scRNAseq clustering tools

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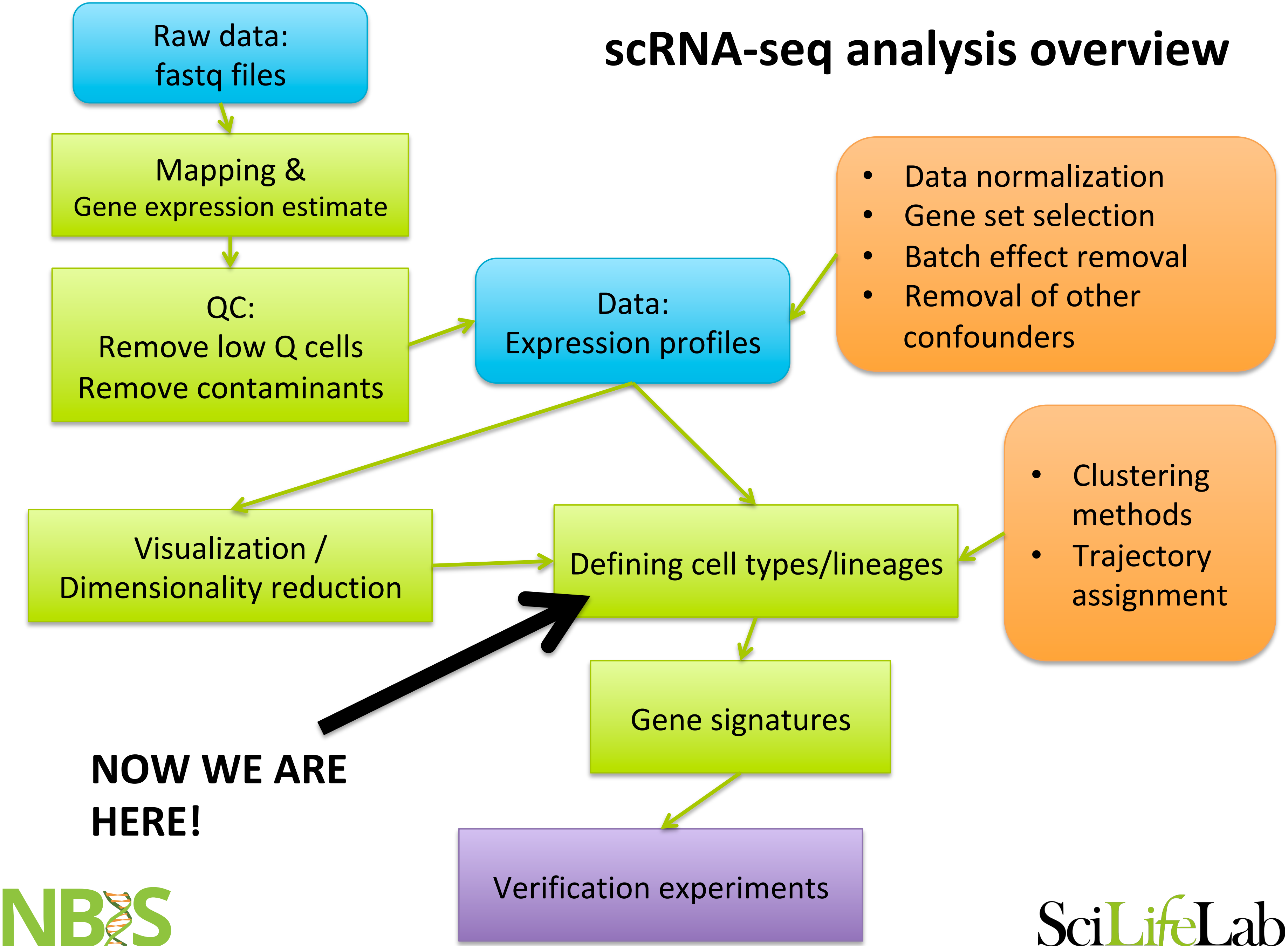
What is a celltype?

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

scRNA-seq analysis overview



NOW WE ARE HERE!

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Outline

- Basic clustering theory
- Graph theory introduction
- Examples of different tools for clustering single cell data

- Other types of analyses on scRNAseq data

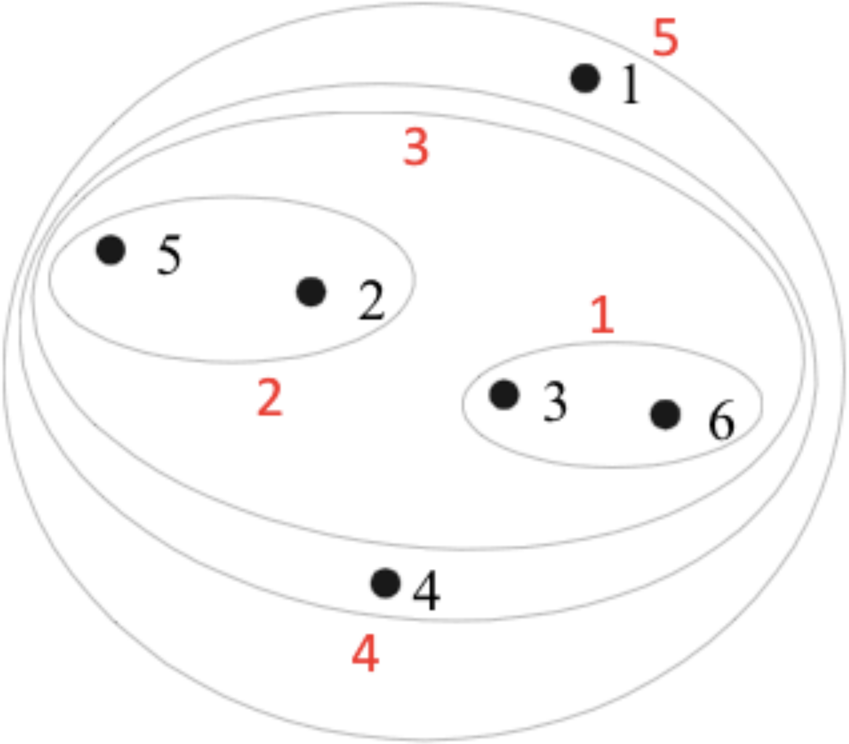
What is clustering?

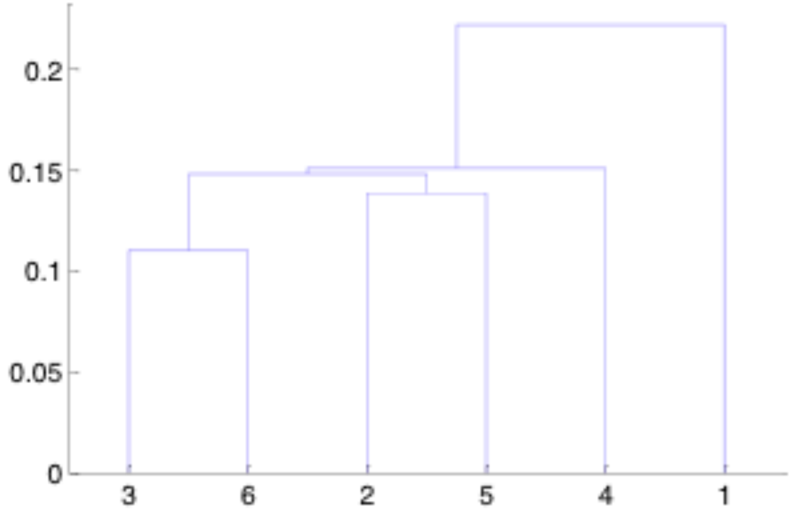
- “The process of organizing objects into groups whose members are similar in some way”
- Typical methods are:
  - Hierarchical clustering
  - K-means clustering
  - Density based clustering
  - Graph based clustering

Hierarchical clustering

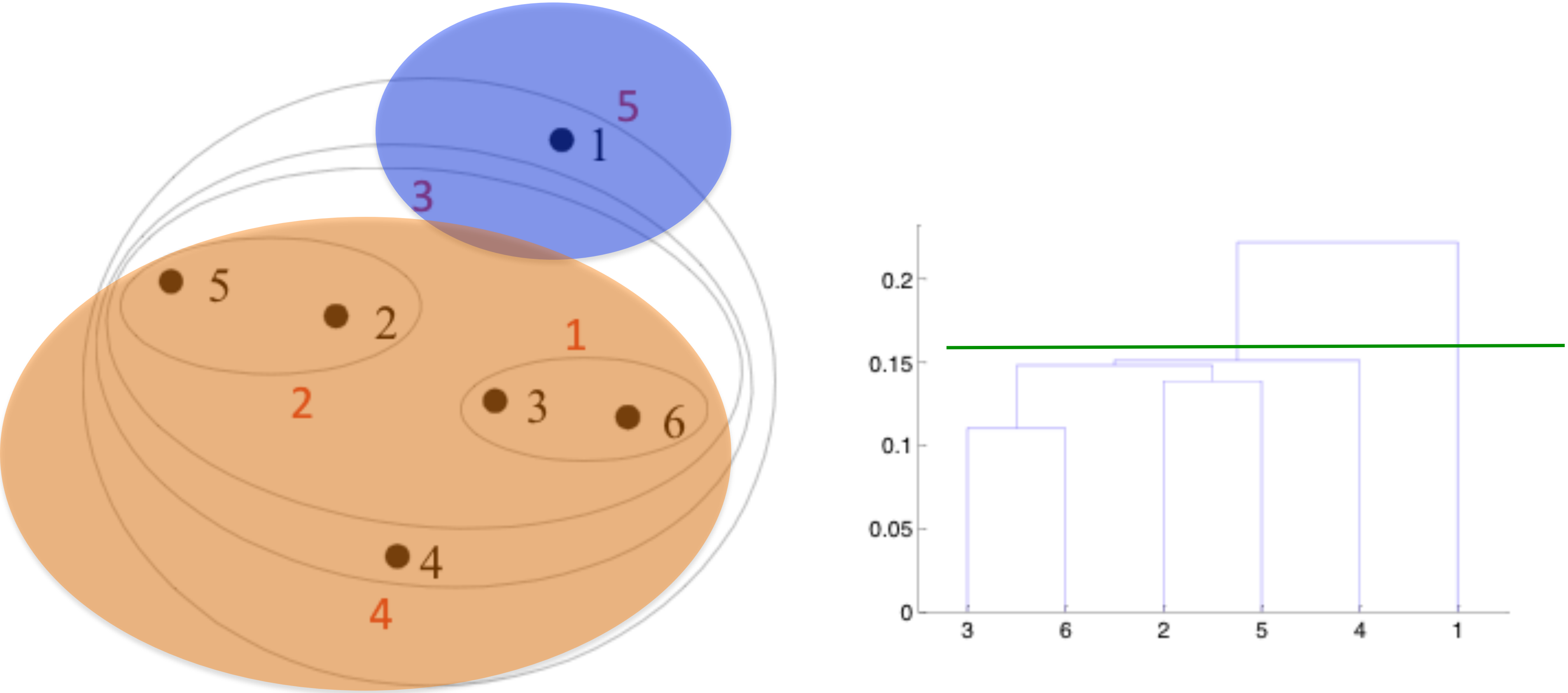
- Builds on **distances** between data points
- **Agglomerative** – starts with all data points as individual clusters and joins the most similar ones in a bottom-up approach
- **Divisive** – starts with all data points in one large cluster and splits it into 2 at each step. A top-down approach
- Final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

