Single cell RNA-seq analysis

Part I: data processing & analysis

BMI/CS 776

www.biostat.wisc.edu/bmi776/

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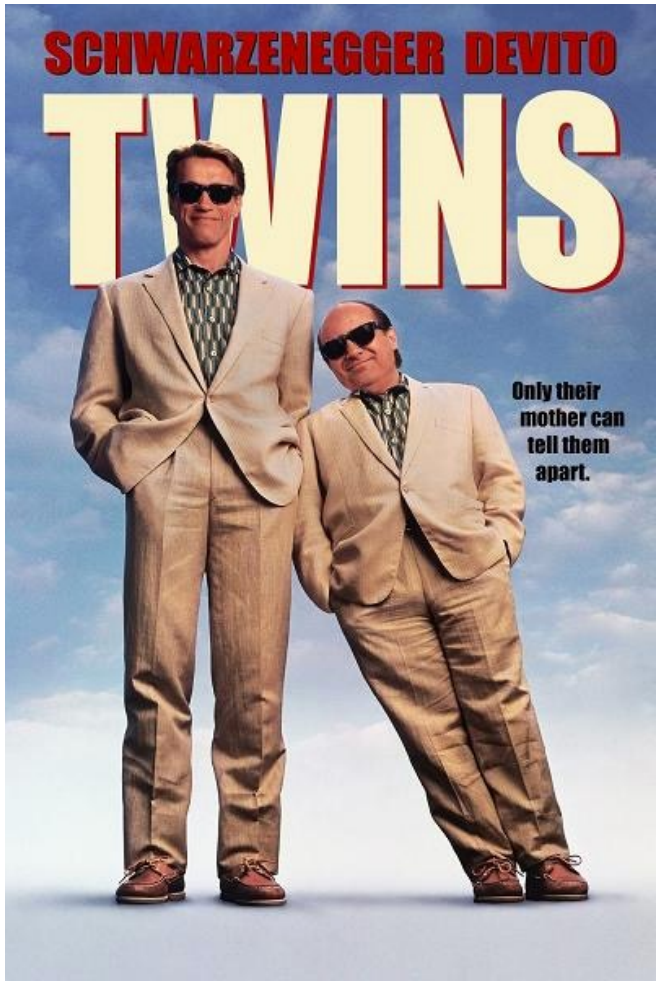
Outline

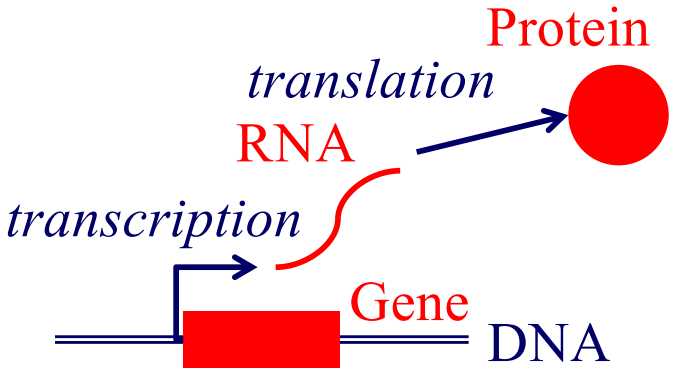
- **Introduction on single cell RNA sequencing**
- Single cell RNA sequencing (scRNA-seq) data processing
- scRNA-seq data analysis

Gene expression and regulation

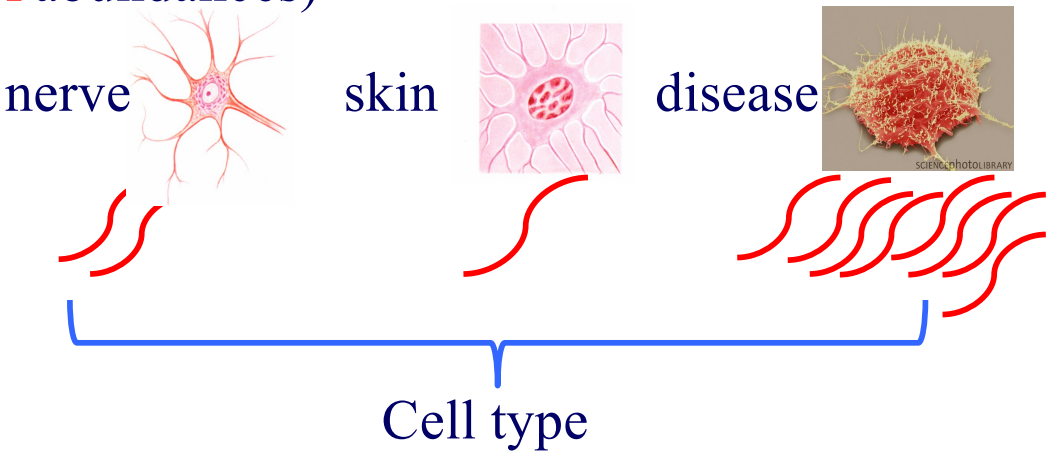
Identical DNA but different gene expression



Central dogma

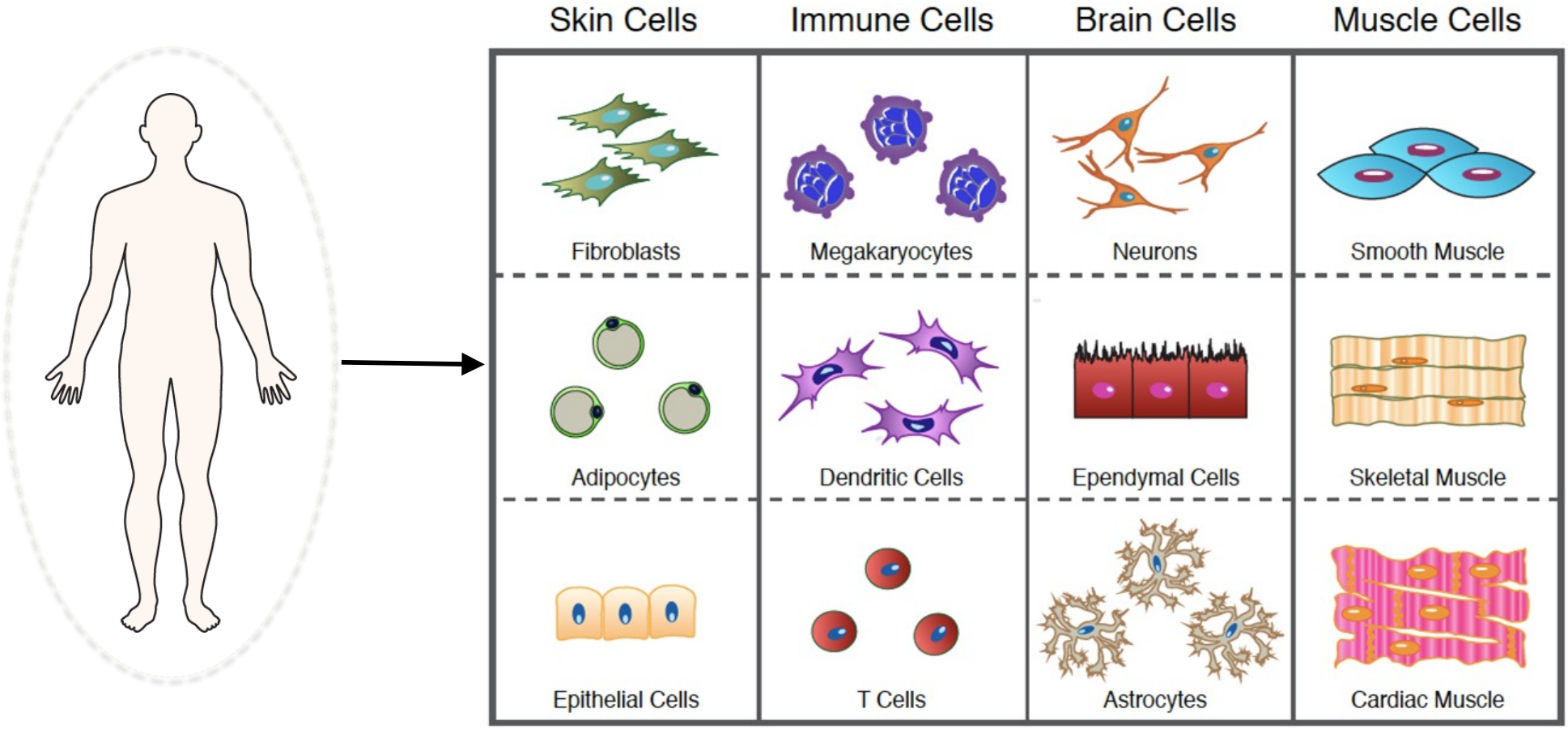


Gene expression levels (e.g., values to quantify RNA abundances)



Gene regulation: which & how genes express?

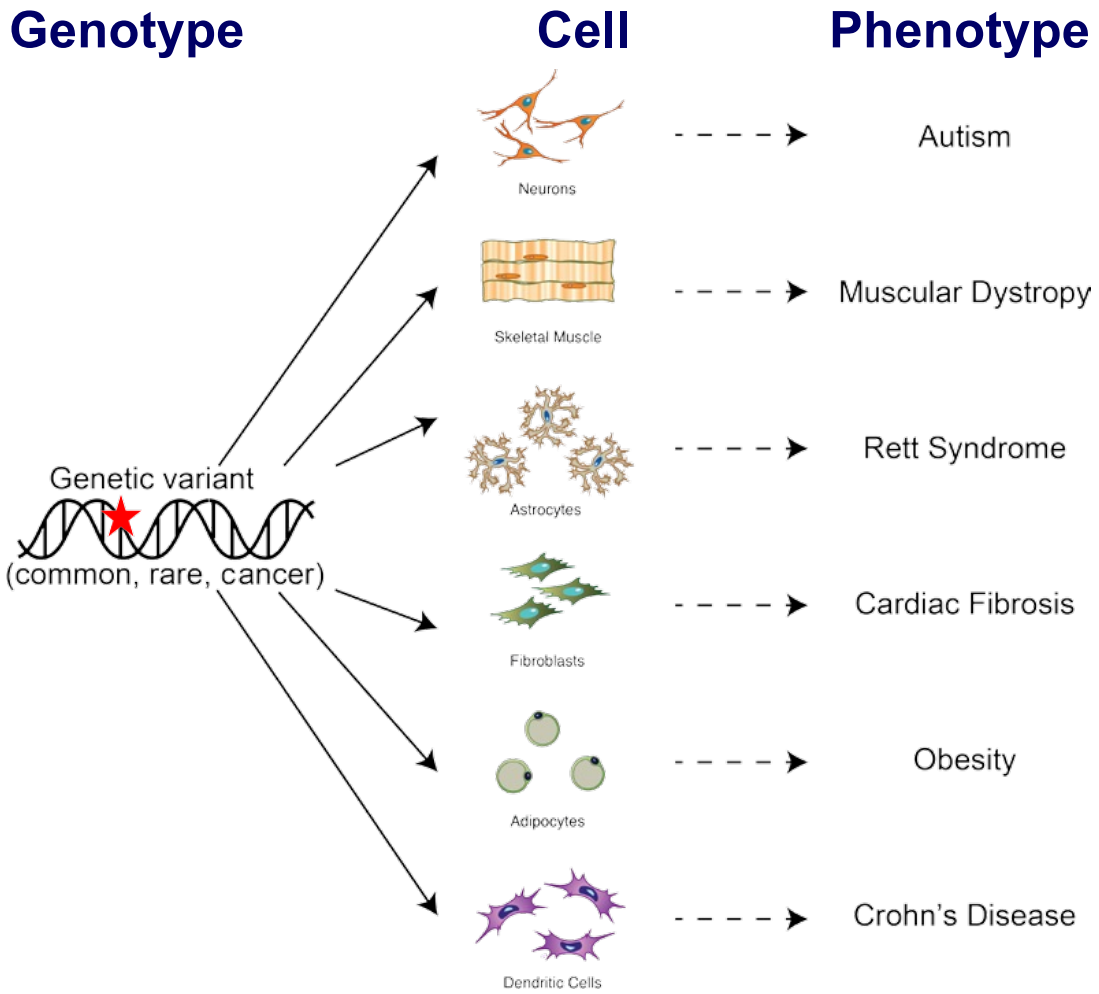
Why study single cells?



Cells are our core constituents, are classified by characteristic molecules, structures, and functions

https://www.youtube.com/watch?v=PRjX3-m16cw

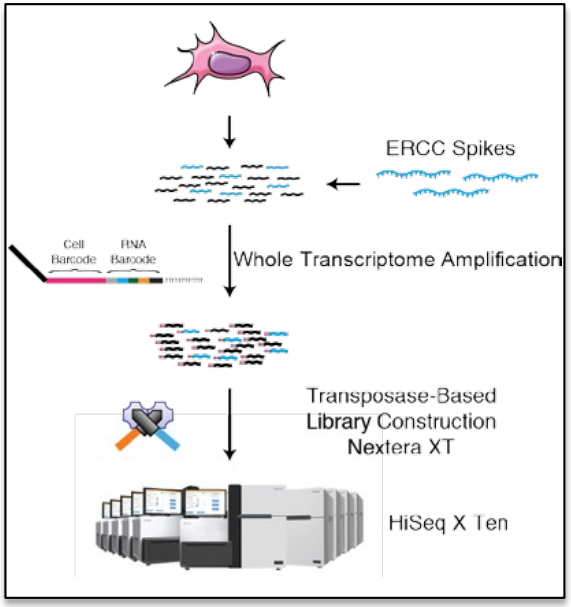
Why study single cells?



