Single cell methods in epigenomics

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SciLifeLab (Long-term Bioinformatics Support)

Single cell RNAseq data analysis, February 14 2024

Outline

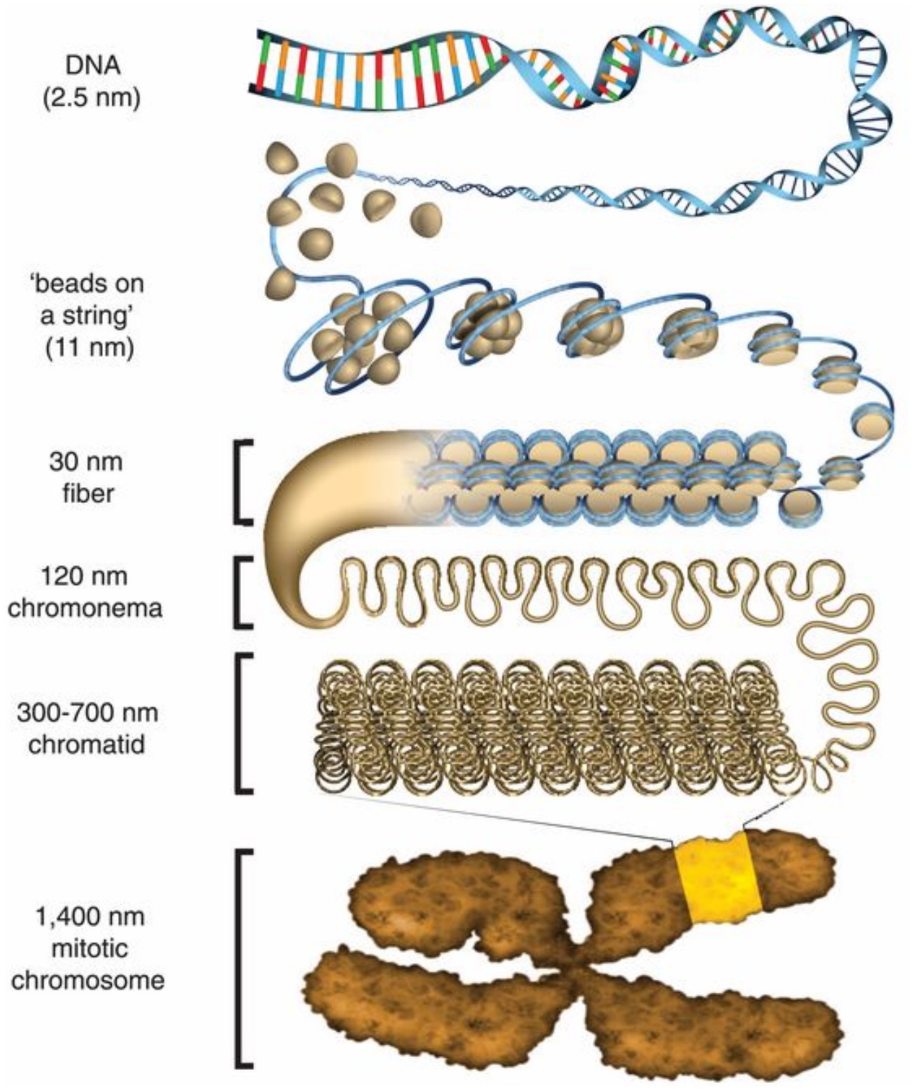
- Background on epigenomics & ATAC-seq
- Single cell ATAC-seq
- Single cell CUT & TAG
- Single cell DNA-methylation
- Multi-omics
- Spatial methods in epigenomics

- Broad overview
- Focus on concepts over details.

Epigenomics

- The epigenome is made up of chemical compounds and proteins that can attach to DNA and direct such actions as turning genes on or off, controlling the production of proteins in particular cells
- In a field of study known as epigenomics, researchers are trying to chart the locations and understand the functions of all the chemical tags that mark the genome.

https://www.genome.gov/about-genomics/fact-sheets/Epigenomics-Fact-Sheet



(Ou et al, 2017, Science)

Epigenomics, continued

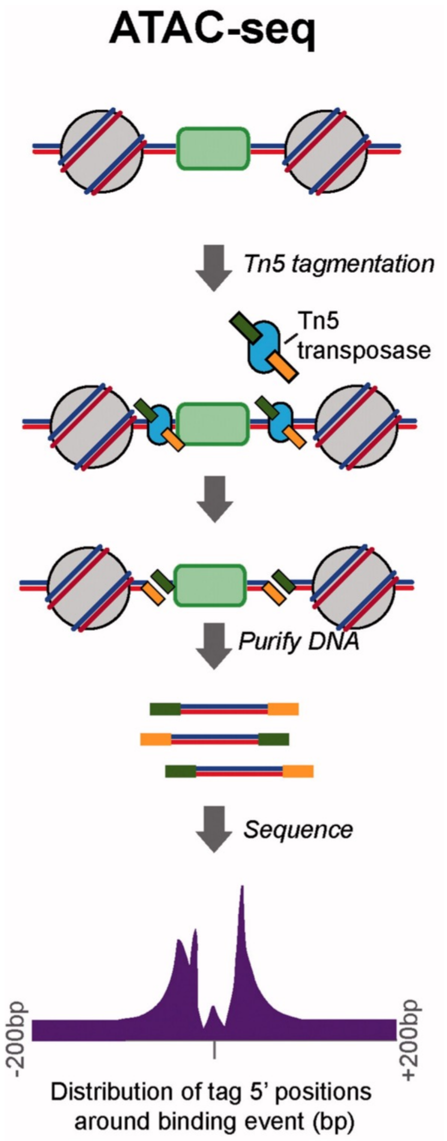
- The epigenome is involved in many processes, e.g. development, cancer, aging and more

- Things to measure:
  - DNA methylation (e.g. bilsulphite sequencing)
  - Chromatin accessibility (e.g. ATAC-seq)
  - DNA – protein interactions (e.g. ChIP-seq, CUT&TAG)
    - Histones, histone modifications
    - Transcription factor binding
    - Other proteins

- Such methods were first developed for bulk samples, but have been adapted for single cell assays.

ATAC-seq

- **A**ssay for **T**ransposase-**A**ccessible **C**hromatin using sequencing.
- Measures chromatin that is accessible, i.e. not bound by any big molecules or folded into compact structures.
- Basic steps:
  - Transfect cells with Tn5 transposase
  - This inserts sequencing adaptors into regions of the chromatin that it can access.
  - These adaptors can be used to purify DNA from open chromatin, and create sequencing libraries.



(Pugh 2015)

Why ATAC-seq?

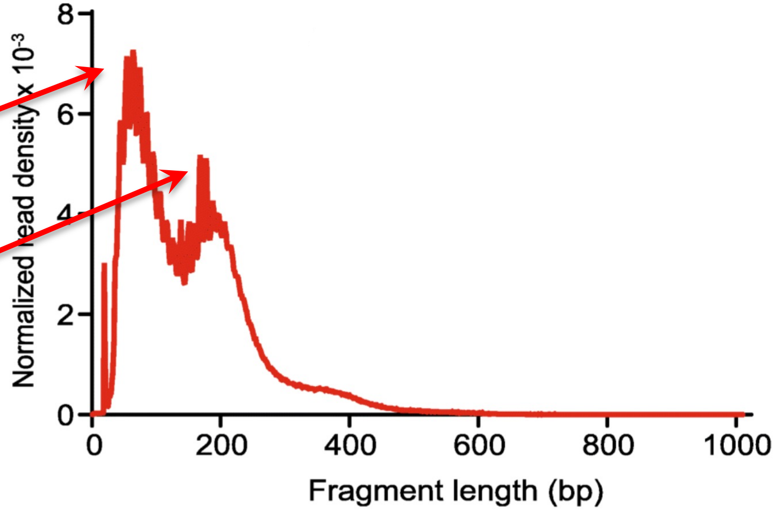
- Accessible chromatin can tell us about:
  - Where promoters are
  - How active they are or if they are poised for activation
  - Where enhancers are
  - If chromatin is open around specific transcription factor binding sites
  - Spread of heterochromatin
  - …

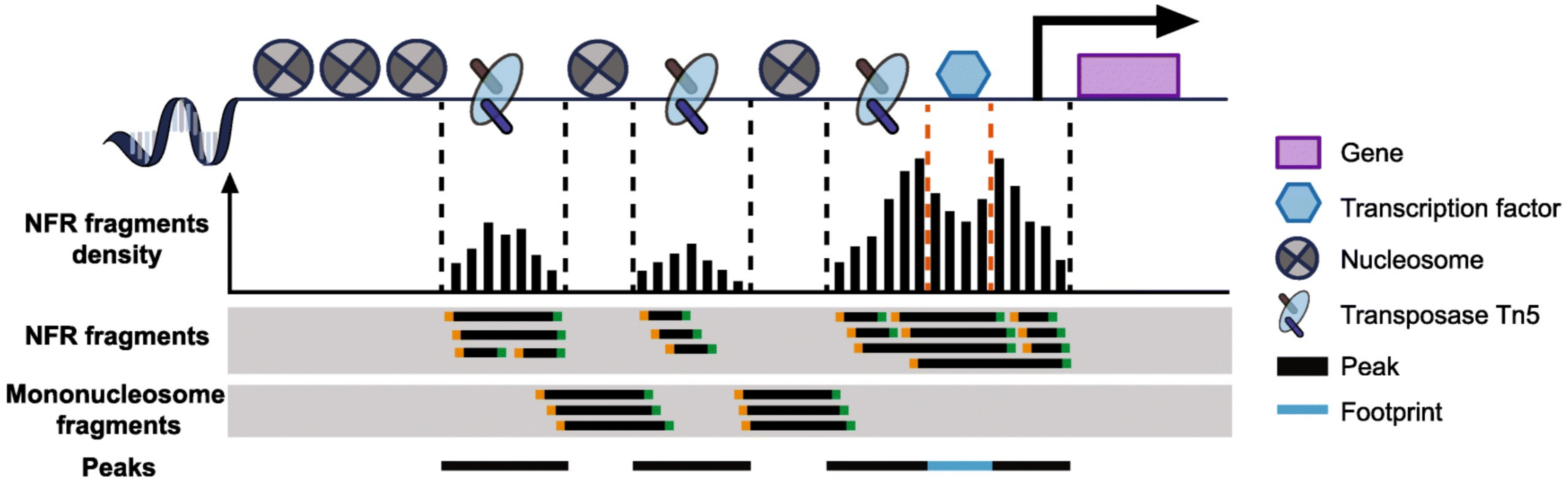
- ATAC-seq is simple to use, and works with very little starting material (even single cells).

