

# Introduction to Single-cell Omics Analysis

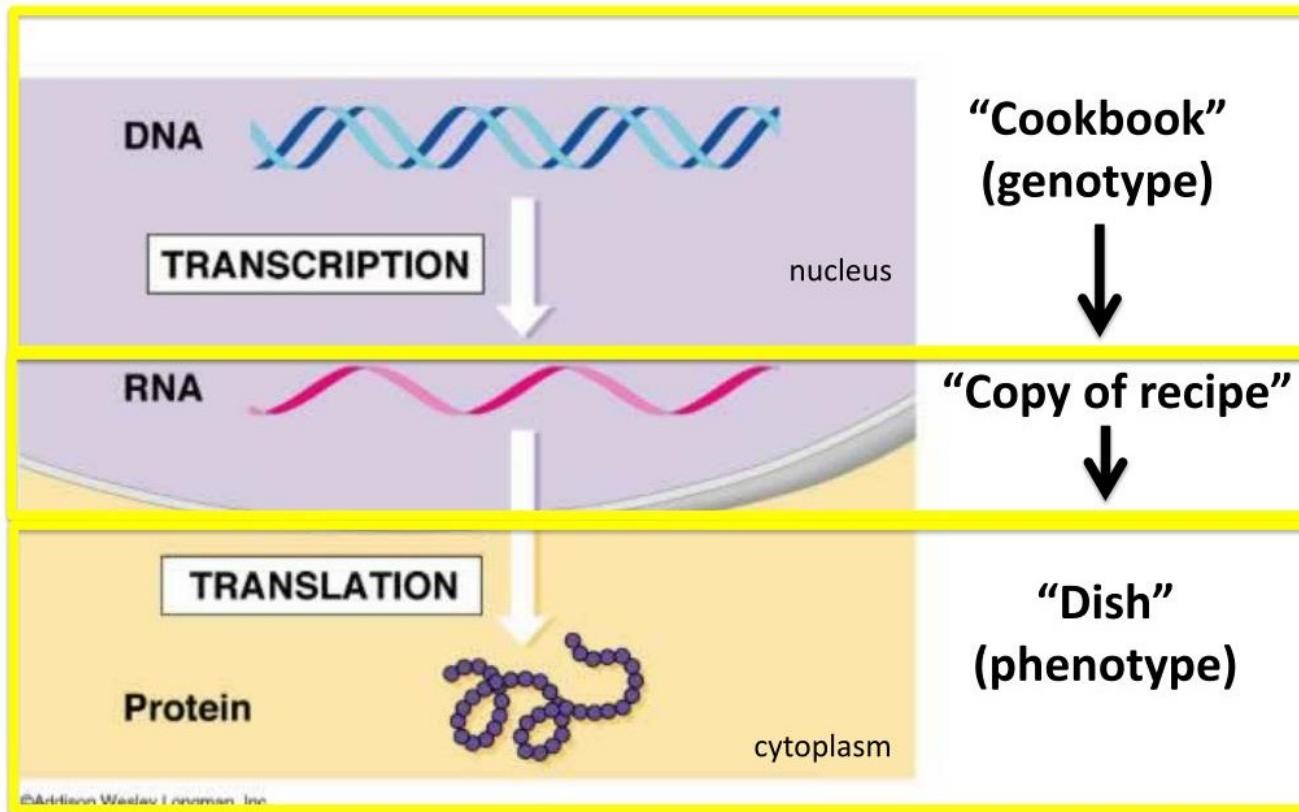
Joel Gruber

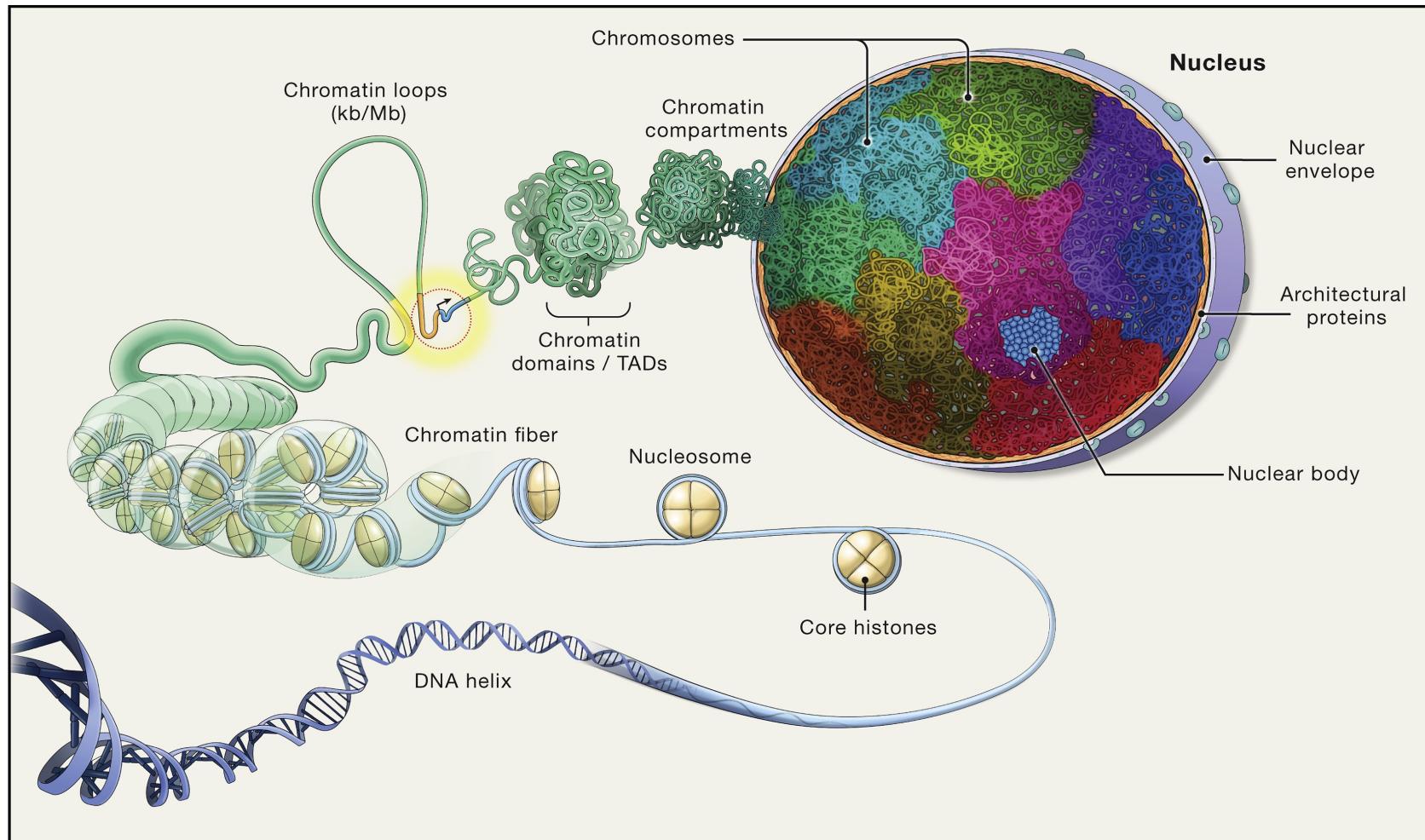
Senior Staff Scientist

Director, Comparative Genomics and Data Science Core

MDI Biological Laboratory

# Basics of Gene Expression: the Central Dogma





# What can we measure?

- Nucleic Acid Sequence
  - hundreds of millions of reads at 100-150 bases in length
  - We mostly sequence as DNA
  - Bulk samples ( $10^8$ ), single-cell ( $10^7$ )
- Proteins/Metabolomics
  - 1000s of fragments per sample
- Question: Why is nucleic acid sequencing easier than protein?
- Answer: because nature did much of the work for us

# Sequencing by synthesis

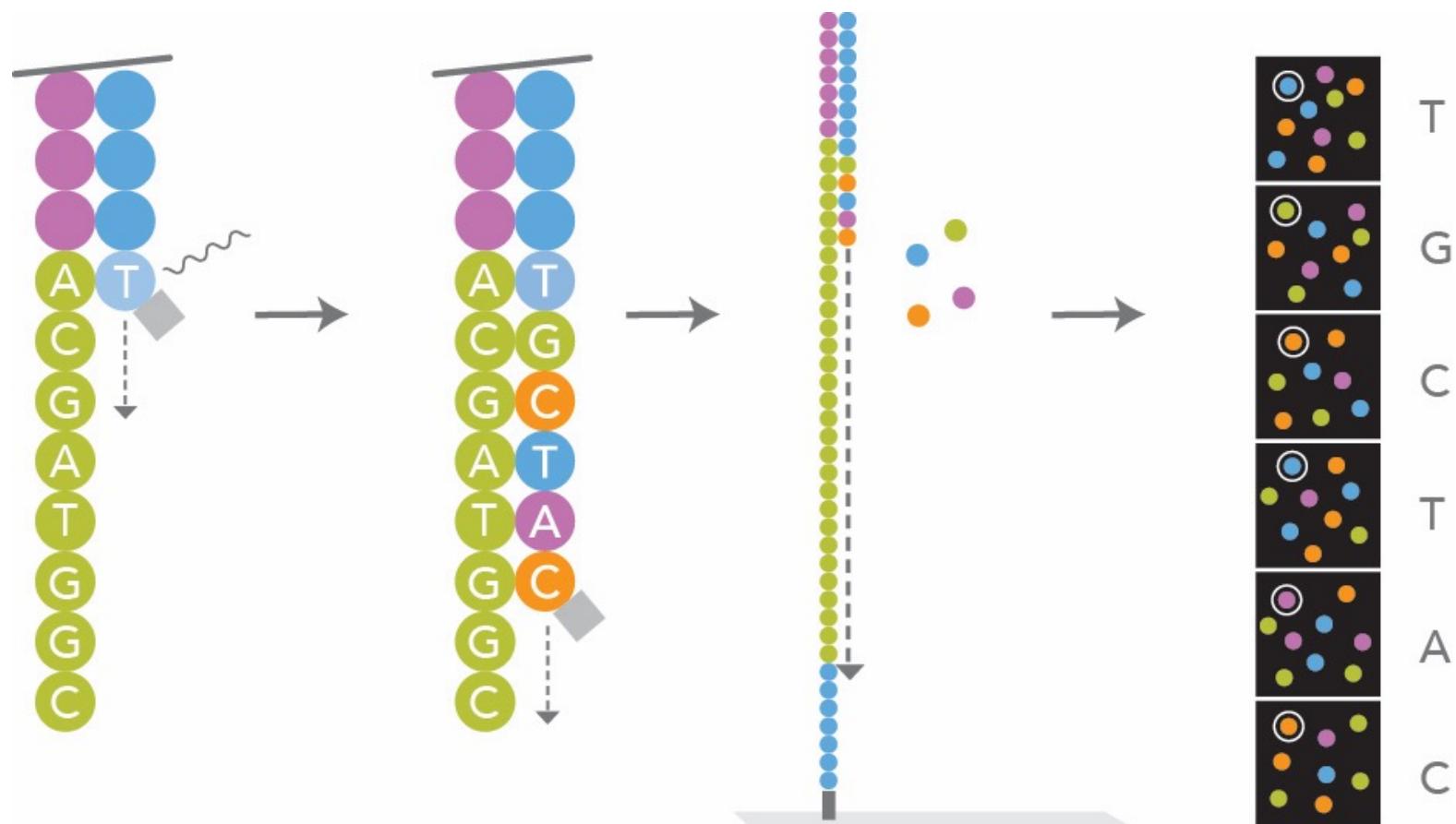


Image source: <https://www.lexogen.com/rna-lexicon-next-generation-sequencing/>

# A conceptual RNAseq experimental workflow

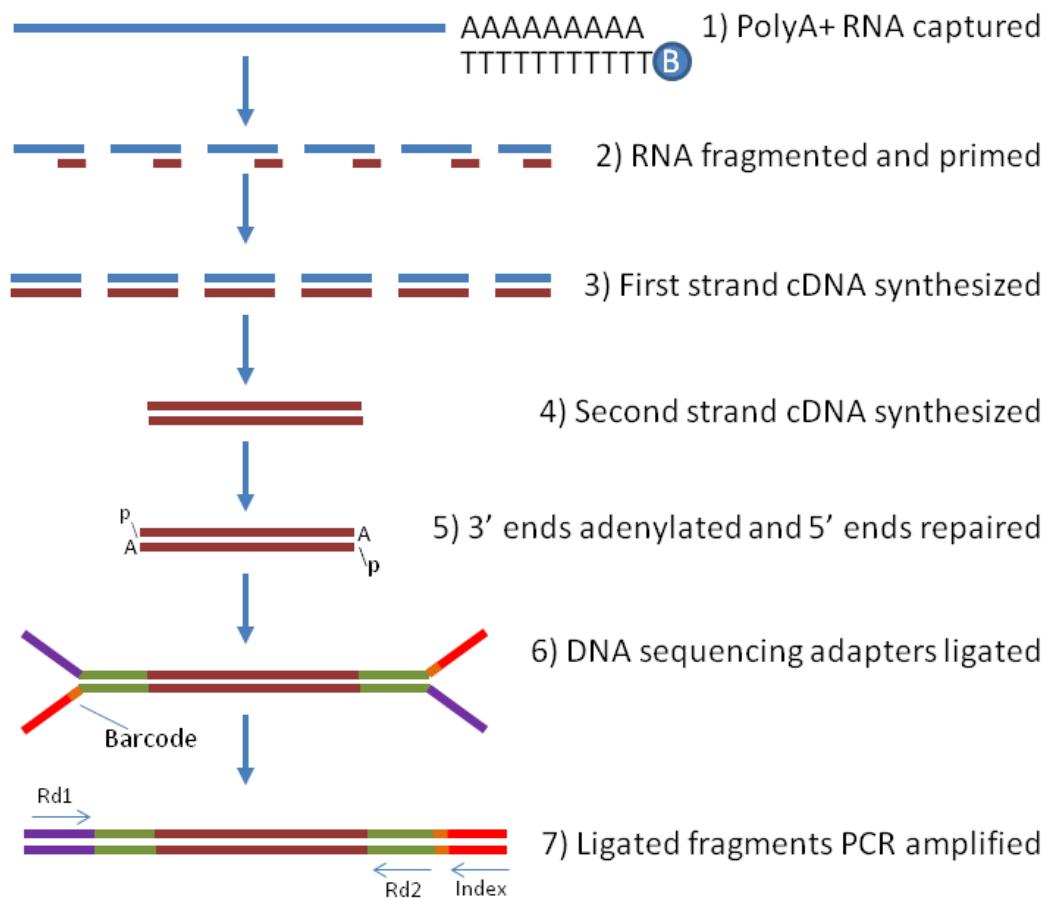
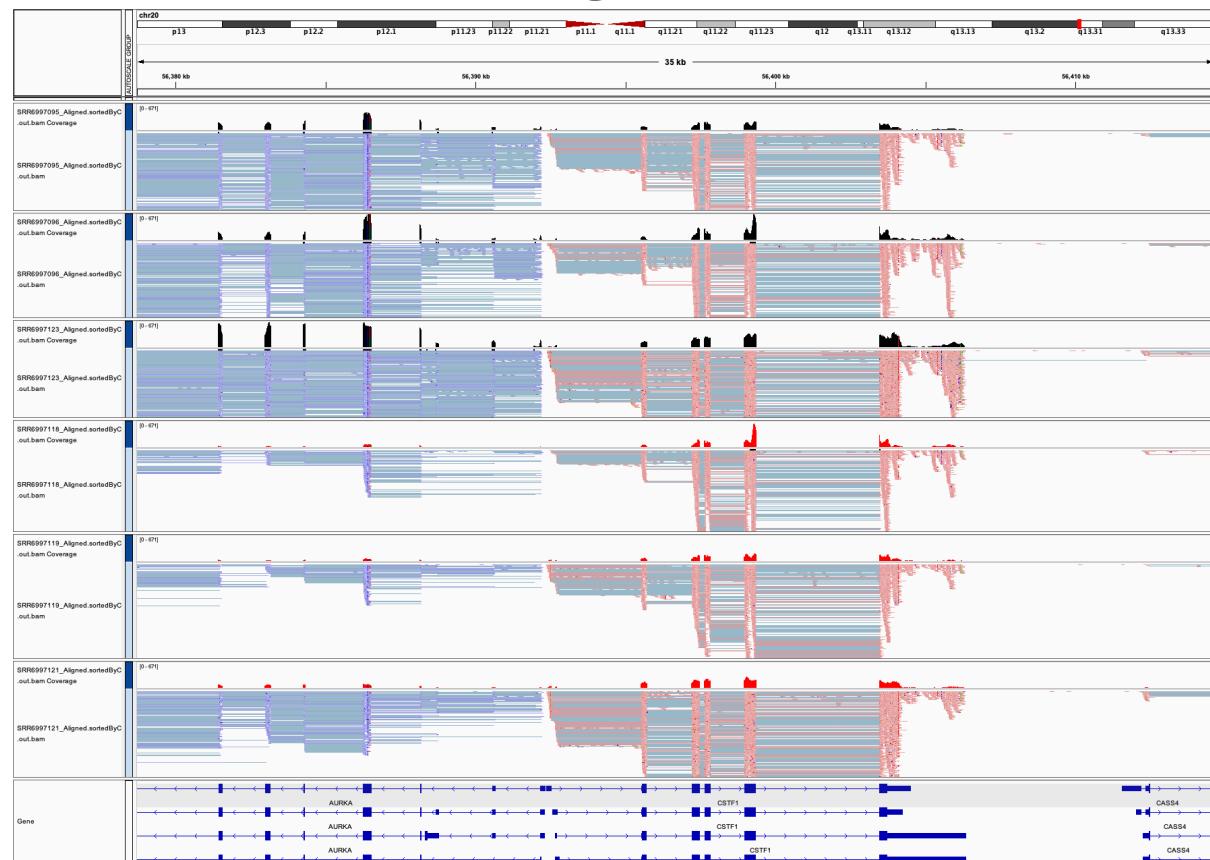


