

# Material for this workshop

- [This presentation](#)
- ArchR\_demo\_1\_create\_arrow\_files.Rmd
- ArchR\_demo\_2\_analysis.Rmd
- [Input data](#)

Please follow the pre-workshop instructions on the [workshop webpage](#)

# Introduction to scATAC-seq Data Analysis

April 17-18 2025

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Michela Traglia  
Ayushi Agrawal

**GLADSTONE** INSTITUTES  
*SCIENCE OVERCOMING DISEASE*

# Introductions

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Bioinformatician III

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Bioinformatician I

# Aim of the workshop

- To give an overview of the biological insights using scATAC-seq analysis
- Highlight assumptions and limitations underlying the methods
- Understand the relevance and impact of difference between scRNAseq and scATAC-seq workflow
- Experience how to analyze scATAC-seq data in ArchR

# Workshop organization

- **Session 1 (Thursday, 9am-12pm)**

1. Cell regulome and ATAC-seq
2. Technology
3. From sequencer to fragments file
4. Pre-processing and QC
  - Break
5. Normalization, Dimensionality reduction, embedding
6. Clustering and cell type annotation based on feature markers
  - Break
7. Advance analysis: Calling Peaks and Motif enrichment

- **Session 2 (Friday, 9am-12pm)**

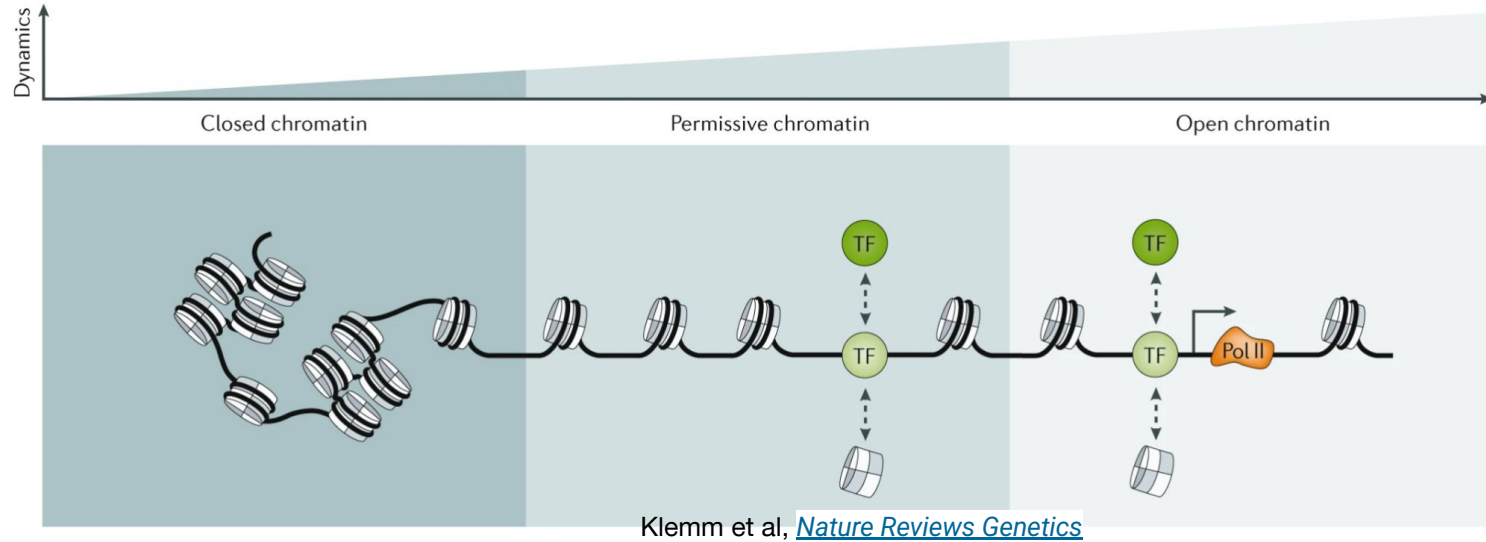
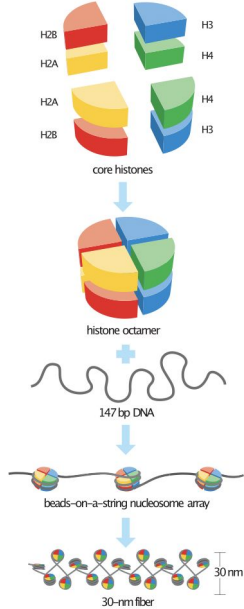
8. Intro to ArchR
  - Demo

scATAC-seq and scRNA-seq Data Integration workshop - April 24 at 1pm

Register [here](#)

# 1. Chromatin architecture of the regulatory regions

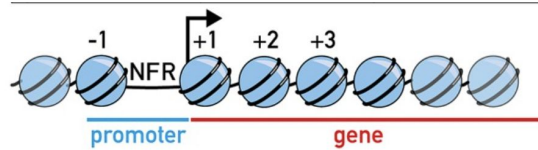
# Chromatin accessibility



- Nucleosome is an octamer of histone proteins wrapped by ~147 bp of DNA.
- Organized into chromatin with limited accessibility to external factors.
- Accessibility is dynamic and depends by the occupancy and topological organization of nucleosomes.

# Non uniform organization of nucleosomes

- Nucleosome depletion at regulatory loci (promoters, enhancers, etc.)
- Nucleosome-free regions (NFRs) are localized just upstream of the transcription start site (TSS).

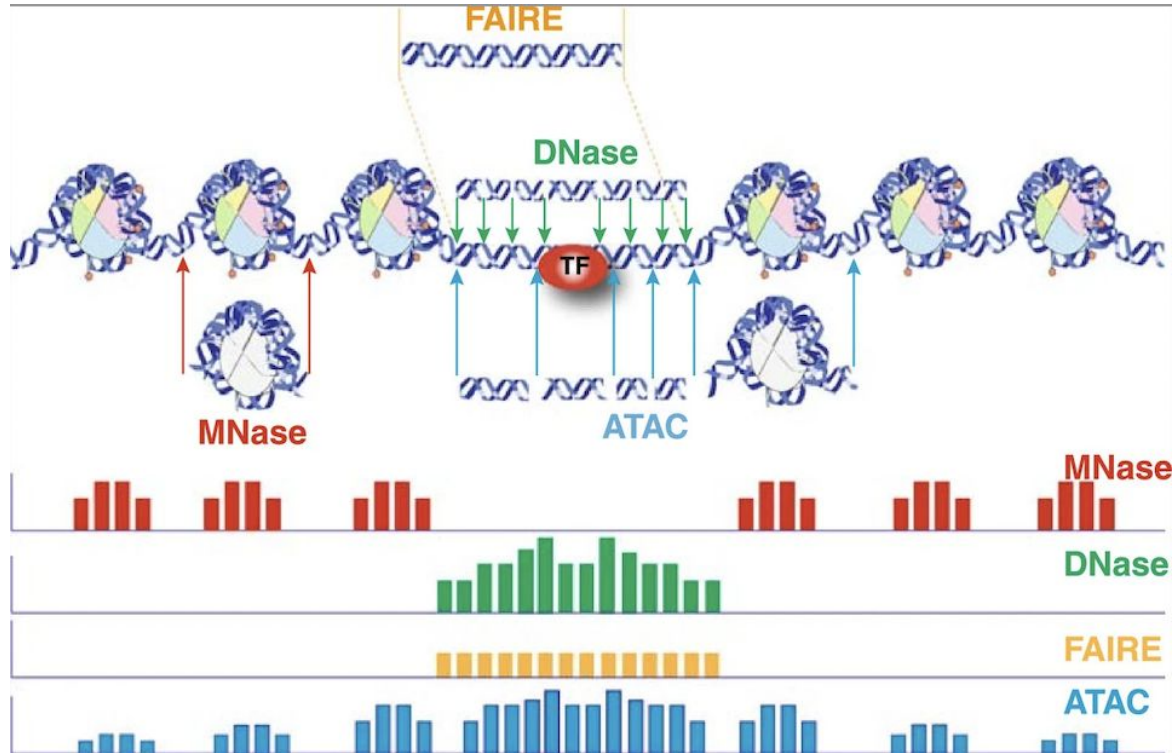


- First nucleosome downstream of the TSS (the + 1 nucleosome) is strongly localized at the same location across cells.
- Accessible genome comprises ~2–3% of total DNA sequence
- > 90% of regions bound by Transcription Factors (TF).

Genome-wide profiling of chromatin accessibility to identify **candidate regulatory genomic regions** in a tissue or cell type



# Which regulatory region are you interested in?



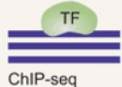
**MNase:** indirect study of accessibility - nucleosome occupancy

**DNase:** DNase I hypersensitive site - nucleosome depleted regions

**FAIRE:** easy but background

**ATAC:**

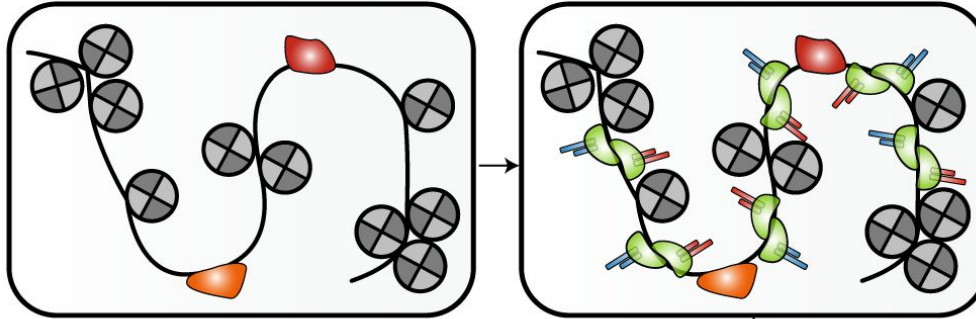
- simple and fast two-step protocol
- high sensitivity
- low starting cell number (500 to 50,000 cells)
- multiple aspects of chromatin architecture simultaneously at high resolution.



**ChIP-seq** : binding sites of DNA-associated proteins and can be used to map global binding sites for a given protein.

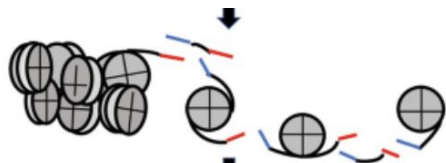
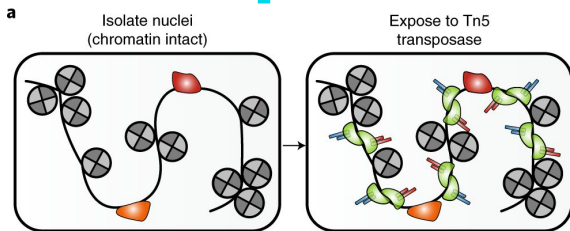
# ATAC is based on the transposition system

Assay for Transposase-Accessible Chromatin with high-throughput sequencing

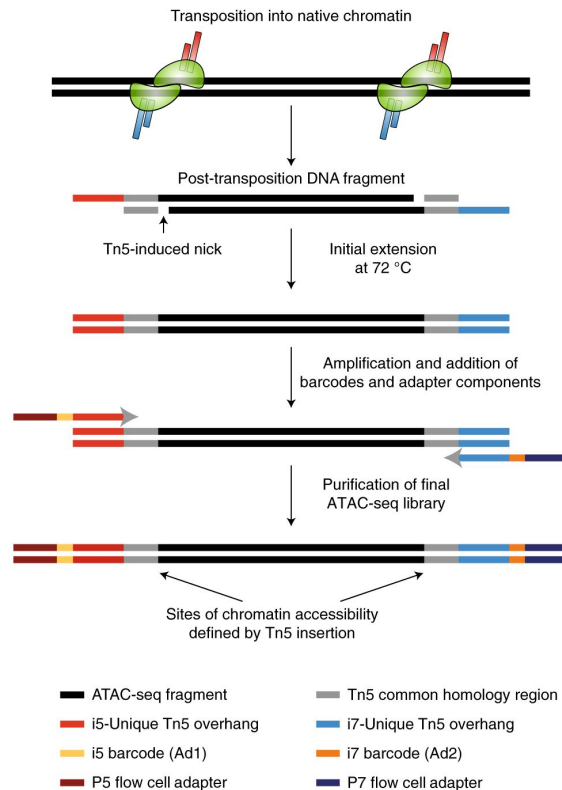


- Transposons are genetic elements that can “jump” to different locations within a genome.
- Bacterial Tn5 - encode a hyperactive Tn5 transposase (Tnp) that can simultaneously cut accessible DNA fragments and ligate sequencing adapters to both strands.
- Fast “cut and paste” and “copy and paste” functions.

# Transposition events create fragments



- Binds as a homodimer with **9-bp of DNA between the two Tn5 molecules.**
- Two transposition events = **FRAGMENT**



- The central point of the “accessible” site is in the **very center of the Tn5 dimer**, not the location of each Tn5 insertion.
- **Adjusting** plus-stranded insertion events by **+4 bp** and minus-stranded insertion events by **-5 bp**.
- Paired-end sequencing.

# Tn5 application

## 2D structure:

- Bulk ATAC-seq
- scATAC-seq to study the dynamically regulated chromatin in a cell type-specific manner

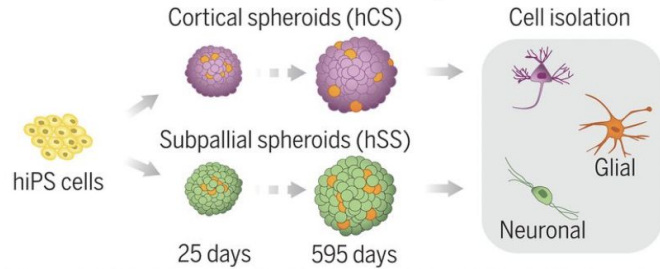
## Spatial proximity within the nucleus by analysing contacts between genomic regions:

- High-throughput sequencing for the study of chromatin 3D structure - chromatin conformation capture assays 3C (one vs one) and Hi-C (all vs all).

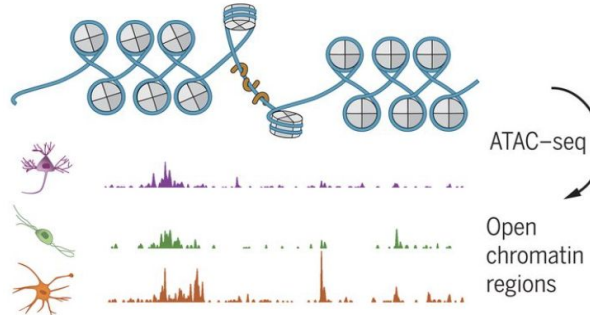
# Experimental design is fundamental (I)

- Which epigenetic mechanism do you need to study?
- Is single cell suitable for my purpose?

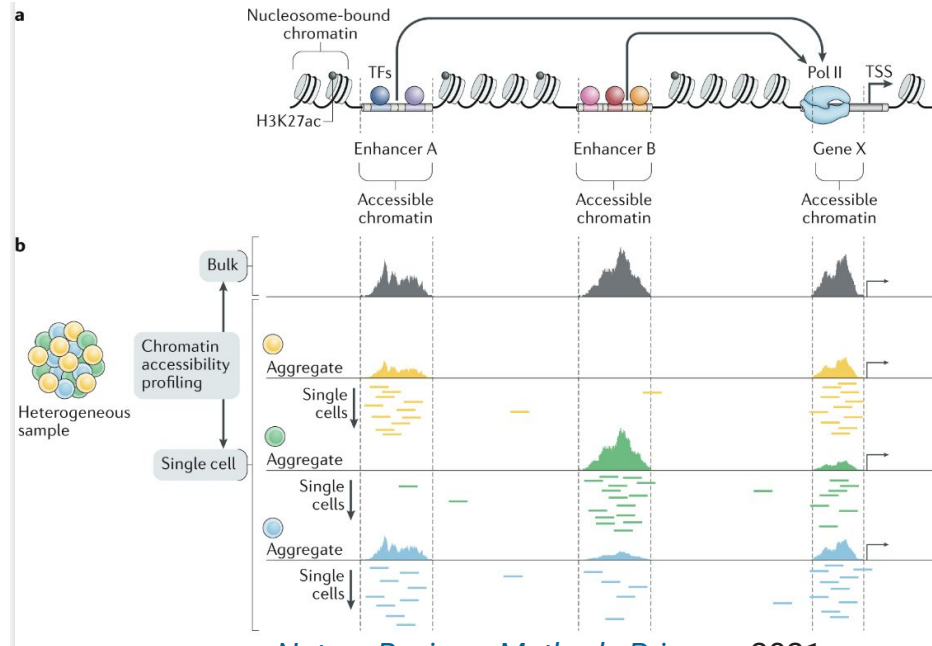
## Human cellular model of forebrain development



