Quality Control, Normalization, and Feature Selection

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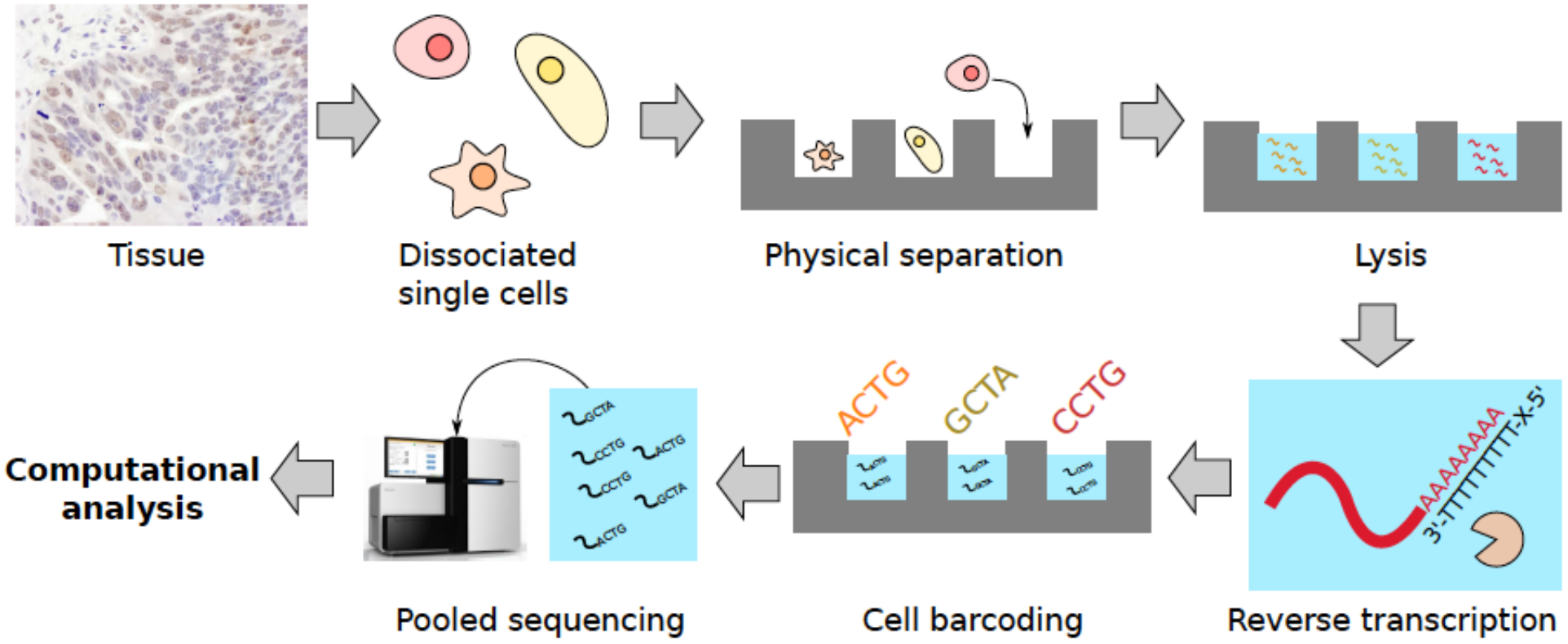
Single cell RNA-sequencing (scRNA-seq)

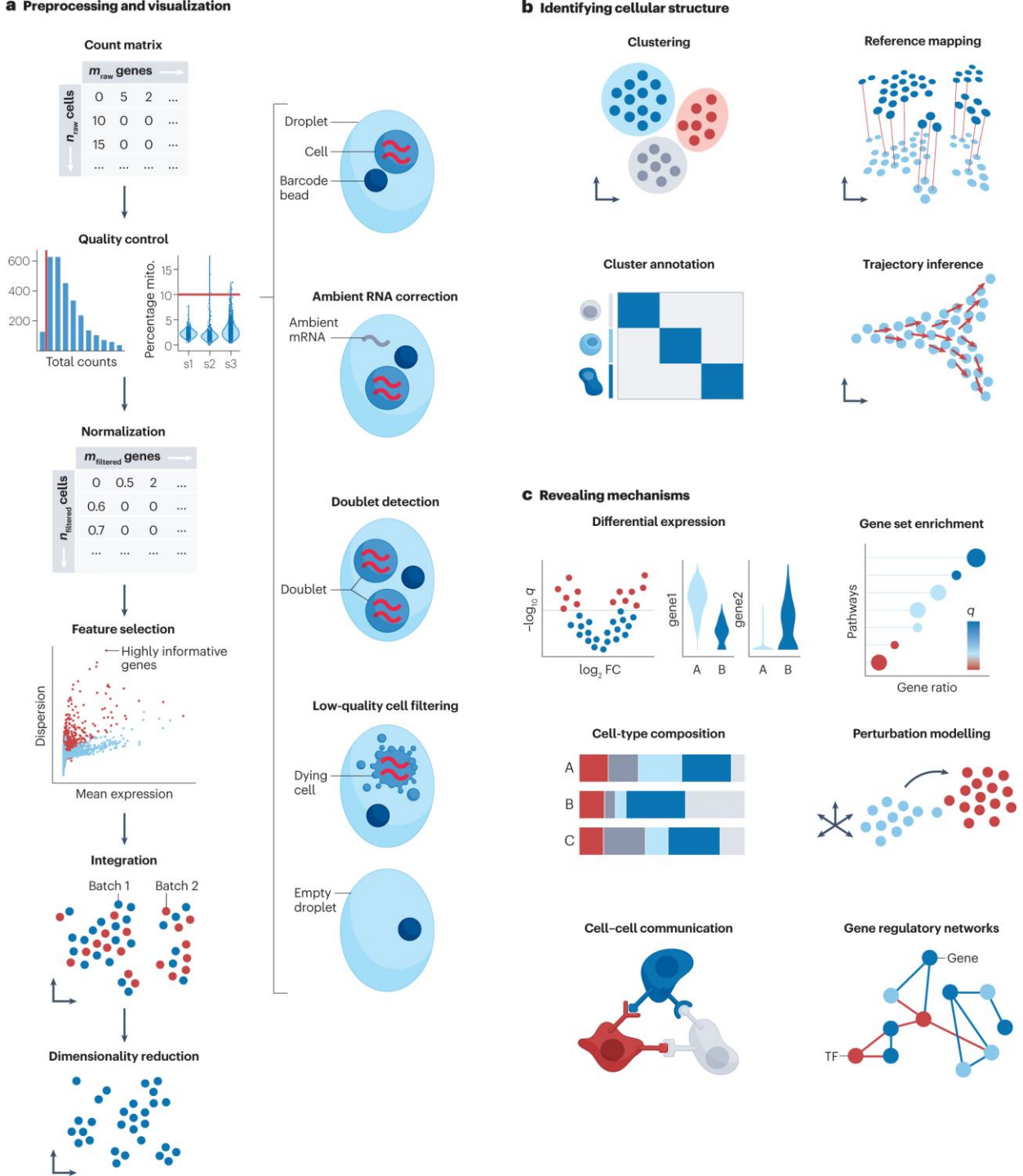


scRNA-seq Data Analysis

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



Heumos, Schaar, et al (Nat Rev Genetics 2023)

scRNA-seq Data Analysis

- Preprocessing:
  - Reads to count matrix ✓
  - Quality control (QC)
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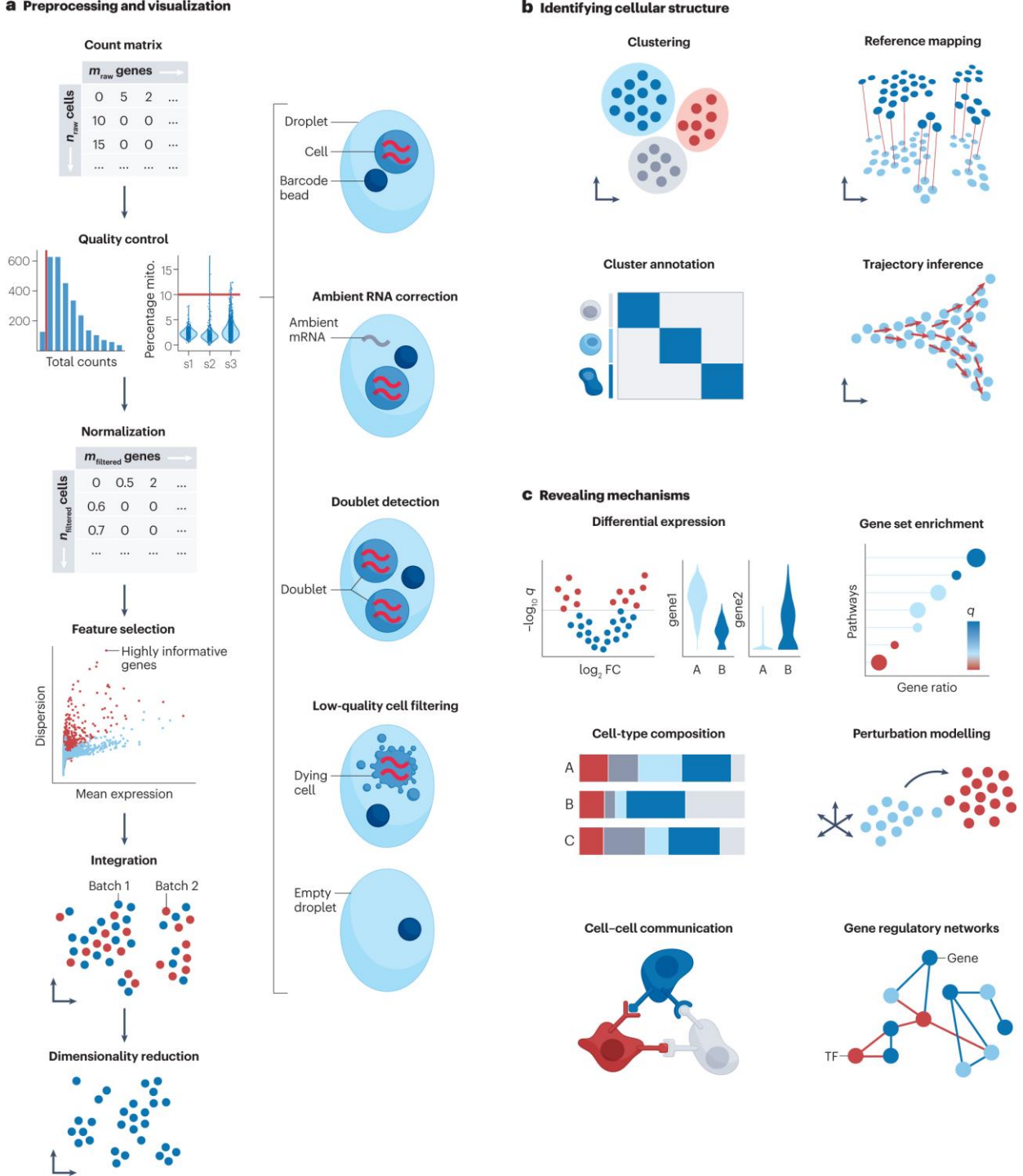
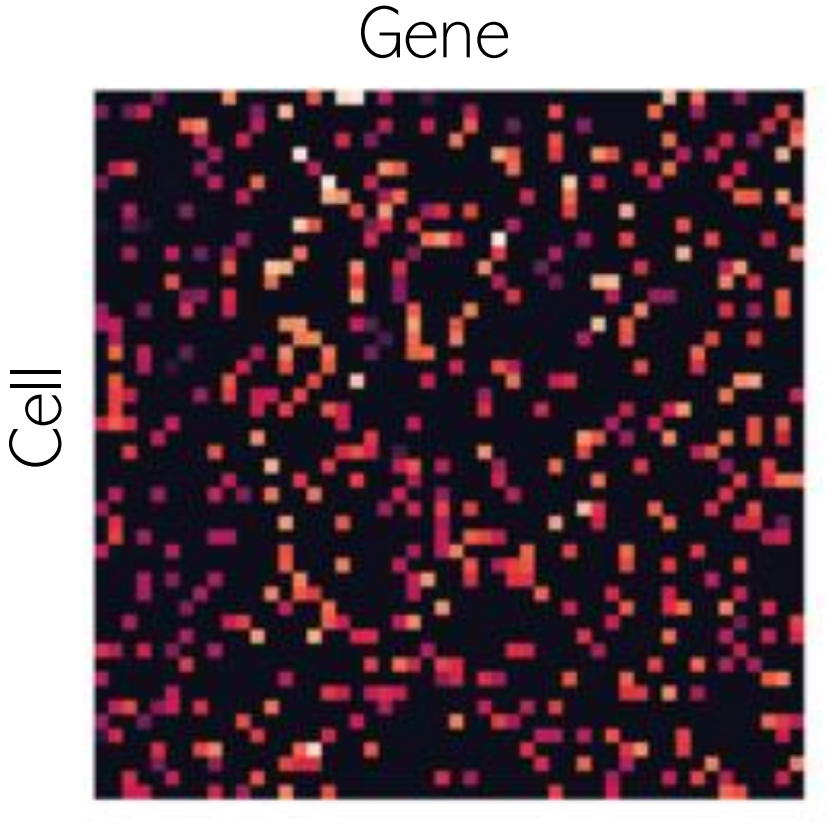


