Lecture 13. RNAseq

Michael Schatz

March 9, 2020

Applied Comparative Genomics

Assignment 5: Due Wed Mar 11

Assignment 5: Annotations and RNA-seq

Assignment Date: Wednesday, March 4, 2020
Due Date: Wednesday, March 11, 2020 @ 11:59pm

Assignment Overview

In this assignment, you will analyze gene expression data and learn how to make several kinds of plots in the environment of your choice. (We suggest Python or R.) **Make sure to show your work/code in your writeup!** As before, any questions about the assignment should be posted to Piazza.

Question 1. Gene Annotation Preliminaries [10 pts]

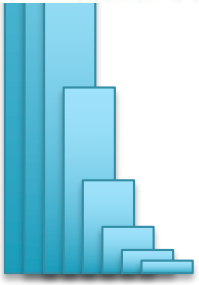
Download the annotation of build 38 of the human genome from here: ftp://ftp.ensembl.org/pub/release-87/gtf/homo_sapiens/Homo_sapiens.GRCh38.87.gtf.gz

- Question 1a. How many annotated protein coding genes are on each autosome of the human genome? [Hint: Protein coding genes will have "gene" in the 3rd column, and contain the following text: gene_biotype "protein_coding"]
- Question 1b. What is the maximum, minimum, mean, and standard deviation of the span of protein coding genes? [Hint: use the genes identified in 1b]
- Question 1c. What is the maximum, minimum, mean, and standard deviation in the number of exons for protein coding genes? [Hint: you should separately consider each isoform for each protein coding gene]

Question 2. Sampling Simulation [10 pts]

A typical human cell has ~250,000 transcripts, and a typical bulk RNA-seq experiment may involve millions of cells. Consequently in an RNAseq experiment you may start with trillions of RNA molecules, although your sequencer will only give a few million to billions of reads. Therefore your RNAseq experiment will be a small sampling of the full composition. We hope the sequences will be a representative sample of the total population, but if your sample is very unlucky or biased it may not represent the true distribution. We will explore this concept by sampling a small subset of transcripts (500 to 50000) out of a much larger set (1M) so that you can evaluate this bias.

In data1.txt with 1,000,000 lines we provide an abstraction of RNA-seq data where normalization has been performed and the number of times a gene name occurs corresponds to the number of transcripts in the sample.



Project Proposal

Project Proposal

Assignment Date: Monday March 9, 2020
Due Date: Monday, March 16 2020 @ 11:59pm

Review the Project Ideas page

Work solo or form a team for your class project (no more than 3 people to a team).

The proposal should have the following components:

- Name of your team
- List of team members and email addresses
- Short title for your proposal
- 1 paragraph description of what you hope to do and how you will do it
- References to 2 to 3 relevant papers
- References/URLs to datasets that you will be studying (Note you can also use simulated data)
- Please add a note if you need me to sponsor you for a MARCC account (high RAM, GPUs, many cores, etc)

Submit the proposal as a 1 to 2 page PDF on GradeScope (each team member should submit the same PDF). After submitting your proposal, we will schedule a time to discuss your proposal, especially to ensure you have access to the data that you need. The sooner that you submit your proposal, the sooner we can schedule the meeting. No late days can be used for the project.

Later, you will present your project in class during the last week of class. You will also submit a written report (5-7 pages) of your project, formatting as a Bioinformatics article (Intro, Methods, Results, Discussion, References). Word and LaTeX templates are available at https://academic.oup.com/bioinformatics/pages/submission_online

Please use Piazza to coordinate proposal plans!

Goal: Genome Annotations

aatgcatgcggctatgctaatgcatgcggctatgctaagctgggatccgatgacaatgcatgcggctatgctaat
gcatgcggctatgcaagctgggatccgatgactatgctaagctgggatccgatgacaatgcatgcggctatgct
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Outline

1. Alignment to other genomes
2. Prediction aka "Gene Finding"
3. Experimental & Functional Assays

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Very Similar Sequences

```
Query: HBA_HUMAN Hemoglobin alpha subunit
Sbjct: HBB_HUMAN Hemoglobin beta subunit

Score =  114 bits (285),  Expect = 1e-26
Identities = 61/145 (42%), Positives = 86/145 (59%), Gaps = 8/145 (5%)

Query  2   LSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHF------DLSHGSAQV 55
           L+P +K+ V A WGKV  +  E G EAL R+ + +P T+ +F  F      D   G+ +V
Sbjct  3   LTPEEKSAVTALWGKV--NVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKV 60

Query  56  KGHGKKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPA 115
           K HGKKV  A ++ +AH+D++    + LS+LH  KL VDP NF+LL + L+  LA H
Sbjct  61  KAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGK 120

Query  116 EFTPAVHASLDKFLASVSTVLTSKY 140
           EFTP V A+  K +A V+  L  KY
Sbjct  121 EFTPPVQAAYQKVVAGVANALAHKY 145
```

Outline

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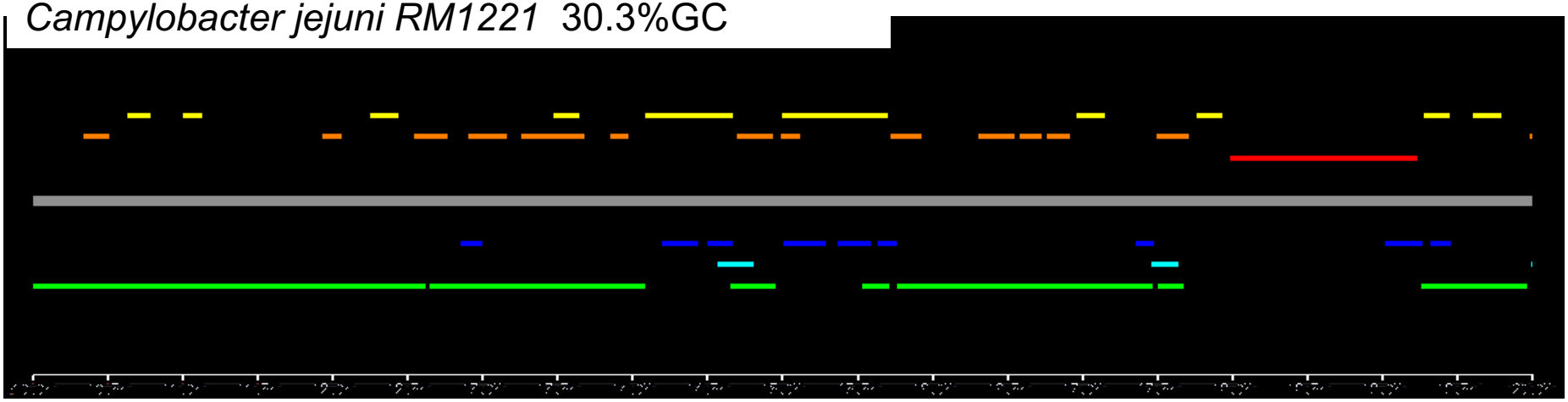
2. Prediction aka "Gene Finding"
3. Experimental & Functional Assays

Campylobacter jejuni RM1221 30.3%GC



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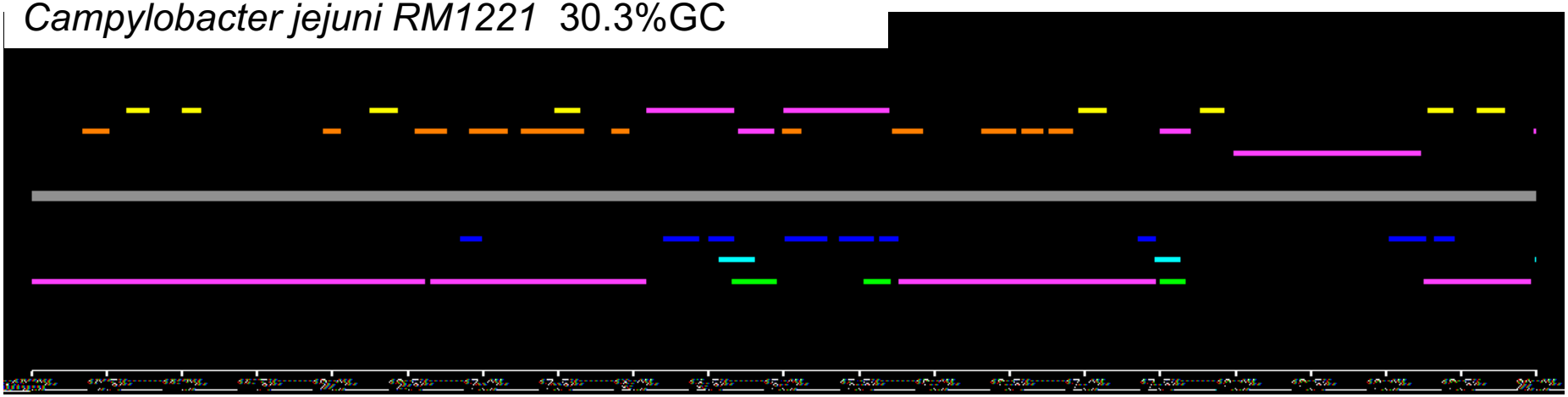
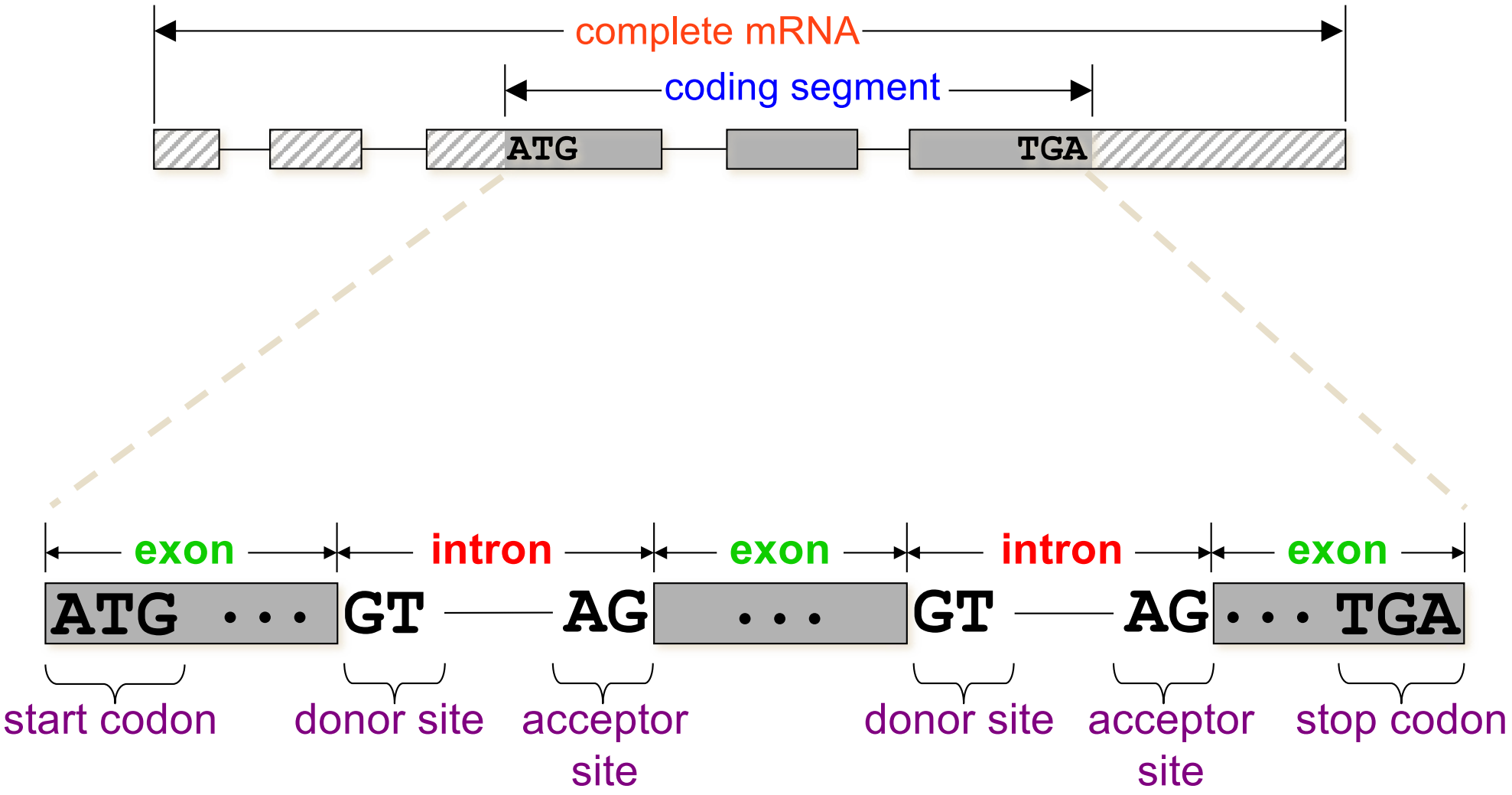
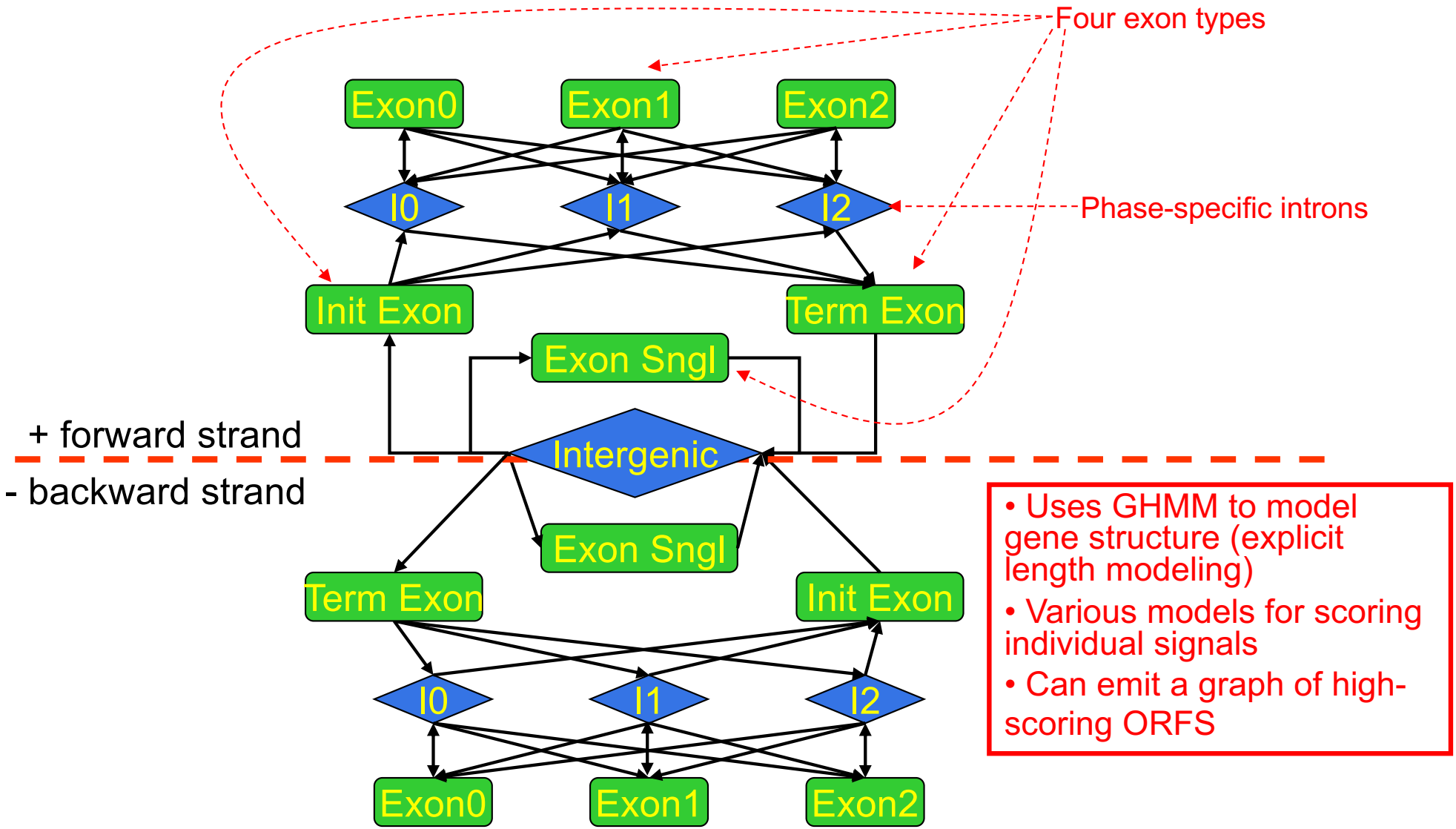


