# UBC Bioinformatics Workshop

## Topic 6: RNAseq and analysis of differential gene expression

# Lecture outcomes

Explain how RNAseq is generated and used

Identify the basic steps to align and analyze RNAseq data

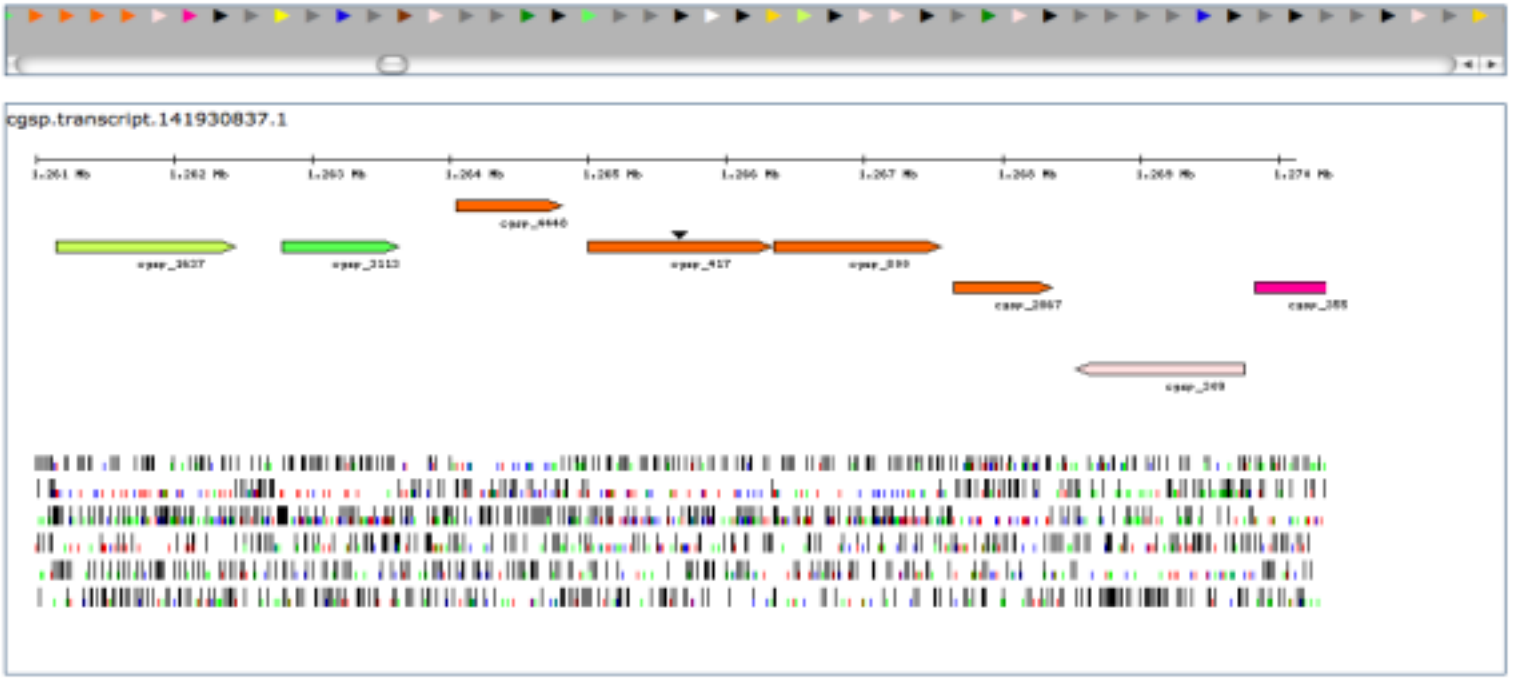
# Outline

1. Introduction and background
2. Overview of the methods and workflow
3. Quantifying expression levels
4. Analyzing patterns in expression
5. Technical considerations

# Introduction and background

## Why use RNAseq?

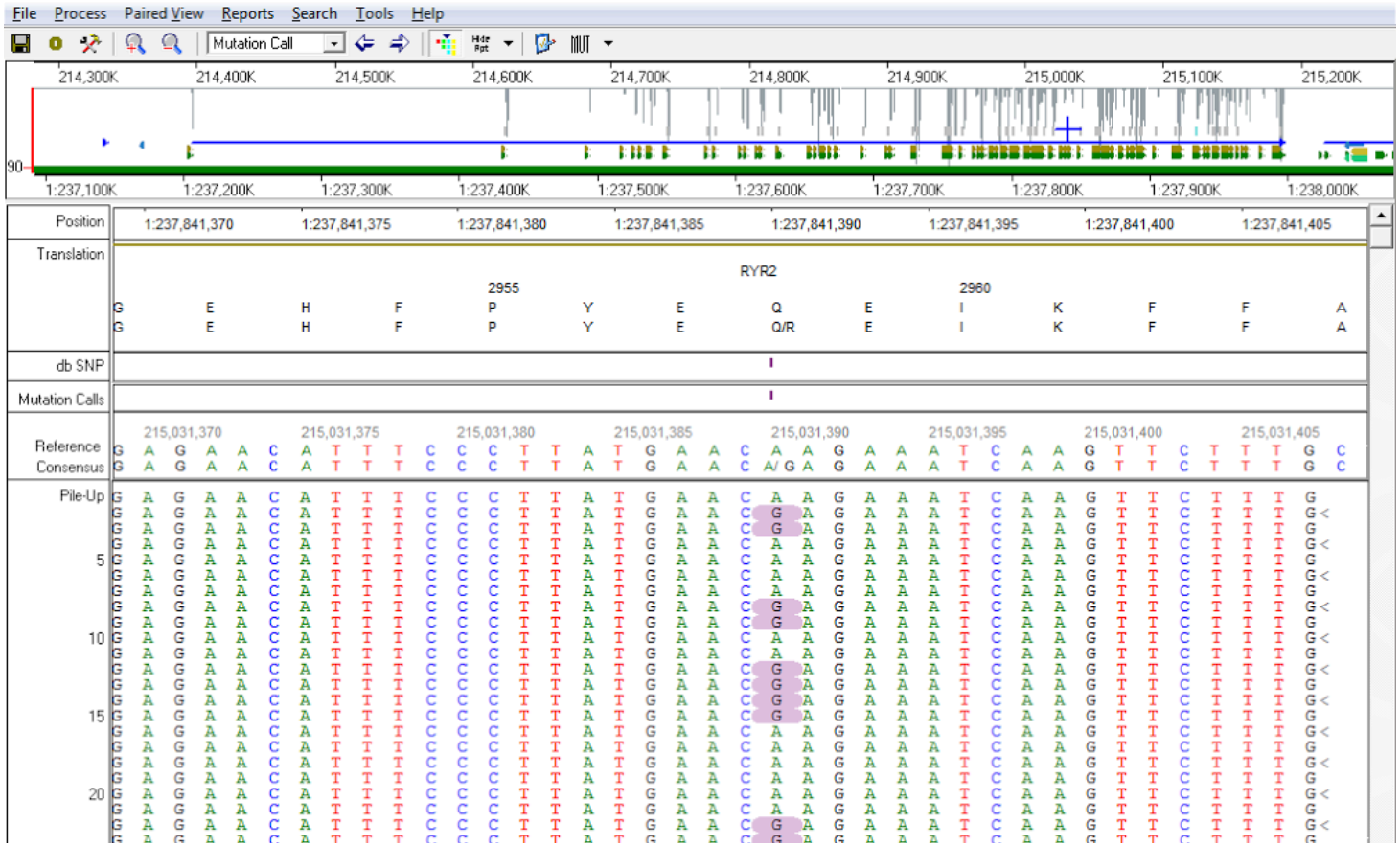
- Assembling gene space and genome annotation



# Introduction and background

## Why use RNAseq?

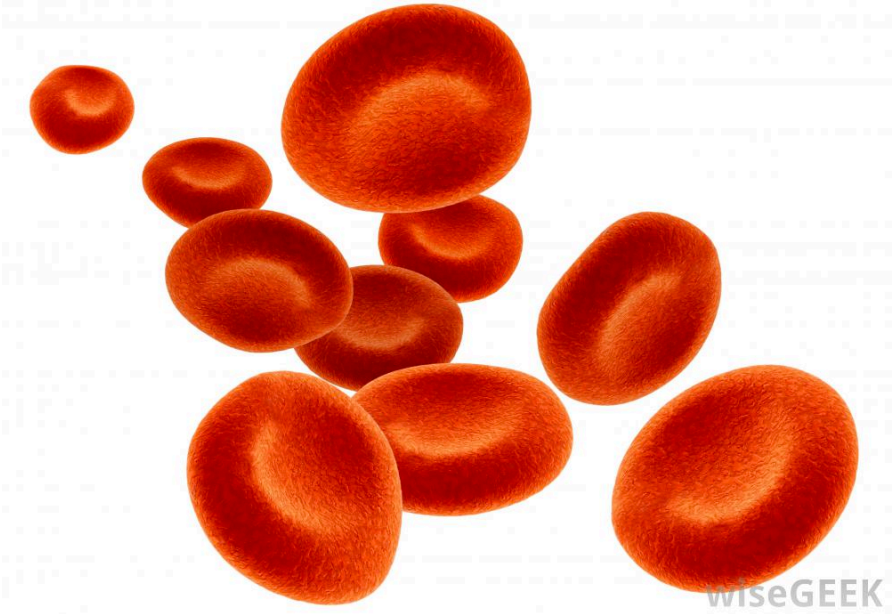
- Genotyping within the transcribed regions



# Introduction and background

Why use RNAseq?

