



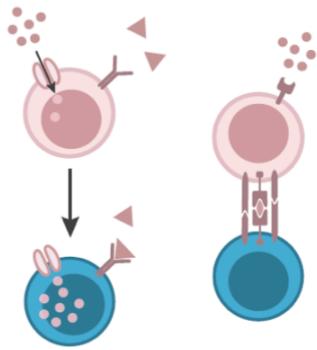
# scRNAseq clustering tools

Åsa Björklund

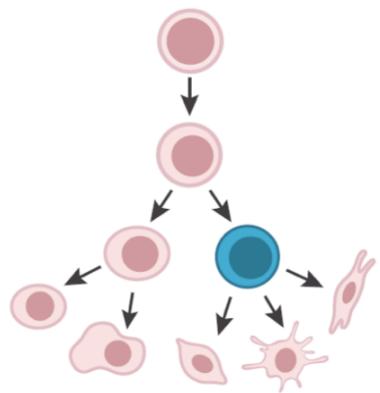
[asa.bjorklund@scilifelab.se](mailto:asa.bjorklund@scilifelab.se)

# Cell identity

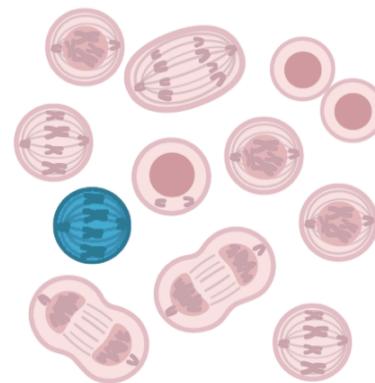
Environmental stimuli



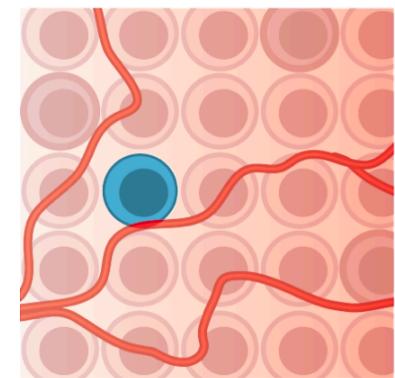
Cell development



Cell cycle



Spatial context



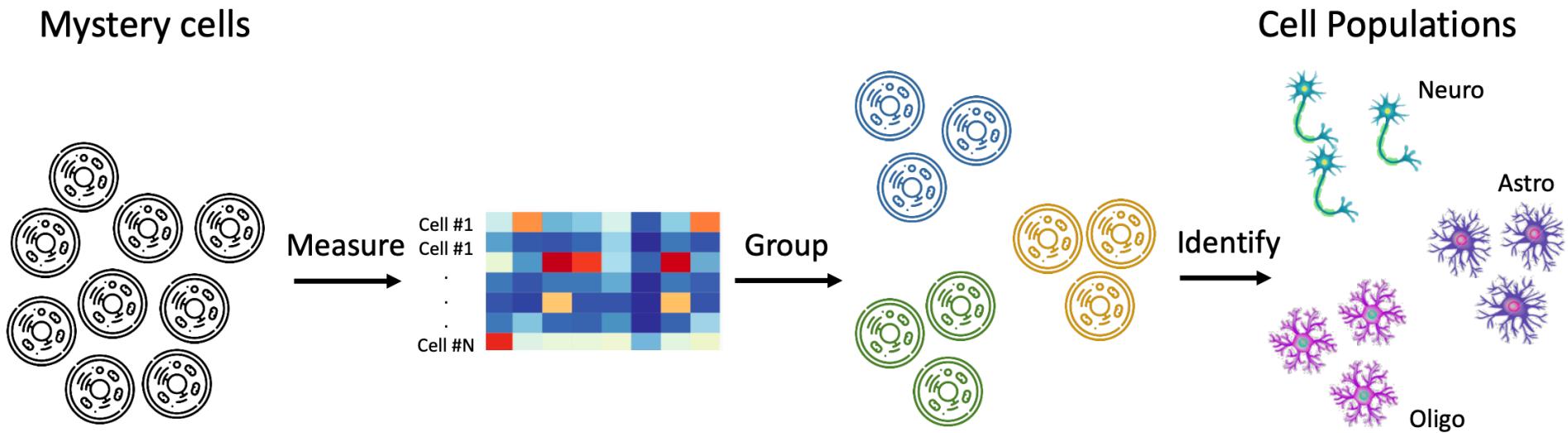
# What is a cell type?

- A cell that performs a specific function?
- A cell that performs a specific function at a specific location/tissue?
- Not clear where to draw the line between cell types and **subpopulations** within a cell type.
- Also important to distinguish between **cell type** and **cell state**.
  - A cell state may be infected/non infected
  - Metabolically active/inactive
  - Cell cycle stages
  - Apoptotic

# Outline

- Basic clustering theory
- Graph theory introduction
- scRNAseq clustering with graphs
- Examples of different tools

# How can we identify populations?



# Considerations for clustering

- Hypotheses:
  - What is a cell type? What cell types are in my tissue?
  - What is the number of clusters  $k$ ?
- Choices:
  - Gene set selection
  - Similarity measure / Space to calculate similarity
  - Algorithm and hyper parameters of that algorithm.
- Different choice leads to different results. Validate, interpret and repeat steps.

# What is clustering?

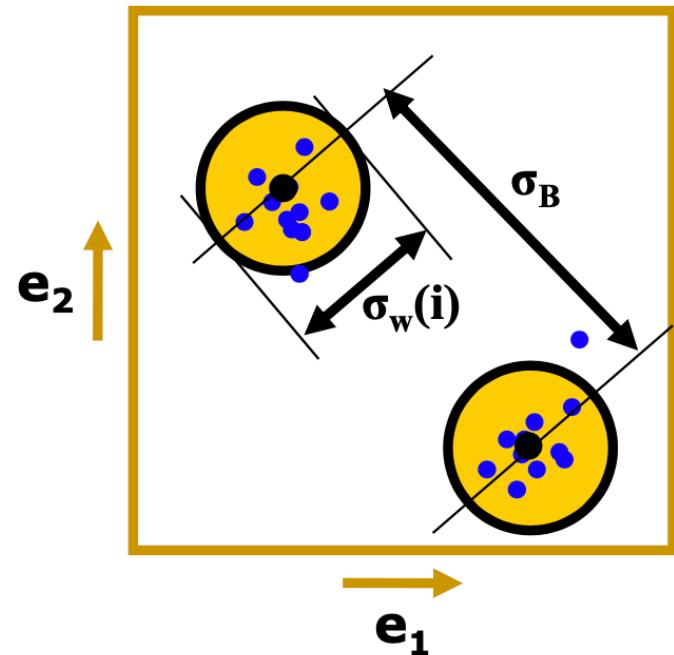
- “The process of organizing objects into groups whose members are similar in some way”
- Typical methods are:
  - Hierarchical clustering
  - K-means clustering
  - Density based clustering
  - Graph based clustering

# The main idea

- Structure when:
  - 1) Samples within cluster resemble each other (*within variance,  $\sigma_W(i)$* )
  - 2) Clusters deviate from each other  
(*between variance,  $\sigma_B$* )

Group samples such that:

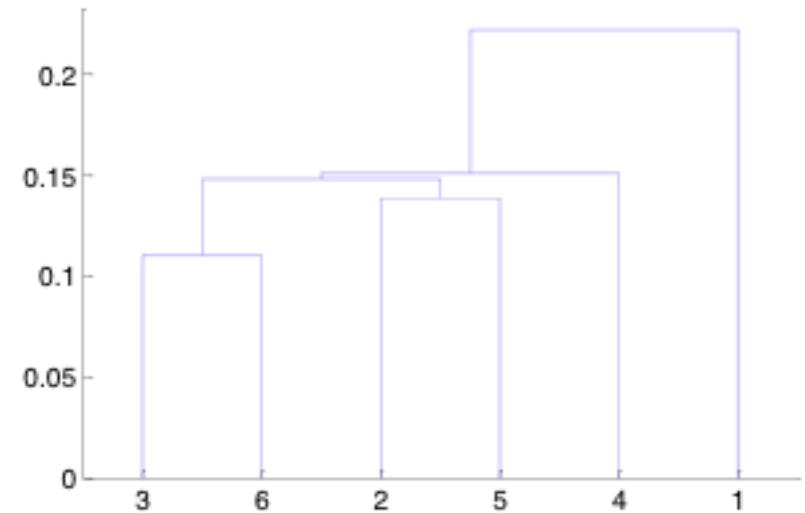
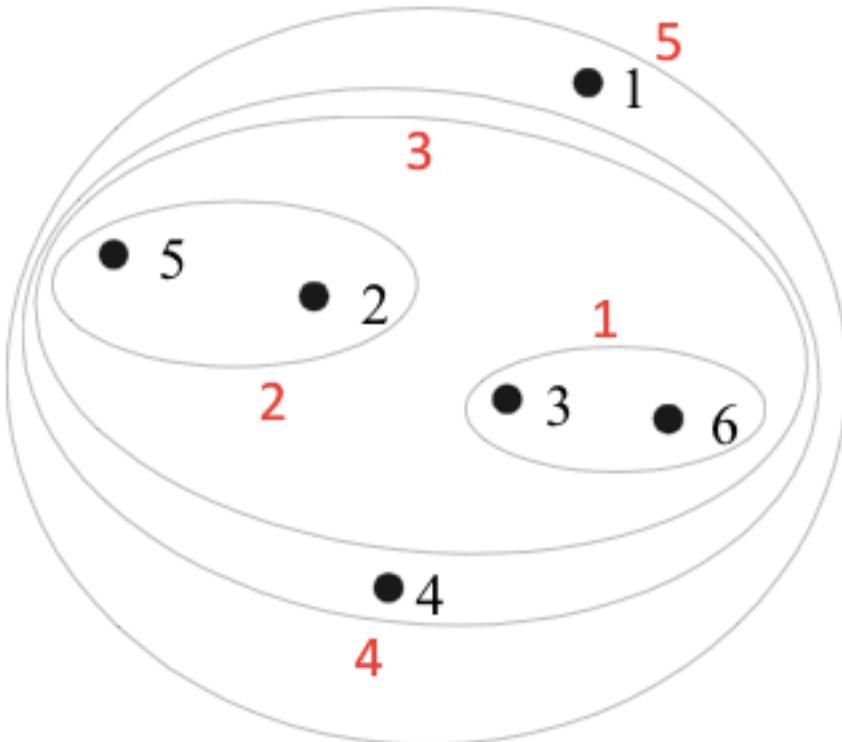
$$\min \left( \frac{\sum_{\text{clusters}} \sigma_w(i)}{\sigma_B} \right) \rightarrow \begin{matrix} \sigma_w: \text{small} & \& \\ \& \& \sigma_B: \text{large} \end{matrix}$$



