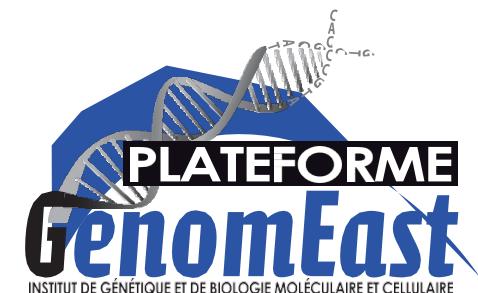


Epigenetics assays

Analysis of gene expression regulation

Stéphanie Le Gras
GenomEast Platform, Illkirch, France

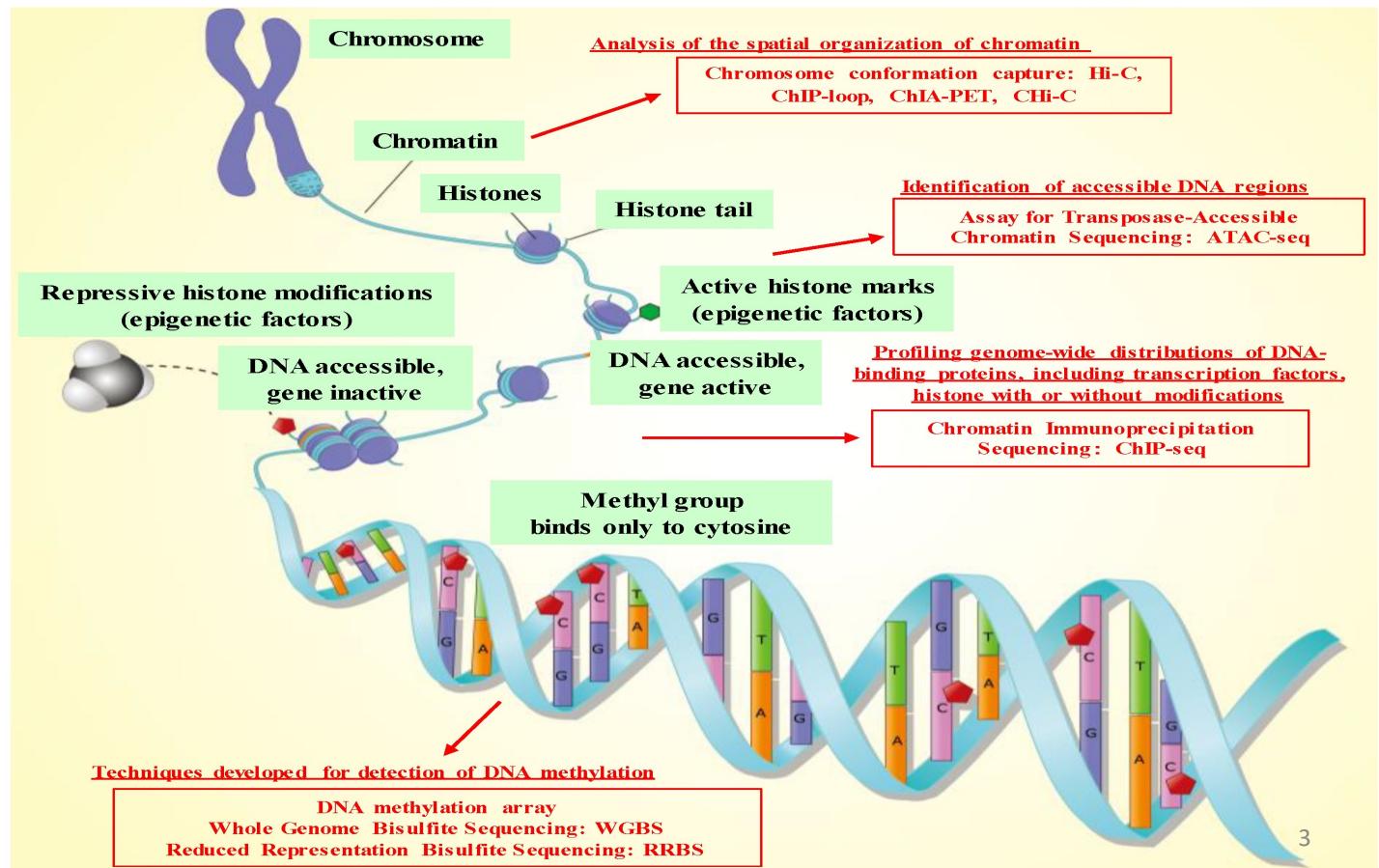


Epigenetics

- Epigenetics is the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence. (Wu et al, 2001)
- Epigenetic mechanisms such as DNA methylation, Histone Post-Translational Modification (PTM), Nucleosome positioning are affected by development (in utero, childhood), environmental chemicals, drugs/pharmaceuticals, aging, diet,...

Technologies for epigenetics analysis

Hamamoto et al, 2020



ChIP-seq

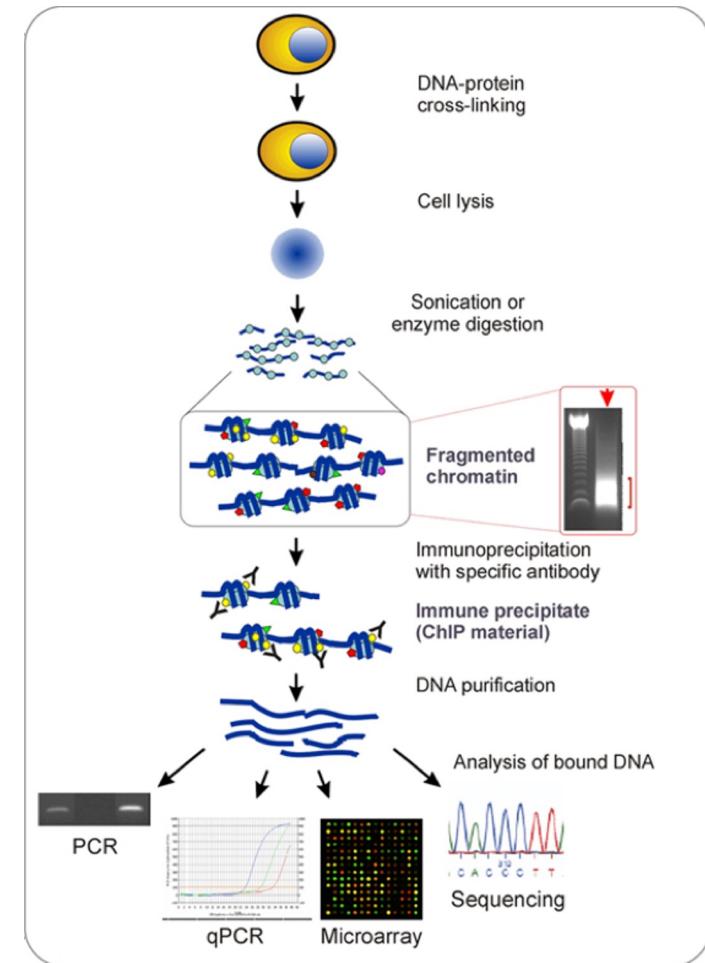
Chromatin ImmunoPrecipitation followed by sequencing

ChIP-seq

ChIP (=Chromatin Immuno-Precipitation)

differences in methods to detect the bound DNA

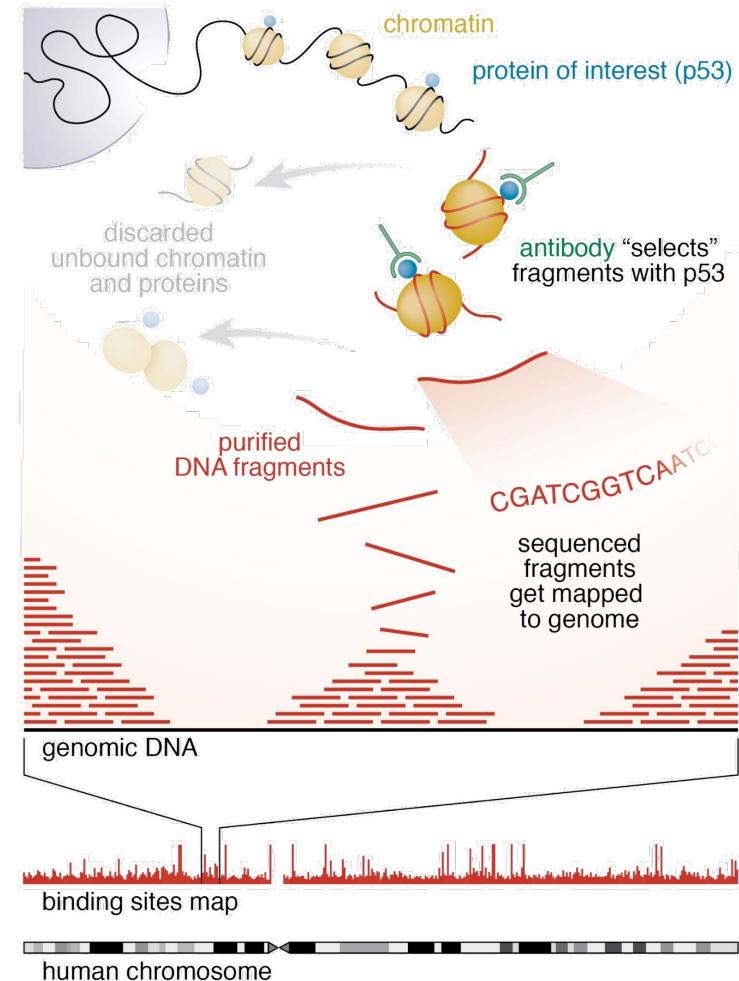
- small-scale: PCR / qPCR
- large-scale:
- microarray = ChIP-on-chip
- sequencing = ChIP-seq



<http://www.chip-antibodies.com/>

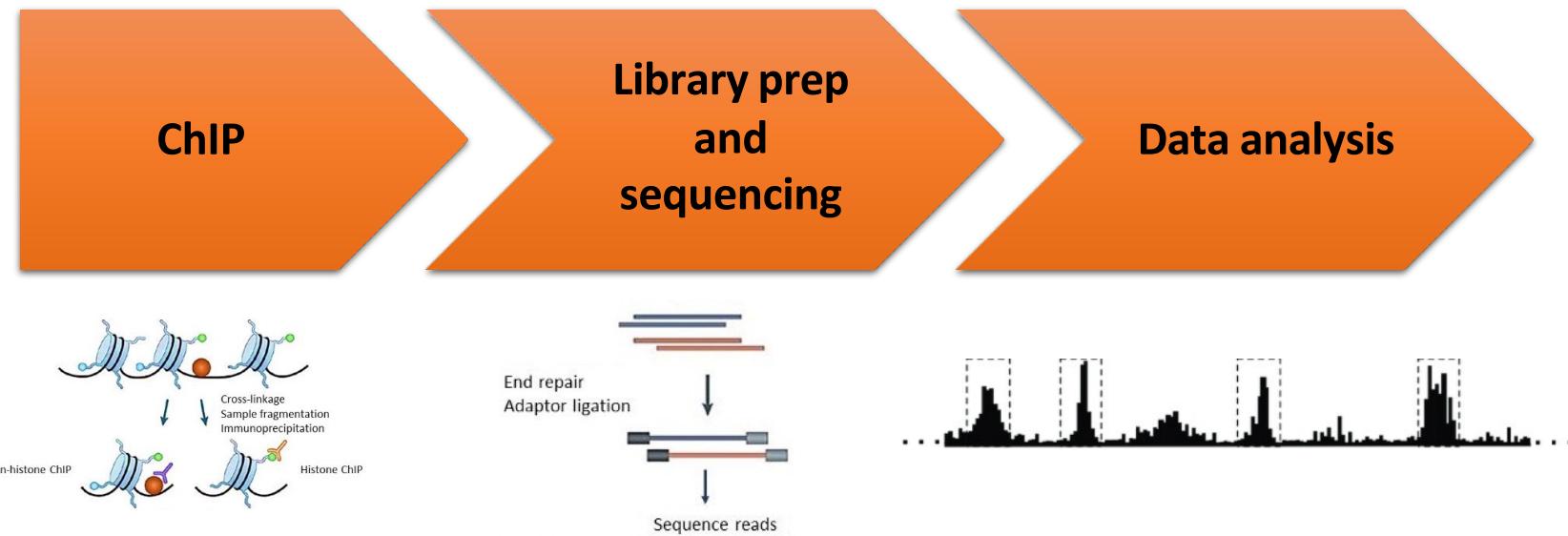
ChIP-seq

- Johnson et al, 2007
- Alternative to ChIP-on-chip (hybridization technique)
- ChIP-seq combines chromatin immunoprecipitation and sequencing to analyze protein interactions with DNA
- It can be used to detect and analyze
 - Binding sites of various proteins bound to DNA such as transcription factors (TF ChIP-seq)
 - Position of histone post translational modifications (Histone ChIP-seq)
 - Nucleotide modification such methylation (MeDIP-seq)
- Expected results of a ChIP-seq experiment are genomic regions with significant sequenced read enrichments (also called peaks)

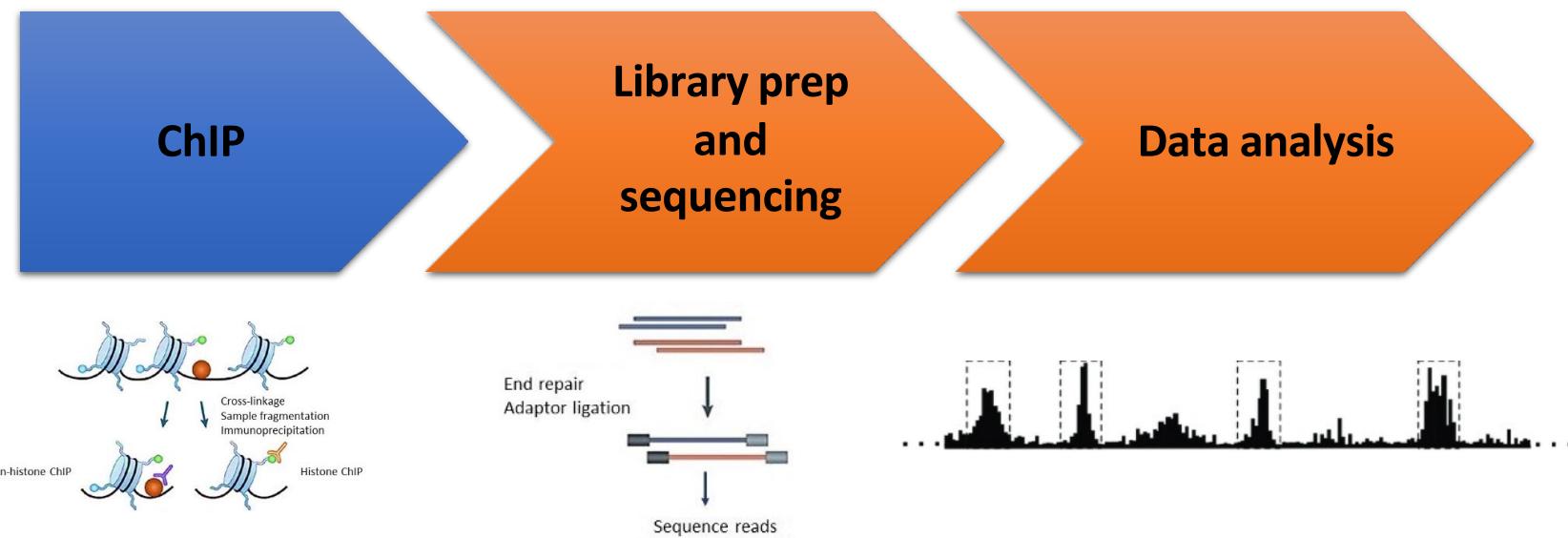


https://hbctraining.github.io/Intro-to-ChIPseq/lessons/01_Intro_chipseq_data_organization.html

ChIP-seq process

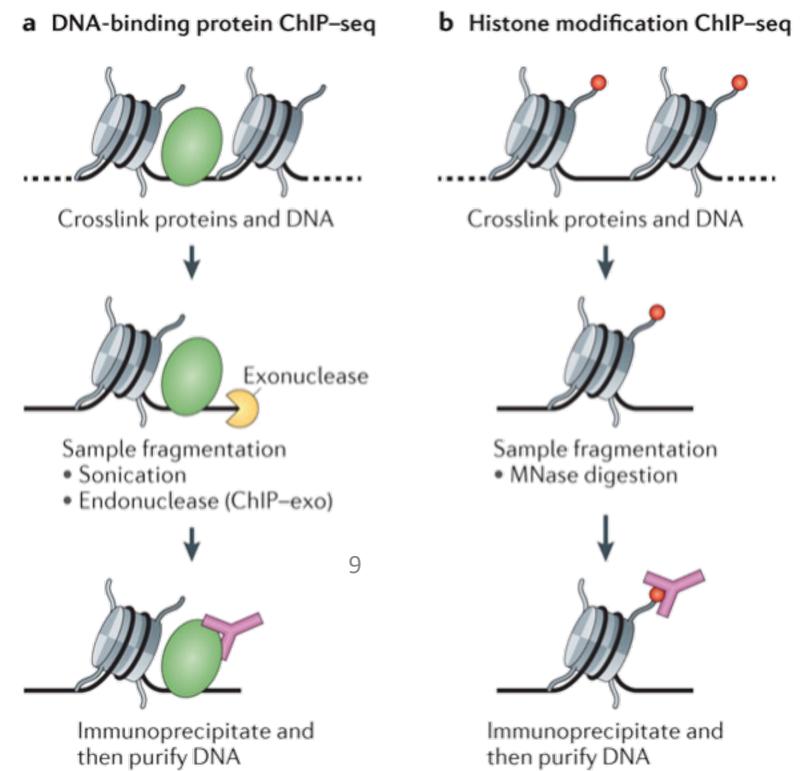


Chromatin ImmunoPrecipitation



Chromatin ImmunoPrecipitation

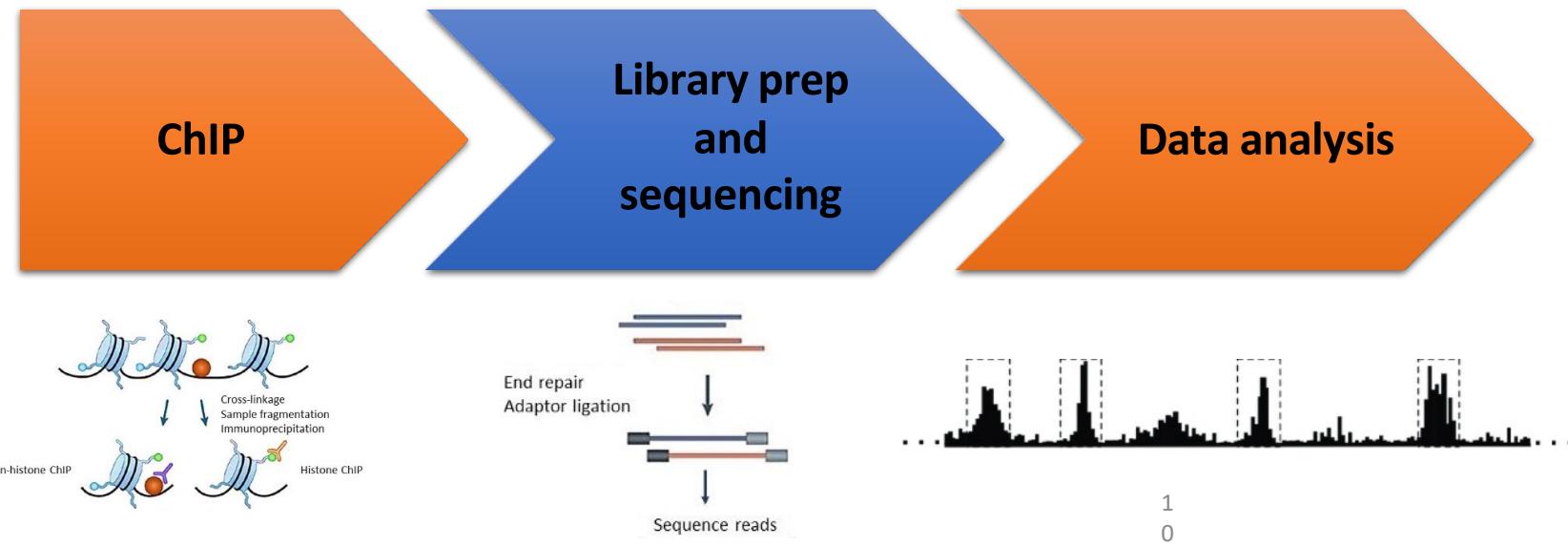
- ChIP is used to isolate DNA fragments bound by proteins or modified histones of interest using an antibody specifically design to target them
- It requires a high number of cells (1-10 million cells)
- It requires highly tested and verified antibody working specifically for ChIP



9

Furey, 2012

Library prep and sequencing

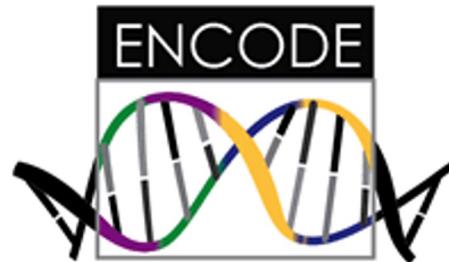




Experimental design

ENCODE

- The Encyclopedia of DNA Elements (ENCODE) Consortium has carried out thousands of ChIP-seq experiments and has used this experience to develop a set of working standards and guidelines

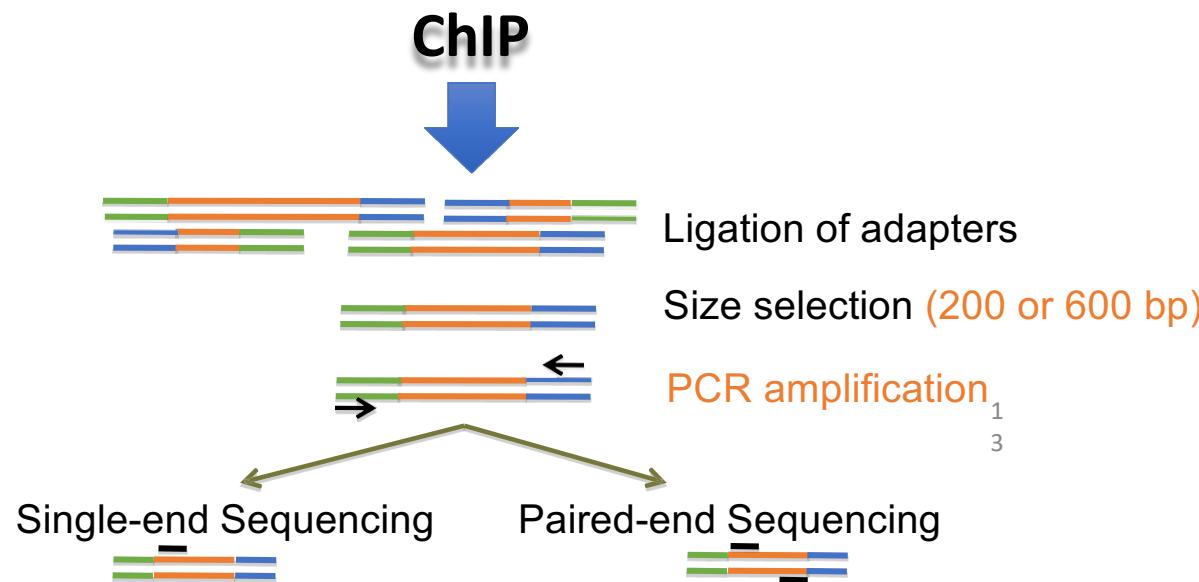


Landt SG, Marinov GK, Kundaje A *et al.* (2012) ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. *Genome research* **22**, 1813–1831.

See: <https://www.encodeproject.org/about/experiment-guidelines/>

Library prep

- Starting material: ChIP sample (1-10ng of sheared DNA)

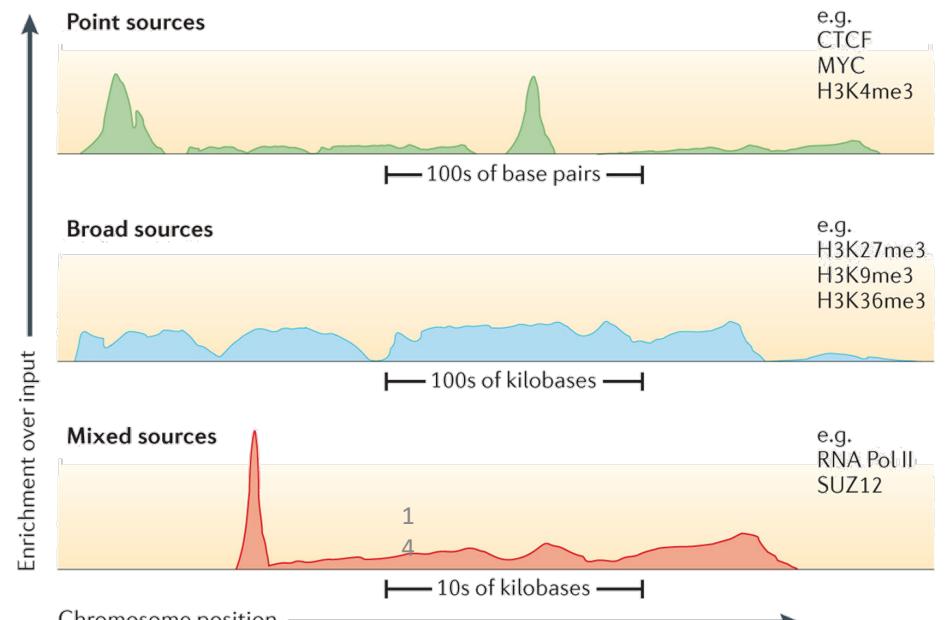


Sequencing

- Paired-end or single-end
- Short reads (50nt)
- Number of reads needed depends on:
 - chipped protein,
 - type of expected profile,
 - number of expected binding sites,
 - size of the genome of interest.

Ex:

- For human genomes, 20 million uniquely mapped read sequences are suggested for point-source peaks, or 40 million for broad-source peaks.
- For fly genome: 8 million reads
- For worm genome: 10 million reads



Park, 2009

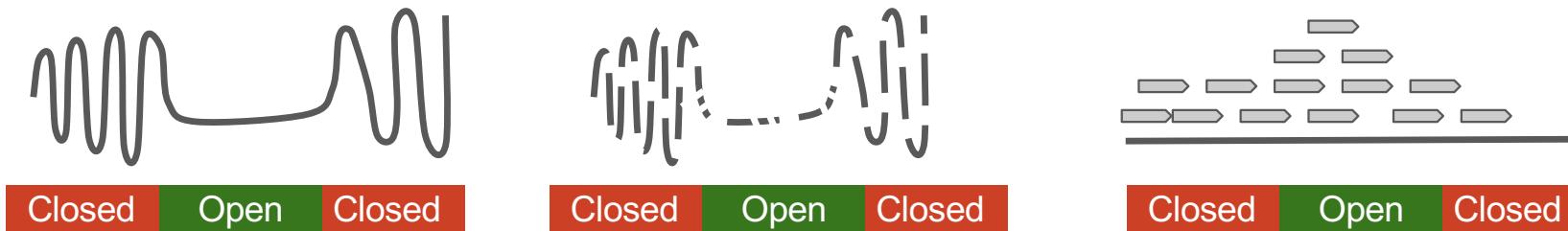
Nature Reviews | Genetics

Controls

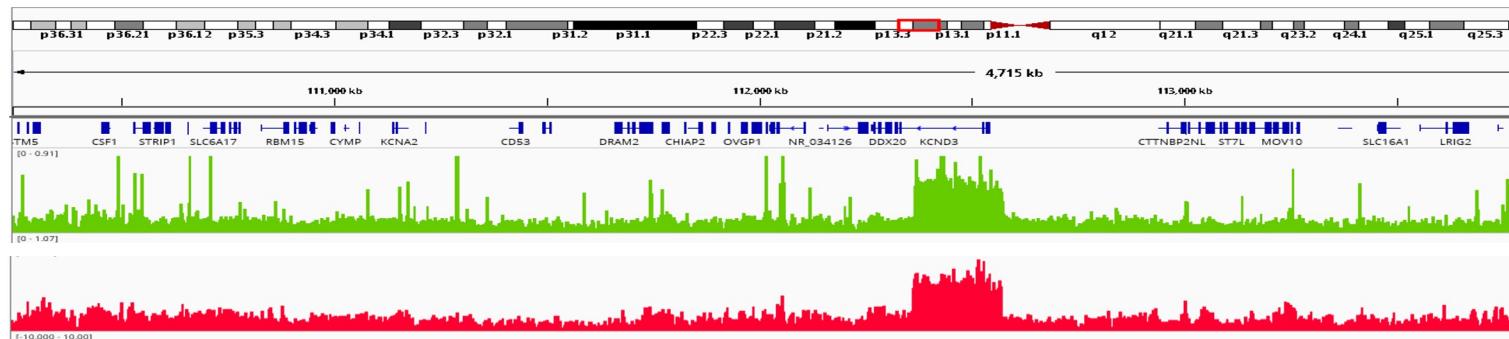
- Used mostly to filter out false positives (high level of noise)
 - Idea: potential false positive will be enriched in both treatment and control.
- A control will fail to filter out false positives if its enrichment profile is very different from the enrichment profile of false positive regions in the treatment sample
- Most commonly used control: Input DNA (a portion of DNA sample removed prior to IP)
- Choice of control is extremely important
- It is recommended to cover the control in a higher extent than the IPs

Why an Input is required ?

- The input is used to model **local noise level**
 - Accessible regions are expected to produce **more reads**

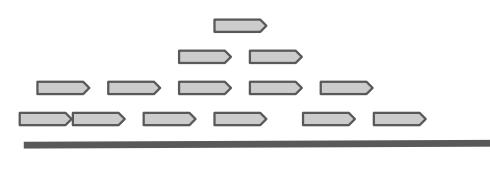
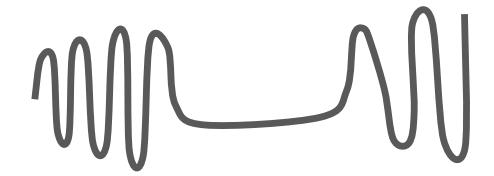


- Amplified regions (CNV) are expected to produce **more reads**



Why an Input is required ?

- The input is used to model **local noise level**
 - Accessible regions are expected to produce **more reads**



- Amplified regions (CNV) are expected to produce **more reads**
- Moreover, most peak callers are configured with an **input as control**

Other controls

- IgG (mock IP): controls for non-specific IP enrichment
 - Problem : low-complexity library (few reads)
- Histone H3 (for H3 variants)
- Uninduced condition (for inducible TFs)
 - Example : Glucocorticoid Récepteur
 - Induced by Dexamethasone (Dex)
 - Control vehicle = Ethanol (EthOH)
- KO of your protein of interest
- Non flagged cell lines
- ...

Replicates

- A minimum of **two** replicates should be carried out per experiment.
- Each replicate should be a **biological** rather than a technical replicate; that is, it results from an independent cell culture, embryo pool or tissue sample.

