RNA-sequencing

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Based on materials by:
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Next Generation Sequencing Bioinformatics Course
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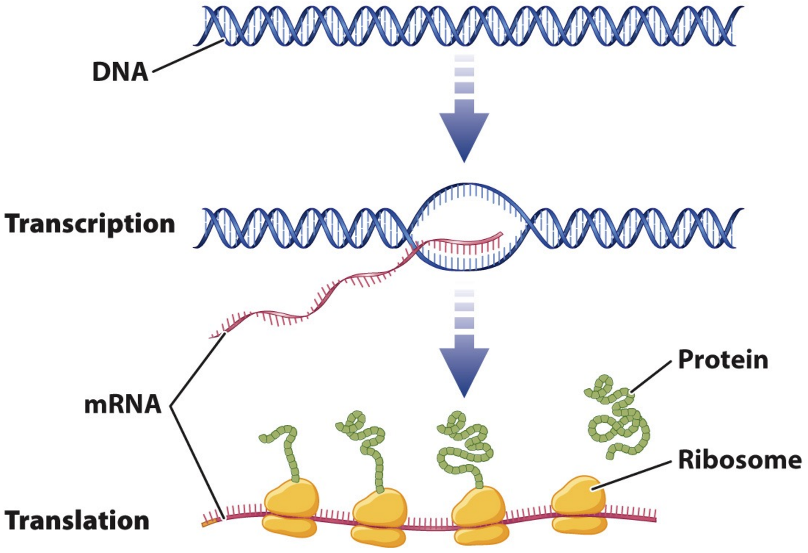
Overview

- RNA-seq background.
- Experimental design.
- Data alignment.
- Quantification.
- Normalisation.
- Differential expression.
- Interpretation of results.



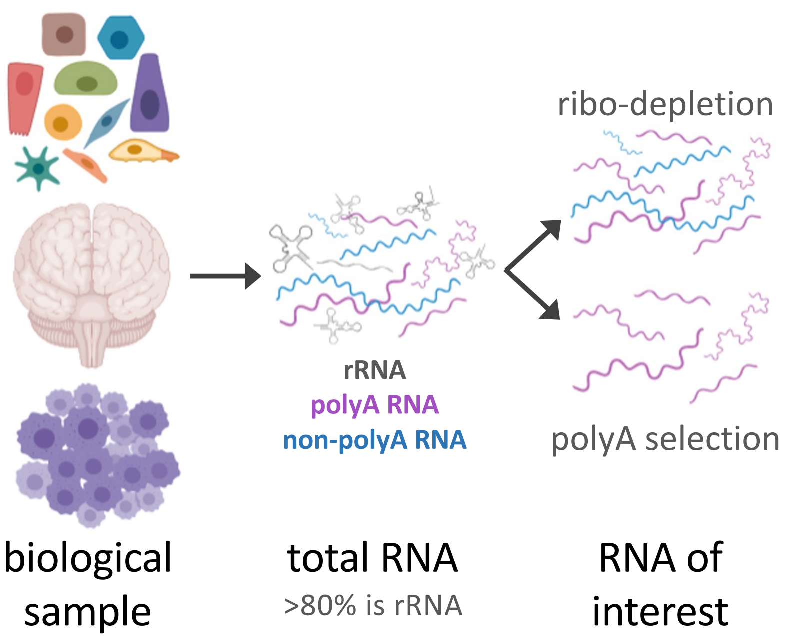
http://www.macmillanhighered.com/BrainHoney/Resource/6716/digital_first_content/trunk/test/morris2e/asset/img_ch3/morris2e_ch03_fig_03_03.html

RNA-sequencing

- Uses high-throughput sequencing to profile the **transcriptome** of a biological sample at a given time.
  - Transcriptome = set of RNA molecules present in a cell.
- Allows to
  - **Catalogue** all species of transcripts (messenger, small, non-coding).
  - Annotate the **structure** of genes (start, end, splice isoforms, UTRs).
  - Compare the **types and quantities** of the RNA molecules across time and conditions.

Wang, Gerstein and Snyder, *Nat Rev Genetics* 10 (2009) doi.org/10.1038/nrg2484

RNA-sequencing



ribo-depletion

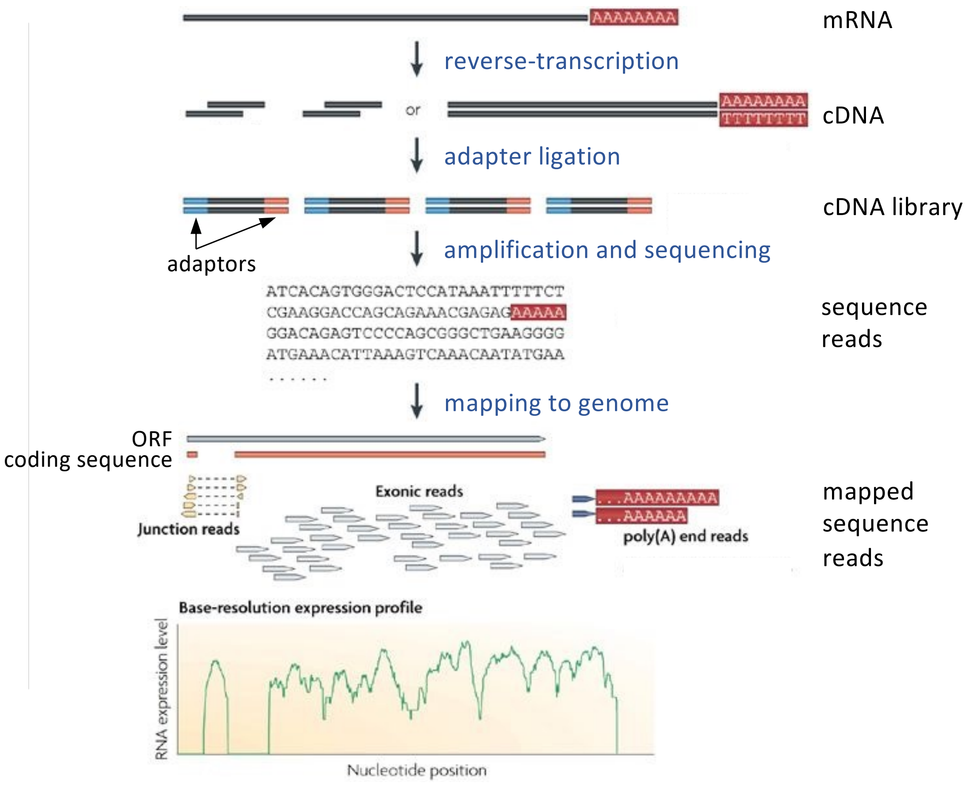
rRNA
polyA RNA
non-polyA RNA

polyA selection

biological sample

total RNA
>80% is rRNA

RNA of interest



mRNA

reverse-transcription

cDNA

adapter ligation

cDNA library

adaptors

amplification and sequencing

sequence reads

```
ATCACAGTGGGACTCCATAAATTTTTCT
CGAAGGACCAGCAGAAACGAGAGAAAAA
GGACAGAGTCCCCAGCGGGCTGAAGGGG
ATGAAACATTAAAGTCAAACAATATGAA
......
```

mapping to genome

ORF
coding sequence

mapped sequence reads

Modified from Wang, Gerstein and Snyder, *Nat Rev Genetics* 10 (2009)
doi.org/10.1038/nrg2484

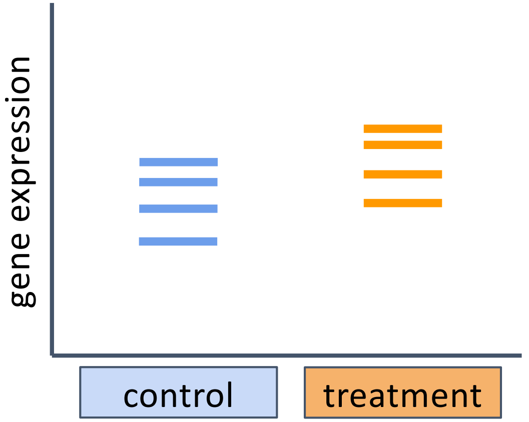
Experimental design

- Start by identifying **what is the question to answer** and what type of information is required.
  - **Single vs paired-end** (isoform analysis).
  - **Stranded vs unstranded** (antisense and overlapping transcripts).
  - **Sequencing depth** (detection of low abundance transcripts).
  - **Number of replicates**:
    - **Biological** = independent, biologically distinct samples.
    - **Technical** = repeated measurement of the same sample.
    - ➢ For differential expression analysis, increasing the number of replicates is better than increasing depth.

