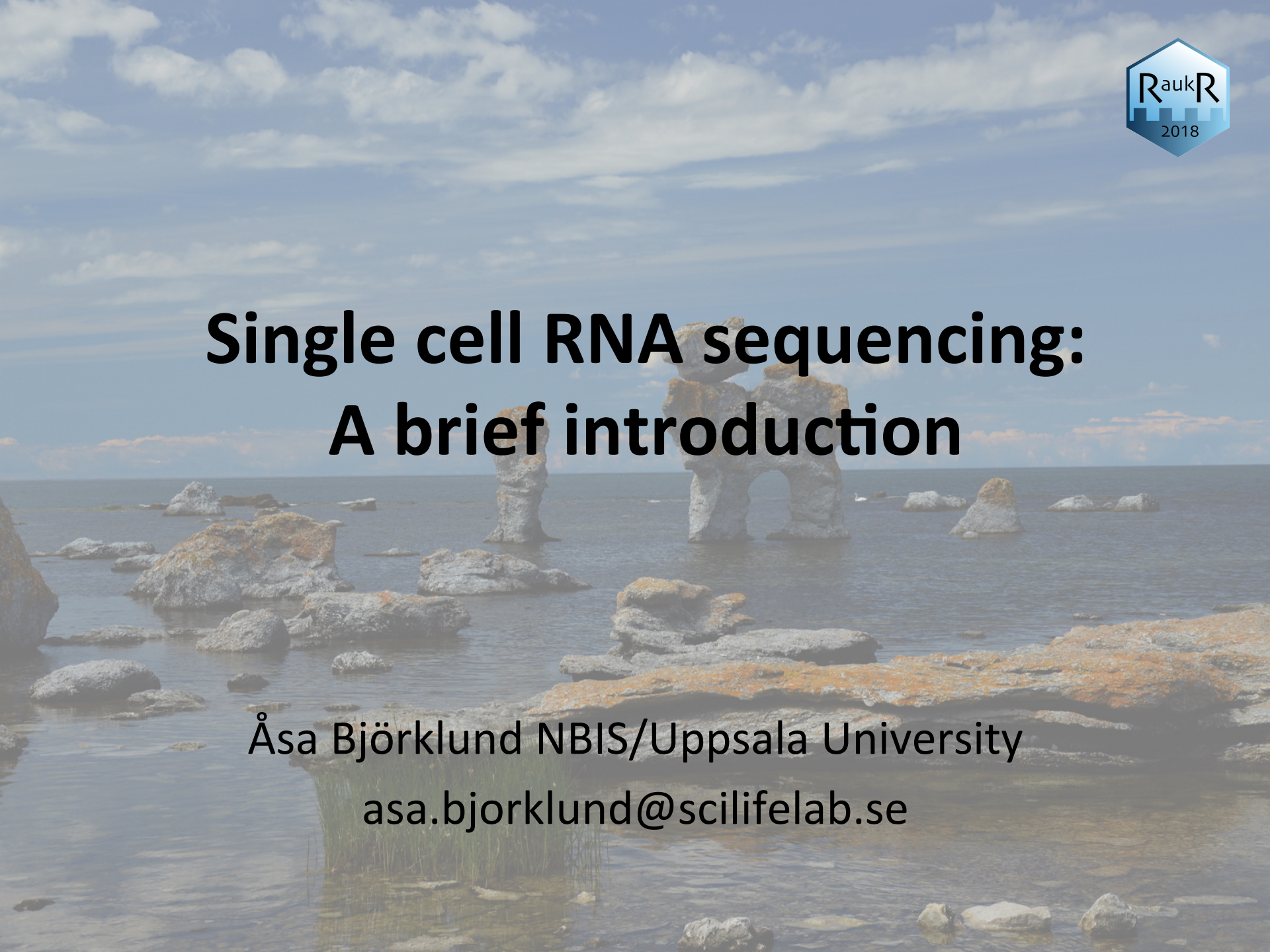


Single cell RNA sequencing: A brief introduction

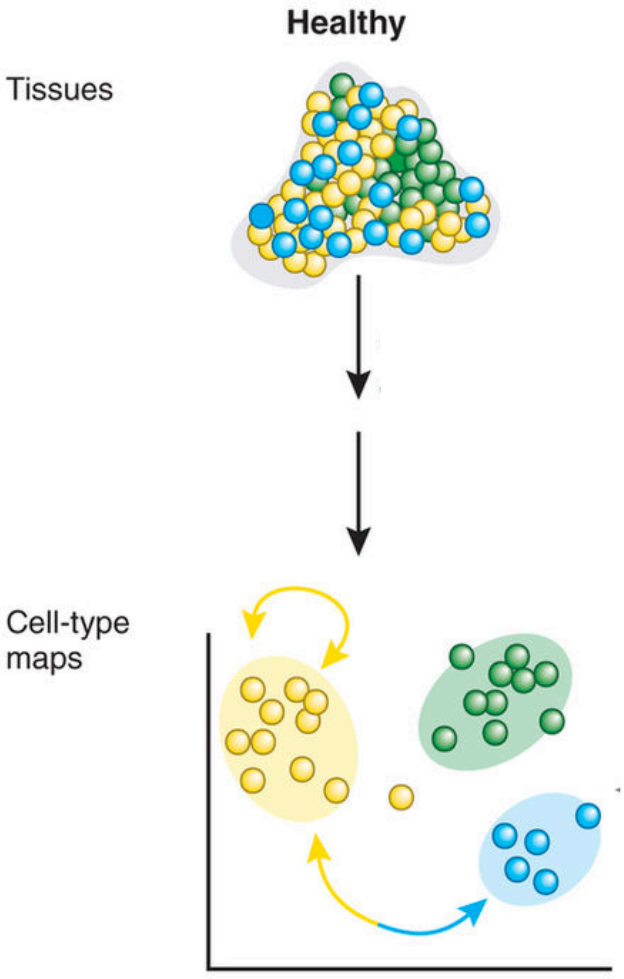
Åsa Björklund NBIS/Uppsala University

asa.bjorklund@scilifelab.se

Outline

- Overview of scRNA-seq:
  - Why scRNAseq?
  - How is it done?
  - Transcriptional bursting
  - Spike-in RNAs
- Data analysis:
  - Overview of main steps
  - Examples of tools
- Recent developments

Why single cell?



Between cell types

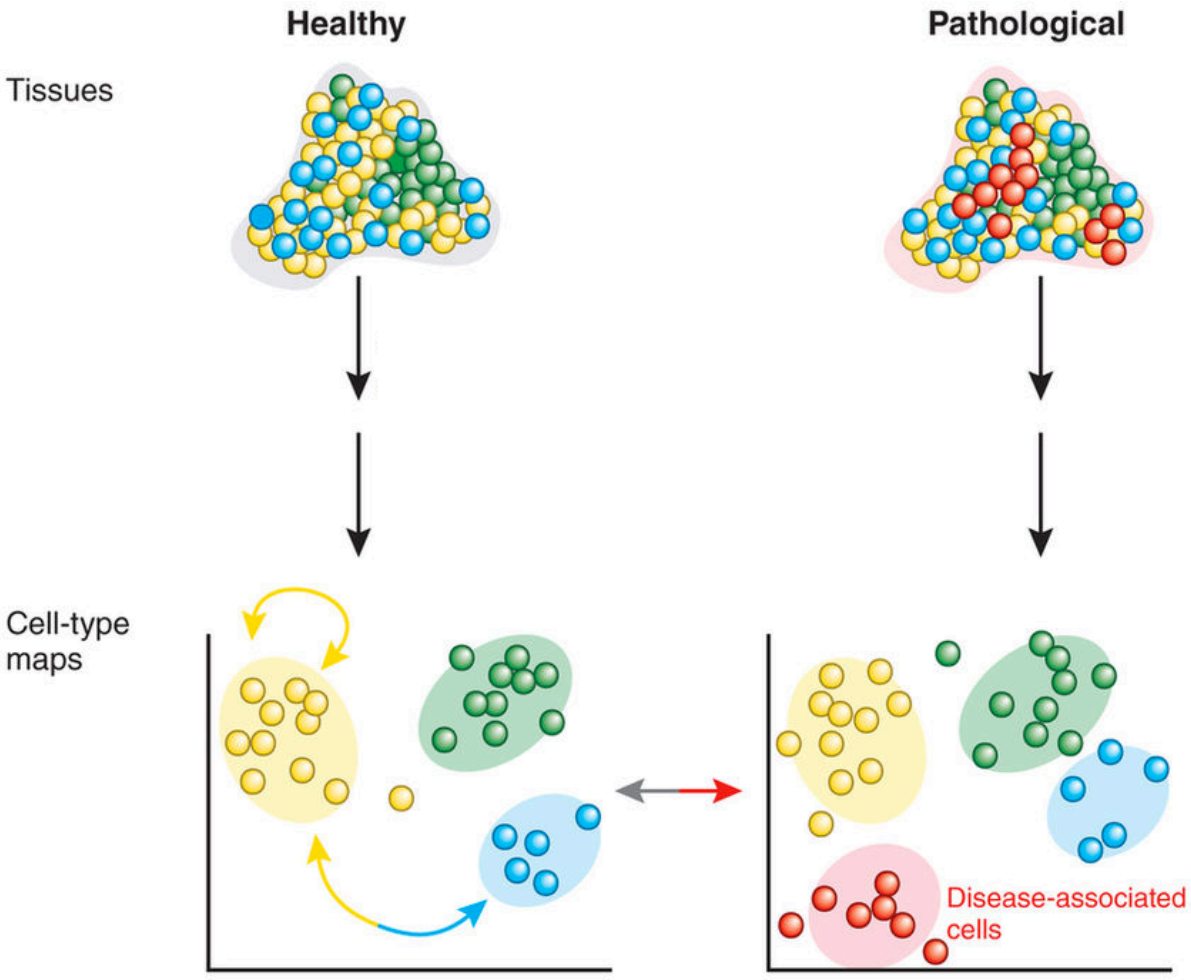
- Identify biomarkers
- Compare regulatory networks

Within cell type

- Transcriptional mechanisms
- Regulatory networks
- Allelic expression patterns
- Cell cycle progression

(Sandberg, *Nature Methods* 2014)

Why single cell?



Between tissues

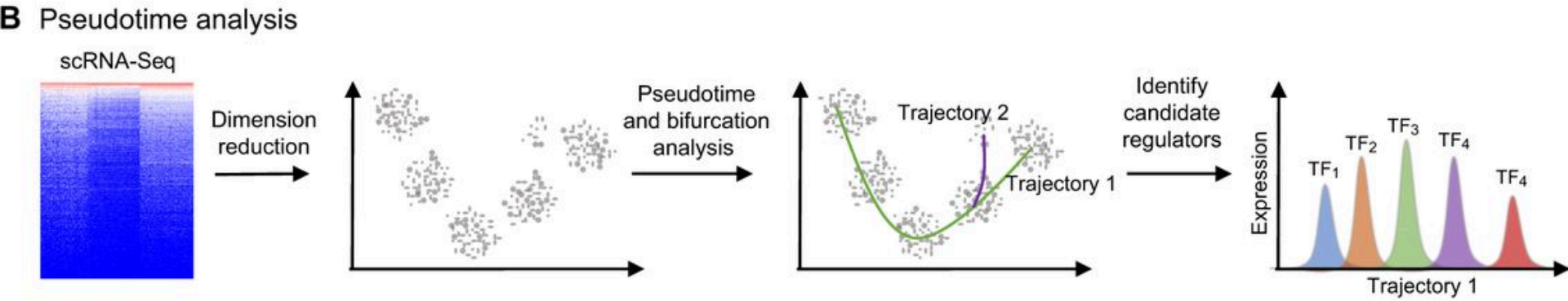
- Cell-type compositions
- Altered transcription in matched celltypes

(Sandberg, *Nature Methods* 2014)

Why single cell?

Developmental progression

- Transitions during differentiation
- Lineage decisions



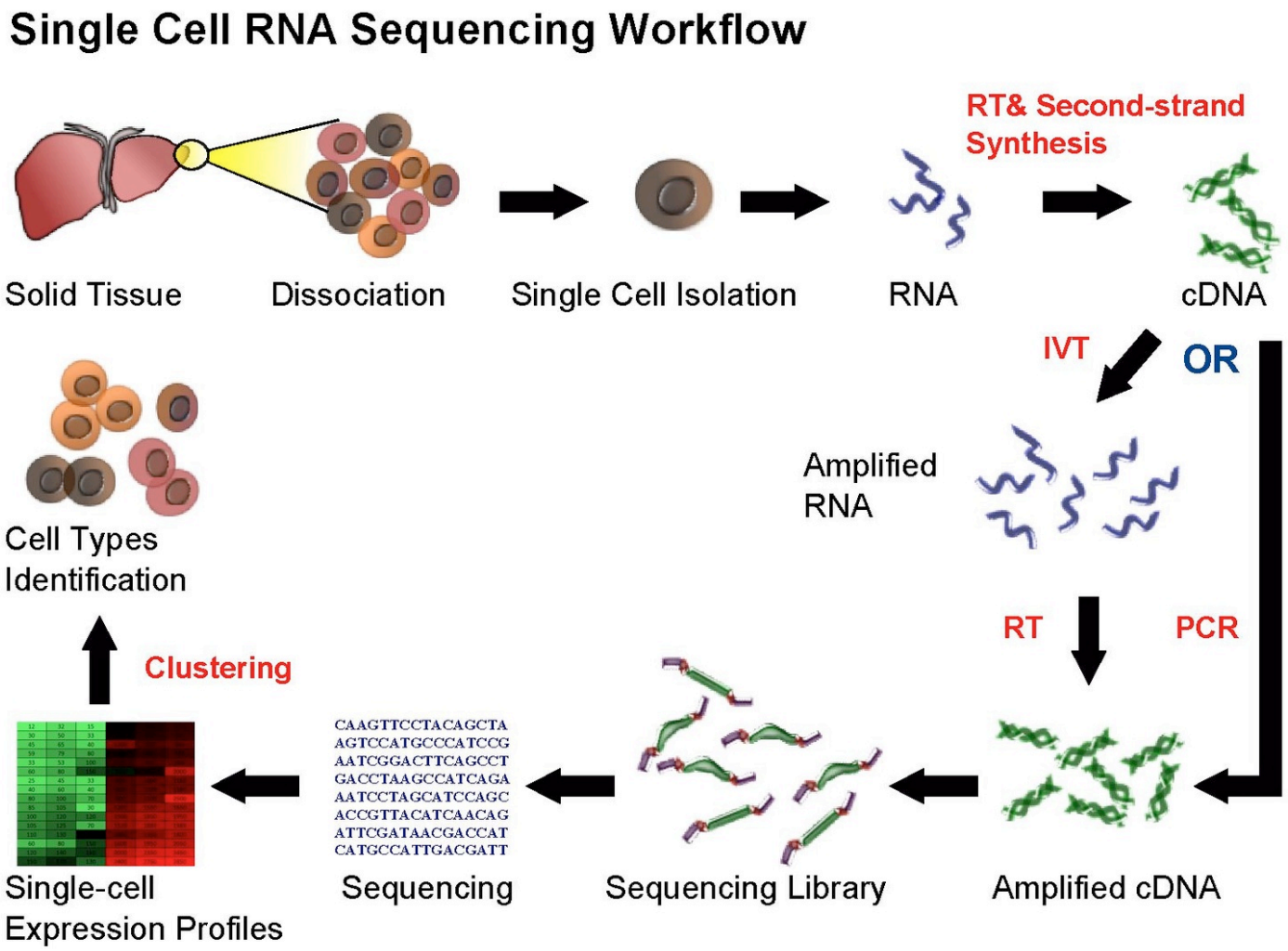
(Kuman *et al. Development*, 2017)

Why single cell?

Main applications:

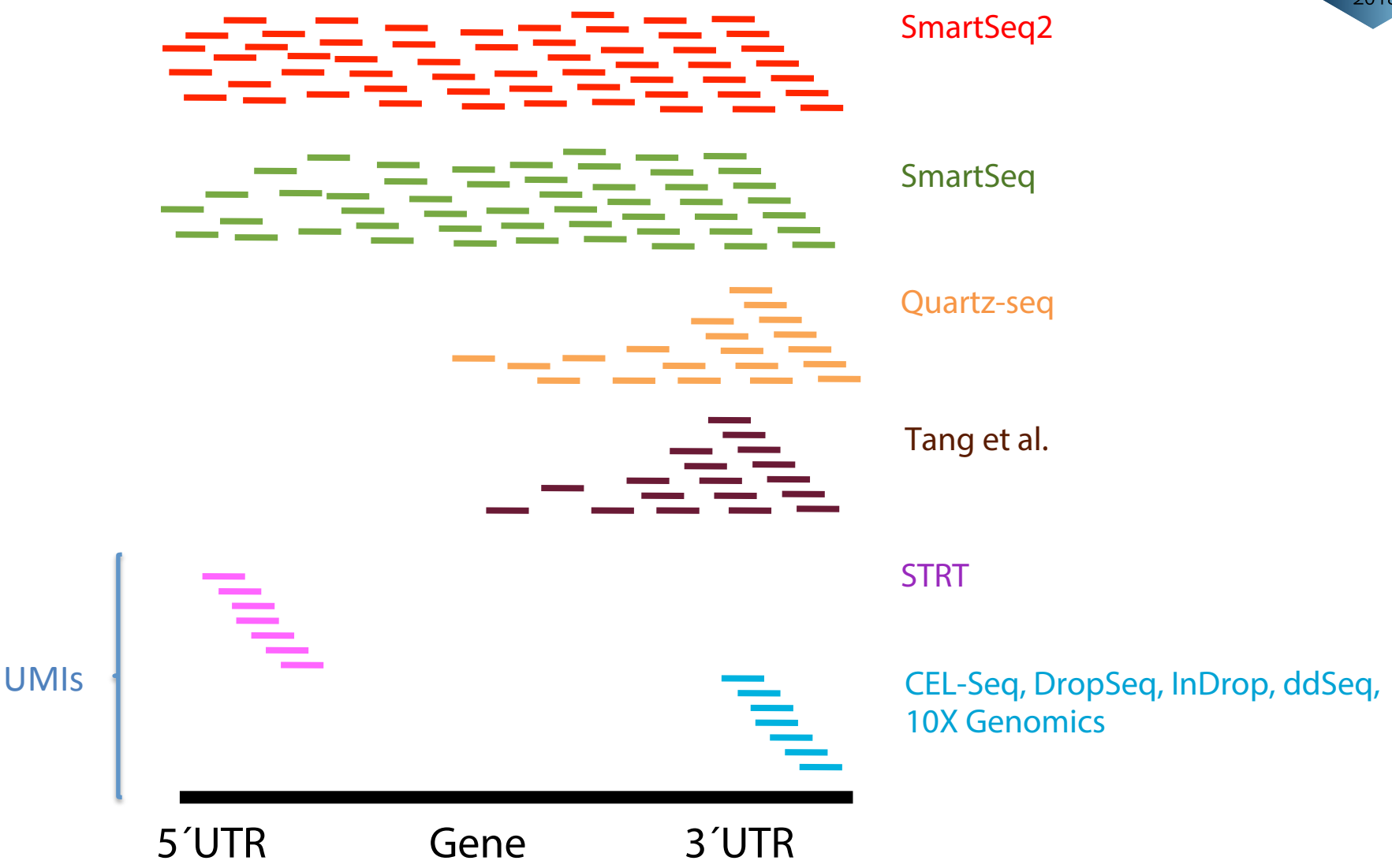
- Understanding heterogeneous tissues - Atlas
  - Identifying cell types in a tissue
- Changes in cellular composition and cellular states.
  - Upon a treatment
  - With time
  - Between healthy and disease
- Dissection of temporal changes − Trajectories
  - Understand the development of cell types

How is it done?

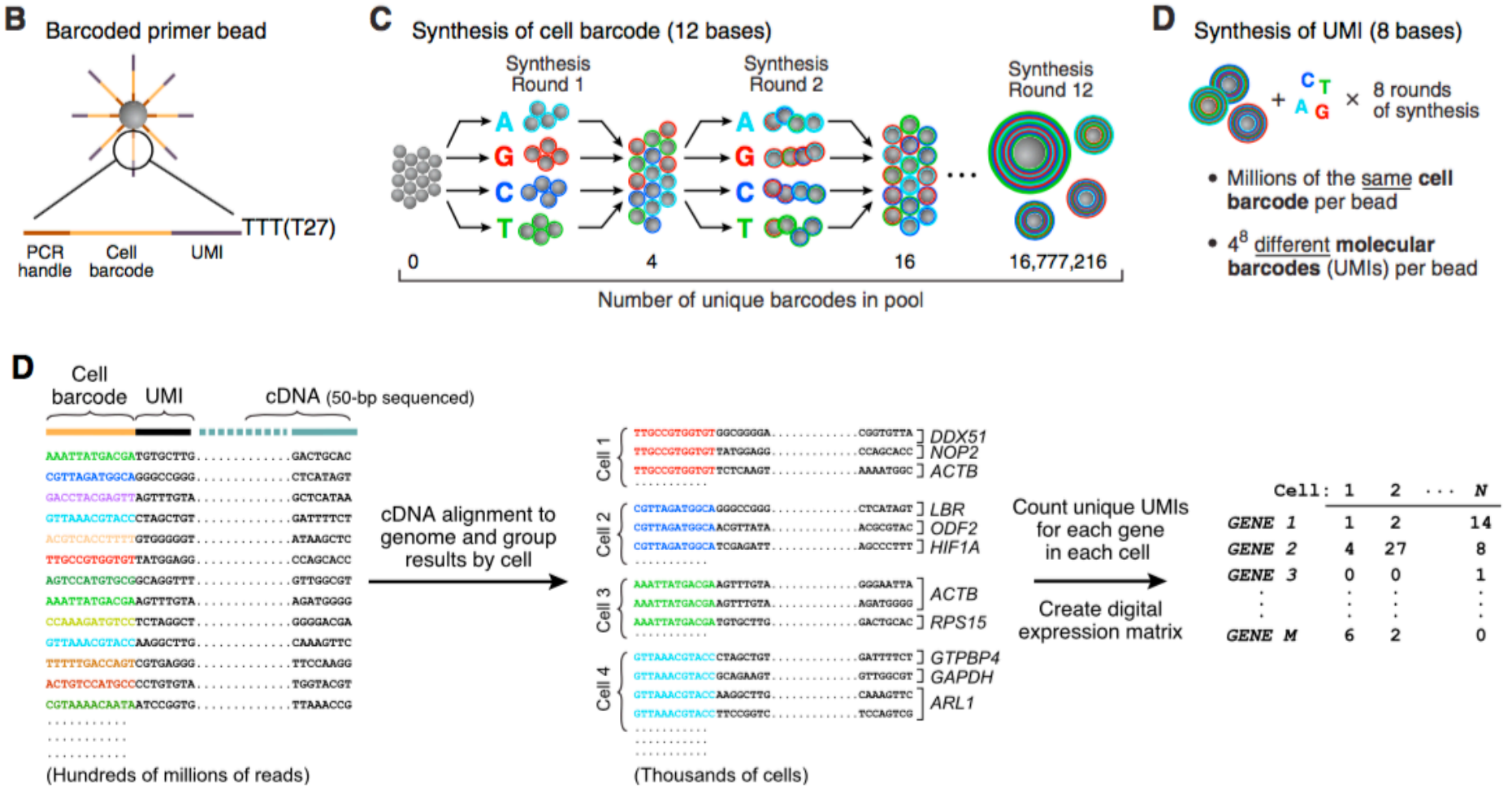


(Wikipedia)

