Introduction to Chromatin IP – sequencing (ChIP-seq) data analysis

Introduction to Bioinformatics using NGS data

Linköping, 21 April 2016

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BILS Bioinformatics Infrastructure for Life Sciences

SciLifeLab

NBIS NATIONAL BIOINFORMATICS INFRASTRUCTURE SWEDEN

Chromatin state and gene expression

PEV
Position effect variegation
in Drosophila eye
(nature.com)

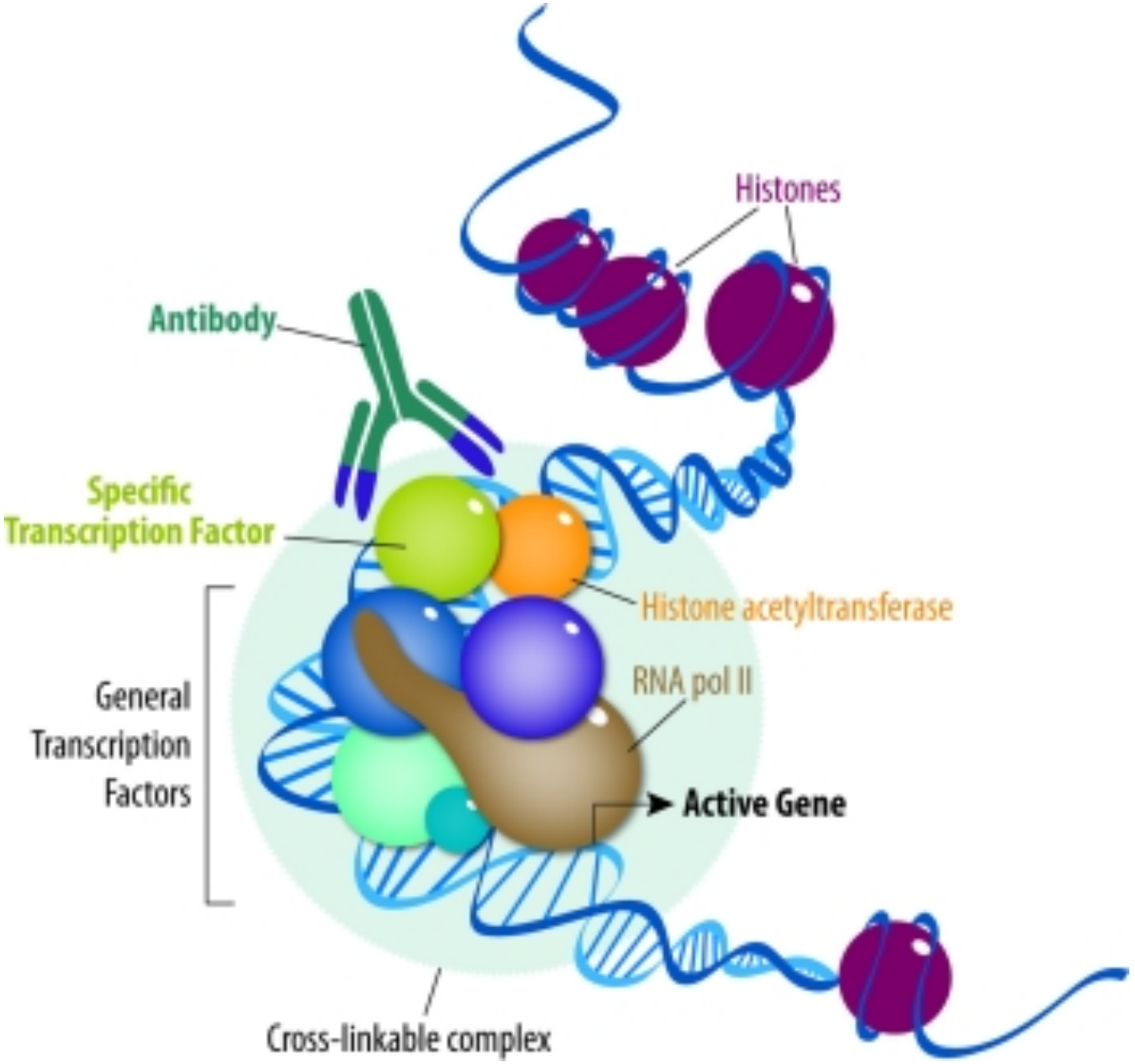
First observed by
H. Muller
1930

Juxtaposition of eye colour genes with heterochromatin results in the “mottled” eye colouration (red and white).

Proteins, which bind heterochromatin, act to “spread” the silencing signal by providing a forward feedback loop.

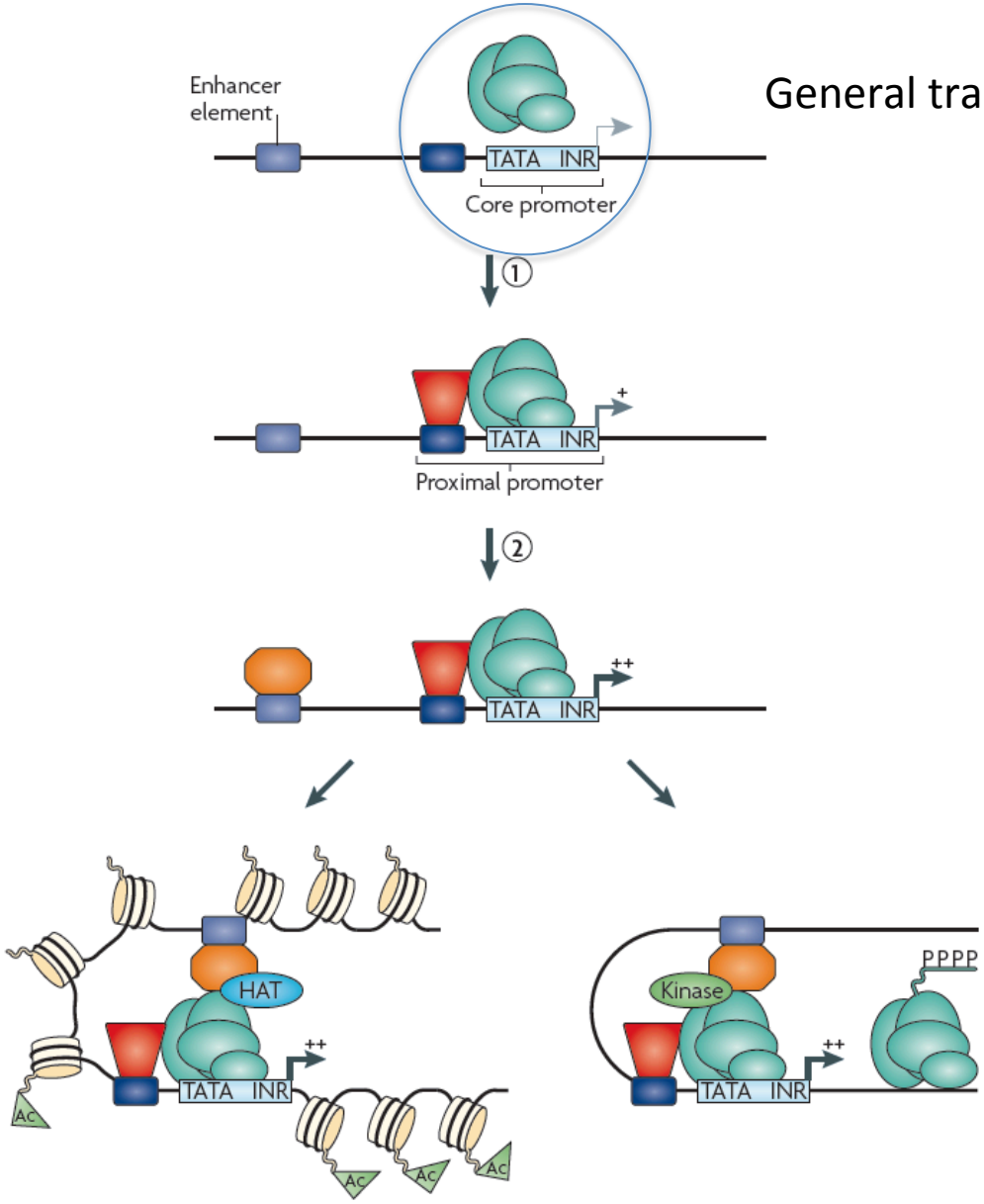
Heterochromatin Protein 1; Histone methyltransferase Su(var)3-9; H3K9 methylation

Chromatin immunoprecipitation



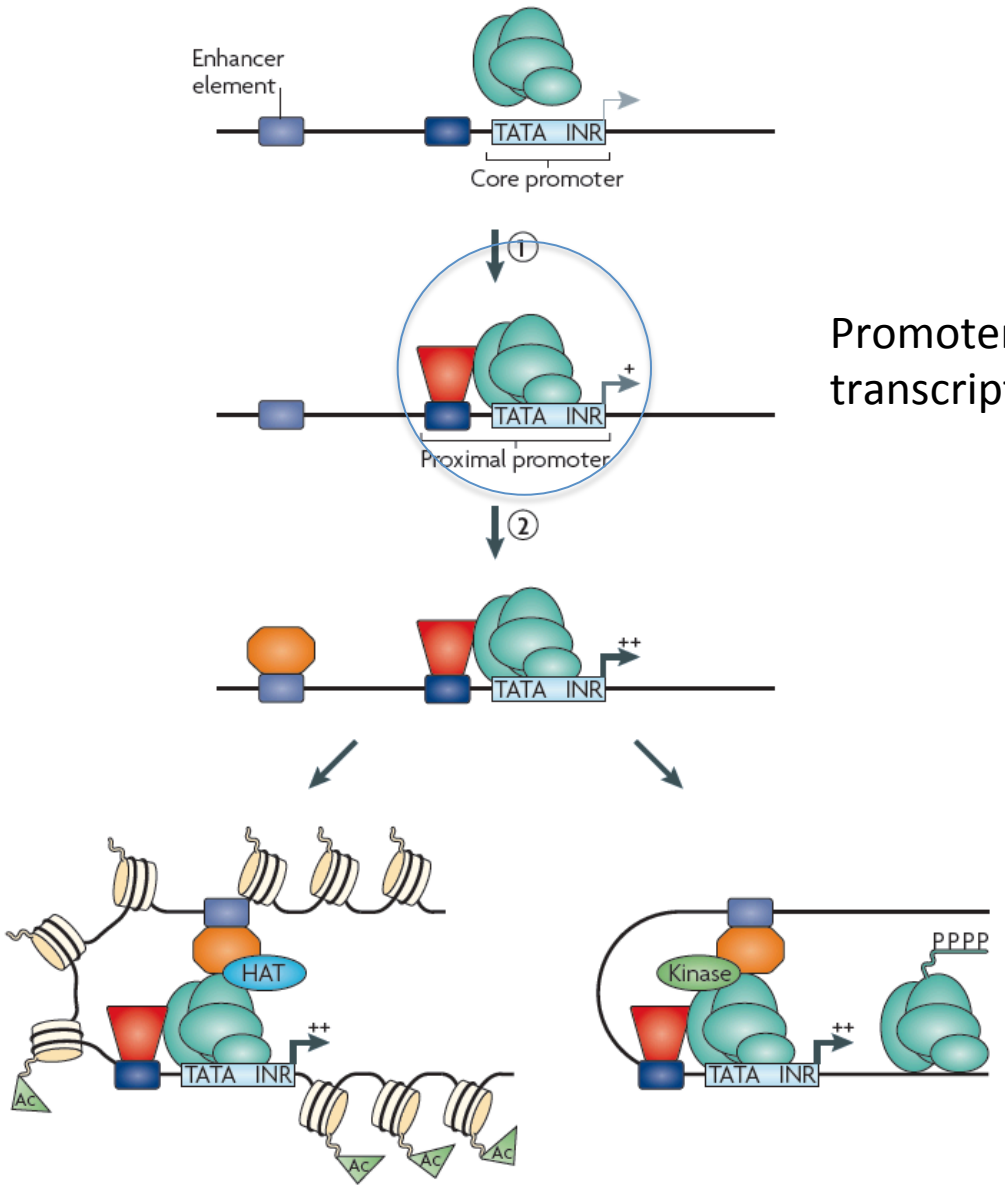
RnDsystems

Applications



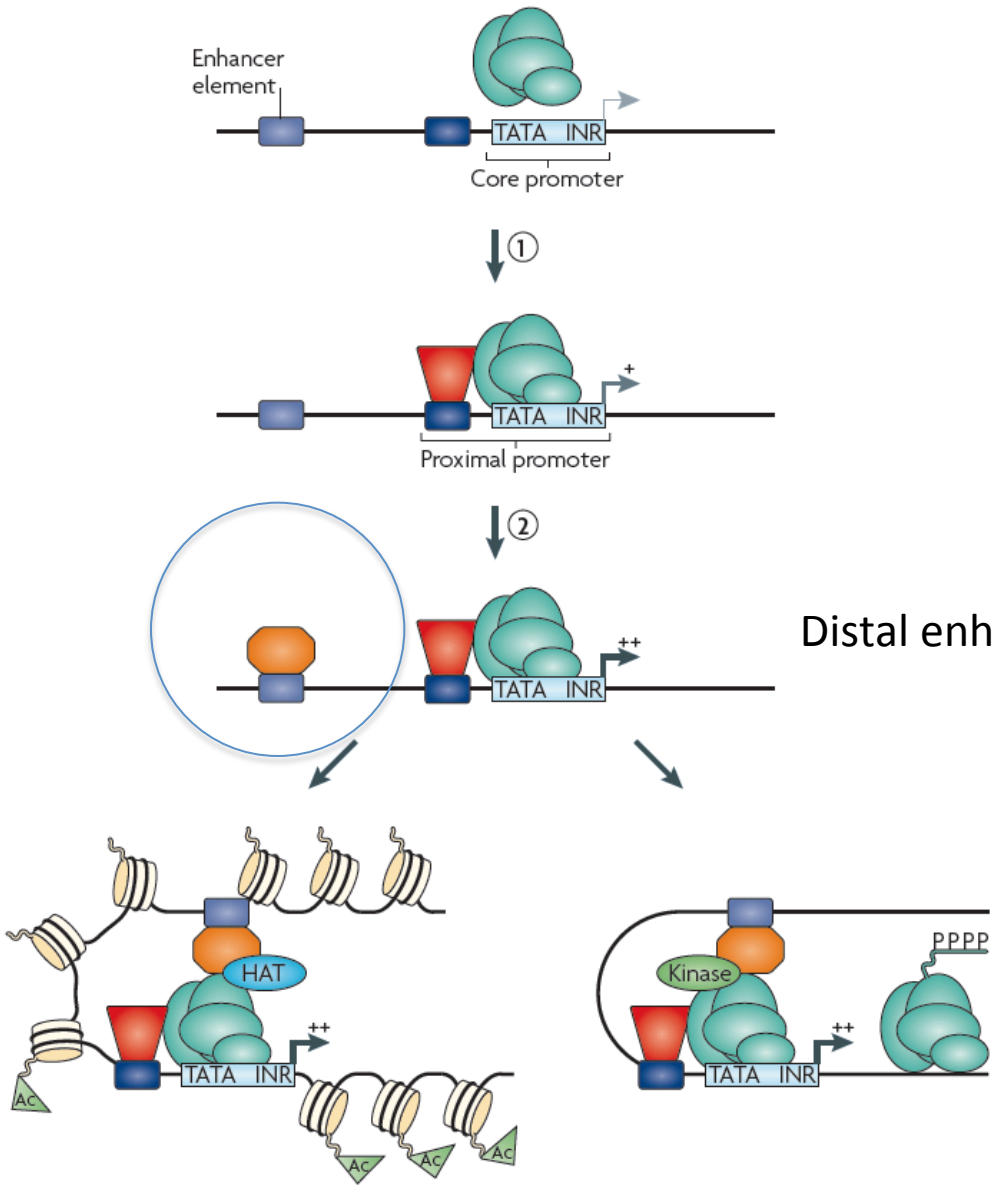
General transcription machinery

Applications



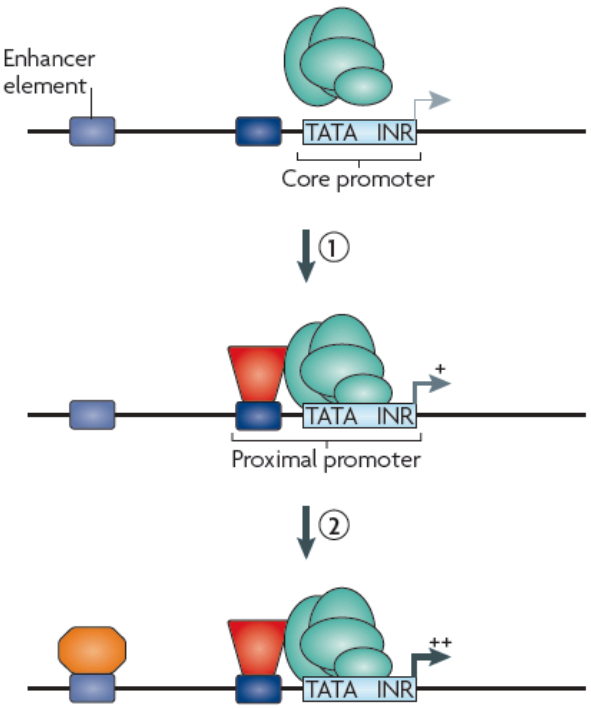
Promoter-associated transcription factors

