BIMM 143

Genome Informatics II

Lecture 14

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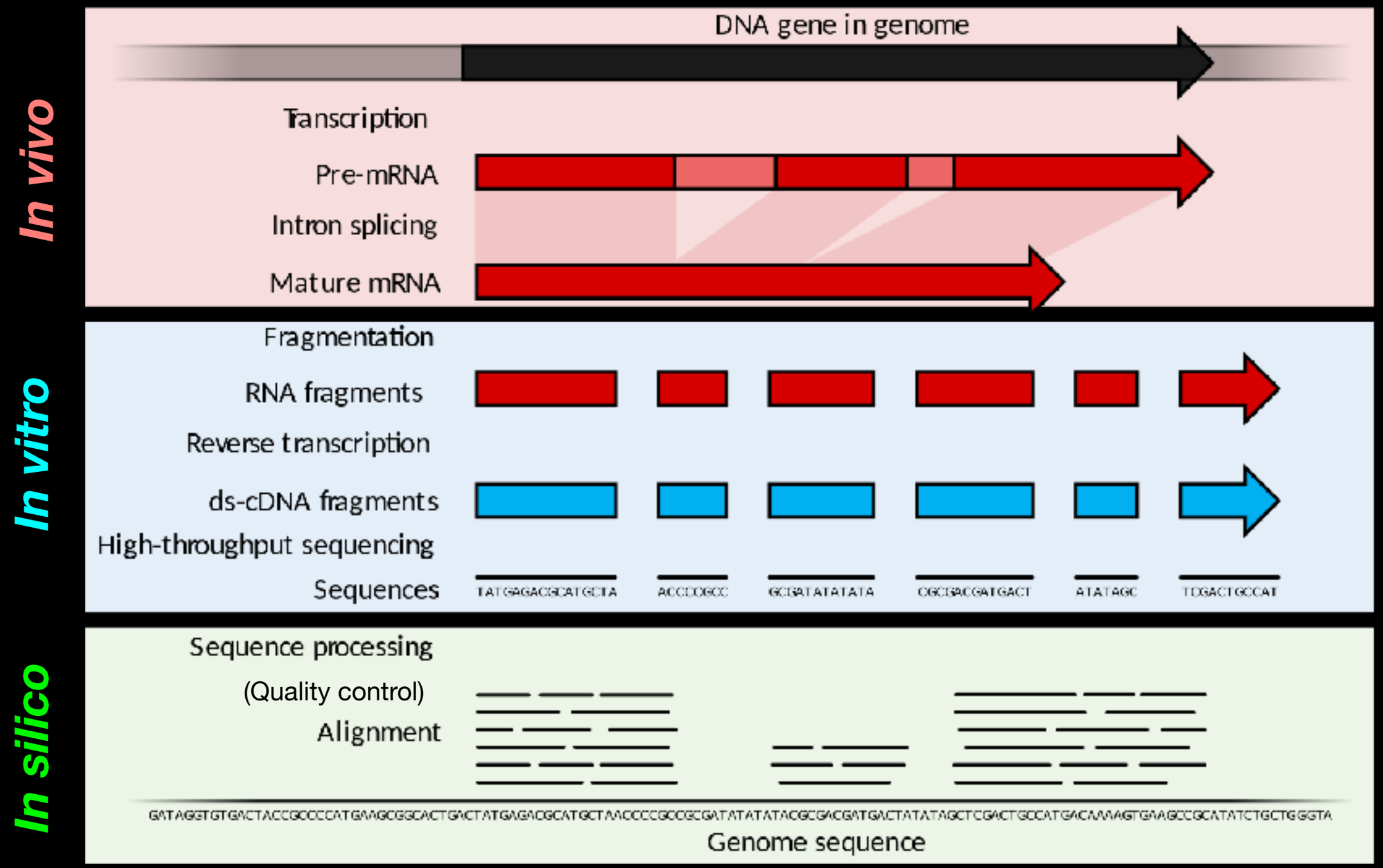
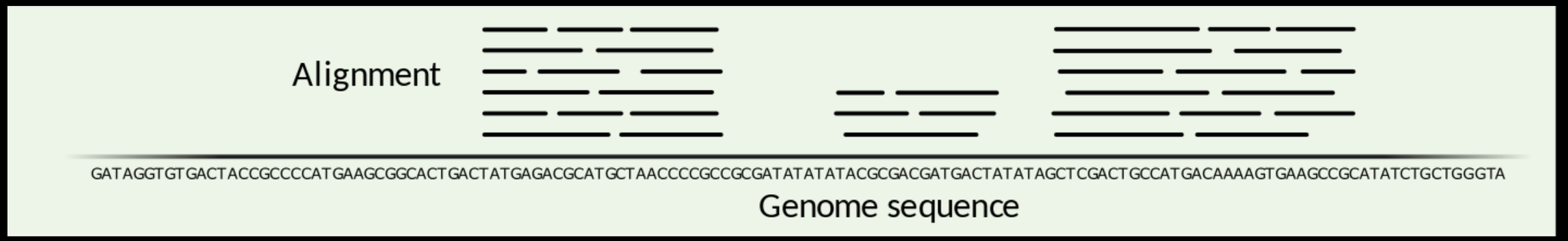
http://thegrantlab.org/bimm143

