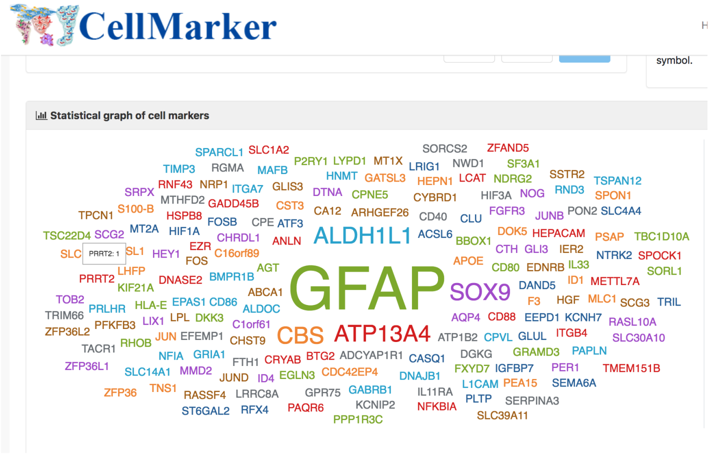
Databases with cell type marker genes

- PanglaoDB https://panglaodb.se/ (mouse and human)

  Check out https://cran.r-project.org/web/packages/rPanglaoDB/index.html

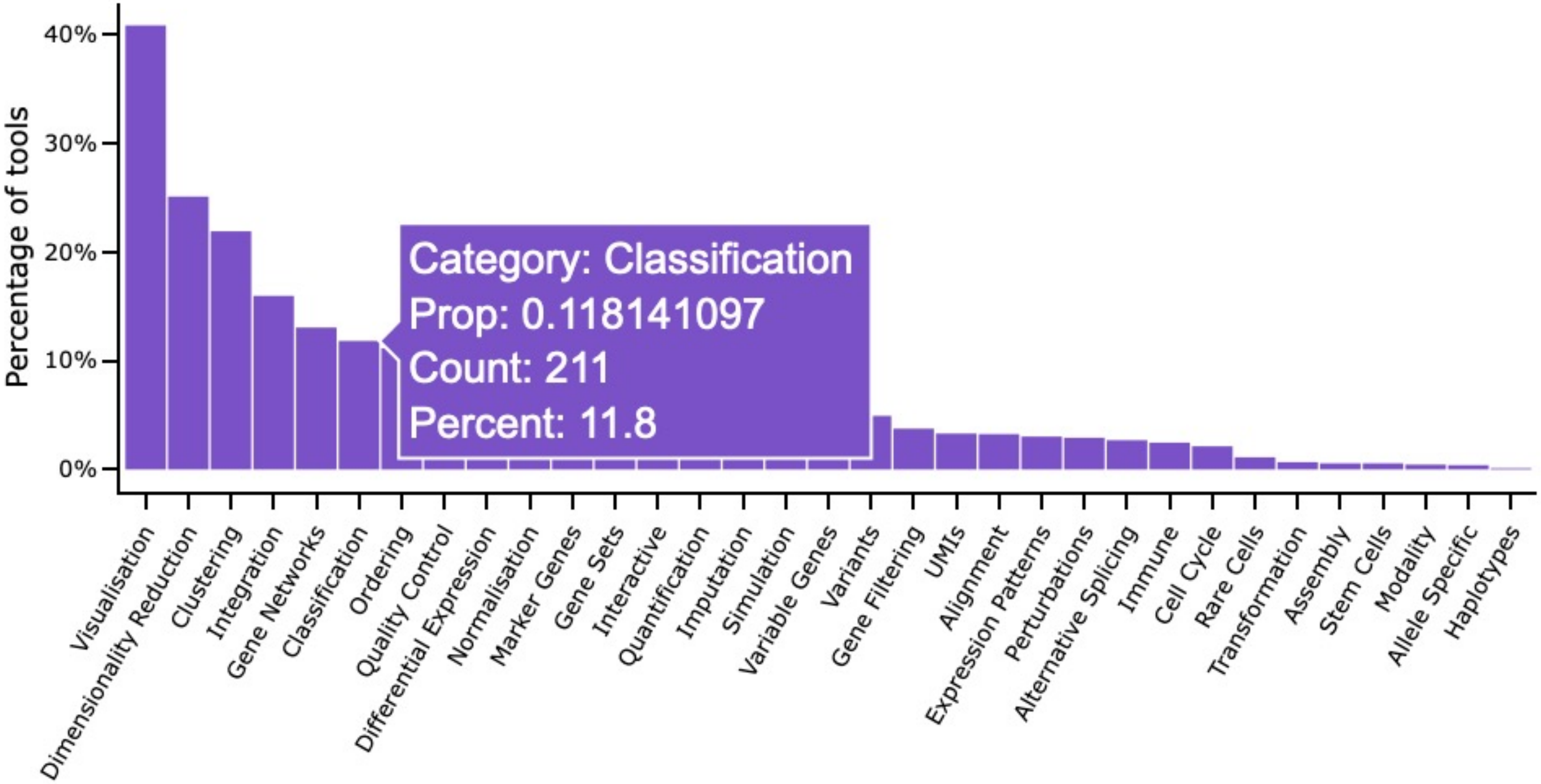
- CellMarker (mouse and human)

