Quality Control and Normalization of Single Cell RNA-seq Data

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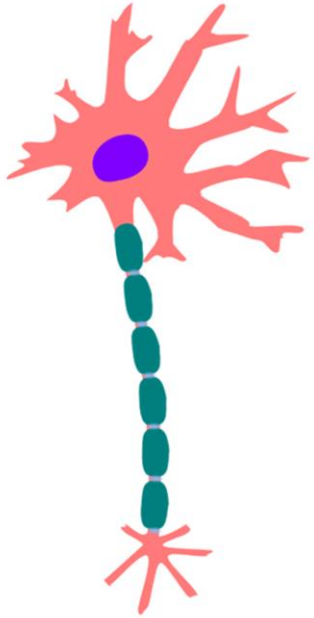
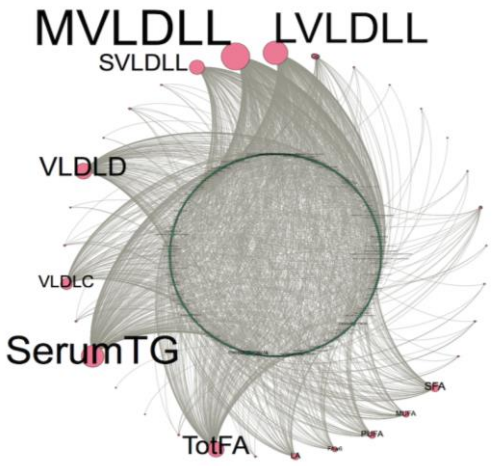
Spatio-Temporal Omics

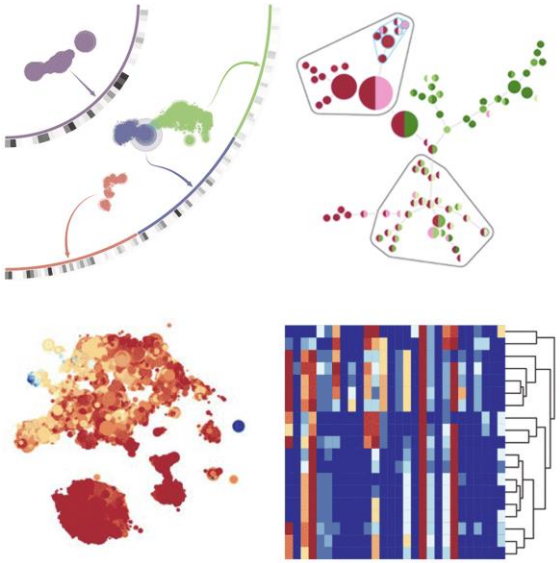
Singe Cell Omics



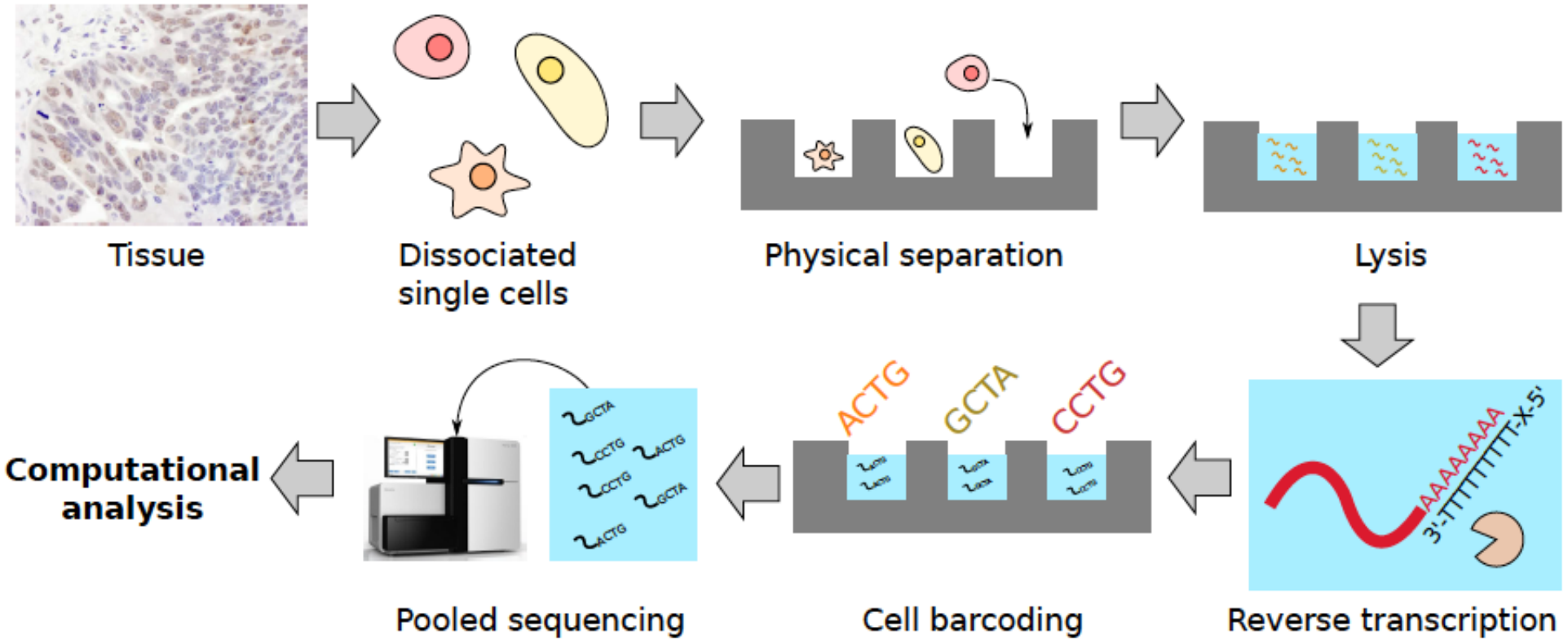
Multi-Omics Integration



Visualization & Visual Analytics



Single cell RNA-sequencing (scRNA-seq)



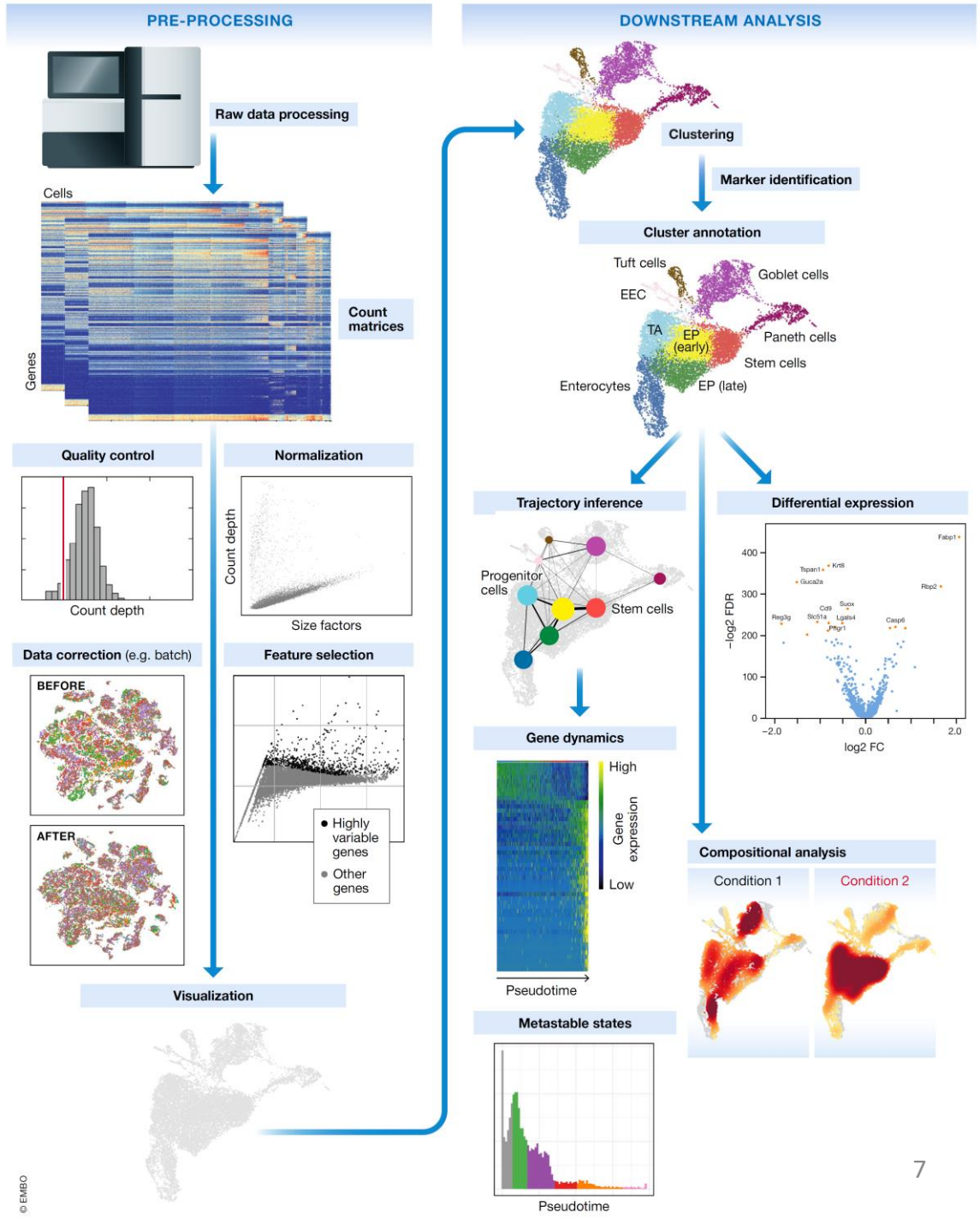
Aaron Lun (Marioni Group, EMBL-EBI)

