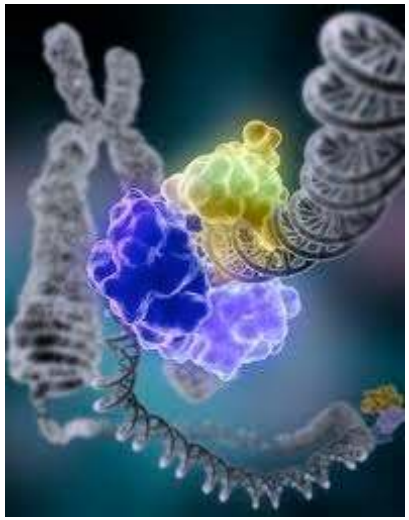
Sequence analysis with Scripture

Manuel Garber

The dynamic genome

The dynamic genome



Copied and
Repaired

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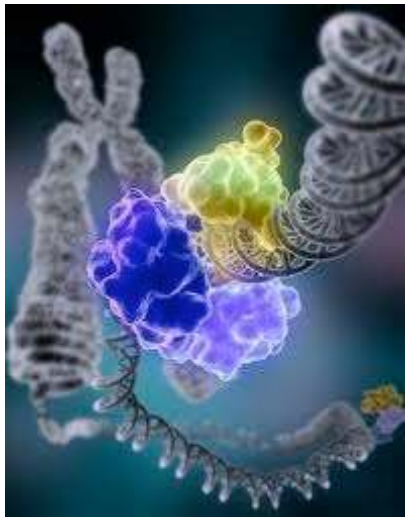

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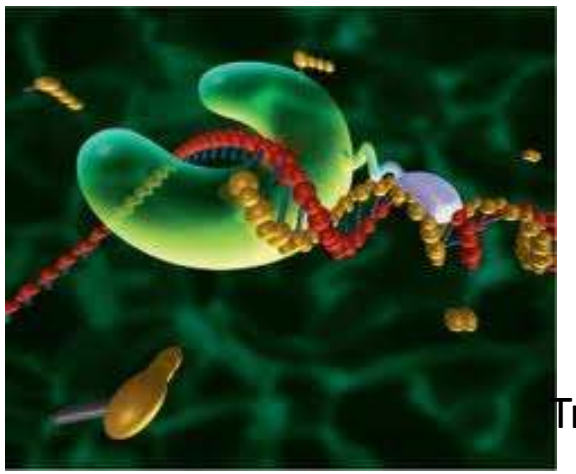

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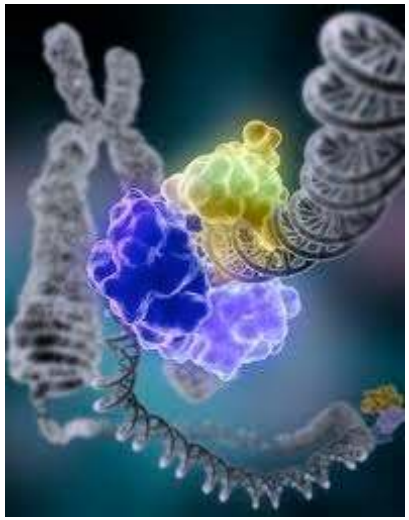

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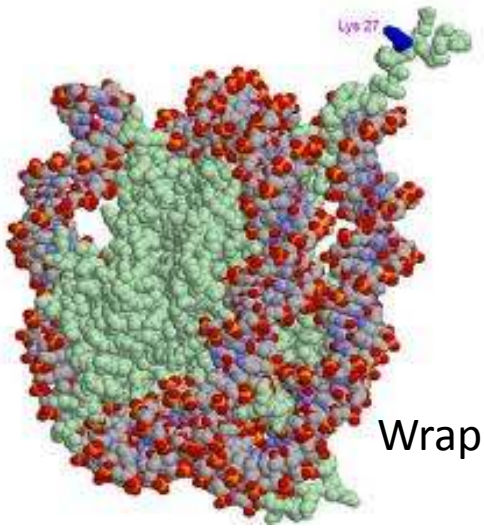
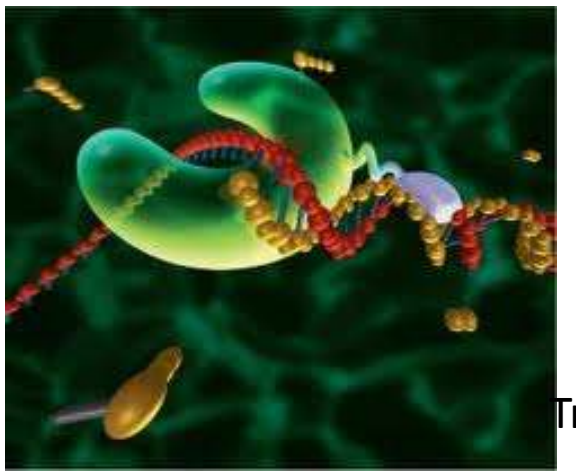


Wrapped in marked histones



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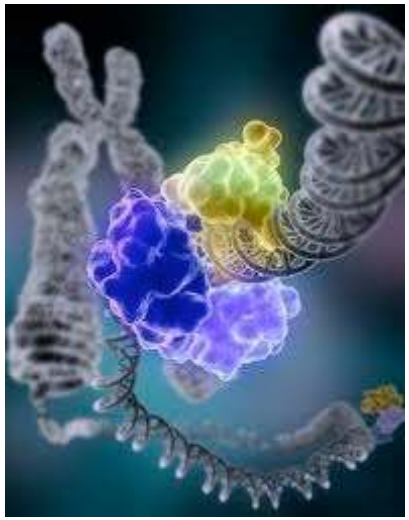

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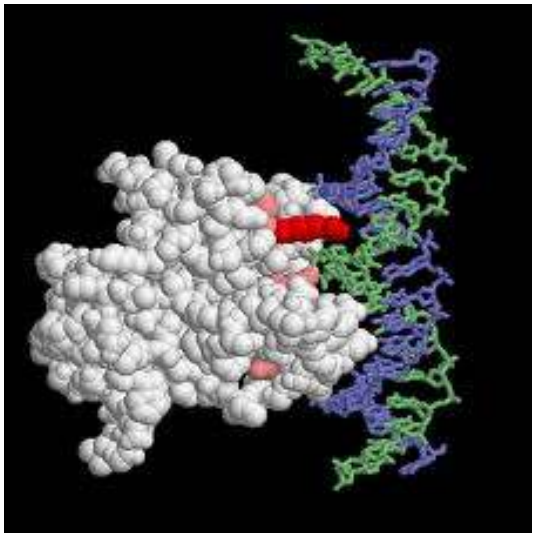
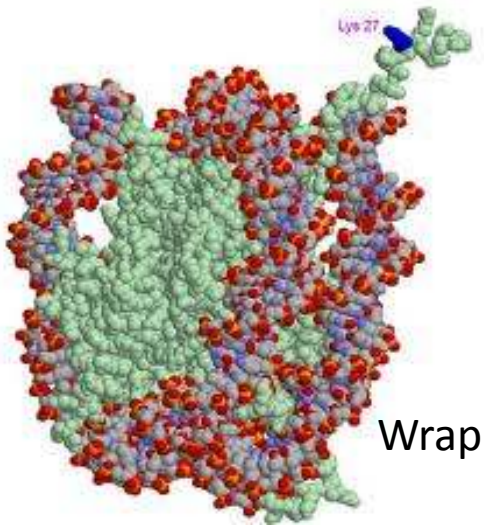
Bound by Transcription Factors





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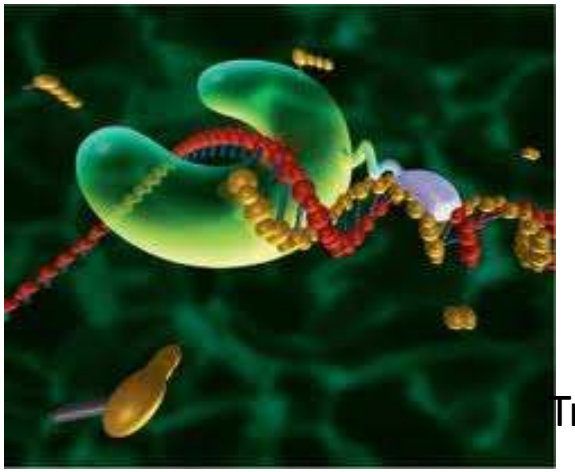

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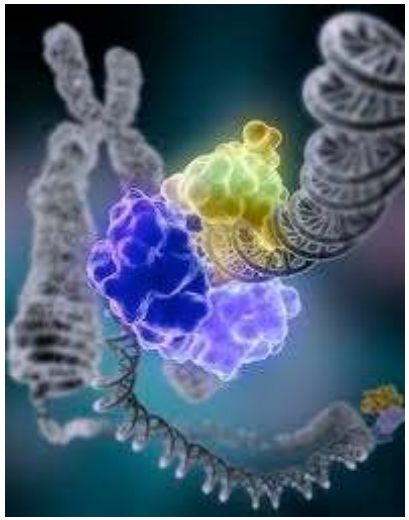

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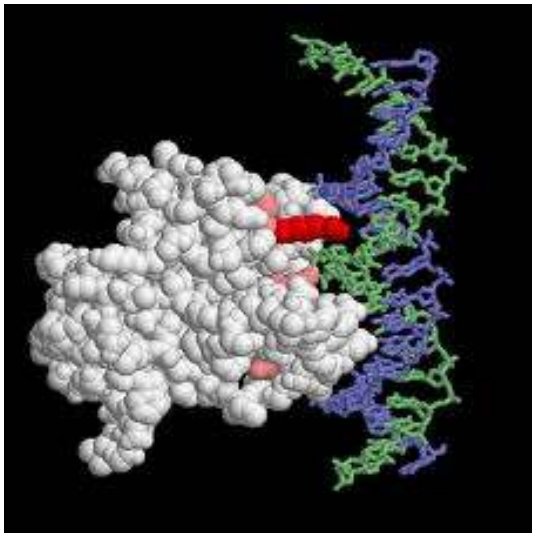

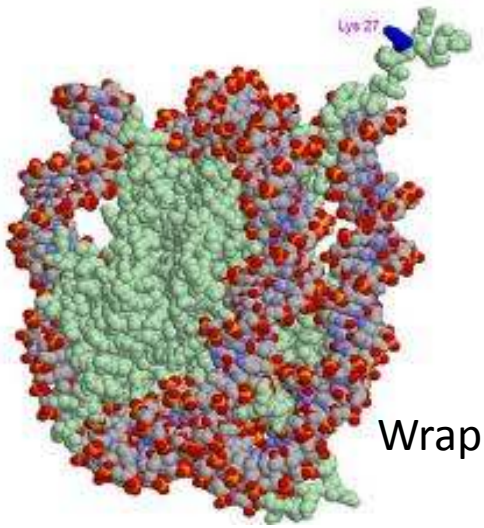

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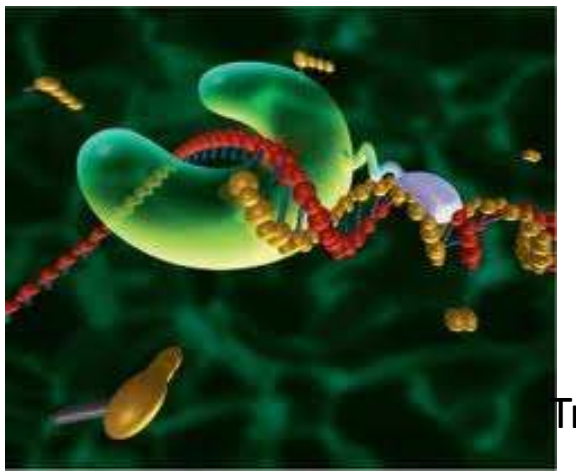
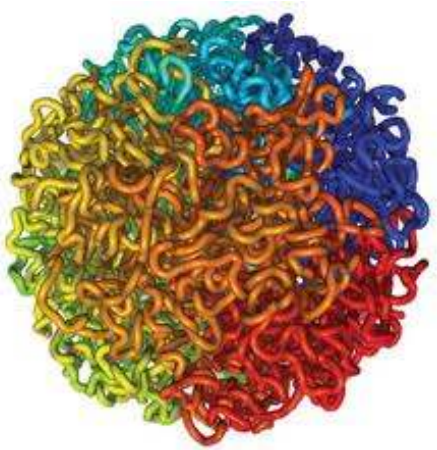
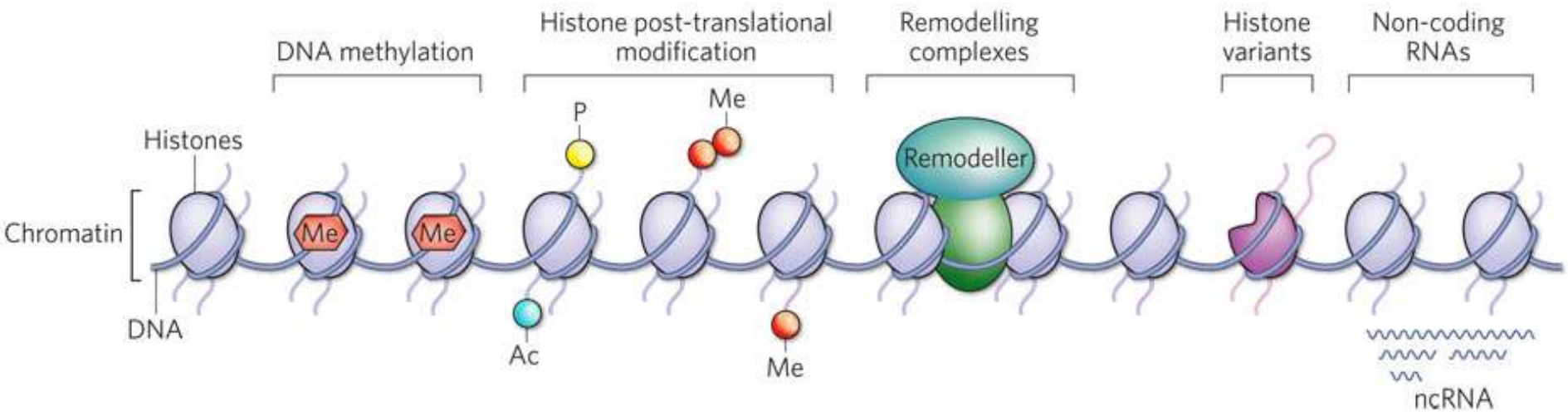


Folded into a fractal globule



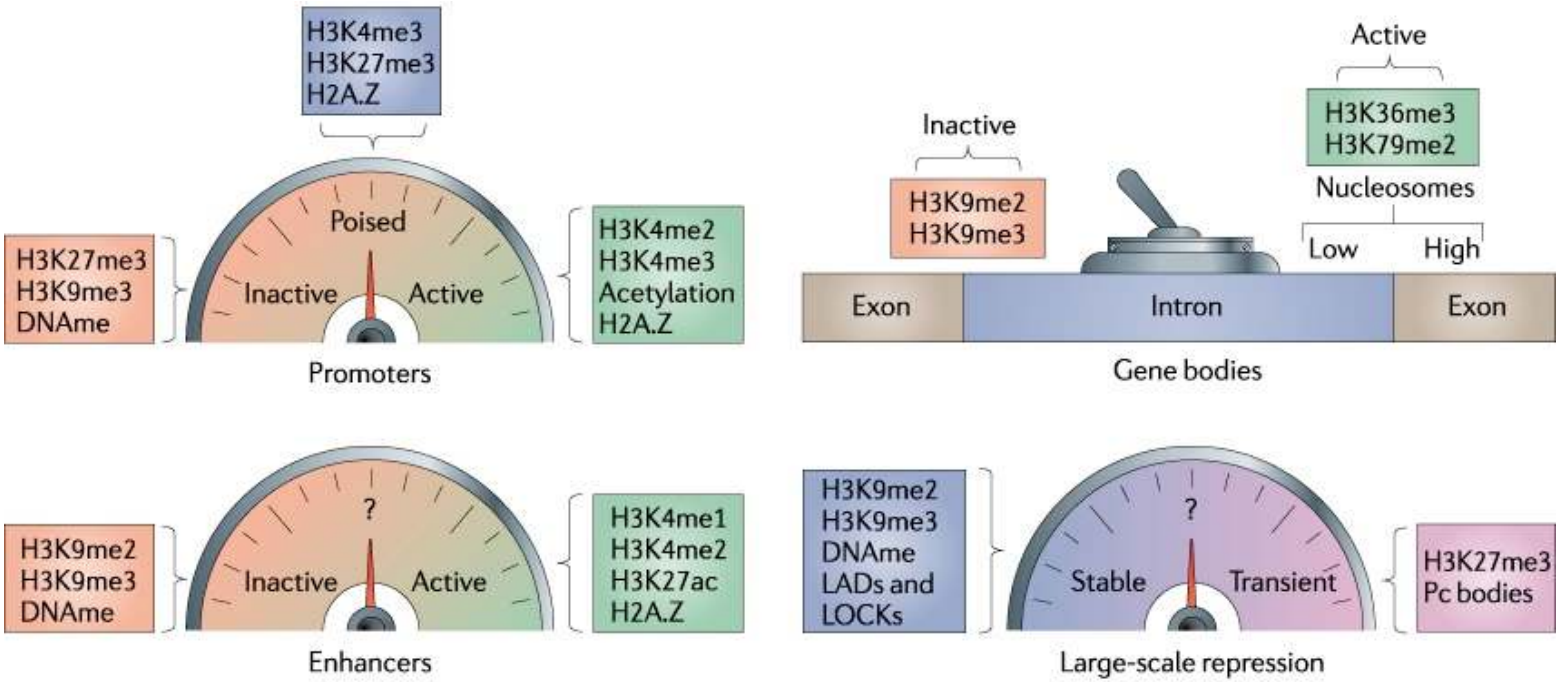
Transcribed

With a very dynamic epigenetic state



Catherine Dulac, Nature 2010

Which define the state of its functional elements



Zhou, Goren, Berenstein, **Nature Reviews** | Genetics

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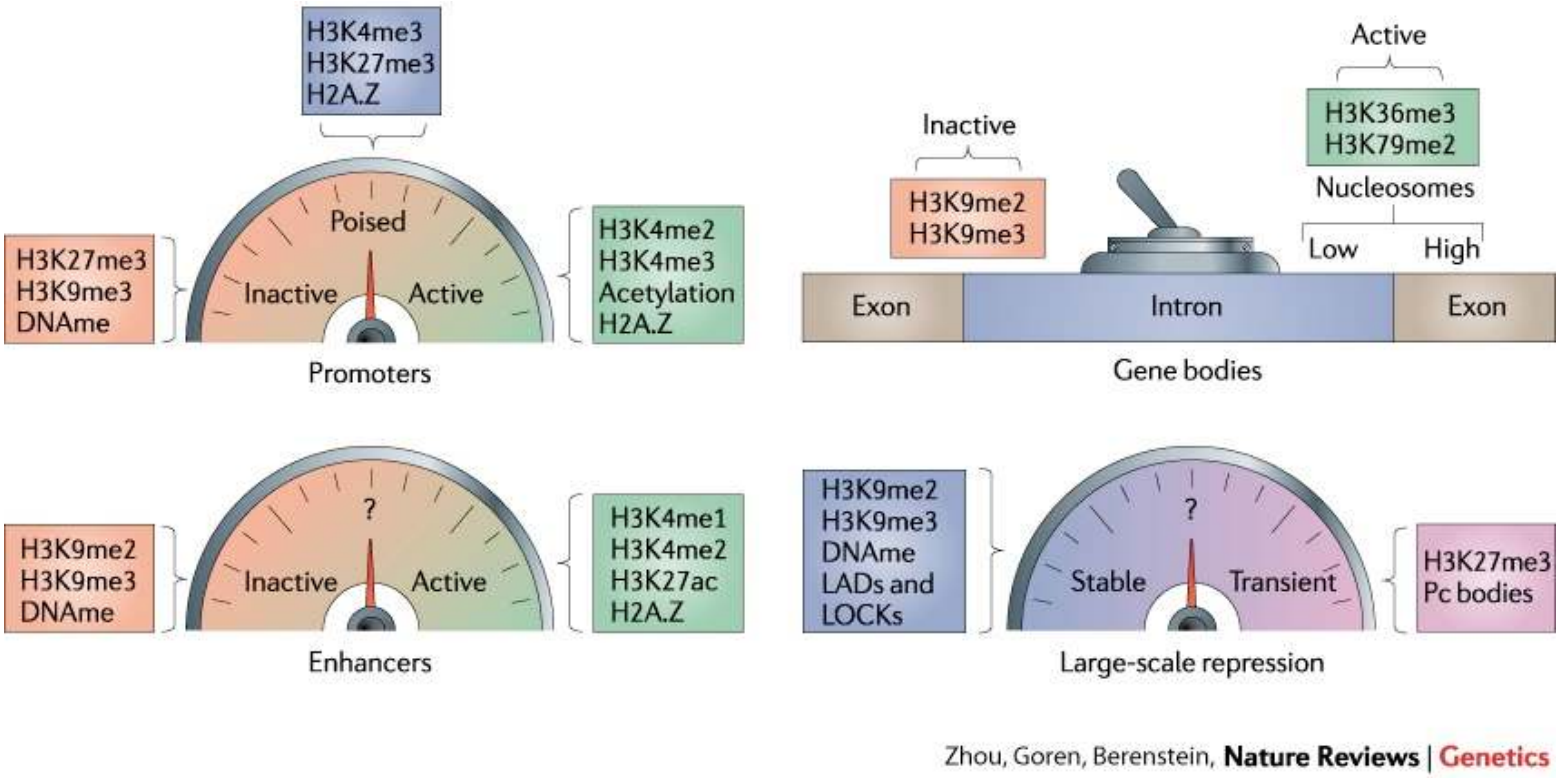
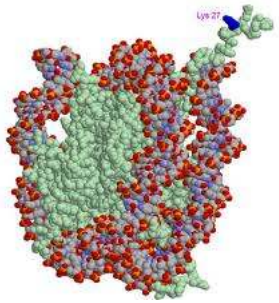


Zhou, Goren, Berenstein, **Nature Reviews** | Genetics

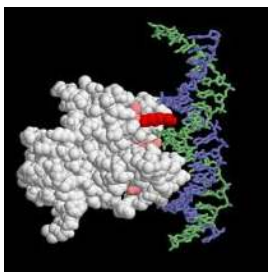
Motivation: find the genome state using sequencing data

We can use sequencing to find the genome state (Protein-DNA)

Histone Marks



Transcription Factors



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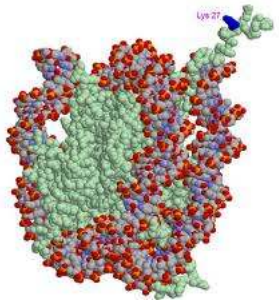

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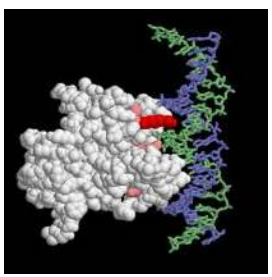
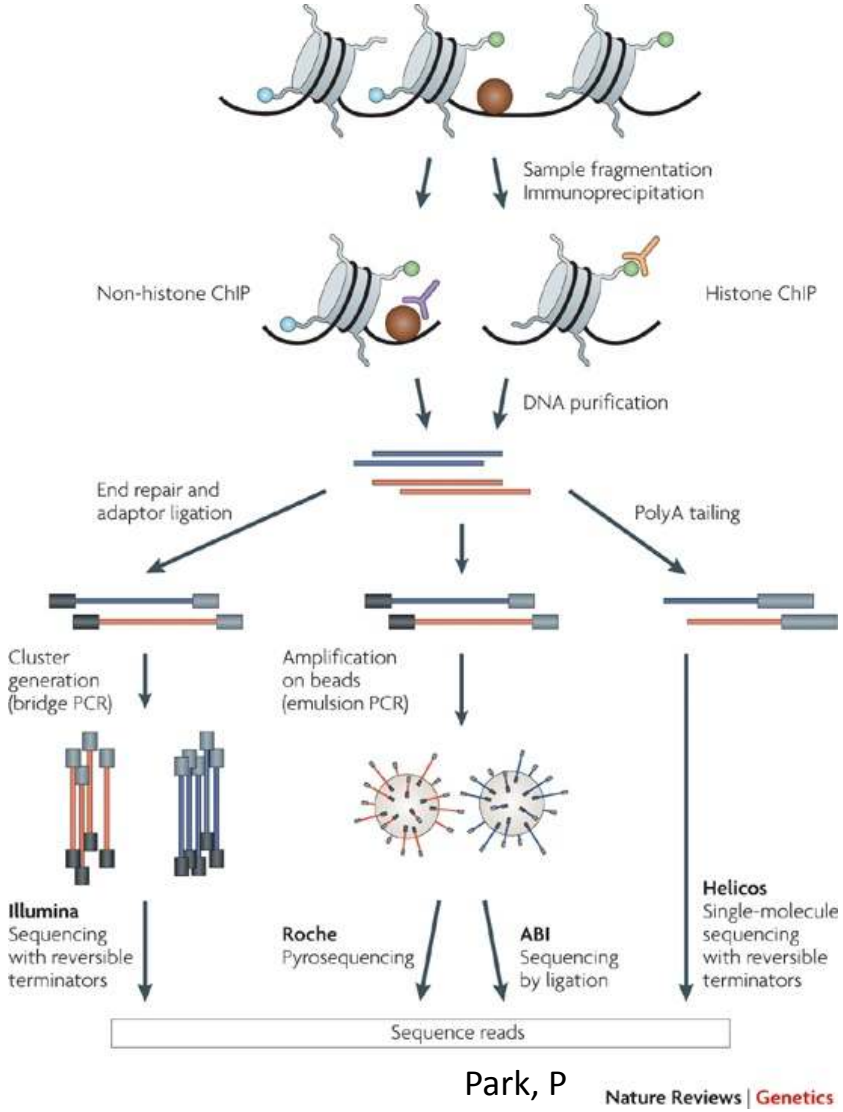


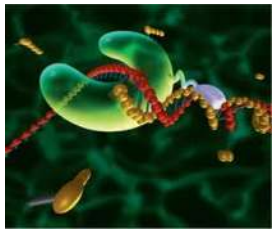
ChIP-Seq



Park, P

Nature Reviews | Genetics

We can use sequencing to find the genome state



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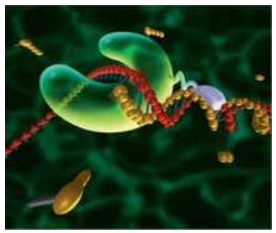
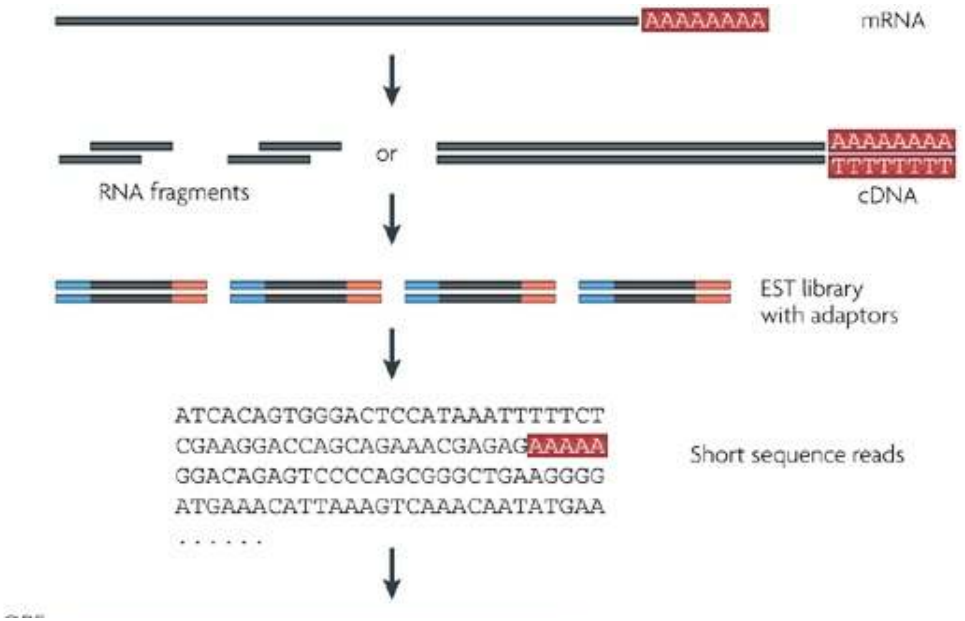


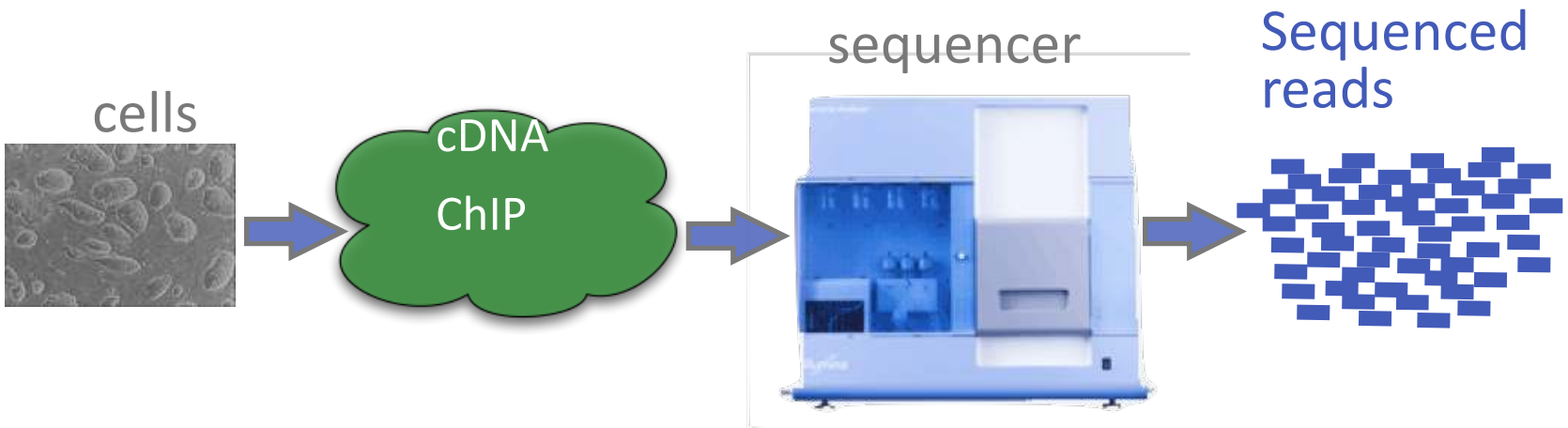
Transcription

RNA-Seq



Wang, Z Nature Reviews Genetics 2009

Once sequenced the problem becomes computational



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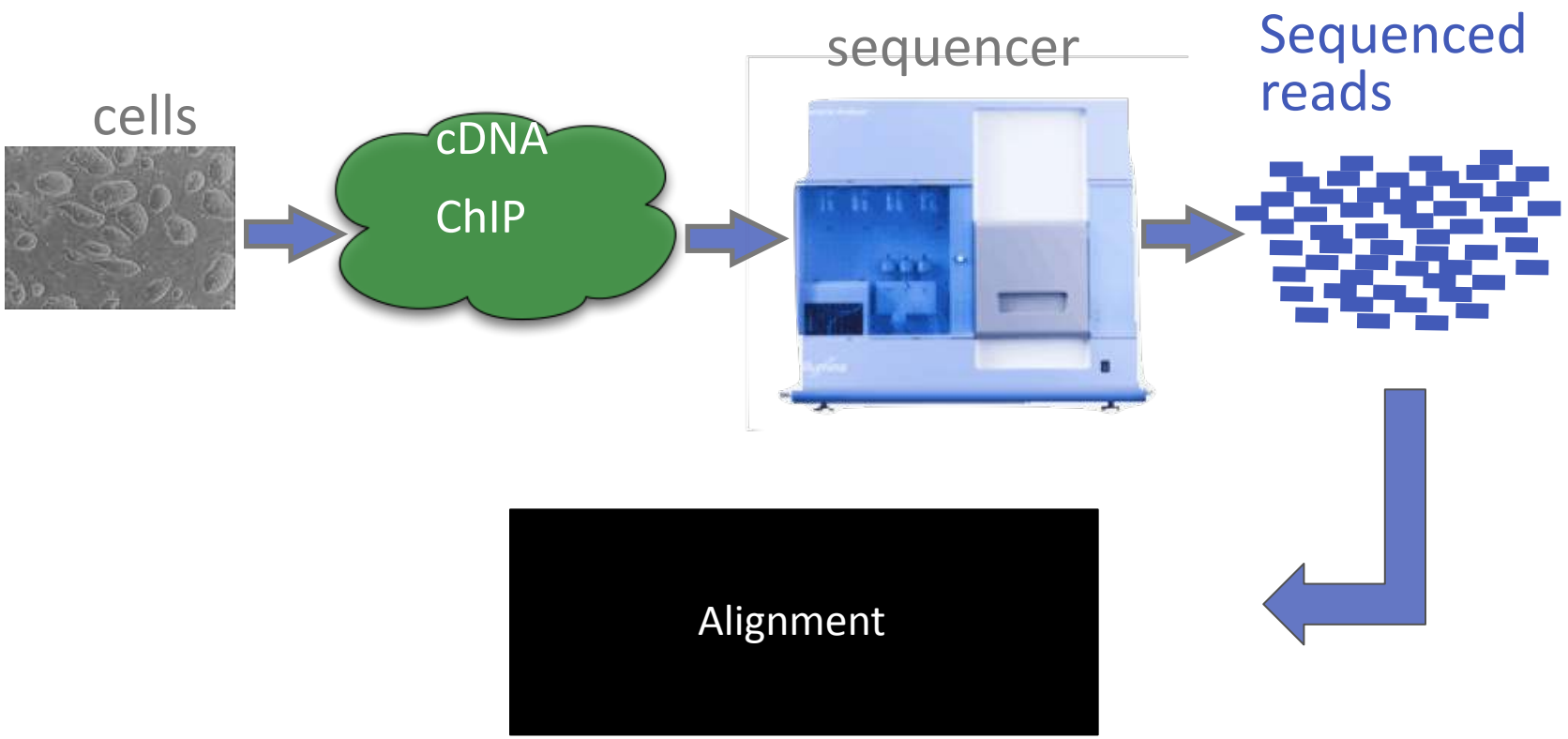

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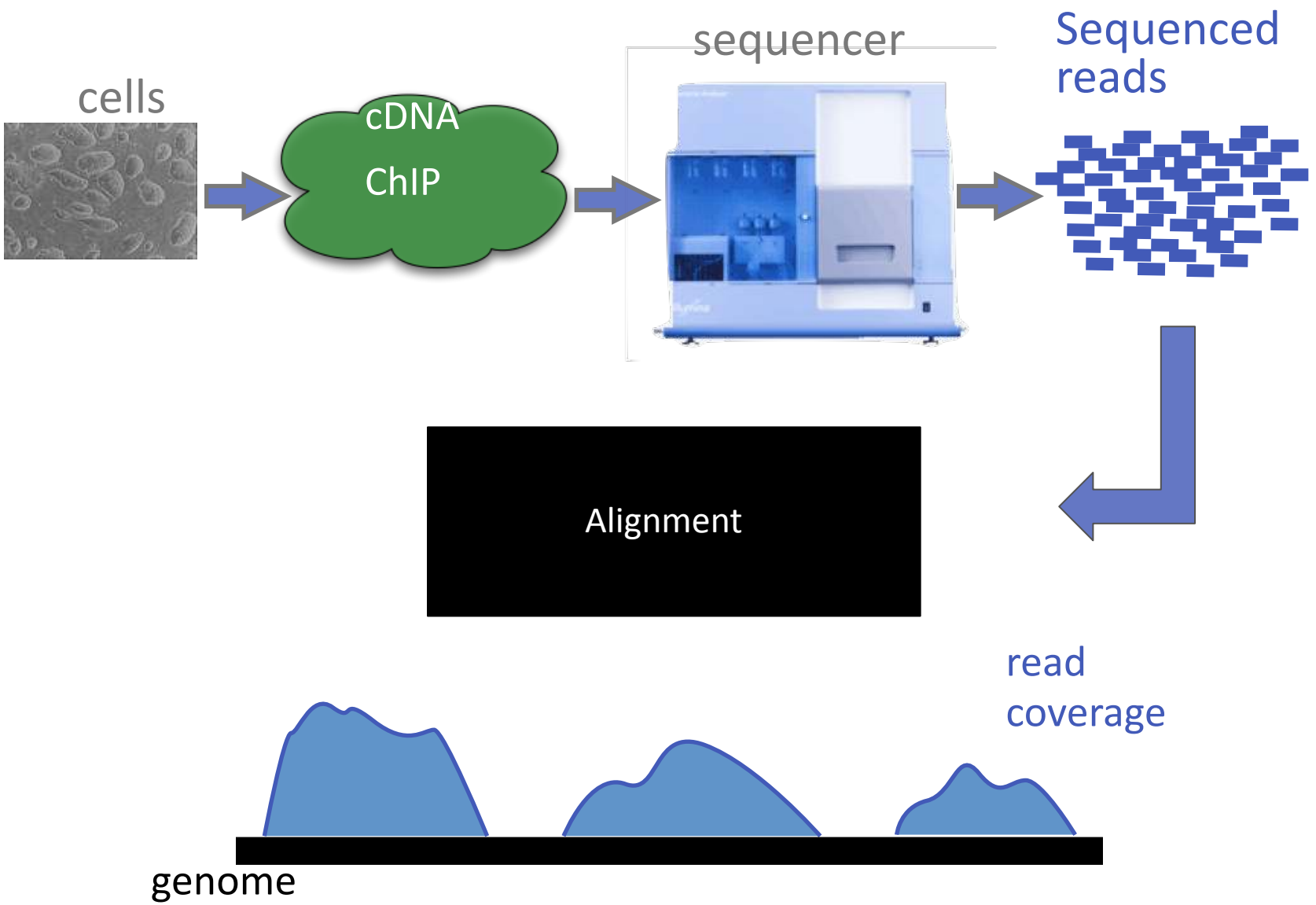

Overview of the session

We'll cover the 3 main computational challenges of sequence analysis for *counting applications*:

- Read mapping (alignment): Placing short reads in the genome
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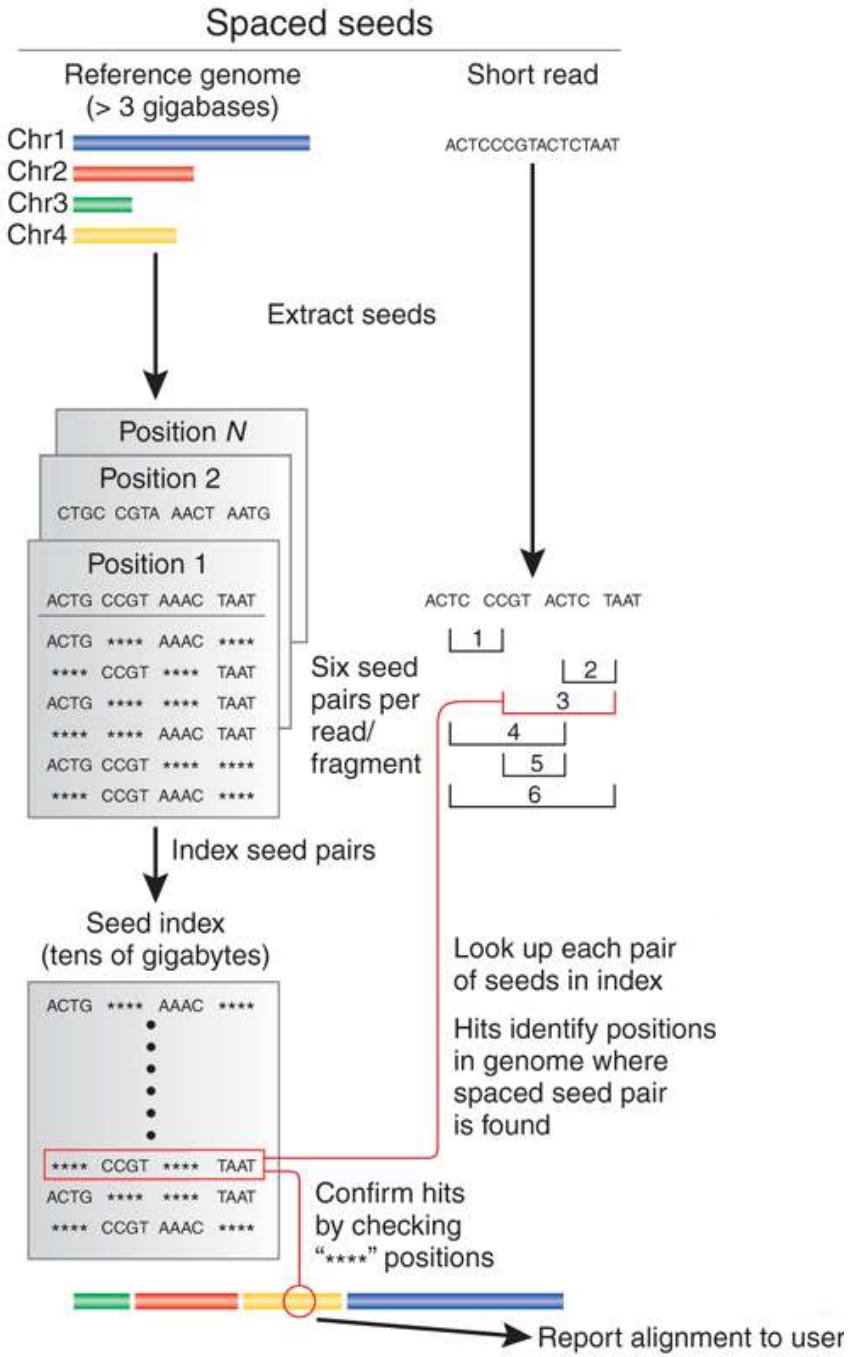
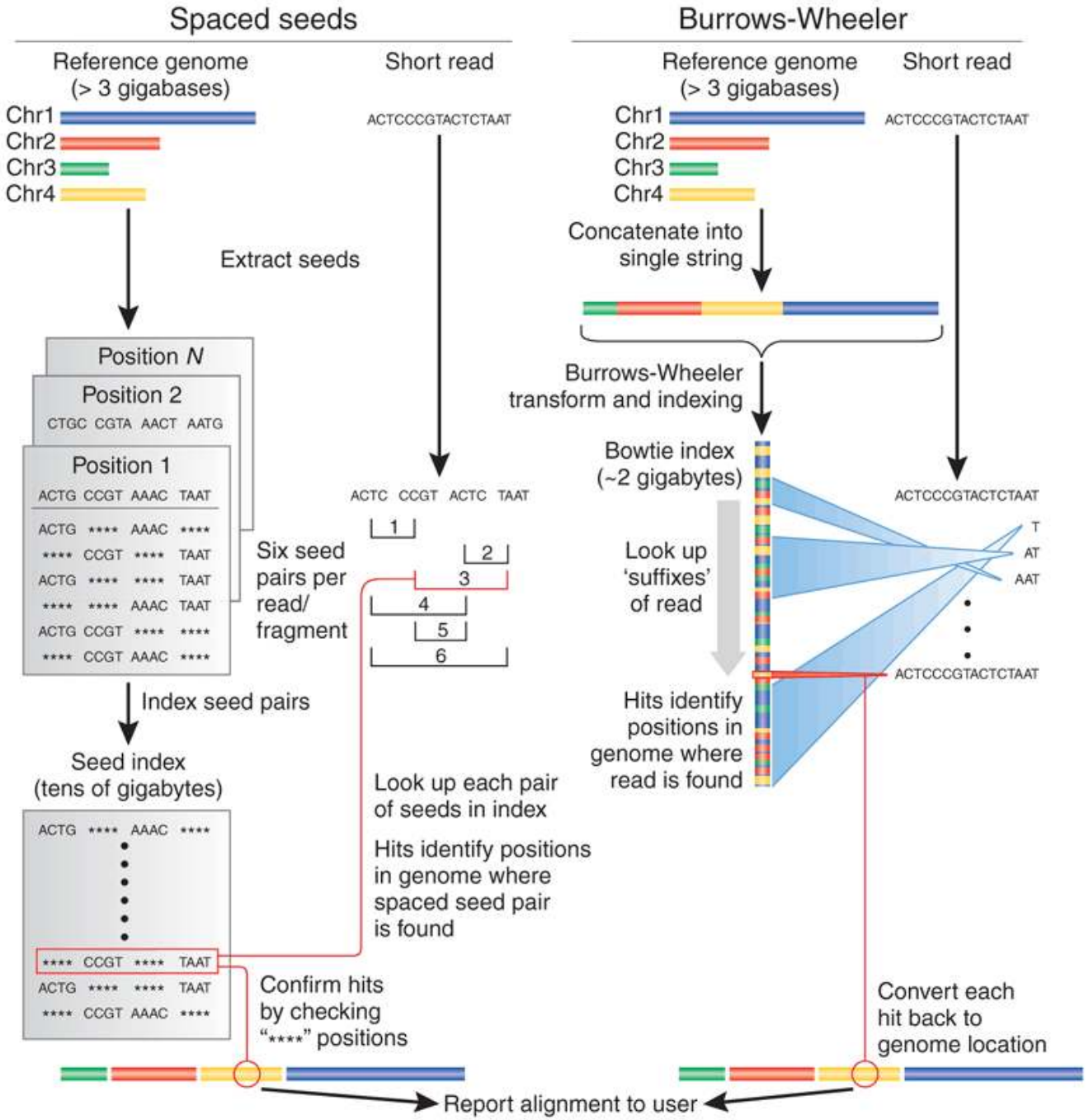


Trapnell, Salzberg, Nature Biotechnology 2009



Trapnell, Salzberg, Nature Biotechnology 2009

Short read mapping software for ChIP-Seq

Seed-extend

	Short indels	Use base qual
Maq	**No**	**YES**
BFAST	Yes	NO
GASSST	Yes	NO
RMAP	Yes	**YES**
SeqMap	Yes	NO
SHRiMP	Yes	NO

BWA

	Use Base qual
BWA	**YES**
Bowtie	NO
Soap2	NO
Stampy*	YES

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Stampy is a hybrid approach which first uses BWA to map reads then uses seed-extend only to reads not mapped by BWA

RNA-Seq read mapping is more complex

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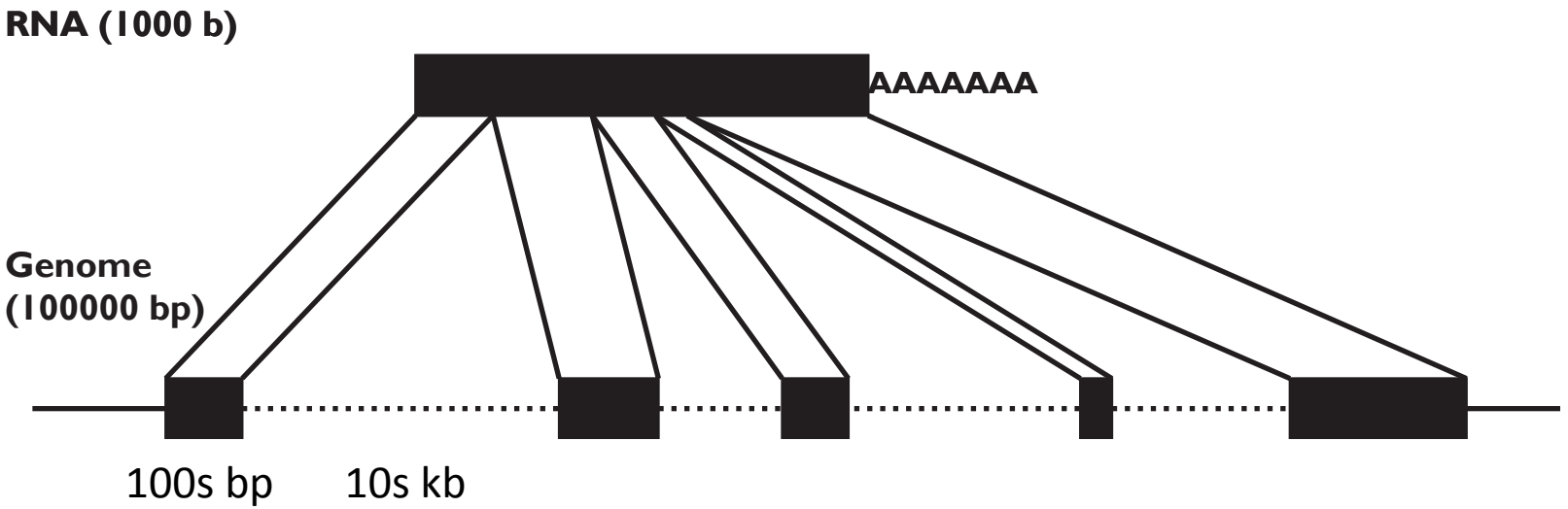

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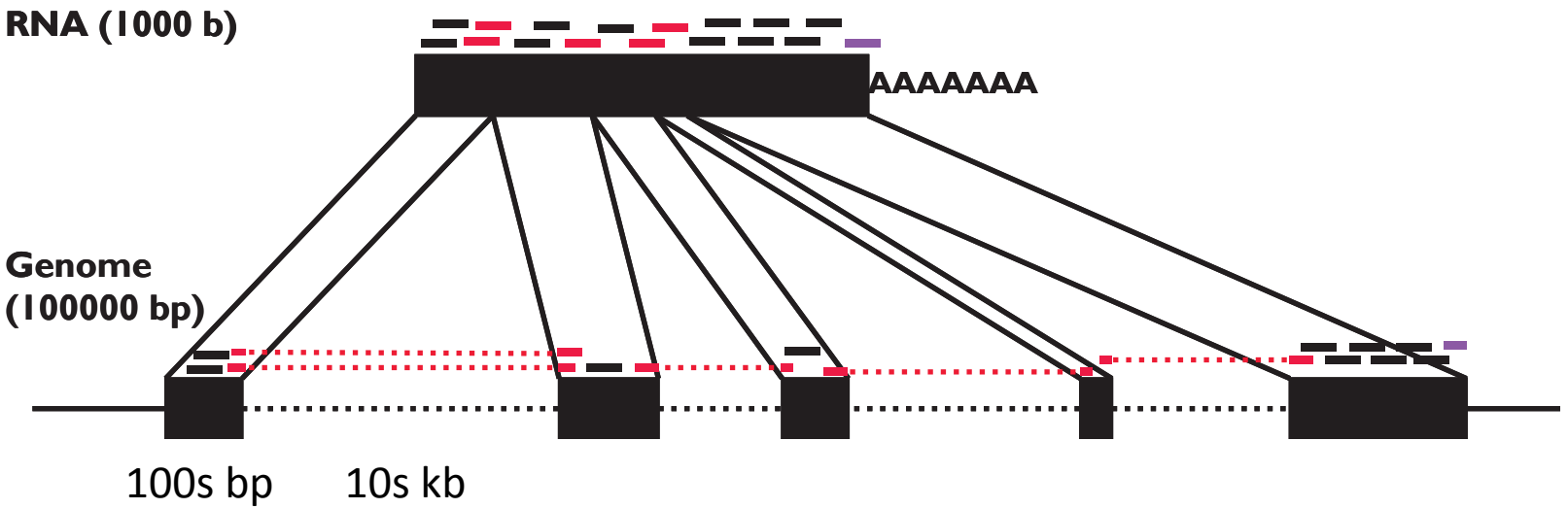

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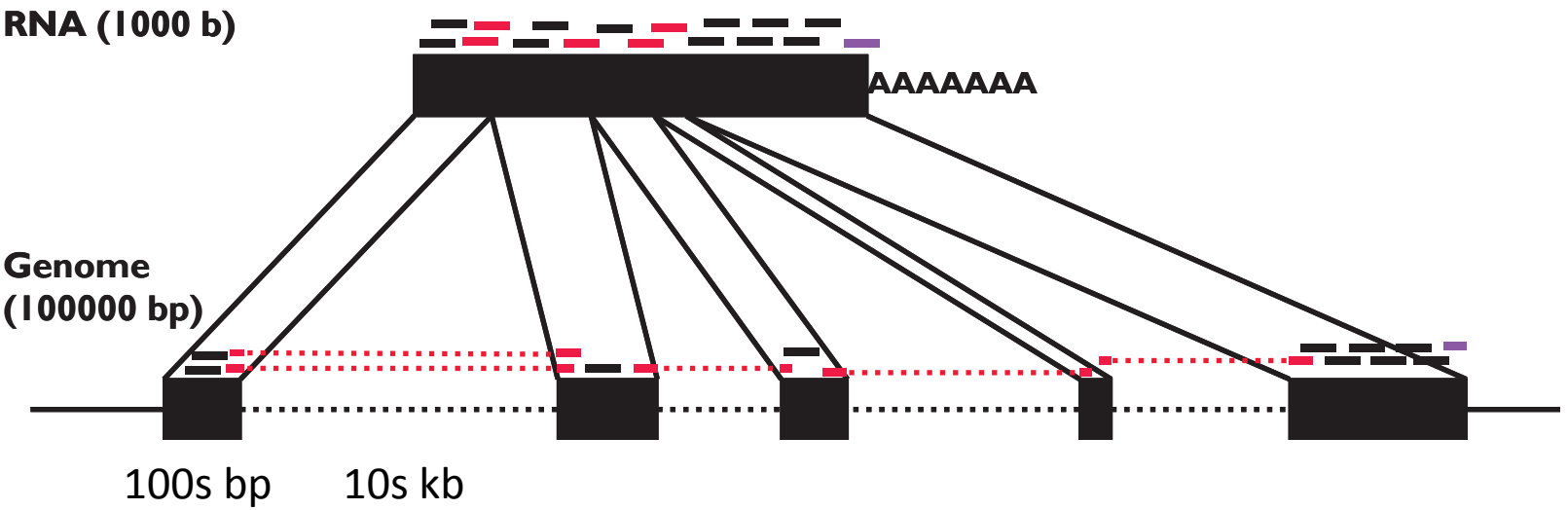

RNA-Seq reads can be spliced, and spliced reads are most informative

Method 1: Seed-extend spliced alignment

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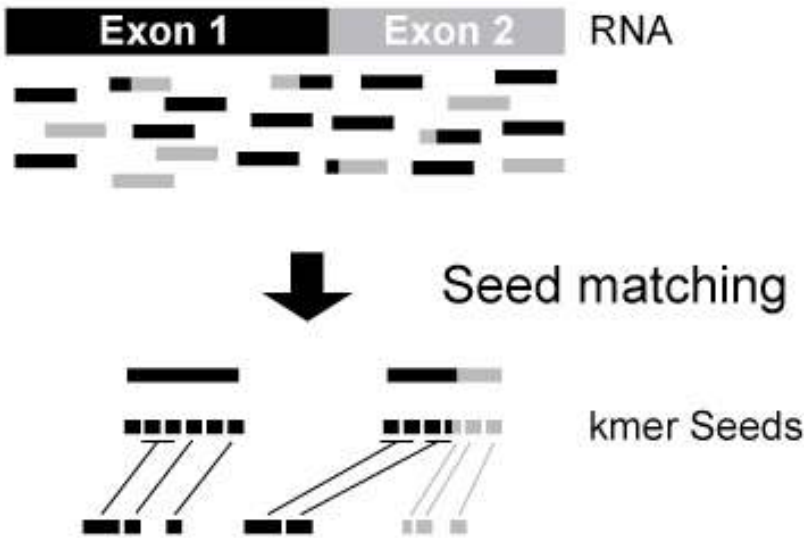

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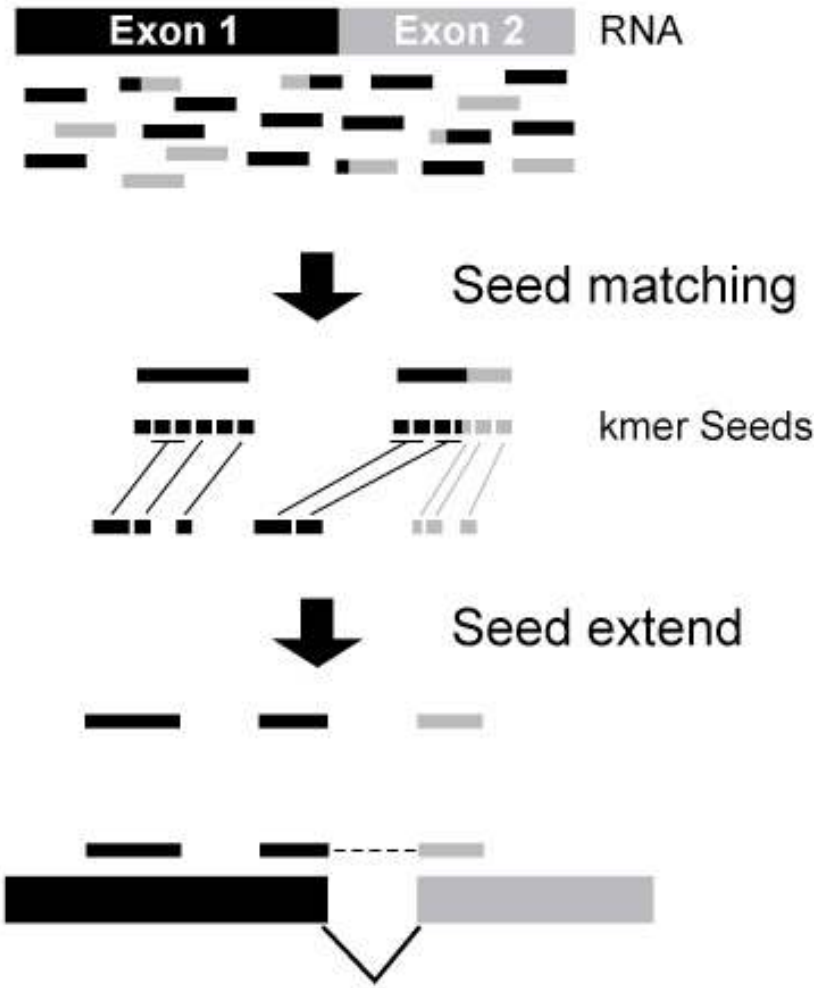

Method 11: Exon-first spliced alignment

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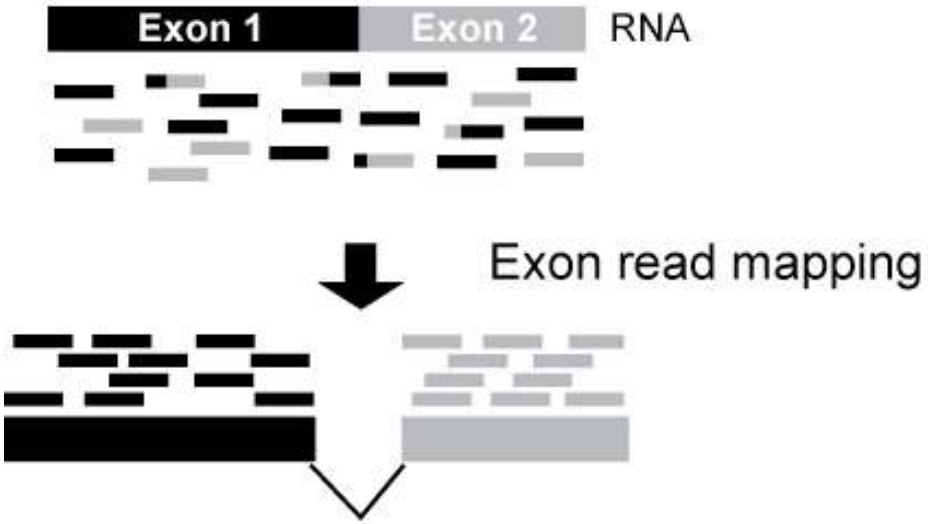

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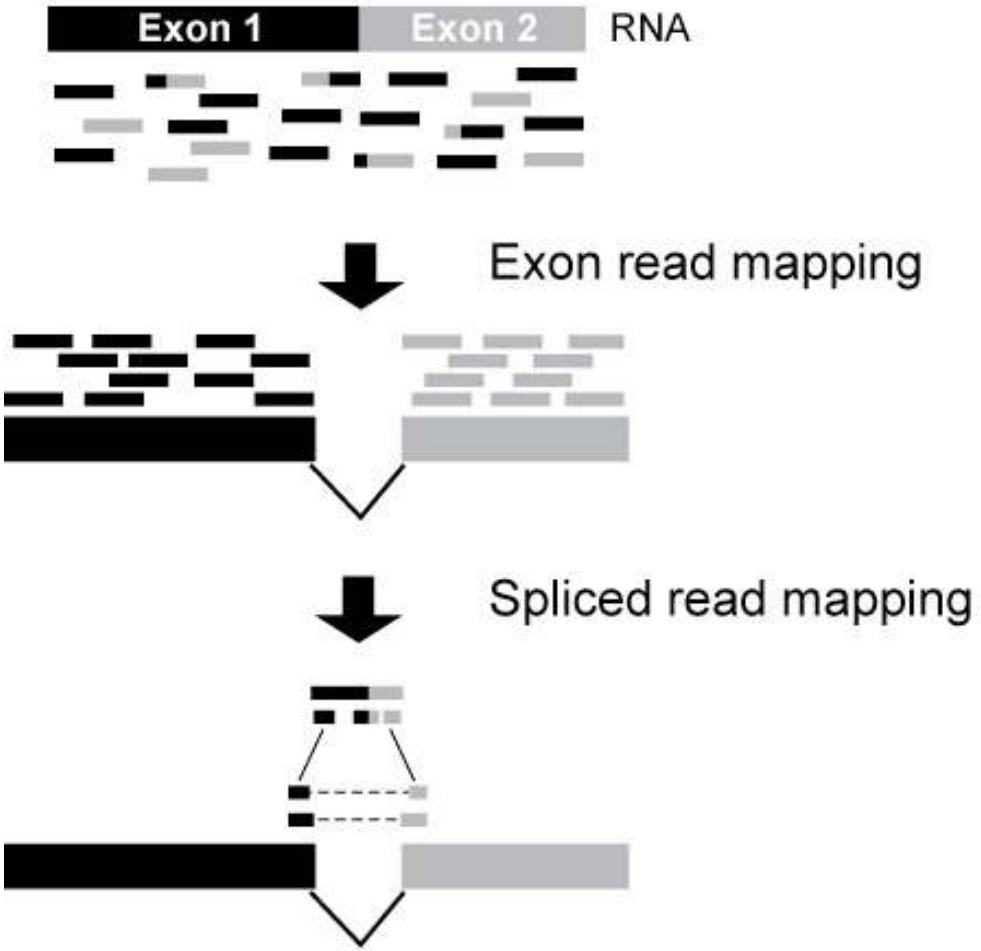

