Materials for this workshop

- Concepts: this presentation
- Hands-on session:
  - Sept2022handson.R
  - targets.txt
  - GSE60450_Lactation-GenewiseCounts.txt.gz

Please install the following library in Rstudio

```
Install.packages(magrittr)
Install.packages(edgeR)
Install.packages(org.Mm.eg.db)
Install.packages(tidyverse)
Install.packages(ggplot2)
Install.packages(statmod)
```

Intermediate RNA-seq data analysis

Gladstone Institutes

Michela Traglia and Min-Gyoung Shin
Bioinformatics Core, GIDB
13 September 2022

Assumed background

- Familiarity with R and RStudio
- Familiarity with RNA-seq protocol
- Familiarity with basic concepts of statistics and hypothesis testing

Introductions

Min-Gyoung Shin

Bioinformatician II

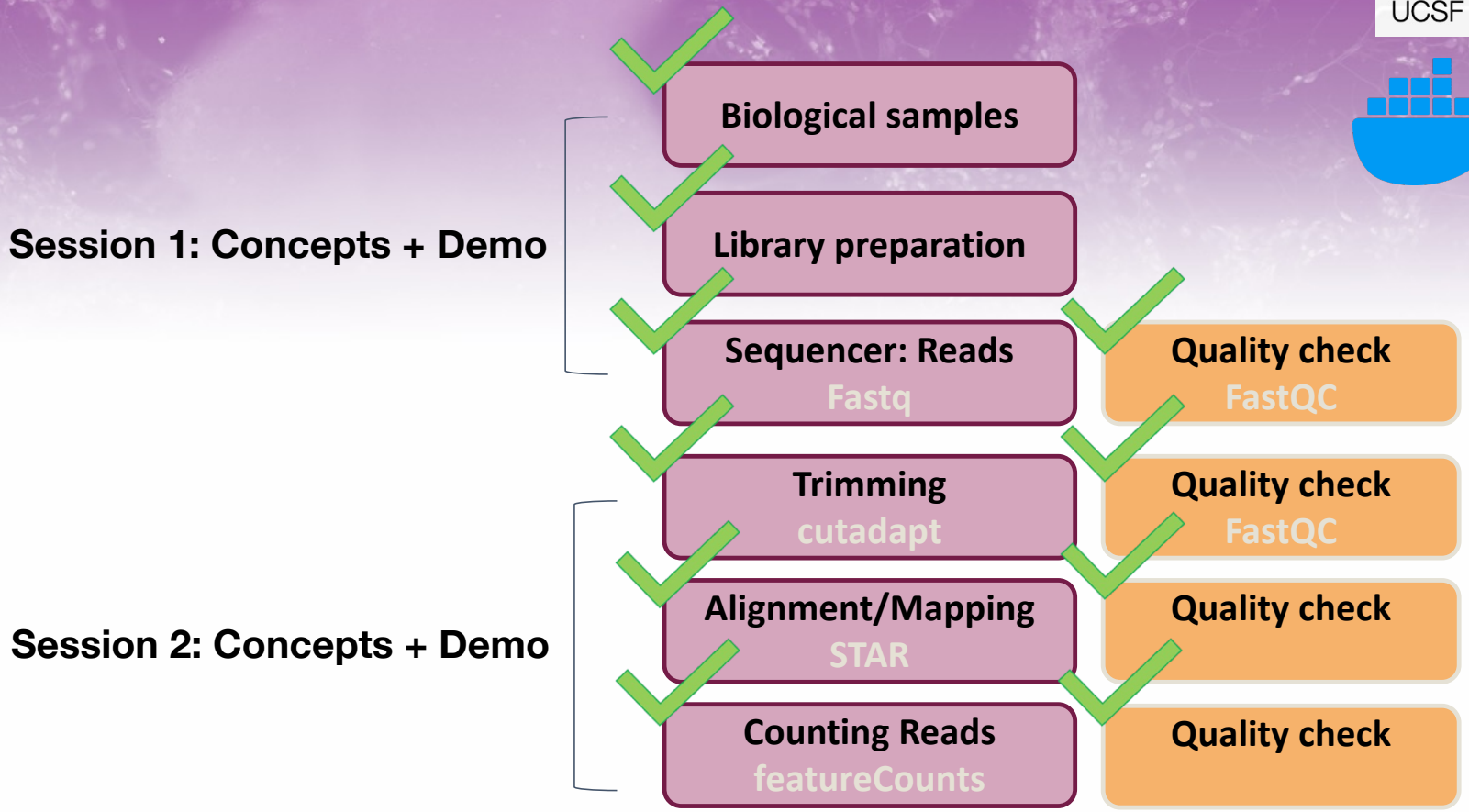
Michela Traglia

Statistician III

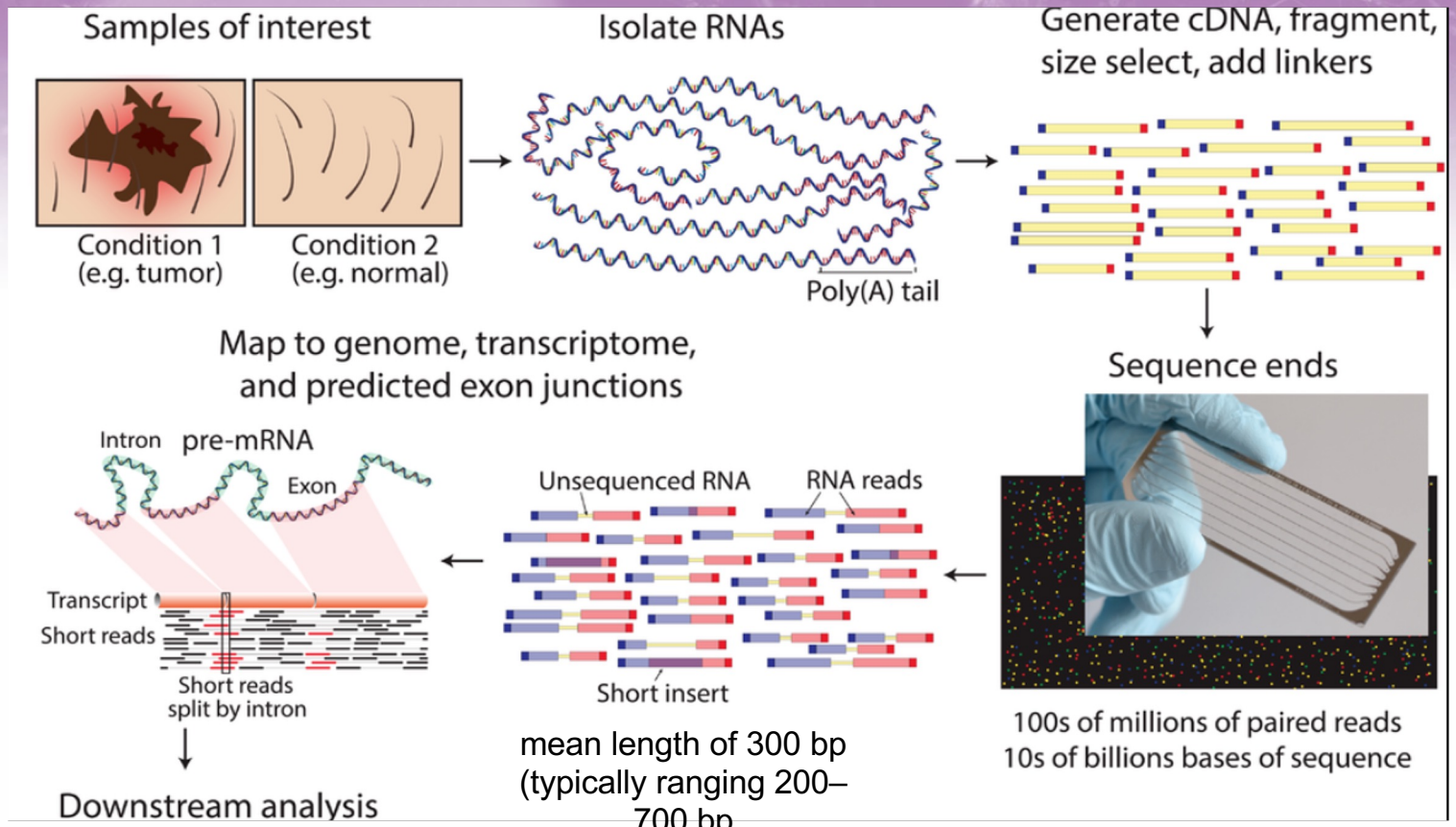
RNA-seq - analysis workflow

WYNTON
UCSF Research Computing at Scale

docker

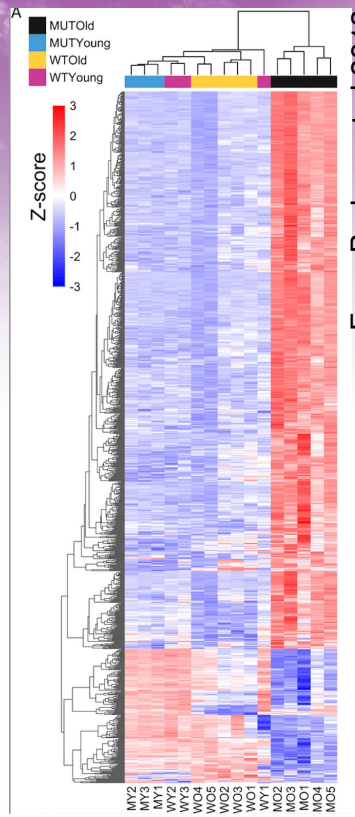


Typical RNA-seq protocol



https://www.wikiwand.com/en/RNA-Seq

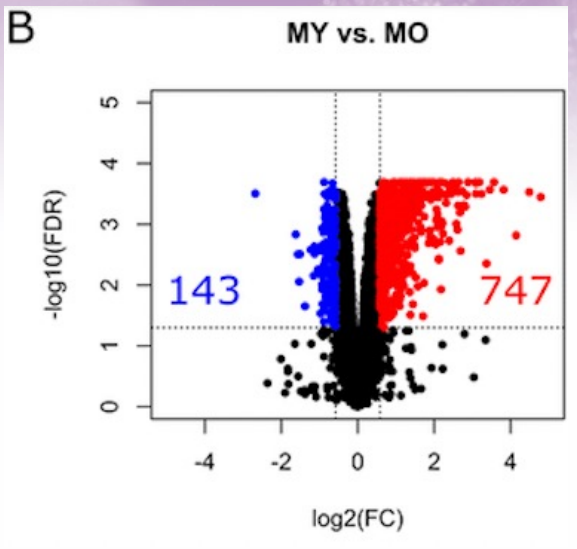
Goal of DEG analysis



From Barham et al 2013

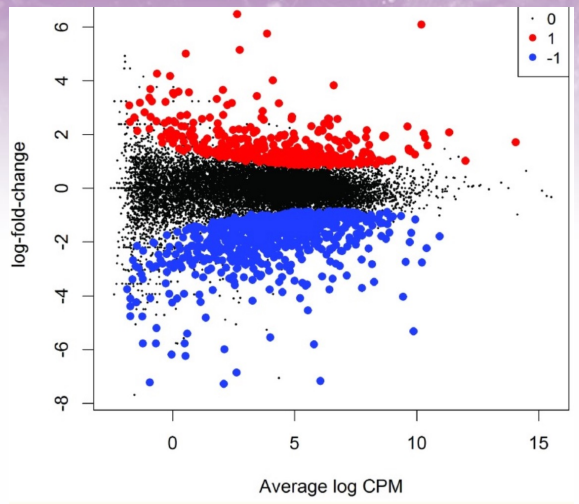
Heatmap

Volcano plot



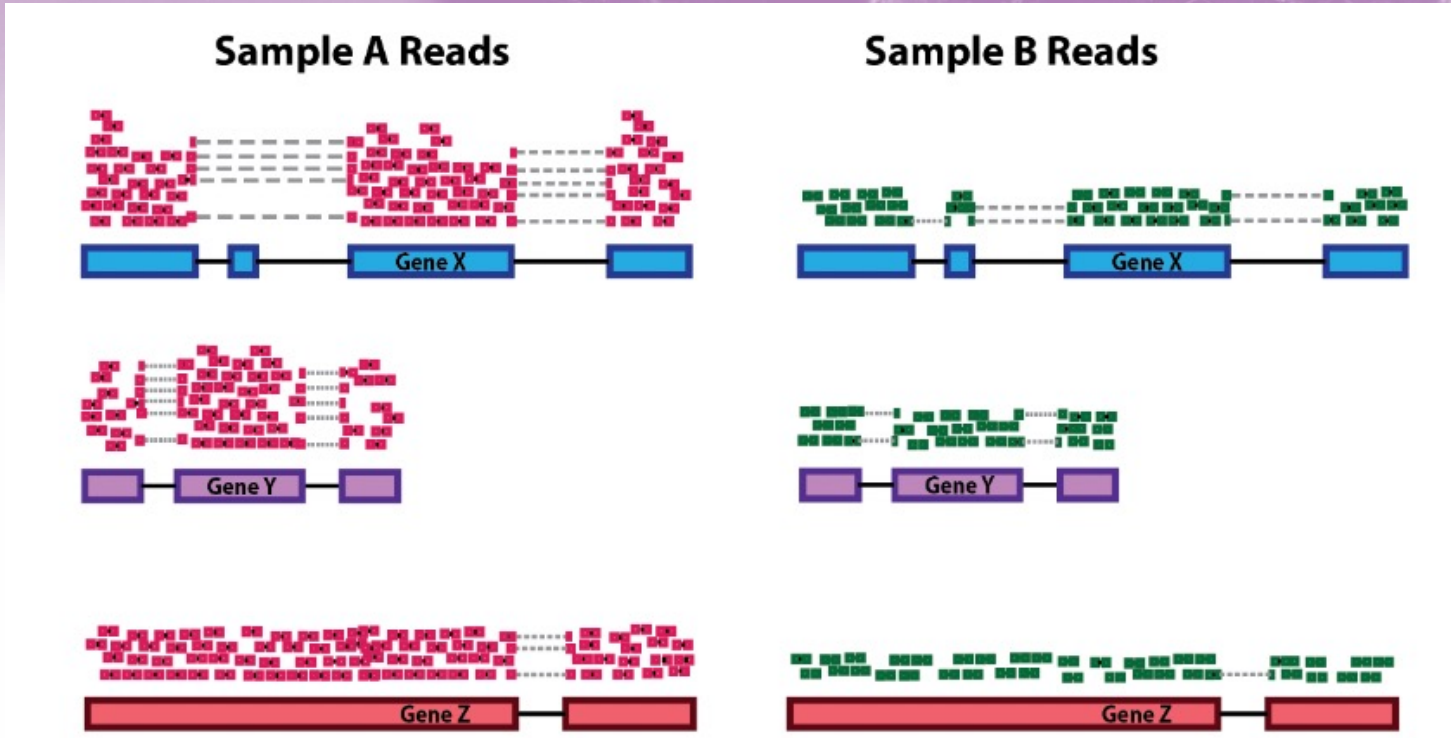
From Barham et al 2013

MD (mean-difference) plot



Identify genes (and molecular pathways) that are differentially expressed (DE) between two or more biological conditions

Many steps to calculate the Differentially Expressed Genes (DEG) between sample A and B



We need to make the counts **comparable** across samples

Workshop outline

- Intro to a real experiment – Demo