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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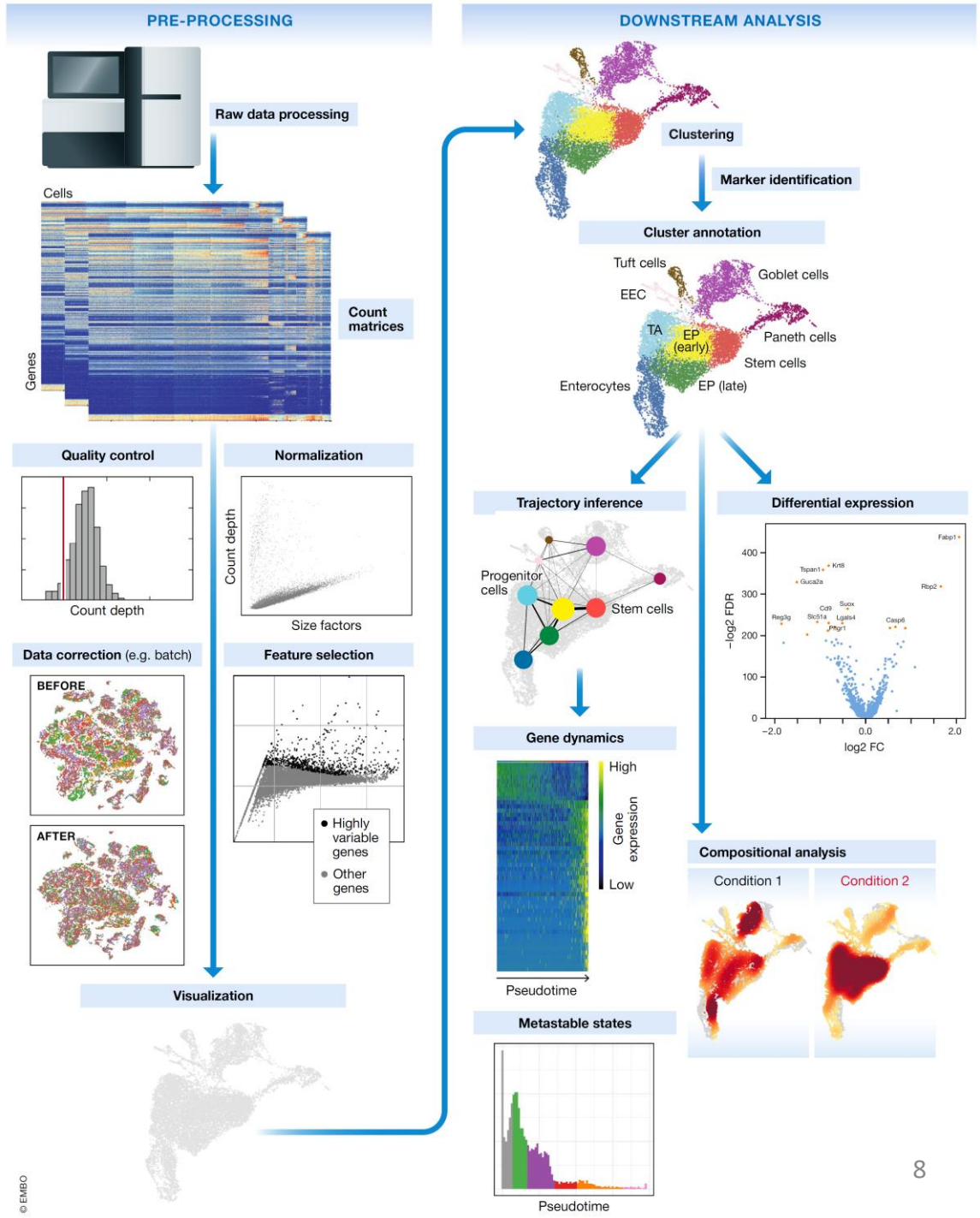


Luecken and Theis (MSB 2019)

Course materials

https://github.com/LeidenCBC/MGC-SingleCellAnalysis2019

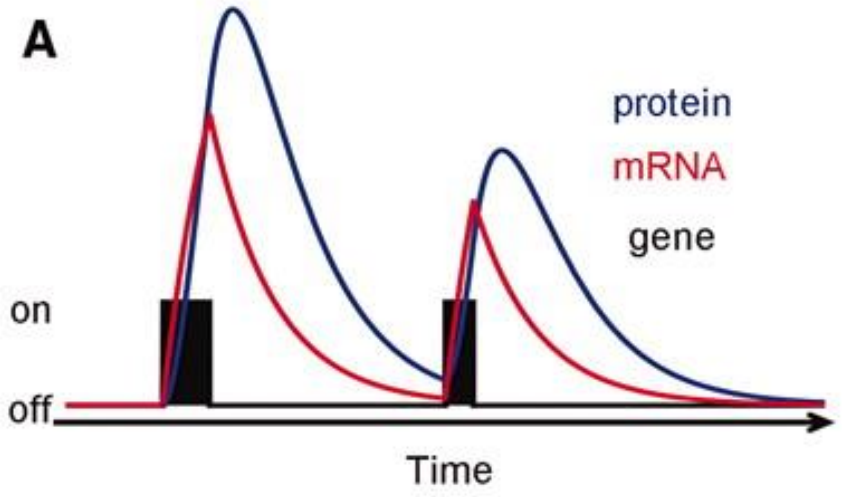
Credits: Åsa Björklund (NBIS, SciLifeLab)

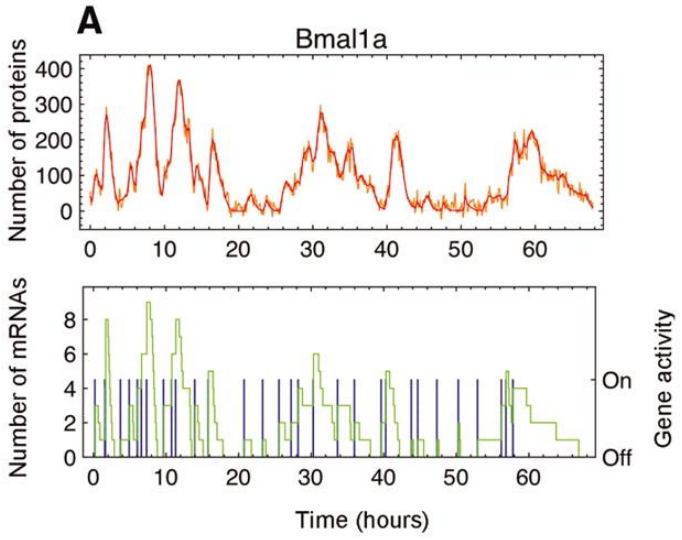
Our agenda

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

Transcriptional bursting

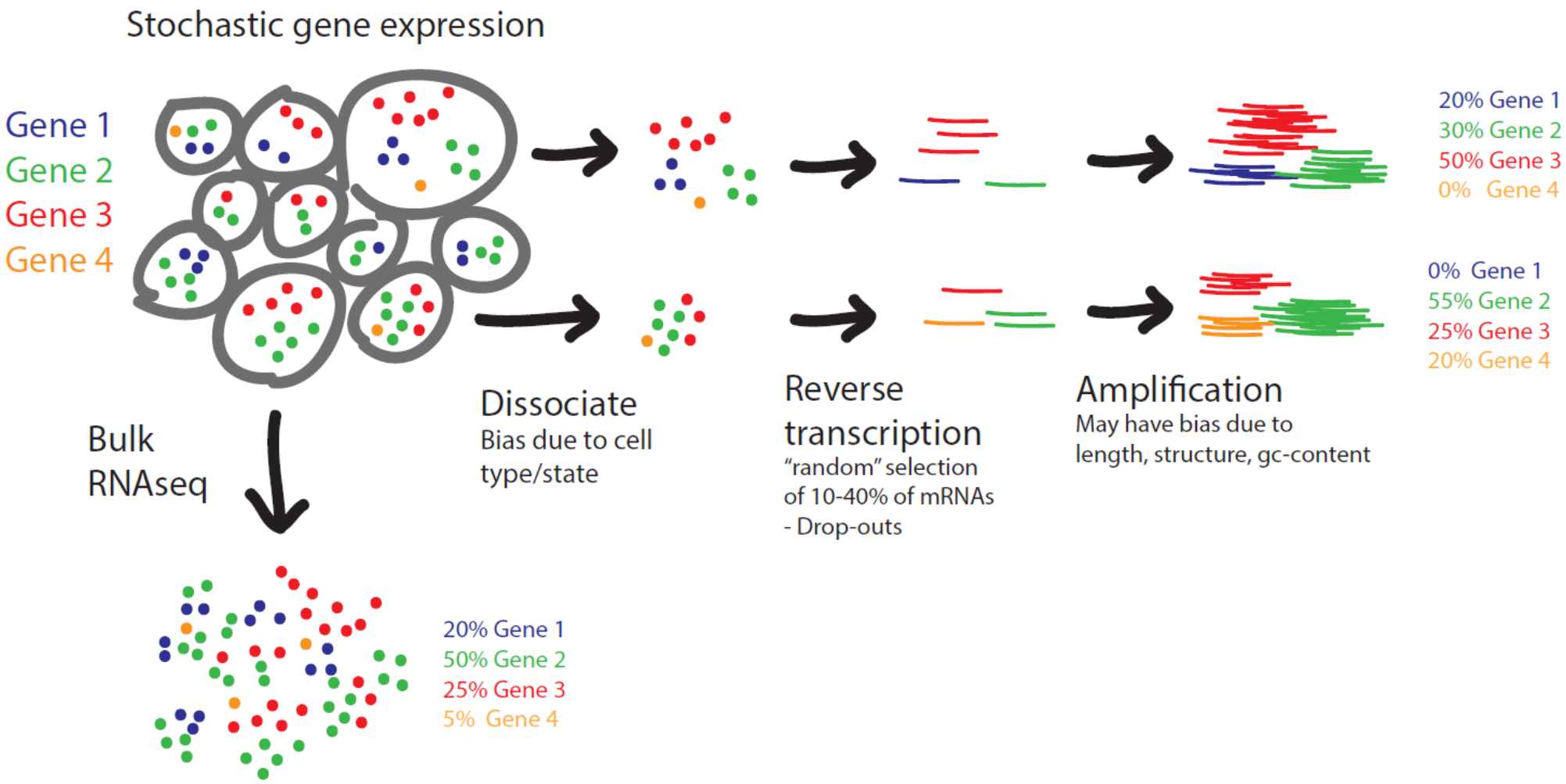
- Burst frequency and size is correlated with mRNA abundance
- Many TFs have low mean expression (and low burst frequency) and will only be detected in a fraction of the cells