scRNAseq methods

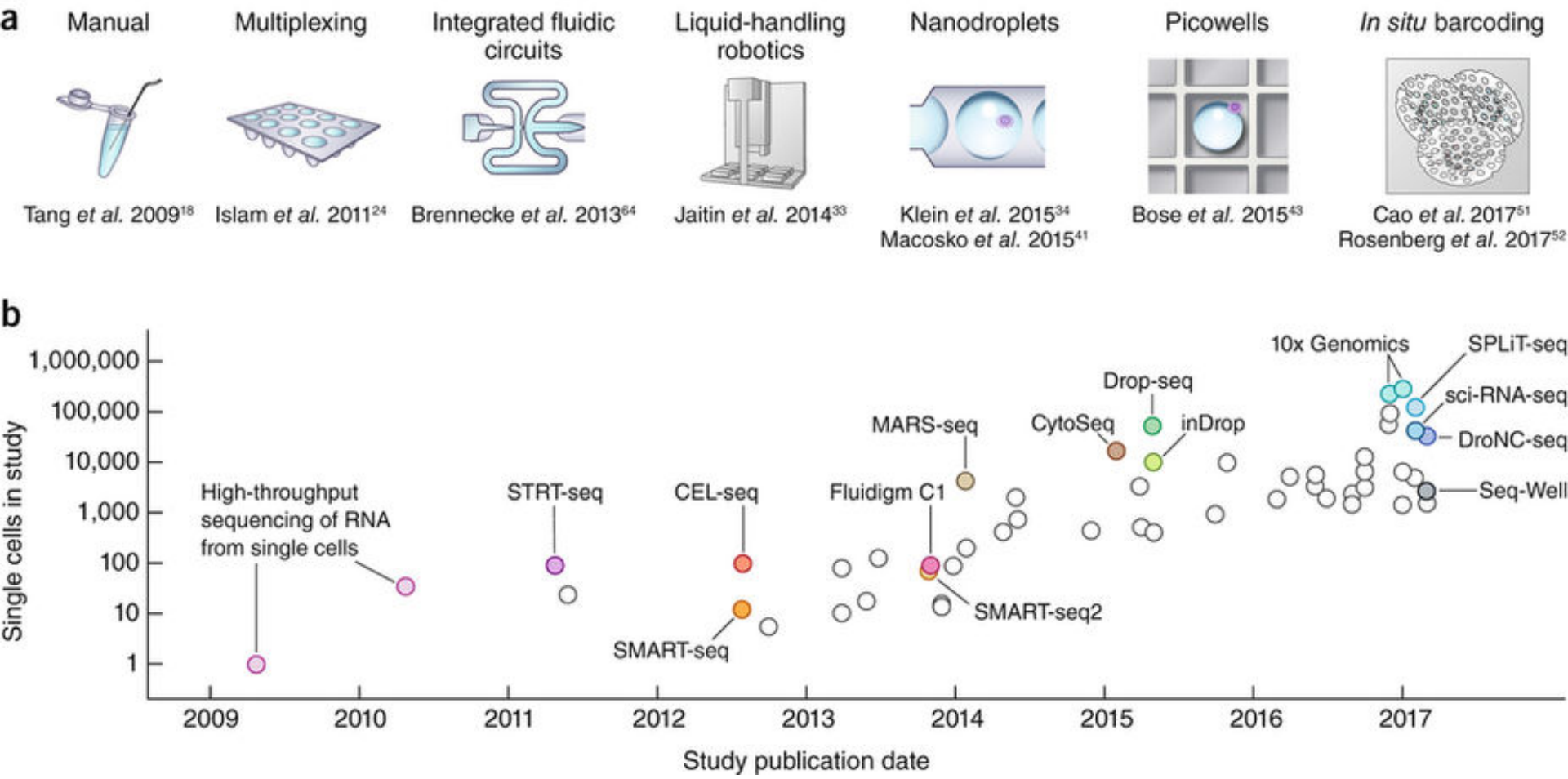


Unique molecular identifiers - UMIs



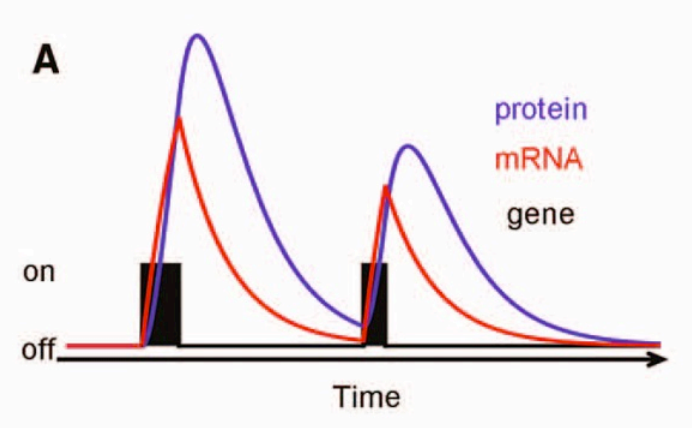
(http://mccarrolllab.com/dropseq/)

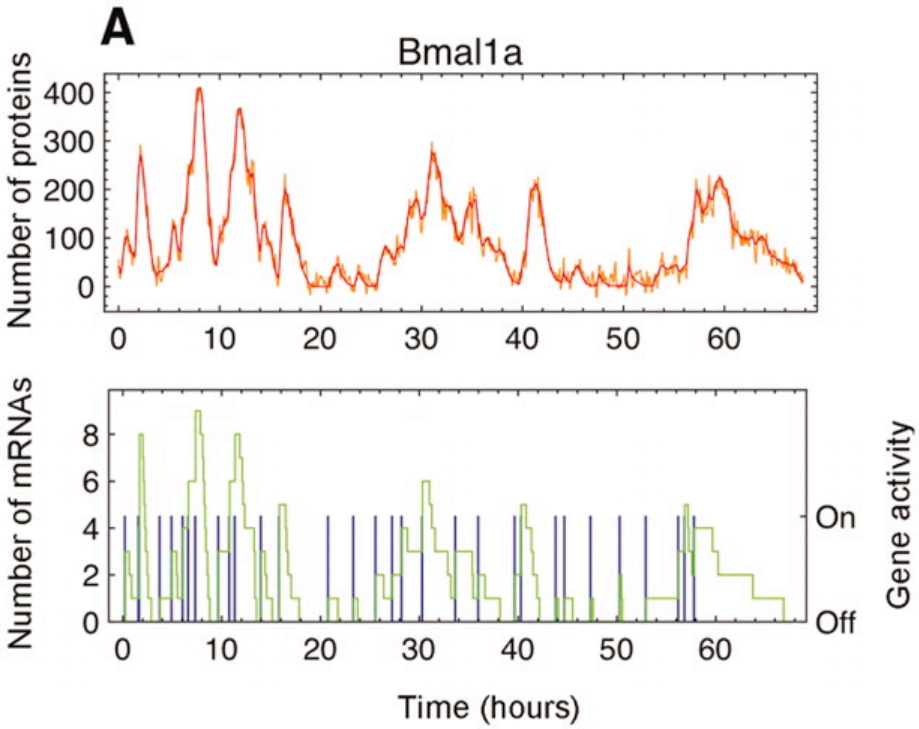
Method developments



(Svensson *et al. Nature Protocols* 2018)

Transcriptional bursting

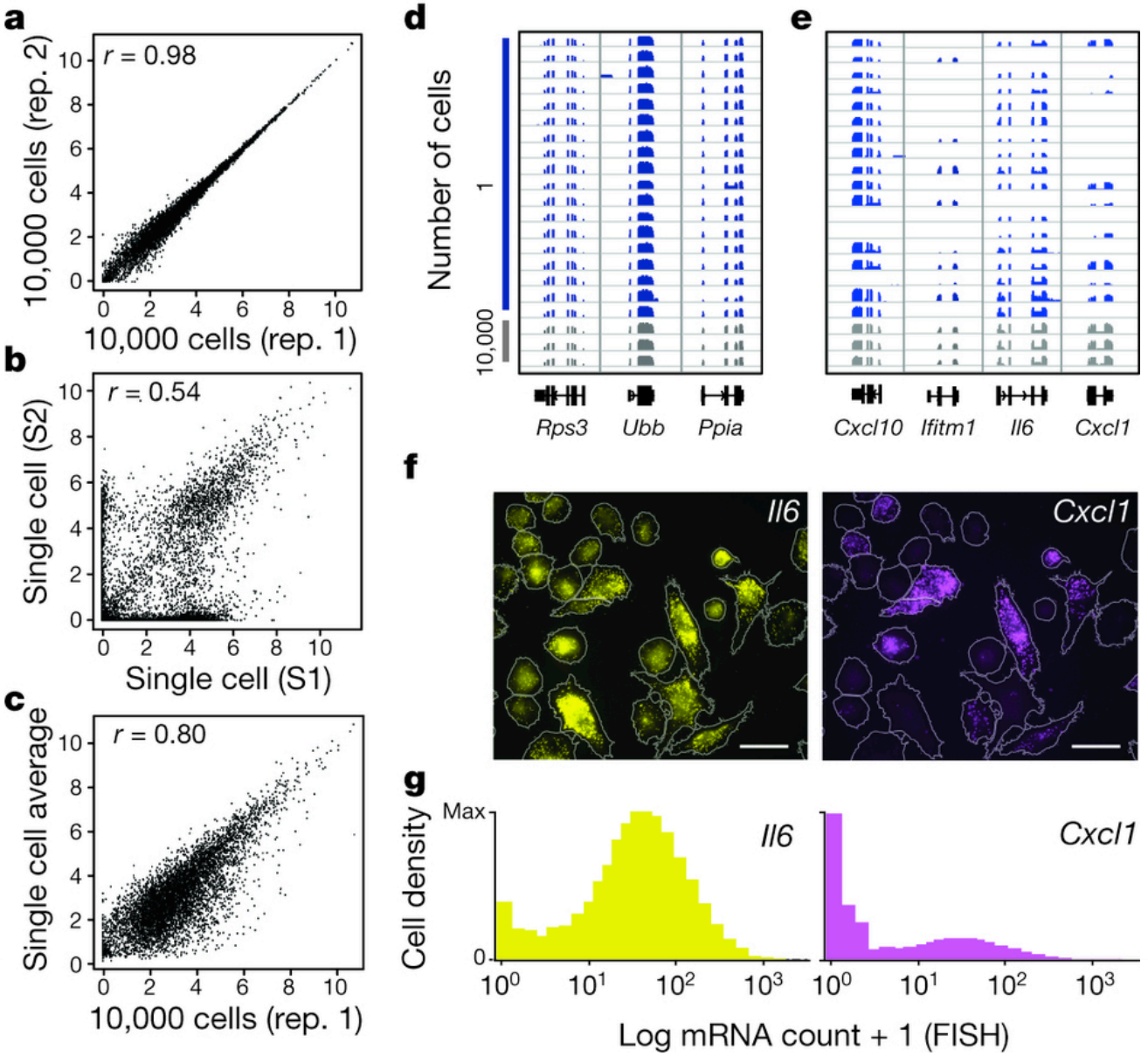




- Burst frequency and size is correlated with mRNA abundance but may differ per gene
- Many TFs have low expression and low burst frequency and will only be detected in a fraction of the cells

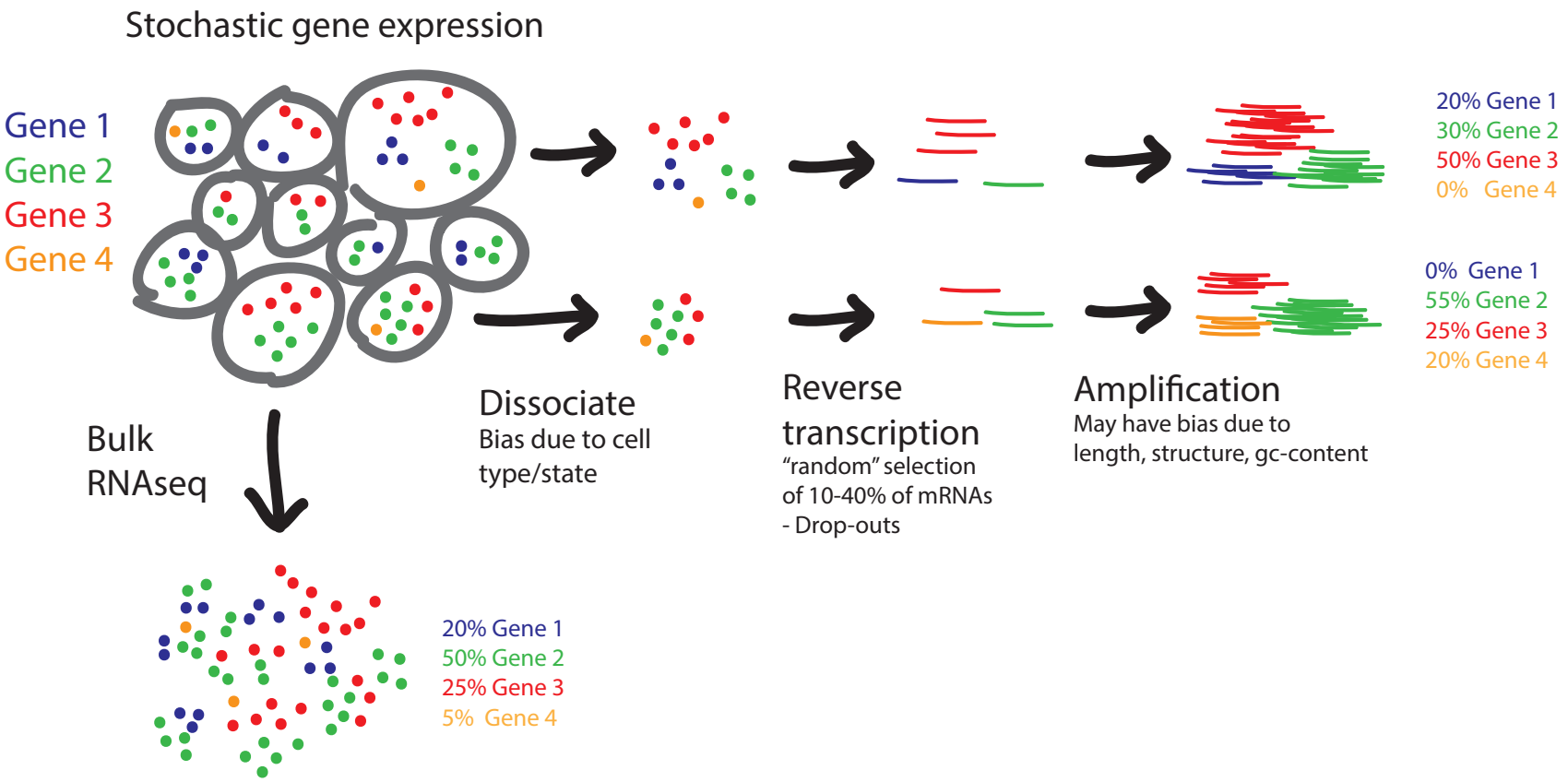
(Suter *et al. Science* 2011)

Example data - mouse bone-marrow-derived dendritic cells (BMDCs)

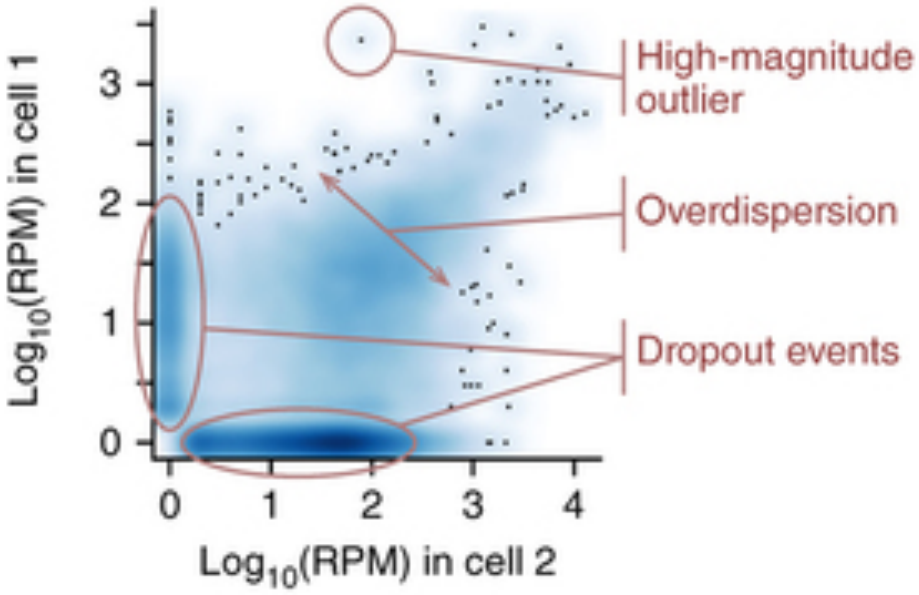


(Shalek *et al. Nature* 2014)

Bursting, drop-outs and amplification bias



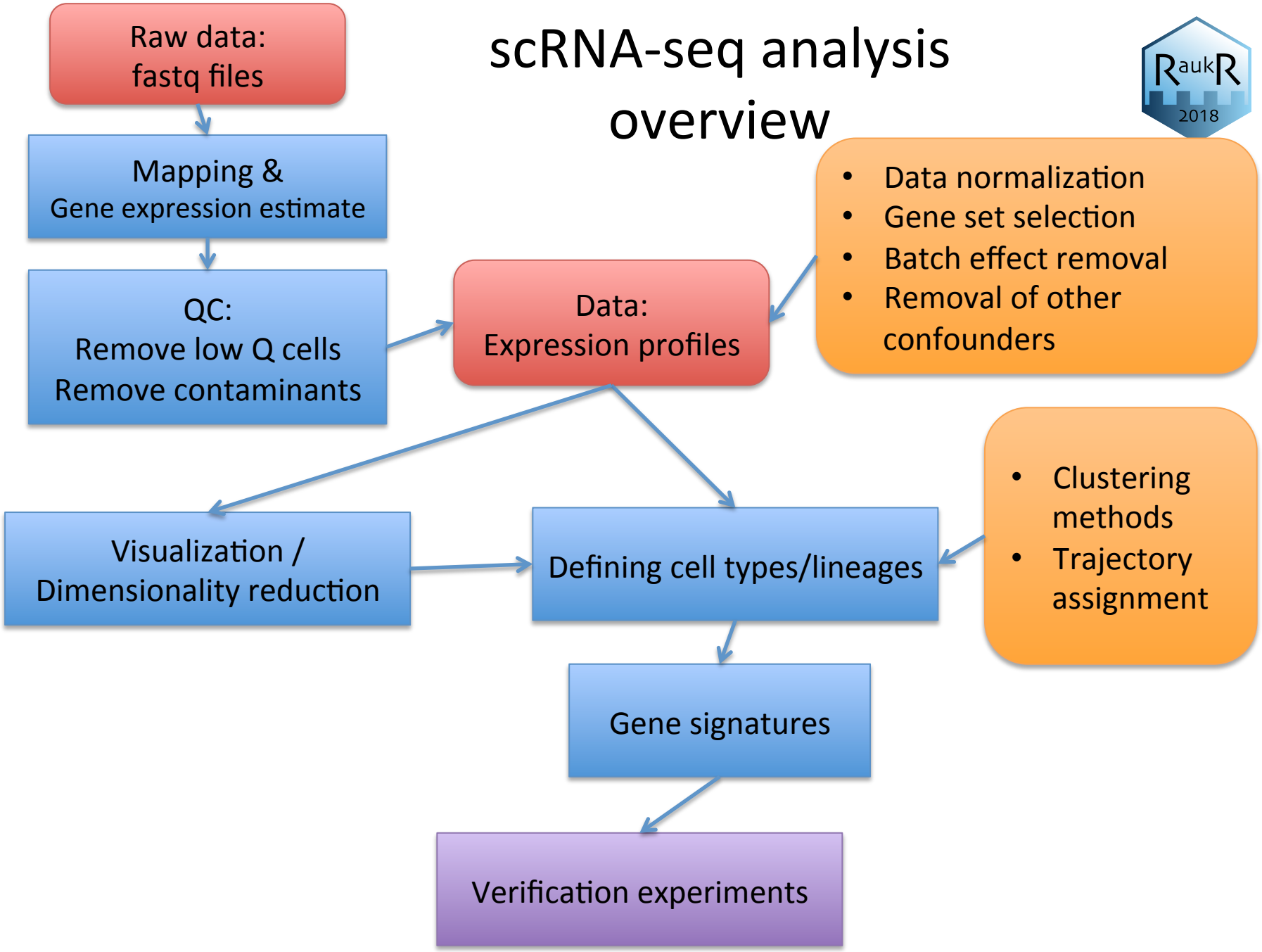
Problems compared to bulk RNA-seq



- Transcriptional bursting
- Drop-out rates
- Amplification bias
- Background noise
- Bias due to cell-cycle, cell size and other factors
- Often clear batch effects

- Zero-inflated data − different distribution compared to bulk RNAseq

(Karchenko *et al. Nature Methods* 2014)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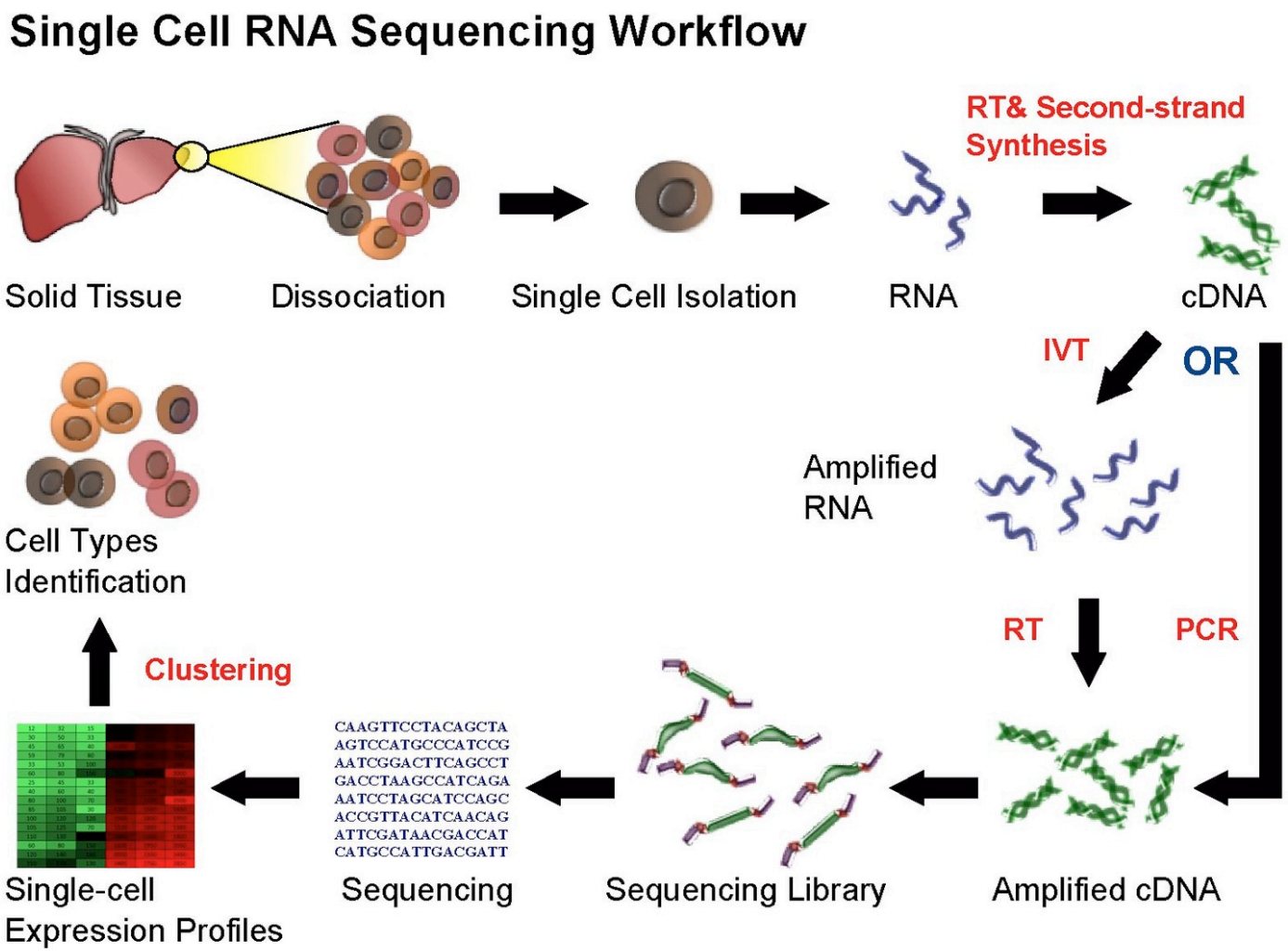
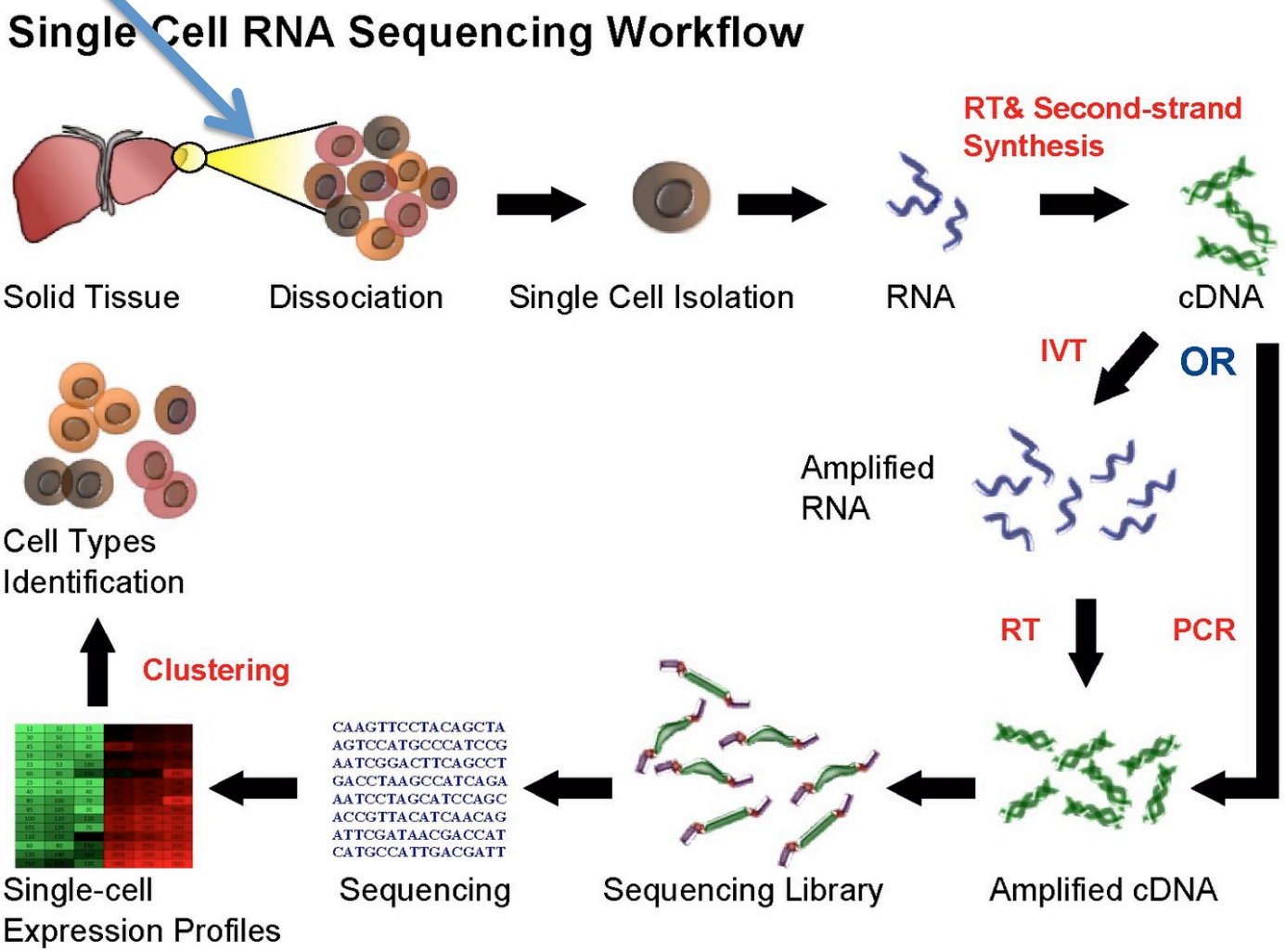
What can go wrong?