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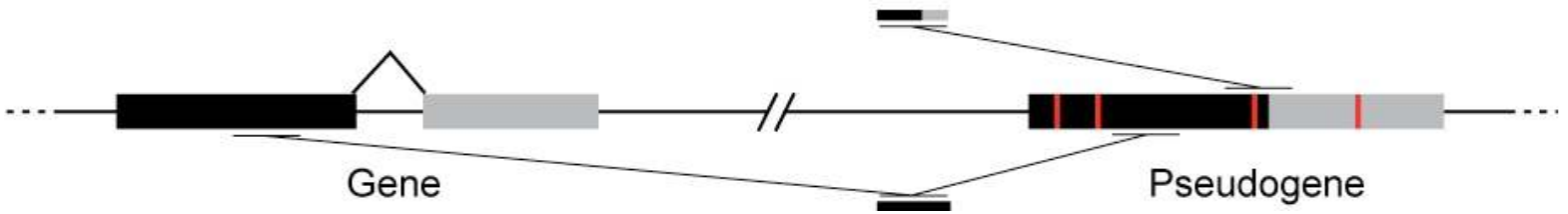
Exon-first alignments will map contiguous first at the expense of spliced hits

Exon-first aligners are faster but at cost



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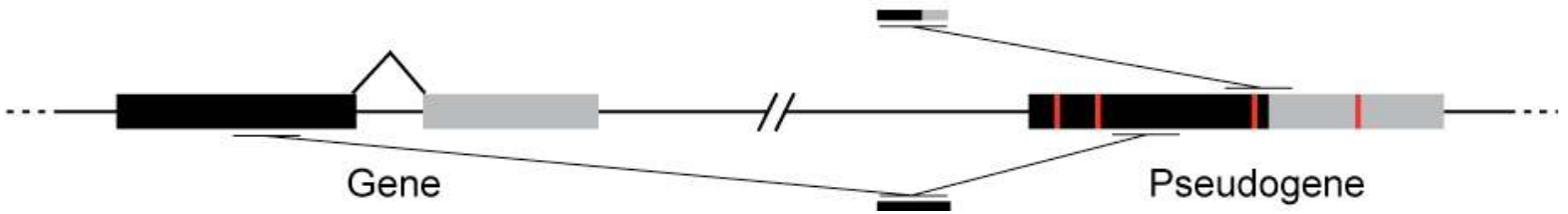


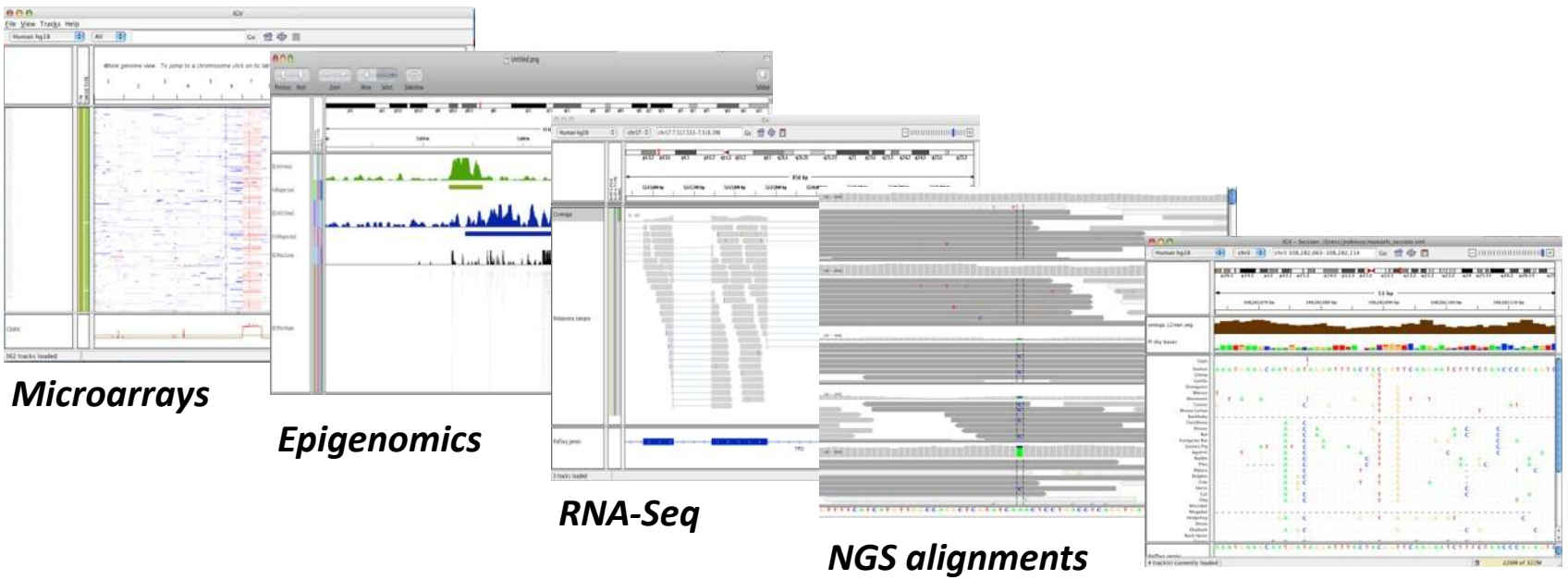
How do we visualize the results of these programs

IGV: Integrative Genomics Viewer

A desktop application

for the visualization and interactive exploration

of genomic data



Microarrays

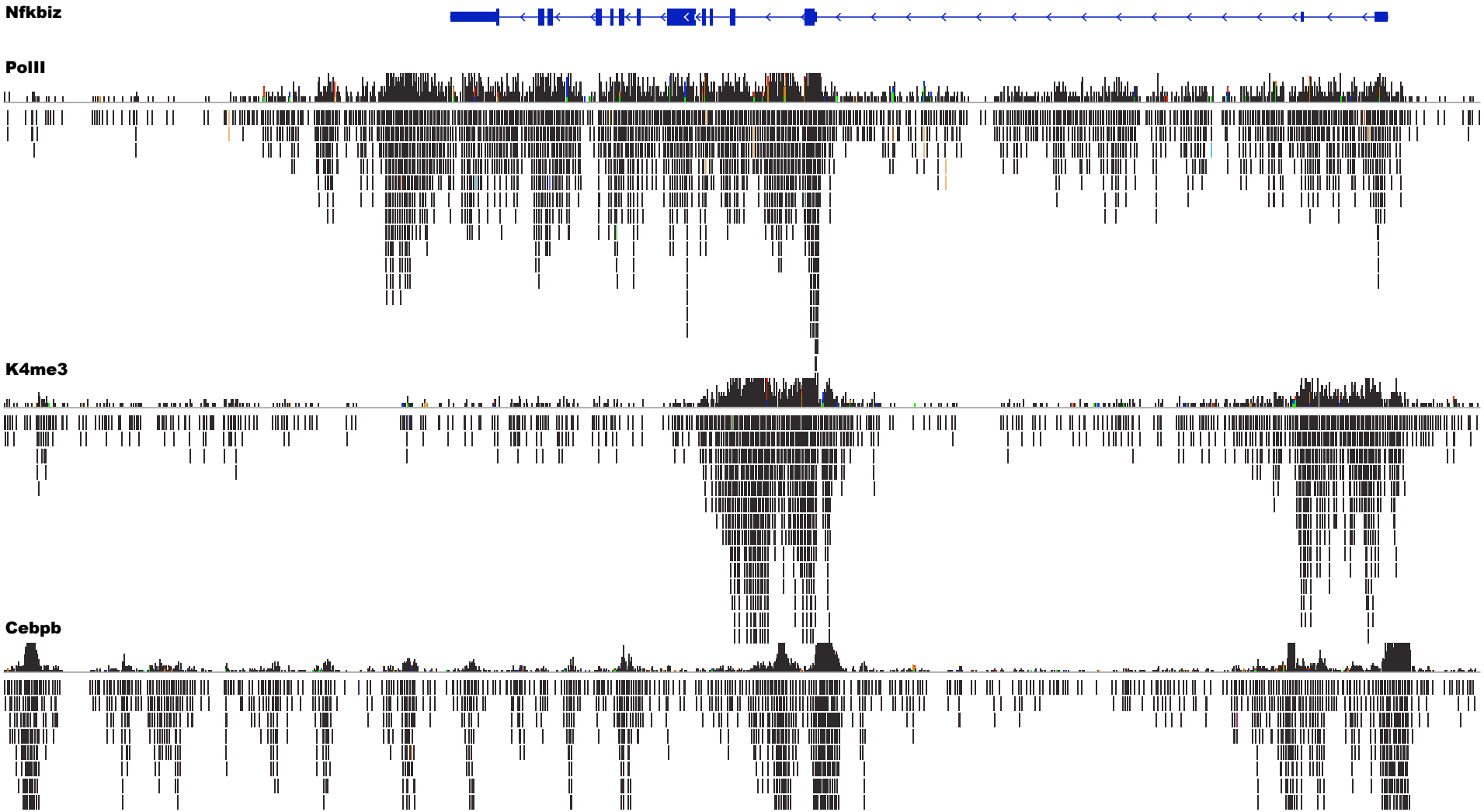
Epigenomics

RNA-Seq

NGS alignments

Comparative genomics

Visualizing read alignments with IGV

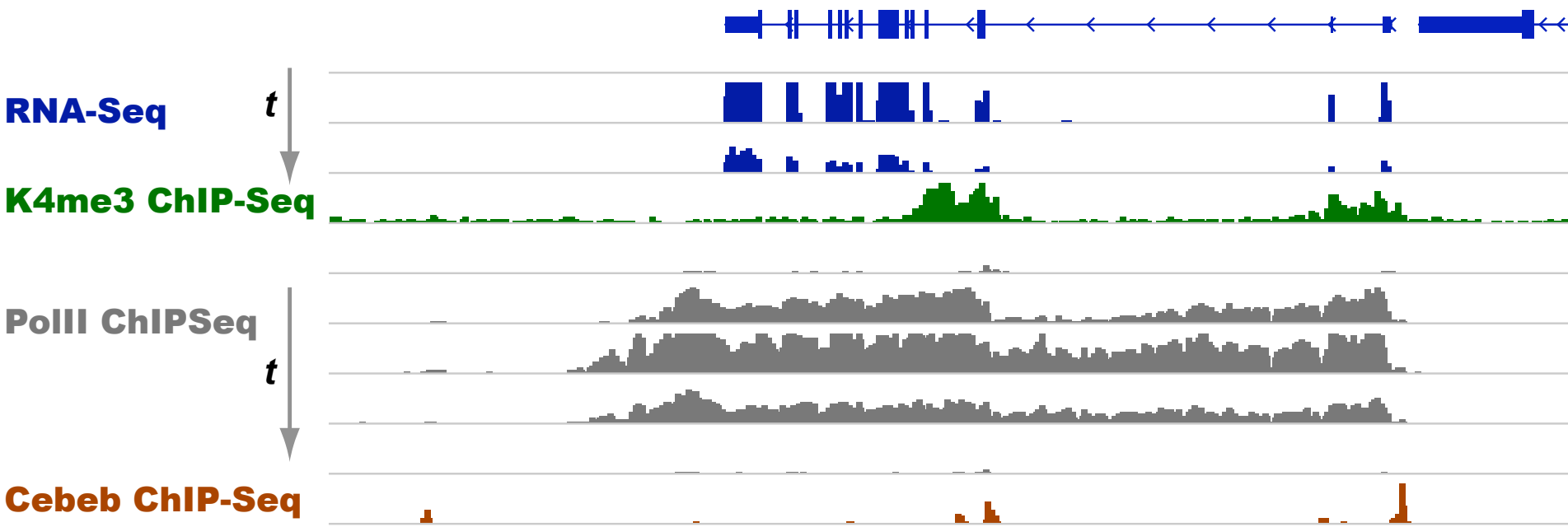


Visualizing read alignments with IGV — RNASeq

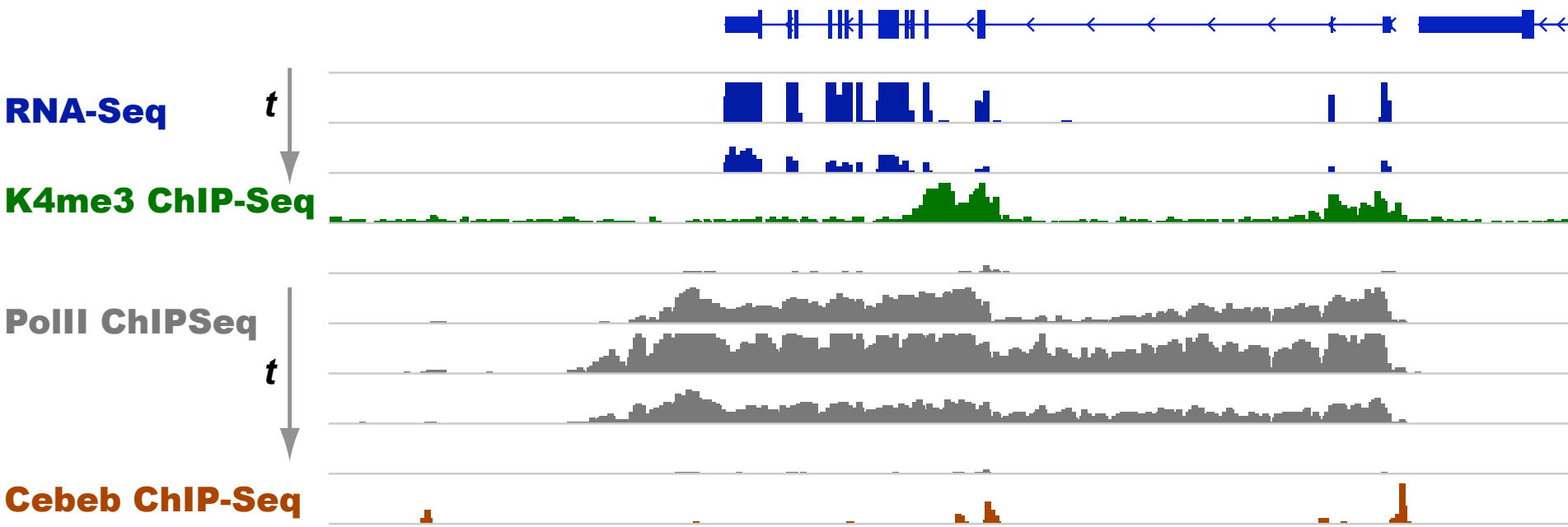
Visualizing read alignments with IGV — RNASeq

Visualizing read alignments with IGV — RNASeq close-up

Visualizing read alignments with IGV — zooming out



Visualizing read alignments with IGV — zooming out



How can we identify regions enriched in sequencing reads?

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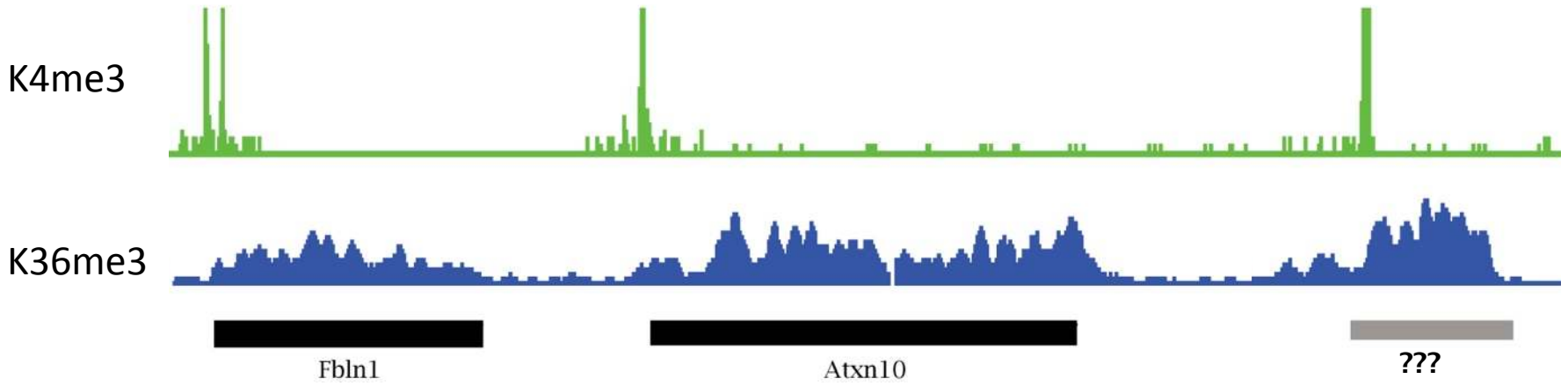
Chromatin domains demarcate interesting surprises in the transcriptome



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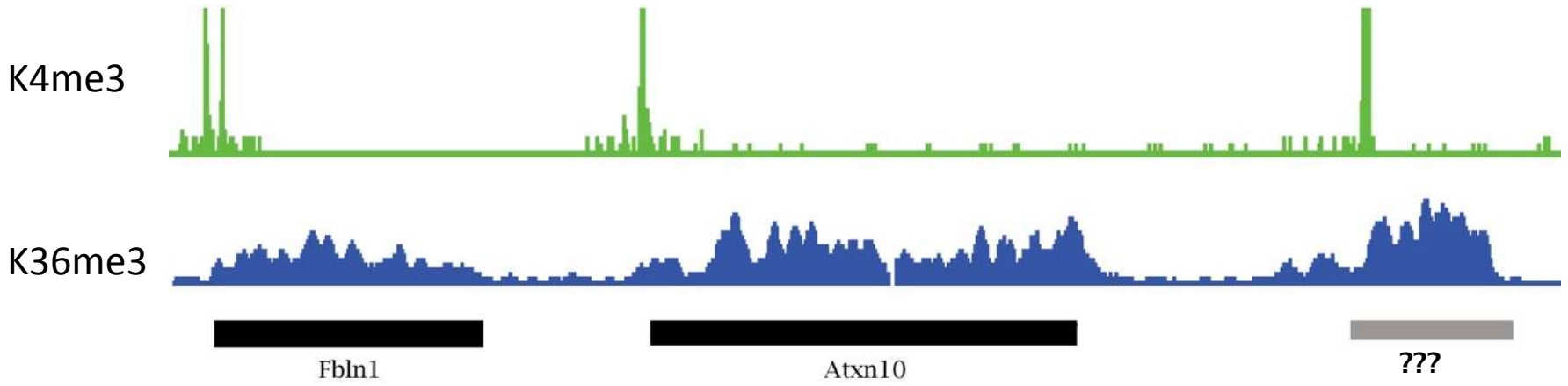

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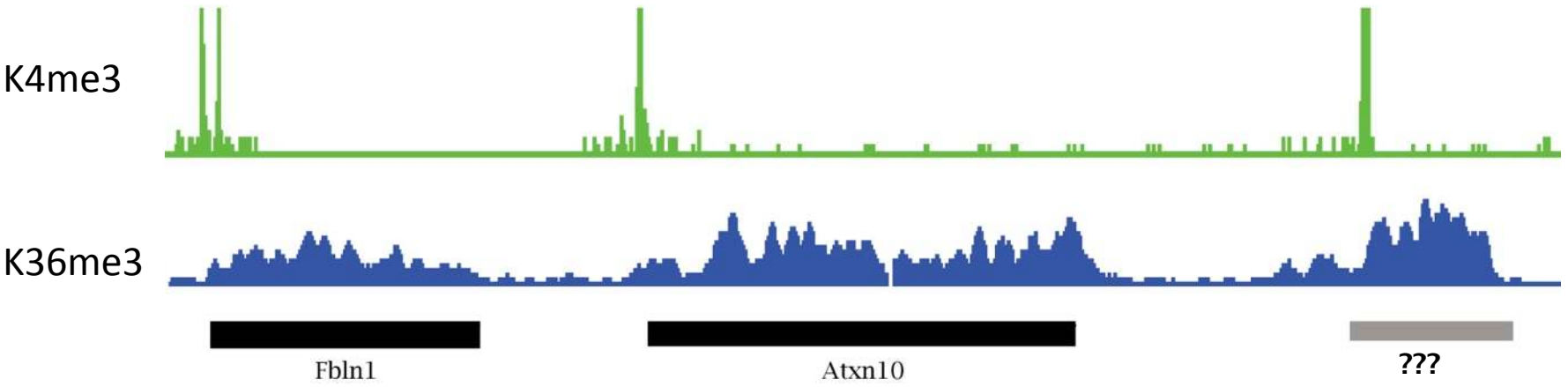
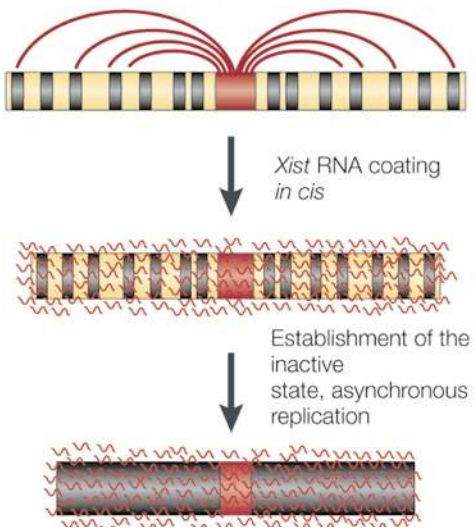
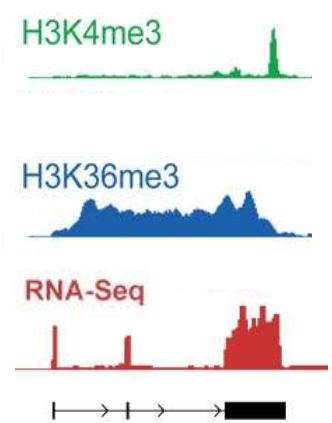


XIST



How can we identify these chromatin marks and the genes within?



Short modification

Long modification

Discontinuous data

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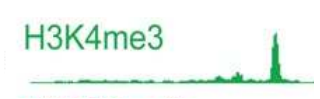
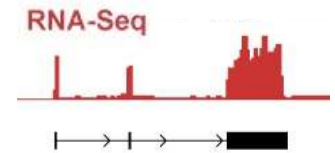
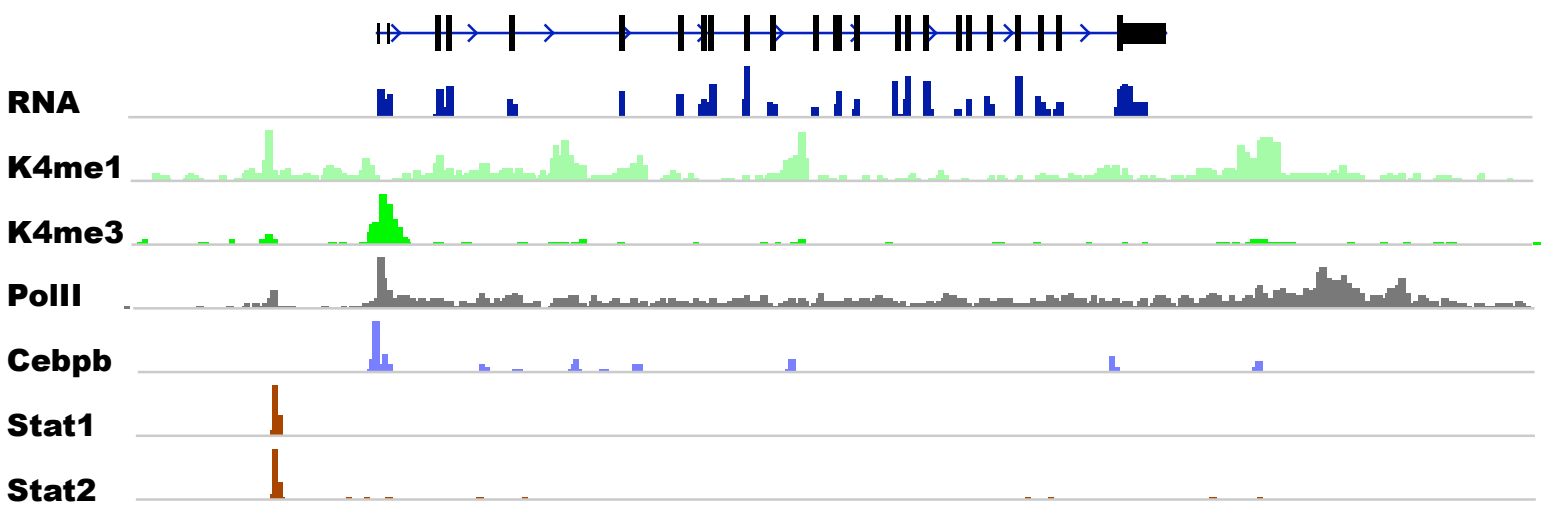


Short modification

Long modification



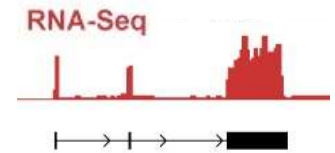
Discontinuous data



How can we identify these chromatin marks and the genes within?

Short modification

Long modification



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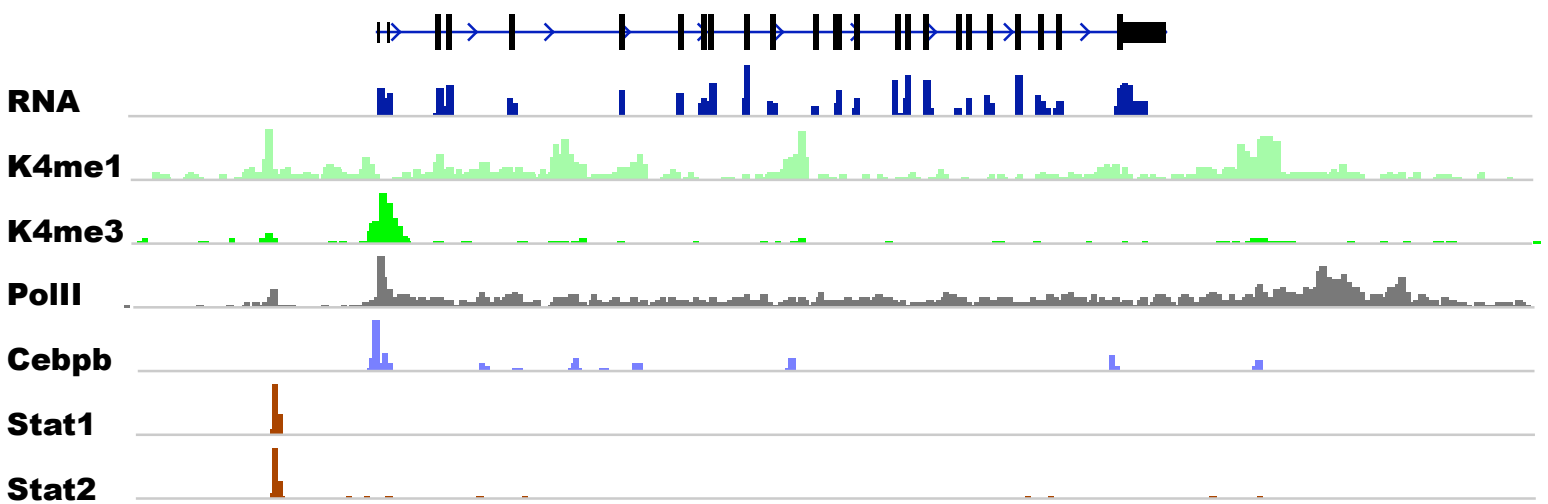
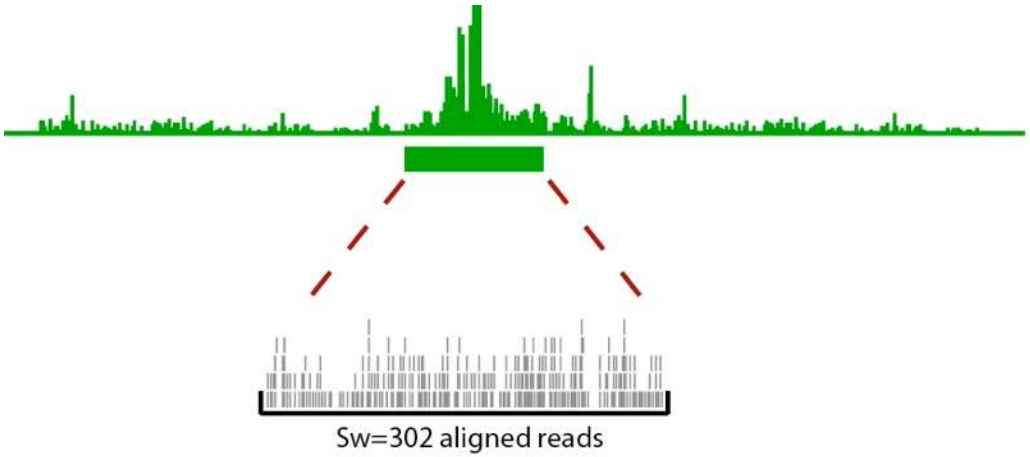


Scripture is a method to solve this general question

Our approach

Our approach



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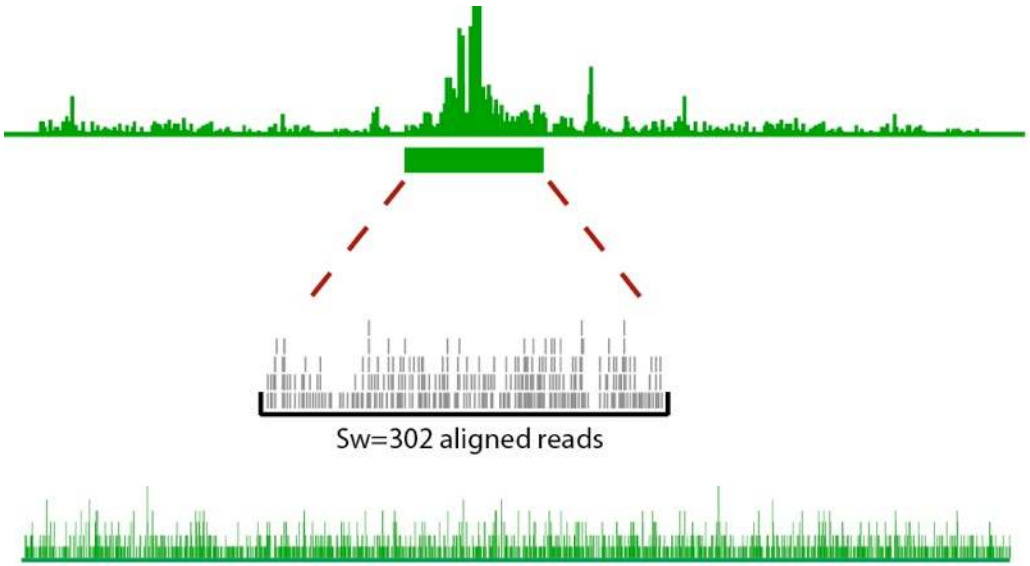

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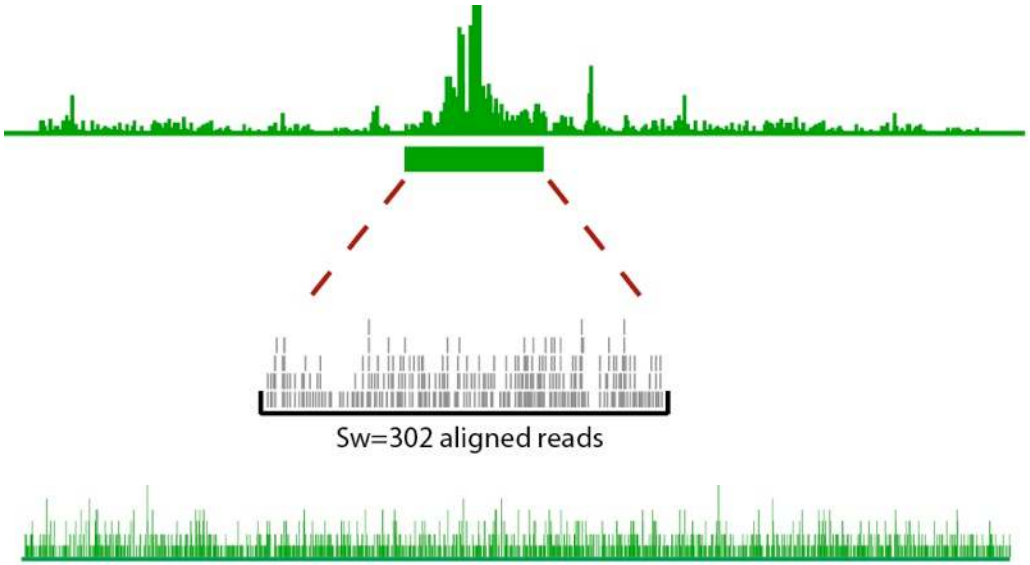
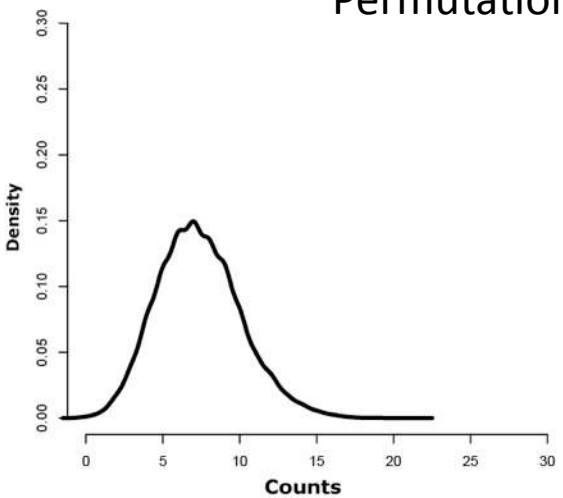


Permutation



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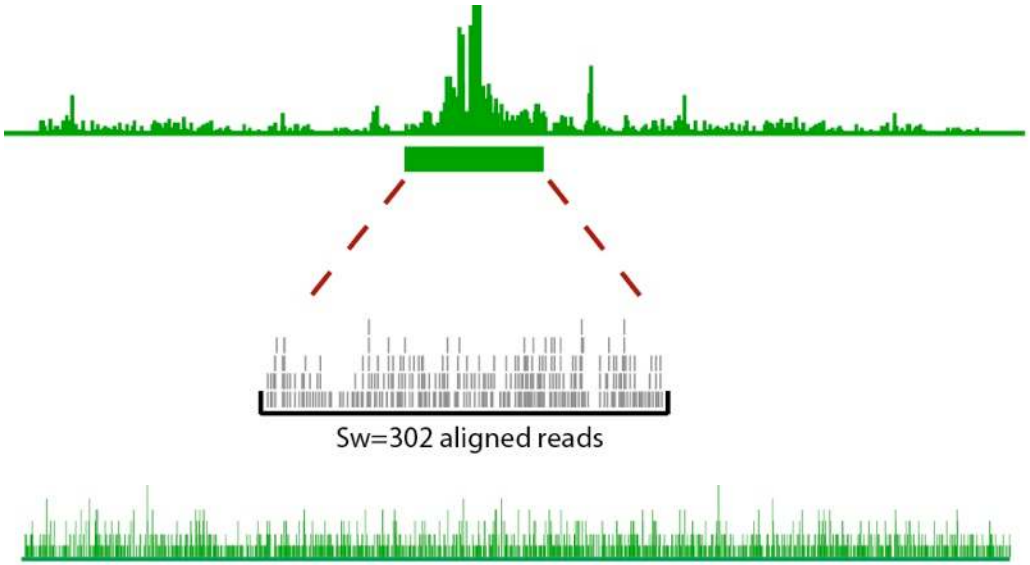
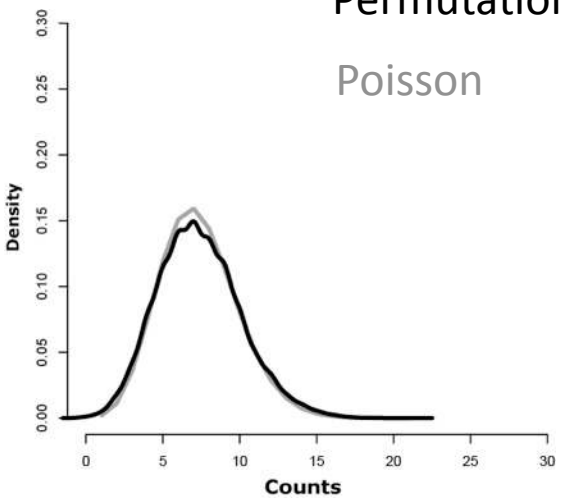


Permutation

Poisson



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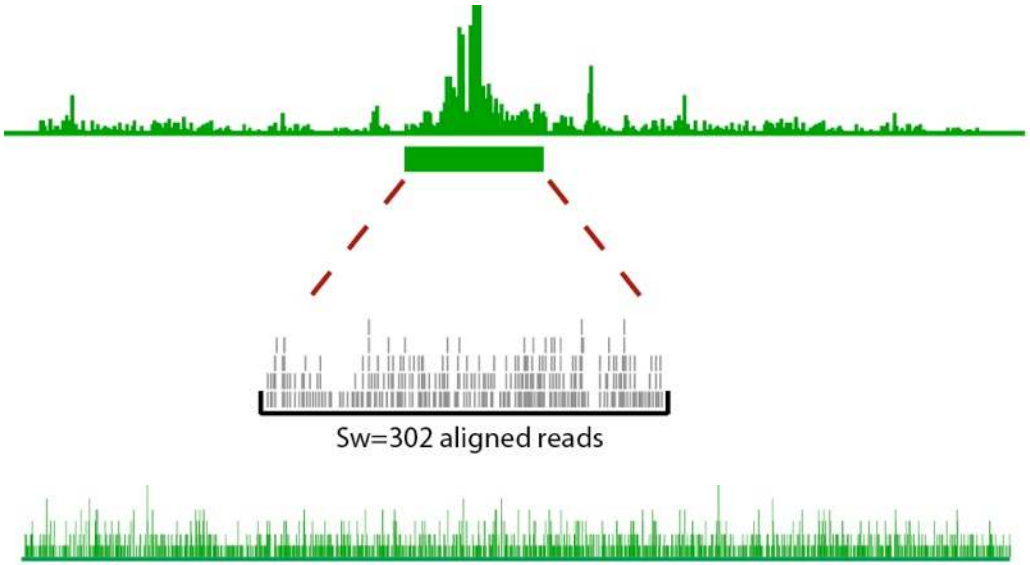

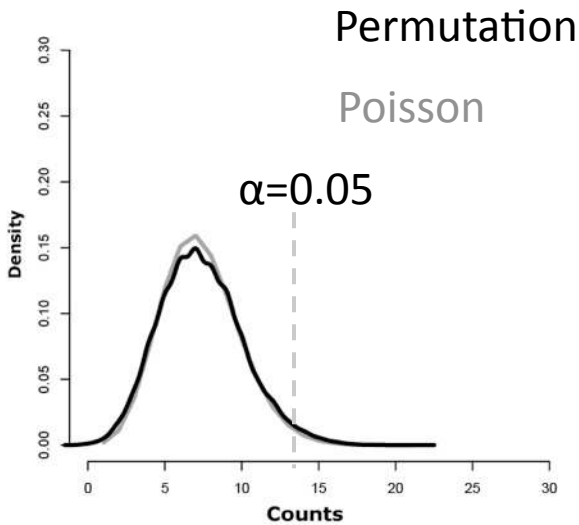

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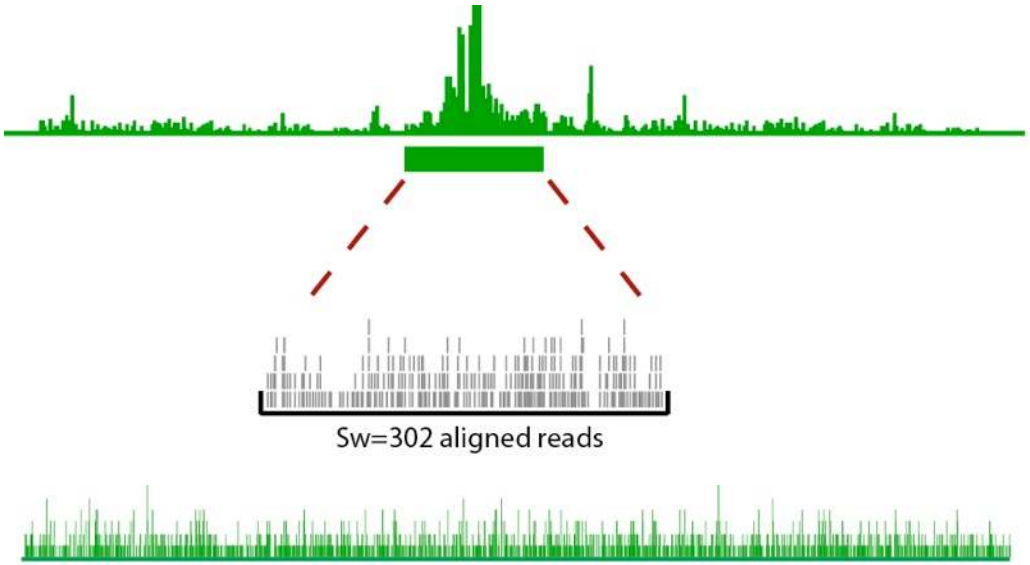
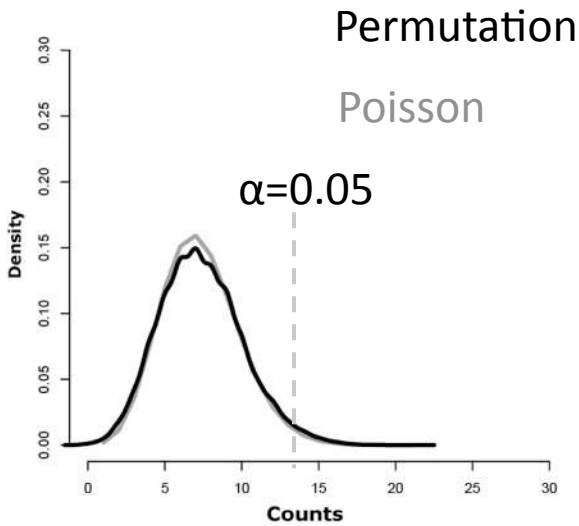
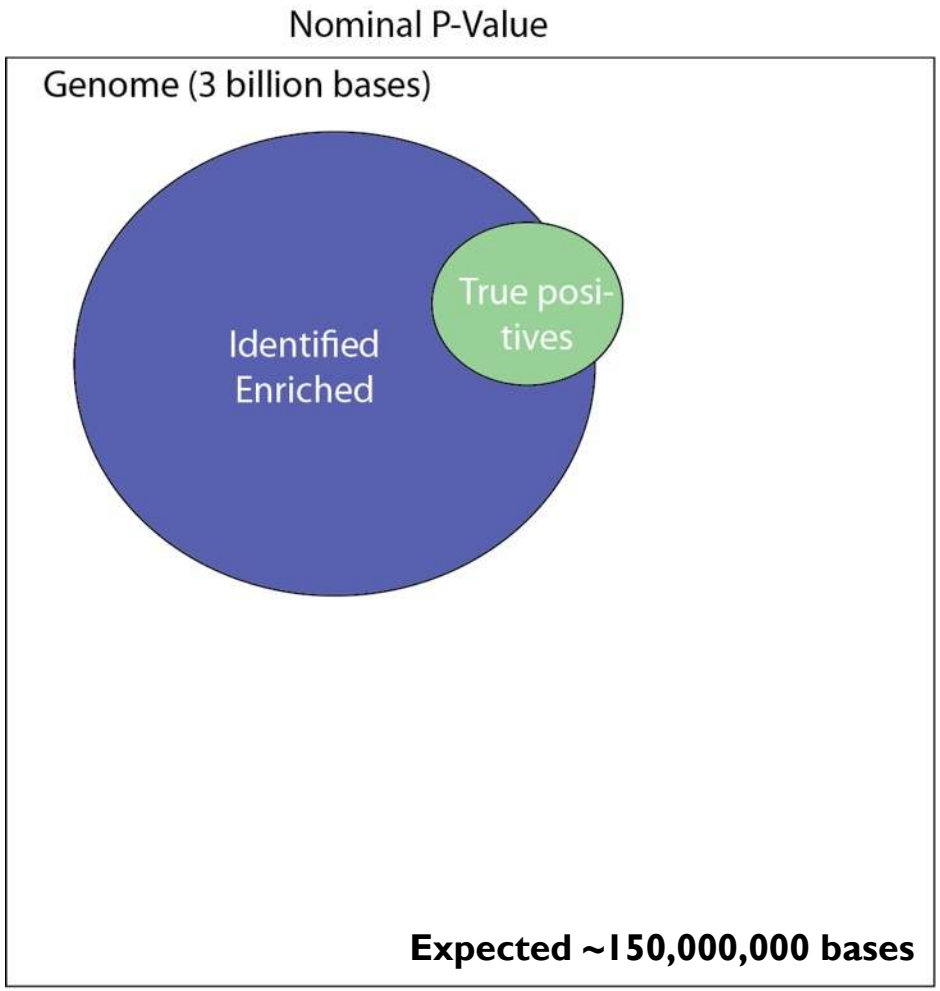




We have an efficient way to compute read count p-values …

The genome is big: A lot happens by chance



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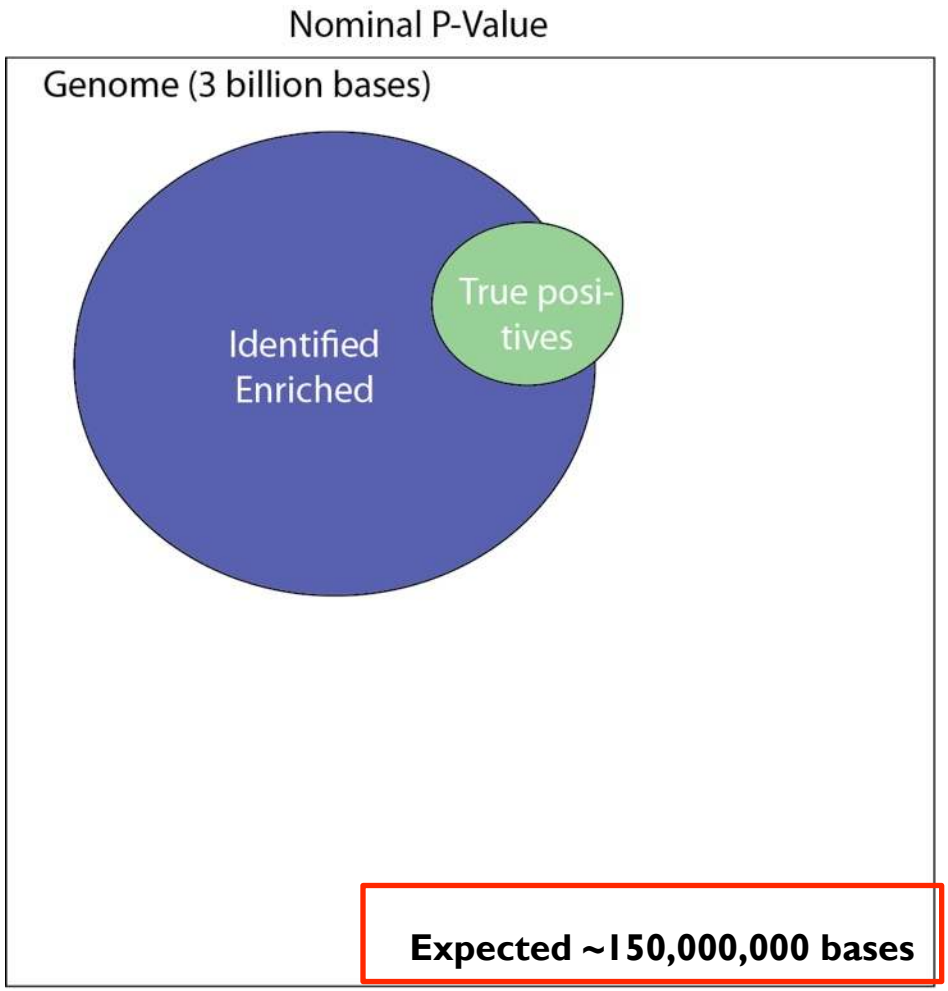

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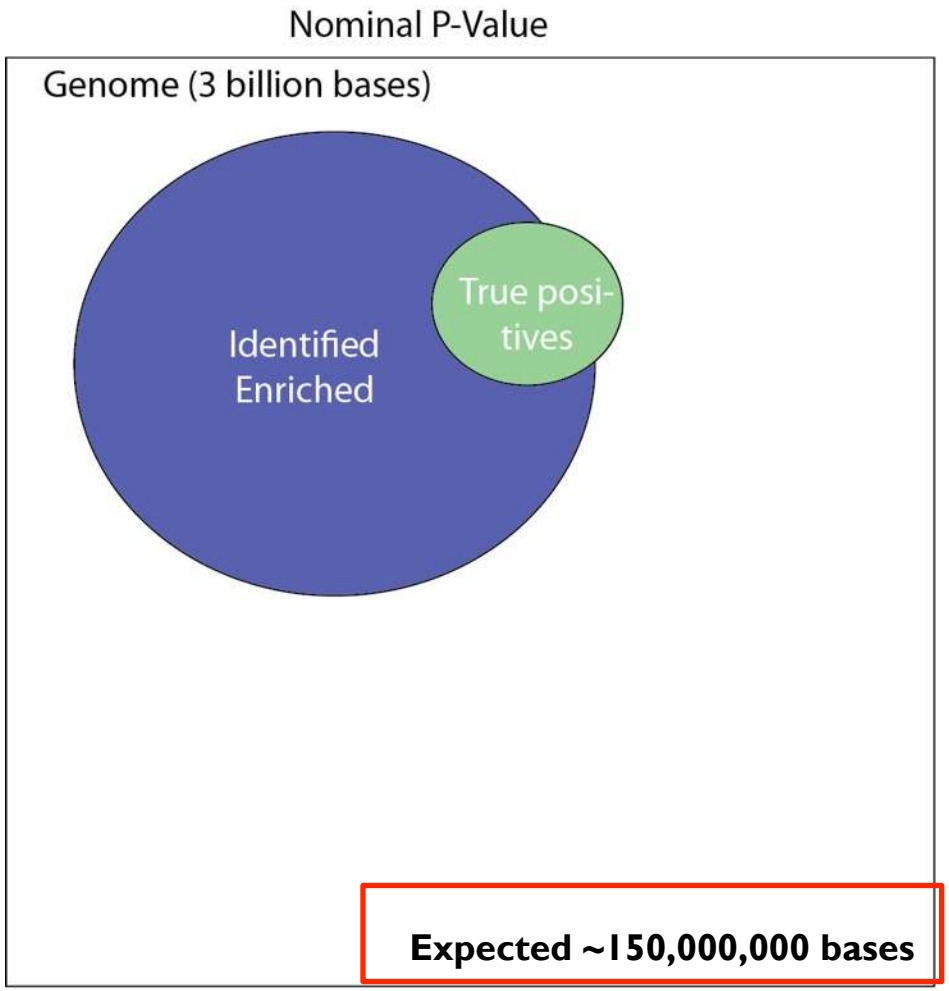
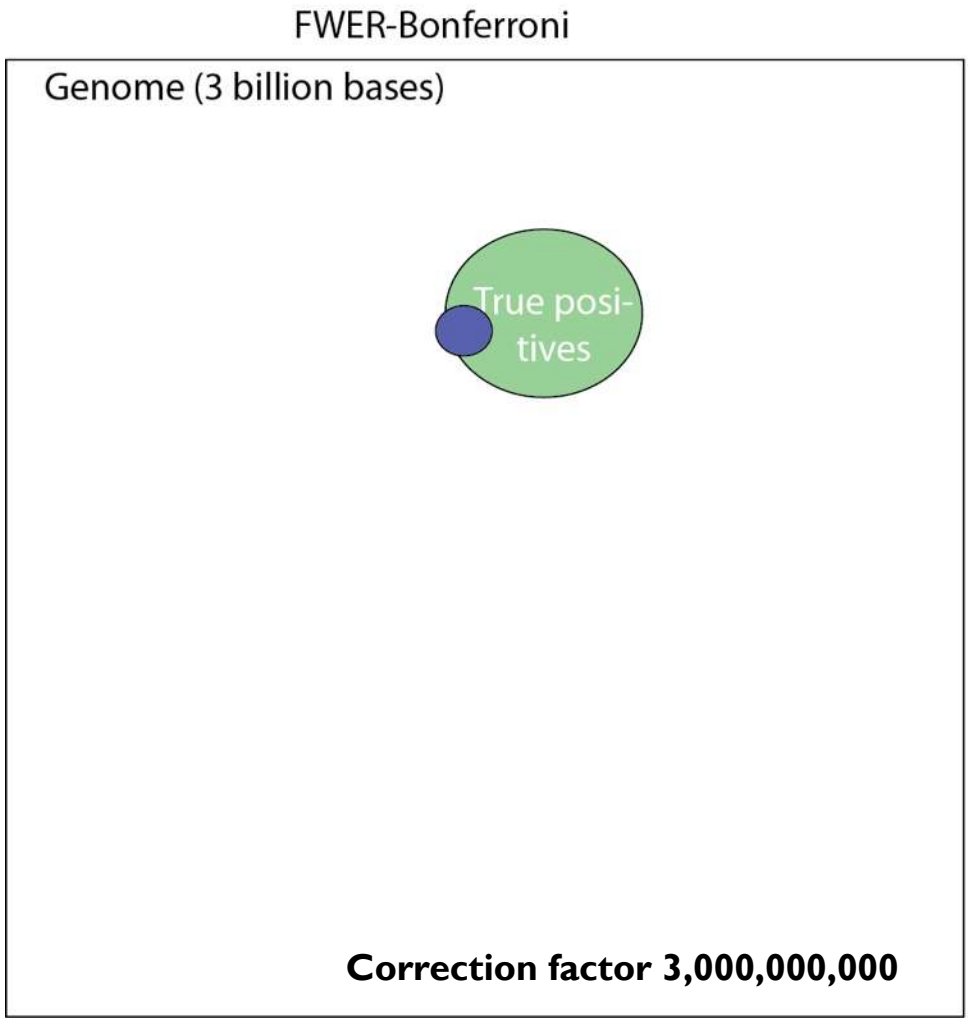


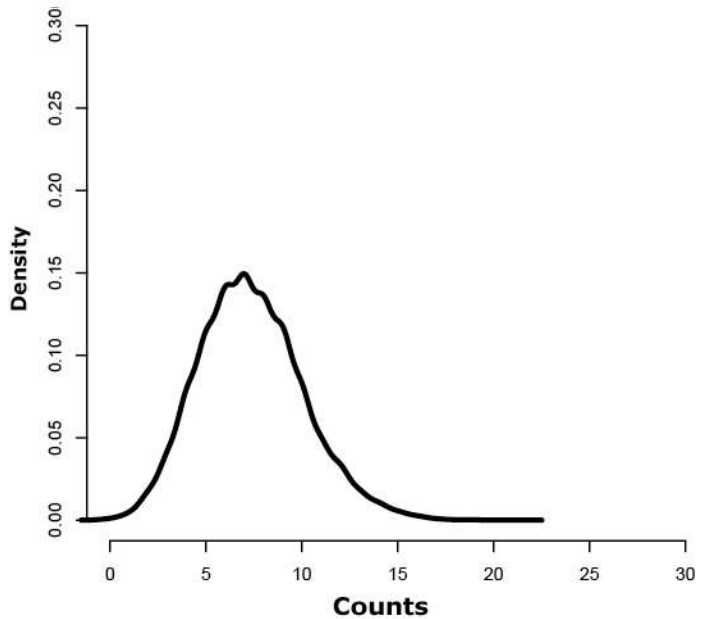
We need to correct for multiple hypothesis testing

Bonferroni correction is way to conservative



Bonferroni corrects the number of hits but misses many true hits because its too conservative– How do we get more power?

Controlling FWER



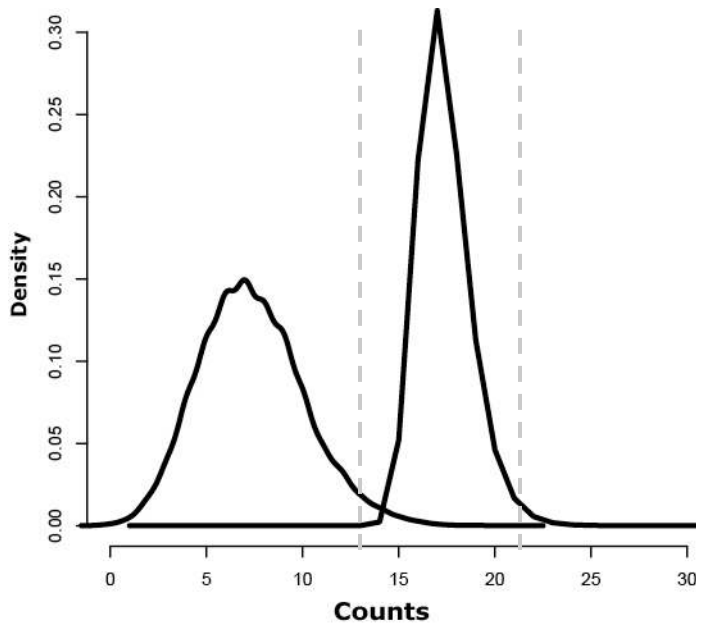
Count distribution (Poisson)

Given a region of size w and an observed read count n. What is the probability that one or more of the $3x10^9$ regions of size w has read count >= n under the null distribution?

Controlling FWER

Max Count distribution

α=0.05 α_{FWER}=0.05



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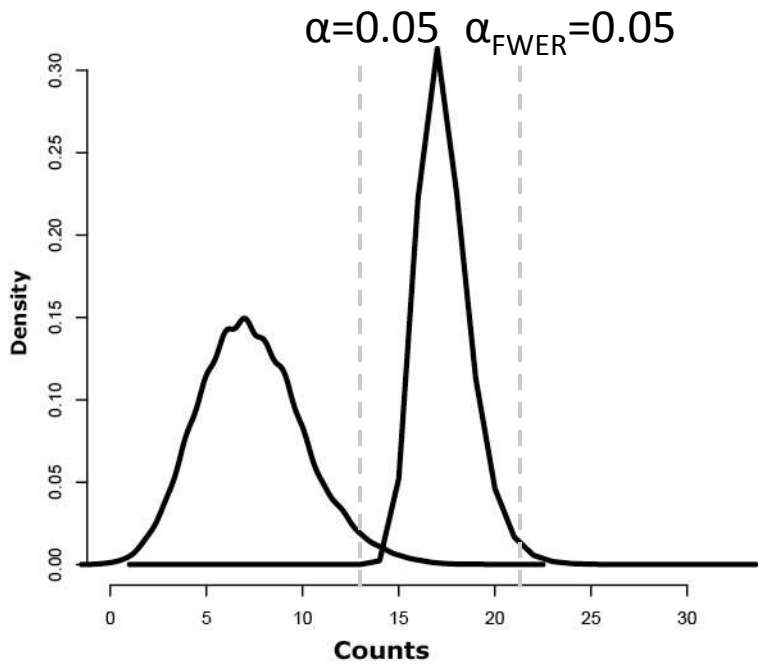
We could go back to our permutations and compute an FWER: **max of the genome-wide distributions of same sized region**)→ but really really really slow!!!

Scan distribution, an old problem

- Is the observed number of read counts over our region of interest high?
- Given a set of Geiger counts across a region find clusters of high radioactivity
- Are there time intervals where assembly line errors are high?



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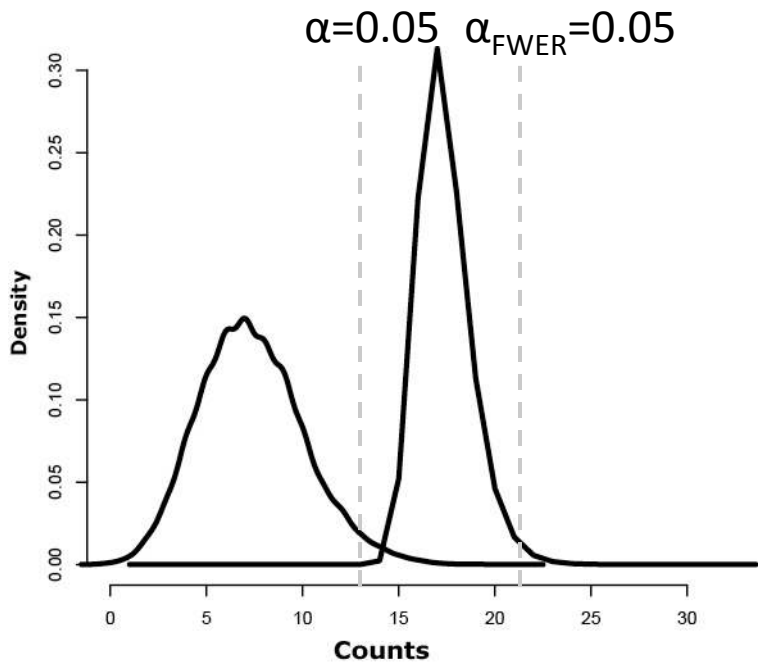

Thankfully, there is a distribution called the Scan Distribution which computes a closed form for this distribution.

ACCOUNTS for dependency of overlapping windows thus more powerful!

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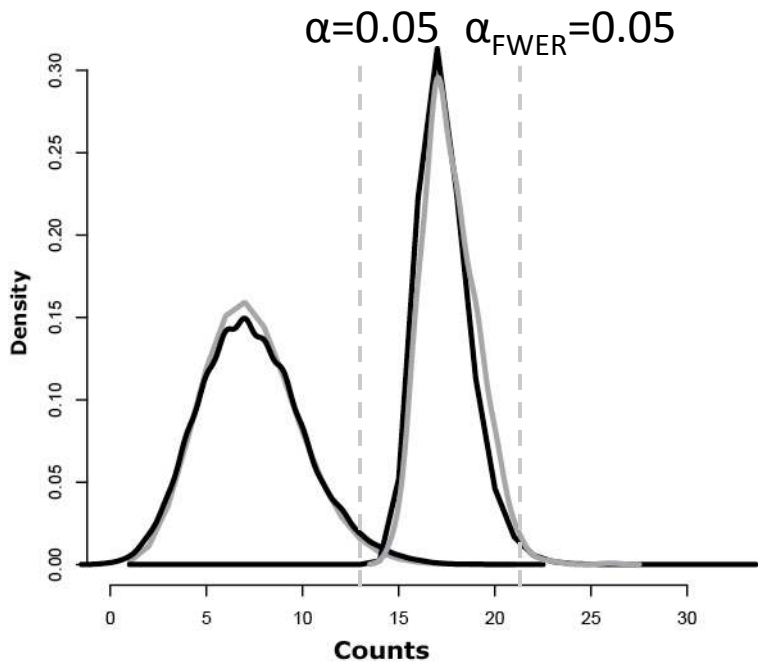

Poisson distribution

Thankfully, there is a distribution called the Scan Distribution which computes a closed form for this distribution.