Eukaryotic Gene Syntax



Regions of the gene outside of the CDS are called *UTR*'s (*untranslated regions*), and are mostly ignored by gene finders, though they are important for regulatory functions.

GlimmerHMM architecture



- Uses GHMM to model gene structure (explicit length modeling)
- Various models for scoring individual signals
- Can emit a graph of high-scoring ORFS

Gene Finding Overview

- Prokaryotic gene finding distinguishes real genes and random ORFs
  - Prokaryotic genes have simple structure and are largely homogenous, making it relatively easy to recognize their sequence composition

- Eukaryotic gene finding identifies the genome-wide most probable gene models (set of exons)
  - "Probabilistic Graphical Model" to enforce overall gene structure, separate models to score splicing/transcription signals
  - Accuracy depends to a large extent on the quality of the training data

Outline

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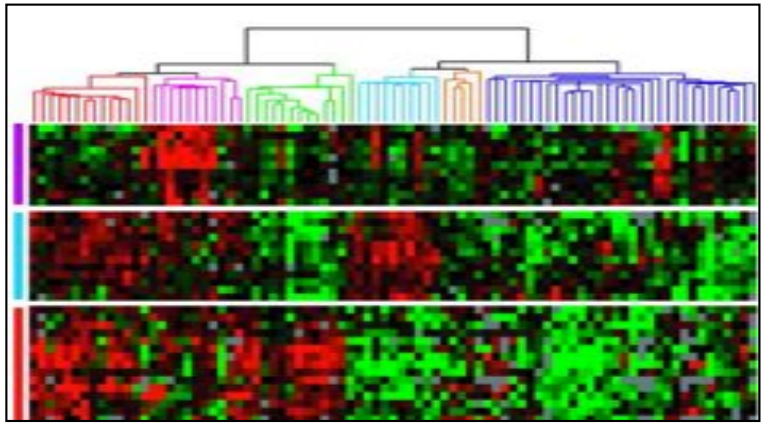
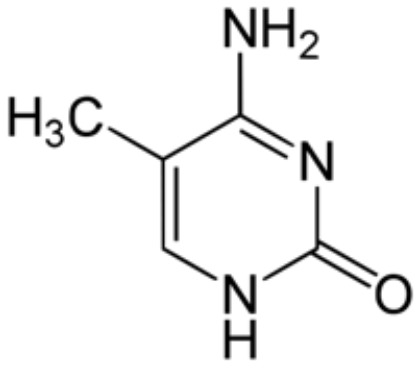
2. Prediction aka "Gene Finding"
3. Experimental & Functional Assays

