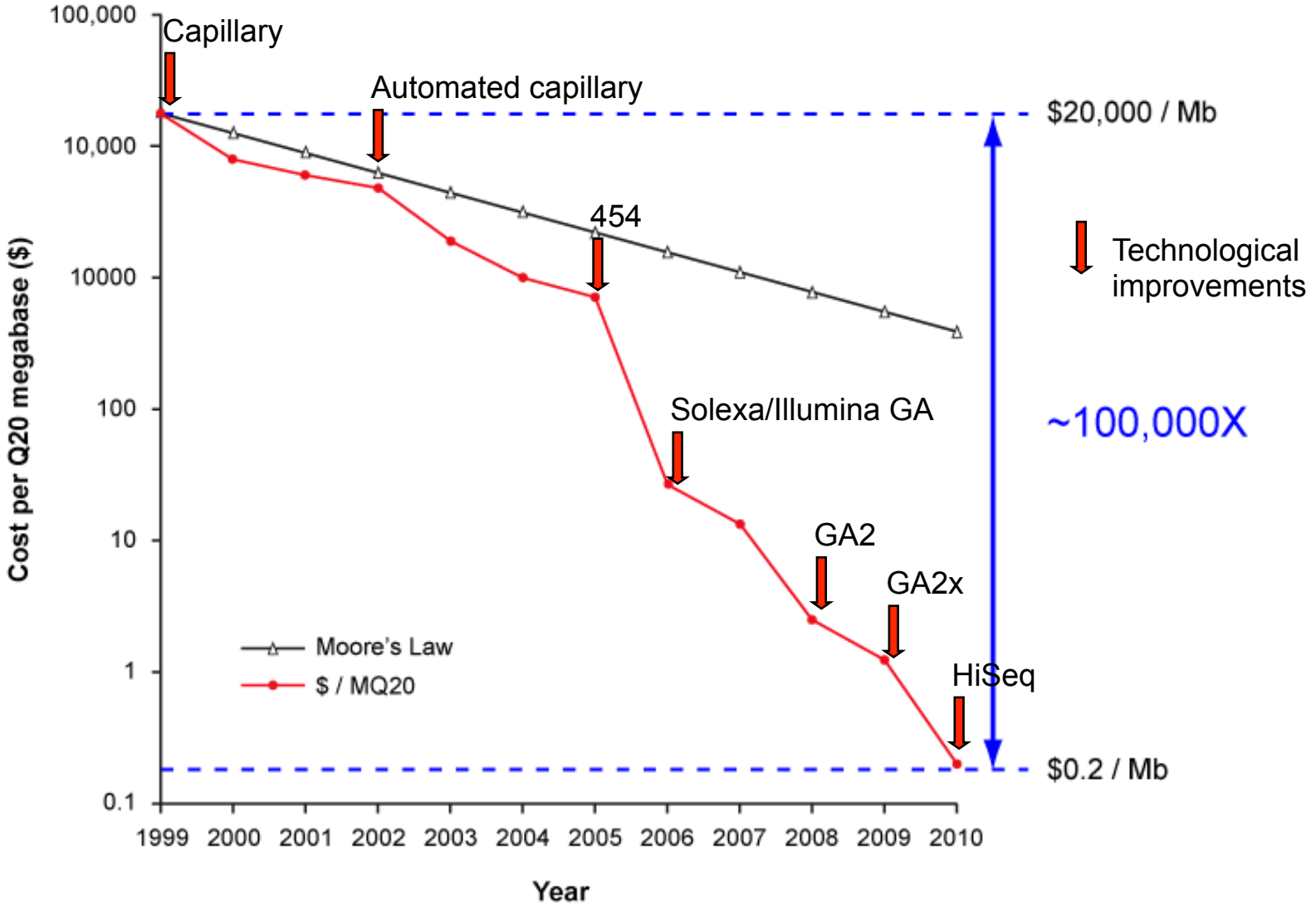
University of Massachusetts Medical School

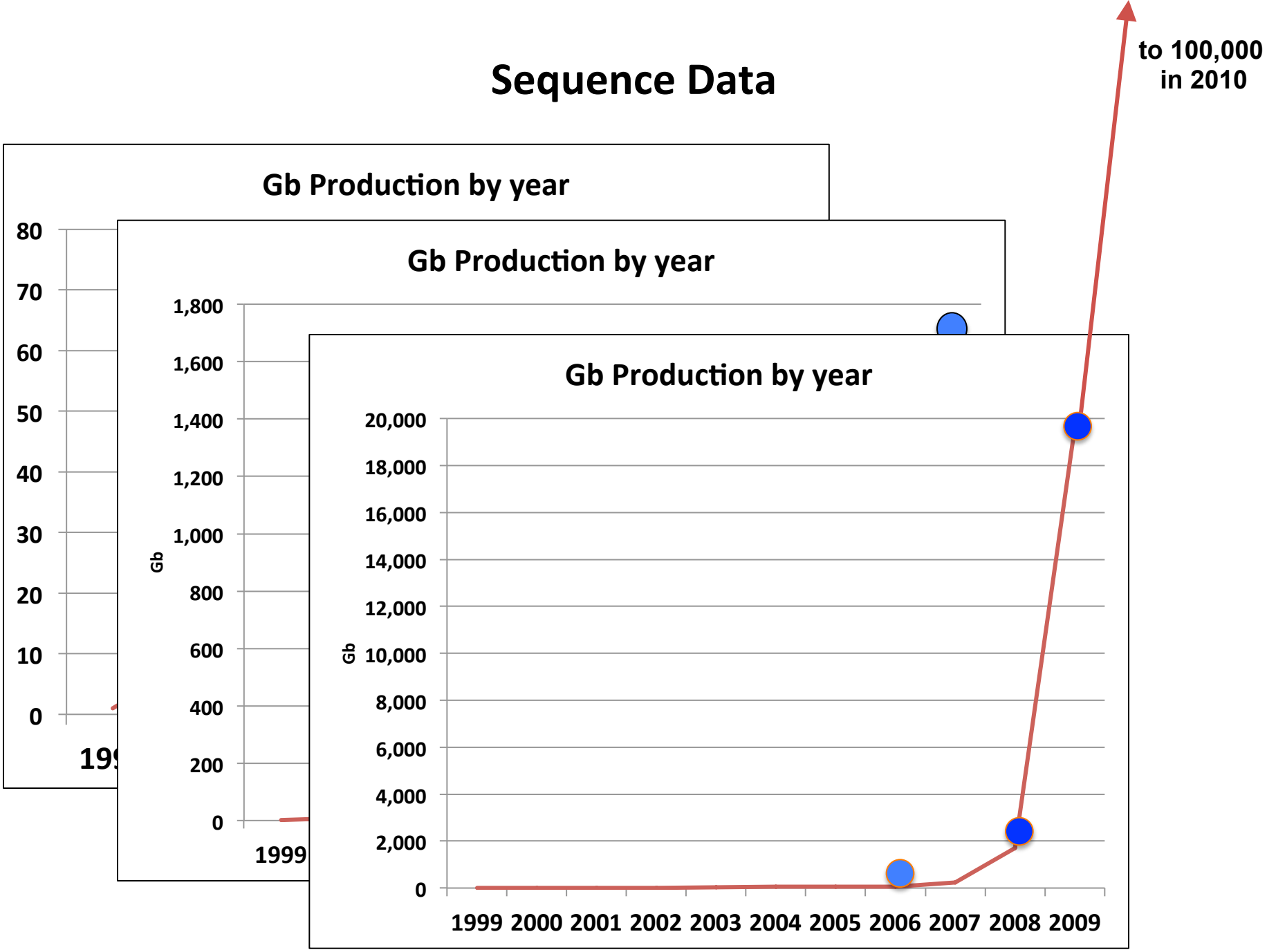
Short read sequence analysis

Manuel Garber

Plummeting cost of DNA sequencing



Sequence Data



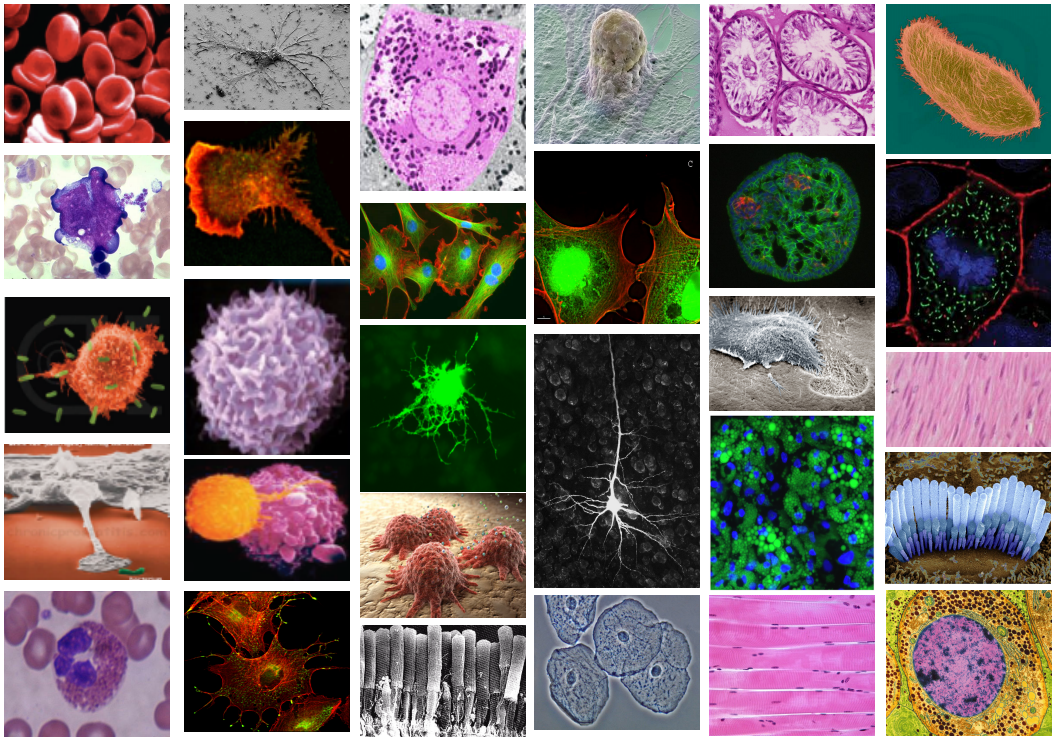
Sequencing: applications

Counting applications
- Profiling
  - microRNAs
  - Immunogenomics
  - Transcriptomics
- Epigenomics
  - Map histone modifications
  - Map DNA methylation

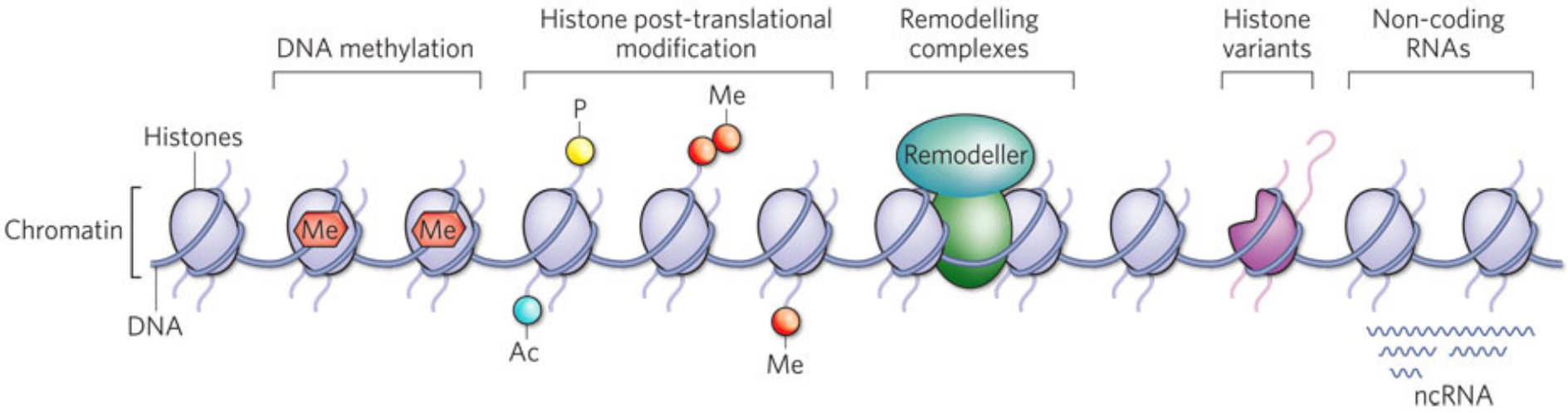
- Genome assembly
- Ancient DNA (Neanderthal)
- Pathogen discovery
- Metagenomics

Polymorphism/mutation discovery: Whole genome OR Targeted
  - Bacteria
  - Genome dynamics
  - Exon (and other target) sequencing
  - Disease gene sequencing
- Normal human variation and association studies
- Human genetics and gene discovery
- Cancer genomics
  - Map translocations, CNVs, structural changes
  - Profile somatic mutations

How does a single genome gives rise to more than 200 different cells?

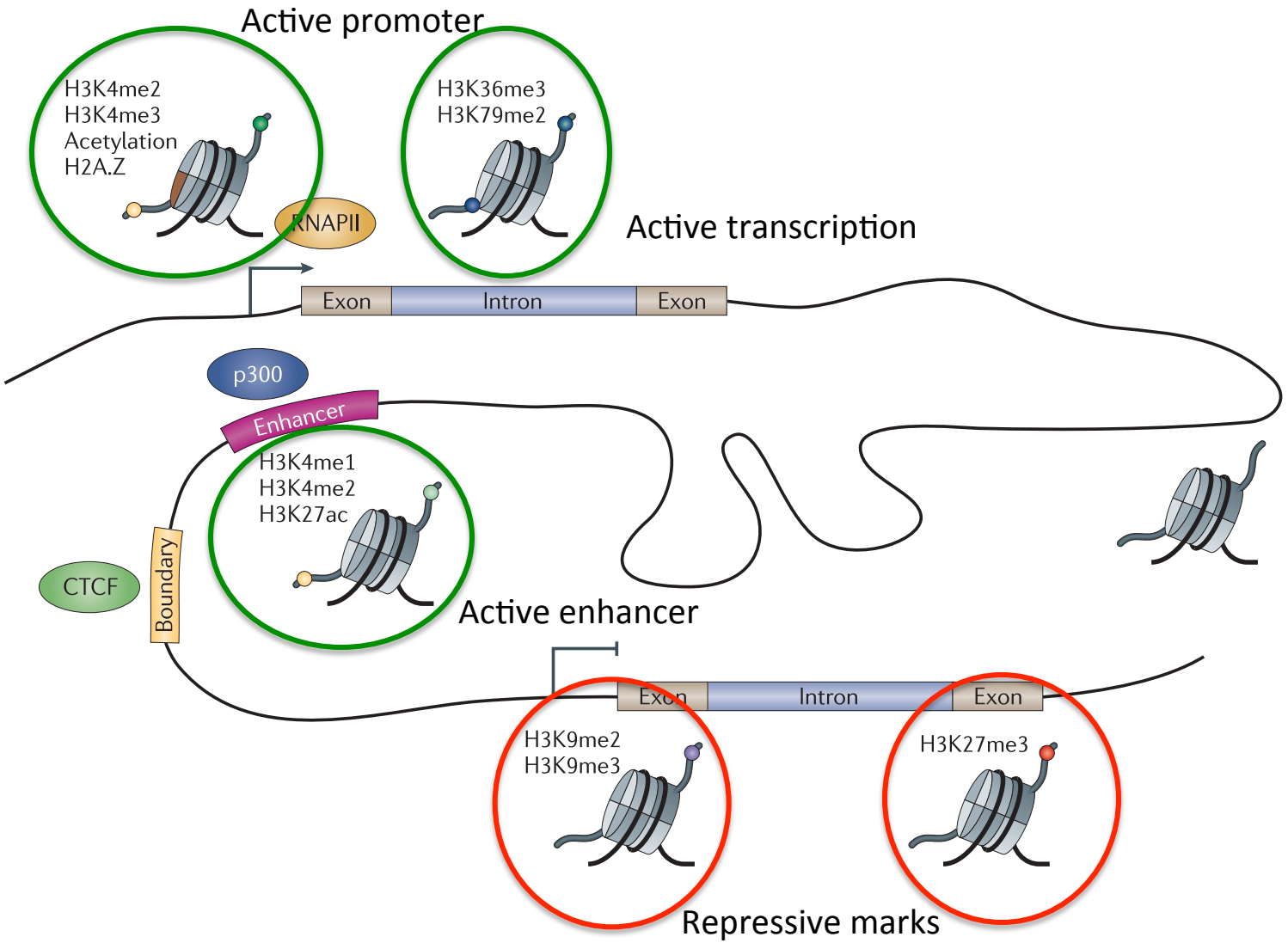


Cell identity is determined by its epigenetic state



Catherine Dulac, Nature 2010

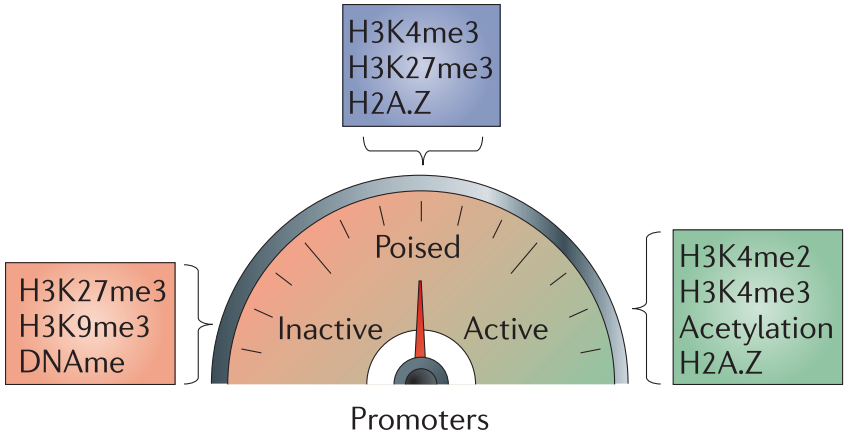
Which controls the genome functional elements

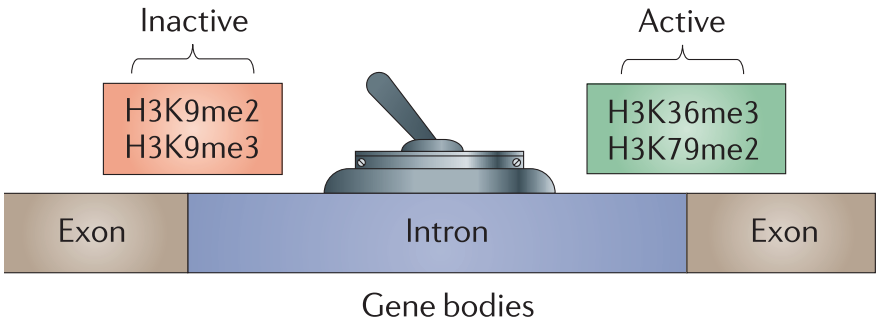


Motivation: find the genome state using sequencing data

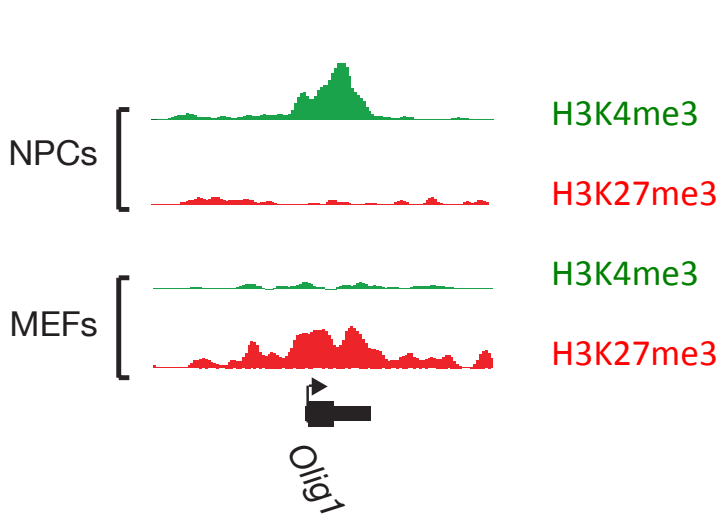
Zhou, Goren Berenstein, Nature Rev. Genetics 2011

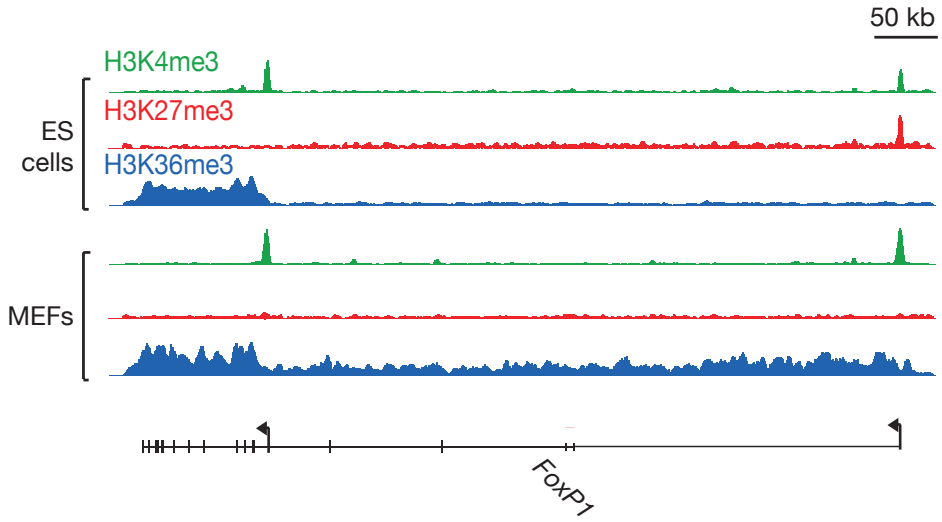
How does it work?





Zhou, Goren Berenstein, Nature Rev. Genetics 2011

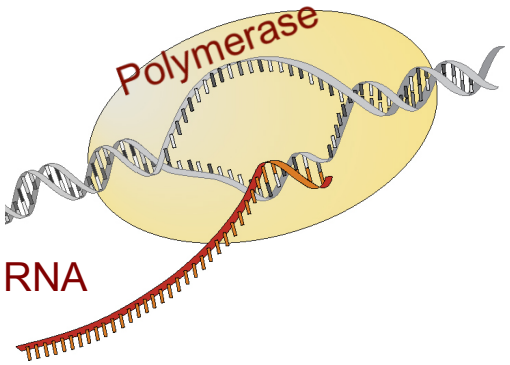




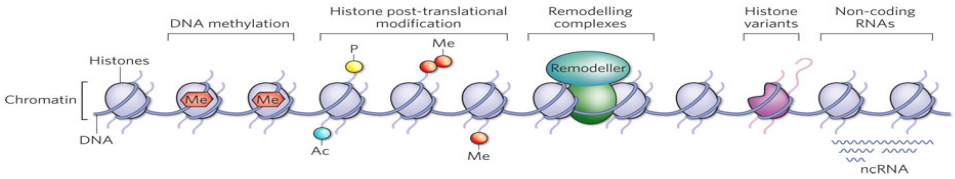
Mikkelsen et al, Nature 2007

Goal: Find the genome state and output

- Transcriptomics (output)



- Epigenomics (state)
  - Open promoters (H3K4me3)
  - Active enhancers (H3K4me1, H3K27Ac)
  - Transcribed regions (PolII, H3K36me3)
  - Repressed genes (H3K27me3)



Catherine Dulac, Nature 2010

The goal of this session is to survey computational tools to analyze sequencing data to measure state and output

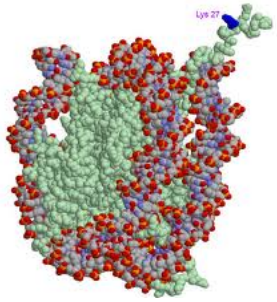
Which require three main approaches

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

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

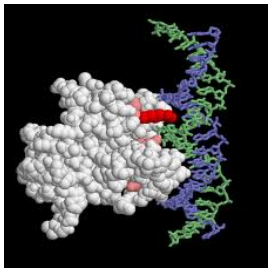
1. ChIP-Seq: Genome state

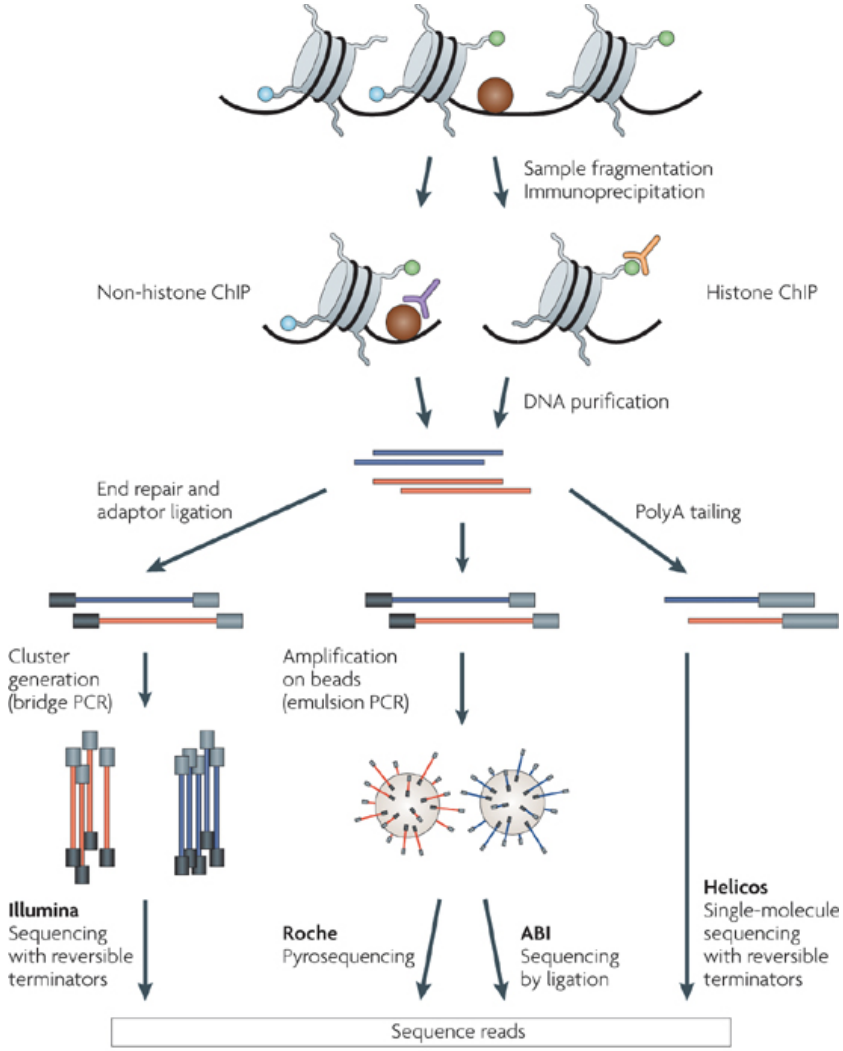
Histone Marks



ChIP-Seq

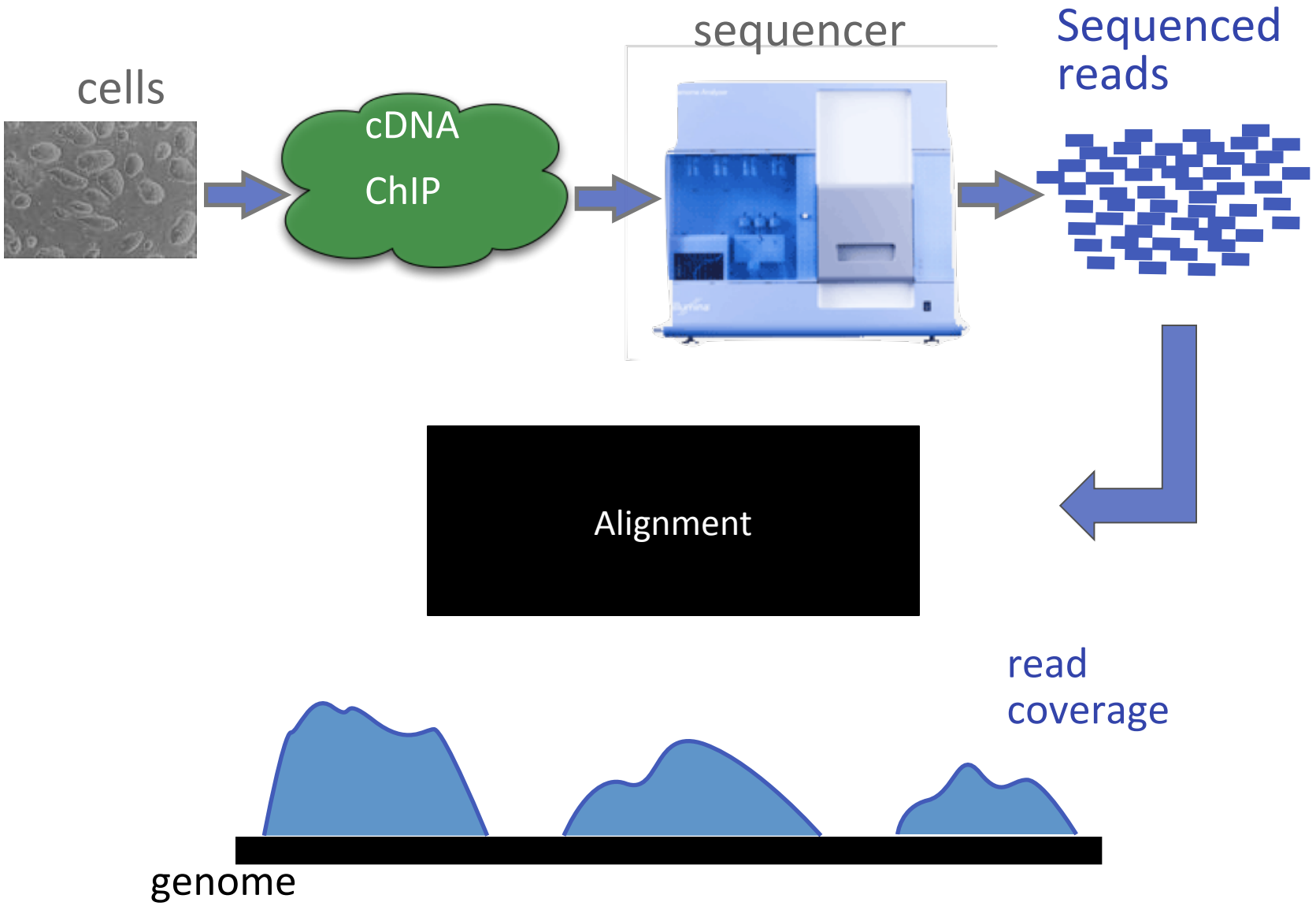
Transcription Factors





Park, P Nature Reviews | Genetics

Once sequenced the problem becomes computational



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
- Reconstruction: Finding the regions that originated the reads
- Quantification:
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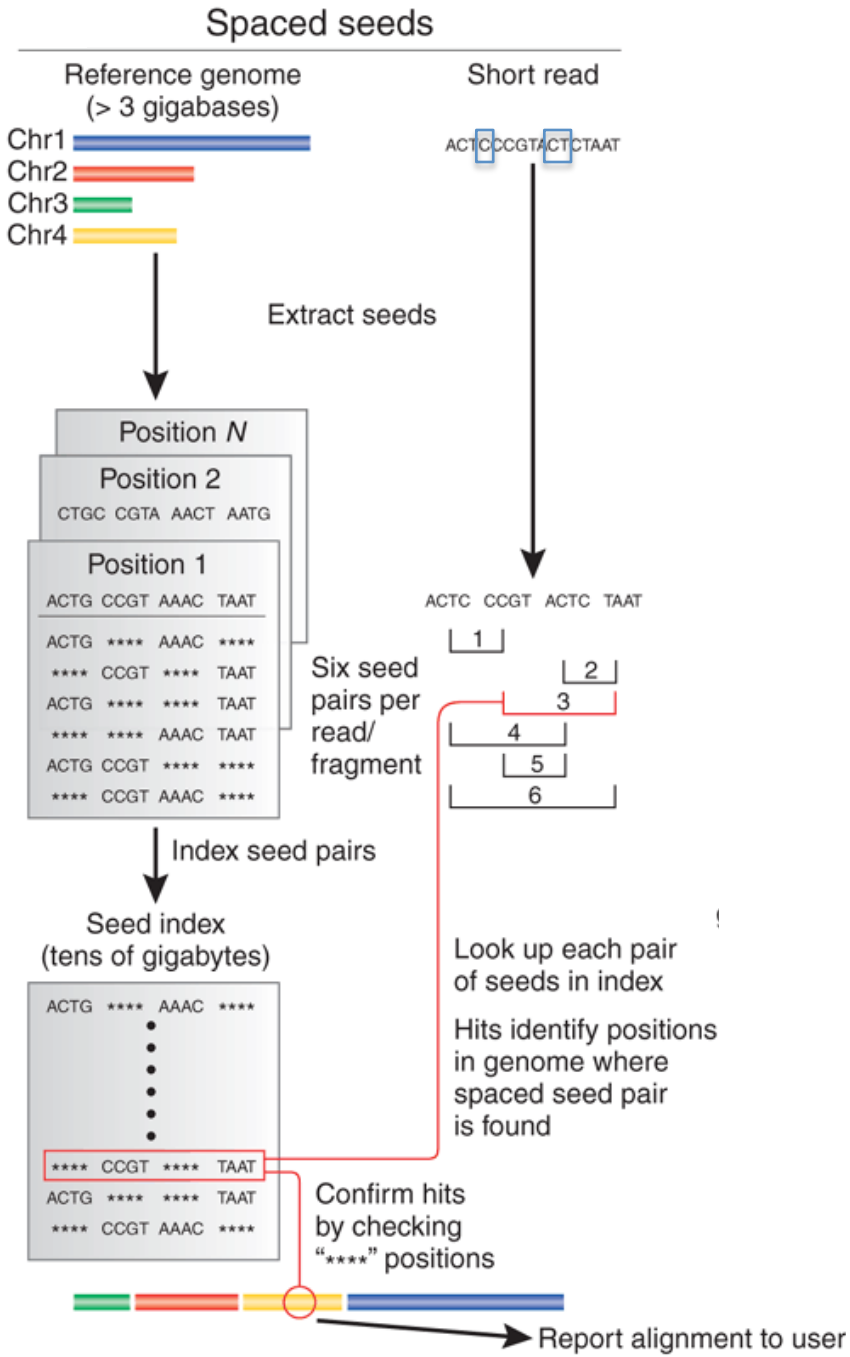