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Scan distribution for a Poisson process

The probability of observing k reads on a window of size w in a genome of size L given a total of N reads can be approximated by (Alm 1983):

$$P(k|\lambda w, N, L) \approx 1 - F_p(k-1|\lambda w)e^{-\frac{k-w\lambda}{k}\lambda(T-w)P(k-1|\lambda w)}$$

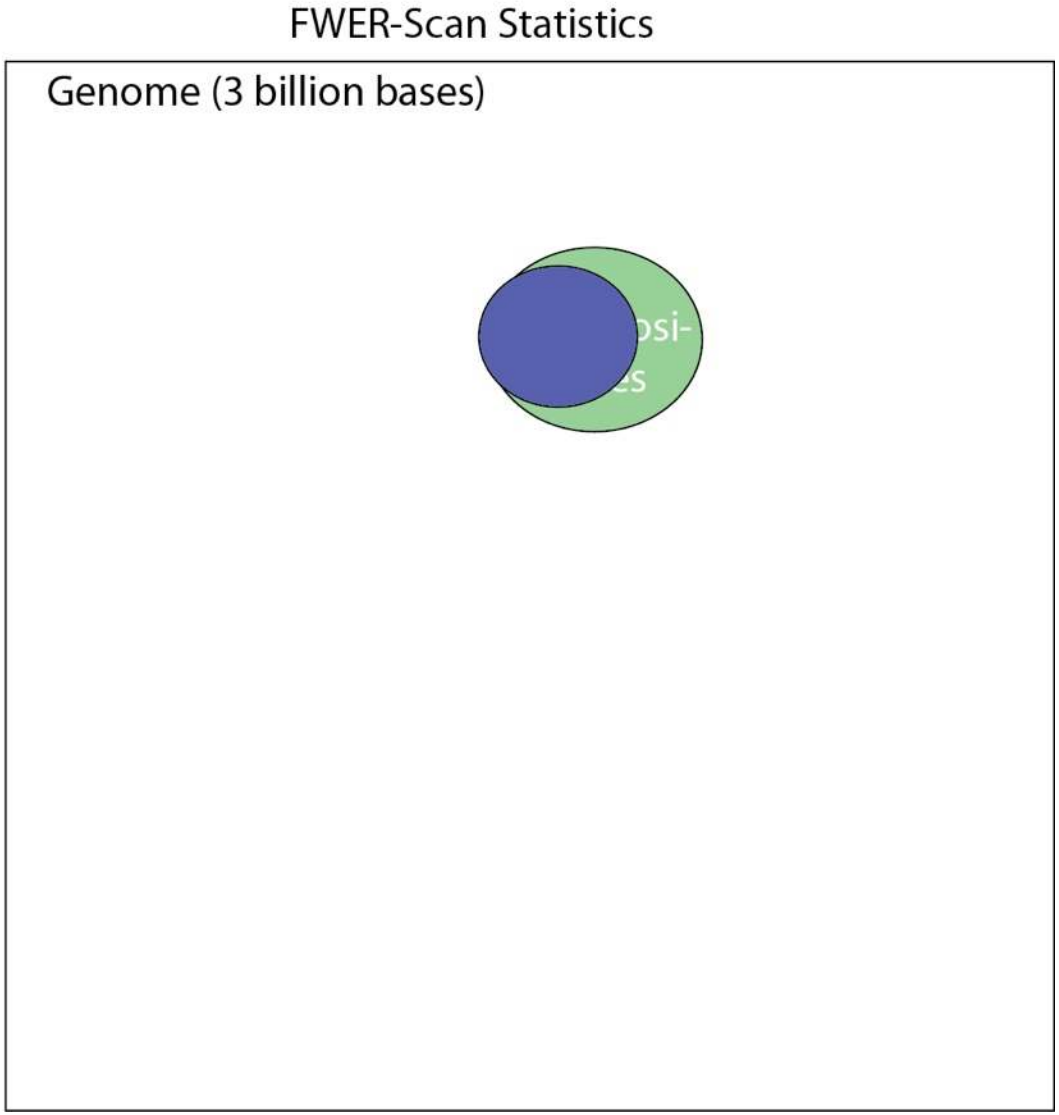
where

$P(k-1|\lambda w)$ is the Poisson probability of observing $k-1$ counts given an expected count of λw

and

$F_p(k-1|\lambda w)$ is the Poisson probability of observing $k-1$ or fewer counts given an expectation of λw reads

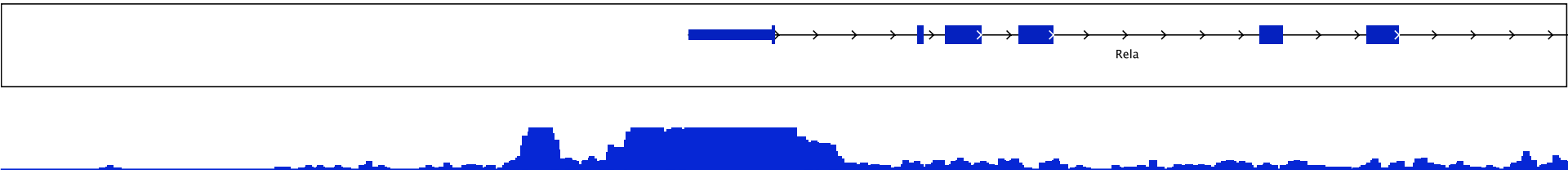
The scan distribution gives a computationally very efficient way to estimate the FWER



By utilizing the dependency of overlapping windows we have greater power, while still controlling the same genome-wide false positive rate.

Segmentation method for contiguous regions

Example : PolII ChIP



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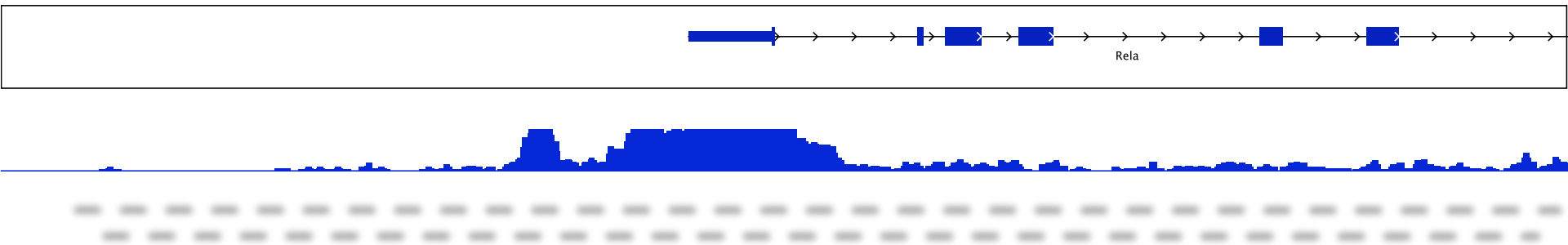

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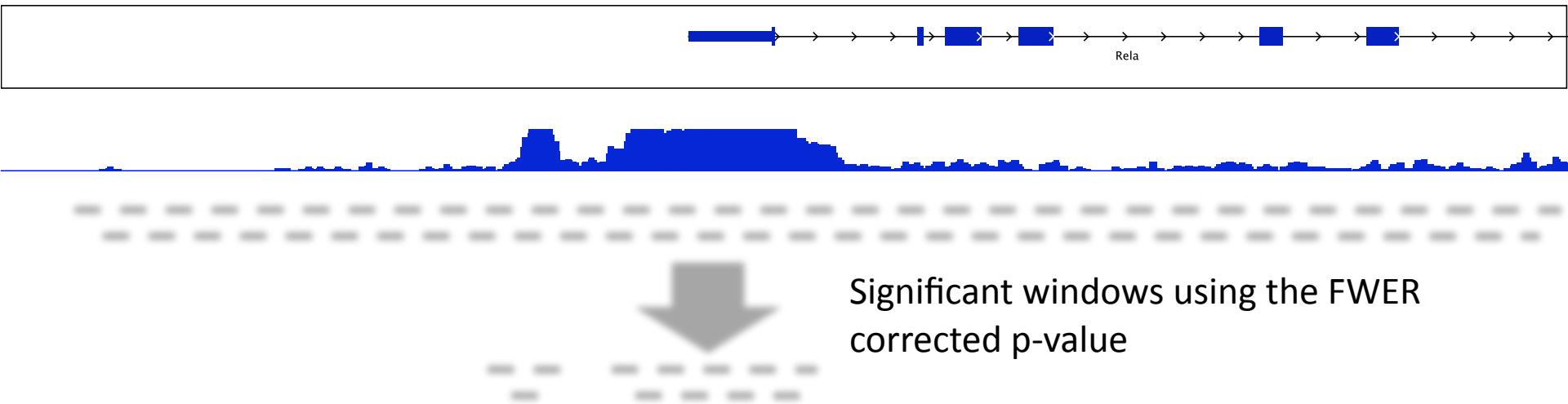
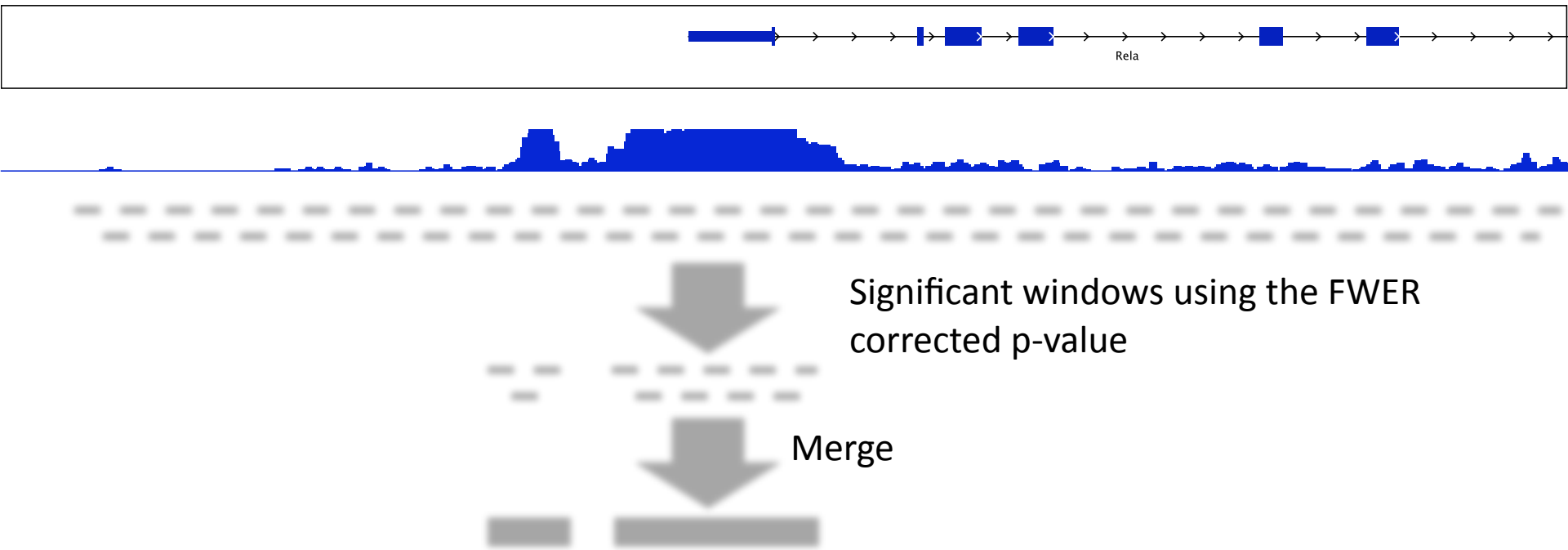


Significant windows using the FWER corrected p-value

Segmentation method for contiguous regions

Example : PolII ChIP



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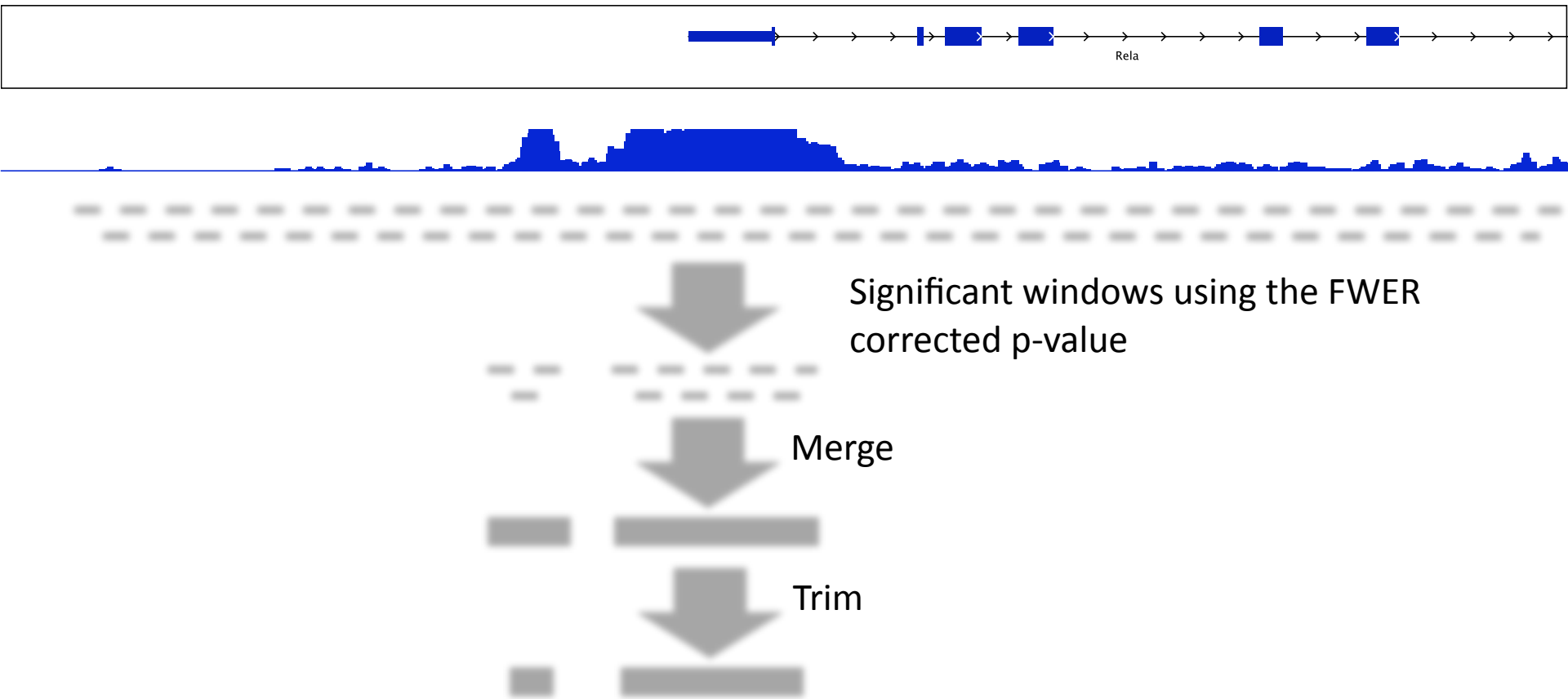

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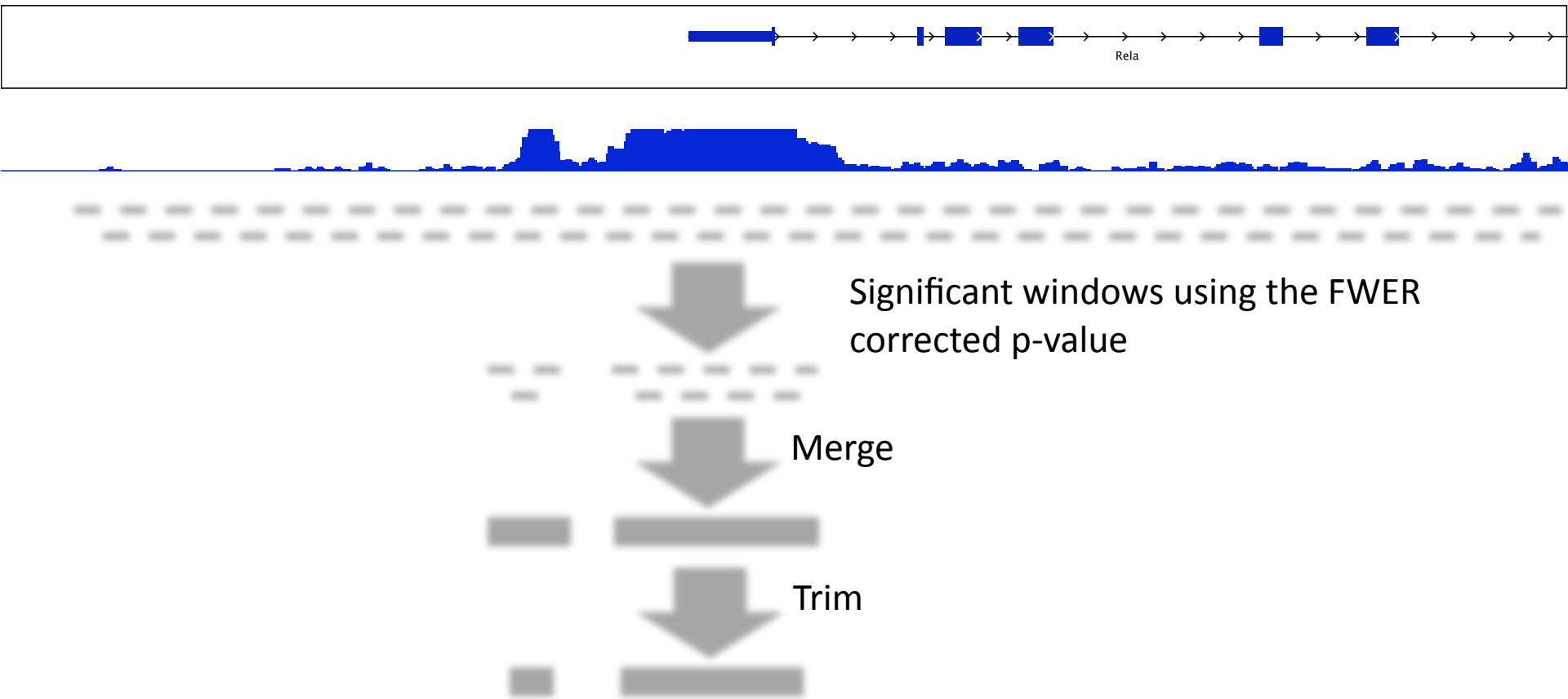
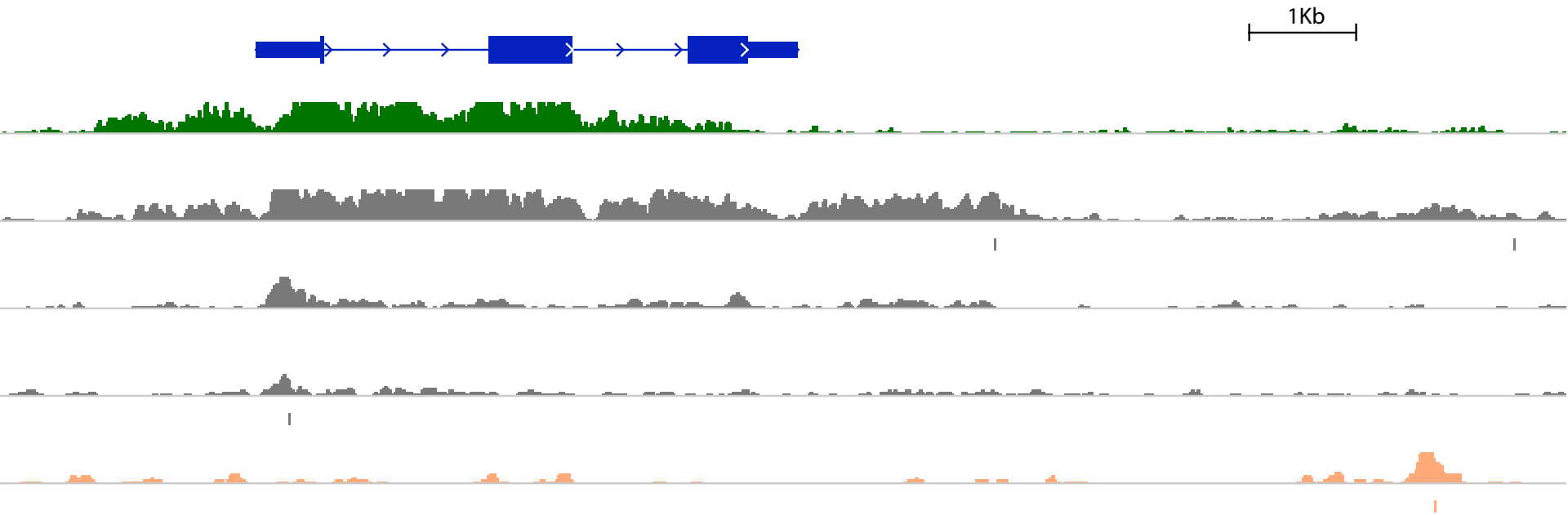


But, which window?

We use multiple windows

- Small windows detect small punctuate regions.
- Longer windows can detect regions of moderate enrichment over long spans.
- In practice we scan different windows, finding significant ones in each scan.
- In practice, it helps to use some prior information in picking the windows although globally it might be ok.

Applying Scripture to a variety of ChIP-Seq data



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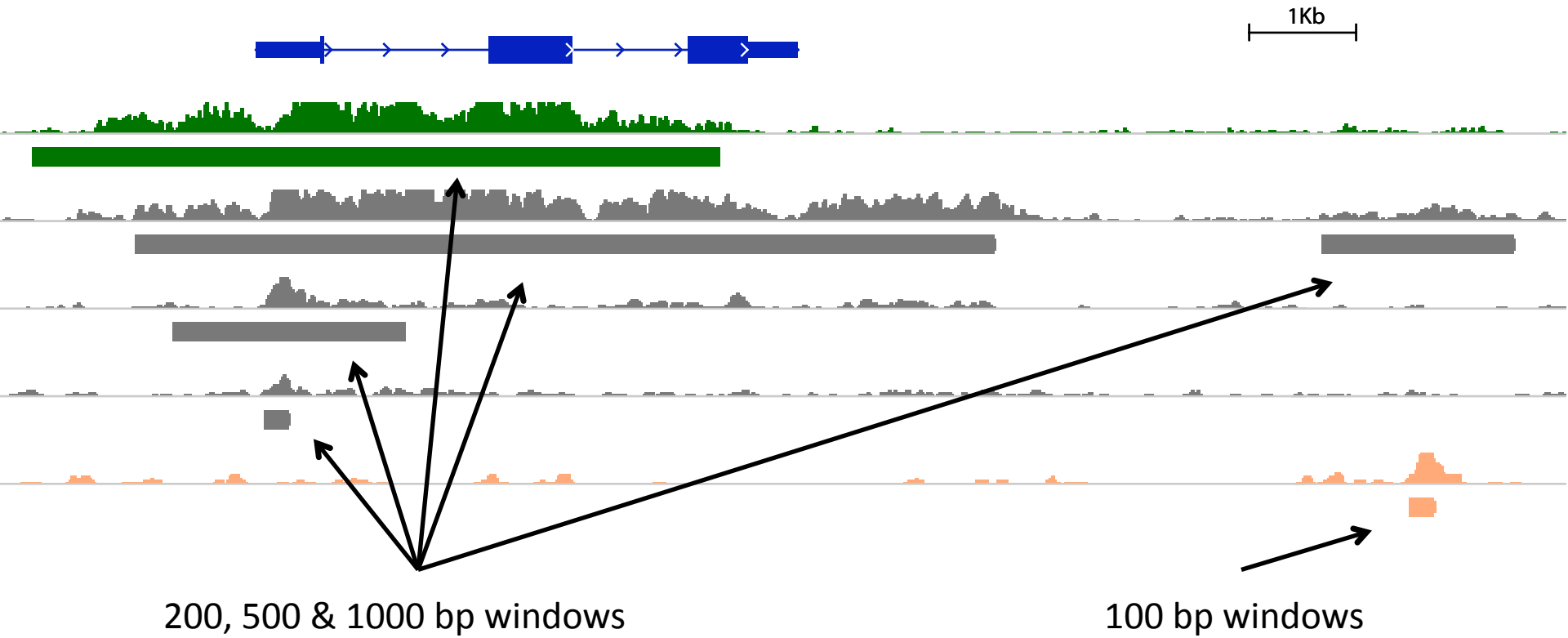
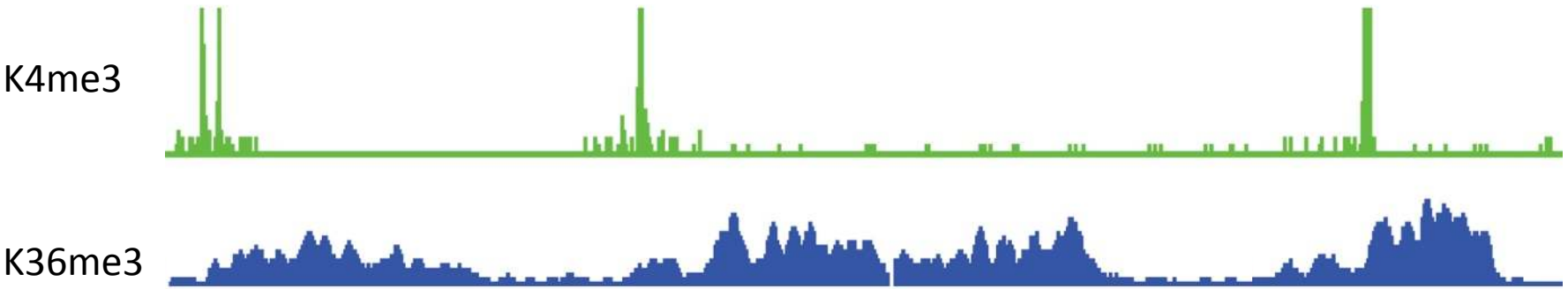


Application of scripture to mouse chromatin state maps



Mitch Guttman

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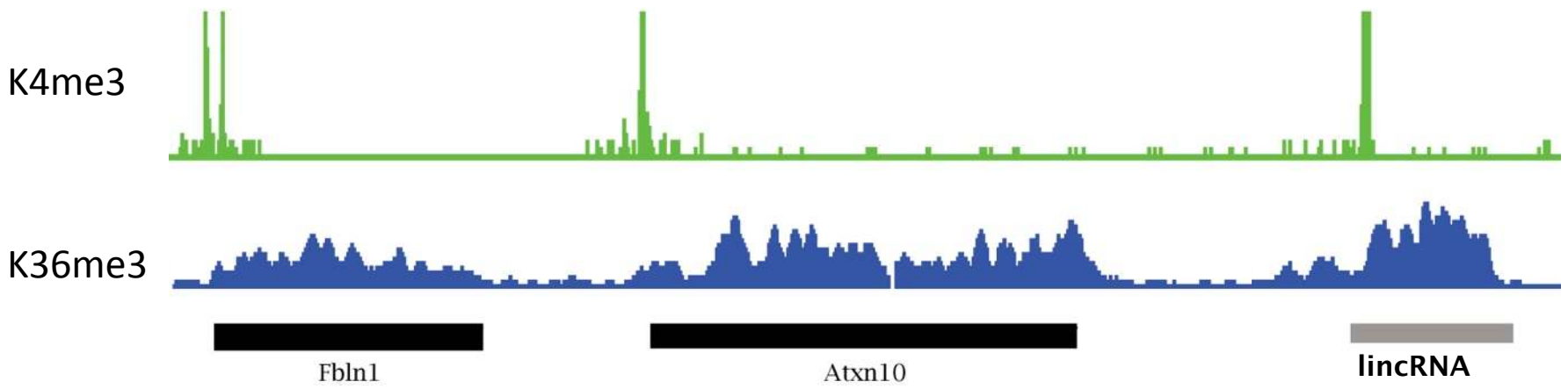

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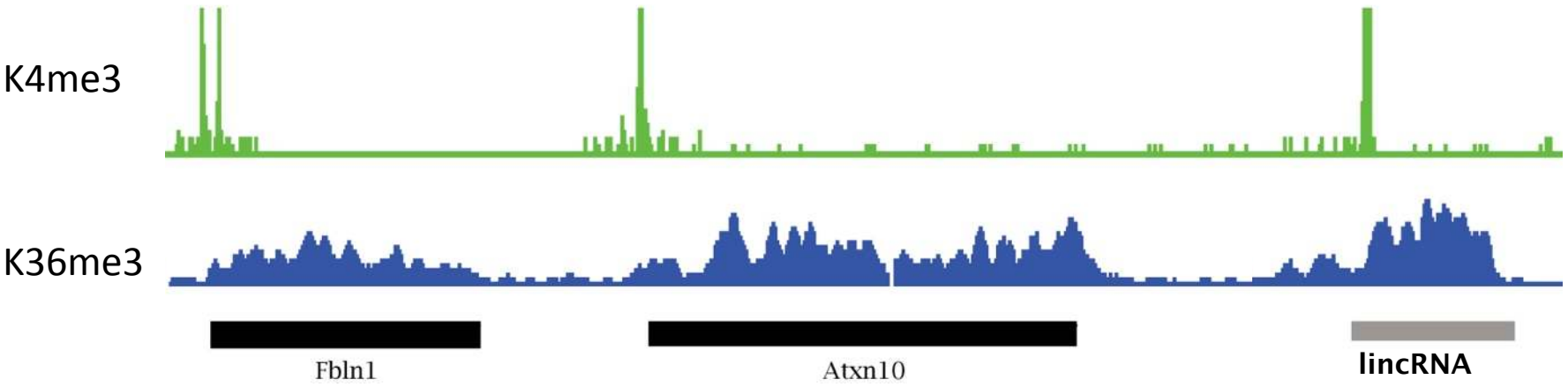

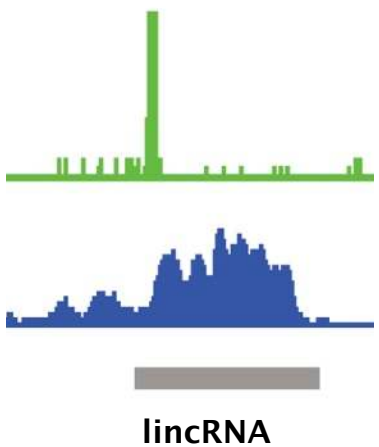

Identifed

- ~1500 lincRNAs
  - Conserved
  - Noncoding
  - Robustly expressed

Mitch Guttman

Can we identify enriched regions across different data types?

Short modification ✓

Long modification ✓

Can we identify enriched regions across different data types?

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Using chromatin signatures we discovered hundreds of putative genes.
What is their structure?

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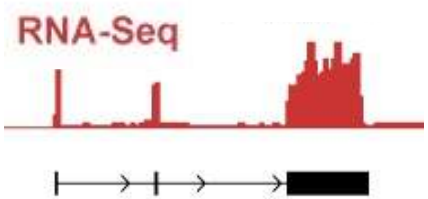
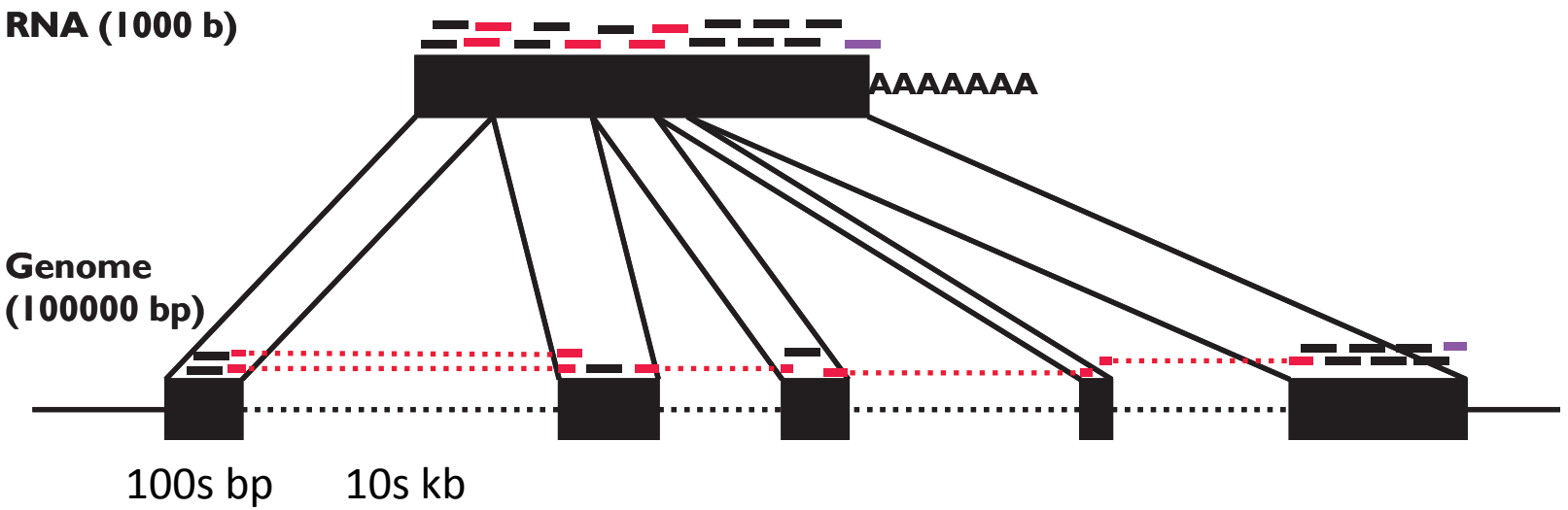


Discontinuous data: RNA-Seq to find gene structures for this gene-like regions

Scripture for RNA-Seq:
Extending segmentation to discontiguous regions

The transcript reconstruction problem as a segmentation problem



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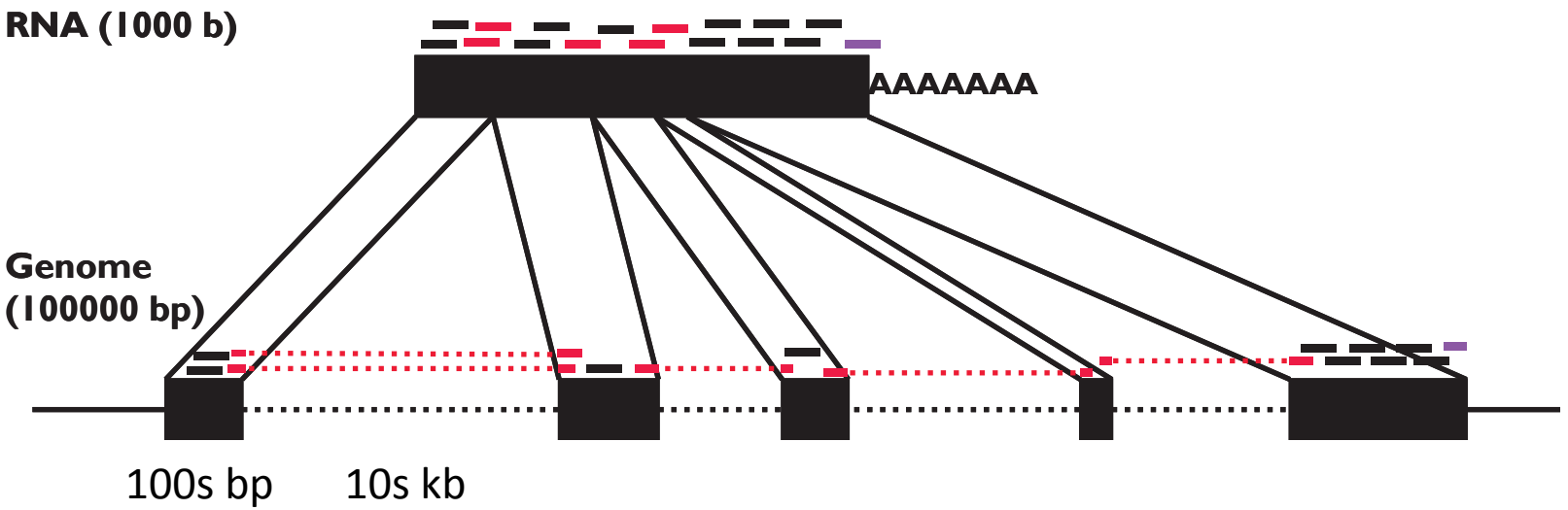
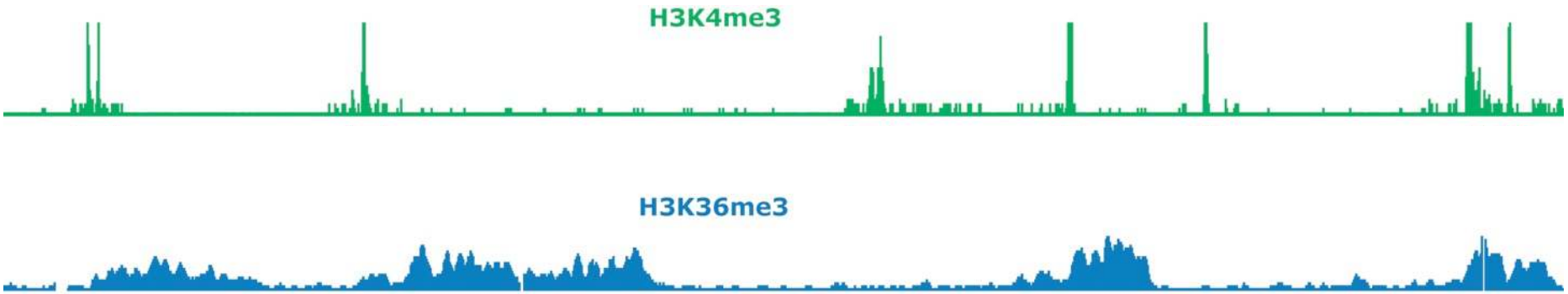


Challenges:

- Genes exist at many different expression levels, spanning several orders of magnitude.
- Reads originate from both mature mRNA (exons) and immature mRNA (introns) and it can be problematic to distinguish between them.
- Reads are short and genes can have many isoforms making it challenging to determine which isoform produced each read.

Scripture: A statistical genome-guided transcriptome reconstruction



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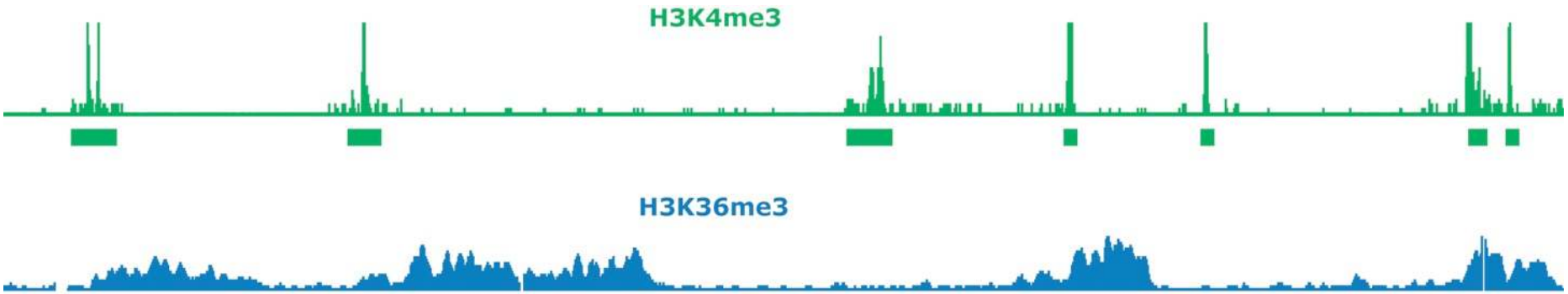

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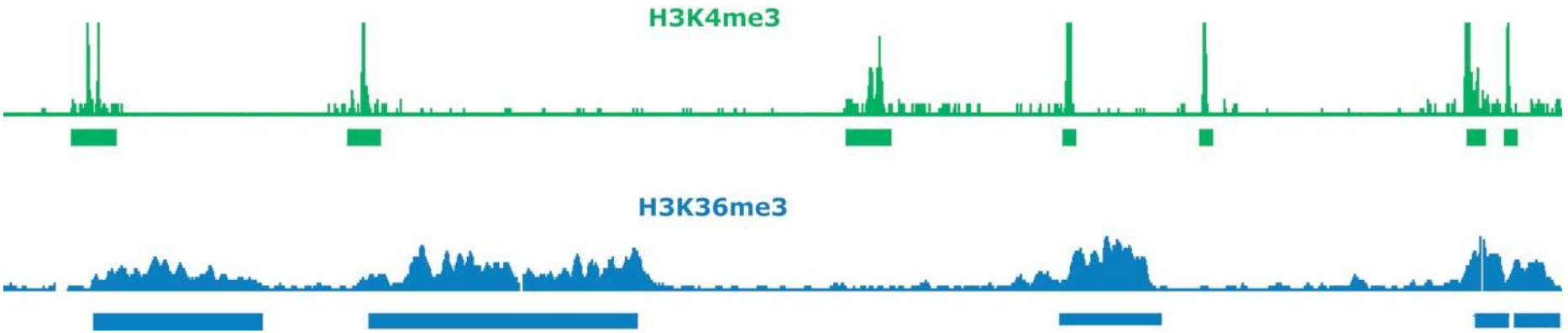

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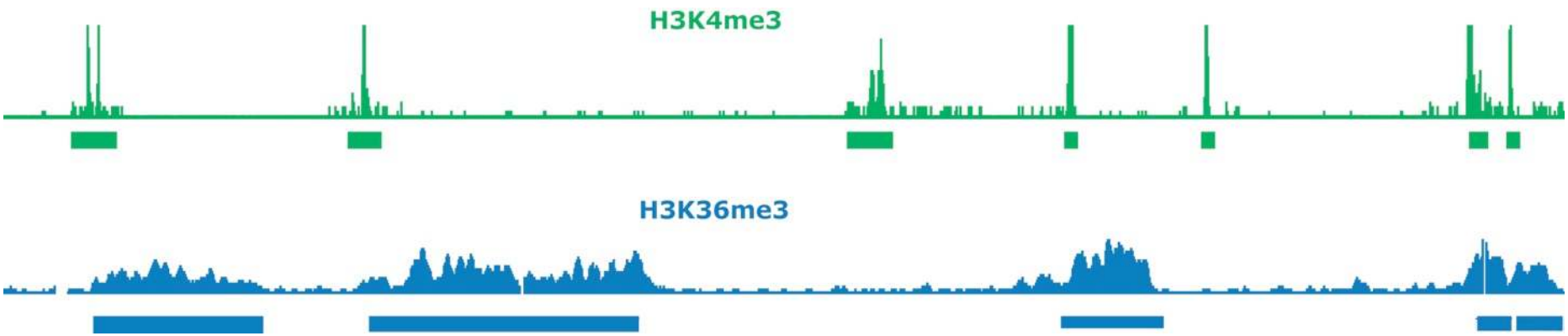

Statistical segmentation of chromatin modifications uses continuity of segments to increase power for interval detection

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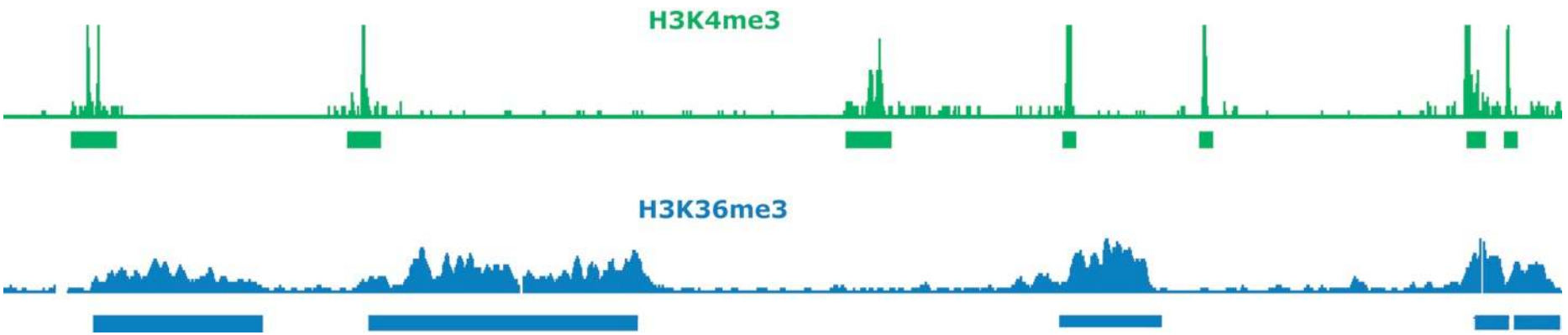

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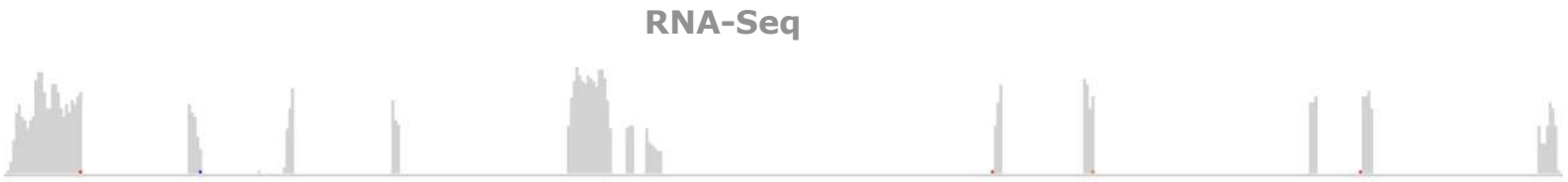

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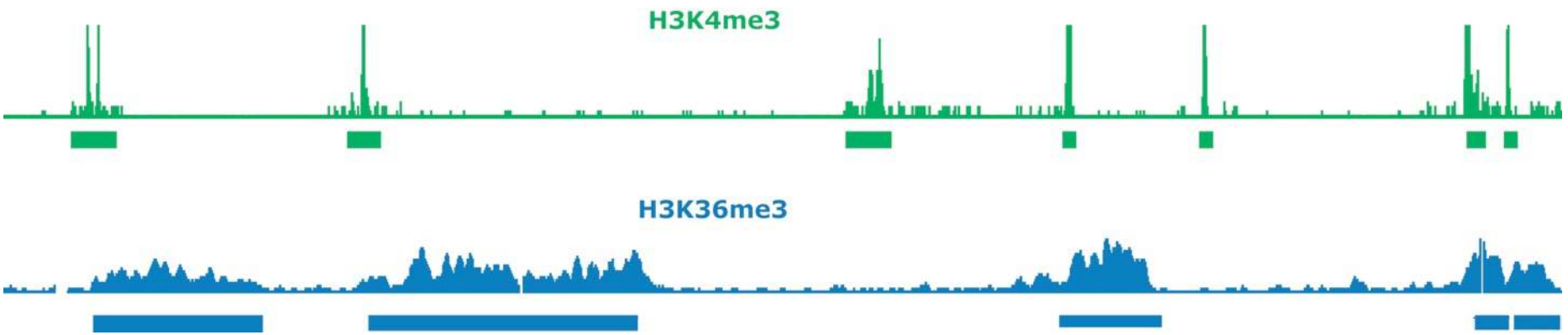

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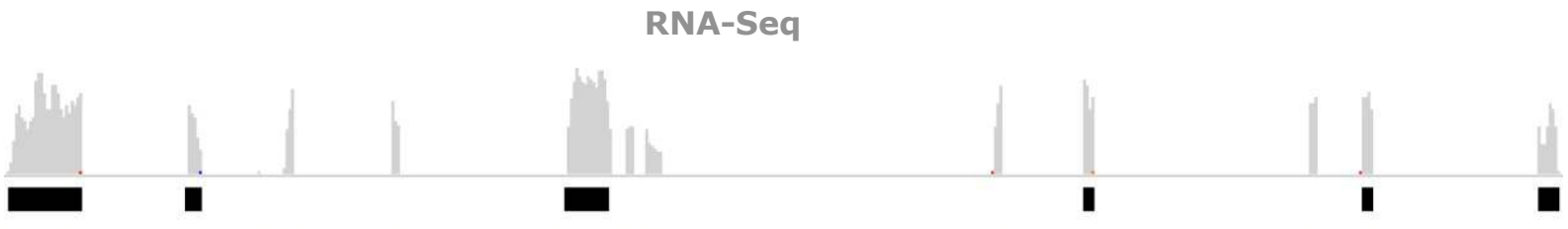

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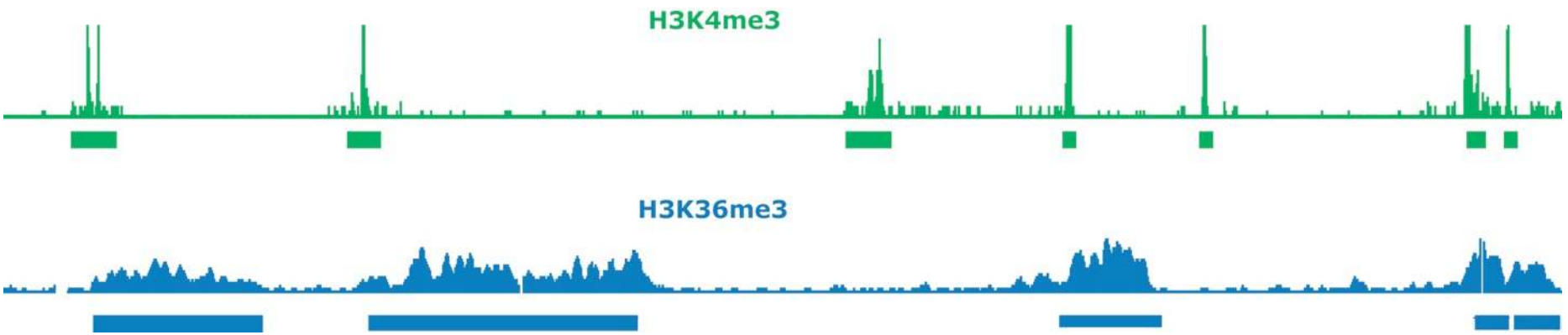

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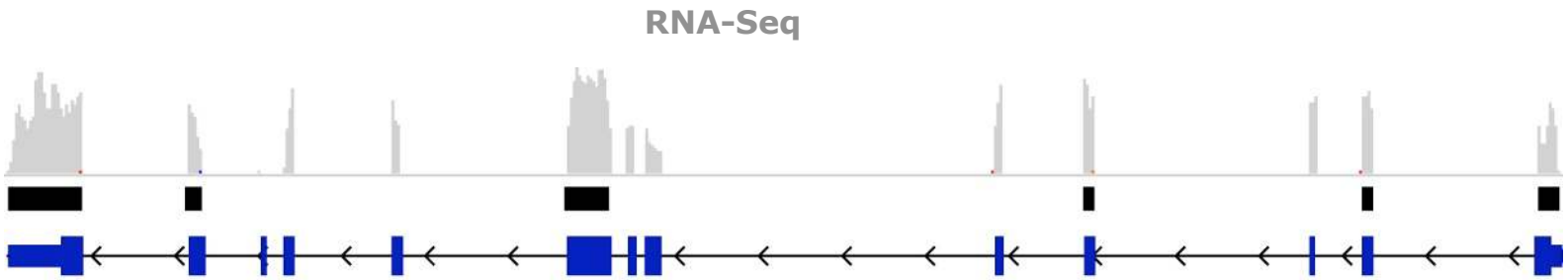

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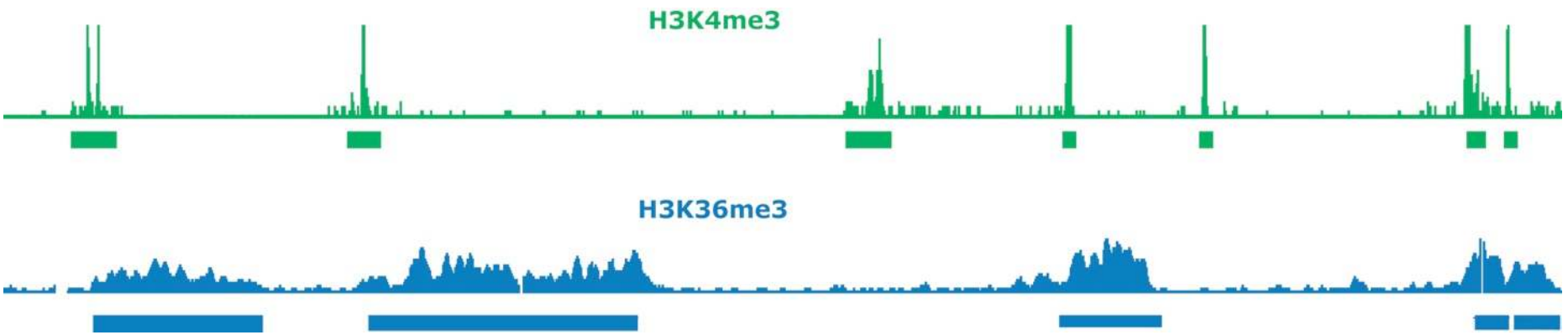
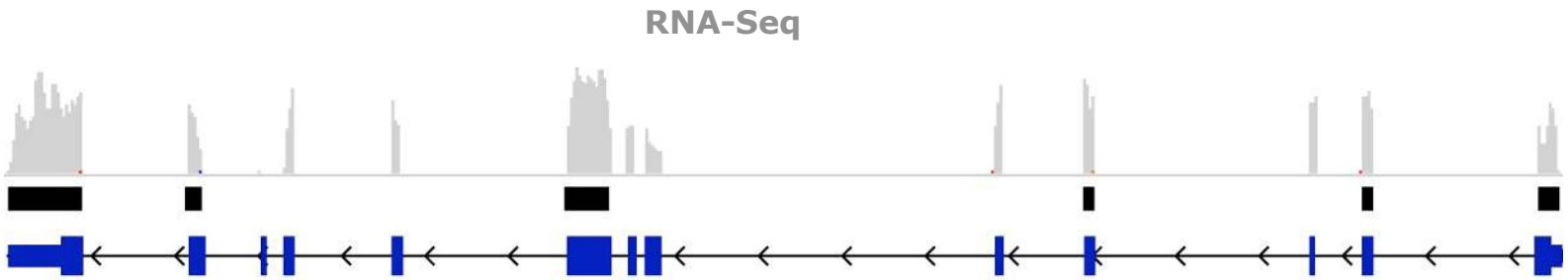


Statistical segmentation of chromatin modifications uses continuity of segments to increase power for interval detection



If we know the connectivity of fragments, we can increase our power to detect transcripts

Longer (76) reads provide increased number of junction reads

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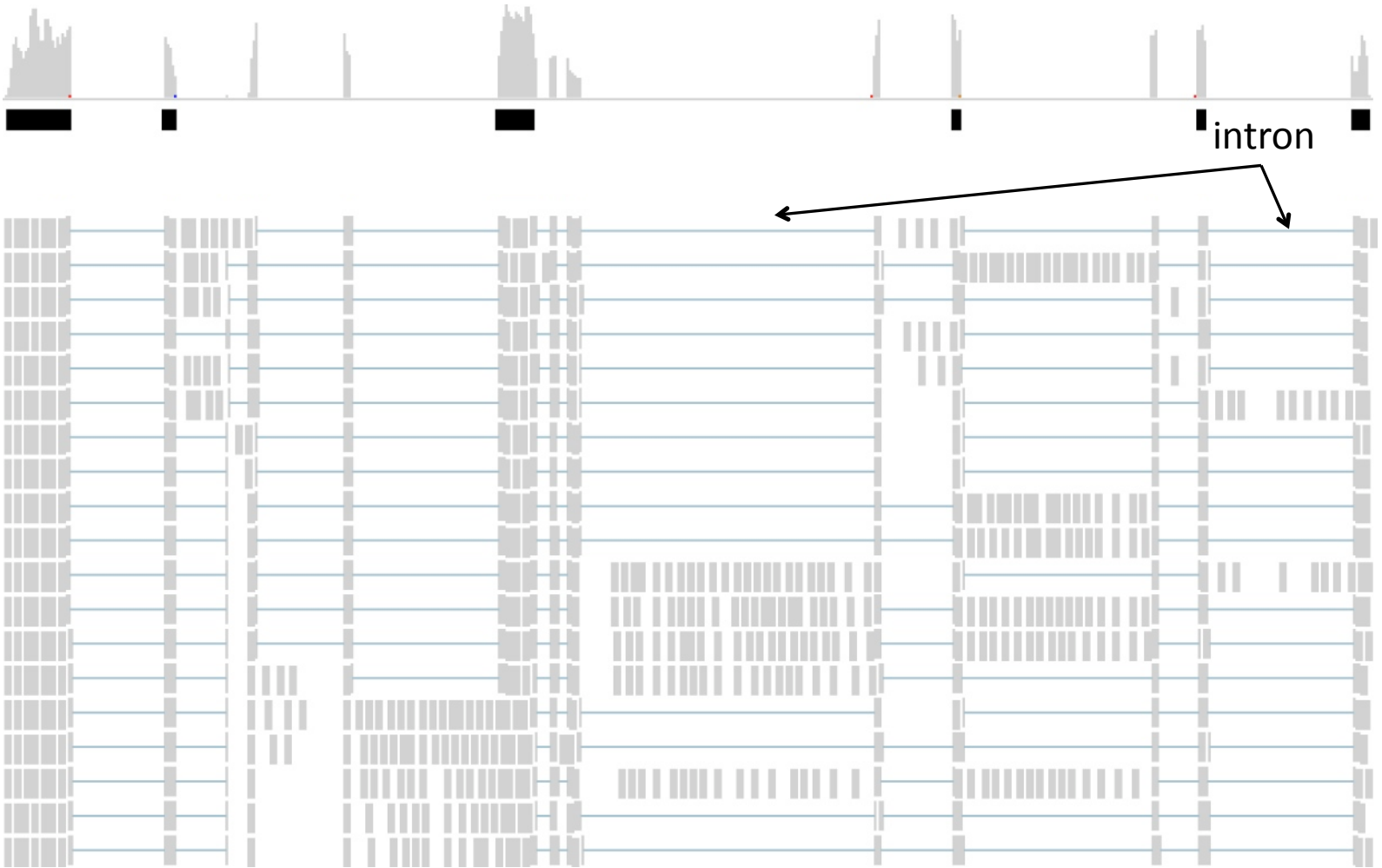

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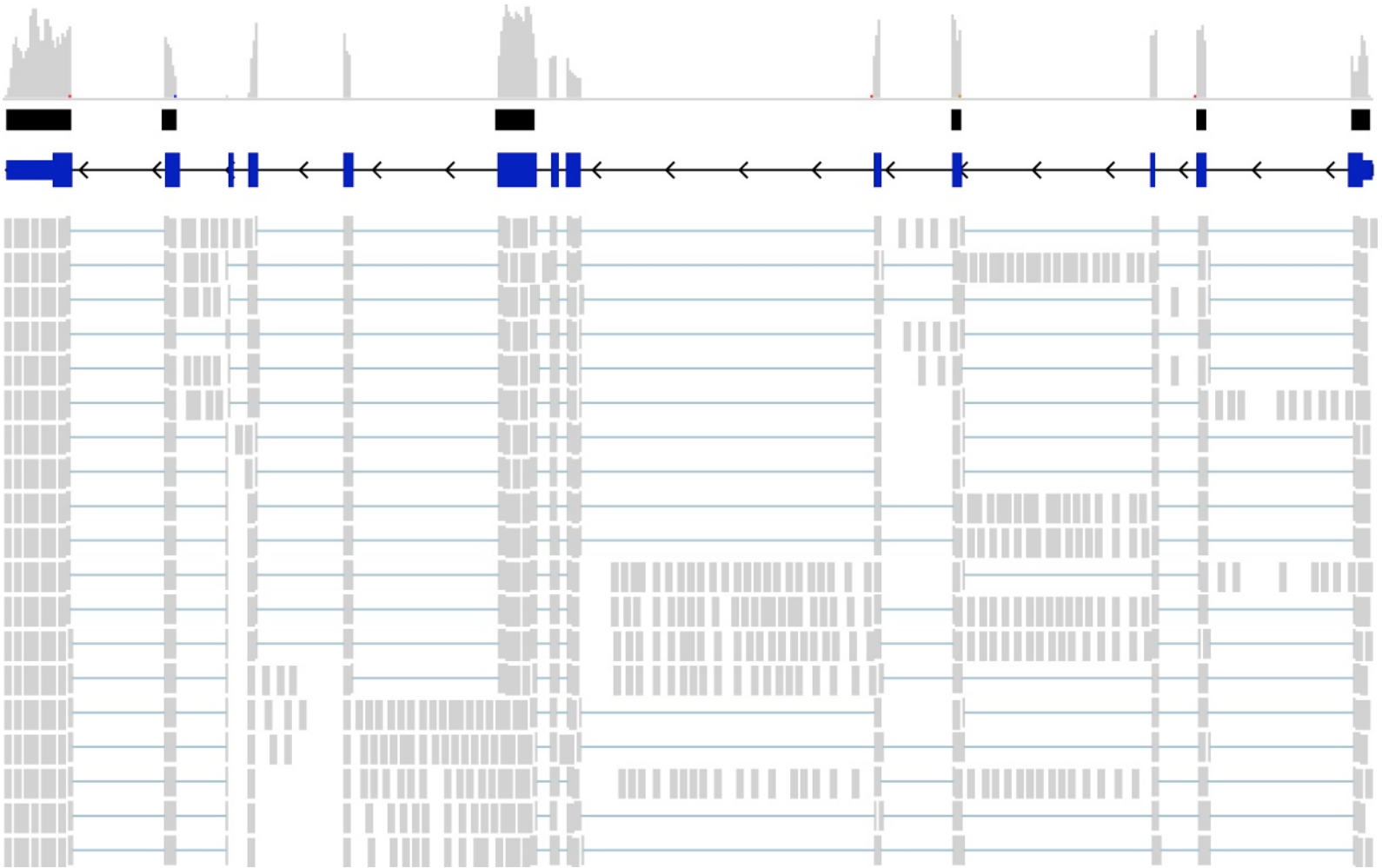

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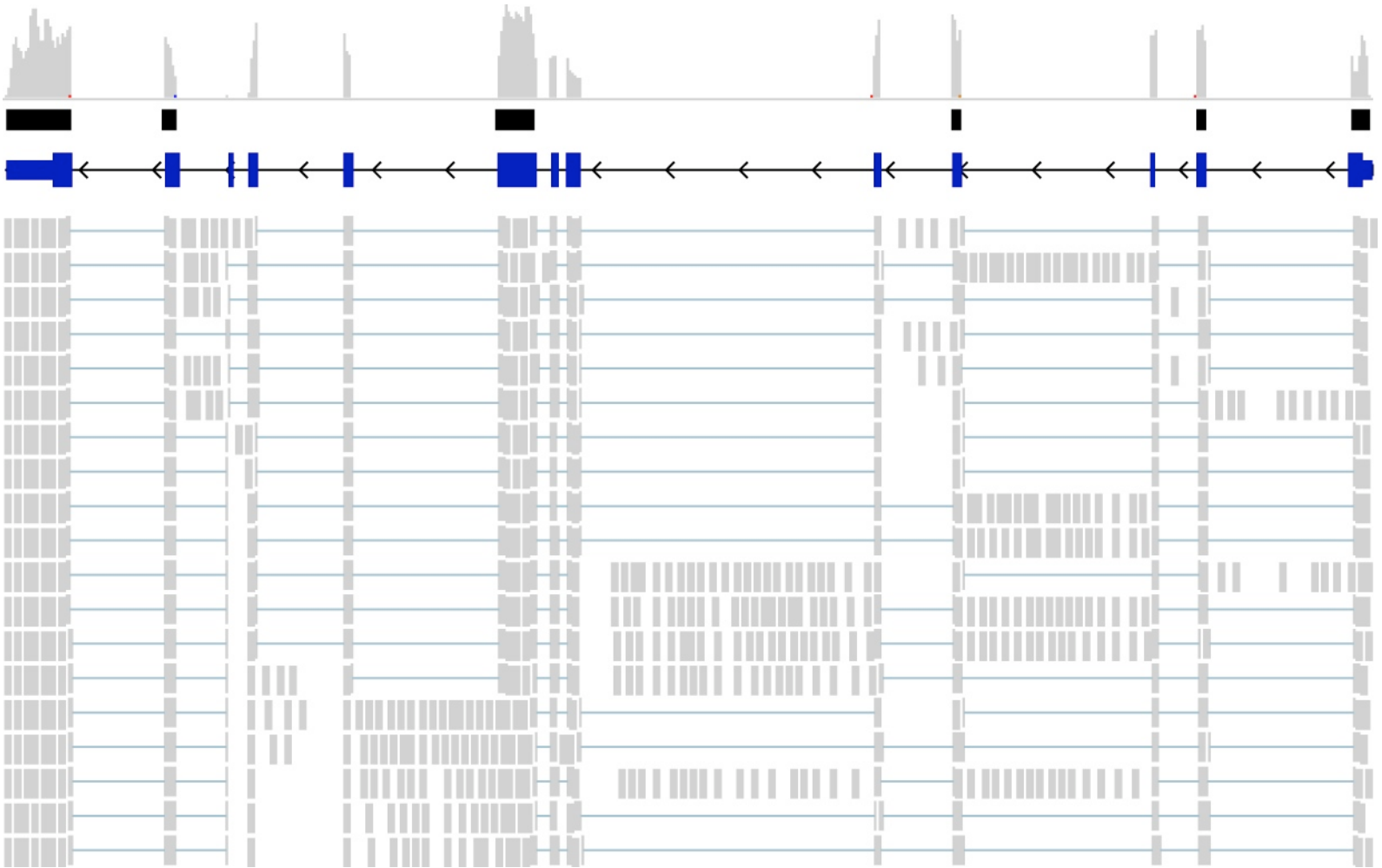
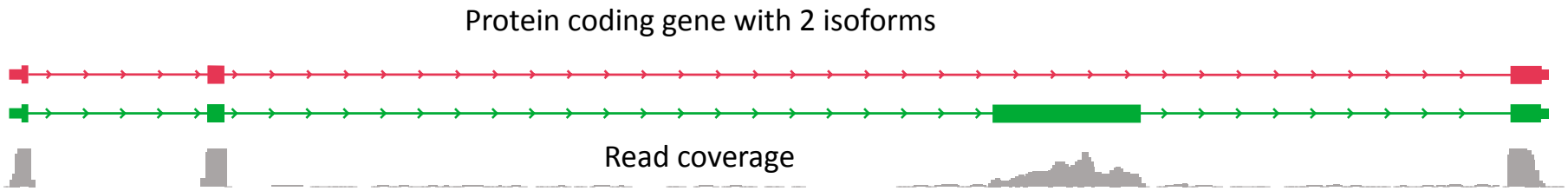
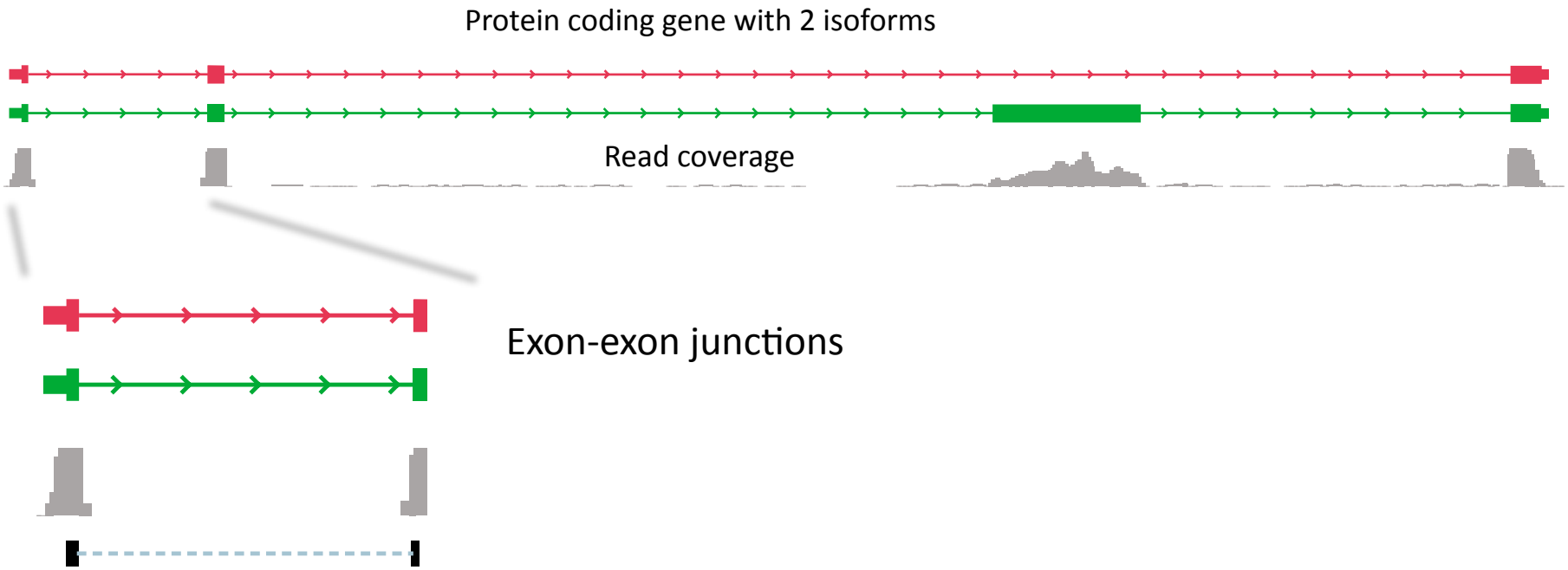


Exon junction spanning reads provide the connectivity information.