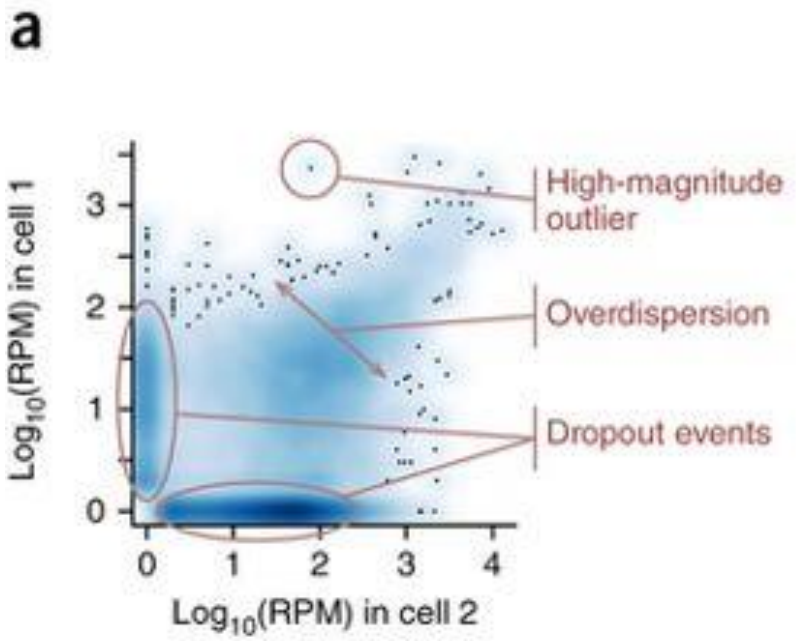
Suter et al. (Science 2011)

Bursting, drop-outs and amplification bias



Problems compared to bulk RNA-seq

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



Karchenko et al. (Nature Methods 2014)

What could go wrong?

Cell Dissociation

Single cell capture

Single cell lysis

Reverse transcription

Preamplification

Library preparation and sequencing

Cell dissociation

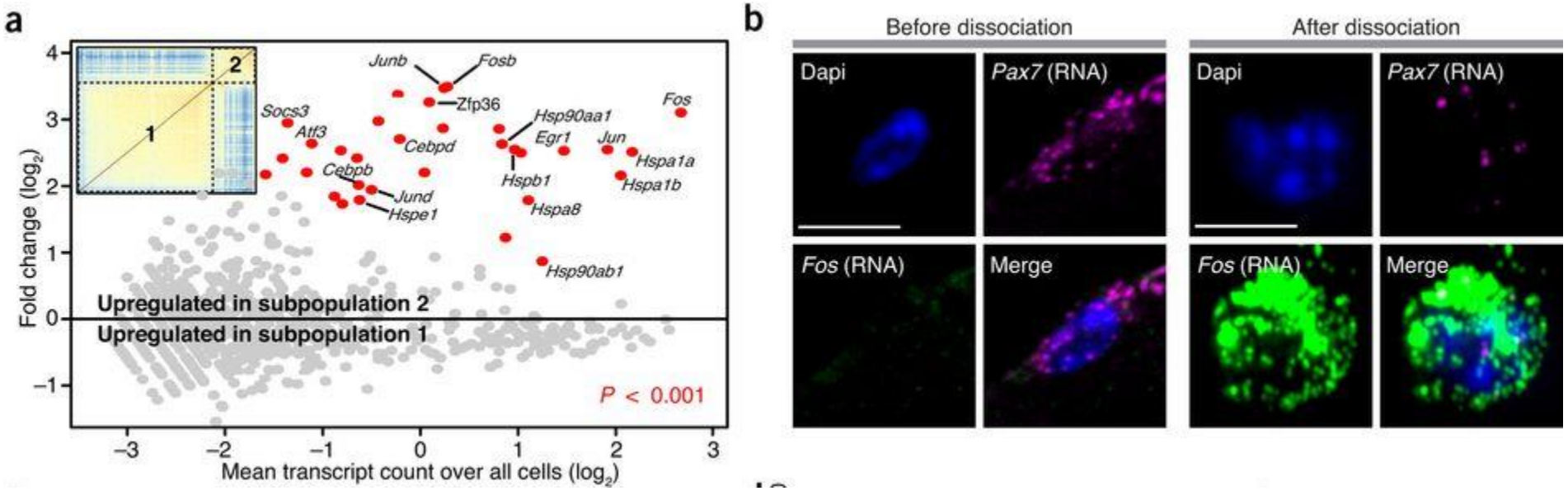
- It is critical to have healthy whole cells with no RNA leakage. Short time from dissociation to cell!

- 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.

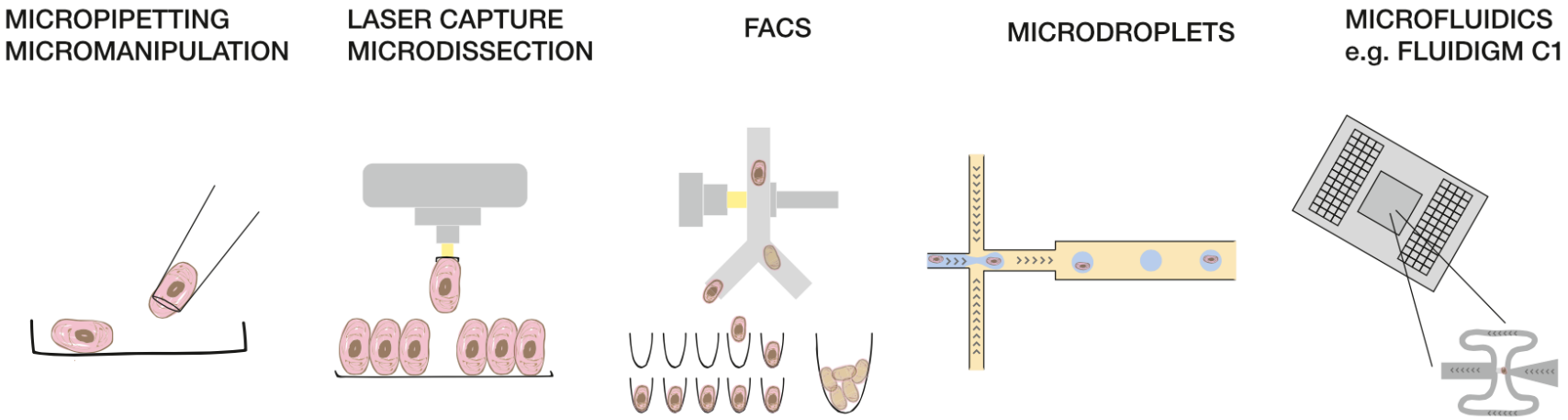
Dissociation artifacts

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



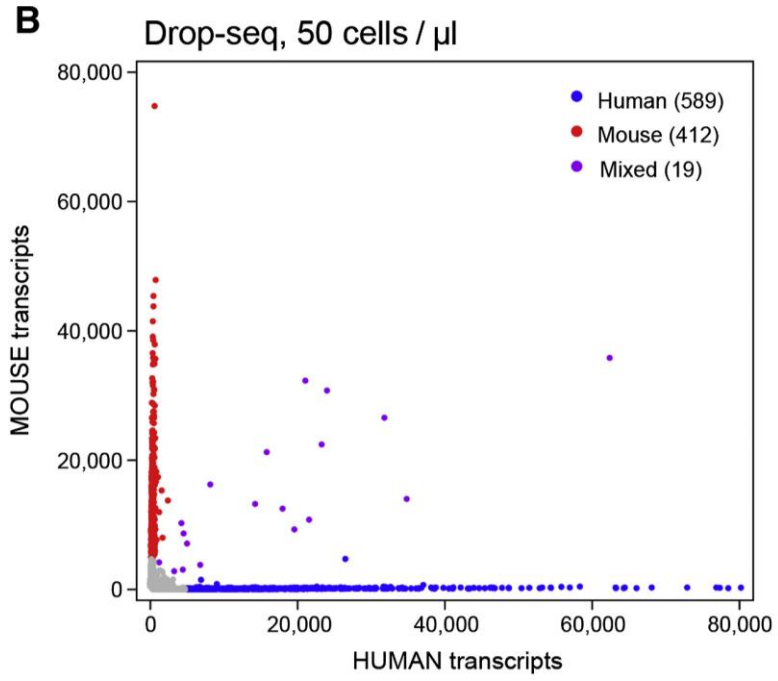
van den Brink et al. (Nature methods 2017)