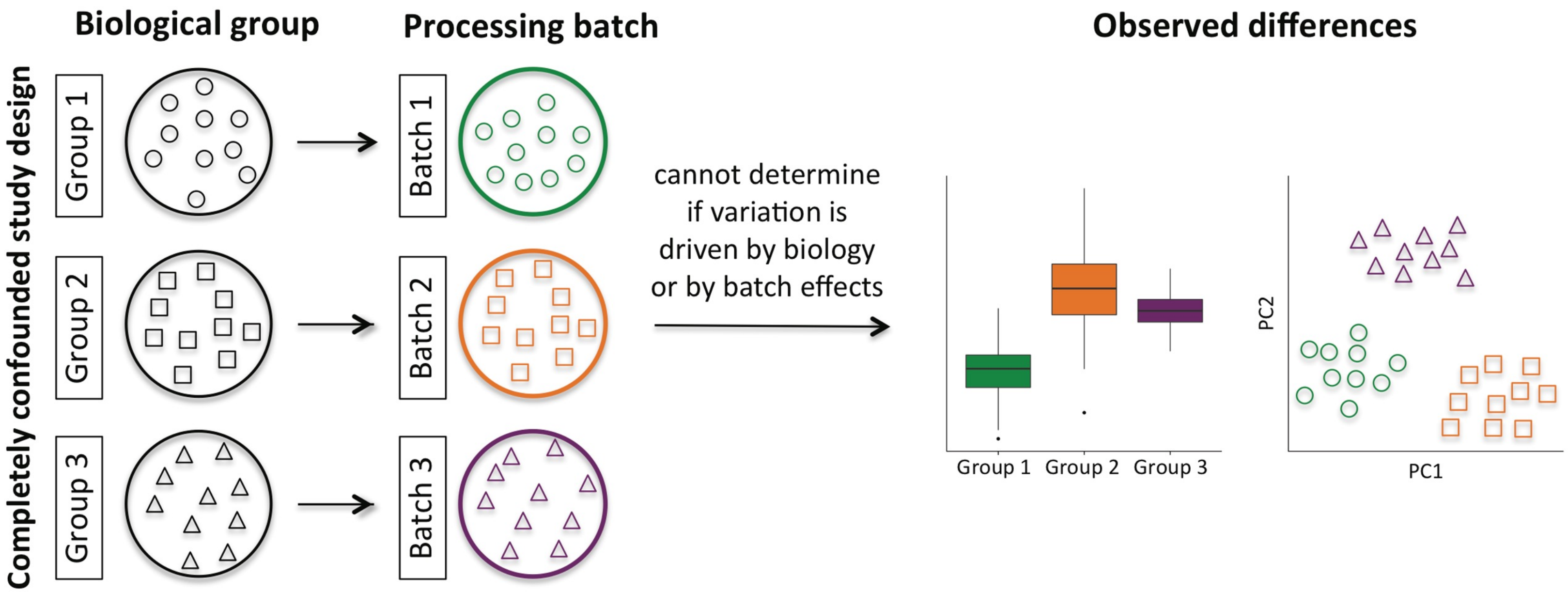
Liu, Zhou and White, *Bioinformatics* 30 (2014) doi:10.1093/bioinformatics/btt688

Experimental design

- Start by identifying **what is the question to answer**.
- Consider what are possible **sources of variation**.
  - Biological: sex, age, genetic background, ethnicity…
  - Technical: sample processing date, reagent’s batch, time of sample collection…
- To estimate variation we need **biological replicates**.
- **Power** calculations.
  - Number of replicates needed to observe an effect.

