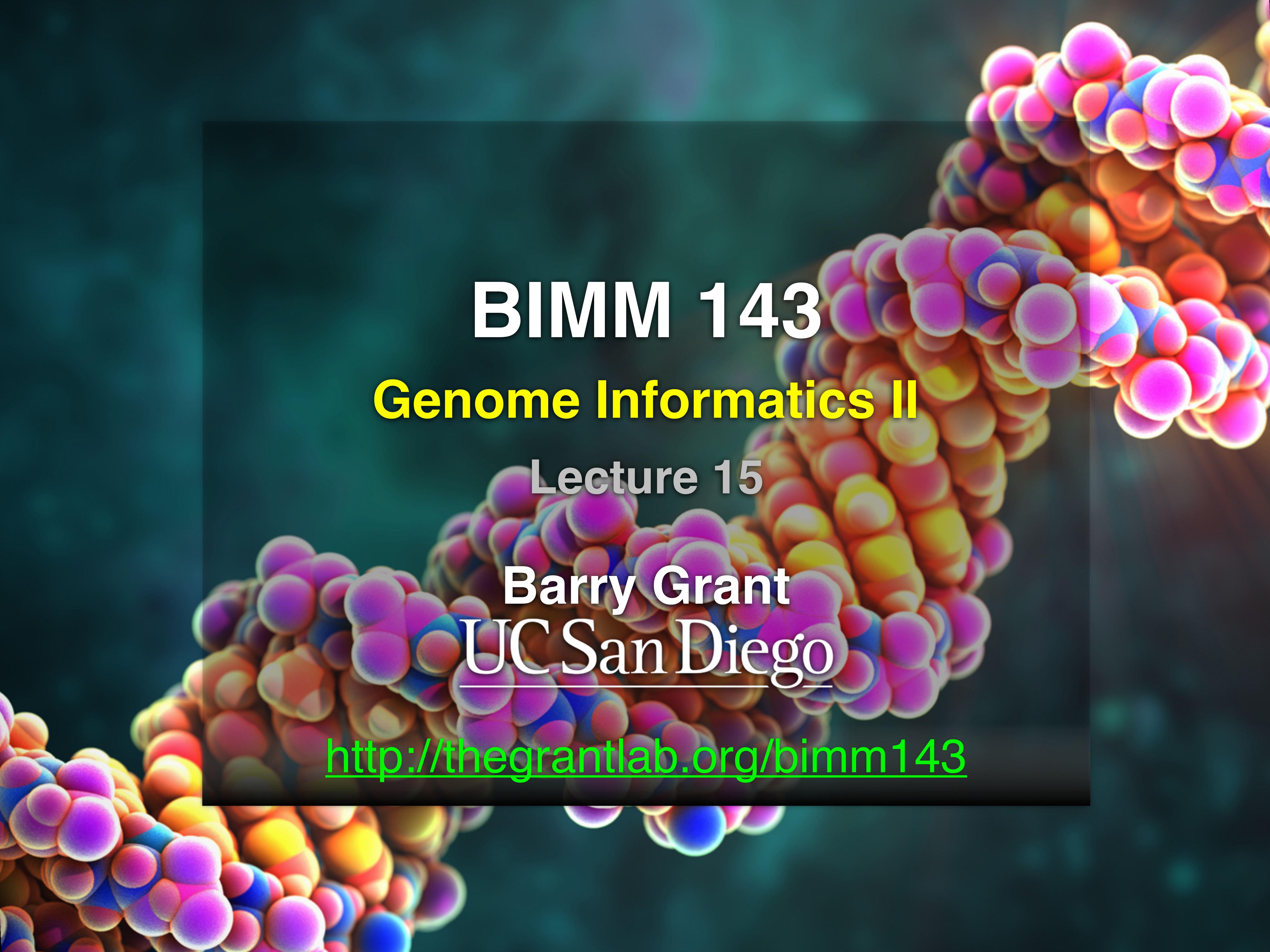


BIMM 143

Genome Informatics II

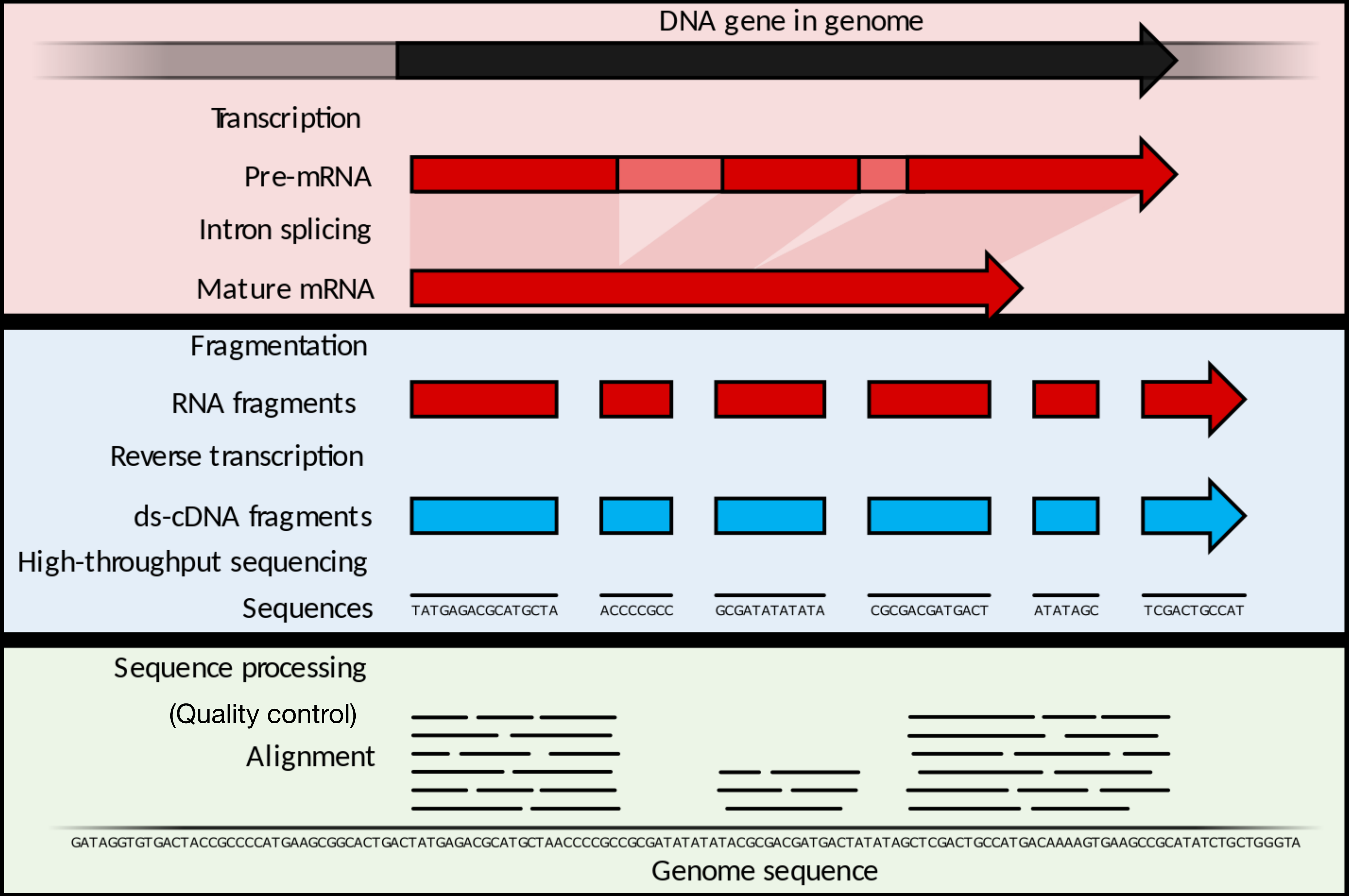
Lecture 15

Barry Grant

UC San Diego

http://thegrantlab.org/bimm143

RNA sequencing overview



In vivo

DNA gene in genome

Transcription

Pre-mRNA

Intron splicing

Mature mRNA

In vitro

Fragmentation

RNA fragments

Reverse transcription

ds-cDNA fragments

High-throughput sequencing

Sequences

TATGAGACGCATGCTA ACCCCGCC GCGATATATATA CGCGACGATGACT ATATAGC TCGACTGCCAT

In silico

Sequence processing