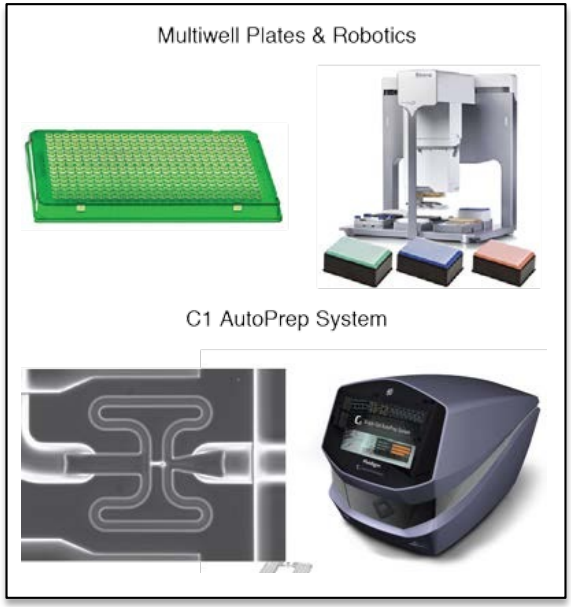
Cells are key intermediate from genotype to phenotype, e.g., essential functional dissection of genetic variants

https://bioinformatics-core-shared-training.github.io/cruk-summer-school-2018/SingleCell/slides/2018-07-25_CRUK_CI_summer_school-scRNAseq.pdf https://www.youtube.com/watch?v=PRjX3-m16cw

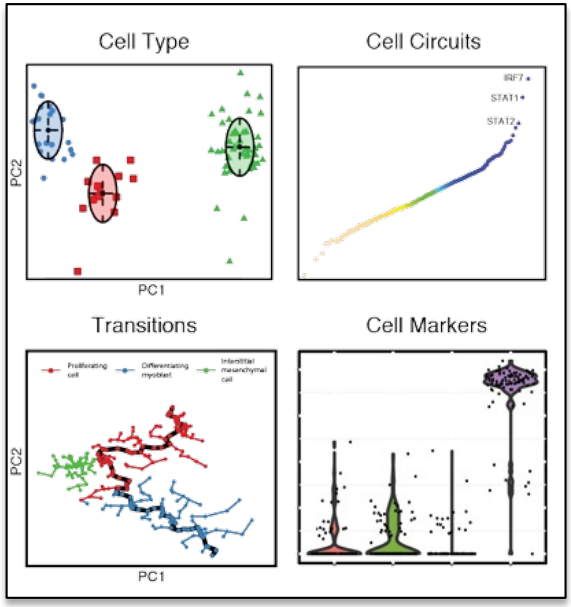
Sequencing RNAs in singe cells



✓ **Core technology**



✓ **Sample prep**



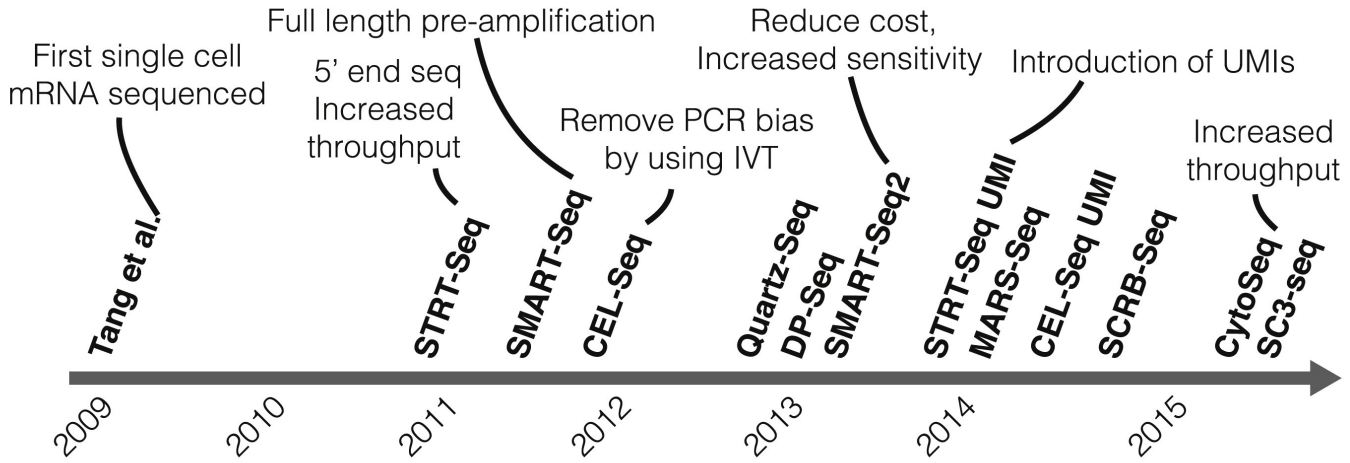
✓ **Computation**

2012: 18 cells → **2020: ~100,000 cells**

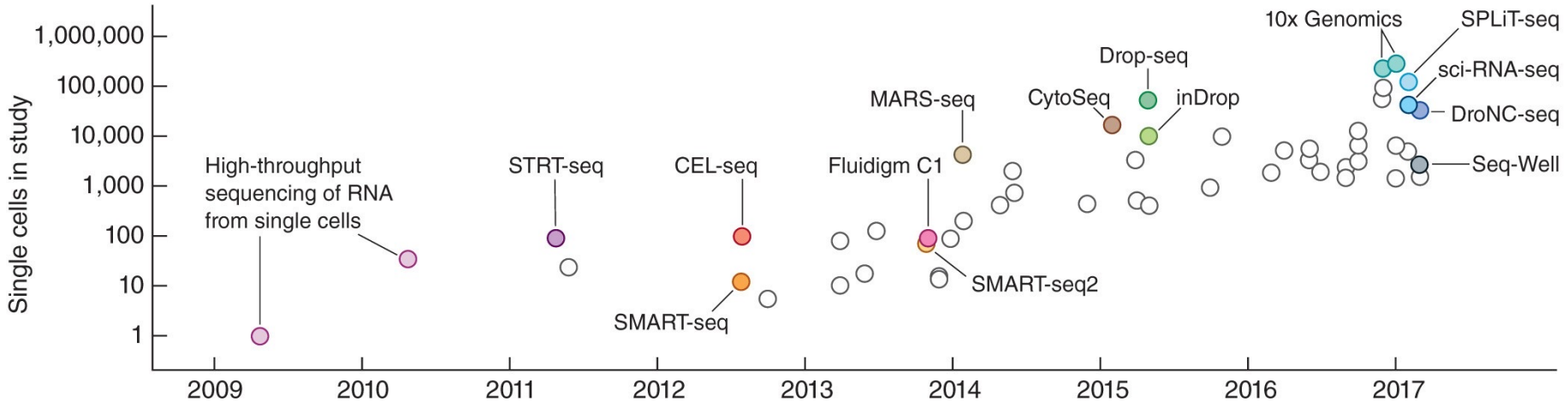
https://www.youtube.com/watch?v=PRjX3-m16cw

Evolution of single cell sequencing

- High-throughput technologies enable the profiling of thousands of cells in parallel, providing an unbiased view of the heterogeneity of single cells within a population. *Fan et al., 2015*

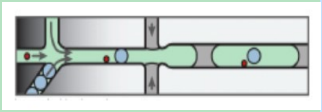
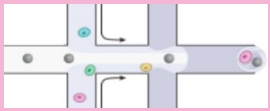
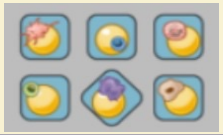


- Cell numbers reported in representative publications by publication date



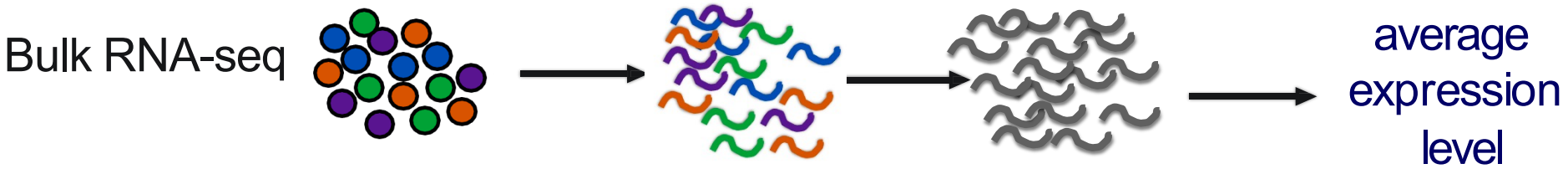
Kolodziejczyk et al, Mol Cell (2015)
Svensson V, Vento-Tormo R, Teichmann SA. Nat Protoc. 2018 Apr;13(4):599-604

Single cell RNA sequencing (scRNA-seq) technology

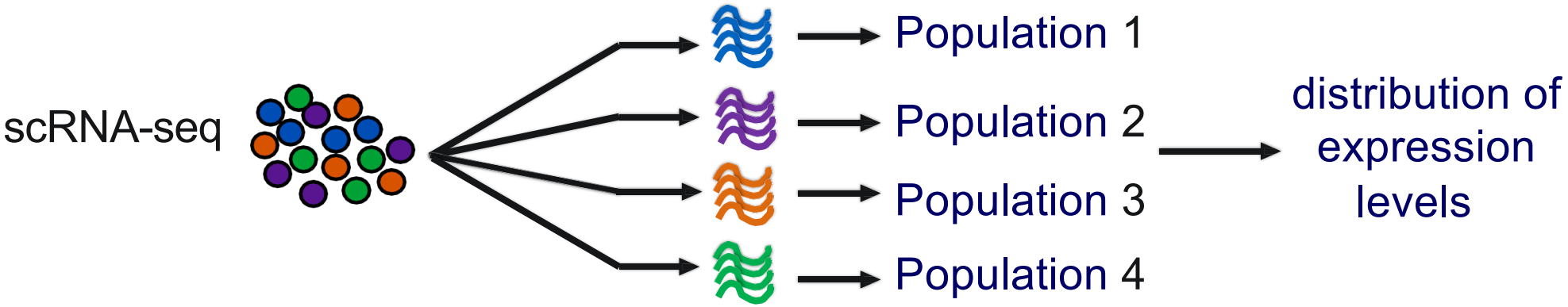
	inDrops	10x Genomics	Drop-seq	Seq-well (Honeycomb)	SMART-seq
Cell capture efficiency	~70-80%	~50-70%	~10%	~80%	~80%
Time to capture 10k cells	~30min	10min	1-2 hours	5-10min	--
Encapsulation type	Droplet 	Droplet 	Droplet 	Nanolitre well 	Plate-based 
Library prep	CEL-seq Linear amplification by IVT	SMART-seq Exponential PCR based amplification	SMART-seq Exponential PCR based amplification	SMART-seq Exponential PCR based amplification	SMART-seq Exponential PCR based amplification
Commercial	Yes	Yes	--	Yes (Summer 2020)	Yes
Cost (~$ per cell)	~0.06	~0.2	~0.06	~0.15	1
Strengths	• Good cell capture • Cost-effective • Real-time monitoring • Customizable	• Good cell capture • Fast and easy to run • Parallel sample collection • High gene / cell counts	• Cost-effective • Customizable	• Good cell capture • Cost-effective • Real-time monitoring • Customizable	• Good cell capture • Good mRNA capture • Full-length transcript • No UMI
Weaknesses	Difficult to run	Expensive	Difficult to run & low cell capture efficiency	Available Soon	Expensive

https://github.com/hbctraining/scRNA-seq/blob/master/slides/Single_Cell_2_27_20.pdf

Bulk vs scRNA-seq



- quantifying expression signatures from ensembles
- insufficient for studying heterogeneous system



- inference of gene regulatory networks across the cells
- heterogeneity of cell responses
- cell type identification

Outline

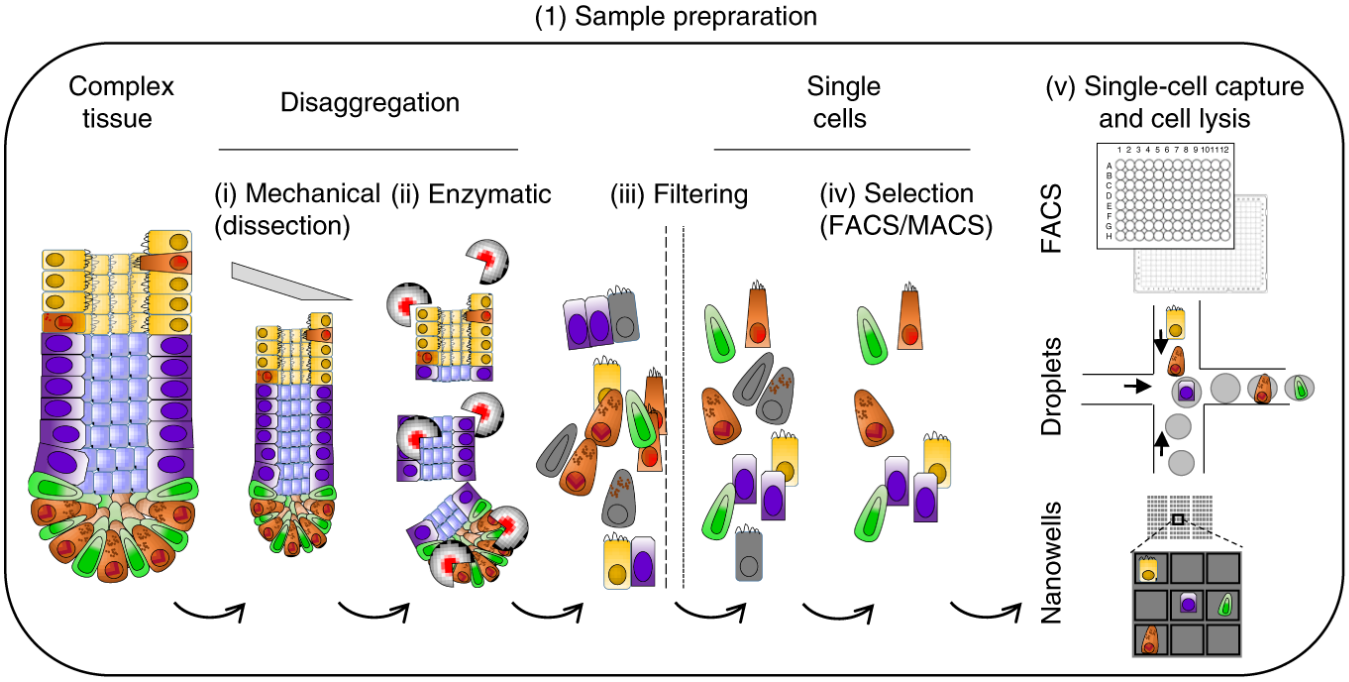
- Introduction on single cell RNA sequencing
- **Single cell RNA sequencing (scRNA-seq) data processing**
- scRNA-seq data analysis

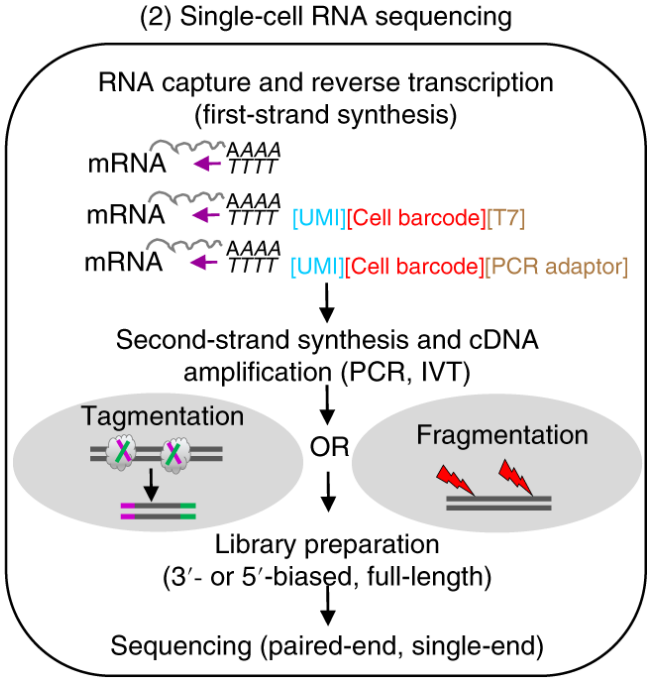
Single-cell RNA sequencing (scRNA-seq) process