RNA sequencing overview



Goal: RNA quantification, transcript discovery, variant identification

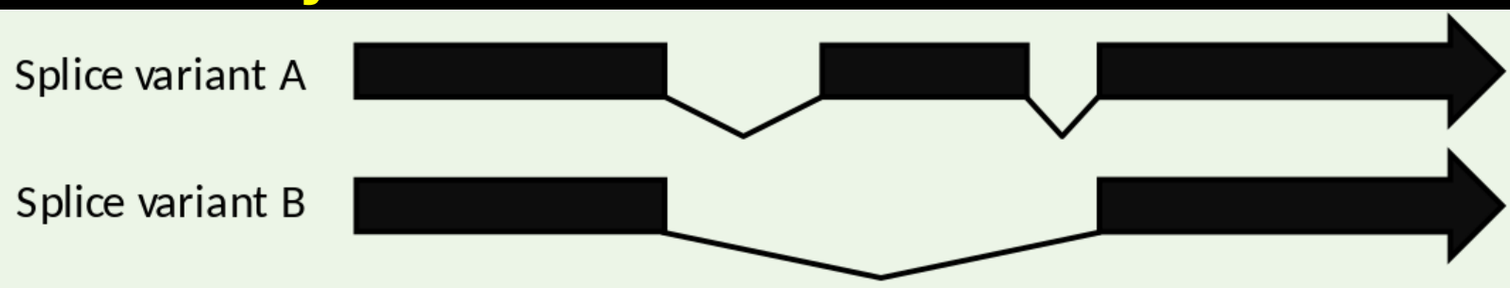
Mapping/Alignment



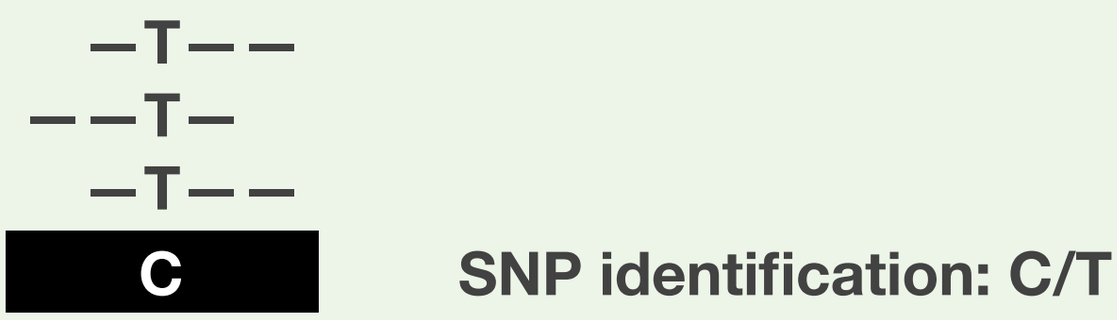
Quantification

Absolute read counts	**15**	**5**	**15**	**(35)**
Normalized read counts	$RPKM = \frac{totalTranscriptReads}{mappedReads(millions)\ x\ transcriptLength(Kb)}$			**(0.7)**