(Wikipedia)

Biased selection of cells.
Quality of cells

can go wrong?



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Biased selection of cells.
Quality of cells

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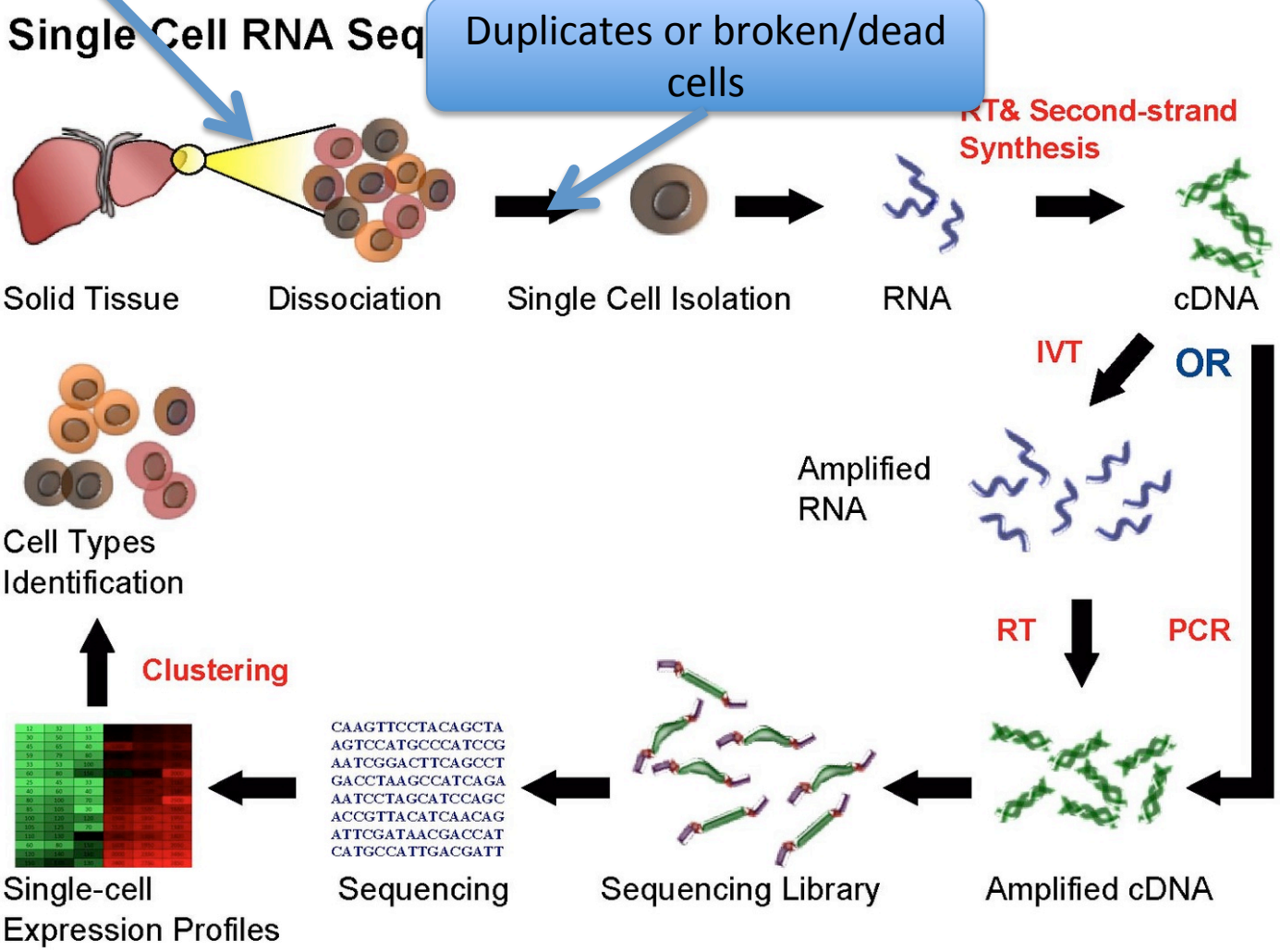
Duplicates or broken/dead cells



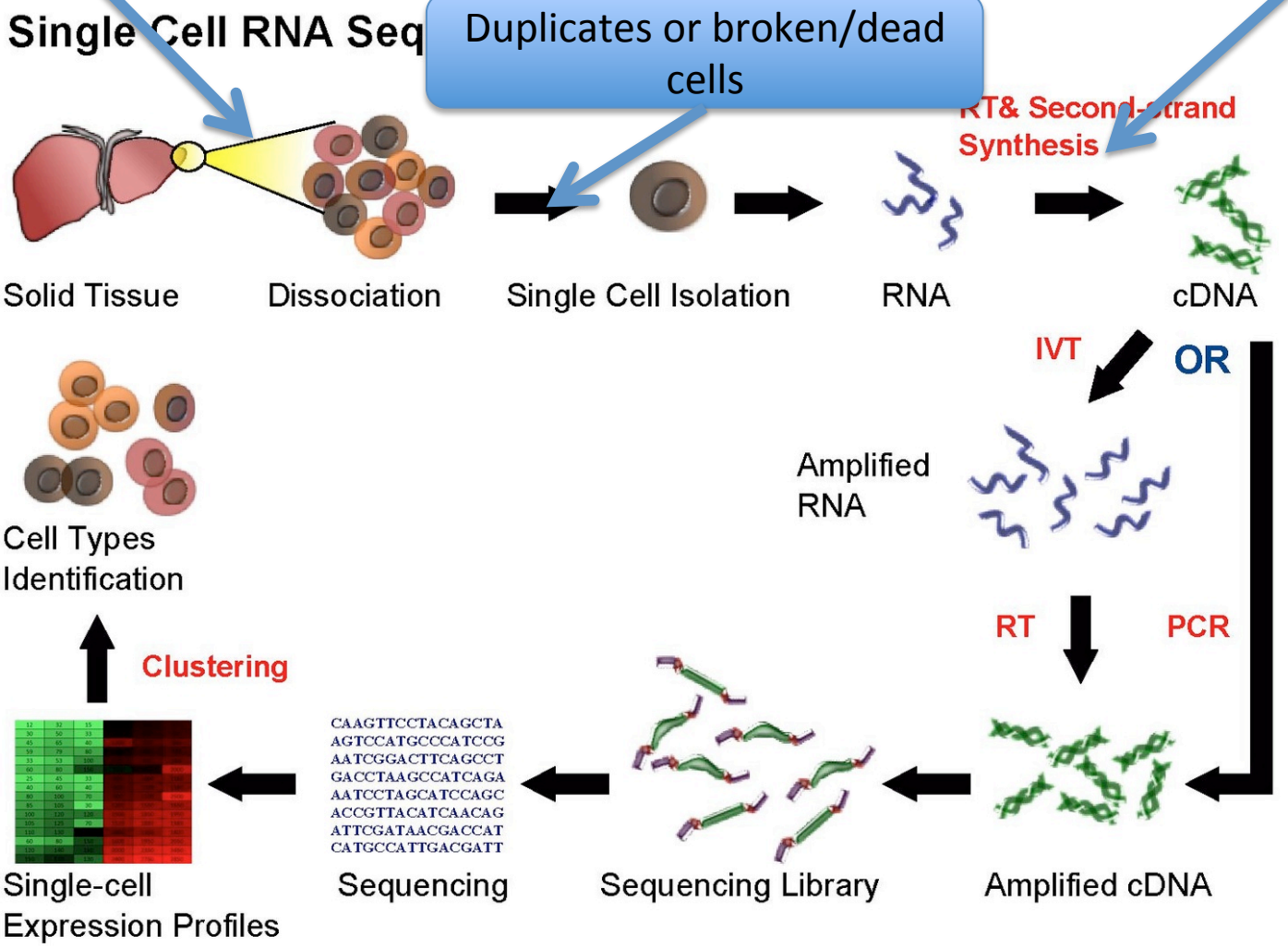
(Wikipedia)

can go wrong?

Biased selection of cells.
Quality of cells

Duplicates or broken/dead cells

Drop-outs
60-90%



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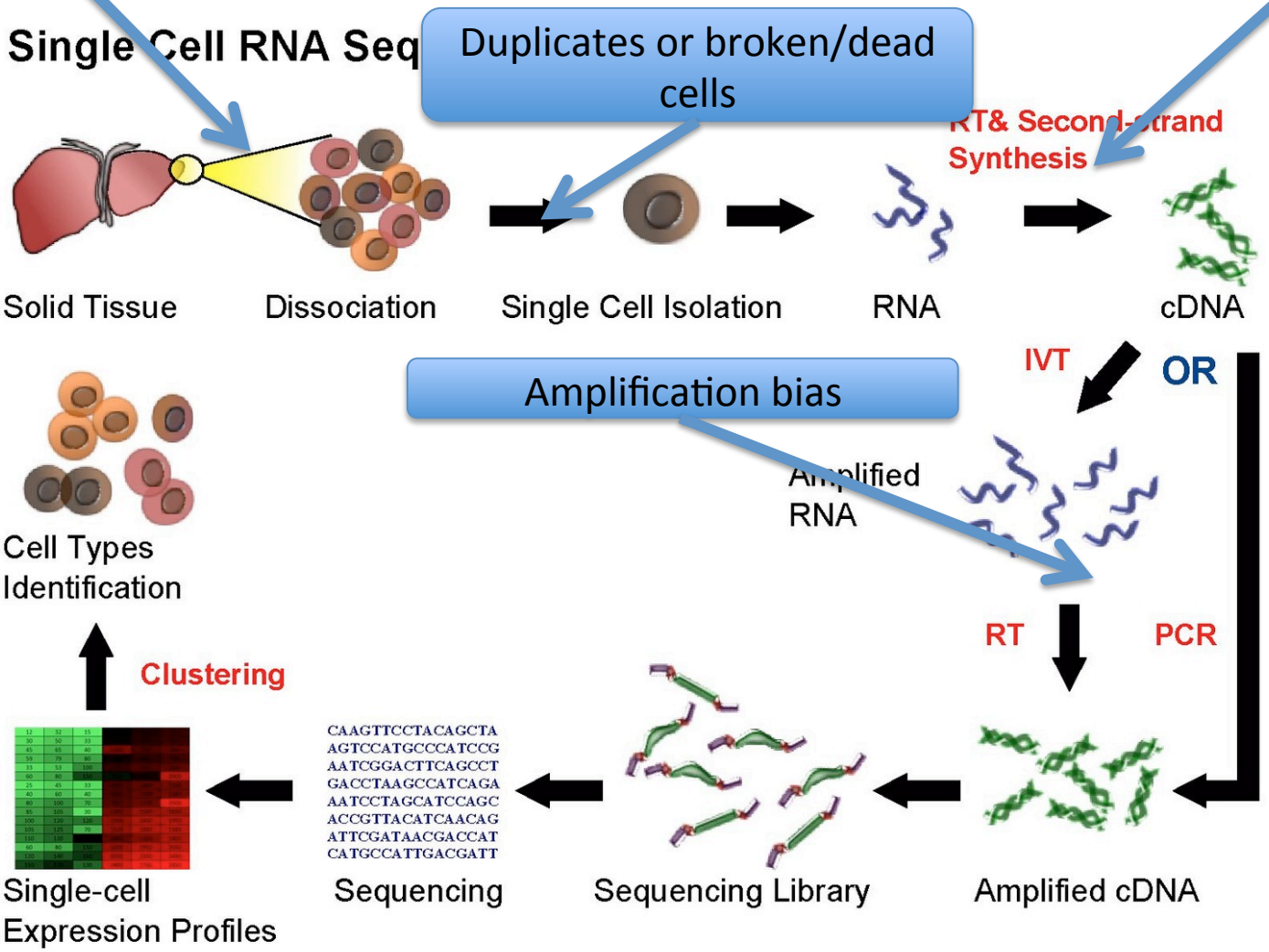

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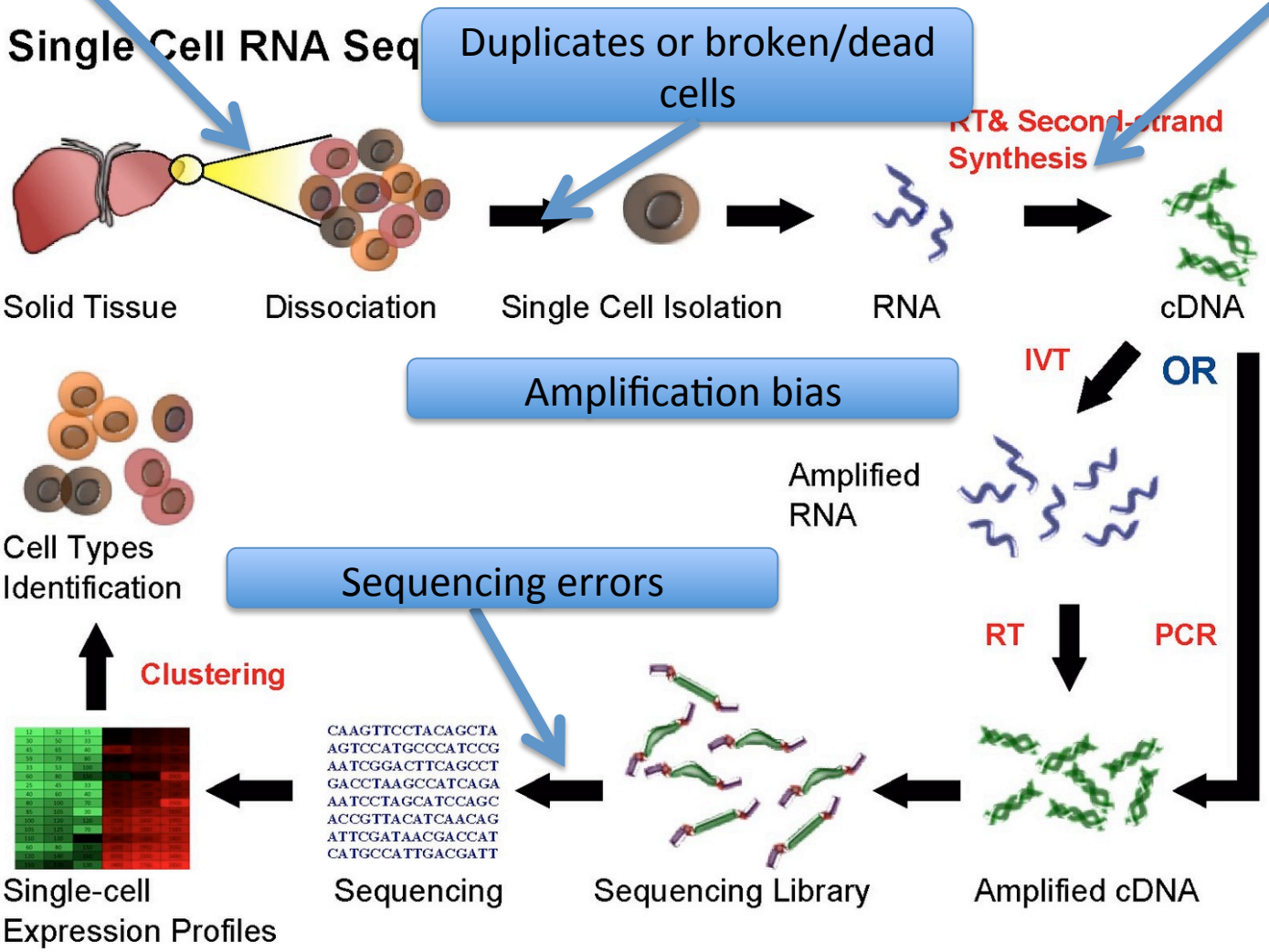
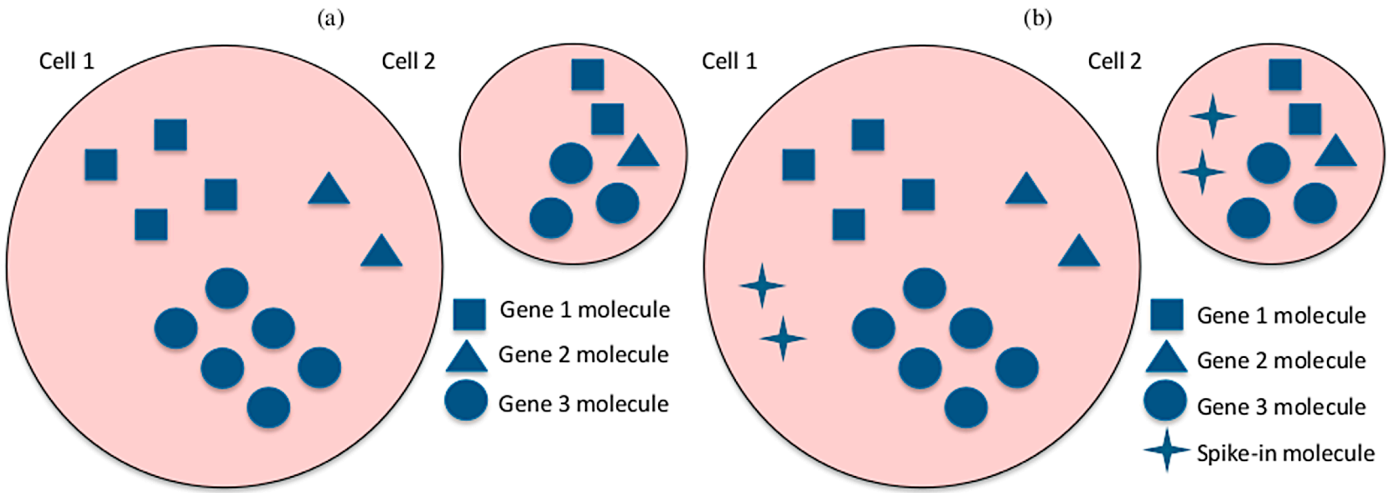


(Wikipedia)

Spike-in RNAs

- Addition of external controls
- ERCC spike-in most widely used, consists of 48 or 96 mRNAs at 17 different concentrations.
- Important to add equal amounts to each cell, preferably in the lysis buffer.



