BIOINFORMATICS PIPELINE FOR SCRNA-SEQ: FROM RAW DATA TO INSIGHTS

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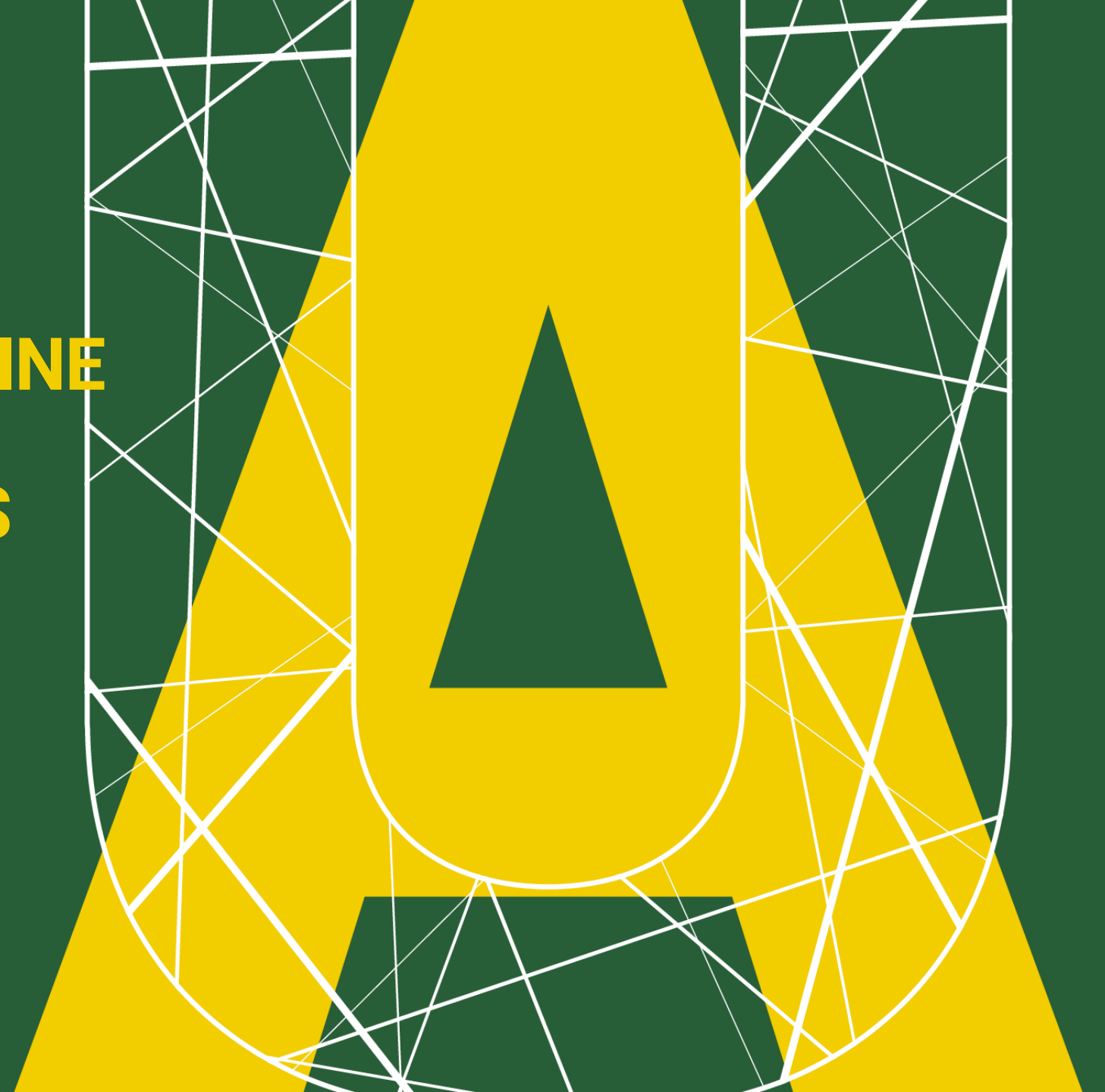


Outline

- Introduction
- Input Data preprocessing
- QC
- Normalized expression
- Clustering
- Marker genes and cell-type annotation

Objectives

- Understand the principles and workflow of single-cell RNA sequencing (scRNA-seq)
- Learn the importance of quality control in scRNA-seq and the rationale behind it
- Gain hands-on experience running one of the most widely used analysis pipelines

Note

- The slides can be found in Github:
  https://github.com/ualberta-rcg/scRNA-seq

- Apptainer container and sample FASTQ:
  https://drive.google.com/drive/folders/18vXOcOPEPUGW85fM6ZbCxKQJQuHnanQD

- Workshop Cluster
  https://tinyurl.com/UofA-scRNA-seq

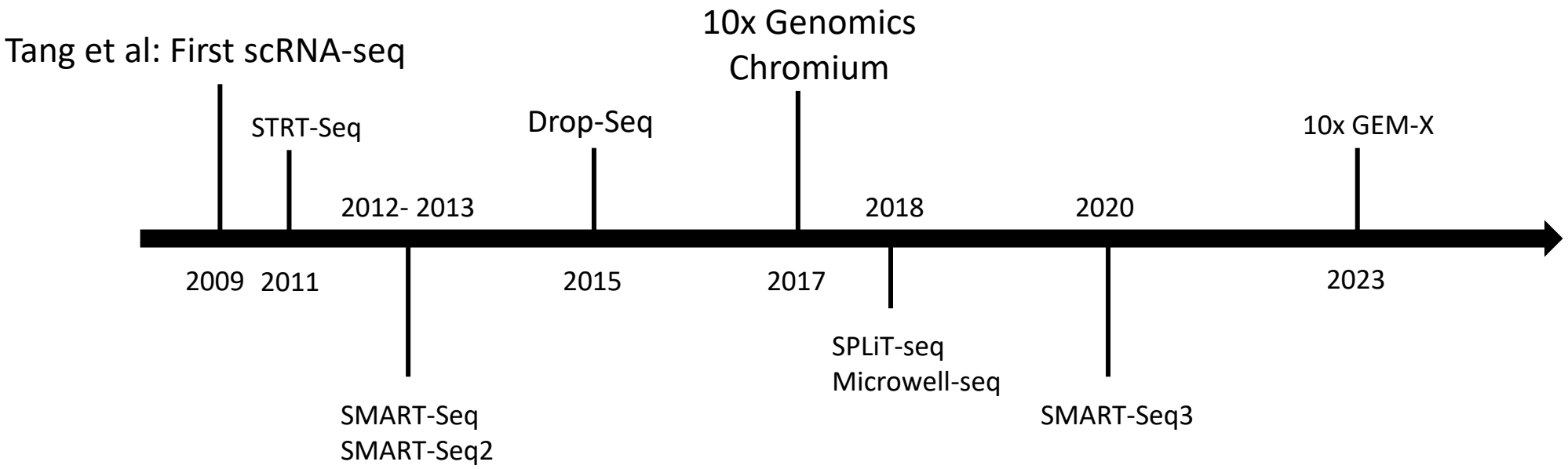
Login by ssh

- Our workshop cluster

```
ssh user000@fall2025-uofa.c3.ca
```

Introduction

The Milestone of scRNA-seq



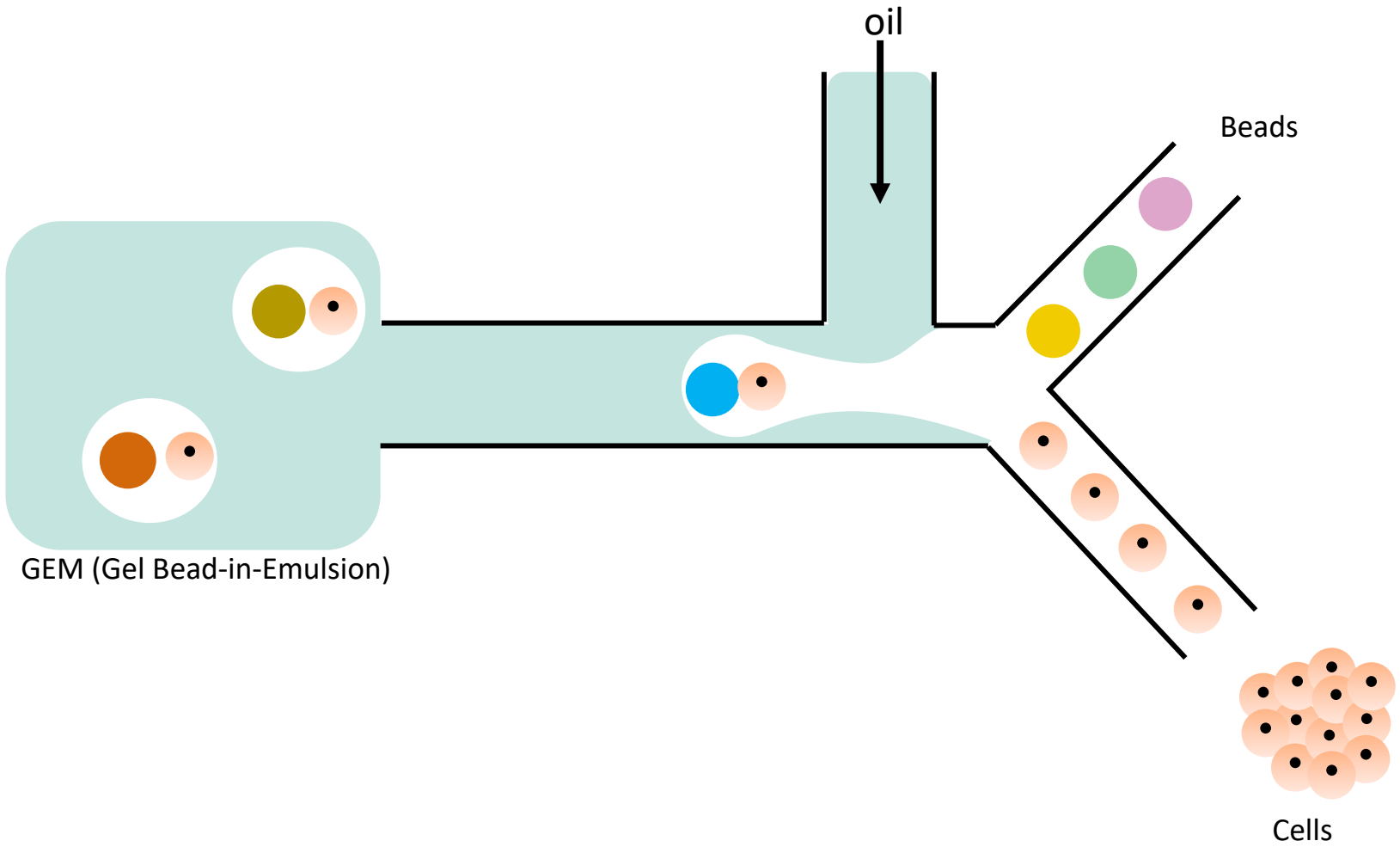
scRNA-seq Wet Lab Pipeline

Protocol	Type	Transcript Coverage	UMI Support	Throughput	Cost per Cell	Special Features / Use Cases
10x Chromium	Droplet-based	3’ end or 5’ end	Yes	Very High (> 1M cells)	$0.10–$0.50	Standardized, reliable and reproducible
SPLiT-seq	Plate-based	3’ end	YES	Very High	~$0.01	Cost-friendly
Microwell-seq	Microwell-based	3’ end	Yes	Medium	$0.01-$0.05	Used in Mouse Cell Atlas; optimized for bulk processing

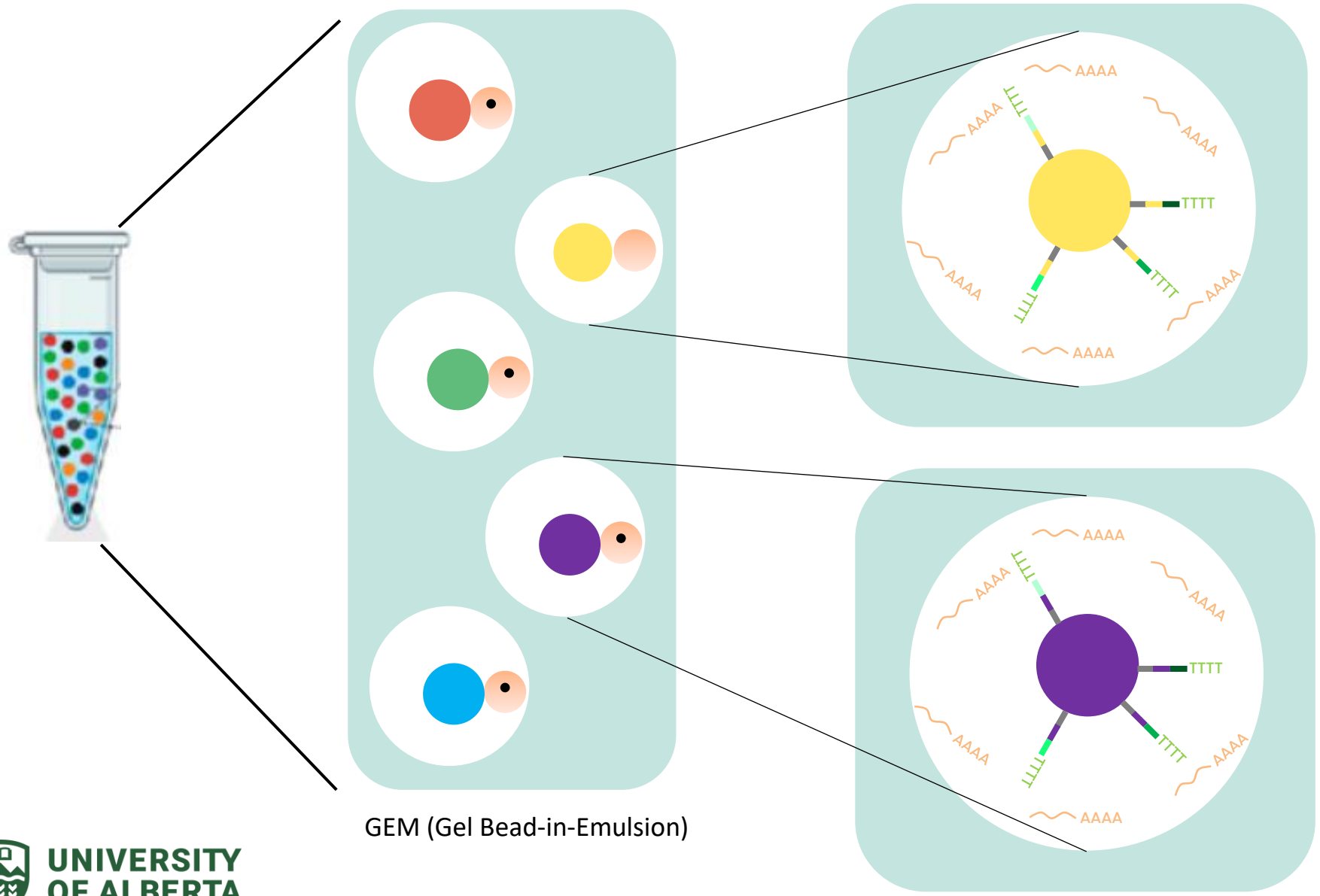
Why 10x Genomics is preferred over others?

- Fully automated
- Consistent and well support
- Can be integrated to multi-modal data such as Assay for Transposase-Accessible Chromatin (ATAC)
- Ease to use and no need custom setup

10x Chromium 3’ scRNA-seq – GEM Formation



10x Chromium 3’ scRNA-seq



GEM (Gel Bead-in-Emulsion)

Reverse Transcription

ds-cDNA synthesis

PCR with sample-specific primers

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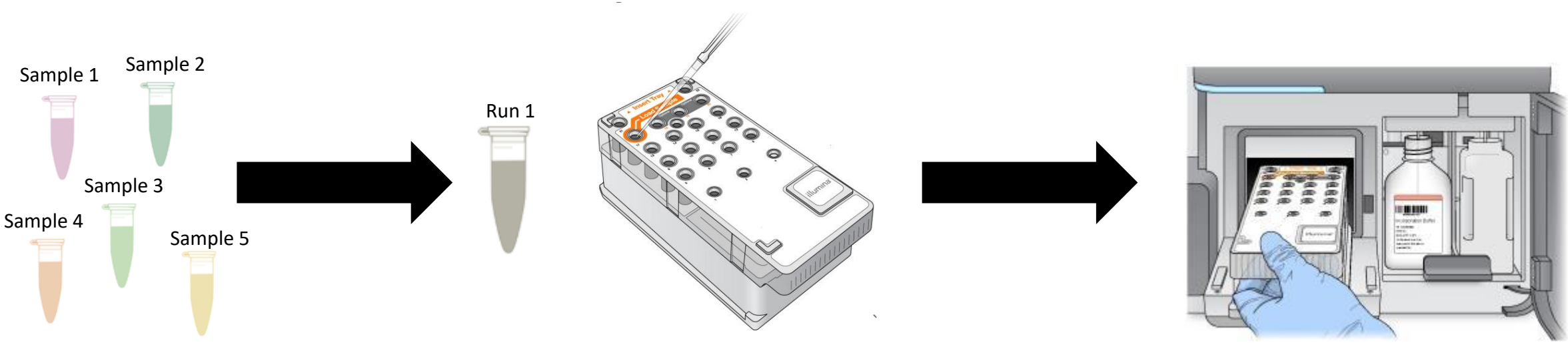
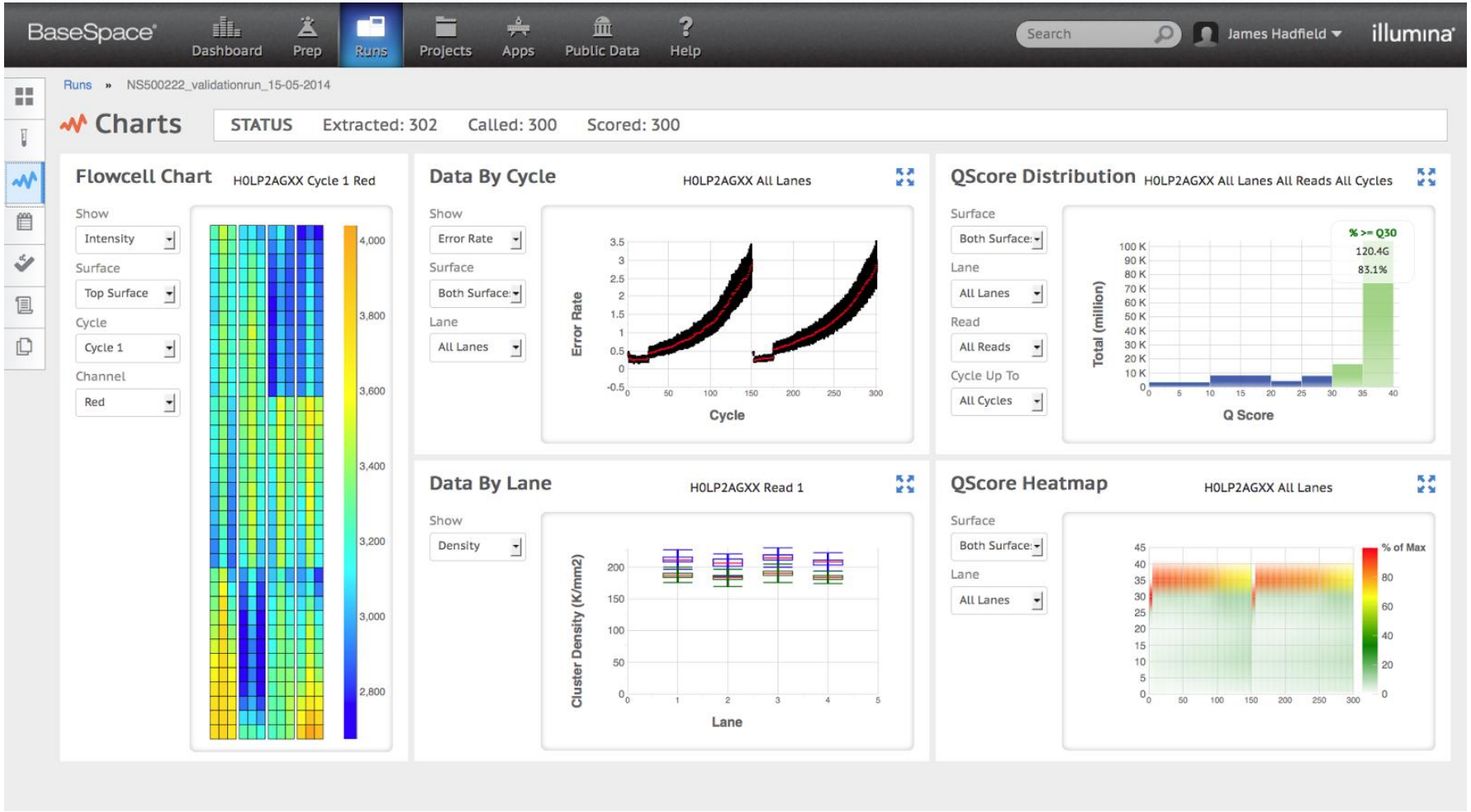
10x Chromium 3’ scRNA-seq



