Feature selection, dimensionality reduction, clustering, marker gene identification, and visualization in scRNA-Seq

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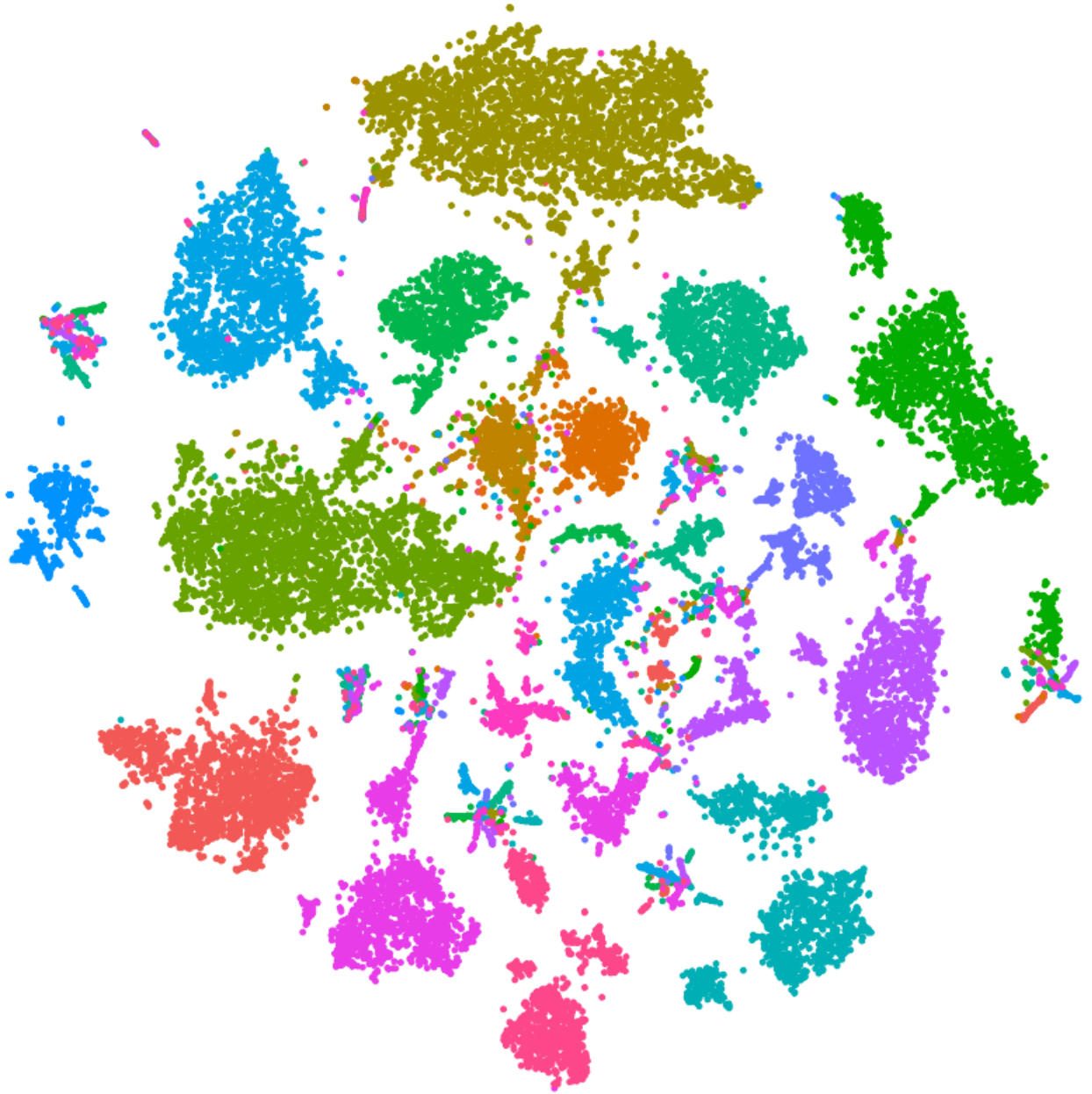
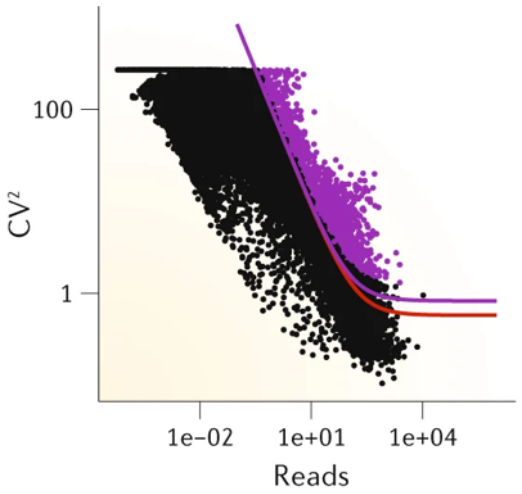
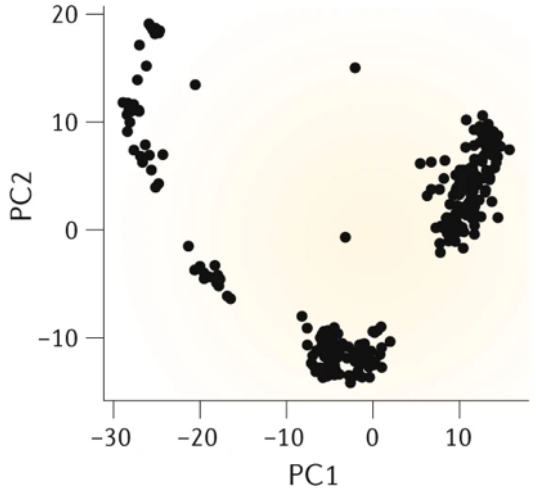
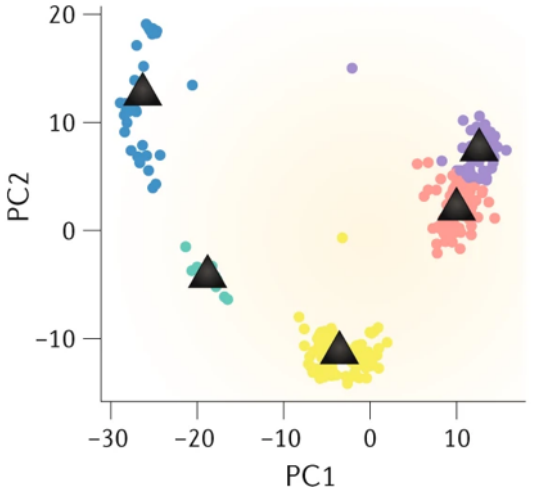
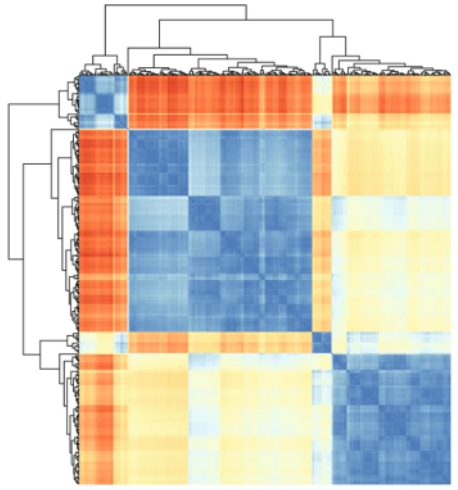


Feature (gene) selection → Dimensionality reduction → Clustering → Marker gene identification

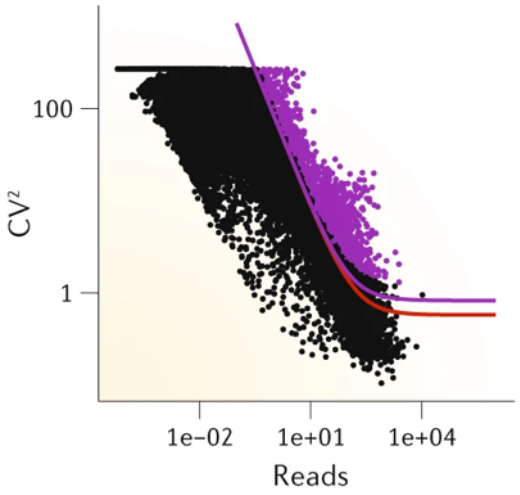


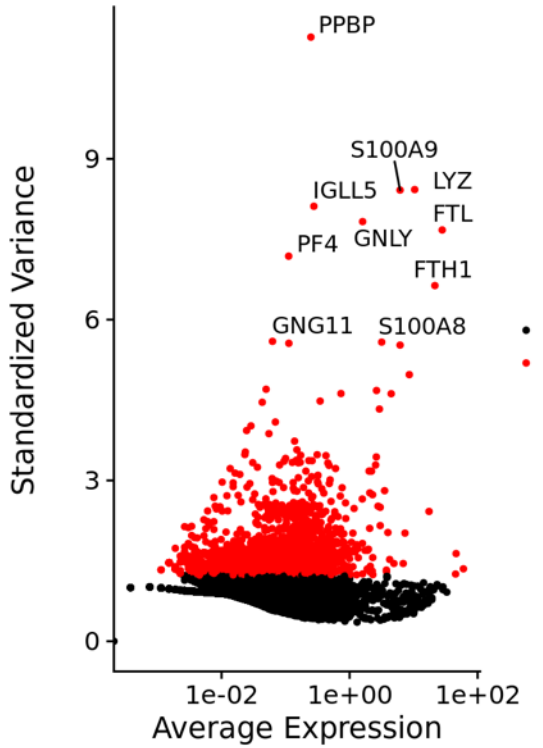






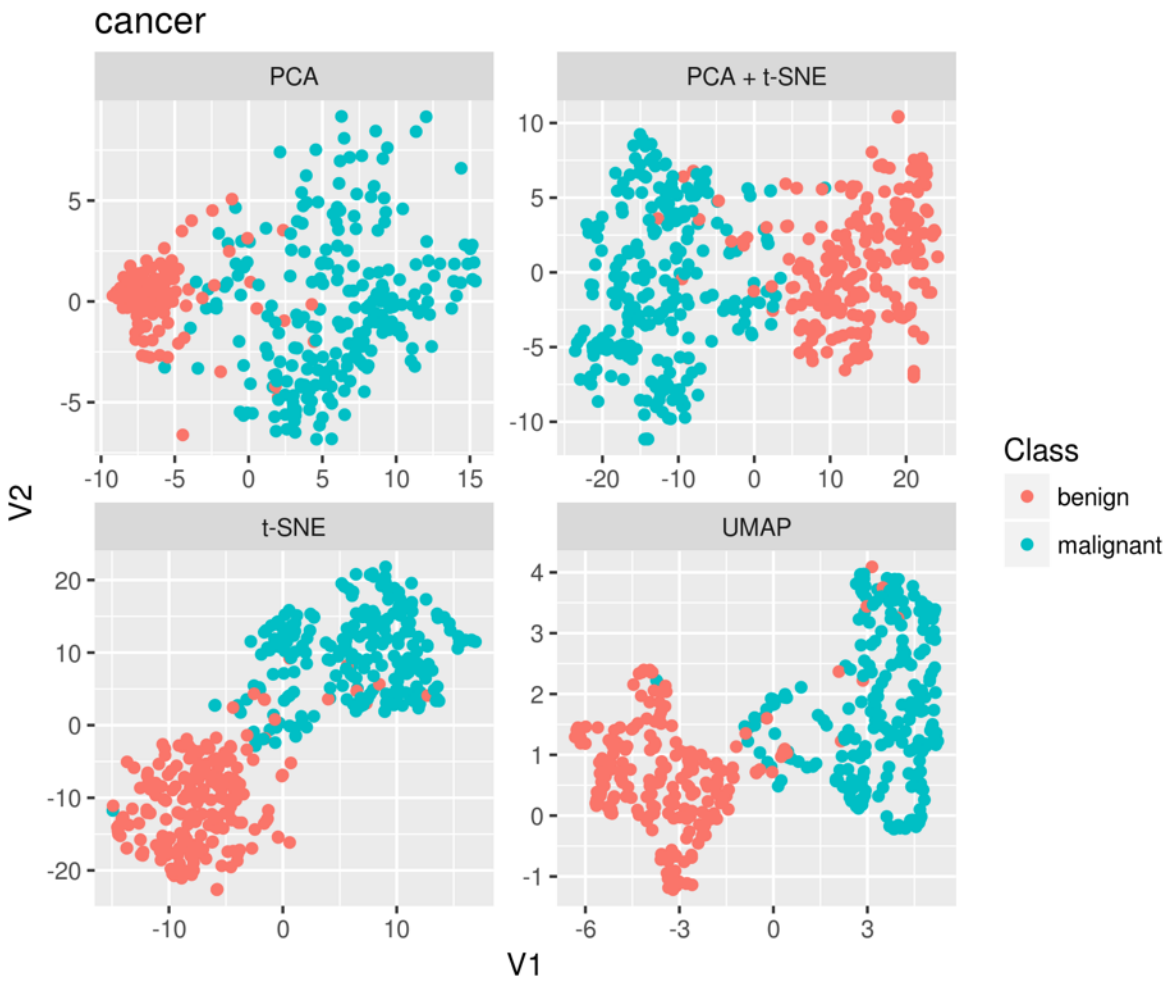
Feature (gene) selection





- Prior to dimensionality reduction, genes with highest expression variability (with enough read counts/above background) are identified.
- Typical input: Data normalized with the total expression in each cell, multiplied by a factor (e.g., 10,000) and log-transformed (not scaled data).
- 1000-5000 genes with the highest expression variability are selected
- In robust workflows (e.g., Seurat and Scanpy), downstream analysis is not very sensitive to the exact number of selected genes.
- Expanded selection can help identify novel clusters with the risk of introducing additional noise into downstream analysis.
- Ideally, gene selection is done after batch correction.
- The goal is making sure genes variable only among batches (rather than cell groups within batches) do not dominate downstream results.