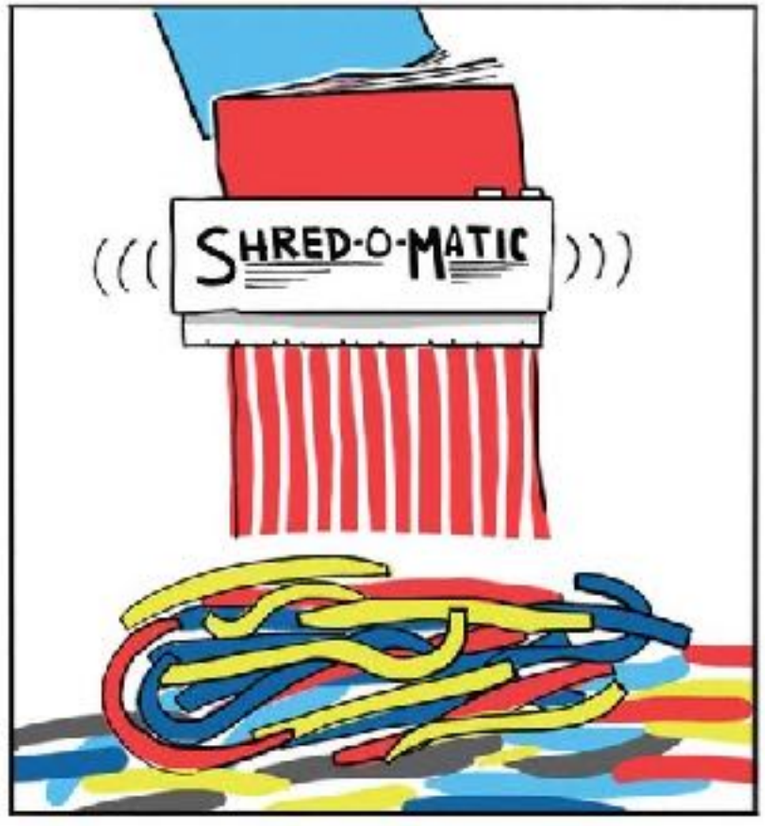
Transcript discovery



Variant discovery

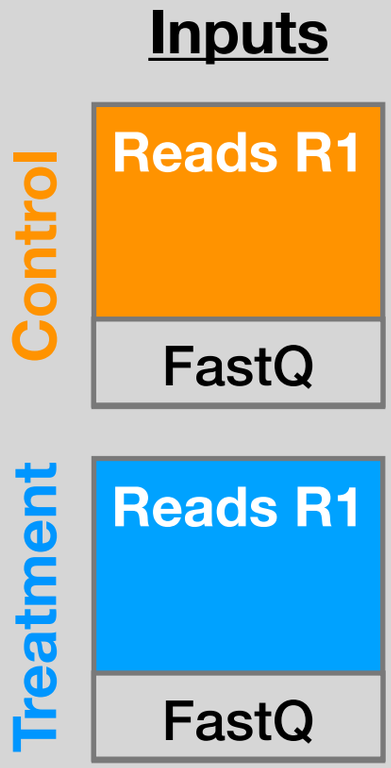


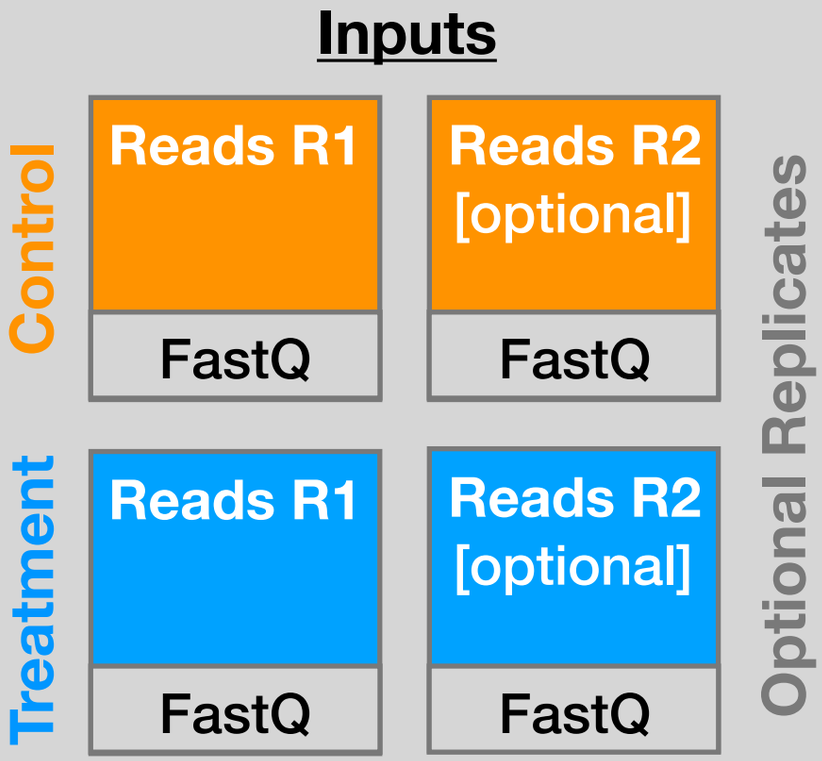

TRANSCRIPTOME
I'LL TAKE ALL OF THEM!

