

Requirements for the demo

Please install the following library in Rstudio

```
install.packages("magrittr")
```

```
install.packages("statmod")
```

```
install.packages("tidyverse")
```

```
install.packages("ggplot2")
```

```
install.packages("BiocManager")
```

```
BiocManager::install("edgeR")
```

```
BiocManager::install("org.Mm.eg.db")
```

Verify the installation:

```
library(magrittr)
```

```
library(statmod)
```

```
library(tidyverse)
```

```
library(ggplot2)
```



```
library(edgeR)
```

Reference for the workshop



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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Upcoming workshop

- **February 24-25** | Introduction to Statistics, Experimental Design and Hypothesis Testing
- **March 3** | Introduction to Machine Learning

Winter workshops schedule - [Data Science Training Program](#)

Intermediate RNA-seq data analysis using R

Michela Traglia, Min-Gyoung Shin
Bioinformatics Core, GIDB

February 20th, 2025

GLADSTONE
INSTITUTES

Introductions

Min-Gyoung Shin

Bioinformatician III

Michela Traglia

Senior Statistician

Assumed background

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

Poll 1

How familiar you are:

1. I attended the 'Introduction to RNA-seq' Gladstone workshop or I similar courses
2. I attended 'Introduction to R' Gladstone workshop/experience with
3. I have experience in analysing the RNASeq data
4. No experience with RNAseq
5. No experience with R

Materials for this workshop

Please download the compressed file **2025Feb_intermediatRNAseq.zip**

Double click on the file, the unzipped folder includes:

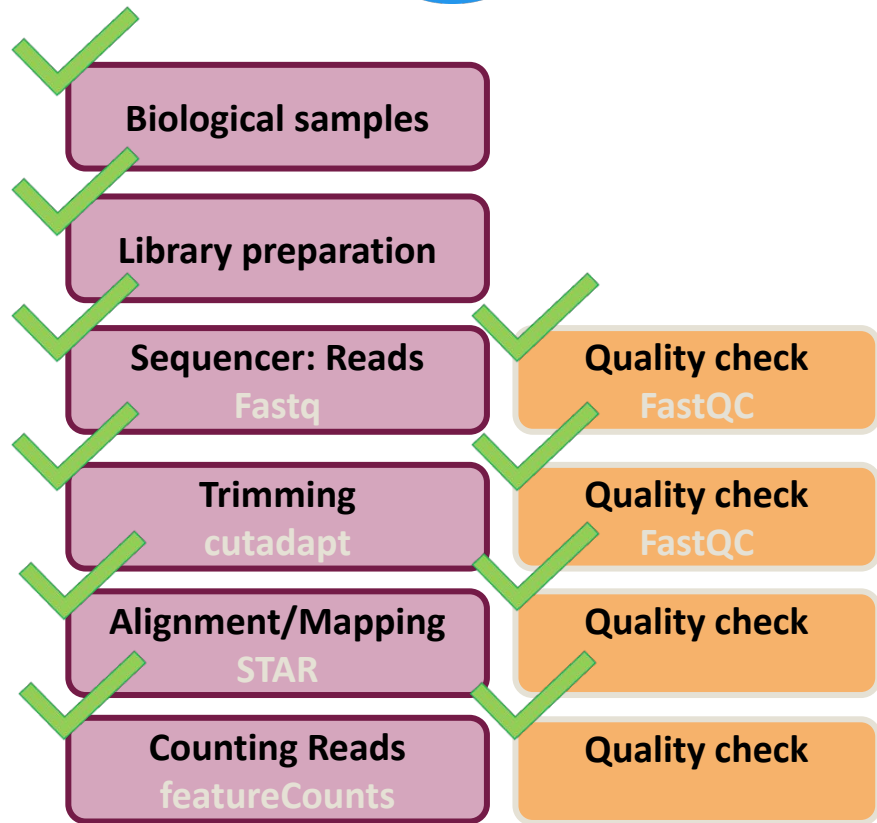
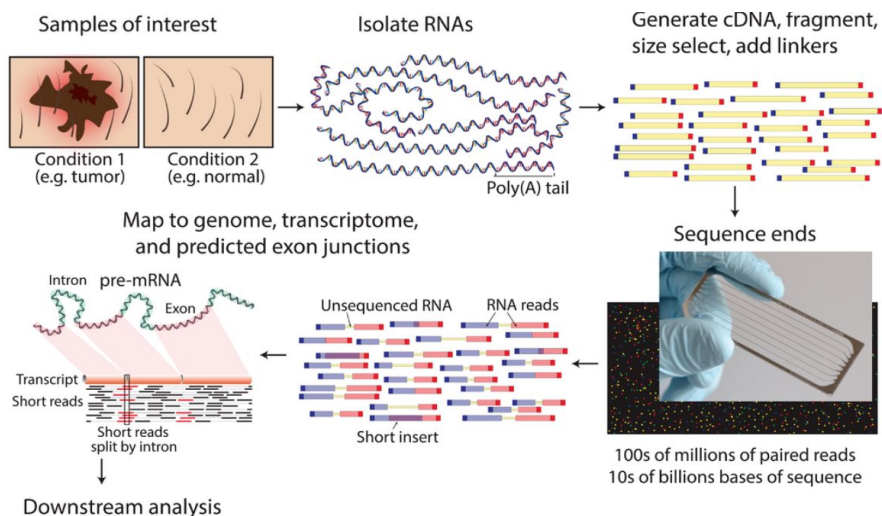
- Hands-on session files:
 - handson.R
 - targets.txt
 - DE_resutls.txt
 - GSE60450_Lactation-GenewiseCounts.txt.gz

This [presentation](#) with concepts

Workshop outline

- Intro to a real experiment
- Approach for Differentially Expressed Gene analysis: edgeR
- Filtering genes
- Normalization
 - Demo I
- Exploratory visualization: MDS – PCA
- Fit the model for DEG
- Compare groups and visualize the DEG
 - Demo II