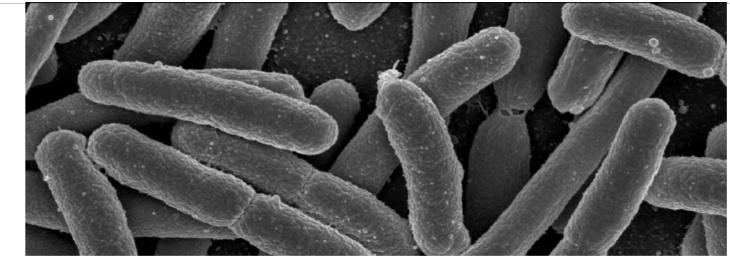


Data analysed in this course

Dataset used

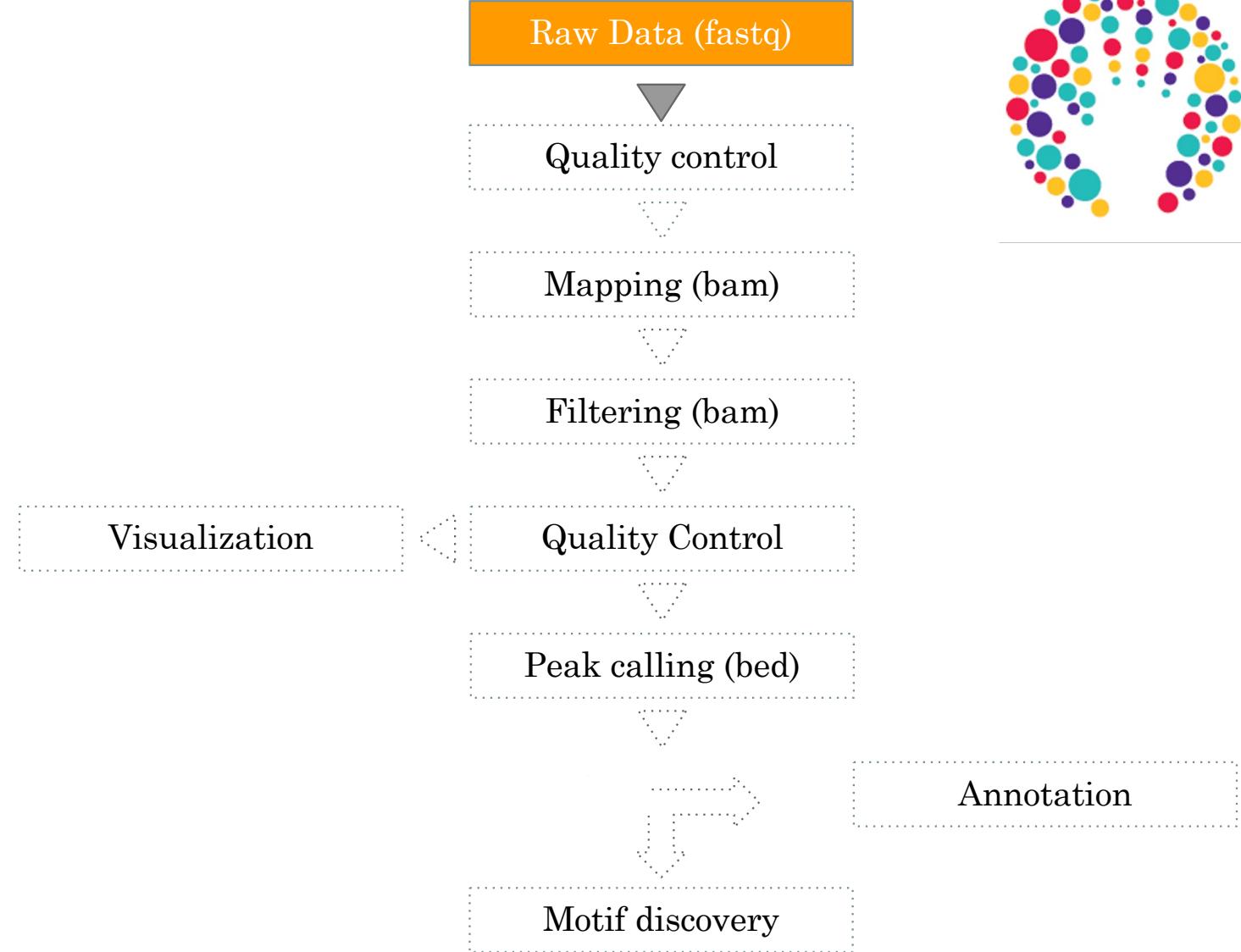
Genome-scale Analysis of *Escherichia coli* FNR Reveals Complex Features of Transcription Factor Binding

Kevin S. Myers^{1,2}, Huihuang Yan^{3✉a}, Irene M. Ong³, Dongjun Chung^{4✉b}, Kun Liang^{4,5✉c}, Frances Tran^{6✉d}, Sündüz Keleş^{4,5}, Robert Landick^{3,6,7*}, Patricia J. Kiley^{2,3*}



- All experiments (GEO): GSE41187
- Experiment: FNR IP ChIP-seq Anaerobic A (SRX189773 - SRR576933)
- Control: anaerobic INPUT DNA (SRX189778 - SRR576938)

Protocol

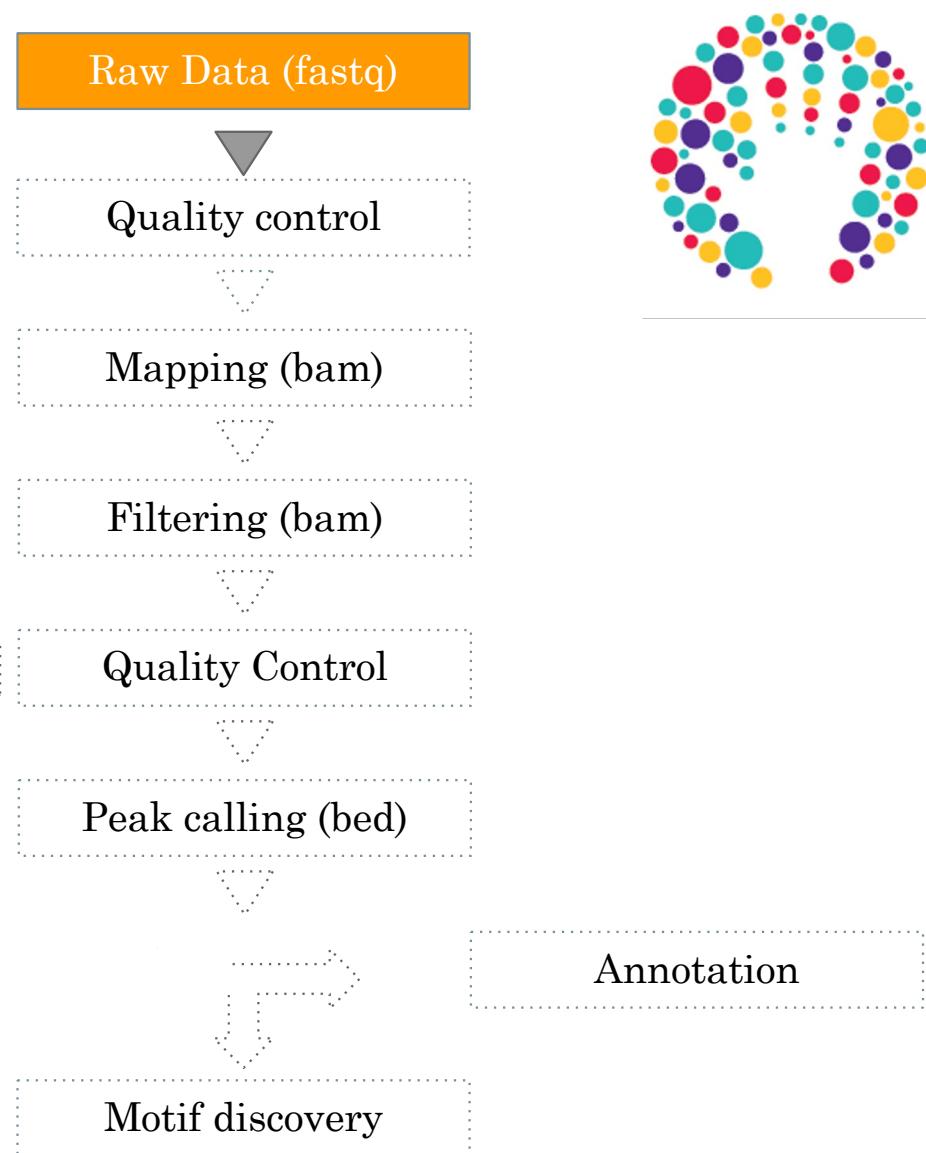


TIPS

- **Keep track of all command lines you run.** You can for example, create a text file in which you write every commands you run.
- **Give content-explicit names** to the files you're generating.
- **Give to files the right extension.**
- **Create directories with explicit names!!**
- **Compress big files** (with gzip for instance).

Protocol

- [Downloading ChIP-seq reads from NCBI](#)



Protocol



- Connect to the server and set up your environment



Quality control of the reads

Quality control of the reads

- As for any NGS datasets
- FastQC program (c.f. Course “preprocessing” Denis Puthier Monday afternoon)

Protocol

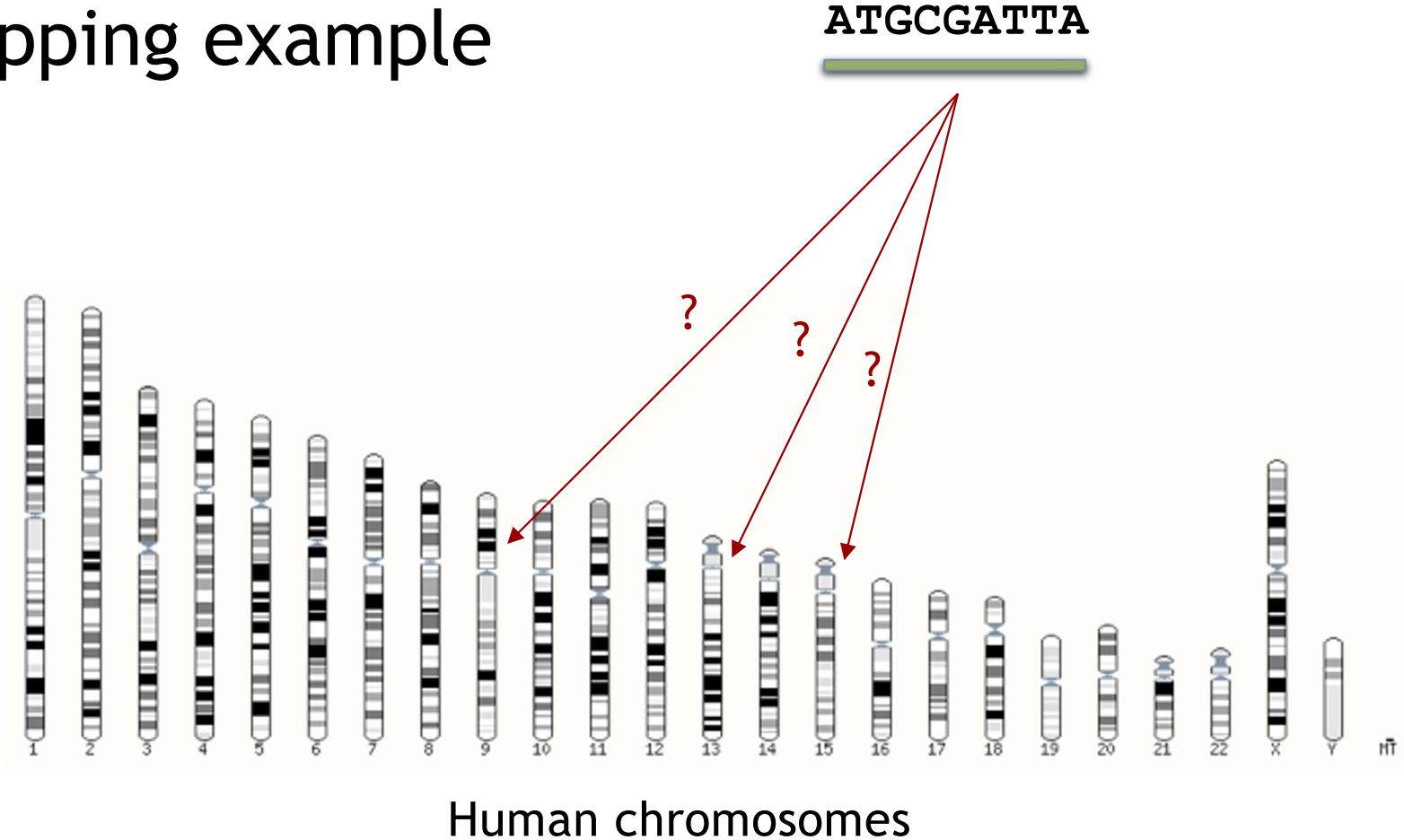
- Quality control of the reads and statistics



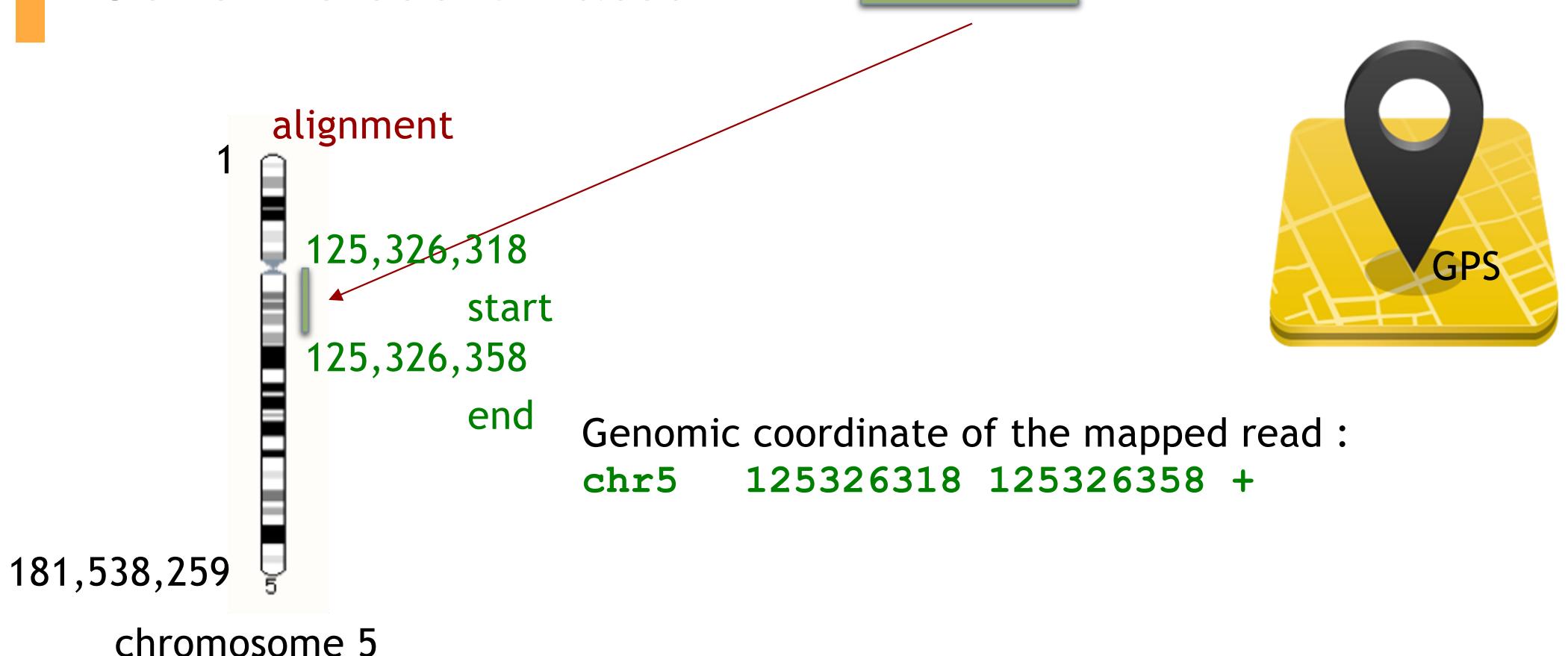


Mapping

Mapping example



Genomic coordinates



Mapping

- Find out the position of the reads within the reference genome

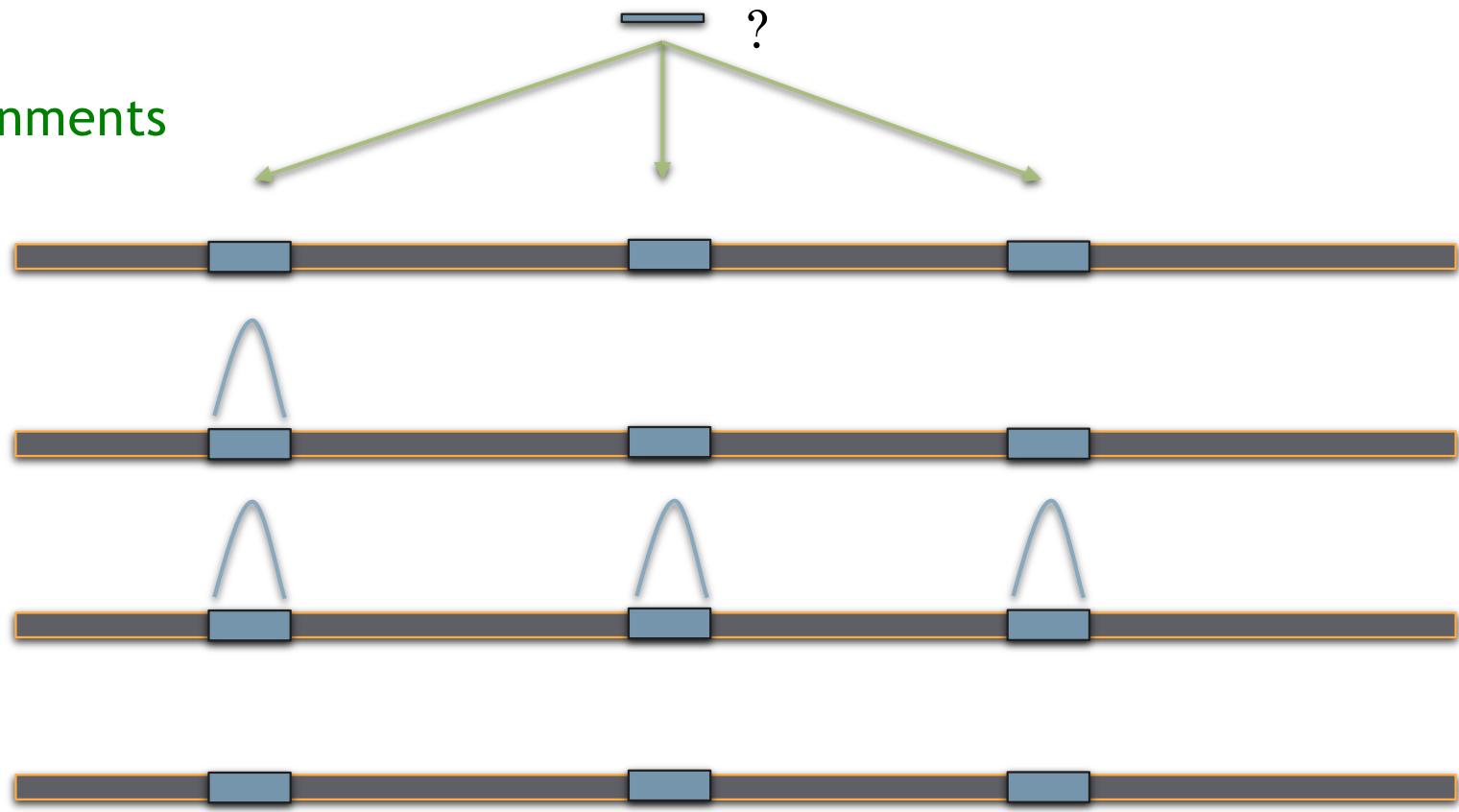


Mapping tool used: Bowtie

- (c.f. Course “mapping” Denis Puthier Monday afternoon)
- Designed to align reads if:
 - many of the reads have at least one good, valid alignment,
 - many of the reads are relatively **high-quality**
 - the number of alignments reported per read is small (close to 1)
- Langmead B. et al, Genome Biology 2009
- Langmead B (2010) Aligning short sequencing reads with Bowtie. Curr Protoc Bioinformatics Chapter 11: Unit 11 17

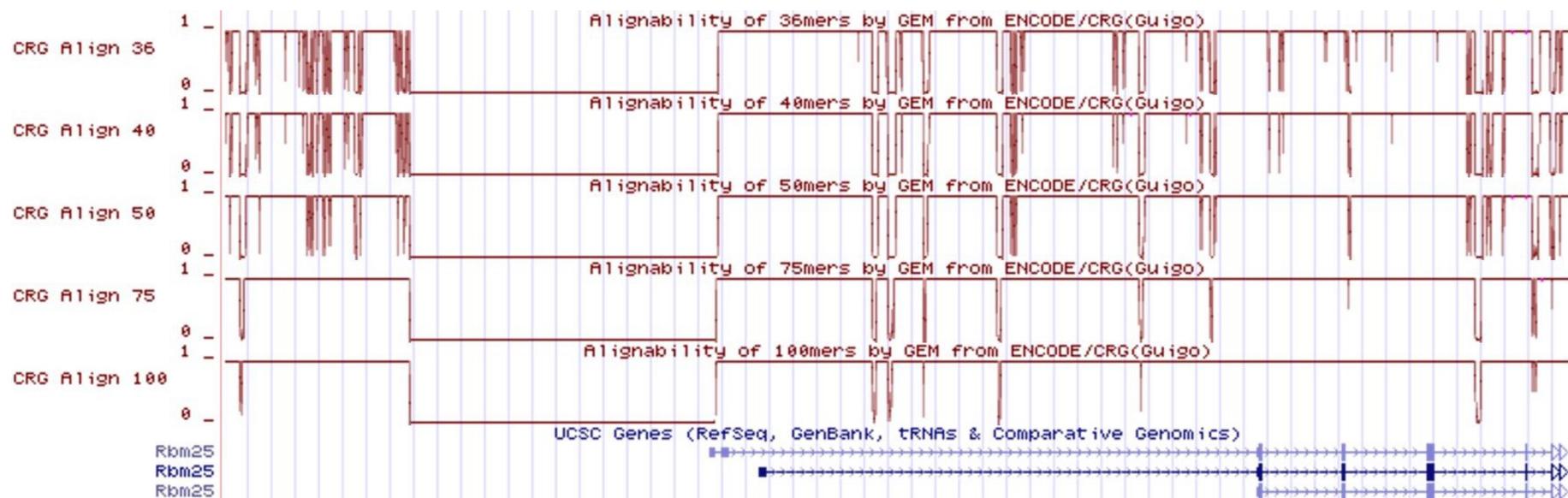
Duplicated genomic regions

3 possible alignments



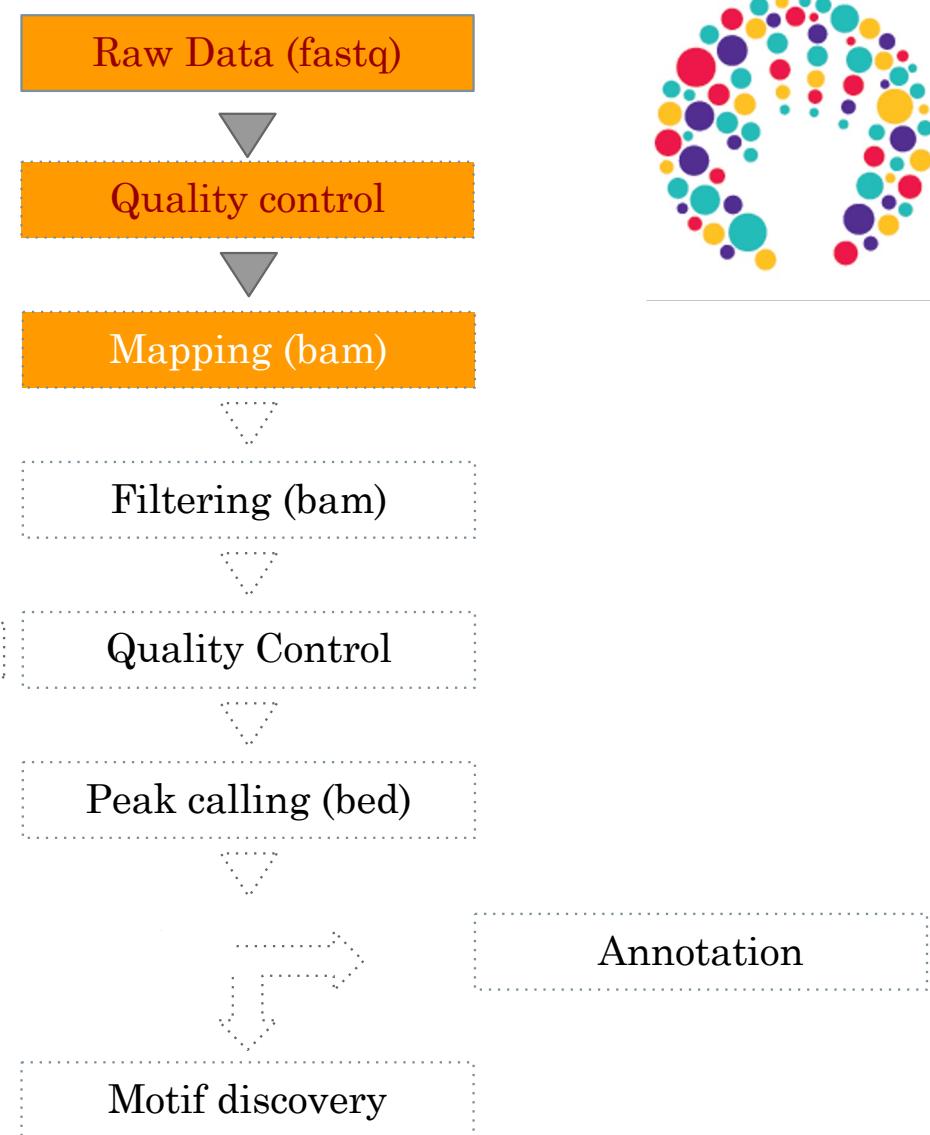
Mappability

- Mappability (a): how many times a read of a given length can align at a given position in the genome
 - $a=1$ (read align once)
 - $a=1/n$ (read align n times)



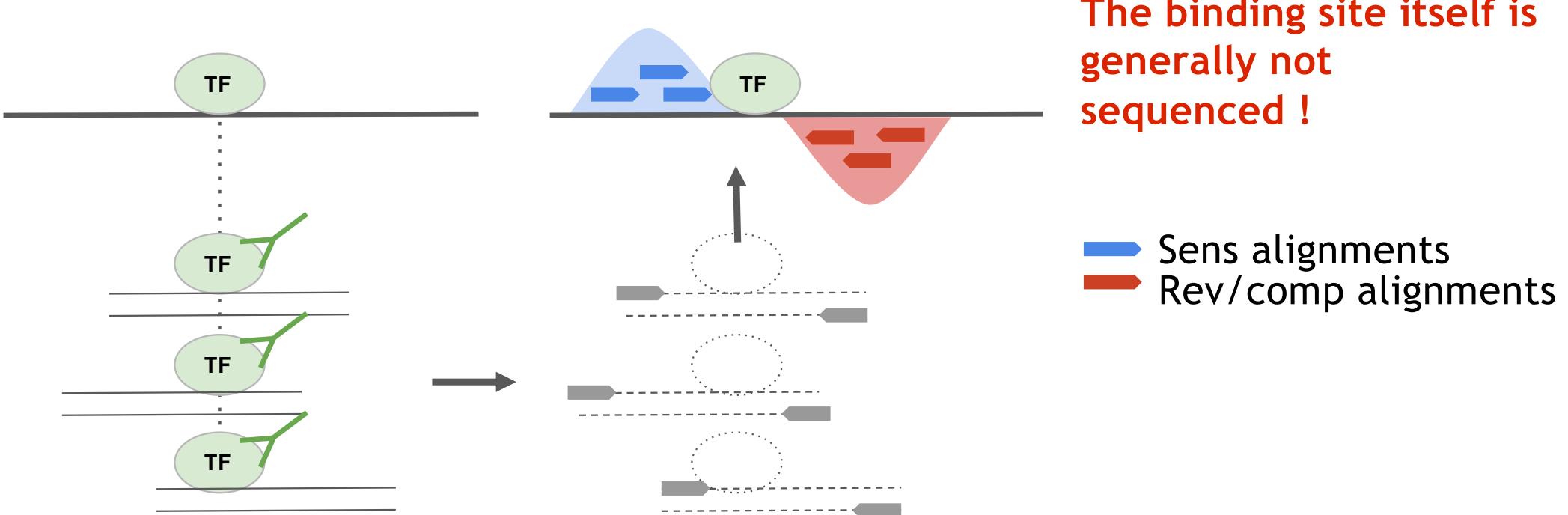
Protocol

- Mapping the reads with Bowtie



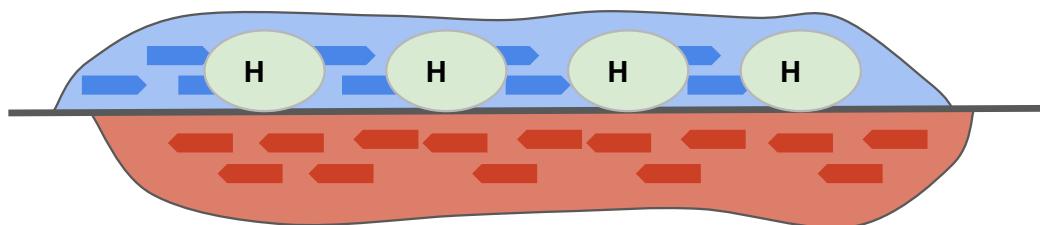
Mapping: expected signal

- For a transcription factor signal is expected to be sharp

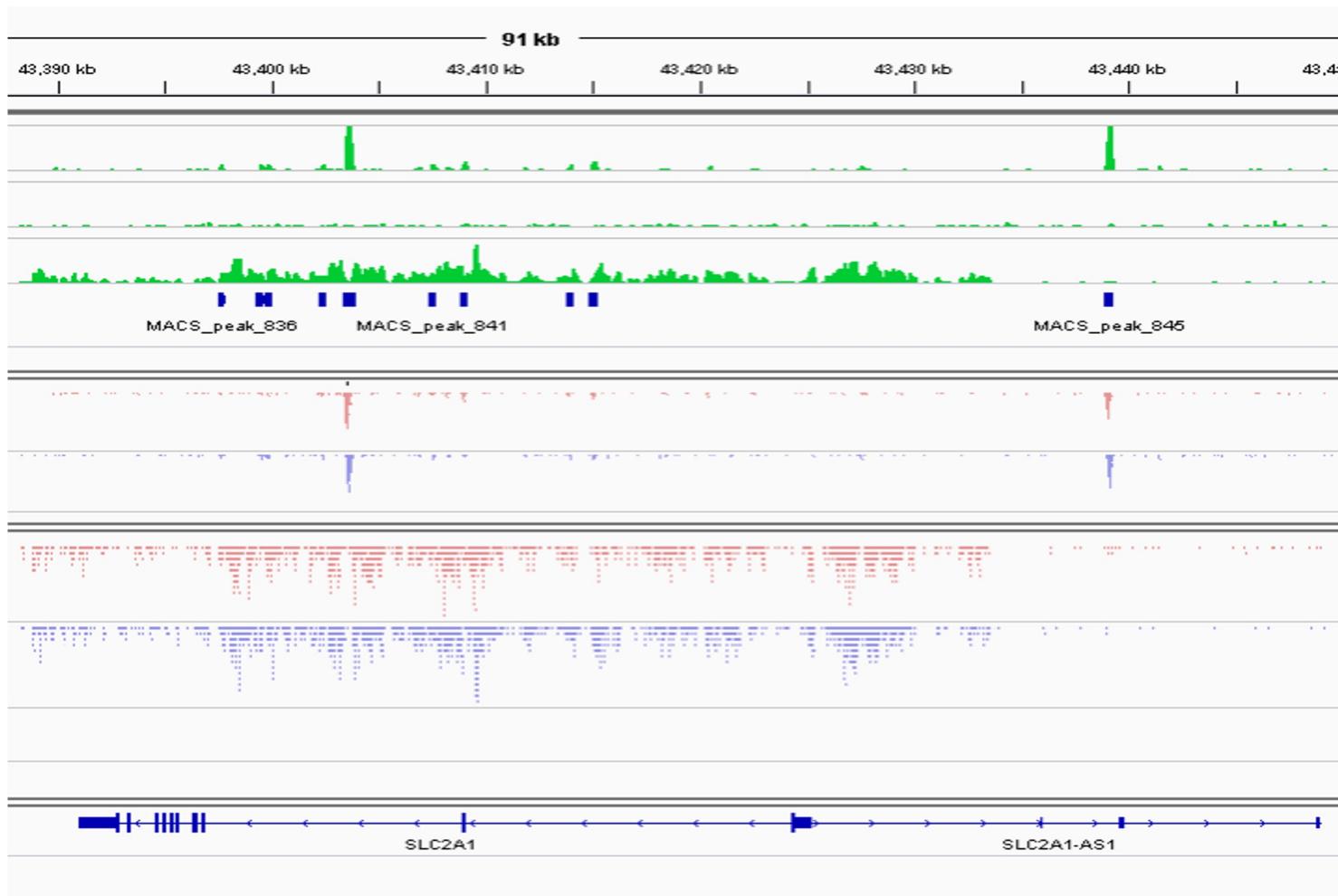


Mapping: the expected signal

- For most **histone marks** the signal is expected to be **broad**
- Asymmetry is less/not pronounced
- Peak calling algorithms need to adapt to these various signals



Mapping: observed signal



Trans. Factor
(ESR1)

Histone mark
(H3K4me1)



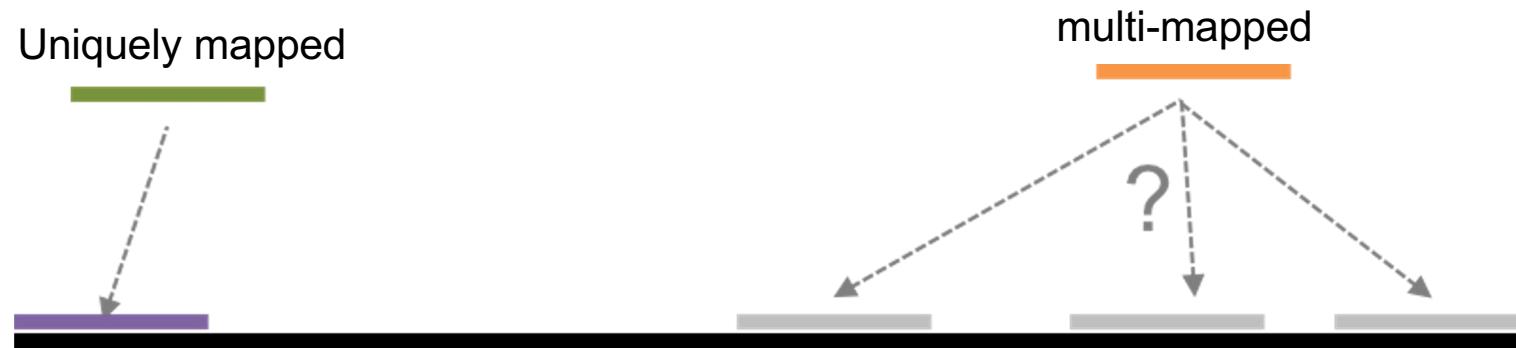
Filtering mapped reads

Which reads to filter ?

