Single Cell RNA-seq: Characteristics, Preprocessing and QC

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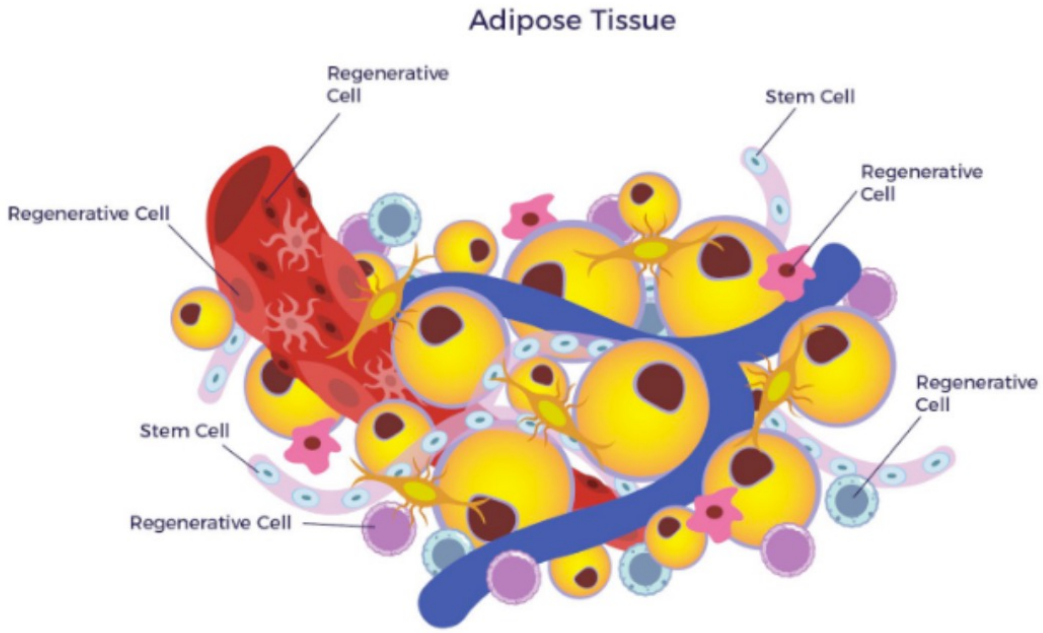
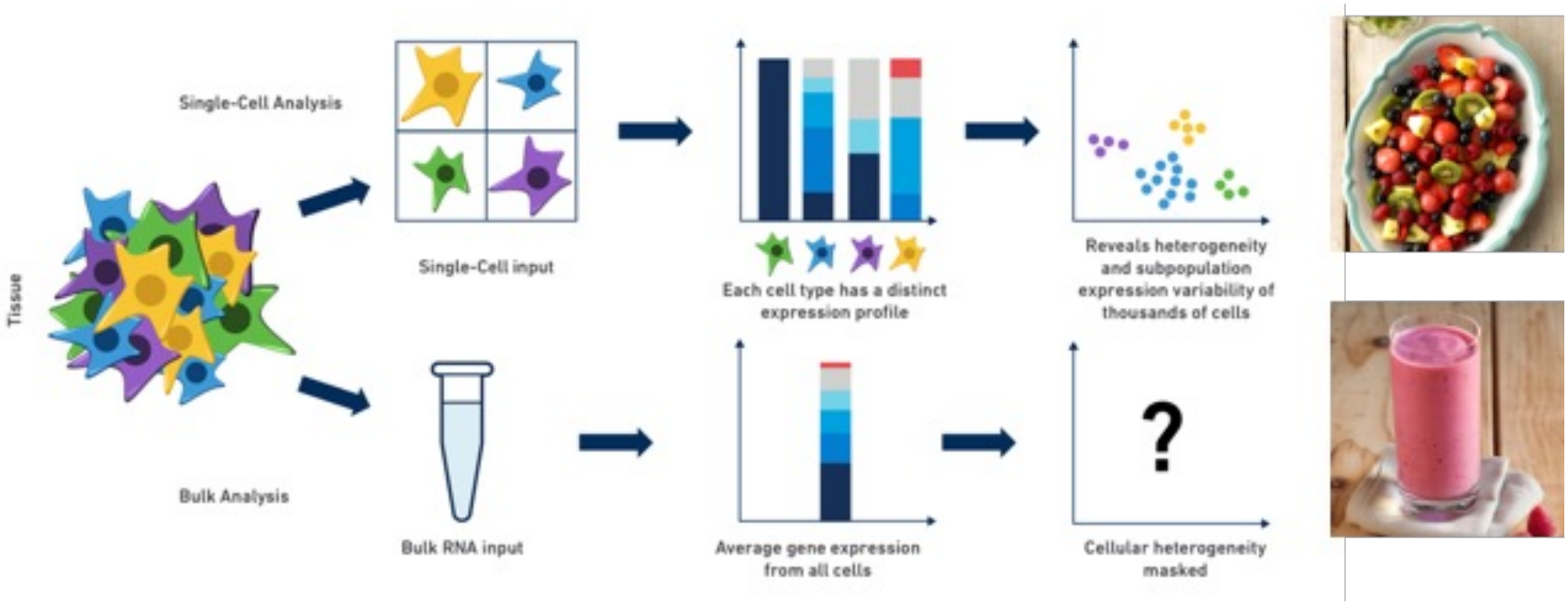
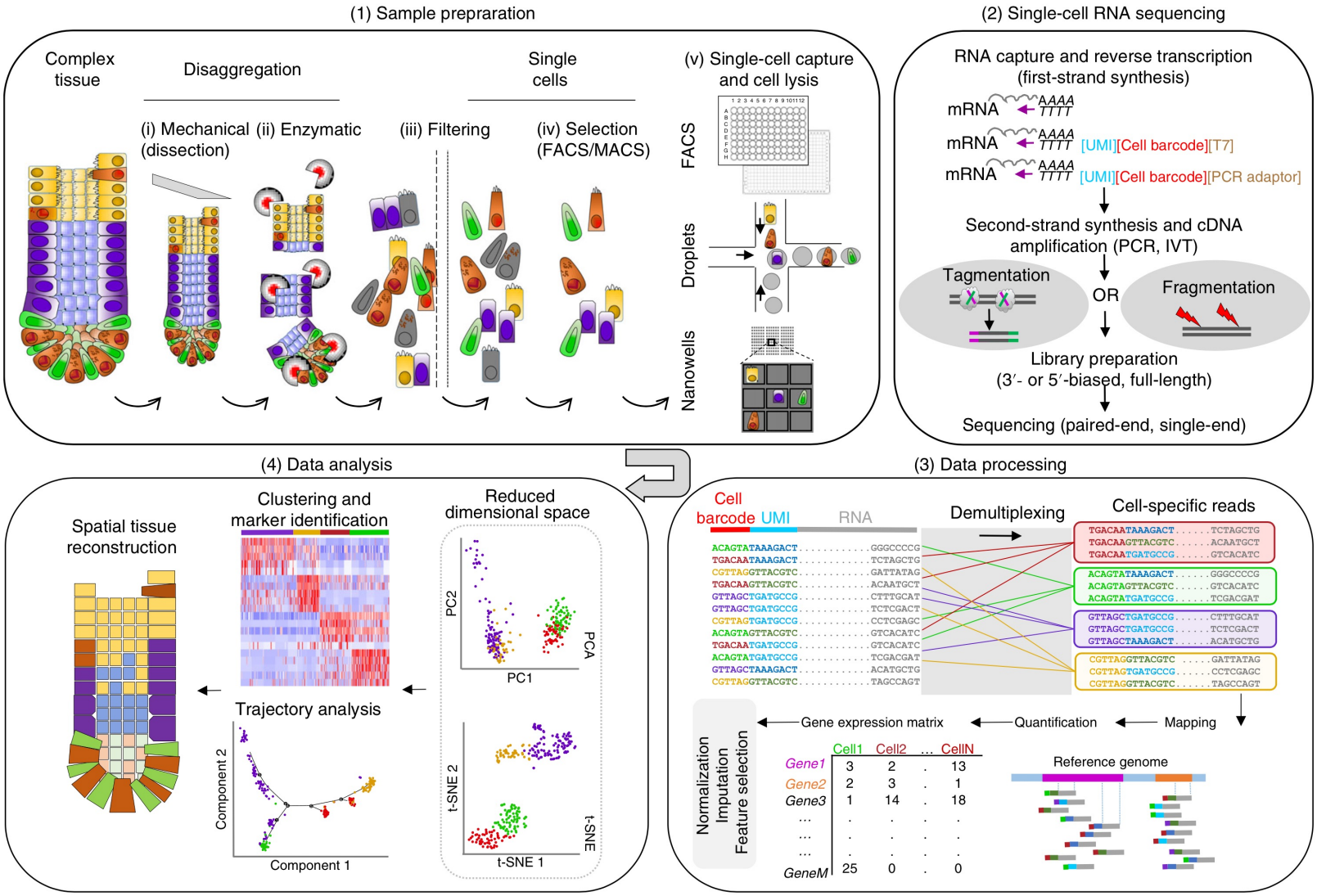
Tissues are heterogeneous



levelanatomyandphysiology.com


Single-Cell Analysis
Tissue
Single-Cell input
Each cell type has a distinct expression profile
Reveals heterogeneity and subpopulation expression variability of thousands of cells
Bulk Analysis
Bulk RNA input
Average gene expression from all cells
?
Cellular heterogeneity masked

