Preprocessing

Prepping the count matrix

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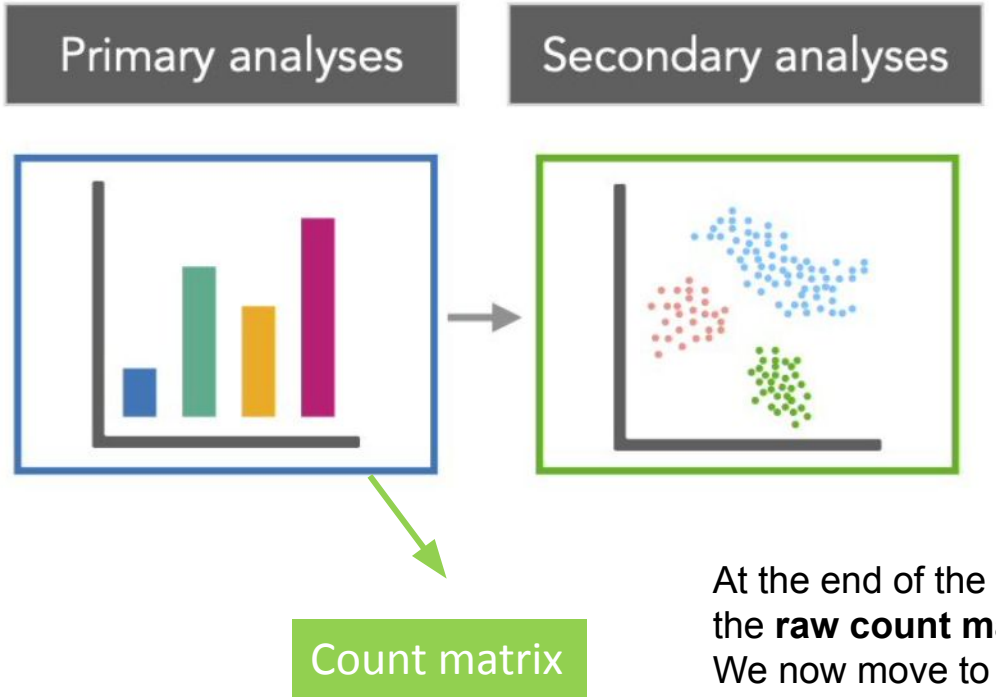
École de bioinformatique EB3I 2025

Organization of this session



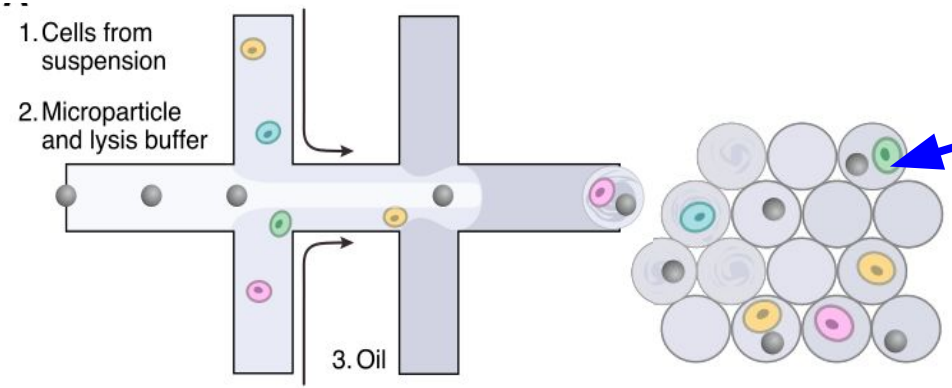
At the end of the primary analysis, the **raw count matrix** is produced. We now move to the downstream analysis

Organization of this session

- Prepping the raw counts matrix
  - Filtering out low quality / “empty” droplets
  - Assessing, removing ambient RNA
  - Generating technical and signature metrics (counts, %mito, %ribo, cell cycle phase, …)
    - Filtering out extreme “bad” cells on these
    - Lowering bias *(later)*
  - Identifying, filtering out cell doublets

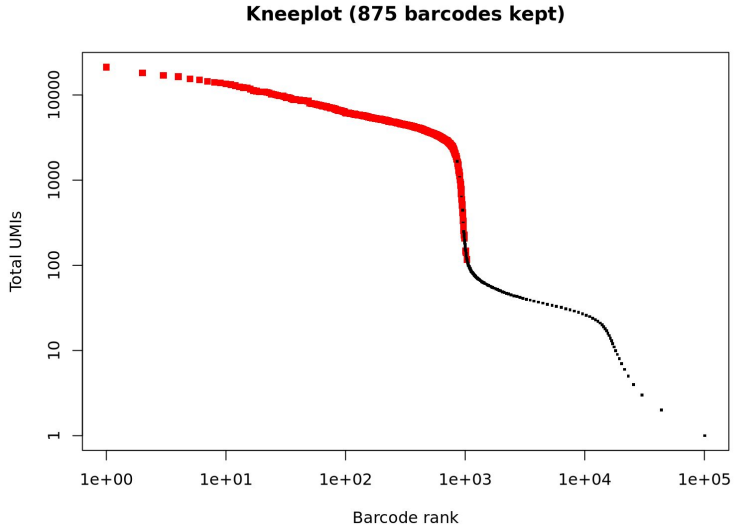
- Your input : a [**feature**] -by- [**droplet barcode**] count matrix

