Pre-processing and Quality Control of Single Cell RNA-seq Data

Daniel Chafamo
Klarman Cell Observatory
Broad Institute

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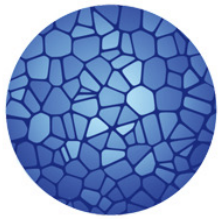

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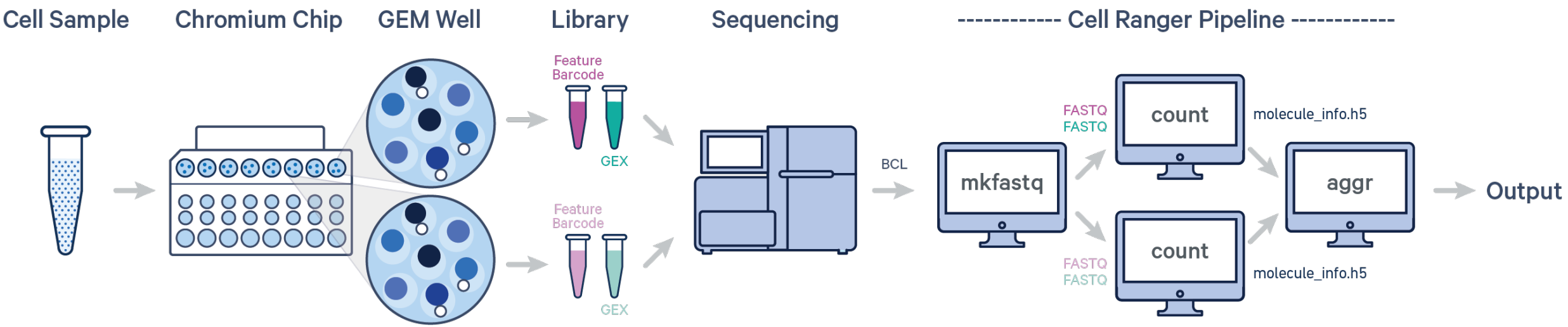
Topics

- Overview of steps to go from raw sequencing files to count matrices
- Demo of Terra: a scalable platform for preprocessing single cell data
- Quality control for single cell RNA-Seq Data
- Normalization of single cell RNA-Seq data

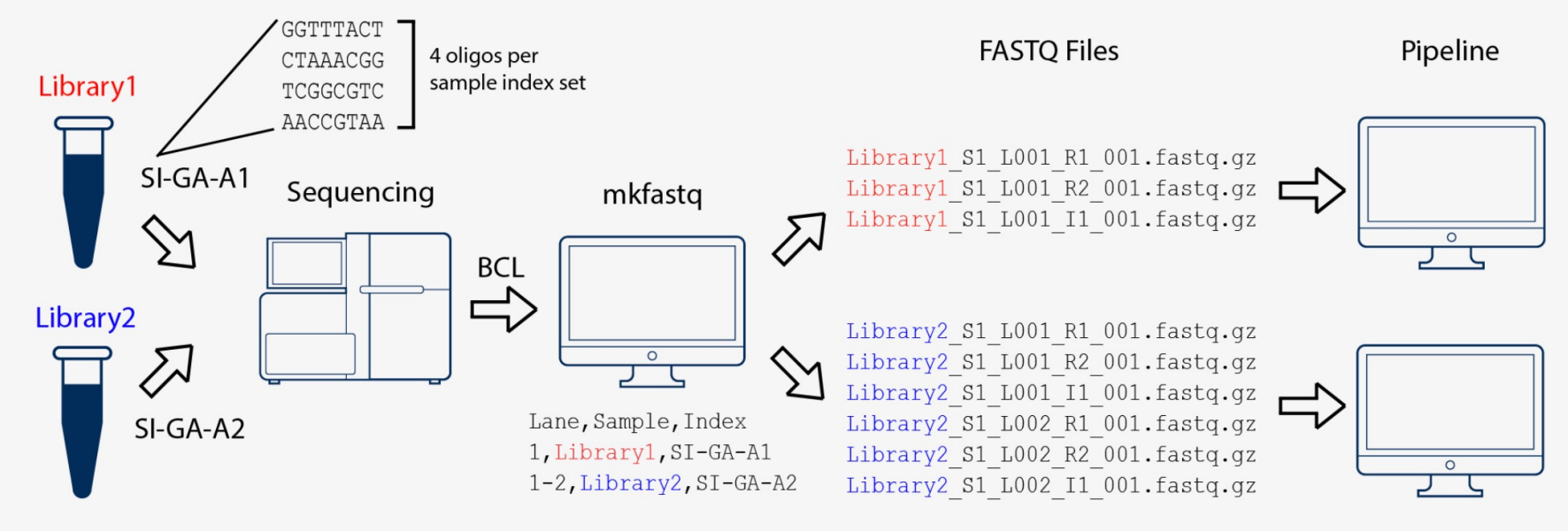
Raw sequencing files to count matrices



Source: 10X Genomics

Raw sequencing files to count matrices
Step 1: BCL to FASTQ

- The primary output of Illumina sequencing instruments are per-cycle base call files in BCL format.
- The first step is to convert these BCL files to fastq files. Steps include separating reads into individual fastq files based on their barcode (demultiplexing) and moving unique molecular identifier (UMI) bases from the read to the fastq header.
- Tools: cellranger mkfastq, bcl2fastq or BCL Convert



Source: 10X Genomics

Raw sequencing files to count matrices
Step 1: BCL to FASTQ

- A FASTQ file is a text file that contains the sequence data which consists of:

1. A sequence identifier with information about the sequencing run and the cluster.
2. The sequence (the base calls; A, C, T, G and N).
3. A separator, which is simply a plus (+) sign.
4. The base call quality scores as phred scores $P = 10^{\frac{-Q}{10}}$.

- Example sequence in a FASTQ

```
@HWI-ST808:130:H0B8YADXX:1:1101:2088:2222:CELL_GGTCCA:UMI_CCCT
AGGAAGATGGAGGAGAGAAGGCGGTGAAAGAGACCTGTAAAAAGCCACCGN
+
@@@DDBD>=AFCF+<CAFHDECII:DGGGHGIGGIIIEHGIIIGIIDHII#
```

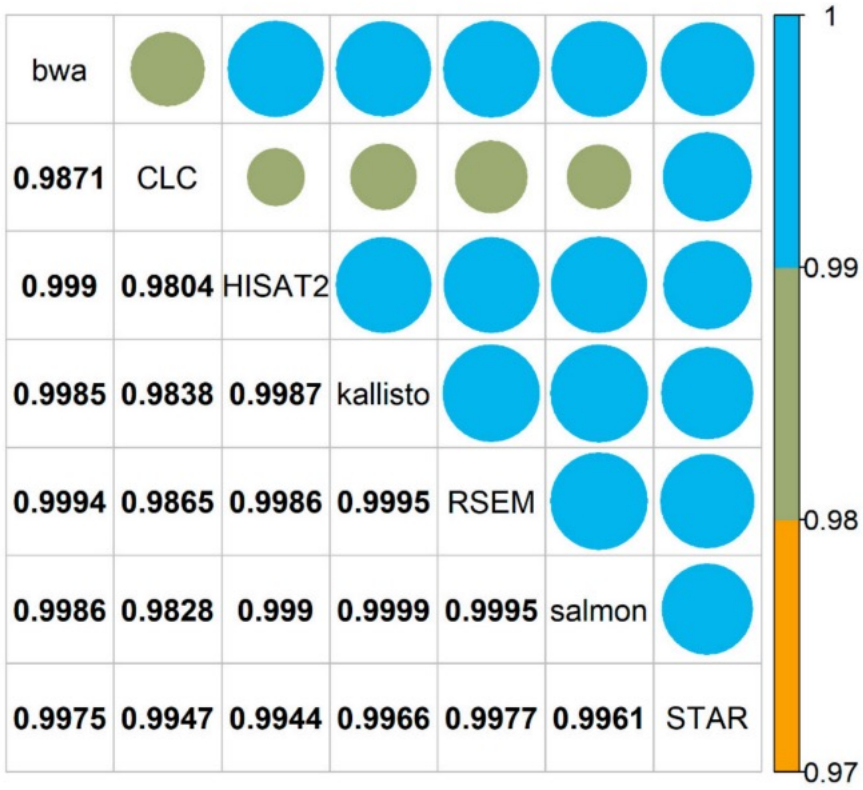
Raw sequencing files to count matrices
Step 2: Alignment to reference genome

- The next step is to determine which gene each read originated from. In order to do this the read sequences are mapped to a precompiled genome reference.

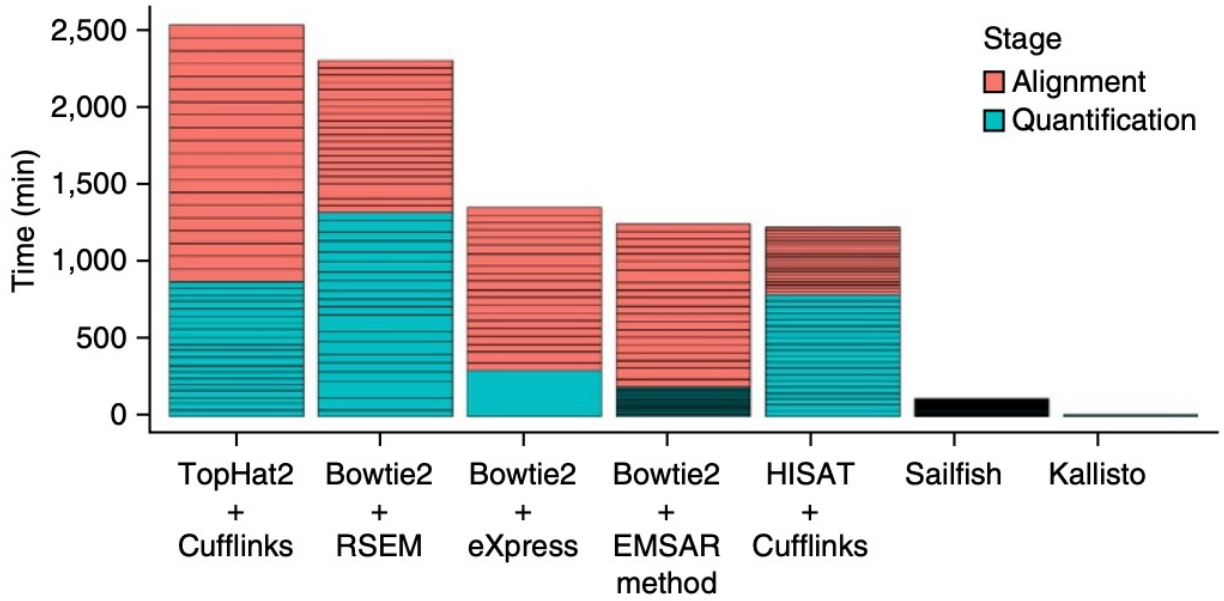
- Be mindful of reference versions and sources. Use latest when possible.
  - Human reference, GRCh38 (GENCODE v32/Ensembl 98)
  - Mouse reference, mm10 (GENCODE vM23/Ensembl 98)

Raw sequencing files to count matrices
Step 2: Alignment to reference genome

- The choice of aligner is often a personal preference and also dependent on the computational resources that are available to you.