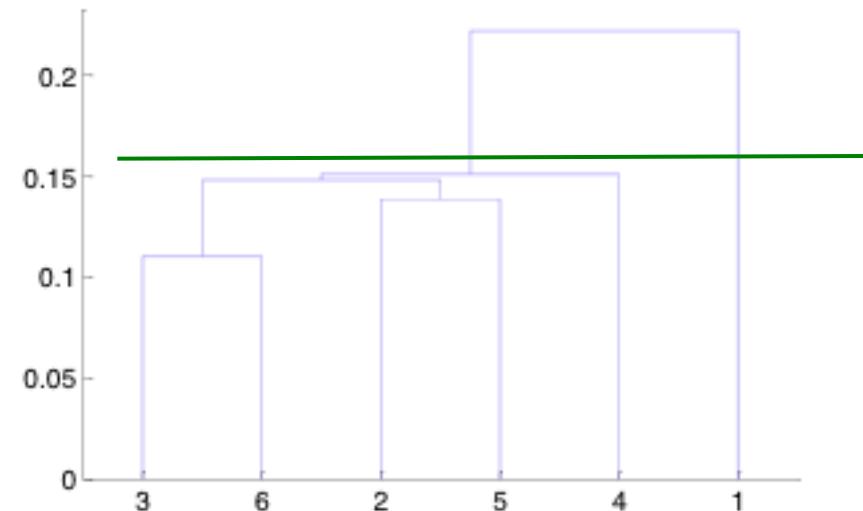
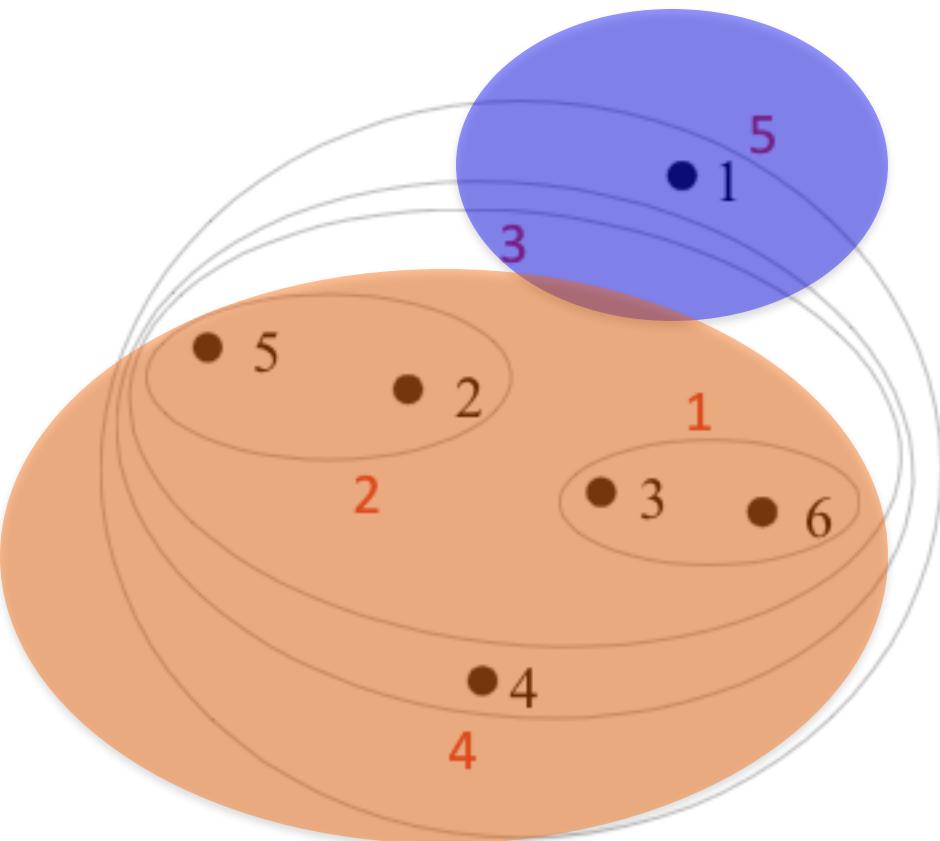
# Hierarchical clustering

- Builds on **distances** between data points
- **Agglomerative** – starts with all data points as individual clusters and joins the most similar ones in a bottom-up approach
- **Divisive** – starts with all data points in one large cluster and splits it into 2 at each step. A top-down approach
- Final product is a **dendrogram** representing the decisions at each merge/division of clusters

# Hierarchical clustering

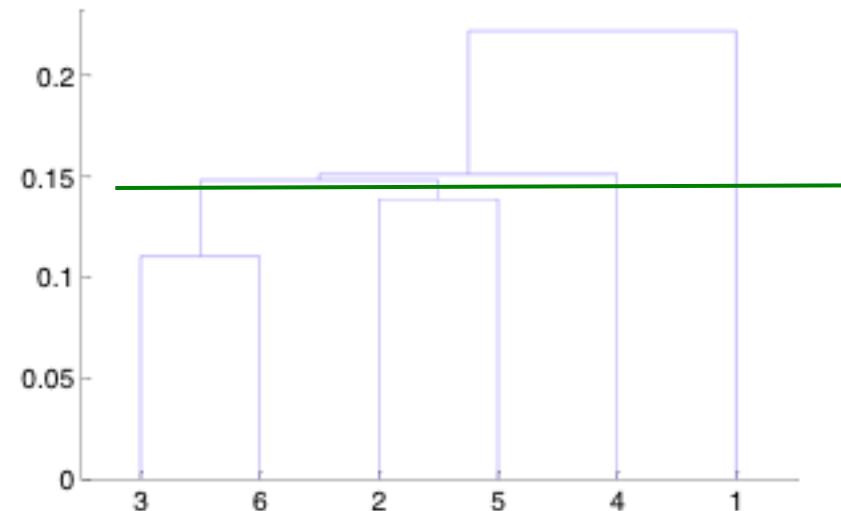
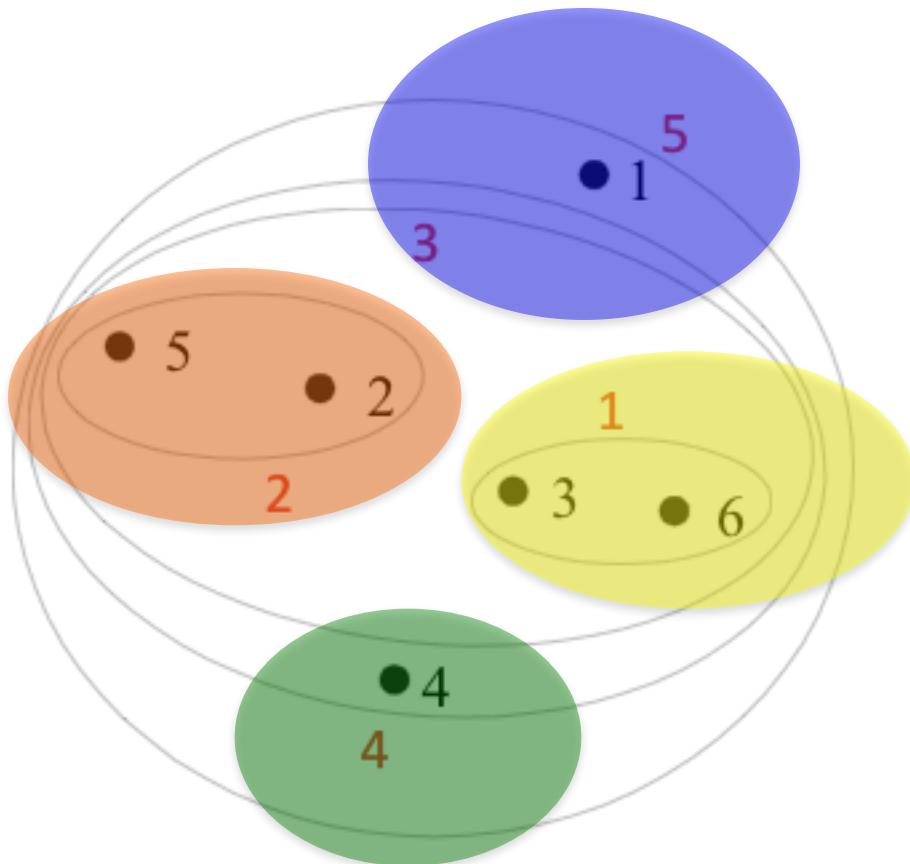


# Hierarchical clustering



Clusters are obtained by cutting the tree at a desired level

# Hierarchical clustering

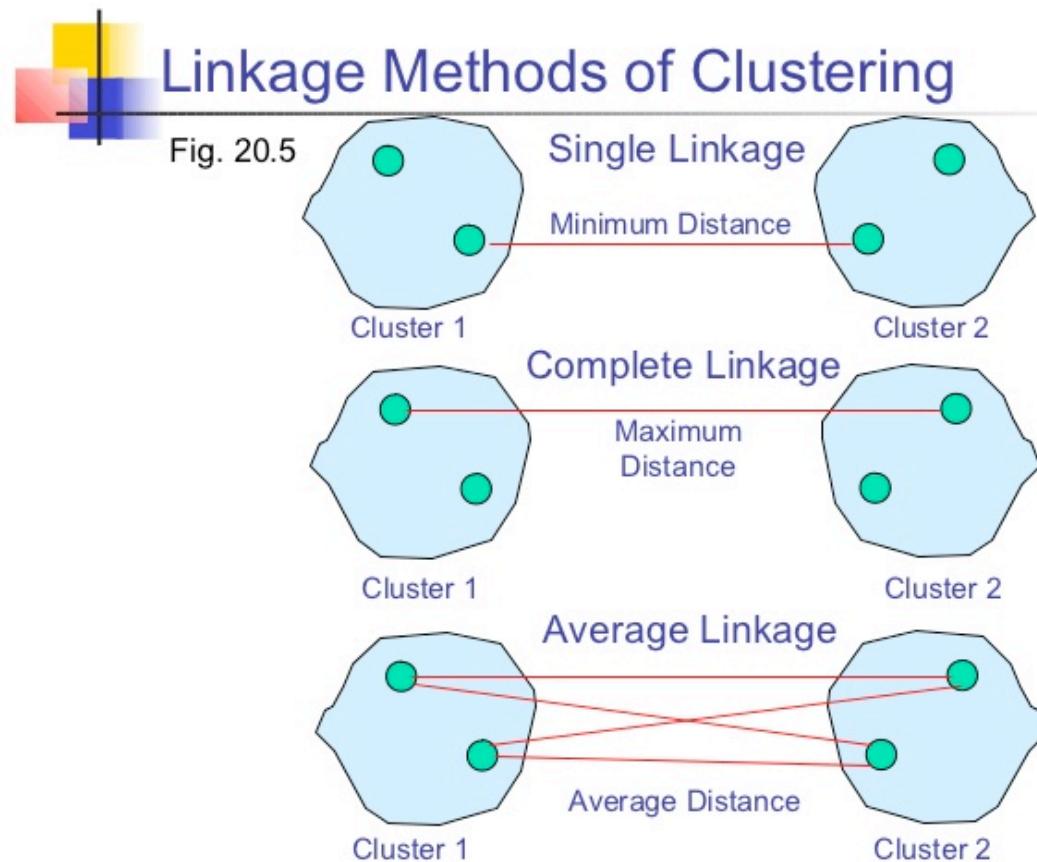


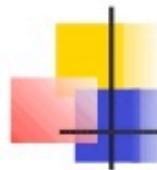
Clusters are obtained by cutting the tree at a desired level

# Linkage criteria

- Calculation of similarities between 2 clusters (or a cluster and a data point)

20-17

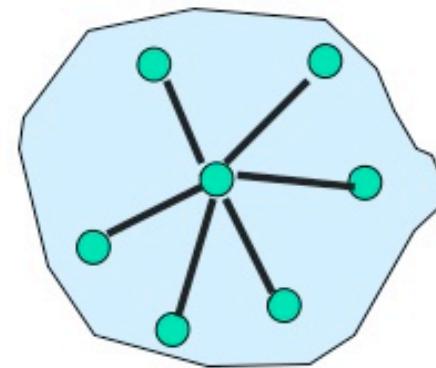
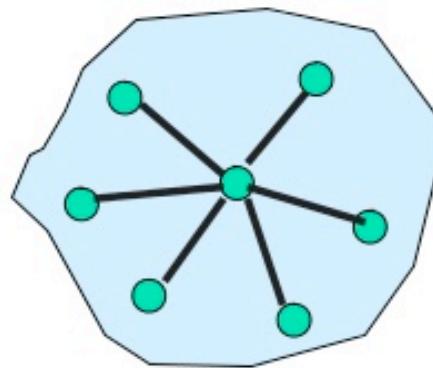