Empty droplets filtering



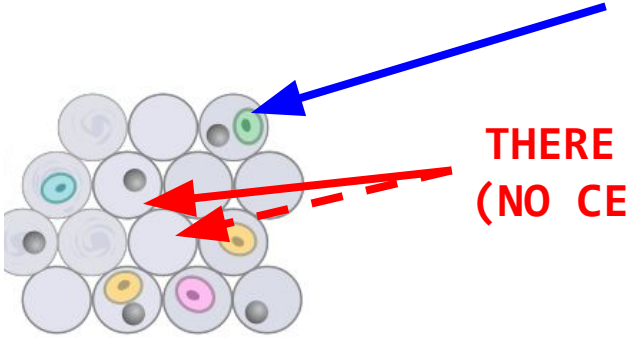
THERE IS RNA HERE
(CELL IN GEM RNA)

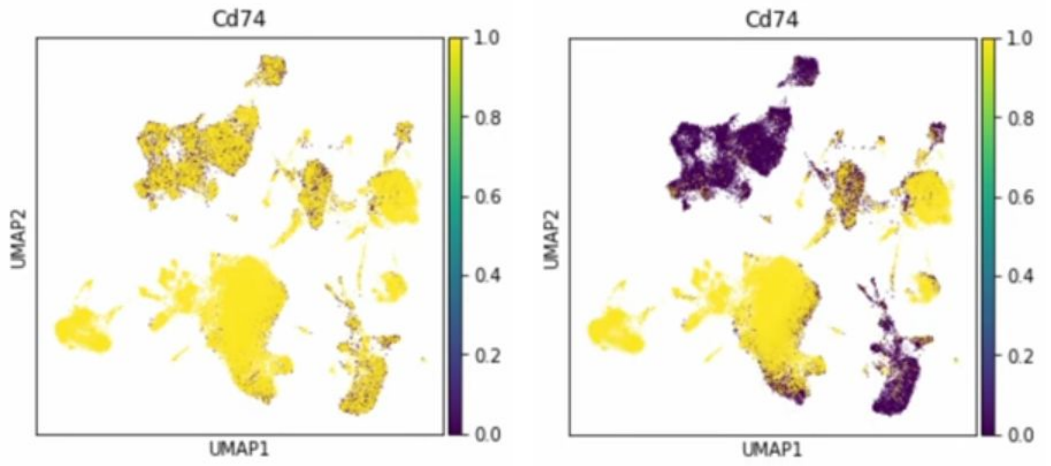
- The (double) "**kneeplot**"
- Counts = f(ranked.dropplets)
- "steep cliff" => best transition from "true cells" to empty droplets
- Actually a bit more complex …
- R tool : DropletUtils::*emptyDrops*



Ambient RNA filtering (SoupX)

- emptyDrops : removed empty droplets (contained only ambient RNA)
- ***BUT*** *non-empty droplets* ***ALSO*** *have ambient RNA !*
- ***soupX*** determines the amount of ambient RNA in counts, removes it





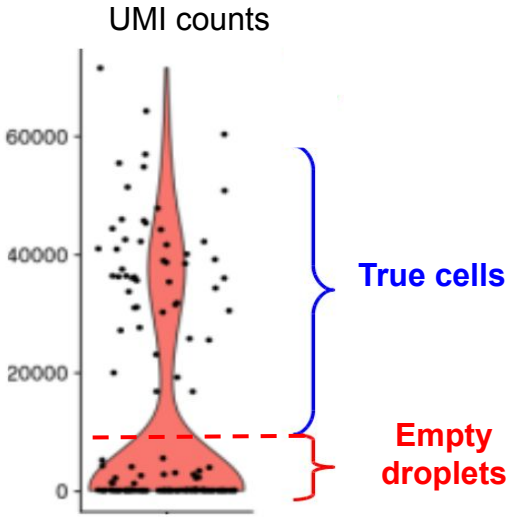
Young et al. GigaScience (2020)

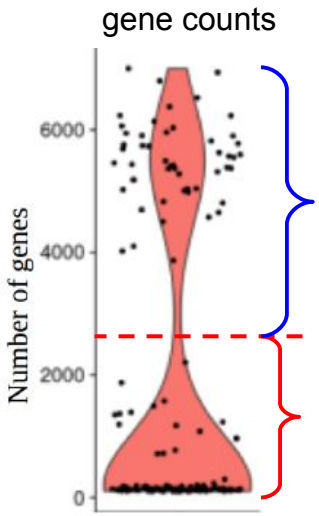

To Be Continued

QC and filtering (counts, features)