Trapnell, Salzberg, Nature Biotechnology 2009

Spaced seed alignment – Hashing the genome

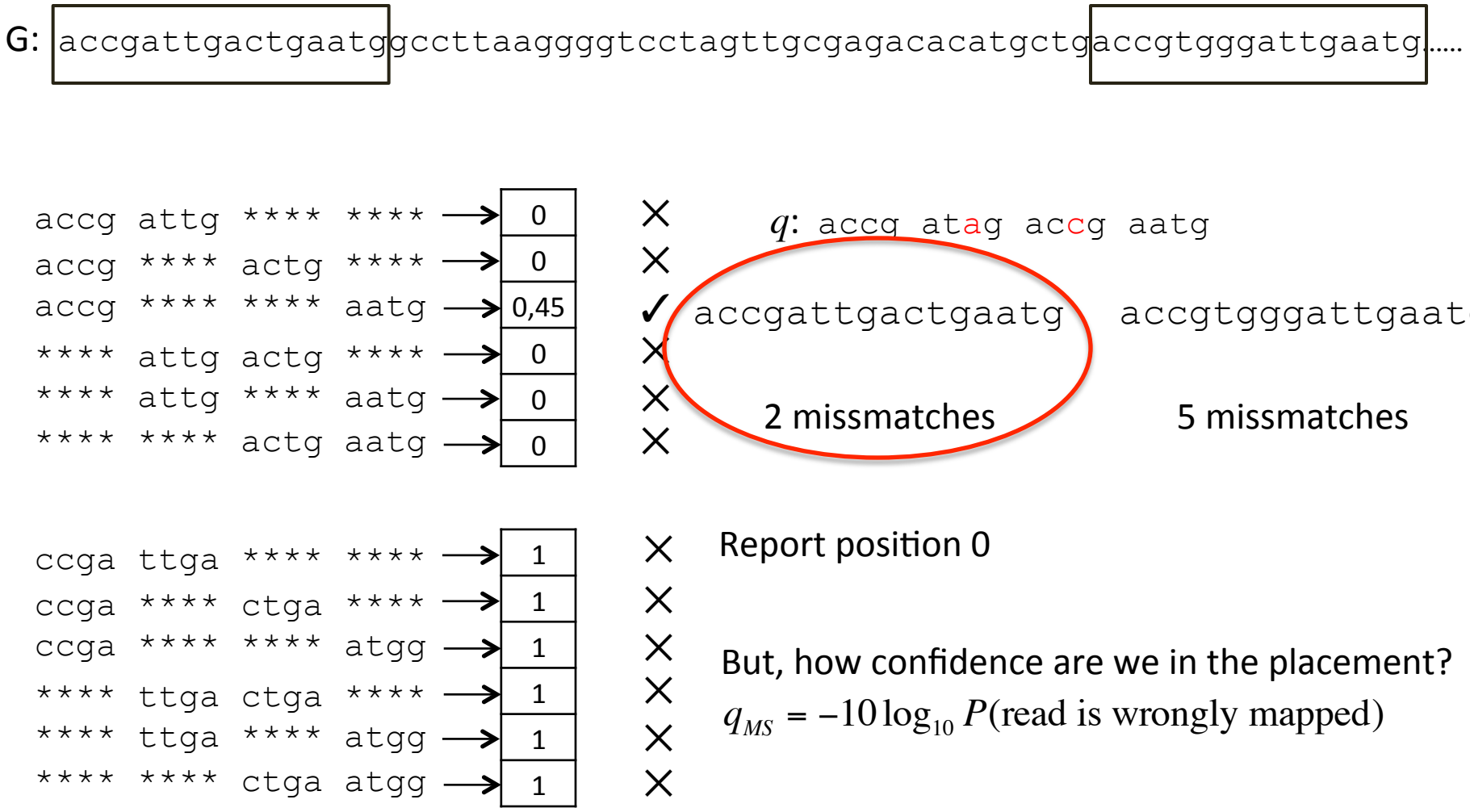
G: accgattgactgaatggccttaaggggtcctagttgcgagacacatgctgaccgtgggattgaatg……

Store spaced seed positions

accg attg **** **** →	0
accg **** actg **** →	0
accg **** **** aatg →	0,45
**** attg actg **** →	0
**** attg **** aatg →	0
**** **** actg aatg →	0

ccga ttga **** **** →	1
ccga **** ctga **** →	1
ccga **** **** atgg →	1
**** ttga ctga **** →	1
**** ttga **** atgg →	1
**** **** ctga atgg →	1

Spaced seed alignment – Mapping reads



Mapping quality

What does $q_{MS} = -10\log_{10} P(\text{read is wrongly mapped})$ mean?

Lets compute the probability the read originated at genome position i

q: accg atag accg aatg

q_s: 30 40 25 30 30 20 10 20 40 30 20 30 40 40 30 25

$q_s[k] = -10\log_{10} P(\text{sequencing error at base k})$, the PHRED score. Equivalently:

$$P(\text{sequencing error at base k}) = 10^{-\frac{q_s[k]}{10}}$$

So the probability that a read originates from a given genome position i is:

$$P(q \mid G,i) = \prod_{j\ \text{match}} P(q_j \text{good call}) \prod_{j\ \text{missmatch}} P(q_j \text{bad call}) \approx \prod_{j\ \text{missmatch}} P(q_j \text{bad call})$$

In our example

$$P(q \mid G,0) = \left[(1-10^{-3})^6 (1-10^{-4})^4 (1-10^{-2.5})^2 (1-10^{-2})^2\right]\left[10^{-1}10^{-2}\right] = [0.97]*[0.001] \approx 0.001$$

Mapping quality

What we want to estimate is $q_{MS} = -10 \log_{10} P(\text{read is wrongly mapped})$

That is, the posterior probability, the probability that the region starting at i was sequenced *given* that we observed the read *q*:

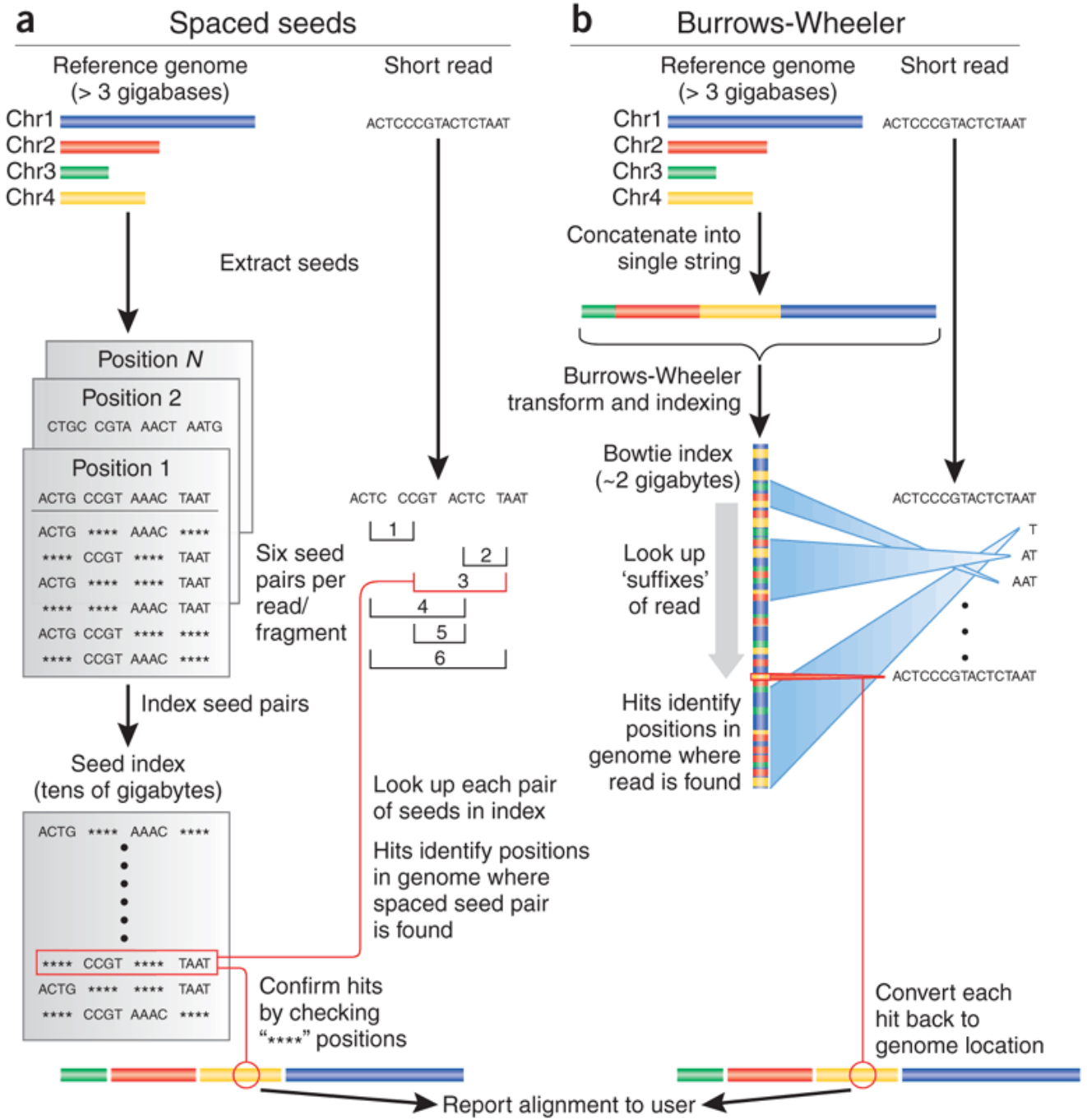
$$P(i \mid G,q) = \frac{P(q \mid G,i)P(i \mid G)}{P(q \mid G)} = \frac{P(q \mid G,i)P(i \mid G)}{\sum_j P(q \mid G,j)}$$

Fortunately, there are efficient ways to approximate this probability (see Li, H *genome Research* 2008, for example)

$$q_{MS} = -10 \log_{10}(1 - P(i \mid G,q))$$

Considerations

- Trade-off between sensitivity, speed and memory
  - Smaller seeds allow for greater mismatches at the cost of more tries
  - Smaller seeds result in a smaller tables (table size is at most 4^k), larger seeds increase speed (less tries, but more seeds)



Trapnell, Salzberg, Nature Biotechnology 2009

Considerations

- BWT-based algorithms rely on perfect matches for speed
- When dealing with mismatches, algorithms "backtrack" when the alignment extension fails.
- Backtracking is expensive
- As read length increases novel algorithms are required

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

BWT

	Use Base qual
BWA	**YES**
Bowtie	NO
Soap2	NO
Stampy*	YES
Bowtie2*	(NO)

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

***Bowtie2 breaks reads into smaller pieces and maps these "seeds" using a BWT genome.**

RNA-Seq

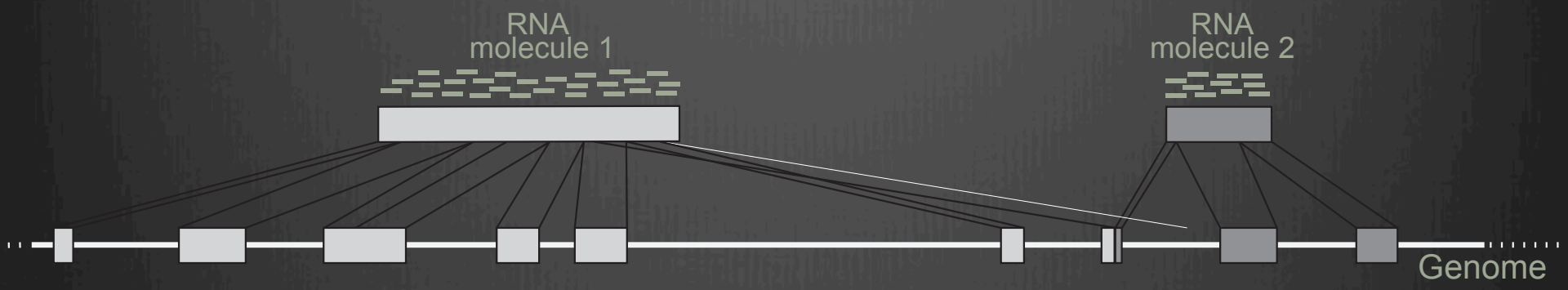
What's the fuss

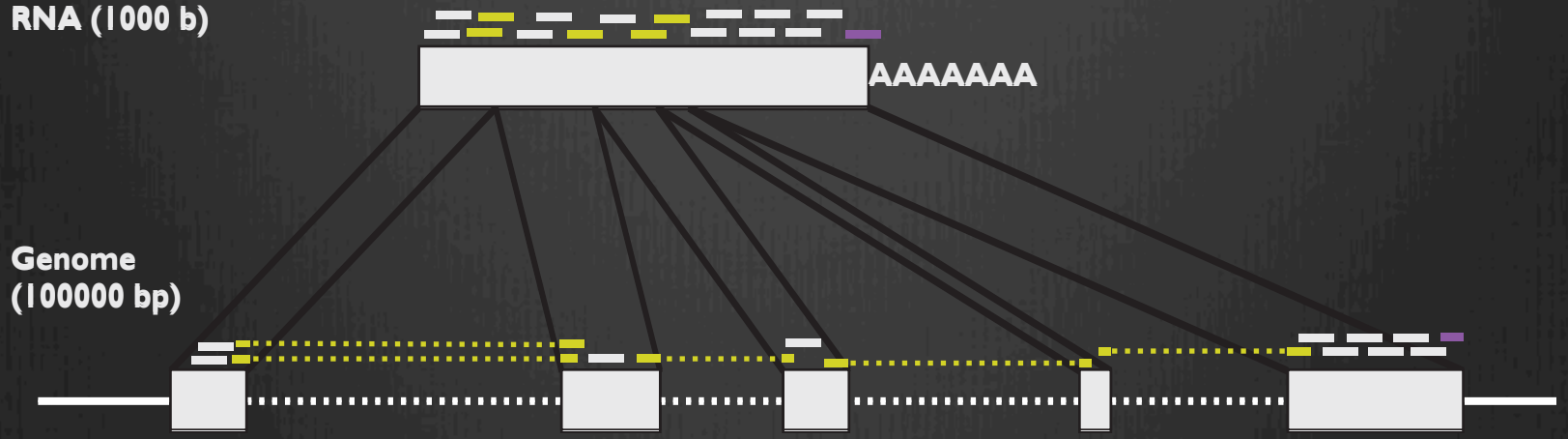
	Expression arrays	Exon Arrays	Tiling Arrays	**RNASeq**
	✓	✗	✗	✓
	✗	✓	✗	✓
	✗	✗	✓	✓

- ~~Until recently transcriptomics required:~~
  - ~~A "finished" grade genome~~
  - ~~A clone based cDNA and EST annotation~~

RNASeq requires none

RNA-Seq Read mapping





Mapping RNA-Seq reads: Seed-extend spliced alignment (e.g. GSNAP)

Mapping RNA-Seq reads: Exon-first spliced alignment (e.g. TopHat)

Short read mapping software for RNA-Seq

Seed-extend

	Short indels	Use base qual
GSNAP	**Yes**	?
QPALMA	Yes	NO
BLAT	Yes	NO

Exon-first

	Use base qual
MapSplice	NO
SpliceMap	NO
TopHat	NO

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

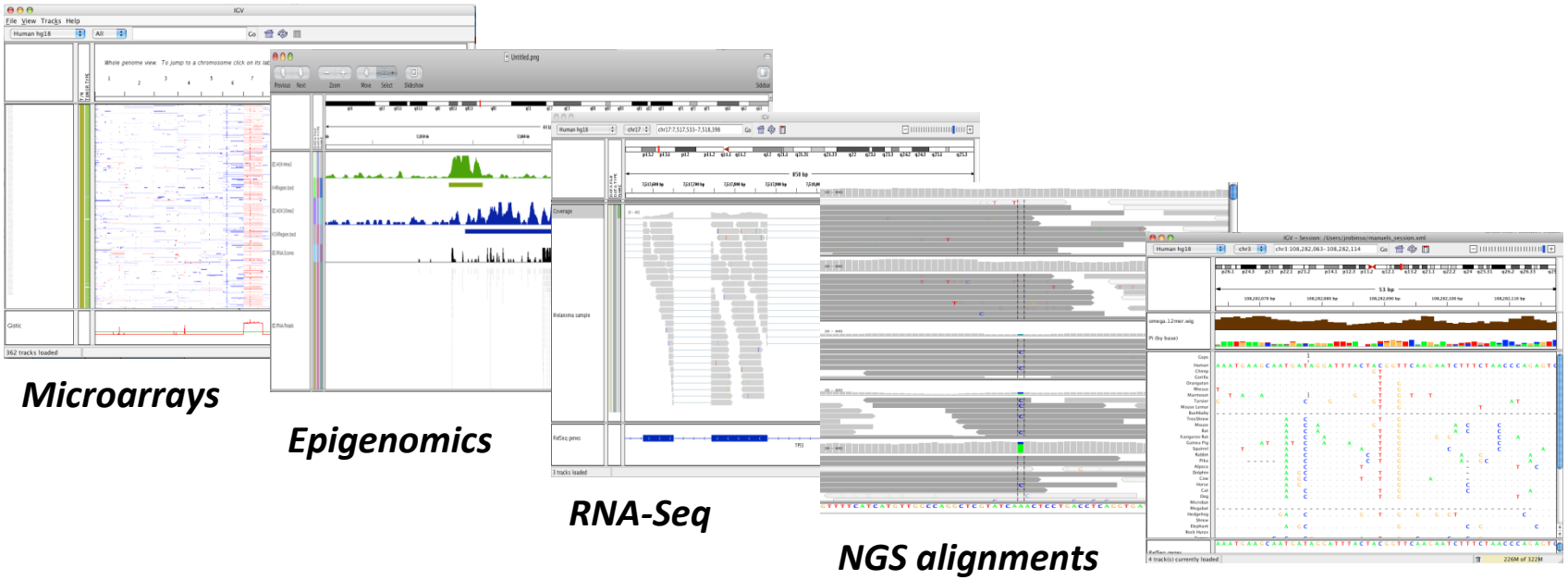
IGV: Integrative Genomics Viewer

Integrative Genomics Viewer

A desktop application

for the visualization and interactive exploration

of genomic data



Microarrays

Epigenomics

RNA-Seq

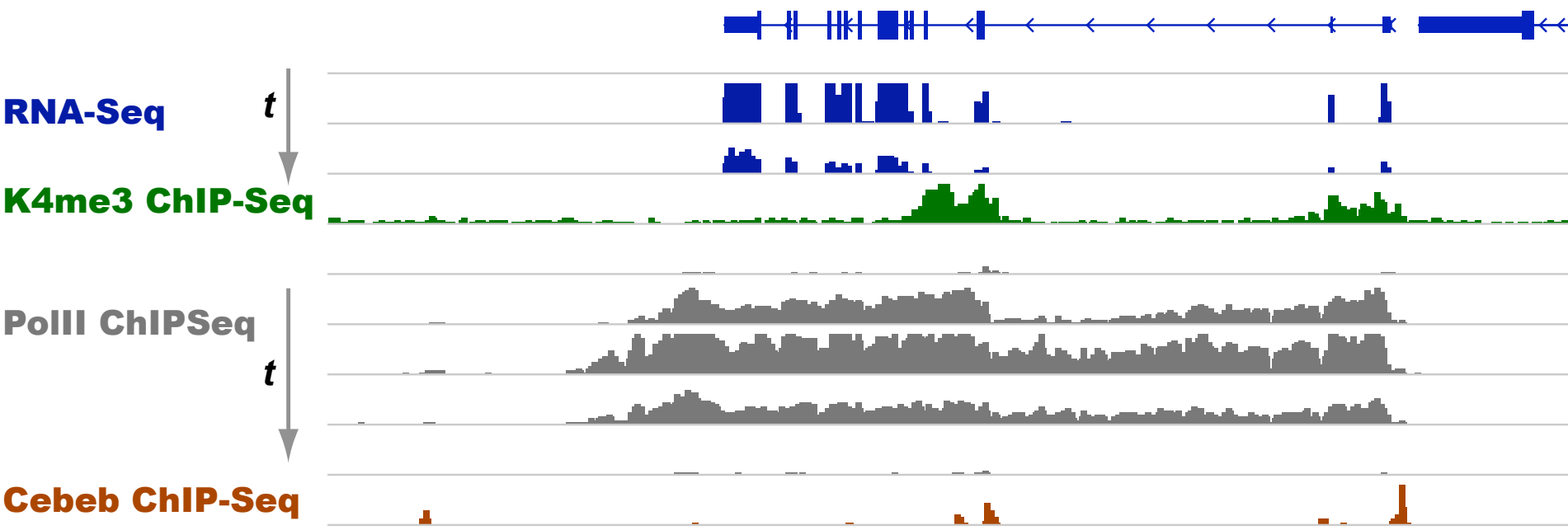
NGS alignments

Comparative genomics

BROAD INSTITUTE

Visualizing read alignments with IGV — RNASeq

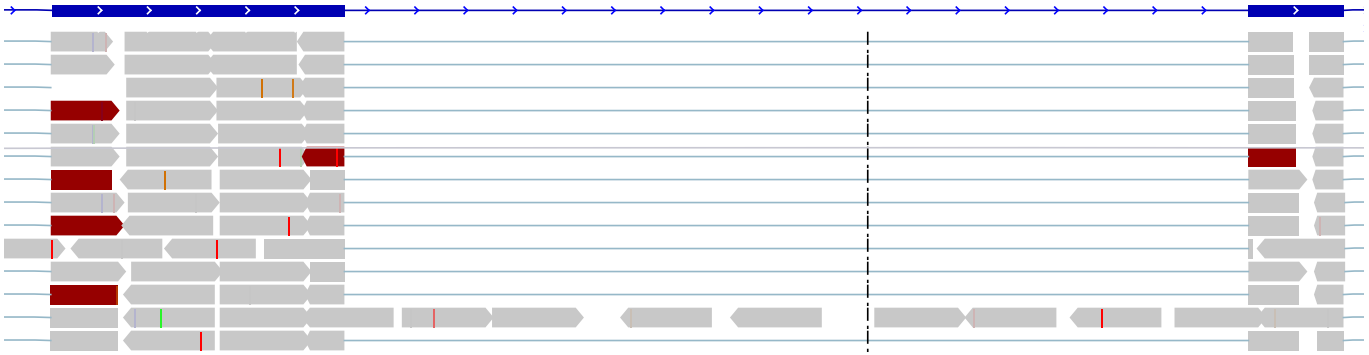
Visualizing read alignments with IGV — zooming out



Mapping longer reads

MiSeq “Bench” sequencer
~15 Million 2x250 base reads.
Ideal for **deep annotation of Targeted RNA**

Large number of expected mismatches
Given sequencing errors (>1.5%) + SNPs
expect many reads with >4 missmatches



Short (76b) reads

Long (250b) reads

Longer, reads mapping cannot be done with standard BWT based aligners

How do "short" read aligners responded to read increase?

- Break reads into seeds (e.g. 16nt every 10nt)
- Use BWT or HashTable to find candidate positions
- Prioritize candidates
- Extend top candidates using classical alignment techniques.

Aligner	Technique
TopHat2 (Bowtie2)	BWT
GSNAP	Hash Table

Overview of the session

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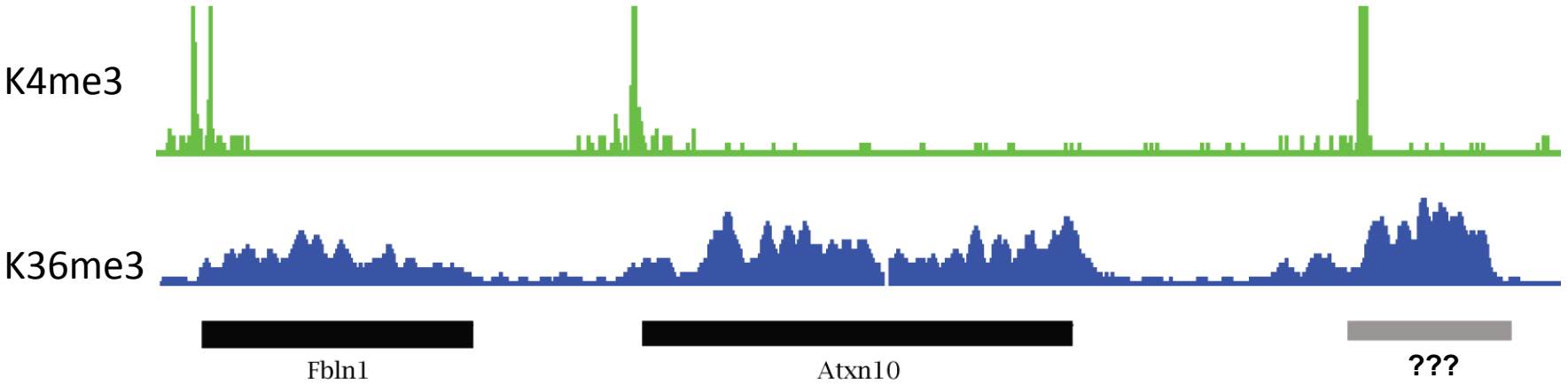
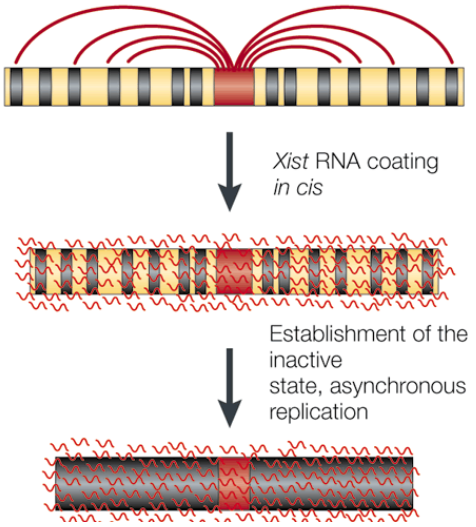
Chromatin domains demarcate interesting surprises in the transcriptome



XIST



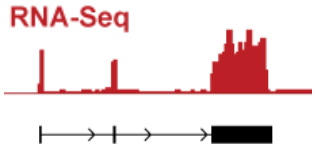
These regions likely contain similar non-coding RNA genes

Mitch Guttman

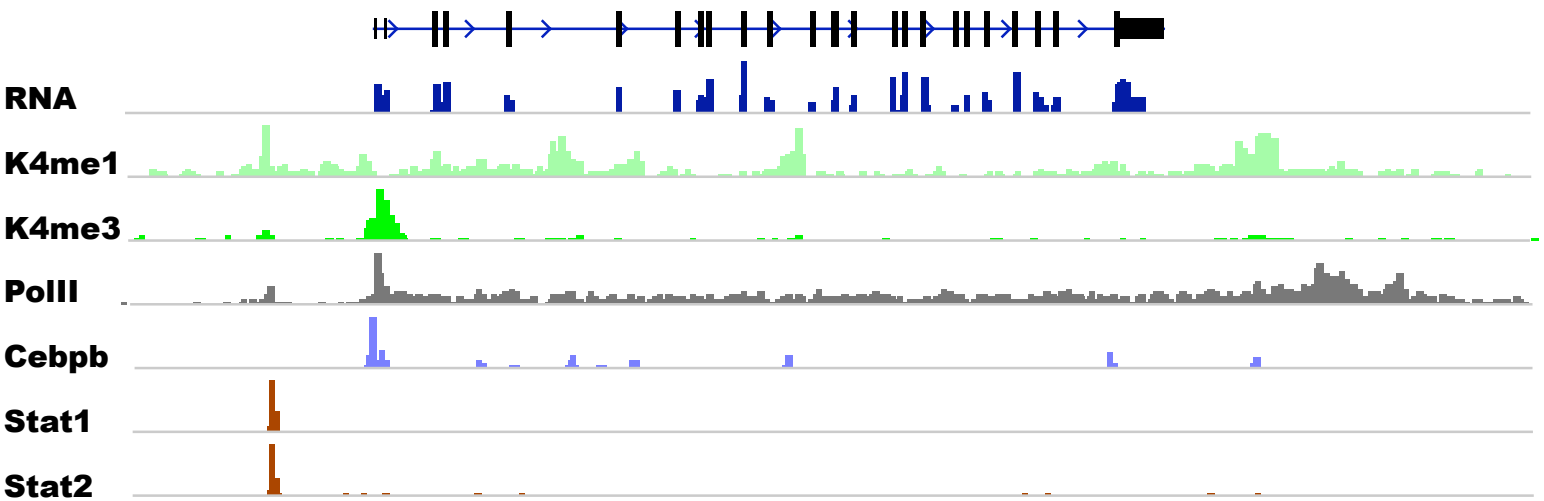
How can we identify these chromatin marks and the genes within?