Image source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

# RNA-seq analysis: alignment/quantification



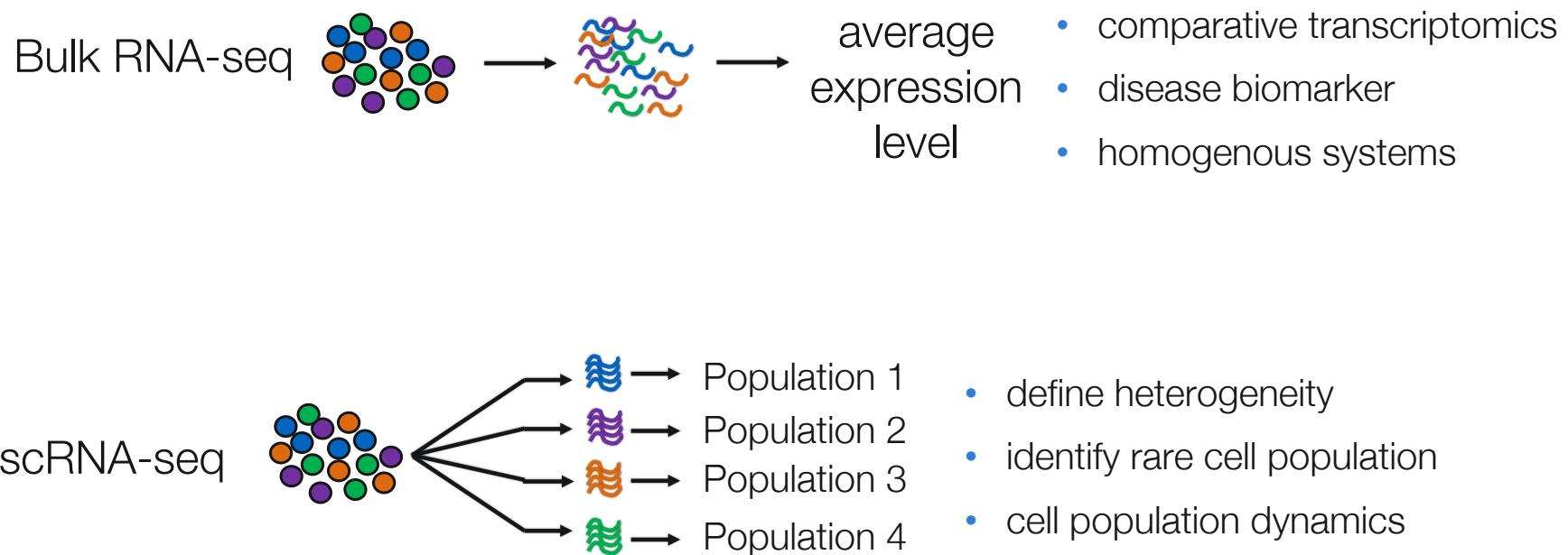
After expression is assessed in each sample,  
they are merged into a “count matrix”

gene_id	SL94881	SL94882	SL94883	SL94884	SL94885	SL94886
ENSDARG000000000001	33	40	36	38	58	40
ENSDARG000000000002	136	126	156	167	170	158
ENSDARG000000000018	319	356	345	368	357	334
ENSDARG000000000019	1174	1390	1430	1356	1130	1237
ENSDARG000000000068	522	468	590	622	506	528
ENSDARG000000000069	1622	1622	1546	1494	1546	1561
ENSDARG000000000086	413	536	474	489	290	476
ENSDARG000000000103	1212	1390	1266	1296	1012	1390
ENSDARG000000000142	118	97	99	110	94	126
...						

# Fundamental ideas of transcriptome profiling

- The activity or state of a sample can be defined by a “snapshot” (profile) of molecules
  - In a transcriptome, the molecules are the RNA transcripts
- Perturbations/changes to a system induce systemic (and predictable and reproducible) responses
- Comparison of molecular profiles can
  - delineate the mechanisms/pathways involved
  - Identify and classify related samples

# Bulk vs Single Cell RNA-seq (scRNA-seq)



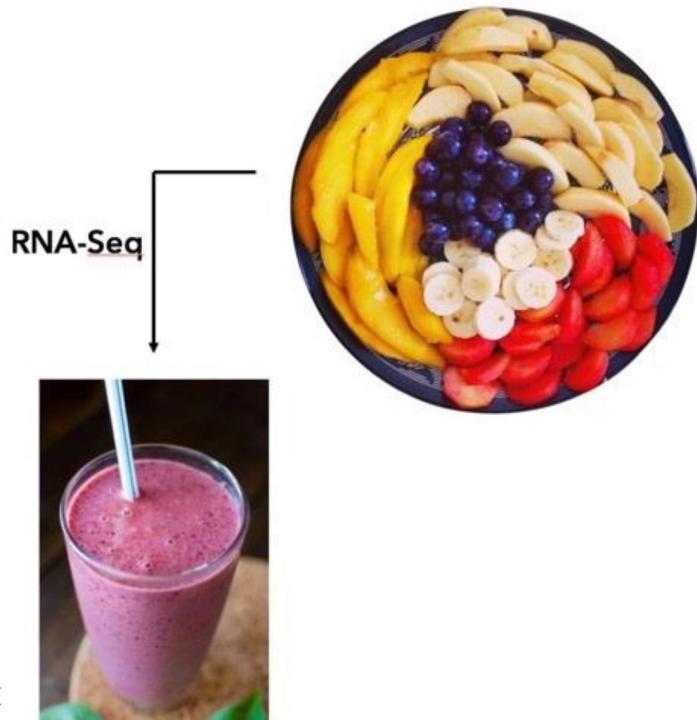
Slide source: <https://www.sambuz.com/doc/introduction-to-single-cell-rna-sequencing-ppt-presentation-754293>

# BULK VS SINGLE CELL RNA-SEQ



Average expression level

- Comparative transcriptomics
- Disease biomarker
- Homogenous systems



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# ● BULK VS SINGLE CELL RNA-SEQ

## 1. mRNA: TruSeq RNA-Seq (Gold Standard)

- ~20,000 transcripts
  - More when consider splice variants / isoforms
- Observe 80-95% of transcripts depending on sequencing depth

## 2. Low input methods ~3000 cells / well

- 4000-6000 transcripts per sample
  - Limiting to transcripts observed across all samples
- Observe 20-60% of the transcriptome

## 3. Single Cell Methods

- 200 -10,000 transcripts per cell
- Observe 10-50% of the transcriptome
- Many transcripts will show up with zero counts in every cell. (even GAPDH)
- If you only looked at transcripts observed in all cells numbers drop dramatically.

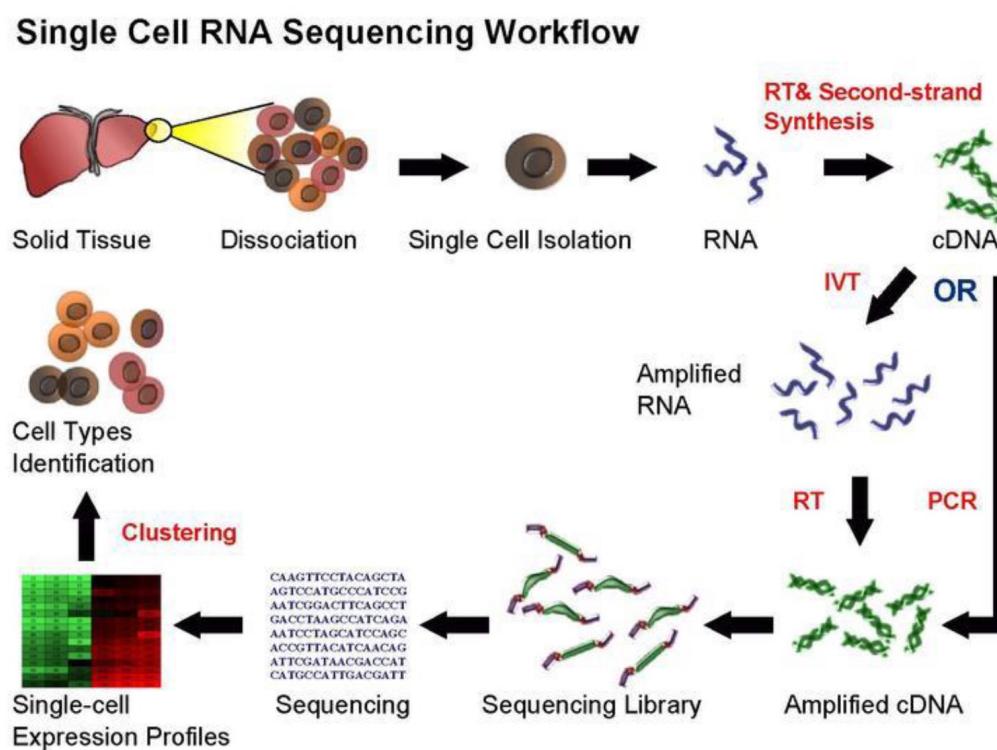
Source: Sarah Boswell, Harvard Medical School, September 2020

Slide source: [https://bioinformatics-core-shared-training.github.io/SingleCell\\_RNASeq\\_Jan23/UnivCambridge\\_ScRnaSeqIntro\\_Base/Slides/01\\_Introduction.pdf](https://bioinformatics-core-shared-training.github.io/SingleCell_RNASeq_Jan23/UnivCambridge_ScRnaSeqIntro_Base/Slides/01_Introduction.pdf)

# WORKFLOW



Good sample preparation is key to success!

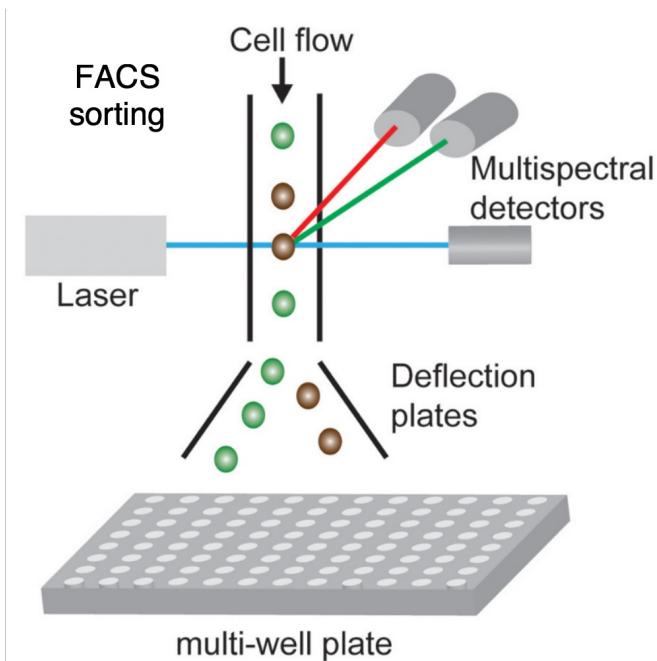


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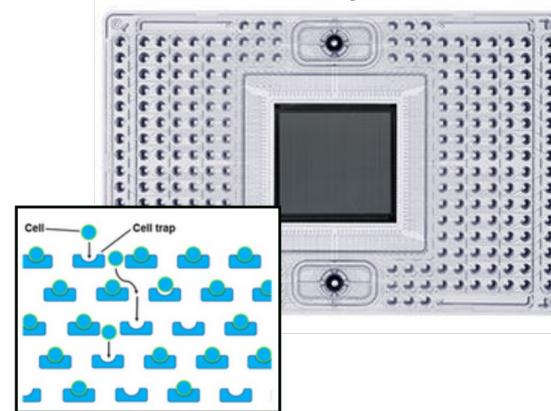
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# Multiple techniques have been developed

**Microtitre Plates**



**Microfluidic Arrays**



**Microfluidic Droplets**

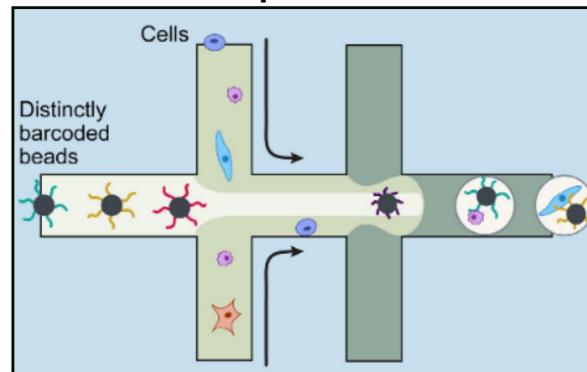


Image source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

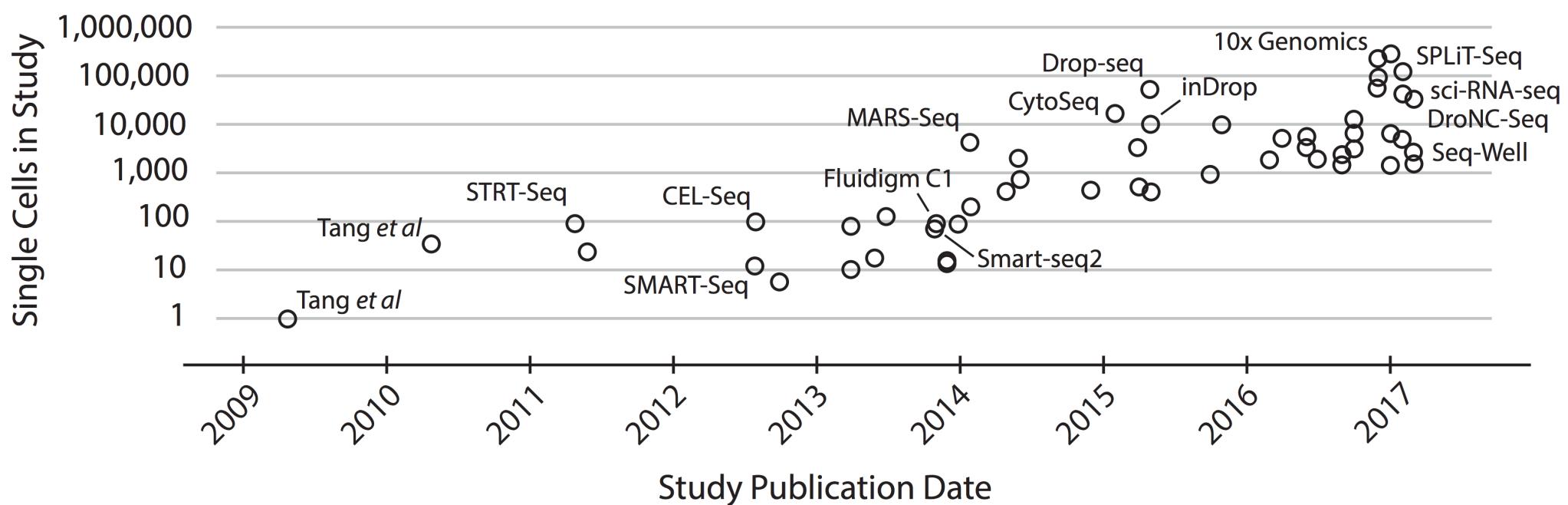
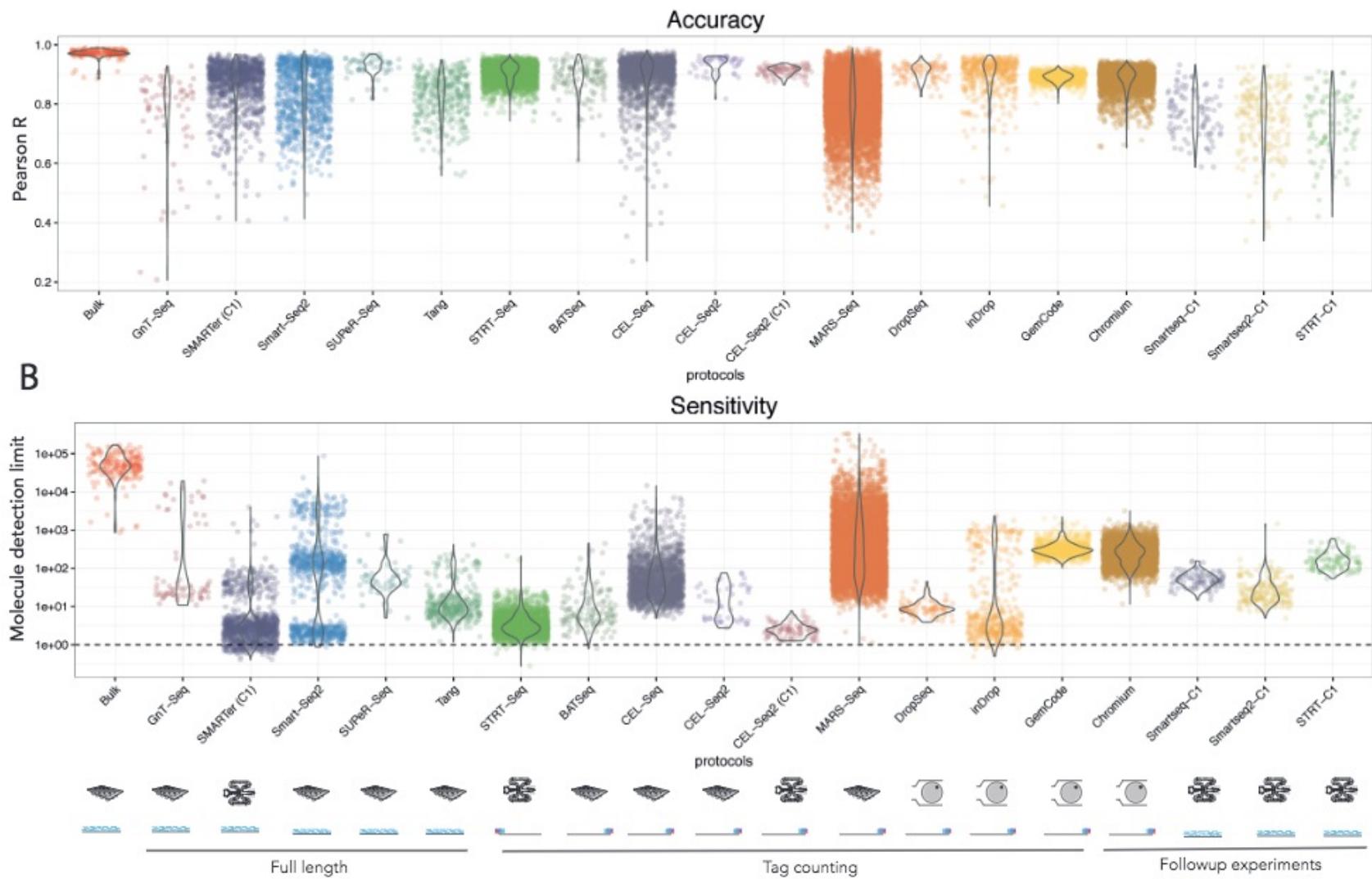