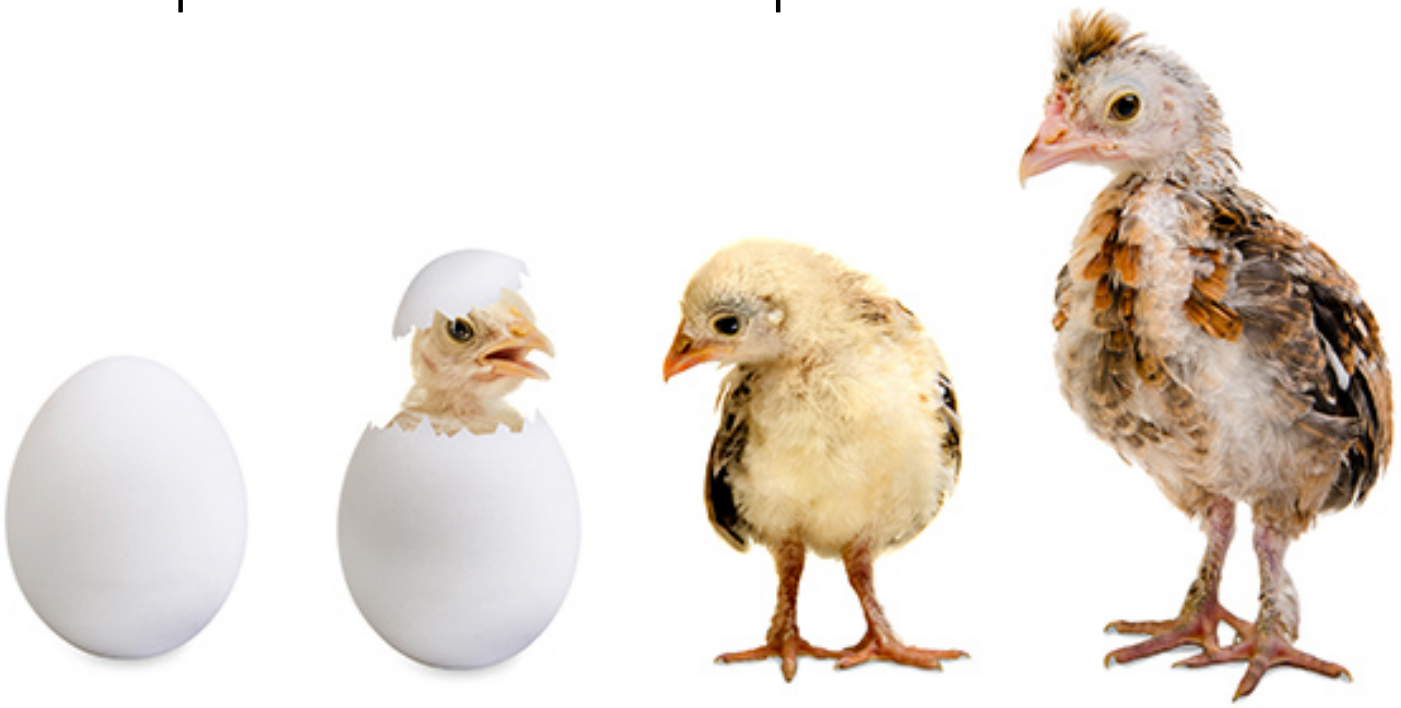
- Quantify patterns of gene expression
  - Organ, tissue, or cell types



# Introduction and background

Why use RNAseq?

- Quantify patterns of gene expression
  - Timepoints and development



# Introduction and background

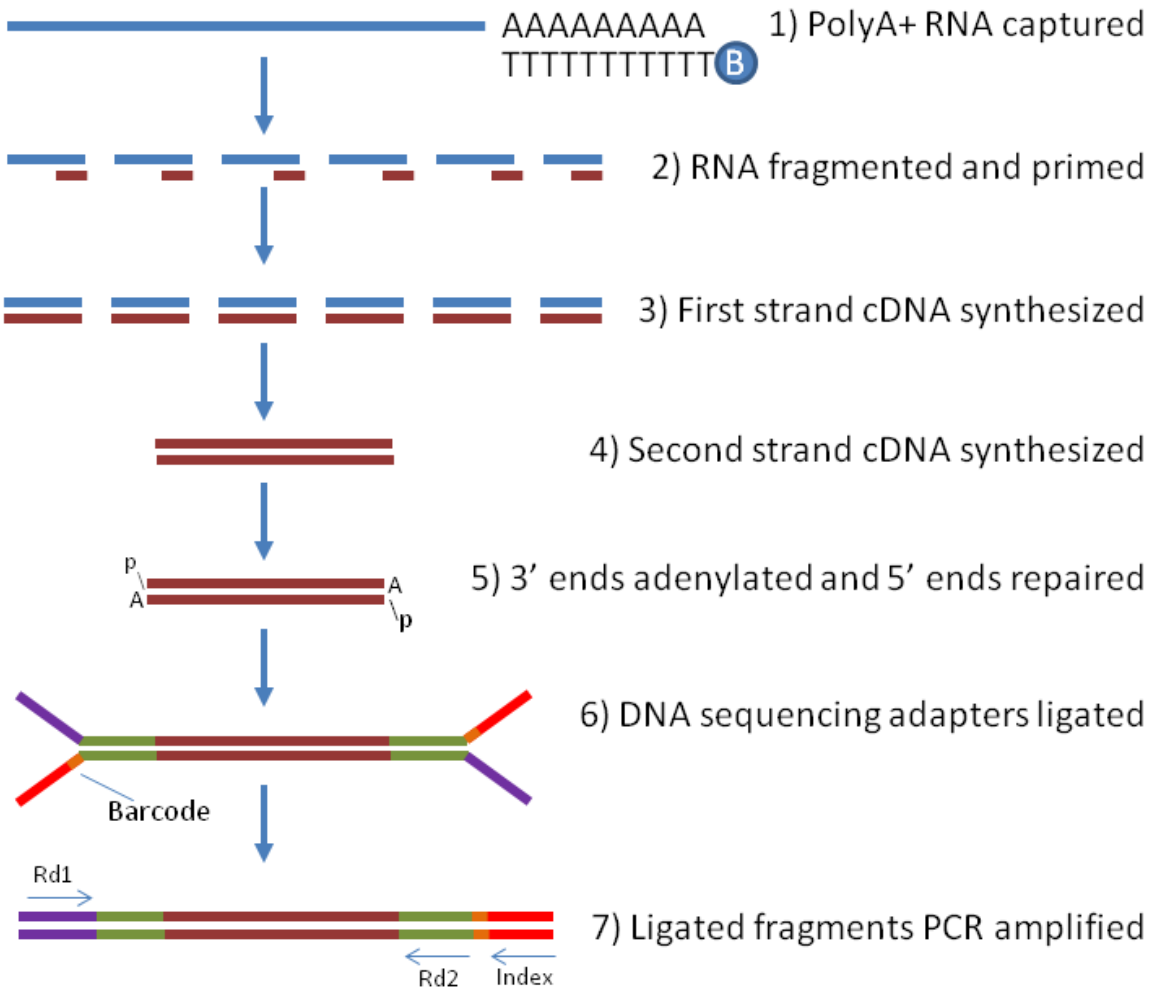
## Why use RNAseq?

- Quantify patterns of gene expression
  - Experimental treatments or observational categories

# Introduction and background

## How is RNAseq data generated?

- mRNA is isolated, fragmented, and cDNA is synthesized and sequenced
- Standard Illumina paired-end data will thus represent a snapshot of the mRNA present in your sample




http://aws.labome.com/

# Overview of the methods

Quantifying patterns of gene expression:

1. RNAseq extraction protocol & sequencing
2. Clean and filter reads
3. Map reads to a reference
4. Count number of reads per gene in each individual
5. Statistical analysis of differences in read counts

# Overview of the methods

- Cleaning and filtering is particularly important for de novo transcriptome assembly

- For expression analysis discard outlier samples

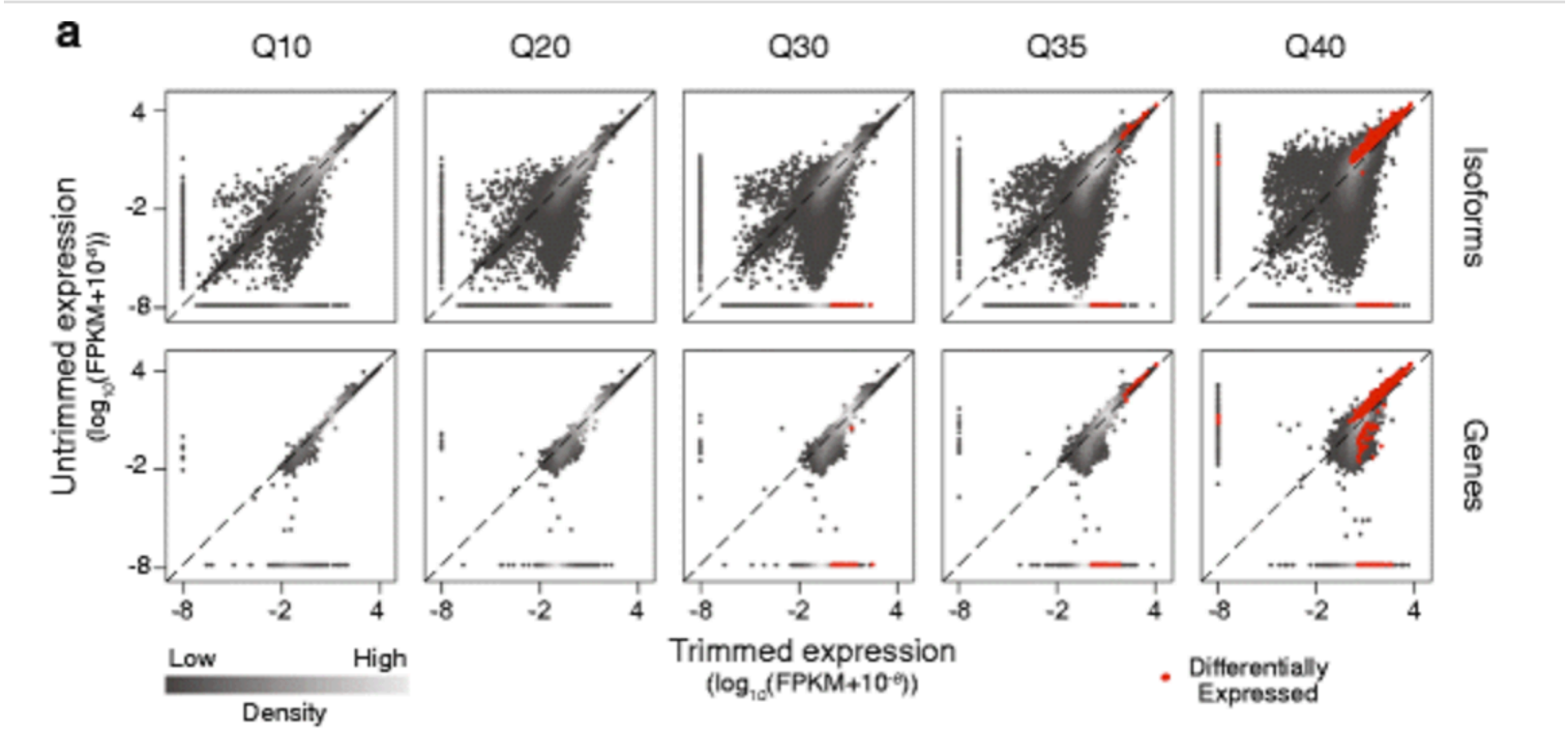
Recommended tools

QC: Fastqc

Trimming/filtering: Trimmomatic, Fastx, SnoWhite

# Overview of the methods

- Aggressive trimming and spurious alignments of short reads can lead to inaccurate estimates of gene expression