Applications

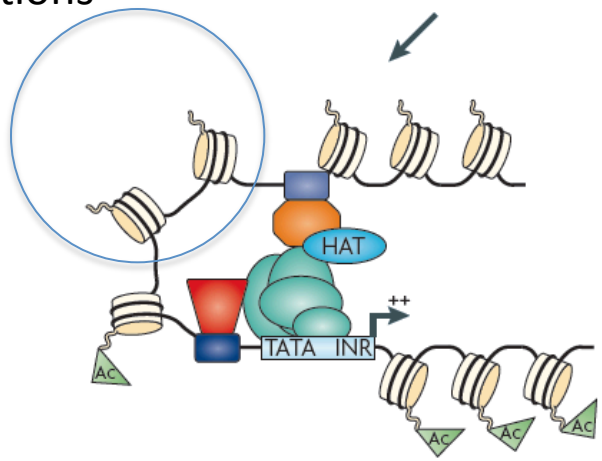


Distal enhancers

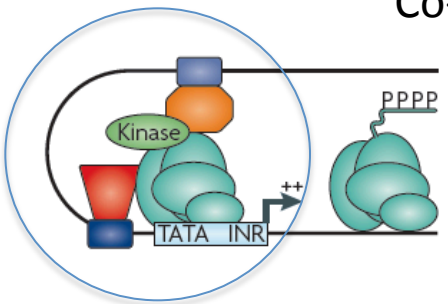
Applications



Histone modifications and variants



Activation states
Co-factors



Workflow of a ChIP-seq study

design study

obtain input chromatin

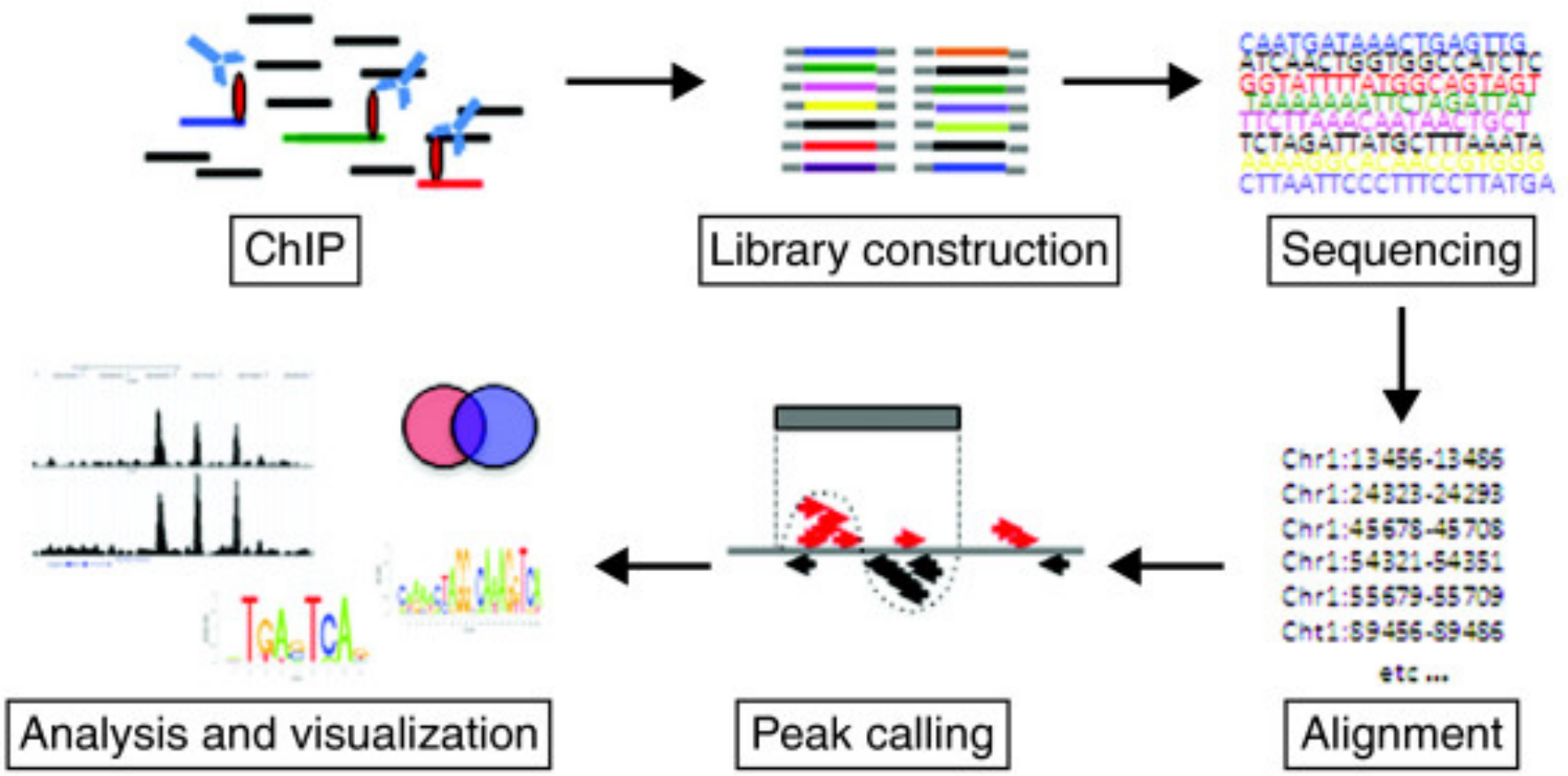
perform precipitation

construct library

sequence library

bioinformatic analysis

ChIP-seq workflow



Liu, Pott and Huss, BMC Biology 2010

Critical factors

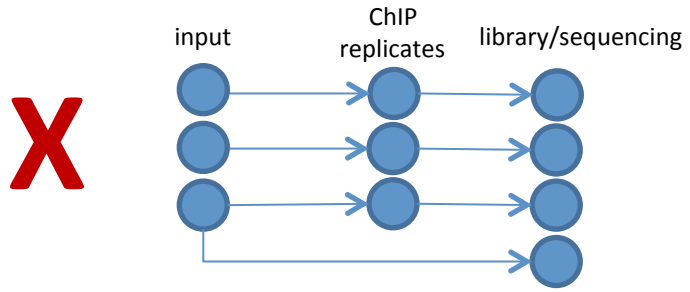
- Antibody selection
- Library cloning and sequencing
- Algorithm for peak detection
- Proper control sample (input chromatin or mock IP)

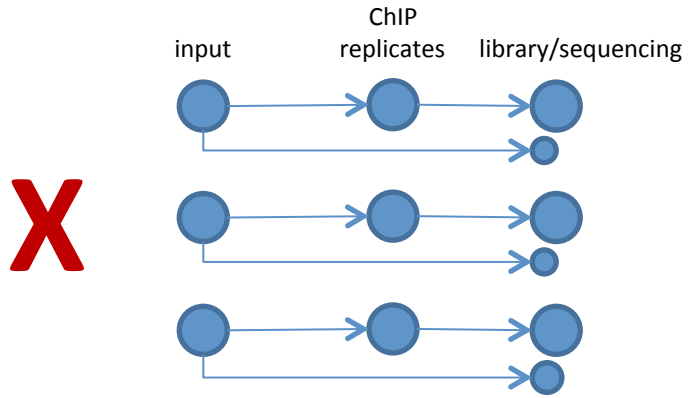
- Reproducibility in chromatin fragmentation
- Cross-linker choice
- Enough material and biological replicates

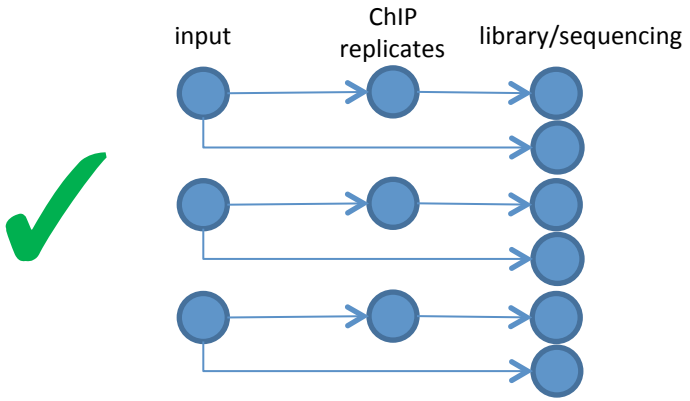
Experiment design

- Sound experimental design: replication, randomisation and blocking (R.A. Fisher, 1935)
- In the absence of a proper design, it is essentially impossible to partition biological variation from technical variation
- Sequencing depth: depends on the structure of the signal; cannot be linearly scaled to genome size
- Single- vs. paired-end reads: PE improves read mapping confidence and gives a direct measure of fragment size, which otherwise has to be modelled or estimated

Experiment design



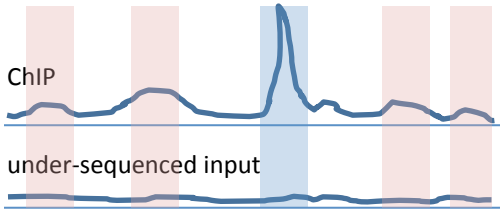


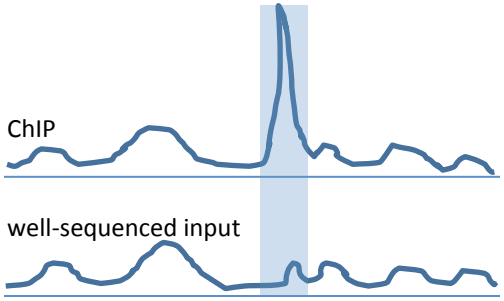


Ideal design:

Each sample has a matched input
Input sequenced to a comparable depth as IP sample

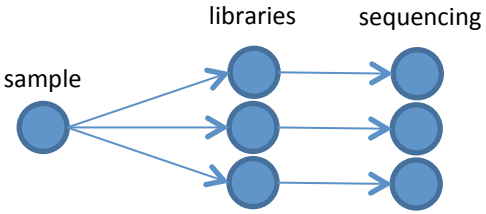
≥2 biological replicates for site identification
≥3 biological replicates for differential binding





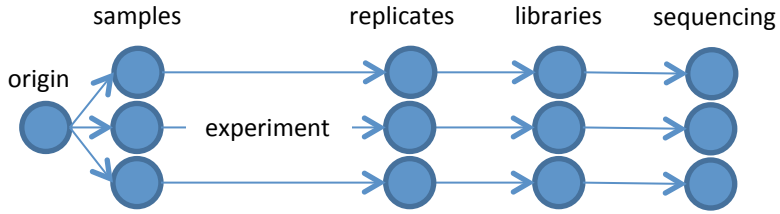
Importance of biological replicates

X



technical replicates are generally a waste of time and money

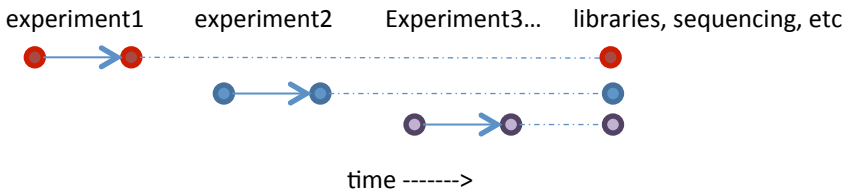
X



many studies do not account for batch effects

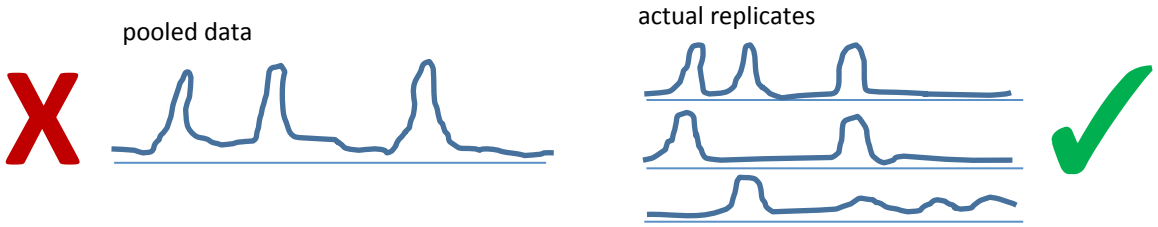
i. time
ii. origin

✓

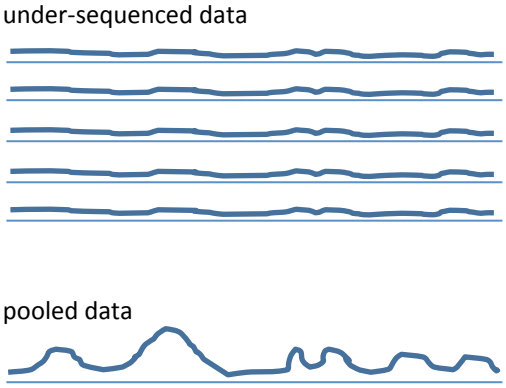


so if you care about reproducibility

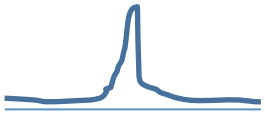
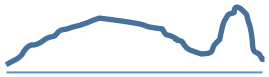
Importance of sequencing depth



if you need to pool your data, then it is under-sequenced



Sequencing depth depends on data type

	Transcription Factors	Chromatin Remodellers Histone marks	Chromatin Remodellers Histone marks RNA polymerase II
			
	point-source	mixed signal	broad signal
Human:	TF: 20 M H3K4me3: 25 M	? H3K36me3: 35 M	? H3K27me3: 40 M H3K9me3: >55 M

No clear guidelines for mixed and broad type of peaks

Source: The ENCODE consortium; Jung et al, NAR 2014

The ***ENCODE*** (Encyclopedia of DNA Elements) Consortium and the ***Roadmap Epigenomics*** Consortium are a vast resource of various kinds of functional genomics data (as well as RNA-seq data).

- ChIP – sequencing: introduction from a bioinformatics point of view
- Principles of analysis of ChIP-seq data
- ChIP-seq: downstream analyses
- Resources
- Exercise overview

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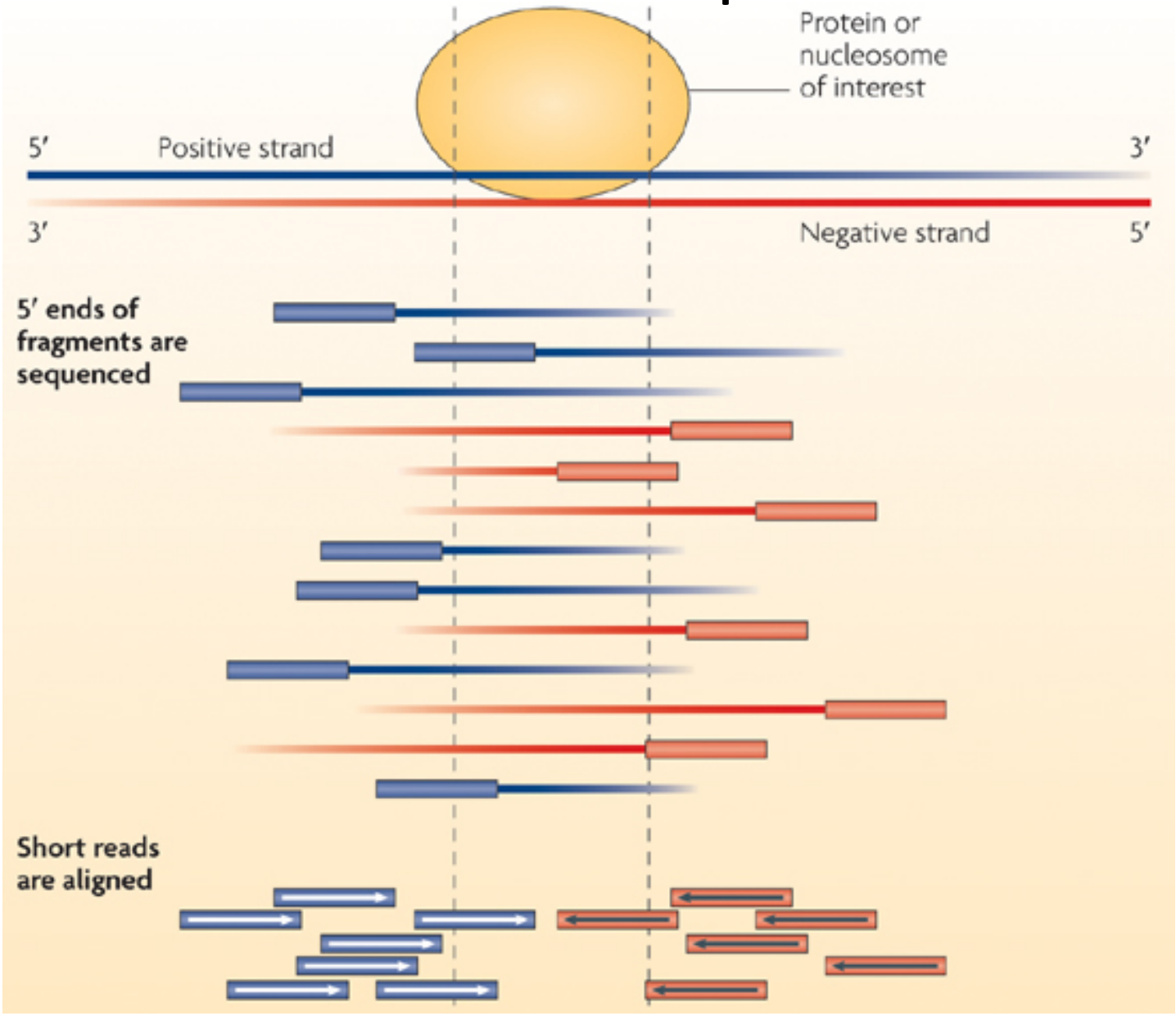
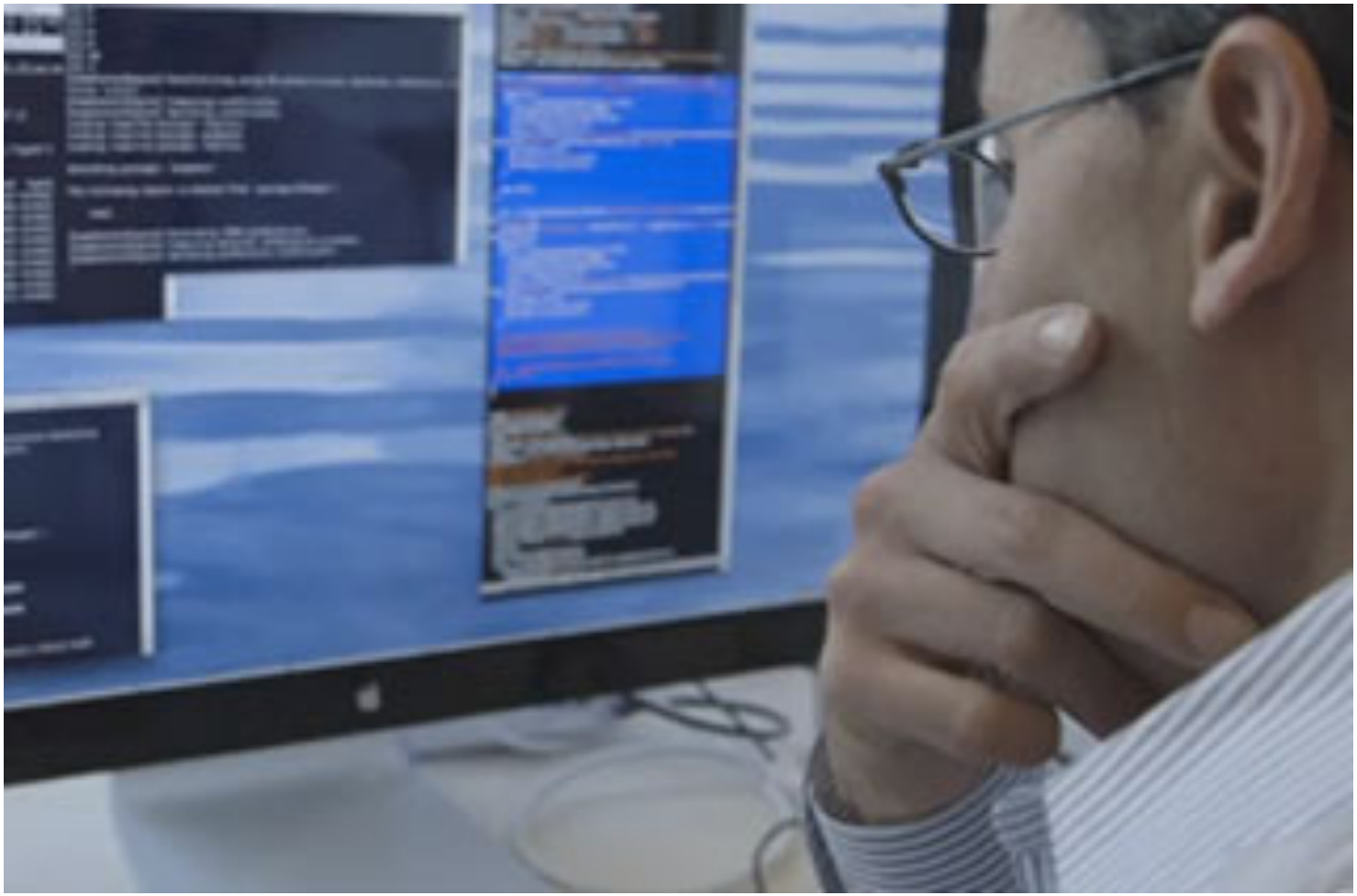
Chromatin = DNA + proteins