http://xteam.xbio.top/CellMarker/index.jsp


CellMarker
symbol.
Statistical graph of cell markers
PRRT2: 1
GFAP
ALDH1L1
SOX9
CBS
ATP13A4
SPARCL1 SLC1A2 SORCS2 ZFAND5
TIMP3 RGMA MAFB P2RY1 LYPD1 MT1X LRIG1 NWD1 SF3A1
SRPX RNF43 NRP1 ITGA7 GLIS3 HNMT GATSL3 HEPN1 LCAT NDRG2 SSTR2 TSPAN12
MTHFD2 GADD45B CST3 DTNA CPNE5 CYBRD1 HIF3A NOG RND3 SPON1
TPCN1 S100-B HSPB8 FOSB CPE ATF3 CA12 ARHGEF26 CD40 CLU FGFR3 JUNB PON2 SLC4A4
TSC22D4 SCG2 MT2A HIF1A CHRDL1 ANLN ACSL6 BBOX1 DOK5 HEPACAM PSAP TBC1D10A
SLC SL1 HEY1 EZR FOS C16orf89 CTH GLI3 IER2 NTRK2 SPOCK1
LHFP AGT APOE CD80 EDNRB IL33 SORL1
PRRT2 KIF21A DNASE2 BMPR1B ABCA1 DAND5 ID1 METTL7A
TOB2 PRLHR HLA-E EPAS1 CD86 ALDOC F3 HGF MLC1 SCG3 TRIL
TRIM66 LIX1 LPL DKK3 C1orf61 AQP4 CD88 EEPD1 KCNH7 RASL10A
ZFP36L2 PFKFB3 JUN EFEMP1 CHST9 ATP1B2 CPVL GLUL ITGB4 SLC30A10
TACR1 RHOB NFIA GRIA1 FTH1 CRYAB BTG2 ADCYAP1R1 CASQ1 DGKG GRAMD3 PAPLN
ZFP36L1 SLC14A1 MMD2 JUND ID4 EGLN3 CDC42EP4 DNAJB1 FXYD7 IGFBP7 PER1 TMEM151B
ZFP36 TNS1 RASSF4 LRRC8A GPR75 GABRB1 IL11RA L1CAM PEA15 SEMA6A
ST6GAL2 RFX4 PAQR6 KCNIP2 NFKBIA PLTP SERPINA3
PPP1R3C SLC39A11