## Cell type-specific chromatin accessibility

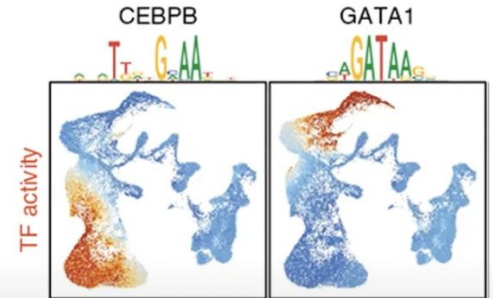
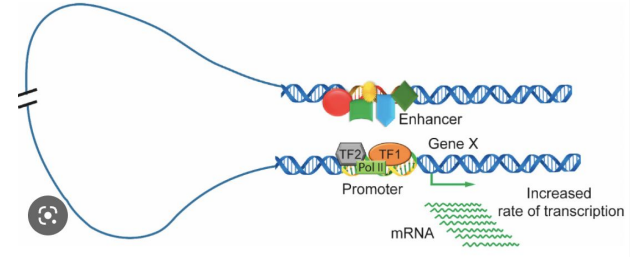
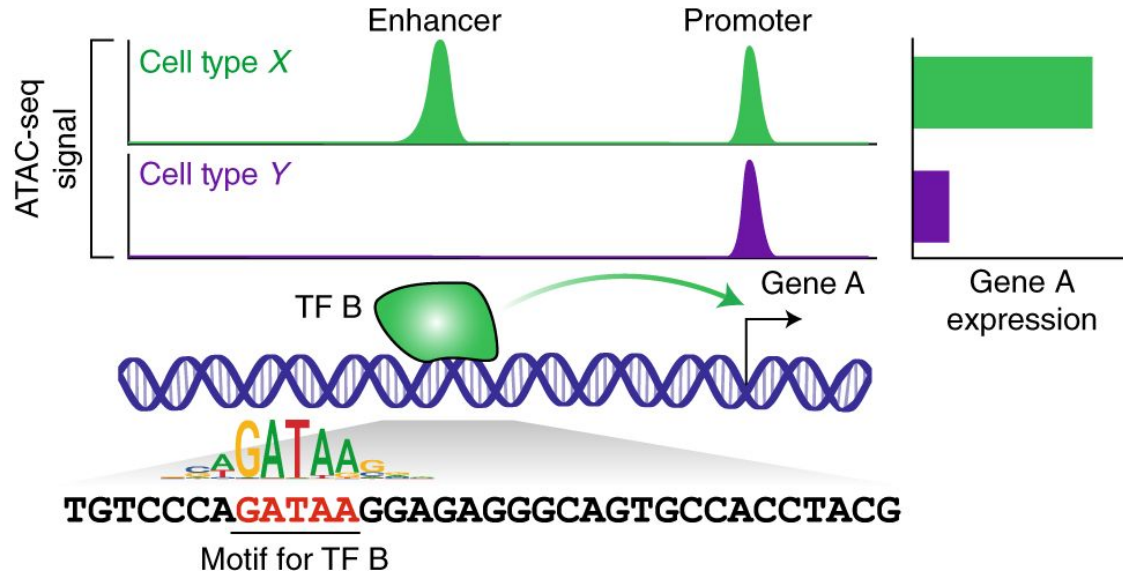


Trevino et al, Science 2020



*Nature Reviews Methods Primers*, 2021

# What biological insight we can get from scATAC-seq



- One step further than scRNA-seq:
  - In trajectory analysis, identify variable TF and enhancers
  - Gene regulatory networks between transcription factor gene and accessible target genes

# scATAC-seq workflow

**From bench**

**Through the bioinformatic analysis**

**To the biological interpretation**

Your cell's regulome question

Fragments count

Embedding

Library preparation & sequencing

QC filtering low quality cells

Clustering

Demultiplex, read alignment and  
quantification

Layered dimensionality reduction

Find Markers - cell annotation

**For more biological insight in cell's regulome**

Peak calling

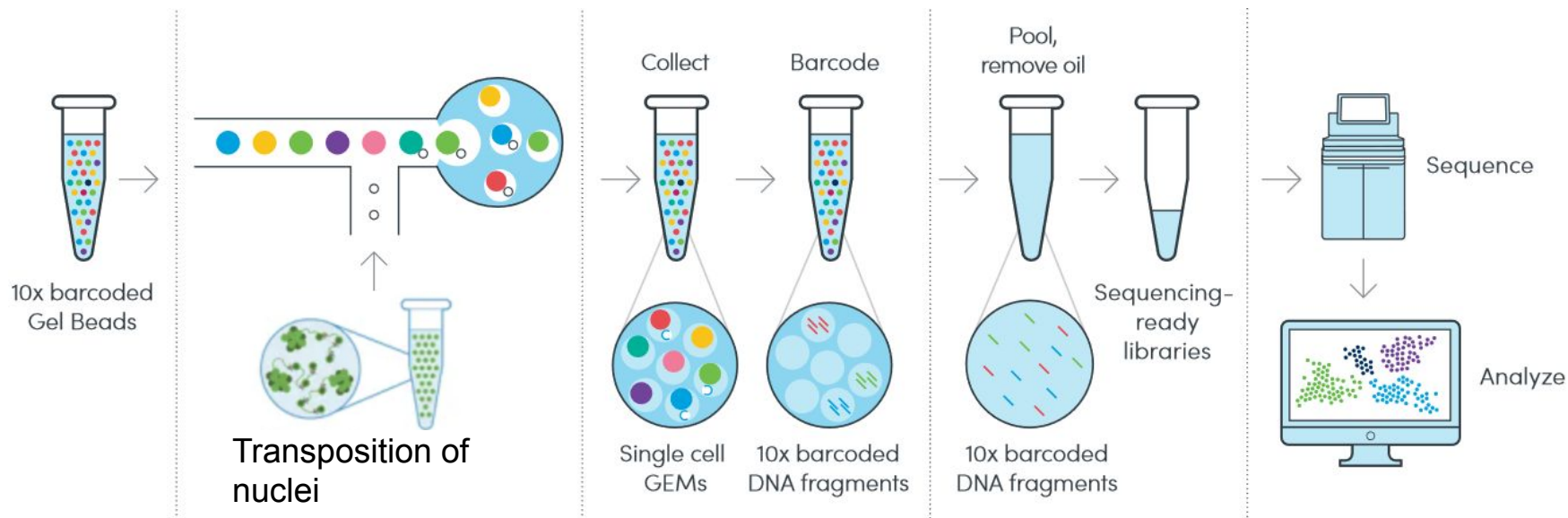
Motif Enrichment

Motif Footprinting

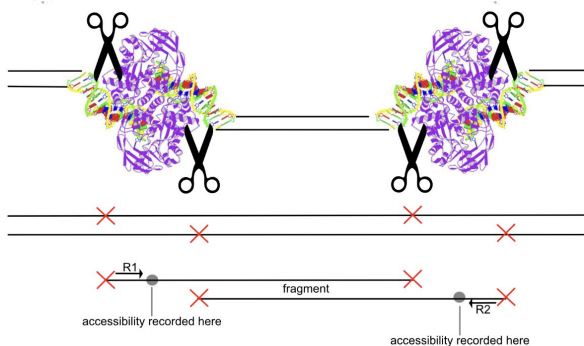
## 2. scATACseq technology



# Single cell ATAC-seq protocol



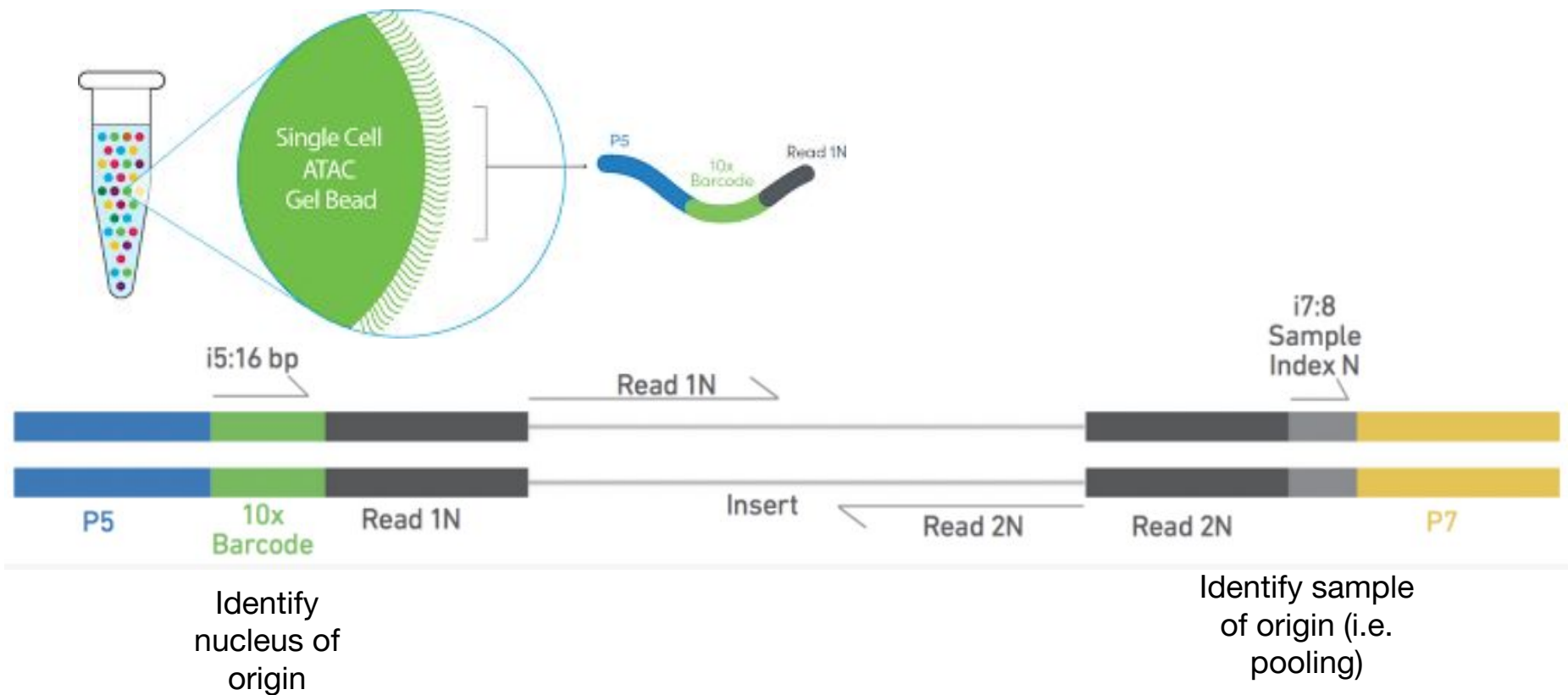
10X Genomics scATAC-seq website



doublets

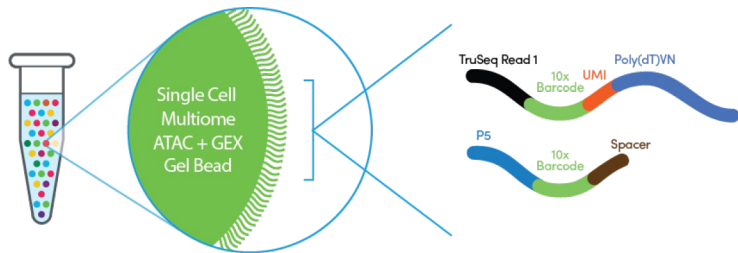
Tn5 enters the nuclei, fragments DNA in open chromatin regions, and adds the indexing adapters

# Single cell ATAC-seq protocol



# Experimental design is fundamental (II)

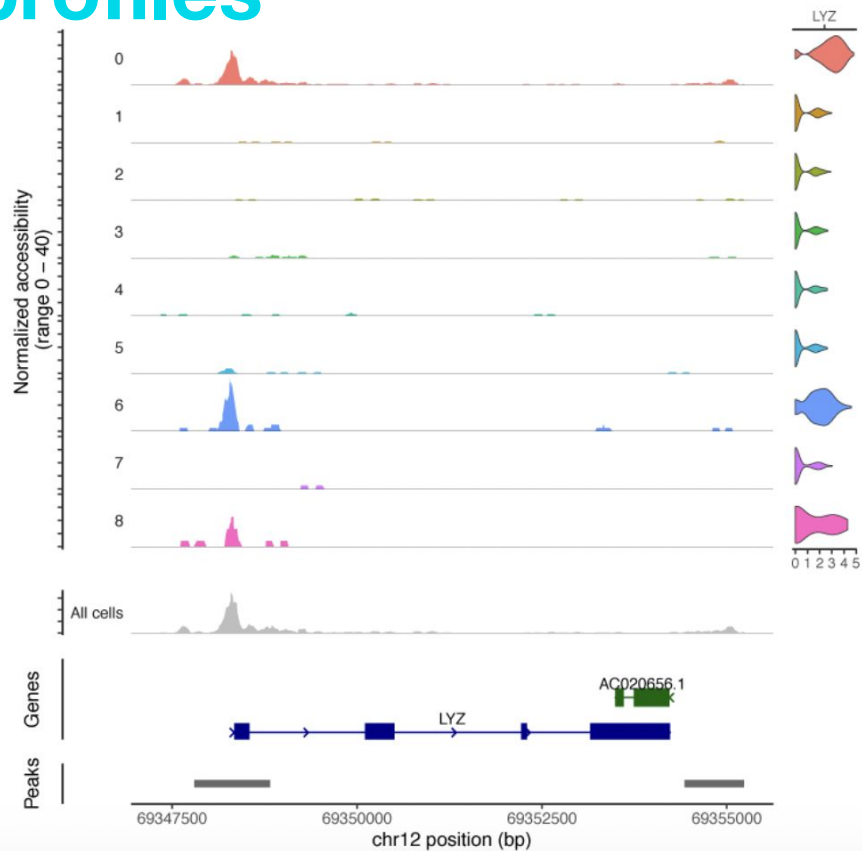
- Do I need to perform scRNA-seq and scATAC-seq separately or Multiome analysis (scATAC+scRNAseq)?



## Multiome:

- + Simultaneously profiling gene expression and open chromatin from the same cell.
- + More straightforward downstream analysis.
- + No need to integrate scRNA and scATAC seq data.
- Costs

# Simultaneously expression and accessibility profiles



Clusters of gene expression for LYZ

Recommendation: whether the budget allows, use Multiome

# Caveats of scATAC-seq

A typical human scATAC-seq dataset contains 100 - 10,000 cells and 1,000 - 100,000 sequence reads per cell.



- **Drops with no cell/doublets**
- **Low detection efficiency** - limited # of accessible regions from each cell (5–15% of peaks detected)
- In a cell, **most cis-REs don't have mapped read** - cis-REs in the genome >> 100,000.
- Activities of all cis-regulatory elements is a continuous steady-state activity in a cell.  
-> scATAC-seq give a **picture of a specific timepoint.**

# Knowledge check 1

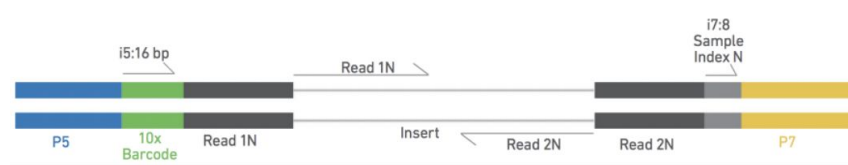
Using scATAC-seq, it is possible to identify:

1. An enhancer active in different cell types.
2. A gene poorly expressed in a particular cell type.
3. Cell-specific peaks of accessible chromatin
4. All of the above.

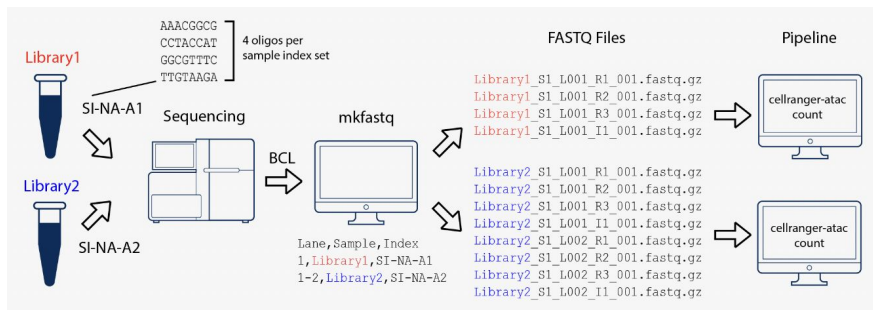
### 3. From raw sequencing data to the fragments file

Tool: Cellranger-ATAC

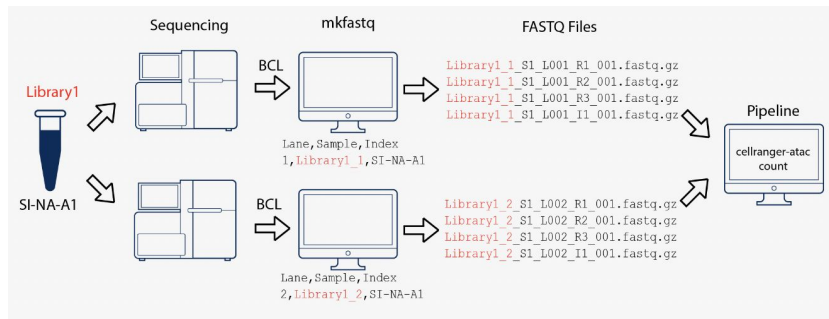
# Cellranger ATAC



## Two 10x Genomics libraries - multiplexed on a single flow cell



## One 10x Genomics library sequenced on two flow cells



1. Demultiplex (BCL to FASTQ)
  - **cellranger-atac mkfastq**
2. Alignment, filtering, barcode counting, and additional steps
  - **cellranger-atac count**
3. Aggregate counts from multiple runs
  - **cellranger-atac aggr**

Reads mapped to chrM, specified as non-nuclear contigs, are filtered out



# Cell Ranger-ATAC outputs

- BAM files with aligned reads
- **web\_summary.html**
- **Fragments and bulk peak files.**
- Secondary analysis (e.g., dimensionality reduction, clustering, etc.) -> discard
- Loupe file to interactively view secondary analysis results with Loupe Browser from 10x Genomics
- Molecule info

# Cell Ranger outputs

(more detail on [HTML output](#))

## atac\_pbmc\_1k\_nextgem - Peripheral blood mononuclear cells (PBMCs) from a healthy donor

1,004

Estimated number of cells

16,214

Median high-quality fragments per cell

70.1%

Fraction of high-quality fragments  
overlapping peaks

Summary

[Data Quality](#)

### Sample

Sample ID	atac_pbmc_1k_nextgem
Sample description	Peripheral blood mononuclear cells (PBMCs) from a healthy donor
Pipeline version	cellranger-atac-2.0.0
Reference path	...a-cellranger-arc-GRCh38-2020-A-2.0.0
Chemistry	ATAC
Organism	Homo_sapiens

### Sequencing ?

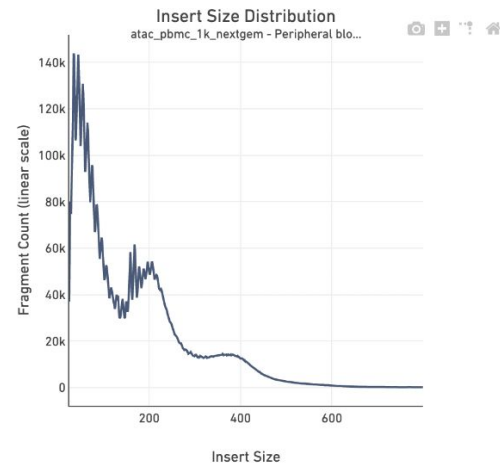
Sequenced read pairs	52,413,244
Valid barcodes	98.1%
Q30 bases in barcode	86.1%
Q30 bases in read 1	95.3%
Q30 bases in read 2	95.4%
Q30 bases in sample index i1	85.3%

# Cell Ranger outputs

(more detail on HTML output)

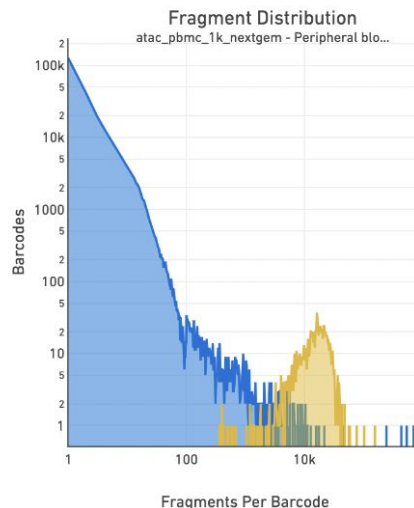
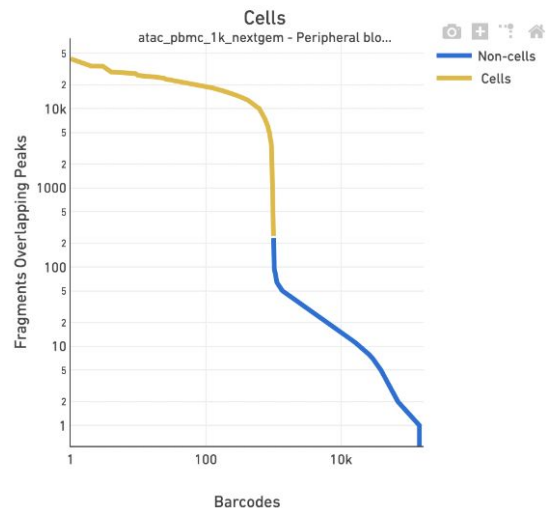
## Mapping ?