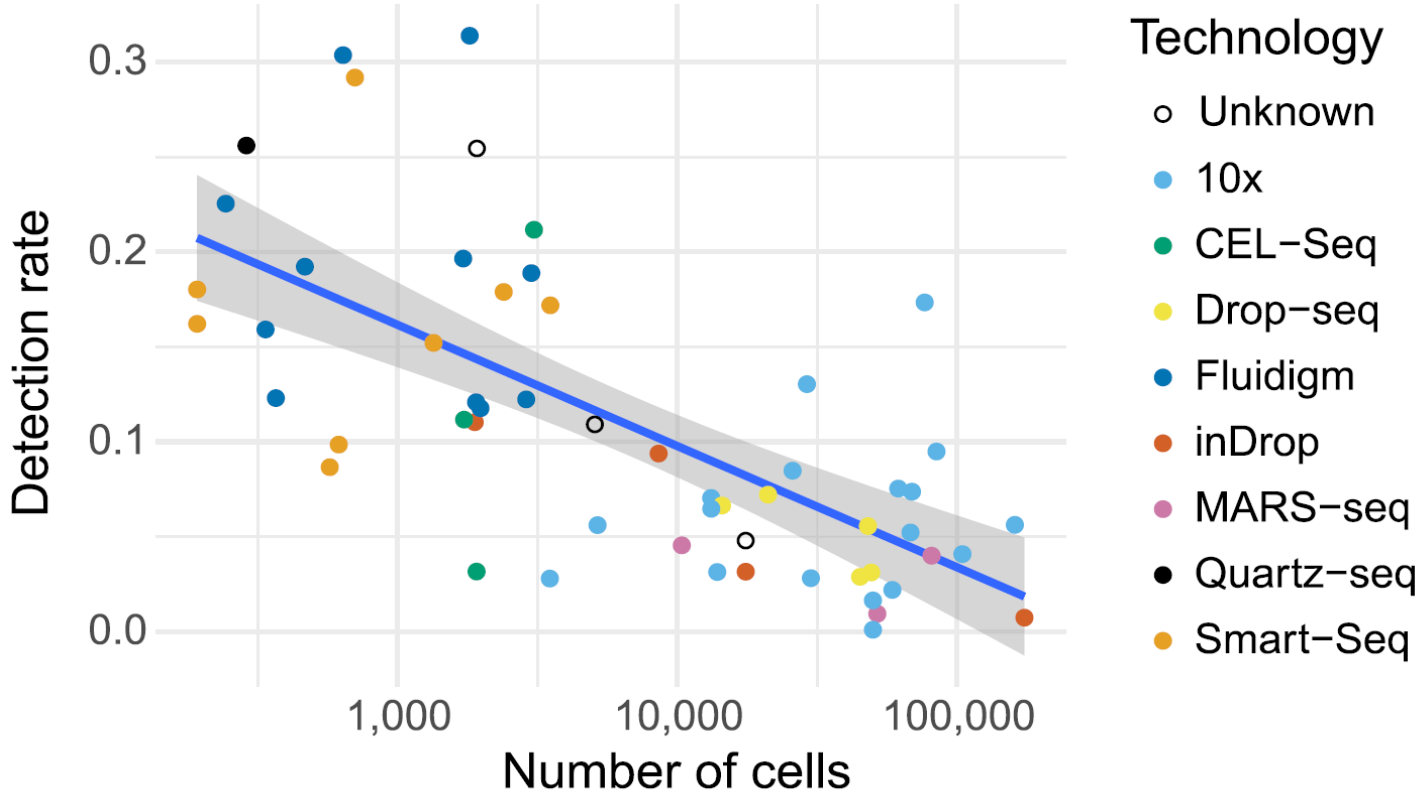
Heumos, Schaar, et al (Nat Rev Genetics 2023)

Our agenda

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

scRNA-seq data is mainly zeros

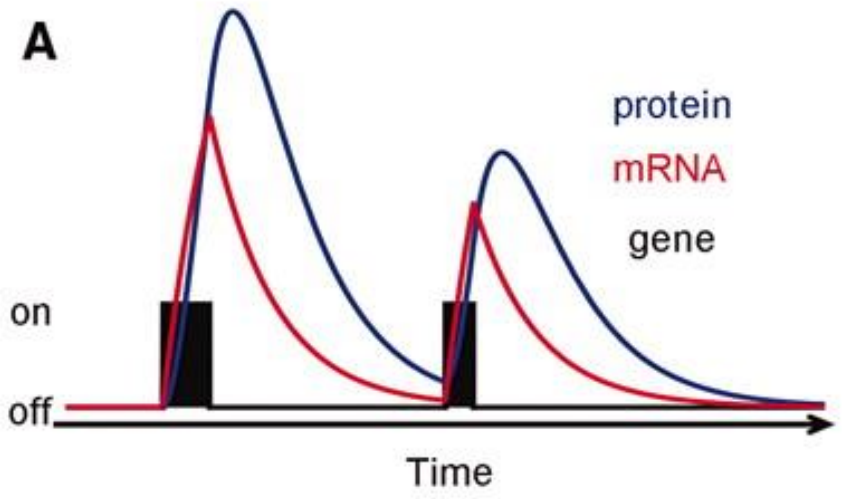


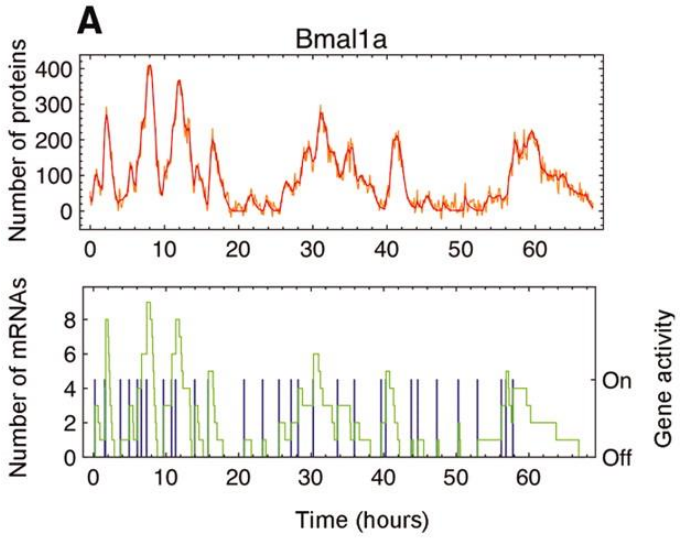


Bouland et al. (Genome Biology 2023)

Transcriptional bursting

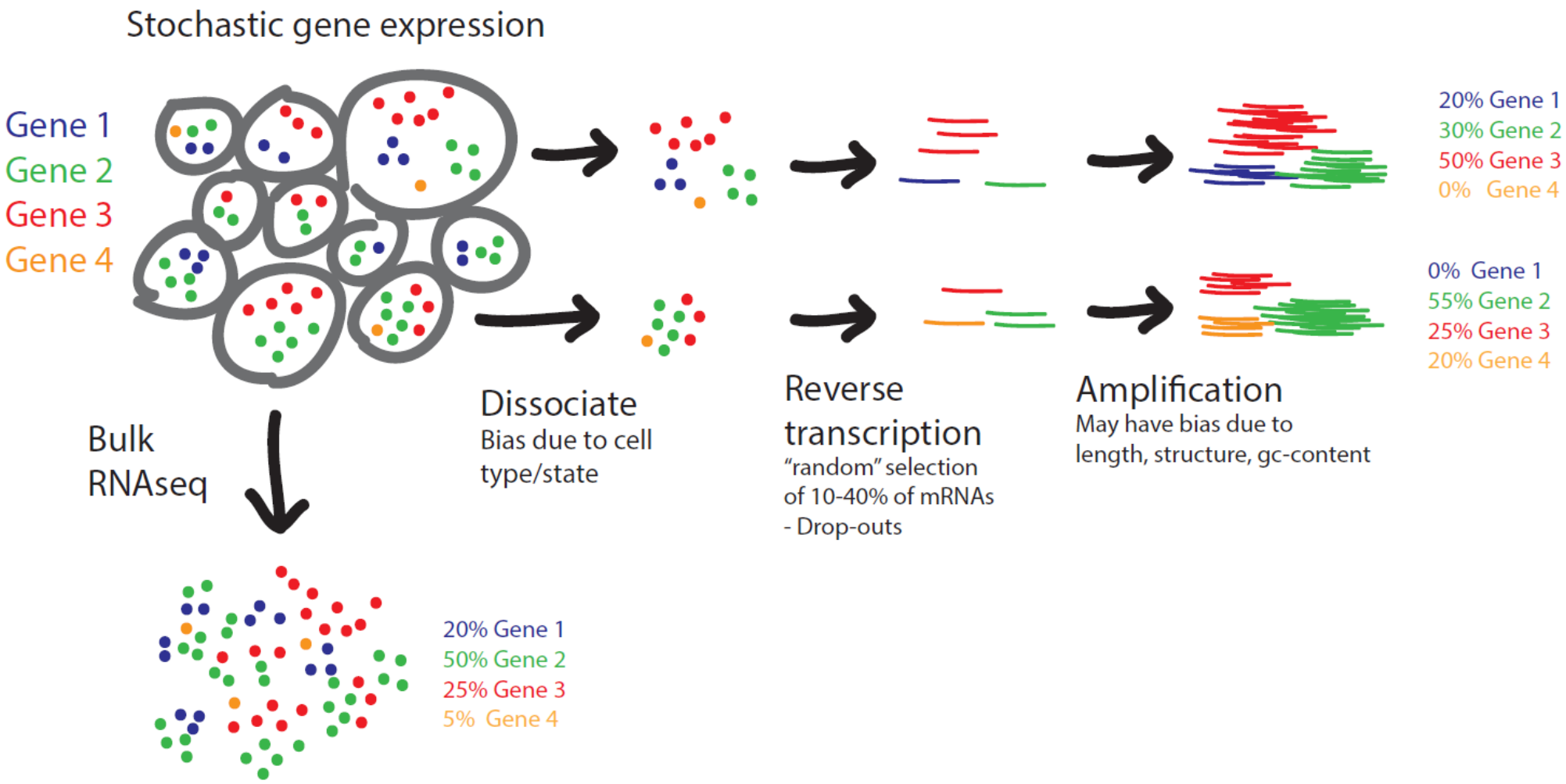
- Burst frequency and size is correlated with mRNA abundance
- Many TFs have low mean expression (and low burst frequency) and will only be detected in a fraction of the cells





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

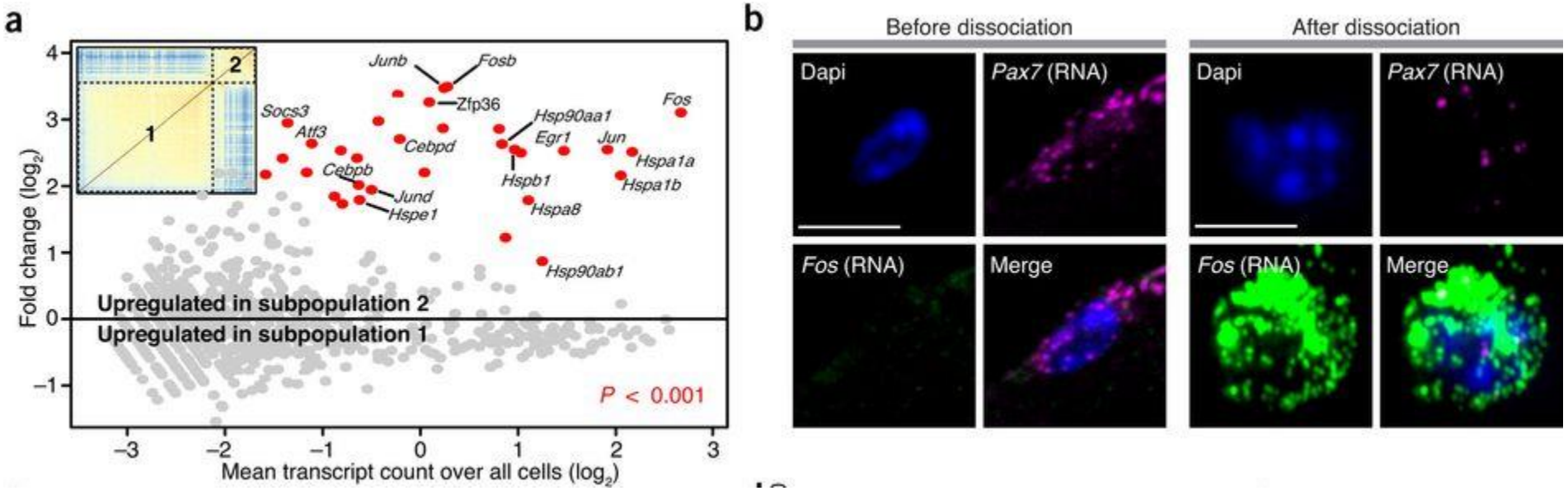
- It is critical to have healthy whole cells with no RNA leakage. Short time from dissociation to cell!

- 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.

