# From reads mapping to count matrix

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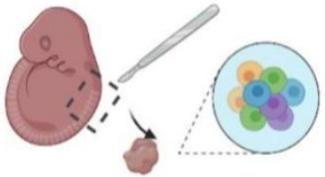
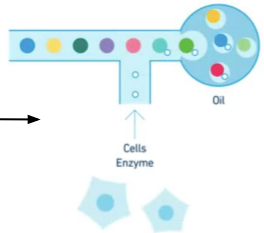
# Recap of 10x scRNAseq library preparation

Tissue dissection + cell dissociation

Cell partitioning + mRNA capture

Library preparation + sequencing



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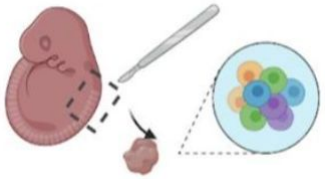

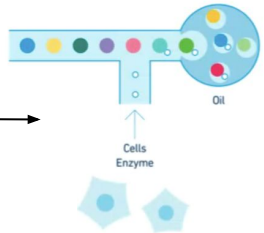


BCL

FASTQs

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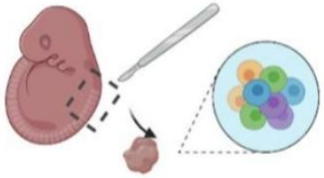
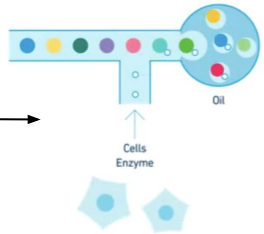


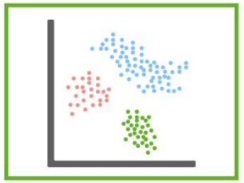



BCL

FASTQs

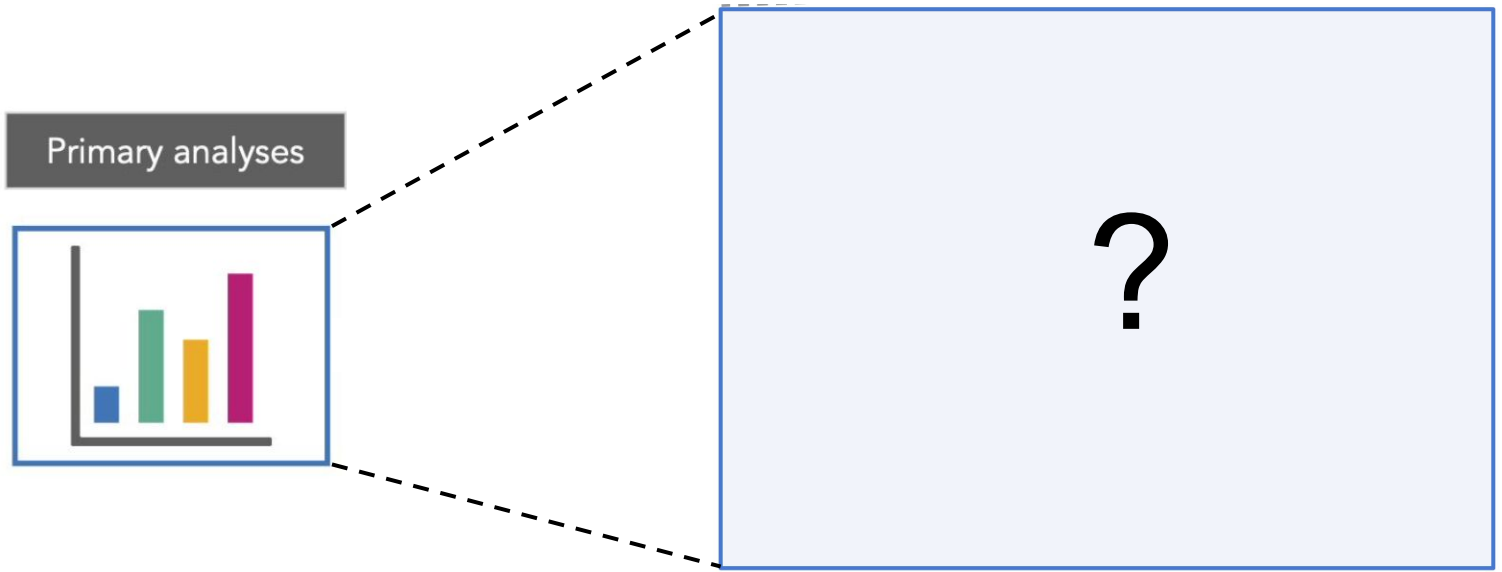
Primary analyses

Secondary analyses



# Quizz 1

## What are the main steps before getting to the count matrix ?



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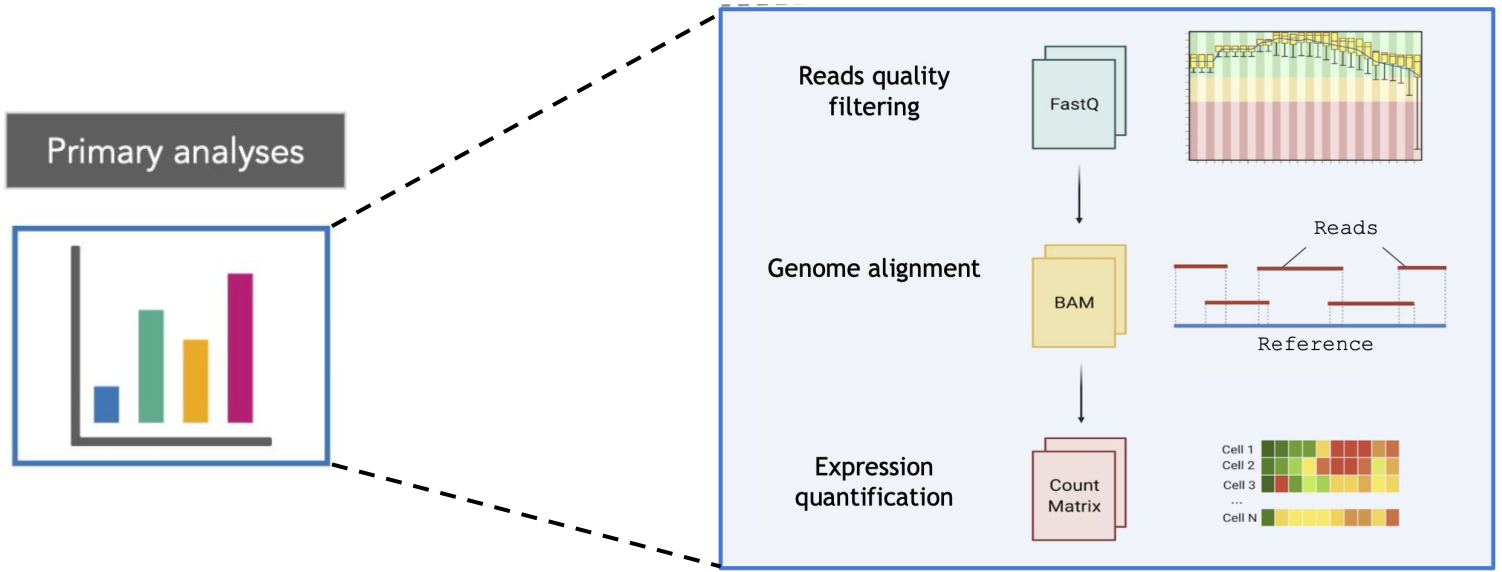
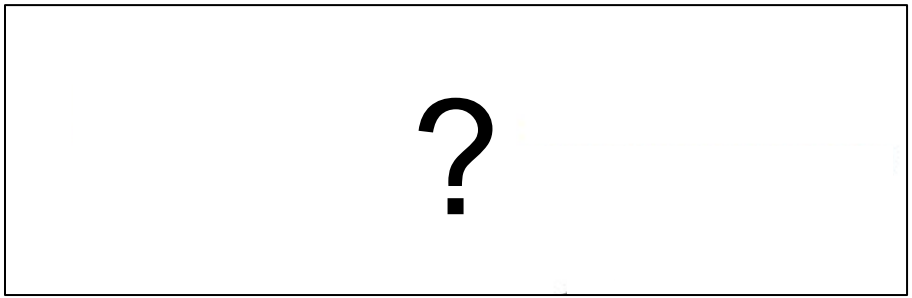


