Quality Control and Normalization of Single Cell RNA-seq Data

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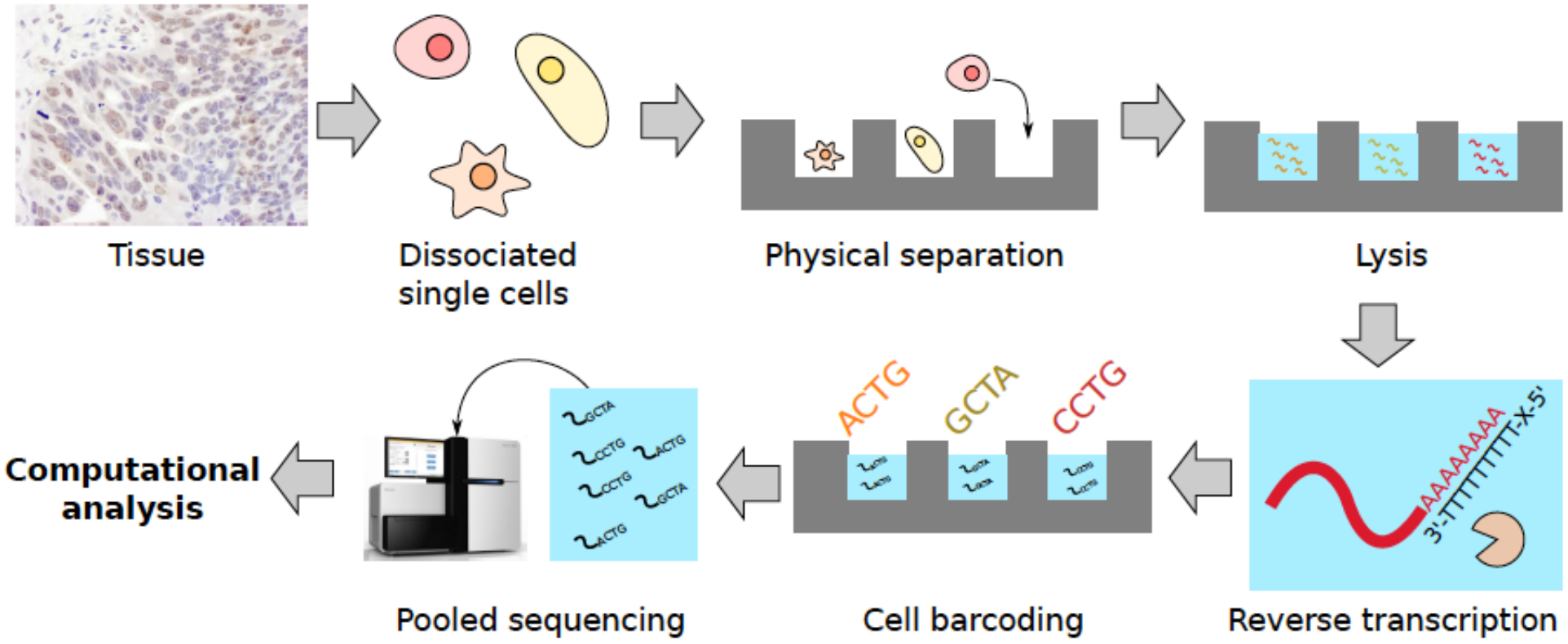
Single cell RNA-sequencing (scRNA-seq)

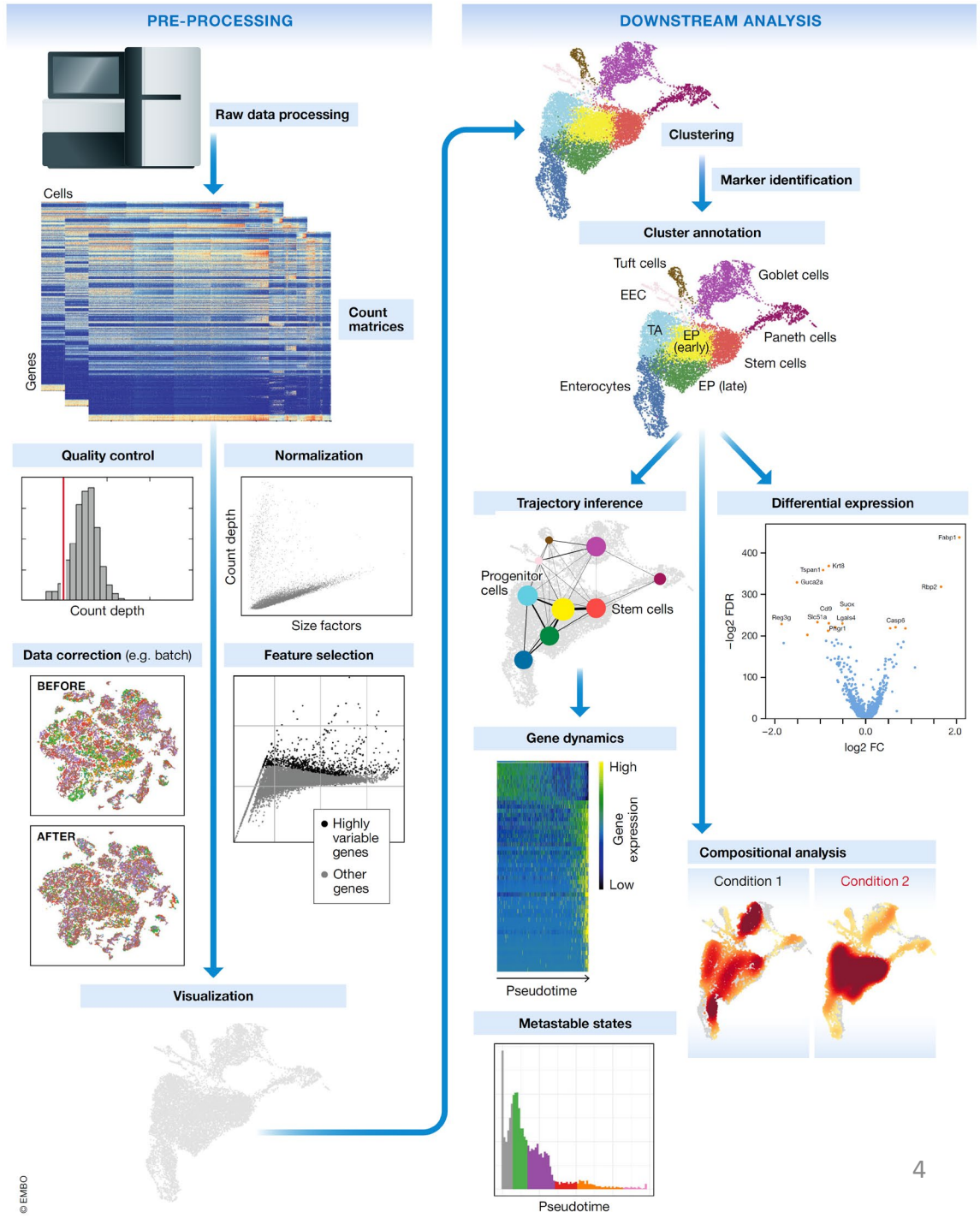


scRNA-seq Data Analysis

Our goal is to derive/extract real biology from technically noisy data

scRNA-seq Data Analysis

- Preprocessing:
  - Reads to count matrix
  - Quality control (QC)
  - Normalization
  - Batch correction
  - Feature selection
- Downstream
  - Cell type identification (clustering/classification)
  - Trajectory inference
  - Differential expression
  - Compositional analysis
  - Co-expression network analysis



Luecken and Theis (MSB 2019)

scRNA-seq Data Analysis

- Preprocessing:
  - Reads to count matrix ✔
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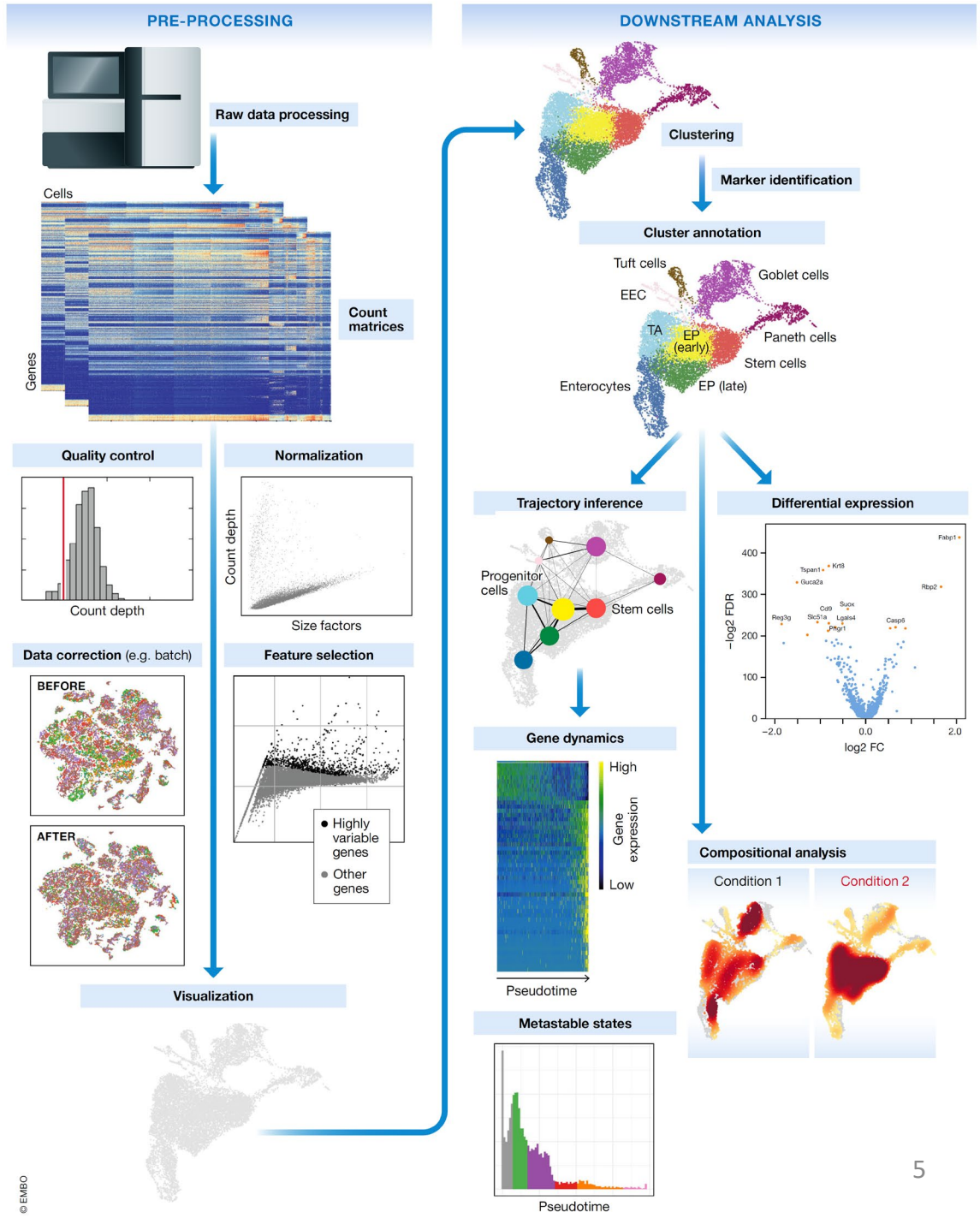


Luecken and Theis (MSB 2019)

Course materials

https://github.com/LeidenCBC/MGC-BioSB-SingleCellAnalysis2022

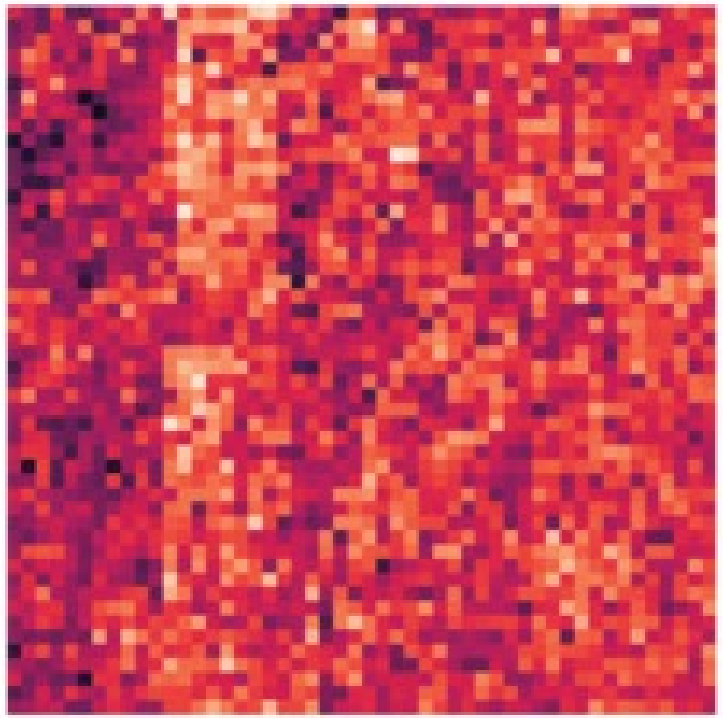
Credits: Åsa Björklund (NBIS, SciLifeLab)

Our agenda

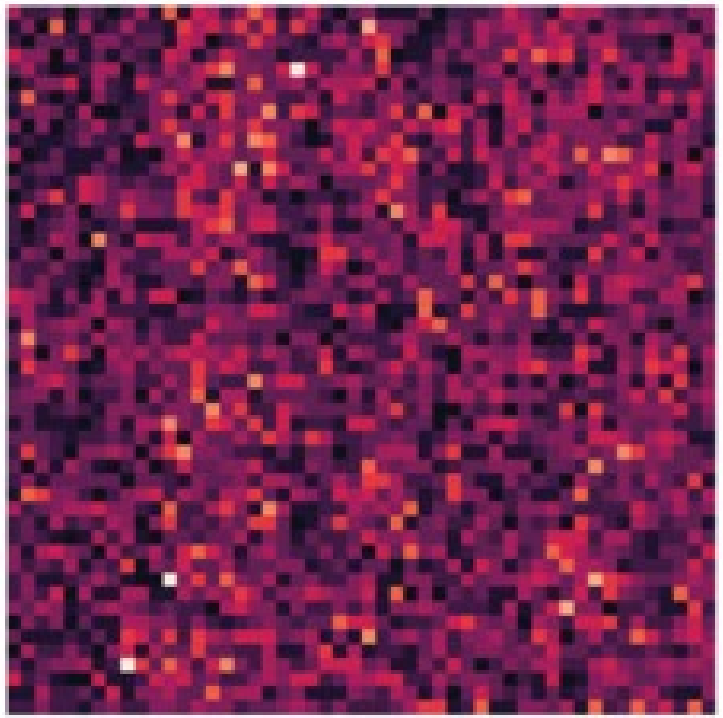
- Background on transcriptional bursting & drop-outs
- Experimental setup – what could go wrong?
- Quality control
- Normalization
- Feature selection

Which matrix best resembles scRNA-seq data?