Filtering of empty / bad quality cells

- Visualize data and **deduce** thresholds
- Possible visualization: **Violin** Plot : Distribution of a cell feature. Can add points to visualize cells exactly (1 point = 1 cell) // **Histogram**
- Ideal distribution should be normal. In practice, it is **bimodal**



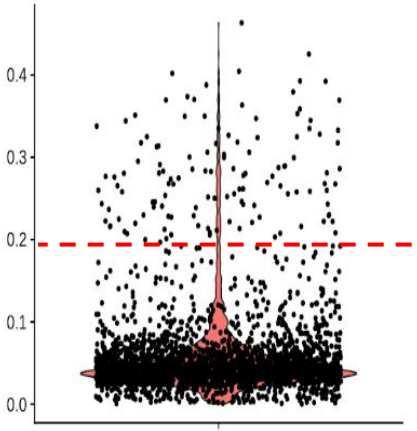


QC and filtering (%mito, %ribo, …)

Filtering of empty / bad quality cells

- Visualize data and **deduce** thresholds
- Possible visualization: **Violin** Plot : Distribution of a cell feature. Can add points to visualize cells exactly (1 point = 1 cell) // **Histogram**
- Distribution of features that **capture** a large part of expression (mito genes, riboproteins, …)

Mitochondrial (mt) genes expression

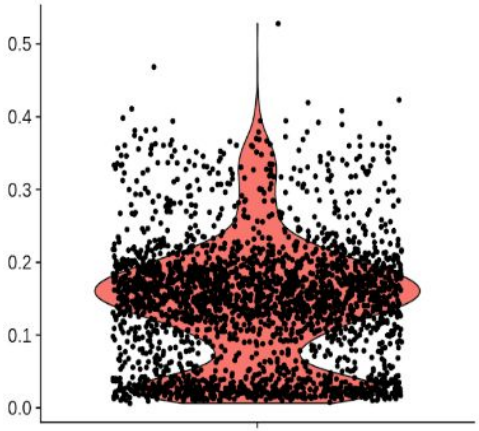


High % of mt genes may be due to apoptotic/dead or over-lyzed cells

Here the distribution has a long right tail.

Depending on dataset, remove cells > 5, 10, 20, 25% mtRNAs… (tl,tb)

Ribosomal protein genes expression ?



Reflects cell stress or cellular activity? Cell cycle?

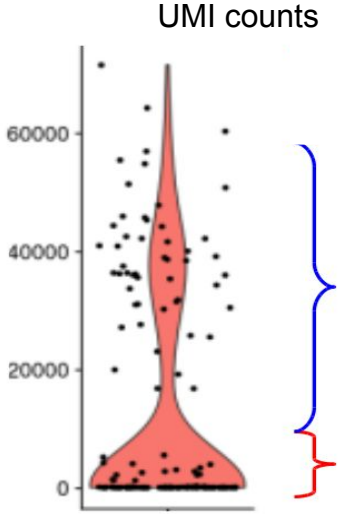
Is it a good marker: community debate.

+ Mechanical stress

QC and filtering

Filtering of empty / bad quality cells

- Visualize data and deduce thresholds
- Possible visualization: Violin Plot : Distribution of a cell feature. Can add points to visualize cells exactly (1 point = 1 cell)