# Quizz 2

## How are the reads from 10x Genomics organised ?

The sequenced library



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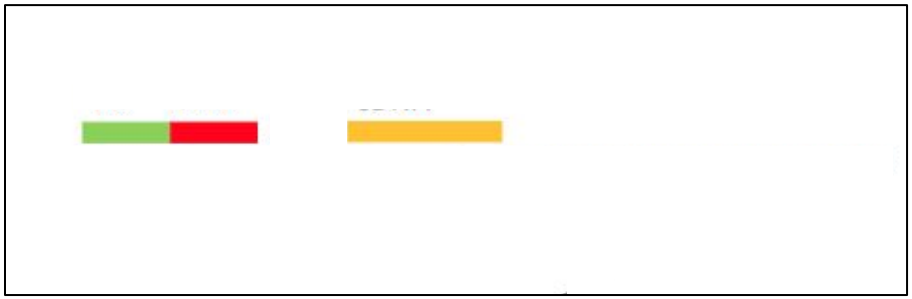

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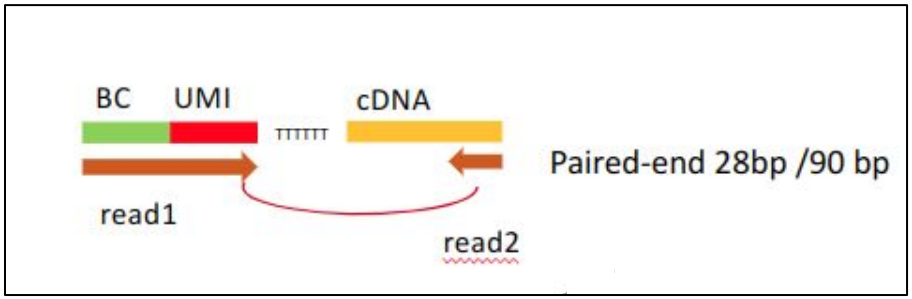


Read 1: unique cell barcode (16 nt) + UMI (12 nt)

Read 2: RNA 3' sequence

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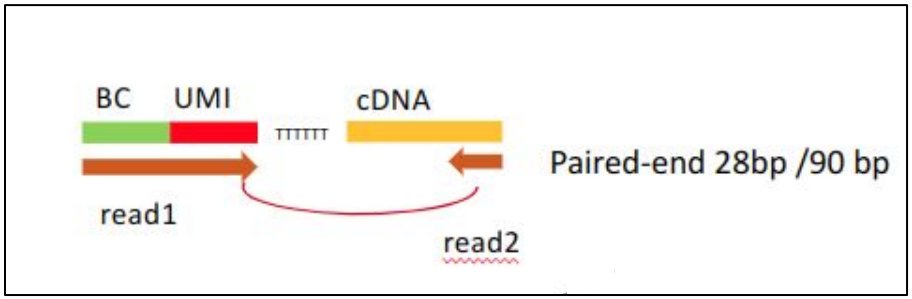
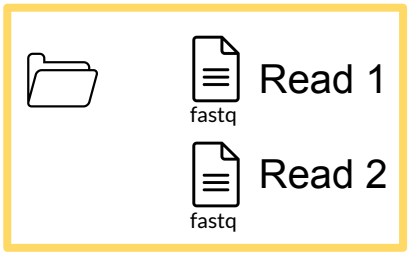


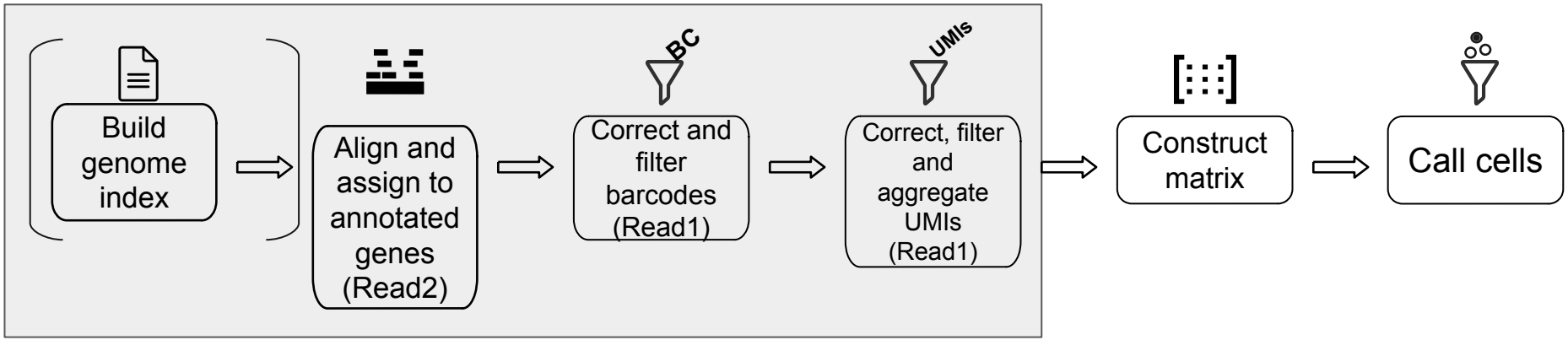

10x GENOMICS




Read 1: unique cell barcode (16 nt) + UMI (12 nt)