Dimensionality reduction of scRNA-Seq data



- scRNA-Seq data is **inherently low-dimensional**.
- Information in the data (expression variability among genes/cells) can be reduced from the number of total genes (1000s) to a much lower number of dimensions (10s).
- Dimensionality reduction generates linear/non-linear combinations of gene expression vectors for clustering & visualization.
- Major dimensionality reduction techniques for scRNA-Seq:
  - Principal component analysis (PCA)
  - Most commonly used ones: UMAP and t-SNE (inputs: PCA results)
  - UMAPs typically preserve more of global structure with shorter run times
  - Other alternatives: Diffusion Maps & force-directed layout with k-nearest neighbors

Scaling normalized data & performing PCA

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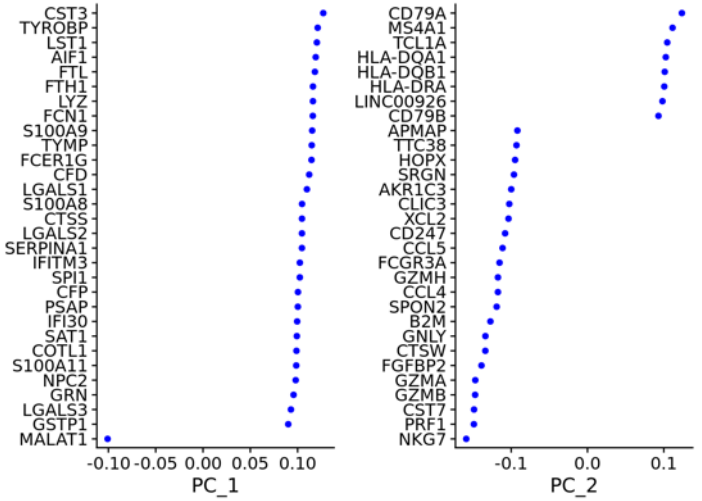
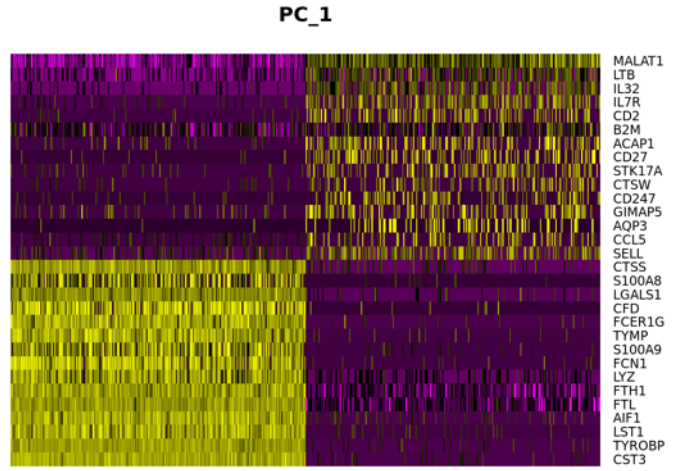
- PCA is performed on the scaled data.
- Scaled data represented as z-scores.
- Mean=0 & variance=1 for each gene.
- z-scoring makes sure that highly-expressed genes do not dominate.

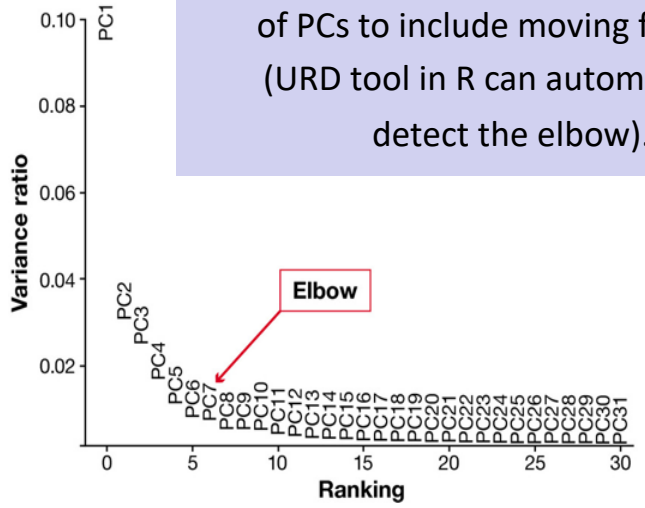


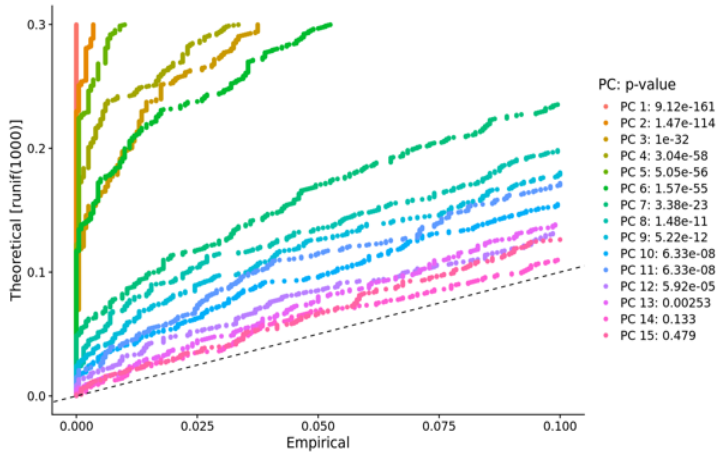
PC score plots show genes that dominate each PC



PC heatmaps visualize anti-correlated gene sets (yellow: higher expression)

Elbow plots show the number of PCs to include moving forward (URD tool in R can automatically detect the elbow).

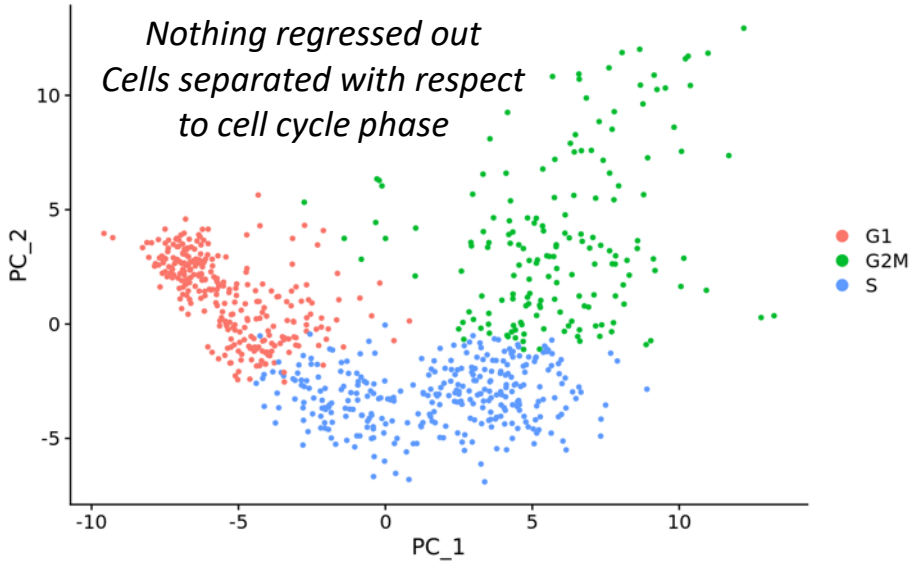


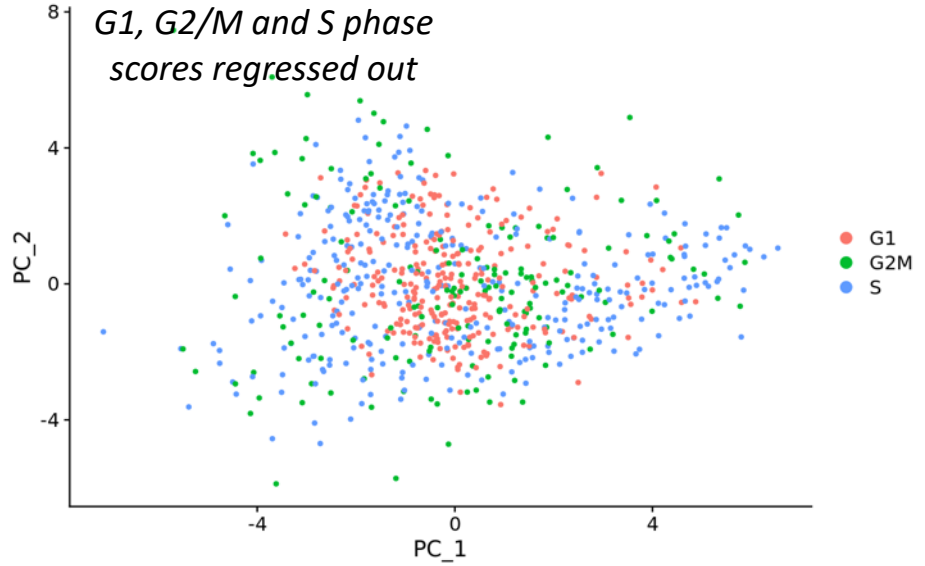


Jackstraw analysis generates a p-value (significance) of each PC 1% of the data is randomly permuted, PCA is rerun, 'null distribution' of gene scores constructed (these steps repeated many times).

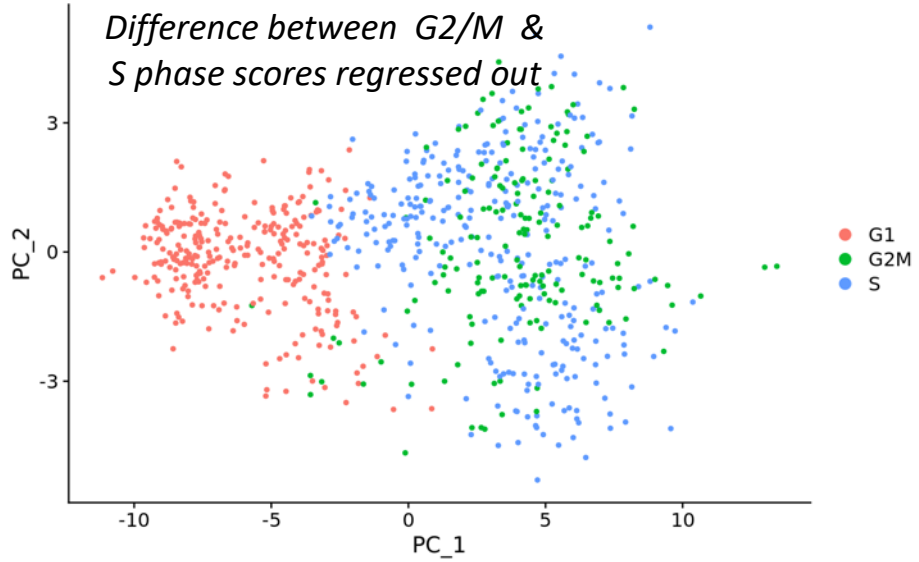
'Significant' PCs have a strong enrichment of low p-value genes.