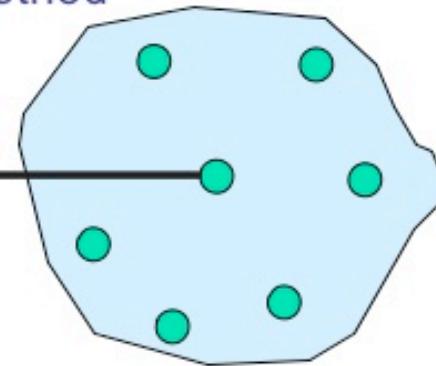
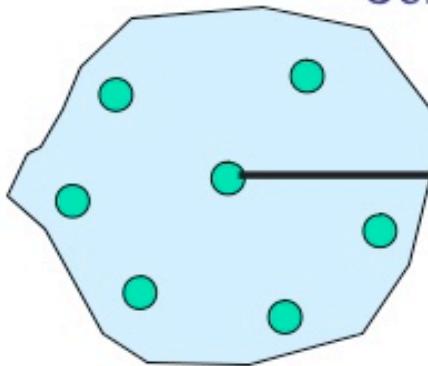
## Other Agglomerative Clustering Methods

Fig. 20.6

Ward's Procedure



Centroid Method

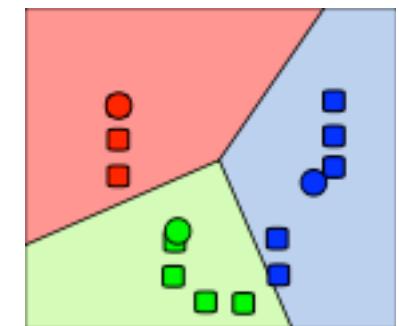
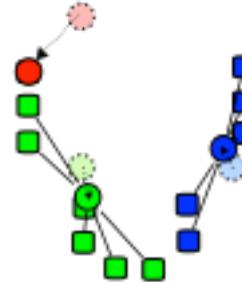
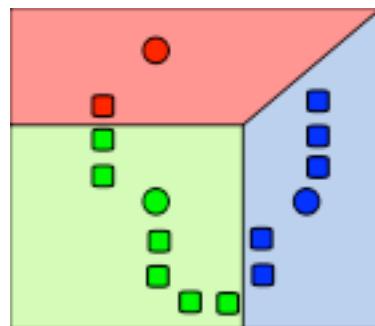
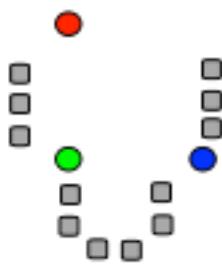


- Ward (minimum variance method). Similarity of two clusters is based on the increase in squared error when two clusters are merged.

# K-means clustering

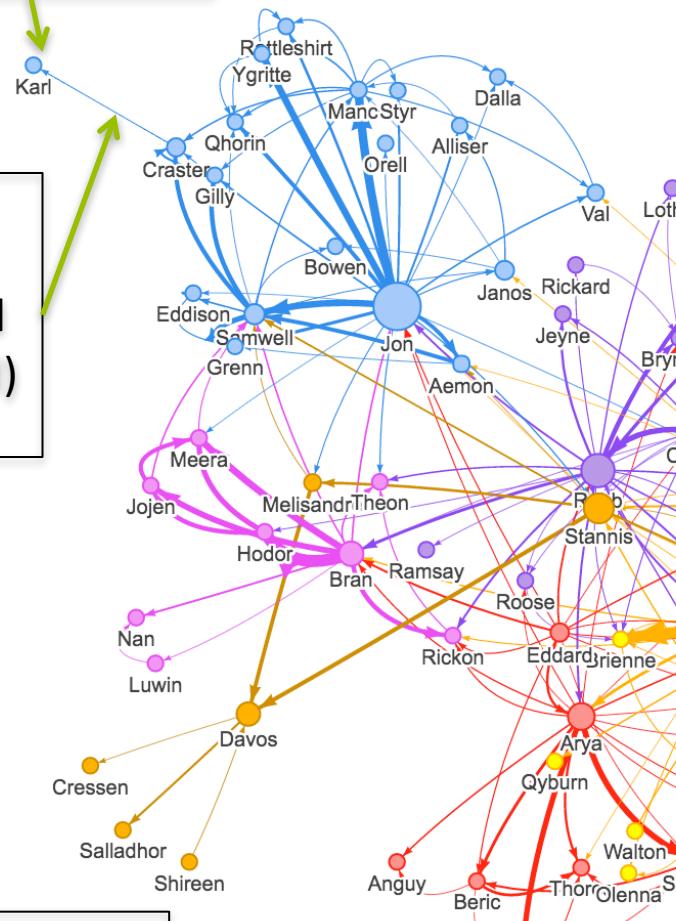
1. Starts with random selection of cluster centers (centroids)
2. Then assigns each data points to the nearest cluster
3. Recalculates the centroids for the new cluster definitions
4. Repeats steps 2-3 until no more changes occur.

Can use same distance measures as in hclust.



# Network/graph clustering

Node/Vertex



Edge –  
(weighted  
& directed)

Community

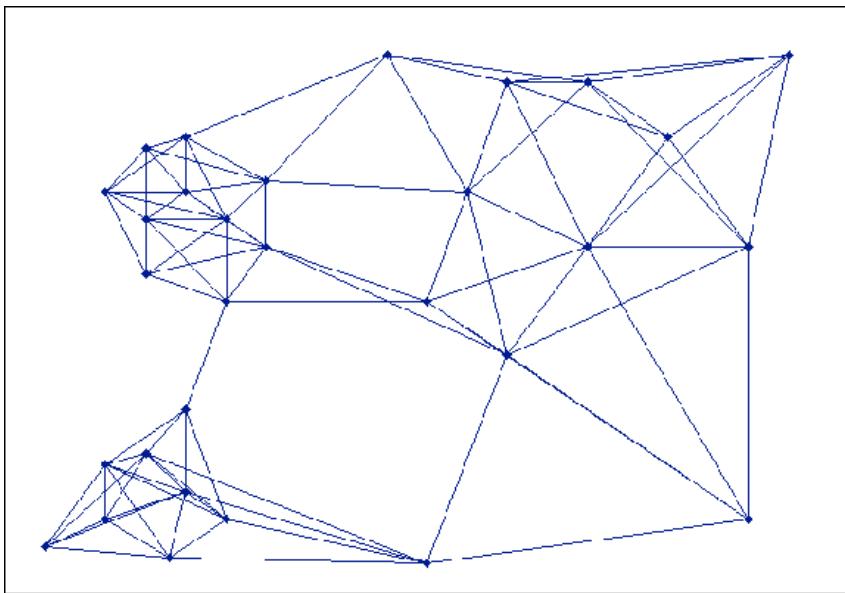
Hubs

Connectivity  
- # of edges

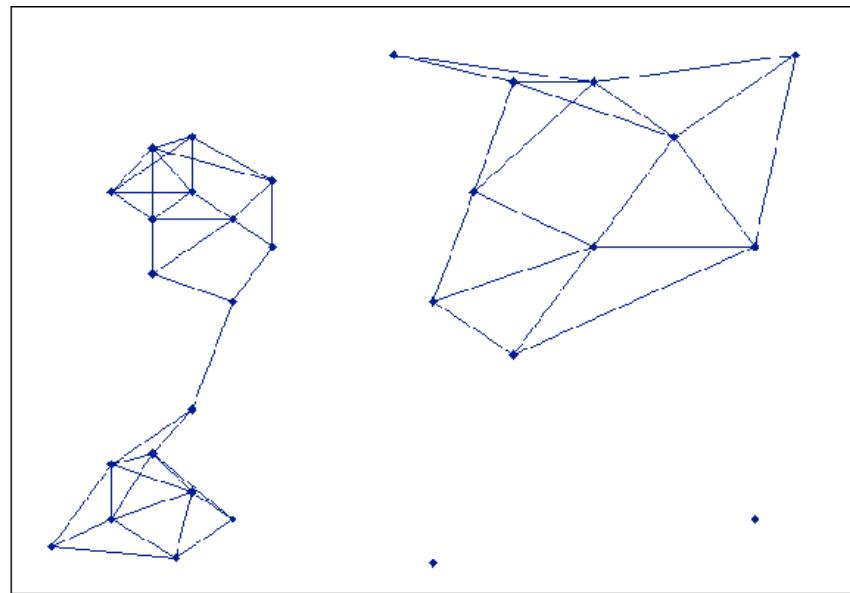
# Types of graphs

- The  **$k$ -Nearest Neighbor ( $k$ NN)** graph is a graph in which two vertices  $p$  and  $q$  are connected by an edge, if the distance between  $p$  and  $q$  is among the  $k$ -th smallest distances from  $p$  to other objects from  $P$ .
- The **Shared Nearest Neighbor (SNN)** graph has weights that defines proximity, or similarity between two edges in terms of the number of neighbors (i.e., directly connected vertices) they have in common.

# SNN graph



**Figure 2. Near Neighbor Graph**

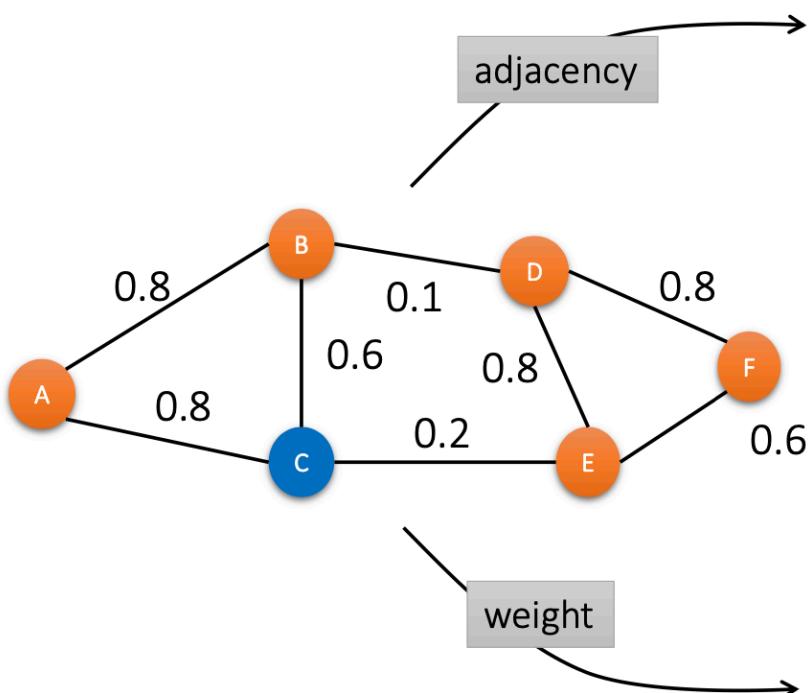


**Figure 3. Unweighted Shared Near Neighbor Graph**

# SNN graph

- A common measure of shared neighbors is the Jaccard index:
  - Shared neighbors / Total neighbors for both.  $J(A,B) = \frac{|A \cap B|}{|A \cup B|}$
- Other measures includes rank, number of shared neighbors, overlap coefficient
- Common to do pruning – remove all edges between nodes with e.g. Jaccard similarity < cutoff.