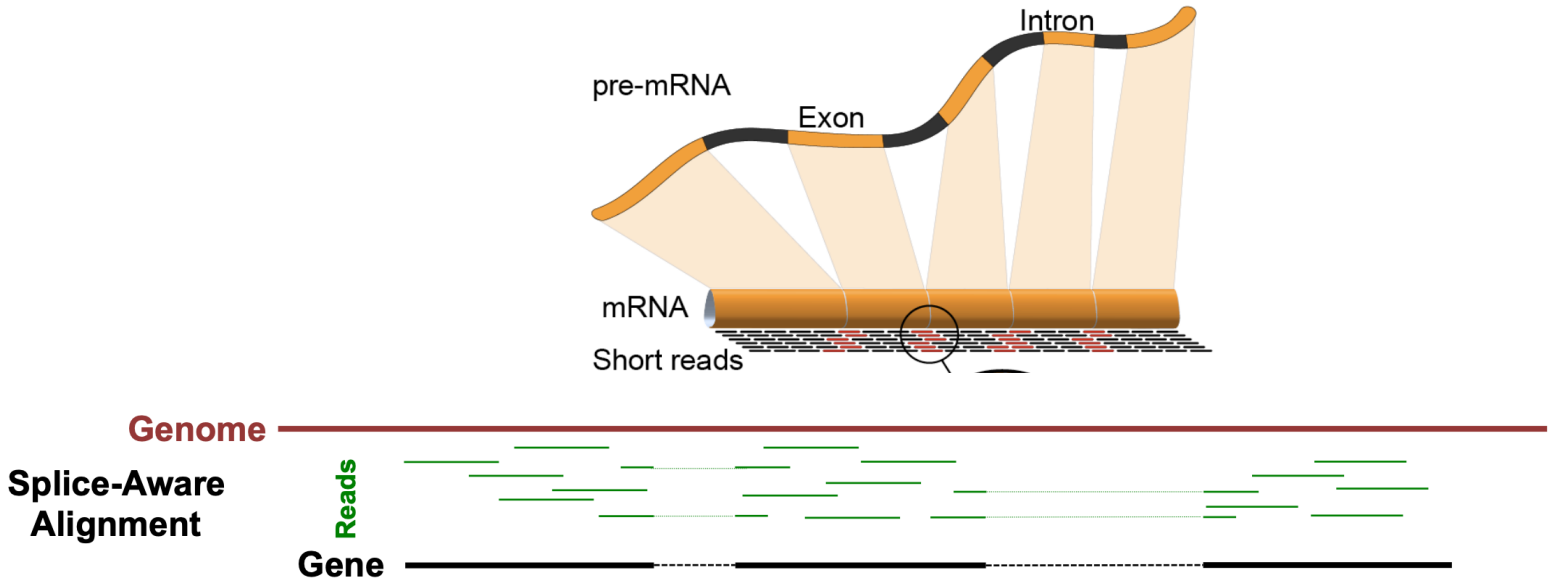
Mapper comparison based on raw count distributions.



Source: https://hbctraining.github.io/

Raw sequencing files to count matrices
Step 2: Alignment to reference genome

- 10X Cellranger uses the STAR aligner.
- Because of widespread splicing in animal genomes, read alignment against a genome should be done with a splice-aware aligner.



Source: https://hbctraining.github.io/

Raw sequencing files to count matrices
Step 3: Counting reads

- Reads that have been confidently mapped to the transcriptome are then assigned to cells based on their barcode (**cell barcode demultiplexing**) and the the number of unique RNA molecules corresponding to each gene within each cell are counted (**UMI deduplication**).

- The result is the gene x cell matrix that is the starting point for downstream analysis:

	Cell1	Cell2	...	CellN
Gene1	3	2	.	13
Gene2	2	3	.	1
Gene3	1	14	.	18
...	.	.	.	.
...	.	.	.	.
...	.	.	.	.
GeneM	25	0	.	0

Terra: a scalable platform for biomedical research

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Access public and access-controlled datasets

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Bring together data and tools into secure, shareable units

Workflows

Run workflows at scale; bring your own or explore community favorites

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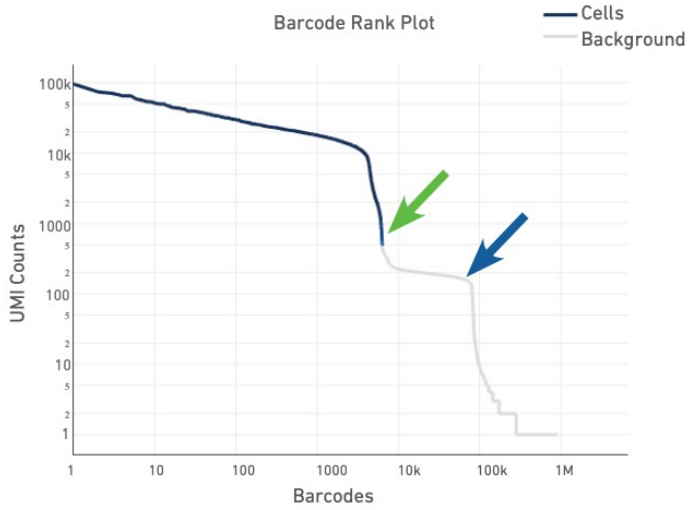
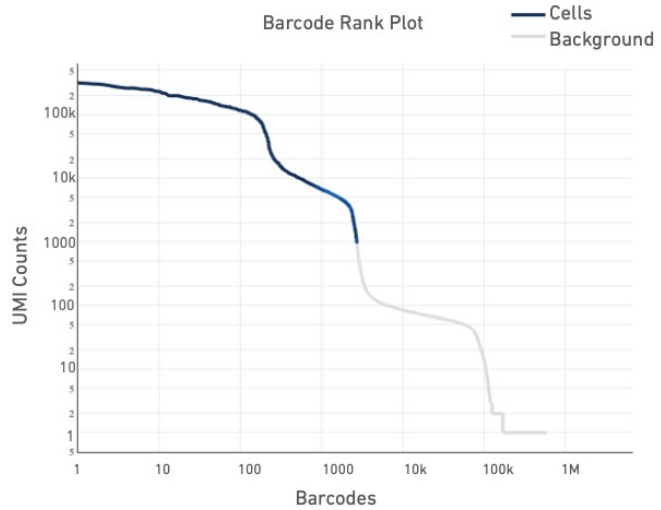
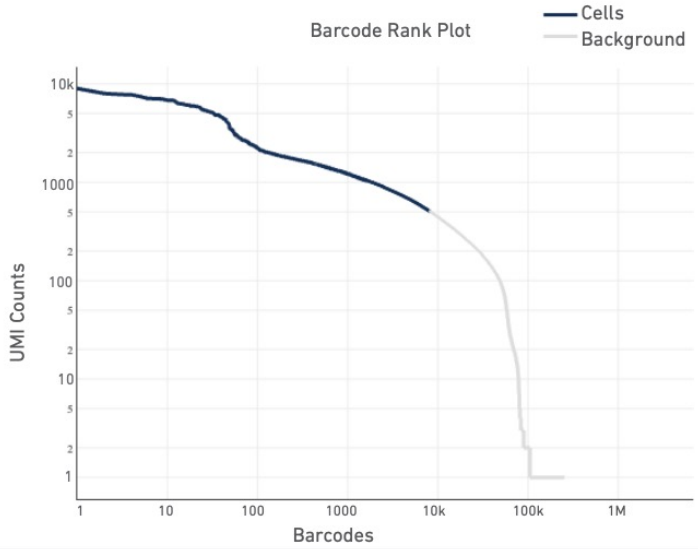
Typical Sample: A steep drop-off is indicative of good separation between the cell-associated barcodes and the barcodes associated with empty GEMs. An ideal Barcode Rank plot has a distinctive shape, which is referred to as a "cliff and knee". The blue-to-gray transition (green arrow) is referred to as the cliff; the solid gray is referred to as the knee (blue arrow).



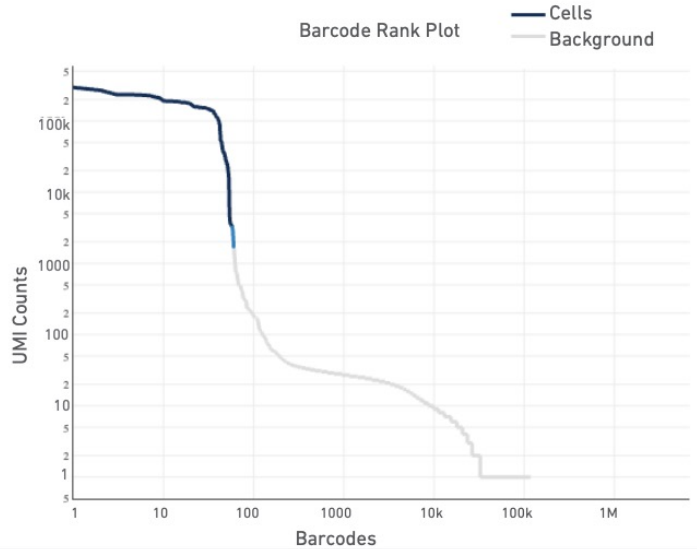
Heterogeneous Sample: Occasionally and based on sample type, there can be heterogeneous populations of cells in a sample that may result in a bimodal plot. In these situations, the cell-associated 10x Barcodes will have two "cliff and knee" distributions. However, there should still be clear separation between the barcodes called as 'cells' and barcodes called as 'background'.



Compromised Sample: Round curve and lack of steep cliff may indicate low sample quality or loss of single-cell behavior. This can be due to a wetting failure, premature cell lysis, or low cell viability.



Compromised Sample: Defined cliff and knee, but the total number of barcodes detected may be lower than expected. This can be caused by a sample clog or inaccurate cell count.



Source: 10X Genomics

Read 10X hdf5 file

Source: `R/preprocessing.R`

Read count matrix from 10X CellRanger hdf5 file. This can be used to read both scATAC-seq and scRNA-seq matrices.

```
Read10X_h5(filename, use.names = TRUE, unique.features = TRUE)
```

Arguments

filename	Path to h5 file
use.names	Label row names with feature names rather than ID numbers.
unique.features	Make feature names unique (default TRUE)

Value

Returns a sparse matrix with rows and columns labeled. If multiple genomes are present, returns a list of sparse matrices (one per genome).