→ Often a useful complement to RNA-seq.

ATAC-seq data

- Sequencing reads mapped to the genome the can be used to find
  - Open chromatin (from short DNA fragments)
  - Nucleosomes (from longer DNA fragments)
- Programs, like MACS3, are used to find peaks, i.e. regions with many DNA fragments mapping.

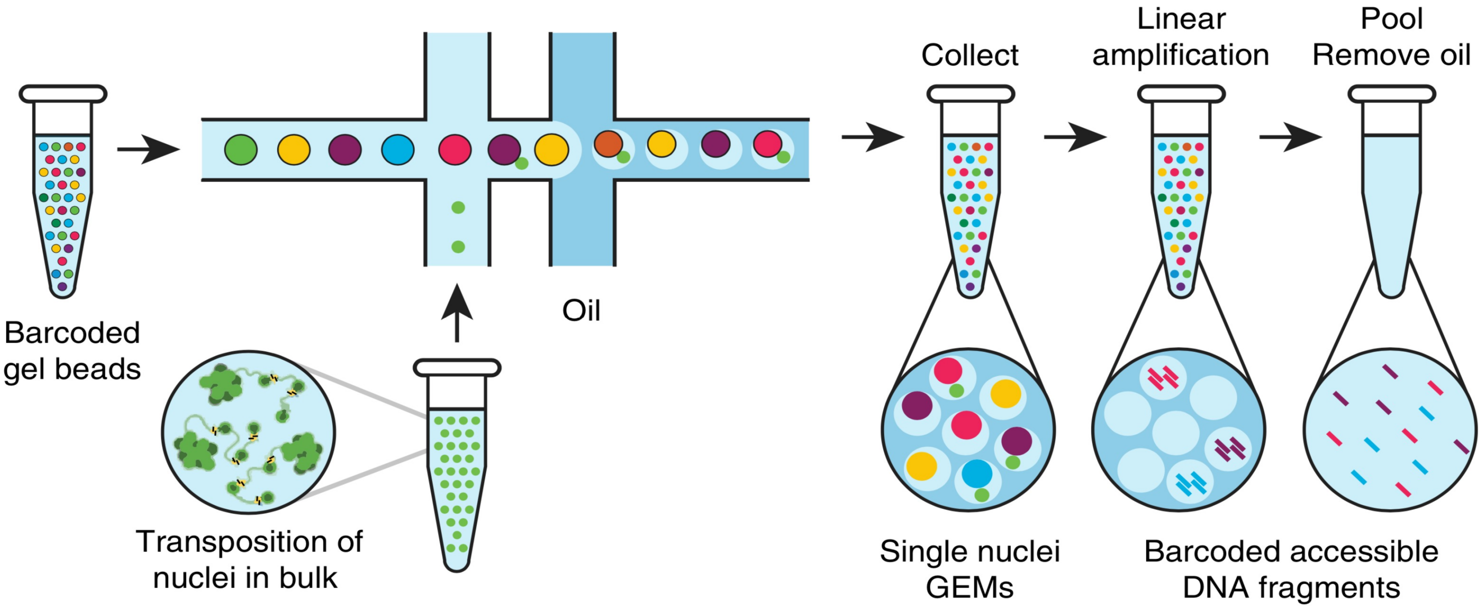




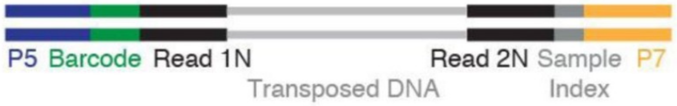
(Yan, 2020, Genome Biology)

Single cell ATAC-seq

- First paper, from Greenleaf lab: (Buenrostro et al. 2015, Nature)
- Now available as a kit from 10X Genomics
  - Each cell is attached to a bead containing a different barcode, inside an oil droplet.
  - These barcodes are attached to the DNA fragments, making it possible to assign each sequenced DNA fragment to a cell.

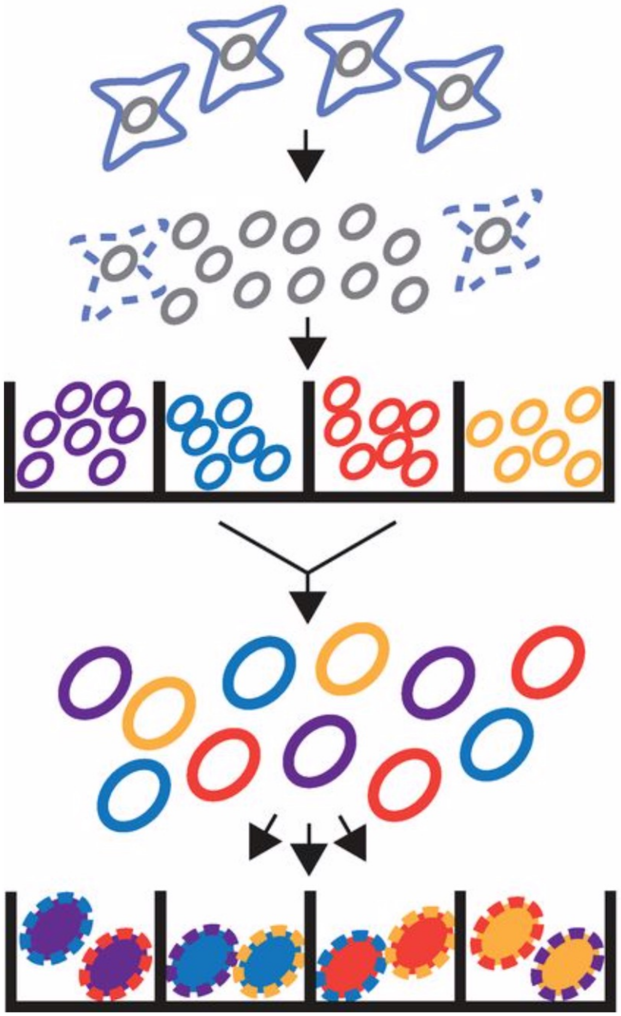


(Satpathy et al. 2019 Nature Biotechnology)