Image source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

# Capabilities, depth, and characteristics vary among approaches

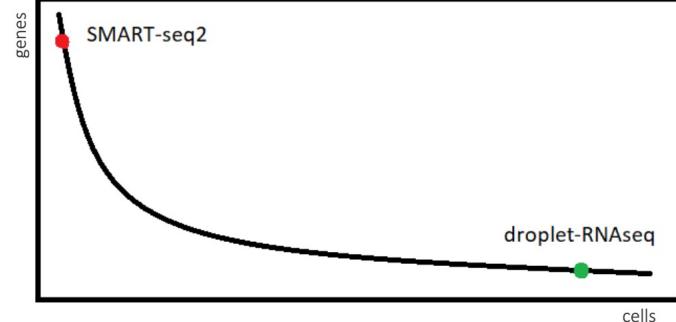
	SMART-seq2	CEL-seq2	STRT-seq	Quartz-seq2	MARS-seq	Drop-seq	inDrop	Chromium	Seq-Well	sci-RNA-seq	SPLiT-seq
Single-cell isolation	FACS, microfluidics	FACS, microfluidics	FACS, microfluidics, nanowells	FACS	FACS	Droplet	Droplet	Droplet	Nanowells	Not needed	Not needed
Second strand synthesis	TSO	RNase H and DNA pol I	TSO	PolyA tailing and primer ligation	RNase H and DNA pol I	TSO	RNase H and DNA pol I	TSO	TSO	RNase H and DNA pol I	TSO
Full-length cDNA synthesis?	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes
Barcode addition	Library PCR with barcoded primers	Barcoded RT primers	Barcoded TSOs	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers and library PCR with barcoded primers	Ligation of barcoded RT primers
Pooling before library?	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Library amplification	PCR	In vitro transcription	PCR	PCR	In vitro transcription	PCR	In vitro transcription	PCR	PCR	PCR	PCR
Gene coverage	Full-length	3'	5'	3'	3'	3'	3'	3'	3'	3'	3'
Number of cells per assay											
	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>4</sup>



Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

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# MORE CELLS OR MORE GENES?

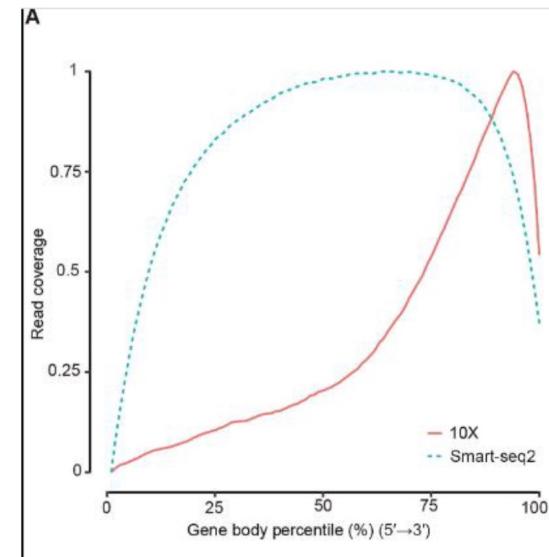


## SMART-seq2

- 100 cells
- Full-length libraries
- 1M reads per cell

## Droplet-RNAseq

- 10000 cells
- 50k reads per cell
- 3'/5' bias



Source: Wang, et al. Genom. Proteom. Bioinform. 19(2), 253-266 (2021).



- Required number of cells increases with complexity of the sample.
- Number of reads will depend on biology of sample
- Cell-type classification of a mixed population usually requires lower read depth
- You can always re-sequence your samples.

Slide source: [https://bioinformatics-core-shared-training.github.io/SingleCell\\_RNASeq\\_Jan23/UnivCambridge\\_ScRnaSeqIntro\\_Base/Slides/01\\_Introduction.pdf](https://bioinformatics-core-shared-training.github.io/SingleCell_RNASeq_Jan23/UnivCambridge_ScRnaSeqIntro_Base/Slides/01_Introduction.pdf)

# A conceptual RNAseq experimental workflow

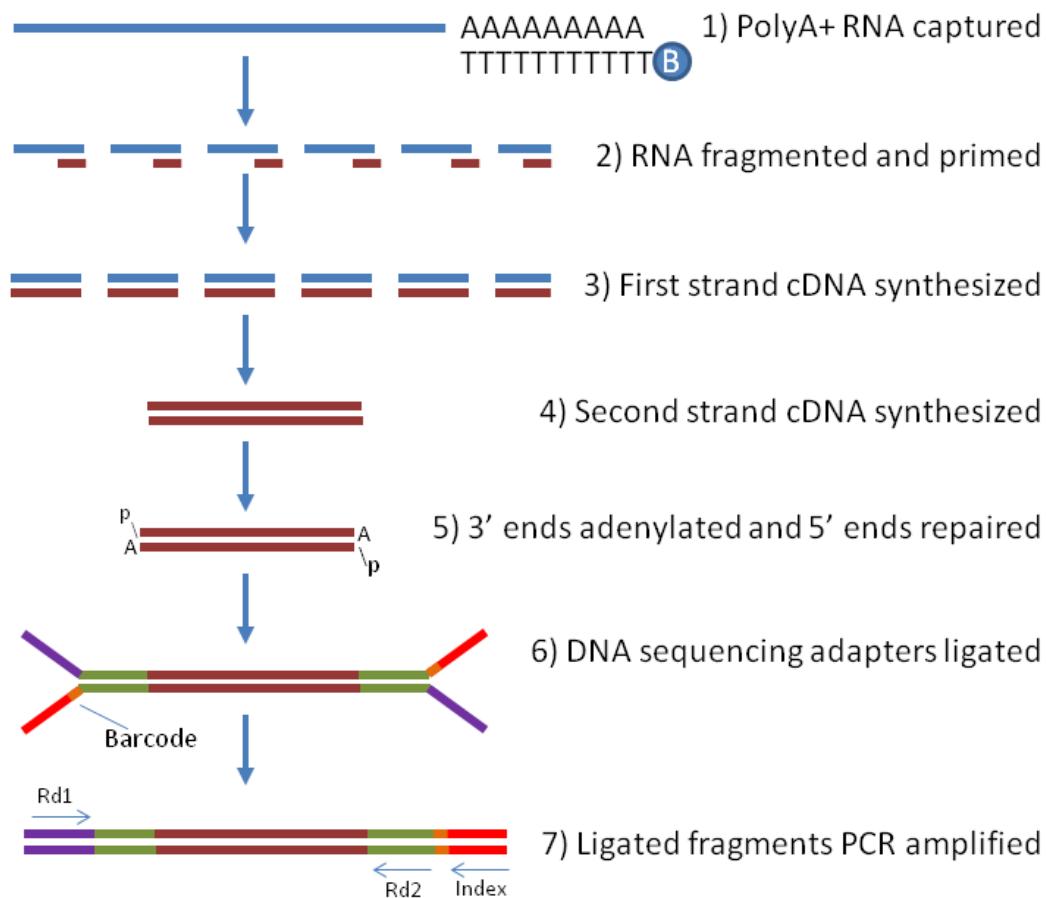
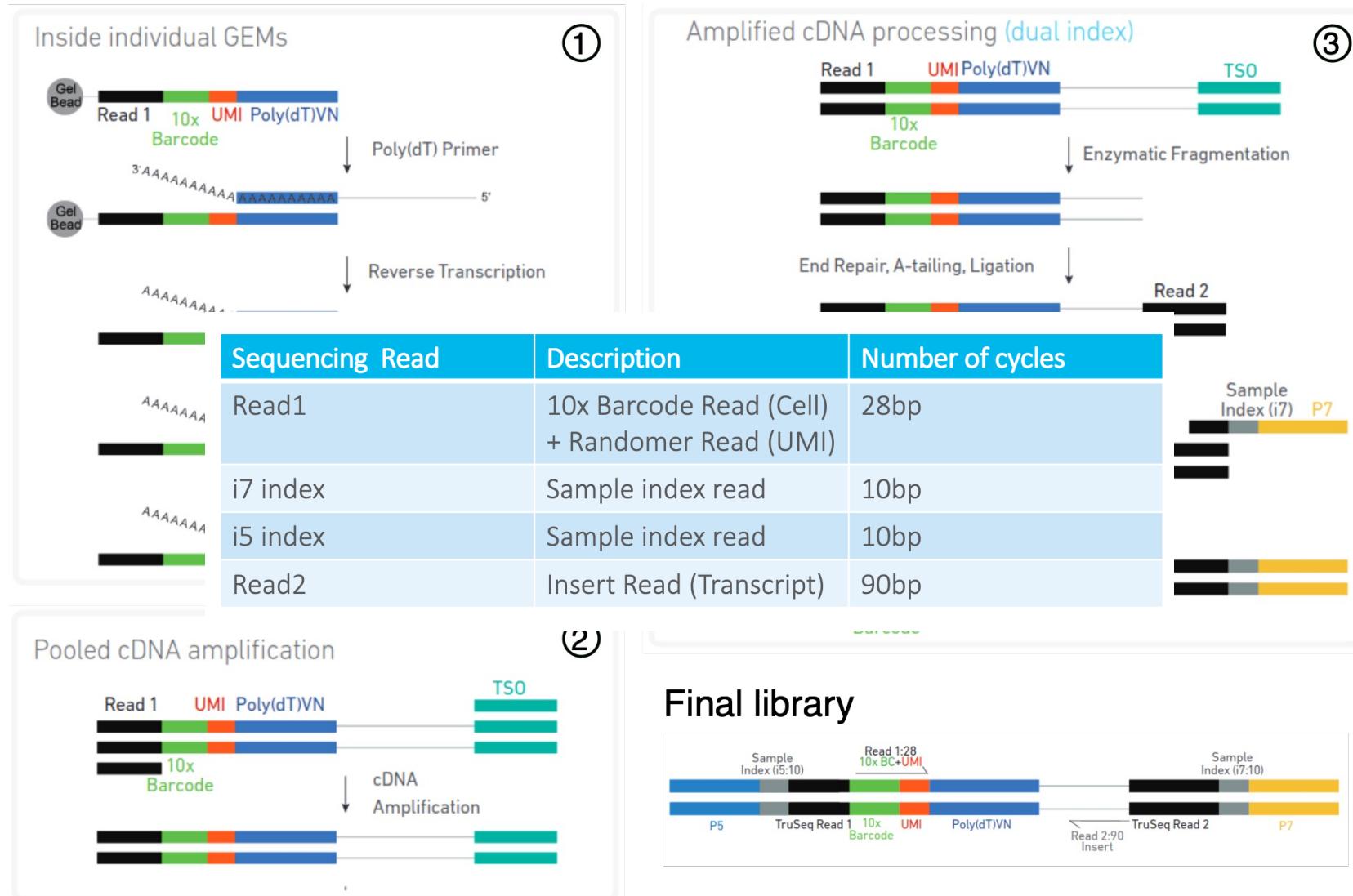
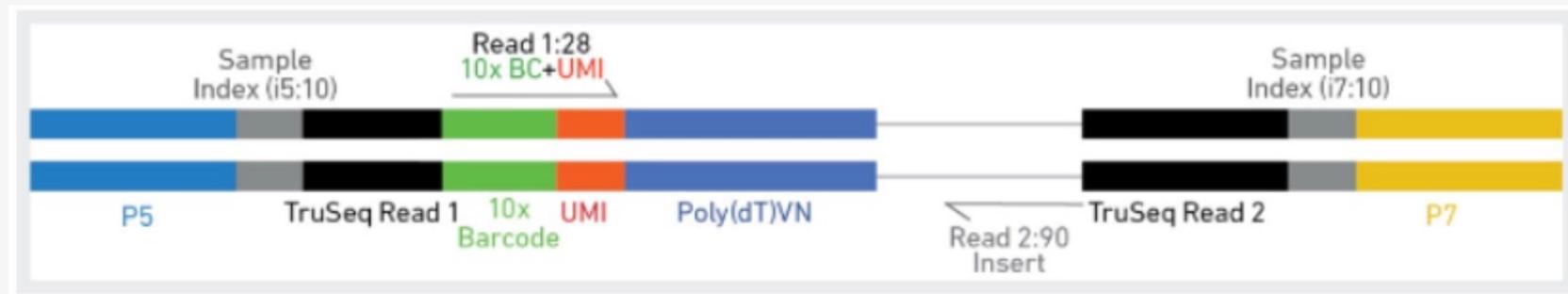


Image source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>



Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

### Single Cell 3' v3.1(Dual Index) Gene Expression Library:



### Single Cell 5' v2 Gene Expression Library:



Image Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

# Sequencing is imperfect: Barcodes must be checked and corrected

Chemistry	CB, bp	UMI, bp	Whitelist file
10x Chromium Single Cell 3' v1	14	10	737K-april-2014_rc.txt
10x Chromium Single Cell 3' v2	16	10	737K-august-2016.txt
10x Chromium Single Cell 3' v3	16	12	3M-february-2018.txt
10x Chromium Single Cell 3' v3.1 (Next GEM)	16	12	3M-february-2018.txt
10x Chromium Single Cell 5' v1.1	16	10	737K-august-2016.txt
10x Chromium Single Cell 5' v2 (Next GEM)	16	10	737K-august-2016.txt
10x Chromium Single Cell Multiome	16	12	737K-arc-v1.txt

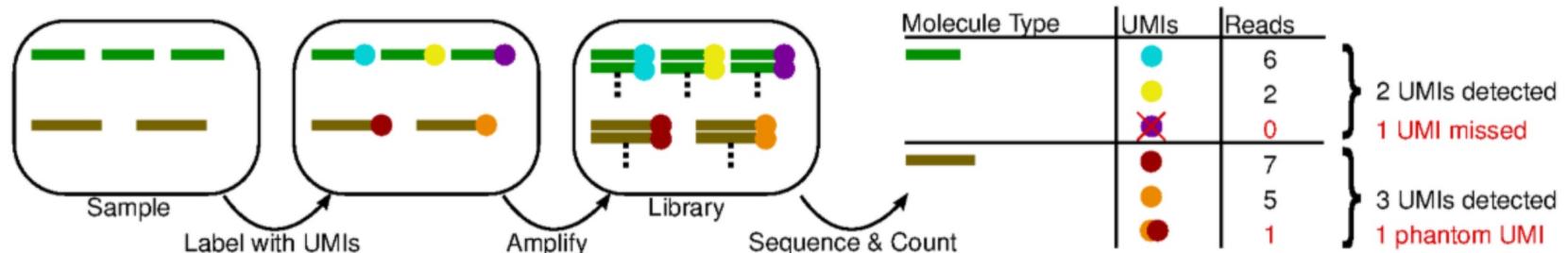
Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

# UMI – UNIQUE MOLECULAR IDENTIFIERS

After PCR enrichment, without UMIs, one can not distinguish if multiple copies of a fragment are caused by PCR clones or if they are real biological duplicates.

By using UMIs, PCR clones can be found by searching for non-unique fragment-UMI combinations, which can only be explained by PCR clones.

