RNA-seq Quantification

Outline

- short-read RNA-seq
- quantification:
  - read counts
  - generative models; relative abundances
  - pseudo-mapping
- the RNA-seq setting
- normalizing fractions

Typical setting for bulk RNA-seq

Given a tissue or cell line:

- ~25’000 genes in the genome
- ~10 – 18’000 genes expressed at any given time; expression level of the different genes differs largely

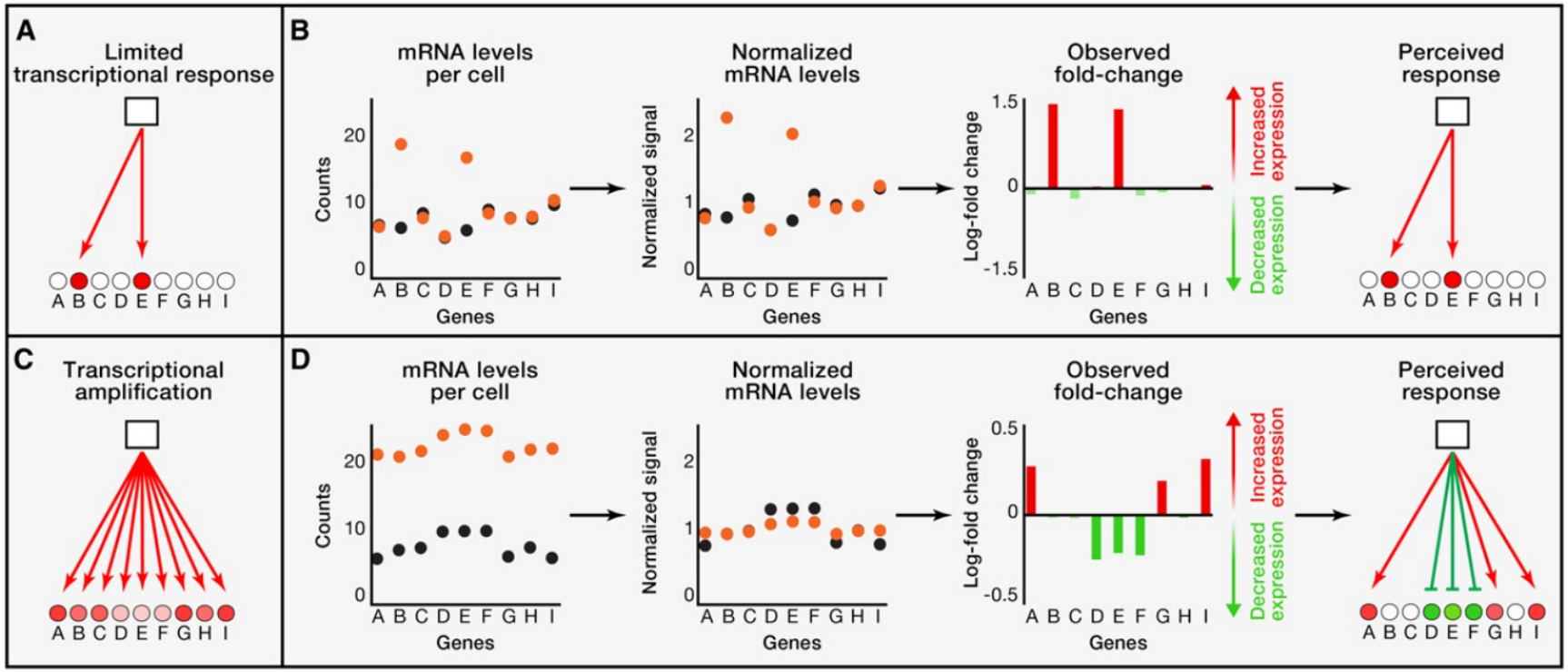
Working hypotheses:

- relative prevalence of a gene reflects activity of associated pathways; more prevalent, more active
- baseline abundance is not known → **perturbation experiments**: treatment vs control
- informative quantity: relative abundance of the genes in a tissue

Limitations:

- genes vs protein
- alternative explanations …. cell size effects; other side effects

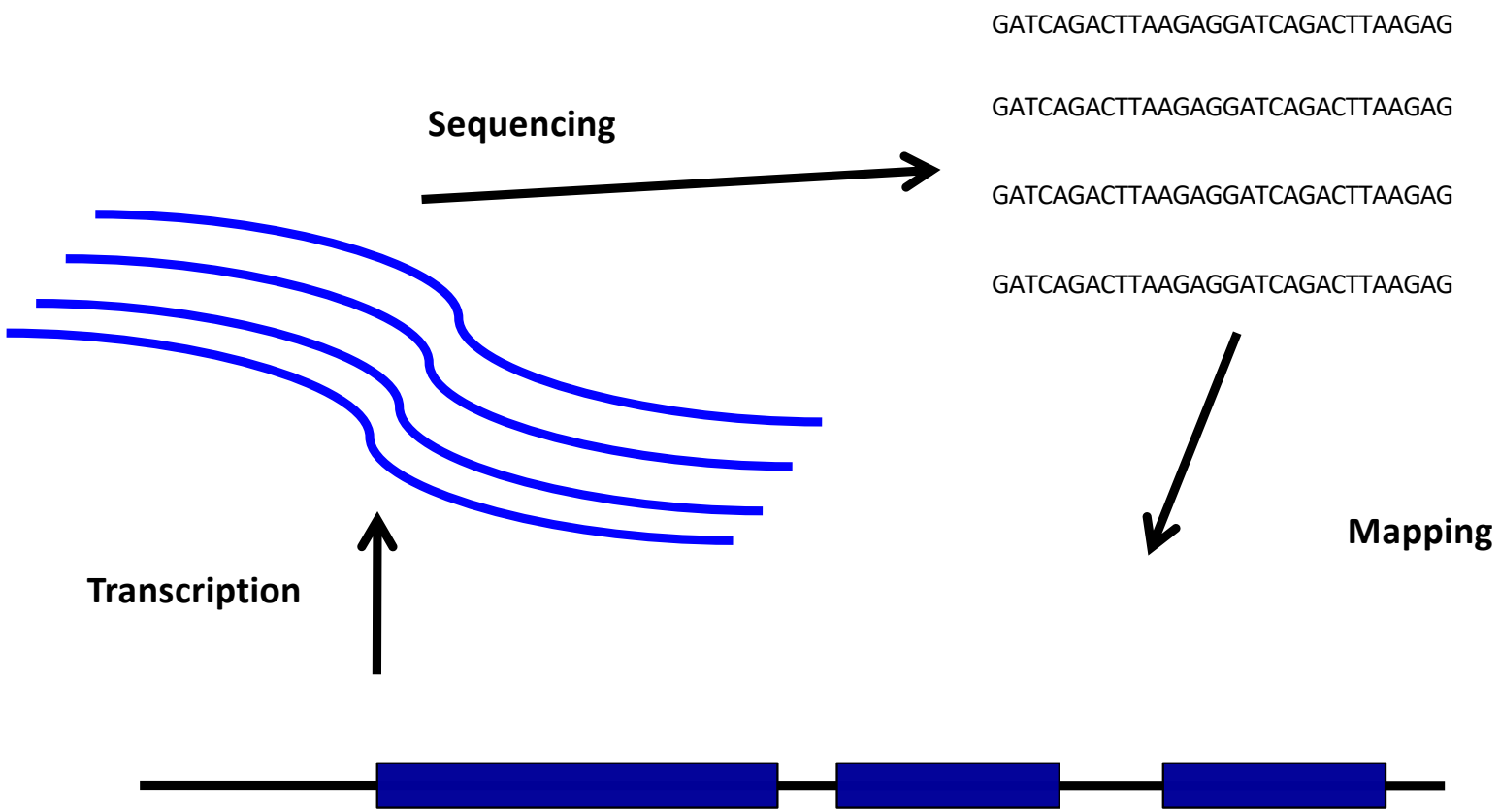
Perturbation Experiments



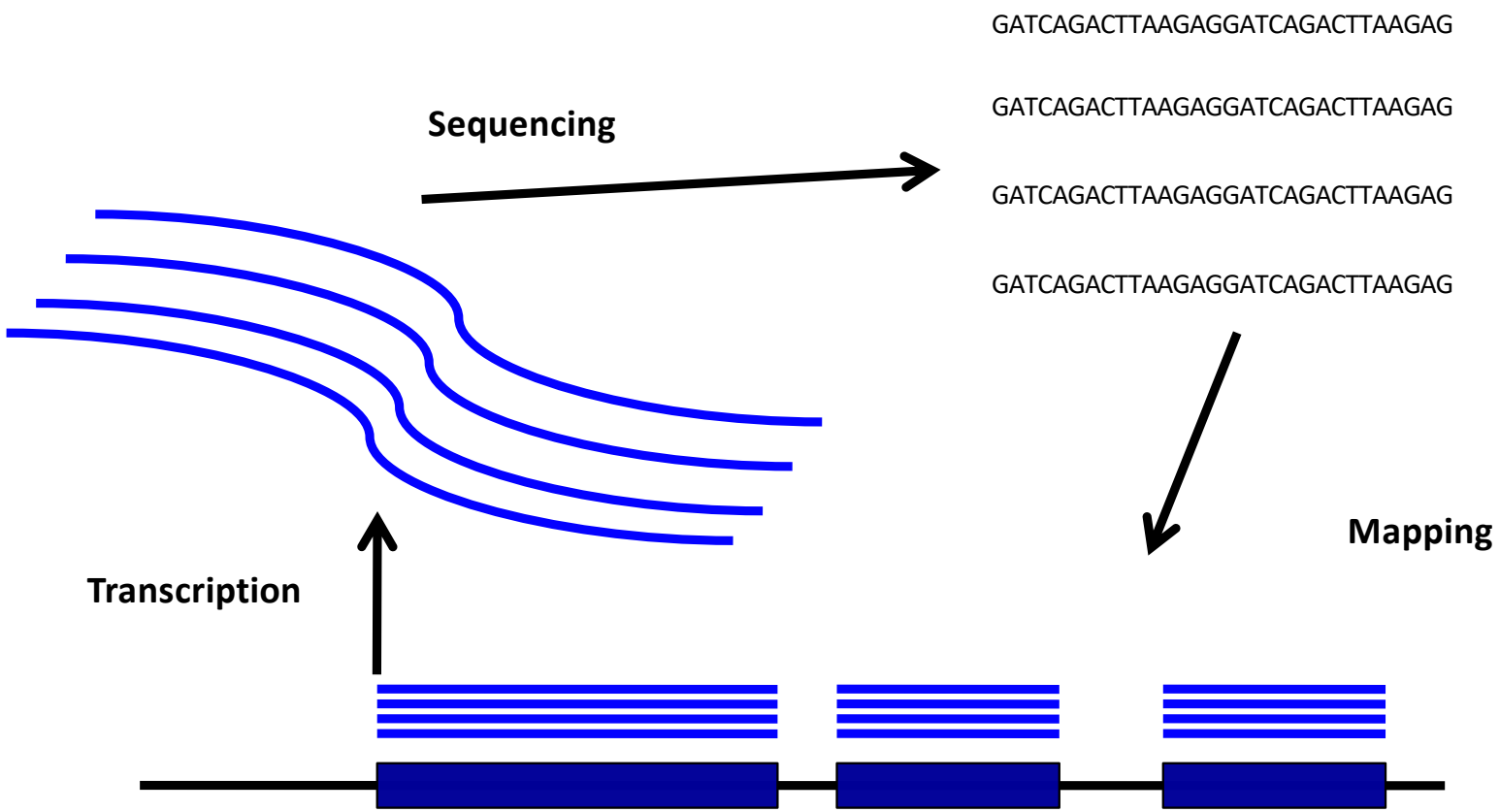
- The top row shows the assumption how a baseline expression profile (black) changes as a response (red) to a perturbation
- Note: in this example, expression counts of individual genes are around ~10, in practice they range from 0 – 1 Mio