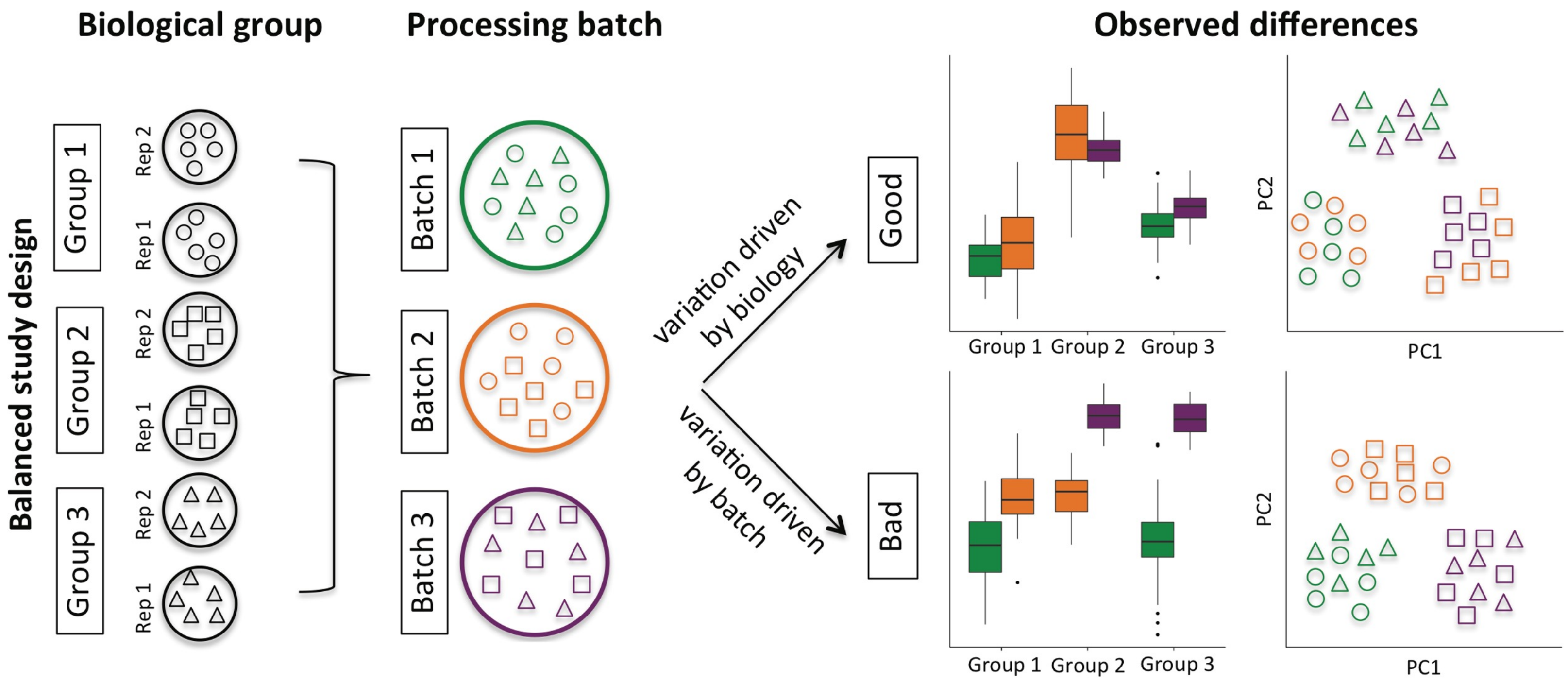


Experimental design



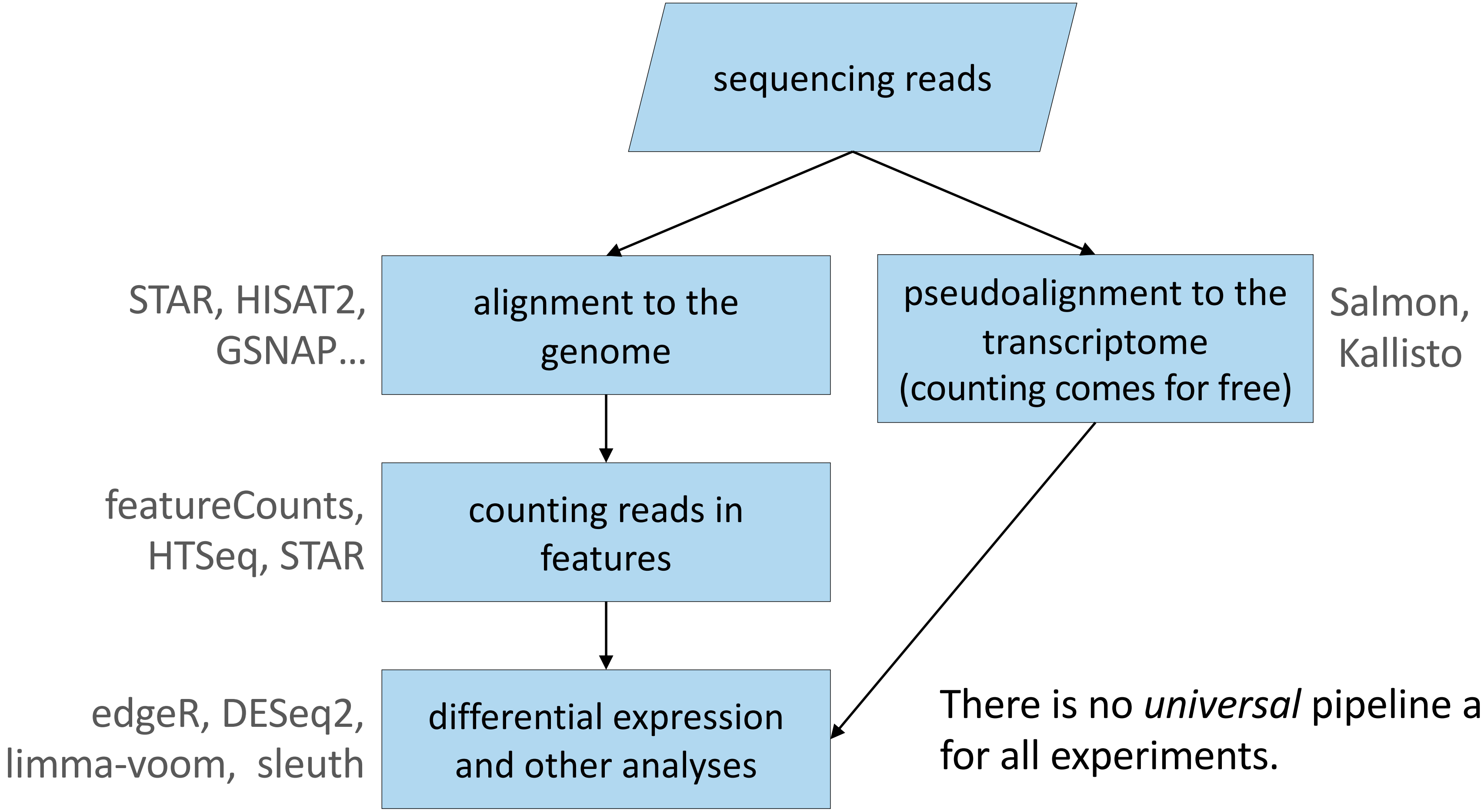
Adapted from Hicks et al., BioRxiv, version from December 27, 2015 - 06:15
doi.org/10.1101/025528

Experimental design



Adapted from Hicks et al., BioRxiv, version from December 27, 2015 - 06:15
doi.org/10.1101/025528

RNA-seq data analysis pipeline



sequencing reads

STAR, HISAT2, GSNAP…

alignment to the genome

pseudoalignment to the transcriptome (counting comes for free)

Salmon, Kallisto

featureCounts, HTSeq, STAR

counting reads in features

edgeR, DESeq2, limma-voom, sleuth

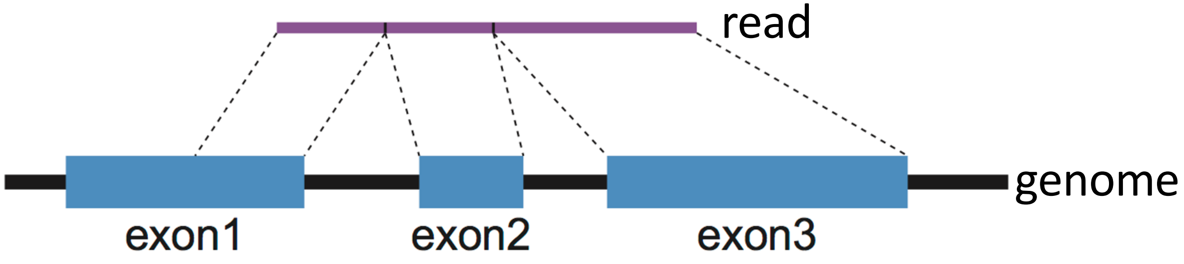
differential expression and other analyses

There is no *universal* pipeline adequate for all experiments.

Different datasets and biological questions will require different analyses and will influence which programs and parameters are chosen.

RNA-seq data alignment

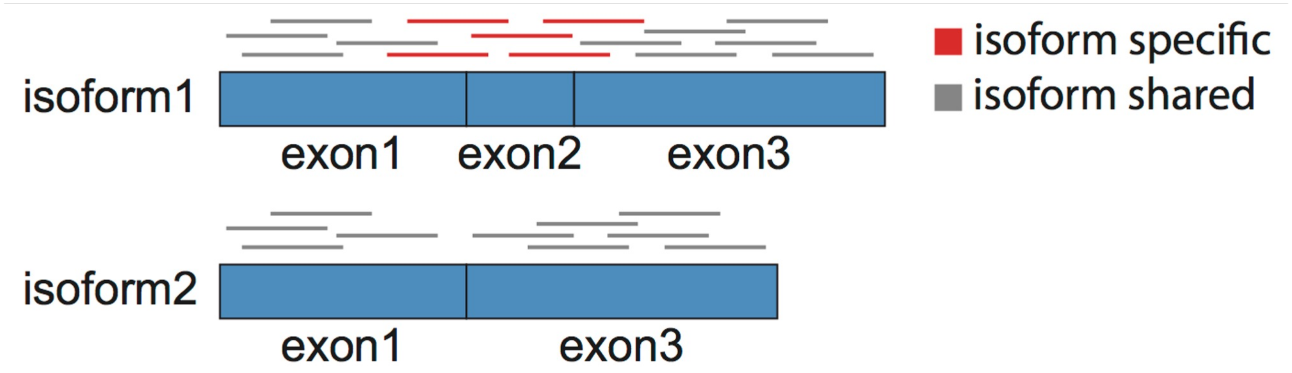
- RNA-seq reads come from spliced mRNAs.
- Therefore, their alignment in the genome is interrupted by introns.



- Two solutions:
  - Map reads to the transcriptome instead of the genome.
  - Allow gapped alignments.

Map to the transcriptome

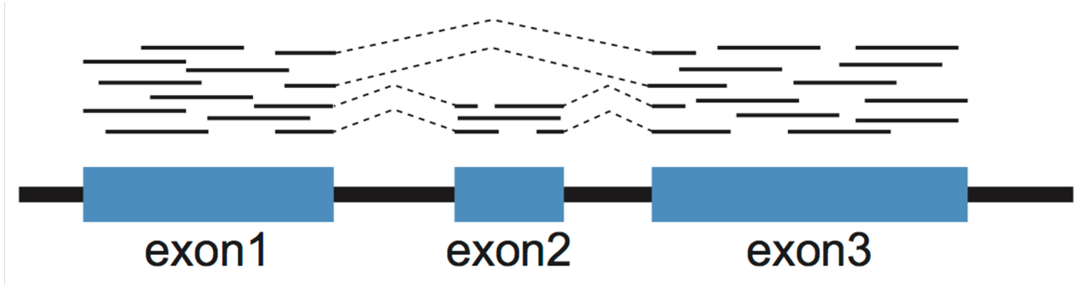
- If the RNA-seq reads are mapped to the transcriptome, reads in exons that are shared across transcript isoforms will map multiple times.