# Graphs, adjacency and weight matrices

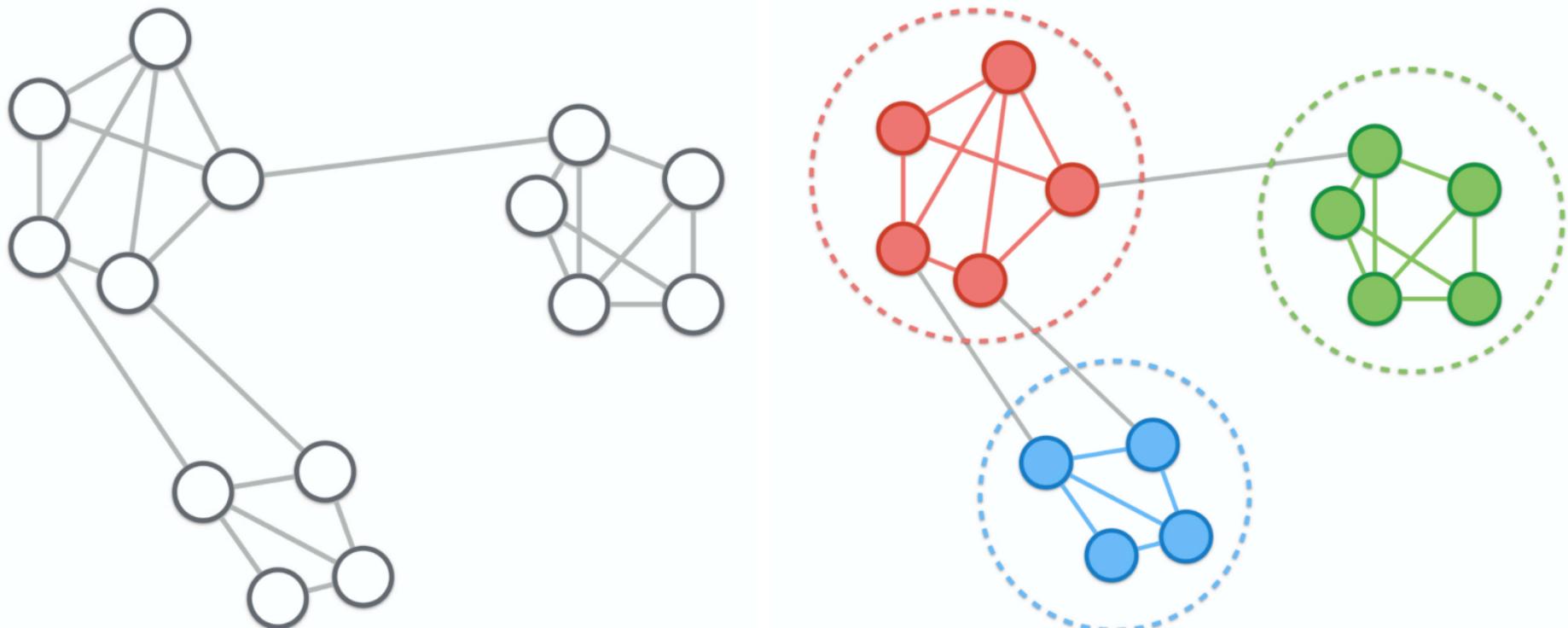


$$A = \begin{matrix} & \begin{matrix} A & B & C & D & E & F \end{matrix} \\ \begin{matrix} A \\ B \\ C \\ D \\ E \\ F \end{matrix} & \left( \begin{array}{cccccc} 0 & 1 & 1 & 0 & 0 & 0 \\ 1 & 0 & 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 & 1 & 0 \\ 0 & 1 & 0 & 0 & 1 & 1 \\ 0 & 0 & 1 & 1 & 0 & 1 \\ 0 & 0 & 0 & 1 & 1 & 0 \end{array} \right) \end{matrix}$$

$$W = \begin{matrix} & \begin{matrix} A & B & C & D & E & F \end{matrix} \\ \begin{matrix} A \\ B \\ C \\ D \\ E \\ F \end{matrix} & \left( \begin{array}{cccccc} 0 & 0.8 & 0.8 & 0 & 0 & 0 \\ 0.8 & 0 & 0.6 & 0.1 & 0 & 0 \\ 0.8 & 0.6 & 0 & 0 & 0.2 & 0 \\ 0 & 0.1 & 0 & 0 & 0.8 & 0.8 \\ 0 & 0 & 0.2 & 0.8 & 0 & 0.6 \\ 0 & 0 & 0 & 0.8 & 0.6 & 0 \end{array} \right) \end{matrix}$$

# Community detection

Communities, or clusters, are usually groups of vertices having higher probability of being connected to each other than to members of other groups.

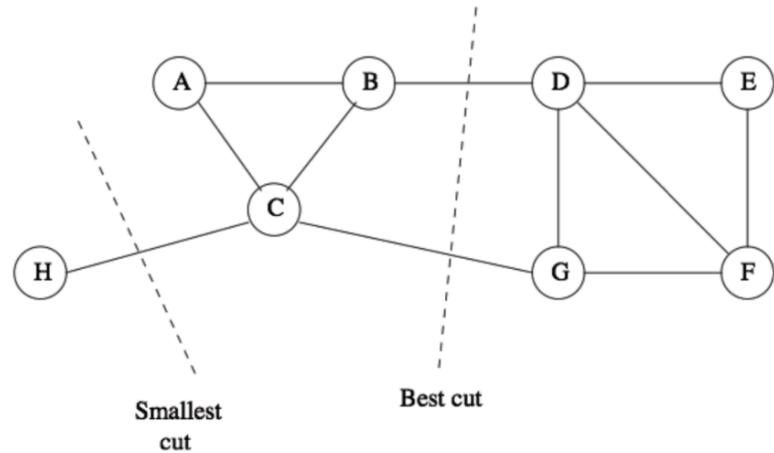


# Community detection

- Main objective is to find a group (community) of vertices with more edges **inside** the group than edges linking vertices of the group with the rest of the graph.

# Graph cuts

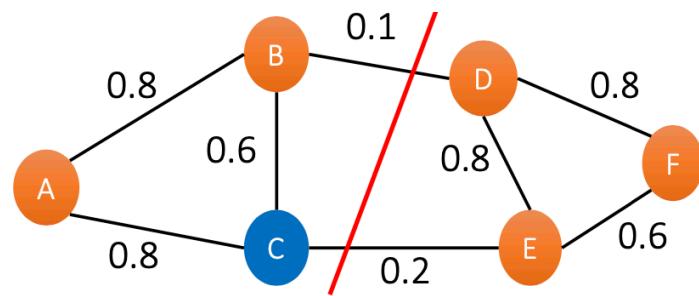
- Graph cut partitions a graph into subgraphs
- Cut cost is the sum of weights of the edges.
- Clustering by graph cuts: find the smallest cut that bi-partitions the graph
- The smallest cut is not always the best cut – may give many small disjoint clusters



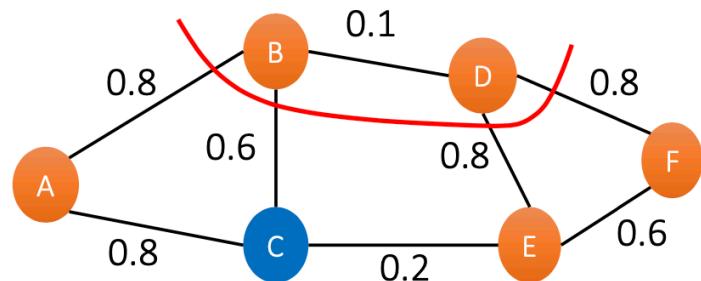
# Normalized cut

- Normalized cut computes the cut cost as a fraction of the total edge connections to all the nodes in the graph.

- $\text{cut}(S,T) = 0.1 + 0.2 = 0.3$
- $\text{vol}(S) = 0.3 + 0.6 + 0.8 + 0.8 = 2.5$
- $\text{vol}(T) = 0.3 + 0.8 + 0.8 + 0.6 = 2.5$
- $\text{Ncut}(S,T) = 0.3/2.5 + 0.3/2.5 = 0.24$



- $\text{cut}(S,T) = 0.8 + 0.6 + 0.8 + 0.8 = 3.0$
- $\text{vol}(S) = 3.0 + 0.1 = 3.1$
- $\text{vol}(T) = 3.0 + 0.8 + 0.2 + 0.6 = 4.6$
- $\text{Ncut}(S,T) = 3.0/3.1 + 3.0/4.6 = 1.62$



# Normalized cut

- Searching for the best normalized cut is NP-hard
- We need a heuristic method to solve the problem:
  - Spectral clustering
  - Markov clustering
  - Louvain
  - Leiden
  - ...

## For single cell data

- Can start with distances based on correlation, euklidean distances in PCA space etc. Same as for hclust/k-means.
- Build a KNN graph with cells as vertices.
  - Find  $k$  nearest neighbors to each cell.
  - The size of  $k$  will strongly influence the network structure.
- Can create weighted network based on shared neighbors (SNN).
- Find clusters with community detection method.
- Graphs can also be used for trajectory analysis etc.

# How to work with networks

- Igraph package – implemented for both R, python and Ruby
- Has most commonly used layout optimization methods and community detection methods implemented.
- Simple R example at:

<https://jef.works/blog/2017/09/13/graph-based-community-detection-for-clustering-analysis/>

- Tutorial to igraph at:  
<http://kateto.net/networks-r-igraph>
- Example how to build your own graph with scRNASeq data:  
<https://github.com/NBISweden/workshop-scRNASeq/blob/master/oldlabs/igraph.md>

# Distance between cells

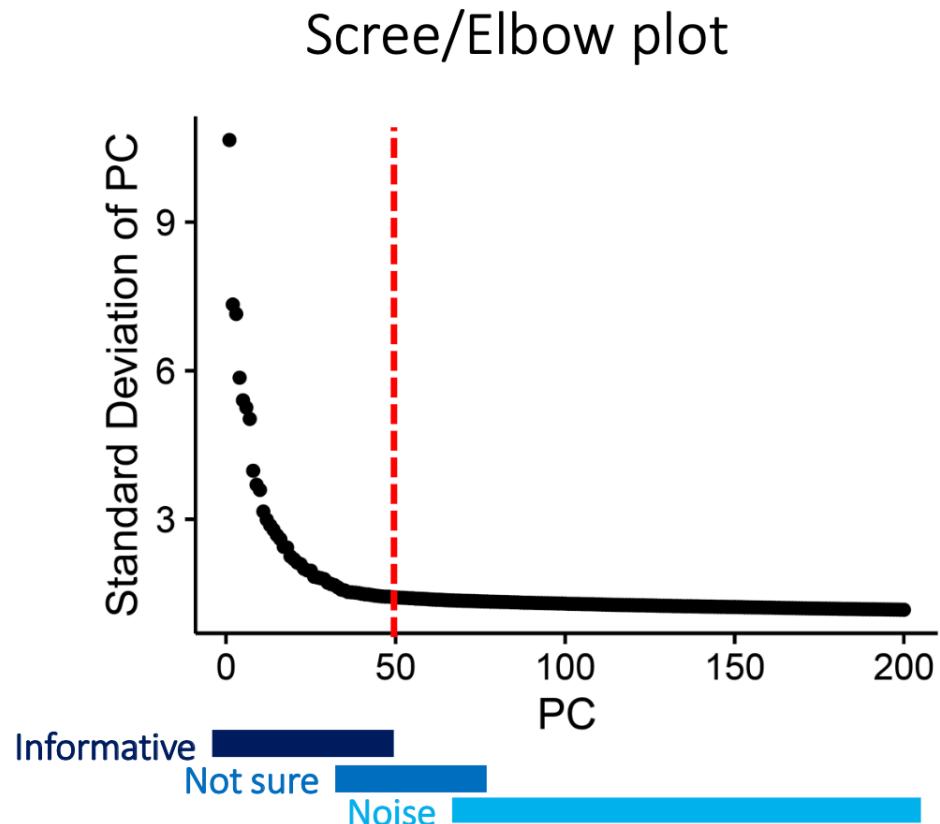
- All clustering methods need to define distances between cells. Things to consider are:
  - What gene set should be included?
    - Commonly used: Highly variable genes
  - What space to calculate distance in?
    - Commonly done in PCA space
    - Can also be full space, tSNE, UMAP etc.
  - How many dimensions to include?
  - What distance measure?