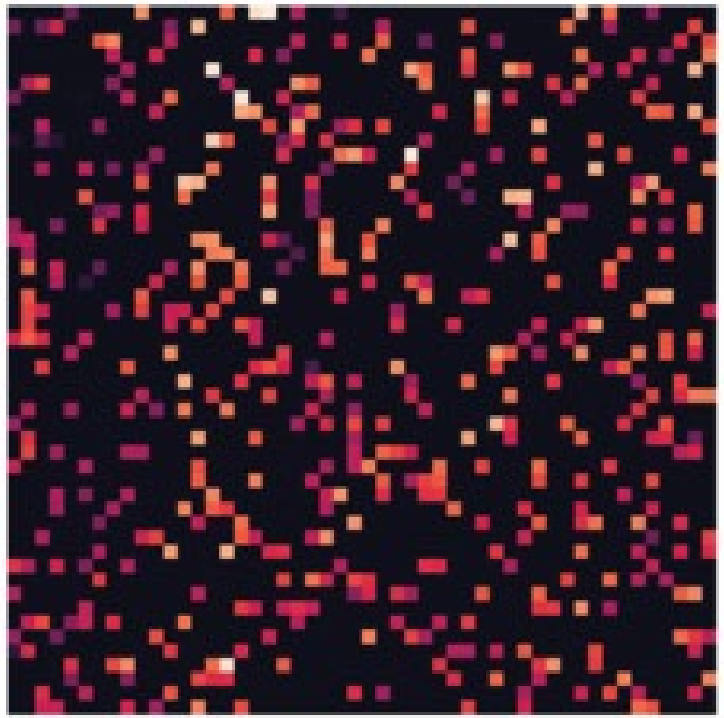
Original matrix



80% of the molecules are not counted



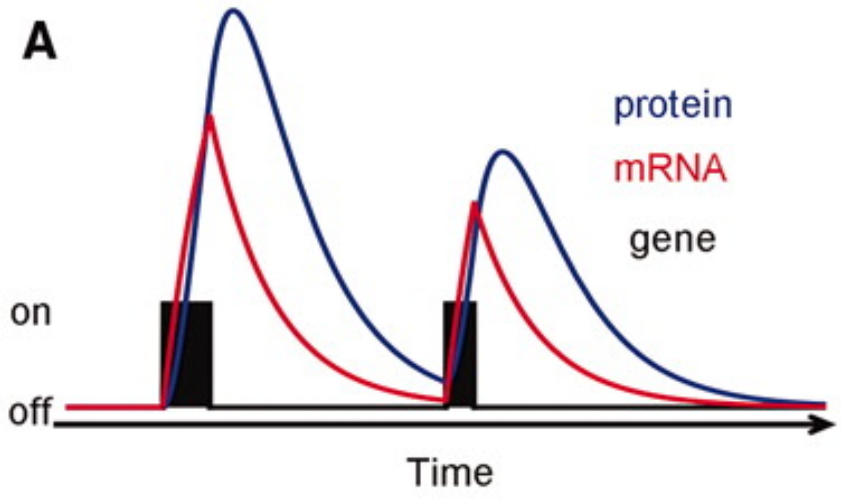
80% zeros

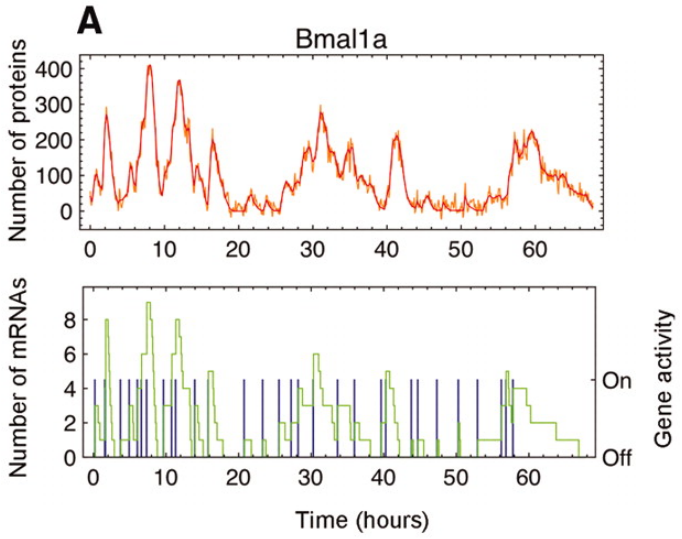


[Blog post](#) by David van Dijk

Transcriptional bursting

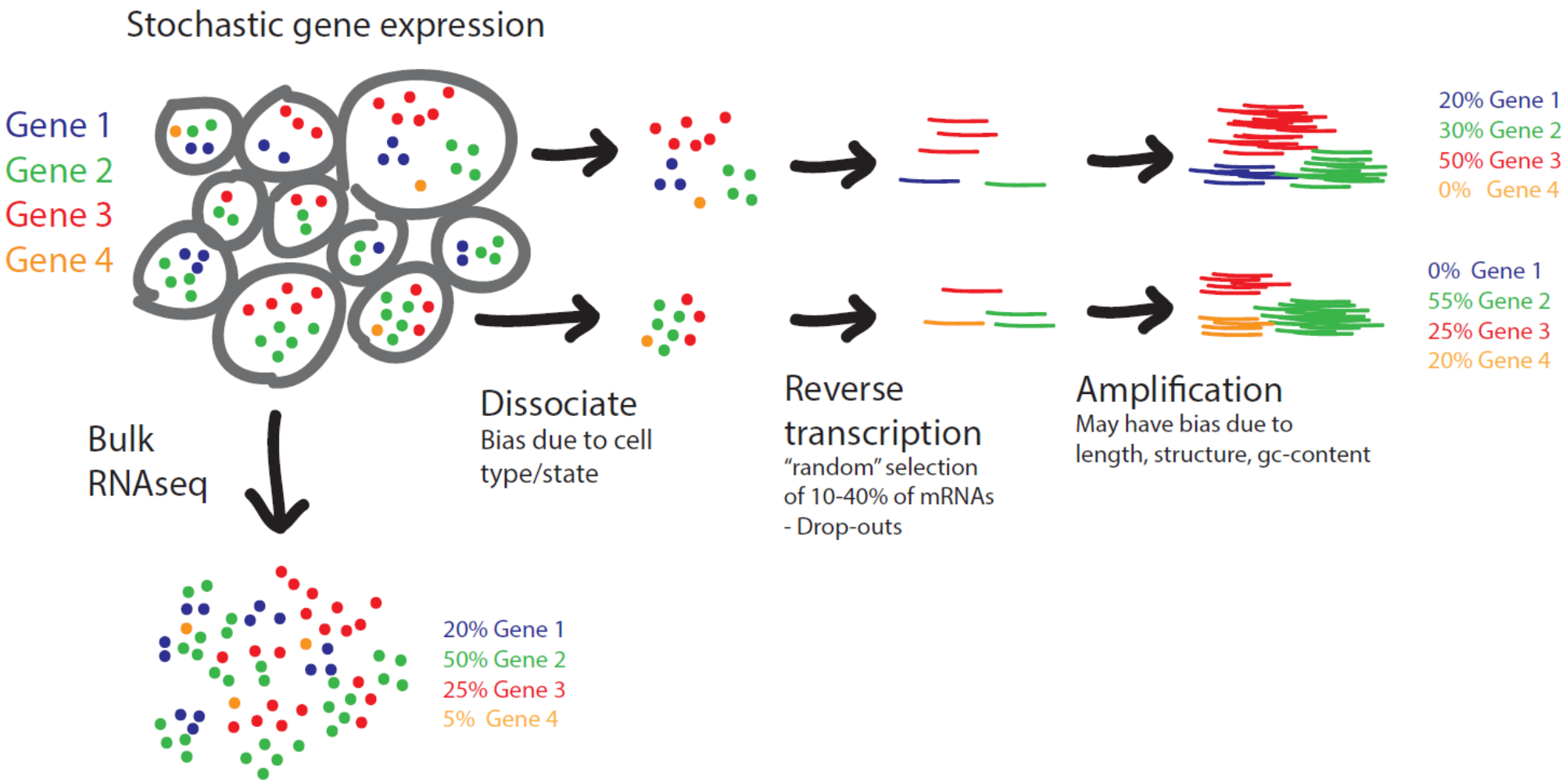
- Burst frequency and size is correlated with mRNA abundance
- Many TFs have low mean expression (and low burst frequency) and will only be detected in a fraction of the cells





Suter et al. (Science 2011)

Bursting, drop-outs and amplification bias



What could have gone wrong?

- Cell dissociation
- Cell capture
- Cell lysis
- Reverse transcription
- Preamplification
- Library preparation and sequencing

Cell dissociation

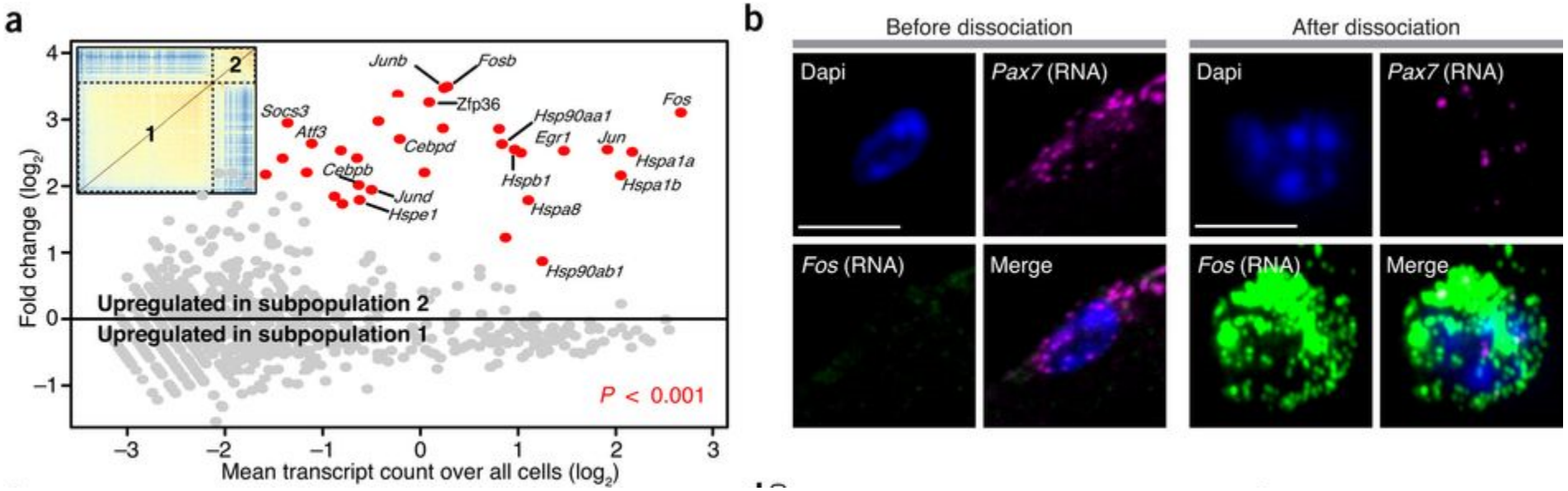
- It is critical to have healthy whole cells with no RNA leakage. Short time from dissociation to cell!

- Tissues that are hard to dissociate:
  - Laser capture microscopy (LCM)
  - Nuclei sorting

- PROBLEMS:
  - Incomplete dissociation can give multiple cells sticking together.
  - Too harsh dissociation may damage cells -> RNA degradation and RNA leakage.
  - Leakage of RNA – background signal.

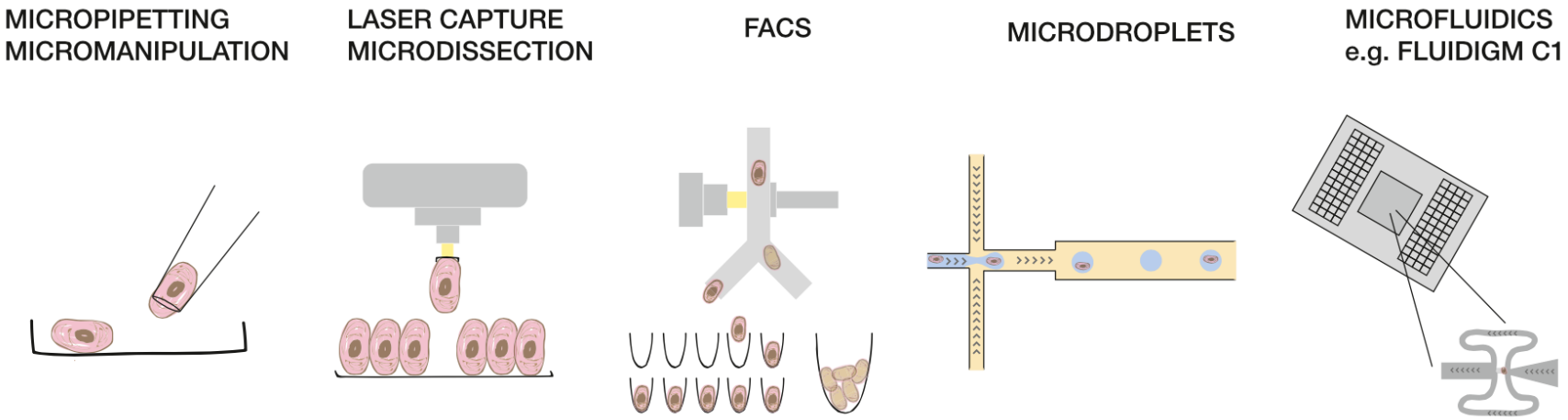
Dissociation artifacts

- Dissociation may bias your cell populations
- Dissociation protocols may introduce transcriptional changes.