(Quality control)

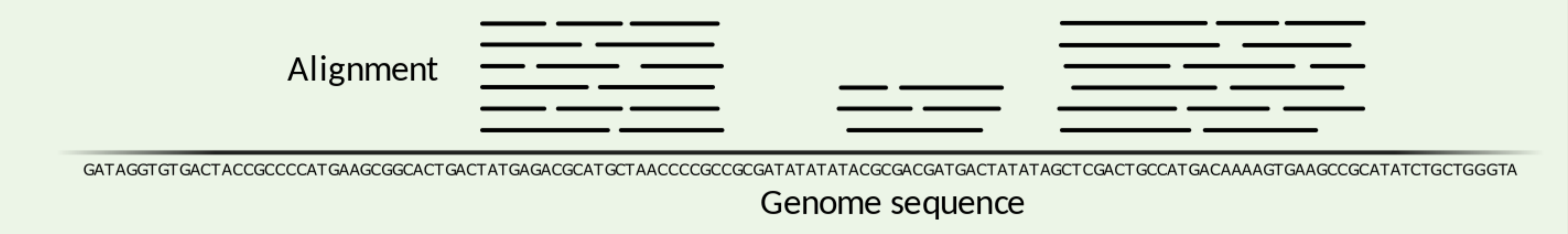
Alignment

GATAGGTGTGACTACCGCCCCATGAAGCGGCACTGACTATGAGACGCATGCTAACCCCGCCGCGATATATATACGCGACGATGACTATATAGCTCGACTGCCATGACAAAAGTGAAGCCGCATATCTGCTGGGTA

Genome sequence

Goal: RNA quantification, transcript discovery, variant identification

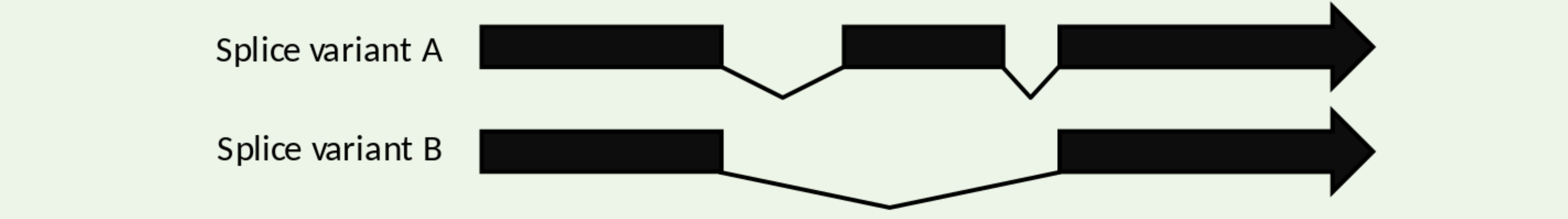
Mapping/Alignment


Alignment
GATAGGTGTGACTACCGCCCCATGAAGCGGCACTGACTATGAGACGCATGCTAACCCCGCCGCGATATATATACGCGACGATGACTATATAGCTCGACTGCCATGACAAAAGTGAAGCCGCATATCTGCTGGGTA
Genome sequence