Lovén et al, 2012, https://doi.org/10.1016/j.cell.2012.10.012

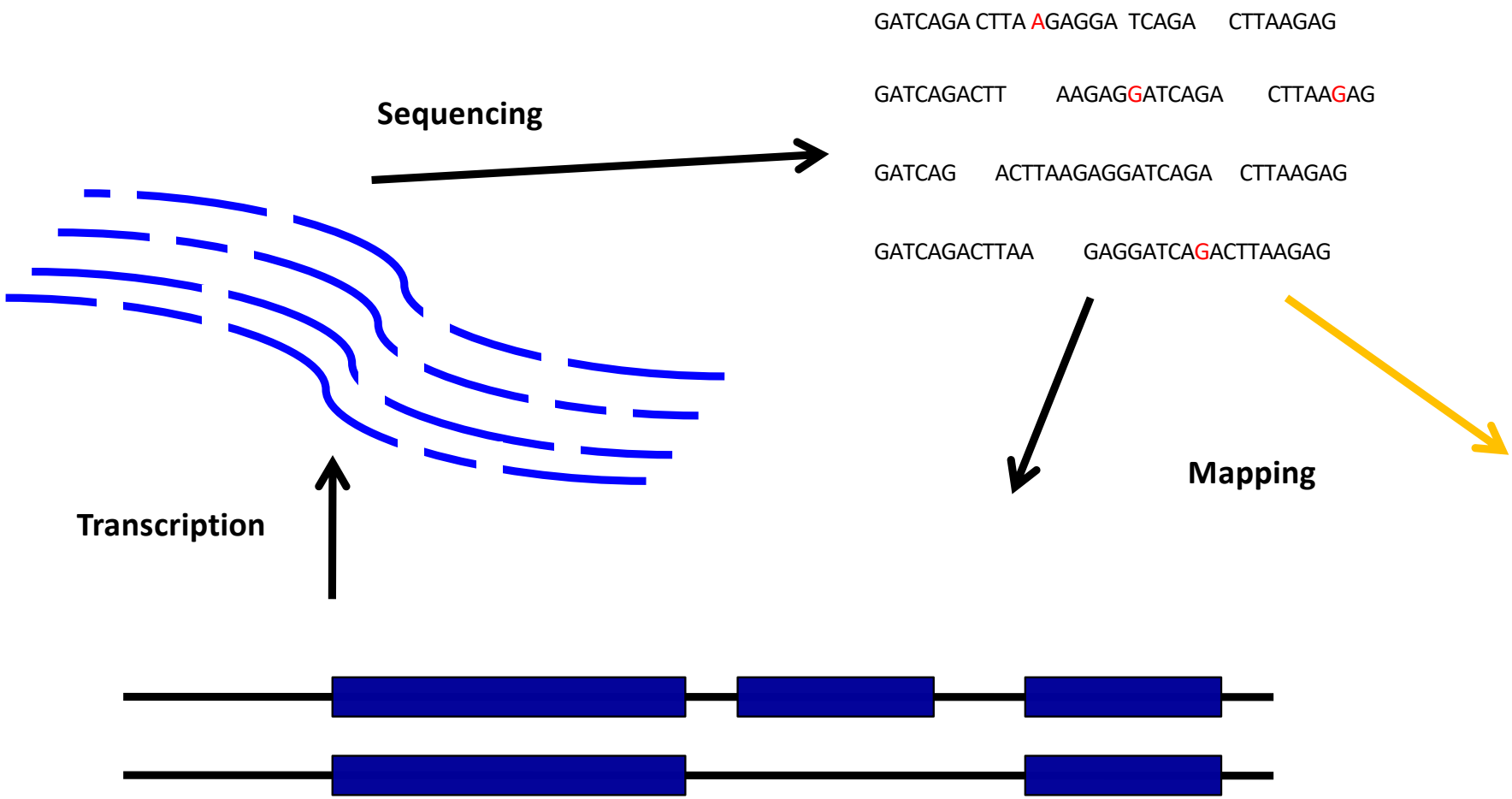
Transcript Expression with NGS



Read mapping and counting (ideal)



Read mapping and counting (short reads with SNPs and sequencing errors)



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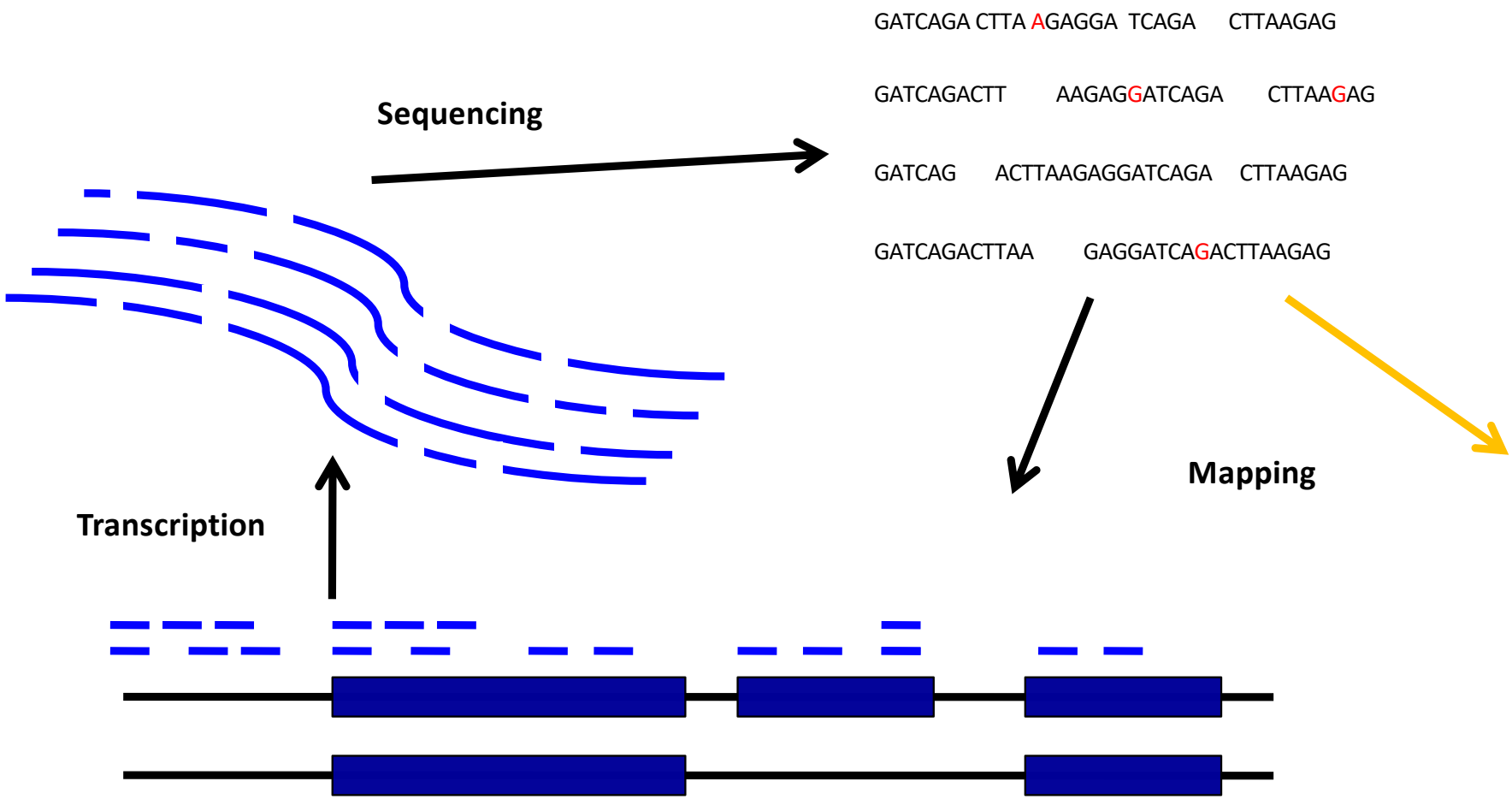


Abundance estimates

Abundance of what???

- Biologically relevant:
  - **gene level:**
    - # molecules transcribed from one gene locus (per cell)
  - **isoform level:**
    - # molecules of a specific isoform transcribed from one gene (per cell)
- Feasible with RNA-seq:
  - **relative fractions that indicate the abundance relative to all other genes/isoforms**

Gene-level Read Counts

- rather straightforward to compute:
  - **number of reads** that uniquely map to a gene locus
    → biased by length, discards information in multi-mappers
  - **number of reads** that map to gene locus (including multi-mappers)
    → disambiguation is not possible if you do not have abundance estimates of the isoforms
    → needs to resort to heuristics to assign multi-mappers
    - randomly assign to one of the matching genes
    - do a fractional assignment with a with 1/#genes mapped

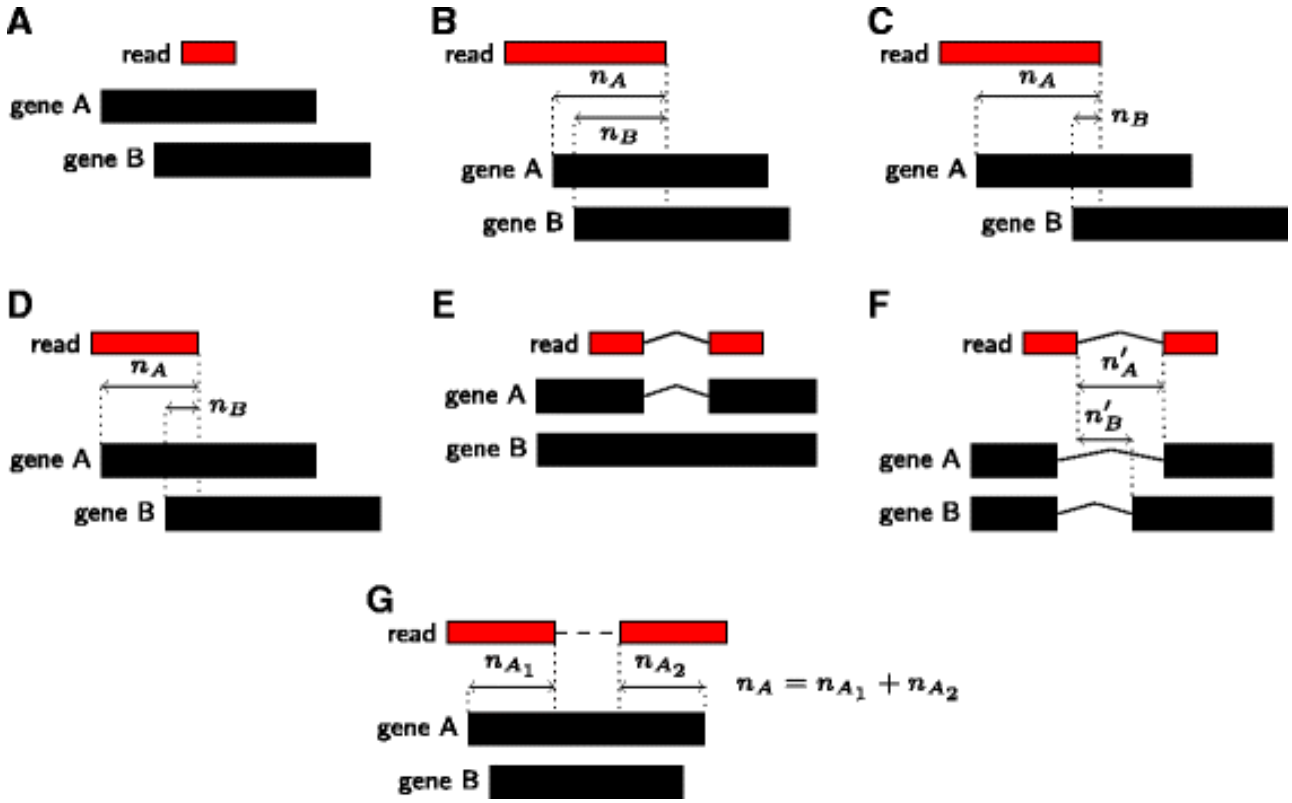
Rsubread::featureCounts – assigning reads to genes

- versatile function to count reads towards exons, transcripts, genes, …
- implements many different counting modes
- covers different aspects of overlap situations
  - partial overlap
  - overlapping multiple features at the same alignment position
  - overlapping multiple features at different alignment positions