Single cell ATAC-seq II

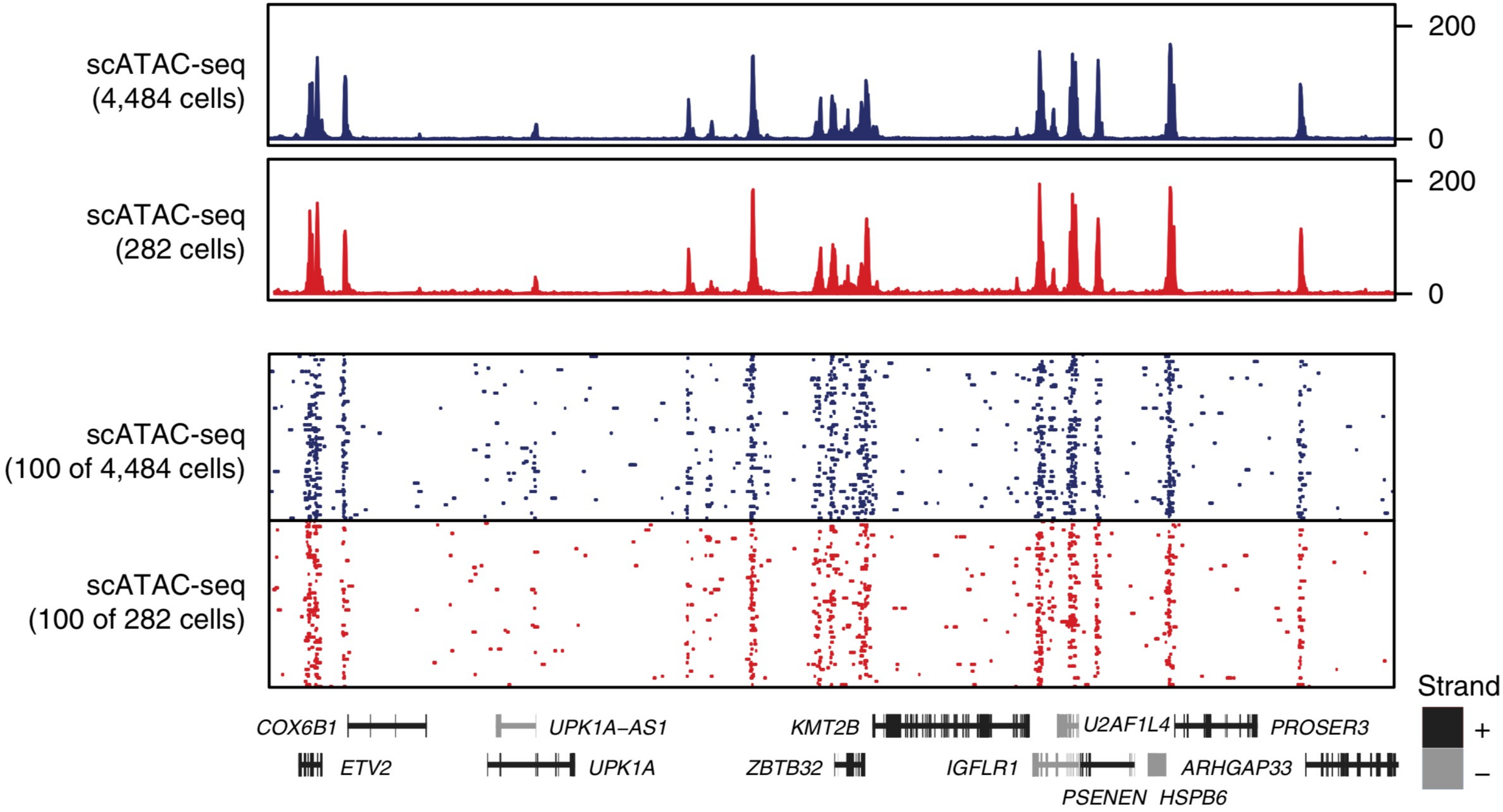
- An alternative to the droplet based method from 10X genomics is **sci-ATAC-seq** (single-cell combinatorial-indexing with ATAC-seq). (Cusanovich et al. 2015, Science)
- Here, cells are split up into e.g. 96 wells, and each well has a different short barcode.
- Cells are then pooled and re-distributed into wells again, adding another short barcode.
- This is repeated enough times so that each cell will eventually have it's own (almost) unique combination of short barcodes.



+ Low cost per cell, enables high throughput
- No commercial solution, harder to set up
- Lower cell recovery, important when there is limited starting material

Single cell ATAC-seq data

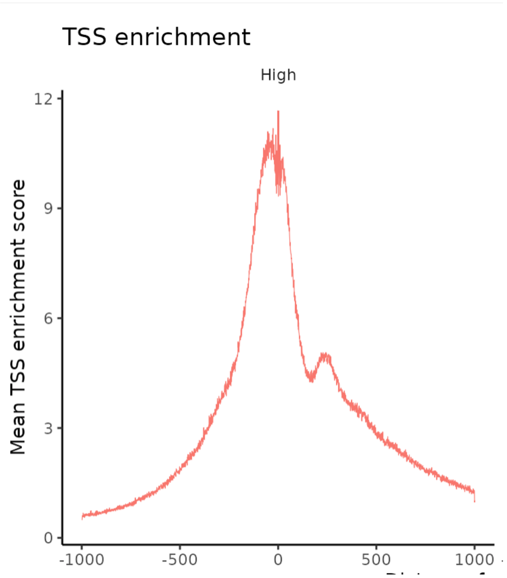
- Looking at each individual cell, scATAC-seq data are **sparse** and **noisy**.
- But combining data from lots of cells gives meaningful signals.



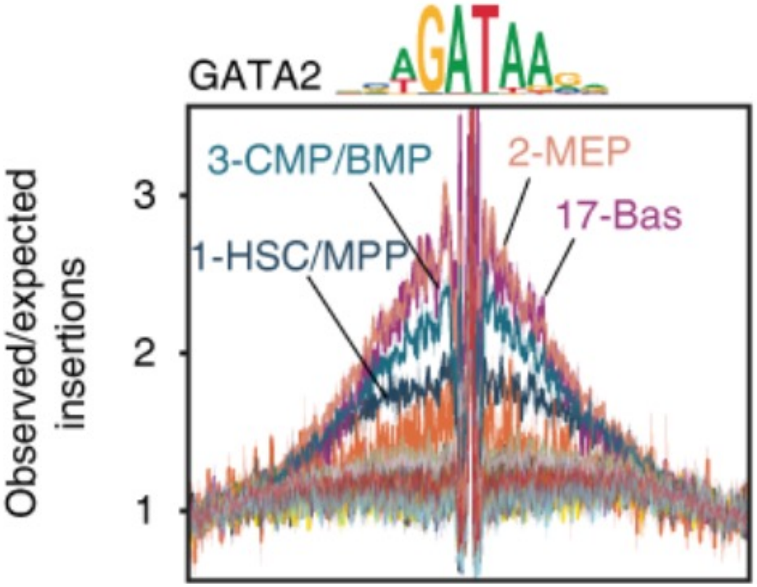
(Satpathy et al. 2019 Nature Biotechnology)

Single cell ATAC-seq data, continued

Combining data from lots of genomic loci can also give meaningful signals.



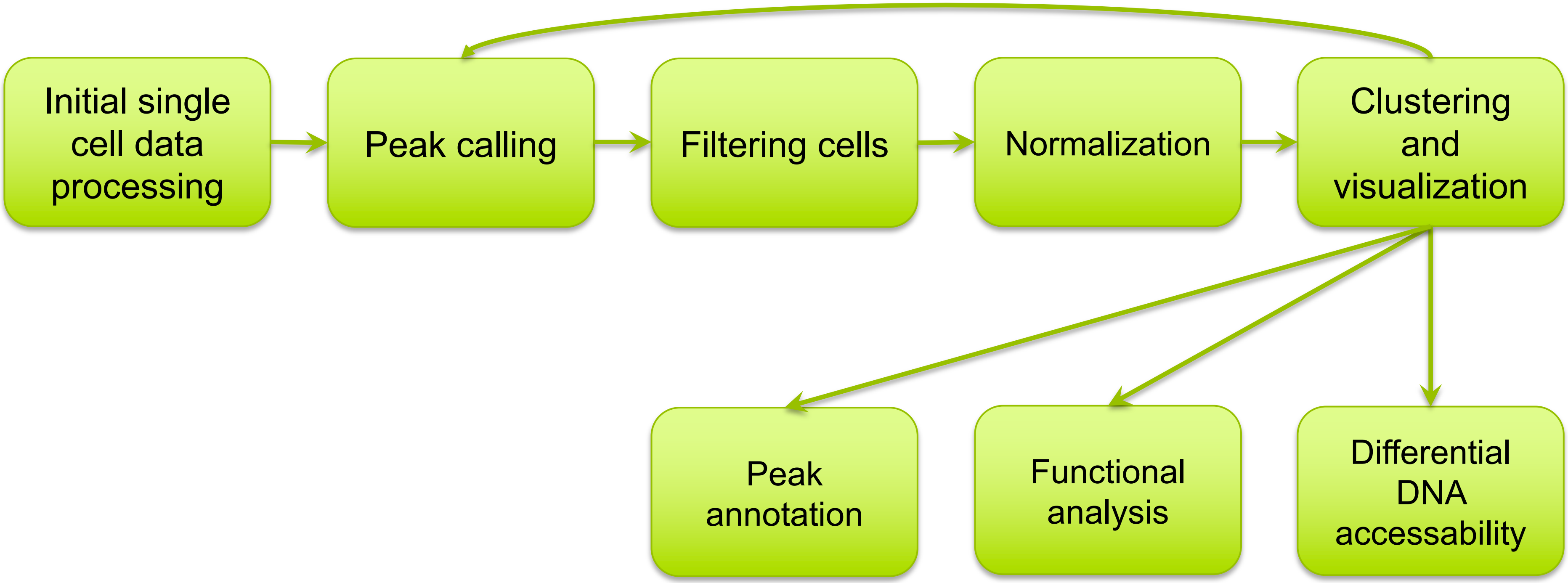
Signac tutorial



(Satpathy et al. 2019 Nature Biotechnology)

Single cell ATAC-seq data analysis

SciLifeLab


Initial single cell data processing
Peak calling
Filtering cells
Normalization
Clustering and visualization
Peak annotation
Functional analysis
Differential DNA accessability

1. Initial single cell data processing

- De-multiplex: Using the cell specific barcodes, assign each read to a cell.
- (Remove primer sequences.)
- Map reads to the genome, e.g. with BWA-MEM.
- Remove duplicates: If several read pairs map to exactly the same coordinates, only one is kept. Such duplicates are assumed to be PCR artifacts.
- Filter out some bad cells already at this stage.

