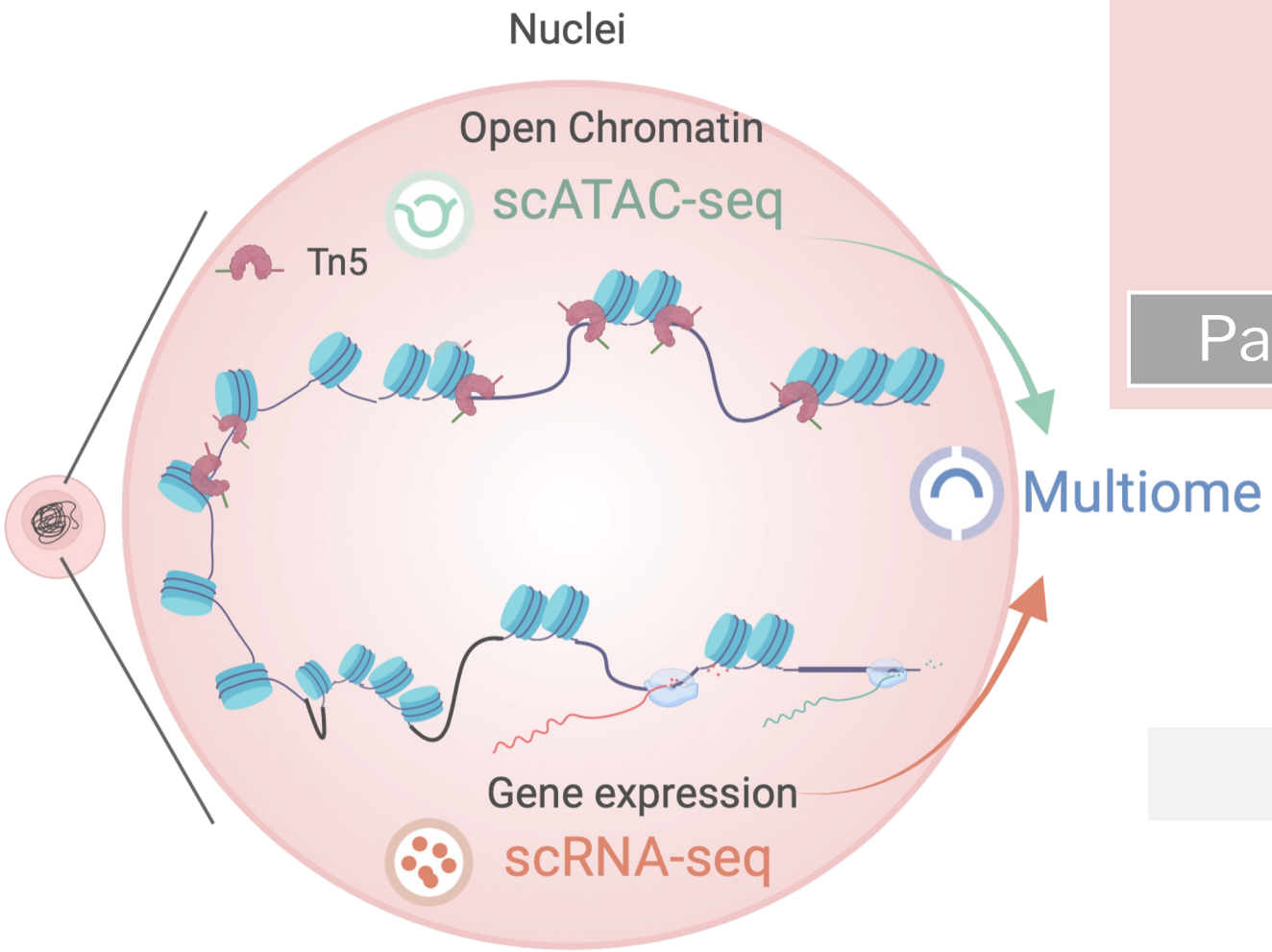

Workshop
Single-cell ATAC-seq

Part I: Introduction to scATAC-seq analysis

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cnag centre nacional d'anàlisi genòmica centro nacional de análisis genómico

CRG Centre for Genomic Regulation

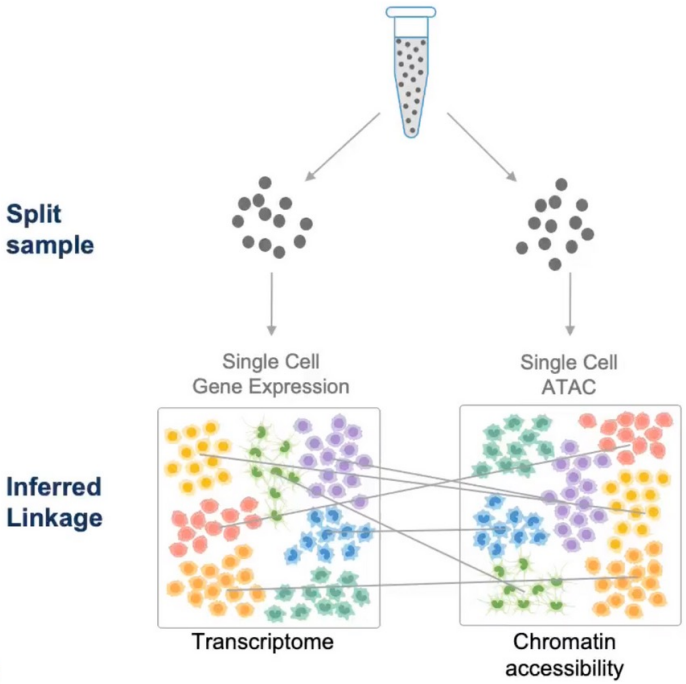
IDIBAPS Institut D'Investigacions Biomèdiques August Pi i Sunyer

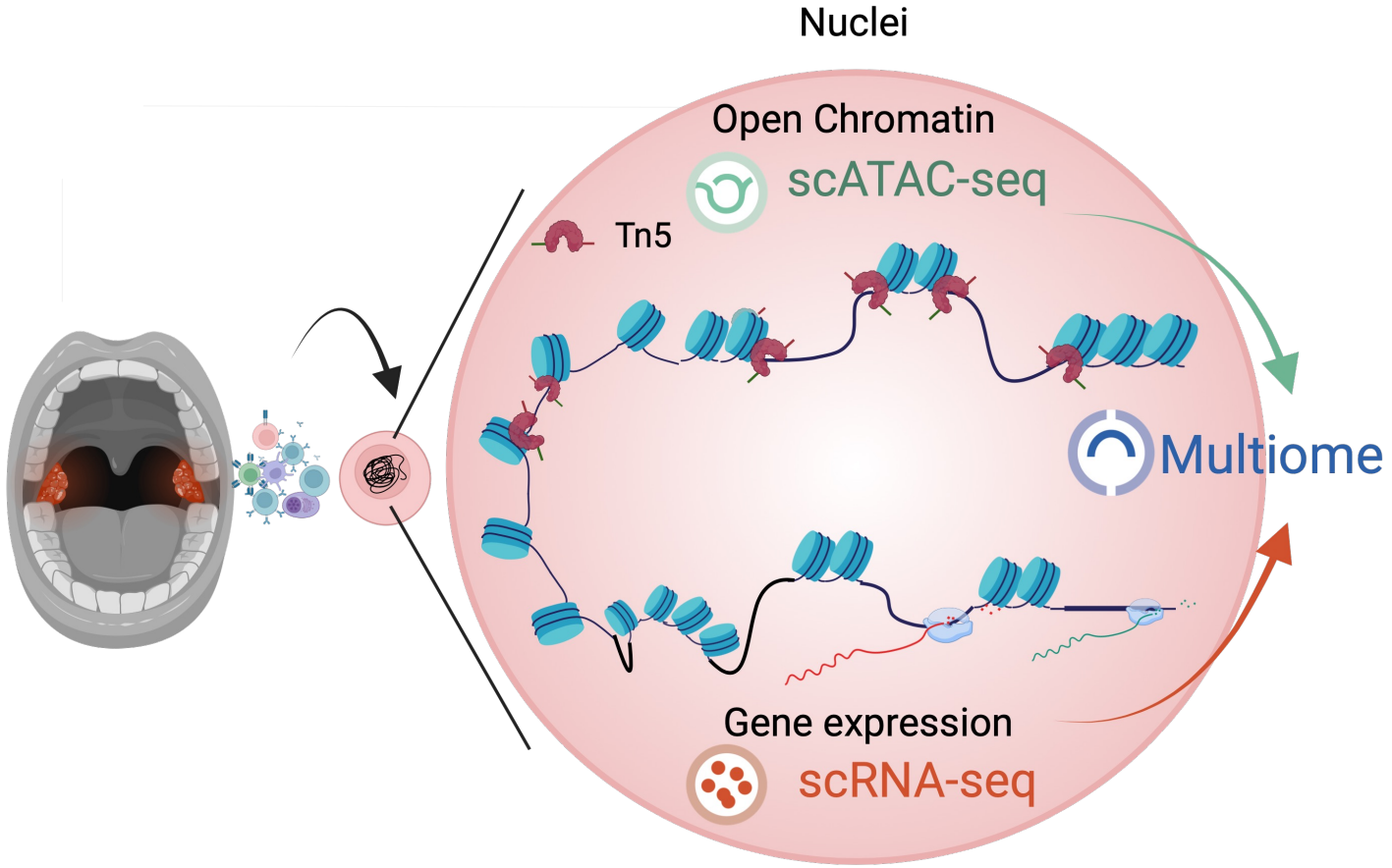
What are we going to learn?

1. **Multiome (scRNA-seq+ scATAC-seq)**
   Why Multiome?
2. **What Single-Cell ATAC-seq (scATAC-Seq) is**
   What kind of information scATAC-seq can provide us?
3. **How Multiome works:**
   Focused on scATAC-seq
4. **scATAC-seq Pipeline workflow analysis**
5. **Next steps & Let's get to work**

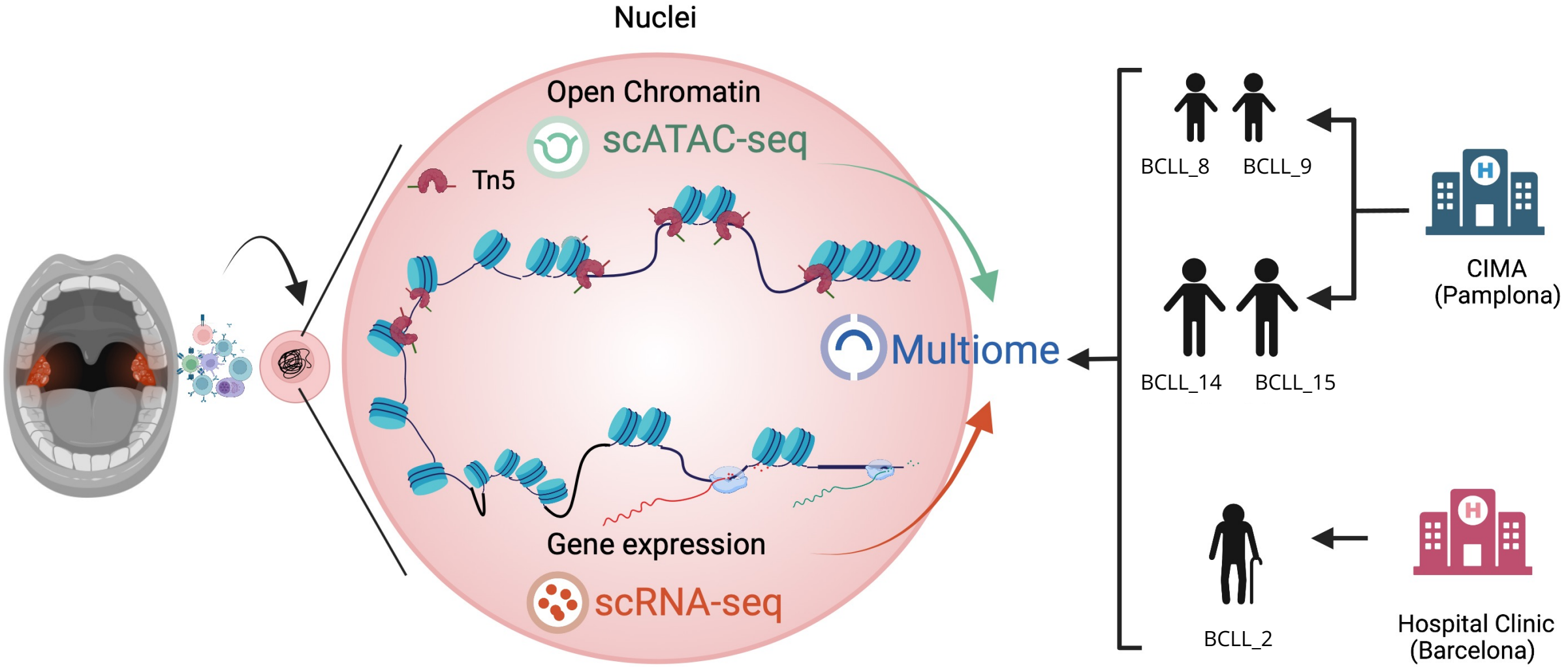
Single cell Multiome (Gene expression + ATAC-seq)

Consist in the simultaneous assessment of single cell RNA-seq and ATAC-seq for each individual cell



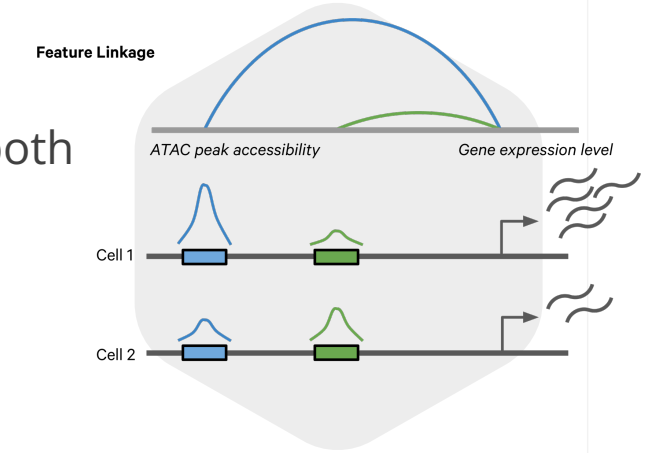


Single cell Multiome
(Gene expression + ATAC-seq)



Why Single cell Multiome?