(Vallejos et al. *PLOS Comp Biol* 2015)

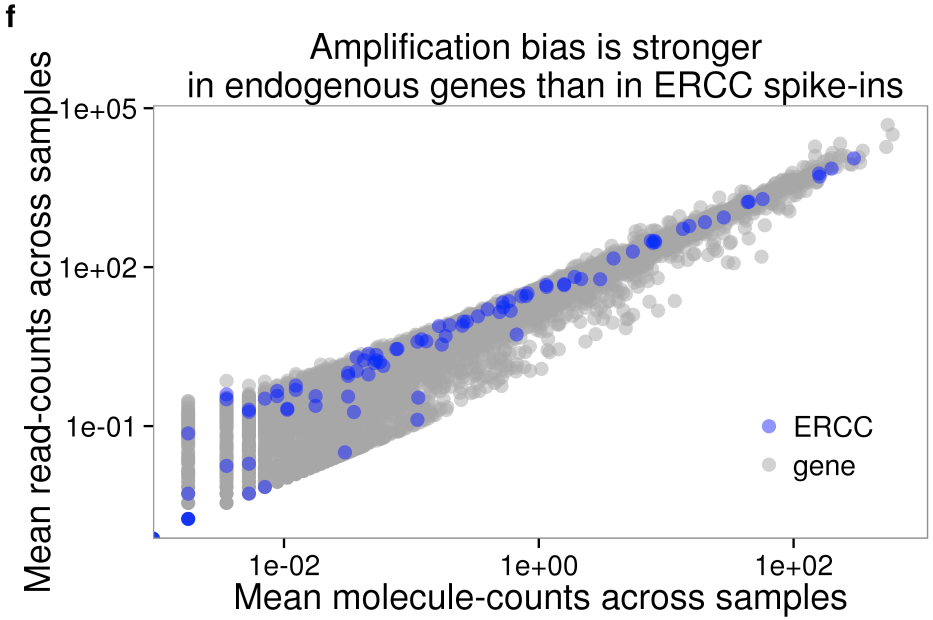
Spike-in RNAs

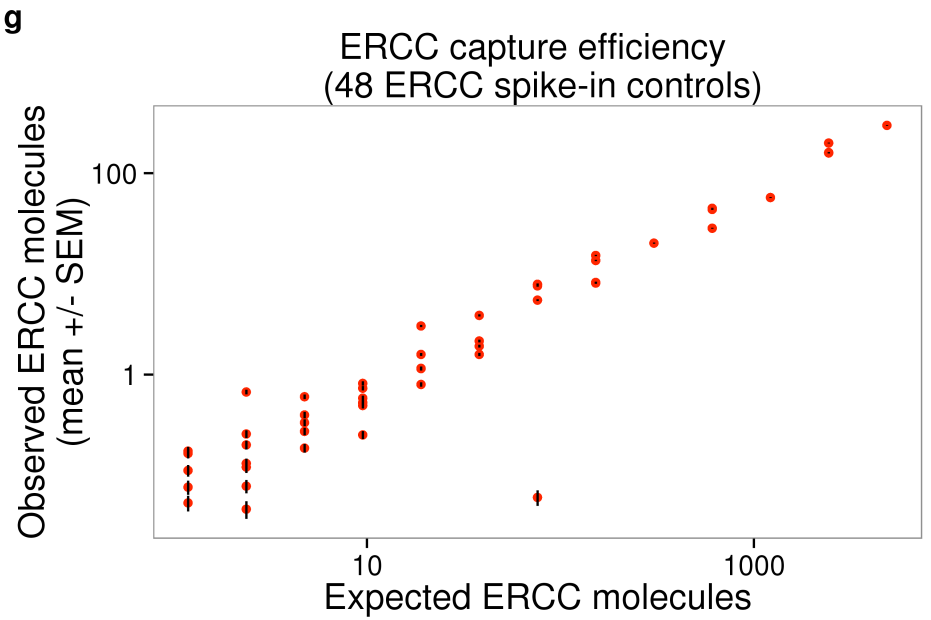
Spike-ins can be used to model:

- Technical noise
- Drop-out rates
- Starting amount of RNA in the cell
- Data normalization

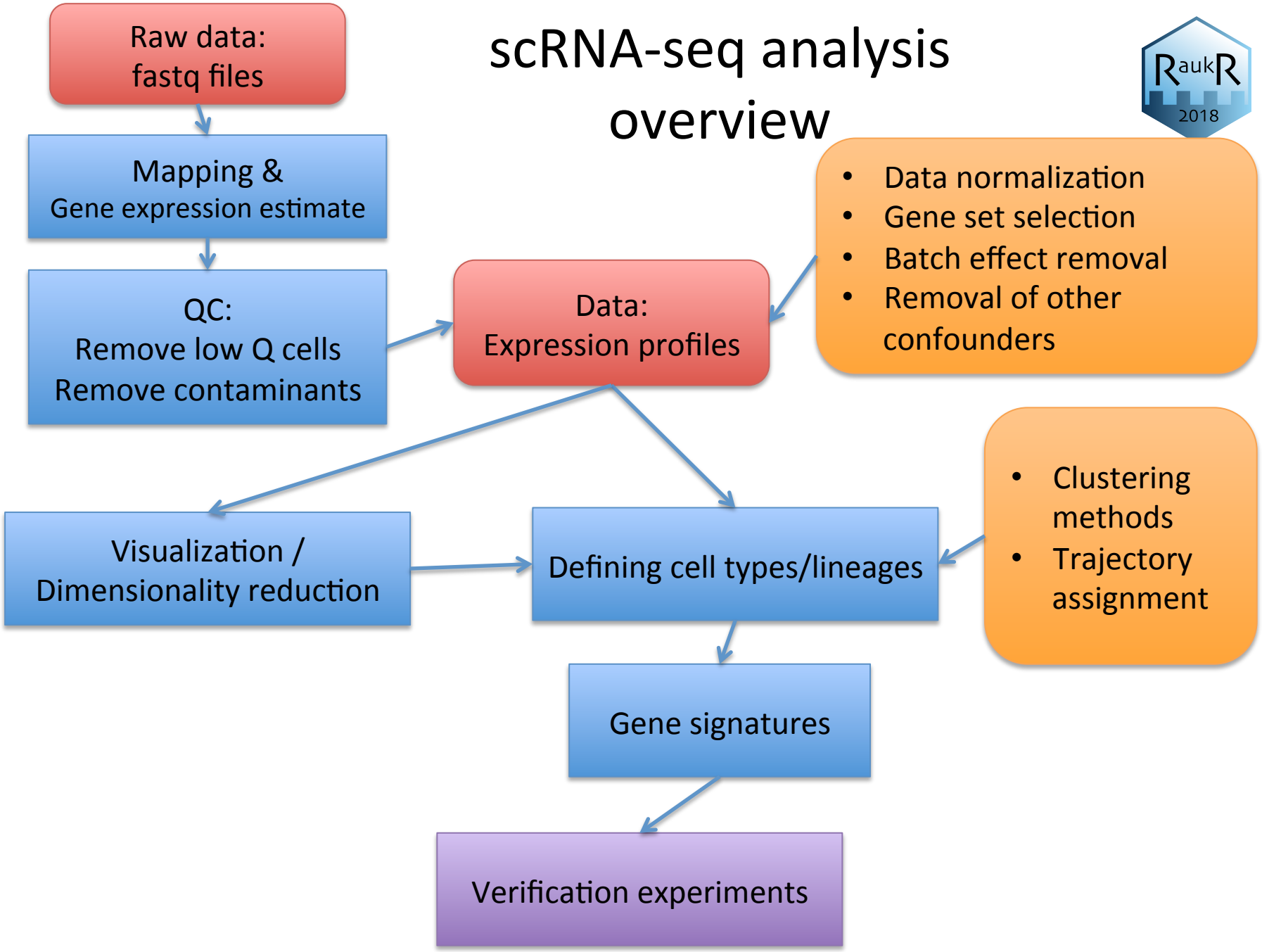
(Vallejos et al. *PLOS Comp Biol* 2015)

Spike-in RNAs





(Tung et al. *Scientific Reports* 2017)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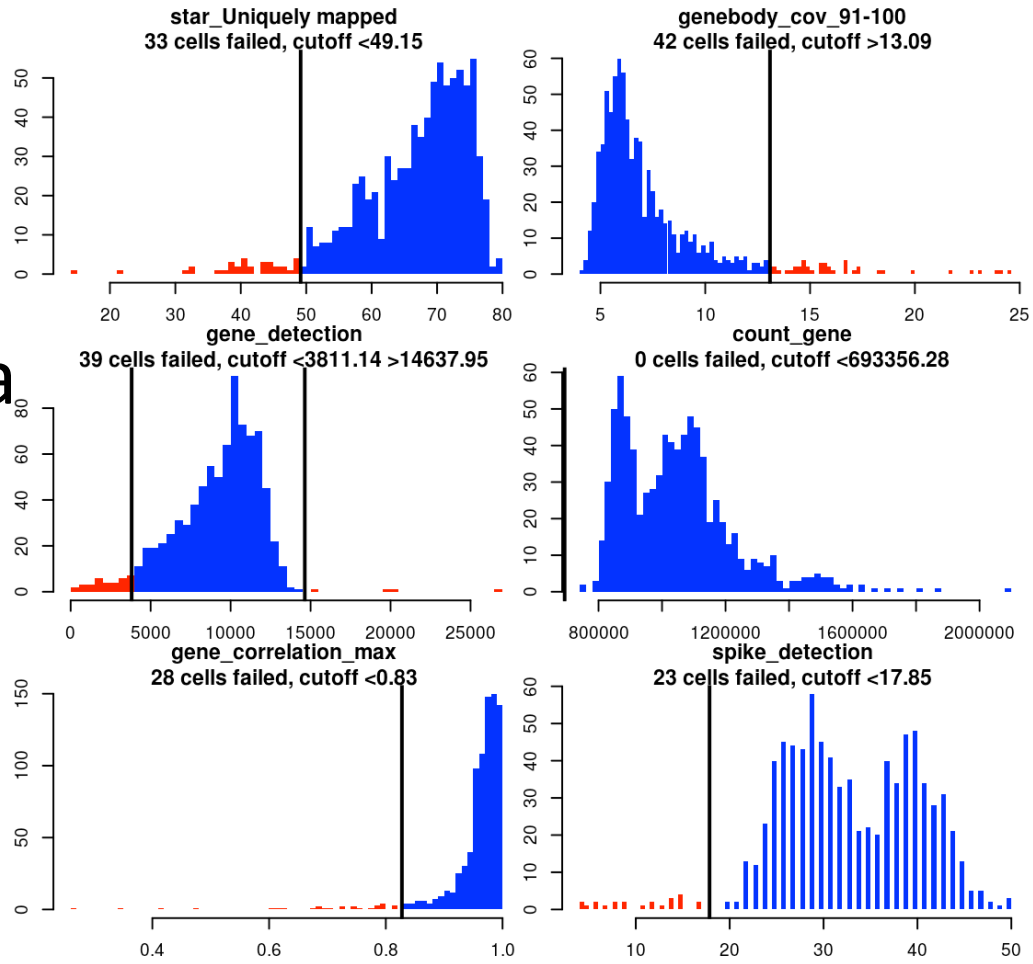
QC-metrics

- Starting amount of RNA
  - Number of reads
  - Mapping statistics (% uniquely mapping, fraction of exon mapping etc.)
  - Number of detected genes
  - Spike-in ratio
- RNA degradation:
  - 3’ bias – for full length methods like SmartSeq2
- Success of library prep:
  - Spike-in detection
- Quality of the cell
  - Mitochondrial read fraction
  - rRNA read fraction
  - Pairwise correlation to other cells
- Duplicate/multiple cells
  - High number of detected genes

How to filter cells

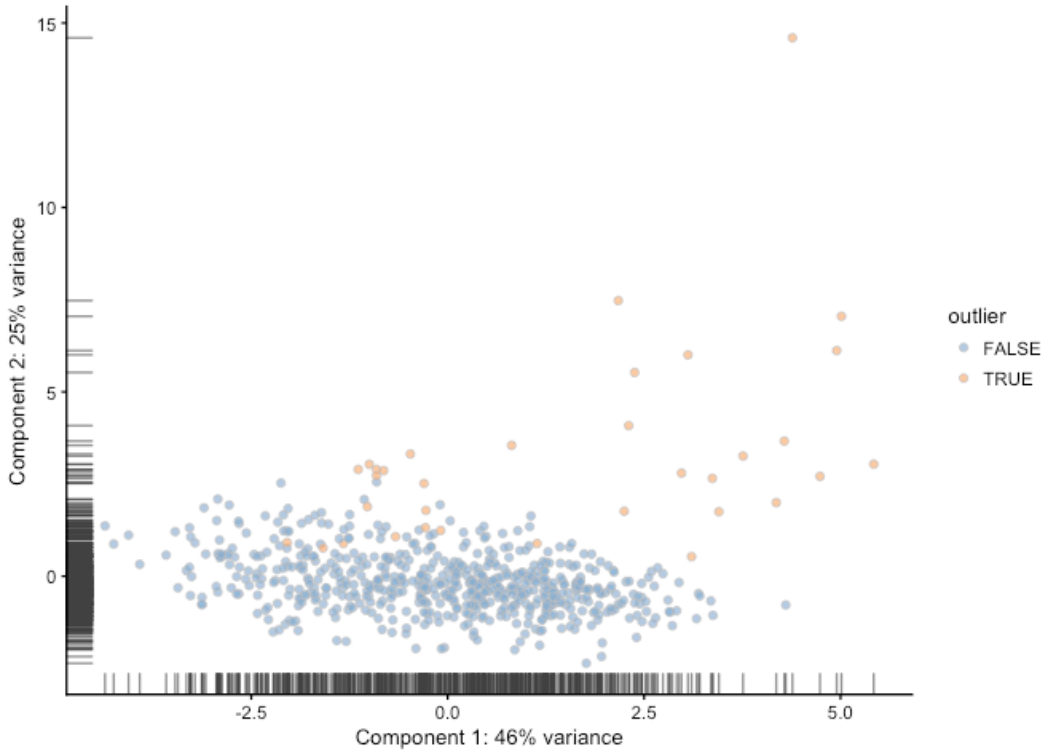
- Normally, most of these qc-metrics will show the same trends, so it could be sensible to use a combination of measures.
- Look at the distributions before deciding on cutoffs.



How to filter cells

- Can use PCA based on all QC-metrics to identify outlier cells.

  (Scater package)



Deciding on cutoffs for filtering

- Do you have a homogeneous population of cells with similar sizes?
- Is it possible that you will remove cells from a smaller celltype (e.g. red blood cells, t-cells)
- Examine PCA/tSNE before/after filtering and make a judgment on whether to remove more/less cells.

Detecting duplicate/multiple cells

- High number of detected genes – can be a sign of multiple cells
  - But, beware so that you do not remove all cells from a larger celltype.
- After clustering – check if you have cells with signatures from multiple clusters.
- A combination of those 2 features would indicate duplicates.

Data normalization

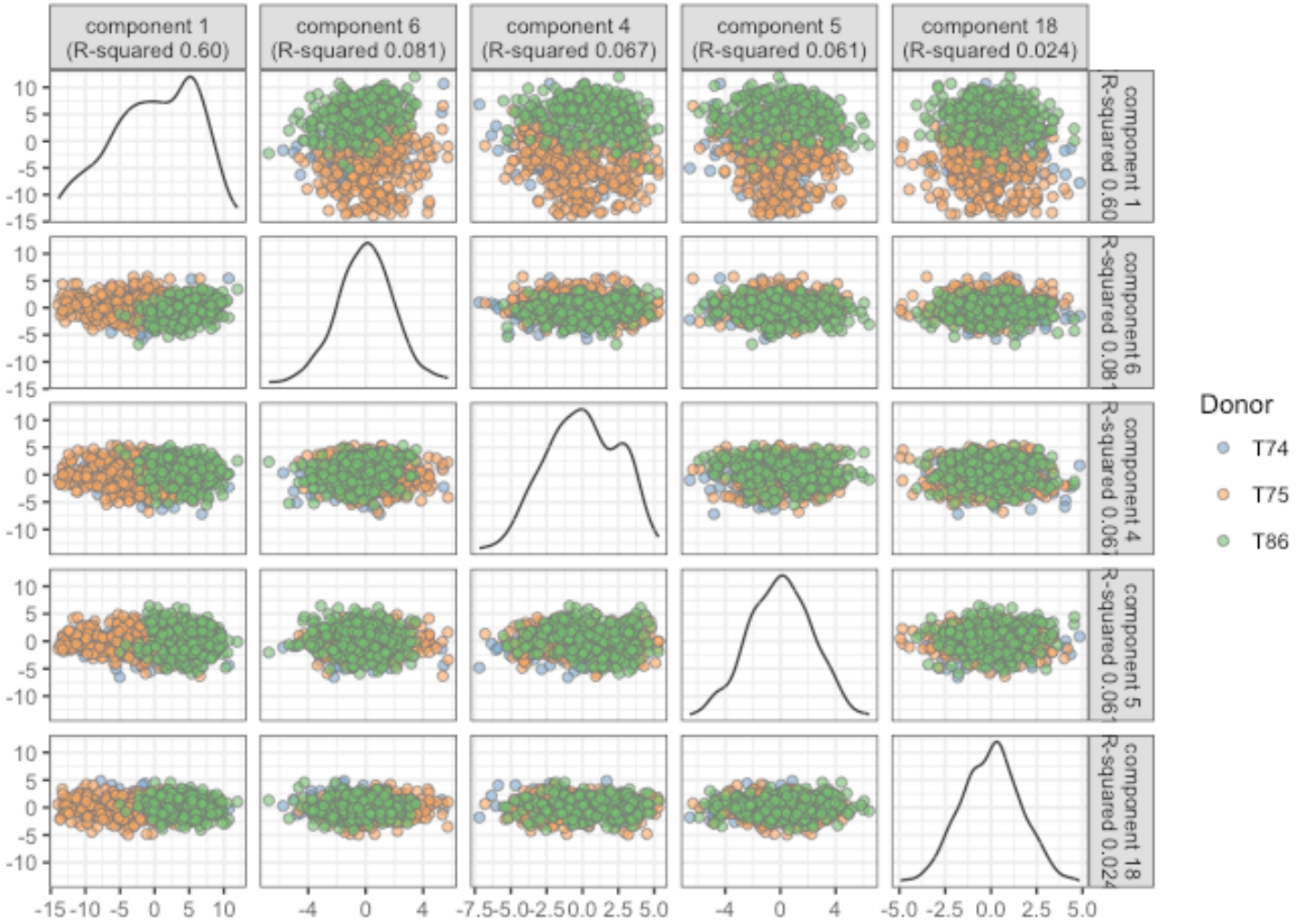
- Controlling for gene length - RPKMs, FPKM, TPMs
- Size factors from:
  - DESeq TMM, CPM
  - Deconvolution method (scran package) - Deals with the problem of vary large numbers of zeros by pooling cells together calculating a normalization for the sum of each pool.
- Error models in SCDE package − based on pairwise comparisons between cells, drop-out rates are estimated.
- Census algorithm to convert TPM to relative transcript counts.

Batch normalization

- Technical batches:
  - Dissociation batch
  - Library prep
  - Sequencing run
- Biological batches:
  - Cell culture
  - Animal
  - Patient

- Often clear biological variation between human samples.

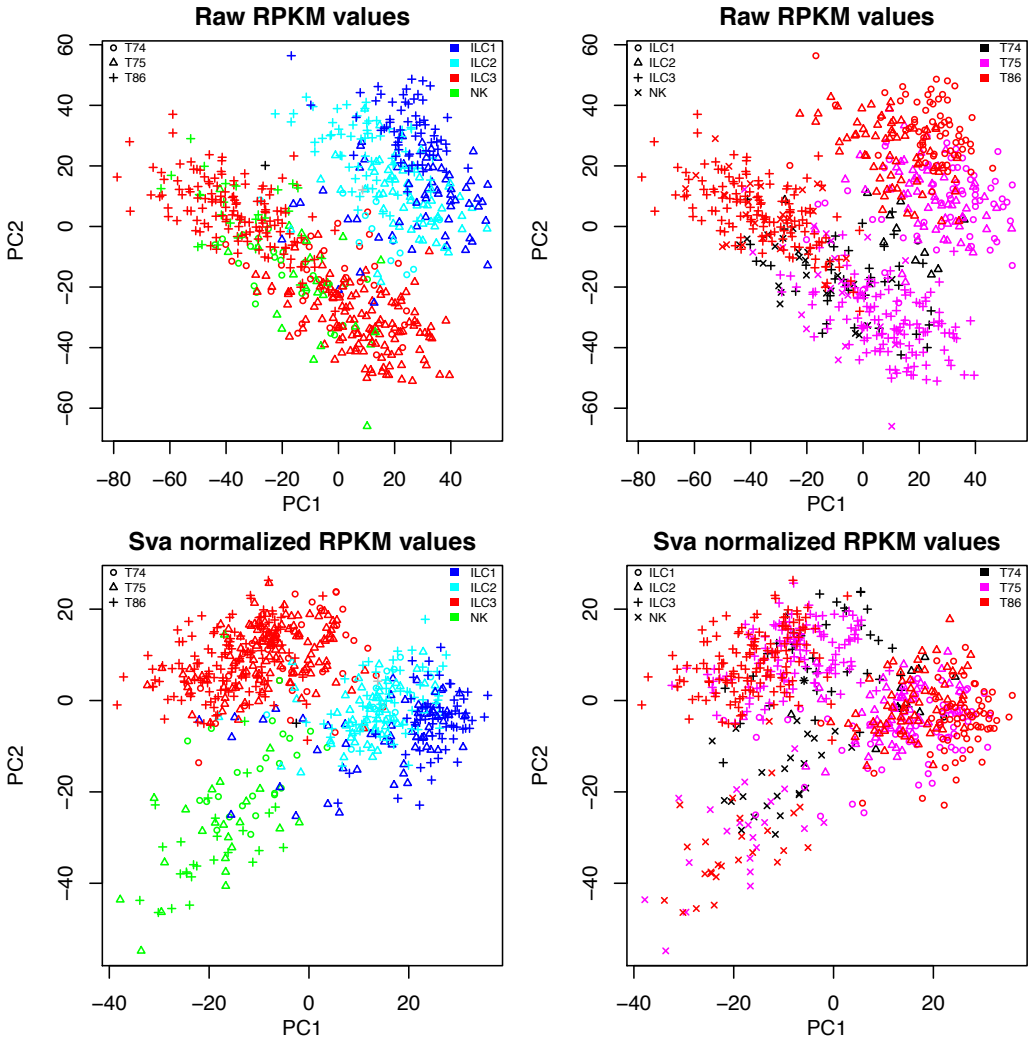
Check for batch effects in PCA



Scater package

Batch normalization with SVA function ComBat

Color by celltype



Color by donor

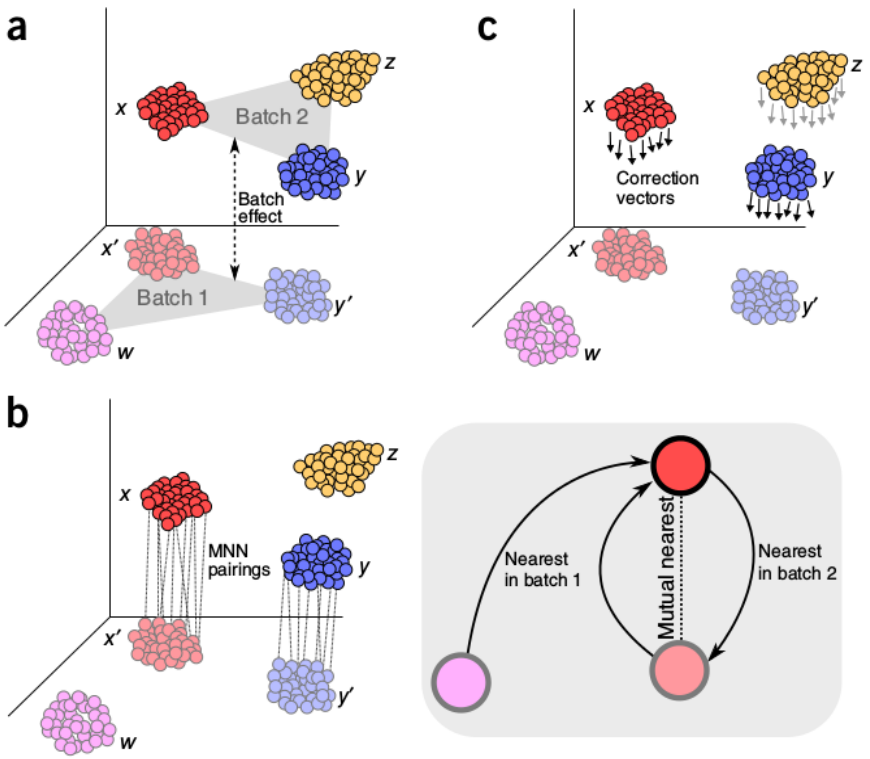
(Björklund *et al*. *Nature Immunology* 2016)

Combining different batches

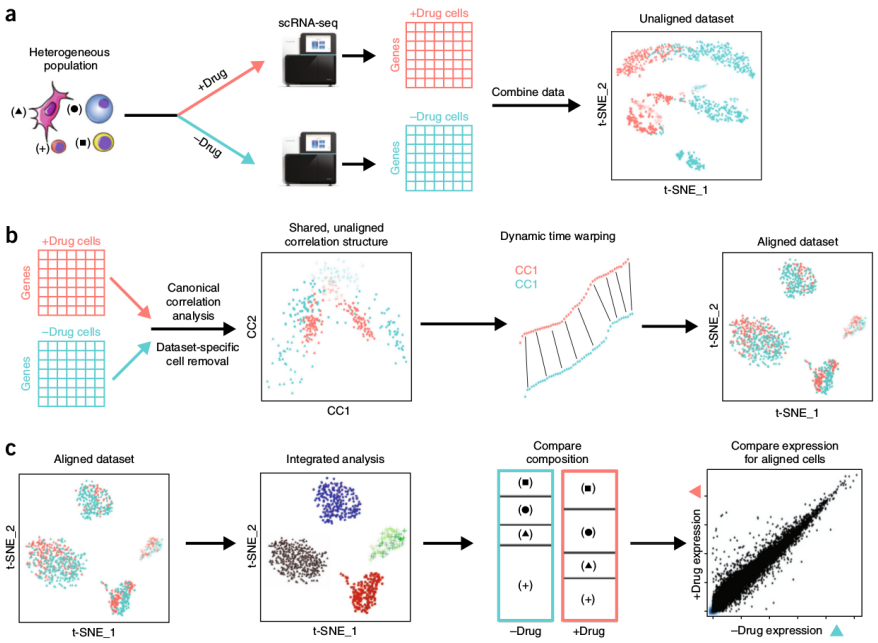
- SVA - ComBat function
- Seurat
  - ScaleData − linear model or generalized linear model (poisson, negative binomial)
  - Correlated Component Analysis (CCA)
- SCDE - pagoda.subtract.aspect − removes weighted projection of the expression matrix onto a specified aspect
- mnnCorrect - based on the detection of mutual nearest neighbors in the high-dimensional expression space

Other Methods for Batch Effects Corrections

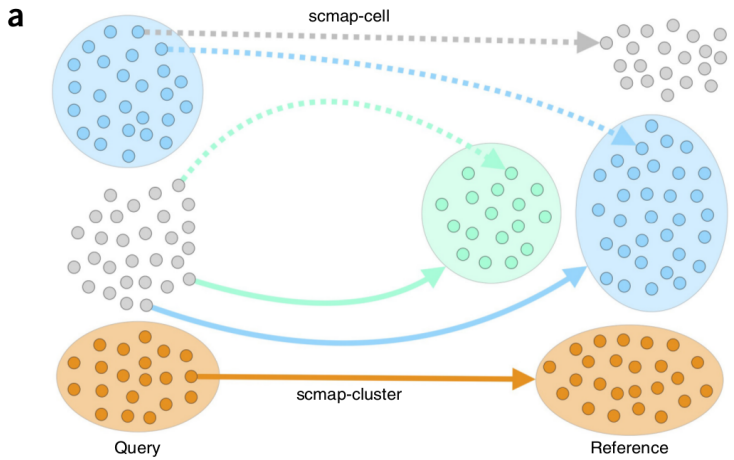
MNN



SEURAT

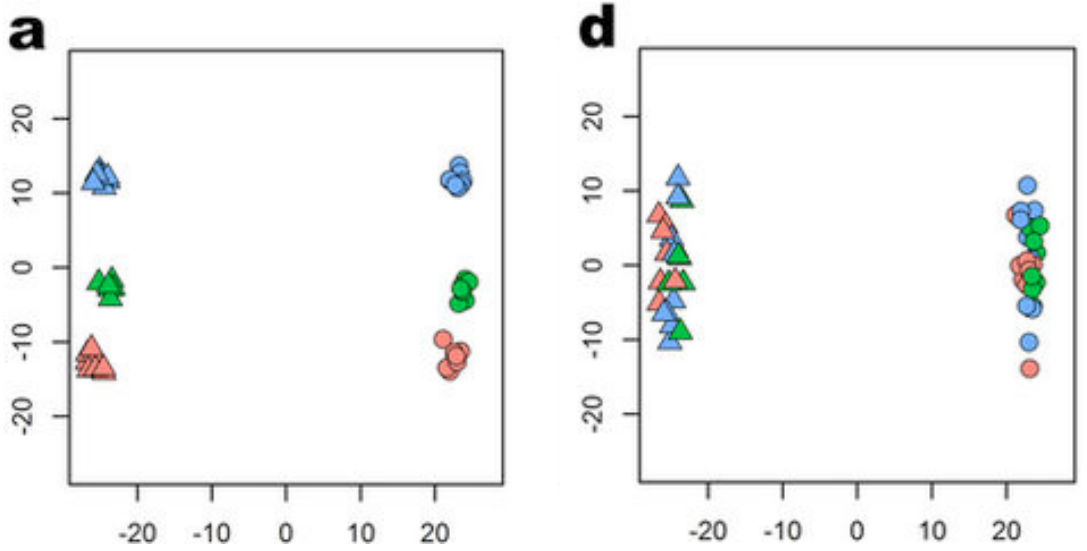


SCMAP



Remove other unwanted confounders

- Cell size / Number of detected genes
- Cell cycle
- Most methods use linear regression, implemented in SCDE, Seurat, scran etc.