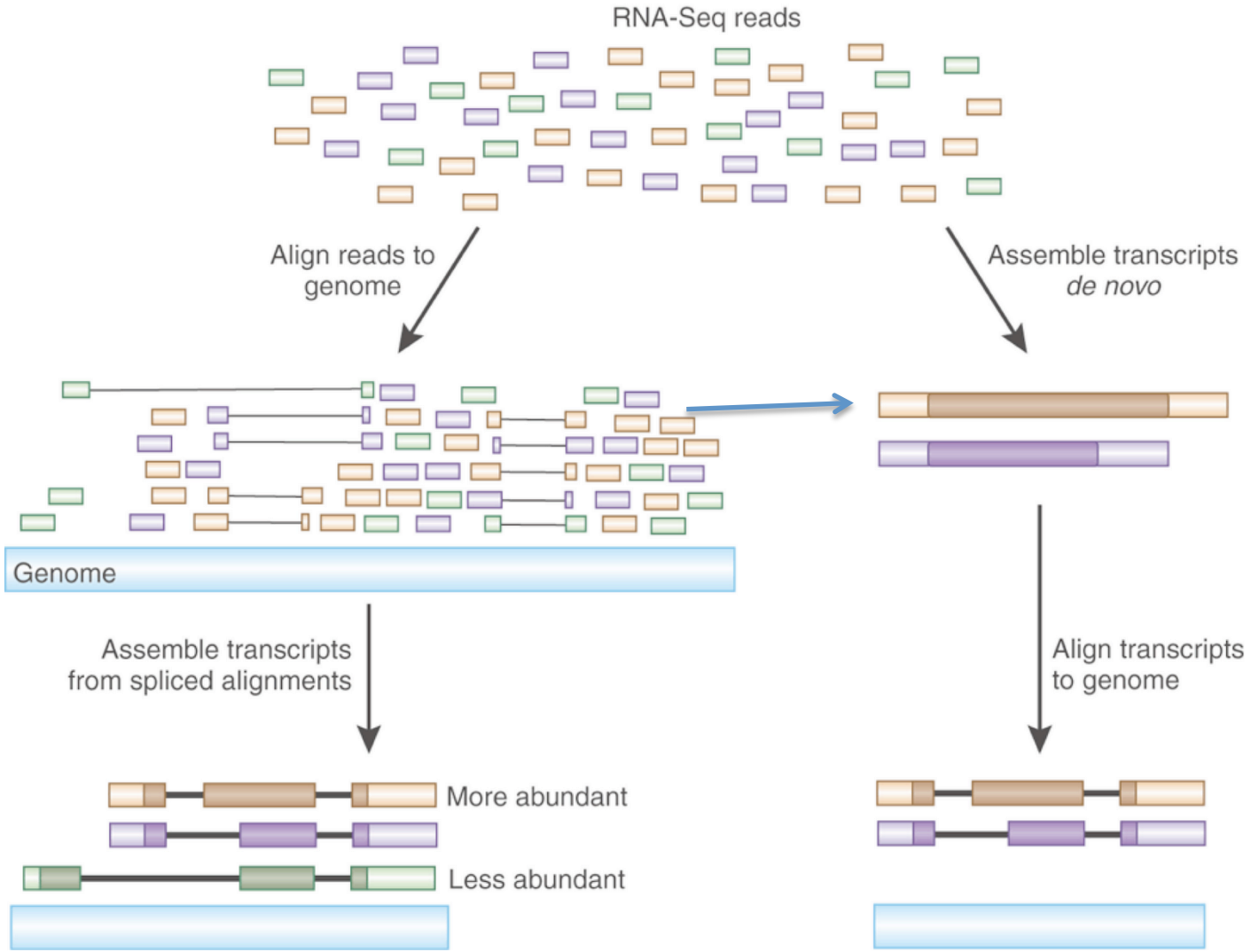

Williams et al 2016

# Overview of the methods

Quantifying patterns of gene expression:

1. RNAseq extraction protocol & sequencing
2. Clean and filter reads
3. Map reads to a reference
4. Count number of reads per gene in each individual
5. Statistical analysis of differences in read counts

# Quantifying expression levels

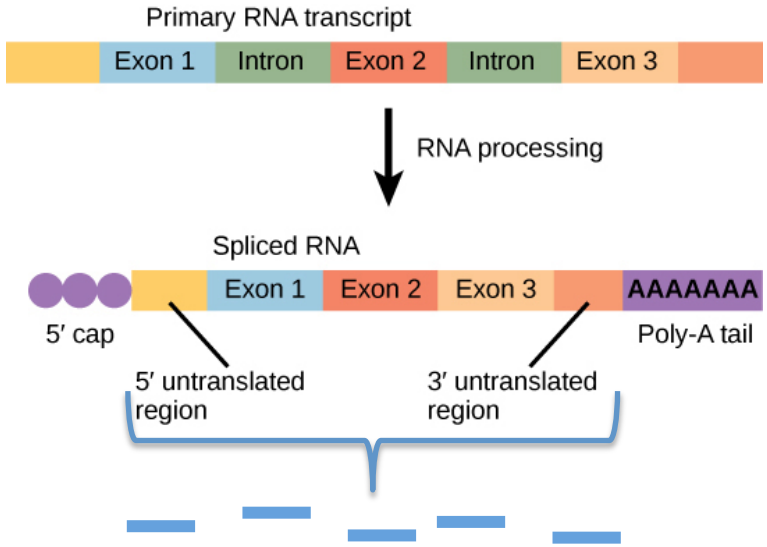



## Assembling and Aligning

Haas & Zody 2010 Nature Biotechnology

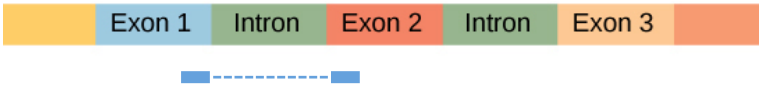
# Quantifying expression levels

## Challenge 1: Mapping reads across intron-exon boundaries



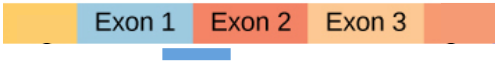

Our filtered RNAseq reads come from the mature transcript

**The genome sequence looks like this:**




Read fails to map across intron

**The transcriptome sequence looks like this:**




Read maps correctly

Some reads span two exons, and would not map to the genome using conventional approaches

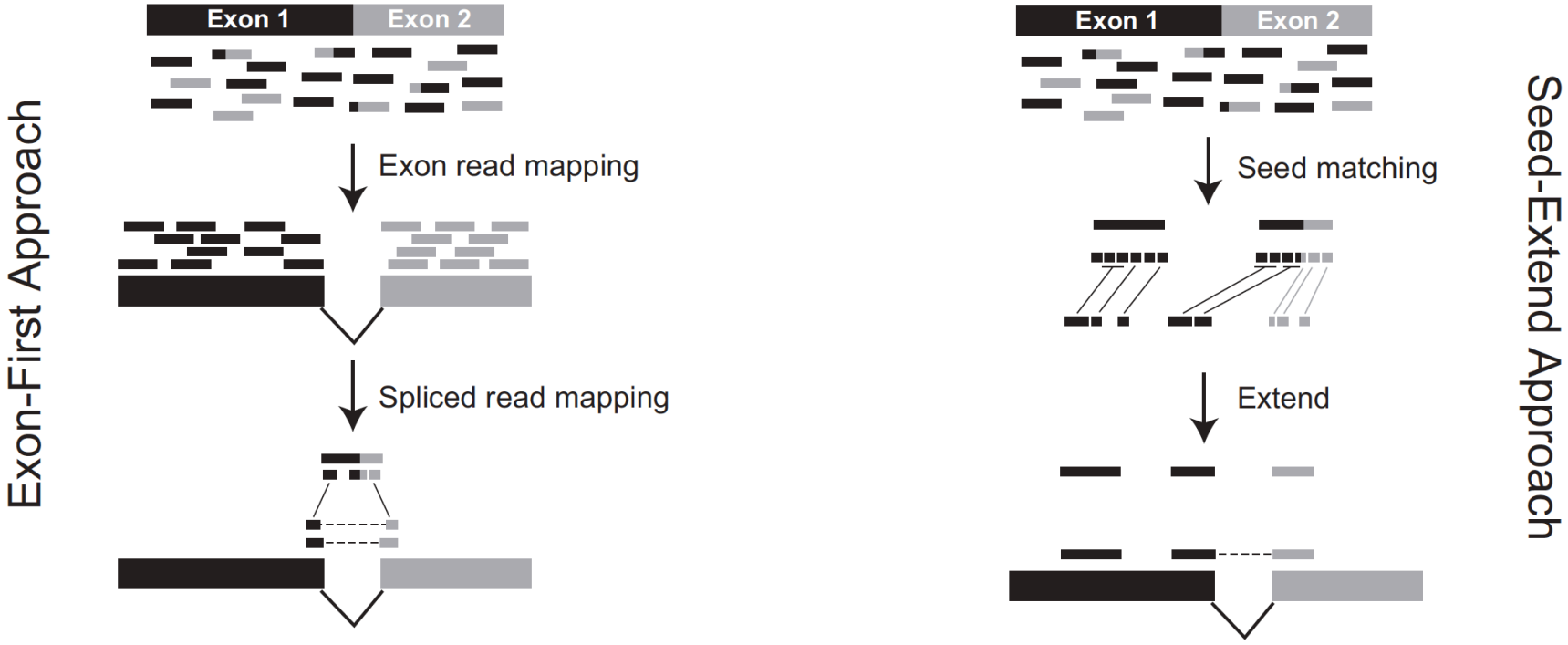
# Quantifying expression levels

Challenge 1: Mapping reads across intron-exon boundaries

Solutions:

- Map reads to a transcriptome (e.g. RSEM)
- Exon first mapping to genome (e.g. TopHat)
  - Use an “unspliced read aligner” to map the reads within a single exon
  - Split unmapped reads into shorter segments and attempt to re-map
- Seed and extend methods map small chunks to the genome and extend to splice sites (e.g. GSNAP)

# Quantifying expression levels



# Quantifying expression levels

## Challenge 2: Identifying abundance of alternatively spliced transcripts


**Splice variant #1**


**Splice variant #2**

If there are two known splice variants, a read spanning exon 1 & 2 or 1 & 3 will identify which variant is present

**Splice variant #1**

**Splice variant #2**

If a read aligns to exon 2 then differential expression of isoforms can be inferred, relative to the expression levels of other isoforms

# Quantifying expression levels

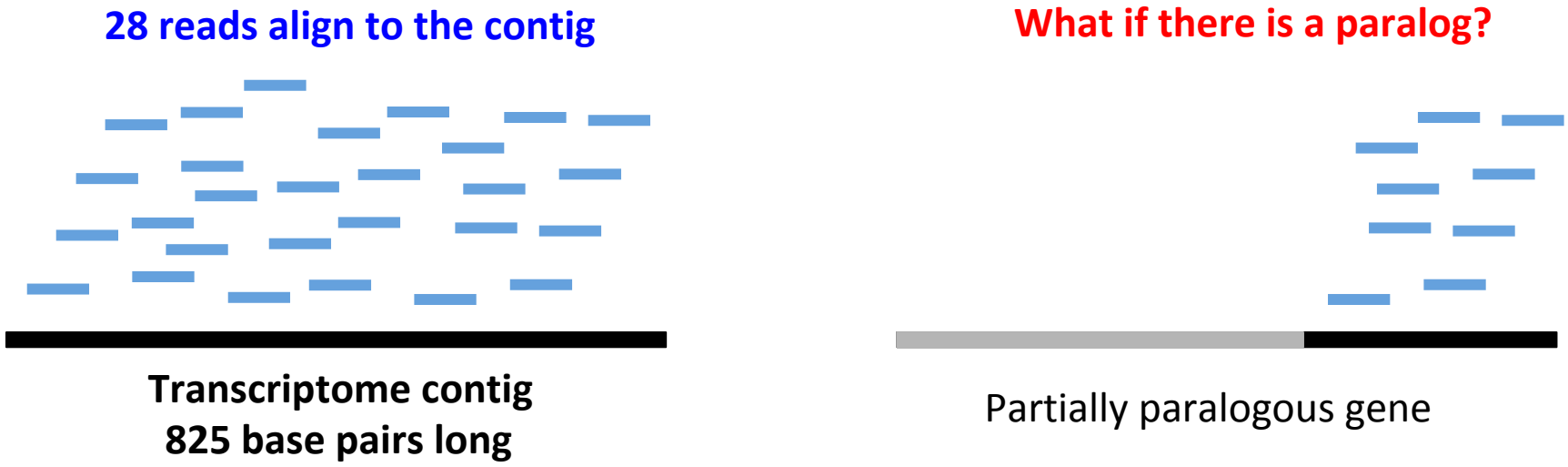
Challenge 2: Identifying abundance of alternatively spliced transcripts

Solutions:

- Identify expression levels for reads spanning diagnostic splice sites, relative to expression levels in non-diagnostic exons
- Multiple complex algorithms for sorting reads based on compatibility with different isoform models (e.g. Cufflinks)

# Quantifying expression levels

## Challenge 3: Dealing with multireads at the gene- and isoform-level




Both paralogs and alternatively spliced transcripts (isoforms) can give the problem of “multireads”: a read that maps with high score to several places

Li et al. (2010) found that 17% (mouse) or 52% (maize) of reads were multireads

# Quantifying expression levels

Challenge 3: Dealing with multireads at the gene- and isoform-level

Solutions:

- Discard (only use uniquely mapping reads)
- “rescue” multireads by allocating fractions of them in proportion to the number of uniquely mapping reads mapping to each contig
- ML algorithms to assign multireads and sum across all isoforms for gene-level estimates (e.g. RSEM)

# Quantifying expression levels

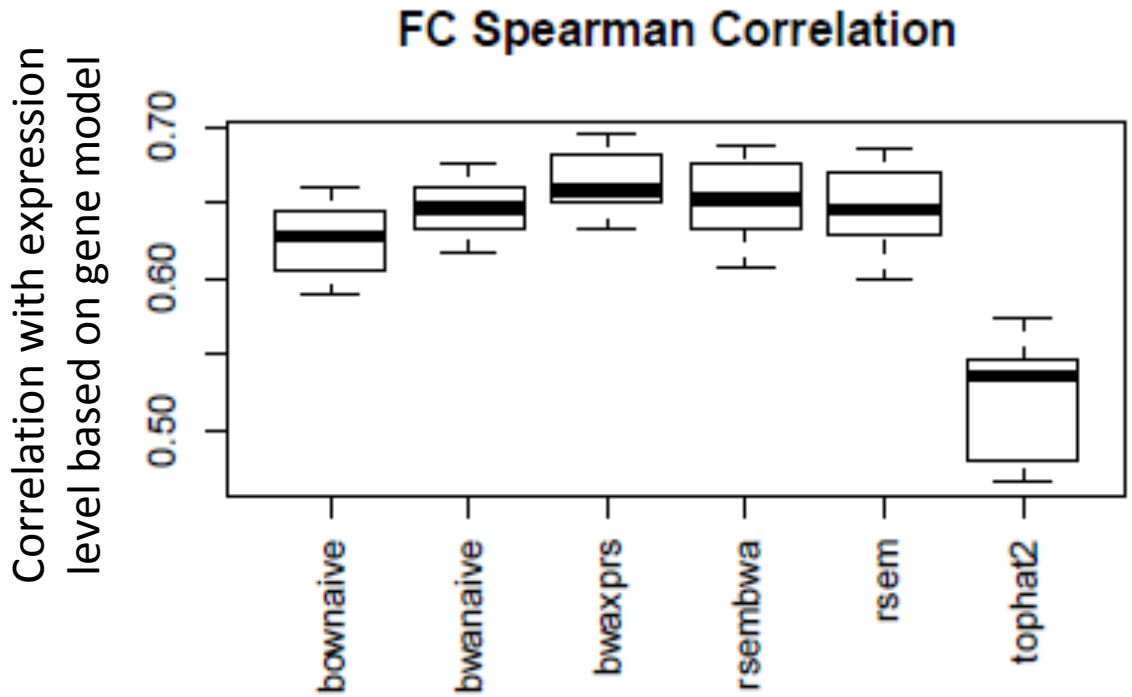
Practical approaches: RSEM

- Single pipeline to align and estimate expression
- Will estimate isoform-level expression counts (if isoforms for each gene are identified)
- No sequenced genome needed (a reasonable reference transcriptome can be built de novo using Trinity in non-model organisms)
- In the exercise following lecture, we will work through a simple example dataset with RSEM

# Quantifying expression levels

Practical approaches: TopHat + Cufflinks

- TopHat + Cufflinks provide a joint approach to mapping reads to the genome and require a good reference genome
- Tophat may be less accurate than RSEM:




Courtesy of Eric Aronesty

# Overview of the methods

Quantifying patterns of gene expression:

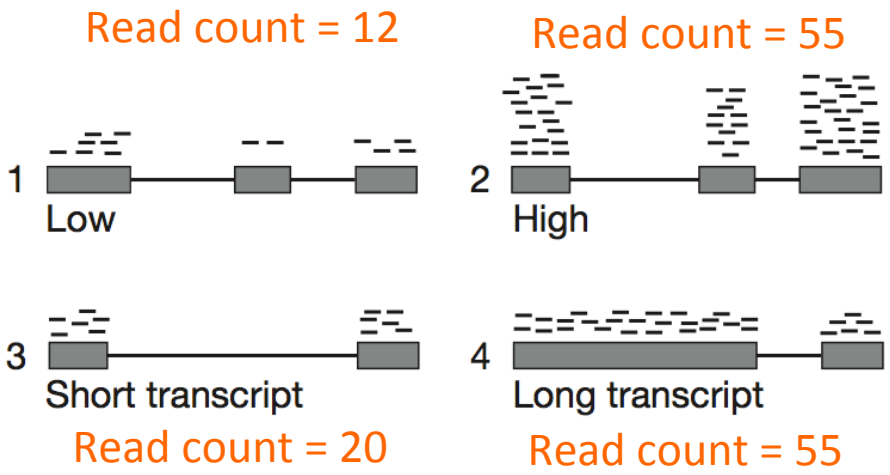
1. RNAseq extraction protocol & sequencing
2. Clean and filter reads
3. Map reads to a reference
4. Count number of reads per gene in each individual
5. Statistical analysis of differences in read counts

# Analyzing patterns in expression

RNAseq normalization needed due to two systematic causes of variation:

1) Differences in the amount sequenced among individuals

2) More reads from a long transcript than from a short transcript



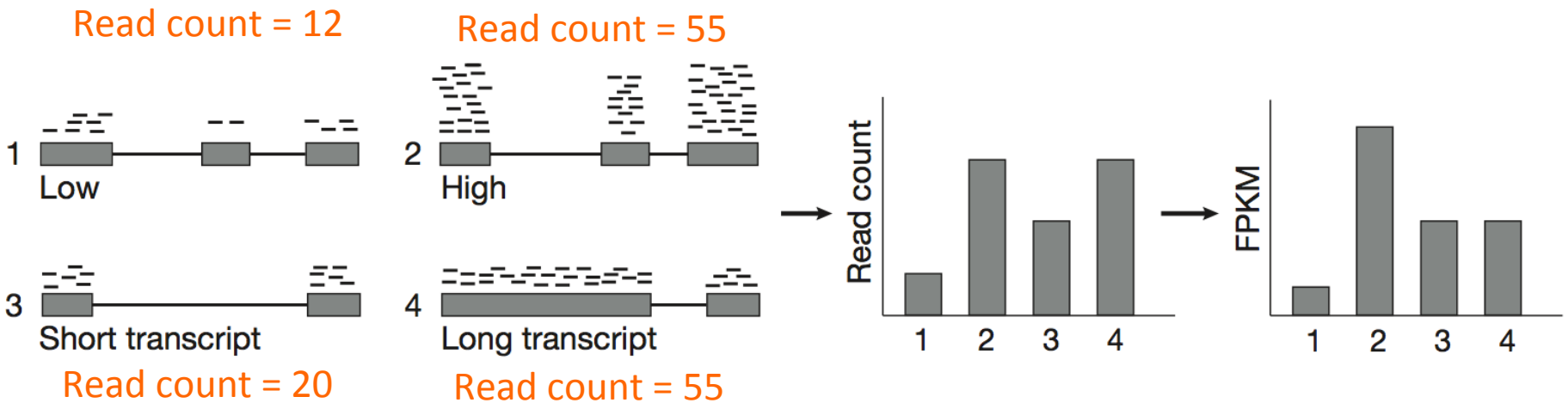

Garber et al. 2011

# Analyzing patterns in expression

RNAseq normalization needed due to two systematic causes of variation:

1) Differences in the amount sequenced among individuals

2) More reads from a long transcript than from a short transcript




Garber et al. 2011

# Analyzing patterns in expression

FPKM: Fragments Per Kilobase of transcript per Million reads mapped

- normalizes by transcript length and the total size of the mapped library
- correct both issues

Garber et al. 2011

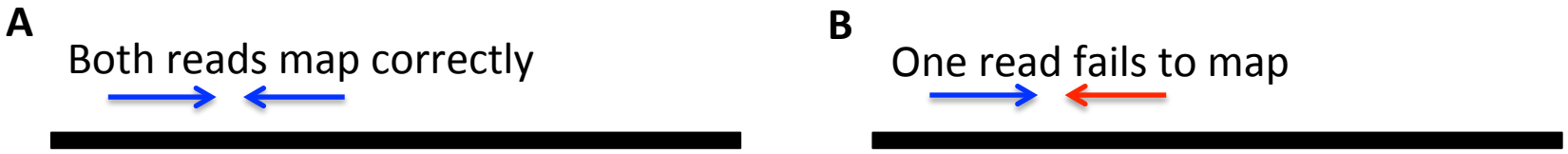
# Analyzing patterns in expression

RPKM vs. FPKM

FPKM: Fragments Per Kilobase of transcript per Million reads mapped

RPKM: Reads Per Kilobase of transcript per Million reads mapped

FPKM corrects for the non-independence of two reads when you have paired-end data:




RPKM would count that A had 2x more expression than B, giving an underestimate for B. FPKM adjusts this count for paired end data

# Analyzing patterns in expression

## Practical implementation

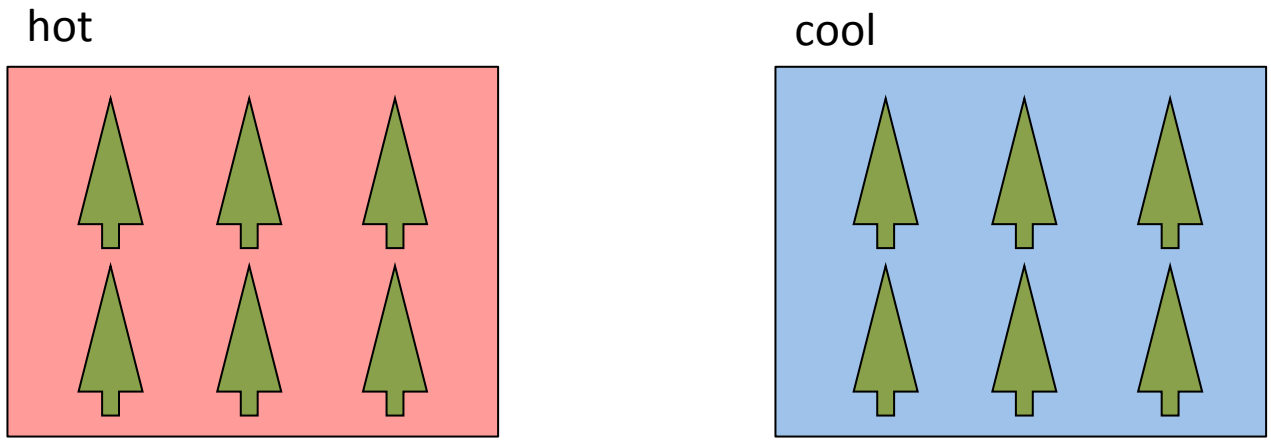
## Simple: most programs will estimate FPKM or RPKM for you

### Sample output from RSEM

| gene_id | transcript_id(s) | length | effective_length | expected_count | TPM | FPKM | |
|---|---|---|---|---|---|---|---|
| comp10000_c0 | comp10000_c0_seq1 | 1502 | 1299.85 | 3 | 60.1 | 34.36 | |
| comp100017_c0 | comp100017_c0_seq1 | 735 | 532.87 | 1 | 48.87 | 27.94 | |
| comp10002_c0 | comp10002_c0_seq1 | 4182 | 3979.85 | 7 | 45.8 | 26.19 | |
| comp100037_c0 | comp100037_c0_seq1 | 1921 | 1718.85 | 0 | 0 | 0 | |
| comp100052_c0 | comp100052_c0_seq1 | 679 | 476.89 | 0 | 0 | 0 | |
| comp10005_c0 | comp10005_c0_seq1 | 1764 | 1561.85 | 0 | 0 | 0 | |
| comp100064_c0 | comp100064_c0_seq1 | 631 | 428.92 | 0 | 0 | 0 | |
| comp10006_c0 | comp10006_c0_seq1 | 2680 | 2477.85 | 4 | 42.04 | 24.04 | |

# Tutorial part I

Align reads and estimate expression levels for three pine samples to the transcriptome reference



# Tutorial part I

1. Follow the instructions on the github Topic 6 page to align and assess transcript abundance with RSEM (PART I)

3. Answer the following questions:

What is the expected count of comp996_c0 for each individual?

What expression measure would you use to compare gene expression between different genes and why (expected counts versus FPKM)?

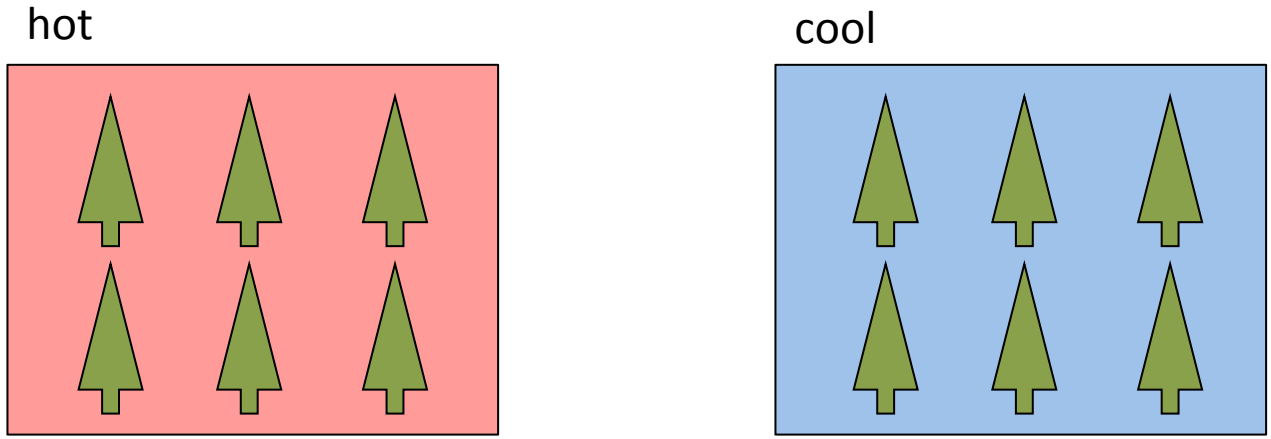
# Overview of the methods

Quantifying patterns of gene expression:

1. RNAseq extraction protocol & sequencing
2. Clean and filter reads
3. Map reads to a reference
4. Count number of reads per gene in each individual
5. Statistical analysis of differences in read counts

# Analyzing patterns in expression

Fitting models to expression data




6 individuals per treatment (1 library/ind)

What genes are differentially expressed in response to temperature?

# Analyzing patterns in expression

How to go from raw expression counts

| | | | | |
|---|---|---|---|---|
| comp10109_c2 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c20 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c22 | 176.00 | 13.00 | 5.00 | 9.00 |
| comp10109_c23 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c25 | 0.00 | 0.00 | 2.00 | 2.00 |
| comp10109_c31 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c32 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c33 | 1.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c35 | 148.00 | 403.87 | 327.20 | 117.14 |
| comp10109_c36 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c37 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c38 | 1.00 | 1.00 | 0.00 | 0.00 |
| comp10109_c40 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp10109_c41 | 96.00 | 51.00 | 61.00 | 24.00 |
| comp10109_c42 | 15.00 | 0.00 | 0.00 | 1.00 |
| comp10109_c7 | 0.00 | 0.00 | 0.00 | 0.00 |
| comp1010_c0 | 483.00 | 2125.91 | 2397.11 | 526.00 |

To biologically meaningful results?