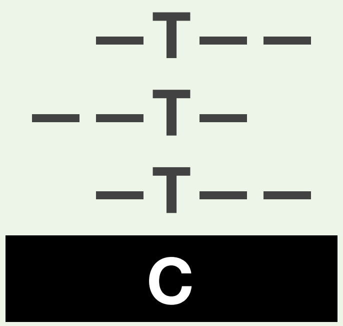
Quantification

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

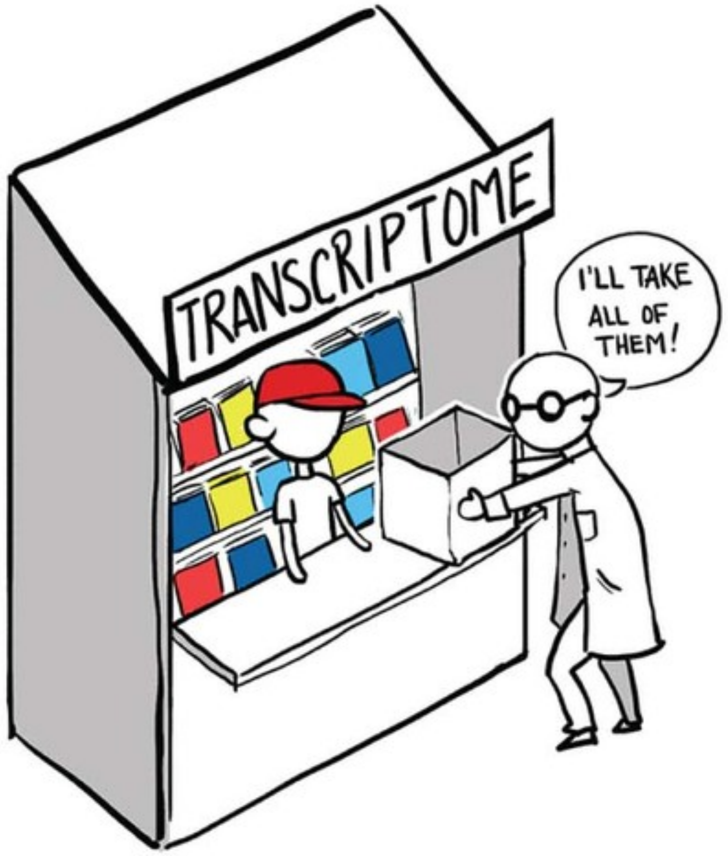
Transcript discovery

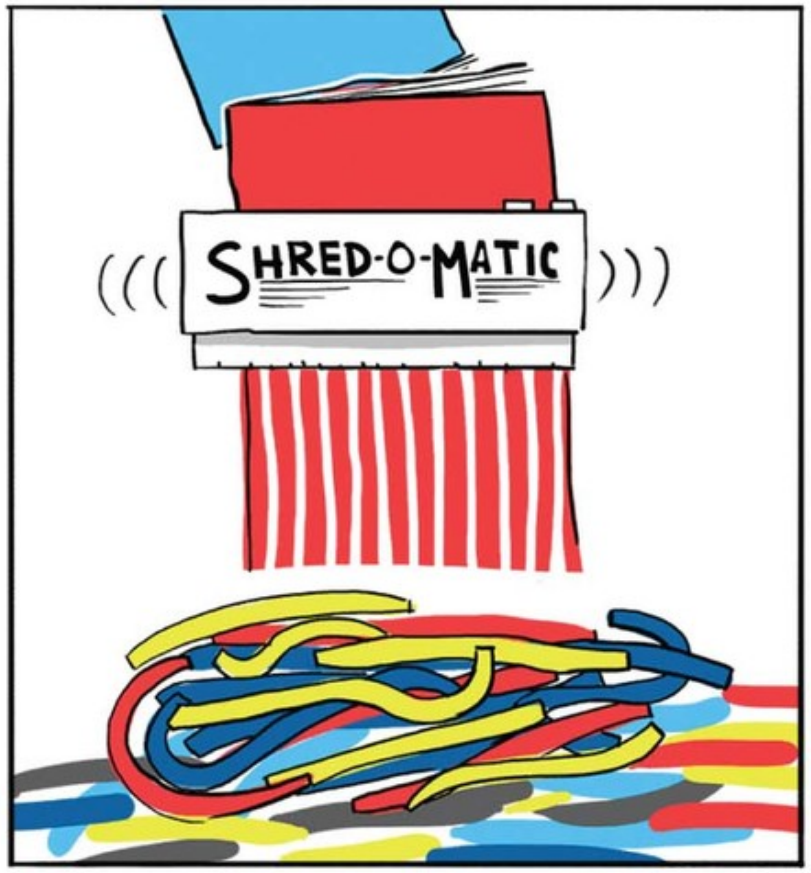

Splice variant A
Splice variant B

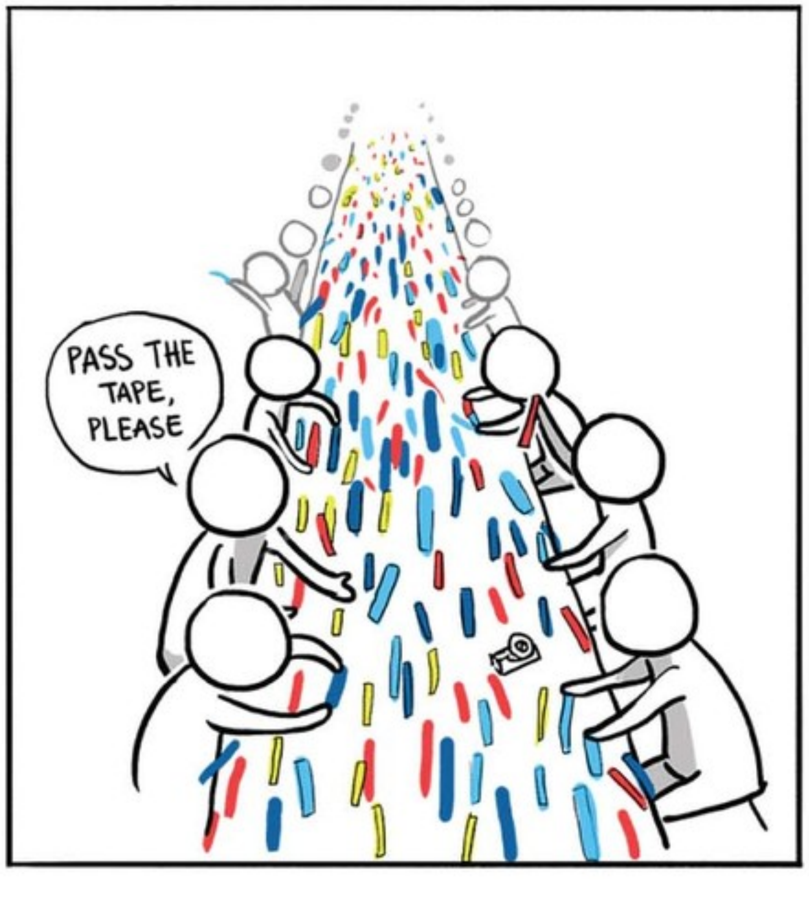
Variant discovery

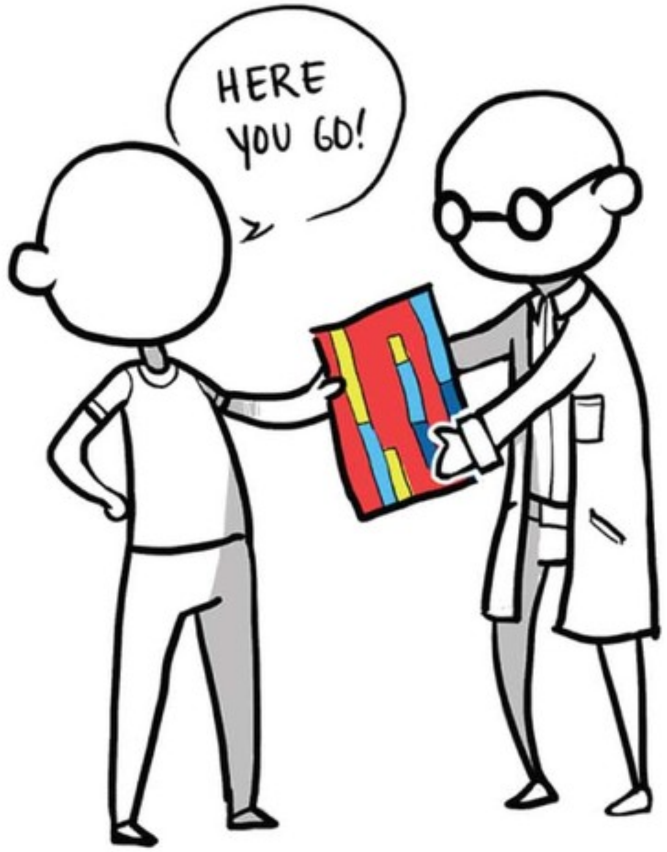


SNP identification: C/T


TRANSCRIPTOME
I'LL TAKE ALL OF THEM!


SHRED-O-MATIC


PASS THE TAPE, PLEASE


HERE YOU GO!