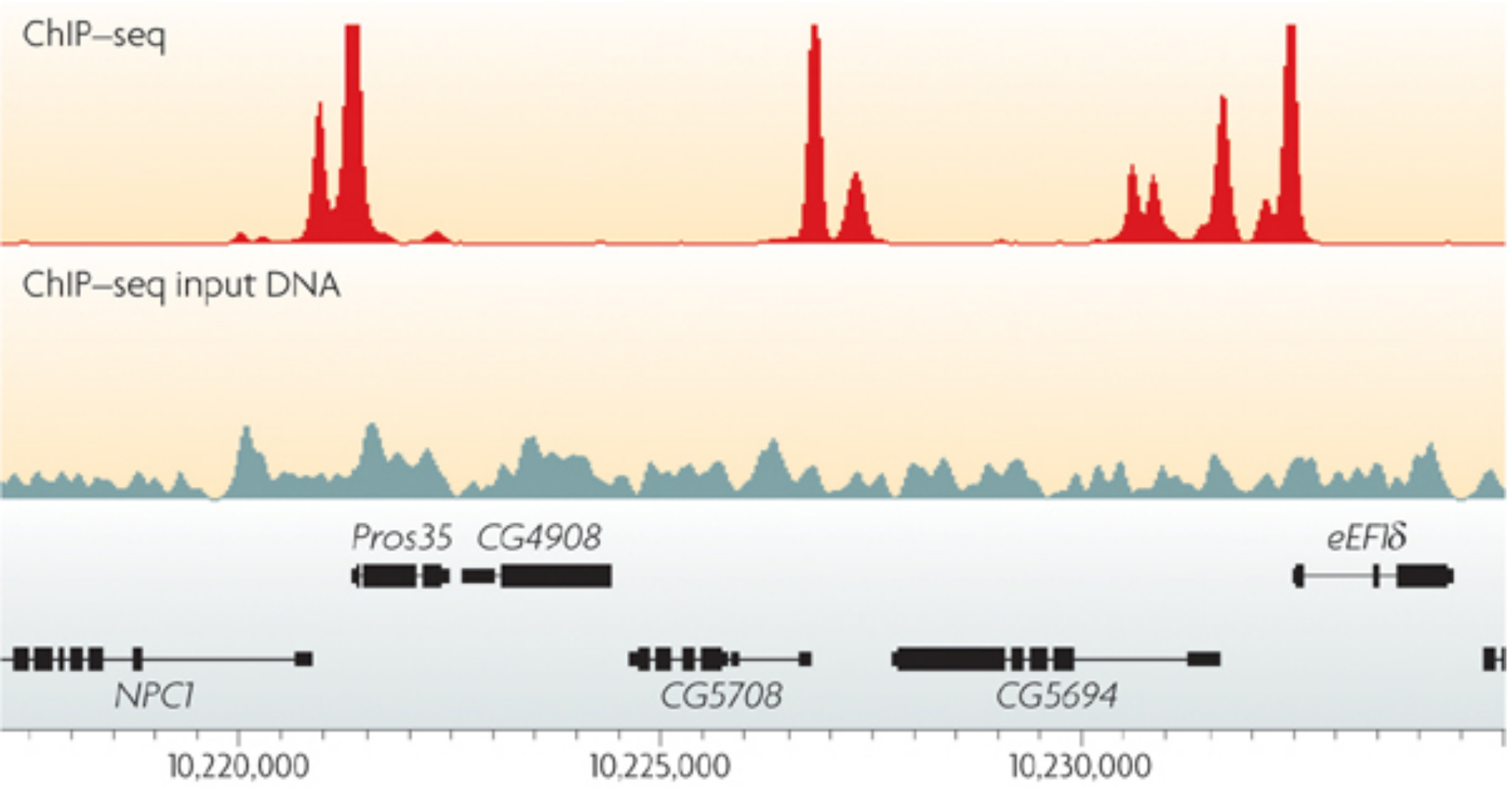


Park, Nature Rev Genetics, 2009

Data analysis



Profile of protein binding sites vs. input



Chromator (*Drosophila*) – protein binding methylated histones

Park, Nature Rev Genetics, 2009

Workflow of a ChIP-seq study

design study

obtain input chromatin

perform precipitation

construct library

sequence library

library quality control
filter sequences

align sequences

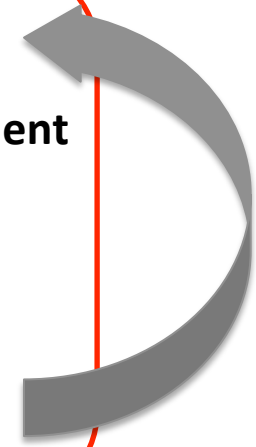
filter alignments

identify peaks / regions of enrichment

assess data quality

understand the data / results

downstream analyses



Iterative process

- ChIP – sequencing: introduction from a bioinformatics point of view
- Principles of analysis of ChIP-seq data
- ChIP-seq: downstream analyses
- Resources
- Exercise overview

Two questions to address

- 1. Did the ChIP part of the ChIP-seq experiment work? Was the enrichment successful?

- 2. Where are the binding sites (of the protein of interest)?

Word of caution!

ChIP-seq experiments are more unpredictable than RNA-seq!

Error sources:

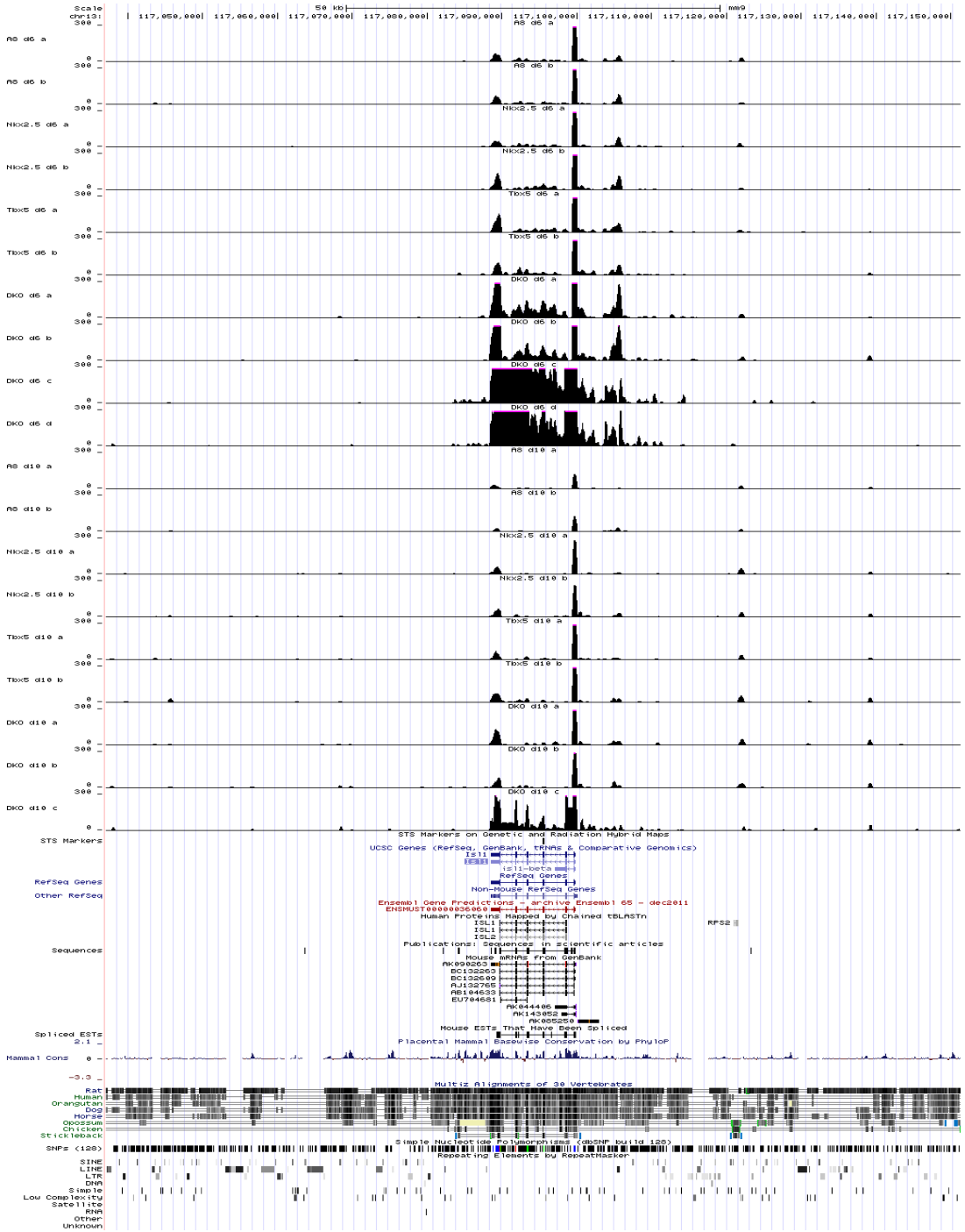
- chromatin structure
- PCR over-amplification
- non-specific antibody
- other things?

ChIP-seq QC: did the ChIP work?

- 1. Inspect the signal (mapped reads, coverage profiles) in genome browser

- 2. Compute peak-independent quality metrics (cross correlation, cumulative enrichment)

- 3. Assess replicate consistency (correlations between replicates of the same condition)



tag density distribution

reproducibility

similarity of coverage

signal at known sites

…

Spotting inconsistencies

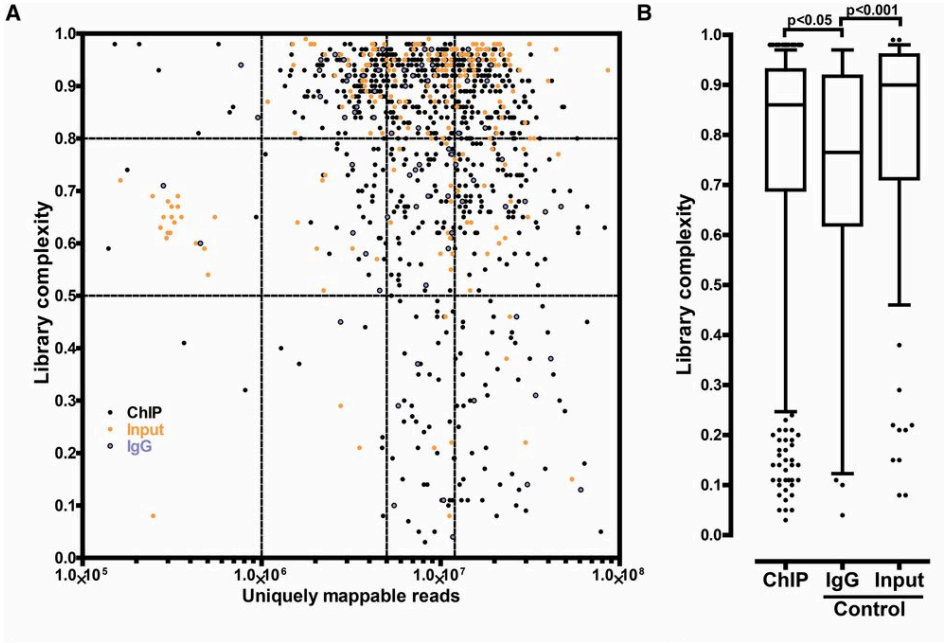
Confounding factors

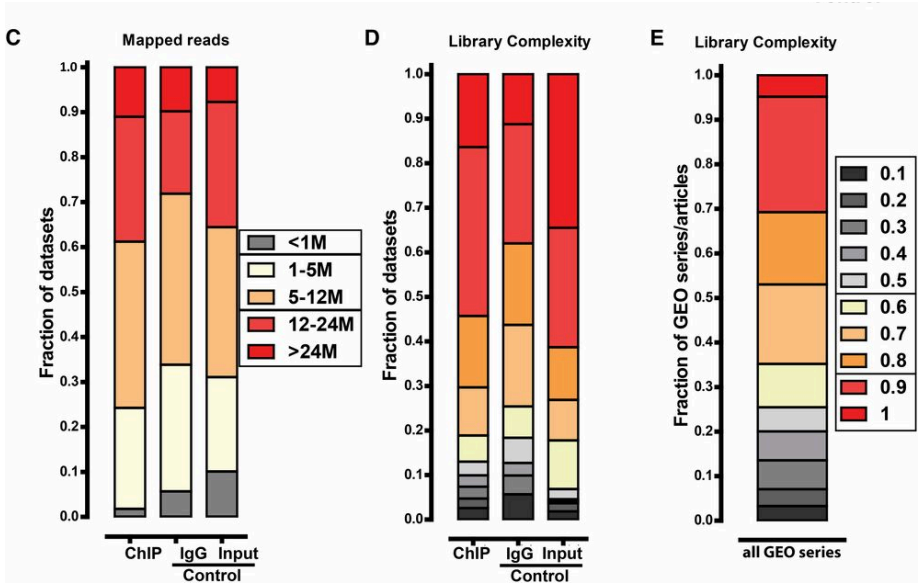
Under-sequenced libraries

…

How do I know my data is of good quality?

Library complexity

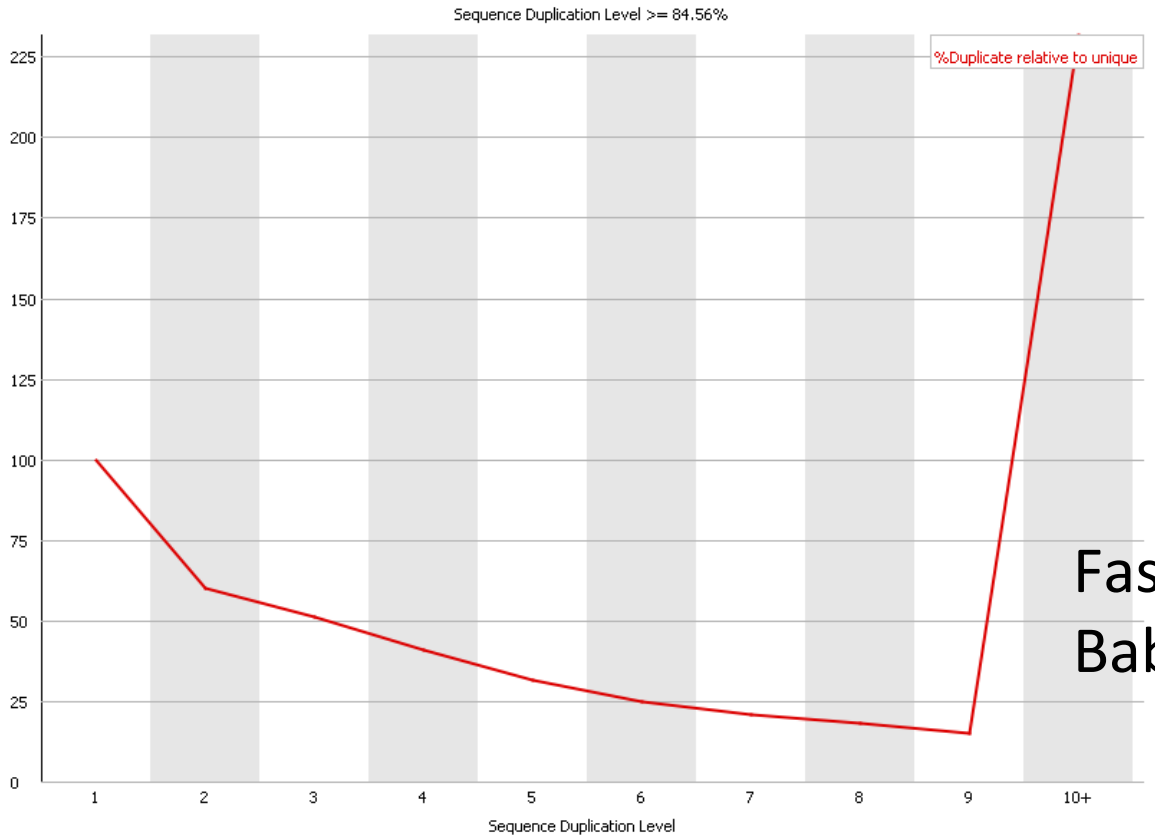




Marinov et al, G3 2013

Quality control: tag uniqueness – library complexity metric

Sequence duplication level > 80% (low complexity library)



FastQC
Babraham Institute

NRF: Non-redundant fraction (of reads): proportion of unique tags / total

<u>less than 20% of reads should be duplicates for 10 million reads sequenced (ENCODE)</u>

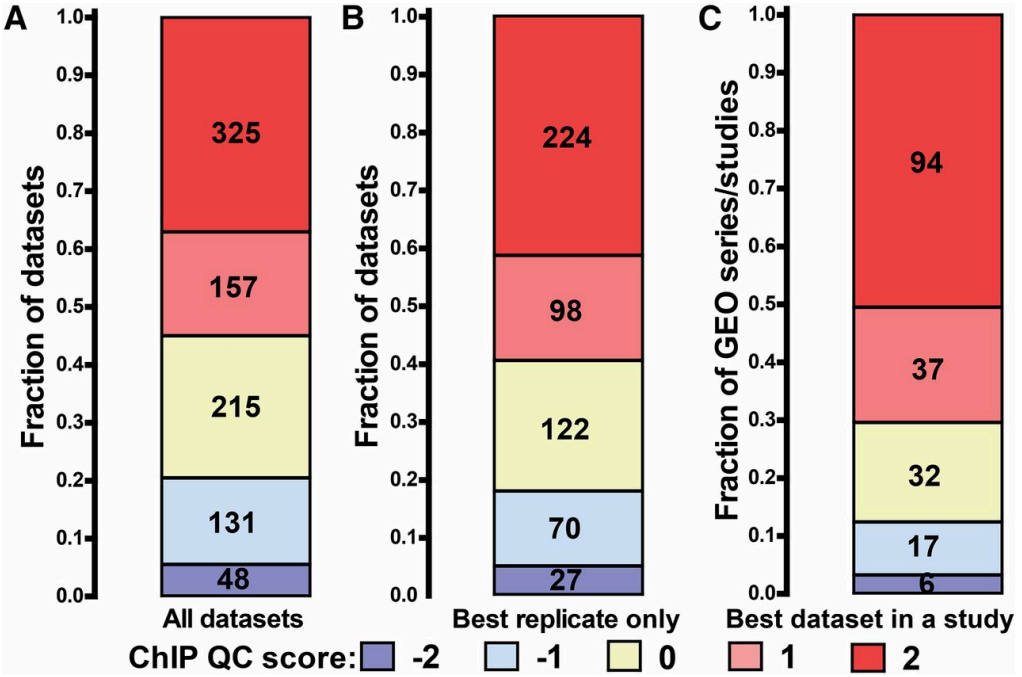
How do I know my data is of good quality?