Confidently mapped read pairs	92.3%
Unmapped read pairs	1.2%
Non-nuclear read pairs	0.2%
Fragments in nucleosome-free regions	53.0%
Fragments flanking a single nucleosome	33.0%



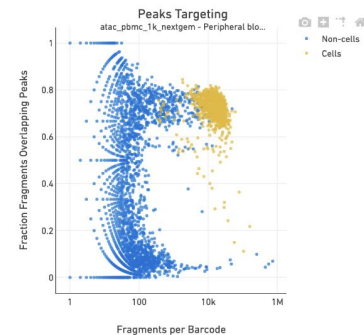
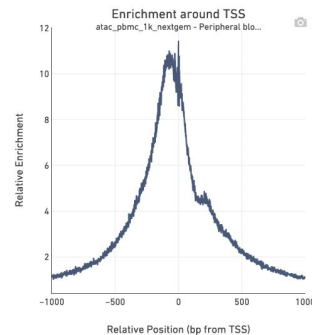
## Cells ?

Estimated number of cells	1,004
Mean raw read pairs per cell	52,204.43
Fraction of high-quality fragments in cells	79.6%
Fraction of transposition events in peaks in cells	66.6%
Median high-quality fragments per cell	16,214



## Targeting ?

Number of peaks	82,579
Fraction of genome in peaks	2.3%
TSS enrichment score	11.45
Fraction of high-quality fragments overlapping TSS	55.2%
Fraction of high-quality fragments overlapping peaks	70.1%



# Output cellranger -> fragments linked to the cell ID

`atac_fragments.tsv.gz`

Accessible Regions (Fragments)

chr	start	stop	cell id
chr1	1895645	1895786	AGACA...
		⋮	
		⋮	

chr1	10073	10209	TTTAGCAAGGTAGCTT-1	1
chr1	10079	10285	GCCTTTGTTGTTCT-1	1
chr1	10079	10333	AGCCGGTTCCGGAACC-1	1
chr1	10089	10560	TGATTAGTCTACCTGC-1	1
chr1	10090	10346	ATTGACTCAATCCTGA-1	1
chr1	10096	10344	CGTTAGGTCATTAGTG-1	1

- Header lines: # record information about the sample, the reference used and primary contigs in the reference.
- Each scATAC-seq fragment and the corresponding cell ID, one fragment per line.

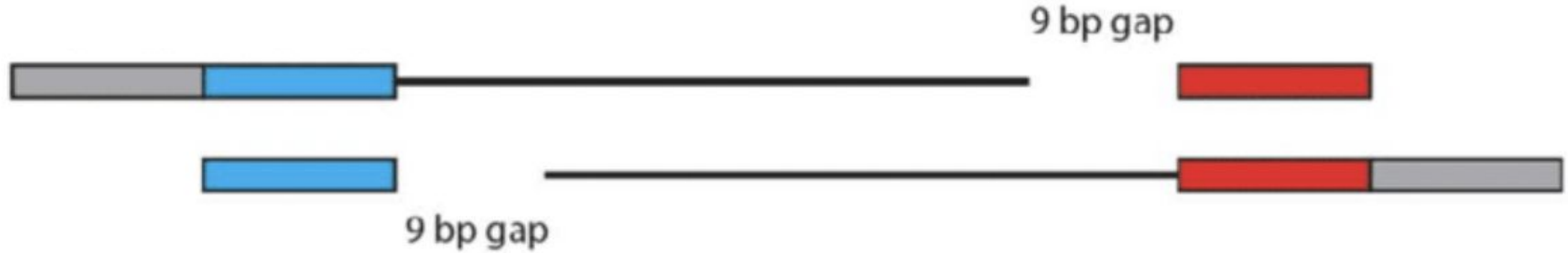
# Different frameworks to process the fragments and perform the analyses downstream.

- SnapATAC - [Nature Comm 2021](#) and SnapATAC2 - [Nature Methods 2024](#)
- [Signac](#) - extension of Seurat for the analysis of single-cell chromatin data
- [ArchR](#) - shown during this workshop [[paper](#)]

## 4. Pre-processing and cell QC

```
ArchR: ArchRProject(); createArrowFiles();  
      addDoubletScores(); filterDoublets()  
plotGroups(); plotFragmentSizes()
```

# Fragments position need to be adjusted for 9bp -> insertions



In ArchR for rapid access, **fragment files** chunked and converted to a compressed temporary HDF5-formatted file including:

- Chromosome
- offset-adjusted chromosome start position
- offset-adjusted chromosome end position
- Cellular barcode ID.

**insertions**-> the offset-adjusted single-base position at the very center of an accessible site.

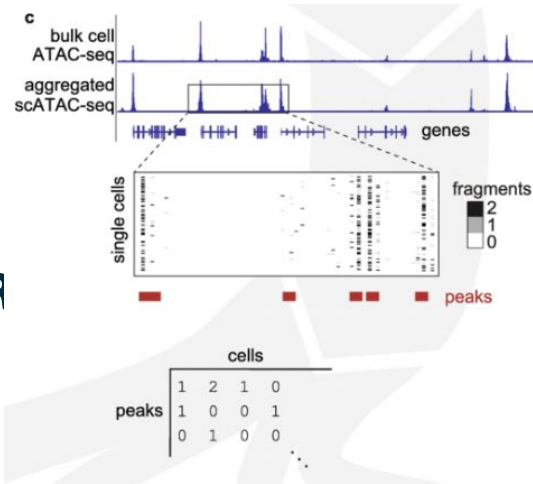
# Sparsity of data issue

- Transposition is rare.
- Majority of accessible regions are not transposed -> many loci having 0 accessible alleles.
- 1= accessible; 0=???
- 0 in scATAC-seq could mean “not-accessible” **or** “not sampled”
- Binarized scATAC-seq data matrix mostly 0s.



# Input for analyses downstream

- **Count matrix on a set of peaks** (Signac - Stuart lab)
  - Bulking cells to call peak -> no rare cells peaks
- **Genome-wide tiles** (SnapATAC, SnapATAC2 and **ArchR**)
  - 5kb - SnapATAC
  - Accessibility regions are long bp  $\ll$  5kb
  - ArchR + SnapATAC2 implement **insertion counts** across **genome-wide 500bp tiles**
    - Better resolution
    - Allows for the identification of clusters prior to calling peaks
    - 3B bp in 500bp tiles: 6 million features to be included in the cell by tile matrix



```
createArrowFiles(..., addTileMat = TRUE)
```

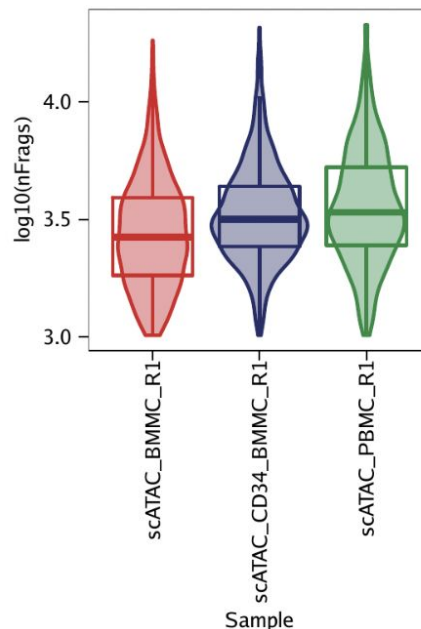
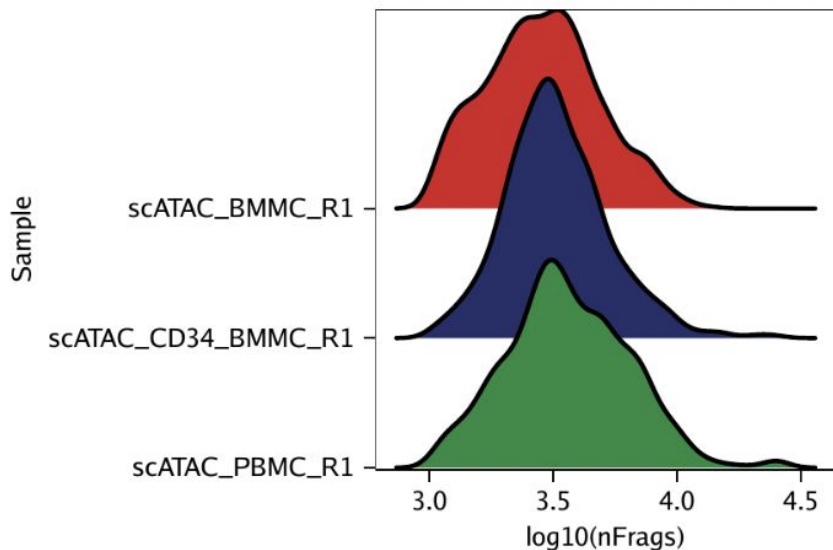
# Knowledge check 2

What is the best input matrix for sparse data to preserve cell specificity:

1. Bulk peak matrix (across cells)
2. Insertion counts across genome-wide tiles

# Filtering out low quality cells

## 1. Number of unique nuclear fragments > 1000

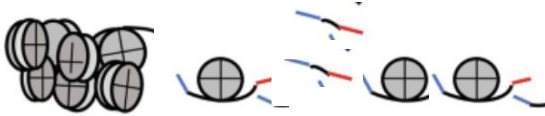


```
plotGroups(..., name = "log10(nFragments)", ...)
```

```
plotGroups(..., name = "log10(nFragments)", plotAs = "violin")
```

# Filtering out low quality cells

## 2. Signal-to-background ratio - > TSS enrichment

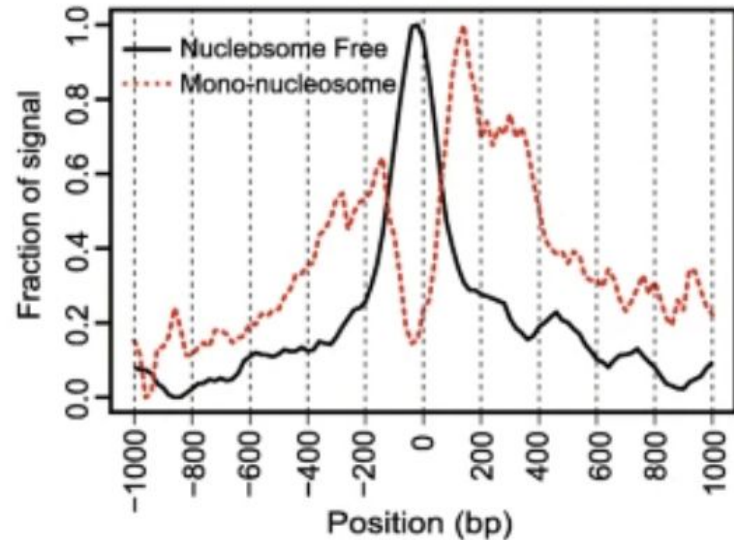


Nucleosome-free regions (NFR) ( $< 100$  bp)

Mono-, di-, and tri-nucleosomes regions ( $\sim 200$ ,  $400$ ,  $600$  bp, respectively)

Fragments from the NFR enriched around the transcription start site (TSS) of genes.

Fragments from nucleosome-bound regions depleted at TSS + enrichment of flanking regions around TSS.

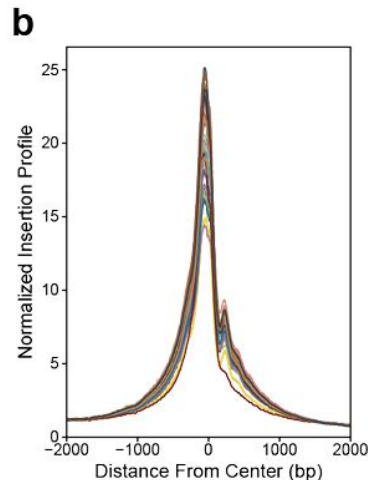


# Filtering out low quality cells

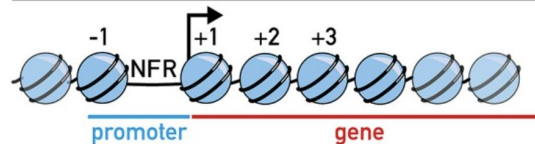
## 2. Signal-to-background ratio - > TSS enrichment

NFR enriched at gene TSS regions compared to other genomic regions.

1. Aggregated distribution of reads centered on the TSSs - avg accessibility
2. Extension of 2000 bp in either direction.
3. Normalized by the average read depth in the 100 bps at each of the end flanks.
4. Fold change at each position over that average read depth.
5. Increase in signal up to a peak in the middle.
6. **TSS enrichment metric** is the signal value at the center of the distribution after this normalization.



```
plotTSSEnrichment()
```



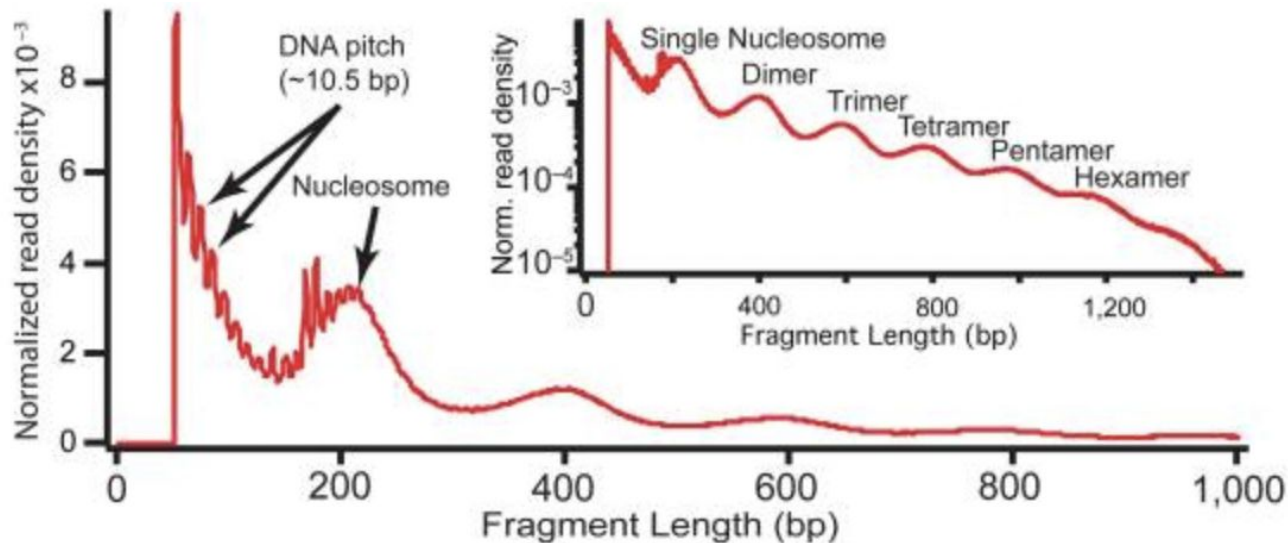
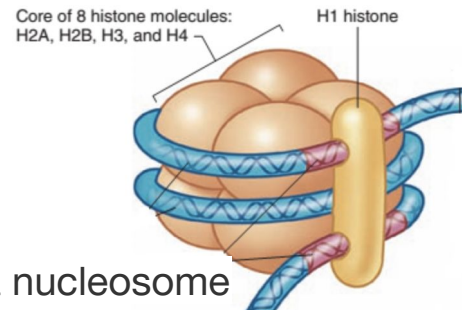
Good quality cells TSS > 4

**Low signal-to-background ratio** -> dead or dying cells (de-chromatinized DNA allows for random transposition genome-wide).

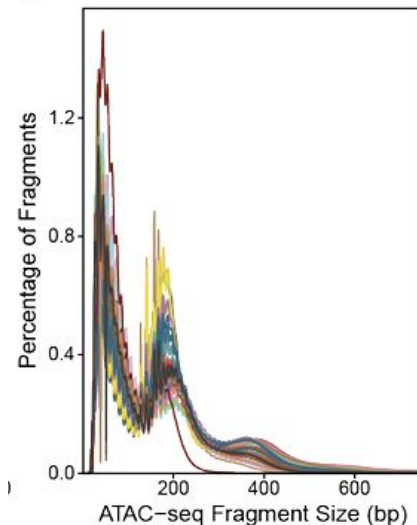
# Filtering out low quality cells

## 3. Fragment size distribution

- Nucleosomal periodicity in the distribution of fragment sizes.
- Depletion of fragments that are the length of DNA wrapped around a nucleosome (approximately 147 bp).