# Different distance measures

- Most commonly used in scRNA-seq:
  - Euclidean distance
  - Inverted pairwise correlations (1-correlation)
- Other common methods are:
  - Manhattan distance
  - Mahalanobis distance
  - Maximum distance

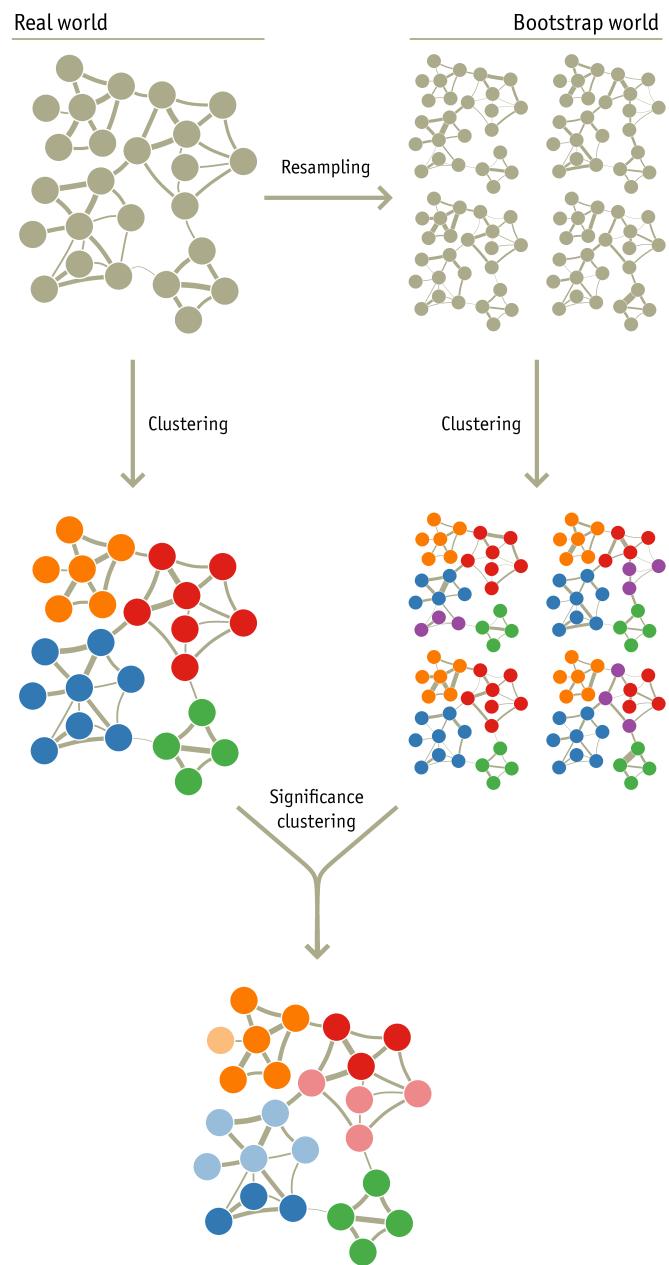
# Selection of principal components

- To overcome the extensive technical noise in scRNA-seq data, it is common to cluster cells based on their PCA scores
- Each PC represents a ‘metagene’ that (linearly) combines information across a correlated gene set
- Depending on the heterogeneity of your data more/less PCs should be selected.



# Bootstrapping

- How confident can you be that the clusters you see are real?
- You can always take a random set of cells from the same cell type and manage to split them into clusters.
- Most scRNAseq packages do not include any bootstrapping.  
Scran has function **bootstrapCluster**.



# Seurat clustering

- FindNeighbors:
  - First construct a KNN (k-nearest neighbor) graph – default is based on the euclidean distance in PCA space
  - Then SNN graph the edge weights between any two cells based on the shared overlap in their local neighborhoods (Jaccard distance) and pruning of distant edges.
- Important parameters:
  - reduction: default is “pca”
  - dims: number of PCs
  - k.param: Number of neighbors in KNN graph
  - prune.snn: Cutoff for pruning

# Seurat clustering

- FindClusters: To cluster the cells, modularity optimization:
  - Louvain
  - Louvain with multilevel refinement
  - Leiden
  - SLM
- Important parameters:
  - resolution: default 0.8, larger values give more communities, smaller gives less.
  - Algorithm

# Scran clustering

- `buildKNNGraph`: Constructs the KNN graph
- `buildSNNGraph`: Constructs KNN and then SNN graph. OBS! Adds weighted edges to cells that shares neighbors.
  - Allows for different similarity measures, default is “rank” but also includes “jaccard” or “number”
- Important parameters:
  - `use.dimred`: dimensionality reduction to use
  - `k`: number of neighbors
  - `type`: weighting method

# Scran clustering

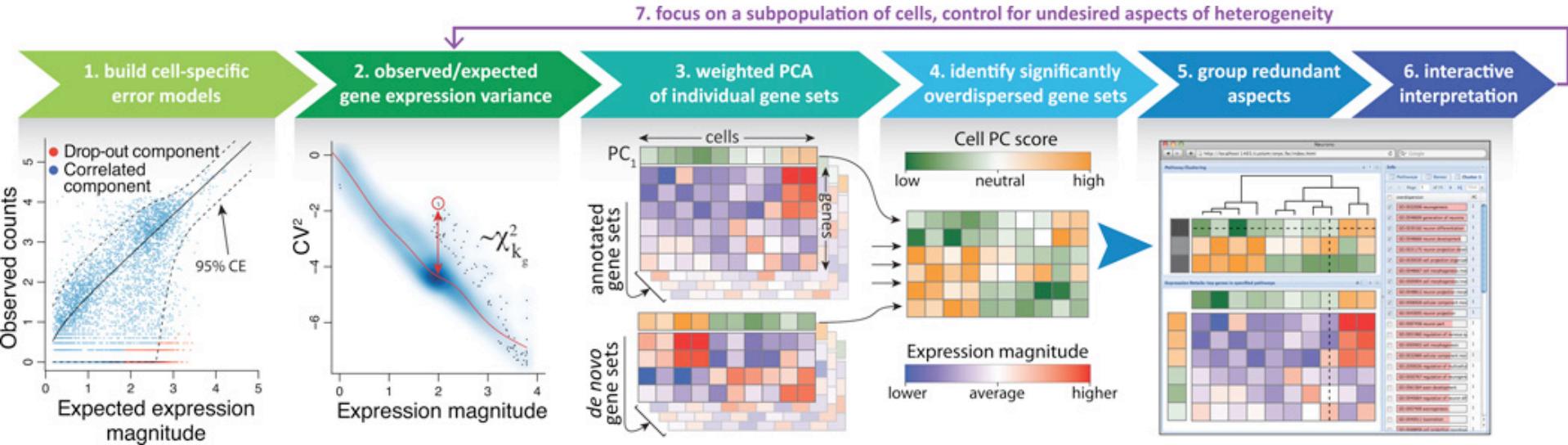
- Community detection is done with the igraph package.
  - cluster\_louvain
  - cluster\_leiden
  - cluster\_infomap
  - Many more...
- No resolution parameters, can mainly tweak cluster number with the k parameter.

# Scanpy clustering

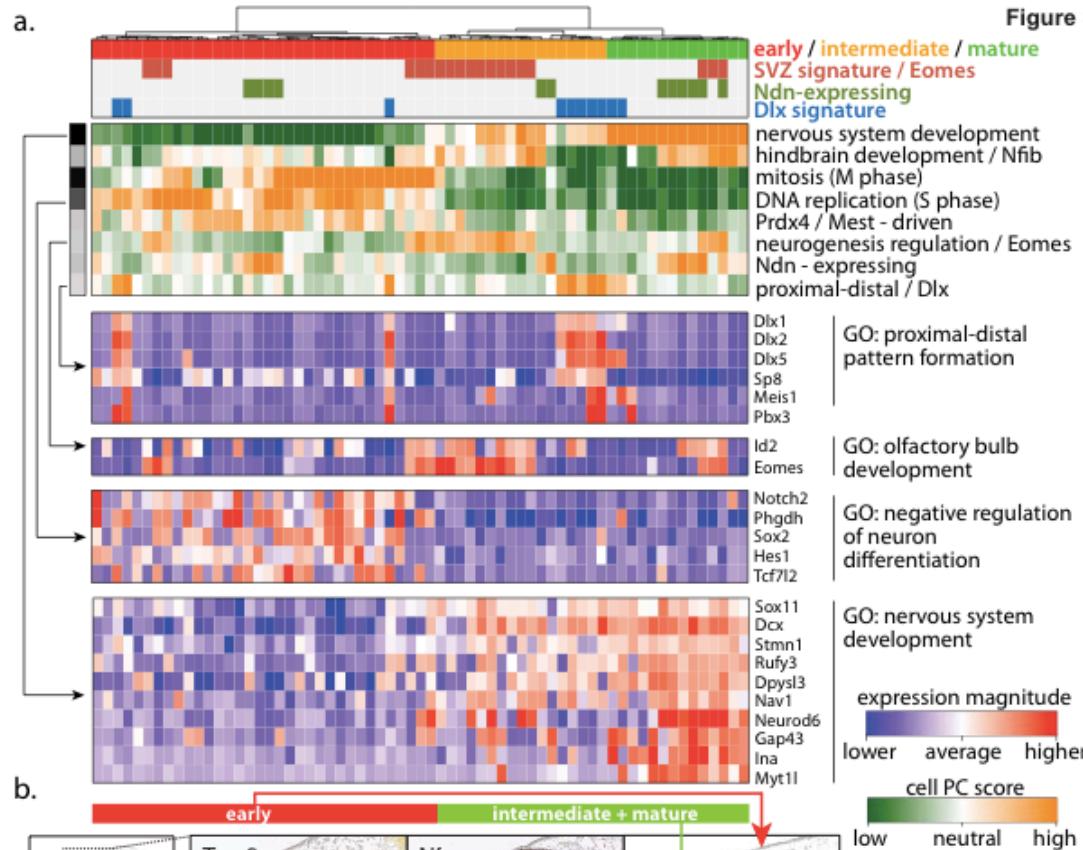
- `sc.pp.neighbors` – creates KNN graph
  - Has many different options for distance calculation, default is euclidean.
  - No SNN graph construction
- Clustering:
  - `sc.tl.leiden`
  - `sc.tl.louvain`
  - Can specify resolution like in Seurat.