RNA-seq - analysis workflow



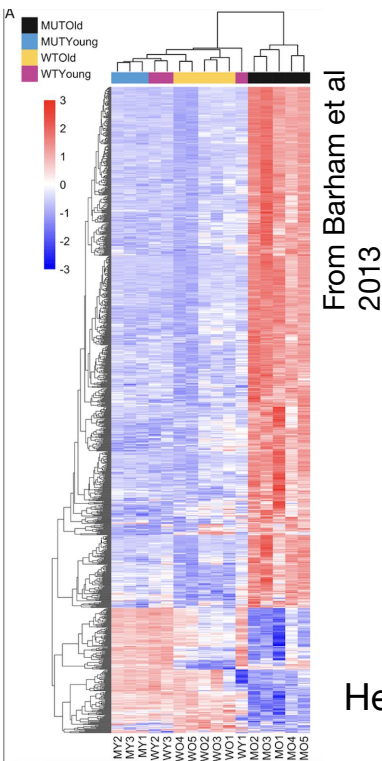
Today: From the count matrix to the DEG

Each column is a sample

Each row is a gene

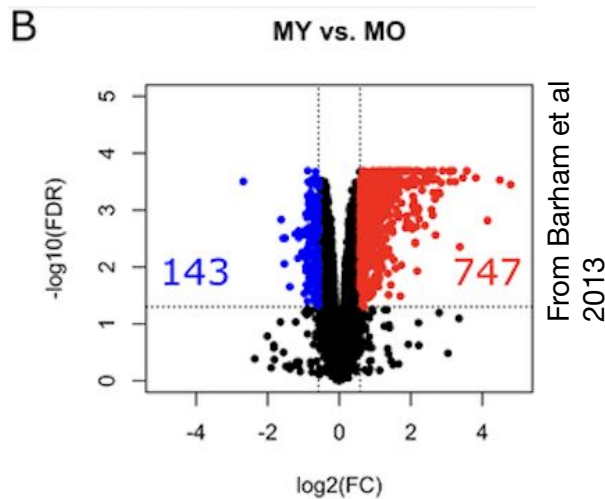
GENE ID	KD.2	KD.3	OE.1	OE.2	OE.3	IR.1	IR.2	IR.3
1/2-SBSRNA4	57	41	64	55	38	45	31	39
A1BG	71	40	100	81	41	77	58	40
A1BG-AS1	256	177	220	189	107	213	172	126
A1CF	0	1	1	0	0	0	0	0
A2LD1	146	81	138	125	52	91	80	50
A2M	10	9	2	5	2	9	8	4
A2ML1	3	2	6	5	2	2	1	0
A2MP1	0	0	2	1	3	0	2	1
A4GALT	56	37	107	118	65	49	52	37
A4GNT	0	0	0	0	1	0	0	0
AA06	0	0	0	0	0	0	0	0
AAA1	0	0	1	0	0	0	0	0
AAAS	2288	1363	1753	1727	835	1672	1389	1121
AACS	1586	923	951	967	484	938	771	635
AACSP1	1	1	3	0	1	1	1	3
AADAC	0	0	0	0	0	0	0	0
AADACL2	0	0	0	0	0	0	0	0
AADACL3	0	0	0	0	0	0	0	0
AADACL4	0	0	1	1	0	0	0	0
AADAT	856	539	593	576	359	567	521	416
AAGAB	4648	2550	2648	2356	1481	3265	2790	2118
AAK1	2310	1384	1869	1602	980	1675	1614	1108
AAMP	5198	3081	3179	3137	1721	4061	3304	2623
AANAT	7	7	12	12	4	6	2	7
AARS	5570	3323	4782	4580	2473	3953	3339	2666
AARSD	4454	2323	3281	3121	1340	2488	2074	1657

Goal of DEG analysis



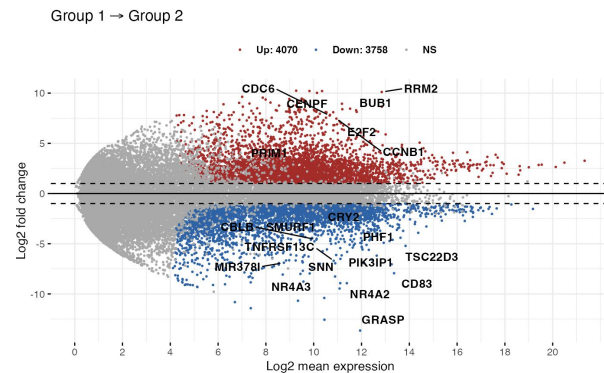
Heatmap

Volcano plot



From Barham et al
2013

MA (mean-difference) plot



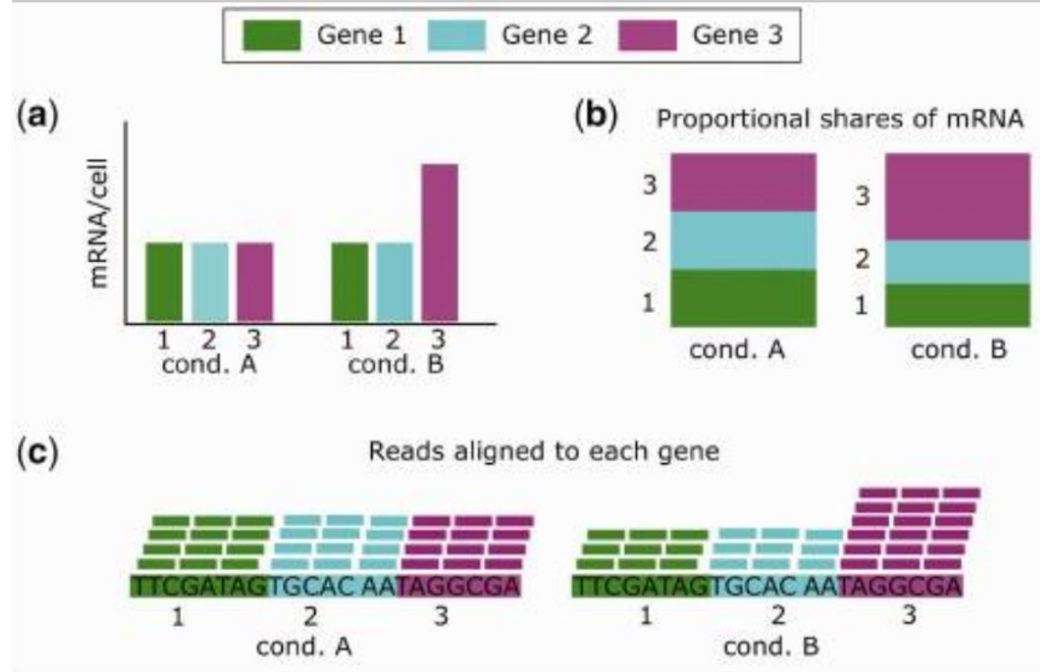
Identify genes (and molecular pathways) that are differentially expressed (DE) between two or more biological conditions