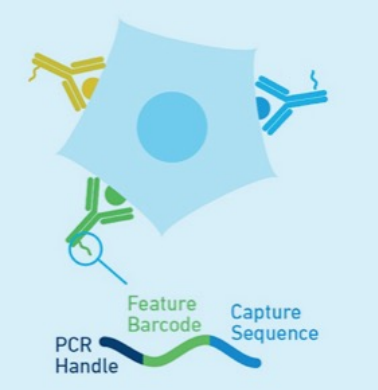
scRNA-seq

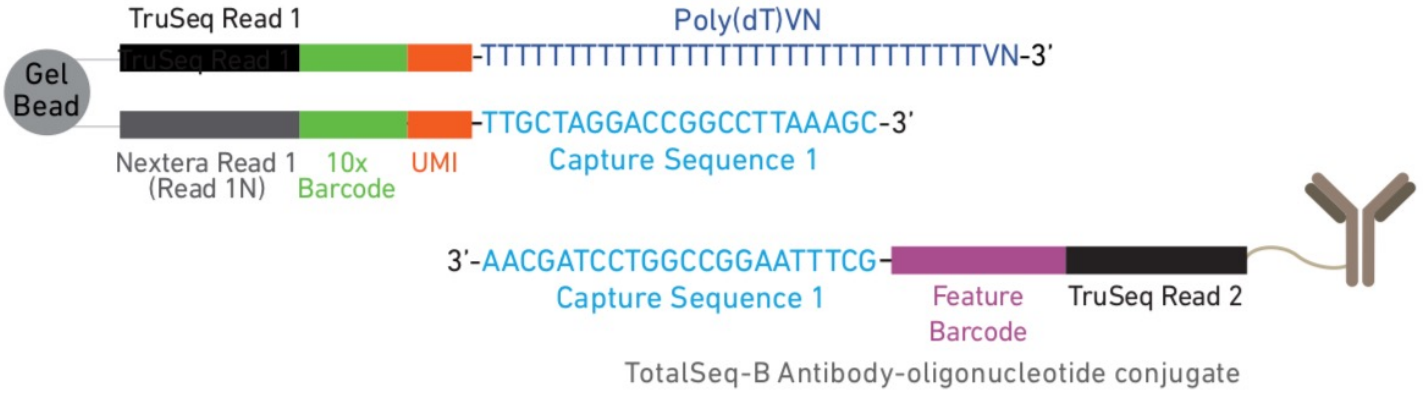


Lafzi et al. https://www.nature.com/articles/s41596-018-0073-y

Cell hashing (feature barcoding)

CITE-seq





- Can be used
  - to combine cells from different pools in one experiment
  - detect presence of surface markers of cells
  - detect doublets

Molecule numbers in cells

Table 1. RNA content of a typical human cell

Total RNA per cell	~10–30 pg
Proportion of total RNA in nucleus	~14%
DNA:RNA in nucleus	~2:1
mRNA molecules	$2 \times 10^5 - 1 \times 10^6$

Table 2. RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80–85%
tRNAs, snRNAs, low MW species	15–20%
mRNAs	1–5%

Table 3. mRNA classification based on abundance

Abundance class	Copies/cell	Number of different messages/cell	Abundance of each message
Low	5–15	11,000	<0.004%
Intermediate	200–400	500	<0.1%
High	12,000	<10	3%

https://www.gene-quantification.de/

From the cell to the RNA library

- ~ 30% of the cells are lost in the fluidics …
- Major loss point: Reverse transcription (RT)
  - Estimate: 5 – 20% of the mRNA make it to the cDNA
- Full length cDNA amplification:
  - 11 – 13 cycles
- Fragmentation & PCR amplification:
  - Depending on the amount of generated cDNA: 6 – 15 cycles

→Many duplicates; UMIs are essential to improve accuracy

→Many dropouts for low abundance mRNA

Data characteristics

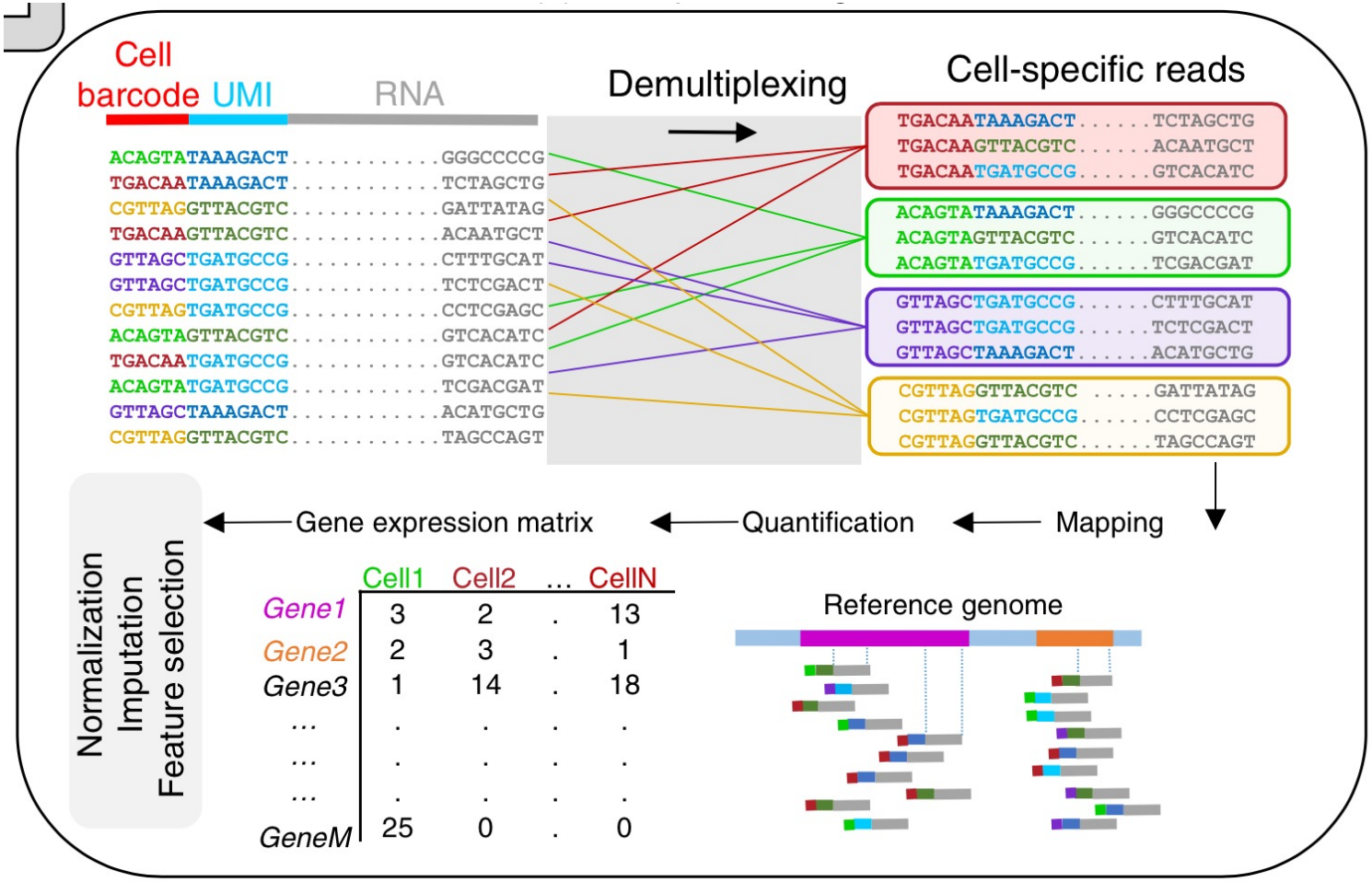
- bulk RNA-seq:
  - 10k to 10Mio cells → billions of mRNA molecules → 100s of billions of fragments after fragmentation and amplification → sequencing samples ~20 Mio reads out of a much larger pool of fragments
  - 10-20k different genes detected in a sample
- single cell RNA-seq
  - 1 cell → 200k mRNA molecules
  - 70-90% of mRNA molecules are typically lost during library prep; precise numbers are unknown
  - sample prep **overamplifies deliberately**, i.e. sequencing is expected to capture multiple reads that originate from the same mRNA fragments; without strong amplification the detection sensitivity of current technologies is too low
  - protocols include Unique Molecular Identifiers (UMIs) that allow to identify and deduplicate reads
  - 200 – 8000 different genes detected in a cell
  - transcription happens in **transcriptional bursts** → considerable cell-to-cell variability

UMIs and 3'-tagging

- The use of UMIs implies that only one end of the transcript can be captured! Expression quantification is only at the **gene level** not at the isoform level
- bulk RNA-seq:
  - typically, coverage of entire transcript body
- single cell RNA-seq
  - transcript end tagging and UMI deduplication

  or:
  - whole transcript coverage but increased variability caused by clonal reads

Generating the single cell count matrix

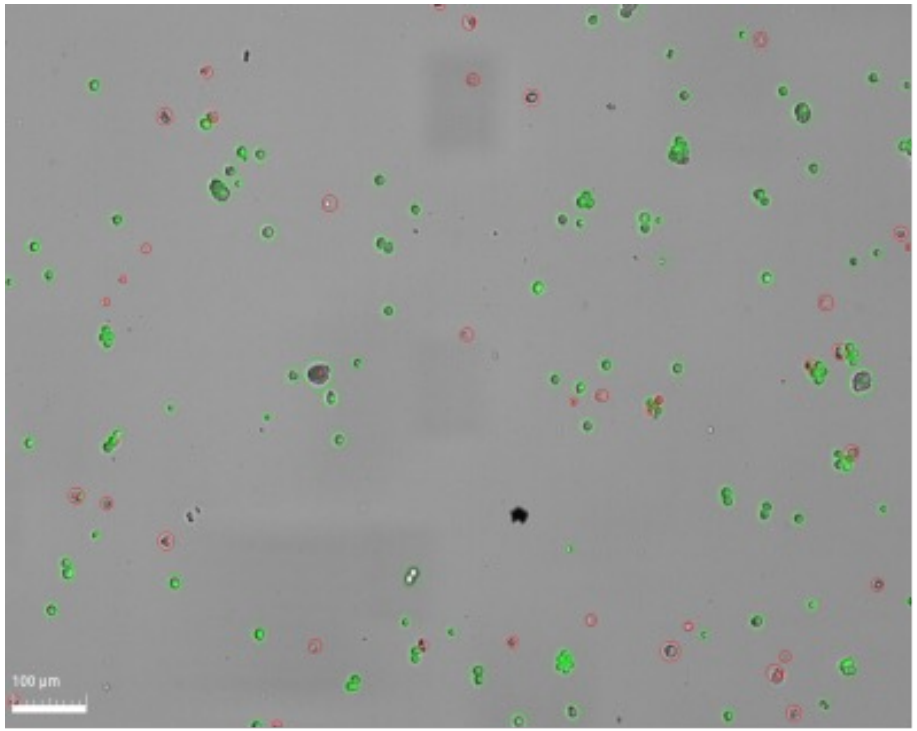


- Expression matrix cells vs genes
- Measurement does not capture the identity of the cells
- **Identity of cells needs to be derived from gene expression**
- Up to 95% of the expression matrix may be zero