Hierarchical clustering



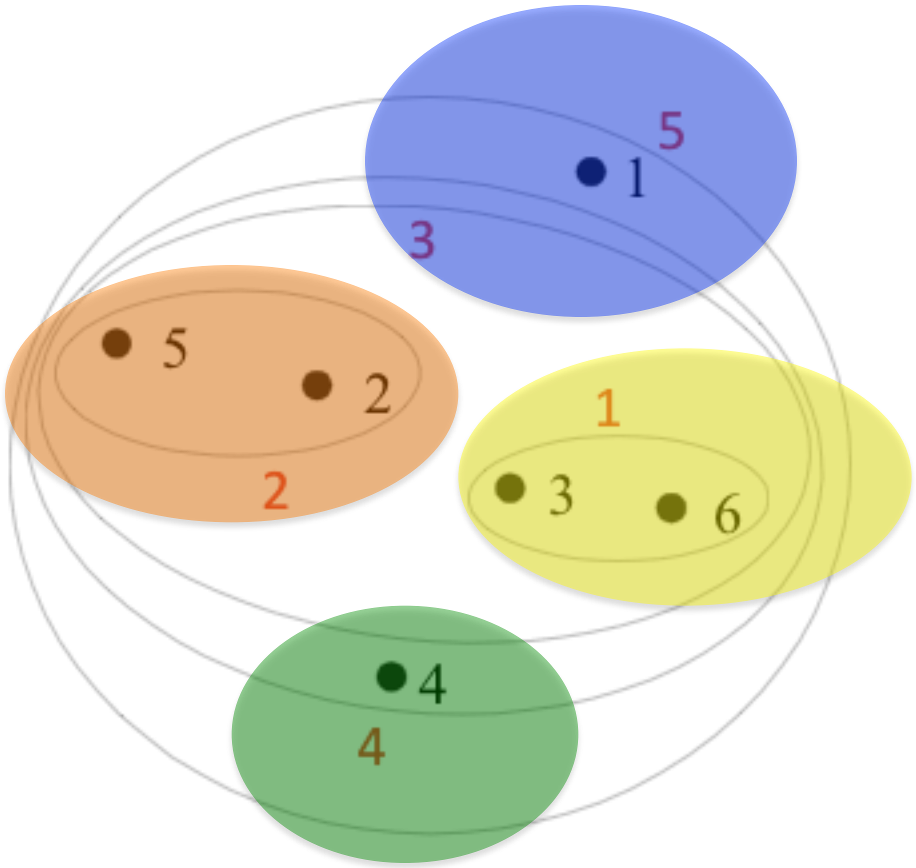


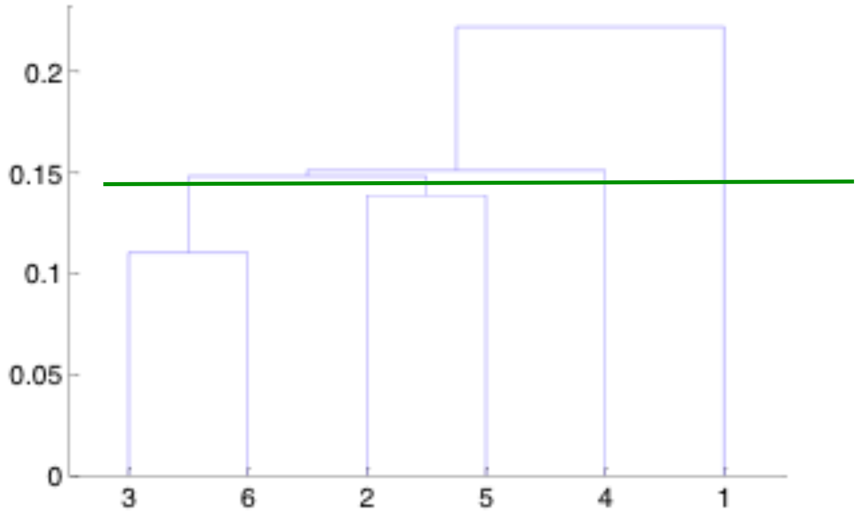
Hierarchical clustering



Clusters are obtained by cutting the tree at a desired level

Hierarchical clustering





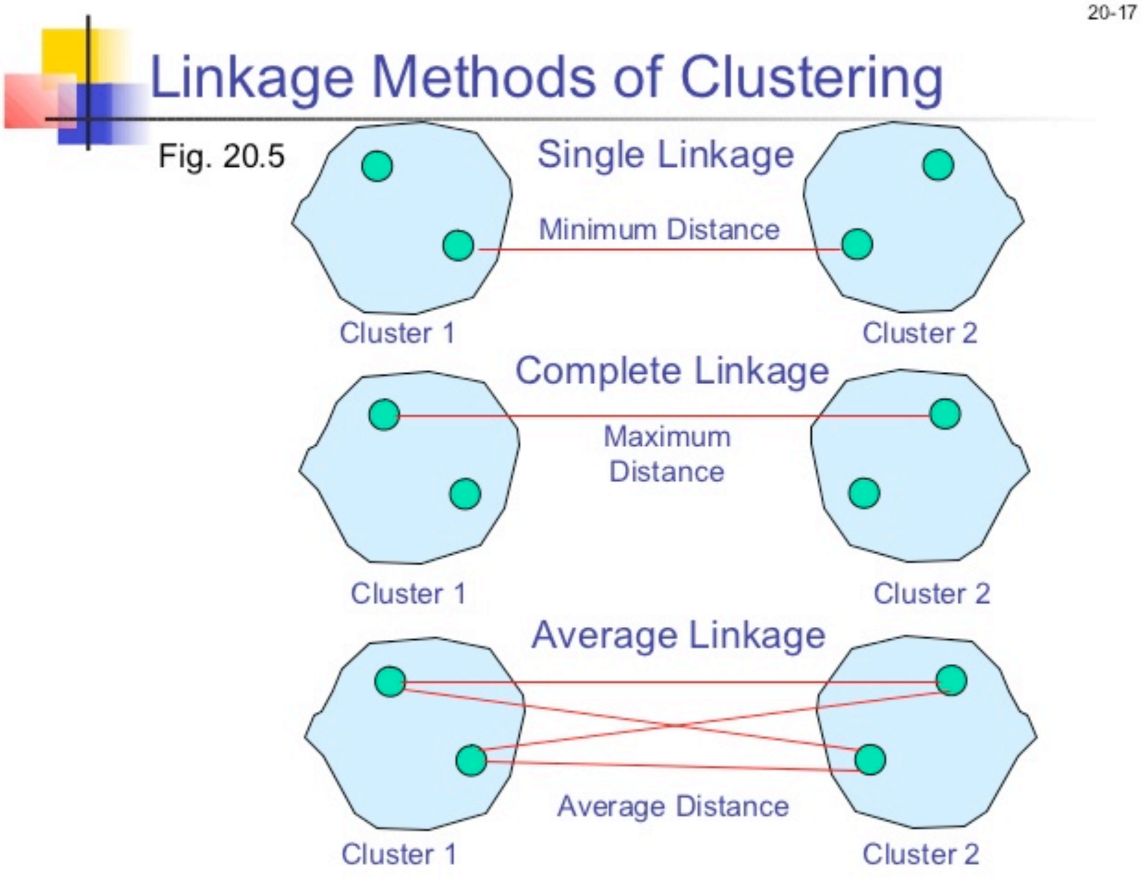
Clusters are obtained by cutting the tree at a desired level

Different distance measures

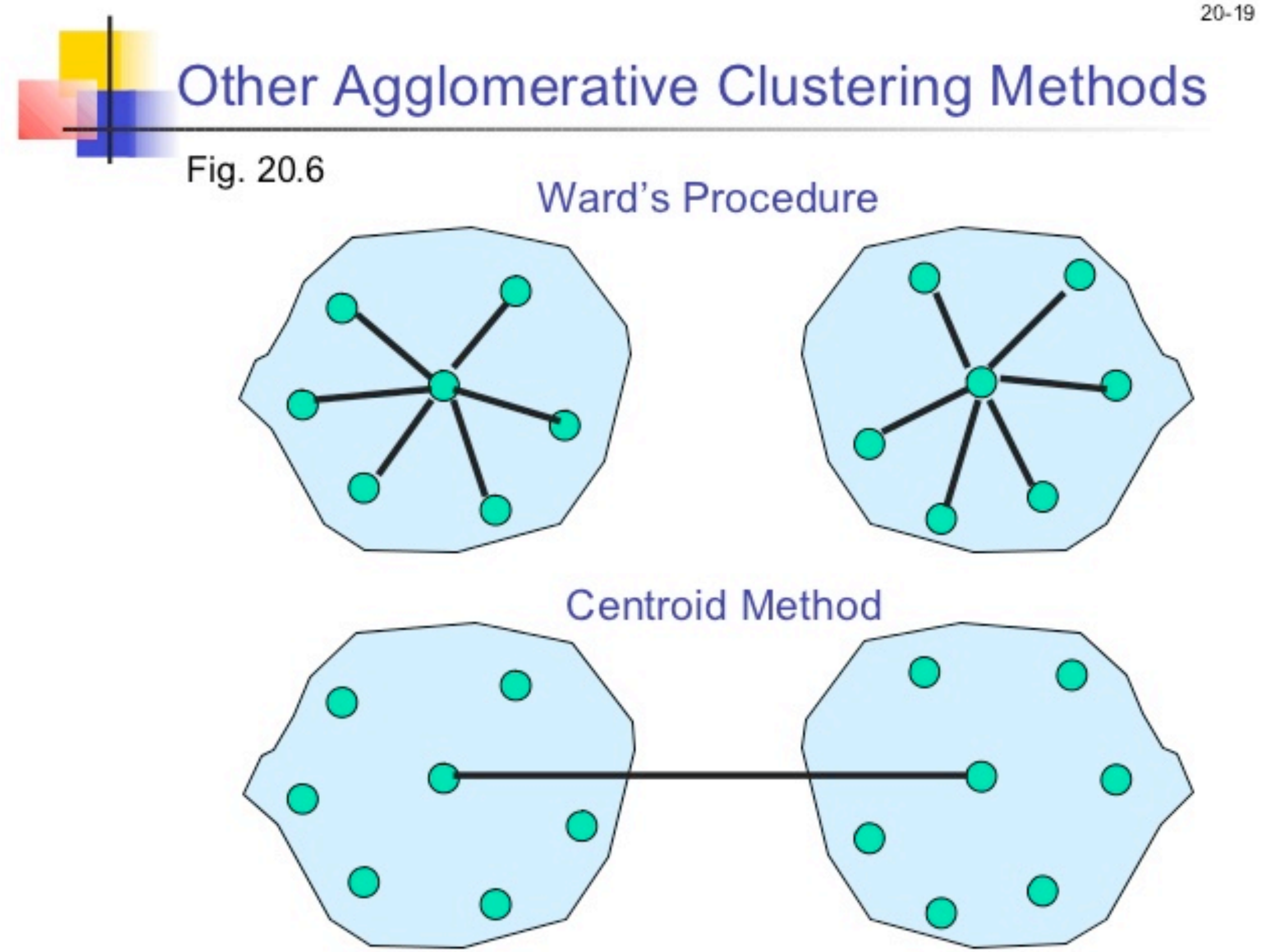
- Most commonly used in scRNA-seq:
  - Euclidean distance
    - In multidimensional space
    - In PCA/tSNE or other reduced space
  - Inverted pairwise correlations (1-correlation)
- Others include:
  - Manhattan distance
  - Mahalanobis distance
  - Maximum distance