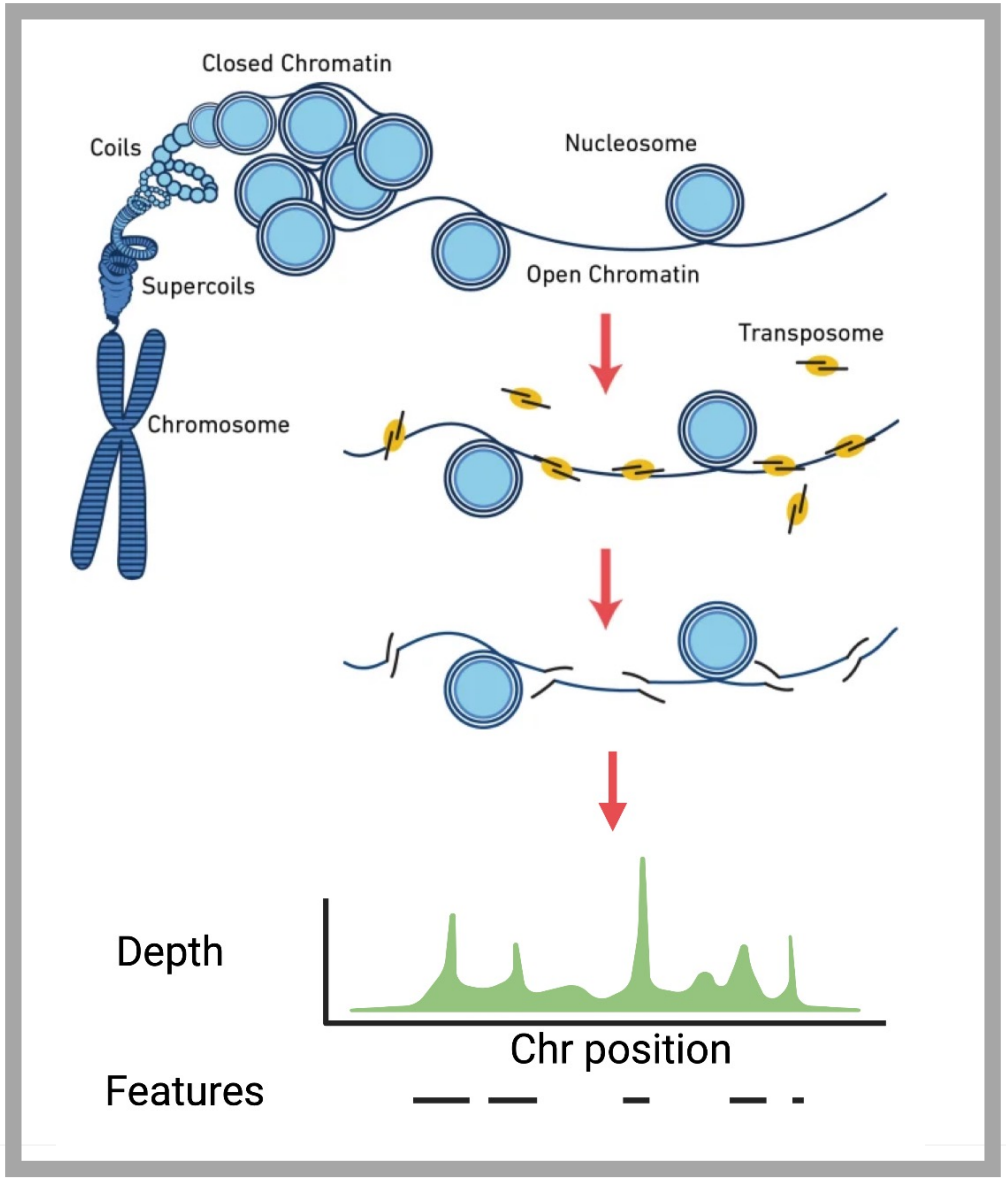
- Perform a **Peak-Gene Linkage analysis**:
  - Link gene expression to peaks (open chromatin regions) without inferring both methodology information



- Discover cells with **similar transcriptional** profiles but functionally **different chromatin landscapes**
- Discover new potential ***cis*-regulatory elements (CREs)** of target genes involved in cell differentiation, development and disease. (Need use chromatin state information)

- **Increase resolution** of complex and heterogeneous cell population
  - Improve the separation of cell subsets & identify rare population
  - Differentiate cell type depended on the cell state.

What scATAC-Seq is



Assay for Transposase Accessible Chromatin –

Allows the assessment of open and accessible chromatin region of the genome and assess the physical structure of the genome by identifying regions of open chromatin.

This technique uses a hyperactive transposase enzyme that cuts and inserts sequencing adapters into exposed DNA. The resulting sequencing library produces reads that are enriched in open chromatin regions.

What kind of information scATAC-seq can provide us?

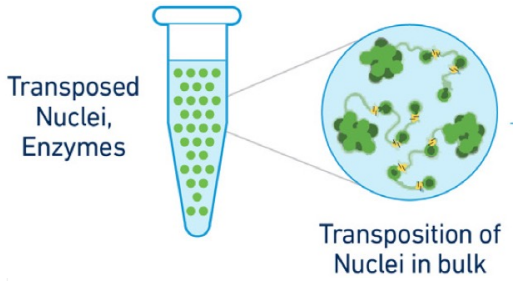
- Study the **single cell epigenetic states**:
  - Providing information about chromatin accessibility can **reveal areas of open chromatin that may correlate with gene expression (it should be validated by histone marks activity).**
  - According to Signac: We can distinguish different cell subtype by identify cell-type-specific **TF enrichment.** **According to me:** it is really difficult to differentiate cell subtypes.
- Increase our understanding of complex **regulatory networks**:
  - Where transcription factors binds and which genes TFs turn on or off depends on both the presence of a binding site in the DNA and whether that binding site is accessible. (Important to differentiate between active chromatin and open chromatin)
- Reveal **regulatory patterns** that are complementary to gene expression
- It enables the computational inference of **developmental.**
  - We can determine the temporal order of cells in a differentiation process.

What are the limitation of scATAC-seq?

- Only information of chromatin accessibility. Association with gene expression so difficult.
- Low cell subtype discriminatory power.
- More sensitive to number of principal component in lineal dimensionality reduction (LSI). (The first component correlate with sequencing depth).
- We need the histone marks bulk data to validate potential *cis*-Regulatory elements (CREs).

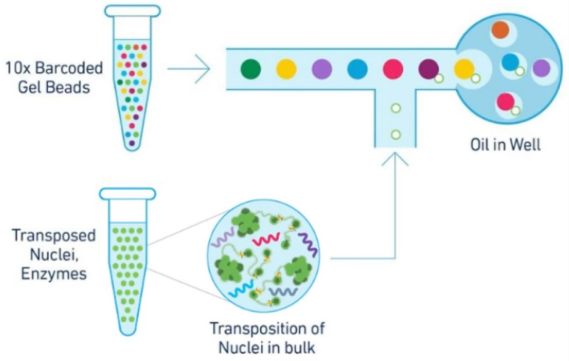
How single cell Multiome works

1. Bulk Nuclei



2. Chromium

(Reaction in GEM, Pre-amplification)



3. Library construction in parallel

4. Pre-processing data

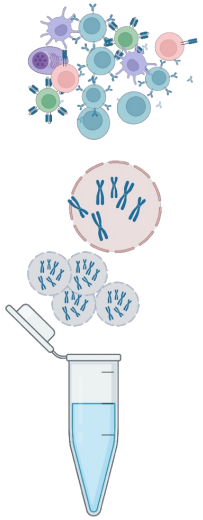
(Sequencig, CellRanger)

scATAC-seq

1. Bulk Nuclei

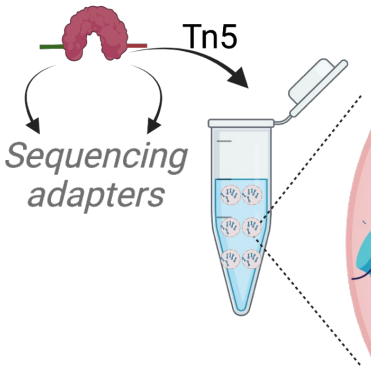
1

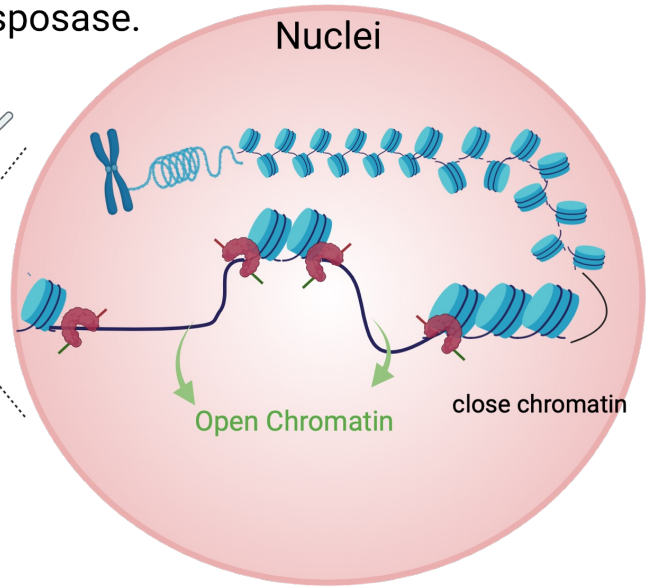
Assolation nuclei from 500 to 10,000 cells

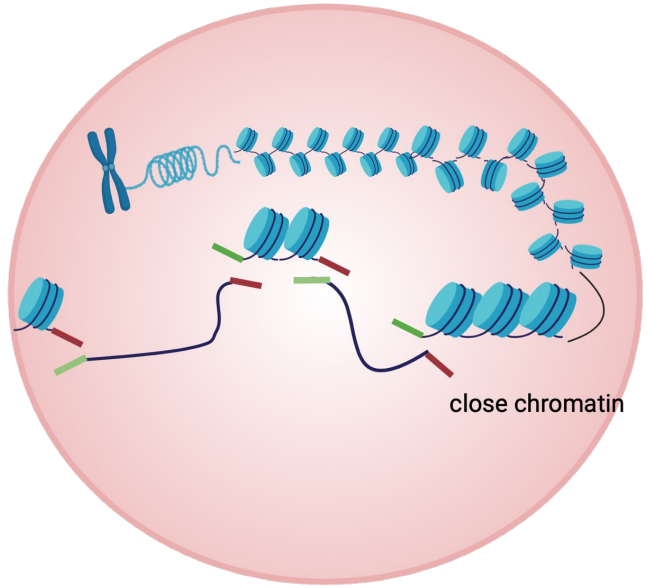


2

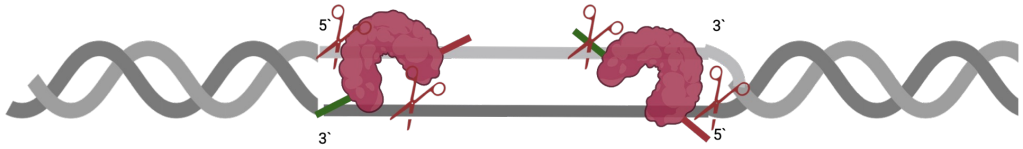
Nuclei are incubated with Tn5 transposase.







Tn5 transposase targets chromatin open regions



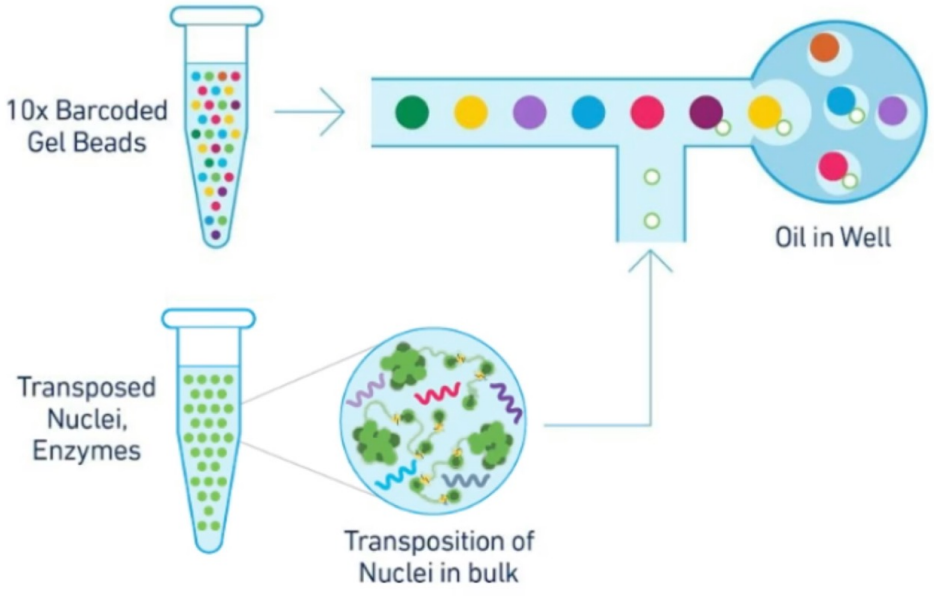
Creating adapter tagged fragments

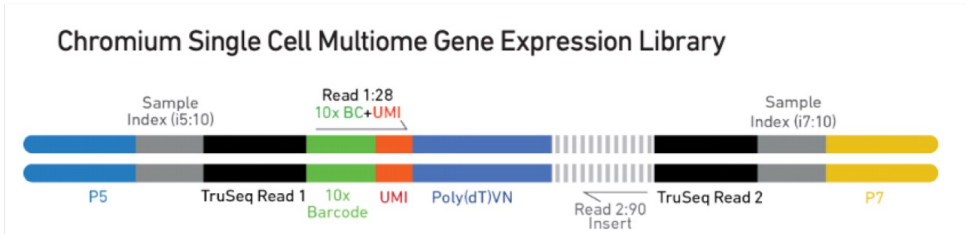
Multiome workflow

1. Nuclei

2. Chromium
(Reaction in GEM, Pre-amplification)

3. Library construction in parallel





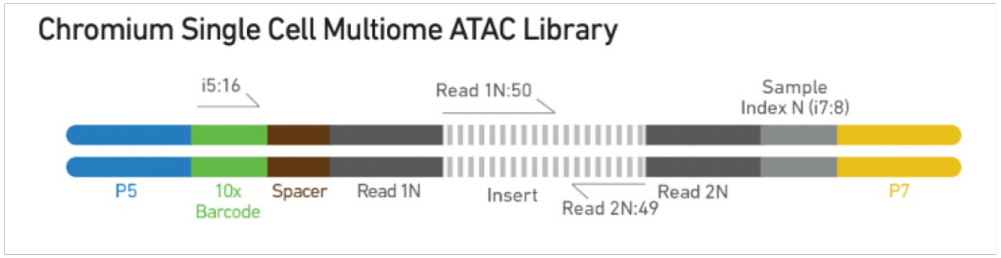
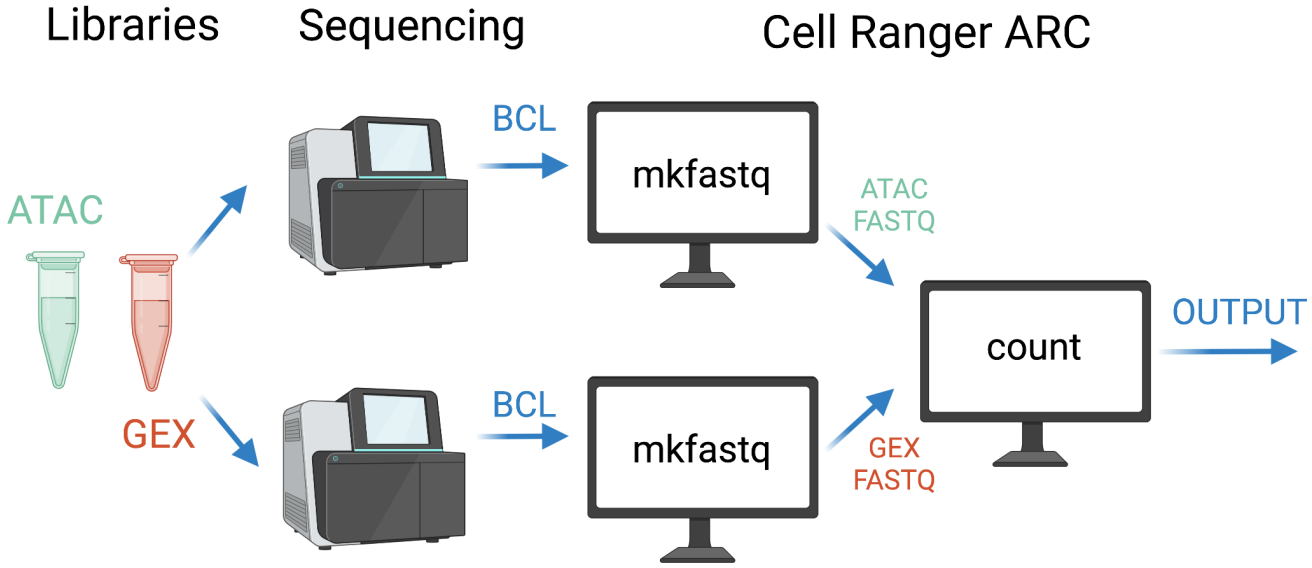