Inputs

Control

Reads R1

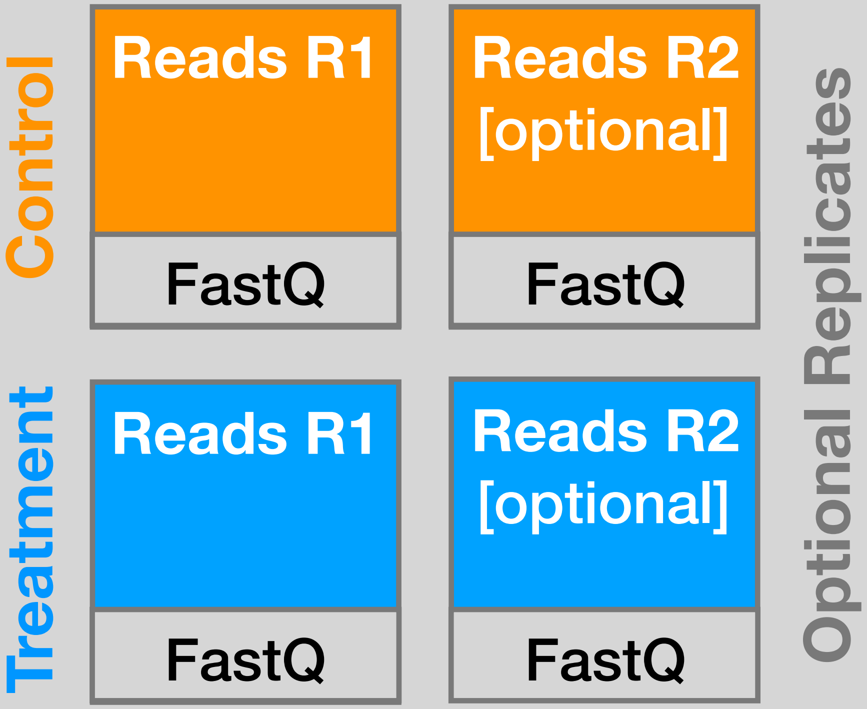
FastQ

Treatment

Reads R1

FastQ

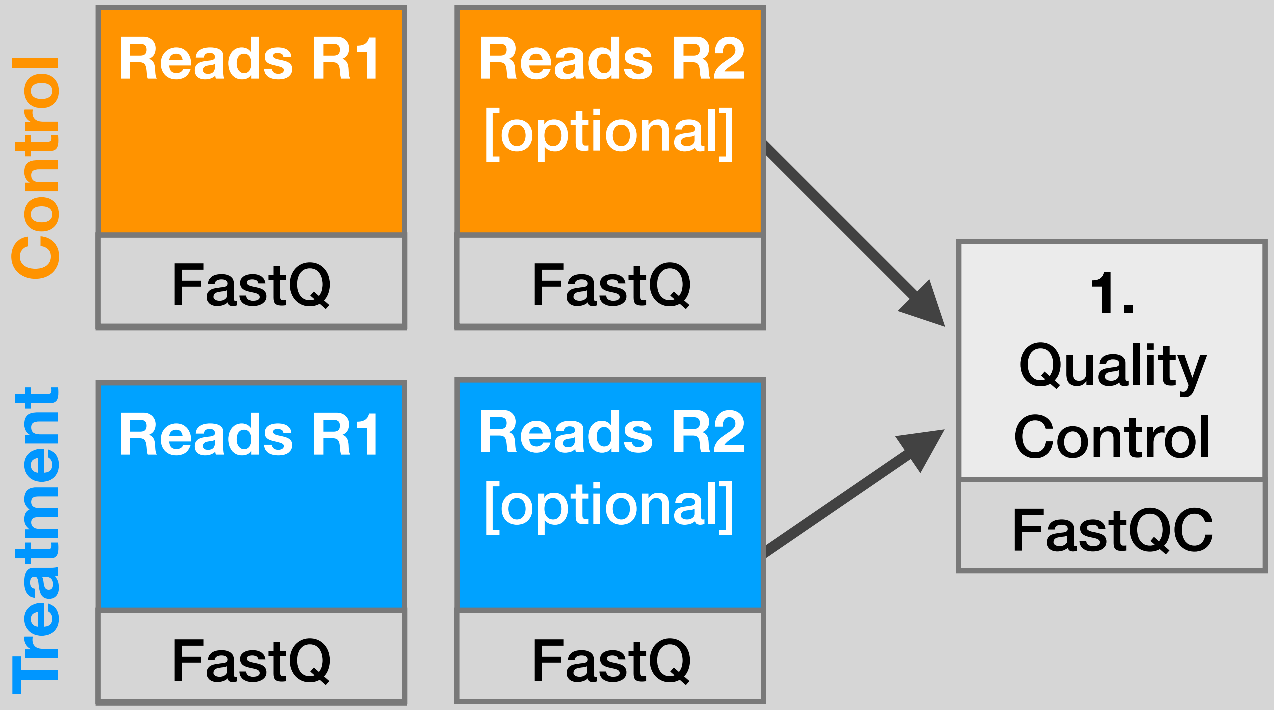
Inputs



Control	Reads R1 FastQ	Reads R2 [optional] FastQ
Treatment	Reads R1 FastQ	Reads R2 [optional] FastQ

Optional Replicates

Inputs **Steps**



Control

Reads R1

FastQ

Reads R2 [optional]