- Steps for Differentially Expressed Gene analysis
- Approach for DEG: edgeR
- Filtering genes
- Dealing with noisy data -> Normalization
- Quality check - Exploratory visualization: MDS – PCA
- Fit the model for DEG – dispersion estimate
- Which comparison and how to visualize the DEG

Reference for the workshop

F1000Research F1000Research 2016, 5:1438 Last updated: 06 DEC 2018

SOFTWARE TOOL ARTICLE

REVISED From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline [version 2; referees: 5 approved]

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[4]Department of Mathematics and Statistics, The University of Melbourne, Victoria, 3010, Australia

Dataset

Transcriptome analysis of luminal and basal cell subpopulations in the lactating versus pregnant mammary gland

- GEO (gene expression omnibus) accession: **GSE60450**
- Tissue of origin: Mammary glands of mouse
- Cell types: Basal stem-cell enriched cells (B) and committed luminal cells (L)
- Biological conditions: Virgin, Lactating (2 day) and Pregnant (18.5 day)
- # of groups: 2 cell types (B/L) x 3 conditions (V/L/P) = 6 groups
- # of replicates: 2 of each group
- Illumina Hiseq sequencer - about 30 million 100bp single-end reads for each sample.

https://www.ncbi.nlm.nih.gov/geo/

Files for the hands-on session

	GEO	SRA	CellType	Status
MCL1.DG	GSM1480297	SRR1552450	B	virgin
MCL1.DH	GSM1480298	SRR1552451	B	virgin
MCL1.DI	GSM1480299	SRR1552452	B	pregnant
MCL1.DJ	GSM1480300	SRR1552453	B	pregnant
MCL1.DK	GSM1480301	SRR1552454	B	lactating
MCL1.DL	GSM1480302	SRR1552455	B	lactating
MCL1.LA	GSM1480291	SRR1552444	L	virgin