- Simple overlap is not sufficient, read must be compatible with exon structure

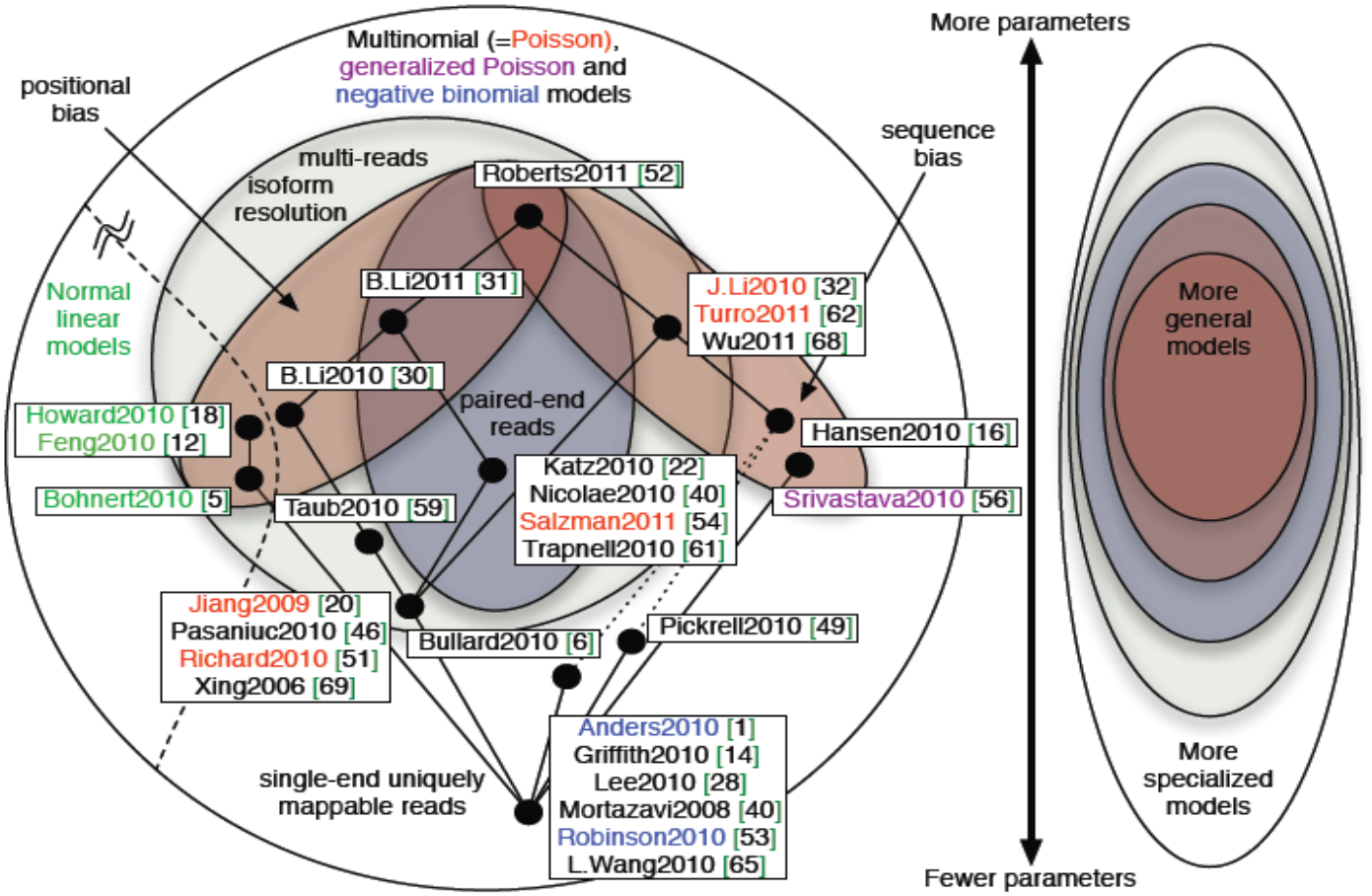
Model-free Counting of Overlapping reads – Count Modes

mmquant: resolve multi-mapping reads based on heuristics



https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-017-1816-4

Generative Models for RNA-seq quantification



Pachter 2011, https://doi.org/10.48550/arXiv.1104.3889

RNA-seq model

Likelihood that a read/fragment from transcript *t* is generated:

$$\alpha_t = \mathrm{P}[\text{read from transcript } t] = \frac{1}{Z}\rho_t l_t$$

with:

ρ_t expression level / abundance / fraction

l_t transcript length

$Z = \sum_t \rho_t l_t$ normalization factor

The normalization factor is the weighted mean length of the transcripts.

RNA-seq model

Estimate of the probability that a read from a specific transcript is generated:

$$\hat{\alpha}_t = \frac{X_t}{N} = \frac{\#\text{reads mapping to transcript } t}{\#\text{mappable reads in total}}$$

Abundance estimates:

$$\hat{\rho}_t \propto \frac{\hat{\alpha}_t}{l_t}$$

Maximum Likelihood Estimation

- The estimated abundances represent unique MLE estimates

$$\text{with } \alpha = \{\alpha_t\}_{t \in T}$$

$$L[\alpha] = \prod_{t \in T} \prod_{f \in F_t} P[f \in t] \frac{1}{l_t}$$

$$= \prod_{t \in T} \prod_{f \in F_t} \alpha_t \frac{1}{l_t}$$

$$= \prod_{t \in T} \left(\frac{\alpha_t}{l_t} \right)^{X_t}$$

Effective Transcript Length

- Since fragments have a non-zero length the read probabilities depend actually on an *effective* length:

$$l_t := \text{transcript length - fragment length + } 1$$

- For simplicity, we continue to use the symbol without tilde but will always assume it is the effective length
- The effective length represents the stretch of the transcript from which I can get a fragment that I can then map back to the transcript
- → The effective length should also consider mappability!
- → Mappability does depend on mapping algorithm, mutations, …

Multi-reads

- Reads that cannot be uniquely assigned to one transcript were ignored so far
- Multi-reads can occur
  - if a read aligns more than once in the genome
  - if at an alignment position there is more than one transcript defined
- Multi-reads do occur due to homology not due to pure chance

Considering Multi-reads

- Define a compatibility matrix

$$\mathbf{Y} = \{y_{ft}\}_{f \in F, t \in T}$$

with

$$y_{ft} = \begin{cases} 1 \text{ if read } f \text{ aligns to transcript } t \\ 0 \text{ else} \end{cases}$$

- The likelihood is now:

$$\mathrm{L}[\alpha] = \prod_f \left(\sum_t y_{ft} \frac{\alpha_t}{l_t} \right)$$

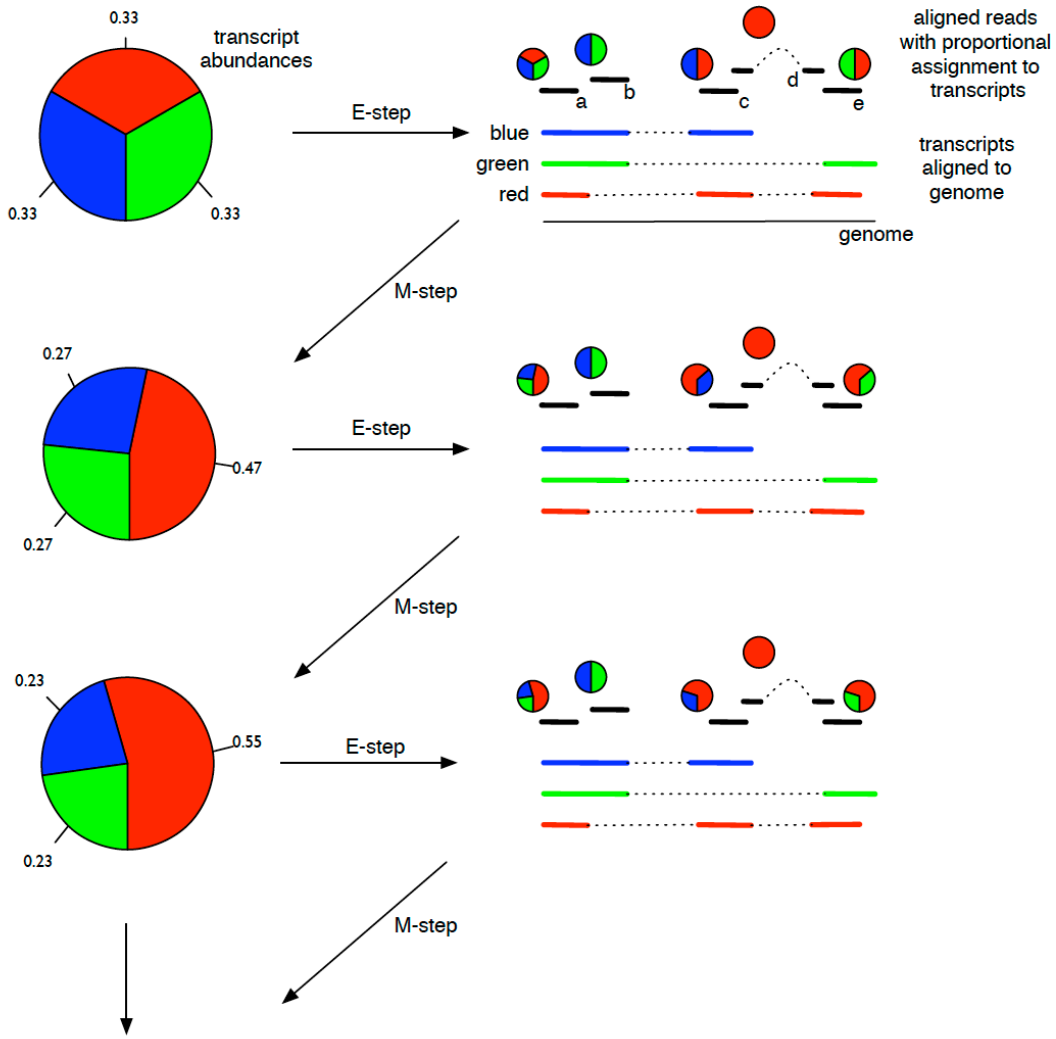
- but now abundances must be estimated iteratively

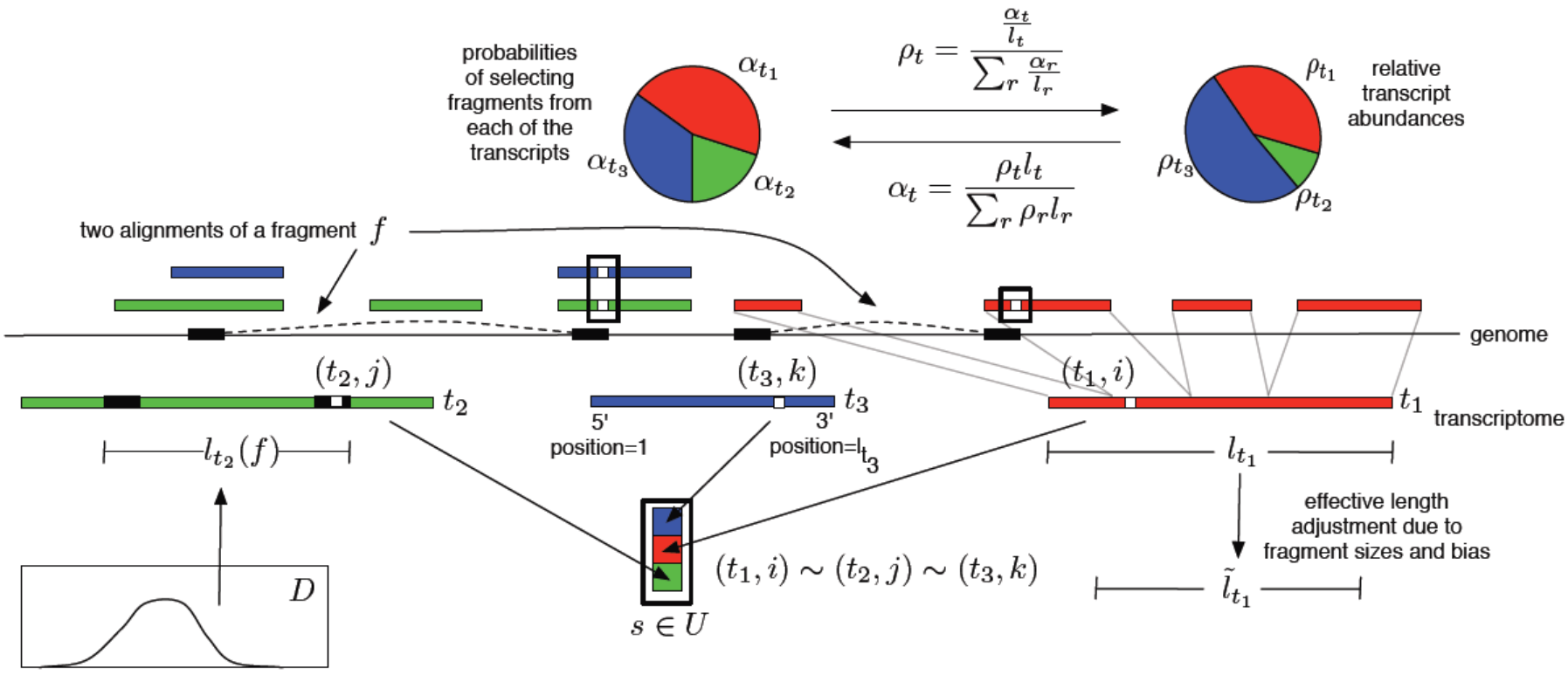
Iterative Estimation

Three step algorithm

1. Estimate abundances based on uniquely mapping reads only
2. For each multi-read, divide it between the transcripts to which it maps, proportionally to their abundances estimated in the first step
3. Recompute abundances based on updated counts for each transcript
4. Continue with Step 2

Expectation-Maximization Estimation




probabilities of selecting fragments from each of the transcripts
α_{t_1}
α_{t_2}
α_{t_3}
$\rho_t = \frac{\frac{\alpha_t}{l_t}}{\sum_r \frac{\alpha_r}{l_r}}$
$\alpha_t = \frac{\rho_t l_t}{\sum_r \rho_r l_r}$
ρ_{t_1}
ρ_{t_2}
ρ_{t_3}
relative transcript abundances
two alignments of a fragment f
genome
(t_2, j)
t_2
(t_3, k)
t_3
(t_1, i)
t_1
transcriptome
5'
position=1
3'
position=l_{t_3}
$l_{t_2}(f)$
l_{t_1}
effective length adjustment due to fragment sizes and bias
D
$(t_1, i) \sim (t_2, j) \sim (t_3, k)$
$s \in U$
$\tilde{l}_{t_1}$

