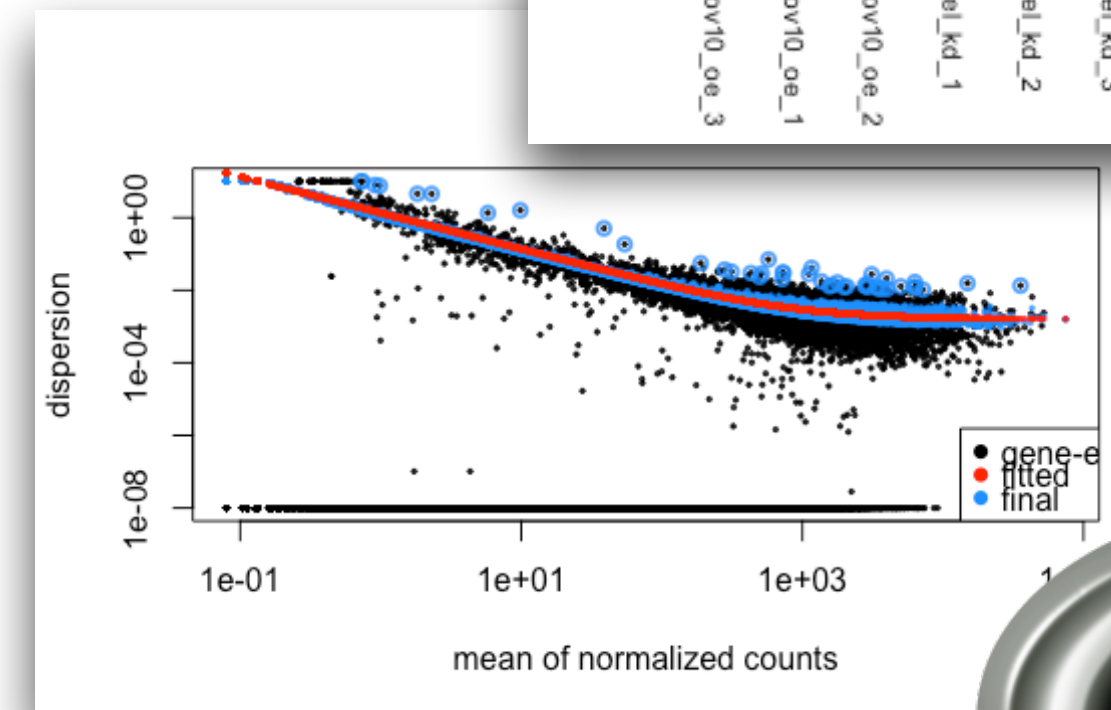
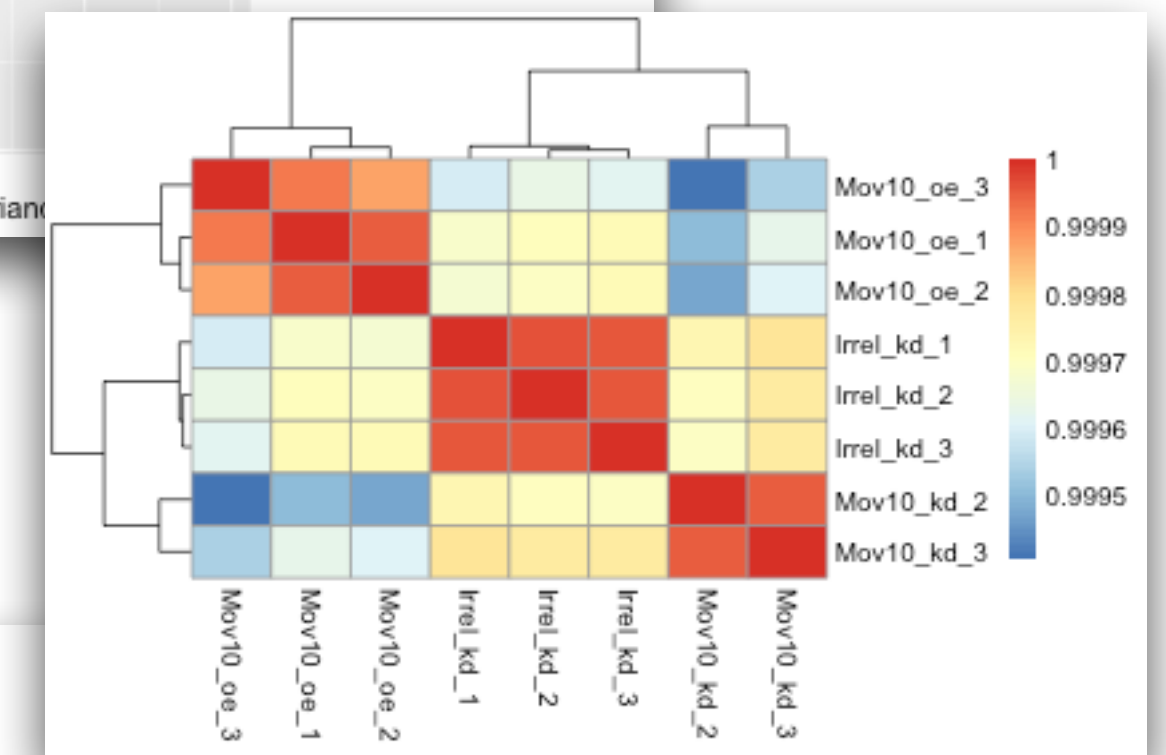
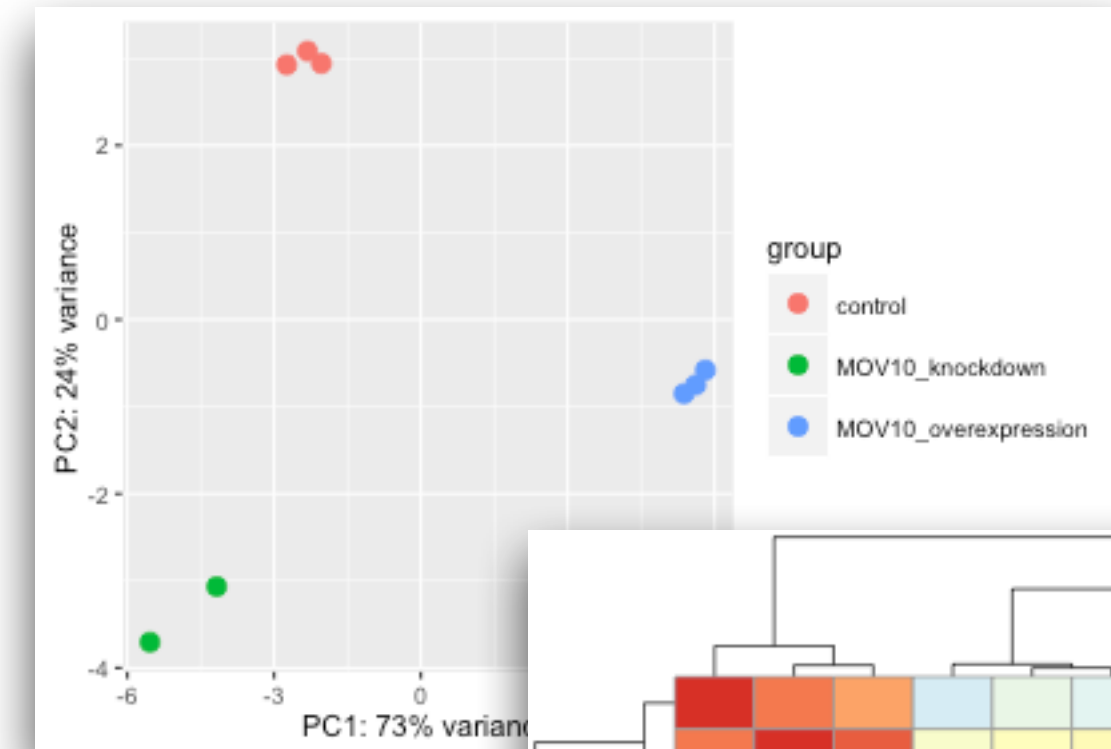


# Using R for Differential Gene Expression Analysis

Harvard Chan Bioinformatics Core

October 20, 2016

<http://tinyurl.com/hbc-DGEworkshop>



# Learning Objectives

- ✓ Understand the considerations for performing statistical analysis on RNA-Seq data
- ✓ Utilize R knowledge to perform a standard differential expression analysis
  - ✓ Starting with Gene Counts (after alignment and counting), perform basic QC on the count data
  - ✓ Use DESeq2 to perform differential expression (DE) analysis on the count data and obtain a list of significantly different genes
  - ✓ Visualize expression patterns of DE genes

The image shows handwritten mathematical derivations on a blackboard. The top part shows the derivative of the log-likelihood function with respect to the parameter  $\tau$ :

$$\frac{d\ell}{d\tau} = \frac{1}{\tau} - \frac{1}{P} \frac{dP}{d\tau}$$

Below this, there are two expressions for the derivative of the log-likelihood function with respect to the parameter  $P$ :

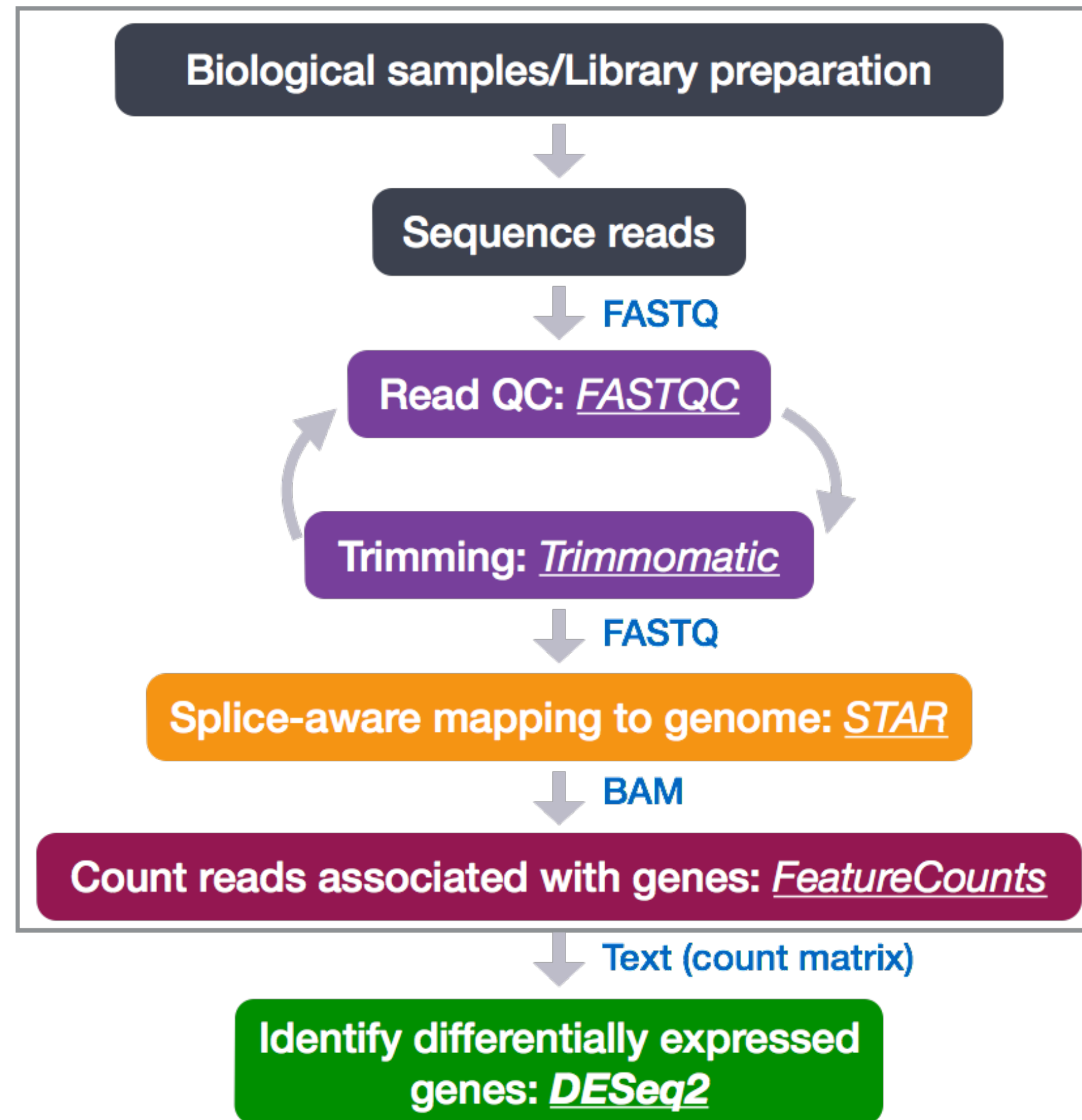
$$\frac{d\ell}{dP} = \frac{P_0 - P}{P} \sim \frac{1}{P}$$
$$\frac{d\ell}{dP} = \frac{P_0 - P}{P_0} \sim \frac{1}{P_0}$$