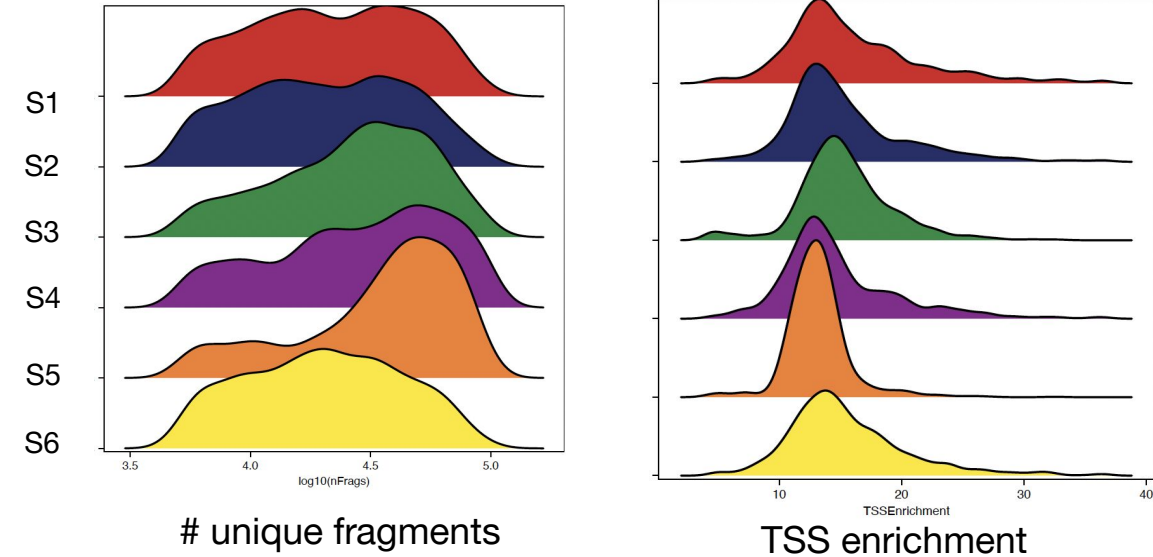


**c**

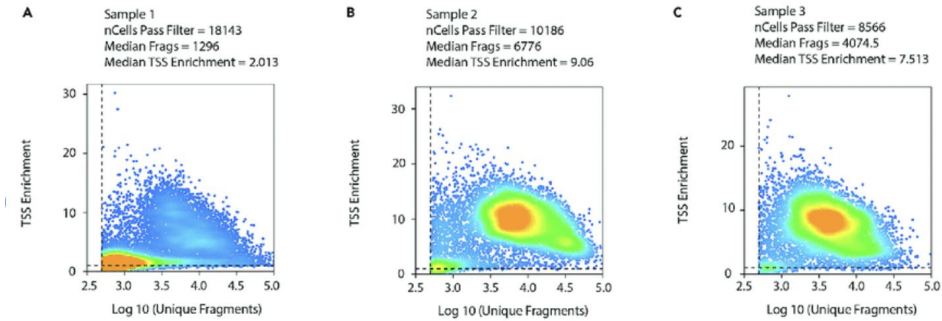
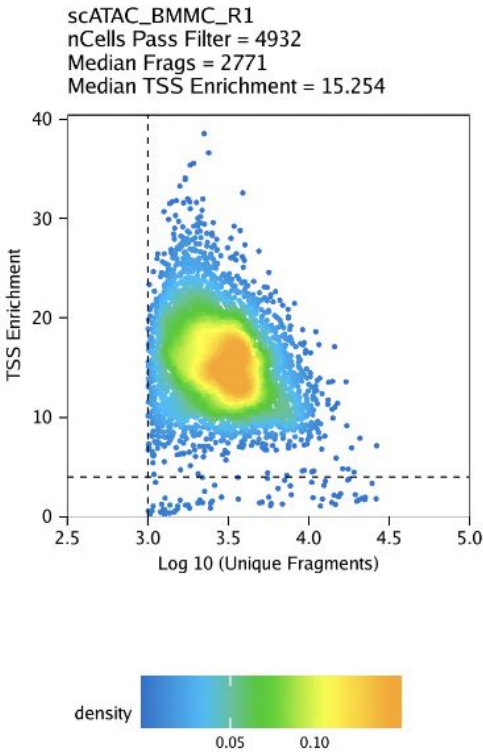


`plotFragmentSizes(...)`

# Visualize the quality of your data



TSS enrichment vs unique fragments



# To summarize the pre-processing

- Create a ArchR project using the fragments.tsv file
- In ArchR command specify the thresholds for min fragment size and TSS enrichment
- Specify to add the Tile Matrix (we will add other matrices to the same object)
- Plots to verify the trend across samples



**Break ~ 5 minutes**

# Workflow

**From bench**

**Through the bioinformatic analysis**

**To the biological interpretation**

Your cell's regulome question

Fragments count

Embedding

Library preparation & sequencing

QC filtering low quality cells

Clustering

Demultiplex, read alignment and  
quantification

Layered dimensionality reduction

Find Markers - cell annotation

**For more biological insight in cell's regulome**

Peak calling

Motif Enrichment

Motif Footprinting

## 5. Normalization and visualization on lower dimensional space

ArchR: `addIterativeLSI()` ; `addUMAP()` ; `addTSNE()`

# For dimensionality reduction we need to identify features that can separate cells

- Highly variable genes ??
- Most accessible features ??

# Alternative normalization and dimensionality reduction

$$L = D - A$$

L: Graph Laplacian

D: Degree of each node

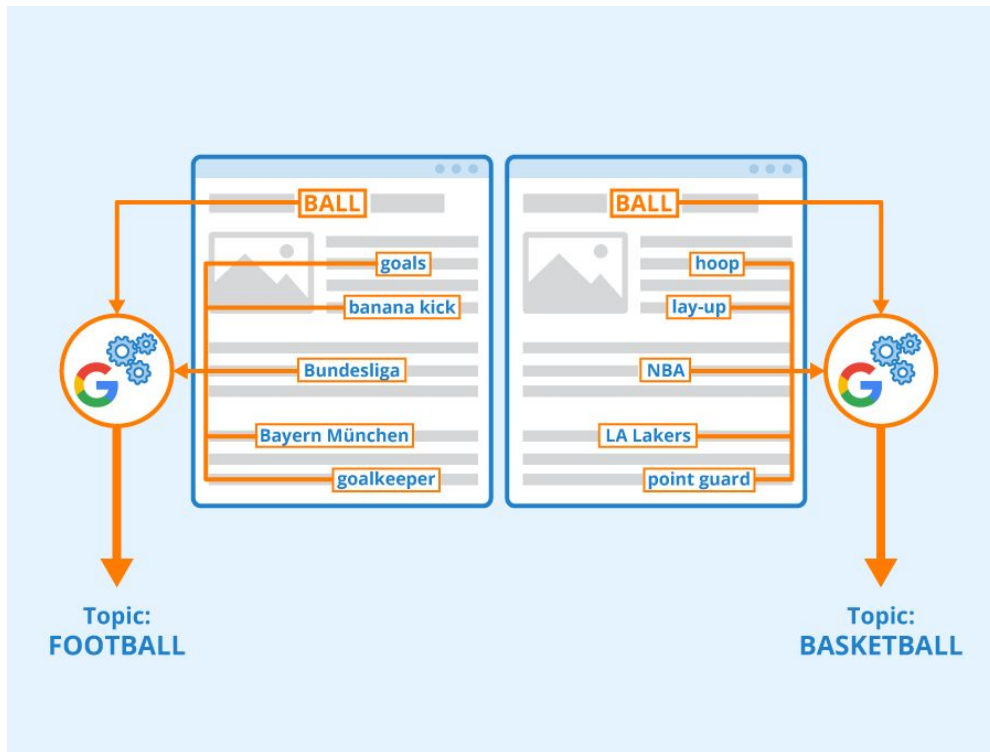
A: Node adjacency matrix

Consideration: cells look very similar.

- Lots of 0s, bias in the PCs -> misleading similarity.
- **Spectral embedding** (using eigenvectors from Graph Laplacian - created from cell-cell similarity matrix)
  - Introduced in 2021 SnapATAC, with recent 2024 updates SnapATAC2
- **Latent Semantic Indexing (LSI)**
  - Introduced in 2015, implemented in different ways by Signac and 2021 by ArchR.

# Latent semantic Indexing (LSI)

To find co-occurrences of words in documents to give insights into the **topics** of those documents



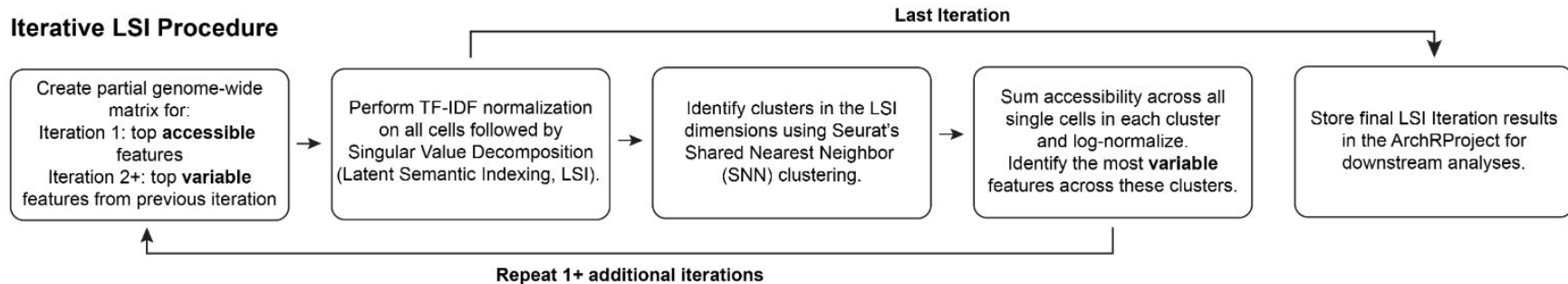
- How many times a word appears in a document?
- Term frequency of a word in a document
- Are common words (this, what, and if ) informative? -> low ranking
- Inverse document frequency of the word across a set of documents.
- Use singular value decomposition (SVD) to identify patterns between words and documents.

# LSI to normalize scATAC-seq data

- Documents -> cells; Words -> regions/peaks
- Help to identify features that are more “**specific**” rather than **commonly accessible**.
- Normalize for term frequency followed by depth norm to a constant 10,000
- Normalized by the inverse document frequency -> weighted features
- The term frequency-inverse document frequency (TF-IDF) matrix reflects how important a *region/peak* is to a *sample/cell*
- Low dimensional space -> singular value decomposition (SVD) of the most *valuable* information across cells/samples

[Excel sheet](#) illustrating LSI computations

# LSI to process scATAC-seq data

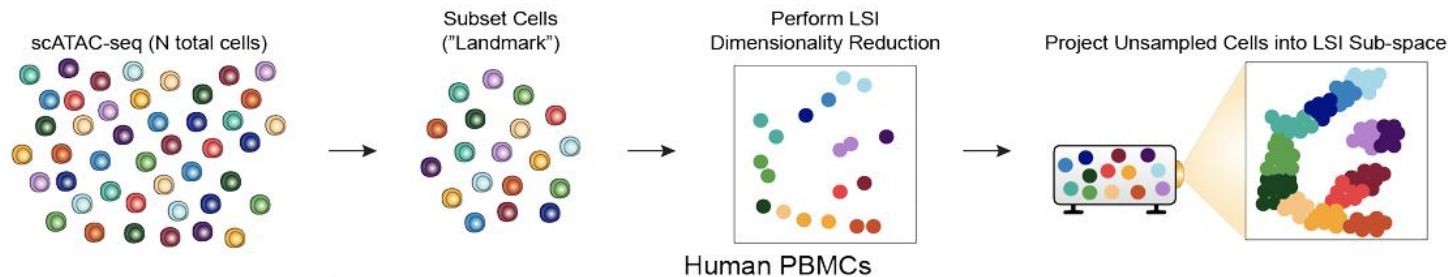


- Identify low specific clusters of cells (set a low resolution) based on the most accessible 500bp tiles using TF-IDF normalization and the Seurat clustering approach
- Derive the most variables features across clusters based on averaged accessibility within a cluster
- Use the most variable feature as in scRNA-seq and iterate N times
- Tune the N iterations if batch effects are present

```
addIterativeLSI ( )
```



# Estimated LSI approach for large dataset



Similar to Iterative LSI but

- Use a subset of cells to define the sub-space
- Normalize the remaining cells and project to the sub-space.

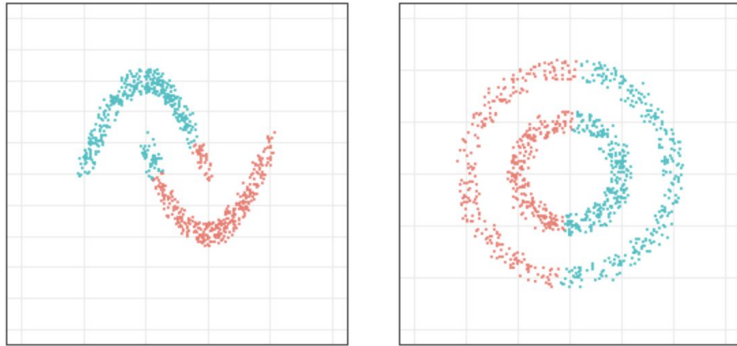
For very large datasets

- Speeds up dimensionality reduction
- Decrease the granularity of the data
- Similar to landmark diffusion maps (LDM) implemented by SnapATAC but required for > 25k cells
- ArchR required for >200k cells otherwise Iterative LSI is good

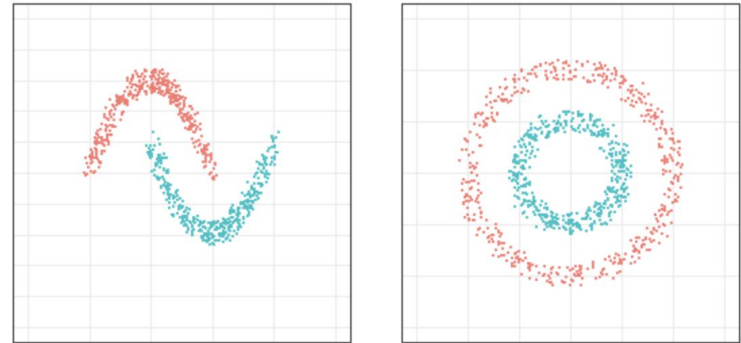
# Spectral embedding

- Cells are considered nodes in a graph
- Edges between cells reflect cells that are immediately to each other
- Spectral embedding is a partitioning technique of the resulting graph using the eigenvalue decomposition of the Graph Laplacian
- The embedding has been used for clustering

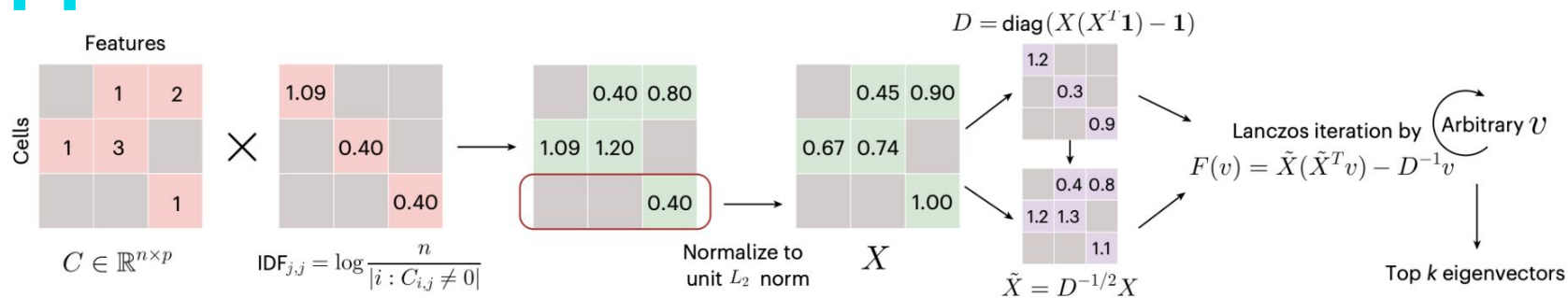
## K-Means clustering



## Spectral clustering



# SnapATAC2 spectral embedding approach



feature scaling with inverse term frequency (*Downweights features that are accessible in all cells*)

row-wise L2 norm normalization

Identification of eigenvectors of Graph Laplacian created using normalized matrix  $X$

Graph-based spectral embedding leverages eigenvalues and eigenvectors of the pairwise cell similarity matrix to perform dimensionality reduction - > time consuming

Matrix-free spectral embedding -> computes eigenvectors using the Lanczos algorithm wo constructing a full similarity matrix

# Knowledge check 3

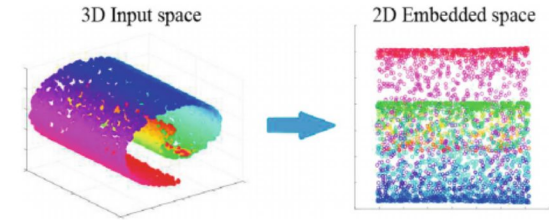
Which dimensionality reduction method is able to preserve the **local structure of cells (neighborhood)** in the low dimensional space?