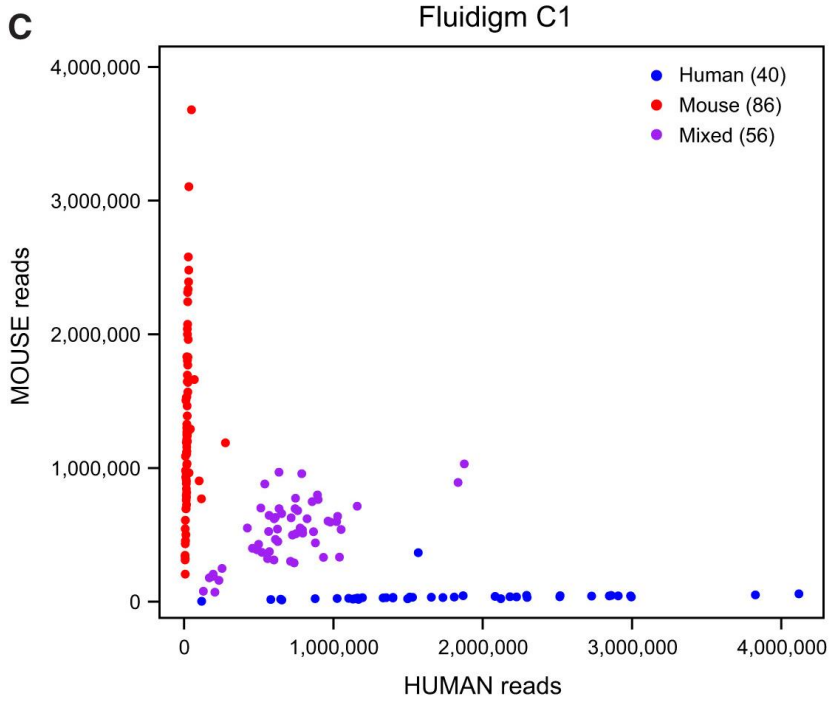
Single cell capture



- 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 celltype(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 celltype.

- 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

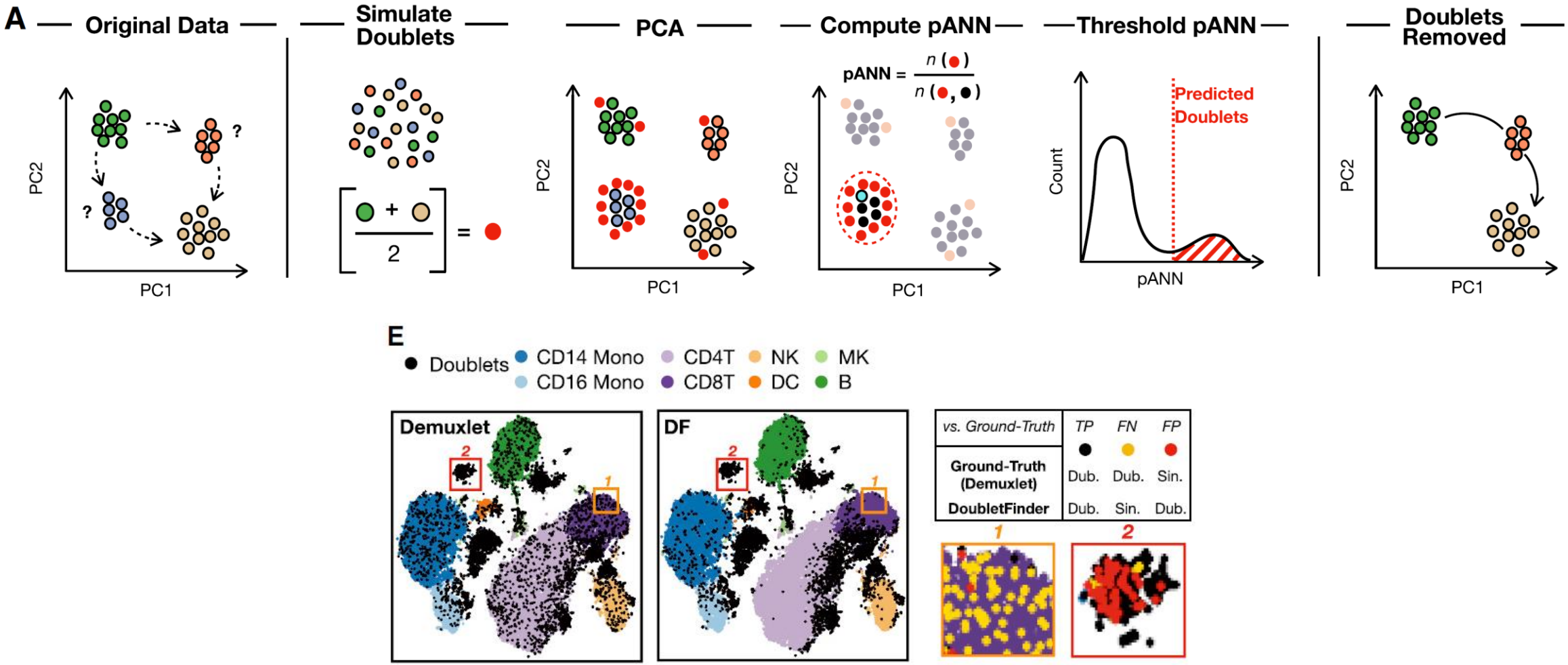
https://github.com/AllonKleinLab/scrublet

- DoubletDecon

https://github.com/EDePasquale/DoubletDecon

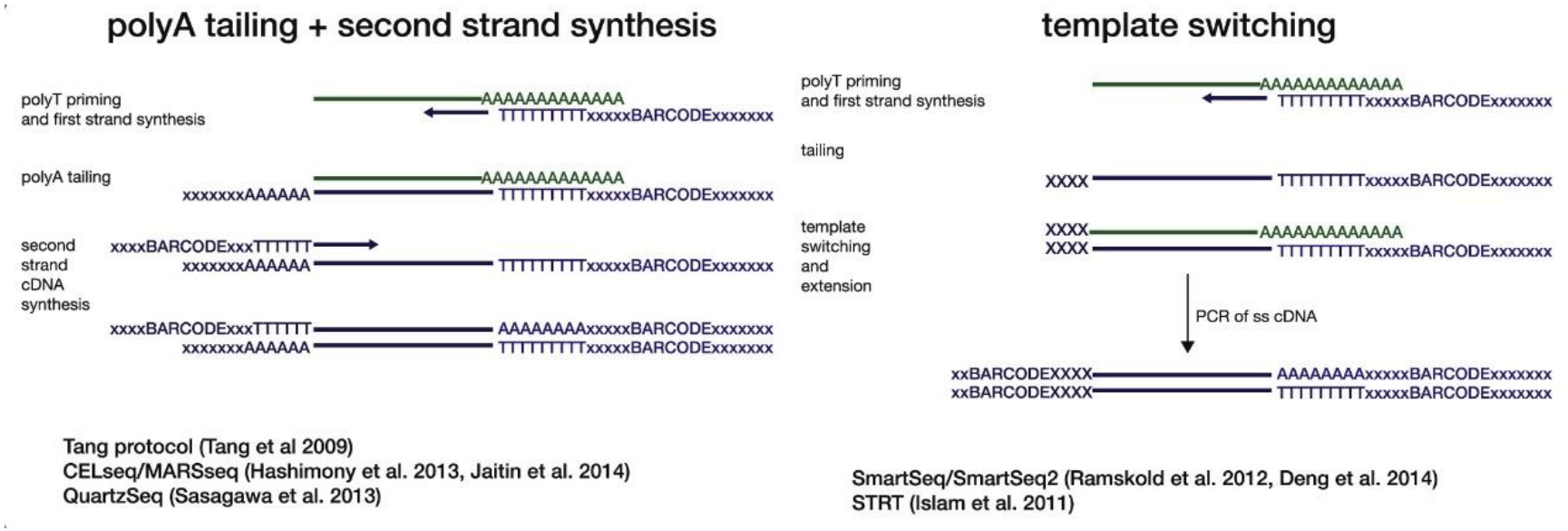
- DoubletCluster / DoubletCell in Scran

DoubletFinder



McGinnis et al. (Cell Systems 2019)

Reverse transcription



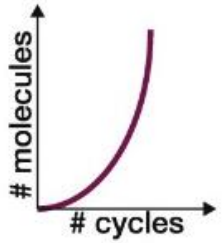
- Efficiency of reverse transcription is the key to high sensitivity.
- Drop-out rate is around 90-60% depending on the method used.
- Two libraries with the same method using the same cell type may have very different drop-out rates.