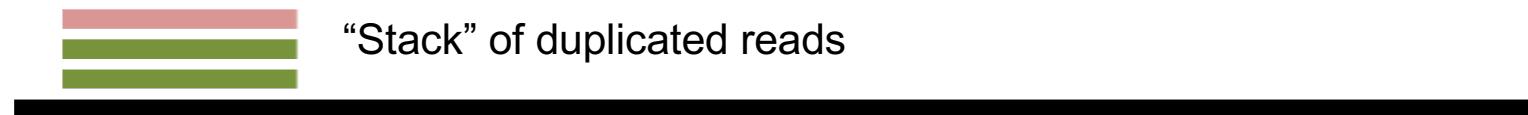
- Low-quality read alignments
 - Tool : samtools
- Multi-mapped reads (unless removed during the mapping step)
 - Tool : samtools
- Duplicated reads (PCR duplicates)
 - Tool : Picard MarkDuplicates

Source of confusion

uniquely mapped reads = reads that “matches” only 1 region in the genome



duplicated reads = reads that “match” at the SAME location (same start, strand)

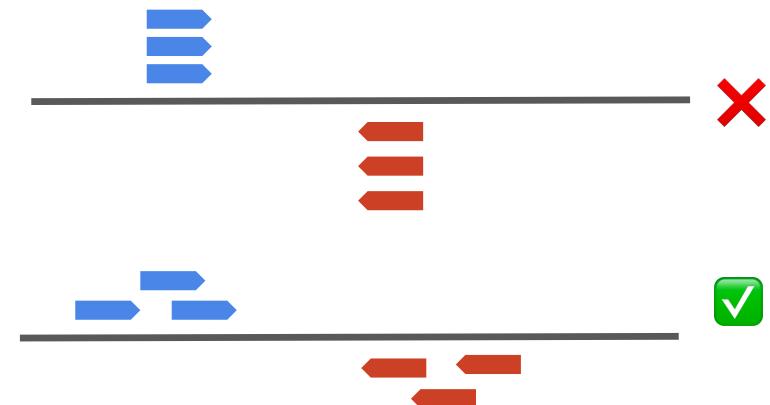


PCR duplicates

- PCR duplicates
 - Related to poor library complexity
 - The same set of fragments are amplified, may indicates that immuno-precipitation failed
 - Tools to check for
 - FastQC report (duplicate diagram)
 - PCR bottleneck metric (ENCODE)

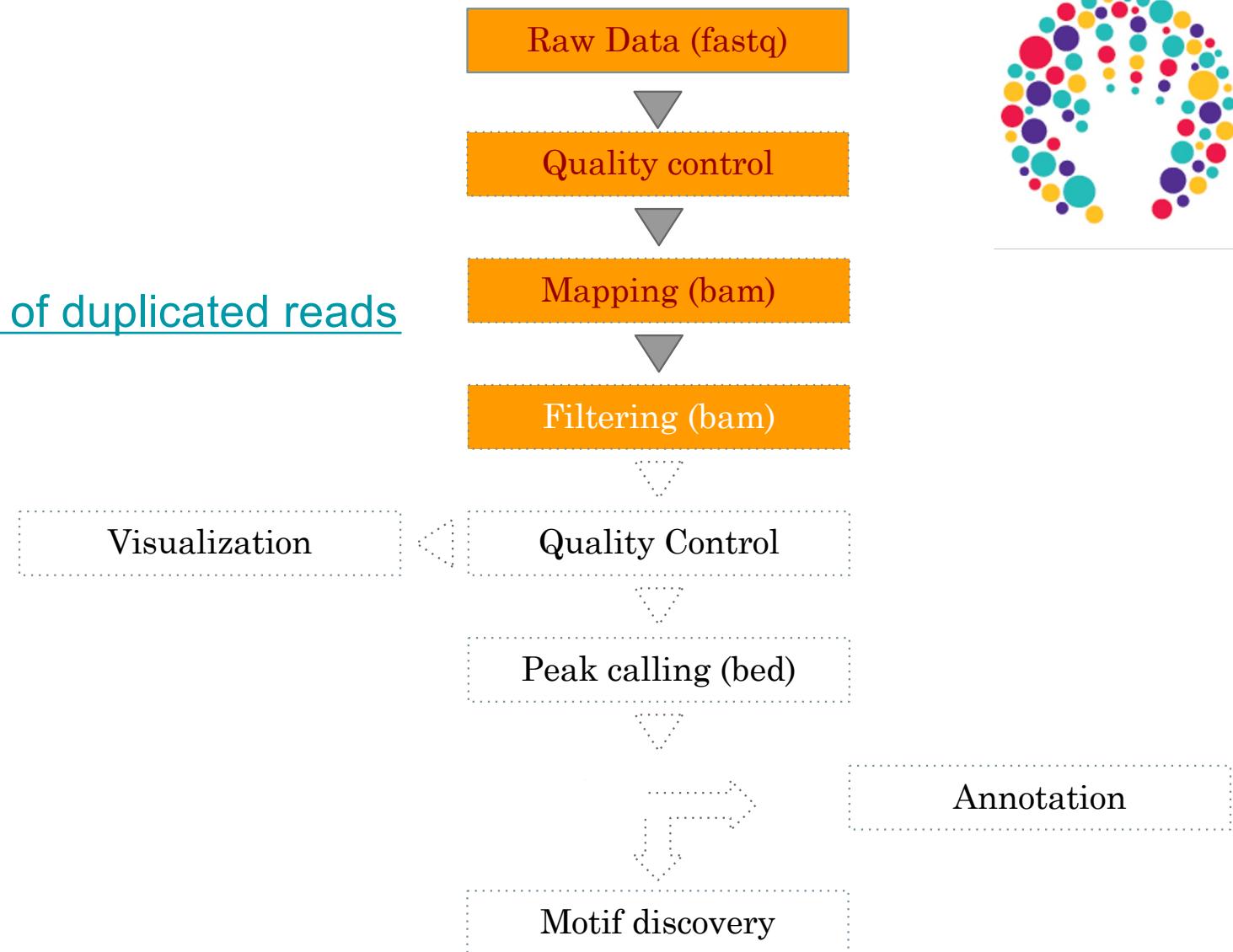
QC : PBC (PCR Bottleneck Coefficient)

- An approximate measure of library complexity
- $PBC = N_1/N_d$
 - N_1 = Genomic position with 1 read aligned
 - N_d = Genomic position with ≥ 1 read aligned
- Value :
 - 0-0.5: severe bottlenecking
 - 0.5-0.8: moderate bottlenecking
 - 0.8-0.9: mild bottlenecking
 - 0.9-1.0: no bottlenecking



Protocol

- Estimating the number of duplicated reads





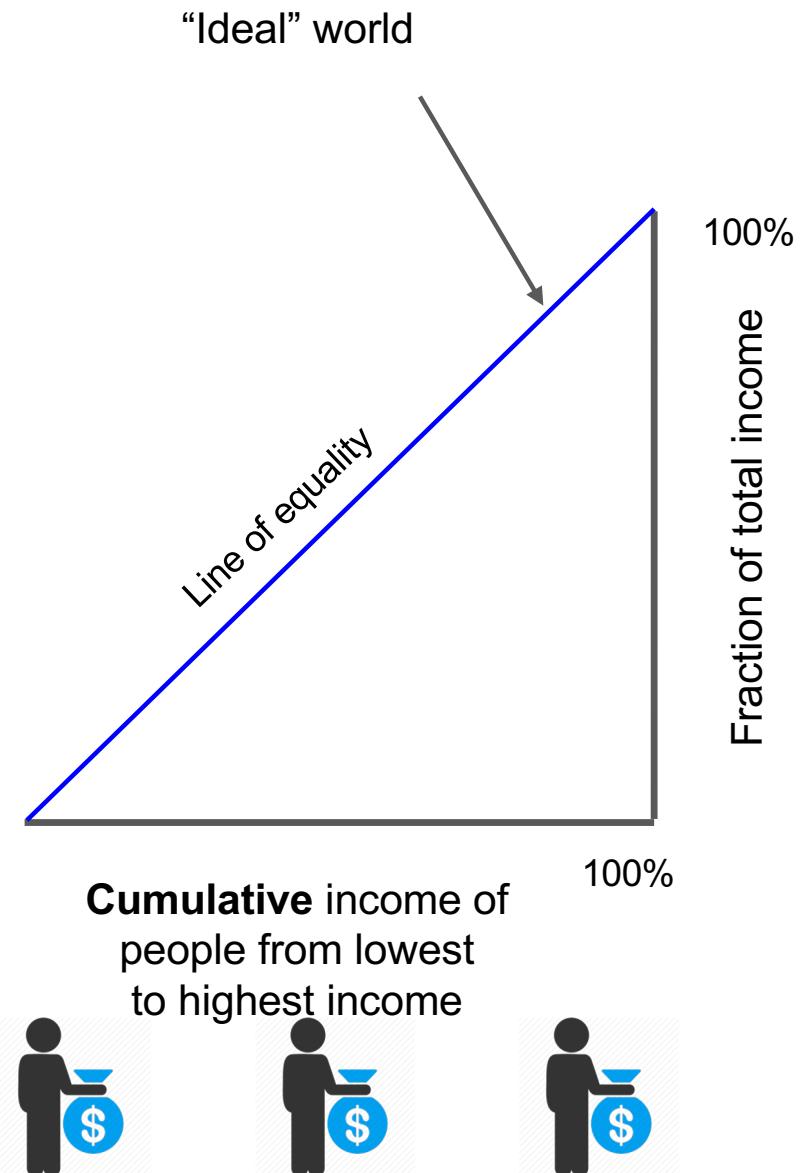
Quality Control on mapped reads

Assessing ChIP quality

- Guidelines from ENCODE
- Various metrics
 - Check **duplicate** rate (see previous Filtering section)
 - Use a **Lorenz Curve** (implemented in **Deeptools fingerprint**)
 - Look at **strand cross-correlation** (implemented in **SPP BioC package** and **phantompeakqualtools**)
 - Fraction of reads in peaks (**FRiP**, as proposed by ENCODE), but requires to find peaks.

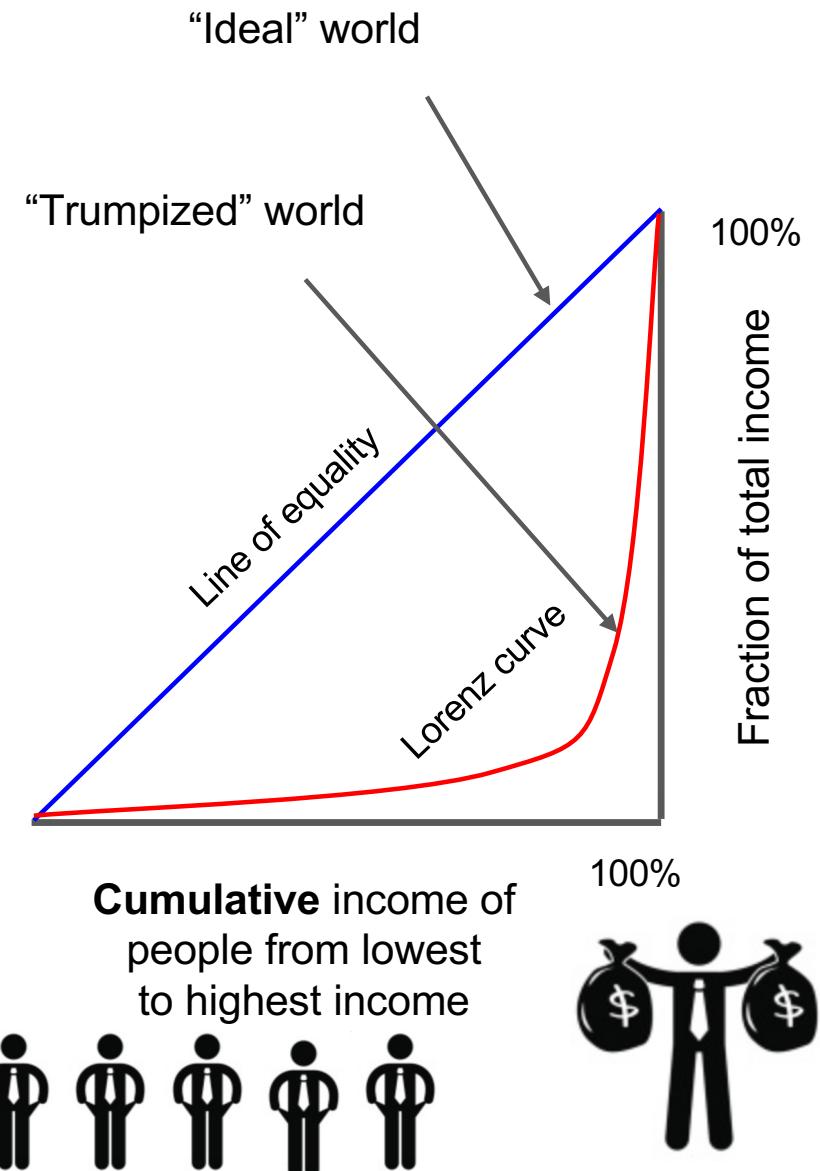
Lorenz curve

- Analyze income among workers by computing cumulative sum.
 - If uniform income distribution :
 - Straight line



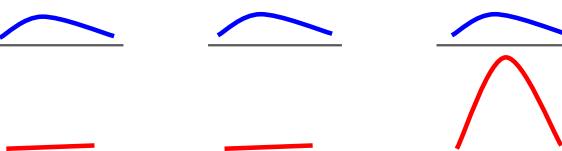
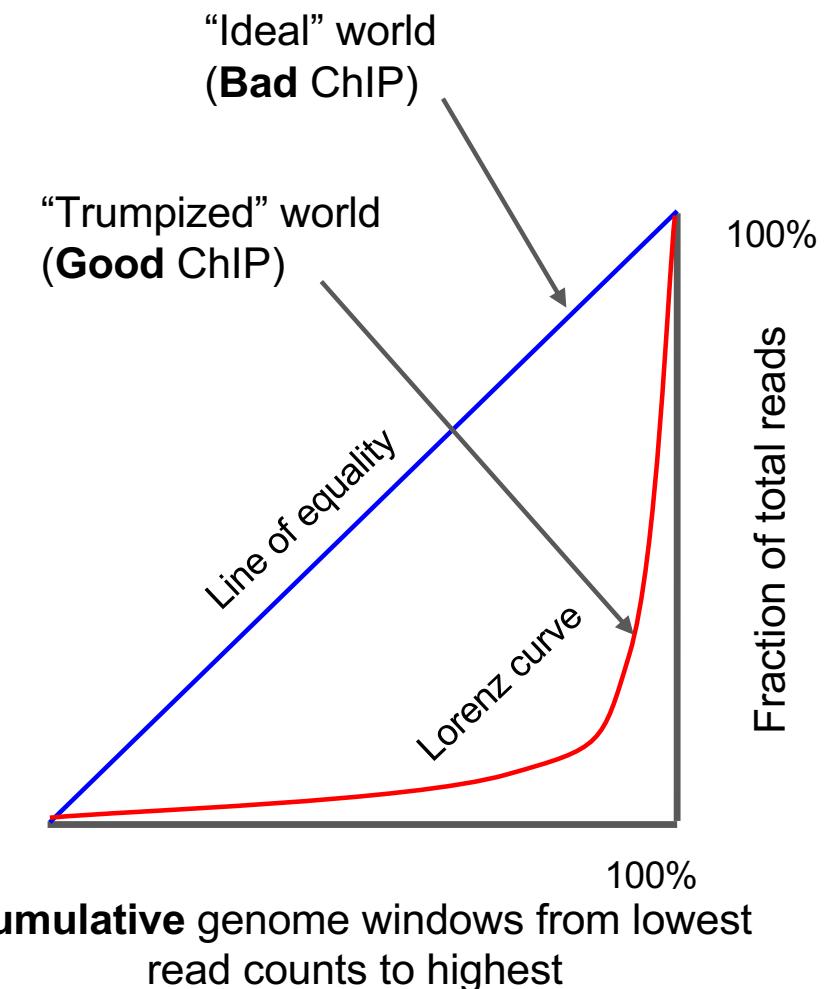
Lorenz curve

- Analyze income among workers by computing cumulative sum.
 - If uniform income distribution :
 - Straight line
 - If they were trumpized
 - Lorenz curve



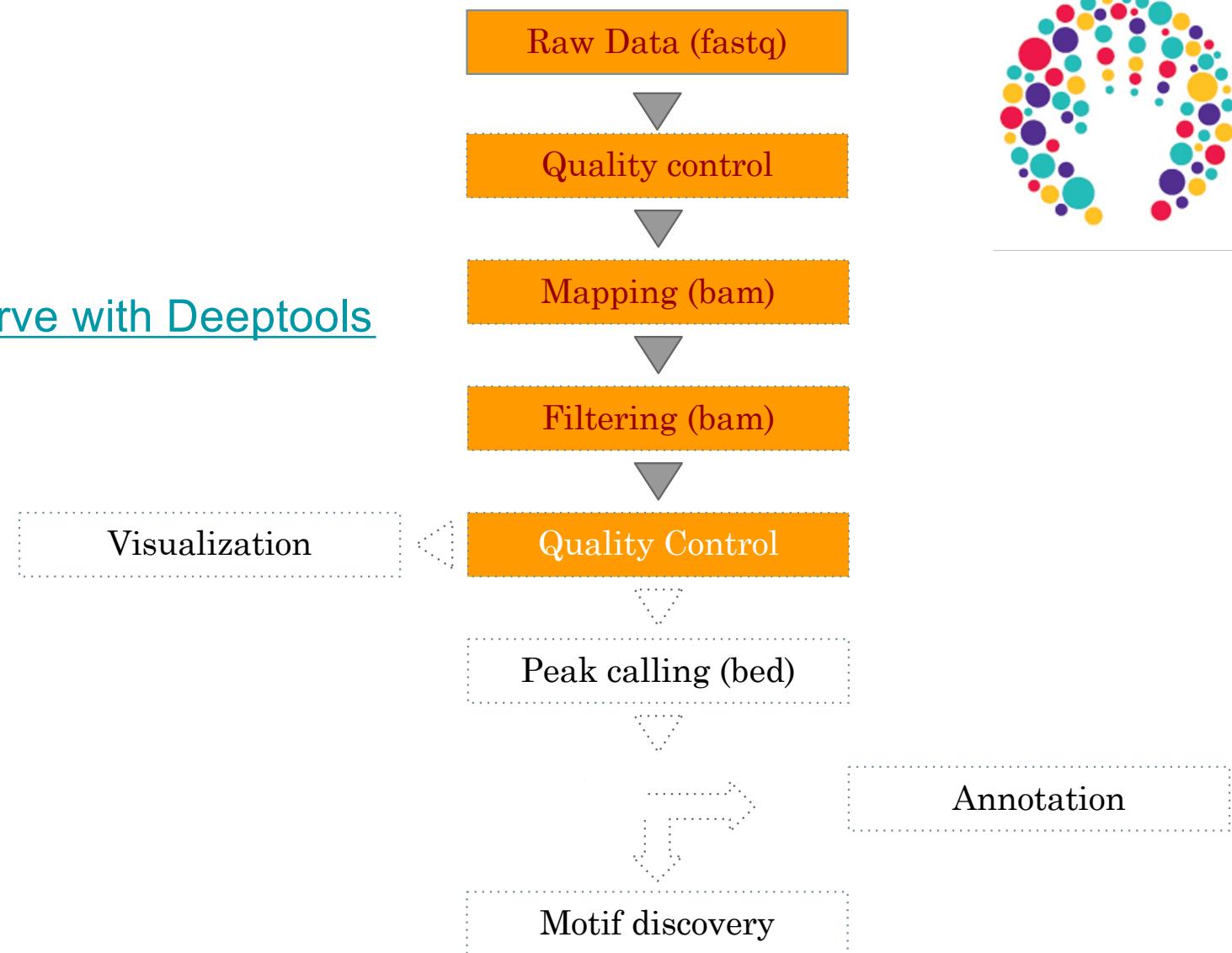
Lorenz curve

- Analyze income among workers by computing cumulative sum.
 - If uniform income distribution :
 - Straight line
 - If they were trumpized
 - Lorenz curve
- Here the workers are the genome windows and incomes are reads



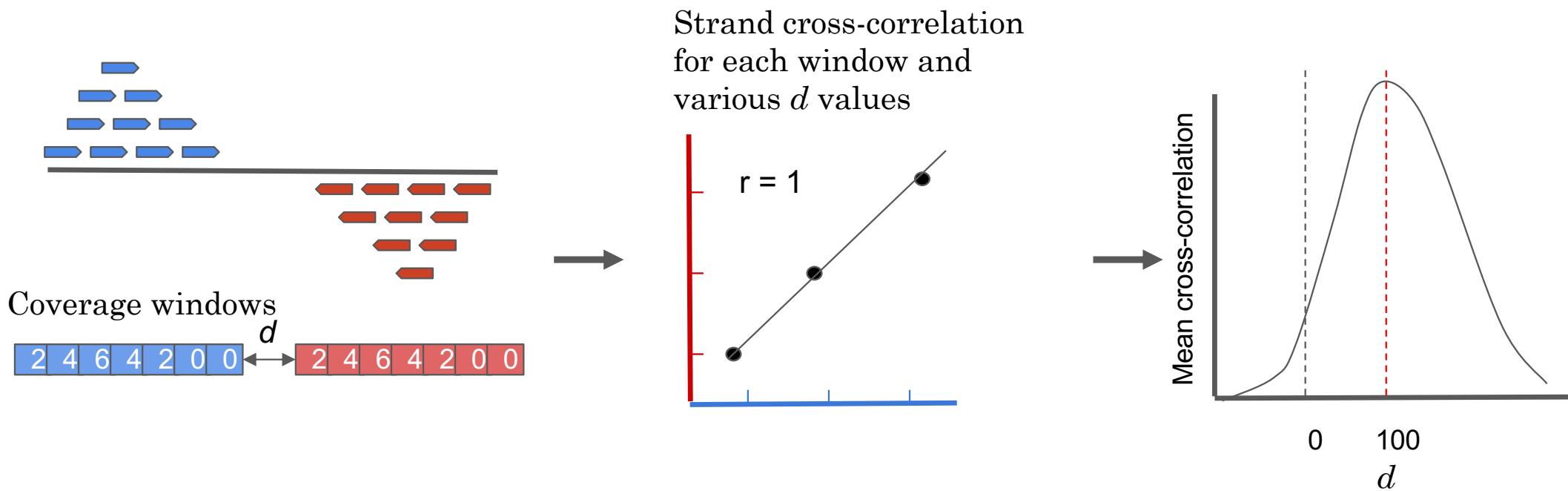
Protocol

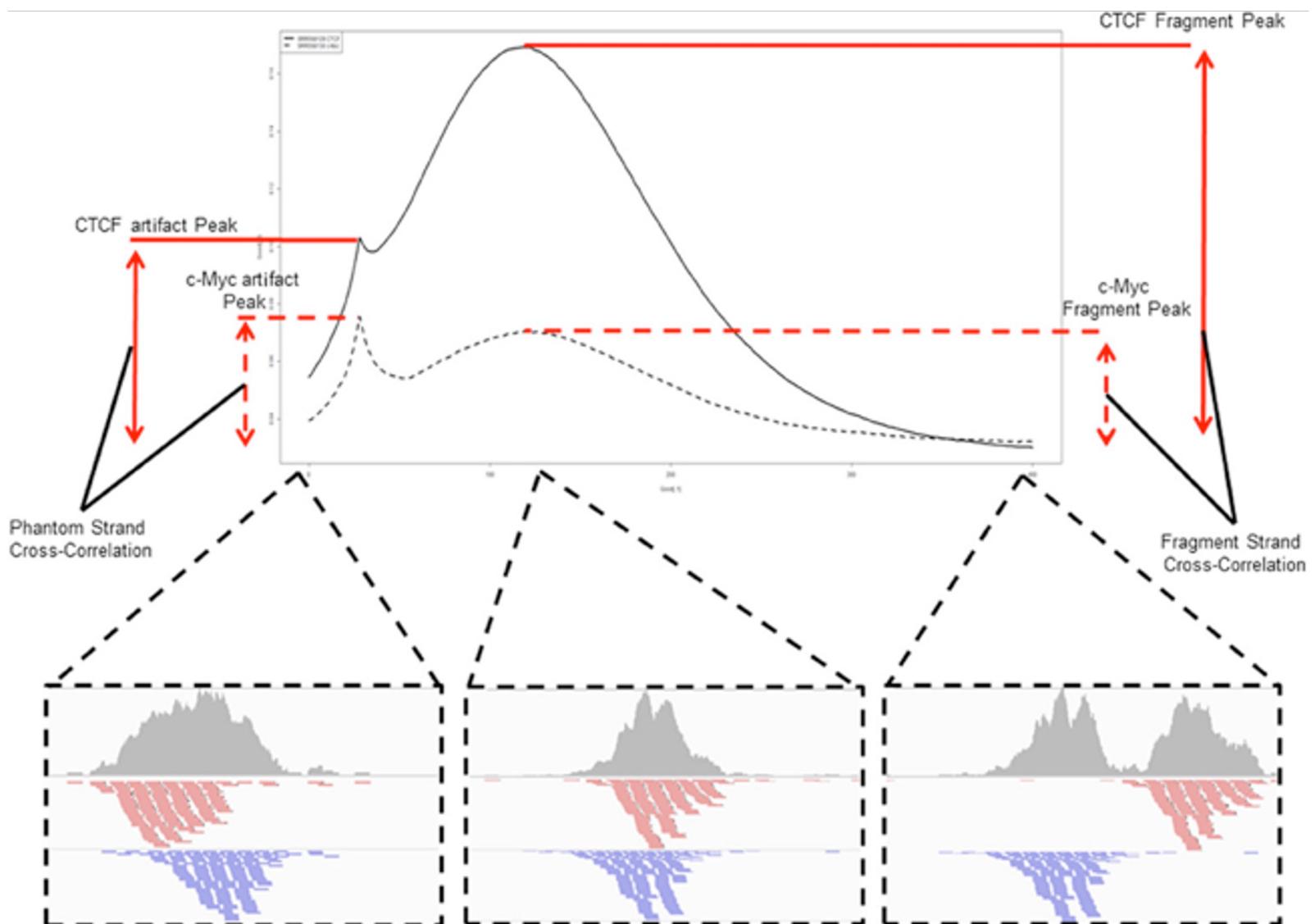
- Plot the Lorenz curve with DeepTools



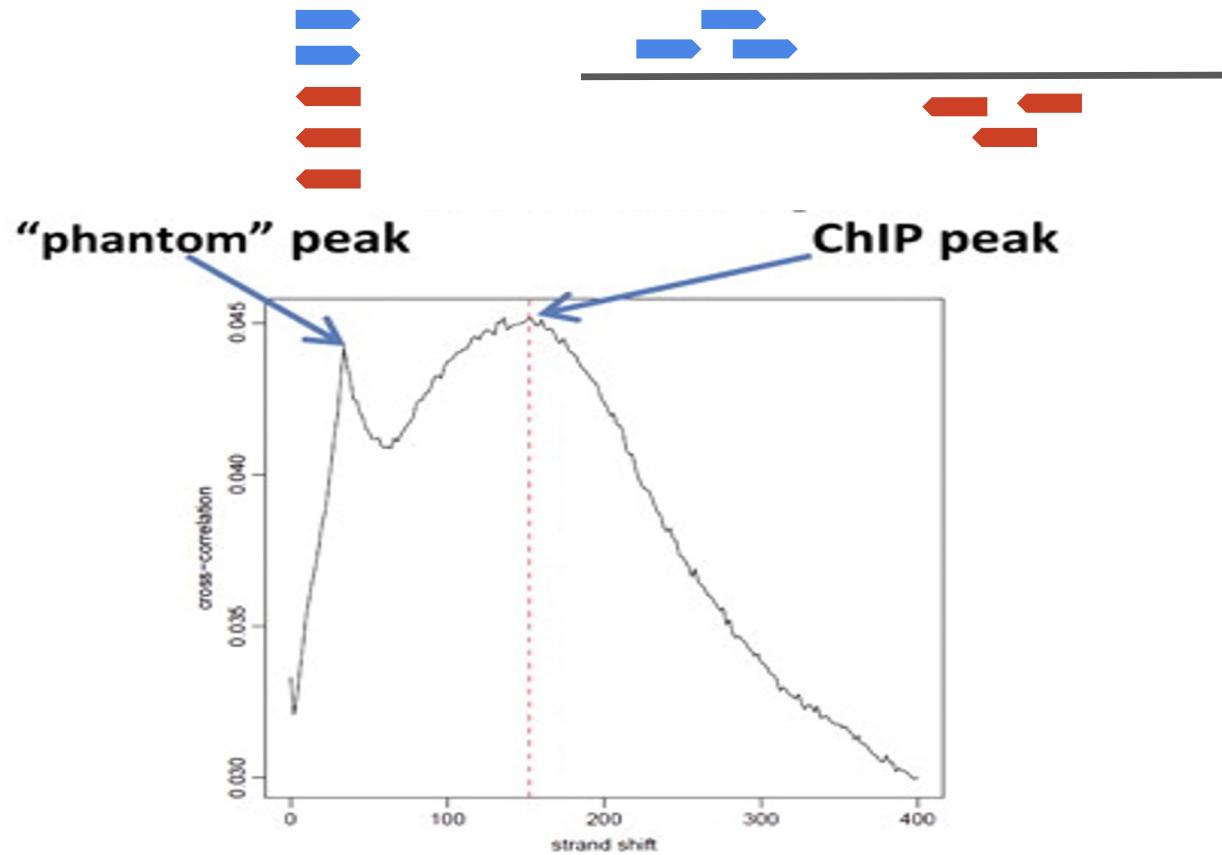
Strand cross-correlation

- Compute strand cross correlation for each window w across the genome.
- Use various distance d and compute the mean cross-correlation observed

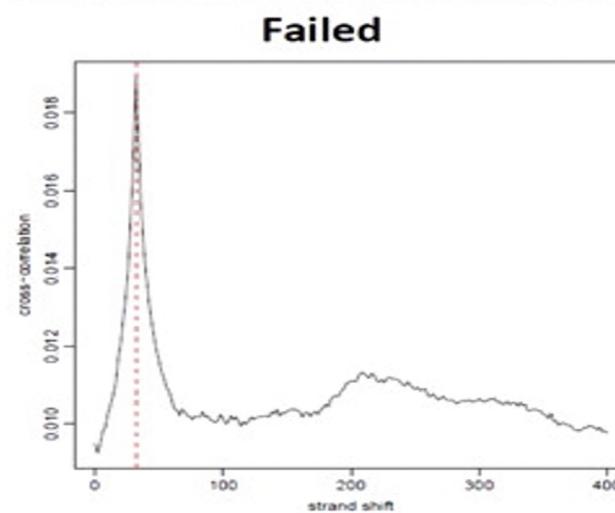
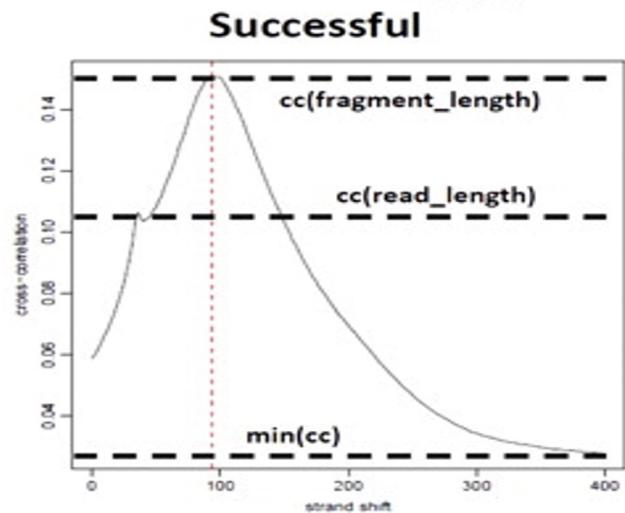




Strand cross-correlation



Strand cross-correlation



NSC: normalized strand coefficient

$$NSC = \frac{cc(fragment\ length)}{\min(cc)}$$

$NSC \geq 1.05$ is recommended

Relative strand correlation (RSC)

$$RSC = \frac{cc(fragment\ length) - \min(cc)}{cc(read\ length) - \min(cc)}$$

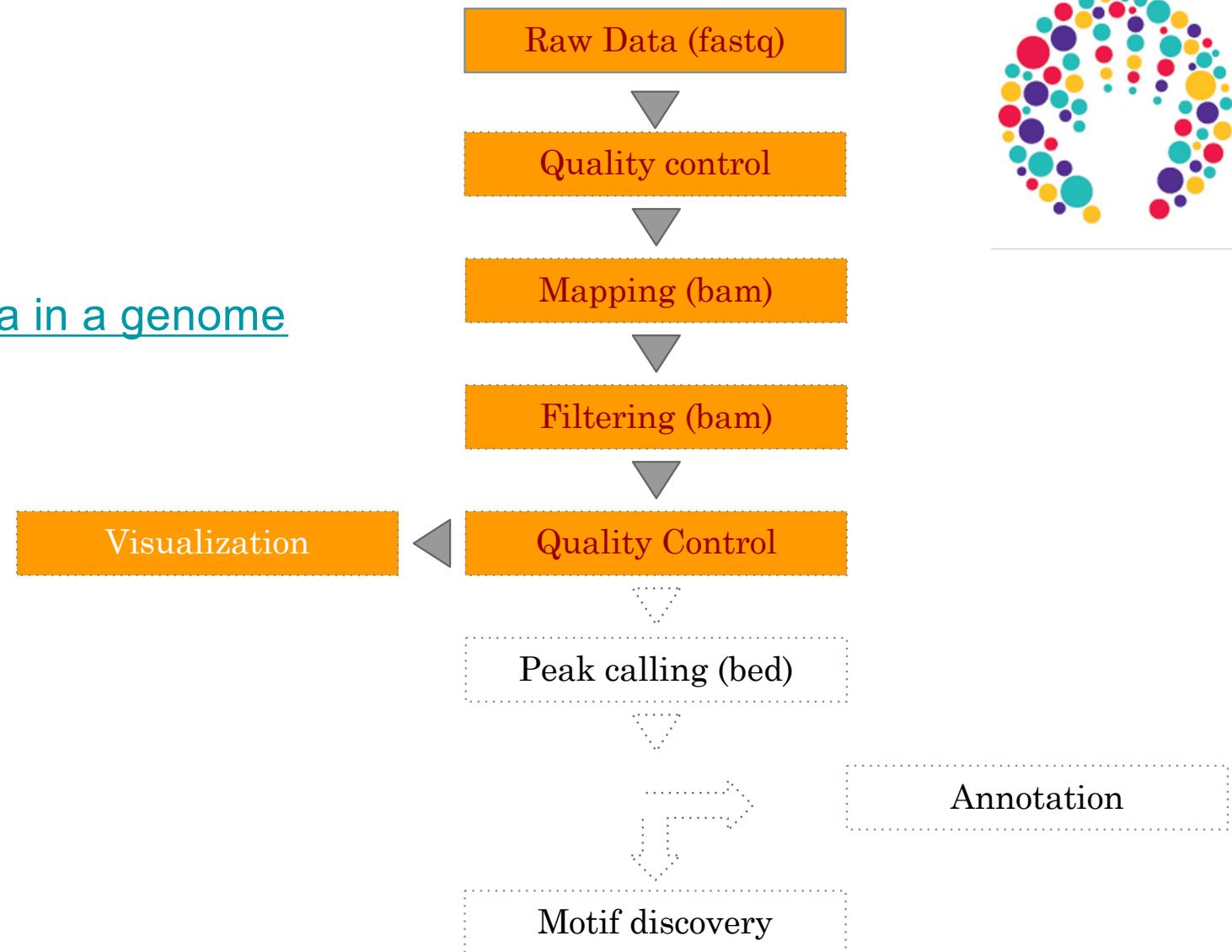
$RSC \geq 0.8$ is recommended



Visualization: computing a genomic coverage file

Protocol

- Visualizing the data in a genome browser

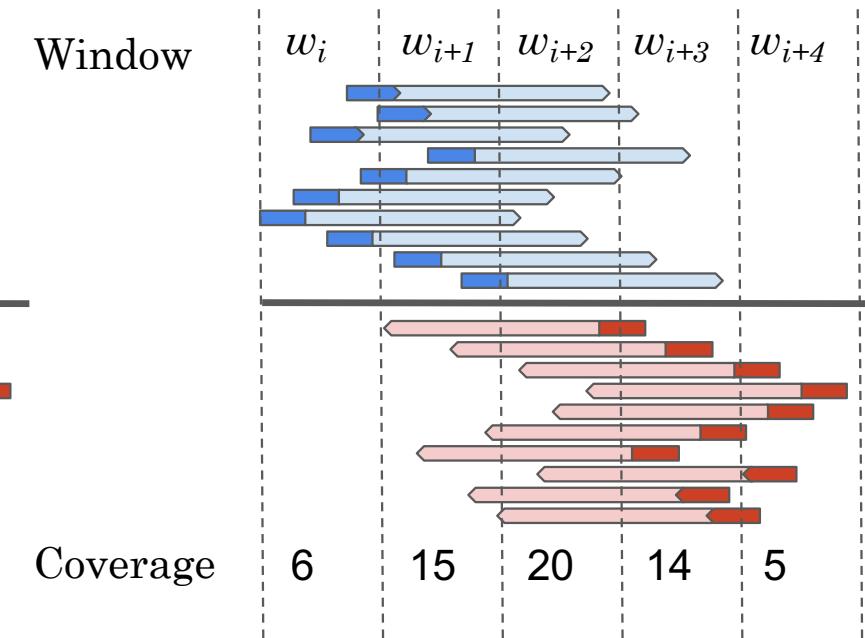
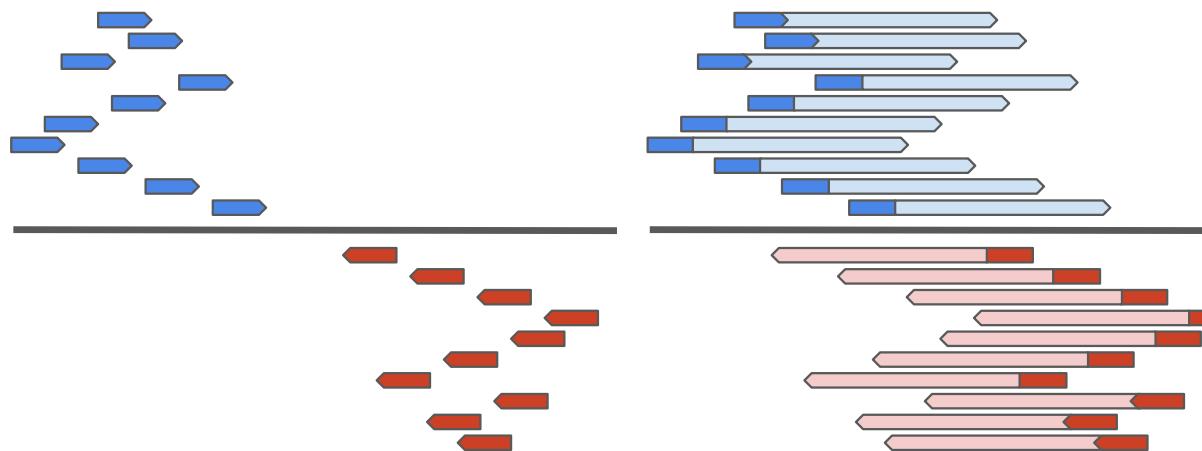