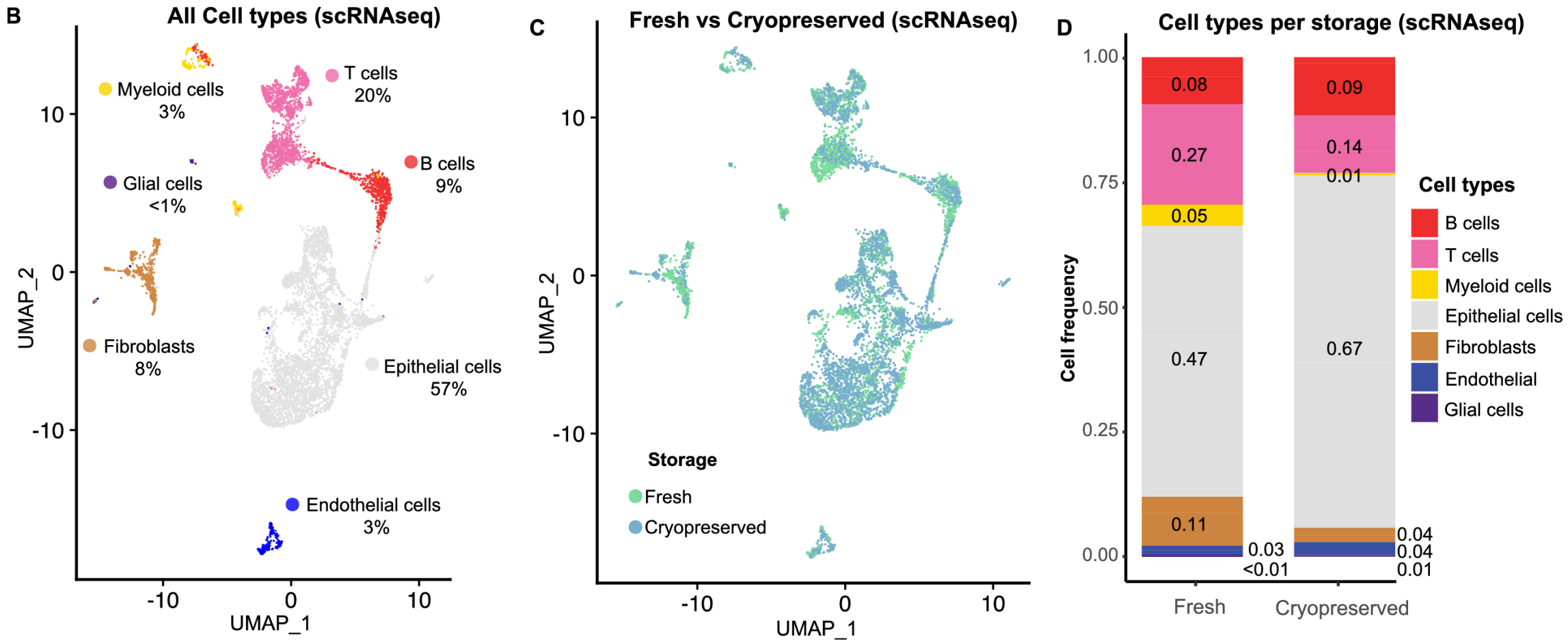
Dissociation artifacts

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



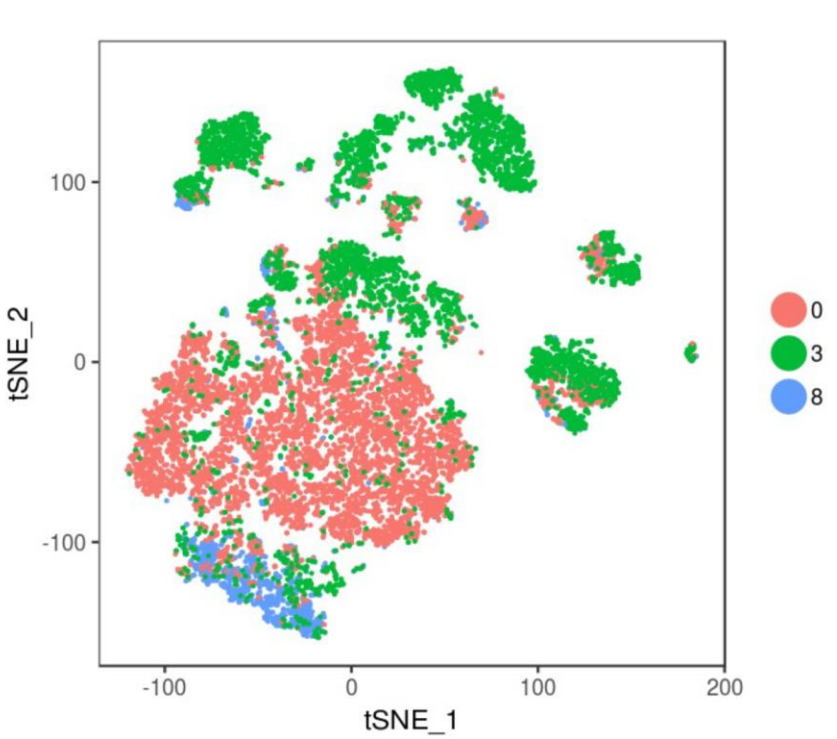
van den Brink et al. (Nature methods 2017)

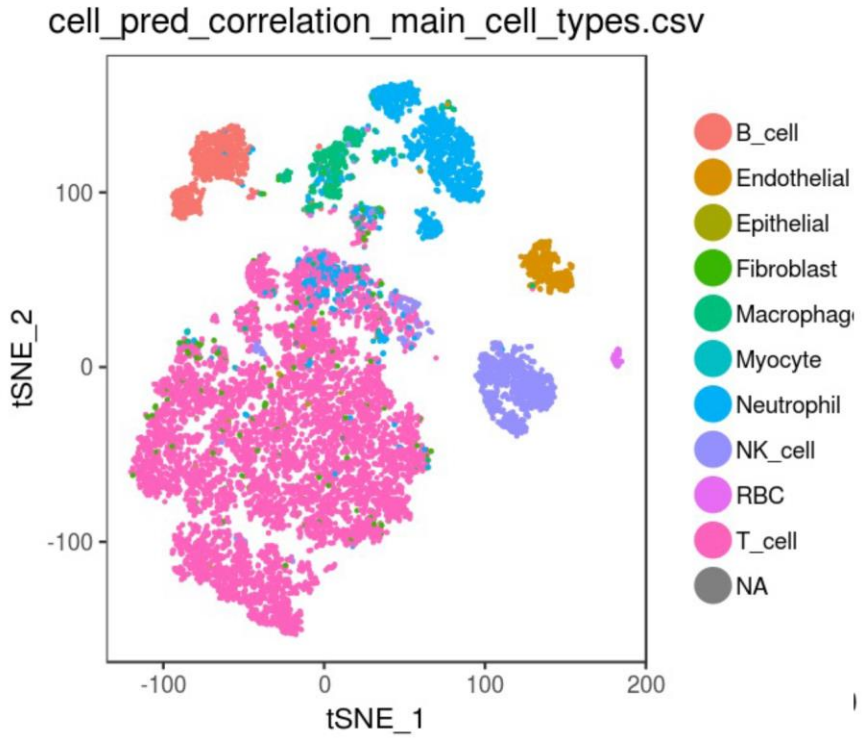
Biased distribution due to dissociation

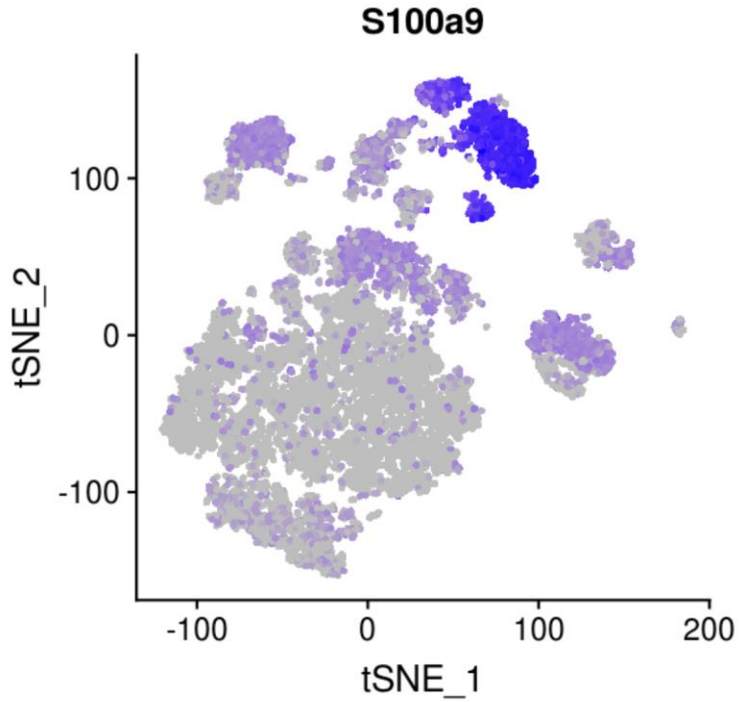


Uniken Venema *et al.* Sci. Rep. 2022

Ambient RNA



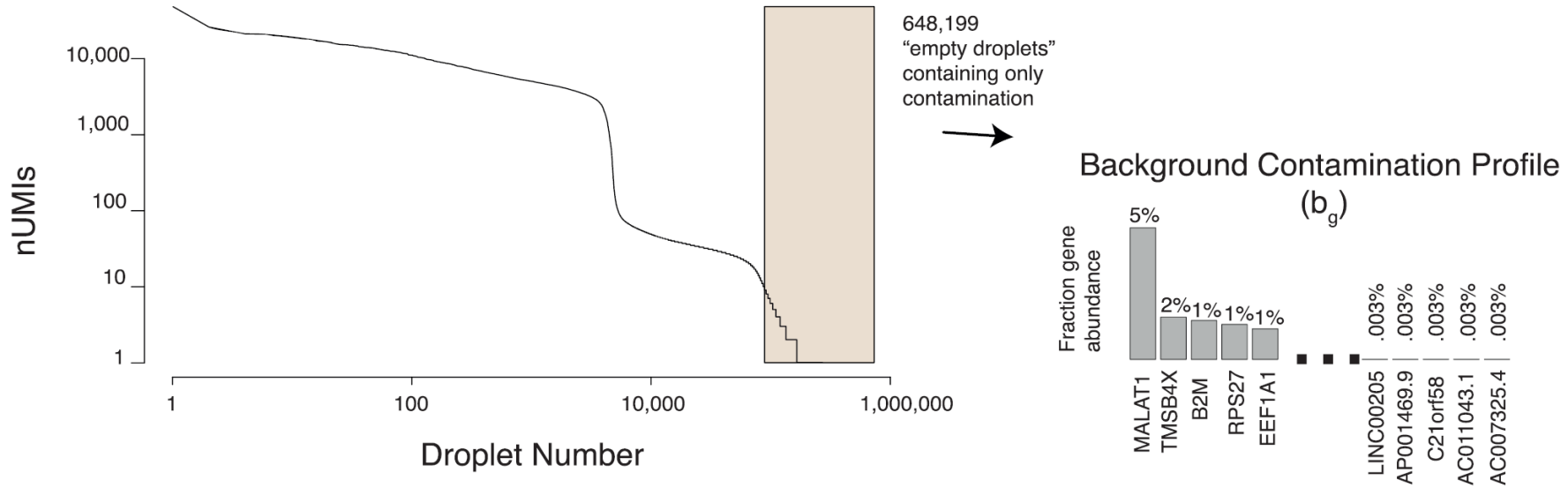




Sample from Day3 have detection of Neutrophil markers in all cells -> possibly contamination from ambient RNA.

Ambient RNA

- Using empty droplets, estimate background signal
- SoupX (Young MD, GigaScience 2020)
- Cellbender (Flemming et al. BiorXiv 2022)



Ambient RNA

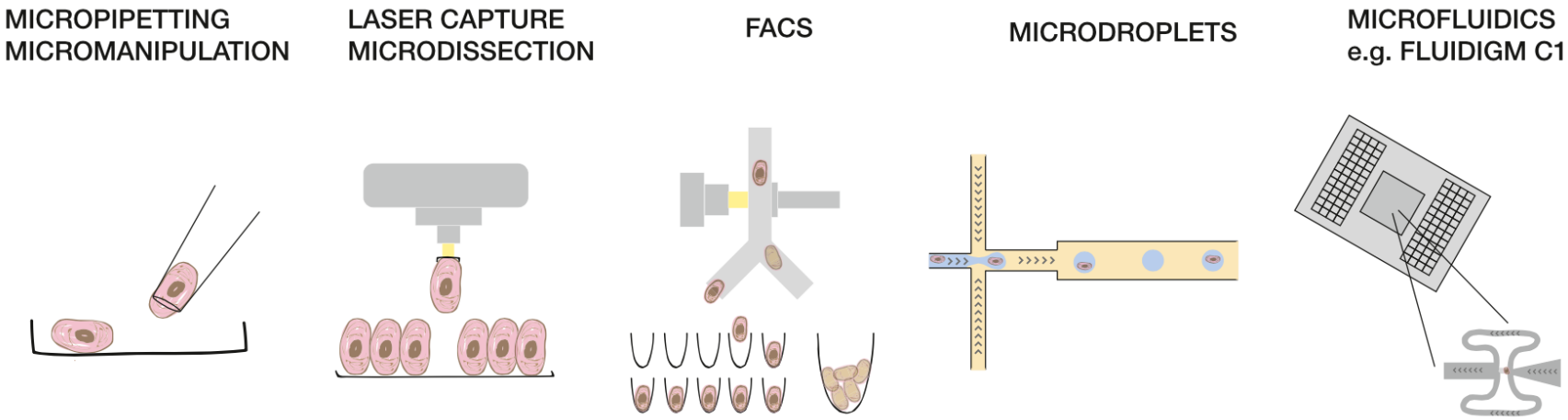
- Instead of removing/subtracting ambient counts, we can:
- Exclude genes with significant ambient contribution (e.g. >10%)
- Report mean contaminating percentage in DE analysis

```
## DataFrame with 6 rows and 6 columns
##             logFC    logCPM         F       PValue          FDR contamination
##         <numeric> <numeric> <numeric>    <numeric>    <numeric>     <numeric>
## Xist     -7.55561   8.21232   6439.91  0.00000e+00  0.00000e+00     0.0605735
## Hbb-bh1  -8.09110   9.15972  10479.99  0.00000e+00  0.00000e+00     0.9900717
## Hbb-y    -8.41561   8.35705   7129.23  0.00000e+00  0.00000e+00     0.9674483
## Hba-x    -7.72481   8.53284   7658.59  0.00000e+00  0.00000e+00     0.9945348
## Hba-a1   -8.59664   6.74429   2748.74  0.00000e+00  0.00000e+00     0.8626846
## Hba-a2   -8.86621   5.81300   1468.41 4.31982e-301 7.66768e-298     0.7351403
```

Empty droplets contain important information!!