Further down, there are two expressions for the log-likelihood function:

$$10^{-53}$$
$$10^{-26}$$

At the bottom, there are two expressions for the log-likelihood function:

$$10^8 \text{ (} 10^{11} \text{)}$$
$$10^{10} \text{ (} 10^{11} \text{)}$$



From sequence data to count matrix


# High-throughput sequencing data

	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516
ENSG000000000003	679	448	873	408	1138
ENSG000000000005	0	0	0	0	0
ENSG000000000419	467	515	621	365	587
ENSG000000000457	260	211	263	164	245
ENSG000000000460	60	55	40	35	78

# High-throughput sequencing data

samples: want to see if differences across  
condition are significant  
(w.r.t. biological and technical variation)

features (e.g. genes)




	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516
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# High-throughput sequencing data

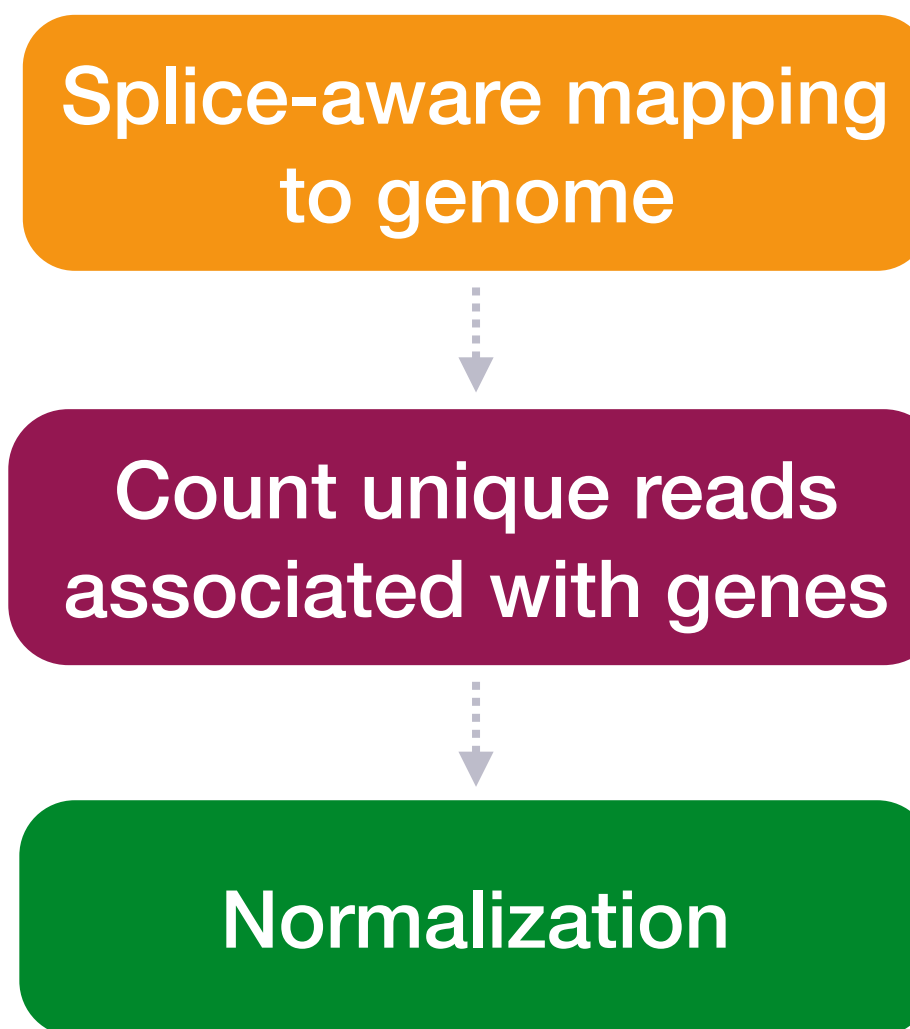
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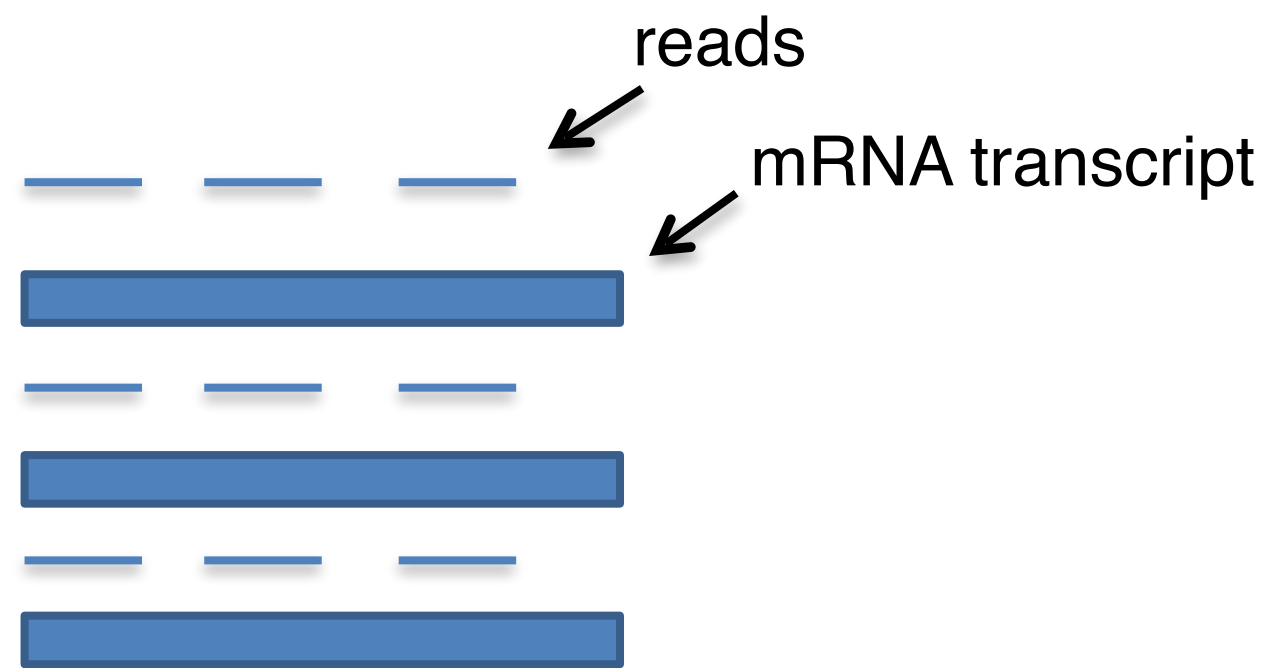
- counts need an appropriate statistical model (normalization and variance modeling)