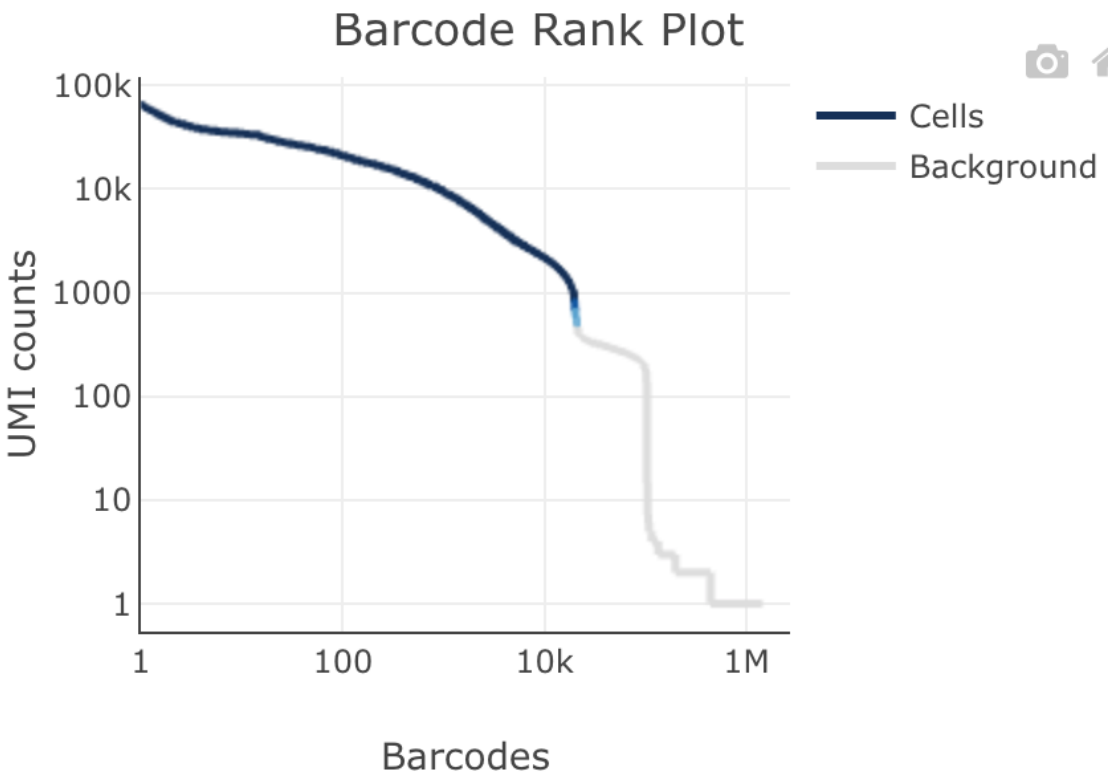
Cell viability

- Microscopy of cell suspension
  - Green: live
  - Red: dead
  - Unstained: debris
- Cell viability is the key parameter that drives the quality (ideally above 90%)

Low quality example with ~80% viability



EmptyDrops algorithm



- In a typical single-cell 10X experiment >1Mio barcodes are detected
- These are error-corrected barcodes; error-correction is done by matching barcode reads against *known-good* barcodes from 10X
- Vast majority do not represent cells but are empty drops capturing few ambient mRNA molecules

Empty drops

- The expression matrix contains also columns/barcodes that correspond to empty drops
  - Reads in empty drops correspond to free-floating RNA in the suspension of single cells
  - Empty drops are expected to have few reads and the reads in empty drops all come from the **same distribution of ambient RNA**
- Implemented in Bioconductur package *DropletUtils* and 10X Genomics CellRanger software

Method | Open Access | Published: 22 March 2019

EmptyDrops: distinguishing cells from empty droplets in droplet-based single-cell RNA sequencing data

Aaron T. L. Lun, Samantha Riesenfeld, Tallulah Andrews, The Phuong Dao, Tomas Gomes, participants in the 1st Human Cell Atlas Jamboree & John C. Marioni

Genome Biology **20**, Article number: 63 (2019) | Cite this article

EmptyDrops algorithm

Steps:

1. Select y% of the barcodes with lowest total UMI counts (those that are for sure background)
   → assume they represent empty cells and build ambient RNA model
   → call barcodes that deviate from ambient model as valid cells
2. Additionally, call all cells above the *knee* point as valid cells

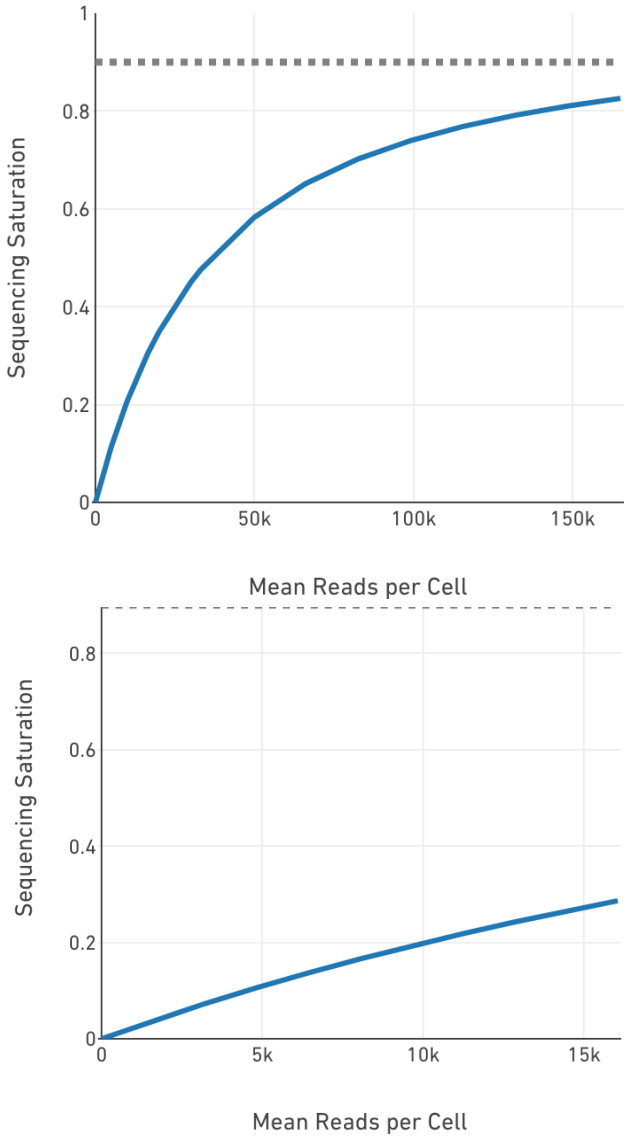
In essence barcodes are called *cells* if they

- have either sufficient reads (above knee-point)
- if they are significantly different from background distribution

Cellranger implements a similar approach, Cellranger is often too optimistic and includes too many barcodes as cells

Sequencing Depth

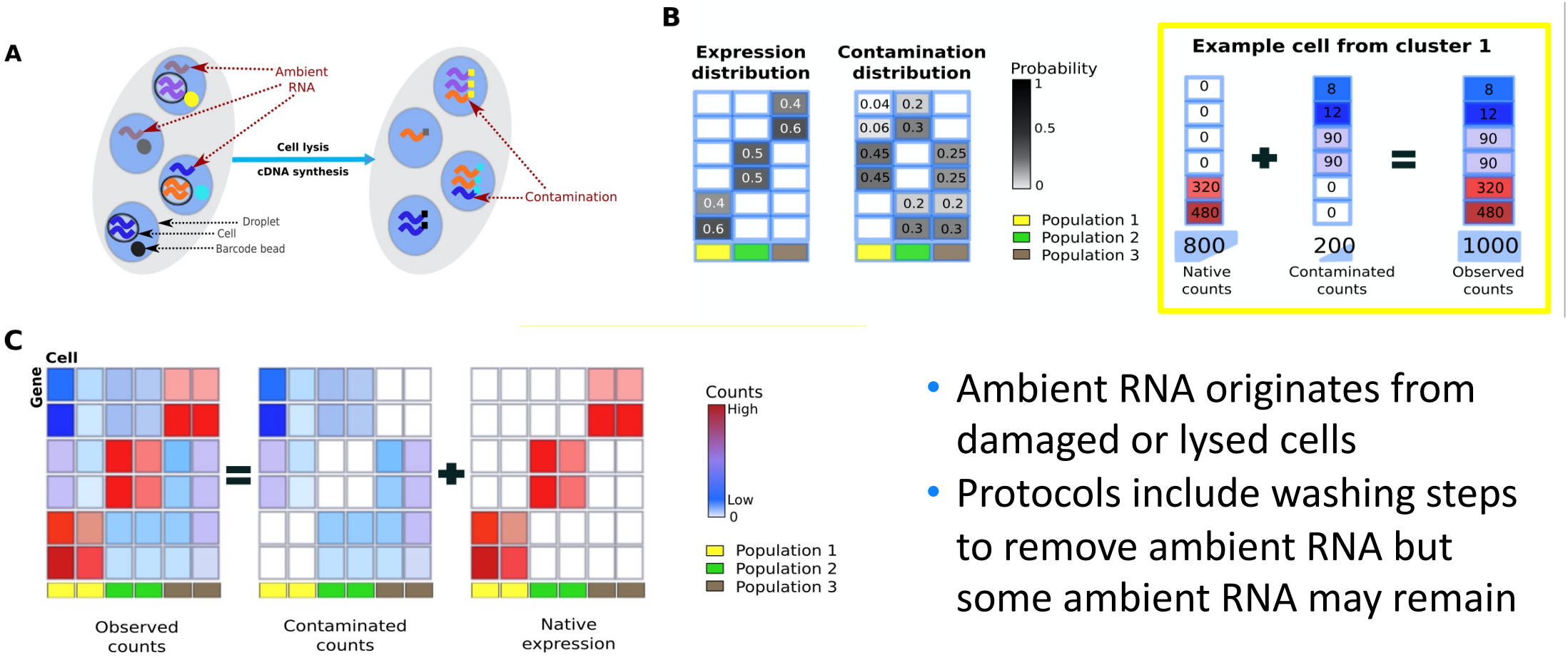
- Based on resampling one can estimate how many genes would be additionally detected if more reads were sequenced
- Typically, 50’000 reads per cell are targeted, since the yield in terms of detected cells may vary the actual number of reads per cell can be very different