Highly expressed genes bias the real DEG

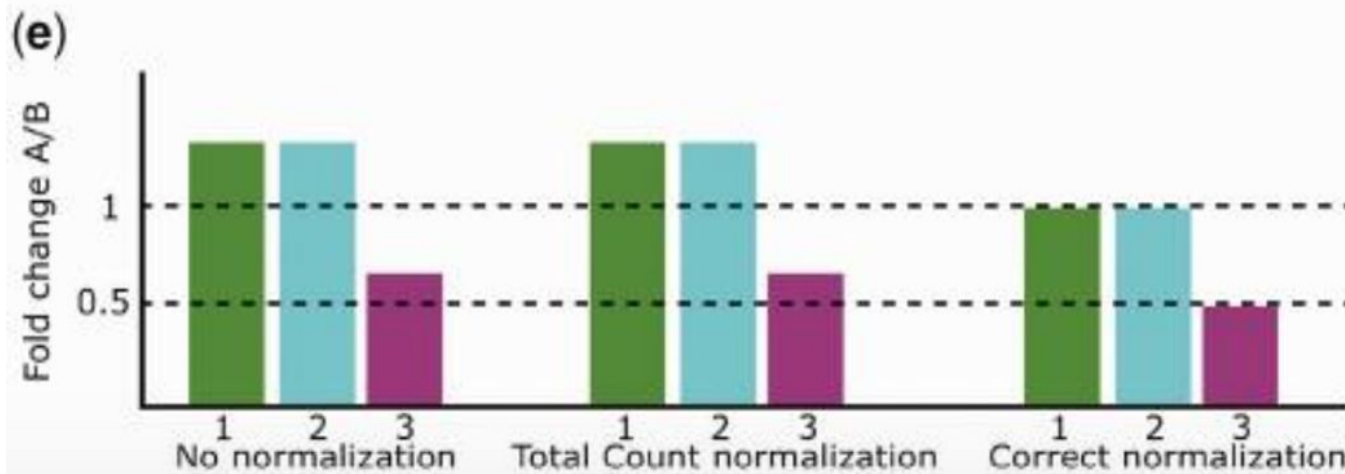
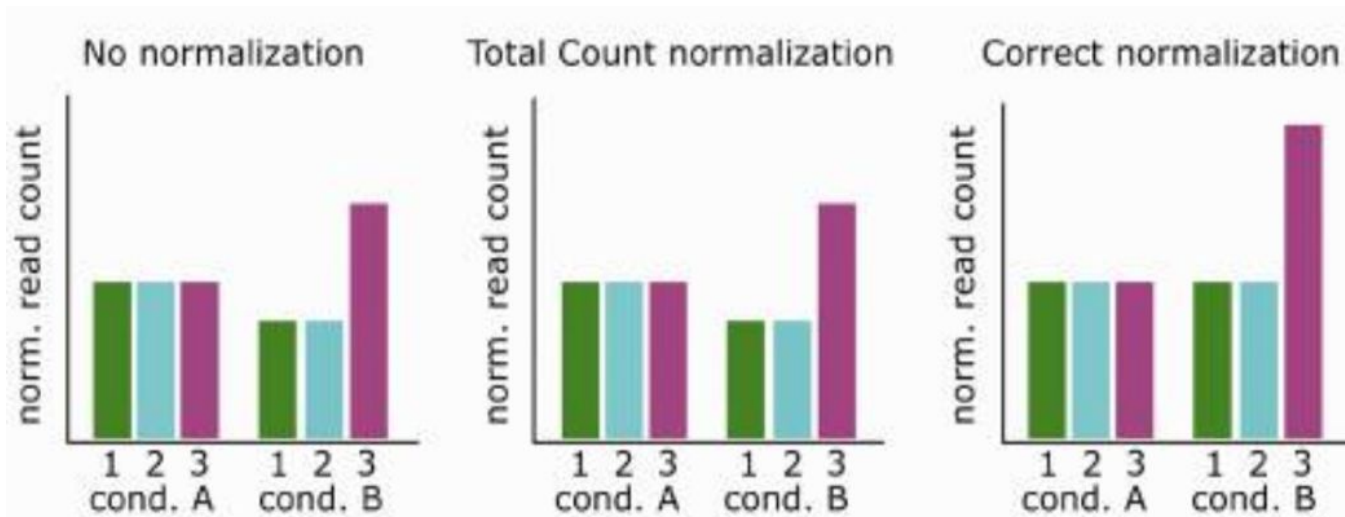
Total read count for each sample is the same.

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](https://doi.org/10.1093/bib/bbx008)



Gene 1 and 2 not-DE
Gene 3 is 2X DE

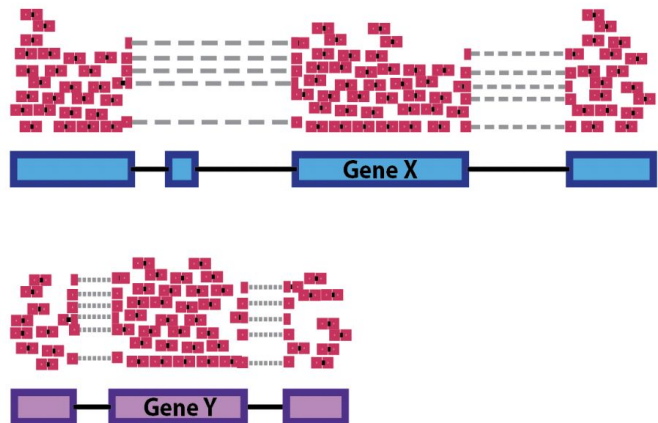
Please find more details on our wiki page

[Introduction-to-RNA-Seq-Analysis](#)

Possible analyses

Within a sample

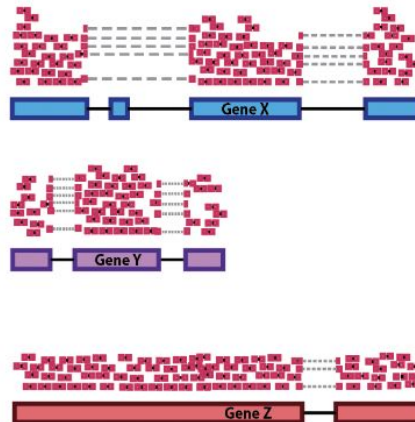
Sample A Reads



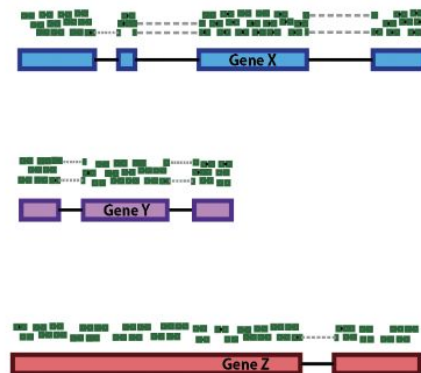
Estimate and compare gene expressions
across genes (features)