Read 2: RNA 3' sequence

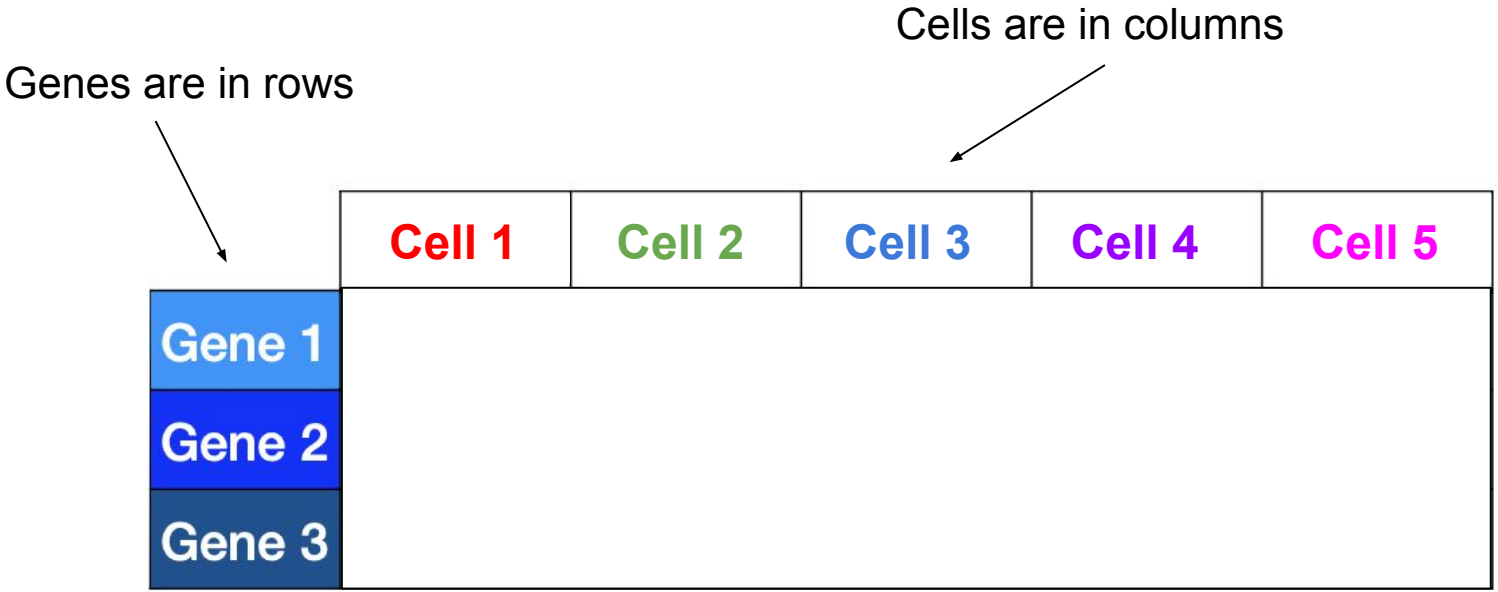
# Primary analysis : overview of the workflow



# Organisation of the scRNA-seq course

- From cells to nucleotide sequences (reads)
  - focus on the 10X genomics technology
  - how are the reads organised
- Preprocessing : from reads to raw count matrix
  - quality check (FASTQC)
  - mapping (STAR)
  - how is annotation used
  - barcode and UMI treatment
  - visualizing the reads
  - constructing the count matrix
  - call cells / empty droplets filtering