Kolodziejczyk et al. (Molecular Cell 2015)

Preamplification

PCR

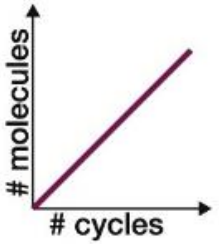
- exponential amplification
- PCR base specific biases



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



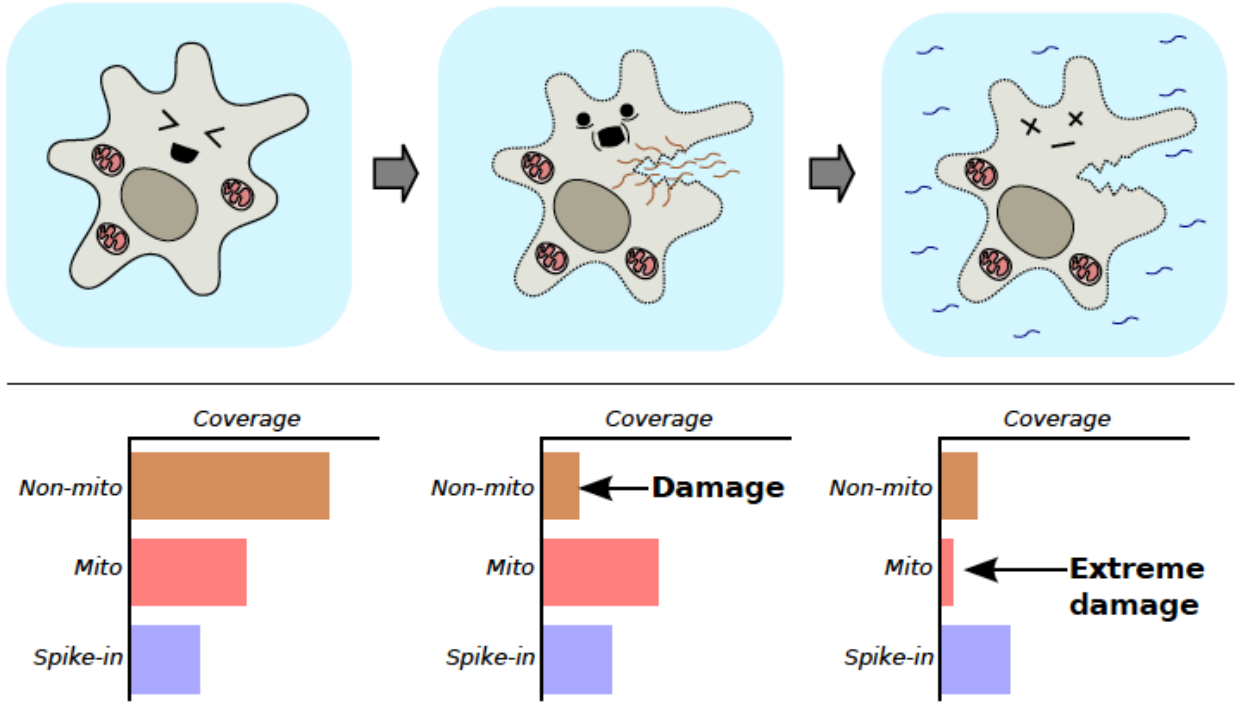
CELseq/MARSseq (Hashimony et al. 2013, Jaitin et al. 2014)

- 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)

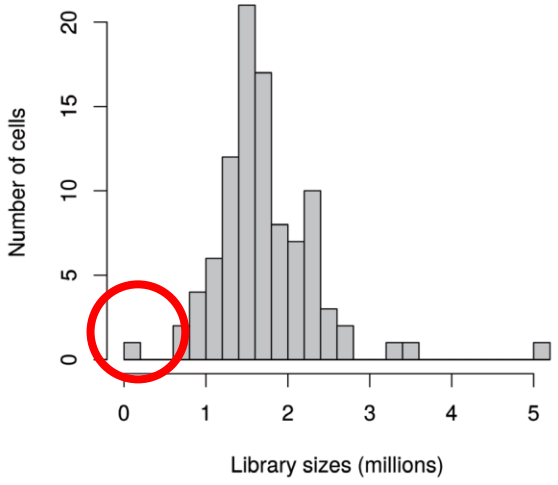
Quality control of cells (1)

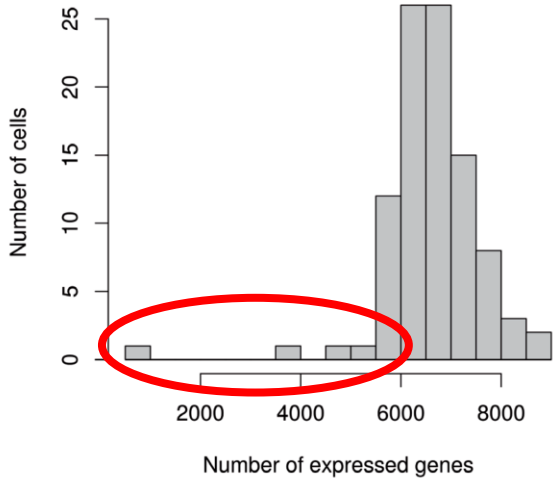
- Low sequencing depth
- Low numbers of expressed genes (i.e. any nonzero count)
- High spike-in (if present) or mitochondrial content



Quality control of cells (2)

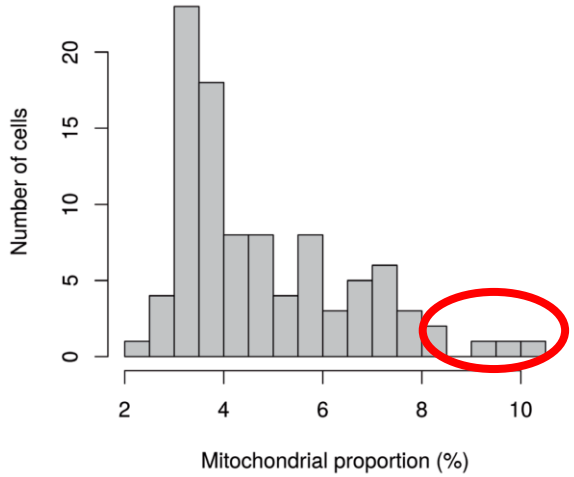
RNA has not been efficiently captured during library preparation

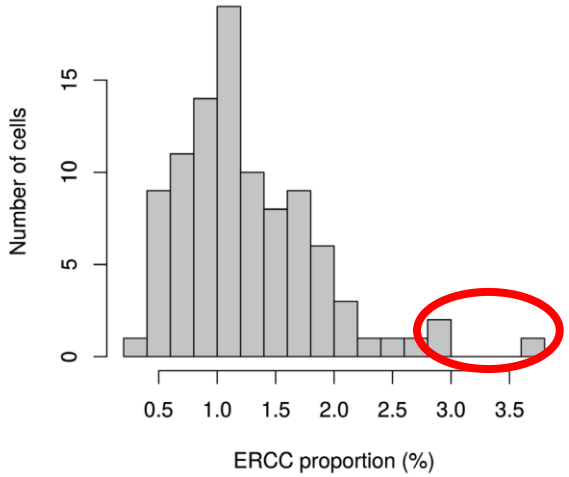




Diverse transcript population not captured

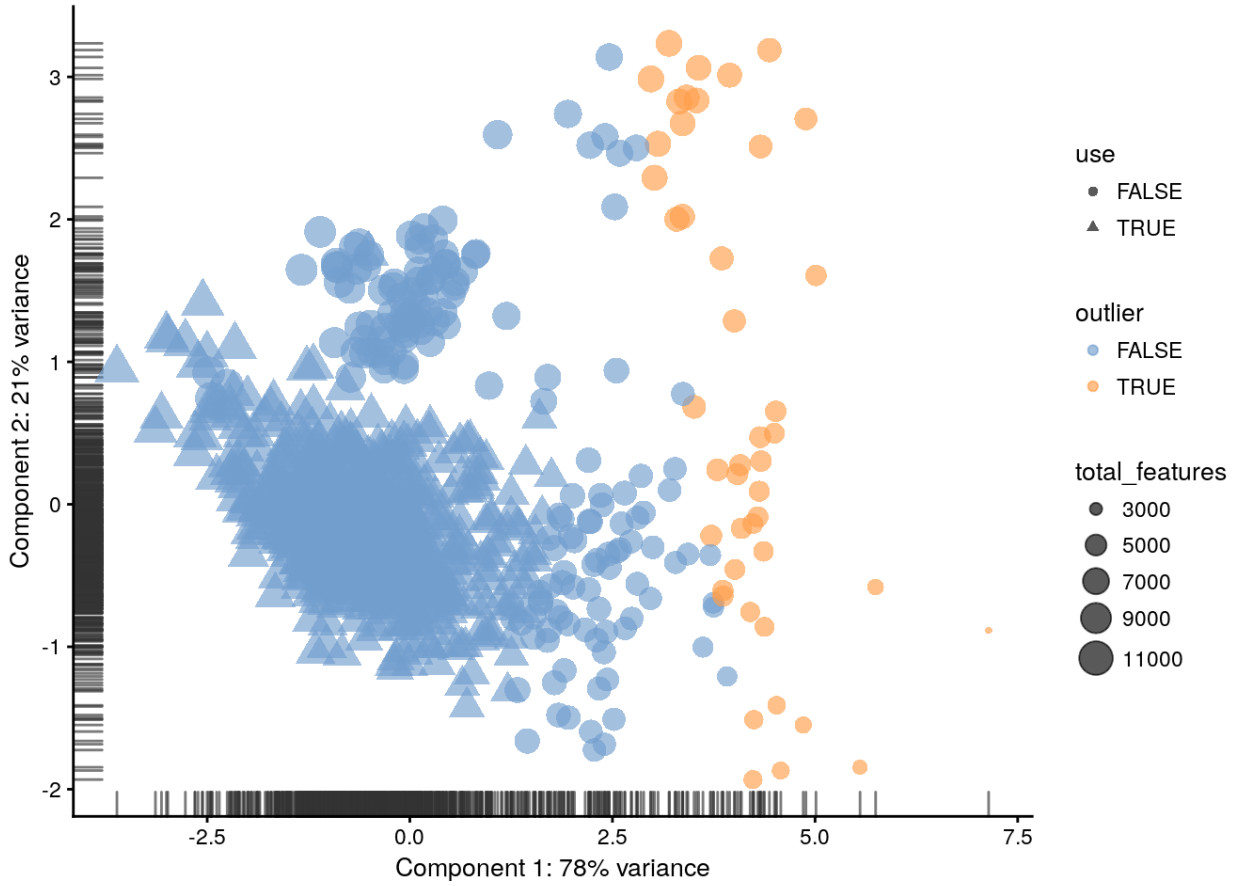
Possibly because of increased apoptosis and/or loss of cytoplasmic RNA from lysed cells