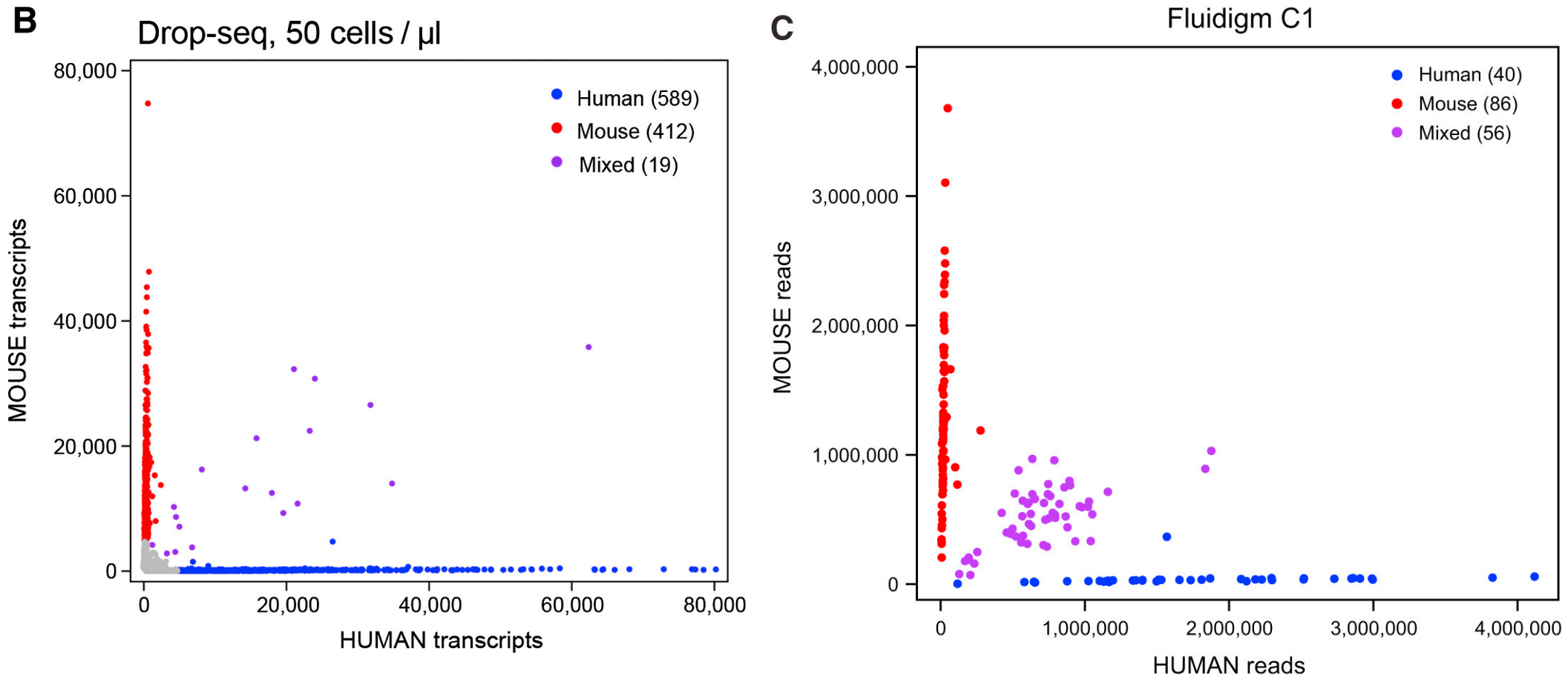
van den Brink et al. (Nature methods 2017)

Single cell capture



- PROBMLEMS:
  - All these methods may give rise to empty wells/droplets, and also duplicates or multiples of cells.
  - Size selection bias for many of the methods – dropseq has upper limit for cell size.
  - Biased selection of certain cell type(s)
  - Long time for sorting may damage the cells

scRNA-seq is not always single-cell



Macosko et al. (Cell 2015)

10x doublet rate

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~870	~500
~0.8%	~1700	~1000
~1.6%	~3500	~2000
~2.3%	~5300	~3000
~3.1%	~7000	~4000
~3.9%	~8700	~5000
~4.6%	~10500	~6000
~5.4%	~12200	~7000
~6.1%	~14000	~8000
~6.9%	~15700	~9000
~7.6%	~17400	~10000

10x Genomics

Doublets

- High number of detected genes or UMIs – can be a sign of multiples
  - But, beware so that you do not remove all cells from a larger cell type.

- After clustering – check if you have cells with signatures from multiple clusters.

- A combination of those 2 features would indicate duplicates.

- With 10X you should have a feeling for your doublet rate based on how many cells were loaded

Doublet detection

- DoubletFinder

https://github.com/chris-mcginnis-ucsf/DoubletFinder

- Scrublet

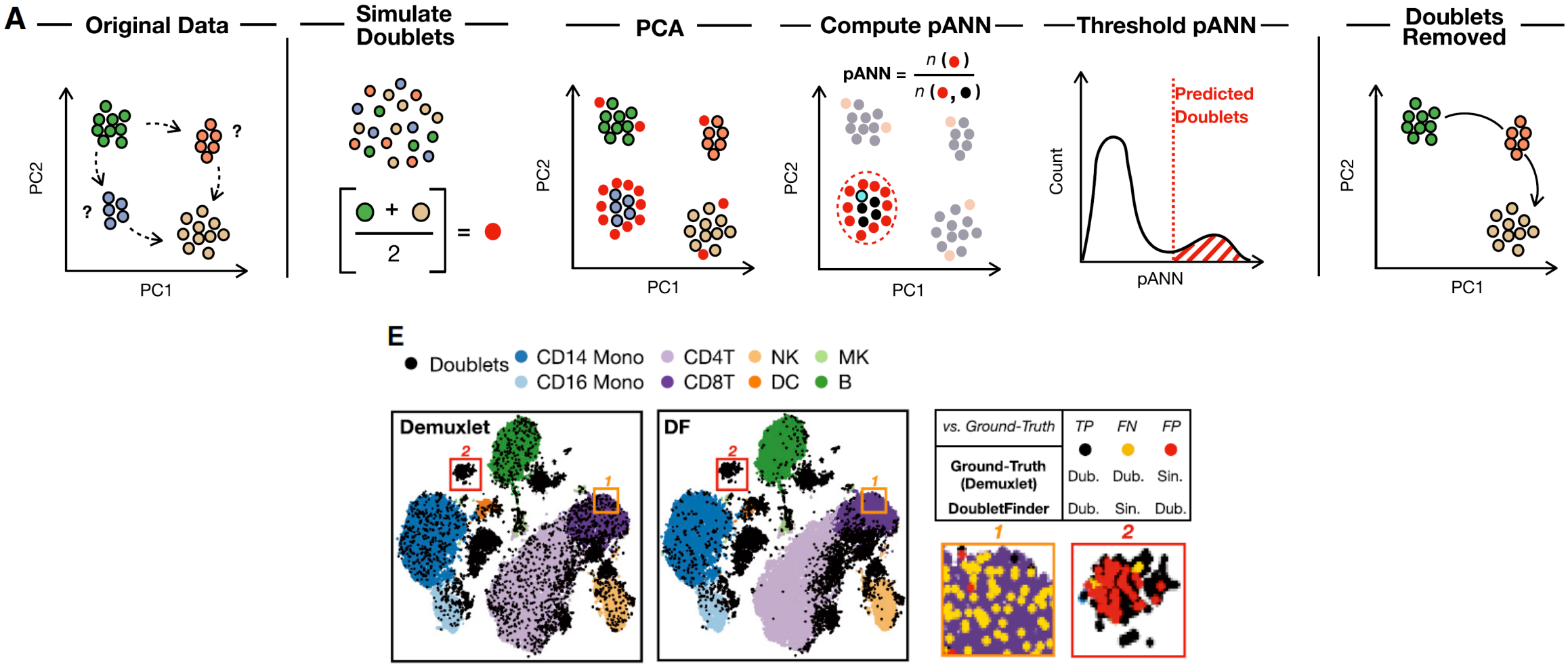
https://github.com/AllonKleinLab/scrublet

- DoubletDecon

https://github.com/EDePasquale/DoubletDecon

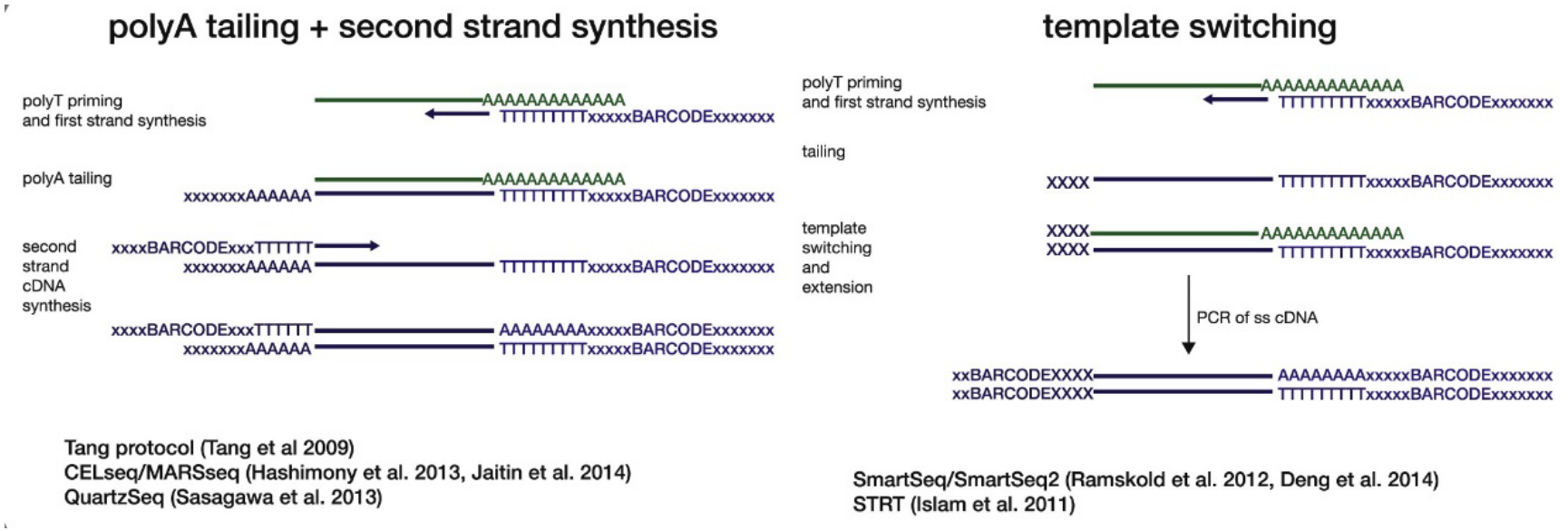
- DoubletCluster / DoubletCell in Scran

DoubletFinder



McGinnis et al. (Cell Systems 2019)

Reverse transcription



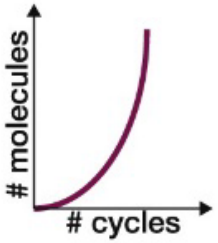
- Efficiency of reverse transcription is the key to high sensitivity.
- Drop-out rate is around 90-60% depending on the method used.
- Two libraries with the same method using the same cell type may have very different drop-out rates.

Kolodziejczyk et al. (Molecular Cell 2015)

Preamplification

PCR

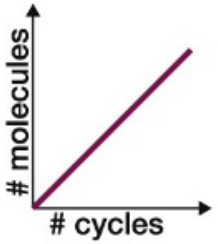
- exponential amplification
- PCR base specific biases



Tang protocol (Tang et al. 2009)
STRT (Islam et al. 2011)
SmartSeq/SmartSeq2 (Ramskold et al. 2012, Deng et al. 2014)

IVT

- linear amplification
- 3' bias due to two rounds of reverse transcription



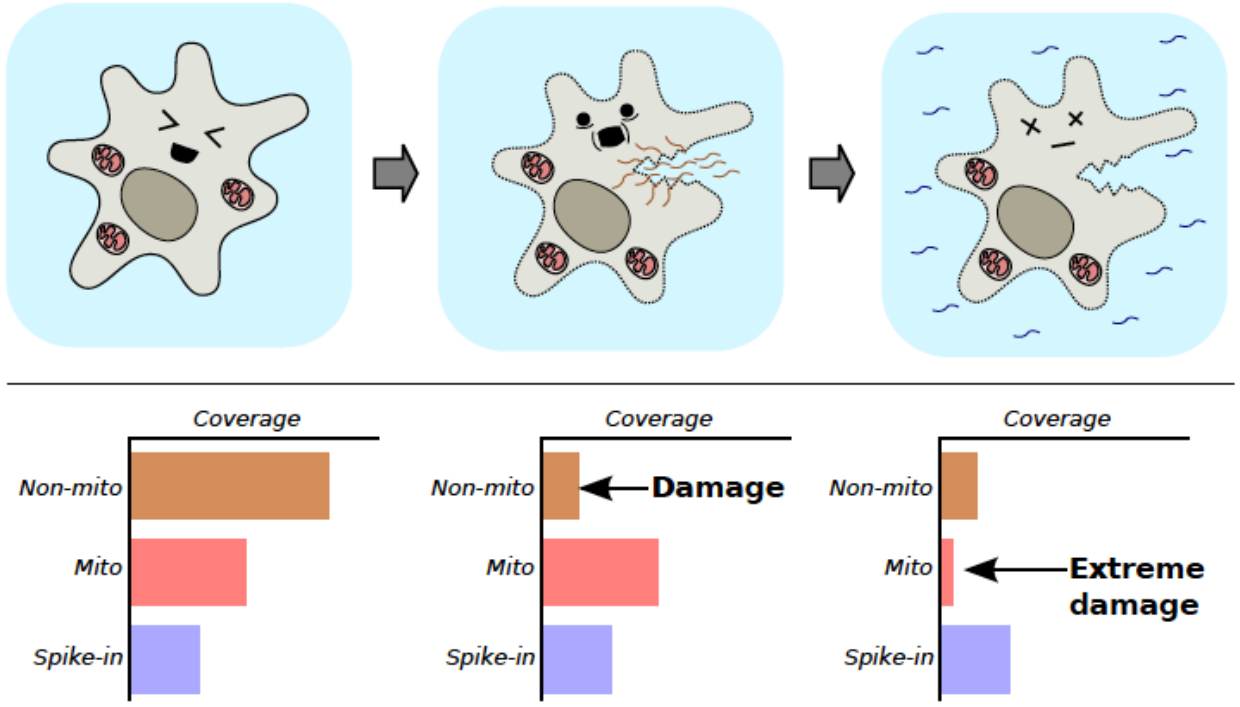
CELseq/MARSseq (Hashimony et al. 2013, Jaitin et al. 2014)

- Any amplification step will introduce a bias in the data.
- Methods that uses UMIs will control for this to a large extent, but the chance of detecting a transcript that is amplified more is higher.
- Full length methods like SmartSeq2 has no UMIs, so we cannot control for amplification bias.