Ambient RNA

- EmptyDrops removes barcodes that do contain only ambient RNA
- Ambient RNA still may affect expression of true cells by an additive contribution
- This may be relevant for cells that have low transcriptional activity
- Ambient RNA contributions can be estimated (and removed)
  - SoupX
  - DecontX
  - CellBender

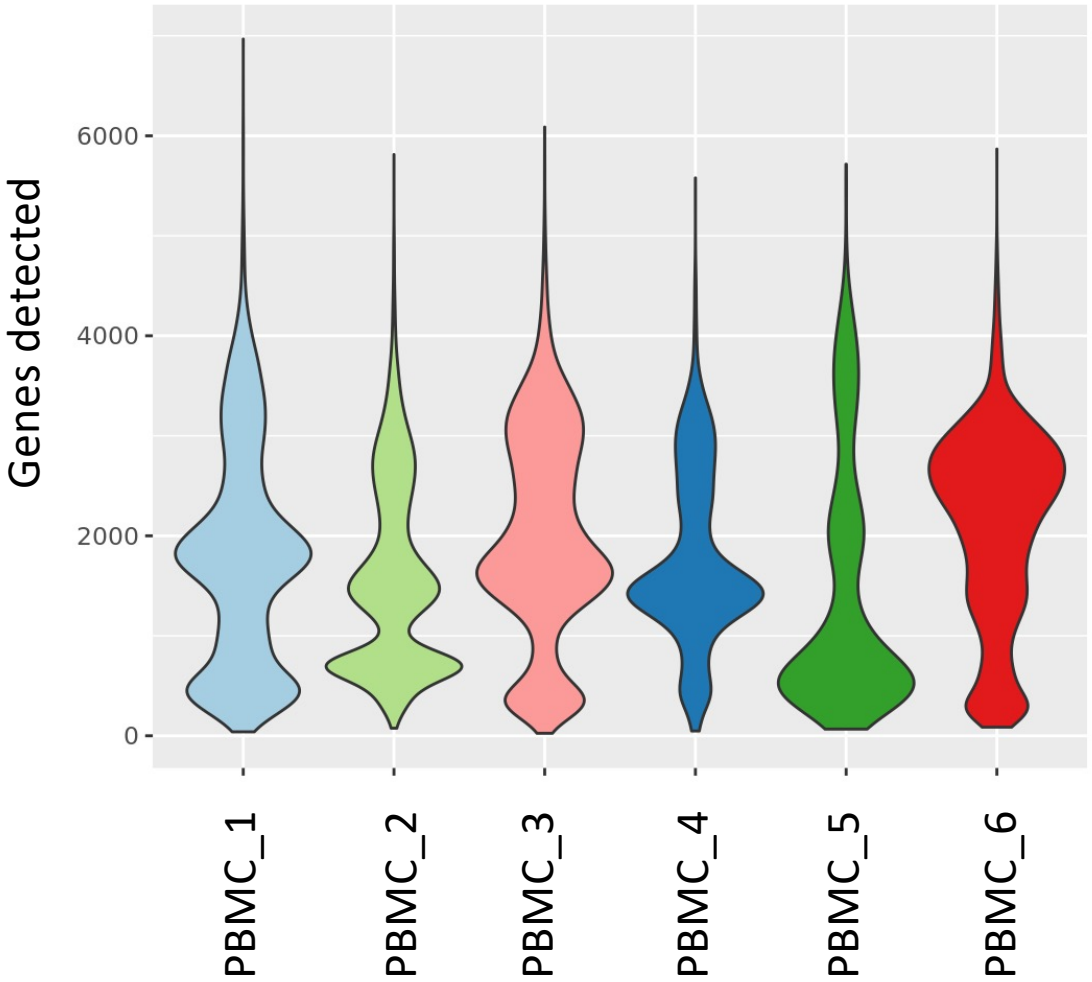
Ambient RNA



- Ambient RNA originates from damaged or lysed cells
- Protocols include washing steps to remove ambient RNA but some ambient RNA may remain

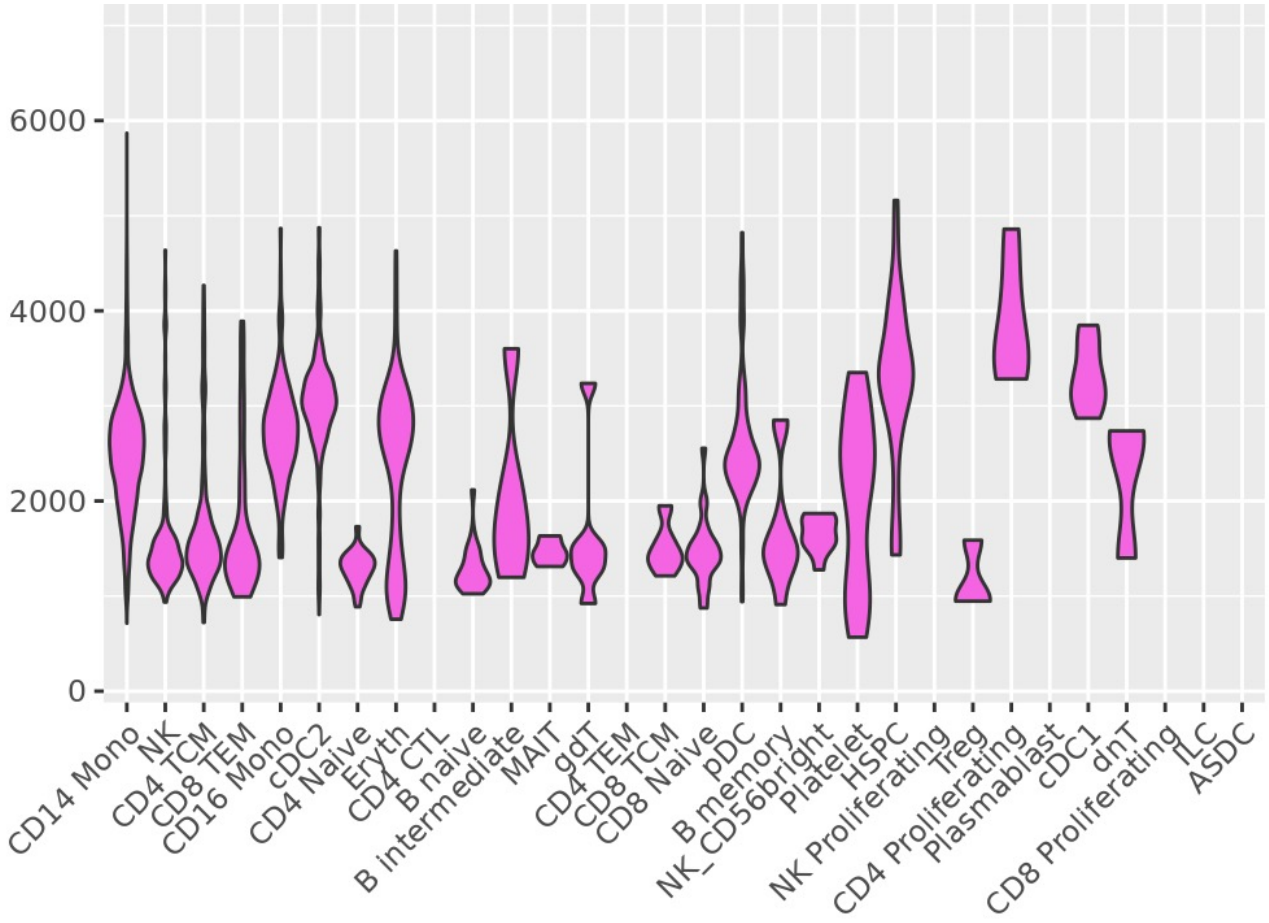
Yang et al., DecontX, https://genomebiology.biomedcentral.com/articles/10.1186/s13059-020-1950-

Filtering example: Peripheral Blood Mononuclear Cells (PBMCs)



- distribution
  - is multi-modal
  - differs between samples

Filtering example: PBMCs



- PBMCs consist of different cell types with varying overall transcriptional activity
- Risk: cell types with “few” genes expressed may be filtered out, e.g. neutrophils

Quality control: Stressed or dying cells

Look at specific gene groups (no hard thresholds available):

- **Mitochondrial genes:** Apoptosis, membrane leakage, damaged cells
- Other gene groups
  - Ribosomal protein genes (RPS*/RPL*)
  - Stress response genes (FOS, FOSB, …)
  - Apoptosis markers
  - Cell cycle markers: “M-phase only”
  - MALAT1: long-noncoding RNA that can have poly-A