10x Chromium 3’ scRNA-seq – Sequencing Report



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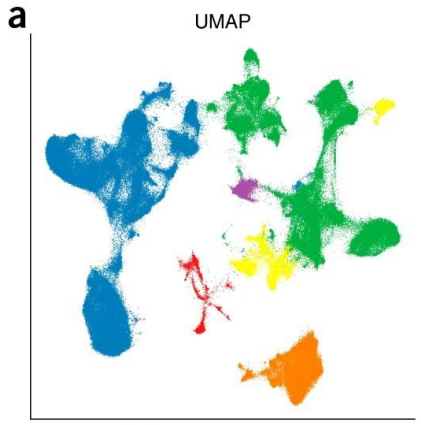
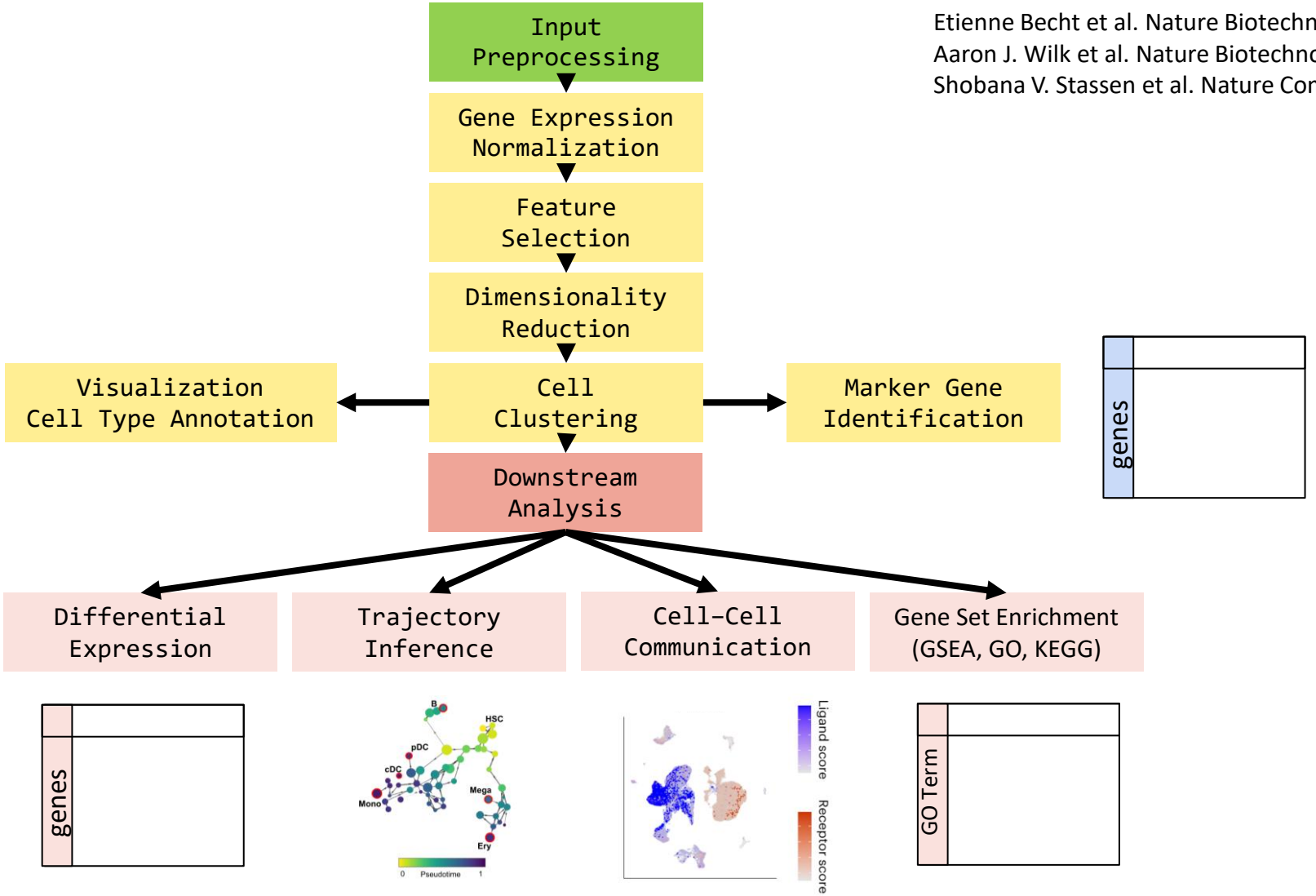
illumina

The Pipeline of scRNA-seq Analysis





Etienne Becht et al. Nature Biotechnology. 37: 38–44 (2019)
Aaron J. Wilk et al. Nature Biotechnology. 42: 470–483 (2024)
Shobana V. Stassen et al. Nature Communications. 12: 5528 (2021)

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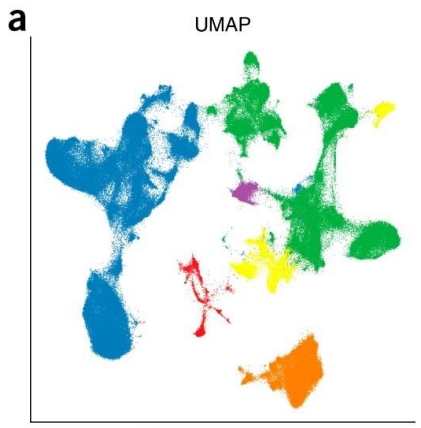
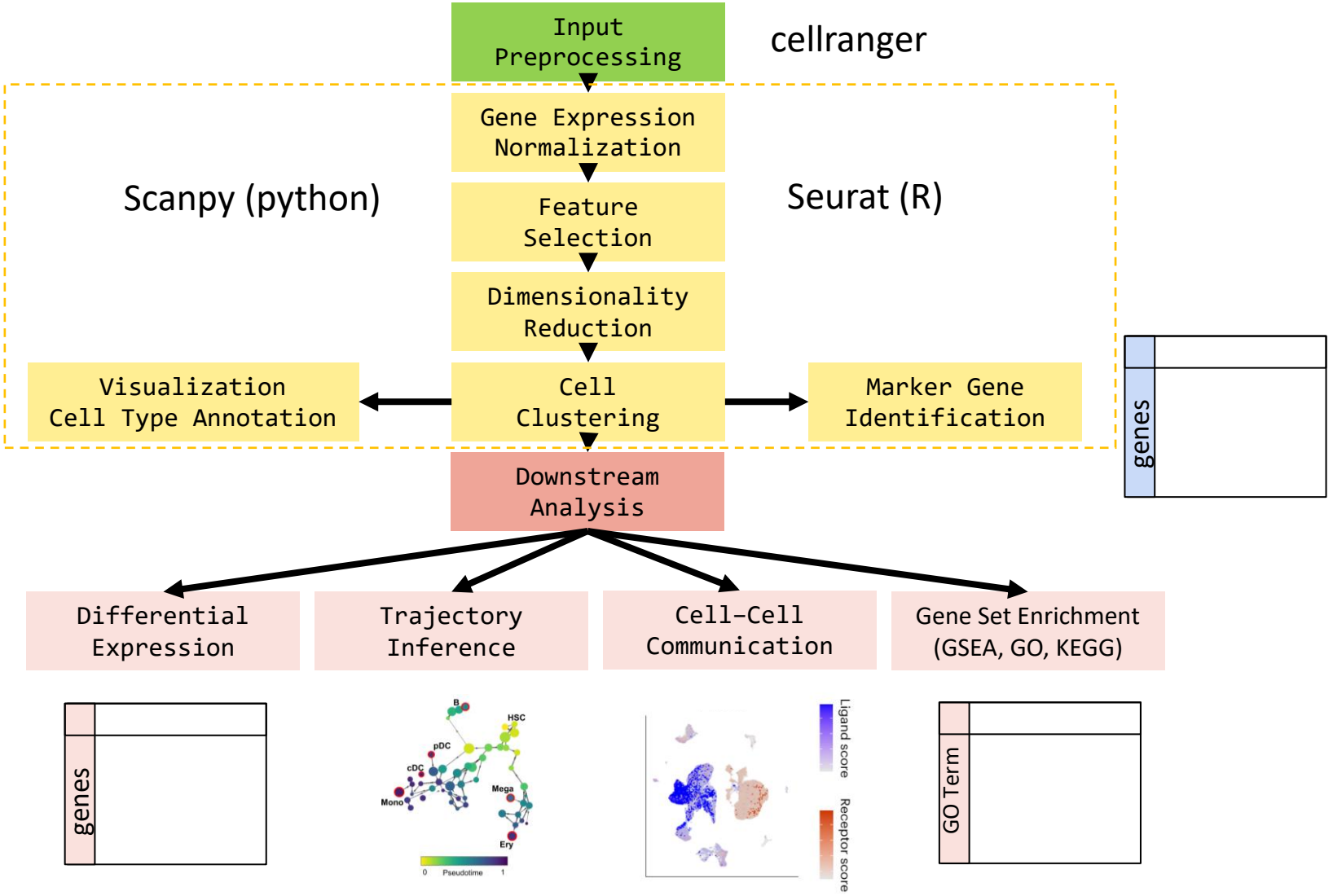
The Pipeline of scRNA-seq Analysis





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Question?

Input Processing

Input Preprocessing by Cell Ranger

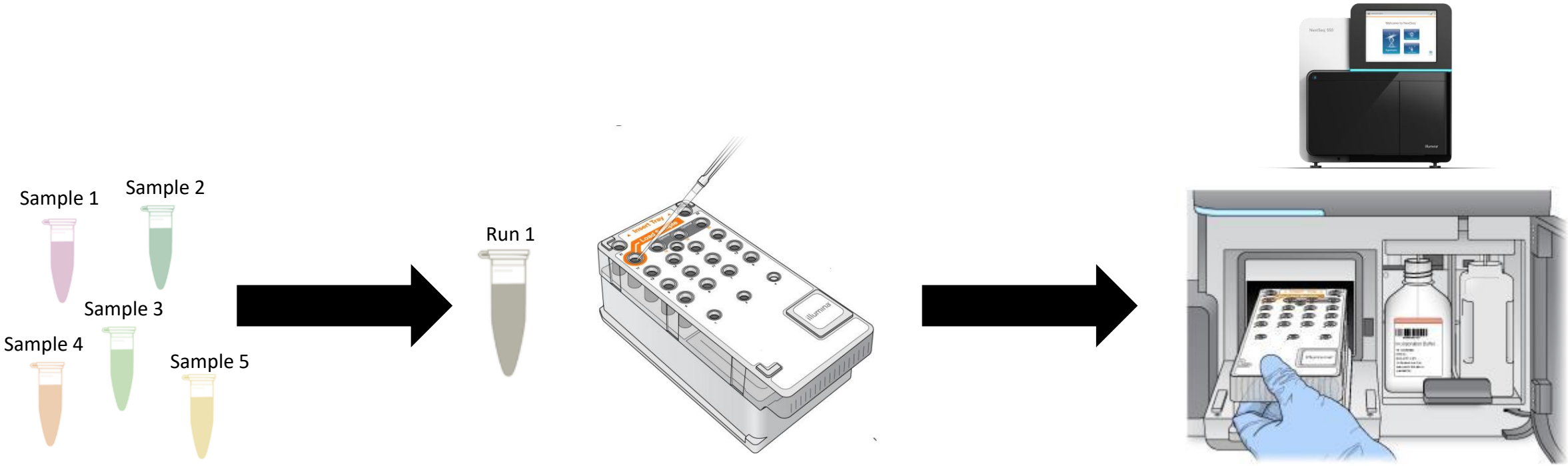
Input Preprocessing

- Base calling & demultiplexing
- Alignment & quantification

Optional Tools:

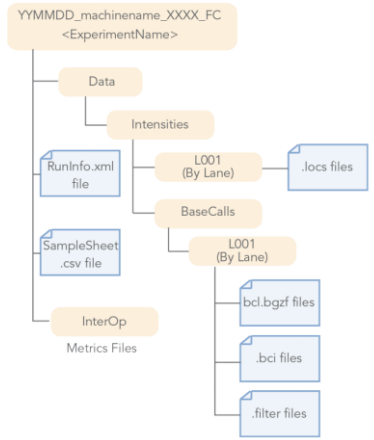
Cell Ranger	**Most popular**
STARsolo	Open source so you can adjust the parameters, like the tolerance of mismatches
Alevin	Super fast

10x Chromium 3’ scRNA-seq



Base calling & demultiplexing

Figure 2 BCL Conversion Input Files from the MiniSeq or NextSeq System



SampleName1_S1_L001_R1_001.fastq.gz — 10x barcode+UMI
SampleName1_S1_L001_R2_001.fastq.gz — cDNA sequence
SampleName1_S1_L001_I1_001.fastq.gz — index sequence

SampleName2_S2_L001_R1_001.fastq.gz — 10x barcode+UMI
SampleName2_S2_L001_R2_001.fastq.gz — cDNA sequence
SampleName2_S2_L001_I1_001.fastq.gz — index sequence

Split chunk of reads (4M in default)

Sample Name (given by you when filling the sample sheet

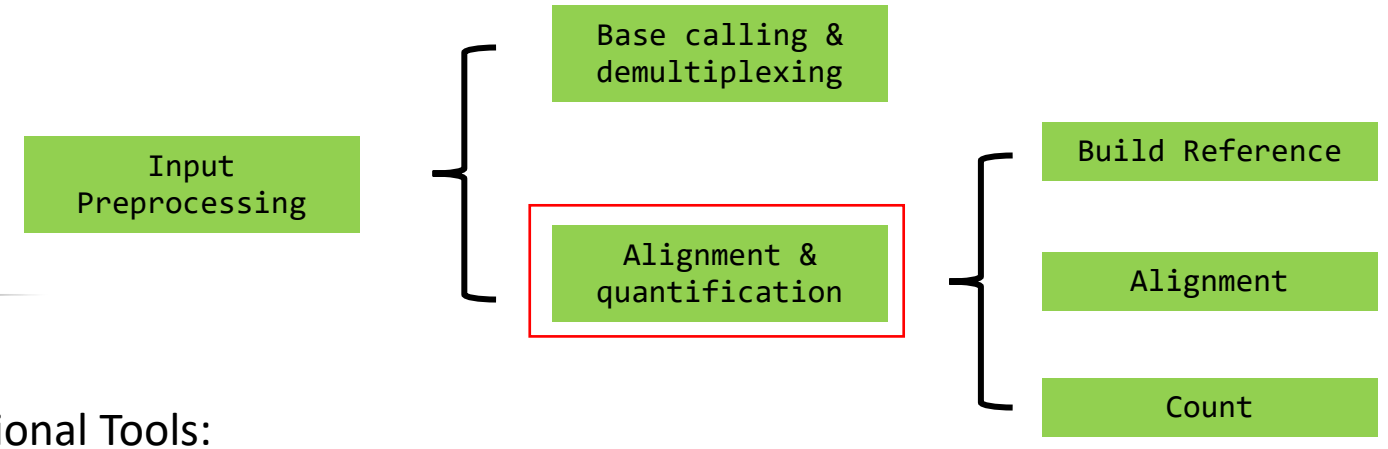
```
cellranger mkfastq \
  --id=sample_name_fastq \
  --run=/path/to/bcl_folder \
  --csv=sample_sheet.csv \
  --output-dir=/path/to/fastq/
```

Base calling & demultiplexing

- You need to do base calling & demultiplexing **ONLY WHEN** you have 10x genomics machine and sequencer.
- However, if you do need to run this step, here is a guide of computing power needed for your reference

Run size (rough input)	Typical reads	CPUs to request	RAM to request	Ballpark runtime*
Small (MiSeq/mini-runs; BCL ~30–50 GB)	~20–40 M	**8** cores	**16–32 GB**	**0.5–1.5 h**
Medium (NextSeq lane; BCL ~60–120 GB)	~80–150 M	**16** cores	**32–48 GB**	**1–3 h**
Large (NovaSeq X/6k/10k lane; BCL ~200–400 GB)	~300–600 M	**24–32** cores	**48–64 GB**	**2–6 h**
Very large (≥2 lanes; BCL ~400–800 GB)	~0.6–1.2 B	**32–48** cores	**64–128 GB**	**4–10 h**

Input Preprocessing by Cell Ranger



Optional Tools:

Cell Ranger	**Most popular**
STARsolo	Open source so you can adjust the parameters, like the tolerance of mismatches
Alevin	Super fast

Install Cellranger

https://www.10xgenomics.com/support/software/cell-ranger/downloads#download-links

Download and unzip

```
cd
cp -r projects/def-sponsor00/scRNA-seq/Sample_FASTQ/ .
```

Alignment & quantification – Build The Reference

```
cd
cp -r projects/def-sponsor00/scRNA-seq/Sample_FASTQ/ .
export PATH=/project/def-sponsor00/scRNA-seq/cellranger-9.0.1/bin:$PATH
cd Sample_FASTQ/ref
cellranger mkref --genome tp53 --fasta tp53.fa --genes tp53.gtf
```

Tips & best practices:

- For human reference genome: https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest

- For big genome that 10x Genomics doesn't have, run this step in a batch job

Genome (approx size)	Examples	CPUs to request	RAM to request	Runtime (ballpark)
Bacteria/virus (≤10 Mb)	E. coli, SARS-CoV-2	2–4	1–2 GB	minutes
Fish/amphib (~1–2 Gb)	Zebrafish	8	16–24 GB	~30–90 min
Human/Mouse (~3 Gb)	GRCh38, GRCm39	8–16	**32 GB**	**~1–3 h**
Large plant/complex (6–20 Gb)	Maize, wheat	16–32	**64–128 GB**	6-24 hours

Alignment & quantification

```
cd $HOME/Sample_FASTQ
vi cellranger.sh            # edit the job name
```

```
cellranger count \
  --id output_tp53 \                                    # Name of the output folder
  --transcriptome ref/tp53 \                            # Path to reference genome built for Cell Ranger
  --fastqs fastq \                                      # Folder containing FASTQ files
  --sample tp53test \                                   # Sample ID in FASTQ file names
  --localcores 1 \                                      # Number of CPU cores
  --localmem 2  \                                       # Amount of memory (GB)
  --create-bam true \                                   # whether create a bam file
  --chemistry=SC3Pv4                                    # only for testing in this case, delete it when
                                                          you run your analysis
```

```
sbatch cellranger.sh
```

The job will take a few minutes to finish

Alignment & quantification – check the output

```
cd $HOME/Sample_FASTQ/output_tp53
du -h | tail -1
du -h ../fastq
```

The output could be 1000x larger than the input!

Factors affect the output size:

1. Number of cells
2. Sequencing depth
3. Size of the reference genome
4. Introns included?
5. Bam files generated?

Alignment & quantification – Sequencing Depth

For typical human tissue studies	
Cells per sample	5,000 – 20,000
Reads per cell	20,000 – 100,000
Total reads per sample	100M – 1B
Genes detected per cell	A few hundreds to thousands

Factors affecting the sequencing depth:

- Transcriptome size and complexity
- The nature of sample: FFPE, fresh, frozen?
- Genes of interest: highly or lowly expressed? Isoform-specific transcripts?
- Your budget

Alignment & quantification – Computational Resources

Cells × Reads	Cores	RAM	Time
~3k cells, 100M reads	8 cores	32 GB	~1–1.5 hrs
~10k cells, 500M reads	16 cores	64 GB	~2–4 hrs
~50k cells, 1B+ reads	32 cores	128 GB	4–8+ hrs

Understand The Output

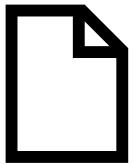
```
cd $HOME/Sample_FASTQ/output_tp53/outs
```

Gene Expression Profile

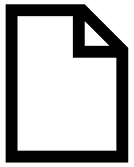
sample_raw_feature_bc_matrix

sample_filetered_feature_bc_matrix

Summary and Quality Assessment



web_summary.html



matrix_summary.csv

https://github.com/ualberta-rcg/scRNA-seq

Go to Google Drive link -> scRNA-seq -> 10x_data

Quick Assessment Of Cellranger Outputs

- Number of cells

Cells
4,999

- Median genes / cell

Cell Type / Sample	Expected Range
Fresh PBMCs	1,000–2,500
Solid tissue	500–1,500
FFPE or nuclei	200–800

Median genes per cell
696

- Median UMI Counts per Cell
  - Fresh PBMCs: ~5,000–15,000
  - FFPE or nuclei: ~1,000–5,000

Median UMI counts per cell
1,145

- Sequencing Saturation
  - <50% You can benefit from deeper sequencing
  - 50-70% Still can get some new UMIs
  - >70% No need to sequence more

Sequencing saturation
83.69%

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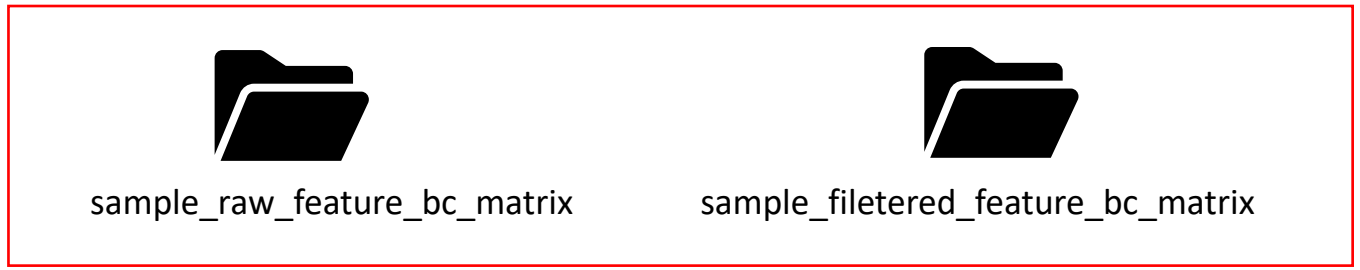
Understand The Output

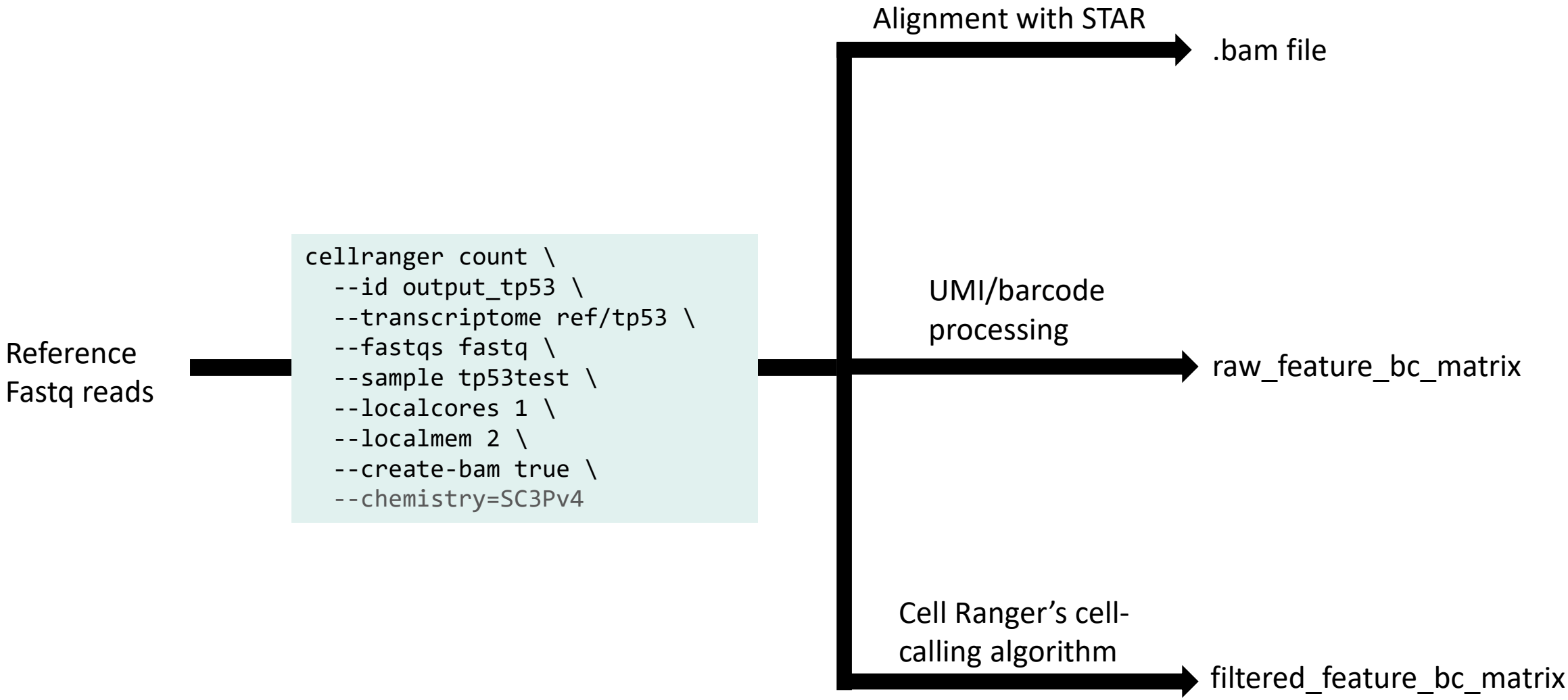
```
cd $HOME/Sample_FASTQ/output_tp53/outs
```



https://github.com/ualberta-rcg/scRNA-seq

Go to Google Drive link -> scRNA-seq -> 10x_data

Understand The Output



Understand The Output

What we want:

	Cell1	Cell2	Cell3
Gene1	0	20	3
Gene2	104	1	1

What we got:

```
cd $HOME/Sample_FASTQ/output_tp53/outs/raw_feature_bc_matrix
zcat matrix.mtx.gz
zcat barcodes.tsv.gz
zcat features.tsv.gz
```

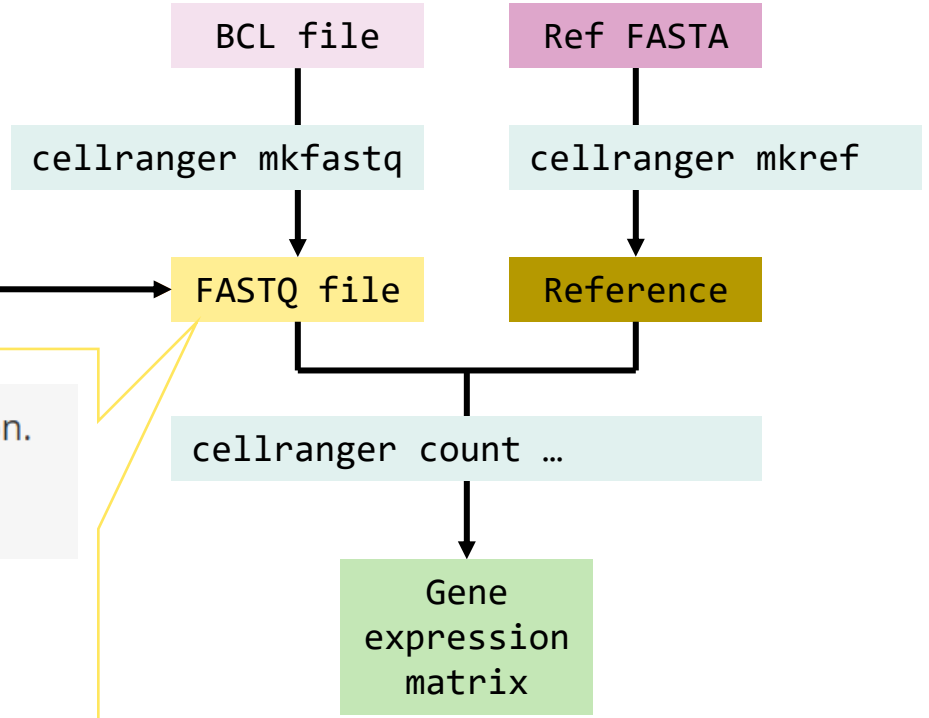
```
# matrix.mtx.gz
1 8 8              # In total, there are 1 gene, 8 cells, and 8 non-zero numbers
1 1 1              # Gene1  Cell1  1 read
1 2 1              # Gene1  Cell2  1 read
1 3 1              # Gene1  Cell3  1 read
1 4 1              # Gene1  Cell4  1 read
1 5 1              # Gene1  Cell5  1 read
1 6 1              # Gene1  Cell6  1 read
1 7 1              # Gene1  Cell7  1 read
1 8 1              # Gene1  Cell8  1 read
```

```
zcat ~/projects/def-sponsor00/scRNA-seq/10x_data/sample_raw_feature_bc_matrix/matrix.mtx.gz |head
zcat ~/projects/def-sponsor00/scRNA-seq/10x_data/sample_raw_feature_bc_matrix/barcodes.tsv.gz |head
zcat ~/projects/def-sponsor00/scRNA-seq/10x_data/sample_raw_feature_bc_matrix/features.tsv.gz |head
```

Summary of input preprocessing

```
SRR9291388_1.fastq.gz       # Read1
SRR9291388_2.fastq.gz       # Read2
SRR9291388_3.fastq.gz       # Index
```

NCBI SRA



Cell Ranger requires FASTQ file names to follow the `bcl2fastq` file naming convention.

`[Sample Name]`_S1_L00`[Lane Number]`_`[Read Type]`_001.fastq.gz

```
SRR9291388_S1_L001_R1_001.fastq.gz    # Read1
SRR9291388_S1_L001_R2_001.fastq.gz    # Read2
SRR9291388_S1_L001_I1_001.fastq.gz    # Index
```

https://kb.10xgenomics.com/hc/en-us/articles/115003802691-How-do-I-prepare-Sequence-Read-Archive-SRA-data-from-NCBI-for-Cell-Ranger

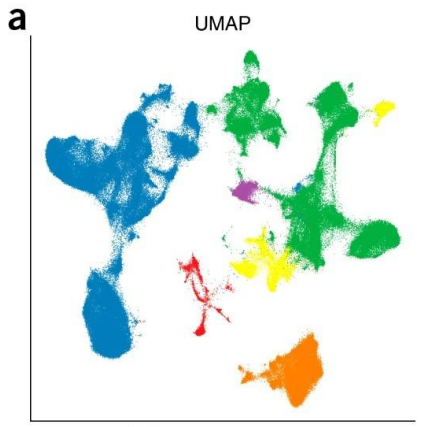
Question?

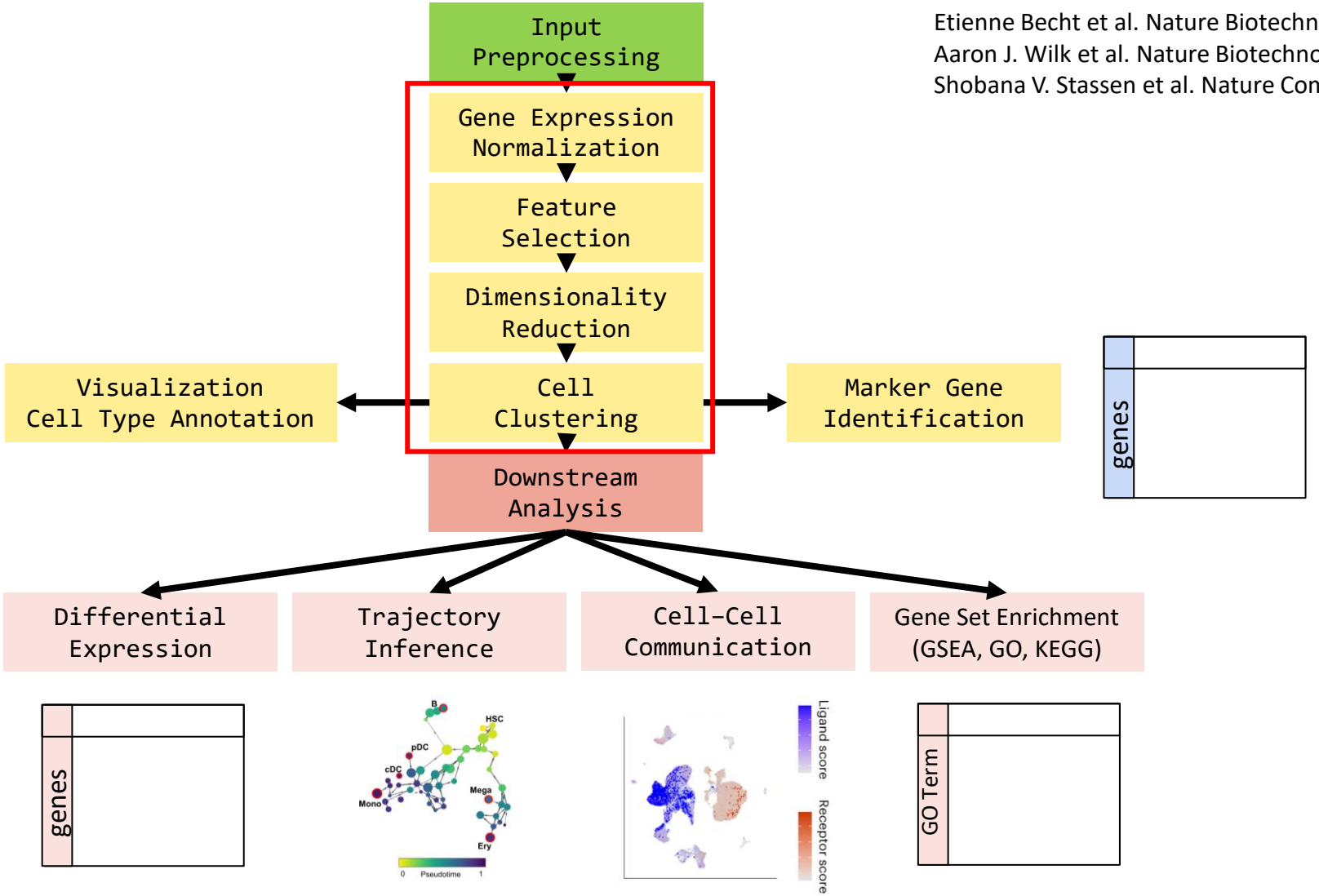
Quality Control

Start Rstudio

- https://github.com/ualberta-rcg/scRNA-seq
- Open the file “Analysis_with_Seurat.md”

The Pipeline of scRNA-seq Analysis





Etienne Becht et al. Nature Biotechnology. 37: 38–44 (2019)
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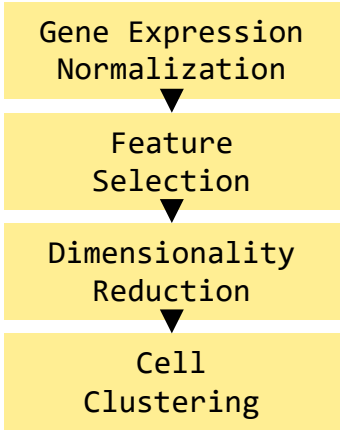
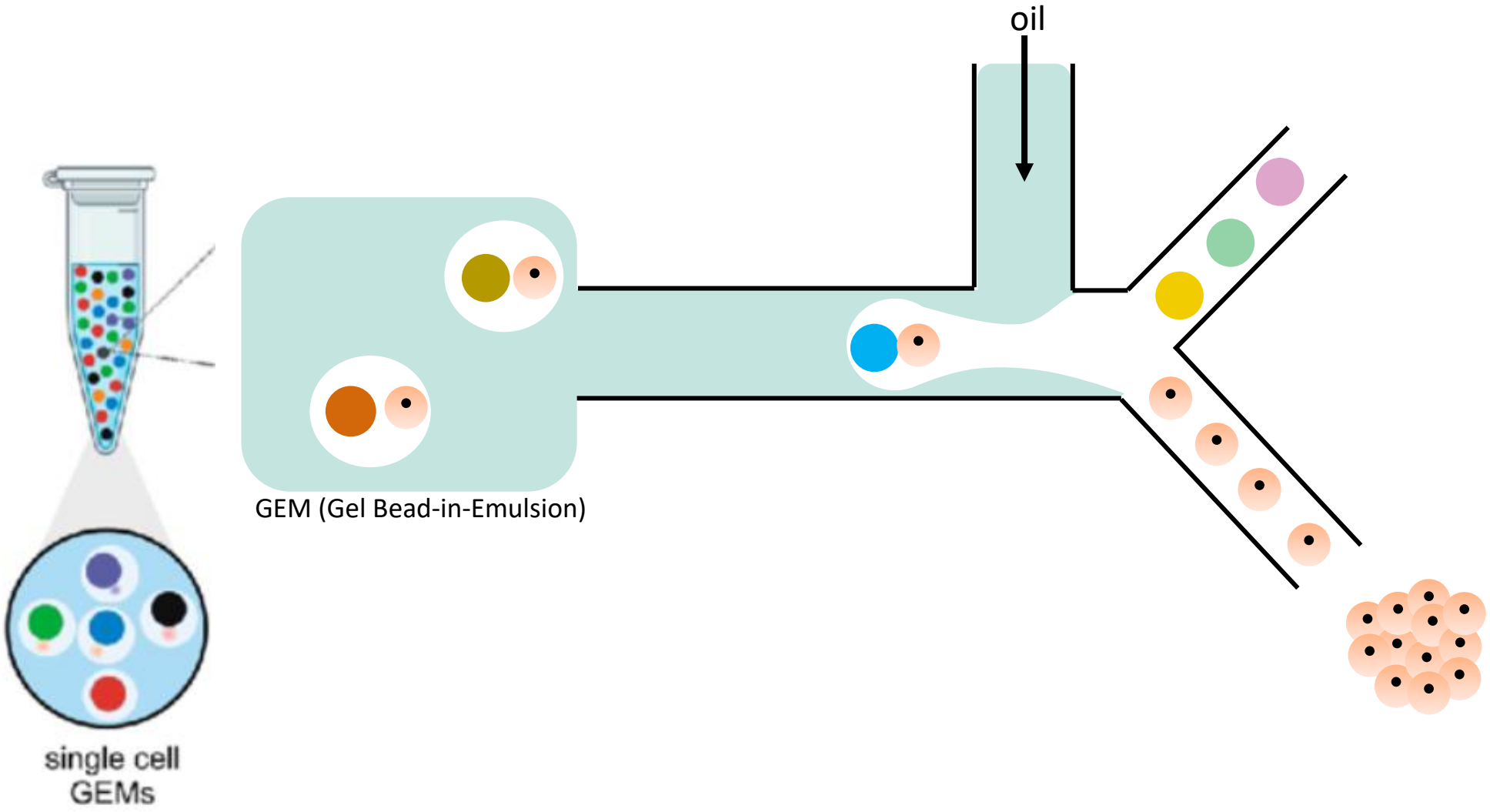
The Pipeline of scRNA-seq Analysis



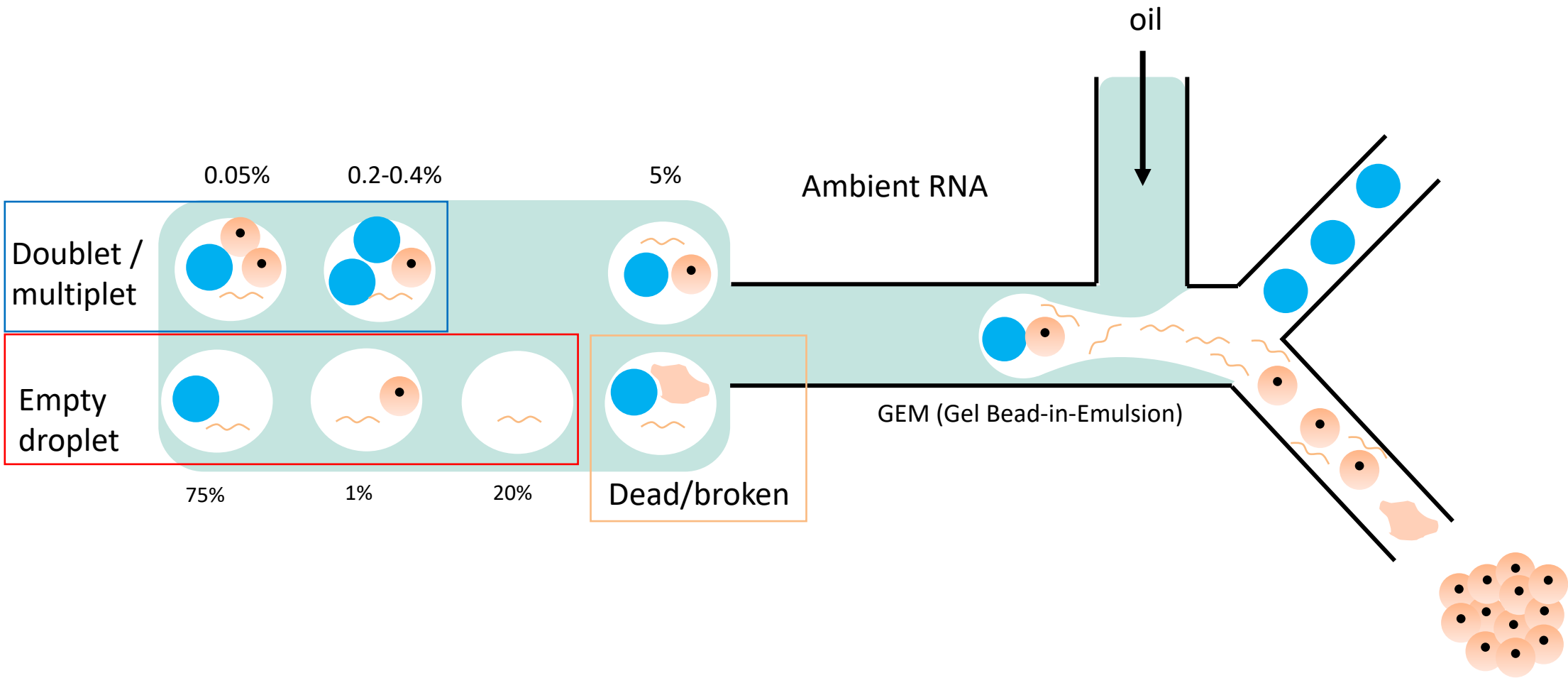

```
filter_dat <- Seurat::Read10X("filtered_feature_bc_matrix/")
seur_obj <- Seurat::CreateSeuratObject(filter_dat, min.cells=5, min.features=100)
seur_obj <- Seurat::NormalizeData(seur_obj)
seur_obj <- Seurat::FindVariableFeatures(seur_obj)
seur_obj <- Seurat::ScaleData(seur_obj)
seur_obj <- Seurat::RunPCA(seur_obj)
seur_obj <- Seurat::FindNeighbors(seur_obj)
seur_obj <- Seurat::FindClusters(seur_obj)
```

- It is straight forward if the web lab experiment is ideal

10x Chromium 3’ scRNA-seq – GEM Formation



10x Chromium 3’ scRNA-seq – In Real World



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Lareau et al. (2020) doi: 10.1038/s41467-020-14667

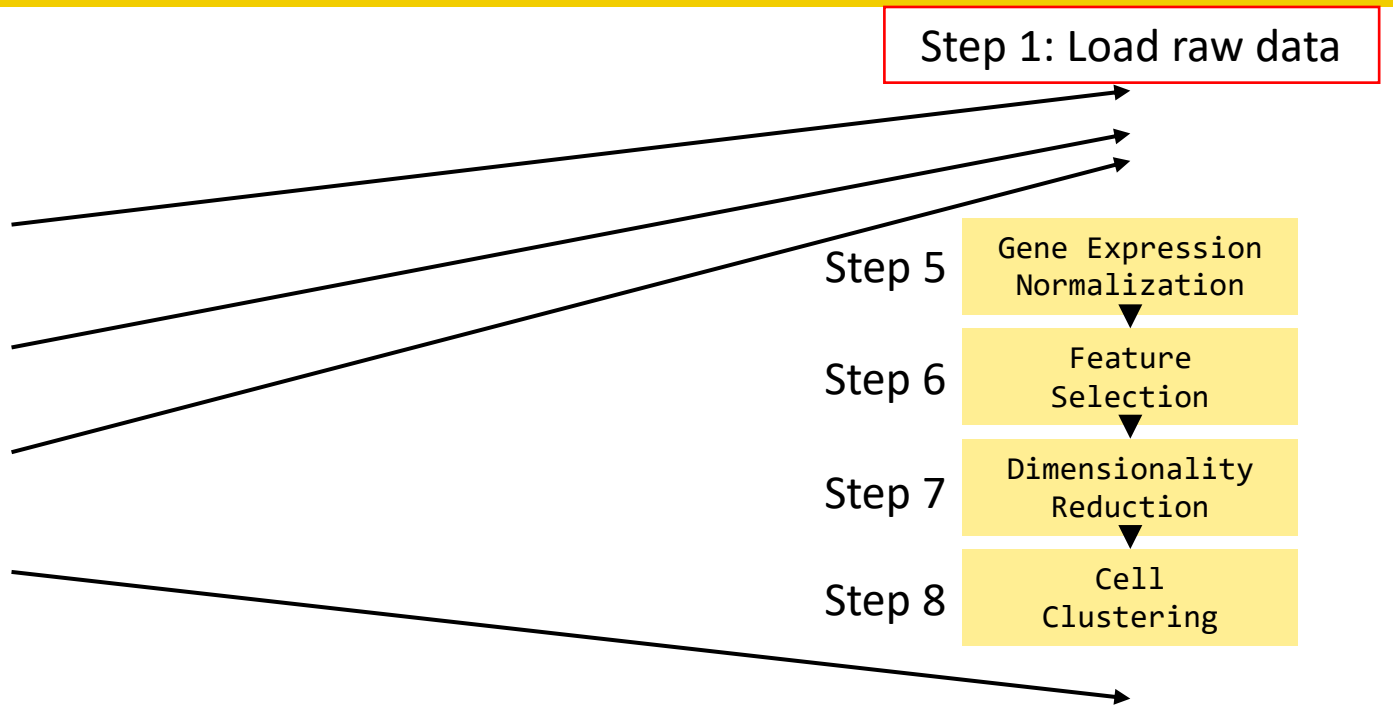
Quality Control

Step 2 • Remove empty droplets (QC1)

Step 3 • Filter out Ambient RNA (QC2)

Step 4 • Identify dead/broken cells (QC3)

Step 9 • Identify doublets (QC4)



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(Code) Step 1: Load the raw matrix

What we want:

	Cell1	Cell2	Cell3
Gene1	0	20	3
Gene2	104	1	1

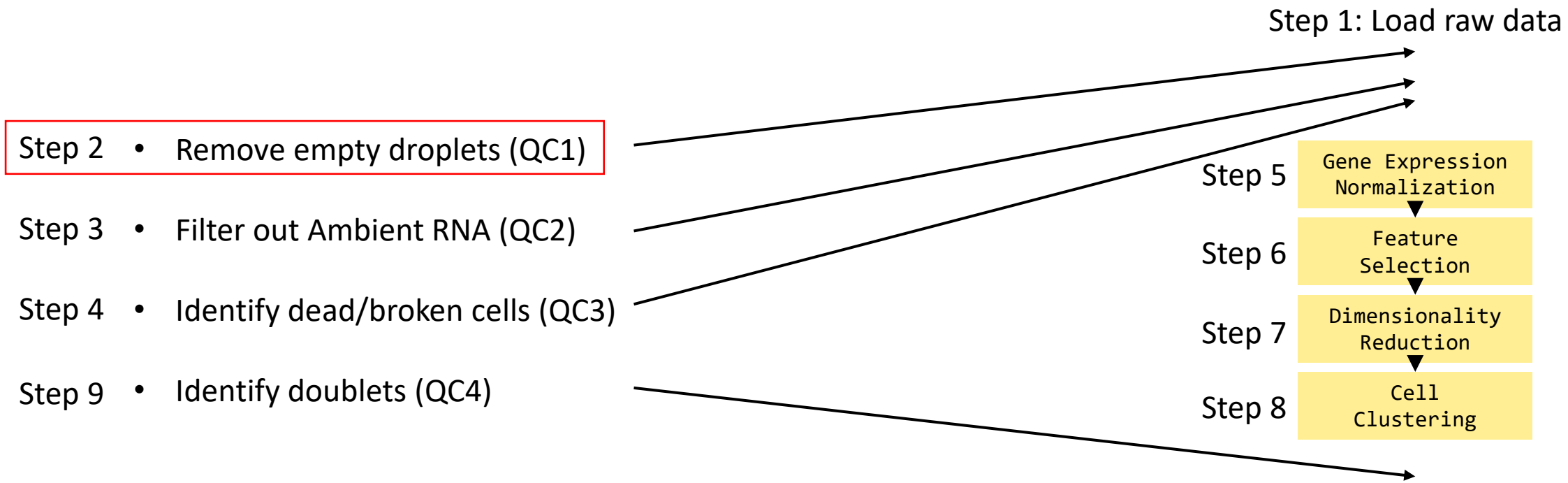
What we got:

```
# matrix.mtx.gz
1 8 8                # In total, there are 1 gene, 8 cells, and 8 non-zero numbers
1 1 1                # Gene1  Cell1  1 read
1 2 1                # Gene1  Cell2  1 read
1 3 1                # Gene1  Cell3  1 read
1 4 1                # Gene1  Cell4  1 read
1 5 1                # Gene1  Cell5  1 read
1 6 1                # Gene1  Cell6  1 read
1 7 1                # Gene1  Cell7  1 read
1 8 1                # Gene1  Cell8  1 read
```

```
......
raw_dat <- Seurat::Read10X(data.dir = "/usr/local/10x_data/sample_raw_feature_bc_matrix")
......
```