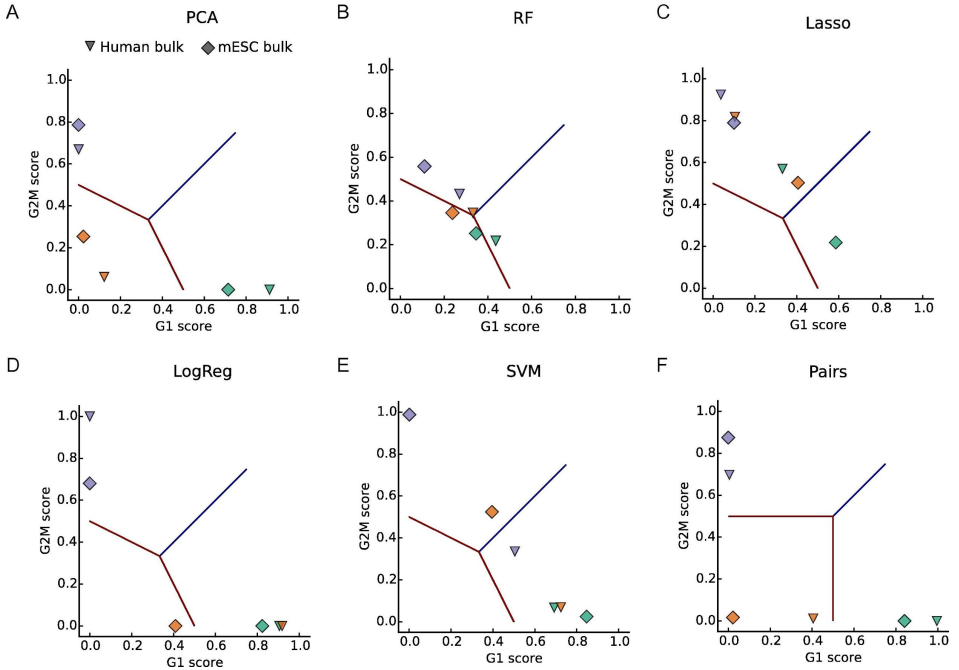


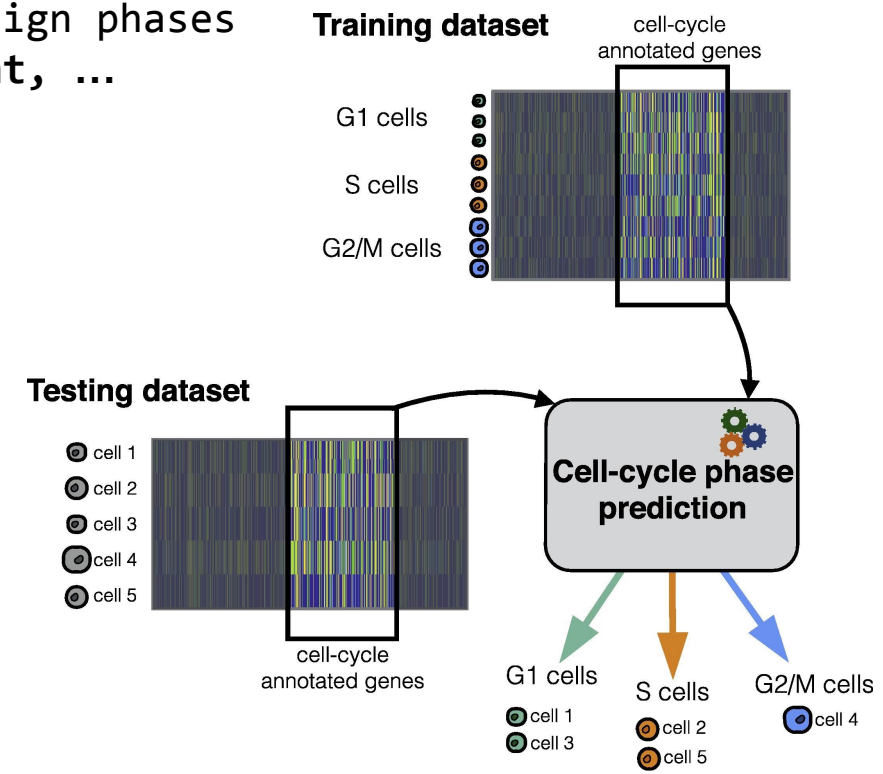
Select the thresholds carefully if you expect a population with a **small transcriptome**: e.g. immune cells (B especially), stem cells, …


To Be Continued

Cell cycle phase estimation

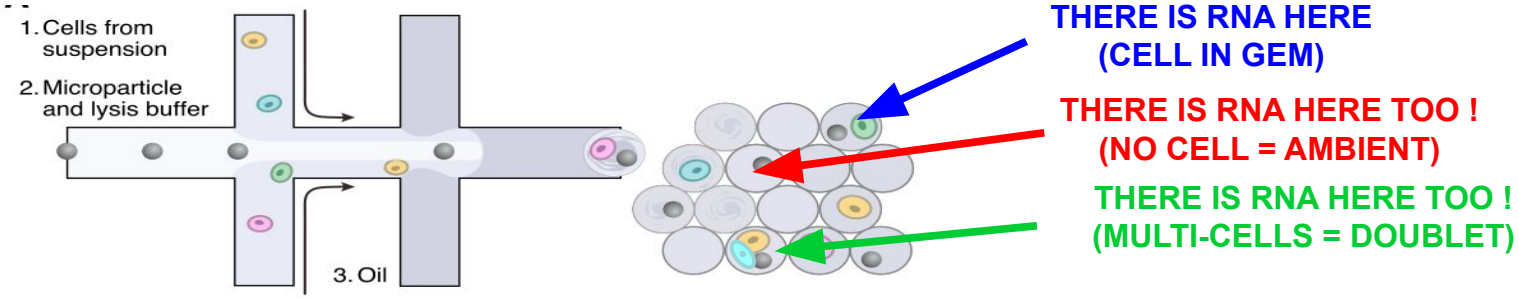
- Variational expression due to cell phase may be strong !
- Training on reference set with the 3 phases identified
- Use pairs of differential genes
- Apply model pairs to new dataset, assign phases
- Implemented in **cyclone** *(scran)*, **Seurat**, …