Quality control on cells (3)

PCA on a set of QC metrics



Possible features

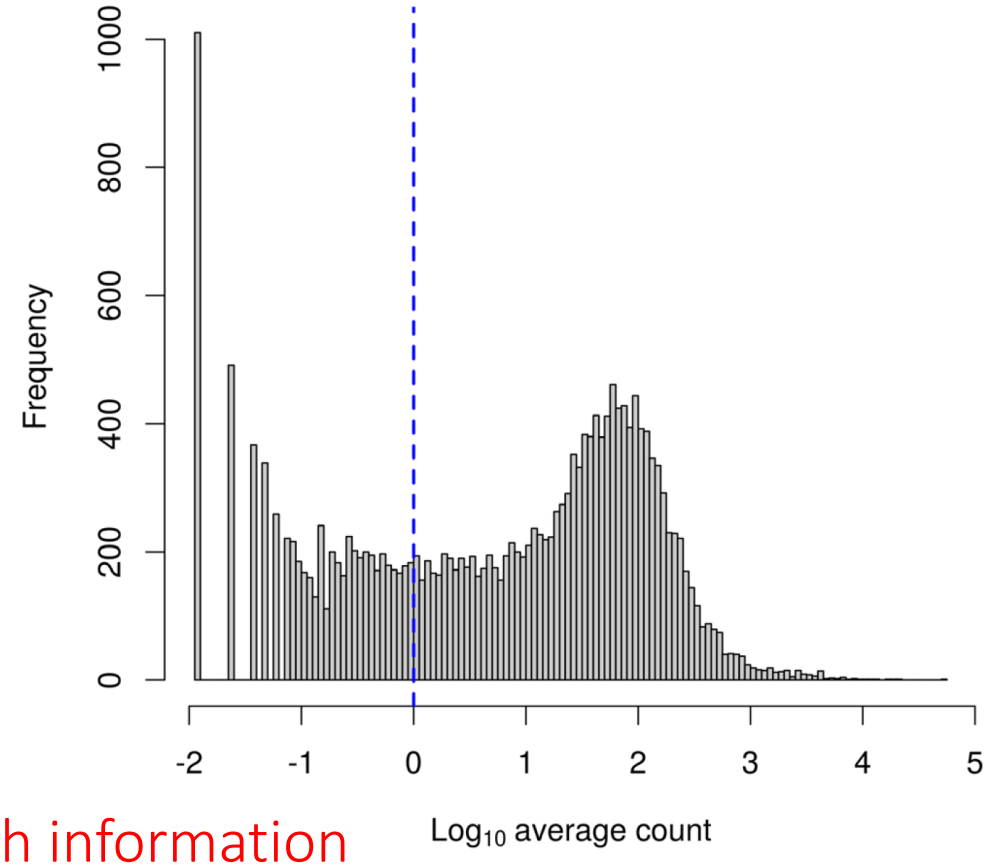
- total number of reads
- total number of features
- proportion of mitochondrial reads
- …

Interpretation!

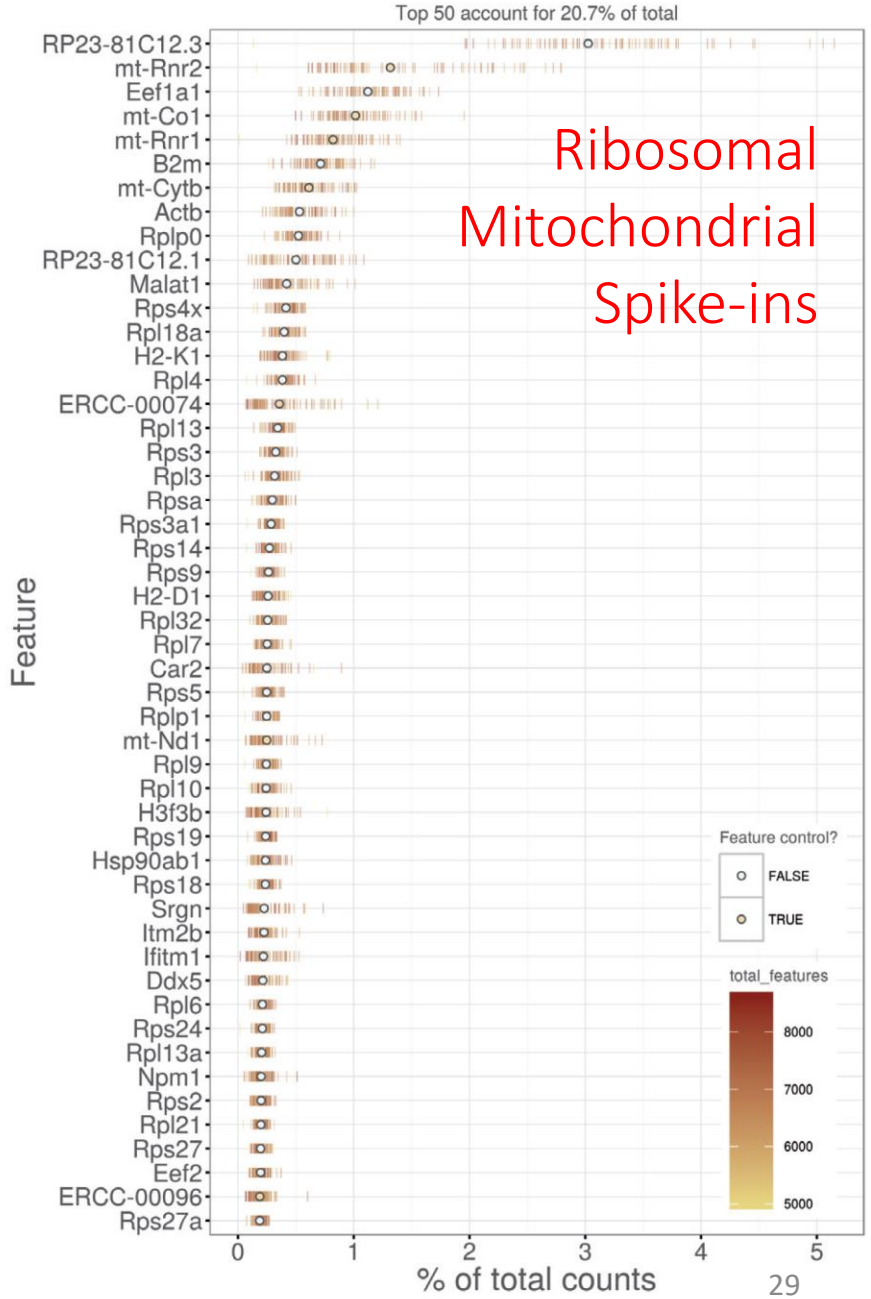
Deciding on cutoffs for filtering

- Do you have a homogeneous population of cells with similar sizes?
- Is it possible that you will remove cells from a smaller celltype?
- 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).

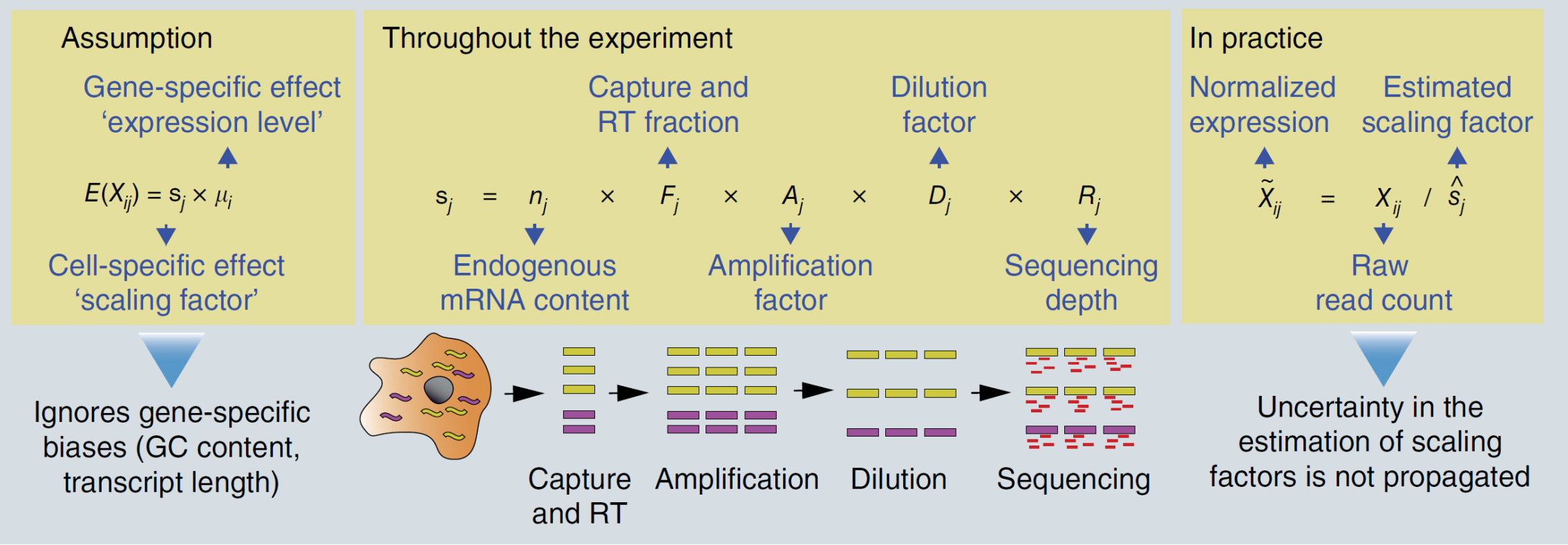
Luecken and Theis (MSB 2019)