The effect of regressing out cell cycle phase scores on PCA results



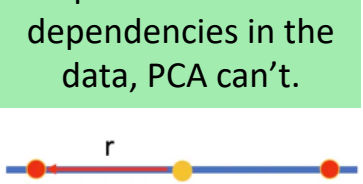


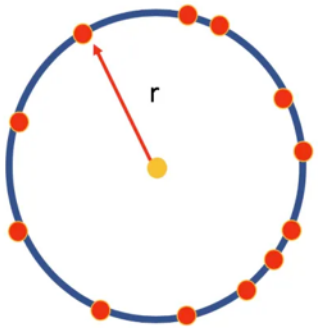
- What if majority of variance in top PCs are dominated by a specific set of genes that are not of biological interest?
- Example: Cell cycle heterogeneity in a murine hematopoietic progenitor data set.
- Scores computed for each phase based on canonical markers.
- Each cell mapped to a cell cycle phase (highest scoring phase)
- To differentiate between cycling and non-cycling cells, |G2/M-S| score difference is regressed out.

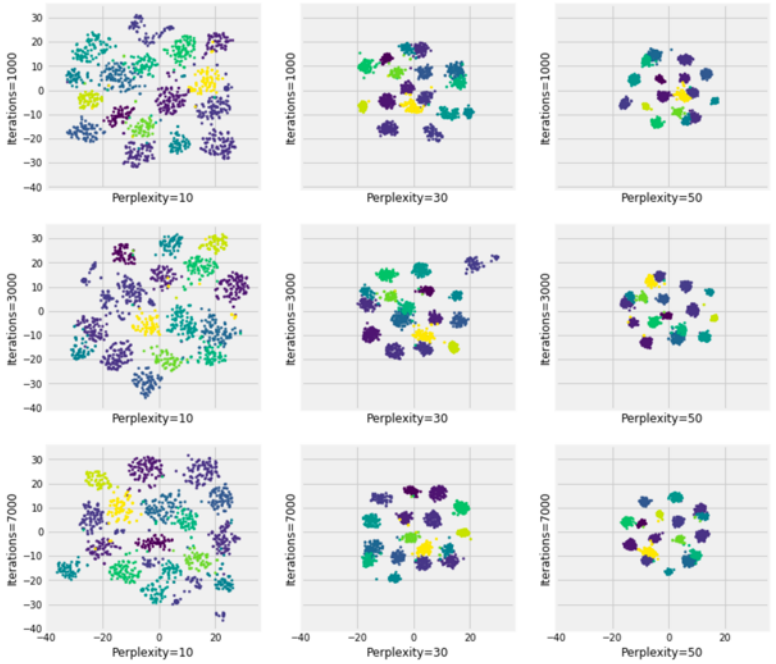


t-SNE: t-Distributed Stochastic Neighbor Embedding

t-SNE can capture capture non-linear dependencies in the data, PCA can't.



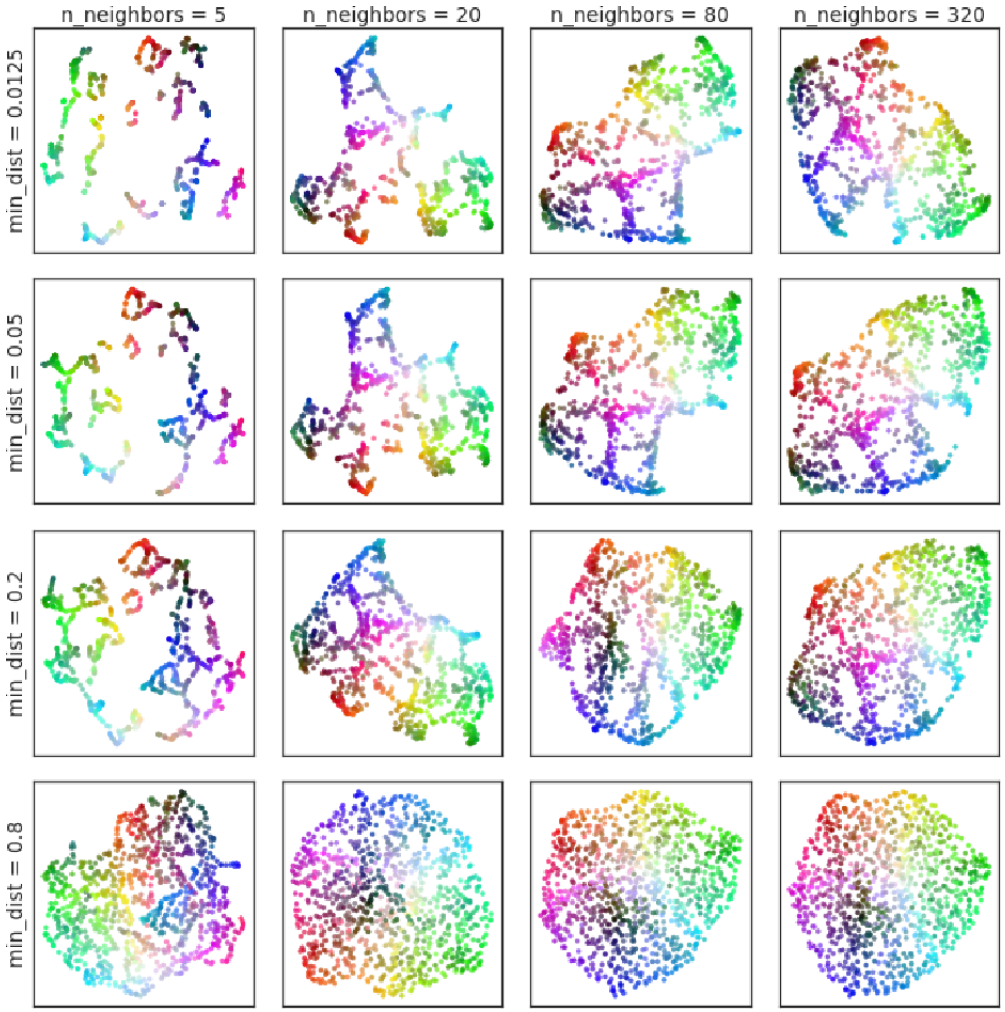




- One of the two most commonly used nonlinear dimensionality reduction techniques
- Used for <u>embedding</u> high-dimensional data for onto a low-dimensional space of 2 or 3 dimensions
- Can reveal local data structures efficiently **<u>without overcrowding</u>**
- t-SNE creates a probability distribution using the Gaussian distribution (defines the relationships between points in high-dimensional space).
- Uses the Student t-distribution to recreate the probability distribution in low-dimensional space.
- This prevents the crowding problem (points tend to get crowded in low-dimensional space/curse of dimensionality).
- t-SNE optimizes the embeddings directly using gradient descent.
- Cost function is non-convex with risk of getting stuck in local minima (t-SNE avoids poor local minima).
- Perplexity parameter serves as a knob that sets the number of nearest neighbors

t-SNE tries to recreate a low dimensional space that follows the probability distribution dictating the relationships between various neighboring points in higher dimensions

UMAP: Uniform Manifold Approximation and Projection



- UMAP is another manifold learning technique for dimensionality reduction
- Four major UMAP parameters control its topology

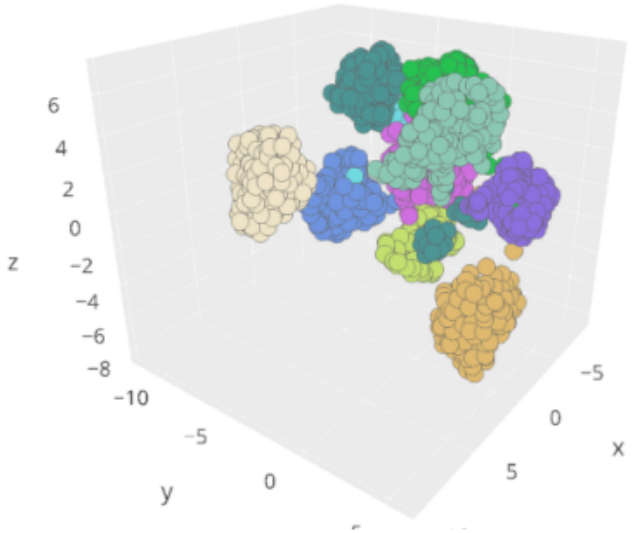
Number of neighbors per cell on the UMAP

- Balances local vs global structure in the data by constraining the size of the local neighborhood
- Low values force UMAP to concentrate on very local structure (potentially to the detriment of the big picture),
- Large values will push UMAP to look at larger neighborhoods of each point when estimating the manifold structure of the data, losing fine detail structure

Minimum distance between cells on the UMAP

- Controls how tightly cells are packed together
- Effective minimum distance between embedded points
- Low values lead to clumped nearby cells (finer topological structure)
- High values prevent packing points together (clusters get closer)
- High values preserves broad topological structure at the expense of finer topological details

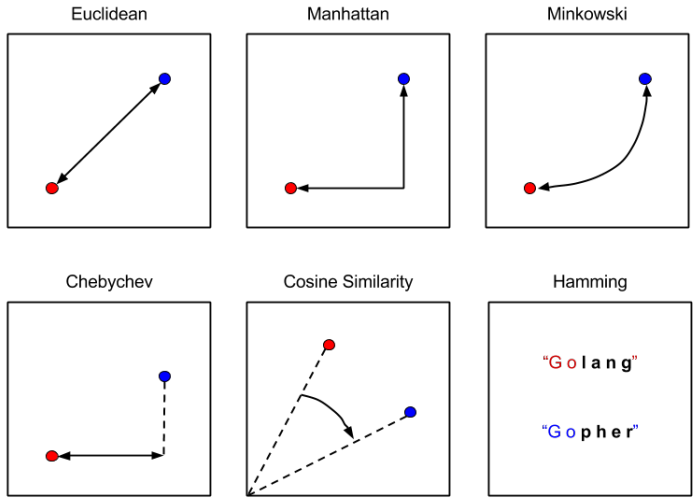
More UMAP parameters



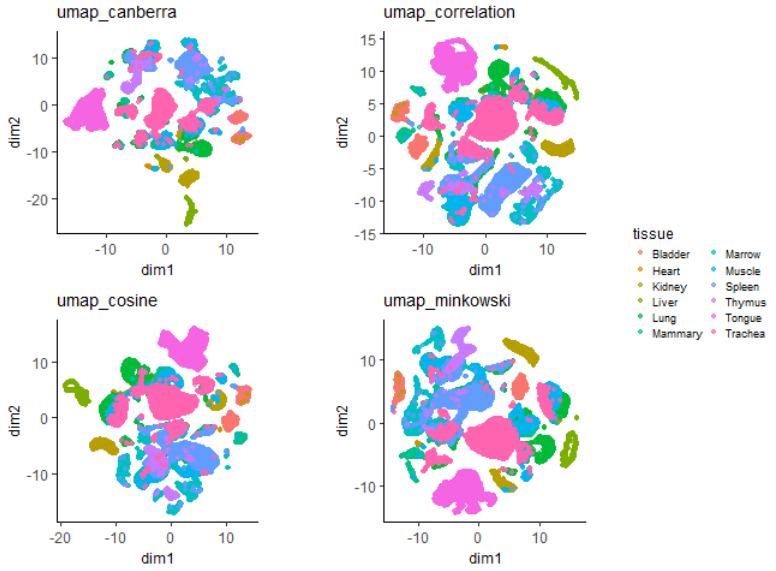
Number of UMAP dimensions

- Reduced data can be embedded into 2, 3, or higher dimensions

Cosine similarity & Pearson/Spearman correlation are scale invariant (driven by relative differences between cells, robust to library or cell size differences)



UMAP distance metric (cell to cell)



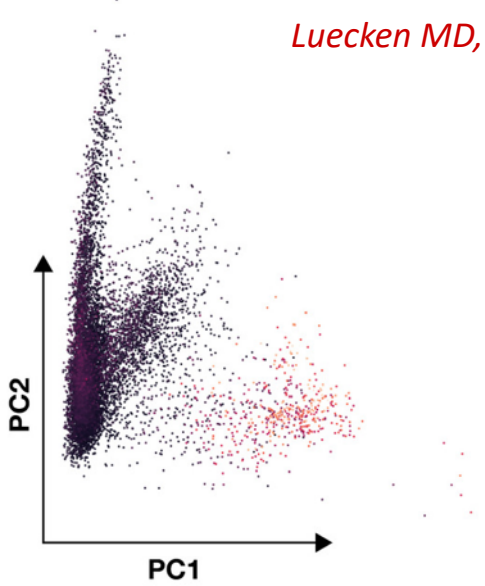
- The metric used to measure distance between cells in the input space
- Examples: Euclidean, Manhattan, and Minkowski
- Angular metric: Cosine similarity
- Pearson and Spearman correlation based metrics