Linkage criteria

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



http://www.slideshare.net/uzairjavedsiddiqui/malhotra20



20-19

Other Agglomerative Clustering Methods

Fig. 20.6

Ward's Procedure

Centroid Method

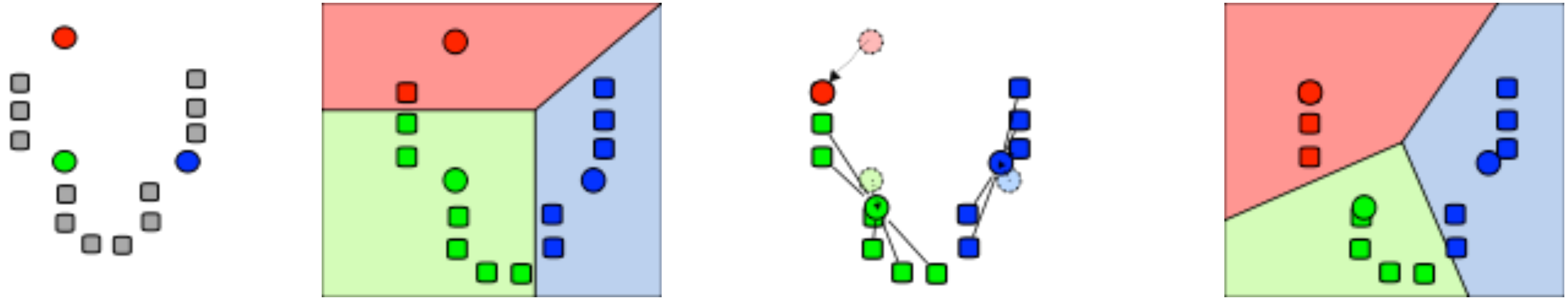
- Ward (minimum variance method). Similarity of two clusters is based on the increase in squared error when two clusters are merged.

http://www.slideshare.net/uzairjavedsiddiqui/malhotra20

K-means clustering

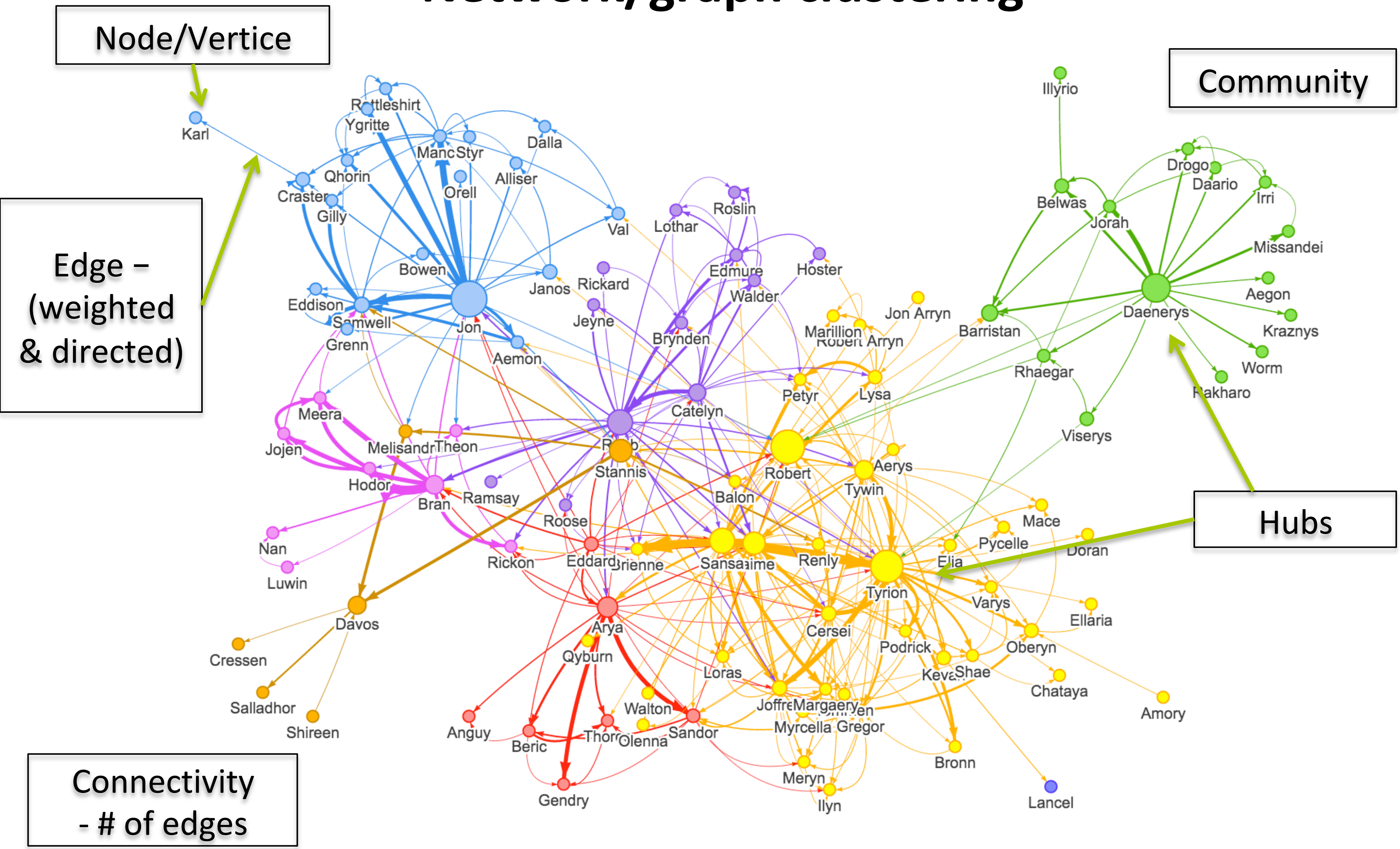
1. Starts with random selection of cluster centers (centroids)
2. Then assigns each data points to the nearest cluster
3. Recalculates the centroids for the new cluster definitions
4. Repeats steps 2-3 until no more changes occur.

Can use same distance measures as in hclust.



https://en.wikipedia.org/wiki/K-means_clustering

Network/graph clustering



Node/Vertice

Edge – (weighted & directed)

Community

Hubs

Connectivity - # of edges

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(http://www.lyonwj.com/2016/06/26/
graph-of-thrones-neo4j-social-network-analysis/)

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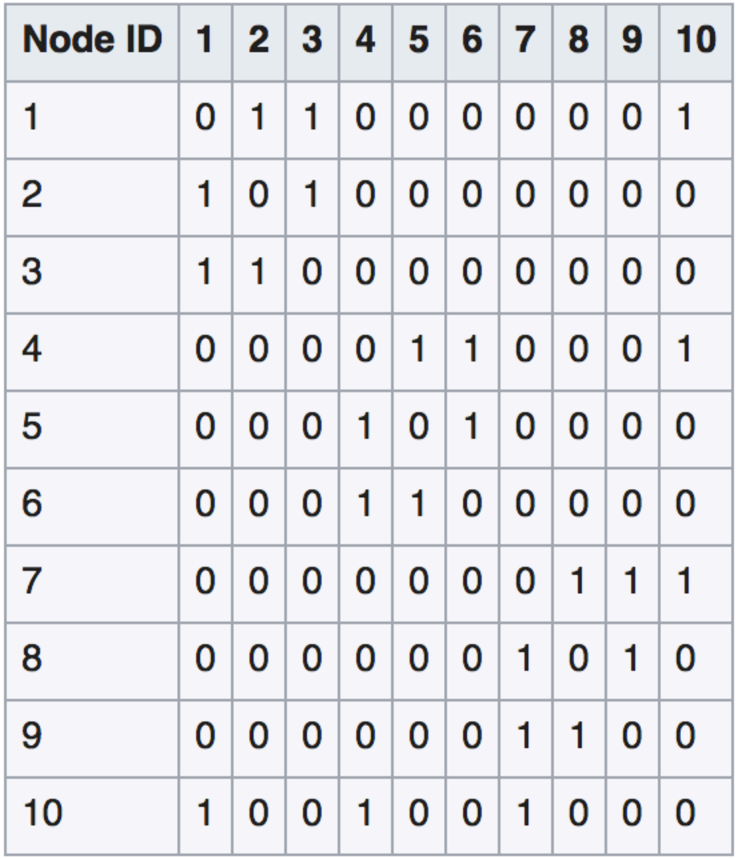
Network/graph clustering



Node ID	1	2	3	4	5	6	7	8	9	10
1	0	1	1	0	0	0	0	0	0	1
2	1	0	1	0	0	0	0	0	0	0
3	1	1	0	0	0	0	0	0	0	0
4	0	0	0	0	1	1	0	0	0	1
5	0	0	0	1	0	1	0	0	0	0
6	0	0	0	1	1	0	0	0	0	0
7	0	0	0	0	0	0	0	1	1	1
8	0	0	0	0	0	0	1	0	1	0
9	0	0	0	0	0	0	1	1	0	0
10	1	0	0	1	0	0	1	0	0	0

Adjacency matrix

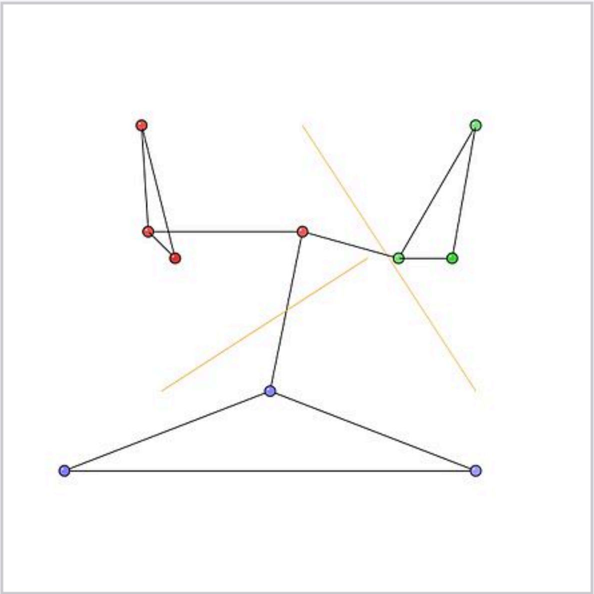


Fig 2. Network partitions that maximize Q. Maximum Q=0.4896

https://en.wikipedia.org/wiki/Modularity_(networks)
#Example_of_multiple_community_detection