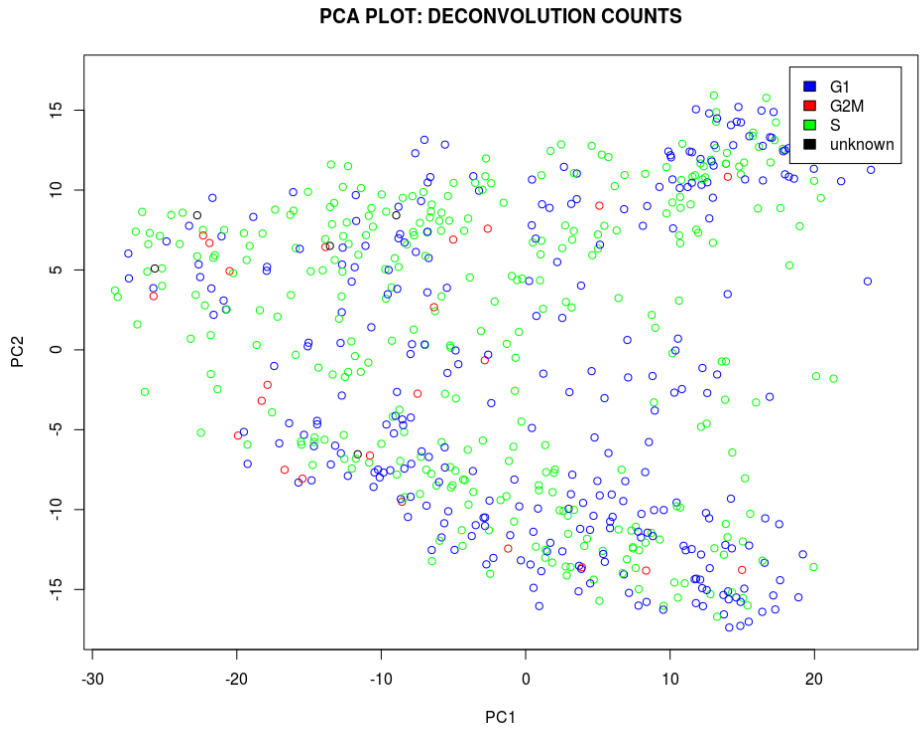


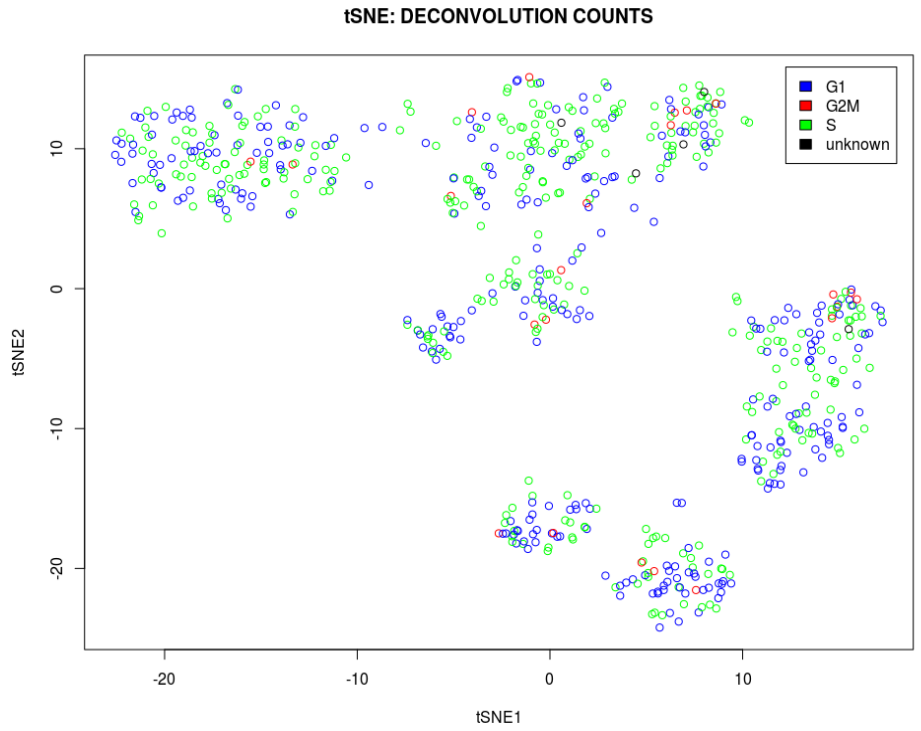
Color = CC phase
Shape = cell type

(ccRemover − Barron & Li, *Sci. Reports* 2016)

Cell Cycle Phase Assignment

Cyclone - Pre-trained classifier looks at pairs of genes having difference in expression that changes sign from phase to phase of cell cycle

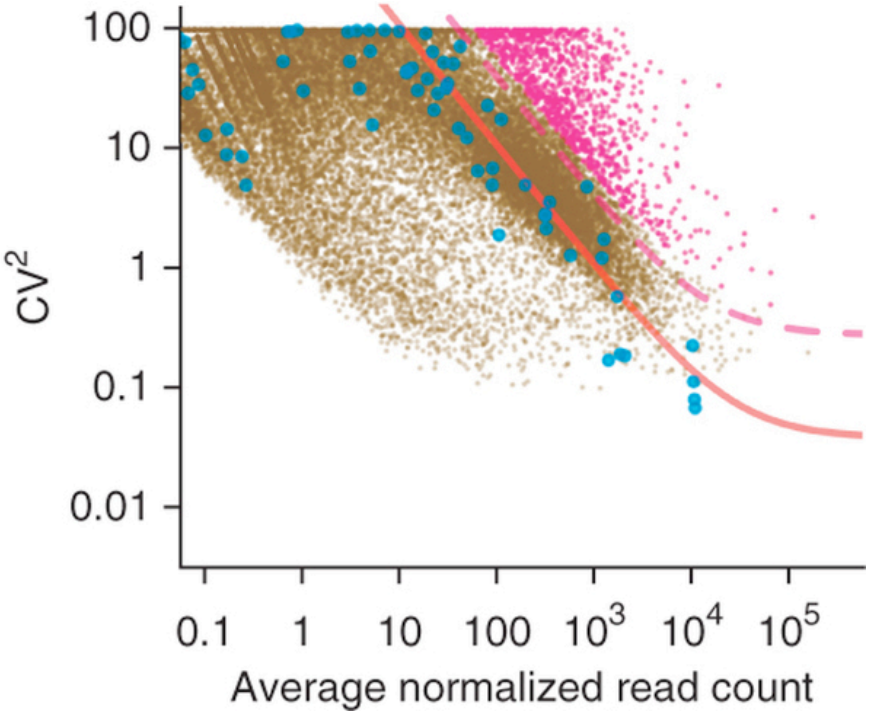




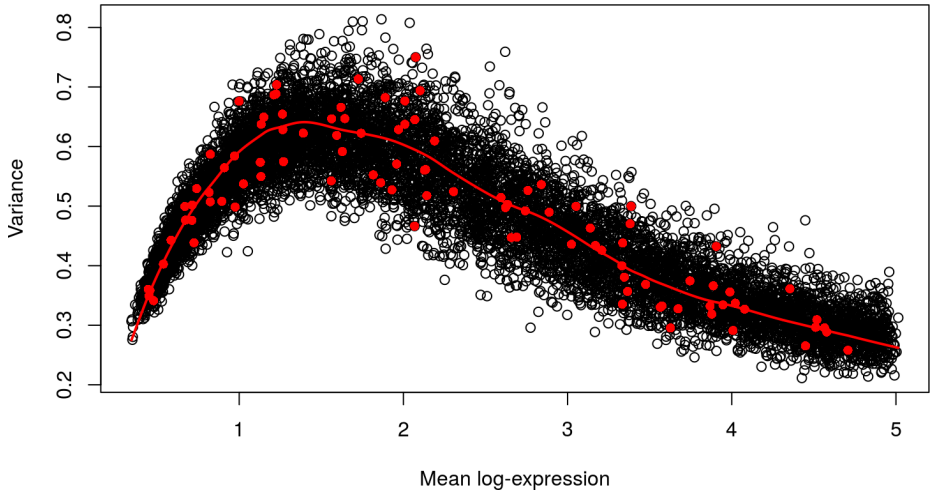
Feature (gene) selection

- In most cases, all genes are not used in analysis.
- Filtering based on:
  - Genes expressed over X in Y cells.
  - **Top variable genes** – implemented in most packages
  - Filter out genes with correlation to few other genes
  - Prior knowledge / annotation
  - DE genes from bulk experiments
  - Top PCA loadings
- Especially for trajectory analysis – important to build the trajectories on a relevant gene set

Spike-in RNAs
Finding biologically variable genes



Decompose variance of each gene into biological and technical component. Using spike-ins to model technical variation



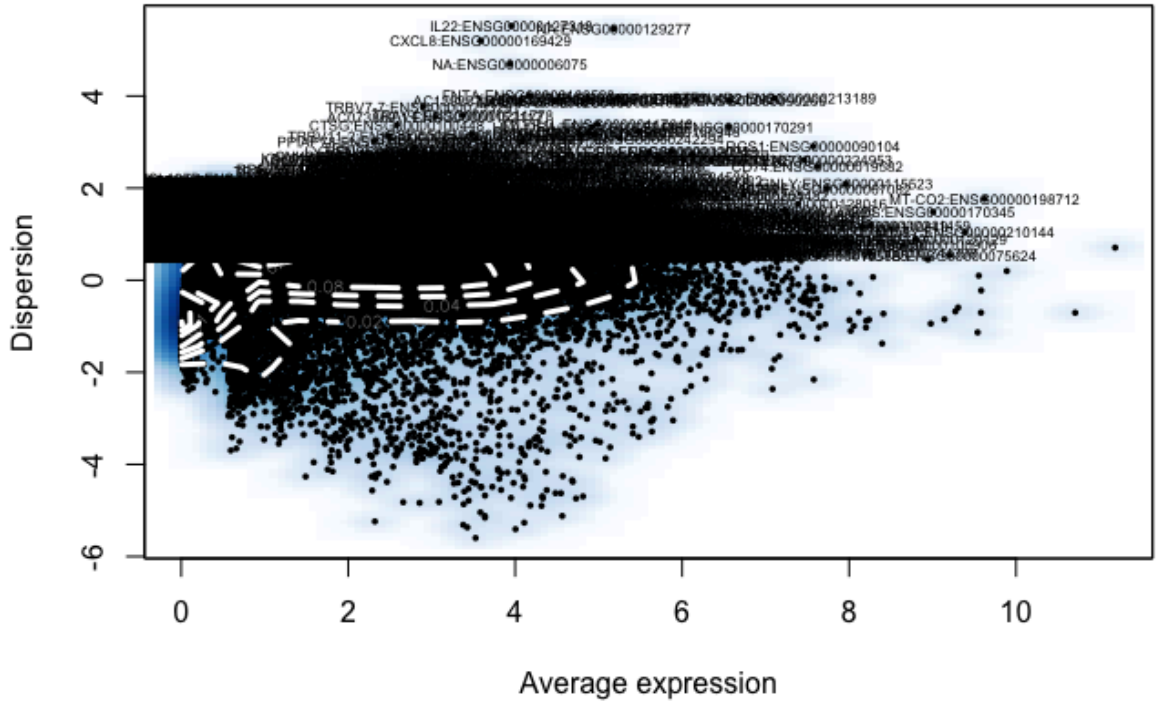
Coefficient of variation^2:

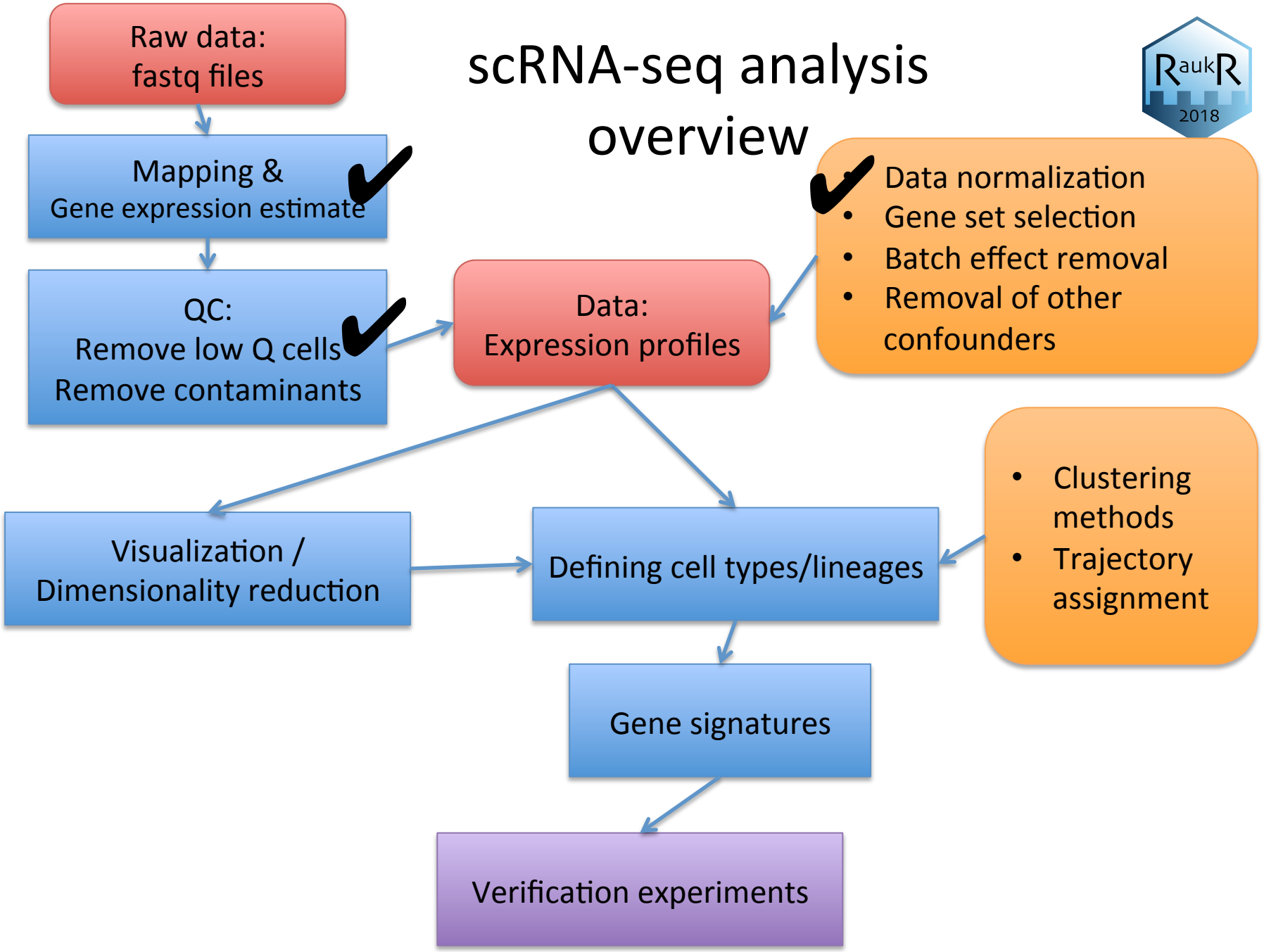
CV^2 = standard deviation / mean ^2

(Brennecke et al. *Nature Methods* 2013, Scran tutoral)

Top variable genes

Seurat uses Dispersion vs Average expression and you define cutoffs for x & y to select genes

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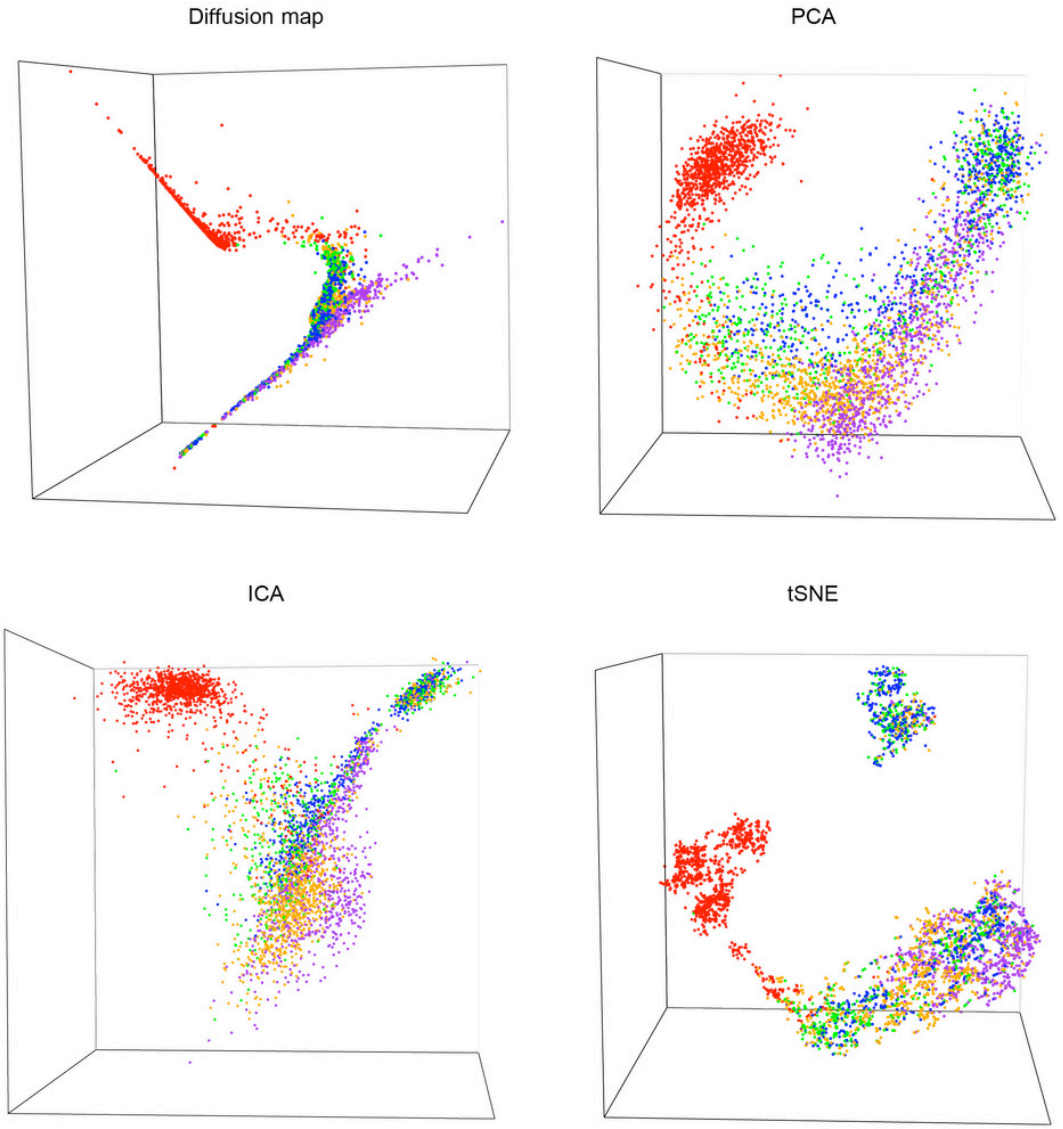
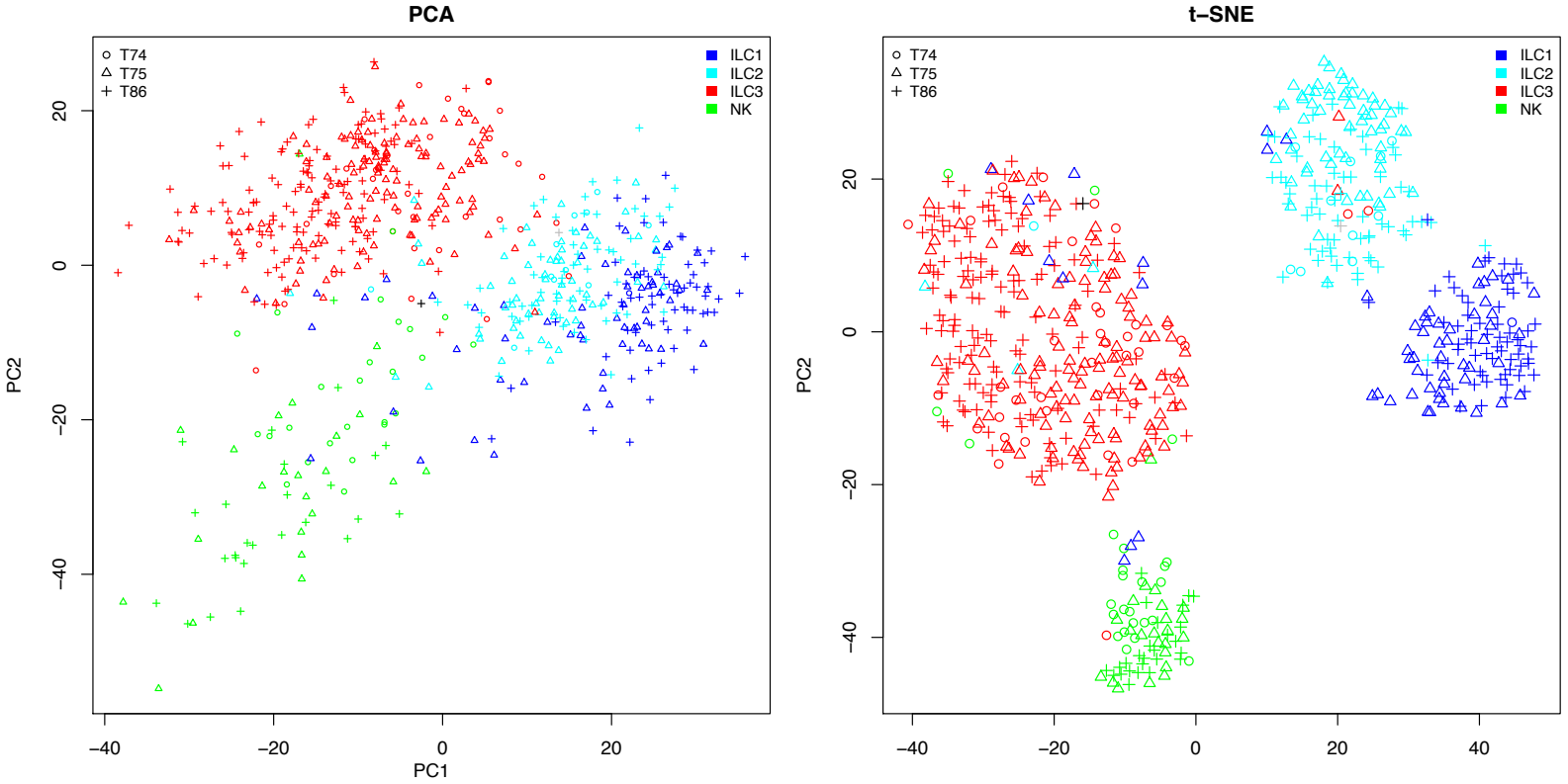