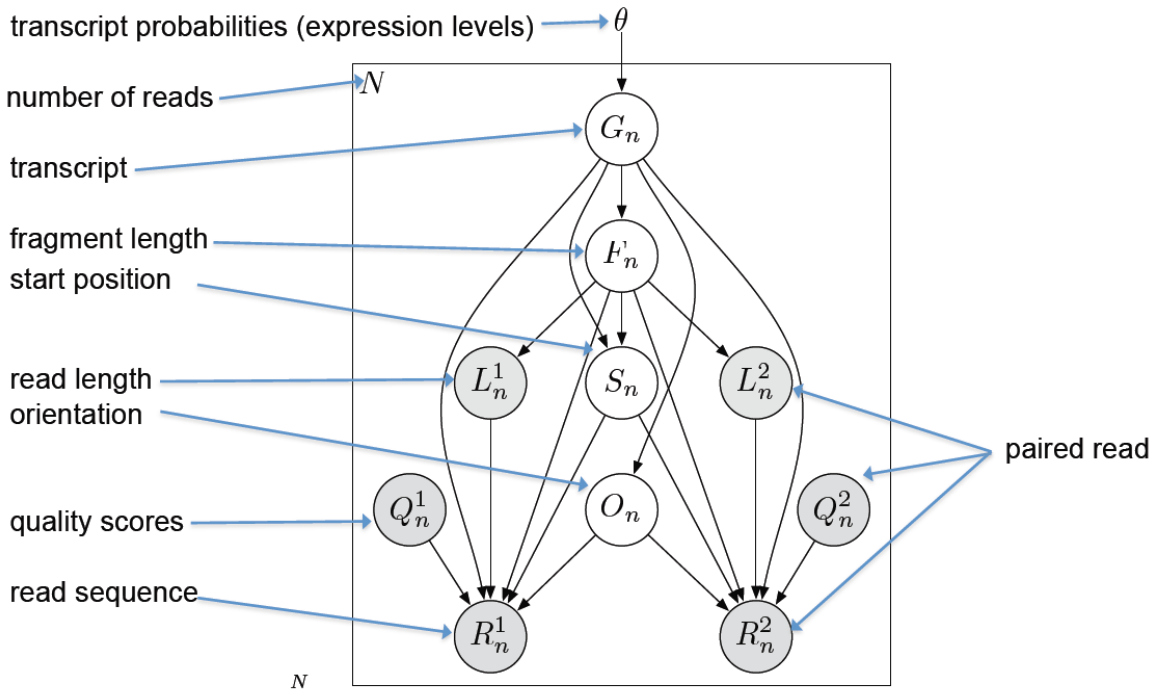
General Formulation of Abundance Estimation

A full model for the abundance estimation would ideally consider:

- position bias
- fragment-length distribution
- sequencing errors
- site-specific bias
- …

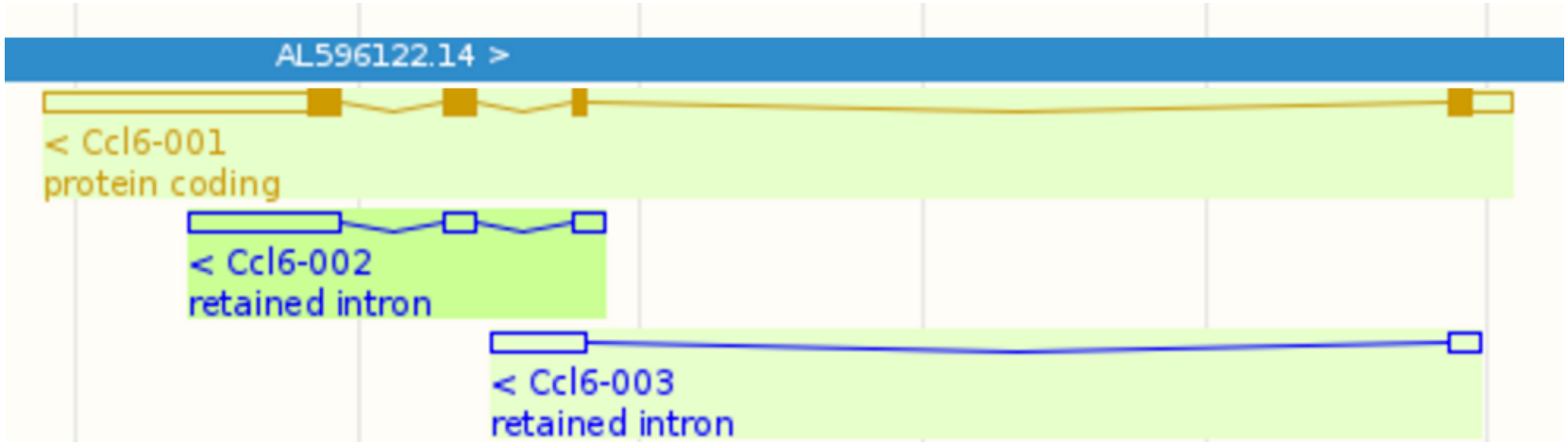
RSEM: Model specification

Dewey: RSEM



$$P(\mathbf{g},\mathbf{f},\mathbf{s},\mathbf{o},\ell,\mathbf{q},\mathbf{r}|\theta)=\prod_{n=1}^{N}P(g_n|\theta)P(f_n|g_n)P(s_n|f_n,g_n)P(o_n|g_n)P(q_n)P(\ell_n|f_n)P(r_n|g_n,f_n,s_n,o_n,\ell_n,q_n)$$

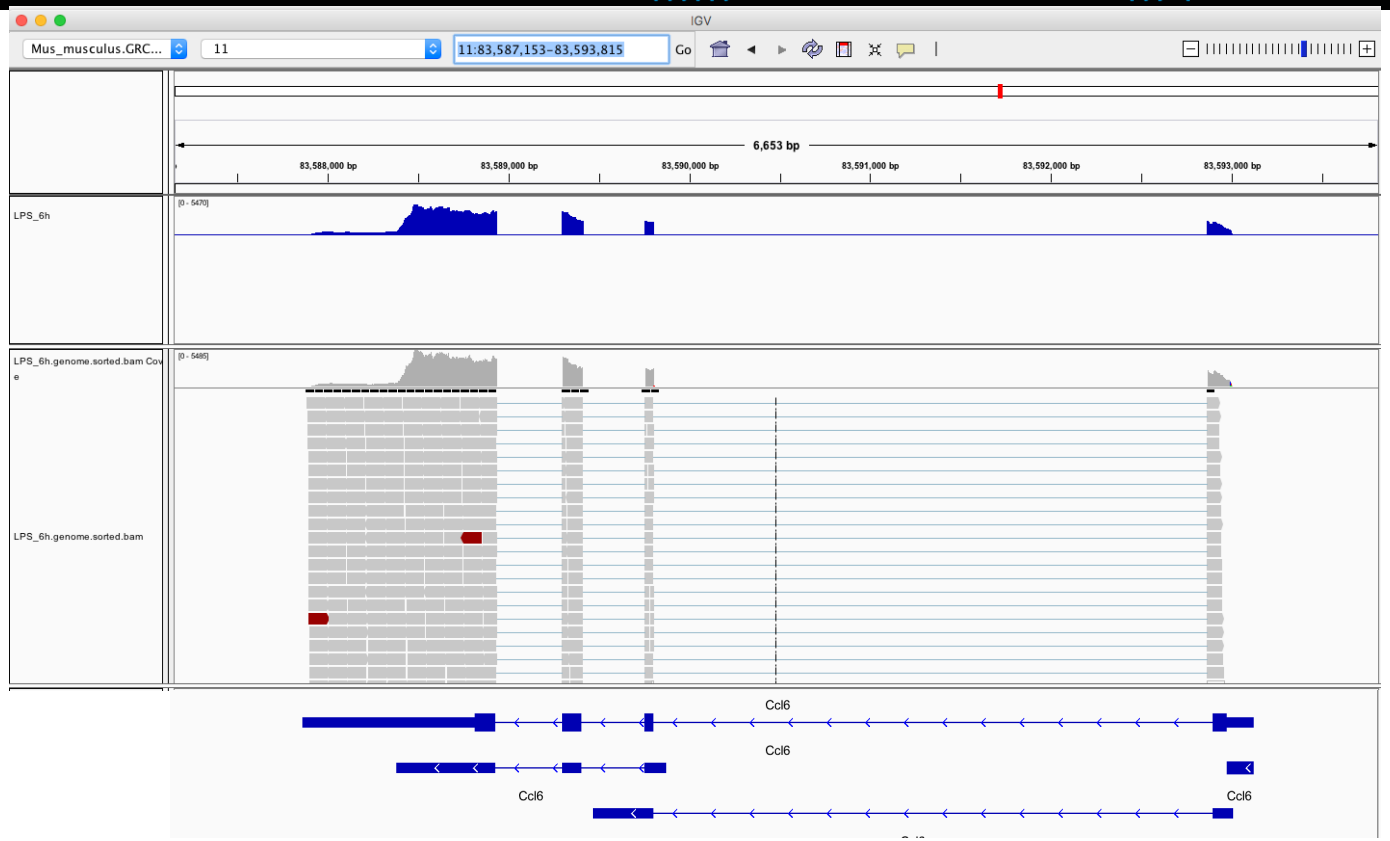
Example: RSEM



Ccl6 gene locus with 3 isoforms

follows the example:

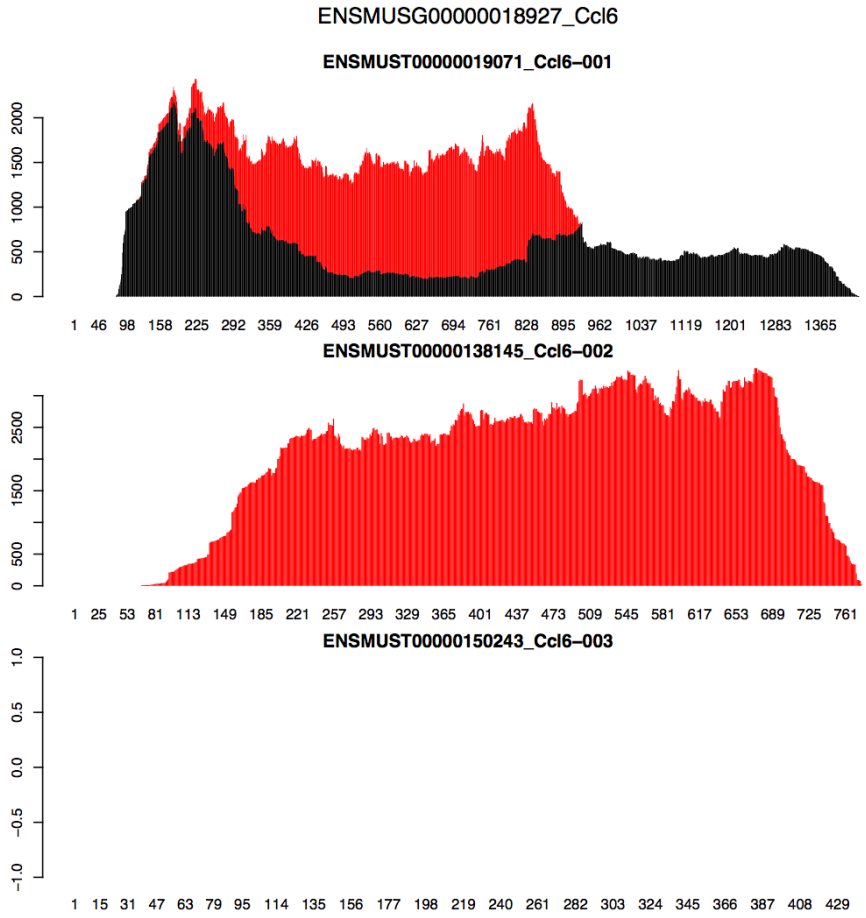
https://github.com/bli25broad/RSEM_tutorial



RSEM result:

transcript_id	gene_id	length	effective_length	expected_count	TPM	FPKM	IsoPct		
ENSMUST00000019071_Ccl6-001	ENSMUSG00000018927_Ccl6	1440	1194.85	7805.95	8862.10	9334.46	31.00		
ENSMUST00000138145_Ccl6-002	ENSMUSG00000018927_Ccl6	776	530.94	7719.05	19721.39		20772.55		69.00
ENSMUST00000150243_Ccl6-003	ENSMUSG00000018927_Ccl6	442	202.64	0.00	0.00	0.00	0.00		

Ccl6 coverage in transcript space



- orientation is flipped because gene is on negative strand
- black: unique alignments
- red: expected depth from multi-mapping reads

Limitations of Generative Models

- Estimates can not be correct if underlying model of transcripts are incorrect or incomplete
- Abundance estimates are fractions; these can be used to get estimates of the number of reads generated by a given gene; error distribution of estimated read counts may be unclear

Implementation of Generative Models

- **RSEM:**
  Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).