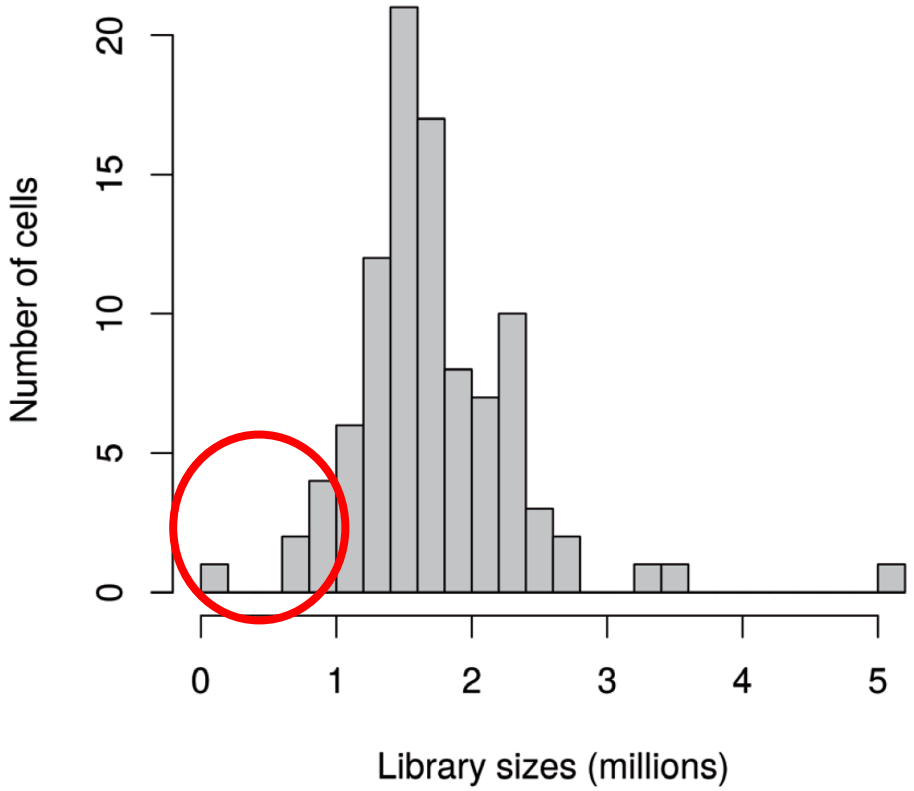
Kolodziejczyk et al. (Molecular Cell 2015)

Quality control of cells (1)

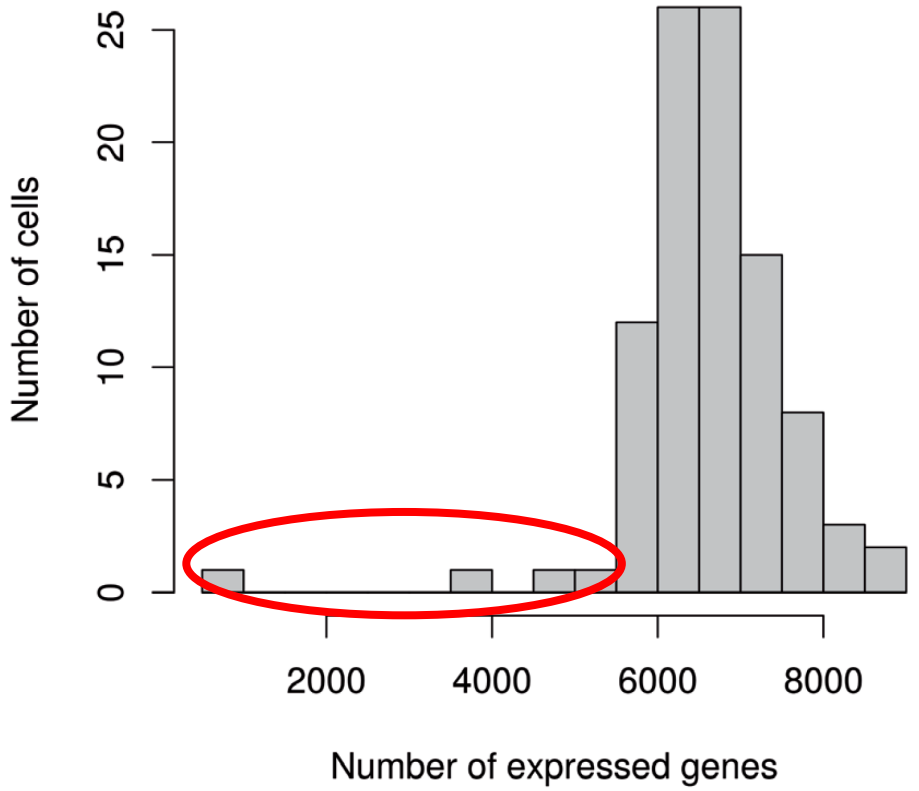
- Low sequencing depth
- Low numbers of expressed genes (i.e. any nonzero count)
- High mitochondrial content



Quality control of cells (2)

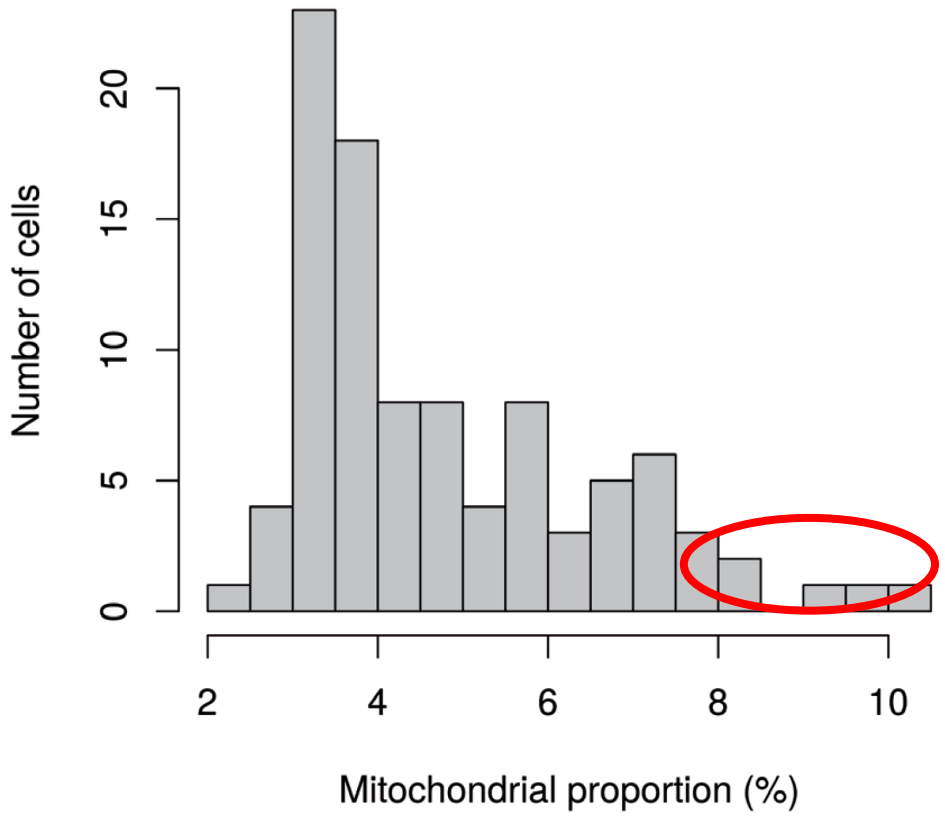


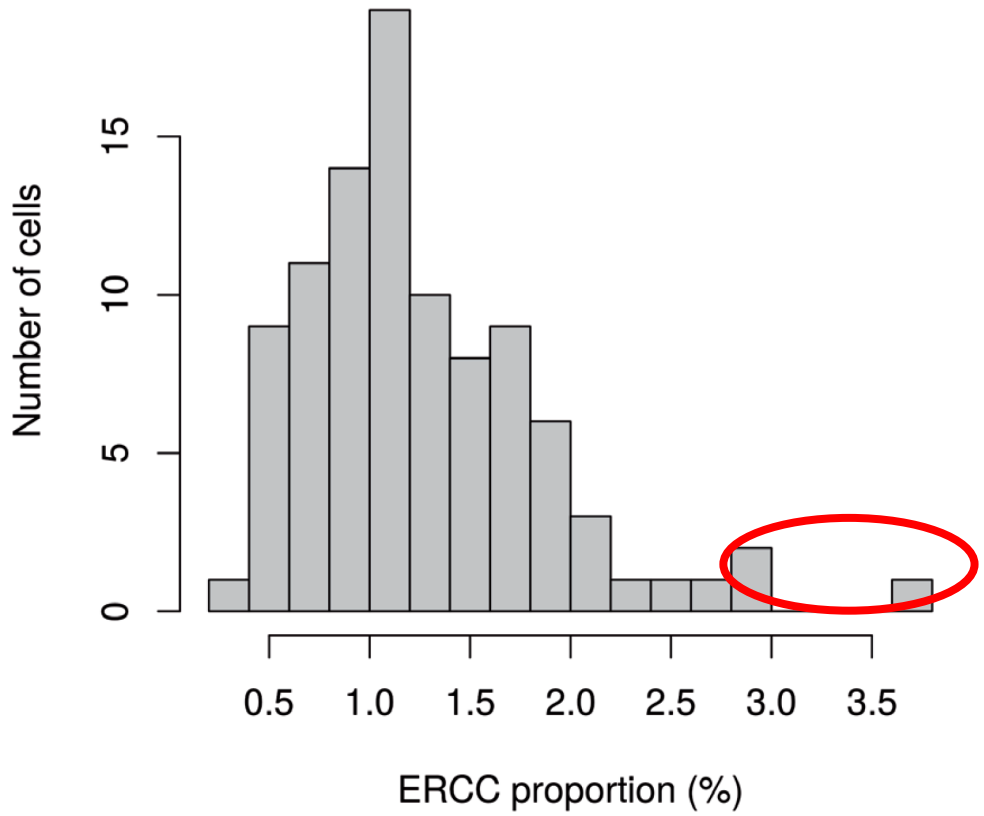
RNA has not been efficiently captured during library preparation



Diverse transcript population not captured

Quality control of cells (2)



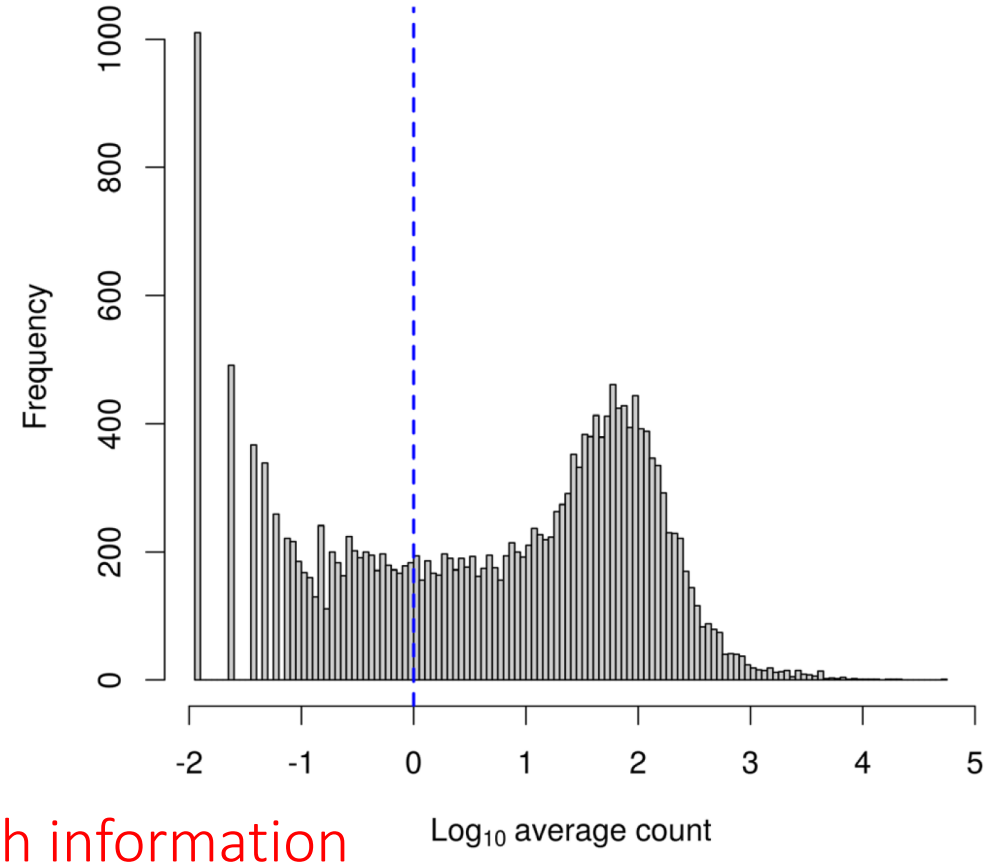


Possibly because of increased apoptosis
and/or loss of cytoplasmic RNA from lysed cells