Objective (i.e. peak independent) metrics to quantify enrichment in ChIP-seq;

for TF in mammalian systems:

Normalised Strand Correlation NSC
Relative Strand Correlation RSC

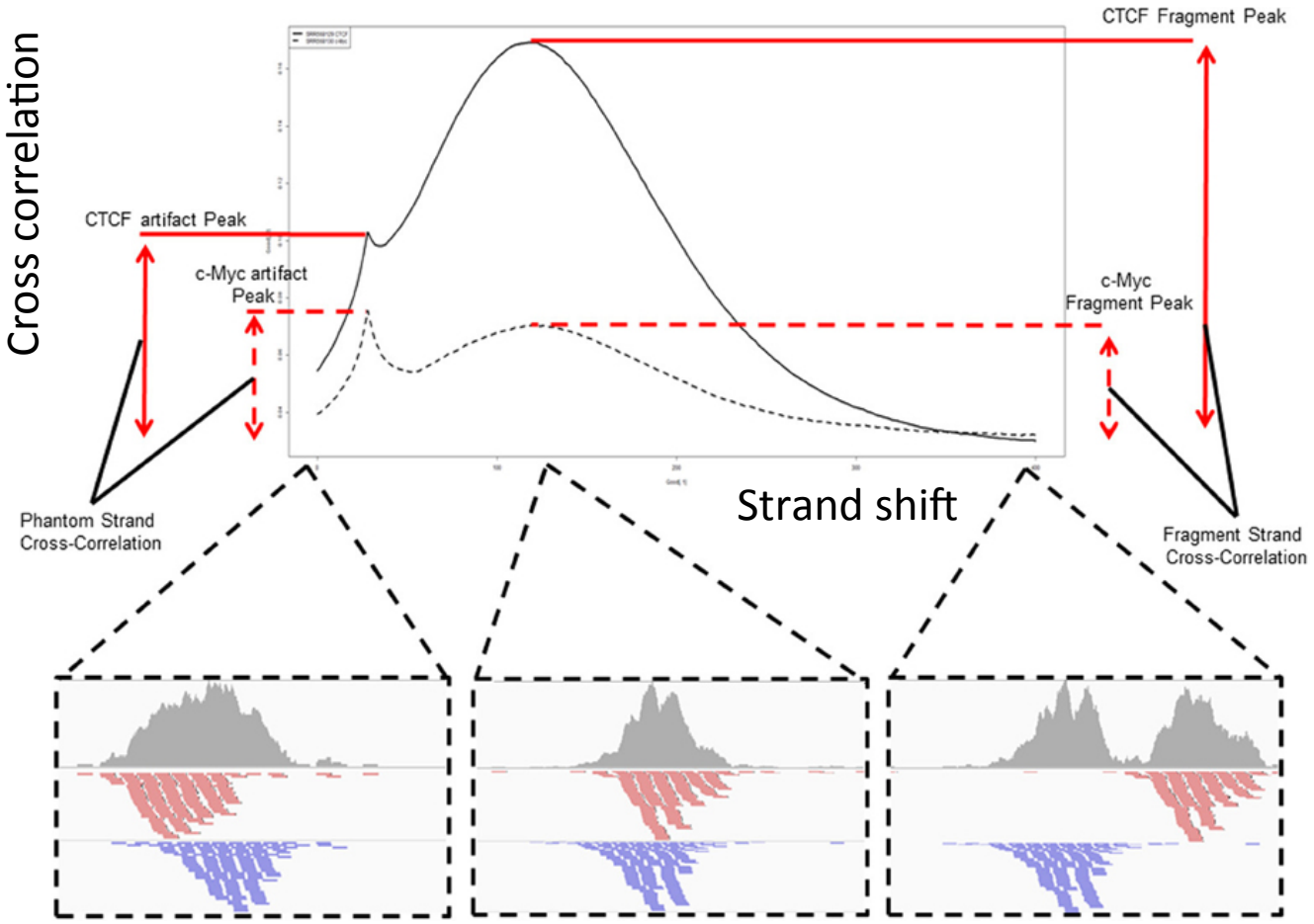
Large-scale quality analysis of published ChIP-seq data sets:
20% low quality
25% intermediate quality
30% inputs have metrics similar to IPs



Marinov et al, G3 2013

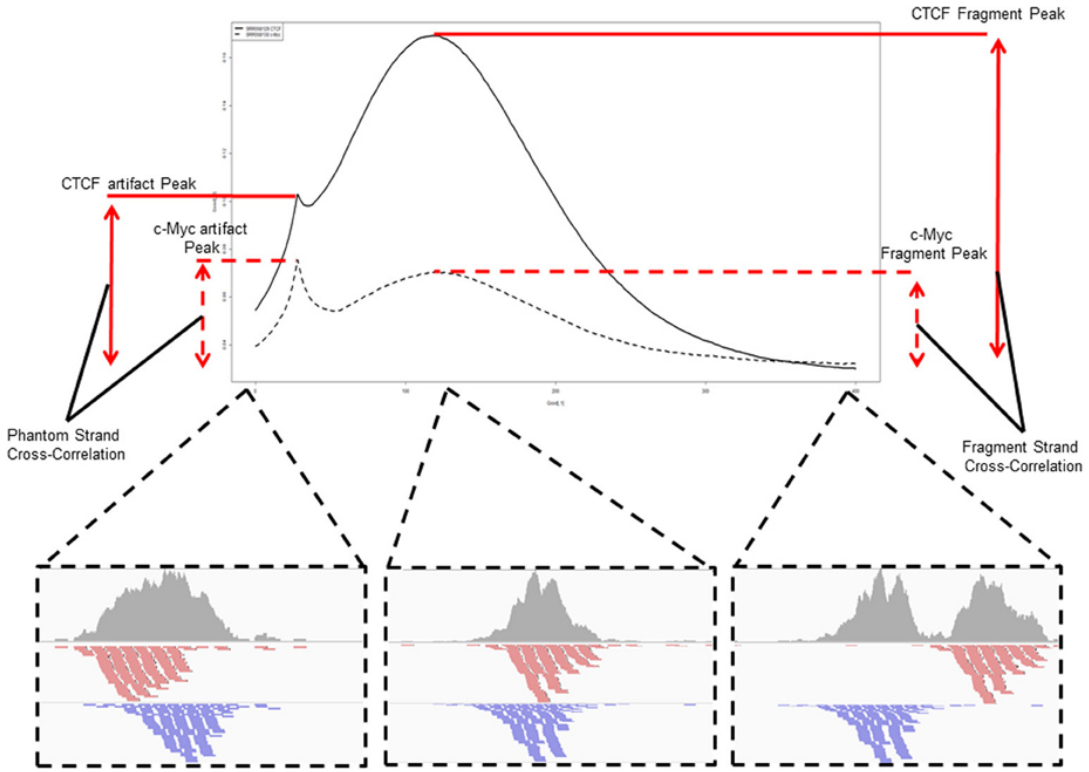
Strand cross-correlation

The correlation between signal of the 5’ end of reads on the (+) and (-) strands is assessed after successive shifts of the reads on the (+) strand and the point of maximum correlation between the two strands is used as an estimation of fragment length.



Carroll et al, Front Genet 2014

Strand cross-correlation



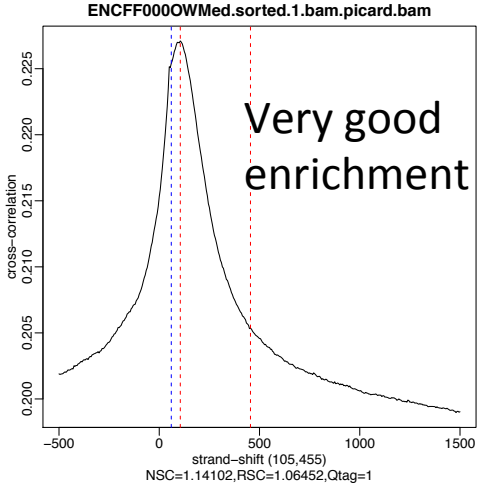
$$NSC = \frac{\text{Max CC value (fLen)}}{\text{Min CC}}$$

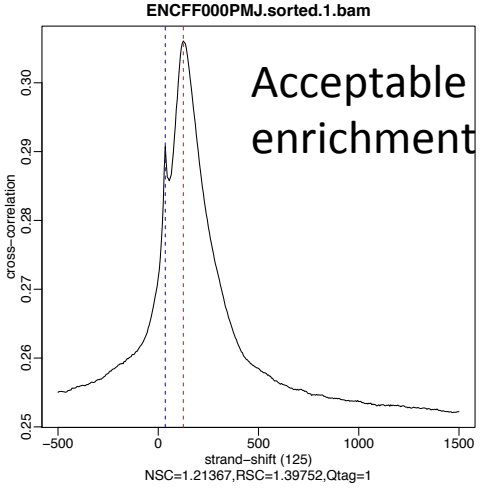
$$RSC = \frac{\text{Max CC} - \text{Min CC}}{\text{Phantom CC} - \text{Min CC}}$$

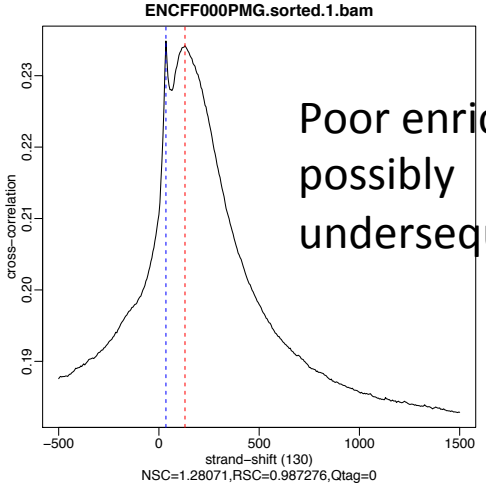
Carroll et al, Front Genet 2014

Cross-correlation plots

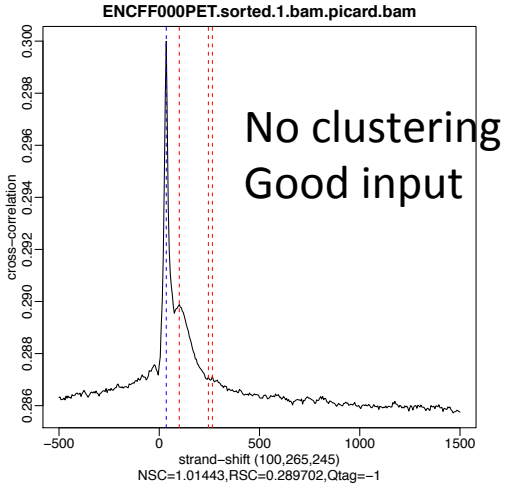
ChIP

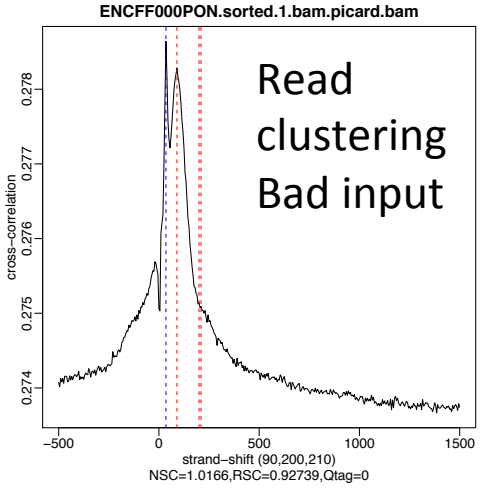




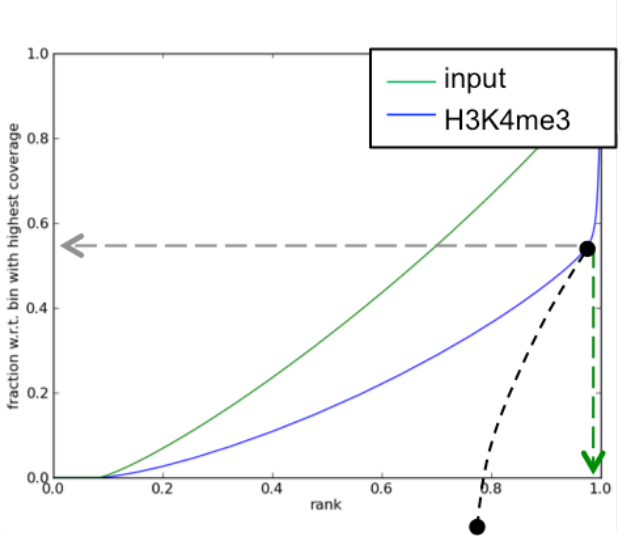


Input



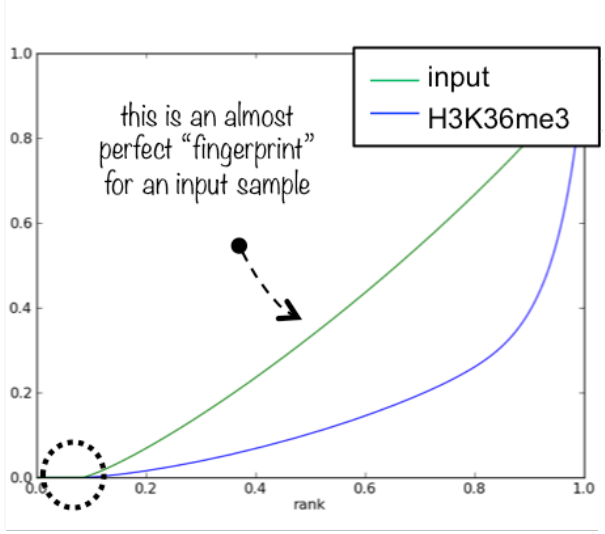


Cumulative enrichment aka “Fingerprint” is another metric for successful enrichment

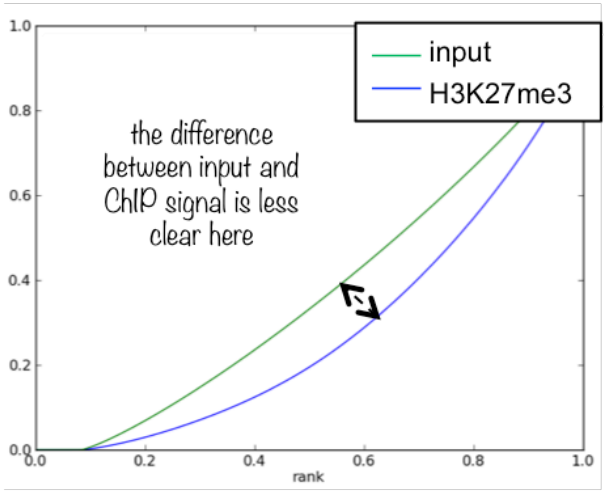


when counting the reads contained in **97%** of all genomic bins, only ca. **55%** of the maximum number of reads are reached, i.e. 3% of the genome contain a very large fraction of reads!

→ this indicates very localized, very strong enrichments!
(as every biologist hopes for in a ChIP for H3K4me3)



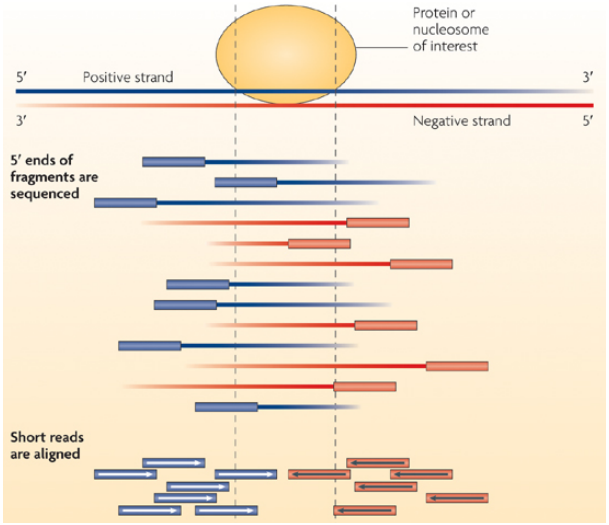
pay attention to where the curves start to rise – this already gives you an assessment of how much of the genome you have not sequenced at all (i.e. bins containing zero reads – for this example, ca. 10% of the entire genome do not have any read)