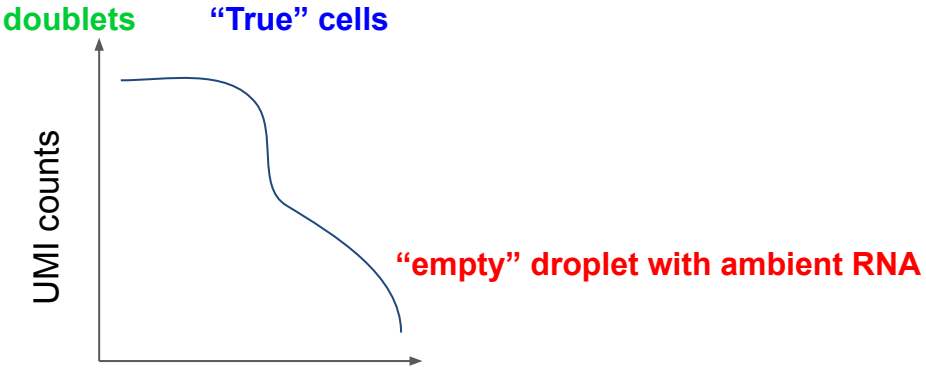




Scialdone et al., Methods (2015)

Filtered matrix composition : Doublets

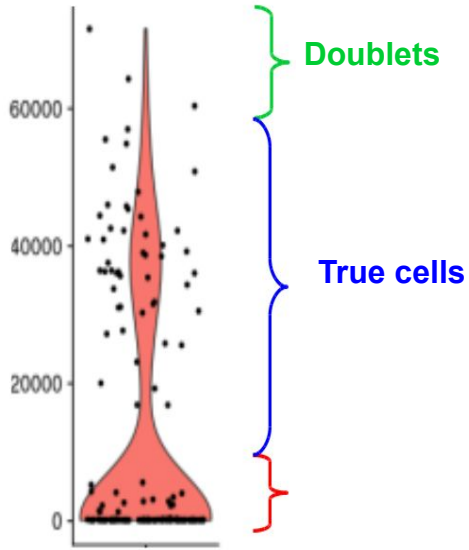




- True cells
- Empty, low quality droplets
- Doublets:
  - 1% for 1000 cells
  - 5% for 10 000 cells

Doublets detection

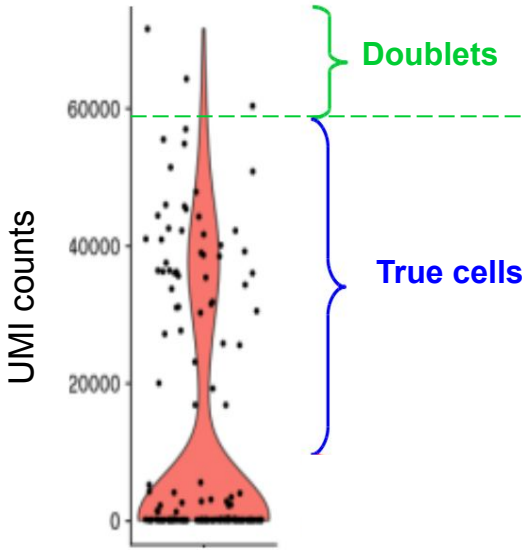
- Visualize nb UMIs (nCount) as a Violin Plot and set a threshold



- Doublets harbor a non-natural expression :
  - Higher level but same profile for doublets of the same cell type *(homotypic)*
  - Artificial profile for doublets of different cell types *(heterotypic)*
- This may have a **major impact** on the structure of signal in the data

Doublets detection

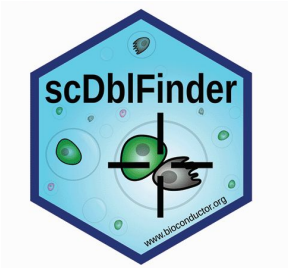
- Visualize nb UMIs as a Violin Plot and set a threshold



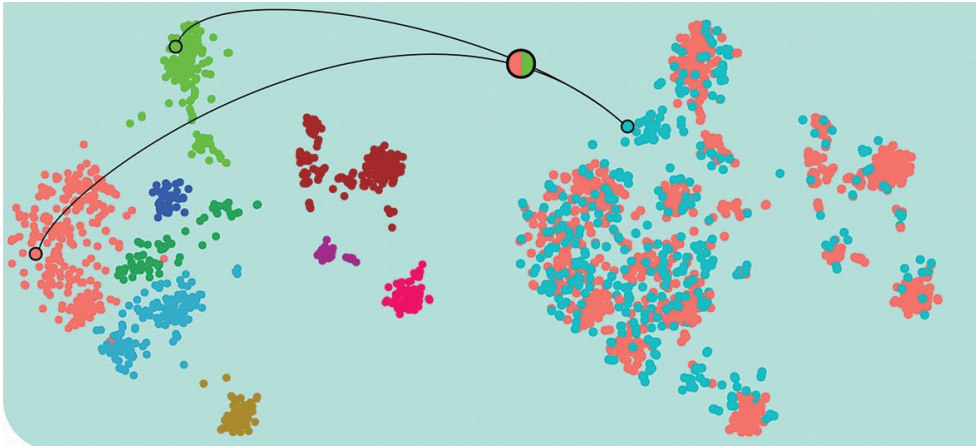
This threshold might be hard to tell
+
There may be a true cell subpopulation with higher expression ?