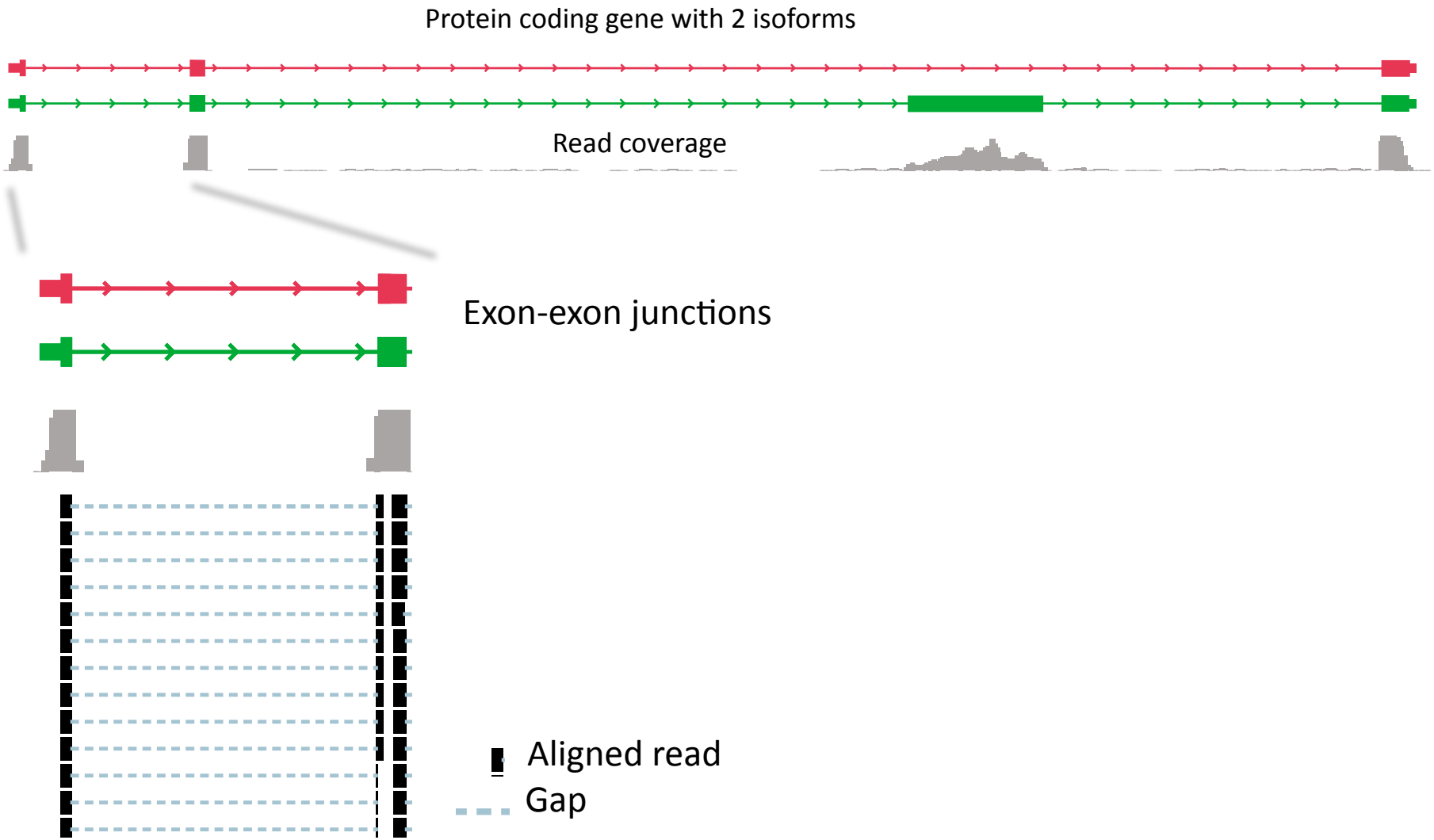
The power of spliced alignments



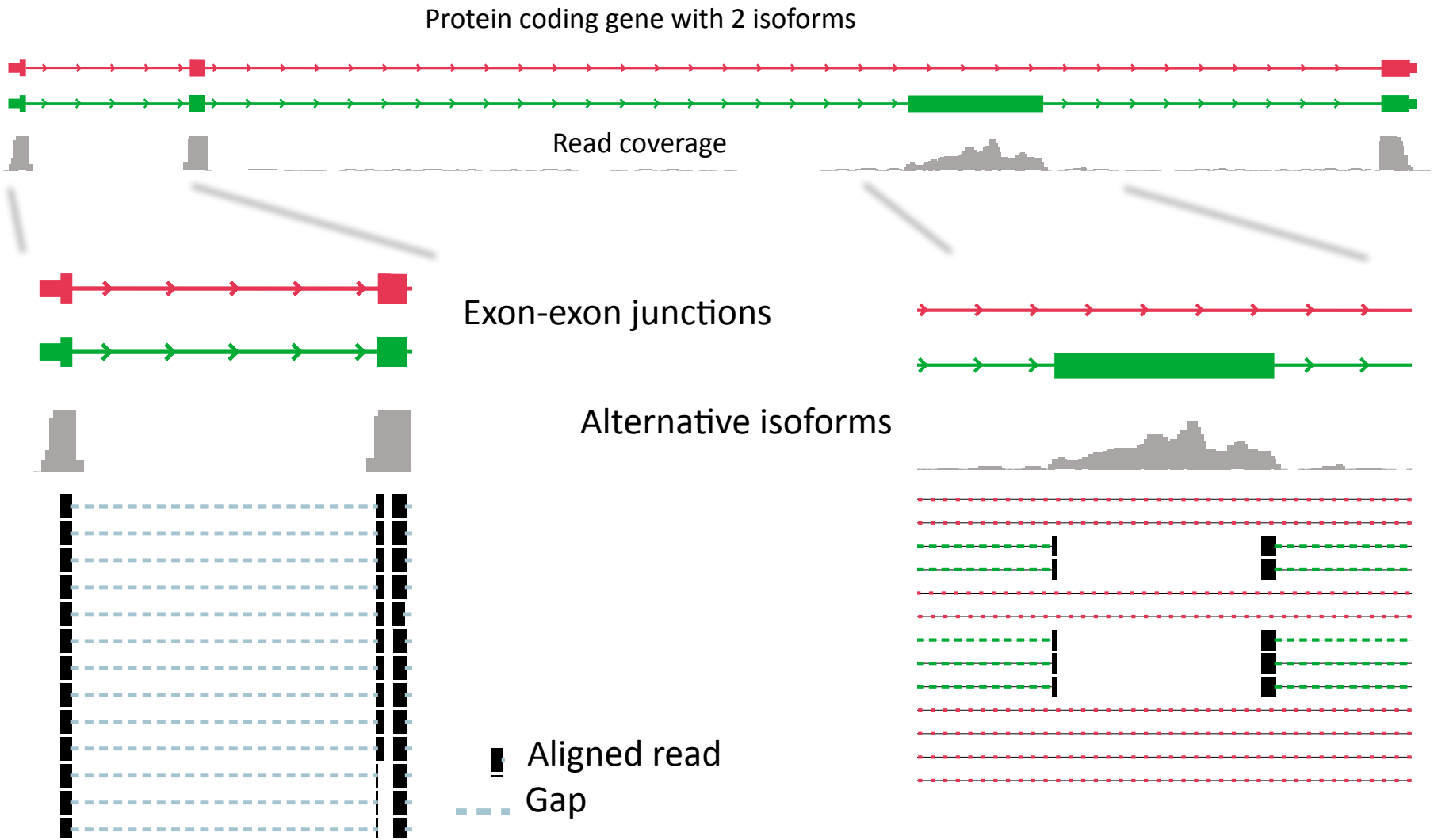
The power of spliced alignments



The power of spliced alignments

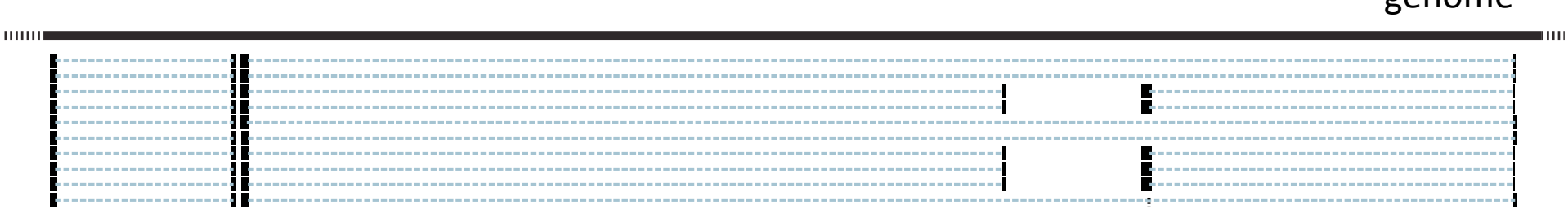


The power of spliced alignments



Statistical reconstruction of the transcriptome

Step 1: Align Reads to the genome allowing gaps flanked by splice sites



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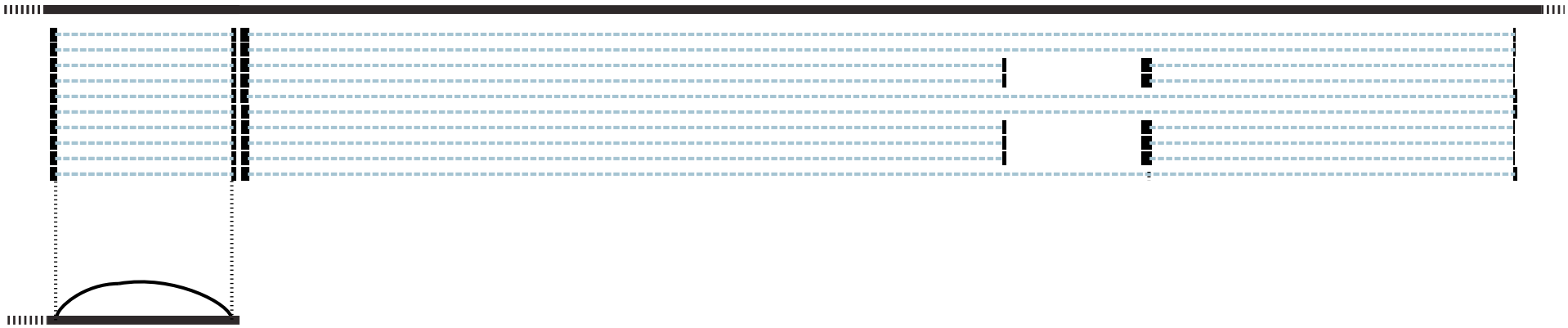

Step 2: Build an oriented connectivity graph using every spliced alignment and orienting edges using the flanking splicing motifs

Statistical reconstruction of the transcriptome

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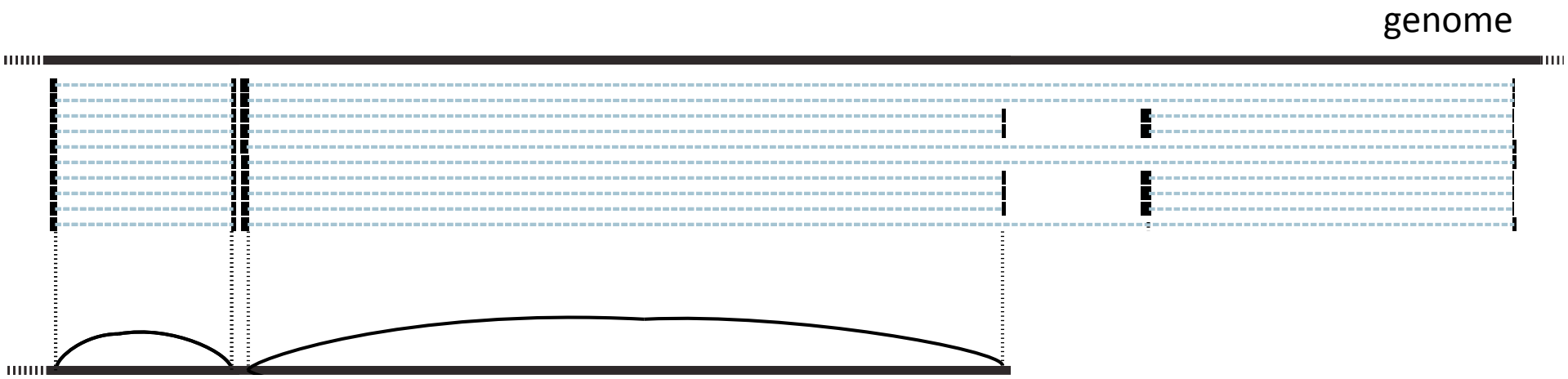

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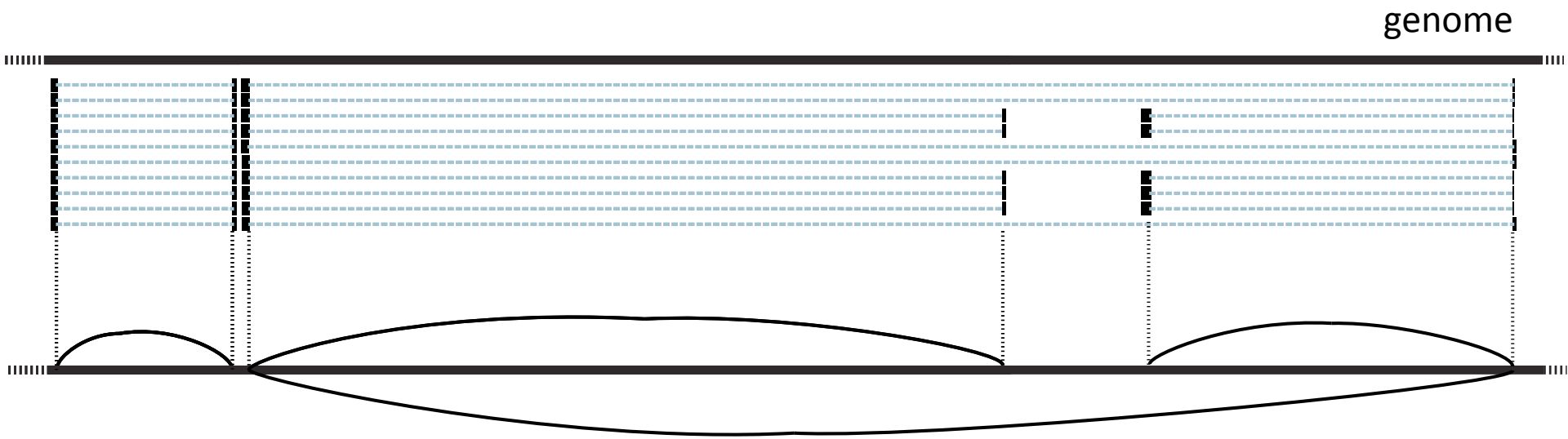

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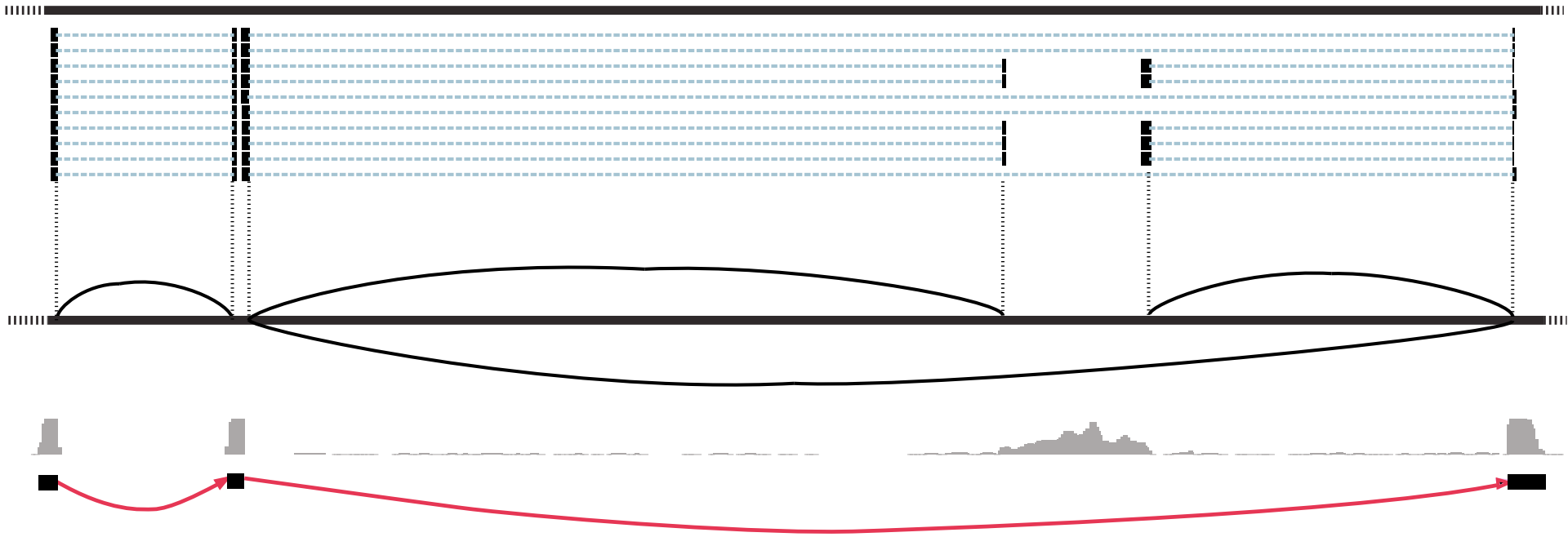
The "connectivity graph" connects all bases that are directly connected within the transcriptome

Statistical reconstruction of the transcriptome

Step 3: Identify “segments” across the graph



Statistical reconstruction of the transcriptome

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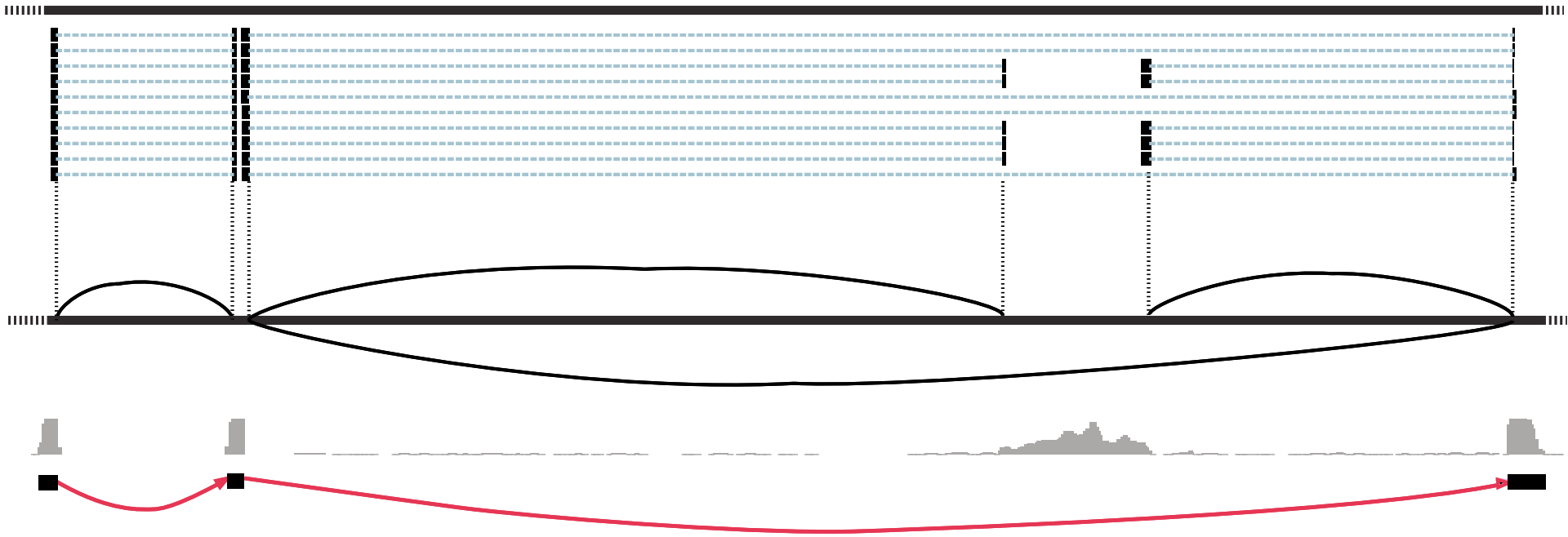
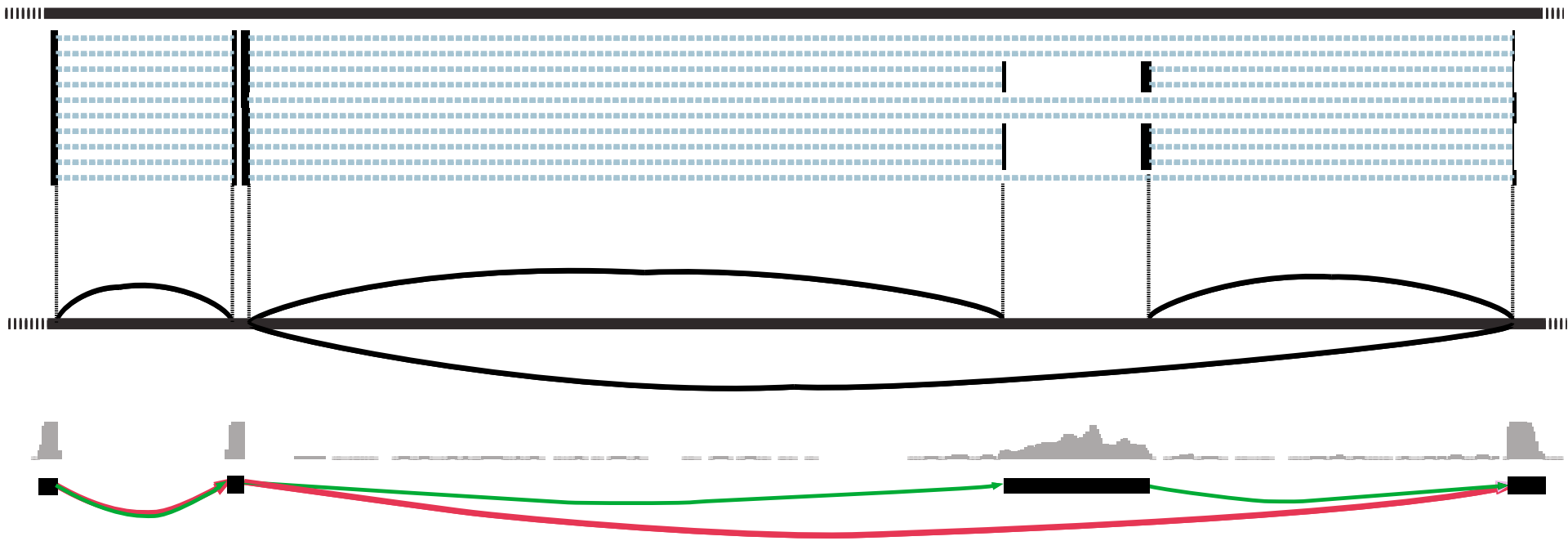


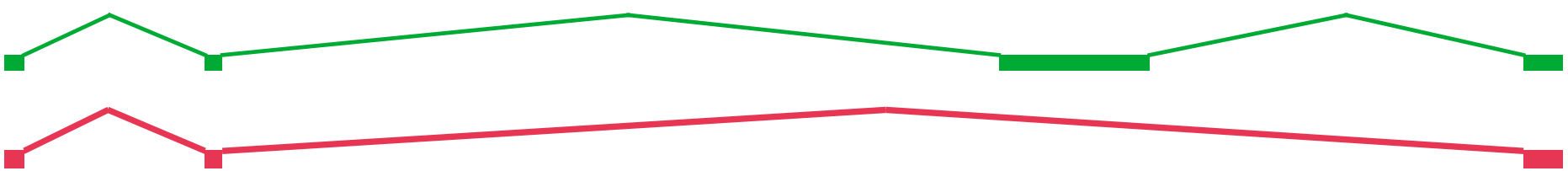
Step 4: Find significant segments

Statistical reconstruction of the transcriptome

Step 3: Identify “segments” across the graph



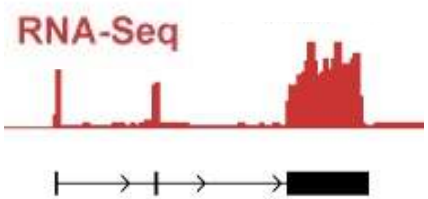
Step 4: Find significant segments



Can we identify enriched regions across different data types?

Short modification ✓

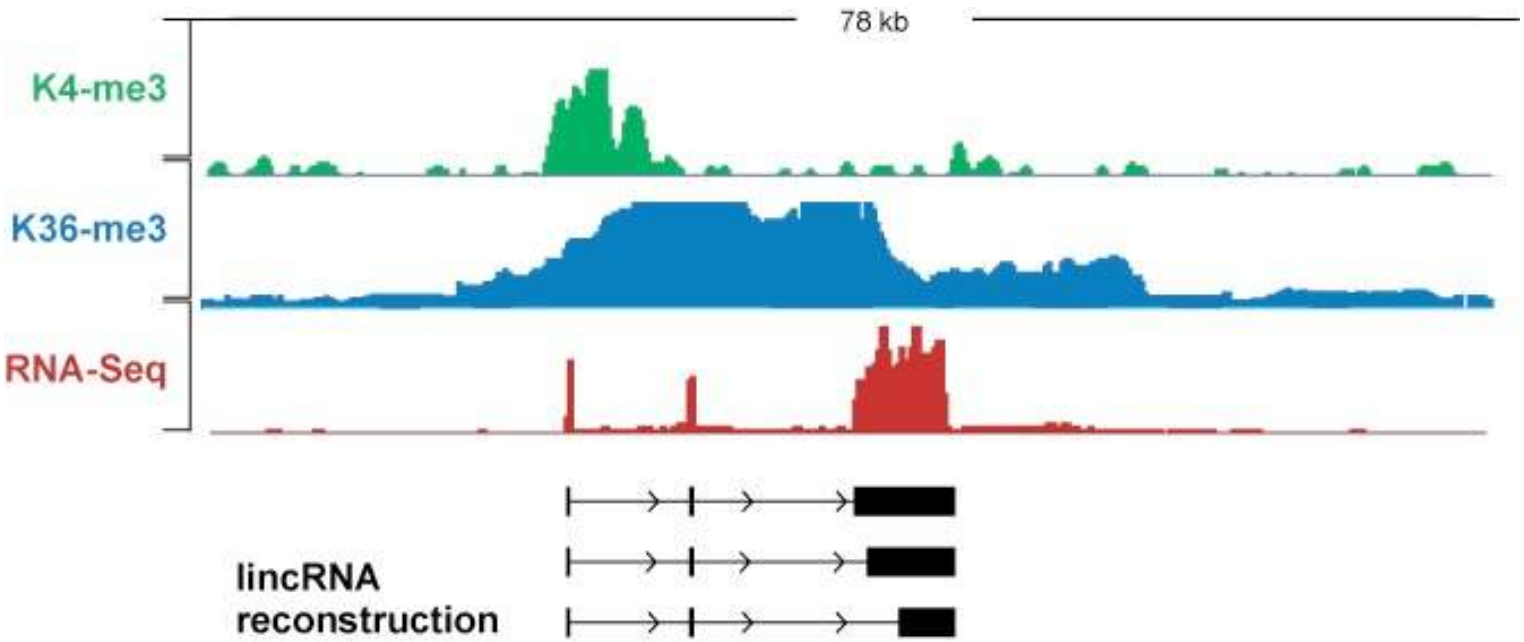
Long modification ✓



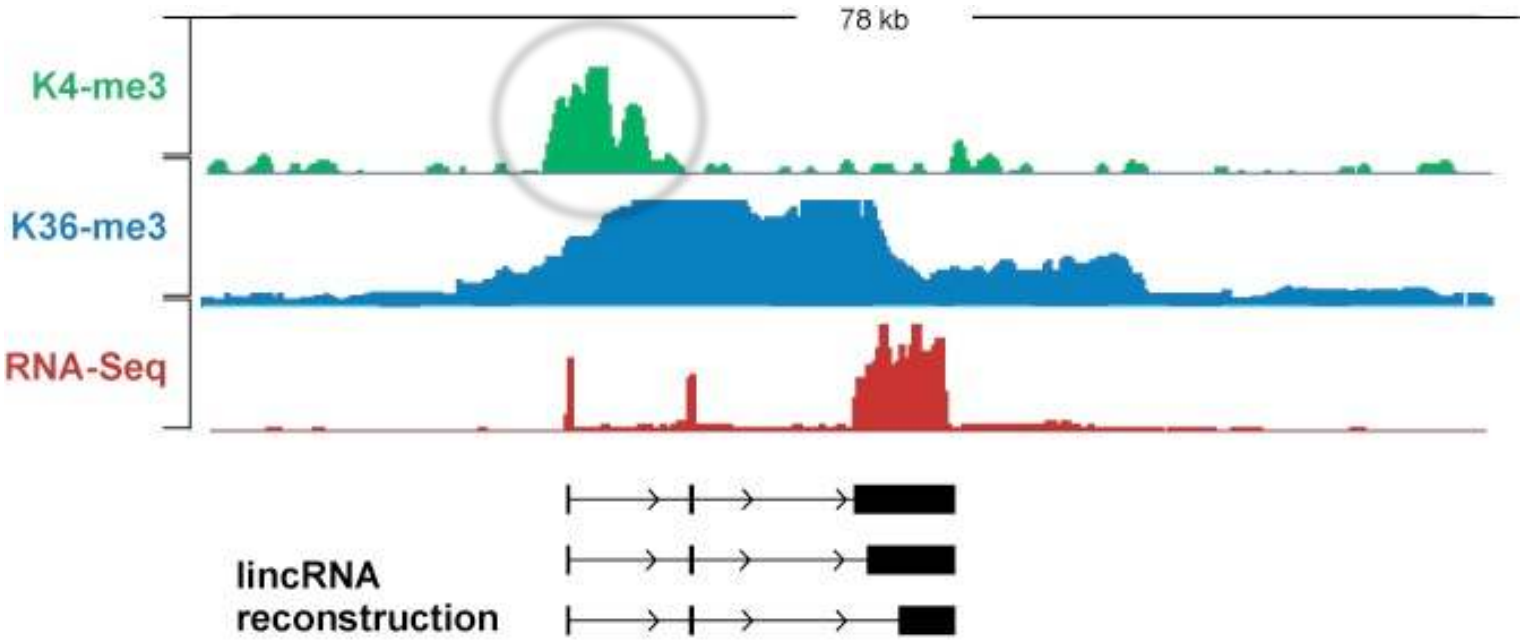
Discontinuous data ✓

Are we really sure reconstructions are complete?

RNA-Seq data is incomplete for comprehensive annotation



RNA-Seq data is incomplete for comprehensive annotation



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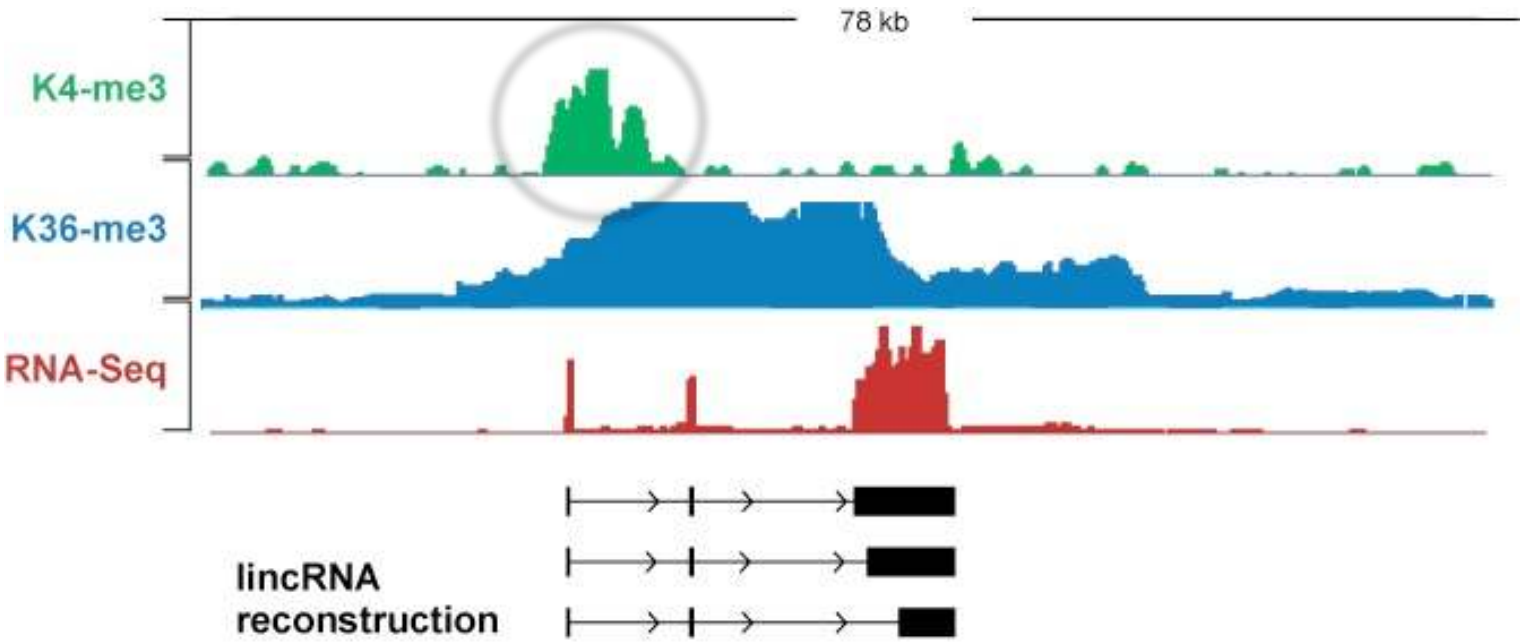


Library construction can help provide more information. More on this later

Applying scripture: Annotating the mouse transcriptome

Reconstructing the transcriptome of mouse cell types

Mouse Cell Types

Sequence

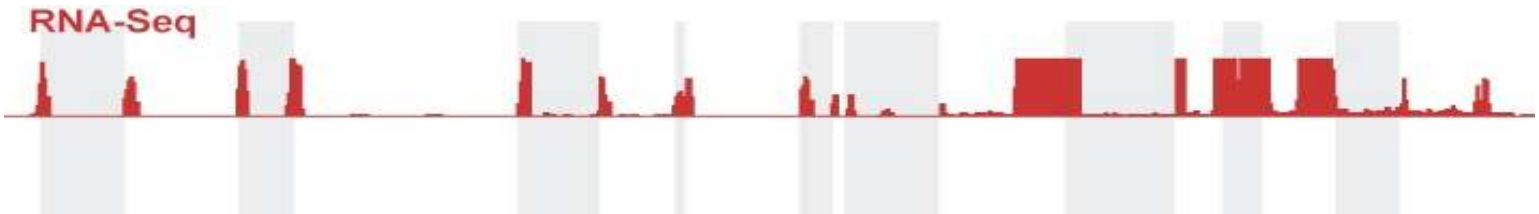
Reconstruct

Reconstructing the transcriptome of mouse cell types

Mouse Cell Types

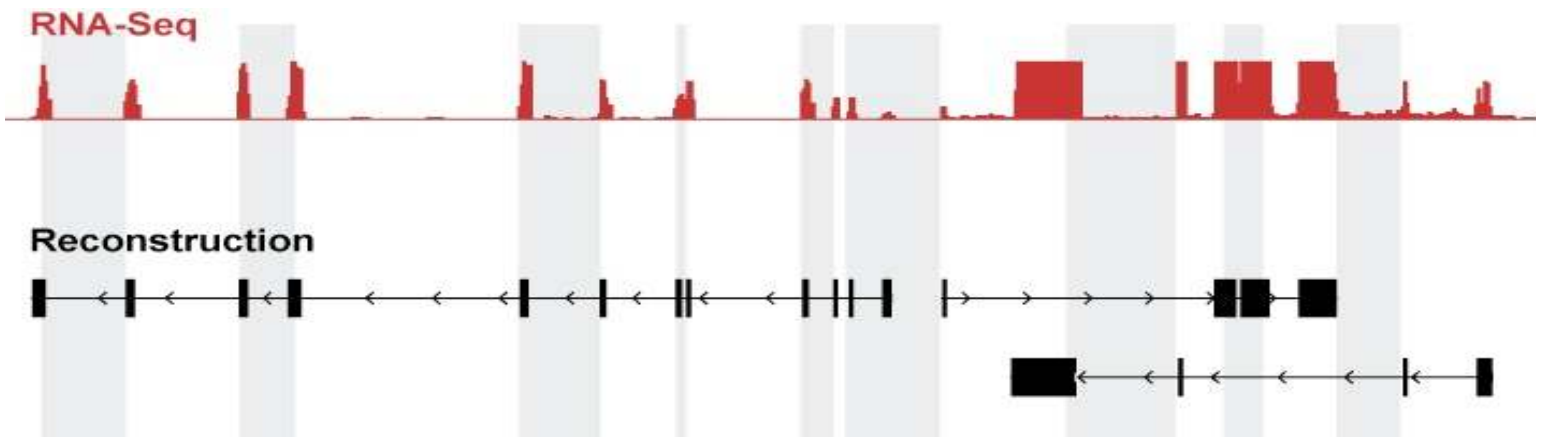
Sequence

Reconstruct



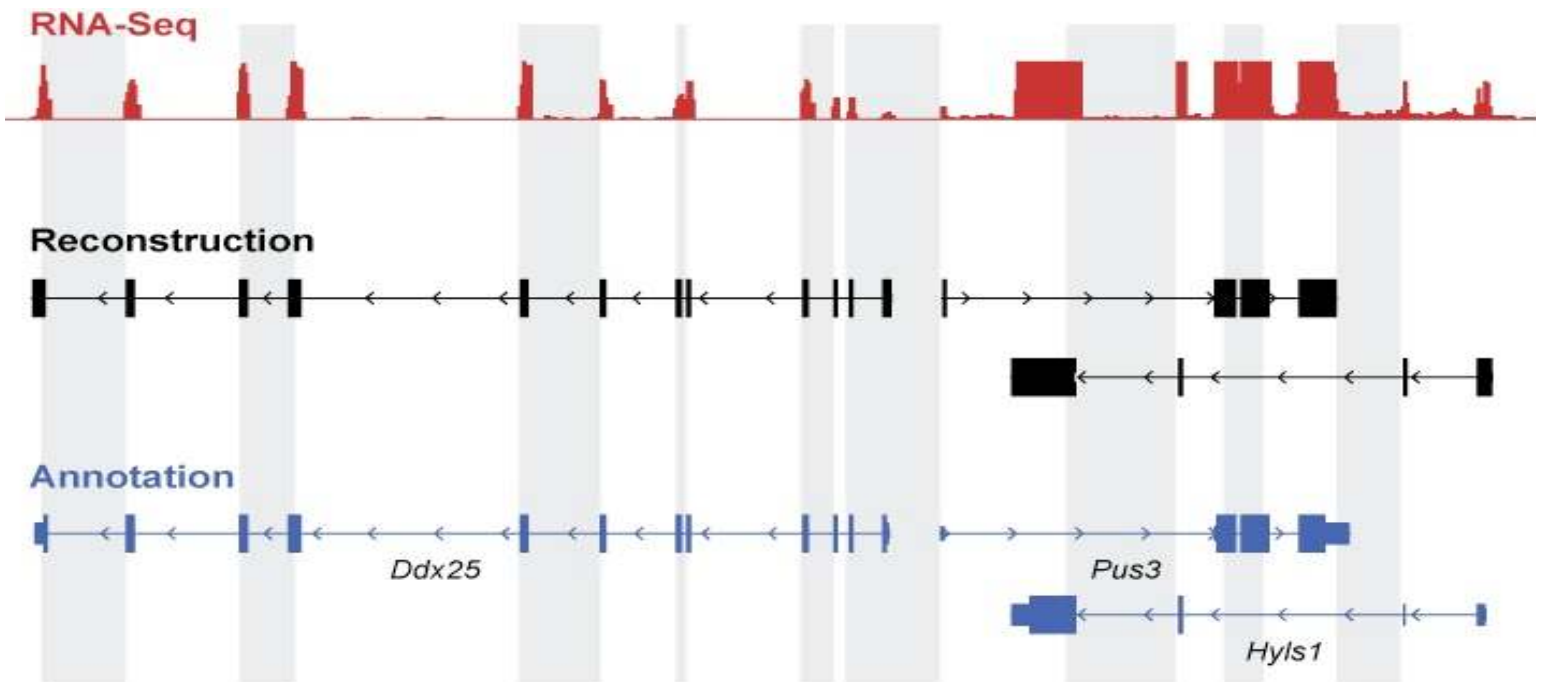
Reconstructing the transcriptome of mouse cell types

Mouse Cell Types **Sequence** **Reconstruct**



Reconstructing the transcriptome of mouse cell types

Mouse Cell Types **Sequence** **Reconstruct**



Reconstructing the transcriptome of mouse cell types

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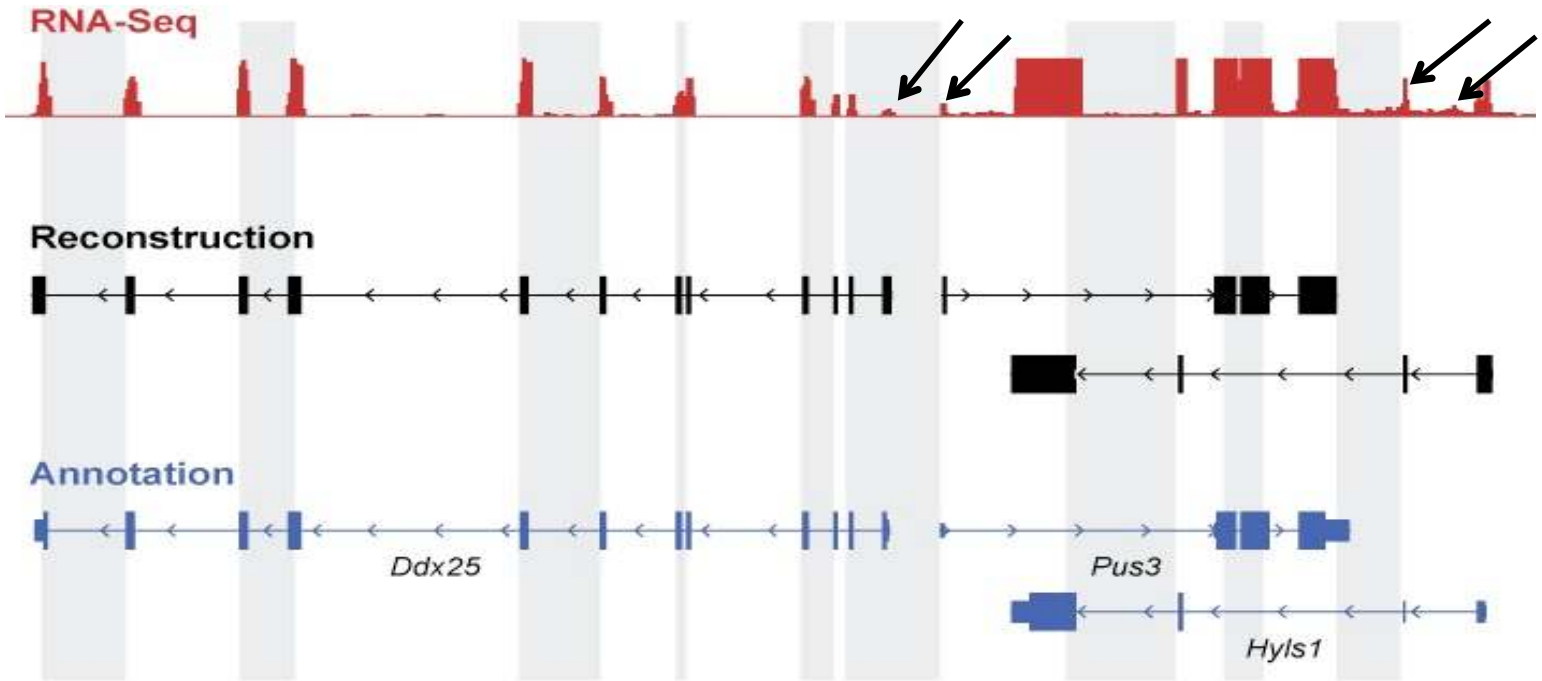
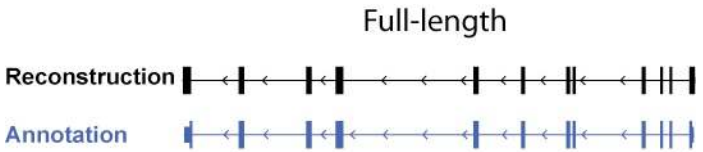
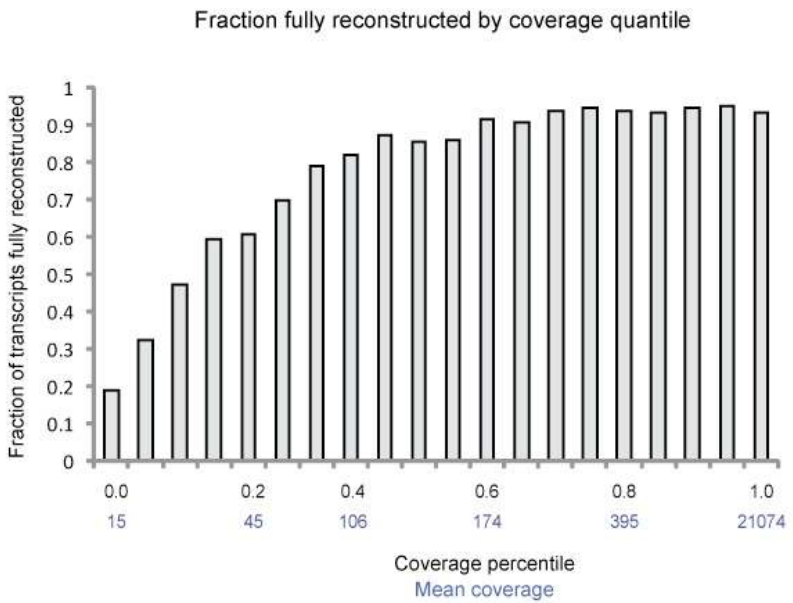


Sensitivity across expression levels





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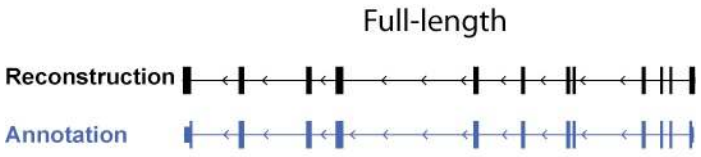

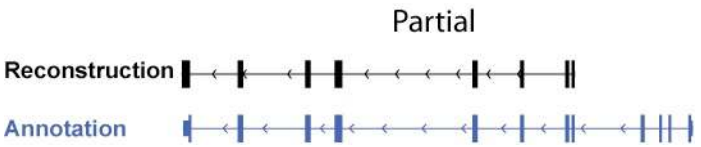

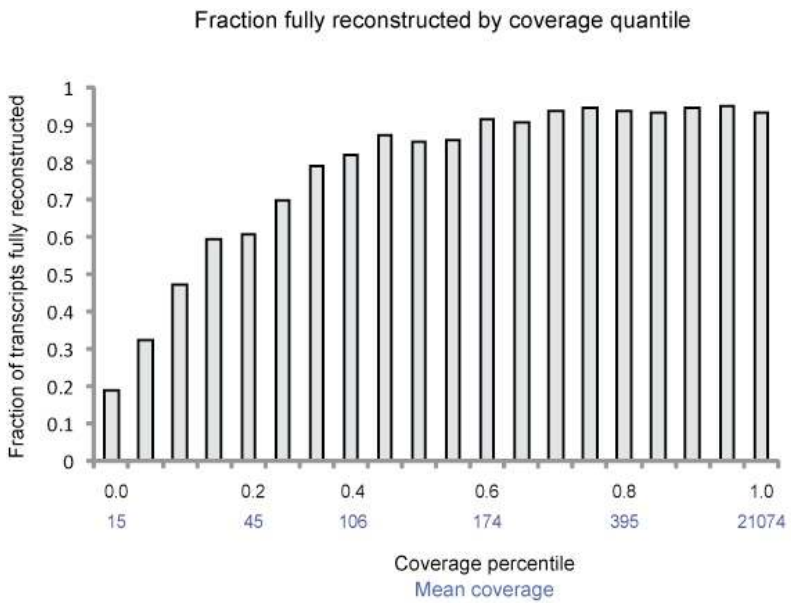

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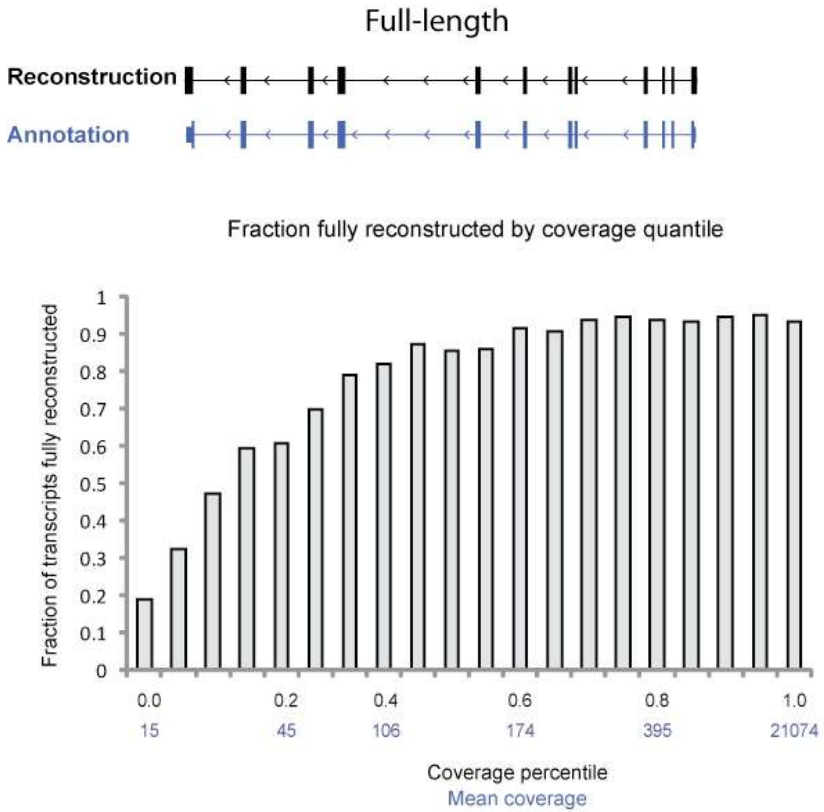

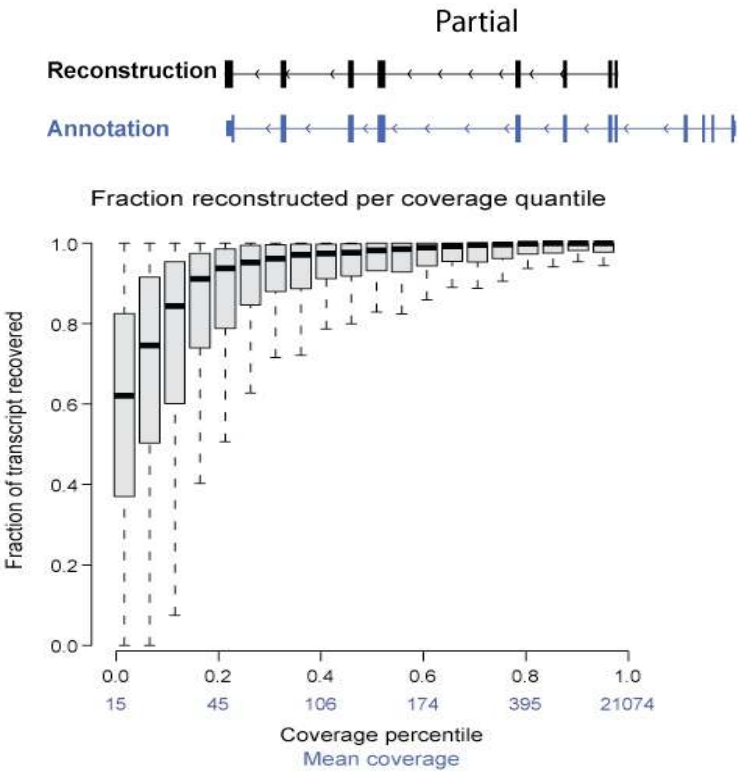

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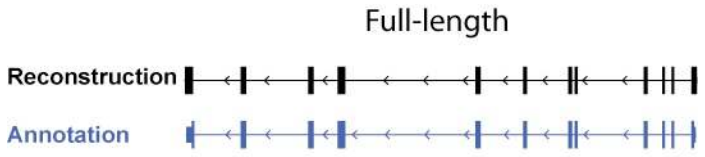
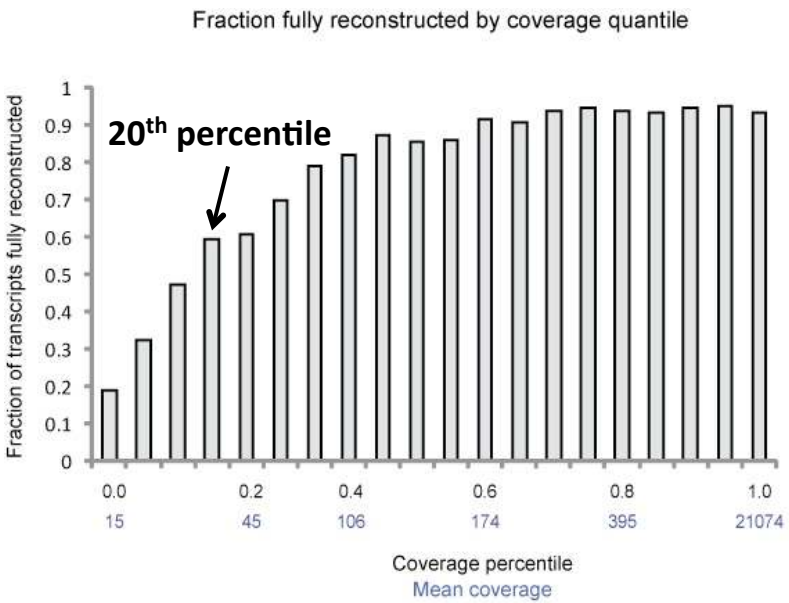
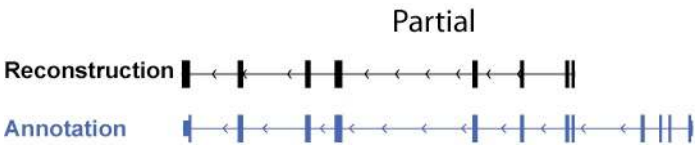
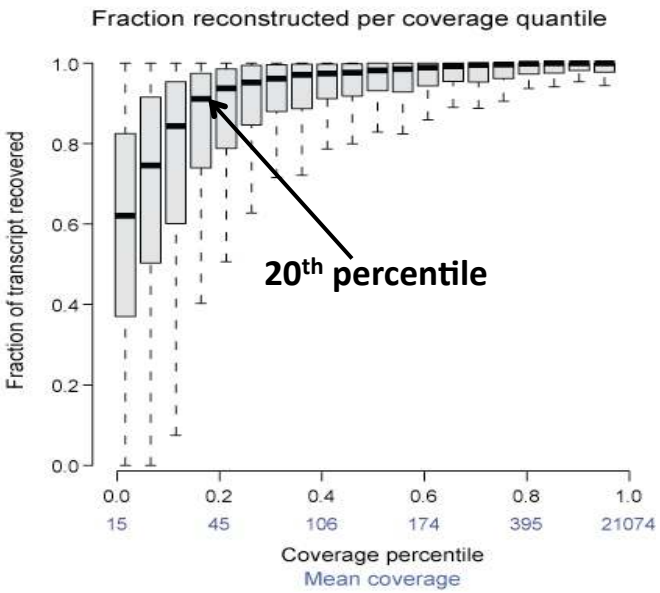


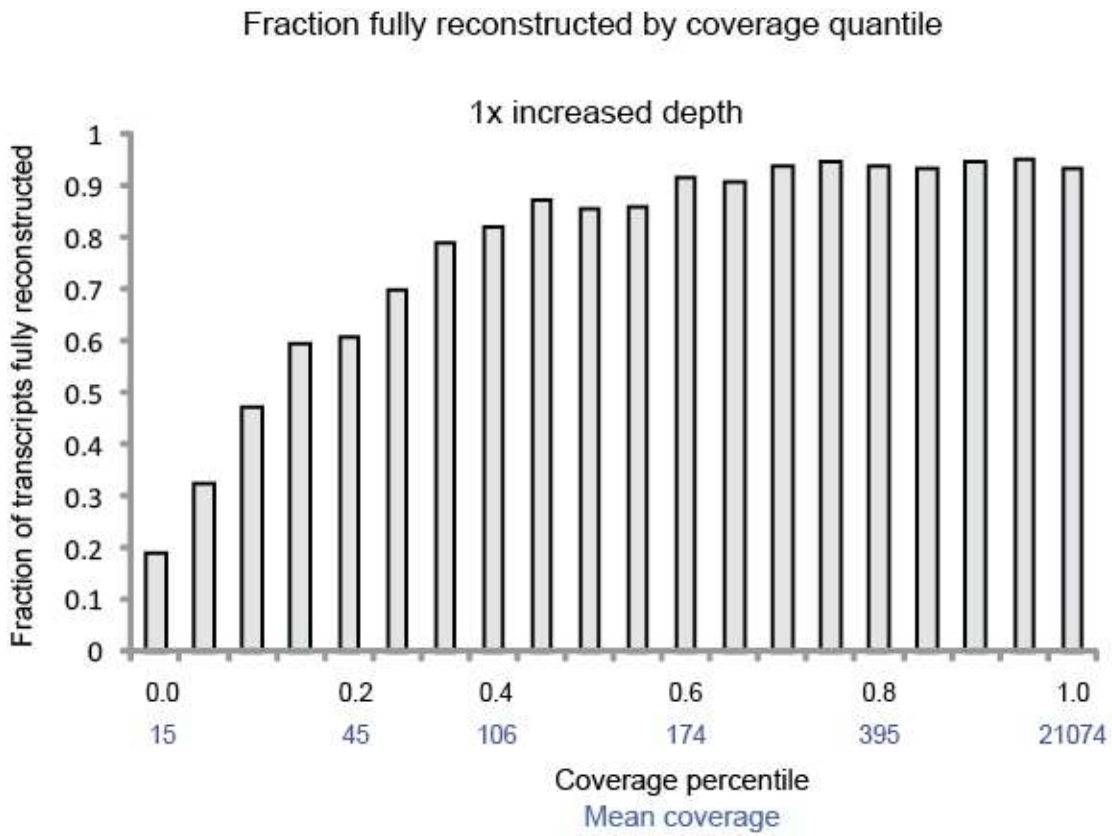






Even at low expression (20th percentile), we have:
average coverage of transcript is ~95% and 60% have full coverage