- Step1: Sample preparation
- Step2: Single-cell RNA sequencing
- Step3: Data processing
- Step4: Data analysis

scRNA-seq process

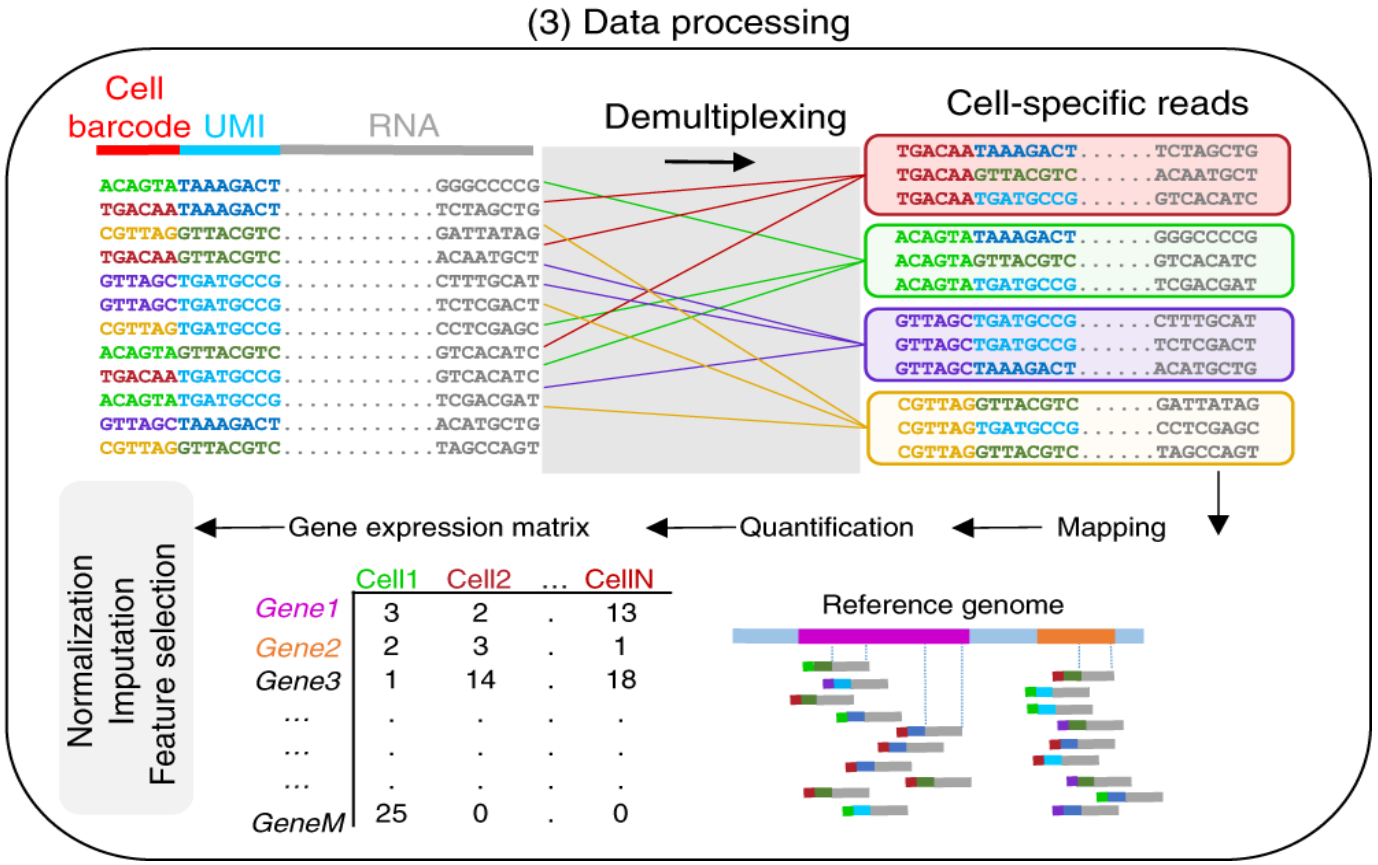
- Step 1 - Sample preparation: cells are physically separated into a single-cell solution from which specific cell types can be enriched or excluded
- Step 2 - Single-cell RNA sequencing





scRNA-seq process

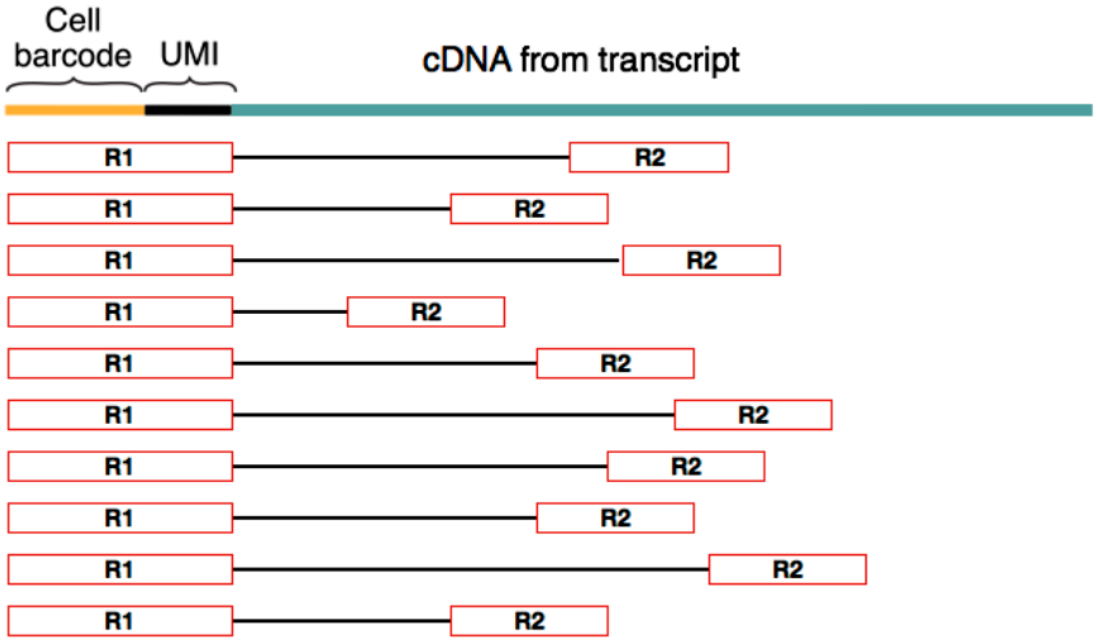
- Step 3 - Data processing
  - Unique molecular identifier (UMI)
  - Gene counts
  - Drop-outs in single cell
  - Imputation method: MAGIC



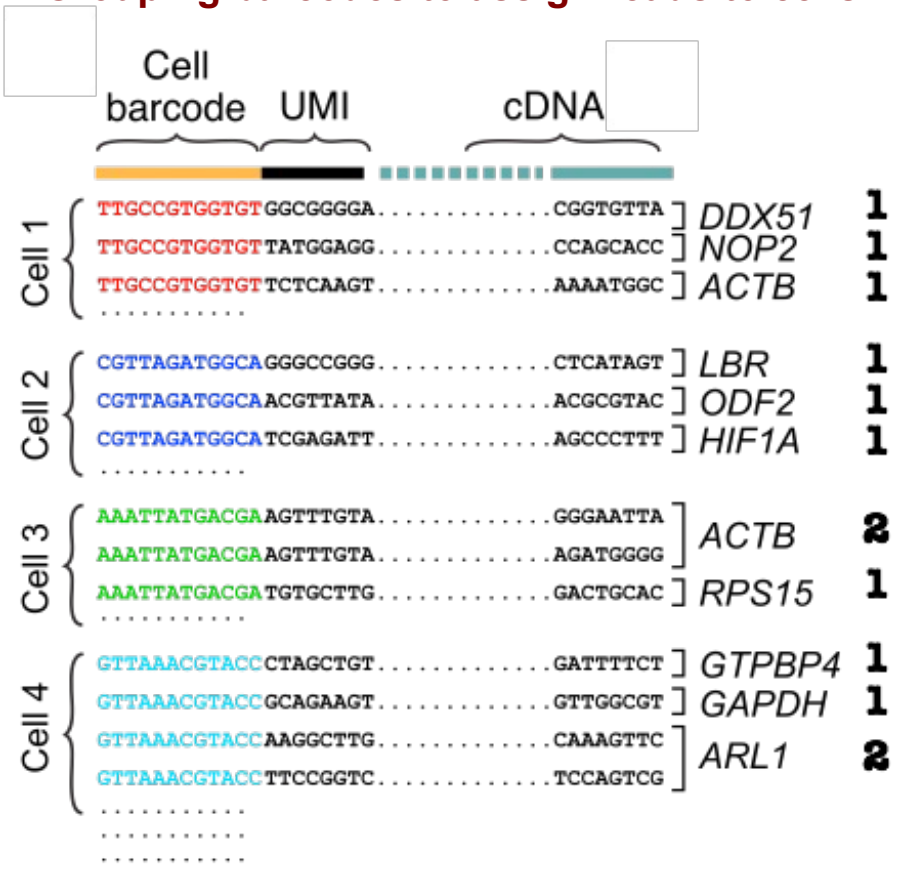
Unique molecular identifier (UMI)

- UMIs are short (4-10bp) random barcodes added to transcripts during reverse-transcription.

Biased paired-end reads



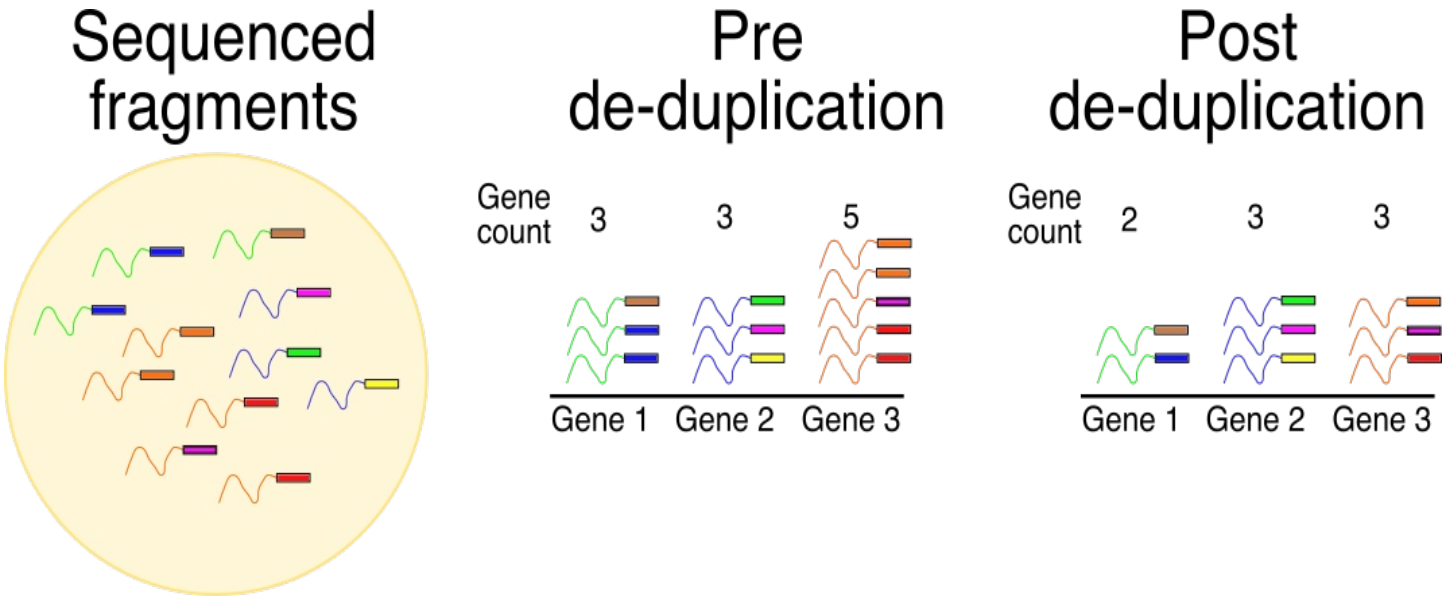
Grouping barcodes to assign reads to cells



http://data-science-sequencing.github.io/Win2018/lectures/lecture16/
https://bioinformatics-core-shared-training.github.io/cruk-summer-school-2018/SingleCell/slides/2018-07-25_CRUK_CI_summer_school-scRNAseq.pdf

Unique molecular identifier (UMI)

- They enable sequencing reads to be assigned to individual transcript molecules and thus the removal of amplification noise and biases from scRNA-Seq data
- They reduce the amplification noise by allowing (almost) complete de-duplication of fragments



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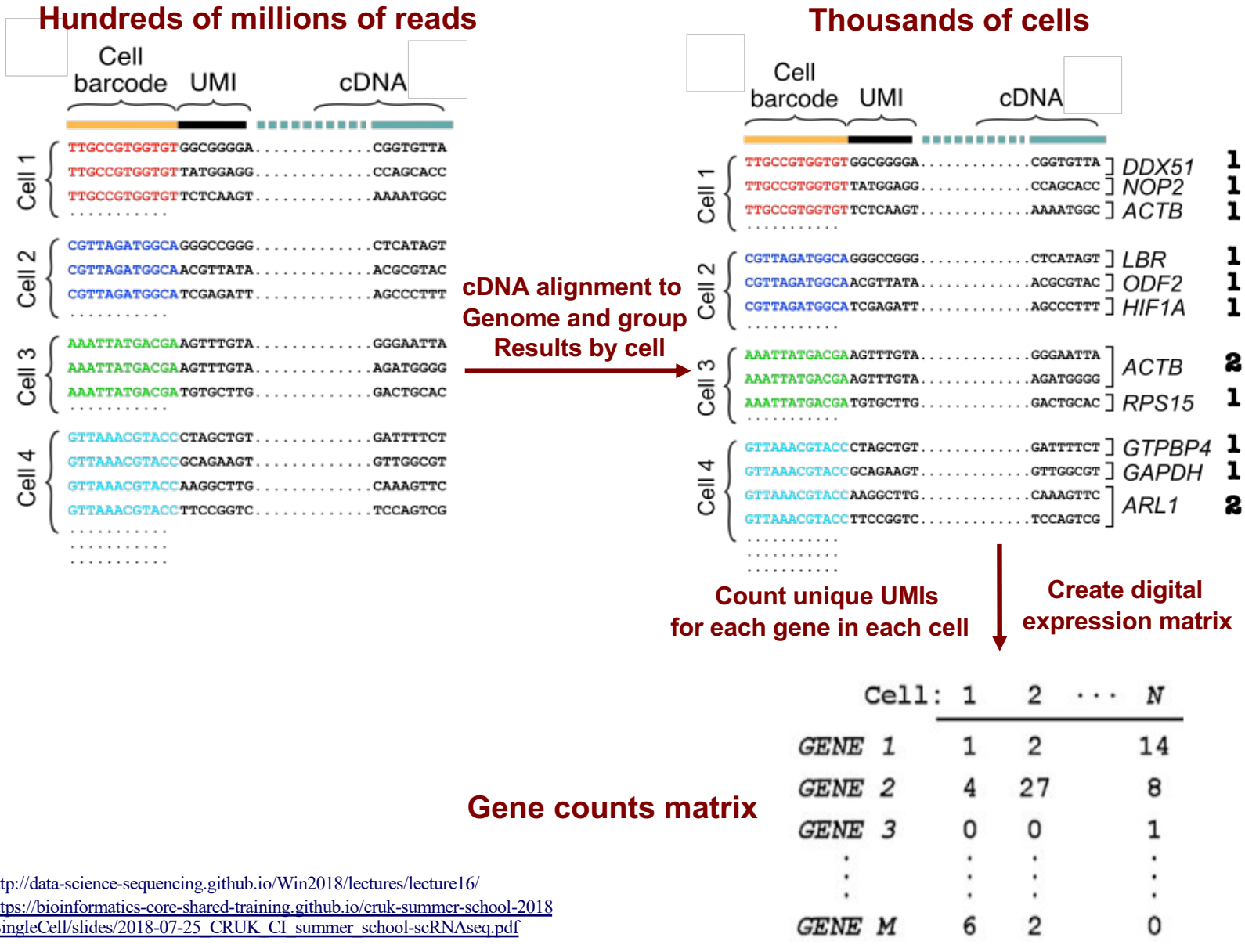
https://bioinformatics-core-shared-training.github.io/cruk-summer-school-2018/SingleCell/slides/2018-07-25_CRUK_CI_summer_school-scRNAseq.pdf

Gene Counts

- In each gene, within each cell, the total number of unique UMI is counted and reported as the number of transcripts of that gene for a given cell.