Quality control: Low quality cell

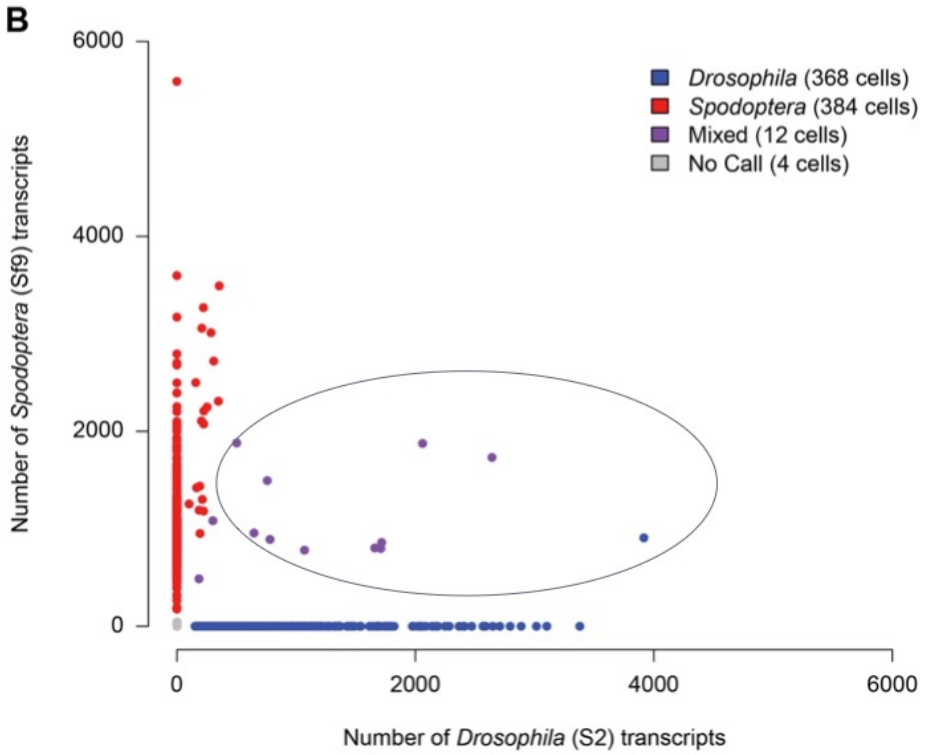
- Remove barcodes with
  - high fraction of mitochondrial genes (in apoptotic or partially lysed cells)
  - zero mitochondrial genes (cells that have lost the cytoplasm?)

- Potential confounder: number of mitochondria differs between cells
  - red blood cells: no mitochondria
  - liver cells: up to 2000 mitochondria

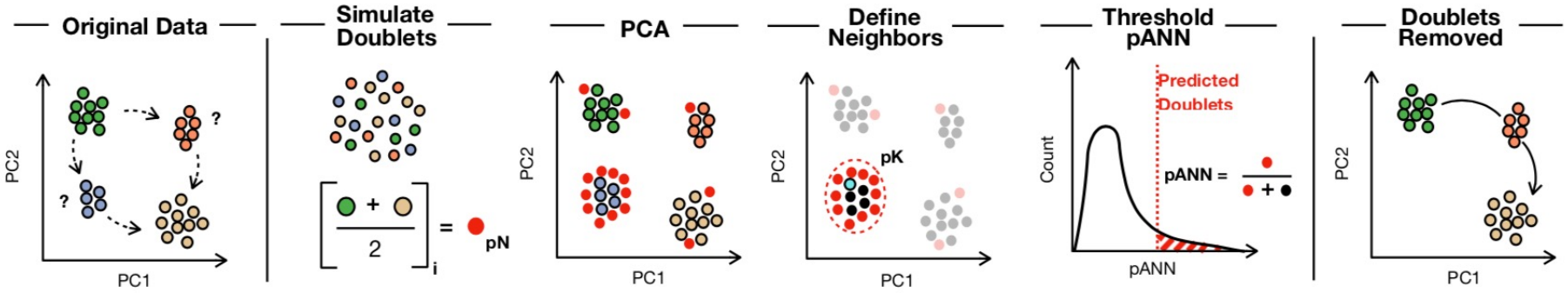
Quality control: Doublets

- Barcode collisions: not enough barcodes
- Technical doublets: two cells in the same droplet; tech specs of 10X Gennomics: +1% per 1000 cells
- Biological doublets: two cells sticking tightly together and form a unit; need to do single-nucleus RNA-seq

- Test datasets for doublets consist of cells from two species



Doublet Detection



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

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

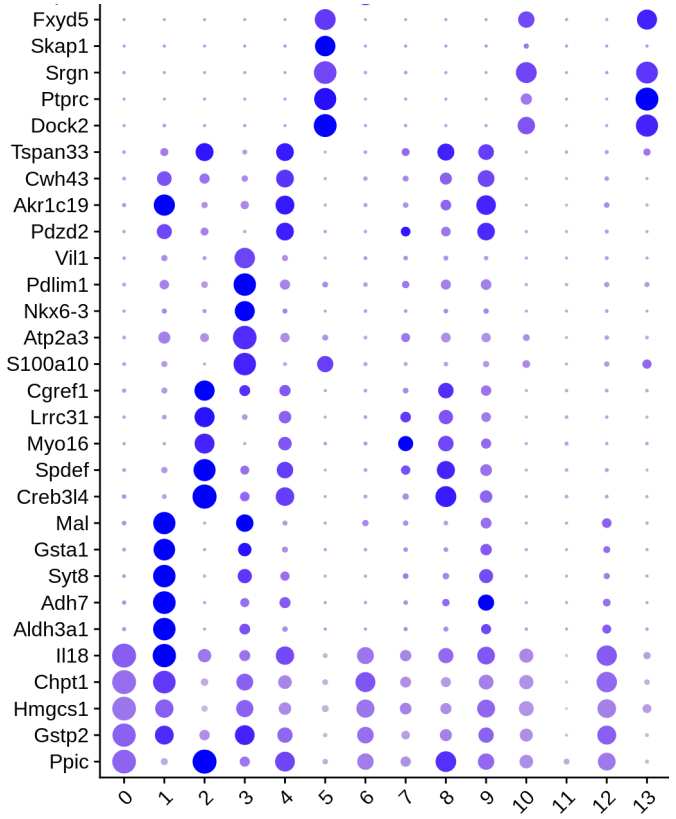
Quality control thresholds

- Quality metrics, like the number of reads or genes detected per cell, do depend on factors like
  - cell type
  - sequencing depth
  - overall quality
  - …
- As a consequence thresholds for these metrics need to be adapted to individual samples and cell types

Cluster Marker genes are not specific

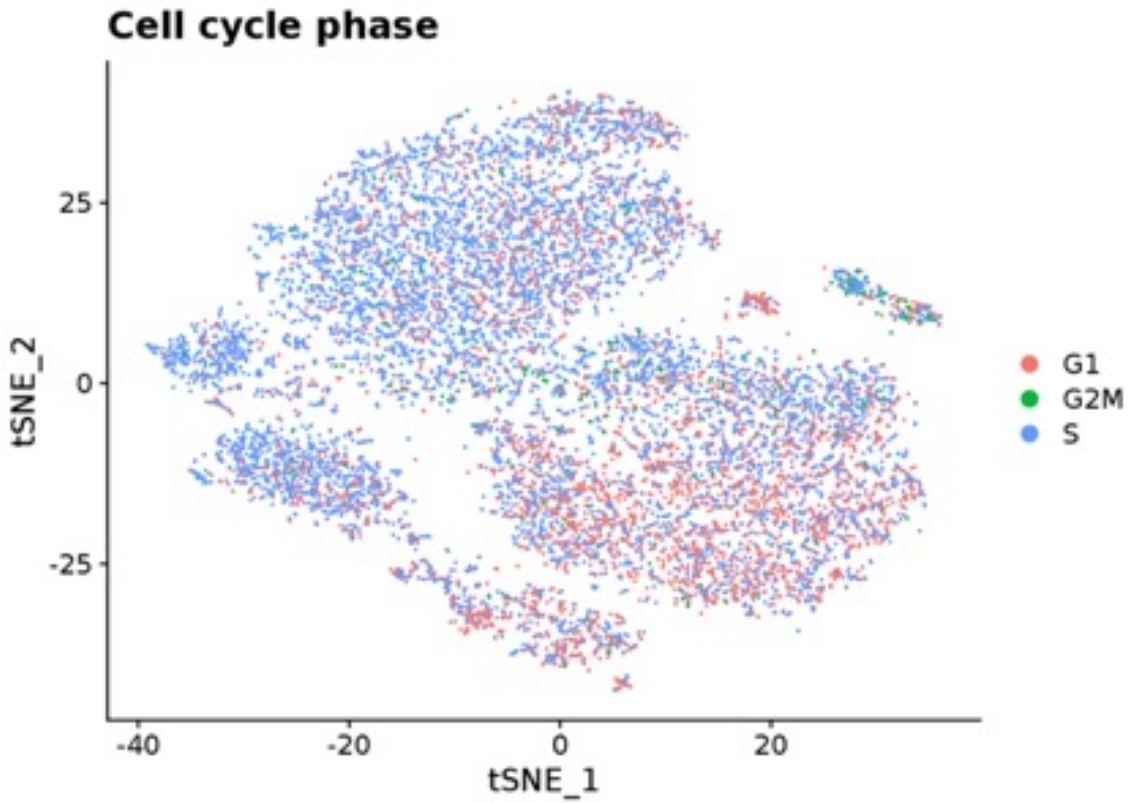
- If we compute cell clusters (== cell types) and the marker genes for these clusters (genes higher expressed in one cluster relative to all other clusters, then the top marker genes should be **cluster-specific** (only expressed in one cluster)
- Reasons for unspecific markers:
  - Too many clusters
  - Ambient RNA

Example of cluster marker genes, where cluster zero represents low count, low quality cell fragments or ambient RNA



Cell cycle: Cause of variation in gene expression

- Cell cycle can be a quality indicator too. Cells in M-phase might indicate dying or damaged cells
- Depending on the research goal, cell-cycle associated variation
  - can be used to discriminate cells
  - is unwanted variation and cell-cycle should be considered as latent variable