(Moignard *et al. Nature Biotech* 2015)

t-SNE – t-distributed stochastic neighbor embedding

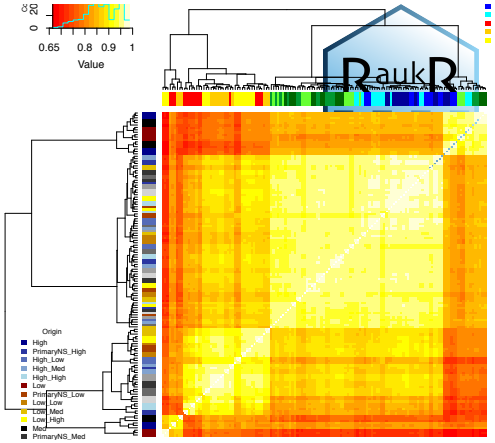
- Conserve pairwise distances in n-dimensional PCA space to 2D in local neighborhoods

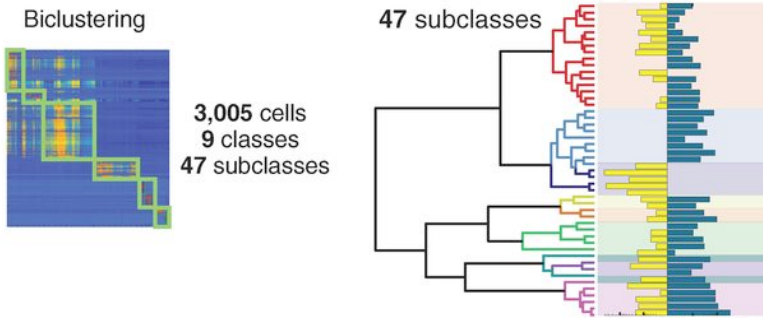


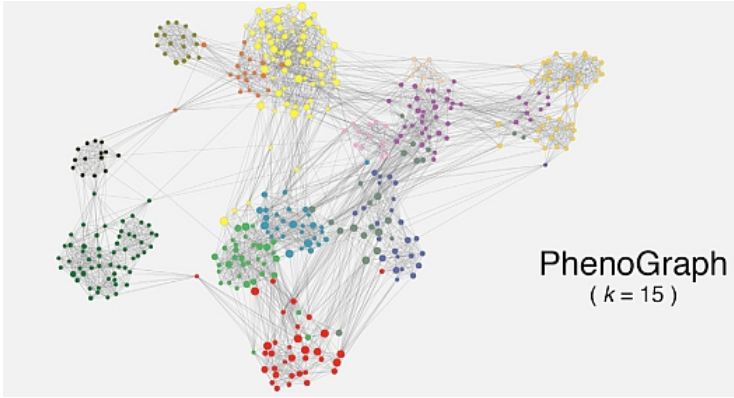
(Björklund *et al*. *Nature Immunology* 2016)

Clustering

- Classical clustering methods
  - Hierarchical
  - k-means clustering
- Biclustering
  - WGCNA
  - BackSPIN
- Graph based clustering
  - SNNCliq
  - Seurat
  - Pagoda2







Different distance measures

- Most commonly used in scRNA-seq:
  - Euclidean distance in multidimensional space (often with a reduced gene set)
  - Euclidean distance in PCA, tSNE or other reduced space
  - Inverted pairwise correlations (1-correlation)

Clustering

- About 1 paper/week with a new method for clustering single cells
- Consensus from several methods increases confidence in separation.
- Key challenges:
  - Deciding on the true number of clusters
  - Separation of true clusters from technical artifacts

Some toolkits for scRNAseq analysis

- Scater
- Scran
- Seurat
- SCDE
- Monocle

Scater

- Single Cell Experiment class for scRNAseq data:
  - Assays − counts, logcounts, tpms etc.
  - Reduced dimensions − PCA, tSNE, diffusion map
  - Spike in controls
  - Meta data
  - Feature data
- Contains functions for:
  - Visualization
  - Quality control, check for batch effects.
  - Normalization

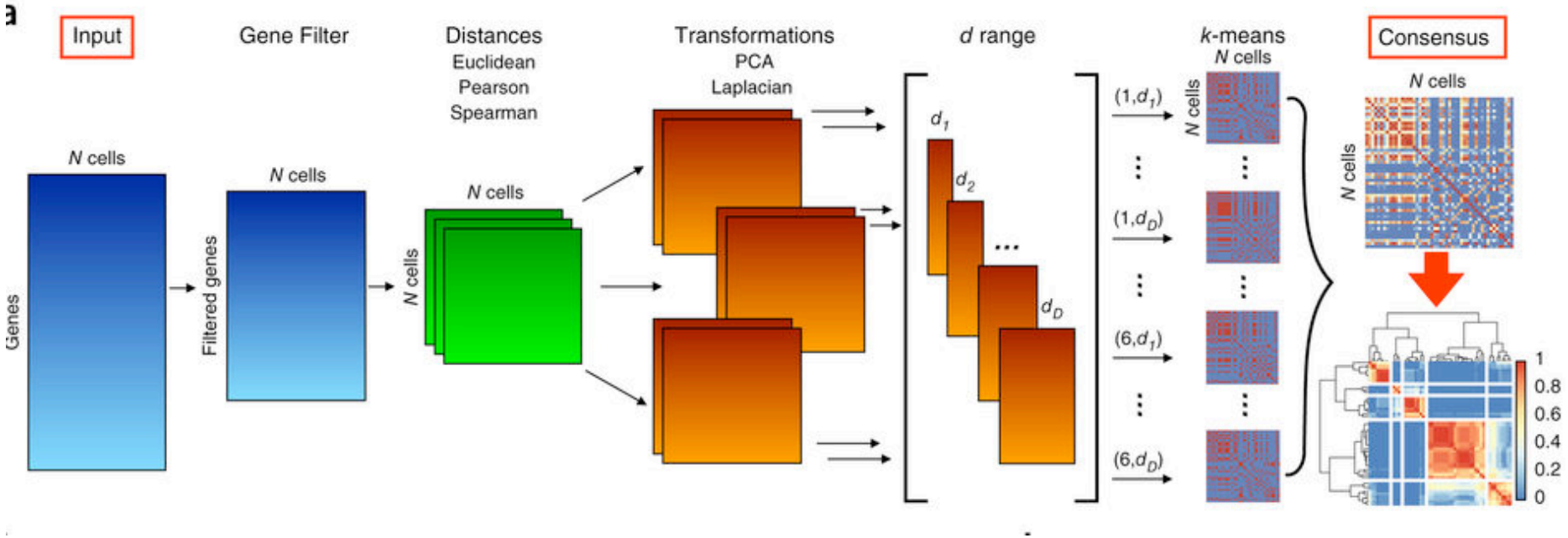
(McCarthy et al. Bionformatics 2017)

SCRAN – Single Cell RNA ANalisys

- Uses SingleCellExperiment class
- Includes cyclone method for predicting cell cycle phase.
- Includes Basics deconvolution strategy for size factors.
- Batch effect correction with mnnCorrect.
- Detection of variable genes by deconvolution of technical and biological variance.
- Also contains method for SNN graphs

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.

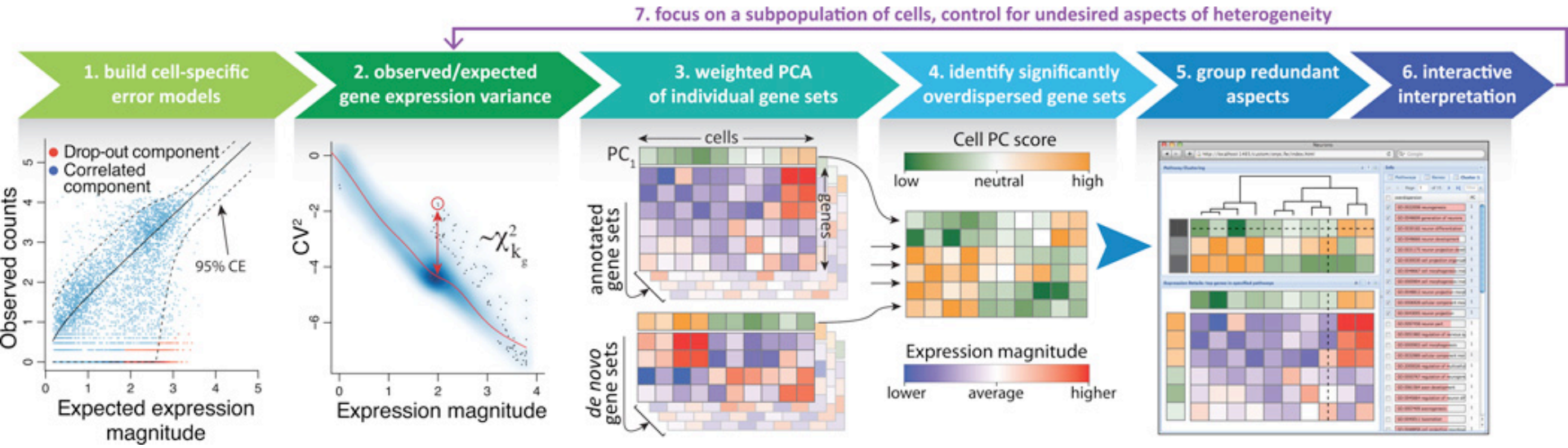
Differential expression with nonparametric Kruskal–Wallis test.

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)

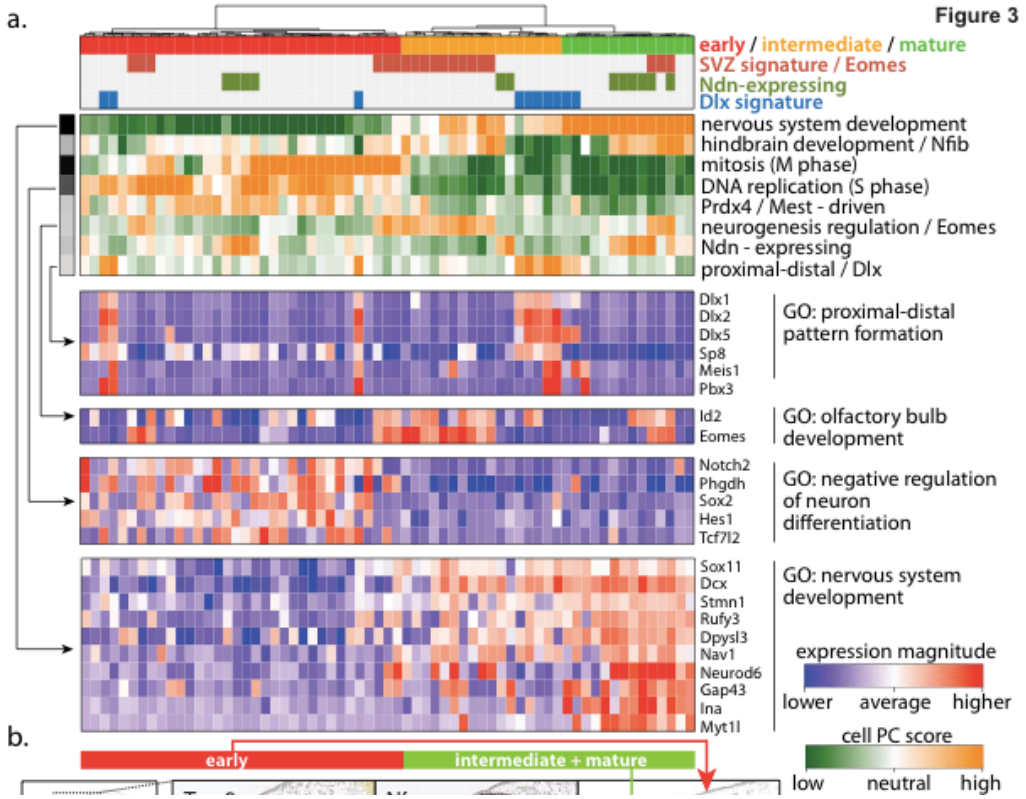
Pagoda – Pathway And Geneset OverDispersion Analysis

Implemented in the SCDE package



(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
- Can visualize gene sets.
- https://github.com/hms-dbmi/pagoda2

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
  - 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.

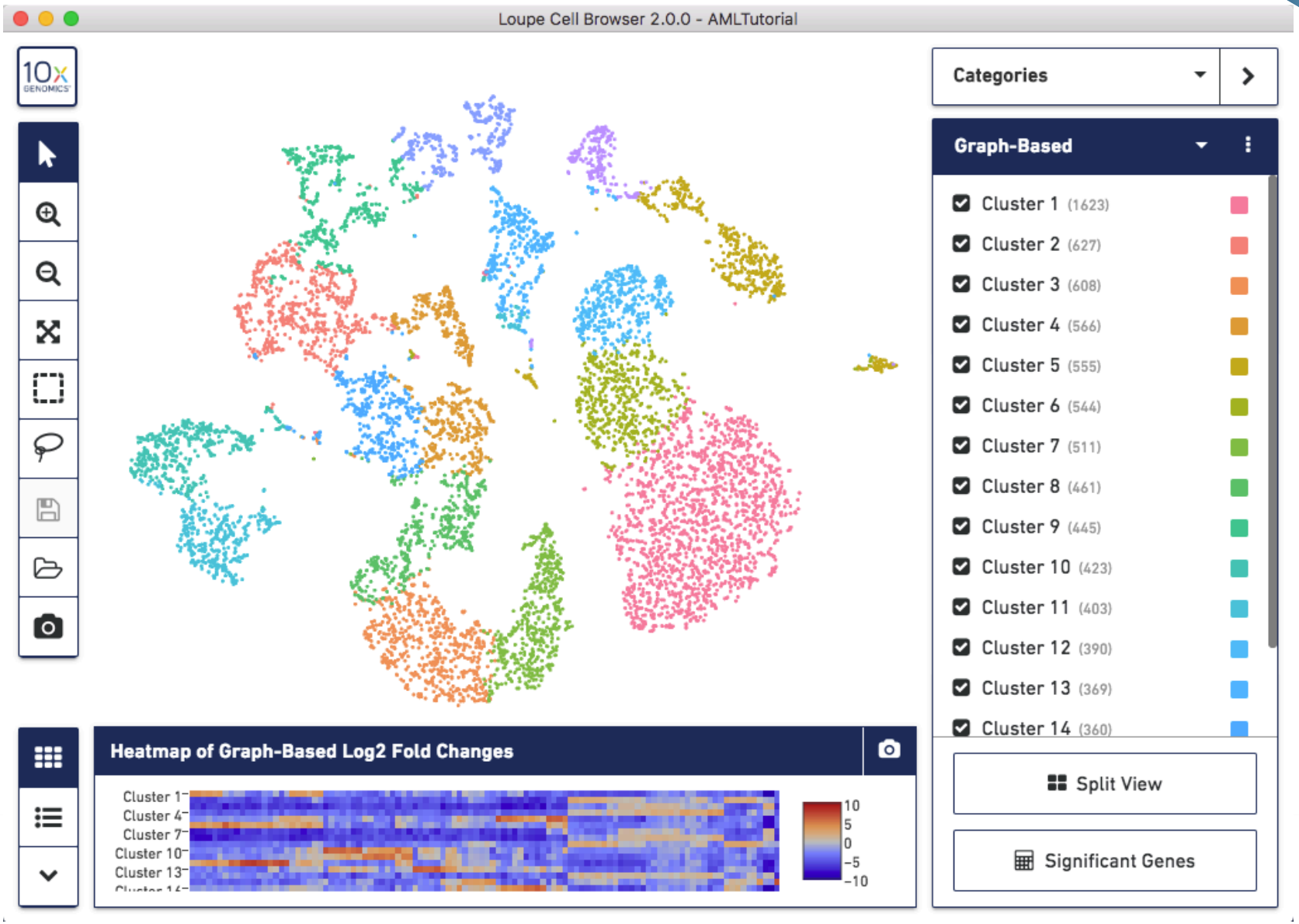
(http://satijalab.org/seurat/)

Seurat

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

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

Loupe – Cell Browser, from 10X Genomics



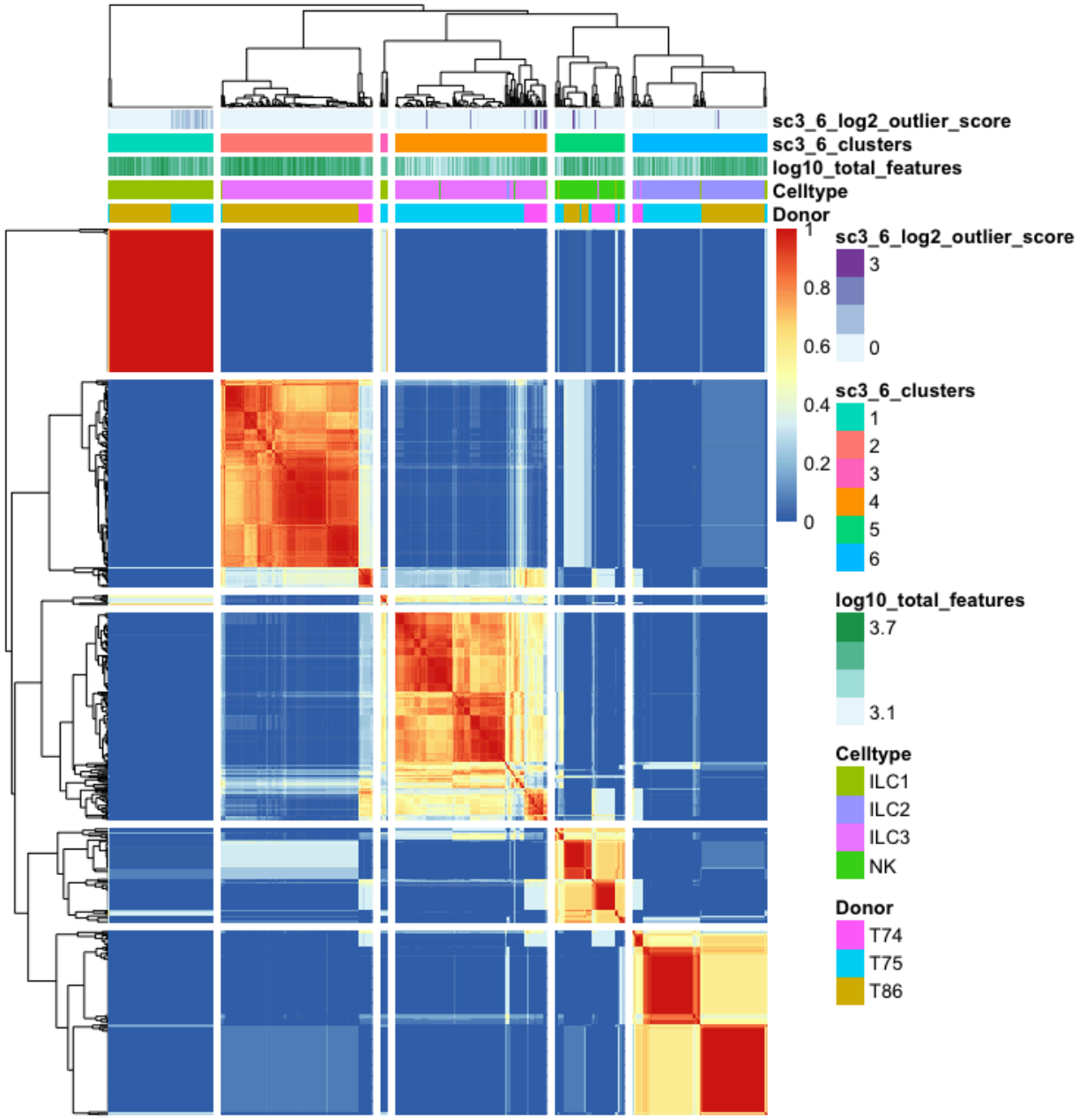
Which clustering method is best?

- 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 euklidean or correlation distances will work fine.

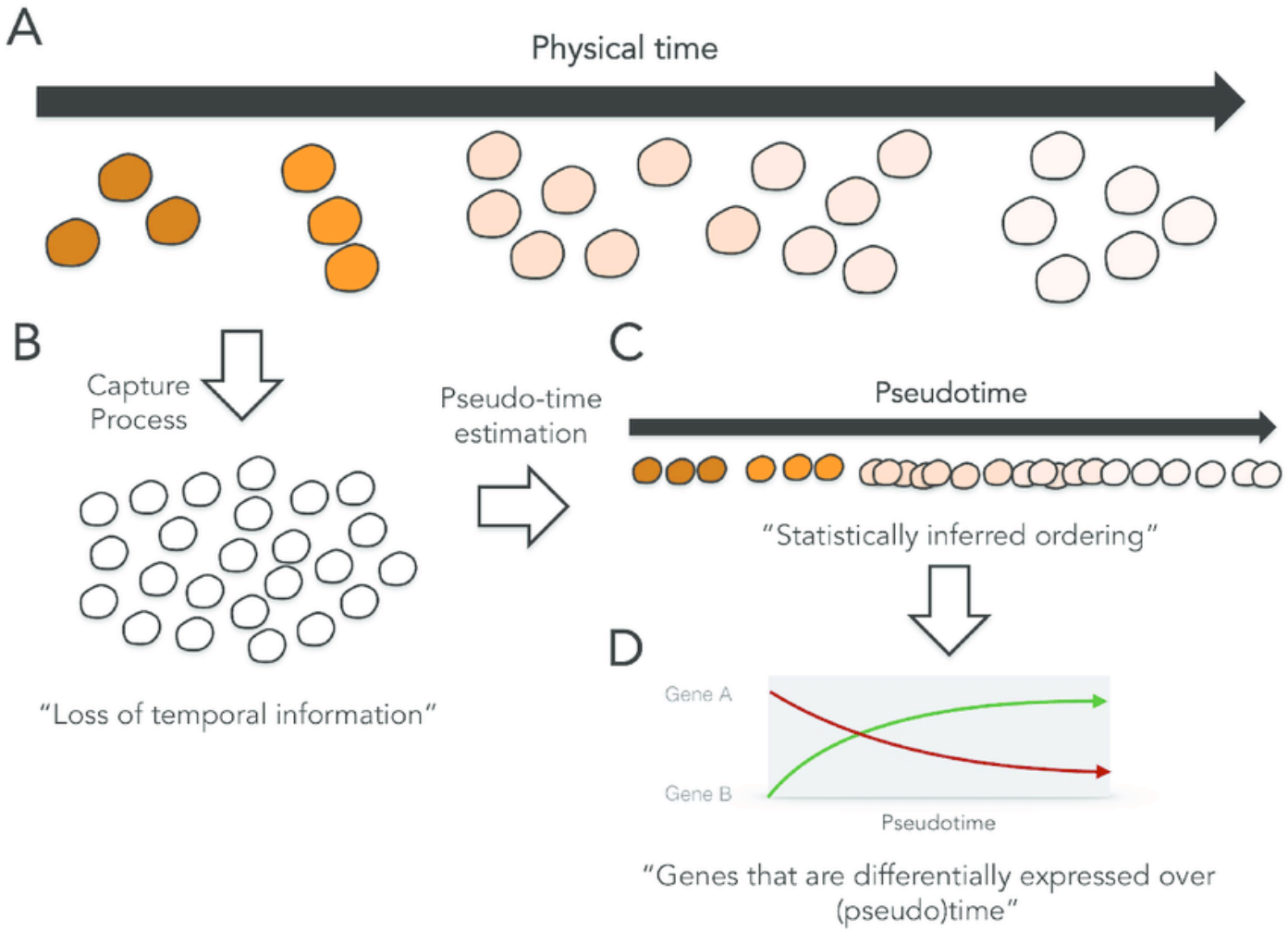
How many clusters do you really have?