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

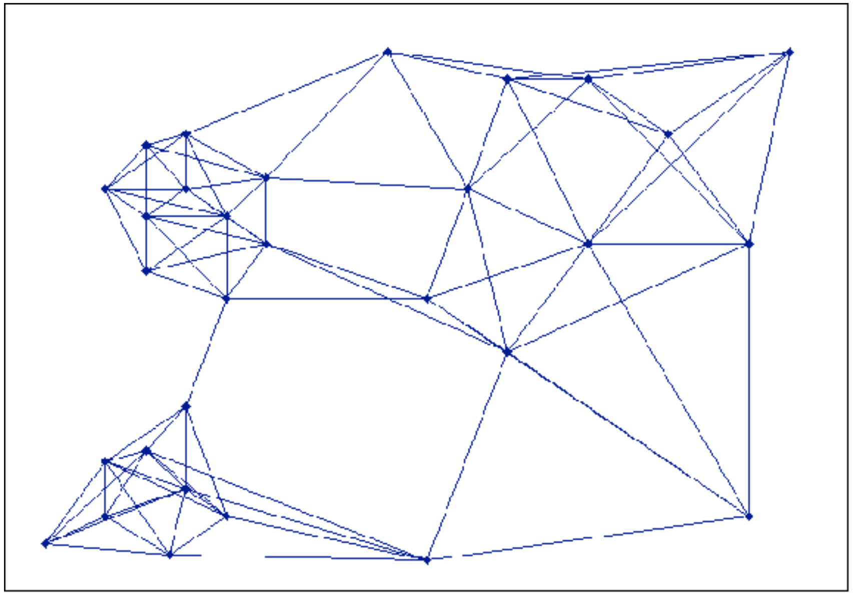


Figure 2. Near Neighbor Graph

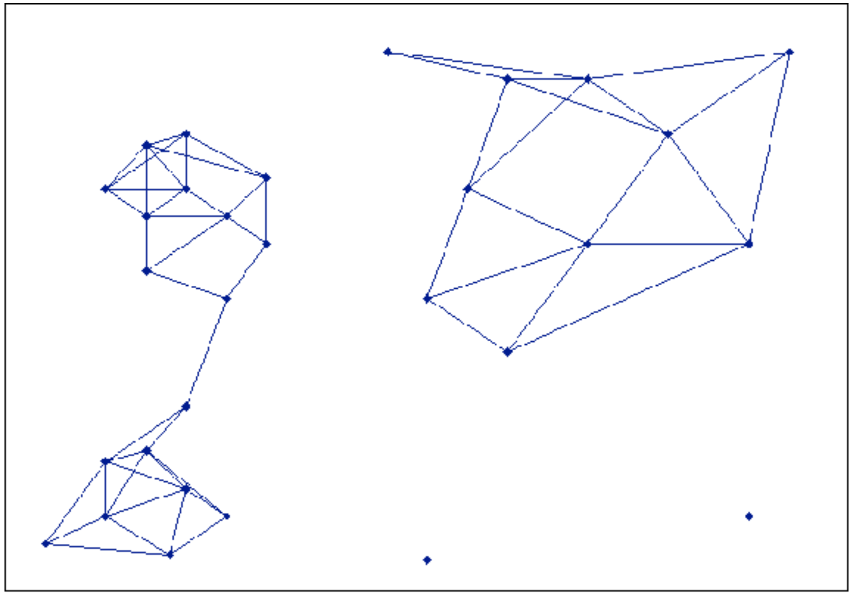
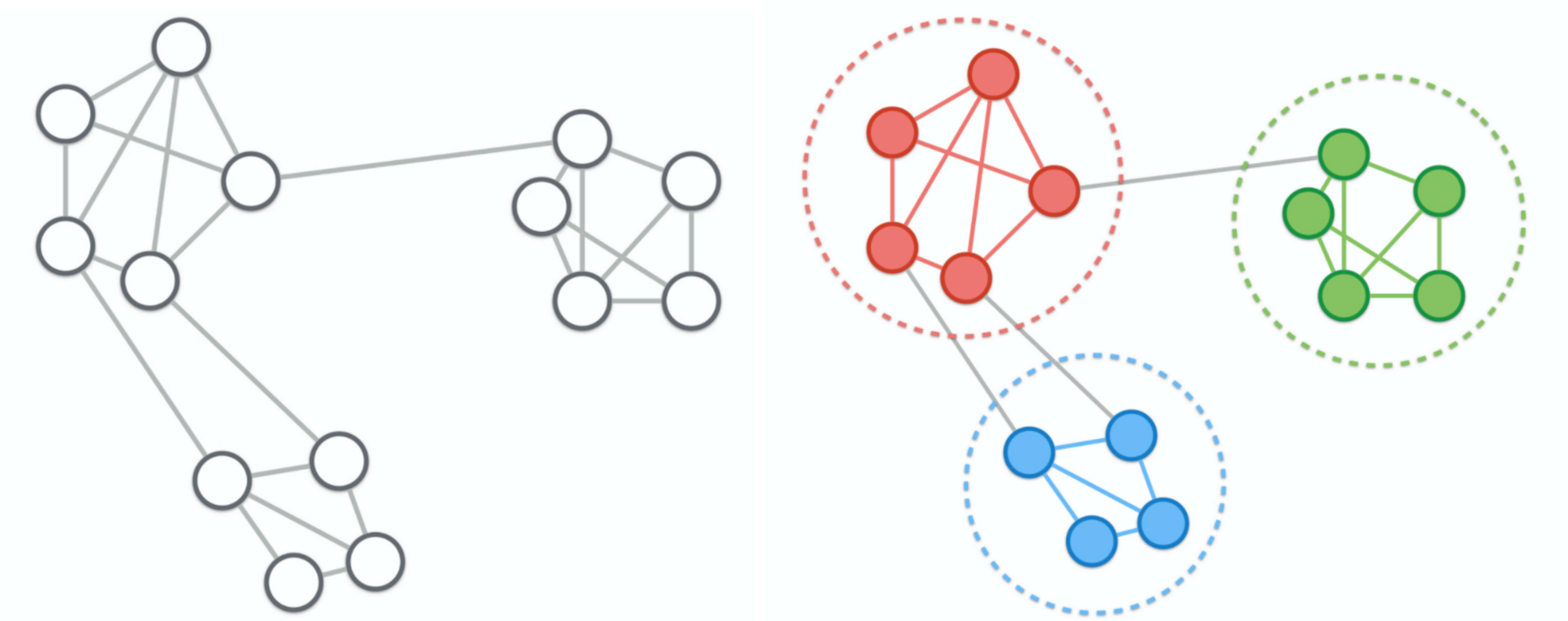


Figure 3. Unweighted Shared Near Neighbor Graph

(Ertöz et al. Semantic scholar, 2002)

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.
- Many implemented algorithms to this problem:
  - Different methods of Modularity optimization
  - Infomap
  - Walktrap
  - etc.
- Most methods will automatically define the number of clusters based on some user parameters.

For single cell data

- Can start with distances based on correlation, euklidean distances in PCA space etc. Same as for hclust/k-means.
- Buld 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 reduce network based on shared neighbors.
- Find clusters with community detection method.
- Graphs can also be used for trajectory analysis

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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:

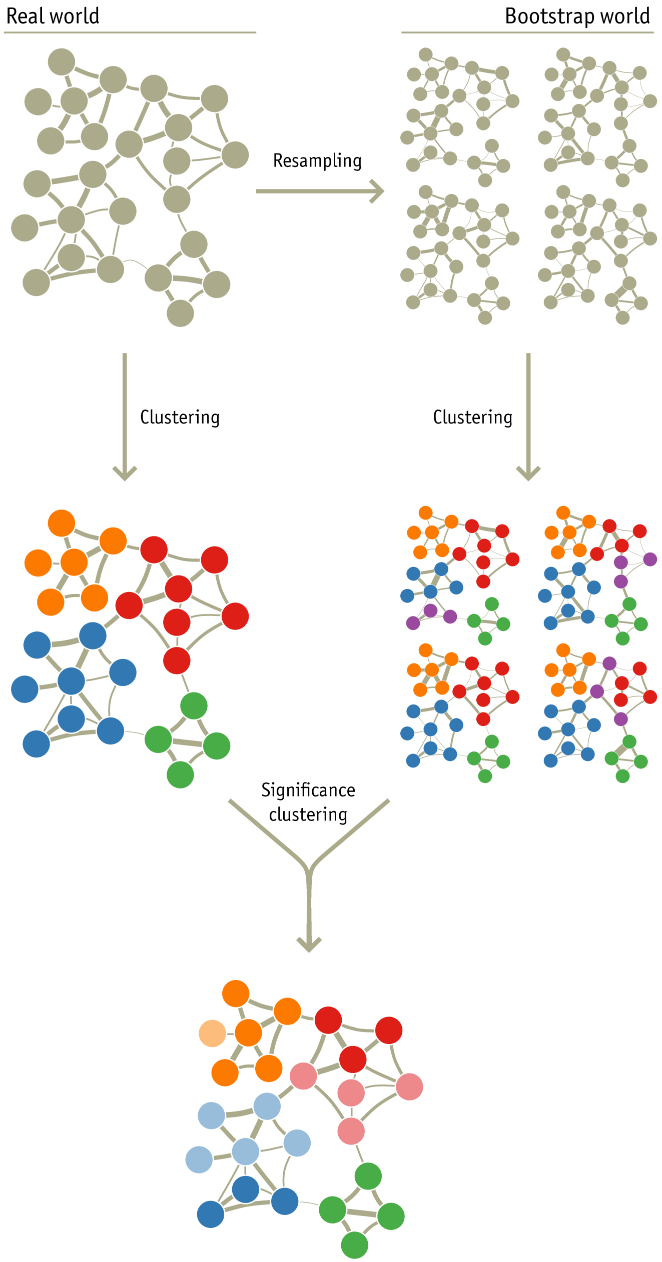
https://jef.works/blog/2017/09/13/graph-based-community-detection-for-clustering-analysis/

- Tutorial to igraph at:

http://kateto.net/networks-r-igraph

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



(Rosvall et al. *Plos One* 2010)

scRNAseq clustering

- Easy case with distinct celltypes:
  - rpkms/counts – Euklidean or correlation distances
  - PCA, tSNE or other dimensionality reduction method
- Examples of programs for clustering (many more out there):
  - BackSPIN
  - Pagoda
  - SC3
  - Seurat
  - pcaReduce
  - SNNcliq