Sensitivity at low expression levels improves with depth



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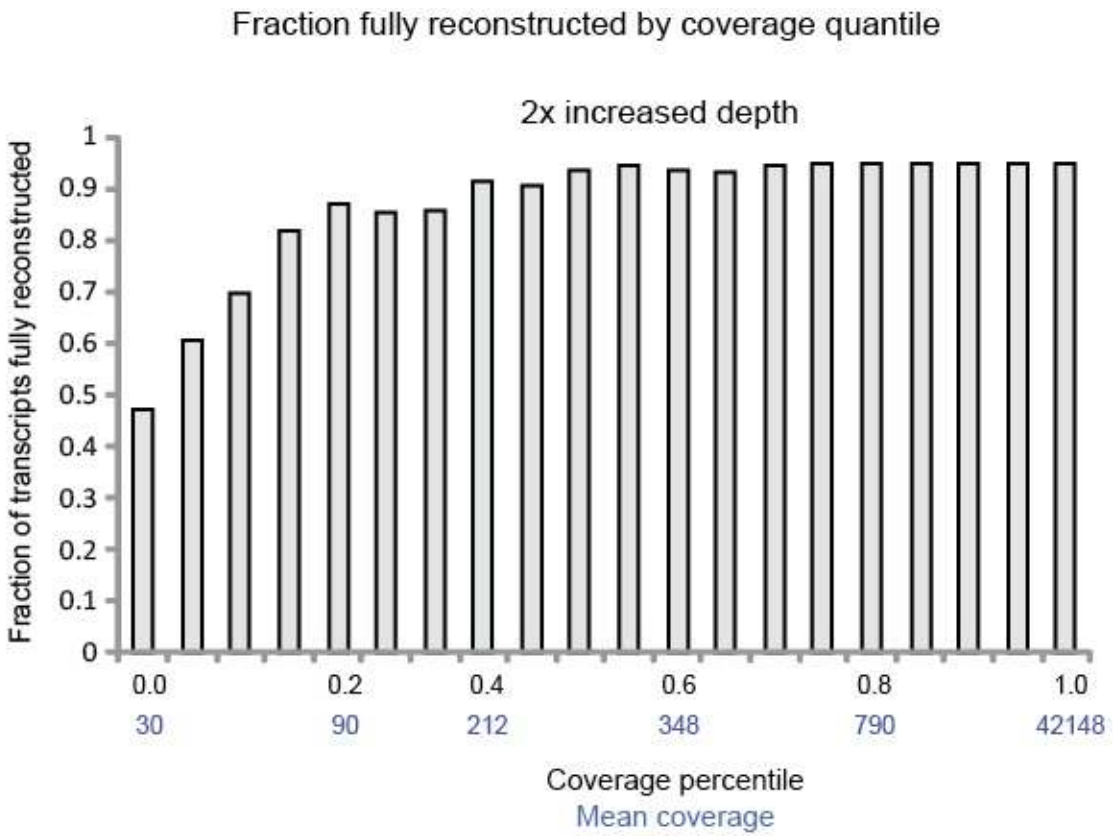

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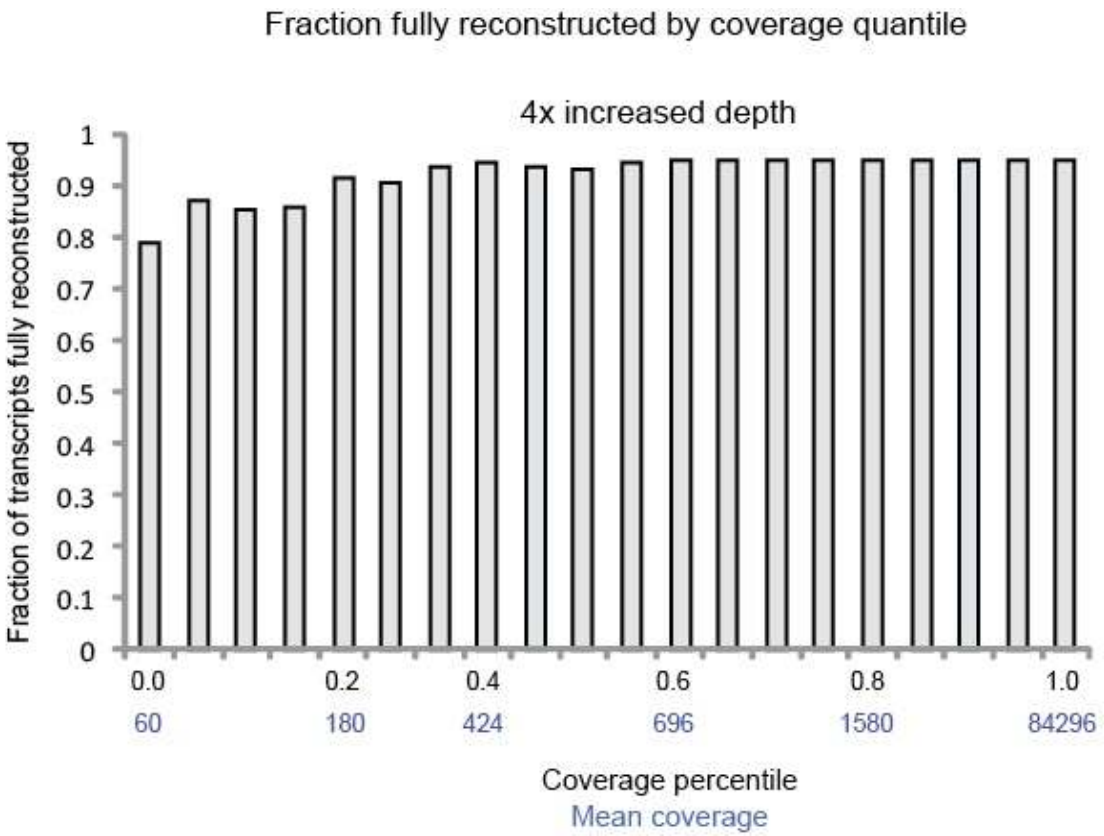

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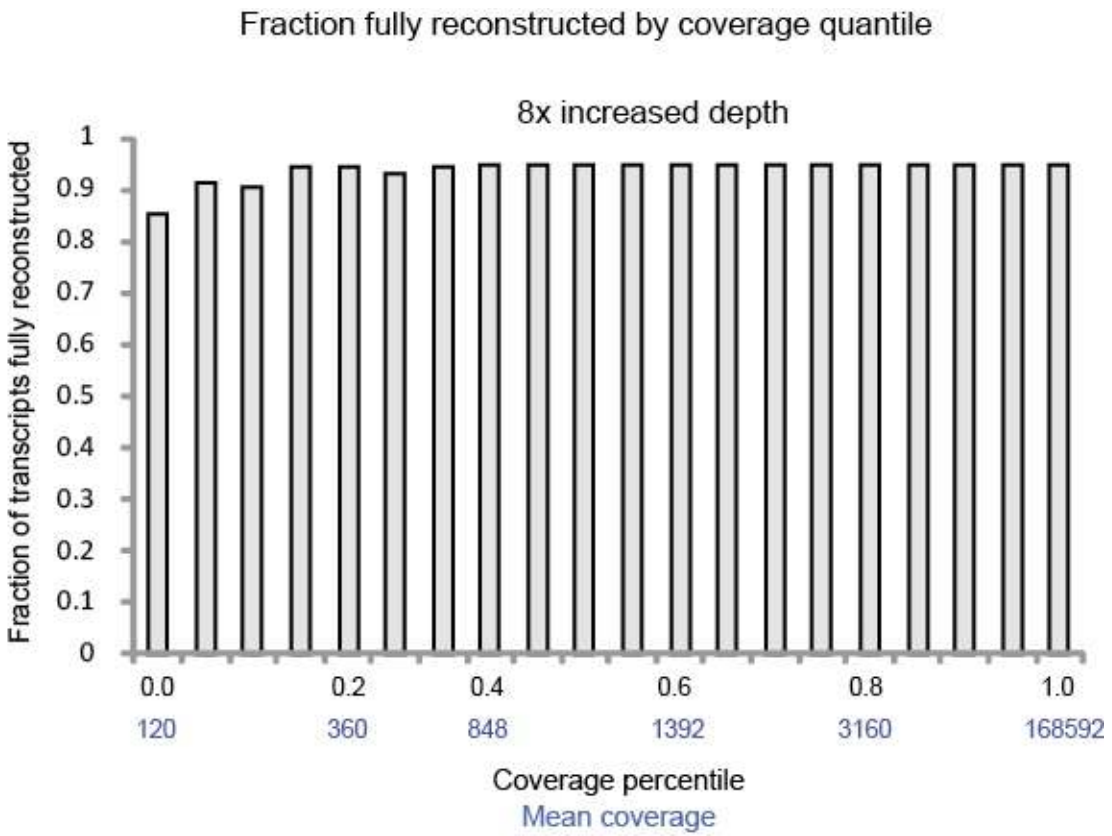

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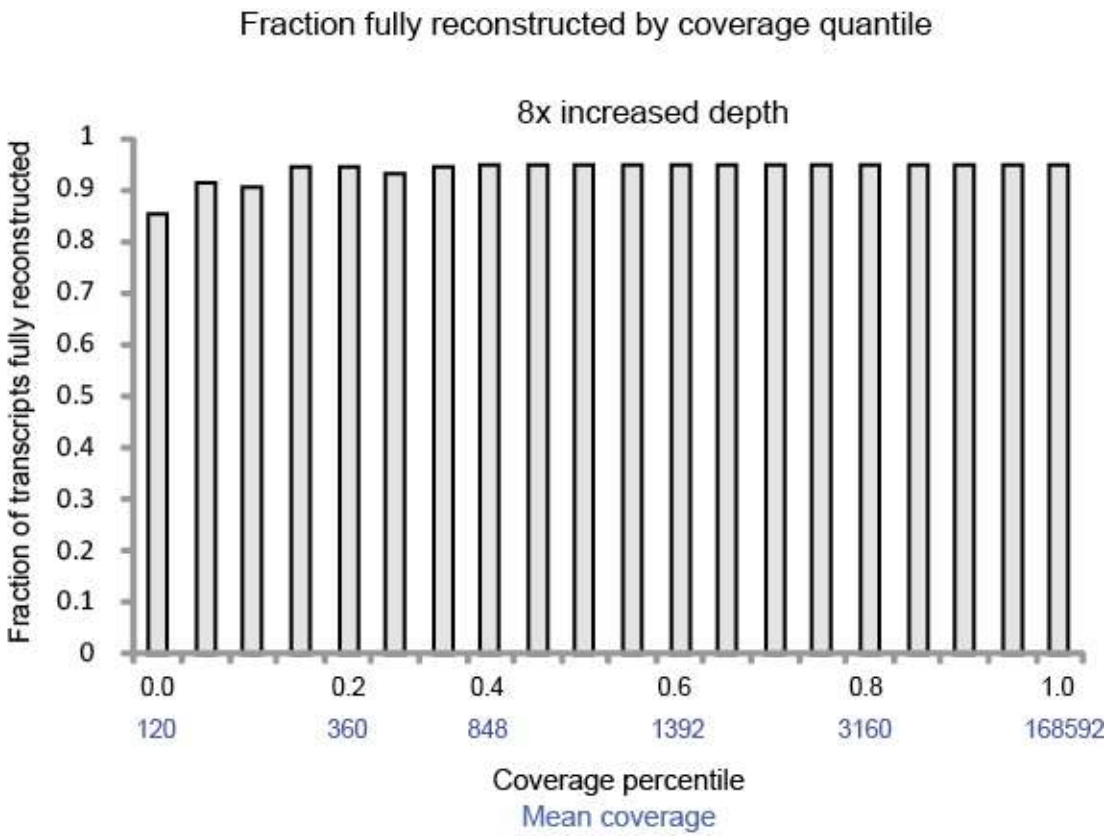
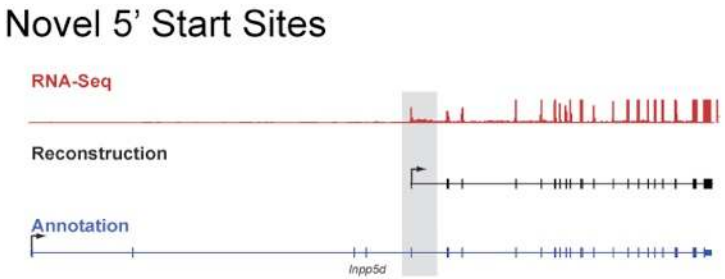


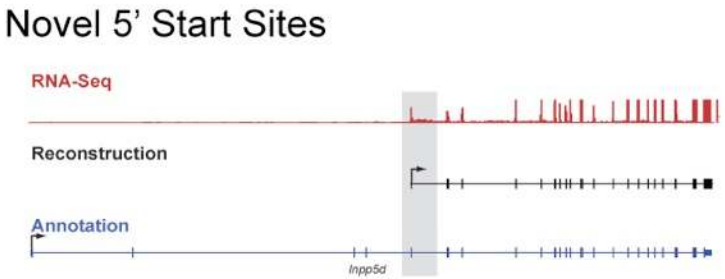
As coverage increases we are able to fully reconstruct a larger percentage of known protein-coding genes

Novel variation in protein-coding genes

ES cells



Novel variation in protein-coding genes



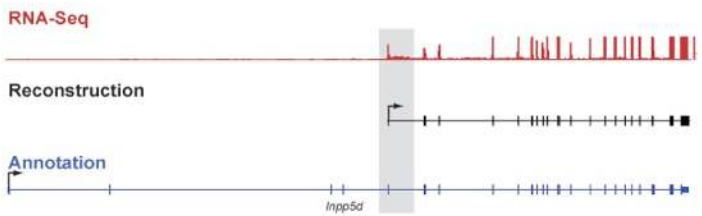
ES cells

1,804

Novel variation in protein-coding genes

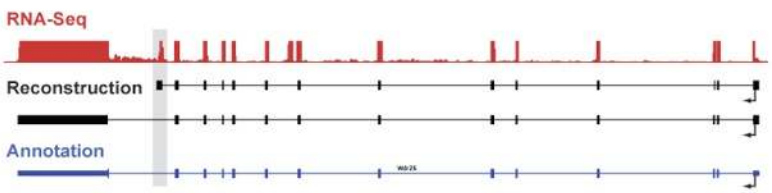
ES cells

Novel 5' Start Sites



1,804

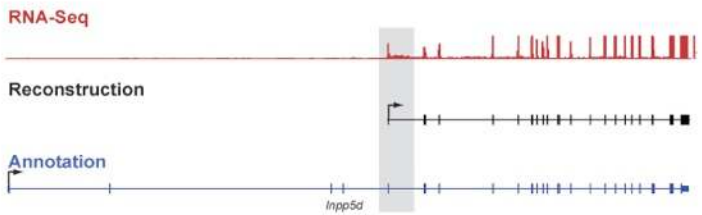
Novel 3' End



Novel variation in protein-coding genes

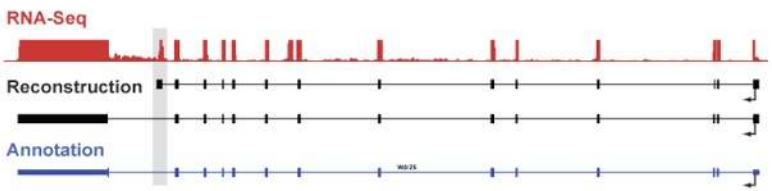
ES cells

Novel 5' Start Sites



1,804

Novel 3' End

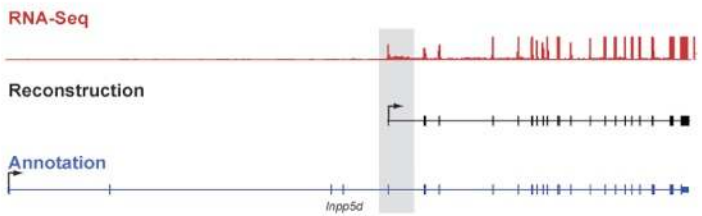


1,310

Novel variation in protein-coding genes

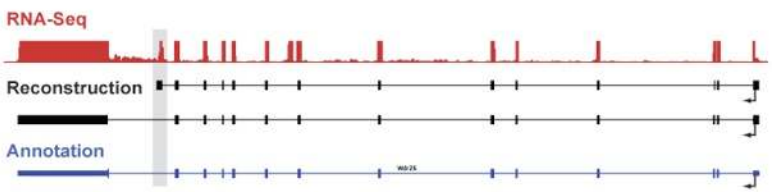
ES cells

Novel 5' Start Sites



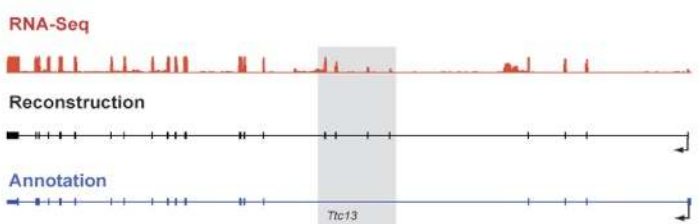
1,804

Novel 3' End



1,310

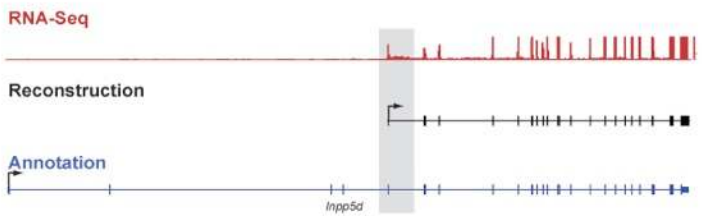
Novel Coding Exons



Novel variation in protein-coding genes

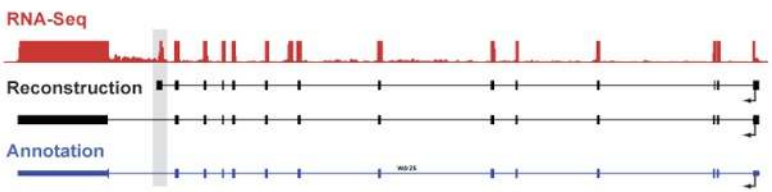
ES cells

Novel 5' Start Sites



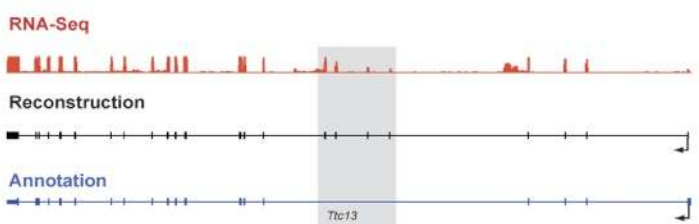
1,804

Novel 3' End



1,310

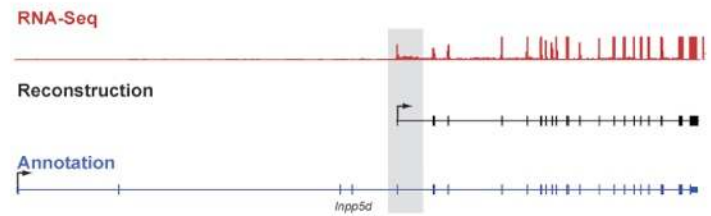
Novel Coding Exons

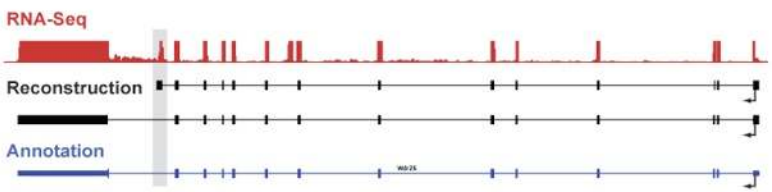


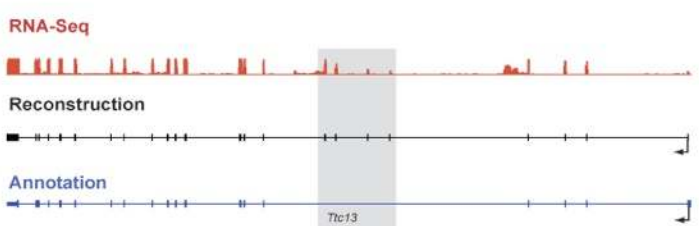
588

Novel variation in protein-coding genes

	ES cells	3 cell types
Novel 5' Start Sites	1,804	3,137
Novel 3' End	1,310	2,477
Novel Coding Exons	588	903

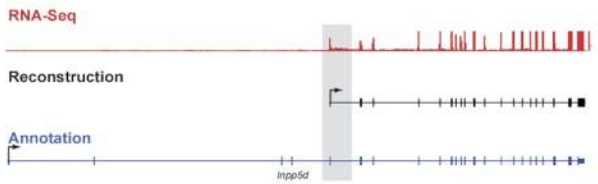




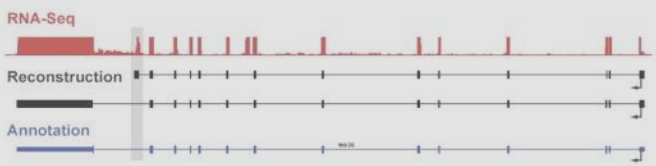


Novel variation in protein-coding genes

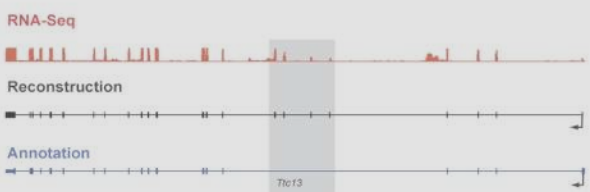
Novel 5' Start Sites

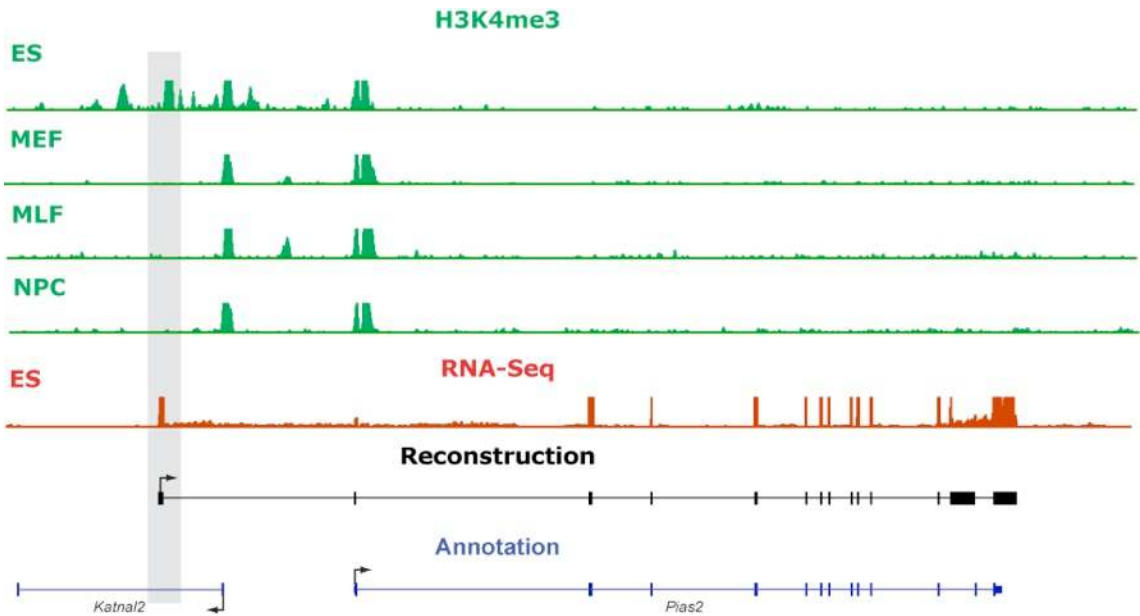


Novel 3' End



Novel Coding Exons





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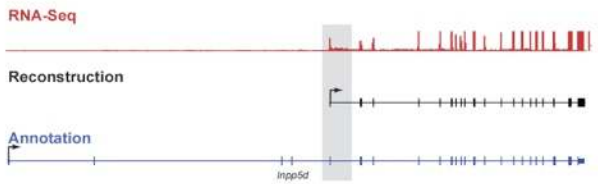
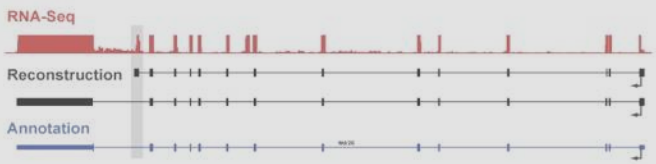
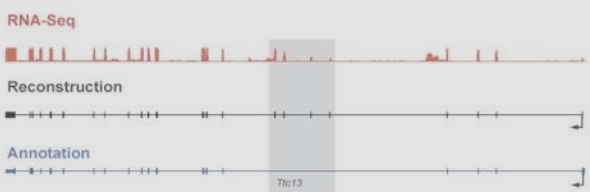
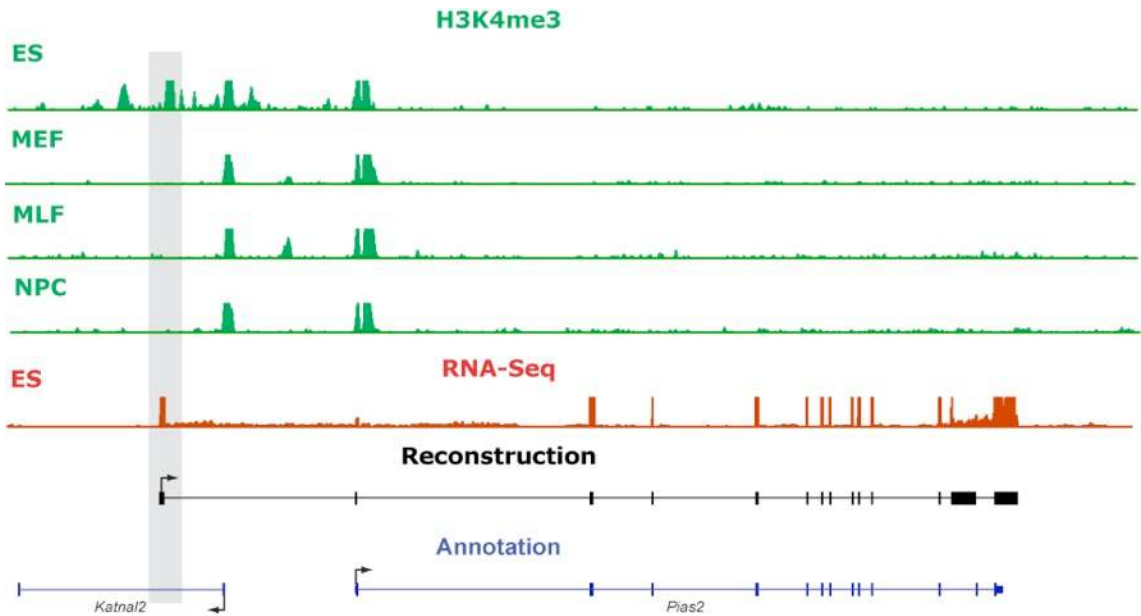


Novel 3' End



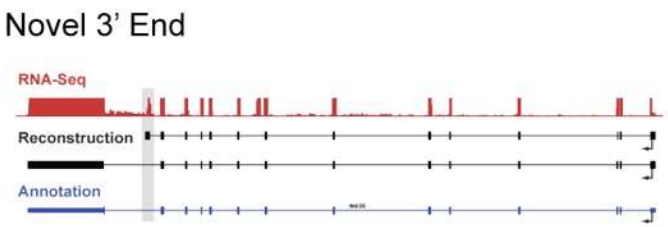
Novel Coding Exons

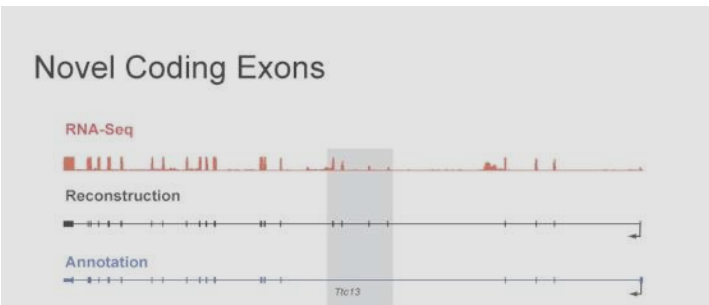




~85% overlap K4me3

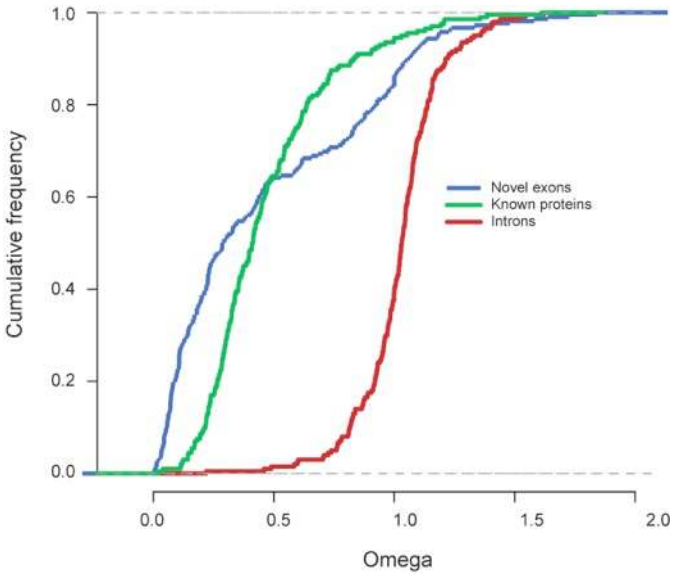
Novel variation in protein-coding genes

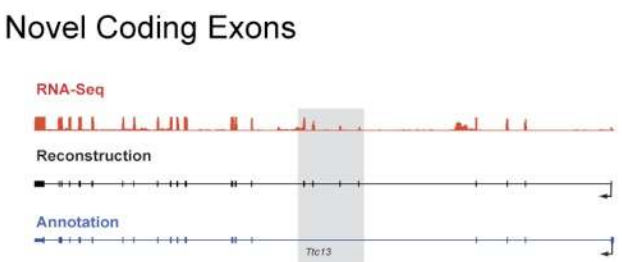




~50% contain polyA motif Compared to ~6% for random

Novel variation in protein-coding genes





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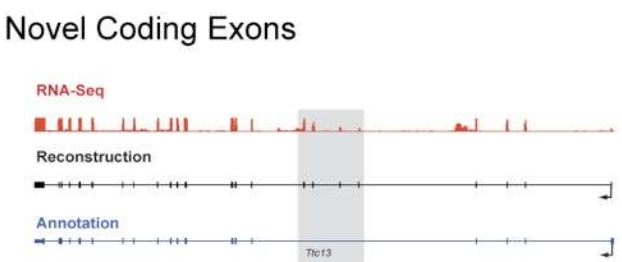
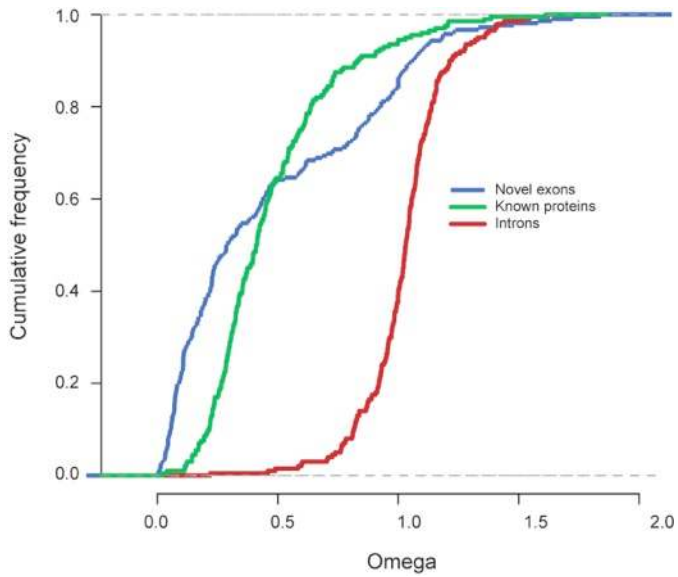




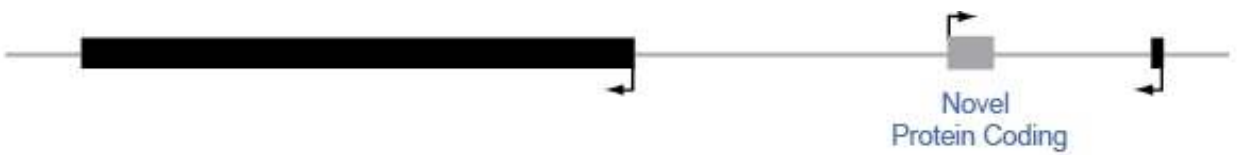
~80% retain ORF

What about novel genes?

Class 1: Overlapping ncRNA

Class 2: Large Intergenic ncRNA (lincRNA)

Class 3: Novel protein-coding genes



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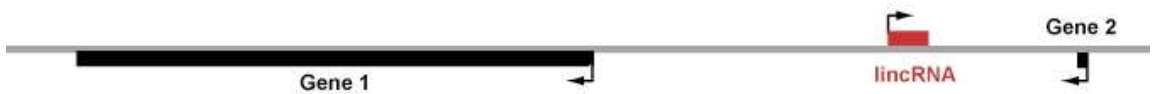
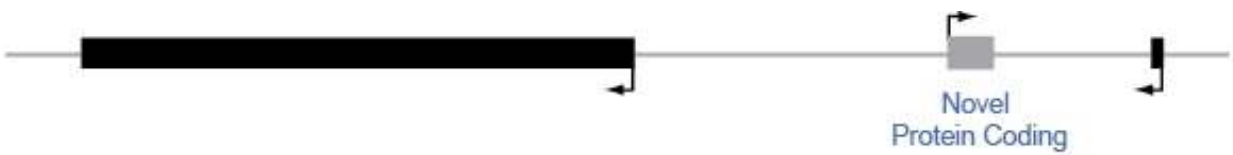
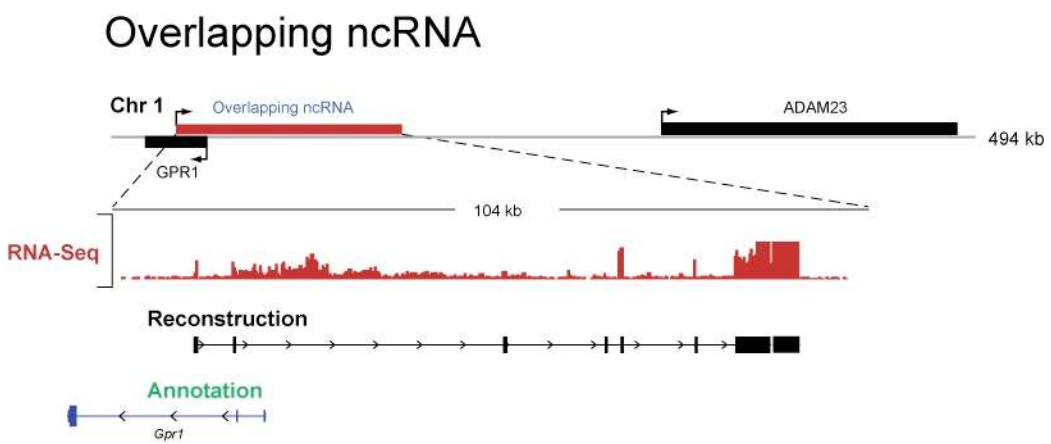


Class 3: Novel protein-coding genes



Class 1: Overlapping ncRNA

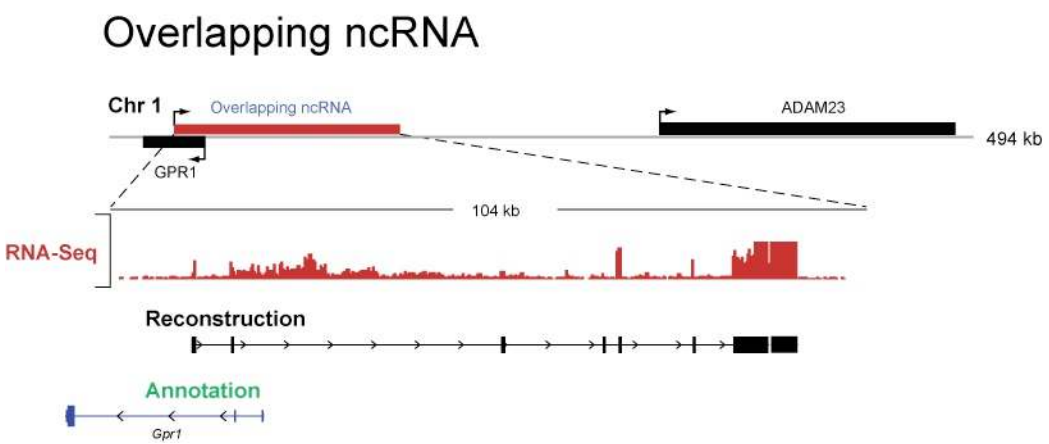


ES cells

3 cell types

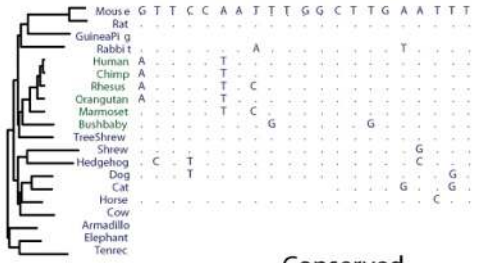
201

Class 1: Overlapping ncRNA

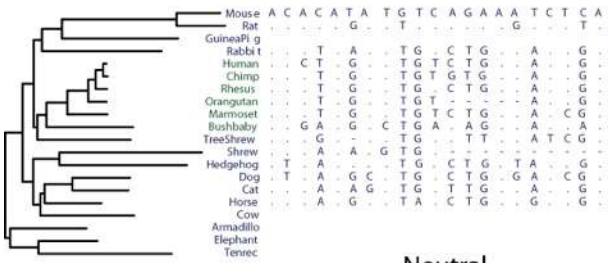


ES cells	3 cell types
201	**446**

Overlapping ncRNAs: Assessing their evolutionary conservation



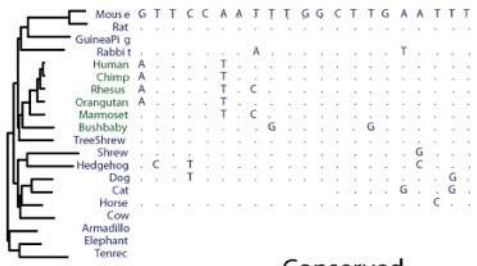
Conserved



Neutral

SiPhy-Garber et al. 2009

Overlapping ncRNAs: Assessing their evolutionary conservation



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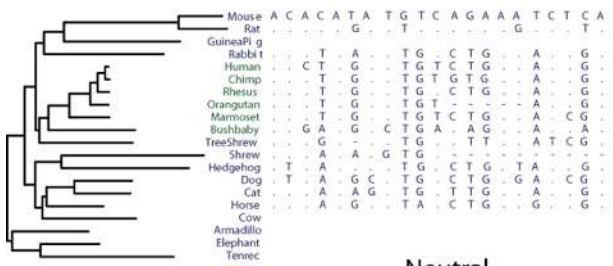
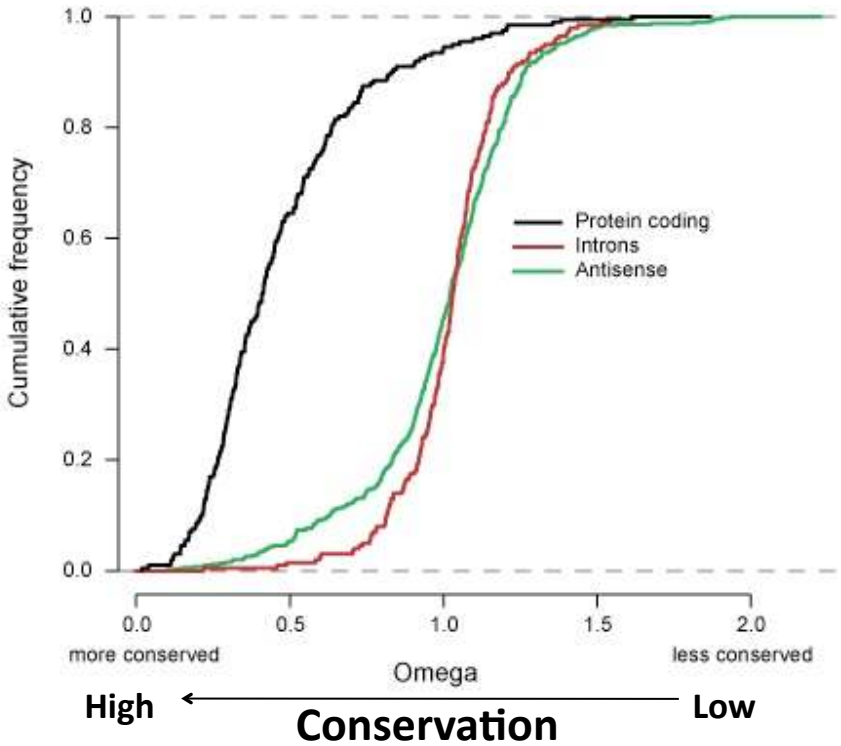


Neutral

SiPhy-Garber et al. 2009



Overlapping ncRNAs show little evolutionary conservation

What about novel genes?

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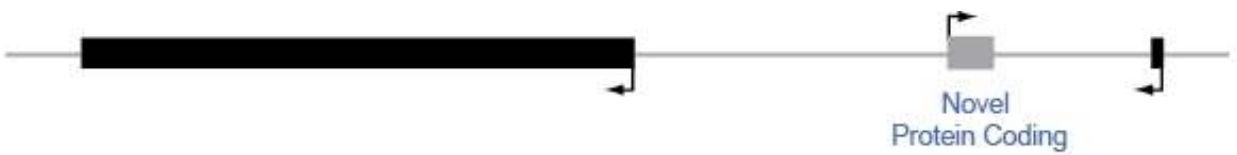

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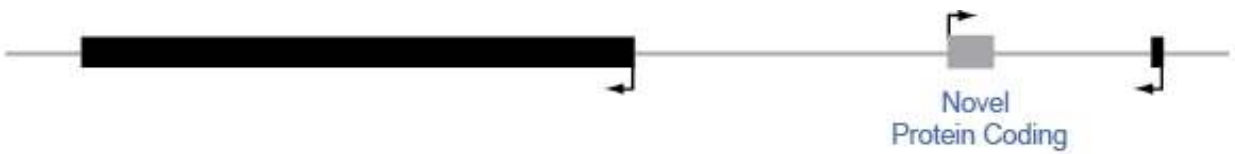
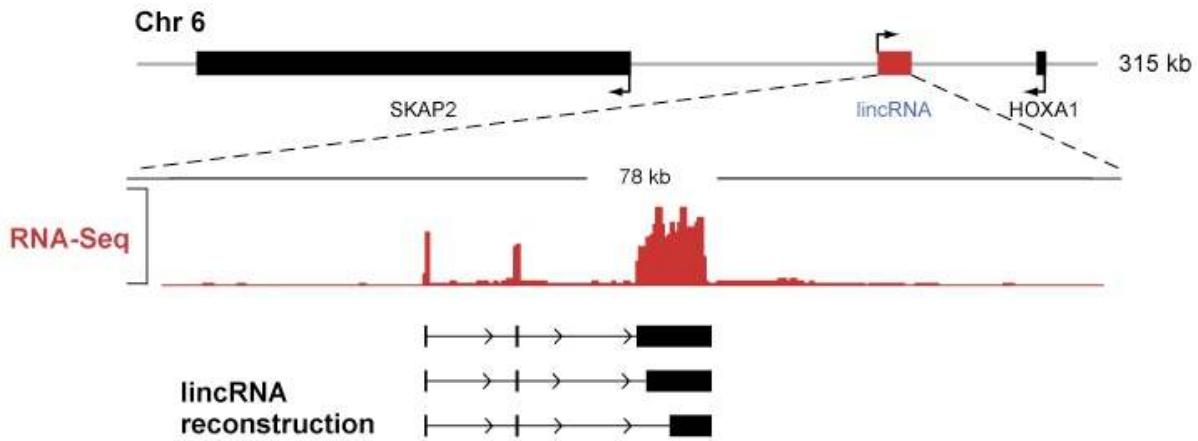


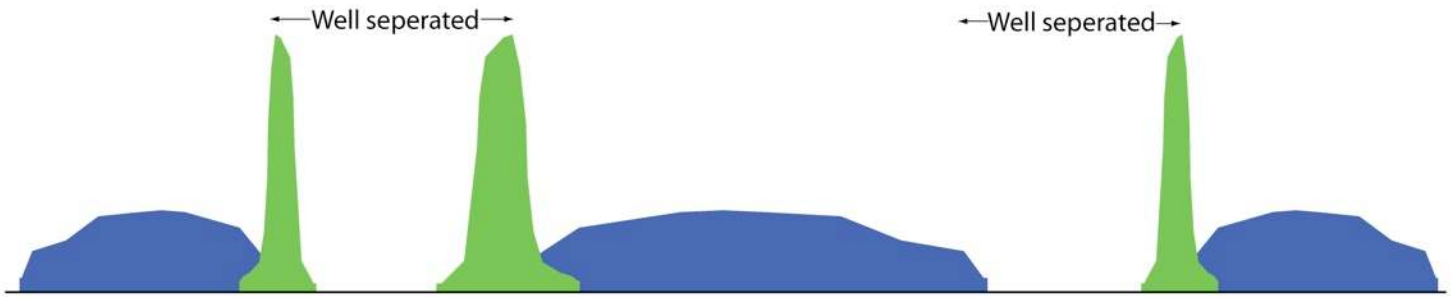
Class 2: Intergenic ncRNA (lincRNA)



ES cells	3 cell types
~500	**~1500**

lincRNAs: Comparison to K4-K36

Chromatin Approach



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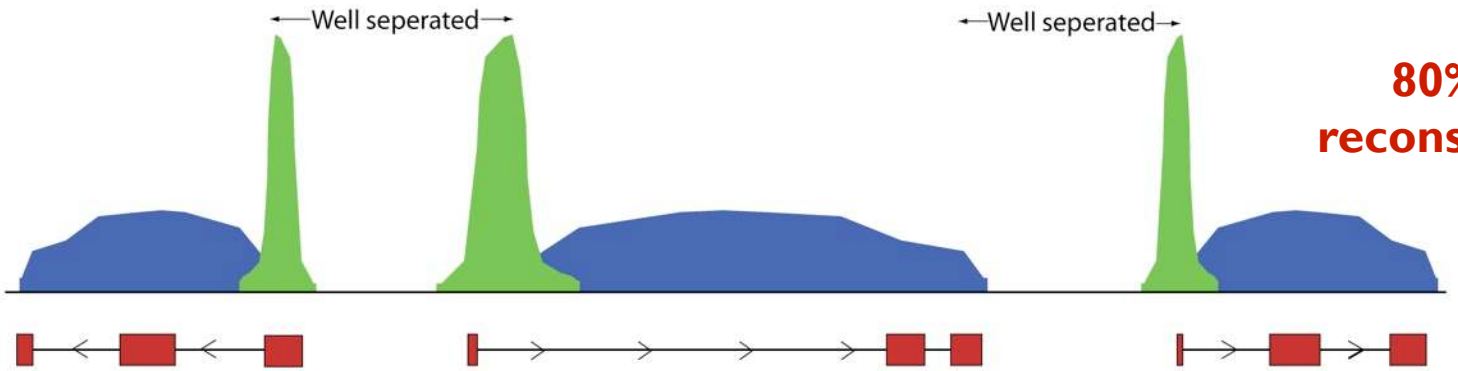
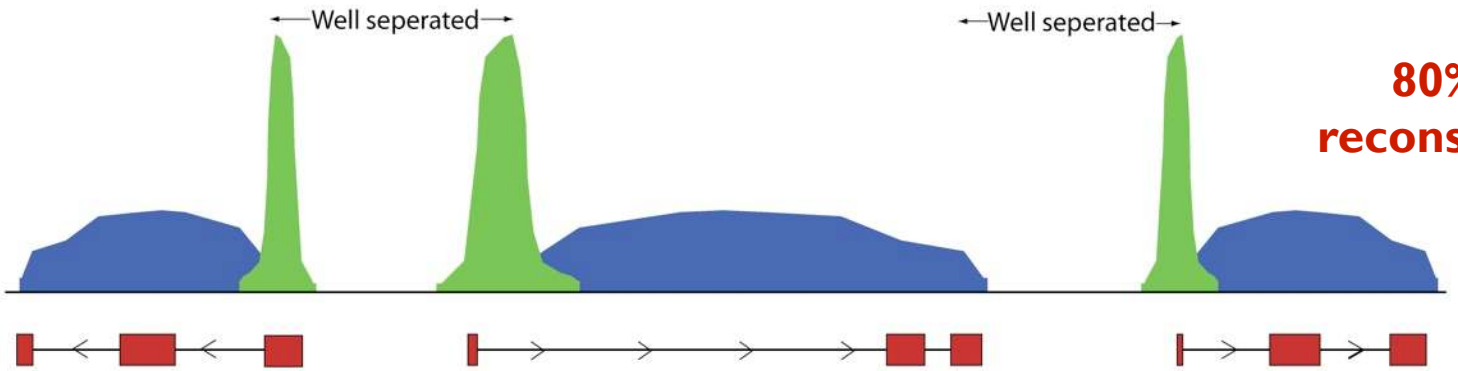


80% have reconstructions

lincRNAs: Comparison to K4-K36

Chromatin Approach

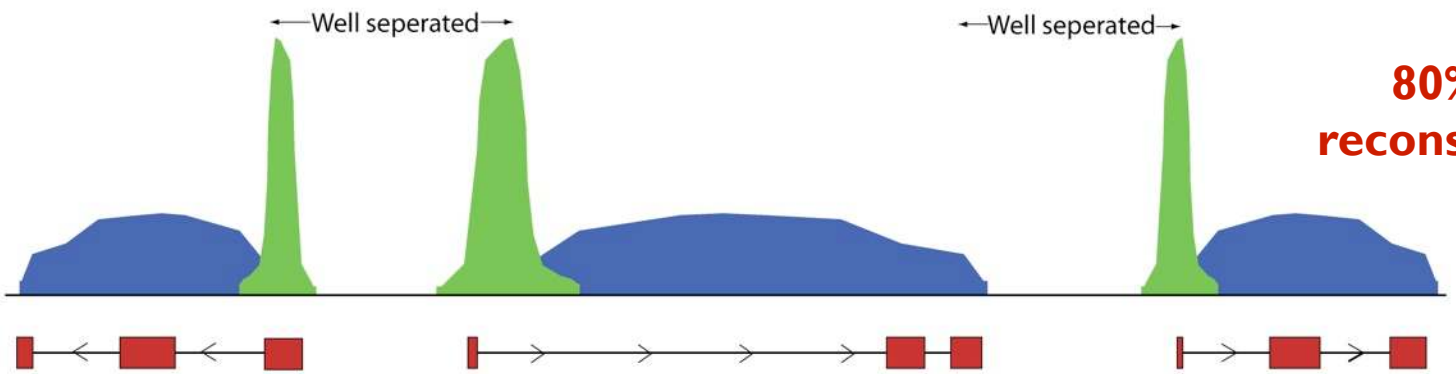


80% have reconstructions

RNA-Seq First Approach

lincRNAs: Comparison to K4-K36

Chromatin Approach



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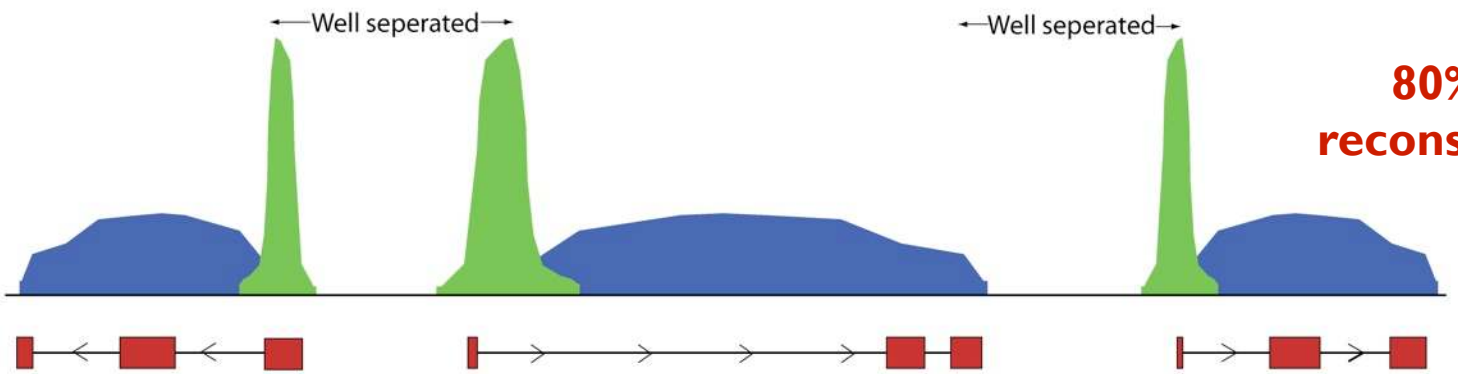
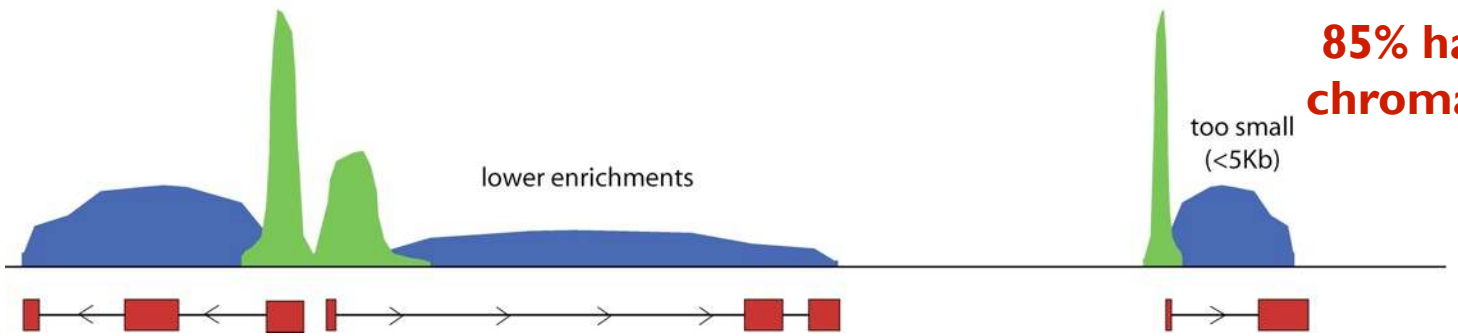


80% have reconstructions

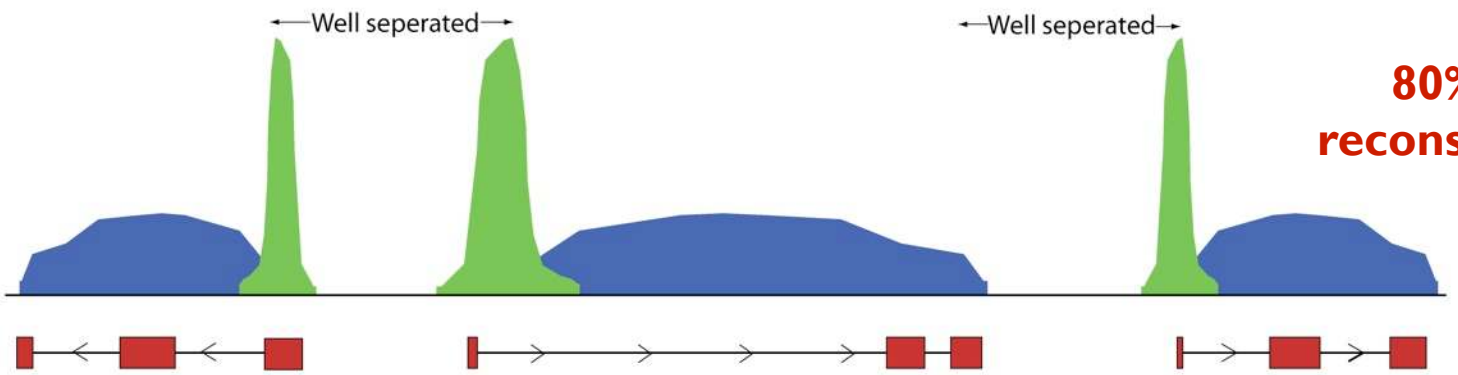
RNA-Seq First Approach



85% have chromatin

lincRNAs: Comparison to K4-K36

Chromatin Approach



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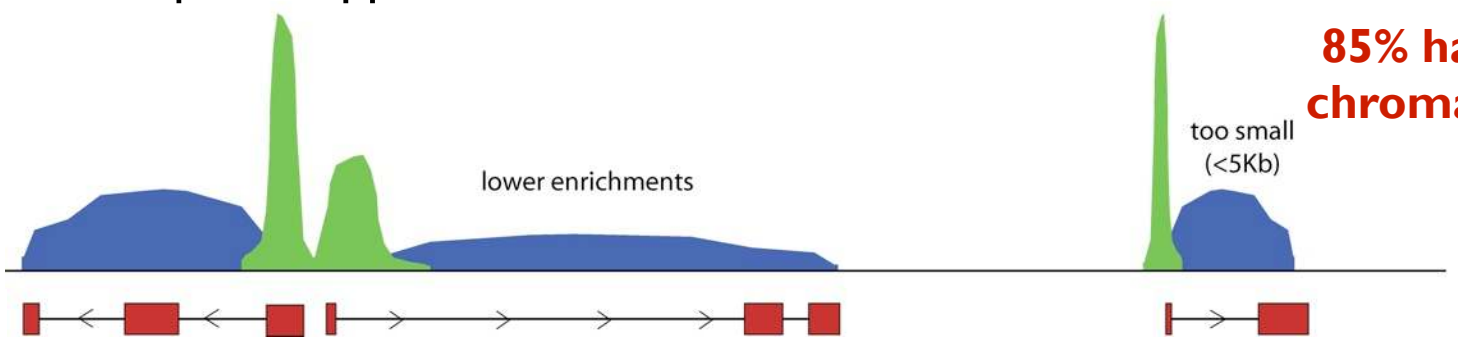
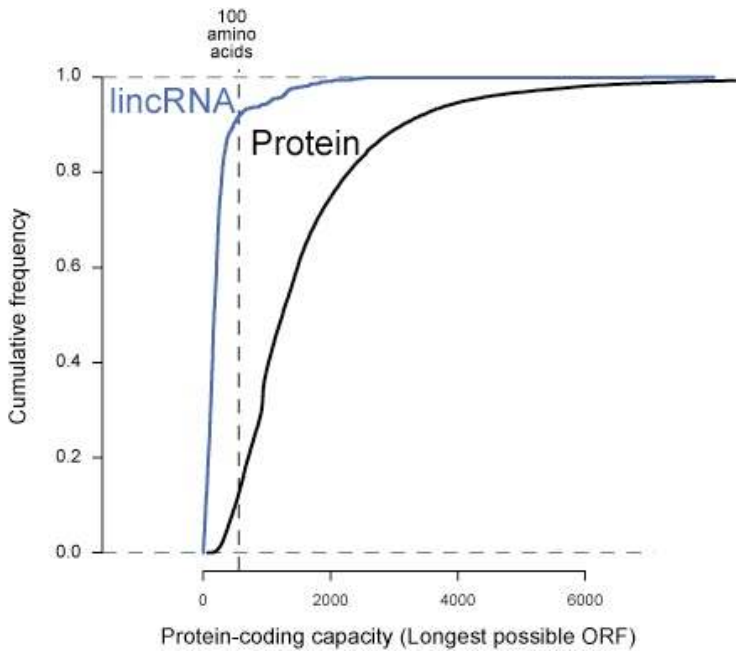


85% have chromatin

RNA-Seq reconstruction and chromatin signature synergize to identify lincRNAs

lincRNAs: How do we know they are non-coding?