Image of 10x Genomics

- **Input:** Transposed nuclei
- **Output:**
  - One ATAC library and one Gene Expression library from each sample
  - Paired ATAC and Gene Expression data for each nucleus

4. Pre-processing



Pre-procesed data

ATAC Per fragment information file:
atac_fragments.tsv.gv

Chrom	ChromStart	ChromEnd	barecode	readSopport
ch1	30015	31224	ATGGCTAATCG	3
ch1	30126	31242	ATGGCTCATGG	1

ATAC Per fragment information index:
atac_fragments.tsv.gv.tbi

Filtered feature barcode matrix HDF5:
filtered_feature_bc_matrix.h5

scRNA-seq

Gene count matrix

	Cell 1	Cell 1	...	Cell n
Gene 1	0	2	...	0
Gene 2	5	0		7
Gene m	0	20	...	0

scATAC-seq

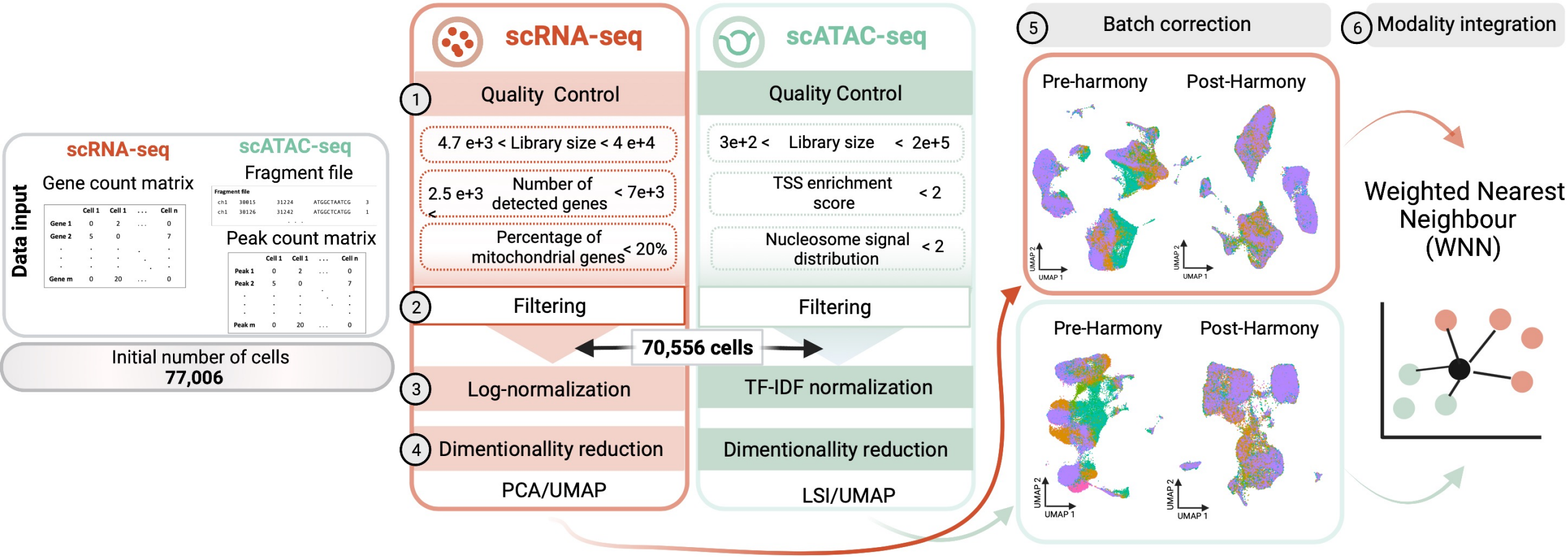
Peak count matrix

	Cell 1	Cell 1	...	Cell n
Peak 1	0	2	...	0
Peak 2	5	0		7
Peak m	0	20	...	0

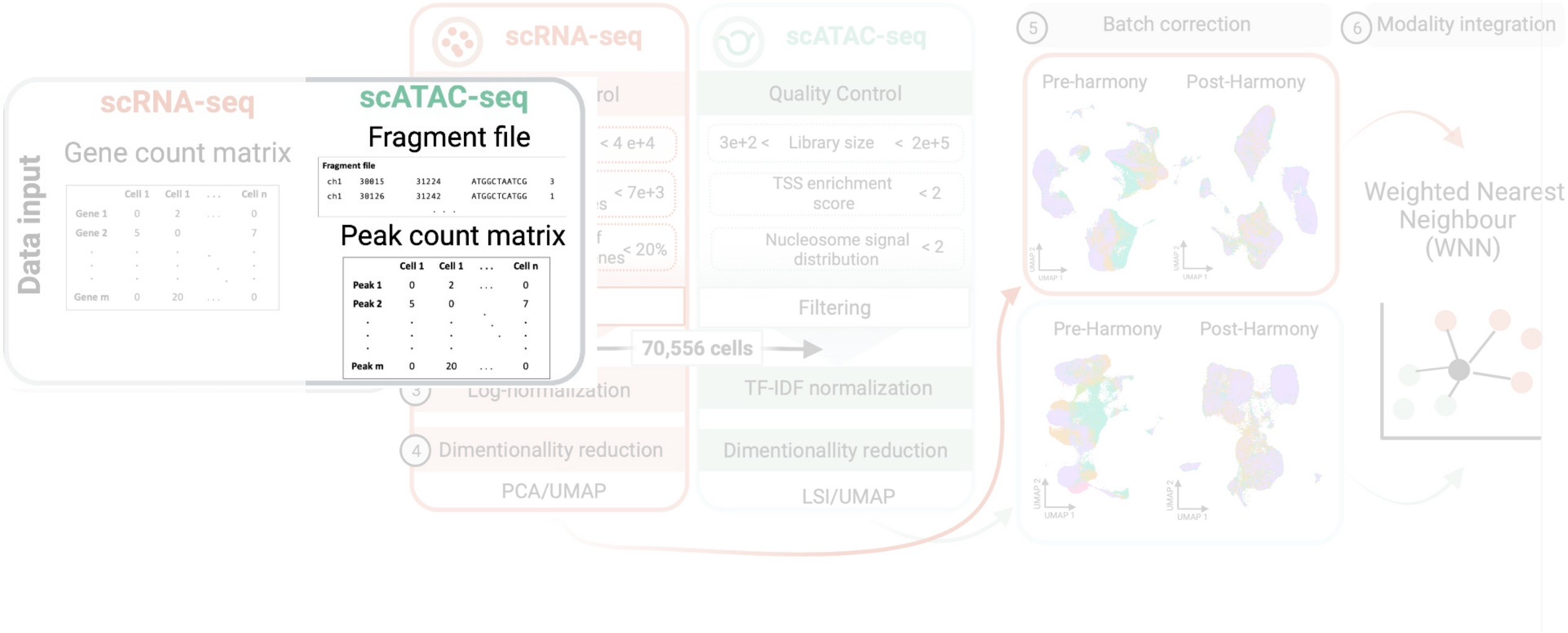
Remember: ***tbi*** *fragment file* has to be in the same directory that as you fragment file

Multiome analysis workflow

(Tools: Seurat, Signac, Harmony, Scrublet)



scATAC-seq analysis workflow



Data input

scATAC-seq

Fragment file

Chrom	ChromStart	ChromEnd	barcode	readSopport
ch1	30015	31224	ATGGCTAATCG	3
ch1	30126	31242	ATGGCTCATGG	1

BED-like format

Each line represets a unique **ATAC-seq fragment** captured by asssay

Peak count matrix

	Cell 1	Cell 1	...	Cell n
Peak 1	0	2	...	0
Peak 2	5	0		7
.	.	.		.
.	.	.		.
.	.	.		.
Peak m	0	20	...	0

Data input

scATAC-seq

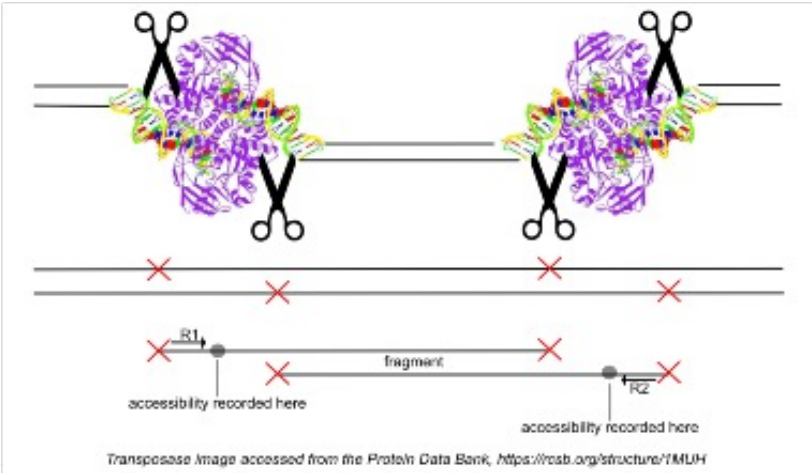
Fragment file

Chrom	ChromStart	ChromEnd	barcode	readSopport
ch1	30015	31224	ATGGCTAATCG	3
ch1	30126	31242	ATGGCTCATGG	1

BED-like format

Each line represets a unique **ATAC-seq fragment** captured by asssay

Each fragment was created by 2 separated transposition events



Transposase image accessed from the Protein Data Bank, https://rcsb.org/structure/1MUH

Peak count matrix

	Cell 1	Cell 1	...	Cell n
Peak 1	0	2	...	0
Peak 2	5	0		7
.	.	.		.
.	.	.		.
.	.	.		.
Peak m	0	20	...	0

Data input

scATAC-seq

Fragment file

Chrom	ChromStart	ChromEnd	barcode	readSopport
ch1	30015	31224	ATGGCTAATCG	3
ch1	30126	31242	ATGGCTCATGG	1

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Each line represets a unique **ATAC-seq fragment** captured by asssay

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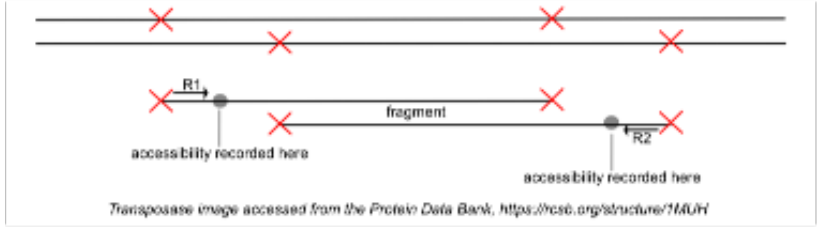


Duplicate reads are collapsed into a single fragment record (readSupport)

Peak count matrix

	Cell 1	Cell 1	...	Cell n
Peak 1	0	2	...	0
Peak 2	5	0		7
.	.	.		.
.	.	.		.
.	.	.		.
Peak m	0	20	...	0

Data input

scATAC-seq

Fragment file

Chrom	ChromStart	ChromEnd	barcode	readSopport
ch1	30015	31224	ATGGCTAATCG	3
ch1	30126	31242	ATGGCTCATGG	1

Peak count matrix

	Cell 1	Cell 1	. . .	Cell n
Peak 1	0	2	. . .	0
Peak 2	5	0		7
Peak m	0	20	. . .	0

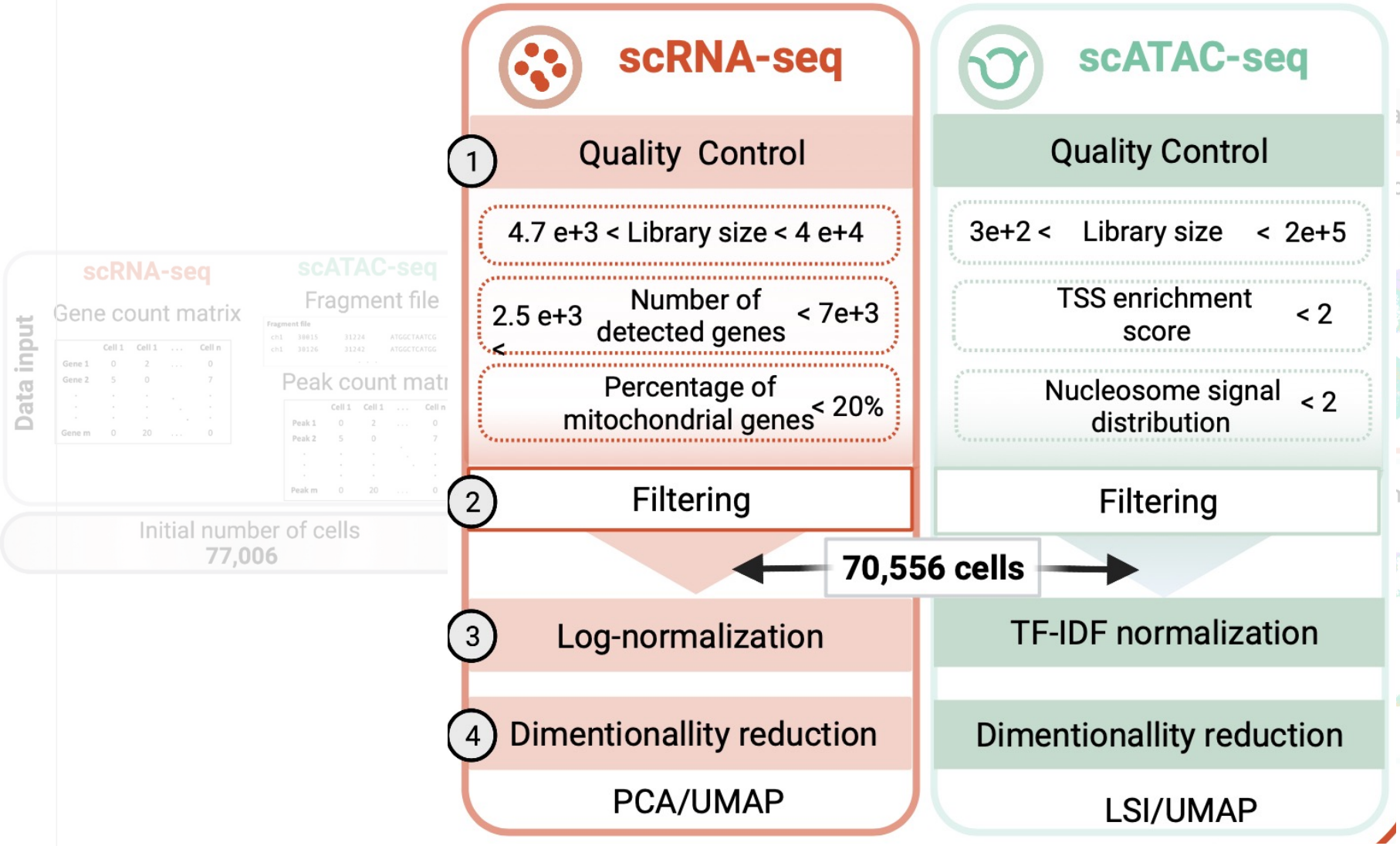
Each row represents a open chromatin region of the genome (a peak).