# Pagoda – Pathway And Geneset OverDispersion Analysis

Implemented in the SCDE package



# Pagoda – Pathway And Geneset OverDispersion Analysis



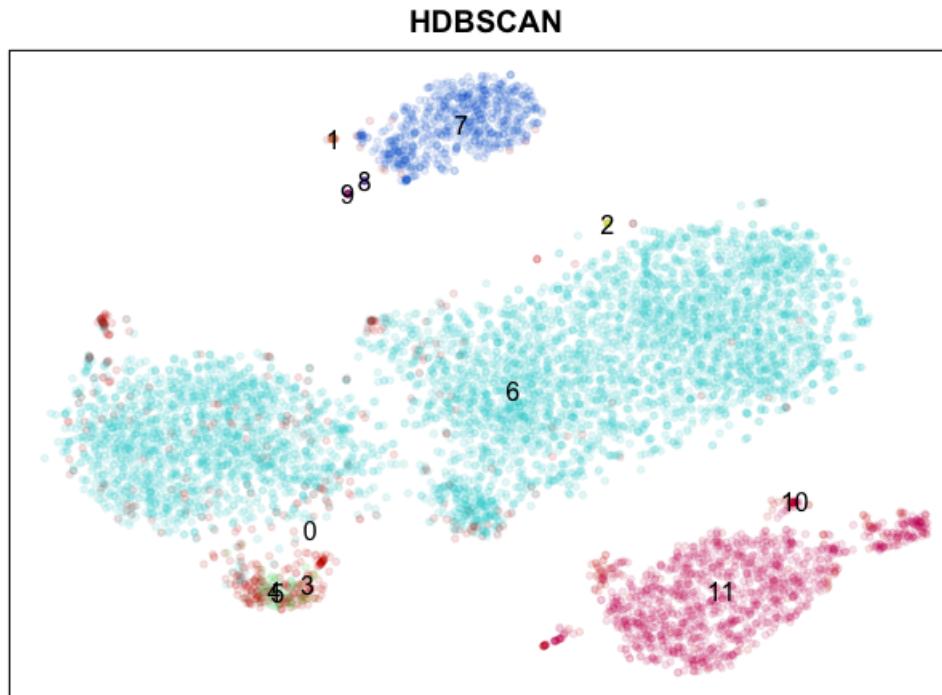
- Helps with biological interpretation of data
- Important to have good and relevant gene sets
- High memory consumption when running Pagoda
- Also has methods for removing batch effect, detected genes, cell cycle etc

# Pagoda2

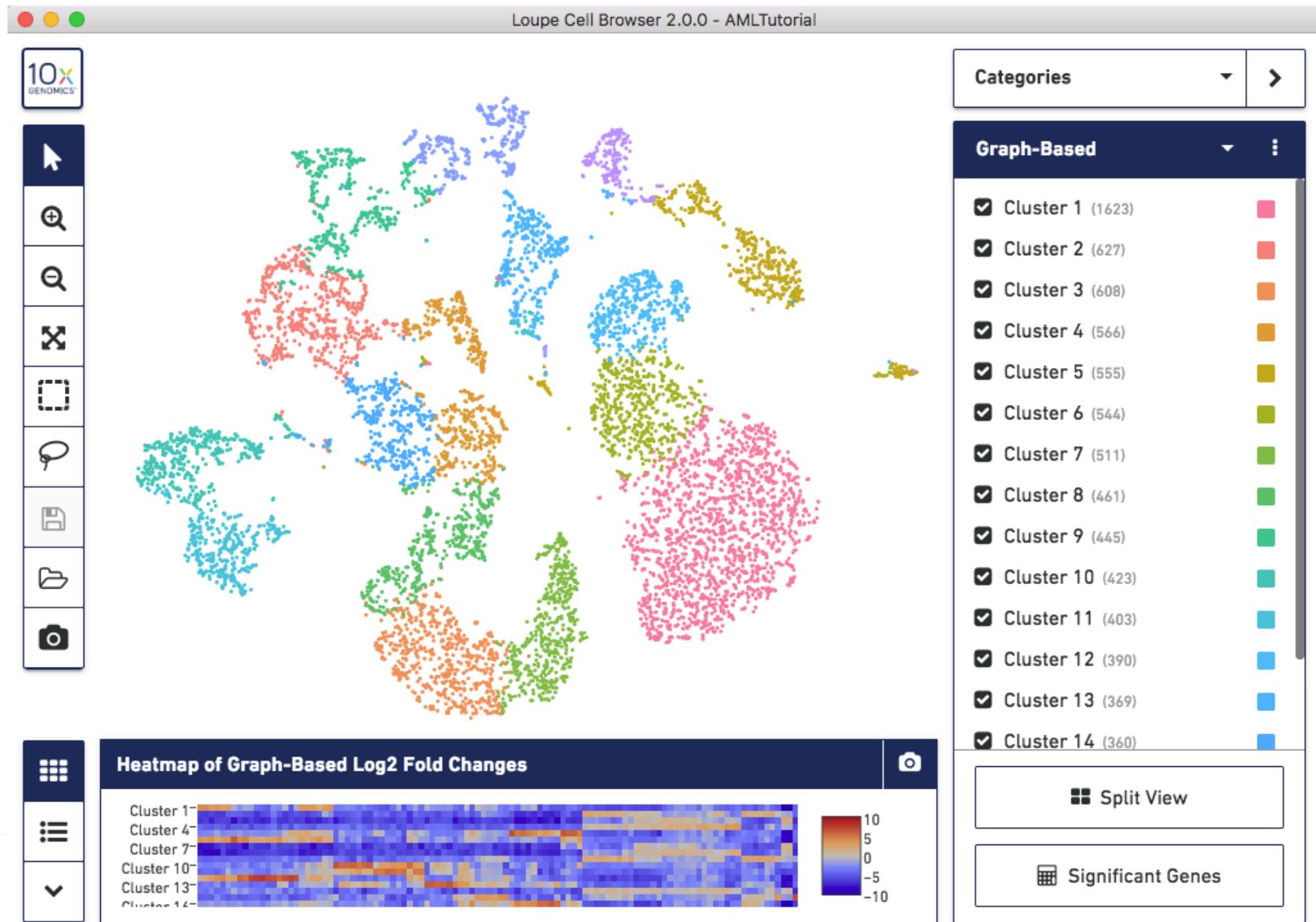
- Similar error modelling
- Now include KNN graph clustering
- largeViz for dimensionality reduction
- Can visualize gene sets.
- <https://github.com/hms-dbmi/pagoda2>

# HDBSCAN

- Hierarchical DBSCAN – density based clustering on tSNE / Umap



# Loupe – Cell Browser, from 10X Genomics



# Which clustering method is best?

- Depends on the input data
- Consistency between several methods gives confidence that the clustering is robust
- The clustering method that is most consistent – best bootstrap values is not always best
- In a simple case where you have clearly distinct celltypes, simple hierarchical clustering based on euclidean or correlation distances will work fine.

# Comparison of clustering methods

F1000Research

F1000Research 2018, 7:1141 Last updated: 11 SEP 2018



Check for updates

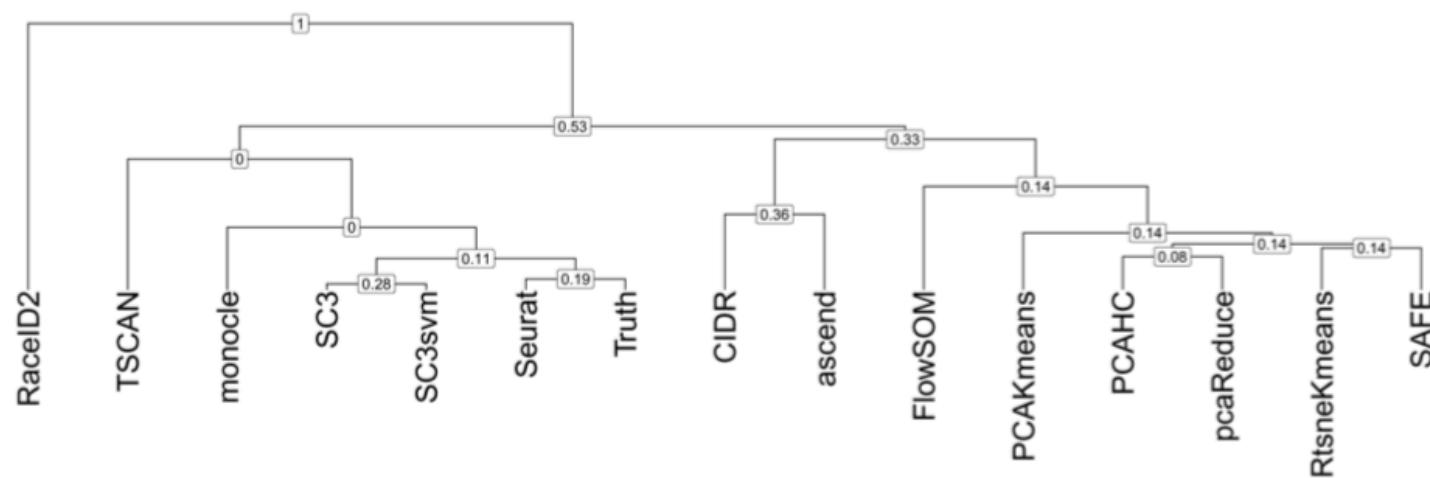
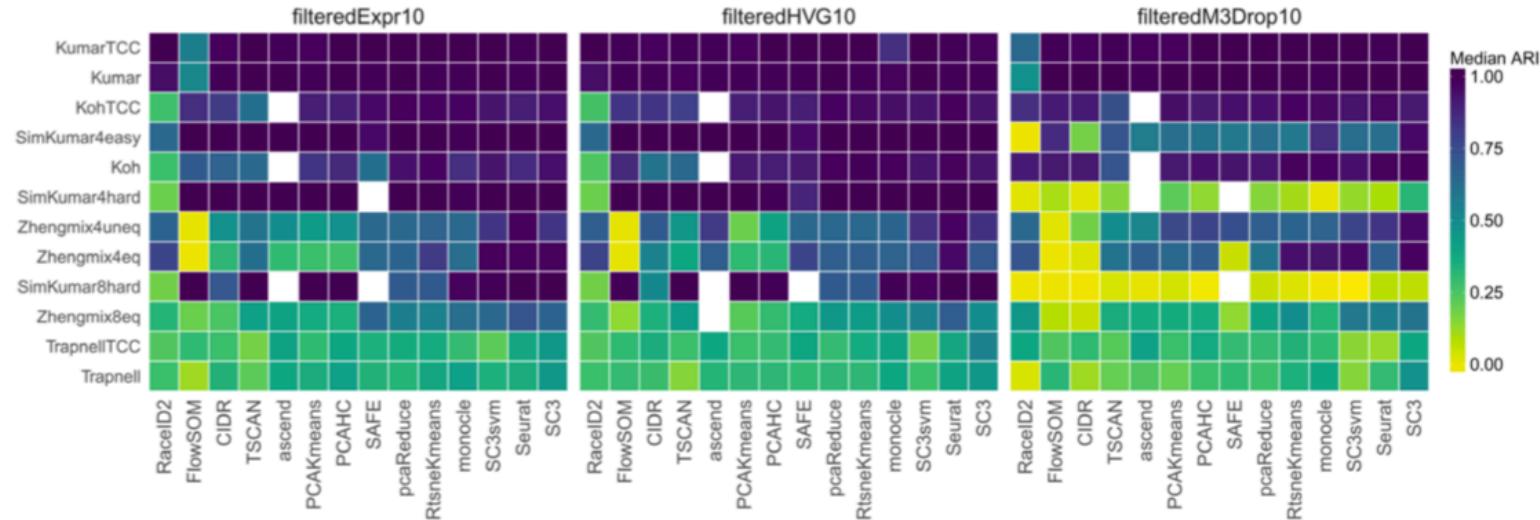
RESEARCH ARTICLE