DE workflow :: normalization

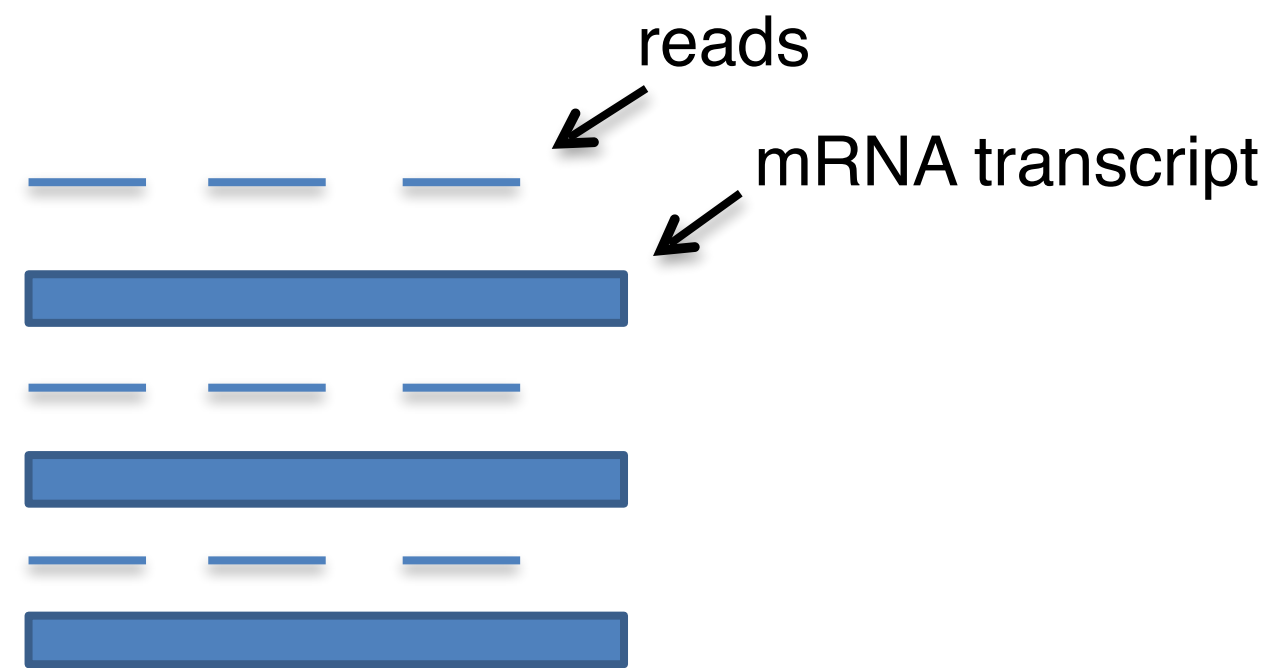


# mRNAs to reads





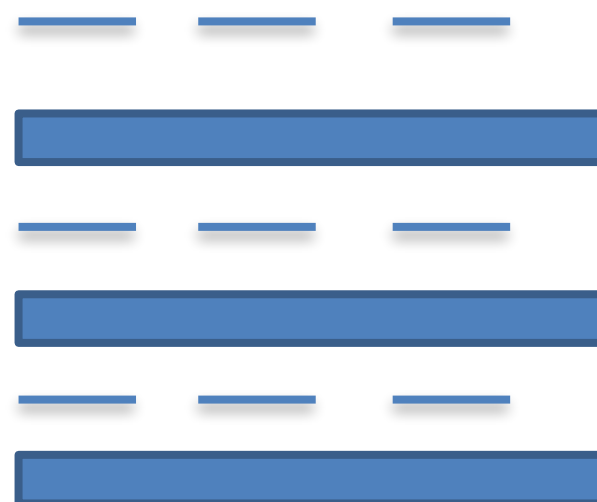
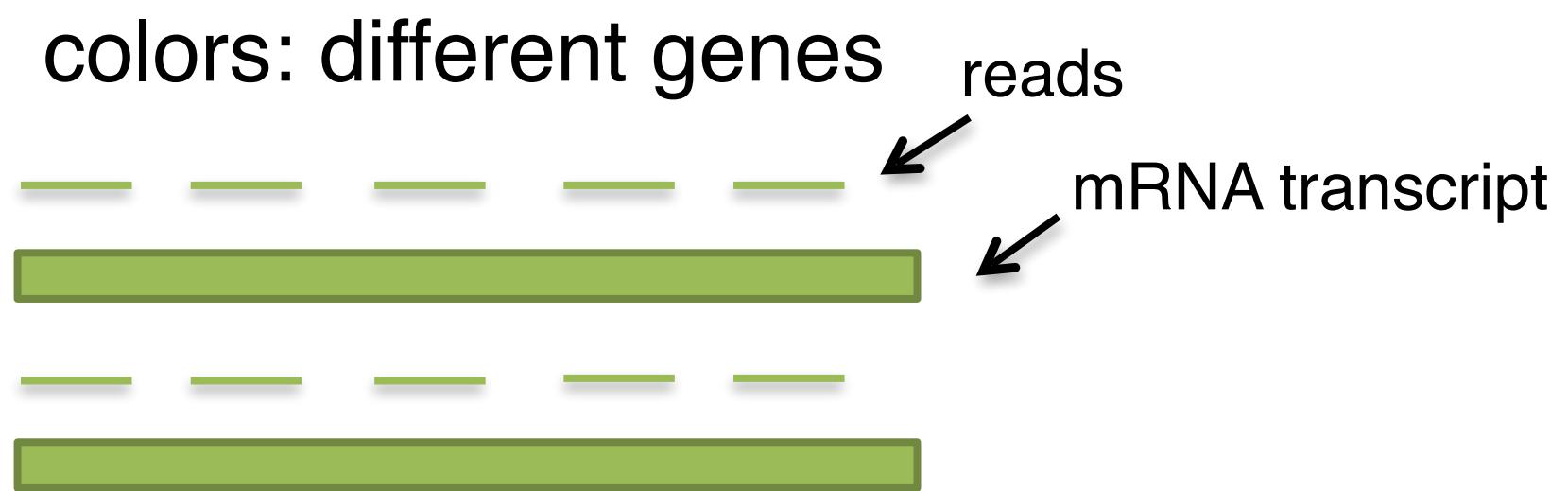
# mRNAs to reads



count of mapped reads proportional to:

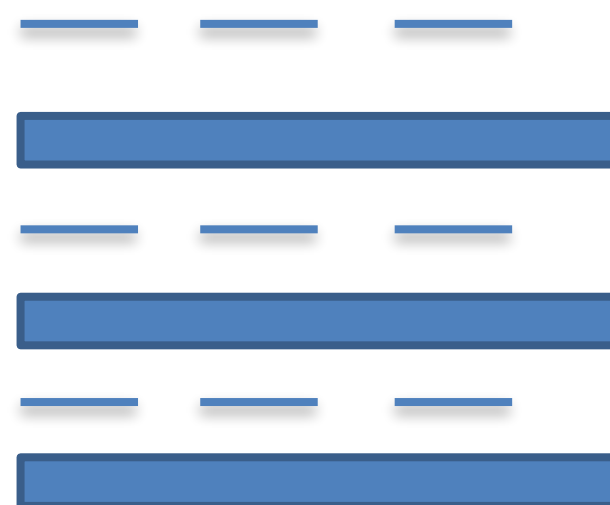
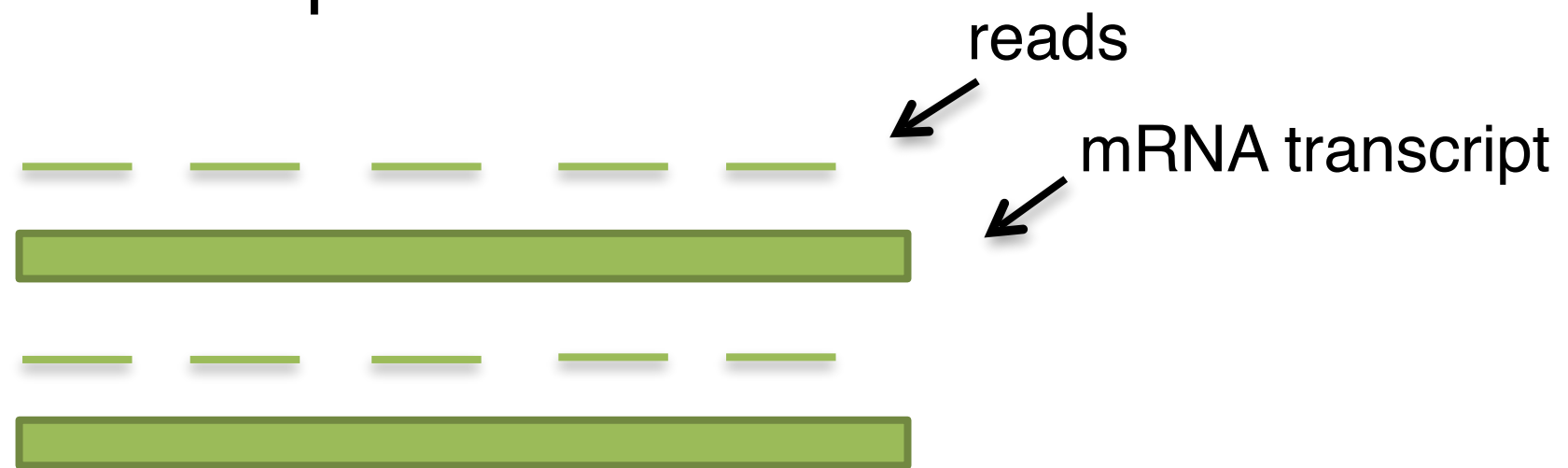
- expression of RNA
- length of gene
- sequencing depth
- library prep. factors (PCR)
- etc...

# Length of gene



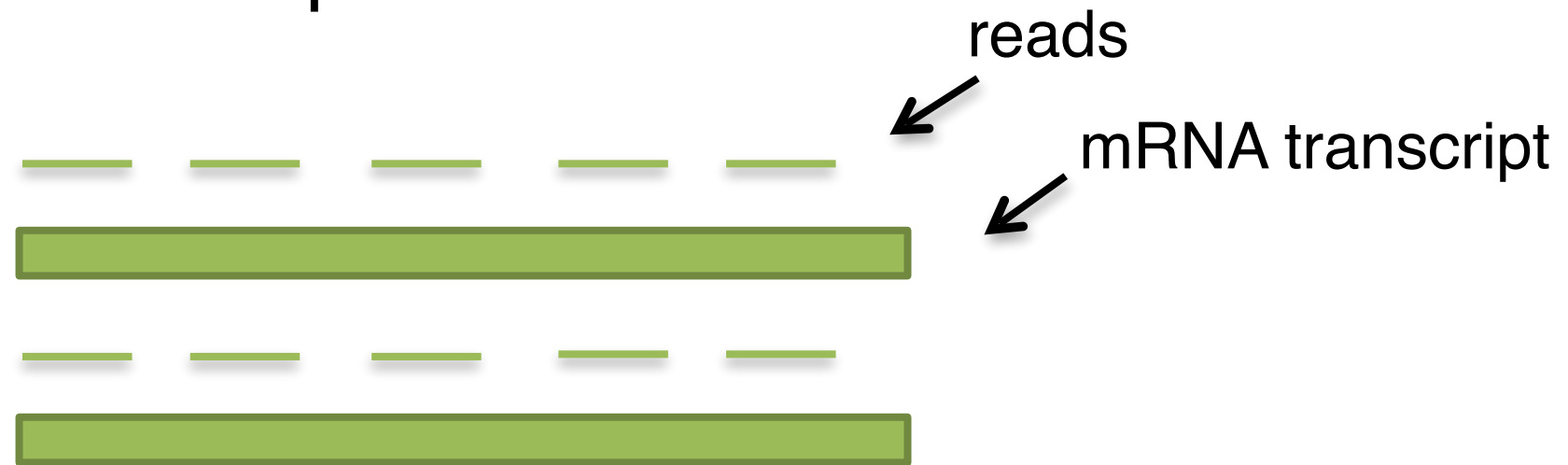
# Sequencing depth

sample 1

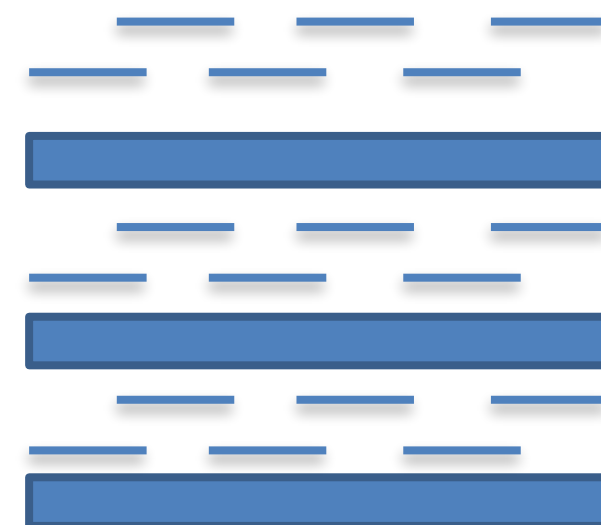
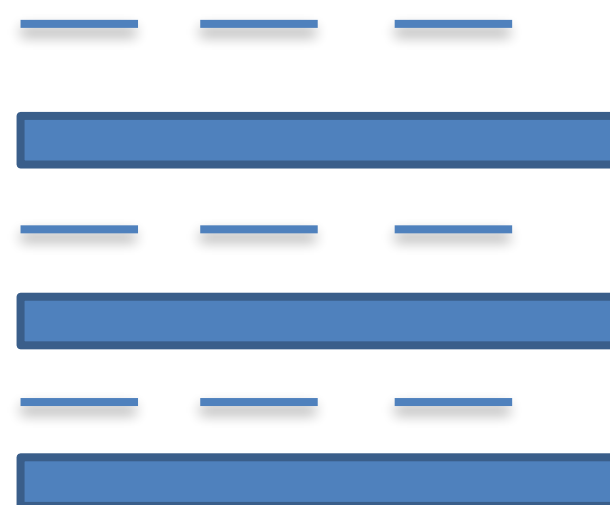