- targets.txt

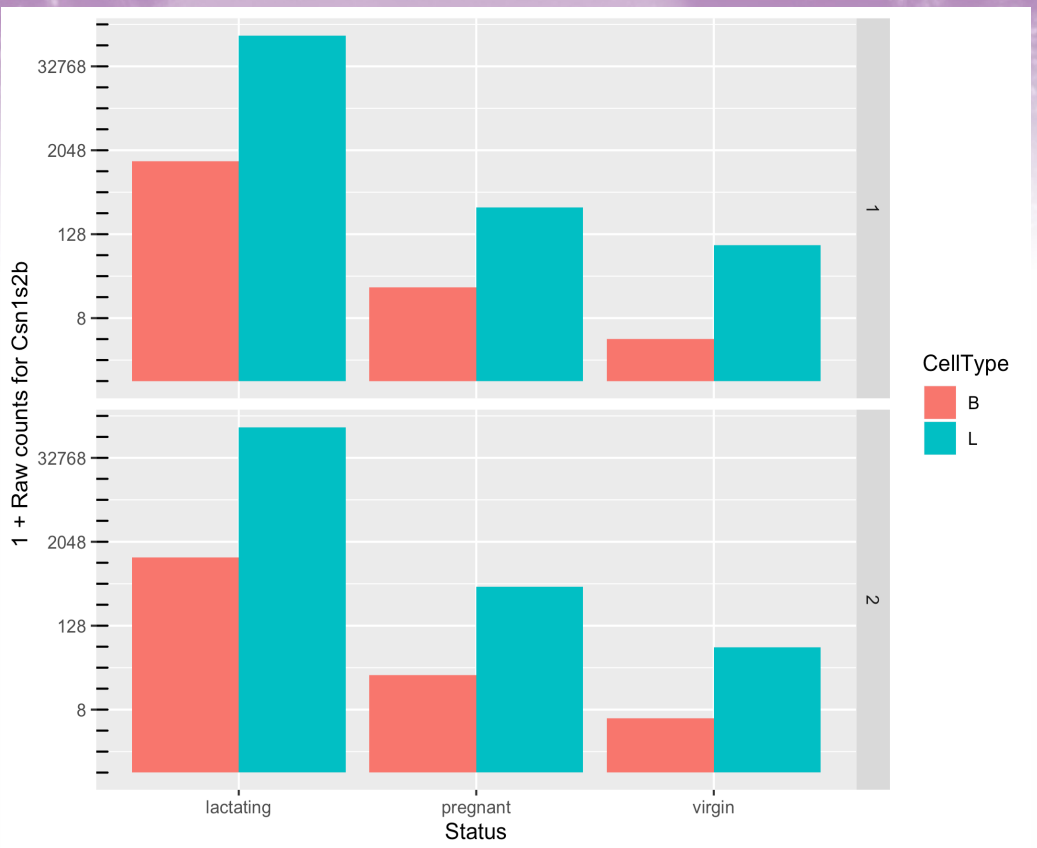
  Phenofile

- GSE60450_Lactation-GenewiseCounts.txt.gz

  Counts for each sample for each gene (Entrez Gene Identifiers)

	Length	MCL1.DG	MCL1.DH	MCL1.DI	MCL1.DJ	MCL1.DK	MCL1.DL	MCL1.LA	MCL1.LB
497097	3634	438	300	65	237	354	287	0	0
100503874	3259	1	0	1	1	0	4	0	0
100038431	1634	0	0	0	0	0	0	0	0
19888	9747	1	1	0	0	0	0	10	3
20671	3130	106	182	82	105	43	82	16	25
27395	4203	309	234	337	300	290	270	560	464

Goal: To identify a set of genes that are differentially expressed across samples

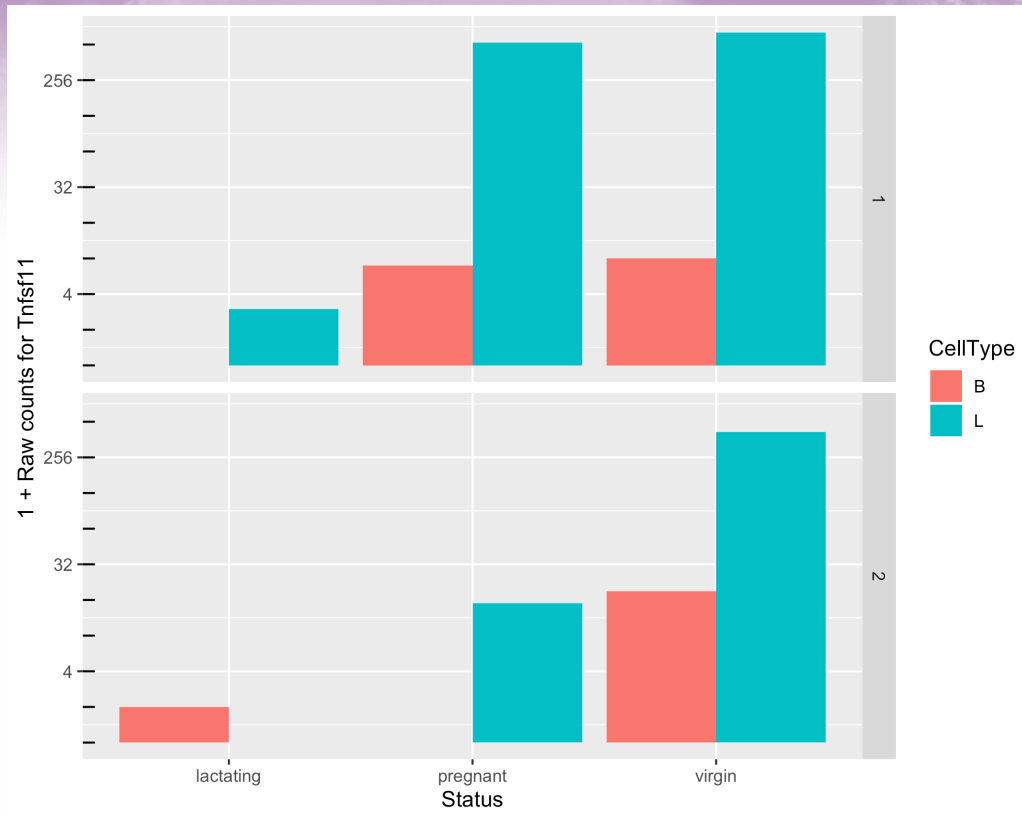


Which comparisons are we interested in?

Example:

1. B vs L,
2. B.lactating vs L.pregnant,
3. ...
4. All of them

Potential issues



How do we ensure high power for detection and high specificity when faced with noise?

Can we make reliable inferences for genes with very low counts? What should we consider “very low”?

Approach to identify DE genes: edgeR

- *Bioconductor package edgeR* utilizes a *theoretical model* that captures some of the *known* processes leading to noise in counts data. (*null model*)
- Assume that the data is generated according to this model.
- Given any observed level of difference in mean expression levels of a gene, compute the probability that the observation will result from the null model (p value)
- If the probability is very low (e.g., $p < 0.05$), infer that something may be happening that we did not account for in the null model. (e.g., biological processes in L cells for milk production)

Need to correct for multiple testing

P value represents the chance that we may be wrong in calling something significantly differential. Example:

- P = 0.01 means 1% chance that we may be wrong.
- P = 0.50 means 50% chance that we may be wrong.

More than 20k genes under consideration

=> if a certain difference in expression levels has only 1% chance of happening given the null model, it might be observed for 200 genes even if the null model were true for all the genes.

=> 200+ false positives

Hence, there is a need to adjust the p-values.

- The more genes we test, the more we must adjust.
- Reduce the number of tests by filtering out “uninteresting” genes.

“Uninteresting” genes
Filtering

- *Biological point of view:* minimal expression level of a gene -> translation into a protein -> biologically relevant

- *Statistical point of view*: low counts -> not enough statistical evidence.

Genes with consistently low counts are very unlikely be assessed as significantly DE

	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516
ENSG00000000003	67	44	87	40	1138
ENSG00000000005	0	0	0	0	0
ENSG00000000419	467	515	621	365	587
ENSG00000000457	260	211	263	164	245
ENSG00000000460	2	5	1	0	1

Normalization of the counts

Why we need to normalize the counts

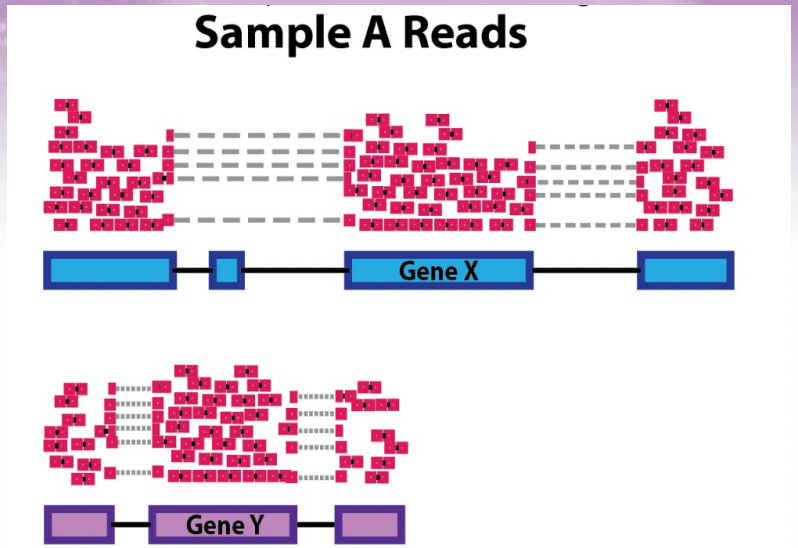
Identify and correct technical biases removing the least possible biological signal