Short modification

Long modification

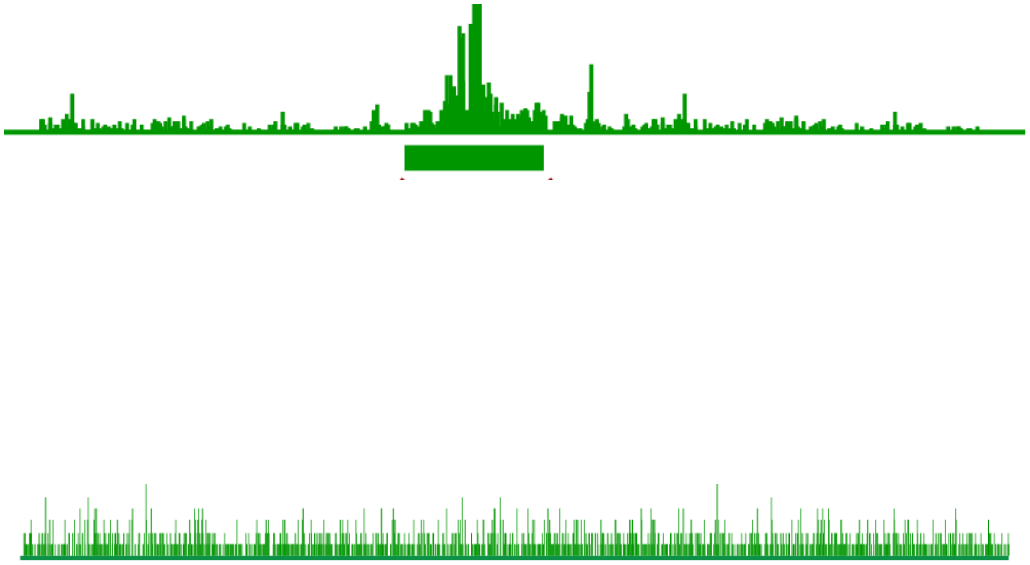


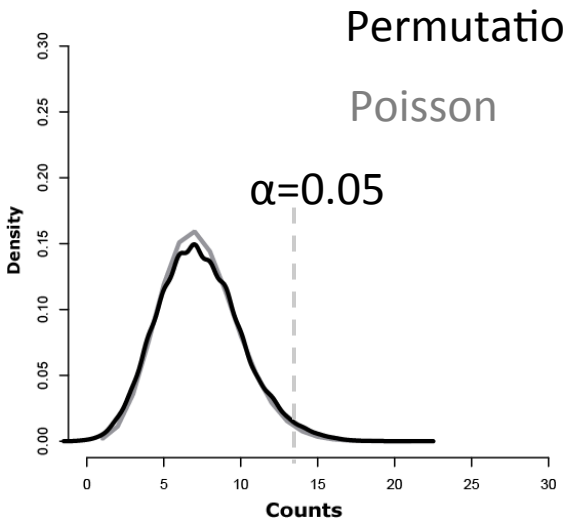
Discontinuous data



Scripture is a method to solve this general question

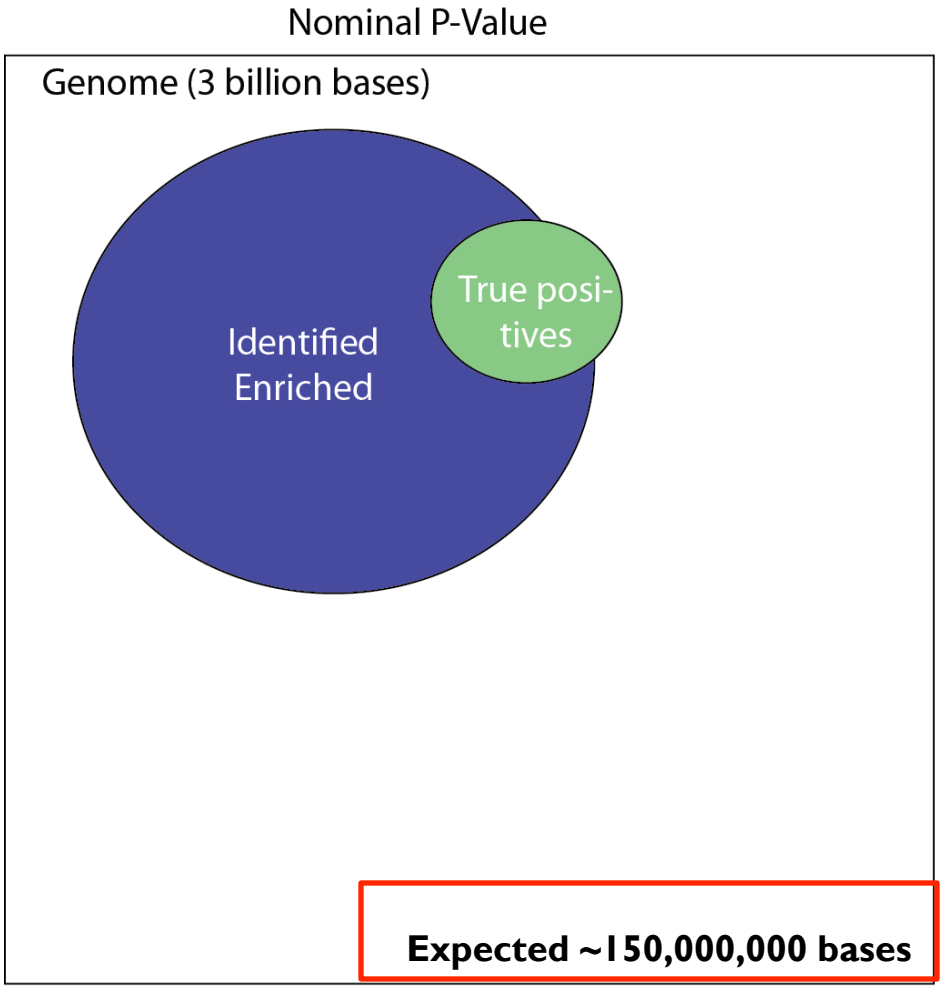
Our approach





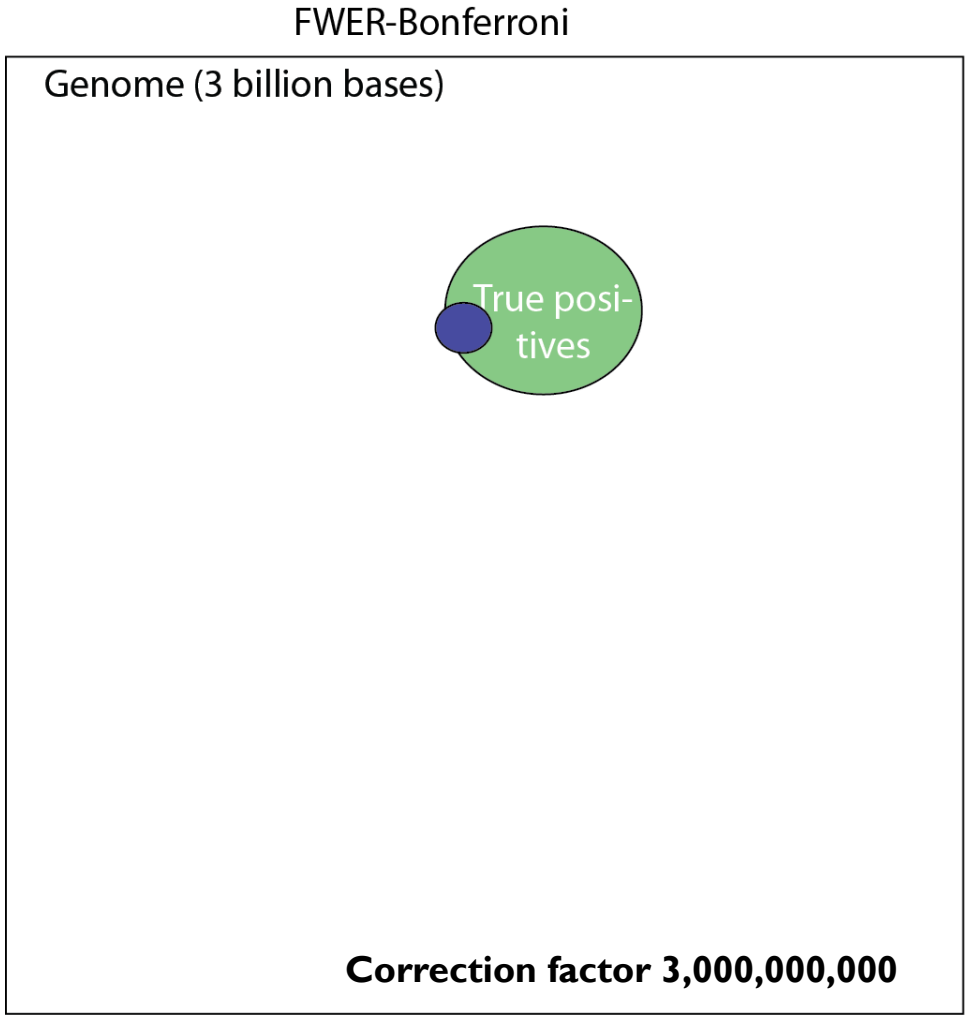
We have an efficient way to compute read count p-values …

The genome is big, many things happen by chance



We need to correct for multiple hypothesis testing

Bonferroni correction is way to conservative

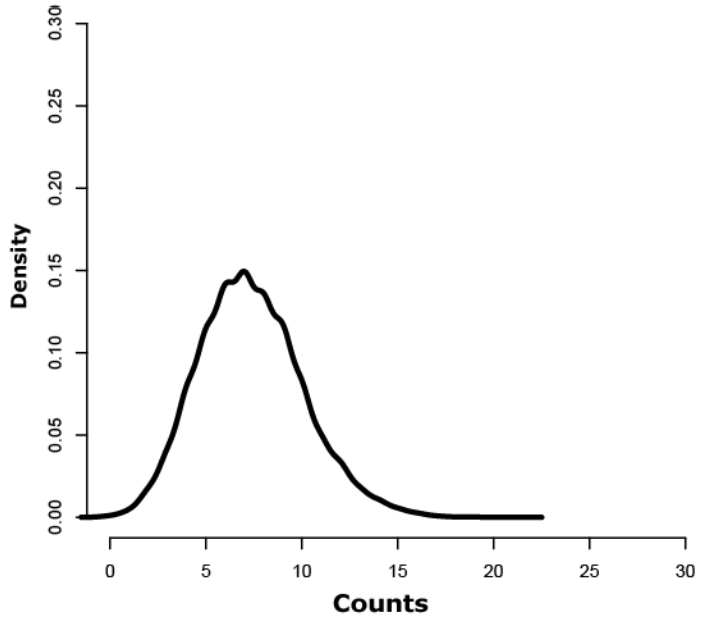


Bonferroni corrects the number of hits but misses many true hits because its too conservative – How do we get more power?

Controlling FWER

Max Count distribution

α=0.05 α_{FWER}=0.05



Count distribution (Poisson)

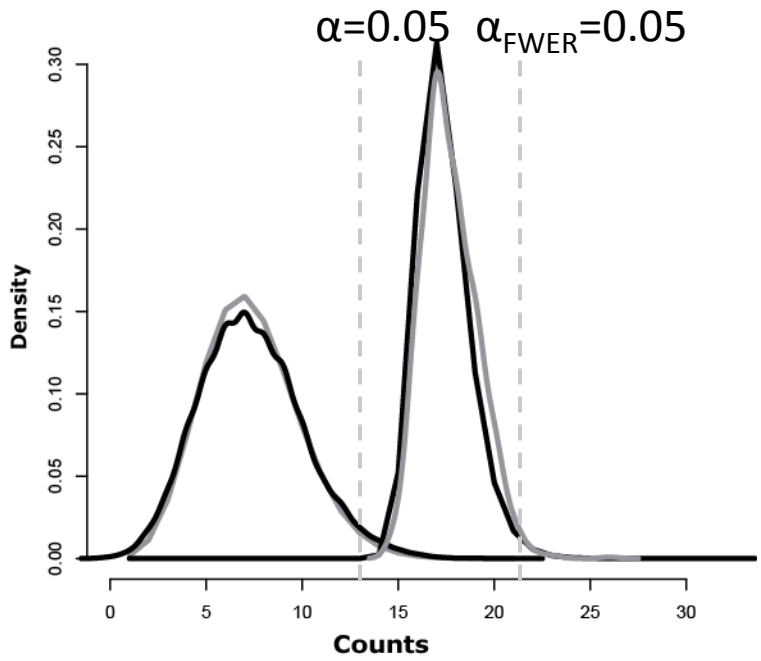
Given a region of size w and an observed read count n. What is the probability that one or more of the $3x10^9$ regions of size w has read count >= n under the null distribution?

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?

Scan distribution



Poisson distribution

Thankfully, the ***Scan Distribution*** computes a closed form for this distribution.

ACCOUNTS for dependency of overlapping windows thus more powerful!

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

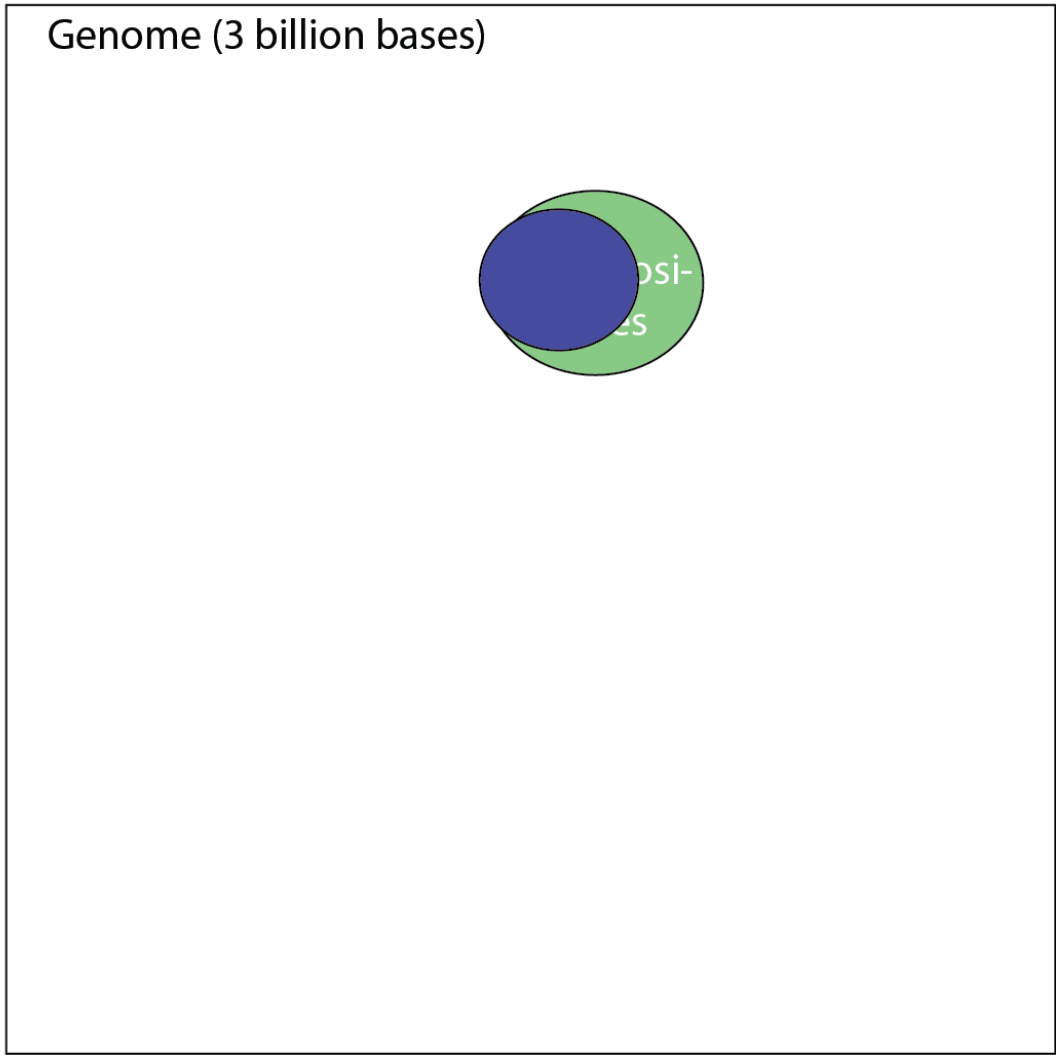
$P(k-1|\lambda w)$ is the Poisson probability of observing $k-1$ counts given an expected count of λw

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

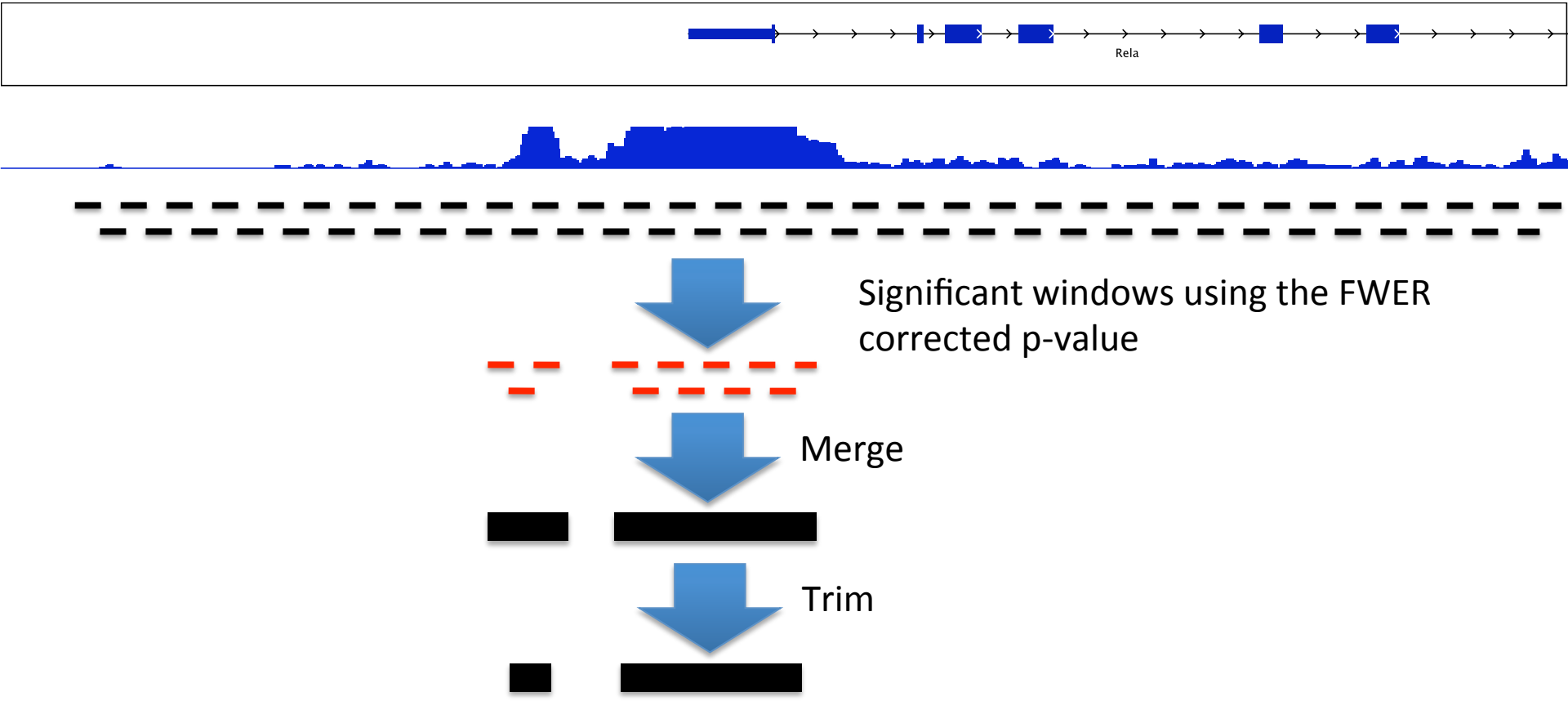
FWER-Scan Statistics



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

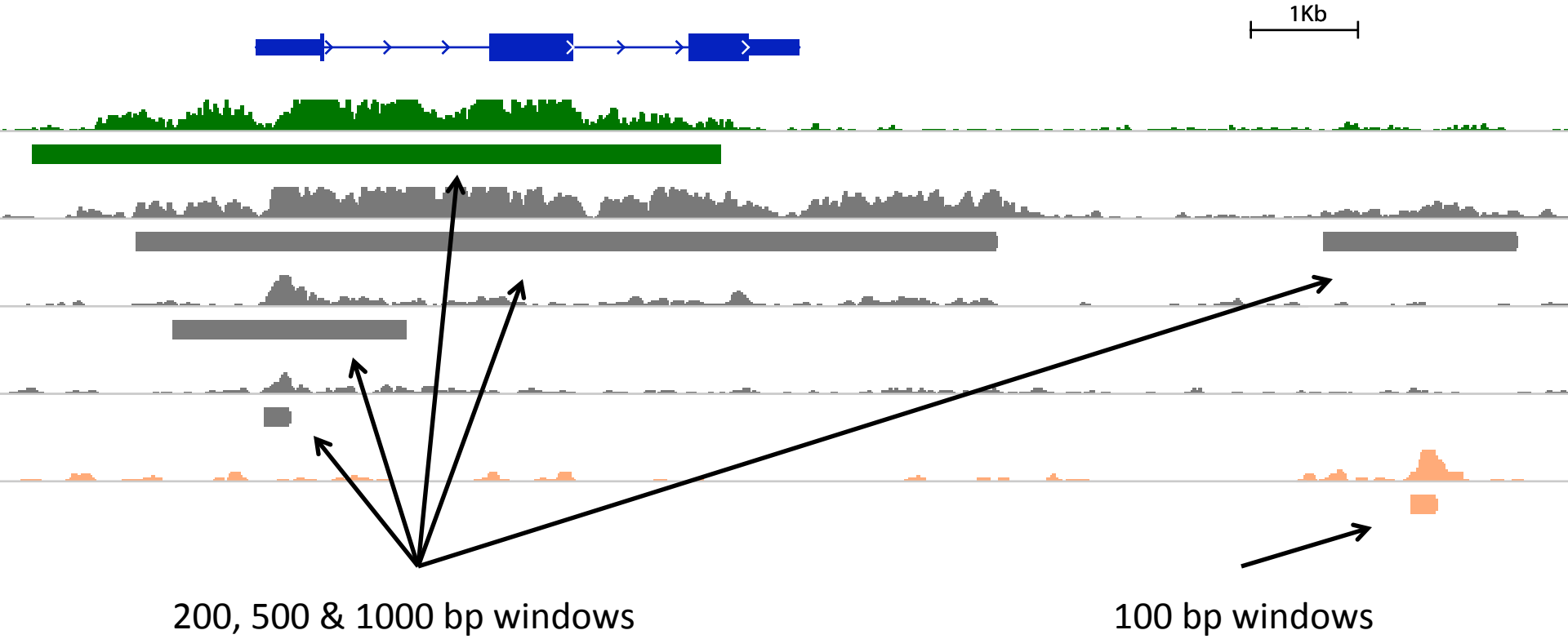


But, which window?

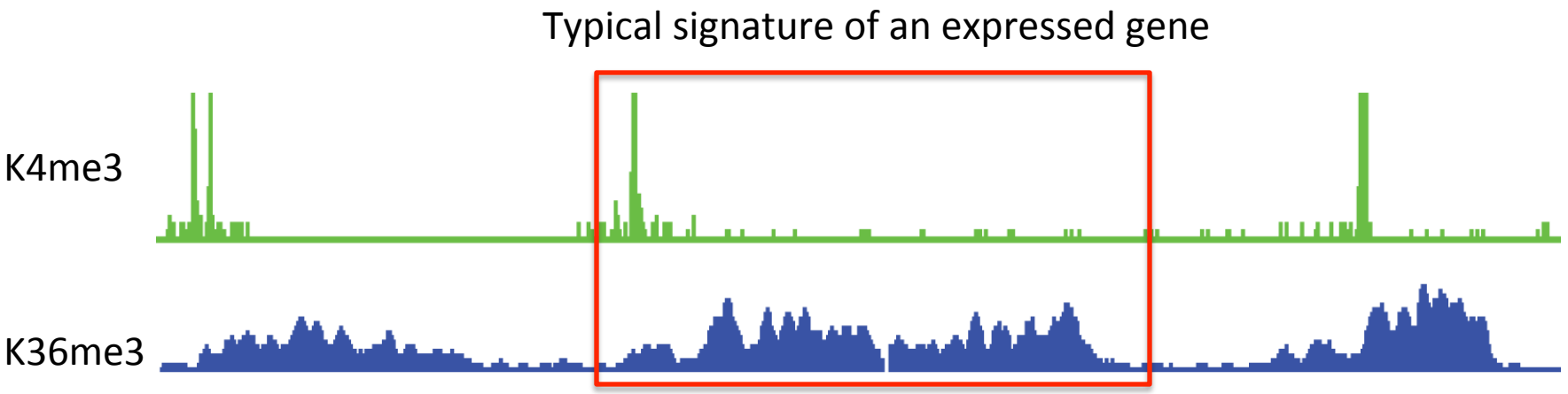
We use multiple windows

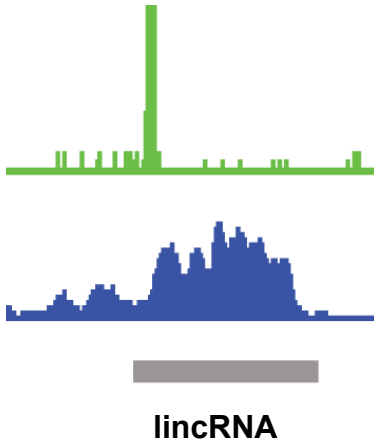
- Small windows detect small punctuate regions.
- Longer windows can detect regions of moderate enrichment over long spans.
- In practice we scan different windows, finding significant ones in each scan.
- In practice, it helps to use some prior information in picking the windows although globally it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

Applying Scripture to a variety of ChIP-Seq data



Application of scripture to mouse chromatin state maps





Identifed
- ~1500 lincRNAs
  - Conserved
  - Noncoding
  - Robustly expressed

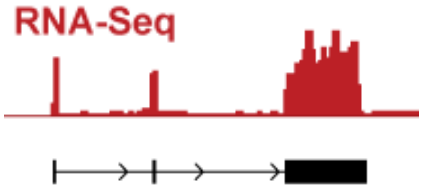
Mitch Guttman

Can we identify enriched regions across different data types?

Short modification ✓

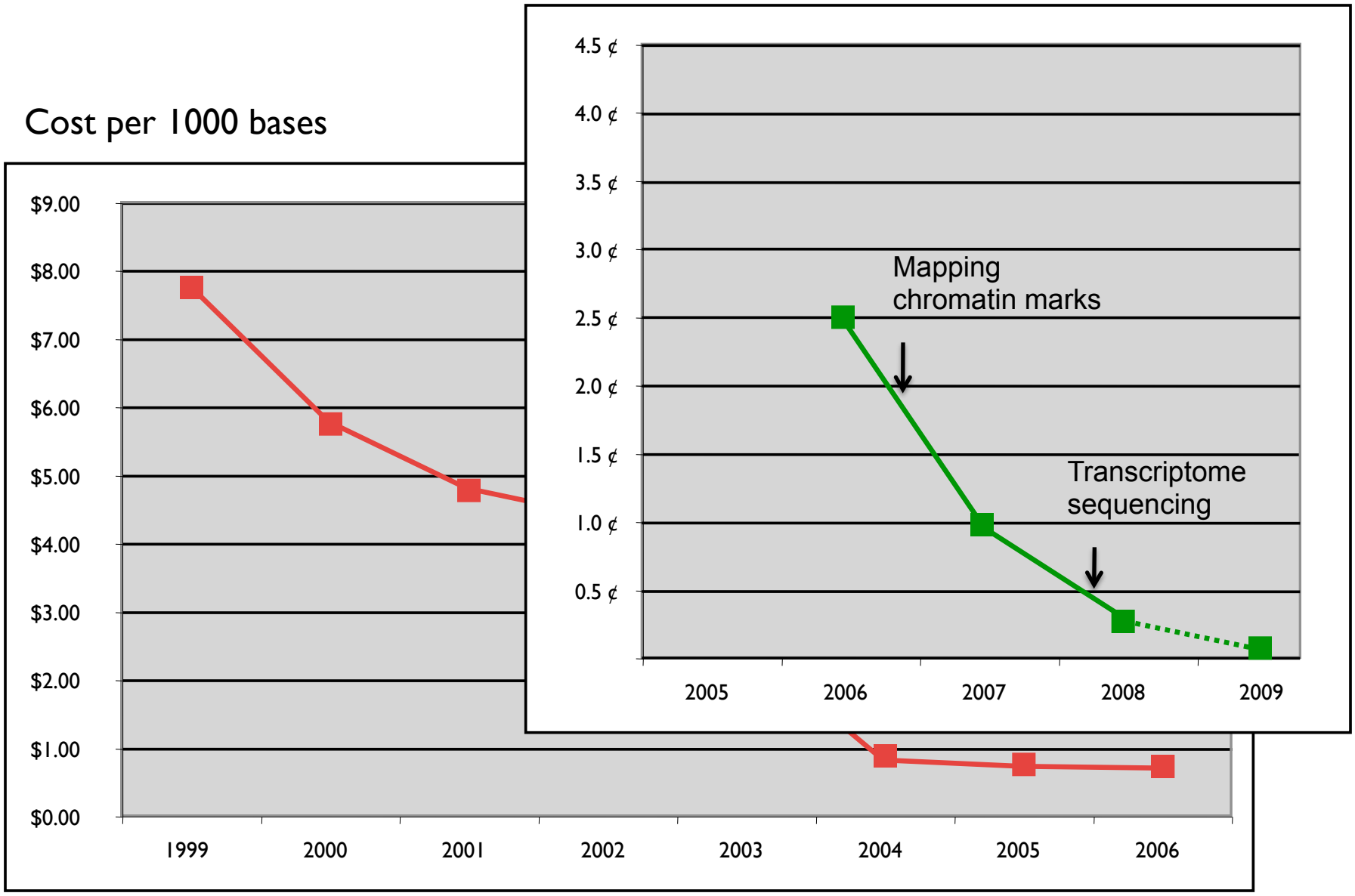
Long modification ✓

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



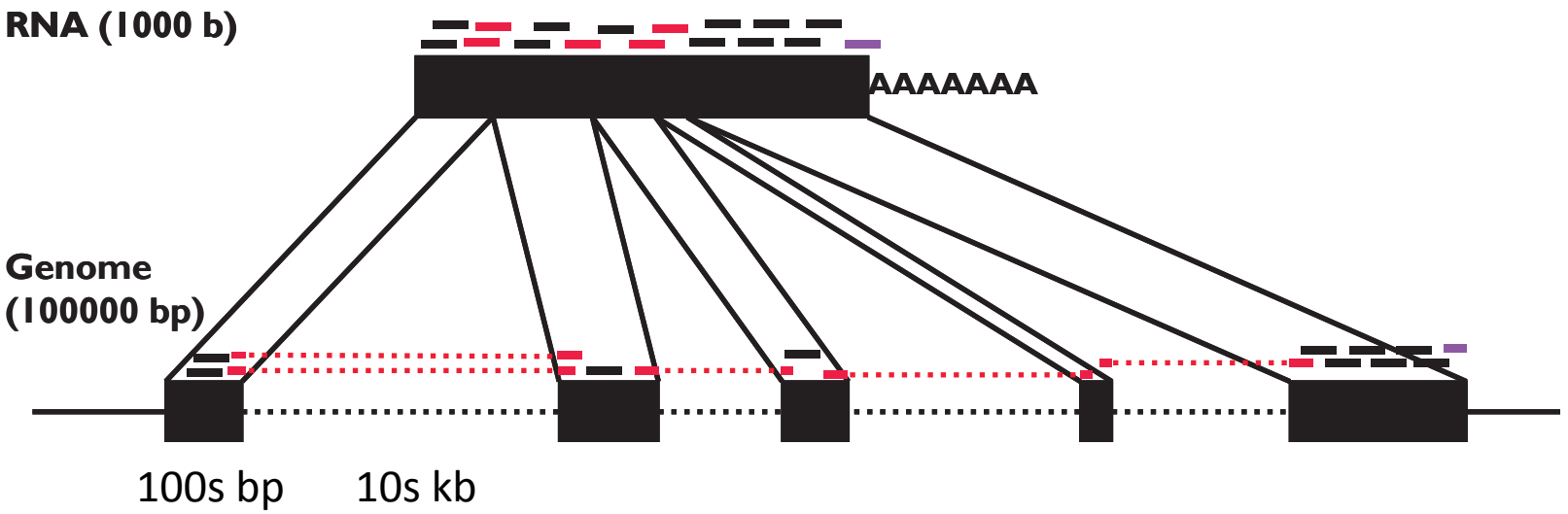
Discontinuous data: RNA-Seq to find gene structures for this gene-like regions

Enabler: Drop in cost of sequening



Scripture for RNA-Seq:
Extending segmentation to discontiguous regions

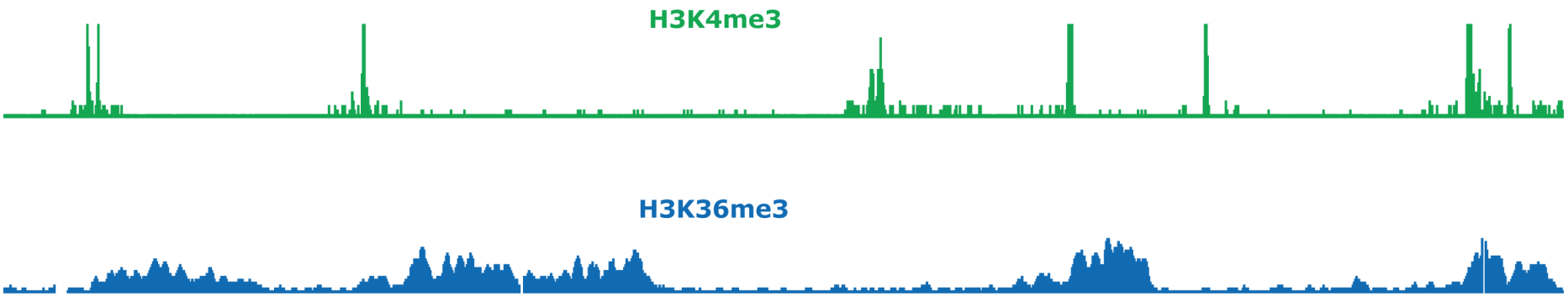
The transcript reconstruction problem as a segmentation problem



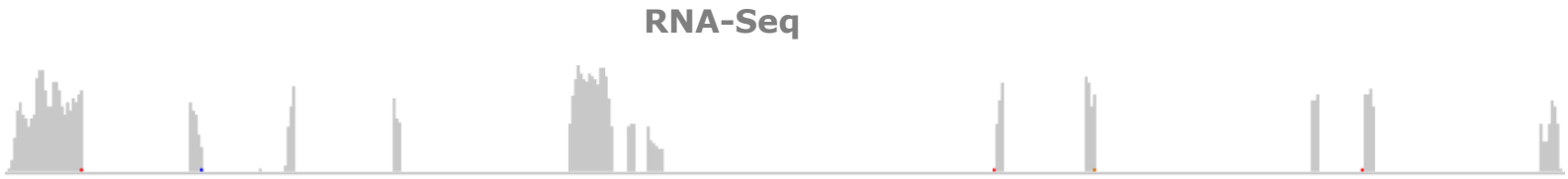
Challenges:

- 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



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

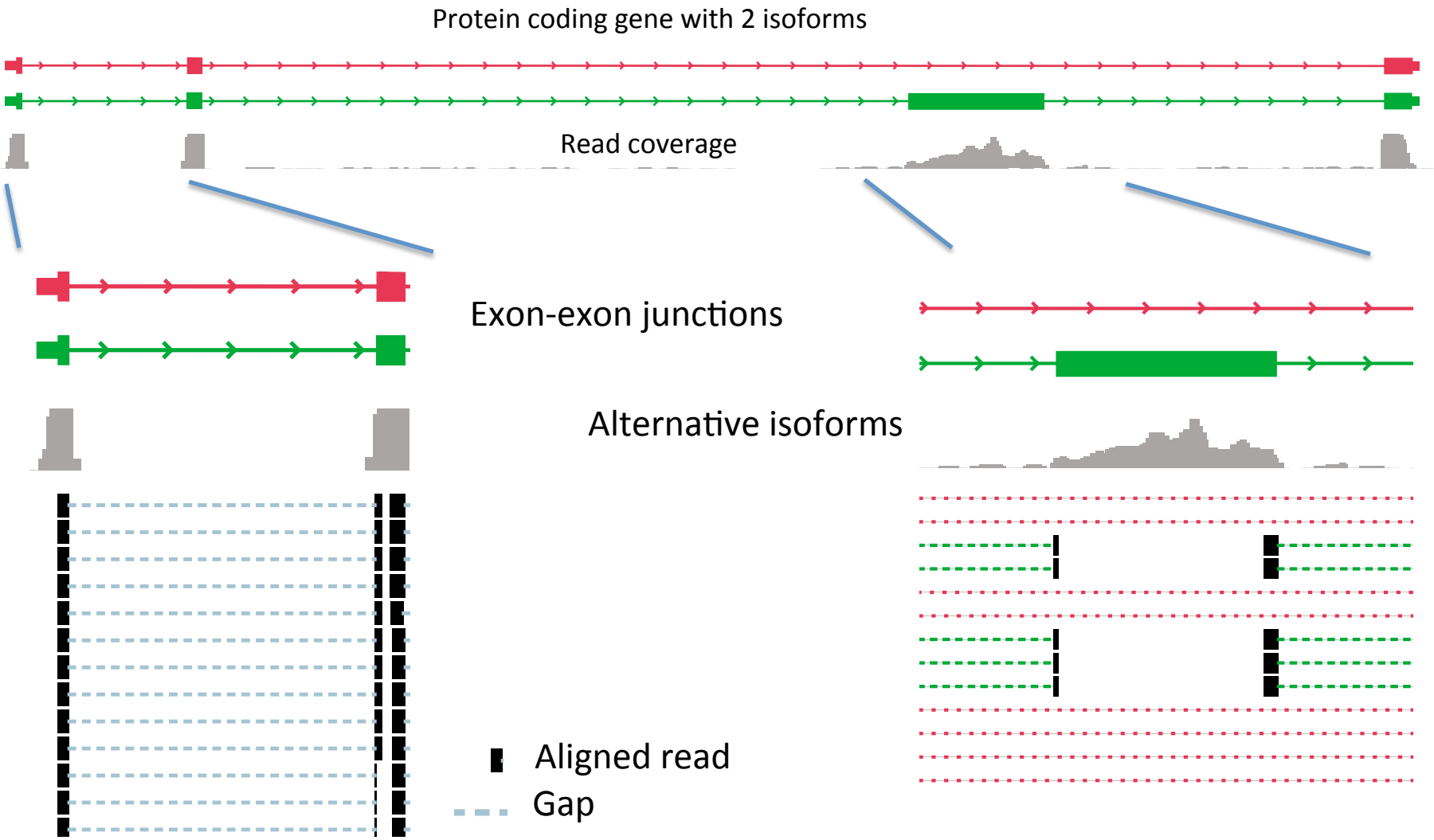


If we know the connectivity of fragments, we can increase our power to detect transcripts

Longer (76) reads provide increased number of junction reads

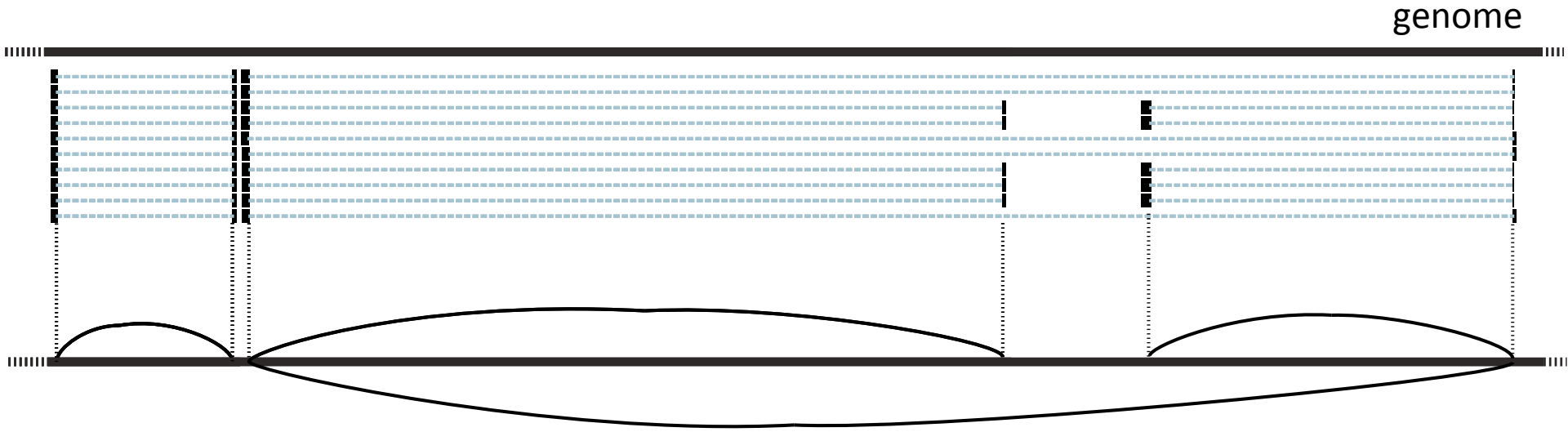
Exon junction spanning reads provide the connectivity information.