- Only possible for organisms with a well-annotated transcriptome.
  - Any novel genes or isoforms will be lost.

Map allowing large gaps

- Instead, we can map to the genome but allowing the alignments to have large gaps.
  - Intron size ranges from 10^2 to ~10^5 in eukaryotes.



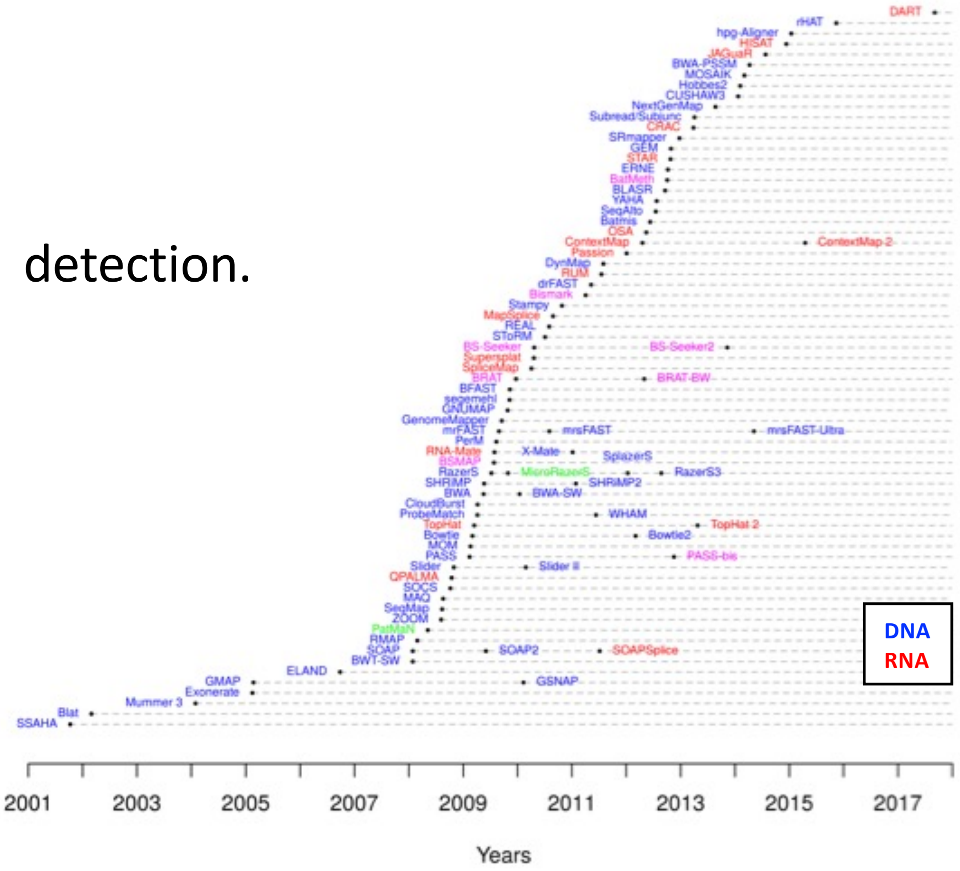
- Many different mappers.
  - STAR, HISAT2, GSNAP, subread, MapSplice.

RNA-seq data aligners

- There are many different programs to align RNA-seq data.

- There is no best aligner.
  - Memory usage.
  - Speed.
  - Accuracy of splice junction detection.

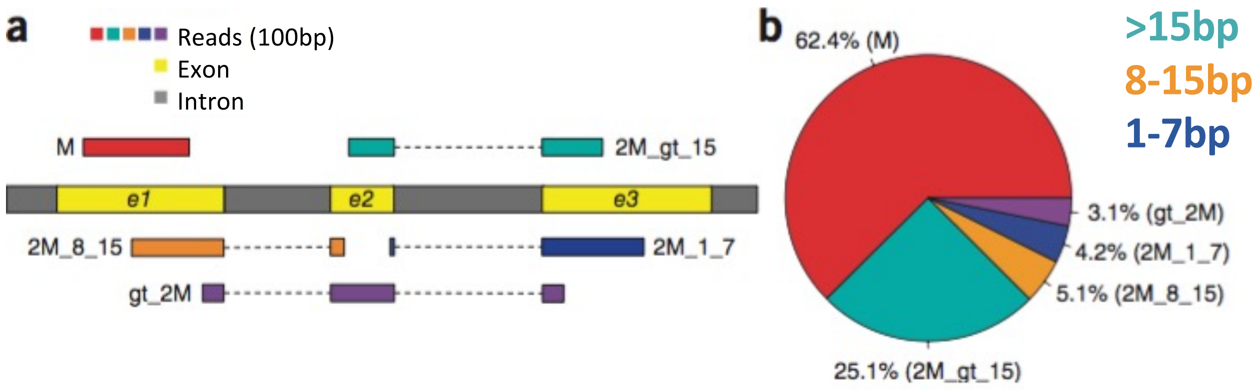
Simulation-based comprehensive benchmarking of RNA-seq aligners.
Baruzzo et al., *Nat Methods* 14 (2017)
doi.org/10.1038/nmeth.4106



HISAT2

- Fast and requires low memory.
  - Combines the Burrows-Wheeler transform and the FM index.
  - Two indices: **one global** FM index of the whole genome.

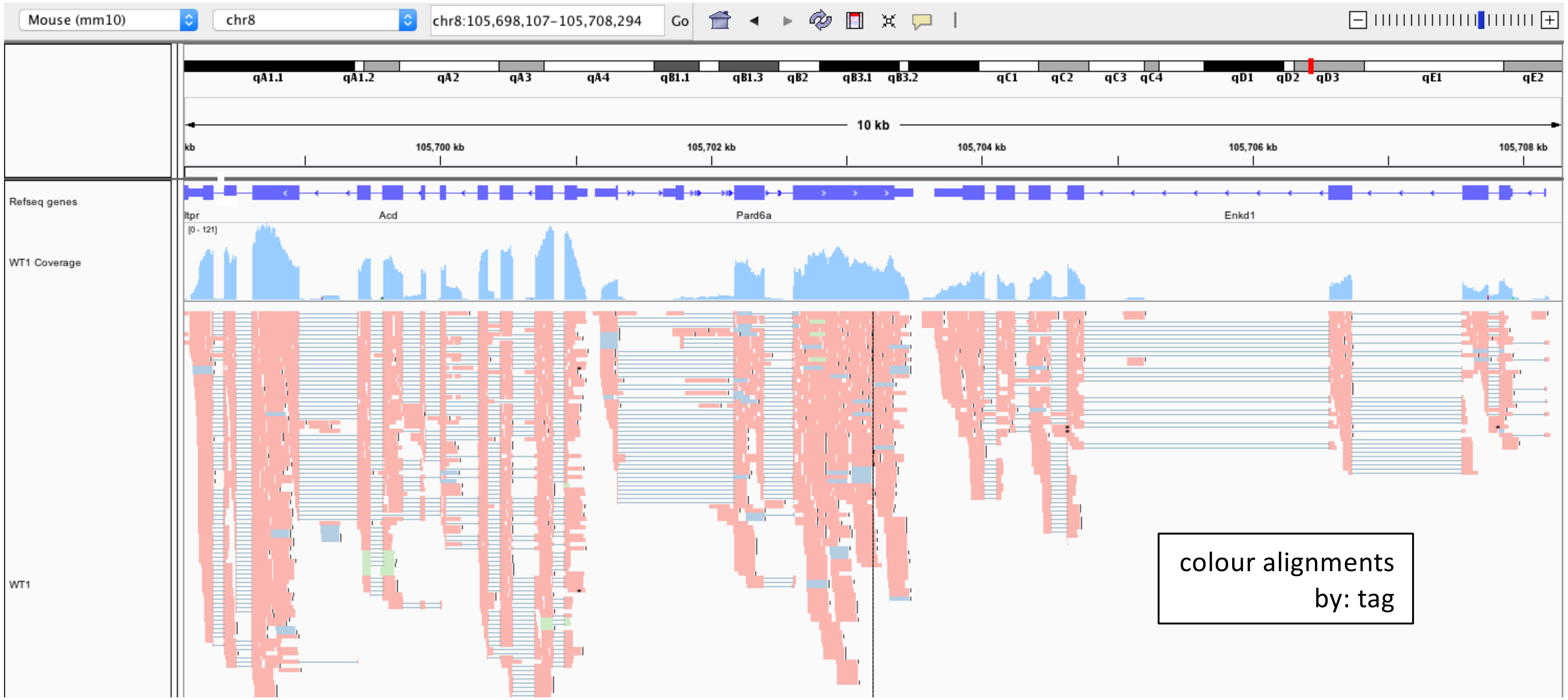
    many small overlapping FM indices of 56kb-long regions.