# Sequencing depth

sample 1



sample 2



# (Pre)-scaled measures of expression

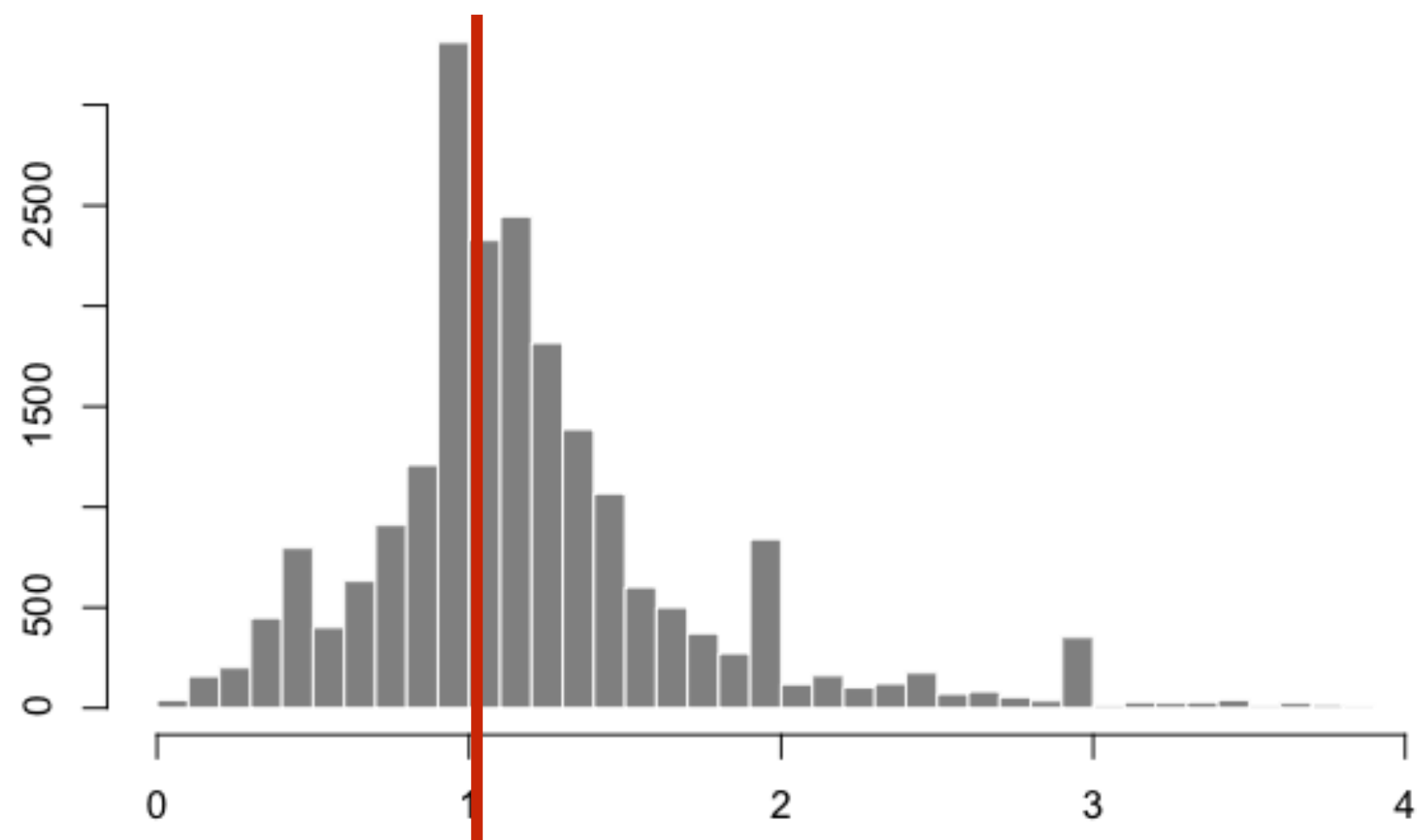
- ▶ CPM (counts per million) counts scaled by the total number of reads
- ▶ TPM (transcripts per million) the proportion of transcripts in your RNA (counts per base)
- ▶ (not recommended) RPKM (reads aligned per kilobase of exon per million reads mapped) – Mortazavi et al 2008
- ▶ (not recommended) FPKM (fragments per kilobase of exon per million fragments mapped). Same idea for paired end sequencing
- ▶ Tool-specific metrics for normalization

Raw counts should be used as input for  
differential expression tools!

# Median of ratios method

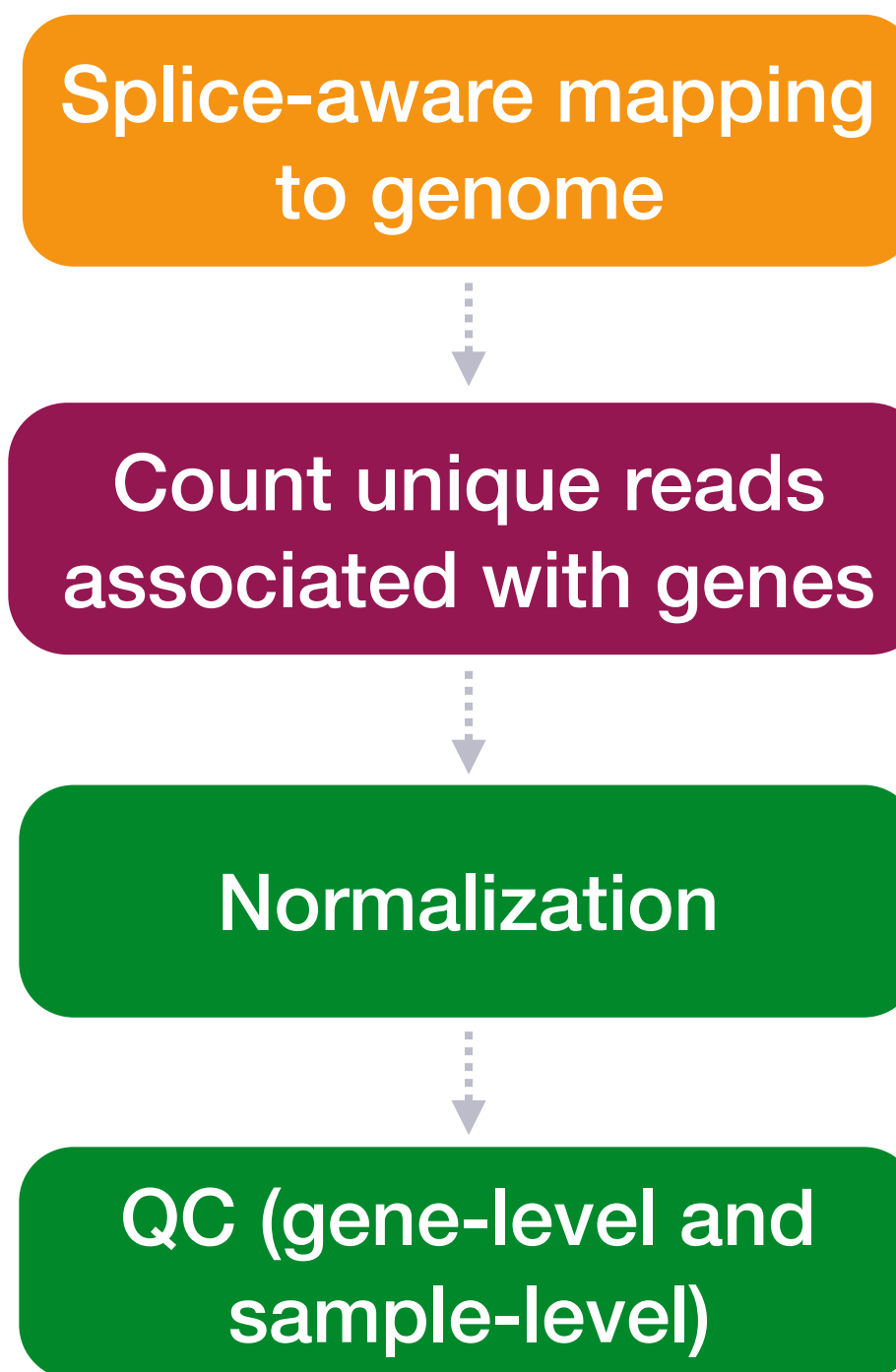
simple approach & works well  
for each gene look at the count ratios:

sample 1 / pseudo-reference sample



- in general: create a pseudo-reference-sample (row-wise geometric mean)
- calculate ratio of each sample to the reference
- take the median value as the normalization factor
- assumes that not *ALL* genes are DE (differentially expressed)
- **robust** to imbalance in up-/down- regulation and large numbers of DE genes