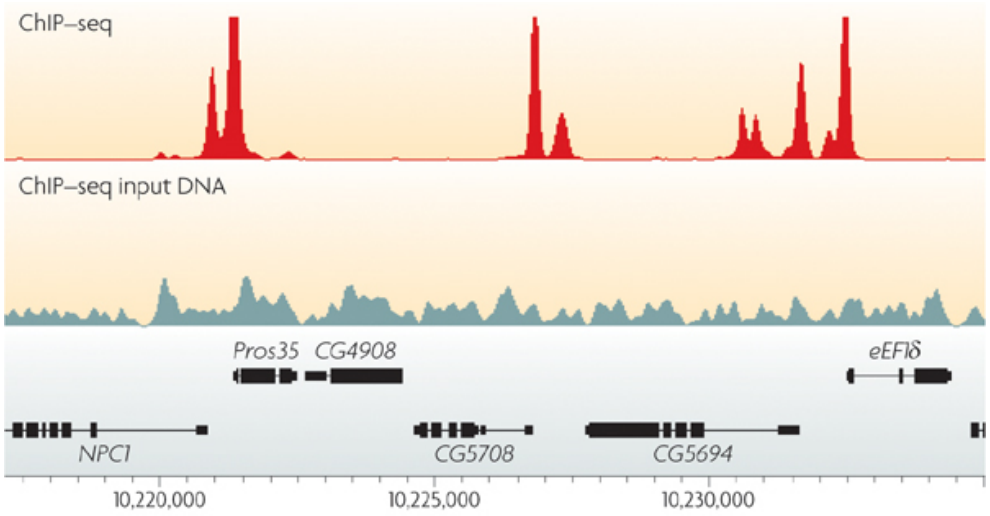


H3K27me3 is a mark that yields broad domains instead of narrow peaks

→ it is more difficult to distinguish input and ChIP, it does not mean, however, that this particular ChIP experiment failed

http://deeptools.readthedocs.org
Diaz et al, Genome Biol 2012



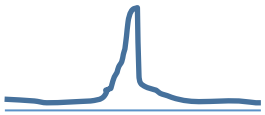


Park, Nature Rev Genetics, 2009

Peak calling

appropriate methodologies depend on data type

Transcription Factors



punctate

SPP

MACS2

Chromatin Remodellers

Histone marks

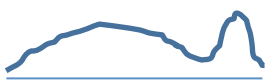
mixed signal

-

Chromatin Remodellers

Histone marks

RNA polymerase II

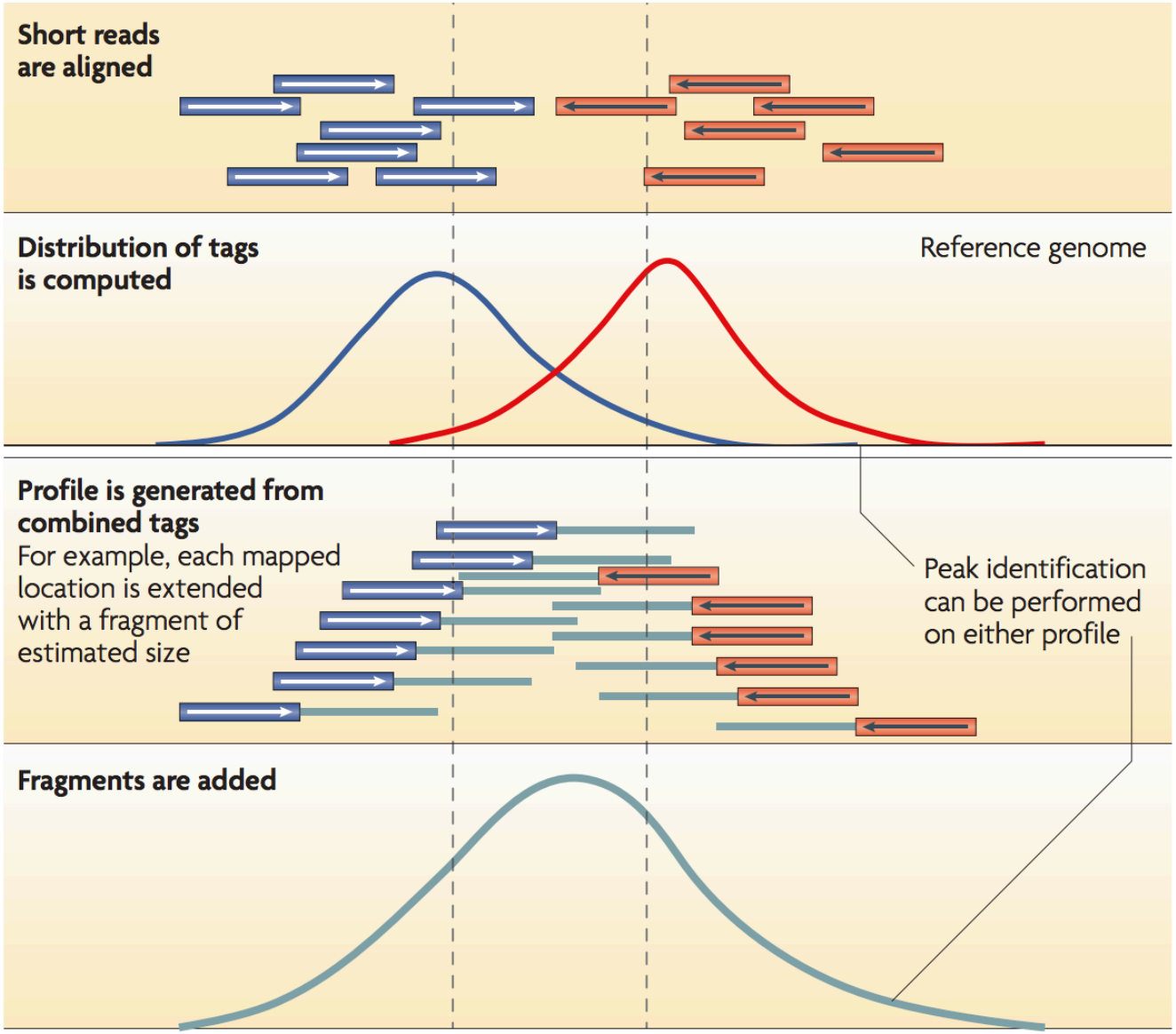


broad signal

-

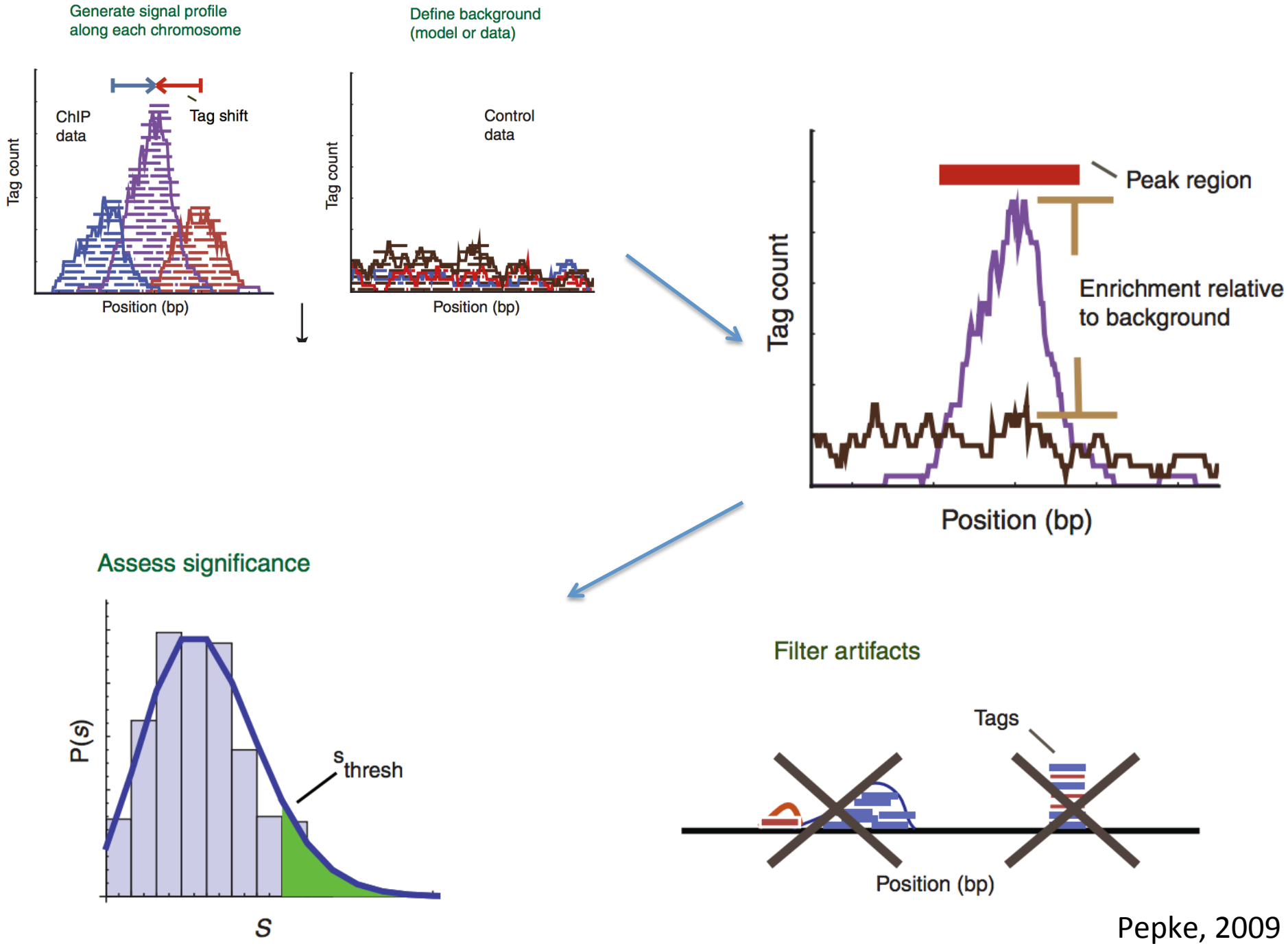
This is an active area of algorithm development

Principle of peak detection



Symmetry in reads mapped to opposite DNA strands

Computation of enrichment model


Generate signal profile
along each chromosome
Define background
(model or data)
ChIP
data
Tag shift
Tag count
Position (bp)
Control
data
Tag count
Position (bp)
Peak region
Enrichment relative
to background
Tag count
Position (bp)
Assess significance
P(s)
s
thresh
S
Filter artifacts
Tags
Position (bp)

Pepke, 2009

Comparison of peak calling algorithms

Program	Reference	Version	Graphical user interface?	Window-based scan	Tag clustering	Gaussian kernel density estimator	Strand-specific scoring	Peak height or fold enrichment (FE)	Background subtraction	Compensates for genomic duplications or deletions	False Discovery Rate	Compare to normalized control data (FE)	Compare to statistical model fitted with control data	Statistical model or test
CisGenome	28	1.1	X*	X				X	X		X		X	conditional binomial model
Minimal ChipSeq Peak Finder	16	2.0.1			X			X				X		
E-RANGE	27	3.1			X			X				X	X	chromsome scale Poisson dist.
MACS	13	1.3.5		X				X			X		X	local Poisson dist.
QuEST	14	2.3				X		X			X**		X	chromsome scale Poisson dist.
HPeak	29	1.1		X				X					X	Hidden Markov Model
Sole-Search	23	1	X	X				X		X			X	One sample t-test
PeakSeq	21	1.01			X			X					X	conditional binomial model
SISSRS	32	1.4		X			X					X		
spp package (wtd & mtc)	31	1.7		X			X		X	X'	X			
				Generating density profiles			Peak assignment		Adjustments w. control data		Significance relative to control data			

X* = Windows-only GUI or cross-platform command line interface

X** = optional if sufficient data is available to split control data

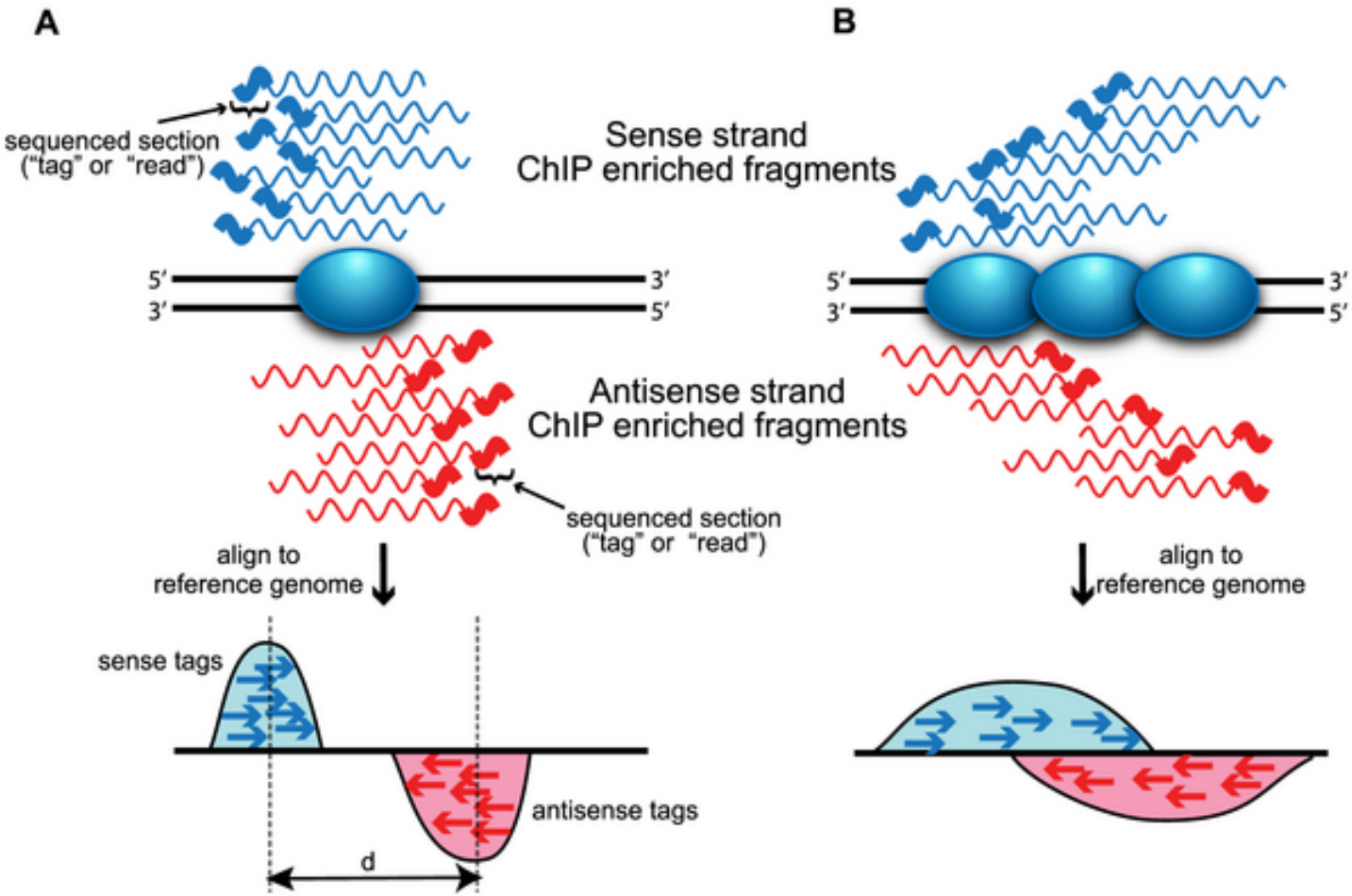
X' = method excludes putative duplicated regions, no treatment of deletions

Wilbanks 2010

Point-source vs. broad peak detection

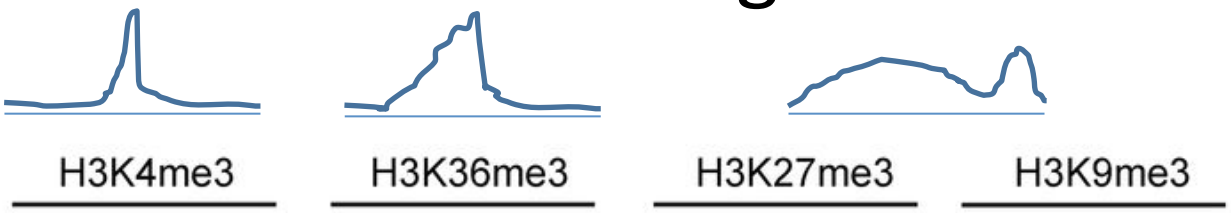
Sequence-specific binding (TFs)

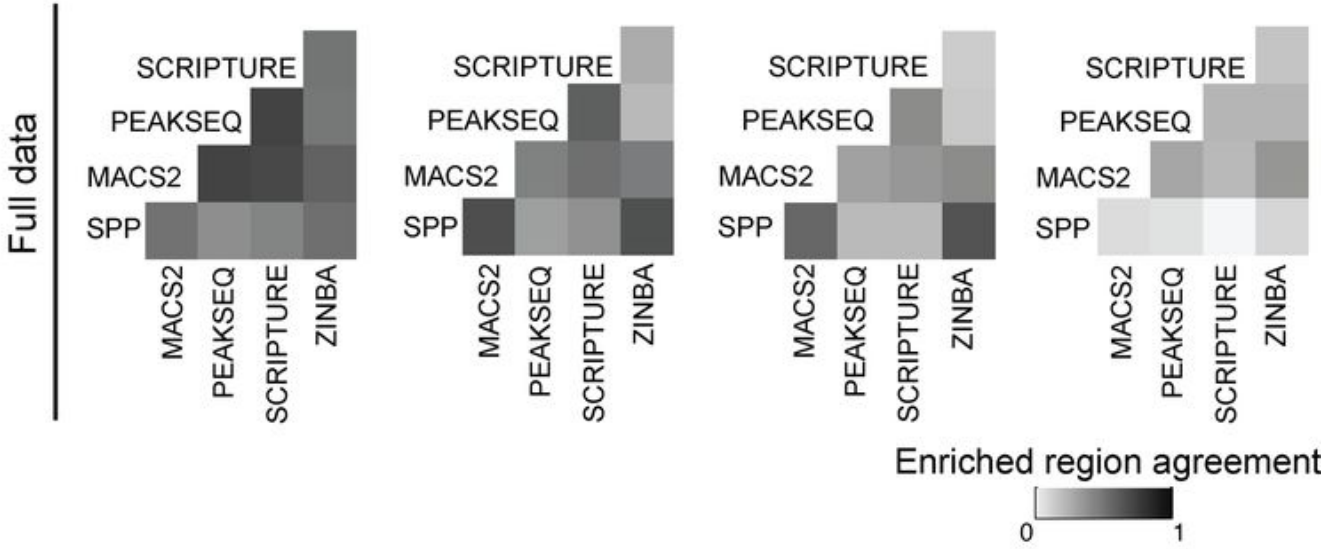
Distributed binding (histones, RNApol2)