*-seq in 4 short vignettes

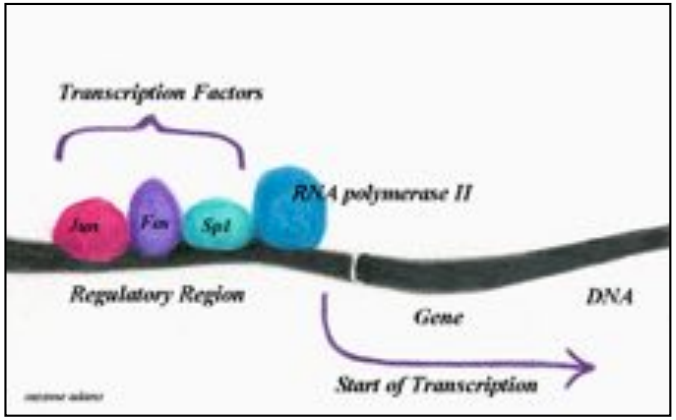
RNA-seq



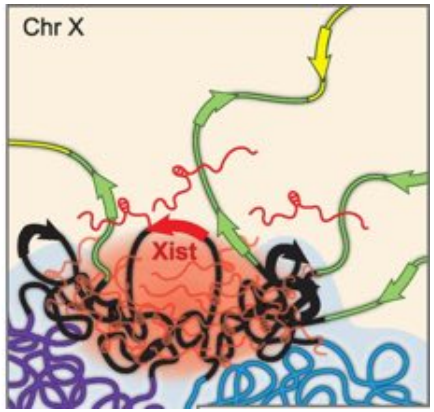
Methyl-seq



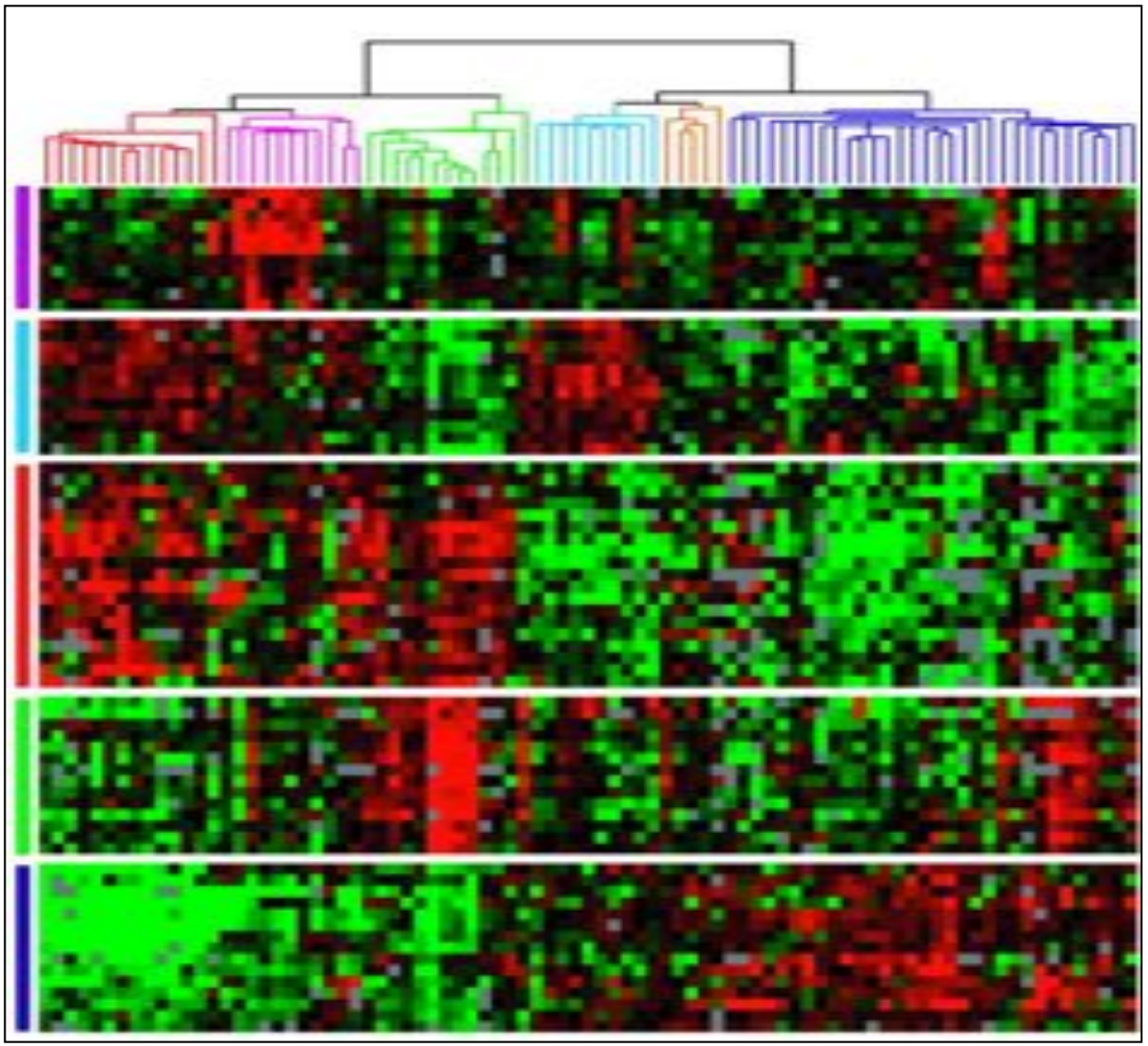
ChIP-seq



Hi-C

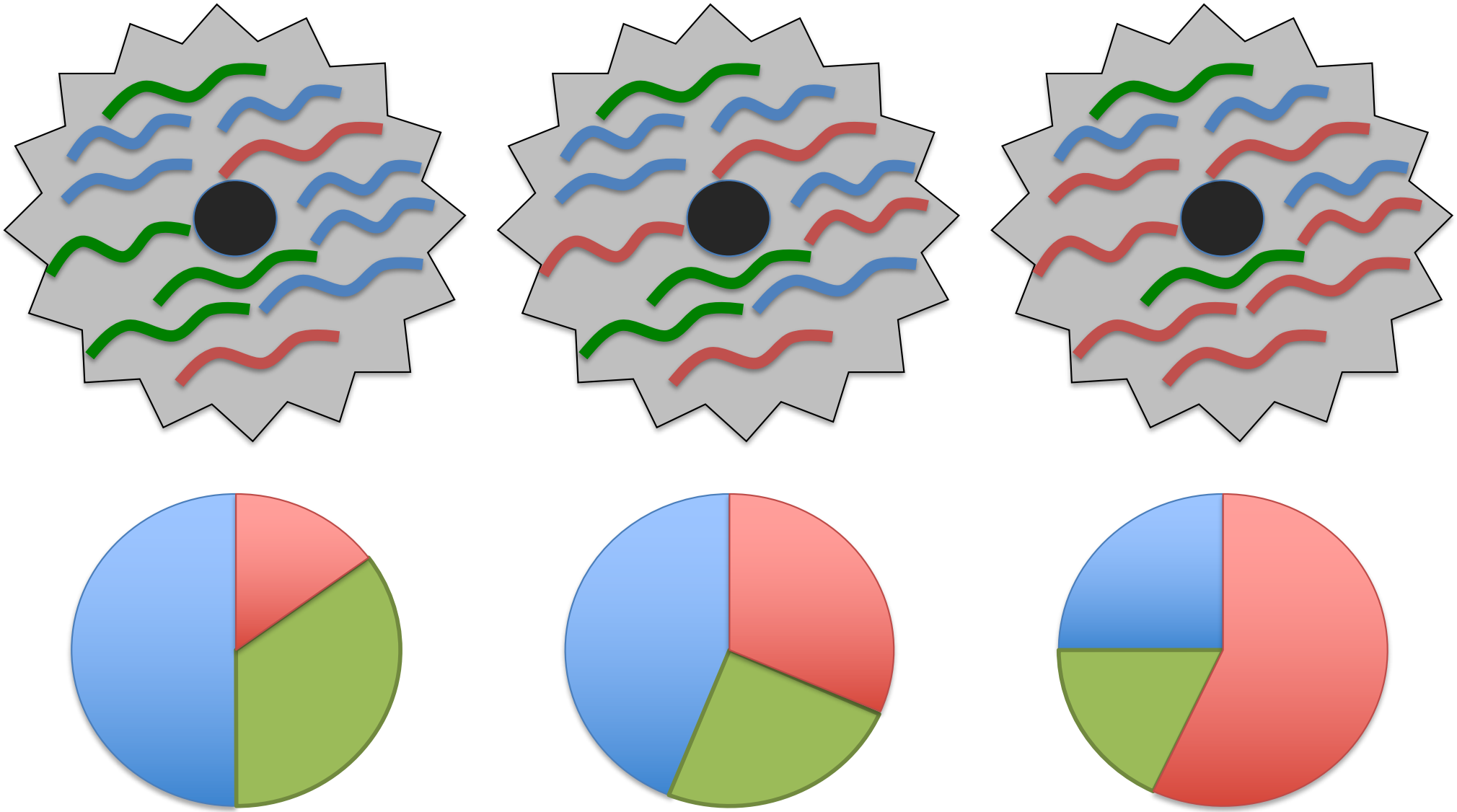


RNA-seq

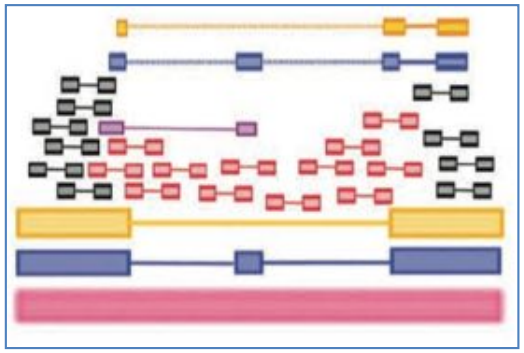


Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.
Sørlie et al (2001) *PNAS*. 98(19):10869-74.

RNA-seq Overview



RNA-seq Challenges



Challenge 1: Eukaryotic genes are spliced

RNA-Seq Approaches

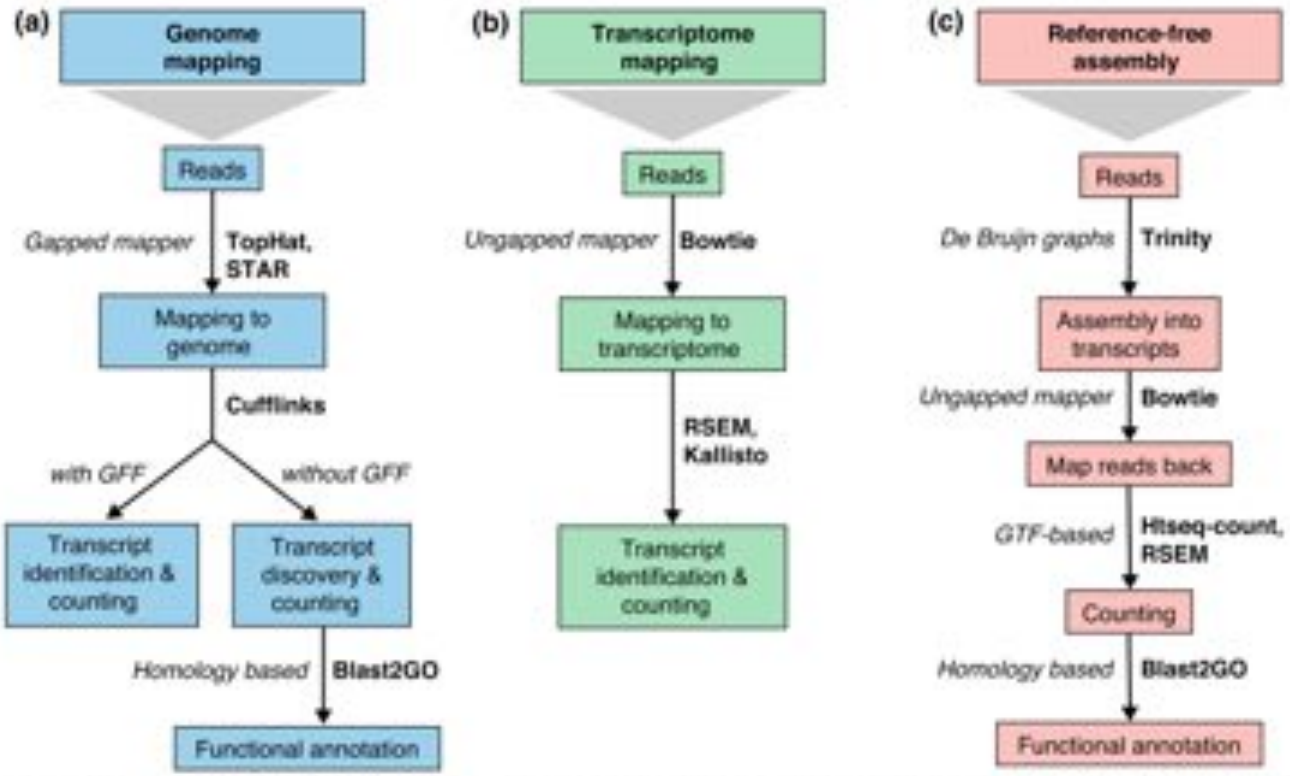
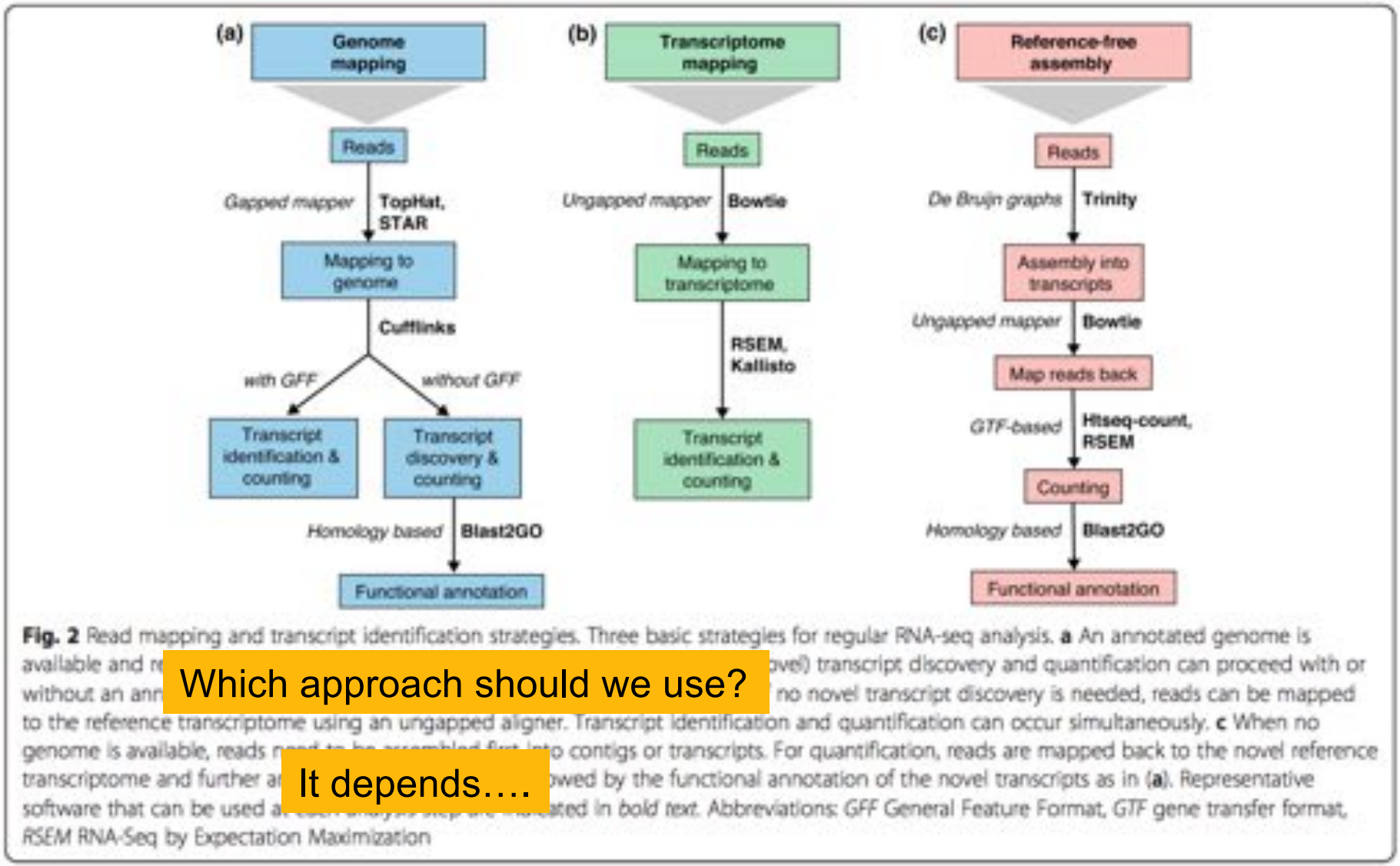


Fig. 2 Read mapping and transcript identification strategies. Three basic strategies for regular RNA-seq analysis. **a** An annotated genome is available and reads are mapped to the genome with a gapped mapper. Next (novel) transcript discovery and quantification can proceed with or without an annotation file. Novel transcripts are then functionally annotated. **b** If no novel transcript discovery is needed, reads can be mapped to the reference transcriptome using an ungapped aligner. Transcript identification and quantification can occur simultaneously. **c** When no genome is available, reads need to be assembled first into contigs or transcripts. For quantification, reads are mapped back to the novel reference transcriptome and further analysis proceeds as in (**b**) followed by the functional annotation of the novel transcripts as in (**a**). Representative software that can be used at each analysis step are indicated in *bold text*. Abbreviations: *GFF* General Feature Format, *GTF* gene transfer format, *RSEM* RNA-Seq by Expectation Maximization

A survey of best practices for RNA-seq data analysis
Conesa et al (2016) Genome Biology. doi 10.1186/s13059-016-0881-8

RNA-Seq Approaches



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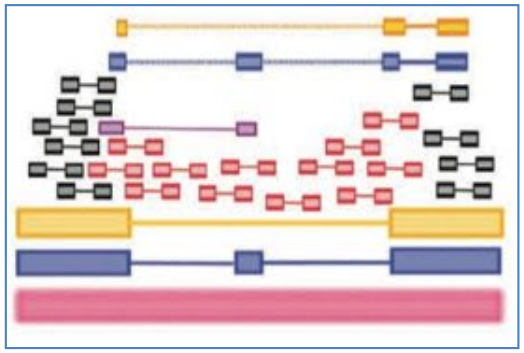
Which approach should we use?

It depends….