1. PCA
2. UMAP
3. Both

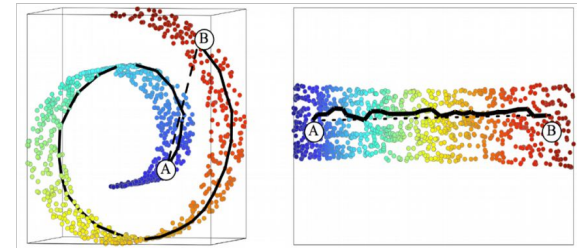
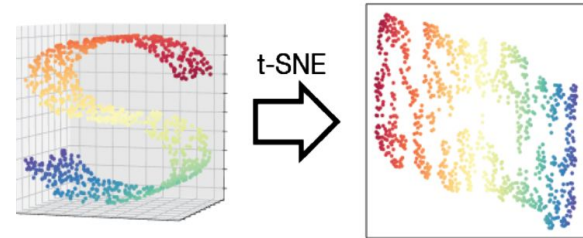
# Embeddings methods for visualization: UMAP or t-SNE

- Visualize in low dimensional space preserving the local vs global structure (i.e distance) as much as possible.
- Linear (PCA, MDS) - Euclidean distance
- Non linear combinations methods: t-SNE (t-Distributed Stochastic Neighbor Embedding) and UMAP (Uniform Manifold Approximation and Projection) - transforming Euclidean distances into “probability of being neighbor”

Linear projection

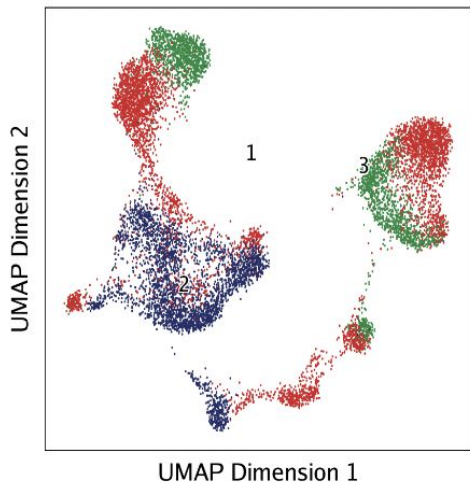


Non linear projection

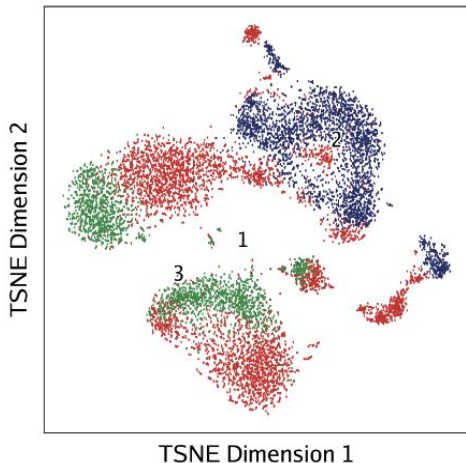


# UMAP/tSNE visualization of IterativeLSI data

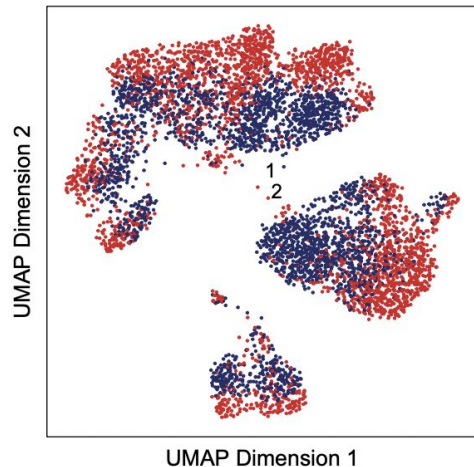
UMAP of IterativeLSI colored by colData : Sample



TSNE of IterativeLSI colored by colData : Sample



UMAP of IterativeLSI colored by colData : Sample



color 1-scATAC\_BMMC\_R1 2-scATAC\_CD34\_BMMC\_R1 3-scATAC\_PBMC\_R1

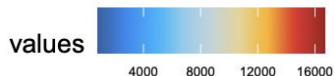
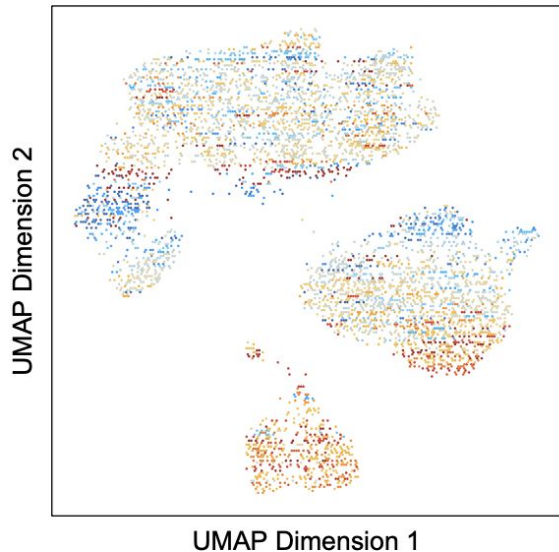
`addUMAP ( )`

color 1-scATAC\_BMMC\_R1 2-scATAC\_CD34\_BMMC\_R1 3-scATAC\_PBMC\_R1

`addTSNE ( )`

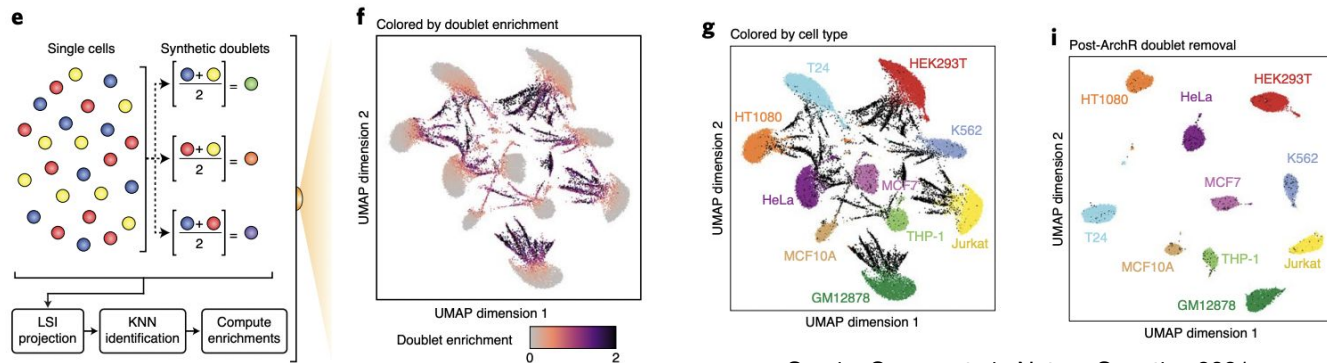
# ArchR generates metrics plots

UMAP of IterativeLSI colored by  
colData : ReadsInTSS

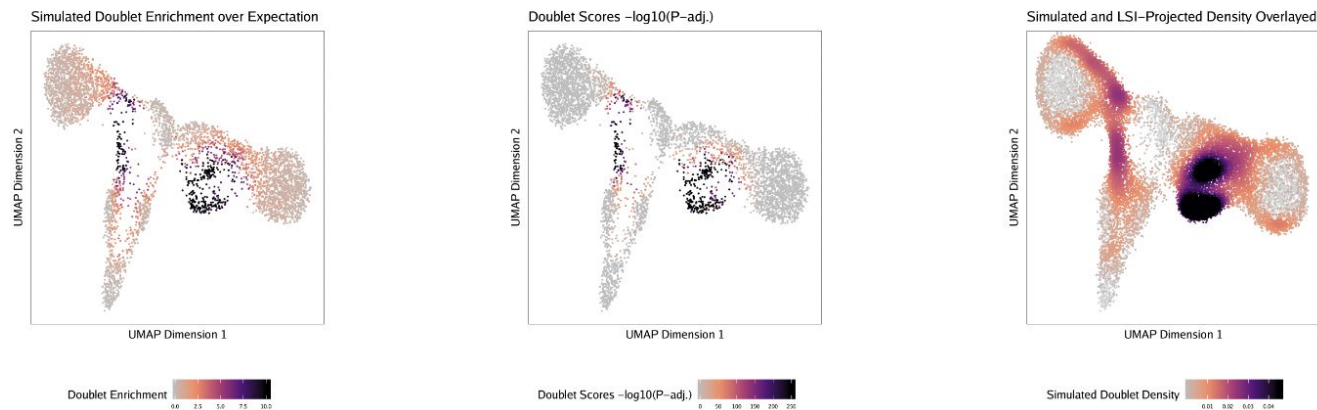


- Reads in Promoter
- Reads in [Blacklist](#)
- NucleosomeRatio
  - >  $(nDiFrag + nMultiFrag) / nMonoFrag$
- PromoterRatio

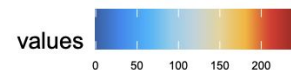
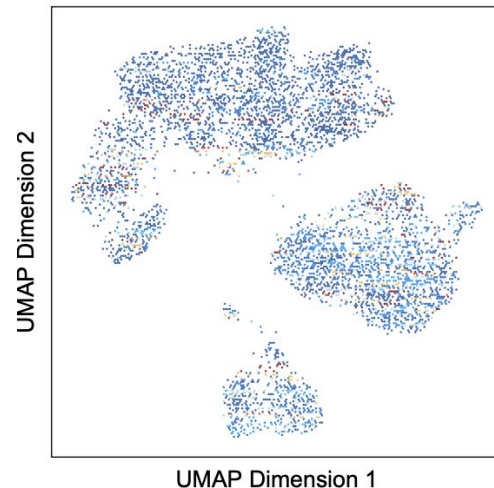
# Doublets detection and removal



Granja, Corces et al., Nature Genetics 2021



UMAP of IterativeLSI colored by colData : DoubletScore



**Suggestion:** check data after and before doublet removal

```
addDoubletScores() ; filterDoublets()
```



# Workflow

**From bench**

**Through the bioinformatic analysis**

**To the biological interpretation**

Your cell's regulome question

Fragments count

Embedding

Library preparation & sequencing

QC filtering low quality cells

Clustering

Demultiplex, read alignment and  
quantification

Layered dimensionality reduction

Find Markers - cell annotation

**For more biological insight in cell's regulome**

Peak calling

Motif Enrichment

Motif Footprinting

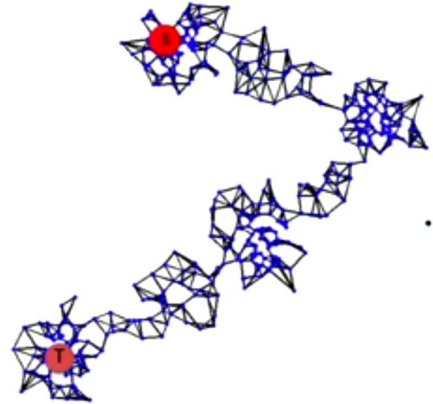
## 6. Clustering and cell type annotation based on feature markers

ArchR: `addClusters () ; getMarkerFeatures ()`

# ArchR use Seurat approach to find clusters

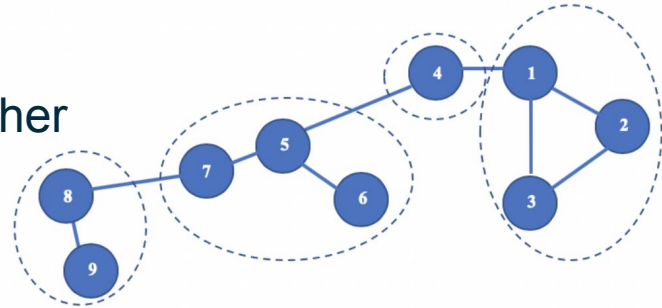
## 1. Build shared nearest neighbor graph

- Build a k-nearest neighbor graph
  - Keep only edges between cells that share a neighbor
  - Adjust the edge weights between any two cells based on similarity
- = Shared nearest neighbor graph

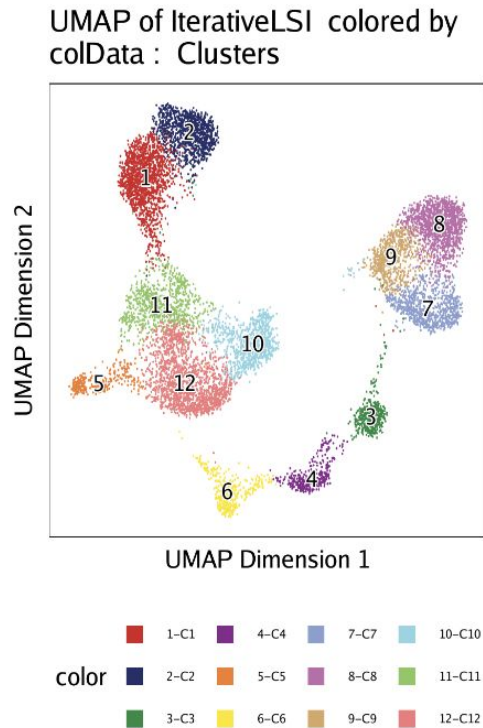


## 2. Community detection

- Cluster of cells more connected than with cells of other communities
- graph-based clustering detects clusters of arbitrary structures
- optimizes modularity - **resolution** - 0.4-1.2 typically  
returns good results for 3K single-cell datasets



# Clustering of IterativeLSI data using Seurat clusters

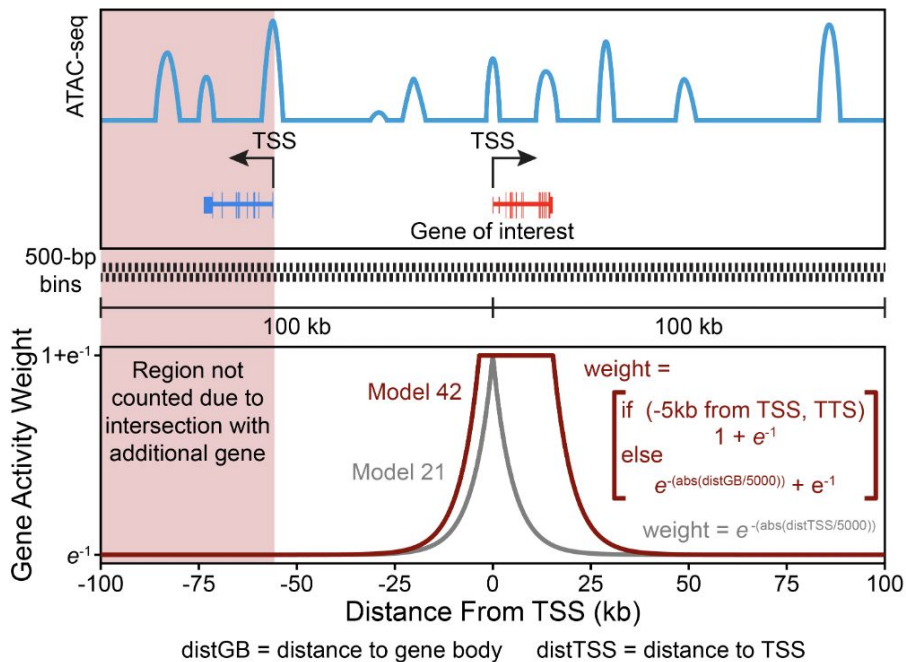


```
addClusters(..., reducedDims = "IterativeLSI", method = 'Seurat',  
resolution = N) N[0.4-1.2]
```

# How to identify marker genes having accessibility data

- Gene expression is not known.
- **Are the regulatory elements of a gene accessible?**
  - -> high gene expression, high activity.
- **Infer the gene expression using the distance between the tile and the gene -> *GENE SCORE MATRIX***
- Validate on scRNAseq and scATACseq datasets

# Best model to infer gene score matrix



Granja, Corces et al., Nature Genetics 2021

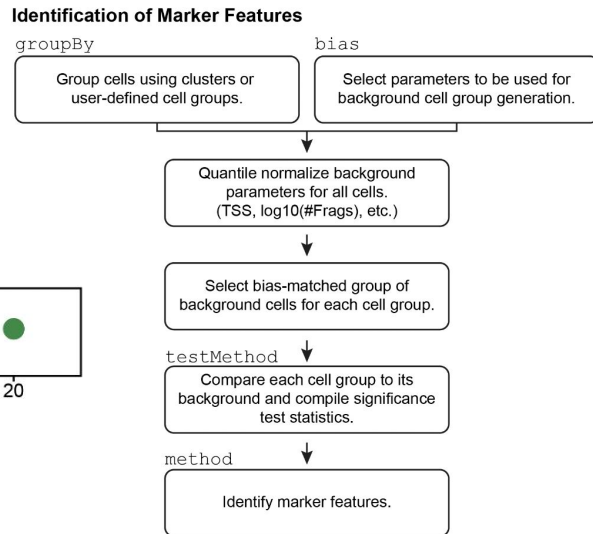
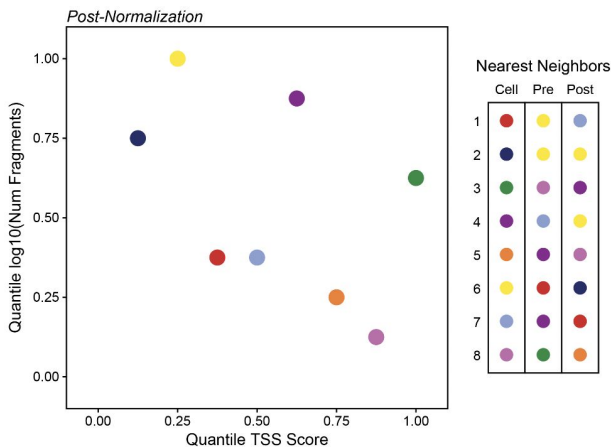
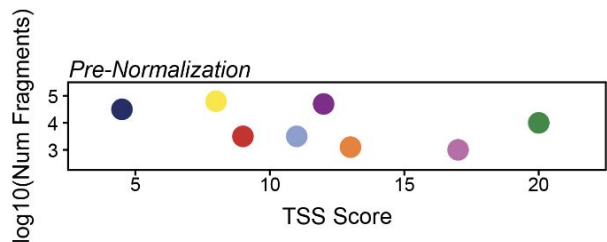
Inferring **gene score** using 56 models.