2. Peak calling

- Done on aggregated data from all cells. (There is not enough data in a single cell to call peaks.)
- If we have a rare cell type with e.g. 50 out of 2000 cells, peaks specific to this cell type can be missed when we use the aggregated data for peak calling.
  - We can go back and redo the peak calling later, only looking at specific groups of cells.
- We then count the reads from every cell in every peak:

	Cell 1	Cell 2	Cell 3	…	Cell M
Peak 1	0	1	1		0
Peak 2	0	0	0		0
Peak 3	0	0	0		1
…					
Peak N	1	0	0		0

Mostly 0s

3. Filtering cells

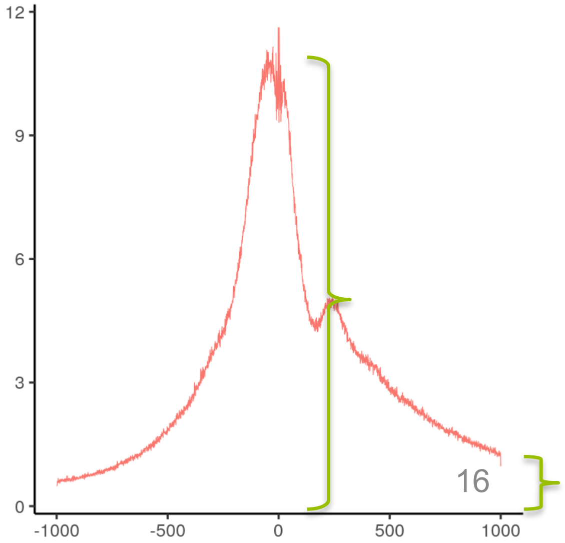
- There are many things that could go wrong in a single cell ATAC-seq experiment
  - No cell in a droplet
  - Several cells in a droplet
  - Dead cells
  - Few reads from a cell
  - No transfection in a cell
- Therefore we use several quality measures to identify and remove problematic cells/barcodes:
  - Number of fragments in peaks: Cells with very few reads may need to be excluded due to low sequencing depth. Cells with extremely high levels may represent doublets, nuclei clumps, or other artefacts.
  - Fraction of fragments in peaks: Cells with low values (i.e. <15-20%) often represent low-quality cells or technical artifacts that should be removed.

3. Filter cells II

- Fragment sizes: Open chromatin corresponds to short DNA fragments, so we want to remove cells with too few short DNA fragments, coming from open chromatin.

- Reads in blacklist regions: The ENCODE project has provided a list of blacklist regions, i.e. regions with artefactual signal. Cells with many reads mapping to these blacklist regions (compared to reads mapping to peaks) often represent technical artifacts and should be removed.

- Transcriptional start site (TSS) enrichment score. TSS are associated with open chromatin, so a low level of chromatin enrichment would suggest poor ATAC-seq experiments.



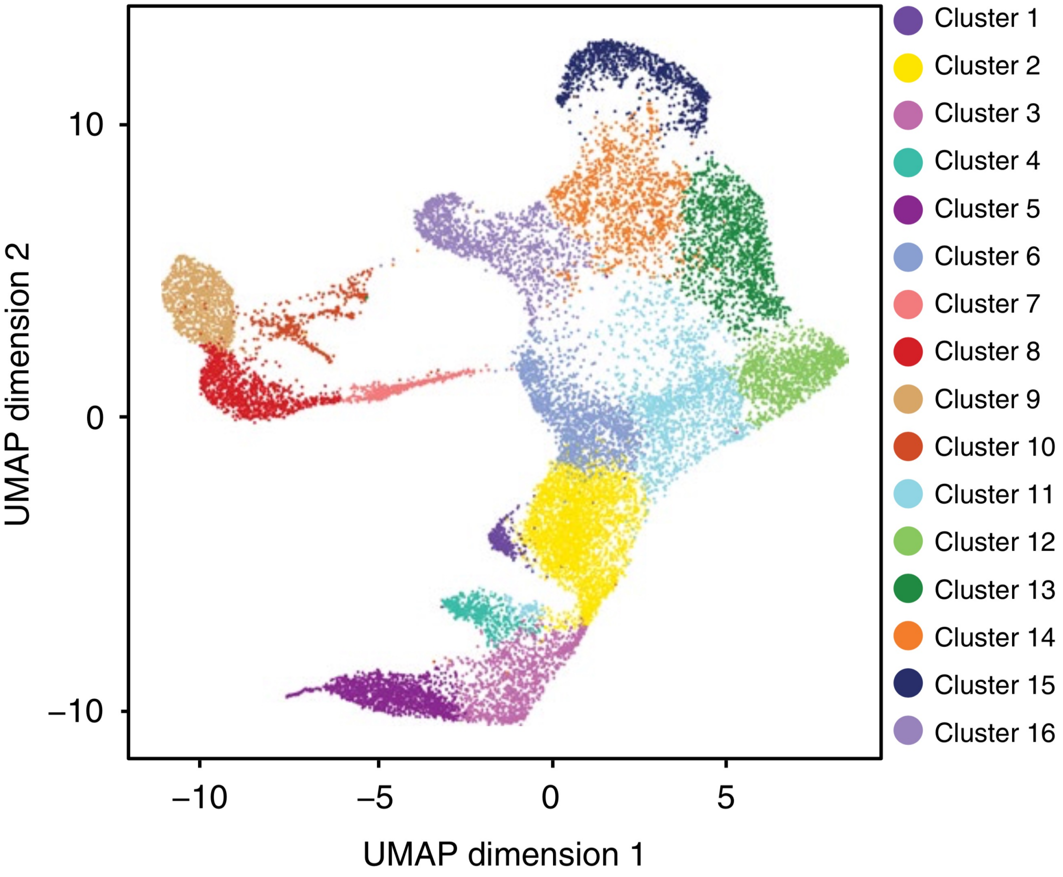
4. Normalization

- Account for different sequencing depth in different cells
- Create a simplified representation of the data, using dimension reduction (singular value decomposition). This is similar to principal component analysis (PCA).
  - The idea behind this is to reduce noise, and to select informative features to improve clustering of cells and visualization
  - Typically, the first component correlates with sequencing depth, so by removing it we get rid of artefactual signal.
  - Reducing dimensionality is often good in itself.
  - Results are often better when we select only some features (peaks)
    - Those with highest signal
    - Those with highest variability

5. Cluster and visualize cells

- Similar to single cell RNA seq:
  - Project many dimensions down to 2.
  - UMAP algorithm

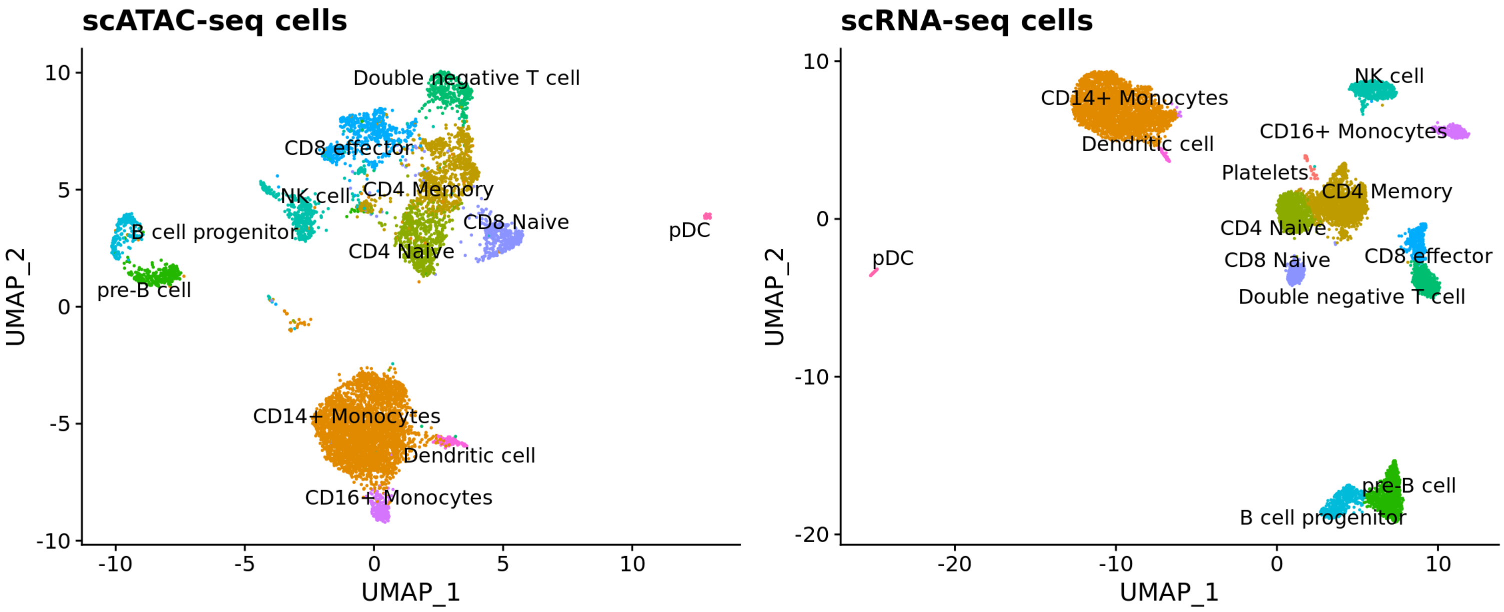
- Clustering, to identify groups of similar cells (representing different cell types or cell states).



(Satpathy et al. 2019 Nature Biotechnology)

5. Cluster and visualize cells II

- It's often not clear which cell types etc. these clusters represent.
  - In single cell RNA-seq we can look at marker genes, unique to a specific cell type. In single cell ATAC-seq, this is harder.
  - If it's possible to get RNA-seq data from a similar set of cells, these can be annotated and then used to annotate the ATAC-seq clusters.
  - For this we can use gene activity scores (level of open chromatin around genes), as a proxy for gene expression.