	AAACCTGAGATAGGAG-1	AAACCTGAGATCCTGT-1	AAACCTGAGATTACAA-1	...
TP53	6	0	2	
KRAS	3	0	7	
…				

Quality Control

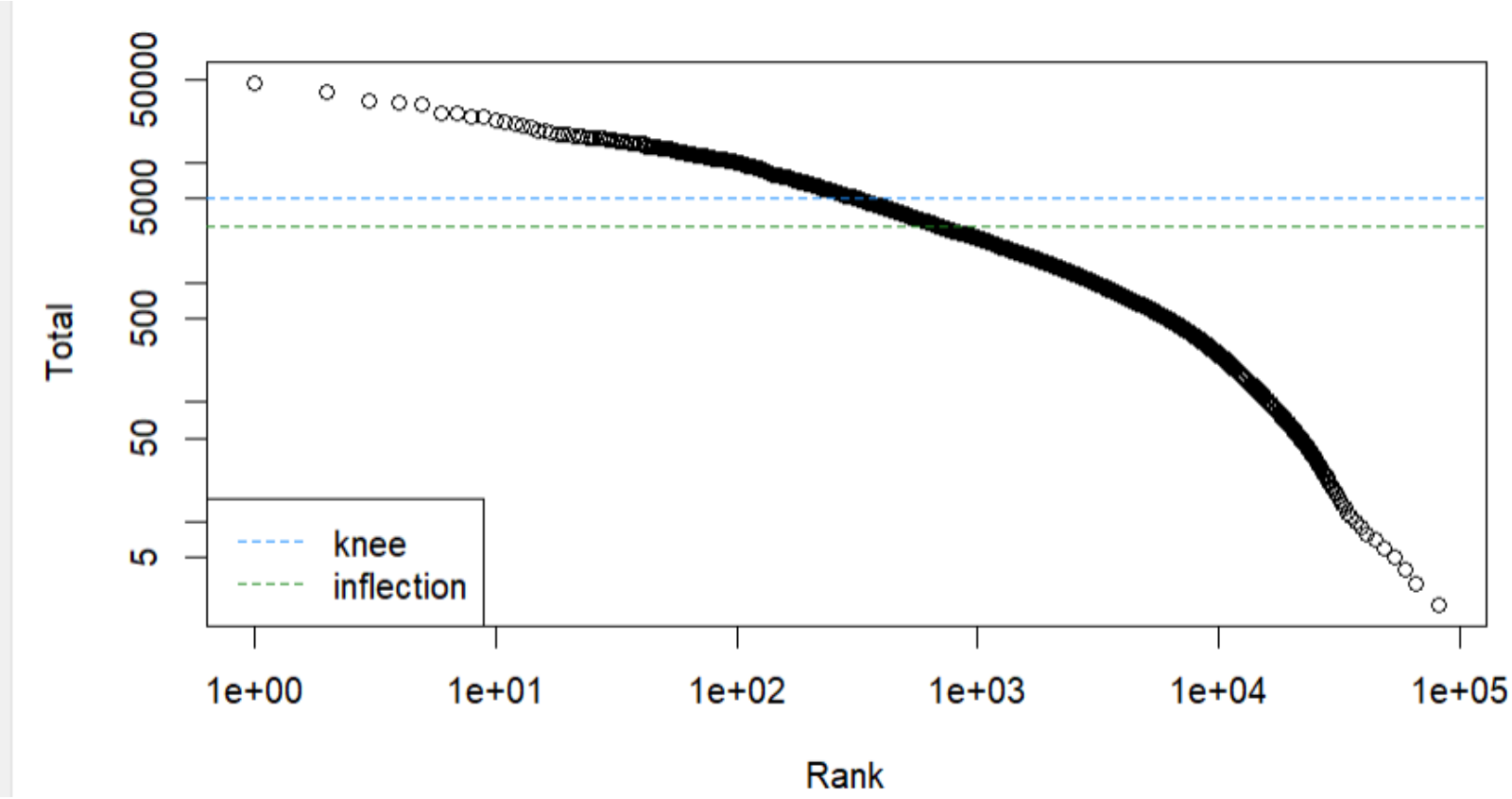


Step 2: Remove the empty droplets

- Three methods:
  - Cell Ranger Strategy
  - Knee/Inflection
  - Poisson

Step 2: Remove the empty droplets

- Barcode Rank Plot



Knee: min 2nd derivative (Max curve bending)

Inflection: 2nd derivative = 0

Step 2: Remove the empty droplets

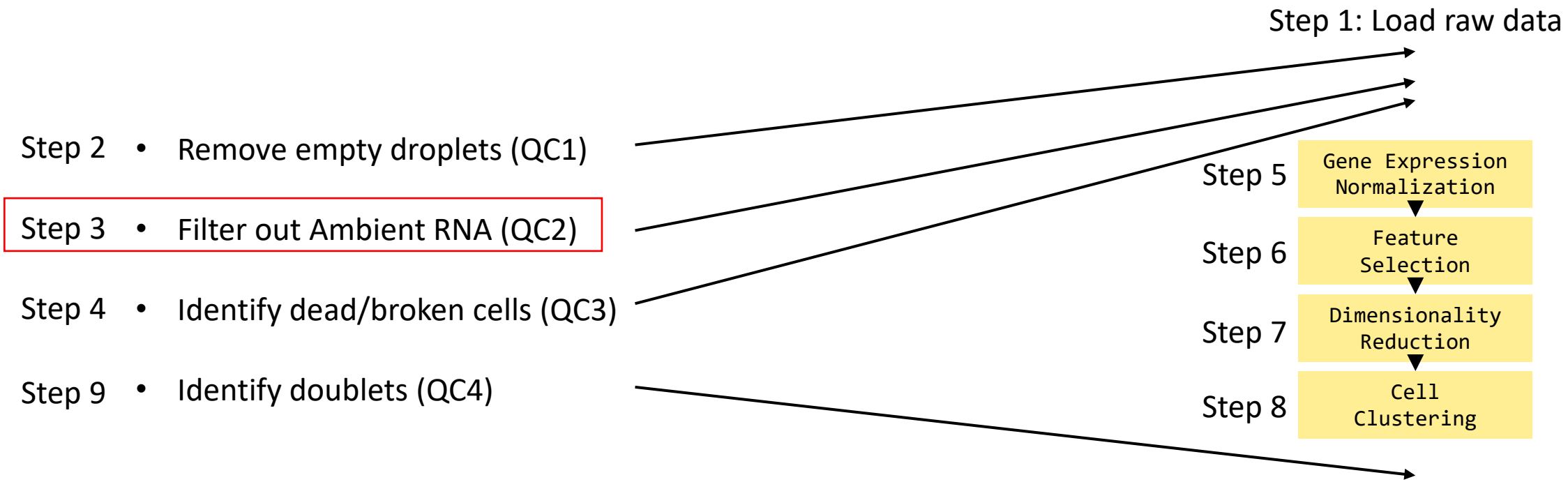
- Summary of three methods

Method	Based on...	Sensitivity	Specificity	Best for…
Cell Ranger Strategy	Proprietary + knee-like model + internal heuristics	High	Variable	Large cell recovery
Poisson	Statistical background noise model	Low	High	Low quality sample (FFPE)

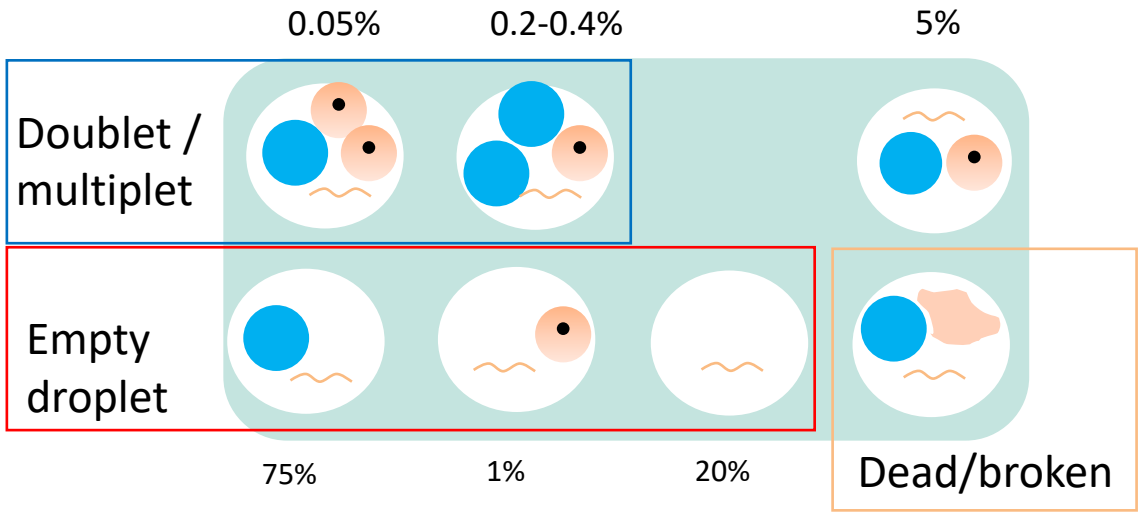
Step 2: Remove the empty droplets

```
e.out <- emptyDrops(raw_dat, lower=100, niters=10000, ignore=NULL, retain=2*br.out$knee)
```

Step 3: Filter Out Ambient RNA (QC2)



Step 3: Filter out ambient RNA (QC2)



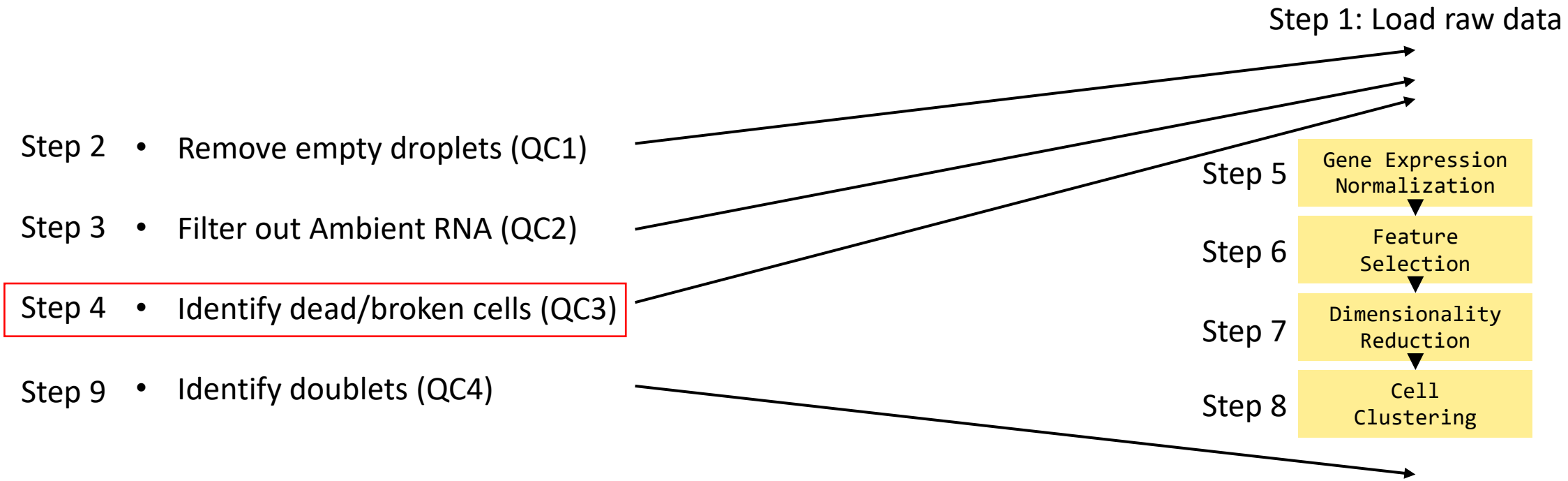
- There are multiple tools/ways.
  We are using DecontX today because:
  - It is easy to be integrated into the pipeline.
  - It doesn't need GPU.
  - The syntax is simple and clean.

- What DecontX does:
  - Group cells into clusters and estimates cluster-specific expression profiles
  - For each cell, tune the cell-specific contamination fraction to make the gene expression profile match the observation and the cluster as much as possible.

Step 3: Filter out ambient RNA (QC2)

	Cell A	Profile A	Ambient <- B	Cell B	Profile B	Ambient <- A
Gene1	90	0.545	0.046 x 15	6	0.046	0.545 x 10
Gene2	60	0.363	0.030 x 15	4	0.030	0.363 x 10
Gene3	10	0.060	0.538 x 15	70	0.538	0.060 x 10
Gene4	5	0.030	0.385 x 15	50	0.385	0.030 x 10
Total	165		165 x 9.1% = 15	130		130 x 7.7% = 10
Contamination with Bayesian Mixture model	9.1%			7.7%		

Step 4: Identify Dead/Broken Cells (QC3)

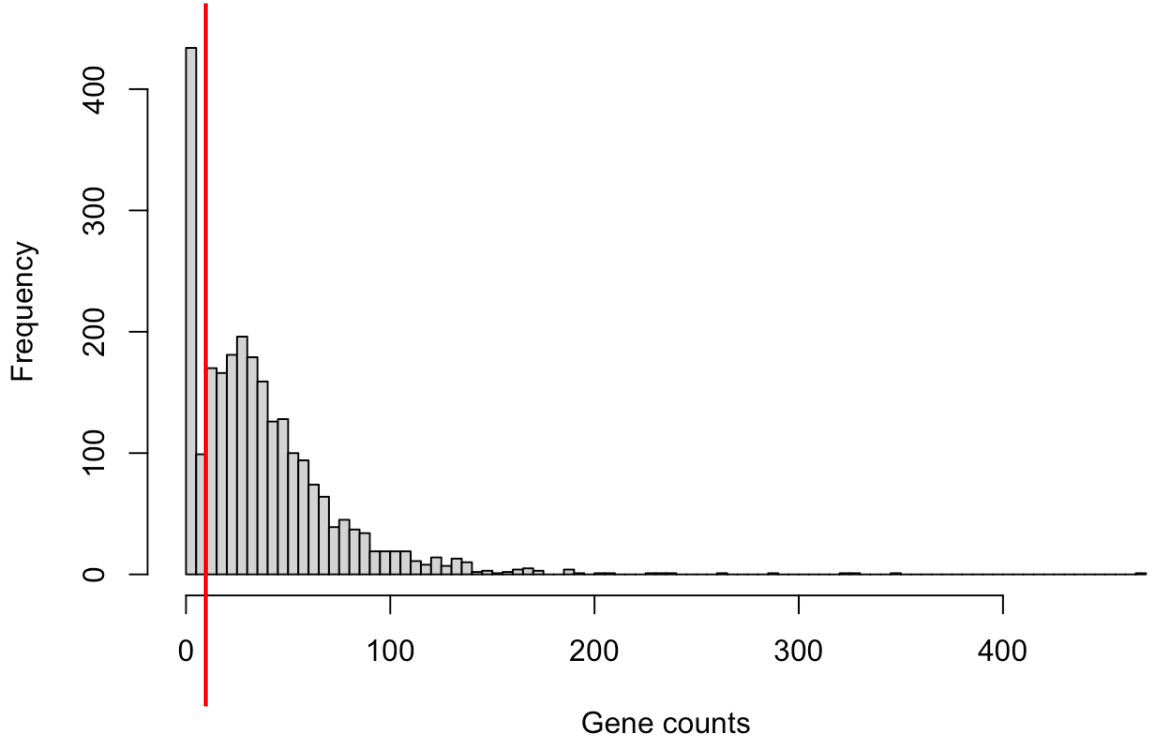


Step 4: Identify the dead / broken cells (QC3)

- Through Mitochondrial genes.

Consideration	Guidance
Human PBMCs / tumors / organs	200–500 gene threshold is typical. MT% cutoff: 30%
Low-input / fragile tissues (e.g., FFPE, brain)	Use a **lower threshold** (100–300). MT% cutoff: 40-60%
Visual inspection	Use hist(sce$detected) or violin plots to **see natural separation**

Choose your genes based on your project, for example, for PBMC sample, you may want to remove those red blood cells with high hemoglobin mRNAs (>0.5-1%).



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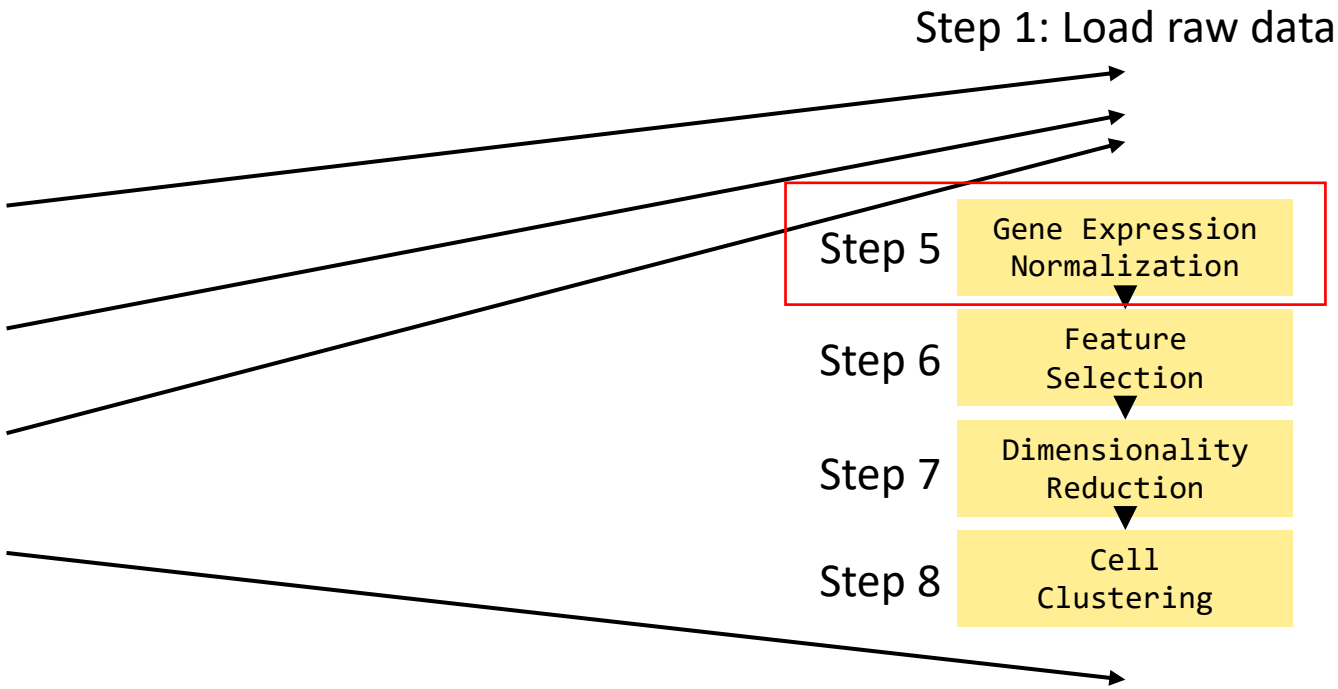
Step 4: Identify dead/broken cells (QC3)

```
......
......
is.mt <- grepl("^MT-", rowData(sce)$Symbol)
......
......
......
cell_filter_detect <- sce$detected < 100
cell_filter_MT <- sce$subsets_Mito_percent > 30
```

Step 5: Gene Expression Normalization

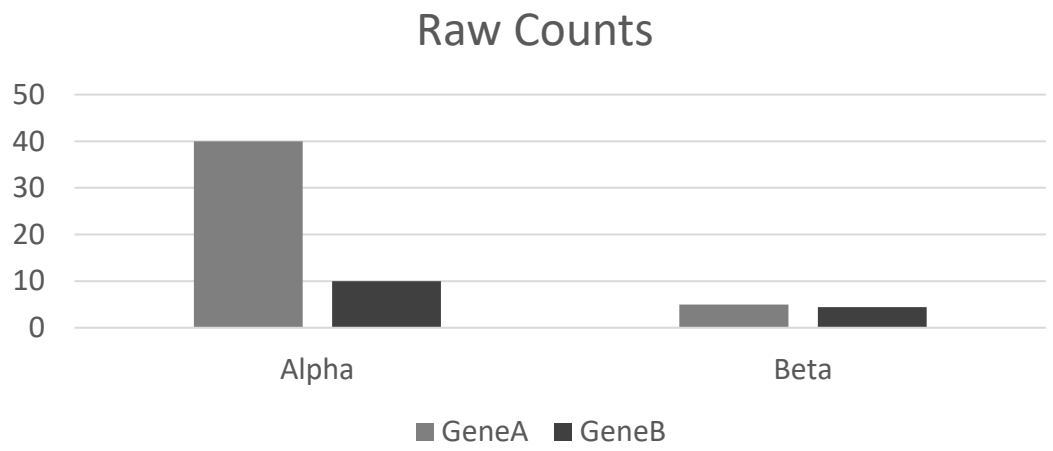
- Step 2 • Remove empty droplets (QC1)
- Step 3 • Filter out Ambient RNA (QC2)
- Step 4 • Identify dead/broken cells (QC3)
- Step 9 • Identify doublets (QC4)