FastQ

Treatment

Reads R1

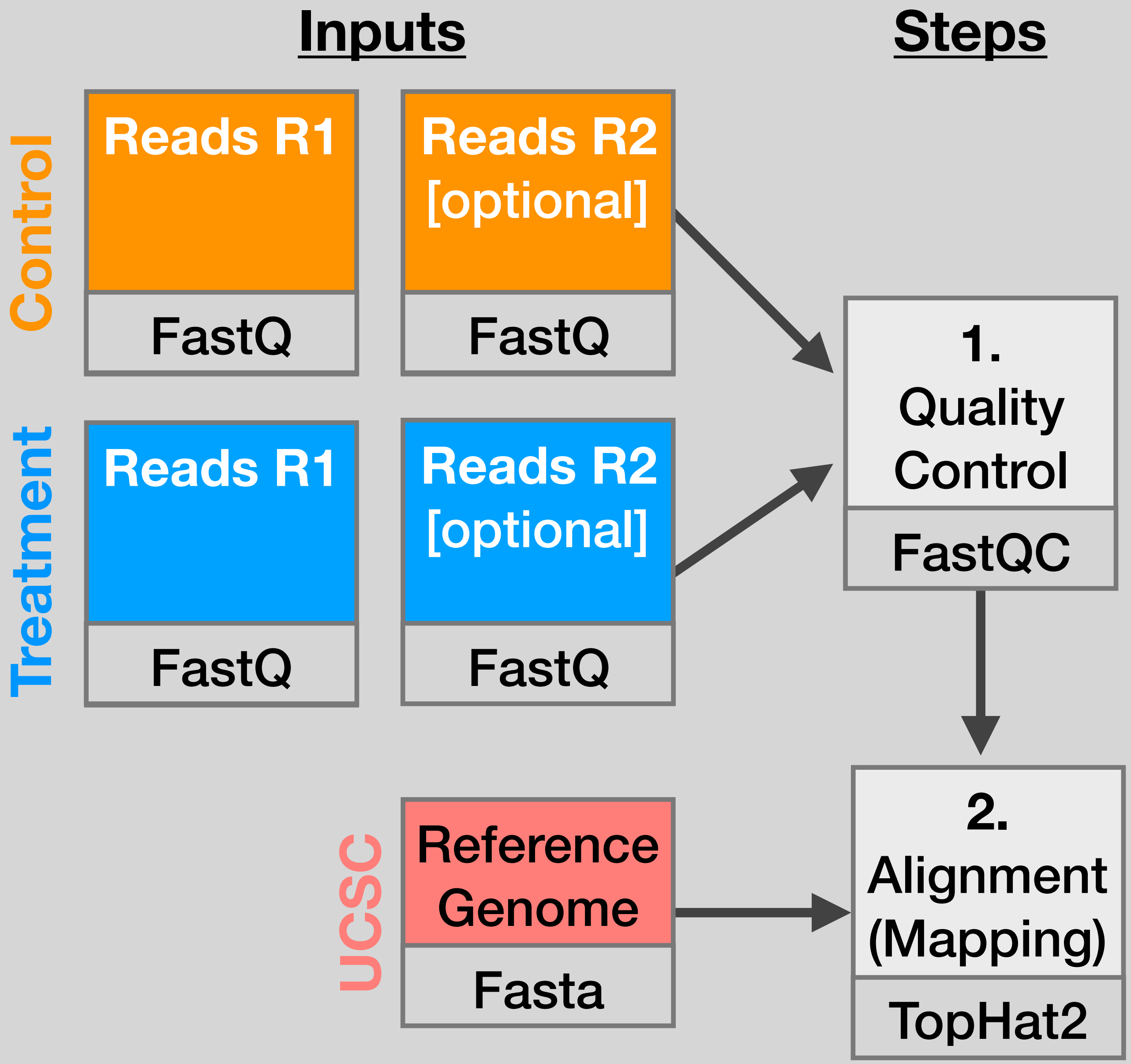
FastQ

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

UCSC

Reference Genome

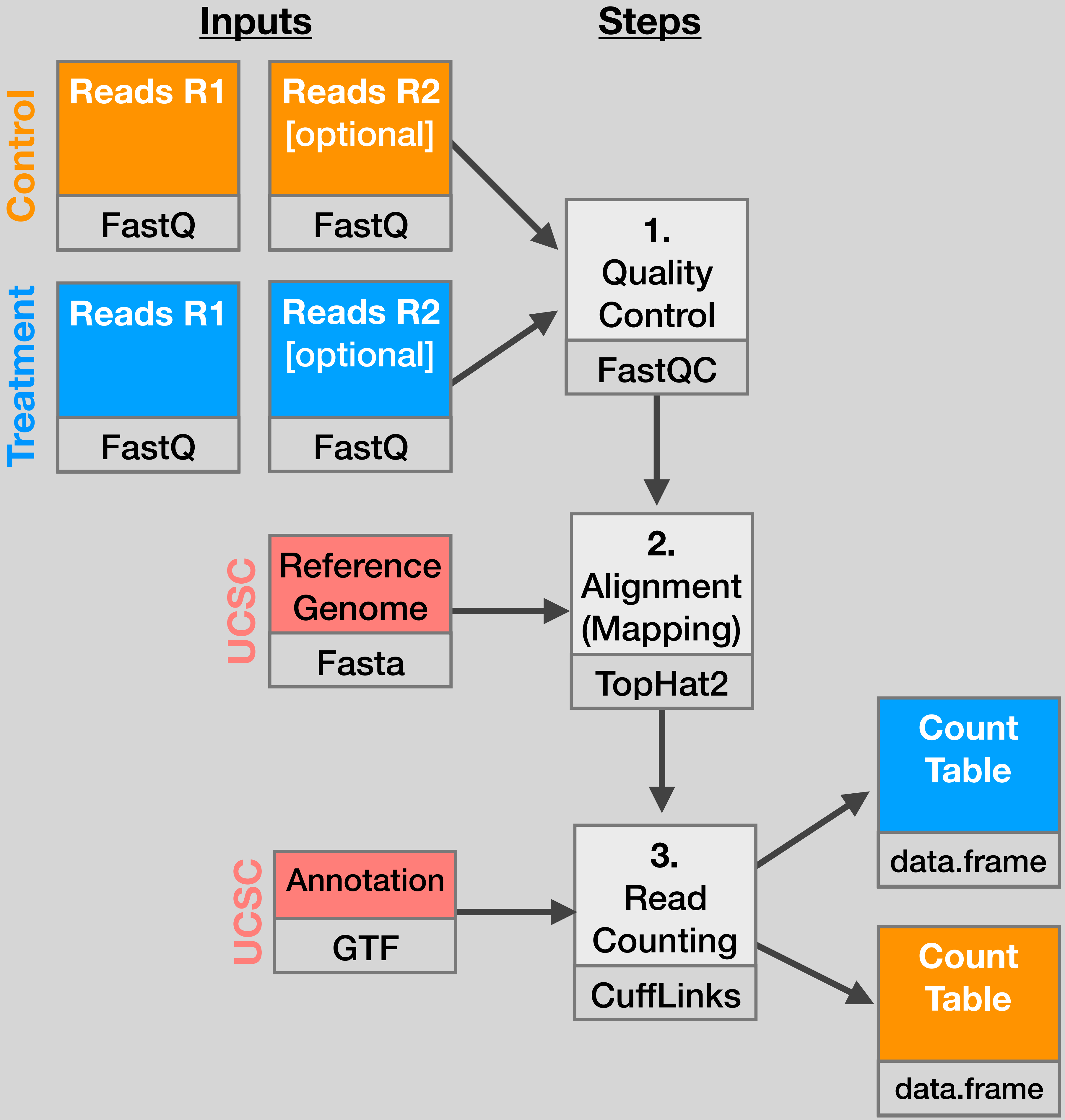
Fasta

1.
Quality Control

FastQC

2.
Alignment (Mapping)

TopHat2



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

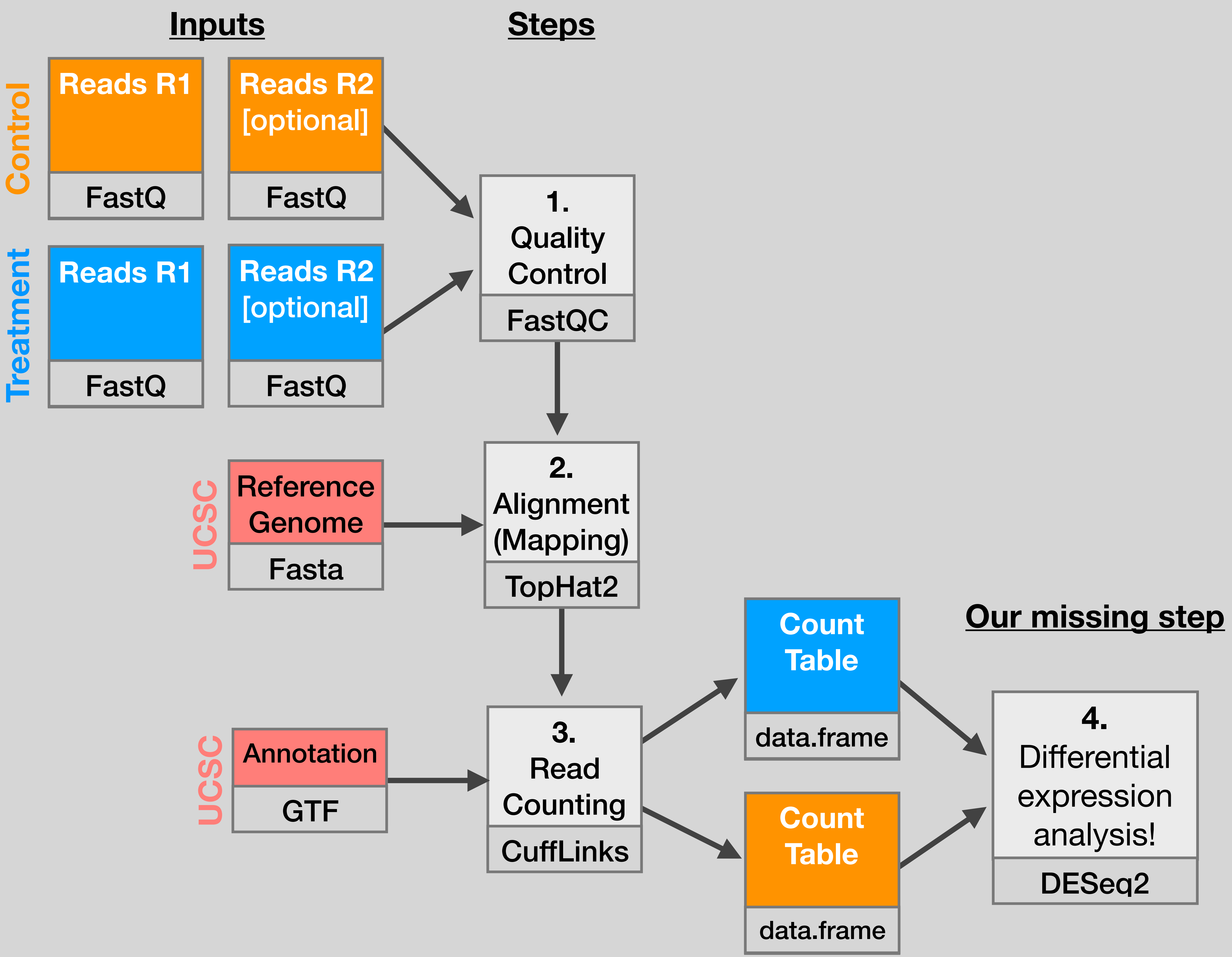
3. Read Counting