Bam files are fat

- BAM files are fat as they do contain exhaustive information about read alignments
 - Memory issues (can only visualize fraction of the BAM)
- Need a more **lightweight** file format containing **only genomic coverage** information:
 - **✗ Wig** (not compressed, not indexed)
 - **✓ TDF** (compressed, indexed)
 - **✓ BigWig** (compressed, indexed)

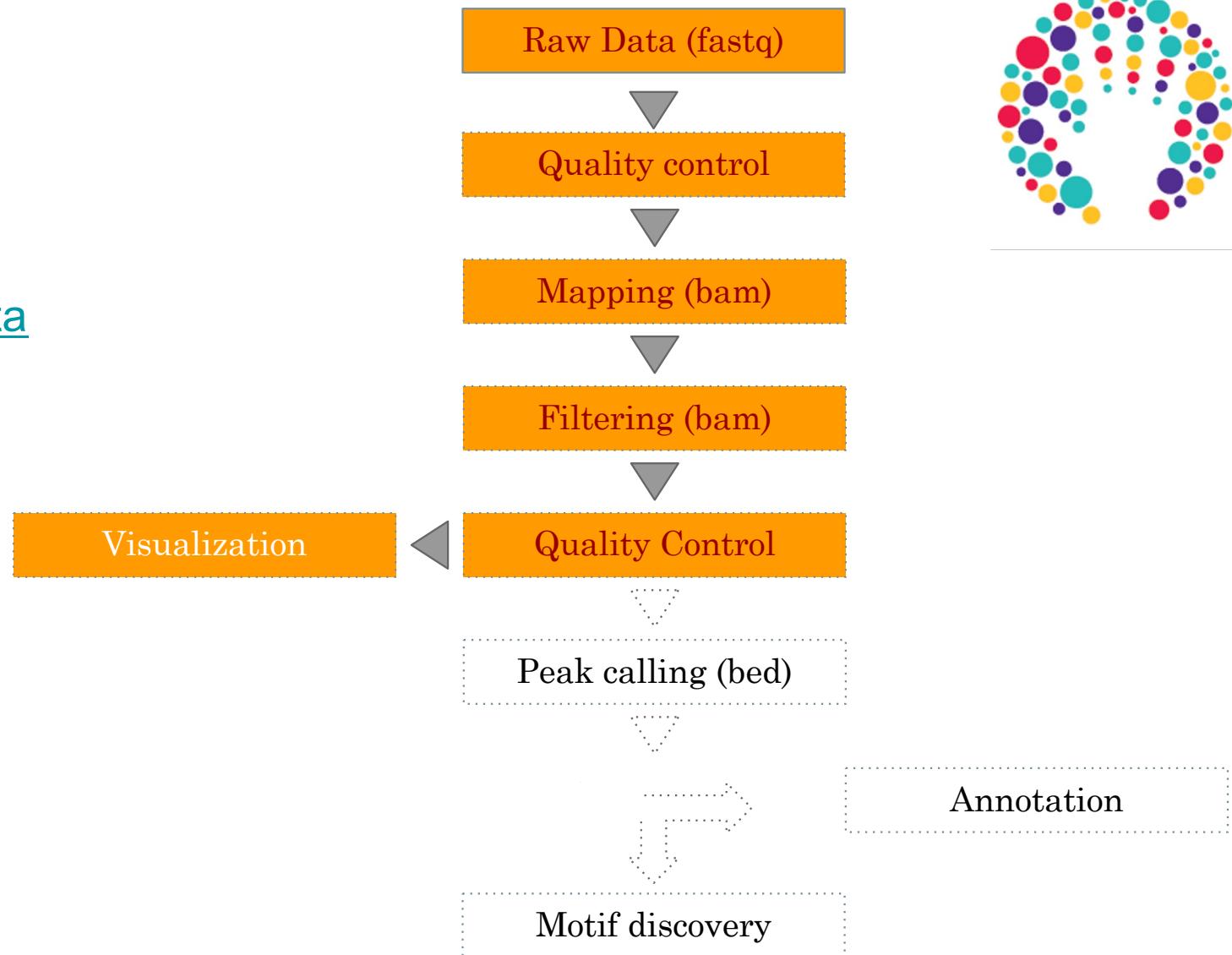
Coverage file and read extension

- BAM files do not contain fragment location but read location
- We need to extend reads to compute fragments coordinates before coverage analysis
- Not required for PE



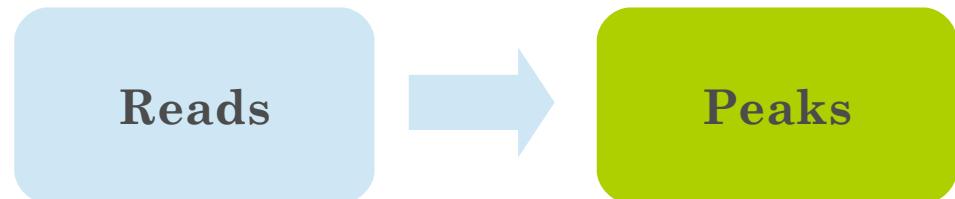
Protocol

- Viewing scaled data



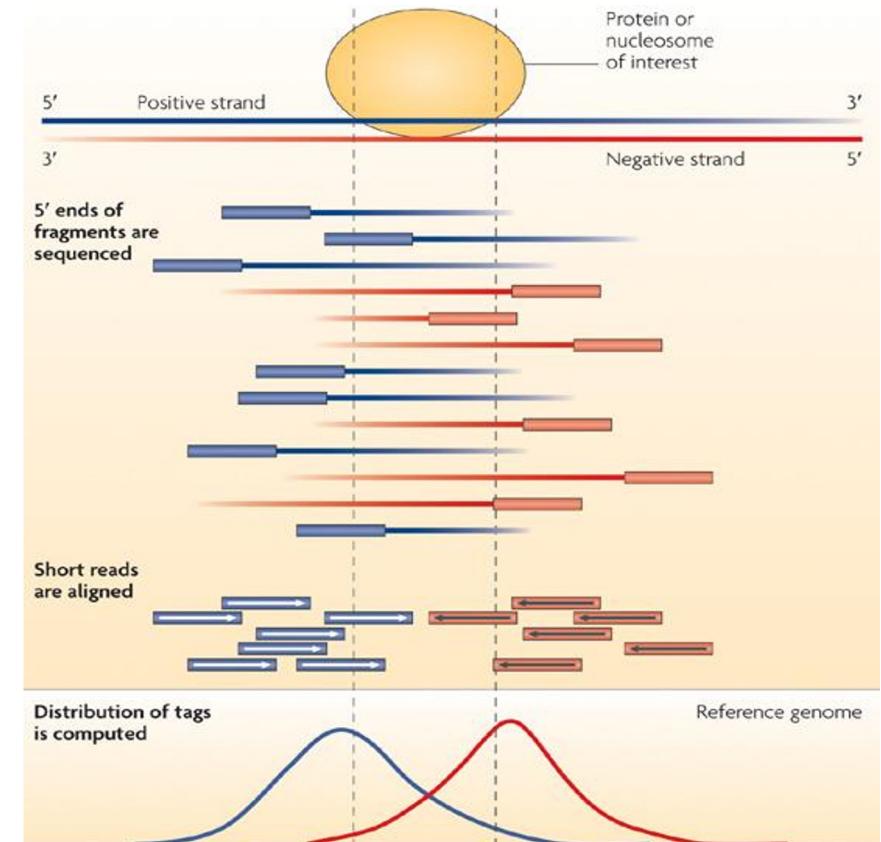


Peak Calling



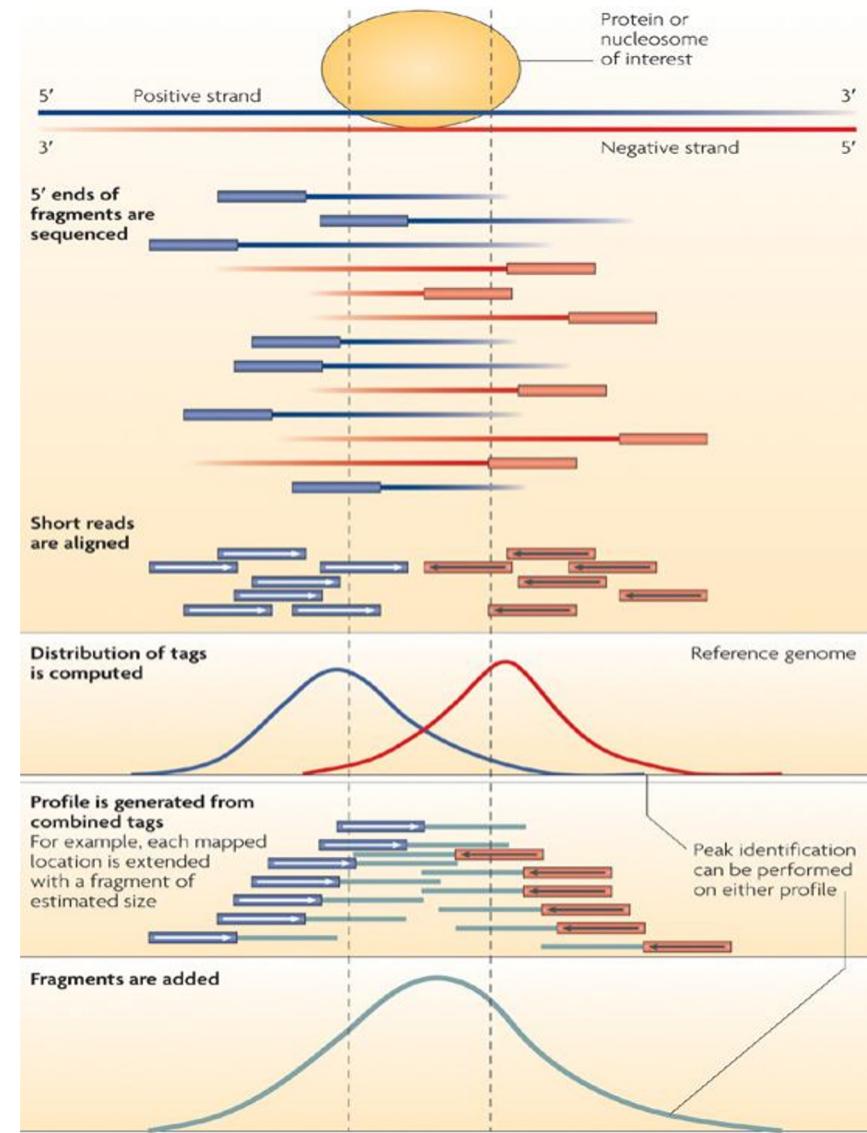
From reads to peaks

- Chip-seq peaks are a mixture of two signals:
 - + strand reads (Watson)
 - - strand reads (Crick)
- The sequence read density accumulates on forward and reverse strands centered around the binding site



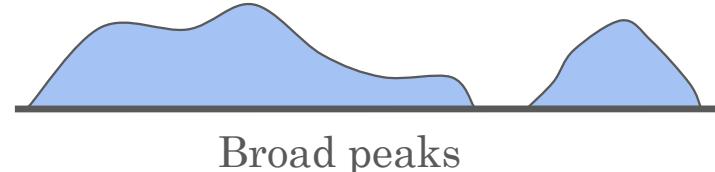
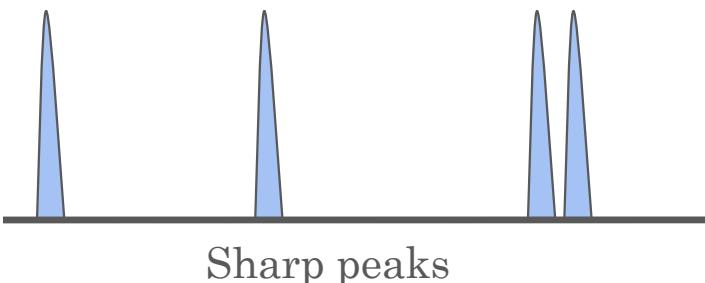
From reads to peaks

- Get the signal at the right position
 - Read shift
 - Extension
- Estimate the fragment size
- Do paired-end



Peak callers

- The **peak caller** should be **chosen** based on
 - Experimental design
 - SE or PE (E.g MACS1.4 vs MACS2)
 - Expected signal
 - Sharp peaks (e.g. Transcription Factors).
 - E.g. MACS
 - Broad peaks (e.g. epigenetic marks).
 - E.g MACS, SICER,...



A variety of peak callers

- 60 programs listed on OMICTOOLS
- Most support a control

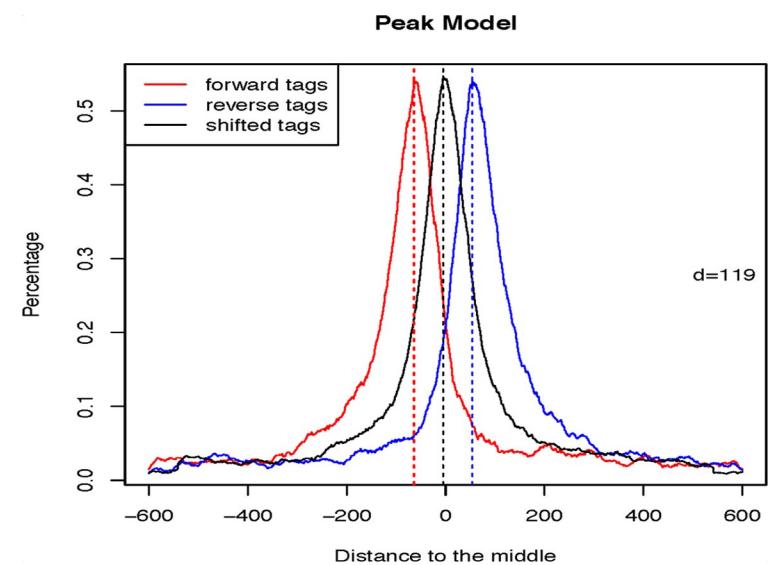
The screenshot shows the OMICTOOLS website interface. At the top, there's a navigation bar with the OMICTOOLS logo and a search bar containing the placeholder "Find the best of bioinformatics". Below the navigation, a breadcrumb trail shows "HIGH-THROUGHPUT SEQUENCING > CHIP-SEQ ANALYSIS > PEAK CALLING". The main content area is titled "PEAK CALLING SOFTWARE TOOLS | CHIP SEQUENCING DATA ANALYSIS". A sub-section title "Identification of genomic regions of interest in ChIP-seq data, commonly referred to as peak-calling, aims to find the locations of transcription factor binding sites, modified histones or nucleosomes. Source text:(Cairns et al., 2011) BayesPeak-an... > Read more" is present. A "FILTERS" button is visible. Below this, five software tools are listed in cards:

- MACS**: Model-based Analysis for ChIP-Seq. Rating: ★★★★★ (1). Discussions: 1. Favorites: 4 times. Description: A software to analyze data generated by short read sequencers. MACS empirically models the shift size of ChIP-Seq tags, and uses it to improve the spatial resolution of predicted binding sites. It...
- HOMER**: Hypergeometric Optimization of Motif EnRichment. Rating: ★★★★★ (5). Discussions: 0. Favorites: 4 times. Description: A suite of tools for Motif Discovery and next-gen sequencing analysis. HOMER contains many useful tools for analyzing ChIP-Seq, GRO-Seq, RNA-Seq, DNase-Seq, Hi-C and numerous other types of...
- SICER**: A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. Rating: ★★★★★ (1). Discussions: 0. Favorites: 1 time. Description: An R package for analysis of ChIP-seq and other functional sequencing data. SPP has been designed to detect protein binding positions with high accuracy. SPP can also examine the saturation level of...
- SPP**: An R package for analysis of ChIP-seq and other functional sequencing data. Rating: ★★★★★ (0). Discussions: 0. Favorites: 0 times. Description: An R package for analysis of ChIP-seq and other functional sequencing data. SPP has been designed to detect protein binding positions with high accuracy. SPP can also examine the saturation level of...
- Scripture**: A method for transcriptome reconstruction that relies solely on RNA-Seq reads and an assembled genome to build a transcriptome ab initio. The statistical methods to estimate read coverage... Rating: ★★★★★ (0). Discussions: 0. Favorites: 0 times. Description: A method for transcriptome reconstruction that relies solely on RNA-Seq reads and an assembled genome to build a transcriptome ab initio. The statistical methods to estimate read coverage...

MACS [Zhang et al, 2008]

1. Modeling the shift size of ChIP-Seq tags

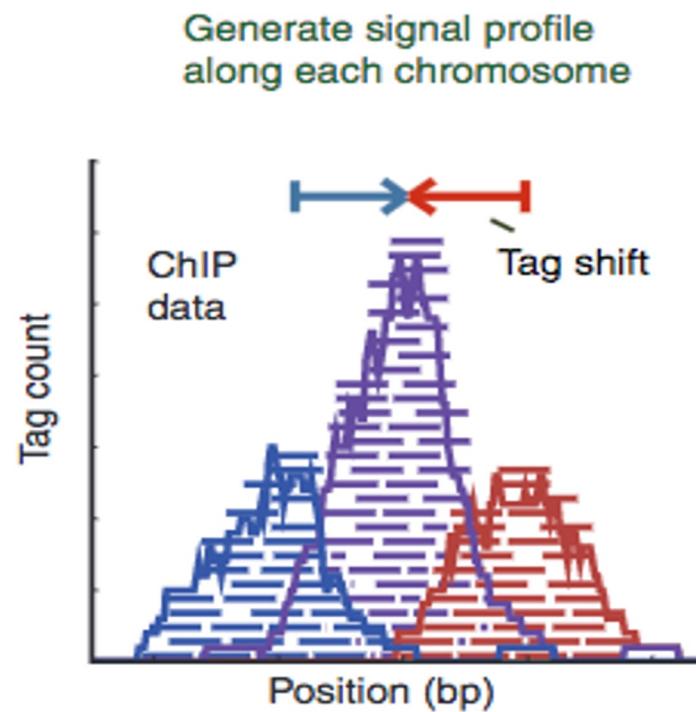
- slides $2 * \text{bandwidth}$ windows across the genome to find regions with tags more than $mfold$ enriched relative to a random tag genome distribution
- randomly samples 1,000 of these highly enriched regions
- separates their + and - reads, and aligns them by the midpoint between their + and - read centers
- define d as the distance in bp between the summit of the two distribution



MACS [Zhang et al, 2008]

2. Peak detection

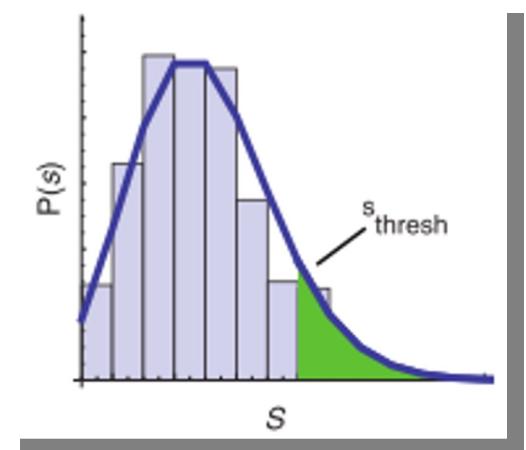
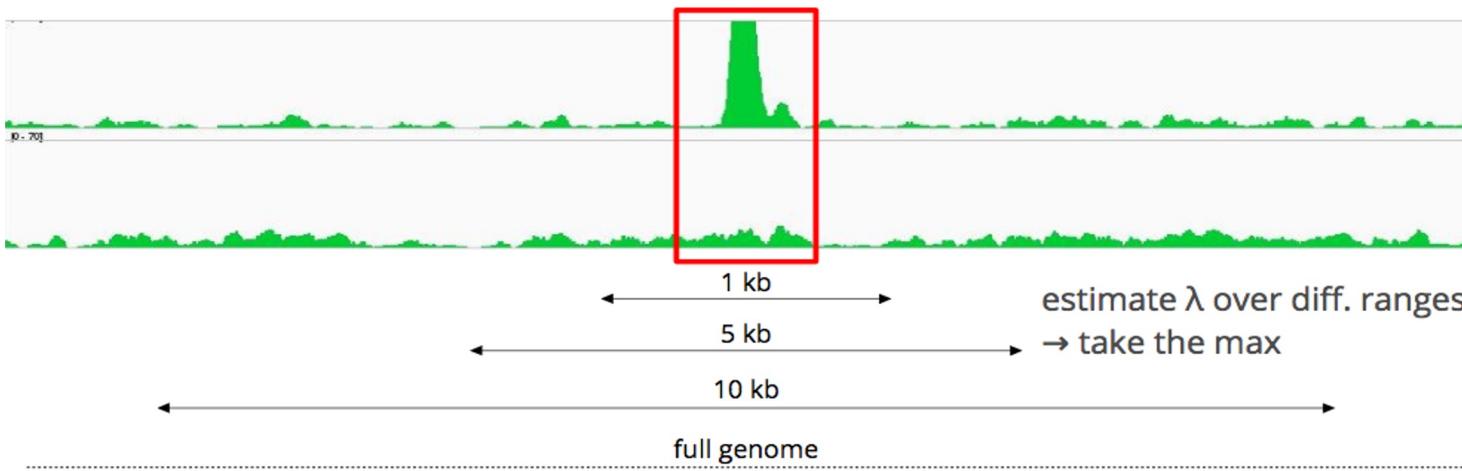
- Scales the total Input read count to be the same as the total ChIP read count
- Duplicate read removal
- Reads are shifted by $d/2$
(d value is the model obtained in step 1)



Pepke et al, 2009

MACS [Zhang et al, 2008]

- Slides 2d windows across the genome to find candidate peaks with a significant read enrichment (Poisson distribution p -value based on λ_{BG} , default 10^{-5})
- Estimate parameter λ_{local} of Poisson distribution
- Keep peaks significant under λ_{BG} and λ_{local} and with p -value < threshold

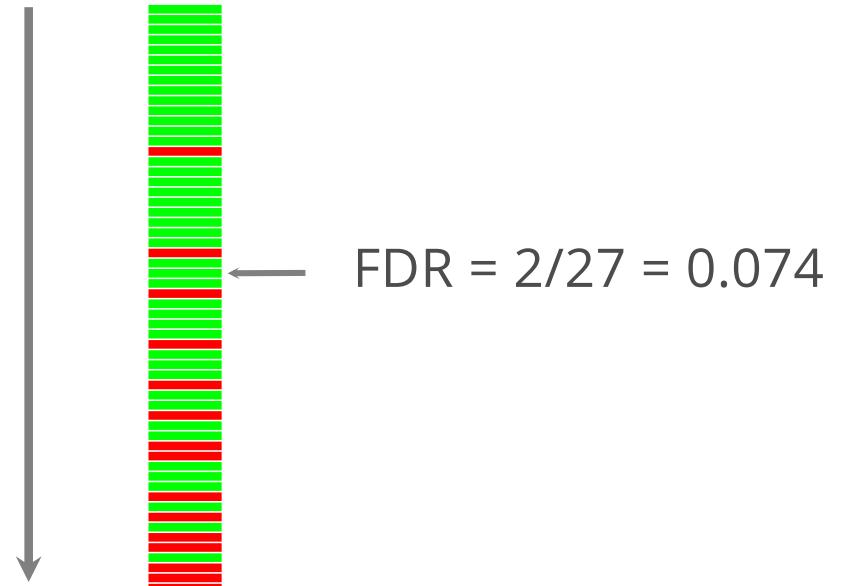


MACS [Zhang et al, 2008]

3. Multiple testing correction (FDR)

- Swap treatment and input and call negative peaks
- Take all the peaks (neg + pos) and sort them by increasing p-values

$$FDR(p) = \frac{\text{# Negative peaks with } p\text{-value} < p}{\text{# Selected peaks}}$$

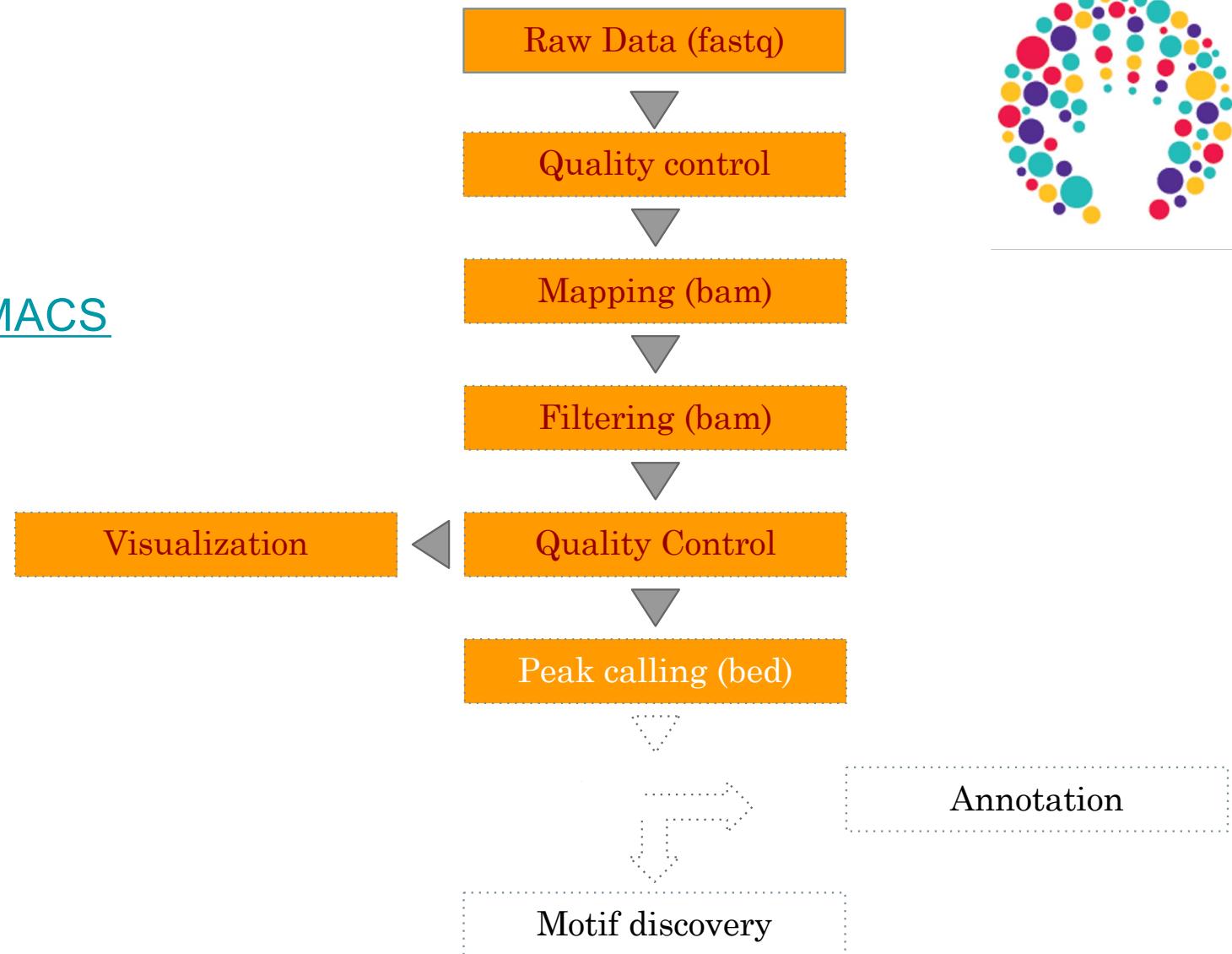


MACS in summary

- Step 1 : search for candidate regions that look like good peaks, to produce a fine-tuned **model** of the peaks (d value) to search in Step 2
- Step 2 : actual peak calling
 - **sliding window** length = $2*d$
 - In each window : test if the region is a peak, by comparing the number of reads in the treatment and the expected number of reads
 - Comparison is based on a **statistical test** with a Poisson distribution, keeping only regions with **p-value < threshold**
- Step 3 : correction for multiple testing (many windows were tested), calculation of **FDR**

Protocol

- Peak calling with MACS
(stop after step 5)

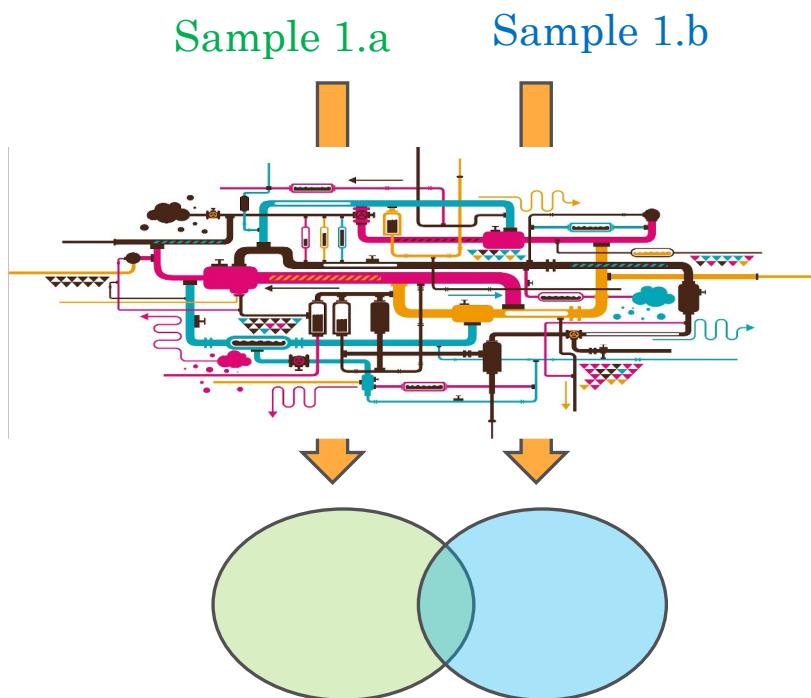




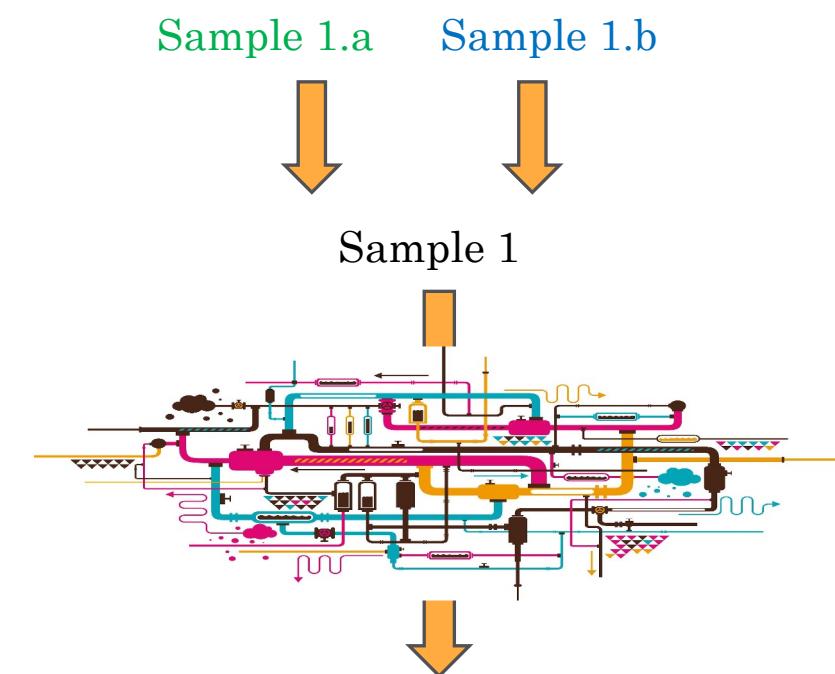
How to deal with replicates

How to deal with replicates

Analyze samples separately
and takes union or intersection
of resulting peaks



Merge samples prior to the peak
calling (e.g recommended by
MACS) => “pooling”