Between samples

Sample A Reads



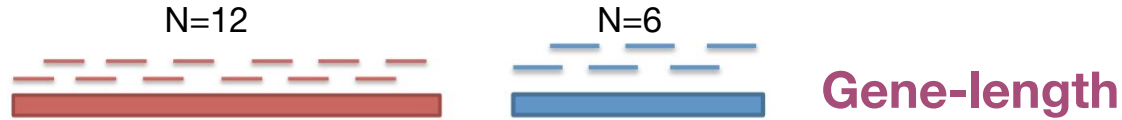
Sample B Reads



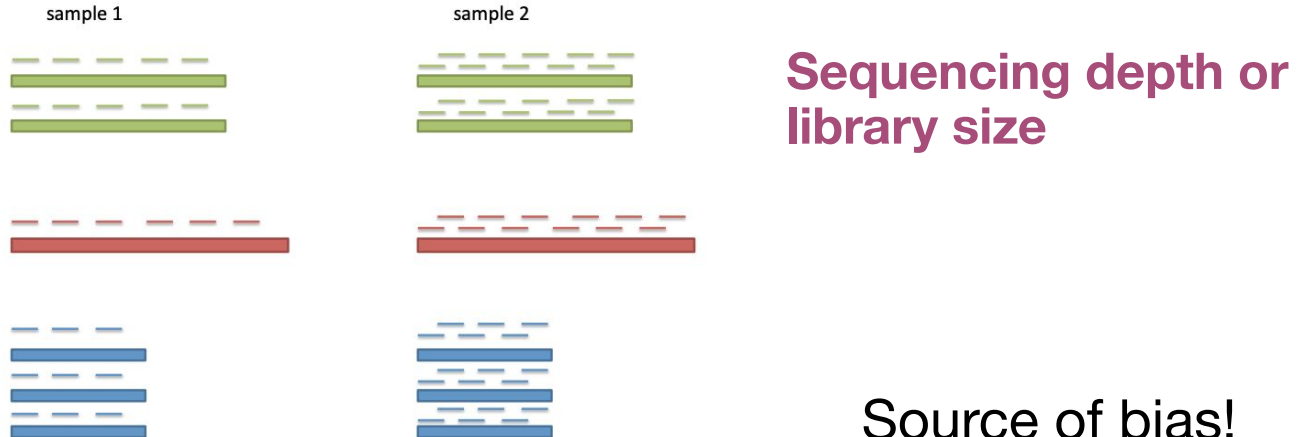
DEG: Compare gene expressions across samples

Sequence depth - total number of reads

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



Higher sequencing depth, higher counts

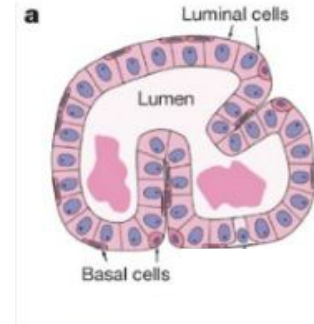


Different sequence depths cause variation in counts

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

Source of technical variability

Identify and correct technical biases removing the least possible biological signal.

- Gene length
- Library size or sequence depth (number of mapped reads)
- RNA sample composition
- Batch effects
- ...

Poll 2

What is the source of bias when quantifying the gene expression in one mouse only for each gene?

1. Gene length
2. Sequencing depth
3. Both

Poll 3

What is the source of bias when comparing the gene expression of one gene between treated mice and control mice?

1. Gene length
2. Sequencing depth
3. Both

Several normalization methods to remove technical bias

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

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

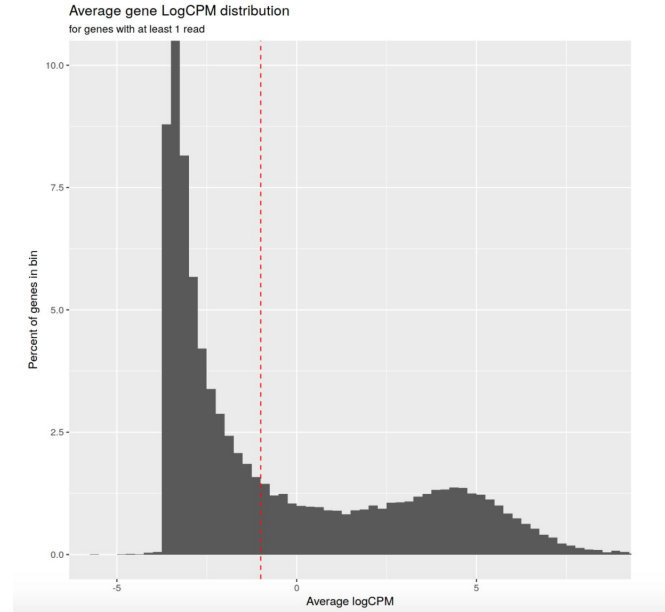
5000 reads matched to a given gene

$$\text{RPM or CPM} = \frac{5000 \times 10^6}{4 \times 10^6} = 1250$$

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 -> Identifying the CPM that corresponds to 10-15 counts

For comparison within replicates or same group, NOT for within sample, NOT for DEG



Common methods to normalize the counts considering gene length

Commonly used normalization method that includes sequence depth and gene length correction:

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

RPKM: seq depth and gene length bias

Reads/Fragments Per Kilobase per Million

Gene A 600 bases

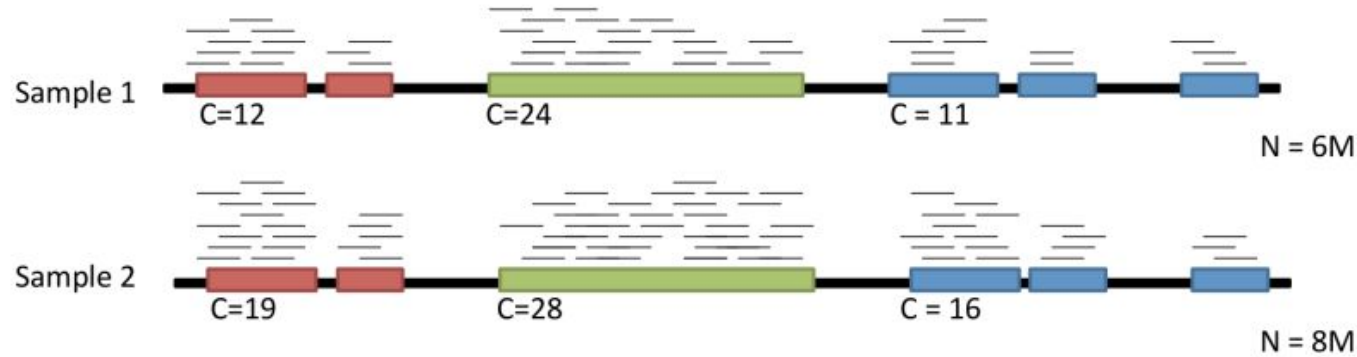
Gene B 1100 bases

Gene C 1400 bases

$$\text{RPKM} = 12 / (0.6 * 6) = 3.33$$

$$\text{RPKM} = 24 / (1.1 * 6) = 3.64$$

$$\text{RPKM} = 11 / (1.4 * 6) = 1.31$$



$$\text{RPKM} = 19 / (0.6 * 8) = 3.96$$

$$\text{RPKM} = 28 / (1.1 * 8) = 1.94$$

$$\text{RPKM} = 16 / (1.4 * 8) = 1.43$$

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

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.*

$$\text{RPKM} = \frac{5000 \times 10^3 \times 10^6}{4 \times 10^6 \times 2000} = 625$$