- 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)

Check QC data

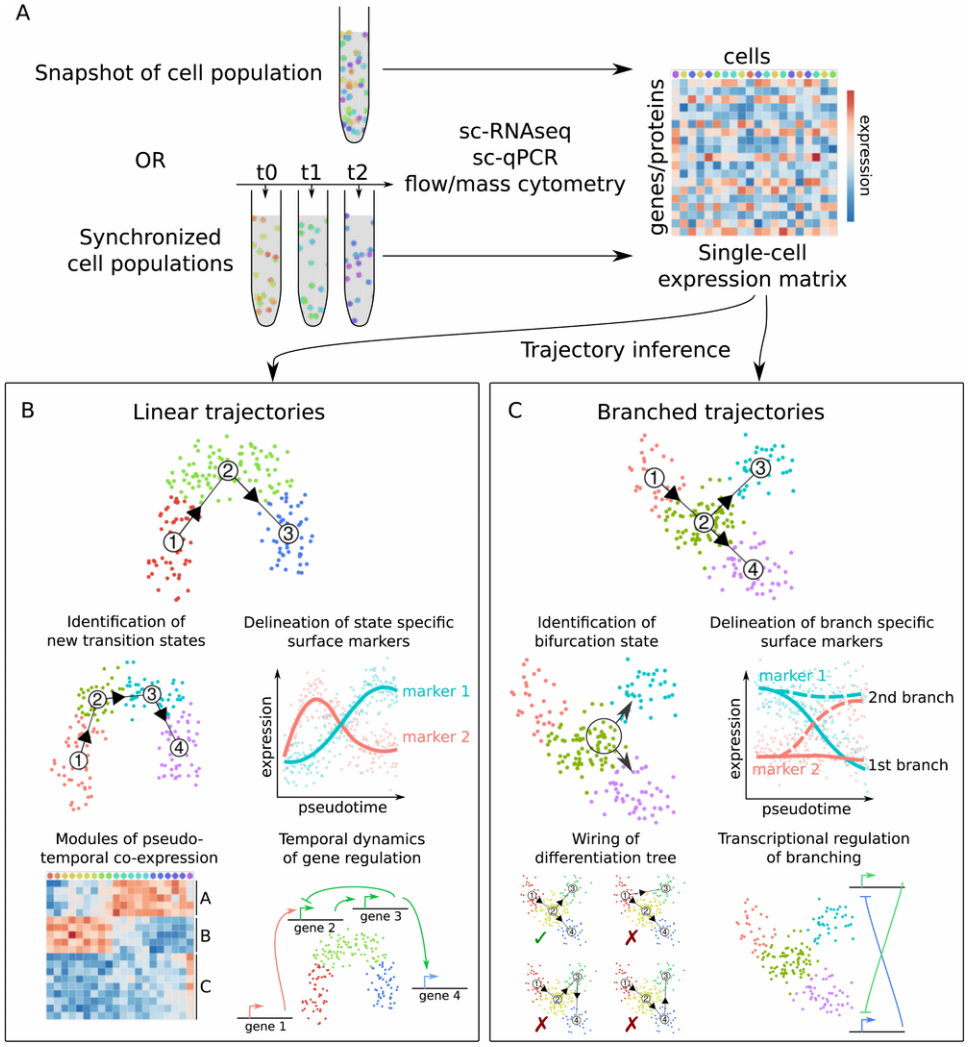


Pseudotime/trajectory analysis



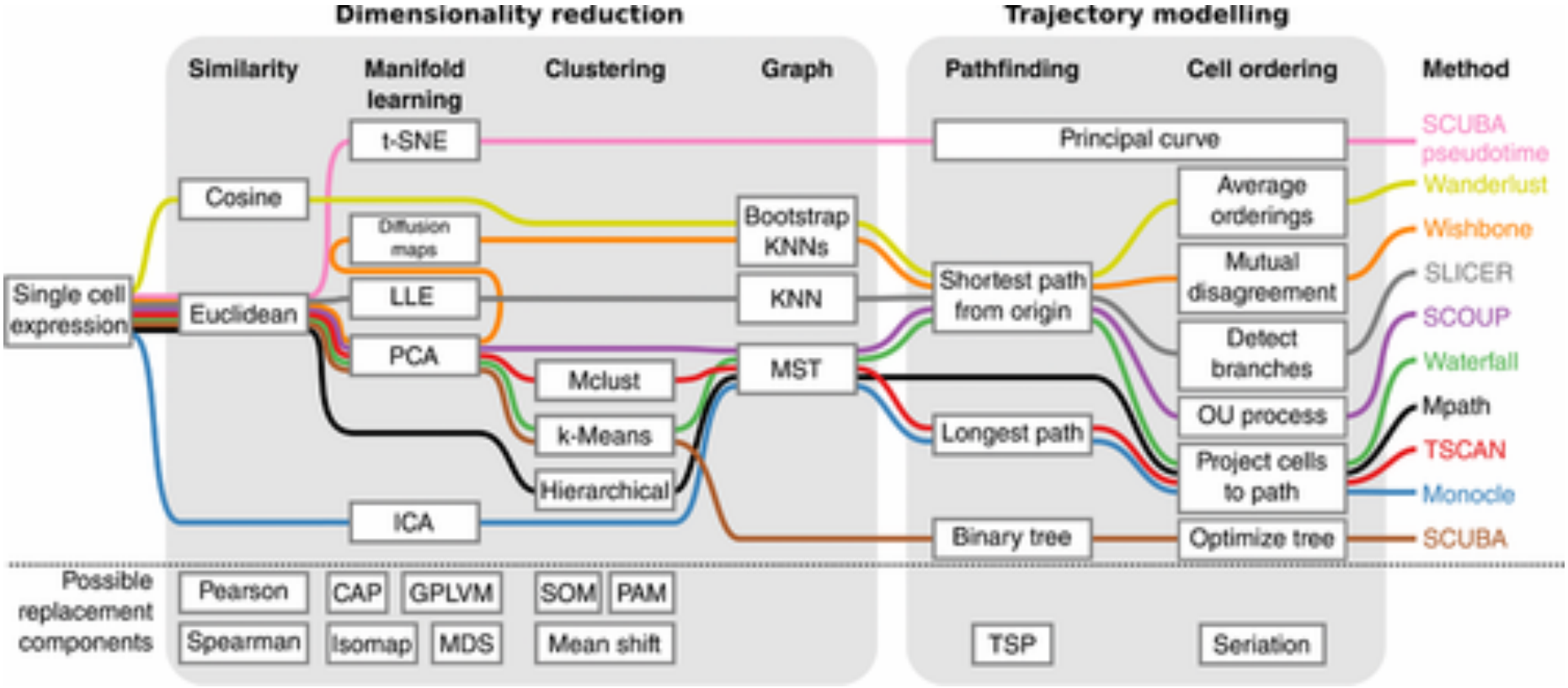
(Kieran *et al*. *Plos Comp Biol*. 2017)

Pseudotime/trajectory analysis



(Cannoodt *et al*. *EJI* 2016)

Trajectory tools



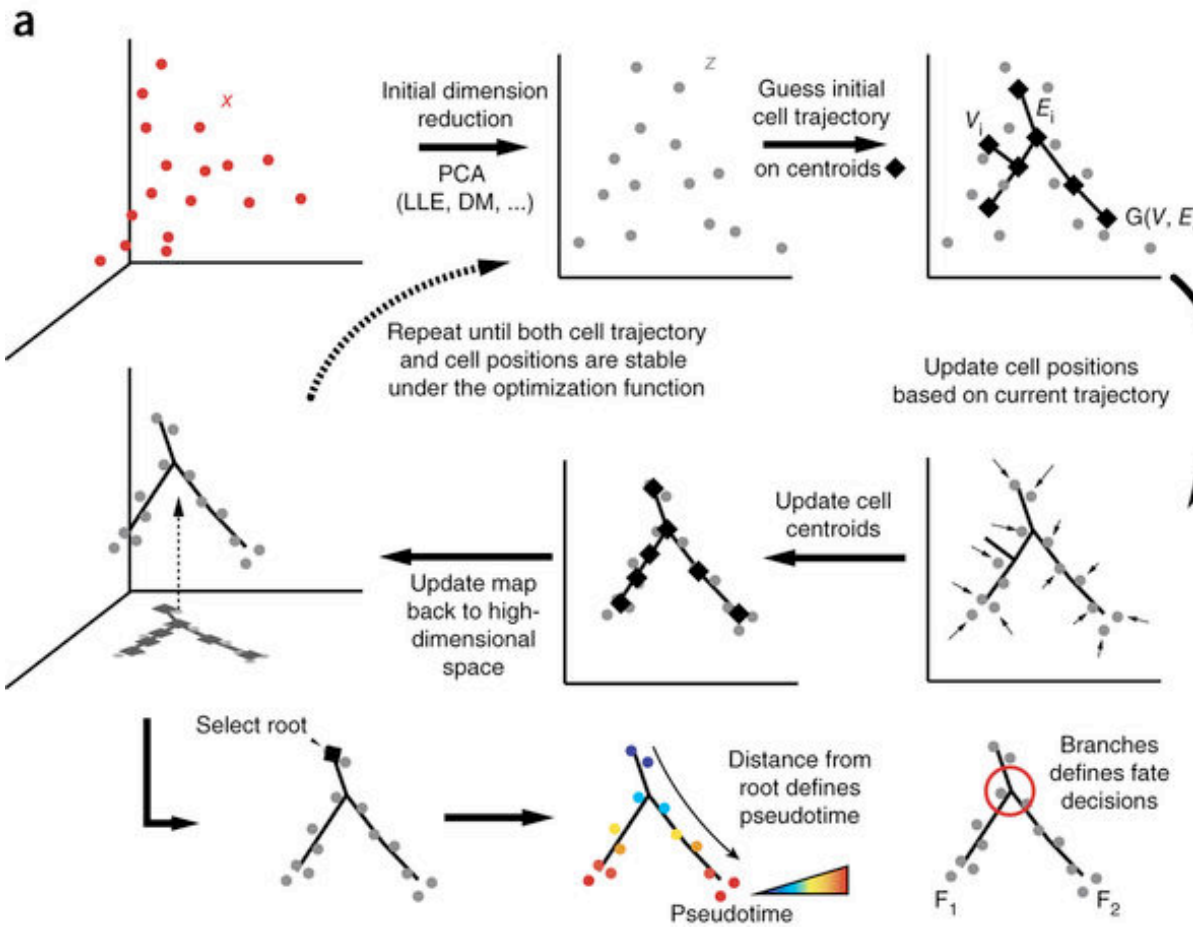
(Cannoodt *et al*. *EJI* 2016)

Should you run trajectory analysis?

- Are you sure that you have a developmental trajectory?
- Do you believe that you have branching in your trajectory?
- Do you have enough cells along the trajectory?

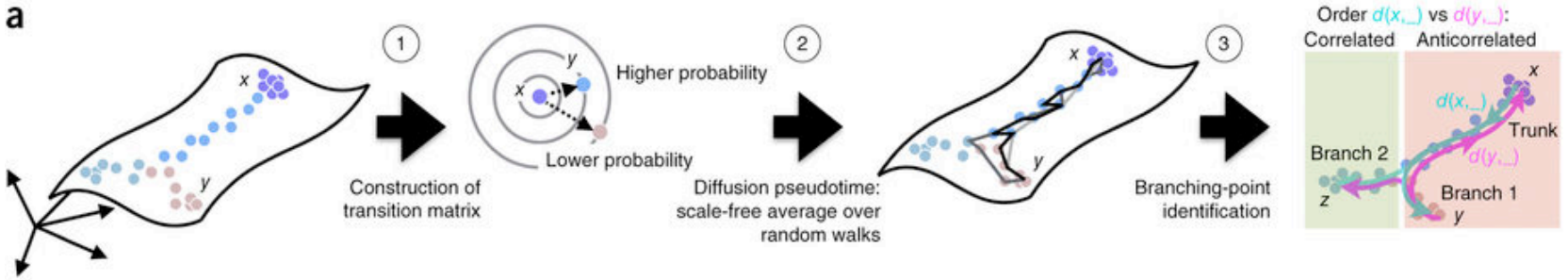
- Be aware, any dataset can be forced into a trajectory without any biological meaning!
- First make sure that gene set and dimensionality reduction captures what you expect.

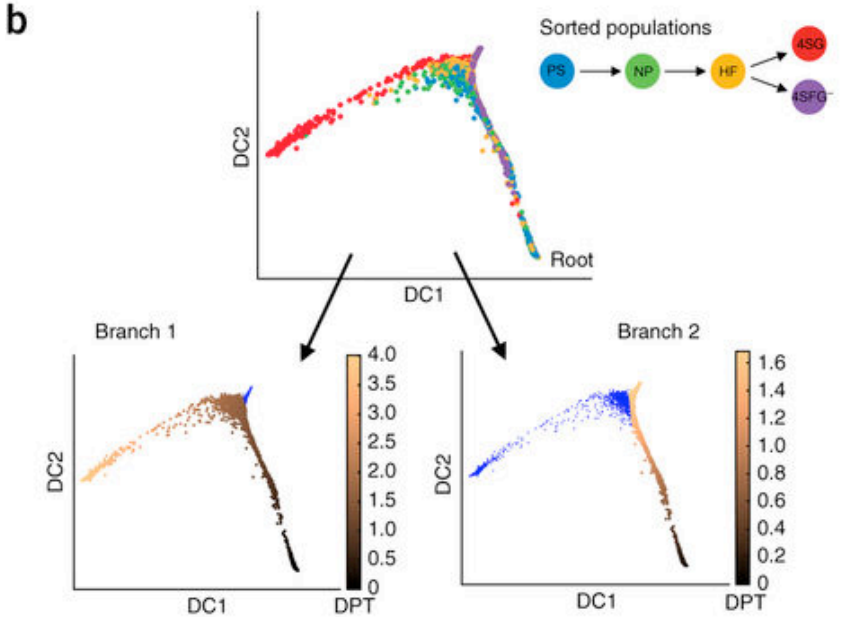
Monocle2 – reversed graph enbedding

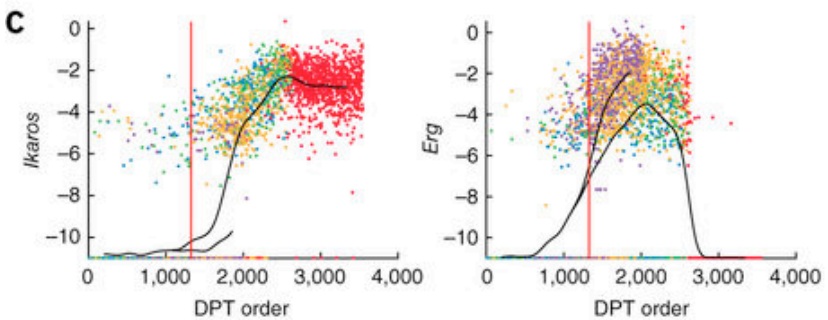


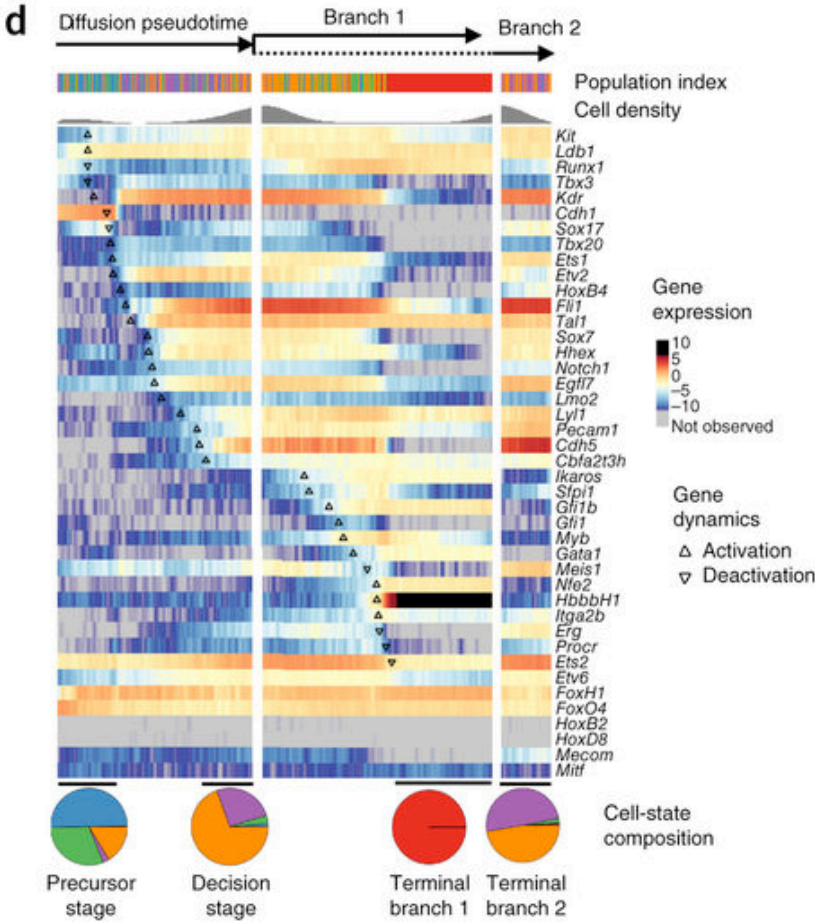
(Qiu et al. *Nat Methods* 2017)

Diffusion pseudotime (DPT) (Haghvedi *et al.* *Nature Methods* 2016)





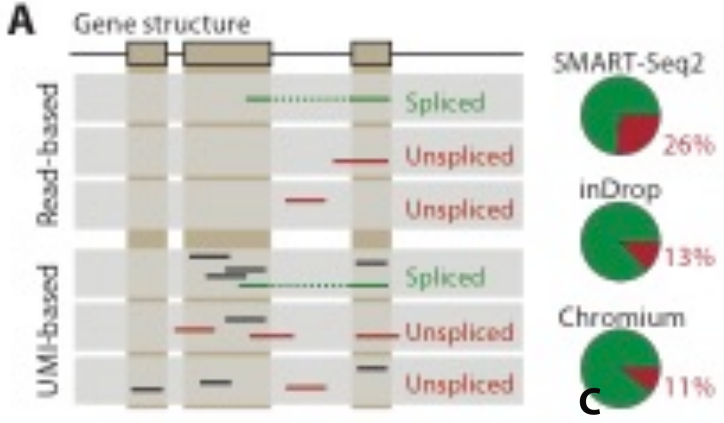


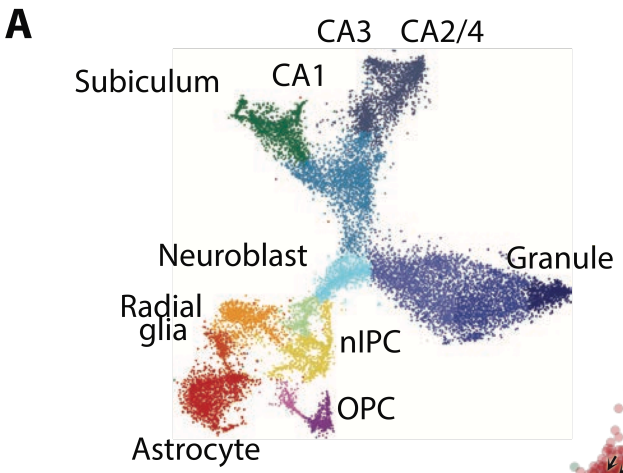


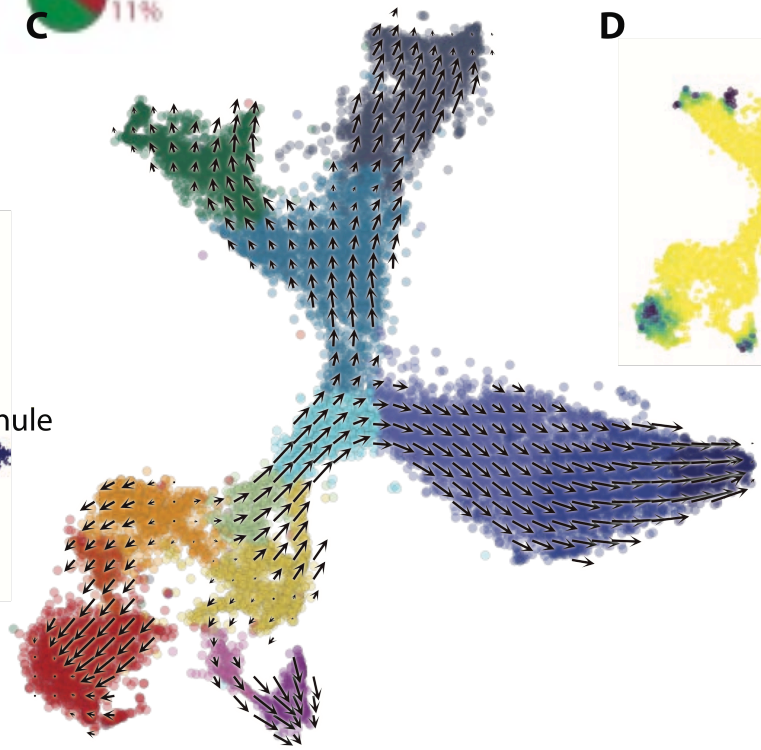
RNA velocity

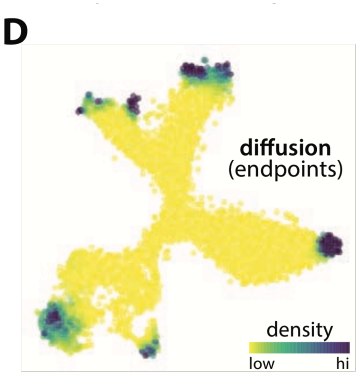
Uses proportion of unspliced reads to identify recently activated genes.

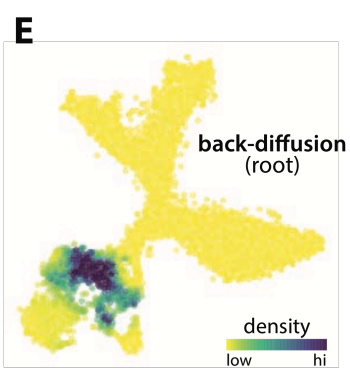
Predicts future fate of the cells.