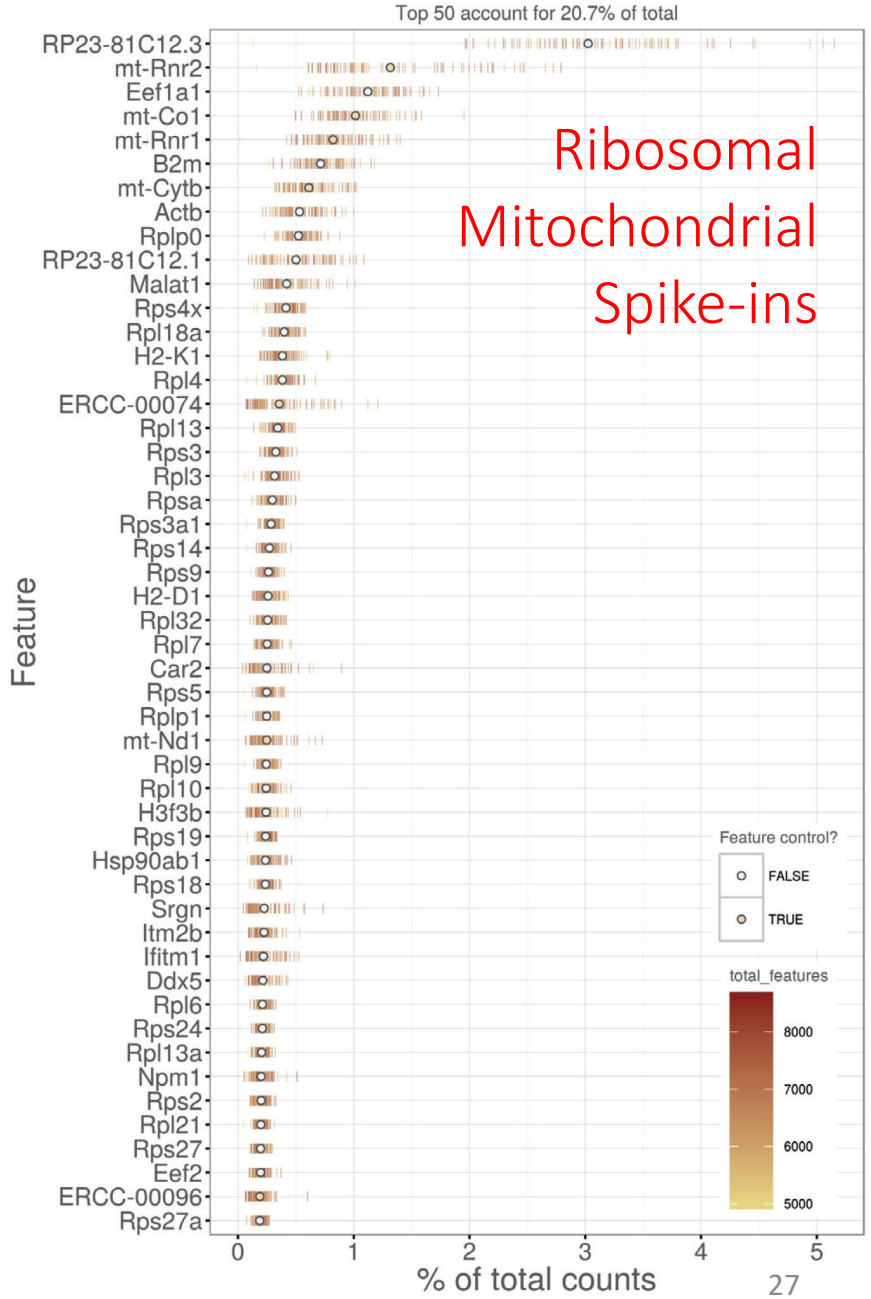
Deciding on cutoffs for filtering

- Do you have a homogeneous population of cells with similar sizes?
- Is it possible that you will remove cells from a smaller cell type?
- Examine PCA/tSNE/UMAP before and after filtering and make a judgment on whether to remove more or less cells.

Quality control of genes



Not enough information for reliable statistical inference



Ribosomal
Mitochondrial
Spike-ins

QC (pitfalls and recommendations)

- Perform QC by finding outlier peaks in the number of genes, the count depth and the fraction of mitochondrial reads. Consider these covariates jointly instead of separately.

- Be as permissive of QC thresholding as possible, and revisit QC if downstream clustering cannot be interpreted.

- If the distribution of QC covariates differ between samples, QC thresholds should be determined separately for each sample to account for sample quality differences as in Plasschaert et al (2018).

Luecken and Theis (MSB 2019)

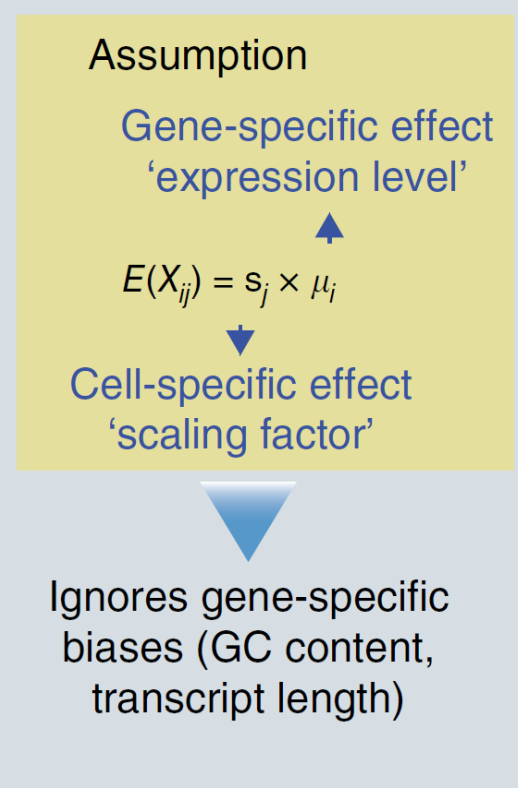
Check!

- Always go back to QC-stats after doing downstream analysis (clustering/lineage analysis etc.)

- Are your findings correlated with technical factors?

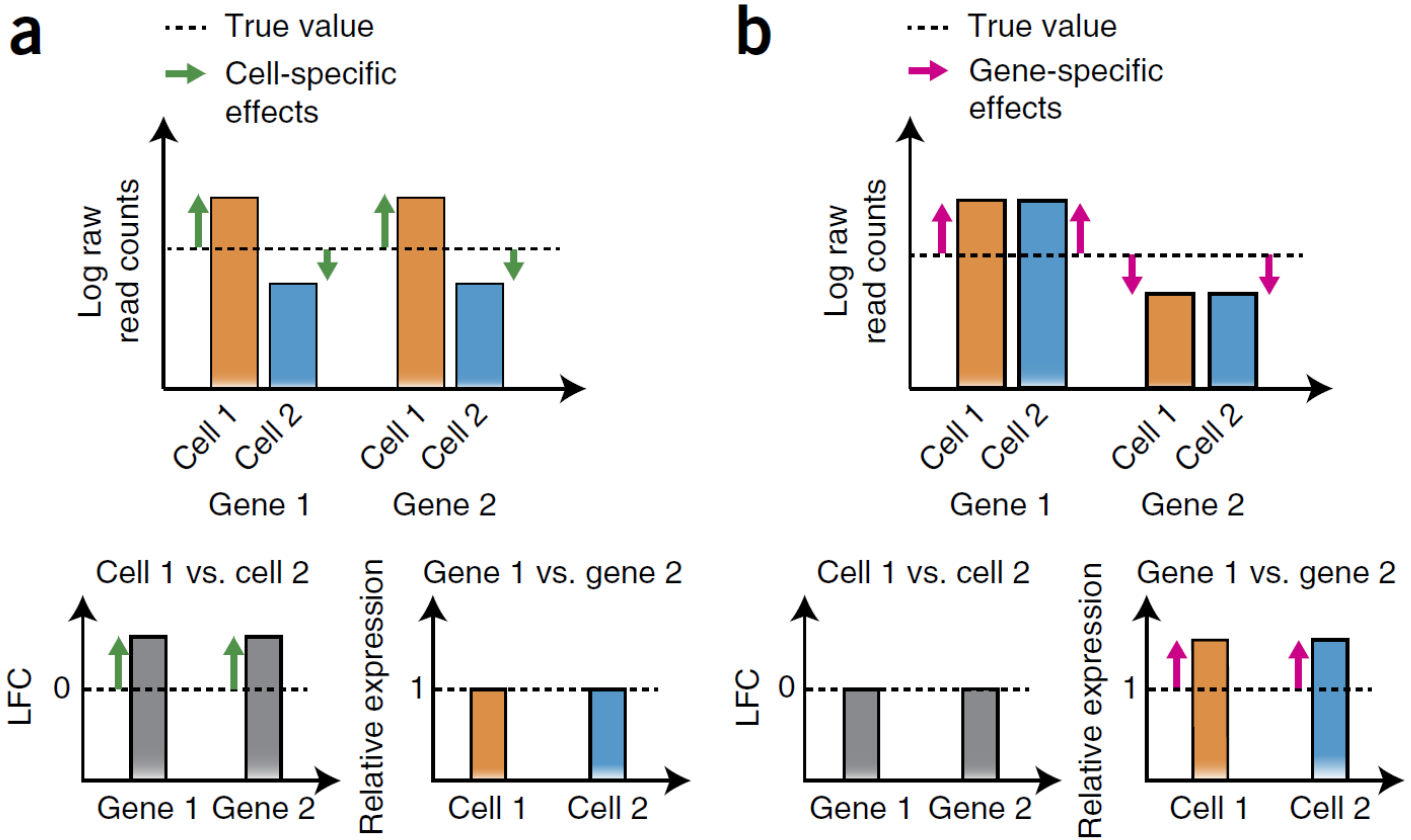
Normalization

Normalization (1)



Vallejos et al. (Nat Methods 2017)

Cell- and gene-specific effects in RNA-seq experiments



Vallejos et al. (Nat Methods 2017)

Which effects are NOT removed by UMIs?

c

	Cell-specific effects	Gene-specific effects	Not removed by UMIs
Sequencing depth	✓		✓
Amplification	✓	✓	
Capture and RT efficiency	✓	✓	✓
Gene length		✓	
GC content	✓	✓	✓
mRNA content	✓		✓

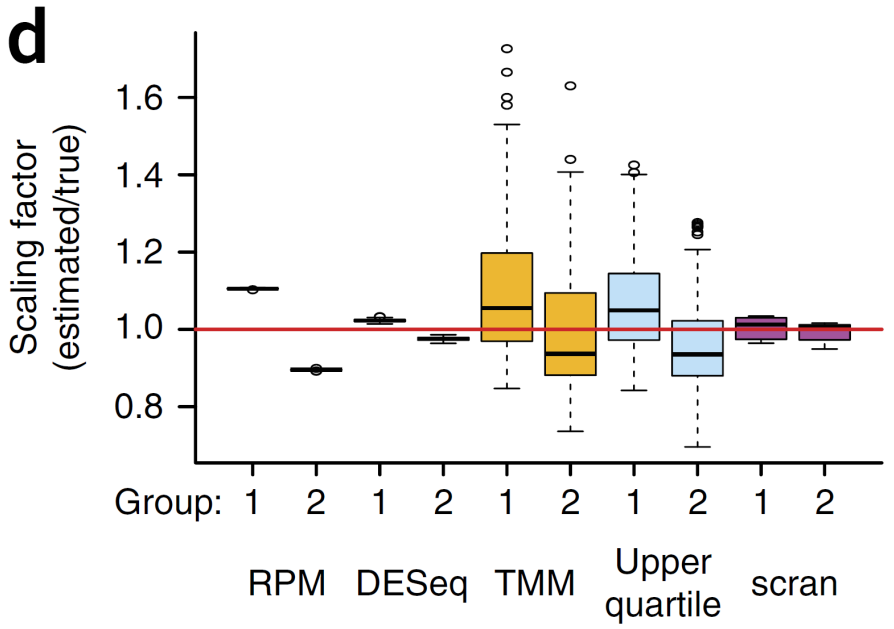
Normalization (2)

- The aim is bring all cells onto the same distribution to remove biases
- We want to preserve biological variability, not introduce new technical variation
- Primary source of bias is sequencing depth – scale down counts accordingly
- Need a method that is robust to sparsity and composition bias

Vallejos et al. (Nat Methods 2017)

What is different from bulk RNA-seq?