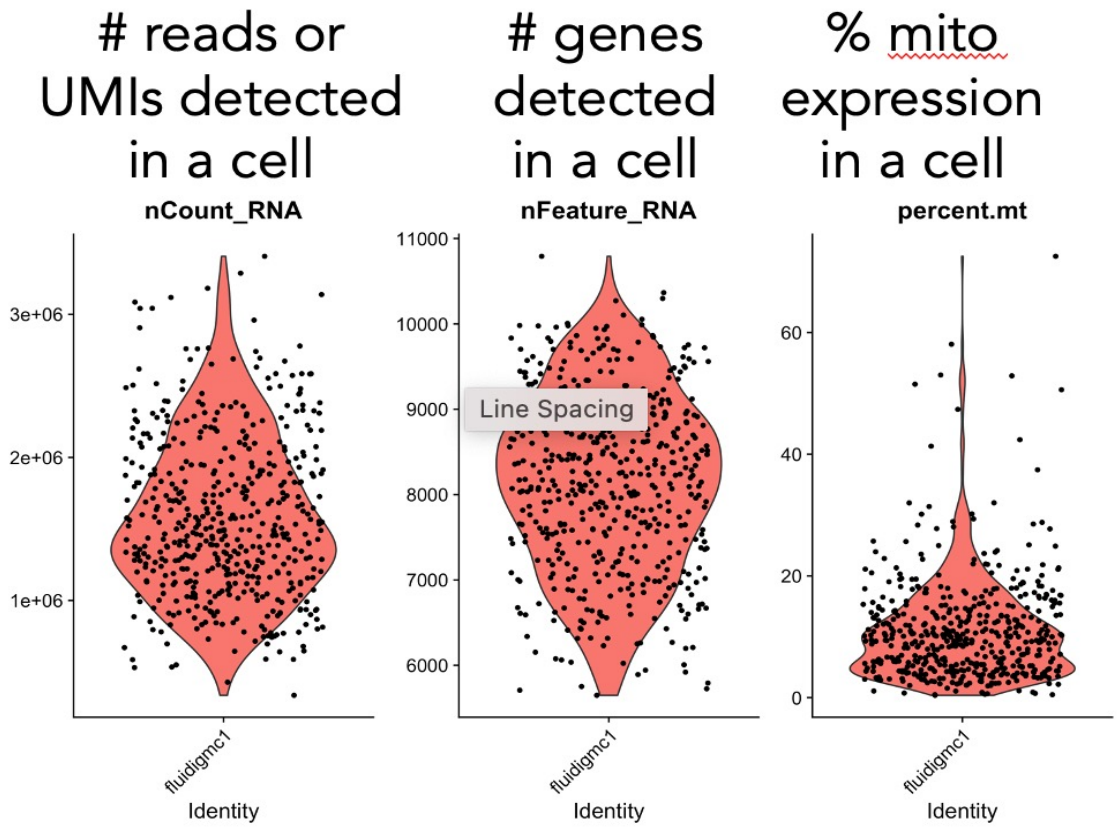
Quality Control

- Goals:
  - Filter the data to only include true cells that are of high quality
    - Remove empty droplets
    - Remove doublets
    - Remove dead or damaged cells
    - Remove ambient RNA
  - Identify any failed samples and either try to salvage the data or remove from analysis

- Challenges:
  - Delineating cells that are **poor quality** from **less complex cells**
  - Choosing **appropriate thresholds** for filtering, to keep high quality cells without removing biologically relevant cell types

Fundamental QC metrics

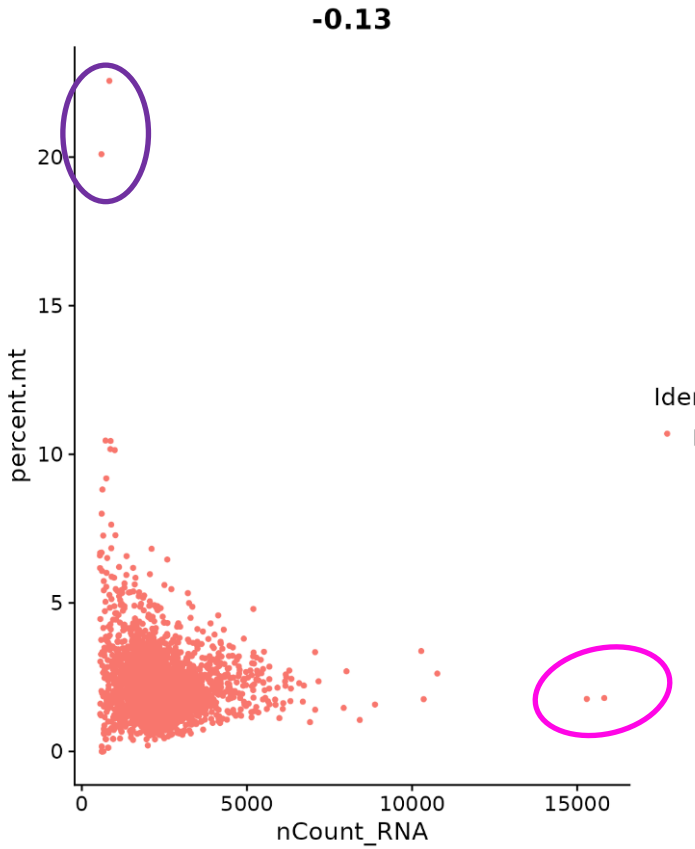
- Cell QC is commonly performed based on three principal QC covariates:
  - the number of counts per barcode (count depth),
  - the number of genes per barcode, and
  - the fraction of counts from mitochondrial genes per barcode

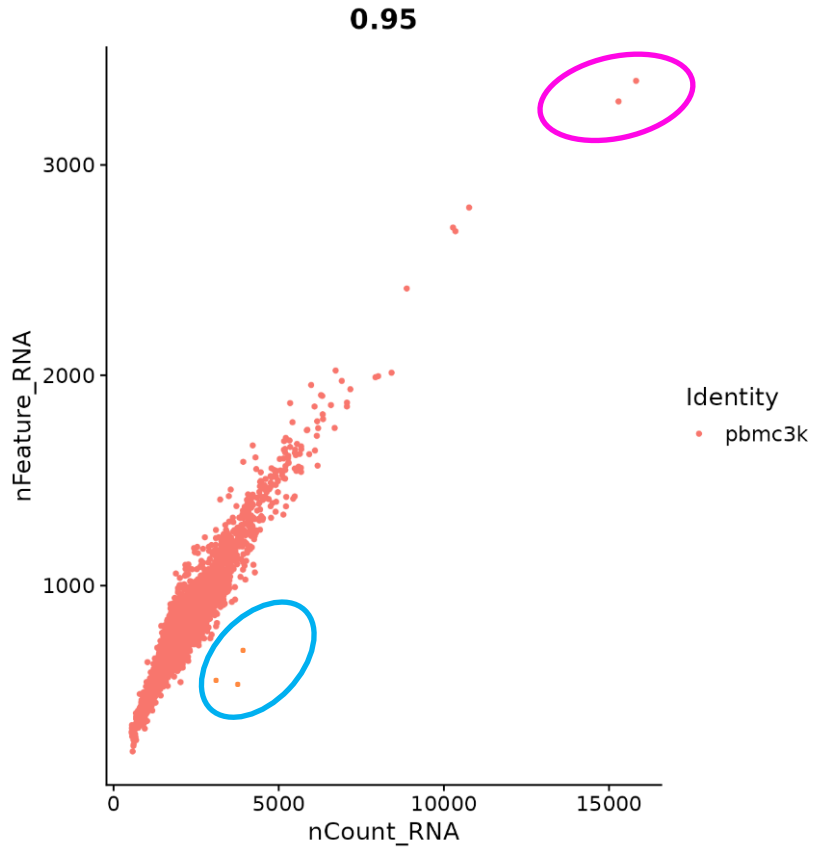


Fundamental QC metrics

It is sometimes helpful to use a combination of these metrics to filter out cells.

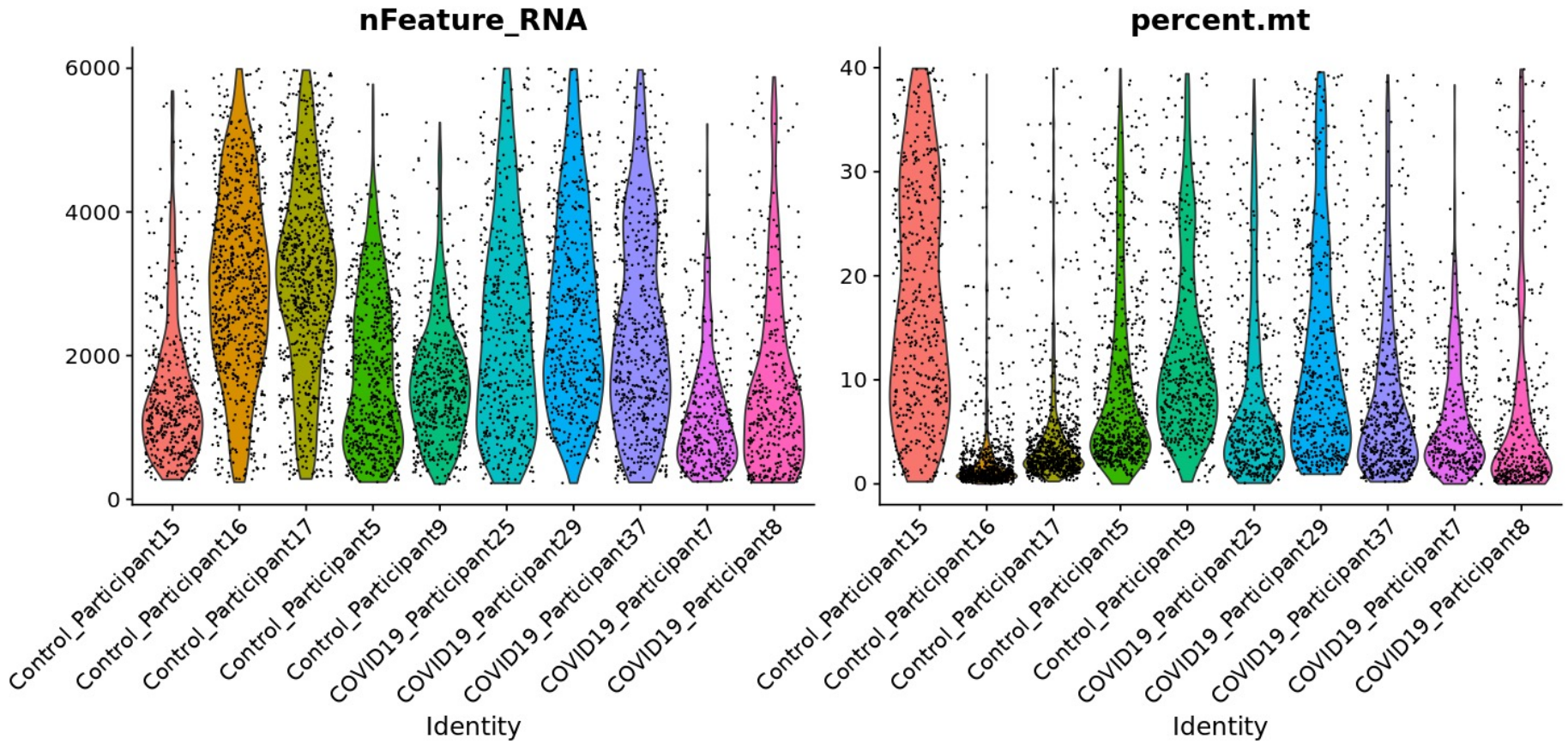
- **Low nUMI and high % mitochondrial -** Cells captured but lost a lot of the mRNA, and the mitochondrial genes were protected and retained.
- **High nUMI & low nGene ratio –** low quality library or capture rate
- **High nUMI & high nGene –** doublets