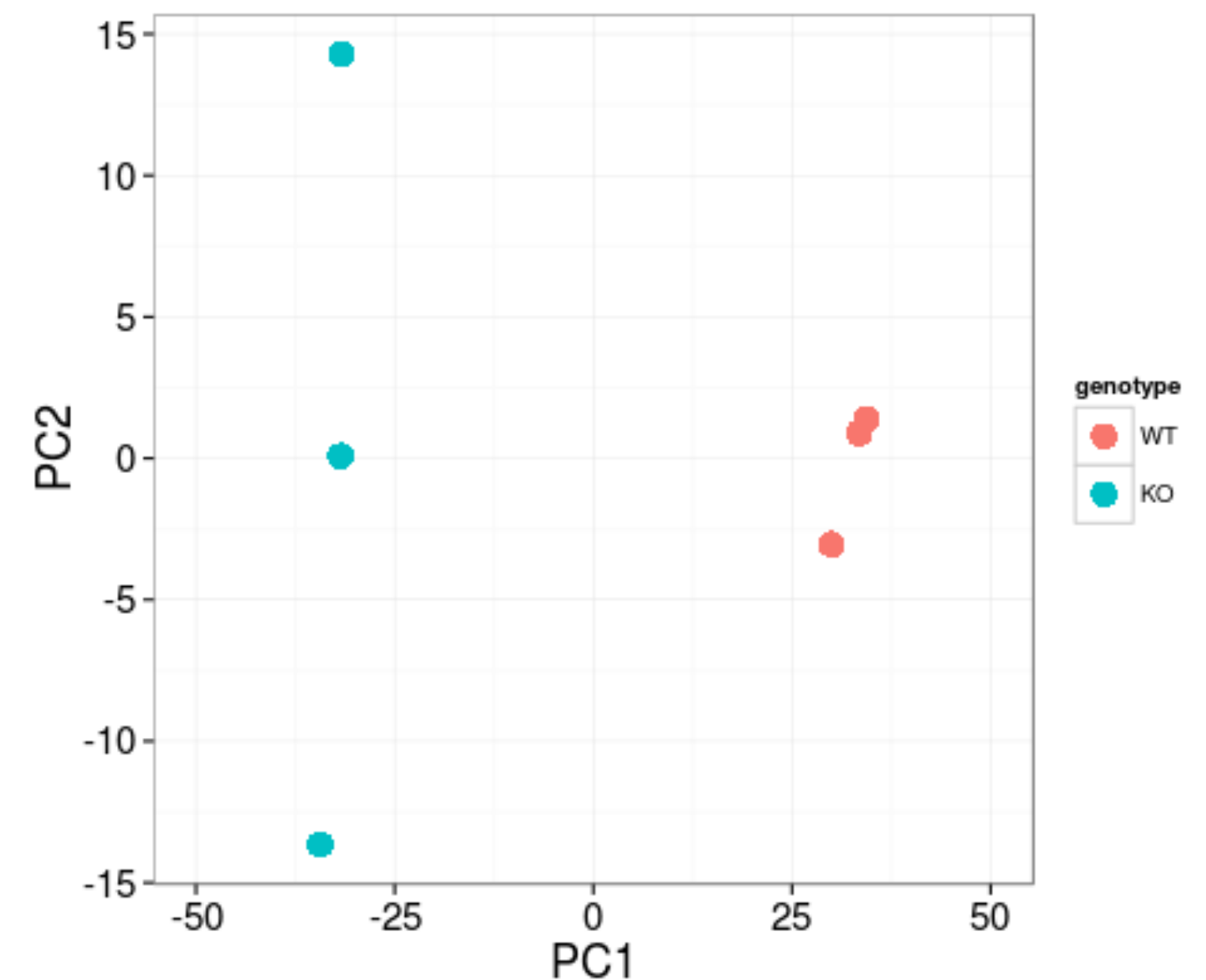




DE workflow :: quality control

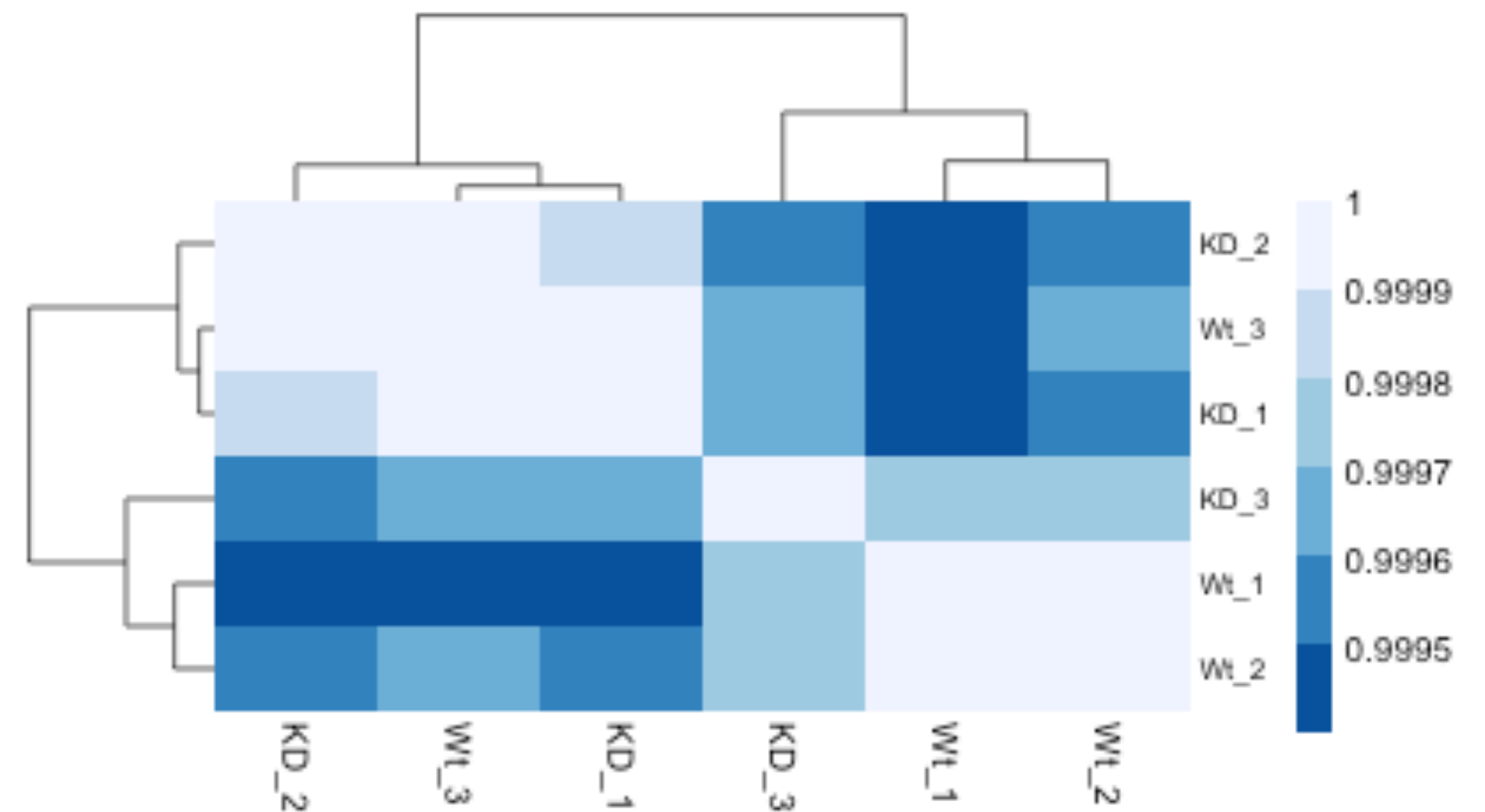
# QC: Sample-level

- ▶ **Principal Component Analysis (PCA):** A technique used to emphasize variation and bring out strong patterns in a dataset (dimensionality reduction)
- ▶ Project a line through the data points in  $n$  dimensional space ( $n = \text{genes}$ )
- ▶ Measure how much variance there is from that line (the distance from each point to the line).
- ▶ PC1 explains highest variance, PC2 next highest etc.



# QC: Sample-level

- ▶ Identify strong patterns in a dataset and potential outliers
- ▶ Correlation or distances for all pairwise combinations of samples.
- ▶ Generally high correlations with each other (values higher than 0.80)
- ▶ 'Blocks' indicate substructure in the data



# QC: Gene-level filtering

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ENSG000000000003	679	448	873	408	1138
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Genes with  
zero counts

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Genes with extreme  
count outlier



Genes with  
zero counts



# QC: Gene-level filtering

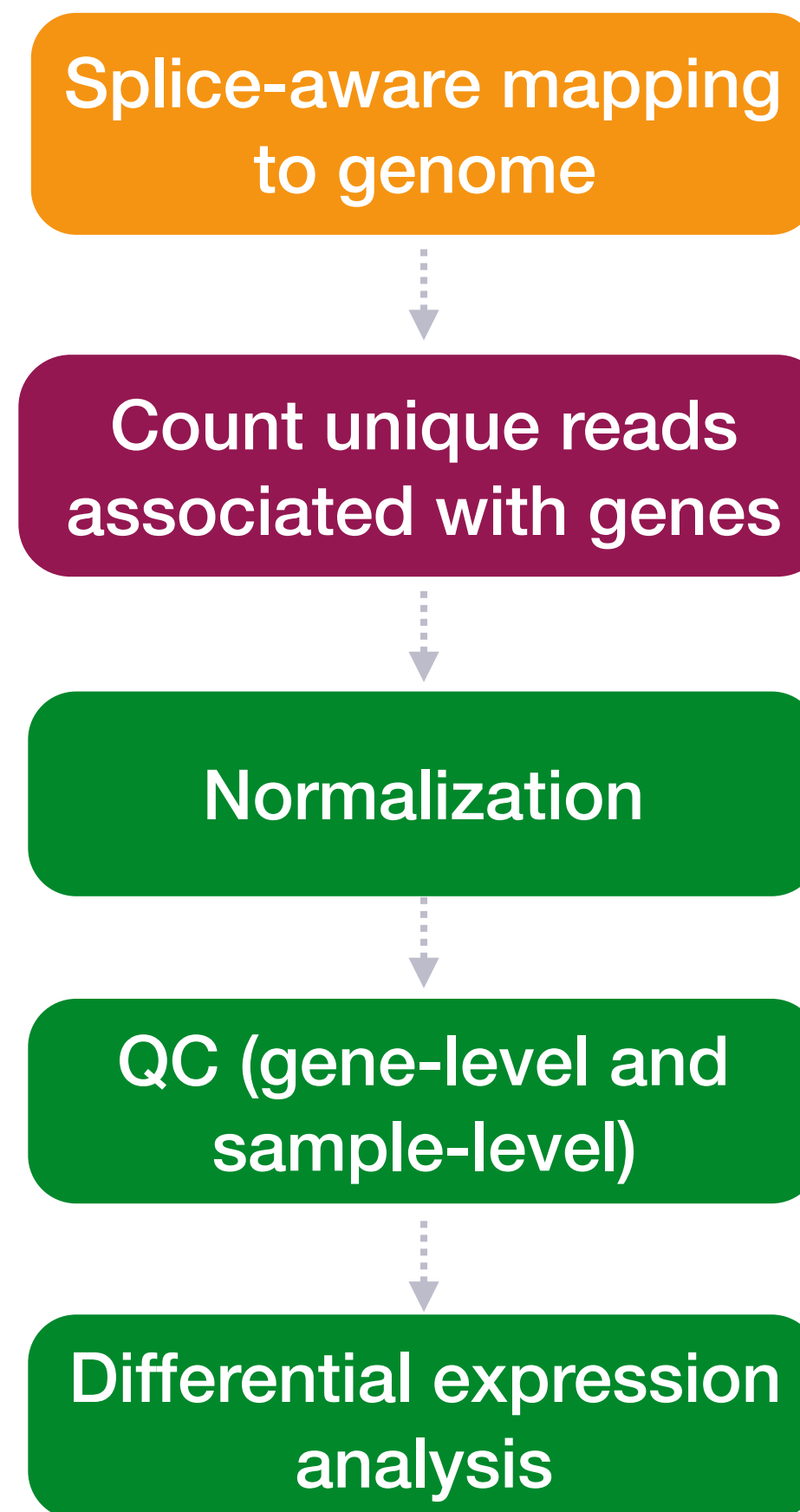
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Genes with extreme  
count outlier