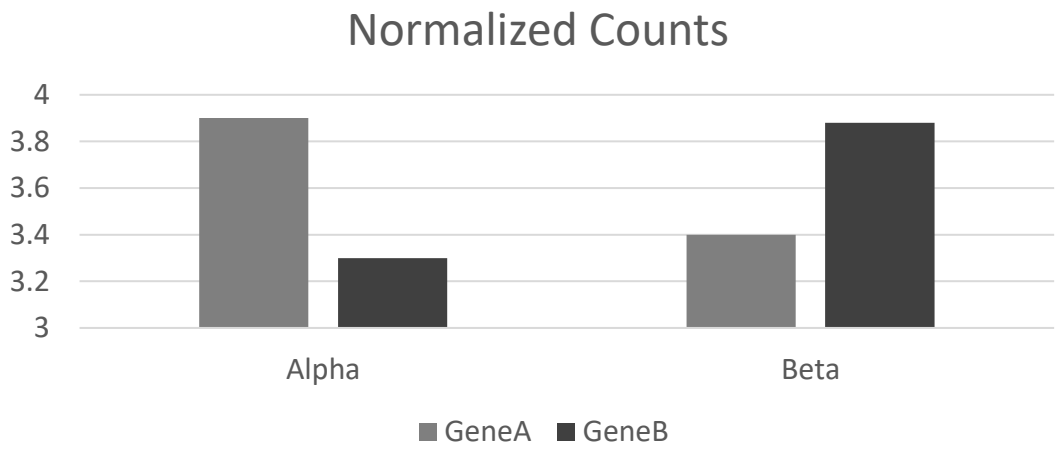


Step 5: Gene Expression Normalization

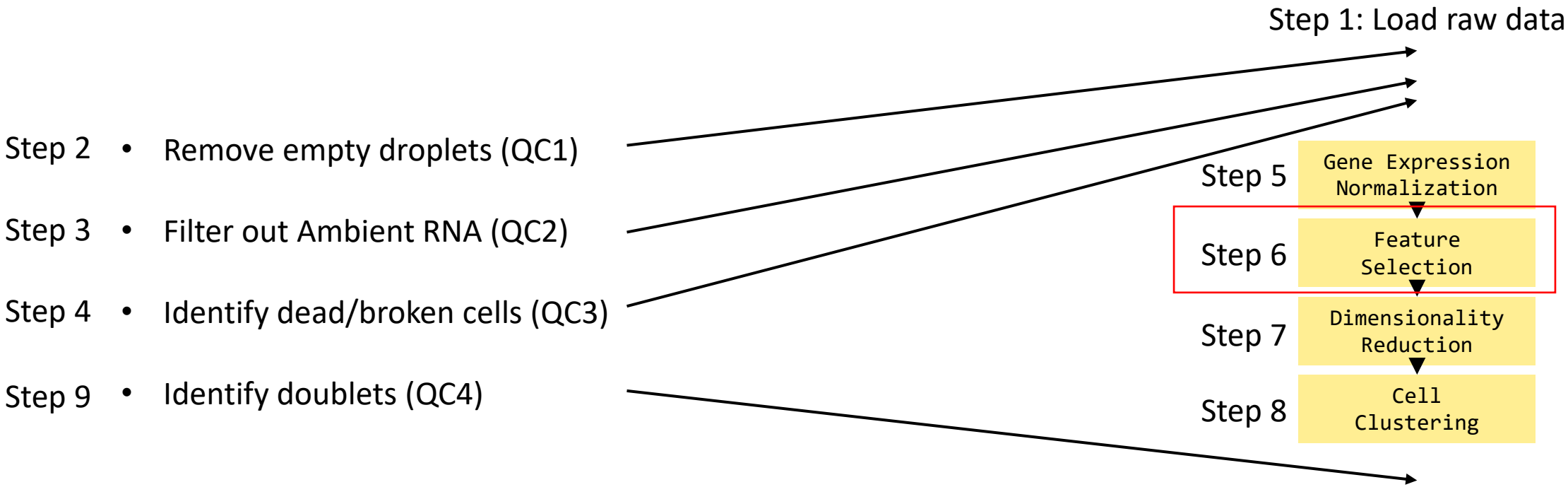
```
seur_filtered <- NormalizeData(seur_filtered, normalization.method = "LogNormalize", scale.factor = 10000)
```

Cell	Gene	UMIs	Scaling	Log Transformation
Alpha	A	40	40/(40+10)*10000 = 8000	log(1+8000) = 3.90
	B	10	10/(40+10)*10000 = 2000	log(1+2000) = 3.30
Beta	A	5	5/(5+15)*10000 = 2500	log(1+2500) = 3.40
	B	15	15/(5+15)*10000 = 7500	log(1+7500) = 3.88





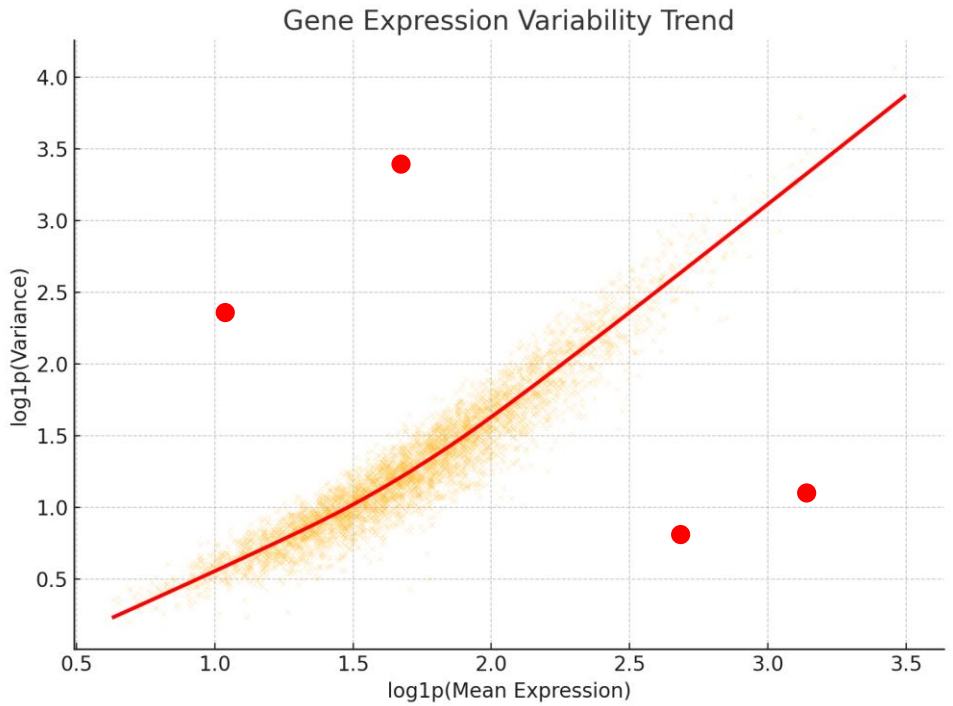
Step 6: Feature Selection



Step 6: Feature Selection - Identify highly variable genes

```
seur_filtered <- FindVariableFeatures(seur_filtered, selection.method = "vst", nfeatures = 500)
```

Identify 500 most variable genes through “Variance Stabilizing Transformation” (VST).



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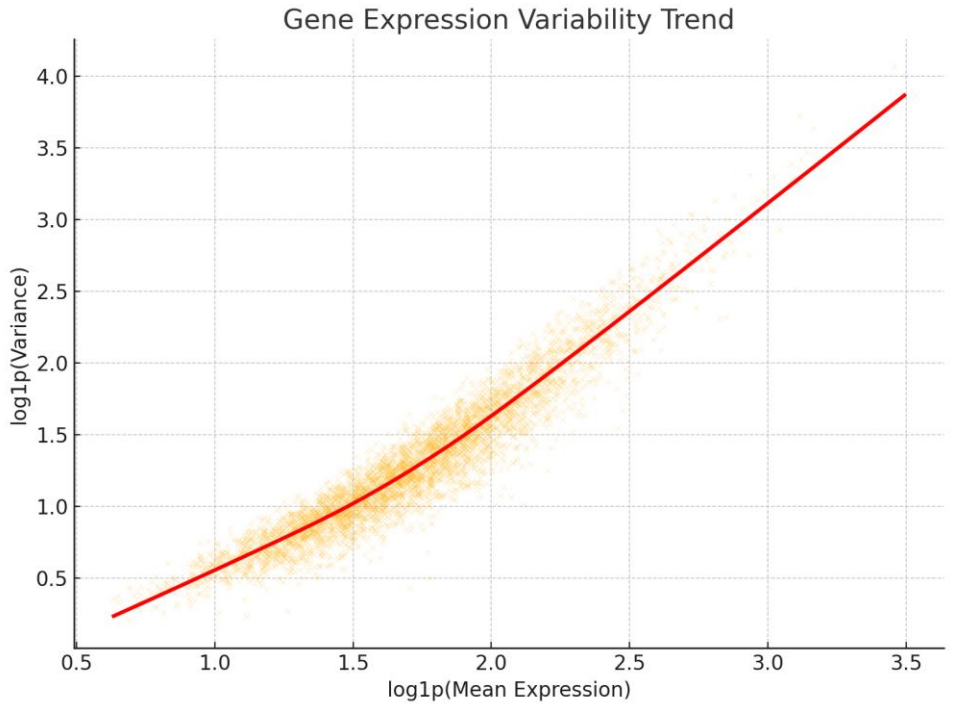
Step 6: Feature Selection - Identify highly variable genes

```
seur_filtered <- FindVariableFeatures(seur_filtered, selection.method = "vst", nfeatures = 500)
```

Identify 500 most variable genes through “Variance Stabilizing Transformation” (VST).



How to determine “nfeatures”?

Total genes detected per cell	Suggested *nfeatures*
< 500	300–500
~1000	500–1000
>2000	Up to 2000

```
summary(seur_filtered$nFeature_originalexp)
```

Step 6: Feature Selection - Identify highly variable genes

```
seur_filtered <- FindVariableFeatures(seur_filtered, selection.method = "vst", nfeatures = 500)
```

Identify 500 most variable genes through “Variance Stabilizing Transformation” (VST).

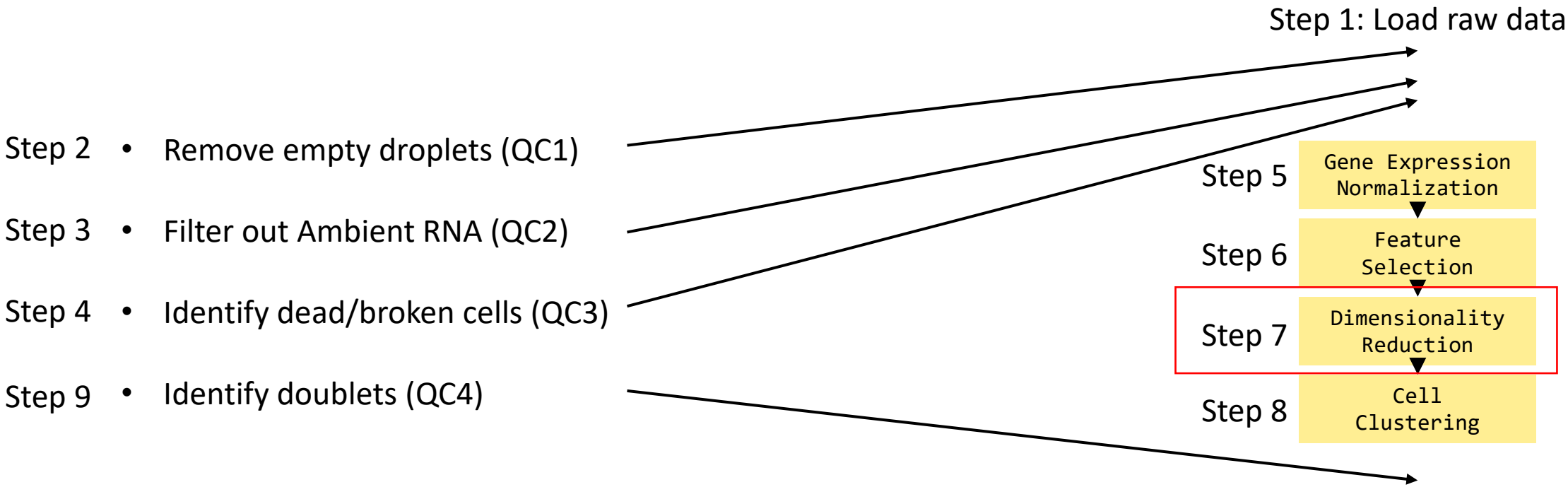
- This is arbitrary.
- You can play with the number and see which makes more sense.
- Usually, it wouldn’t change much from 2000 to 2100, but it is worth testing from 500 to 400/600.

How to determine “nfeatures”?

Total genes detected per cell	Suggested *nfeatures*
< 500	300–500
~1000	500–1000
>2000	Up to 2000

```
summary(seur_filtered$nFeature_originalexp)
```

Step 7: Dimensionality Reduction



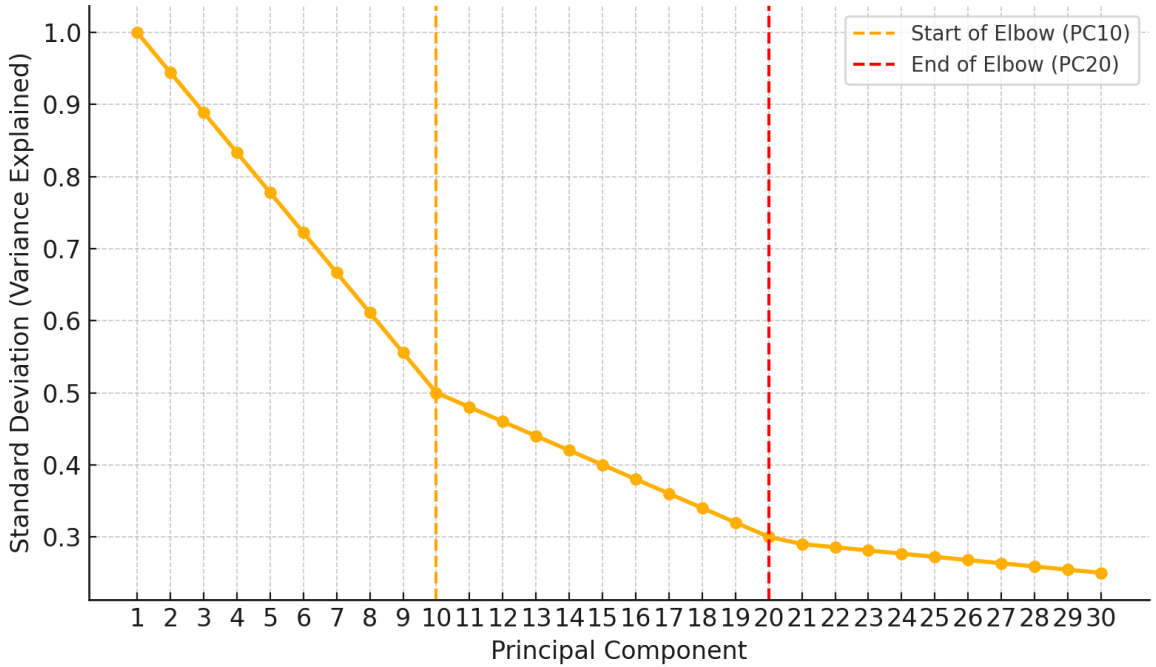
Step 7: Dimensionality Reduction

- Principle Component Analysis (PCA) is the most popular way to reduce dimensionality.

- Why do we want to reduce dimensionality?
  - Remove noise
  - Speed up computation
  - Visualize the data
  - Reveal biological structure

- Determine how many PCs via ElbowPlot

```
ElbowPlot(seur_filtered)
PCs <- 7
```



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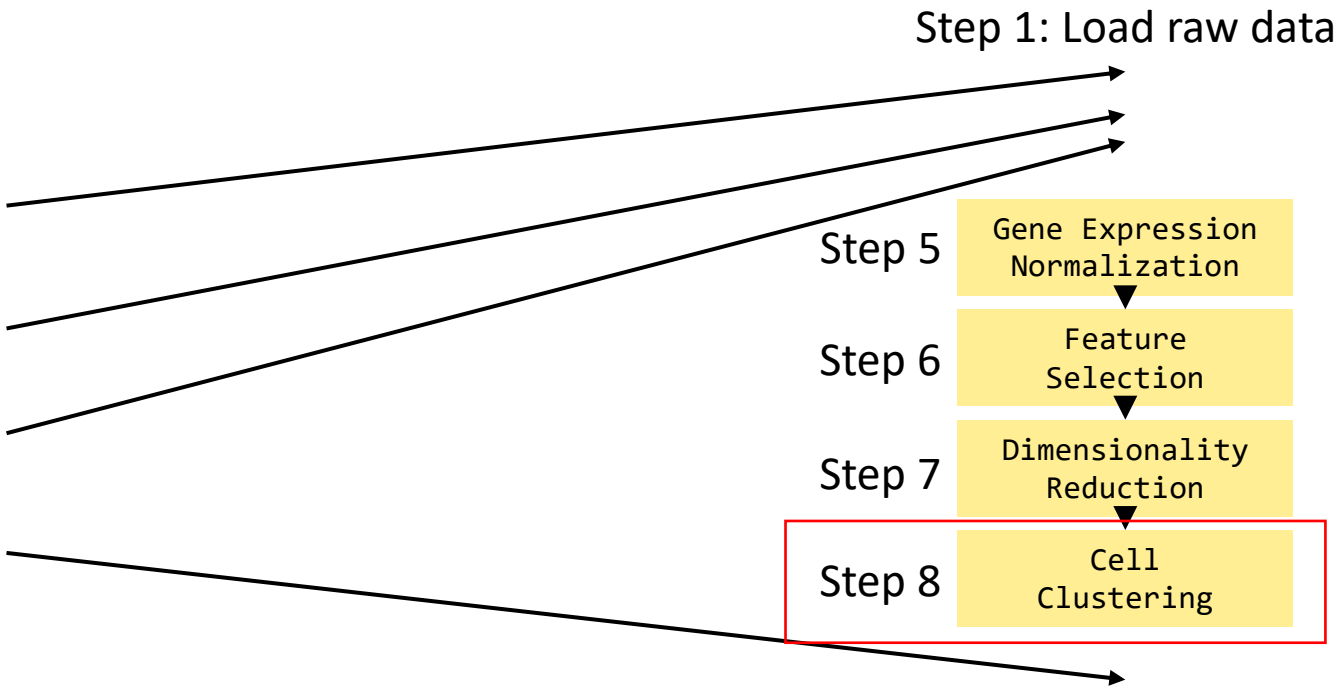
Step 8: Cell Clustering

Step 2 • Remove empty droplets (QC1)

Step 3 • Filter out Ambient RNA (QC2)

Step 4 • Identify dead/broken cells (QC3)

Step 9 • Identify doublets (QC4)

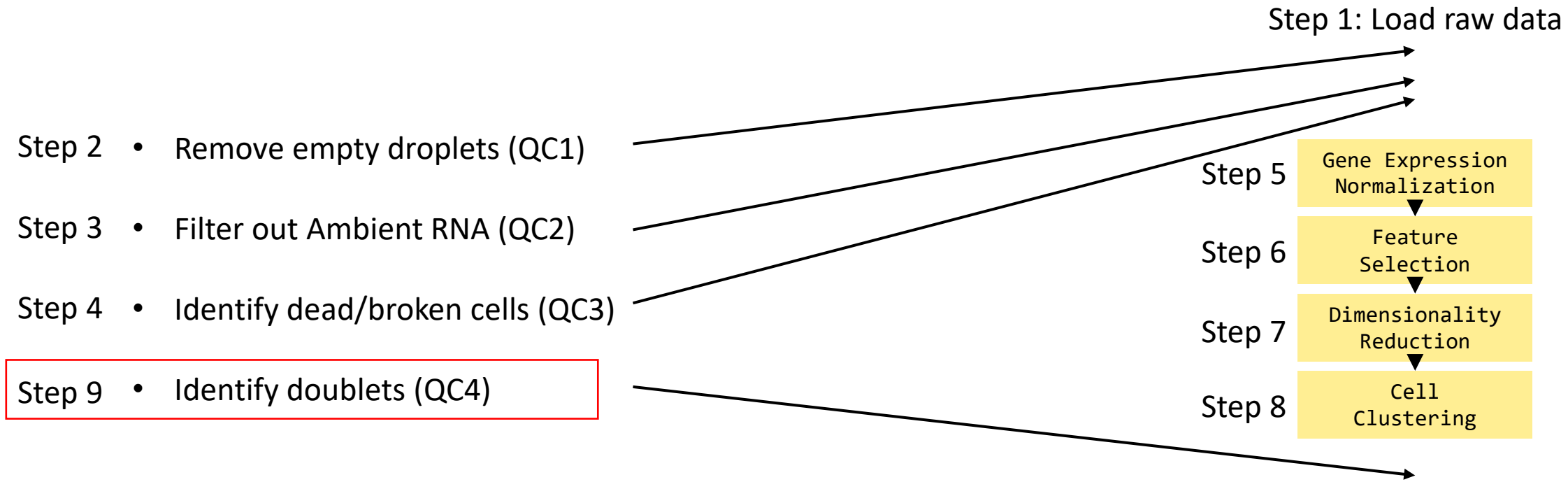


Step 8: Cell Clustering

```
seur_filtered <- FindNeighbors(seur_filtered, dims = 1:PCs)
seur_filtered <- FindClusters(seur_filtered, resolution = 0.3)
```

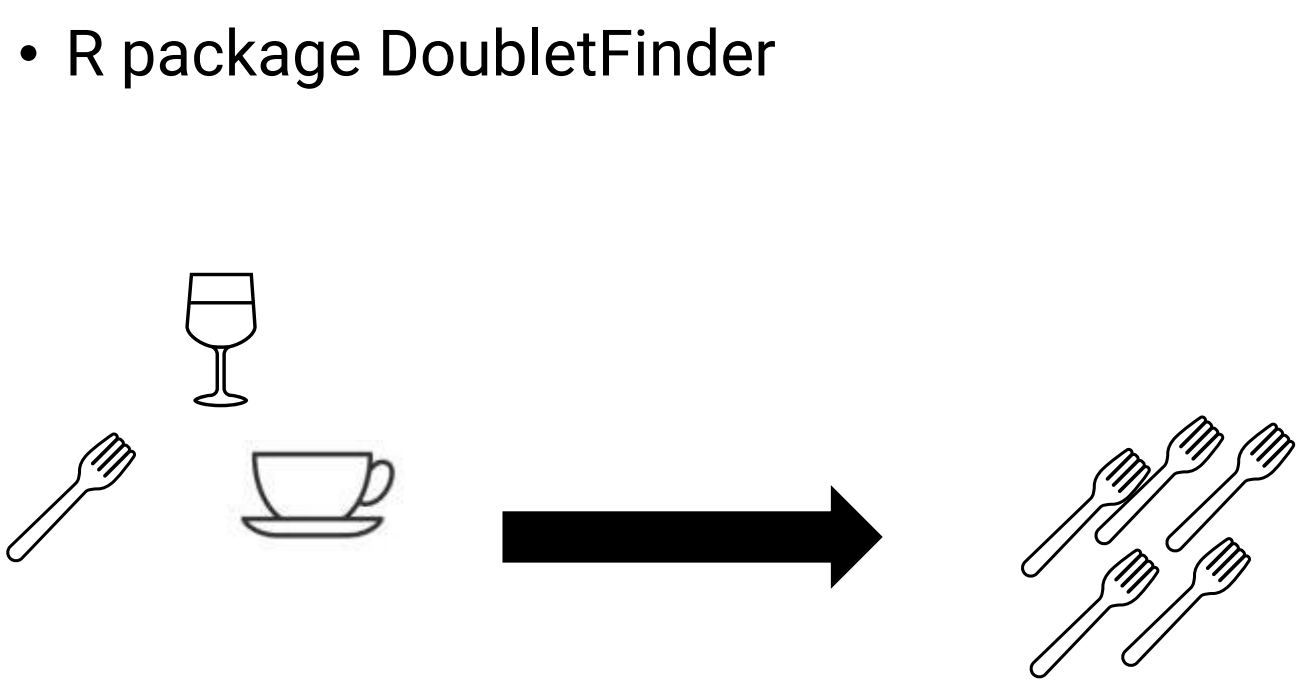
Resolution value	Effect
0.1	Large, coarse clusters
0.3	Moderate clusters (default-ish)
0.8	Smaller, finer clusters
≥1.0	Many small clusters (may over-split)