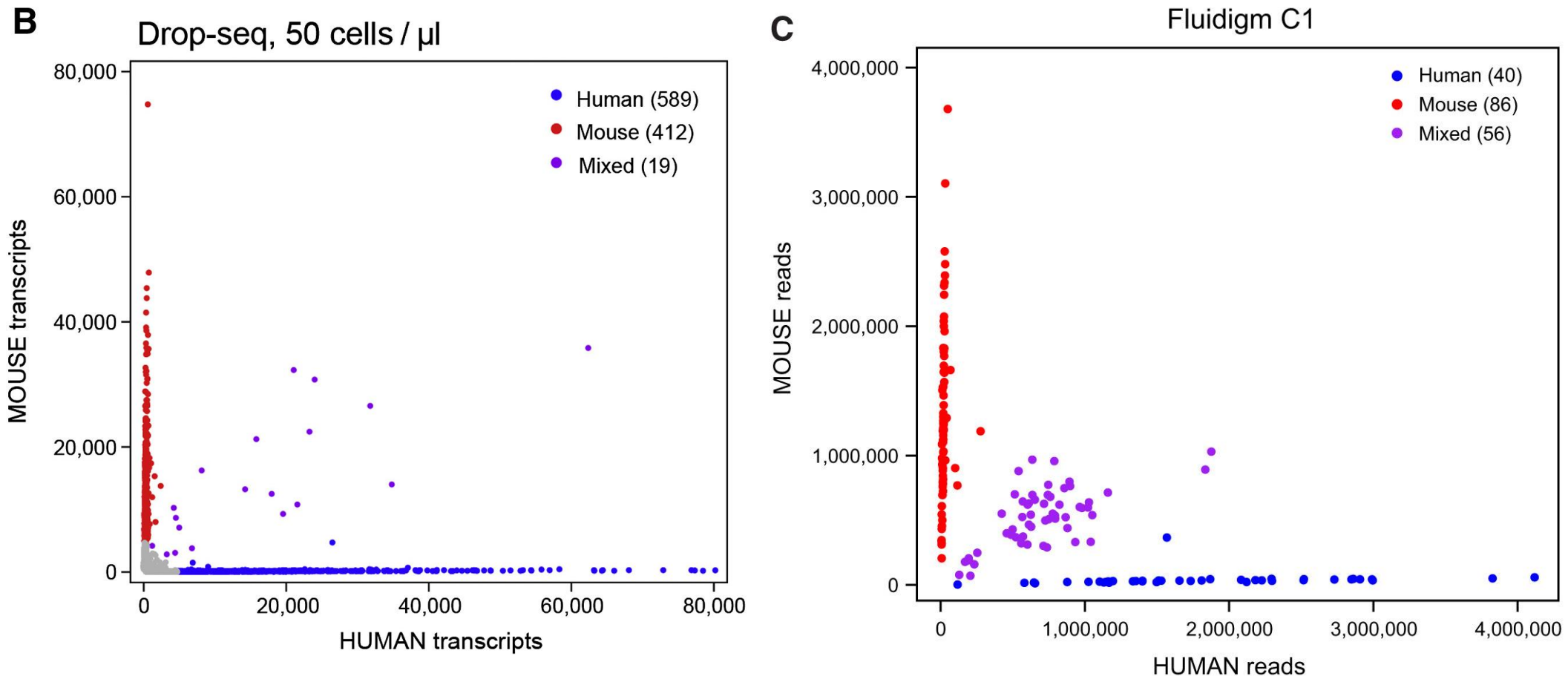
[Chapter 5 Problems with ambient RNA | Multi-Sample Single-Cell Analyses with Bioconductor]

Single cell capture



- 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

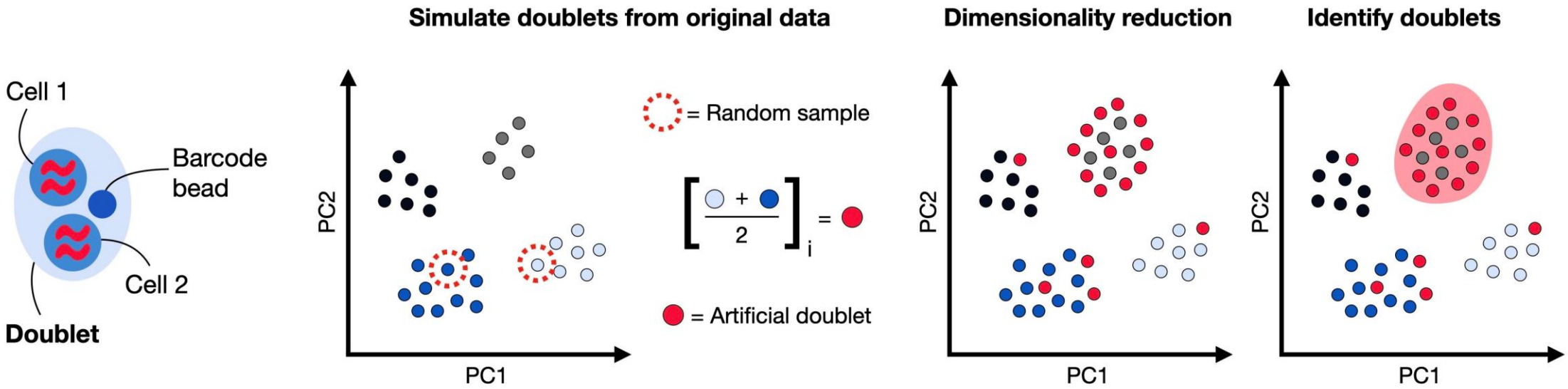
https://github.com/AllonKleinLab/scrublet

- DoubletDecon

https://github.com/EDePasquale/DoubletDecon

- scDblFinder
  https://github.com/plger/scDblFinder

scDblFinder



Germain et al (F1000Res) 2022

Other factors affecting quality

- Cell Lysis:
  - Optimal lysis conditions may vary from celltype to celltype and for nuclei vs cells.
- Reverse transcription:
  - Drop-out rate is around 90-60% depending on the method used
- Preamplification
  - Methods that uses UMIs will control for amplification bias to a large extent, but the chance of detecting a transcript that is amplified more is higher.

Cells vs nuclei

snRNAseq pros

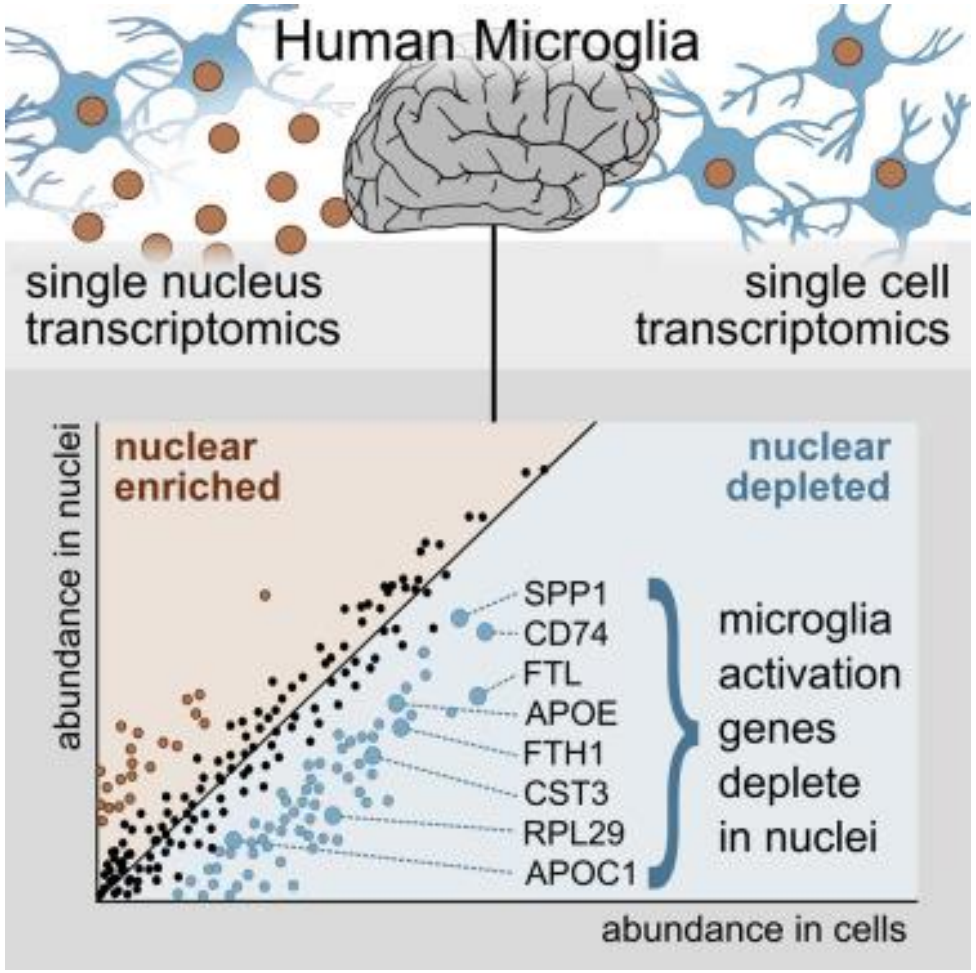
- Can avoid some biases due to dissociation.
- Hard to dissociate celltypes (e.g. neurons, muscle fibres,
- adipocytes)
- Frozen tissues

snRNAseq cons

- Less mRNA per nuclei
- More dominated by nuclear lincRNAs
- Internal priming of polyA stretches in introns

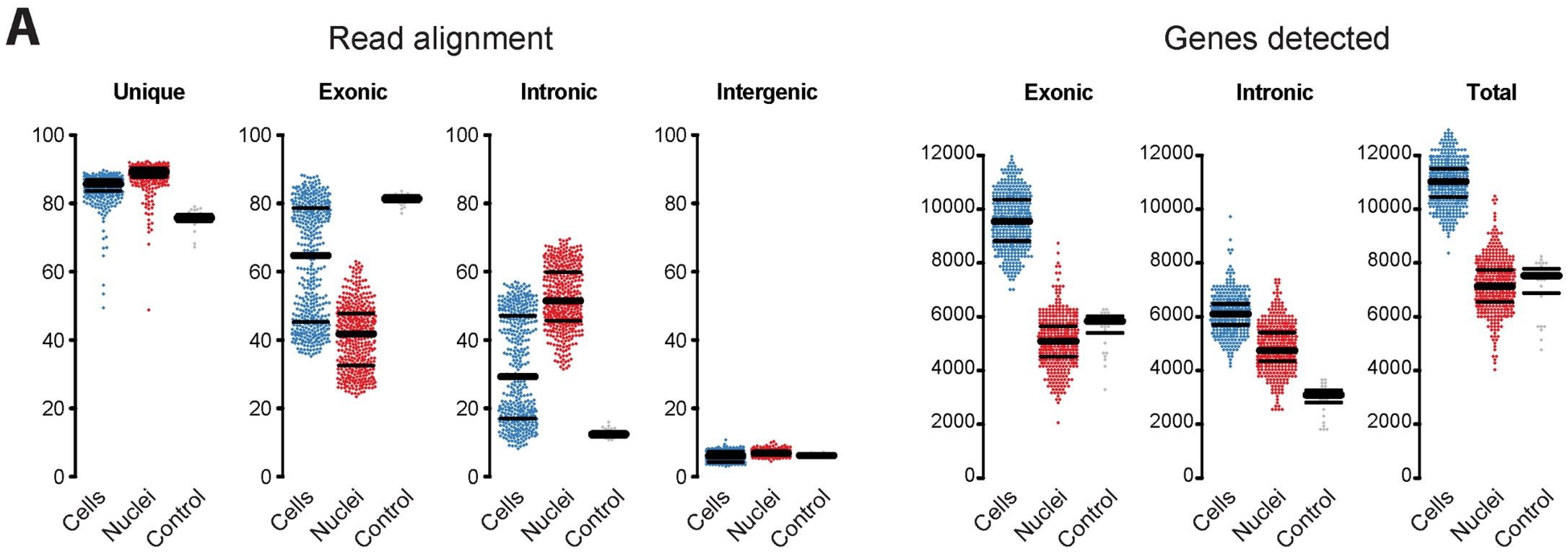
Cells vs nuclei

- For some cell types there may be biased detection of genes in nuclei vs cell.



Thrupp et al. Cell Rep. 2020

Cells vs nuclei

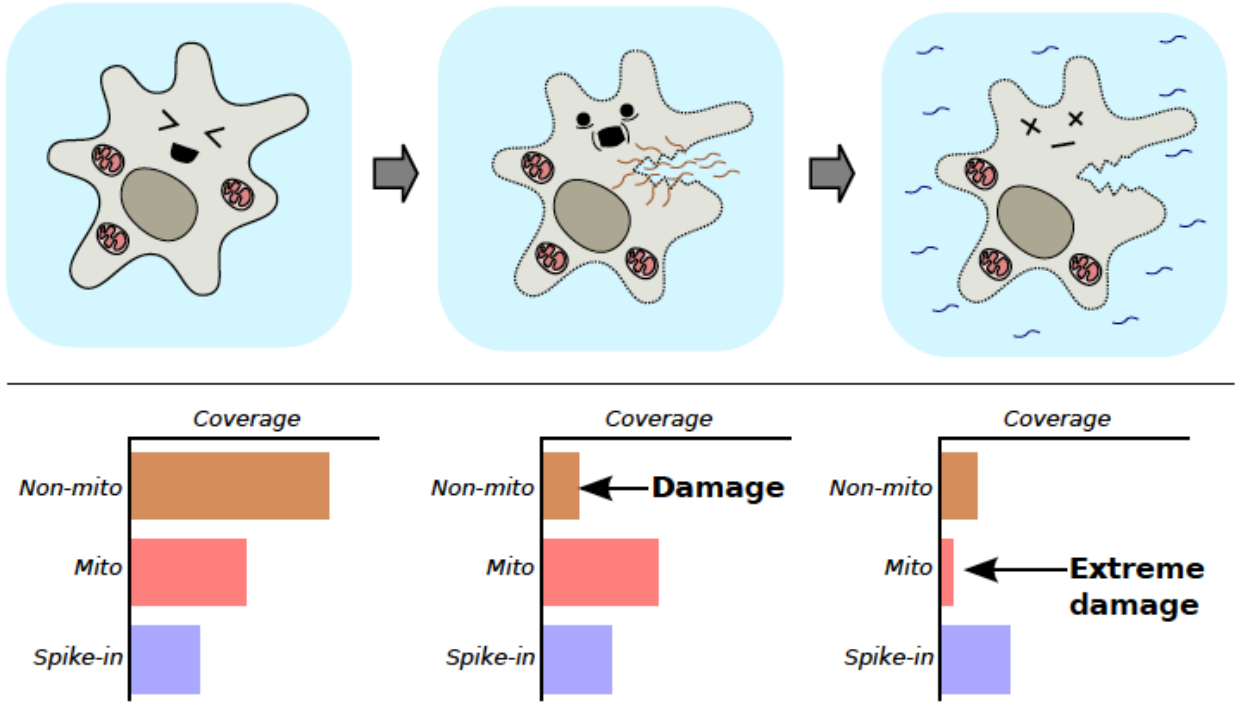


- Usually need to include intronic counts to increase transcript detection in snRNAseq
- Is now default in CellRanger also for scRNAseq