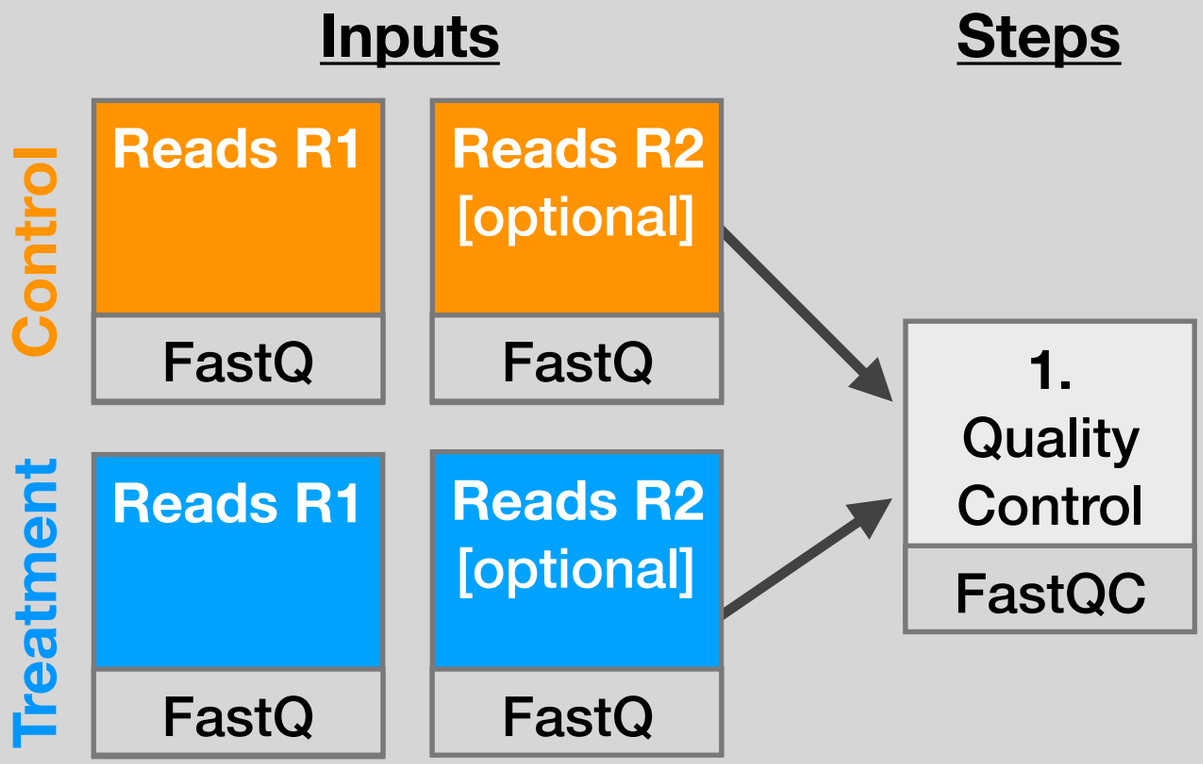

SHRED-O-MATIC

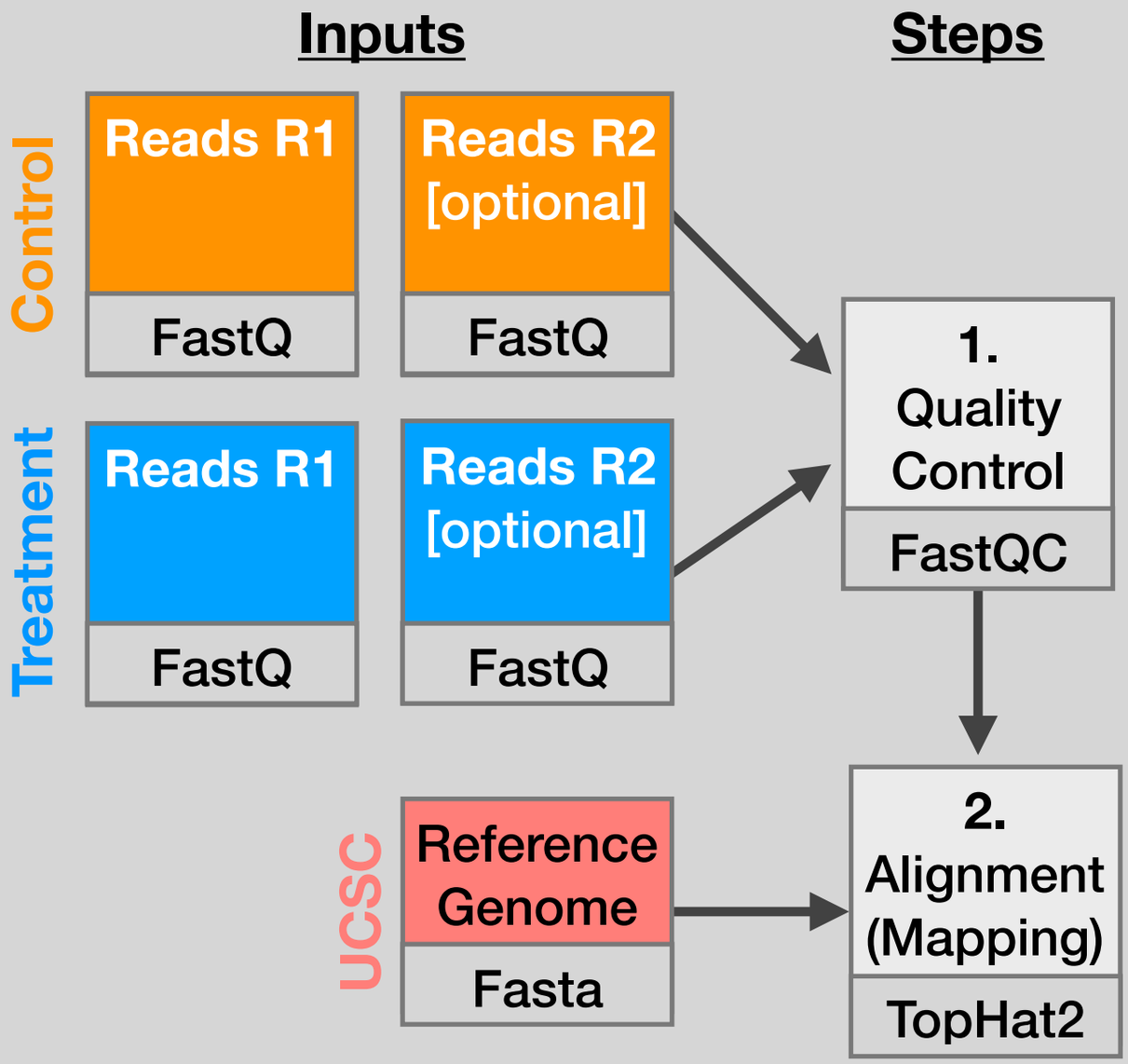