Wilbanks 2010

Comparison of enriched regions detected by various algorithms

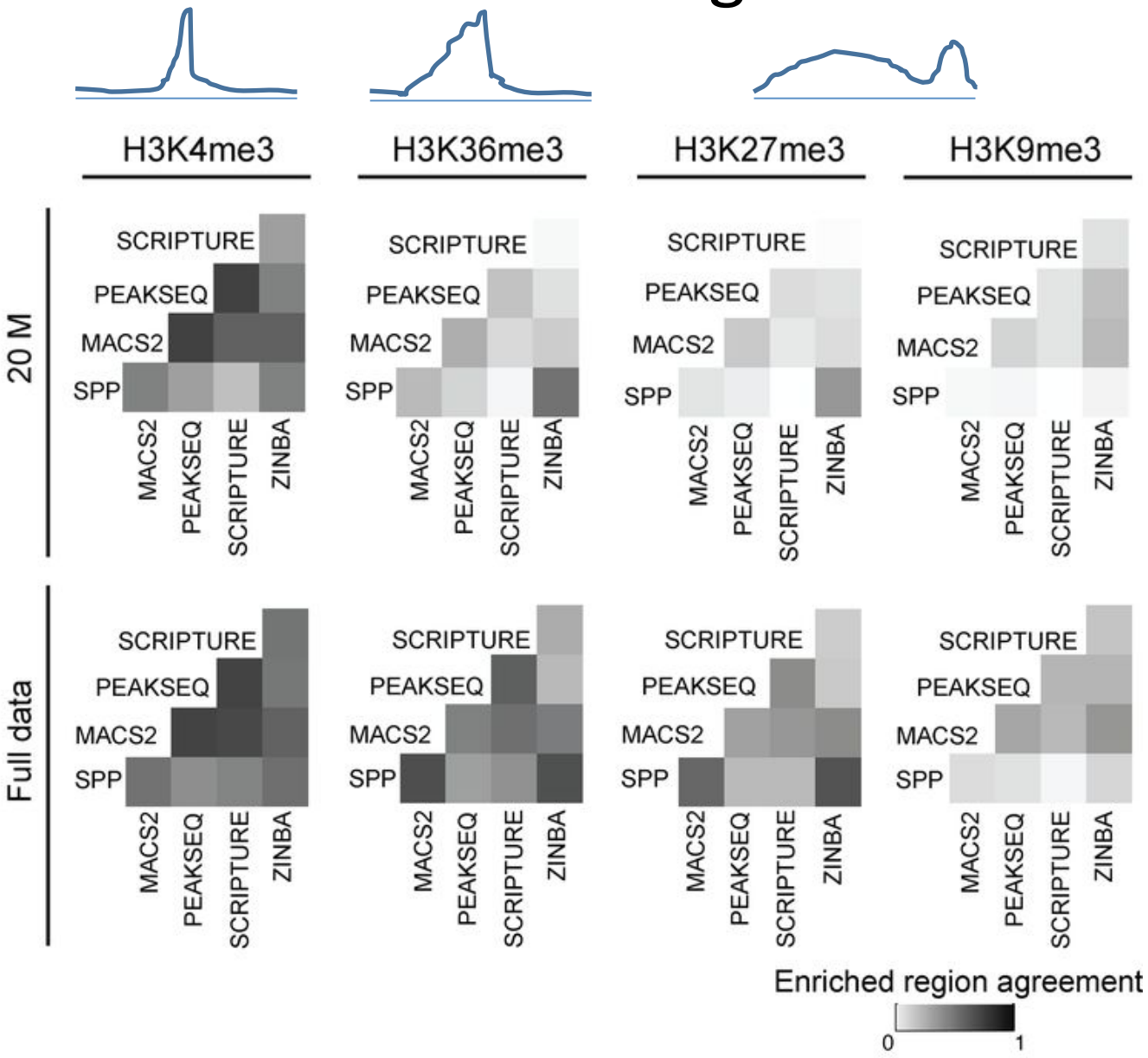




55M human

Jung 2014

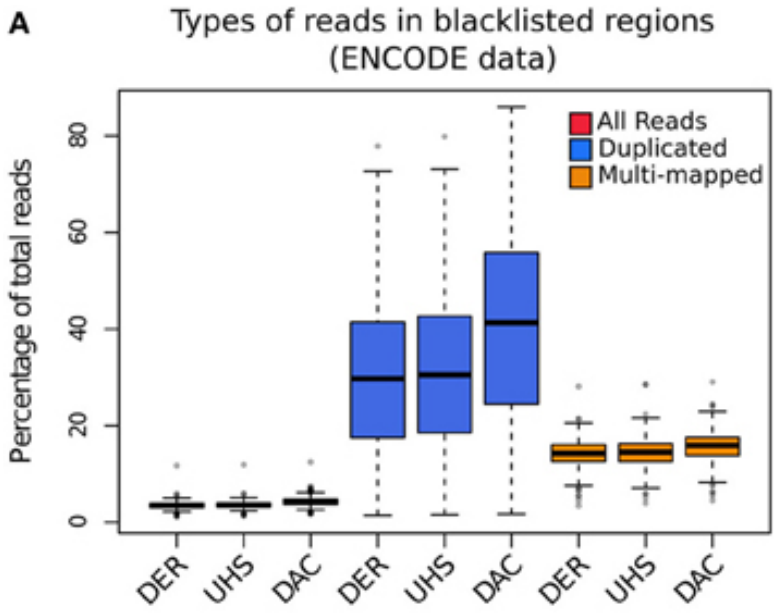
Comparison of enriched regions detected by various algorithms



55M human

Jung 2014

“Hyper-chippable” regions



Reads mapped to these regions should be filtered out prior to peak calling

Tracks available from UCSC for human, mouse, fly and worm

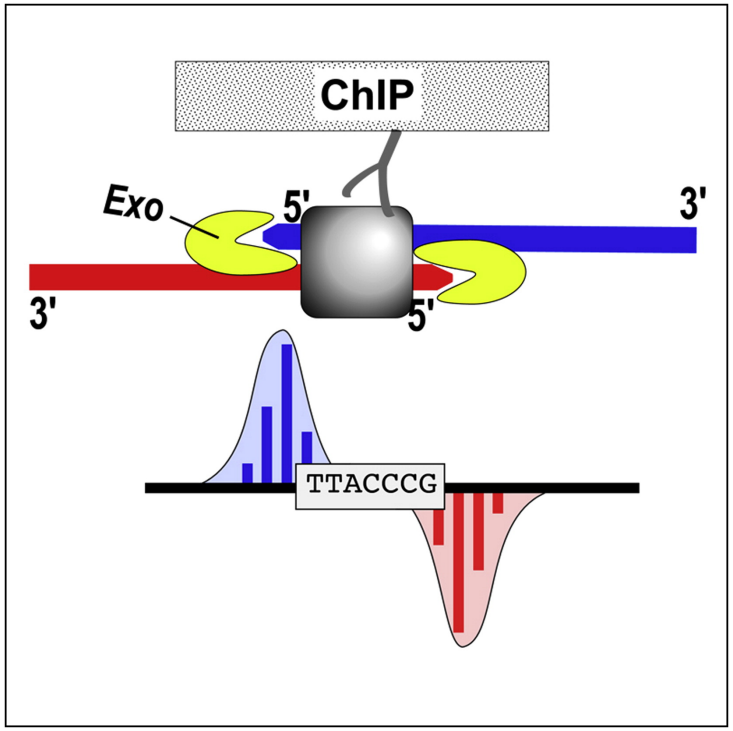
DER – Duke Excluded Regions
(11 repeat classes)
UHS – Ultra High Signal
(open chromatin)
DAC – consensus excluded regions

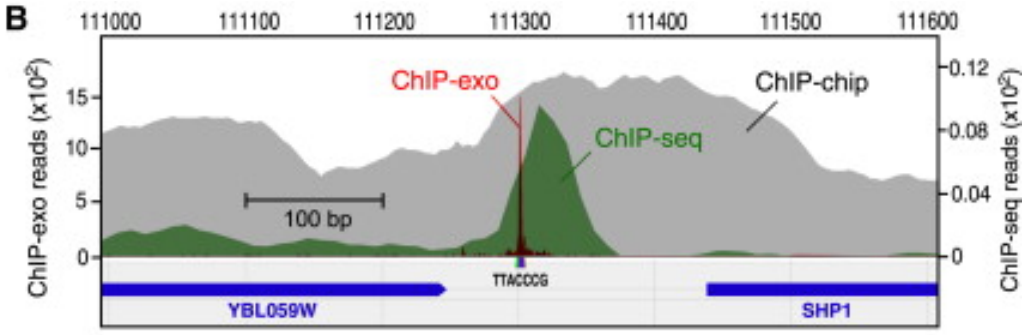
Carroll et al, Front Genet 2014

Quality considerations

- ChIP-seq quality guidelines from the ENCODE project (Relative strand cross-correlation, Irreproducible discovery rate)
- Antibody validation
- Appropriate sequencing depth (depending on genome size and peak type). For human genome and broad-source peaks, min. 40-50M reads is required.
- Experimental replication
- Fraction of reads in peaks (FRiP) > 1%
- Cross correlation (correlation of the density of sequences aligned to opposite DNA strands after shifting by the fragment size)
- Experimental verification of known binding sites (and sites not bound as negative controls)

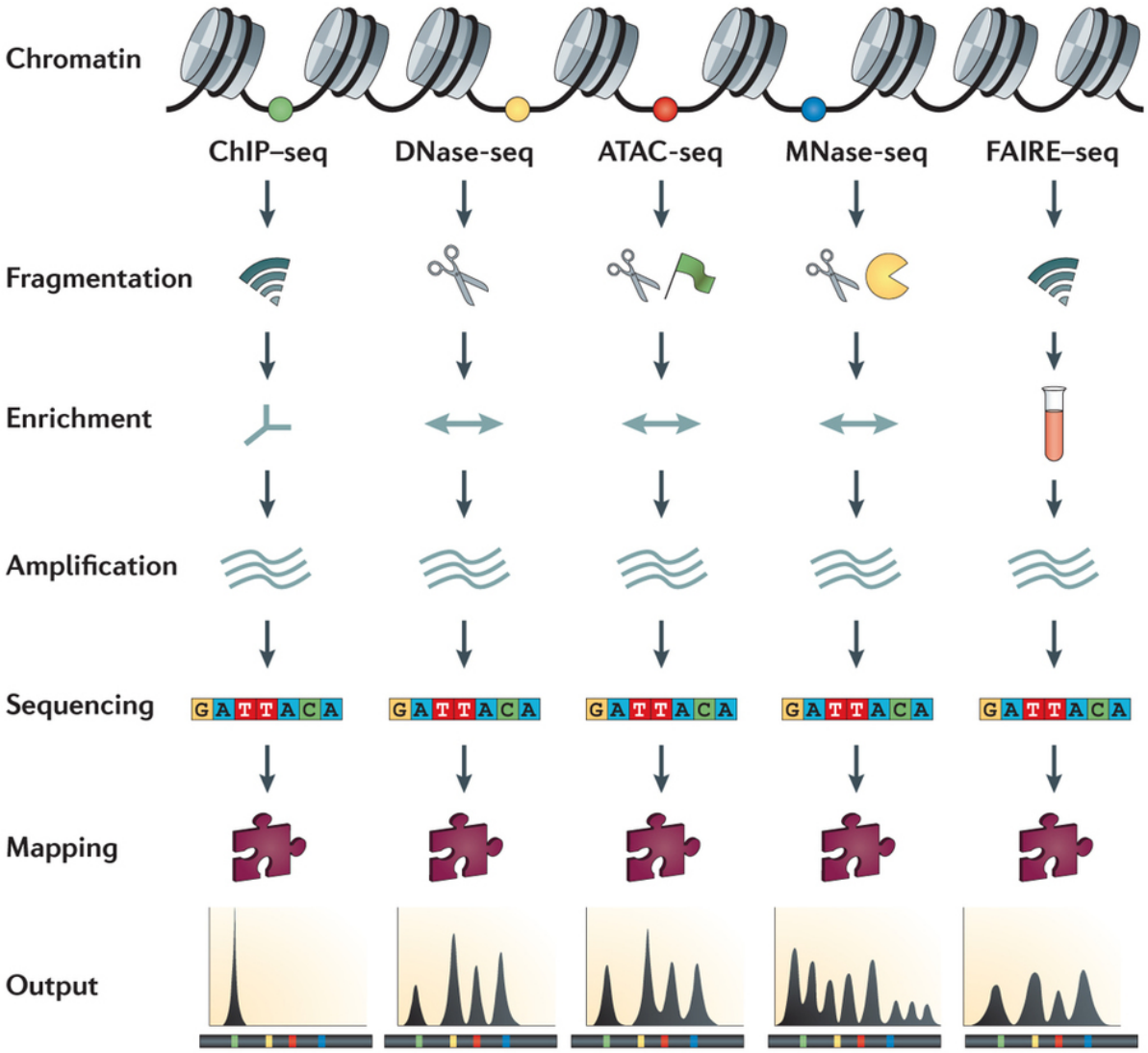
ChIP-exo: improvement in binding site identification





Rhee and Pugh, Cell 2011

Other functional genomics techniques



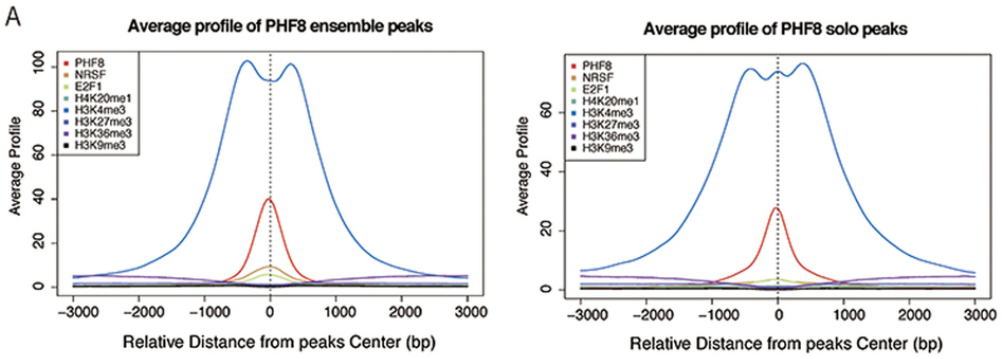
Clifford et al, Nature Rev Genet, 2014

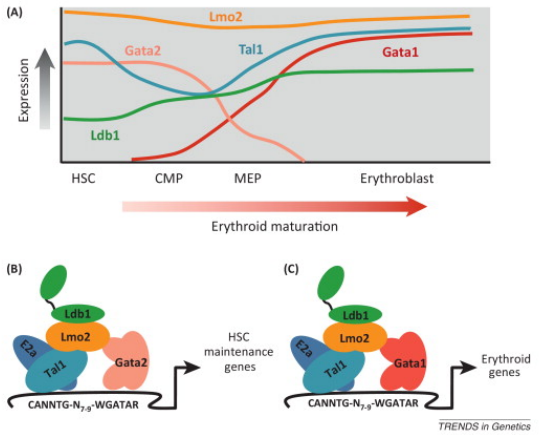
- ChIP – sequencing: introduction from a bioinformatics point of view
- Principles of analysis of ChIP-seq data
- ChIP-seq: downstream analyses
- Resources
- Exercise overview

ChIPseq downstream analyses

- Validation (wet lab)

- Downstream analysis
  - Motif discovery
  - Annotation
  - Integration of binding and expression data
  - Integration of various binding datasets
  - Differential binding





Peak annotation

Identification of nearest genomic features

- BEDtools,
- BEDops,
- PeakAnnotator,
- CisGenome,
- In R / Bioconductor: ChIPpeakAnno

Motif detection

- Enrichment of known sequence motifs (CEAS, Transfac Match, HOMER, RSAT)
- *De novo* motif detection (MEME, CisFinder, HMS, DREME, ChIPMunk, HOMER, RSAT)

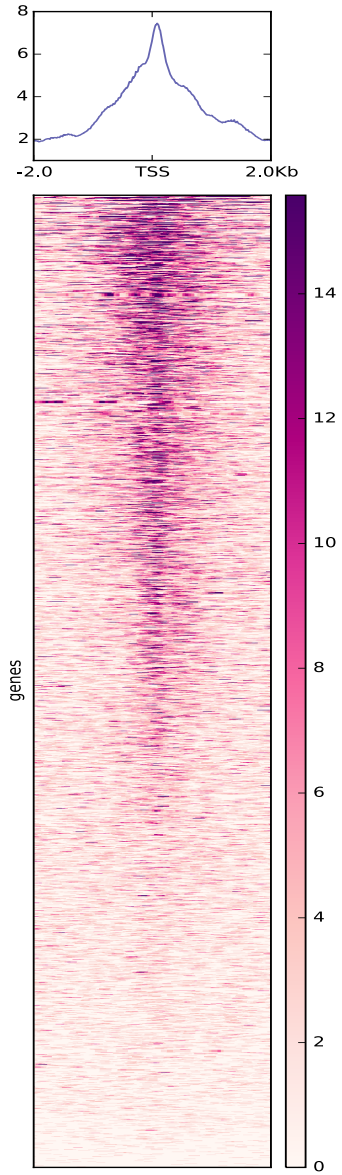
Enrichment of known motifs (Homer):

Homer Known Motif Enrichment Results

Homer *de novo* Motif Results
Gene Ontology Enrichment Results
Known Motif Enrichment Results (txt file)
Total Target Sequences = 900, Total Background Sequences = 45419

Rank	Motif	Name	P-value	log P-pvalue	q-value (Benjamini)	# Target Sequences with Motif	% of Targets Sequences with Motif	# Background Sequences with Motif	% of Background Sequences with Motif	Motif File	PDF
1		Lhx3(Homeobox)/Forebrain-p300-ChIP-Seq/Homer	1e-178	-4.114e+02	0.0000	512.0	56.89%	6985.5	15.38%	motif file (matrix)	pdf
2		Sox3(HMG)/NPC-Sox3-ChIP-Seq(GSE33059)/Homer	1e-128	-2.955e+02	0.0000	515.0	57.22%	9264.1	20.40%	motif file (matrix)	pdf

Signal visualisation and interpretation



deepTools
ngsplots
seqMiner

- Clustering
- Heatmaps
- Profiles
- Comparison of different datasets

Binding profile of a TF in relation to the transcription start site

Differential occupancy

- Use algorithms developed for differential expression and summarise reads mapped in peaks; normalisation; statistical testing; R environment
  - edgeR / csaw
  - DiffBind (implements several normalisation methods)
- Calculate enrichment in sliding windows
  - DROMPA
  - Diffreps

- ChIP – sequencing: introduction from a bioinformatics point of view
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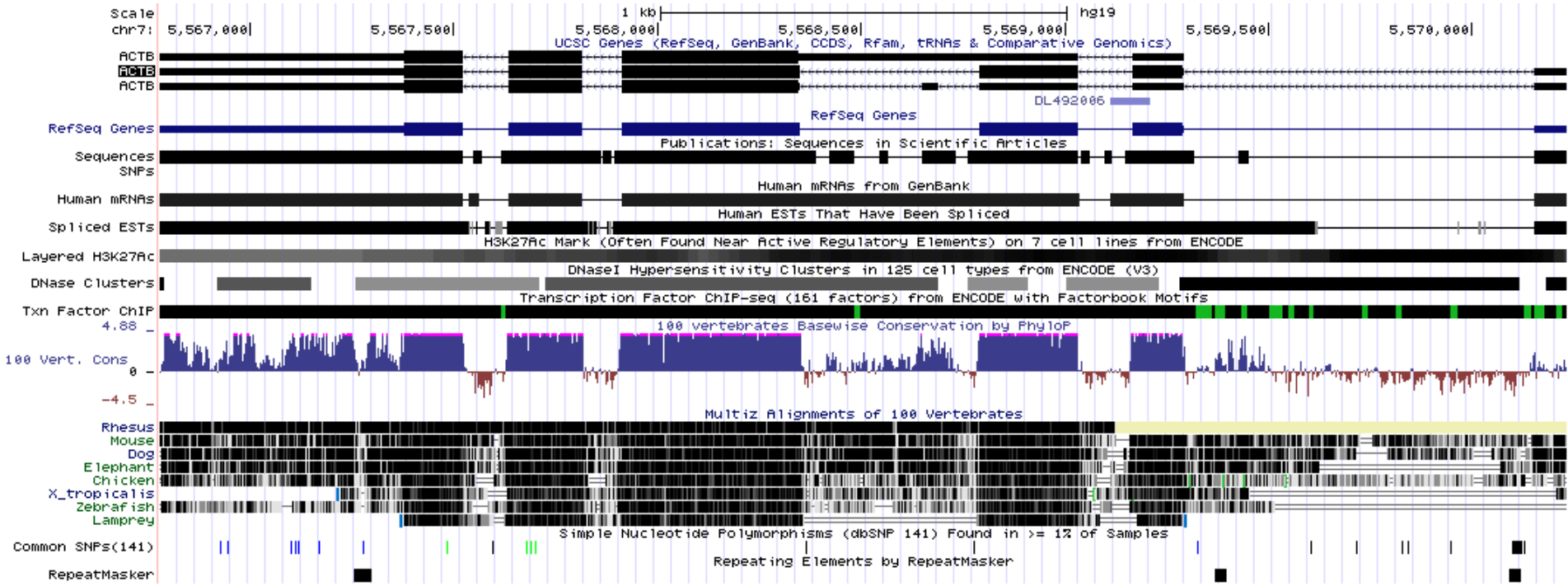
Where to obtain data?