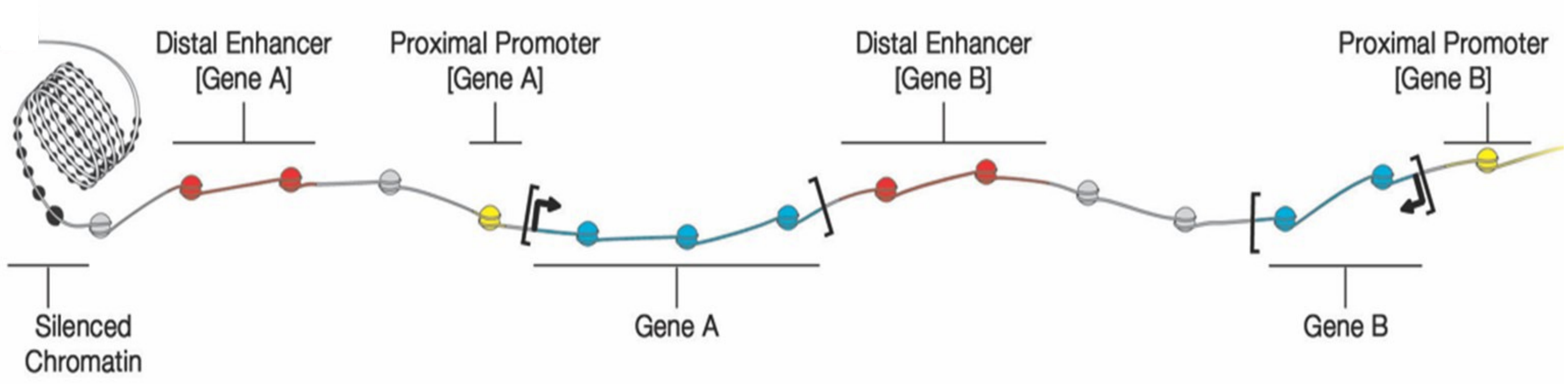


Example from Seurat web site

6. Peak annotation

- To easier interpret the peaks, it's useful to note their location with regard to the nearest gene (or the nearest transcription start site).

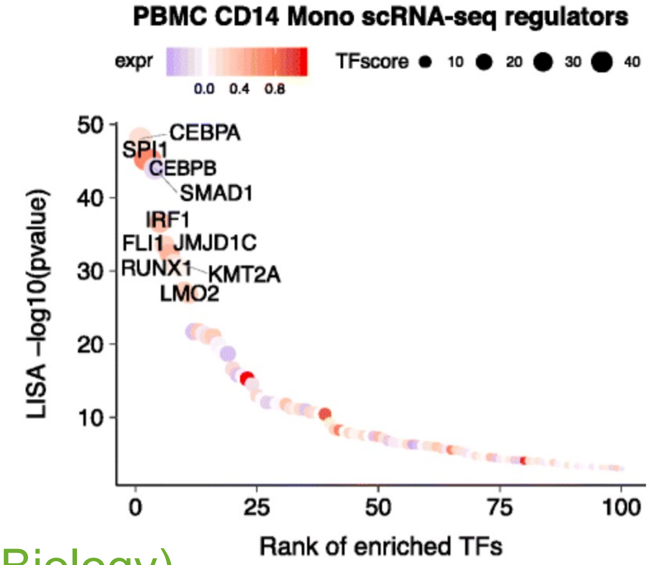
- A region might not interact with the nearest gene, this is just a starting guess!



(Hinman & Cary. 2017, eLife)

7. Functional analysis

- Regions with open chromatin can be further analyzed, to see with transcription factors might bind there. This can give important information on which signaling pathways drive gene expression in different cells.
  - Looking for enriched motifs
  - Cross-referencing open chromatin regions against public ChIP-seq data on different TFs.
- This can be done for each cell or cluster of cells



(Wang et al. 2020 Genome Biology)

8. Differential DNA accessibility

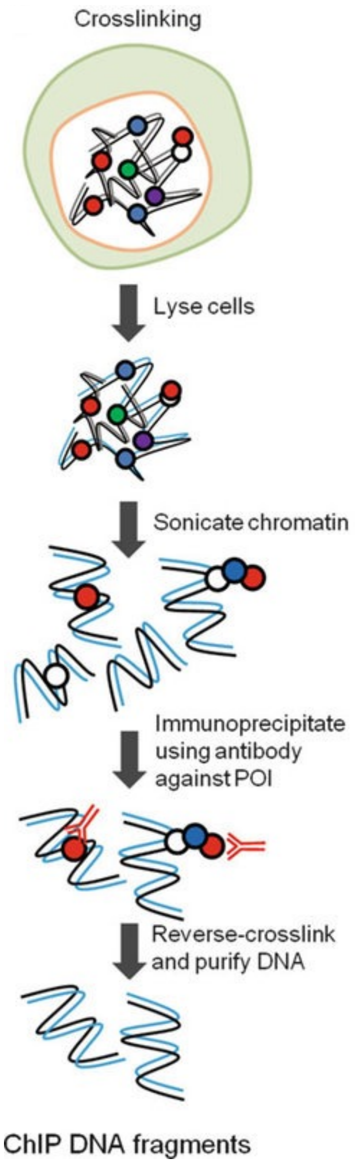
- It’s often interesting to know which chromatin regions differ in accessibility between cell types etc.
- This is a similar problem to differential gene expression (for RNA-seq data)
- Examples of methods:
  - Logistic regression
  - Negative binomial generalized linear model

Single cell ATAC-seq analysis tools

- **ATAC Cell Ranger**
  - Computational pipeline from 10X genomics, does (more or less) all of the analysis steps described here
- **Seurat/Signac (Archer)**
  - R packages originally developed for single cell RNA-seq: Filtering cells, normalization, clustering, visualization, differential DNA accessibility. Data integration.
- **episcanpy**
  - Python package, originally developed for single cell RNA-seq. Similar functionality to Seurat/Signac
- **ChromVar**
  - R package, mostly useful for motif analysis. (Can do clustering, visualization, differential DNA accessibility too..)
- **Giggle**
  - Command line tool for cross-refencing genomic regions against public data sets.

ChIP-seq

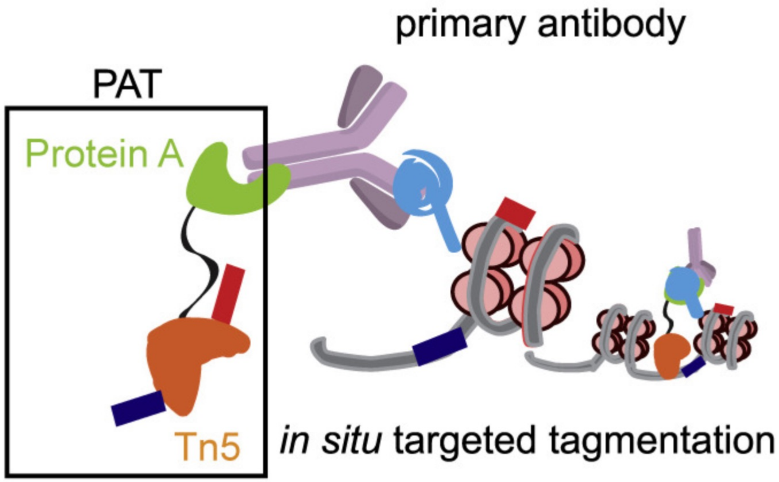
- **Ch**romatin **I**mmuno-**P**recipitation, followed by sequencing
- Measures interactions between a protein of interest and DNA
- Uses an antibody towards the protein of interest to enrich for bound DNA.
- Analysis similar to ATAC-seq: finding peaks (regions with many reads mapping)
- ChIP on single cells, e.g. using droplets, is hard.
  - (Rotem et al. 2015, Nature Biotechnology) had around 800 reads/cell. Still enough to distinguish different cell types.
  - (Grosselin et al. 2019, Nature Genetics) had around 1600 reads/cell.


Crosslinking
Lyse cells
Sonicate chromatin
Immunoprecipitate using antibody against POI
Reverse-crosslink and purify DNA
ChIP DNA fragments