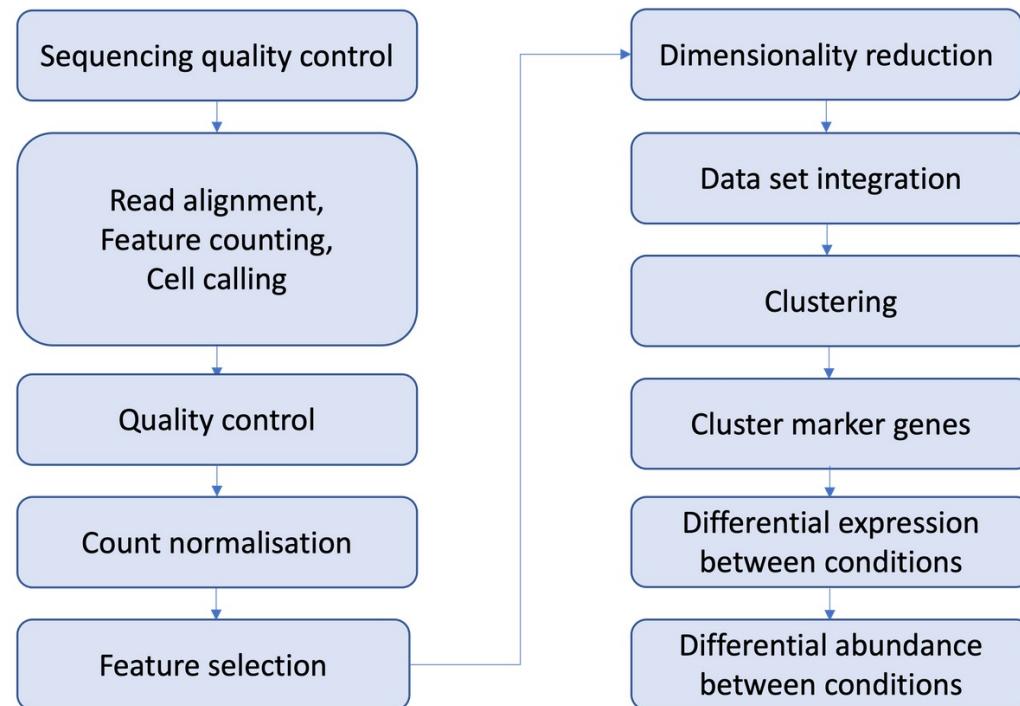
When performing variant analyses, these falsely overrepresented fragments can result in incorrect calls and thus wrong diagnostic findings



Source: Pflug et al. Bioinformatics (2018)

	Cellular barcode	UMI	
Cell 1	TTGCCGTGGTGT	GGCGGGGA.....	CGGTGTTA ] <i>DDX51</i>
	TTGCCGTGGTGT	TATGGAGG.....	CCAGCACC ] <i>NOP2</i>
	TTGCCGTGGTGT	TCTCAAGT.....	AAAATGGC ] <i>ACTB</i>
Cell 2	CGTTAGATGGCA	GGGCCGGG.....	CTCATAGT ] <i>LBR</i>
	CGTTAGATGGCA	ACGTTATA.....	ACGCGTAC ] <i>ODF2</i>
	CGTTAGATGGCA	TCGAGATT.....	AGCCCTTT ] <i>HIF1A</i>
Cell 3	AAATTATGACGA	AGTTTGTA.....	GGGAATTA ] <i>ACTB</i> ← 2 reads, 1 molecule
	AAATTATGACGA	AGTTTGTA.....	AGATGGGG ]
	AAATTATGACGA	TGTGCTTG.....	GACTGCAC ] <i>RPS15</i>
Cell 4	GTTAACGTACC	CTAGCTGT.....	GATTTCT ] <i>GTPBP4</i>
	GTTAACGTACC	GCAGAAAGT.....	GTTGGCGT ] <i>GAPDH</i>
	GTTAACGTACC	AAGGCTTG.....	CAAAGTTC ] <i>ARL1</i> ← 2 reads, 2 molecules
	GTTAACGTACC	TTCCGGTC.....	TCCAGTCG ]
(Thousands of cells)			

# Typical Computational Workflow



# Alignment and assignment depend upon gene knowledge (GFF/GTF)

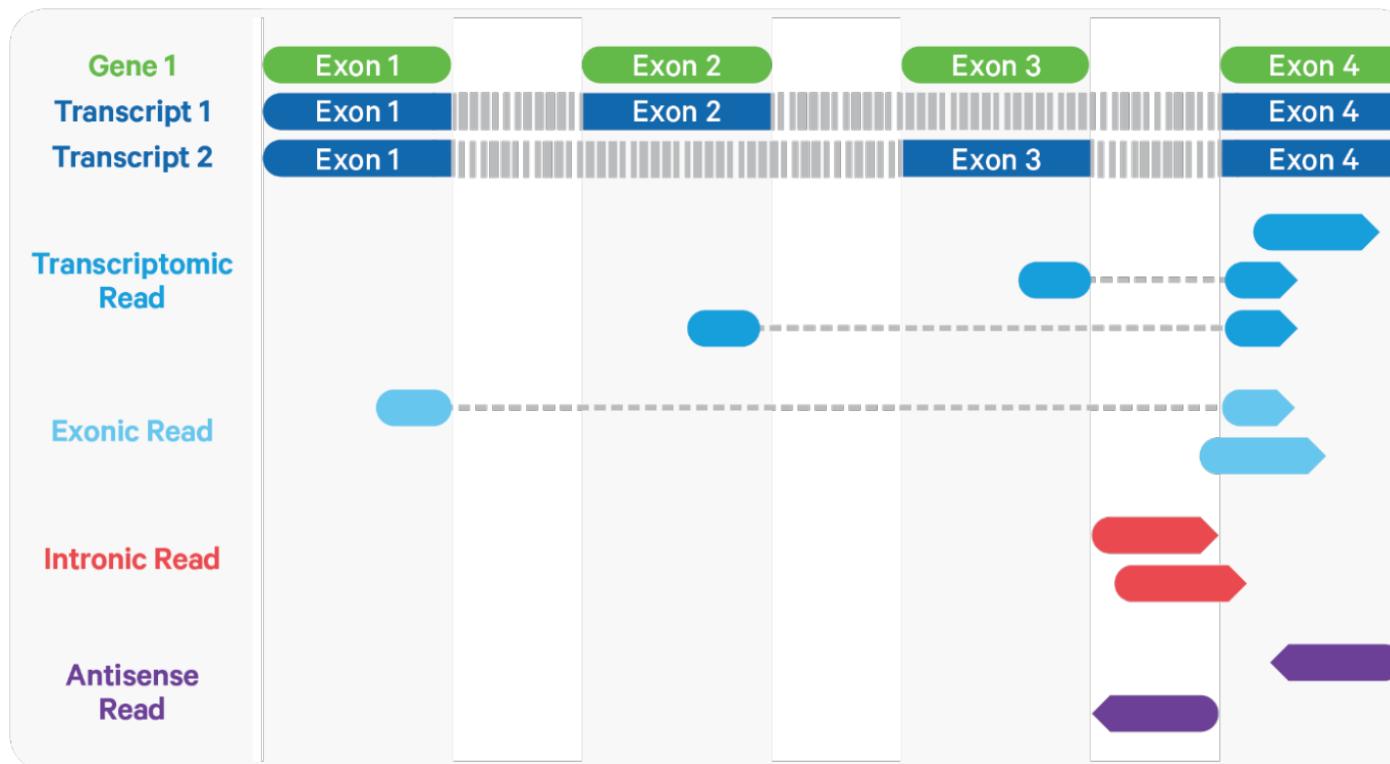


Image Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

# Typical Chromium Gene Coverage plots

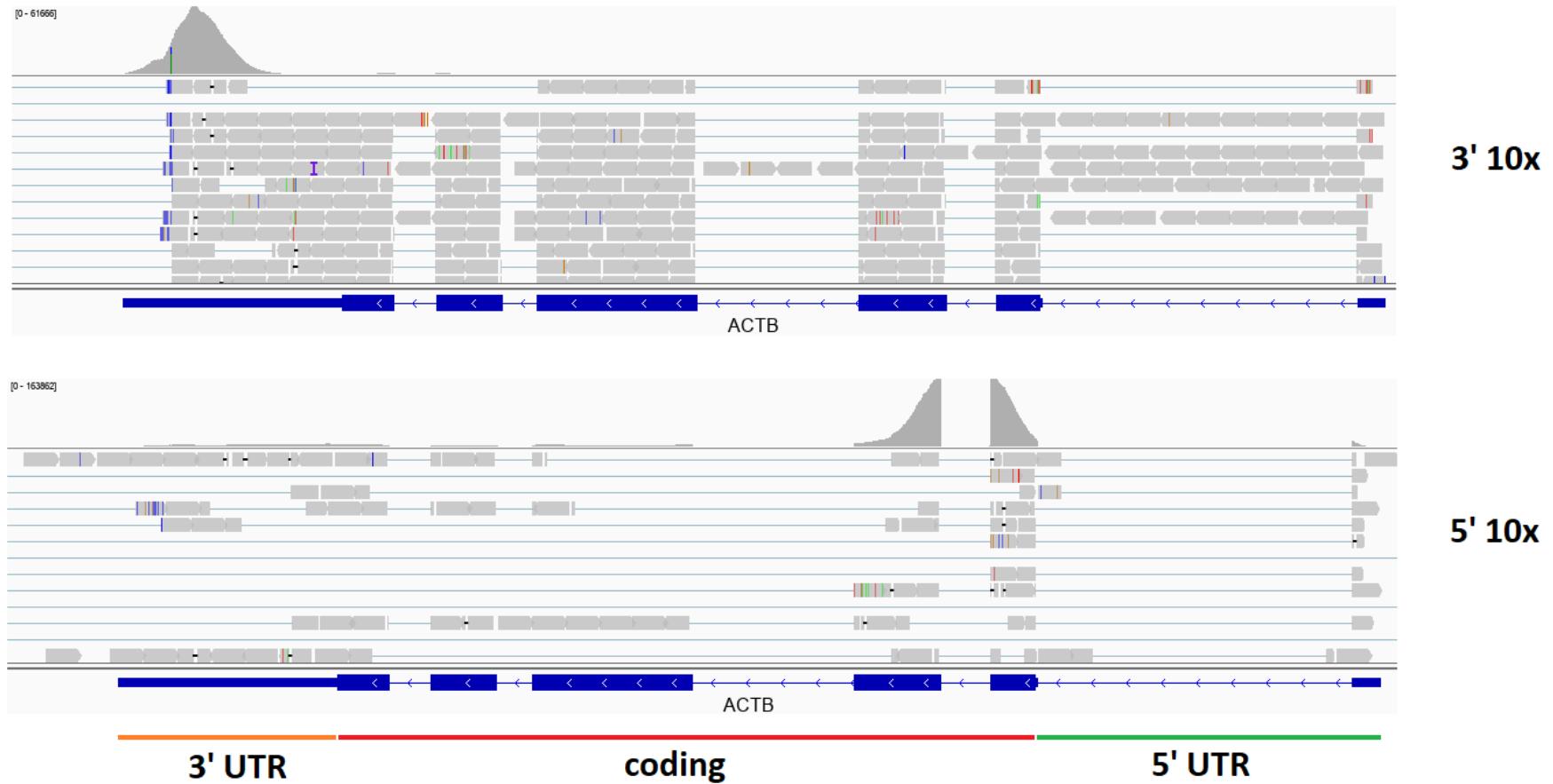


Image Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

## Not every droplet is useable



A single happy cell in a droplet is ideal

- Complex transcriptome
- Average number of genes detected



Empty droplet: No cell in a droplet

- No genes detected



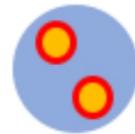
Droplet with ambient RNA

- Low complex transcriptome
- Genes detected much lower than average genes per cell



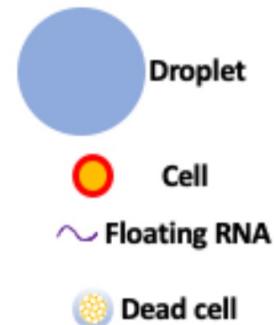
Droplet with dead cell

- Enriched for mitochondrial genes



Droplet with multiple cell

- Very complex transcriptome
- Genes detected much higher than average genes per cell



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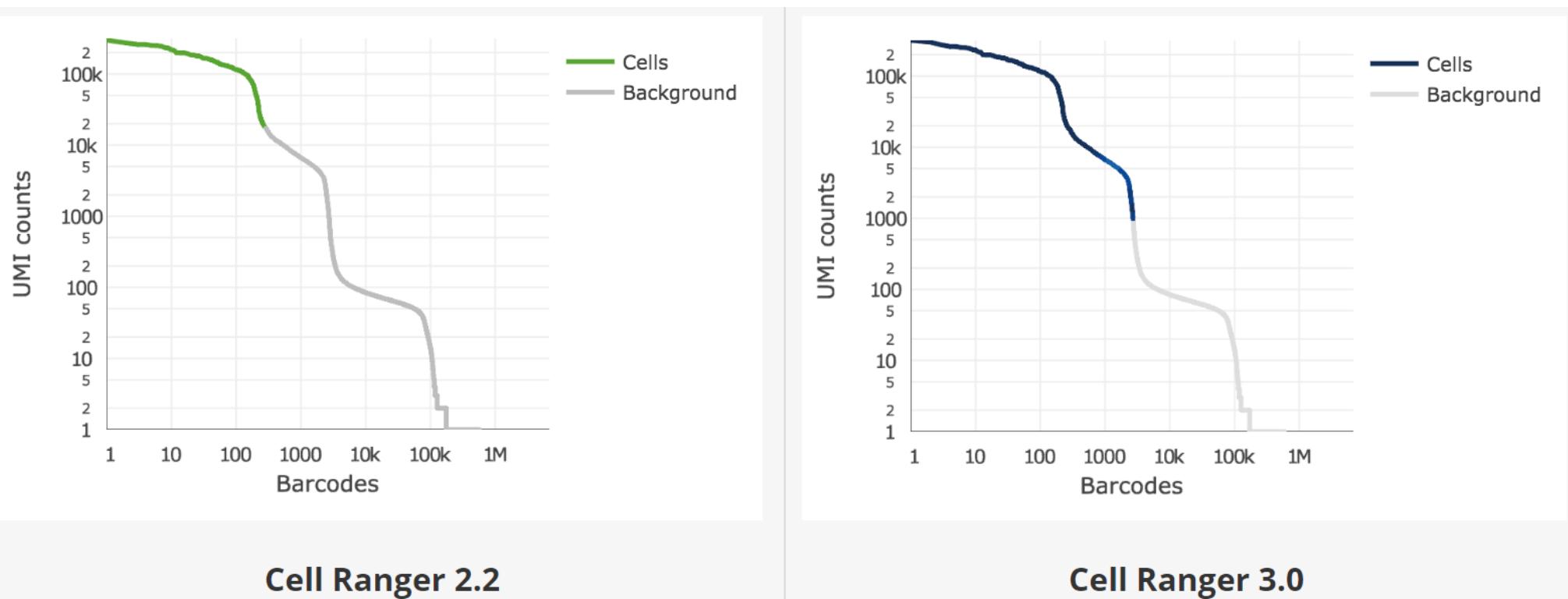


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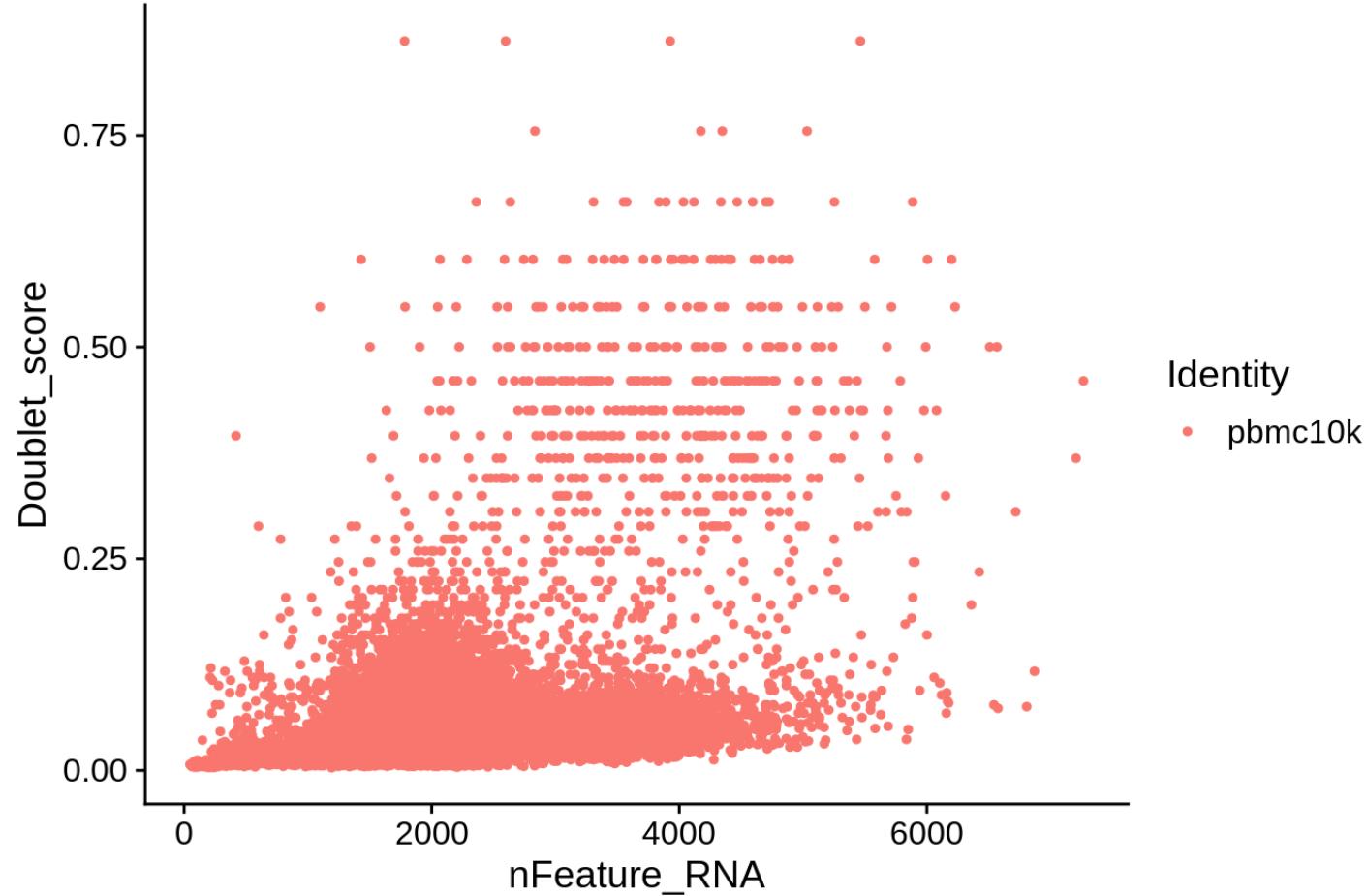
9/13