ORF Length



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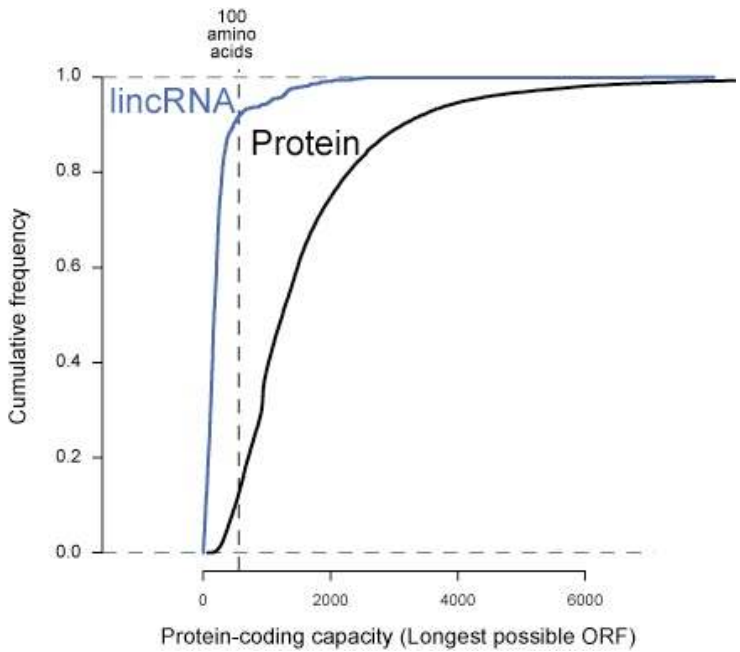
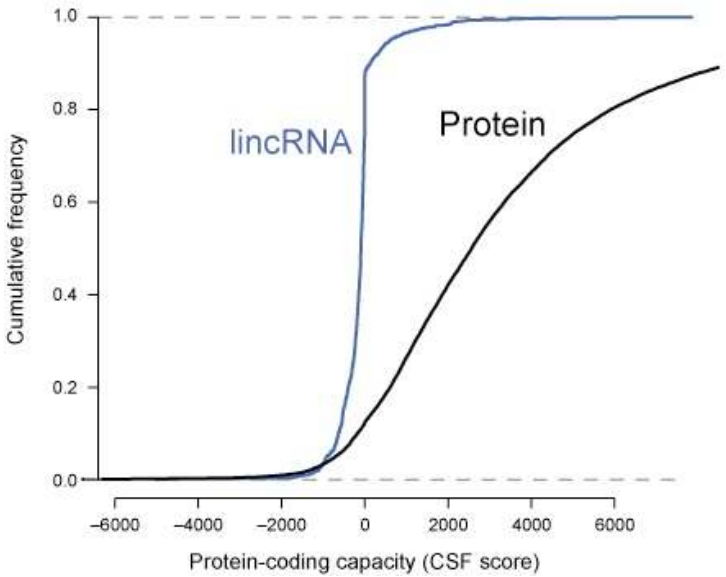


CSF (ORF Conservation)



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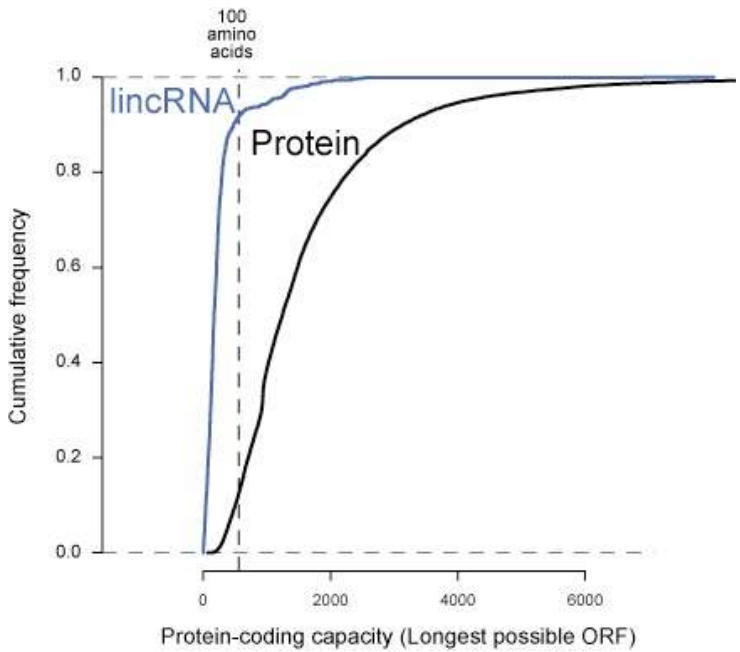

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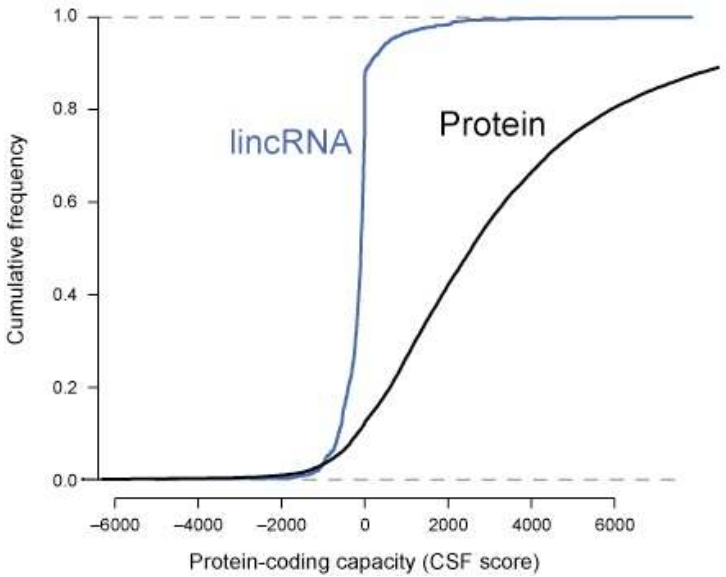
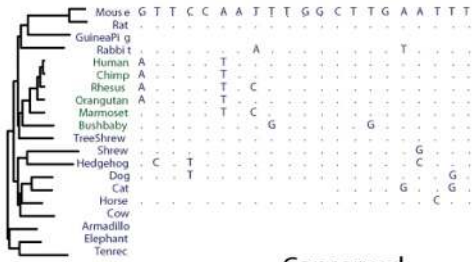


>95% do not encode proteins

lincRNAs: Assessing their evolutionary conservation



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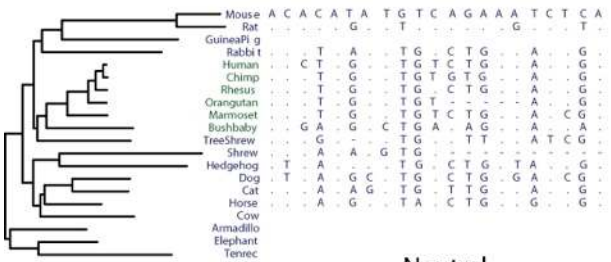
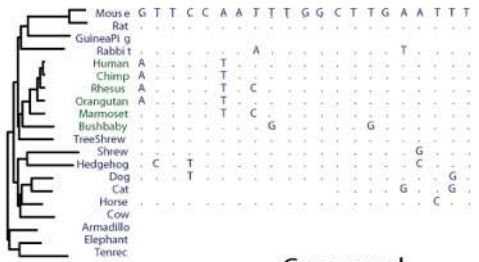


Neutral

lincRNAs: Assessing their evolutionary conservation



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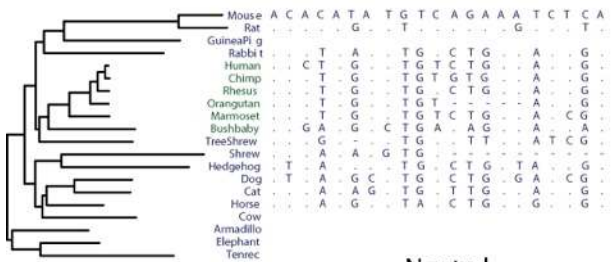

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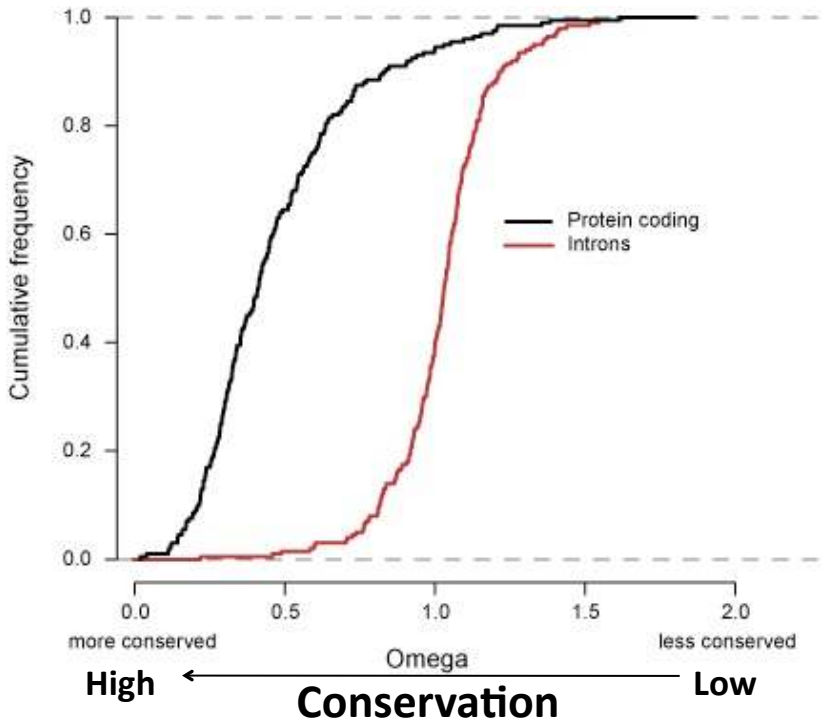
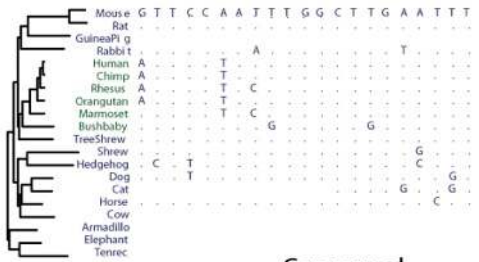


lincRNAs: Assessing their evolutionary conservation



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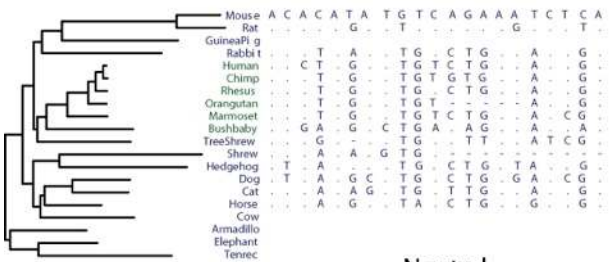

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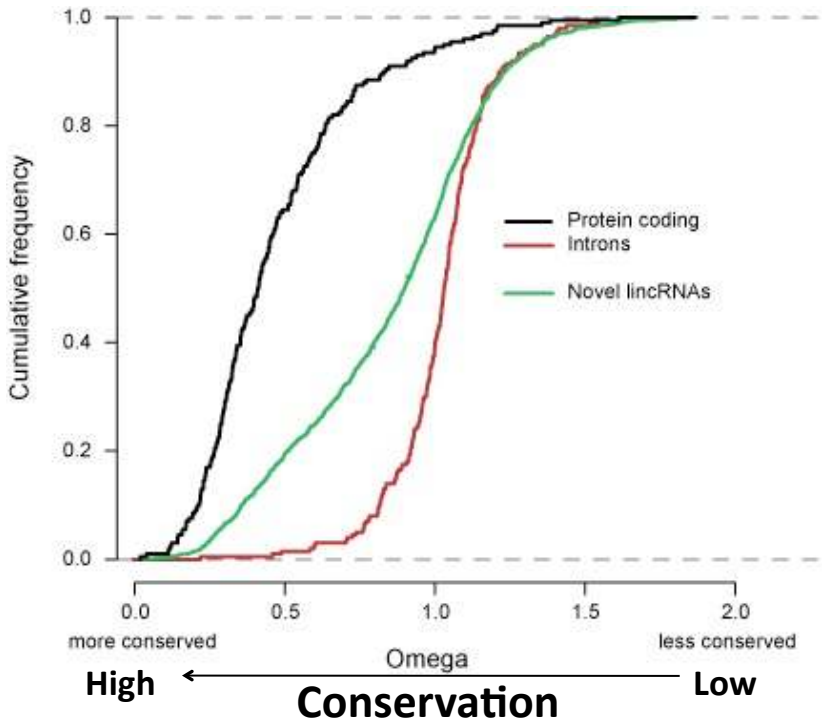

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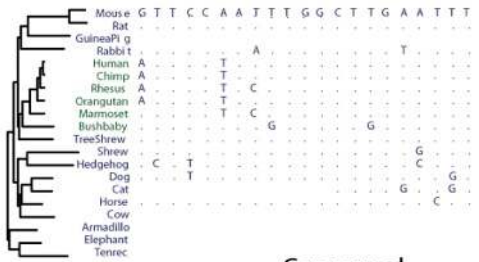

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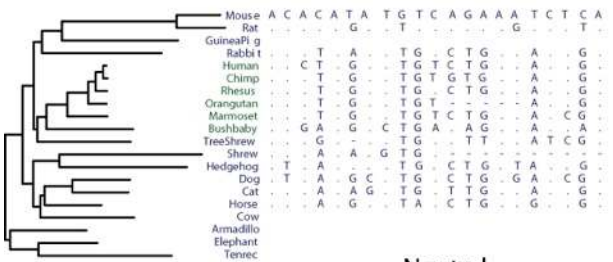

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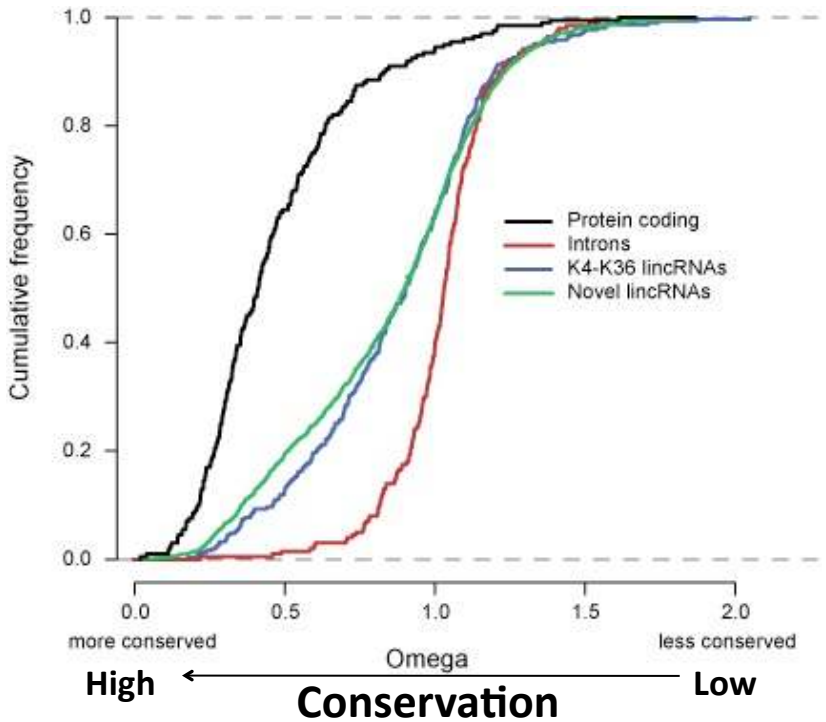

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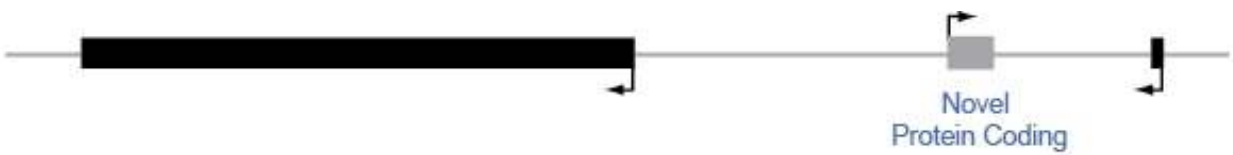
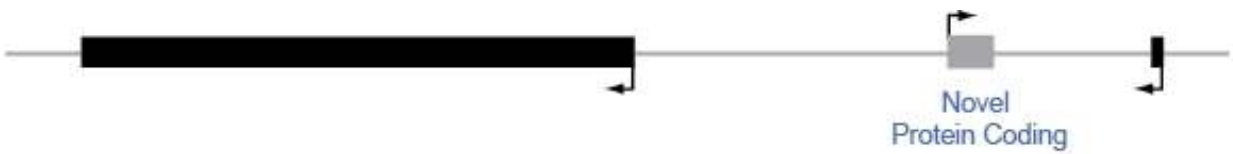


What about novel coding genes?

Class 1: Overlapping ncRNA

Class 2: Large Intergenic ncRNA (lincRNA)

Class 3: Novel protein-coding genes



Finding novel coding genes

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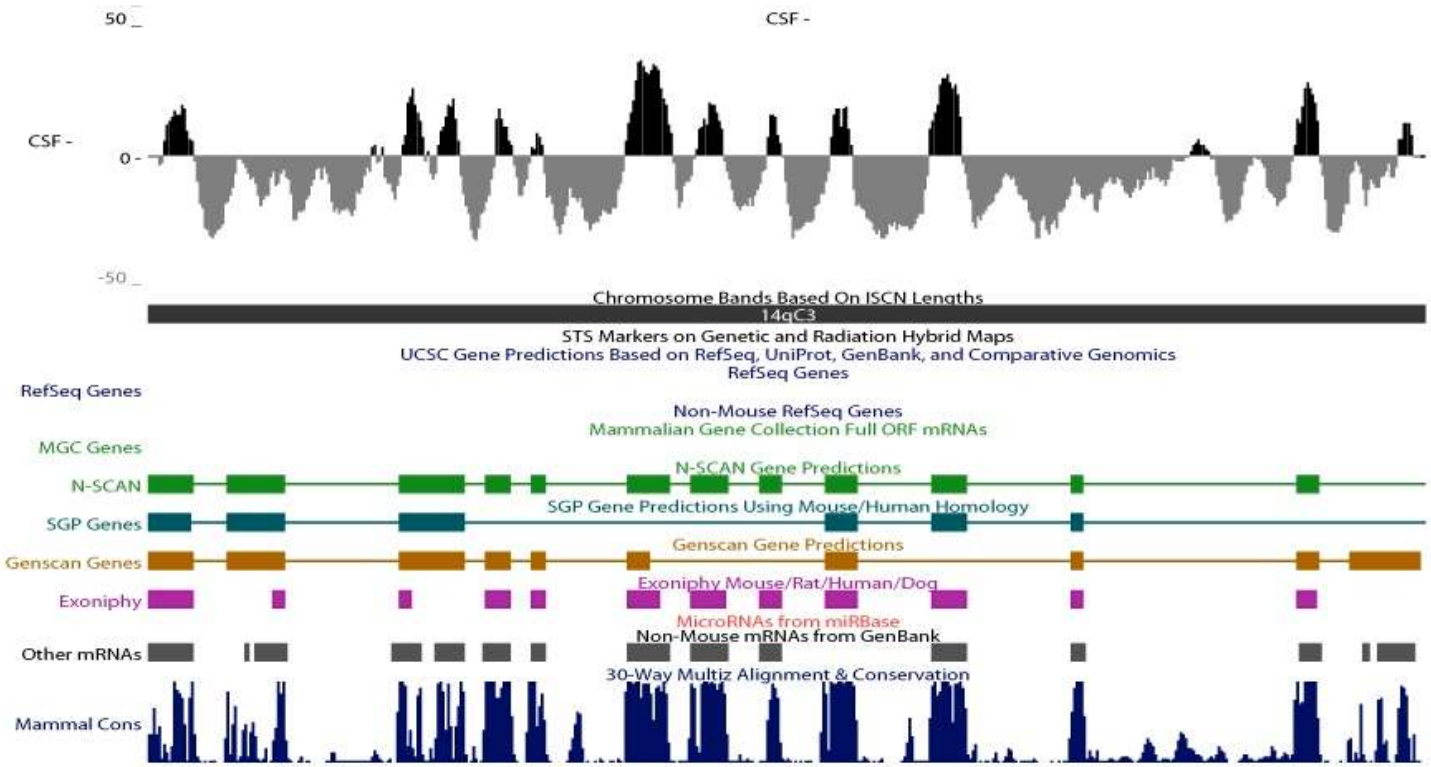

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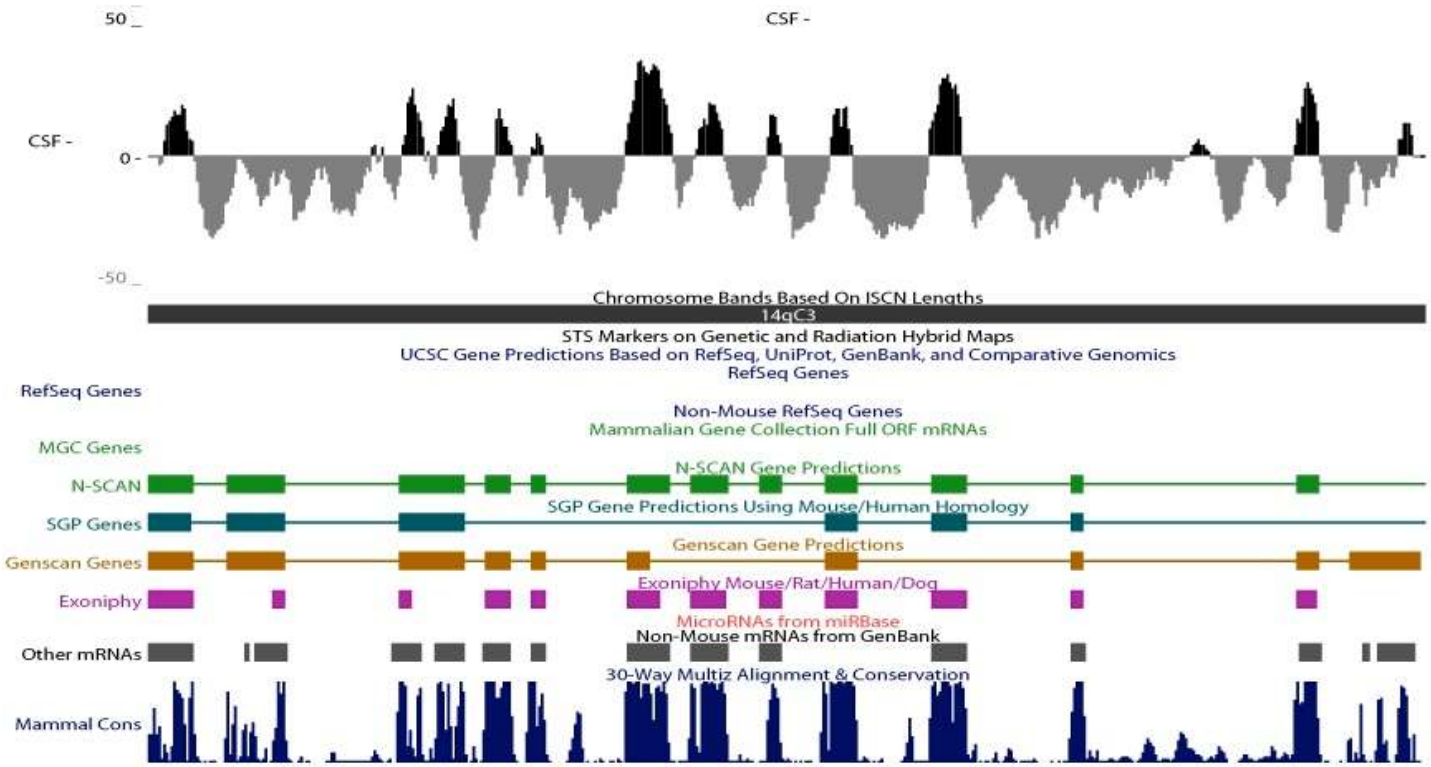

~40 novel protein-coding genes

Other transcript reconstruction methods

Method I: Direct assembly

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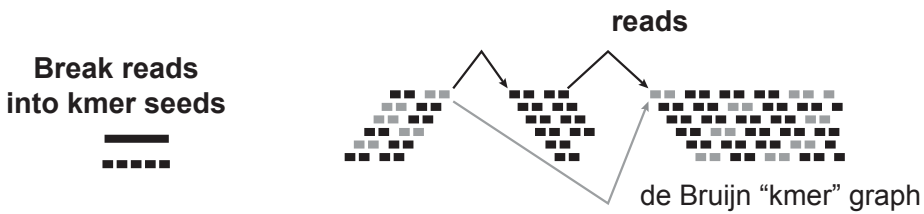

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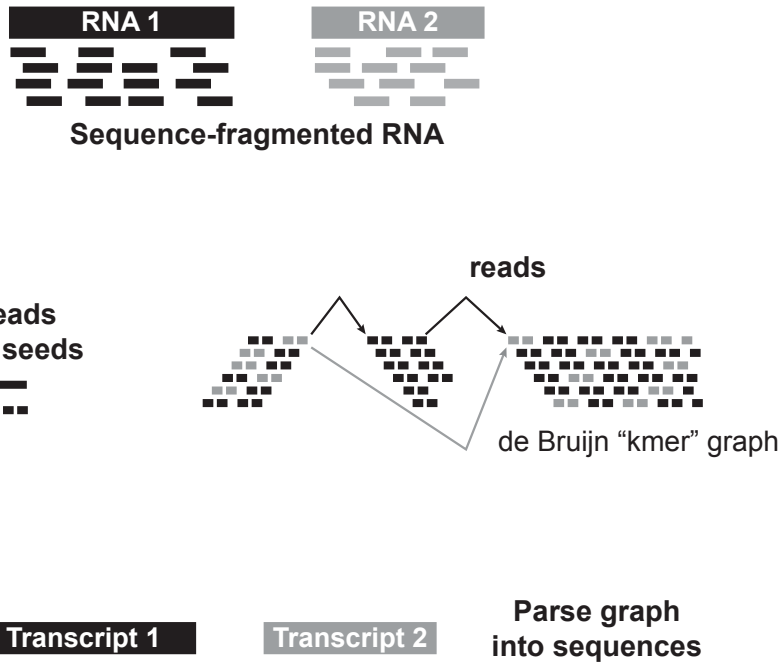
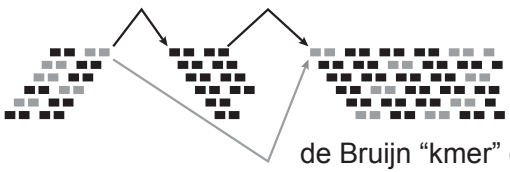


Method I: Direct assembly

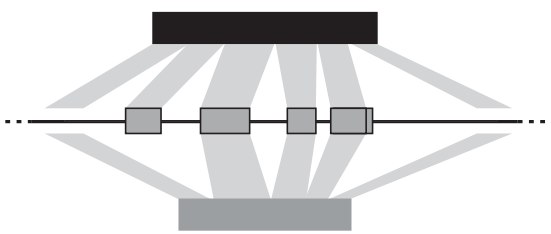
Sequence-fragmented RNA

Break reads into kmer seeds



de Bruijn “kmer” graph

Parse graph into sequences



Align sequences to genome

Method II: Genome-guided

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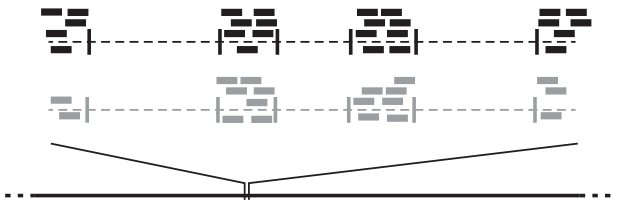
Sequence-fragmented RNA



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Fragments aligned to genome

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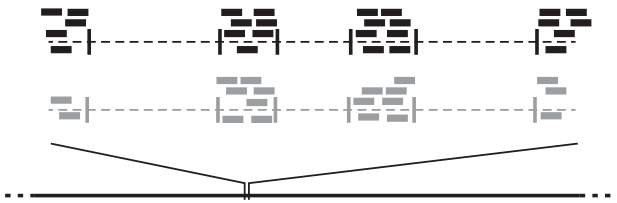

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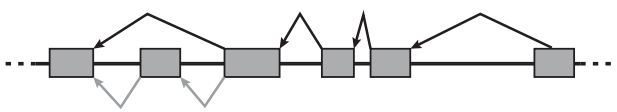

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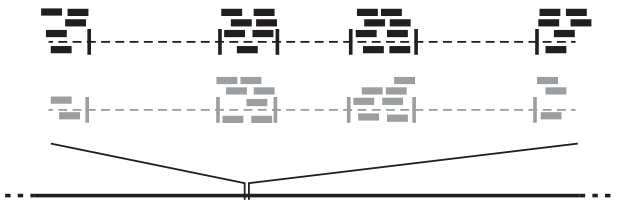

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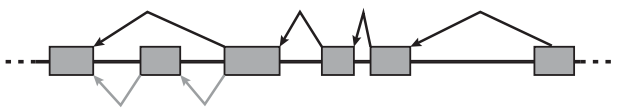

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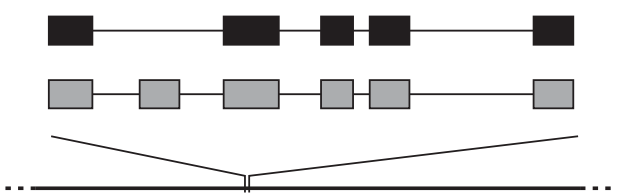
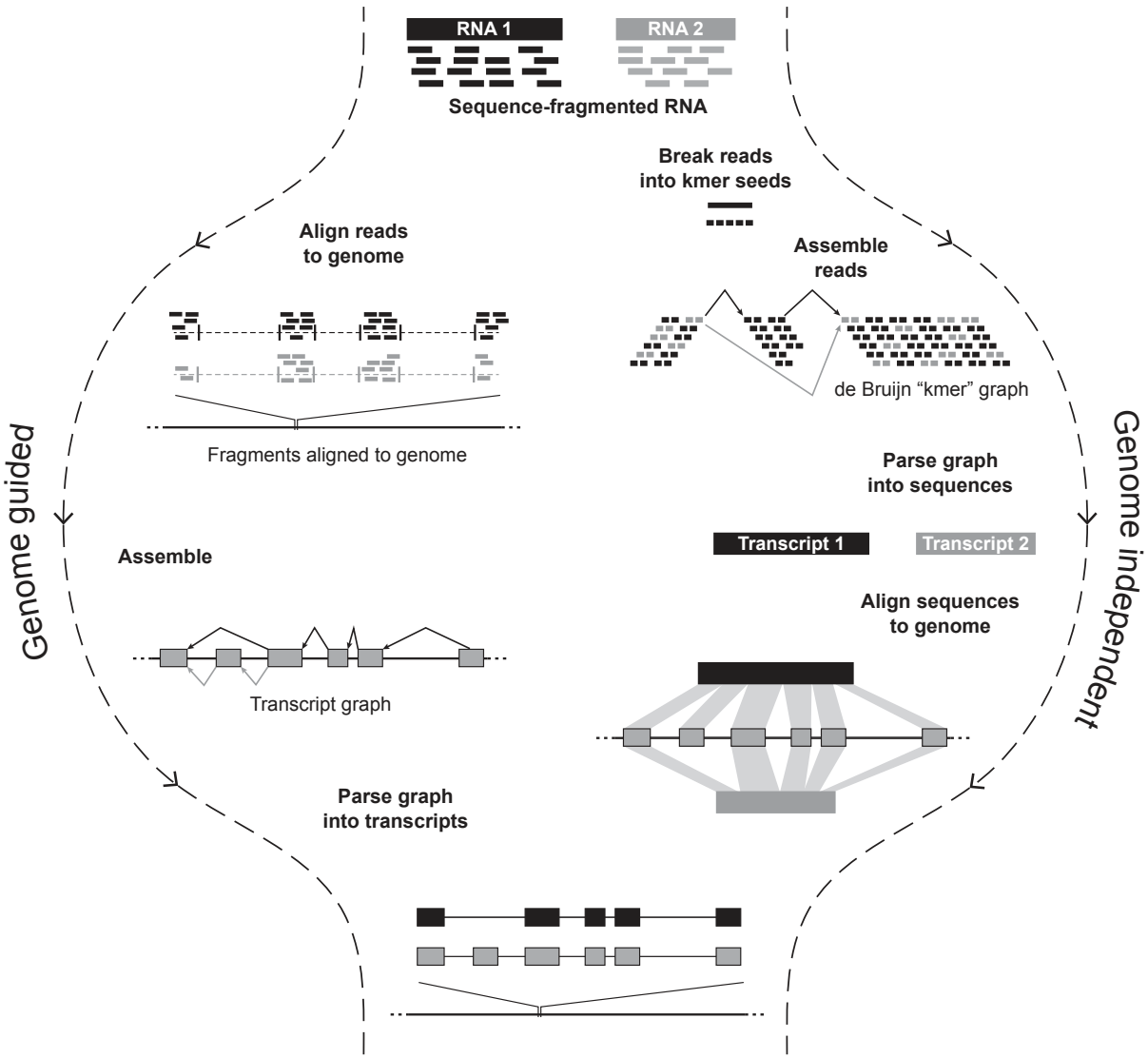


Transcriptome reconstruction method summary



Pros and cons of each approach

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- Hybrid approaches for lesser quality or transcriptomes that underwent major rearrangements, such as in cancer cell.
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- Genome guided methods are very sensitive to alignment artifacts.

RNA-Seq transcript reconstruction software

Assembly	Genome Guided
Oasis	Cufflinks
Trans-ABySS	Scripture
Trinity	

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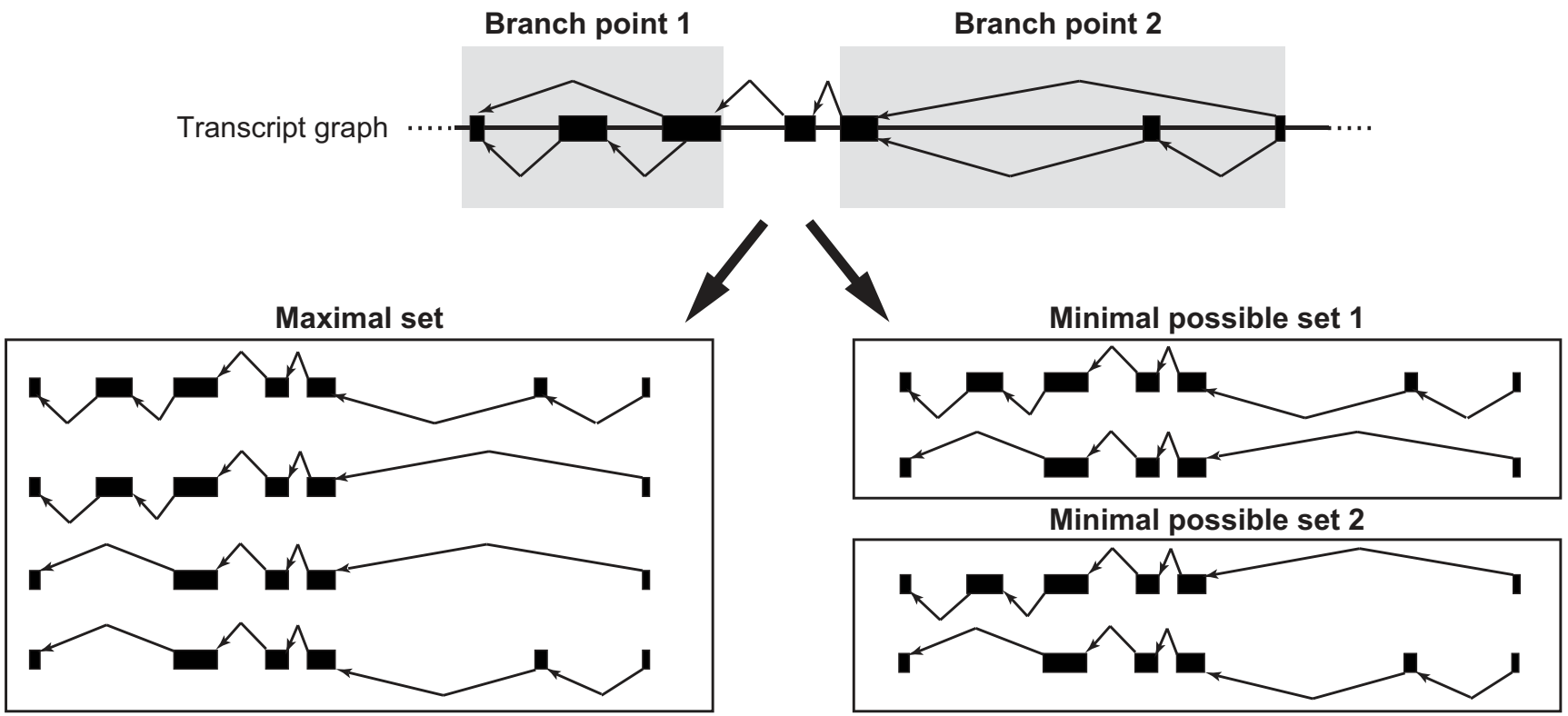
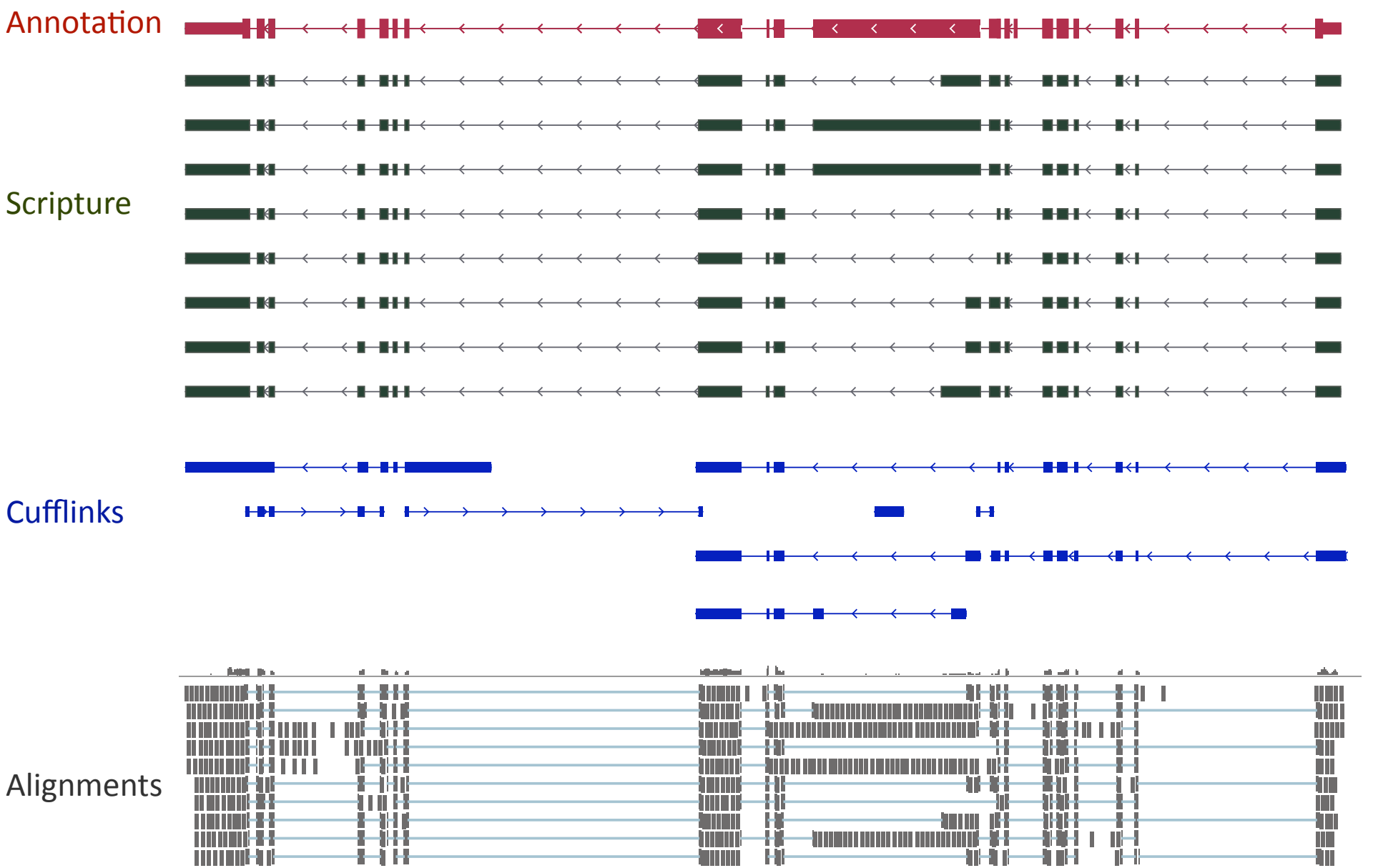
- Cuffllinks was designed with quantification in mind. It limits reported isoforms to the minimal number that explains the data (*Maximum precision*).

Maximum sensitivity vs. maximal precision



Differences between Cufflinks and Scripture – Example



Comparing reconstructions

	CPU Hours Total Memory	Genes fully reconstructed	Mean isoforms per reconstruction	Mean fragments per known annotation	Number of fragments predicted
Cufflinks	10	1.4 G	5,994	1.2	1.4	**159,856**
Scripture	16	3.5 G	6,221	**1.6**	1.3	61,922
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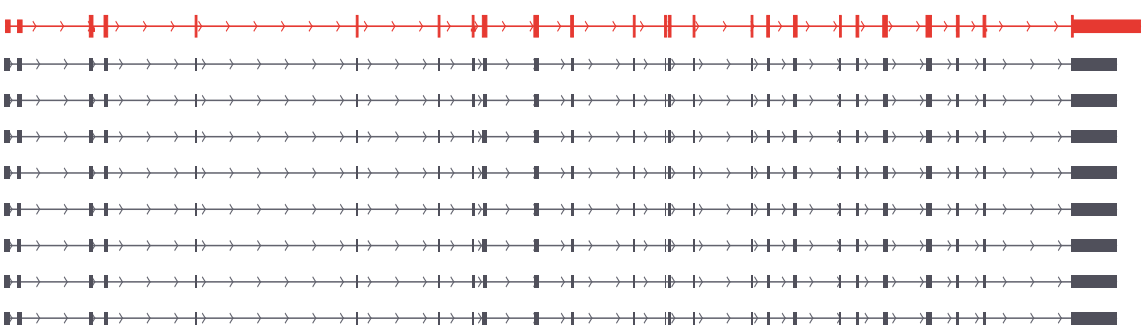
Many of the bogus locus and isoforms are due to alignment artifacts

Why so many isoforms

Annotation

Reconstructions



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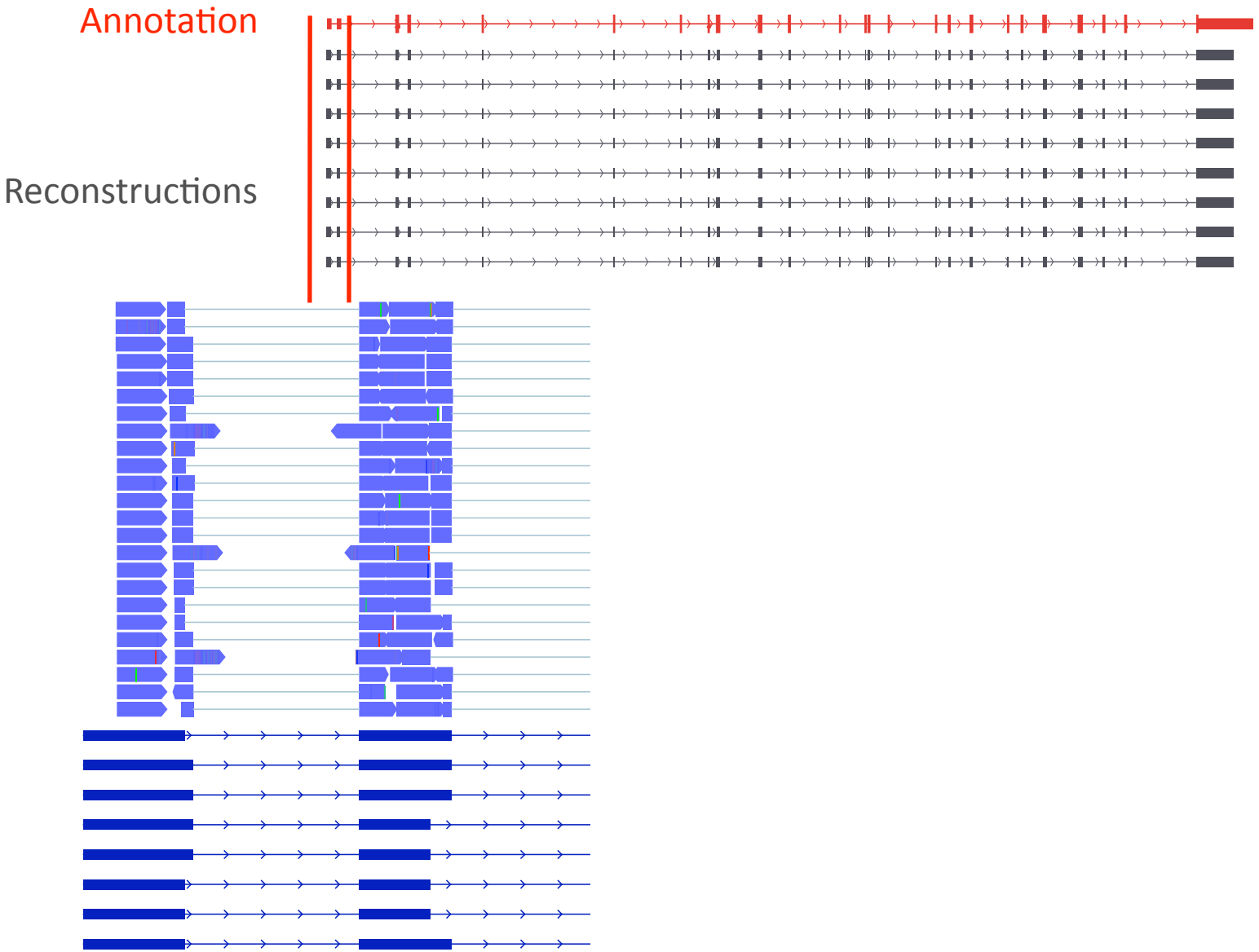

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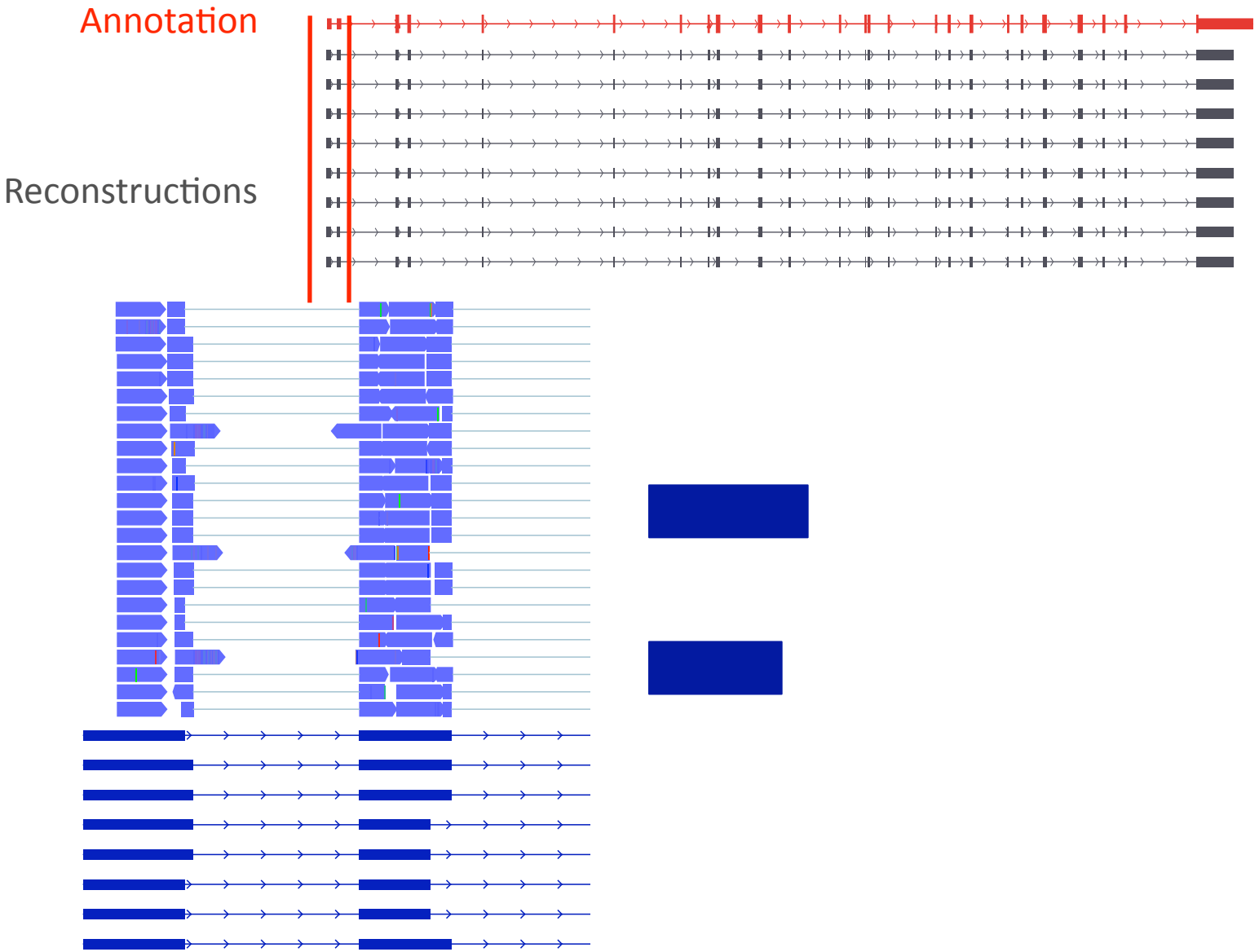

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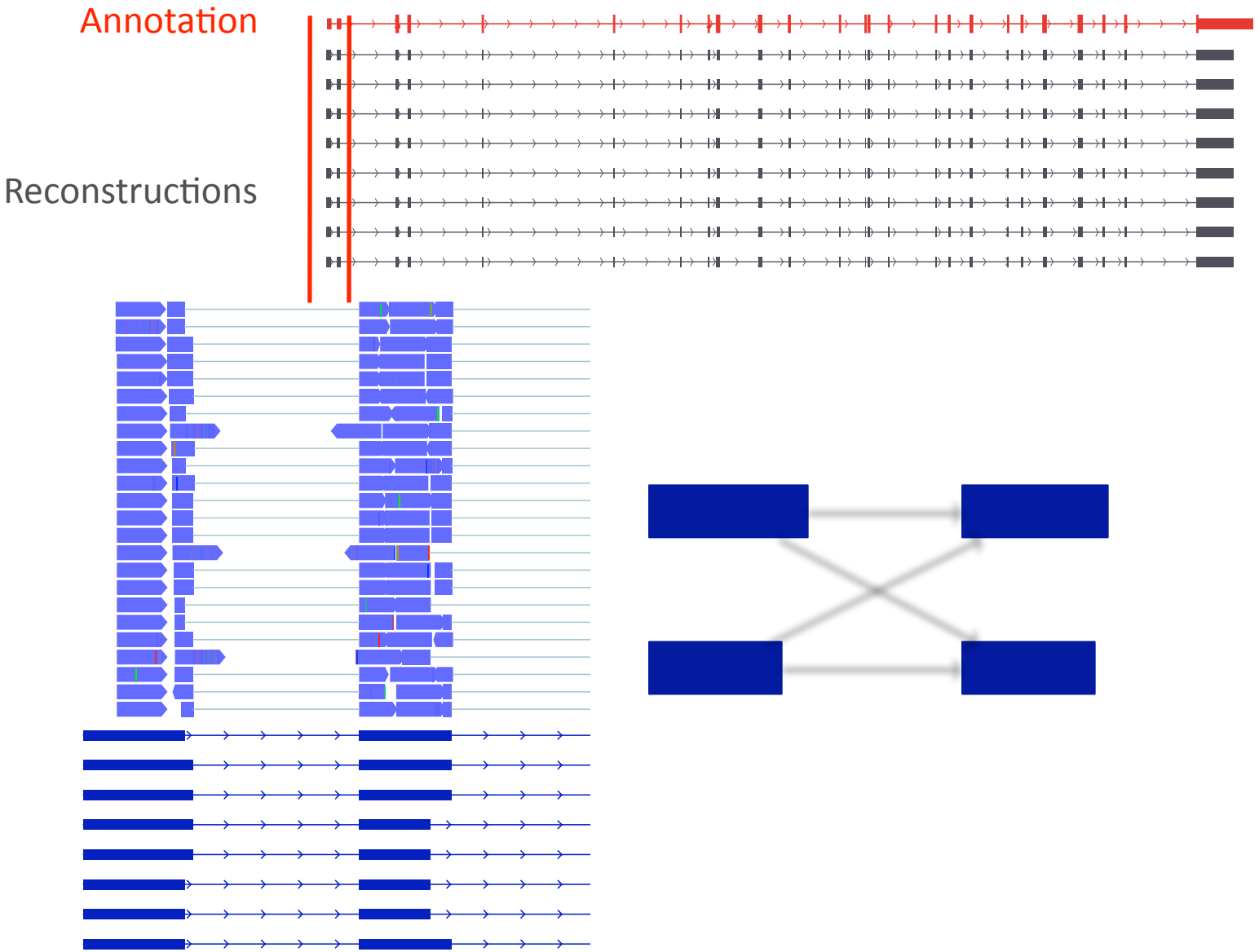

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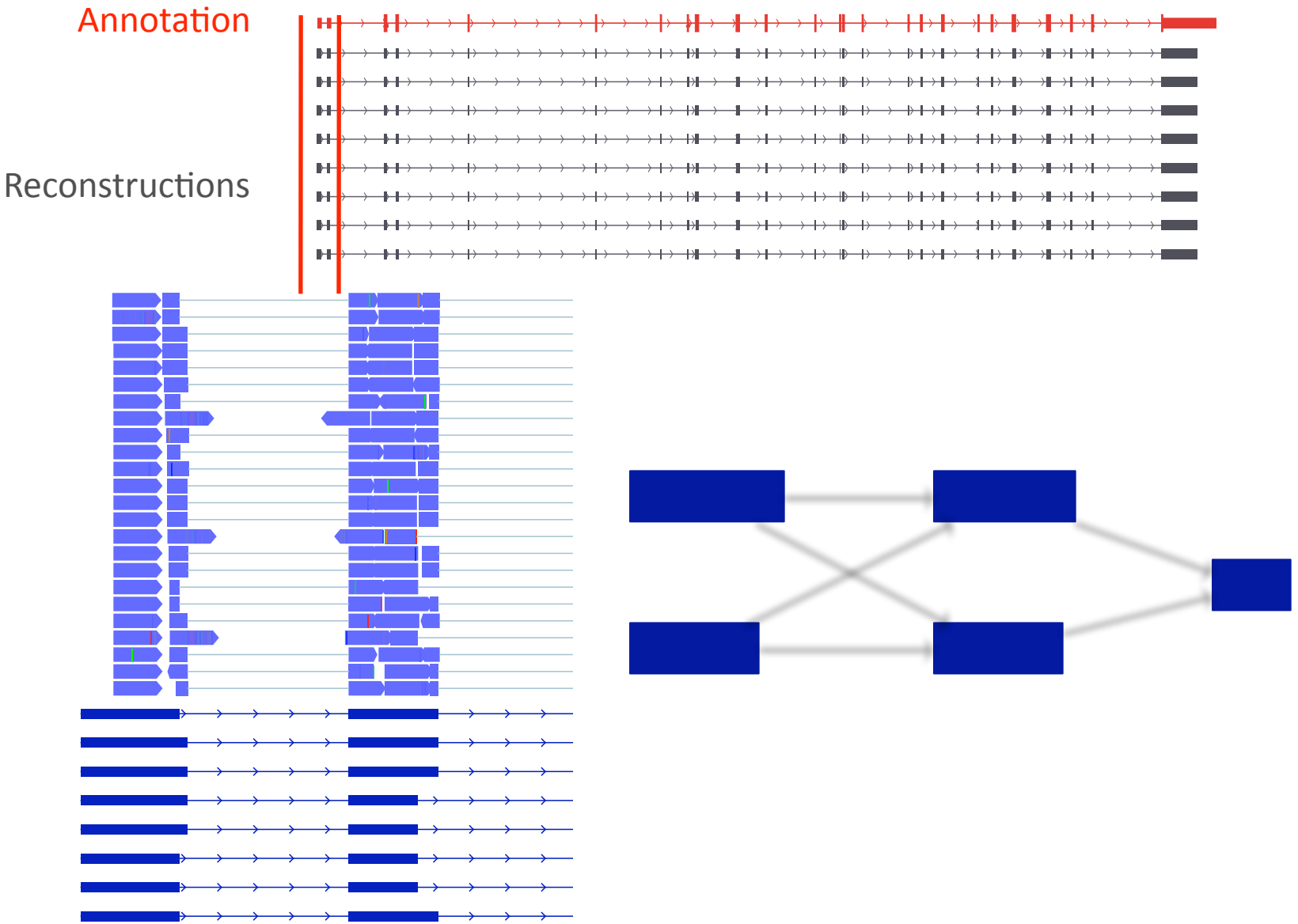

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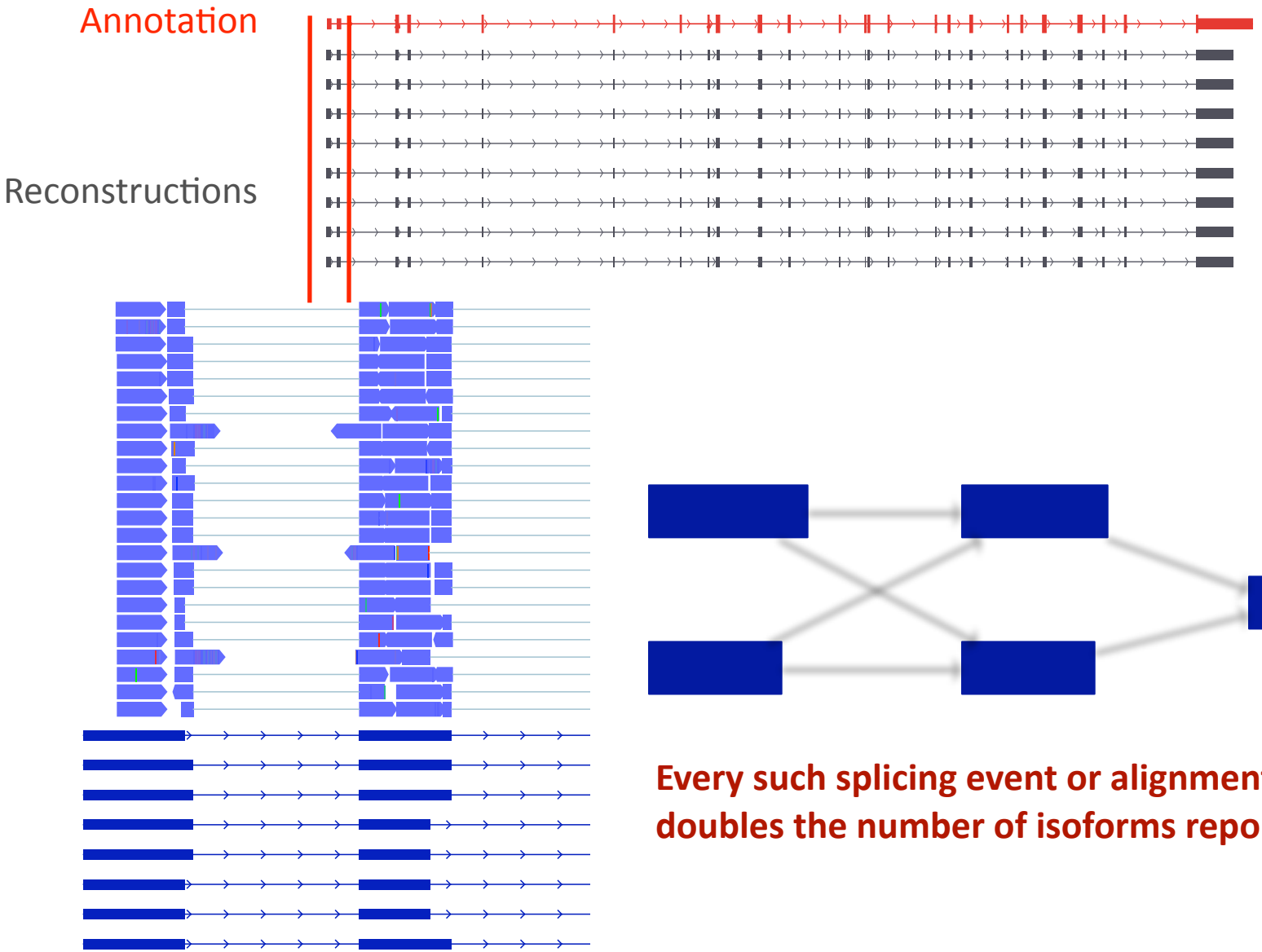
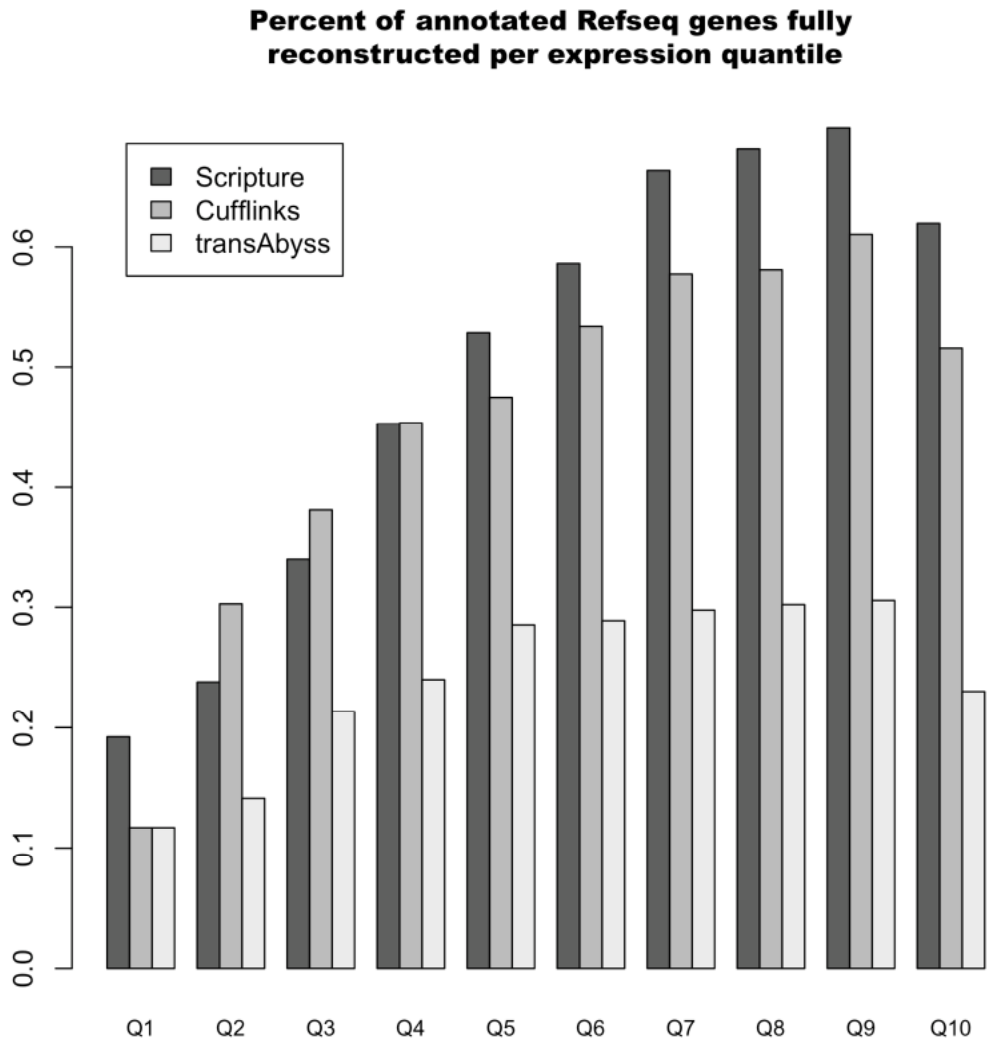


Every such splicing event or alignment artifact doubles the number of isoforms reported