Genes with  
zero counts

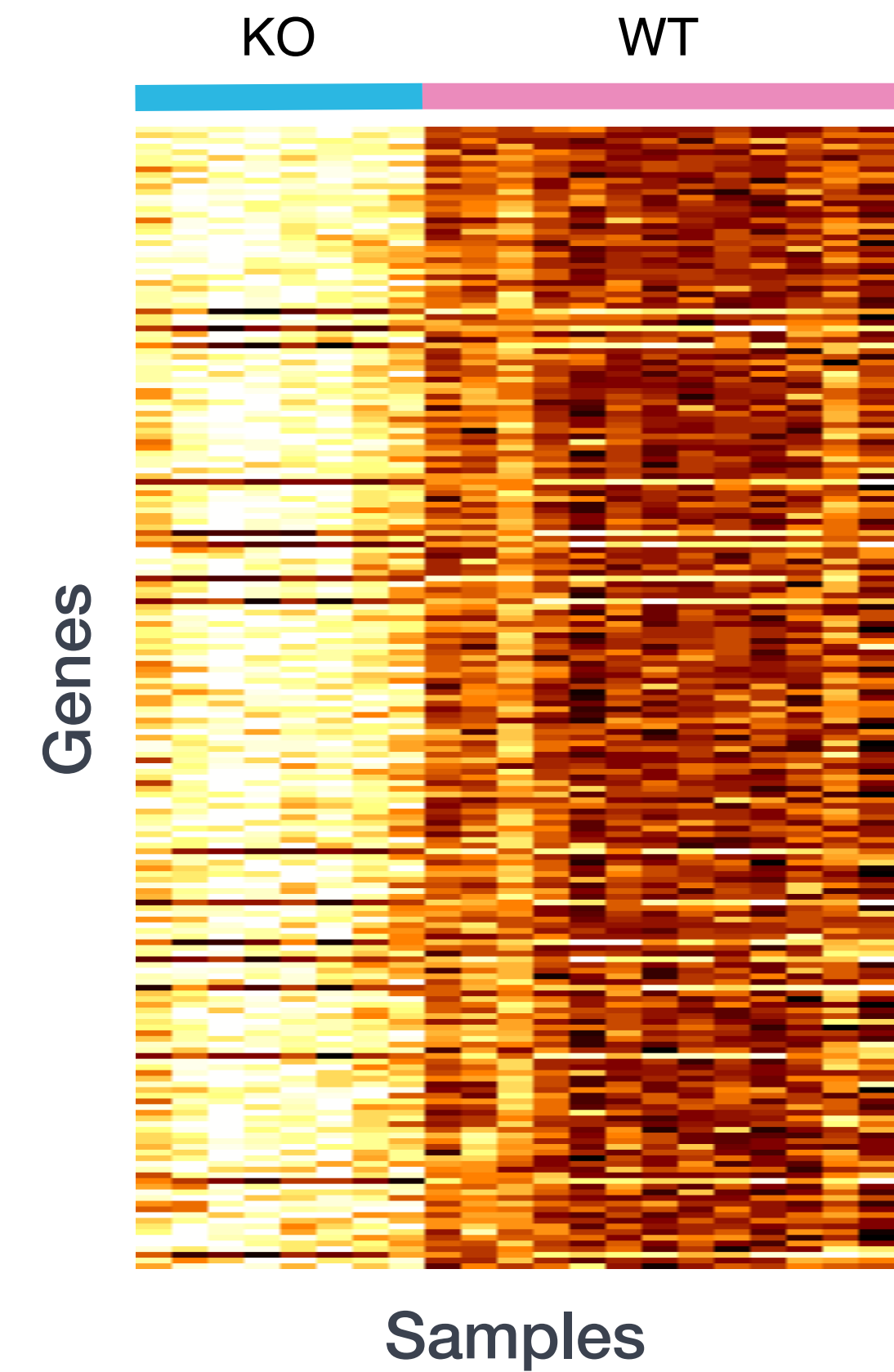
Genes with low mean  
normalized counts  
(‘Independent filtering’)





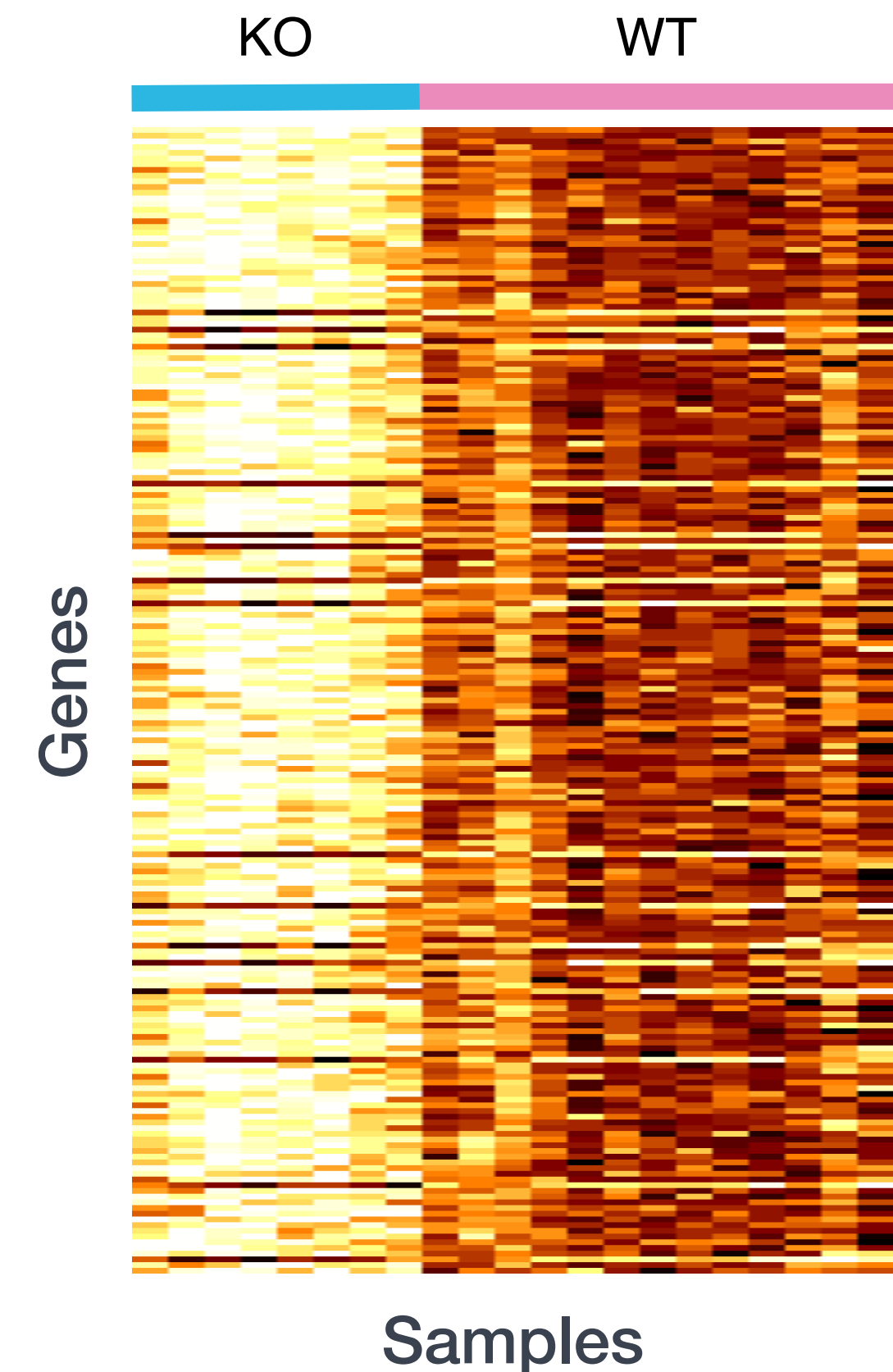
DE workflow :: differential expression

# Identifying differences in gene expression



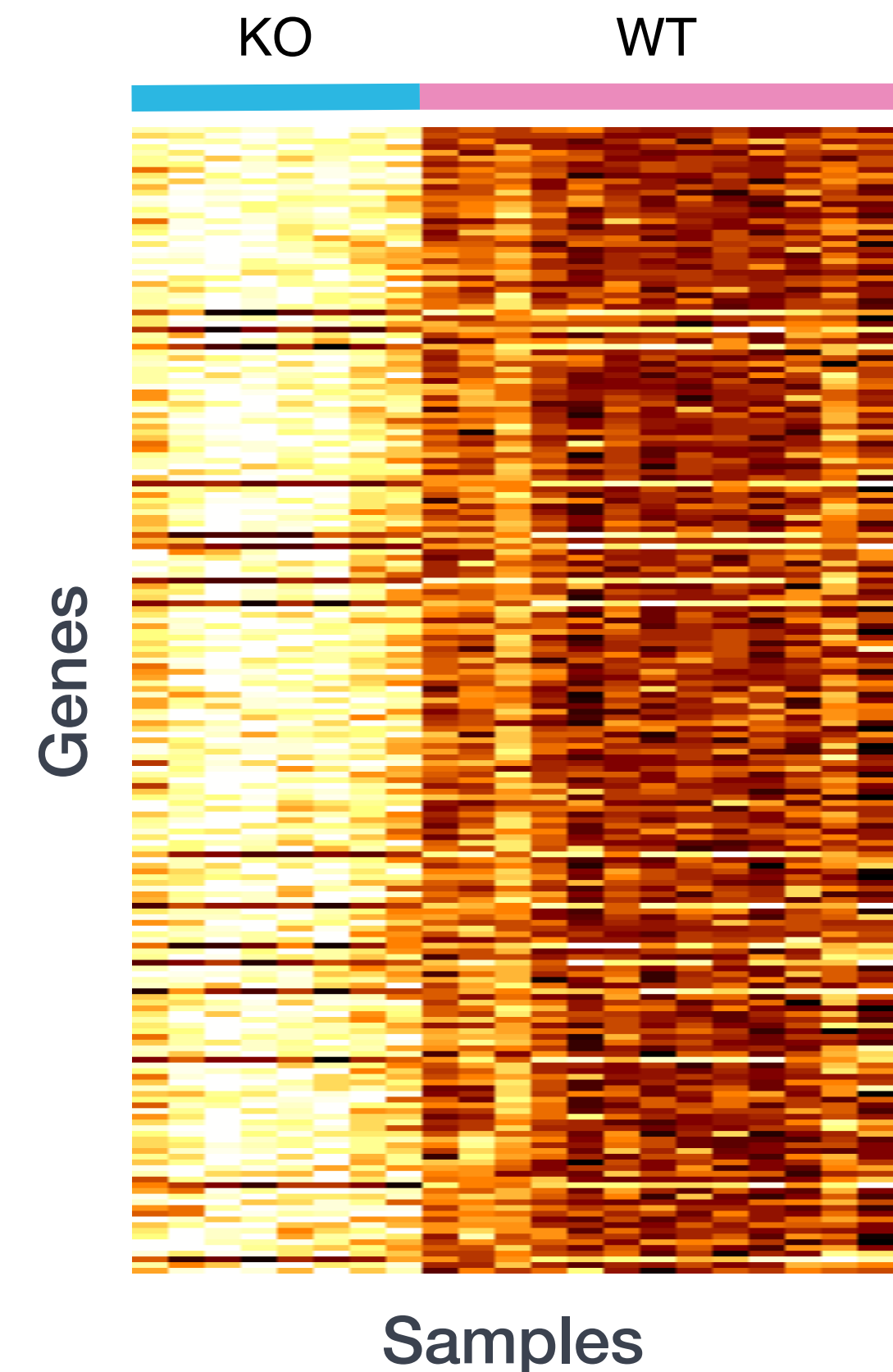
# Identifying differences in gene expression

- ▶ Looking for genes that change in expression between two or more groups
  - ▶ case vs. control
  - ▶ correlation of expression with some variable or clinical outcome