Processing tools will catch some of the problematic barcodes for you



Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

## Typical OC data can help ID problem data



Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

# Finally- you have (sparse) count matrix(es)

- CellRanger outputs: gives two output folders raw and filtered
- Each folder has three zipped files
  - features.tsv.gz, barcodes.tsv.gz and matrix.mtx.gz
  - raw\_feature\_bc\_matrix
    - All valid barcodes from GEMs captured in the data
    - Contains about half a million to a million barcodes
    - Most barcodes do not actually contain cells
  - filtered\_feature\_bc\_matrix
    - Excludes barcodes that correspond to this background
    - Contains valid cells according to 10x cell calling algorithm
    - Contains 100s to 1000s of barcodes

```
%h%-$ ls SRR9264343/outs/raw_feature_bc_matrix  
barcodes.tsv.gz  
features.tsv.gz  
matrix.mtx.gz
```



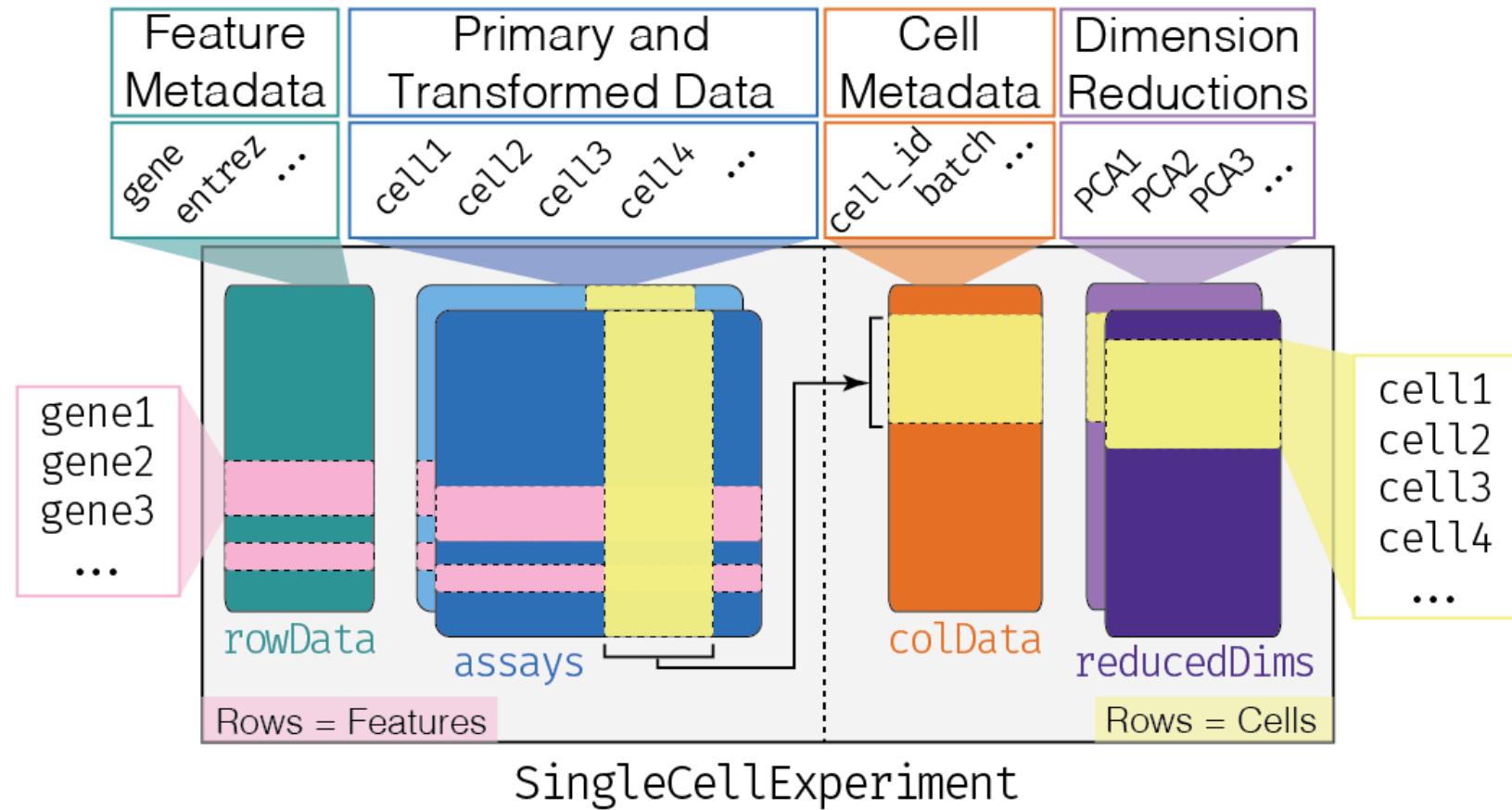
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# Computation tools have specialized data structures for single-cell analysis

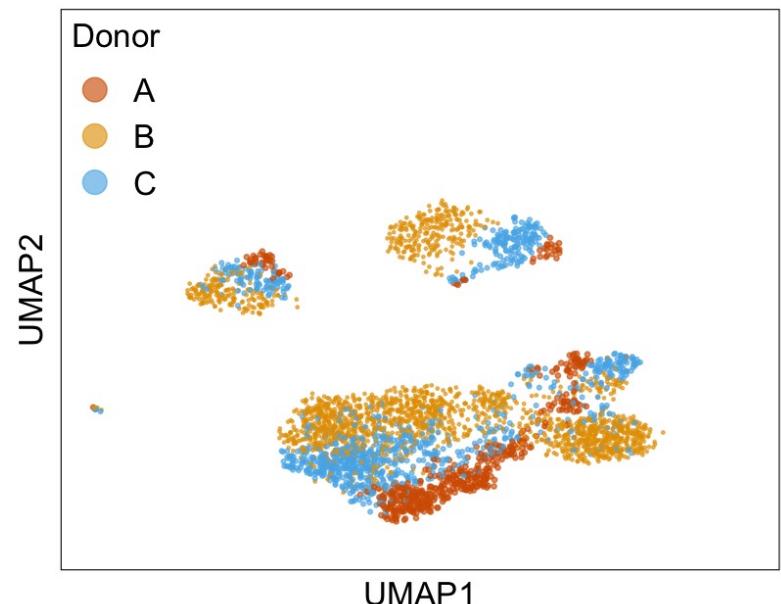
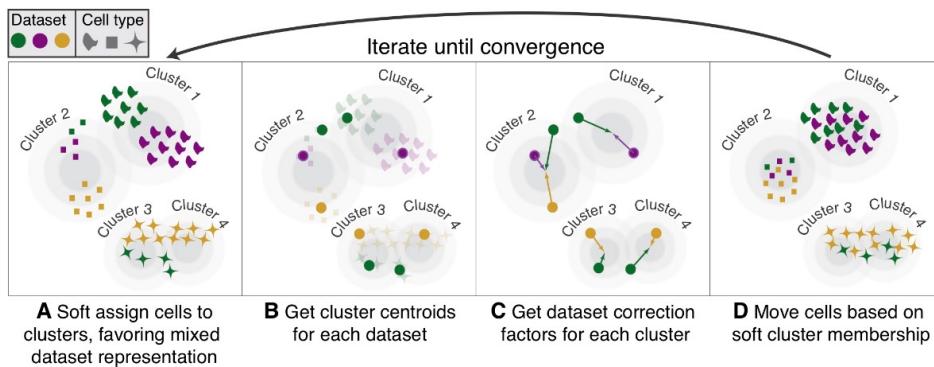


## Following QC reduction, issues can still remain

- Common systematic effects that can obscure biological effects
  - Batch effects
  - Cell cycle

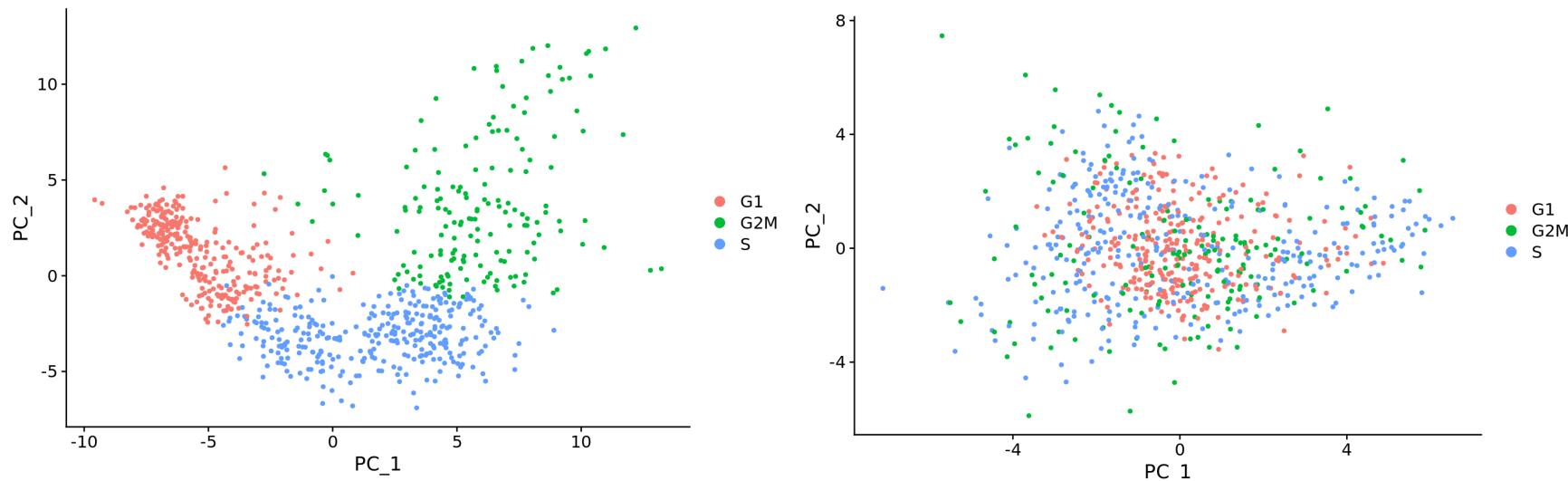
# Harmony

- **Harmony** : An algorithm that projects cells into clusters based on their cell identity rather than dataset specific conditions.
- Harmony applies a transformation to the principal component values. The algorithm then determines if there is a balanced quantity of cells from each batch within the clusters. Each cell is then evaluated to see how much its batch identity influences its PC coordinates. The cells position is corrected by shifting it towards the centroid of its cluster.



# Cell cycle has a systematic (but known) measurable effect on expression patterns

- Characteristic genes have been identified
- Cells can be modeled to estimate cell cycle stage

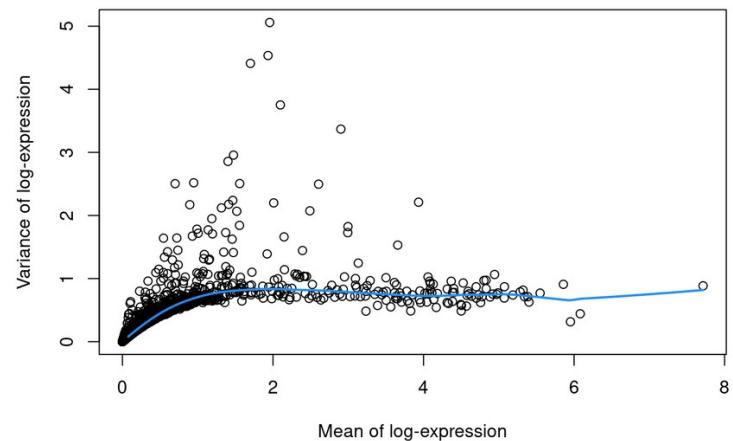


Source: [https://satijalab.org/seurat/archive/v3.1/cell\\_cycle\\_vignette.html](https://satijalab.org/seurat/archive/v3.1/cell_cycle_vignette.html)

# Which genes should we use for downstream analysis?

Select genes which capture biologically-meaningful variation, while reducing the number of genes which only contribute to technical noise

(Image Source)



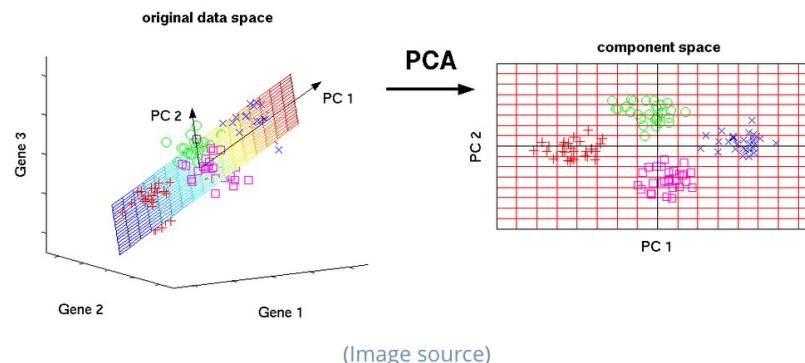
- Model the gene-variance relationship across all genes to define a data-driven “technical variation threshold”
- Select **highly variable genes (HVGs)** for downstream analysis (e.g. PCA and clustering)

# Why do high-dimensional data pose a problem?

In single-cell data we typically have thousands of genes across thousands (or millions!) of cells.

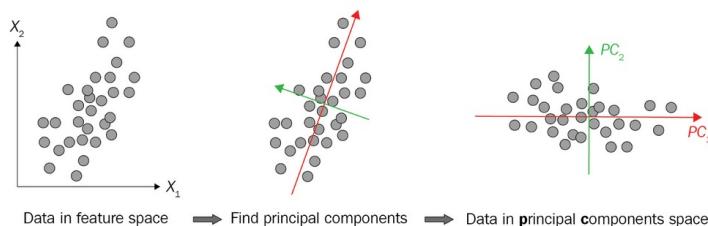
- Interpretation/visualisation beyond 2D is hard.
- As we increase the number of dimensions, our data becomes more sparse.
- High computational burden for downstream analysis (such as cell clustering)

Solution: collapse the number of dimensions to a more manageable number, while preserving information.



# All downstream analysis is based on “reduced” data

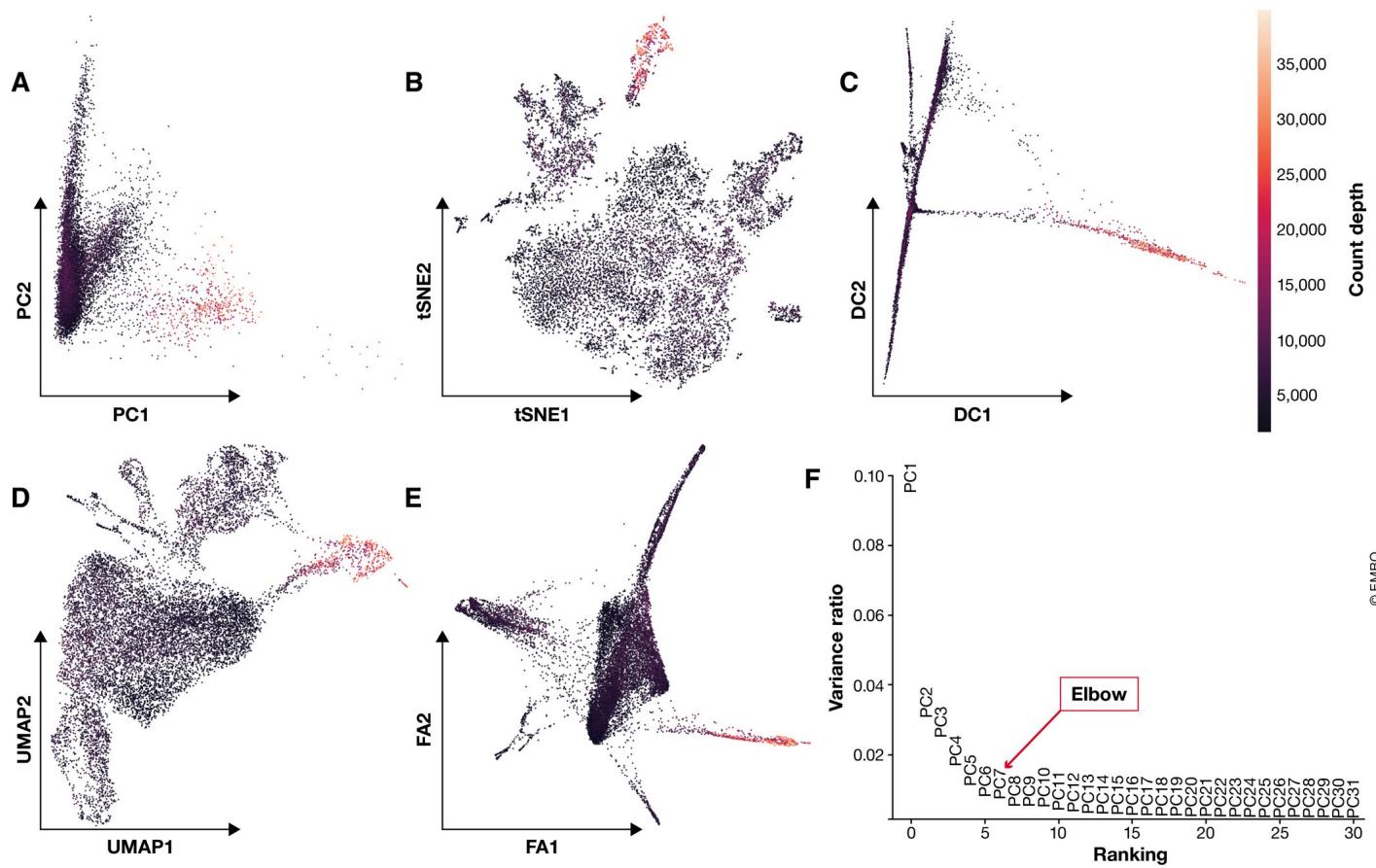
## Principal Components Analysis (PCA)



(Image Source)

- It's a linear algebraic method of dimensionality reduction
- Finds principal components (PCs) of the data
  - Directions where the data is most spread out (highest variance)
  - PC1 explains most of the variance in the data, then PC2, PC3, etc.
  - PCA is primarily a dimension reduction technique, but it is also useful for visualization
  - A good separation of dissimilar objects is provided
  - Preserves the global data structure