Main pipelines

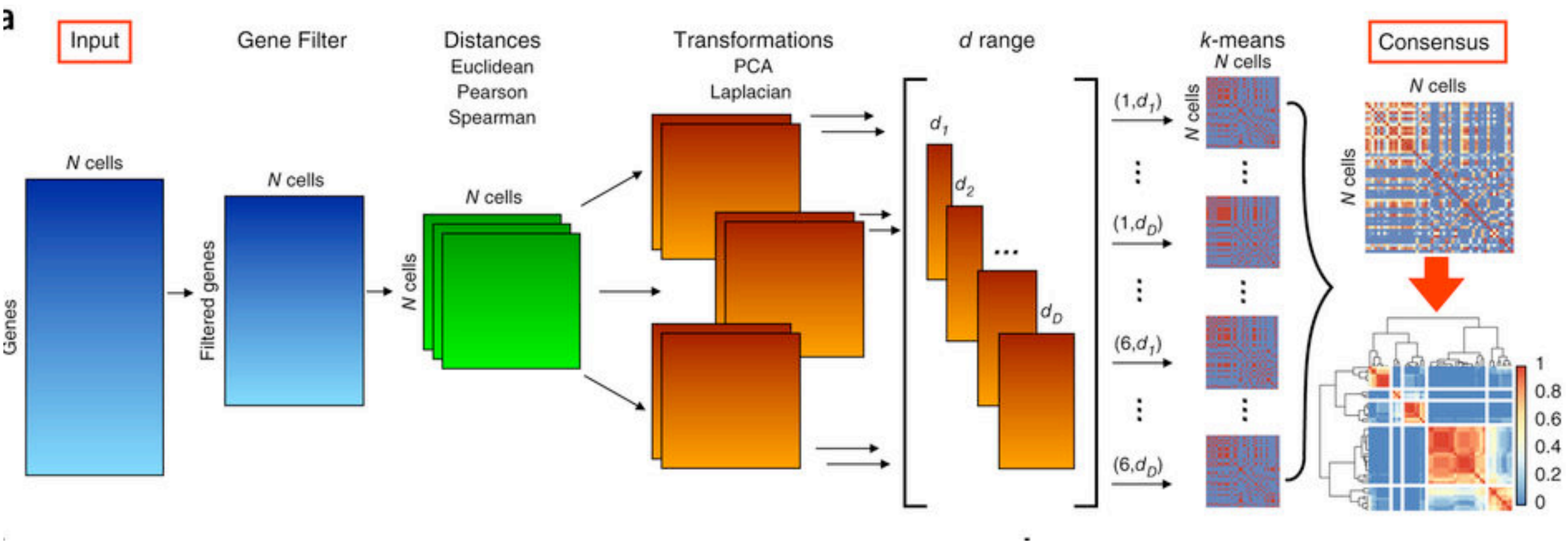
- Scater + Scran – EBI groups, Marioni, Lun, McCarthy
- Seurat – Satija lab
- Monocle – Trapnell lab
- Pagoda – Kharchenko lab

SCRAN – Single Cell RNA ANalisys

- Uses SingleCellExperiment class – same as in Scater package
- Cyclone method for predicting cell cycle phase.
- Basics deconvolution strategy for size factors.
- Detection of variable genes by deconvolution of technical and biological variance.
- MNNCorrect for batch correction
- Also contains method for SNN graphs and community detection.

http://bioconductor.org/packages/devel/bioc/vignettes/scran/inst/doc/scran.html

Single Cell Consensus Clustering – SC3



(Kiselev et al *Nat. Methods* 2017)

Single Cell Consensus Clustering – SC3

1. Gene filtering – rare and ubiquitous genes
2. Distance matrices (DM) – Euklidean, Spearman, Pearson
3. Transformation of DM with PCA or Laplacian
4. K-means clustering with first *d* eigenvectors
5. Consensus clustering – distance 1/0 for cells in same/ different clusters -> hierarchical clustering on average distances.

Differential expression with nonparametric Kruskal–Wallis test.

Marker genes with areas under the ROC curve (AUROC) from 100 permutations of cell cluster labels and P-values from Wilcoxon signed-rank test.

(Kiselev et al *Nat. Methods* 2017)

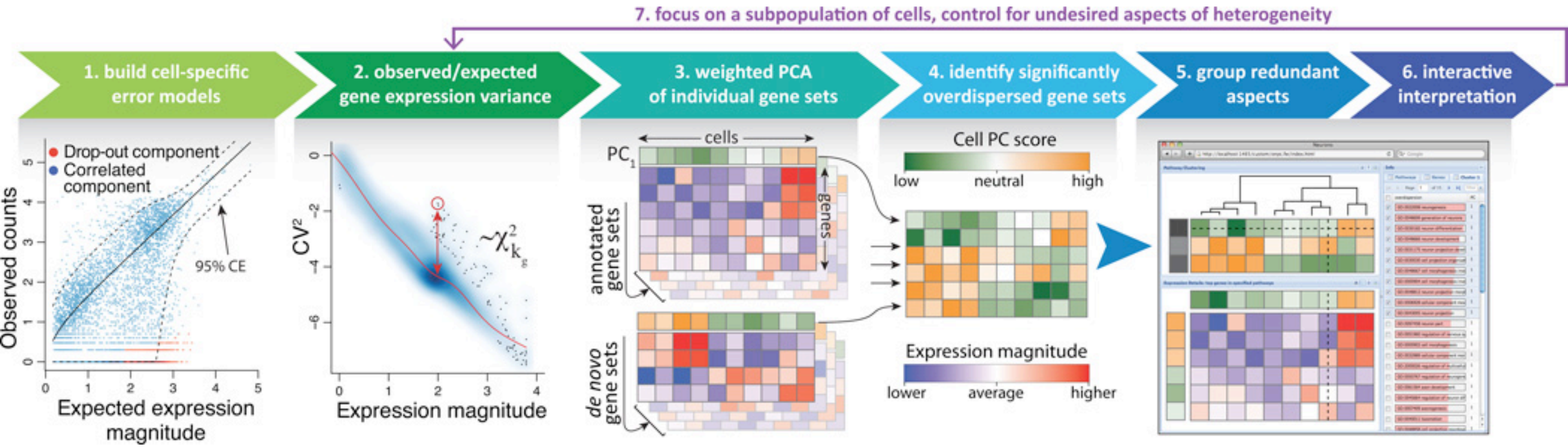
Shared nearest neighbor (SNN)-Cliq

- Similarity matrix using Euclidean distance (can use other distances)
- List the *k*-nearest-neighbors (KNN)
- Edge between cells if at least one shared neighbor
- Weights based on ranking of the neighbors
- Graph partition by finding cliques
- Identify clusters in the SNN graph by iteratively combining significantly overlapping subgraphs
- Implemented in Matlab and Python

(Xu et al *Bioinformatics* 2015)

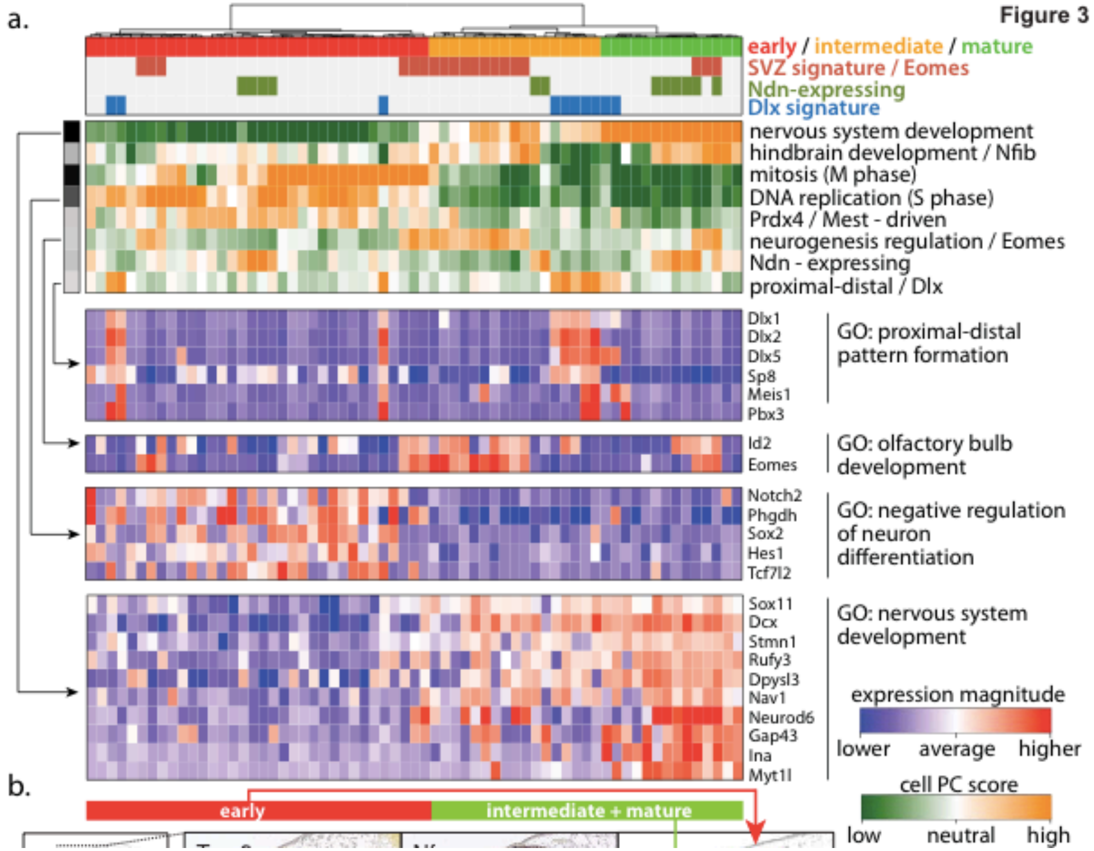
Pagoda – Pathway And Geneset OverDispersion Analysis

Implemented in the SCDE package


7. focus on a subpopulation of cells, control for undesired aspects of heterogeneity
1. build cell-specific error models
2. observed/expected gene expression variance
3. weighted PCA of individual gene sets
4. identify significantly overdispersed gene sets
5. group redundant aspects
6. interactive interpretation
Drop-out component
Correlated component
95% CE
Observed counts
Expected expression magnitude
0 1 2 3 4 5
CV²
0 -2 -4 -6
~χ²k_g
Expression magnitude
0 1 2 3 4
cells
PC1
genes
annotated gene sets
de novo gene sets
Cell PC score
low
neutral
high
Expression magnitude
lower
average
higher

(Fan et al. *Nature Methods* 2016)

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

(Fan et al. *Nature Methods* 2016)

Pagoda2

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

BackSPIN - Biclustering

- Simultaneous clustering genes and cells.
- An iterative, biclustering method based on sorting points into neighborhoods (SPIN) to find shapes in a reduced space
  1. ordering of samples using genes as features,
  2. ordering of genes using samples as features and
  3. zooming in on subsets of the original expression matrix to order objects in a reduced subspace.
- Clusters both genes and cells to identify subpopulations as well as potential markers for each subpopulations.
- Implemented in Python.

(Zeisel et al. *Science* 2015)

Seurat

- Developed for drop-seq analysis – compatible with 10X output files. But works also for other types of data.
- Contains function for
  - Data normalization
  - Detection of variable genes
  - Regression of batch effects and other confounders
  - Prediction of cell cycle score
  - JackStraw to detect significant principal components
  - tSNE and other dimensionality reduction techniques
  - Clustering based on SNN graphs
  - Many different methods for Differential expression

(http://satijalab.org/seurat/)

Seurat - FindClusters

- First construct a KNN (k-nearest neighbor) graph based on the euclidean distance in PCA space.
  - Select which principal components to include
- Refine the edge weights between any two cells based on the shared overlap in their local neighborhoods (Jaccard distance).
- To cluster the cells, modularity optimization techniques to iteratively group cells together.
- **OBS!** Earlier versions of Seurat uses “spectral tSNE” and DBScan density clustering.