# What is the count matrix ?



# What is the count matrix ?

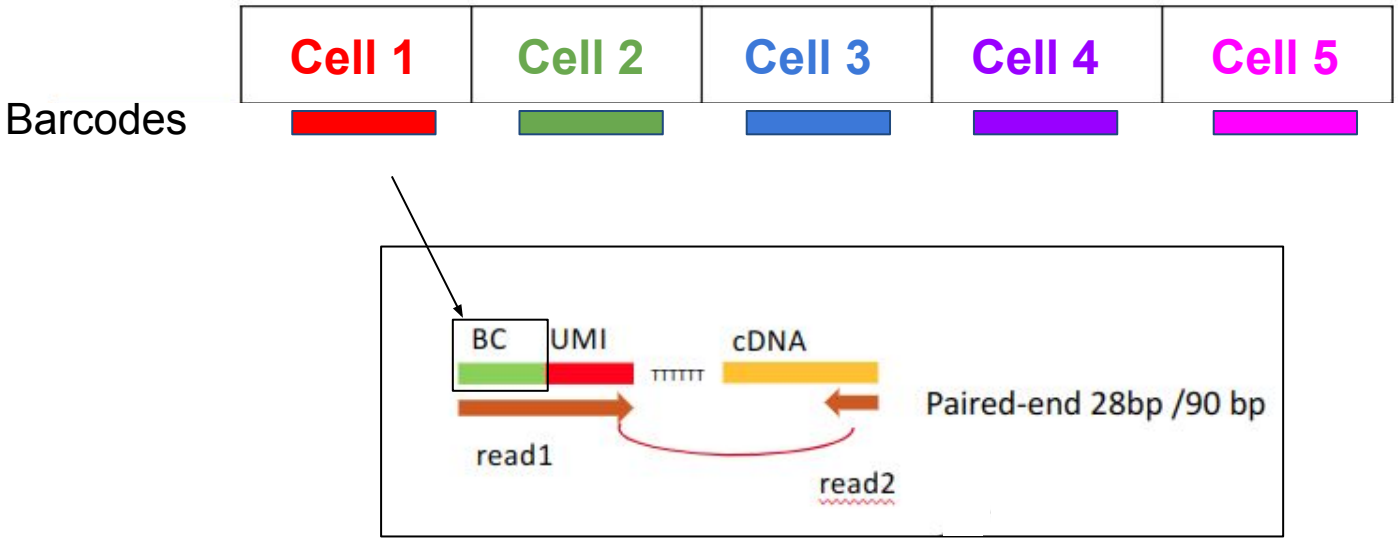
Cells are in columns

Genes are in rows

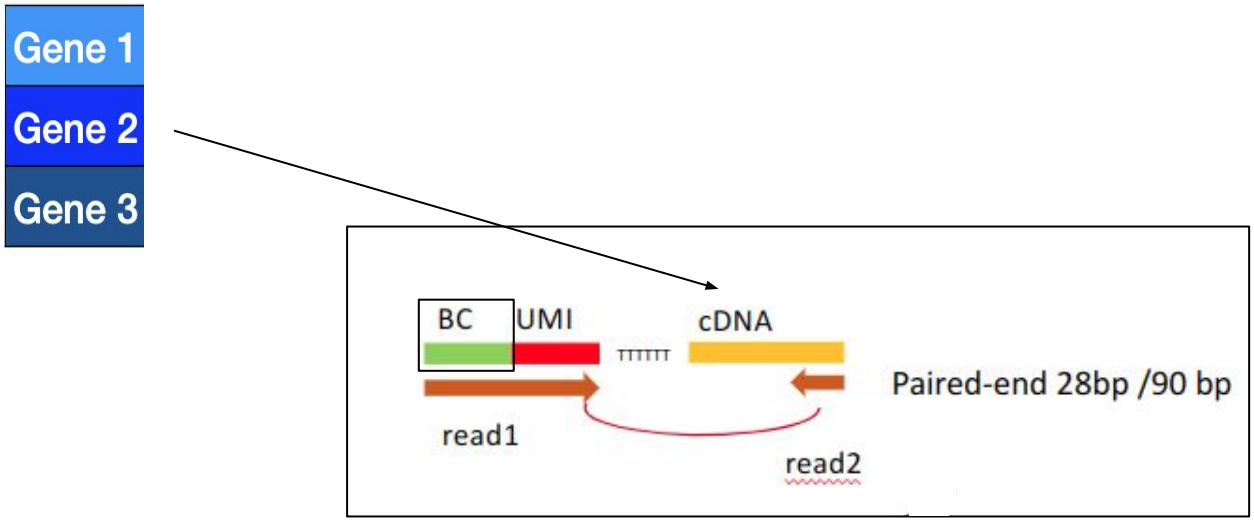
| | Cell 1 | Cell 2 | Cell 3 | Cell 4 | Cell 5 |
|---|---|---|---|---|---|
| Gene 1 | 0 | 0 | 0 | 0 | 0 |
| Gene 2 | 1 | 0 | 4 | 2 | 0 |
| Gene 3 | 0 | 8 | 1 | 0 | 1 |

Content of the table :
gene counts (expression levels)

# Each cell is represented by a valid barcode (read1)

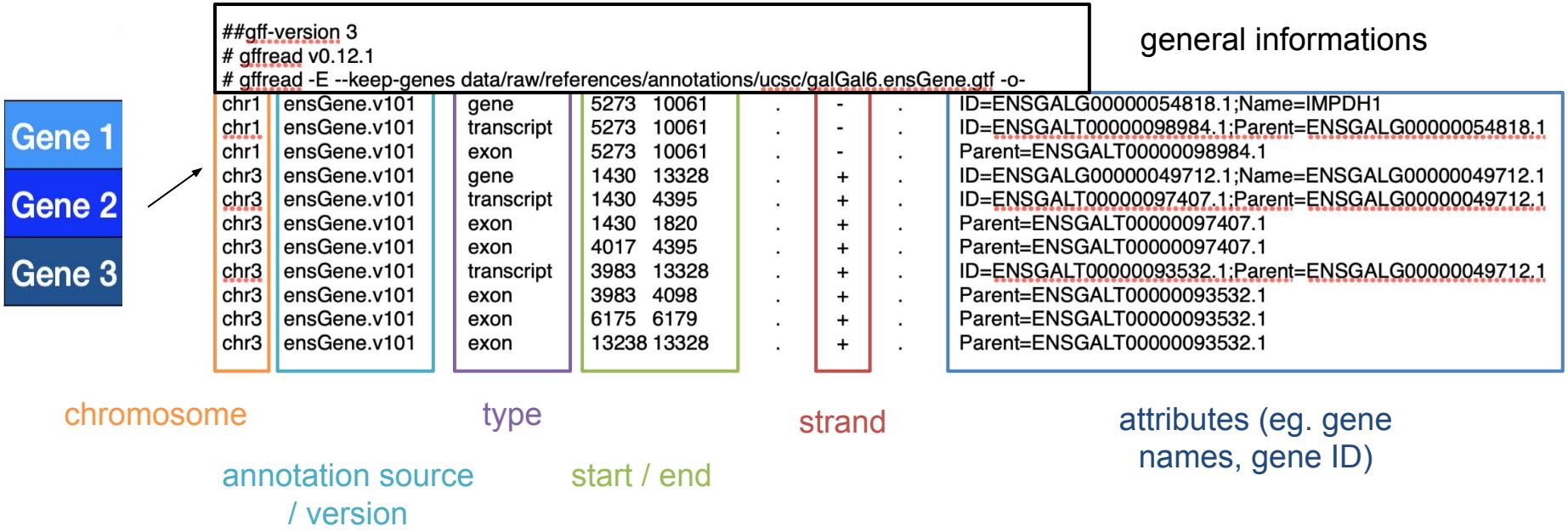


# Each read2 is assigned to a gene after the mapping

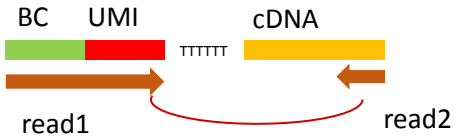


# Gene names are taken from your annotation

## Structure of a GFF3 file (annotation file)



# How are the reads counted ?



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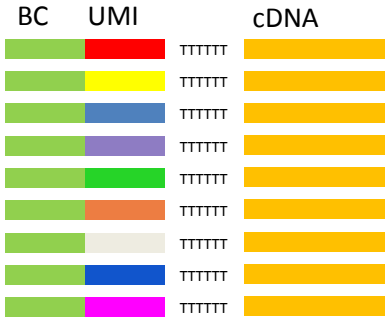


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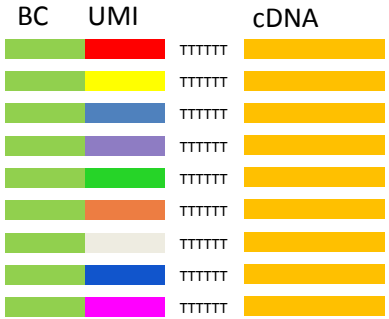


One cell (1 BC)

One gene, detected 9 times
(1 cDNA - 9 UMI)

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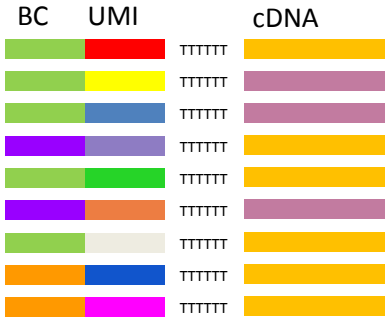


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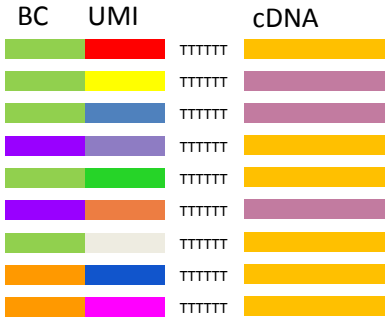