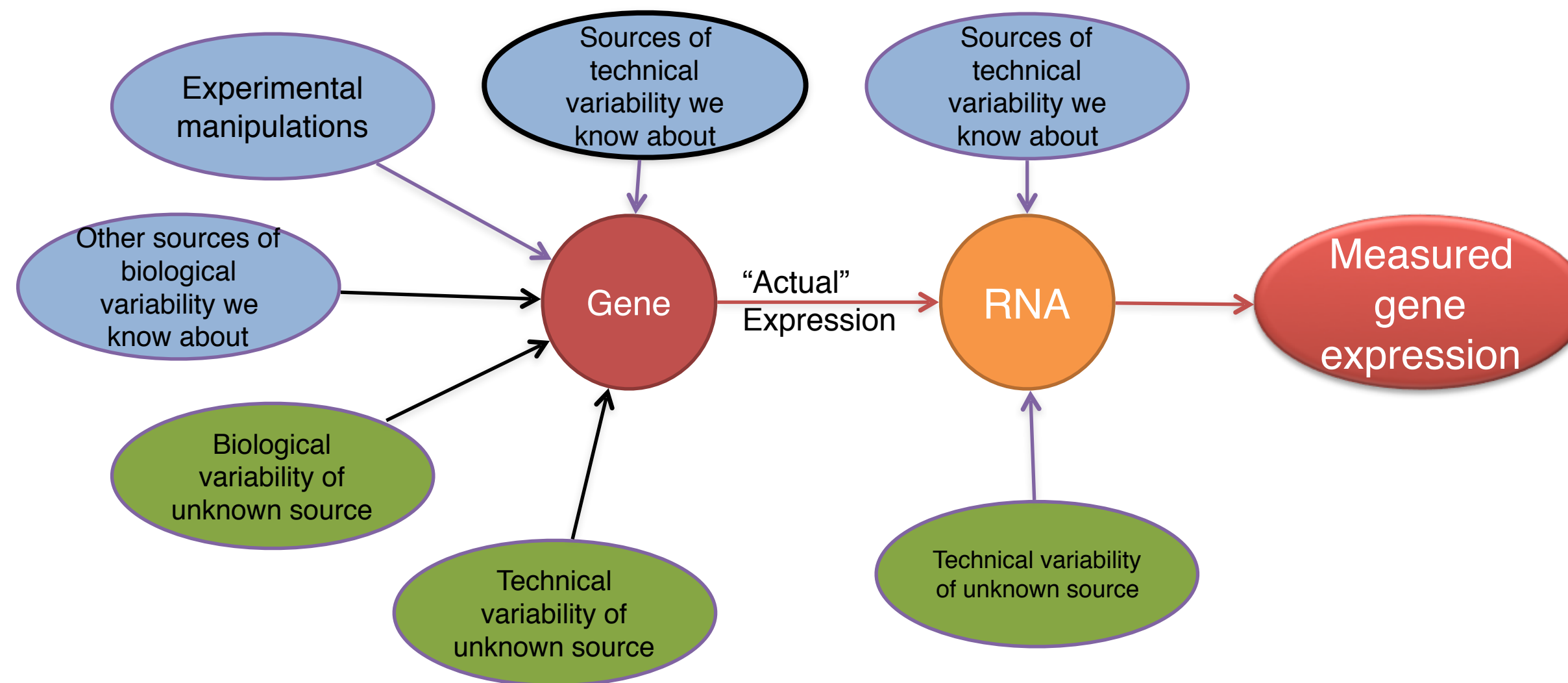


# Identifying differences in gene expression

- ▶ Looking for genes that change in expression between two or more groups
  - ▶ case vs. control
  - ▶ correlation of expression with some variable or clinical outcome
- ▶ Rank the genes by how different they are between the two groups (based on fold change values). **Why does this not work?**



The measurement is the “sum” of many effects

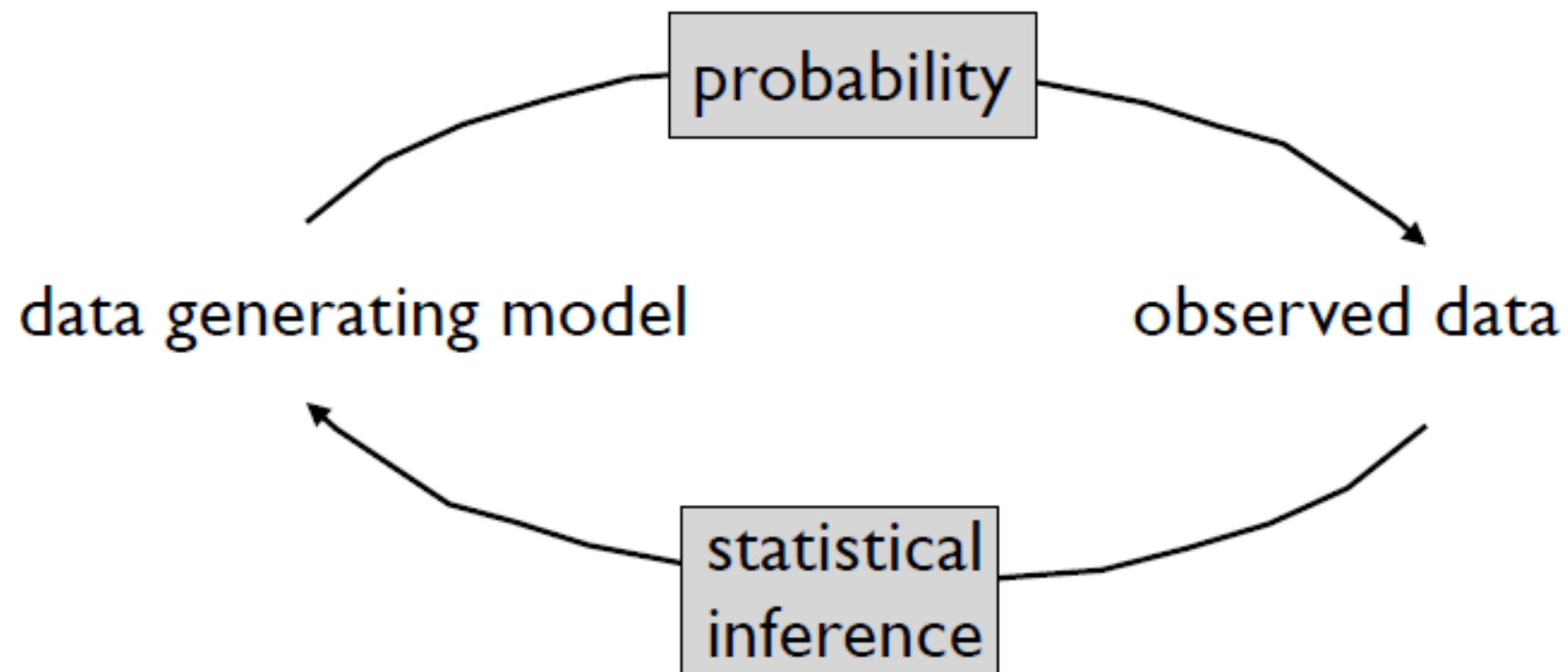


# Modeling gene-level data

Courtesy of Paul Pavlidis, UBC

# Making sense of data

Data is what we observe. We want to infer something about “where it came from”



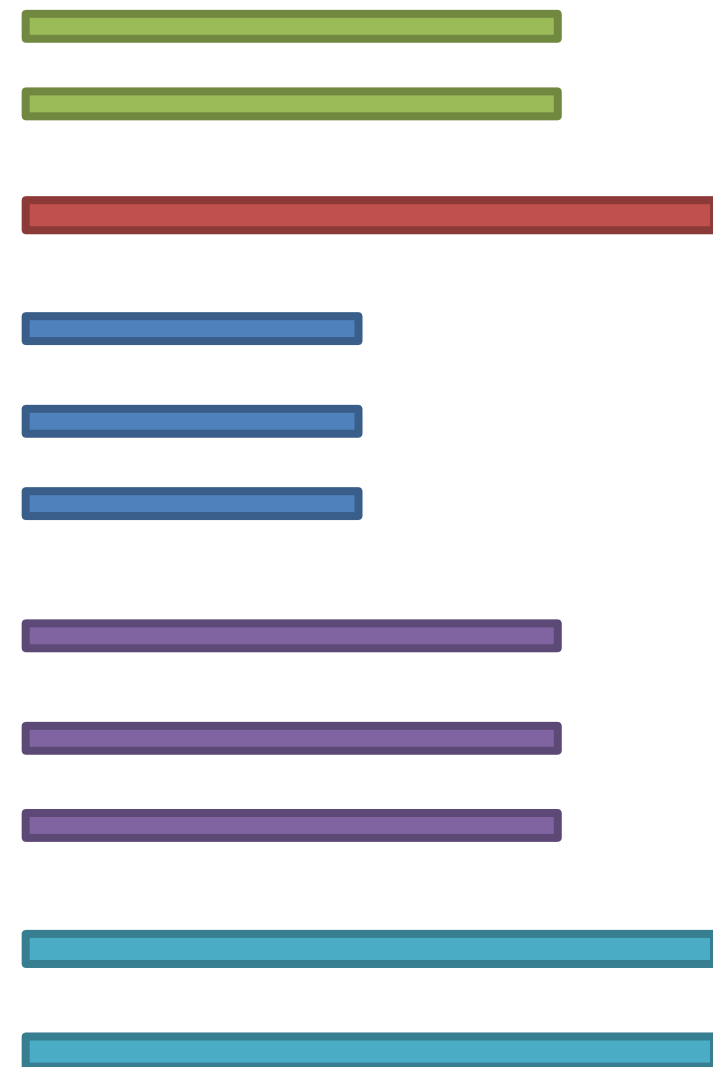
# Characteristics of count data

- Discrete measurement for each gene
- Large dynamic range
- Not normally distributed
- Need regression models specific for count data; generalized linear models



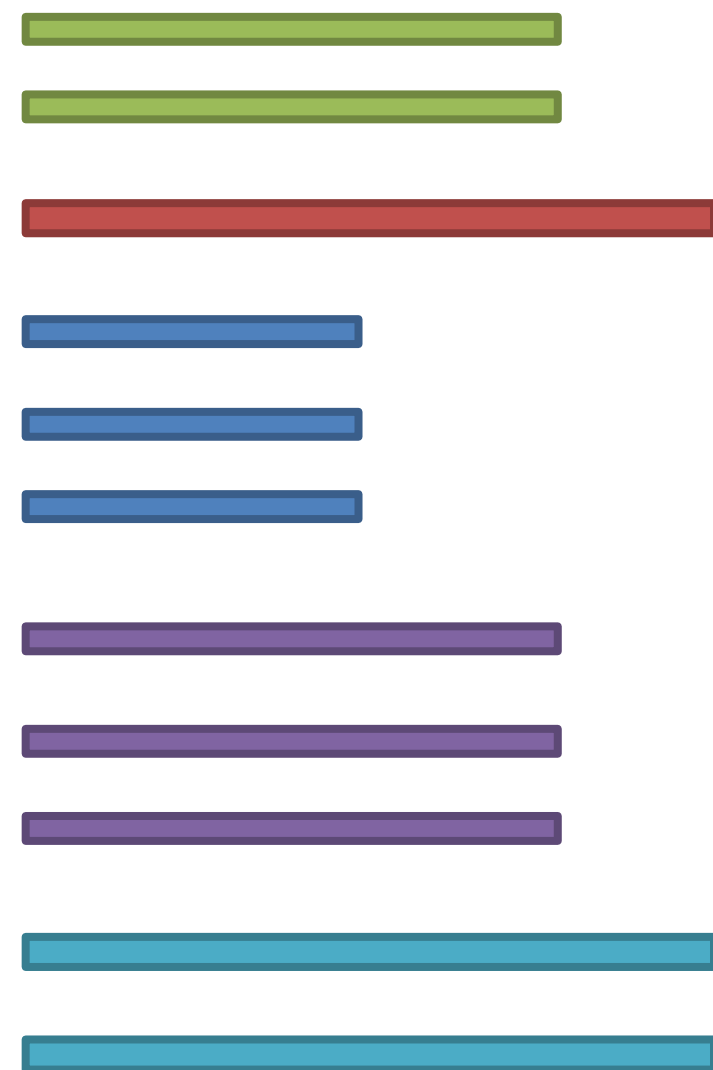
# Distribution of Counts: Poisson versus Negative Binomial

If the proportions of mRNA stays exactly constant between biological replicates we can expect **Poisson distribution**.