Reconstruction comparison



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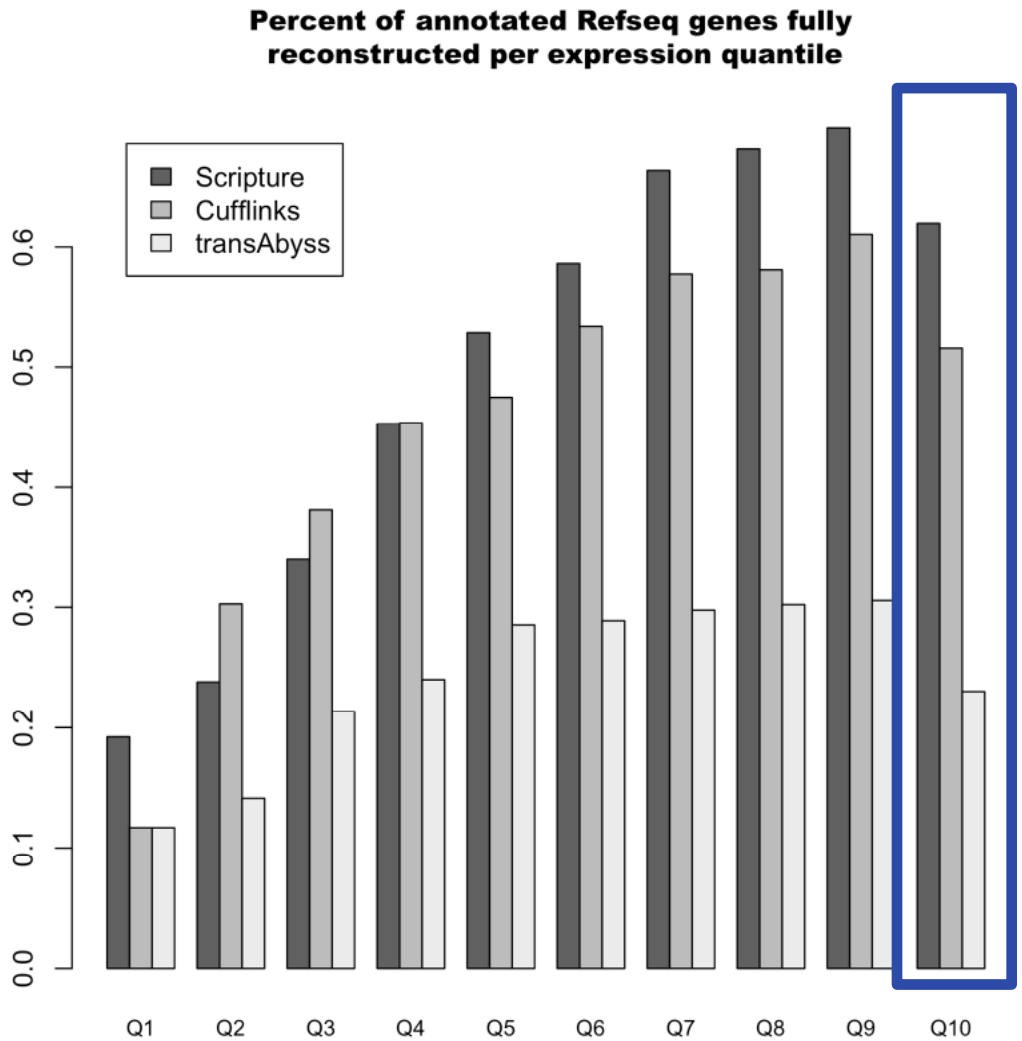
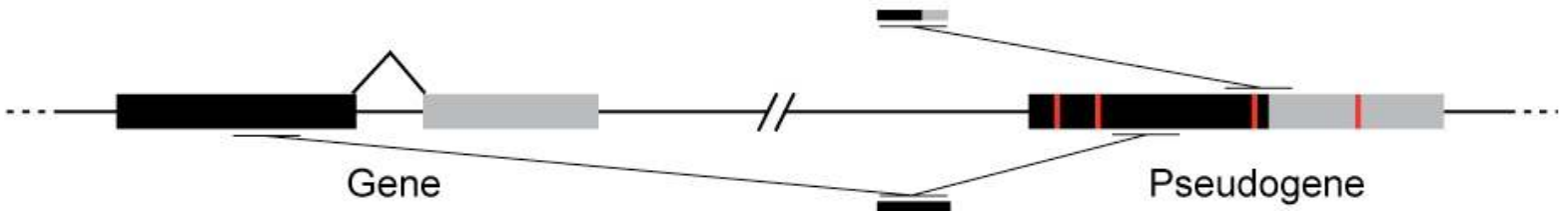


Alignment revisited — spliced alignment is still work in progress

Exon-first aligners are faster but at cost



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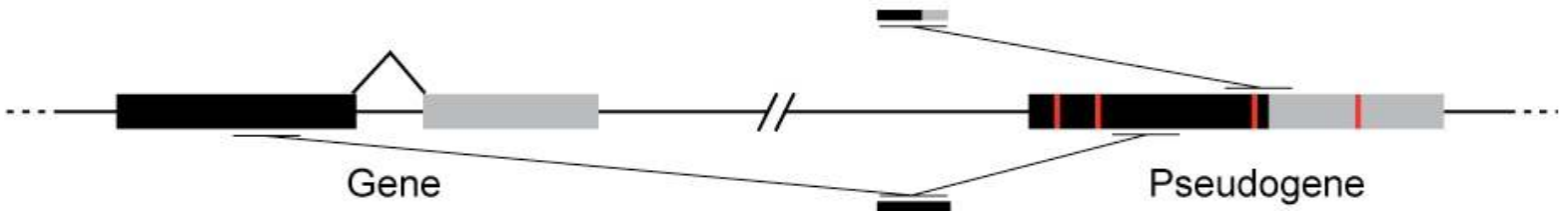
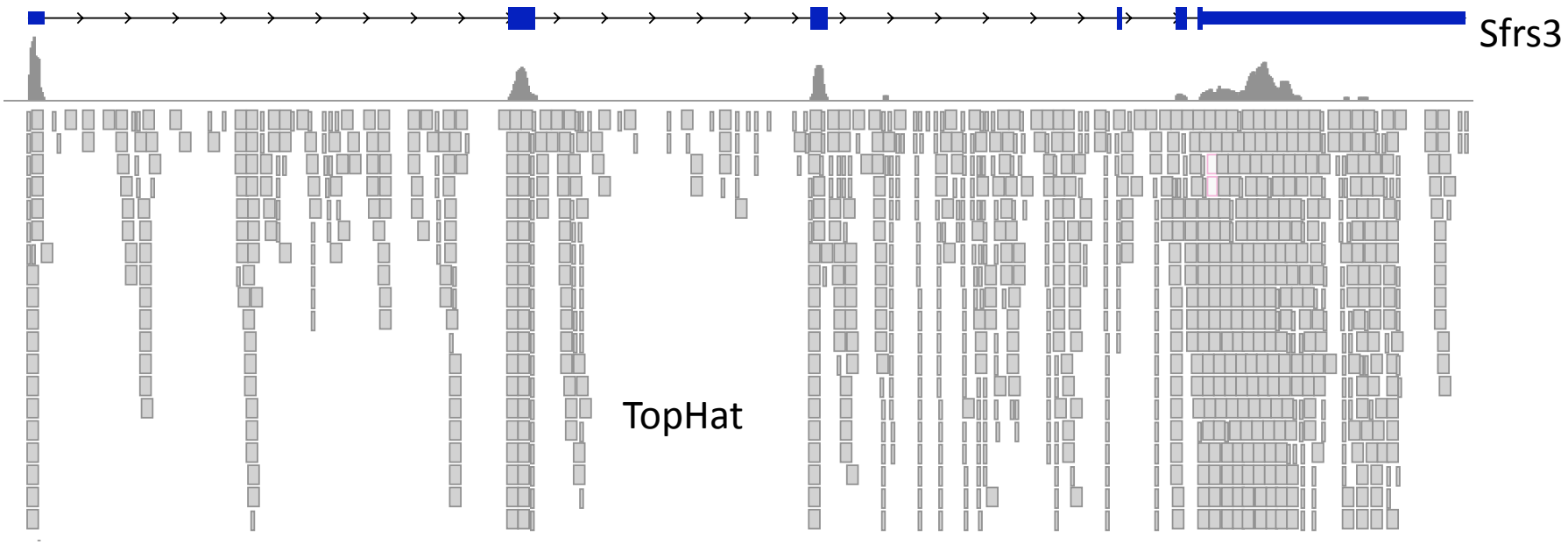


Alignment artifacts can also decrease sensitivity

Missing spliced reads for highly expressed genes



Read mapped uniquely

Read ambiguously mapped

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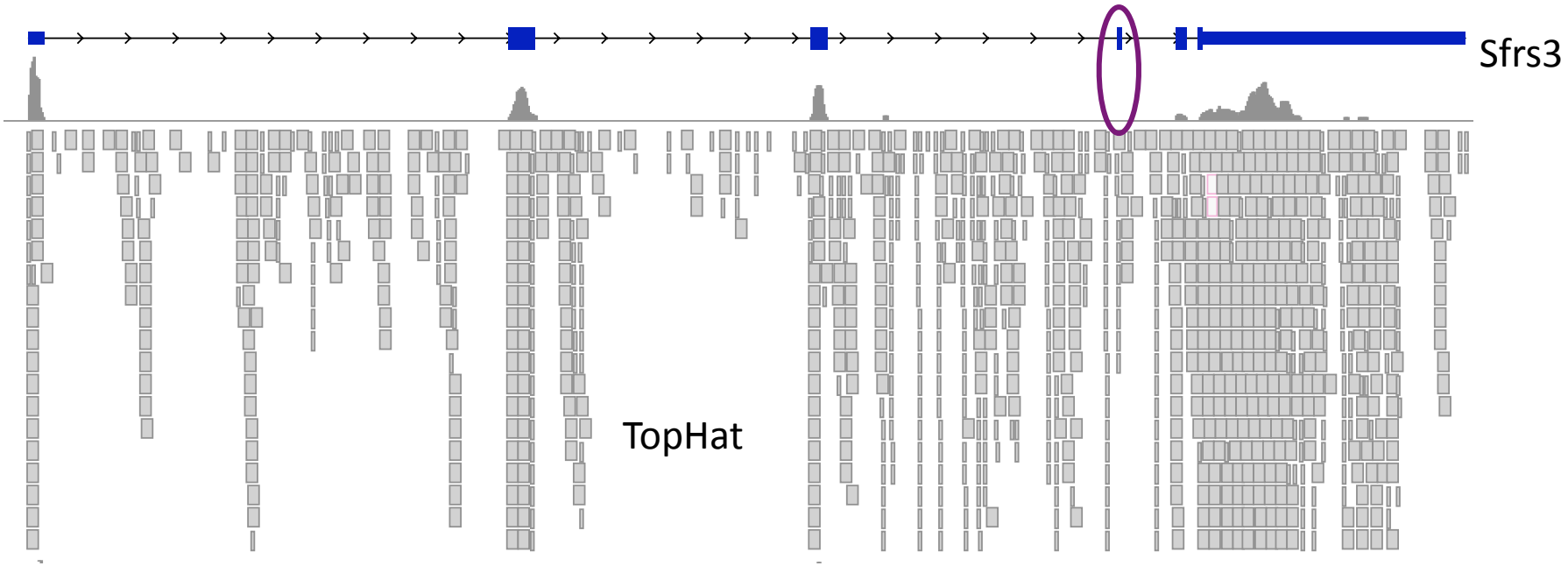

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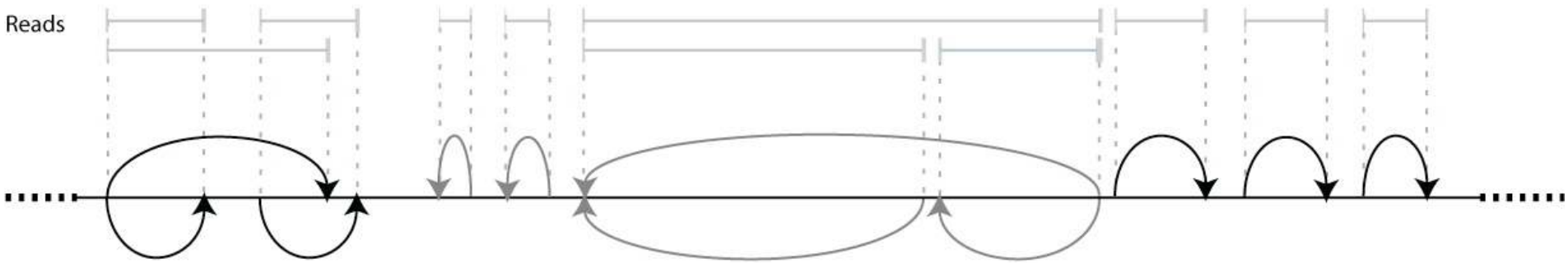
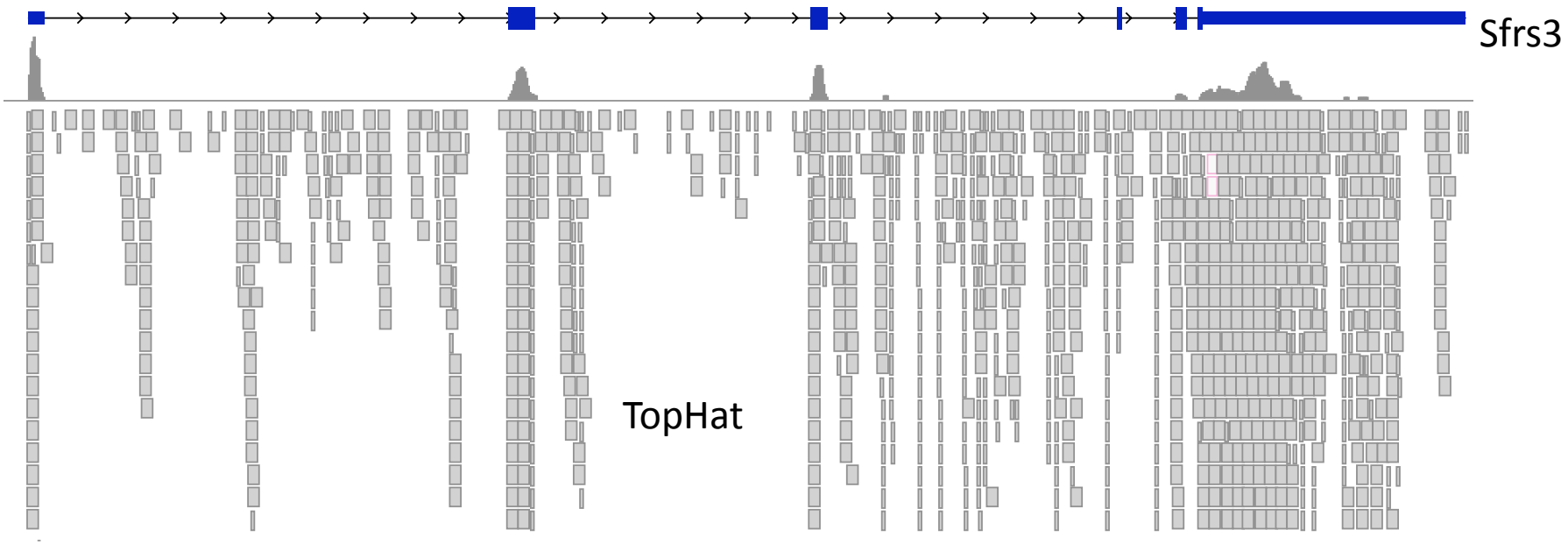


ScriptAlign: Can increase alignment across junctions



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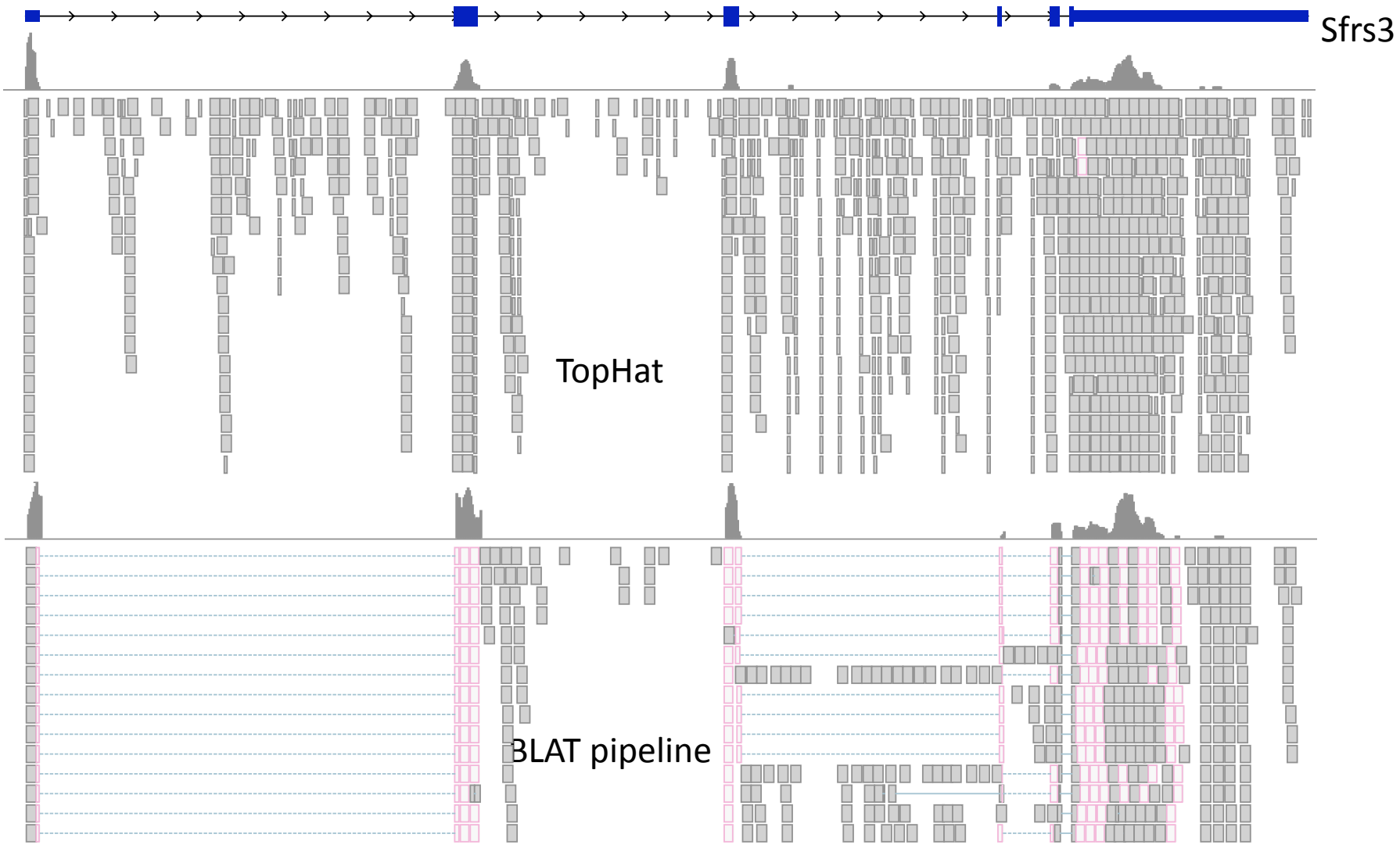

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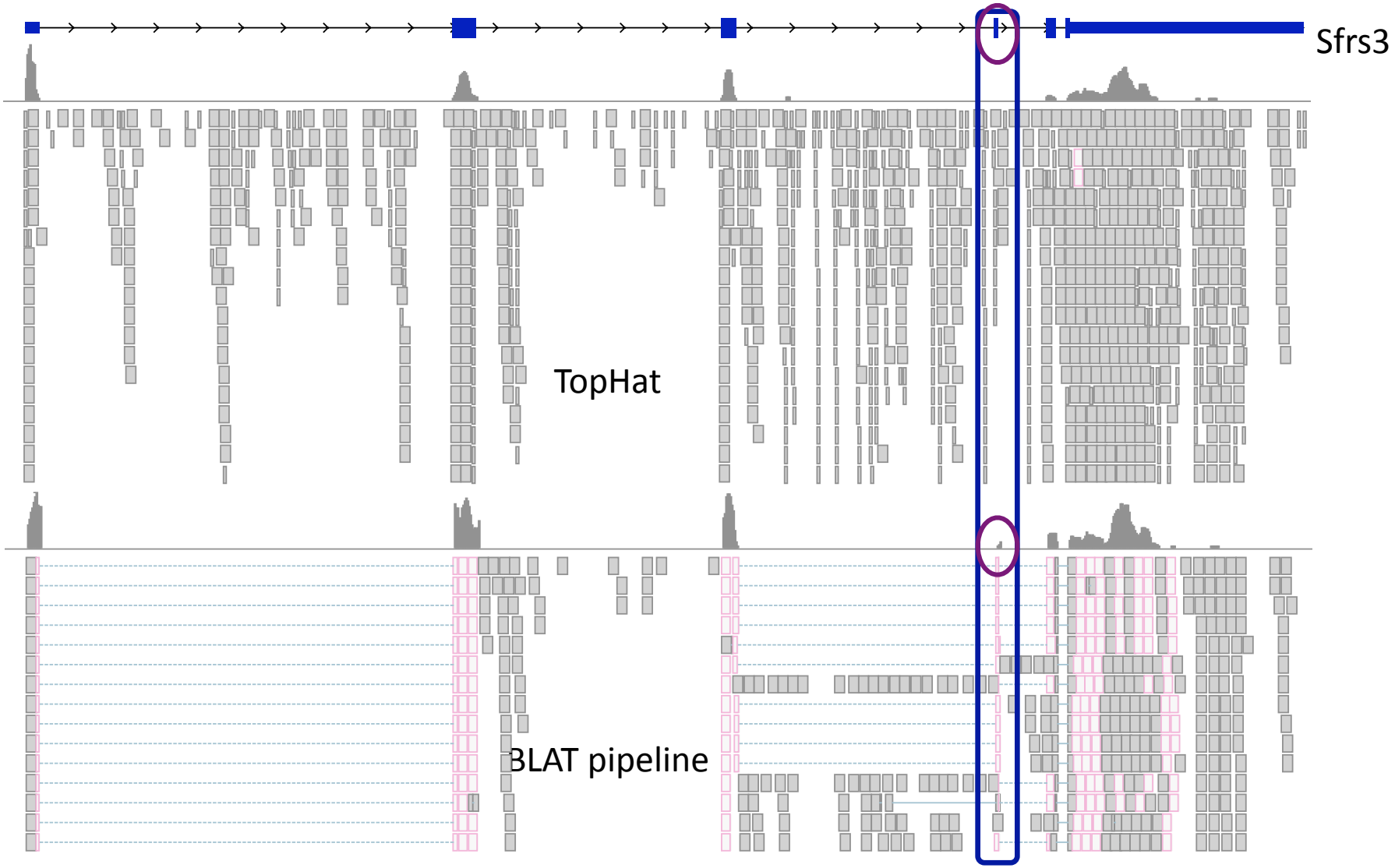

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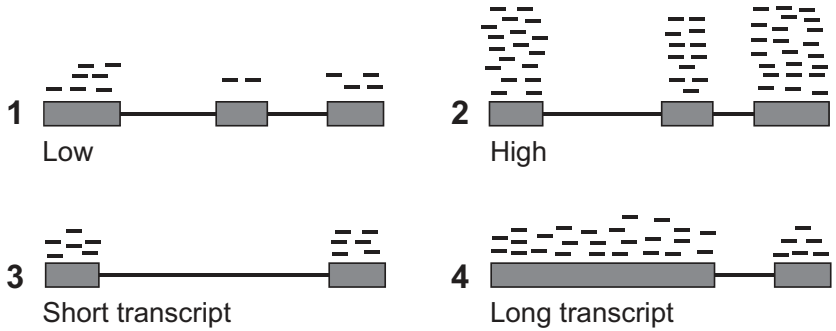
"Map first" reconstruction approaches directly benefit with mapping improvements
We even get more uniquely aligned reads (not just spliced reads)

Overview of the session

The 3 main computational challenges of sequence analysis for *counting applications*:

- Read mapping: Placing short reads in the genome
- Reconstruction: Finding the regions that originate the reads
- Quantification:
  - Assigning scores to regions
  - Finding regions that are differentially represented between two or more samples.

Quantification: only one isoform



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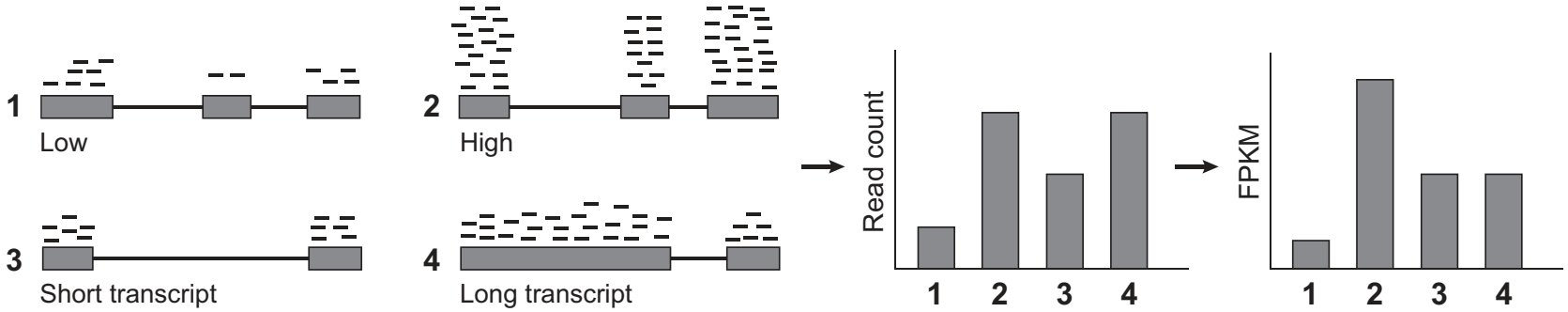

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Reads per kilobase of exonic sequence per million mapped reads

(*Mortazavi* et al Nature methods 2008)

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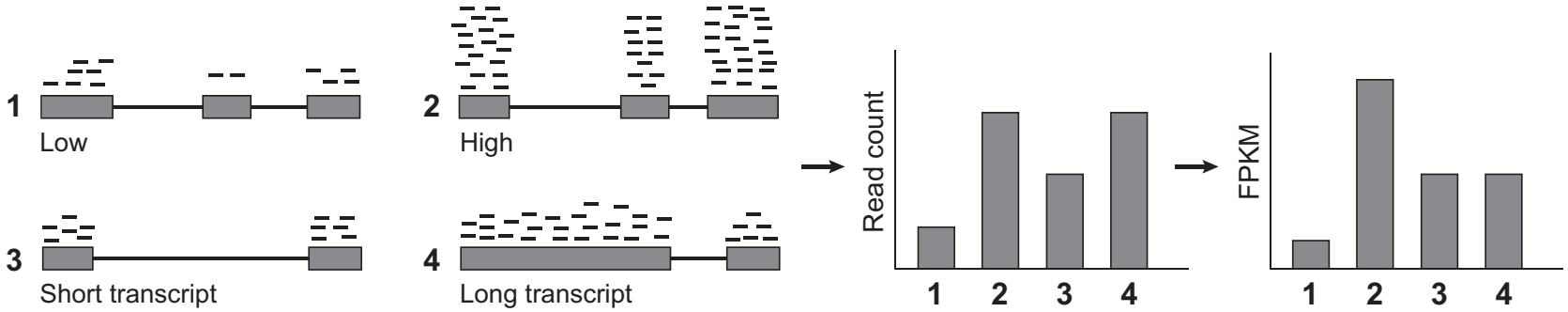

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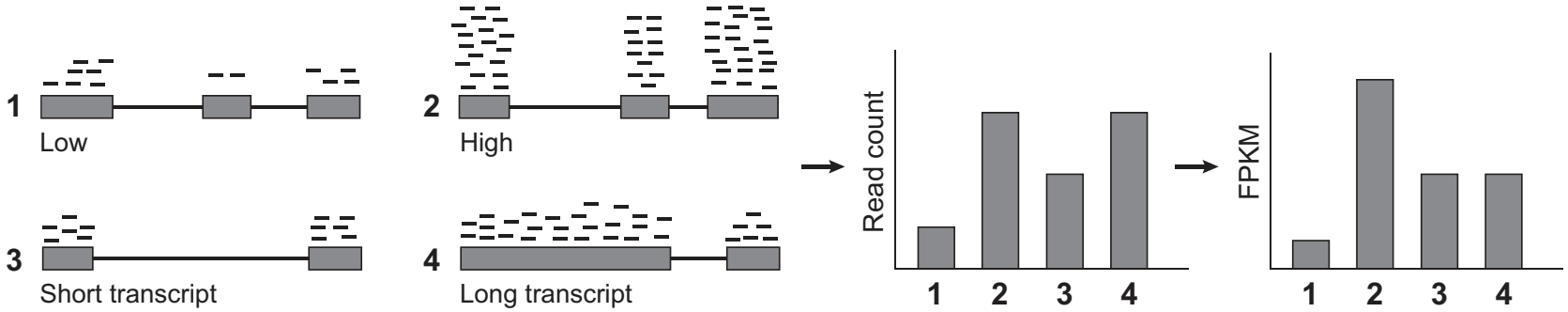

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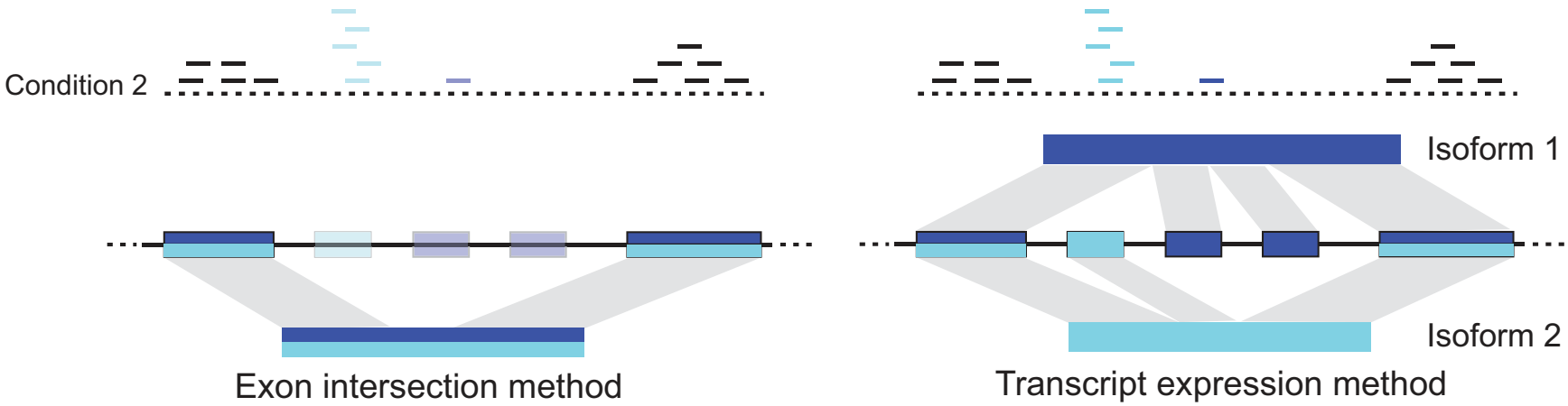
This is all good when genes have one isoform.

Quantification: gene expression with multiple isoforms

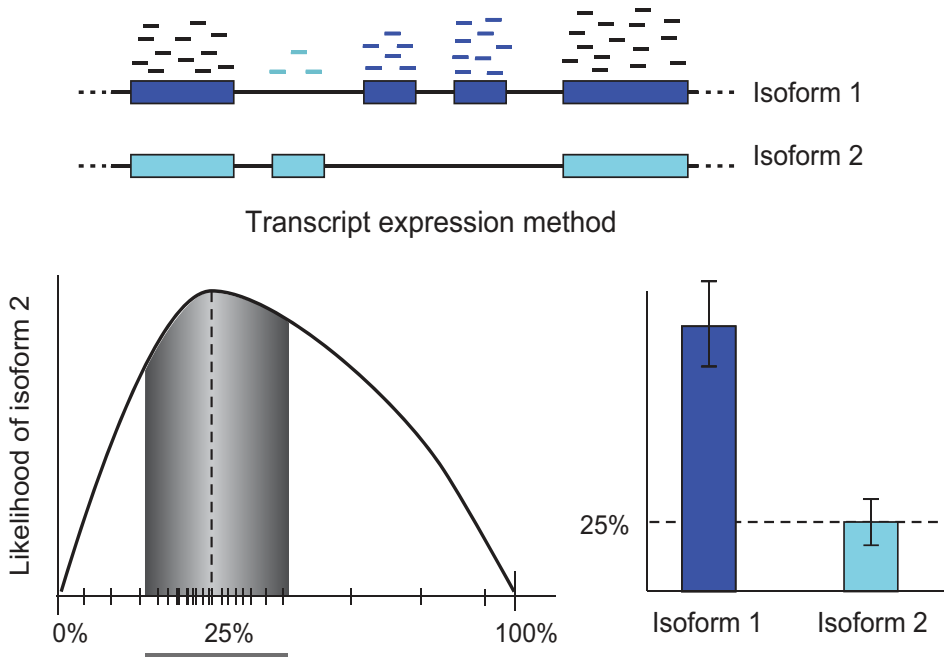


Exon intersection model: Score constituent exons

Exon union model: Score the the “merged” transcript

Transcript expression model: Assign reads uniquely to different isoforms. *Not a trivial problem!*

Quantification: read assignment method



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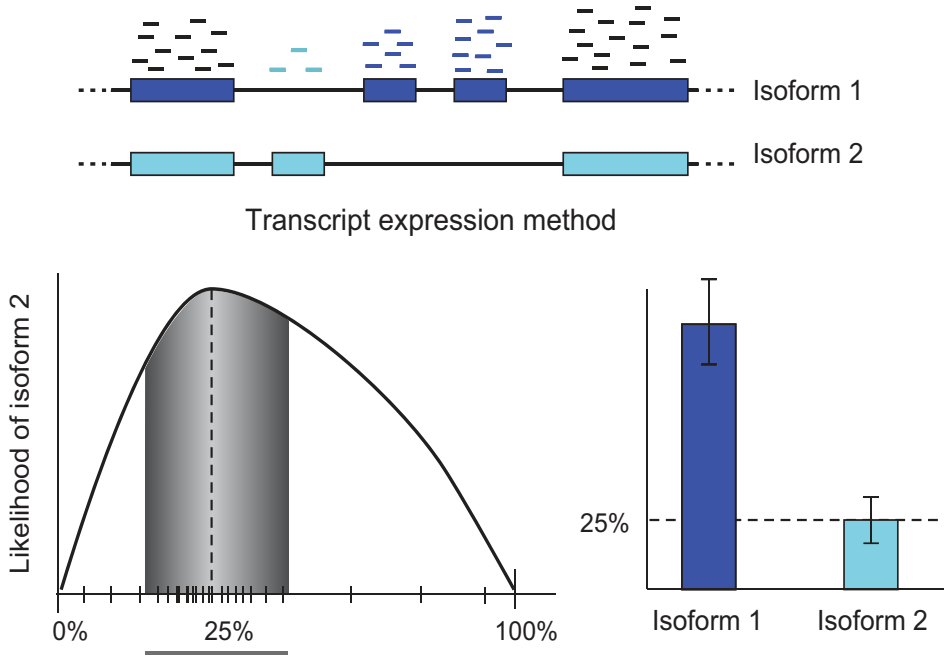

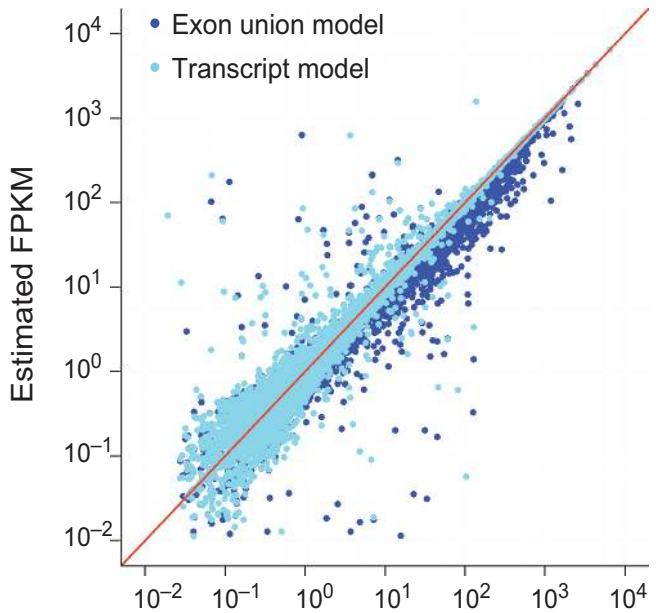

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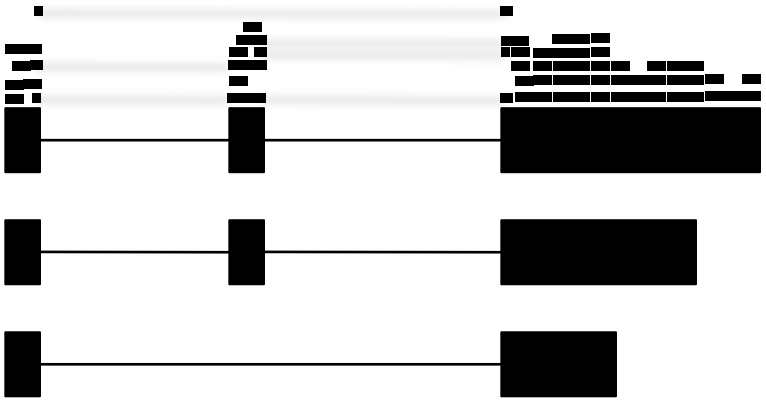

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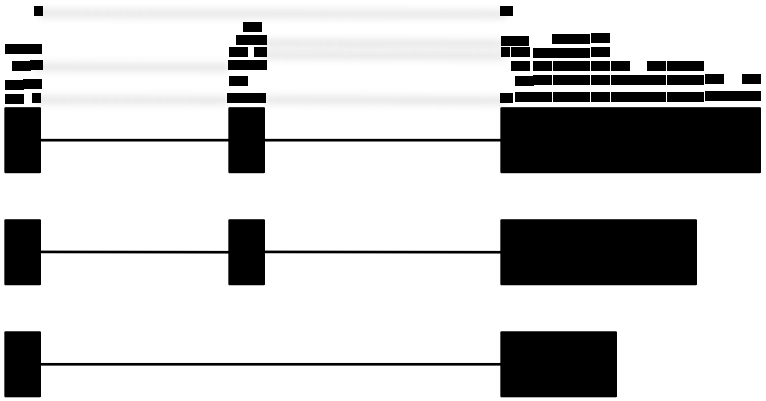
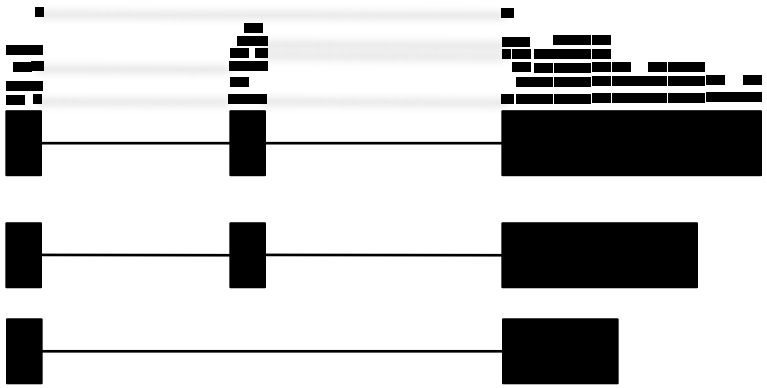


How do we define the gene expression?
How do we compute the expression of each isoform?

Computing gene expression



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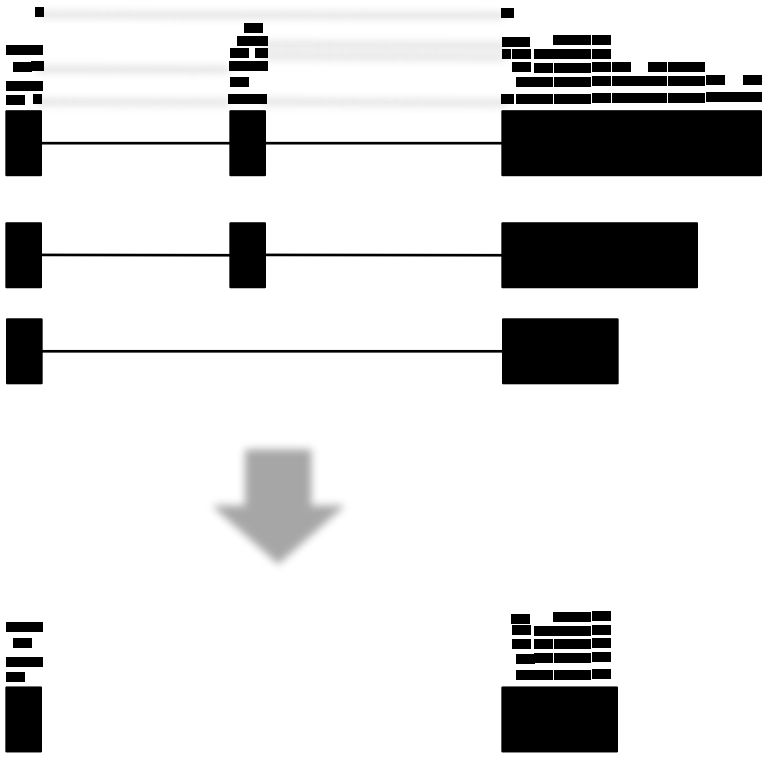
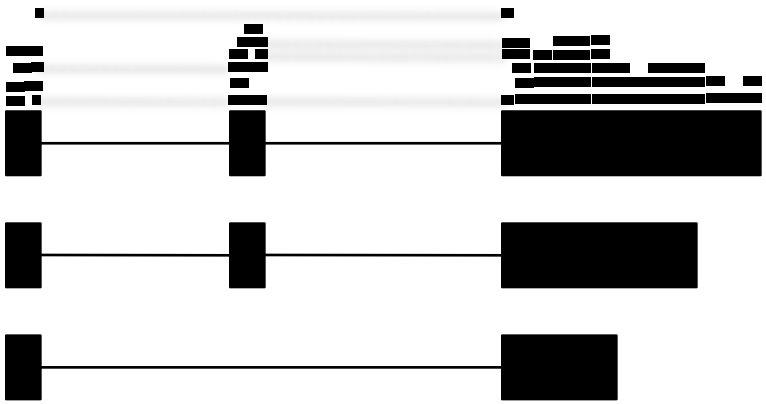


Idea1: RPKM of the constitutive reads (Neuma, Alexa-Seq, Scripture)

Computing gene expression — isoform deconvolution



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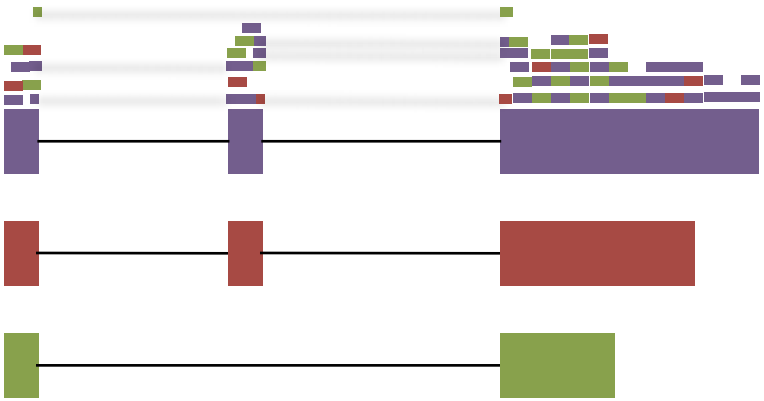

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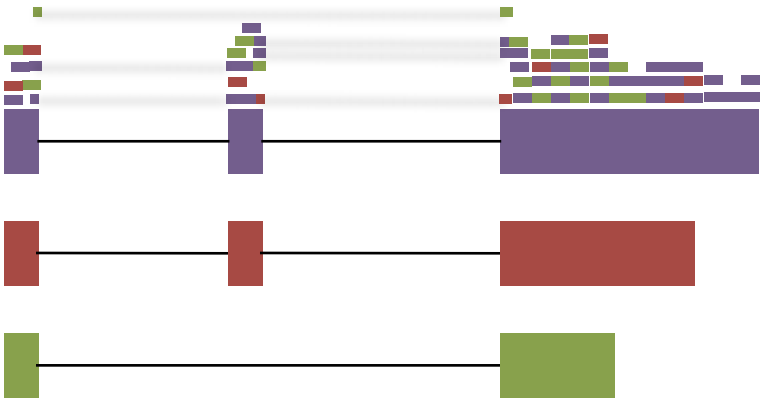

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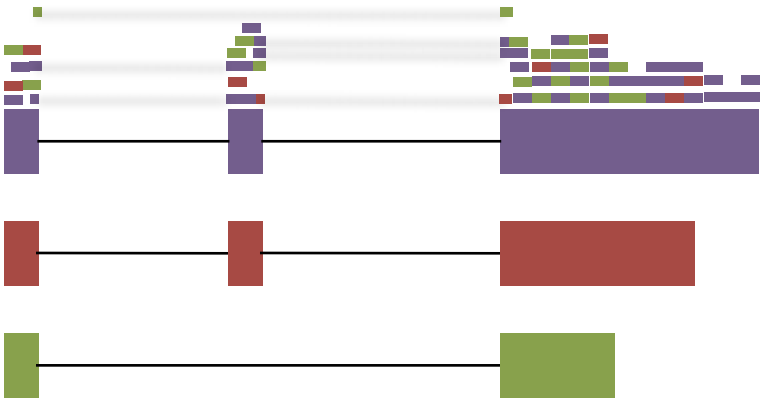

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$$E = RPKM_1 + RPKM_2 + RPKM_3$$

Programs to measure transcript expression

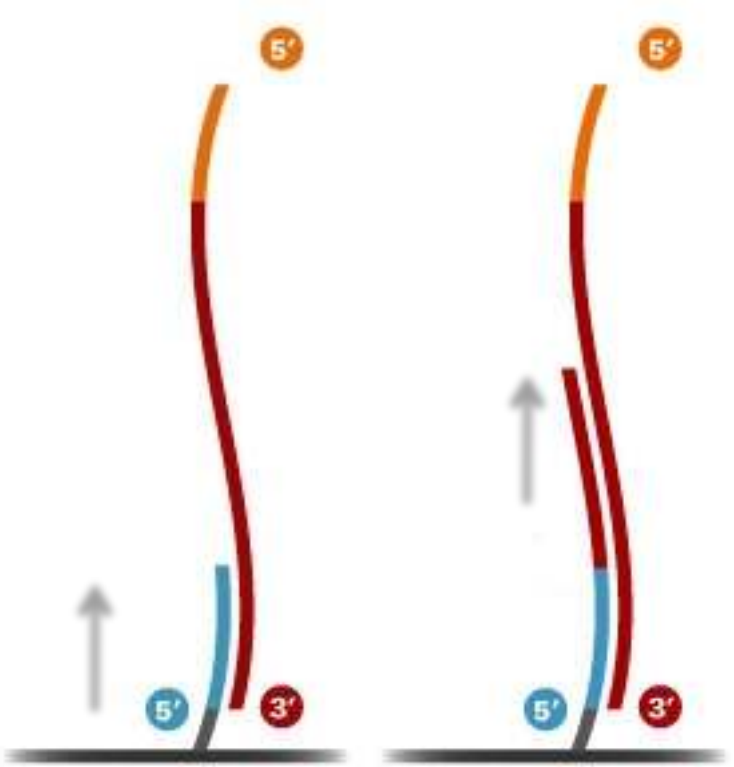
	Implemented method
Alexa-seq	Gene expression by constitutive exons
ERANGE	Gene expression by using all Exons
Scripture	Gene expression by constitutive exons
Cufflinks	**Transcript deconvolution by solving the maximum likelihood problem**
MISO	**Transcript deconvolution by solving the maximum likelihood problem**
RSEM	**Transcript deconvolution by solving the maximum likelihood problem**

Impact of library construction methods

Library construction improvements — Paired-end sequencing

Adapted from the Helicos website

Library construction improvements — Paired-end sequencing



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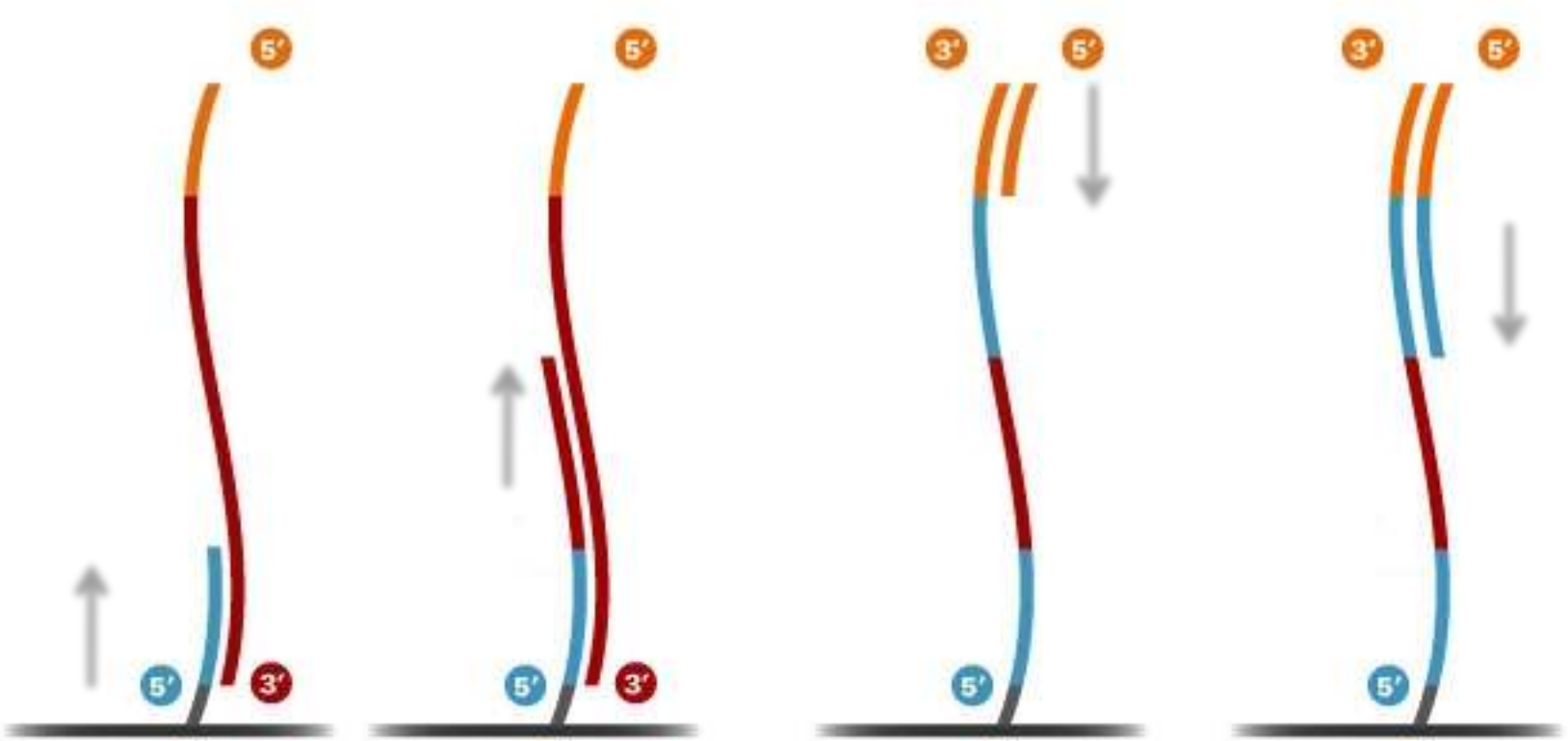

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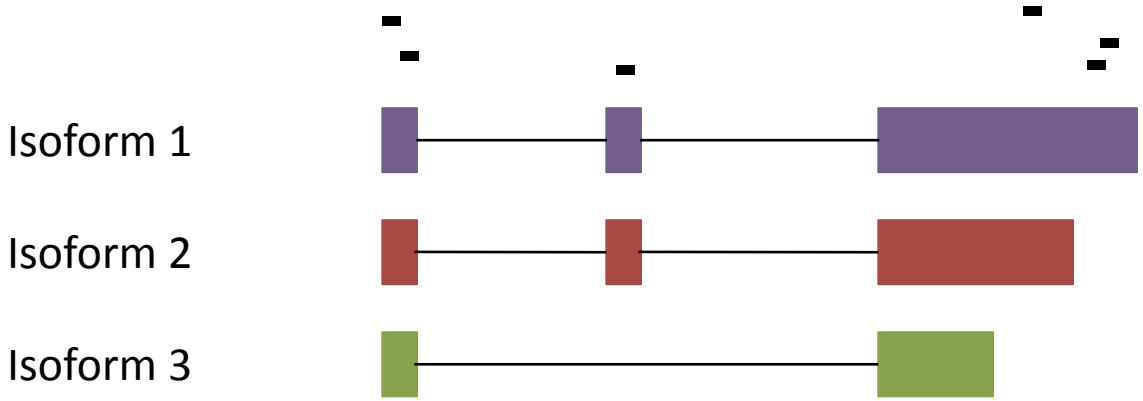
Paired-end reads are easier to associate to isoforms



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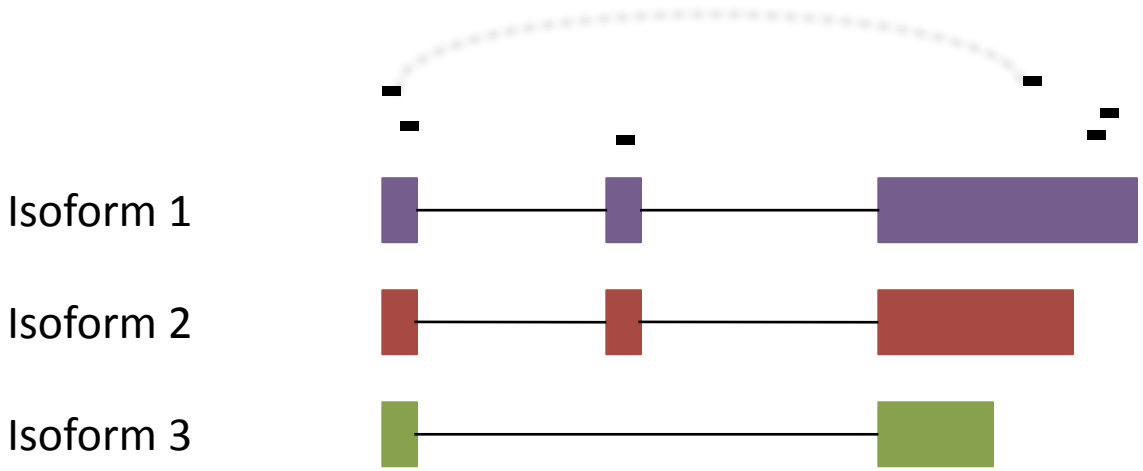

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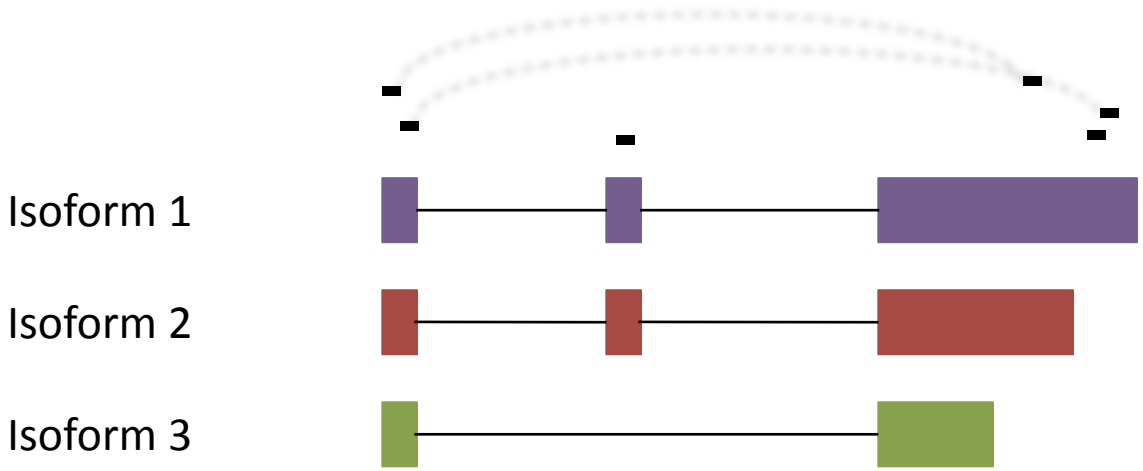

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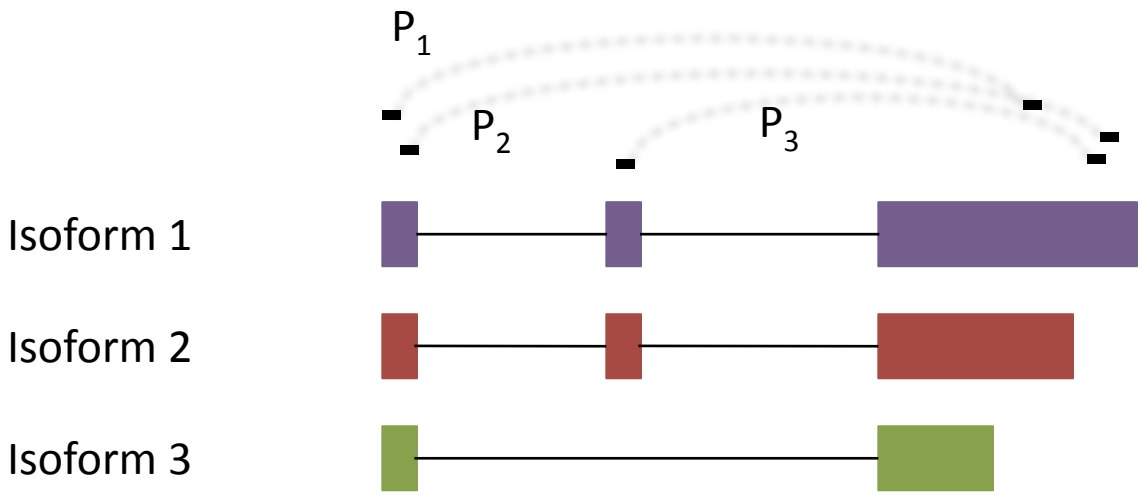

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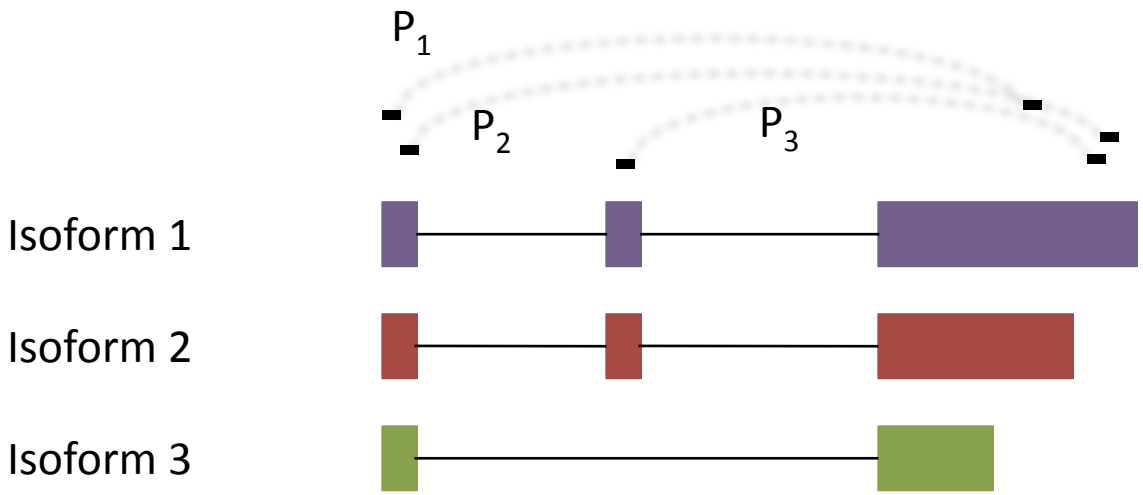

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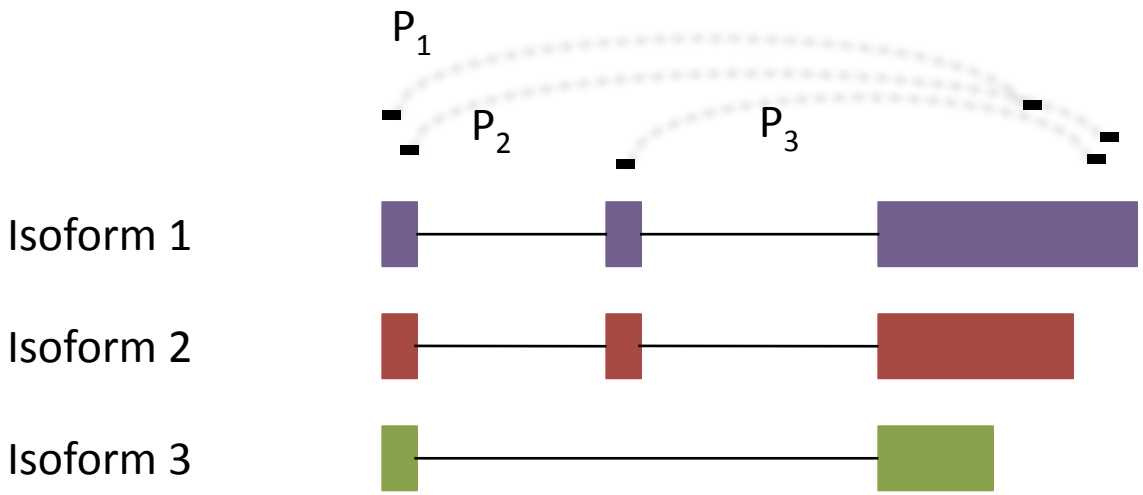

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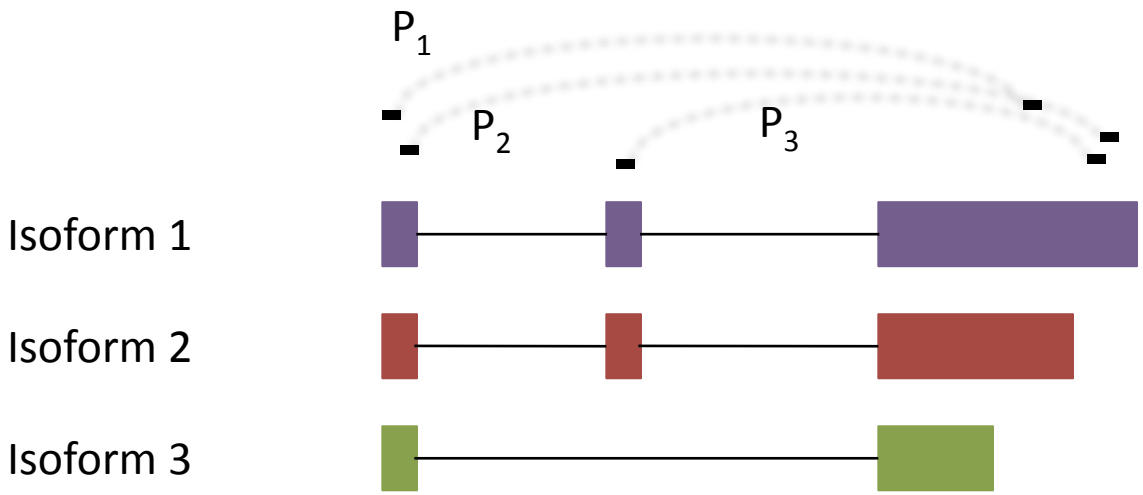

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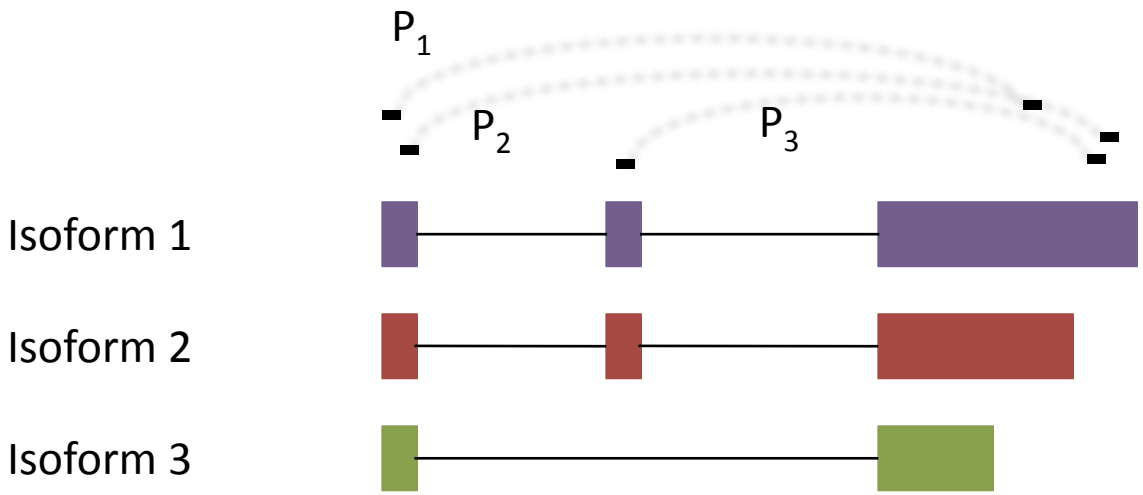

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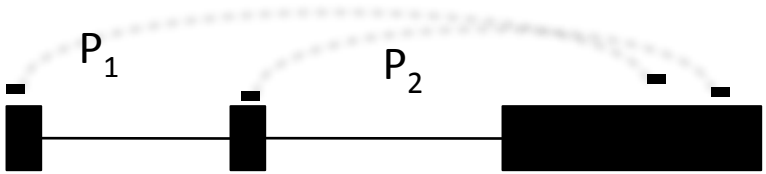
Do paired-end reads also help identifying reads originating in isoform 3?