3 cells (3 BC)

2 genes (2 cDNA)

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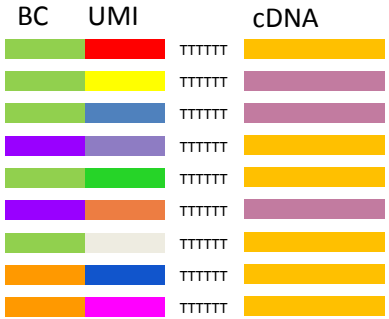
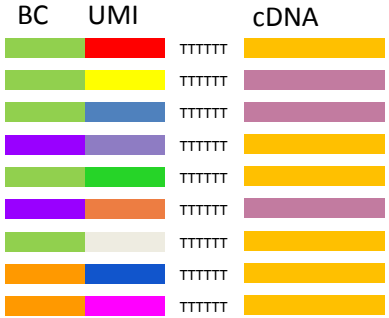



3 cells (3 BC)

2 genes (2 cDNA)

In practice, the count in the matrix corresponds to the number of **UMI** per barcode per gene

# How are the reads counted ?




3 cells (3 BC)

2 genes (2 cDNA)

| | Cell 1 | Cell 2 | Cell 3 |
|---|---|---|---|
| **Gene 1** | | | |
| **Gene 2** | | | |

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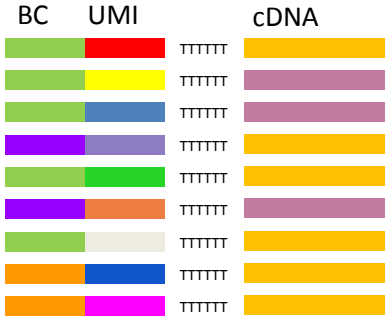


3 cells (3 BC)

2 genes (2 cDNA)

| | Cell 1 | Cell 2 | Cell 3 |
|---|---|---|---|
| **Gene 1** | **3** | **1** | **2** |
| **Gene 2** | **2** | **1** | **0** |

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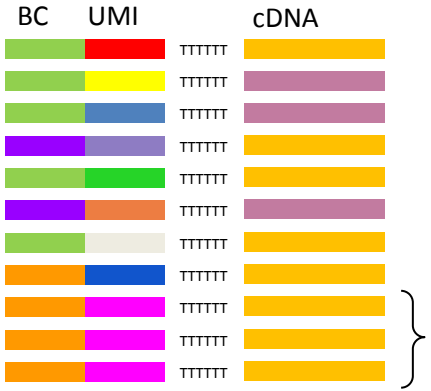


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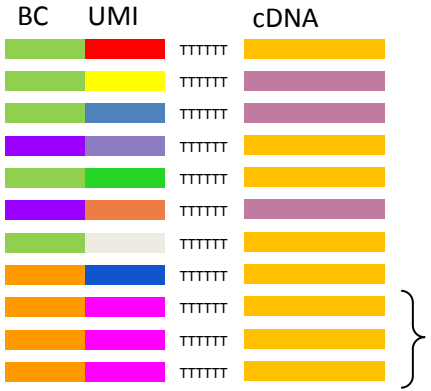


Reads with the same BC+UMIs are assigned to the same gene (originate from 1 unique RNA molecule) :
**they count as 1**

The UMIs are used to correct for
**amplification artefacts**

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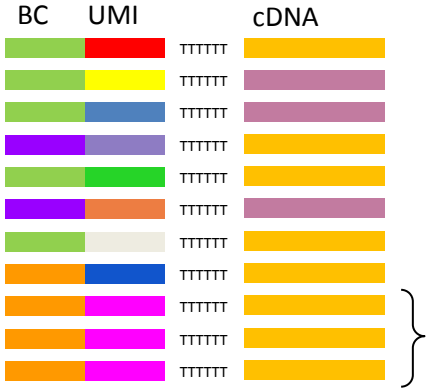


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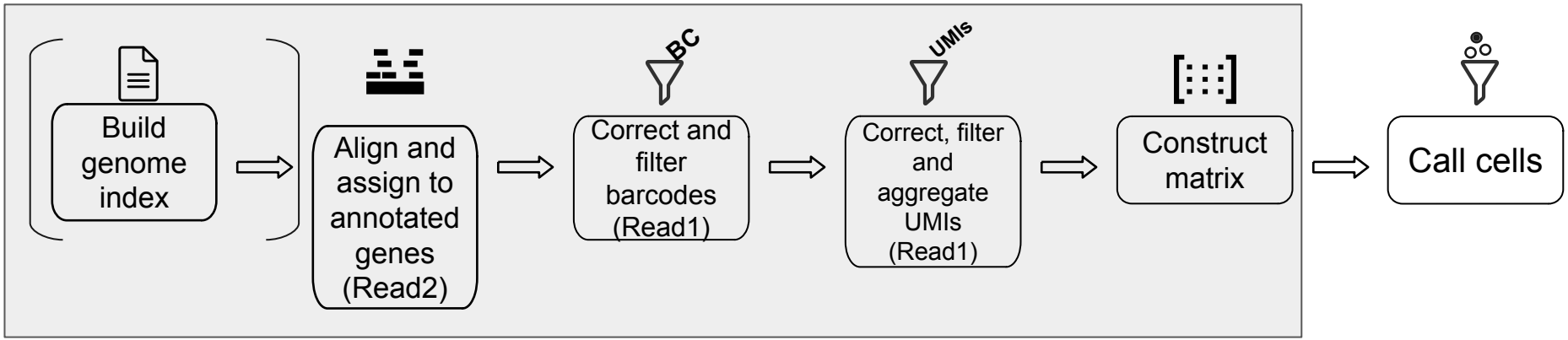
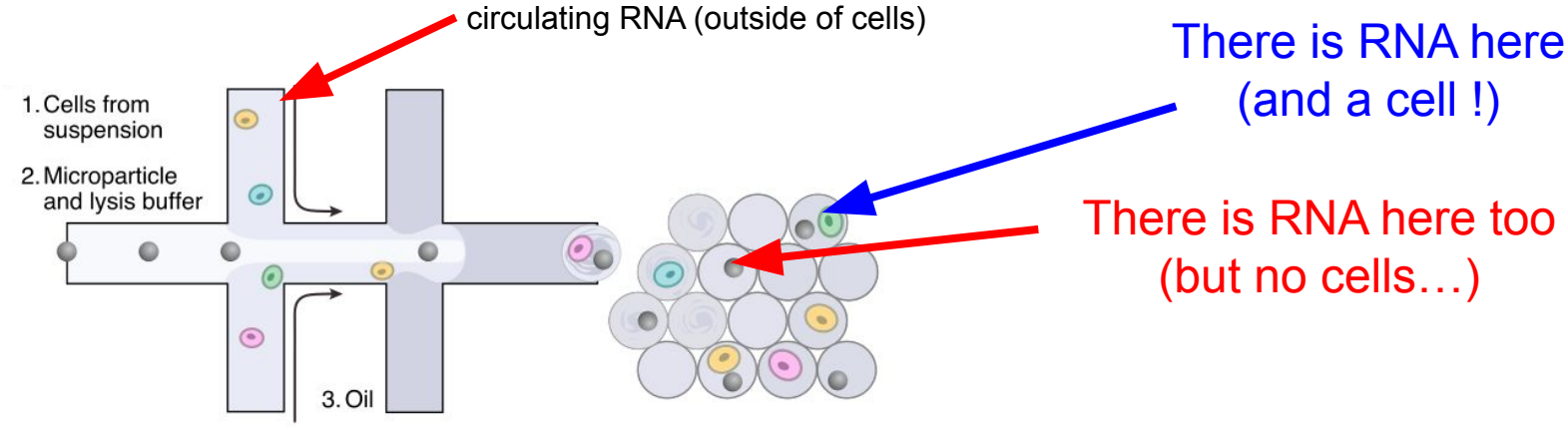
*Same count matrix as before*