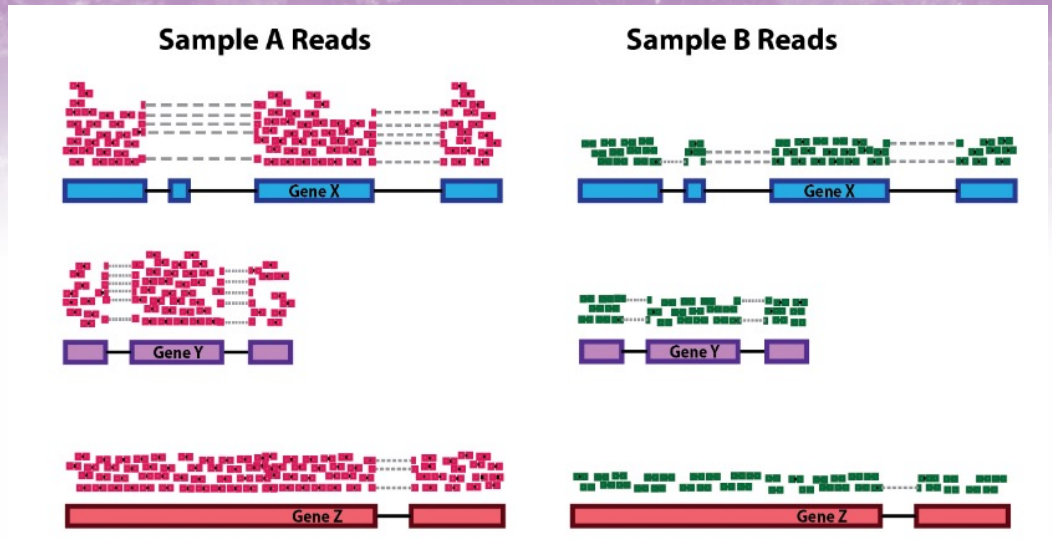
- library prep, sequencing technology, …

It is an essential step in the analysis of gene expression:

- to accurately estimate gene expressions in a sample
- to compare genes from different samples (differential analysis)

Why we need to normalize the counts





Compare gene expressions across genes (features)

DEG: Compare gene expressions across samples

Technical source of variability to account for

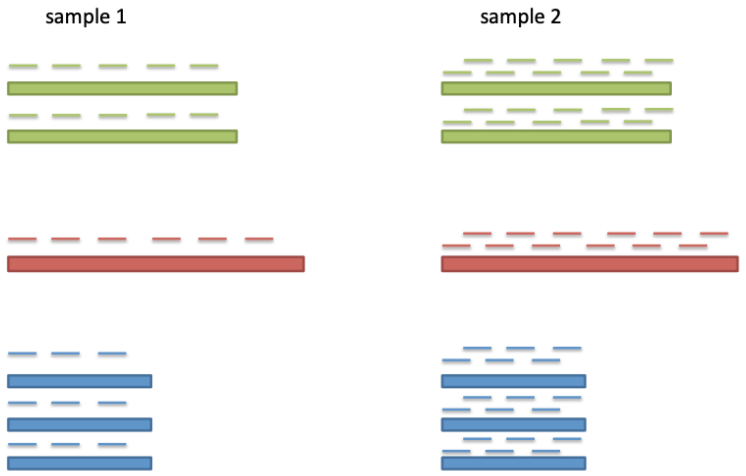
Source of technical variability

- Gene length
- Library size or sequence depth (number of mapped reads)
- Batch effects
- …

Gene-length and sequence depth

At the same expression level, a long gene will have more reads than a shorter gene

Higher sequencing depth, higher counts



Different sequence depths cause variation in counts

- Variation in sequencing depths => Need to normalize counts

Group	Total counts
B.virgin	23085177
B.virgin	21628857
B.pregnant	23919152
B.pregnant	22490570
B.lactating	21382233
B.lactating	19884434

Group	Total counts
L.virgin	20213223
L.virgin	21509988
L.pregnant	22073815
L.pregnant	21837341
L.lactating	24638939
L.lactating	24581591

Library size about 20M

Several normalization methods to remove technical bias

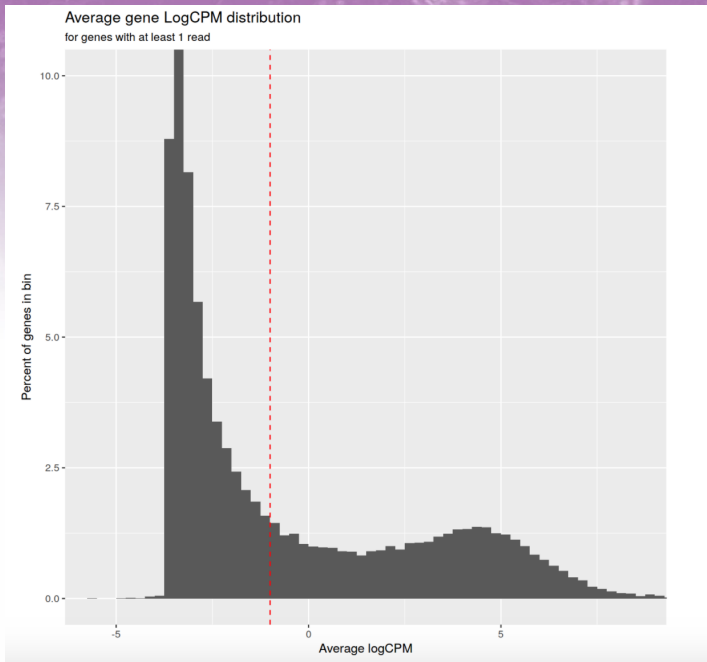
- Various *gene expression units* such as RPM(o CPM), RPKM, FPKM, TPM, TMM, *DESeq*, Scnorm
- Measure of the abundance of gene or transcripts.
- Normalized expression units are necessary to remove technical biases
  - *Depth of sequencing* correction
  - *Depth of sequencing* and *gene length* correction
- To remove batch effects

Within sample normalization methods

Sequence depth/Library size - Count Per Million mapped reads

$$\text{RPM or CPM} = \frac{\text{Number of reads mapped to gene} \times 10^6}{\text{Total number of mapped reads}}$$

Sequenced one library with 5 million(M) reads.
Total 4 M matched to the genome sequence
5000 reads matched to a given gene



Filter on count-per-million (CPM) values to avoid favoring genes that are expressed in larger libraries over those expressed in smaller libraries

A gene at least 10–15 counts in at least some libraries before it is considered to be expressed

Common methos to normalize the counts considering gene length

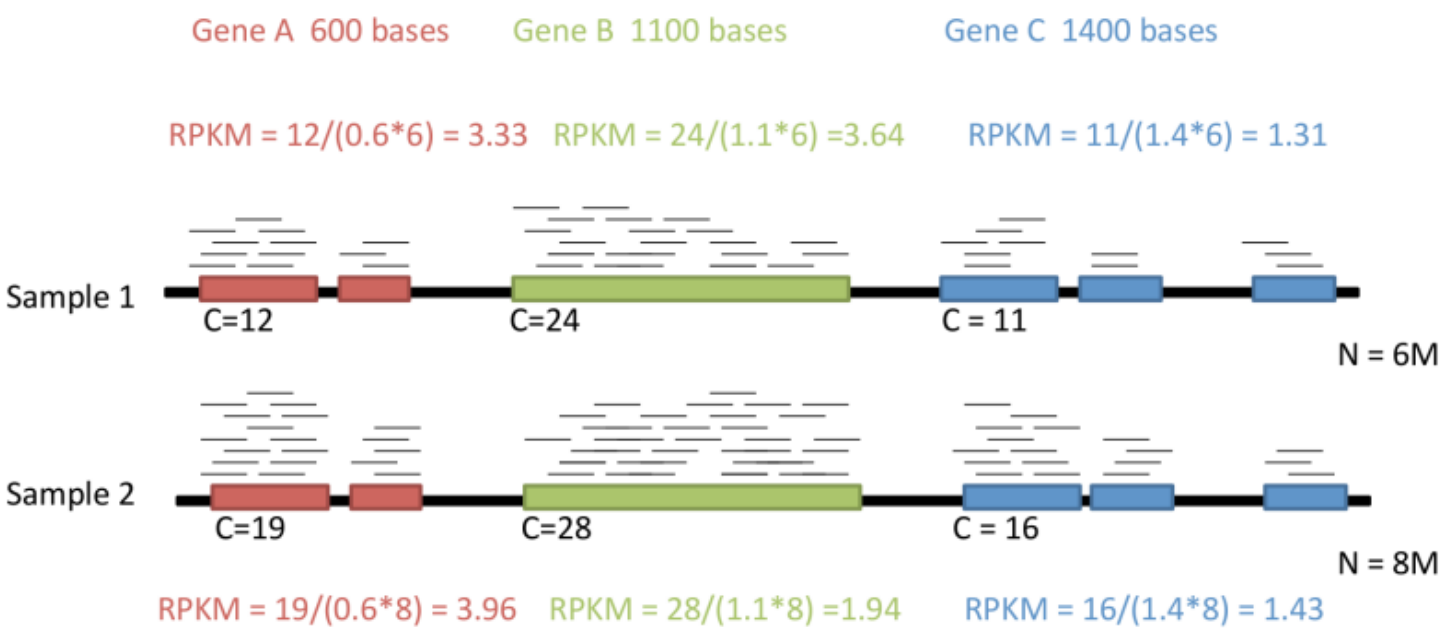
Commonly used normalization method that includes gene length correction