# Common visualization options



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## **Clustering and Biology: What do you want to learn from the experiment?**

- Classify cells and discover new cell populations
- Compare gene expression between different cell populations
- Reconstruct developmental 'trajectories' to reveal cell fate decisions of distinct cell subpopulations



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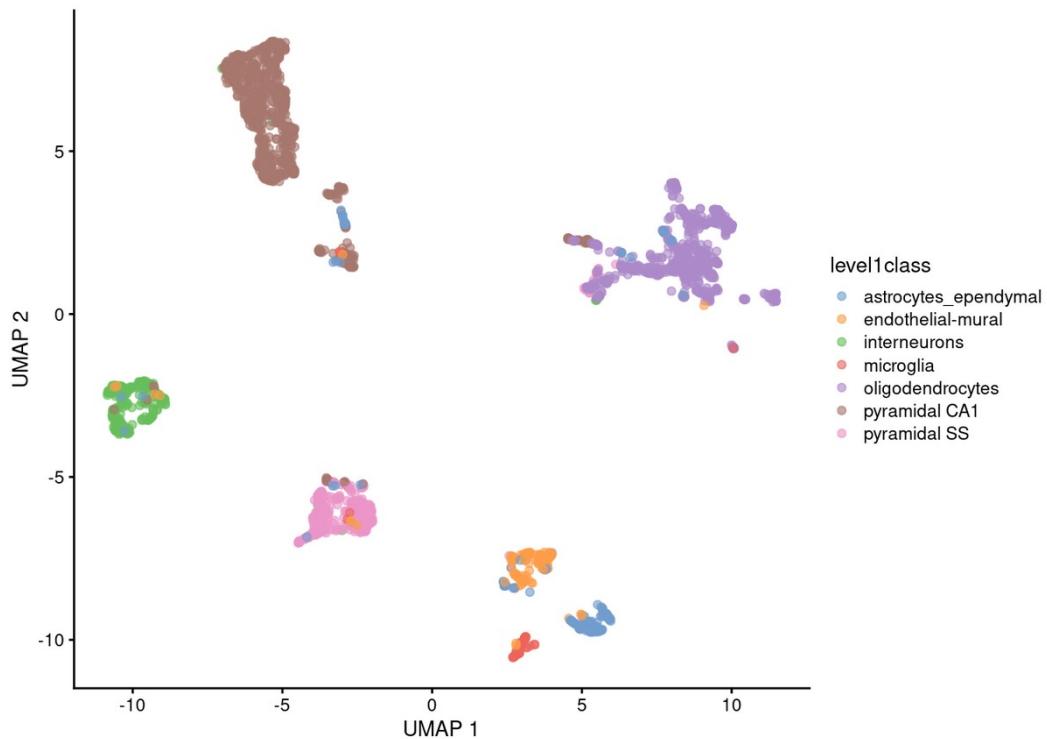
# UMAP

Main parameter in UMAP is **n\_neighbors** (the **number of neighbours** used to construct the initial graph).

Another common parameter is **min\_dist** (minimum distance between points)

- Together they determine balance between preserving local vs global structure
- For practical simplicity, we usually only tweak **n\_neighbors**, although playing with both parameters can be beneficial

Exploring different number of neighbours that best represent the biological diversity of cells is recommended.



## Is there a “correct” clustering?

Clustering, like a microscope, is a tool to explore the data.

We can zoom in and out by changing the resolution of the clustering parameters, and experiment with different clustering algorithms to obtain alternative perspectives on the data.

Asking for an unqualified “best” clustering is akin to asking for the best magnification on a microscope.

A more relevant question is “how well do the clusters approximate the cell types or states of interest?”. Do you want:

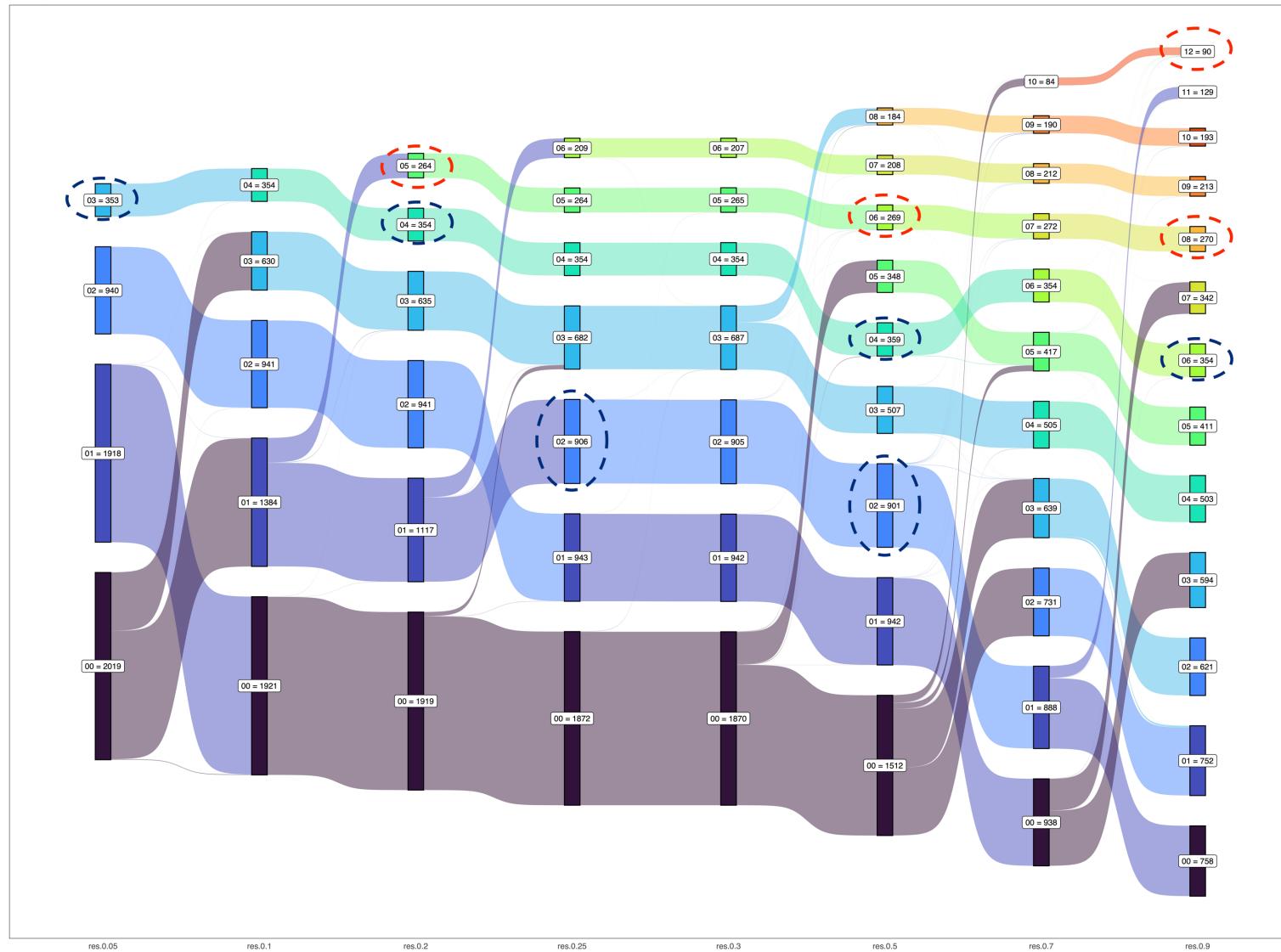
- resolution of the major cell types?
- Resolution of subtypes?
- Resolution of different states (e.g., metabolic activity, stress) within those subtypes?

Explore the data, use your biological knowledge!

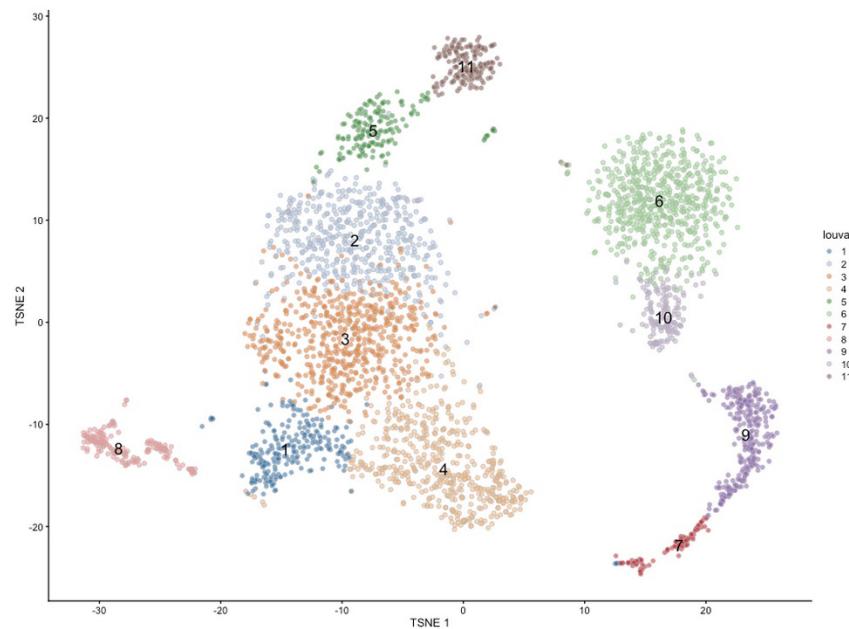


Image by Les Chatfield from Brighton, England - Fine rotative table Microscope 5, CC BY 2.0, <https://commons.wikimedia.org/w/index.php?curid=32225637>

Sankey: Macro Subset Dataset



# Identifying Cluster Marker Genes



Our goal is to identify genes that are differently expressed between clusters

Calculate effect sizes that capture differences in:

- mean expression level
- rank of expression
- proportion of cells expressing the gene

These are calculated in pairwise cluster comparisons.



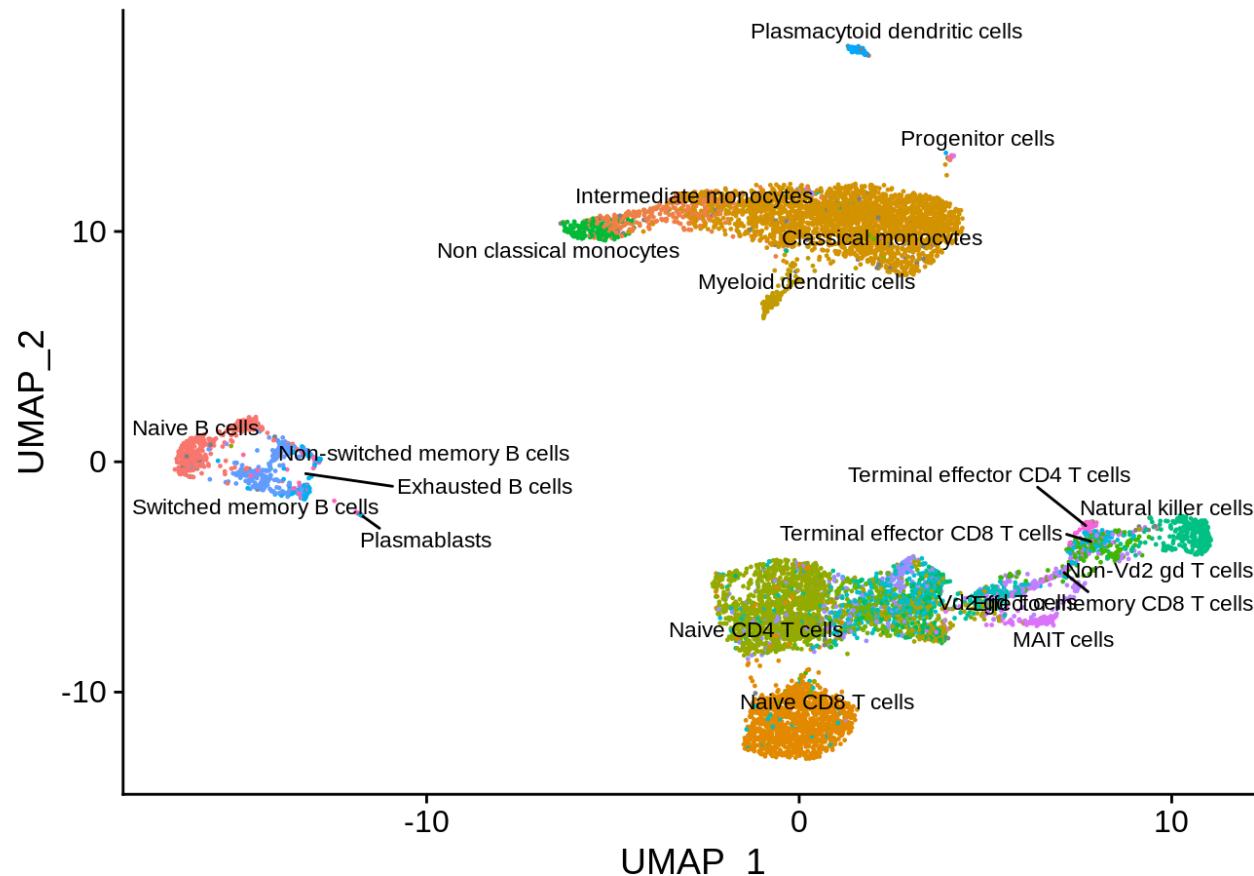
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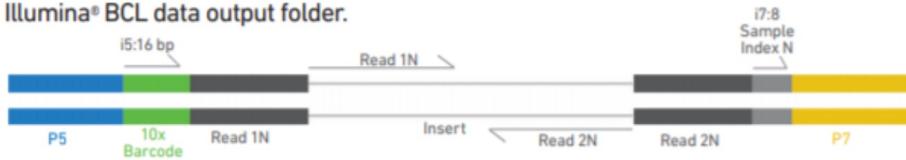
# Cell identification has generally been based upon known marker genes



Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

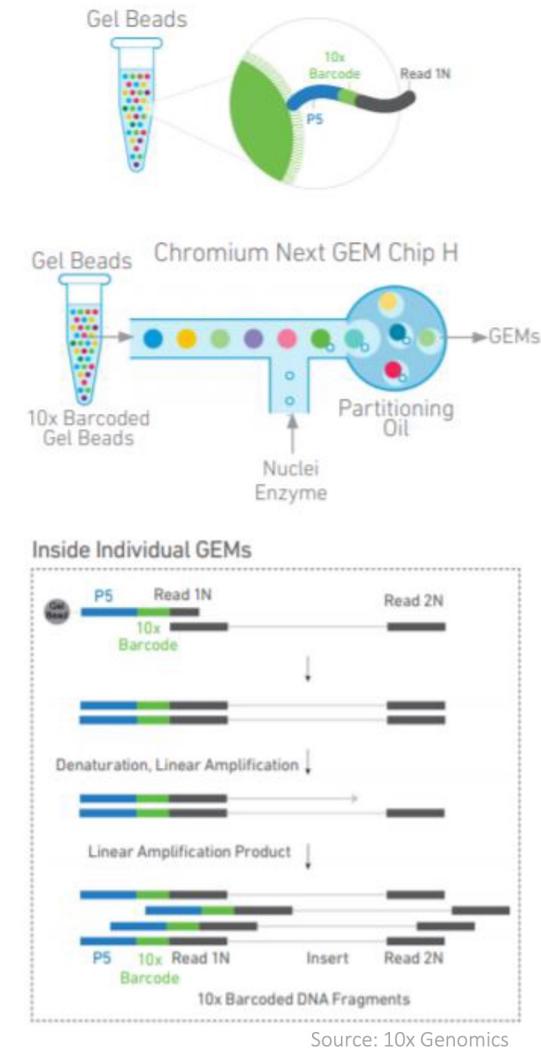
# 10X ATAC

Chromium Single Cell ATAC libraries comprise double stranded DNA fragments which begin with P5 and end with P7. Sequencing these libraries produces a standard Illumina® BCL data output folder.