**REVISED A systematic performance evaluation of clustering methods for single-cell RNA-seq data [version 2; referees: 2 approved]**

Angelo Duò<sup>1,2</sup>, Mark D. Robinson 1,2, Charlotte Soneson 1,2

<sup>1</sup>Institute of Molecular Life Sciences, University of Zurich, Zurich, 8057, Switzerland

<sup>2</sup>SIB Swiss Institute of Bioinformatics, Zurich, 8057, Switzerland



Dimension reduction

- PCA
- tSNE
- Various
- None

Clustering method

- Hierarchical
- Kmeans
- ModelBased
- Kmedoids
- Graph
- SOM
- Density
- Various



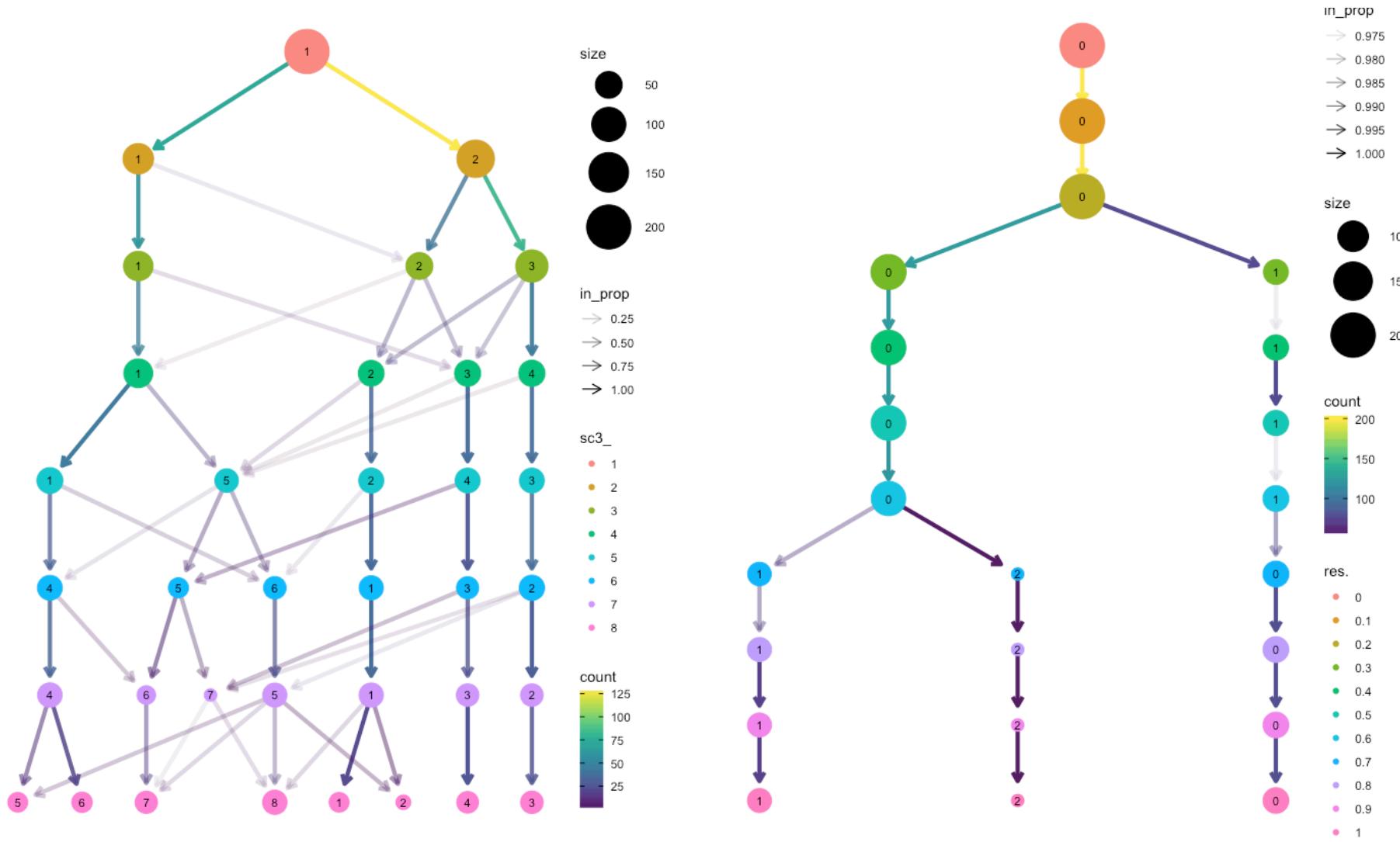
Input

- Raw
- LogNorm
- Various

# How many clusters do you really have?

- It is hard to know when to stop clustering – you can always split the cells more times.
- Can use:
  - Do you get any/many significant DE genes from the next split?
  - Some tools have automated predictions for number of clusters – may not always be biologically relevant
- Always check back to QC-data – is what your splitting mainly related to batches, qc-measures (especially detected genes)

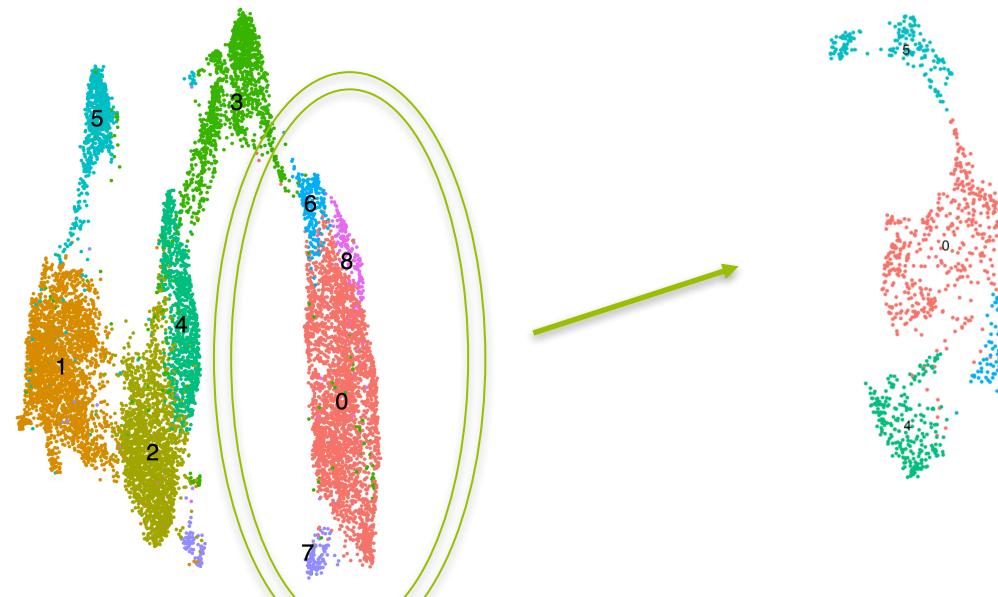
# Clustree – R package



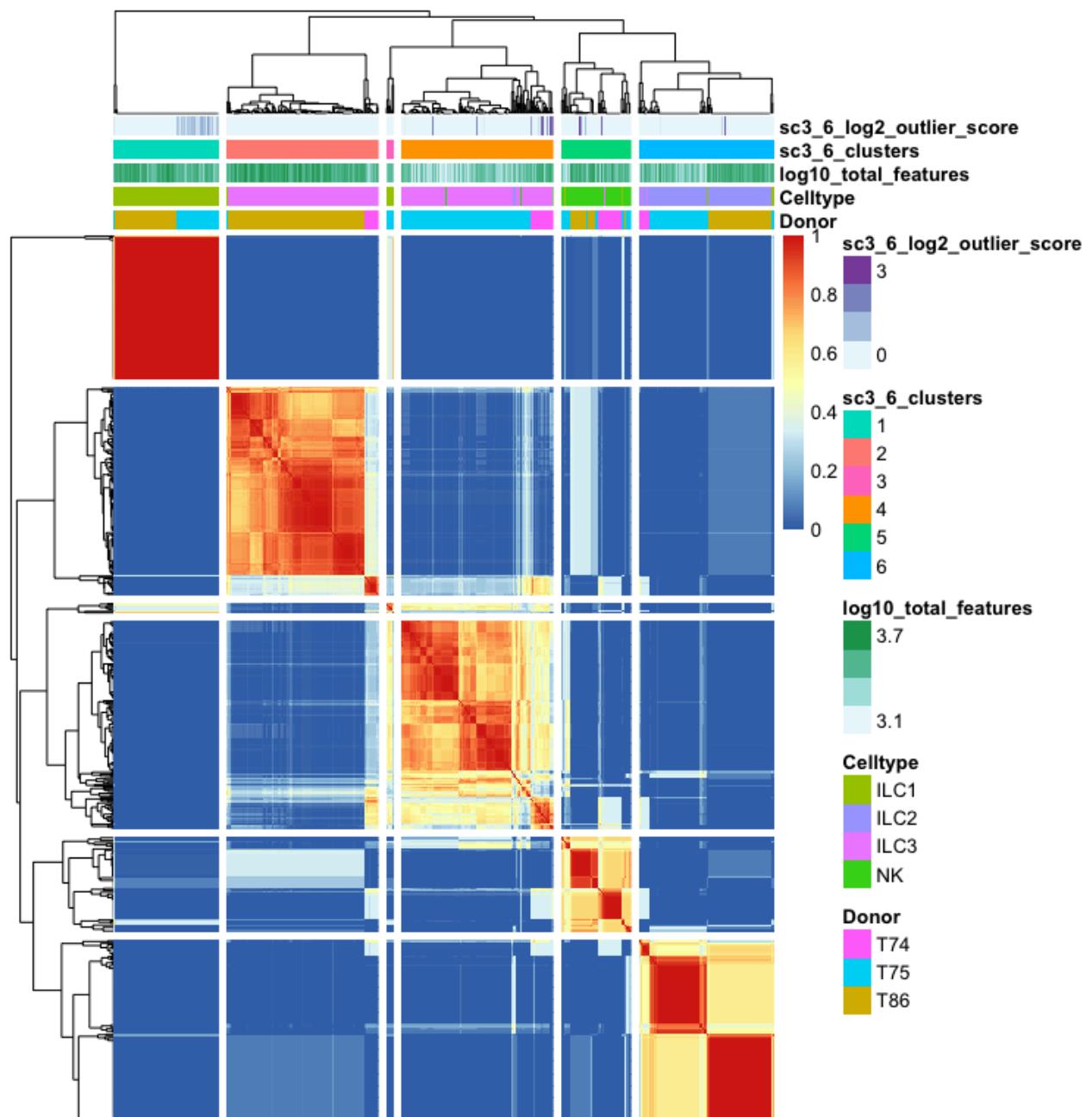
<https://cran.r-project.org/web/packages/clustree/vignettes/clustree.html>

# Subclustering

- Most of the variation in a heterogeneous data set will be between broad celltypes.
- By selecting one celltype and rerunning HVG-selection and PCA – most of the variation will be differences between subtypes.