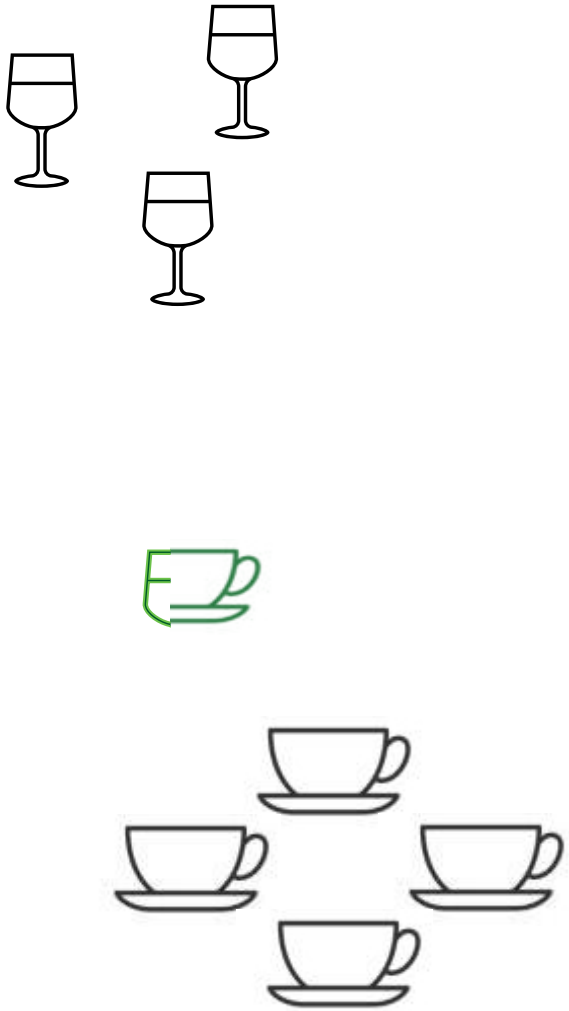
Step 9: Identify Doublets (QC4)



Step 9: Identify the doublets (QC4)

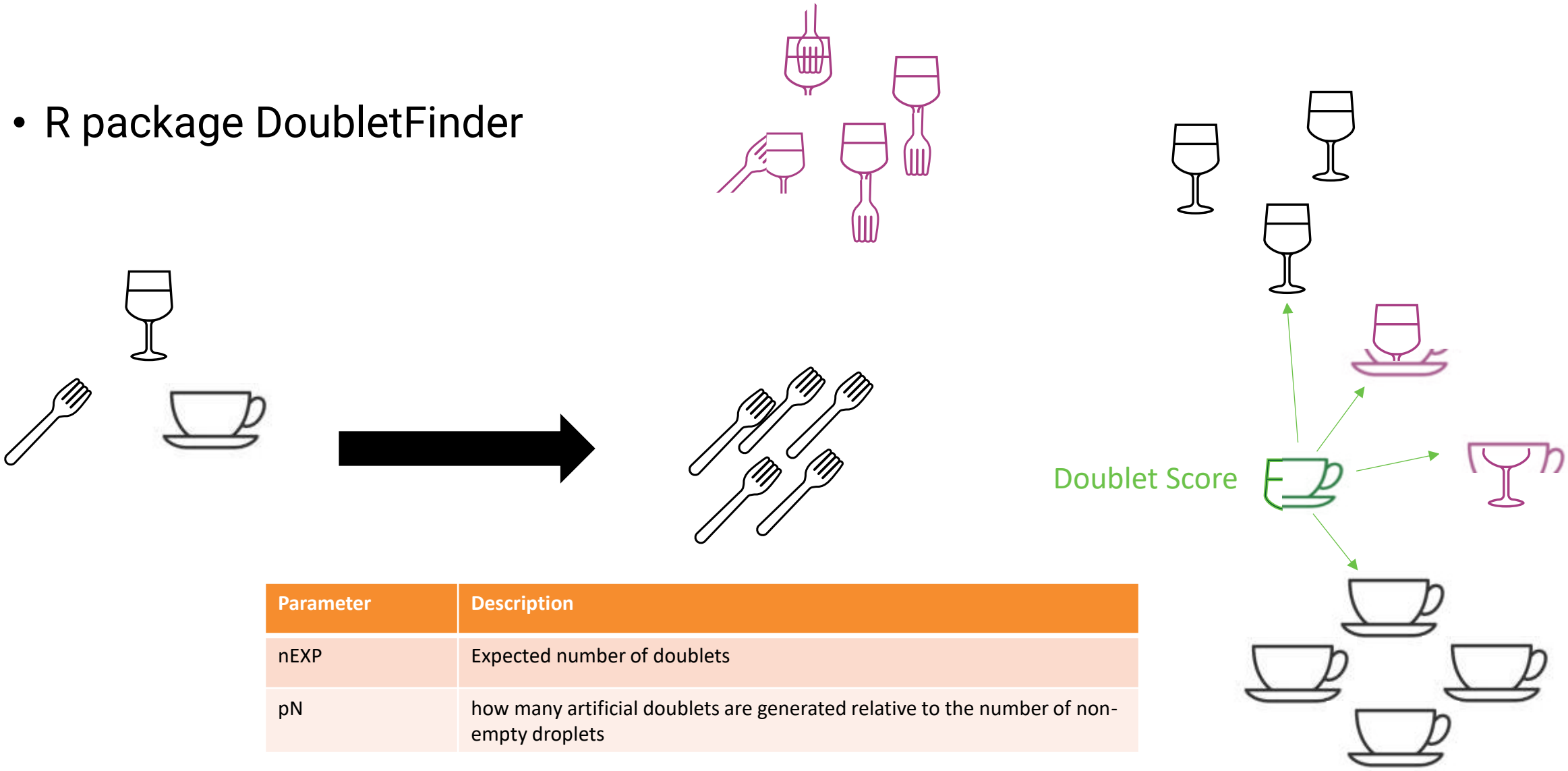
- R package DoubletFinder





Step 9: Identify the doublets (QC4)

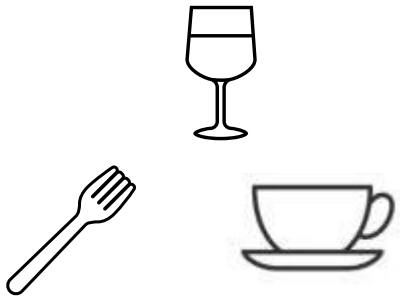
- R package DoubletFinder

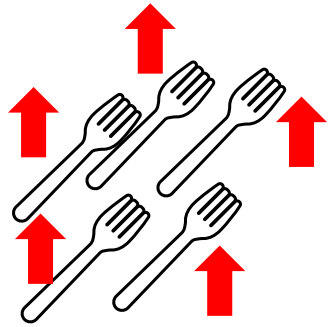


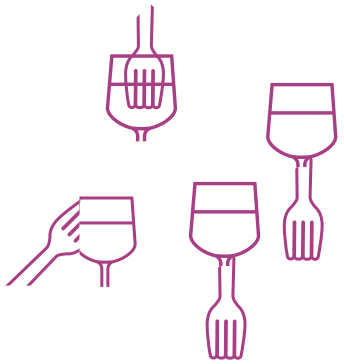
Parameter	Description
nEXP	Expected number of doublets
pN	how many artificial doublets are generated relative to the number of non-empty droplets

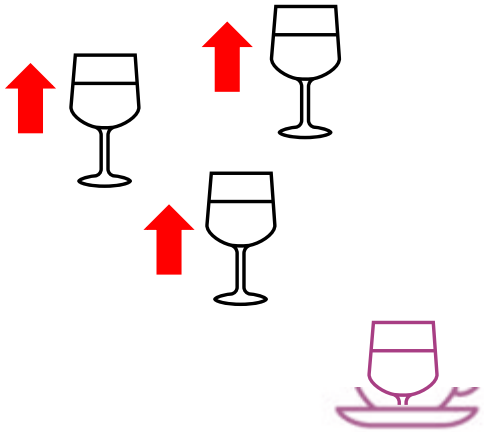
Step 9: Identify the doublets (QC4)

- R package DoubletFinder









Parameter	Description
nEXP	Expected number of doublets
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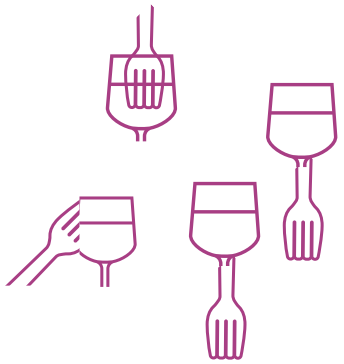
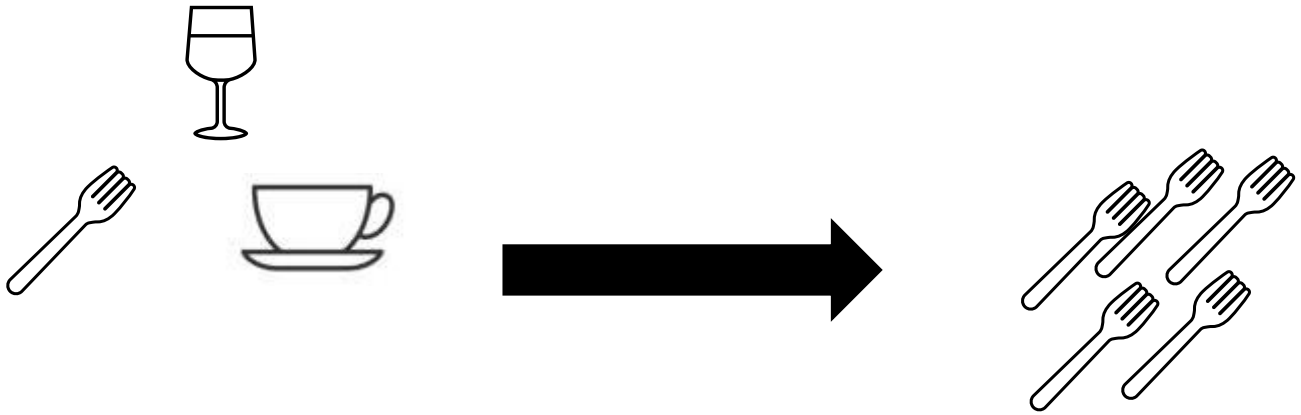
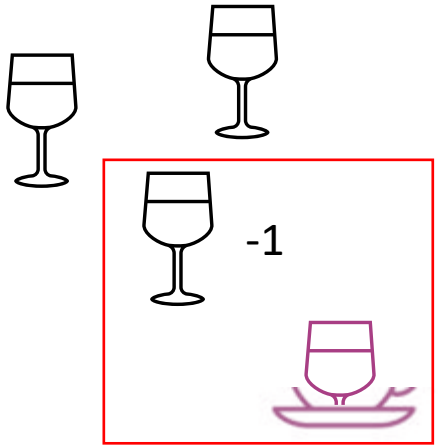
Step 9: Identify the doublets (QC4)

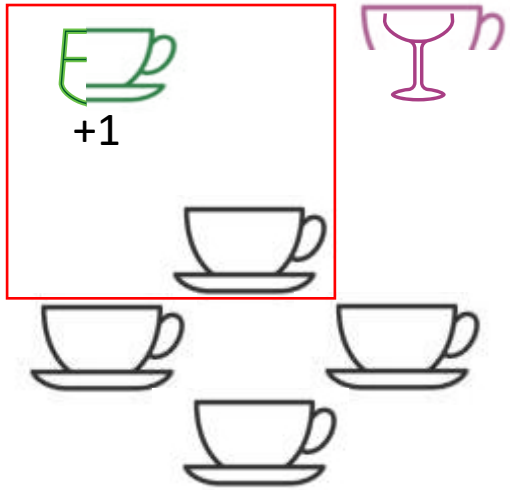
- R package DoubletFinder





pK is too small





Parameter	Description
nEXP	Expected number of doublets
pN	how many artificial doublets are generated relative to the number of non-empty droplets
pK	the neighborhood size parameter, which determines for a droplet, how many nearby droplets are included to calculate its doublet score

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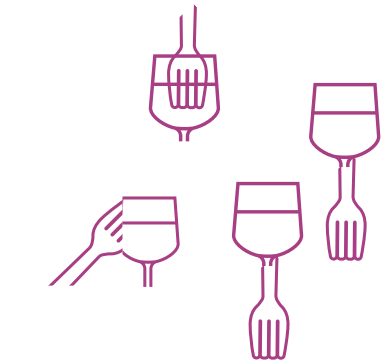
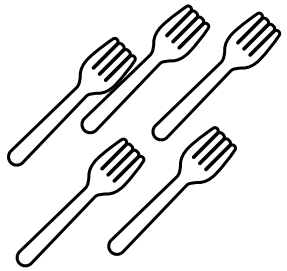
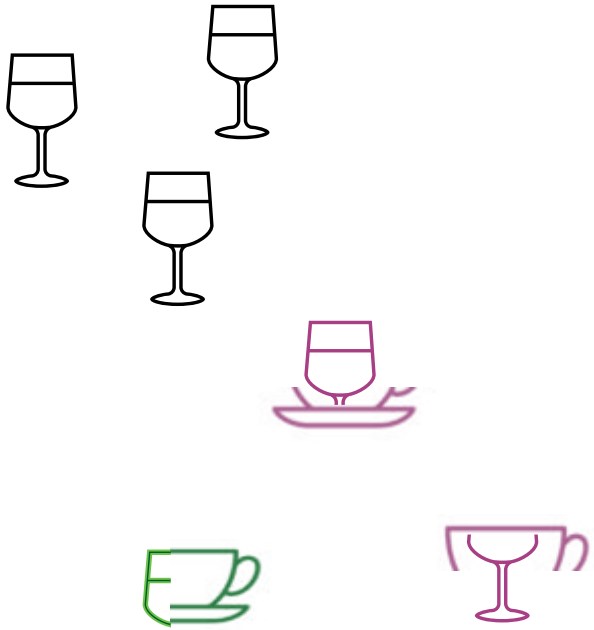
Step 9: Identify the doublets (QC4)

- R package DoubletFinder





pK is too large



Parameter	Description
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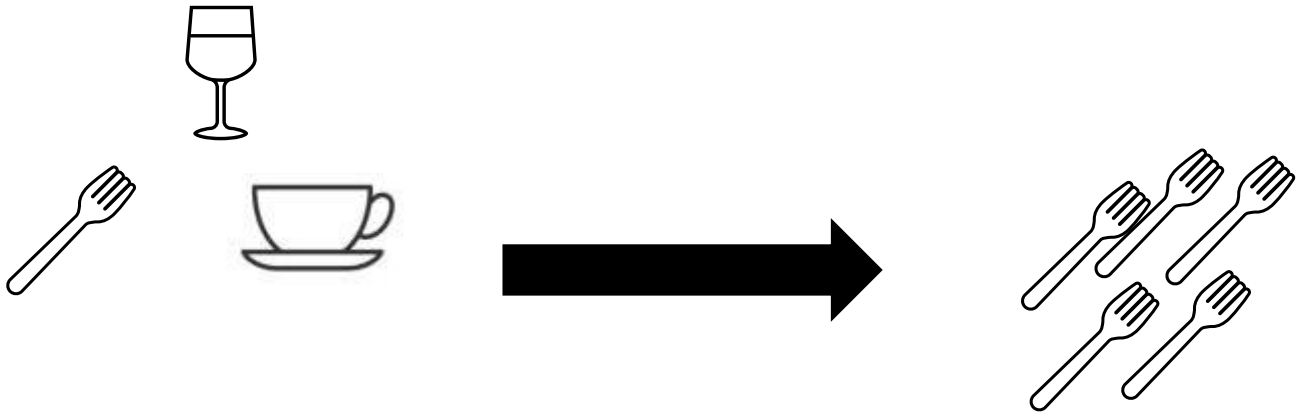
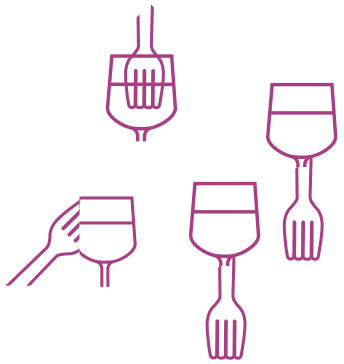
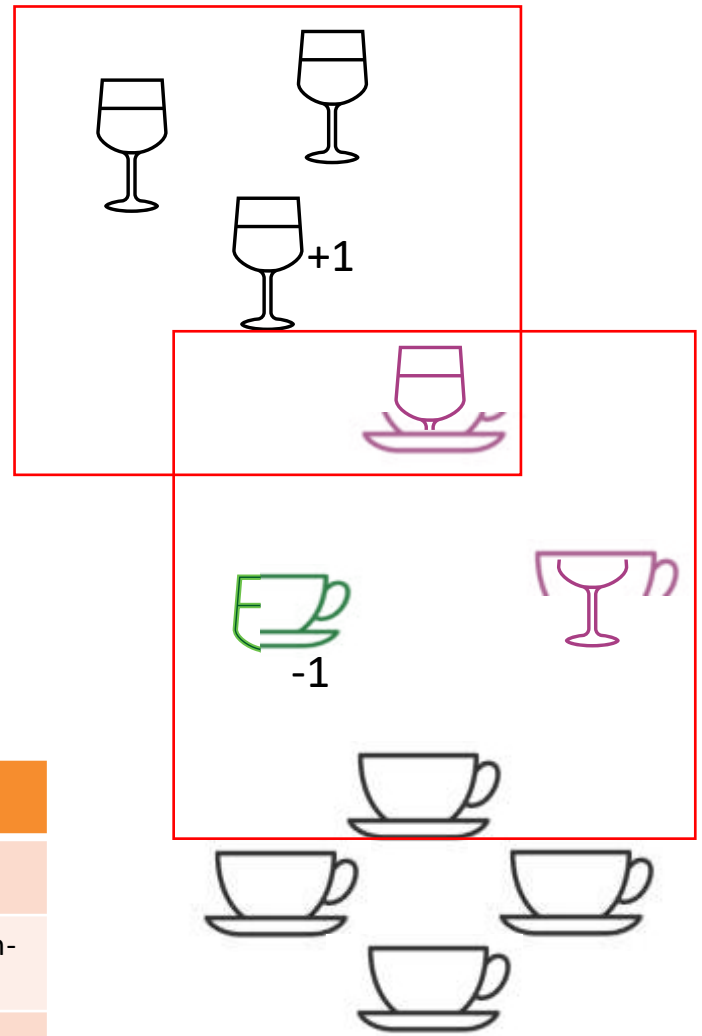
Step 9: Identify the doublets (QC4)

- R package DoubletFinder





pK is just right



Parameter	Description
nEXP	Expected number of doublets
pN	how many artificial doublets are generated relative to the number of non-empty droplets
pK	the neighborhood size parameter, which determines for a droplet, how many nearby droplets are included to calculate its doublet score