# Primary analysis : overview of the workflow



# Counting the cells




- A million of droplets to recover ~10k cells
- Problem : RNA from dead cells circulates and is encapsulated in droplets
- **Question : how to differentiate between “real cells” and “droplets with RNA” ?**

# Empty droplets filtering

- Need to **filter** the count matrix to retain the droplets most likely containing a true cell, removing the “empty” droplets containing only ambient RNA

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- **Problem**: we have no prior knowledge about whether a barcode corresponds to cell-containing or empty droplets. We need to call cells from empty droplets based on the observed expression profiles.

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# Empty droplets filtering

- Need to **filter** the count matrix to retain the droplets most likely containing a true cell, removing the “empty” droplets containing only ambient RNA

- **Problem**: we have no prior knowledge about whether a barcode corresponds to cell-containing or empty droplets. We need to call cells from empty droplets based on the observed expression profiles.

- **Principle** : true cells will contain many different RNA molecules, compared to empty droplets containing few ambient RNA
  => translates into : barcodes associated to many UMI are more likely to be true cells than barcodes associated to few UMIs

# Identification of the “true” cells depends on UMI diversity

| | Cell 1 | Cell 2 | Cell 3 |
|---|---|---|---|
| Gene 1 | 3 | 1 | 2 |
| Gene 2 | 2 | 1 | 0 |
| Total | 5 | 2 | 2 |

1 UMI = 1 single RNA molecule

# Identification of the “true” cells depends on UMI diversity

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gene 1** | **30** | **1** | **2** | **1** | **0** | **6** | **7** | **0** | **0** | **2** | **0** | **9** | **2** | **0** | **0** | **1** | **2** |
| **Gene 2** | **8** | **1** | **0** | **4** | **0** | **2** | **1** | **4** | **1** | **3** | **0** | **3** | **15** | **1** | **0** | **1** | **0** |
| **Total** | **38** | **2** | **2** | **5** | **0** | **8** | **8** | **4** | **1** | **5** | **0** | **12** | **17** | **1** | **0** | **2** | **2** |

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| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
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“Low UMI cells” ~ “empty droplets”

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| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
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| **Gene 2** | **8** | **1** | **0** | **4** | **0** | **2** | **1** | **4** | **1** | **3** | **0** | **3** | **15** | **1** | **0** | **1** | **0** |
| **Total** | **38** | **2** | **2** | **5** | **0** | **8** | **8** | **4** | **1** | **5** | **0** | **12** | **17** | **1** | **0** | **2** | **2** |

“Low UMI cells” ~ “empty droplets”

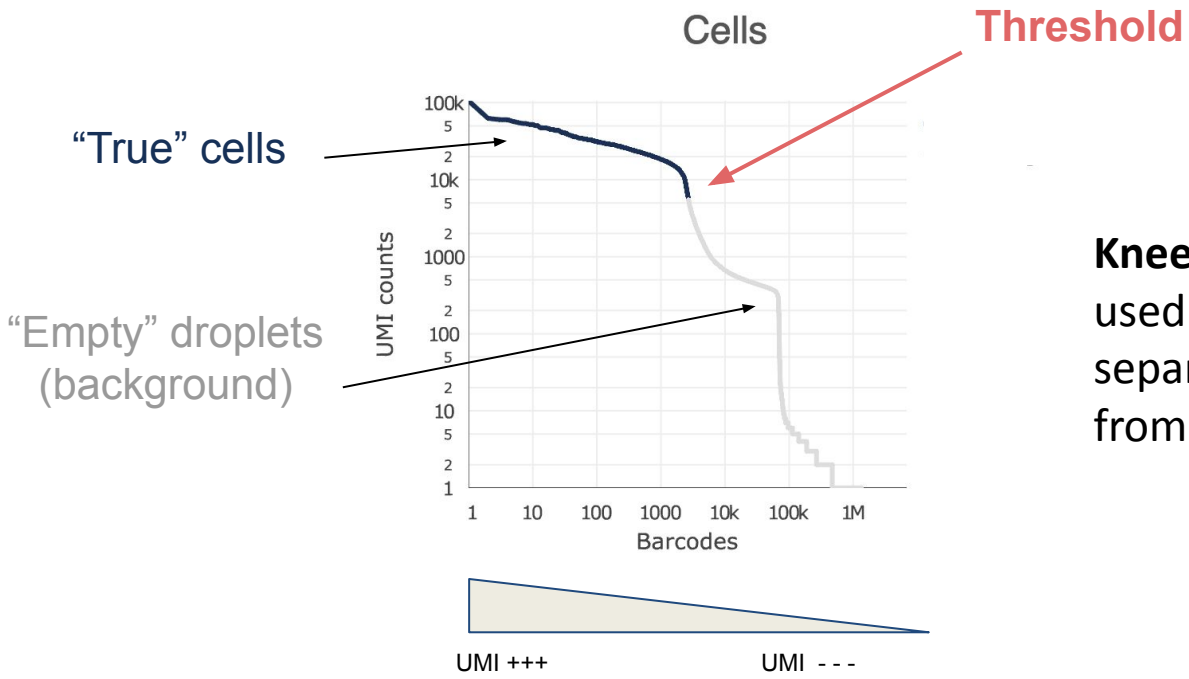
# Identification of the “true” cells depends on UMI diversity

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gene 1** | 30 | 1 | 2 | 1 | 0 | 6 | 7 | 0 | 0 | 2 | 0 | 9 | 2 | 0 | 0 | 1 | 2 |
| **Gene 2** | 8 | 1 | 0 | 4 | 0 | 2 | 1 | 4 | 1 | 3 | 0 | 3 | 15 | 1 | 0 | 1 | 0 |
| **Total** | 38 | 2 | 2 | 5 | 0 | 8 | 8 | 4 | 1 | 5 | 0 | 12 | 17 | 1 | 0 | 2 | 2 |

“Low UMI cells” ~ “empty droplets”... ???