Each value represents the number of Tn5 integration sites for each single barcode (i.e. a cell) that map within each peak.

Multiome analysis workflow

Quality Control

(Tools: Seurat, Signac, Harmony, Scrublet)



Quality control metrics

scATAC-seq

Quality Control

3e+2 < Library size < 2e+5

TSS enrichment score > 2

Nucleosome signal distribution < 2

Filtering

TF-IDF normalization

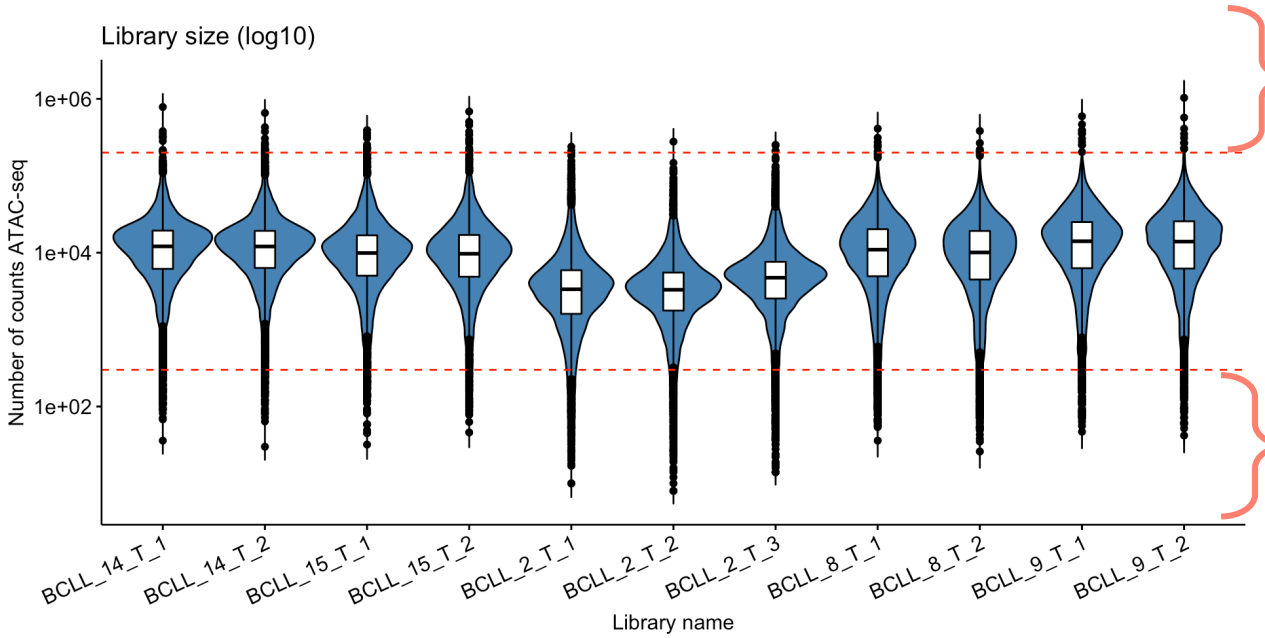
Dimentionallity reduction

LSI/UMAP

The expected range of values for these parameters will vary depending on your biological system, cell viability, and other factors.

Library sizes = sequencing depth / complexity

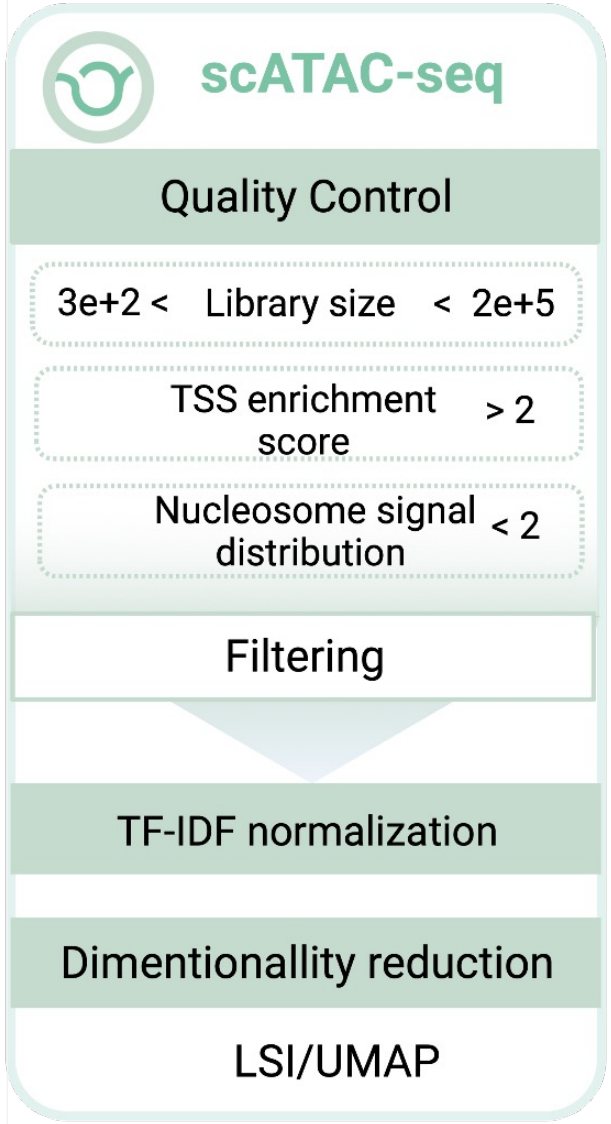
Total number of fragments in peaks:



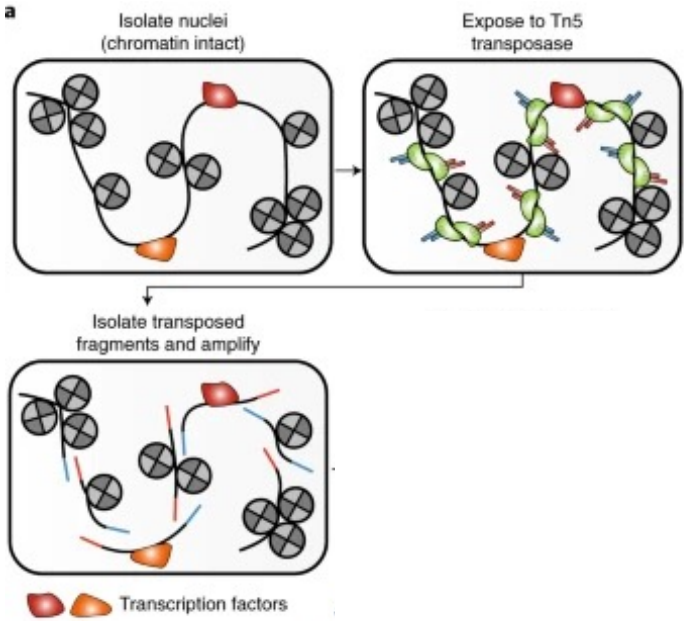
Cells with **extremely high levels of reads**→ may represent **doublets, nuclei clumps, or other artefacts**.

Cells with **very few reads** → Exclude due to **low sequencing depth**.

Quality control metrics



Nucleosome signal distribution



- It quantifies the approximate ratio of Di-nucleosome (DI) and mono-nucleosome (MONO) to nucleosome-free region (NFR) fragments.
- <u>It is used to evaluate the quality of transposase reaction:</u> We expect to **find half or more of the of fragments within the NFR** to confirm that our data is high quality and the transposase worked properly.

Quality control metrics

scATAC-seq

Quality Control

3e+2 < Library size < 2e+5

TSS enrichment score > 2

Nucleosome signal distribution < 2

Filtering

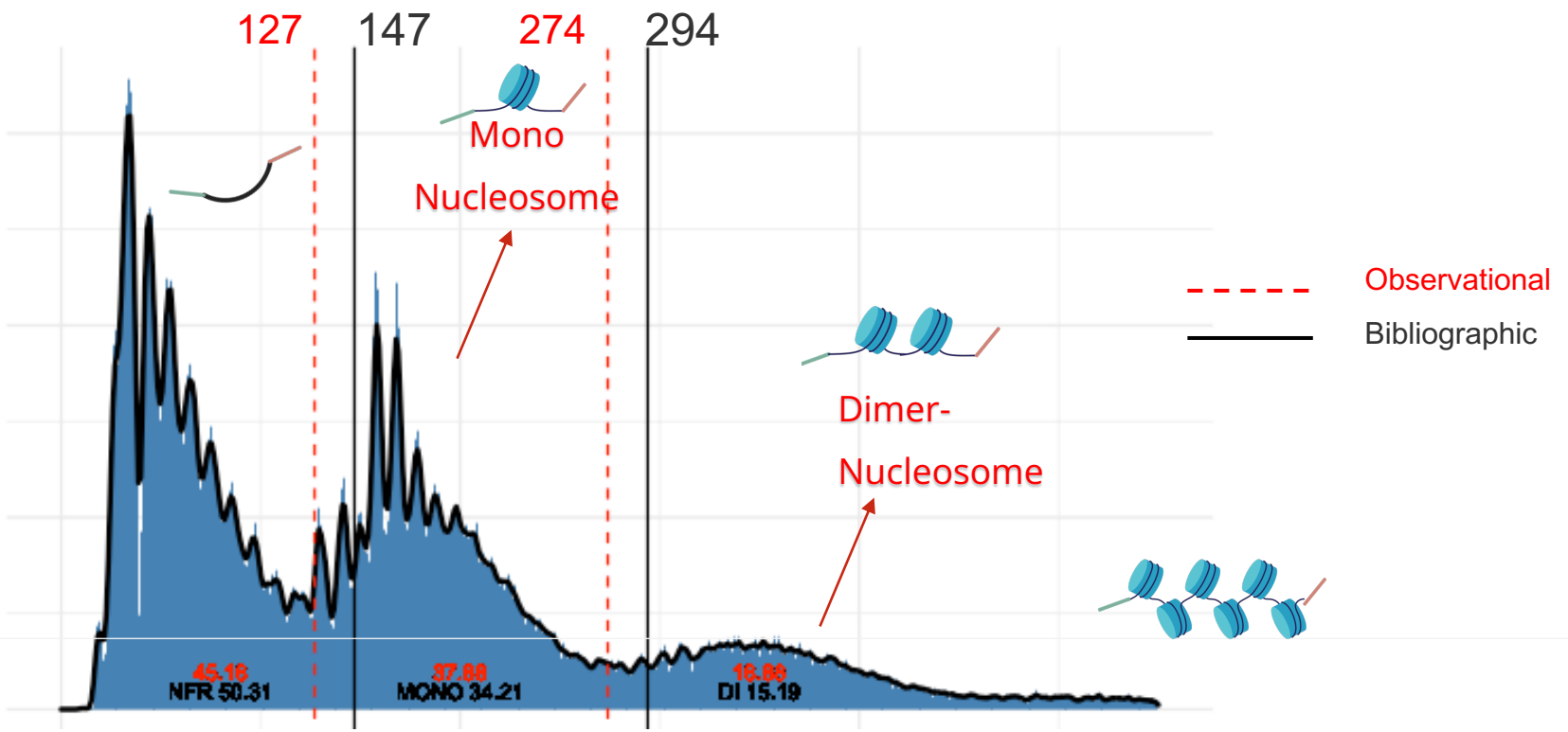
TF-IDF normalization

Dimentionallity reduction

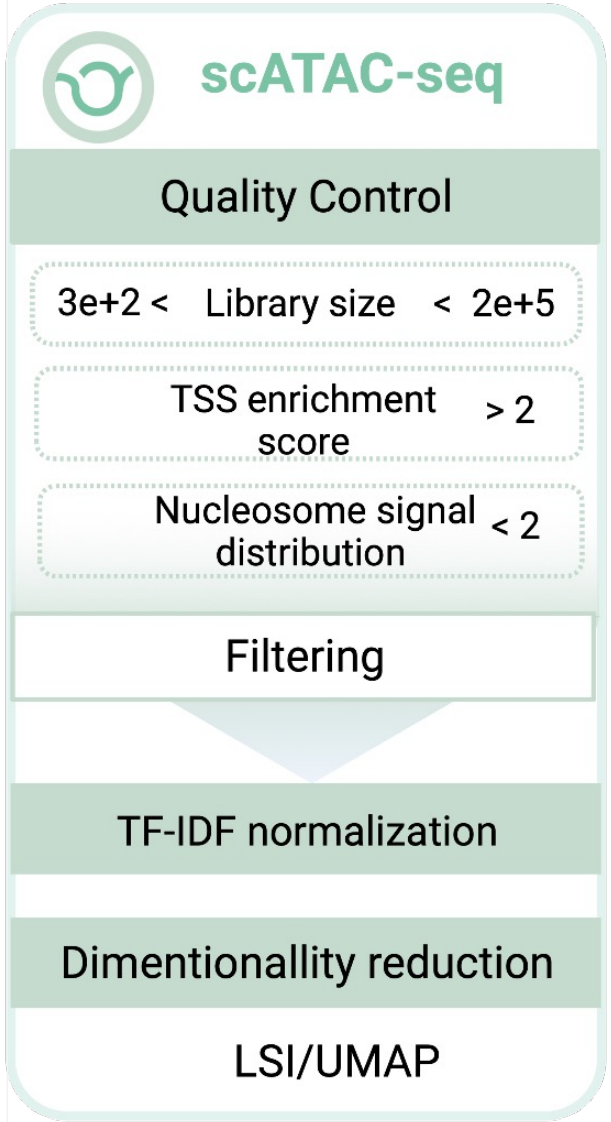
LSI/UMAP

Nucleosome signal distribution

The histogram of DNA fragment sizes should exhibit a strong nucleosome banding pattern corresponding to the length of DNA wrapped around a single nucleosome.