(http://satijalab.org/seurat/)

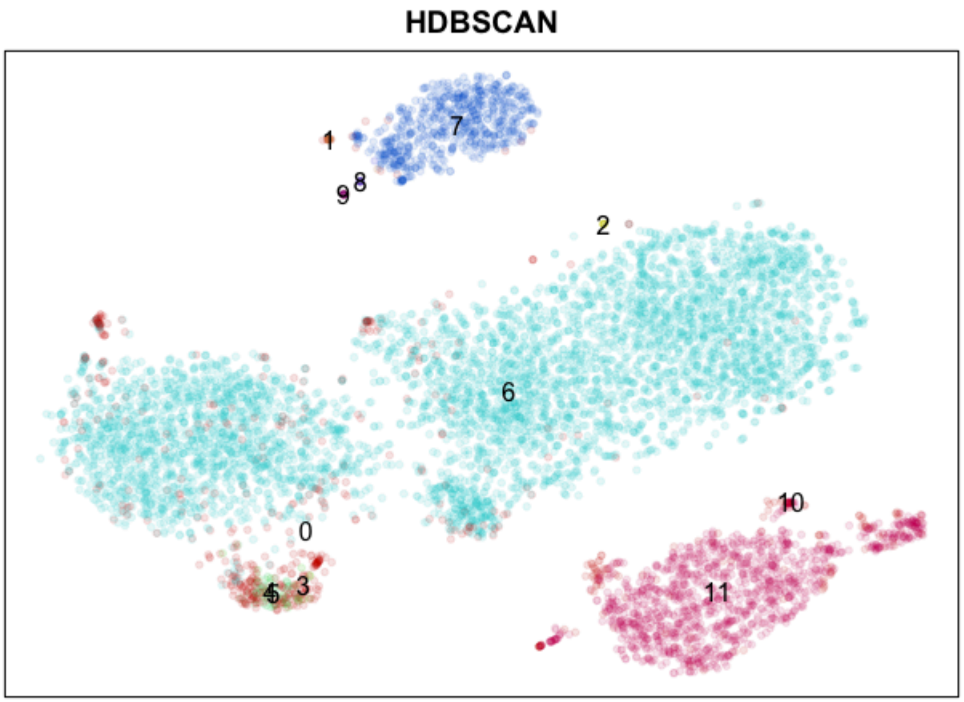
Seurat

- Also contains functions for:
  - Spatial reconstruction of single cell data using *in situ* references (Zebrafish embryos)
  - Integrated analysis across platforms
  - Analysis of multimodal datasets (e.g. RNA + protein)

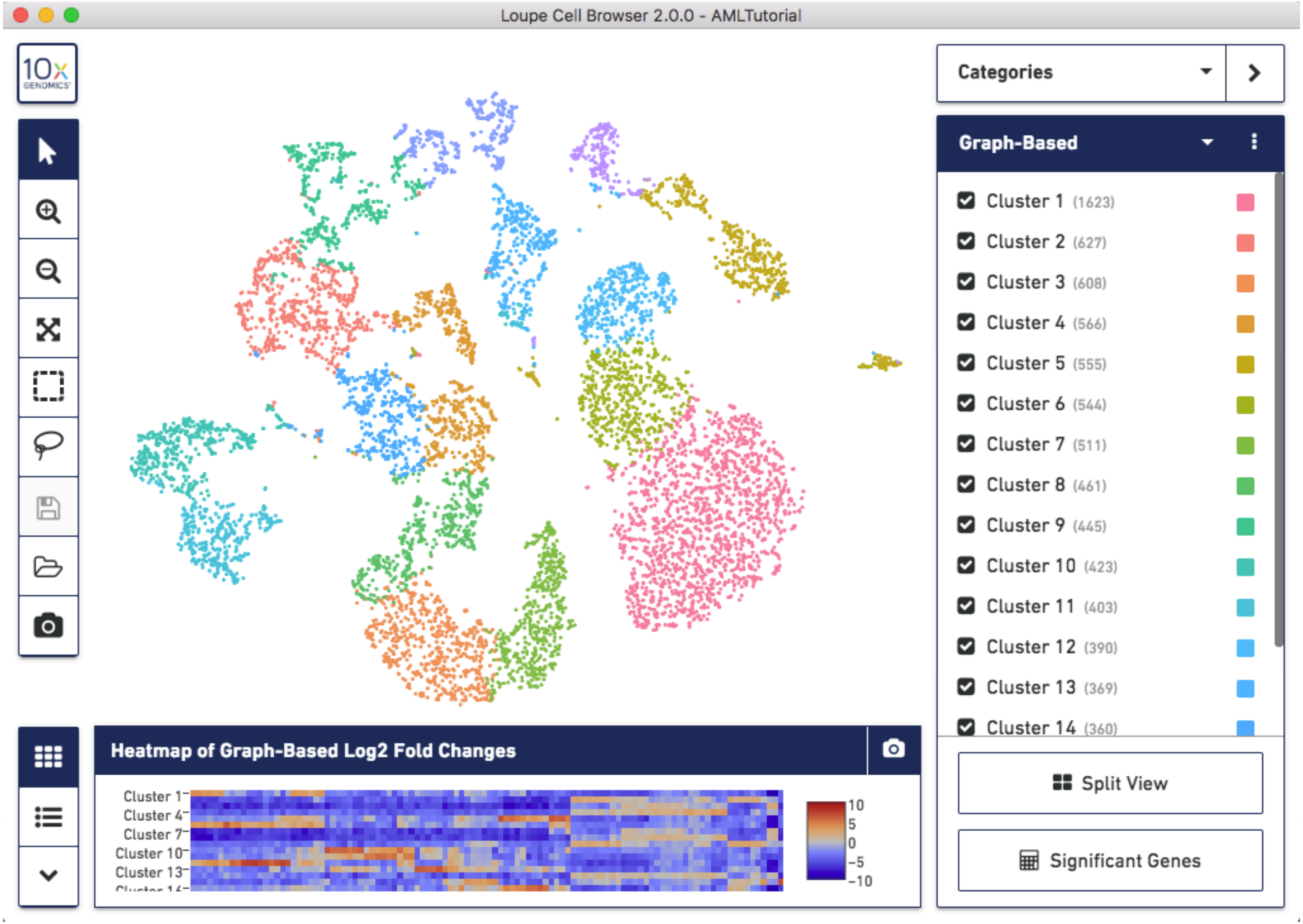
(http://satijalab.org/seurat/)

HDBSCAN

- Hierarchical DBSCAN – density based clustering on tSNE



Loupe – Cell Browser, from 10X Genomics



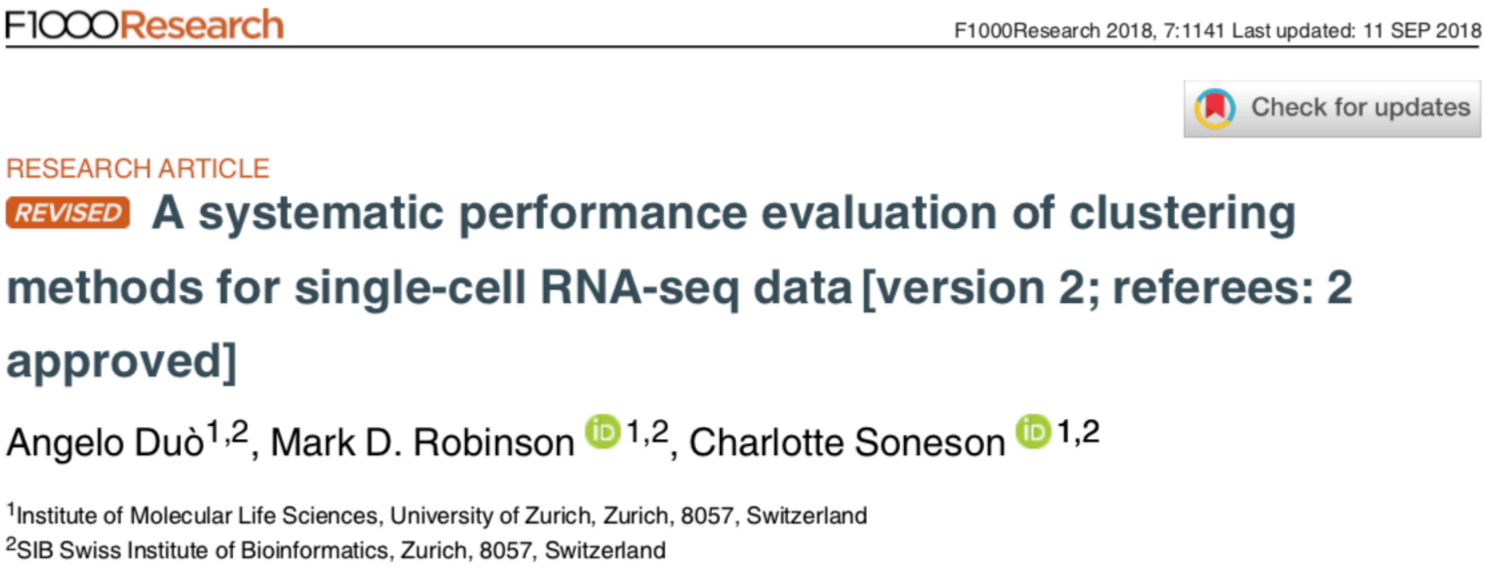
Loupe Cell Browser 2.0.0 - AMLTutorial

☑ Cluster 1 (1623)
☑ Cluster 2 (627)
☑ Cluster 3 (608)
☑ Cluster 4 (566)
☑ Cluster 5 (555)
☑ Cluster 6 (544)
☑ Cluster 7 (511)
☑ Cluster 8 (461)
☑ Cluster 9 (445)
☑ Cluster 10 (423)
☑ Cluster 11 (403)
☑ Cluster 12 (390)
☑ Cluster 13 (369)
☑ Cluster 14 (360)

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 2018, 7:1141 Last updated: 11 SEP 2018