The power of spliced alignments



Statistical reconstruction of the transcriptome

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

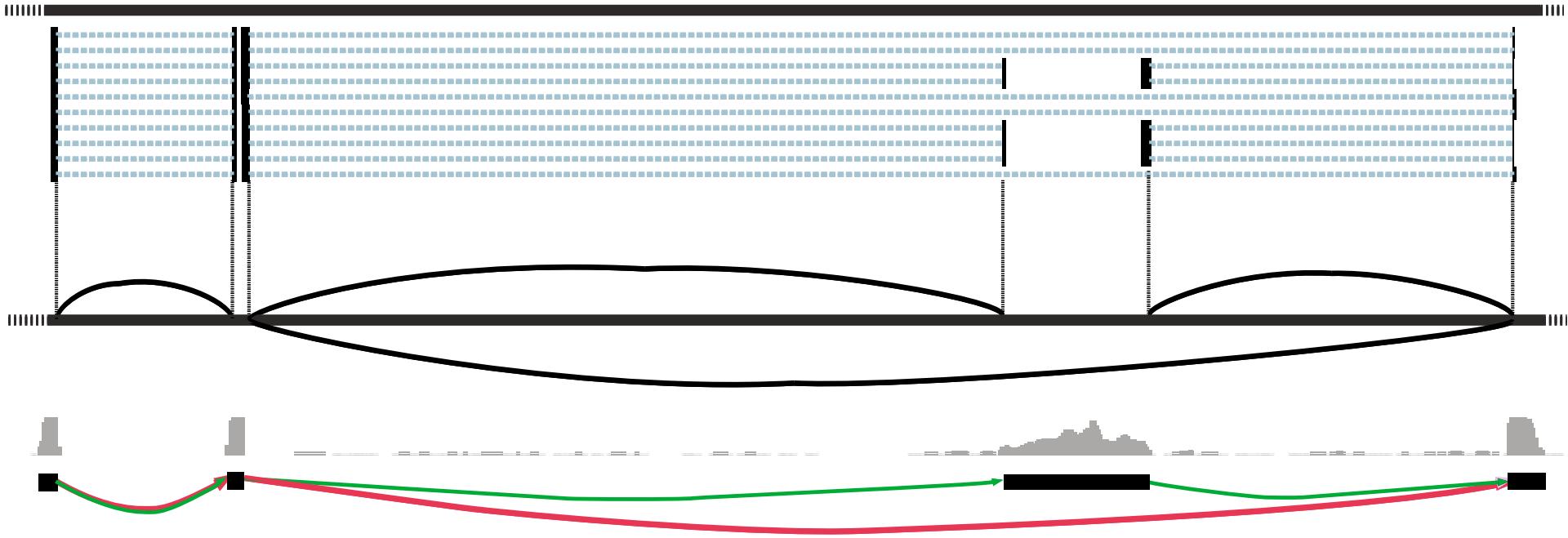


Step 2: Build an oriented connectivity graph using every spliced alignment and orienting edges using the flanking splicing motifs

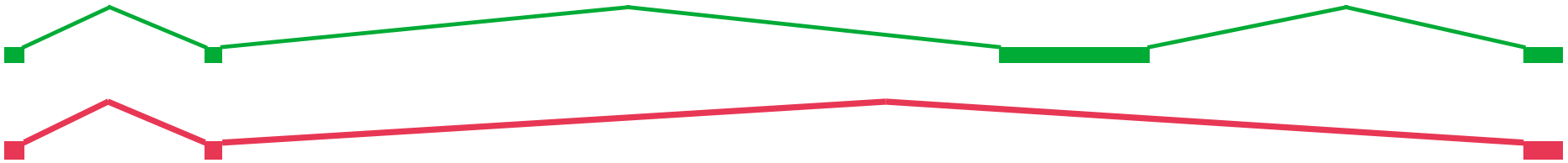
The "connectivity graph" connects all bases that are directly connected within the transcriptome

Statistical reconstruction of the transcriptome

Step 3: Identify “segments” across the graph



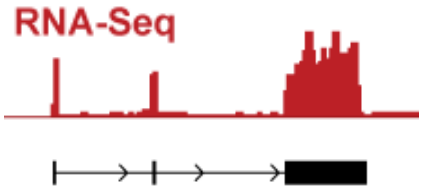
Step 4: Find significant segments



Can we identify enriched regions across different data types?

Short modification ✓

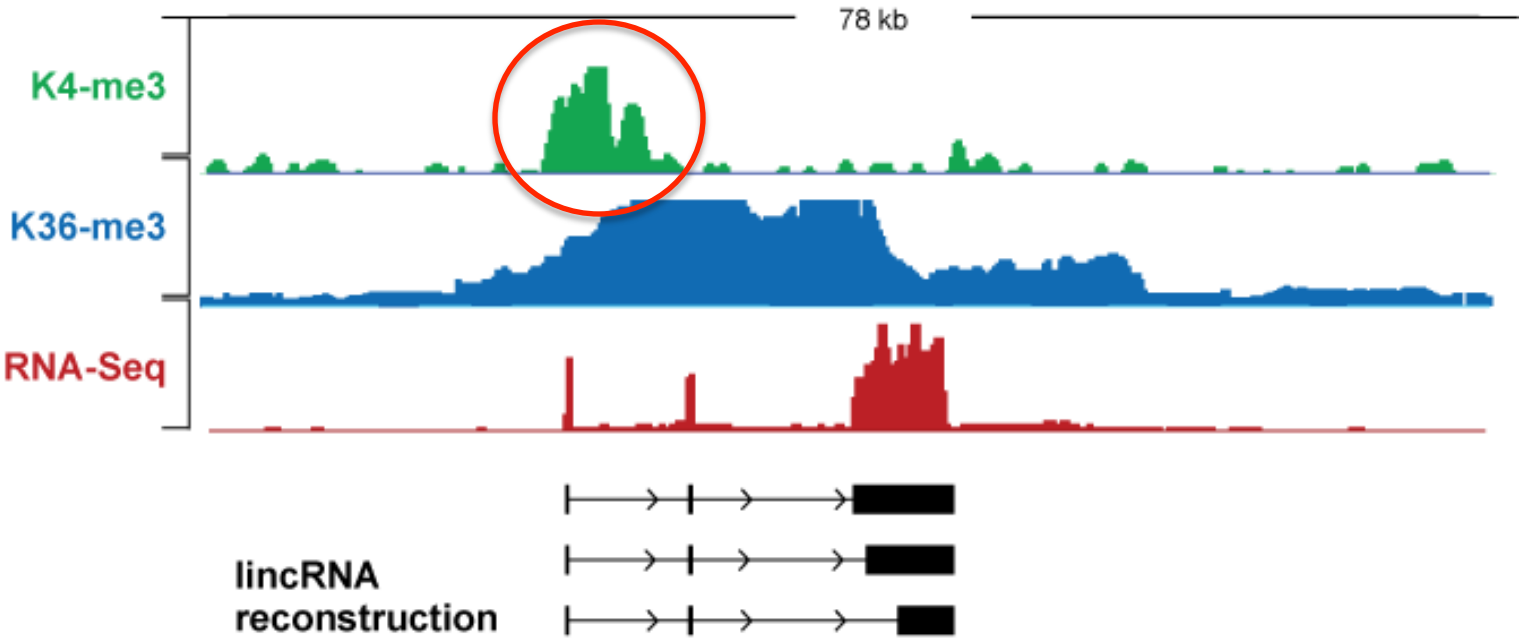
Long modification ✓



Discontinuous data ✓

Are we really sure reconstructions are complete?

RNA-Seq data is incomplete for comprehensive annotation

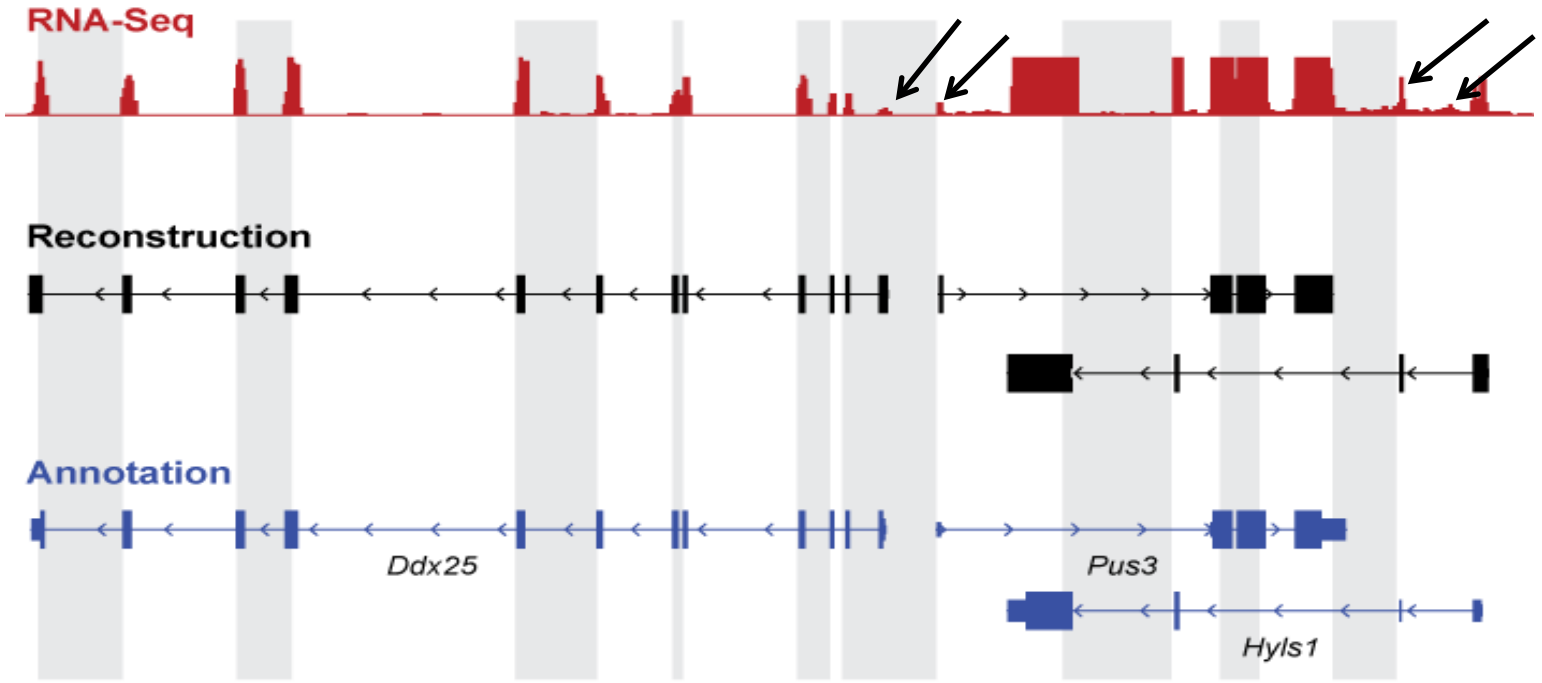


Library construction can help provide more information. More on this later

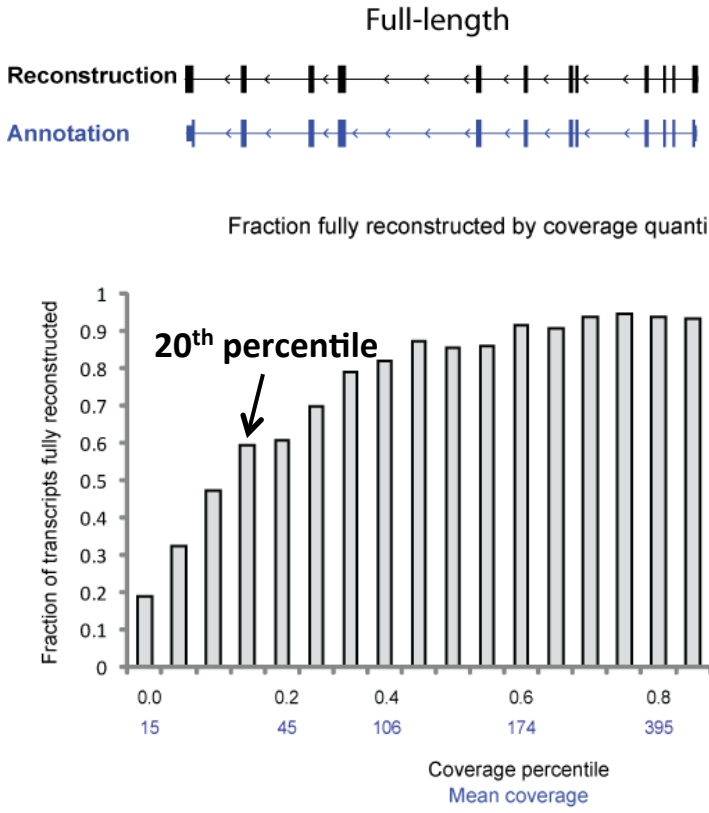
Applying scripture: Annotating the mouse transcriptome

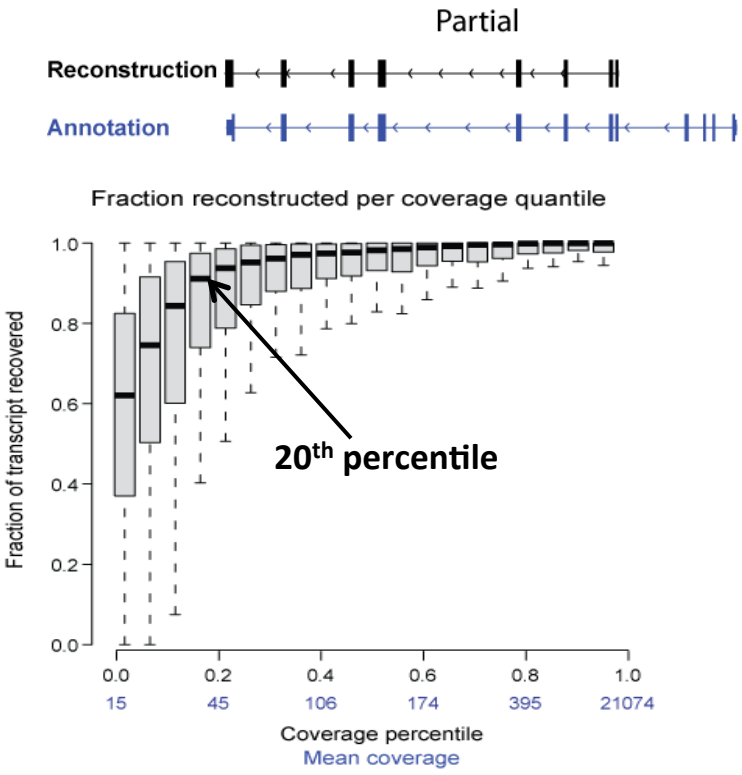
Reconstructing the mouse transcriptome (45M paired reads)

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



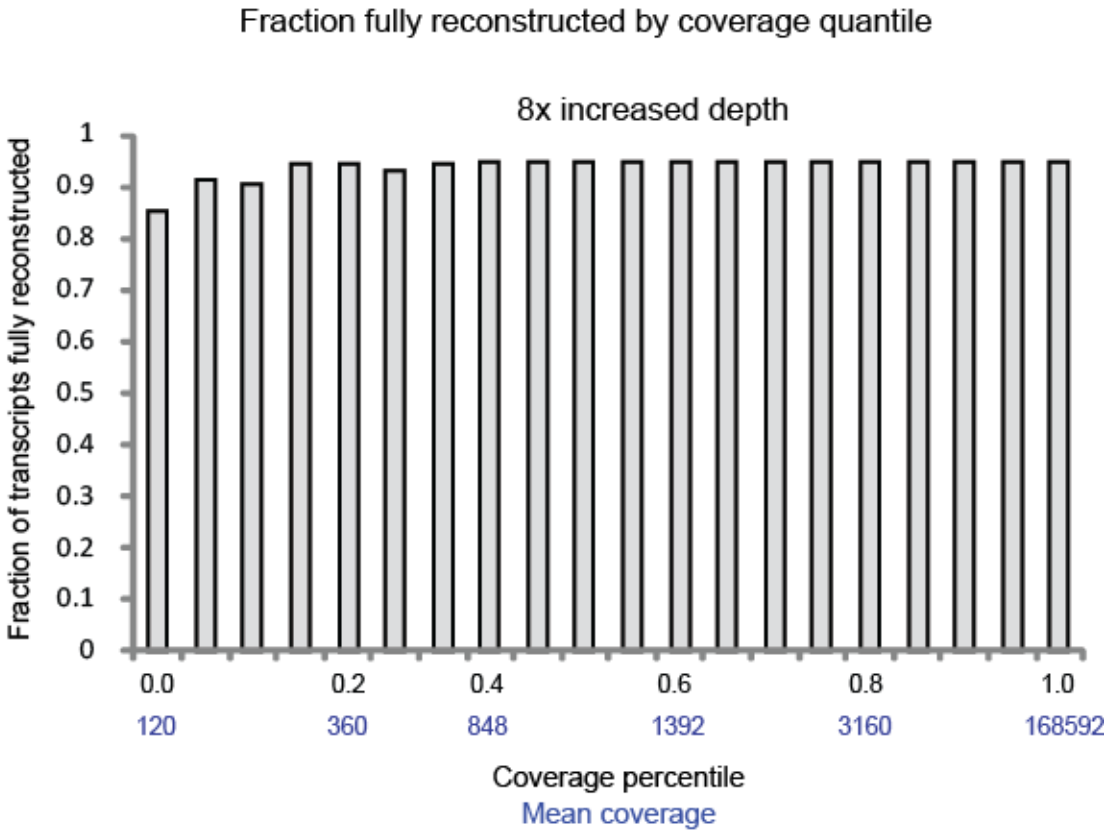
Sensitivity across expression levels





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

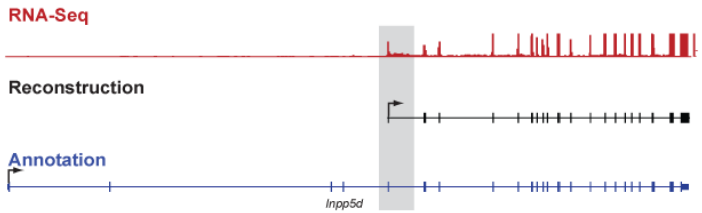
Sensitivity at low expression levels improves with depth



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

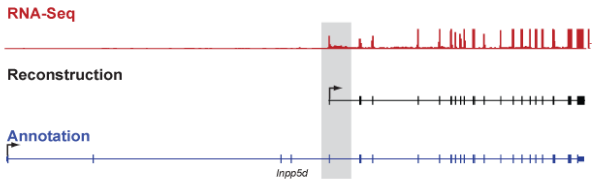
Novel variation in protein-coding genes

	ES cells	3 cell types
Novel 5’ Start Sites	**1,804**	**3,137**
	1,310	**2,477**
	588	**903**

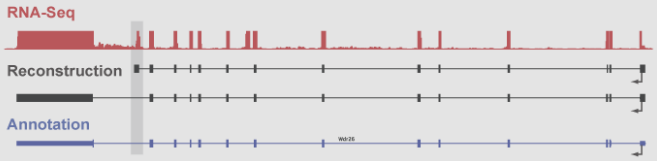


Novel variation in protein-coding genes

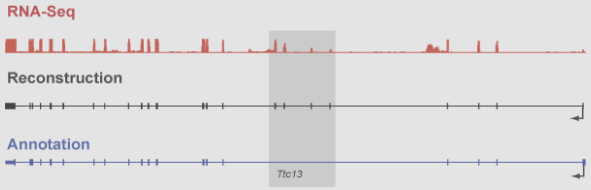
Novel 5’ Start Sites

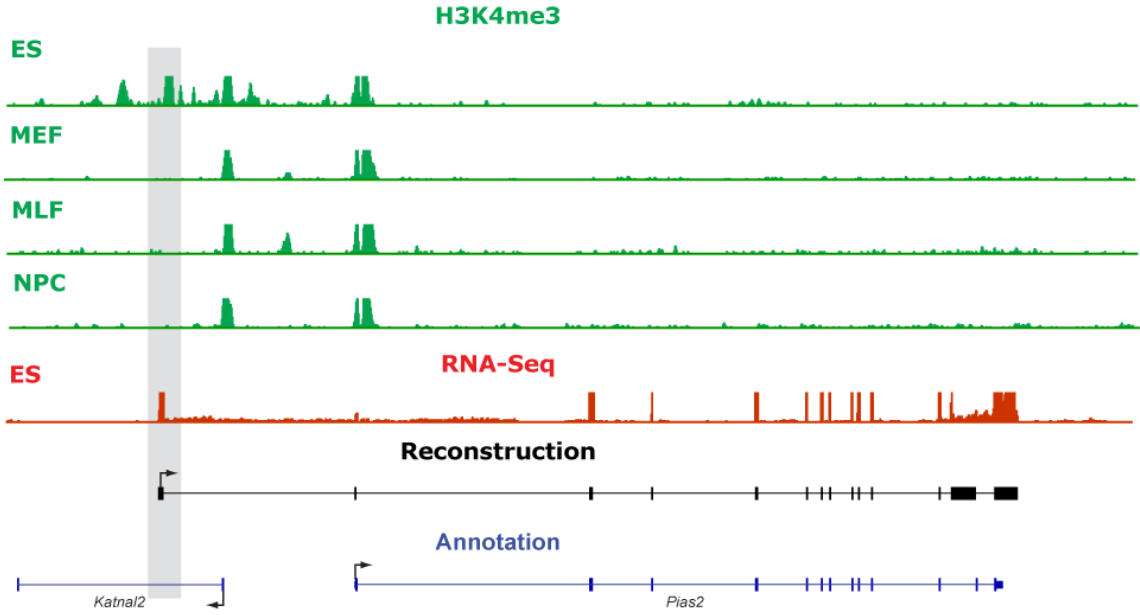


Novel 3’ End



Novel Coding Exons

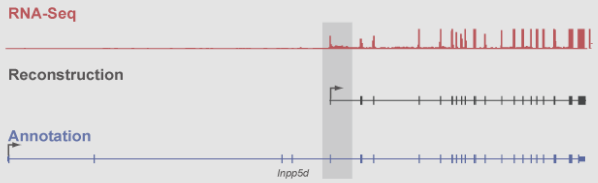




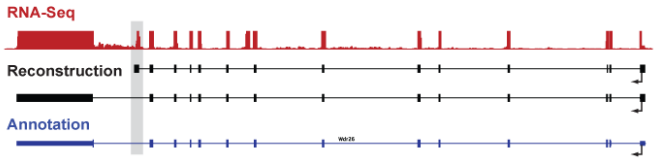
~85% overlap K4me3

Novel variation in protein-coding genes

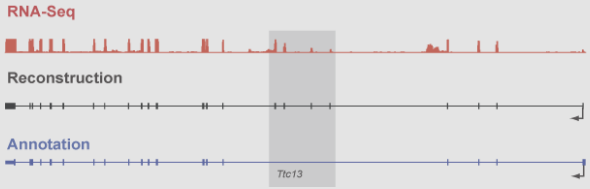
Novel 5’ Start Sites



Novel 3’ End

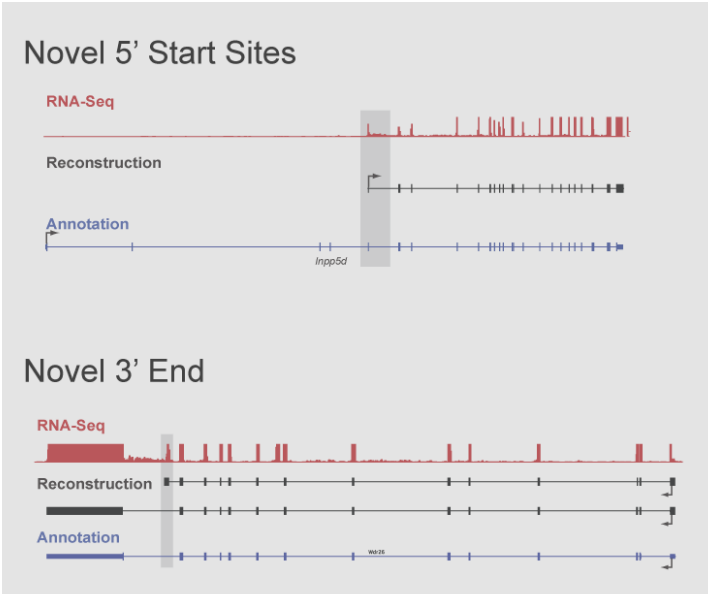


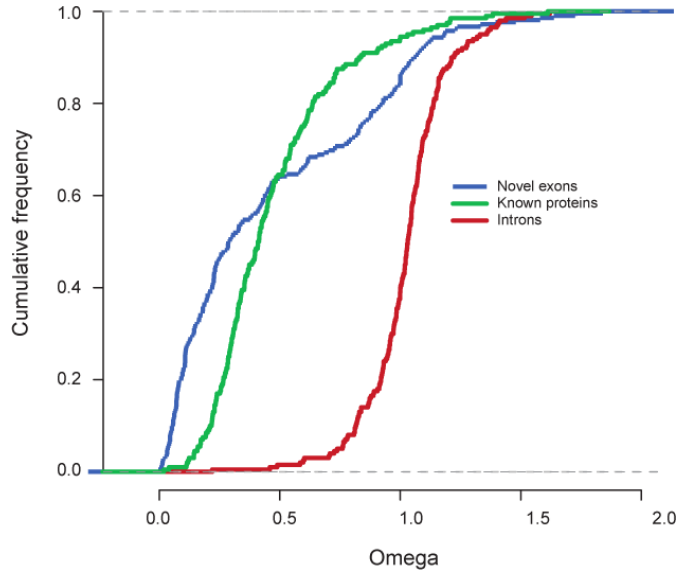
Novel Coding Exons

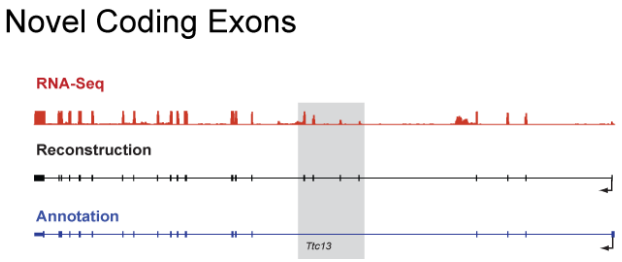


~50% contain polyA motif
Compared to ~6% for random

Novel variation in protein-coding genes







~80% retain ORF

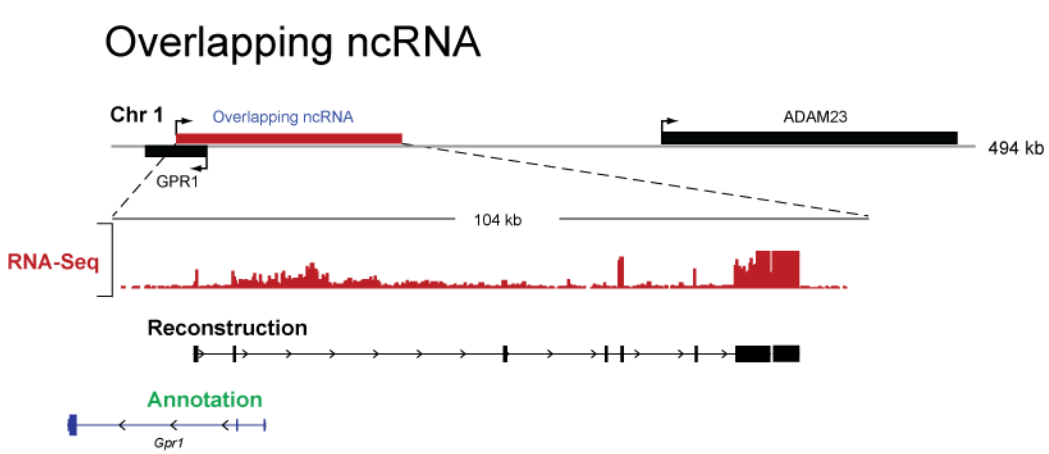
What about novel genes?

Class 1: Overlapping ncRNA

Class 2: Large Intergenic ncRNA (lincRNA)

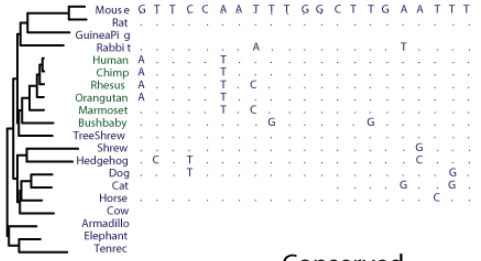
Class 3: Novel protein-coding genes

Class 1: Overlapping ncRNA

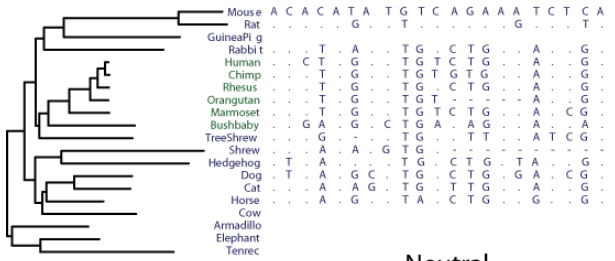


ES cells	3 cell types
201	**446**

Overlapping ncRNAs: Assessing their evolutionary conservation

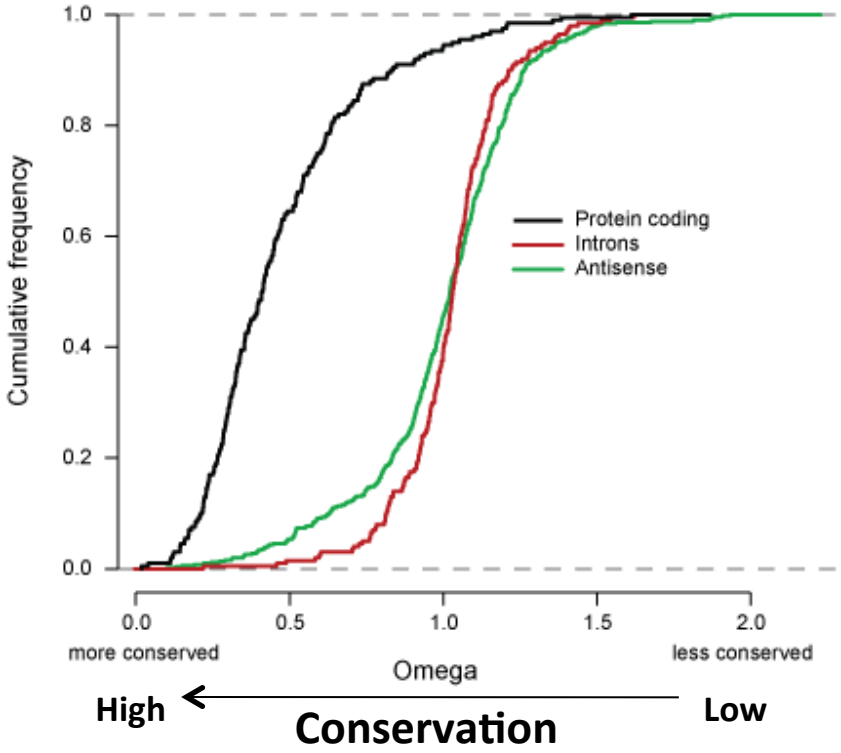


Conserved



Neutral

SiPhy – (Garber et al. *Bioinformatics*, 2009)



Overlapping ncRNAs show little evolutionary conservation

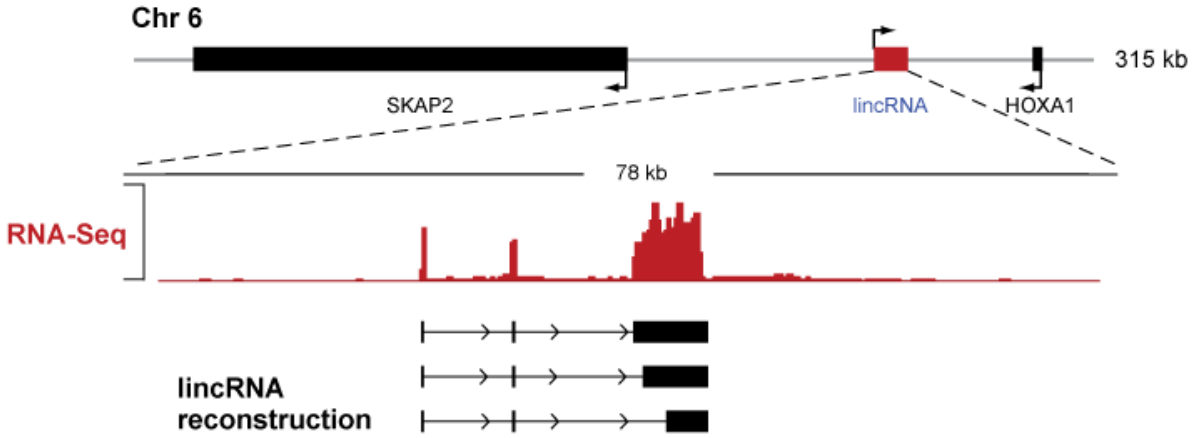
What about novel genes?