- RPKM /FPKM (Reads/Fragments Per Kilobase per Million)
- TPM (Transcripts Per kilobase Million)

Not very relevant for samples comparisons

Proved to be inadequate and biased

RPKM: seq depth and gene length bias



One library with 5 M reads
Total 4 M matched to the genome sequence
5000 reads matched to a given gene *with a length of 2000 bp.*

$$\text{RPKM} = \frac{\text{Number of reads mapped to gene} \times 10^3 \times 10^6}{\text{Total number of mapped reads} \times \text{gene length in bp}}$$

Biased: differentially expressed genes as the total normalized counts for each sample will be different

TPM: seq depth and gene length bias

Normalize for gene length first, and then normalize for sequencing depth

TPM average is constant and is proportional to the relative RNA molar concentration

The sum of all TPMs in each sample are the same -> to compare the proportion of reads that mapped to a gene in each sample.

RPKM and FPKM -> the sum of the normalized reads in each sample may be different, and this makes it harder to compare samples directly.

Between sample normalization methods

Between samples variation: observed counts depend on total reads sequenced and sample composition

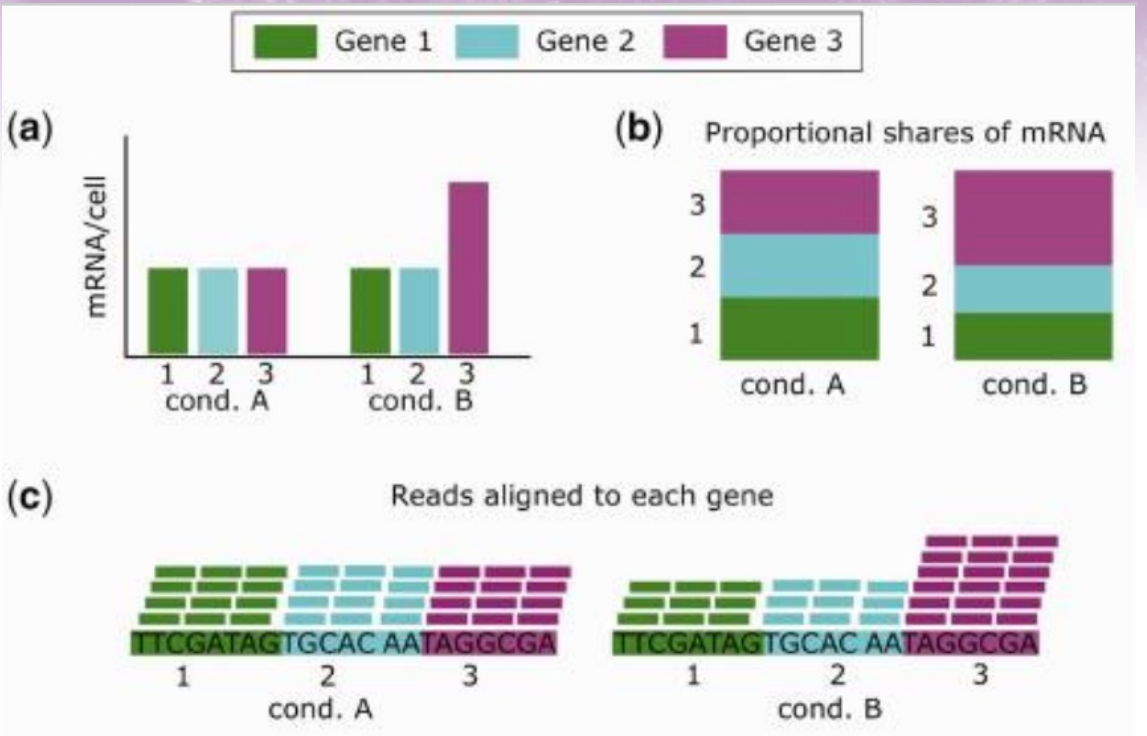
Gene expression values should be compared among samples

- Number of reads for GENE1 = $\frac{\text{Amount of nucleic acid from GENE1}}{\text{Total nucleic acid in sample}}$ x Total reads
- Need to normalize for difference in total reads between samples.
  - Might be enough if total nucleic acid is the same in both samples.
  - Example: technical replicates
- Need to account for difference in sample composition

Proportion of mRNA corresponding to a given gene may change across biological conditions

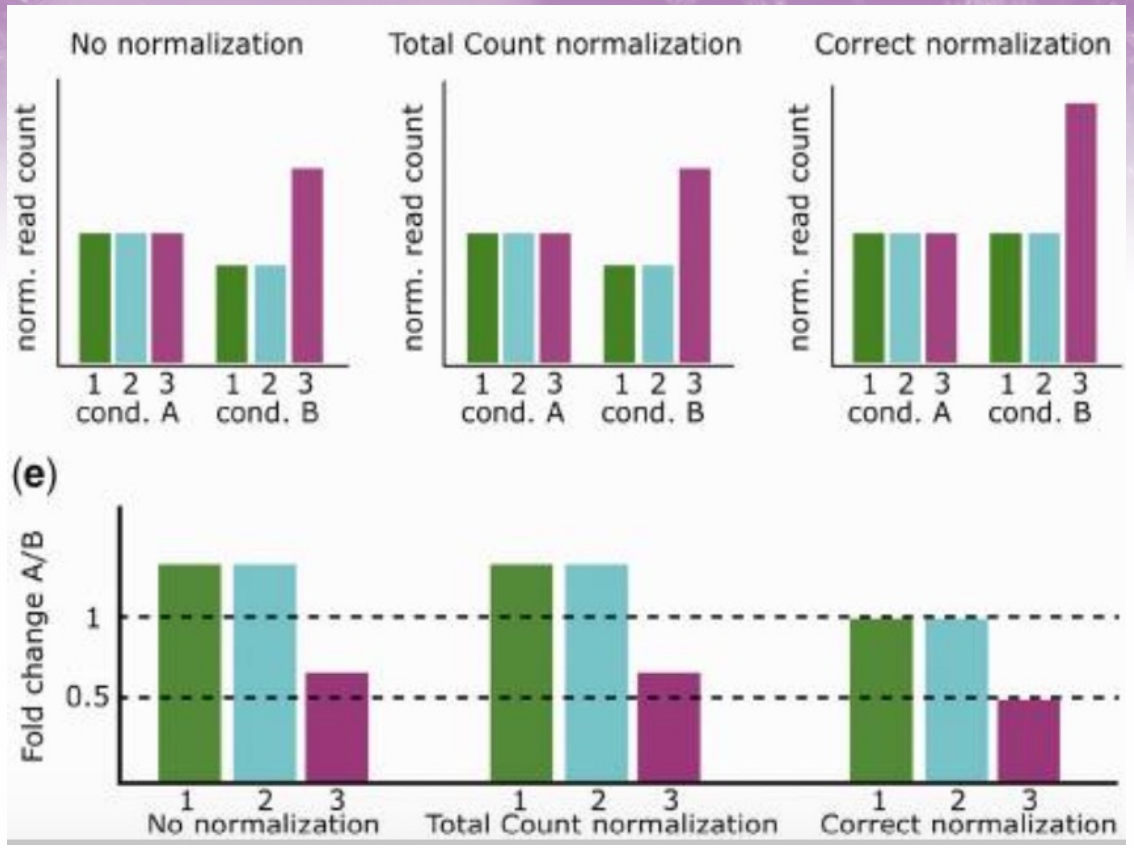
Few highly expressed genes make up a greater share of the total molecules (total library size) of samples B

Smaller fraction of the reads will be left for the other genes for that samples (undersampling)



doi: 10.1093/bib/bbx008

The goal is for differences in normalized read counts to represent differences in true expression



Total read count for each sample is the same

doi: 10.1093/bib/bbx008

Scaling factors

Normalize for RNA composition differences by scaling factor

A few highly differentially expressed genes may have a strong influence on read counts -> minimizing effect of such genes

Assumption: A majority of transcripts is not differentially expressed

Adjust counts such that for most genes, counts are not differential.

	group	lib.size	norm.factors
Sample1	1	10880519	1.17
Sample2	1	9314747	0.86
Sample3	1	11959792	1.32
Sample4	2	7460595	0.91
Sample5	2	6714958	0.83

Between sample normalization

Normalize for RNA composition by a set of scaling factors that minimize the log-fold changes between the samples for most genes

- *Reference sample*: have the closest average expressions to the mean of all samples
- *Test samples*: other samples

Scaling factor: weighted mean of log ratios between the test and reference, from a gene set removing most/lowest expressed genes (avg read counts) and genes with highest/lowest log ratios (differences in expression)

Normalization: Trimmed Mean of M-values

1. Choose a reference sample.
2. Compute the M and A values for all genes.
   M=log FC between ref and test; A=avg count gene between ref and test
3. Filter genes that fall in the tails of M and A distributions.
4. Estimate variance of M values.
5. Estimate TMM --- the weighted average of trimmed M-values.
6. Size factor is 2^{TMM}.
7. Adjust such that these multiply to 1.