The ENCODE project

www.encodeproject.org

- Encyclopedia of DNA elements
- Identification of regulatory DNA elements in human (and mouse) genome
- 240 human and 55 mouse DNA binding proteins
- 1464 human and 432 mouse samples
- RNA profiling, protein-DNA interaction, chromatin condensation, DNA methylation, …
- 2009 - ongoing

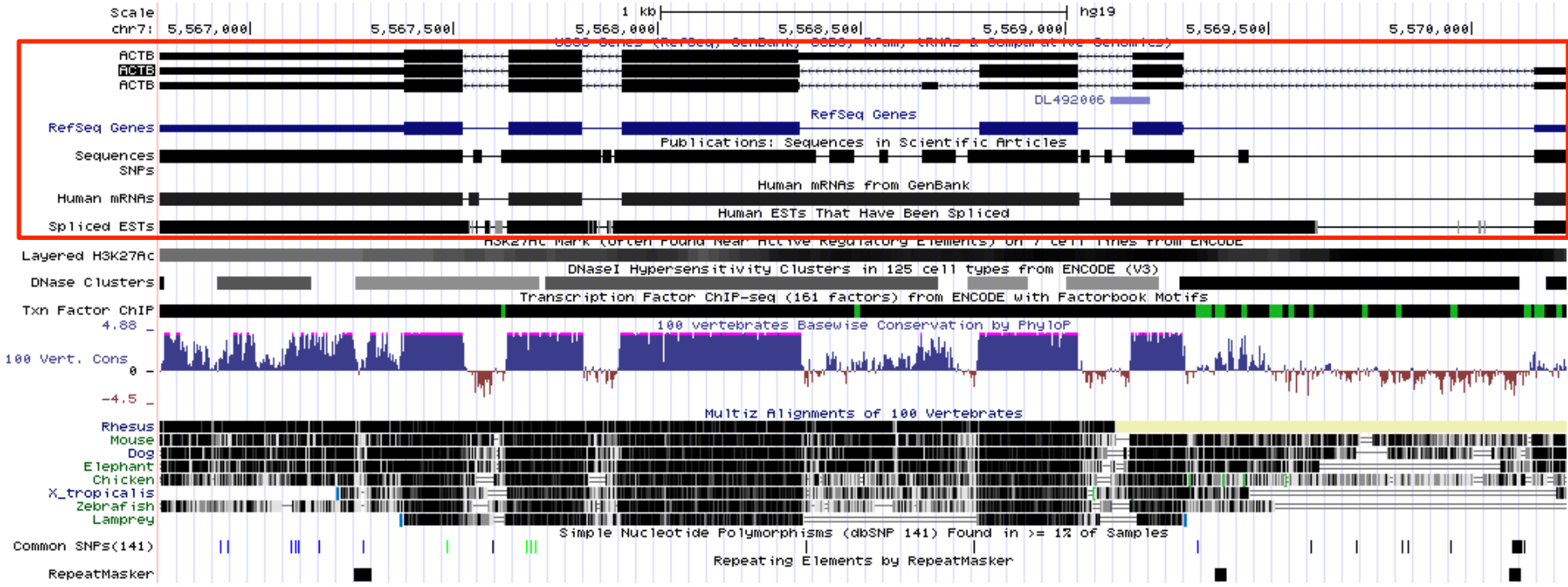
Human ACTB locus as seen in the UCSC Genome Browser



Gene model
Alternative transcripts
Histone modifications
Chromatin structure

Transcription factor binding sites
DNA conservation
Single nucleotide polymorphisms (SNP)
Repeats

Human ACTB locus as seen in the UCSC Genome Browser



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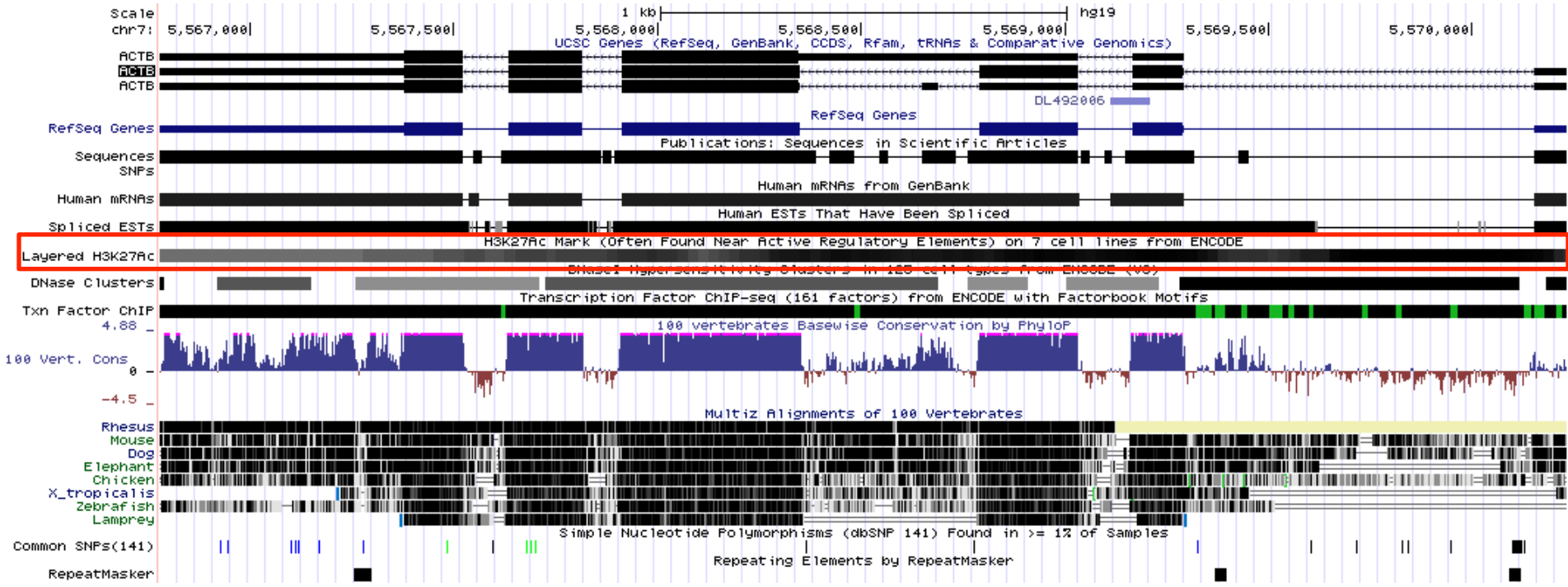

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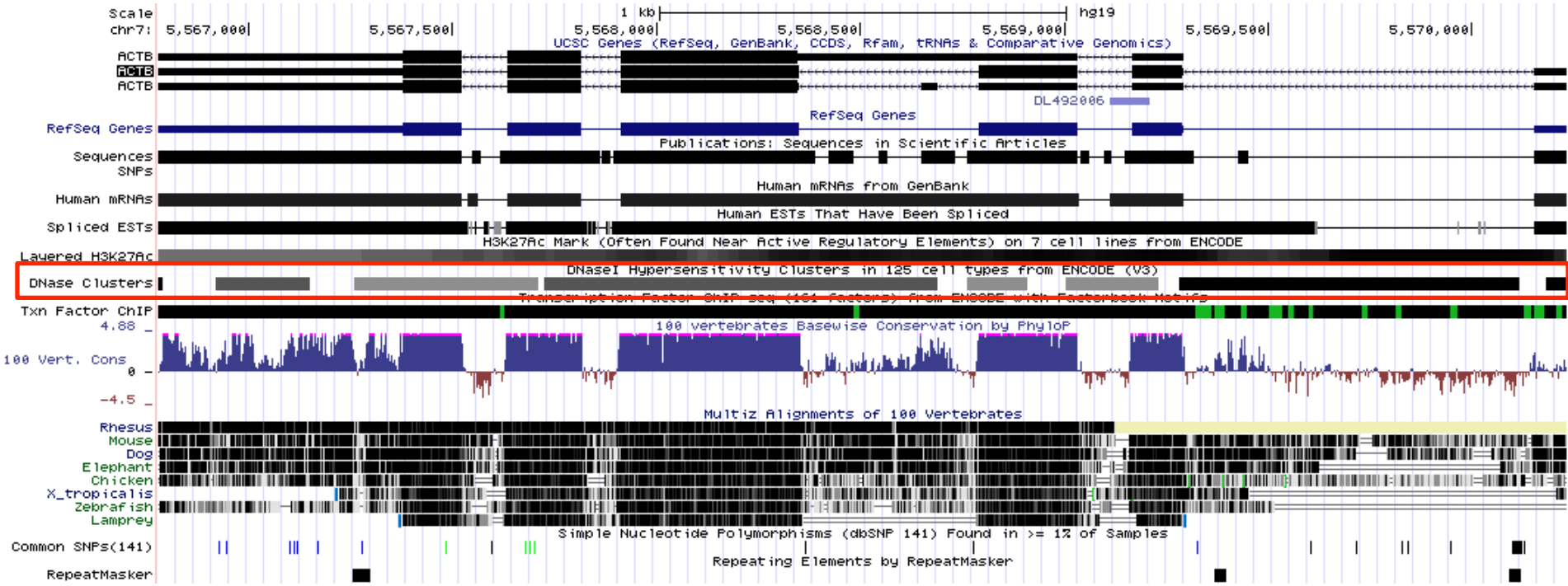

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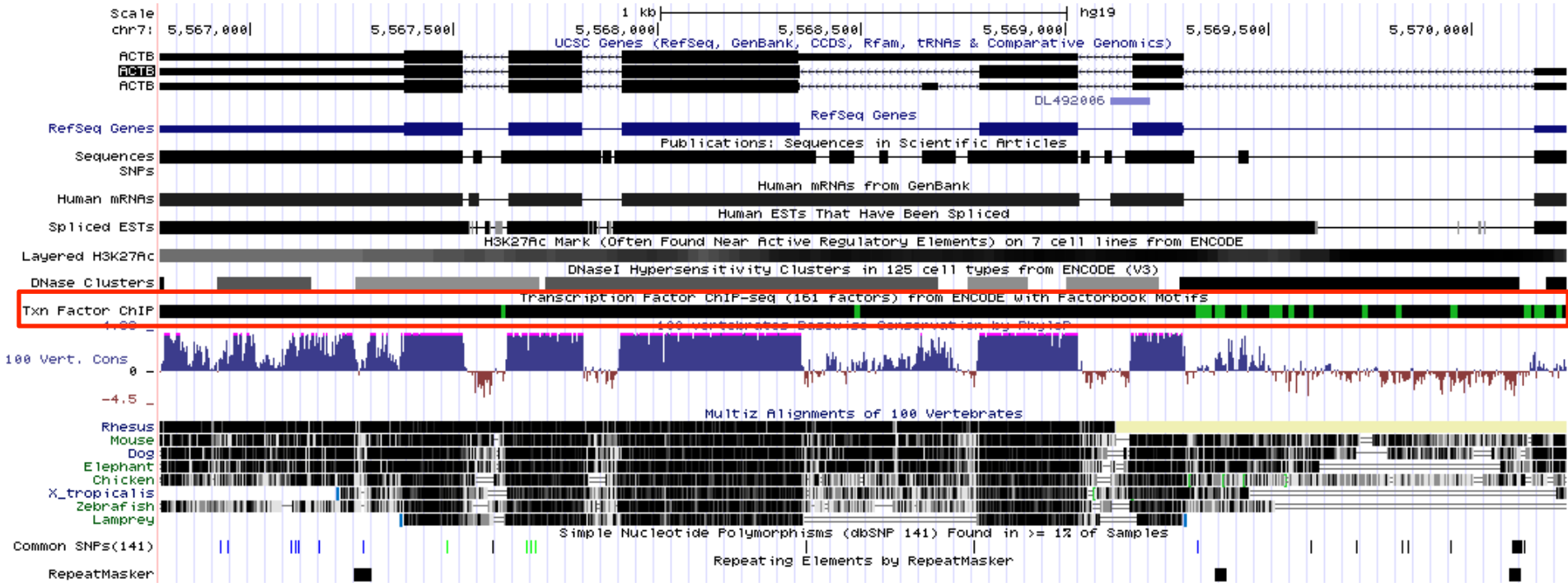

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DNA conservation

Single nucleotide polymorphisms (SNP)

Repeats

The Epigenomics Roadmap Project

http://www.roadmapepigenomics.org/

- Reference human epigenomes
- DNA methylation, histone modifications, chromatin accessibility and small RNA transcripts
- Stem cells and primary *ex vivo* tissues
- 111 tissue and cell types
- 2,804 genome-wide datasets

Further reading

- Impact of artifact removal on ChIP quality metrics in ChIP-seq and ChIP-exo data. Carrol et al, Front. Genet. 2014
- Impact of sequencing depth in ChIP-seq experiments. Jung et al, NAR 2014
- ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. Landt et al, Genome Res. 2012
- http://genome.ucsc.edu/ENCODE/qualityMetrics.html#definitions
- https://www.encodeproject.org/data-standards

Bioconductor ChIP-seq resources

- General purpose tools:
  - Rsubread (read mapping; not ideal for global alignment)
  - Rbowtie (global alignment)
  - GenomicRanges (tools for manipulating range data)
  - Rsamtools (SAM / BAM support)
  - htSeqTools (tools for NGS data; post-alignment QC)
  - chipseq (utilities for ChIP-seq analysis)
  - Csaw (a pipeline for ChIP-seq analysis, including statistical analysis of differential occupancy)
- Peak calling
  - SPP
  - BayesPeak (HMM and Bayesian statistics)
  - MOSAiCS (model-based one and two Sample Analysis and Inference for ChIP-Seq)
  - iSeq (Hidden Ising models)
  - ChIPseqR (developed to analyse nucleosome positioning data)
- Quality control
  - ChIPQC
- Differential occupancy
  - edgeR
  - DESeq, DESeq2
  - DiffBind (compatible with objects used for ChIPQC, wrapper for DESeq and edgeR DE functions)
- Peak Annotation
  - ChIPpeakAnno (annotating peaks with genome context information)

- ChIP – sequencing: introduction from a bioinformatics point of view
- Principles of analysis of ChIP-seq data
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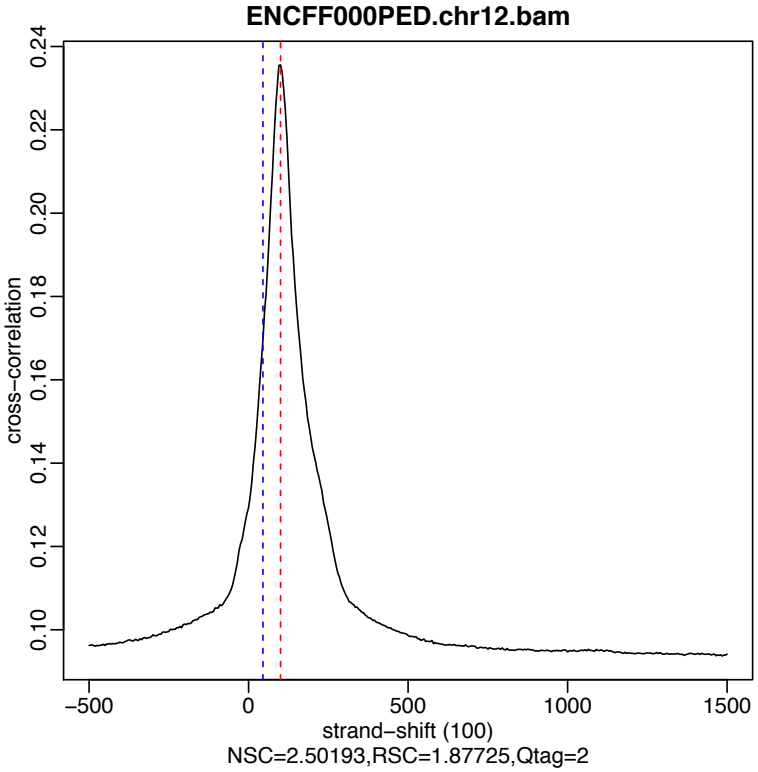
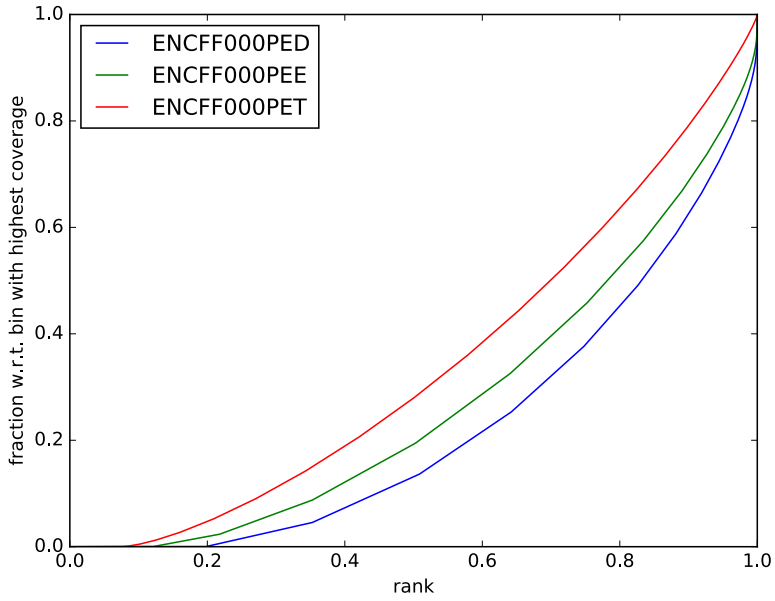
Exercise

- 1. Quality control
- 2. Read preprocessing
- 3. Peak calling
- 4. Exploratory analysis (sample clustering)
- 5. Visualisation
- 6. Statistical analysis of differential occupancy

Did my ChIP work?