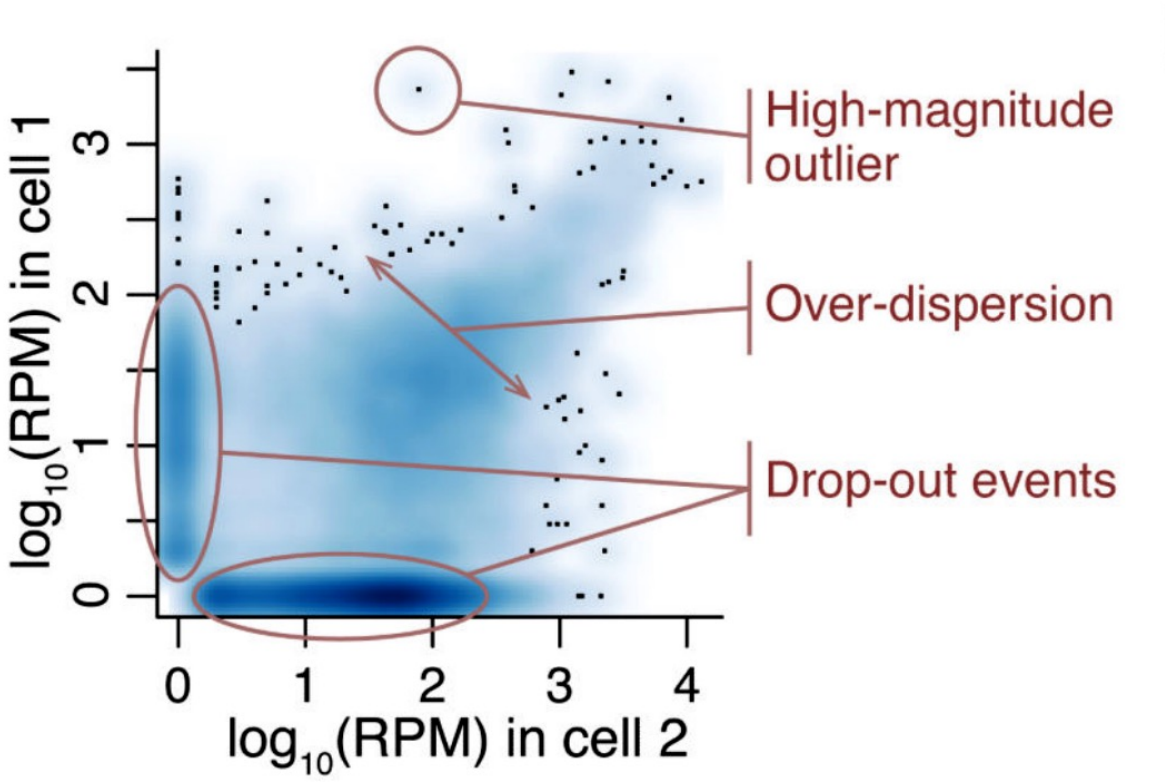
http://data-science-sequencing.github.io/Win2018/lectures/lecture16/
https://bioinformatics-core-shared-training.github.io/cruk-summer-school-2018/SingleCell/slides/2018-07-25_CRUK_CI_summer_school-scRNAseq.pdf

Drop-outs in single cell

- a gene is observed at a moderate or high expression level in one cell but is not detected in another cell

Why do dropouts occur in single cell?

- technical artifacts
- cell type differences
- statistical sampling
- biological factors



Kharchenko, P., Silberstein, L. & Scadden, D. Bayesian approach to single-cell differential expression analysis. Nat Methods 11, 740–742 (2014).

Drop-outs in single cell

What should we do about dropouts?

- Ignore zero inflation
- Preprocess/reduce dimensions
- Impute scRNA-seq gene count matrix before analysis

Why do we need imputation methods?

- Downstream analyses relying on the accuracy of gene expression measurements

Imputation Methods

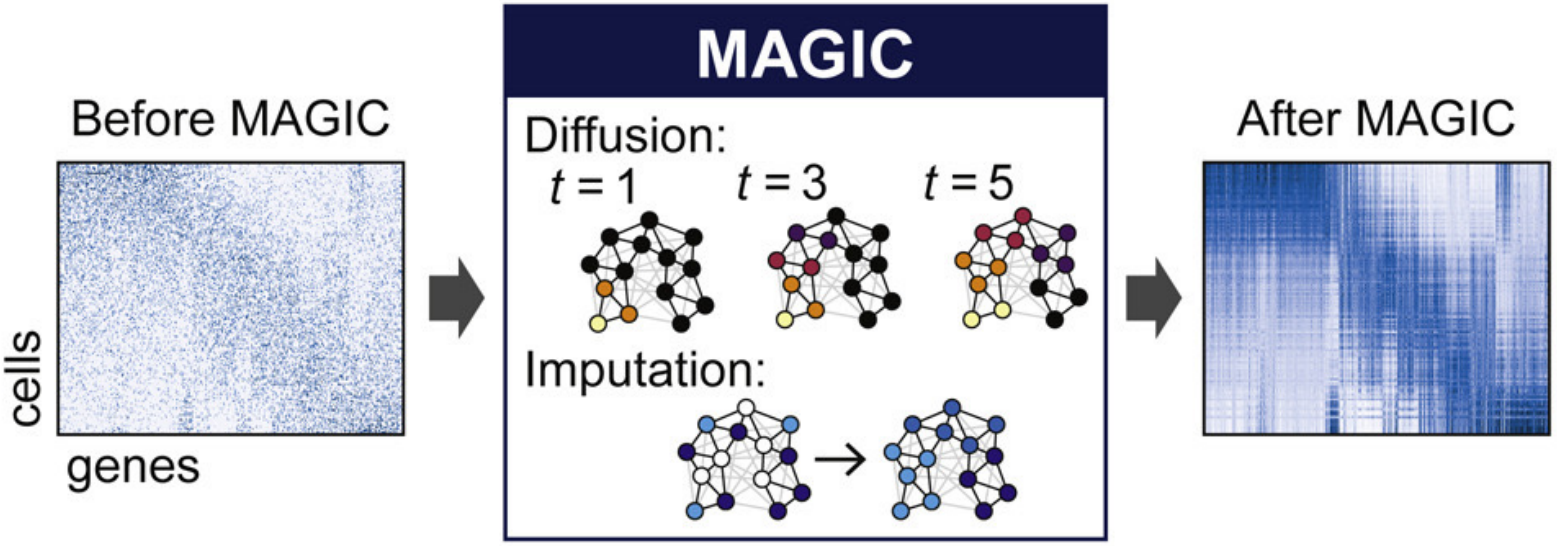
- MAGIC
- Droplet
- DrImpute
- scDoc

MAGIC

Markov **a**ffinity-based **g**raph **i**mputation of **c**ells

- Denoise high-dimensional scRNA-seq data
- Impute missing expression values by sharing information across similar cells

- Similarity between two cells $A_{ij} = e^{-(\frac{Distance_{ij}}{\sigma})^2}$
- Transform the similarity matrix $\boldsymbol{A}$ into a Markov transition matrix $\boldsymbol{M}$
- Raise the Markov matrix to the power of t: $\boldsymbol{M}^t$, which determines the weights of cells

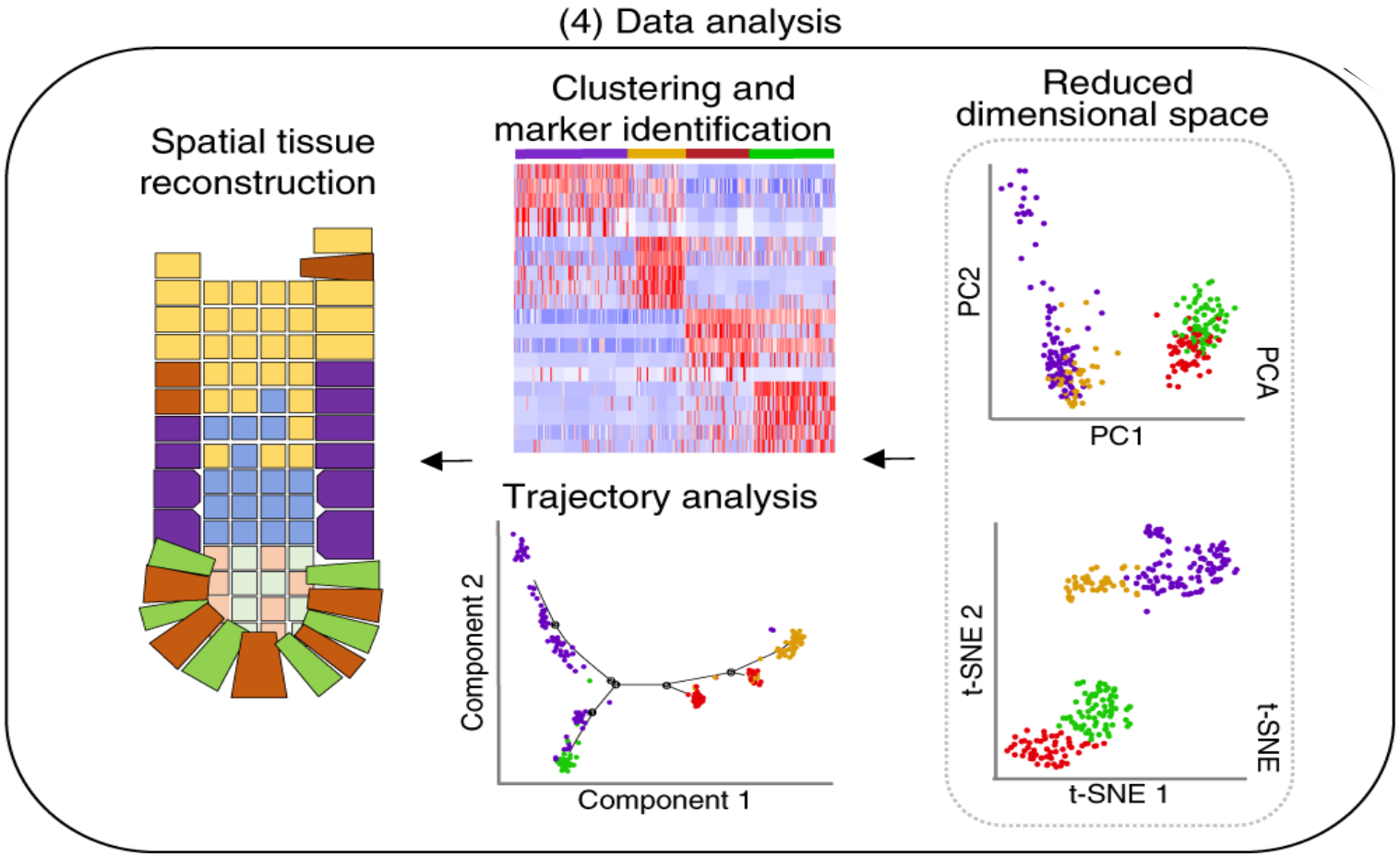


http://jsb.ucla.edu/sites/default/files/scImpute.pdf

van Dijk D, Sharma R, Nainys J, Yim K, Kathail P, Carr AJ, Burdziak C, Moon KR, Chaffer CL, Pattabiraman D, Bierie B, Mazutis L, Wolf G, Krishnaswamy S, Pe'er D. Recovering Gene Interactions from Single-Cell Data Using Data Diffusion. Cell. 2018 Jul 26;174(3):716-729.e27. doi: 10.1016/j.cell.2018.05.061. Epub 2018 Jun 28. PMID: 29961576; PMCID: PMC6771278.

scRNA-seq process

- Step 4 - Data analysis
  - Dimensionality reduction
  - Clustering and marker identification
  - Trajectory analysis



Lafzi et al. Tutorial: guidelines for the experimental design of single-cell RNA sequencing studies, Nature Protocols 2018 (https://doi.org/10.1038/s41596-018-0073-y)

Outline

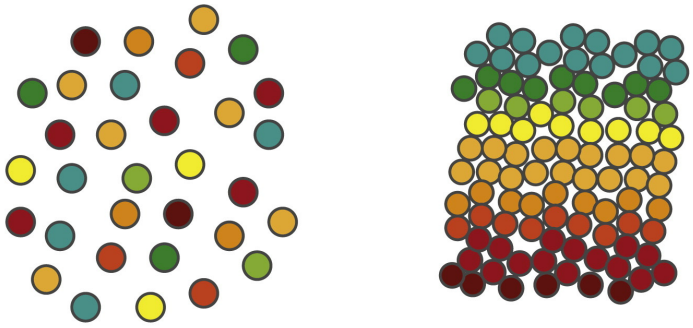
- Introduction on single cell RNA sequencing
- Single cell RNA sequencing (scRNA-seq) data processing
- **scRNA-seq data analysis**

scRNA-seq data analysis

- **Introduction of single cell analysis**
- Dimensionality reduction
  - Linear: PCA
  - Non-linear: t-SNE, UMAP
- Cell clustering
  - K-means
  - hierarchical clustering
  - graph-based clustering

Why single-cell analysis ?

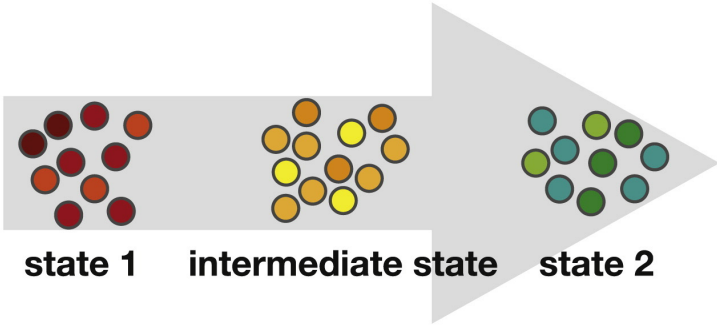
- Single-cell data opens the door to several types of biological questions:
  - What cell types exist within a population of cells in a particular sample?



e.g. blood cells

e.g. intestinal crypt,
developing cerebral cortex

  - What does this high-resolution view of cellular transitions tell us about switches in cell states?

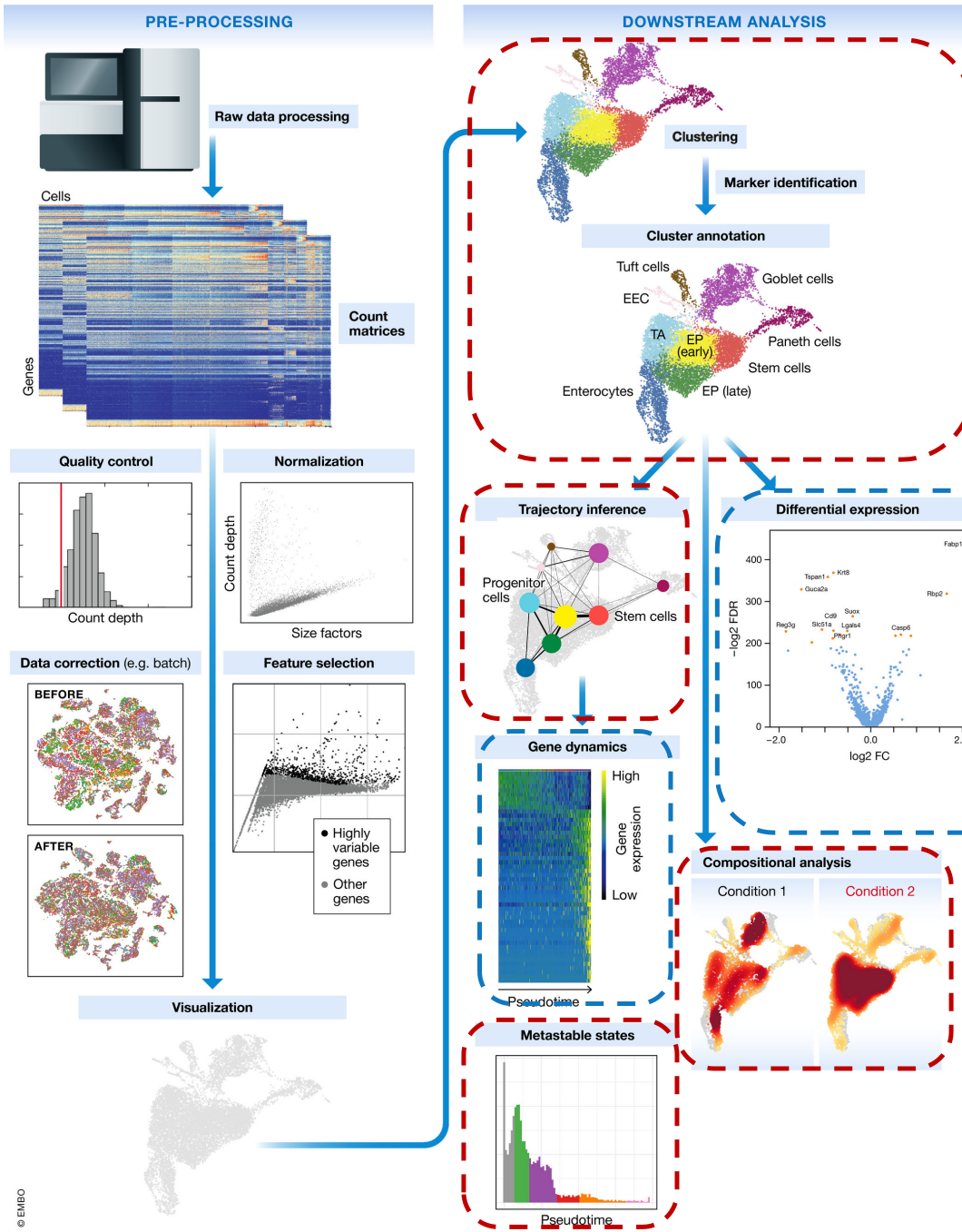


e.g. dendritic cells response to LPS

  - How can gene regulatory networks drive cell types or states?