Class 1: Overlapping ncRNA

Class 2: Large Intergenic ncRNA (lincRNA)

Class 3: Novel protein-coding genes

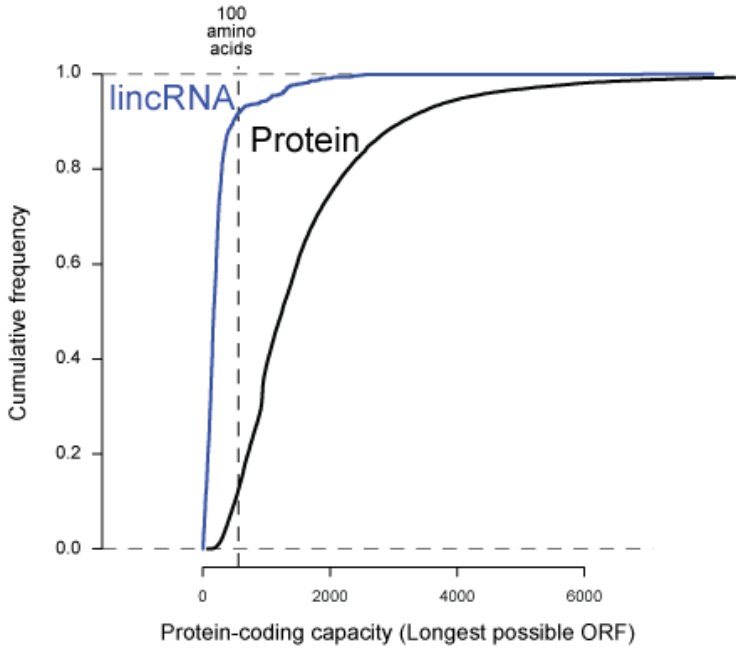
Class 2: Intergenic ncRNA (lincRNA)



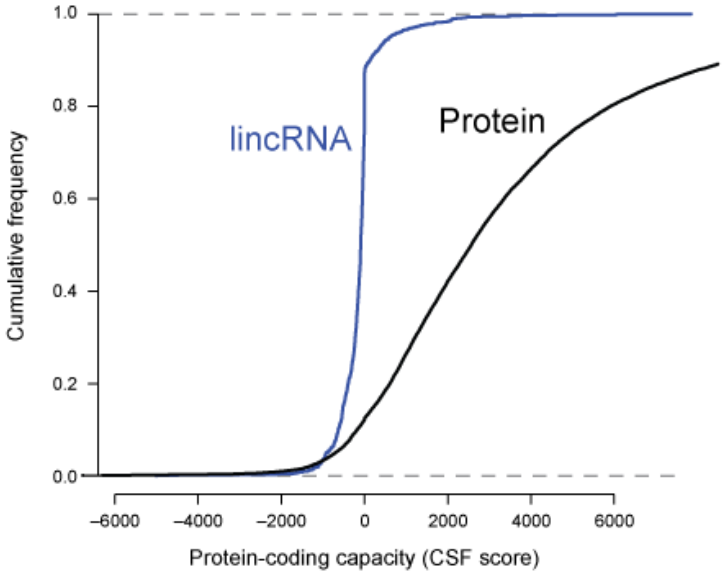
ES cells	3 cell types
~500	**~1500**

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

ORF Length

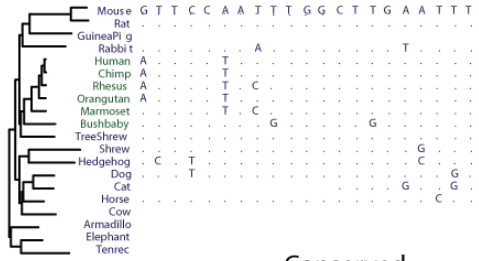


CSF (ORF Conservation)

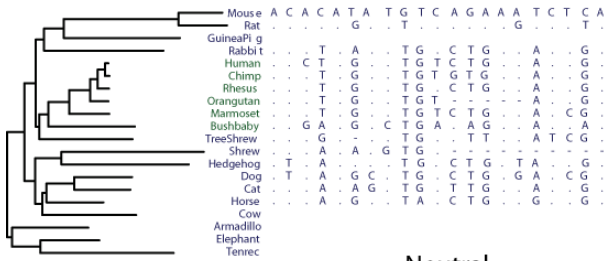


>95% do not encode proteins

lincRNAs: Assessing their evolutionary conservation



Conserved



Neutral

High ← **Conservation** **Low**

What about novel coding genes?

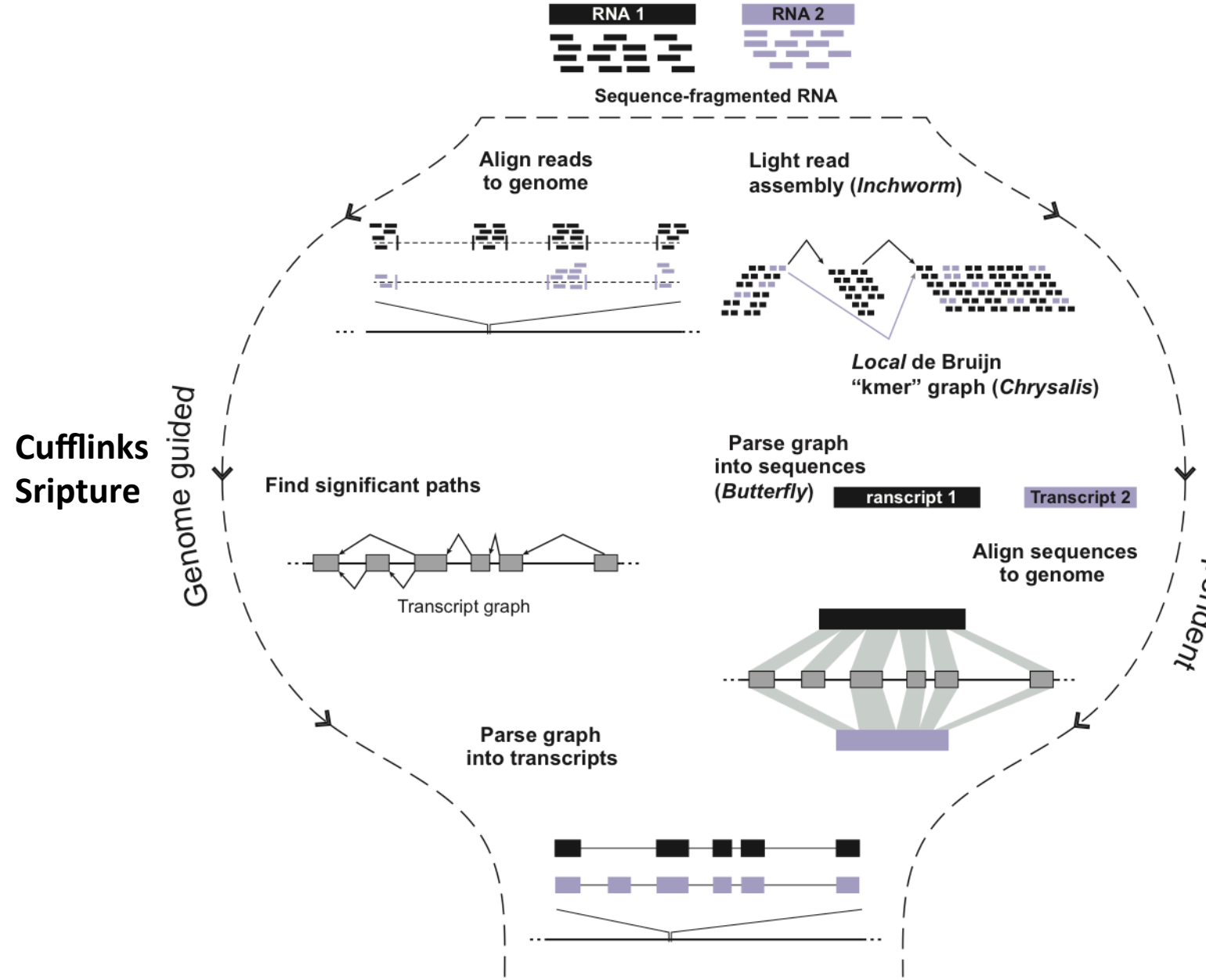
Class 1: Overlapping ncRNA

Class 2: Large Intergenic ncRNA (lincRNA)

Class 3: Novel protein-coding genes

~40 novel protein-coding genes

If there is no reference genome!
Genome independent methods



Rayan Chikhi lecture

Cufflinks
Sripture

Abyss
Trinity
Velvet

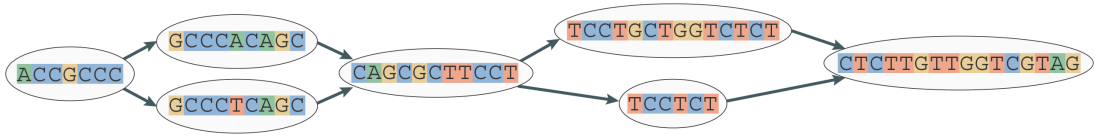
Garber et al, Nature Methods 2011

Assembly approach

1) Extract all substring of length k from reads

Assembly approach

3) Collapse graph



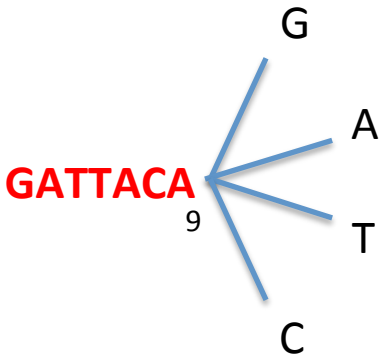
But this challenging already with DNA and RNA has many different challenges

The Trinity approach: Localize

Decompose all reads into overlapping Kmers (25-mers)

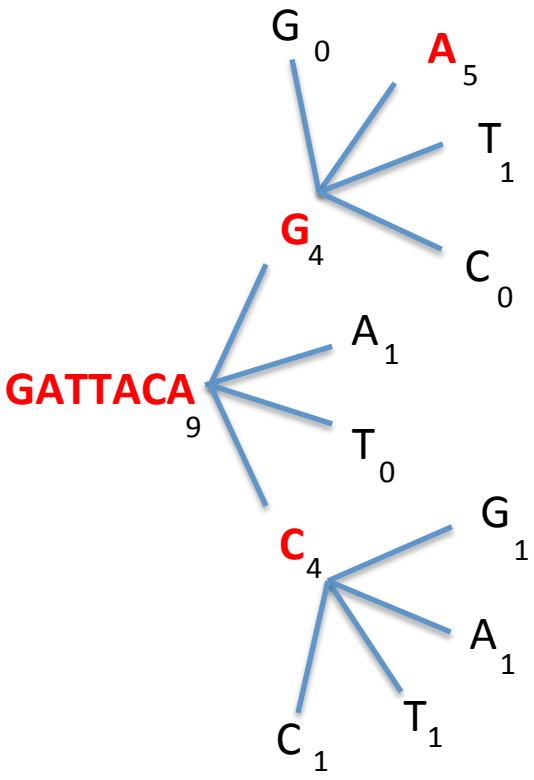
Identify seed kmer as most abundant Kmer, ignoring low-complexity kmers.

Extend kmer at 3' end, guided by coverage.



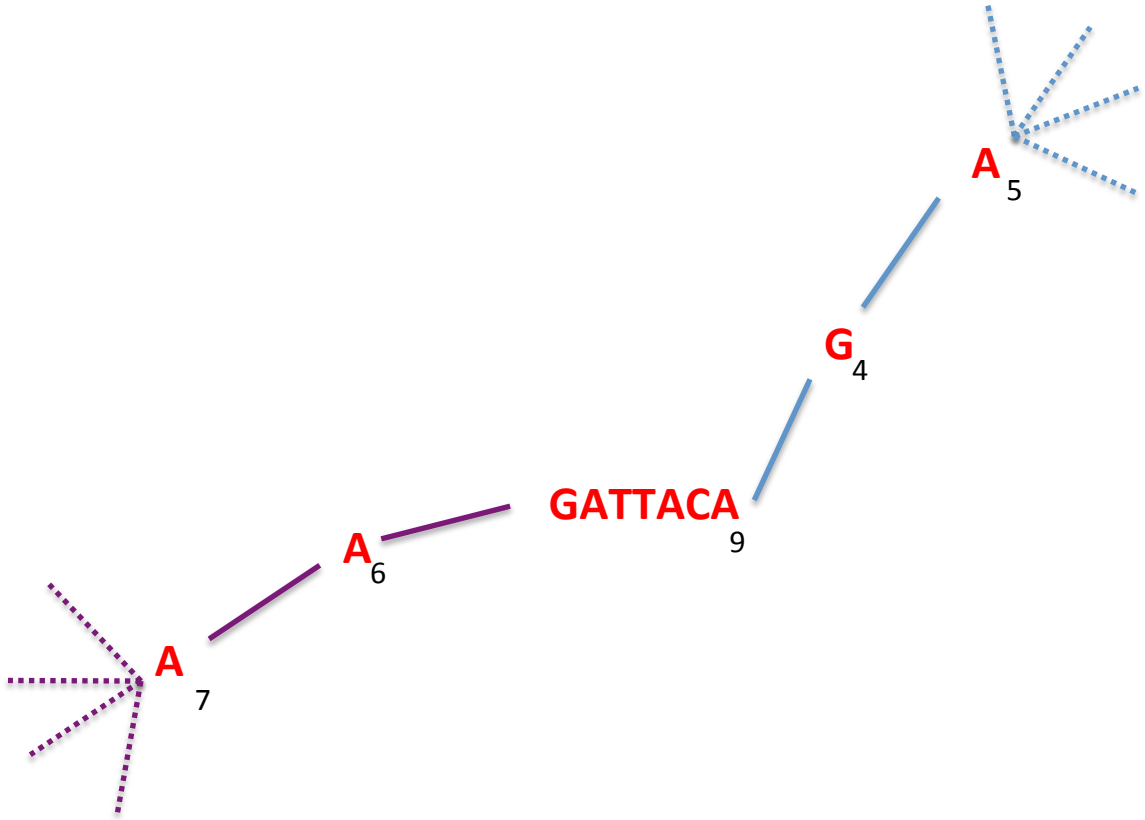
Briah Haas

The Trinity approach: Localize



Briah Haas

The Trinity approach: Localize



Report contig: ....AAGATTACAGA....

Remove assembled kmers from catalog, then repeat the entire process.

Briah Haas

Trinity approach: Assemble

RNA-Seq reads

Group similar contigs

key: localize the assembly problem

Pros and cons of each approach

- Transcript assembly methods are the obvious choice for organisms without a reference sequence.
- Genome-guided approaches are ideal for annotating high-quality genomes and expanding the catalog of expressed transcripts and comparing transcriptomes of different cell types or conditions.
- Hybrid approaches for lesser quality or transcriptomes that underwent major rearrangements, such as in cancer cell.
- More than 1000 fold variability in expression leves makes assembly a harder problem for transcriptome assembly compared with regular genome assembly.
- Genome guided methods are very sensitive to alignment artifacts.

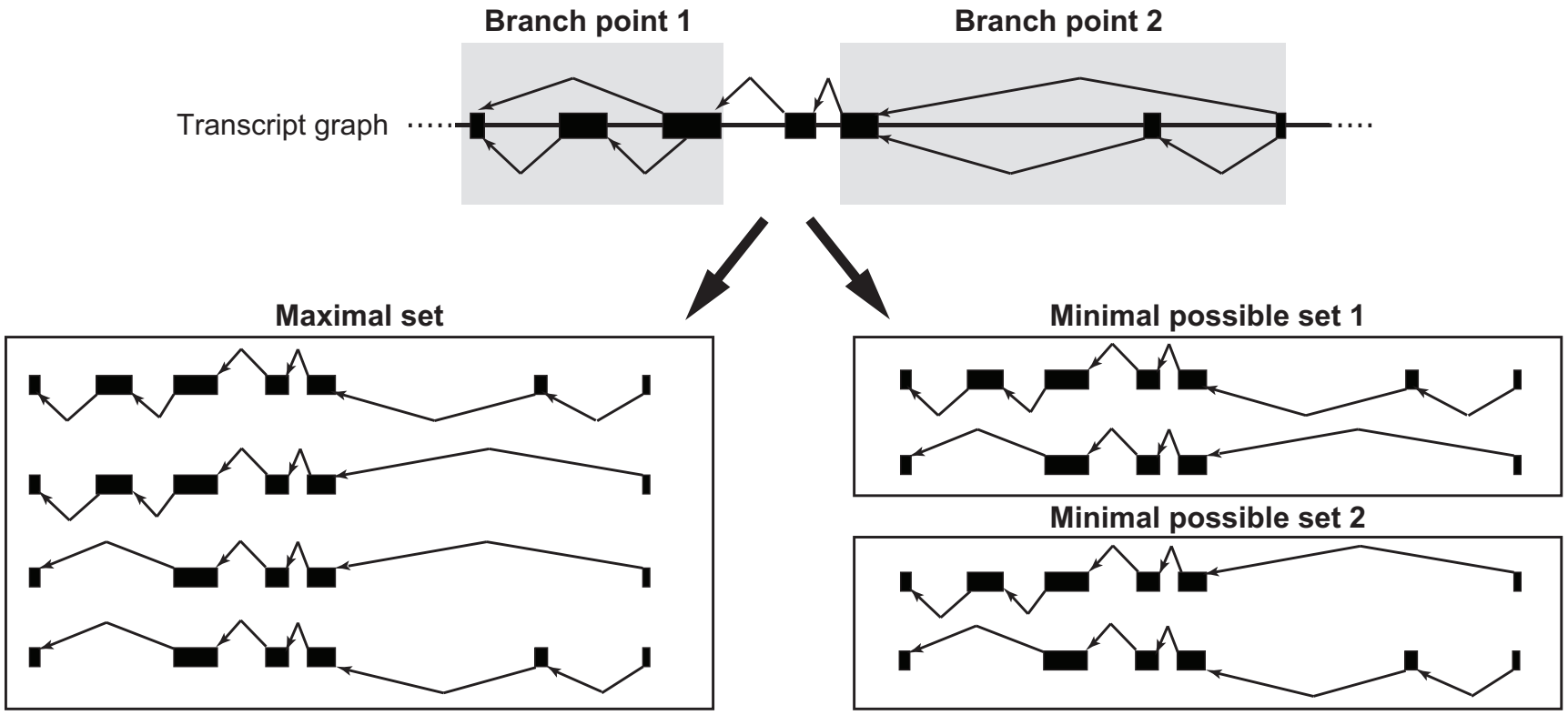
RNA-Seq transcript reconstruction software

Assembly	Genome Guided
Oasis (velvet)	Cufflinks
Trans-ABySS	Scripture
Trinity	

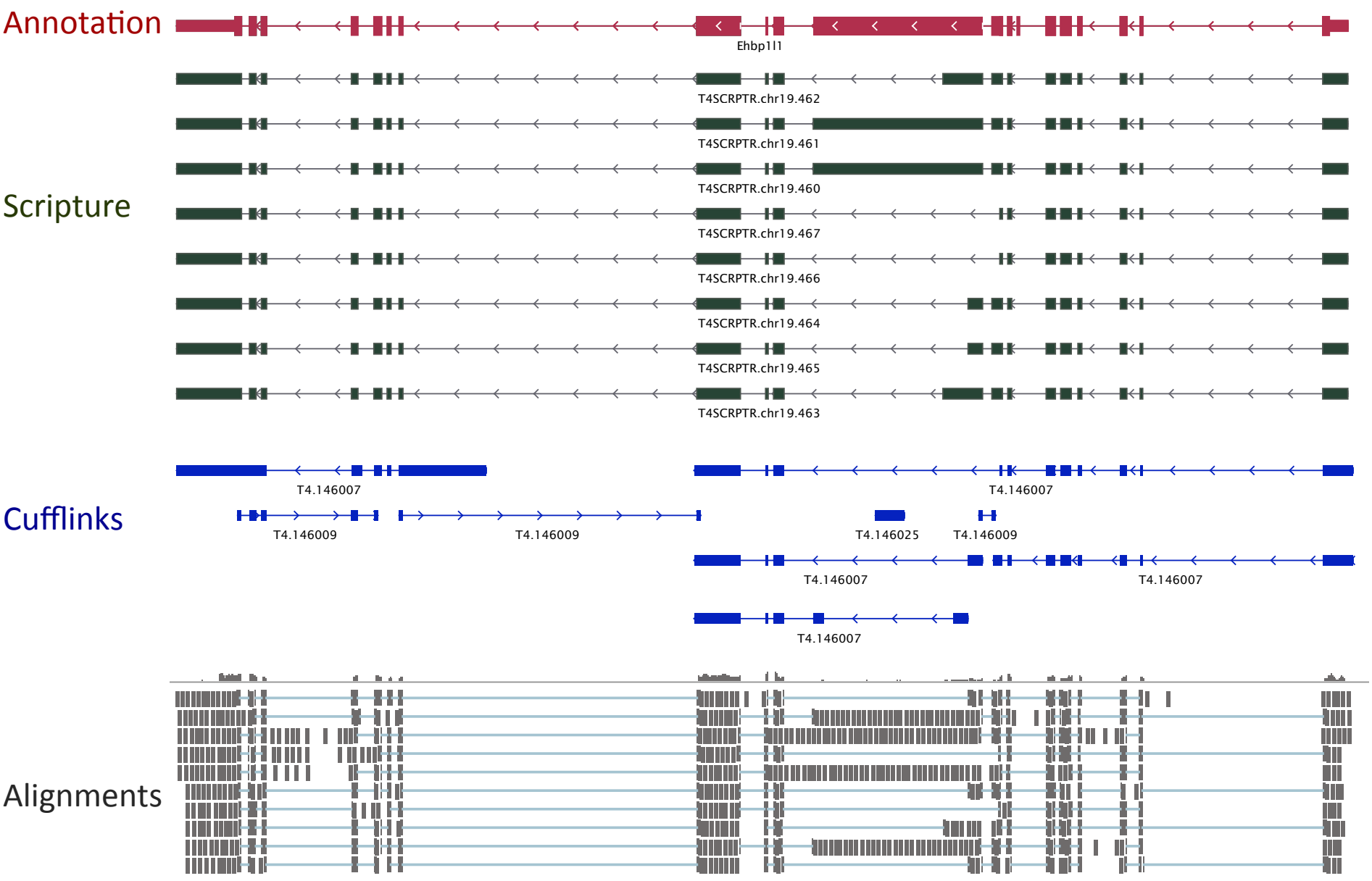
Differences between Cufflinks and Scripture

- Scripture was designed with annotation in mind. It reports all possible transcripts that are *significantly expressed* given the aligned data (*Maximum sensitivity*).
- 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



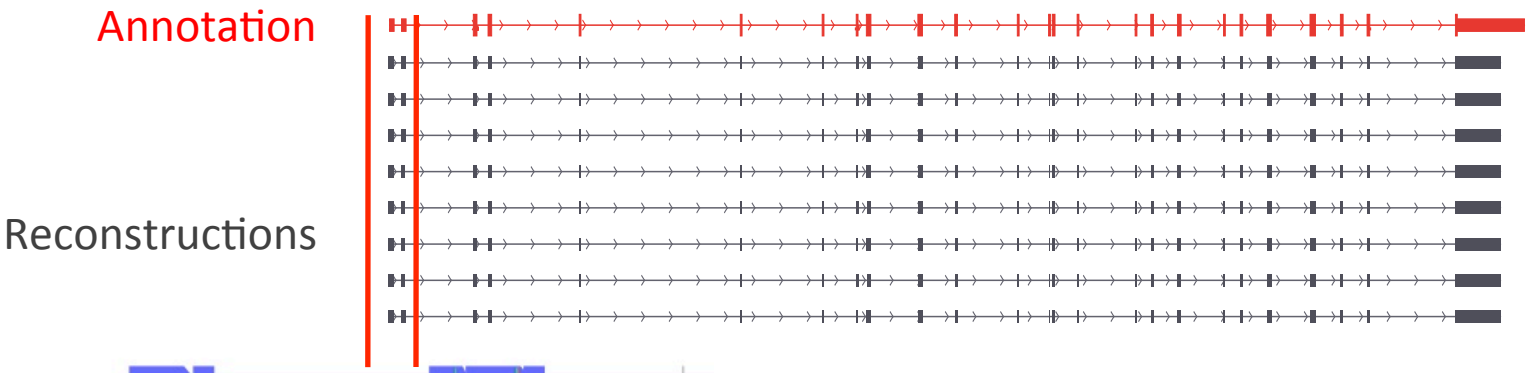
Comparing reconstructions

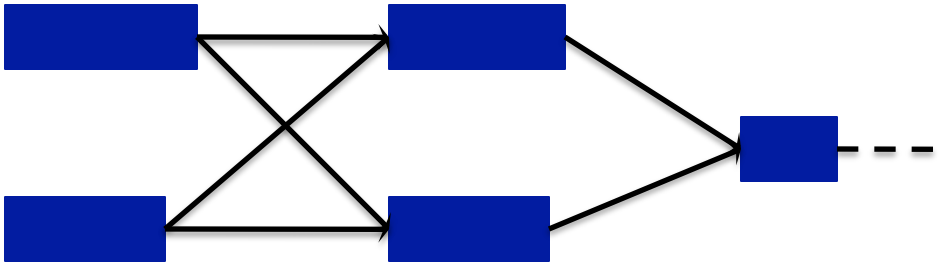
	CPU Hours	Total Memory	Genes fully reconstructed	Mean isoforms per reconstruction	Mean fragments per known annotation	Number of fragments predicted
Cufflinks	10	1.4 G	5,994	1.2	1.4	**159,856**
Scripture	16	3.5 G	6,221	**1.6**	1.3	61,922
Trans- Abyss	650	120 G[4]	3,330	4.7	2.6	3,117,238

Many of the bogus locus and isoforms are due to alignment artifacts

Garber et al, Nature Methods 2011

Why so many isoforms

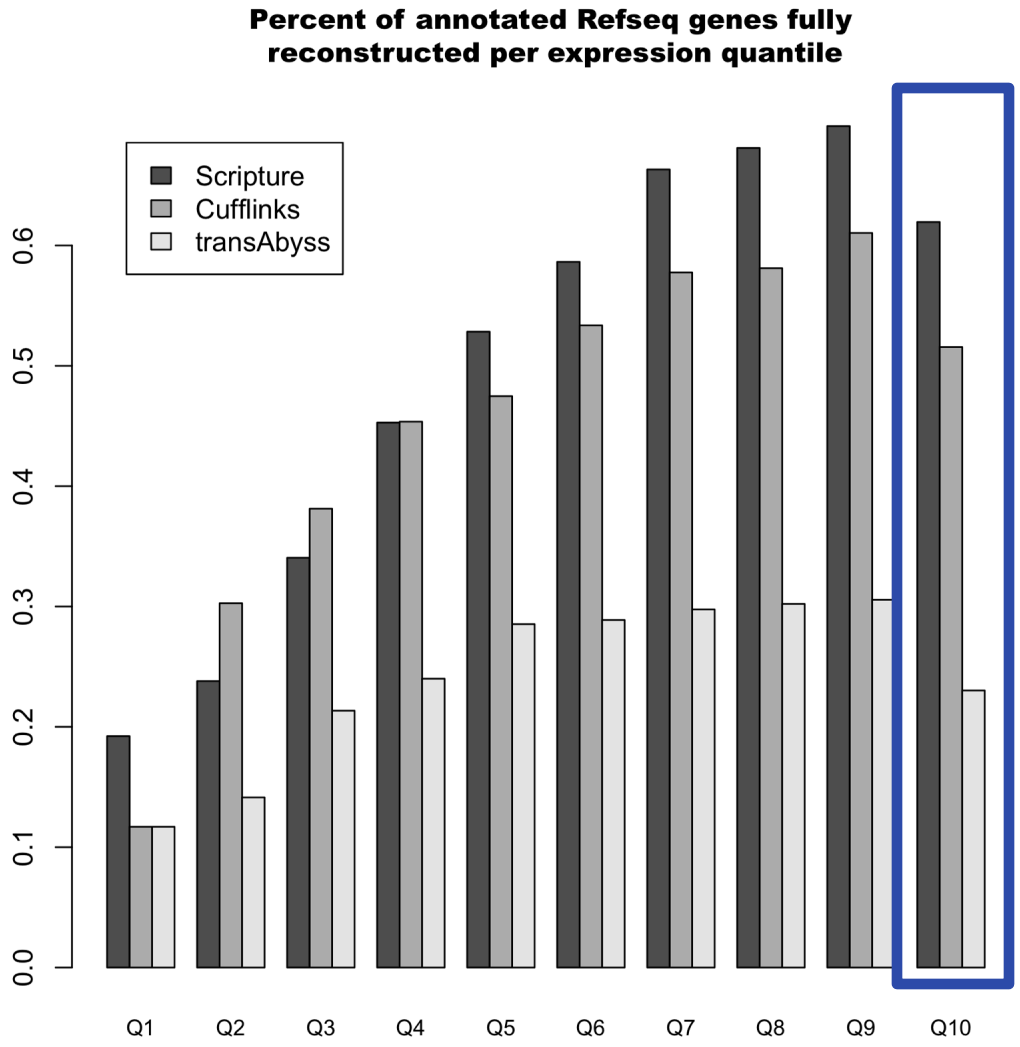




Every such splicing event or alignment artifact doubles the number of isoforms reported

Longer reads (already possible) will reduce the uncertainty and possibilities

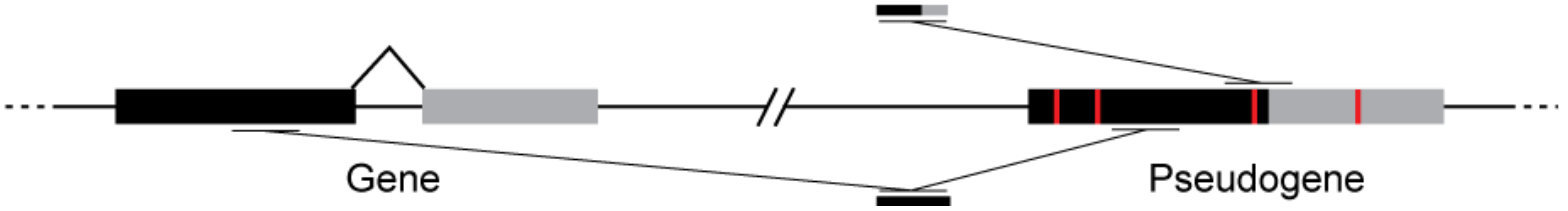
Reconstruction comparison



Too much of a good thing is not handled well by most reconstruction methods

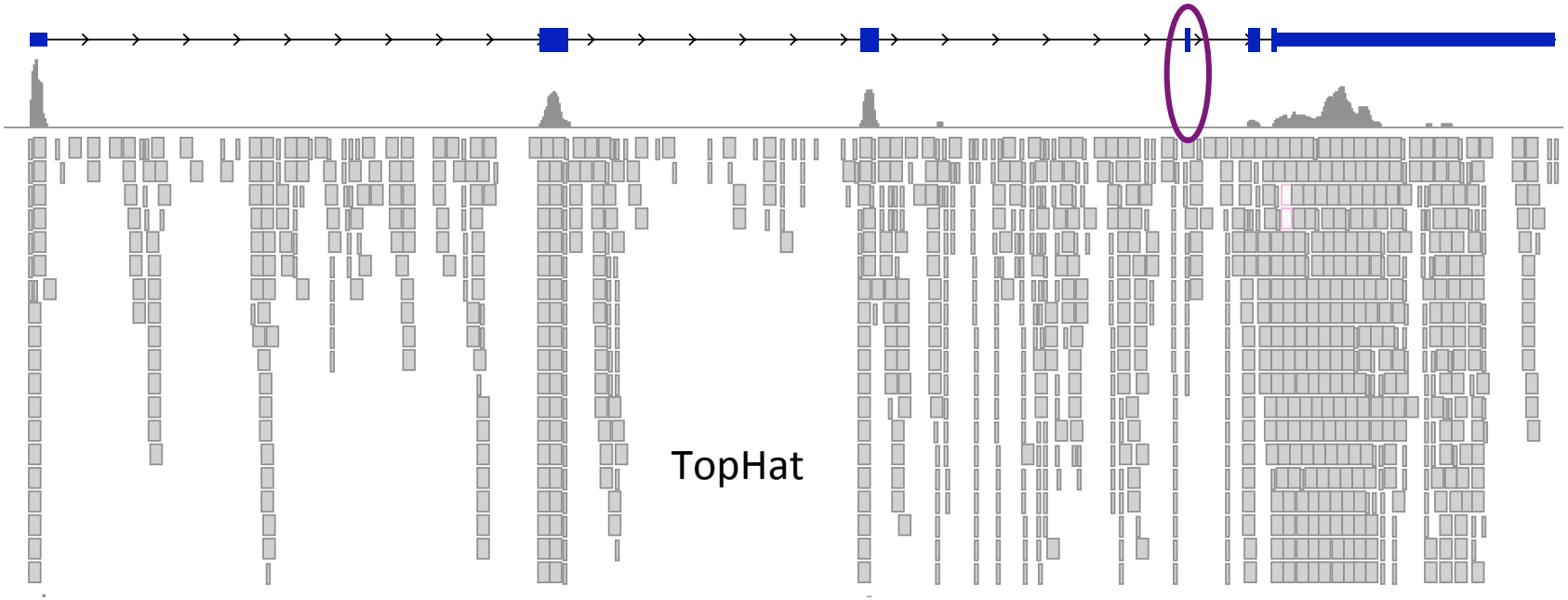
Alignment revisited — spliced alignment is still work in progress