-> Check whether your marker genes are part of any of the cluster marker genes

Automated cell type annotation



May 24th, 2024
https://www.scrna-tools.org/

Several methods are available

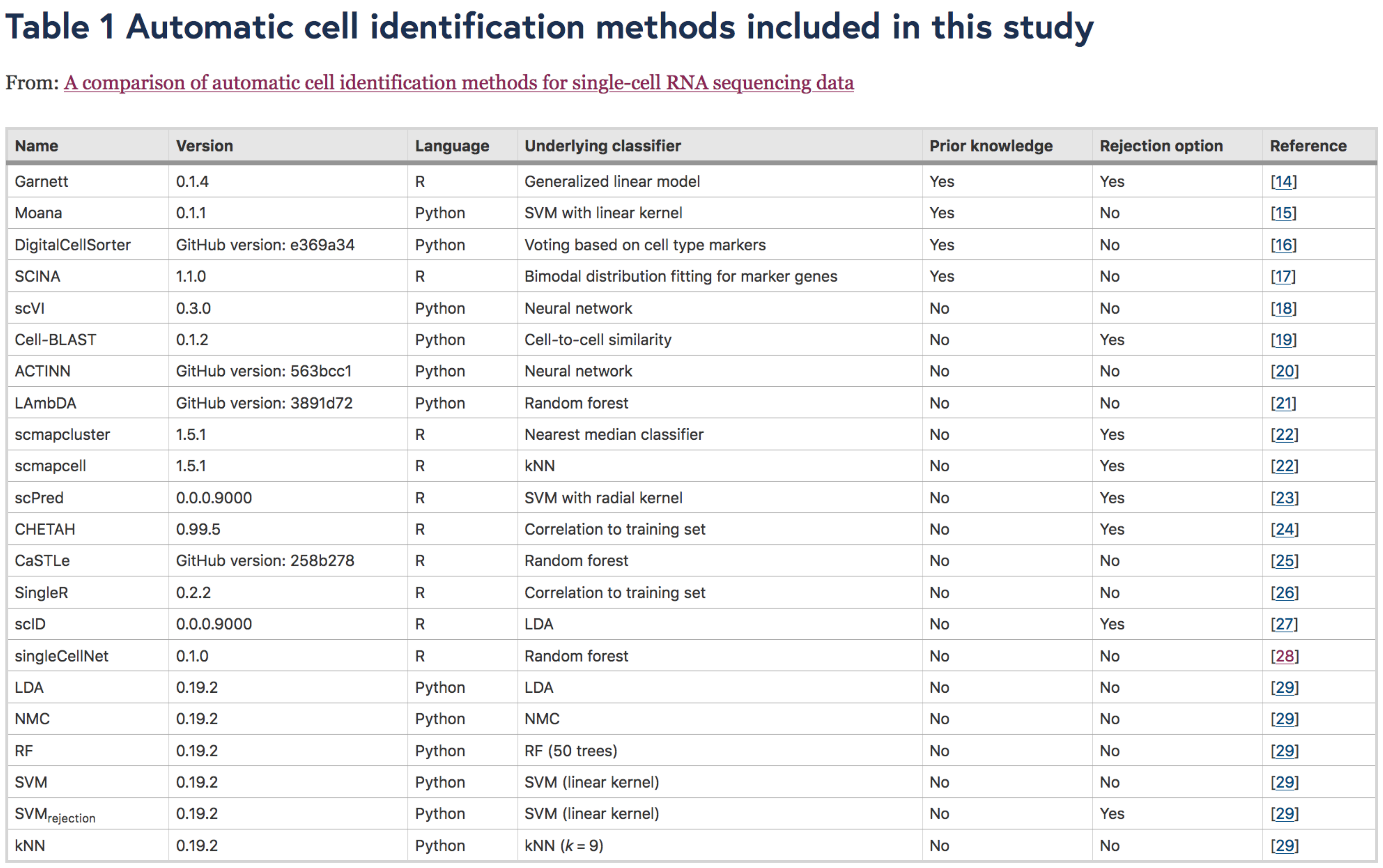


Table 1 Automatic cell identification methods included in this study

From: A comparison of automatic cell identification methods for single-cell RNA sequencing data