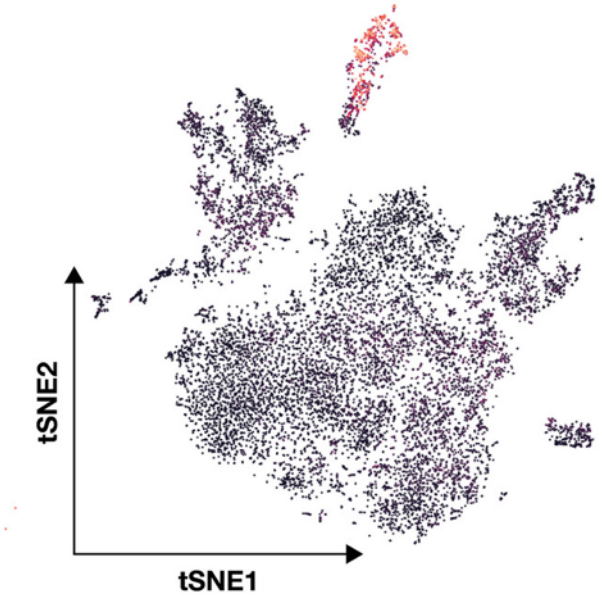
Euclidean $\sqrt{\sum_{i=1}^{k}(x_i - y_i)^2}$

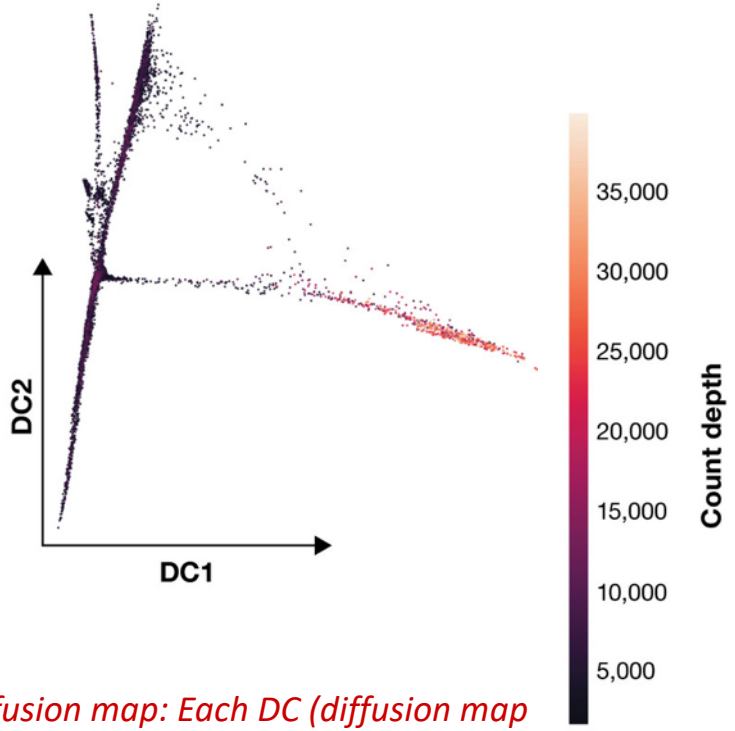
Manhattan $\sum_{i=1}^{k}|x_i - y_i|$

Minkowski $\left(\sum_{i=1}^{k}(|x_i - y_i|)^q\right)^{1/q}$

Luecken MD, Theis FJ. Current best practices in single-cell RNA-seq analysis: a tutorial. Molecular systems biology (2019).

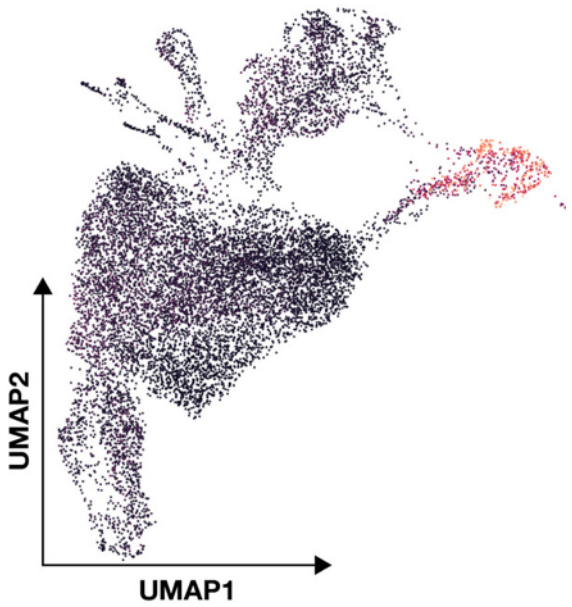


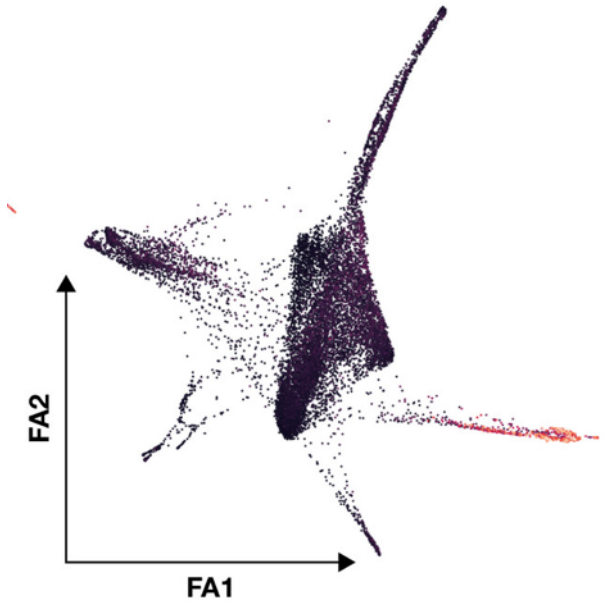




Diffusion map: Each DC (diffusion map dimension) highlights the heterogeneity of a different cell population.

Connectivity ~ probability of walking between the points in one step of a random walk (diffusion)





Force-directed graph layout via ForceAtlas2
Nodes repulse each other like charged particles, while edges attract their nodes, like springs.

Various dimensionality reduction methods applied to mouse intestinal epithelium data

Clustering cells with similar expression profiles together

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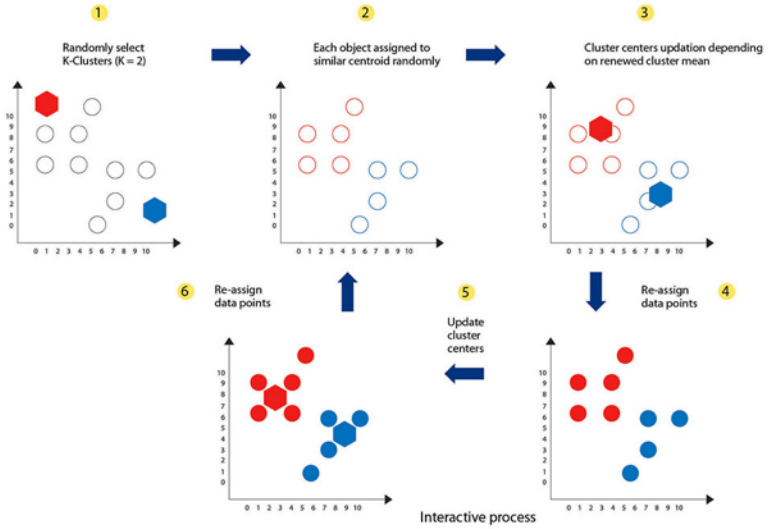

- Unsupervised machine learning problem
  - Input: distance matrix (cell-cell distances)
  - Output: Cluster membership of cells

- Cells grouped based on the similarity of their gene expression profiles
  - Distance measured in dimensionality-reduced gene expression space (scaled data)

- k-means clustering divides cells into k clusters
  - Determines cluster centroids
  - Assigns cells to the nearest cluster centroid
  - Centroid positions iteratively optimized (MacQueen, 1967).
  - Input: number of expected clusters (heuristically calibrated)

- k-means can be utilized with different distance metrics
- Alternatives to standard Euclidean distance:
  - Cosine similarity (Haghverdi et al, 2018)
  - Correlation-based distance metrics (Kim et al, 2018)
  - SIMLR method learns a distance metric using Gaussian kernels (Wang et al, 2017)

Using community detection & modularity optimization for finding clusters

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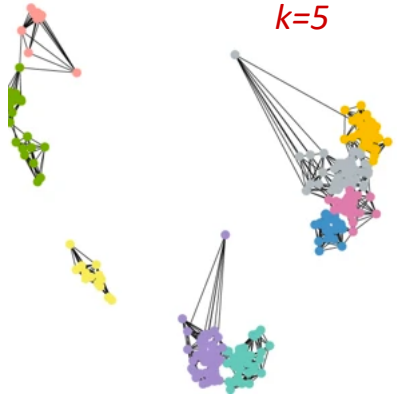
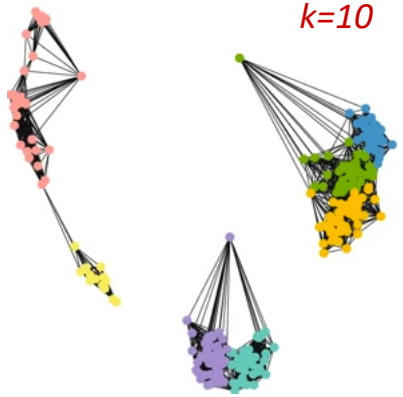
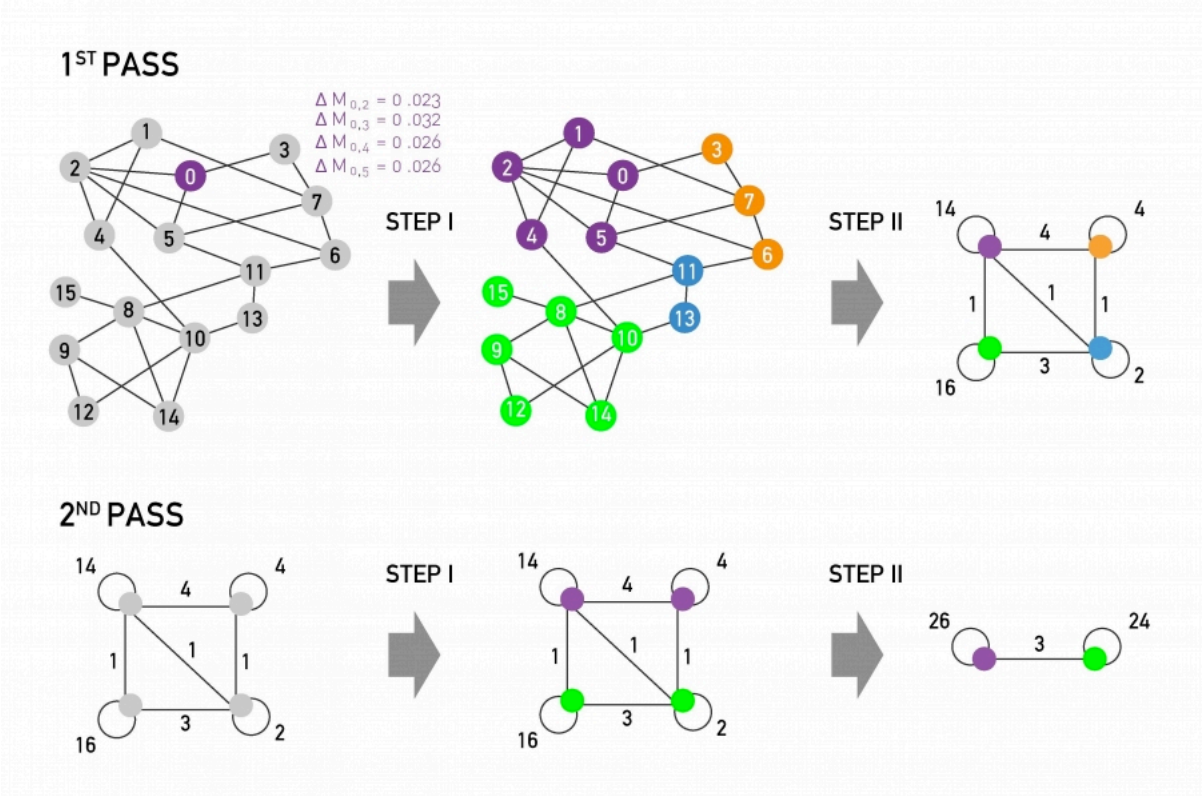
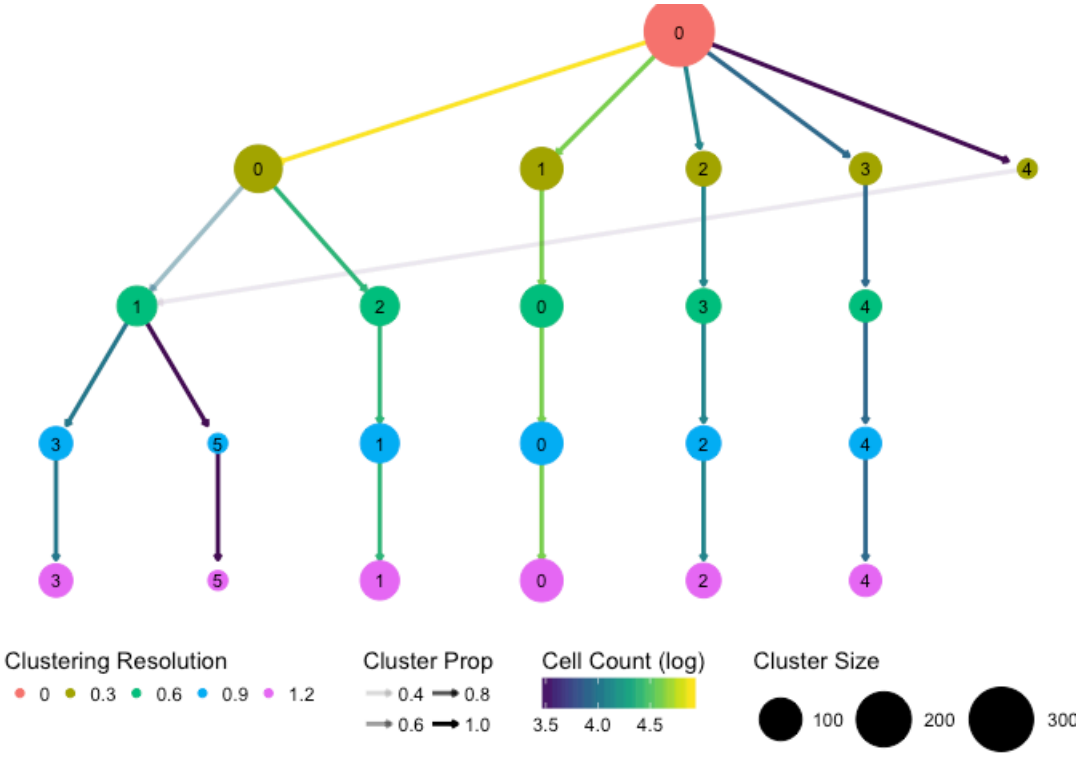