PASS THE TAPE, PLEASE

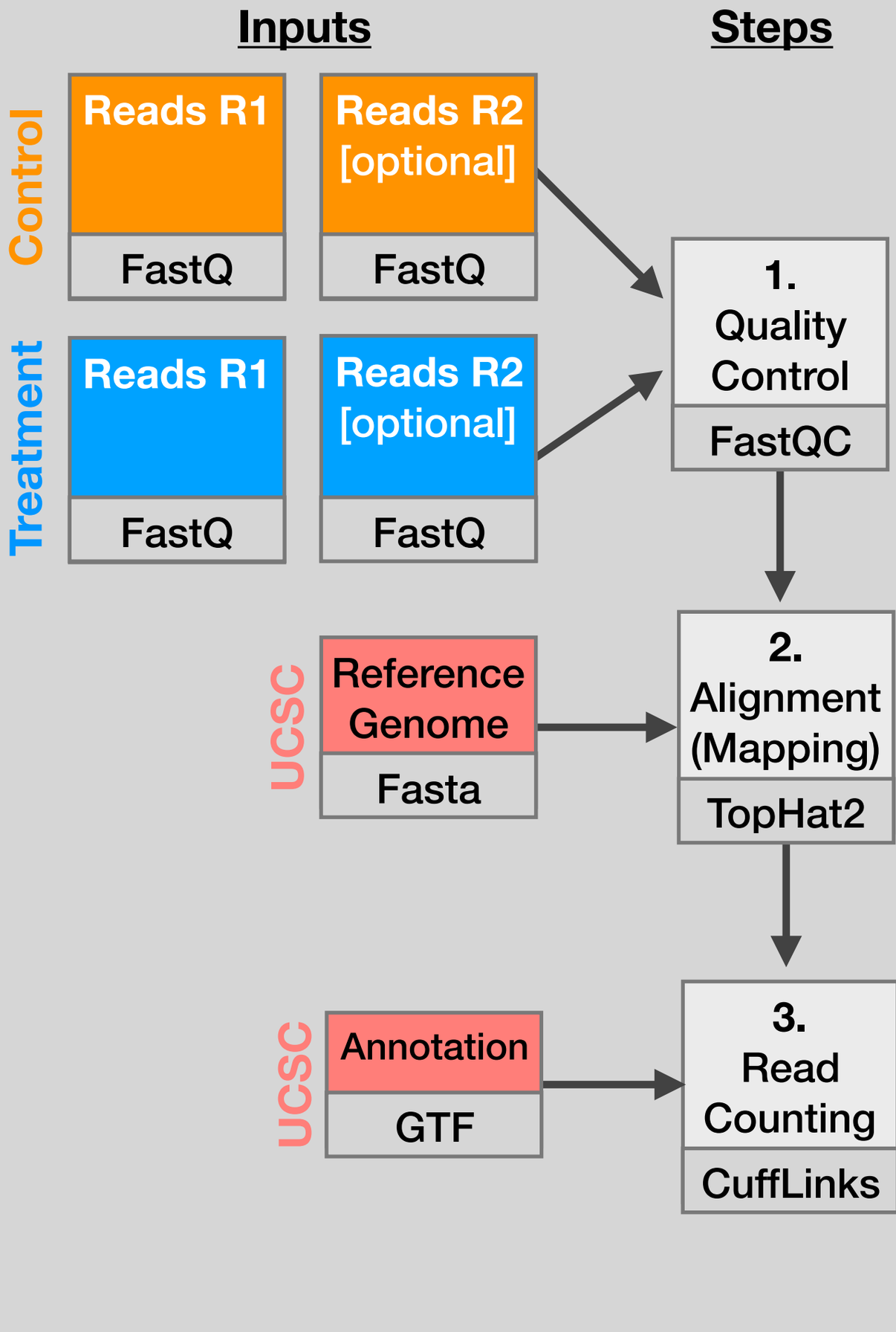

HERE YOU GO!