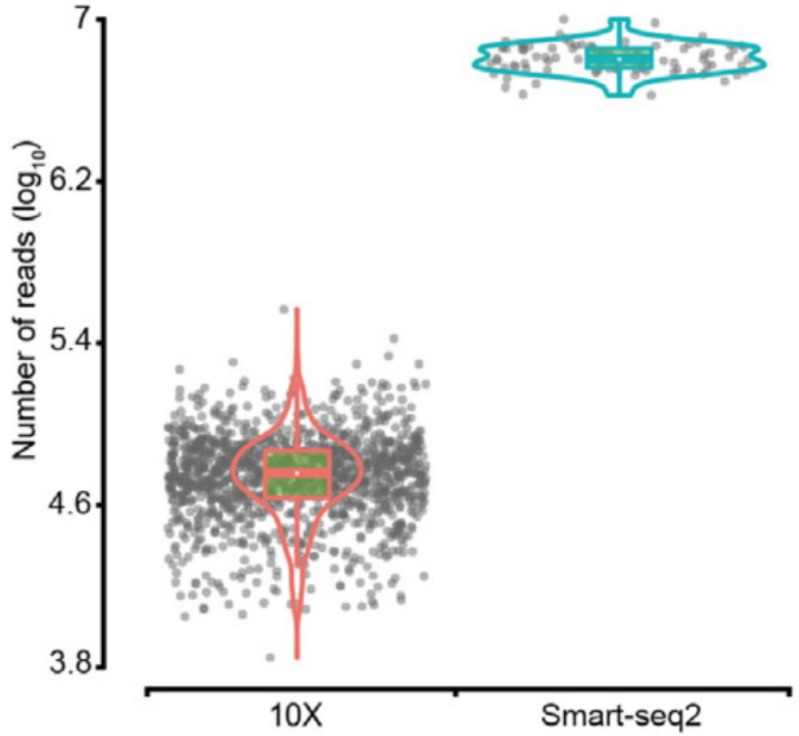
Fundamental QC metrics

It is often helpful to visualize per group QC metrics



Appropriate quality control filters vary with platform and cell types

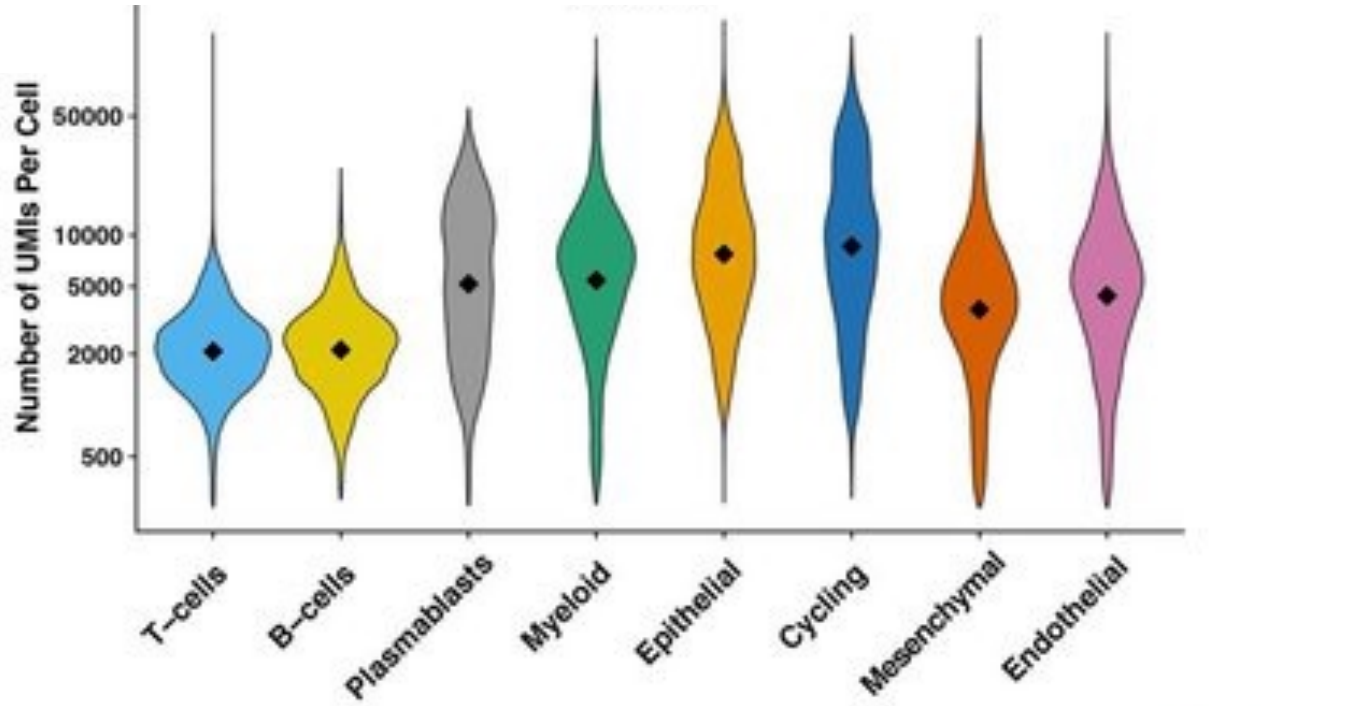
- Different platforms set different expectations
  - Example:. Smart-Seq2 often yields more genes detected per cell than 10x Chromium.



Source: Galow et al, Cellular and Molecular Life Sciences

Appropriate quality control filters vary with platform and cell types

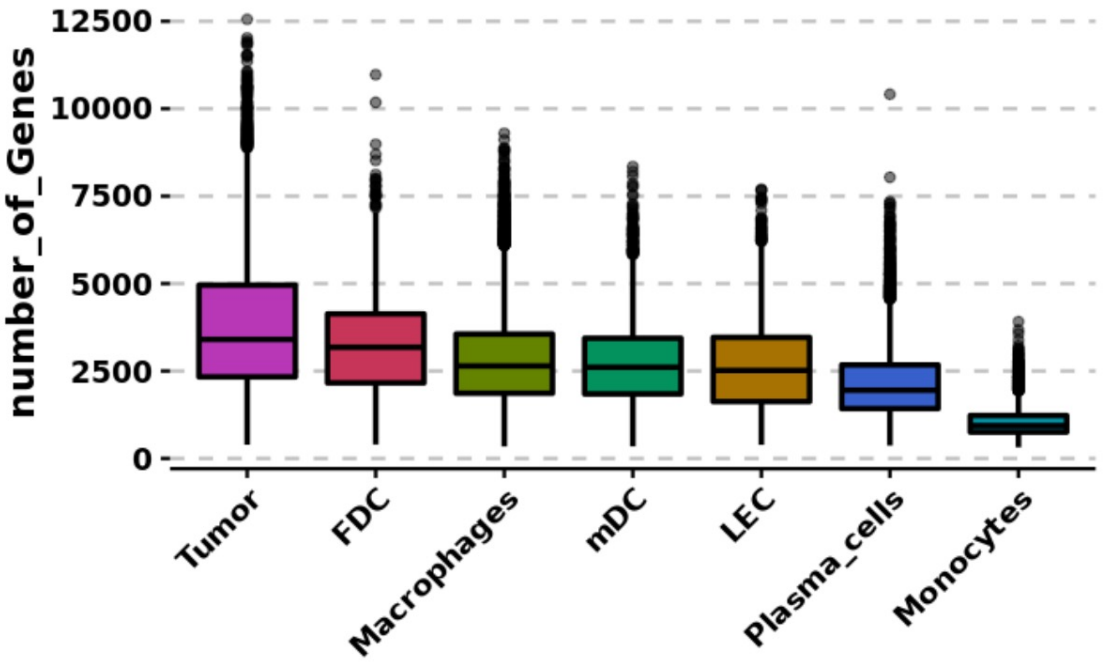
- Different cell types set different expectations
  - Immune cells normally have fewer genes detected per cell than non-immune cells



Source: Wu et al, Nature Genetics

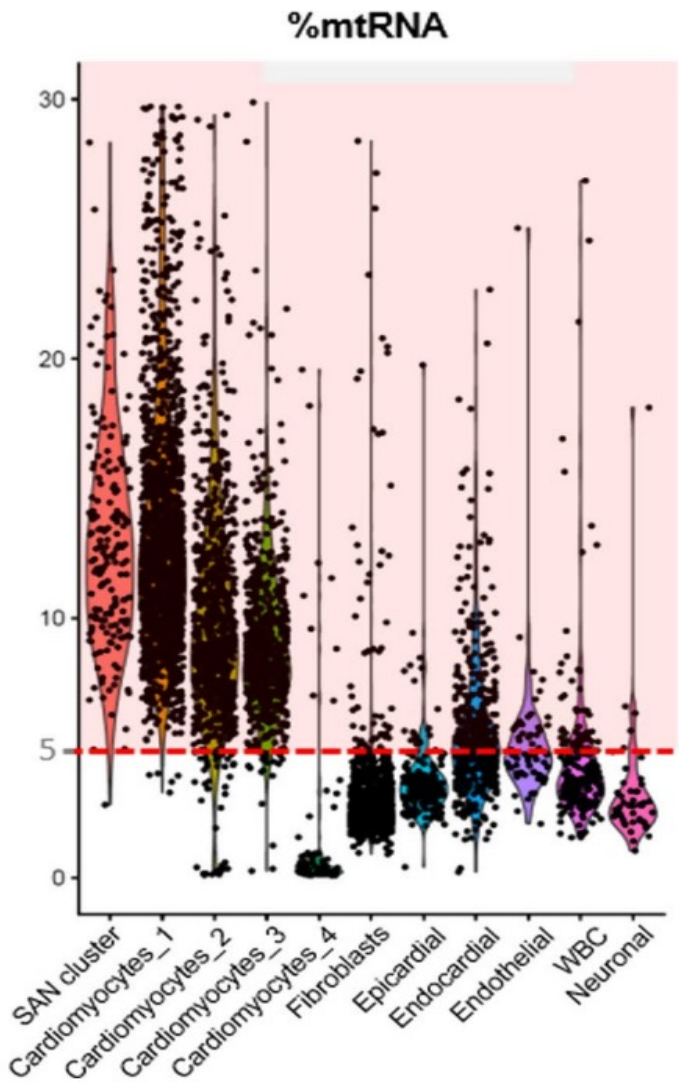
Appropriate quality control filters vary with platform and cell types