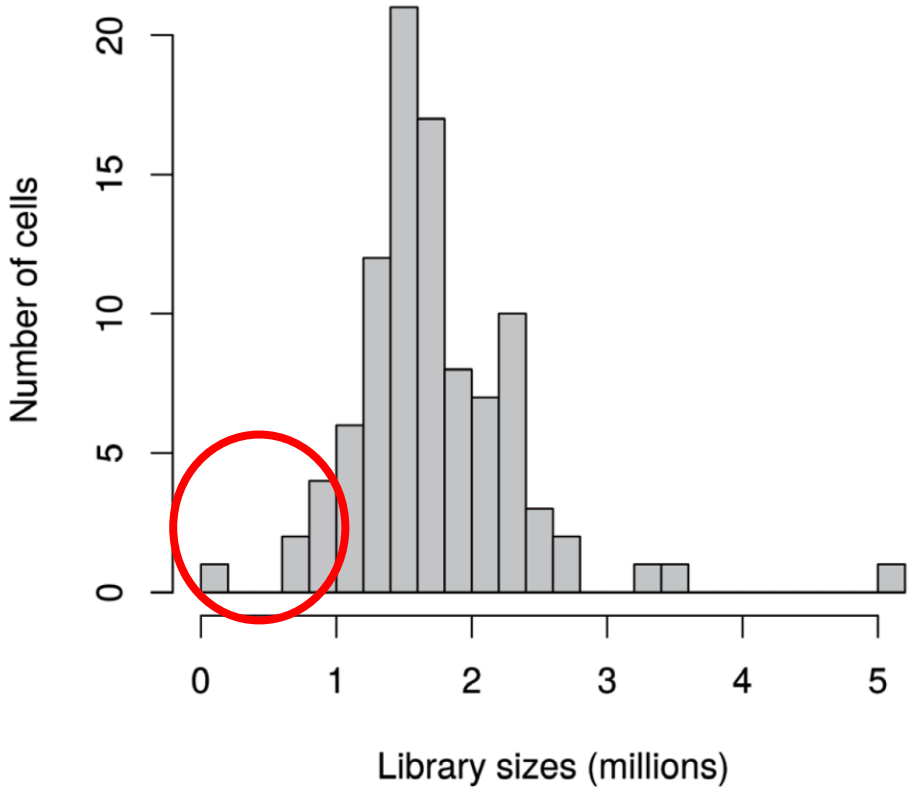
Bakken et al. Plos One 2018

Quality control of cells (1)

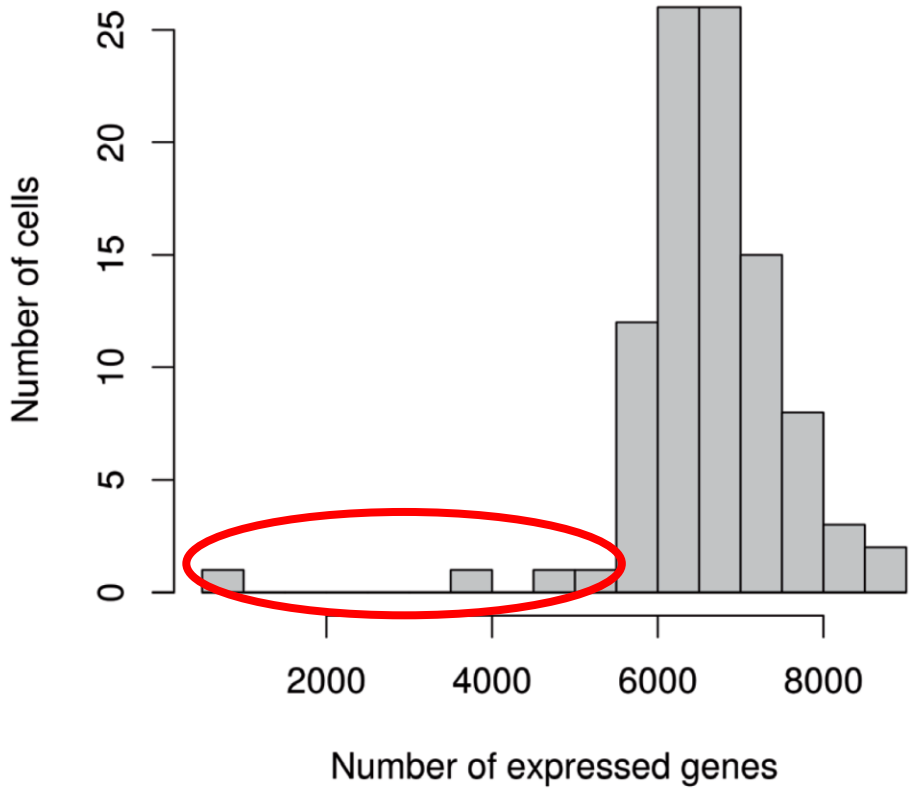
- Low sequencing depth/library size
- Low numbers of expressed/detected genes (i.e. any nonzero count)
- High mitochondrial content



Quality control of cells (2)

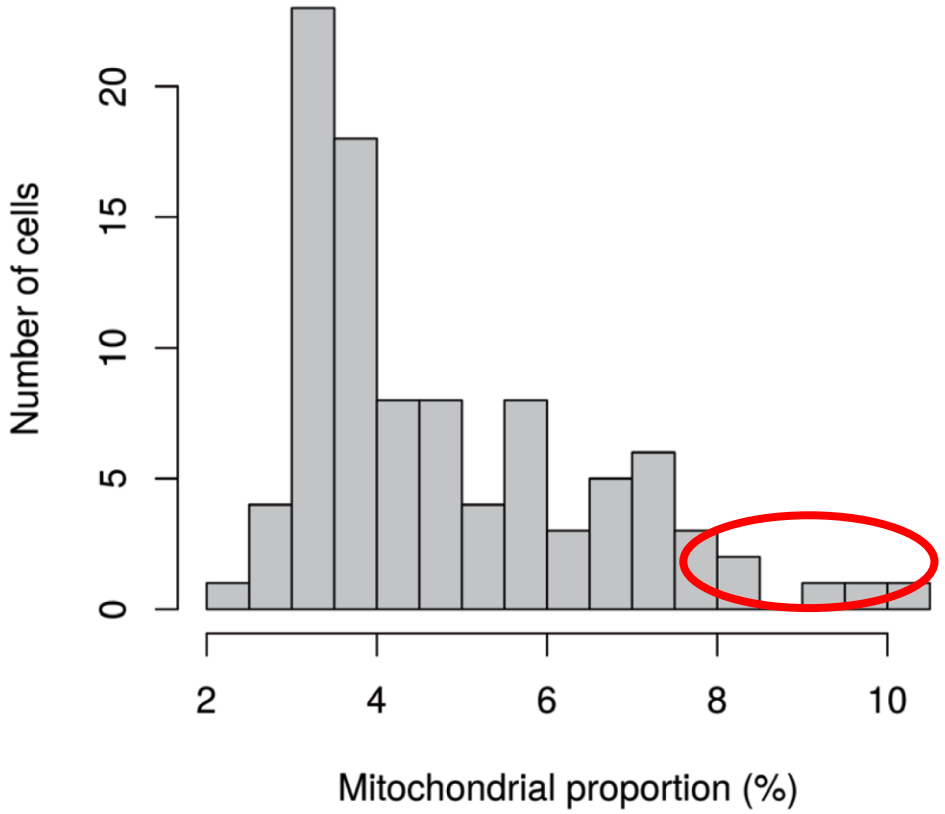


RNA has not been efficiently captured during library preparation



Diverse transcript population not captured

Quality control of cells (3)



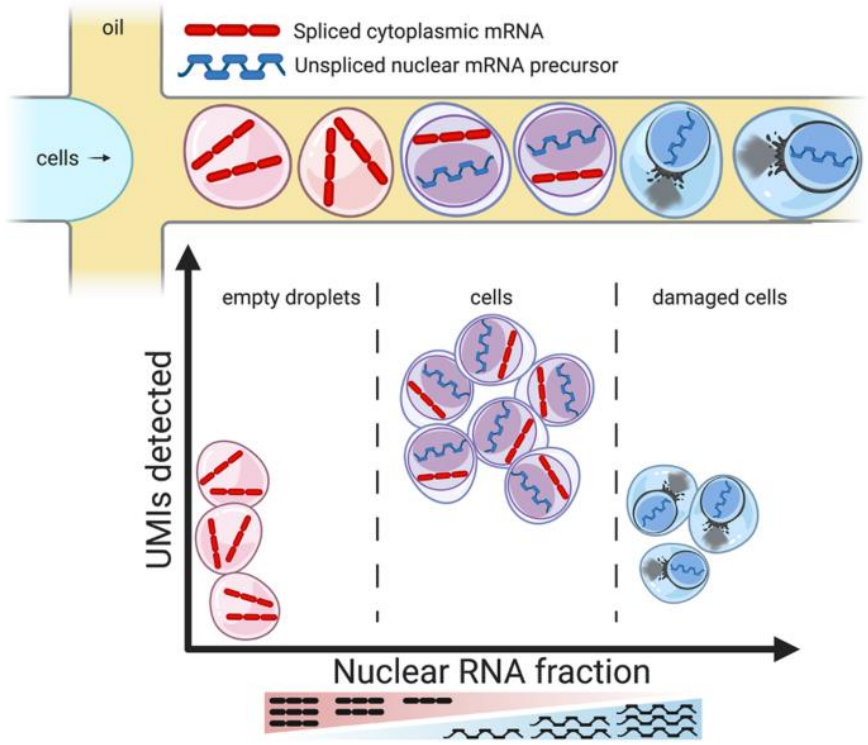
Possibly because of increased apoptosis and/or loss of cytoplasmic RNA from lysed cells

Ribosomal RNA read fraction

Possible that degradation of RNA leads to more templating of rRNA-fragments.

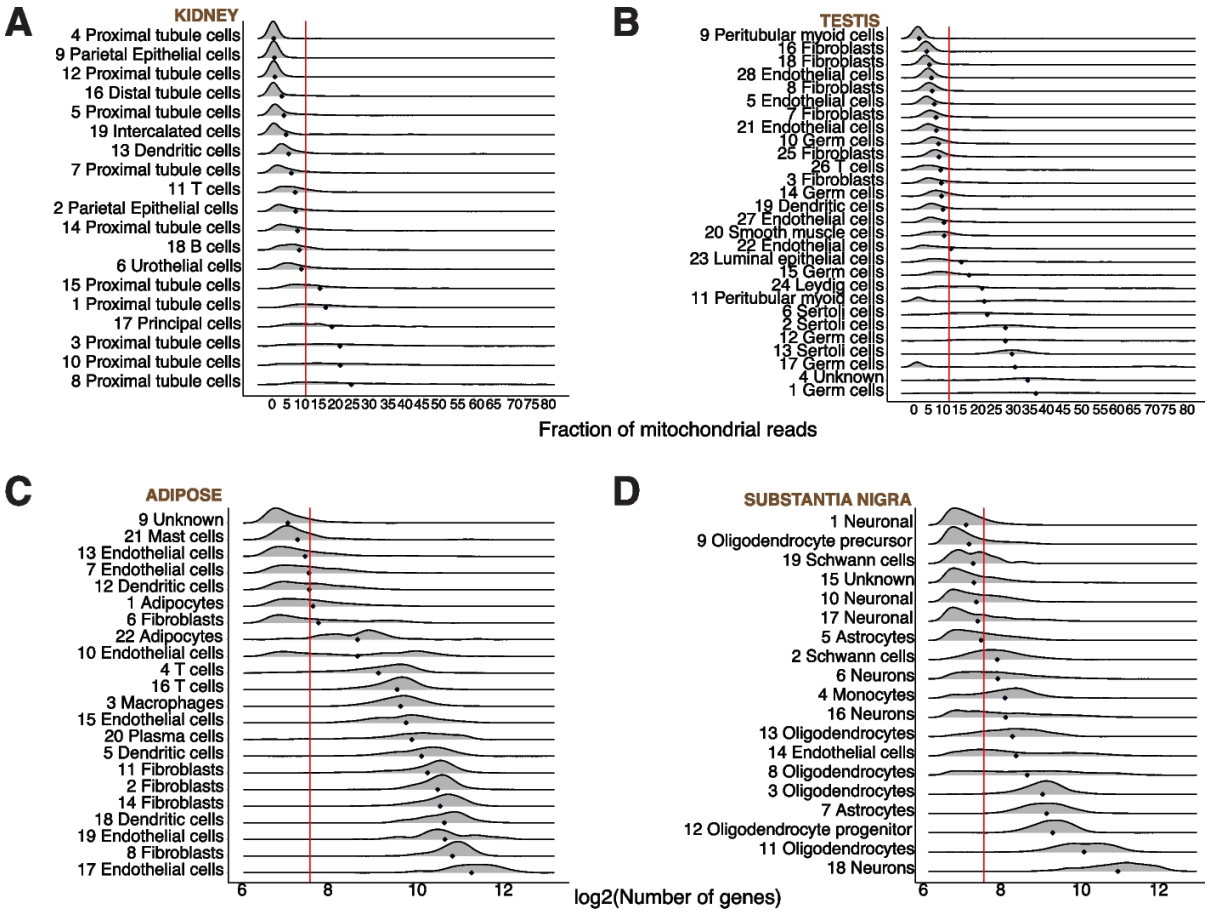
Additional QC metrics

- Nuclear RNA (unspliced transcripts) fraction
- *MALAT1* expression (if nuclear fraction values not available)



Muskovic, Powell (Genome Biology) 2021

QC metrics vary by celltype



Subramanian et al (Genome Biology) 2022

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.

Adaptive thresholds

- Assumes most cells are of acceptable quality
- Mark cells as outlier if >3 MAD (median absolute deviation)
  - $\mathrm{MAD} = \mathrm{median}(|X_i - \mathrm{median}(X)|)$
  - Retain 99% non-outlier values

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/batches, 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?
- Did you inadvertently discard an entire cell type?