A survey of best practices for RNA-seq data analysis
Conesa et al (2016) Genome Biology. doi 10.1186/s13059-016-0881-8

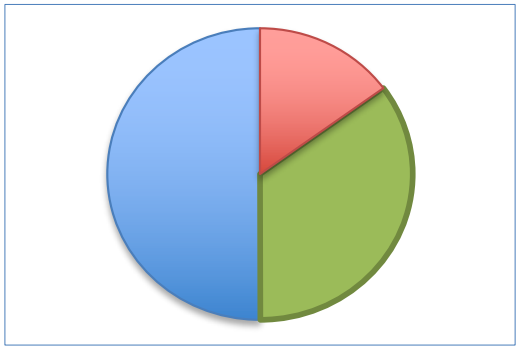
RNA-seq Challenges



Challenge 1: Eukaryotic genes are spliced

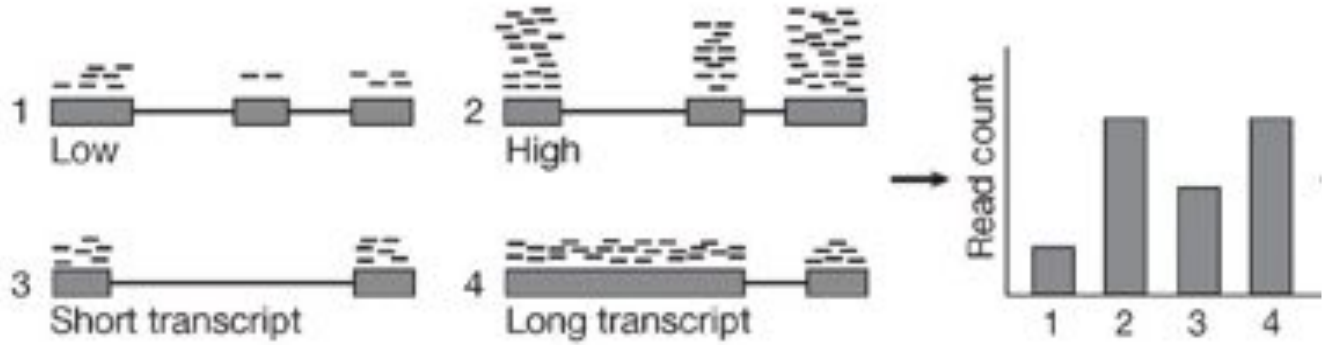
Solution: Use a spliced aligner, and assemble isoforms

TopHat: discovering spliced junctions with RNA-Seq.
Trapnell et al (2009) *Bioinformatics*. 25:0 1105-1111



Challenge 2: Read Count != Transcript abundance

RPKM, FPKM, TPM

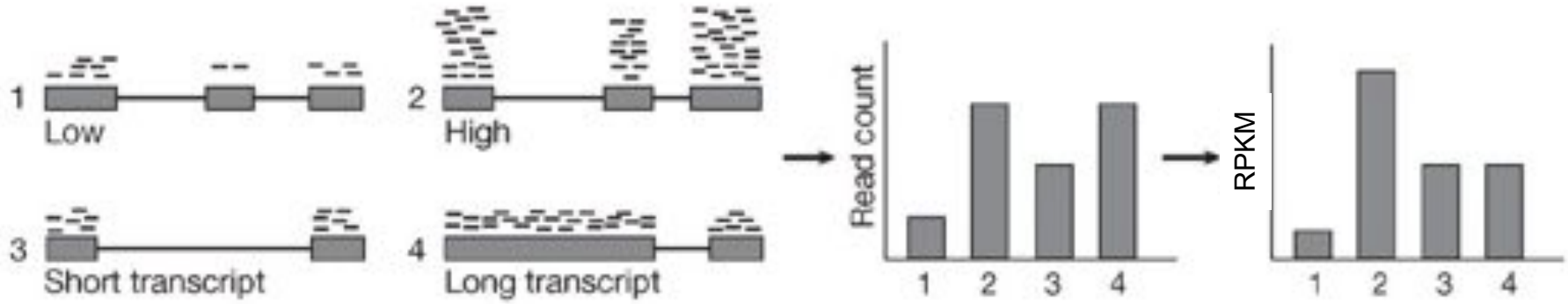


Counting Reads that align to a gene DOESN'T work!

- Overall Coverage: 1M reads in experiment 1 vs 10M reads in experiment 2
- Gene Length: gene 3 is 10kbp, gene 4 is 100kbp

1. RPKM: Reads Per Kilobase of Exon Per Million Reads Mapped (Mortazavi et al, 2008)

RPKM, FPKM, TPM



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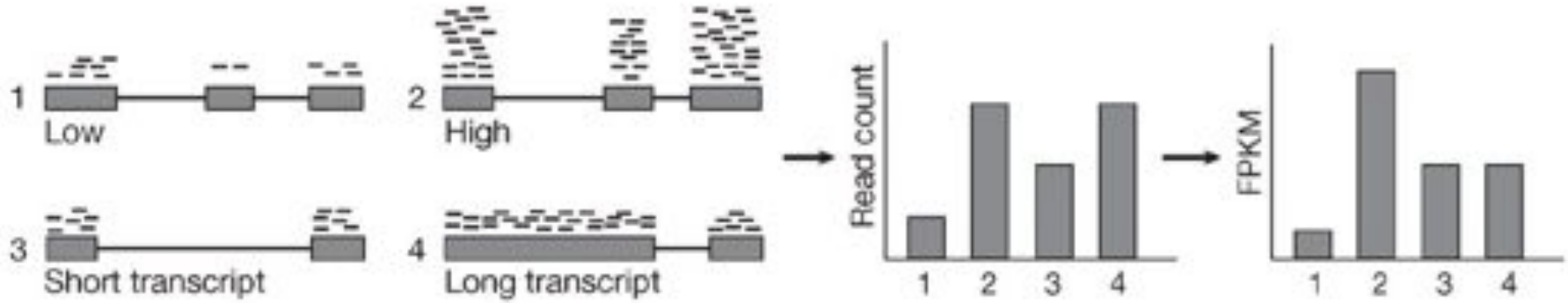
(Count reads aligned to gene) / (length of gene in kilobases) / (# millions of read mapped)

=> Wait a second, reads in a pair arent independent!

RPKM, FPKM, TPM



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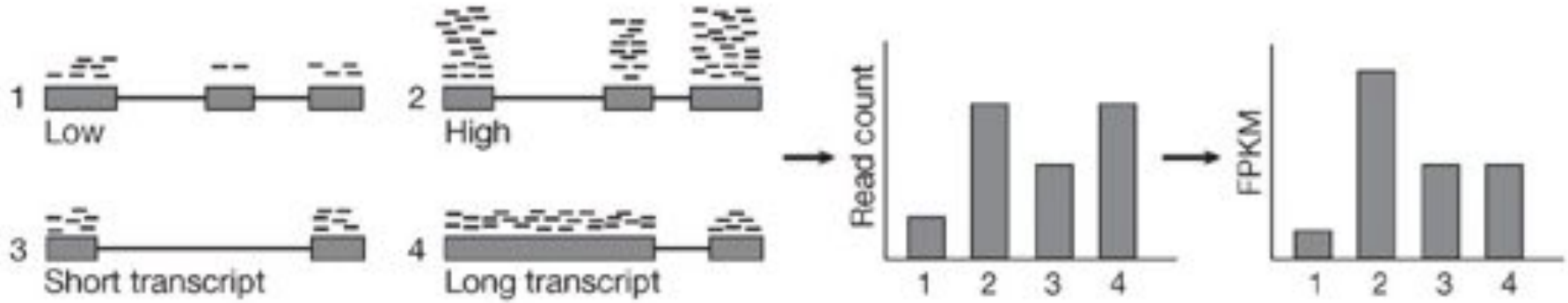
=> Wait a second, reads in a pair arent independent!

2. FPKM: Fragments Per Kilobase of Exon Per Million Reads Mapped (Trapnell et al, 2010)

$\Rightarrow$ Does a much better job with short exons & short genes by boosting coverage

$\Rightarrow$ Wait a second, FPKM depends on the average transcript length!

RPKM, FPKM, TPM



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2. FPKM: Fragments Per Kilobase of Exon Per Million Reads Mapped (Trapnell et al, 2010)

=> Wait a second, FPKM depends on the average transcript length!

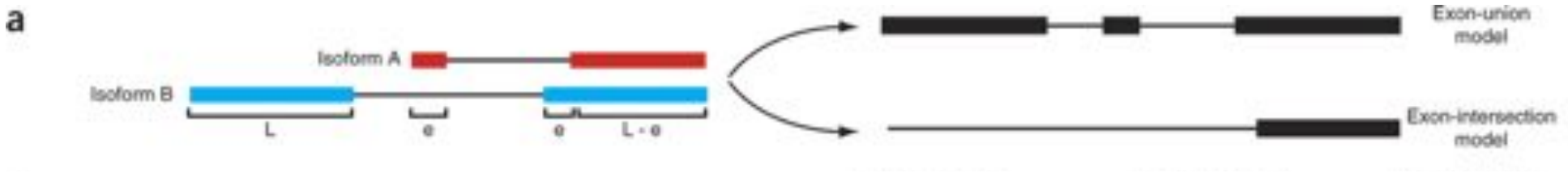
3. TPM: Transcripts Per Million (Li et al, 2011)

⇒ If you were to sequence one million full length transcripts, TPM is the number of transcripts you would have seen of type i, given the abundances of the other transcripts in your sample

=> Recommend you use TPM for all analysis, easy to compute given FPKM

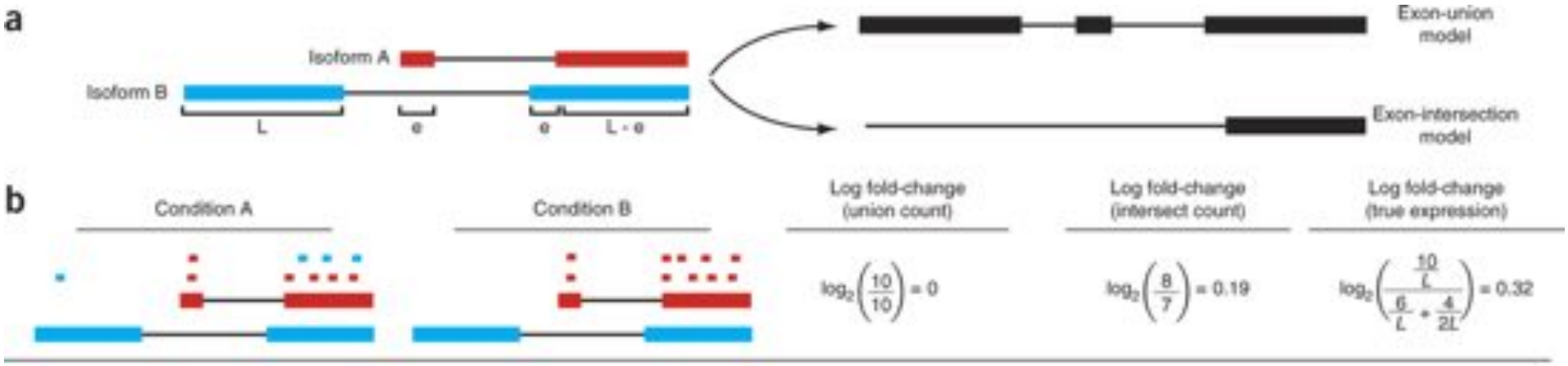
$$\mathrm{TPM}_i = \left(\frac{\mathrm{FPKM}_i}{\sum_j \mathrm{FPKM}_j} \right) \cdot 10^6$$

Gene or Isoform Quantification?



Differential analysis of gene regulation at transcript resolution with RNA-seq
Trapnell et al (2013) Nature Biotechnology 31, 46–53. doi:10.1038/nbt.2450

Gene or Isoform Quantification?