Doublets detection

- doublet detection by simulation



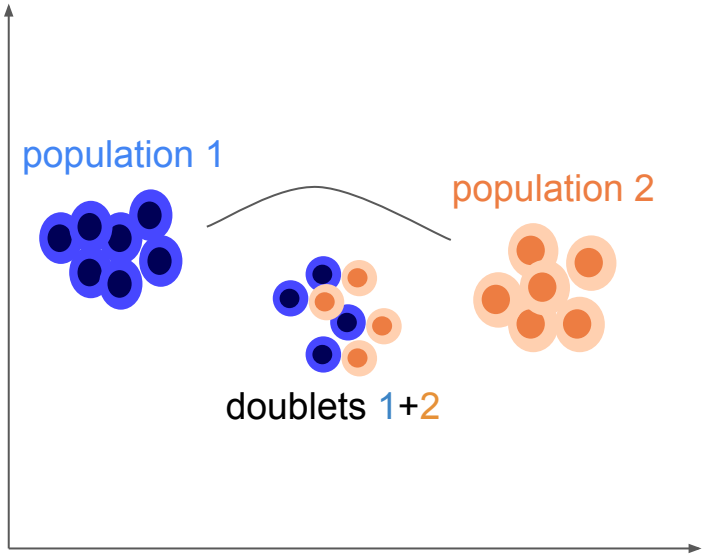
Pick 2 random cells ⇨ Generate artificial doublets ⇨ train doublet classifier ⇨ exclude doublet

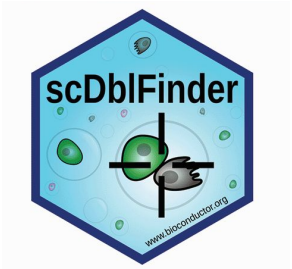


if a droplet looks like many artificial doublets, it has a high doublet score

Doublets detection

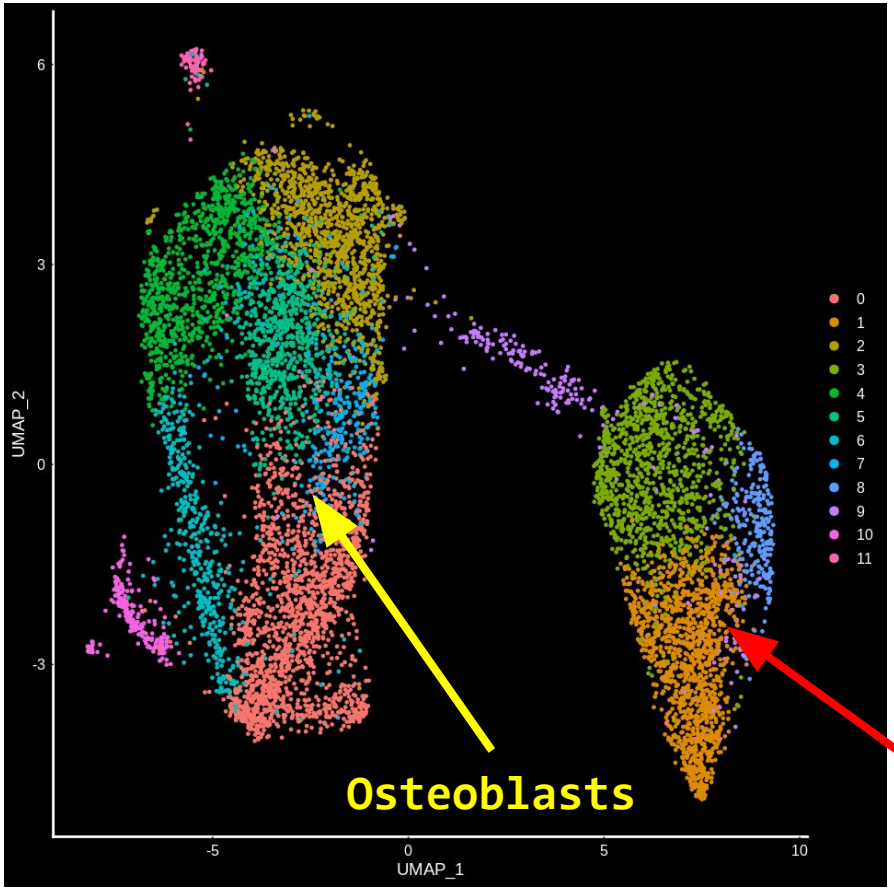
- doublet detection by clustering:
  - doublets composed of two cell types cluster between these cell types
  - check differentially expressed genes between putative doublets cluster and pop1 + pop2: there should not be many