- Community detection methods utilize graph representation derived from k-nearest neighbors (kNN)
- Then, the modularity function is optimized to determine clusters.
- Typical range of k is 5-100
- Densely sampled regions of expression space are represented as densely connected regions of the graph.
- Community detection is often faster than clustering as only neighboring cell pairs have to be considered as belonging to the same cluster.
- Optimized modularity function includes a resolution parameter, which allows the user to determine the scale of the cluster partition.



Number of clusters and biological context



- Number of clusters is a function of the resolution parameter.
- Multiple resolution values can be explored to see the interplay between resolution and UMAP or t-SNE plots for a given data set.
- Biological context can be used for guidance.
- Examples: Expected number of major cell types or subtypes.
- Isolating a cluster to identify sub-clusters can generate useful biological insights (e.g., differential expression between cellular subtypes in a cluster).
- If cluster-specific markers for multiple clusters overlap (e.g., ribosomal genes), these clusters can be merged without losing much information regarding cell subtypes.

Clustering methods for scRNA-Seq data

Name	Year	Method type	Strengths	Limitations
scanpy[4]	2018	PCA + graph-based	Very scalable	May not be accurate for small data sets
Seurat (latest)[3]	2016			
PhenoGraph[32]	2015			
SC3[22]	2017	PCA + *k*-means	High accuracy through consensus, provides estimation of *k*	High complexity, not scalable
SIMLR[24]	2017	Data-driven dimensionality reduction + *k*-means	Concurrent training of the distance metric improves sensitivity in noisy data sets	Adjusting the distance metric to make cells fit the clusters may artificially inflate quality measures
CIDR[25]	2017	PCA + hierarchical	Implicitly imputes dropouts when calculating distances	
GiniClust[75]	2016	DBSCAN	Sensitive to rare cell types	Not effective for the detection of large clusters
pcaReduce[27]	2016	PCA + *k*-means + hierarchical	Provides hierarchy of solutions	Very stochastic, does not provide a stable result
Tasic et al.[28]	2016	PCA + hierarchical	Cross validation used to perform fuzzy clustering	High complexity, no software package available
TSCAN[41]	2016	PCA + Gaussian mixture model	Combines clustering and pseudotime analysis	Assumes clusters follow multivariate normal distribution
mpath[45]	2016	Hierarchical	Combines clustering and pseudotime analysis	Uses empirically defined thresholds and a priori knowledge
BackSPIN[26]	2015	Biclustering (hierarchical)	Multiple rounds of feature selection improve clustering resolution	Tends to over-partition the data
RaceID[23], RaceID2[115], RaceID3	2015	*k*-Means	Detects rare cell types, provides estimation of *k*	Performs poorly when there are no rare cell types
SINCERA[5]	2015	Hierarchical	Method is intuitively easy to understand	Simple hierarchical clustering is used, may not be appropriate for very noisy data
SNN-Cliq[80]	2015	Graph-based	Provides estimation of *k*	High complexity, not scalable

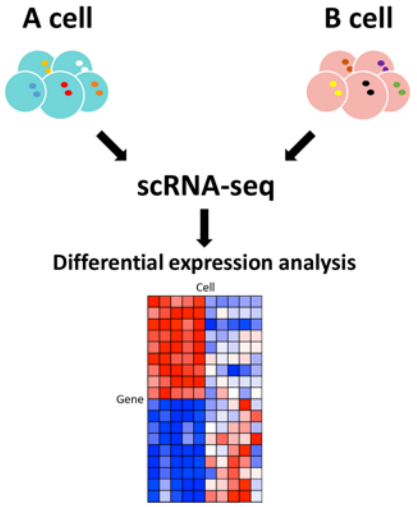
Kiselev, Vladimir Yu, Tallulah S. Andrews, and Martin Hemberg. "Challenges in unsupervised clustering of single-cell RNA-seq data." Nature Reviews Genetics (2019).

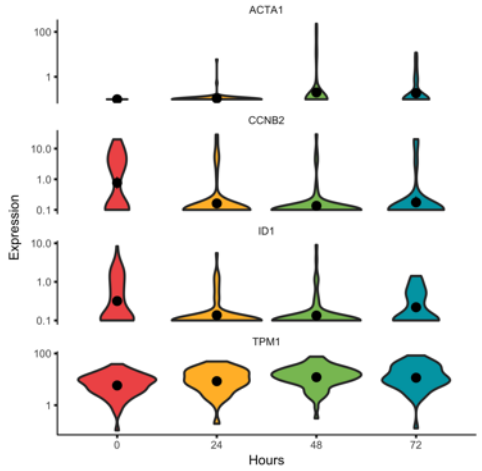
Each method with own strengths & limitations.

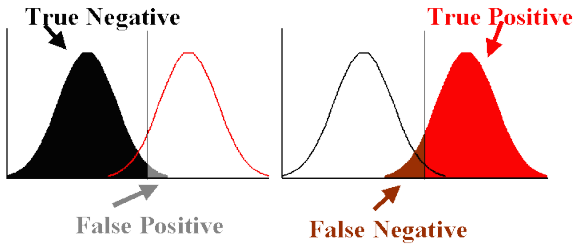
Seurat, Phenograph, and scanpy are the most popular methods (only limitation: accuracy for small data sets)

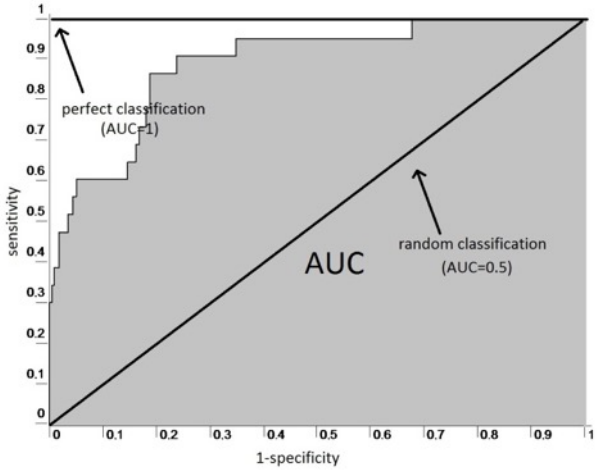
Other methods are mainly limited in their scalability, stability (stochastic), and ability to handle very noisy data.

Marker gene identification









Differential expression approaches for marker identification:

- Wilcoxon rank sum test and student's t-test
- Logistic regression
- DESeq2: Negative binomial generalized linear models (read counts) & Wald test for significance.
- MAST : GLMs in which cellular detection rate is treated as a covariate
- GLMs are flexible and do not make assumptions (homogenous distributions of residuals/fitting errors or normally distributed variances).

Classifier based approach for marker identification:

- Classifiers built with normalized expression levels (one classifier per gene).
- Genes ranked with respect to their ability of each gene to distinguish between two groups of cells (e.g. KO vs WT, cluster 1 vs 2, or cluster 1 vs all clusters).
- Area under each ROC curve represents the predictive power of the gene.

Butler et al. Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nature biotechnology. 2018 May;36(5):411.

MAST (Model-based Analysis of Single-cell Transcriptomics)

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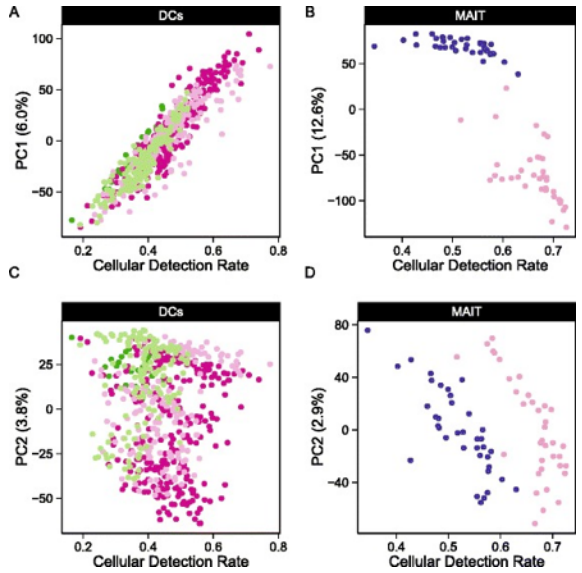
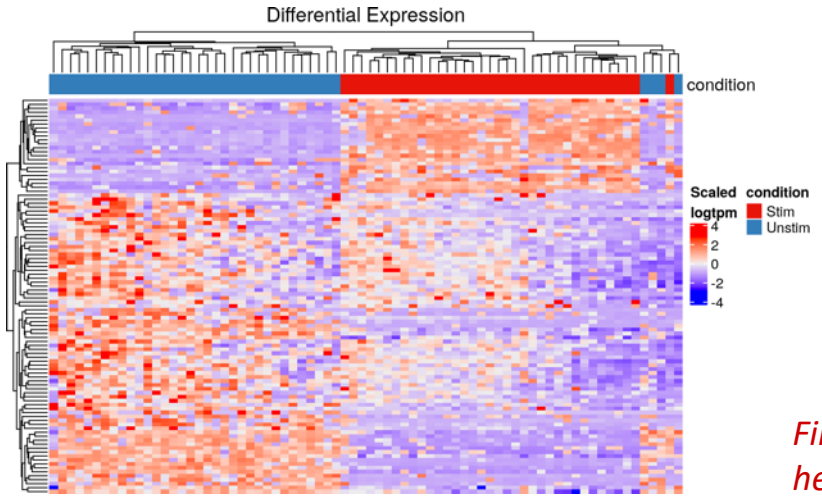




- Accounts for the fact that the number of cells expressing a gene varies from gene to gene.
- The fraction of genes expressed, or cellular detection rate (CDR) correlates with top PCs of of variation.
- Modeling CDR as a covariate controls for differences in abundance due to cell size and other extrinsic biological and technical effects.
- MAST has been tested against differential expression methods developed for bulk RNA-Seq (limma, edgeR, and DESeq) in Finak et al (2015).
- MAST was found to generate GO enrichment profiles biologically more relevant to mucosal-associated invariant T cell activation and LPS-stimulated myeloid dendritic cells in Finak et al (2015).