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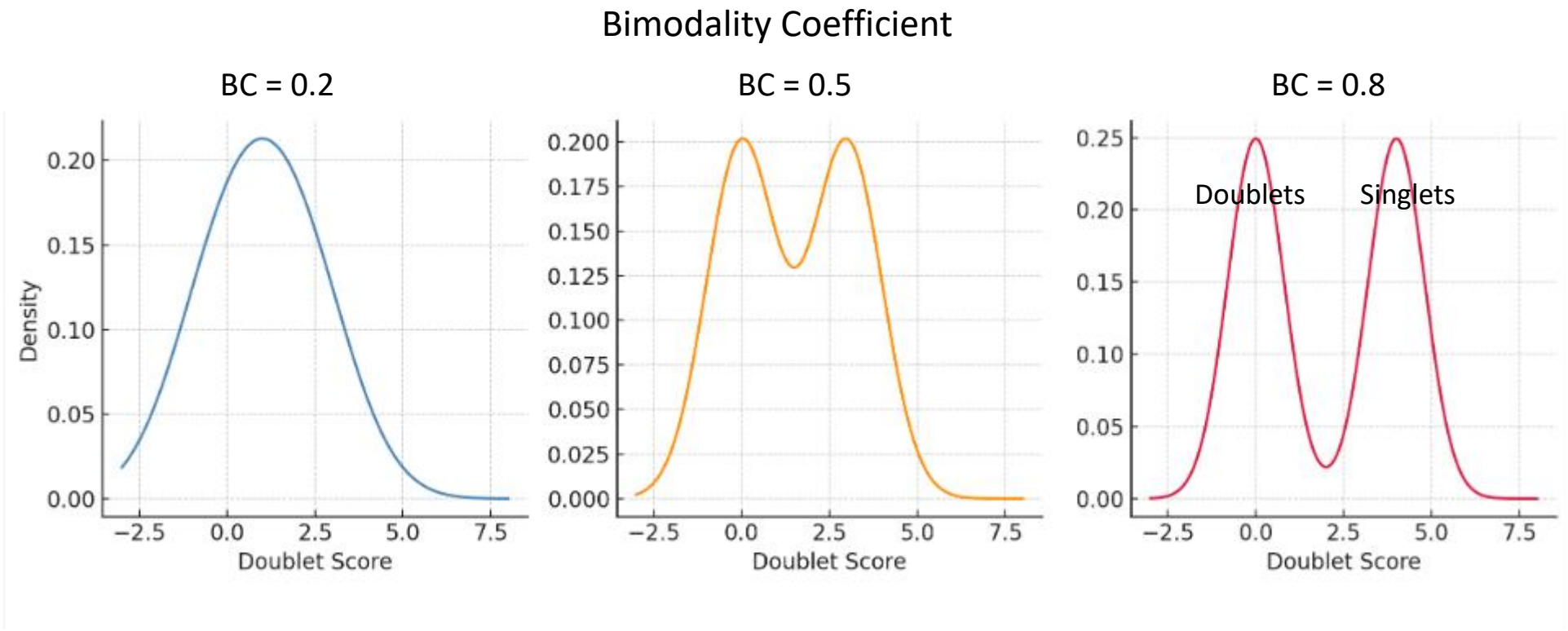
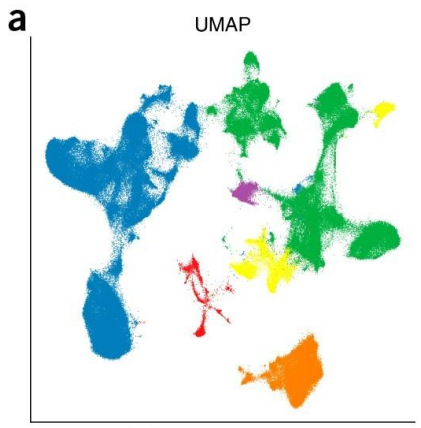
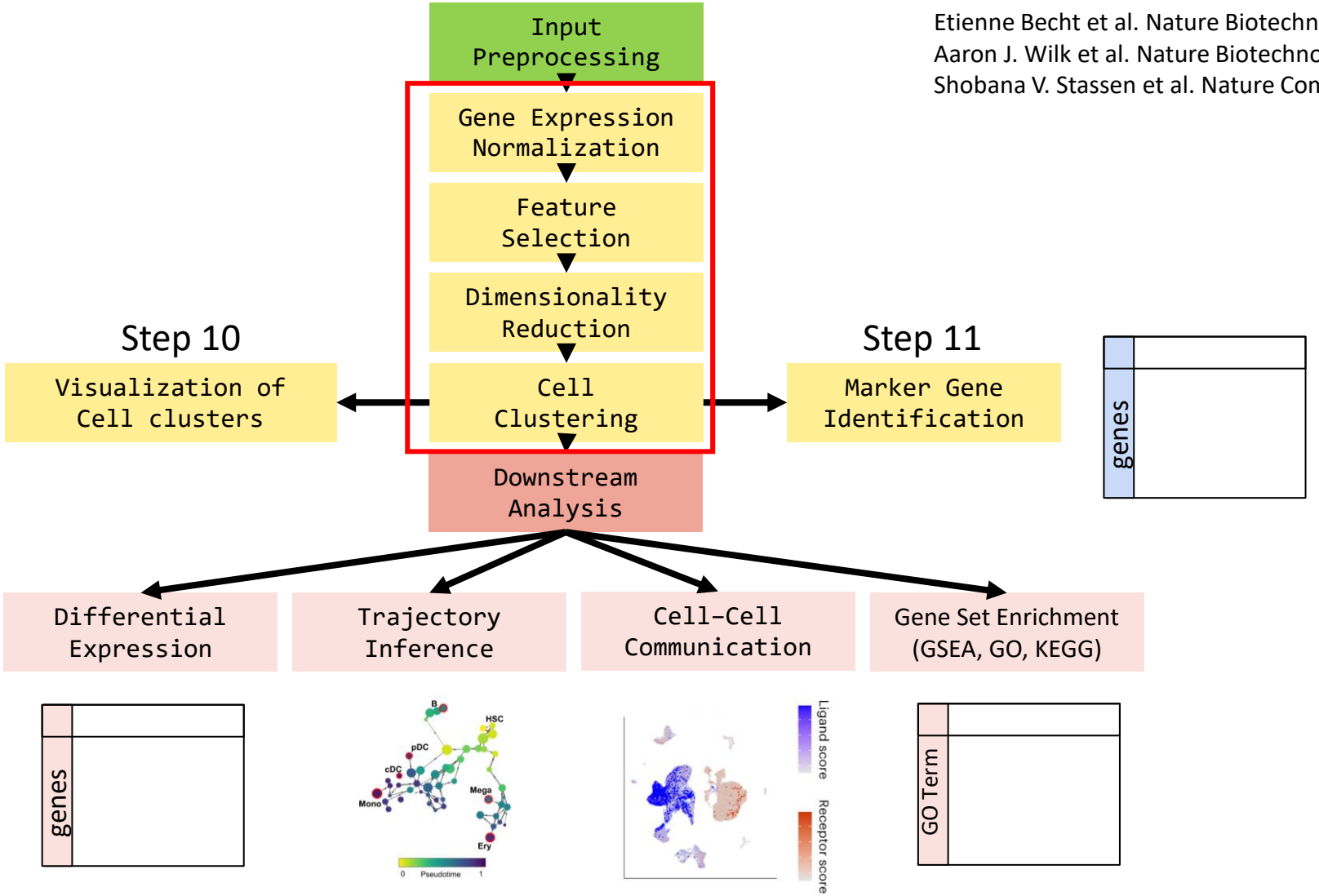
Step 9: Identify the doublets (QC4)

- A series of pK are tested. The best pK is the one that generates highest BC.



Cell Type Annotation





Etienne Becht et al. Nature Biotechnology. 37: 38–44 (2019)
Aaron J. Wilk et al. Nature Biotechnology. 42: 470–483 (2024)
Shobana V. Stassen et al. Nature Communications. 12: 5528 (2021)

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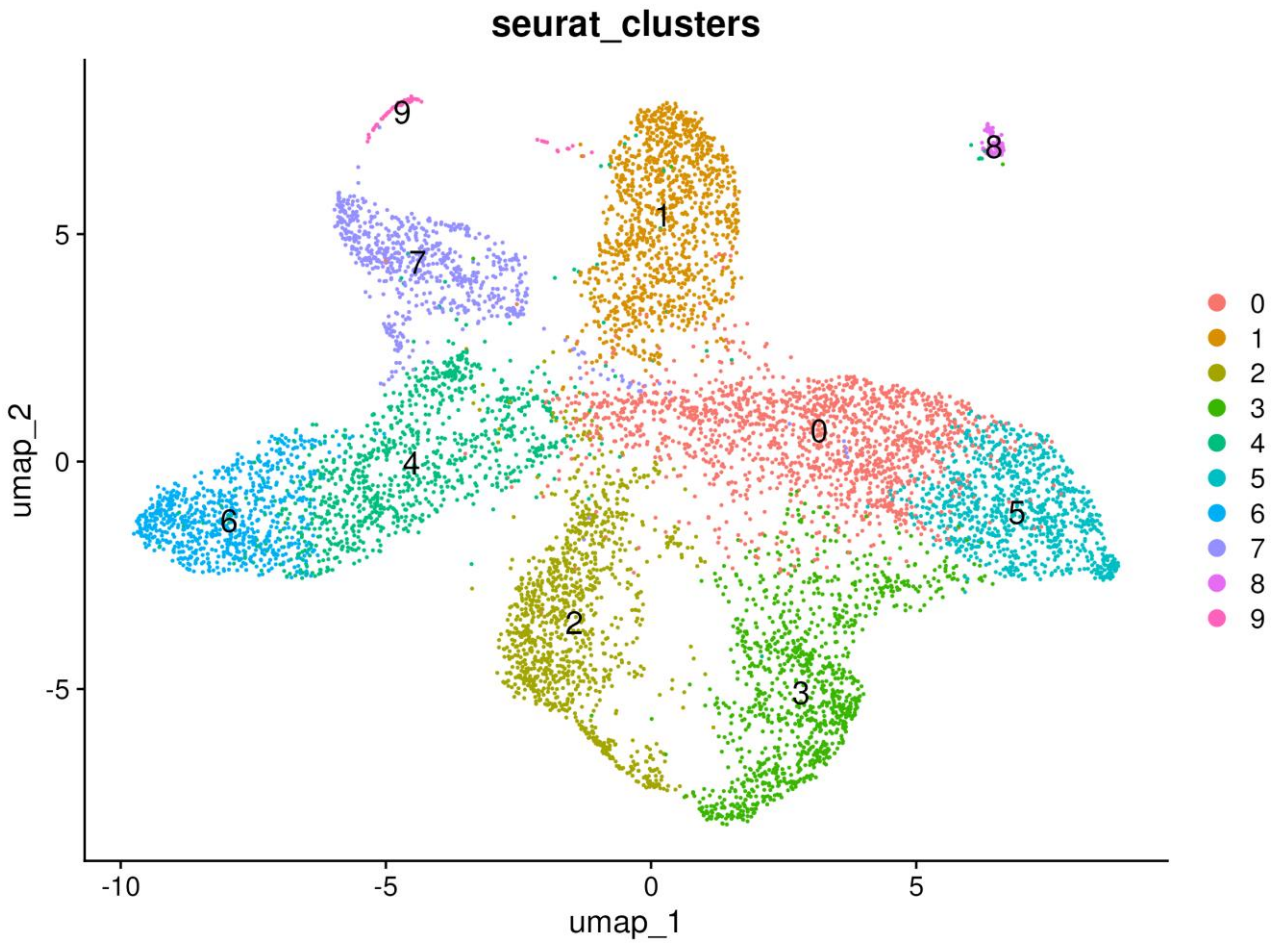
Step 11: Visualization of Cell Clusters

```
seur_filtered <- RunUMAP(seur_filtered, dims = 1:PCs)
picture <- DimPlot(seur_filtered, reduction = "umap", group.by = "seurat_clusters", label = TRUE, label.size = 5)
ggsave("umap_cluster_plot.png", plot = p, width = 8, height = 6, dpi = 300)
```



Step 12: Marker Gene Identification

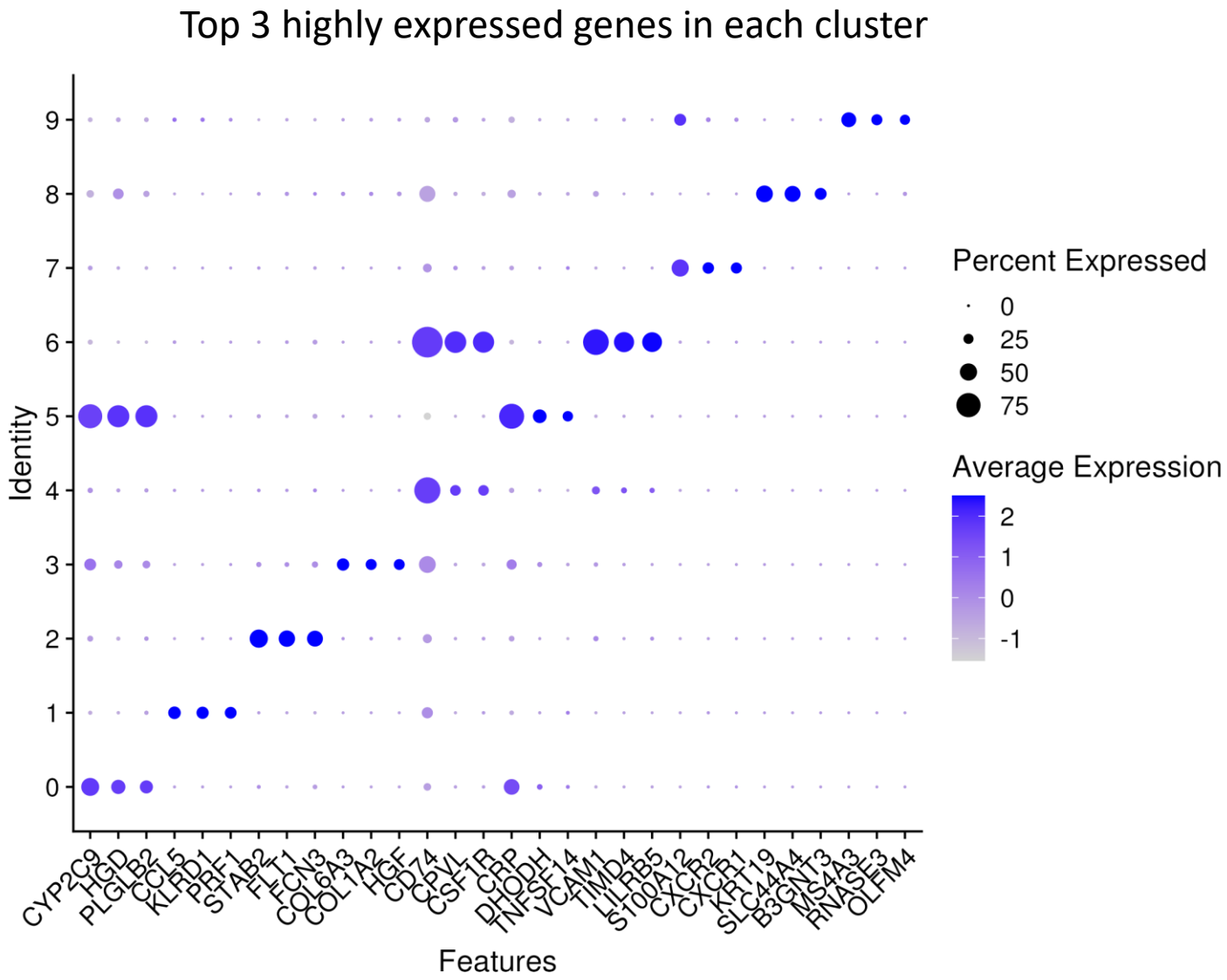
```
markers <- FindAllMarkers(seur_filtered, only.pos = TRUE, min.pct = 0.25, logfc.threshold = 0.25)
```

- min.pct = 0.25 — Only test genes expressed in ≥25% of cells in either cluster
- Logfc.threshold = 0.25 — Only report genes with >1.2-fold change in average expression ($log_2X >= 0.25$)

Situation	Suggested Change
You have **rare or small clusters**	Lower min.pct to 0.1
You're interested in **subtle expression differences***	Lower logfc.threshold to 0.1 or 0.15
You're doing exploratory marker discovery	Lower both slightly to get more candidates

* Early development stage, immune cell activation, autoimmune disease, drug responses/resistance etc

Step 12: Marker Gene Identification



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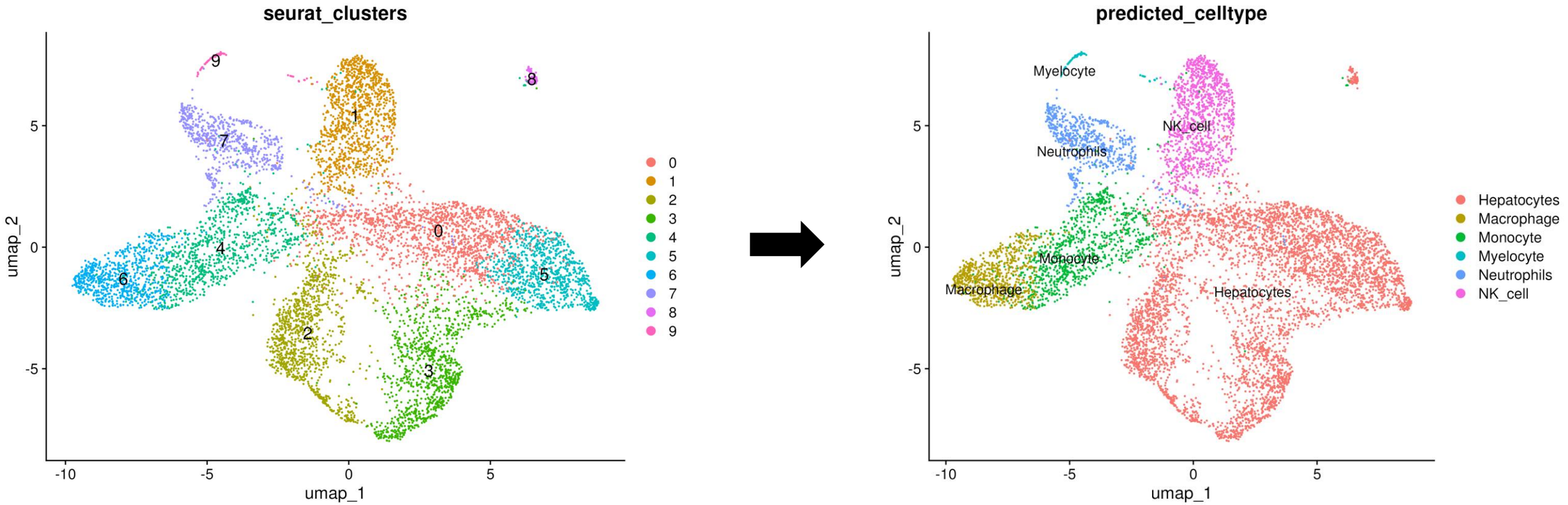
Step 13: Automated Cell-Type Annotation by SingleR



Keep In Mind:

- A good understanding of your sample is essential
  - How was it prepared?
  - What are the expected cell types?
  - What genes should be there and what should not?

- A good understanding of the methodology can help you:
  - Optimize the parameters
  - Assess your results
  - Develop new methods

- There are multiple options/tools for each step. Each has pros & cons with different focus and strength. When choosing the tools, you may want to ask:
  - Does it do a good job with your sample and project?
  - Is it easy to integrated into my pipeline?
  - Does it need customization? If so, does it worth my time?

Question?

Thank you!

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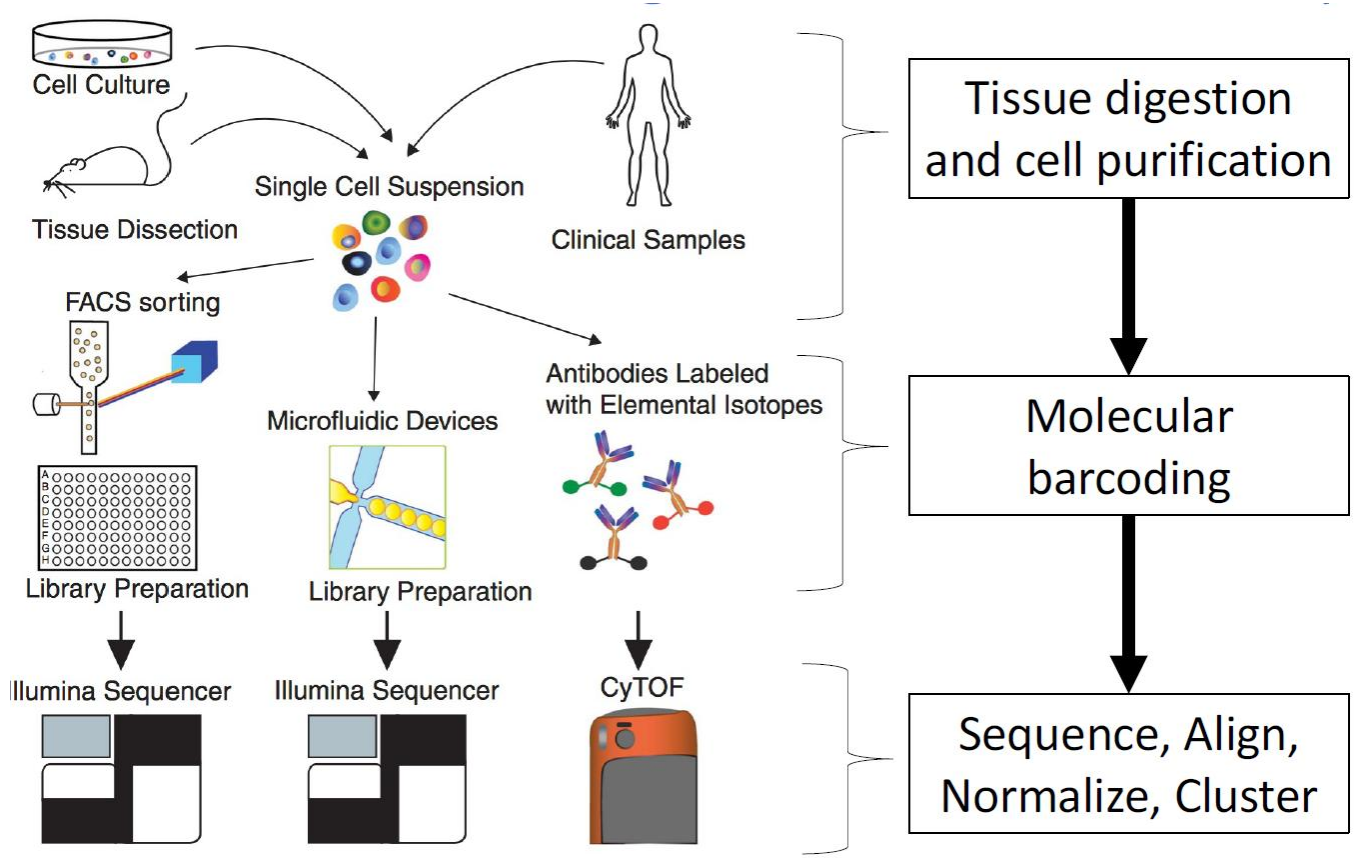
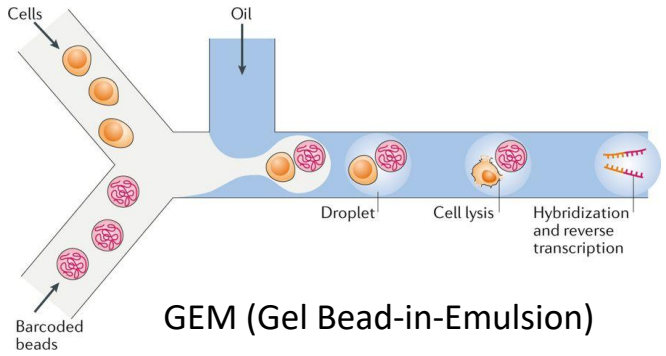
scRNA-seq Wet Lab Pipeline



Proserpio and Lönnberg. Immunol Cell Biol. 2016 Mar;94(3):225-9.