Kolodziejczyk et al, Mol Cell (2015)

scRNA-seq analysis



Cell-level analysis

- Marker gene identification
- Cluster analysis
- Trajectory analysis

Gene-level analysis

- Single-cell differential expression analysis
- Gene set analysis
- Gene regulatory networks

Python for scRNA-seq data analysis

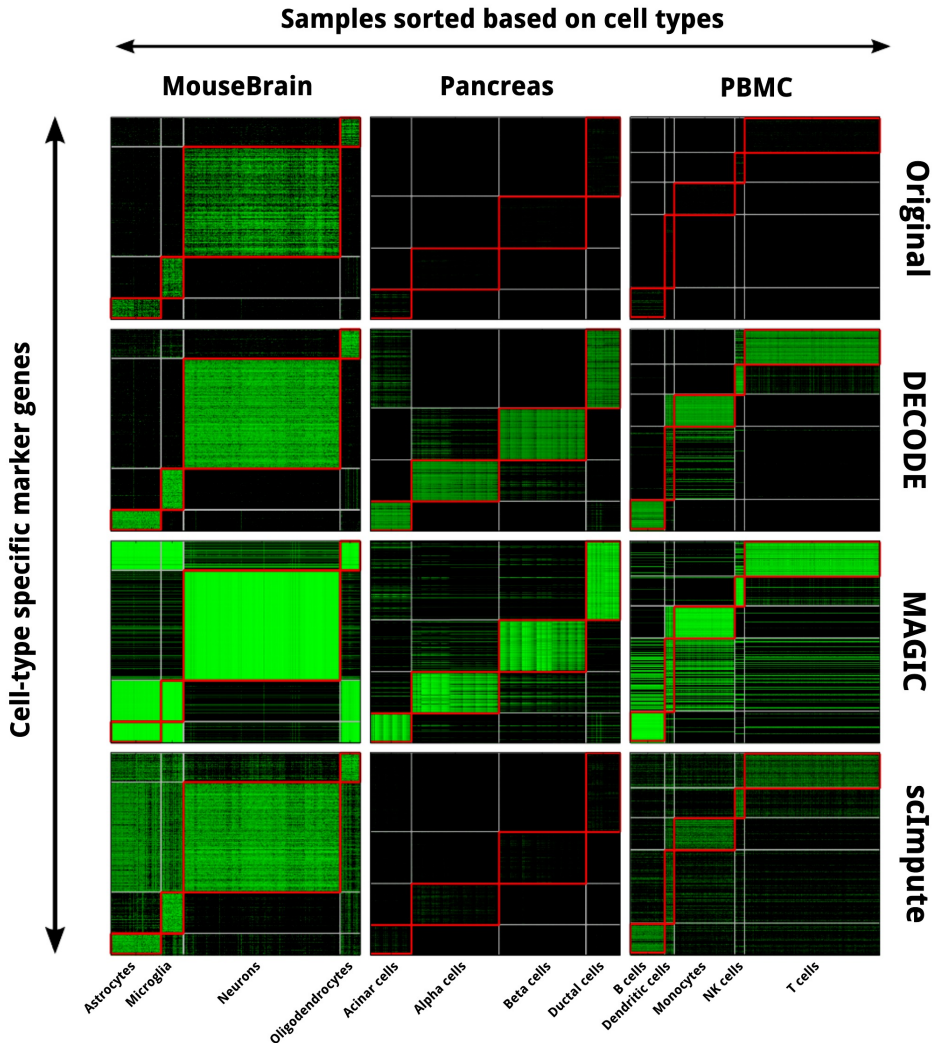
Luecken, MD and Theis, FJ. Current best practices in single-cell RNA-seq analysis: a tutorial, Mol Syst Biol 2019 (doi: https://doi.org/10.15252/msb.20188746)

Single-cell RNA sequencing analysis

- Introduction of single cell analysis
- **Dimensionality reduction**
  - Linear: PCA
  - Non-linear: t-SNE, UMAP
- Cell clustering
  - K-means
  - hierarchical clustering
  - graph-based clustering

Dimensionality Reduction

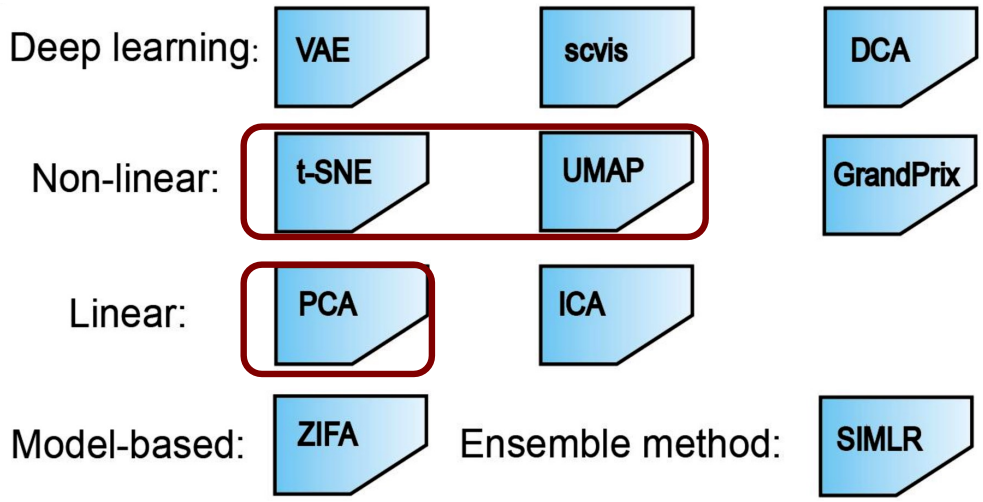
- Dimensionality reduction announces that cells could cluster together into groups.



https://doi.org/10.3389/fgene.2021.646936
https://www.biorxiv.org/content/10.1101/241646v1.full
Luecken, MD and Theis, FJ. Current best practices in single-cell RNA-seq analysis: a tutorial, Mol Syst Biol 2019 (doi: https://doi.org/10.15252/msb.20188746)

Dimensionality Reduction

- Reduce noise, sparsity
- Easier for visualization and processing
- Linear methods:
  - PCA (principal component analysis)
- Non-linear methods:
  - t-SNE
  - UMAP

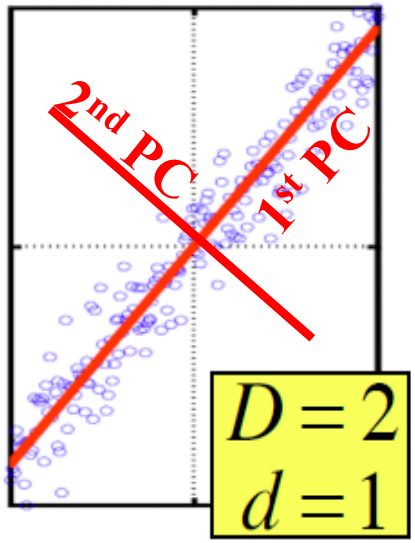


https://doi.org/10.3389/fgene.2021.646936
https://www.biorxiv.org/content/10.1101/241646v1.full

PCA

Principal component analysis

- It is a linear algebraic method of dimensionality reduction
- Any matrix can be decomposed as a multiplication of other matrices
- PC1 represents the most of variance for the data, then PC2, PC3

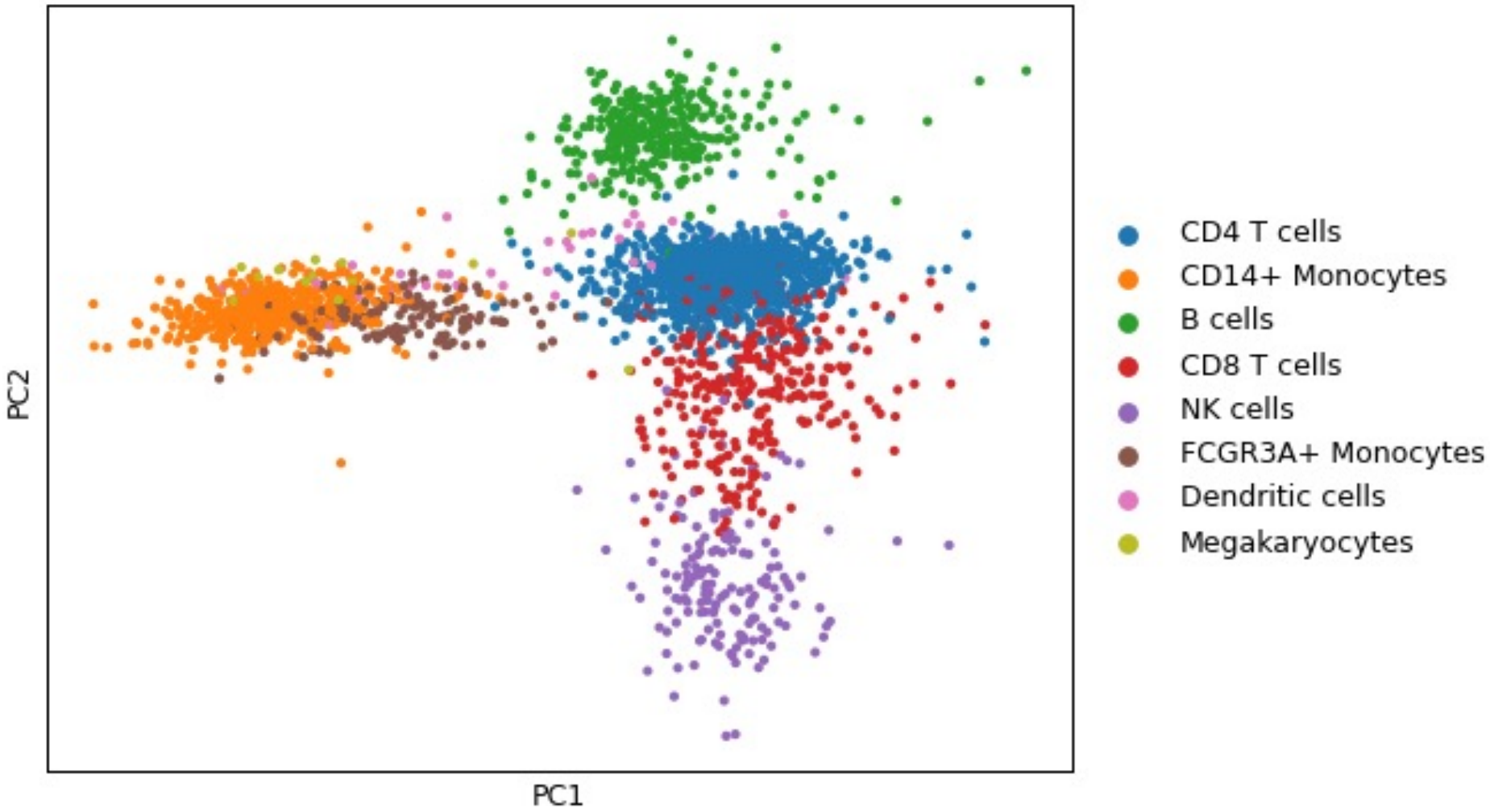


Reduce D genes to d PCs of cells, where $d<<D$

scanpy

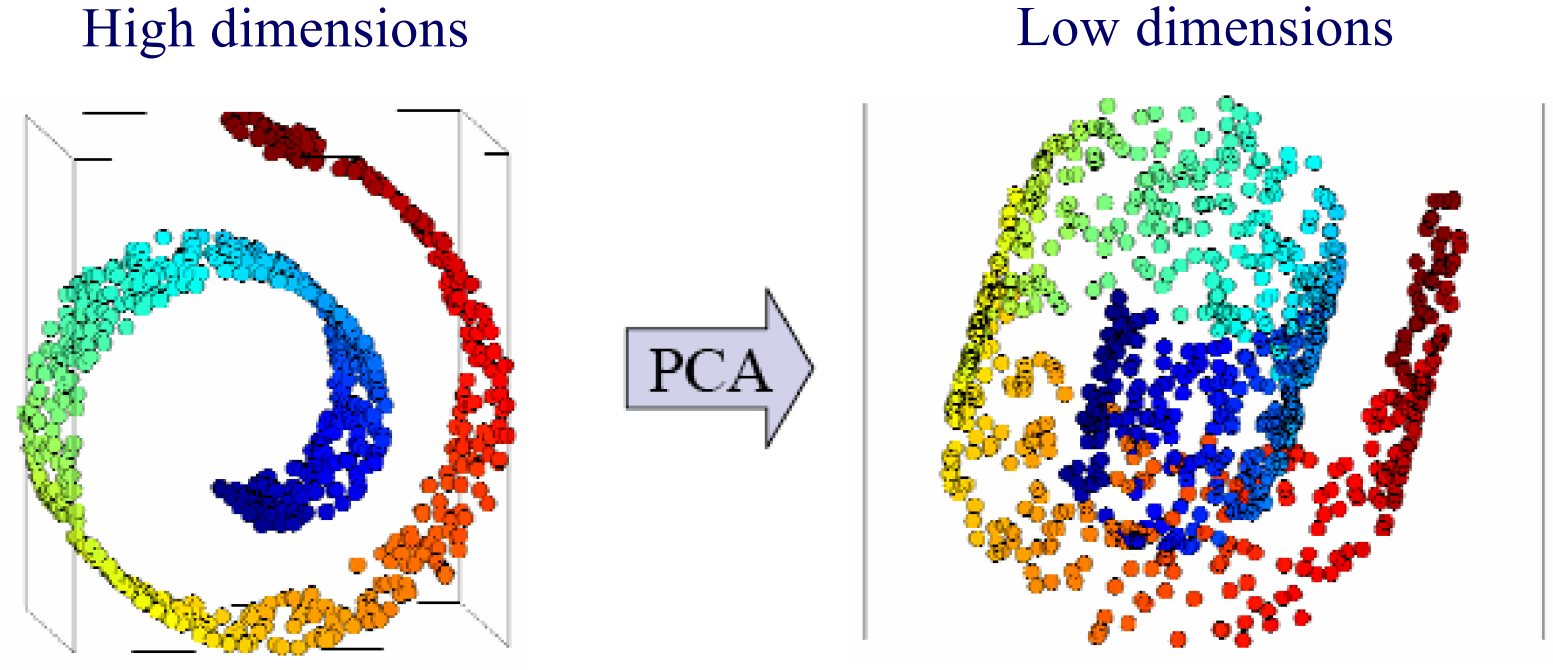
```
sc.pp.pca(adata, n_comps=50)
```

PCA of Peripheral Blood Mononuclear Cells (PBMC)