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

TPM: seq depth and gene length bias

TPM: Normalize for gene length first to get the Reads Per Kilobase, sum up all the RPK in a sample (across all genes), and then normalize for sequencing depth

$$\text{TPM} = 10^6 * \frac{\text{reads mapped to transcript} / \text{transcript length}}{\text{Sum}(\text{reads mapped to transcript} / \text{transcript length})}$$

Represent the relative abundance of a transcript among a population of sequenced transcripts

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

Same denominator -> comparable - NOT for RPKM (different denominator)

Sample1 - gene1 TPM=2.5

Sample2 - gene1 TPM=2.5

Main normalization approaches summary

Normalization method	Description	Accounted factors	Recommendations for use
CPM (counts per million)	counts scaled by total number of reads	sequencing depth	gene count comparisons between replicates of the same sample group; NOT for within sample comparisons or DE analysis
TPM (transcripts per kilobase million)	counts per length of transcript (kb) per million reads mapped	sequencing depth and gene length	gene count comparisons within a sample or between samples of the same sample group; NOT for DE analysis
RPKM/FPKM (reads/fragments per kilobase of exon per million reads/fragments mapped)	similar to TPM	sequencing depth and gene length	gene count comparisons between genes within a sample; NOT for between sample comparisons or DE analysis

Don't use these methods for between condition/groups comparison!

When total RNA composition is similar across samples, these methods could be potentially used.

But you can't assume it!

Cells don't necessarily produce similar levels of RNA/cell between cell types, disease states or developmental stages is not always valid

Sample-to-sample variability in total RNA concentration

Observed counts depend on total reads sequenced AND sample composition

- Number of reads for GENE1 = $\frac{\text{Amount of nucleic acid from GENE1}}{\text{Total nucleic acid in sample}} \times \text{Total reads}$
- 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



Methods for between samples comparisons / DEG

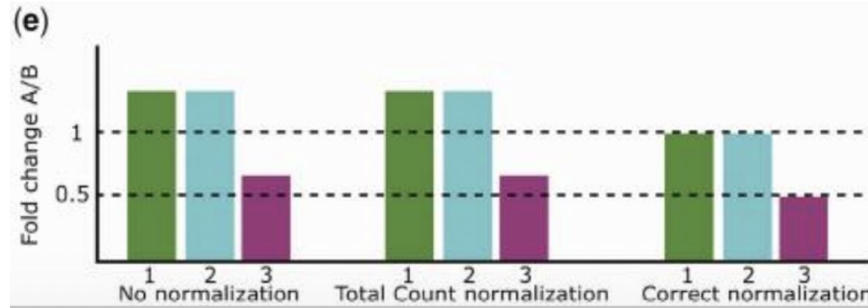
Approach to identify DE genes: edgeR

- *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)

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.



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
ENSG000000000419	467	515	621	365	587
ENSG000000000457	260	211	263	164	245
ENSG000000000460	2	5	1	0	1

Genes with extreme count outlier

Genes with zero counts

Genes with low mean normalized counts

Using cpm to filter out genes

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

Compositional biases: certain genes have much higher read counts due to technical reasons

- > Consider when calculating the library size
- > Scaling factors used to adjust the library size

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

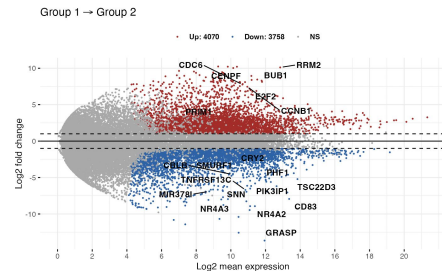
Scaling factor: weighted mean of log ratios between the test and reference, from a gene set removing most/lowest expressed genes (**avg read counts**) and genes with highest/lowest log ratios (**differences in expression**)

```
y <- calcNormFactors(y)
```

Normalization with edgeR: Trimmed Mean of M-values

MA (mean-difference) plot

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, when comparing the samples from different tissues or genotypes.

lib.size	norm.factors
23218026	1.2368993
21768136	1.2139485
24091588	1.1255640
22656713	1.0698261
21522033	1.0359212
20008326	1.0872153
20384562	1.3684449
21698793	1.3653200
22235847	1.0047431
21982745	0.9232822
24719697	0.5291015
24652963	0.5354877

Other approaches to normalization

Relative Log Expression (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* - [tutorial](#)

○ Upper quartile normalization by Bullard et al (2010)

- Normalization factor: 75% quantile of the counts for each sample
- Not recommended in general

○ Control genes (housekeeping genes, spike-in) to estimate technical noise ([RUVSeq](#) – 2014 Remove Unwanted Variation)

Best practice to choose a normalization

An effective normalization should result in a stabilization of read counts across samples (eliminate composition biases between libraries)

- TC, RPKM, UQ - Adjustment of distributions, implies a similarity between RNA molecular 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

Main normalization approaches summary

Normalization method	Description	Accounted factors	Recommendations for use
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RPKM/FPKM (reads/fragments per kilobase of exon per million reads/fragments mapped)	similar to TPM	sequencing depth and gene length	gene count comparisons between genes within a sample; NOT for between sample comparisons or DE analysis
DESeq2's median of ratios [1]	counts divided by sample-specific size factors determined by median ratio of gene counts relative to geometric mean per gene	sequencing depth and RNA composition	gene count comparisons between samples and for DE analysis ; NOT for within sample comparisons
EdgeR's trimmed mean of M values (TMM) [2]	uses a weighted trimmed mean of the log expression ratios between samples	sequencing depth, RNA composition	gene count comparisons between samples and for DE analysis ; NOT for within sample comparisons



Break (5 min)



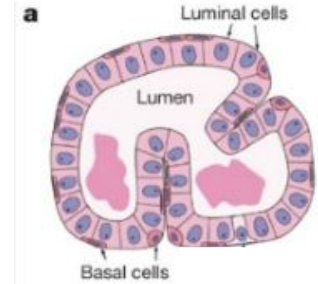
Demo I

Hands-on session

- oLoad and reformat the data
- oExploratory visualization : MA plot
- oCreate DGElist object and retrieve gene symbols
- oFilter genes with inadequate information
- oNormalization
- oExploratory visualization : MDS and PCA plots
- oDefine and fit a model
- oHypothesis testing (four example hypotheses)
- oSave results as a table and explore in Excel

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.