Spliced alignment anchors:
>15bp long
8-15bp intermediate
1-7bp short

Kim, Langmead and Salzberg, *Nat. Methods* 12 (2015) doi.org/10.1038/nmeth.3317

Visualisation of aligned RNA-seq reads



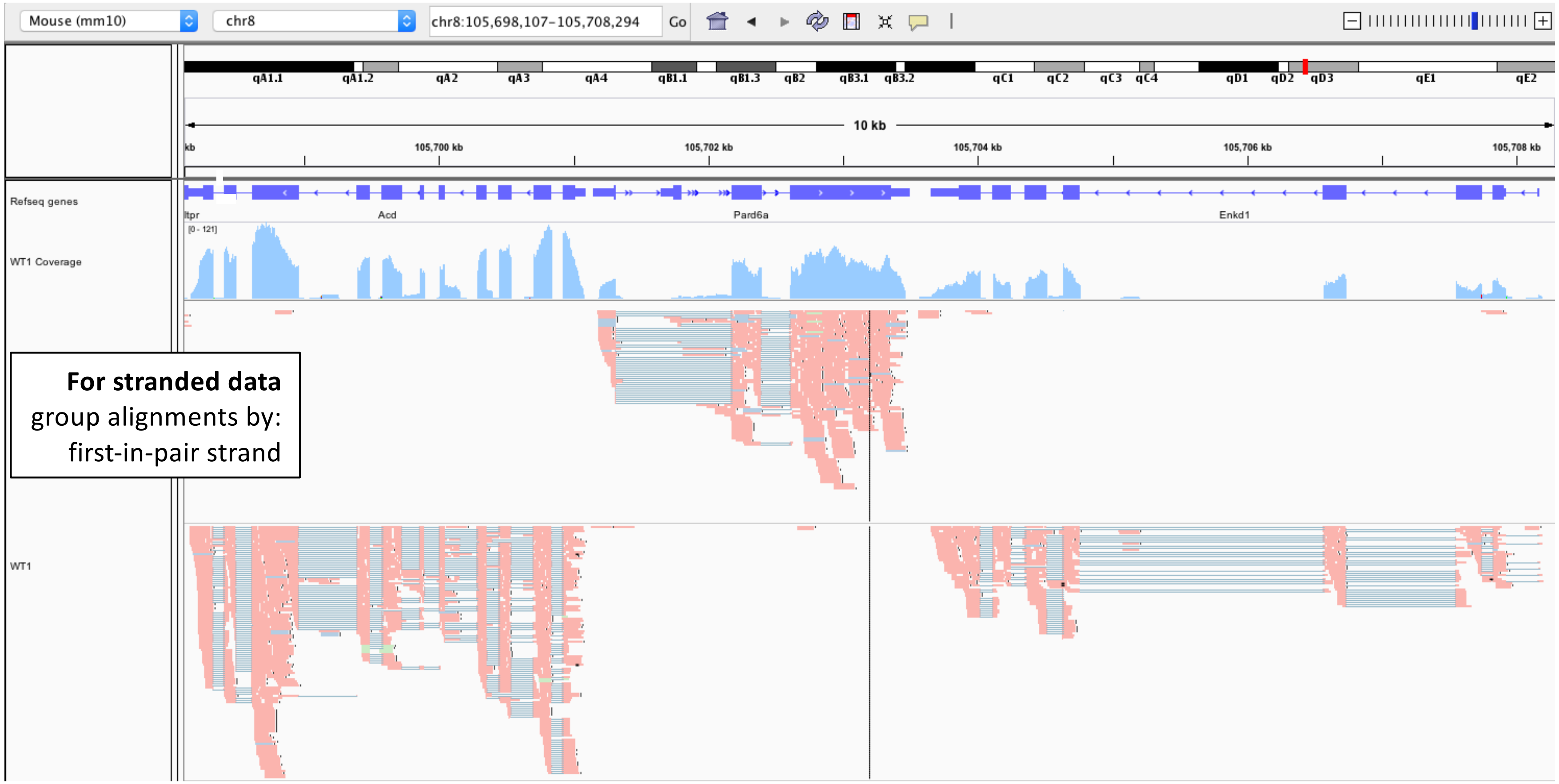
colour alignments by: tag

In this case reads are coloured by the NH tag, indicating the number of alignments:

unique two valid alignments three valid alignments …

http://software.broadinstitute.org/software/igv/

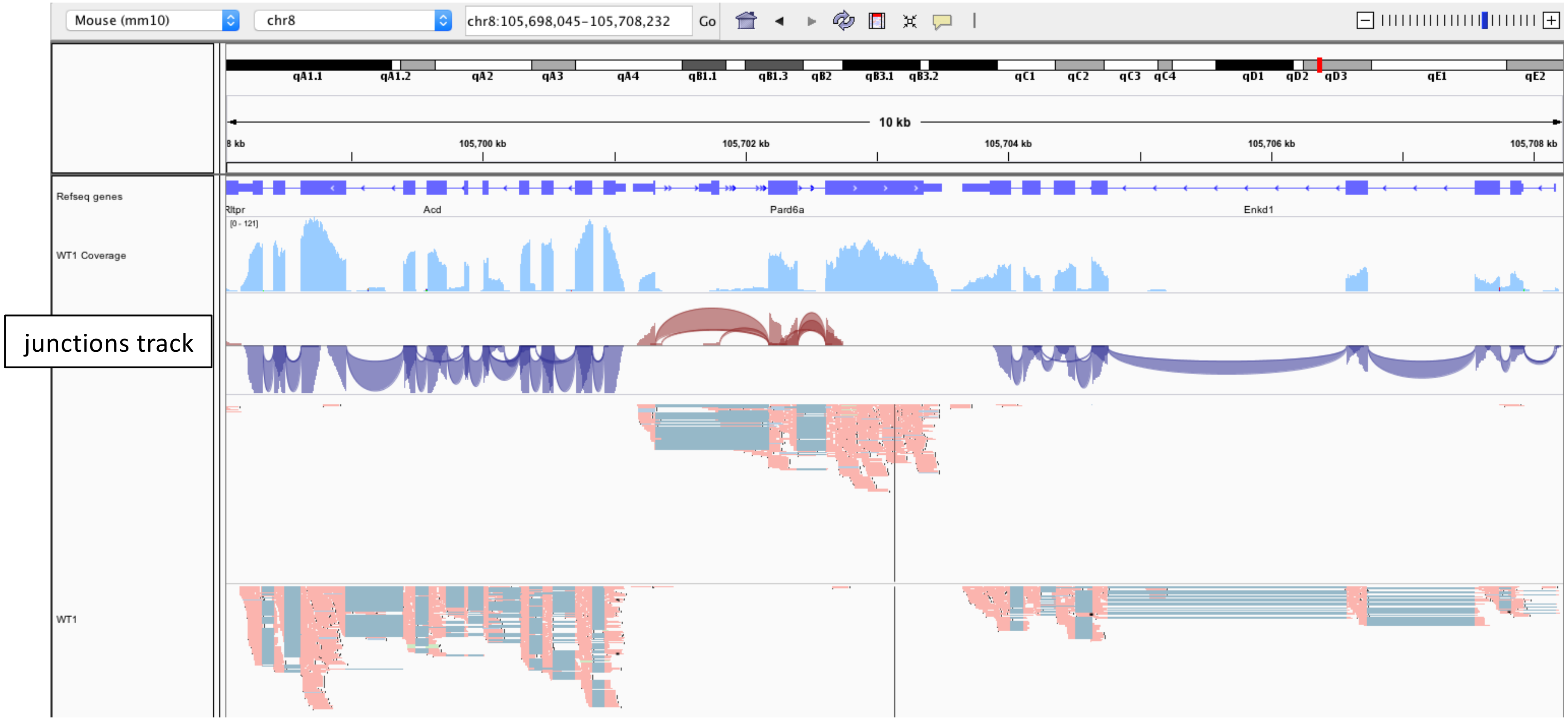
Visualisation of aligned RNA-seq reads



For stranded data
group alignments by:
first-in-pair strand

http://software.broadinstitute.org/software/igv/

Visualisation of aligned RNA-seq reads



junctions track

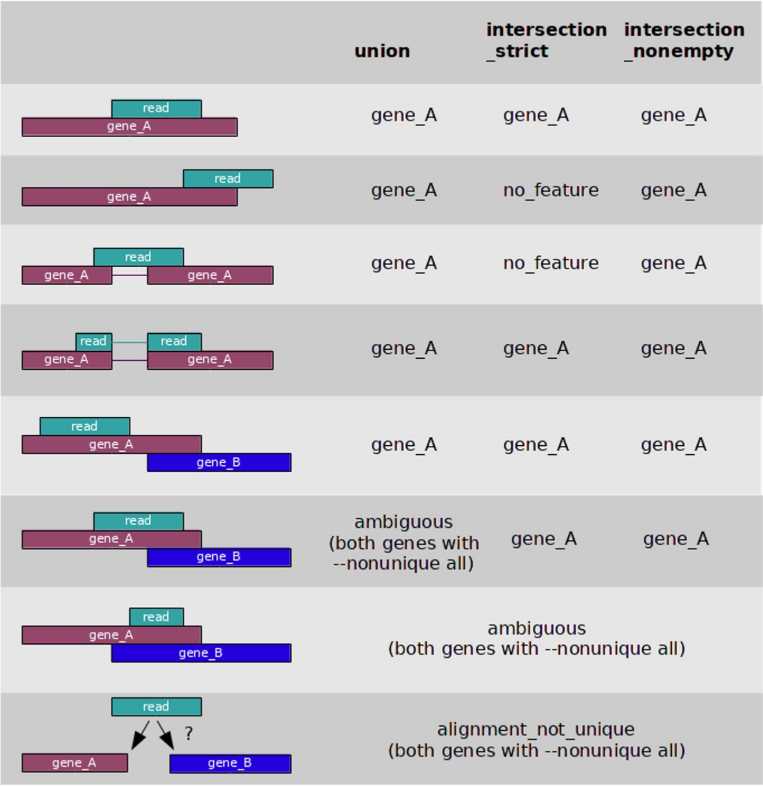
http://software.broadinstitute.org/software/igv/

Quantifying reads in features

- Once reads have been aligned to the genome, we want to quantify how many overlap features of interest (genes).

number of reads ∝ transcript abundance

- Many available programs to do this (take a BAM and an annotation file).
  - HTSeq, FeatureCounts.
- Some aligners and pseudoaligners also do the quantification while mapping.
  - STAR, Kallisto.



	union	intersection _strict	intersection _nonempty
read gene_A	gene_A	gene_A	gene_A
read gene_A	gene_A	no_feature	gene_A
read gene_A gene_A	gene_A	no_feature	gene_A
read read gene_A gene_A	gene_A	gene_A	gene_A
read gene_A gene_B	gene_A	gene_A	gene_A
read gene_A gene_B	ambiguous (both genes with --nonunique all)	gene_A	gene_A
read gene_A gene_B	ambiguous (both genes with --nonunique all)		
read ? gene_A gene_B	alignment_not_unique (both genes with --nonunique all)		

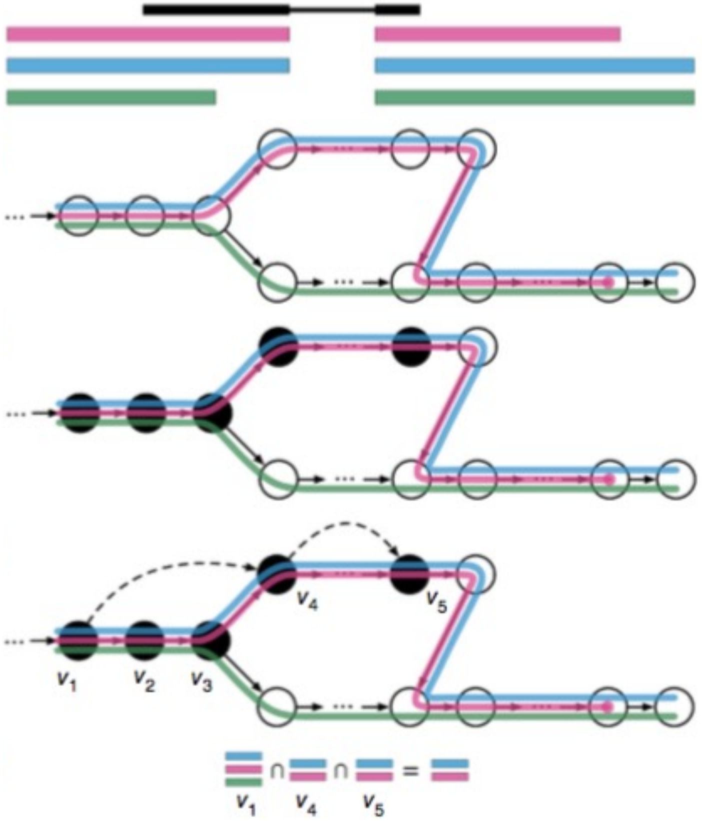
https://htseq.readthedocs.io/en/release_0.9.1/count.html

Quantification through pseudo-alignment

- Quantification of gene expression doesn't require knowing where a read originated from, but which transcripts could have generated it.

- Kallisto uses this principle to pseudo-align RNA-seq reads.
  - de Bruijn graph to represent the transcriptome.
  - exact matching of k-mers from reads to identify their compatibility with a set of transcripts.

- Pseudo-aligned reads are used to quantify the abundance of each compatibility class.



Bray et al., *Nat Biotech* 34 (2016) doi.org/10.1038/nbt.3519

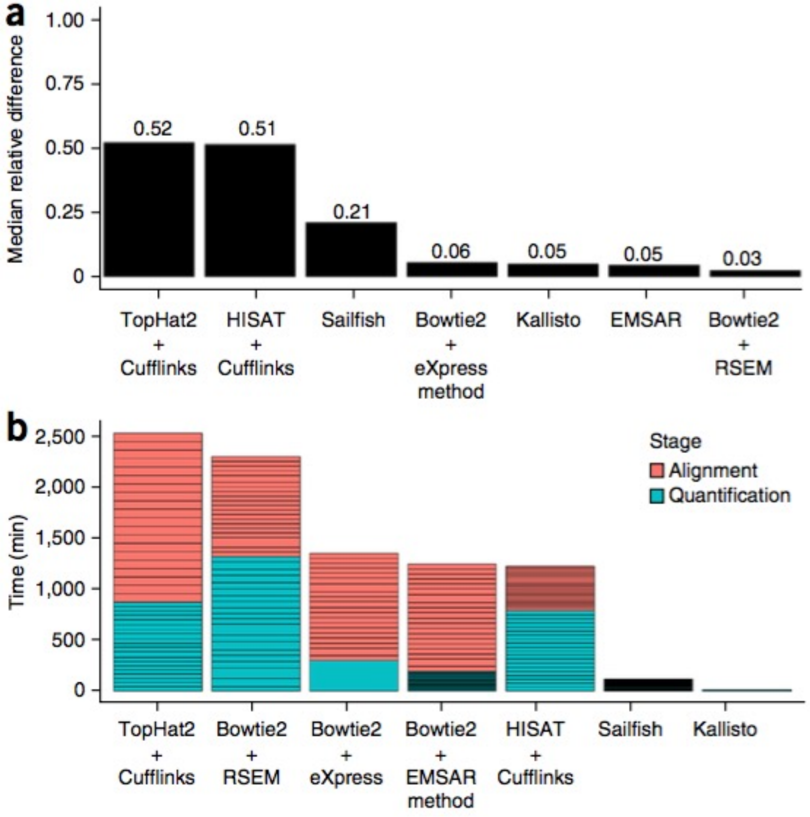
Quantification through pseudo-alignment

- Quantification of gene expression doesn’t require knowing where a read originated from, but which transcripts could have generated it.