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

Is any of your findings correlated with technical factors?

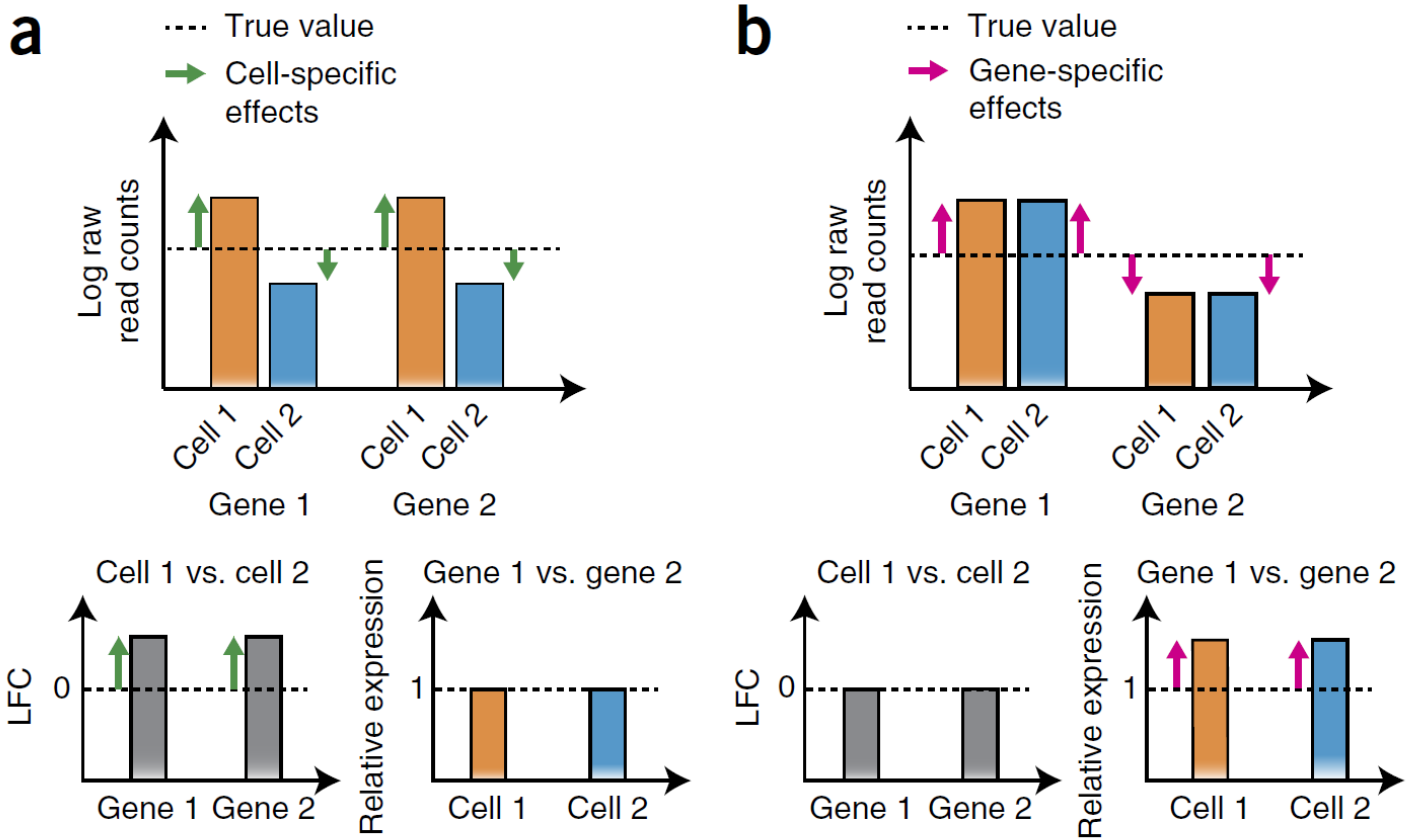
Normalization

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 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	✓		✓

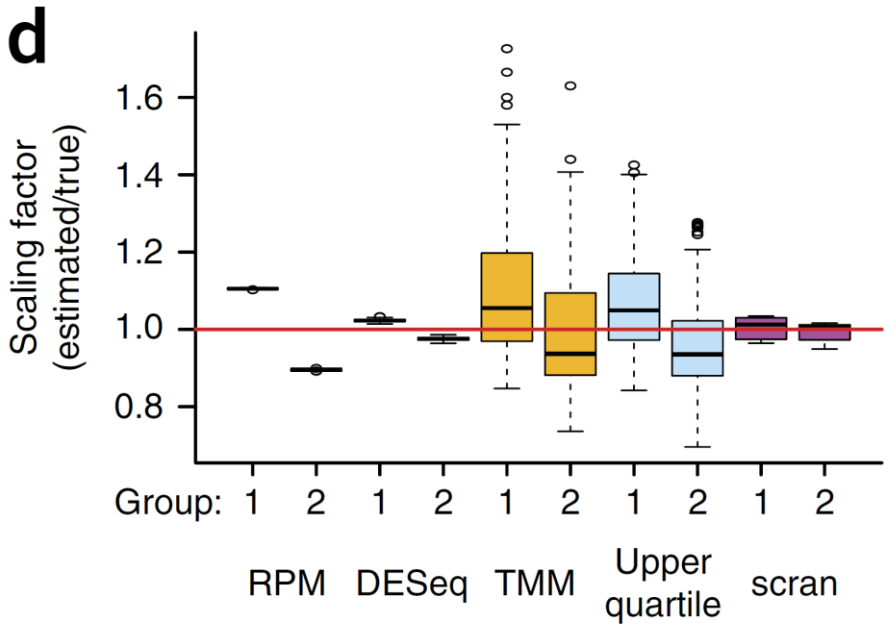
Normalization (2)

- 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

2. Probabilistic methods (Zero-inflated negative binomial (ZINB) models).

   E.g. ZINB-WaVE, Risso et al. (Nature Comm 2018).

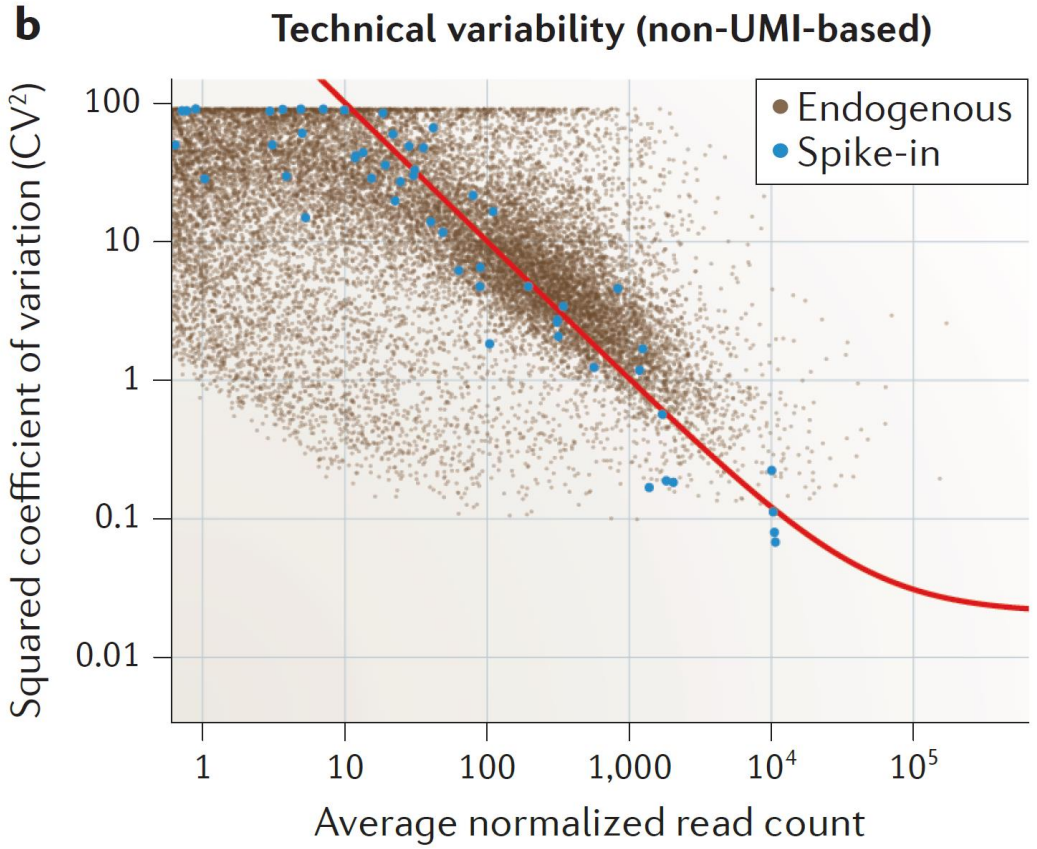
Size factor scaling methods

- 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, 10X Cell Ranger: log-normalization

Using Spike-In RNA

Caveats:

- The same quantity of spike-in RNA may not be consistently added to each sample
- Synthetic spike-in transcripts may not behave in the same manner as endogenous transcripts
- Not easily incorporated in all scRNA-seq protocols (not in droplet-based)



Brennecke et al. (Nat Methods 2013)

Normalization (4)

To spike in or not to spike in?