Normalization

Sources of variation

Biological

Cell type/state

Cell cycle

Cell size

Sex, Age, …

…

Technical

Cell quality

Library prep efficiency

Batch effects

…

Sources of variation

Biological

- Cell type/state
- Cell cycle
- Cell size
- Sex, Age, …
- …

Technical

- Cell quality
- Library prep efficiency
- Batch effects
- …

To identify cell types we would like to remove all other sources of variation

UMIs do not solve everything

c

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

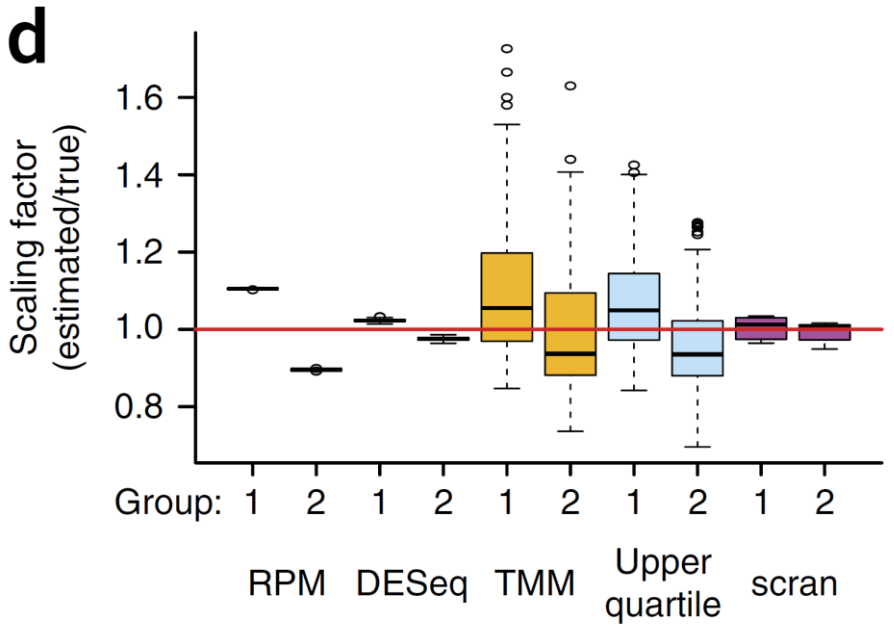
Normalization

- Want to make expression comparable across samples, cells and genes.

- Involves 3 main steps:
  - Scaling
  - Transformation
  - Removal of unwanted variation

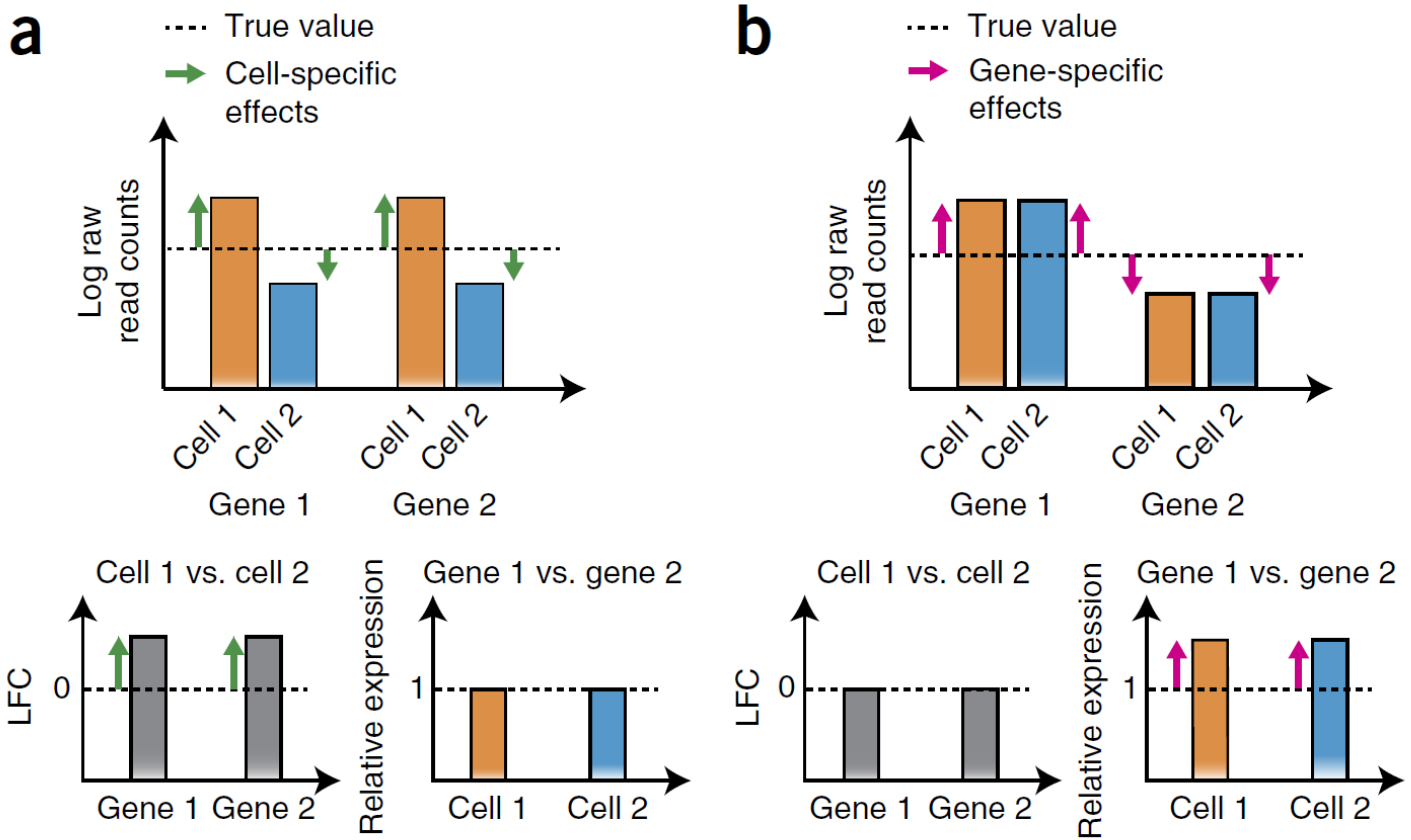
What is different from bulk RNA-seq?

- 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 not suitable



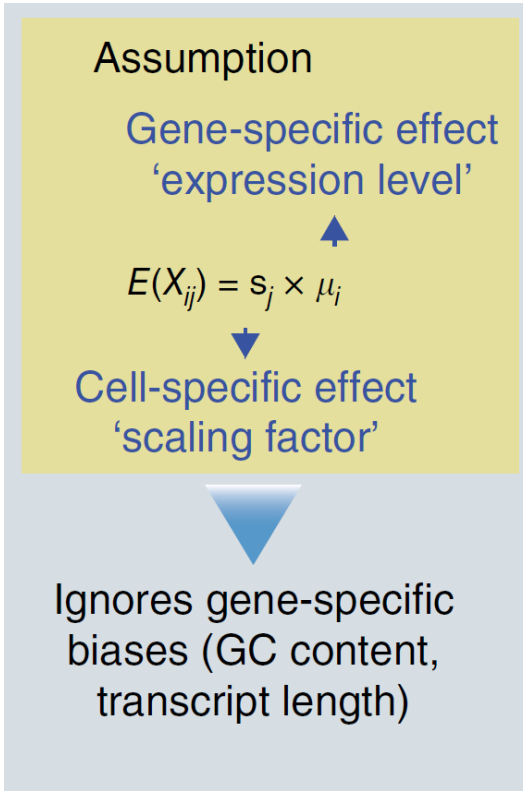
Vallejos et al. (Nat Methods 2017)

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



Vallejos et al. (Nat Methods 2017)

Normalization (1)



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)

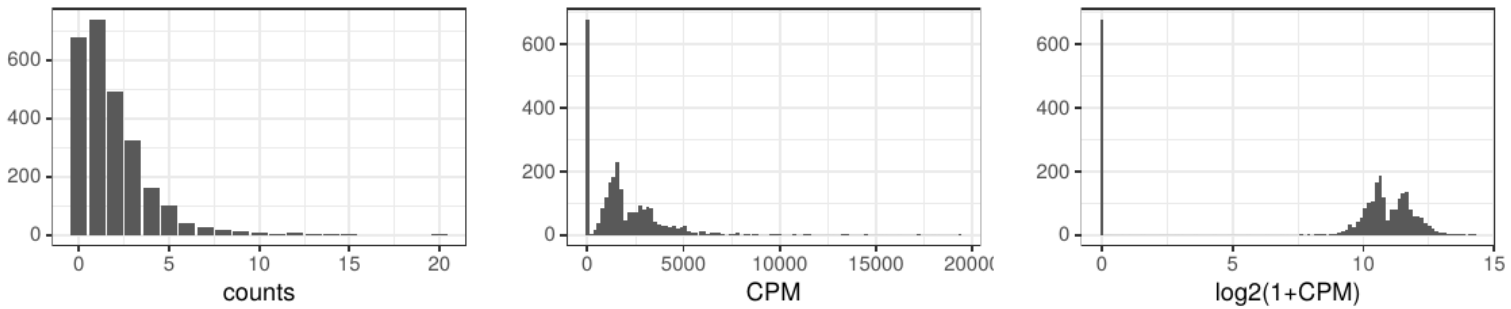
Log-normalization

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

- 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
- A modified version of CPM normalization, i.e. CP10K
- Seurat, scanpy, 10X Cell Ranger: log-normalization

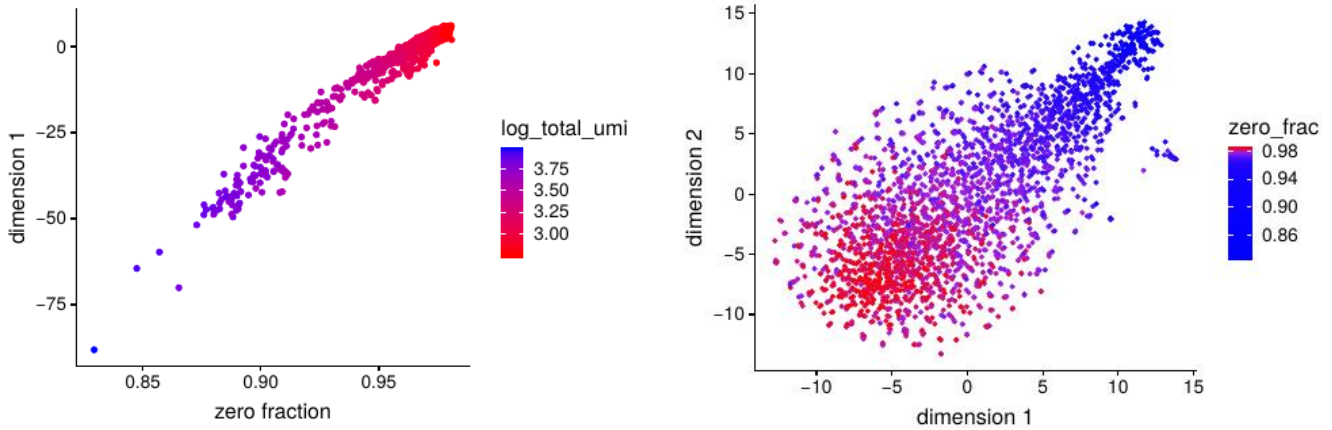
Effect of dropouts on normalization

Inflation of zero counts



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

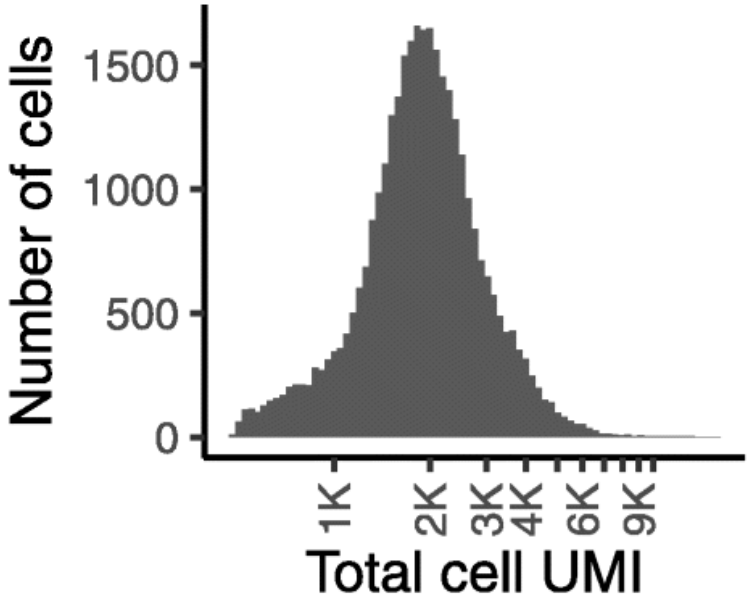
Fraction of zeros become main source of variability