Inputs
Control
Reads R1
FastQ
Treatment
Reads R1
FastQ


Inputs
Control
Reads R1
Reads R2
[optional]
FastQ
FastQ
Treatment
Reads R1
Reads R2
[optional]
FastQ
FastQ
Optional Replicates


Inputs
Steps
Control
Reads R1
FastQ
Reads R2
[optional]
FastQ
Treatment
Reads R1
FastQ
Reads R2
[optional]
FastQ
1.
Quality
Control
FastQC


Inputs
Steps
Control
Reads R1
FastQ
Reads R2
[optional]
FastQ
Treatment
Reads R1
FastQ
Reads R2
[optional]
FastQ
1.
Quality
Control
FastQC
UCSC
Reference
Genome
Fasta
2.
Alignment
(Mapping)
TopHat2



…Now what?

This is where we stoped last day

Inputs **Steps**

Control

Reads R1 — FastQ

Reads R2 [optional] — FastQ

Treatment

Reads R1 — FastQ

Reads R2 [optional] — FastQ

1. Quality Control — FastQC

UCSC

Reference Genome — Fasta

2. Alignment (Mapping) — TopHat2

UCSC

Annotation — GTF

3. Read Counting — CuffLinks

Count Table — data.frame

Count Table — data.frame

Our missing step

4. Differential expression analysis! — DESeq2

Do it Yourself!

Install DESeq2

Bioconductor Setup Link

```
source("http://bioconductor.org/biocLite.R")
biocLite()

# For this class, you'll also need DESeq2:
biocLite("DESeq2")
```

Background to Today's Data

- Data from: Himes *et al.* "RNA-Seq Transcriptome Profiling Identifies CRISPLD2 as a Glucocorticoid Responsive Gene that Modulates Cytokine Function in Airway Smooth Muscle Cells." PLoS ONE. 2014 Jun 13;9(6):e99625.

- Glucocorticoids inhibit inflammatory processes, often used to treat asthma because of their anti-inflammatory effects on airway smooth muscle (ASM) cells.

- RNA-seq to profile gene expression changes in 4 ASM cell lines treated with dexamethasone (a common synthetic glucocorticoid).

- Used Tophat and Cufflinks and found many differentially expressed genes. Focus on CRISPLD2 that encodes a secreted protein involved in lung development

- SNPs in CRISPLD2 in previous GWAS associated with inhaled corticosteroid resistance and bronchodilator response in asthma patients.

- Confirmed the upregulated CRISPLD2 with qPCR and increased protein expression with Western blotting.

Data pre-processing

- Analyzing RNA-seq data starts with sequencing reads.
- Many different approaches, see references on class website.
- Our workflow (previously done):
  - Reads downloaded from GEO (GSE:GSE52778)
  - Quantify transcript abundance (kallisto).
  - Summarize to gene-level abundance (txImport)
- Our starting point is a count matrix: each cell indicates the number of reads originating from a particular gene (in rows) for each sample (in columns).

Data structure: counts + metadata

countData

gene	ctrl_1	ctrl_2	exp_1	exp_1
geneA	10	11	56	45
geneB	0	0	128	54
geneC	42	41	59	41
geneD	103	122	1	23
geneE	10	23	14	56
geneF	0	1	2	0
…	…	…	…	…

countData is the count matrix
(number of reads coming from
each gene for each sample)