- Kallisto
  - Fast and accurate.
  - Cannot discover new genes/transcripts/splice junctions.

- Salmon is a similar program.

  Patro et al., *Nat Methods* 14 (2017)
  doi.org/10.1038/nmeth.4197



Accuracy

Speed

Bray et al., *Nat Biotech* 34 (2016) doi.org/10.1038/nbt.3519

Normalisation

- To compare data from different samples, counts need to be normalised to **remove systematic technical effects**.
- **Sequencing depth bias**: the most obvious difference between samples is how deep they are sequenced.
  - Larger libraries have larger counts. These need to be scaled to be comparable.

	sample 1	sample 2	sample 3
gene 1	6	12	9
gene 2	10	20	15
gene 3	2	4	3
…	…	…	…
library size	18	36	27
size factor	1	2	1.5

$$\xrightarrow{\frac{\text{counts}}{\text{size factor}}}$$

	sample 1	sample 2	sample 3
gene 1	6	6	6
gene 2	10	10	10
gene 3	2	2	2
…	…	…	…
library size	18	18	18

Normalisation

- To compare data from different samples, counts need to be normalised to **remove systematic technical effects**.
- **Sequencing depth bias**: the most obvious difference between samples is how deep they are sequenced.
  - Larger libraries have larger counts. These need to be scaled to be comparable.
- **Gene length bias**: longer genes will also produce more reads.
  - Irrelevant when comparing across samples.
  - Important when comparing across genes, but these comparisons are difficult to interpret.

Measures of normalised counts

- **CPM** – counts per million.
  - Sequencing depth normalisation.
- **RPKM** – reads per kilobase per million mapped.
  - Sequencing depth and length normalisation.
- **FPKM** – fragments per kilobase per million mapped.
  - Equivalent to RPKM but for paired end data, where both reads of the same fragment are only counted once.
- **TPM** – transcripts per million
  - First normalise for length and then scale by library size.
  - Proportion of each transcript in the sample. All samples have the same normalised library size.

RPKM

COUNTS

Gene	Gene length	sample 1	sample 2	sample 3
gene 1	**2,000**	10	12	30
gene 2	**4,000**	20	25	60
gene 3	**1,000**	5	8	15
gene 4	**10,000**	0	0	1
…	**…**	…	…	…

→

RPKM

sample 1	sample 2	sample 3
1.43	1.33	1.42
1.43	1.39	1.42
1.43	1.78	1.42
0	0	0.009
…	…	…

FPKM is for paired-end data.

- Takes into account that two reads come from the same molecule.
- Two reads from the same fragment = 1 count.

Adapted from StatQuest (http://statquest.org)

RPKM vs TPM

RPKM

Gene	sample 1	sample 2	sample 3
gene 1	1.43	1.33	1.42
gene 2	1.43	1.39	1.42
gene 3	1.43	1.78	1.42
gene 4	0	0	0.009
Total	4.29	4.5	4.25

TPM

Gene	sample 1	sample 2	sample 3
gene 1	3.33	2.96	3.326
gene 2	3.33	3.09	3.326
gene 3	3.33	3.95	3.326
gene 4	0	0	0.02
Total	10	10	10

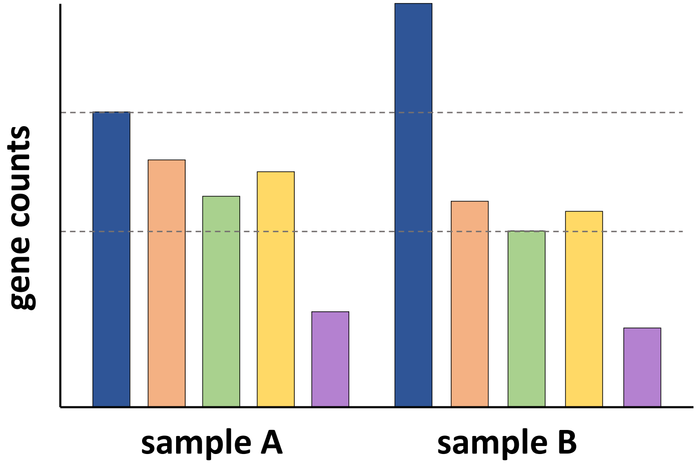
For TPM measurements, the total counts across samples is the same.

- Makes it easier to see the proportion of each gene within a sample.

Adapted from StatQuest (http://statquest.org)

Normalisation

- **Composition biases**: these arise when there is substantial differential expression of a set of transcripts.



Quantification is relative.

Increase in the expression of the blue gene leads to a proportional decrease in all other genes.

Changes in highly expressed genes can have a large impact on total library size.

- FPKM normalisation becomes misleading.

Normalisation

- To account for sequencing depth **and** composition biases, size factors are calculated to normalise systematic differences between samples.
  - The assumption is that the majority of the transcriptome is not differentially expressed.
  - Therefore, any systematic differences must be technical.
- Two popular methods:
  - Median-of-ratios (DESeq2).
  - Trimmed mean of M values (edgeR).

Anders and Huber, *Genome Biology* 11 (2010) doi.org/10.1186/gb-2010-11-10-r106
Robinson and Oshlack, *Genome Biology* 11 (2010) doi.org/10.1186/gb-2010-11-3-r25

Normalisation – DESeq2

counts

	sample 1	**sample 2**	**sample 3**	**pseudo-ref**
gene 1	45	88	66	63.94
gene 2	1268	5072	3804	2903
gene 3	2	4	3	2.88
…	…	…	…	…
gene *n*-2	6	11	8	8.08
gene *n*-1	740	1470	1101	1061.97
gene *n*	39	26	19.5	27.04
library size	1	2	1.5	

size factors

	sample 1	**sample 2**	**sample 3**
gene 1	0.70	1.38	1.03
gene 2	0.44	1.75	1.31
gene 3	0.69	1.39	1.04
…	…	…	…
gene *n*-2	0.74	1.36	0.99
gene *n*-1	0.70	1.38	1.04
gene *n*	1.44	0.96	0.72
median	0.70	1.38	1.04

nonDE gene
DE gene

1. Construct a **pseudo-reference** by taking the geometric mean of each gene across samples.

2. Compute the **fold-change** of each sample over the pseudo-reference.

3. Take the **median** of the fold-changes as the size factors to scale the counts.

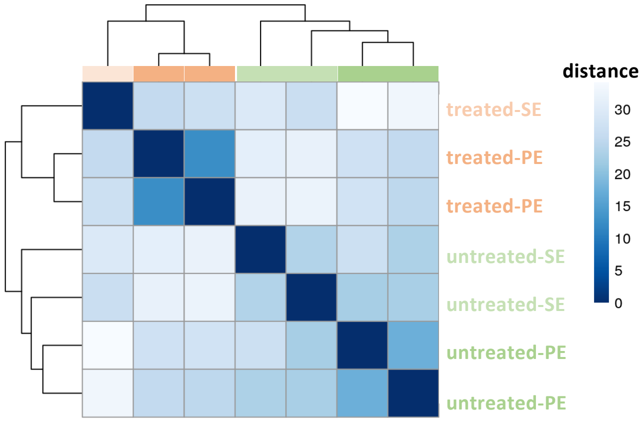
   - The median protects against DE genes.