Exon-first aligners are faster but at cost



Alignment artifacts can also decrease sensitivity

Missing spliced reads for highly expressed genes



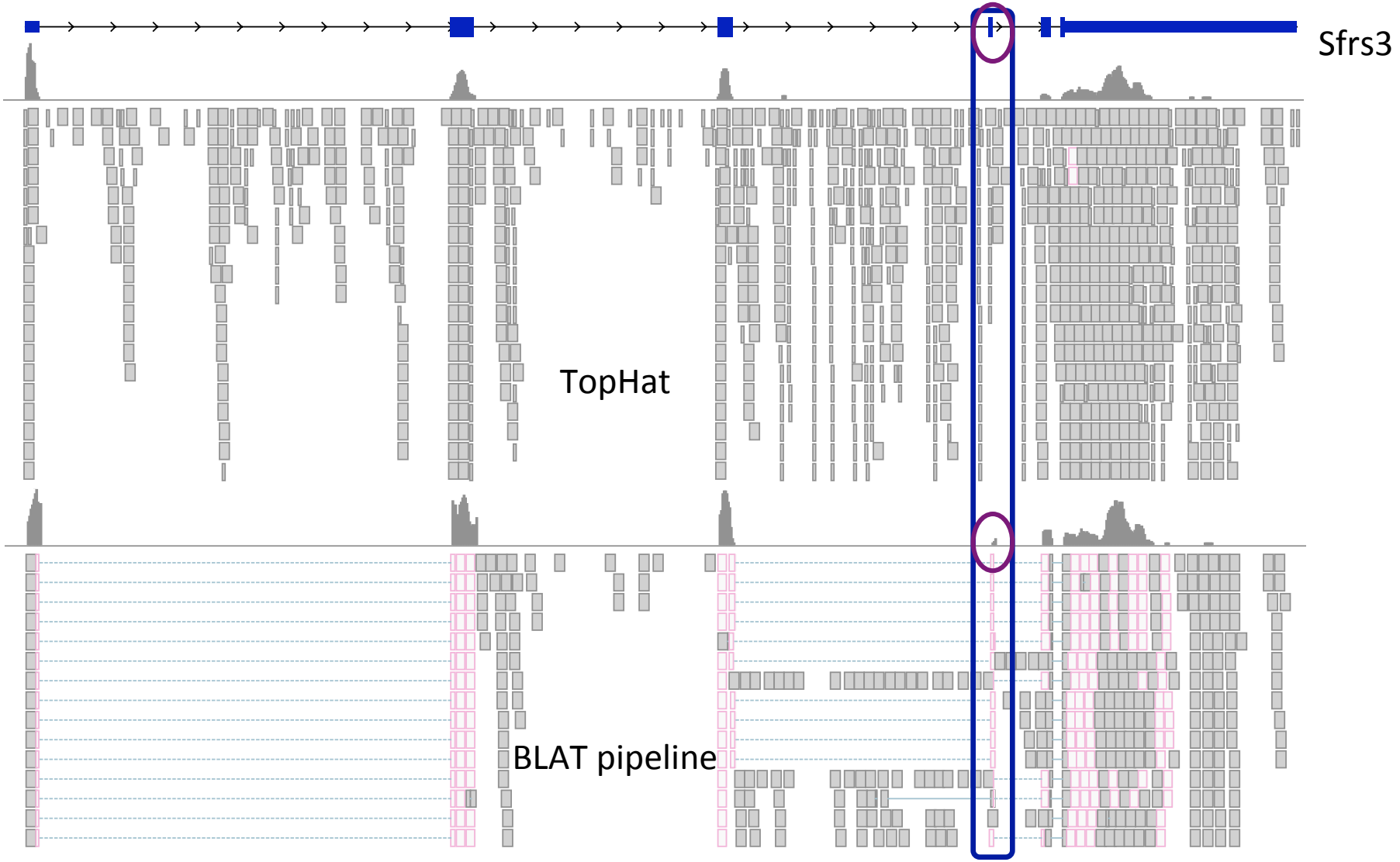
Read mapped uniquely

Read ambiguously mapped

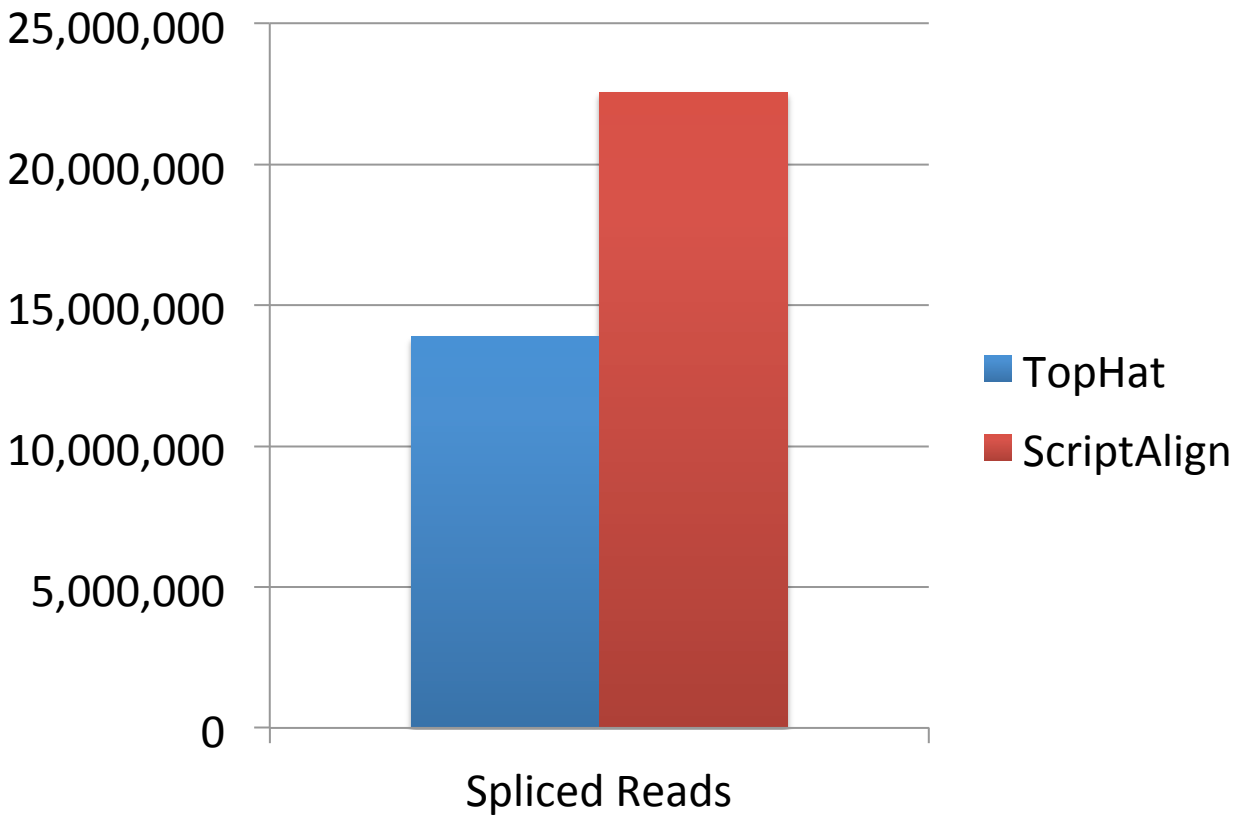
Can more sensitive alignments overcome this problem?

- Use gapped aligners (e.g. BLAT) to map reads
  - Align all reads with BLAT
  - Filter hits and build candidate junction "database" from BLAT hits (Scripture light).
  - Use a short read aligner (Bowtie) to map reads against the connectivity graph inferred transcriptome
  - Map transcriptome alignments back the genome

Many junctions can be rescued



ScriptAlign: Can increase alignment across junctions



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

Alignment strategies for reconstruction

- Use all the information you have.
  - In TopHat use the –G option:

```
tophat --GTF mm9.mrna.10.31.gtf left.reads.fq right.reads.fq
```

- In GNAP use the `--use-splicing` option.
- Align twice:
  - Align first using annotations if available
  - Re-align using both transcripts and junctions found in first run

Overview of the session

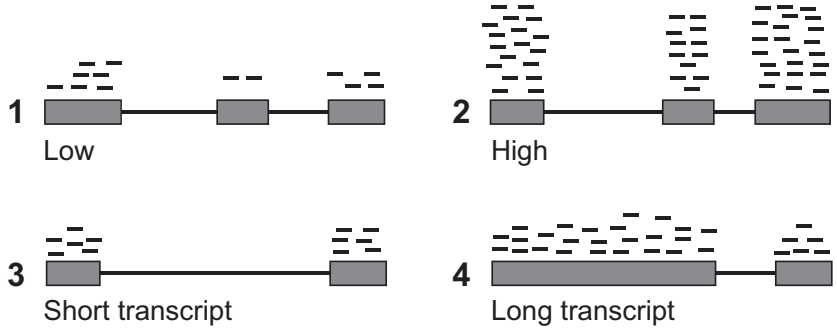
The 3 main computational challenges of sequence analysis for *counting applications*:

- 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.

RNA-Seq quantification

- Is a given gene (or isoform) expressed?
- Is expression gene A > gene B?
- Is expression of gene A isoform a_1 > gene A isoform a_2?
- Given two samples is expression of gene A in sample 1 > gene A in sample 2?

Quantification: only one isoform



$$RPKM = 10^9 \frac{\#reads}{length \times TotalReads}$$

Reads per kilobase of exonic sequence per million mapped reads
(*Mortazavi* et al Nature methods 2008)

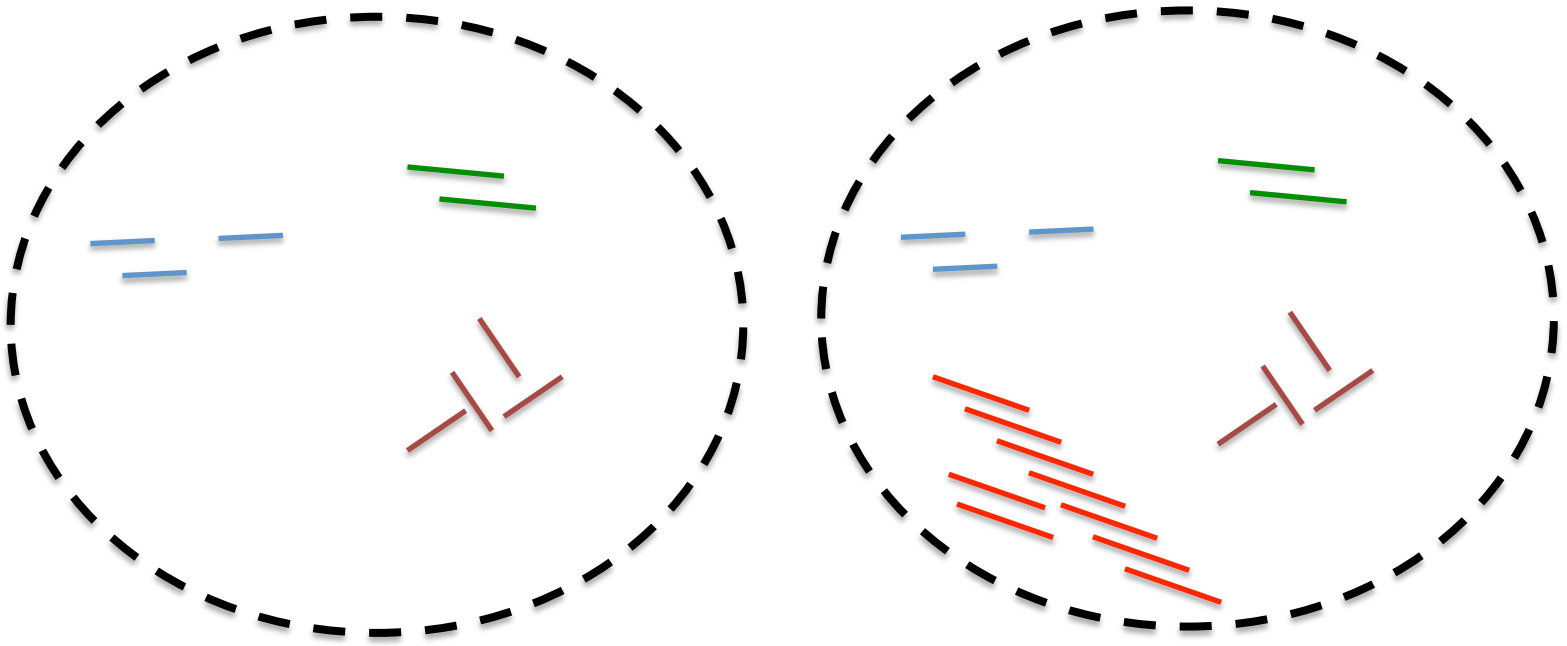
- Fragmentation of transcripts results in length bias: longer transcripts have higher counts
- Different experiments have different yields. Normalization is key for cross lane comparisons

Complexity increases when multiple isoforms exist

Normalization depends on the application

- To compare within a sequence run (lane), RPKM accounts for length bias.
- RPKM is not optimal for cross experiment comparisons.
  - Different samples may have different compositions.

Step 2: Different RNA compositions

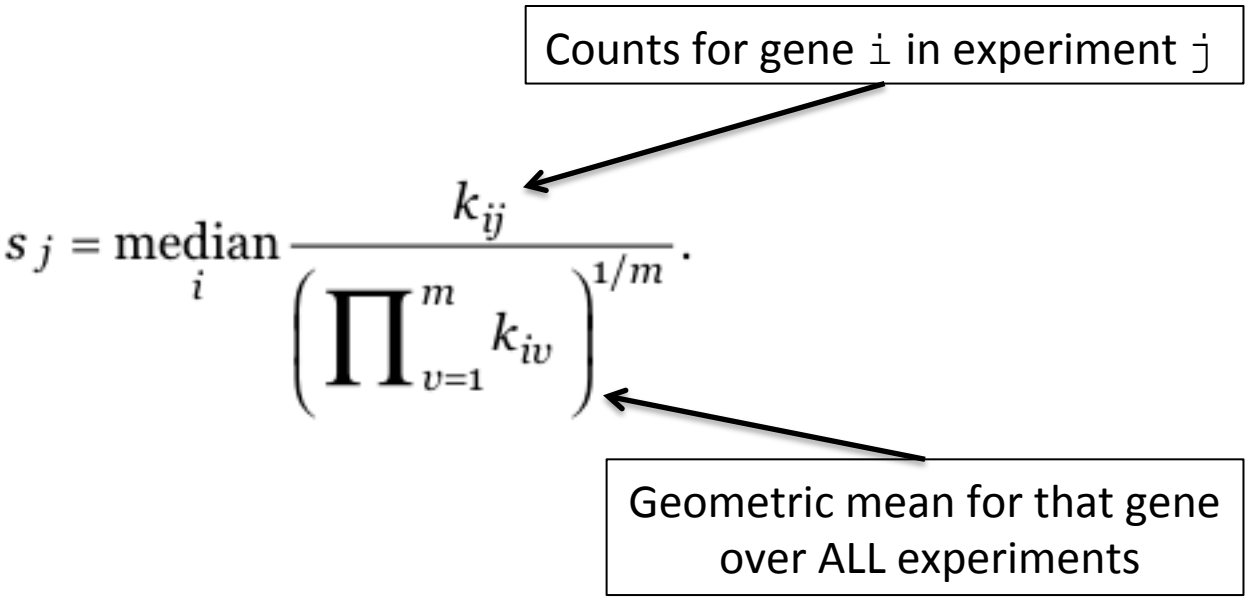


Cell type I

Cell type II

Normalizing by total reads does not work well for samples with very different RNA composition

Step2: More robust normalization



i runs through all *n* genes
j through all *m* samples
k_{ij} is the observed counts for gene *i* in sample *j*
s_j Is the normalization constant

Alders and Huber, 2010

Lets do an experiment (and do a short R practice)

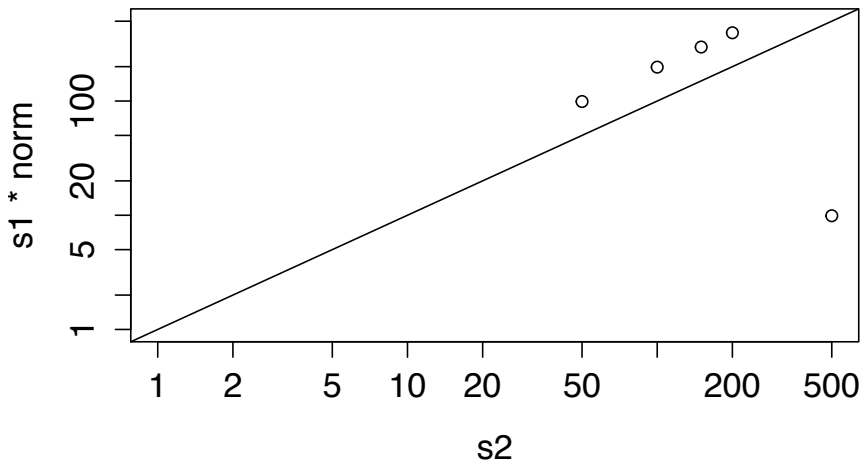
$> s1 = c(100, 200, 300, 400, 10)$
$> s2 = c(50, 100, 150, 200, 500)$

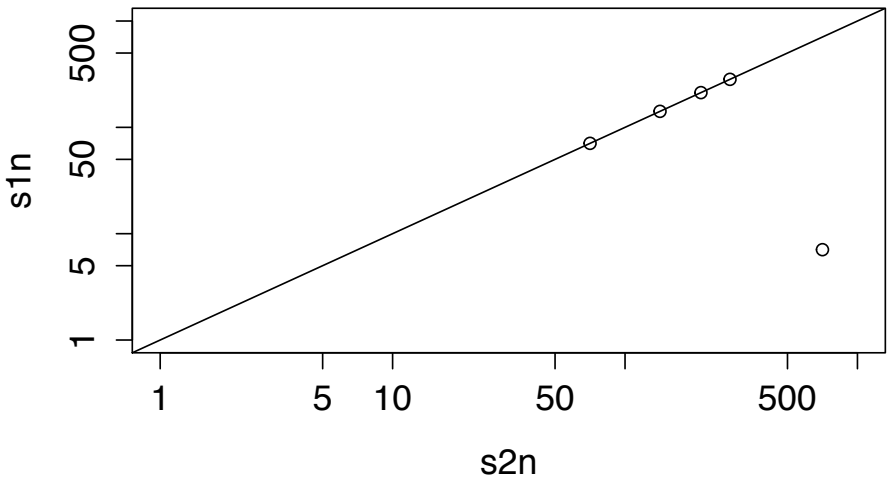
Similar read number,
one transcript many fold changed

$>\text{norm}=\text{sum}(s2)/\text{sum}(s1)$
$>\text{plot}(s2, s1*\text{norm},\text{log}=\text{"xy"})$
$>\text{abline}(a = 0, b = 1)$

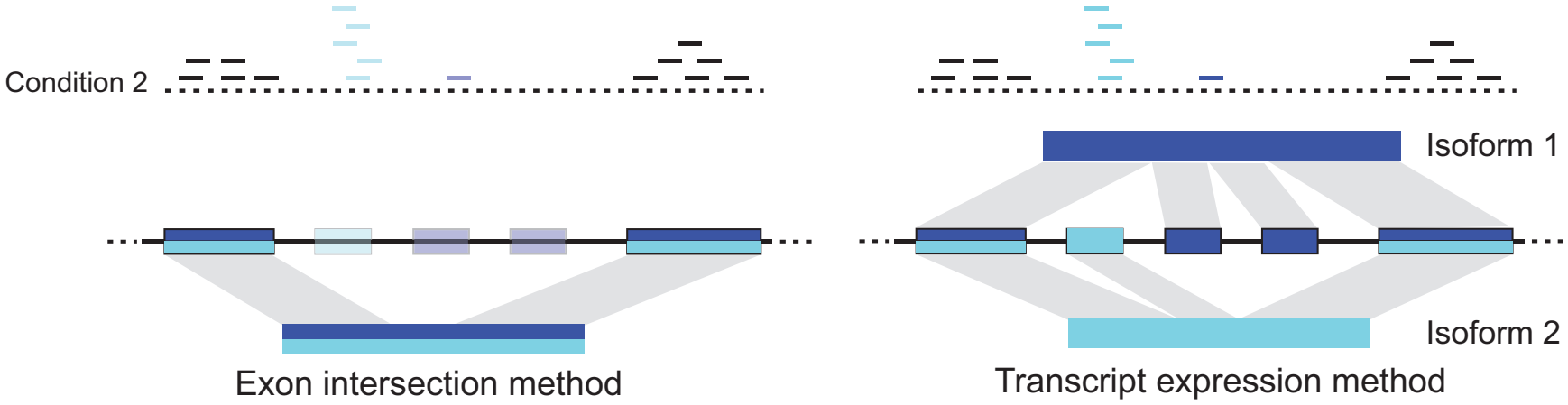
Size normalization results in 2-fold changes in *all* transcripts

$>\text{g} = \text{sqrt}(s1 * s2t)$
$>\text{s1n} = s1/\text{median}(s1/g);\ \ \text{s2n} = s2/\text{median}(s2/g)$
$>\text{plot}(s2n, s1n,\text{log}=\text{"xy"})$
$>\text{abline}(a = 0, b = 1)$





But, how to compute counts for complex gene structures?



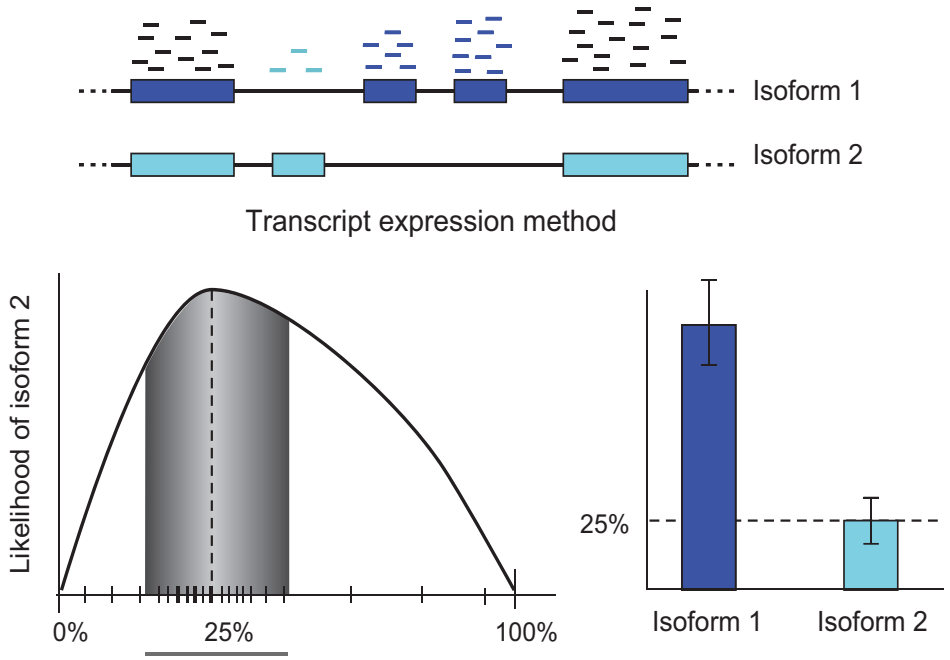
Three popular options:

Exon *intersection* model: Score constituent exons

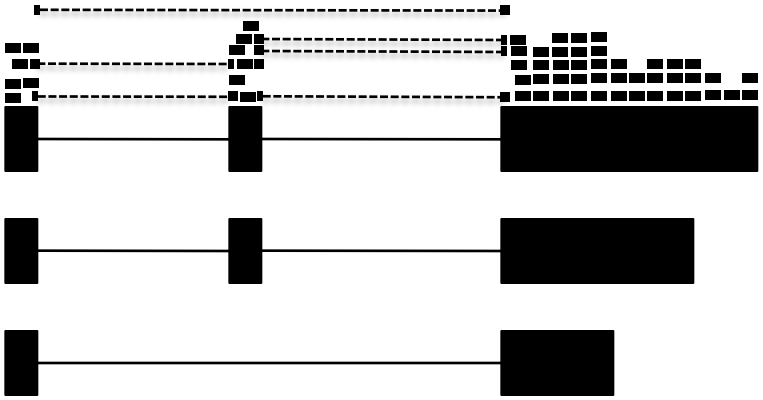
Exon *union* model: Score the the "merged" transcript

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

Quantification: read assignment method

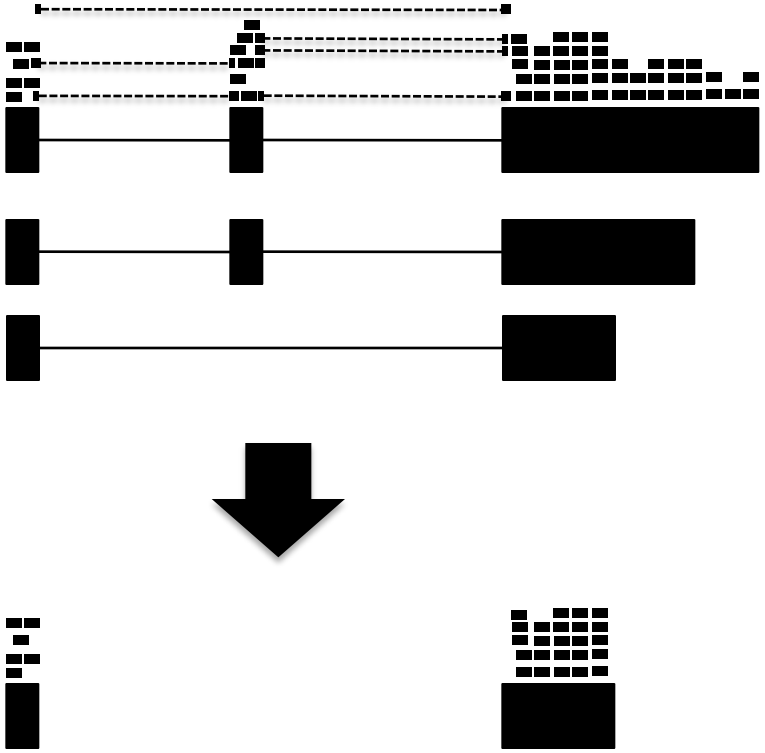


Quantification with multiple isoforms



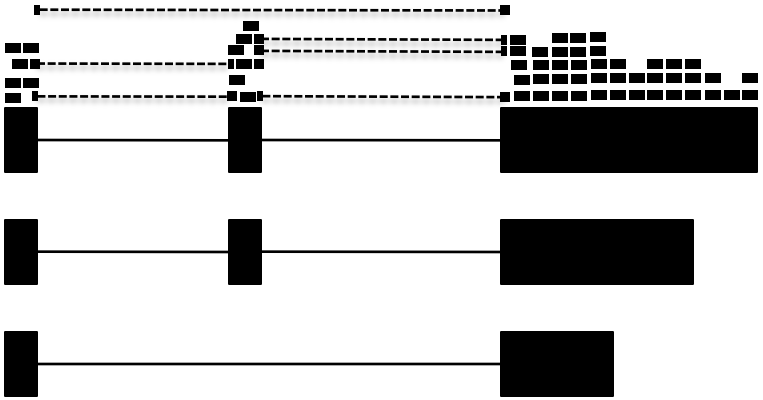
How do we define the gene expression?
How do we compute the expression of each isoform?

Computing gene expression

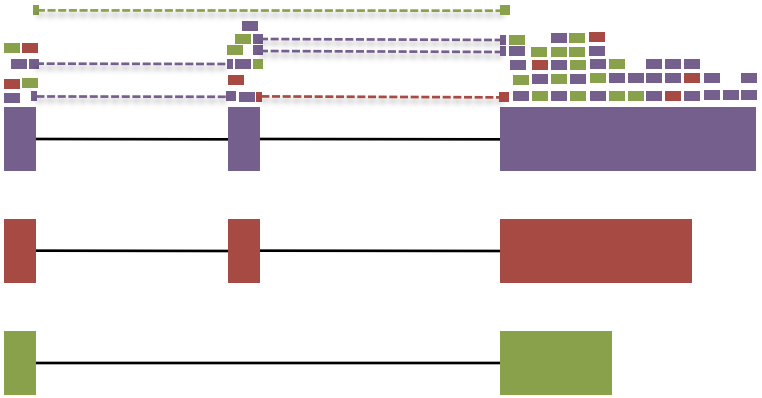


Idea1: RPKM of the constitutive reads (Neuma, Alexa-Seq, Scripture)

Computing gene expression — isoform deconvolution



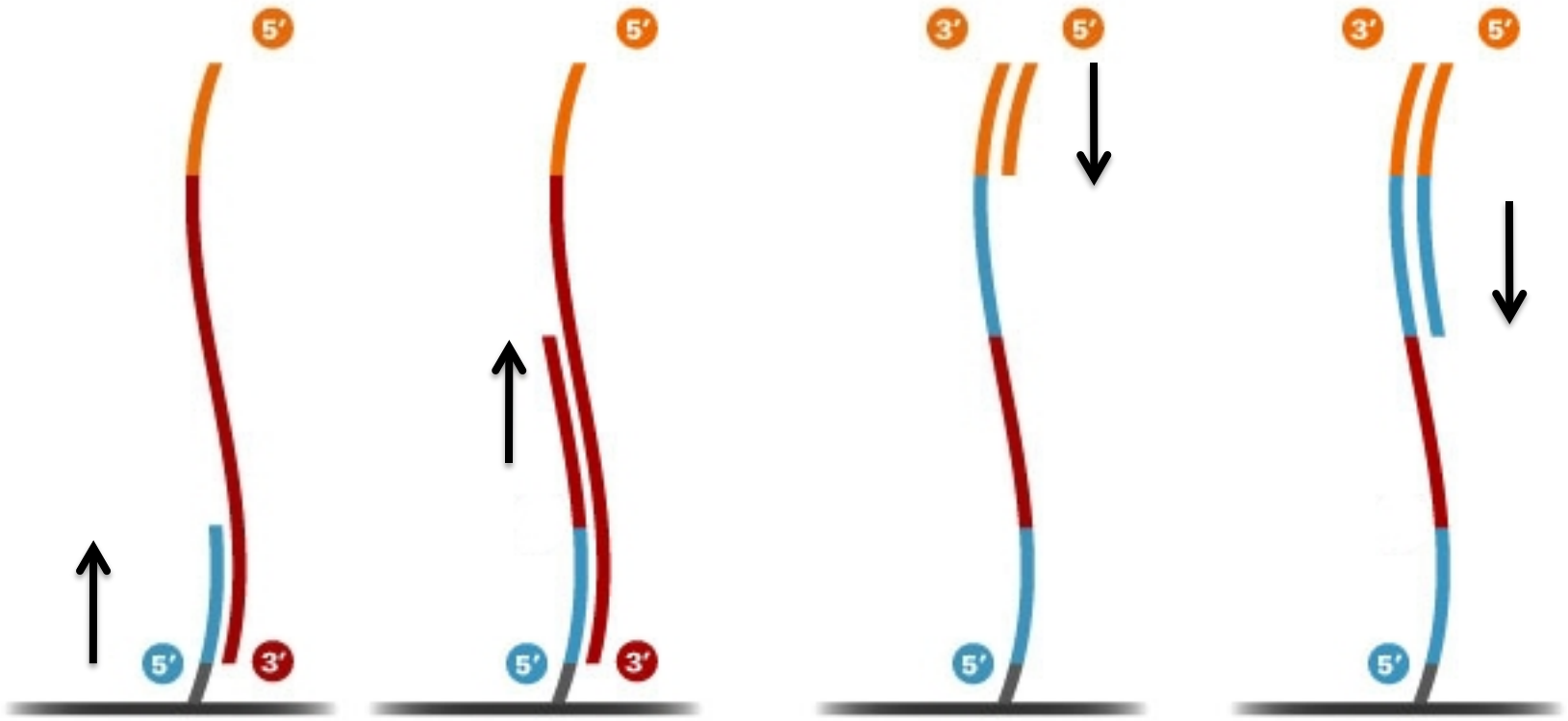
Computing gene expression — isoform deconvolution



If we knew the origin of the reads we could compute each isoform's expression. The gene's expression would be the sum of the expression of all its isoforms.