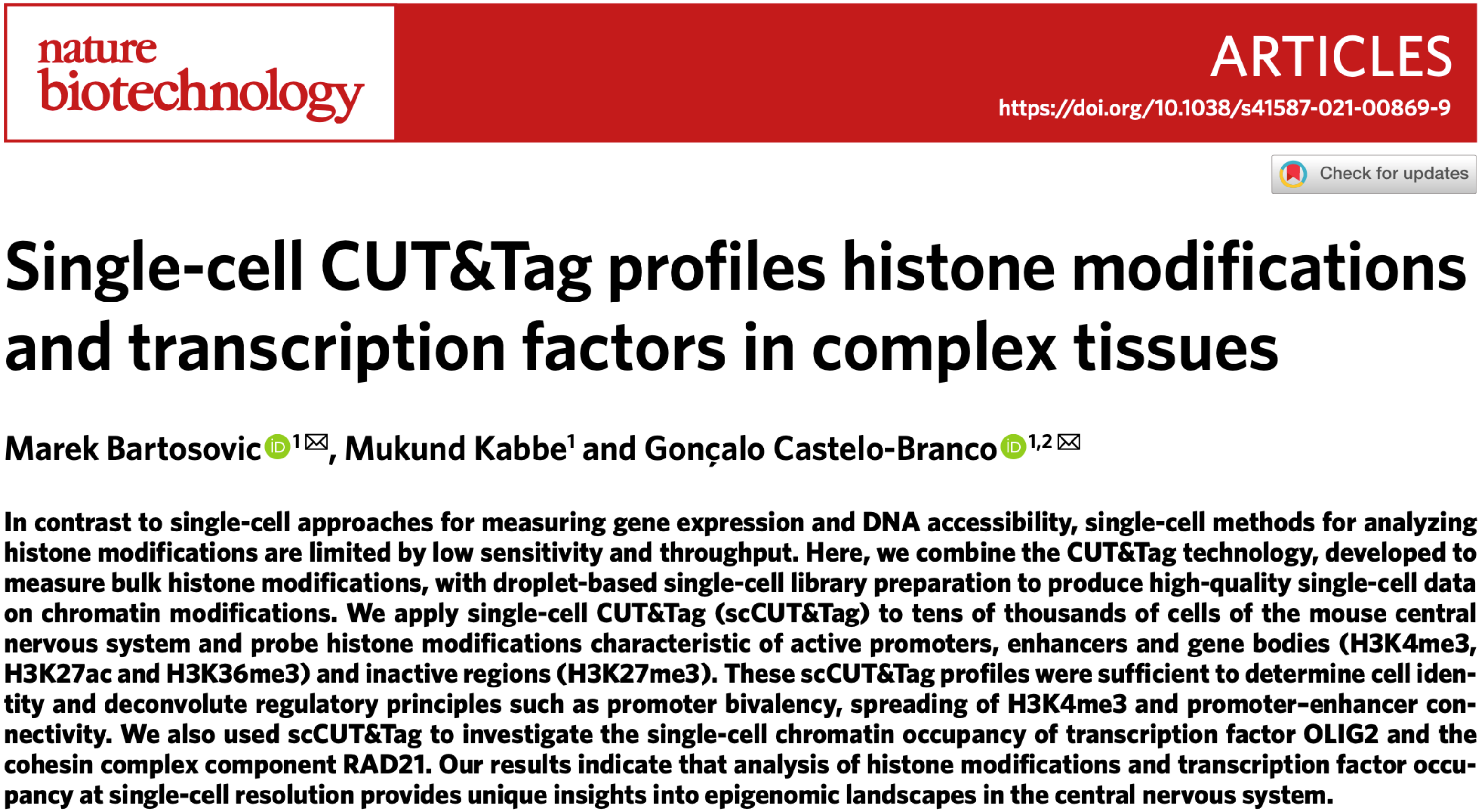
(Narlikar & Jothi, 2011, Next Generation Microarray Bioinformatics)

Single cell ChIP-seq like methods

- ChIP-free methods:
  - (Wang et al. 2019, Molecular Cell) CoBATCH
    - Antibody binds to protein of interest. → This recruits PAT complex with Tn5 → Tagmentation of DNA near protein of interest.
    - 12000 reads/cell
    - Combinatorial indexing (like for sci-ATAC-seq)
    - Quite simple protocol, no ChIP
  - (Kaya-Okur et al. 2019, Nature Communications) CUT&Tag, similar idea. (Used nanowells instead of combinatorial indexing.)



Single cell ChIP-seq like methods III



nature biotechnology

ARTICLES

https://doi.org/10.1038/s41587-021-00869-9

Check for updates

Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues

Marek Bartosovic[1]✉, Mukund Kabbe[1] and Gonçalo Castelo-Branco[1,2]✉

In contrast to single-cell approaches for measuring gene expression and DNA accessibility, single-cell methods for analyzing histone modifications are limited by low sensitivity and throughput. Here, we combine the CUT&Tag technology, developed to measure bulk histone modifications, with droplet-based single-cell library preparation to produce high-quality single-cell data on chromatin modifications. We apply single-cell CUT&Tag (scCUT&Tag) to tens of thousands of cells of the mouse central nervous system and probe histone modifications characteristic of active promoters, enhancers and gene bodies (H3K4me3, H3K27ac and H3K36me3) and inactive regions (H3K27me3). These scCUT&Tag profiles were sufficient to determine cell identity and deconvolute regulatory principles such as promoter bivalency, spreading of H3K4me3 and promoter–enhancer connectivity. We also used scCUT&Tag to investigate the single-cell chromatin occupancy of transcription factor OLIG2 and the cohesin complex component RAD21. Our results indicate that analysis of histone modifications and transcription factor occupancy at single-cell resolution provides unique insights into epigenomic landscapes in the central nervous system.

Single cell ChIP-seq like methods IV

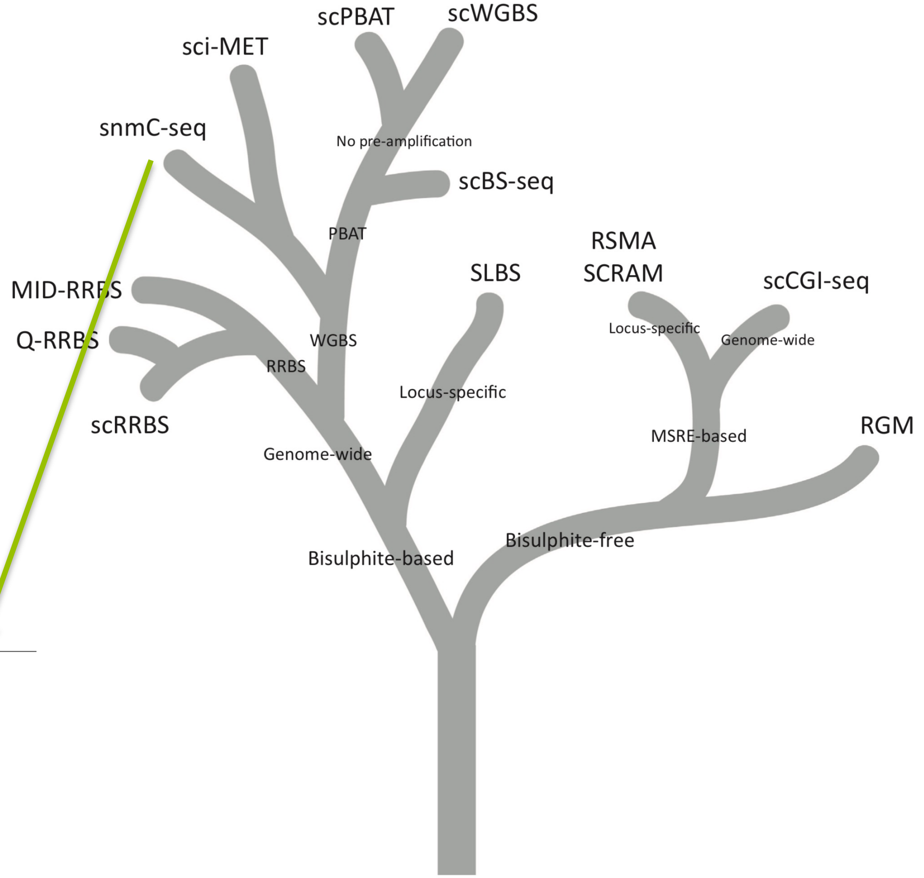
- Data analysis for these methods is similar to single cell ATAC-seq.
- Single cell ChIP-seq is still new, but developing fast. Throughput will likely increase a lot.

DNA methylation

- Methyl group bound to cytosine in DNA, typically at CpG sites.
- Usually associated with repression of gene expression
- Bisulphite sequencing: converts cytosine residues to uracil, except where there is methylation
- Other approaches
  - using methylation-sensitive restriction enzymes
  - fluorescence-based

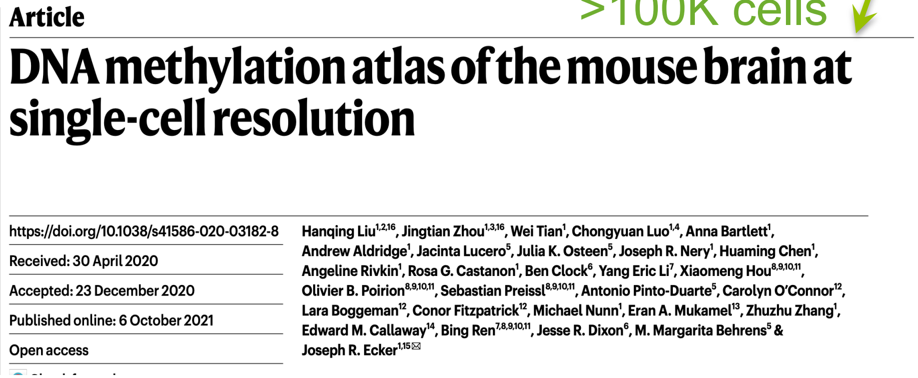
Single cell DNA methylation

- Methods
  - Whole genome vs reduced representation/targeted
  - Bisulphite vs bisulphite-free (methylation-sensitive restriction enzymes)
- Quite hard and expensive
- Data
  - Mostly 5mC
  - Thousands of cells
  - 10^4 -10^7 CpGs per cell
  - Not the same CpGs in all cells.
- Analysis still hard