- **Review:**
  Pachter, L. Models for transcript quantification from RNA-Seq. *arXiv preprint arXiv:1104.3889* (2011).

- **MISO:**
  Katz, Y., Wang, E. T., Airoldi, E. M. & Burge, C. B. Analysis and design of RNA sequencing experiments for identifying isoform regulation. *Nat Methods* **7**, 1009–1015 (2010)

- **MMSEQ:**
  Turro, E. *et al.* Haplotype and isoform specific expression estimation using multi-mapping RNA-seq reads. *Genome Biol* **12**, R13 (2011).
- **NSMAP:**
  Xia, Z., Wen, J., Chang, C.-C. & Zhou, X. NSMAP: a method for spliced isoforms identification and quantification from RNA-Seq. *BMC Bioinformatics* **12**, 162 (2011).

Definition of expression levels

- Goal: Start from read counts and define a quantity that indicates **relative molar concentration of a transcript**

- Transcripts Per Million Transcripts

$$\text{TPM for transcript } t = 10^6 \times Z \times \frac{X_t}{l_t N}$$

- TPM is unitless and is a measure of relative abundance

- TPM assumes that the total sum of all counts (N) can be used to normalize across samples

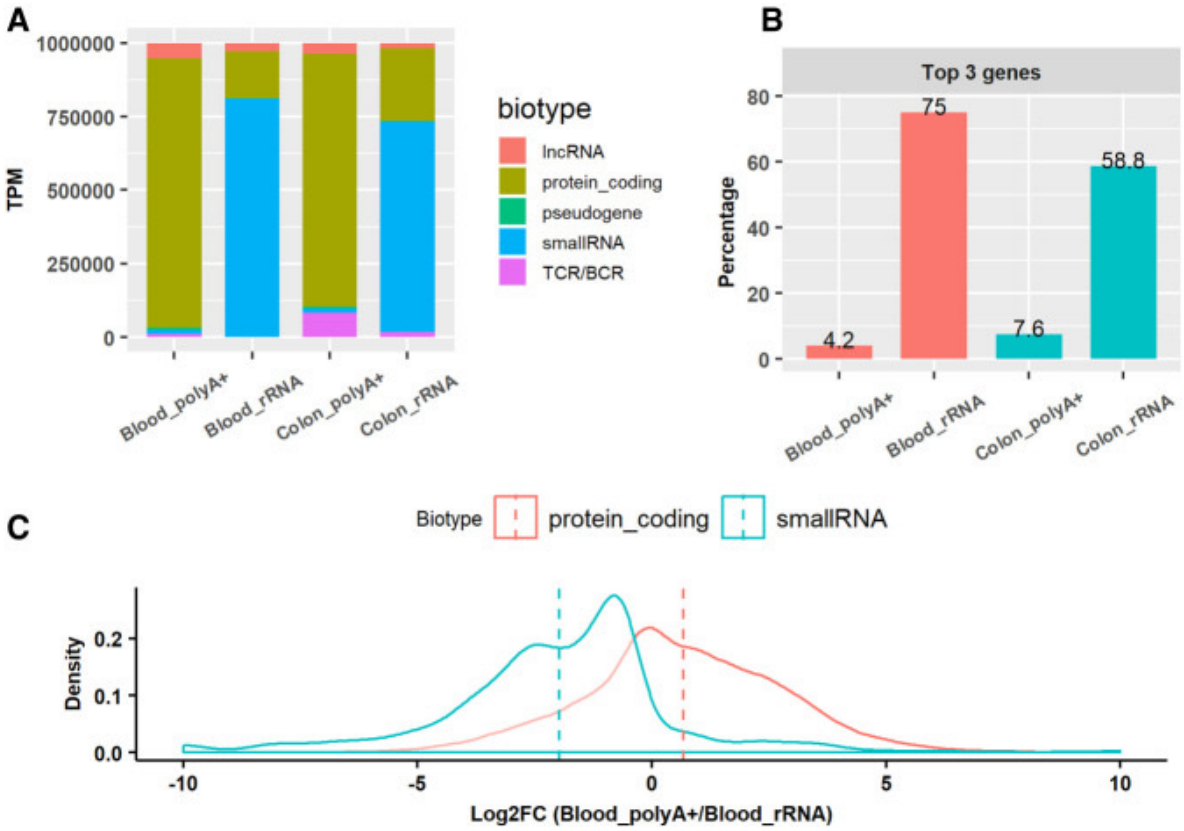
Shortcomings of TPM

- Sum of RPKM varies from sample to sample, i.e. RPKM is not a measure of relative concentration because the measures of relative concentrations would sum up to constant
- TPM is unitless and satisfies this requirement

- **Only TPM should be used!**
- **Limitation of TPM: normalization relies on assumption that the total sum of all counts is a good metric to normalize**
  - **This assumption does not hold true, especially if comparison is across different tissues or different protocols**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7373998/

Comparing samples across protocols

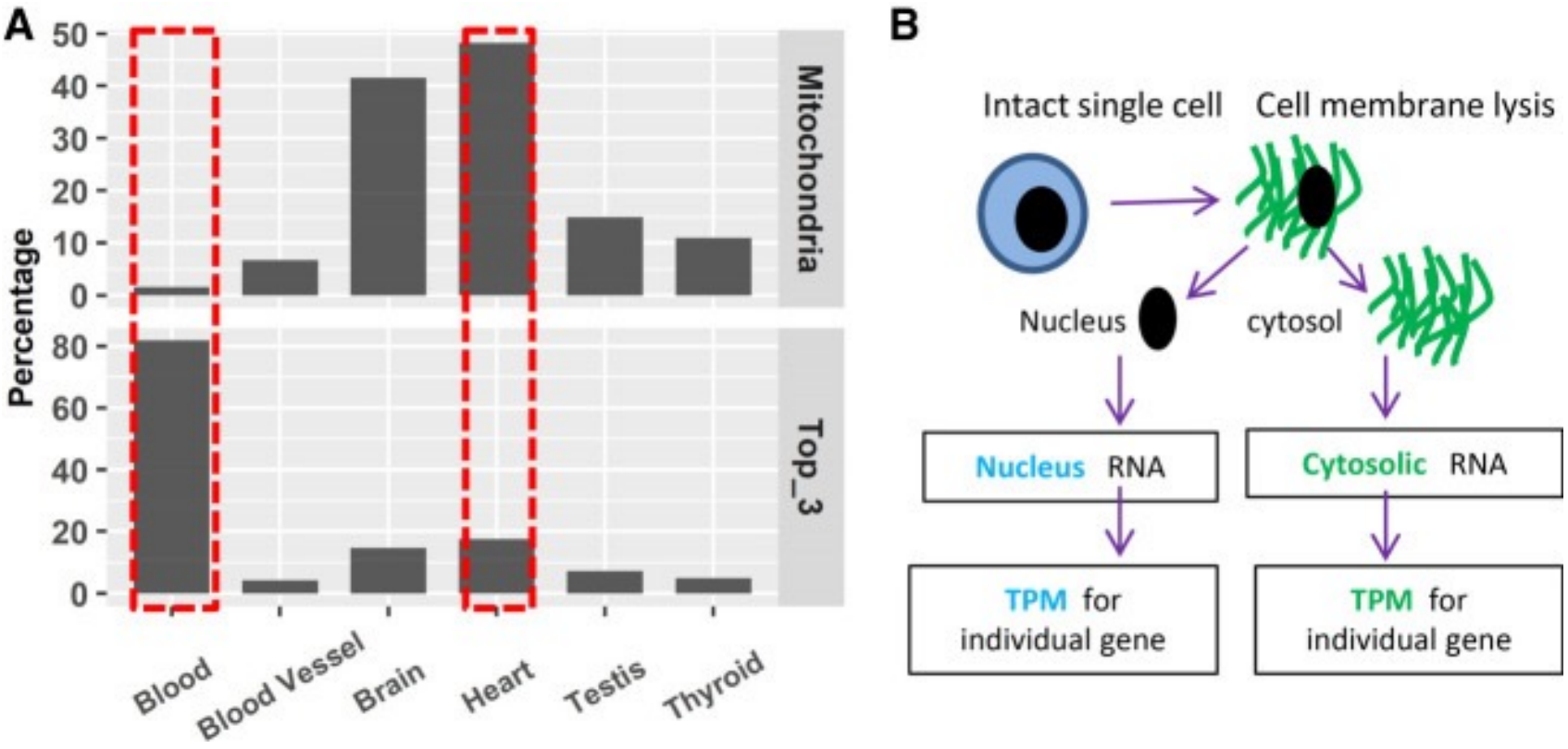


issues:

- surveyed populations are not comparable
- expression of top 3 genes will drive the TPM normalization (because it has a
- major influence on the sum of all reads)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7373998/

Comparing samples across tissues

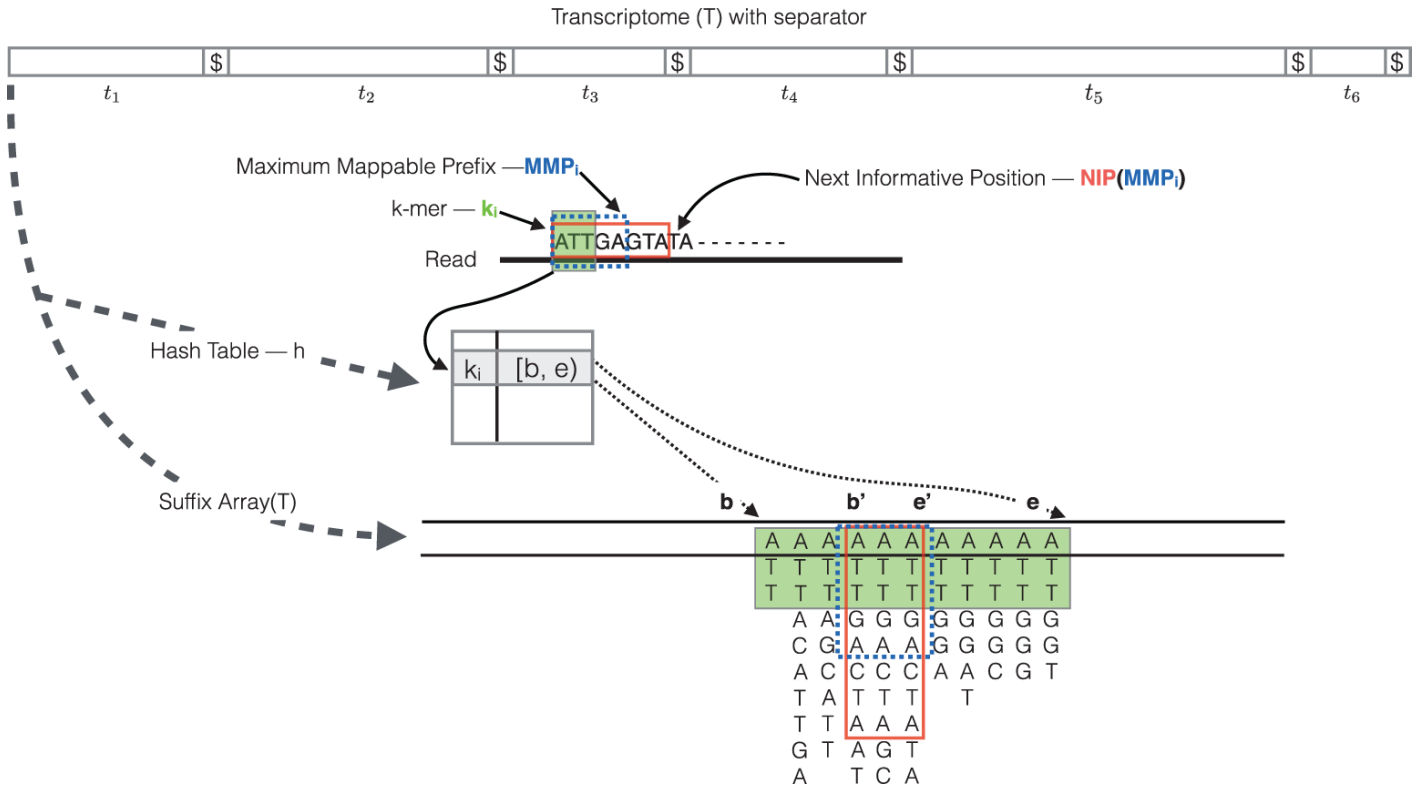


Different tissues may have different populations of genes expressed

Fast approaches to get the Read-Transcript Compatibility Matrix

- Salmon: quasi-mapping
- kallisto: pseudo-alignments

Quasi-mapping



Default k-mer size: 31

https://hbctraining.github.io/Intro-to-rnaseq-hpc-salmon/lessons/04_quasi_alignment_salmon.html