Limitation of PCA

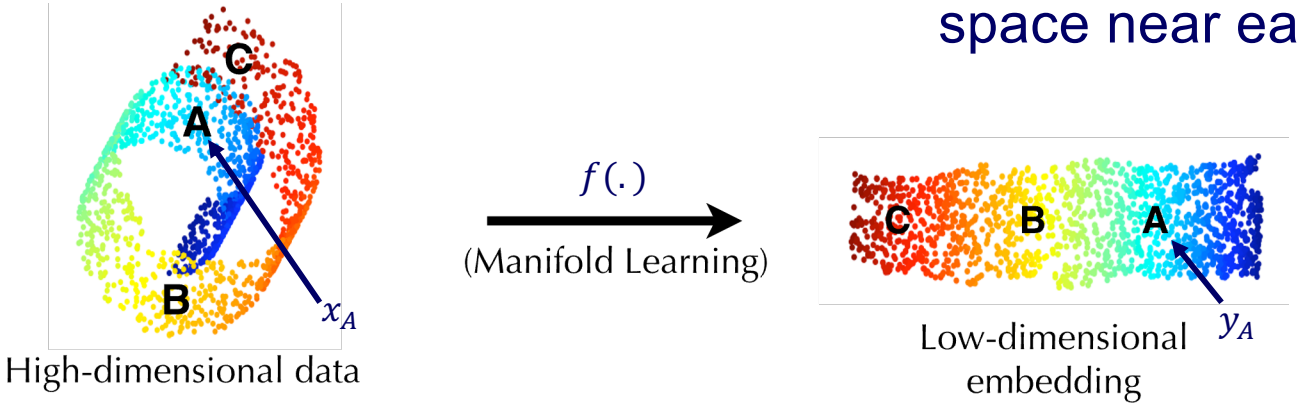
- PCs represent linear combination of individual features (e.g., genes)
- PCA fails to find non-linear structure in the data



https://www.cs.cmu.edu/~bapoczos/Classes/ML10715_2015Fall/slides/ManifoldLearning.pdf

Manifold learning

- A manifold is a topological space that locally resembles Euclidean space near each point



A d dimensional manifold M is embedded in a m dimensional space, and there is an explicit mapping f: $\mathbb{R}^m \rightarrow \mathbb{R}^d$ where $d \leq m$.

Given Sample $x_i \in \mathbb{R}^m$ with noise

$$y_i = f(x_i)$$

$\rightarrow$ find $f(.)$ or y_i from given x_i

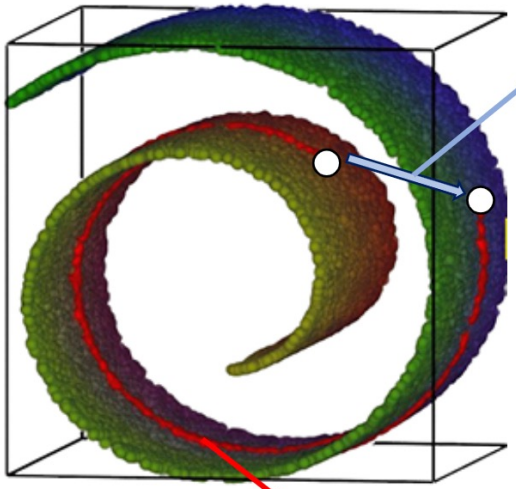
Parametric & generalizable (e.g., linear manifold learning)

Nonparametric & nongeneralizable (e.g., nonlinear manifold learning)

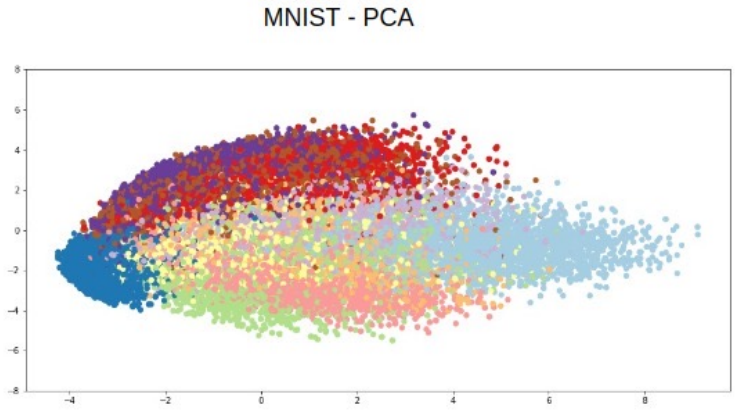
t-SNE

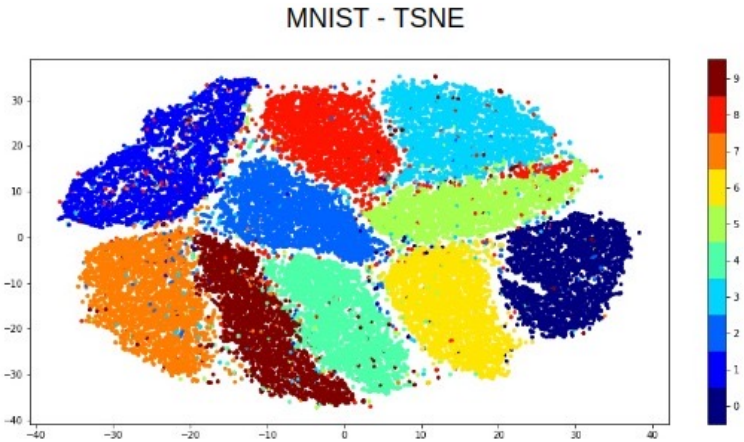
t-Stochastic Neighbor Embedding

- t-SNE is a manifold embedding algorithm for nonlinear dimensionality reduction (”keep manifold structures on low dimension space”)



Src: http://web-ext.u-aizu.ac.jp/~shigeo/home.html

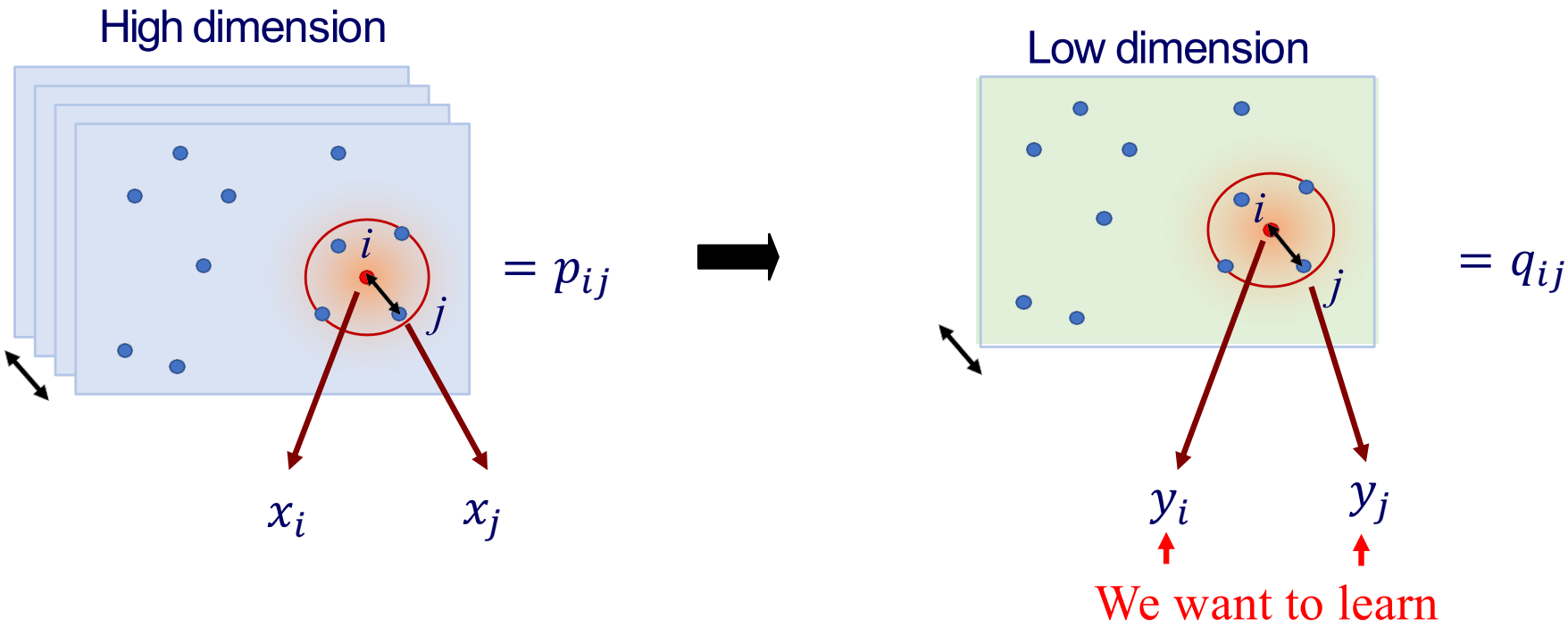




L.J.P. van der Maaten and G.E. Hinton. Visualizing High-Dimensional Data Using t-SNE. JMLR2008
https://nbviewer.org/github/YeoLab/single-cell-bioinformatics/tree/master/
https://mit6874.github.io/assets/sp2020/slides/L11_PCA_tSNE_Autoencoders.pdf

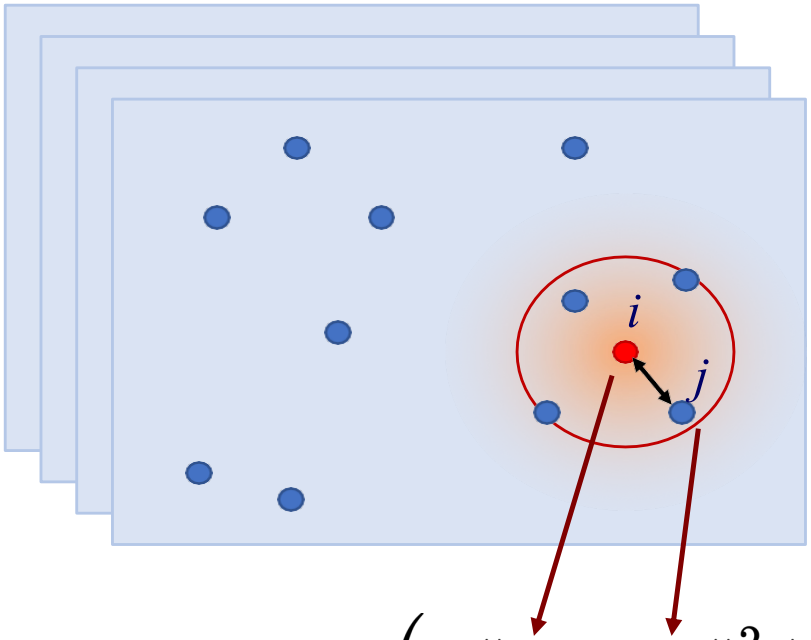
t-SNE

- Given a collection of points $X = \{x_1, \ldots, x_n\} \subset R^d$, find a collection of points $Y = \{y_1, \ldots, y_n\} \subset R^{d'}$, where $d \gg d'$. p_{ij} and q_{ij} measure the conditional probability that a point j would pick point i as it's nearest neighbor, in high (p) and low (q) dimensional space, respectively.



Similarity matrix at high dimension

High dimension



$= p_{ij}$

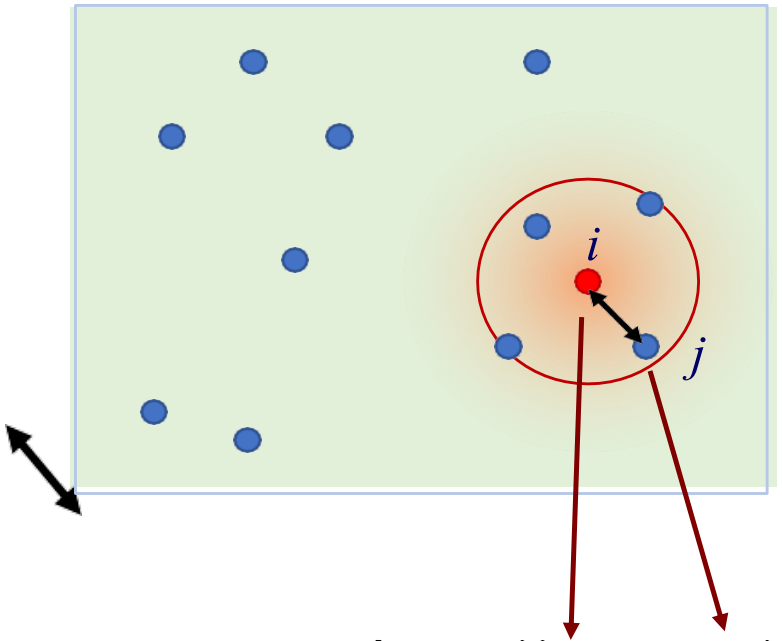
$$p_{j|i} = \frac{\exp\left(-\|x_i - x_j\|^2 / 2\tau_i^2\right)}{\sum_{k \neq i} \exp\left(-\|x_i - x_k\|^2 / 2\tau_i^2\right)}$$

$$p_{ij} = \frac{p_{j|i} + p_{i|j}}{2n}$$

where τ_i^2 is the variance for the Gaussian distribution centered around x_i

Similarity matrix at low dimension

Low dimension



$= q_{ij}$

$$q_{ij} = \frac{(1 + ||y_i - y_j||^2)^{-1}}{\sum_{k \neq l}(1 + ||y_k - y_l||^2)^{-1}}$$

Cost Function

Kullback-Leibler divergence

- Find optimal $\{y_i\}$: Minimize a single Kullback-Leibler divergence between a joint probability P, in the high-dimensional space and a joint probability Q, in the low-dimensional space.

$$C = \sum_i KL(P_i||Q_i) = \sum_{i=1}^{n}\sum_{j=1}^{n} p_{ij} \log \frac{p_{ij}}{q_{ij}}$$

- The gradient is given by

$$\frac{dC}{dy_i} = 4 \sum_{j=1, j\neq i}^{n} (p_{ij} - q_{ij})(1 + ||y_i - y_j||^2)^{-1}(y_i - y_j)$$

- The result of this optimization is a map that reflects the similarities between the high-dimensional inputs.