- Noise
  - Low mRNA content per cell
  - Variable mRNA capture
  - Variable sequencing depth
- Different cell types in the same sample
- Bulk RNA-seq normalization methods (FPKM, CPM, TPM, upperquartile) are based on per-gene statistics –> not suitable for zero-inflated data



Vallejos et al. (Nat Methods 2017)

Normalization methods

1. Size factor scaling methods
   - Log-normalization

2. Probabilistic methods
   - scTransform (Hafemeister & Satija Genome Biol 2019)
   - ZINB-WaVE (Risso et al. Nature Comm 2018)

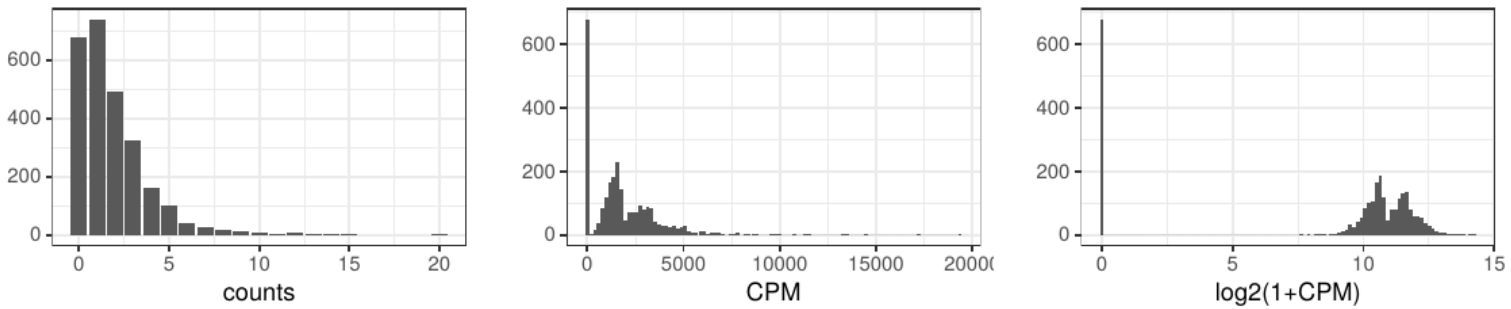
Log-normalization

$$Y_{ij} = log_e((\frac{X_{ij}}{\sum_i X_{ij}} \times 10{,}000) + 1)$$

- Simplest and most commonly used normalization strategy
- Divide all counts for each cell by a cell-specific scaling factor (i.e. size factor)
- Assumes that any cell-specific bias (e.g., in capture or amplification efficiency) affects all genes equally via scaling of the expected mean count for that cell
- Modified CPM normalization
- Seurat, scanpy, 10X Cell Ranger: log-normalization

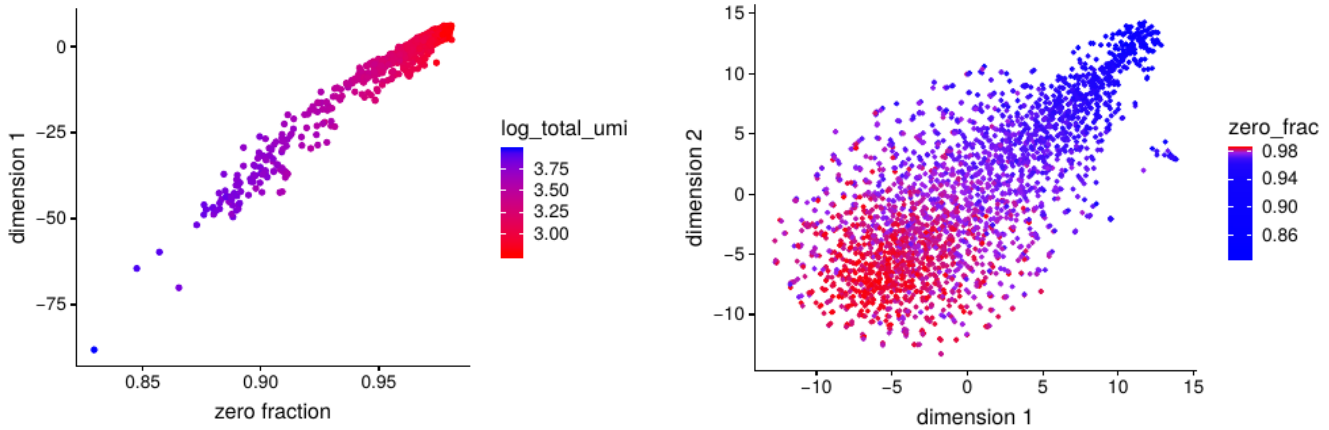
Effect of dropouts on normalization

Inflation of zero counts



(a) UMI counts (b) counts per million (CPM) (c) log of CPM

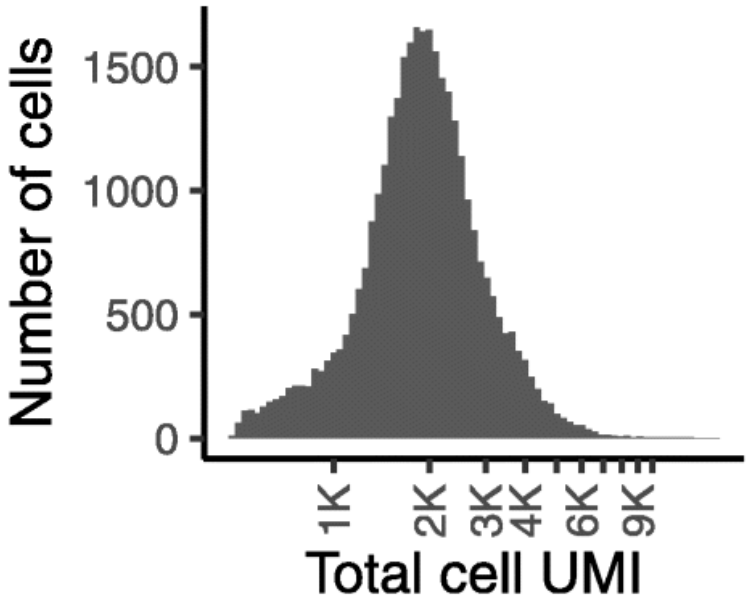
Fraction of zeros become main source of variability

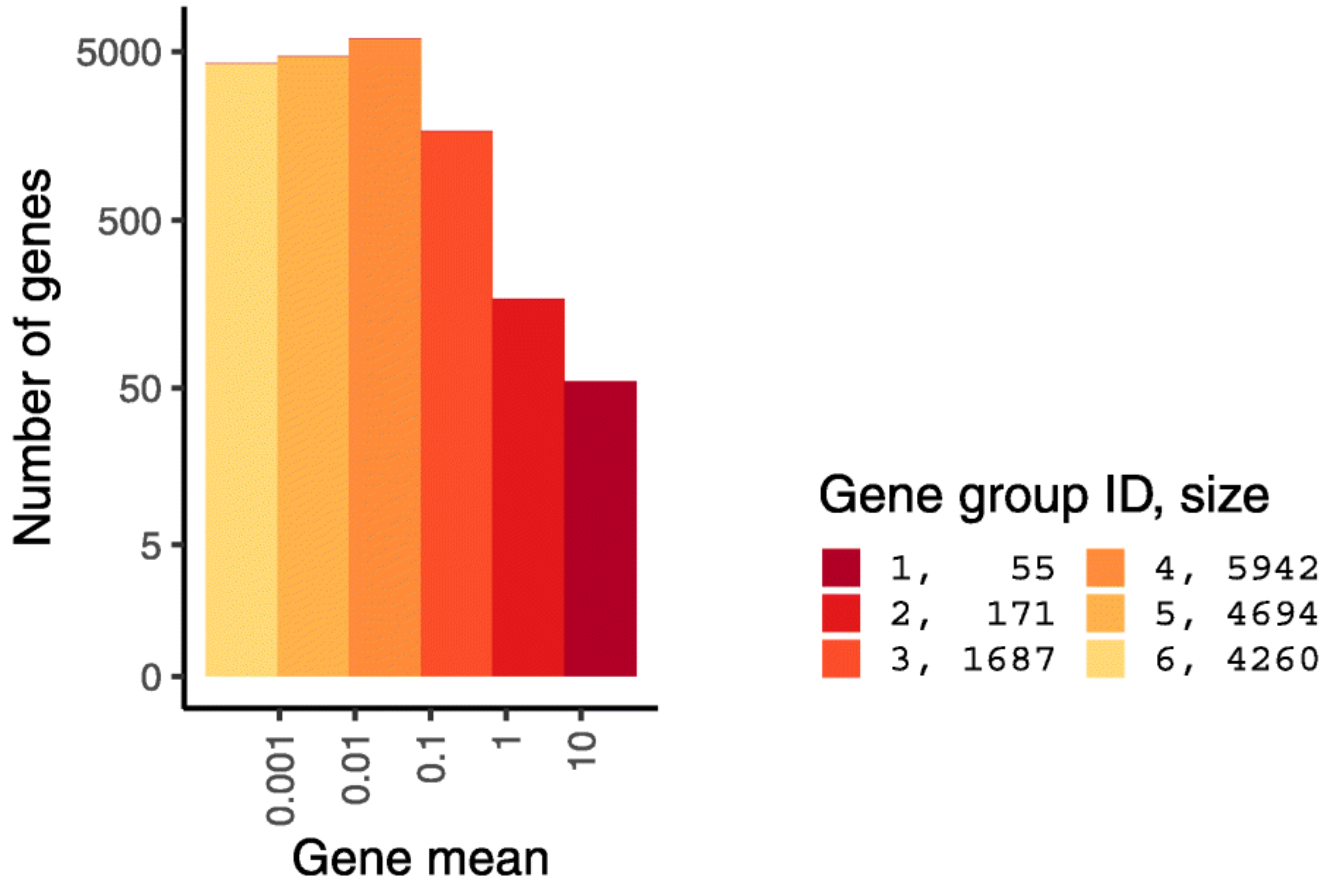


Towns et al. (bioRxiv 2019); Svensson (bioRxiv 2019)

Does log-normalization (scaling) work?

33,148 PBMCs, 10x Genomics
16,809 genes detected ≥ 5 cells

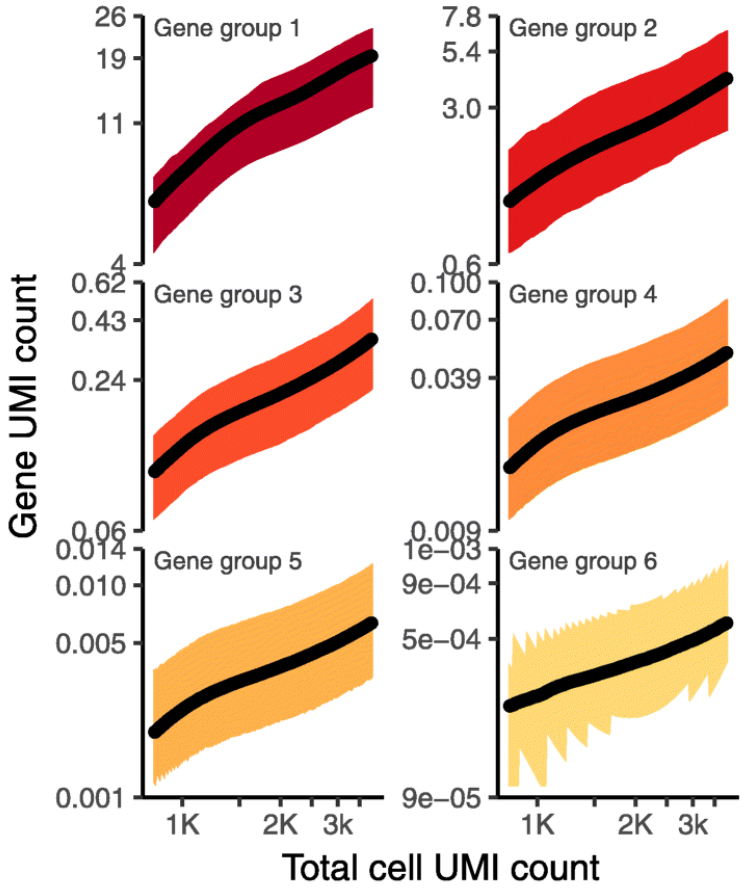




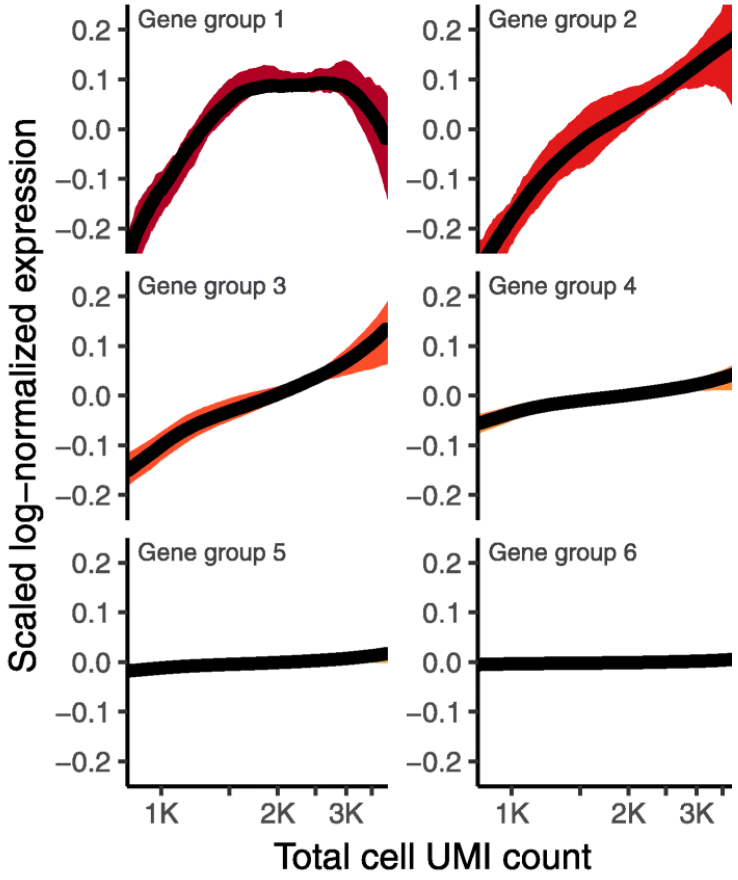
Hafemeister & Satija (Genome Biol 2019)

Does log-normalization (scaling) work?