- Define gene window.
- Overlap tiles.
- Weigh distance and gene size.
- Weights multiplied by #Tn5 insertions within each tile.
- Summed across all tiles within the gene window -> **gene score**
- Depth normalized across genes

```
createArrowFiles(..., addGeneScoreMatrix = TRUE)
```

# Relevant features identification

- Getting the marker features based on the cell specific high activity.
- Marker features based on pairwise comparison of two matched cell groups:
  - Select bias for matching
  - Normalization to equal variance
  - Nearest neighbor
- Significant markers one group vs matched group



```

getMarkerFeatures(useMatrix = "GeneScoreMatrix", groupBy = "Clusters",
bias = c("TSSEnrichment", "log10(nFrgs)"))
    
```

# To summarize so far

- Create a ArchR project using the fragments.tsv file
- In ArchR command specify the thresholds for min fragment size and TSS enrichment
- Specify to add the Tile Matrix, GeneScore matrix
- Plots to verify the trend across samples
- Add Doublets score and filter for doublets, then check!
- Add IterativeLSI for normalization
- AddUMAP, AddClusters using IterativeLSI (on LSI space)
- Use GeneScoreMatrix to get the marker features



**Break ~ 5 minutes**

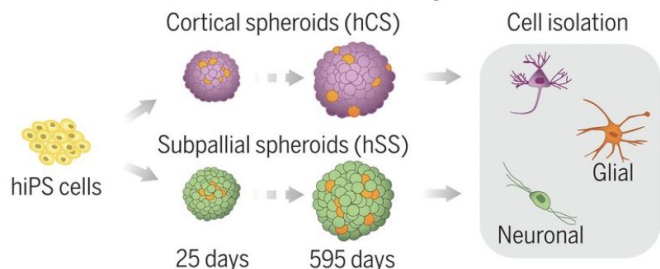
## 7. Advance analysis: Calling Peaks and Motif enrichment

```
ArchR: addGroupCoverages() ; addReproduciblePeakSet() ;  
peakAnnoEnrichment()
```

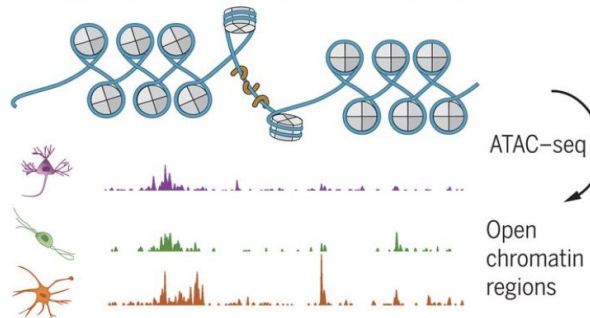
# Where are the cell-type specific enhancers?

- Peak-calling!

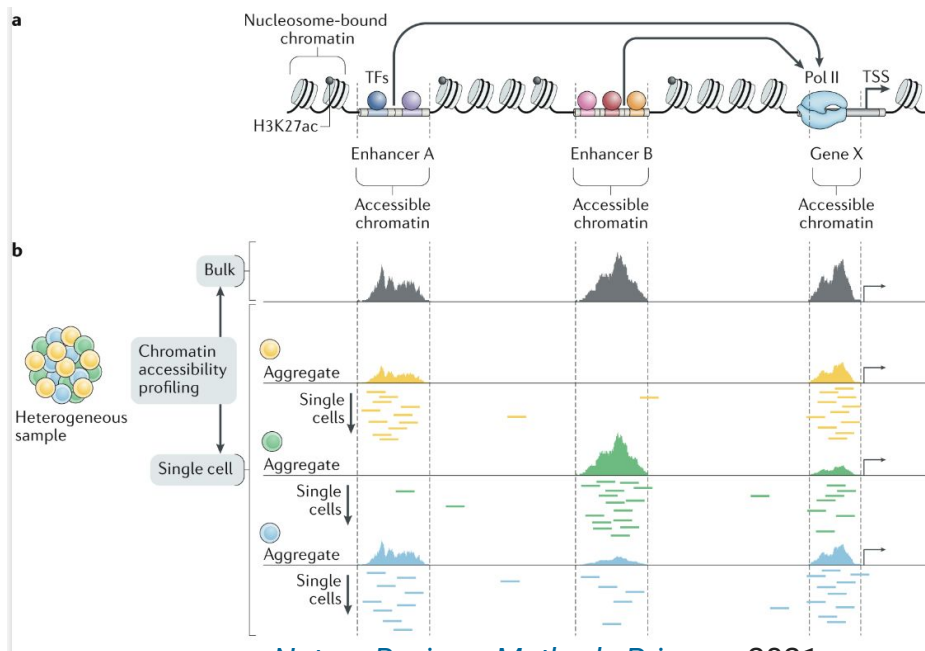
## Human cellular model of forebrain development



## Cell type-specific chromatin accessibility



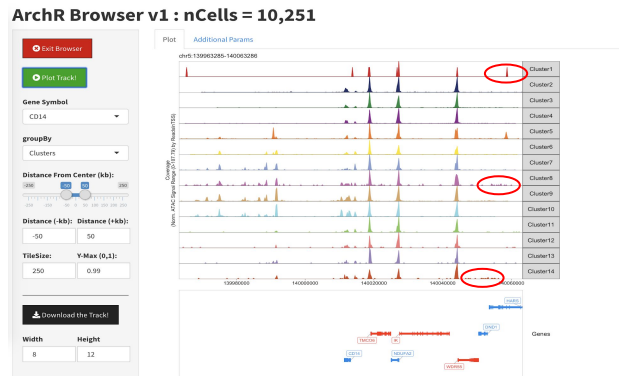
Trevino et al, Science 2020



*Nature Reviews Methods Primers*, 2021

# Motivating Ideas

- We cannot identify enhancer regions for each cell due to the sparsity of signal
- **Assumption**: Cells of the same type share enhancers
- Combining information across multiple cells within a cluster will allow us to identify enhancers
- Distinguish signal from noise?
- Enter Peak calling!



# What is signal and what is noise?

ArchR Browser v1 : nCells = 10,251

[Exit Browser](#)

[Plot Track!](#)

**Gene Symbol**

**groupBy**

**Distance From Center (kb):**

**Distance (-kb):**  **Distance (+kb):**

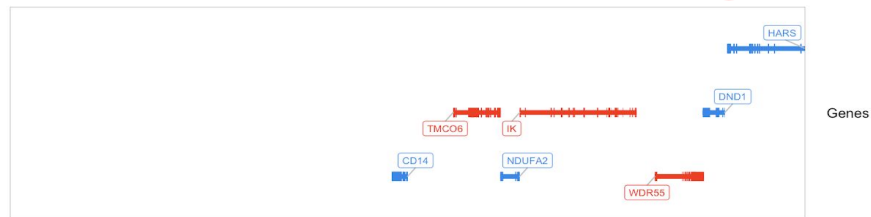
**TileSize:**  **Y-Max (0,1):**

[Download the Track!](#)

**Width**  **Height**

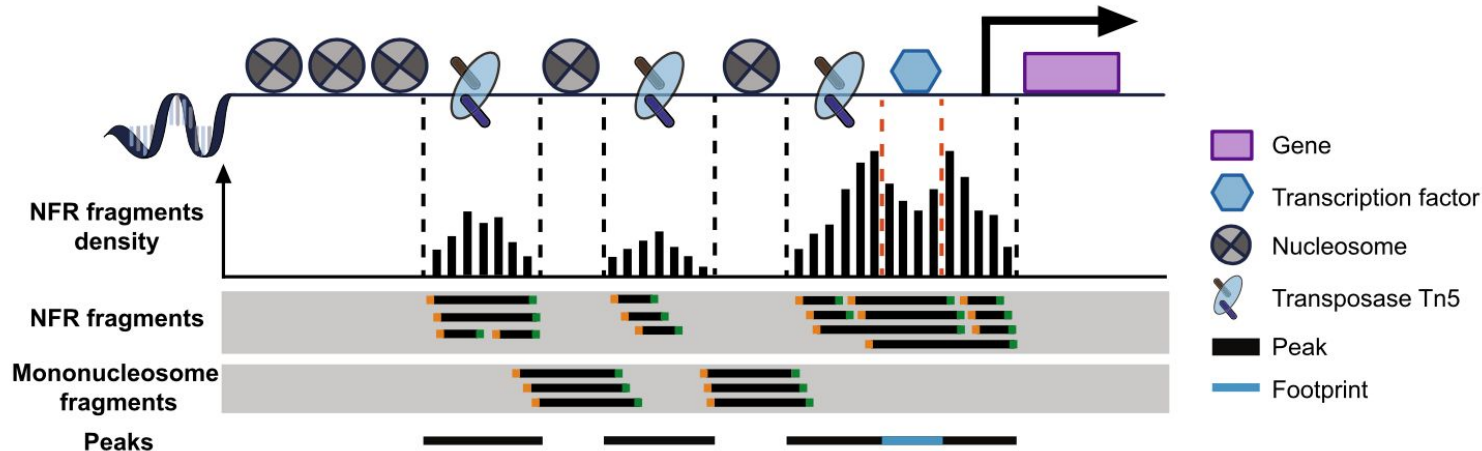
Plot Additional Params

chr5:139963285-140063286

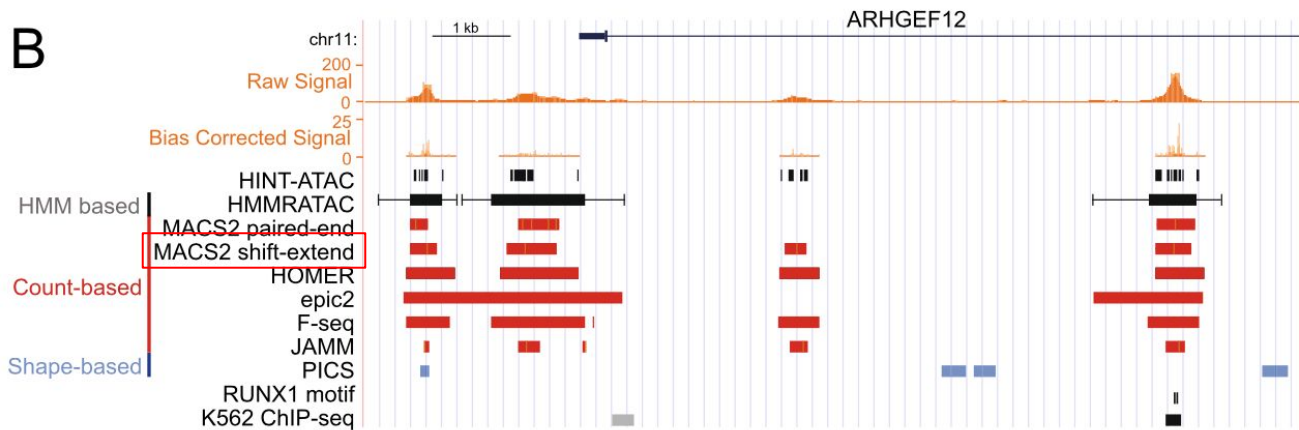


# Peak calling

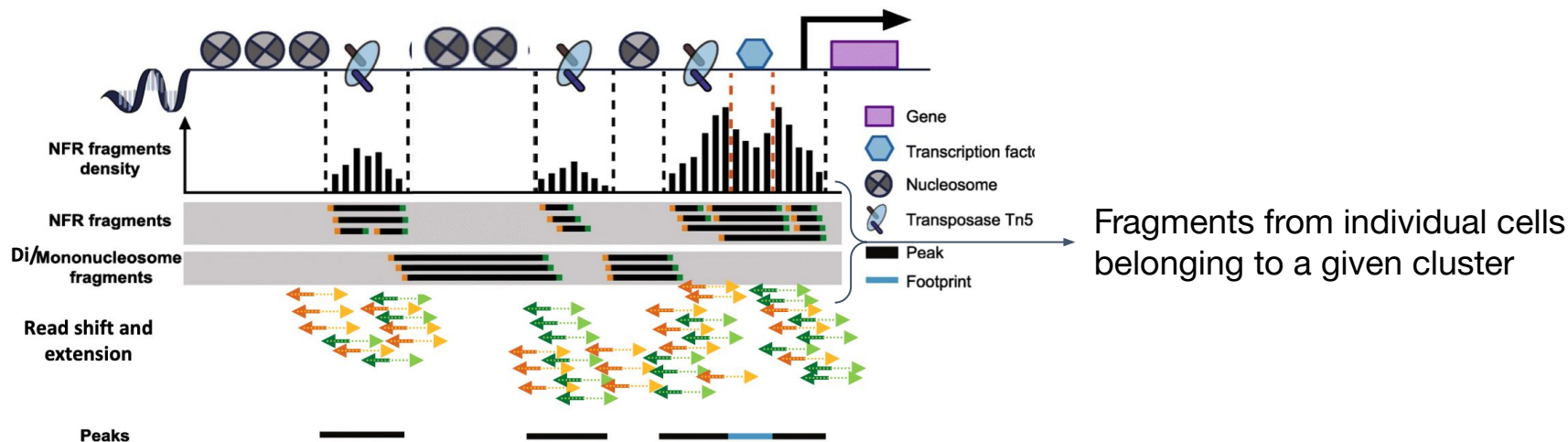
A



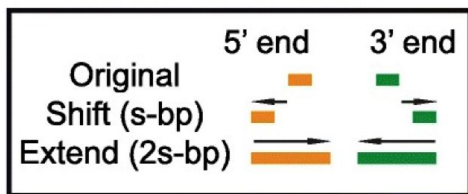
B



# Peak calling: MACS2-shift-extend

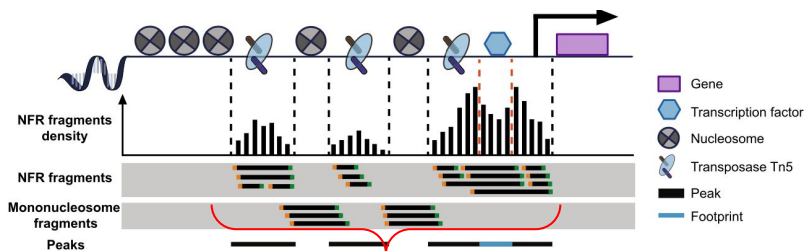


Modified from Feng Yan et.al *Genome Biology* 21 (2020)

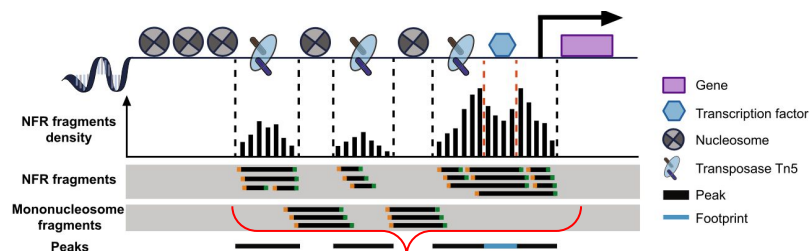


```
addReproduciblePeakSet(ArchRProj = demo_proj,  
  groupBy = "Clusters",  
  pathToMac2 = findMac2(),  
  method = "q")
```

# Reproducibility assessed by calling peaks separately for each sample



Fragments from individual cells of  
**sample1** belonging to a given cluster



Fragments from individual cells of  
**sample2** belonging to a given cluster

```
addGroupCoverages(ArchRProj = demo_proj,  
groupBy = "Clusters")
```

```
addReproduciblePeakSet(ArchRProj = demo_proj,  
groupBy = "Clusters",  
pathToMacs2 = findMacs2(),  
method = "q")
```



# (ArchR) steps to obtain a list of all peaks

- For each cell-type/cluster
  - Keep list of non-overlapping 501 bp peaks that are
    - Reproducible across samples
    - Most significant in terms of enrichment over noise
- Will allow us to identify peak regions either unique to or common across cell-types

# Peak matrix contains accessibility of each peak for each cell

	Cell_1	Cell_2	...	Cell_m
Peak_1	1	0	...	2
Peak_2	0	3	...	1
...			...	
Peak_n	0	0	...	4

Counts of insertions in each cell mapping to a given cell

# Knowledge check 4

Are the counts of the number of insertions in each peak normalized across cells?

1. Yes
2. No

# Knowledge check 5

What could be reasons why the same regions equally accessible in two cells have different numbers of sequenced insertions?

1. Differences in total number of mapped fragments
2. Differences in Signal-to-noise/TSS enrichment
3. Both

# Use Wilcoxon test to identify differentially accessible peak regions

Cells belonging to cluster C1

Cells belonging to cluster C5

	Cells belonging to cluster C1							Cells belonging to cluster C5						
	c1	c2	c3	c4	c5	...	...	c93	c94	c95	c96	c97	c98	c99
p1	1	0	0	0	1	...	...	2	1	2	0	4	1	1
p2	...	...	...	...		...	...							
...						...	...							
pn	...					...	...							...

Cells in clusters C1 and cluster C5 are specifically chosen so they have a similar distribution of  $\log_{10}(\text{nFrag})$  and TSS

```
getMarkerFeatures(ArchRProj = demo proj,  
useMatrix = "PeakMatrix", groupBy = "Clusters",  
bias = c("TSSEnrichment", "log10(nFrag)"),  
testMethod = "wilcoxon")
```

# Use Wilcoxon test to identify differentially accessible peak regions

Cells belonging to cluster C1

Cells belonging to cluster C5

	c1	c2	c3	c4	c5	...	...	c93	c94	c95	c96	c97	c98	c99
p1	1	0	0	0	1	...	...	2	1	2	0	4	1	1
p2	...	...	...	...		...	...							
...						...	...							
pn	...					...	...							...