We can estimate the insert size distribution



Get all single isoform reconstructions

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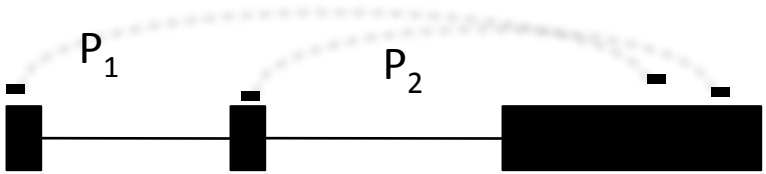

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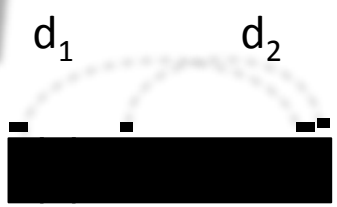

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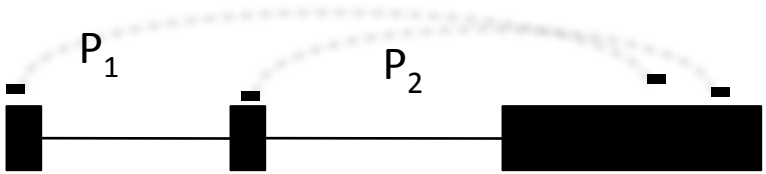

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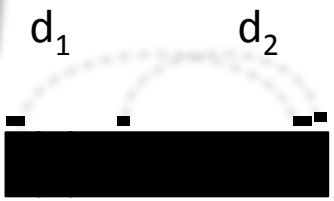

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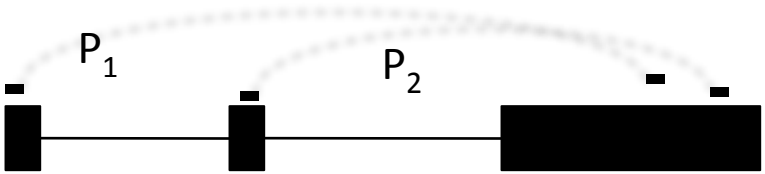

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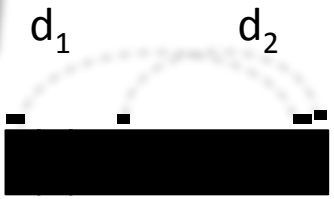

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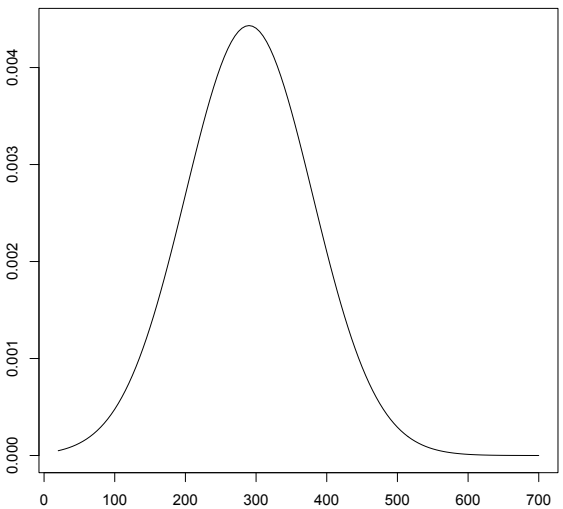

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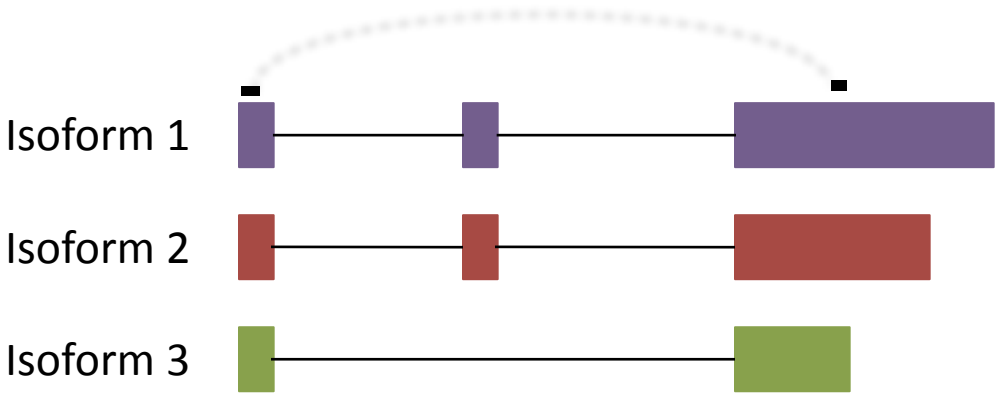

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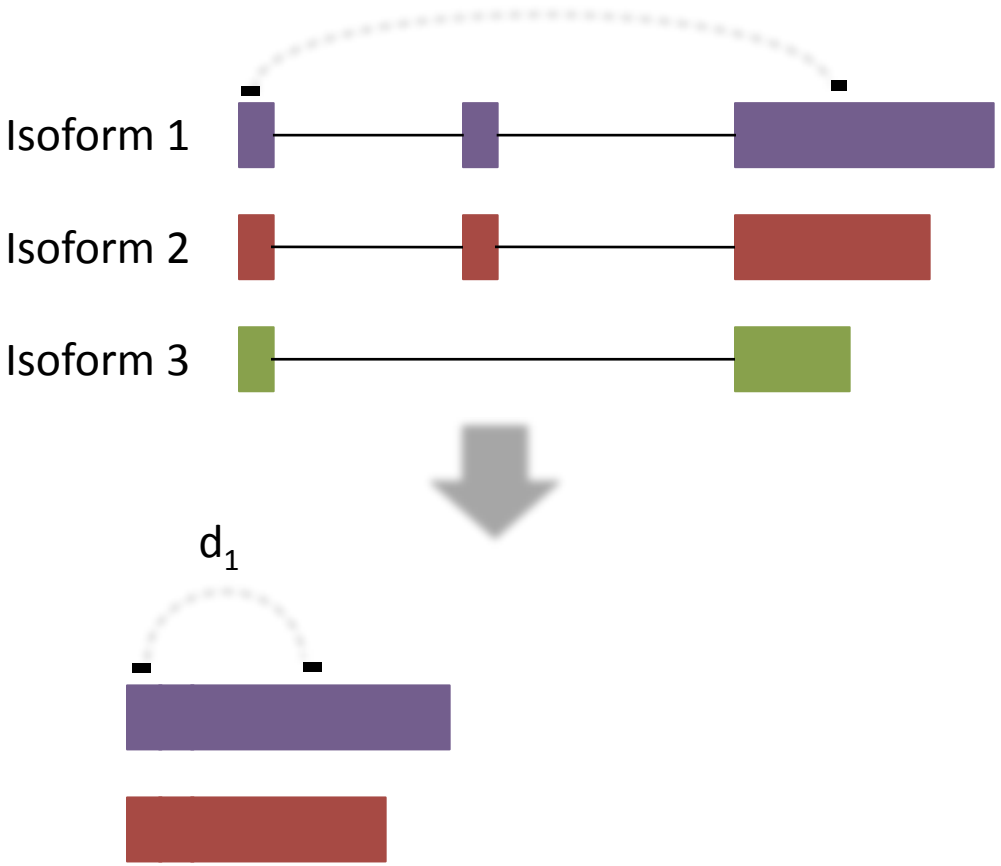

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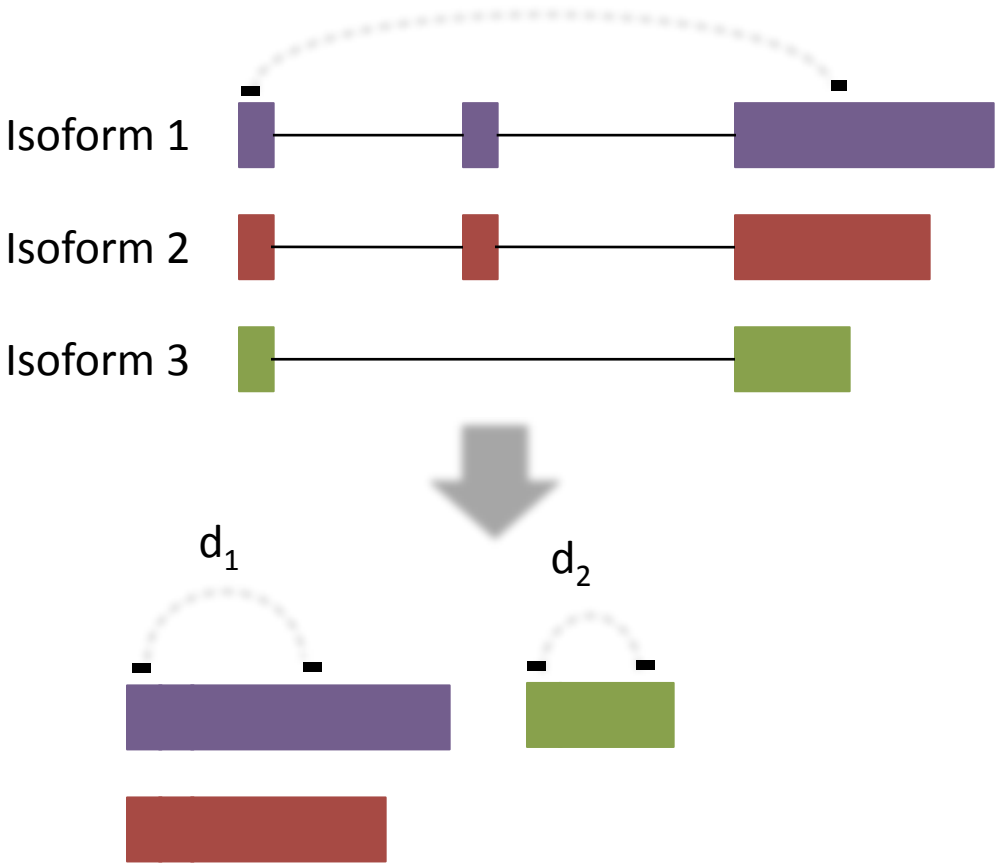

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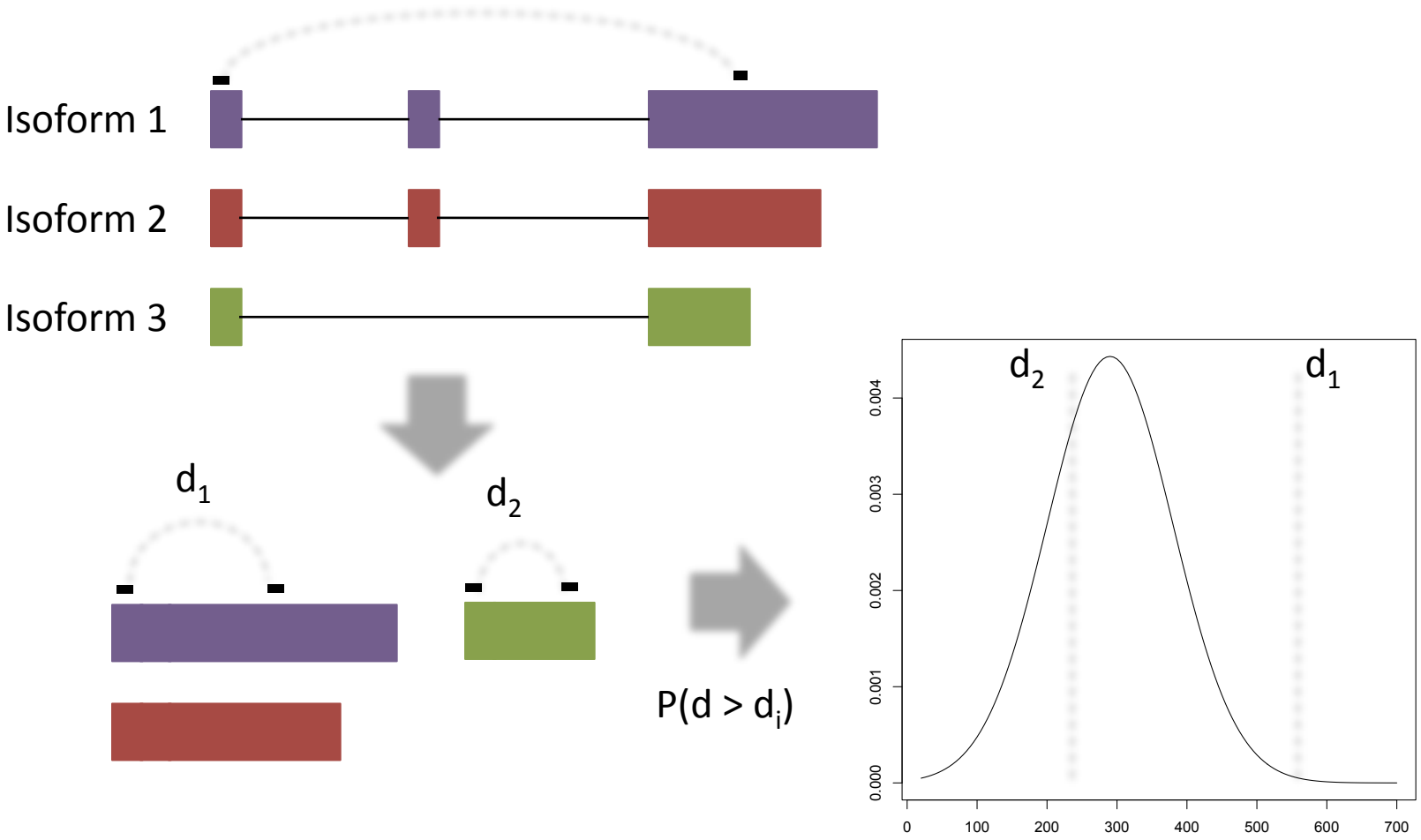
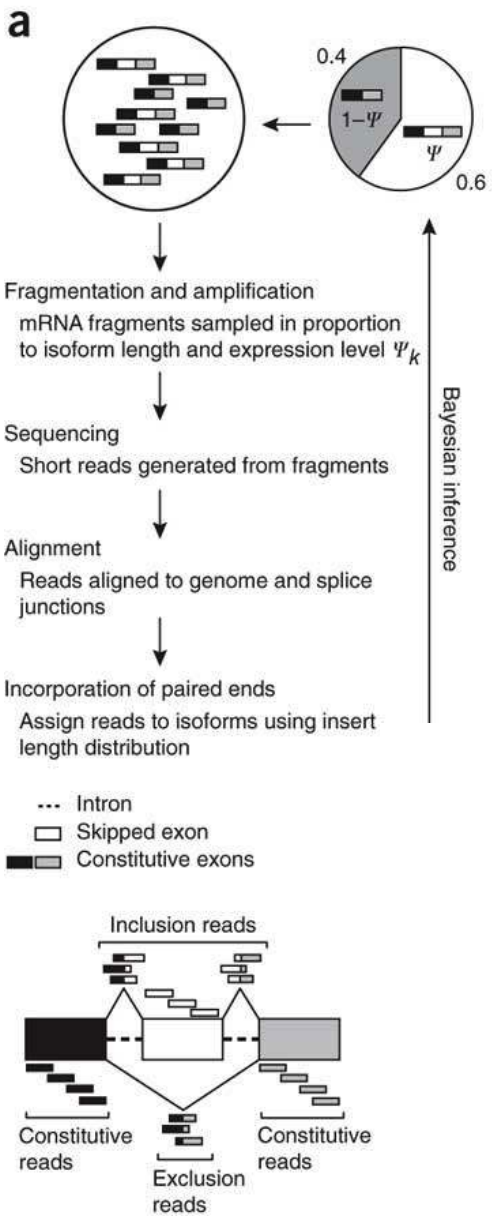


And improve quantification



Katz et al Nature Methods 2008

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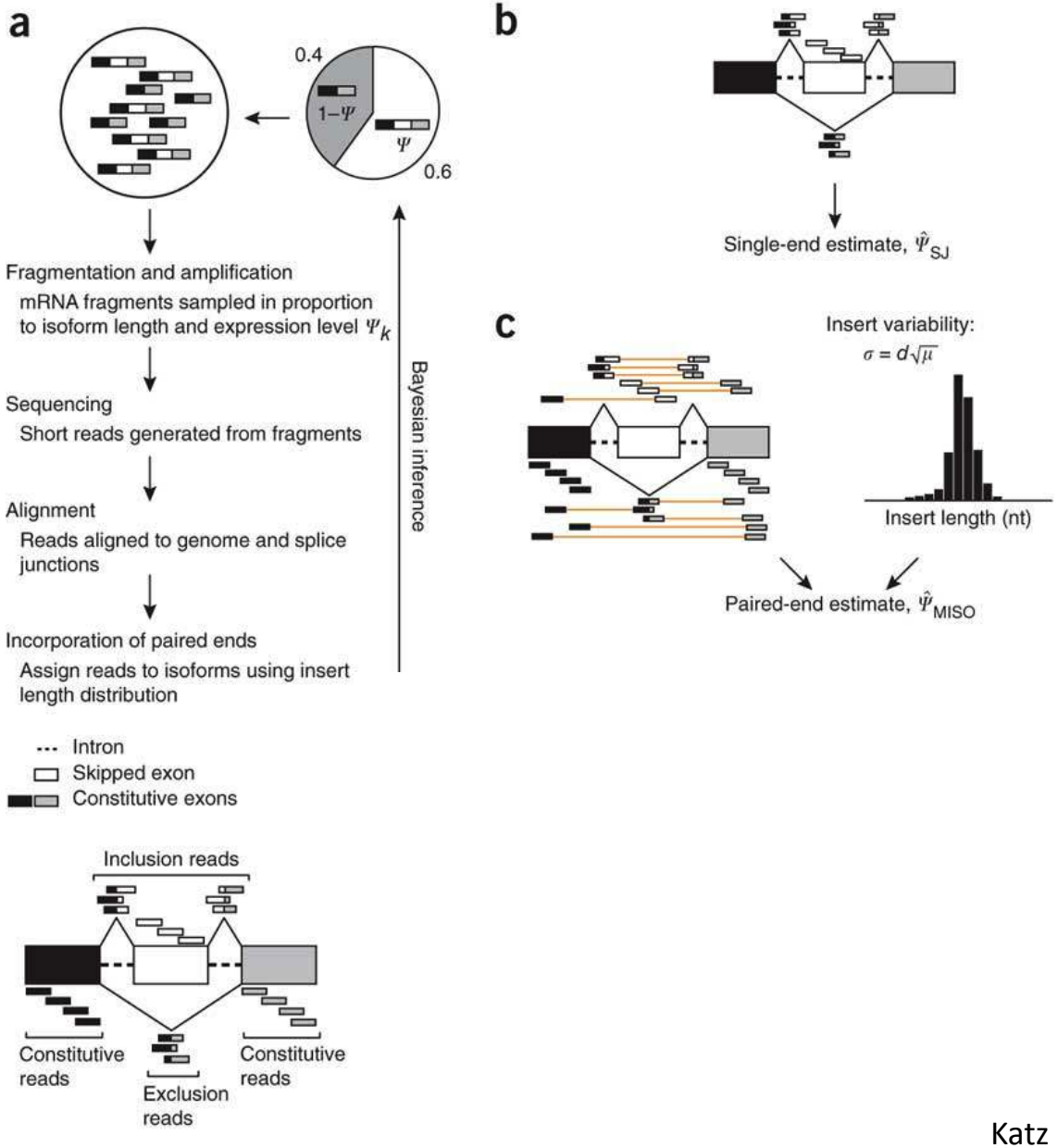

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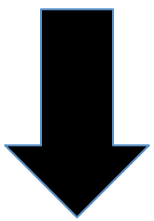
Quantification with paired ends (FPKM)

Cufflinks leverages paired ends to quantify fragments rather than raw reads. The extension of RPKM.

$$RPKM = 10^9 \frac{\#\,reads}{length \times Total\,\mathrm{Re}\,ads}$$



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Fragments per kilobase of exonic sequence per million mapped fragments

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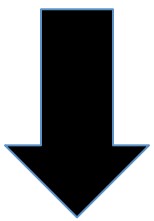

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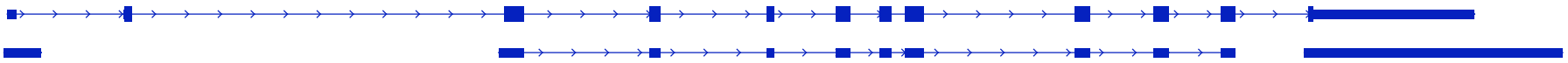
paired-end reads improve quantification accuracy

Paired-end improve reconstructions



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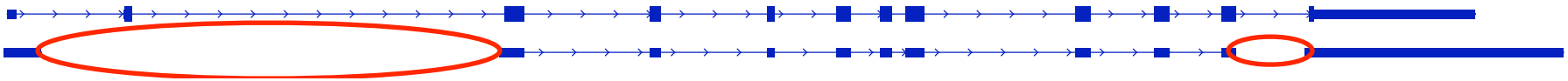

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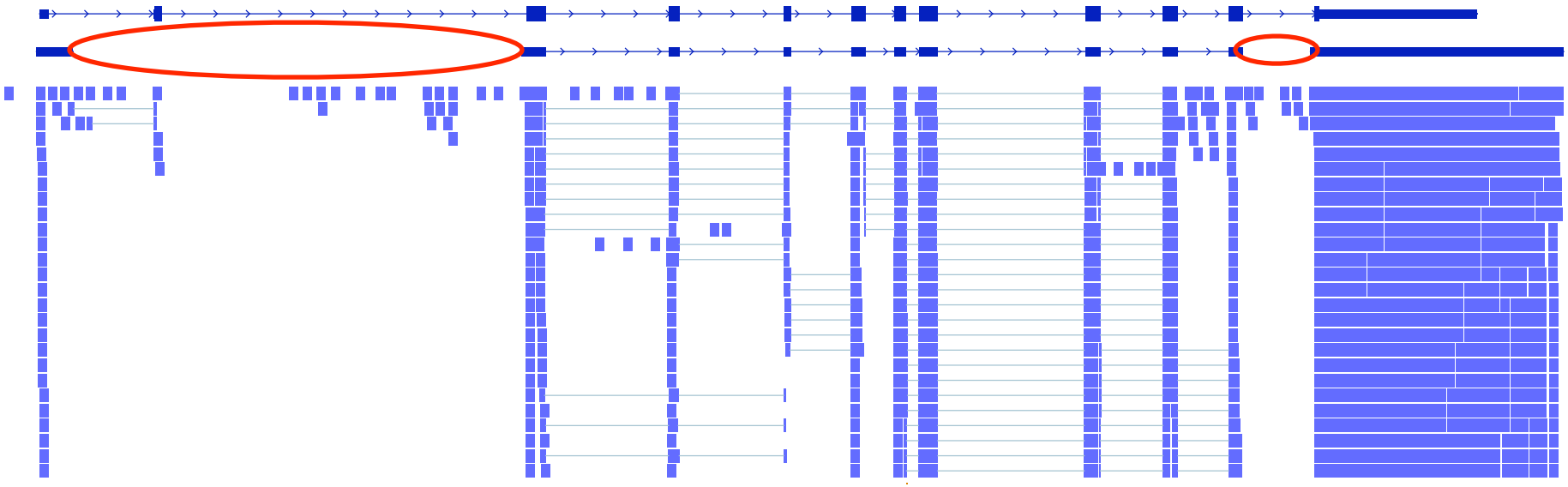

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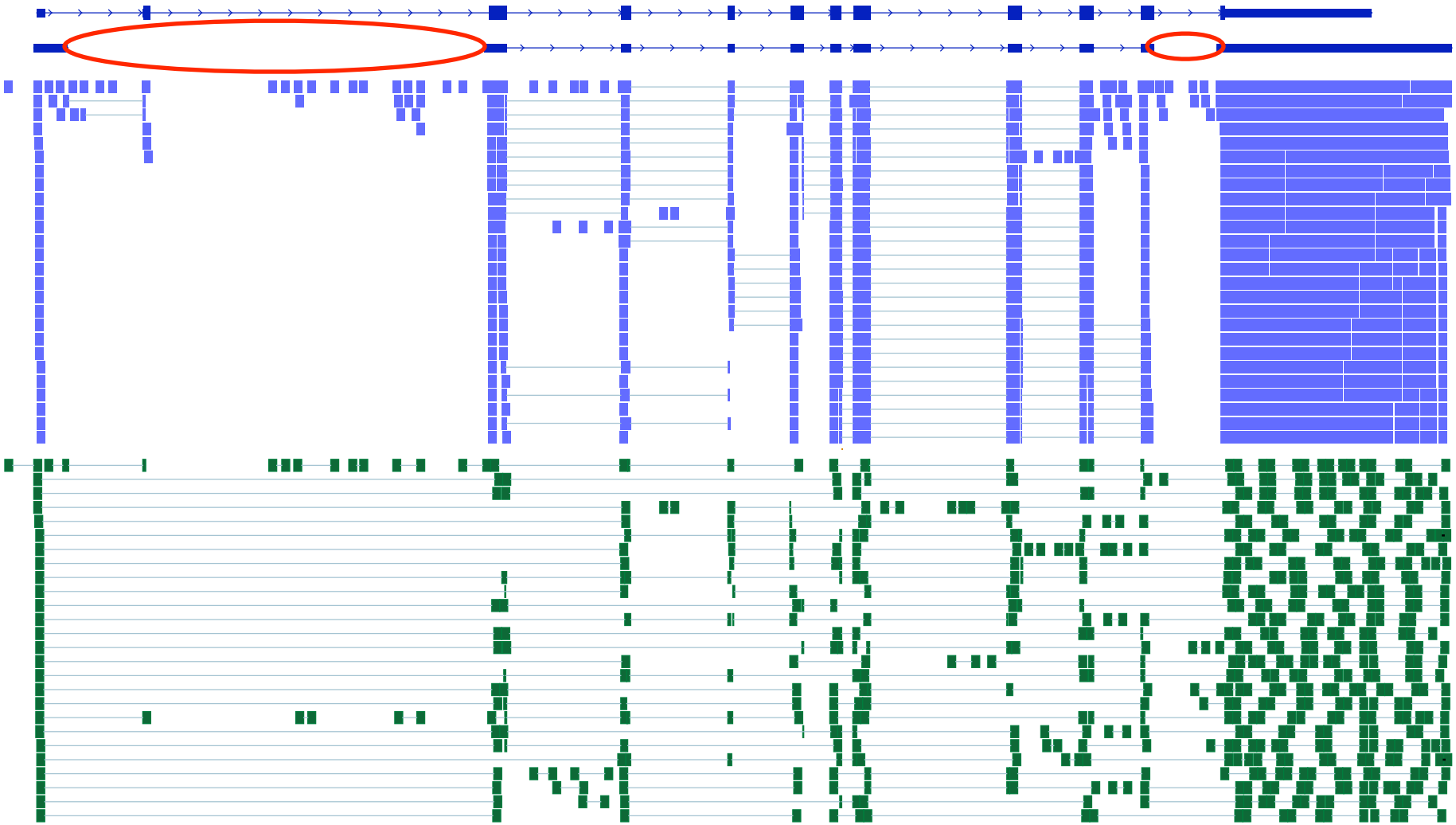

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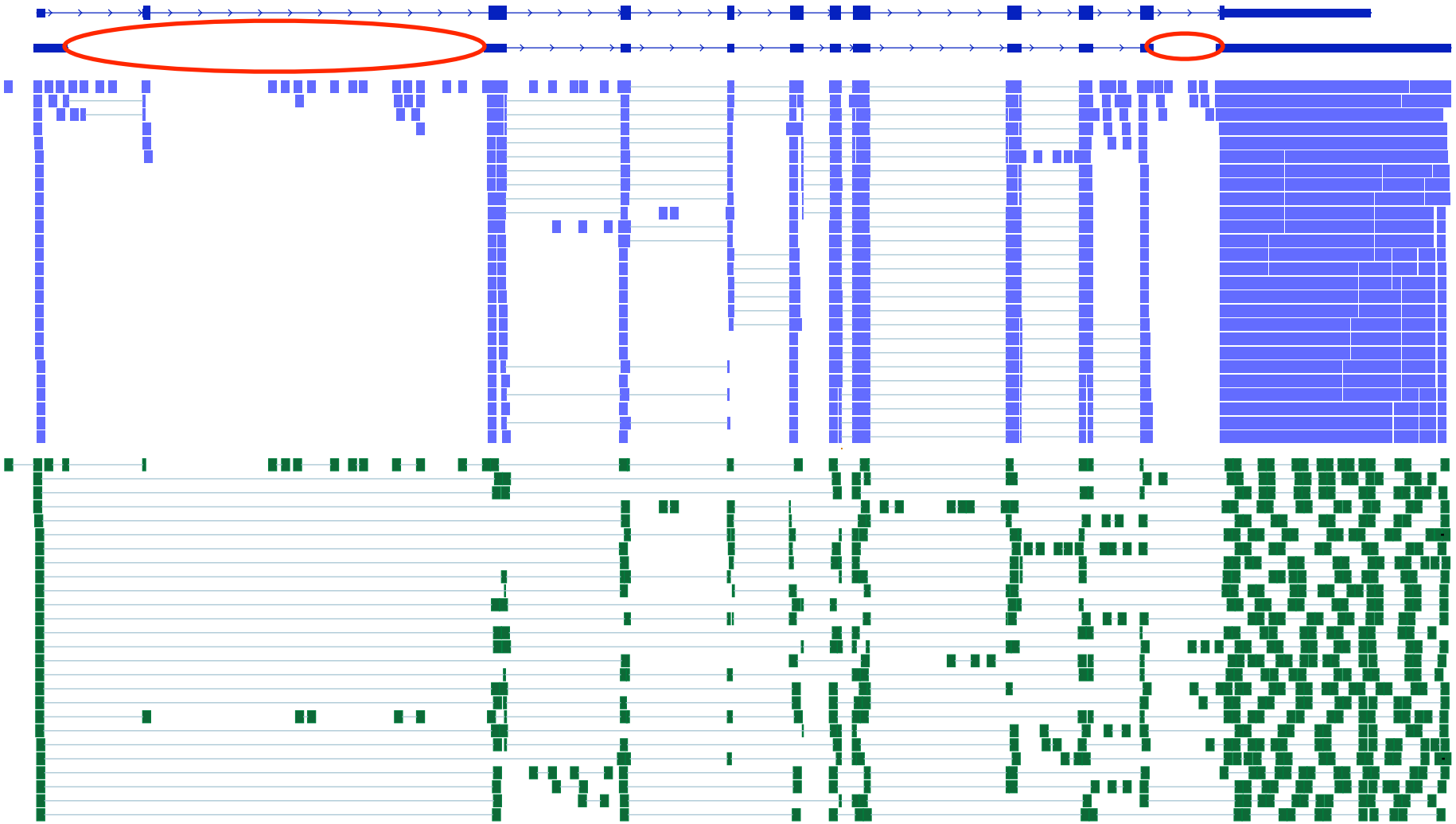
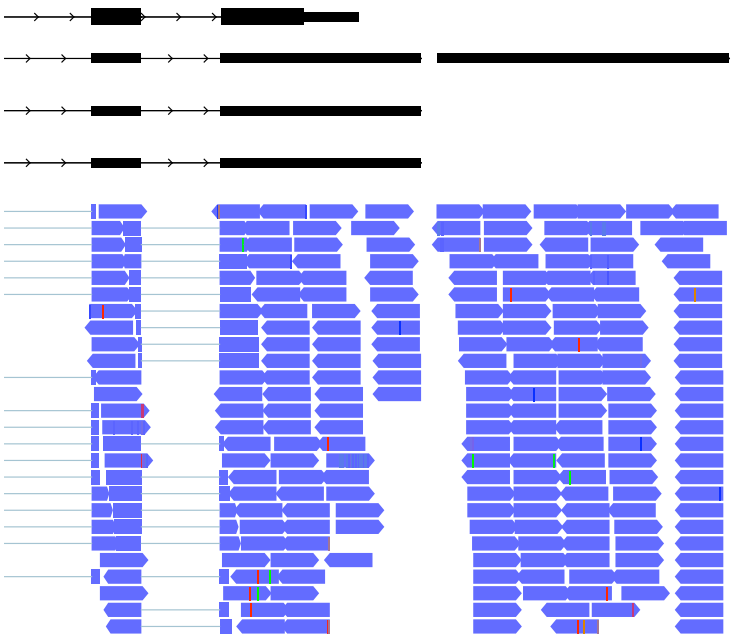


Paired-end data complements the connectivity graph

And merge regions

Single reads



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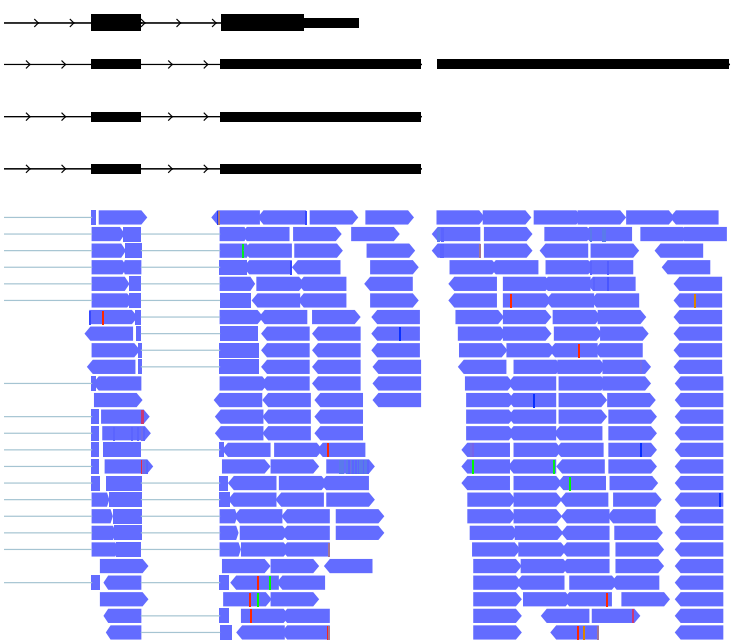

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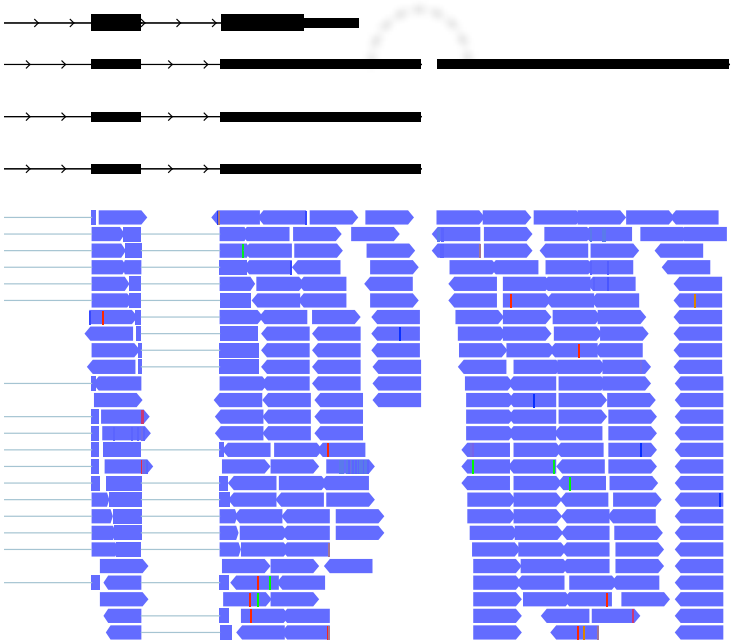

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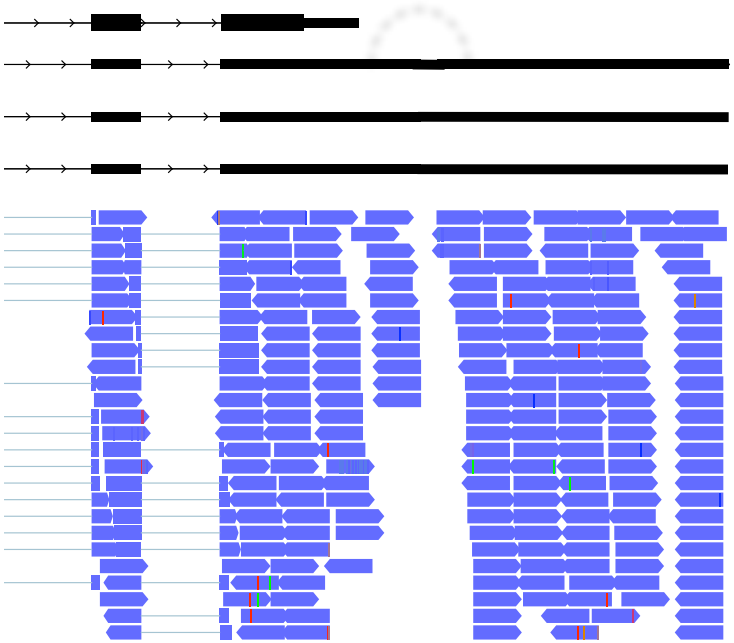

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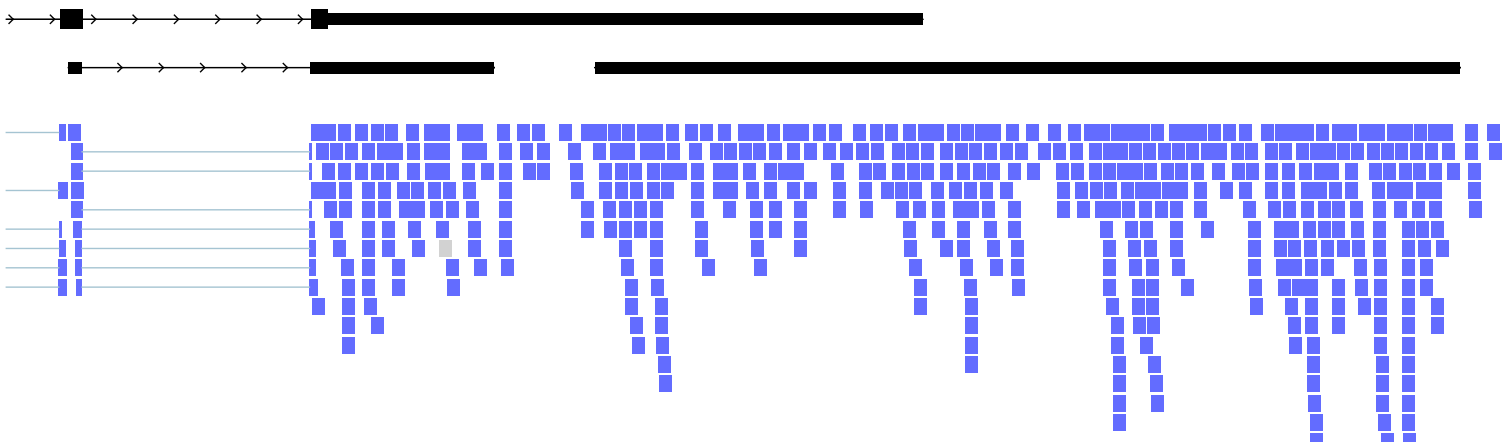

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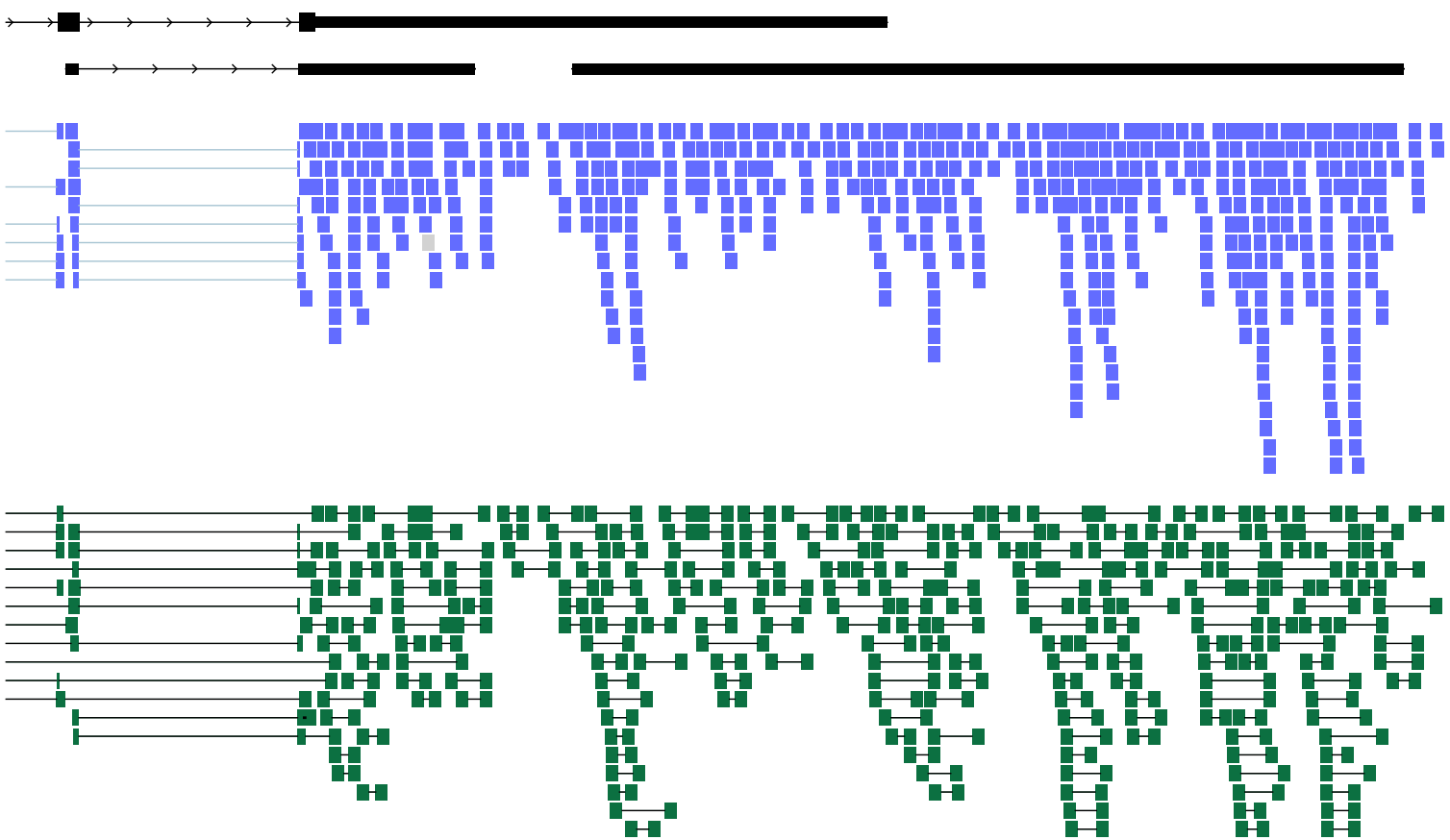

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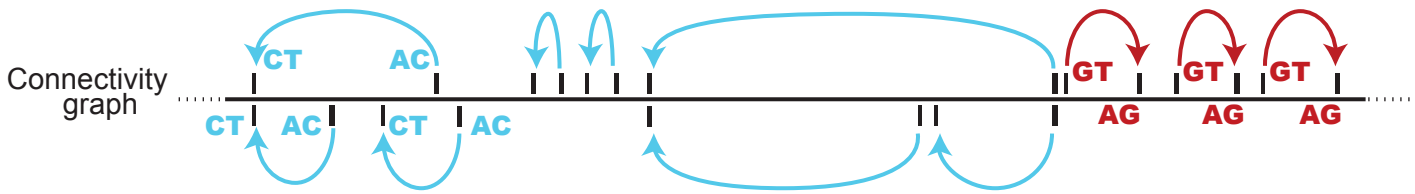
- Paired end alignment is supported by most short read aligners
- Transcript quantification depends heavily in paired-end data
- Transcript reconstruction is greatly improved when using paired-ends (work in progress)

Giving orientation to transcripts — Strand specific libraries

Scripture relies on splice motifs to orient transcripts. It orients every edge in the connectivity graph.



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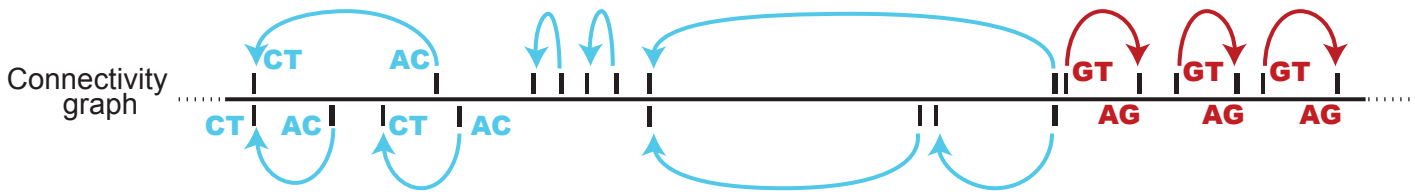


?

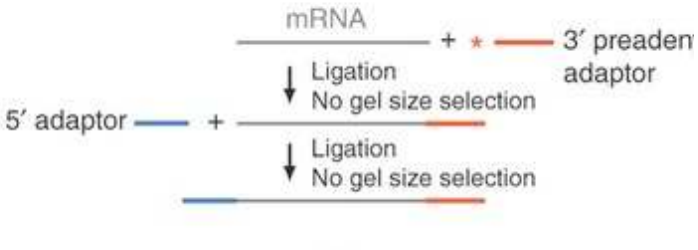
Single exon genes are left unoriented

Strand specific library construction results in oriented reads.

Illumina RNA ligation

3′ preadenylated adaptors and 5′ adaptors ligated sequentially to RNA without cleanup

(S. Luo and G. Schroth, personal communication)



Sequence depends on the adapters ligated

Adapted from Levine et al Nature Methods

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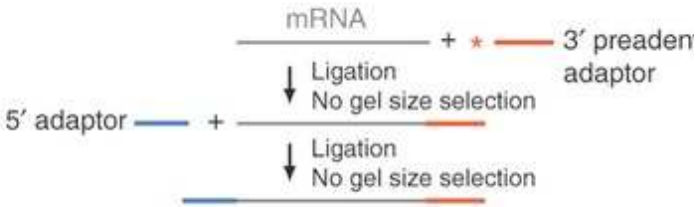

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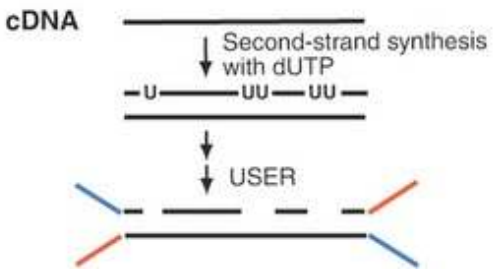
dUTP second strand[13]

Second-strand synthesis with dUTP; remove 'U's after adaptor ligation and size selection



The second strand is destroyed, thus the cDNA read is always in reverse orientation to the RNA

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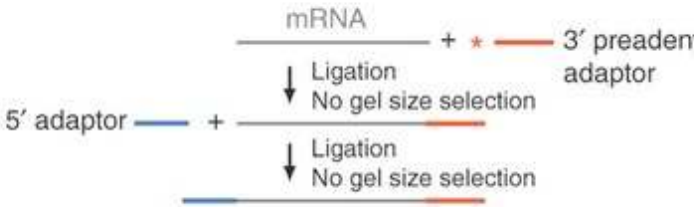

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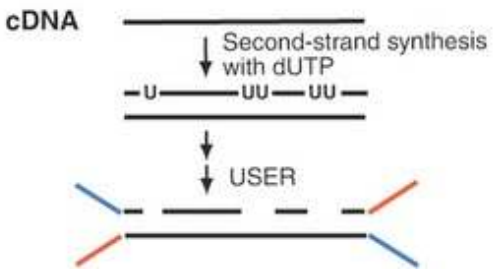

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Adapted from Levine et al Nature Methods

Scripture & Cufflinks allow the user to specify the orientation of the reads.

The libraries we will work with are strand sepcific

Summary

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- Quantification methods support strand specific libraries. For example Scripture will compute expression on both strand if desired.

Overview of the session

The 3 main computational challenges of sequence analysis for *counting applications:*

- Read mapping: Placing short reads in the genome
- Reconstruction: Finding the regions that originate the reads
- Quantification:
    - Assigning scores to regions
    - Finding regions that are differentially represented between two or more samples.

The problem.

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- Finding genes that have different expression between two or more conditions.

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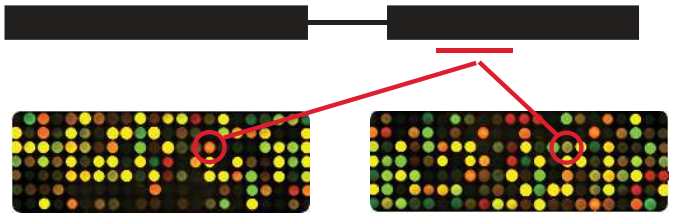
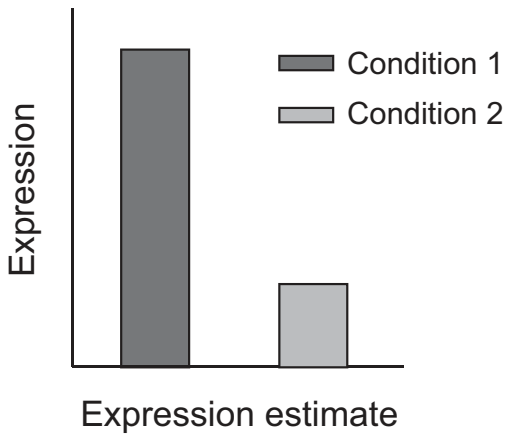
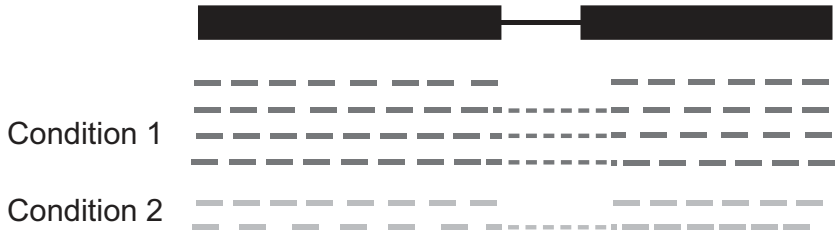
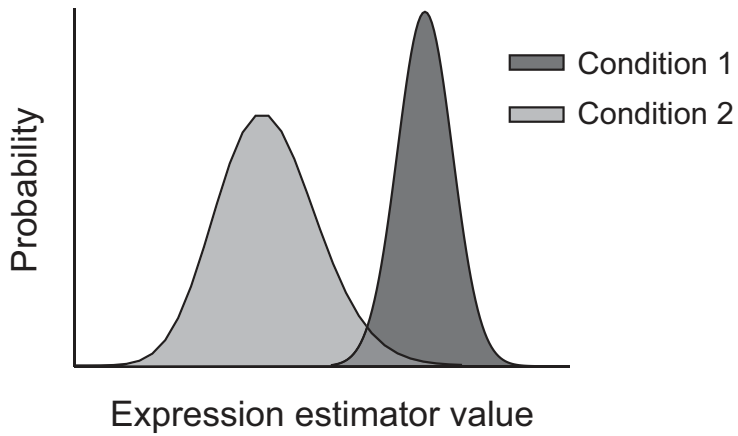
- Finding genes that have different expression between two or more conditions.
- Find gene with isoforms expressed at different levels between two or more conditions.
  - Find differentially used slicing events
  - Find alternatively used transcription start sites
  - Find alternatively used 3' UTRs

Differential gene expression using RNA-Seq









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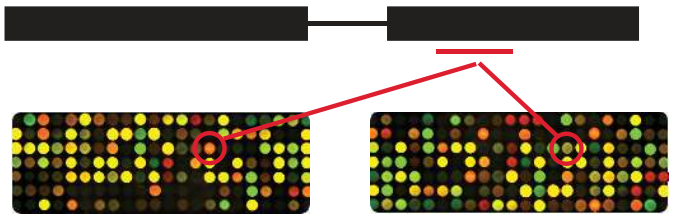

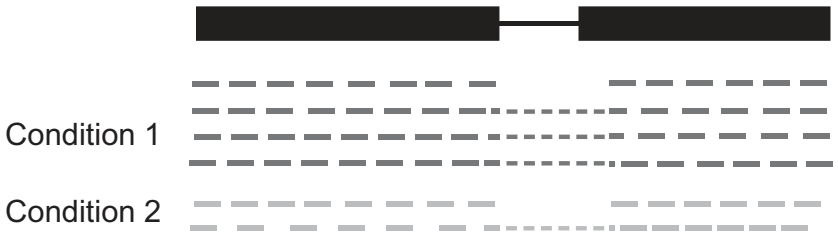

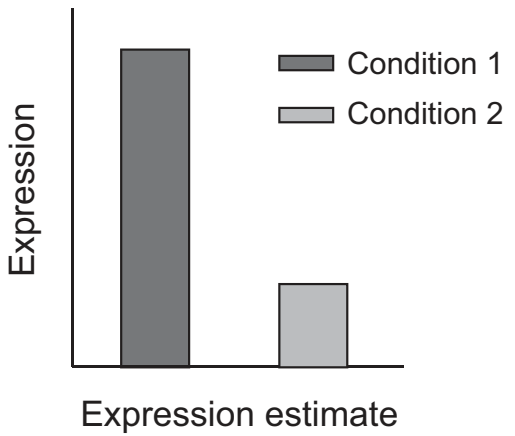

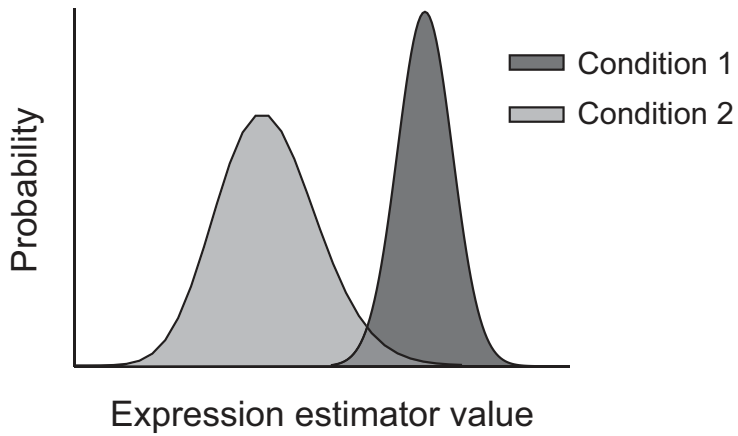

- (Normalized) read counts ←→ Hybridization intensity

Differential analysis strategies

- Use read counts
  - Standard Fisher exact (no preplicates) or χ^2 test (replicates)

	Condition A	Condition B
Gene A reads	n_a	n_b
Rest of reads	N_a	N_b

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- Use a summary statistic and "standard" array

Cufflinks differential issoform ussage

Let a gene G have *n* isoforms and let $p_1, \ldots, p_n$ the estimated fraction of expression of each isoform.

Call this a the isoform expression distribution *P* for G

Given two samples the differential isoform usage amounts to determine whether H_0: $P_1 = P_2$ or H_1: $P_1 \mathrel{!}= P_2$ are true.

To compare distributions Cufflinks utilizes an information content based metric of how different two distributions are called the Jensen-Shannon divergence:

$$JS(p^1, \ldots, p^m) = H\left(\frac{p^1 + \cdots + p^m}{m}\right) - \frac{\sum_{j=1}^{m} H(p^j)}{m}.$$

$$H(p) = -\sum_{i=1}^{n} p_i log p_i.$$

The square root of the JS distributes normal.

RNA-Seq differential expression software

	Underlying model	Notes
DegSeq	Normal. Mean and variance estimated from replicates	Works directly from reference transcriptome and read alignment
EdgeR	Negative Bionomial	Gene read counts table
DESeq	Poisson	Gene read counts table
Myrna	Empirical	Sequence reads and reference transcriptome

Digital gene expression

If all you want is the expression level

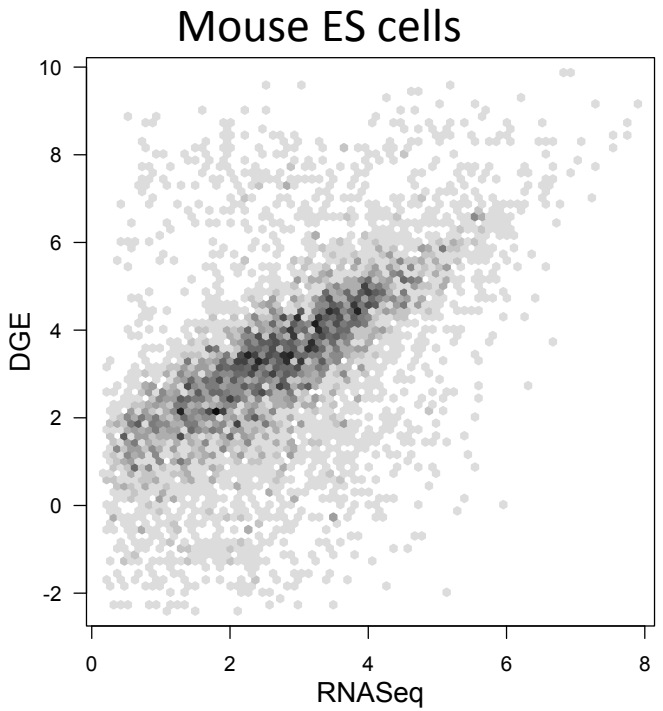
Easy

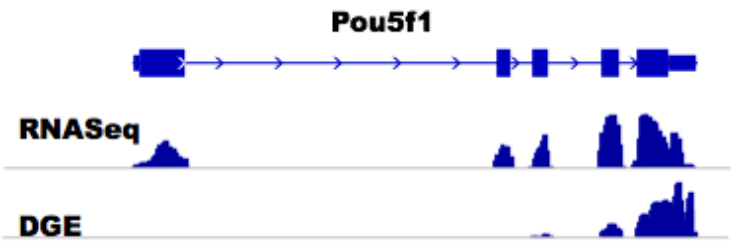
- Fragment RNA (heat)
- PolyA select -> RT -> 2nd strand
- Amplify
- Sequence

Cheap

RNASeq requires 100 mill reads.
DGE requires ~6-10 mill reads.

No size bias





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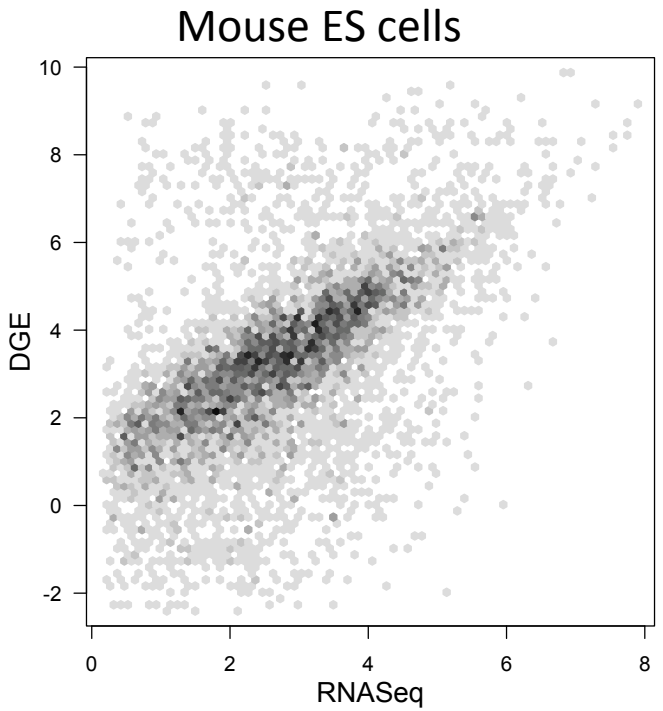

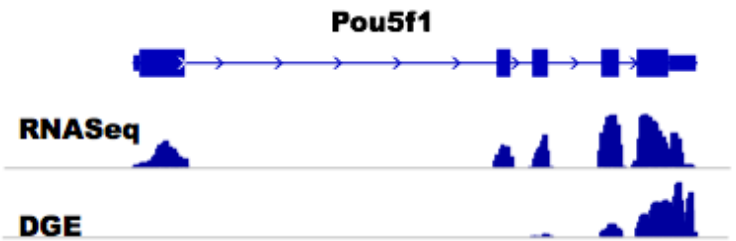

Replicates will be natural and analysis standard

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