Before normalization



After normalization



Hafemeister & Satija (Genome Biol 2019)

Modeling scRNAseq data

- Model the UMI counts for a given gene using a generalized linear model

$$\log\big(\mathbb{E}(x_i)\big) = \beta_0 + \beta_1 \log_{10} m + e_i$$

x_i: vector of UMI counts assigned to gene i

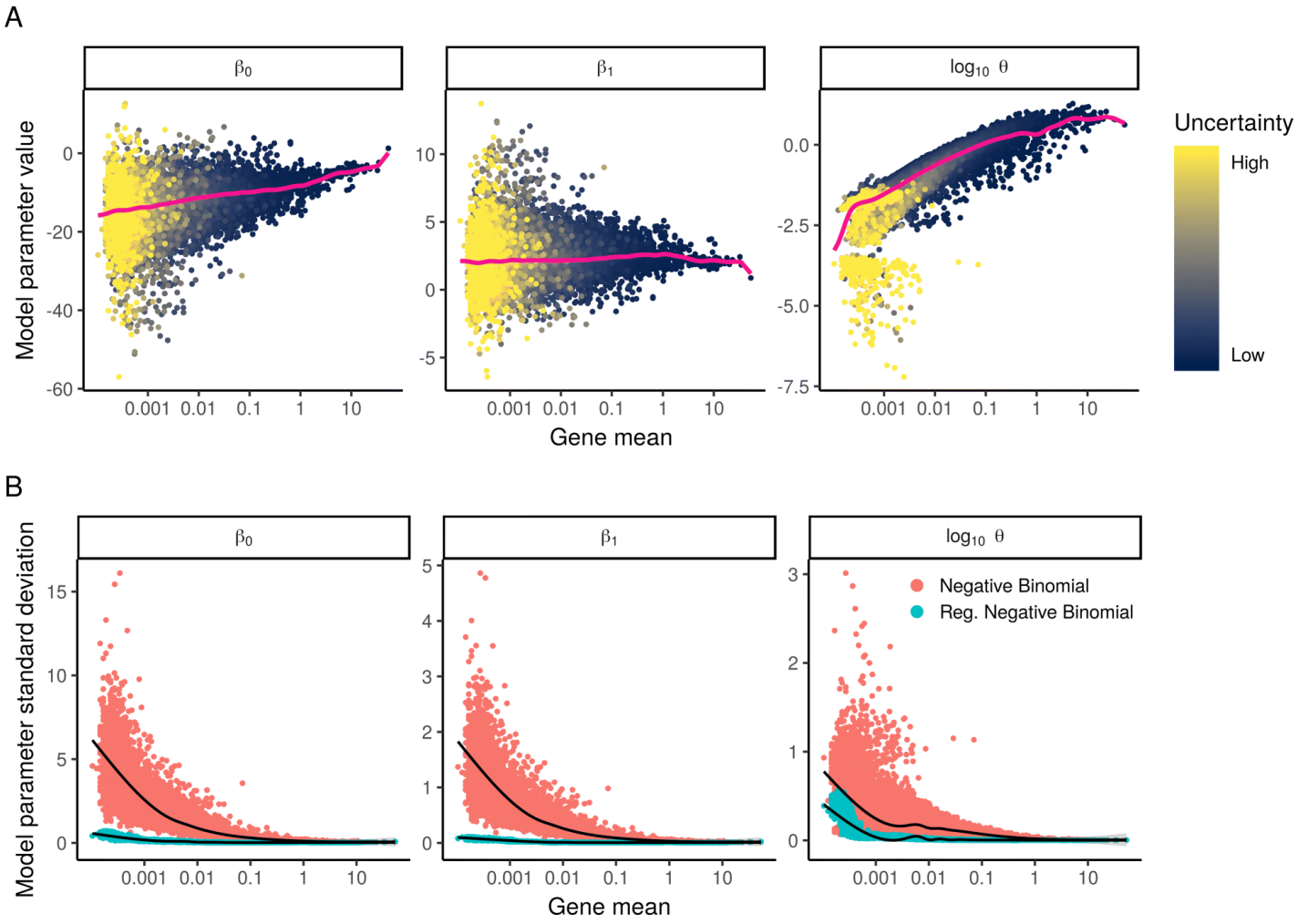
m: vector of molecules assigned to the cells, i.e., $m_j = \sum_i x_{ij}$

e_i: negative binomial (NB) error distribution, parameterized with mean μ and variance $\mu + \frac{\mu^2}{\sigma}$

Hafemeister & Satija (Genome Biol 2019)

Modeling scRNAseq data

- BUT, modeling each gene separately results in overfitting
- Solution: regularize all model parameters, including the NB dispersion parameter θ, by sharing information across genes



Hafemeister & Satija (Genome Biol 2019)

Modeling scRNAseq data

scTransform: Regularized negative binomial regression

Step1: fit independent regression models per gene

Step2: exploit the relationship of model parameter values and gene mean to learn global trends in the data (kernel regression with a normal kernel)

Step3: use the regularized regression parameters to transforms UMI counts into Pearson residuals:

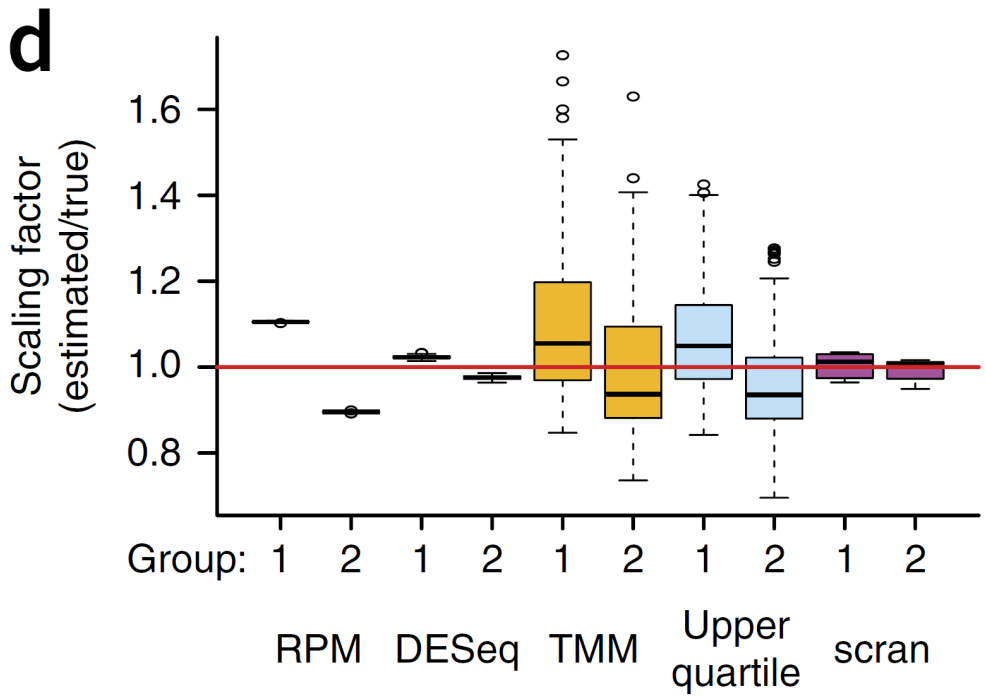
$$z_{ij} = \frac{x_{ij} - \mu_{ij}}{\sigma_{ij}},$$

$$\mu_{ij} = \exp\left(\beta_{0_i} + \beta_{1_i} \log_{10} m_j\right),$$

$$\sigma_{ij} = \sqrt{\mu_{ij} + \frac{\mu_{ij}^2}{\theta_i}},$$

Normalization (5)

- Bulk RNA-based methods: FPKM, CPM, TPM, upperquartile *(NOT APPROPRIATE)*
- Log normalization (Seurat)
- Negative binomial (Monocole)
- Zero-inflated negative binomial (ZINB) models
- scTransform (regularized NB regression)
- …



Performance Assessment and Selection of Normalization Procedures for Single-Cell RNA-Seq
Cole et al, Cell Systems 2019

Vallejos et al. (Nat Methods 2017)

Normalization (pitfalls and recommendations)

- Try both log-normalization (fast) and SCTransform. Are there differeces in downstream results? Use log-normalization if sufficient.

- There is no consensus on scaling genes to 0 mean and unit variance. We prefer not to scale gene expression.

Luecken and Theis (MSB 2019)

More on Normalization…

- Svensson, V. Droplet scRNA-seq is not zero-inflated. *Nat. Biotechnol.* **38**, 147–150 (2020). https://doi.org/10.1038/s41587-019-0379-5

- Sarkar, A., Stephens, M. Separating measurement and expression models clarifies confusion in single-cell RNA sequencing analysis. *Nat Genet* **53,** 770–777 (2021). https://doi.org/10.1038/s41588-021-00873-4

- Lause, J., Berens, P. & Kobak, D. Analytic Pearson residuals for normalization of single-cell RNA-seq UMI data. Genome Biol 22, 258 (2021). https://doi.org/10.1186/s13059-021-02451-7

Normalization vs Batch correction

- **Normalization:** occurs regardless of the batch structure and only considers technical biases.

- **Batch correction:** only occurs across batches and must consider both technical biases and biological differences.

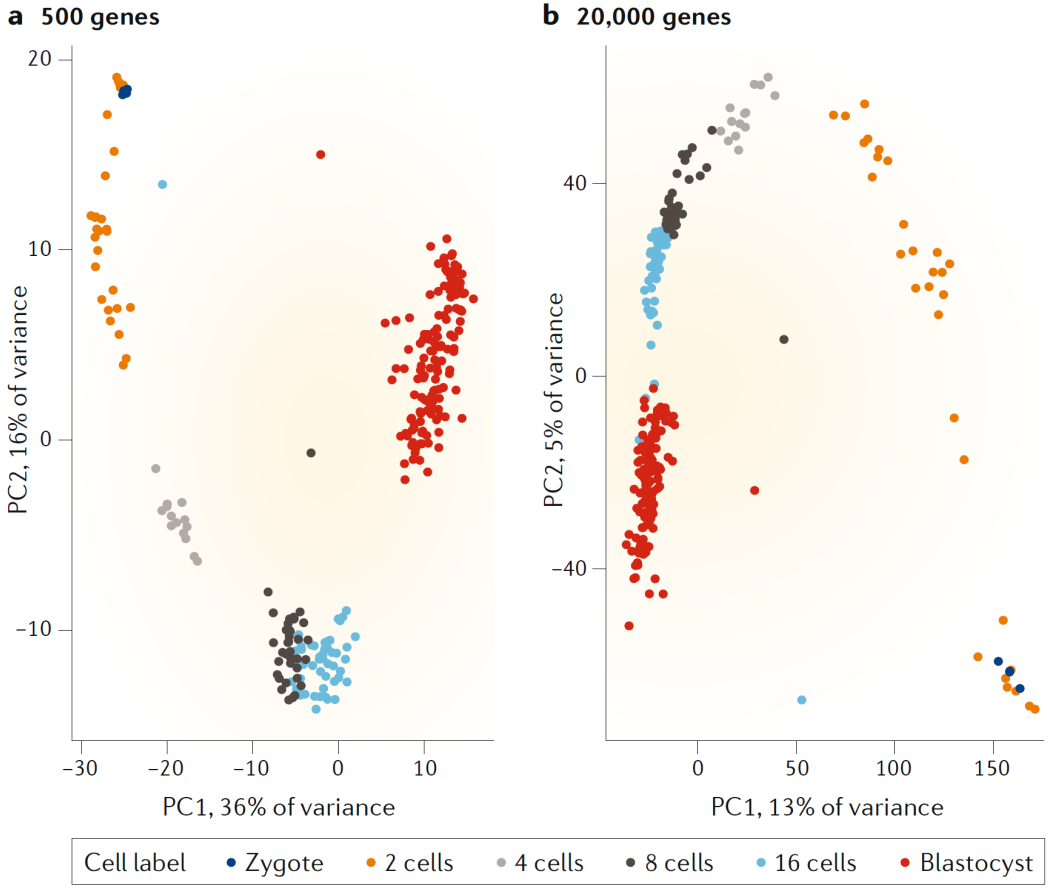
- *Technical biases:* tend to affect genes in a similar manner, or at least in a manner related to their biophysical properties (e.g., length, GC content).

- *Biological differences:* highly unpredictable.

Feature selection

Feature selection

- Curse of dimensionality
  - More features (genes) -> noise dominates distances between samples (cells), effectively all cells get ‘same’ distance
- Remove genes which only exhibit technical noise
  - Increase the signal:noise ratio
  - Reduce the computational complexity



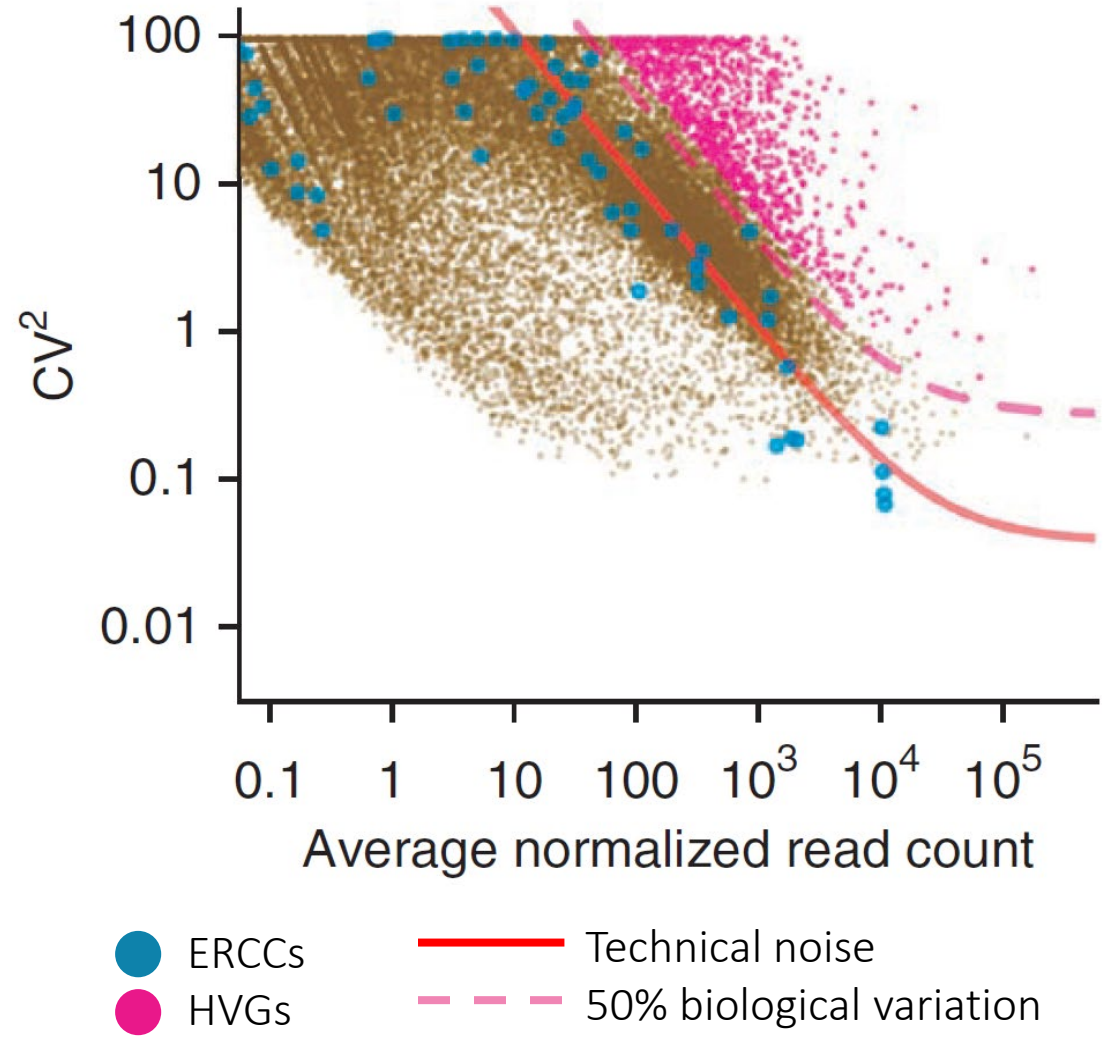
Kiselev et al. (https://doi.org/10.1038/s41576-018-0088-9)

Feature selection

Highly Variable Genes (HVG)

- $CV = \frac{var}{mean} = \frac{\sigma}{\mu}$
- Fit a gamma generalized linear model to spike ins (ERCCs)
- No ERCCs?