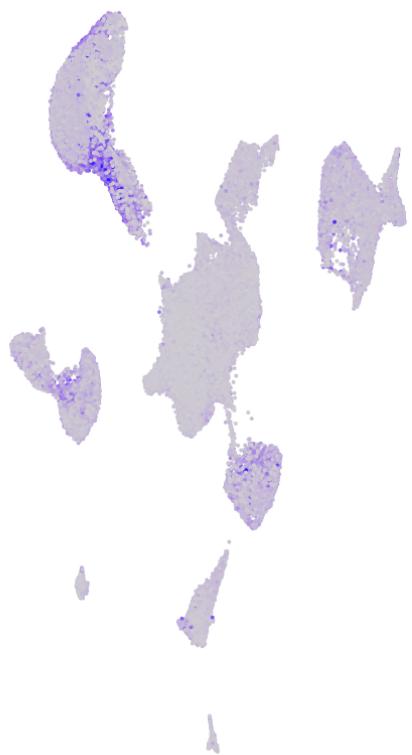


# Check QC data

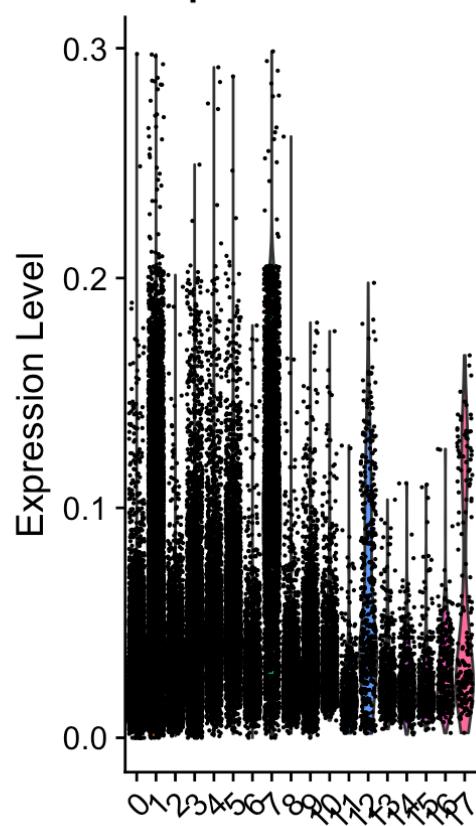


# Check QC data

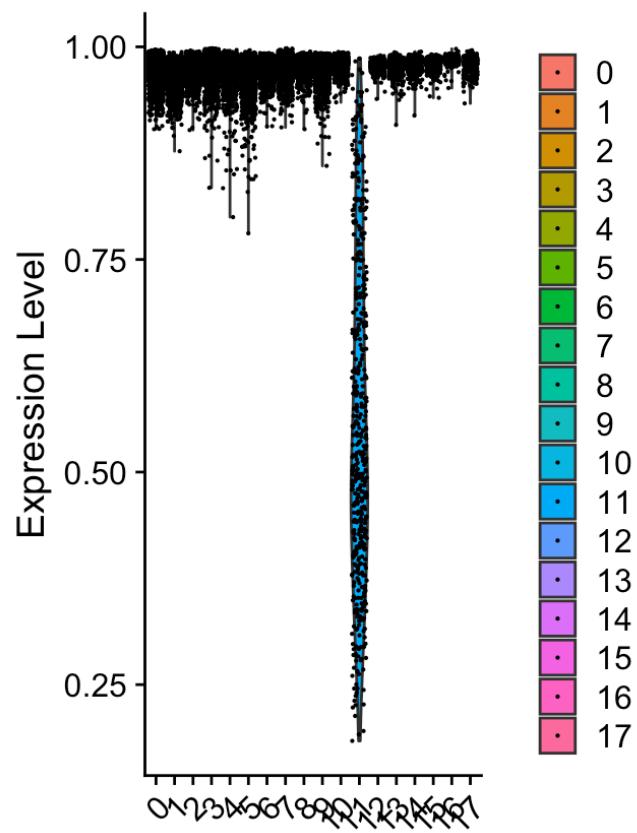
percent.mito



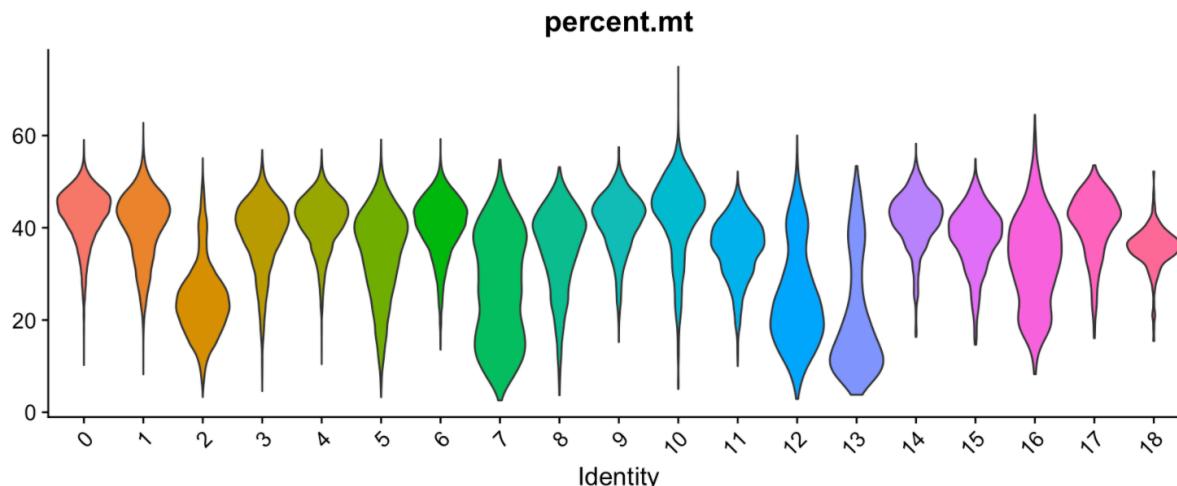
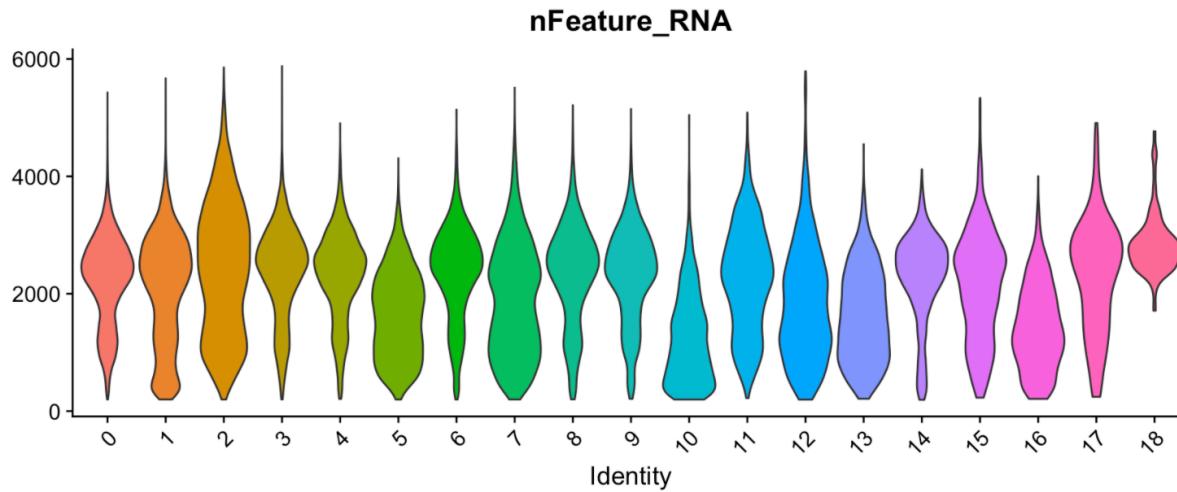
percent.mito



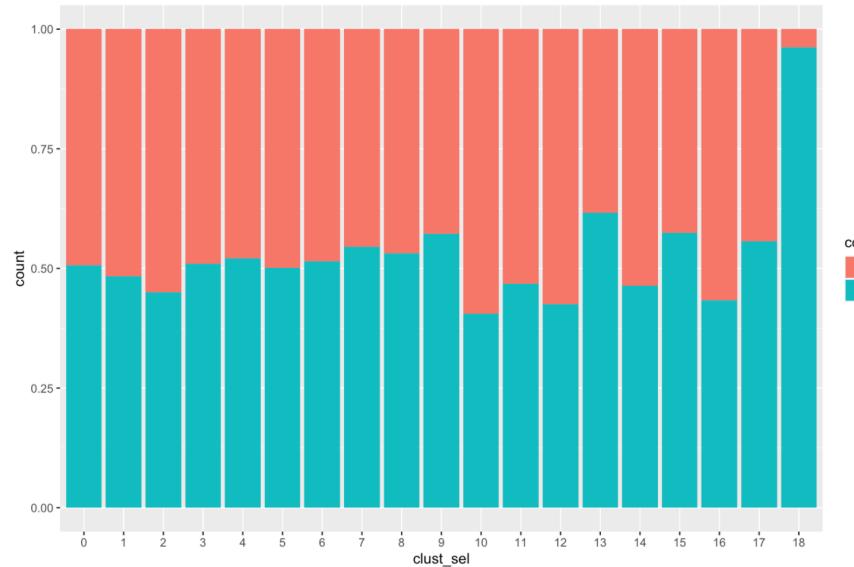
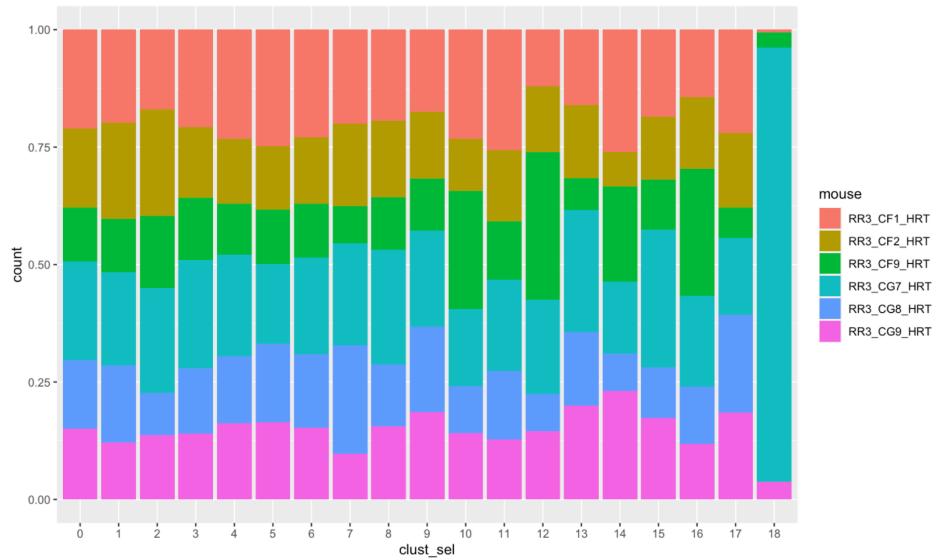
percent.pc



# Check QC data



# Check batches / conditions



# Considerations for clustering

- Hypotheses:
  - What is a cell type? What cell types are in my tissue?
  - What is the number of clusters  $k$ ?
- Choices:
  - Gene set selection
  - Similarity measure / Space to calculate similarity
  - Algorithm and hyper parameters of that algorithm.
- Different choice leads to different results. Validate, interpret and repeat steps.

# Conclusions

- Clearly distinct celltypes will give similar results regardless of method
- Subclustering within celltypes may require careful selection of variable genes, dim reduction etc.
- Consistent results from different methods and agreement with tSNE/UMAP layout is always best!
- Use your biological knowledge to evaluate the results – but try to be unbiased!