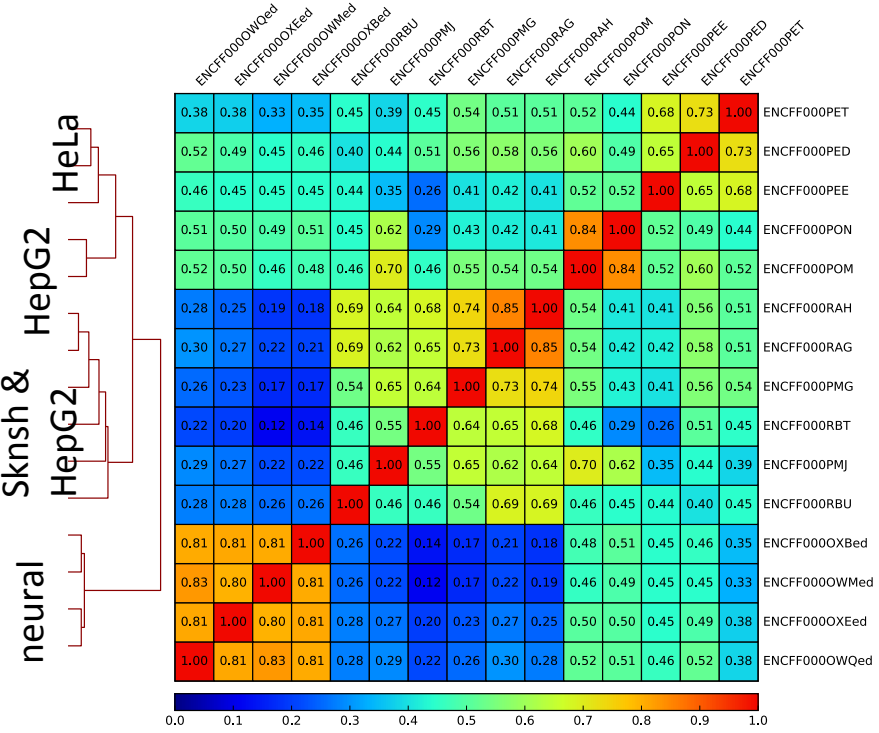


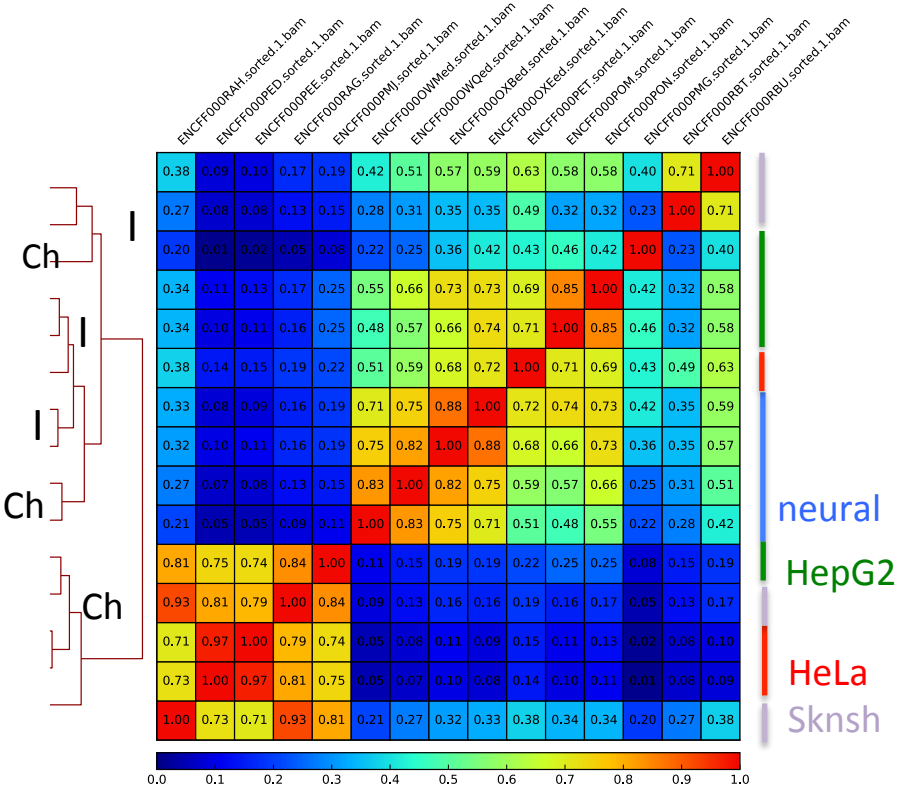
Cross-correlation



Cumulative enrichment

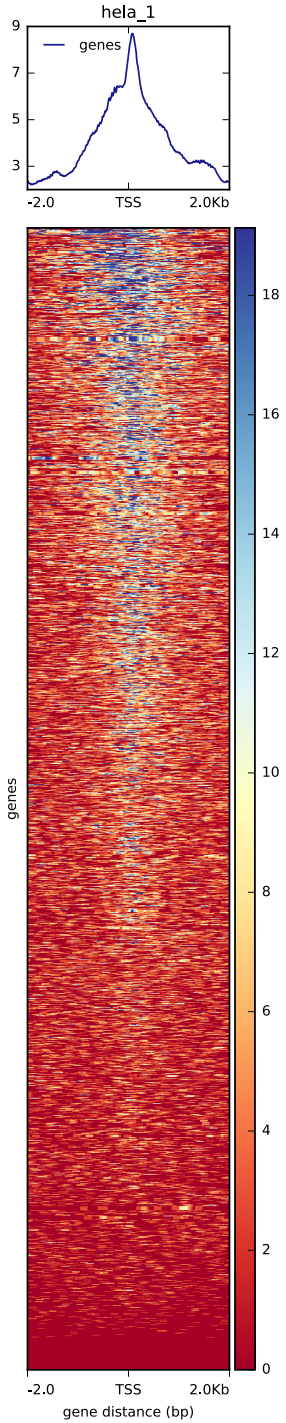
Exploratory analysis





Clustering of libraries
by reads mapped in bins,
genome – wide (spearman)

Clustering of libraries
by reads mapped in peaks
(pearson)



Binding profile around TSS

Questions?

agata.smialowska@bils.se

That’s all for now,

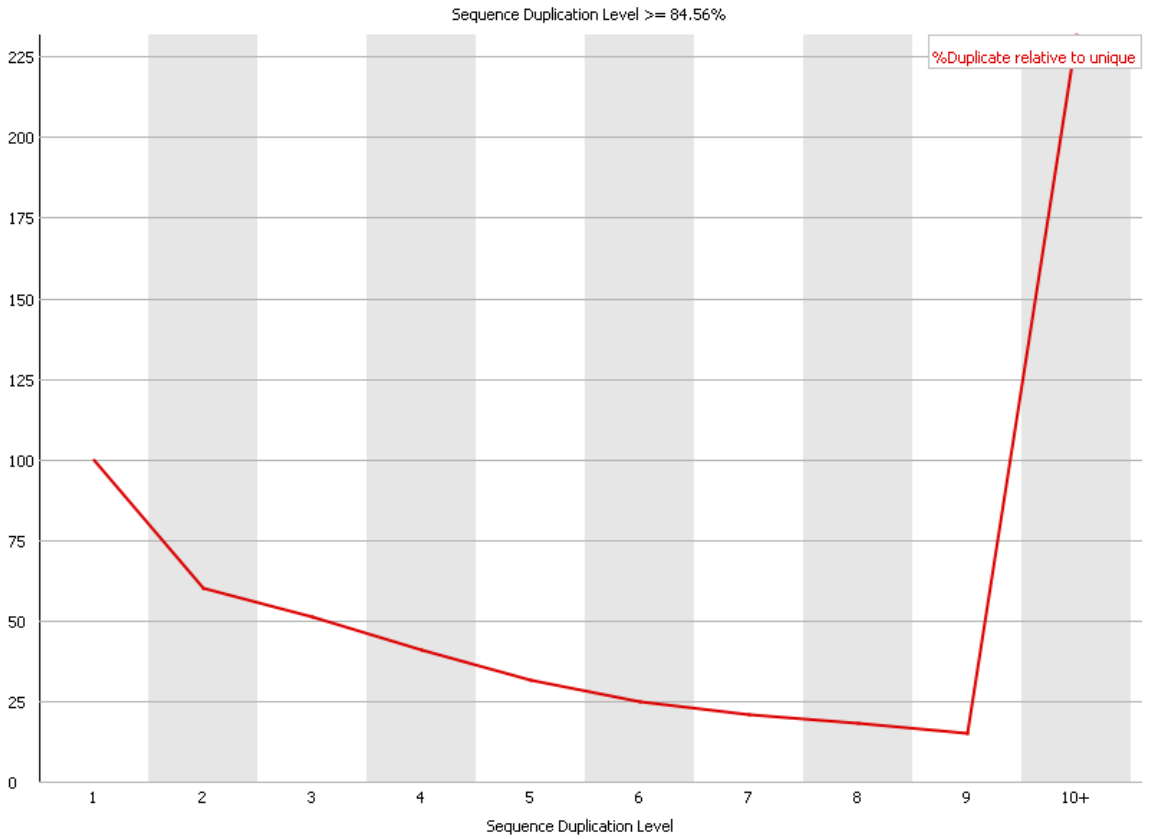
time to do some hands-on work

Library quality control and preprocessing

- FastQC / Prinseq
- Trim adapters if any adapter sequences are present in the reads (as determined by the QC)
- In some cases, you'll observe k-mer enrichment (especially if the data is ChIP-exo, a new variation of ChIP-seq) – it is not necessarily a bad thing, if sequence duplication levels are low; however it may indicate **low complexity of the library** – a warning sign that the enrichment in ChIP was not successful or the libraries are over-amplified (often the latter is the consequence of the former)

Quality control: tag uniqueness – library complexity metric

Sequence duplication level > 70% (low complexity library)



NRF: Non-redundant fraction (of reads): proportion of unique tags / total

<u>less than 20% of reads should be duplicates for 10 million reads sequenced (ENCODE)</u>

Mapping reads to the reference genome

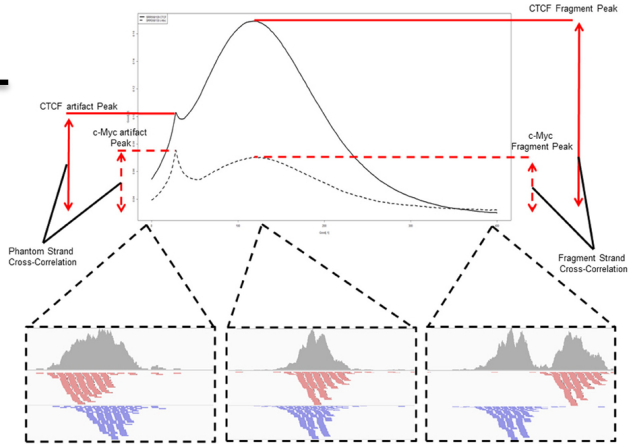
- Choose the right reference: assembly version (not always the newest is best) and type (primary assembly, or assembly from individual chromosome sequences + non-chromosomal contigs; not the top level assembly); choose the matching annotation file (GTF, GFF)
- Read mapping: **global alignment**
- Mappers (= aligners): Bowtie, BWA, BBMap, Novoalign, … (lots of tools are available)

- Visualise data in genome browser
  - BAM files or tracks (wig, bedgraph, bigWig)
  - Local (IGV) or web-based (UCSC genome browser)
  - Data quality assessment

Cross-correlation profiles, RSC and NSC

- Metrics to quantify the fragment length signal and the ratio of fragment length signal to read length signal
- Relative Cross Correlation (RSC) - ChIP to artifact signal

$$\frac{CC(\text{Fragment length}) - \min(CC)}{CC(\text{read length}) - \min(CC)}$$

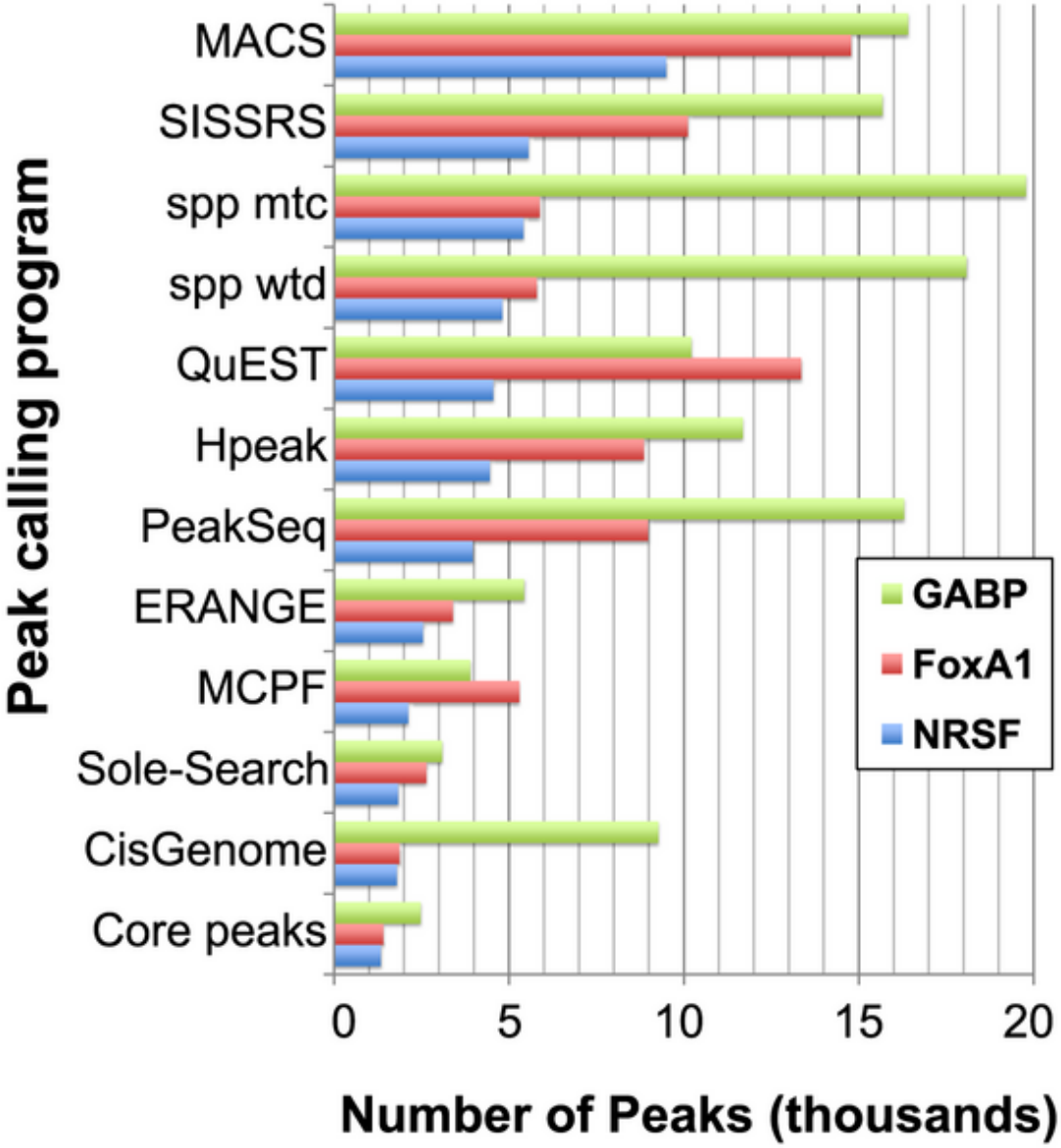


- Normalised Cross Correlation (NSC)

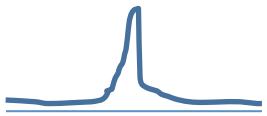
$$\frac{CC(\text{Fragment length})}{\min(CC)}$$

- TFs: fragment lengths are often greater than the size of the DNA binding event, the distinct clustering of (+) and (-) reads around this site is very apparent
- NSC>1.1 (higher values indicate more enrichment; 1 = no enrichment)
- RSC>0.8 (0 = no signal; <1 low quality ChIP; >1 high enrichment
- Broad peaks: this clustering may be more diffuse (fragment length < peak)

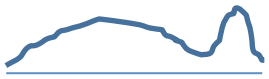
Comparison of peak calling algorithms



Peak overlap (Ho et al, 2012)



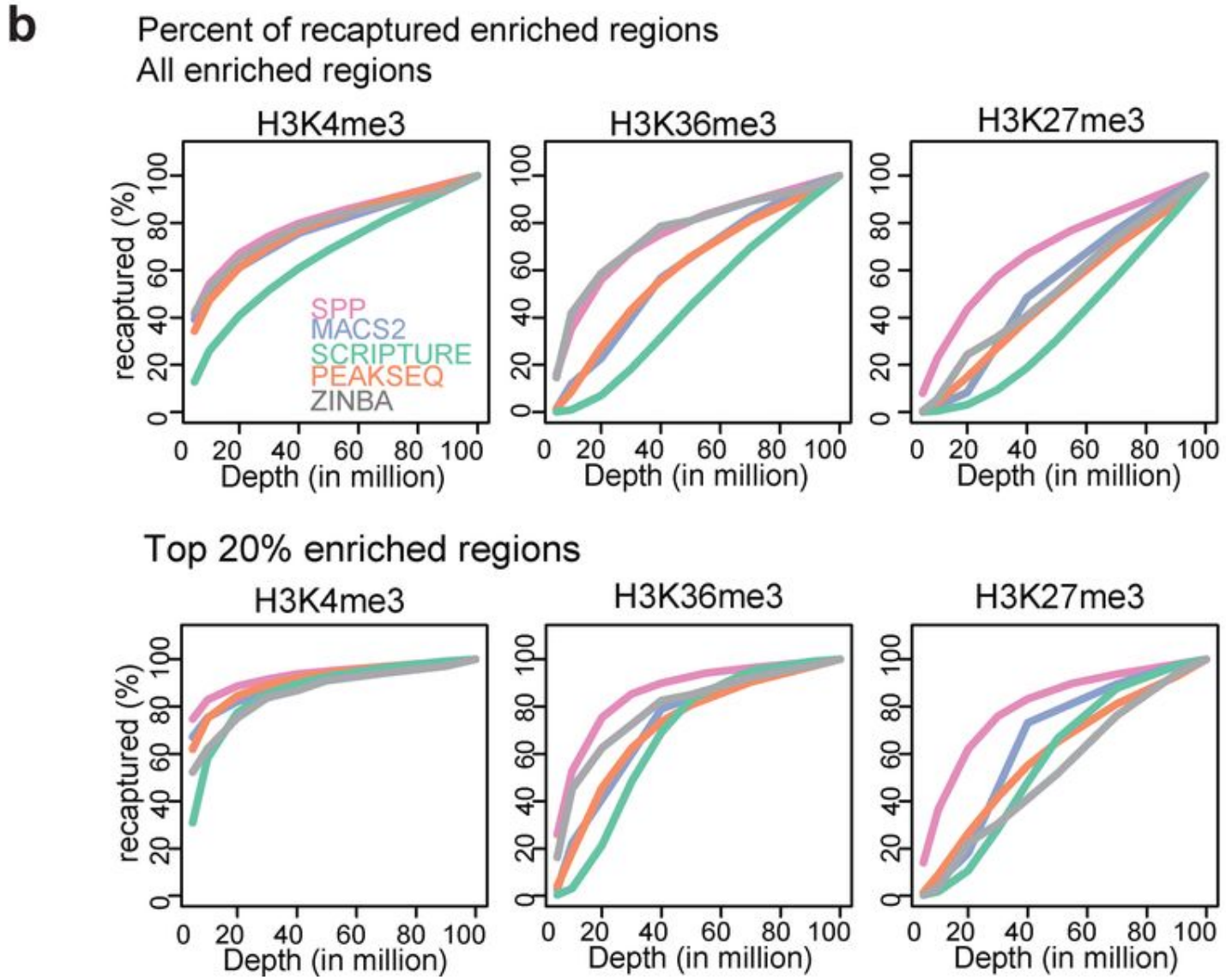
> 50 %



20 %

Wilbanks 2010

Effect of sequencing depth on regions detected by various algorithms



Jung 2014

Fold enrichment = signal / background