# Analyzing patterns in expression

Approaches to analysis:

1. Differential gene expression on gene-by-gene basis (e.g. DESeq, EdgeR, limma)
   - Examine how each gene is affected by a factor (e.g. treatment)
   - Use glms to identify genes with significant expression differences among groups
2. Patterns of gene co-expression
   - Identify clusters of genes that are regulated together

# Analyzing patterns in expression

Biological variation

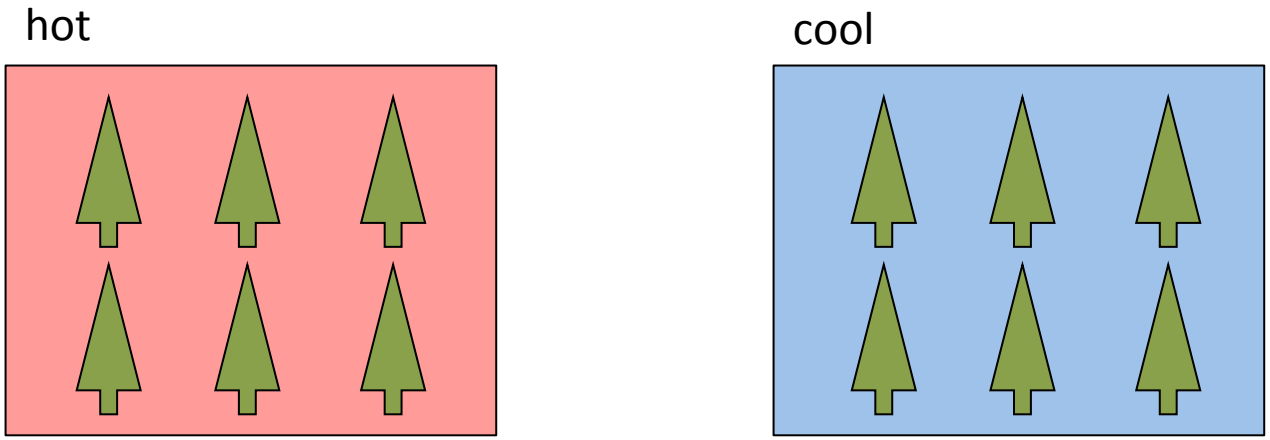
- real differences between samples due to:

1) uncontrolled sources that should be homogenous across treatments

2) controlled sources that arise from experimental treatment/design

Technical variation

- arises from measurement error inherent in the sequencing process (sequencing and library prep)

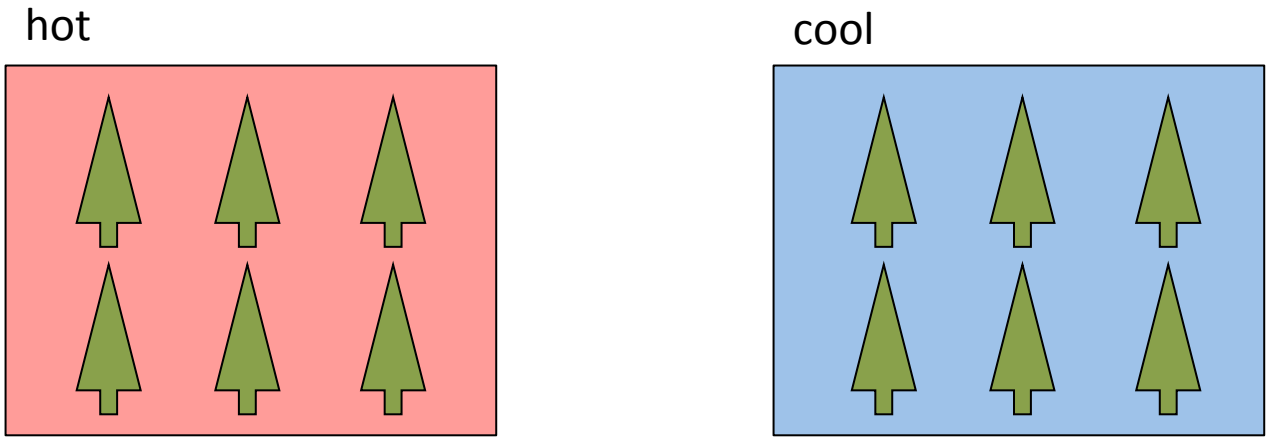


# Analyzing patterns in expression

- biological replication (multiple individuals per treatment)
- technical replication (here, there is no technical replication)

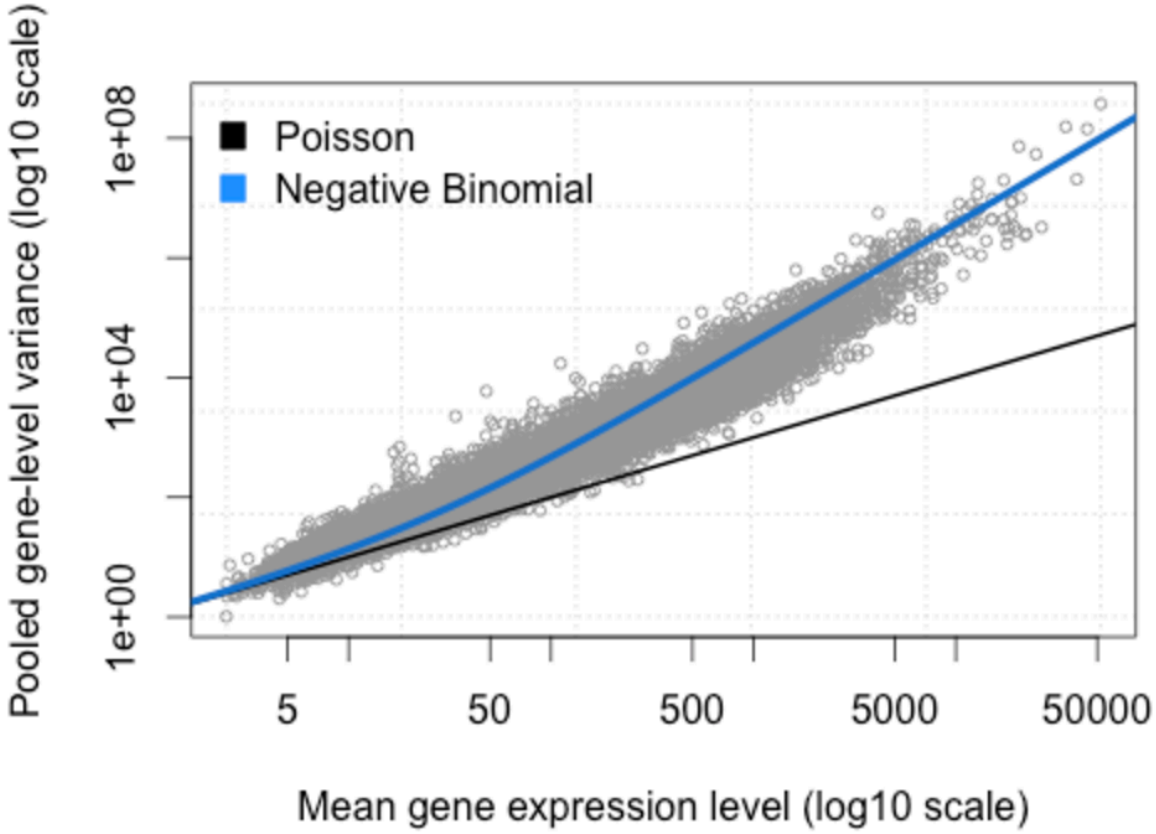
Regression of normalized counts on variable(s) of interest

- fold-change in expression among factor levels (log2(B/A))
- estimates of significance



# Analyzing patterns in expression

- Count data can be modeled using the Poisson distribution (mean=variance)
- Biological variance creates over-dispersion so the mean does not equal the variance



# Analyzing patterns in expression

For the negative binomial:

- $var = \mu + \phi\mu^2$
- $\sqrt{\phi}$ = CV (SD/mean)
- $\phi$ is called the dispersion parameter
- Total $CV^2$ in expression = Technical $CV^2$ + Biological $CV^2$

Biological CV (BCV) is the coefficient of variation with which the (unknown) true abundance of the gene varies between biological replicates.

# Analyzing patterns in expression

## Empirical Bayes for gene expression

- Many RNAseq/microarray approaches use an empirical Bayes method to “borrow” information across genes
- Prevents outliers from driving differential expression

# Who were the best batters?

## the worst batters?

| name | H | AB | average |
|---|---|---|---|
| Frank Abercrombie | 0 | 4 | 0 |
| Horace Allen | 0 | 7 | 0 |
| Pete Allen | 0 | 4 | 0 |
| Walter Alston | 0 | 1 | 0 |
| Bill Andrus | 0 | 9 | 0 |

## the best batters?

| name | H | AB | average |
|---|---|---|---|
| Jeff Banister | 1 | 1 | 1 |
| Doc Bass | 1 | 1 | 1 |
| Steve Biras | 2 | 2 | 1 |
| C. B. Burns | 1 | 1 | 1 |
| Jackie Gallagher | 1 | 1 | 1 |