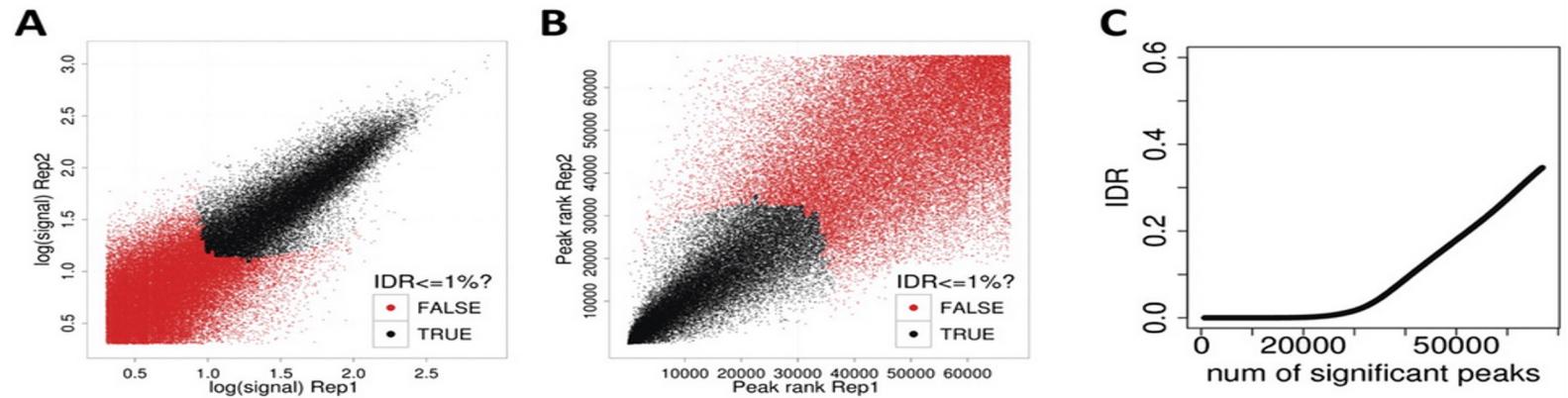
IDR - Irreproducible Discovery Rate (ENCODE)

- Measures consistency between replicates
- Uses reproducibility in score rankings between peaks in each replicate to determine an optimal cutoff for significance.
- Idea:
 - The most significant peaks are expected to have high consistency between replicates
 - The peaks with low significance are expected to have low consistency

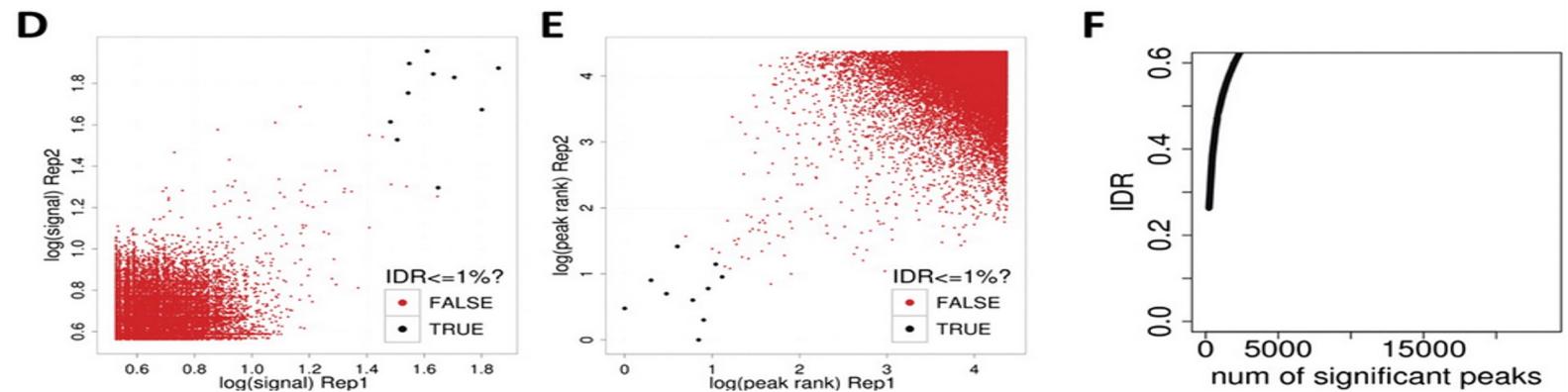
<https://sites.google.com/site/anshulkundaje/projects/idr>

IDR

RAD21 Replicates (high reproducibility)



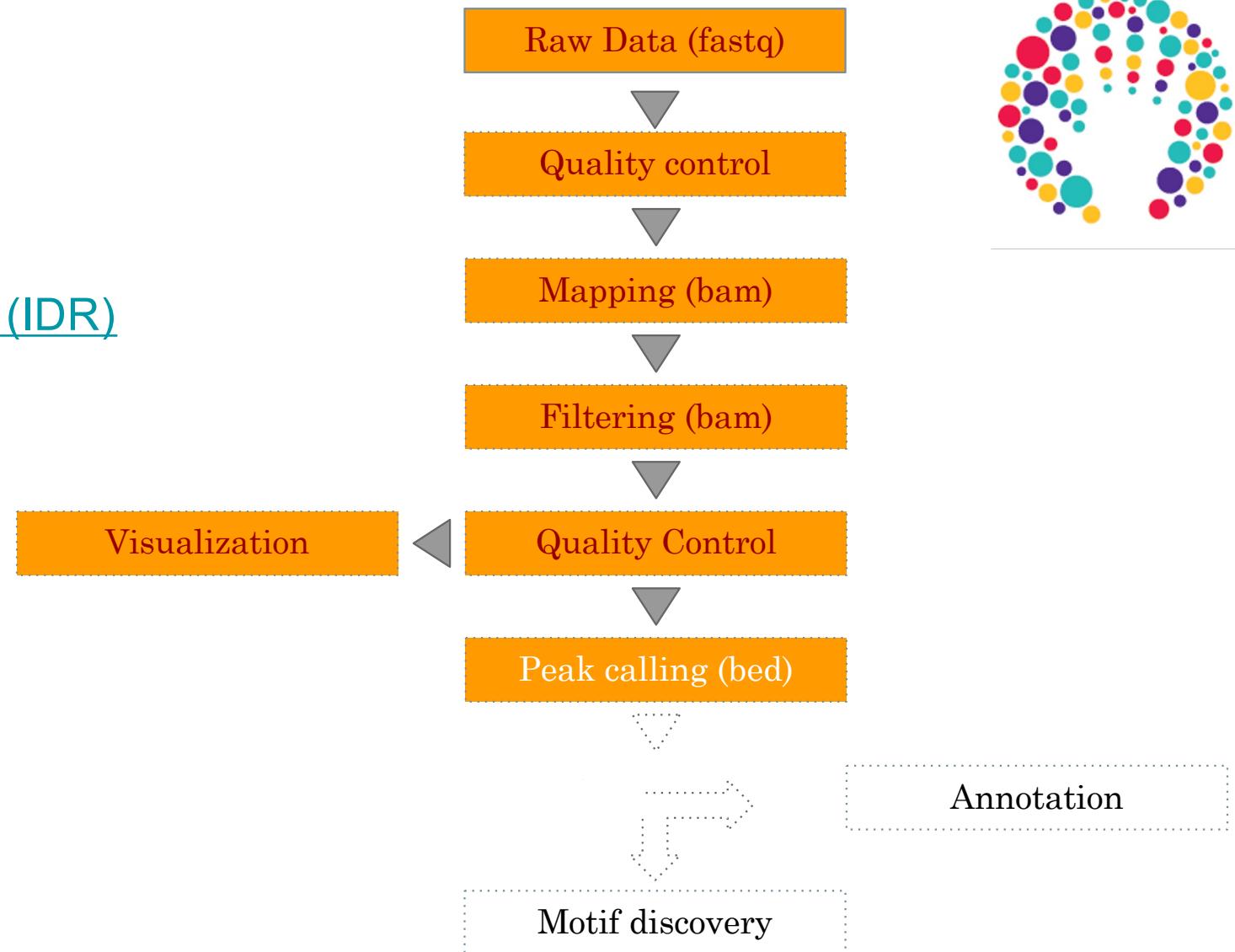
SPT20 Replicates (low reproducibility)



(!) IDR doesn't work on broad source data!

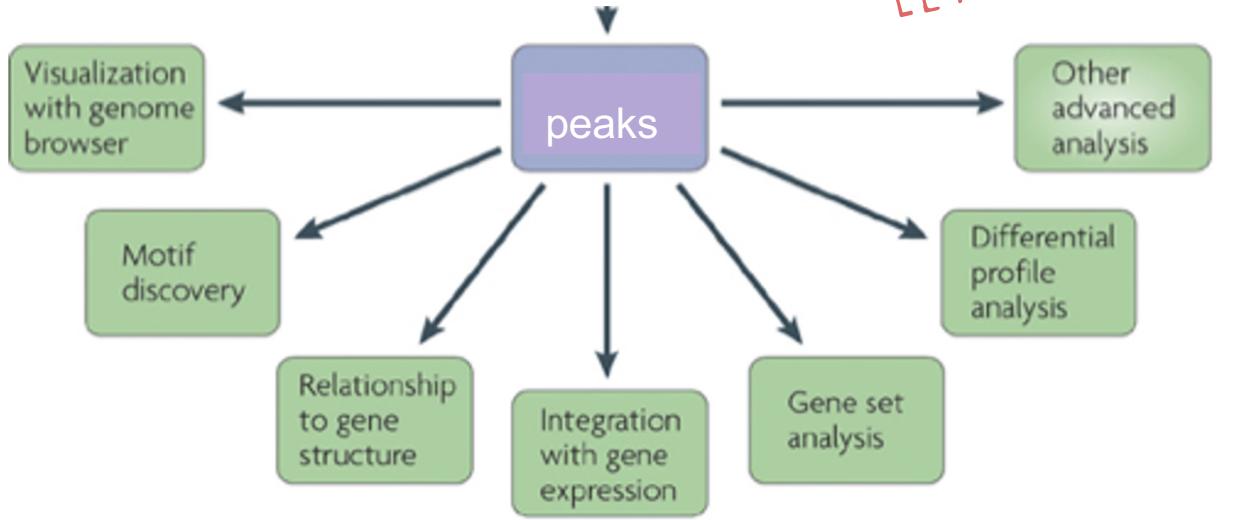
Protocol

- Combine replicate (IDR)



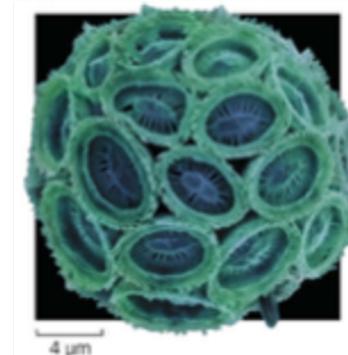
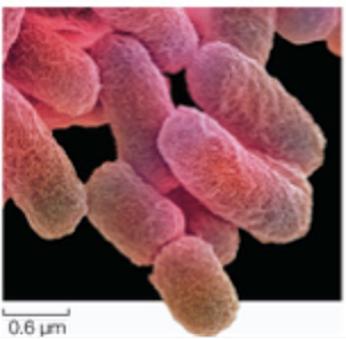
Processing steps are over !

LET'S DO BIOLOGY !!!



Nature Reviews | Genetics

Park, Nature reviews 2009



What is the biological question ?





What is the biological question ?

« see if you can find something in the data »



What is the biological question ?

~~« see if you can find something in the data »~~

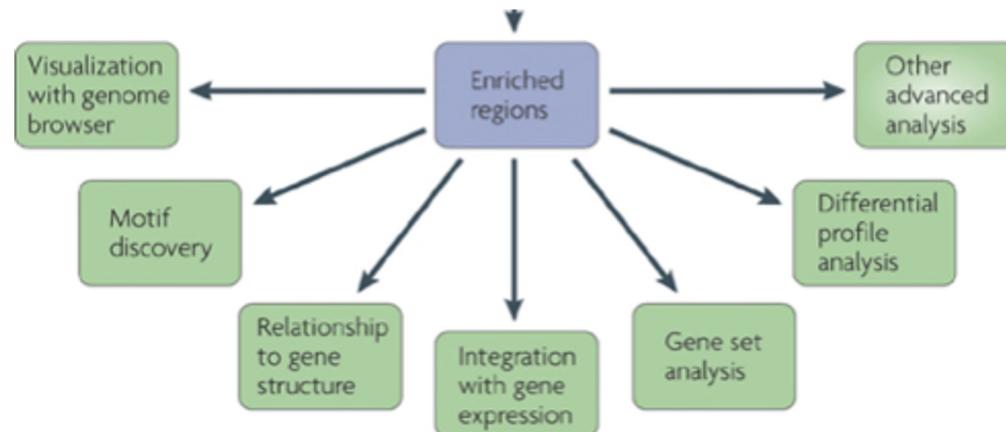
What is the biological question ?

- **Where** do a transcription factor (TF) bind ?
- **How** do a transcription factor (TF) bind ?
 - Which **binding motif(s)** (can be several for a given TF !!)
 - Is the **binding** direct to DNA or via **protein-protein** interactions ?
 - Are there **cofactors** (maybe affecting the motif !!), and if so, identify them
- Which **regulated genes** are directly regulated by a given TF ?
- Where are the **promoters** (PolII) and **chromatin marks** ?

What is the biological question ?

Should drive all « downstream » analyses

Will take time
to « do it all » !!!



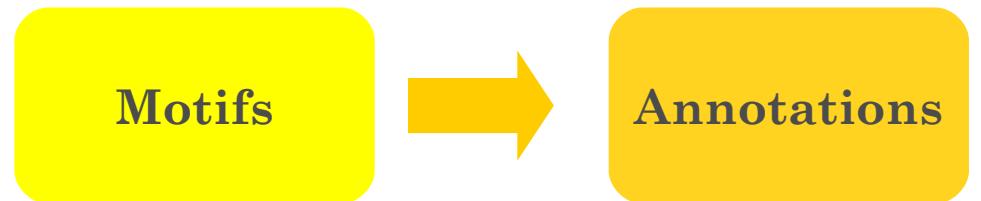


What is the biological question ?

What can be the following experimental work ?

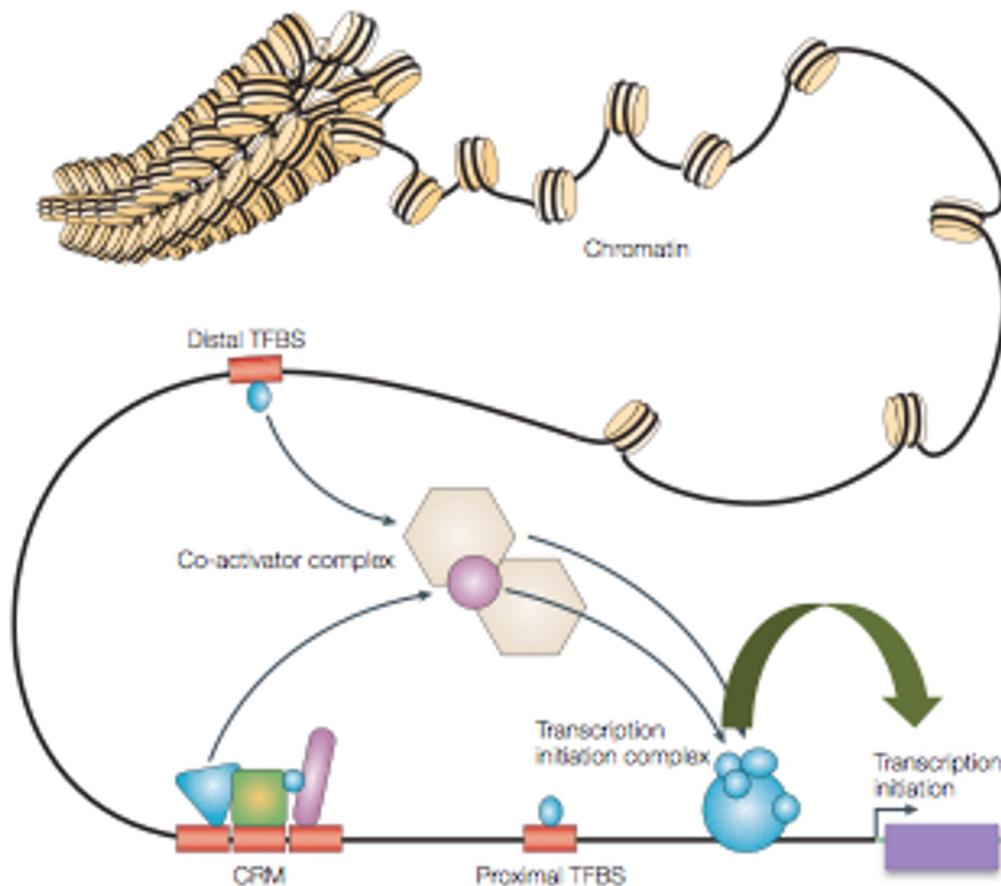
- cell biology (eg: luciferase assay) ?
- in vitro assays (eg: EMSA) ?
- Proteomic (eg: mass spectrometry) ?
- Transgenics ?
- Will depend on
 - the organism
 - available infrastructure

Discovering motifs in peaks



Biological concepts of transcriptional regulation

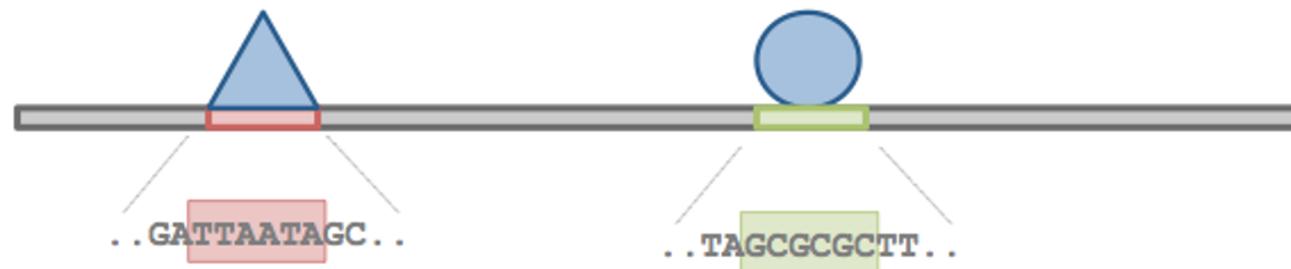
Transcription factors are proteins that modulate (activate/repress) the expression of target genes through the binding on DNA cis-regulatory elements



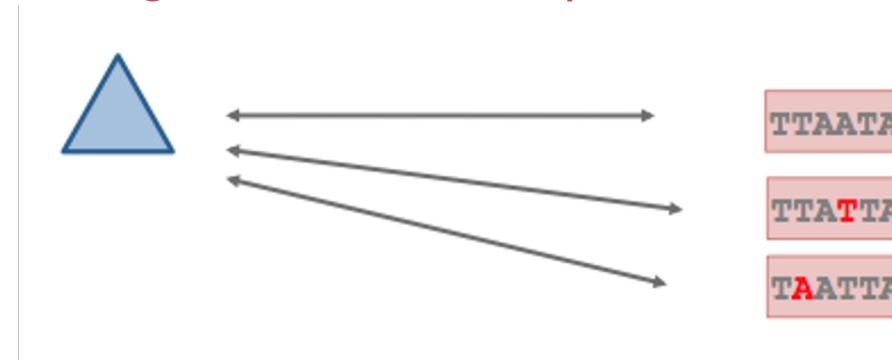
Wasserman et al, *Nat Rev Genet*, 2004

Transcription factor specificity

How do TF « know » where to bind DNA ?

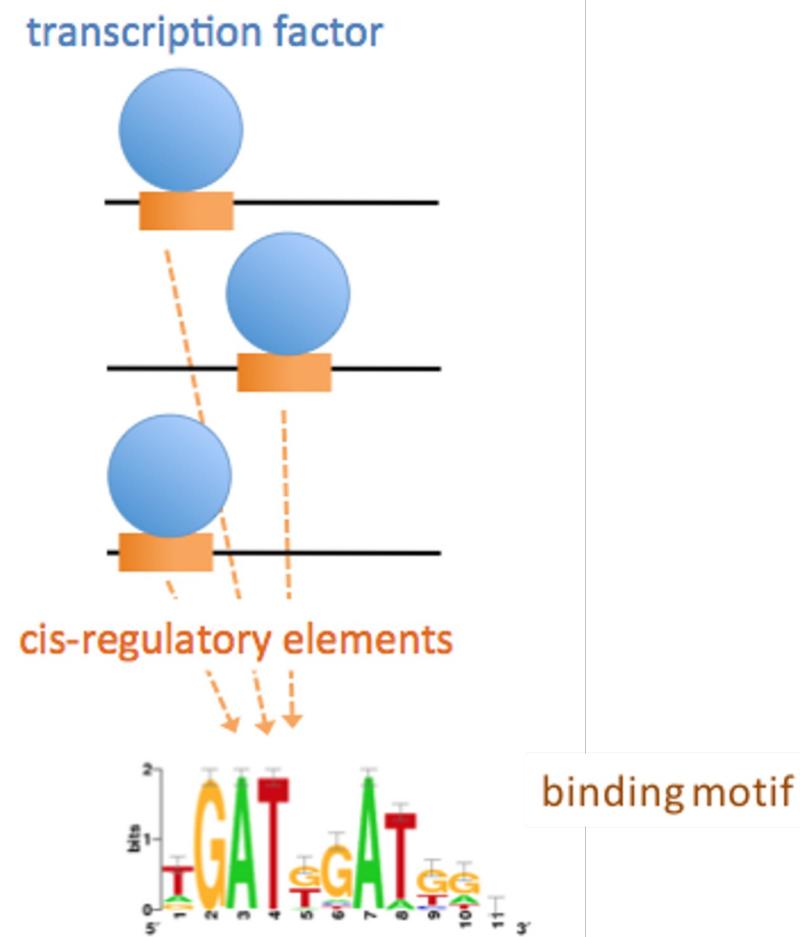


TF recognize TFBS with specific DNA sequences



a given TF is able to bind DNA on TFBSs with different sequences

Binding specificity



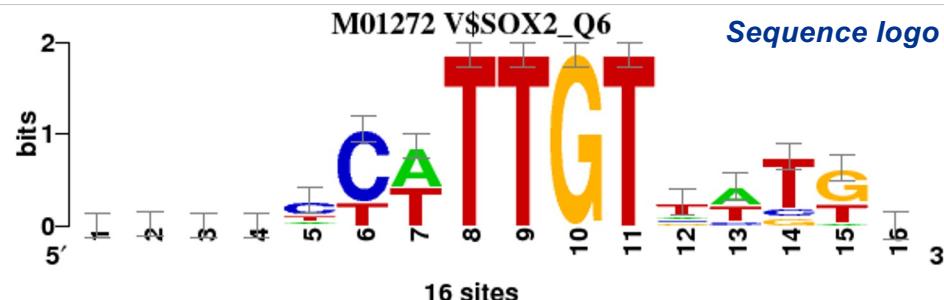
From binding sites to binding motif

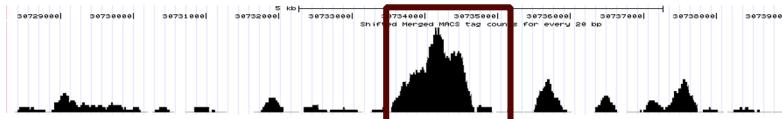
*Collection of binding sites
used to build the Sox2 matrix
(TRANSFAC M01272)*

R15133	GCCCTCATTGTTATGC
R15201	AAACTCTTGTTGGAA
R15231	TTCACCATTGTTCTAG
R15267	GACTCTATTGTCTCTG
R16367	GATATCTTGTTCTT
R17099	TGCACCTTGTTATGC
R19276	AATTCCATTGTTATGA
R19367	AAACTCTTGTTGGAA
R19510	ATGGACATTGTAATGC
R22342	AGGCCTTTGTCCTGG
R22344	TGTGCTTTGTNNNNN
R22359	CTCAACTTGTAATT
R22961	GCAGCCATTGTGATGC
R23679	CACCCCTTGTTATGC
R25928	TTTCTATTGTTTTA
R27428	AAAGGCATTGTGTTTC

Position-specific scoring matrix (PSSM)

A	6	7	4	4	2	0	8	0	0	0	0	2	7	0	1	4
C	2	2	6	5	9	12	0	0	0	0	0	2	2	2	0	6
G	4	3	2	4	1	0	0	0	0	16	0	2	0	2	9	3
T	4	4	4	3	4	4	8	16	16	0	16	9	6	11	5	2





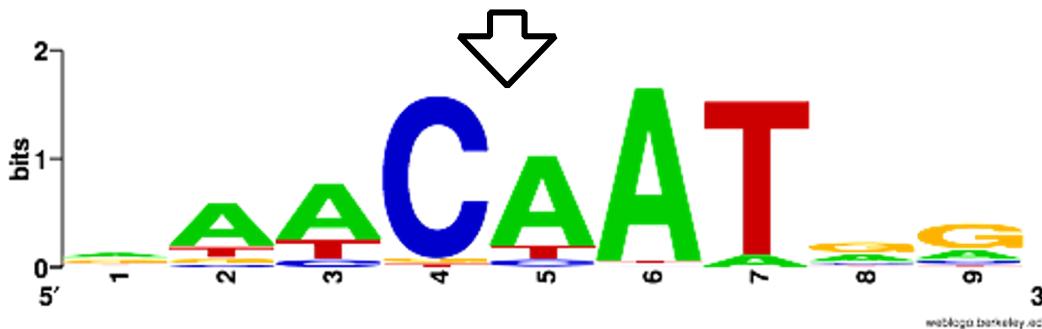
ChIP-seq peaks

>mm9_chr1_39249116_39251316_+
gagaggaaggggagaaaagagggagggagGGTGTAGGTAGCCAGGAG
CCAATGGGGCGTTTCCTGTCCAGGCCACTTGCTGGAATGTGAGATGT
AGAATGACCCAAAGAGAGCTGCCAACAGACAGAGCTCTGCCCAAGGAATTGA
ACTCAAAGGGTGTCAAGAACAGGTGGCCTTGTGCACCTGGCGGGGA
CGTGGCTCCCTCTTCCGGCTGGTCTAGCCAGGtgctgcctgcctgcct
gccGTGATCTCTGGACGCCAGTAGAGGGTTGTTGTGGGTTGGGTGAAAC
ACGCCACCCCTGAGCTTCCGGGGCTAGCAATCTCCCCATCACCCCCA
TTCGCGCTCAGAACCCCTCAGCGATAACAGCAGGCCCTGGTTCCCG

DNA sequence

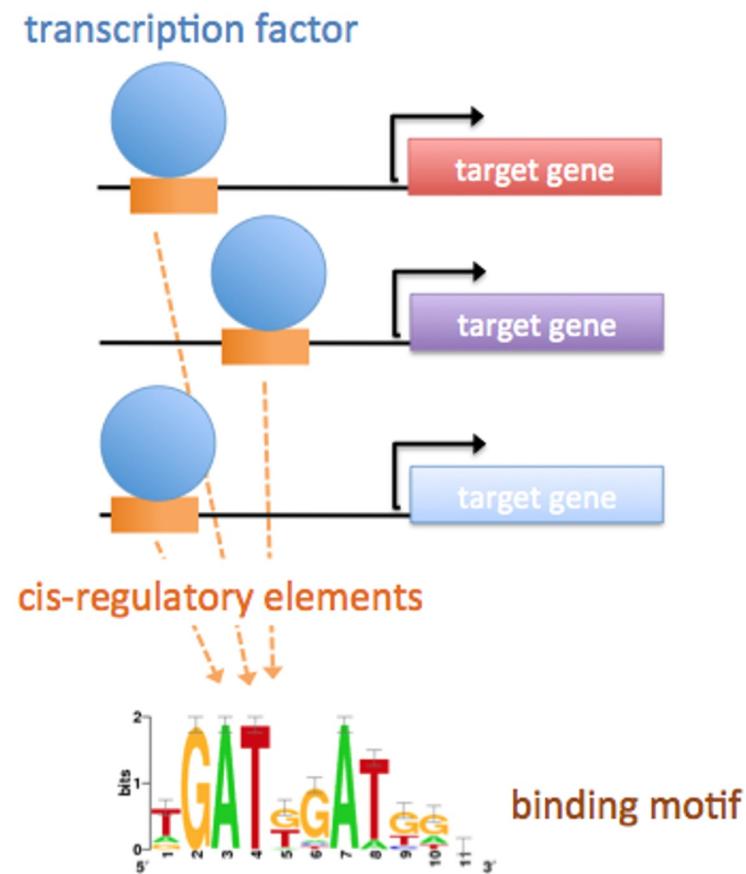
A	[24	54	59	0	65	71	4	24	9]
C	[7	6	4	72	4	2	0	6	9]
G	[31	7	0	2	0	1	1	38	55]
T	[14	9	13	2	7	2	71	8	3]

Discovered motif



Motif logo

De novo motif discovery



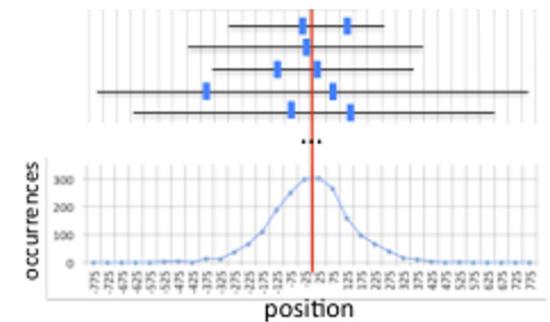
Problem :

*How can we model/describe
the binding specificity of
a given TF ?*

*If there is a common regulating
factor, can we discover its motif
only using these sequences ?*

De novo motif discovery

- Find exceptional motifs based on the sequence only
(No prior knowledge of the motif to look for)
- Criteria of exceptionality:
 - *Over-/under-representation*: higher/lower frequency than expected by chance
 - *Position bias*: concentration at specific positions relative to some reference coordinates (e.g. TSS, peak center, ...).



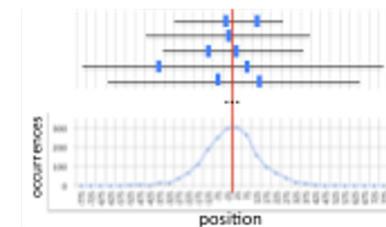
Some motif discovery tools

- MEME (Bailey et al., 1994)
- **RSAT oligo-analysis (van Helden et al., 1998)**
- AlignACE (Roth et al. 1998)
- **RSAT position-analysis (van Helden et al., 2000)**
- Weeder (Pavesi et al. 2001)
- MotifSampler (Thijs et al., 2001)
- ... many others

Why do we need new approaches for genome-wide datasets ?

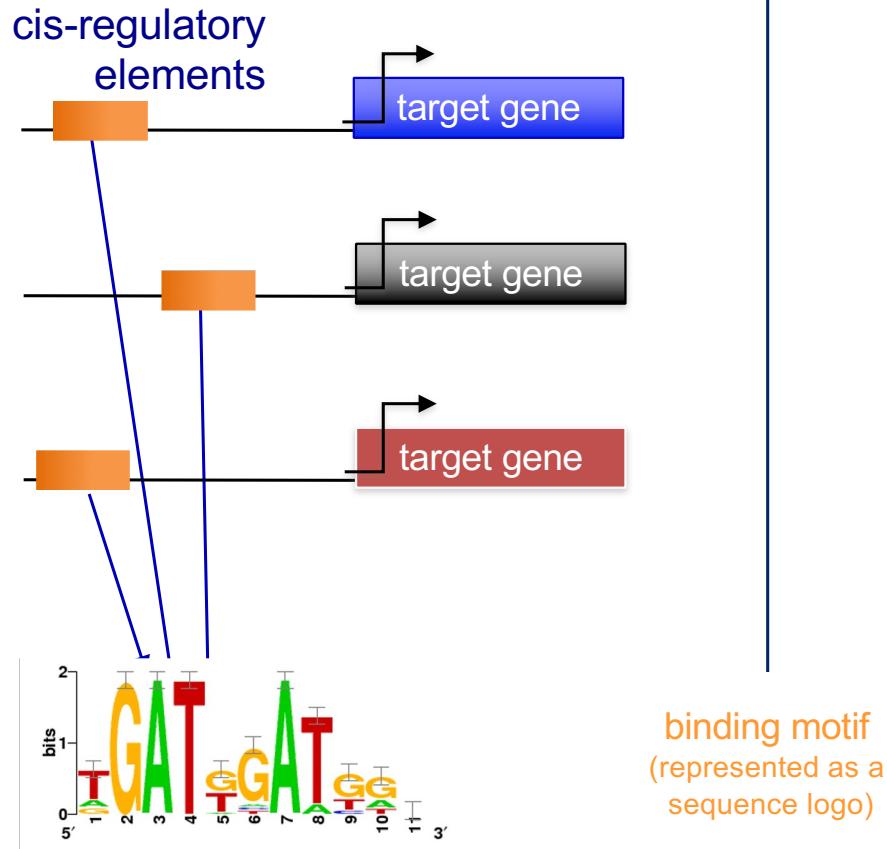
New approaches for ChIP-seq datasets

- **Size, size, size**
 - limited numbers of promoters and enhancers
 - ↓
 - dozens of thousands of peaks !!!!!!
- **the problem is slightly different**
 - promoters: 200-2000bp from co-regulated genes
 - ↓
 - peaks: 300bp, positional bias
- **motif analysis: not just for specialists anymore !**
 - complete user-friendly workflows

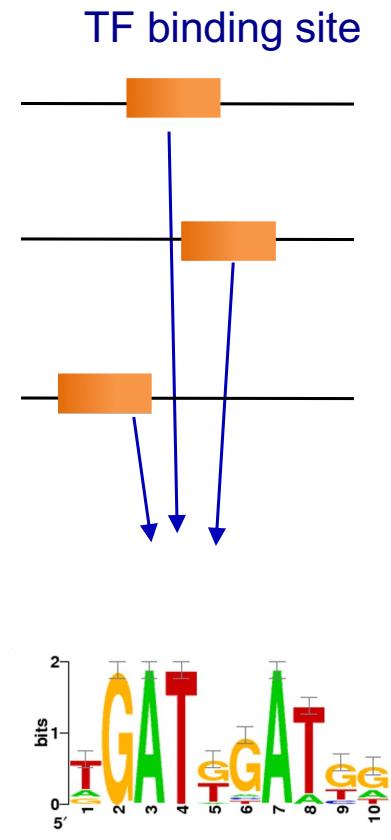


De novo motif discovery

Case 1: promoters of co-expressed genes



Case 2: ChIP-seq peaks



Regulatory sequence Analysis Tools (rsat.eu)

Regulatory Sequence Analysis Tools

Welcome to **Regulatory Sequence Analysis Tools (RSAT)**.

 This web site provides a series of modular computer programs specifically designed for the detection of regulatory signals in non-coding sequences.

RSAT servers have been up and running since 1997. The project was initiated by [Jacques van Helden](#), and is now pursued by the [RSAT team](#).

Choose a server

New ! January 2015: we are in the process of re-organising our mirror servers into taxon-specific servers, to better suit the drastic increase of available genomes.