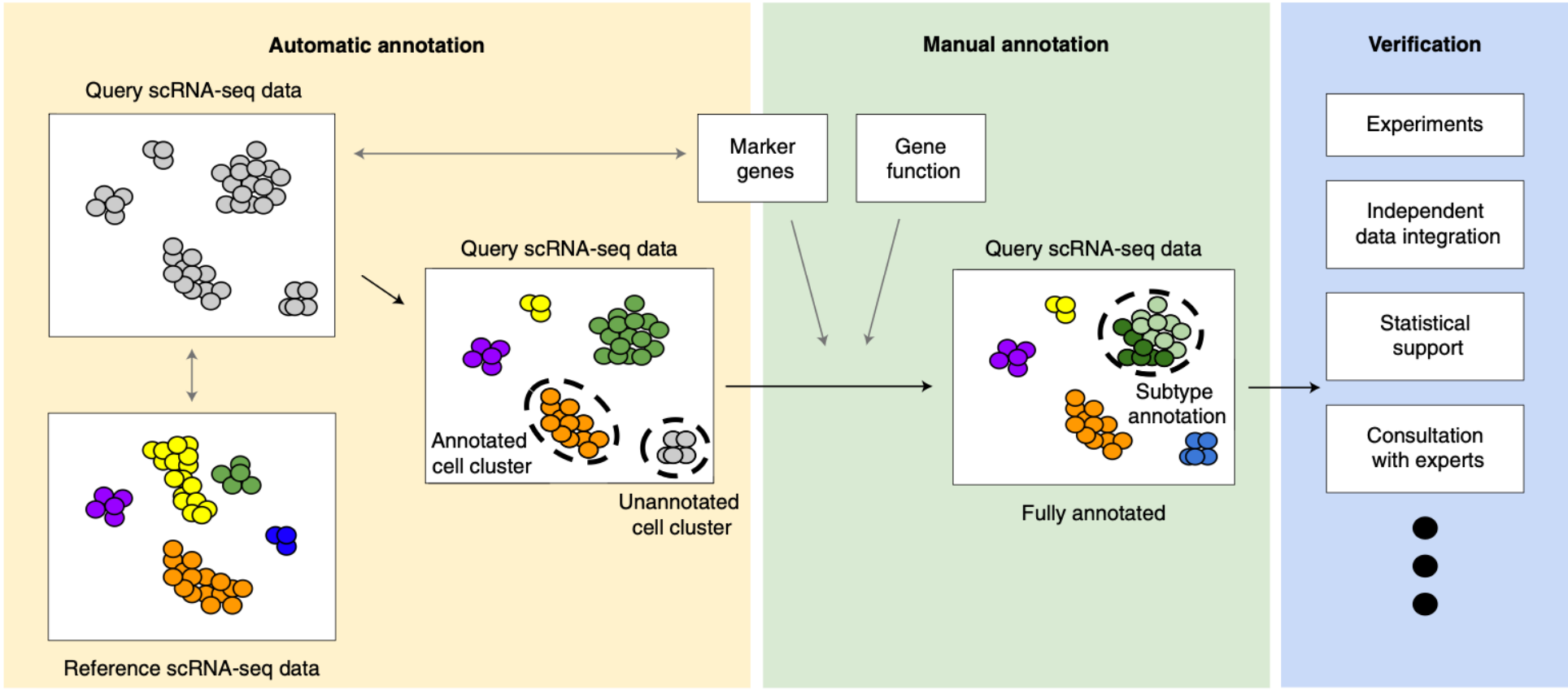


General Quality Measures

- General quality measures
  - Cell viability (%)
  - Knee plot
  - Reads in cells (%)
  - Sequencing depth & sequencing saturation
- Indicate low quality, but are often not actionable

Per Cell Quality Measures

- # UMIs per cell
  - Low: cell fragment; high: doublet
- # genes per cell:
  - Low: cell fragment; high: doublet
- Percent mitochondrial reads:
  - Apoptosis, membrane leakage, damaged cells
- Percent ribosomal protein genes (RPL/RPS)
- Cell-cycle M-phase genes
- Ambient contamination score



Clarke et al. 2021

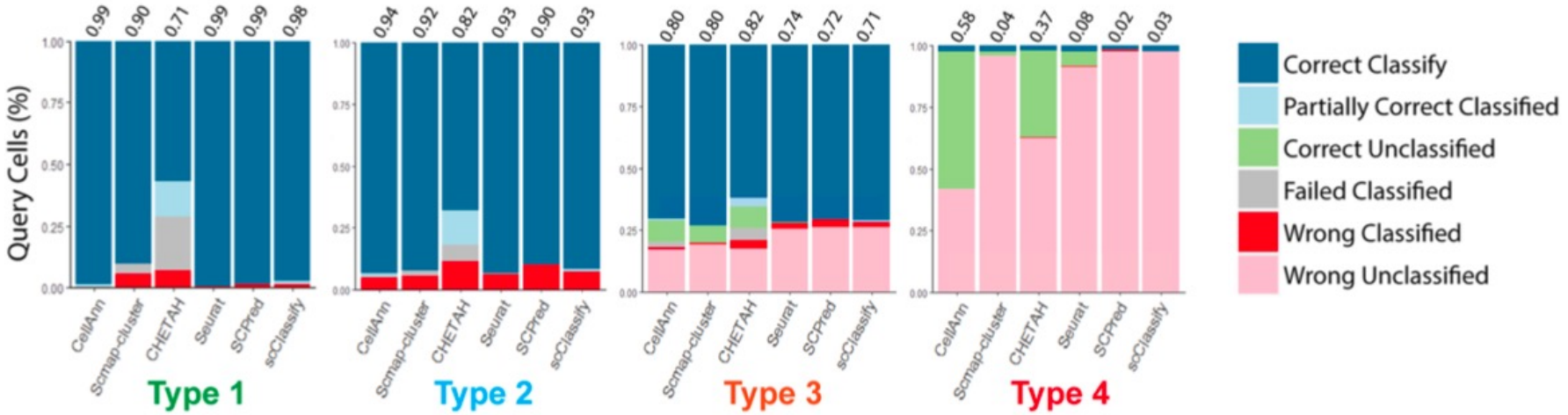
Cell type: annotation approaches and tools

	marker genes	reference expression profiles
per cell	• **AUCell** • scROSHI	• **SingleR** • scArches • **Azimuth** • scmap-cell
per cluster	• **enrichR** • **scType**	• **SingleR** • FR-Match • CellAnn • scmap-cluster

Per-cell vs per-cluster labeling

- generally, both approaches agree for the major cell-types
- discrepancies occur when trying to identify sub-types
- major discrepancies might be explained by
  - cluster resolution inappropriate
  - reference data / marker gene set inappropriate
  - subtype of cells
- discrepancies suggest manual resolution

Reference-based Annotation



- generally good performance
- situation where query and reference do not overlap are of a concern

Lyu, CellAnn paper, 2023

Reference Based Celltype Annotation

- **CellAnn:** compute **correlations** of cluster averages, analyze correlations to define thresholds, compute rank-test to define sub-types
- **Seurat:** integrate data in UMAP space and assign nearby cell type
- **scmap-cell:** approximate **nearest neighbors**
- **SingleR:** identify variable genes in the reference set, **correlate** individual cells with the reference using the variable genes

Single Cell Reference Data

- "Cell-by-gene": https://cellxgene.cziscience.com
- https://atlas.brain-map.org/
- https://www.humancellatlas.org/
- https://www.flycellatlas.org/
- https://azimuth.hubmapconsortium.org/references/

- Single Cell Expression Atlas: https://www.ebi.ac.uk/gxa/sc/home
  - individual studies
- BROAD:
  - https://singlecell.broadinstitute.org/single_cell
- ...

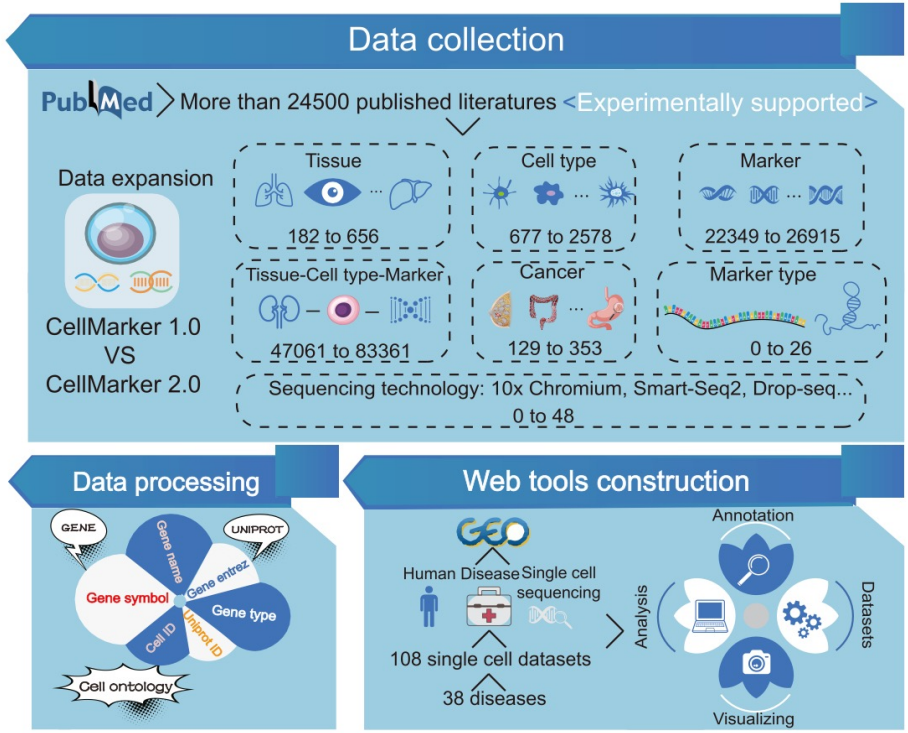
Generally: The more similar the reference is to your system, the better