Single Cell Isolation



GEM (Gel Bead-in-Emulsion)

Droplet-based
S. Steven Potter, Nature Reviews Nephrology volume 14, pages479–492 (2018)

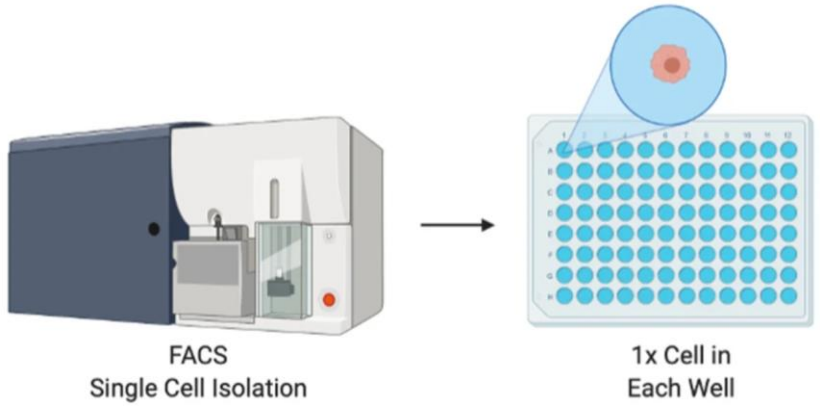
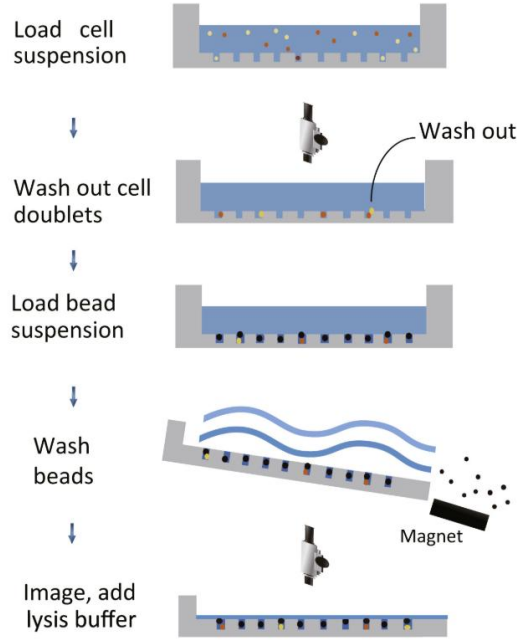
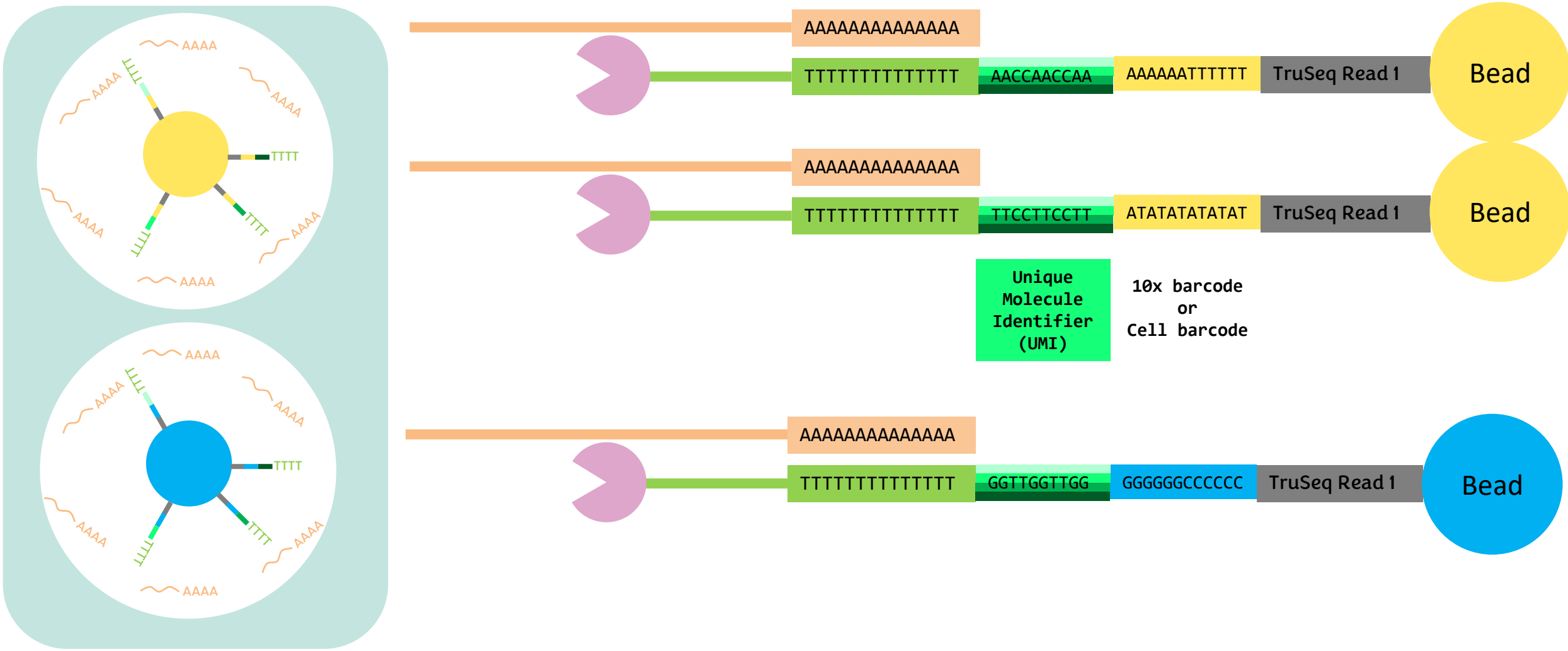


Plate-based
Probst et al., BMC Genomics, 23: 860 (2022)



Microwell-based
Han et al., Cell, 172(5): 1091-1107.e17 (2018)

10x Chromium 3’ scRNA-seq – Reverse Transcription

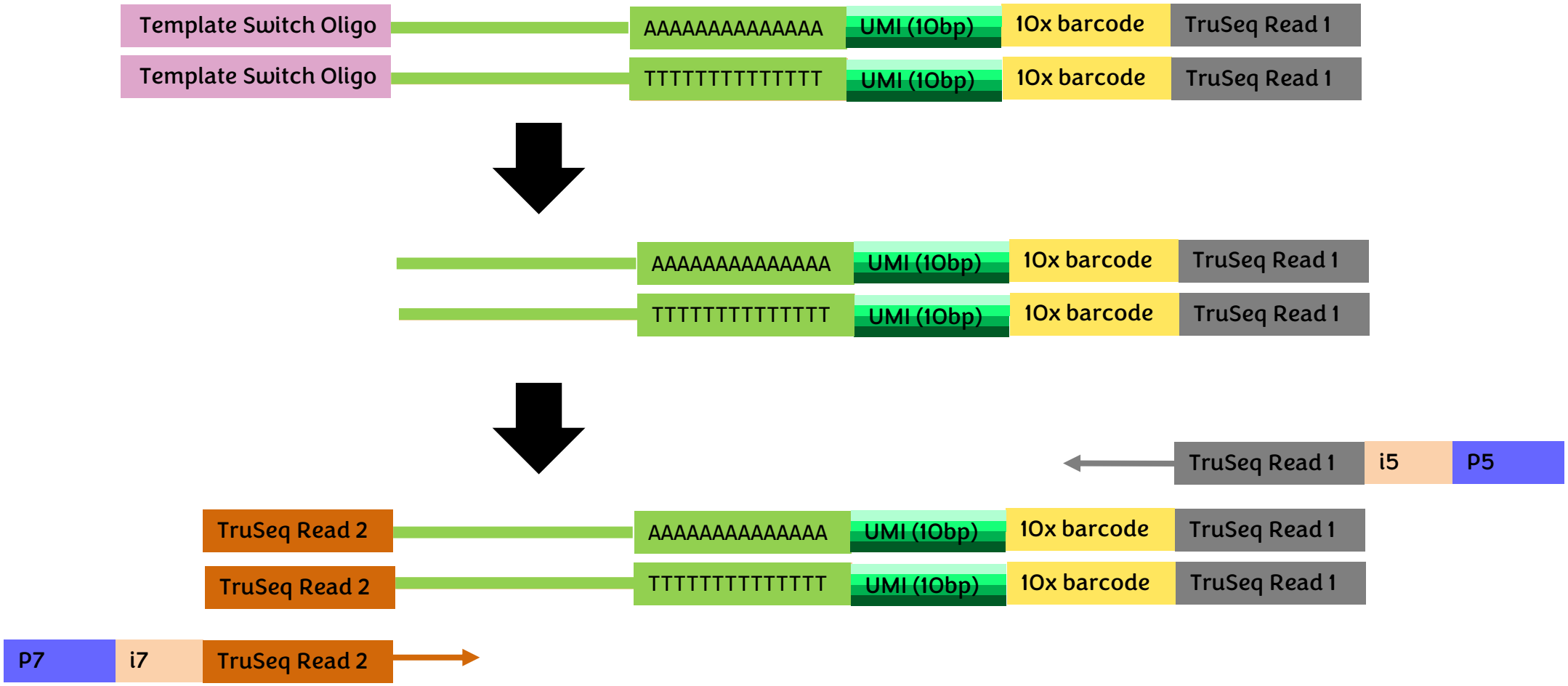


https://www.10xgenomics.com/support/universal-three-prime-gene-expression/documentation/steps/library-prep/chromium-gem-x-single-cell-3-v4-gene-expression-user-guide

10x Chromium 3’ scRNA-seq – Second Strand cDNA

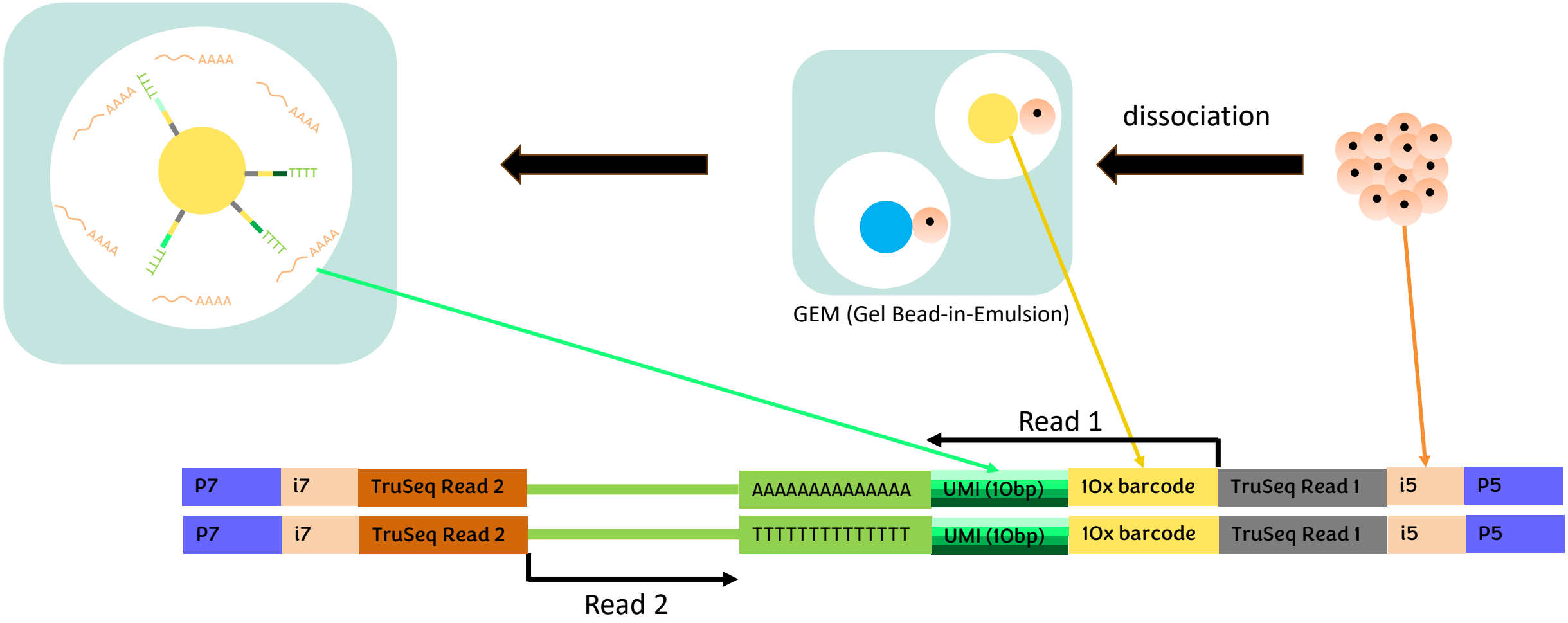
https://www.10xgenomics.com/support/universal-three-prime-gene-expression/documentation/steps/library-prep/chromium-gem-x-single-cell-3-v4-gene-expression-user-guide

10x Chromium 3’ scRNA-seq – Adding sequencing adapter



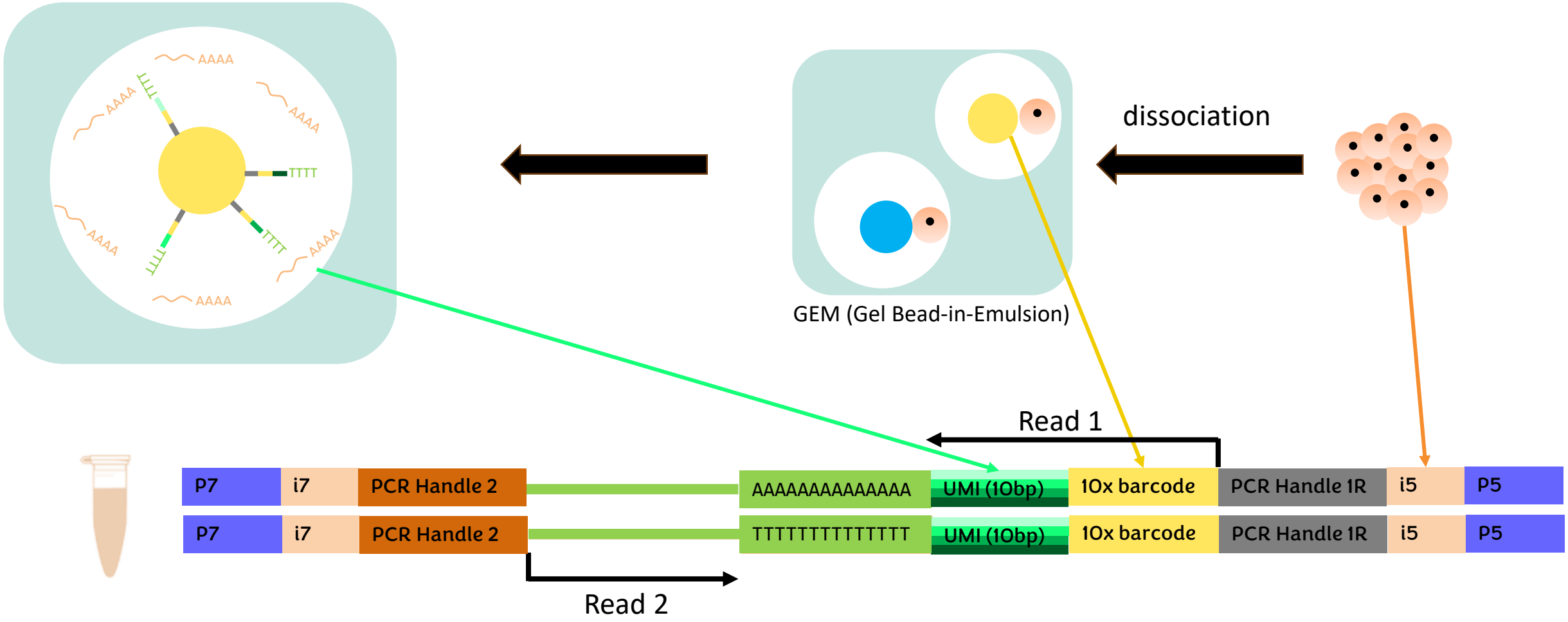
https://www.10xgenomics.com/support/universal-three-prime-gene-expression/documentation/steps/library-prep/chromium-gem-x-single-cell-3-v4-gene-expression-user-guide

10x Chromium 3’ scRNA-seq – Sample, Cell, Molecule

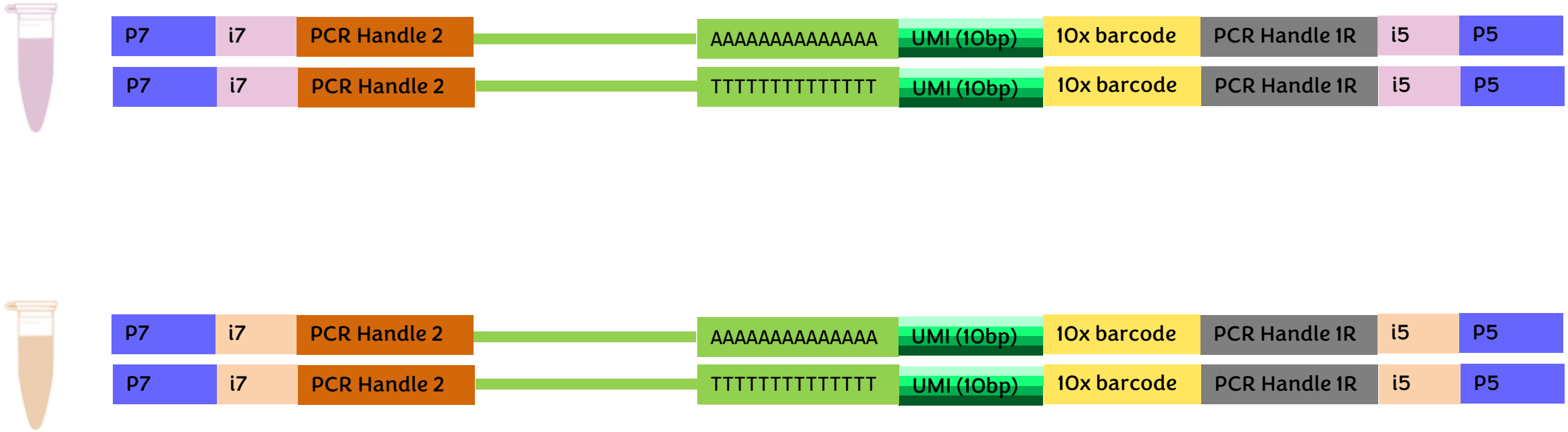


https://www.10xgenomics.com/support/universal-three-prime-gene-expression/documentation/steps/library-prep/chromium-gem-x-single-cell-3-v4-gene-expression-user-guide

10x Chromium 3’ scRNA-seq – One Sample



10x Chromium 3’ scRNA-seq – Multiple Sample



Your Input File: Compressed Fastq

Machine Run ID Lane Tile Coordinate

```
@A00469:87:H5WY2DRX2:1:1101:6247:10324 1:N:0:ATCACG
CCGTATGCGGGGCTCCGATTCCATGTCG
+
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```
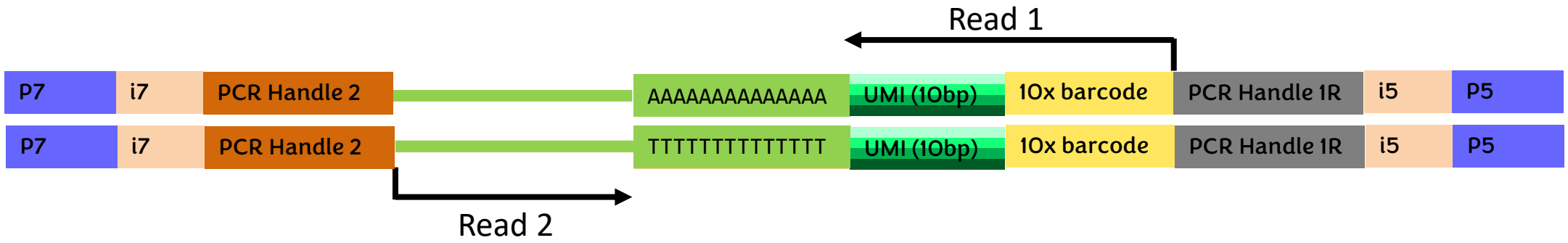
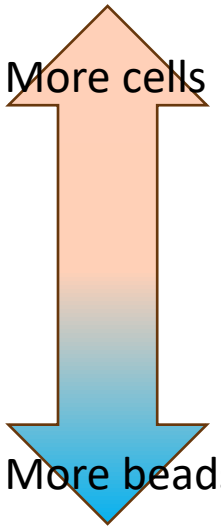




```
@A00469:87:H5WY2DRX2:1:1101:6247:10324 2:N:0:ATCACG
AGACCGGCGGAGGGGCTGGGCGGAGGCTCCGAGAGAGCTGAATGAGGCCTTGGAACTCAAGGATGCCCAGGAGGAGCCGCAGTCAGATCCTAGCGTCGA
+
FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF
```

10x Chromium 3’ scRNA-seq – In Real World

Doublets/multiplets



Empty droplets

Ambient RNA

- Sample type
- Tissue dissociation strategy
- Storage and transport conditions
- Wet-lab strategy
- Whether you did a good job

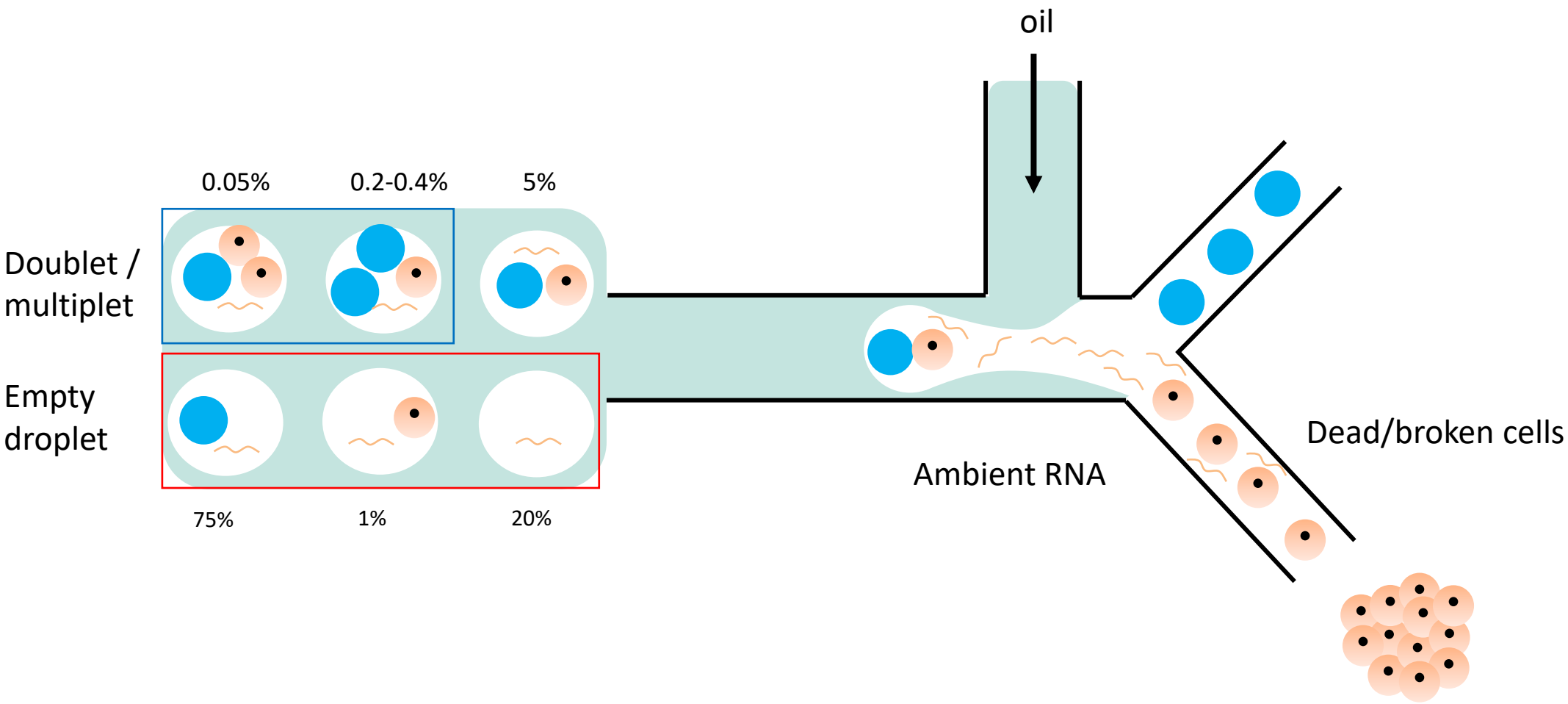
Broken/dead cells

10x Chromium 3’ scRNA-seq – Multiplets vs. Throughput

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~825	~500
~0.8%	~1,650	~1,000
~1.6%	~3,300	~2,000
~2.4%	~4,950	~3,000
~3.2%	~6,600	~4,000
~4.0%	~8,250	~5,000
~4.8%	~9,900	~6,000
~5.6%	~11,550	~7,000
~6.4%	~13,200	~8,000
~7.2%	~14,850	~9,000
~8.0%	~16,500	~10,000

https://kb.10xgenomics.com/hc/en-us/articles/360001378811-What-is-the-maximum-number-of-cells-that-can-be-profiled

Quality Control



Lareau et al. (2020) doi: 10.1038/s41467-020-14667