- Different cell types set different expectations
  - Malignant cells normally have more genes detected per cell than non-malignant cells



Appropriate quality control filters vary with platform and cell types

- Different cell types set different expectations
  - Cardiac cells normally have higher percent of mito genes per cells then other cells



Source: Galow et al, Cellular and Molecular Life Sciences

Tips

- It may be necessary to revisit quality control decisions multiple times when analyzing data. Often it is beneficial to start with permissive QC thresholds and investigate the effects of these thresholds before going back to perform more stringent QC.

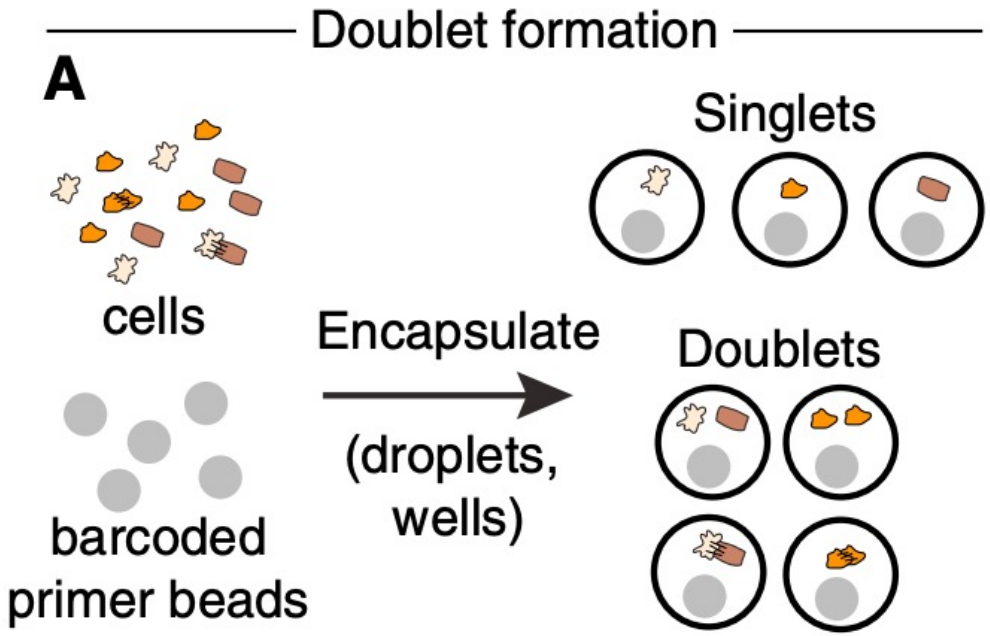
- If the distribution of QC covariates differ between samples, QC thresholds should be determined separately for each sample to account for sample quality differences.

- Always visualize QC metrics per cluster in order to flag any biases in QC filters that have been applied.

Luecken, M. D., & Theis, F. J. (2019). Current best practices in single-cell RNA-seq analysis: a tutorial.

Identifying Doublets in Single-Cell RNA-Seq Data

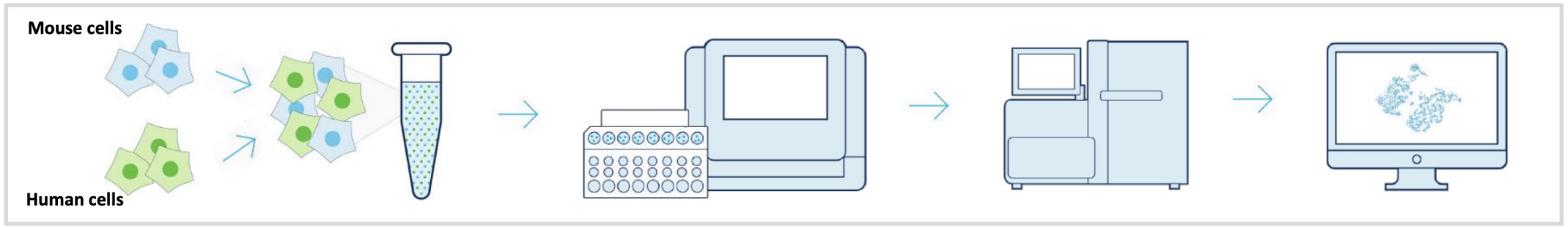
- Doublets = two or more cells captured together. They contain gene counts from the combined cells.

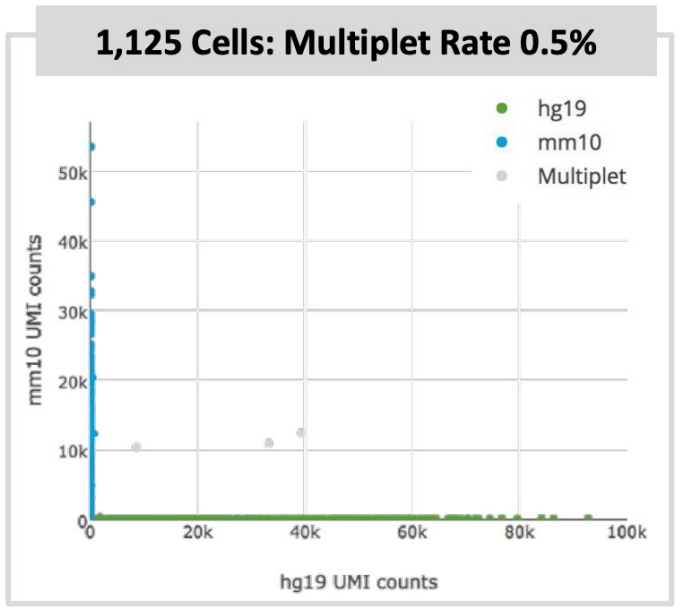


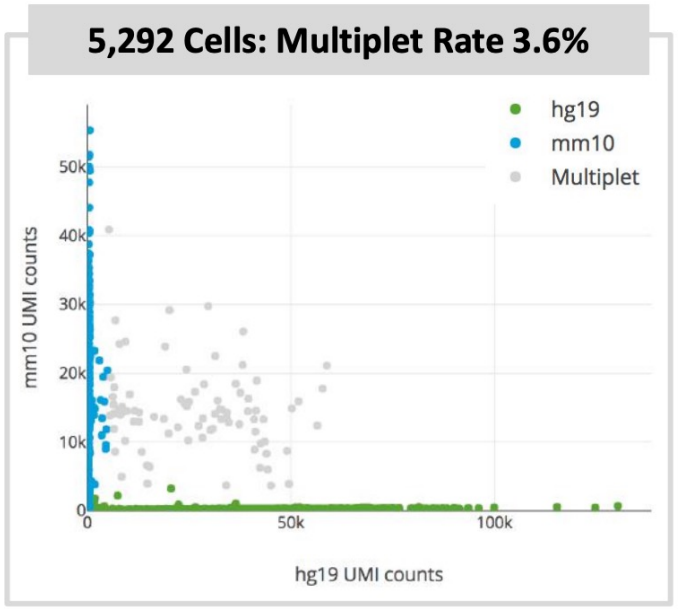
Wolock et al., Cell Syst., 2019

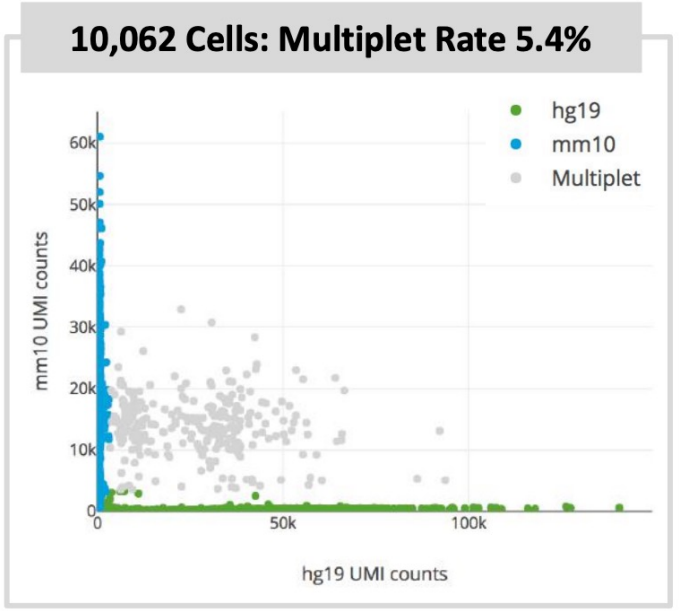
Identifying Doublets in Single-Cell RNA-Seq Data

- Doublet rate often depends on number of cells loaded.