TMM is implemented in edgeR and performs better for between-samples comparisons

Between samples variability

Assumption: most of the genes are not differentially expressed

TMM normalize the total RNA among the samples without considering gene length or library size

TMM good choice when

- comparing the samples from different tissues or genotypes
- RNA population would be significantly different among the samples

Other approaches to normalization

- RLE approach by Anders and Huber (2010)
  - Reference: geometric mean of all samples
  - Normalization factor: median ratio of each sample to the reference
  - RLE and TMM give similar results with real and simulated data
  - *R package DESeq*
- Upper quartile normalization by Bullard et al (2010)
  - Normalization factor: 75% quantile of the counts for each sample
  - Not recommended in general
- Total number of reads : TC (Marioni et al. 2008)
- Control genes (housekeeping genes, spike-in) to estimate technical noise (RUVSeq - 2014)

Best practice to choose a normalization

An effective normalization should result in a stabilization of read counts across samples

- TC, RPKM, UQ - Adjustment of distributions, implies a similarity between RNA repertoires expressed
- DESeq, TMM - More robust ratio of counts using several samples, suppose that the majority of the genes are not DE
- RUVSeq - Powerful when a large set of control genes can be identified

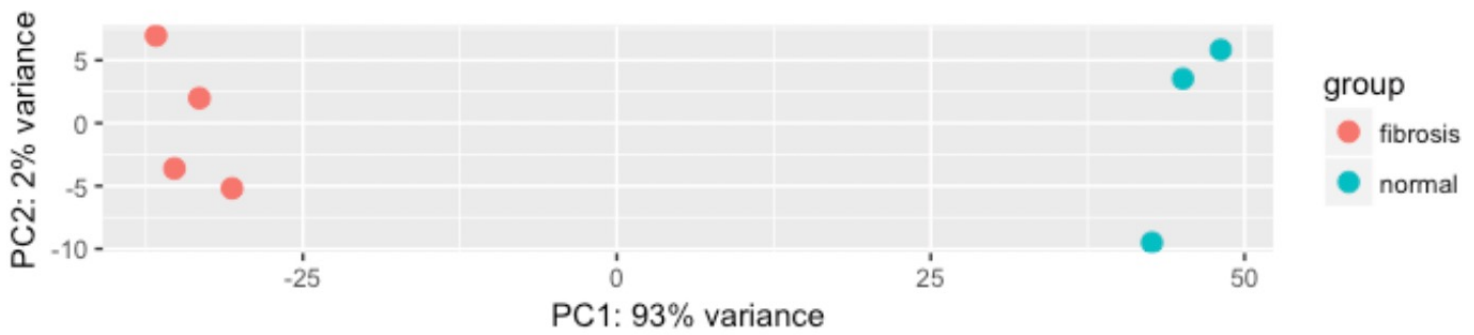
Break (5 min)

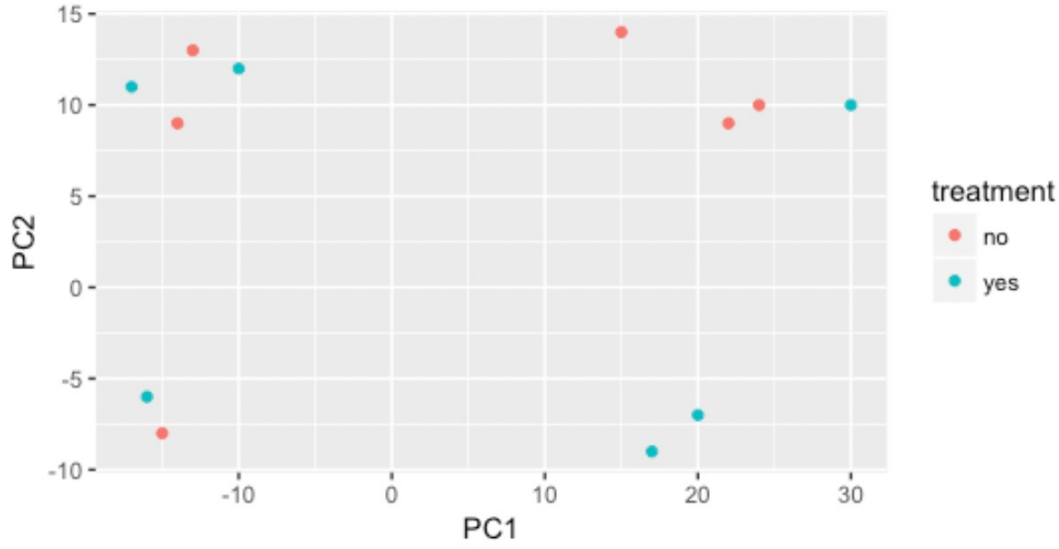
MDS and PCA plots

Assessing overall similarity across samples

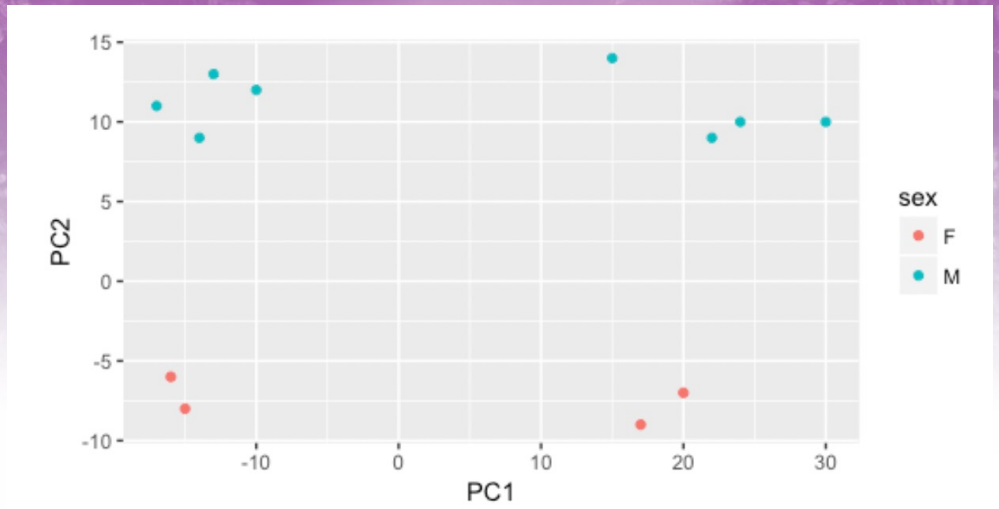
- Which samples are similar to each other, which are different?
- Does this fit to the expectation from the experiment's design?
- What are the major sources of variation in the dataset?