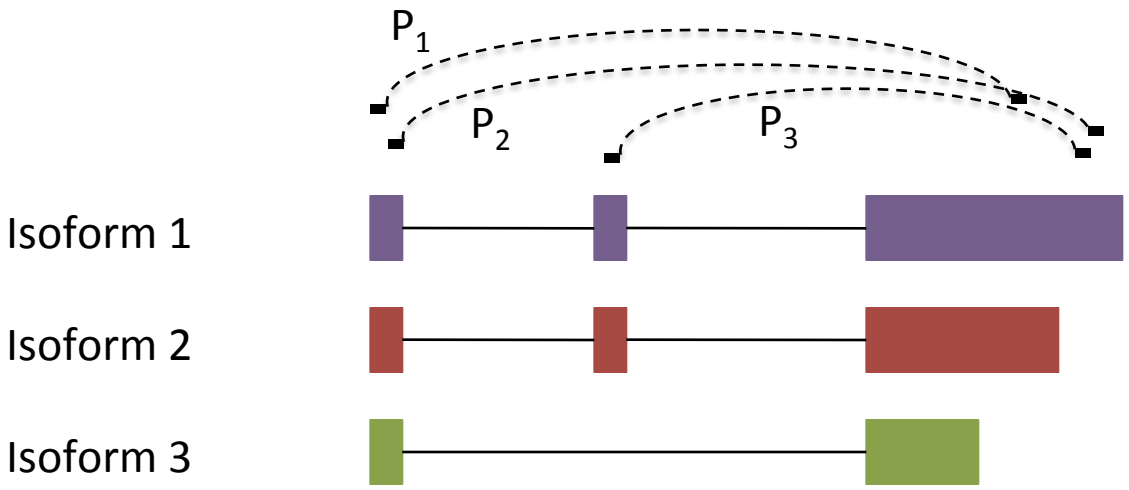
$$E = RPKM_1 + RPKM_2 + RPKM_3$$

Paired-end sequencing is critical for "isoform deconvolution"



Adapted from the Helicos website

Paired-end reads are easier to associate to isoforms

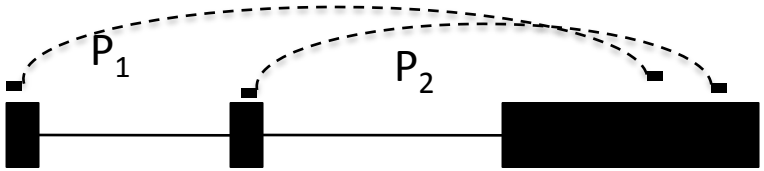


Paired ends increase isoform deconvolution confidence

- P_1 originates from isoform 1 or 2 but not 3.
- P_2 and P_3 originate from isoform 1

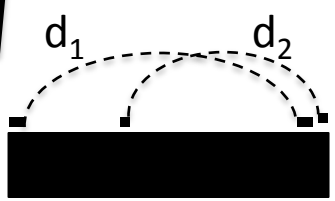
Do paired-end reads also help identifying reads originating in isoform 3?

We can estimate the insert size distribution

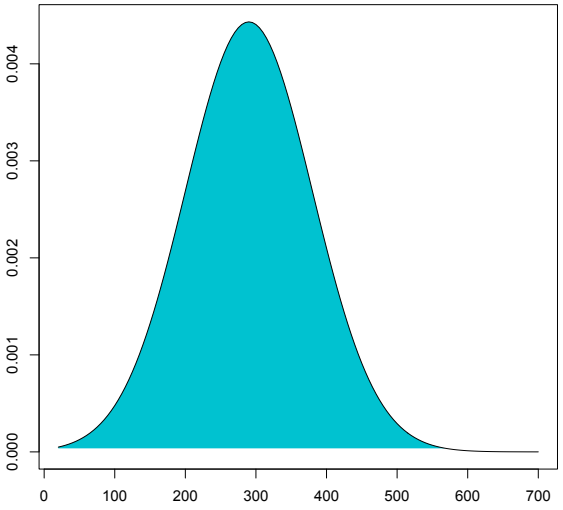


Get all single isoform reconstructions

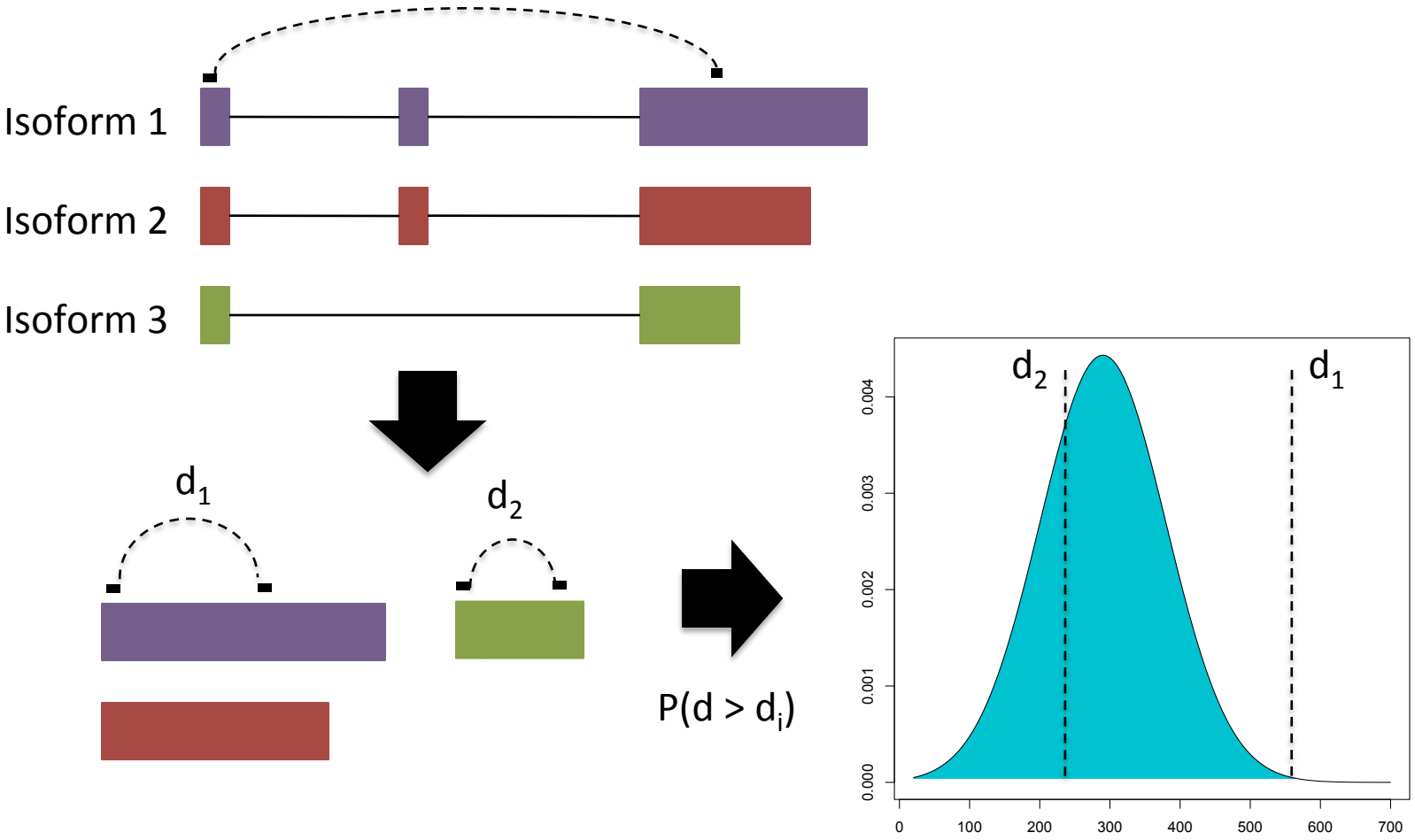
Splice and compute insert distance



Estimate insert size empirical distribution



… and use it for probabilistic read assignment



For methods such as MISO, Cufflinks and RSEM, it is critical to have paired-end data

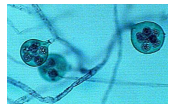
RNA-Seq quantification summary

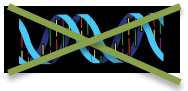
- Counts must be estimated from ambiguous read/transcript assignment.
  - Using simplified gene models (intersection)
  - Probabilistic read assignment
- Counts must be normalized
  - RPKM is sufficient for intra-library comparisons
  - More sophisticated normalizations to account for differences in library composition for inter-library comparisons.

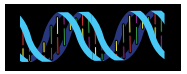
Programs to measure transcript expression

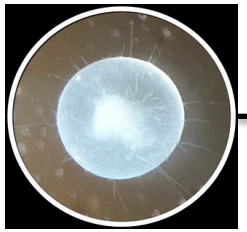
	Implemented method
Alexa-seq	Gene expression using intersection model
ERANGE	Gene expression using union model
Scripture	Gene expression using intersection model
Cufflinks	**Transcript deconvolution by solving the maximum likelihood problem**
MISO	**Transcript deconvolution by solving the maximum likelihood problem**
RSEM	**Transcript deconvolution by solving the maximum likelihood problem**

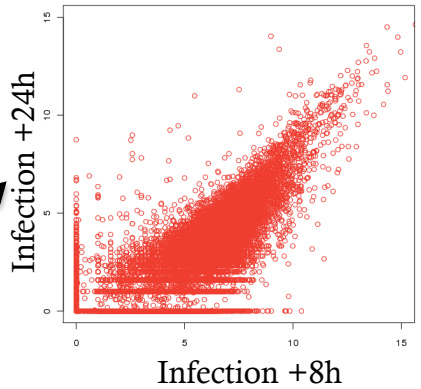
Advantages of RSEM, DESeq



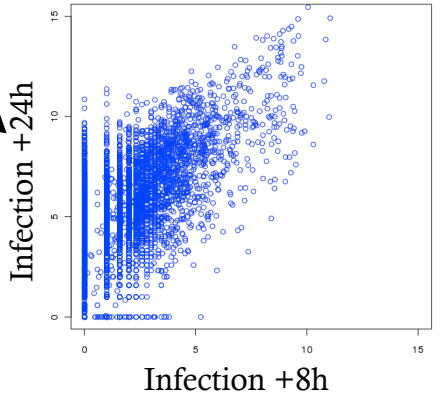








Trout



Saprolegnia

Infected fish cells

Brian Haas, Rays Jiang, Carsten Russ

Overview of the session

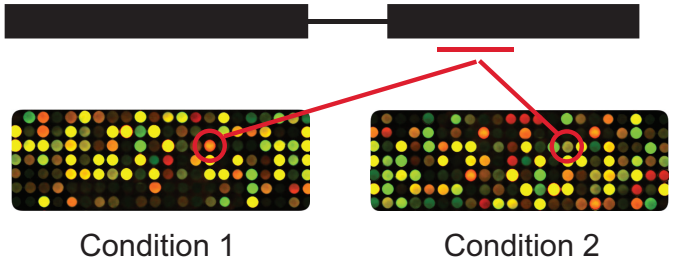
The 3 main computational challenges of sequence analysis for *counting applications:*

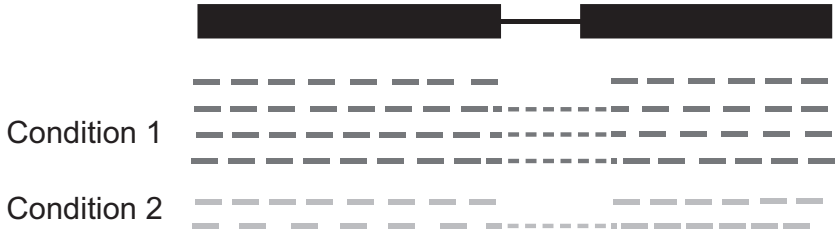
- Read mapping: Placing short reads in the genome
- Reconstruction: Finding the regions that originate the reads
- Quantification:
  - Assigning scores to regions
  - Finding regions that are differentially represented between two or more samples.

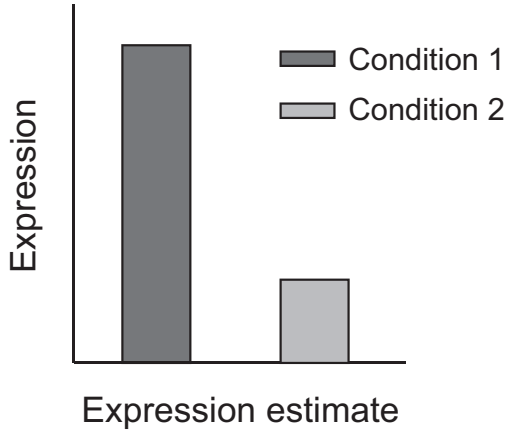
The problem.

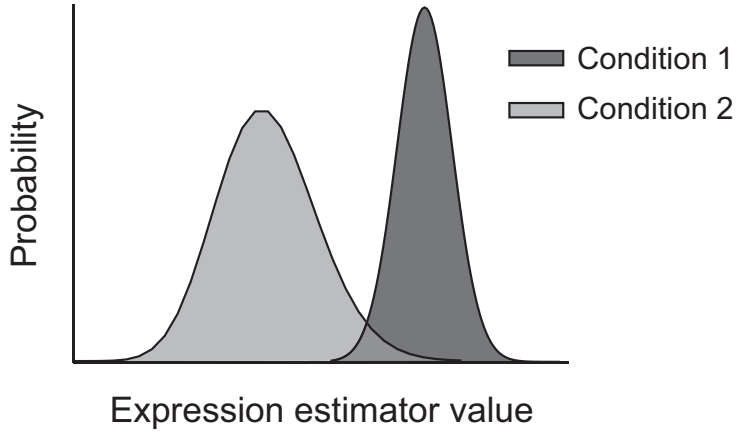
- Finding genes that have different expression between two or more conditions.
- Find gene with isoforms expressed at different levels between two or more conditions.
  - Find differentially used slicing events
  - Find alternatively used transcription start sites
  - Find alternatively used 3’ UTRs

Differential gene expression using RNA-Seq









•(Normalized) read counts ←→ Hybridization intensity

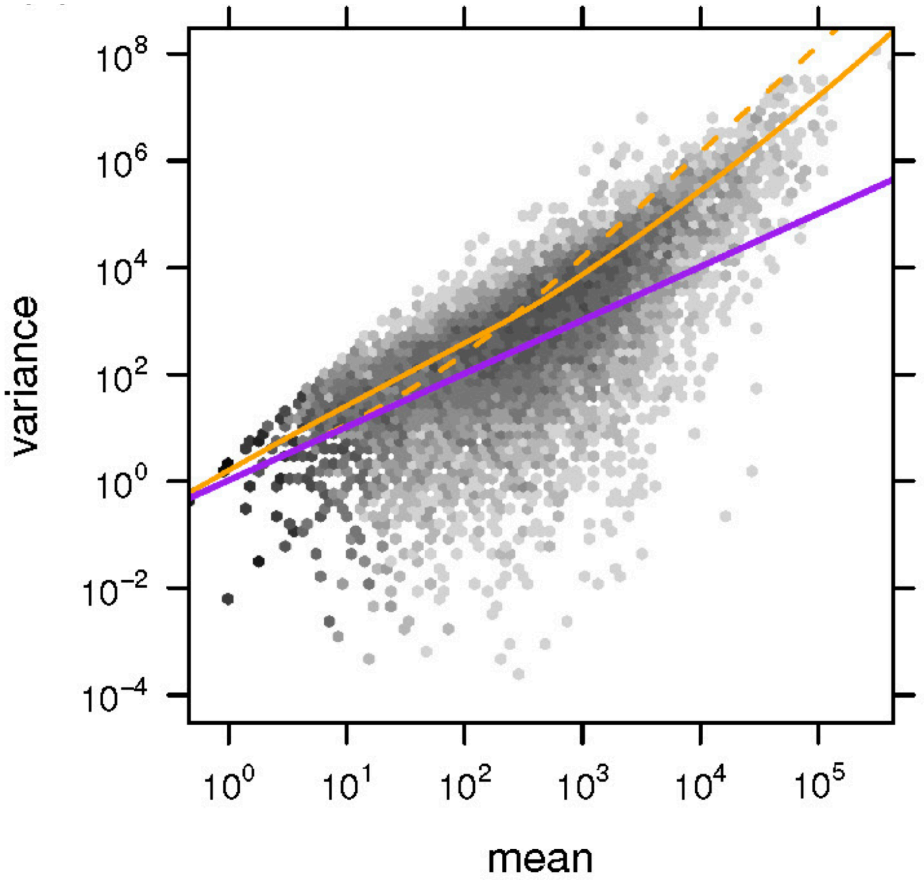
Differential analysis strategies

- 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

  - Model read counts (Poisson, negative binomial) and test whether models are distinct
  - Use empirical approaces that do not rely on parametric assumptions (more on this later

Poisson model does not work



Adapted from Anders, 2010

Biological variance does not follow a Poisson model

Using a parametric model (DESeq, Cufflinks)

Because of overdisperssion DESeq and Cufflinks uses a Negative binomial to model read counts

$$K_{g,s} \sim \mathcal{N}(K_{g,s}, \sigma_{g,s});\ \ \sigma_{g,s} = K_{g,s} + \nu_{g,s}$$

Given observed counts for two samples in replicates

$$k_{g,s_1} \ldots k_{g,s_n};\ \ k_{g,t_1} \ldots k_{g,t_m}$$

DESeq tests the null hypothesis that all counts are sampled from the same distribution

$$P(\sum_i k_{g,s_i} + \sum_j k_{g,t_j} | \mu_s = \mu_t)$$

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 != 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:

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

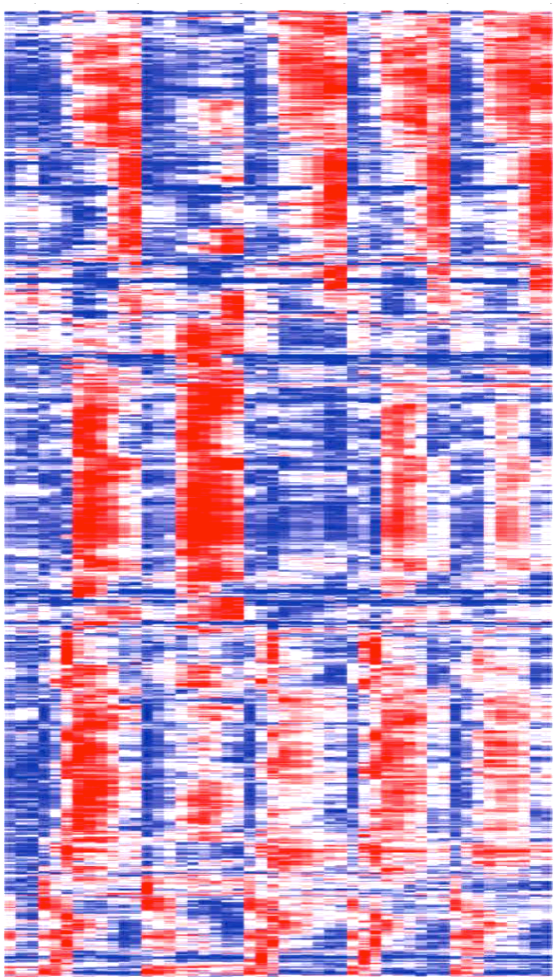
$$H(p) = -\sum_{i=1}^{n} p_i log p_i.$$

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	Negative Bionomial	Gene read counts table
Cufflinks	~~Poisson~~ Negative Bionomial	Works directly from the alignments
Myrna	Empirical	Sequence reads and reference transcriptome

RNA-Seq for traditional gene expression analysis

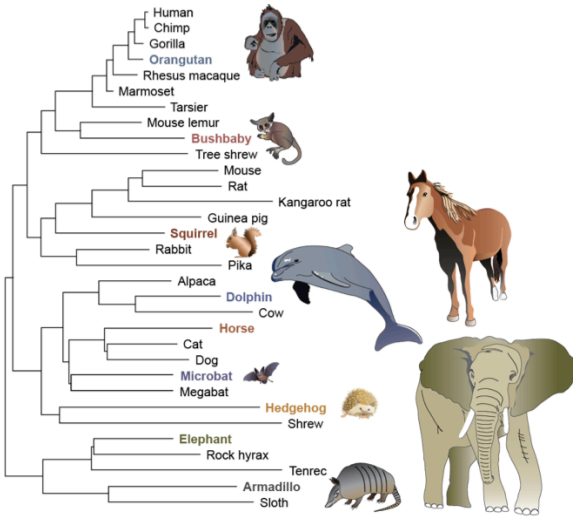


RNASeq is too expensive
for expression assays!

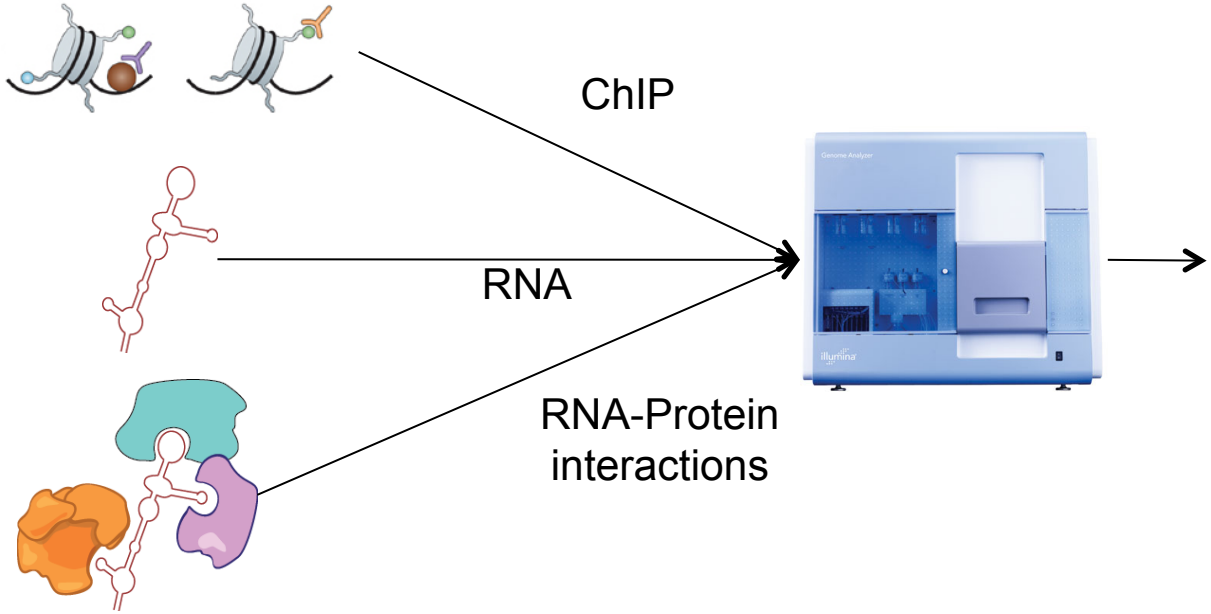
Standard RNA-Seq limitations

- Too expensive to use in HT screens
- Quantification is complex
- Differential expression is biased
  - The larger the transcript the more power to detect DE
- Hard to map alternative 3' or 5' ends of genes

Our work



Estimate the “functional genome” by finding what is under selection



- Develop informatics tools for new methods
- Develop models of transcriptional regulation
- Develop models of epigenetic interactions
- Evolution of large non-coding RNAs

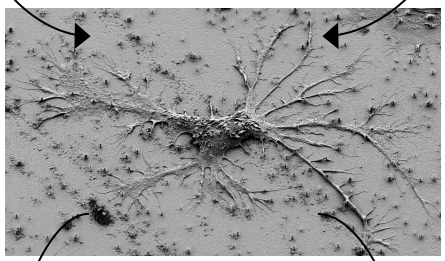
We want to ultimately understand the cell circuits of the cell

For example: Wiring of innate immune cells

Input

Bacteria

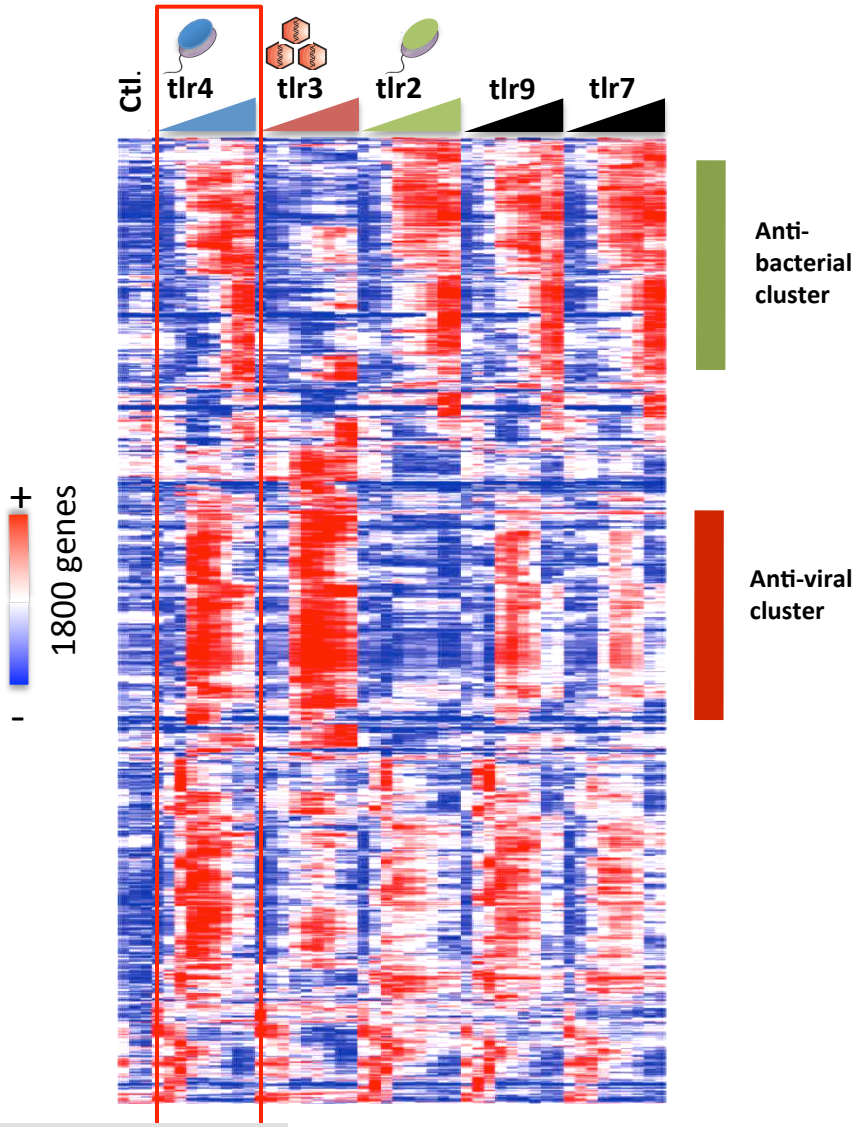
Viruses



Output

Anti-bacterial program (inflammation)

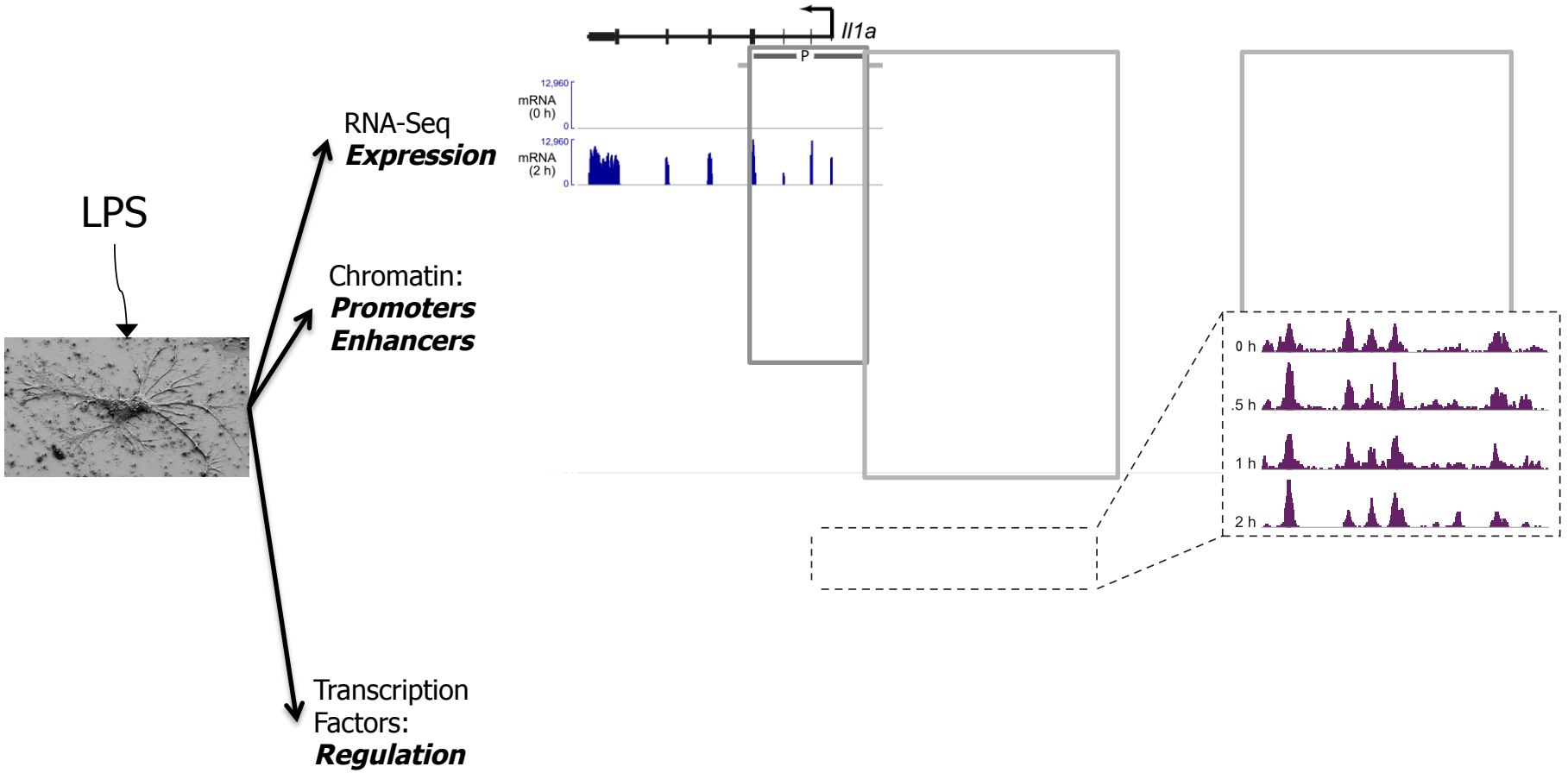
Anti-viral program (interferon)



How is this response controlled?