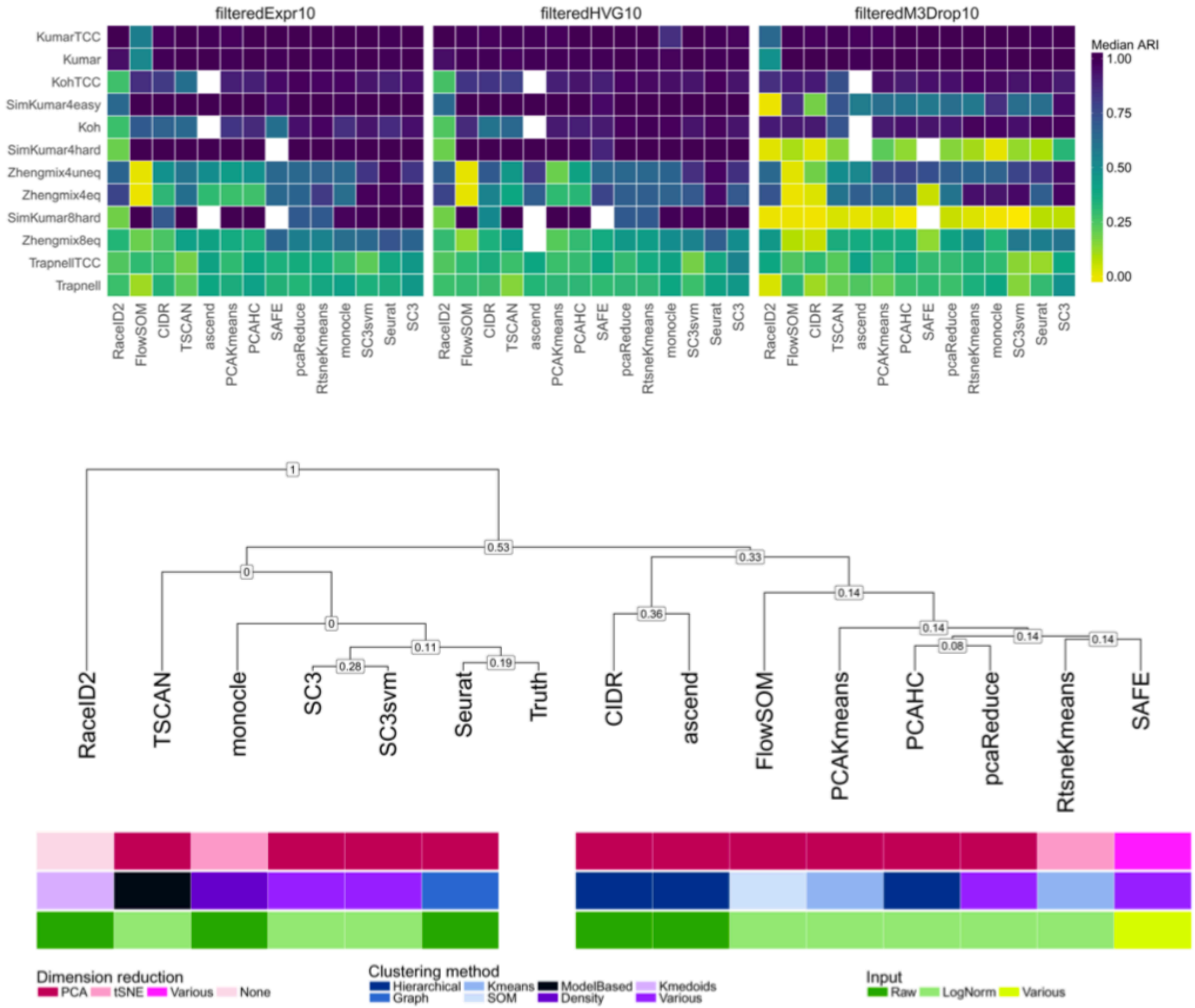
Check for updates

RESEARCH ARTICLE

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

Angelo Duò[1,2], Mark D. Robinson [1,2], Charlotte Soneson [1,2]

[1]Institute of Molecular Life Sciences, University of Zurich, Zurich, 8057, Switzerland
[2]SIB Swiss Institute of Bioinformatics, Zurich, 8057, Switzerland


filteredExpr10
filteredHVG10
filteredM3Drop10
Median ARI
1.00
0.75
0.50
0.25
0.00
KumarTCC
Kumar
KohTCC
SimKumar4easy
Koh
SimKumar4hard
Zhengmix4uneq
Zhengmix4eq
SimKumar8hard
Zhengmix8eq
TrapnellTCC
Trapnell
RaceID2
FlowSOM
CIDR
TSCAN
ascend
PCAKmeans
PCAHC
SAFE
pcaReduce
RtsneKmeans
monocle
SC3svm
Seurat
SC3
1
0.53
0
0
0.11
0.28
0.19
0.33
0.36
0.14
0.14
0.08
0.14
0.14
RaceID2
TSCAN
monocle
SC3
SC3svm
Seurat
Truth
CIDR
ascend
FlowSOM
PCAKmeans
PCAHC
pcaReduce
RtsneKmeans
SAFE
Dimension reduction
PCA
tSNE
Various
None
Clustering method
Hierarchical
Graph
Kmeans
SOM
ModelBased
Density
Kmedoids
Various
Input
Raw
LogNorm
Various

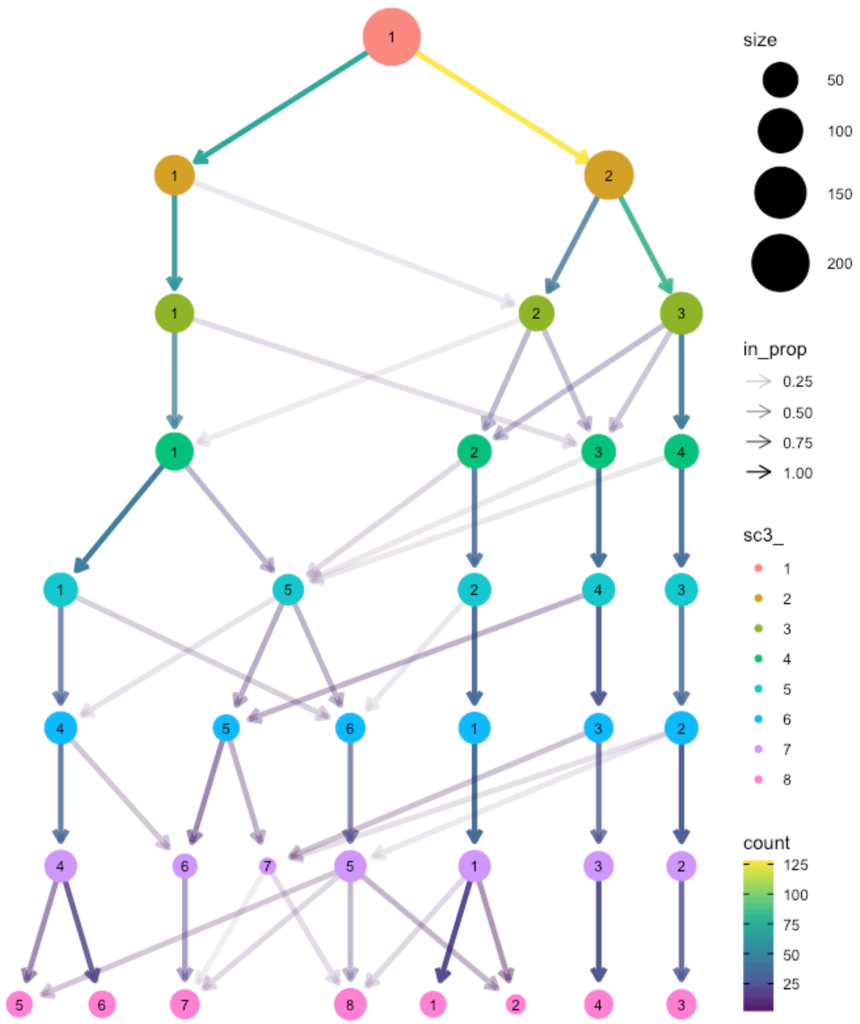
Main pipelines

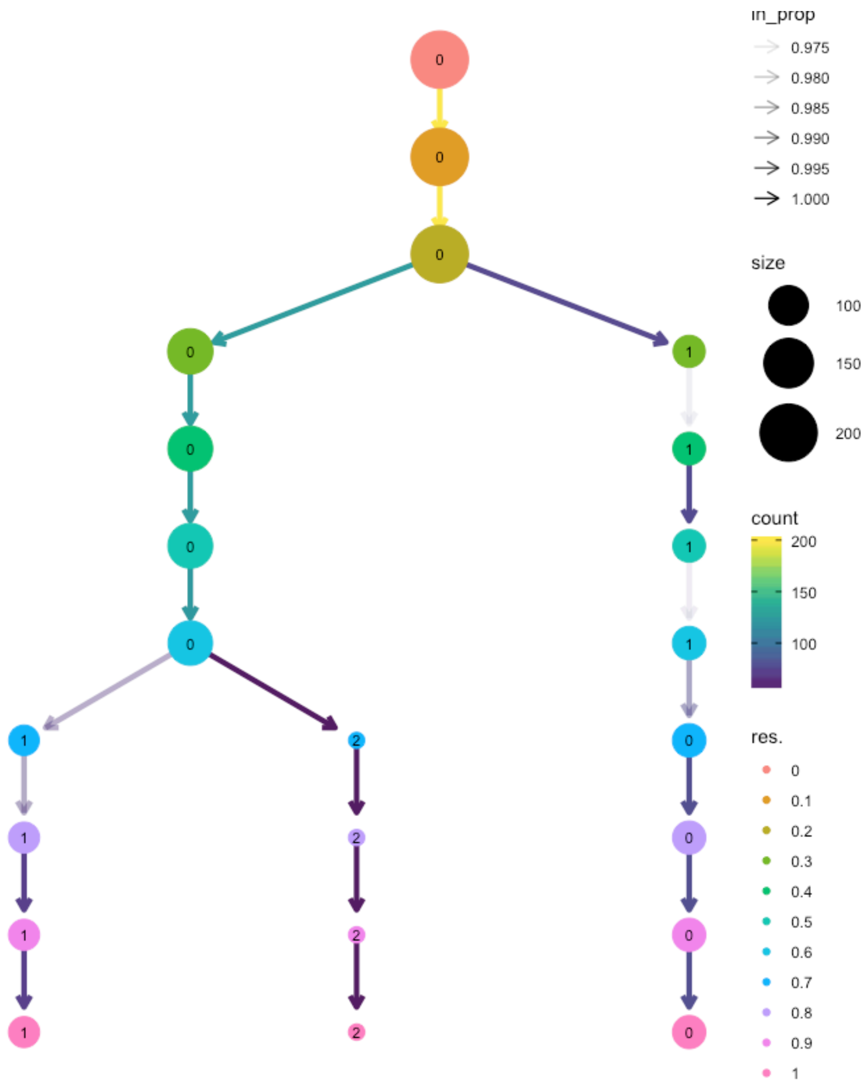
- Scater + Scran – EBI groups, Marioni, Lun, McCarthy
- Seurat – Satija lab
- Monocle – Trapnell lab
- Pagoda – Kharchenko lab

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)