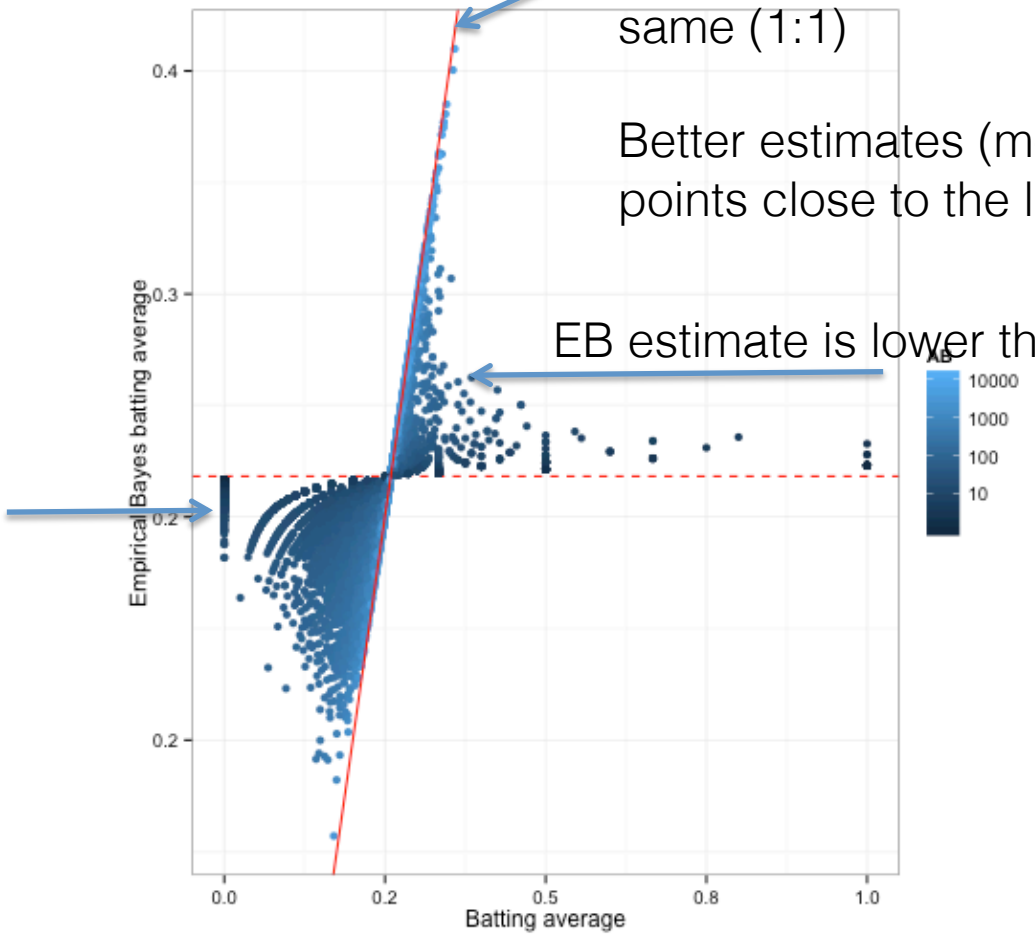
# Shrinkage: EB tends to move estimates towards the mean

Estimate and measured average are the same (1:1)

Better estimates (more data) for those points close to the line

EB estimate is lower than actual estimate

EB estimate is higher than actual estimate




http://varianceexplained.org/r/empirical_bayes_baseball/

# better estimates of batting averages

| name | H | AB | average | eb_estimate |
|---|---|---|---|---|
| Rogers Hornsby | 2930 | 8173 | 0.358 | 0.355 |
| Shoeless Joe Jackson | 1772 | 4981 | 0.356 | 0.350 |
| Ed Delahanty | 2596 | 7505 | 0.346 | 0.343 |
| Billy Hamilton | 2158 | 6268 | 0.344 | 0.340 |
| Harry Heilmann | 2660 | 7787 | 0.342 | 0.339 |

| name | H | AB | average | eb_estimate |
|---|---|---|---|---|
| Bill Bergen | 516 | 3028 | 0.170 | 0.178 |
| Ray Oyler | 221 | 1265 | 0.175 | 0.191 |
| John Vukovich | 90 | 559 | 0.161 | 0.196 |
| John Humphries | 52 | 364 | 0.143 | 0.196 |
| George Baker | 74 | 474 | 0.156 | 0.196 |

# Analyzing patterns in expression

## Estimating Dispersion

- common dispersion: same Biological CV among genes (i.e. proportional relationship between gene-wise standard deviations and gene-wise means is the same for all genes)
  - gene expression levels have non-identical and dependent distribution between genes (common dispersion too naïve)
- tagwise dispersion: the common dispersion estimate is modified for each gene based on a Empirical Bayes estimate of the per-gene relationship between mean and variance

# Analyzing patterns in expression

Using the approach from edgeR as an example

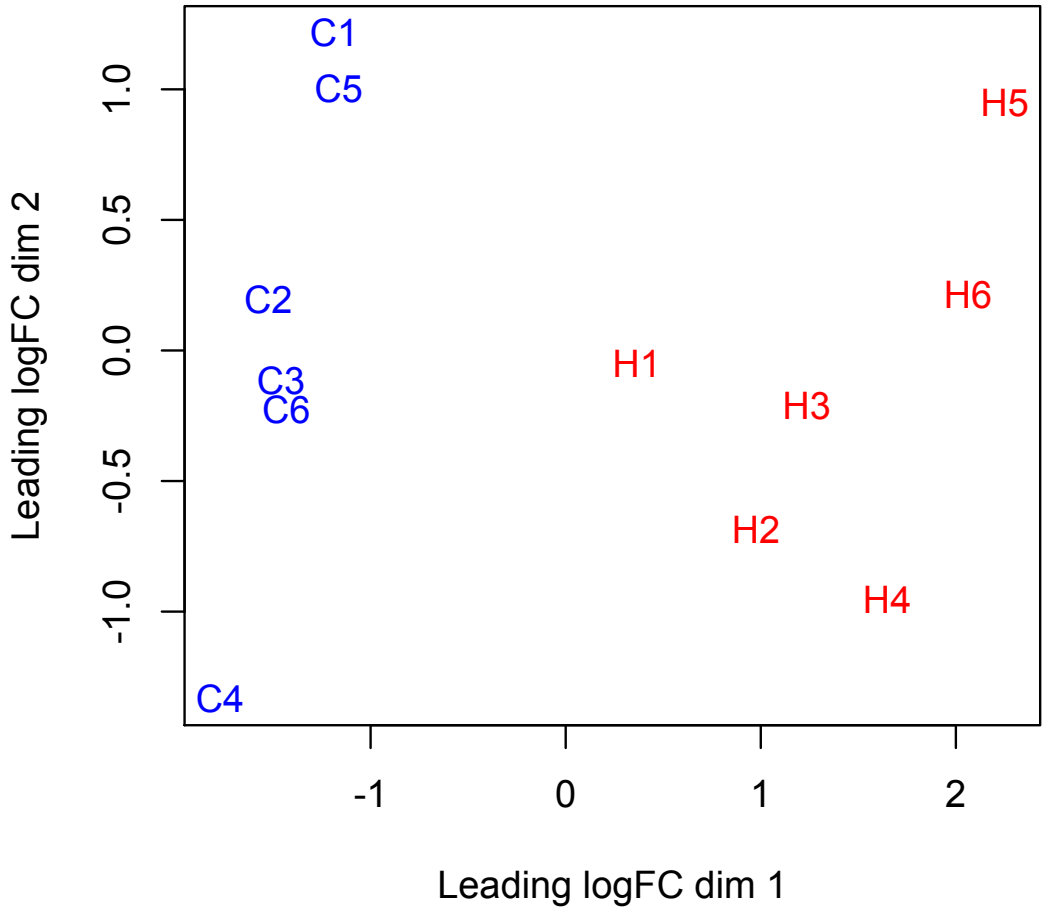
Model fitting results in estimation of log fold change (logFC) in expression, p-value, and estimation of False Discovery Rate (FDR)

| | logFC | logCPM | LR | PValue | FDR |
|---|---|---|---|---|---|
| comp520_c0 | 8.997022 | 10.663572 | 175.7591 | 4.087401e-40 | 7.584581e-36 |
| comp626_c0 | 8.489396 | 8.474038 | 166.4056 | 4.510882e-38 | 4.185197e-34 |
| comp29033_c0 | -3.427787 | 2.914473 | 153.7321 | 2.650165e-35 | 1.639215e-31 |
| comp3737_c0 | 4.121830 | 5.796822 | 134.5117 | 4.222342e-31 | 1.958744e-27 |
| comp6840_c0 | 4.319808 | 5.063555 | 126.0793 | 2.954429e-29 | 1.023962e-25 |
| comp14716_c0 | -2.772885 | 5.115474 | 125.8532 | 3.310934e-29 | 1.023962e-25 |

- EdgeR allows multiple factors for more complex designs (as does Limma)