Karemaker & Vermeulen. 2018, Trends in Biotechnology

>100K cells



Article

DNA methylation atlas of the mouse brain at single-cell resolution

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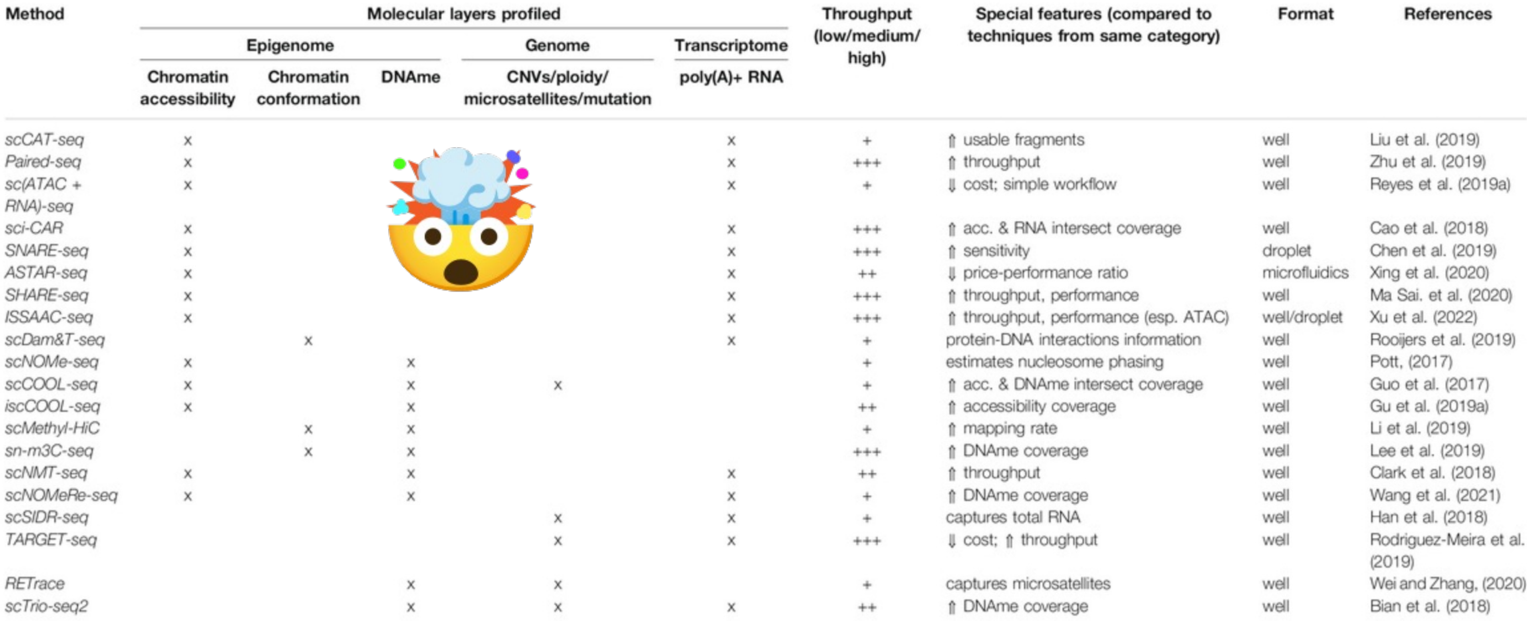
Hanqing Liu[1,2,16], Jingtian Zhou[1,3,16], Wei Tian[1], Chongyuan Luo[1,4], Anna Bartlett[1], Andrew Aldridge[1], Jacinta Lucero[5], Julia K. Osteen[5], Joseph R. Nery[1], Huaming Chen[1], Angeline Rivkin[1], Rosa G. Castanon[1], Ben Clock[6], Yang Eric Li[7], Xiaomeng Hou[8,9,10,11], Olivier B. Poirion[8,9,10,11], Sebastian Preissl[8,9,10,11], Antonio Pinto-Duarte[5], Carolyn O'Connor[12], Lara Boggeman[12], Conor Fitzpatrick[12], Michael Nunn[1], Eran A. Mukamel[13], Zhuzhu Zhang[1], Edward M. Callaway[14], Bing Ren[7,8,9,10,11], Jesse R. Dixon[6], M. Margarita Behrens[5] & Joseph R. Ecker[1,15]✉

Combining assays from the same cells

- Many methods combine several assays from the same cells, e.g.
  - **scRNA-seq and scATAC-seq (10X genomics, SNARE-seq, and many more)**
  - scRNA-seq and sc-protein abundance (CITE-seq)
  - scRNA-seq and scDNA methylation
  - scRNA-seq and scDNA methylation and sc nucleosome (scNMT-seq)
  - scRNA-seq, scATAC-seq, sc-protein abundance and clonal info from mitochondrial DNA (DOGMA-seq)



Method	Molecular layers profiled					Throughput (low/medium/ high)	Special features (compared to techniques from same category)	Format	References
	Epigenome			Genome	Transcriptome				
	Chromatin accessibility	Chromatin conformation	DNAme	CNVs/ploidy/ microsatellites/mutation	poly(A)+ RNA				
scCAT-seq	x				x	+	⇑ usable fragments	well	Liu et al. (2019)
Paired-seq	x				x	+++	⇑ throughput	well	Zhu et al. (2019)
sc(ATAC + RNA)-seq	x				x	+	⇓ cost; simple workflow	well	Reyes et al. (2019a)
sci-CAR	x				x	+++	⇑ acc. & RNA intersect coverage	well	Cao et al. (2018)
SNARE-seq	x				x	+++	⇑ sensitivity	droplet	Chen et al. (2019)
ASTAR-seq	x				x	++	⇓ price-performance ratio	microfluidics	Xing et al. (2020)
SHARE-seq	x				x	+++	⇑ throughput, performance	well	Ma Sai. et al. (2020)
ISSAAC-seq	x				x	+++	⇑ throughput, performance (esp. ATAC)	well/droplet	Xu et al. (2022)
scDam&T-seq		x			x	+	protein-DNA interactions information	well	Rooijers et al. (2019)
scNOMe-seq	x		x			+	estimates nucleosome phasing	well	Pott, (2017)
scCOOL-seq	x		x	x		+	⇑ acc. & DNAme intersect coverage	well	Guo et al. (2017)
iscCOOL-seq	x		x			++	⇑ accessibility coverage	well	Gu et al. (2019a)
scMethyl-HiC		x	x			+	⇑ mapping rate	well	Li et al. (2019)
sn-m3C-seq		x	x			+++	⇑ DNAme coverage	well	Lee et al. (2019)
scNMT-seq	x		x		x	++	⇑ throughput	well	Clark et al. (2018)
scNOMeRe-seq	x		x		x	+	⇑ DNAme coverage	well	Wang et al. (2021)
scSIDR-seq				x	x	+	captures total RNA	well	Han et al. (2018)
TARGET-seq				x	x	+++	⇓ cost; ⇑ throughput	well	Rodriguez-Meira et al. (2019)
RETrace			x	x		+	captures microsatellites	well	Wei and Zhang, (2020)
scTrio-seq2			x	x	x	++	⇑ DNAme coverage	well	Bian et al. (2018)

Dimitriu et al. 2022 Frontiers in Cell and Developmental Biology

Multi-omics analysis

- Seurat/Signac supports analysis of multiomics data, e.g. from 10X
- Best to first analyze each data type separately (QC, filtering, clustering etc.)
- It’s also possible to do a joint neigborhood graph to do clustering and UMAP on all data together
  - WNN: Weighted nearest neighbor
- Another option is to first cluster cells on RNA, then subcluster on e.g. ATAC-seq.

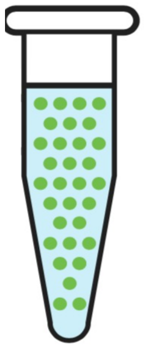
Spatial methods

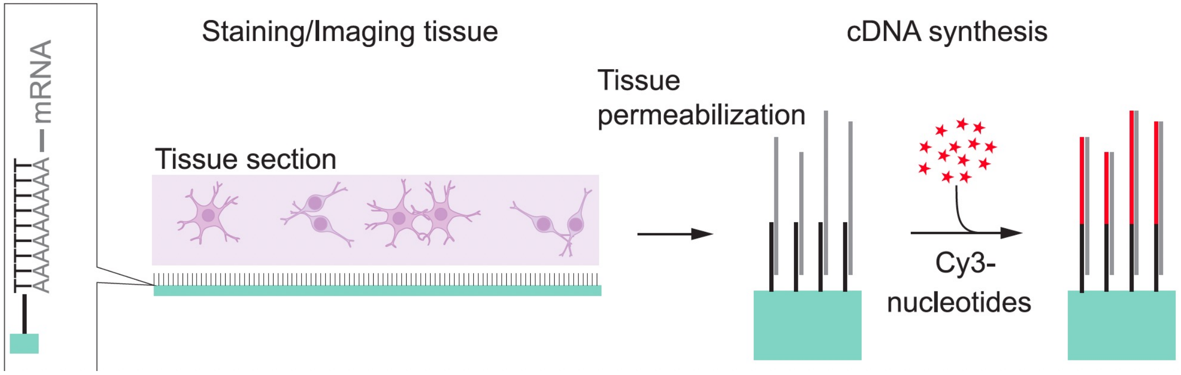
- Sequencing based methods "spatial transcriptomics"