Ideal vs real experiment



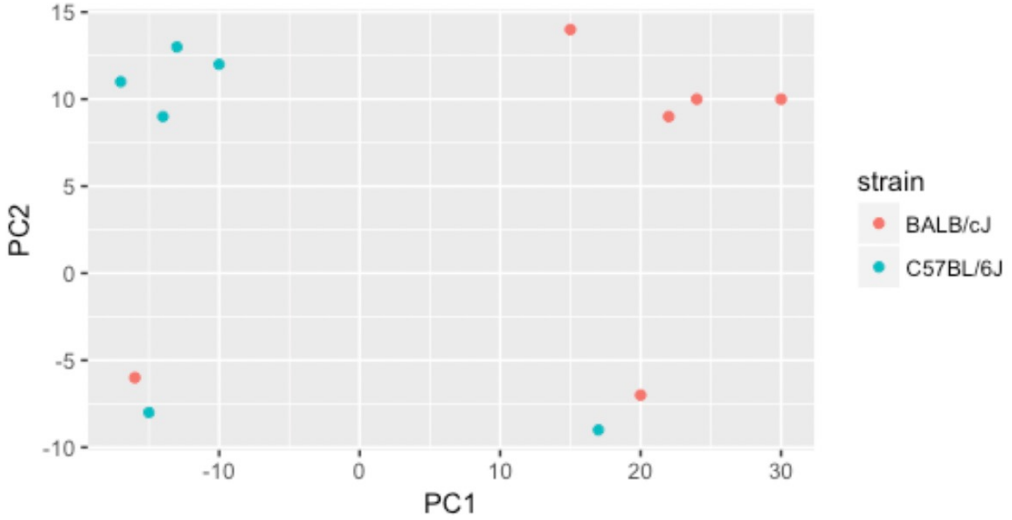


Identifying the source of variability



Variation in PC2

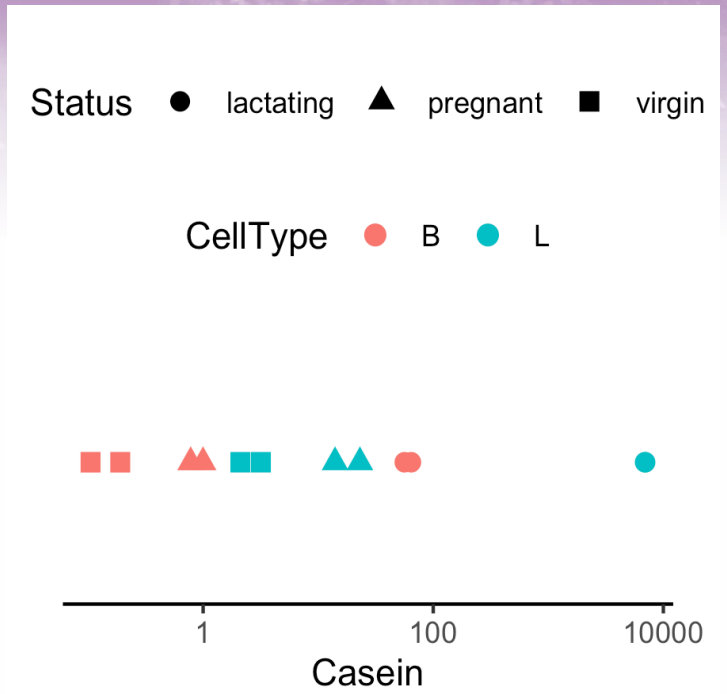
Variation in PC1



Expression level of Casein varies in a way that is strongly indicative of the effect of CellType and Status.

Why are the B.lactating samples not close to B.virgin and B.pregnant samples?

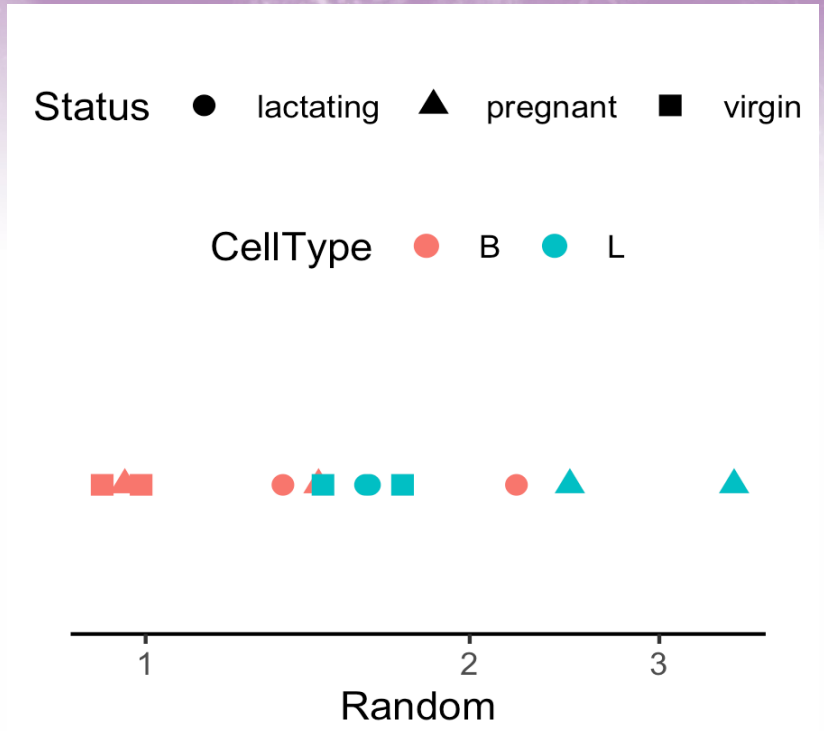
Could it be due to batch effects?



The distance between each pair of samples can be interpreted as the leading log-fold change between the samples for the genes that best distinguish that pair of samples.

Expression appears to vary across samples but…

In general, the way expression appears to vary across samples could be dominated by noise, batch effects, real signal, etc.



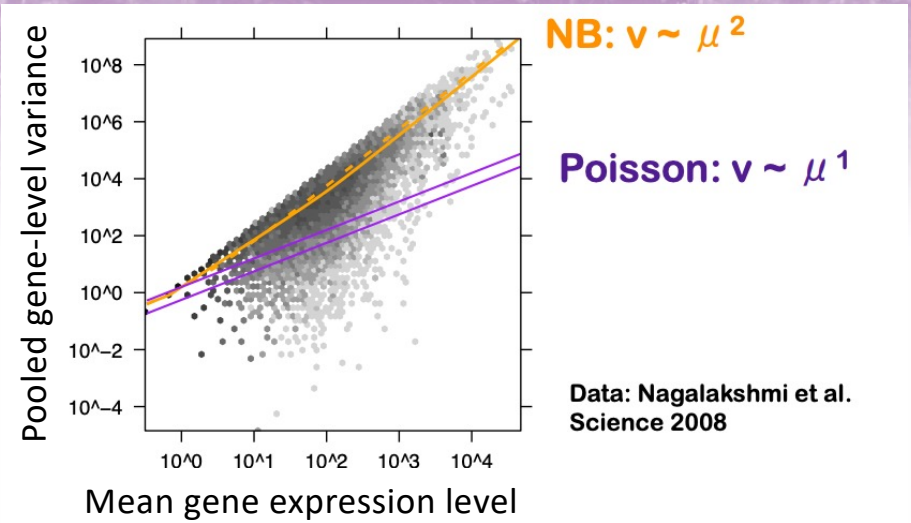
Fitting the model

RNA-seq data and overdispersion between samples

The number of reads that are mapped into a gene was first modeled using a Poisson distribution

Assumption: assumes that mean and variance are the same

The variance grows faster than the mean in RNAseq data.



Overdispersion in RNA-seq data

-> counts from biological replicates tend to have variance exceeding the mean (highly expressed genes)

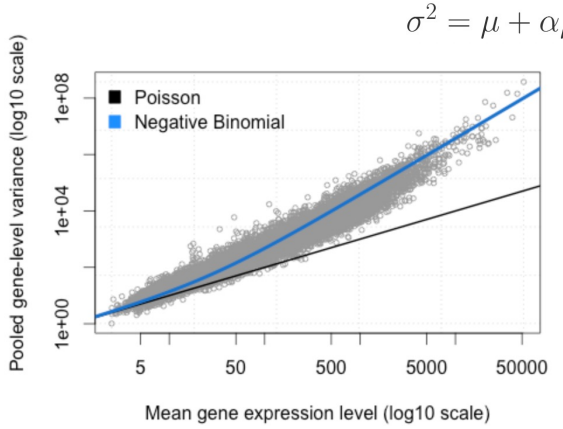
-> underestimation of the biological variance increased type I error rate (probability to falsely declare a gene DE)

Three dispersion estimates

The negative binomial (NB) distribution alternative to model the read counts for each gene *in each sample*

The variance is always larger than the mean for the negative binomial ⇒ suitable for RNA-seq data

$$\sigma^2 = \mu + \alpha\mu^2$$



Many genes, few biological samples - difficult to estimate φ on a gene-by-gene basis
Using information *across all genes* for stable estimates of φ.
3 ways to estimate φ: common, trended, tagwise (moderated)

Empirical Bayes shrinkage moderated dispersion for each individual gene

Empirical Bayes estimates of dispersion parameters: Learning from the experience of others

Dispersion accounts for variability between biological replicates

- Common dispersion: a global dispersion estimate averaged across the genome
- Trended dispersion: dispersion of a gene is predicted from its abundance
- Tagwise dispersion: measure of the degree of consistent inter-library variation for that tag – plotBCV (biological coeff variation)

Empirical Bayes estimates need to be controlled for the possibility of outlier genes with exceptionally large or small individual dispersions (robust=TRUE)

glmQLFit and variance of gene counts

- NB dispersions - higher for genes with very low counts and decrease smoothly with abundance and asymptotically to a constant value for genes with larger counts.

- Extended NB model to account for gene-specific variability from both biological and technical sources (quasi-likelihood)

1) NB dispersion trend is used to describe the overall biological variability across all genes (fit GLM)
2) For each gene-specific variability above and below the overall level (deviance) is picked up by the QL dispersion

edgeR statistical method

- Classic (pairwise comparisons between two or more groups), glm and glmQL
- QL for bulk RNA-seq:
  - + stricter error rate control (more rigorous dispersion and uncertainty)
  - + speed improvement compared to other quasi-methods
  - + appropriate for multiple treatment factors and with small # of biological replicates
  - + relative changes in expression levels between conditions (not absolute)

Limma package for large scale datasets

Get the DE genes - *glmQLFTest*

- Identifies differential expression based on statistical significance regardless of how small the difference might be -> 5000 DE genes between the basal pregnant and lactating groups.

- Interested only in genes with large expression changes -> subset of genes more biologically meaningful.