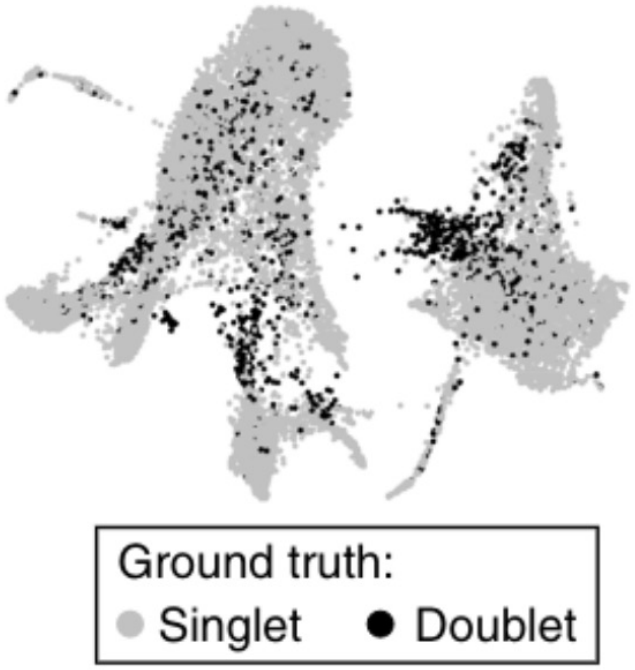


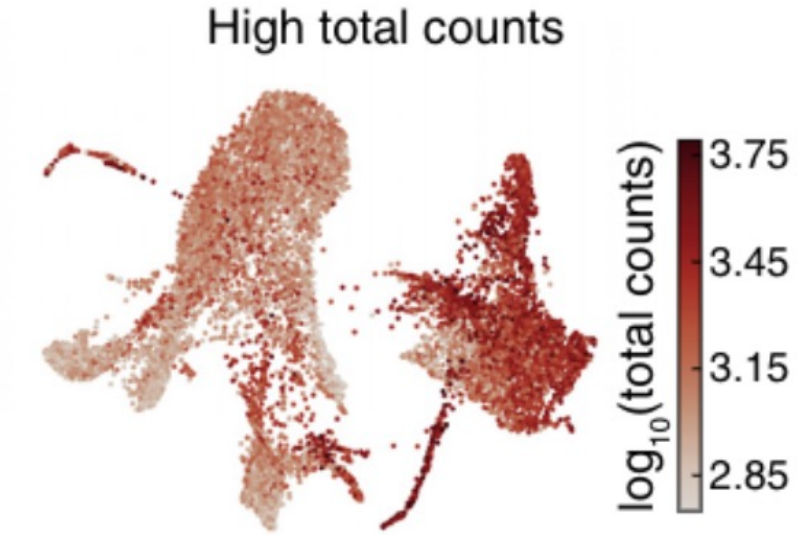


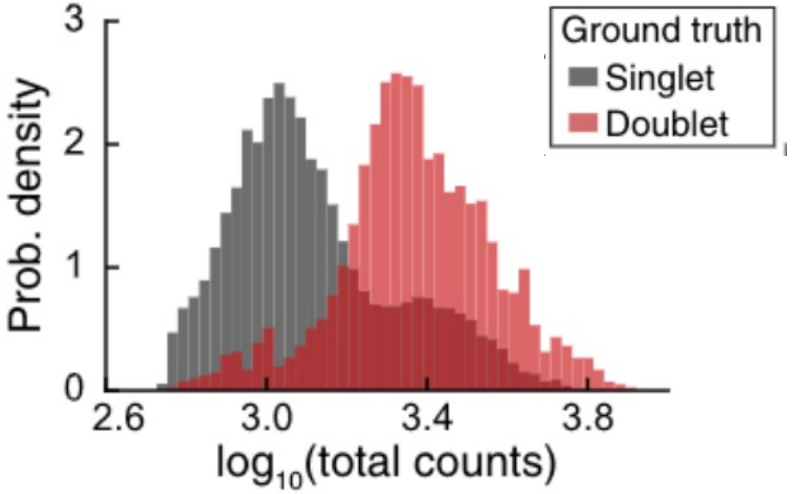
Source: 10X Genomics

Identifying Doublets in Single-Cell RNA-Seq Data

- Some doublets will be filtered by the basic QC steps since doublets will have a high number of genes and number of UMIs



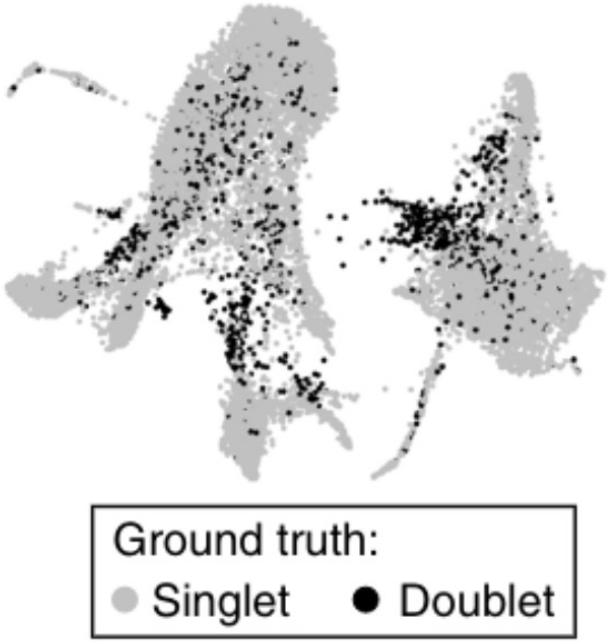


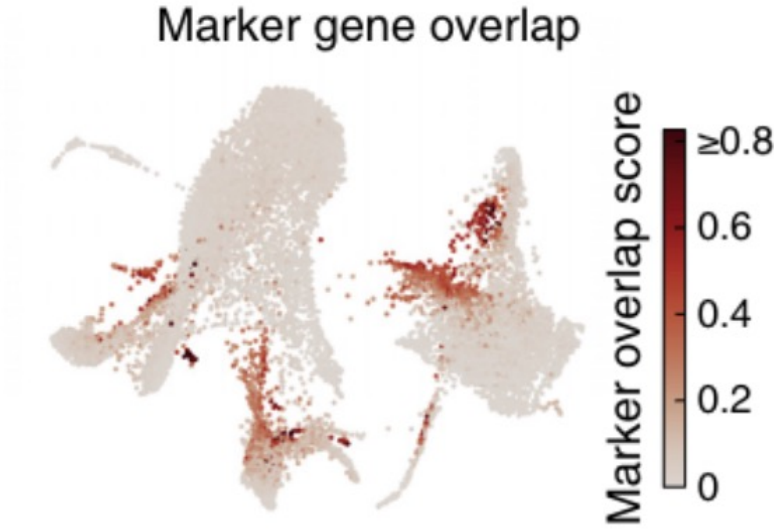


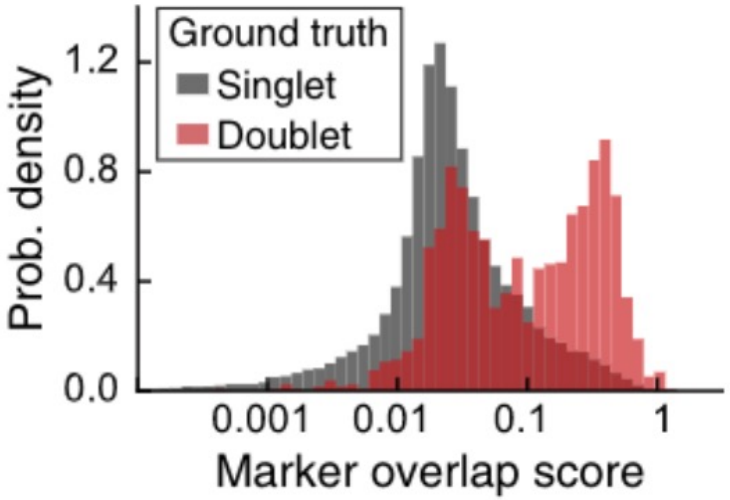
Wolock et al., Cell Syst., 2019

Identifying Doublets in Single-Cell RNA-Seq Data

- A simple heuristic to check for doublet clusters is to see if they express gene markers of two or more disparate cell types.