Quasi-mapping

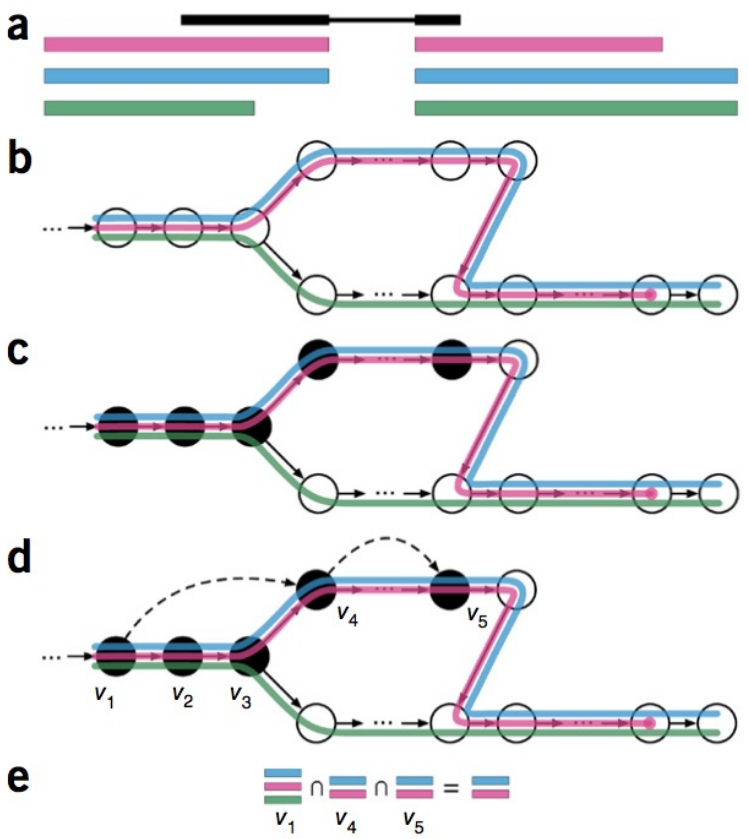
1. The read is scanned from left to right until a k-mer that appears in the hash table is discovered.
2. The k-mer is looked up in the hash table and the SA intervals are retrieved, giving all suffixes containing that k-mer
3. Similar to STAR, the maximal matching prefix (MMP) is identified by finding the longest read sequence that exactly matches the reference suffixes.
4. Salmon identifies the next informative position (NIP), by skipping ahead 1 k-mer (speedup)
5. Repeat above until the end of the read.
6. The final mappings are generated by determining the set of transcripts appearing in all MMPs for the read. The transcripts, orientation and transcript location are output for each read.

Quasi-mapping

- Result: Read-Transcript compatibility matrix
- Only based on compatibility of short k-mers
- Has an optional step to ***validate mappings***:
  - goes through all the read-transcript associations and validates if the entire read is compatible with the transcripts by doing a base-by-base comparison

kallisto: Quantification with pseudo-alignments

- Instead of hashing the transcriptome build a de Bruijn graph
- Find k-mer hits in the de Bruijn graph
- Identifies only transcripts that are consistent with all k-mer hits



Bray et al. Nature Biotechnology, 2016

Performance comparison

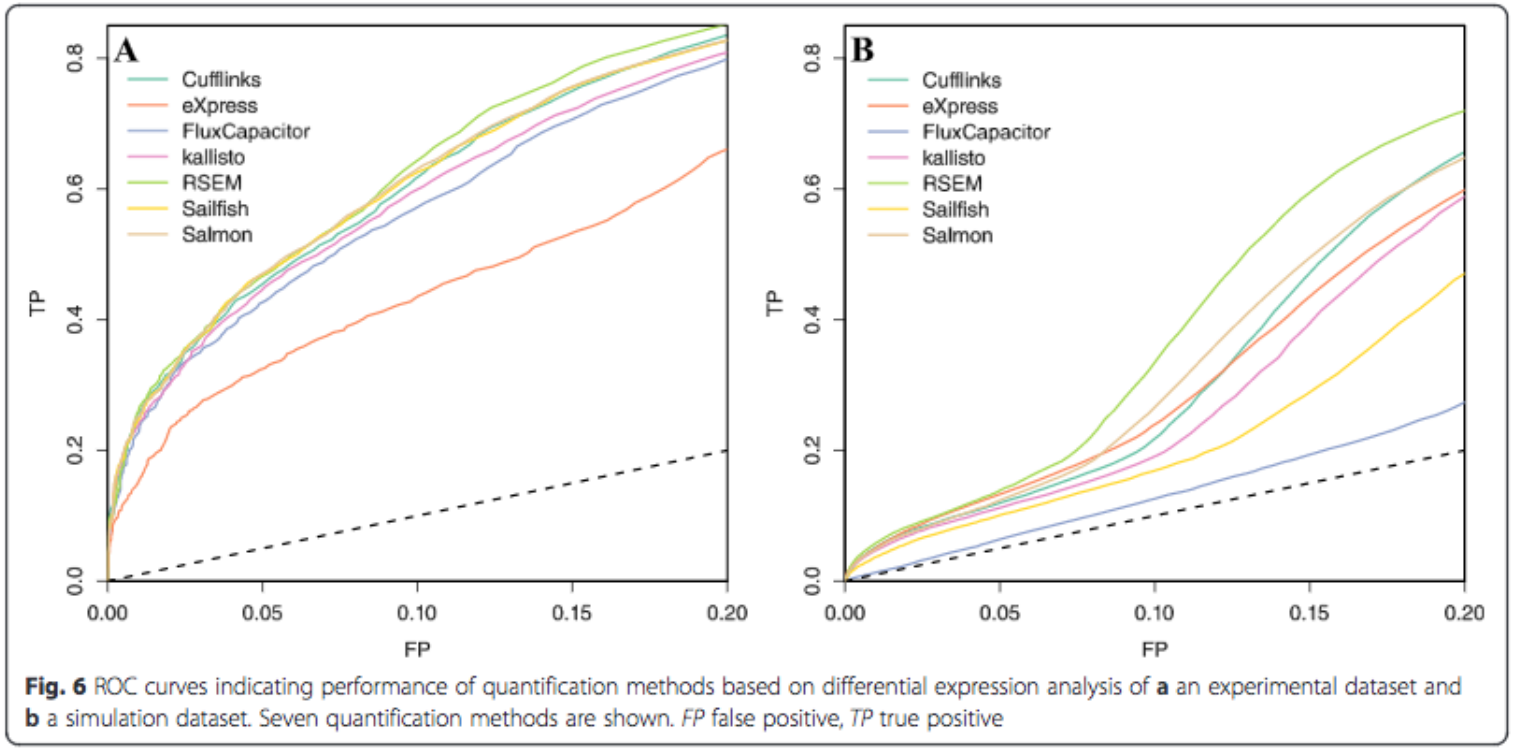
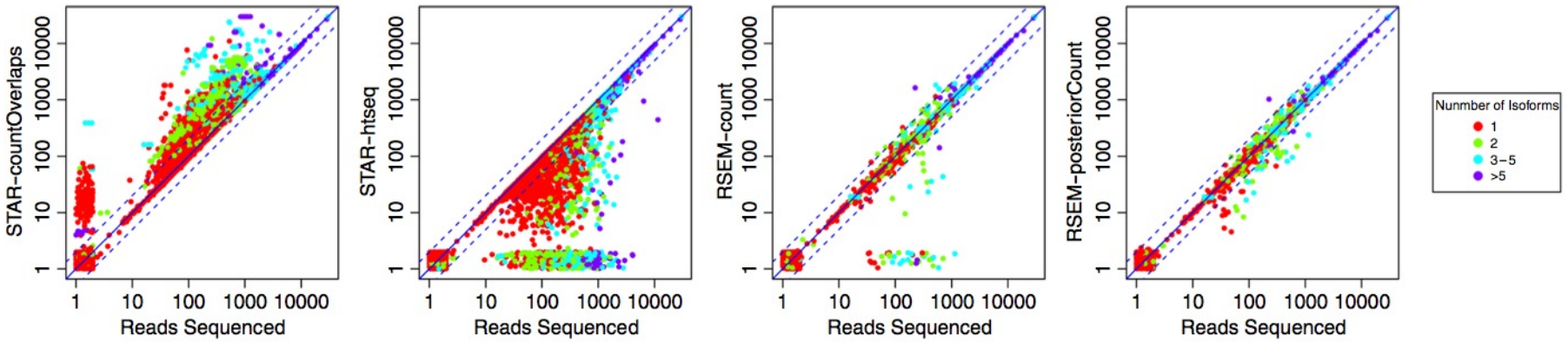


Fig. 6 ROC curves indicating performance of quantification methods based on differential expression analysis of **a** an experimental dataset and **b** a simulation dataset. Seven quantification methods are shown. *FP* false positive, *TP* true positive