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](#)

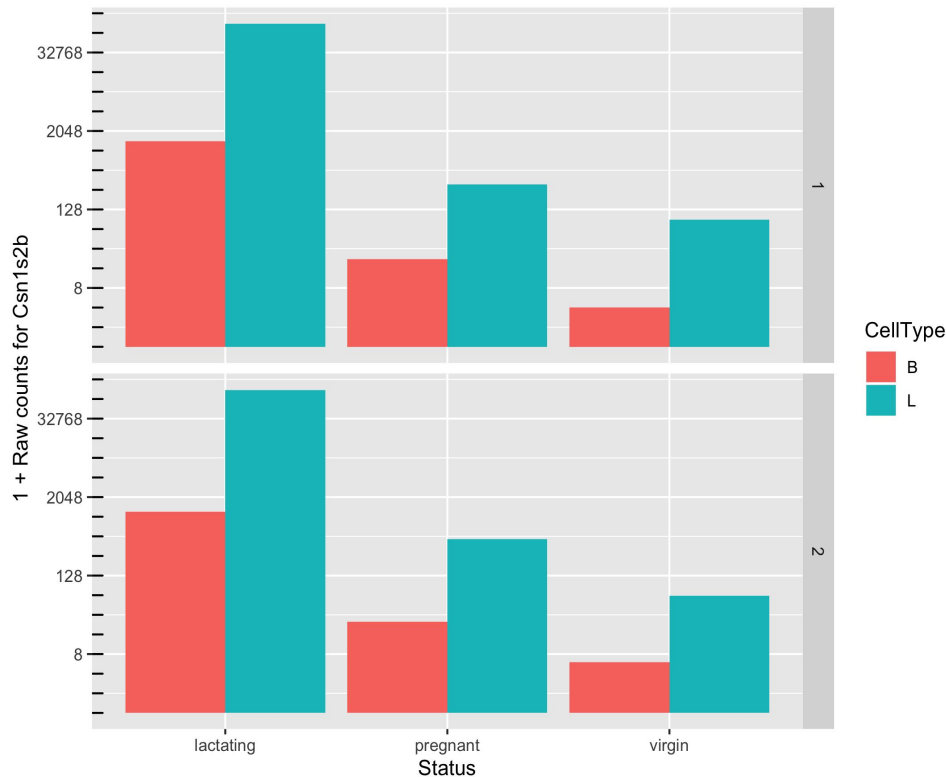
Phenofile

○ [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

Potential biological questions



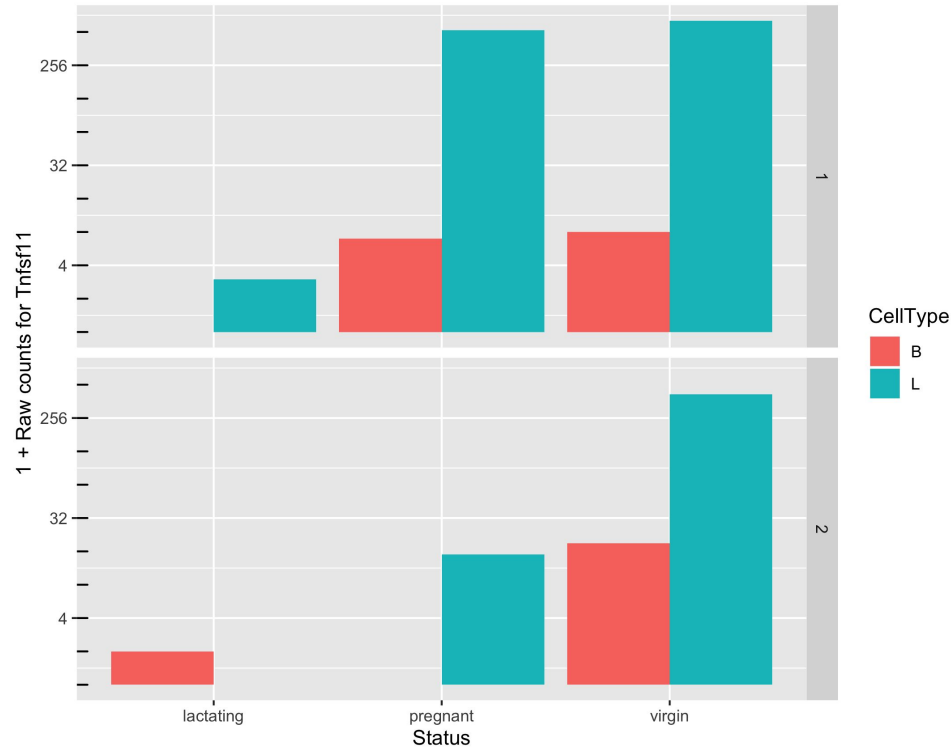
Which comparisons are we interested in?

Example:

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

Or not interested in comparisons but in gene expression of one sample

Potential issues



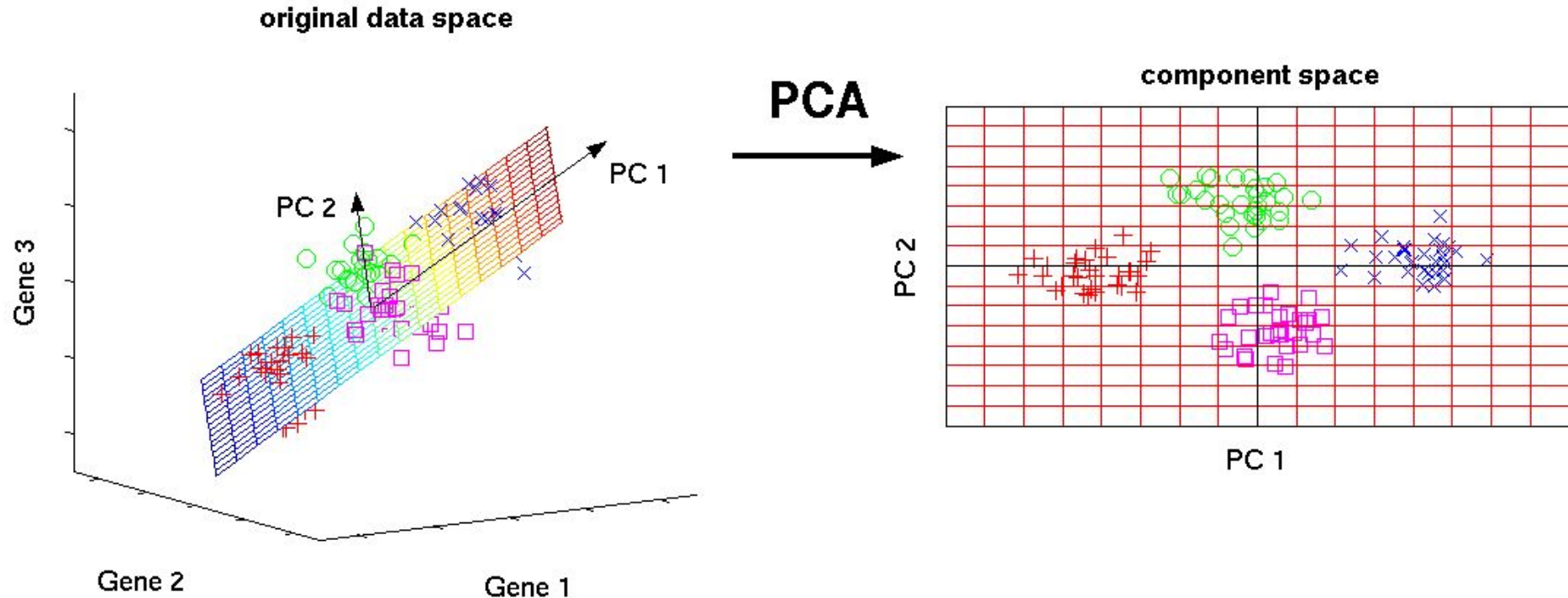
Can we make reliable inferences for genes with very low counts? What should we consider “very low”?