 RSAT 243 Fungi maintained by TAGC - Université Aix Marseilles, France	 RSAT 9638 Bacteria + Archaea maintained by RegulonDB - UNAM, Cuernavaca, Mexico	 RSAT 92 Metazoa maintained by plateforme ABIMS Roscoff, France
 RSAT 186 Protists maintained by Ecole Normale Supérieure Paris, France	 RSAT 39 Plants maintained by Bruno Contreras Moreira, Spain	 RSAT Teaching maintained by SLU Global Bioinformatics Center, Uppsala, Sweden

Citing RSAT complete suite of tools:

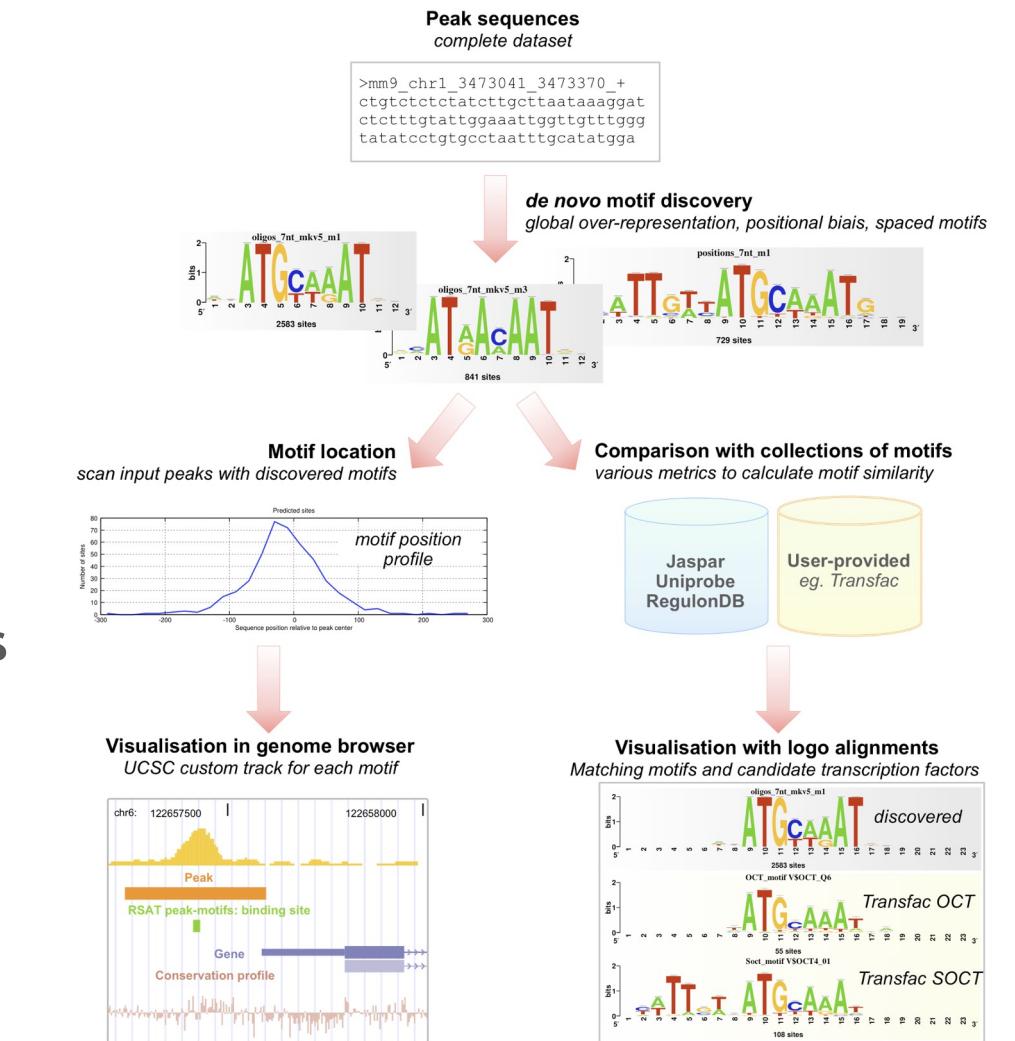
- Thomas-Chollier M, Defrance M, Medina-Rivera A, Sand O, Herrmann C, Thieffry D, van Helden J. (2011) **RSAT 2011: regulatory sequence analysis tools**. Nucleic Acids Res. 2011 Jul;39(Web Server issue):W86-91. [[PubMed 21715389](#)] [[Full text](#)]
- Thomas-Chollier, M., Sand, O., Turatsinze, J. V., Janky, R., Defrance, M., Vervisch, E., Brohee, S. & van Helden, J. (2008). **RSAT: regulatory sequence analysis tools**. Nucleic Acids Res. [[PubMed 18495751](#)] [[Full text](#)]
- van Helden, J. (2003). **Regulatory sequence analysis tools**. Nucleic Acids Res. 2003 Jul 1;31(13):3593-6. [[PubMed 12824373](#)] [[Full text](#)] [[pdf](#)]

For citing individual tools: the reference of each tool is indicated on top of their query form.

RSAT logos designed by Mauricio Guzman (<http://www.altamirastudio.com/>)

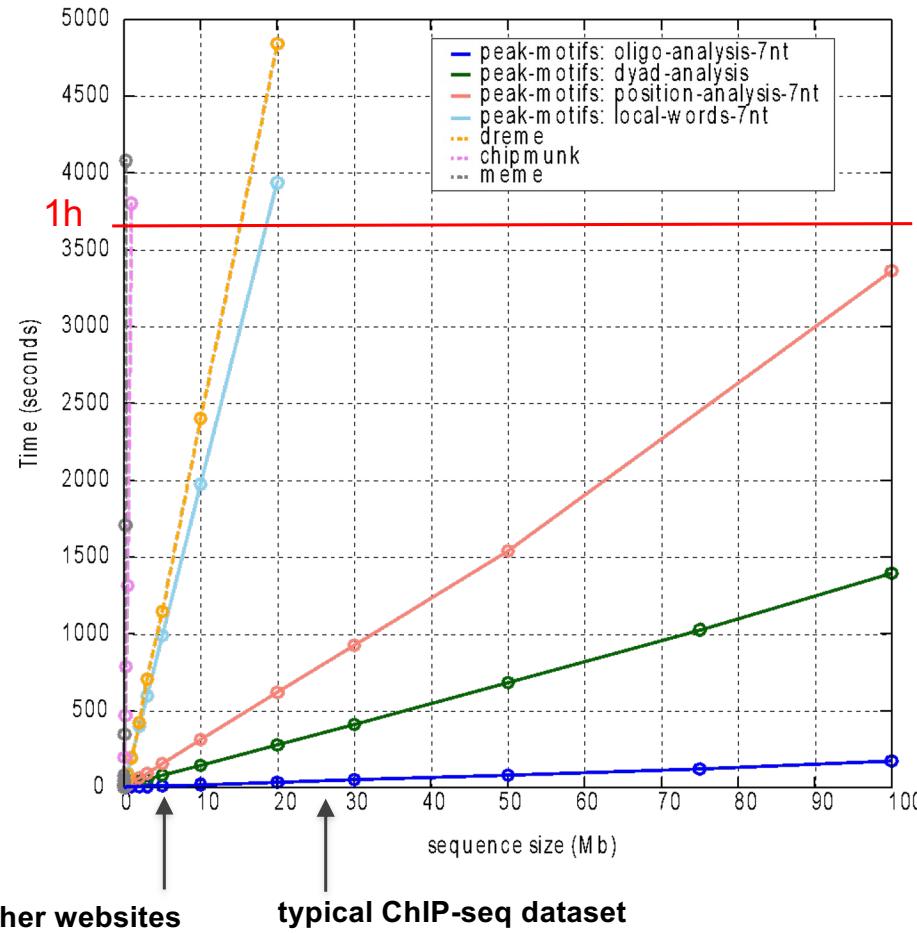
Peak-motifs

- fast and scalable
- treat full-size datasets
- complete pipeline
- web interface
- accessible to non-specialists



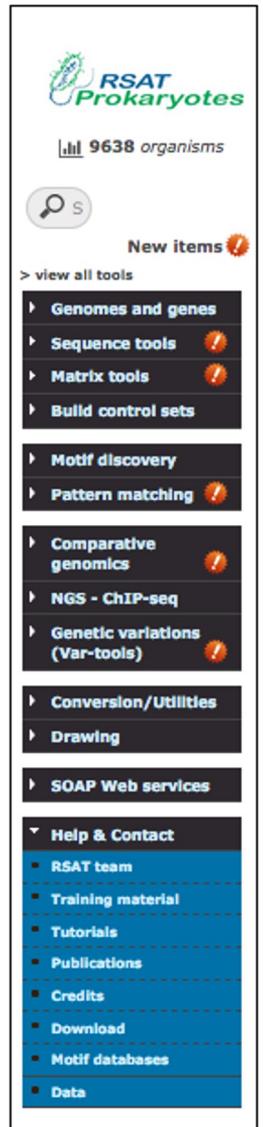
Peak-motifs: why providing yet another tool?

- **fast and scalable**
- **treat full-size datasets**
- **using 4 complementary algorithms**
 - Global over-representation
 - **oligo-analysis**
 - **dyad-analysis**
 - **(spaced motifs)**
 - Positional bias
 - **position-analysis**
 - **local-words**



Thomas-Chollier, Herrmann, Defrance, Sand, Thieffry, van Helden *Nucleic Acids Research*, 2012

RSAT menu



→ 1. Get sequences

→ 2. Run the analysis

→ 3. Visualization

→ Help: tutorials,

RSAT Web forms

RSA-tools - retrieve sequence ←

Returns upstream, downstream or ORF sequences for a list of genes ←

Remark: If you want to retrieve sequences from an organism that is in the Ensembl database, we recommend to use the [retrieve-ensembl-seq](#) program instead.

Single organism Organism: Saccharomyces cerevisiae

Multiple organisms

Genes: all selection

Upload gene list from file

Query contains only IDs (no synonyms)

Feature type: CDS mRNA tRNA rRNA scRNA

Sequence type: upstream From: default To: default

Prevent overlap with neighbour genes (noorf)

Mask repeats (only valid for organisms with annotated repeats)

Admit imprecise positions

Sequence format: fasta

Sequence label: gene name

Output: server display email

GO Reset DEMO MANUAL TUTORIAL

Tool name

Tool description

Tool parameters

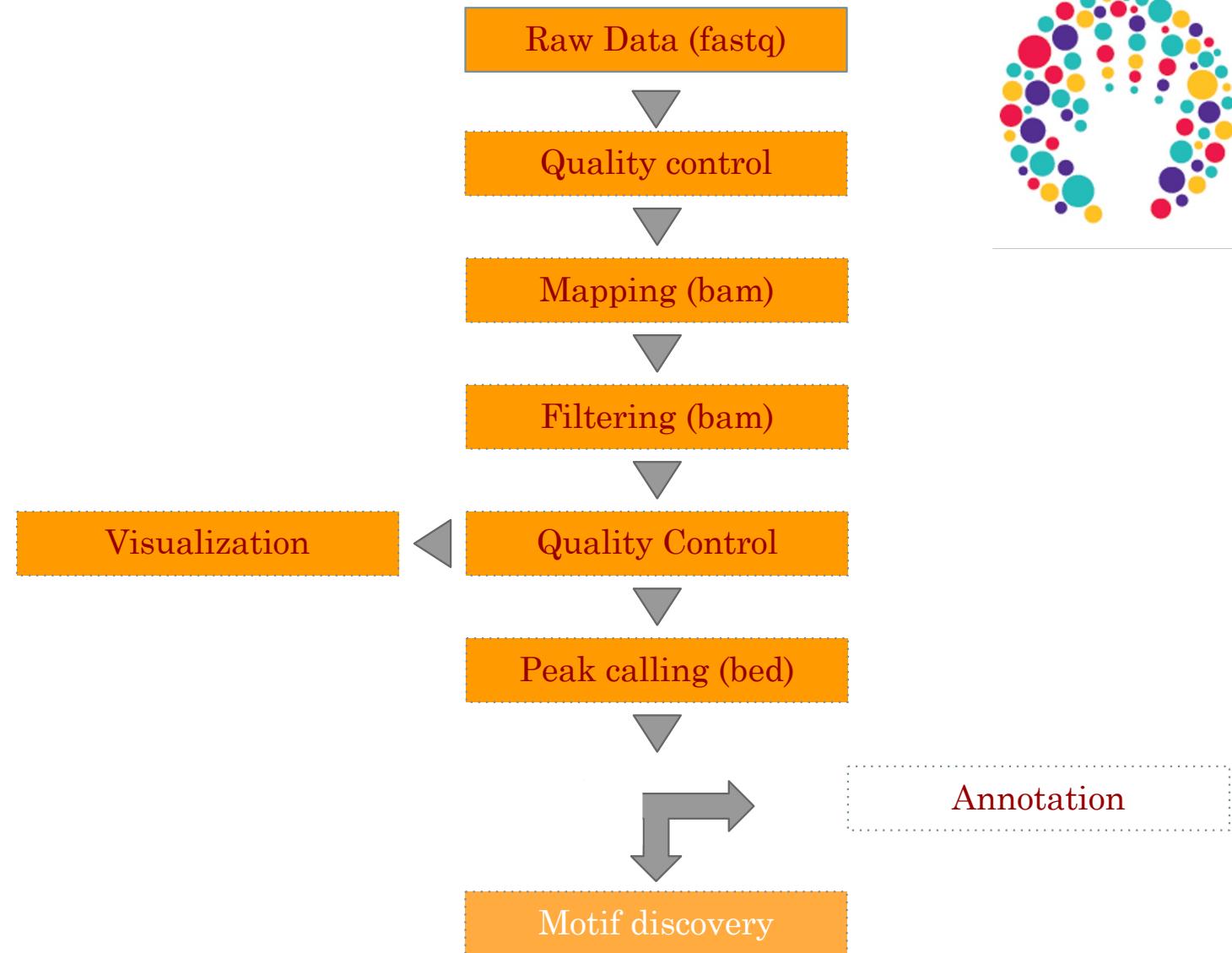
Output

Go button (launches the analysis)
Demo button (fill in the form for test purposes)

Help

Protocol

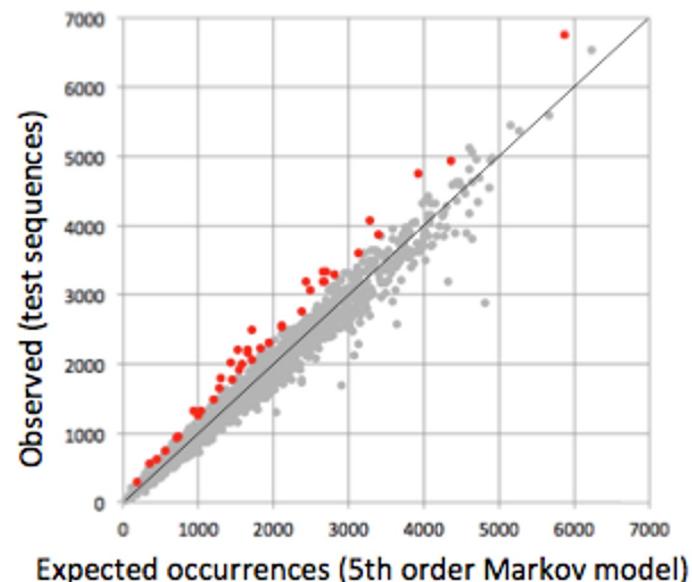
- Motif analysis



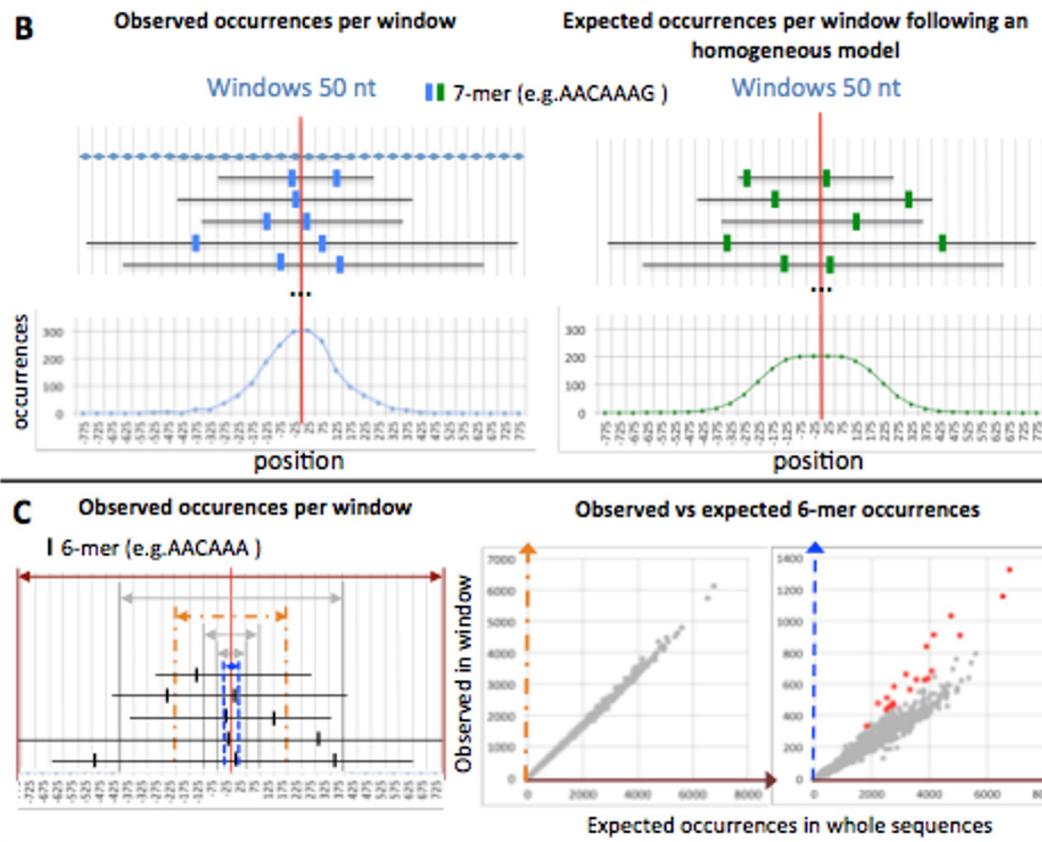
Motif discovery: frequency

Observed 6-mer occurrences computed from:	Expected 6-mer occurrences computed from:
6-mer (e.g. AACAAA)	Background sequences (when available)
Test sequences	
	OR
	Theoretical k-mers frequencies from test sequences
➔ Computation of p-value (binomial) and E-value (multi-testing correction)	

Observed vs expected 6-mer occurrences



Motif discovery: positional bias

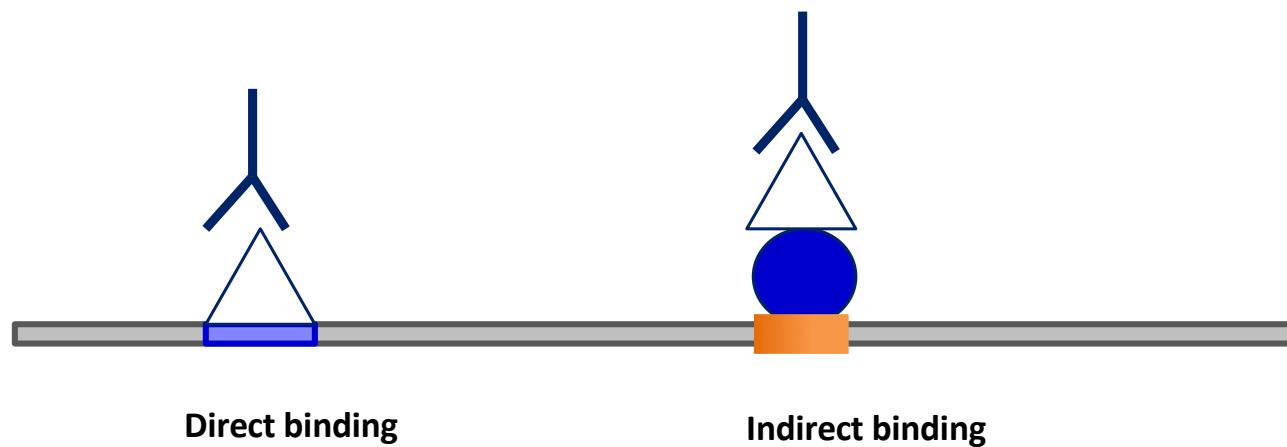


position-analysis

local-words

Direct versus indirect binding

ChIP-seq does not necessarily reveal **direct binding**: The motif of the targeted TF is not always found in peaks!



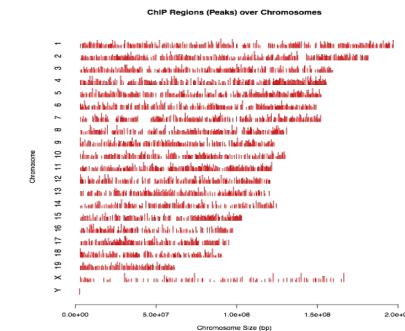
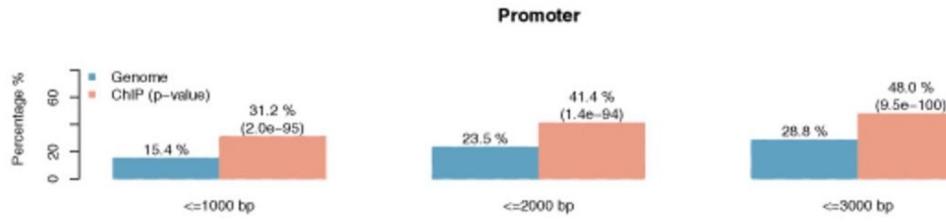


Annotating peaks

Annotations

Are peaks biased towards any genomic features?

- How are the peaks distributed on the chromosomes?
- Are there genomic features (promoters, intergenic, intronic, exonic regions) enriched in the peaks?
- How are the peaks distributed compared to gene structures (TSS, TTS, introns, exons)?
- How are they distributed compared to the genes?



Various tools available

- ChIPseeker (Bioconductor) <https://goo.gl/BemEsw>
- bedtools annotate : <http://bedtools.readthedocs.io>
- HOMER annotatePeaks.pl

Warning : rely on the organism annotation and assembly version

=> not all organisms supported by all programs !

Which are the closest genes?



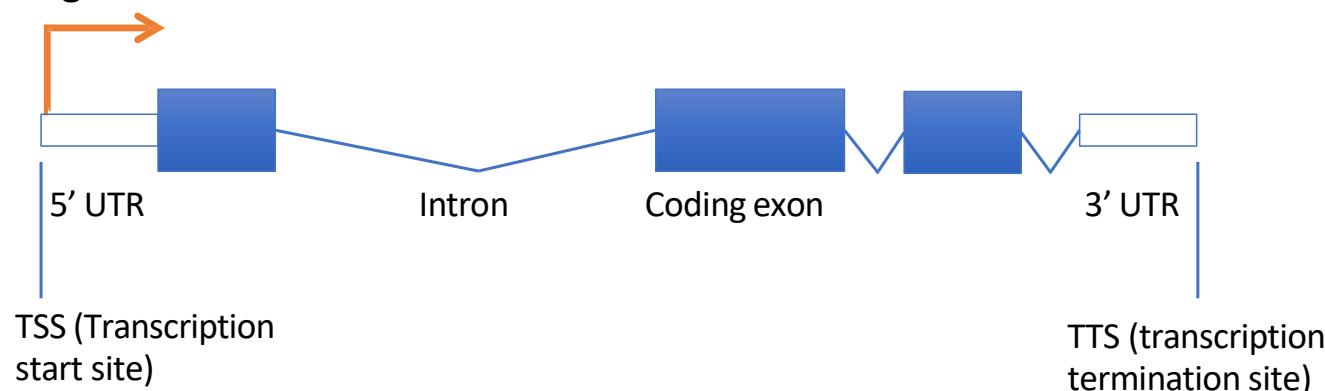
HOMER

Software for motif discovery and ChIP-Seq analysis

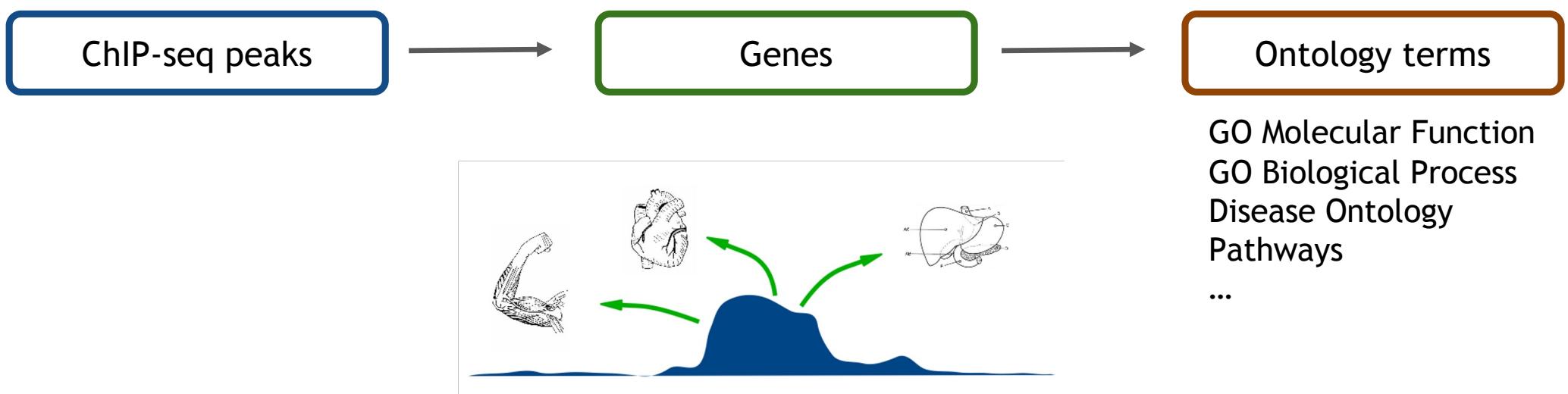
HOMER is a well-maintained suite of tools for functional genomics sequencing data sets. It can perform peak-calling and motif analysis, but we will use it for annotation of the peaks only.

Peak annotation

- Goal: assigning a peak to one or many genome features (genes/transcripts) to understand which genes are possibly regulated by the protein of interest binding
- The name of the gene is important as well as the genic region where the peak is located
- Example of Homer tools:
 - Determines the distance to the nearest Transcription Start Site (TSS) and assigns the peak to that gene
 - Determines the genomic annotation of the region **occupied by the center** of the peak/region. Possible genomic annotation:



What are the genes associated to the peaks ?
Are there some functional categories over-represented ?



Various tools available

These tools work with gene lists

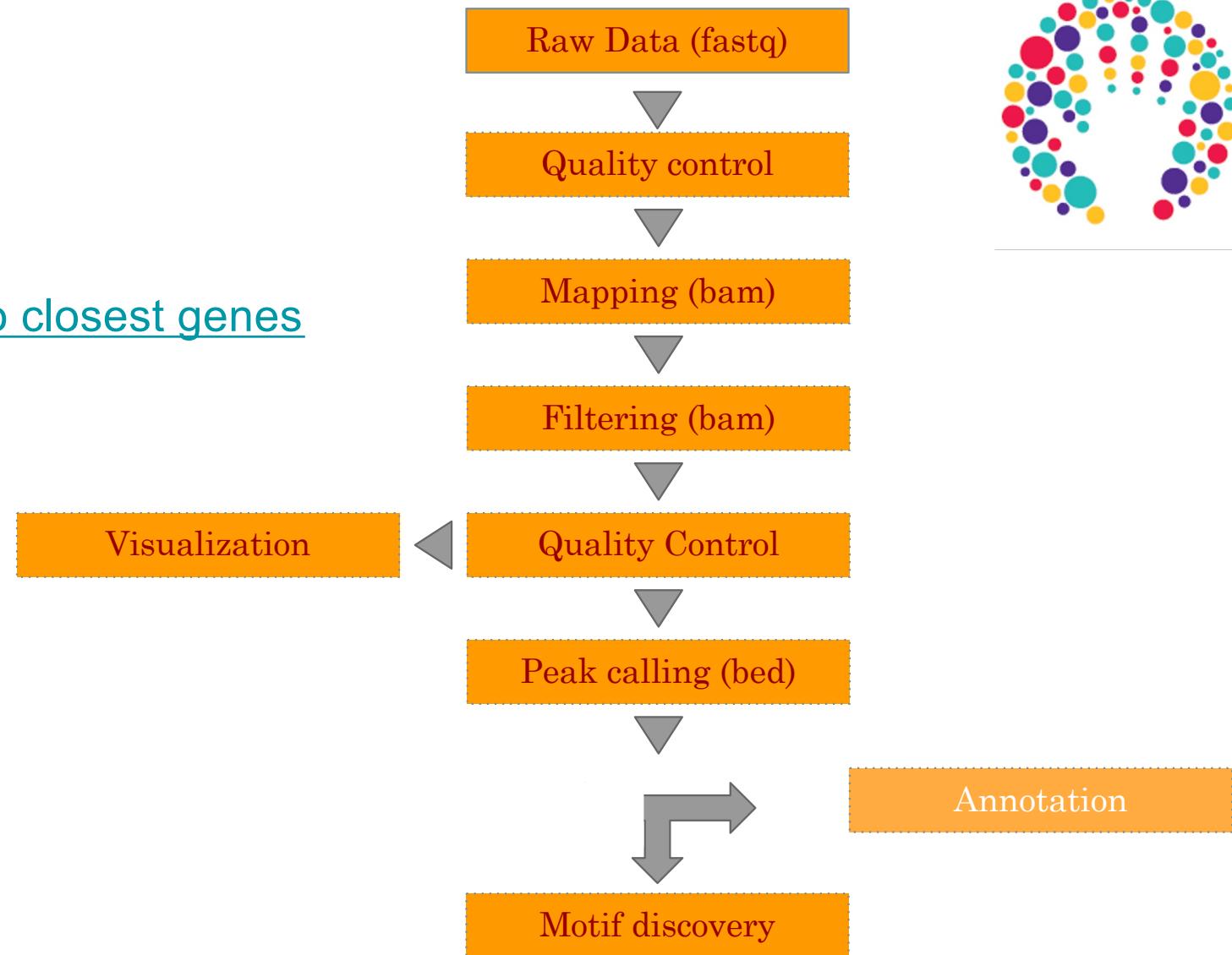
- GSEA: <http://www.broadinstitute.org/gsea>
- gProfiler: <http://biit.cs.ut.ee/gprofiler/gost>
- GSAn: <https://gsan.labri.fr/>
- HOMER: <http://homer.salk.edu/homer>
- DAVID: <http://david.abcc.ncifcrf.gov>

These tools work with regions (BED files)

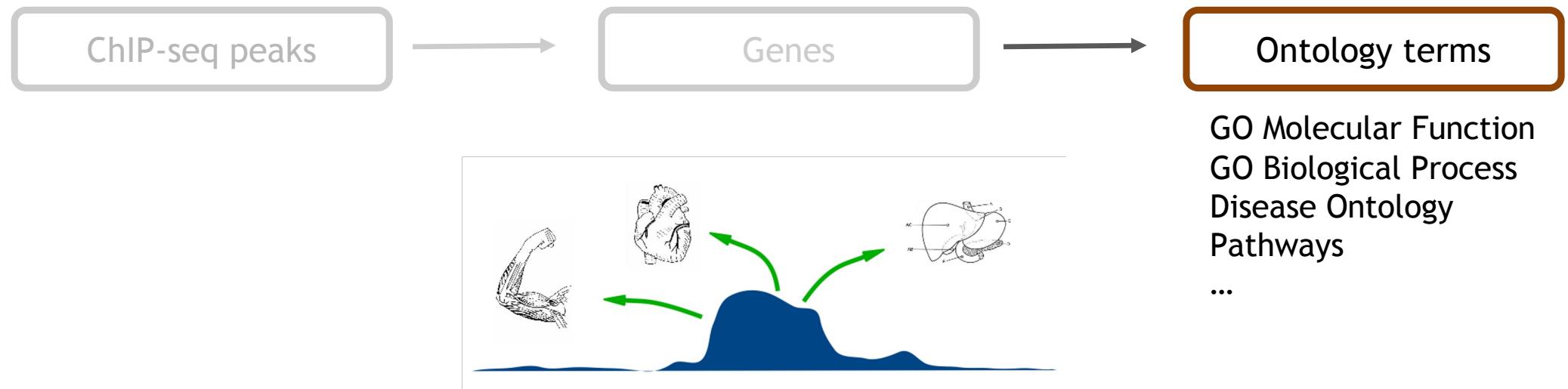
- EnrichR: <http://amp.pharm.mssm.edu/Enrichr/enrich>
- LOLA (Bioconductor) <https://goo.gl/pWDZEs>
- GREAT: <http://great.stanford.edu/public/html/>

Protocol

- Associate peaks to closest genes



Now that we have the genes,
Are there some functional categories over-represented ?