  + Captures all genes

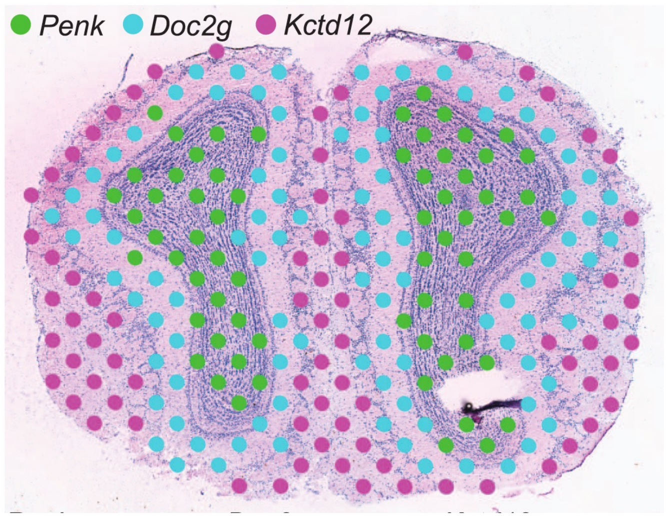
  - not single cell resolution (each spot consists of several cells)

- The basic idea is to hybridize a tissue to an array, where spatial barcodes are added to the RNA molecules





Ståhl et al 2016, Nature



- Imaging based methods "in-situ sequencing"

  - probes a subset of genes (typically 100s)

  + single cell resolution

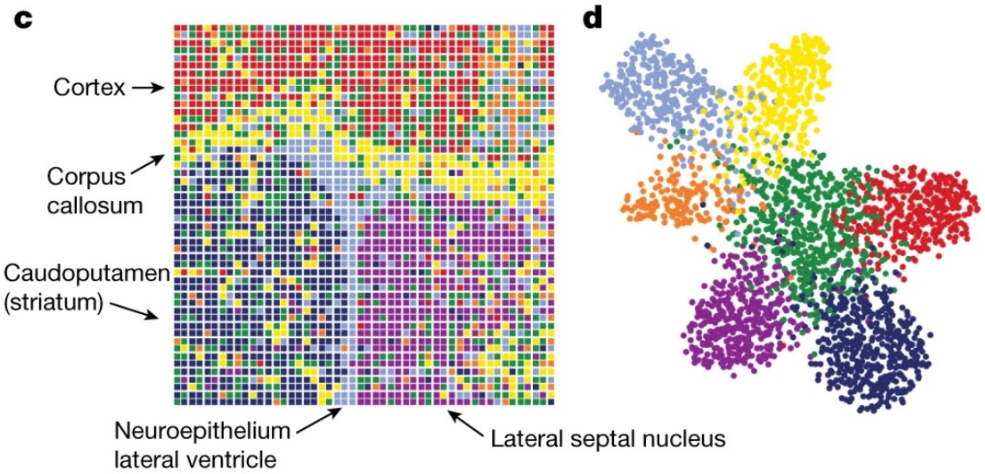
Spatial methods, II

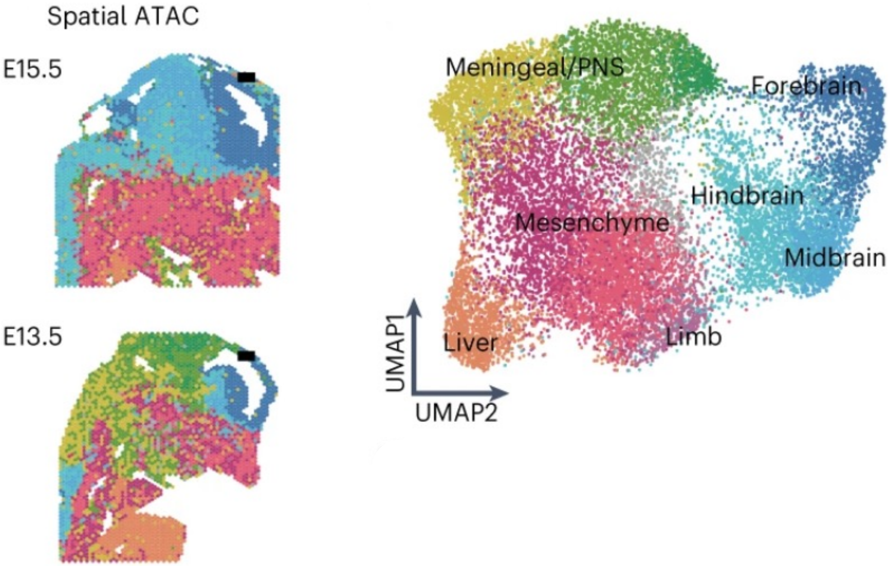
- For sequencing based methods, a lot of the analysis is similar to single cell data, but instead of cells, you work with “spots” (these typically cover several cells).

- Spatial methods are often used together with single cell methods, to get information both on what different cell types looks like (from the single cell data) and where they are located (from the spatial data)

Spatial methods, III

Recently, spatial methods have been adapted to ATAC-seq.



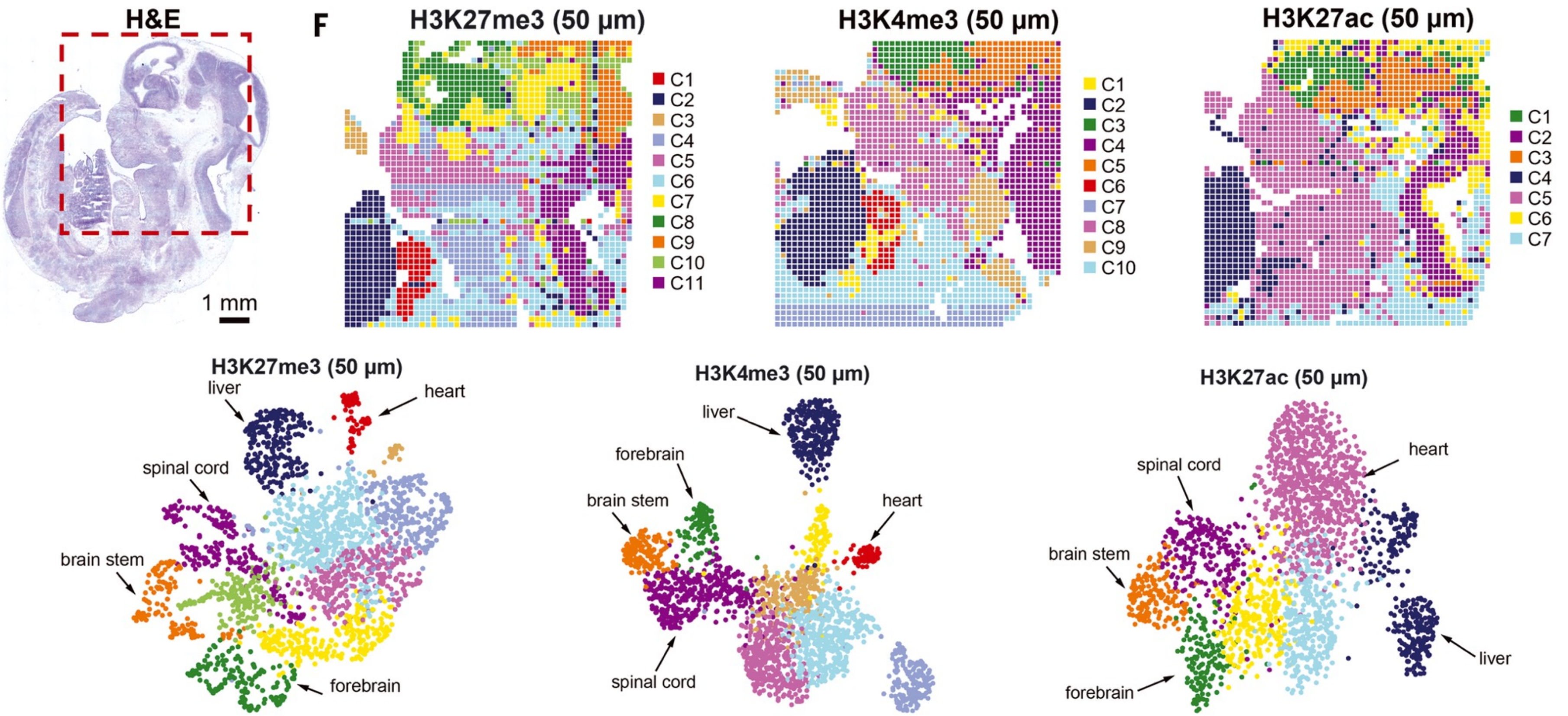


Deng et al 2022, Nature

Llorens-Bobadilla et al 2023, Nature Biotech.

Spatial methods, IV

Spatial methods have also been adapted to CUT & Tag.



Deng et al 2022, Science

Summary

- Single cell ATAC-seq
  - Usually works quite well
  - Commercial kits available
  - Often used together with single cell RNA-seq
- Single cell ChIP-seq/cut & tag etc.
  - Data analysis for all of these methods is similar to single cell ATAC-seq.
  - Still new, but developing fast. Throughput will likely increase a lot.
- Single cell DNA methylation
  - A lot of development happening
  - Useful methods will become more widely available (already scWGBS at NGI/Scilifelab).
- Multi-comics
  - Many method available, a lot of development.
  - Seurat etc. can be used for such data

Some resources

SciLifeLab

- Signac website, lots of tutorials
  - https://stuartlab.org/signac/
- Epigenomics data analysis course
  - https://nbis-workshop-epigenomics.readthedocs.io/en/latest/
  - Next occasion probably fall 2024
- 10X genomics
  - https://www.10xgenomics.com/products/single-cell-atac
  - https://www.10xgenomics.com/products/single-cell-multiome-atac-plus-gene-expression

the End