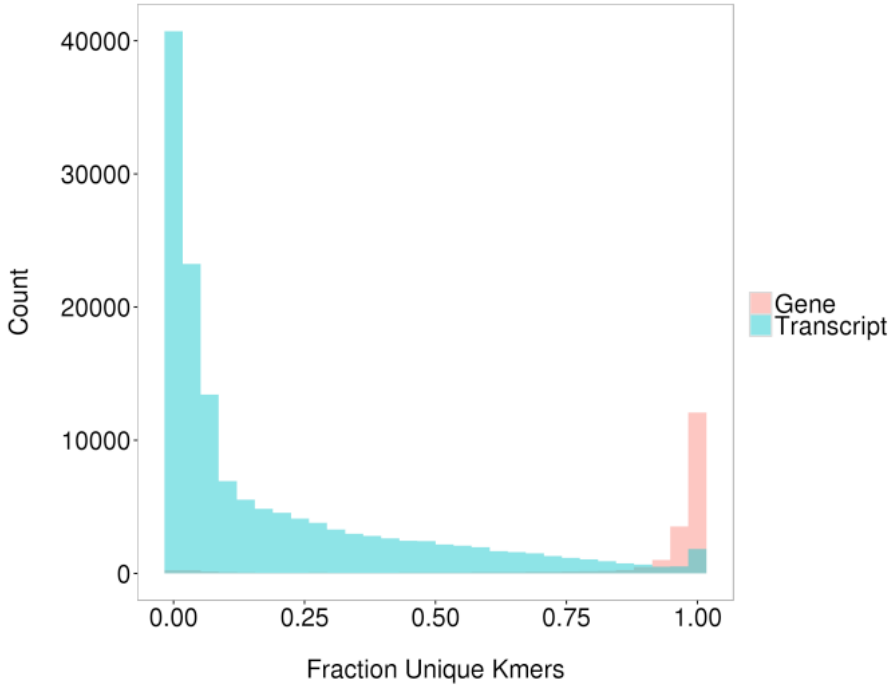
Teng et al. Genome Biology, 2016

Read Counting Accuracy



https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-14-370

Uniqueness: Isoform-level vs gene-level

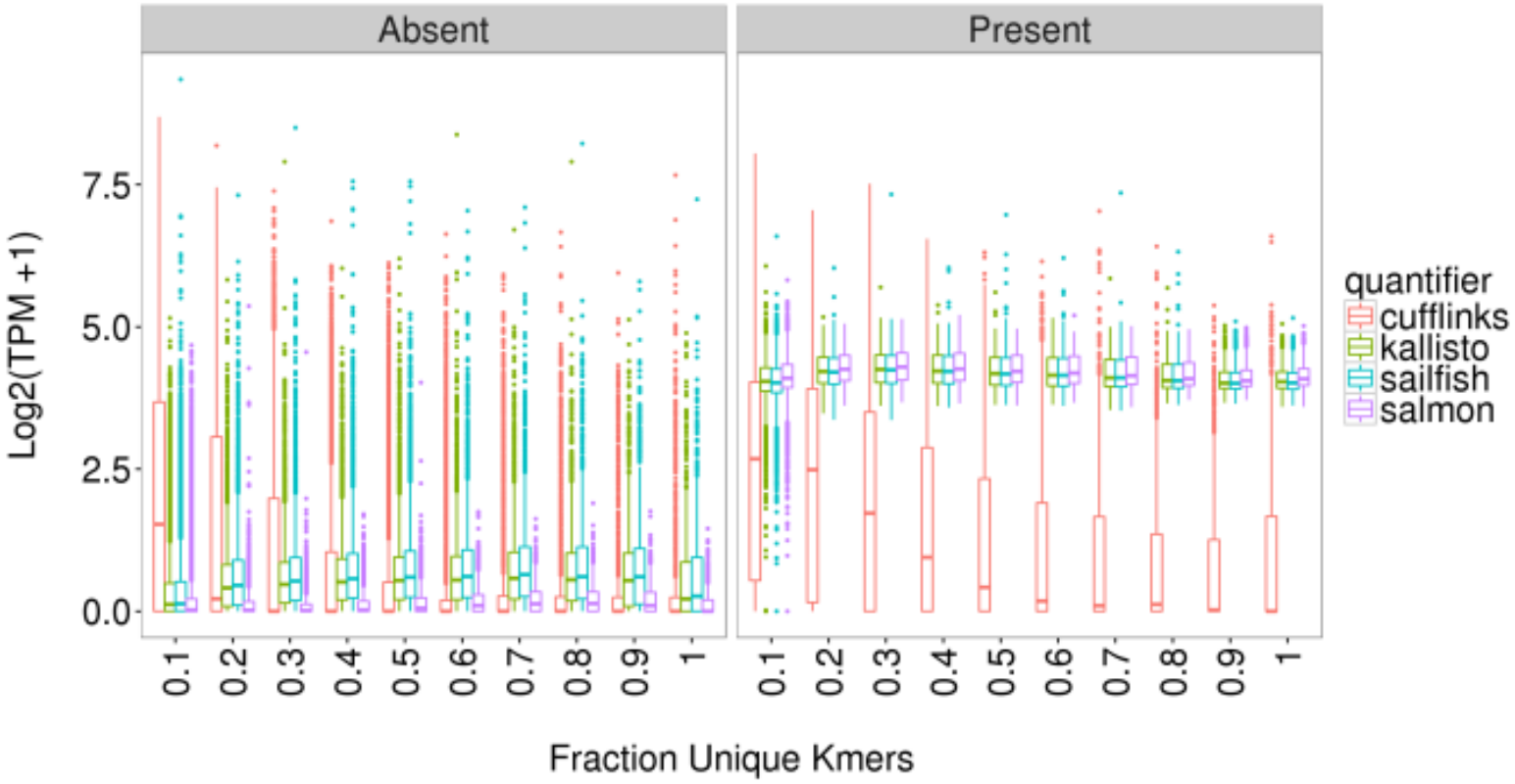


- Fraction of unique k-mer sequences for genes and transcripts
- Ambiguity is mainly between alternative transcripts from the same gene locus

https://cgatoxford.wordpress.com/tag/kallisto/

Misassignments to unexpressed transcripts

- Simulated data show that misassignments do happen



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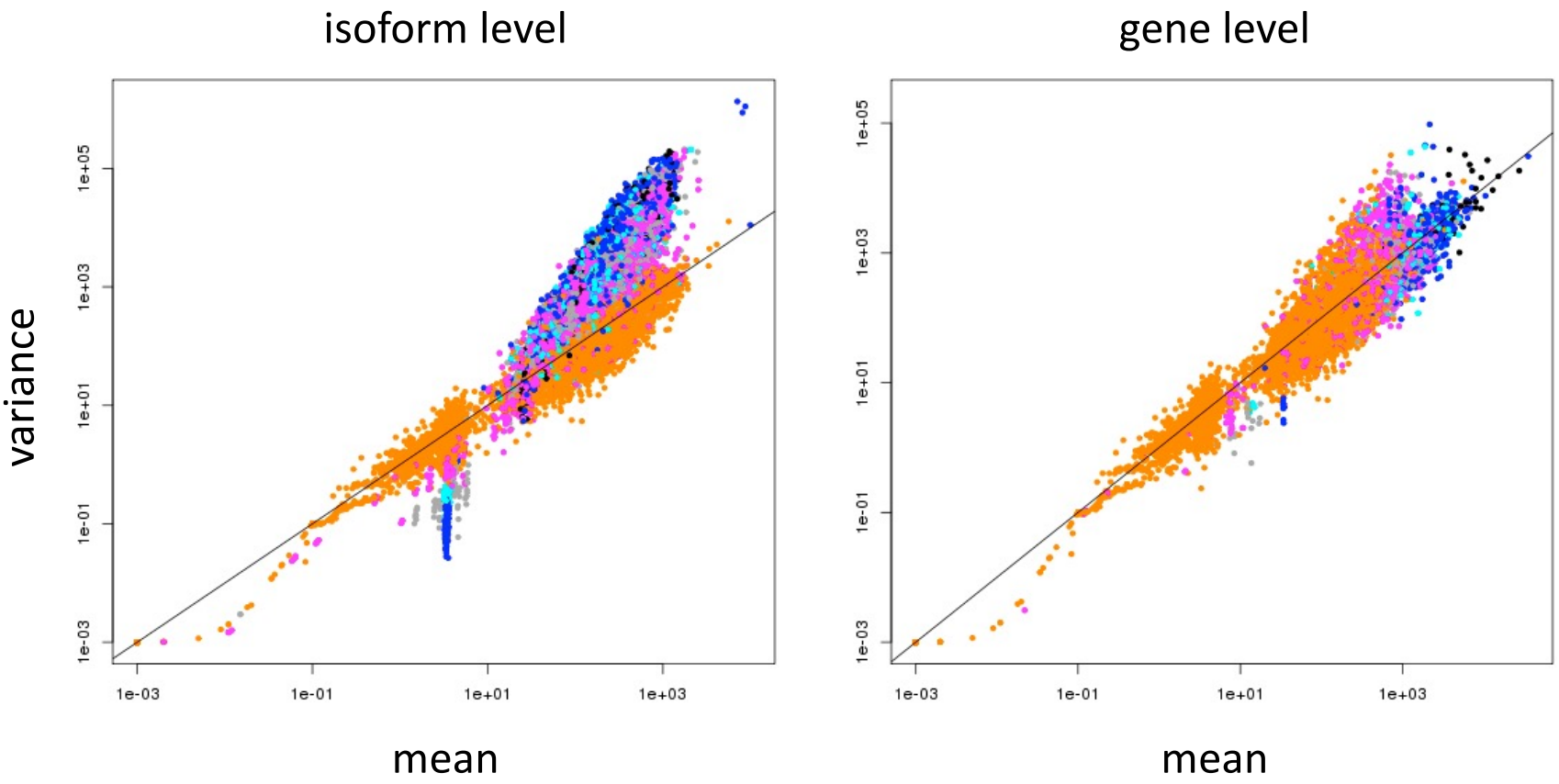
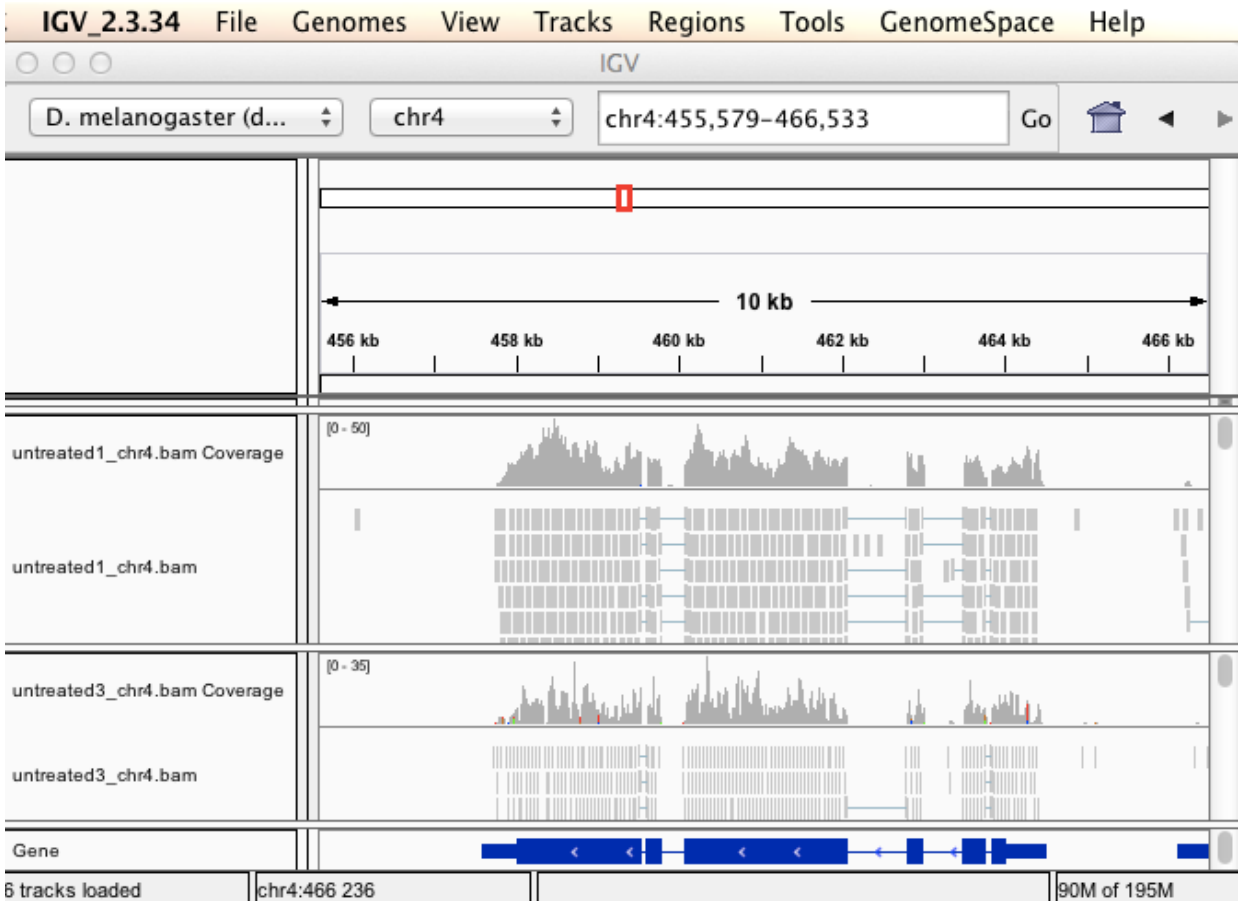
Isoform level has higher variability

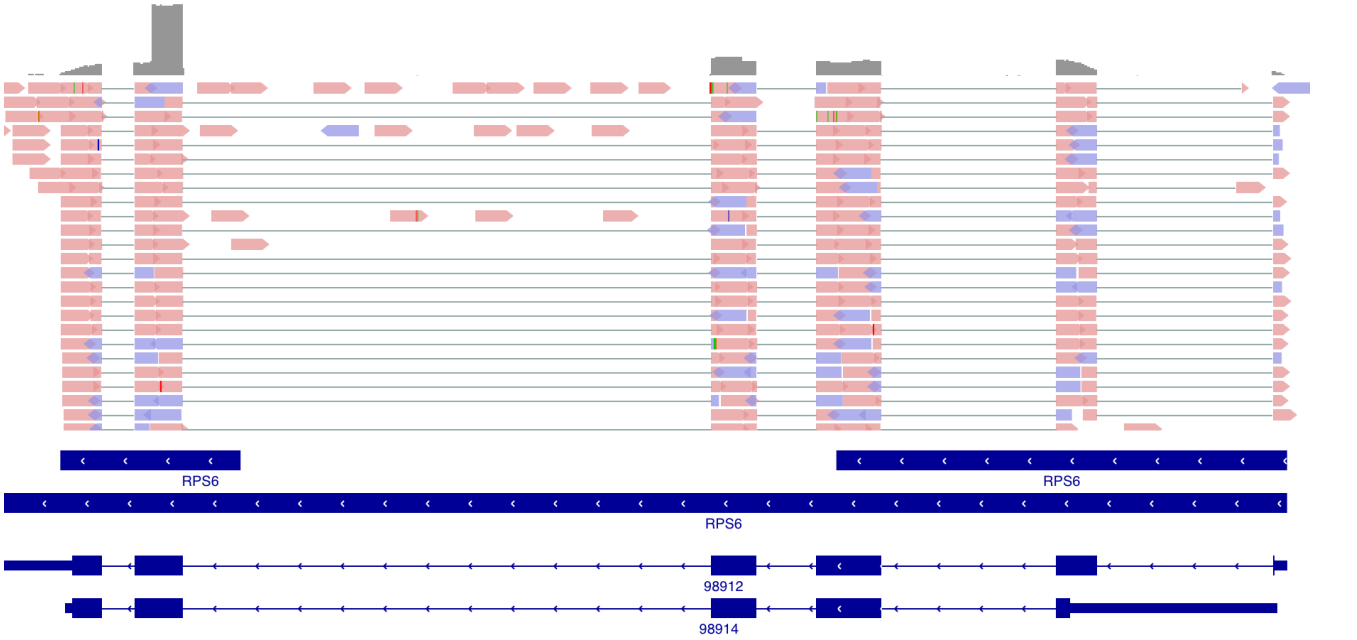


Positional bias of read distributions



Highly Multiplicated Reads

- Mainly a concern for low starting amounts
- <1ng of total RNA



Unspliced transcripts

- Isoform quantification assumes that only spliced transcripts have been measured
- But: unspliced transcripts are also present:
  - these are transcripts from the nucleus that are not yet spliced
  - limited capturing with poly-A based protocols
  - fully captured by random-priming protocols (1 – 10% of mRNA is in the nucleus)