Name	Version	Language	Underlying classifier	Prior knowledge	Rejection option	Reference
Garnett	0.1.4	R	Generalized linear model	Yes	Yes	[14]
Moana	0.1.1	Python	SVM with linear kernel	Yes	No	[15]
DigitalCellSorter	GitHub version: e369a34	Python	Voting based on cell type markers	Yes	No	[16]
SCINA	1.1.0	R	Bimodal distribution fitting for marker genes	Yes	No	[17]
scVI	0.3.0	Python	Neural network	No	No	[18]
Cell-BLAST	0.1.2	Python	Cell-to-cell similarity	No	Yes	[19]
ACTINN	GitHub version: 563bcc1	Python	Neural network	No	No	[20]
LAmbDA	GitHub version: 3891d72	Python	Random forest	No	No	[21]
scmapcluster	1.5.1	R	Nearest median classifier	No	Yes	[22]
scmapcell	1.5.1	R	kNN	No	Yes	[22]
scPred	0.0.0.9000	R	SVM with radial kernel	No	Yes	[23]
CHETAH	0.99.5	R	Correlation to training set	No	Yes	[24]
CaSTLe	GitHub version: 258b278	R	Random forest	No	No	[25]
SingleR	0.2.2	R	Correlation to training set	No	No	[26]
scID	0.0.0.9000	R	LDA	No	Yes	[27]
singleCellNet	0.1.0	R	Random forest	No	No	[28]
LDA	0.19.2	Python	LDA	No	No	[29]
NMC	0.19.2	Python	NMC	No	No	[29]
RF	0.19.2	Python	RF (50 trees)	No	No	[29]
SVM	0.19.2	Python	SVM (linear kernel)	No	No	[29]
$\text{SVM}_{\text{rejection}}$	0.19.2	Python	SVM (linear kernel)	No	Yes	[29]
kNN	0.19.2	Python	kNN ($k = 9$)	No	No	[29]

Abdelaal et al 2019 https://genomebiology.biomedcentral.com/articles/10.1186/s13059-019-1795-z

CellTypist

- Leverages machine learning models trained on large and diverse reference datasets to assign cell type labels in a query dataset.
- Classifiers are trained on the reference database: modelslearn to recognize patterns of gene expression characteristic of cell types.
- Compare the query gene expression profile to the reference profile, **predict** the most likely cell type
- CellTypist can assign broad or specific cell type label (user's choice)
- Obtain confidence score – QC your annotations

https://www.celltypist.org/
https://www.science.org/doi/10.1126/science.abl5197

Sources of references

- **Celltypist** pre-trained models: `models.models_description()`

48 models: https://www.celltypist.org/models

Use your own reference: train the model with `celltypist.train()`

https://colab.research.google.com/github/Teichlab/celltypist/blob/main/docs/notebook/celltypist_tutorial.ipynb#scrollTo=precise-bronze

- **Tabula muris senis**: single-cell RNA-sequencing of different organs across the mouse lifespan, available as .h5ad files:

https://figshare.com/articles/dataset/Tabula_Muris_Senis_Data_Objects/12654728/1

https://github.com/czbiohub-sf/tabula-muris/blob/master/tabula-muris-on-aws.md

- **Single-cell portal**: convert count matrix and cell labels to annData https://singlecell.broadinstitute.org/single_cell
- Some **papers** provide link to .h5ad, eg human lymph node compartments: https://www.nature.com/articles/s41587-021-01139-4