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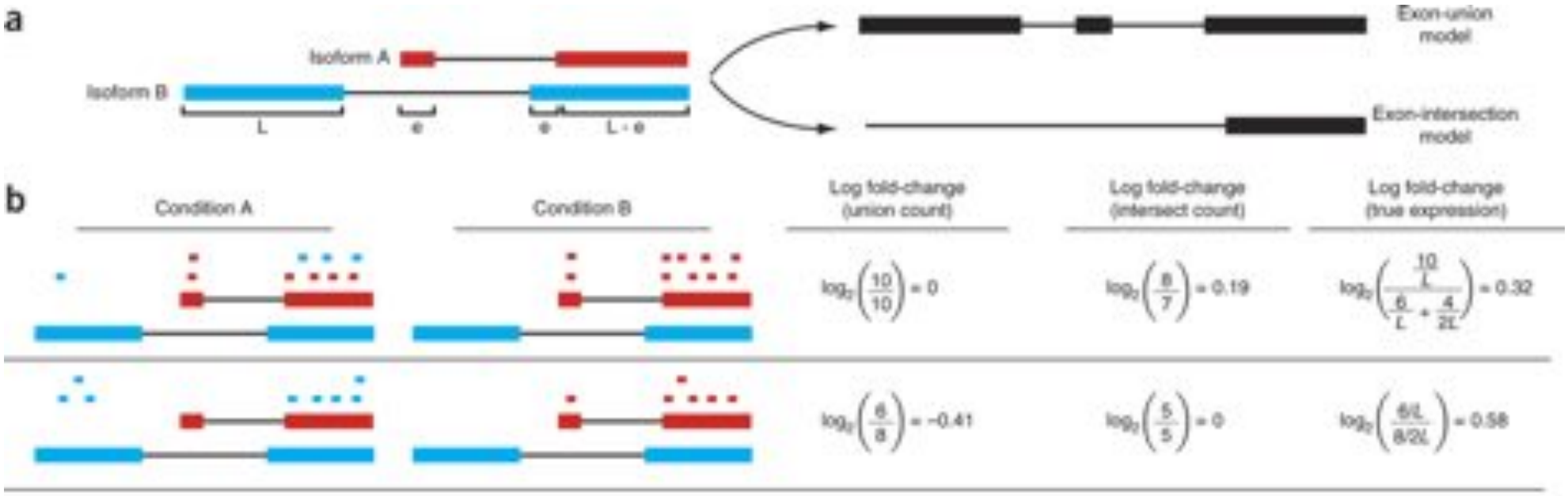

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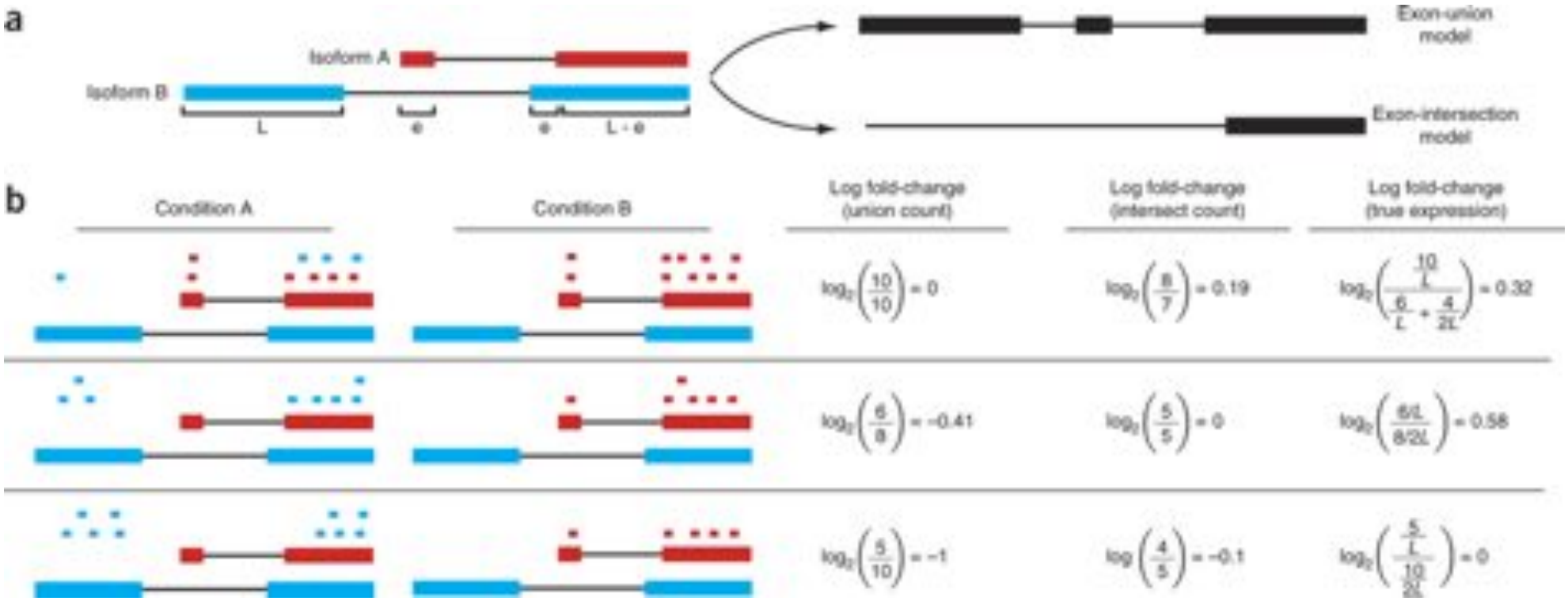
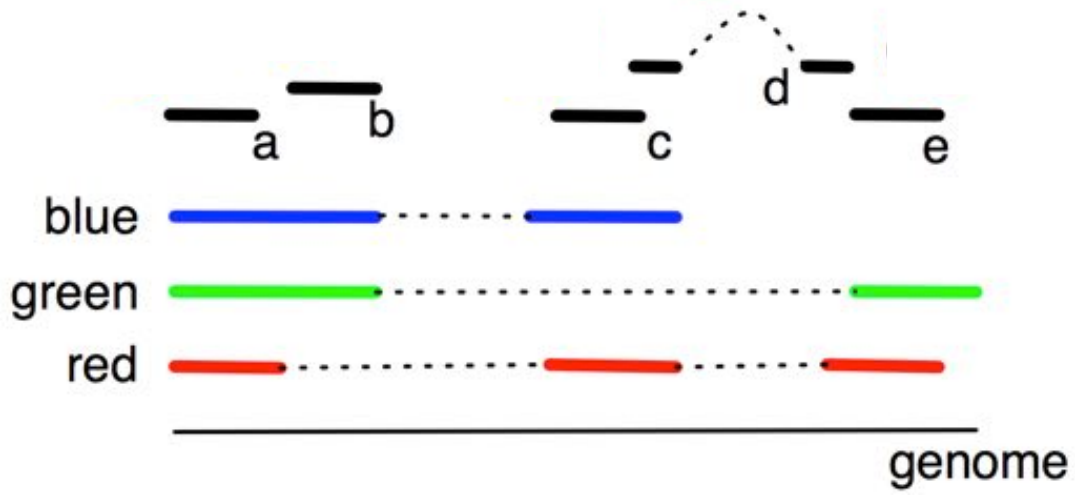


Key point : The length of the actual molecule from which the fragments derive is crucially important to obtaining accurate abundance estimates.

Differential analysis of gene regulation at transcript resolution with RNA-seq
Trapnell et al (2013) Nature Biotechnology 31, 46–53. doi:10.1038/nbt.2450

Multi-mapping? Isoform ambiguity? Expectation Maximization to the Rescue



The gene has three isoforms (red, green, blue) of the same length.
Our initial expectation is all 3 isoforms are equally expressed

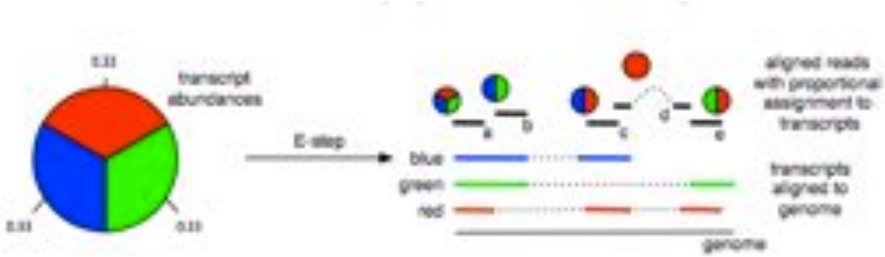
There are five reads (a,b,c,d,e) mapping to the gene.

- Read a maps to all three isoforms
- Read d only to red
- Reads b,c,e map to each of the three pairs of isoforms.

What is the most likely expression level of each isoform?

Models for transcript quantification from RNA-seq
Pachter, L (2011) arXiv. 1104.3889 [q-bio.GN]

Multi-mapping? Isoform ambiguity? Expectation Maximization to the Rescue



The gene has three isoforms (red, green, blue) of the same length. Initially every isoform is assigned the same abundance (red=1/3, green=1/3, blue=1/3)

There are five reads (a,b,c,d,e) mapping to the gene. Read a maps to all three isoforms, read d only to red, and the other three (reads b,c,e) to each of the three pairs of isoforms.

During the expectation (E) step reads are proportionately assigned to transcripts according to the (current) isoform abundances (RGB): a=(.33,.33,.33), b=(0,.5,.5), c=(.5,.5), d=(1,0,0), e=(.5,.5,0)

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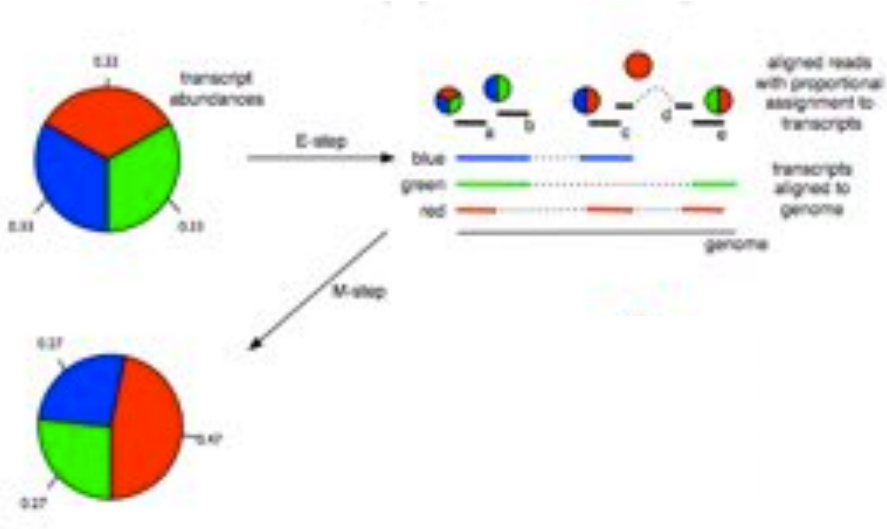

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red: 0.47 = (0.33 + 0.5 + 1 + 0.5)/(2.33 + 1.33 + 1.33)
blue: 0.27 = (0.33 + 0.5 + 0.5)/(2.33 + 1.33 + 1.33)
green: 0.27 = (0.33 + 0.5 + 0.5)/(2.33 + 1.33 + 1.33)

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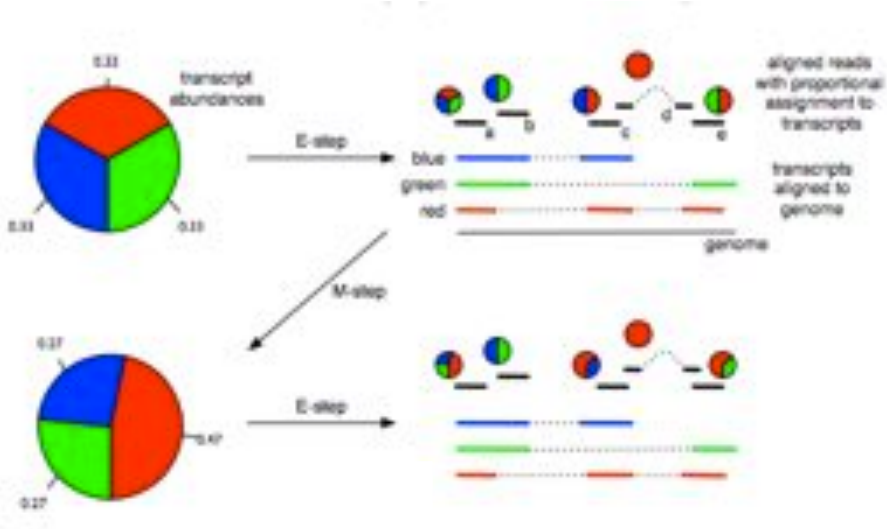