L.J.P. van der Maaten and G.E. Hinton. Visualizing High-Dimensional Data Using t-SNE. JMLR2008

Summary of t-SNE

- t-SNE minimizes the divergence between two similarity distributions:
  - pairwise similarities of the input data points on the high dimensional space $\{p_{ij}\}$
  - pairwise similarities of the corresponding low-dimensional points $\{q_{ij}\}$

Main steps for t-SNE

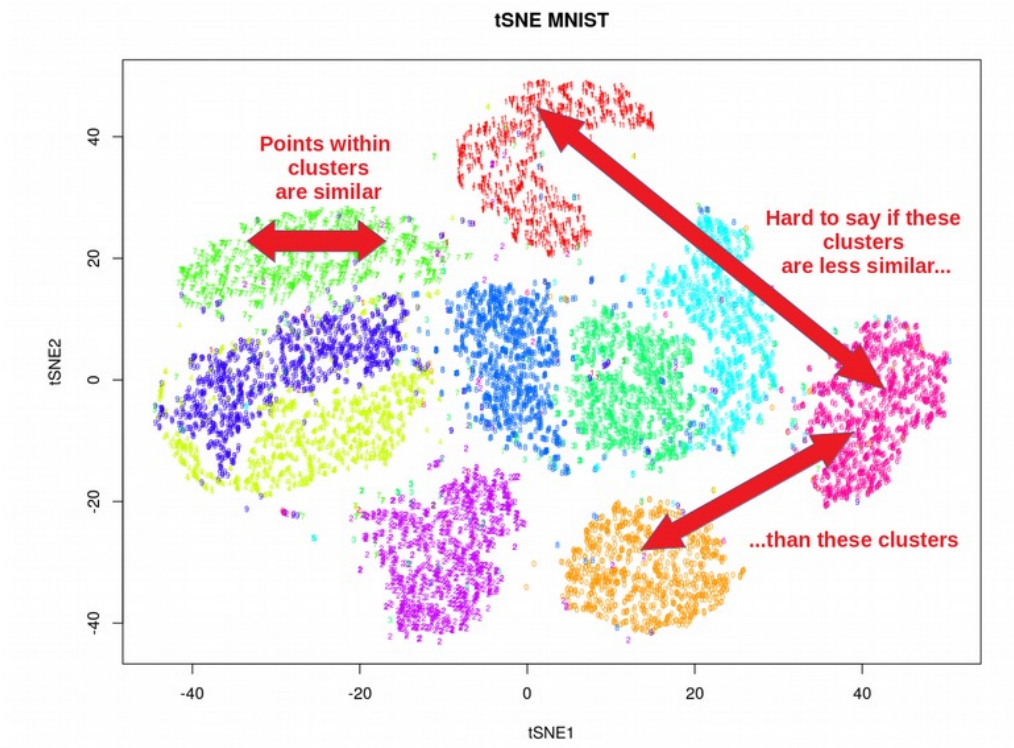
1. Construct a similarity matrix over pairs of high-dimensional points
   - Similar data points are assigned a higher probability, while dissimilar points are assigned a lower probability.

2. Define a similarity matrix in the low-dimensional embedding space

3. Minimize the KL divergence between two similarity distributions using gradient descent to find optimal low dimensional coordinates of data points

Limitation of t-SNE

- t-SNE mainly preserves local similarity structure of data, not global similarity.
- Only within cluster distances are meaningful while between cluster similarities are not guaranteed.



scanpy

```
sc.tl.tsne(adata, use_rep="X_pca")
```

https://towardsdatascience.com/how-exactly-umap-works-13e3040e1668

UMAP

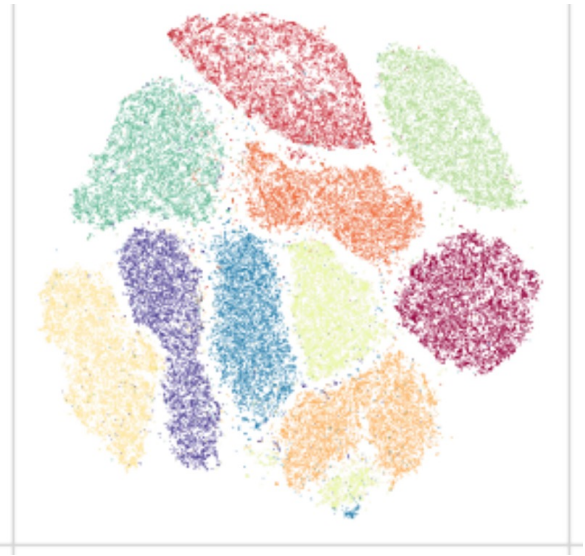
Uniform **M**anifold **A**pproximation and **P**rojection

- Instead of randomly assigning *points* as in t-SNE, UMAP constructs a high dimensional *graph representation* of the data and then optimizes a low-dimensional graph to be as structurally similar as possible

- UMAP could preserve both global and local data structures

UMAP on MNIST dataset

t-SNE on MNIST dataset



- https://www.youtube.com/watch?v=nq6iPZVUxZU
- https://nbisweden.github.io/excelerate-scRNAseq/session-dim-reduction/lecture_dimensionality_reduction.pdf
- https://umap-learn.readthedocs.io/en/latest/how_umap_works.html
- https://pair-code.github.io/understanding-umap/

Main steps for UMAP

1. Construct a weighted k nearest neighbors graph for defining a similarity matrix in high-dimensional space
   - The edge weights represent the likelihood that two data points are connected (i.e., “similarity”)

2. Define a similarity matrix in low-dimensional space

3. Minimize the cross-entropy cost function at each point using gradient descent to find the most *similar graph* in lower dimensions

L.J.P. van der Maaten and G.E. Hinton. Visualizing High-Dimensional Data Using t-SNE. JMLR2008
https://pair-code.github.io/understanding-umap/supplement.html

Similarity matrix at high dimension

- ρ_i, the distance from x_i to the k nearest neighbors in high dimension (e.g., kNN graph)

$$\rho_i = \min\{d(x_i, x_{i_j}) \mid 1 \leq j \leq k, d(x_i, x_{i_j}) > 0\}$$

- UMAP uses an exponential probability distribution in high dimension

Raw distance

Distance to nearest neighbor

$$p_{j|i} = e^{-\frac{d(x_i,x_j) - \rho_i}{\sigma_i}}$$

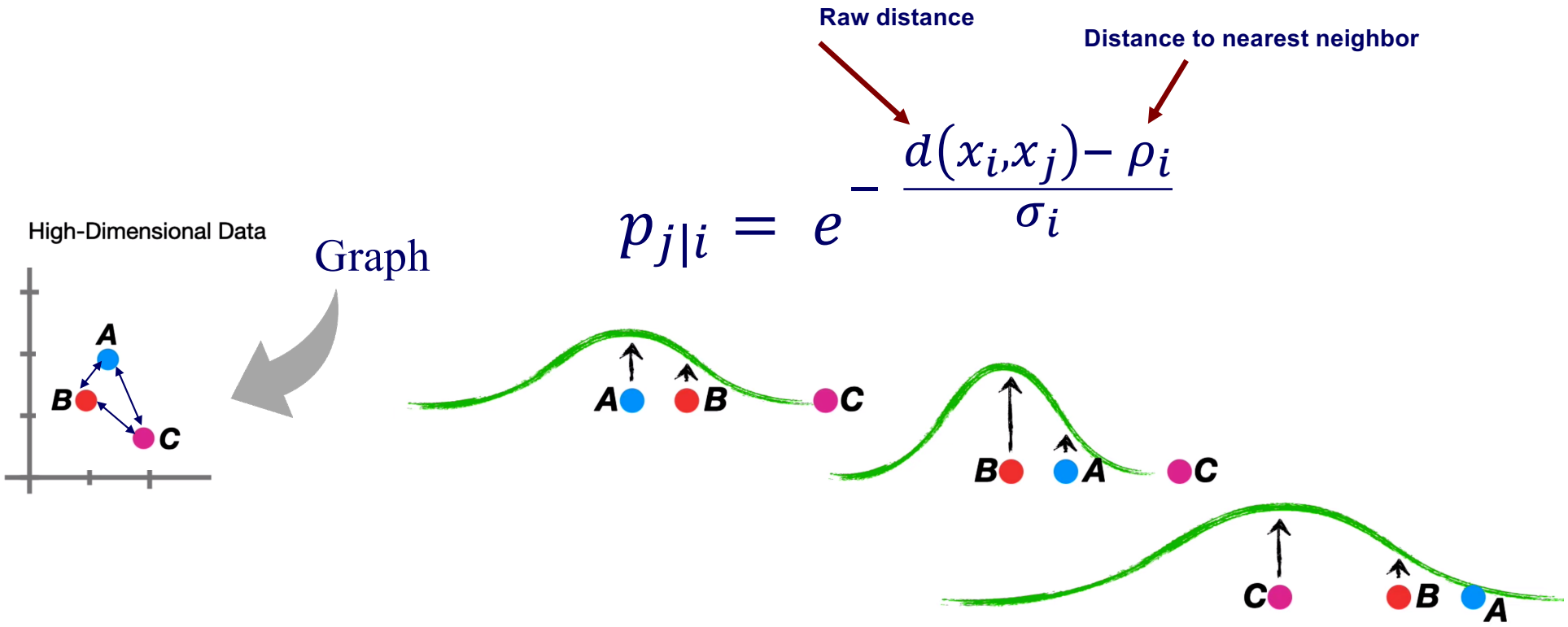
- The symmetrization of high dimension similarity score and the definition of number of nearest neighbors

$$p_{ij} = p_{i|j} + p_{j|i} - p_{i|j}p_{j|i}$$

https://towardsdatascience.com/how-exactly-umap-works-13e3040e1668
arXiv:1802.03426

Similarity matrix at high dimension

- For each data point, it has a locally adaptive exponential kernel, so the distance metric varies from point to point.



https://towardsdatascience.com/how-exactly-umap-works-13e3040e1668
arXiv:1802.03426
https://www.youtube.com/watch?v=jth4kEvJ3P8

Cost Function
binary cross-entropy (CE)

- Similarity matrix at low dimension

$$q_{ij} = \left(1 + a(y_i - y_j)^{2b}\right)^{-1}$$

the family of curves $1/a \times y^{2b}$ for modelling distance probabilities in low dimensions, a and b are hyperparameters

- Cross-entropy cost function makes UMAP to capture the global and local data structures

$$CE(X,Y) = \sum_i \sum_j \left[p_{ij}(X) \log\left(\frac{p_{ij}(X)}{q_{ij}(Y)}\right) + (1 - p_{ij}(X)) \log\left(\frac{1 - p_{ij}(X)}{1 - q_{ij}(Y)}\right) \right]$$

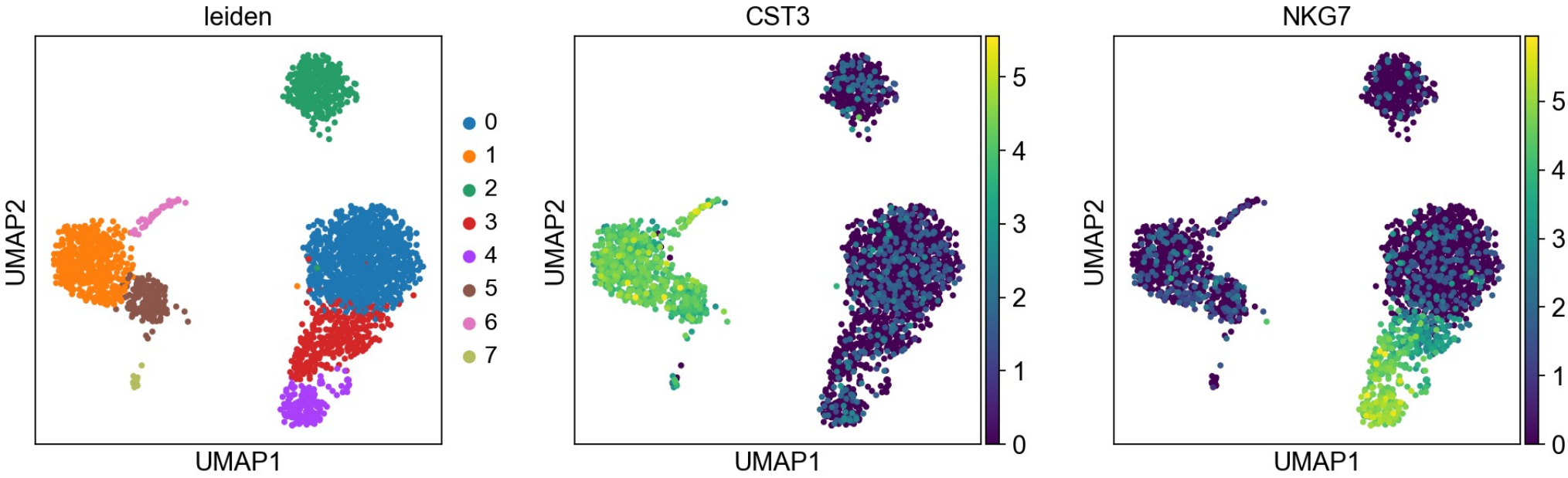
- The gradient of the CE to find optimal $\{y_i\}$

$$\frac{\delta CE}{\delta y_i} = \sum_j \left[\frac{2abd_{ij}^{2(b-1)} P(X)}{1 + ad_{ij}^{2b}} - \frac{2b(1 - P(X))}{d_{ij}^2 (1 + ad_{ij}^{2b})} \right] \quad \text{where } d_{ij} = y_i - y_j$$

arXiv:1802.03426
https://towardsdatascience.com/how-exactly-umap-works-13e3040e1668

UMAP examples

```
sc.pl.umap(adata, color=['leiden', 'CST3', 'NKG7'])
```

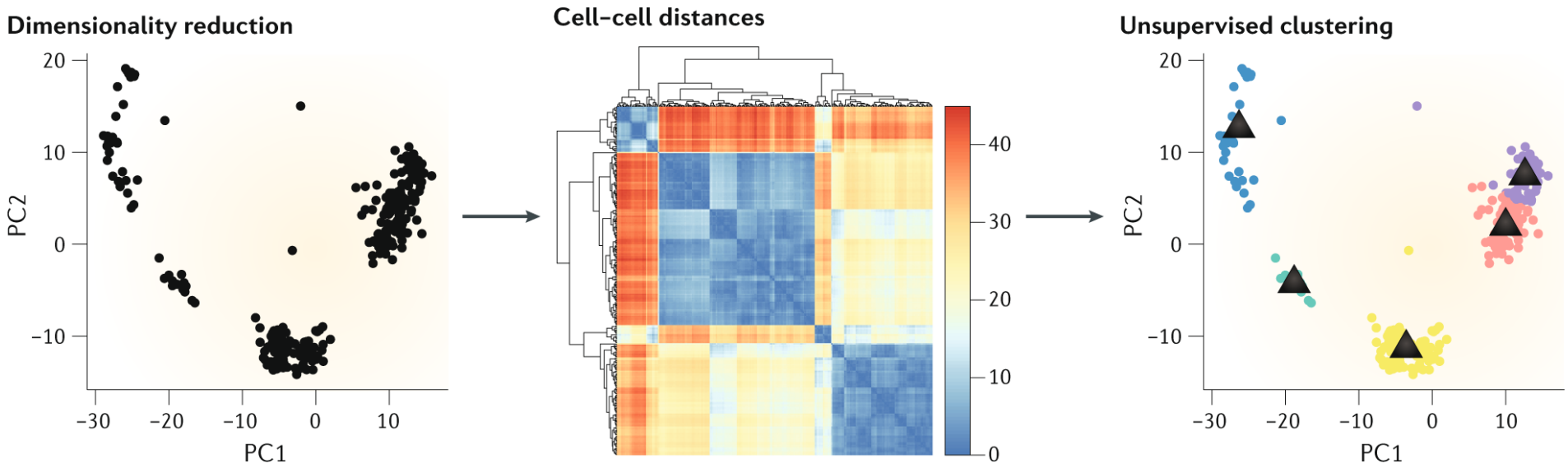


https://scanpy-tutorials.readthedocs.io/en/latest/pbmc3k.html

Single-cell RNA sequencing analysis

- Introduction of single cell analysis
- Dimensional reduction
  - Non-linear: t-SNE, UMAP
  - Linear: PCA
- **Cell clustering**
  - K-means
  - hierarchical clustering
  - graph-based clustering