- Modify the statistical test to evaluate variability as well as the magnitude of change of expression values -> expression changes greater than a specified threshold

- glmTreat() - Not equivalent to a simple fold change cutoff : “the fold-change below which we are definitely not interested in the gene"

- The total number of DE genes identified at an FDR of 5% can be shown with *decideTestsDGE()*

Get the DE genes - *glmQLFTest*

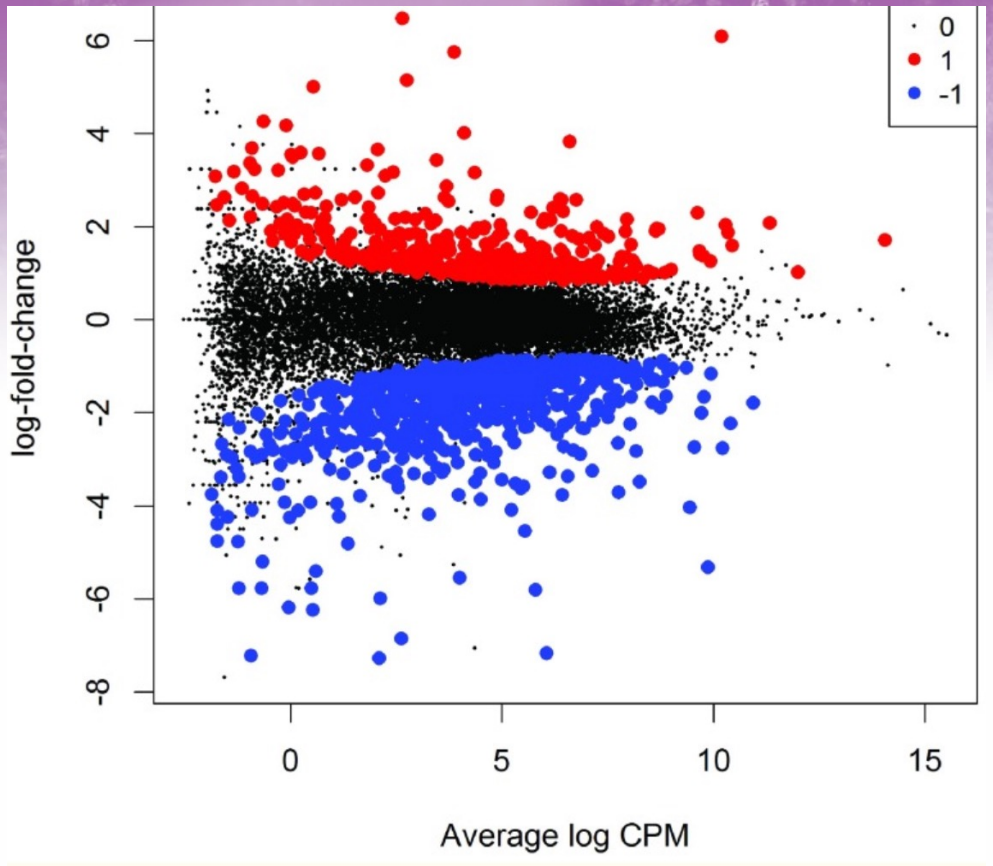
	genes	logFC	logCPM	F	PValue	FDR
PCDHA10	PCDHA10	-3.602	5.676	499.9	8.164e-11	1.354e-06
CHGA	CHGA	2.923	5.976	185.4	1.972e-08	0.0001635
ARRB1	ARRB1	-3.914	5.015	158.3	4.627e-08	0.0002019
TSSC2	TSSC2	3.175	3.301	156.8	4.869e-08	0.0002019

Complicated contrasts - *makeContrasts()*

- between lactating and pregnant mice is the same for basal cells as it is for luminal cells

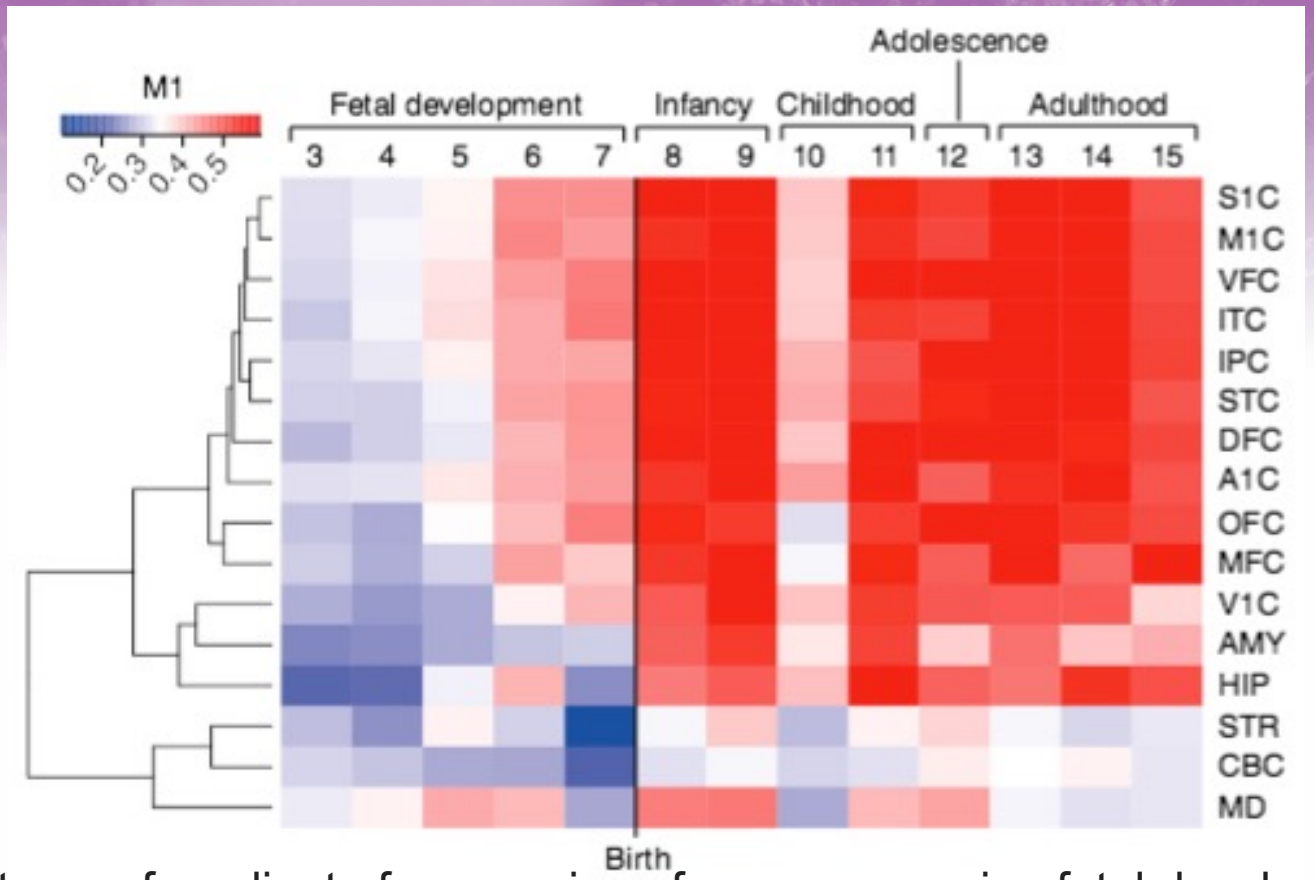
- the interaction effect between mouse status and cell type

MD plot: Over and under expressed genes



Log-fold change and average abundance of each gene

Visualization - heatmap



Pathway analysis

Heatmap of gradient of expression of genes, spanning fetal development to late adulthood and expressed in cortical regions

Workshop outline

- Intro to a real experiment – Demo
- Steps for Differentially Expressed Gene analysis
- Approach for DEG: edgeR
- Filtering genes
- Dealing with noisy data -> Normalization
- Quality check - Exploratory visualization: MDS – PCA
- Fit the model for DEG – dispersion estimate
- Which comparison and how to visualize the DEG

Your feedback is important to us!

At the end of the hands-on session:

Please take the survey ~3 min:
https://www.surveymonkey.com/r/F75J6VZ

Real data might need additional analyses choices that need experience.

Consult with the Gladstone Bioinformatics core for such scenarios and data.

Materials for this workshop

- Concepts: this presentation
- Hands-on session:
  - Sept2022handson.R
  - targets.txt
  - GSE60450_Lactation-GenewiseCounts.txt.gz

Please install the following library in Rstudio

```
Install.packages(magrittr)
Install.packages(edgeR)
Install.packages(org.Mm.eg.db)
Install.packages(tidyverse)
Install.packages(ggplot2)
Install.packages(statmod)
```

Hands-on session

- Load and reformat the data
- Exploratory visualization : MA plot
- Create DGEList object and retrieve gene symbols
- Filter genes with inadequate information
- Exploratory visualization : MDS and PCA plots
- Define and fit a model
- Hypothesis testing (four example hypotheses)
- Save results as a table and explore in Excel


Thank you!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