HOMER [Heinz et al., Mol Cell 2010]

Gene Ontology Analysis of Associated Genes: annotatePeaks go option

P-value	LogP	Term	GO Tree	GO ID	# of Genes in Term	# of Target Genes in Term	# of Total Genes	# of Target Genes	Common Genes
2.912e-26	-5.880e+01	immune response	biological process	GO:0006955	349	35	18091	168	Il10,Cd14,Malt1,Ccl2,Ccl7,Ifh1
3.912e-26	-5.850e+01	immune system process	biological process	GO:0002376	679	45	18091	168	S100a9,Egr1,Il10,Cd14,Malt1,C
1.823e-25	-5.696e+01	cytokine activity	molecular function	GO:0005125	178	27	18371	167	Gdf15,Il10,Csf2,Ccl9,Ccl2,Ccl7
3.372e-23	-5.174e+01	defense response	biological process	GO:0006952	430	35	18091	168	Il10,Cd14,Malt1,Nupr1,Ccl2,C

Genome Ontology: Looking for Enriched Genomic Annotations: annotatePeaks genomeOntology option

Total Input Regions (0.936444051404582.pos): 25961, 33798473 bp

P-value	Log P-value	Annotation	Ann Group	#features	Coverage(bp)	AvgFeatureSize[ref=1301]	Overlap(#peaks)	Overlap(bp)	Expected Overlap(bp, gsize=2.00e+09)	Log Ratio Enrichment	Log P-value(+ underrepresented)	P-value
1e-3660	-8428.50	cpgIsland	basic	28691	21842742	761	9426	5545349	369125	2.71	-8428.50	0.00e+00
1e-2673	-6155.47	promoters	basic	44477	30002652	674	8579	5141062	507021	2.32	-6155.47	0.00e+00
1e-1027	-2363.56	utr5	basic	57703	5423448	93	7002	1516346	91652	2.81	-2363.56	0.00e+00
1e-381	-876.84	exons	basic	503529	73292986	145	9092	3171853	1238595	0.94	-876.84	0.00e+00
1e-341	-783.19	protein-coding	basic	483461	66805131	138	8609	2876255	1128955	0.94	-783.19	0.00e+00
1e-105	-239.83	coding	basic	407555	43508461	106	6592	1482965	735259	0.70	-239.83	6.94e-105
1e-71	-162.42	GC_rich Low_complexity Low_complexity	repeats	13724	552081	40	2716	120042	9329	2.55	-162.42	2.89e-71
1e-60	-136.15	miscRNA	basic	11332	4544003	400	592	287477	76790	1.32	-136.15	7.46e-60
3.14e-20	-44.91	tts	basic	44477	28239519	634	1124	718919	477226	0.41	-44.91	3.14e-20
1.21e-10	-22.84	CGGn Simple_repeat Simple_repeat	repeats	1241	71601	57	313	15761	1210	2.57	-22.84	1.21e-10
1.06e-09	-20.66	C-rich Low_complexity Low_complexity	repeats	9534	1007297	105	673	53335	17022	1.14	-20.66	1.06e-09

GREAT

Species Assembly

- Human: GRCh37 ([UCSC hg19, Feb/2009](#))
- Mouse: NCBI build 37 ([UCSC mm9, Jul/2007](#))
- Mouse: NCBI build 38 ([UCSC mm10, Dec/2011](#))
- Zebrafish: Wellcome Trust Zv9 ([danRer7, Jul/2010](#)) [Zebrafish CNE set](#)

[Can I use a different species or assembly?](#)

Test regions

- BED file: Aucun ...hoisi

- BED data:

[What should my test regions file contain?](#)

[How can I create a test set from a UCSC Genome Browser annotation track?](#)

Background regions

- Whole genome

- BED file: Aucun ...hoisi

- BED data:

[When should I use a background set?](#)

[What should my background regions file contain?](#)

Association rule settings

[Show settings »](#)

[Submit](#)

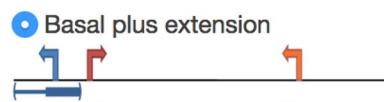
[Reset](#)

Note: Only human (hg19), mouse (mm9, mm10) and zebrafish (danRer7) genomes are supported

GREAT

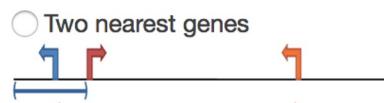
Associating genomic regions with genes

GREAT calculates statistics by associating genomic regions with nearby genes and applying the gene annotations to the regions. Association is a two step process. First, every gene is assigned a regulatory domain. Then, each genomic region is associated with all genes whose regulatory domain it overlaps.



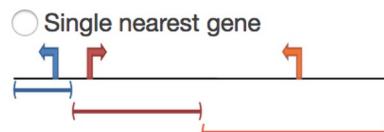
Proximal: 5.0 kb upstream, 1.0 kb downstream, plus Distal: up to 1000.0 kb

Gene regulatory domain definition: Each gene is assigned a basal regulatory domain of a minimum distance upstream and downstream of the TSS (regardless of other nearby genes). The gene regulatory domain is extended in both directions to the nearest gene's basal domain but no more than the maximum extension in one direction.



within 1000.0 kb

Gene regulatory domain definition: Each gene is assigned a regulatory domain that extends in both directions to the nearest gene's TSS but no more than the maximum extension in one direction.



within 1000.0 kb

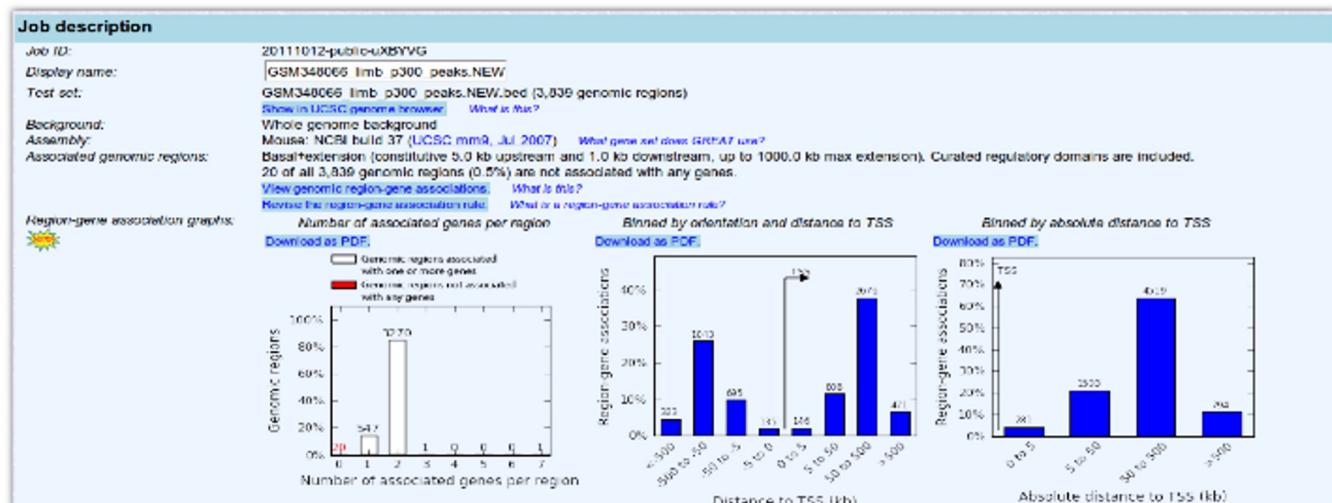
Gene regulatory domain definition: Each gene is assigned a regulatory domain that extends in both directions to the midpoint between the gene's TSS and the nearest gene's TSS but no more than the maximum extension in one direction.

↖ Gene Transcription Start Site (TSS)

Note: Only human (hg19), mouse (mm9, mm10) and zebrafish (danRer7) genomes are supported

GREAT

- Input
 - bed file with peaks
- Output
 - Enriched GO terms and functions



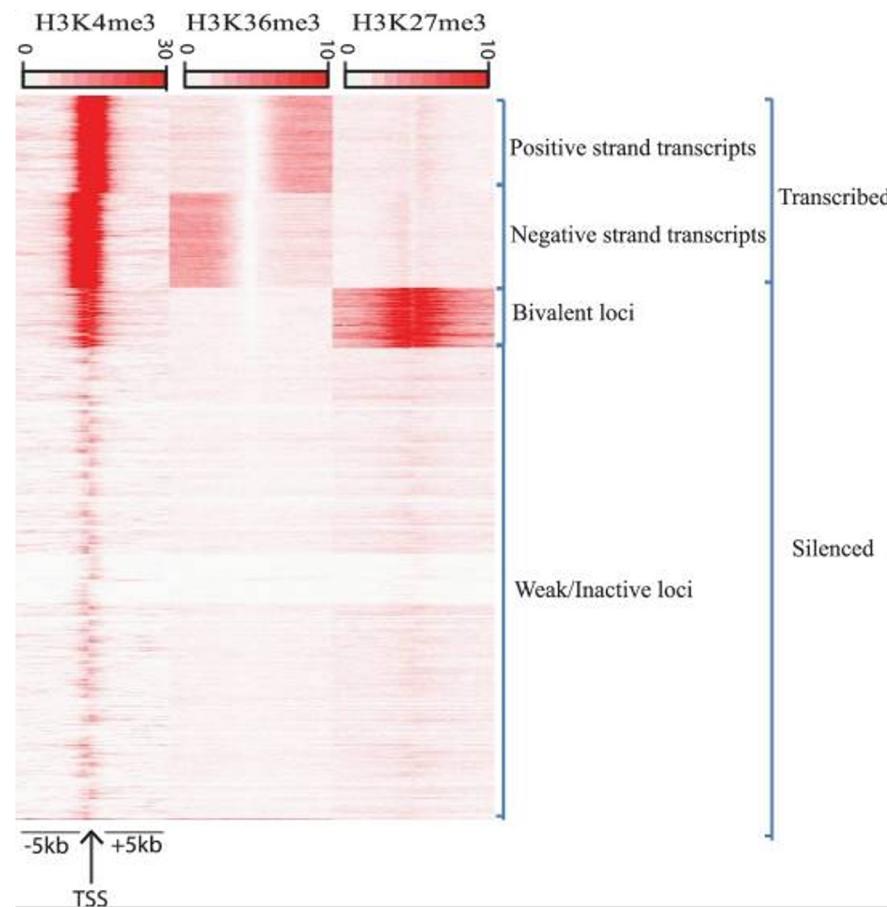
X Mouse Phenotype

Table controls: Export | Shown top rows in this table: 20 | Set | Term annotation count: Min: 1 Max: Inf | Set | Global Controls

Term Name	Binom Rank	Binom Raw P-Value	Binom FDR Q-Val	Binom Fold Enrichment	Binom Observed Region Hits	Binom Region Set Coverage	Hyper Rank	Hyper FDR Q-Val	Hyper Fold Enrichment	Hyper Observed Gene Hits	Hyper Total Genes	Hyper Gene Set Coverage
abnormal limbs/digits/tail morphology	2	2.0559e-91	6.6837e-88	2.1465	780	20.32%	6	2.5295e-40	2.2020	278	681	8.31%
abnormal craniofacial morphology	3	9.3822e-91	2.0334e-87	2.0082	697	23.10%	10	8.8231e-36	2.0382	297	786	8.86%
abnormal limb morphology	5	2.4990e-80	3.2497e-77	2.3077	604	15.73%	9	7.4787e-37	2.4341	292	444	6.04%
abnormal appendicular skeleton morphology	10	3.0255e-70	1.9672e-67	2.3450	517	13.47%	17	3.0549e-30	2.4098	172	385	5.14%
abnormal skeleton extremities morphology	12	3.2687e-69	1.7711e-66	2.3724	499	13.00%	21	7.0557e-29	2.4222	163	363	4.87%
abnormal paw/hand/foot morphology	13	4.0300e-69	2.0156e-66	2.6813	404	10.52%	23	5.4818e-28	2.7186	126	250	3.77%
abnormal head morphology	14	6.4657e-67	3.0028e-64	2.0134	672	17.50%	25	2.9042e-27	2.0562	223	585	6.67%
abnormal digit morphology	18	1.0543e-61	3.8084e-59	2.6982	358	9.33%	36	1.2033e-25	2.7998	109	210	3.26%
abnormal cartilage morphology	23	7.3728e-58	2.0843e-55	2.3432	430	11.20%	29	1.1337e-26	2.5089	140	301	4.19%
abnormal skeleton development	24	3.5769e-56	9.6904e-54	2.0833	530	13.81%	38	5.2377e-25	2.1414	185	466	5.53%
abnormal long bone morphology	25	4.6593e-56	1.2118e-53	2.3374	419	10.91%	43	4.9983e-24	2.3823	140	317	4.19%

Other analyses

- Clustering peaks
(Deeptools, HOMER, seqMINER)



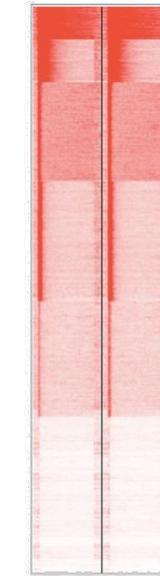
Ye et al, 2011

The darker the red the higher the read enrichment

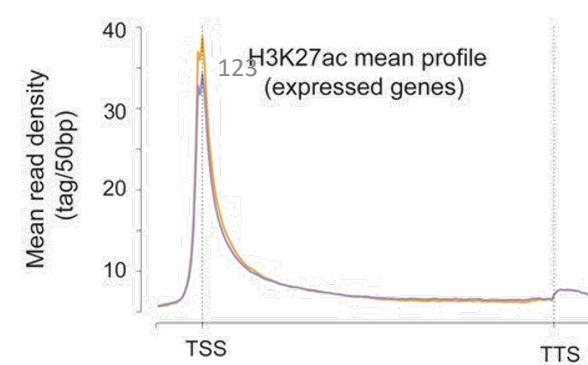
Meta-profiles / Clustering

- Global visualization of the data
- Need:
 - Regions of interest
 - Regions around a reference point e.g all TSS +/- 1Kb,...
 - Scaled regions e.g peaks, gene bodies,...
 - Signal data (mapped reads)
- Data can be grouped together with clustering methods such as k-means.

Heatmap

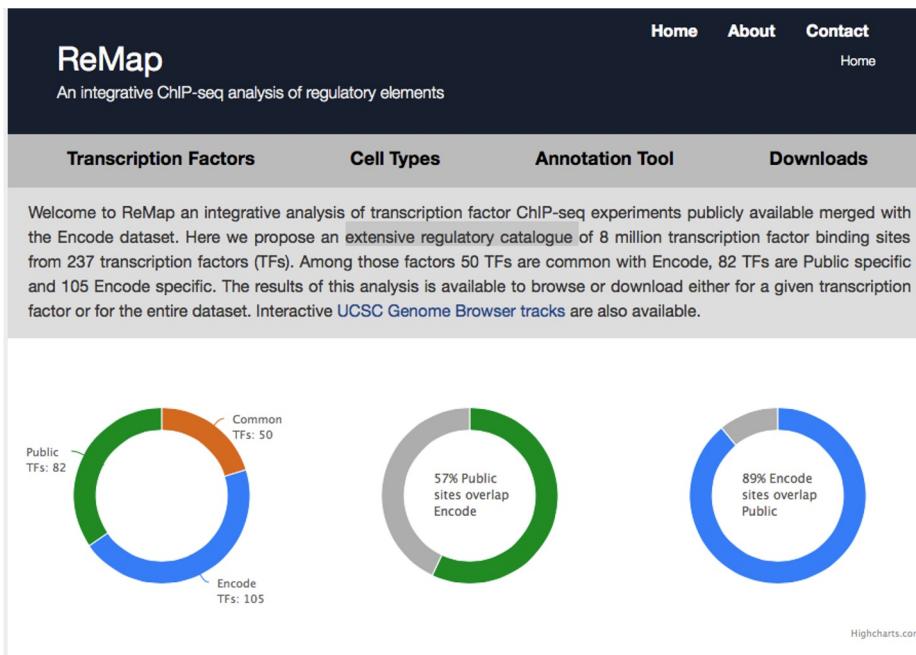


Mean profile

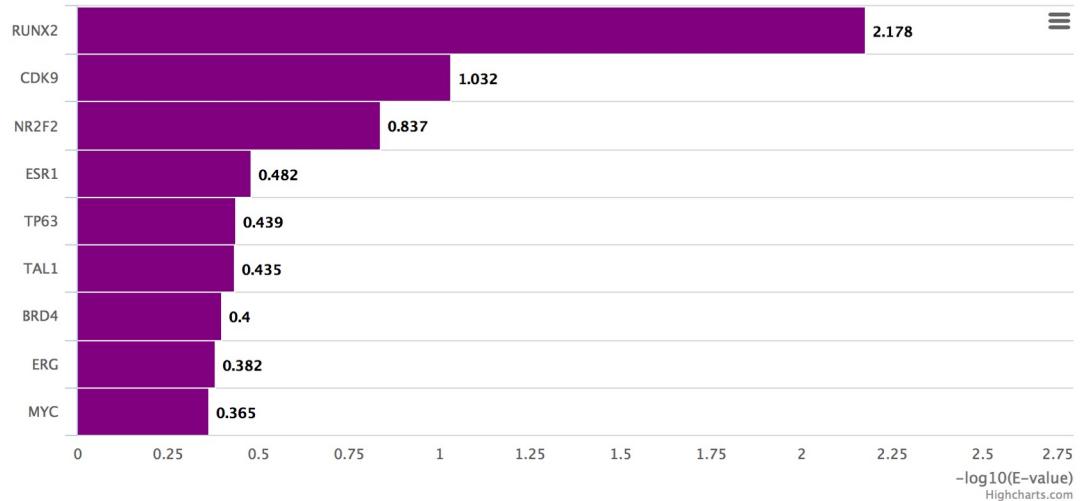


Other analyses

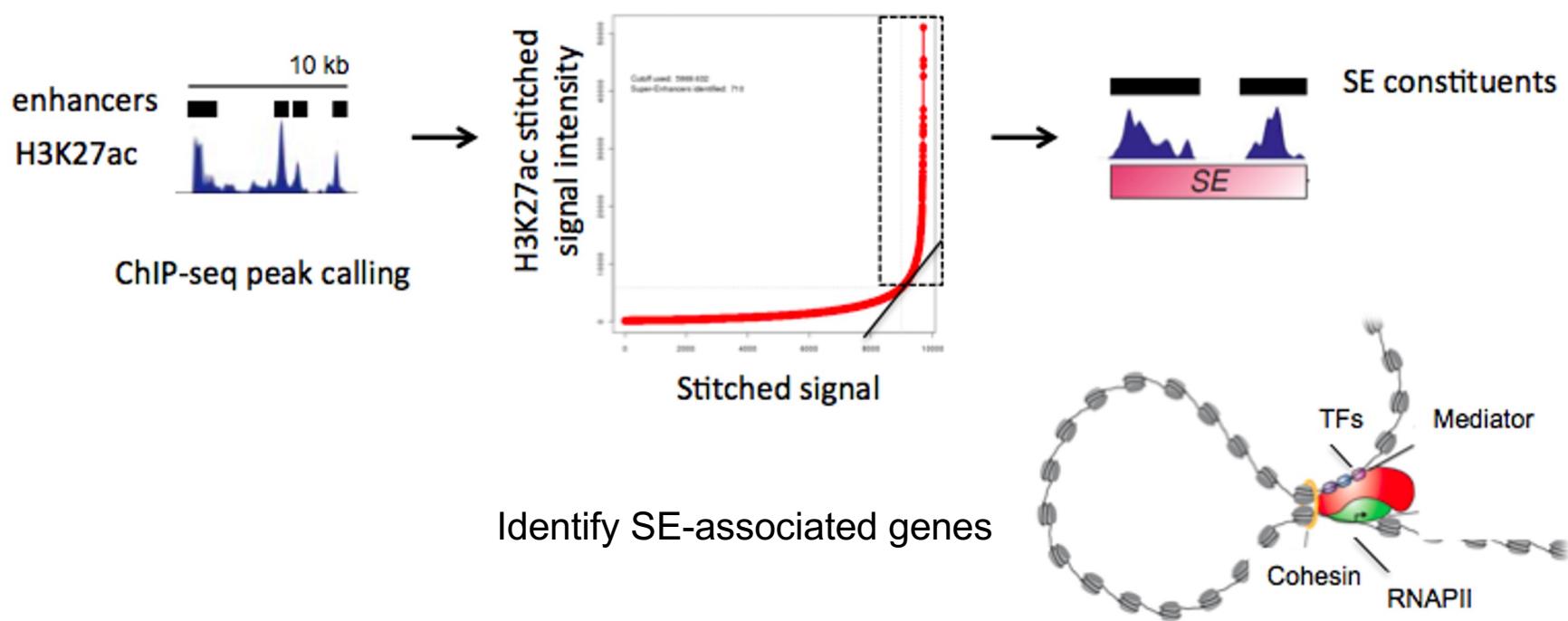
- ReMAP (<http://tagc.univ-mrs.fr/remap/>)
 - Is my peak dataset enriched for known TF peaks?



Enriched TFs in intersection

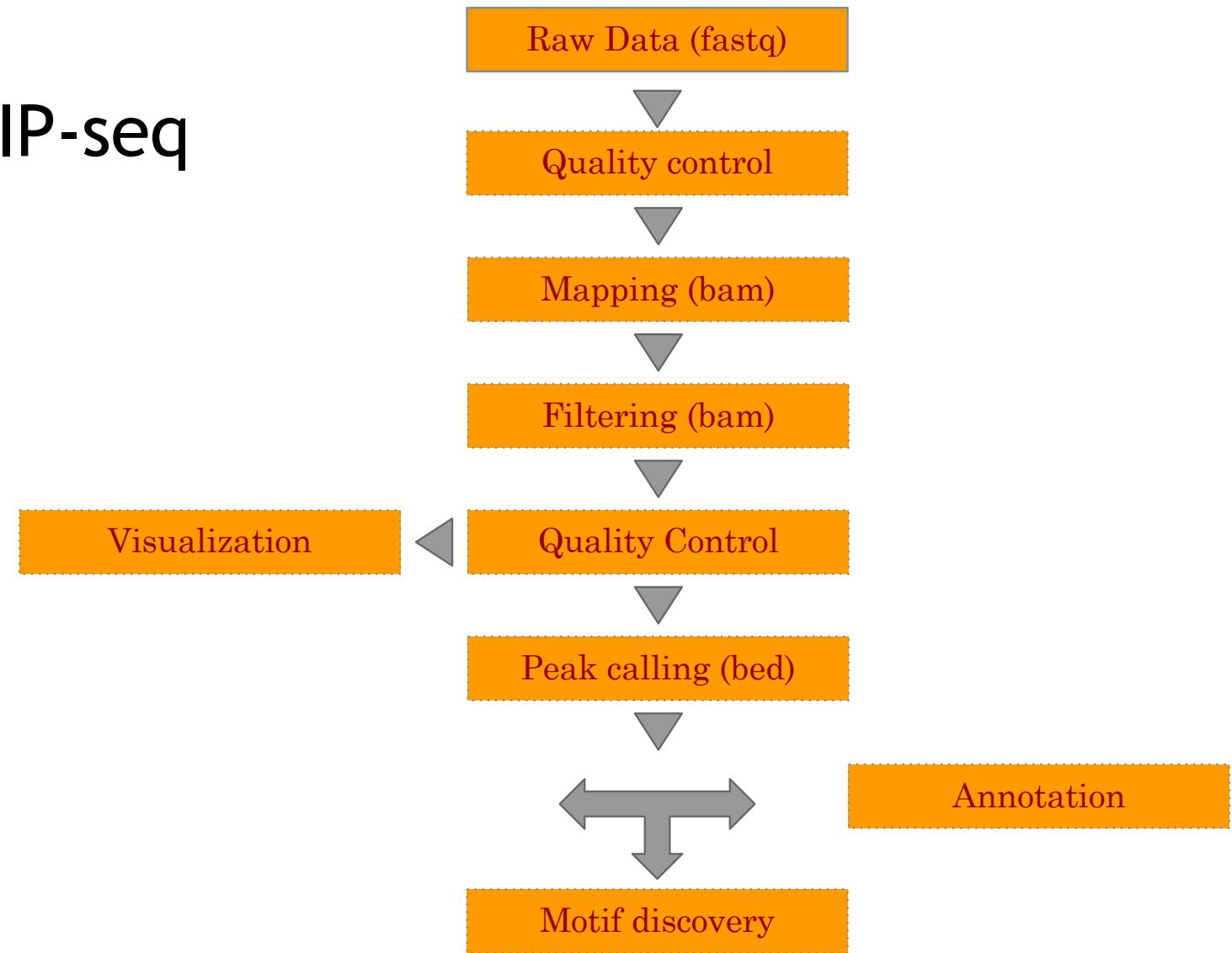


Identification of Super-Enhancers from ChIP-seq peaks with ROSE [Loven et al., Cell 2013]

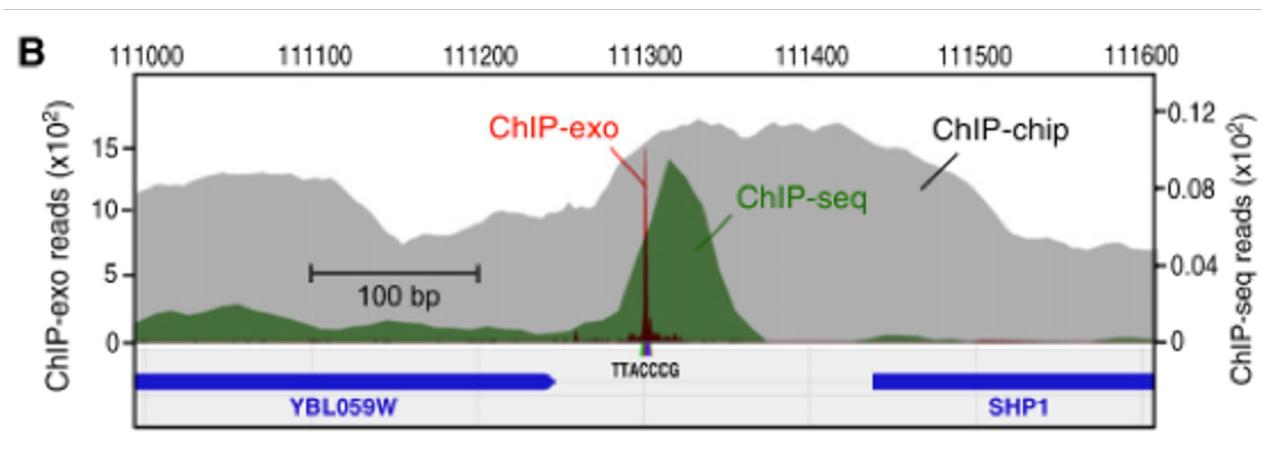


https://bitbucket.org/young_computation/rose

Overview of ChIP-seq Pipeline



Beyond ChIP-seq : ChIP-exo



Rhee, H. S., & Pugh, B. F. (2011).

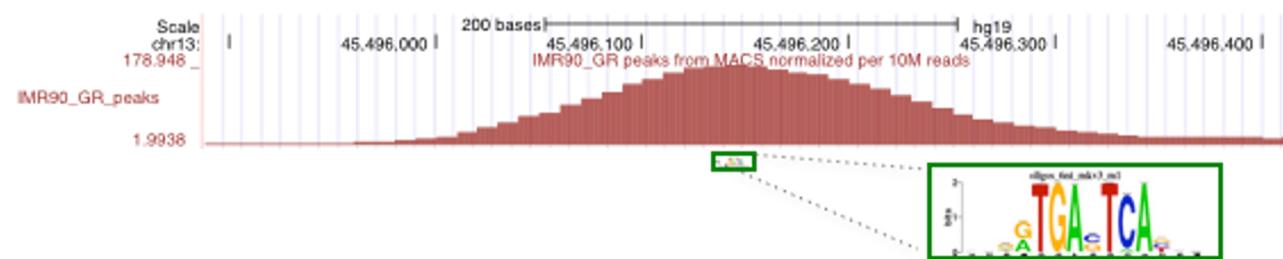
Beyond ChIP-seq

Experimental techniques



Improvement aimed

higher resolution => 300bp to 1bp



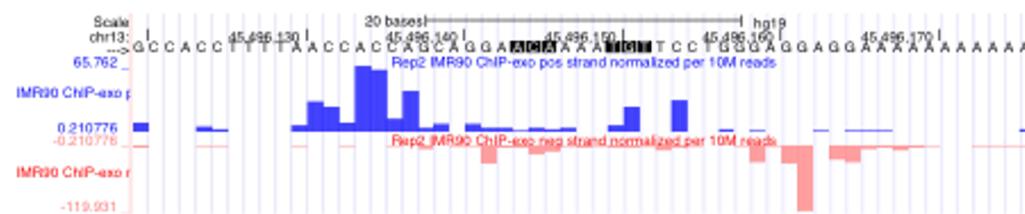
Beyond ChIP-seq : ChIP-nexus

Experimental techniques



Improvement aimed

Get rid of PCR artifacts



Beyond ChIP-seq : native ChIP

Experimental techniques

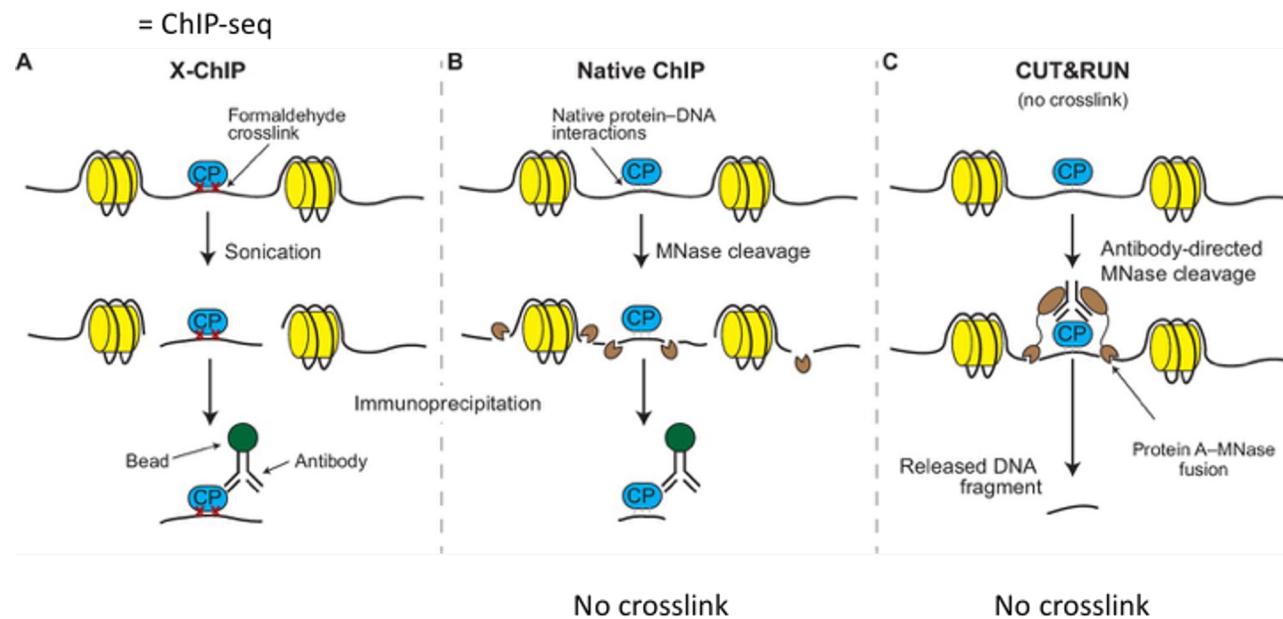


Improvement aimed

Avoid formaldehyde crosslinking

- Formaldehyde crosslinking affects preferentially protein-protein interactions.
- Crosslinking could be the cause of hyper-signaling regions in highly transcribed sites.

Beyond ChIP-seq : native ChIP



CUT&RUN uses the antibodies to guide the cutting activity of the MNase enzyme rather than physically separate wanted from unwanted chromatin fragments

Beyond ChIP-seq : low-input and single-cell

Experimental techniques



Improvement aimed

Reduce the amount of starting material (precious samples)

- Low-input: Optimized ChIP-seq protocols => 100-500 cells

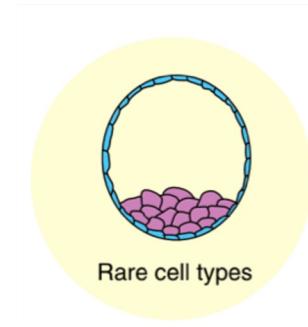
Dahl & Gilfillan, Briefings in Functional Genomics, 2017

- Single-cell ChIP-seq : Only one proof-of-concept study, very low coverage

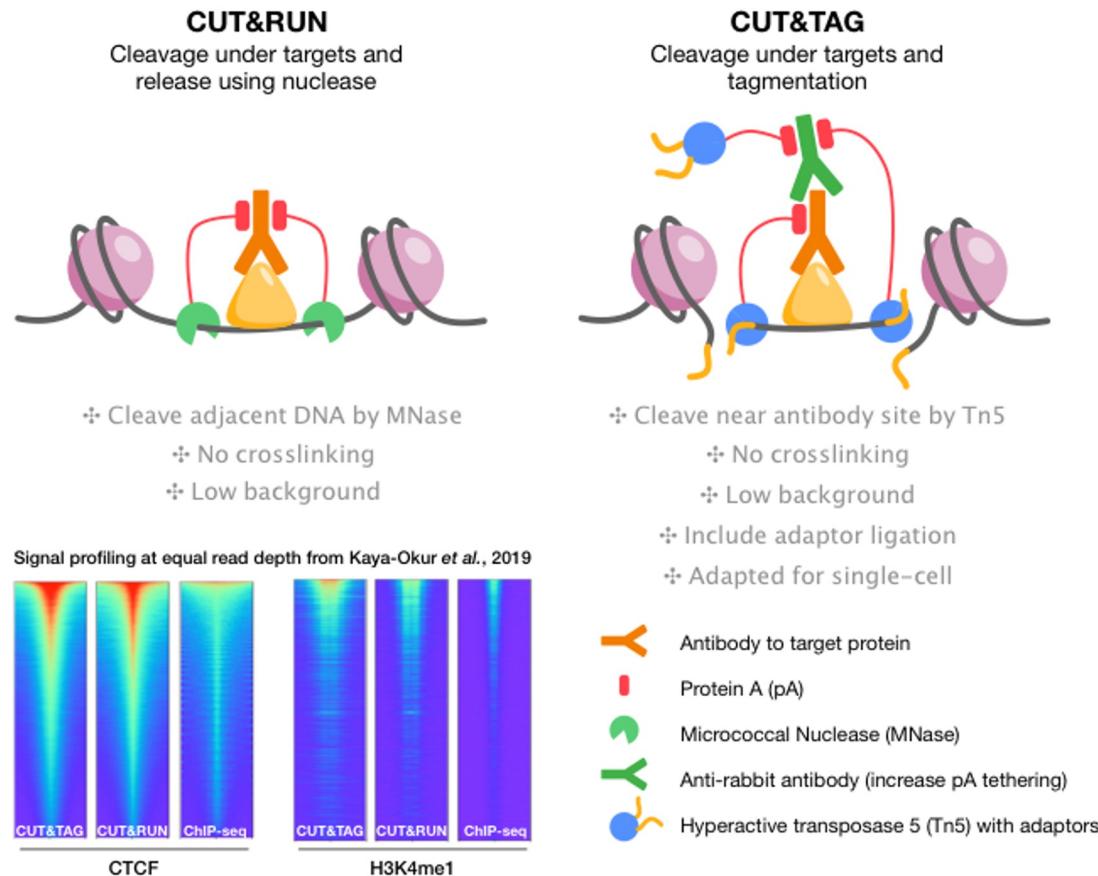
Rotem et al, Nature Biotechnology, 2015

More recent proof-of-concept

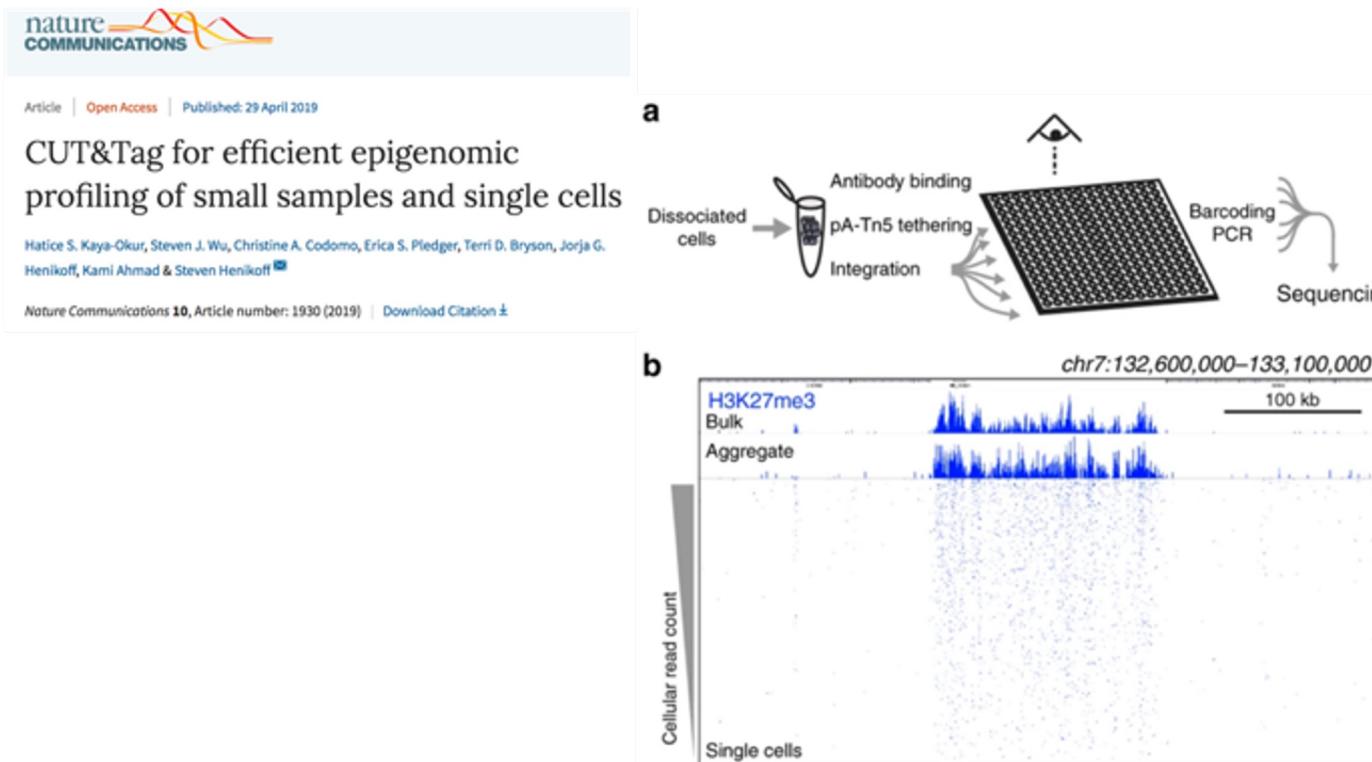
Grosselin et al, Nature Genetics, 2019



Beyond ChIP-seq : Cut&TAG (2019)



Beyond ChIP-seq : Cut&TAG (2019)



Low background => 3 Million reads sufficient for human....