colData

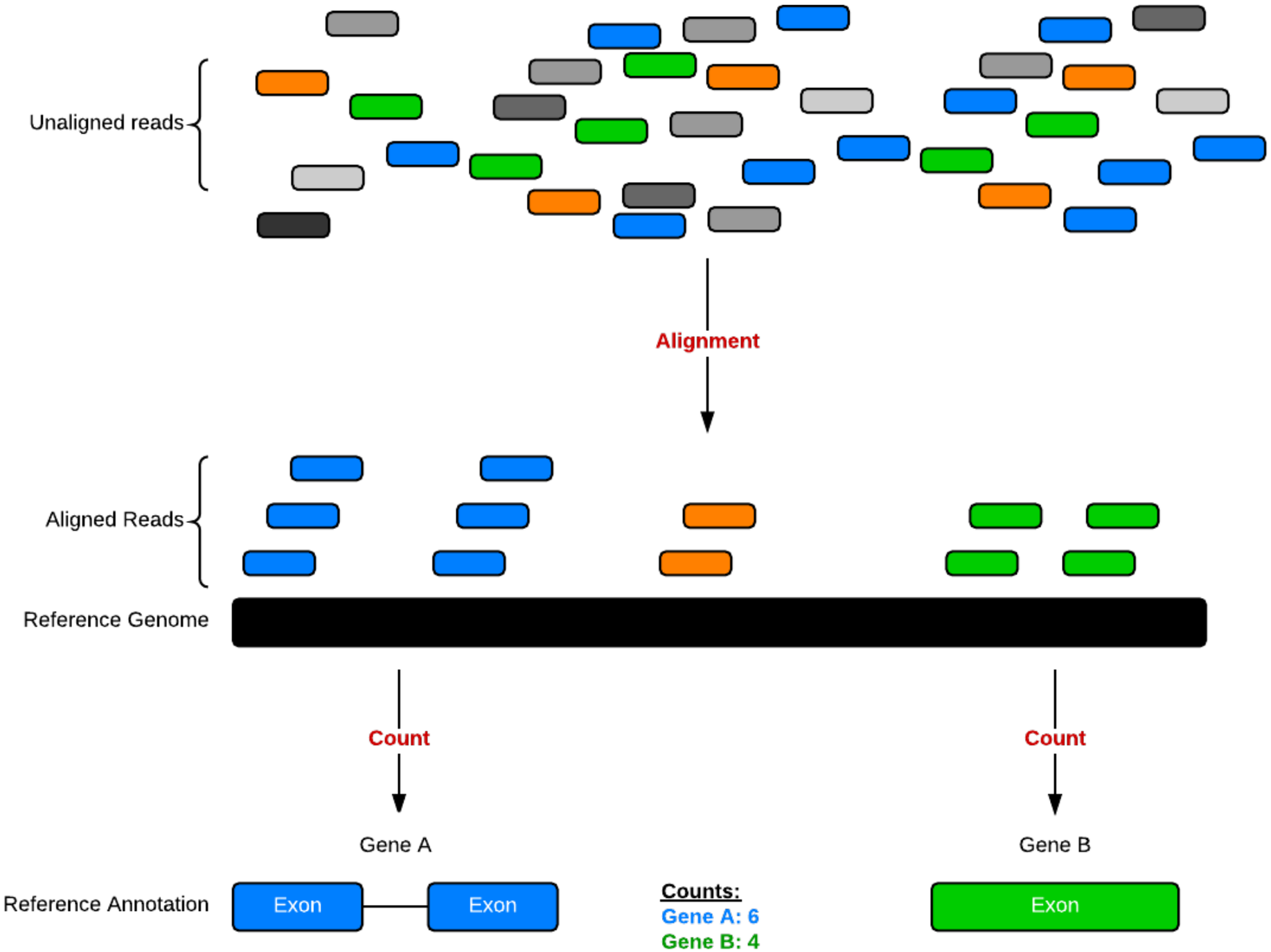
id	treatment	sex	...
ctrl_1	control	male	...
ctrl_2	control	female	...
exp_1	treatment	male	...
exp_2	treatment	female	...

Sample names:
ctrl_1, ctrl_2, exp_1, exp_2

colData describes metadata
about the *columns* of countData

First column of **colData** must match column names of **countData** (-1st)

Counting is (relatively) easy:



Do it Yourself!

Hands-on time!

https://bioboot.github.io/bimm143_W18/lectures/#14

Side-note:

Count Normalization

- Normalization is required to make comparisons in gene expression
    - Between 2+ genes in one sample
    - Between genes in 2+ samples

- Genes will have more reads mapped in a sample with high coverage than one with low coverage
    - 2x depth ≈ 2x expression

- Longer genes will have more reads mapped than shorter genes
    - 2x length ≈ 2x more reads

Normalization: RPKM, FPKM & TPM

Side-note:

- **N.B.** Some tools for differential expression analysis such as edgeR and **DESeq2** want raw read counts - i.e. non normalized input!

- However, often for your manuscripts and reports you will want to report normalized counts

- RPKM, FPKM and TPM all aim to normalize for sequencing depth and gene length. For the former:
    - Count up the total reads in a sample and divide that number by 1,000,000 – this is our “per million” scaling.
    - Divide the read counts by the “per million” scaling factor. This normalizes for sequencing depth, giving you reads per million (RPM)
    - Divide the RPM values by the length of the gene, in kilobases. This gives you RPKM.

Side-note:

- FPKM was made for paired-end RNA-seq
- With paired-end RNA-seq, two reads can correspond to a single fragment
- The only difference between RPKM and FPKM is that FPKM takes into account that two reads can map to one fragment (and so it doesn’t count this fragment twice).