  Estimate technical noise based on all genes



Brennecke et al. (https://doi.org/10.1038/nmeth.2645)

Feature selection

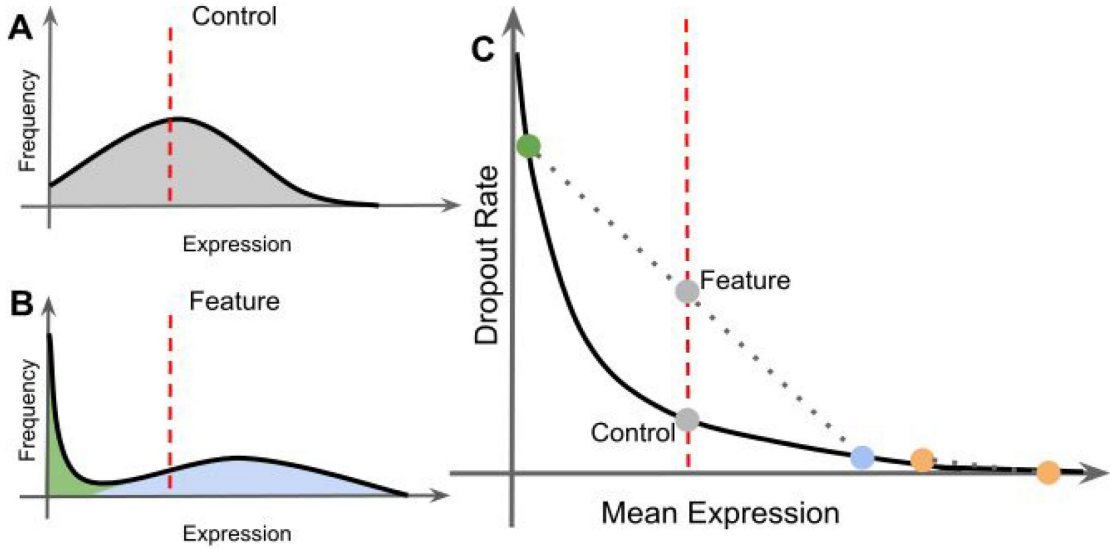
M3Drop: Dropout-based feature selection

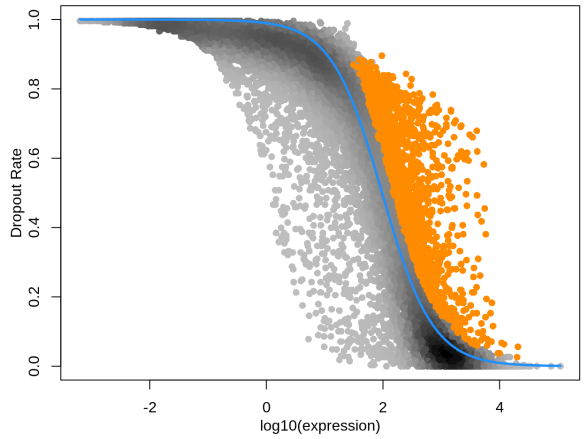
- Reverse transcription is an enzyme reaction thus can be modelled using the Michaelis-Menten equation:

$$P_{dropout} = 1 - \frac{S}{K_M + S}$$

S: average expression

K_M: Michaelis-Menten constant





Brennecke et al. (https://doi.org/10.1038/nmeth.2645)

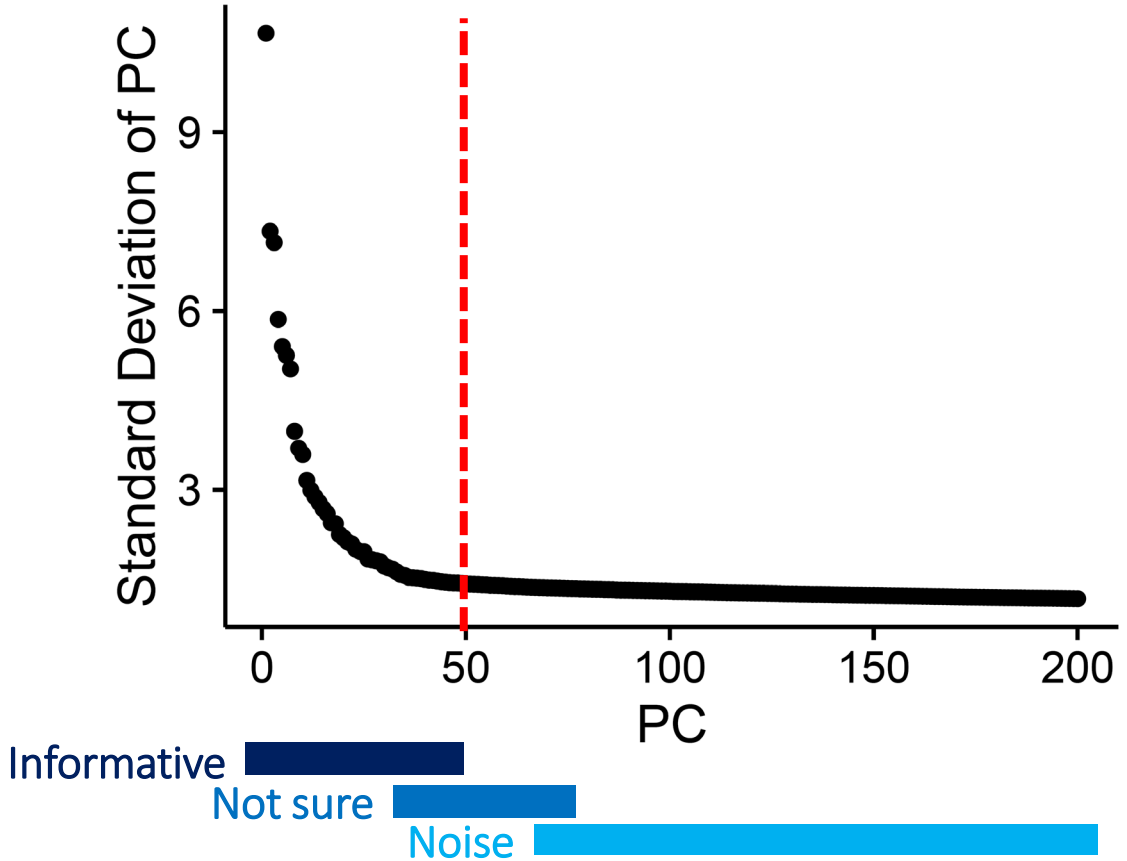
Feature selection

Selecting principal components

- To overcome the extensive technical noise in scRNA-seq data, it is common to cluster cells based on their PCA scores

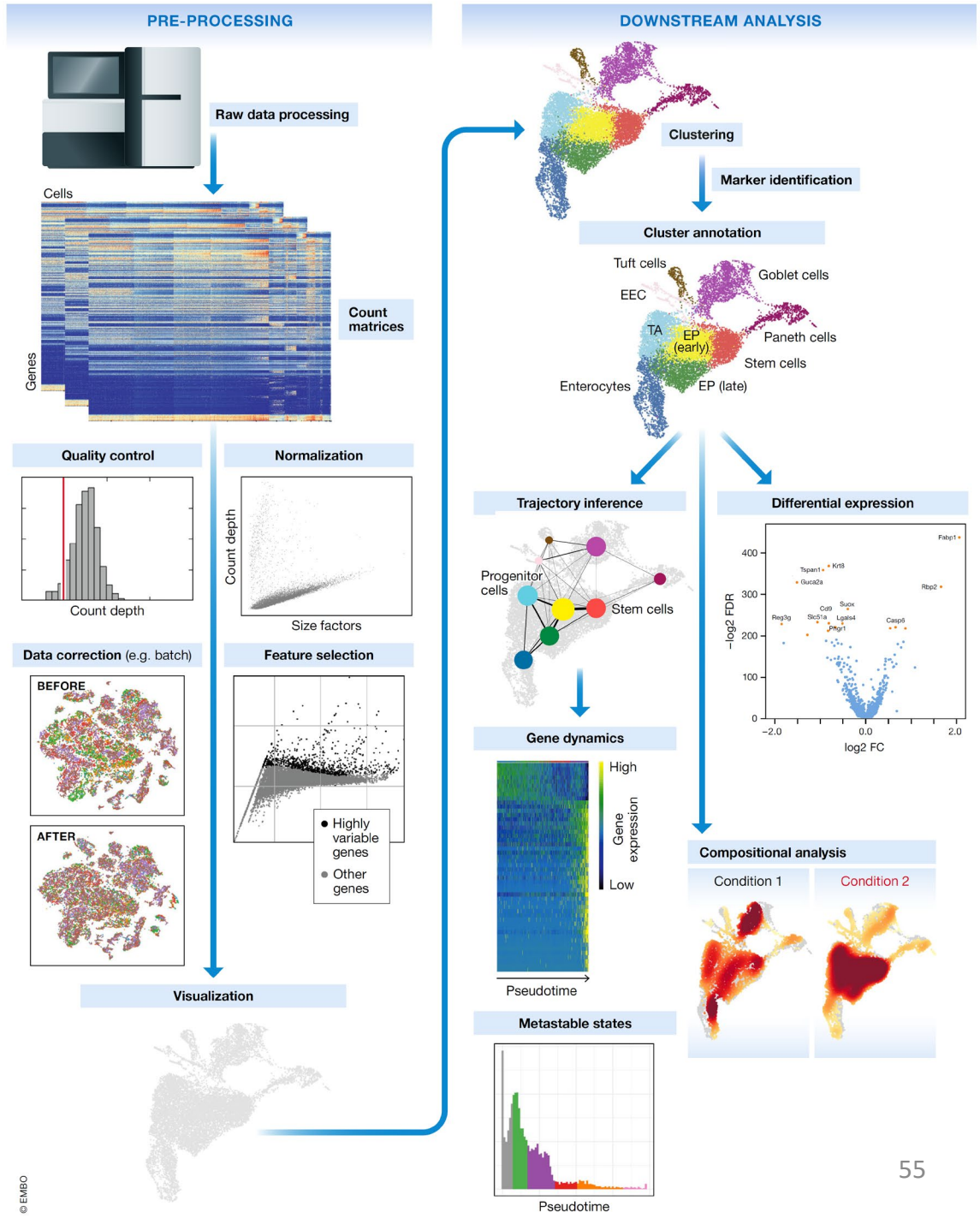
- Each PC represents a ‘metagene’ that (linearly) combines information across a correlated gene set

Scree/Elbow plot



Summary

- Preprocessing:
  - Reads to count matrix ✓
  - Quality control (QC) ✓
  - Normalization ✓
  - Batch correction
  - Feature selection ✓



Luecken and Theis (MSB 2019)

Useful Resources

- NBIS single-cell course:

https://uppsala.instructure.com/courses/52011

- Best practices in single cell RNA-seq analysis (Luecken & Theis, MSB 2019)

https://www.embopress.org/doi/pdf/10.15252/msb.20188746

- Orchestrating Single-Cell Analysis with Bioconductor

https://osca.bioconductor.org/

- Single Cell Course (Martin Hemberg Lab, Welcome Trust Sanger):

http://hemberg-lab.github.io/scRNA.seq.course

- GitHub: Awesome Single Cell

https://github.com/seandavi/awesome-single-cell

Practicals

- You received an invite for RStudio Cloud
- Click on the assignment to start
- Work in pairs!
- RStudio Cloud will be available until 17 Oct 2022

Thank You!

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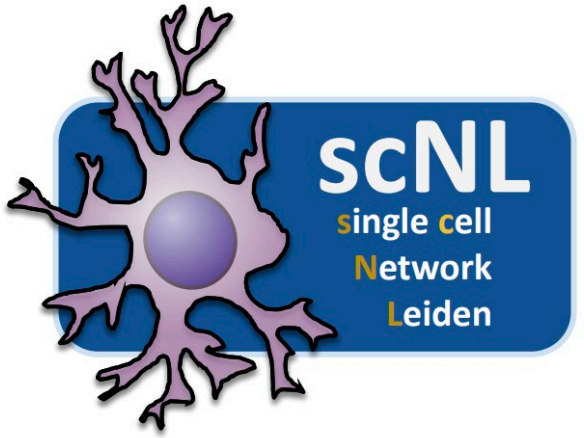

https://www.singlecell.nl/

LUMC TU Delft