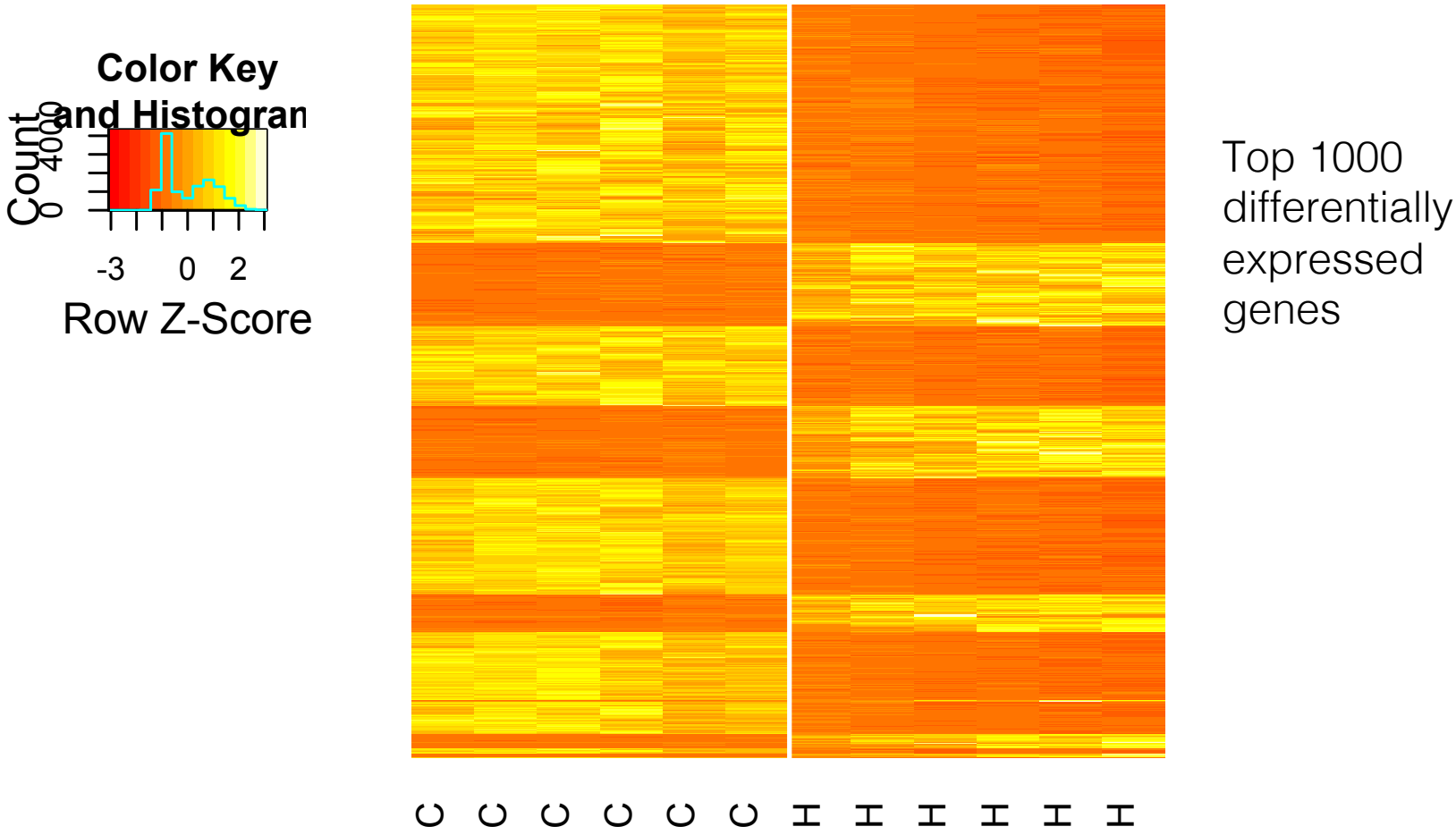
# Analyzing patterns in expression

Approaches to visualizing trends in data: Multi-Dimensional Scaling plot (like principle components, but allows missing data)



# Analyzing patterns in expression

Approaches to visualizing trends in data: Heatmaps to show patterns of expression in the most differentially expressed genes



# Analyzing patterns in expression

Numerous programs have been developed to detect differences in gene expression:

- DESeq
- edgeR
- limmaQN
- limmaVoom
- PoissonSeq
- CuffDiff
- baySeq

Fortunately, they are relatively similar in their power and accuracy; edgeR is consistently found to slightly outperform many others

# Tutorial part II

- Run EdgeR to compare expression between climate treatments (PART II)
- Answer the following questions

1. How many genes are differentially expressed by treatment in the simple contrast of C vs H (using "cold_hot_expression.txt")? How does the choice of FDR cutoff or p-value affect this number? What happens if you include genes in your analysis with low or no expression across all of the samples?

2. How many genes are differentially expressed in the three-way contrast (using "cold_hot_mwh_expression.txt")? Which treatment is driving differential expression here? How do you know?

3. How much does model fitting with common dispersion vs. tagwise dispersion affect the answers you get from the data? (think in terms of the number of DE genes, the evidence for a single gene, etc.)

# Analyzing patterns in expression

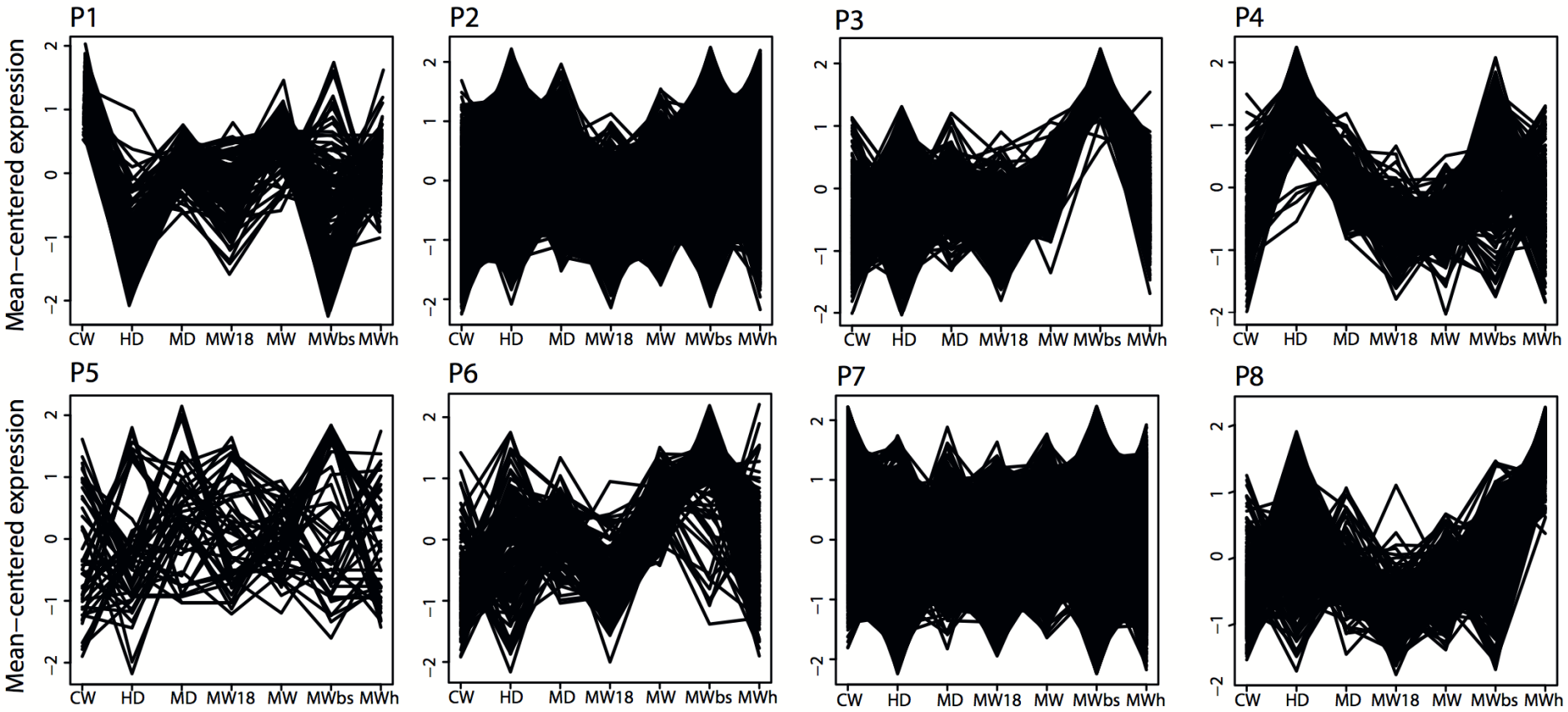
**Gene co-expression networks**: Finding genes that are expressed in the same way across treatments/tissues

Genes that tend to be up-regulated and down-regulated together will have higher correlation in their expression counts across treatments:

- Calculate pairwise correlations between each gene
- Perform clustering algorithm on the correlation table, grouping like with like
- Can also group genes that have opposite patterns of expression
- Requires many treatments to get high power

# Analyzing patterns in expression

Example (from WGCNA): 8 clusters showing gene expression in lodgepole pine over 7




Yeaman et al. (2014)

# Now what?

- As with many approaches in genomics, there is a “too much data” problem
- Annotation of genes
- Useful for identification of genes involved in plasticity and response:
  - are these genes also involved in adaptation
  - do they have signatures of selection?
- Strong experimental design
  - Move from descriptive to biological insight

# Technical considerations

Depth of coverage?

- Dependent on:
  1. study organism
  2. transcriptome size
  3. purpose of your study
- Low power if < 50 counts per million per gene
- Too many individuals per lane can increase your technical variation
- 10 million reads per sample is a benchmark from which to start for most eukaryotes
- Biological replication is often more valuable than higher depth of coverage per individual

# Technical considerations

- Variation among cells of the same type sampled at the same time (single-cell sequencing)
- Variation among cell types of the same tissue (micro-dissection)
- No substitute for biological replication
- Important that replicates be randomized during sample prep and sequencing due to batch effects (RNA extraction, library prep and sequencing).

# Technical considerations

De novo assembly (Illumina reads)

- De novo assembly needs large amounts of RAM
- Lodgepole pine transcriptome assembly:
  40Gbp of sequence data = 200 GB RAM
- Haploid tissue from a single individual is best
- Feasible to pool data from multiple individuals but difficult to know whether putative isoforms are “good” or just different genotypes
- Pooling from multiple tissues, treatments, developmental time points
- Long read transcriptome sequencing (e.g., PacBio) is an alternative (no assembly required)

# Further reading


Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, Szcześniak MW, Gaffney DJ, Elo LL, Zhang X, et al. 2016. A survey of best practices for RNA-seq data analysis. Genome Biology 17: 1–19.

Garber et al. 2011. Computational methods for transcriptome annotation and quantification using RNA-seq. Nature Methods. 8:469-477.

Marinov et al. 2014. From single-cell to cell-pool transcriptomes: Stochasticity in gene expression and RNA splicing. Genome Research. 24:496–510.

Rapaport et al. 2013. Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. Genome Biology. 14:R95.

Seyednasrollah et al. 2013. Comparison of software packages for detecting differential expression in RNA-seq studies. Briefings in Bioinformatics.

Tarazona et al. 2011. Differential expression in RNA-seq: A matter of depth. Genome Res. 21: 2213-2223

http://www.labome.com/method/RNA-seq-Using-Next-Generation-Sequencing.html

http://deweylab.biostat.wisc.edu/rsem/

http://www.mi.fu-berlin.de/wiki/pub/ABI/GenomicsLecture12Materials/rnaseq1.pdf

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

http://rnaseq.uoregon.edu/#analysis-trimming