Side-note:

- **TPM** is very similar to RPKM and FPKM. The only difference is the order of operations:
  - First divide the read counts by the length of each gene in kilobases. This gives you reads per kilobase (RPK).
  - Count up all the RPK values in a sample and divide this number by 1,000,000. This is your “per million” scaling factor.
  - Divide the RPK values by the “per million” scaling factor. This gives you TPM.
- Note, the only difference is that you normalize for gene length first, and then normalize for sequencing depth second.

Side-note:

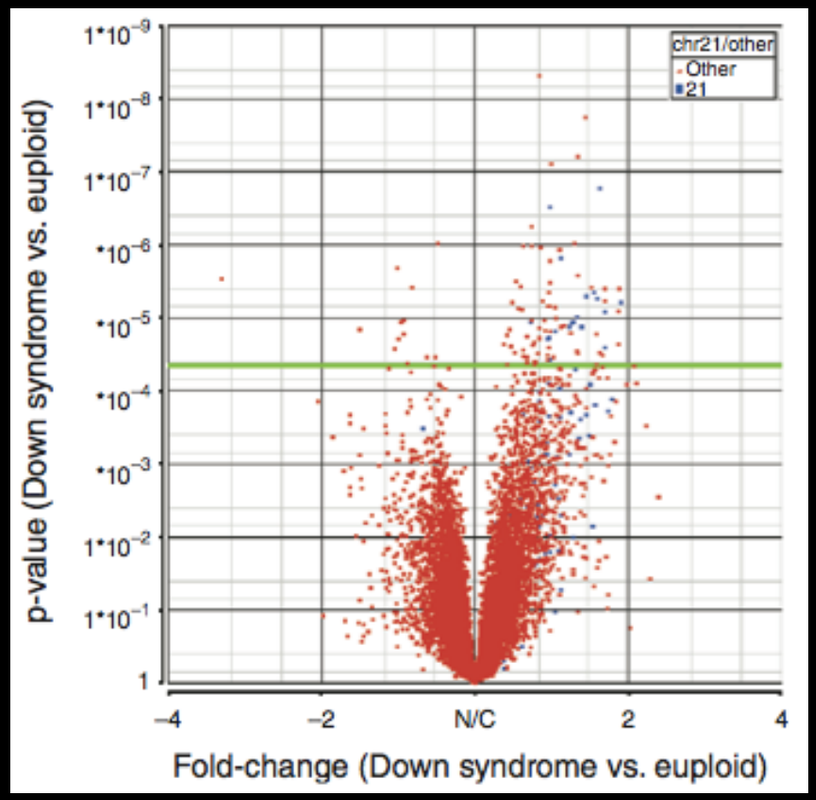
- When you use TPM, the sum of all TPMs in each sample are the same.
- This makes it easier to compare the proportion of reads that mapped to a gene in each sample.
- In contrast, with RPKM and FPKM, the sum of the normalized reads in each sample may be different, and this makes it harder to compare samples directly.

Fold change (log ratios)

- To a statistician fold change is sometimes considered meaningless. Fold change can be large (e.g. >>two-fold up- or down-regulation) without being statistically significant (e.g. based on probability values from a t-test or ANOVA).

- To a biologist fold change is almost always considered important for two reasons. First, a very small but statistically significant fold change might not be relevant to a cell's function. Second, it is of interest to know which genes are most dramatically regulated, as these are often thought to reflect changes in biologically meaningful transcripts and/or pathways.

Volcano plot: significantly regulated genes vs. fold change



- A volcano plot shows fold change (x-axis) versus p value from ANOVA (y-axis). Each point is the expression level of a transcript. Points high up on the y-axis (above the pale green horizontal line) are significantly regulated.

Recent developments in RNA-Seq

- **Long read sequences:**
  - ➡ PacBio and Oxford Nanopore [Recent Paper]

- **Single-cell RNA-Seq**: [Review article]
  - ➡ Observe heterogeneity of cell populations
  - ➡ Detect sub-population

- **Alignment-free quantification**:
  - ➡ Kallisto [Software link]
  - ➡ Salmon [Software link, Blog post]

Public RNA-Seq data sources

- **Gene Expression Omnibus** (GEO):
  - ➡ http://www.ncbi.nlm.nih.gov/geo/
  - ➡ Both microarray and sequencing data
- **Sequence Read Archive** (SRA):
  - ➡ http://www.ncbi.nlm.nih.gov/sra
  - ➡ All sequencing data (not necessarily RNA-Seq)
- **ArrayExpress**:
  - ➡ https://www.ebi.ac.uk/arrayexpress/
  - ➡ European version of GEO
- All of these have links between them

[Muddy Point Feedback Link]