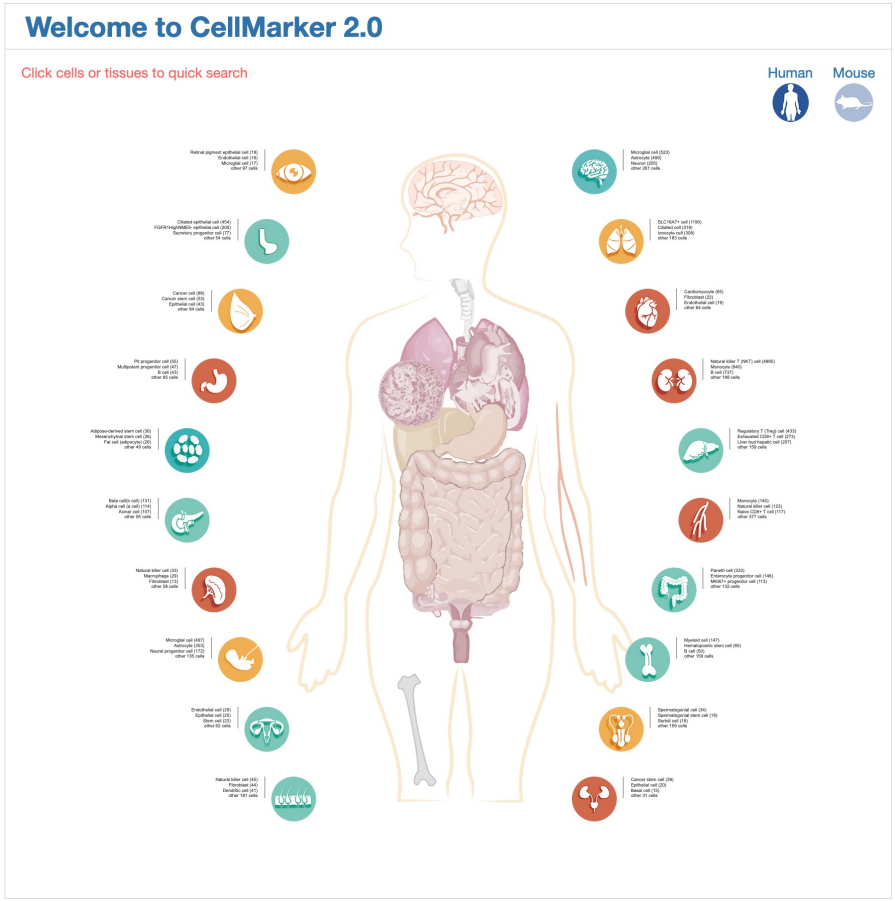
Marker Gene Based Celltype Annotation

Tools:

- **AUCell:** Area und the curve to estimate marker enrichment
- **enrichR:** compute cluster-markers, subsequently compute fuzzy enrichment among cell-type markers
- **scROSHI:** rank test to identify if cell-type markers are higher expressed than others

CellMarker 2.0

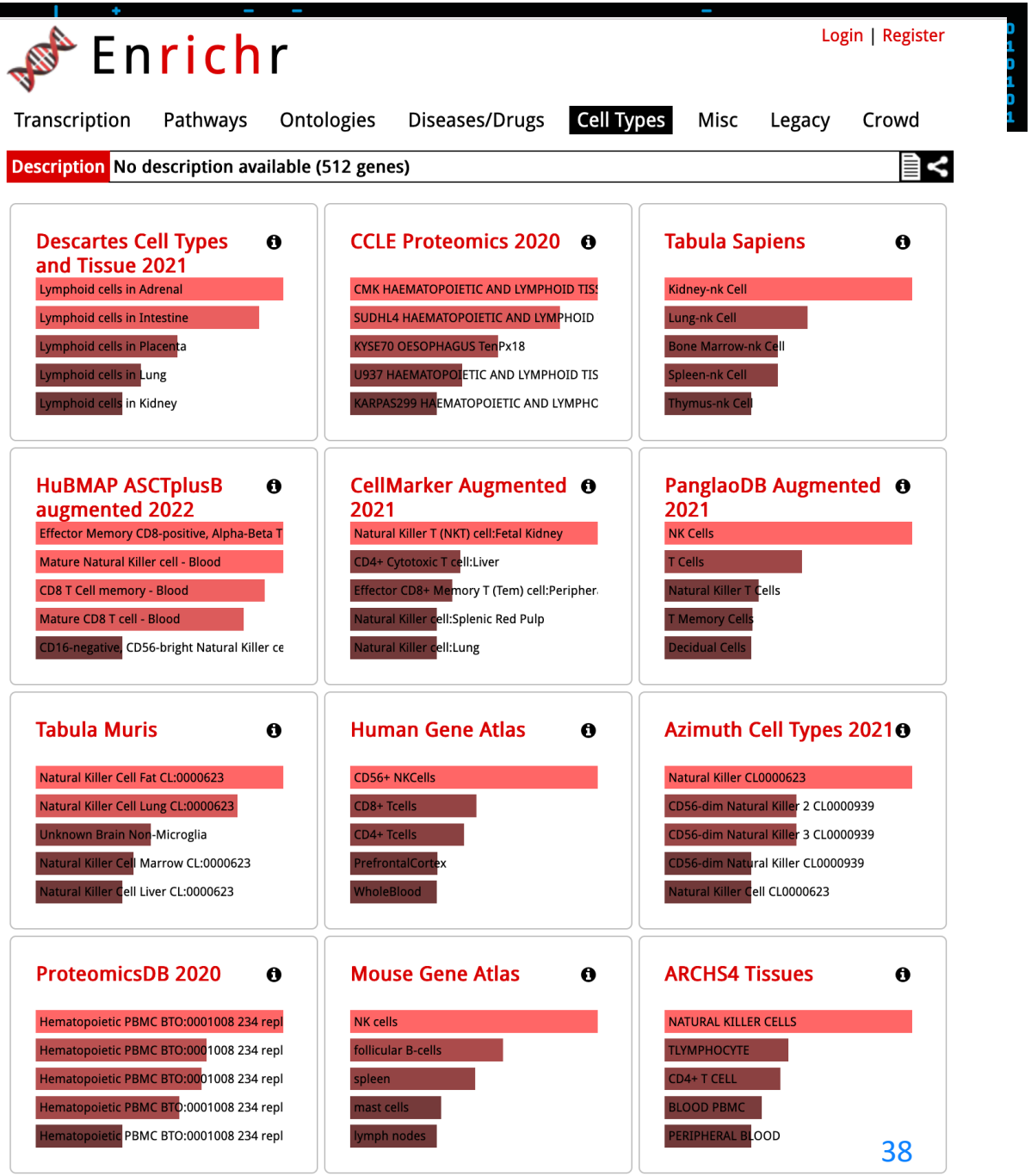




EnrichR

- database of databases
- diverse source of gene lists
- hosts also gene sets specific to cell types
- Gene sets are defined for human/mouse

- EnrichR databases for model organisms exist but do not include cell type specific gene sets

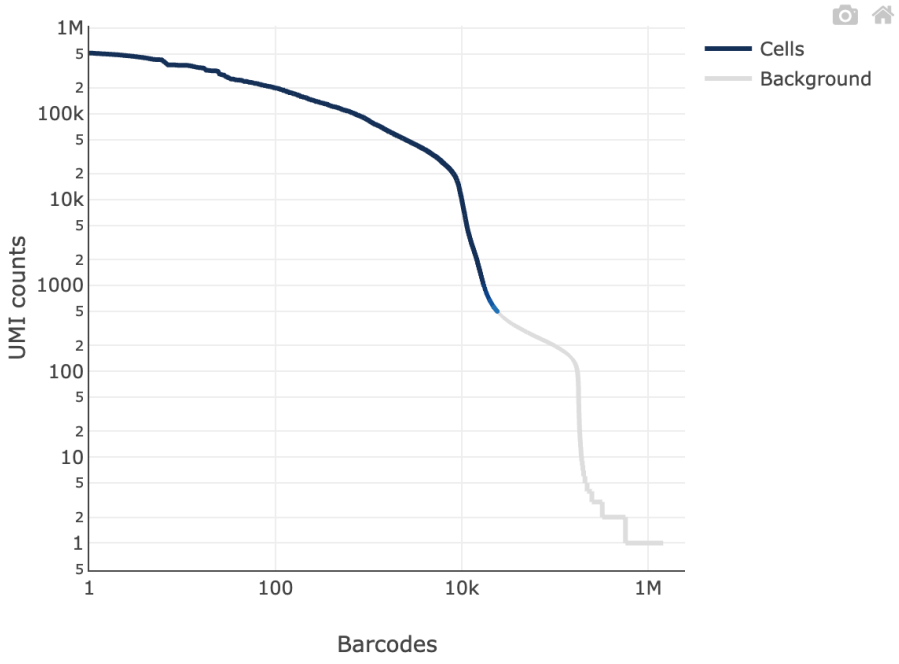


Summary

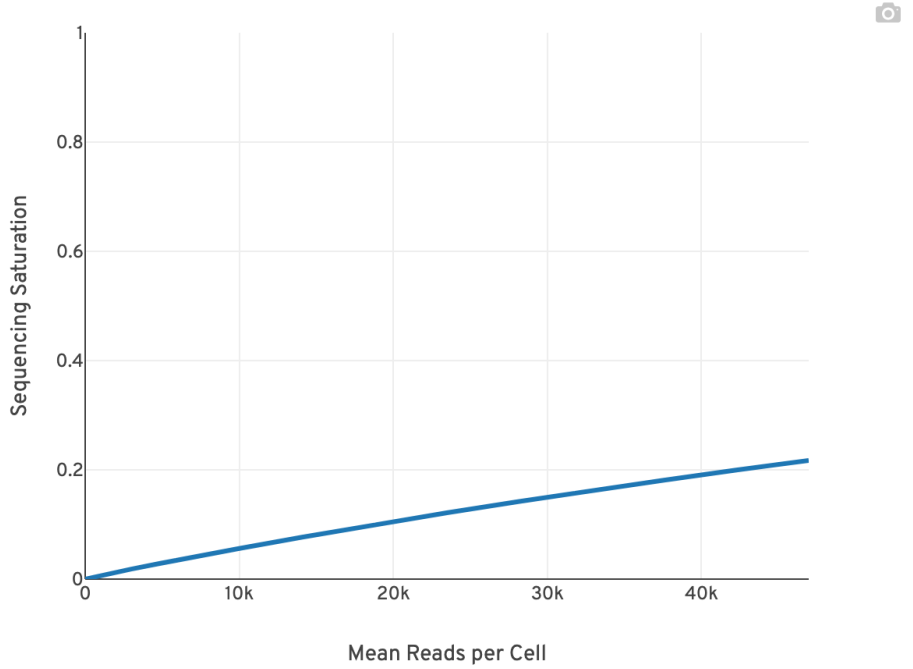
- Quality control is an essential step in single cell processing
- Cell-type annotation typically requires manual curation
- Major cell-type are well automatically detected, subtypes however are often misclustered and misassigned in automatic workflows