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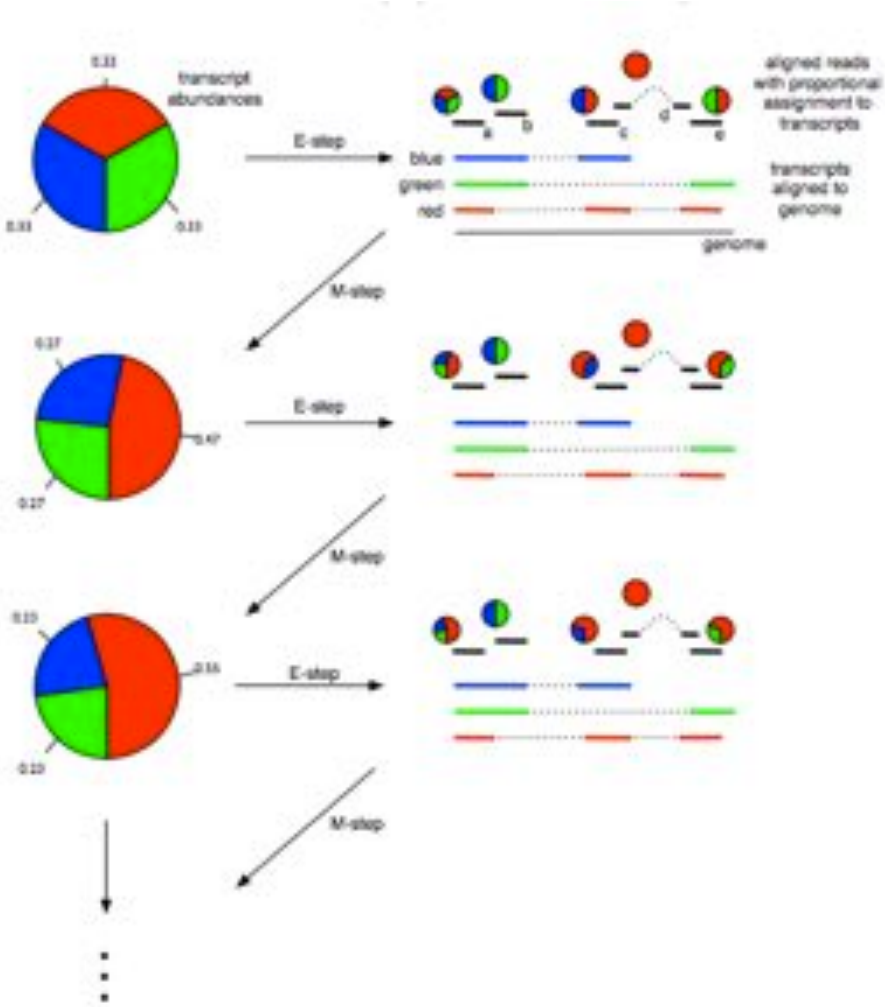

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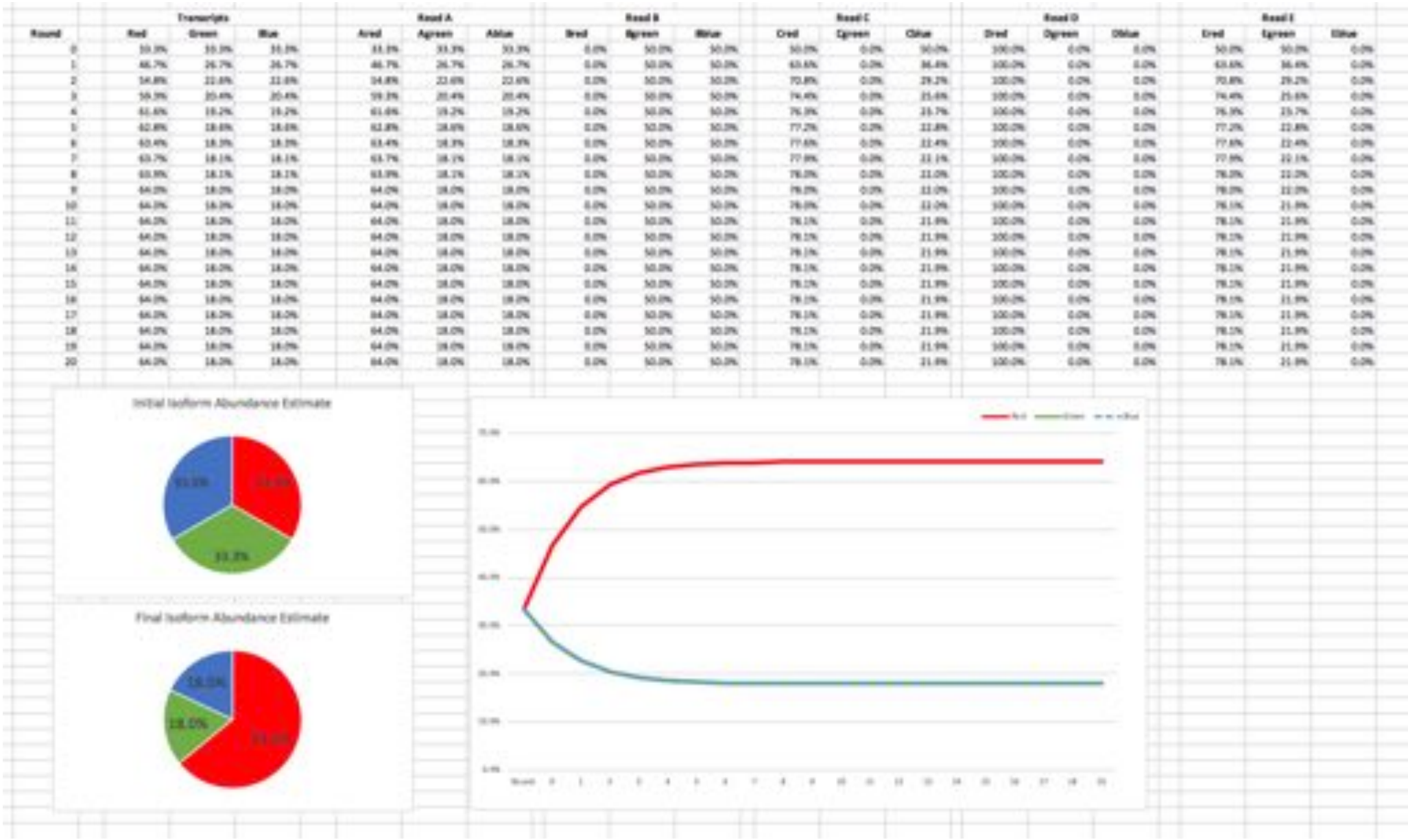

RNAseqExpectationMaximization.xlxs

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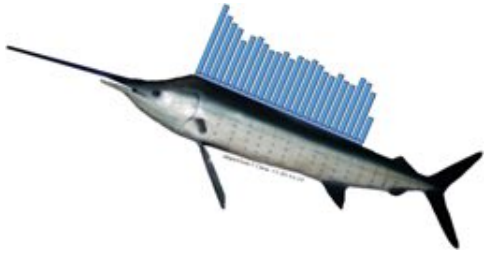
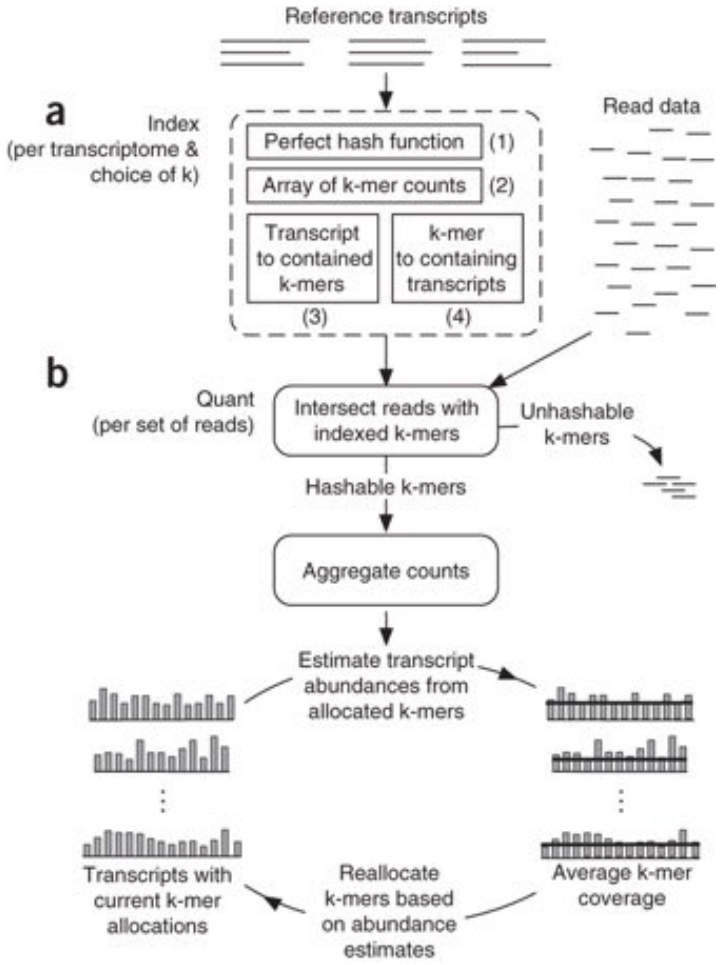
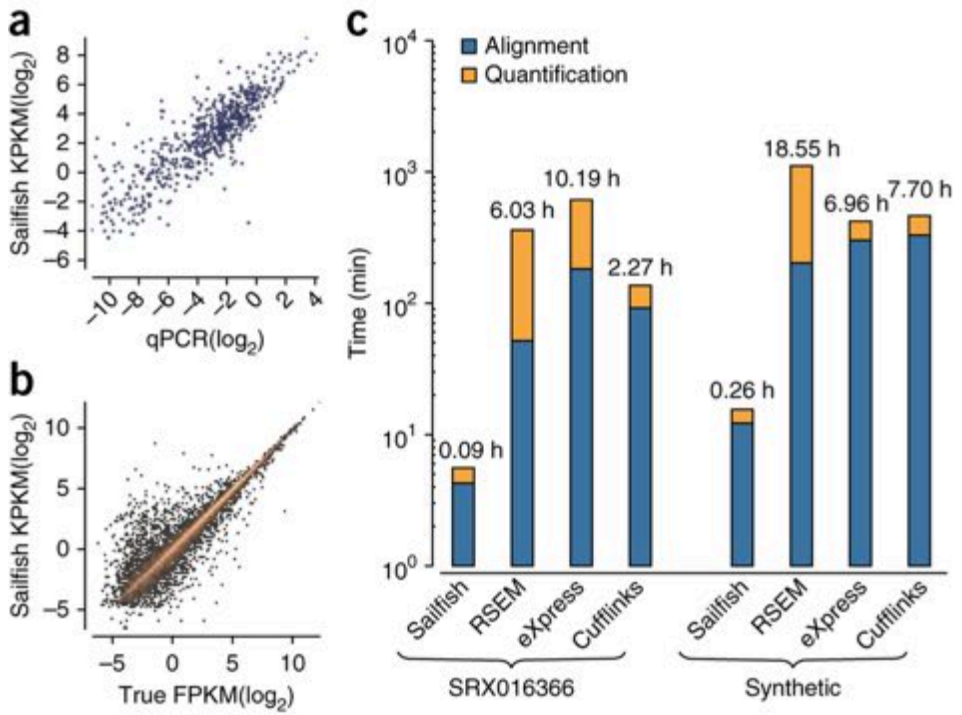
Pachter, L (2011) arXiv. 1104.3889 [q-bio.GN]

Sailfish: Fast & Accurate RNA-seq Quantification





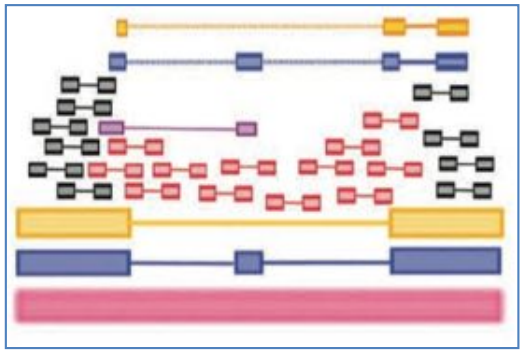


d

	Human brain tissue				Synthetic			
	Sailfish	RSEM	eXpress	Cufflinks	Sailfish	RSEM	eXpress	Cufflinks
Pearson	0.85	0.82	0.85	0.85	0.96	0.96	0.95	0.94
Spearman	0.84	0.80	0.85	0.85	0.76	0.77	0.77	0.75
RMSE	–	–	–	–	6.06	8.90	8.83	10.05
medPE	–	–	–	–	6.50	12.48	14.06	12.42

Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms
Patro et al (2014) Nature Biotechnology 32, 462–464 doi:10.1038/nbt.2862

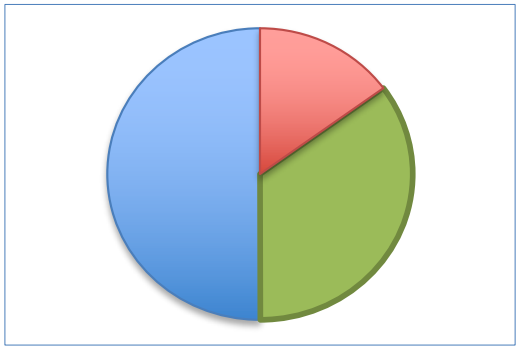
RNA-seq Challenges



Challenge 1: Eukaryotic genes are spliced

Solution: Use a spliced aligner, and assemble isoforms

TopHat: discovering spliced junctions with RNA-Seq.
Trapnell et al (2009) *Bioinformatics*. 25:0 1105-1111



Challenge 2: Read Count != Transcript abundance

Solution: Infer underlying abundances (e.g. TPM)