Peak p1 among cells in cluster C5 appear to be more accessible compared to cells in cluster C1

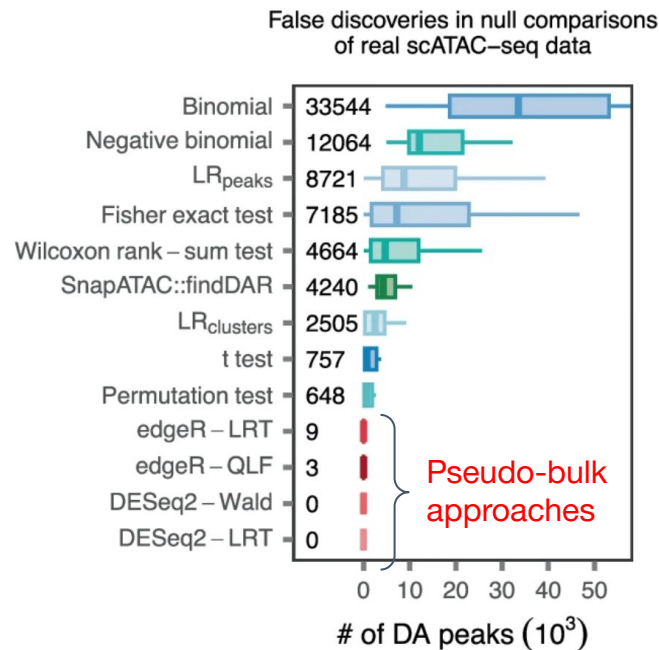
```
getMarkerFeatures(ArchRProj = demo_proj,  
useMatrix = "PeakMatrix", groupBy = "Clusters",  
bias = c("TSSEnrichment", "log10(nFragments)"),  
testMethod = "wilcoxon")
```

# Caution 1: Elevated false-positives with this test

- The previous Wilcoxon test treated all cells as being independent of each other
- Not true!
- Cells are grouped based on the sample from which they are derived
- Ignoring this cell-cell correlation results in elevated false positives
- **Solution:** perform pseudo-bulk of the number of fragments across all cells from each sample
- See details in [scRNAseq workshop](#) and [this paper](#)

# Best practices : differential accessibility analysis in single-cell epigenomics

- Many common DA tests give high false positives by treating cells as independent. Figure shows many spurious peaks.
- The paper shows pseudobulk methods control false positives by aggregating cells per sample. They find very few false peaks.
- Key takeaway:** Use pseudobulk methods for more reliable scATAC-seq DA analysis to reduce incorrect findings.





# Knowledge check 6

Assume sample-wise batch correction was used to obtain updated clusters and visualizations of the data. Will the fragments counts in the peaks also be corrected?

1. Yes
2. No

## Caution 2: Avoid sample-wise batch correction

- You will not be able to distinguish differences in peak accessibility due to the biology you are studying versus those driven by uninteresting technical/batch effects
- See details in our [scRNAseq workshop](#)

# Workflow

**From bench**

**Through the bioinformatic analysis**

**To the biological interpretation**

Your cell's regulome question

Fragments count

Embedding

Library preparation & sequencing

QC filtering low quality cells

Clustering

Demultiplex, read alignment and  
quantification

Layered dimensionality reduction

Find Markers - cell annotation

**For more biological insight in cell's regulome**

Peak calling

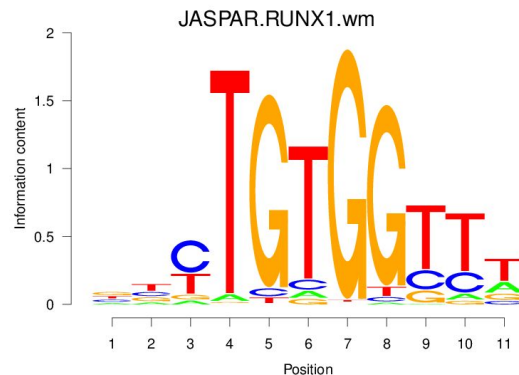
Motif Enrichment

Motif Footprinting

# Motif signatures help us identify important transcription factors

- Transcription factors/proteins bind to DNA sequences with particular sequence/motif patterns
- These motif patterns are available in several databases
- We use these patterns to identify peak regions carrying signatures of binding to particular transcription factors

```
addMotifAnnotations(ArchRProj = demo proj,  
  motifSet = "cisbp", name = "Motif")
```



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**Welcome to CIS-BP, the online library of transcription factors and their DNA binding motifs.**

### Search for a TF

By Identifier

(e.g. Gata\*, YEL009C, I\$FTZ\_01)

### Browse TFs / Restrict Search for TFs

By Model Organism

By Any Species

By Domain Type

By Motif Evidence

By Evidence Type

By Study

Database Build





Database build 2.00 now available!

Last updated: Jan 8th, 2019 Database Build 2.00

Current content: 11491 motifs. 165030 TFs with at least one binding motif (4559 from direct experiments), out of a total of 392333 TFs from 321 families in 741 species

# Identifying motif locations in peak regions by scoring with Position Weight Matrices (PWM)

	1	2	3	4	5	6	7	8	9	10	11	12	13								
A	0	0	6	0	6	5	6	3	1	0	0	0	0								
C	2	0	0	0	0	0	0	0	0	2	2	4	2								
G	4	0	0	0	0	0	0	3	5	4	4	2	4								
T	0	6	0	6	0	1	0	0	0	0	0	0	0								
DNA	G	T	A	T	A	T	A	T	G	G	C	C	G	G	A	T	C	C	A	G	...
N(b,i)	4	6	6	6	6	1	6	0	5	4	2	4	4	$S = \sum_{i=1}^n N(b,i) = 54$							
																					
n																					

Example: TATA-box motif PWM score calculation for given DNA sequence

Estimate the significance of the score assuming the prior expectations of frequencies of each of the 4 nucleotides (e.g., in all promoter regions of the organism)

# Compute Motif presence absence matrix per peak

	Motif_1	Motif_2	...	Motif_k
Peak_1	TRUE	TRUE	...	FALSE
Peak_2	FALSE	FALSE	...	TRUE
...			...	
Peak_n	FALSE	FALSE	...	TRUE

# Fisher's test to identify motifs enriched in one set of peaks versus others

- Universe of all peaks identified
- Subset 1 : peaks identified as differentially present
- Subset 2 : peaks identified to have a given motif
- 
- Is there a significant overlap between these two subsets?
- Answer: Fisher's exact or the hypergeometric test or ORA

```
peakAnnoEnrichment(ArchRProj = demo_proj,  
  seMarker = markersPeaks,  
  ArchRProj = demo_proj,  
  peakAnnotation = "Motif")
```



Tomorrow, at the end of the workshop (or today if not attending)  
please take our survey:

<https://www.surveymonkey.com/r/F75J6VZ>

We use your suggestions to improve!

# Introduction to scATAC-seq Data Analysis

April, 17-18 2025

Reuben Thomas  
Michela Traglia  
Ayushi Agrawal

**GLADSTONE** INSTITUTES  
*SCIENCE OVERCOMING DISEASE*

# Workshop organization

- **Session 1 (Thursday, 9am-12pm)**

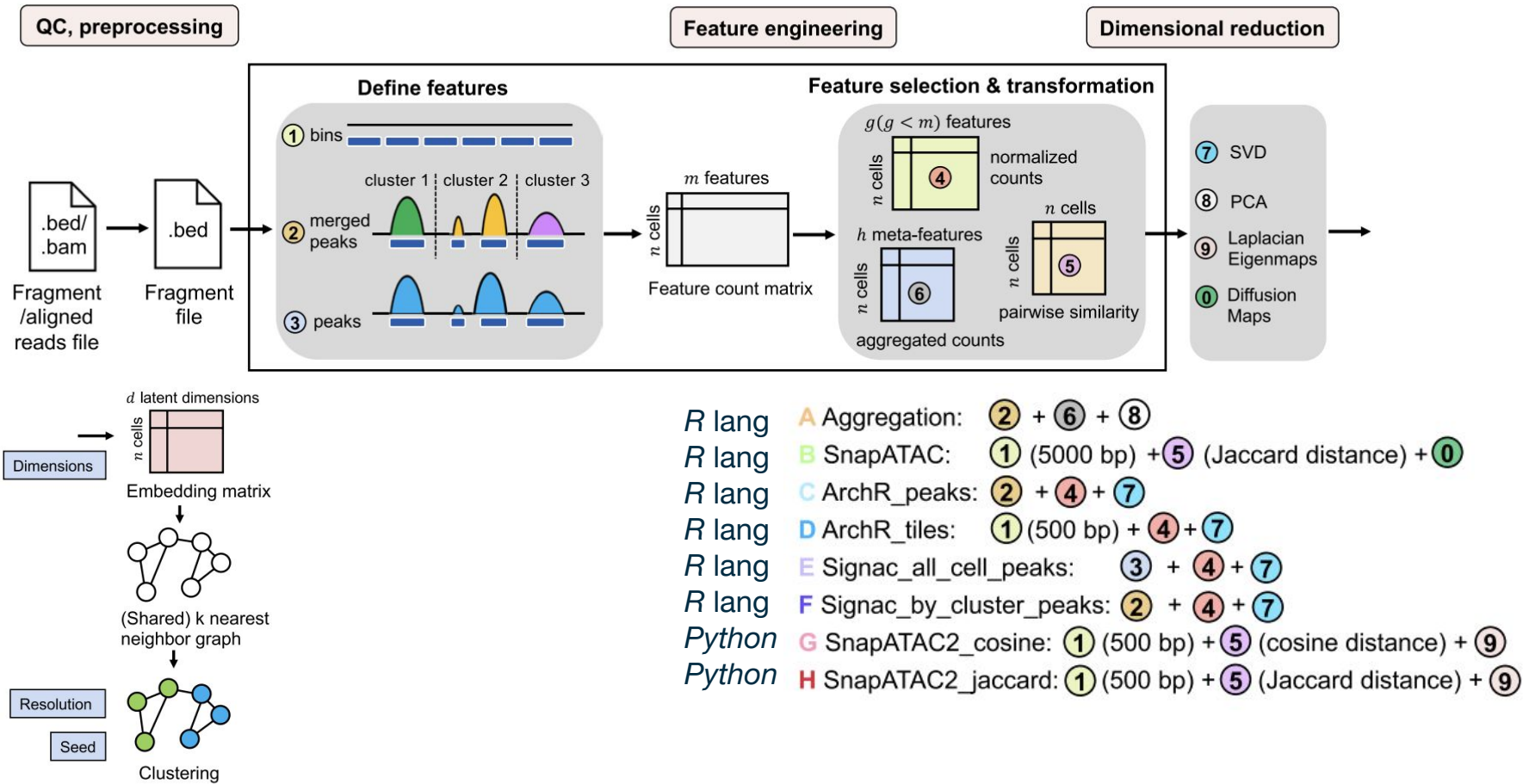
1. Cell regulome and ATAC-seq
2. Technology
3. From sequencer to fragments file
4. Pre-processing and QC
  - Break
5. Normalization, Dimensionality reduction, embedding
6. Clustering and cell type annotation based on feature markers
  - Break
7. Advance analysis: Calling Peaks and Motif enrichment

- **Session 2 (Friday, 9am-12pm)**

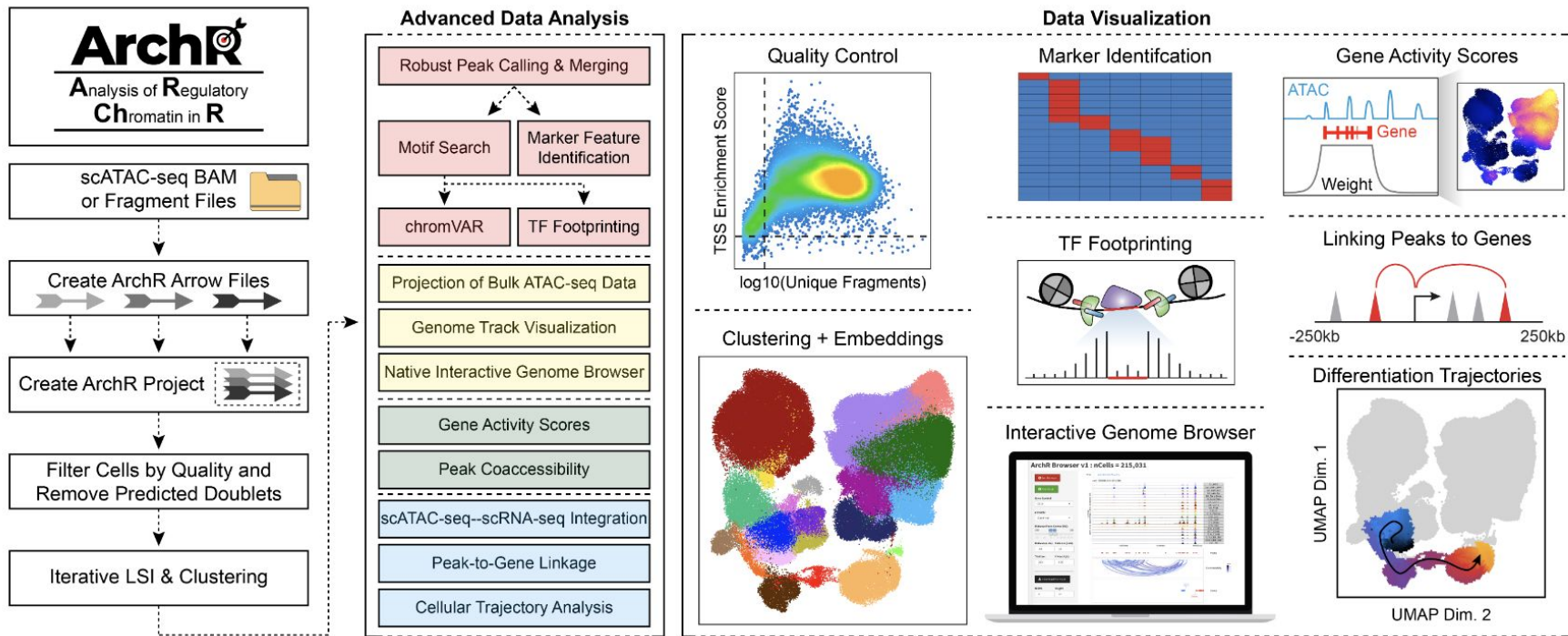
8. Intro to ArchR
  - Demo

Integration scRNA-seq and scATAC-seq workshop - April 24 at 1pm - Register [here](#)

# Tools - overview

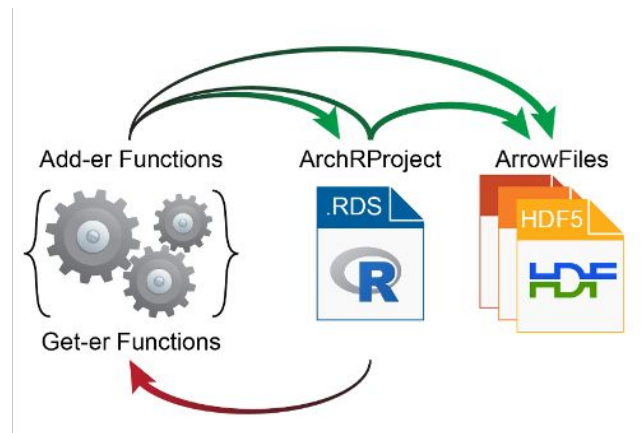


# 8. ArchR - overview

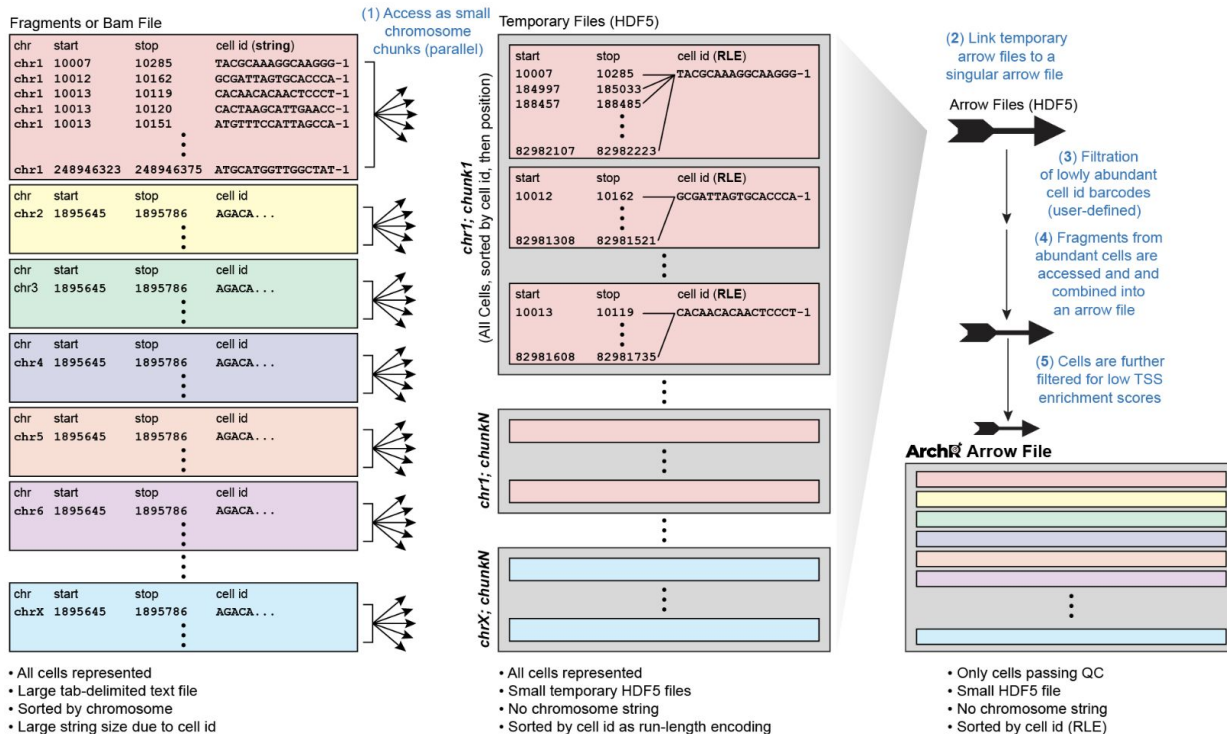


# What is an Arrow file / ArchRProj?

- accessible reads and arrays are organized within
- Used as input for an ArchRProject
- ArchRProject stores the locations of these Arrow files and extracts their cell-centric metadata
- Arrow file - Stored on disk
- ArchRProj - Stored in memory