Sequencing Read	Description	Number of cycles
Read1	Insert Sequence 1N	50bp
i7 index	Sample index read	8bp
i5 index	10x Barcode Read (Cell)	16bp
Read2	Insert Sequence 2N (opposite end)	50bp

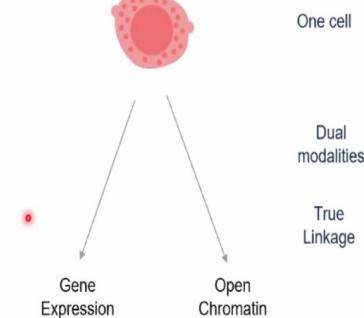
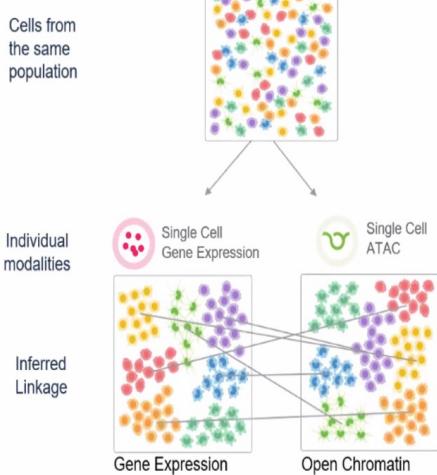
- ASAP-seq is to scATAC-seq what CITE-seq is to scRNA-seq.
- Scale Biosciences – ‘pre-indexing of nuclei through fragmentation’ = 100k nuclei per 10x channel with low number of doublets



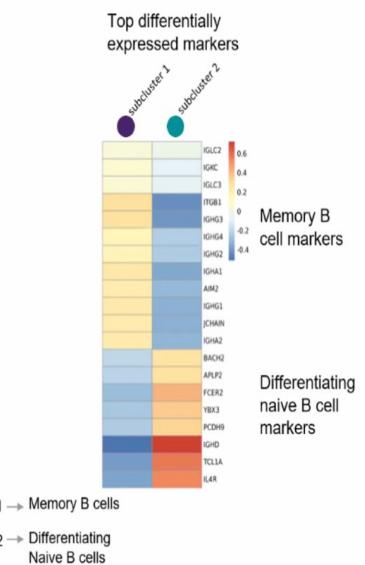
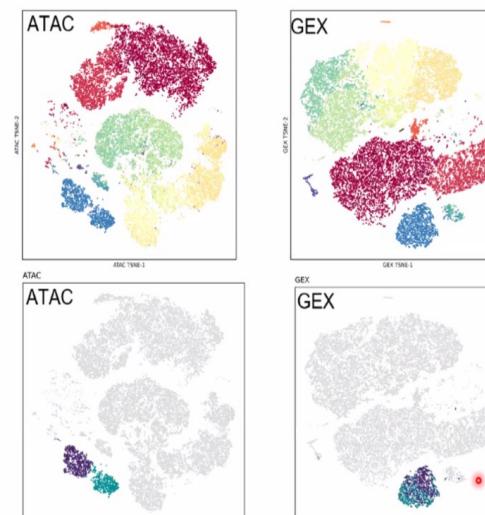
Source: 10x Genomics

# ● 10X MULTIOME (RNA+ATAC)

Profiling Different Modalities To Gain Deeper Insights    Dive Deep Where It Matters

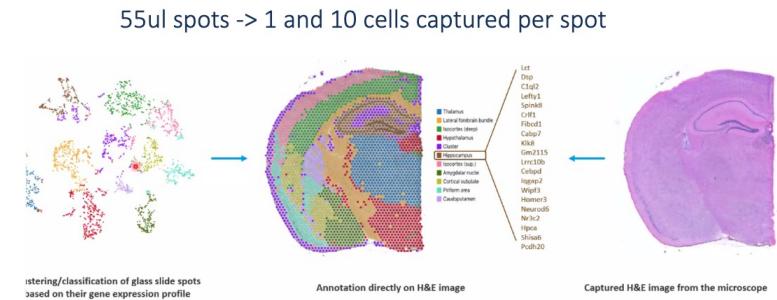
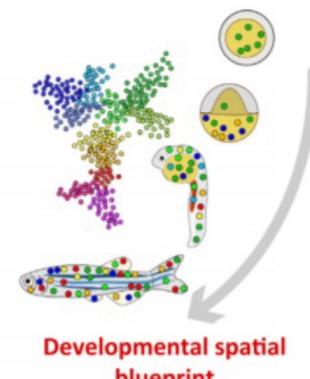
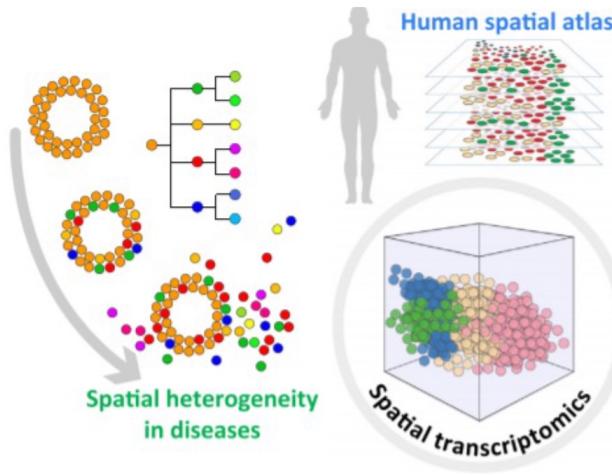


One cell  
Dual modalities  
True Linkage



Source: 10x Genomics

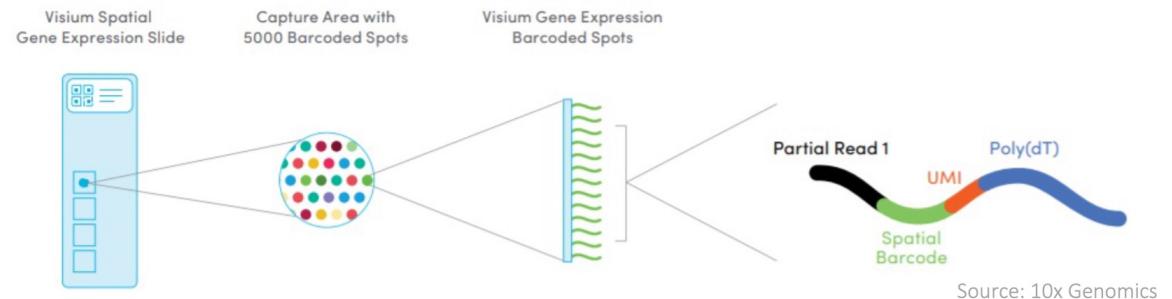
# ● SPATIAL TRANSCRIPTOMICS



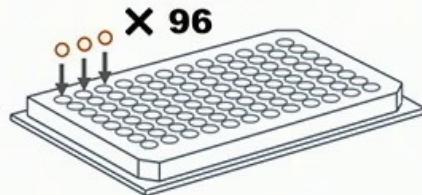
*Trends in Biotechnology*

**Figure 3. Applications for Spatially Resolved Transcriptomics.** Three primary kinds of hot issues can be resolved by spatially resolved transcriptomics: left, discovering spatial heterogeneity of diseases; middle, establishing spatial transcriptome atlases for the human body; and right, delineating an embryonic developmental and spatial blueprint.

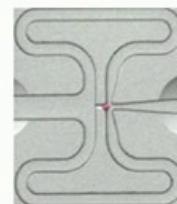
Source: Liao et al. *Trends in Biotechnology*. (2020)



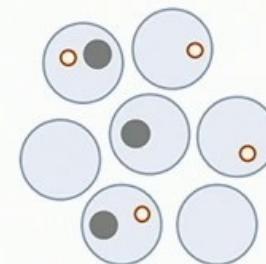
## Most single cell methods rely on physical compartmentalization of cells and barcoding primers



Single cell / single well



Microfluidics Chip  
(e.g. Fluidigm)

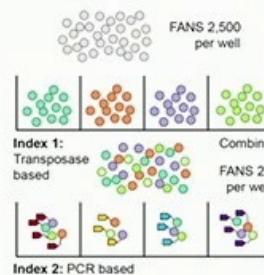


Droplet-based  
(e.g. 10X & BioRad)



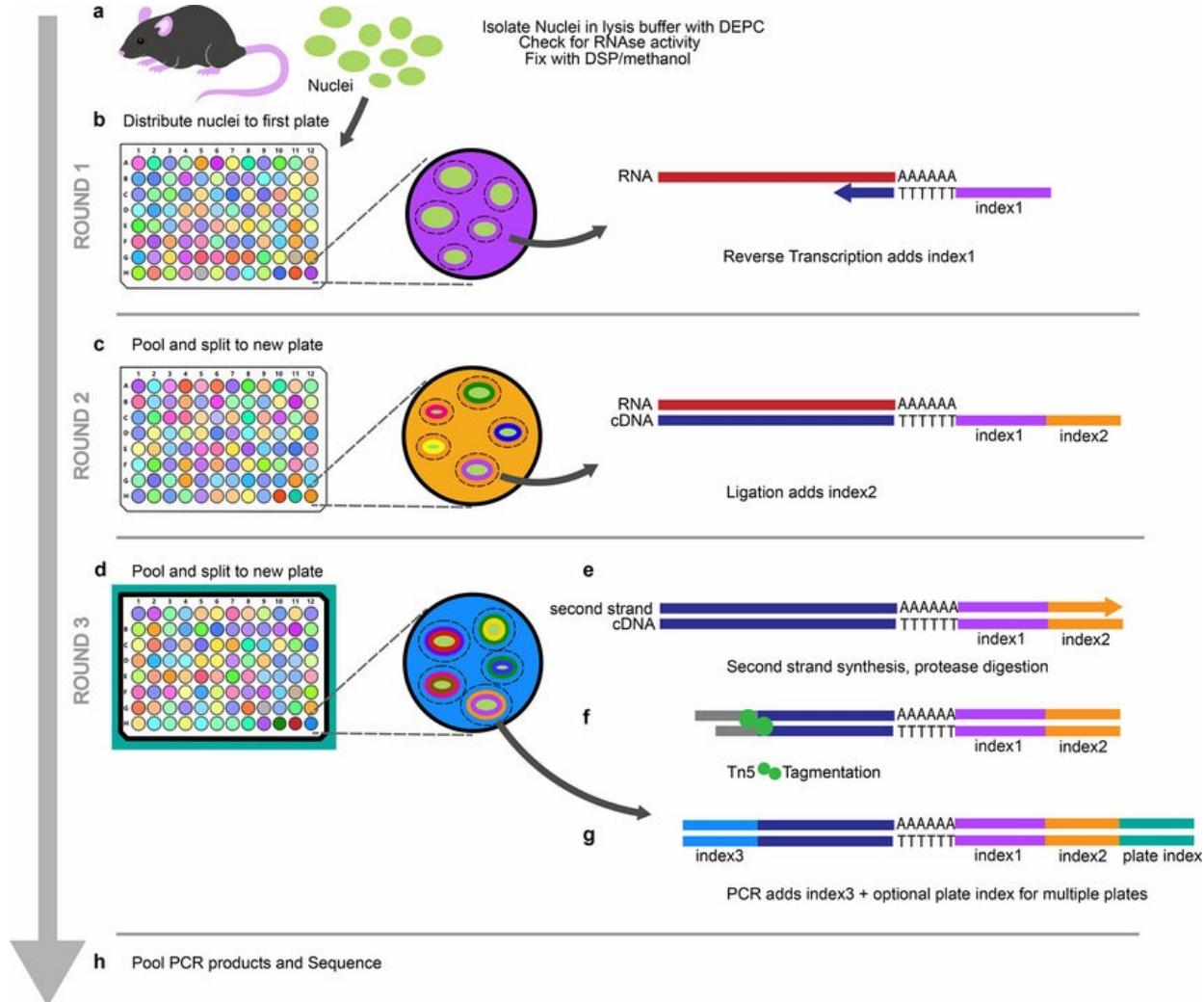
Microwell-based  
(e.g. Takara, CelSee)

## Single-cell combinatorial indexing – an alternative means of high throughput single-cell analysis.

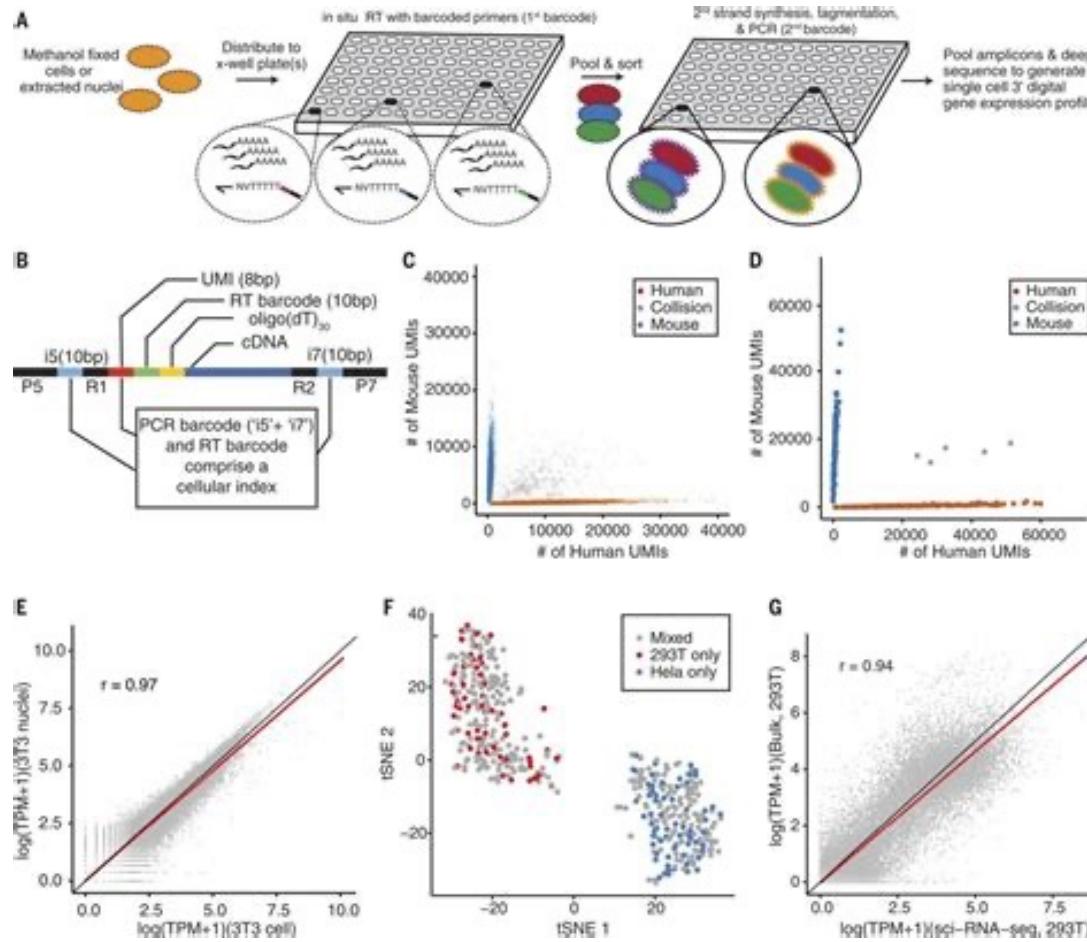


- Combinatorial scaling of throughput
- No specialized equipment required
- Adaptable to a variety of properties
- Inexpensive to perform

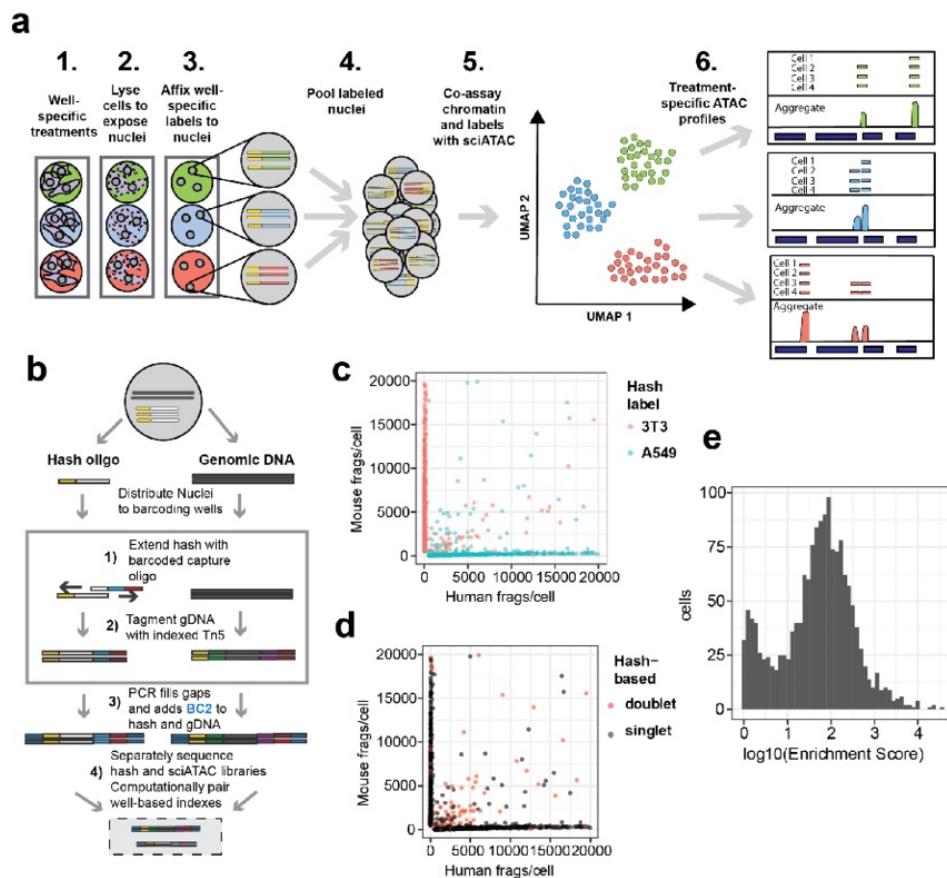
# Combinatorial indexing allows for great cost reduction per cell



# Combinatorial indexing allows for great cost reduction per cell



# Nuclear Oligo Hashing allows both sample labeling and improved normalization



# Questions?



# WHAT PLATFORM SHOULD I USE?

## Choose protocol based on:

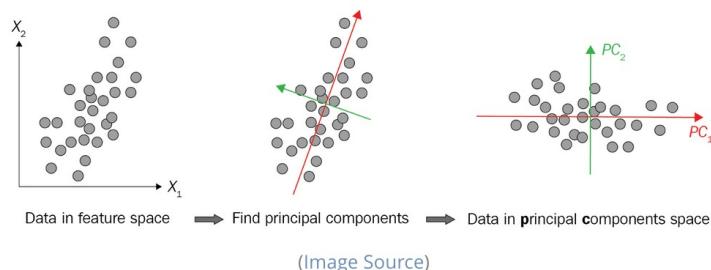
- Throughput (number of cells per reaction)
- Sample of origin
- Cost / Labour / Time limitations
- Gene body coverage: 5' / 3' biased or full-length?
- UMI vs no-UMI
- Sequencing depth per cell

## Examples:

- If you sample is fairly homogeneous – bulk RNAseq
- If your sample is limited in cell number – plate-based method
- If you want re-annotate the transcriptome and discover new isoforms – full-length coverage (SMART-seq2, seqWell)
- If you are looking to classify all cell types in a diverse tissue - high throughput
- If you have only archival human samples – nuclei isolation or 10x fixed RNA profiling

# All downstream analysis is based on PCA reduction of data

## Principal Components Analysis (PCA)



- When data is very highly-dimensional, we can select the most important PCs only, and use them for downstream analysis (e.g. clustering cells)
  - This reduces the dimensionality of the data from ~20,000 genes to maybe 20-50 PCs
  - Each PC represents a robust 'metagene' that combines information across a correlated gene set
- Prior to PCA we scale the data so that genes have equal weight in downstream analysis and highly expressed genes don't dominate

## Making a graph

Nearest-Neighbour (NN) graph:

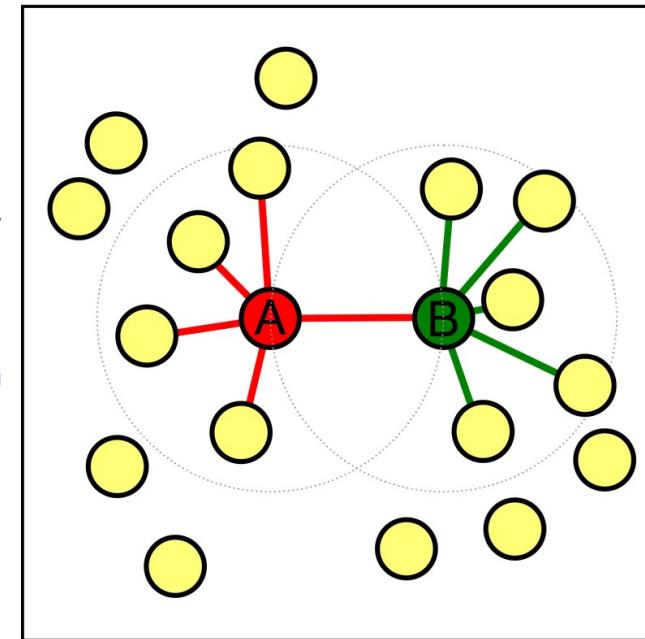
- cells as nodes
- their similarity as edges

In a NN graph two nodes (cells), say A and B, are connected by an edge if:

- the distance between them (in e.g. principal component space) is amongst the  $k$  smallest distances (here  $k = 5$ ) from A to other cells, (KNN)

or

- In a **shared-NN** graph (SNN) two cells are connected by an edge if any of their nearest neighbors are shared (n.b. in Seurat this is different)



Once edges have been defined, they can be weighted. By default the weights are calculated using the 'rank' method which relates to the highest ranking of their shared neighbours.