Anders and Huber, *Genome Biology* 11 (2010) doi.org/10.1186/gb-2010-11-10-r106

Batch effects

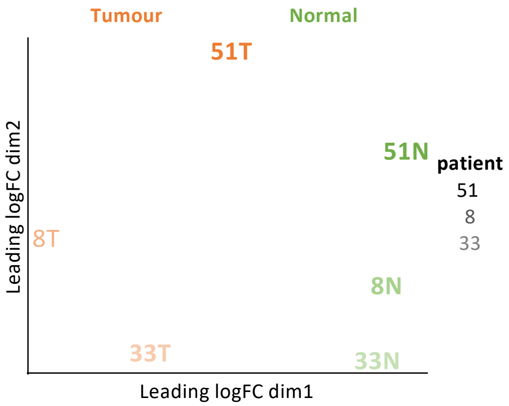
- Once the data has been normalised, it is useful to have an initial quick look at the overall transcriptomes.
  - Make sure samples from different conditions behave as expected.
  - Check if there are still systematic technical effects.

Sample distance heatmap



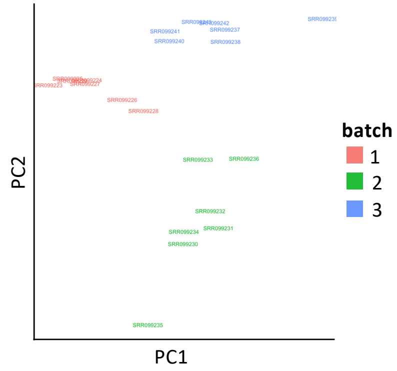
Modified from:
http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html

MDS plot



http://bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf

PCA plot

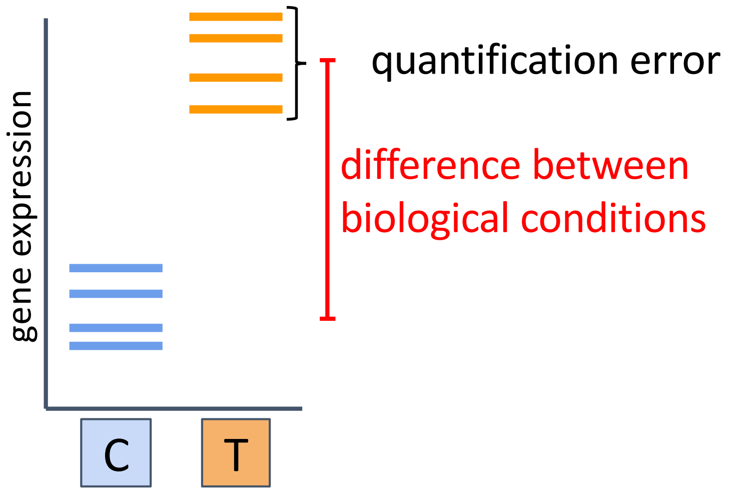


https://pachterlab.github.io/sleuth_walkthroughs/bottomly/analysis.html

Differential expression

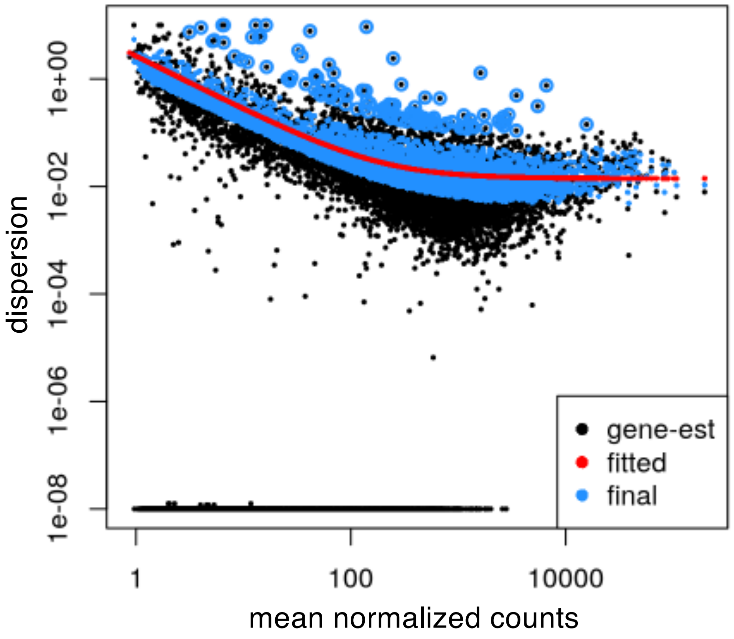
- One of the main applications of RNA-seq is to compare samples under different conditions.
  - Healthy vs diseased.
  - Control vs exposure to treatment/drug/condition.
  - Wild-type vs gene knockout.
  - Changes across development/ageing.

- Differential expression analysis assesses whether the difference in expression levels between groups is larger than quantification error.



Differential expression

- The low number of replicates makes estimating the variation accurately very difficult.
  - This is overcome by pooling information across genes, to make estimates more robust.



There is a strong relationship between the mean expression level and the dispersion.

Genes expressed at lower levels are more variable.

This relationship is not preserved after normalisation.

Always use raw counts for DE testing.

http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html

Differential expression

- There are many programs to perform differential expression analysis.
  - DESeq2
  - edgeR
  - limma-voom

  } extensive documentation

  - sleuth (can use output from kallisto and perform DE analysis at the *transcript* level)

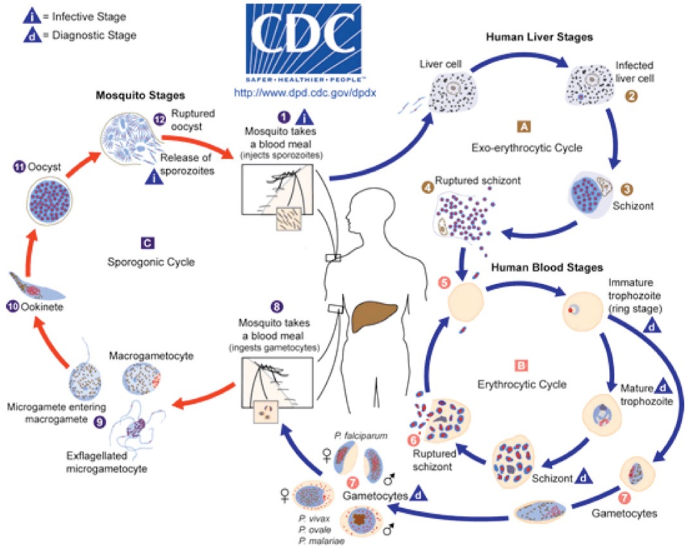
- It is possible to account for complex experimental designs and control for batch effects and confounding factors.

https://www.bioconductor.org/packages/release/bioc/html/DESeq2.html
http://bioconductor.org/packages/release/bioc/html/edgeR.html
http://bioconductor.org/packages/release/bioc/html/limma.html

Interpretation of results

- What to do when you have a list of differentially expressed genes:
  1. If you already have a hypothesis, test it.
  2. Perform gene set enrichment analysis (GSEA, TopGO, InnateDB, Ingenuity Pathway Analysis, etc.)
  3. Work through the list, google, read papers.
  4. Cross-reference with other features/datasets: Pfam domains, chromosomal location, proteome, correlation with ChIP-seq / mutation / GWAS hits…
- Make new hypotheses.
- Go back to the lab.

The exercise



Spence et al., *Nature 498 (*2013)
doi.org/10.1038/nature12231

IS THE TRANSCRIPTOME OF MOSQUITO TRANSMITTED PARASITES DIFFERENT FROM ONE WHICH HAS NOT PASSED THROUGH A MOSQUITO?

- *Plasmodium chabaudi*: a rodent malaria parasite.
  - Exhibits many characteristic associated with the pathogenesis of human infection.
- Serial blood passage (SBP).
  - Direct injection from mouse to mouse.
  - Severe disease.
- Infection with parasite via mosquitos (MT).
  - Lower parasitaemia (presence of parasites in the blood).
  - Mild, chronic disease.