Transcript assembly and quantification by RNA-seq
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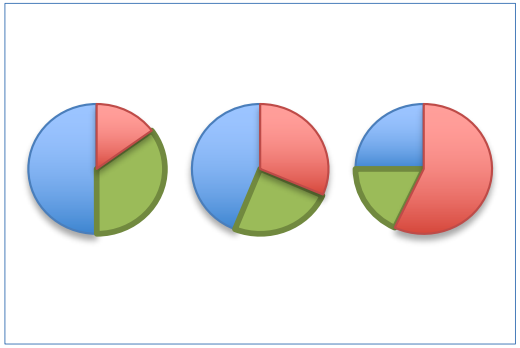
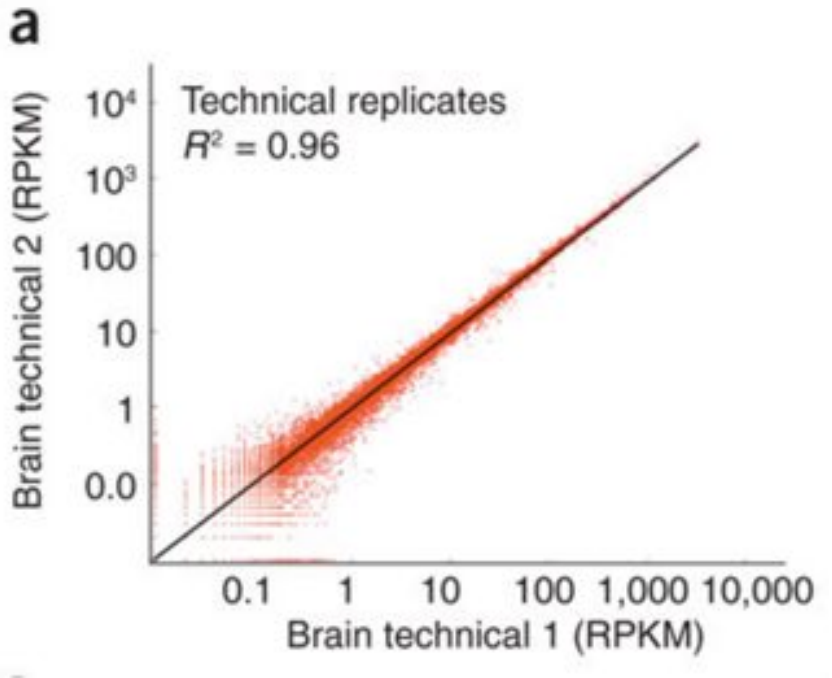
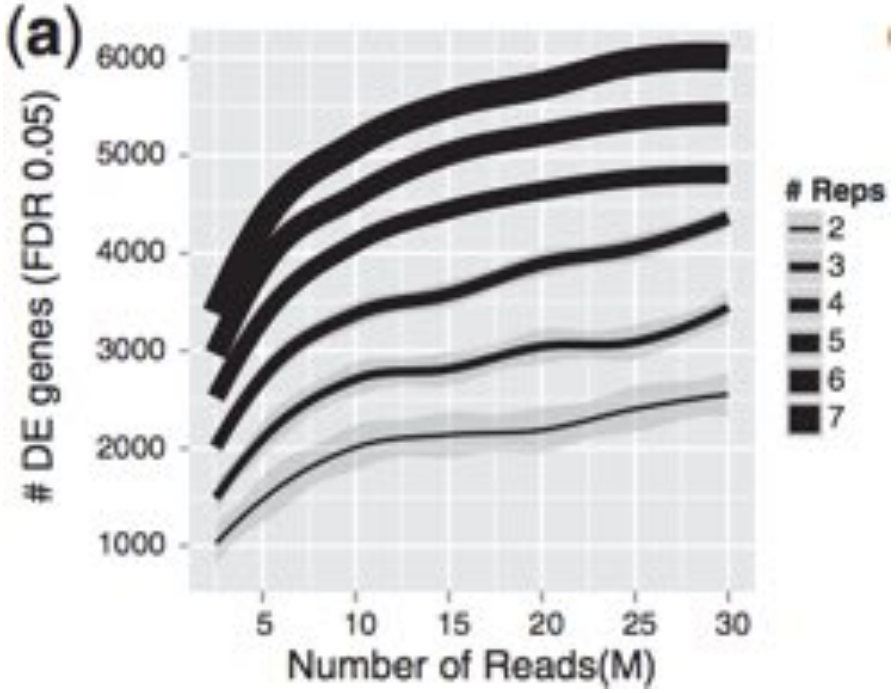


Challenge 3: Transcript abundances are stochastic

How Many Replicates?



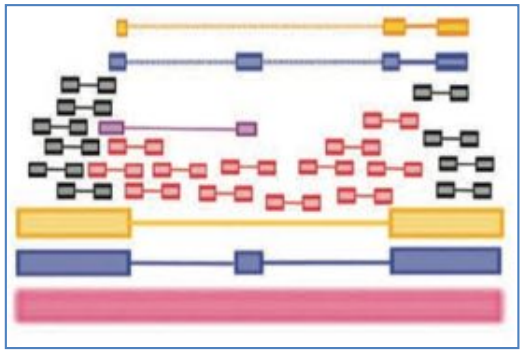


Why don't we have perfect replicates?

Mapping and quantifying mammalian transcriptomes by RNA-Seq
Mortazavi et al (2008) Nature Methods. 5, 62-628

RNA-seq differential expression studies: more sequence or more replication?
Liu et al (2013) Bioinformatics. doi:10.1093/bioinformatics/btt688

RNA-seq Challenges



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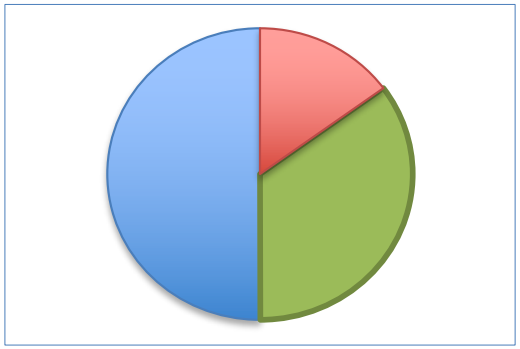

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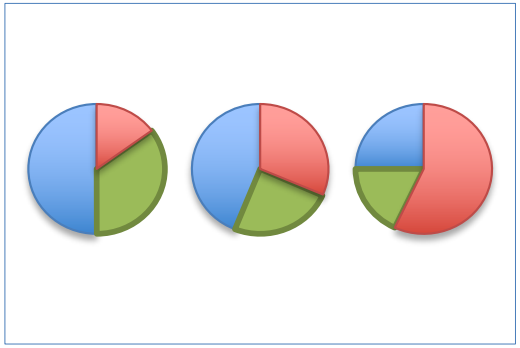
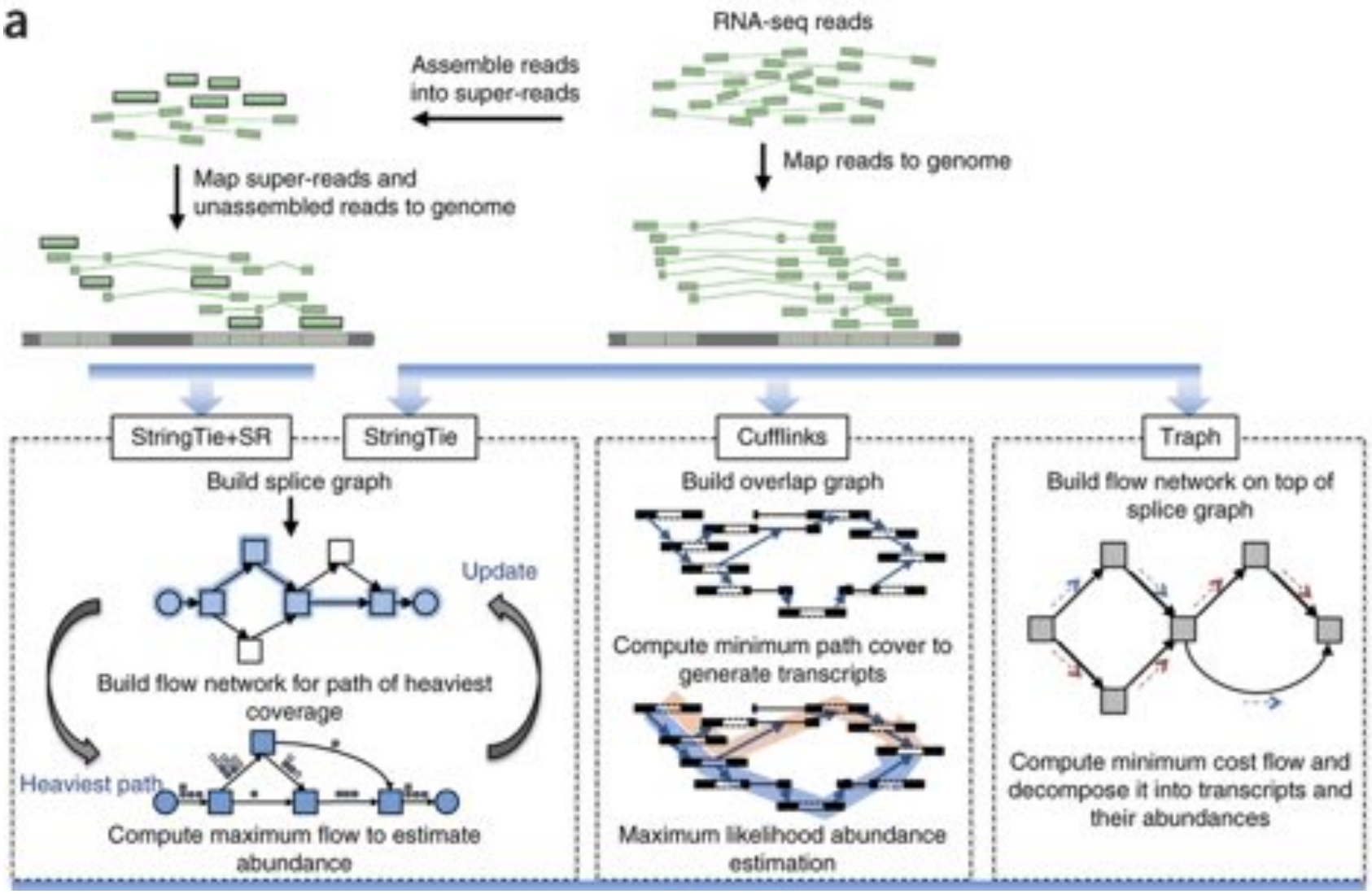


Challenge 3: Transcript abundances are stochastic

Solution: Replicates, replicates, and more replicates

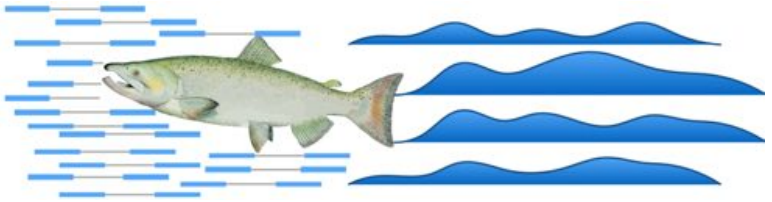
RNA-seq differential expression studies: more sequence or more replication?
Liu et al (2013) *Bioinformatics*. doi:10.1093/bioinformatics/btt688