MDS and PCA plots

Assessing overall similarity across samples

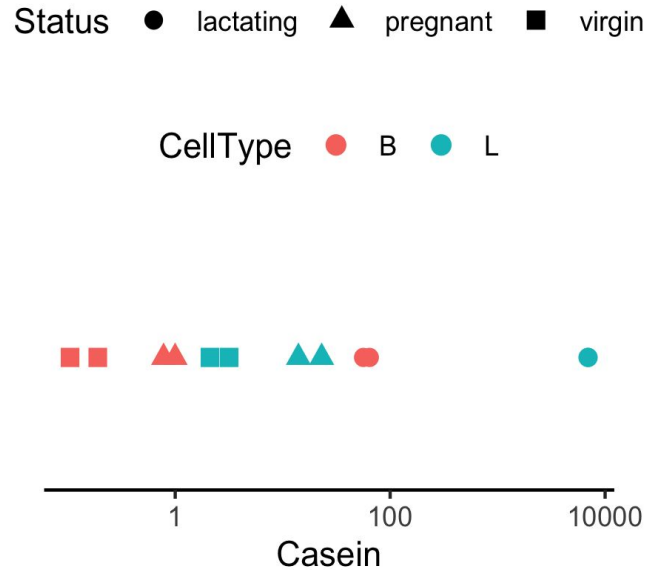
- Which samples are similar to each other, which are different?
- Does this fit to the expectation from the experiment's design?
- What are the major sources of variation in the dataset?



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

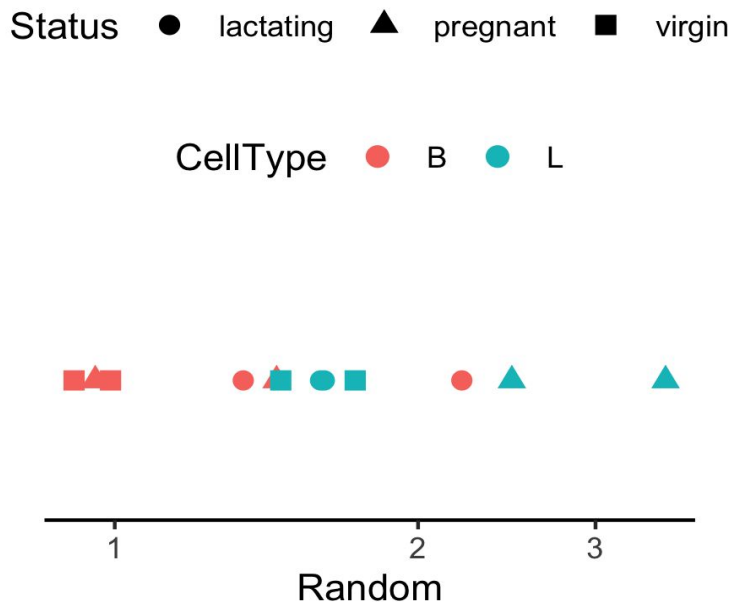
Why are the B.lactating samples not close to B.virgin and B.pregnant samples?

Could it be due to batch effects?



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.



Identify the source for technical variability!



Fitting the model

How to model the normalized RNA-seq read counts

Total number of reads for a sample ~ millions

Counts per gene ~ tens /hundreds /thousands.

The chance of a *given read* to be mapped to any *specific gene* is rather small.

Discrete events sampled out of a large pool with low probability are usually modeled with Poisson distribution

Overdispersion of read counts between samples

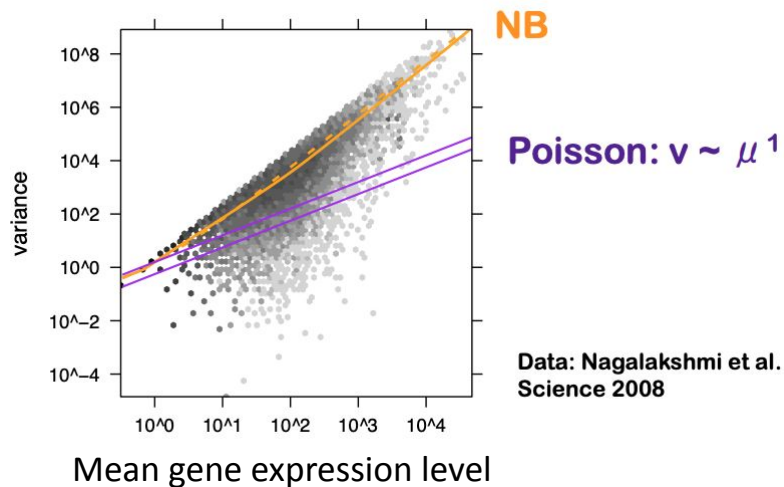
The number of reads mapped to a gene was first modeled using a **Poisson distribution** (Marioni *et al.* (2008))

Assumption: assumes that mean and variance are the same

BUT the variance grows faster than the mean in RNAseq data.

Overdispersion in RNA-seq data

- > counts from biological replicates vary so tend to have variance exceeding the mean (highly expressed genes)
- > underestimation of the biological variance increased the probability to falsely declare a gene DE when it is non-DE (type I error rate)

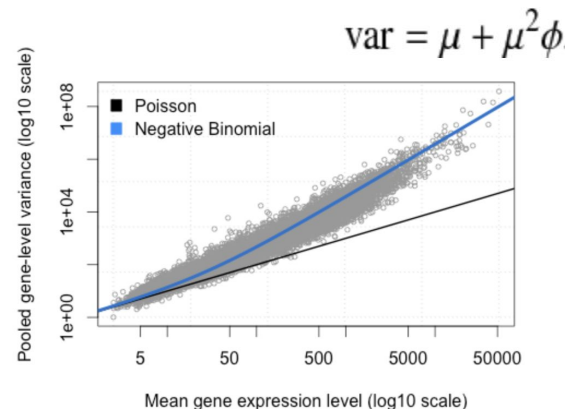


Three dispersion estimates

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 \Rightarrow suitable for RNA-seq data

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 .