Cell clustering



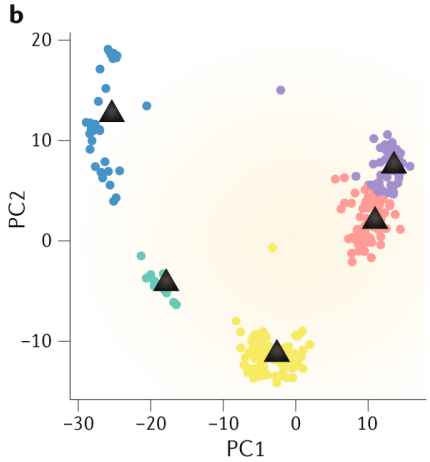
- Feature selection and dimensionality reduction extract the most informative genes and represented features from background noise, respectively
- Cell–cell distances are then calculated in the low dimensional space for clustering cells to clusters

Kiselev, V.Y., Andrews, T.S. & Hemberg, M. Challenges in unsupervised clustering of single-cell RNA-seq data. *Nat Rev Genet* **20,** 273–282 (2019). https://doi.org/10.1038/s41576-018-0088-9

Clustering methods for scRNA-seq

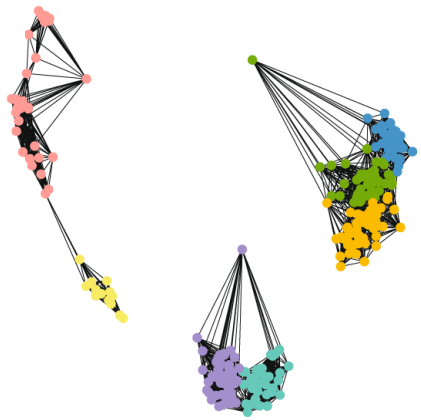
Clustering methods:

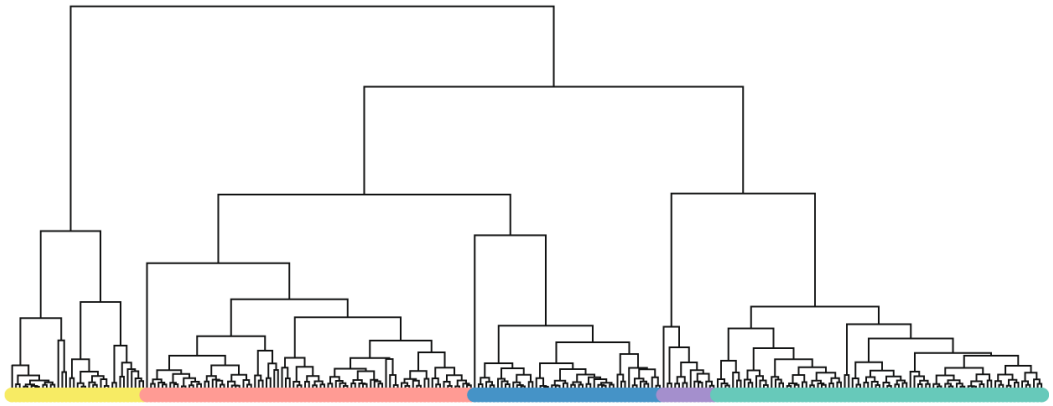
- K-means
- hierarchical clustering
- **graph-based clustering**



Tools for graph-based clustering:

- Seurat: Louvain, Leiden, SLM
- igraph:fast greedy, Louvain, optimal, walktrap, spinglass, infomap

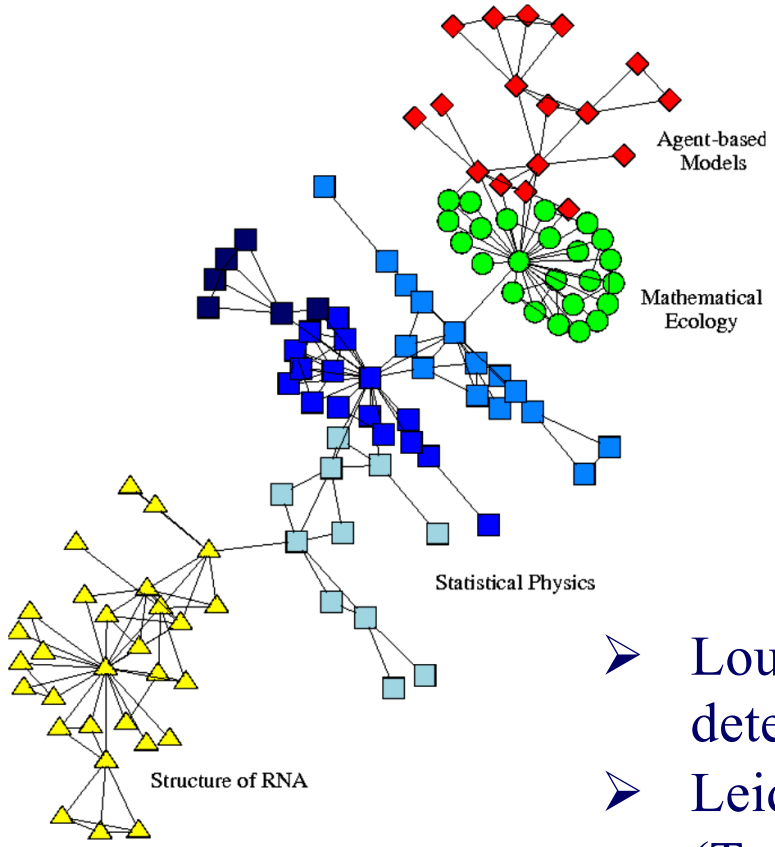




Kiselev, V.Y., Andrews, T.S. & Hemberg, M. Challenges in unsupervised clustering of single-cell RNA-seq data. *Nat Rev Genet* **20,** 273–282 (2019). https://doi.org/10.1038/s41576-018-0088-9

Graph-based clustering

- One of most popular clustering algorithms in scRNA-seq data analysis

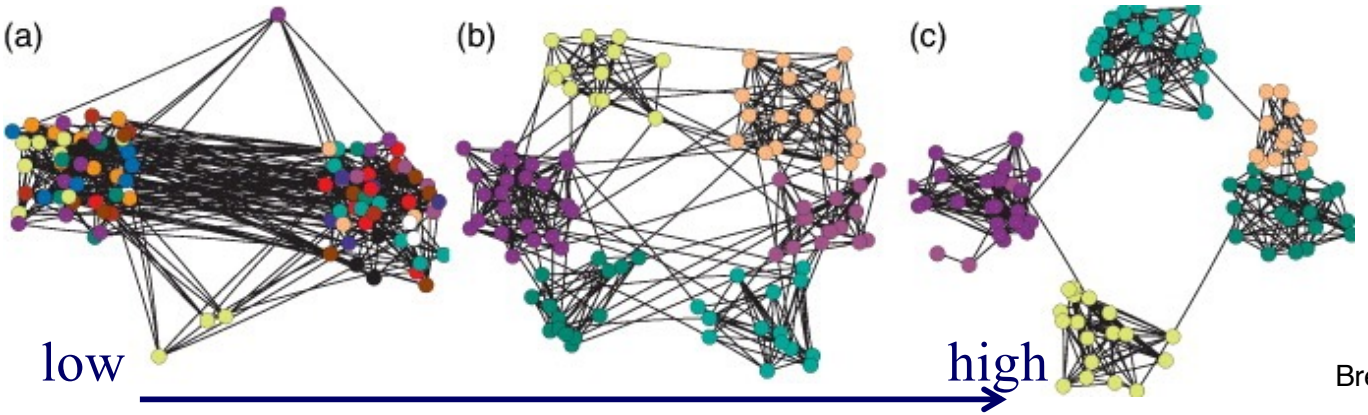


- Node -> cell
- Edge -> similarity between two cells

- Louvain community detection: (Blondel et al. 2008)
- Leiden community detection: (Traag, Waltman, and Eck 2019)

- Freytag, Saskia, Luyi Tian, Ingrid Lönnstedt, Milica Ng, and Melanie Bahlo. 2018. "Comparison of Clustering Tools in R for Medium-Sized 10x Genomics Single-Cell Rna-Sequencing Data." *F1000Research* 7. Faculty of 1000 Ltd.
- Wasserman, S. & Faust, K. (1994) Social Network Analysis (Cambridge Univ. Press, Cambridge, U.K.).
- https://biocellgen-public.svi.edu.au/mig_2019_scrnaseq-workshop/public/clustering-and-cell-annotation.html#ref-freytag2018comparison

Modularity



Brede, Europhysics Letters, 2010.

Modularity Q: measurement on strength of network division

$$Q = \frac{1}{2m}\sum_{i,j}\left(W_{ij} - \frac{k_i k_j}{2m}\right)\delta_{\sigma_i\sigma_j}$$

sum over nodes within a group (module)

normalization
m: total number of edges

edge weight between nodes i and j

$\frac{k_i k_j}{2m} = p_{ij}$ = expected edge weight that would go between i and j

Clustering goal: assign each node a module to maximize "modularity" as an objective function (module is a group of highly connected nodes)

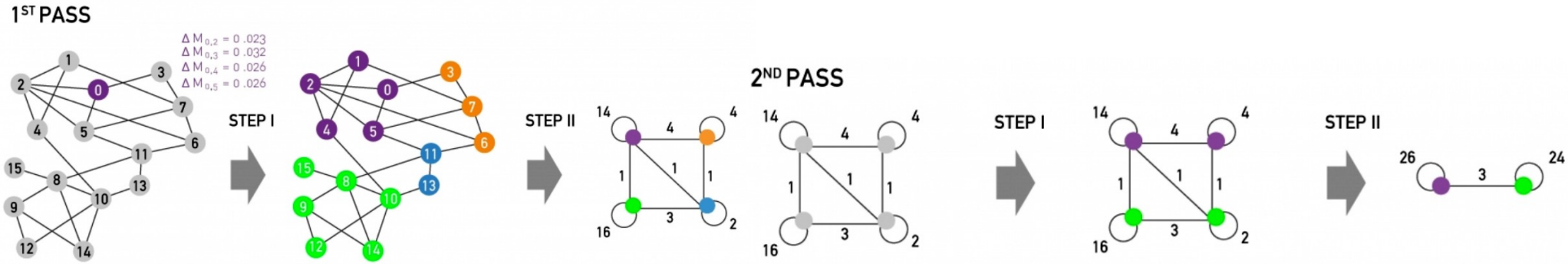
Newman, PNAS, 2006.

Louvain community detection

- Start with every node in its own community (i.e., module/cluster)
- Step1: Modularity optimization
  - Order the nodes and for each node i, move i to the community of neighbor j that leads to maximum ΔQ
  - If all $\Delta Q < 0$ the i remains in its current community
  - Repeatedly cycle through all nodes until $\Delta Q = 0$

- Step2 : Community aggregation
  - Create a weighted network of communities from Step1
  - Nodes : communities in Step1
  - Edge weights : sum of weights of edges between communities
  - Edges within a community become two self-loops

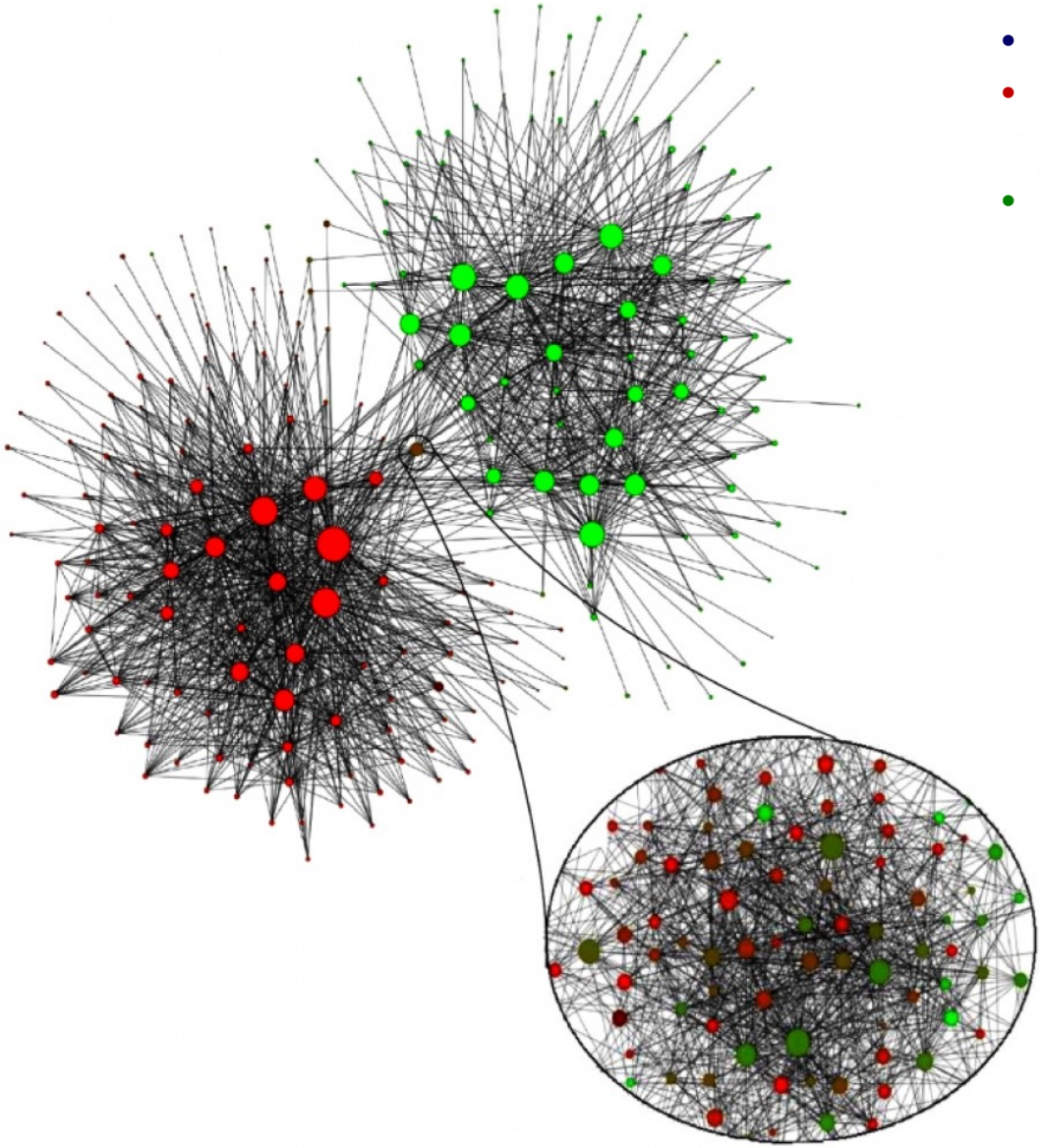
- Repeat: Apply Step1 & Step2 to resulting network, and so on until $\Delta Q = 0$



https://biocellgen-public.svi.edu.au/mig_2019_scrnaseq-workshop/public/clustering-and-cell-annotation.html#ref-freytag2018comparison
https://www.youtube.com/watch?v=QNv7rKWCgM8

Examples of Louvain clustering

Belgian mobile phone network



- 2M nodes
- **Red nodes: French speakers**
- **Green nodes: Dutch speakers**

Blondel et al.2008

Examples of Louvain clustering

The Louvain algorithm clusters millions of cell with reasonable computational complexity.

scanpy

```
sc.tl.louvain(adata, resolution=1.0)
```


Luecken MD, Theis FJ (2019) Current best practices in single-cell RNA-seq analysis: a tutorial. Mol Syst Biol 15:e8746

Han X, Zhou Z, Fei L, Sun H, Wang R, Chen Y, Zhou Y (2020) Construction of a human cell landscape at single-cell level. Nature 581:303–309. https://doi.org/10.1038/s41586-020-2157-4

Seow, J.J.W., Wong, R.M.M., Pai, R. *et al.* Single-Cell RNA Sequencing for Precision Oncology: Current State-of-Art. *J Indian Inst Sci* **100,** 579–588 (2020). https://doi.org/10.1007/s41745-020-00178-1