Finak et al. MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. Genome biology. 2015 Dec;16(1):278.

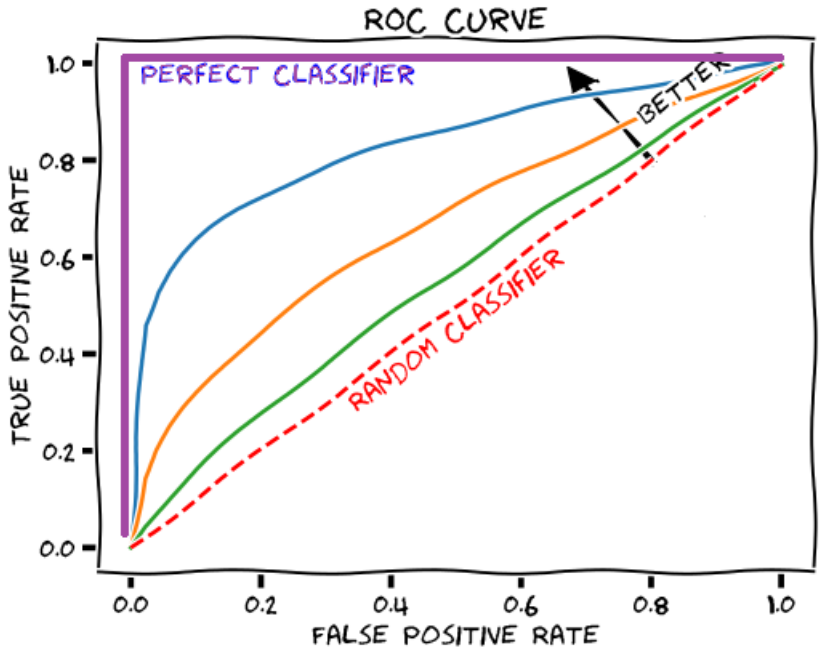
Classifier based-approaches for marker identification

Can the expression level of a gene correctly predict the cluster membership at different expression thresholds?

Cluster 1 vs Cluster 2
Positive class:C1, Negative class:C2

True positive (TP)
True membership: C1, Prediction: C1

False positive (FP)
True membership: C2, Prediction: C1



- Identifying "markers" of clusters using ROC analysis.
- For each gene, a classifier built on that gene alone to classify between two groups of cells.
- Classifier performance evaluated using AUC.
- TP, FP, TN, and FN are computed at different expression thresholds.
- An AUC value of 1 means that expression values for this gene alone can perfectly classify the two groupings.
- All cells in C1 exhibit higher expression than all cells in C2 (AUC=1).
- A value of 0.5 implies that the gene has no predictive power to classify the two clusters.

predicted → / real ↓	*Class_pos*	*Class_neg*
Class_pos	TP	FN
Class_neg	FP	TN

$$\text{TPR (sensitivity)} = \frac{TP}{TP + FN}$$

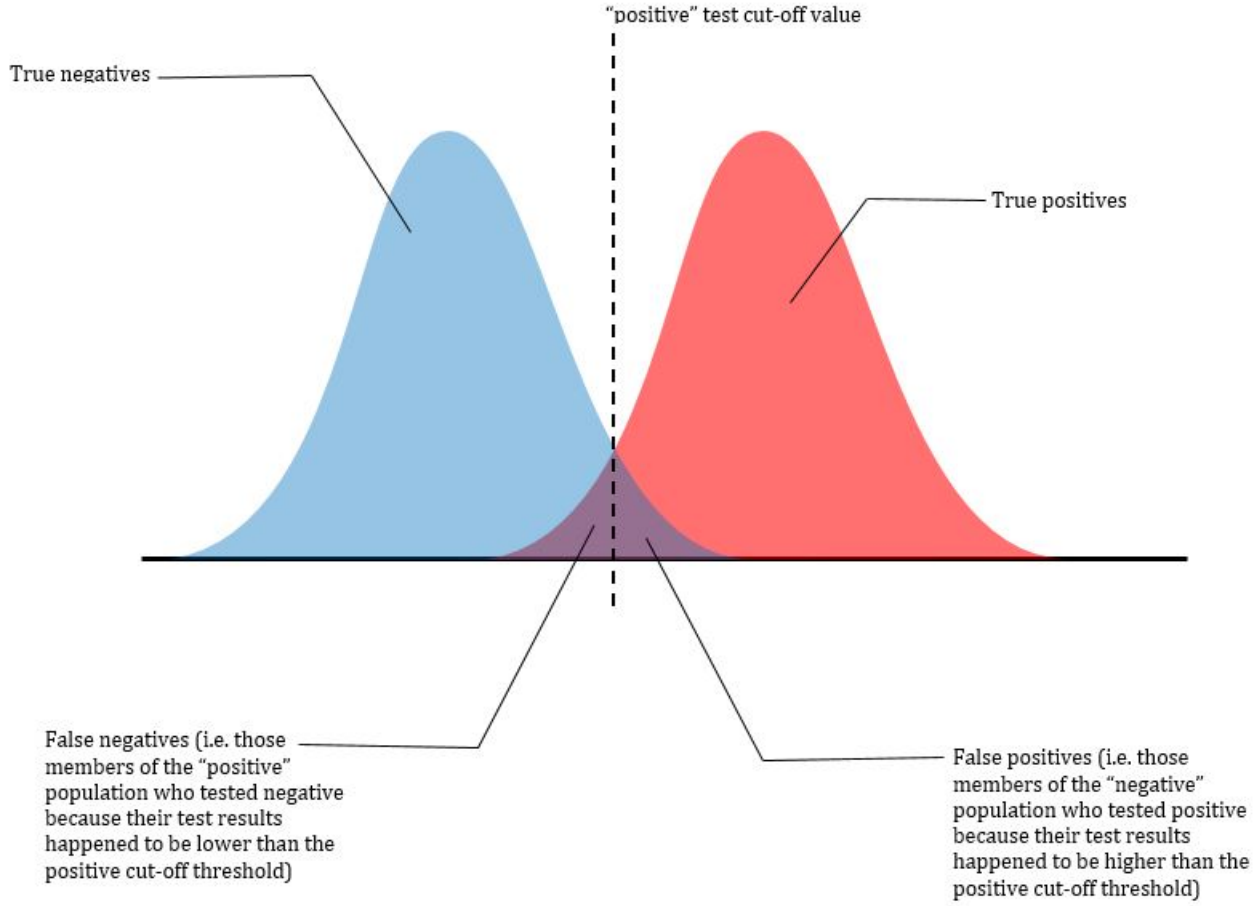
$$\text{FPR (1-specificity)} = \frac{FP}{TN + FP}$$

A closer look at the ROC calculations

Cluster 1 vs Cluster 2
Positive class:C1, Negative class:C2

True positive (TP)
True membership: C1, Prediction: C1

False positive (FP)
True membership: C2, Prediction: C1



A typical differential expression analysis output

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Comparing gene expression in different cell groups:

Cluster 1 vs Cluster 2
Cluster 1 vs all other clusters
Cluster1_KO vs Cluster1_WT

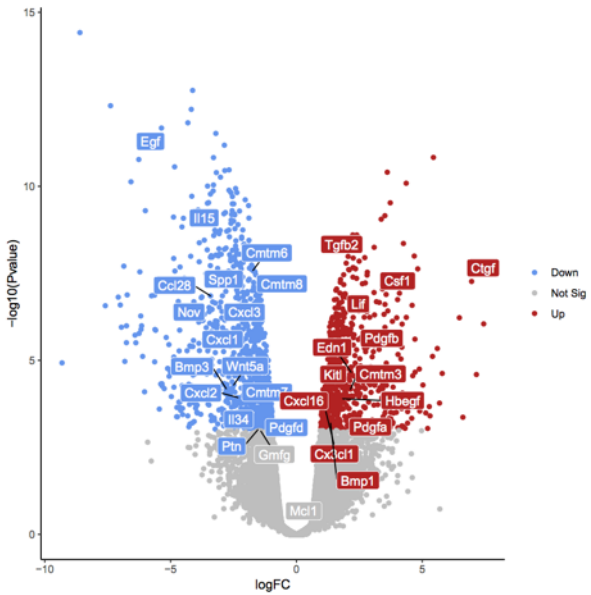
	p_val	avg_logFC	pct.1	pct.2	p_val_adj
S100A9	0	3.860873	0.996	0.215	0
S100A8	0	3.796640	0.975	0.121	0
LGALS2	0	2.634295	0.908	0.059	0
FCN1	0	2.352693	0.952	0.151	0
CD14	0	1.951644	0.667	0.028	0
TYROBP	0	2.111879	0.994	0.265	0

Average log FC
Ratio of expression in log-space

pct.1= percent of cells in Cluster 1 in which the gene is detected

pct.2=percent of cells in Cluster 2

p_val_adj=FDR



Tips for marker identification

Classifier based marker identification: AUC values replace the p-values

- Marker identification can take time with thousands of cells and genes
- Prefiltering cells and genes can reduce the computational time significantly
- Genes rarely detected in either group of cells, are not likely to be differentially expressed
- Genes with small fold-change can also be excluded
- Typically, only upregulated genes (>1 FC) are relevant for cluster-specific marker discovery