CuffLinks

Count Table
data.frame

Count Table
data.frame

…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

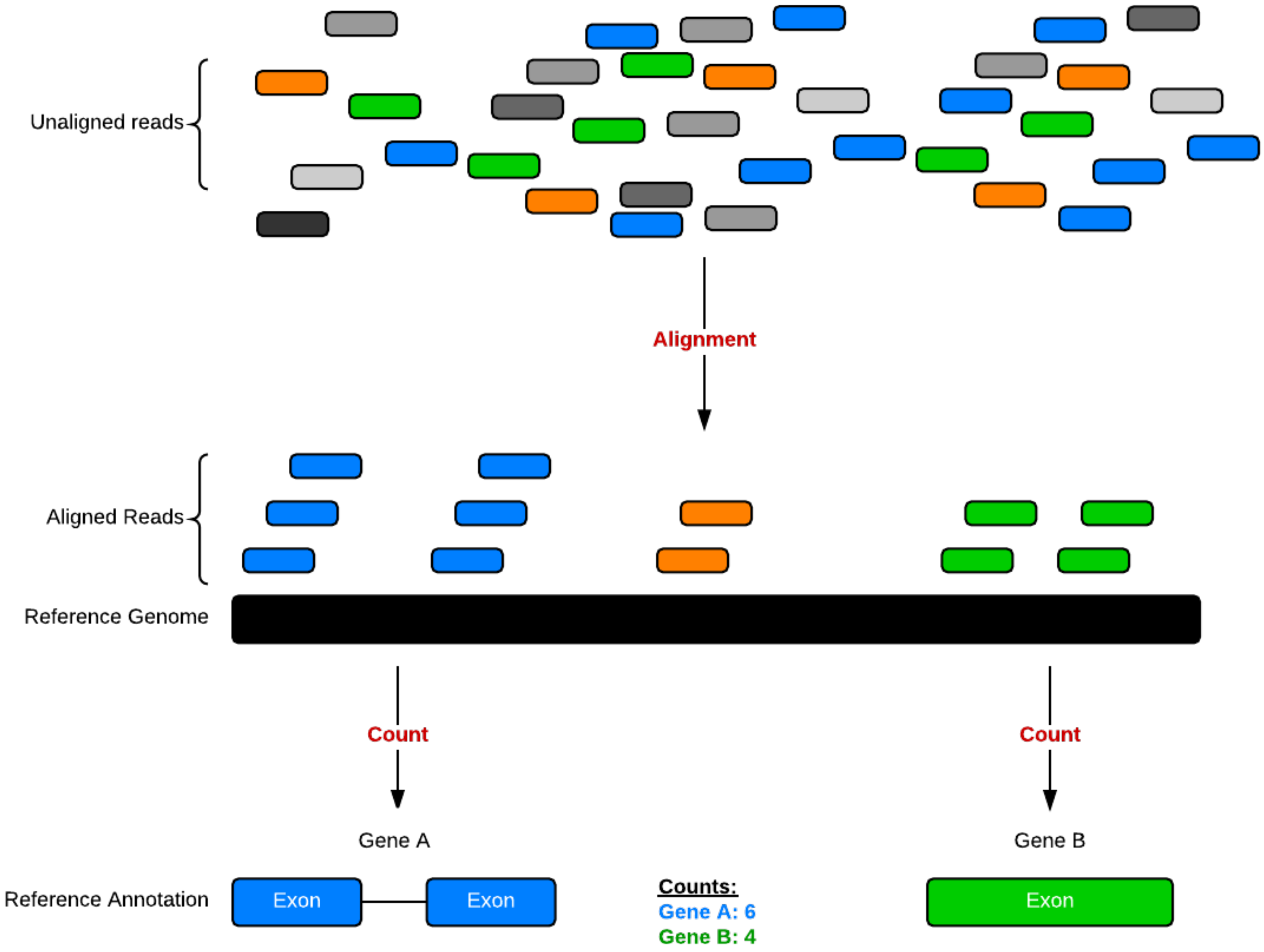
id	**treatment**	**sex**	**...**
ctrl_1	control	male	...
ctrl_2	control	female	...
exp_1	treatment	male	...
exp_2	treatment	female	...

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:


Unaligned reads
Alignment
Aligned Reads
Reference Genome
Count
Count
Gene A
Gene B
Reference Annotation
Exon
Exon
Counts:
Gene A: 6
Gene B: 4
Exon

Do it Yourself!

Hands-on time!

https://bioboot.github.io/bimm143_F18/lectures/#15

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

Side-note:

Normalization: RPKM, FPKM & TPM

- **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:

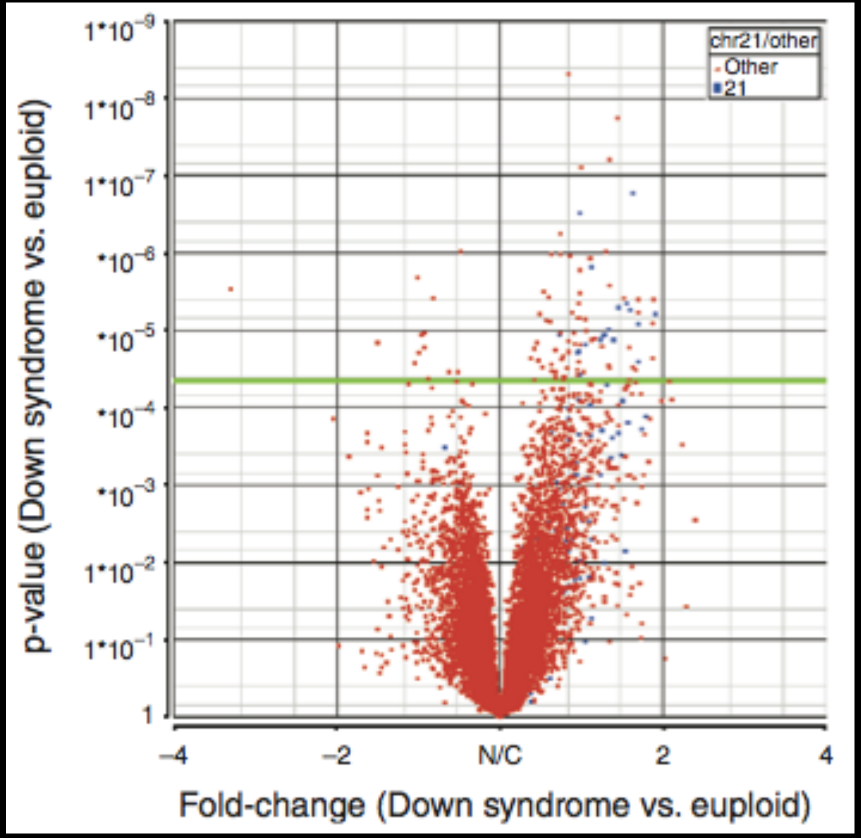
- When you use TPM, the sum of all TPMs in each sample are the same.
- This makes it easier to compare the proportion of reads that mapped to a gene in each sample.
- In contrast, with RPKM and FPKM, the sum of the normalized reads in each sample may be different, and this makes it harder to compare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

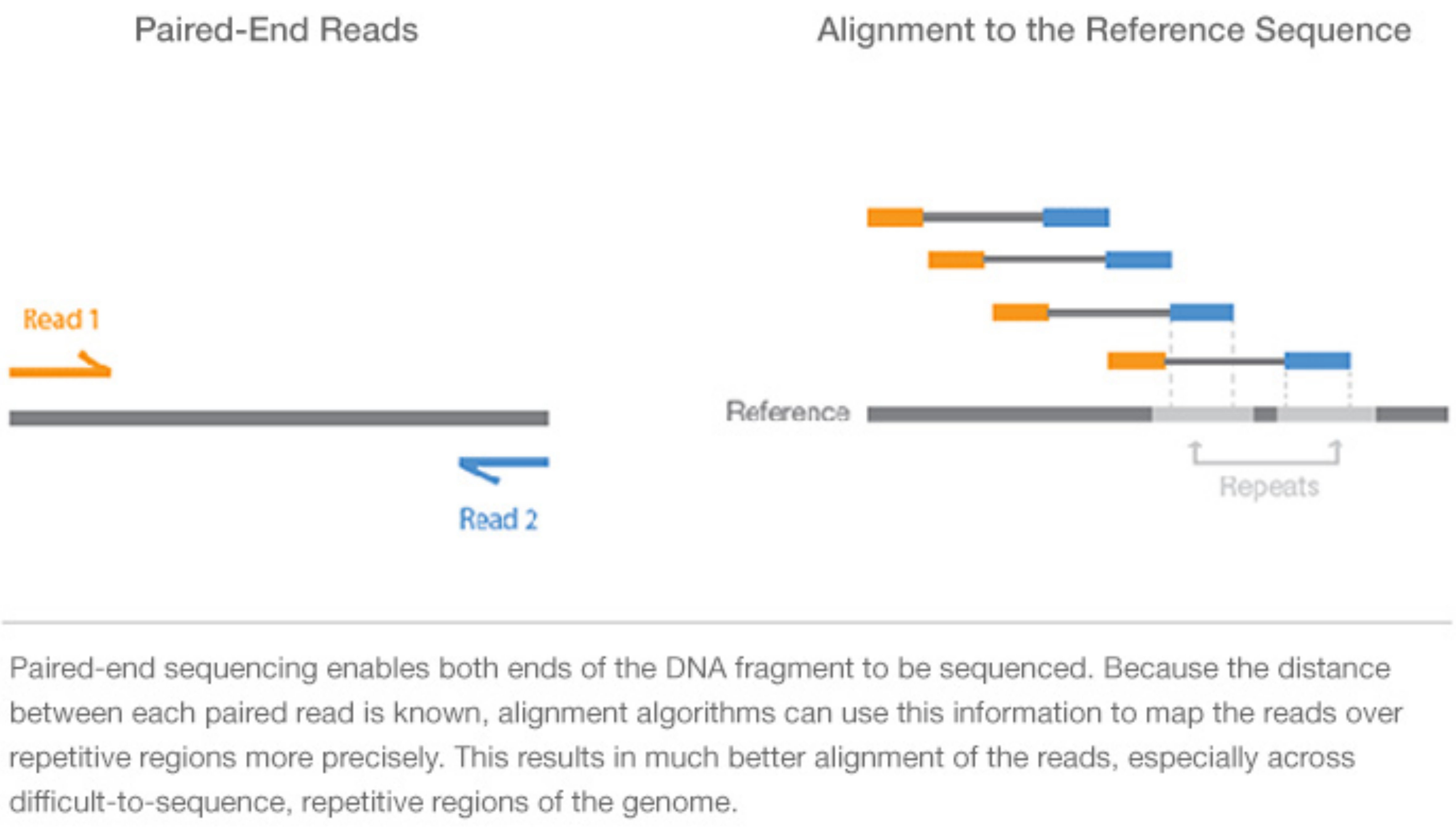
- **Single-cell RNA-Seq**: [Review article]
  - ➡ Observe heterogeneity of cell populations
  - ➡ Detect sub-population

- **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]


Paired-End Reads
Alignment to the Reference Sequence
Read 1
Read 2
Reference
Repeats

Paired-end sequencing enables both ends of the DNA fragment to be sequenced. Because the distance between each paired read is known, alignment algorithms can use this information to map the reads over repetitive regions more precisely. This results in much better alignment of the reads, especially across difficult-to-sequence, repetitive regions of the genome.

Taken From: https://www.illumina.com/science/technology/next-generation-sequencing/paired-end-vs-single-read-sequencing.html