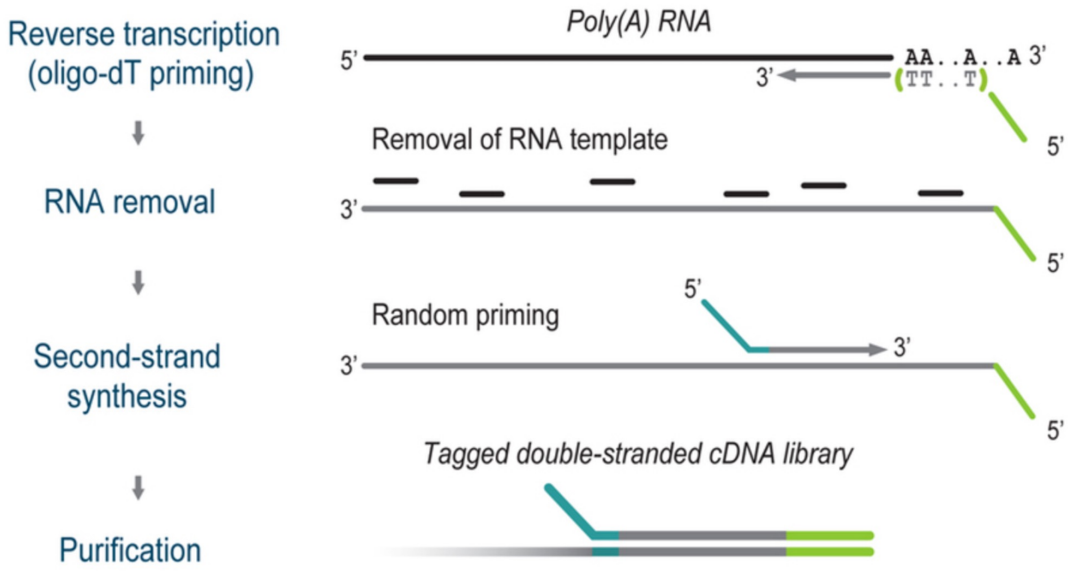
3’-Tagging

Reads are only generated near the 3’-end

Isoforms can not be resolved

Allows counting of the reads at the 3’-end

→ Assignment is to genes



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Given a tissue or cell line:

- ~25’000 genes in the genome
- ~10 – 18’000 genes expressed at any given time; expression level of the different genes differs largely

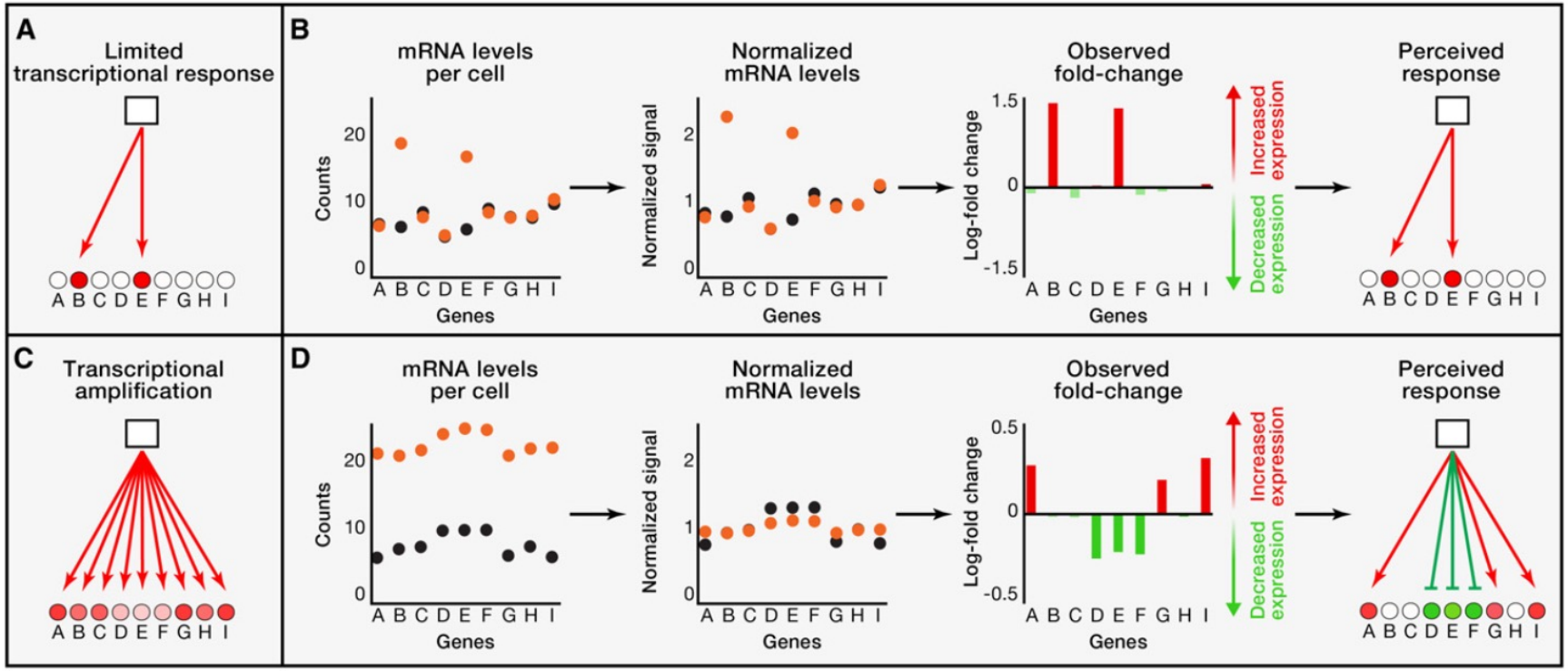
Working hypotheses:

- relative prevalence of a gene reflects activity of associated pathways; more prevalent, more active
- baseline prevalence is not known → **perturbation experiments**: treatment vs control
- the majority of the genes is not affected by the perturbation
- informative quantity: relative abundance of the genes in a tissue
- gene expression has a large dynamic range

Limitations of RNA-seq experiments

- genes vs protein
- alternative explanations …. cell size effects; other side effects
- tissue composition changes

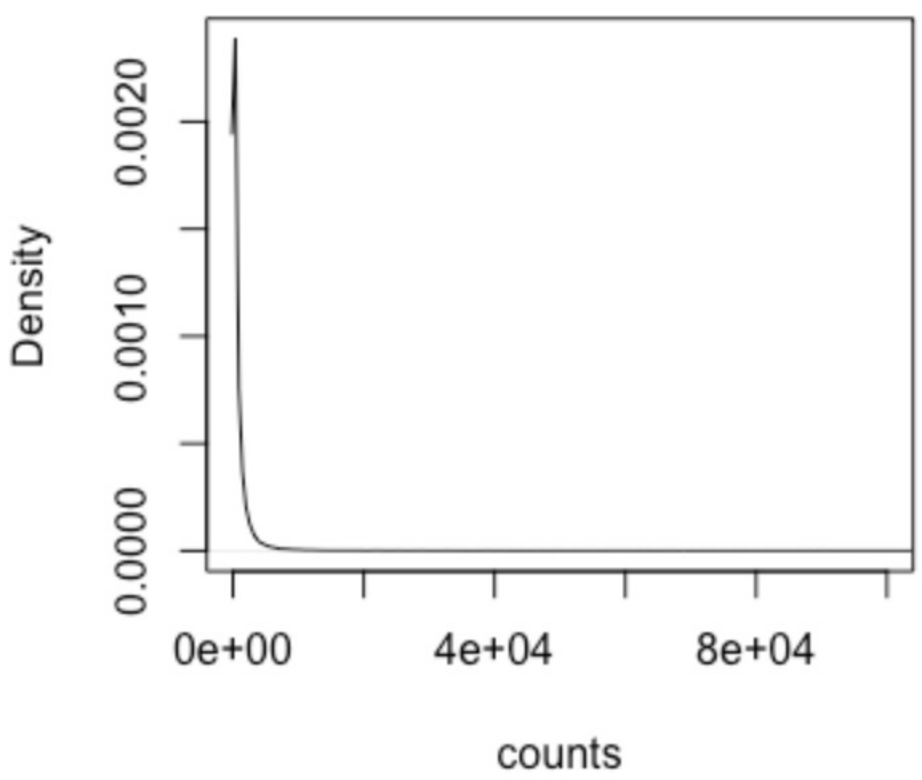
Perturbation Experiments

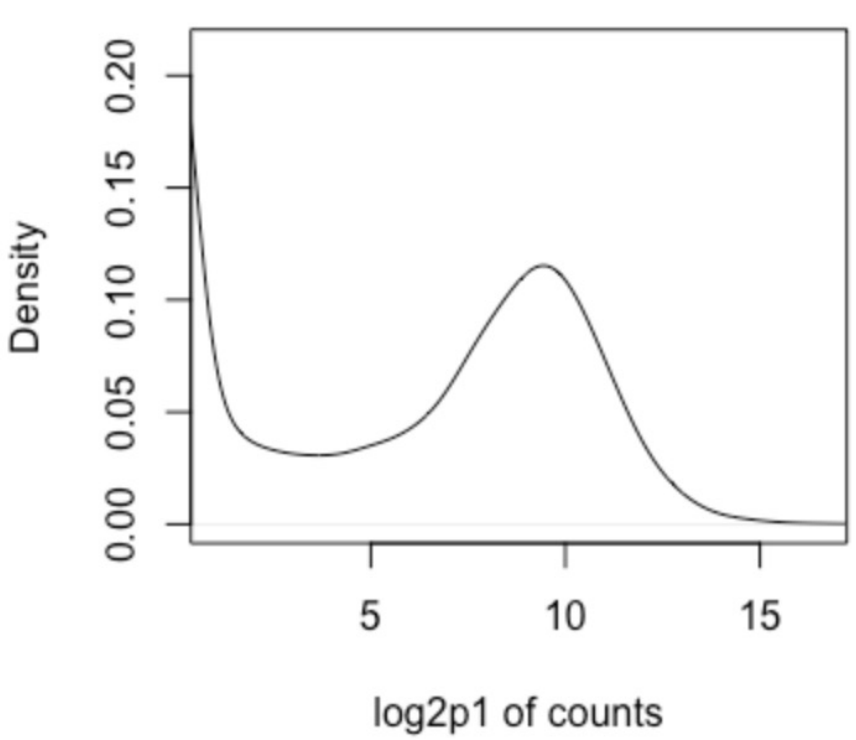


- The top row shows the assumption how a baseline expression profile (black) changes as a response (red) to a perturbation
- Note: expression counts are all around ~10, in practice they range from 0 – 1 Mio

Lovén et al, 2012, https://doi.org/10.1016/j.cell.2012.10.012

Dynamic Range of Expression and Normalization





TPM normalizes to total count. Is the total count a good measure?

Summary

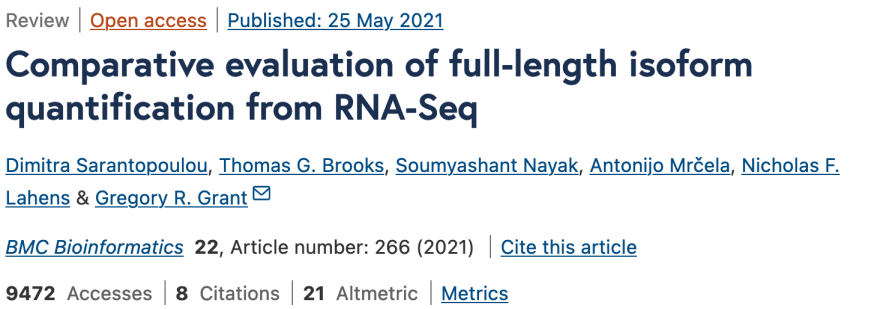
- Alignment + expression estimation using generative models give good results, let you inspect the aligned reads, and can be used to discover new genes and new isoforms
- Pseudo-alignment is reliable and fast but needs as input the accurate and complete set of transcripts
- 3’-end tag sequencing provides only gene-level estimates without isoform resolution
- Full-length transcript sequencing detects isoforms accurately, sequencing depth is typically lower than for short-read sequencing

References

- Compares kallisto, salmon, featureCounts, … https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-021-04198-1

- from the salmon people, performance evaluation and tuning options (especially genomic “decoy”) https://genomebiology.biomedcentral.com/articles/10.1186/s13059-020-02151-8

- from the salmon people, confirm that decoys help https://www.biorxiv.org/content/10.1101/2021.01.17.426996v1



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Dimitra Sarantopoulou, Thomas G. Brooks, Soumyashant Nayak, Antonijo Mrčela, Nicholas F. Lahens & Gregory R. Grant

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Avi Srivastava, Laraib Malik, Hirak Sarkar, Mohsen Zakeri, Fatemeh Almodaresi, Charlotte Soneson, Michael I. Love, Carl Kingsford & Rob Patro

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