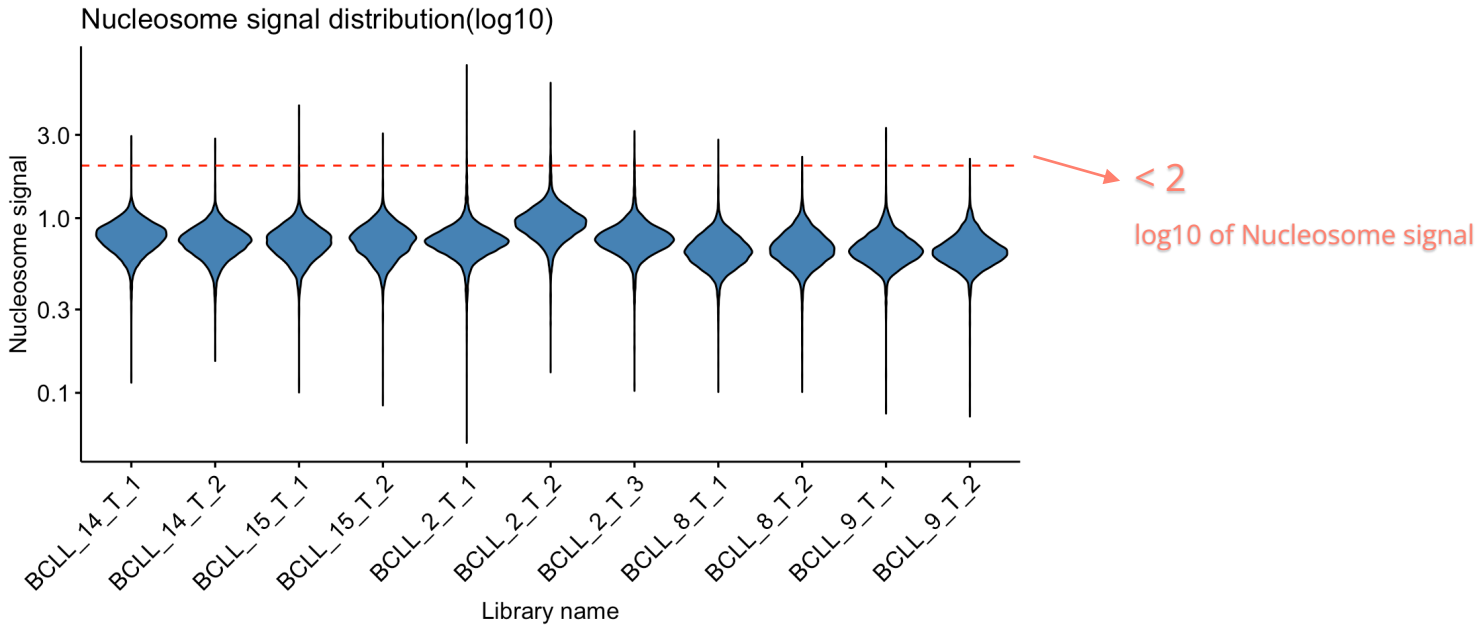
Quality control metrics



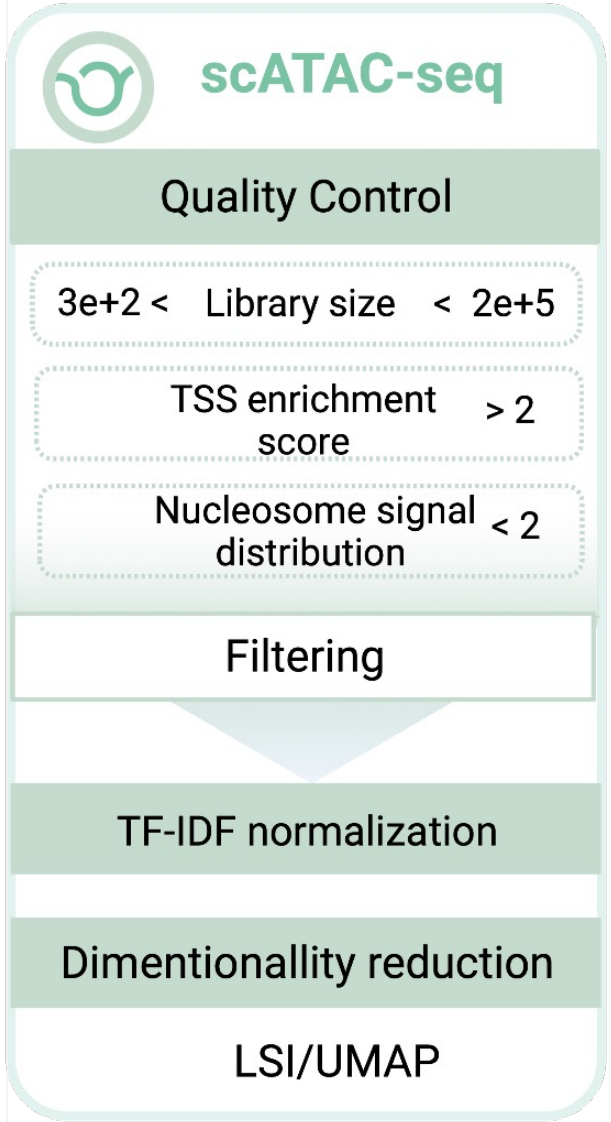
The expected range of values for these parameters will vary depending on your biological system, cell viability, and other factors.

Nucleosome signal distribution

The ratio of mononucleosomal (147–294 bp) to nucleosome-free (<147 bp) fragments sequenced for the cell is a way of quantifying the expected depletion of nucleosome-length DNA fragments.



Quality control metrics

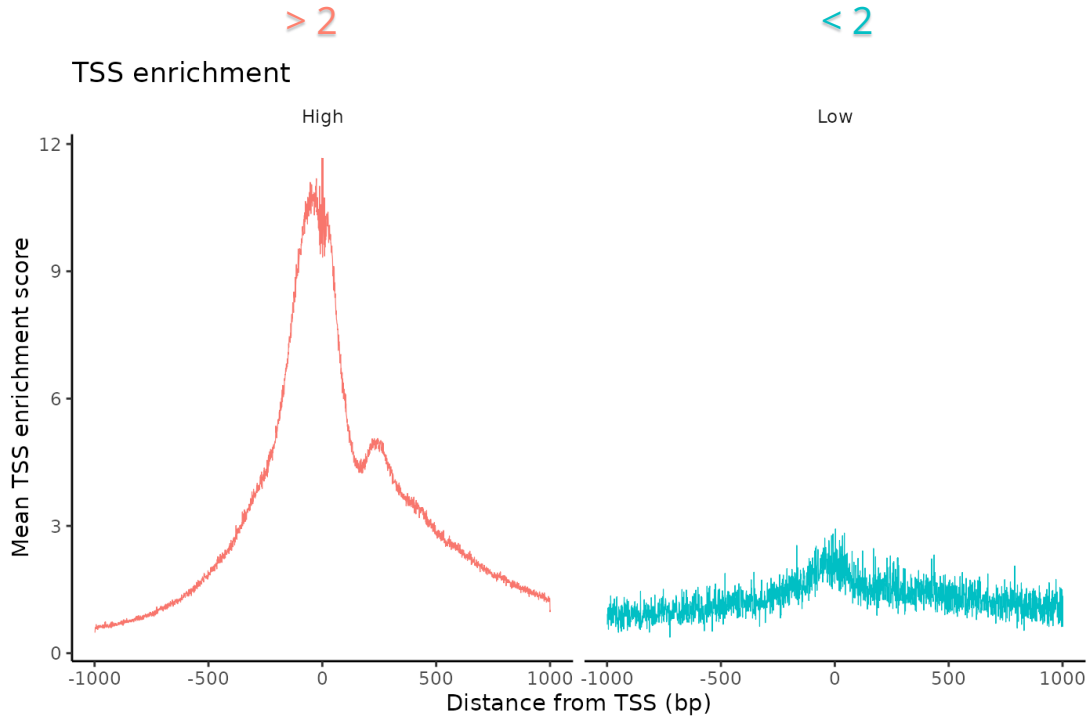


Transcriptional start site (TSS) enrichment score:

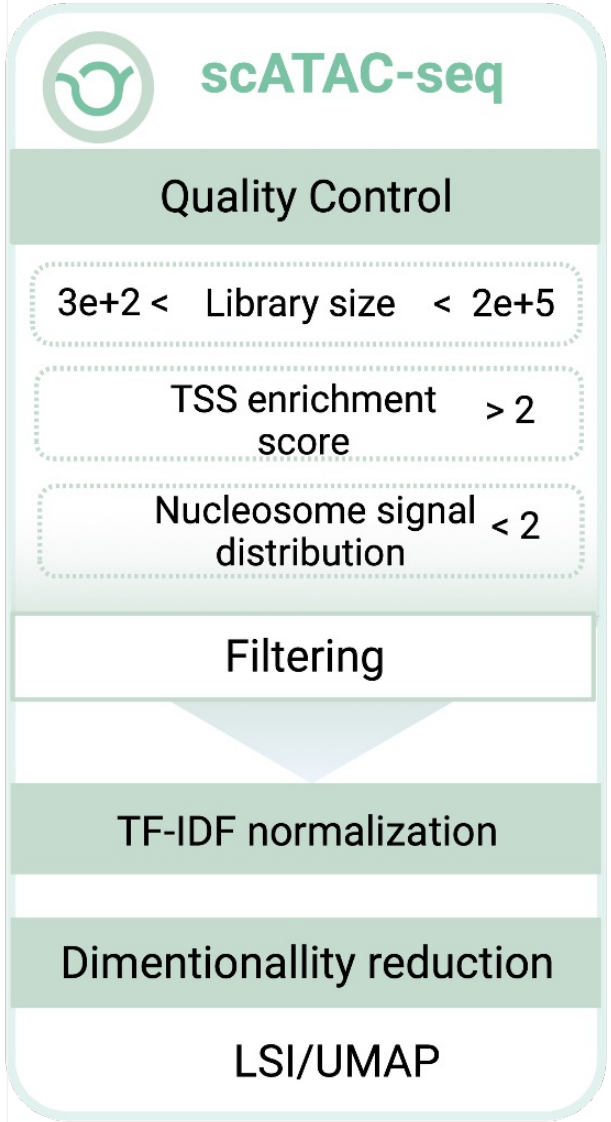
Score based on the ratio of fragments centered at the TSS to fragments in TSS-flanking regions.

- **Low TSS enrichment score** → Poor ATAC-seq experiments.

TSSEnrichment() function: computes this metric → stored in metadata under the column = TSS.enrichment.



Quality control metrics



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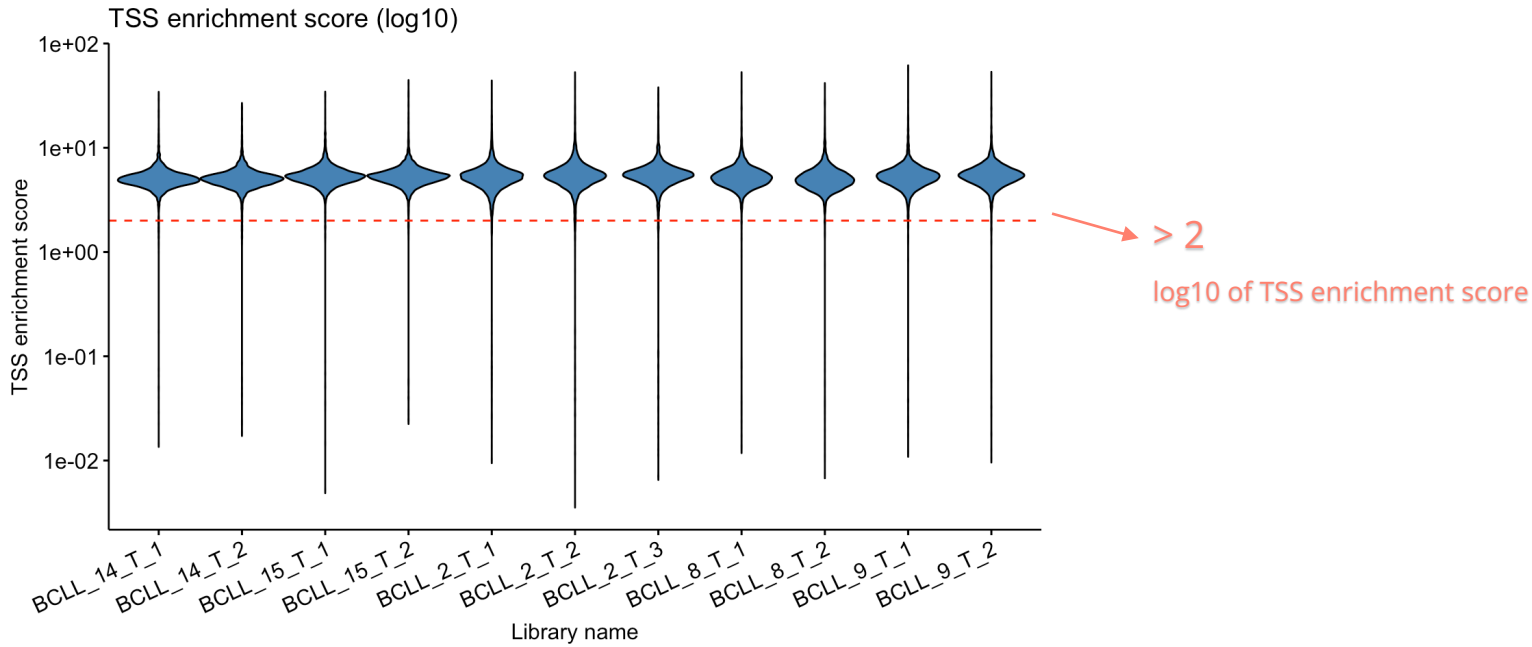
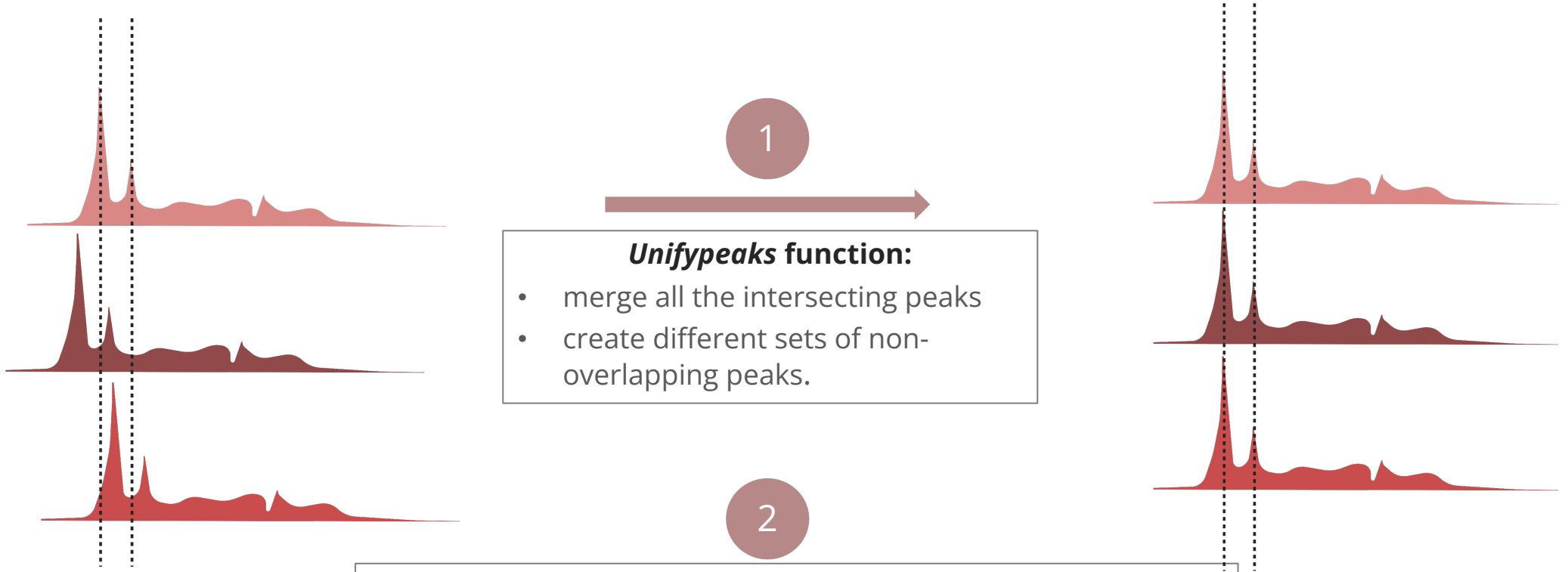