Visualization with violin plots, heatmaps, and ridgeline plots

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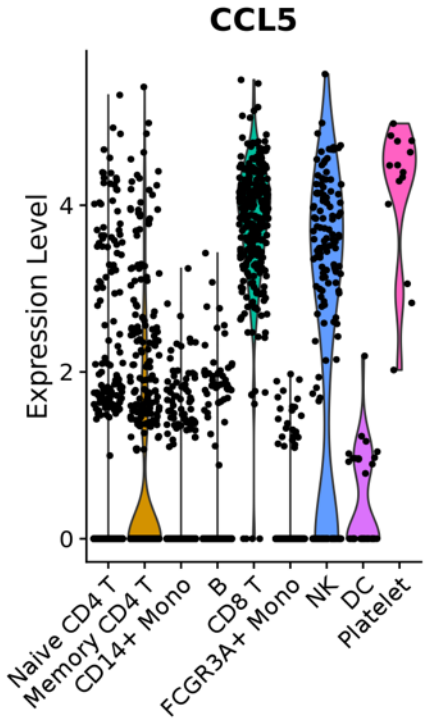
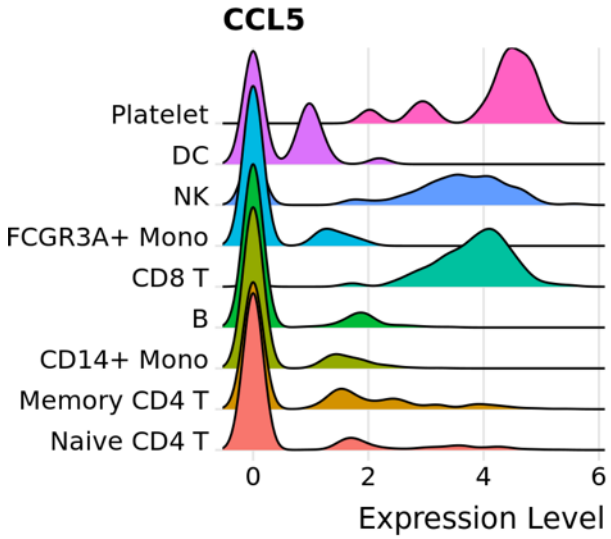
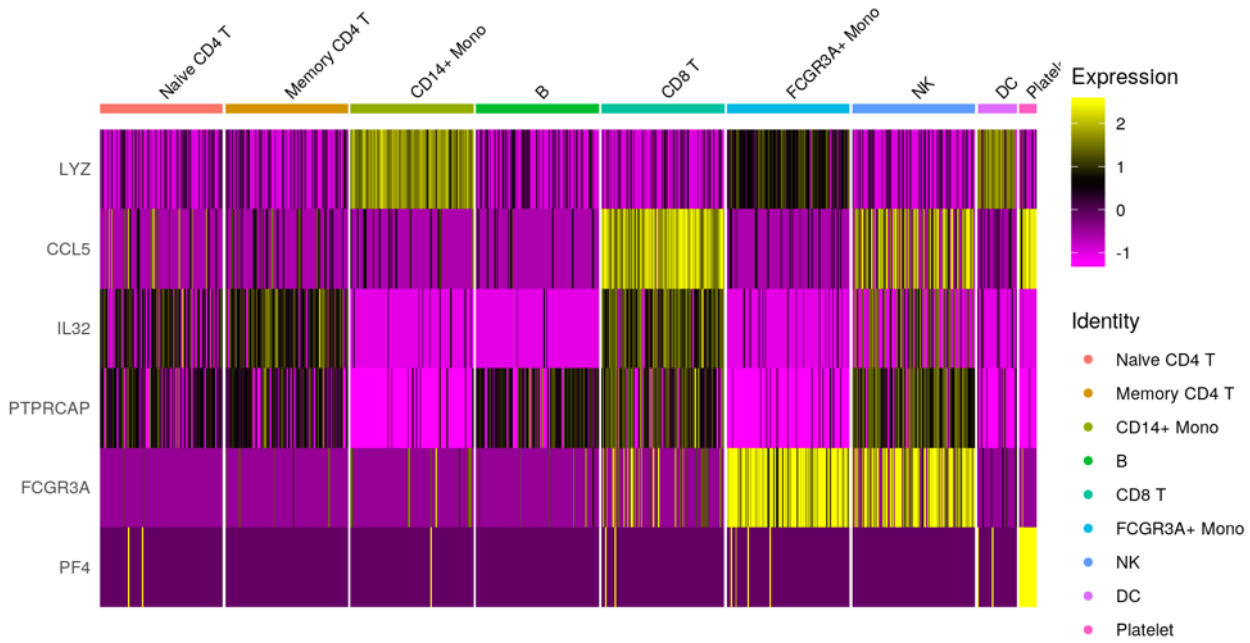


Violin plots show smoothed probability density distributions of expression



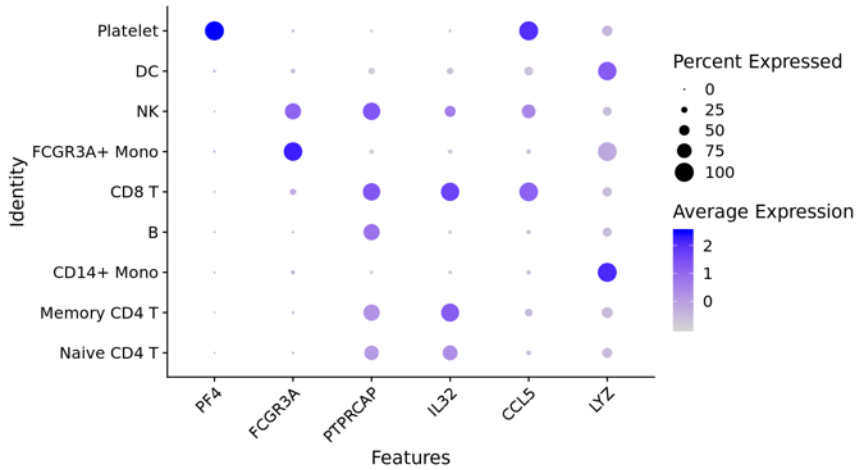
Ridgeline plots visualize finer details (bimodality) of expression distributions



Heatmaps visualize significant expression differences between clusters through contrasting colors

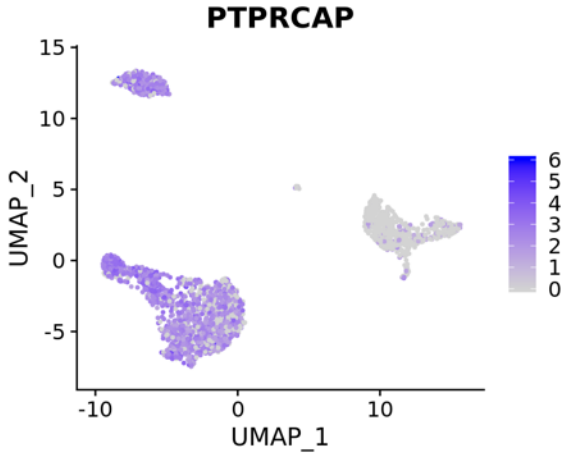
Subsampling of cells: choosing a subset of the whole cell population to avoid having to draw extremely large heatmaps

Visualization with dot plots and feature plots



Dot plots show the average expression levels

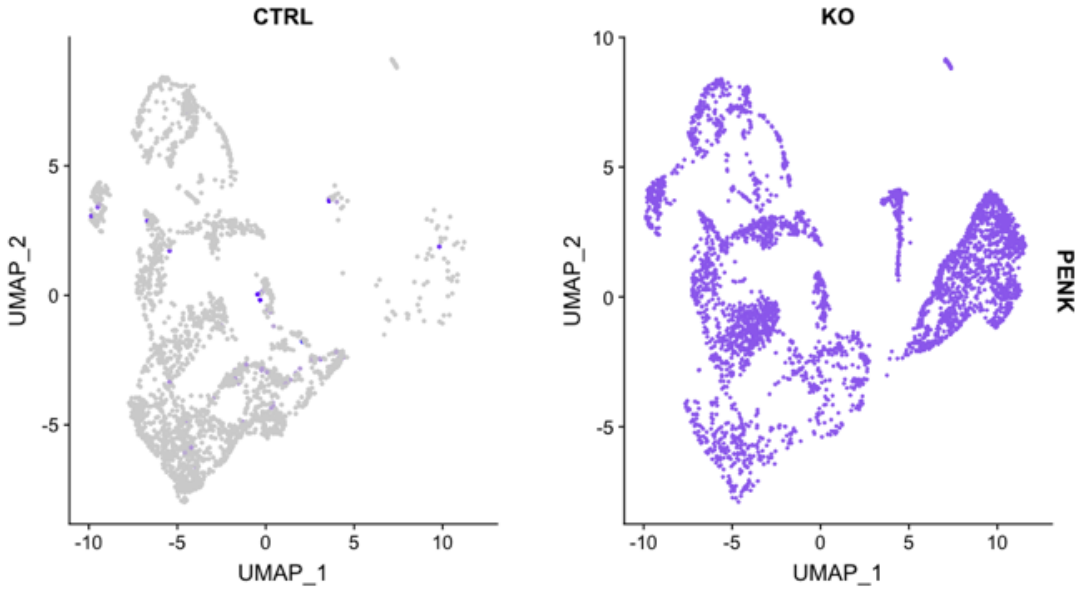
- Visualizing scaled vs normalized expression levels
- Scaled data typically magnifies the differences between clusters
- Size of dots proportional to the percentage of cells that express the gene



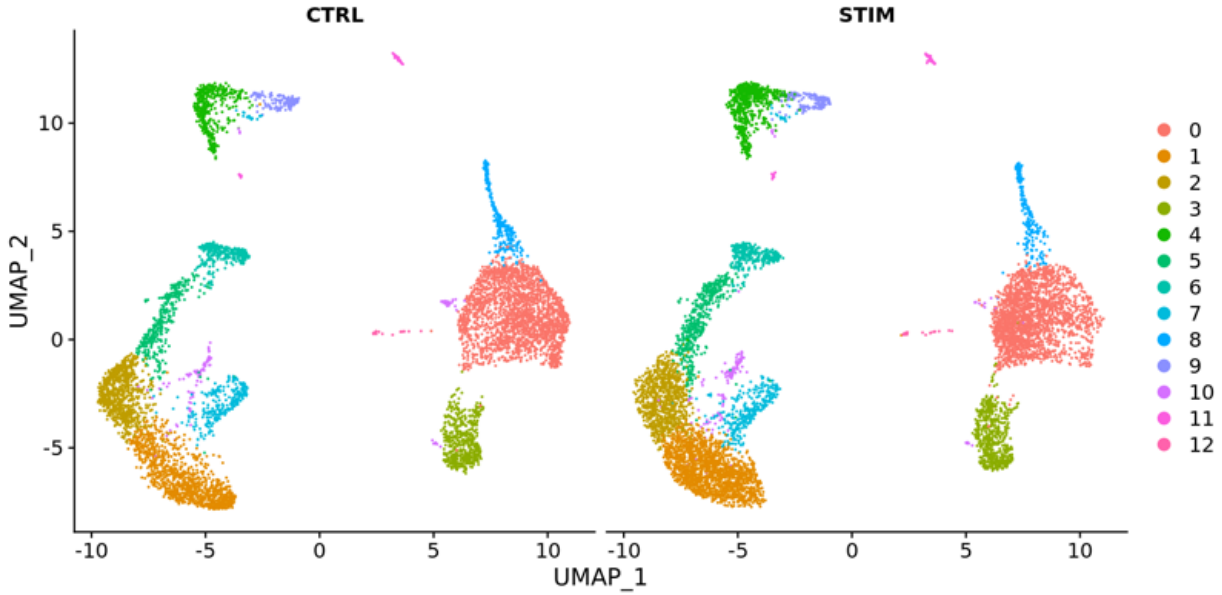
Feature plots

- Visualizing how the expression of the gene is distributed among cells in the reduced space
- Can choose cells to plot, reduction method to use (PCA, t-SNE, or UMAP), or a quantile expression cut-off.

Visualization with dot plots and feature plots



Feature plots split by different experimental conditions



Feature plots split by different experimental conditions & clusters

More ways to visualize cluster-specific expression patterns

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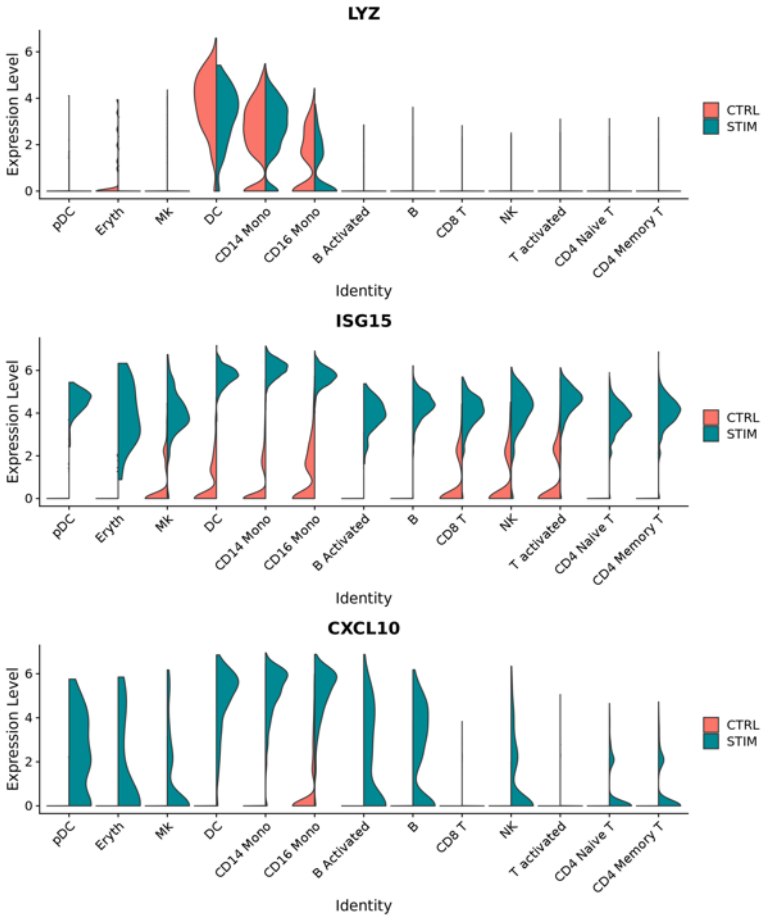
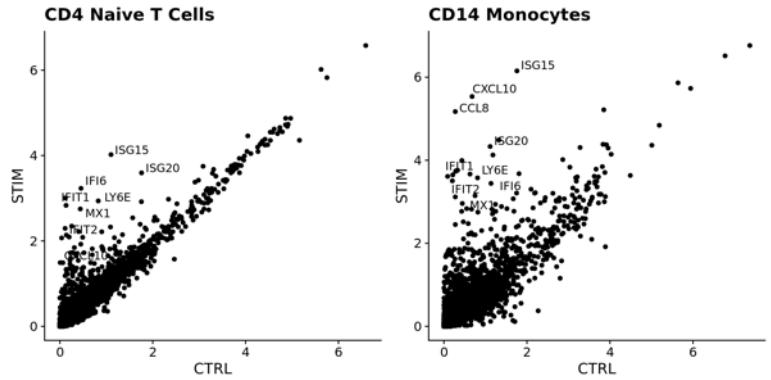
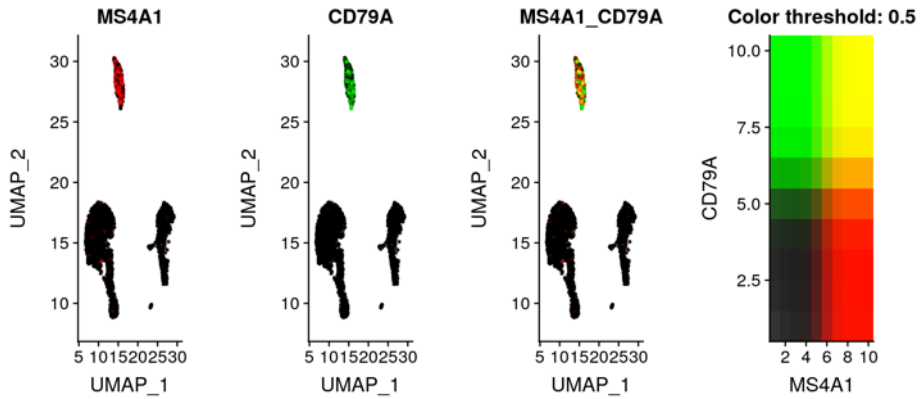