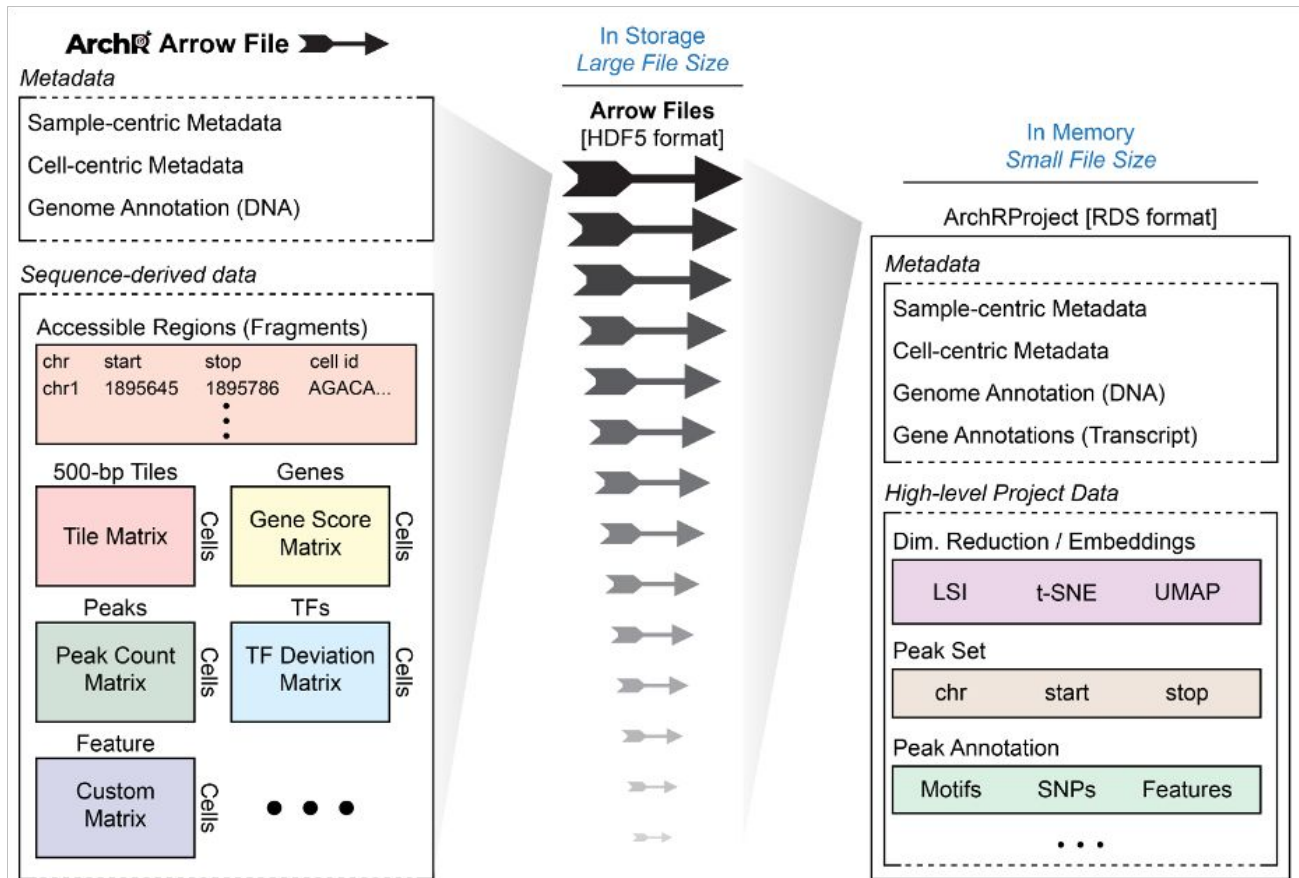


# ArchR Arrow file creation



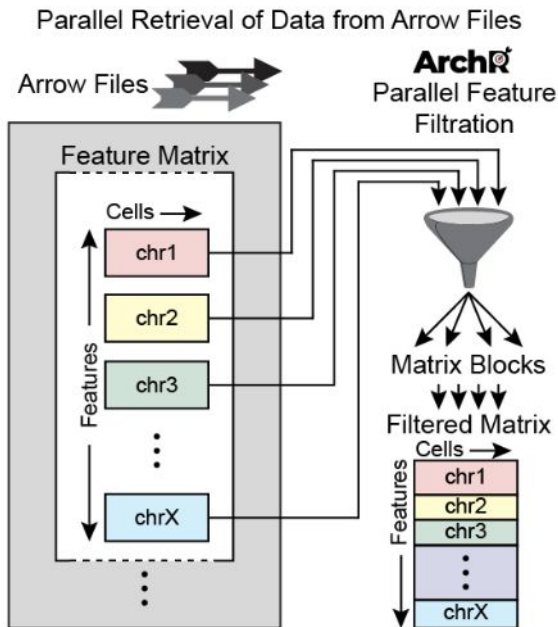
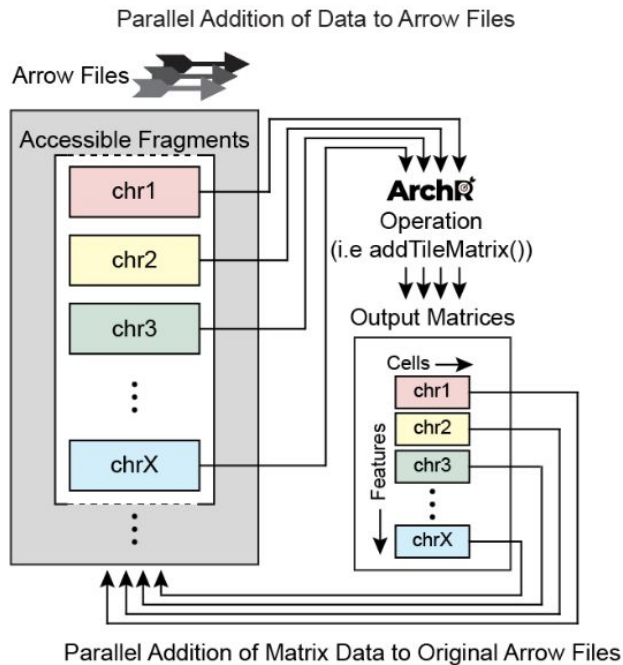


# File infrastructure



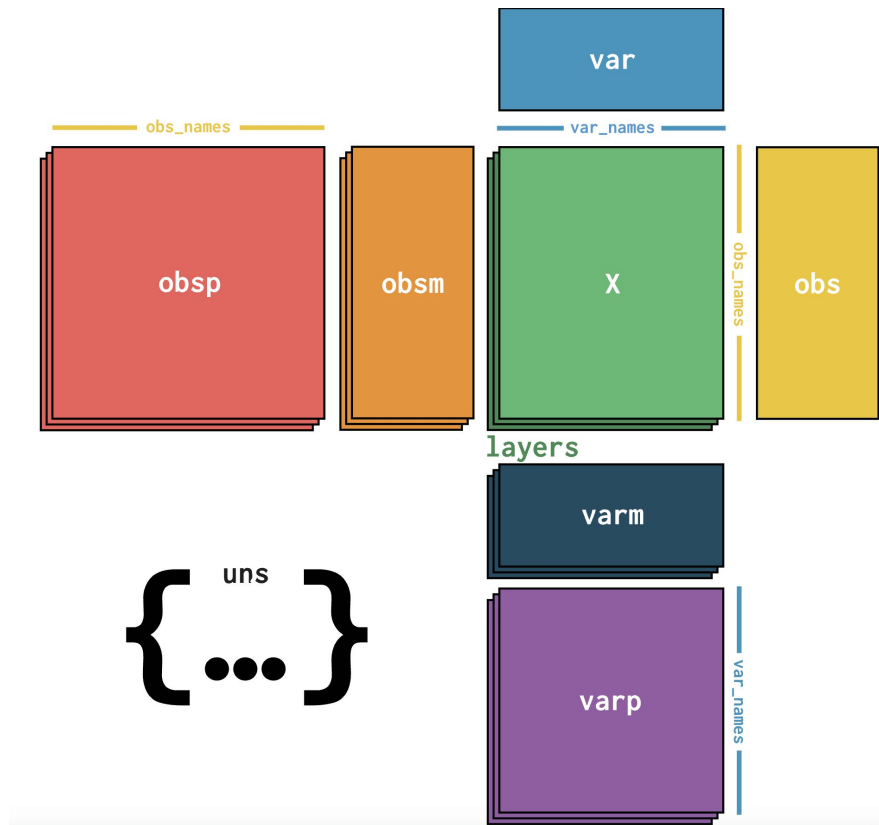


# Information access



Reading and writing into arrows is done in a parallel fashion

# AnnData (used in SnapATAC2) is an alternative to ArrowFiles



# Which tool is the best?

Luo et al. *Genome Biology* (2024) 25:225  
<https://doi.org/10.1186/s13059-024-03356-x>

Genome Biology

## RESEARCH

## Open Access



## Benchmarking computational methods for single-cell chromatin data analysis

Siyuan Luo<sup>1,2</sup>, Pierre-Luc Germain<sup>2,3,4</sup>, Mark D. Robinson<sup>2,3\*</sup> and Ferdinand von Meyenn<sup>1\*</sup> 

- **SnapATAC** - <https://github.com/r3fang/SnapATAC>
- **Signac** - <https://stuartlab.org/signac/index.html>
- **SnapATAC2** - <https://github.com/kaizhang/SnapATAC2>
- **SnapATAC2** - Fastest, high accuracy; ideal if speed/accuracy are top priorities.
- **ArchR** - Most memory-efficient; suitable for large datasets but has library size bias and struggles with identifying rare cell types and distinguishing similar subtypes.
- **ArchR** requires a large amount of time to create the files needed to run an analysis
- **Signac** requires substantially less time for the object creation step.
  
- If speed and accuracy are paramount, SnapATAC2 is recommended. If memory efficiency is critical, ArchR might be a better choice, but consider its limitations in cell type identification and potential lower performance in gene activity prediction.

# Demo

## Input data

- Cryopreserved human peripheral blood mononuclear cells (PBMCs) from a healthy female donor aged 25 were obtained by 10x Genomics.
- Nuclei Isolation for Single Cell Multiome ATAC + Gene Expression Sequencing
- Pbmc\_unsorted\_3k.fragments.tsv.gz
  - all PBMCs
  - 3,009 cells identified by cellranger
- pbmc\_sorted\_3k.fragments.tsv.gz
  - Granulocytes were removed by cell sorting
  - 2,711 cells identified by cellranger

# Helpful resources

- Wynton Slack channel
  - [ucsf-wynton.slack.com](https://ucsf-wynton.slack.com)
- Gladstone Bioinformatics Core slack channel
  - <https://gladstoneinstitutes.slack.com/archives/C0145F1L7QS>
- Wynton tutorials
  - <https://github.com/ucsf-wynton/tutorials/wiki>

## For questions:

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# Please take our survey to improve our workshops

- <https://www.surveymonkey.com/r/F75J6VZ>
- ~3 min.

DATA SCIENCE TRAINING PROGRAM: upcoming workshops

# Questions from participant

- Are the insertion counts NOT normalized in peaks? If I remember correctly, the LSI is implemented for normalization. Yes, insertion counts in peaks in ArchR are NOT normalized. See sample code in next slide.

- When I analyzed the differential peaks for two groups (control vs mutant) using the default function of `getMarkerFeatures()` and then filtering by  $\log_2FC > 0$ , the result was not the same as the pairwise comparison by defining `useGroups` and `bgdGroups` under the function. Theoretically, there should be overlapped peaks between these two methods, but I am confused as to why there were no overlaps.

Need more information. <https://www.archrproject.com/bookdown/pairwise-testing-between-groups.html>

- After identifying differential motifs, is it possible to trace back the corresponding peak locations and target genes of the motifs in ArchR? I found that the outputs of diff motifs from ArchR only contain the genomic location of the motifs rather than the peaks. I'm asking this as I'm interested in identifying the specific motif in a different peak of the target gene/DEG in the mutant.

See sample code in next 3 slides.

# Get info on peak count matrix

```
##get the peak matrix  
peak_data <- getMatrixFromProject(ArchRProj =  
demo_proj, useMatrix = "PeakMatrix")  
  
##view the peak count matrix  
peak_data@assays@data$PeakMatrix[1:10, 1:3]  
  
##get info on genomic ranges for peaks - this  
will also provide info on closest gene  
rowRanges(peak_data)
```



# Access/View the LSI reduction

```
View( (demo_proj@reducedDims@listData[["IterativeL  
SI"]]$matSVD) )
```

# Motif presence in peaks

```
motif_presence <- getMatches(ArchRProj =  
demo_proj, name = "Motif", annoName = NULL)
```

```
motif_presence_matrix <-  
motif_presence@assays@data@listData[["matches"]]  
%>%
```

```
  as.matrix() %>%
```

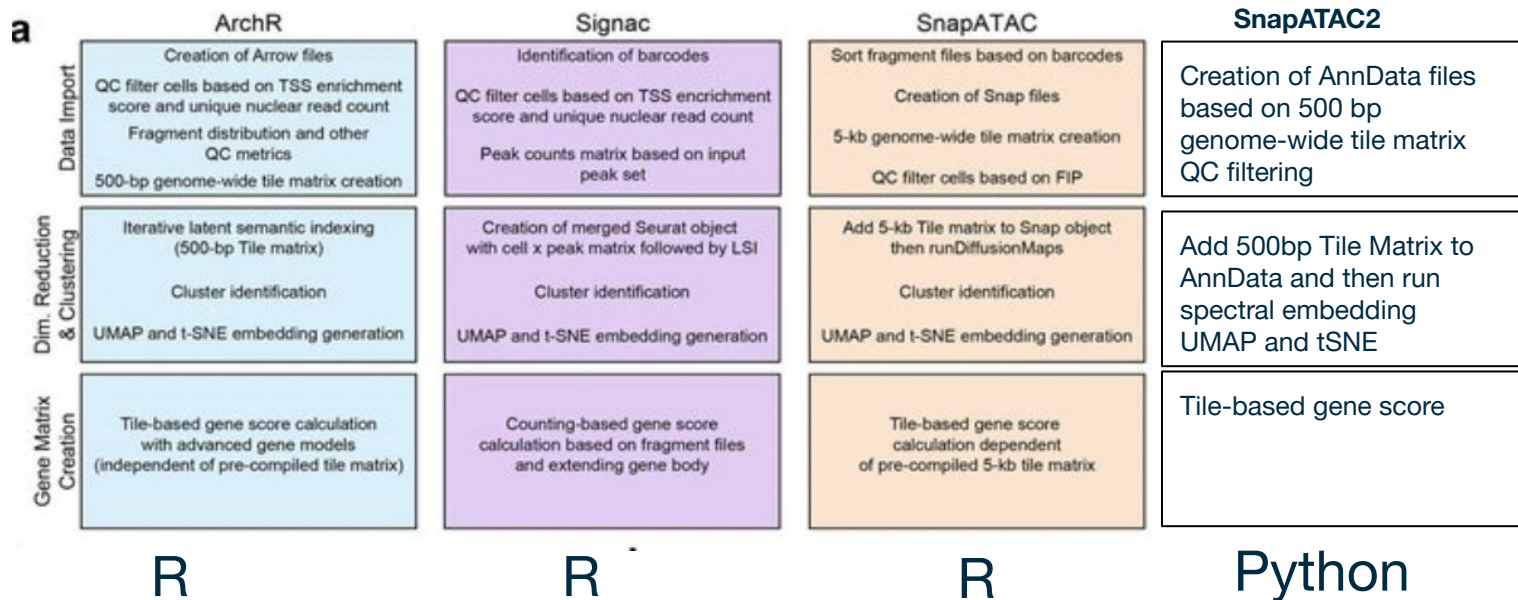
```
  add(0)
```

The background of the image is a deep blue gradient, overlaid with a complex network of glowing, light blue lines that resemble the structure of neurons or a molecular lattice. These lines are interconnected, forming a web-like pattern that fills the entire frame. The lines are slightly brighter at the junctions, giving it a three-dimensional, ethereal appearance.

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# Which tool is the best?



# Why use ArchR?

	ArchR	Signac	SnapATAC	
Pre-processing	NR	NA	✓	
Data import / base file type creation	✓	NA	✓	Data Import
QC filter cells	✓	✓	✓	
Matrix creation	✓ (Tile)	✓ (Peak)	✓ (Tile)	
Doublet removal	✓	NP	NP	Doublet Removal
Data imputation with MAGIC	✓	NP	✓	Gene Scores
Genome-wide gene score matrix	✓	✓	✓	
Dimensionality reduction and clustering	✓	✓	✓	Clustering
UMAP and tSNE plotting	✓	✓	✓	
Cluster peak calling	✓	NP	✓	Standard ATAC-seq Analyses
Cluster-based peak matrix creation	✓	NP	✓	
Motif enrichment	✓	✓	✓	
chromVAR motif deviations	✓	✓	✓	
Footprinting	✓	NP	NP	
Feature set annotation	✓	NP	NP	
Track plotting	✓	✓	NP	Data Visualization
Co-accessibility	✓	NP	NP	
Interactive genome browser	✓	NP	NP	
Cellular trajectory analysis	✓	NP	NP	Advanced ATAC-seq Analyses
Project bulk data into scATAC embedding	✓	NP	NP	
Integration of RNA-seq and ATAC-seq	✓	✓	✓	Integration of RNA-seq and ATAC-seq
Genome-wide peak-to-gene links	✓	NP	NP	

NR = Not Required    NA = Not Applicable    NP = Not Possible

- Comprehensive
- Fast
- Doesn't need to be run on HPC but provides easy export options if desired

# Which tool is the best?

- Benchmarking paper: [Genome Biology 2024](#)
- Evaluated 8 data processing pipelines derived from 5 different scATAC-seq methods.
- SnapATAC2 generally outperforms ArchR in terms of speed, accuracy in cell type identification, and gene activity score prediction.
- **Library Size Bias:** LSI-based methods (Signac, ArchR) are biased by library size; SnapATAC methods mitigate this with linear regression-based normalization.
- **Gene Activity Scores:** SnapATAC and SnapATAC2 outperformed ArchR and Signac for predicting gene activity scores.
- **Strengths & Weaknesses:**
  - **SnapATAC2:** Fastest, high accuracy; ideal if speed/accuracy are top priorities.
  - **ArchR:** Most memory-efficient; suitable for large datasets but has library size bias and struggles with identifying rare cell types and distinguishing similar subtypes.
- **Method performance depends on dataset complexity:**
  - **Simple datasets:** Feature aggregation, SnapATAC and SnapATAC2 performed best.
  - **Complex datasets:** SnapATAC and SnapATAC2 excel.
- **Recommendation:** If speed and accuracy are paramount, SnapATAC2 is recommended. If memory efficiency is critical, ArchR might be a better choice, but consider its limitations in cell type identification and potential lower performance in gene activity prediction.