How do we set the **threshold** between “true” cells and “droplets” ?

# Identification of the “true” cells depends on UMI diversity



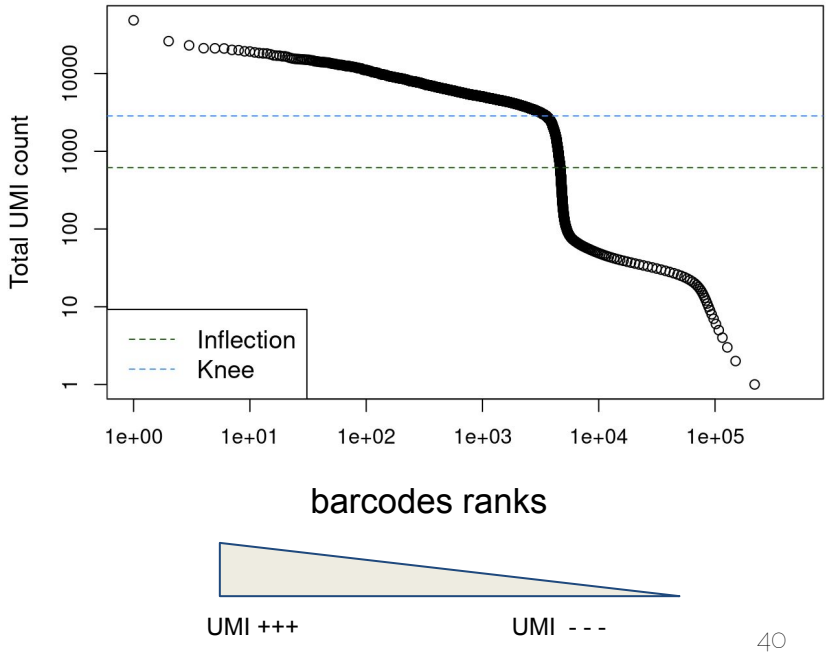

**Knee plot :**
used to find the threshold separating the “true” cells from empty drops

# Knee plot

The knee plot (or *barcode rank plot*) is used for filtering the droplets

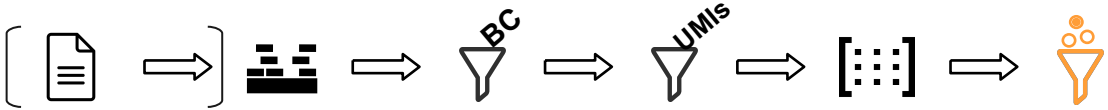
Steps :

1. Keep all barcodes over first knee point
2. Deduce background from low content droplets
3. Select droplets under knee point if the composition is very different from the background (cells with low-content RNA)



# Cell identification with CellRanger

## Cellranger

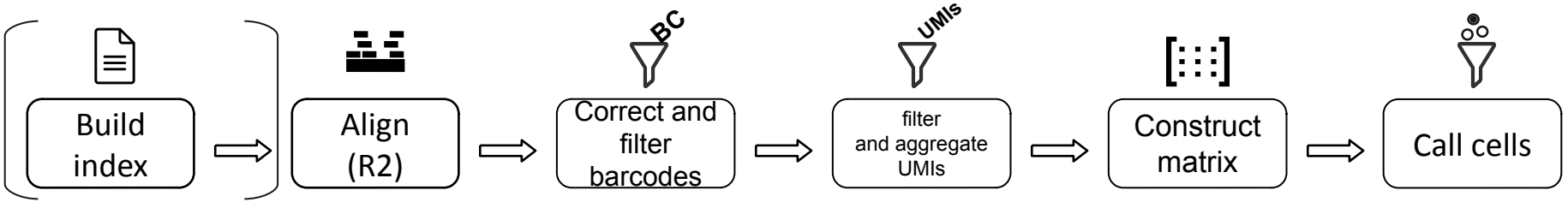



- Final number of cells can be < targeted cells
- With 10x Genomics data, cell capture is usually around 50% - 60%

- A second round of cell filtering step is necessary. It is performed at the beginning of the data analyses (we will see that later in the course)

# Output of CellRanger

## Principle




fastq Read1: unique cell barcode (16 nt) + UMI (12 nt)

fastq Read2: RNA 3' sequence

input

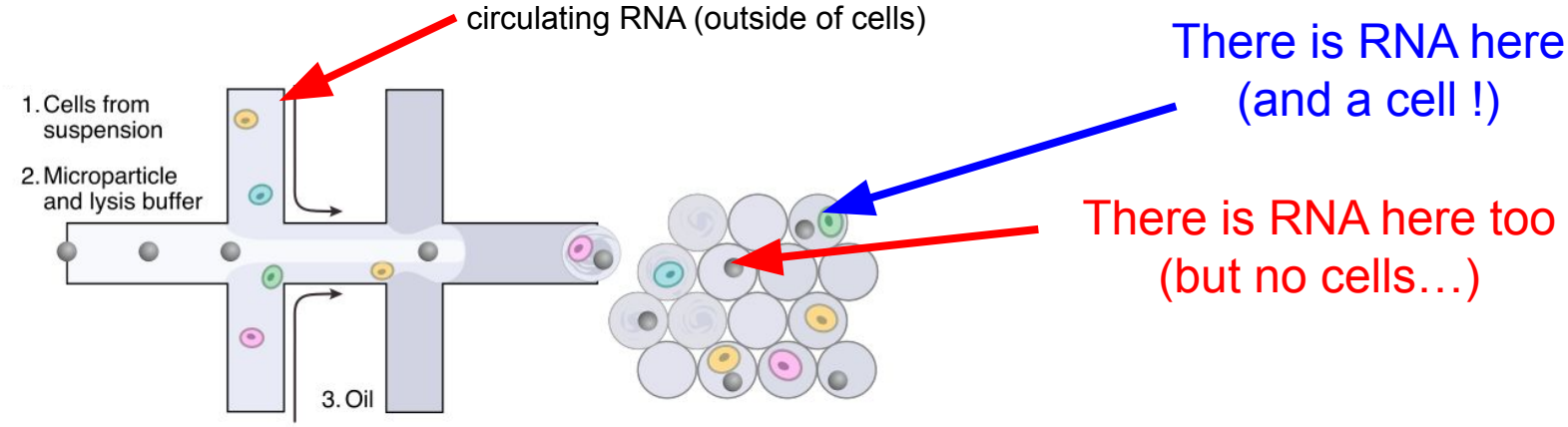
output

tsv barcodes

tsv genes

mtx counts

# Ambient RNA can be present in “true cells”




- Ambient RNA may also be encapsulated within droplets containing a cell (“true cells”)
- There are tools that allow to correct for this biais
- Ex : *SoupX* - that infers ambient RNA “soup” and removes it from the gene counting