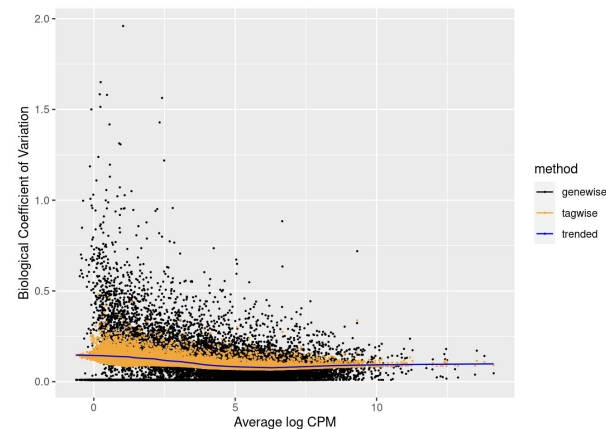
CV(the standard deviation divided by the mean) describes the relative deviation of the gene expression distribution relative to its mean, where a low CV indicates low dispersion with respect to the mean.

Empirical Bayes estimates of dispersion parameters

Dispersion accounts for variability between biological replicates

- Common dispersion: a global dispersion estimate averaged across the genes – not enough
- Trended dispersion: dispersion of a gene is predicted from its abundance – similar abundant genes
- Tagwise dispersion: measure of the degree of consistent inter-library variation for that tag - EB shrinkage to a common (trended) dispersion

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



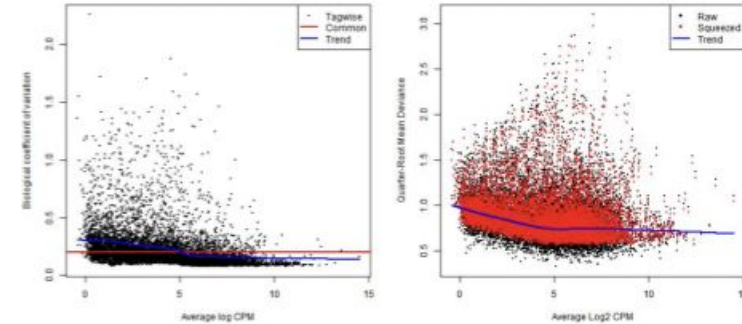
The tag-wise dispersions (orange dots) are the result of shrinking the gene-wise dispersion (black dots) to the trend (blue line)

plotBCV (biological coeff
variation)

```
y <- estimateDisp(y, design)
```

Fitting the model : *glmQLFit*

- NB dispersions - higher for genes with very low counts - 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)
 - 1) For each gene-specific variability above and below the overall level (deviance) is picked up by the QL dispersion



edgeR fitting models

- 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
 - + 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 – high overlap across methods

Get the DE genes - *glmQLFTest*

- Identifies differential expression based on statistical significance regardless of how small the difference might be -> 5000 DE genes between condition and control 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
- 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()* – set cutoff

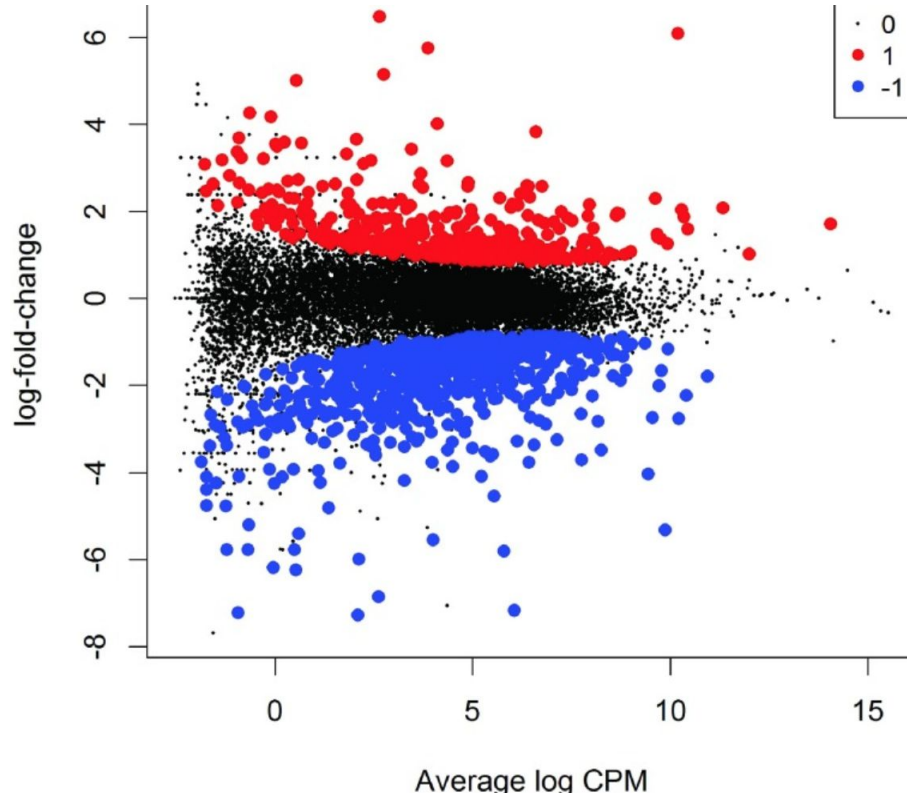
Get the DE genes - *glmQLFTest*

	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



Library size-adjusted log-fold change between two libraries (the difference) vs the average log-expression across those libraries (the mean).

Log-fold change and average abundance of each gene



Demo II

Hands-on session

- oLoad and reformat the data
- oExploratory visualization : MA plot
- oCreate DGElist object and retrieve gene symbols
- oFilter genes with inadequate information
- oNormalization
- oExploratory visualization : MDS and PCA plots
- oDefine and fit a model
- oHypothesis testing (four example hypotheses)
- oSave results as a table and explore in Excel

In summary

- Raw counts are not comparable across samples/genes within a sample
- Several normalization methods: some more suitable for DEG
- Estimate the dispersion, visualize the technical variability
- Fit the model: counts variance exceeding the mean
- Make all the comparisons that you wish using complex contrast
- Visualize the DEG and get a list for pathway analysis

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.

Winter workshops schedule - [Data Science Training Program](#)



Thank you!

The background features a dark teal color with several wavy, overlapping lines in a lighter teal shade. These lines are composed of a fine grid of small dashes or segments, creating a textured, digital effect. The waves flow from the left towards the right, with some peaks and valleys. Centered over this background is the text "GLADSTONE INSTITUTES" in white.

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