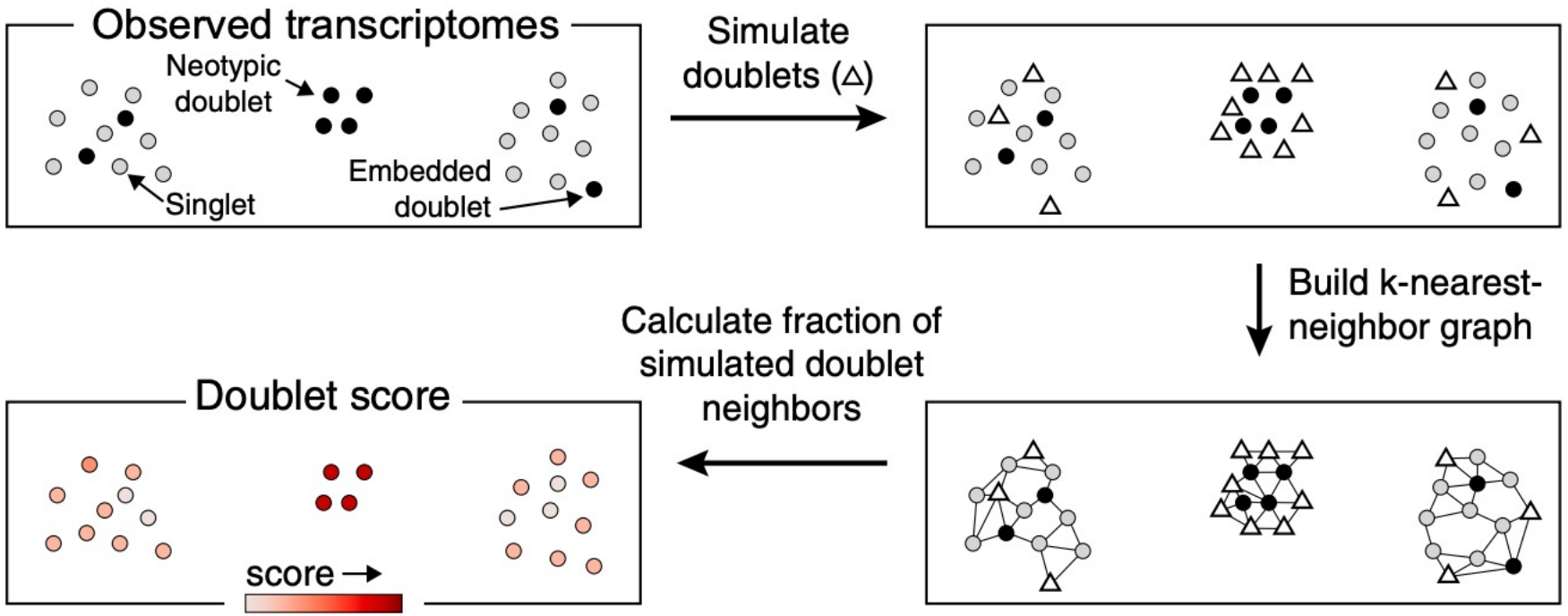




Wolock et al., Cell Syst., 2019

Identifying Doublets in Single-Cell RNA-Seq Data

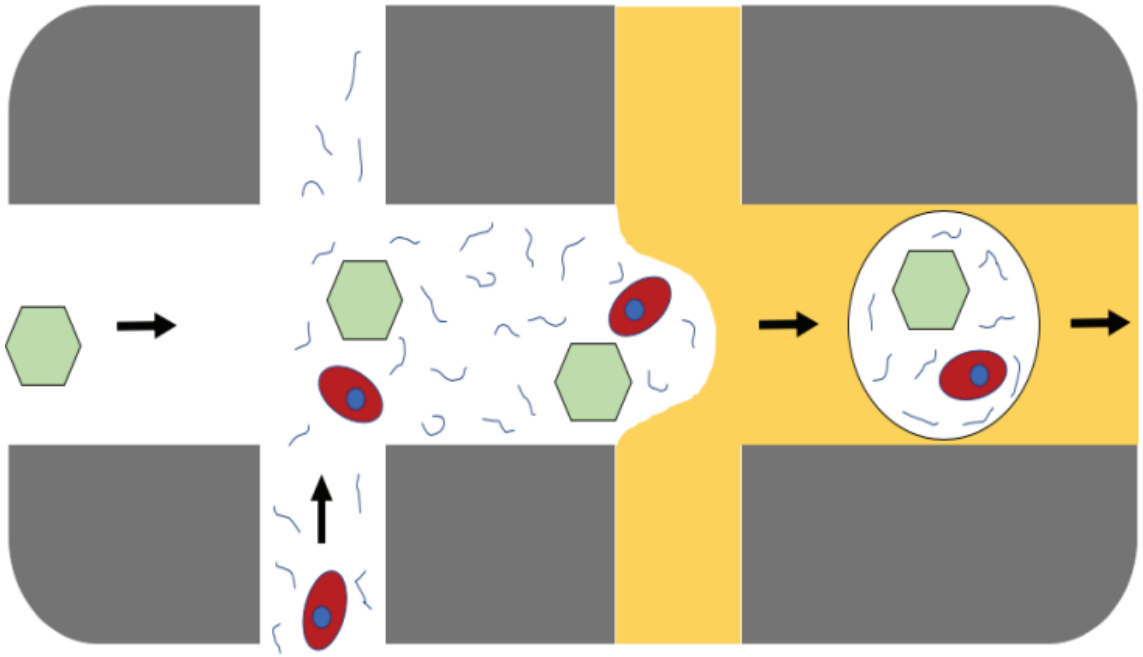
- Advanced methods such as Scrublet and DoubletFinder use simulations to determine doublet scores.



Wollock et al., Cell Syst., 2019

Detecting empty drops and correcting for ambient RNA

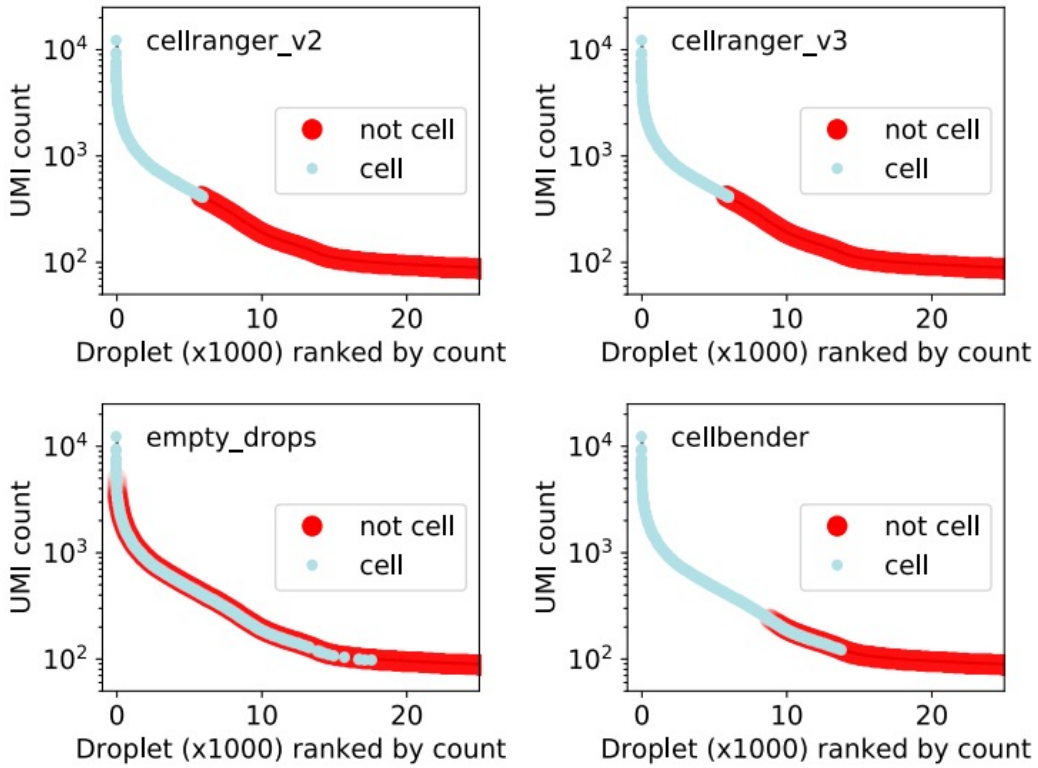
- Ambient gene expression refers to counts that do not originate from a barcoded cell, but from other lysed cells whose mRNA contaminated the cell suspension prior to library construction.
- Sequencing errors in barcodes and Barcode swapping also contribute to ‘ambient’ RNA counts.
- The presence of background RNA can lead to systematic biases and batch effects in various downstream analyses such as differential expression and marker gene discovery.



CellBender: Tool to detect empty drops and correct ambient RNA

CellBender removes ambient background and barcode swapping via deep learning

- successfully learns and subtracts background noise and artifactual counts from non-empty droplets and leads to significantly increased amplitude and specificity of differential gene expression



Fleming et al. *bioRxiv* (2019)

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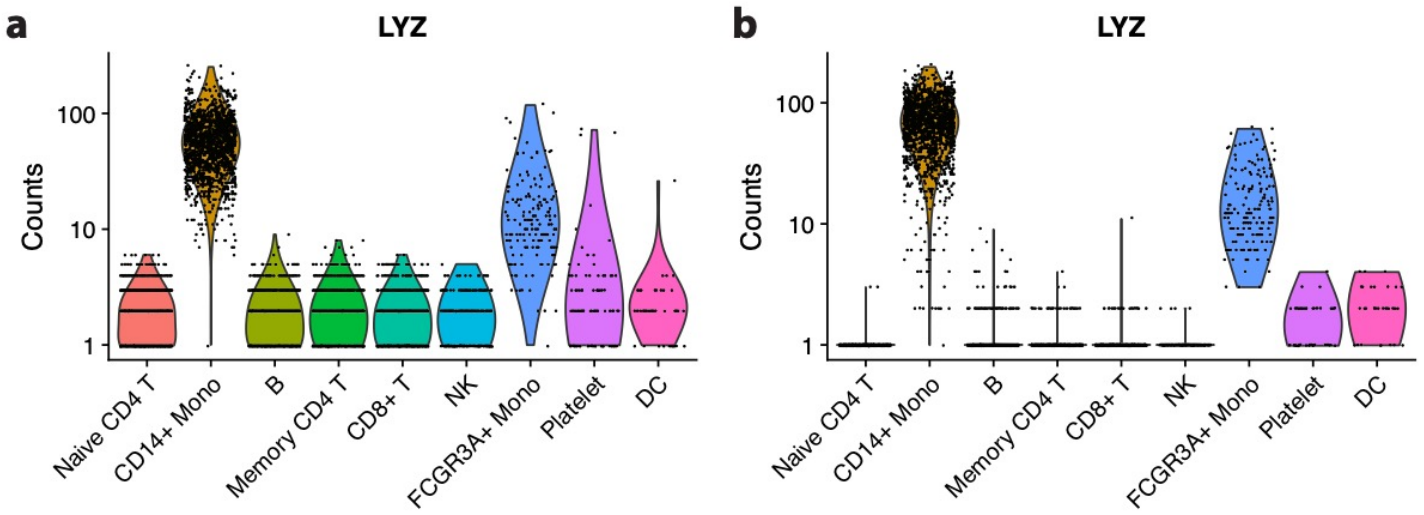
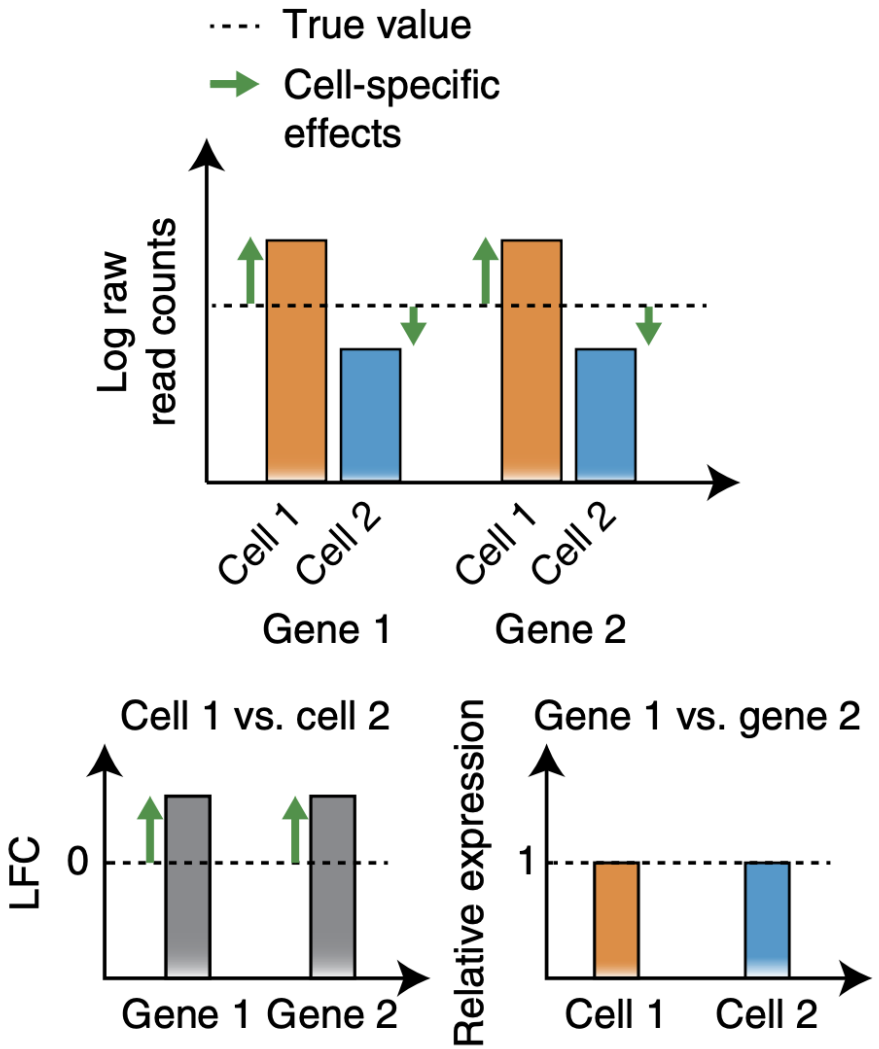