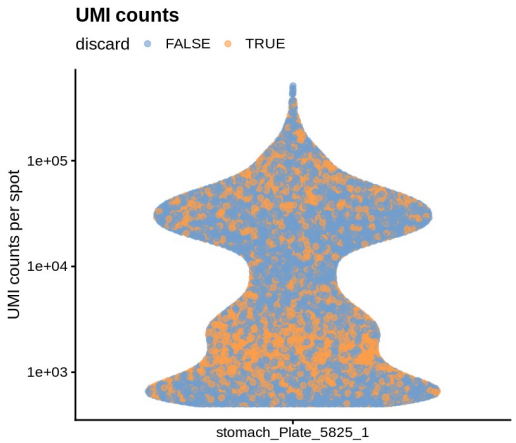
Low Quality Example

GEX Barcode Rank Plot



Sequencing Saturation

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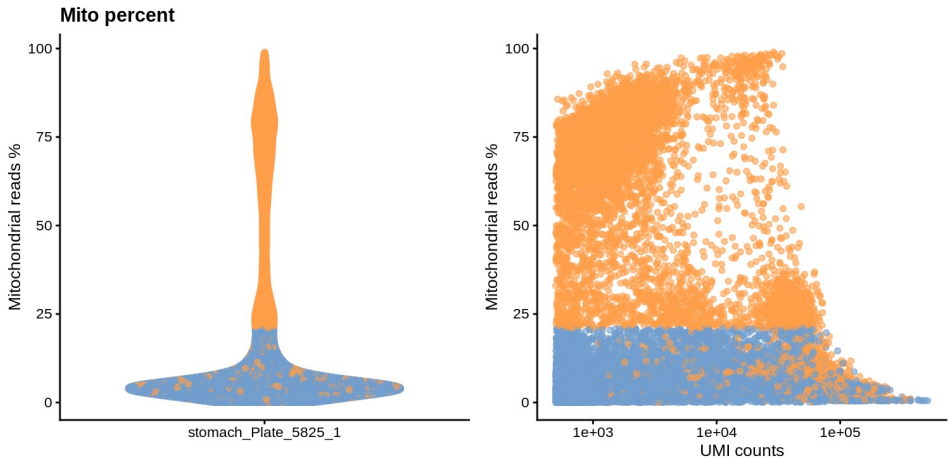
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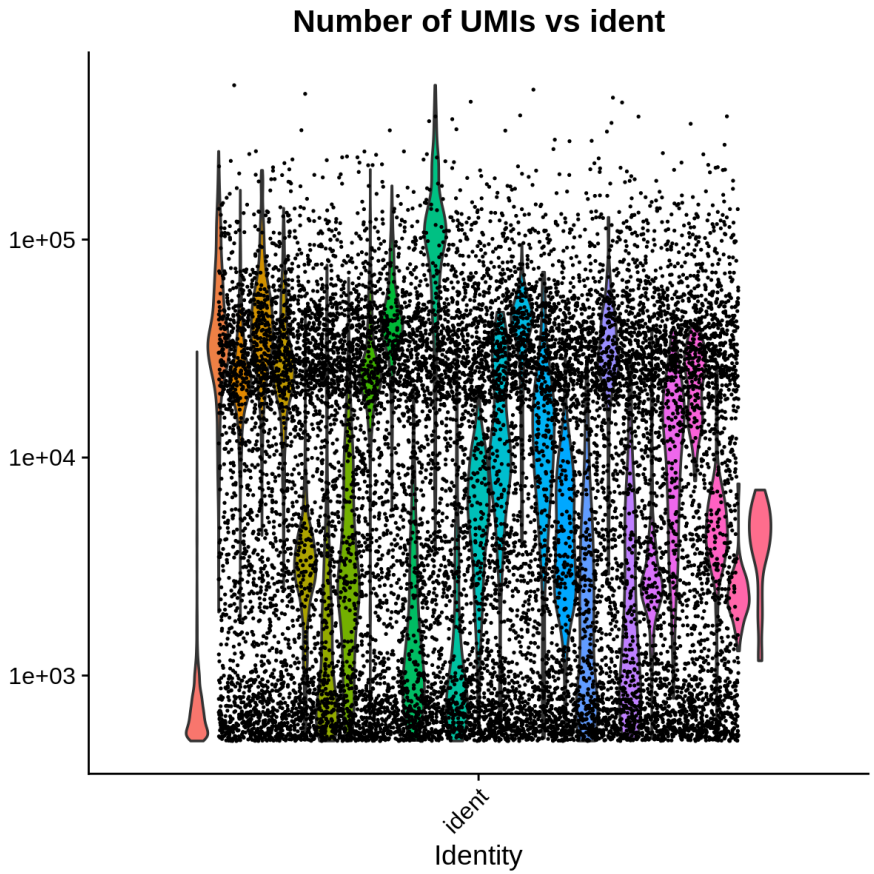
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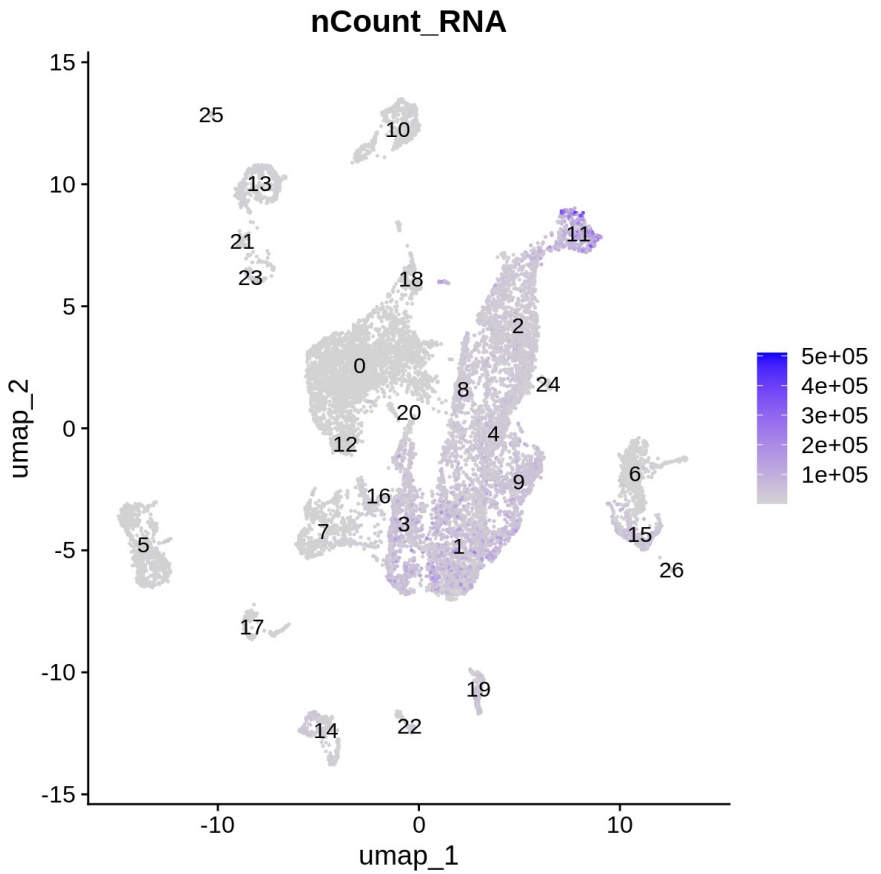
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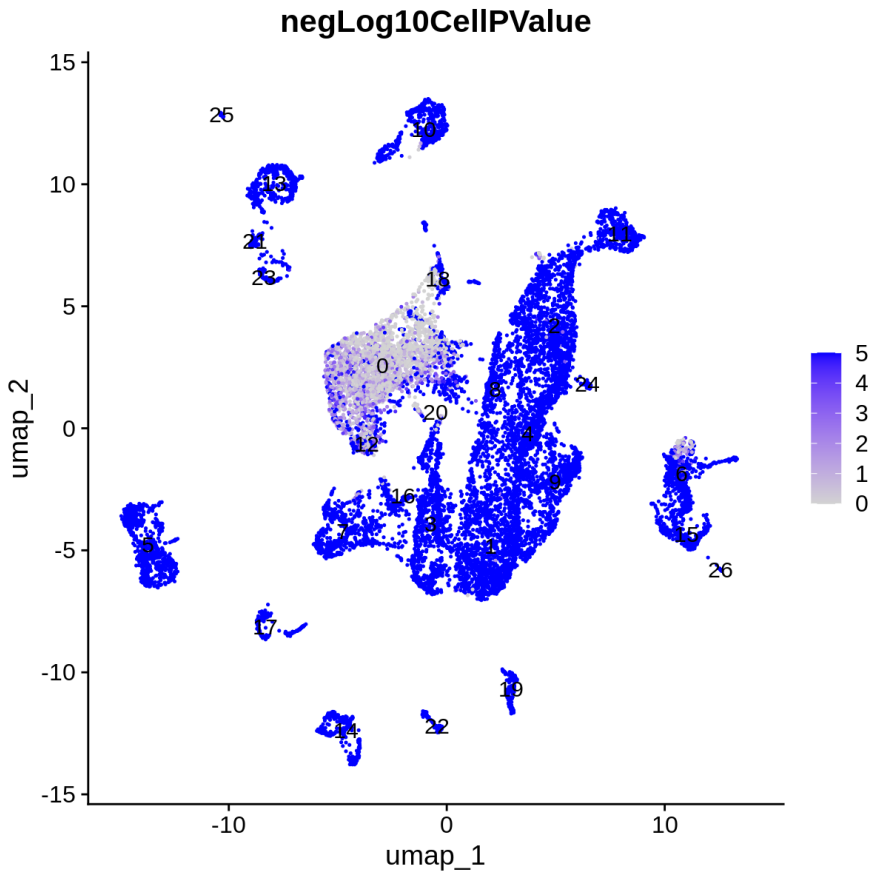
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