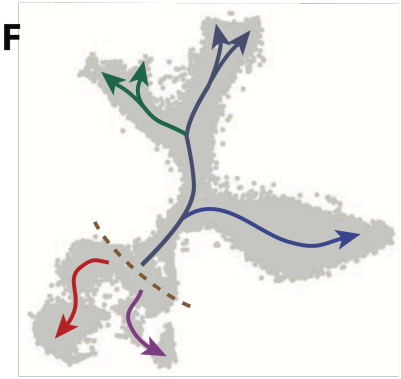




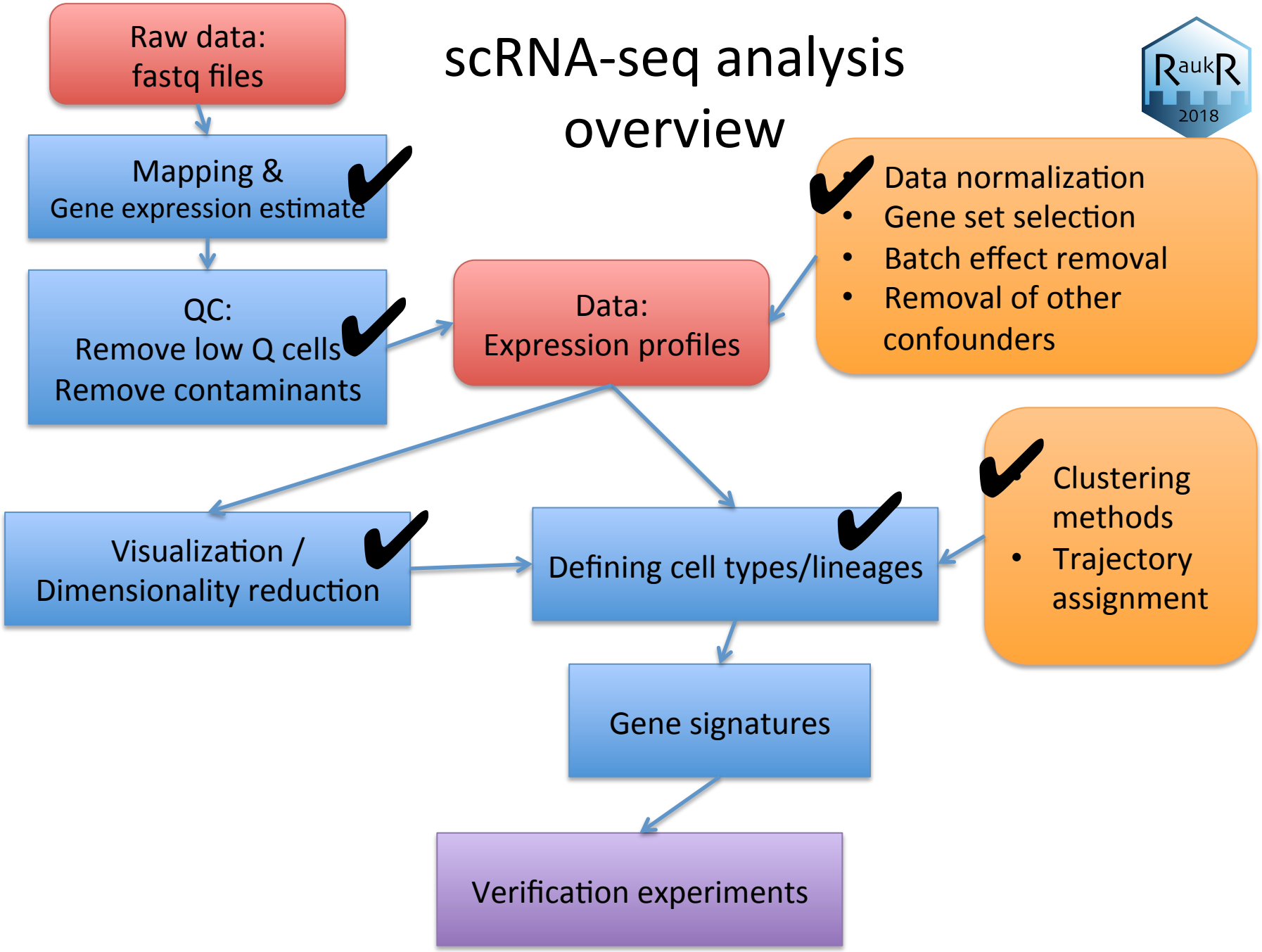








(LaManno *et al*. BioRxiv 2017)

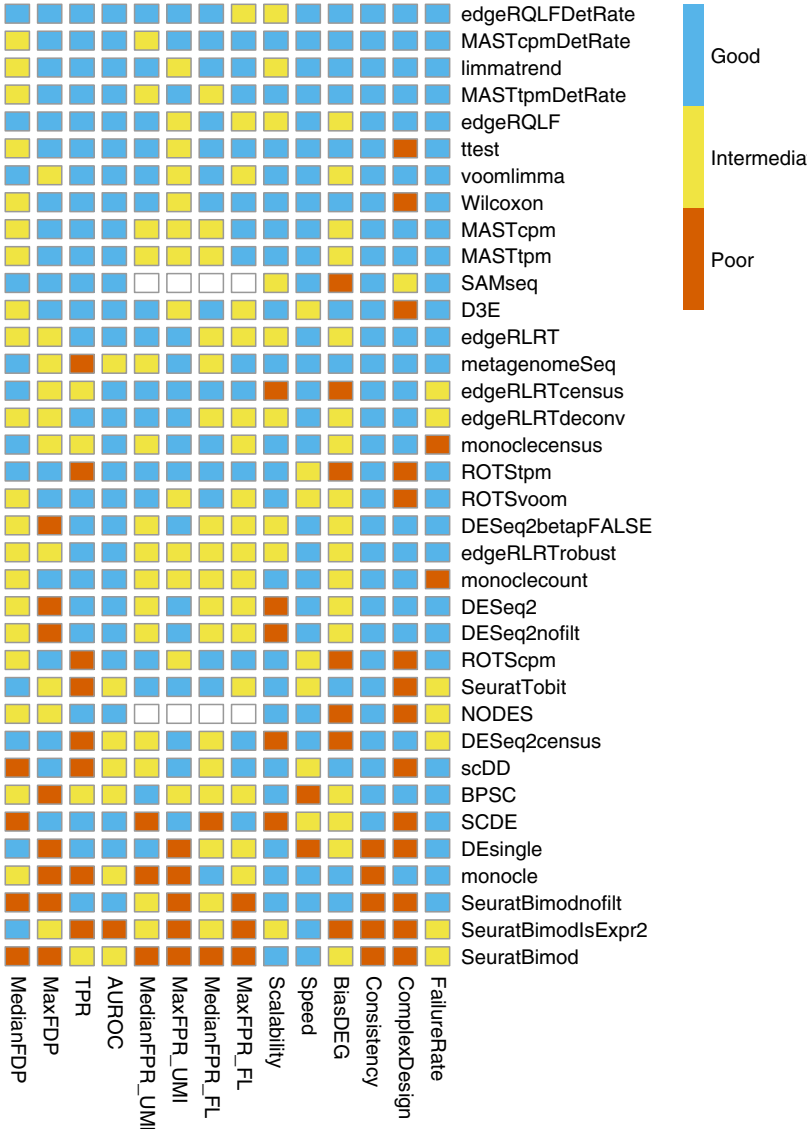

scRNA-seq analysis overview
RaukR 2018
Raw data: fastq files
Mapping & Gene expression estimate
QC: Remove low Q cells Remove contaminants
Data: Expression profiles
Data normalization
Gene set selection
Batch effect removal
Removal of other confounders
Visualization / Dimensionality reduction
Defining cell types/lineages
Clustering methods
Trajectory assignment
Gene signatures
Verification experiments

Differential expression in scRNAseq data

- scRNAseq is affected by higher noise (technical and biological)
- Usually scRNAseq data have many more replicates, can make use of the distributions for each gene, not only the mean + dispersion.
- What is used:
  - Bulk methods: edgeR, limma, DESeq, SAMseq
  - SC methods: MAST, SCDE, Monocle, D3E
  - Classical tests: Kruskal-Wallis, ROTS, t-test, RandomForest classifier, LRT, Kolmogorov-Smirnov test, Wilcox-rank-sum test, etc.

	Short name	Method	Software version	Input	Available from	Reference
	BPSC	BPSC	BPSC 0.99.0/1	CPM	GitHub	[11]
	D3E	D3E	D3E 1.0	raw counts	GitHub	[12]
	DESeq2	DESeq2	DESeq2 1.14.1	raw counts	Bioconductor	[13]
	DESeq2betapFALSE	DESeq2 without beta prior	DESeq2 1.14.1	raw counts	Bioconductor	[13]
	DESeq2census	DESeq2	DESeq2 1.14.1	Census counts	Bioconductor	[13]
	DESeq2nofilt	DESeq2 without the built-in independent filtering	DESeq2 1.14.1	raw counts	Bioconductor	[13]
	DEsingle	DEsingle	DEsingle 0.1.0	raw counts	GitHub	[14]
	edgeRLRT	edgeR/LRT	edgeR 3.19.1	raw counts	Bioconductor	[15–17]
	edgeRLRTcensus	edgeR/LRT	edgeR 3.19.1	Census counts	Bioconductor	[15–17]
	edgeRLRTdeconv	edgeR/LRT with deconvolution normalization	edgeR 3.19.1, scran 1.2.0	raw counts	Bioconductor	[15, 17, 18]
	edgeRLRTrobust	edgeR/LRT with robust dispersion estimation	edgeR 3.19.1	raw counts	Bioconductor	[15–17, 19]
	edgeRQLF	edgeR/QLF	edgeR 3.19.1	raw counts	Bioconductor	[15, 16, 20]
	edgeRQLFDetRate	edgeR/QLF with cellular detection rate as covariate	edgeR 3.19.1	raw counts	Bioconductor	[15, 16, 20]
	limmatrend	limma-trend	limma 3.30.13	log_2(CPM)	Bioconductor	[21, 22]
	MASTcpm	MAST	MAST 1.0.5	log_2(CPM+1)	Bioconductor	[23]
	MASTcpmDetRate	MAST with cellular detection rate as covariate	MAST 1.0.5	log_2(CPM+1)	Bioconductor	[23]
	MASTtpm	MAST	MAST 1.0.5	log_2(TPM+1)	Bioconductor	[23]
	MASTtpmDetRate	MAST with cellular detection rate as covariate	MAST 1.0.5	log_2(TPM+1)	Bioconductor	[23]
	metagenomeSeq	metagenomeSeq	metagenomeSeq 1.16.0	raw counts	Bioconductor	[24]
	monocle	monocle (tobit)	monocle 2.2.0	TPM	Bioconductor	[25]
	monoclecensus	monocle (Negative Binomial)	monocle 2.2.0	Census counts	Bioconductor	[25, 26]
	monoclecount	monocle (Negative Binomial)	monocle 2.2.0	raw counts	Bioconductor	[25]
	NODES	NODES	NODES 0.0.0.9010	raw counts	Author-provided link	[27]
	ROTScpm	ROTS	ROTS 1.2.0	CPM	Bioconductor	[28, 29]
	ROTStpm	ROTS	ROTS 1.2.0	TPM	Bioconductor	[28, 29]
	ROTSvoom	ROTS	ROTS 1.2.0	voom-transformed raw counts	Bioconductor	[28, 29]
	SAMseq	SAMseq	samr 2.0	raw counts	CRAN	[30]
	scDD	scDD	scDD 1.0.0	raw counts	Bioconductor	[31]
	SCDE	SCDE	scde 2.2.0	raw counts	Bioconductor	[32]
	SeuratBimod	Seurat (bimod test)	Seurat 1.4.0.7	raw counts	GitHub	[33, 34]
	SeuratBimodnofilt	Seurat (bimod test) without the internal filtering	Seurat 1.4.0.7	raw counts	GitHub	[33, 34]
	SeuratBimodIsExpr2	Seurat (bimod test) with internal expression threshold set to 2	Seurat 1.4.0.7	raw counts	GitHub	[33, 34]
	SeuratTobit	Seurat (tobit test)	Seurat 1.4.0.7	TPM	GitHub	[25, 33]
	ttest	t-test	stats (R v 3.3)	TMM-normalized TPM	CRAN	[16, 35]
	voomlimma	voom-limma	limma 3.30.13	raw counts	Bioconductor	[21, 22]
	Wilcoxon	Wilcoxon test	stats (R v 3.3)	TMM-normalized TPM	CRAN	[16, 36]

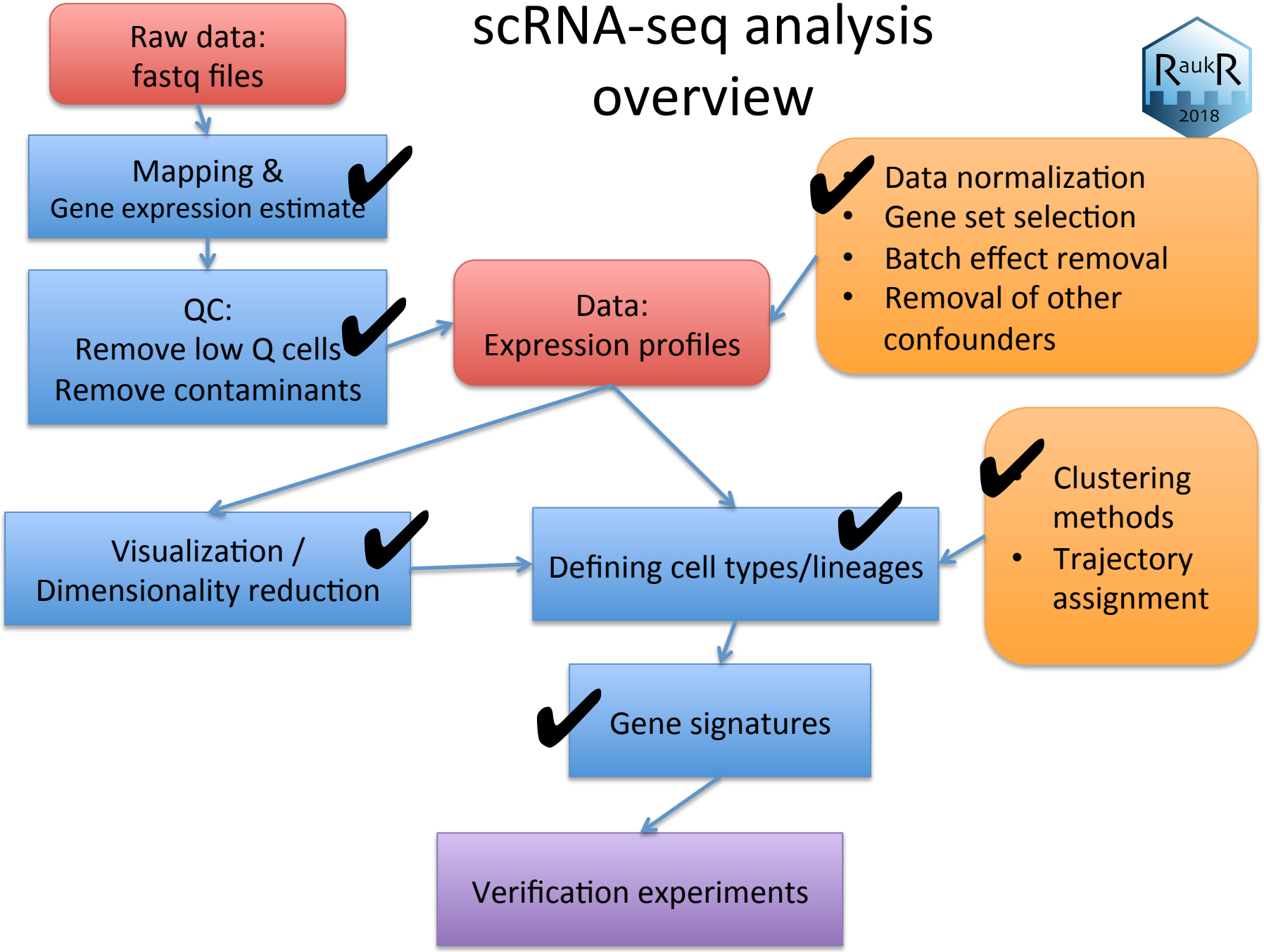
(Soneson & Robinson, *Nature Methods* 2018)



- EdgeR & Limma seem to work well
- MAST ranked high among scRNA-seq methods

(Soneson & Robinson, *Nature Methods* 2018)

scRNA-seq analysis overview



What more can you do?

- Allelic expression
- X chromosome inactivation
- Variant calling
- Copy-number variation
- Alternative splicing

Combination methods

- CITE-seq & REAP-seq − barcoded antibodies to measure protein markers + RNA-seq
- Proximity ligation assay (PLA) + RNA-seq − internal proteins
- T-cell receptor VD(J) profiling + RNA-seq
- Genome + RNA (WGS, ATAC-seq, Bis-seq)
- CRISPR-mediated perturbations + RNA-seq
- Lineage tracing with CRISPR barcodes
- ncRNA methods − single cell small RNA libraries and total RNA libraries
- Combination with spatial methos (*in situs*, smRNA fish, Spatial transcriptomics)

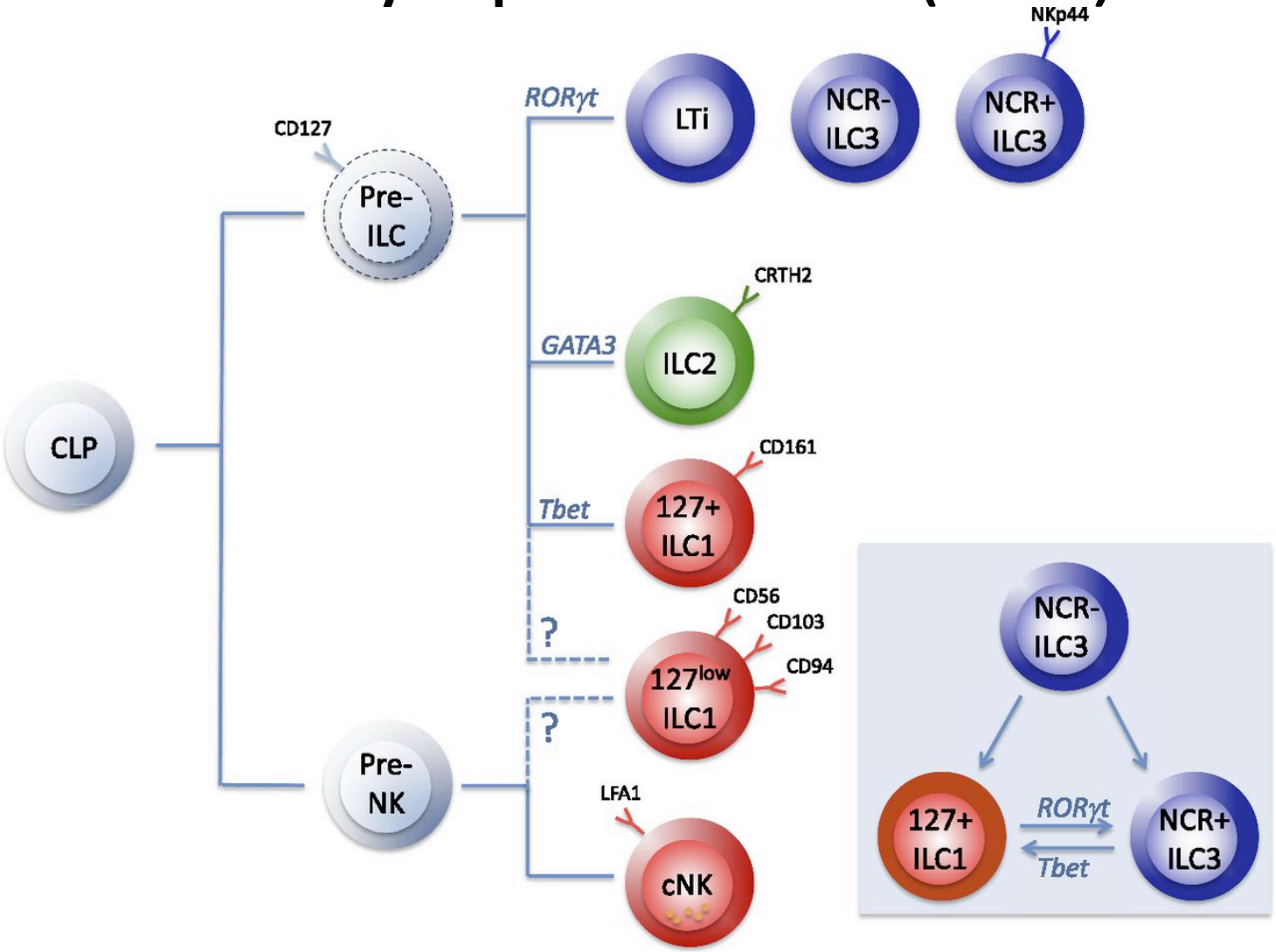
Resources

- Tutorials and lectures from our NBIS course: https://nbisweden.github.io/workshop-scRNAseq/
- Other 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 experiment objects for many datasets: https://hemberg-lab.github.io/scRNA.seq.datasets/
- Conquer datasets - salmon pipeline for many different datasets: http://imlspenticton.uzh.ch:3838/conquer/

Presentation of tutorials

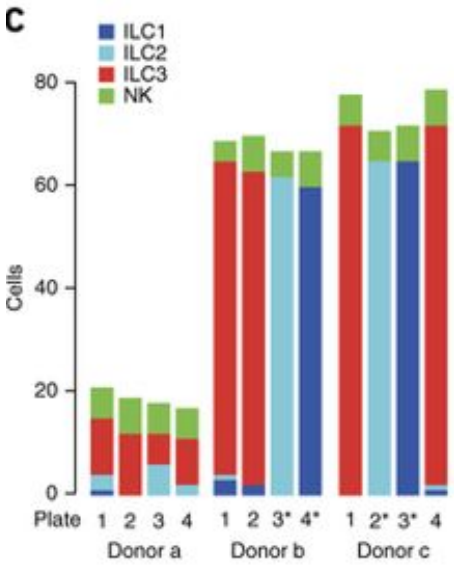
- **Seurat -** Single-cell RNA-seq data workflow with Seurat including filtering, QC and clustering.
- **Scater -** Quality Control with Scater package and batch correction with ComBat
- **SC3 -** Single-cell RNA-seq concensus clustering and with the SC3 package.

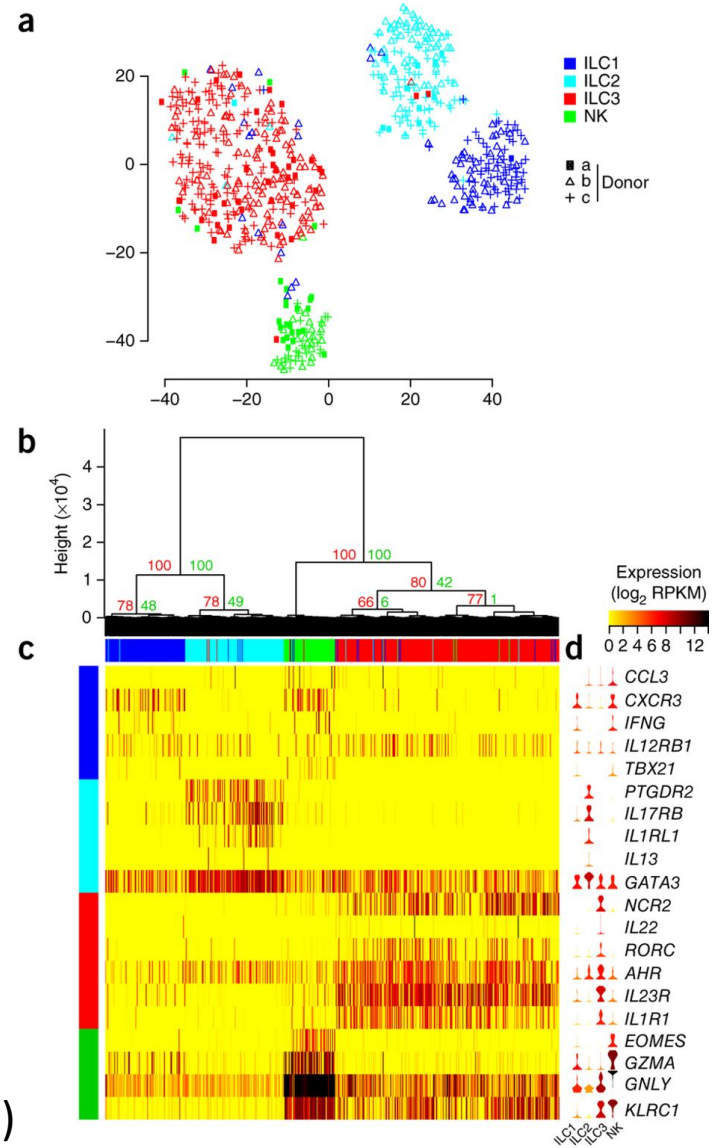
Innate lymphoid cells (ILCs)



(Hazenberg & Spitz, *Blood* 2014)

Innate lymphoid cells (ILCs)





(Björklund *et al. Nature Immunology* 2016)