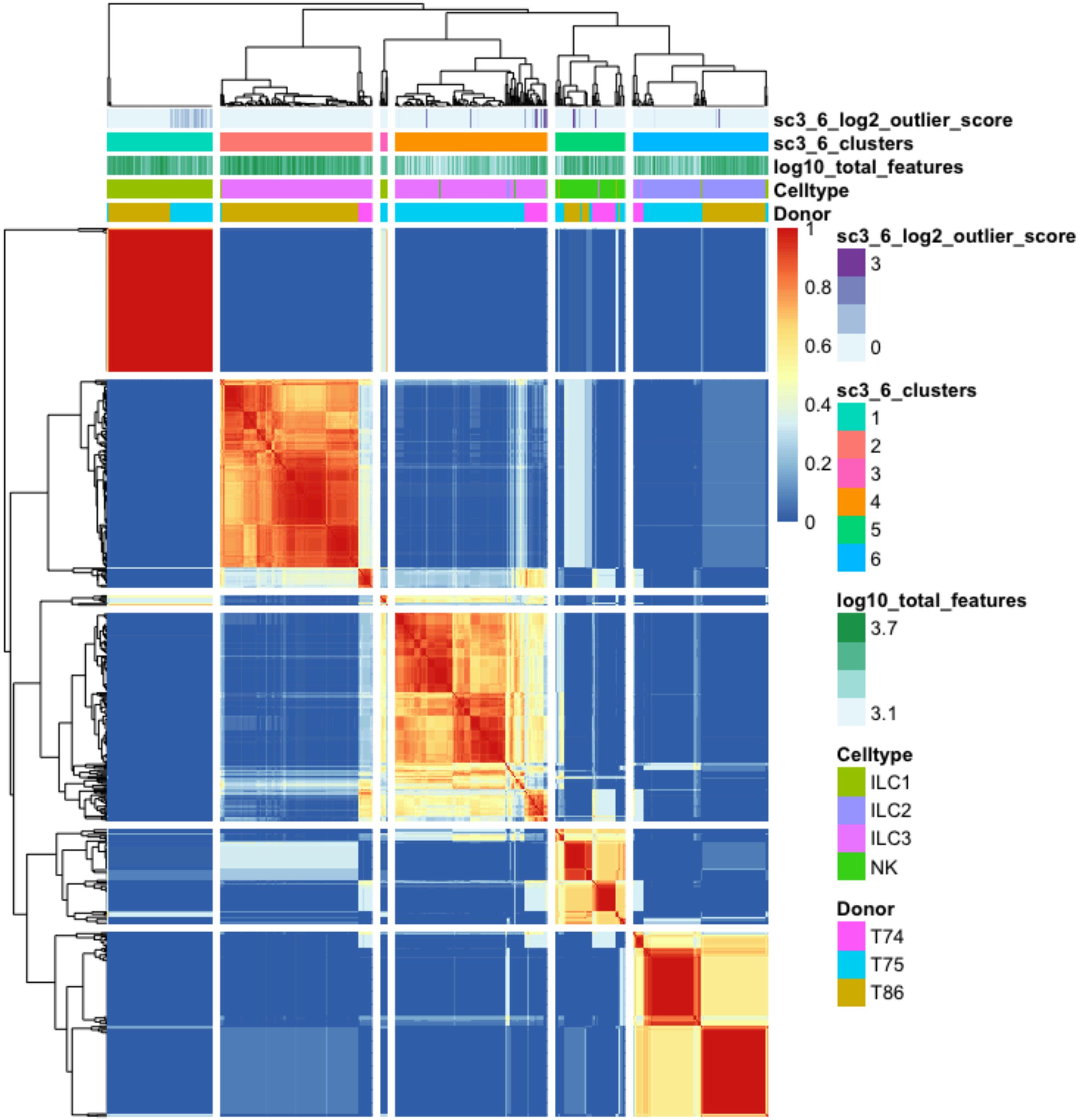
Clustree – R package





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

Check QC data



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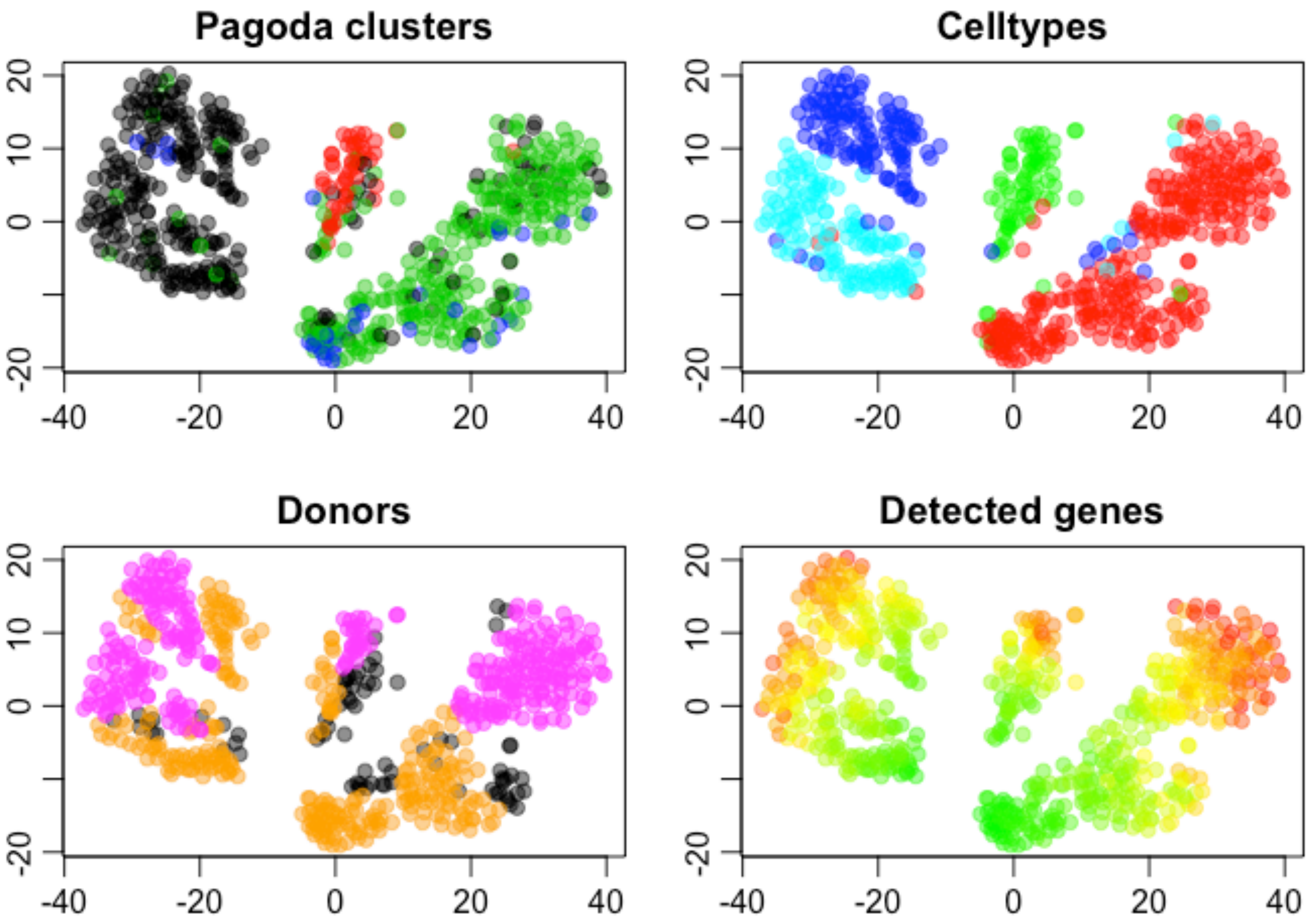
Check QC data



From clusters to celltypes

- Using lists of DE genes and prior knowledge of the biology
- Using lists of DE genes and comparing to other scRNAseq data or sorted cell populations.
- Infer labels from other scRNAseq dataset(s) from the same tissue
  - Correlation between clusters
  - Different data integration methods
  - Programs for inferring celltypes

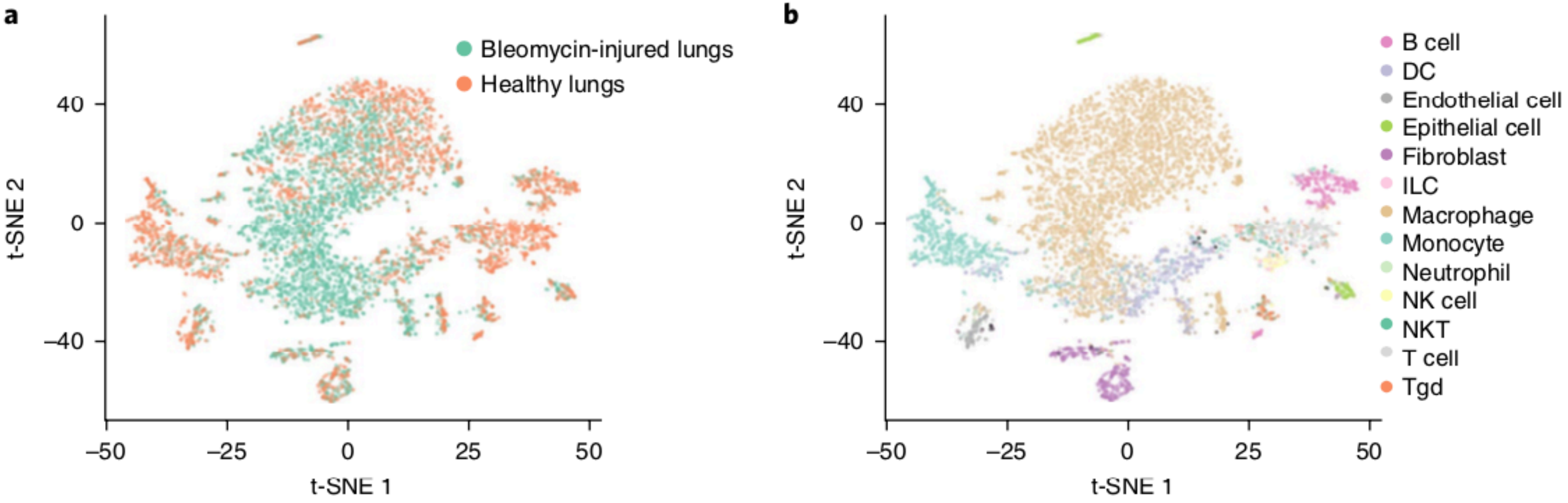
Databases with celltype gene signatures

- PanglaoDB - panglaodb.se
  - Human: 208 samples, 56 tissues, 0.7 M cells
  - Mouse: 798 samples, 147 tissues, 3.3 M cells
  - paper under review

- CellMarker – http://biocc.hrbmu.edu.cn/CellMarker/
  - Human: 13,605 cell markers of 467cell types in 158 tissues
  - Mouse: 9,148 cell makers of 389 cell types in 81 tissues
  - Zhang et al. NAR 2018

singleR

- Annotation of scRNAseq by reference bulk transcriptomes
- Reference from ImmGen, Encode and Blueprint Epigenomics.
- Webportal you can upload your data to.



(Aran et al. Nature Immunol. 2019)

- scPred – Hernandez et al bioRxiv 2018
  - unbiased feature selection from a reduced-dimension space, and machine-learning classification
  - Support vector machine or other models.

- Moana – Wagner & Yanai bioRxiv 2018
  - Hierarchical machine learning framework – classification of celltypes at different levels.
  - PBMC classifier, Pancreas cell type classifier.

- CaSTLe – Lieberman et al PLOS One 2018
  - XGBoost classification model trained on one dataset and predicted onto another dataset

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 layout is always best!
- Use your biological knowledge to evaluate the results – but try to be unbiased!

Resources

- Good course at: https://hemberg-lab.github.io/scRNA.seq.course/
- Many of the packages have very thorough tutorials on their websites
- Repo with scRNA-seq tools: https://github.com/seandavi/awesome-single-cell
- Single cell assay objects for many datasets: https://hemberg-lab.github.io/scRNA.seq.datasets/
- Conquer datasets - salmon pipeline to many different datasets: http://imlspenticton.uzh.ch:3838/conquer/
- EBI Single cell expression atlas: https://www.ebi.ac.uk/gxa/sc