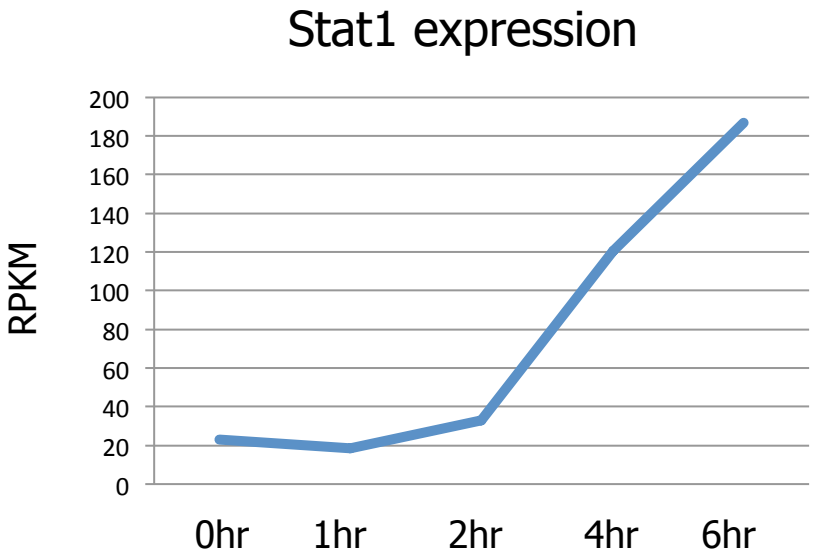
Amit, Garber et al, *Science* 2010

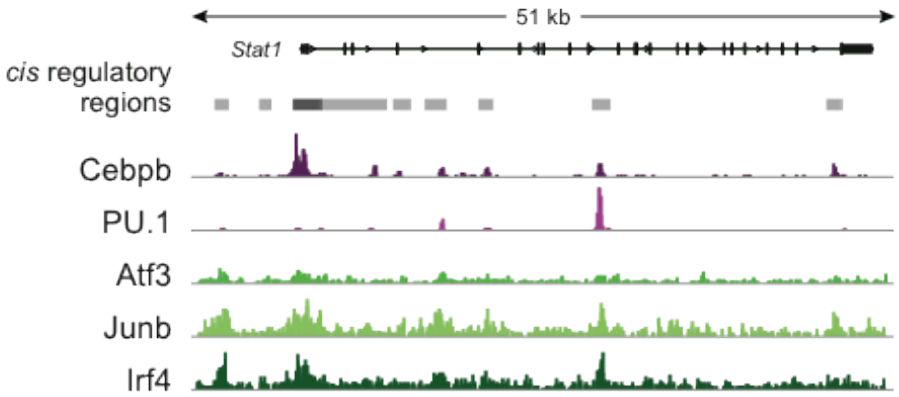
Chip-Seq + RNA-Seq to map and relate components



Sequencing libraries allow us to map output, state and the circuit of the cell

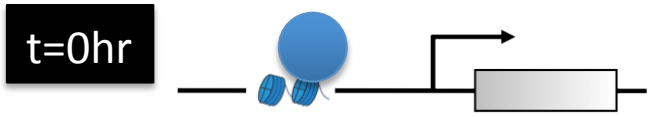
Gene regulation comes in waves



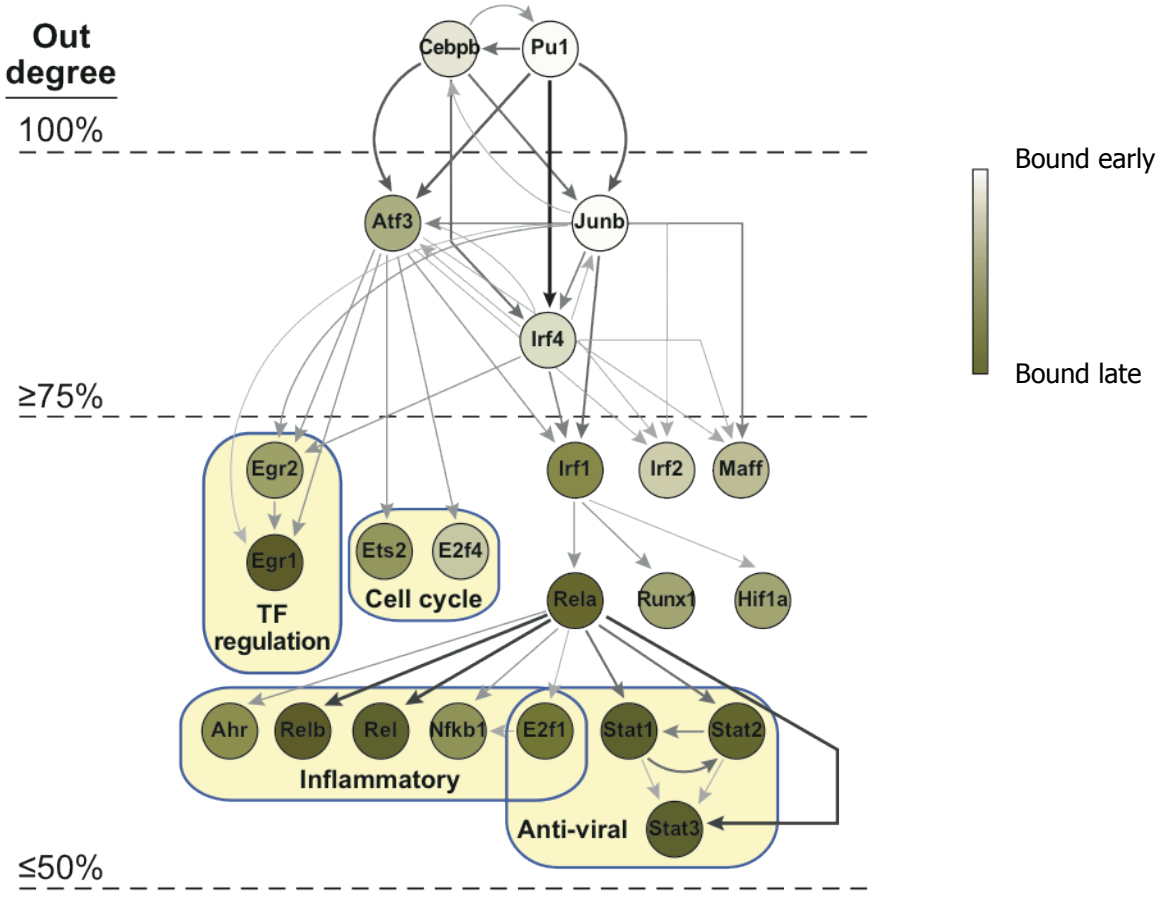


Stat1 expression is a combination of pre-binding and dynamic binding

Regulatory modules are established hierarchically



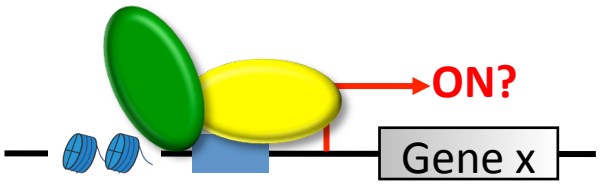
PU.1 coincides with or precedes Stat1 binding



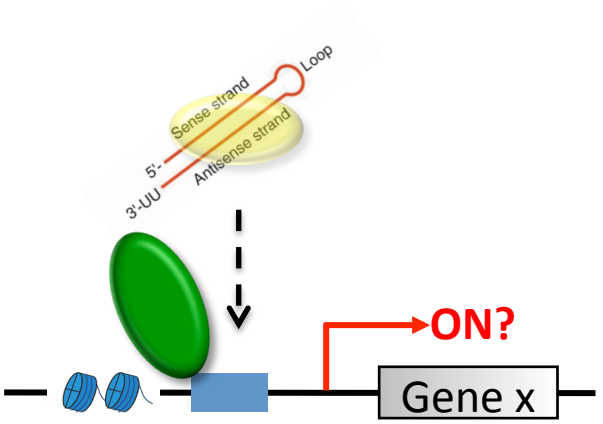
What have we learn from genome state?

- A large fraction of binding exist prior to stimulus
- Immediate vs. late regulation is quite distinct:
  - Early induced genes regulators are more redundant
  - Late induced regulators are less redundant
  - *Are the early inflammation pathways evolutionary more malleable?*
- Factors act in layers, consistent with previous reports
- ***However we can't explain most expression patterns***

What is needed: Perturbing components



TF binding map



Loss of function screen

An expensive proposition…

~100 genes KD * replicates = LARGE NUMBER of samples

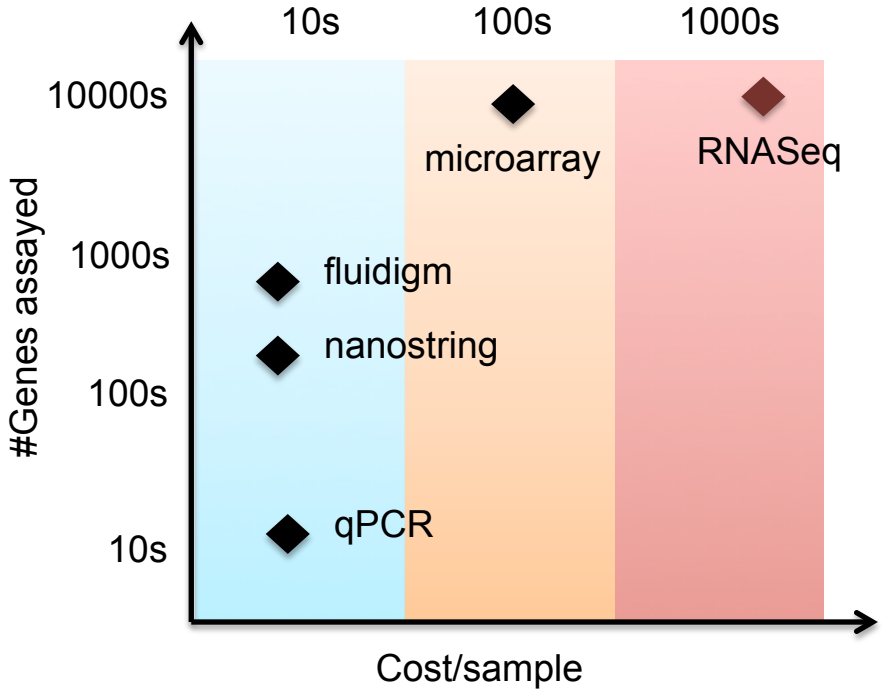
✗ high cost

✗ limited starting material

Previous solution

- qPCR
- Fluidigm
- Luminex
- NanoString
- microarray

Problem: need to chose your genes in advance and limited #genes assayed



Goal: Cheap RNA-Seq for quantification

~100 genes KD * replicates = LARGE NUMBER of samples

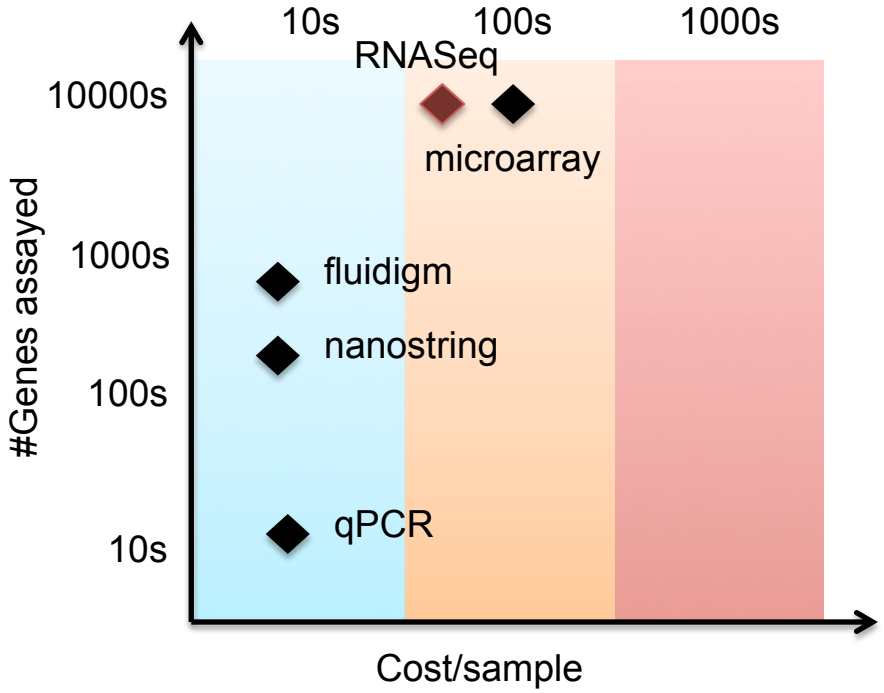
✗ high cost

✗ limited starting material

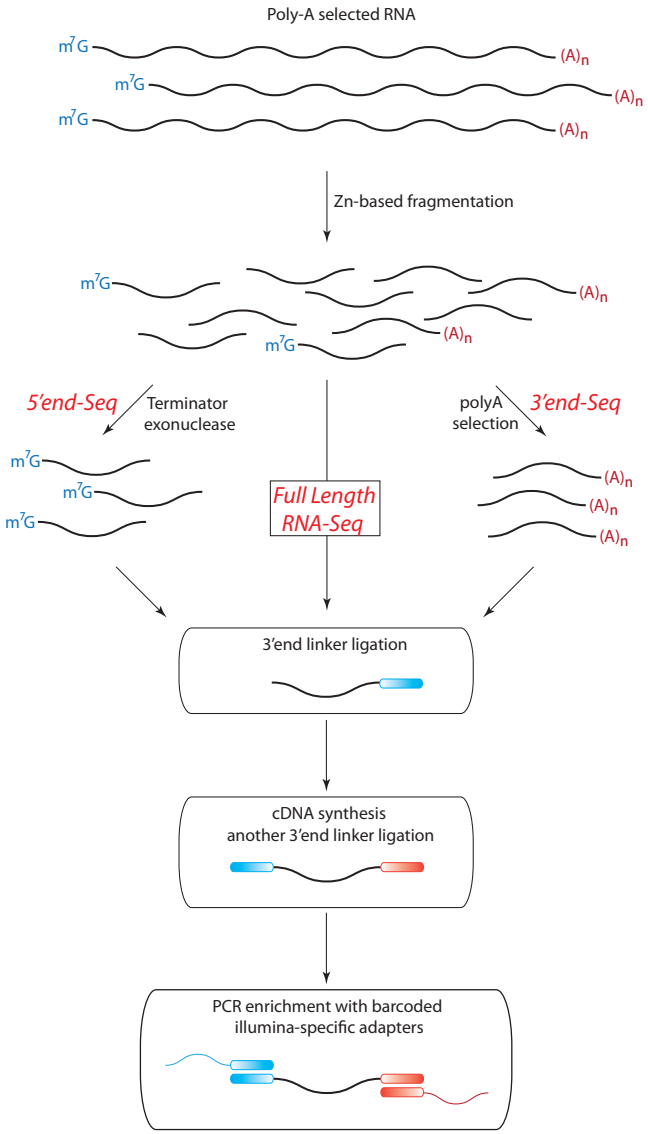
Previous solution

- qPCR
- Fluidigm
- Luminex
- NanoString
- microarray

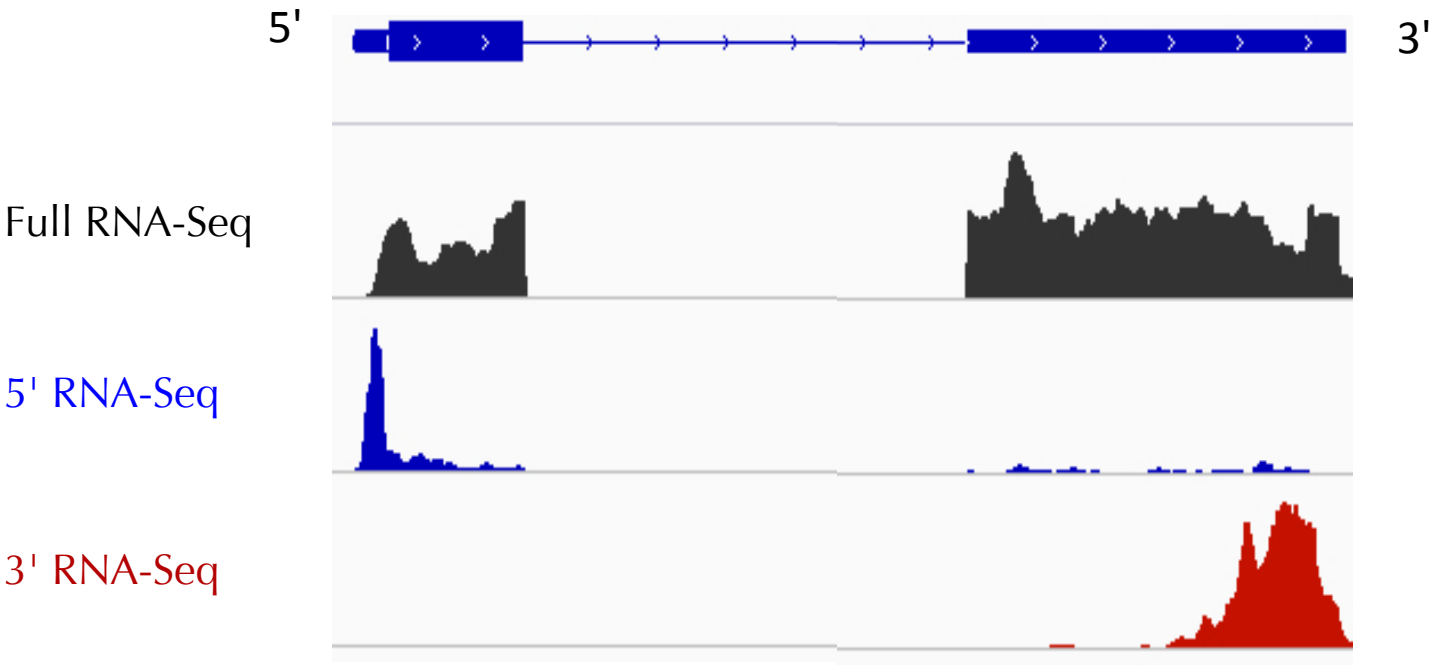
Problem: need to chose your genes in advance and limited #genes assayed

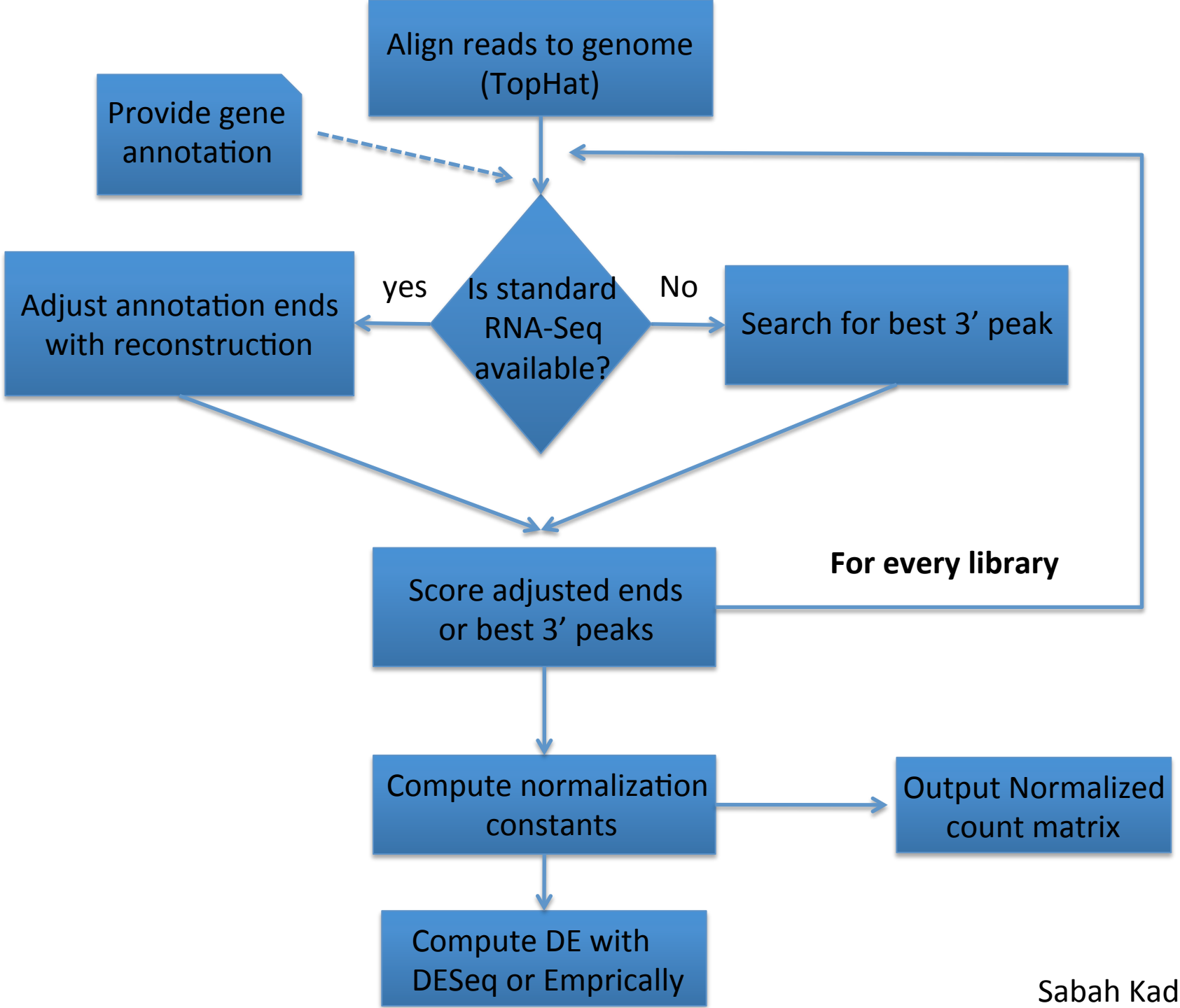


Use different RNA-Seq libraries

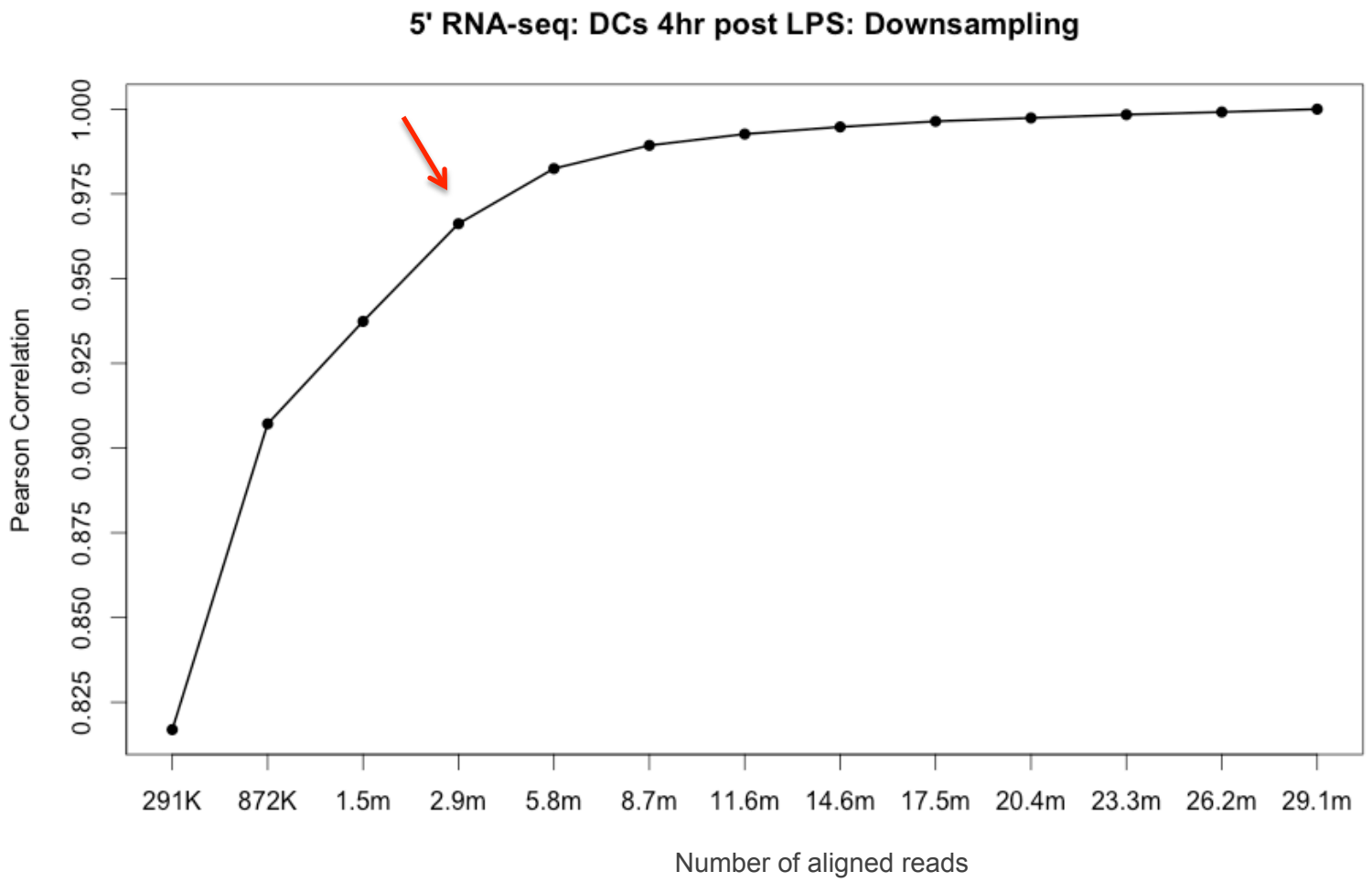


To get libraries that cover the ends and full gene bodies



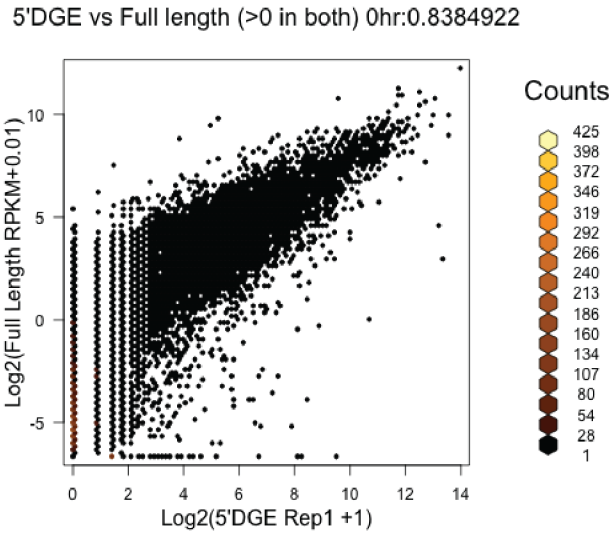

Align reads to genome (TopHat)
Provide gene annotation
Is standard RNA-Seq available?
yes
No
Adjust annotation ends with reconstruction
Search for best 3' peak
Score adjusted ends or best 3' peaks
For every library
Compute normalization constants
Output Normalized count matrix
Compute DE with DESeq or Emprically
Sabah Kadri

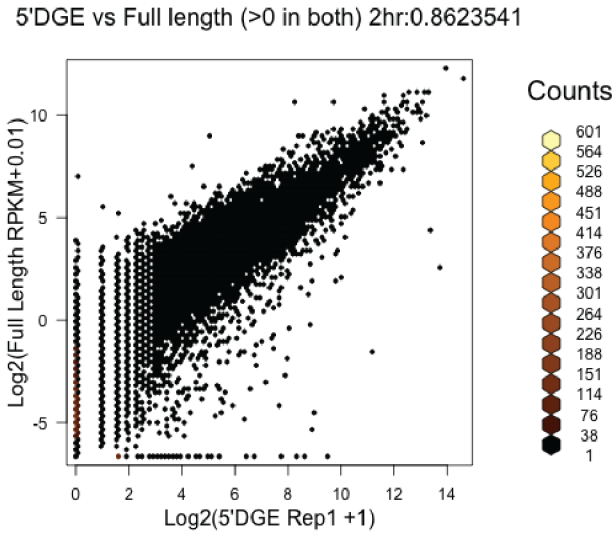
And can provide accurate quantification with small depth

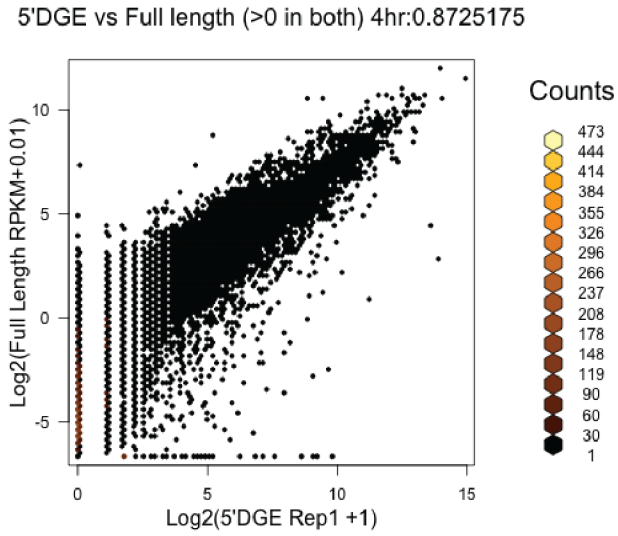


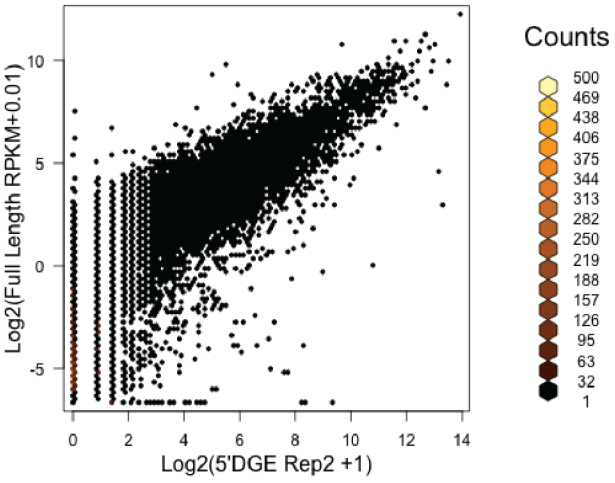
Requires just a fraction of the reads required for RNA-Seq quantification

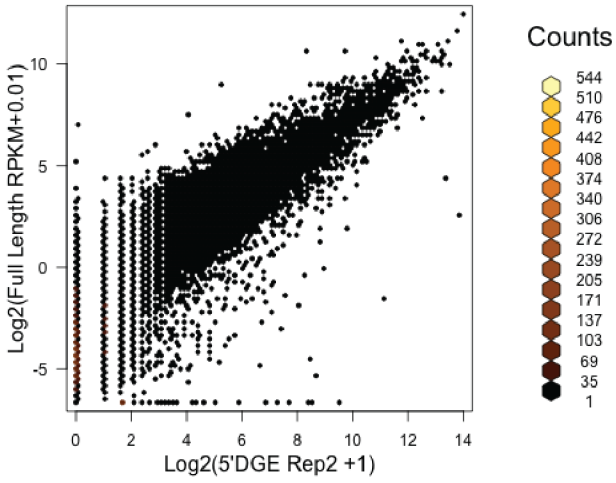
Correlation is good with standard RNA-Seq

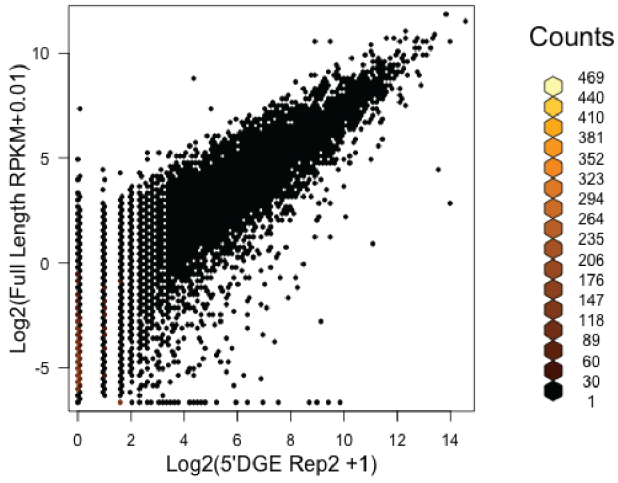












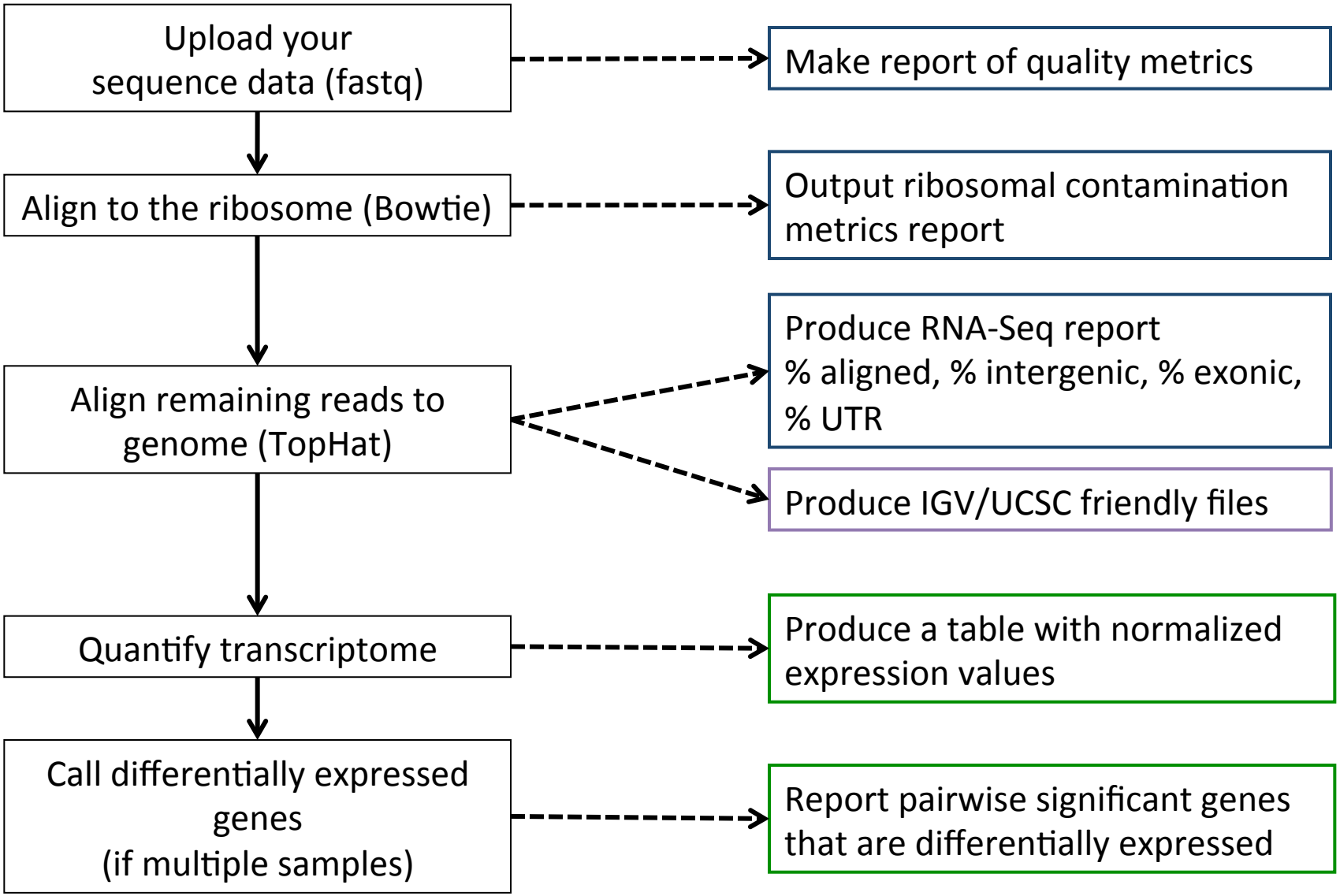
Take home message: RNA-Seq approach depends on your goals

- Full RNA-Seq
  - >90M stranded paired reads for accurate annotation
  - >30M stranded paired reads for accurate quantification at the isoform level of known transcriptome
- End Sequencing for TSS, alternative 3' and quantification
  - 4M-8M 40bp single or 25x2 paired for quantification and annotation (>20x cheaper)

Final considerations: The steps of Sequencing analysis

- Filter reads (fastq file) by removing adapter, splitting barcodes.
  - Evaluate overall quality, look for drop in quality at ends. Trim reads if ends are of low quality
- Alignment to the genome
  - Use transcriptome if available
  - Filter out likely PCR duplicates (reads that align to the same place in the genome
  - Evaluate ribosomal contamination
  - What percent of reads aligned
- Reconstruct(?)
- Quantify
  - Normalize according to application

Our typical pipeline (e.g. RNA-Seq)



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