Merging Seurat Objects

When merging multiple single-cell chromatin datasets, we must be aware **that each dataset is peak-called independently**.
So, their peaks may not overlap perfectly → **create a common set of peaks before merging multiple datasets** using:



1

***Unifypeaks* function:**

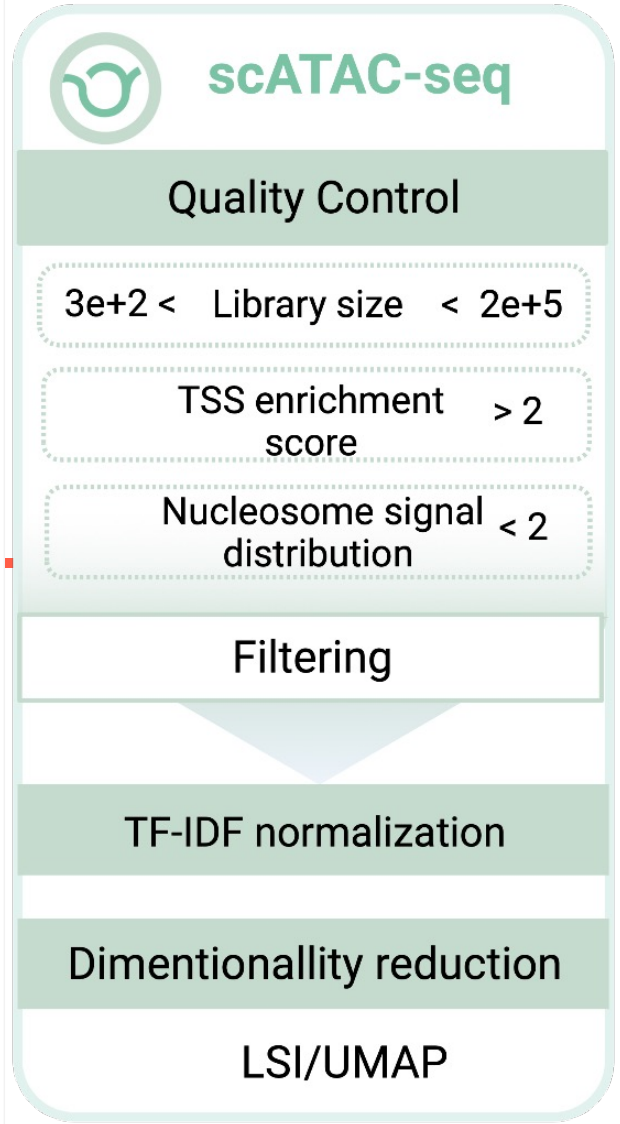
- merge all the intersecting peaks
- create different sets of non-overlapping peaks.

2

***FeatureMatrix* function :**

re-quantify the counts for each data set and it will create a new assay storing the Fragment object for each dataset.

Normalization



Signac performers a two step normalization technique:

TF-IDF: Term Frequency - Inverse Document Frequency (Signac modification where zero counts remain as zero after applying TF-IDF).

1. Normalize the cells: correcting possible differences in sequencing depth between cells
2. Normalizes the peaks: increase the signal of unusual peaks

1 $TF = \frac{C_{i,j}}{F_j}$

$C_{i,j}$ = total number of counts for ***peak i*** in ***cell j.***

F_j = total number of counts for **cell j.**

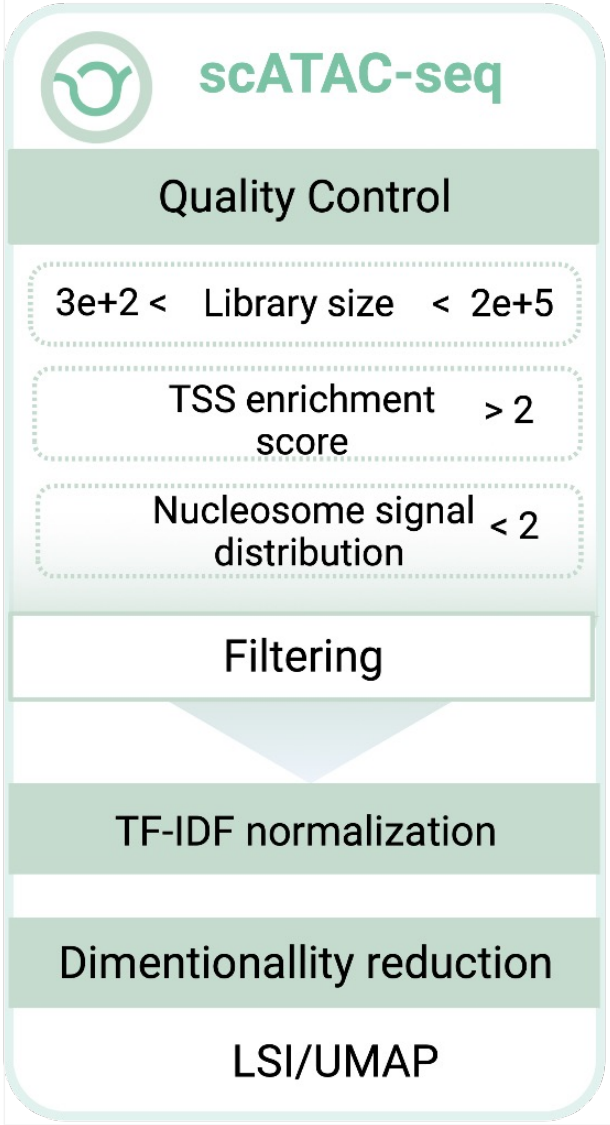
2 $IDF = \frac{N}{n_i}$

N = total number of cells in the dataset

n_i = total number counts for ***peak i*** across all cells

$$\mathrm{TF} - IDF = \log(1 + (TF \times IDF) \times 10^4)$$

Lineal and non-lineal dimensionality reduction (UMAP)



Lineal dimensionality reduction

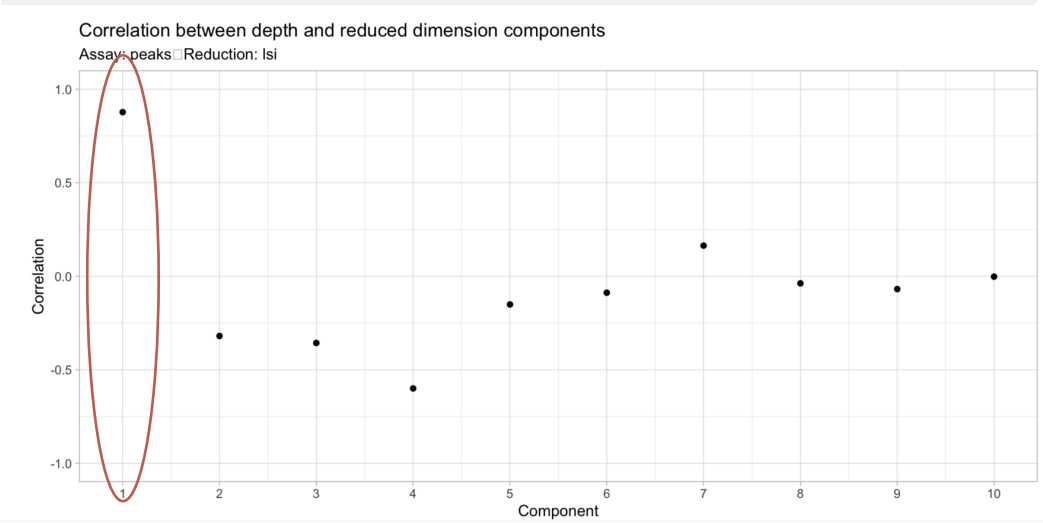
LSI: Latent Semantic Technique

It is scalable to large numbers of cells as it retains the data sparsity.

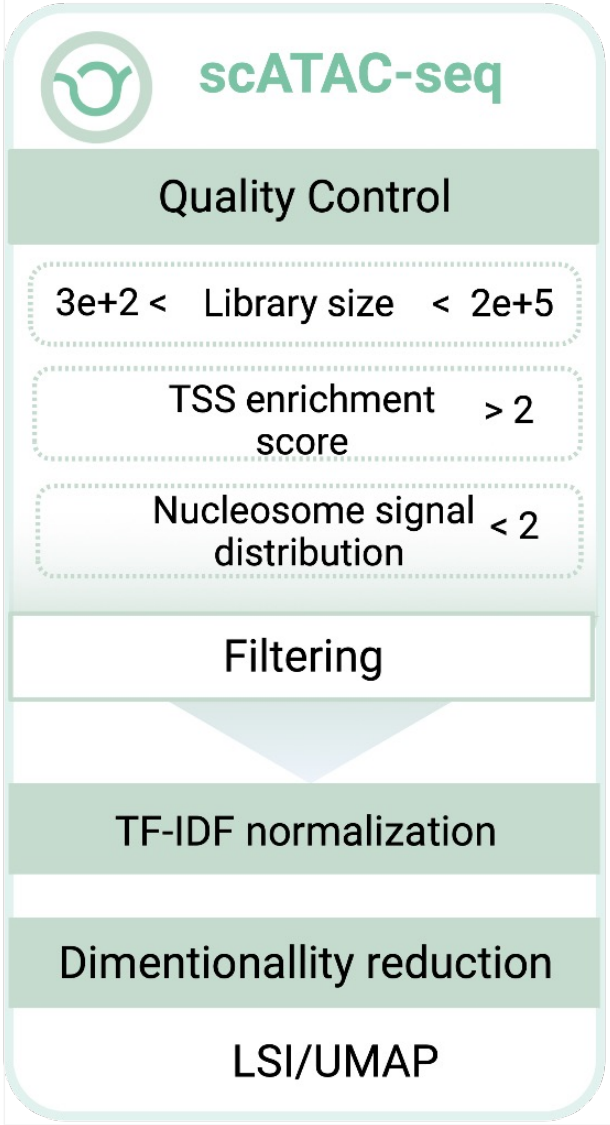
It performs the **SVD** *(Singular Value Decomposition)* on the **TF-IDF transformation matrix.**

LSI technique that combine= TF-IDF step followed by an **SVD step.**

LSI typically captures sequencing depth (technical variance) and not biological variance. We can see the fist component is correlated with the sequence depth $(R \approx 1)$



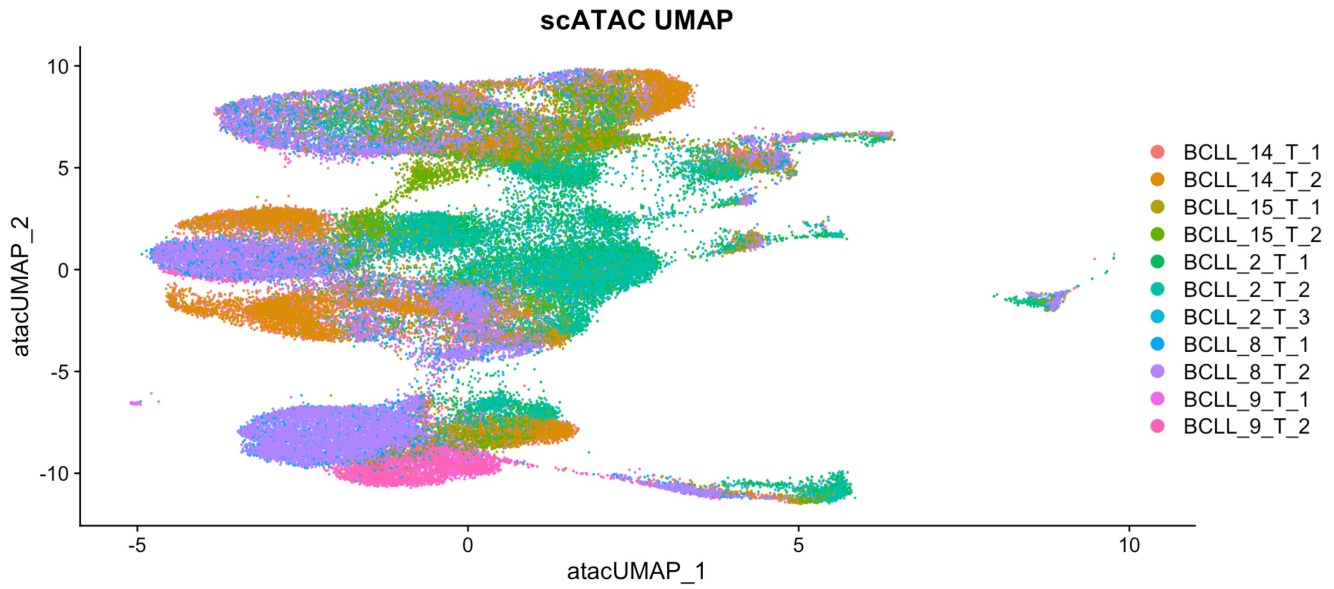
Lineal and non-lineal dimensionality reduction (UMAP)



Non-lineal dimensionality reduction

UMAP: Uniform Manifold Approximation and Projection

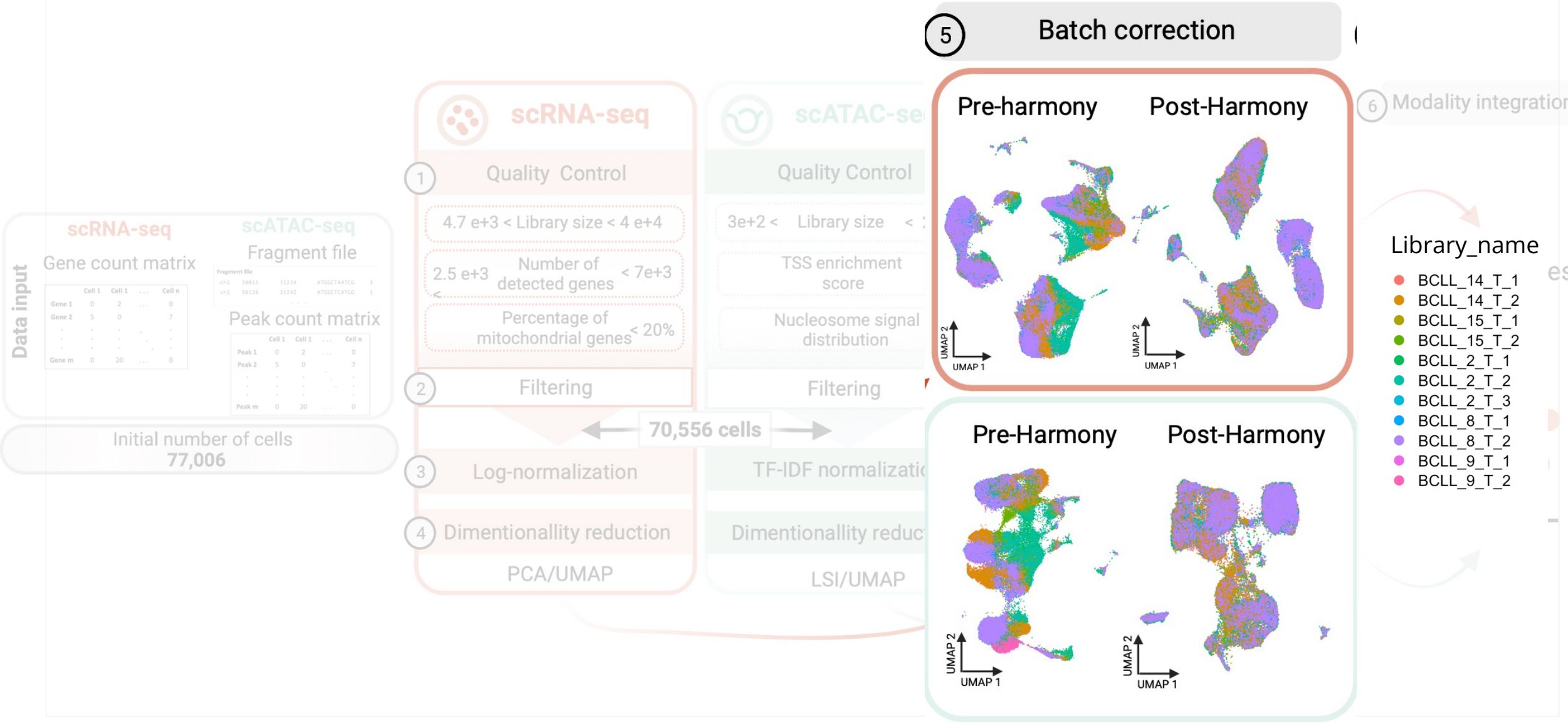
This step is like the scRNA-seq but in this case we will omit the first component of the LSI (dims = 2:40).