ATAC-seq

Assay for Transposase-Accessible Chromatin with highthroughput sequencing

Chromatin accessibility assays

- Chromatin accessibility is the degree to which nuclear macromolecules are able to physically contact chromatinized DNA and is determined by the occupancy and topological organization of nucleosomes as well as other chromatin-binding factors that occlude access to DNA (Klemm et al, 2019)
- Open chromatin regions contains generally transcriptionally active genes
- The accessible genome comprises ~2–3% of total DNA sequence yet captures more than 90% of regions bound by TFs (Thurman et al, 2012)
- Chromatin accessibility is measured by quantifying the susceptibility of chromatin to either enzymatic methylation or cleavage of its constituent DNA
- Chromatin accessibility assays (non exhaustive list)
 - FAIRE-seq
 - DNase-seq
 - MNase-seq
 - ATAC-seq

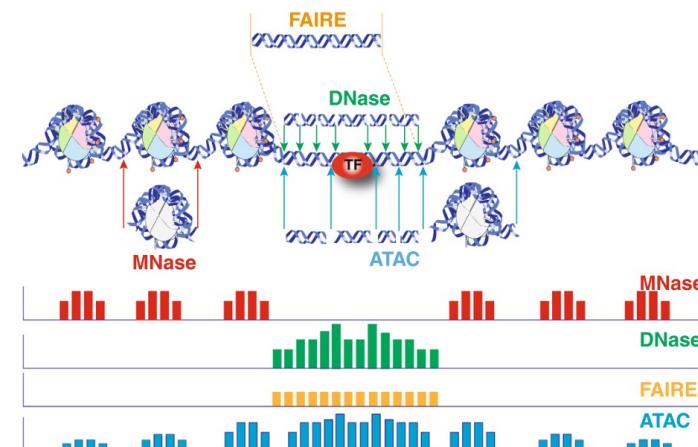
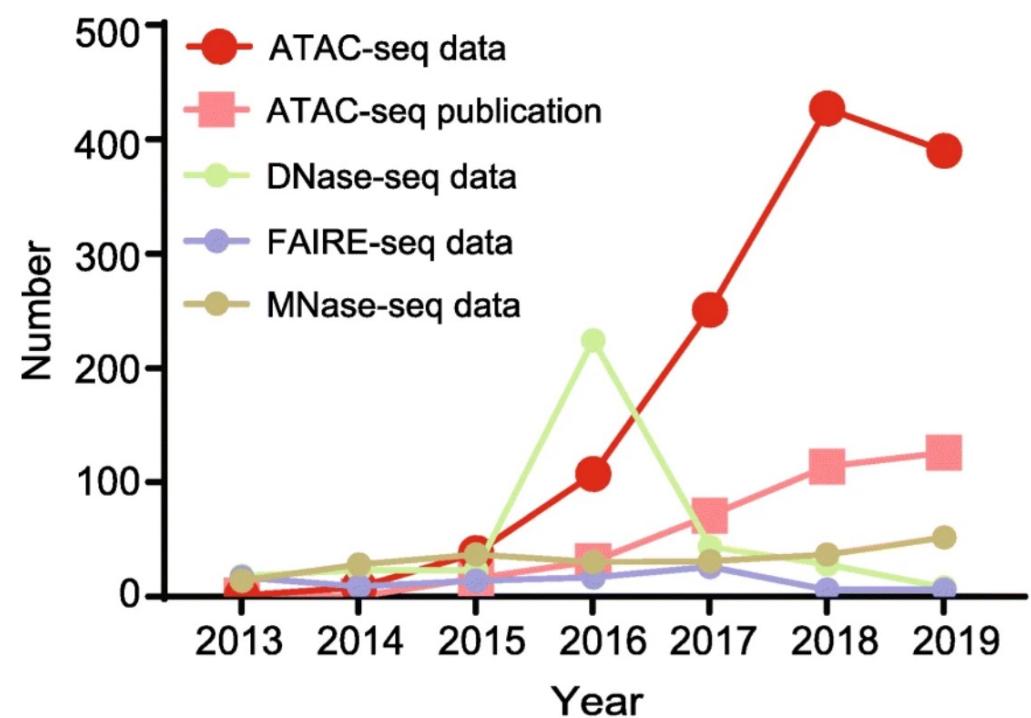


Figure 1 Schematic diagram of current chromatin accessibility assays performed with typical experimental conditions. Representative DNA fragments generated by each assay are shown, with end locations within chromatin defined by colored arrows. Bar diagrams represent data signal obtained from each assay across the entire region. The footprint created by a transcription factor (TF) is shown for ATAC-seq and DNase-seq experiments.

(Tsompana and Buck, 2014)

Chromatin accessibility assays

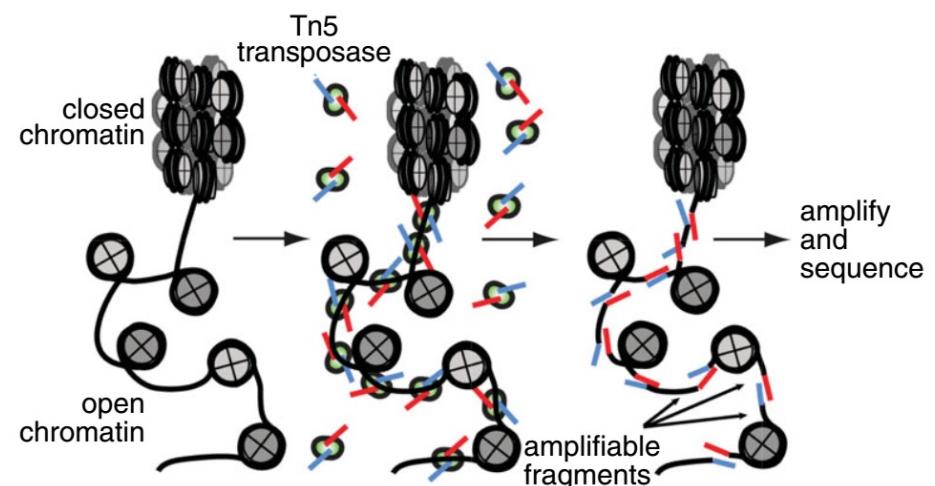
- ATAC-seq has become the most widely used method to detect and analyze open chromatin regions



Yan et al, 2020

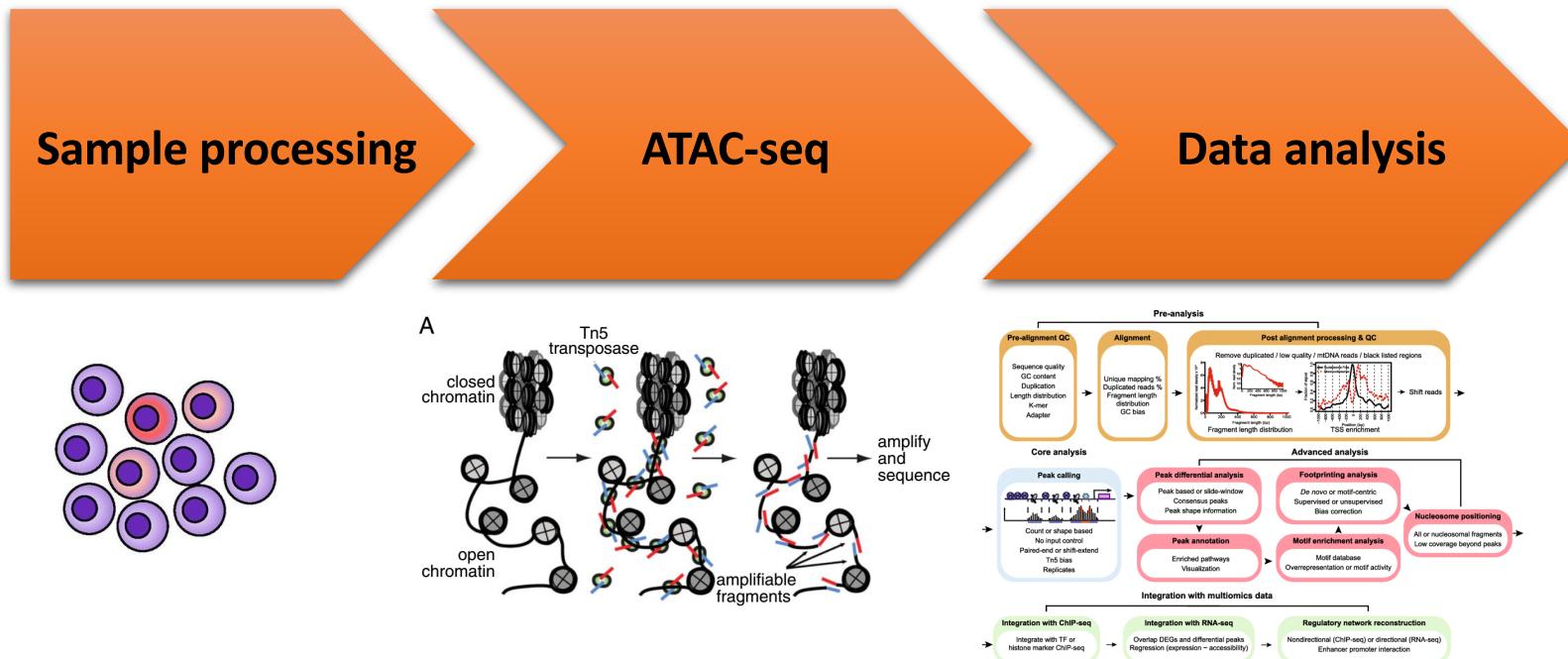
ATAC-seq

- Buenrostro et al, 2013
- ATAC-seq is a method for determining chromatin accessibility across the genome
- Transcription factor binding sites and positions of nucleosomes can be identified from the analysis of ATAC-Seq
- Advantages of ATAC-seq over other chromatin accessibility assays:
 - The sensitivity and specificity are comparable to DNase-seq but superior to FAIRE-seq
 - Straightforward and rapidly implemented protocol. ATAC-seq libraries can be generated in less than 3 hours
 - Low number of cells required (500 - 50,000 cells)
 - single-cell ATAC-seq (scATAC-seq) protocol (Cusanovich et al, 2015)

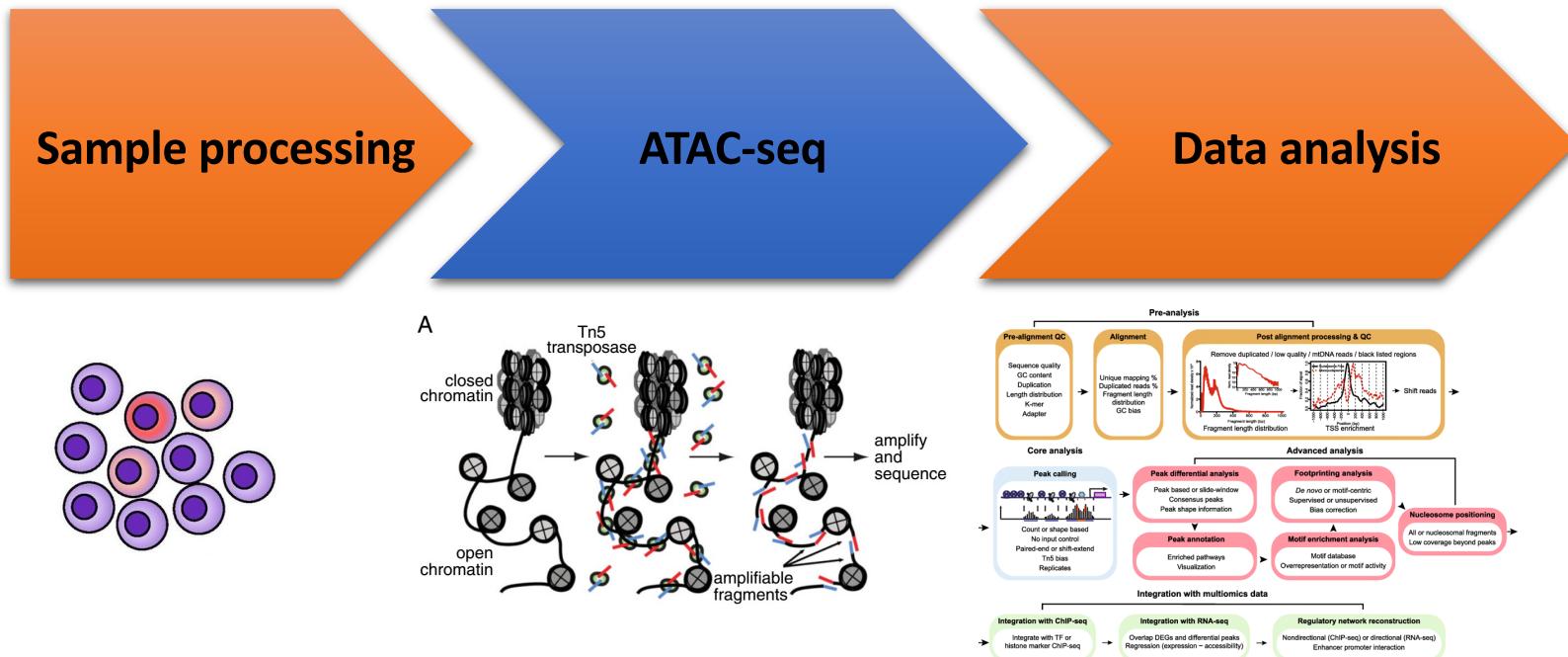


(Buenrostro et al., 2015).

ATAC-seq process

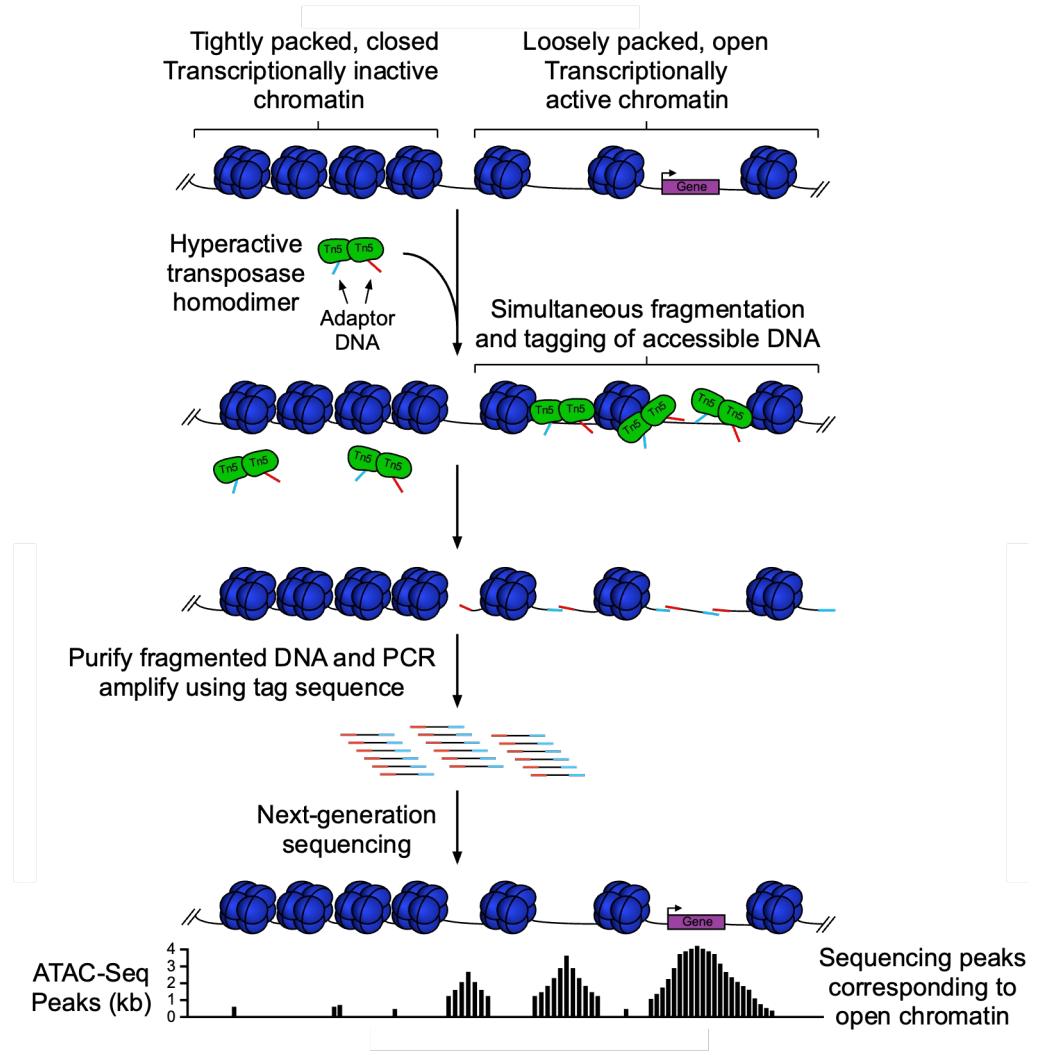


ATAC-seq



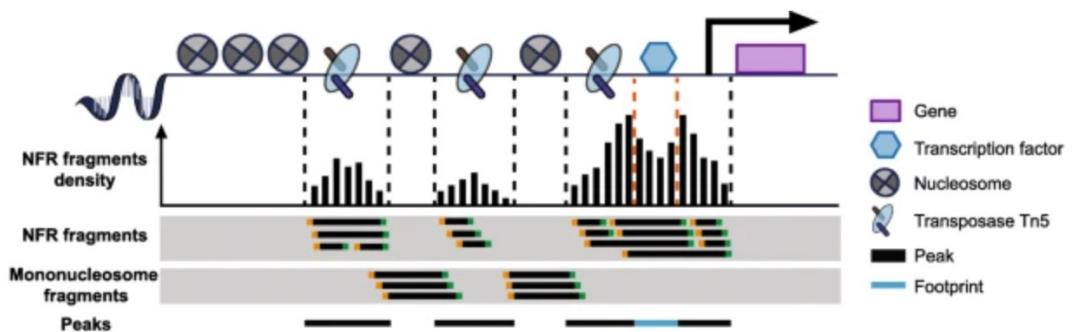
ATAC-seq

- ATAC-seq protocol utilizes a hyperactive Tn5 transposase to insert sequencing adapters into open chromatin regions
- In a process called "tagmentation", Tn5 transposase cleaves and tags double-stranded DNA with sequencing adaptors
- No additional library prep is needed
- Expected results are enrichments of sequenced reads in open chromatin regions as closed chromatin regions, DNA regions bound by TFs or wrapped around nucleosomes should be protected from Tn5 cleavage activity.

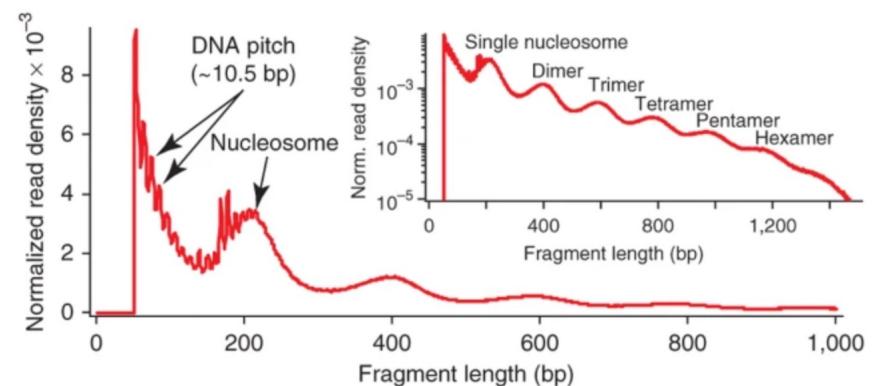


ATAC-seq

- Paired-end sequencing so that by looking at the distance between the two reads of a pair, we know in which the chromatin environment (Nucleosome Free Region (NFR), around a mono, di,-nucleosome, around a TF) of the DNA fragment.

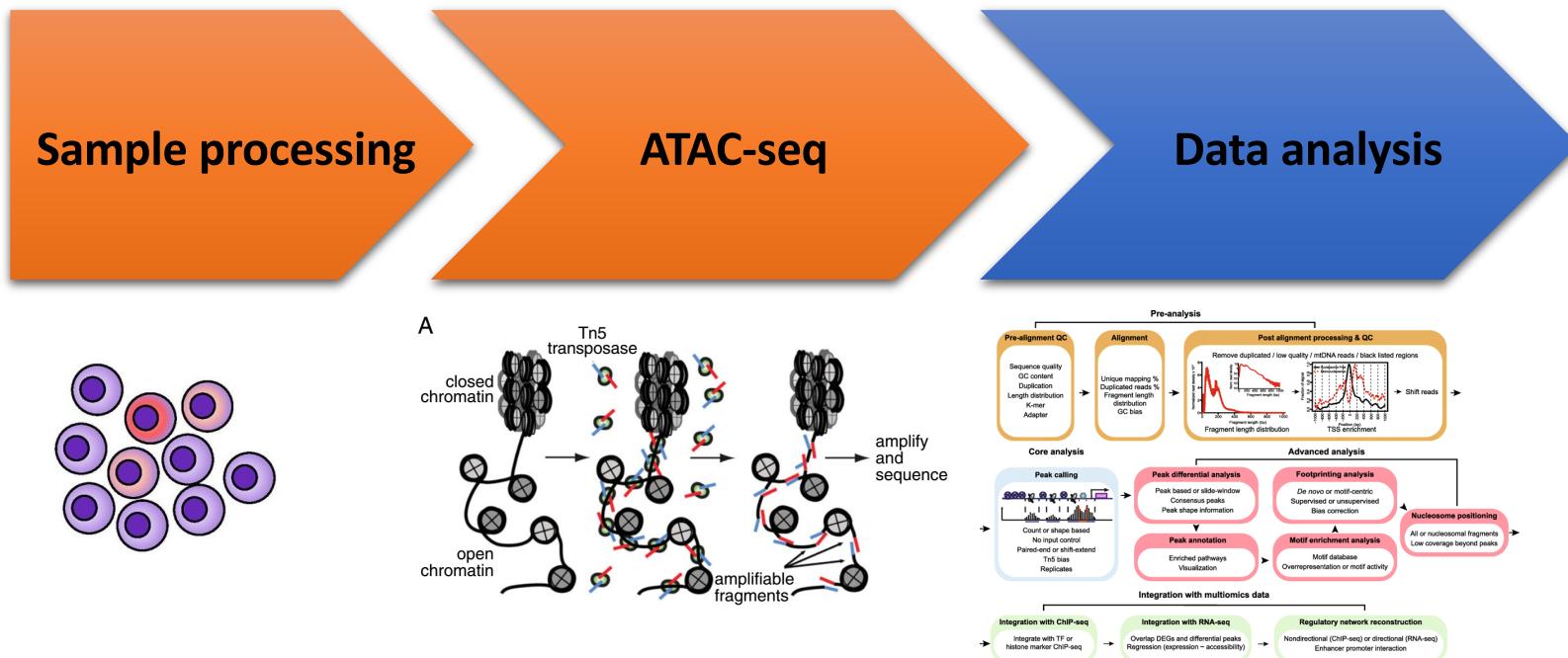


Yan et al, 2020

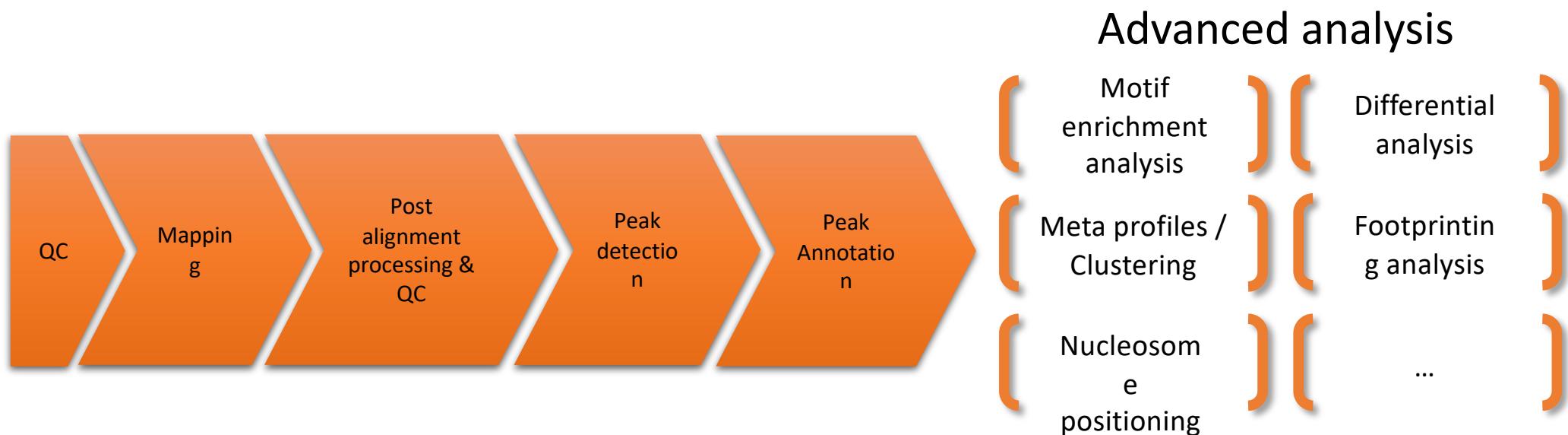


Buenrostro et al, 2023 ¹⁴²

Analysis of ATAC-seq data

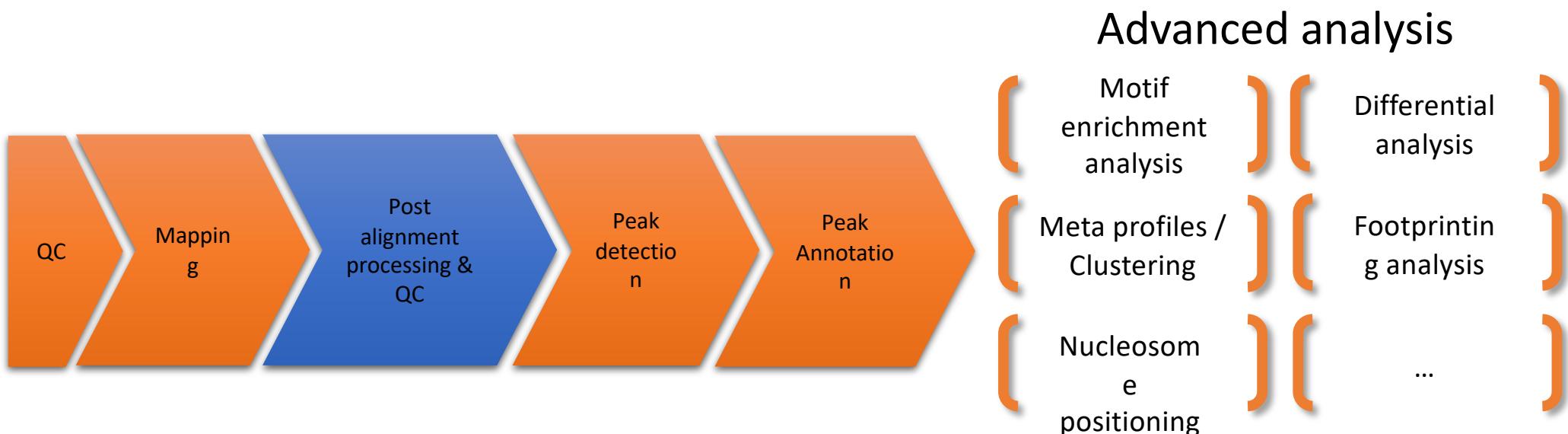


Analysis of ATAC-seq data



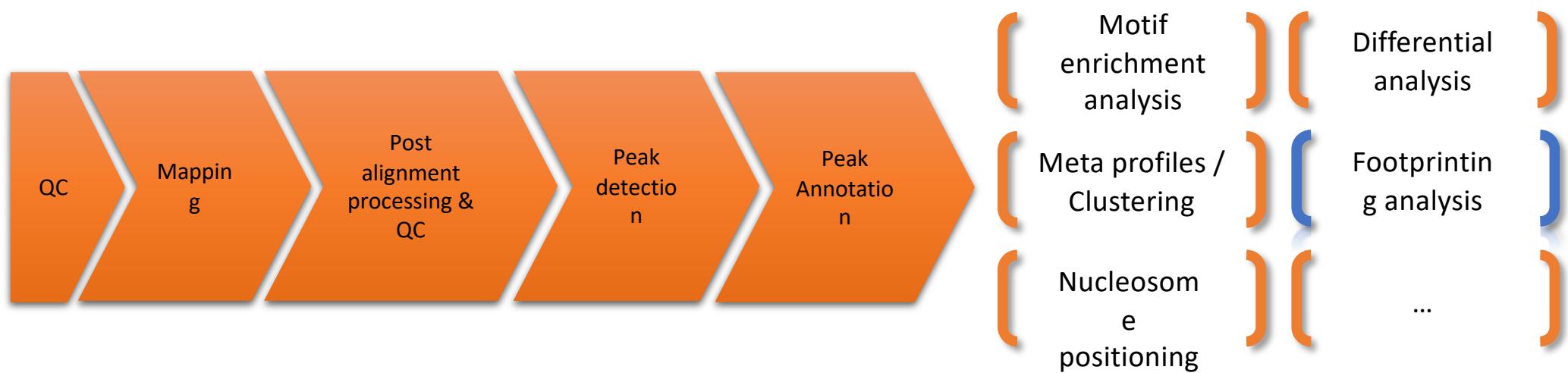
- Overall analysis resemble ChIP-seq data analysis
- Description of particularities of ATAC-seq data analysis

Analysis of ATAC-seq data



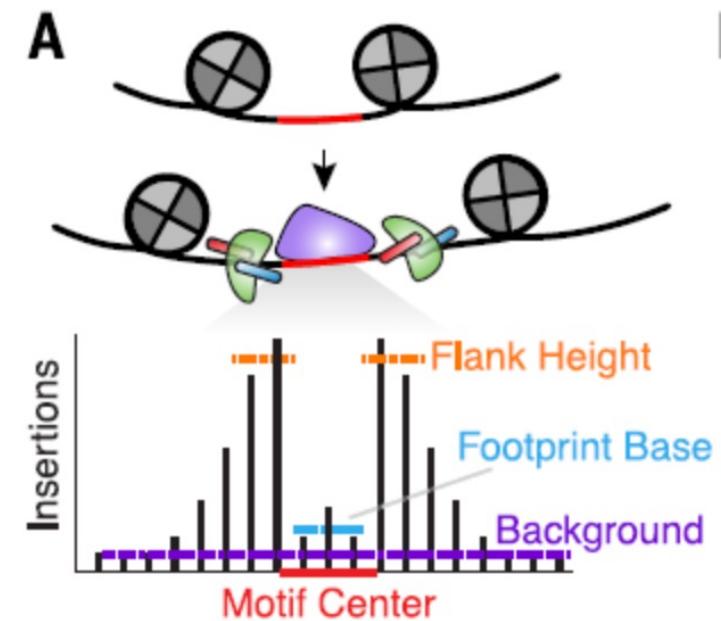
- Some cleaning steps are required for ATAC-seq. For example:
 - A large percentage of reads are derived from mitochondrial DNA. These reads are removed as mitochondrial genome is generally not of interest.
 - Omni-ATAC (Corces et al, 2017)

Analysis of ATAC-seq data



Footprinting analysis

- Tn5 cuts in open chromatin regions
- DNA is protected from cleavage at position of TF binding creating a “notch” in ATAC-seq signal
- Footprinting analysis identifies TF activities
 - Height of the notch reflects TF activity
 - Compare TF activity between different conditions



Corces et al, 2019

Footprinting analysis

- Volcano plots showing differential TF binding activity as predicted by TOBIAS footprinting analysis in ATAC-seq data of NKp, iNK and mNK from Shin et al. (c) iNK vs NKp; (d) mNK vs NKp; (e) mNK vs iNK.
- Each dot represents a TF
- TFs whose activity is changing between the two compared developmental stages are colored (see color legend below volcano plots)