Fleming et al. *bioRxiv* (2019)

Normalization of gene expression data

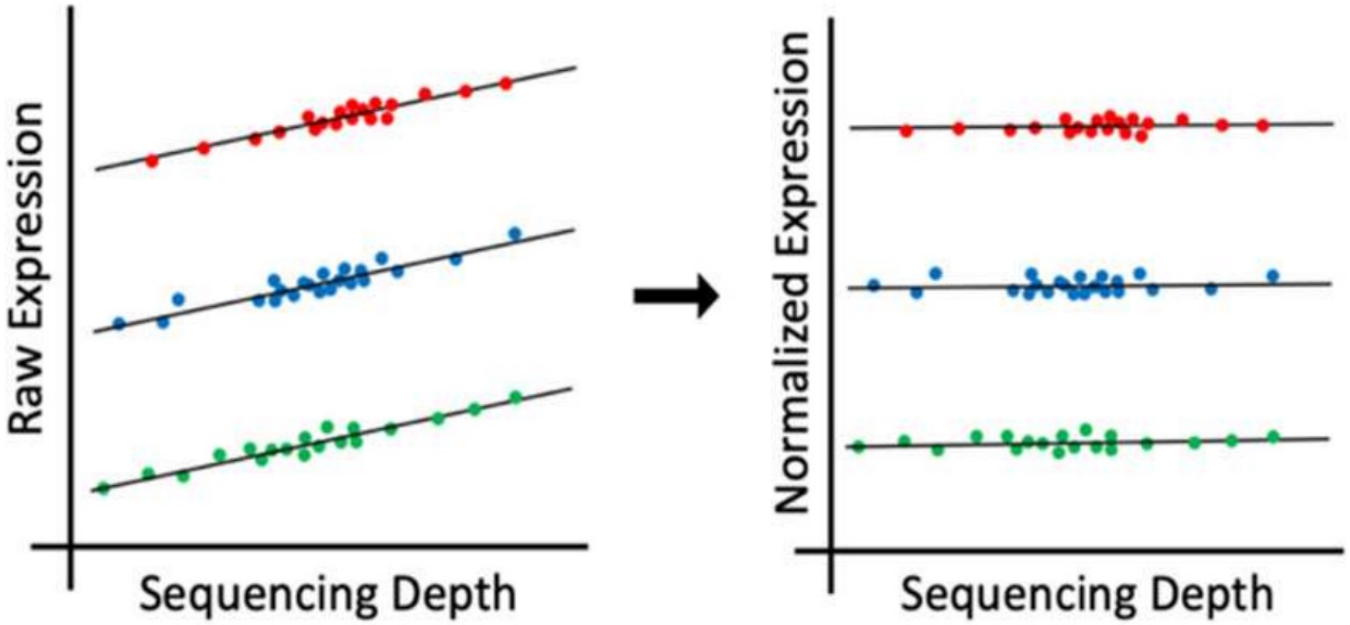
- Normalization is the process of adjusting raw data to account for unwanted factors that prevent direct comparison of expression measures.
- The goal of normalization is for differences in normalized read counts to represent differences in true expression.
- In practice, single cell RNA-Seq normalization involves two steps:
  - Scaling
  - Transformation



Why normalize gene expression within a cell?

1. Many technical factors such as sequencing depth can introduce bias into the raw read counts obfuscating true signal.

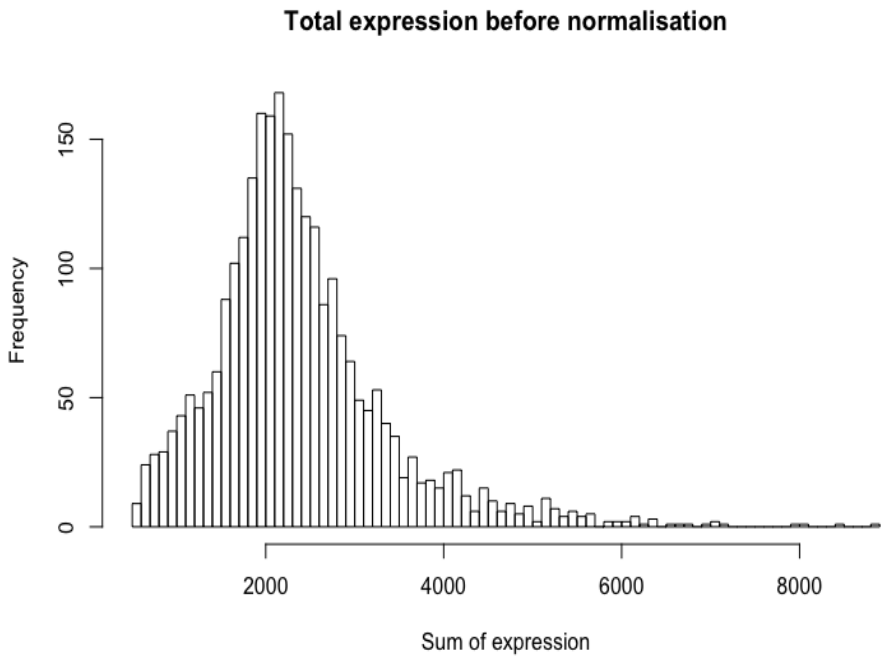
- sequencing depth = total number of molecules sequenced
- When one cell has a higher sequencing depth than another, even non-differentially expressed genes will tend to have higher read counts in that cell

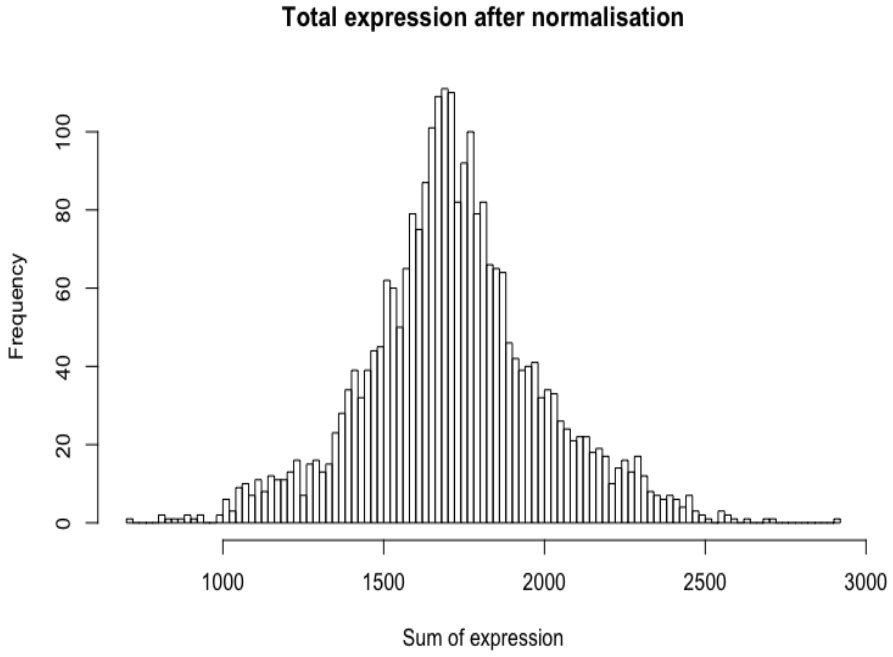


Why normalize gene expression within a cell?

2. There are typically extreme values in distribution of gene expression

- Normalization, especially the transformation step, reduces the skew-ness of the data to approximate the assumption of many downstream analysis tools that the data are normally distributed.





Why normalize gene expression within a cell?

2. There are typically extreme values in distribution of gene expression

- Normalization, especially the transformation step, reduces the skew-ness of the data to approximate the assumption of many downstream analysis tools that the data are normally distributed.
- Also prevents downstream analysis from being completely dominated by differences among the most highly expressed genes. Log transformation for instance

	Raw data			Log_2 transform		
	Cell Type A	Cell Type B	Δ	Cell Type A	Cell Type B	Δ
Gene 1	1	2	1	0	1	1
Gene 2	100	200	100	6.64	7.64	1

Normalization of gene expression data

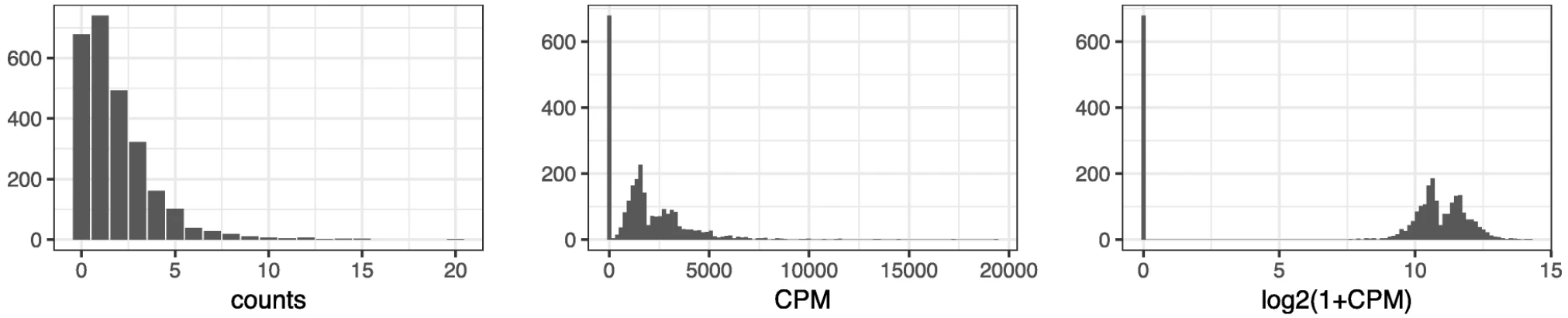
- The most commonly used normalization protocol is count depth scaling, also referred to as “counts per million” or CPM normalization.

- To perform CPM:

  - Gene expression measurements for each cell are normalized by the total gene expression or median gene expression
  - Gene expression values then scaled to sum to 10,000 (typically),
  - Finally, these values are log-transformed: **log(CPM+1).**

Is standard normalization appropriate?

- Normalization methods perform poorly when their assumptions are violated
- Current approaches to normalization and transformation artificially distort differences between zero and nonzero counts.
- Advanced methods like **SCTransform** can address some of the limitations “Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression.” Hafemeister et al. Genome Biology (2019)



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

Questions?