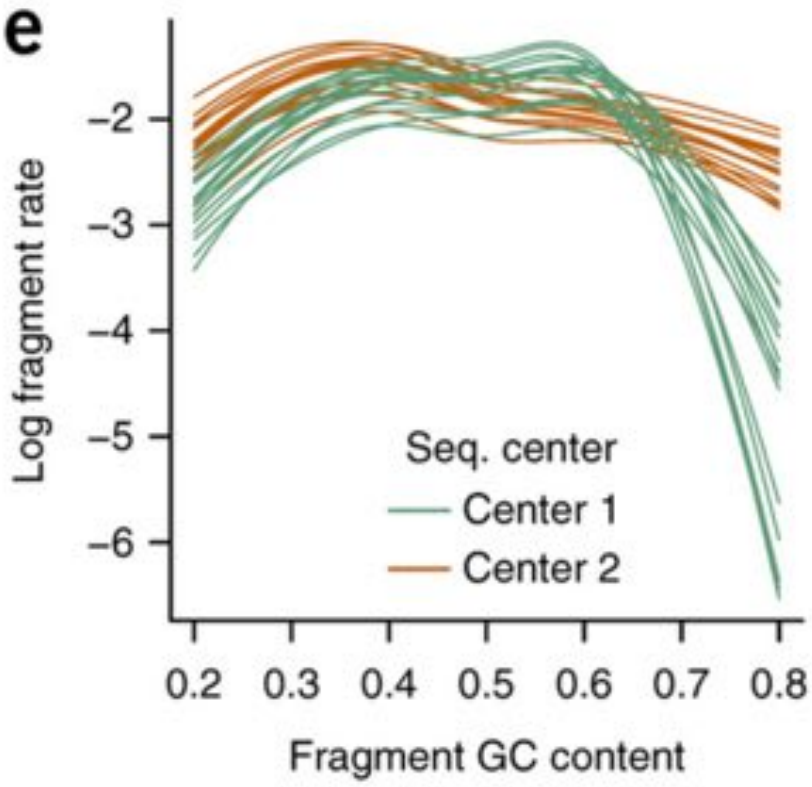
Isoform Quantification Approaches

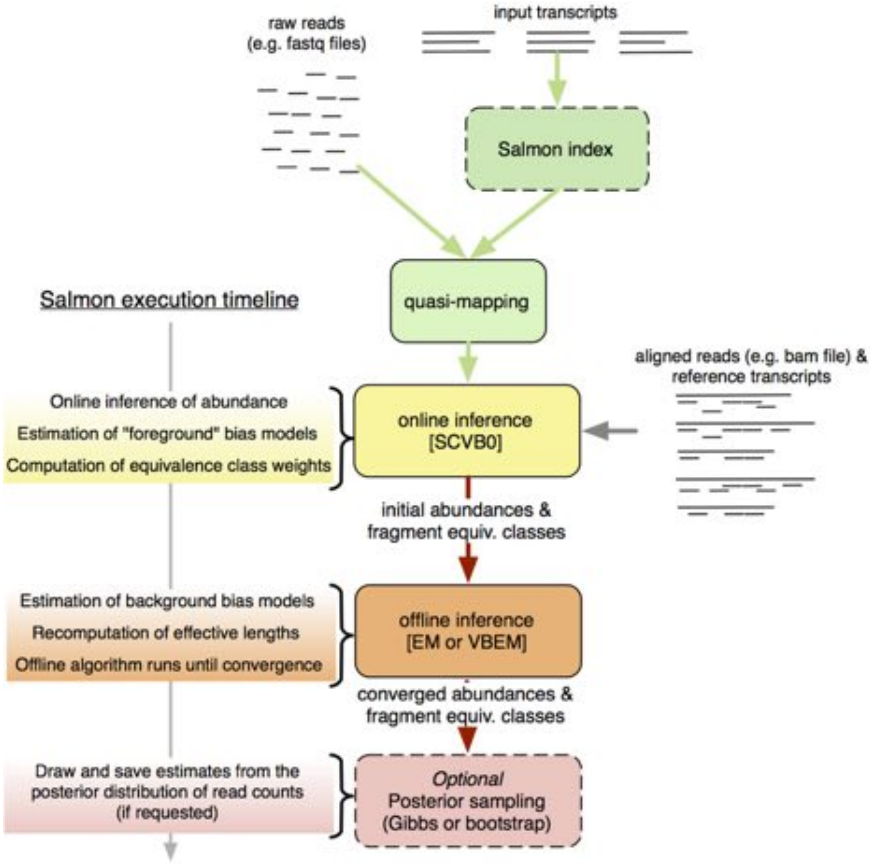


StringTie enables improved reconstruction of a transcriptome from RNA-seq reads.
Pertea M, et al. (2015) Nature Biotechnology. doi: 10.1038/nbt.3122.

Salmon: The ultimate RNA-seq Pipeline?



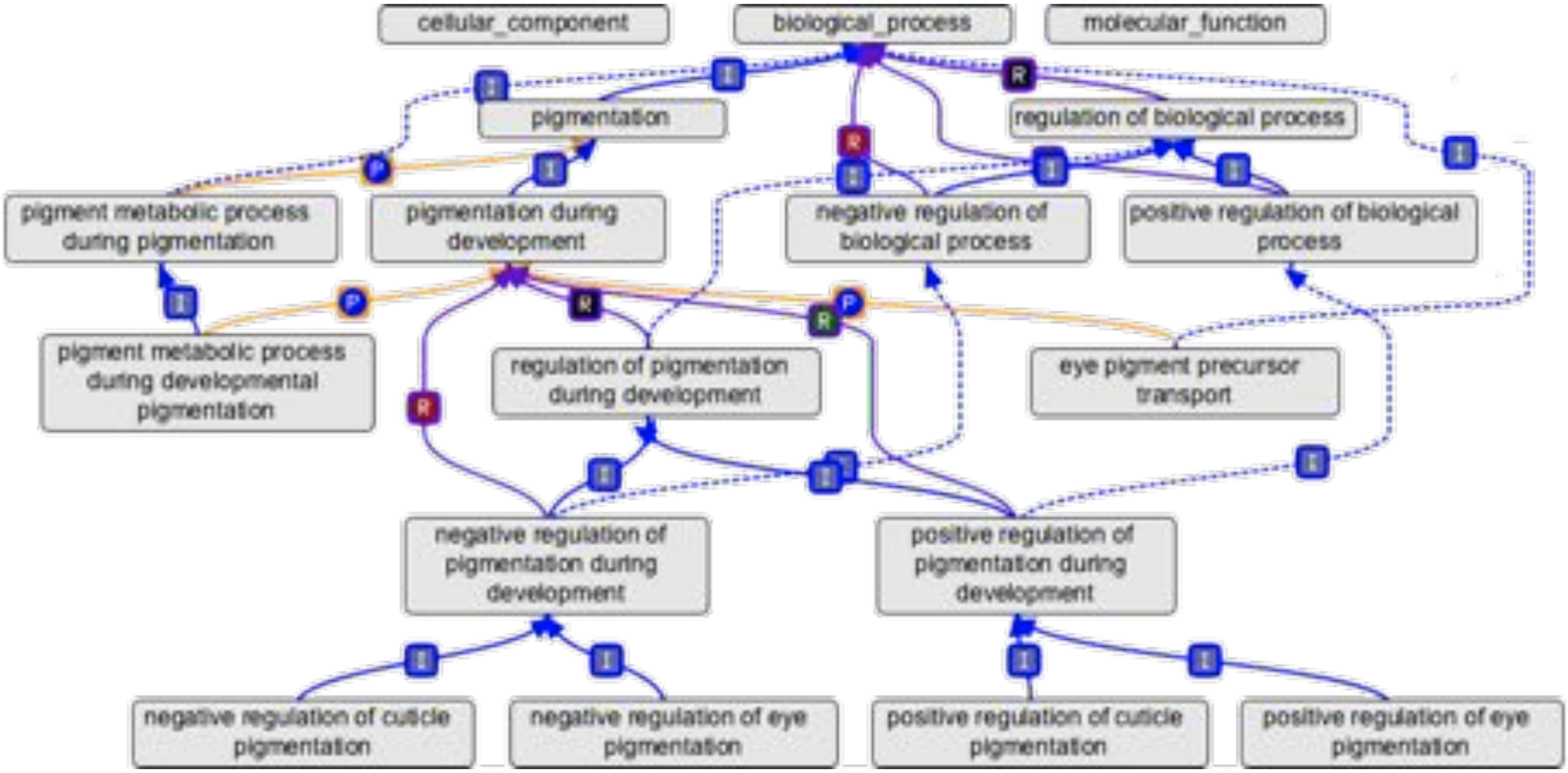




Modeling of RNA-seq fragment sequence bias reduces systematic errors in transcript abundance estimation
Love et al (2016) Nature Biotechnology 34, 1287–1291 (2016) doi:10.1038/nbt.3682

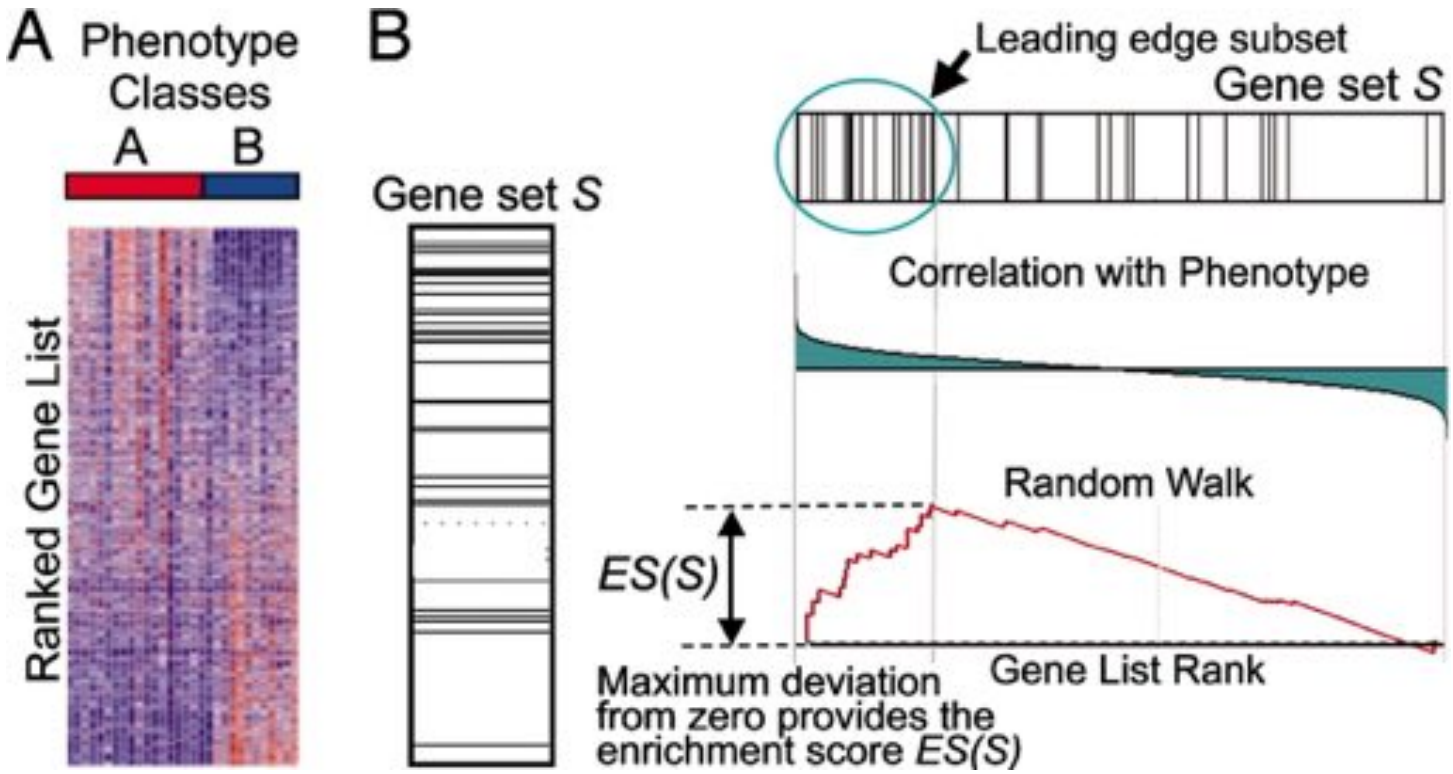
Salmon provides fast and bias-aware quantification of transcript expression
Patro et al (2017) Nature Methods (2017) doi:10.1038/nmeth.4197

Gene Ontology (GO)



AmiGO: online access to ontology and annotation data
Carbon et al (2009) Bioinformatics doi:10.1093/bioinformatics/btn615

GSEA Overview



Collections

The MSigDB gene sets are divided into 8 major collections:

H	**hallmark gene sets** are coherently expressed signatures derived by aggregating many MSigDB gene sets to represent well-defined biological states or processes.
C1	**positional gene sets** for each human chromosome and cytogenetic band.
C2	**curated gene sets** from online pathway databases, publications in PubMed, and knowledge of domain experts.
C3	**motif gene sets** based on conserved cis-regulatory motifs from a comparative analysis of the human, mouse, rat, and dog genomes.
C4	**computational gene sets** defined by mining large collections of cancer-oriented microarray data.
C5	**GO gene sets** consist of genes annotated by the same GO terms.
C6	**oncogenic gene sets** defined directly from microarray gene expression data from cancer gene perturbations.
C7	**immunologic gene sets** defined directly from microarray gene expression data from immunologic studies.

Aravind Subramanian et al. PNAS 2005;102:43:15545-15550

PNAS

Annotation Summary

- Three major approaches to annotate a genome
  1. Alignment:
     - Does this sequence align to any other sequences of known function?
     - Great for projecting knowledge from one species to another
  2. Prediction:
     - Does this sequence statistically resemble other known sequences?
     - Potentially most flexible but dependent on good training data
  3. Experimental:
     - Lets test to see if it is transcribed/methylated/bound/etc
     - Strongest but expensive and context dependent

- Many great resources available
  - Learn to love the literature and the databases
  - Standard formats let you rapidly query and cross reference
  - Google is your number one resource ☺