Assessing the reliability of spike-in normalization for analyses of single-cell RNA sequencing data

Aaron T.L. Lun,[1] Fernando J. Calero-Nieto,[2] Liora Haim-Vilmovsky,[3,4] Berthold Göttgens,[2] and John C. Marioni[1,3,4]

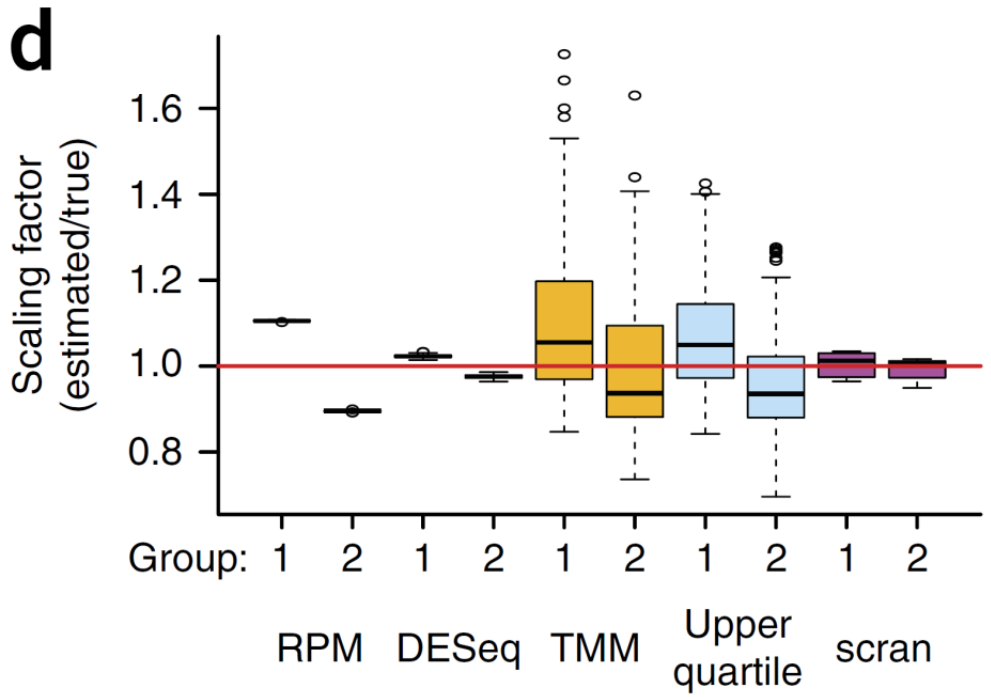
[1]*Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Cambridge CB2 0RE, United Kingdom;* [2]*Wellcome Trust and MRC Cambridge Stem Cell Institute, University of Cambridge, Cambridge CB2 0XY, United Kingdom;* [3]*EMBL European Bioinformatics Institute, Wellcome Genome Campus, Hinxton, Cambridge CB10 1SD, United Kingdom;* [4]*Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom*

By profiling the transcriptomes of individual cells, single-cell RNA sequencing provides unparalleled resolution to study cellular heterogeneity. However, this comes at the cost of high technical noise, including cell-specific biases in capture efficiency and library generation. One strategy for removing these biases is to add a constant amount of spike-in RNA to each cell and to scale the observed expression values so that the coverage of spike-in transcripts is constant across cells. This approach has previously been criticized as its accuracy depends on the precise addition of spike-in RNA to each sample. Here, we perform mixture experiments using two different sets of spike-in RNA to quantify the variance in the amount of spike-in RNA added to each well in a plate-based protocol. We also obtain an upper bound on the variance due to differences in behavior between the two spike-in sets. We demonstrate that both factors are small contributors to the total technical variance and have only minor effects on downstream analyses, such as detection of highly variable genes and clustering. Our results suggest that scaling normalization using spike-in transcripts is reliable enough for routine use in single-cell RNA sequencing data analyses.

Genome Research 27:1795–1806

Normalization (5)

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



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)

- We recommend scran for normalization of non-full-length datasets. An alternative is to evaluate normalization approaches via scone especially for plate-based datasets. Full-length scRNA-seq protocols can be corrected for gene length using bulk methods.

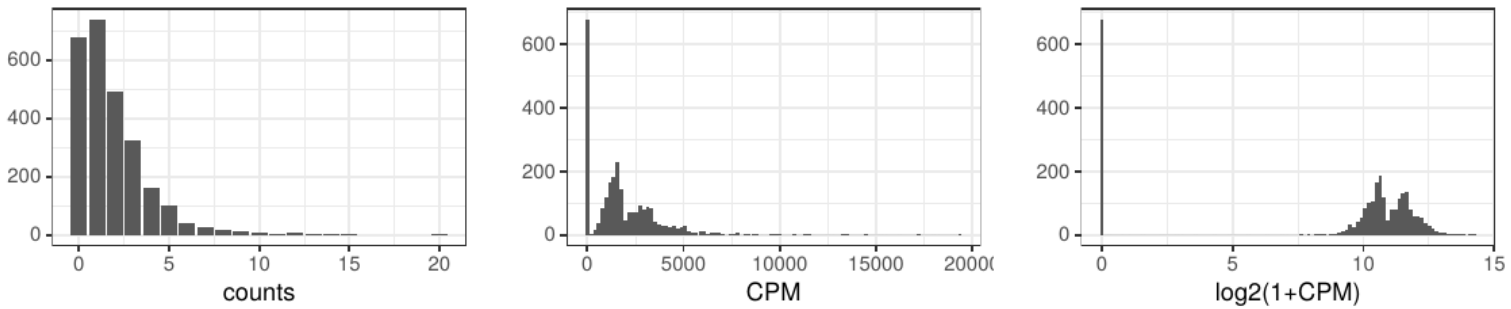
- There is no consensus on scaling genes to 0 mean and unit variance. We prefer not to scale gene expression.

- Normalized data should be log(x+1)-transformed for use with downstream analysis methods that assume data are normally distributed.

Luecken and Theis (MSB 2019)

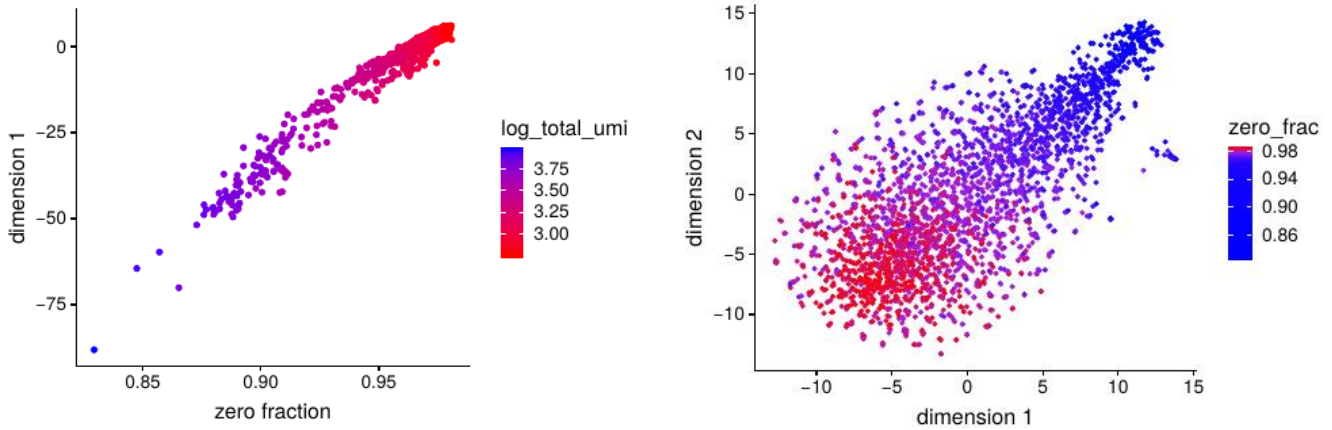
Effect of dropouts on normalization

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)

Useful Resources

- 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

- Aaron Lun's single cell workflow (very detailed):

https://www.bioconductor.org/packages/release/workflows/html/simpleSingleCell.html

- GitHub: Awesome Single Cell

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

- Recent developments in single cell genomics

https://www.dropbox.com/s/woya6ffgq8a3pkw/SingleCellGenomcisDay18_References.pdf?dl=1

Thank You!

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