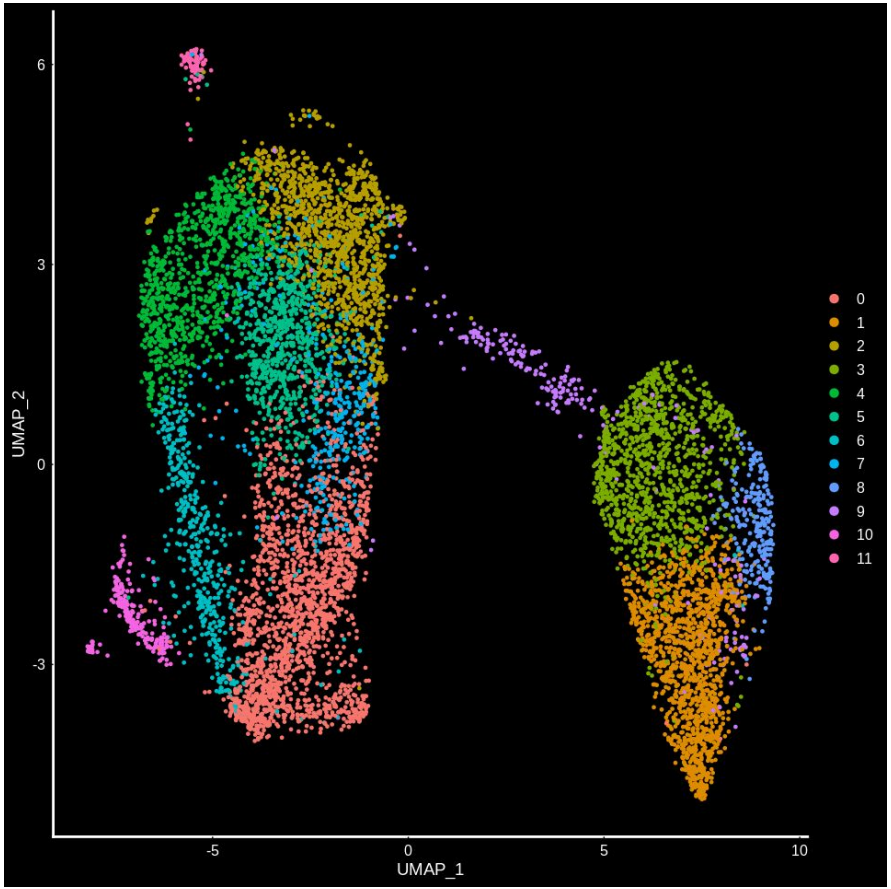
`findDoubletClusters()`

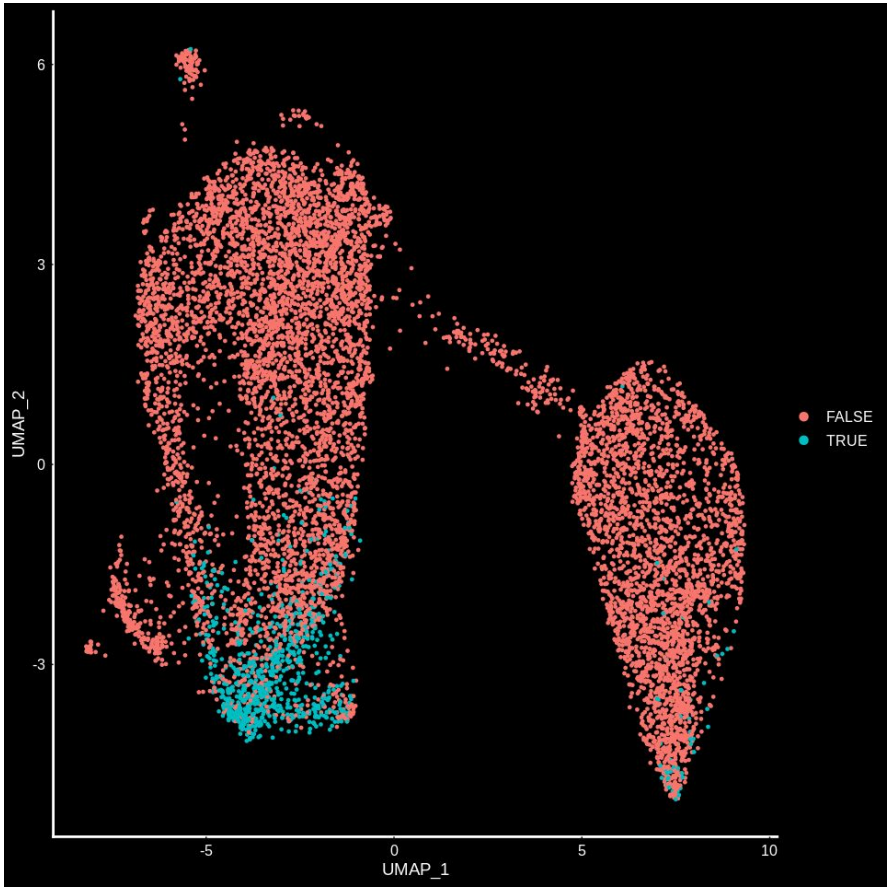
Visualization : a real-life example



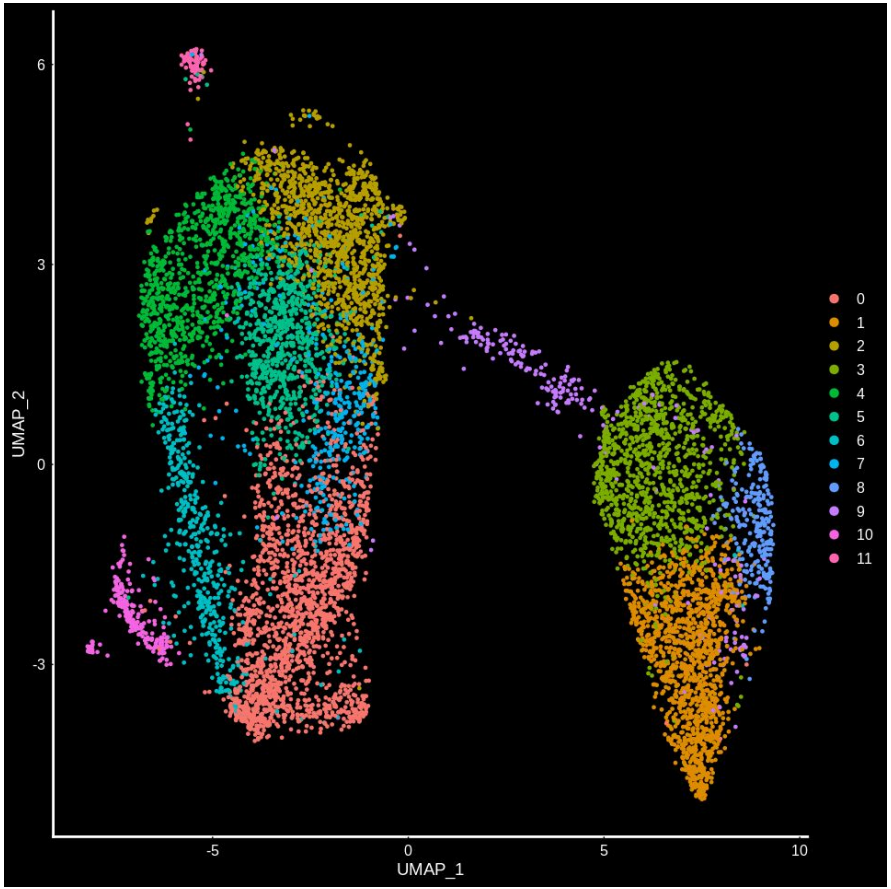
- 10X 3’ scRNAseq v2
- Osteosarcoma metastasis
- 8911 cells x 18613 genes