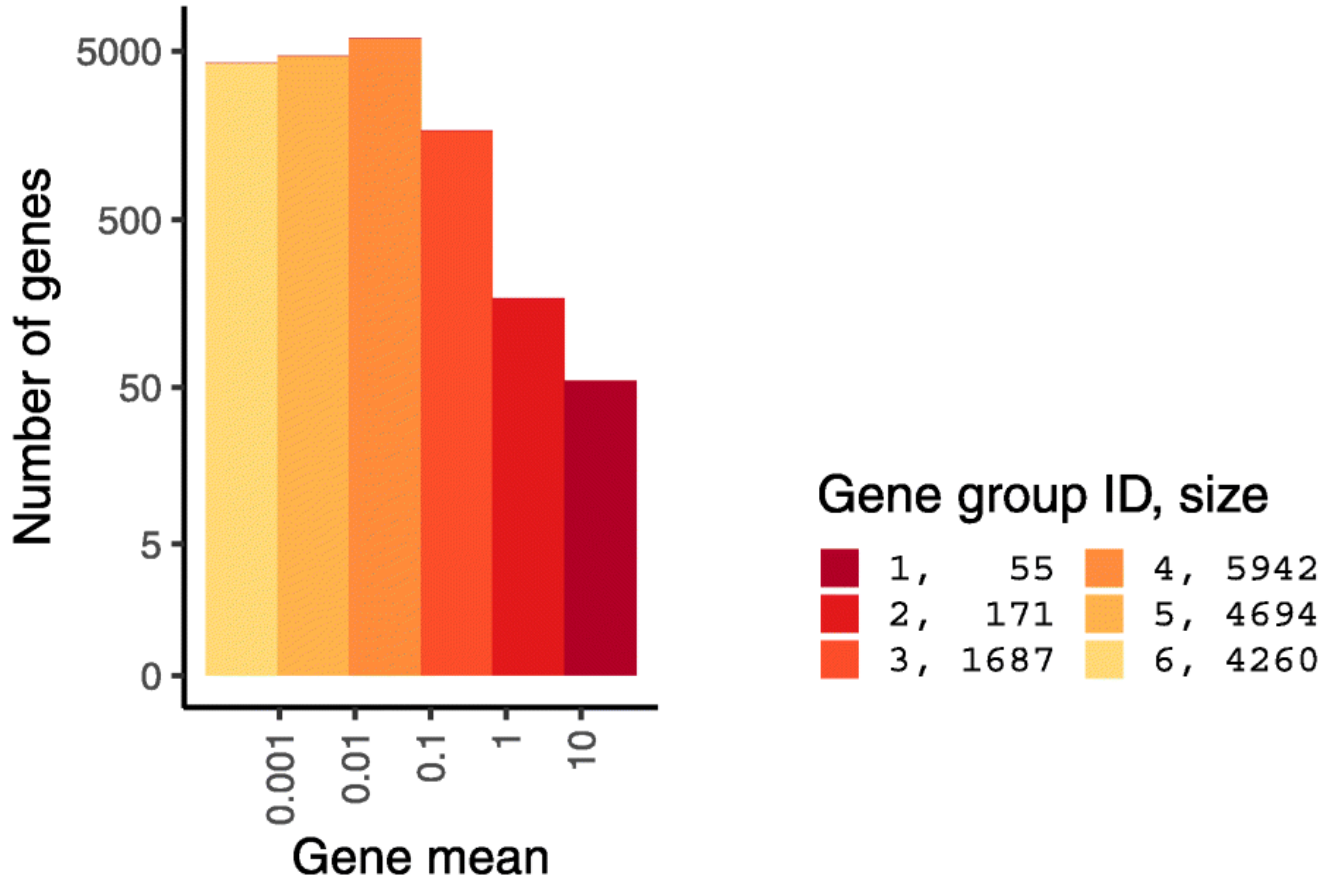


Towns et al. (bioRxiv 2019); Svensson (bioRxiv 2019)

Does log-normalization (scaling) work?

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

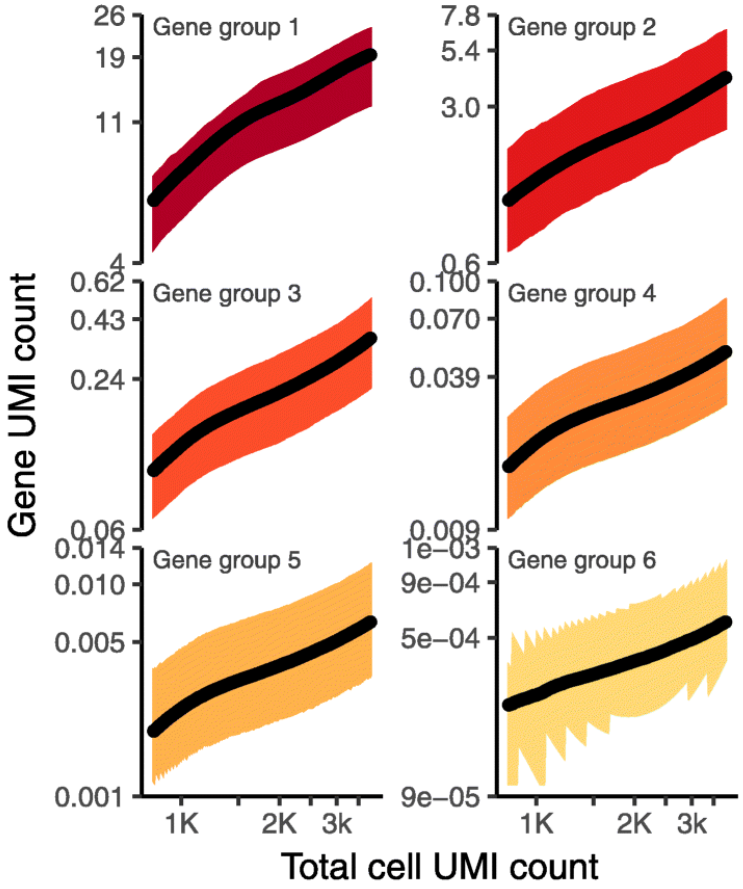




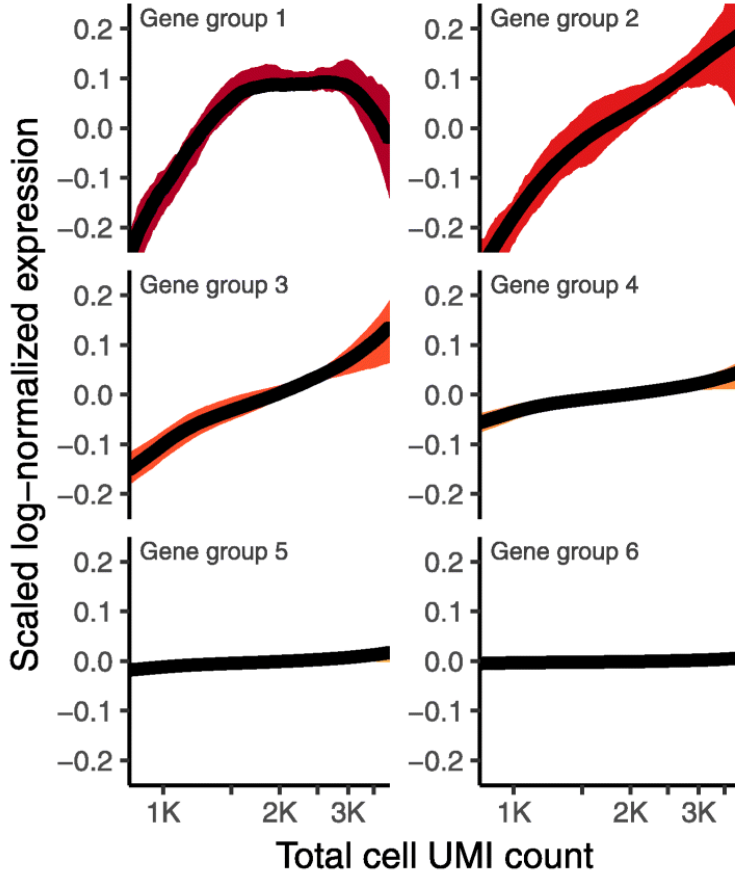
Hafemeister & Satija (Genome Biol 2019)

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)

Scaling data (Z-score transformation)

- Z-score transformation: linearly transform data to a mean of zero and a standard deviation of 1

- PCA or any other type of analysis will be dominated by highly expressed genes with high variance.

- It can be wise to center and scale each gene before performing PCA

Which normalization method to use?

- Normalization has big impact on differential gene expression, but not as much on clustering

- In most cases it is enough to do sequence depth normalization and log-normalization.

- Binning by gene level (SCTransform) helps to remove the effect of different gene detection across cells.

Confounding factors

- Any source of variation that you do not expect to give separation of the cell types:
  - Cell cycle, size, sequencing depth, mitochondrial reads,…
- Regress these sources of variation out from your data
- Tools like SCTransform, ZIMB-WaVE does regression in the same step.
- **BUT,** be careful that your confounders are not related to your biological question!

A different view…

- For cell-based analysis (dimensionality reduction, clustering, cell type identification), binarized data is sufficient

Counts

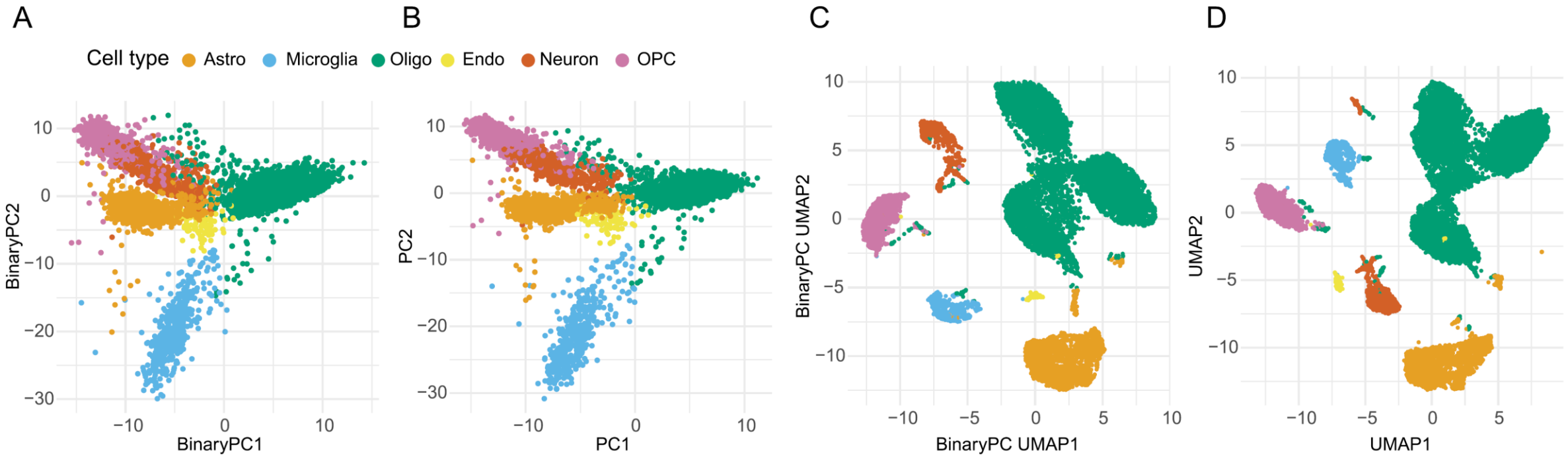
0	0	0	0	0	66	0
3	0	8	24	0	20	15
0	0	0	11	0	102	0
0	4	10	32	0	0	11
1	0	0	49	0	100	2
0	0	10	0	0	75	21
0	1	0	0	0	100	0

Detection

0	0	0	0	0	1	0
1	0	1	1	0	1	1
0	0	0	1	0	1	0
0	1	1	1	0	0	1
1	0	0	1	0	1	1
0	0	1	0	0	1	1
0	1	0	0	0	1	0

Bouland et al. (Genome Biology 2023)

Binary representation of scRNA-seq data



Binarized ~counts

1. Dimensionality reduction
2. Batch correction
3. Cell classification
4. Differential expression

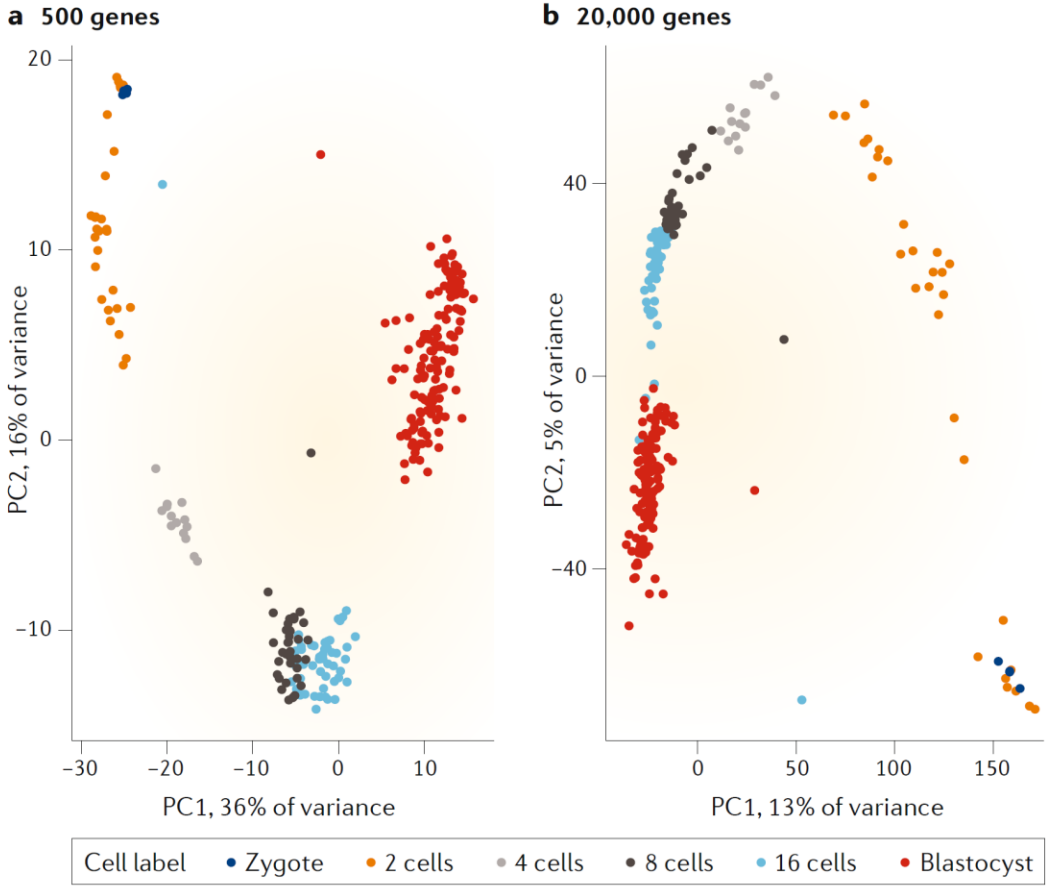
Bouland et al. (Genome Biology 2023)