ingest

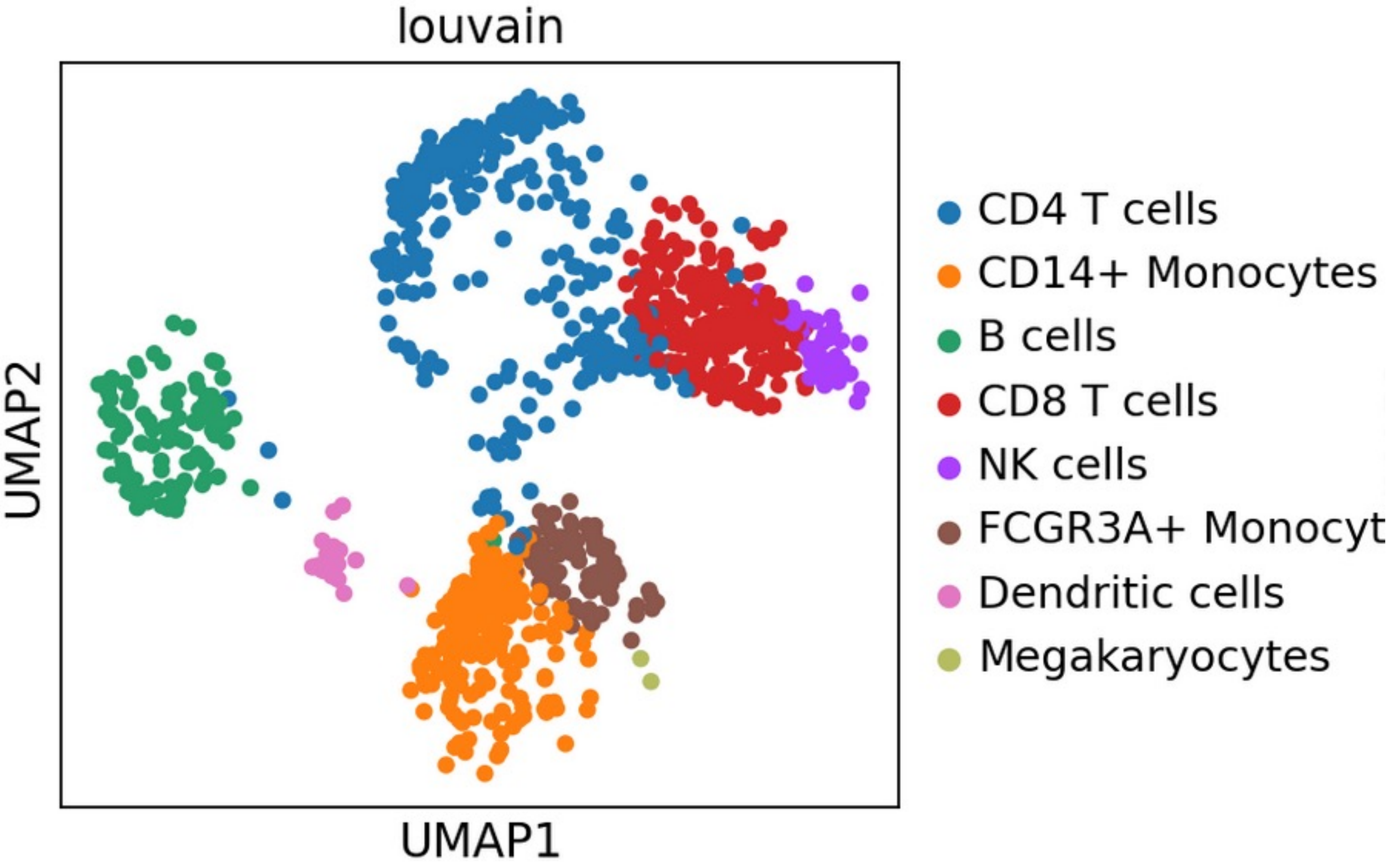
https://scanpy-tutorials.readthedocs.io/en/latest/integrating-data-using-ingest.html

- Uses PCs and neighborhood from reference dataset to infer label information for a new unlabeled dataset.
- Leaves the data matrix invariant
- Solves the label mapping problem
- Maintains a sample-specific embedding that might have desired properties like specific clusters or trajectories
- Look at what genes the different cell types express and use your biological knowledge to decide whether the annotation is good or should be improved.

https://nbisweden.github.io/workshop-scRNAseq/labs/scanpy/scanpy_06_celltyping.html#ingest

```
sc.tl.ingest(adata=adata, adata_ref=adata_ref, obs=”Louvain",
embedding_method='pca')
```

Query after ingest annotation



https://nbisweden.github.io/workshop-scRNAseq/labs/scanpy/scanpy_06_celltyping.html#ingest

Additional links

Review on automated cell annotation, Pasquini et al 2021
https://www.sciencedirect.com/science/article/pii/S2001037021000192

Question on cell type annotation