- PCA (109 PCs retained)
- Louvain clustering
  - 12 clusters
- uMAP representation

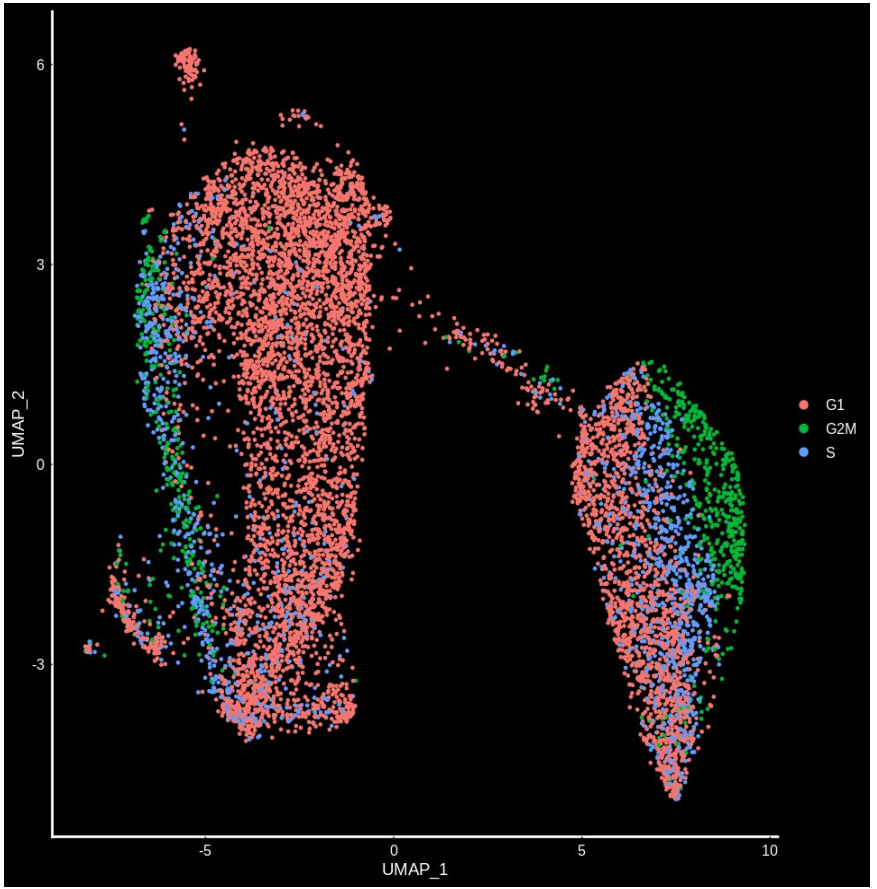
Bias : Dying cells (%mt high) status / score





Bias : Cell cycle phases / scores





Bias : Cell doublet status / score