Feature (Gene) 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



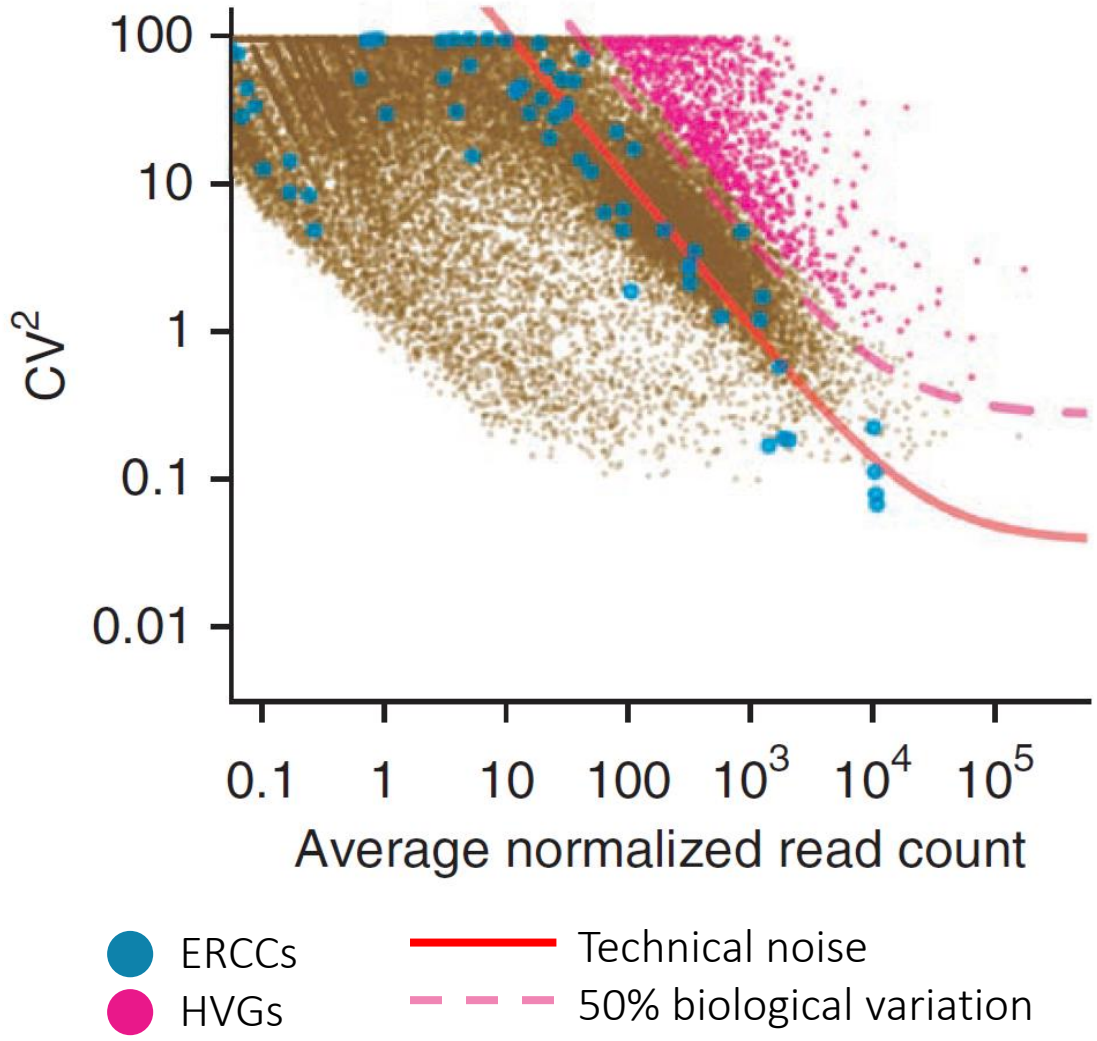
Kiselev et al. (https://doi.org/10.1038/s41576-018-0088-9)

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

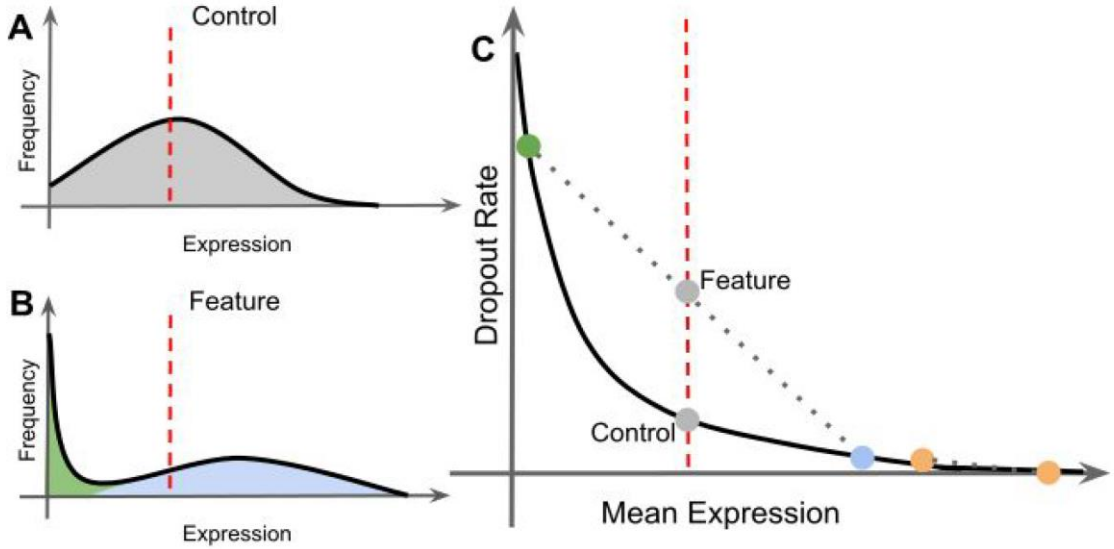
M3Drop: Dropout-based feature selection

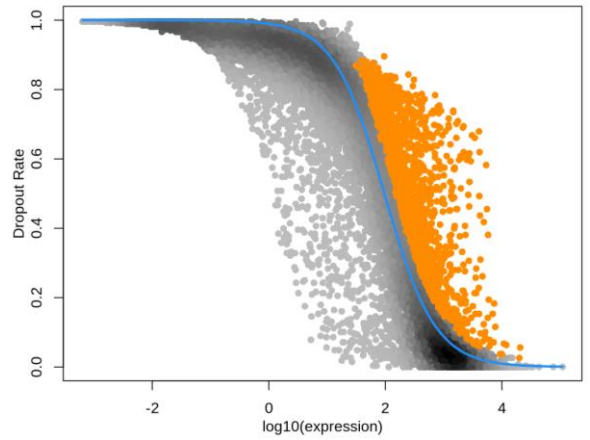
- Reverse transcription is an enzyme reaction thus can be modelled using the Michaelis-Menten equation:

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

S: average expression

K_M: Michaelis-Menten constant





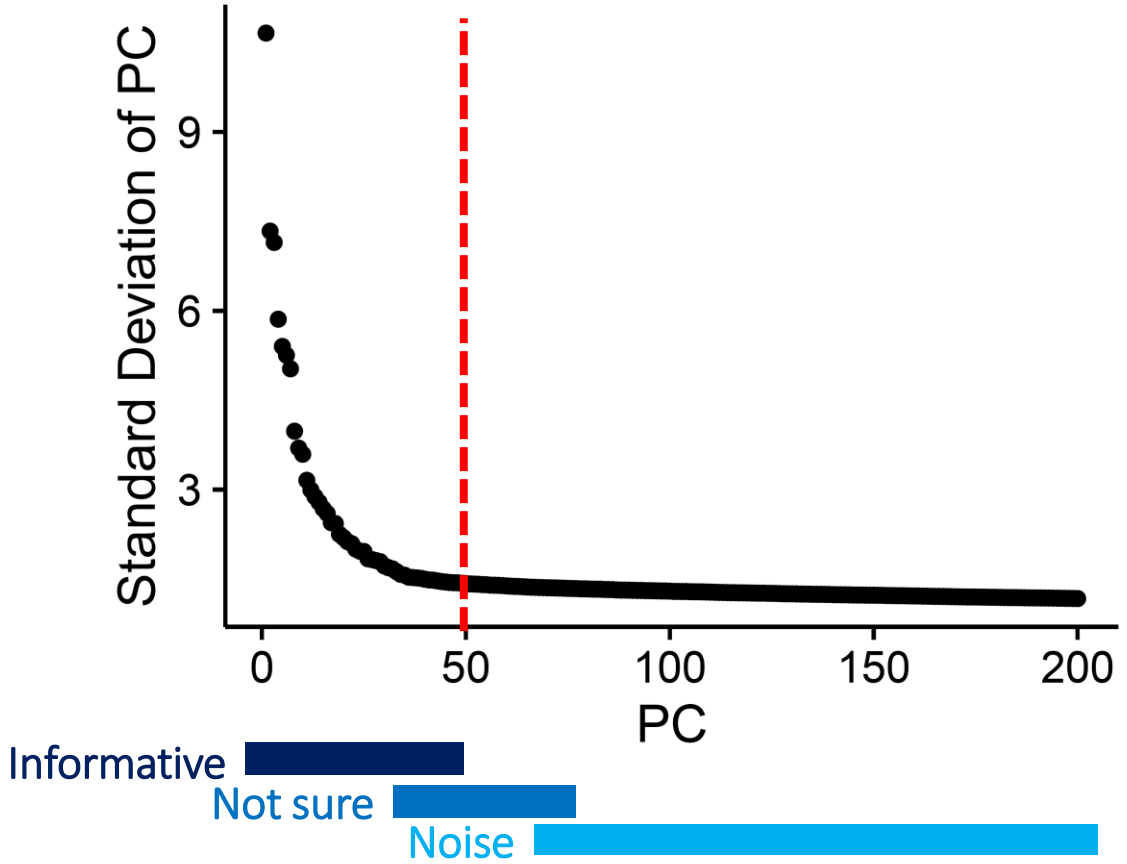
Brennecke et al. (https://doi.org/10.1038/nmeth.2645)

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
- Common to pick <=50 PCs

Scree/Elbow plot



Feature (un)-selection

- Remove genes unlikely to be of interest

- Ribosomal/mitochondrial genes
- Immunoglobulin (Ig) genes
- T cell receptor (TCR) genes

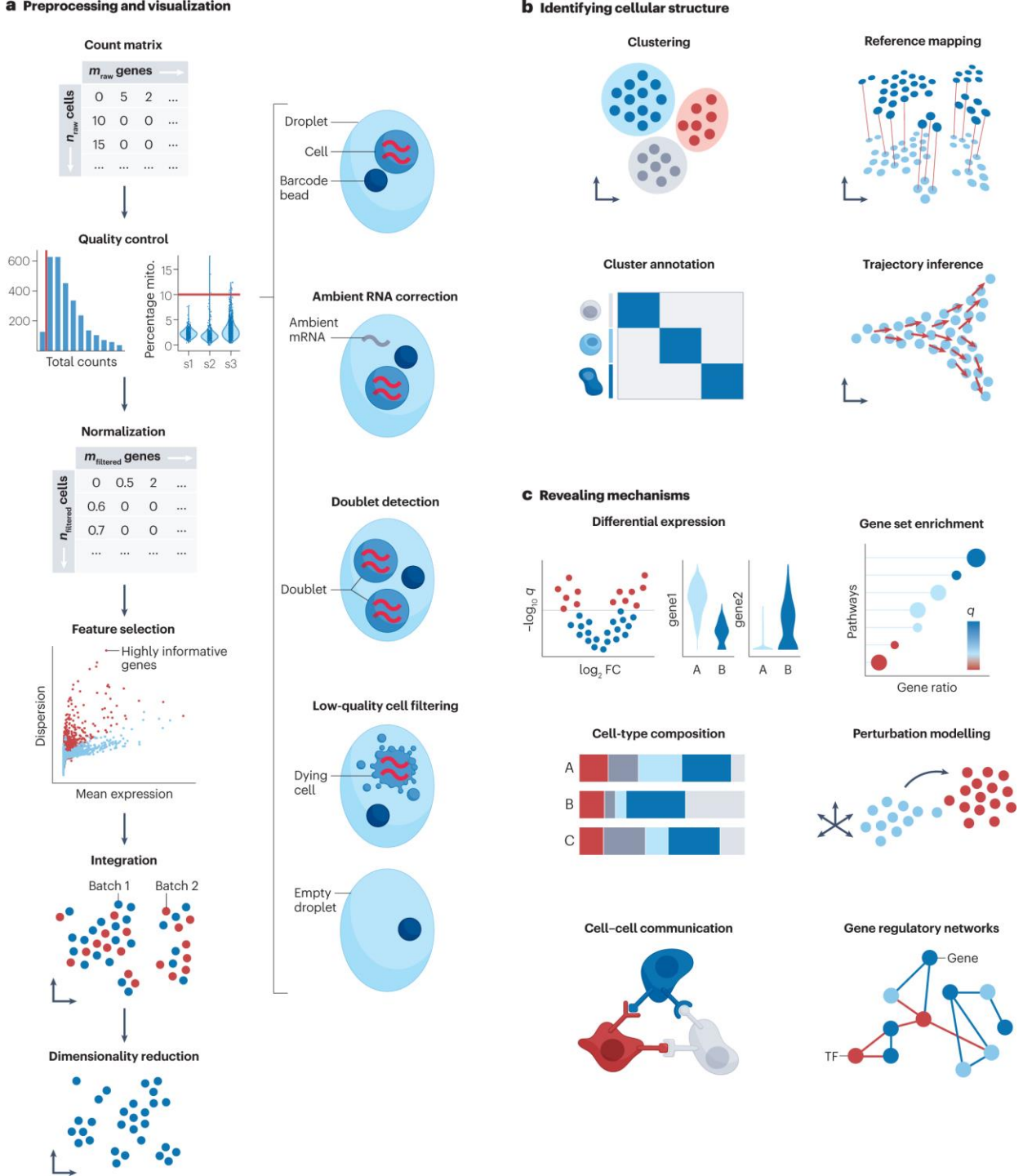
In short…

- If you have distinct celltypes – the clustering will be the same regardless of how you treat the data.

- **But,** for subclustering of similar celltypes normalization and removal of confounders may be crucial.

Summary

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



Heumos, Schaar, et al (Nat Rev Genetics 2023)

Useful Resources

- NBIS single-cell course:

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

- Single-cell best practices

https://www.sc-best-practices.org/

- 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

Thank You!

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