# CellRanger output report

## Estimated Number of Cells

**2,700**

| Mean Reads per Cell | Median Genes per Cell |
|---|---|
| 197,634 | 3,704 |

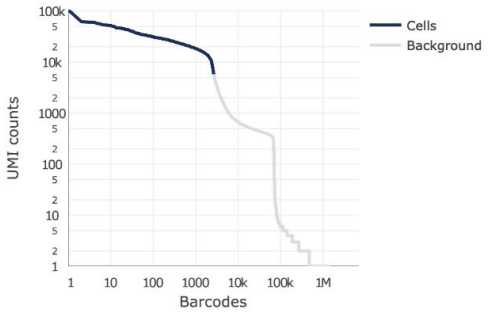
## Sequencing

| | |
|---|---|
| Number of Reads | 533,613,214 |
| Valid Barcodes | 96.0% |
| Sequencing Saturation | 67.7% |
| Q30 Bases in Barcode | 96.1% |
| Q30 Bases in RNA Read | 90.8% |
| Q30 Bases in UMI | 95.2% |

## Mapping

| | |
|---|---|
| Reads Mapped to Genome | 74.6% |
| Reads Mapped Confidently to Genome | 70.5% |
| Reads Mapped Confidently to Intergenic Regions | 8.7% |
| Reads Mapped Confidently to Intronic Regions | 8.4% |
| Reads Mapped Confidently to Exonic Regions | 53.4% |
| Reads Mapped Confidently to Transcriptome | 50.4% |
| Reads Mapped Antisense to Gene | 1.0% |

## Cells




| | |
|---|---|
| Estimated Number of Cells | 2,700 |
| Fraction Reads in Cells | 55.1% |
| Mean Reads per Cell | 197,634 |
| Median Genes per Cell | 3,704 |
| Total Genes Detected | 17,998 |
| Median UMI Counts per Cell | 16,440 |

## Sample

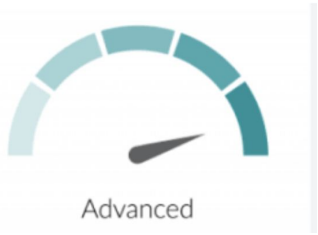
| | |
|---|---|
| Name | CellRanger_Report_1 |
| Description | |
| Transcriptome | cellranger_mkref_output_v3_191003 |
| Chemistry | Single Cell 3' v3 |
| Cell Ranger Version | 3.0.1 |

# CellRanger output report

- Turnkey solution
- Many QC-metrics in 1 html summary
- Some secondary analysis
- More complex experiences : VDJ analysis, feature-barcoding
- Versions for ATAC-Seq, multi-omics

- Proprietary
- Only 10X product (cannot customize BC and UMI patterns)
- Not highly configurable
- (A lot of resource and time)
  but less true for recent versions

# There are other alternatives than CellRanger




**Technical Overview mapper**

| | Cell Ranger | STARsolo | Alevin | Kallisto |
|---|---|---|---|---|
| **Mapping scheme** | Exact alignment | Exact alignment | Pseudo mapping | Pseudo mapping |
| **Internal Mapper** | Star | Star | Salmon | Kallisto |
| **Reference** | Genome | Genome | Transcriptome + Genome | Transcriptome |
| **Supported sequence technology** | 10X Chromium v1 – v3 | 10X Chromium v2;v3, Smart-seq, Drop-seq, inDrop | 10x Chromium v2;v3, Drop-seq, Cel-seq, Cel-seq2, Quartz-seq2 | 10x Chromium v1 – v3, Cel-seq, Cel-seq2, Drop-seq, inDrops v1-v3, SCRB-Seq, SureCell |
| **Barcode correction** | 1-Hamming distance based | 1-Hamming distance based | Edit distance calculation | 1-Hamming distance based |
| **Whitelisting** | Whitelist based | Whitelist based | Frequency based, no whitelist needed | Whitelist based |
| **Alternative Splicing detection** | no | yes | no | no |
| **UMI correction** | Two round correction by barcode, read count and annotation | Two round correction by barcode, read count and annotation | graph based correction | NA |
| **Index** | Suffix array | Suffix array | Colored De-Bruijn Graph | Colored De-Bruijn Graph |
| **Handling of multimapped reads** | discarded | discarded | Distributing read count between genes by EM-algorithm | discarded |
| **Output** | Matrix + Bam-File and summary file as html-file with primary results as well as clustering and DEG analysis | Gene count matrix and primary results summary | Gene count matrix ready for analysis | External software required to create gene count matrix |

**Summary**

| | Cell Ranger | STARsolo | Alevin | Kallisto |
|---|---|---|---|---|
| **Mapping performance** | Lowest runtime | Similar results with Cell Ranger that are accomplished in a shorter time | Whitelisting causes loss or gain of barcodes depending on the data | Fastest runtime with highest mapping rate, more cells are detected with a small gene content |
| **Barcode correction and filtering** | | | Final barcode set included barcodes that are not present in the whitelist | Reports more cells with a low gene content |
| **Gene discovery** | | | | Detection of more genes than all other tools. Highest UMI count for genes not expressed in studied tissue |
| **MT-content** | Highly affected by complete annotation including pseudogenes | See Cell Ranger | Smaller difference of MT-content between the mapping with filtered and unfiltered annotation | See Cell Ranger |
| **Clustering** | Highest Overlap with SCINA classification | Very similar to Cell Ranger with minor differences | Cell types contain lower amount of cells with SCINA classification | Cell types contain the lowest amount of cells with SCINA classification |
| **DEG** | No difference detected | No difference detected | No difference detected | No difference detected |

R. S. Brüning *et al.*, Gigascience (2022)

# Take-home messages

- Take time to **visualize the scRNA-seq signal** in a genome browser (IGV)
- Results can be hugely affected by the annotation
- Exotic /poorly-annotated / non-model organisms : generate a new annotation from bulk data with long-read sequencing (or reconstruct with short-reads)

- Count matrix = nb of UMI per barcode (columns) per gene (row)

- Call the cells : remove the empty droplets containing ambient RNA => use of the knee plot to decide on the threshold and obtain the number of “true” cells
- Sometimes, need to lower this threshold for small cells/low-RNA content
- More filters will be applied in the downstream analysis

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- … and originally taken or inspired from Bastien Job

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  - Morgane Thomas-Chollier