Multiome analysis workflow

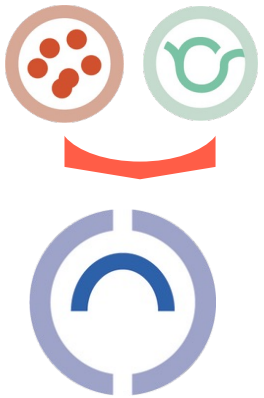
Batch Correction (Harmony)

(Tools: Harmony)

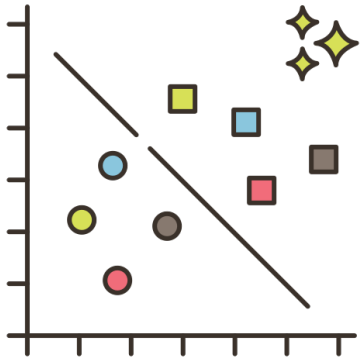


in the following session

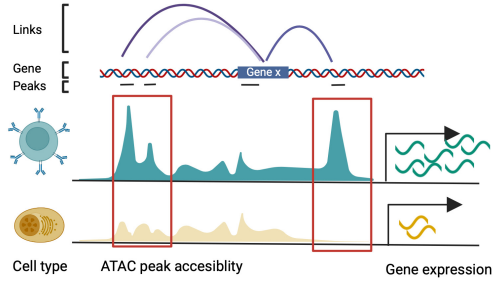
Next steps



Integration of scATAC-seq + scRNA-seq by Weighted Nearest Neigbour (WNN)



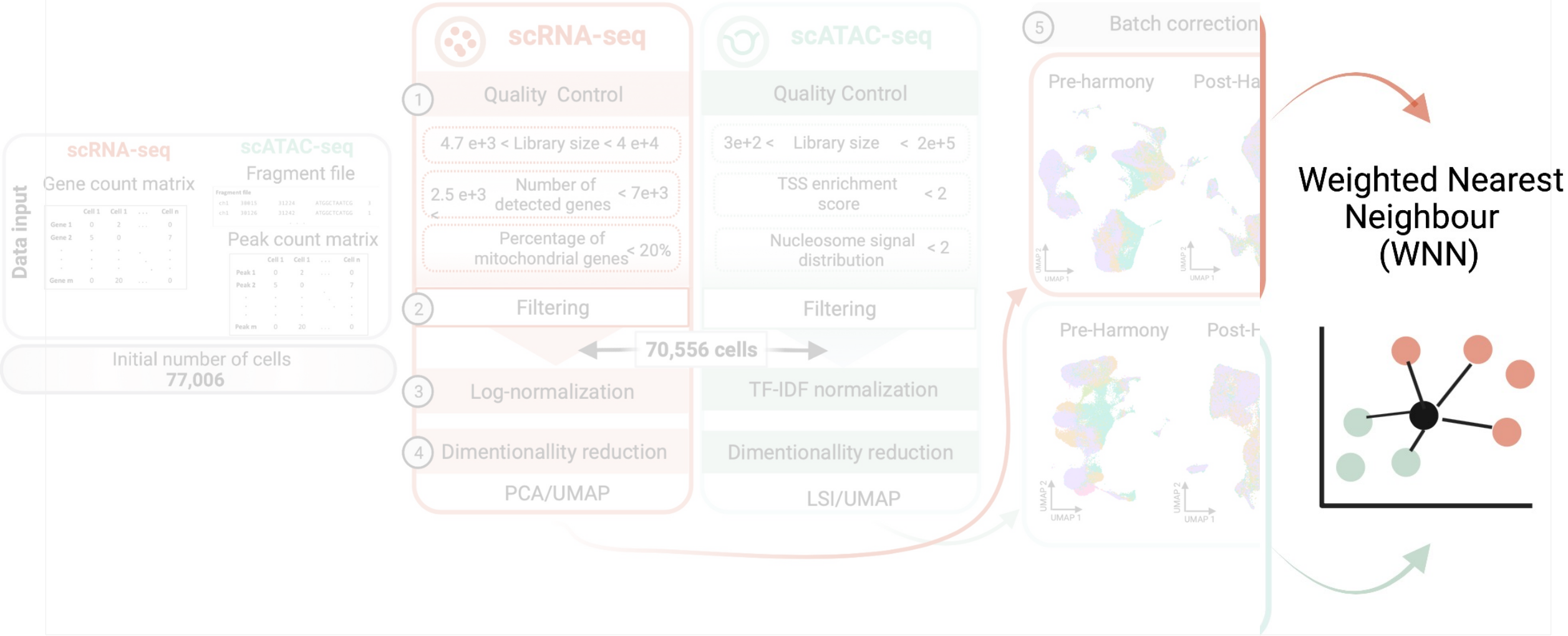
Clustering & Peak calling



Peak-genes Linkages analysis

Multiome analysis workflow

Modality integration (scRNA-seq + scATAC-seq)



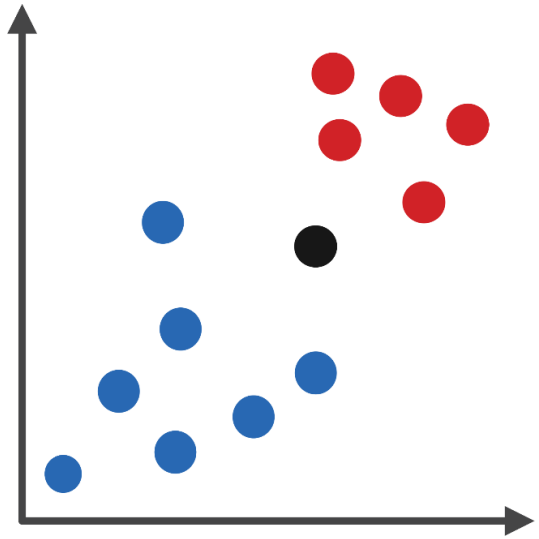
Weighted Nearest Neighbour

It is an unsupervised framework that learns the relative utility of each data type in each cell and allows integrative analysis of multiple modalities.

Weighted Nearest Neighbour is a modified version of k-Nearest Neighbors (kNN) where we take an assumption that the impact of a nearer neighbour on the query point is more than the farther away point

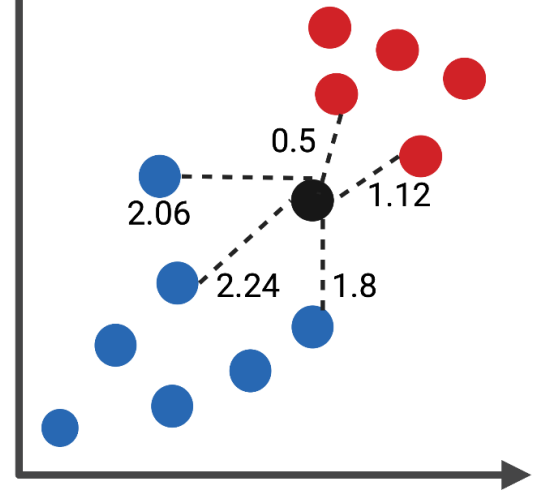
k- Nearest Neighbour (kNN)

1. Look at the data



2. Calculate euclidean distances

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$



k= 5

Euclidean distance

- 0.5 --> 1st NN
- 1.12 --> 2dn NN
- 1.8 --> 3th NN
- 2.06 --> 4th NN
- 2.24 --> 5th NN

3. Vote

# of votes

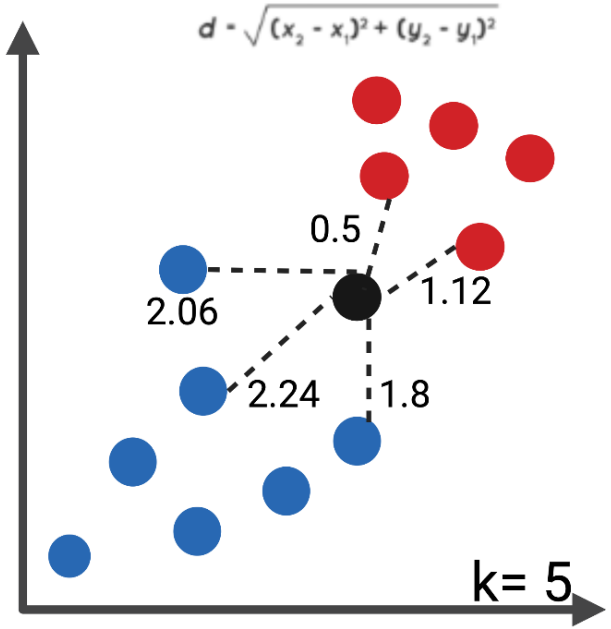
- 2
- 3

Class wins the vote!

Cell is therefore predicted to be of class

Weighted Nearest Neighbour

2. Calculate euclidean distances



3. Weight of each class

Euclidean distance (d)	Weight 1/d	
0.5	2.0	2.0 + 0.89
1.12	0.89	
1.8	0.56	0.56 +0.48 + 0.45 = 1.49
2.06	0.48	
2.24	0.45	

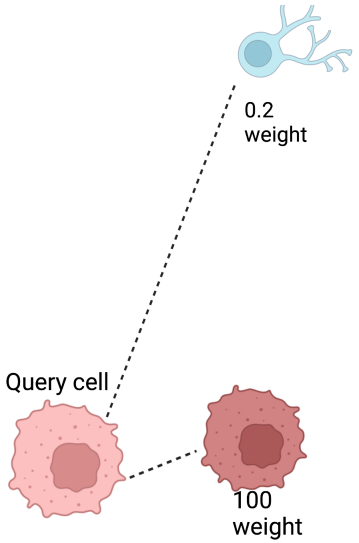
= 2.89

Class ● has more weight.

Cell ● is therefore predicted to be of class ●

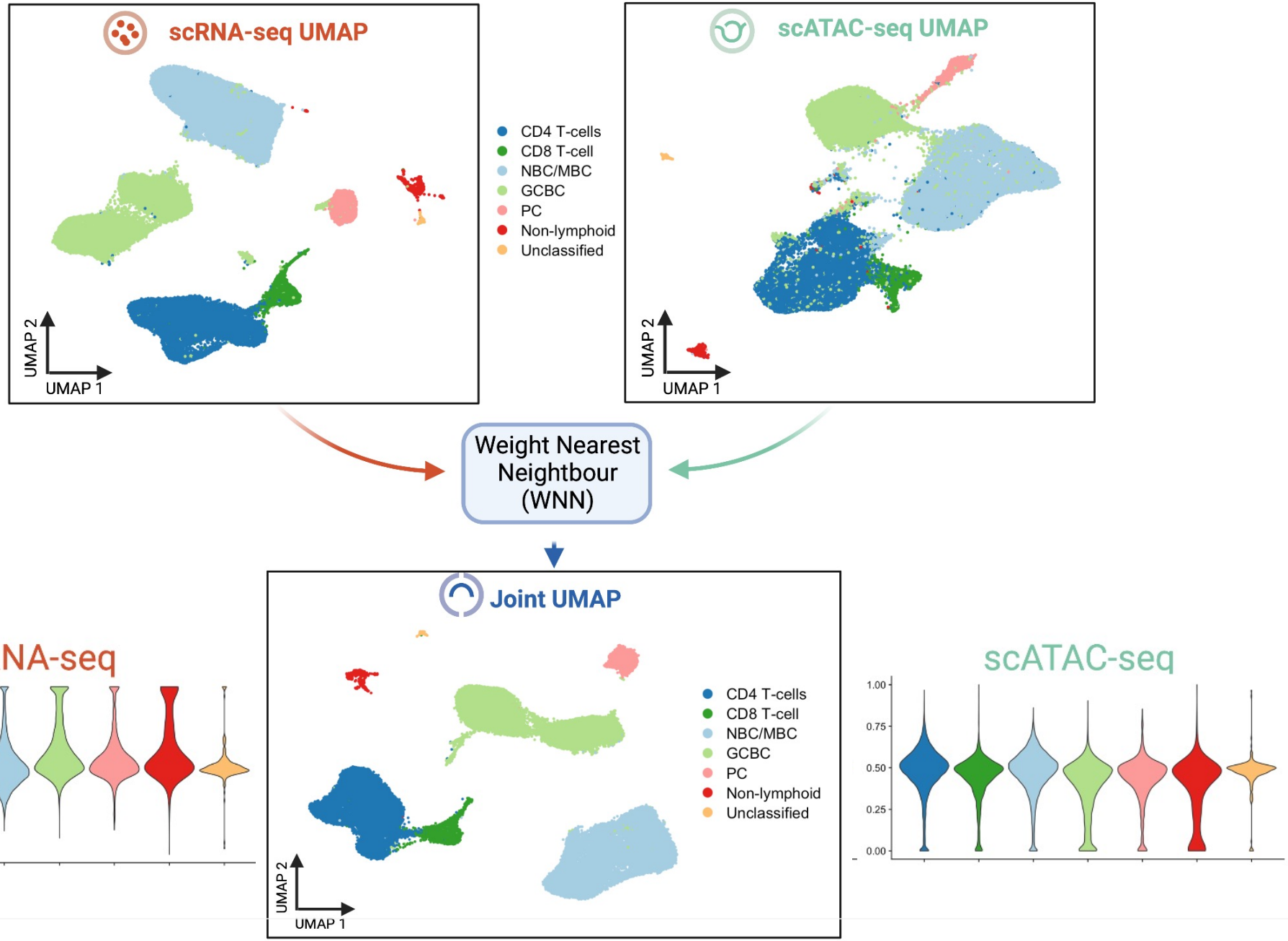
In that way we are **giving more weight to smaller distances**.
So this methods identify similarities depended on the weight:

- **High weight** = more similarity.
- **Low weight** = less similarity



Multiome analysis workflow

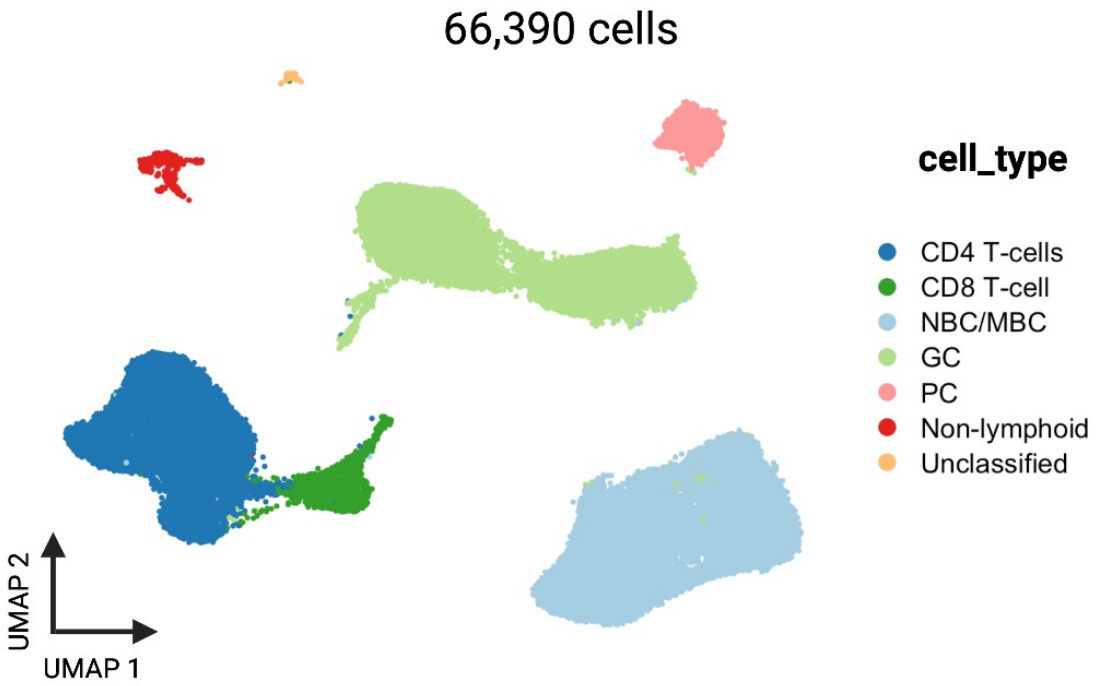
Modality integration
(scRNA-seq + scATAC-seq)

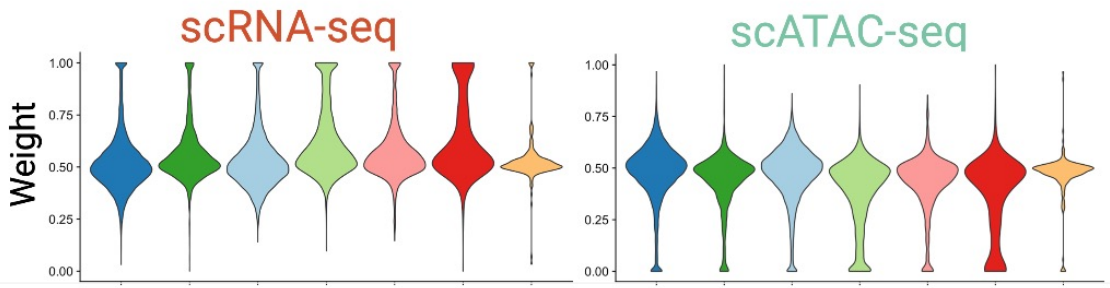


Modality integration

(scRNA-seq + scATAC-seq)

Joint UMAP



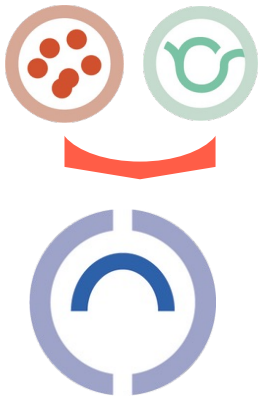


1° ***FindModalNeighbors***
This function will construct a weighted nearest neighbor (WNN) graph.

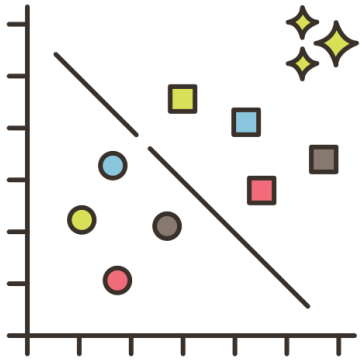
2° ***RunUMAP:*** *as we do in* scRNA-seq

in the following session

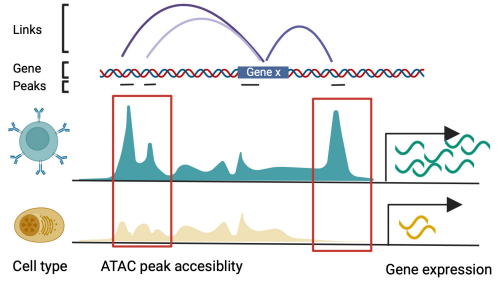
Next steps



Integration of scATAC-seq + scRNA-seq by Weighted Nearest Neigbour (WNN)



Clustering & Peak calling



Peak-genes Linkages analysis

in the following session

Homeworks

You will find 2 pipeline vignette we you will use a data set of 2 tonsil Multiome data and you will see all the functions and outputs to perform step by step all the downstream analysis of Multiome data .

- **Part_I_Seminar_QC_Filtering_Merging.Rmd:** you will perfom the quality control and visualization of the results, the filtering step and the merging of both methodologies (scATAC-seq and scRNA-seq)
- **Part_I _Seminar_I_Normalization_Batch.Rmd :** you will normalize and correct the biases of the data caused by batch effect.

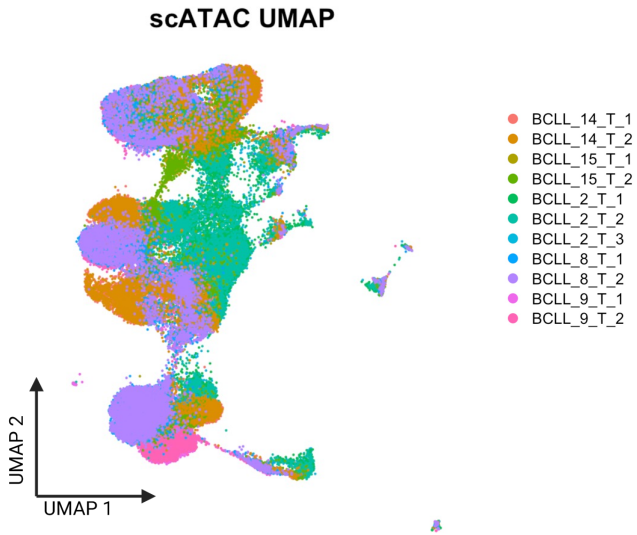
For your attention

(Tools: Harmony)

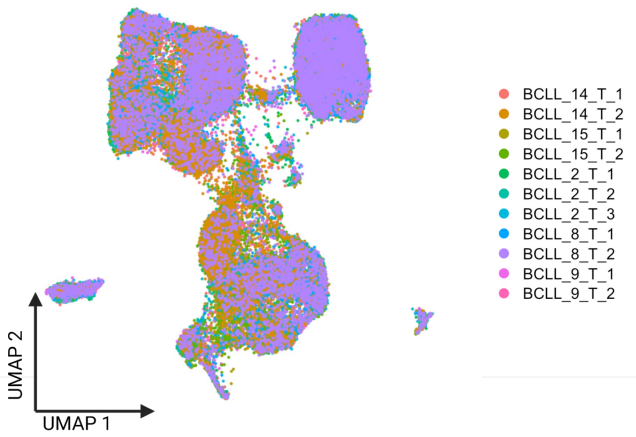
Batch Correction (Harmony)

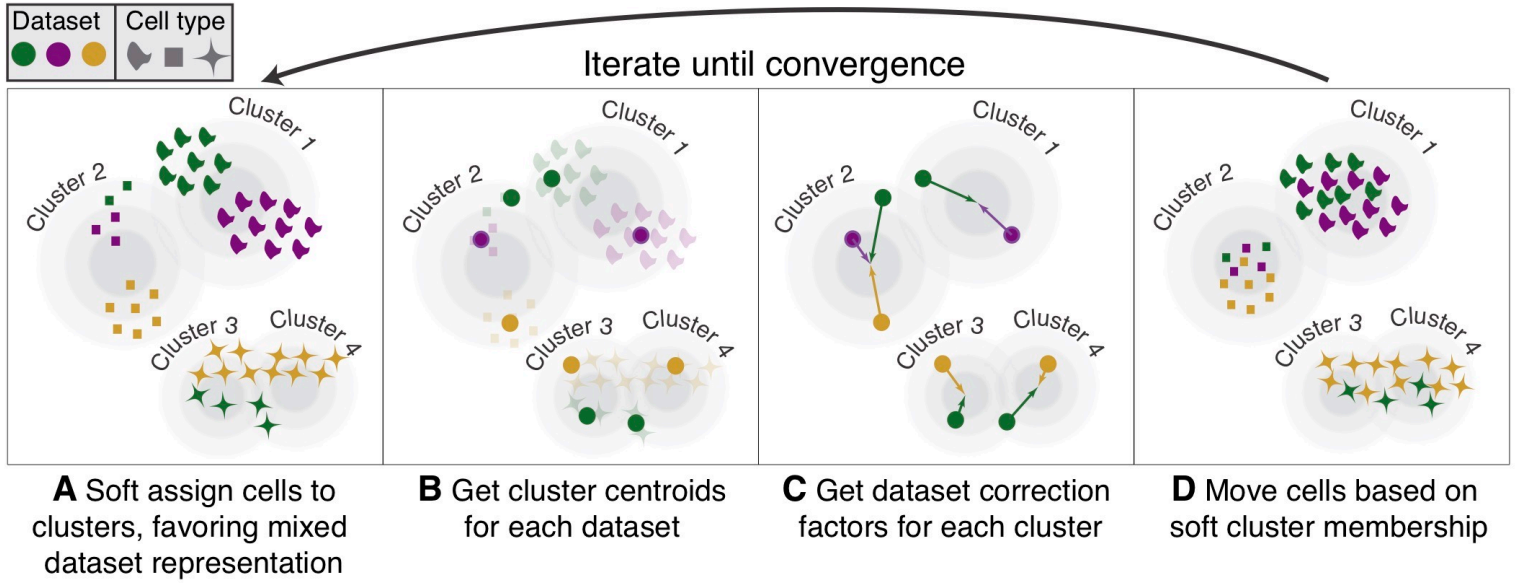
The scATAC-seq UMAP shows a clear batch effect related to the library name sample. So we use this variable to integrate the Seurat objects. Pass the Seurat object to the RunHarmony function and specify which variable to integrate.

Before Harmony

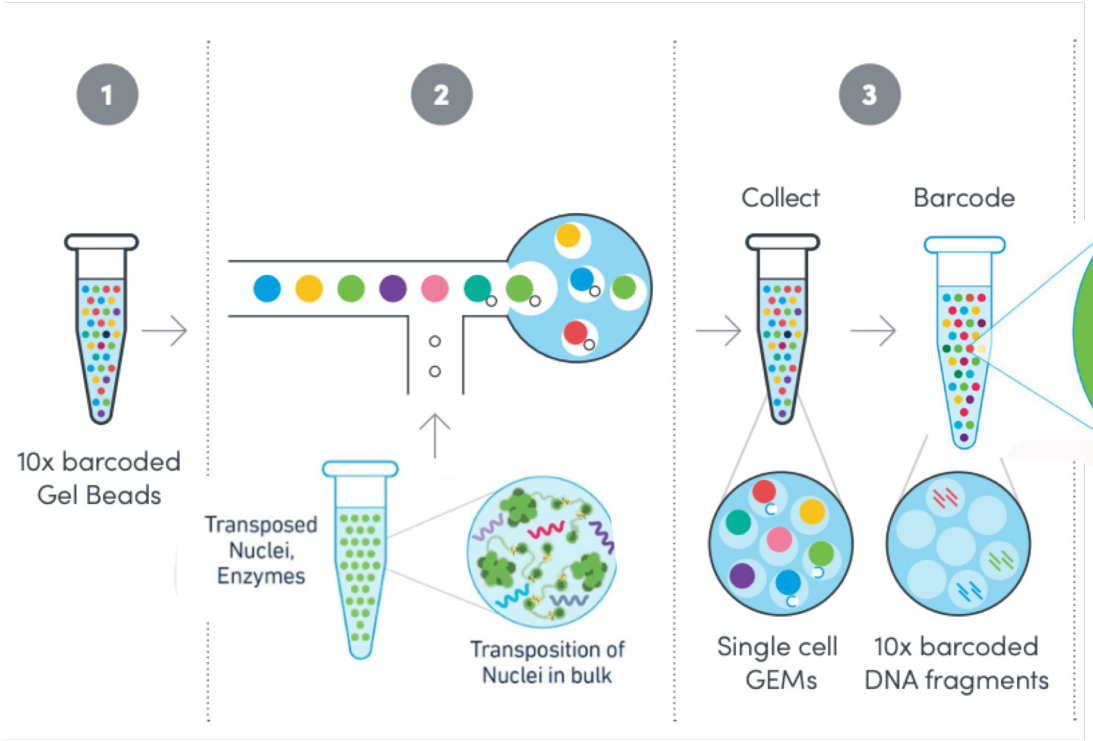


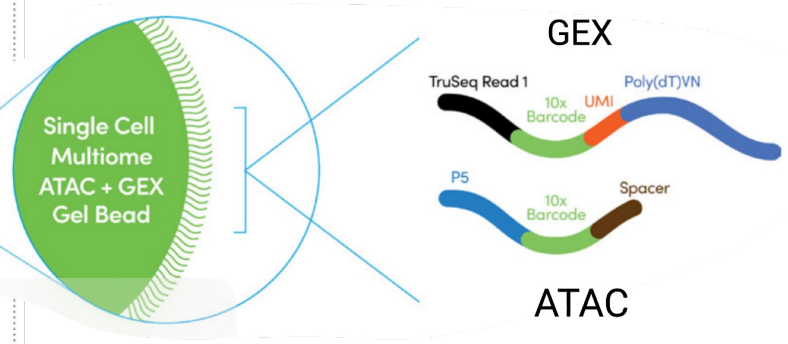
After Harmony

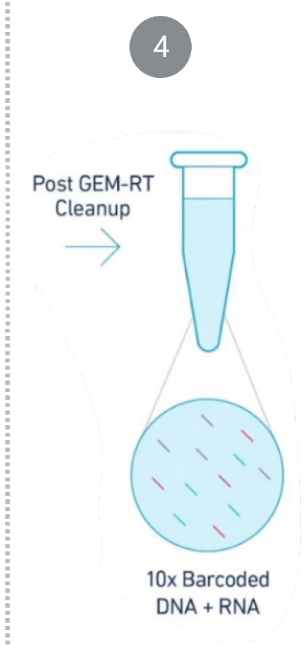




2. Reaction in GEM







Spacer sequence:
enables barcode attachment to transposed DNA fragments for ATAC library.

10x Barcode:
16 nt 10x Genomics Barcode
736k defined barcode sequences

UMI
12 nt Unique Molecular Identifier

Poly(dT)VN
30 nt Poly(dT) sequence (enables capture of polyadenylated mRNA molecules)
sequencing 3' end of mRNA

P5: Ilumina® P5 sequence

GEMs are broken and pooled fractions are recovered.
Cell barcoded are purified from the post GEM-RT reaction mixture.