Violin plots split by clusters and experimental conditions



Changes in average expression in different clusters or varying experimental conditions



Visualizing co-expression among gene pairs

Cluster–specific co-expression of genes can provide insights regarding activation or inhibition of pathways in cell subpopulations

Visualization of differential expression & enrichment results

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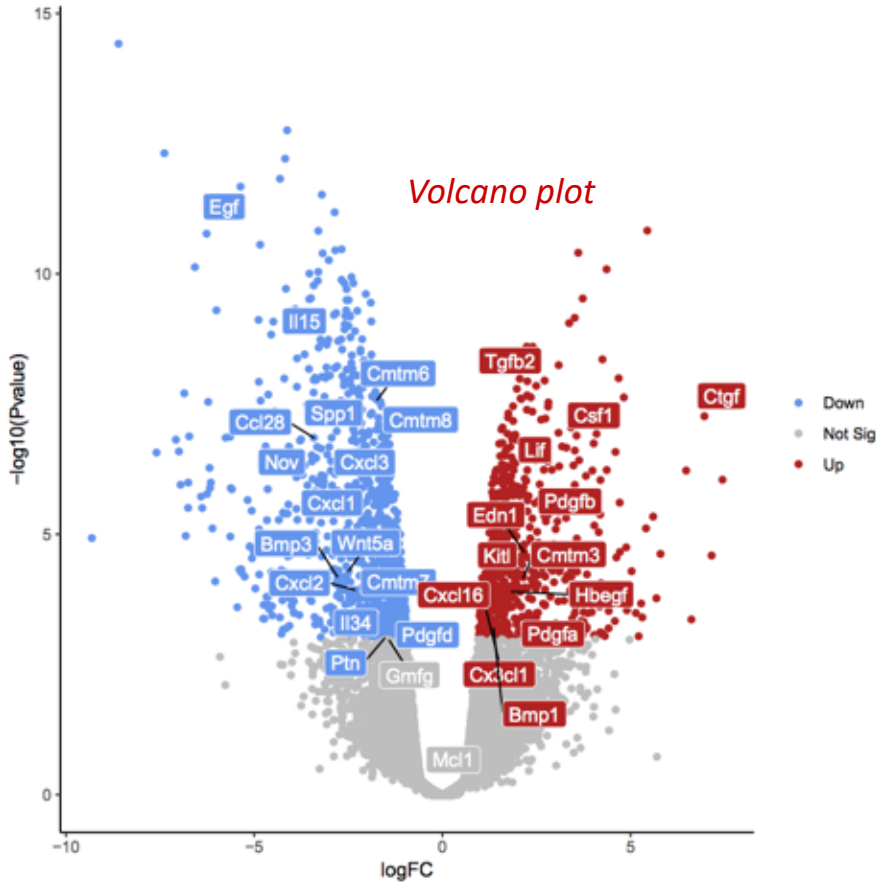
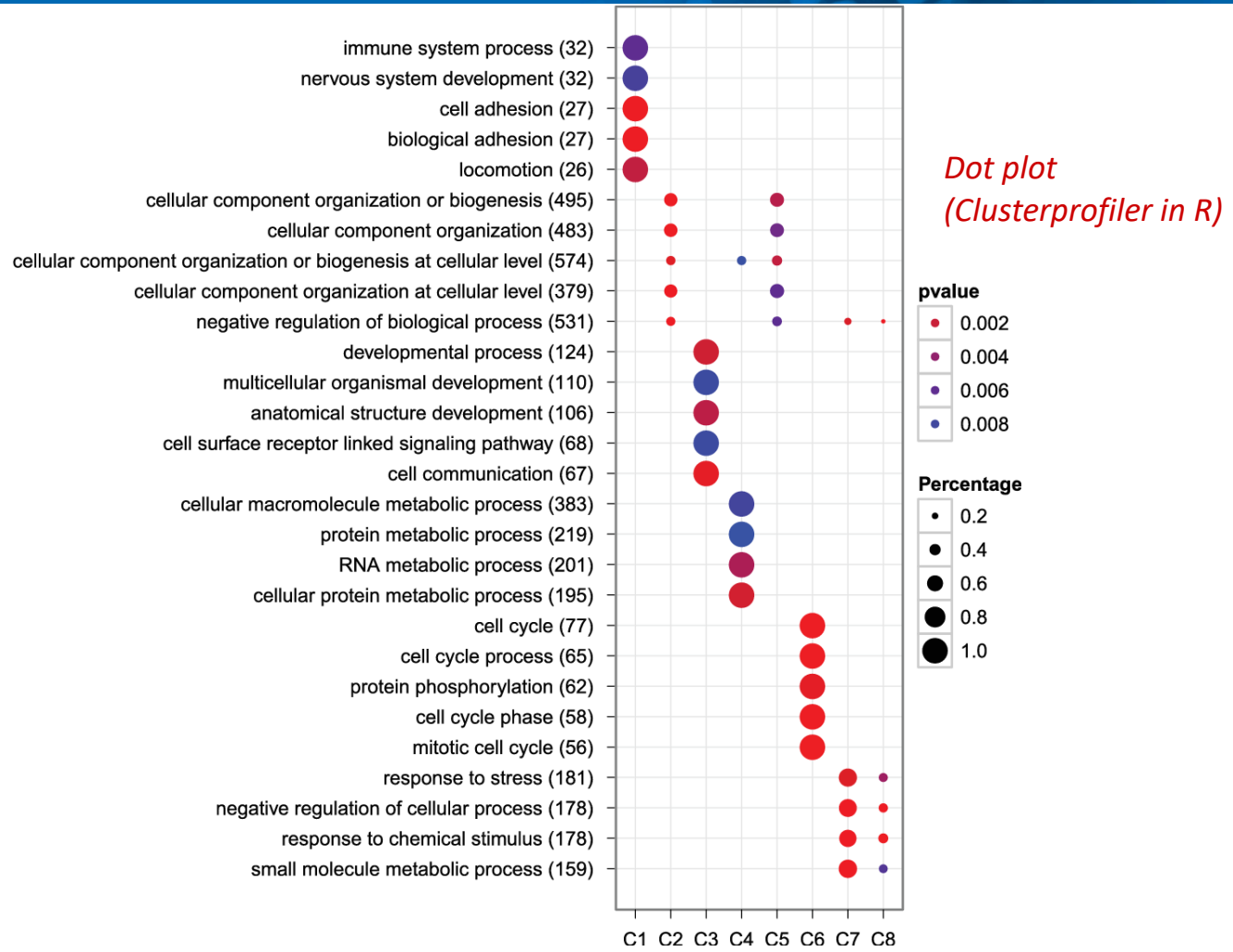


log2FC vs. significance (-log10(p-value))

https://galaxyproject.github.io/training-material/topics/transcriptomics/tutorials/rna-seq-viz-with-volcanoplot/tutorial.html

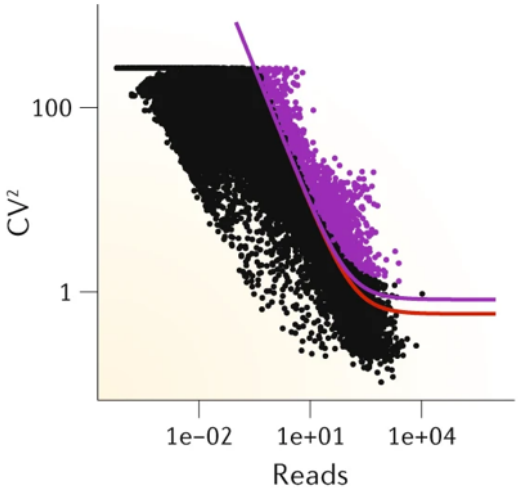


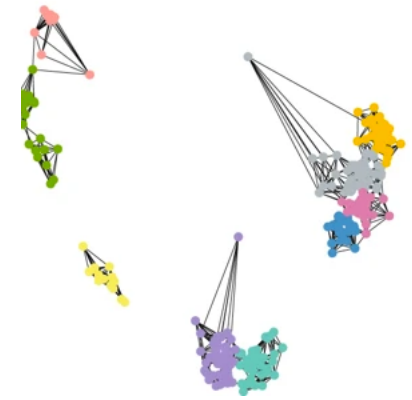
Cluster-specific enriched pathways or GO terms

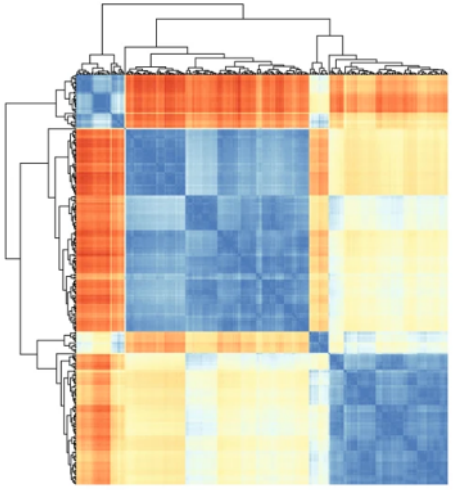
(circle size ~ fraction of cluster-specific markers in the enriched pathway)

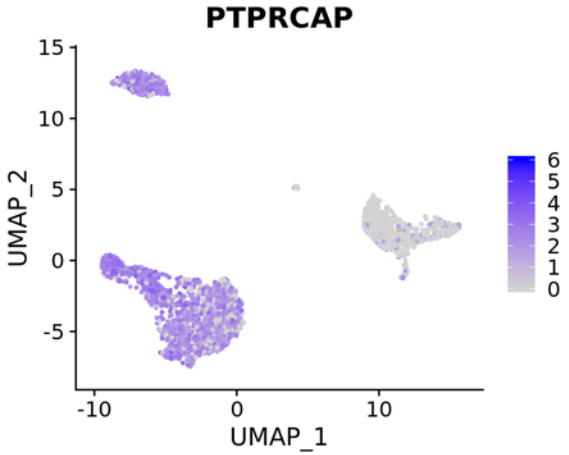
Wrapping-up

- Feature (gene) selection
  - Identifying genes with high expression variance
- Dimensionality reduction (PCA, UMAP, and t-SNE)
- Clustering
  - K-nearest neighbors, community detection, and modularity optimization
- Marker gene identification
  - Differential expression and classifier based approaches
- Visualization
  - Violin plots, dot plots, heatmaps, volcano plots, and scatter plots with the t-SNE/UMAP coordinates of cells









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