## Identifying communities/clusters - Louvain

Nodes are also first assigned their own community.

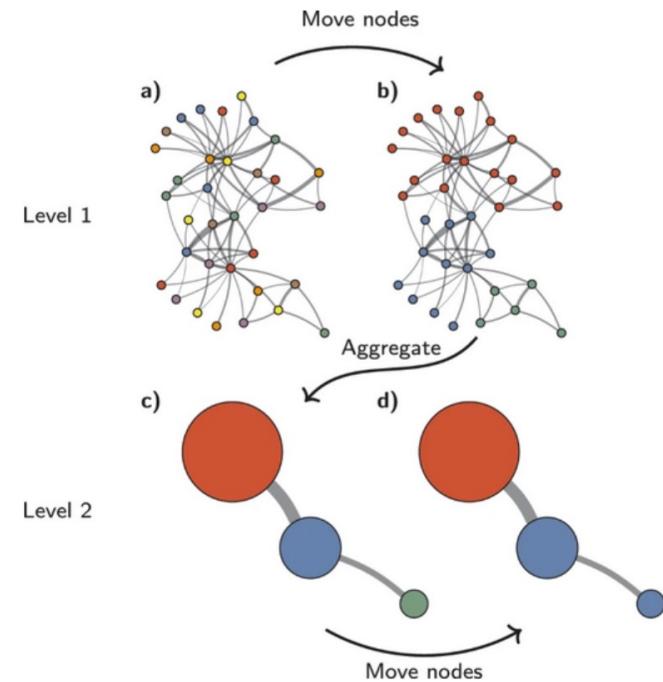
Two-step iterations:

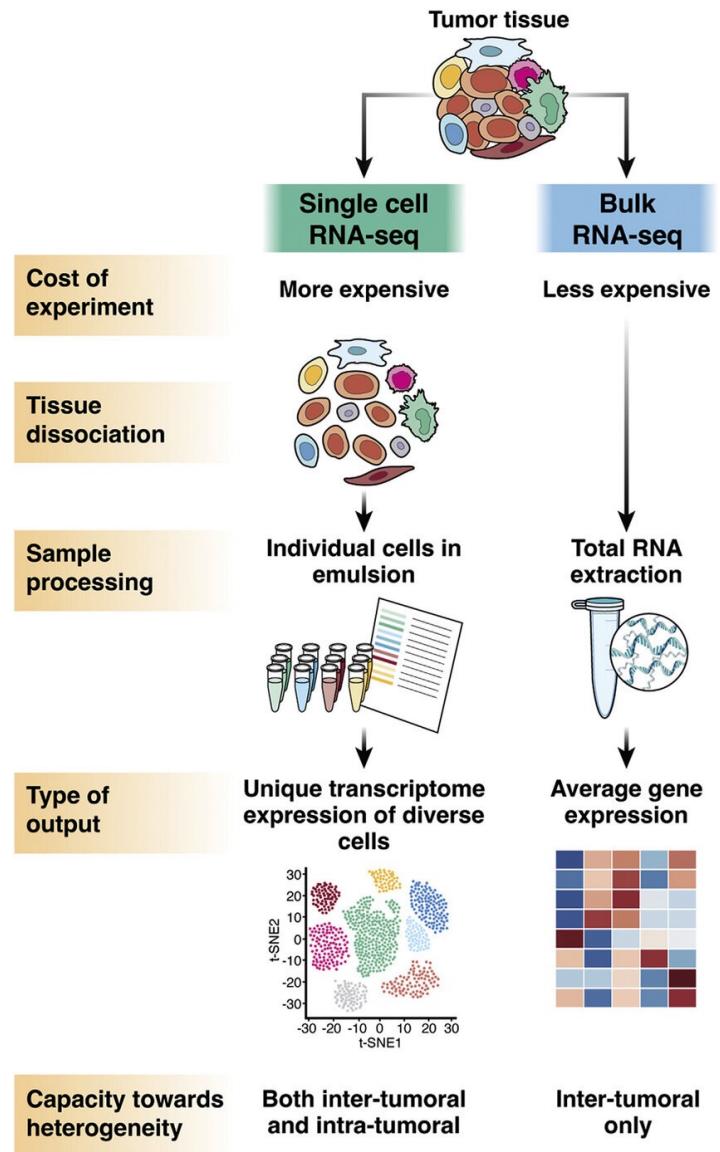
- nodes are re-assigned one at a time to the community for which they increase modularity the most,
- a new, 'aggregate' network is built where nodes are the communities formed in the previous step.

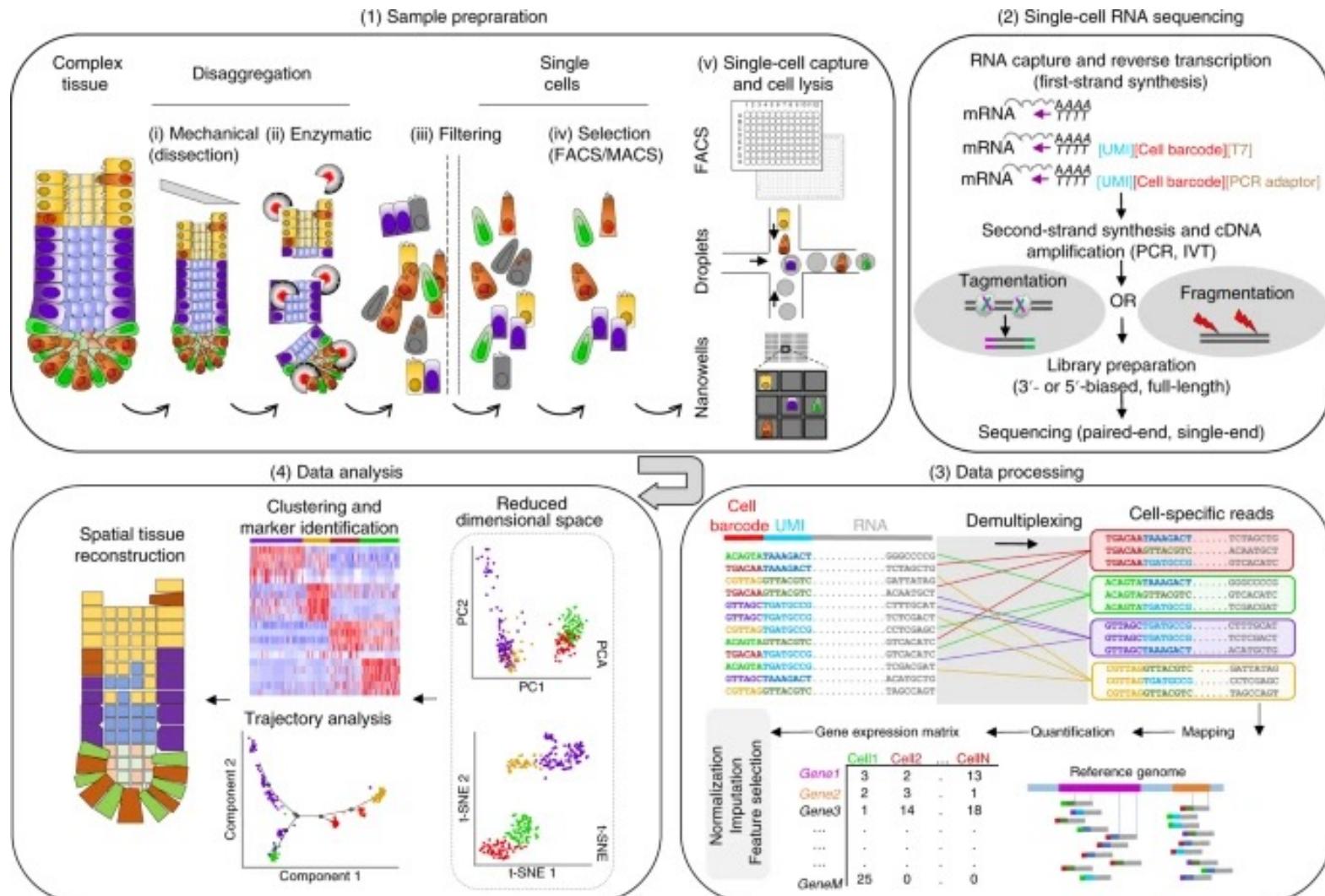
This is repeated until modularity stops increasing.

[\(Blondel et al, Fast unfolding of communities in large networks\)](#)

[\(Traag et al, From Louvain to Leiden: guaranteeing well-connected communities\)](#)

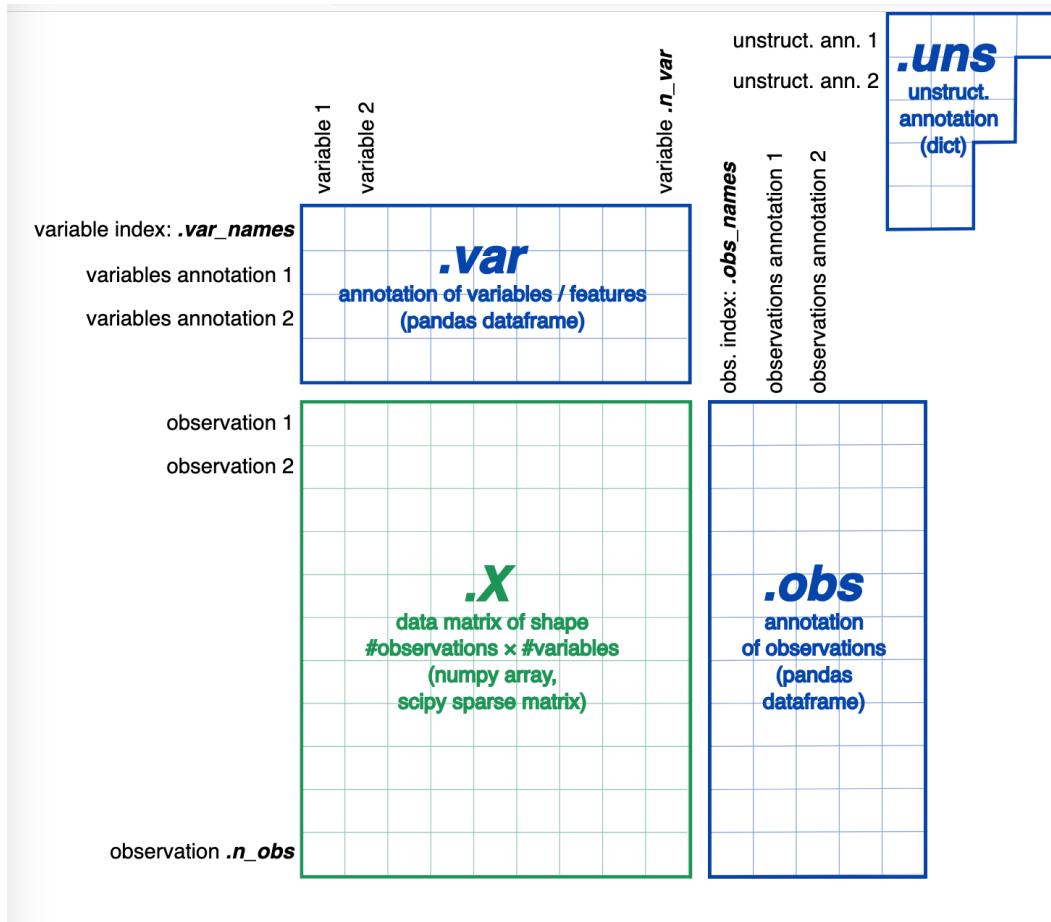






Source: <https://www.singlecellcourse.org/introduction-to-single-cell-rna-seq.html>

# Computation tools have specialized data structures for single-cell analysis

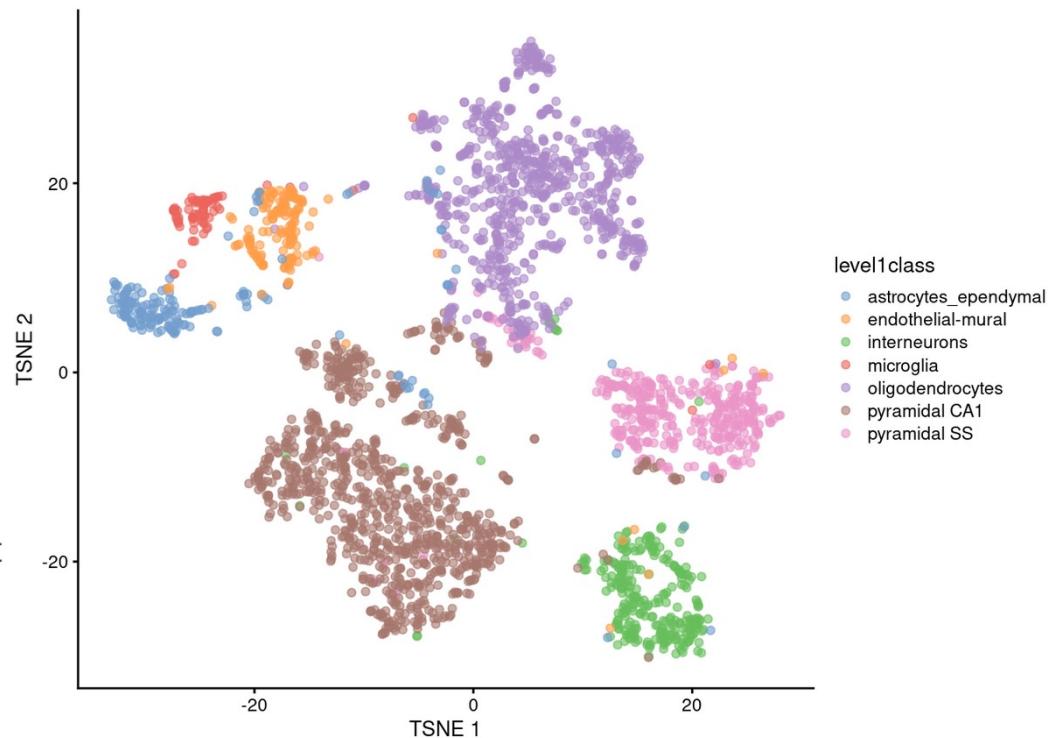


# t-SNE

Main parameter in t-SNE is the **perplexity** (~ number of neighbours each point is “attracted” to)

- Balance between preserving local vs global structure
- Higher values usually result in more compact clusters
- But too high can lead to overlap of clusters, making them harder to distinguish

Exploring different perplexity values that best represent the biological diversity of cells is recommended.



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RESEARCH  
UK

Cambridge  
Institute

# Quality control and filtering

- Quality control
  - Number of reads per cell
  - Number of genes detected per cell
  - Proportion of reads mapping to mitochondrial reads
- Remove cells with poor quality
  - Filter out cells with percentage of mitochondrial reads higher than a cut off
  - Filter out cells with less than a lower threshold on the number of genes or counts per cell
- Remove doublets (two cells captured with one bead in the droplet)
  - Filter out cells with more than an upper threshold on the number of genes or counts per cell in your data
  - More sophisticated way of removing doublets
    - <https://github.com/JonathanShor/DoubletDetection>
    - <https://github.com/AllonKleinLab/scrublet>
    - DoubletFinder

<https://www.sciencedirect.com/science/article/pii/S2405471219300730?via%3Dihub>



Source: [http://barc.wi.mit.edu/education/hot\\_topics/scRNAseq\\_2020/SingleCellRNAseq2020\\_4slidesPerPage.pdf](http://barc.wi.mit.edu/education/hot_topics/scRNAseq_2020/SingleCellRNAseq2020_4slidesPerPage.pdf)