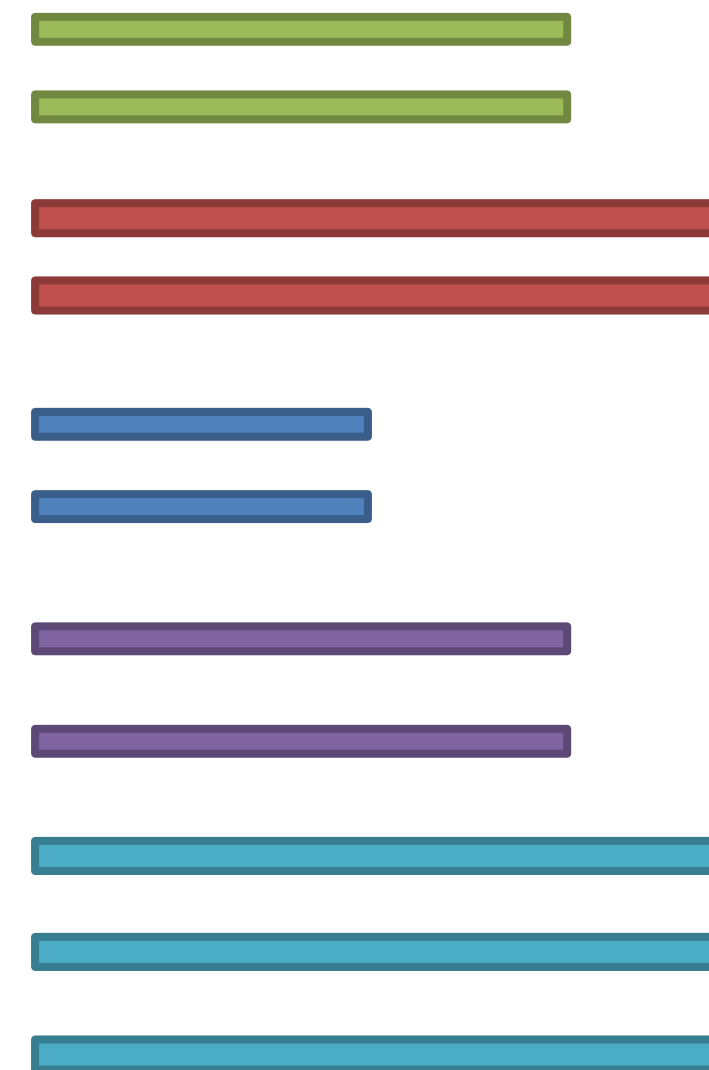


# Distribution of Counts: Poisson versus Negative Binomial

If the proportions of mRNA stays exactly constant between biological replicates we can expect **Poisson distribution**.



But realistically, **biological variation** across sample units is expected and so a **Negative Binomial** distribution is more appropriate.



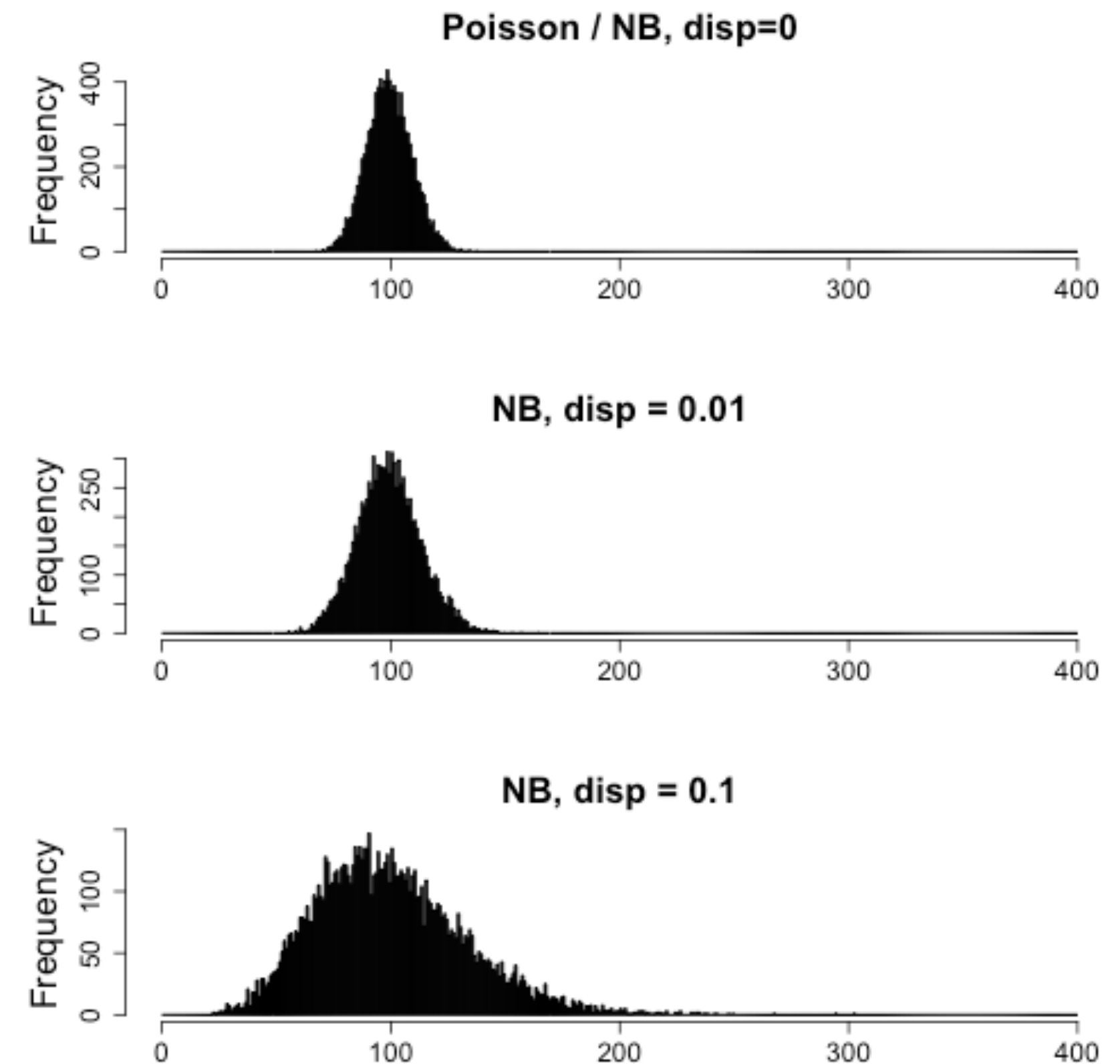
# Biological vs. Technical Replicates

- ▶ **Biological replicates** represent multiple samples representing the same sample class
- ▶ **Technical replicates** represent the same sample but with technical steps replicated
- ▶ Usually **biological variance > technical variance**. They also allow us to make inferences about treatment groups

# Biological replicates produce “over-dispersion” relative to Poisson

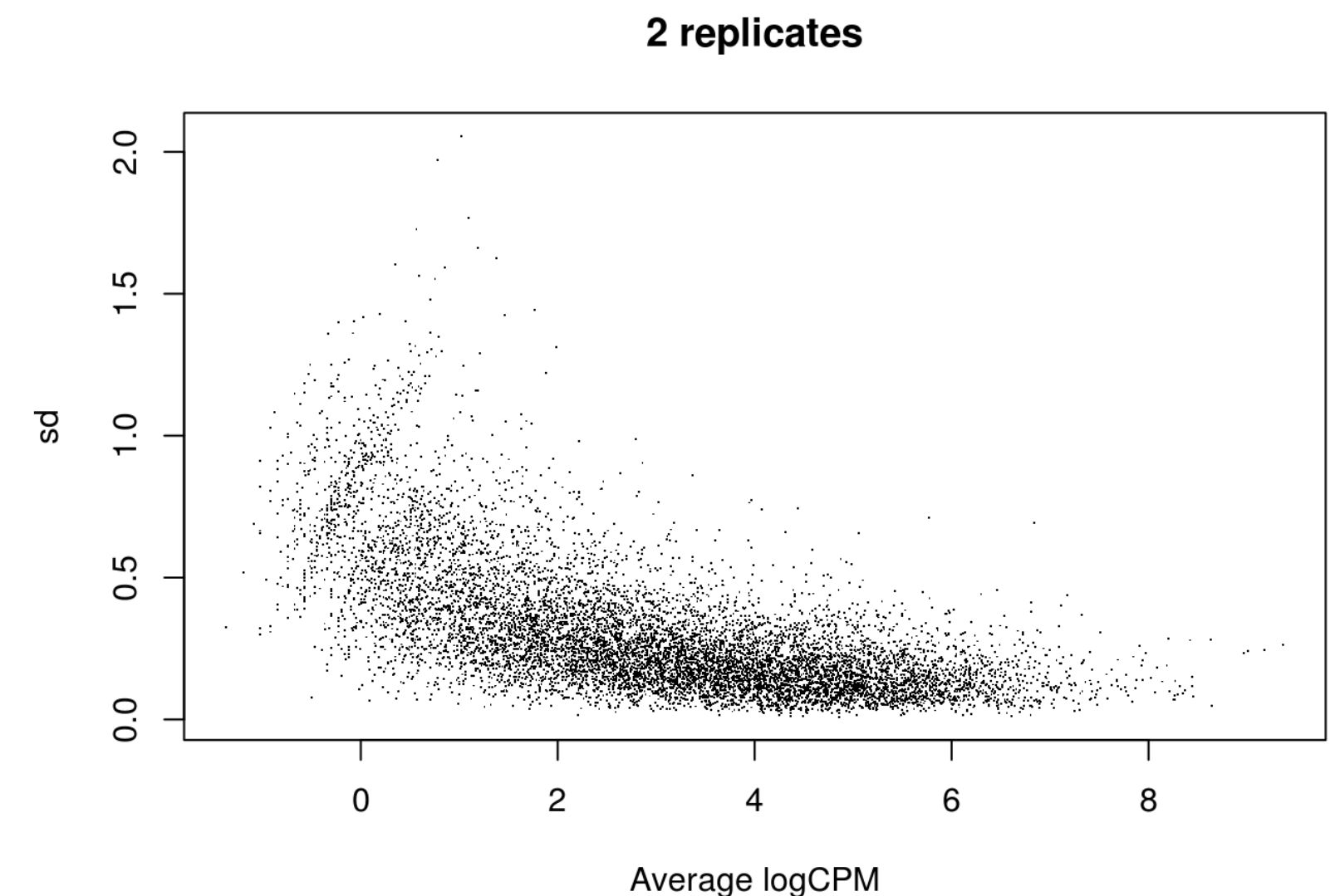
Poisson will underestimate the variability and the effect of the observed differences.

Instead we use the Negative Binomial.



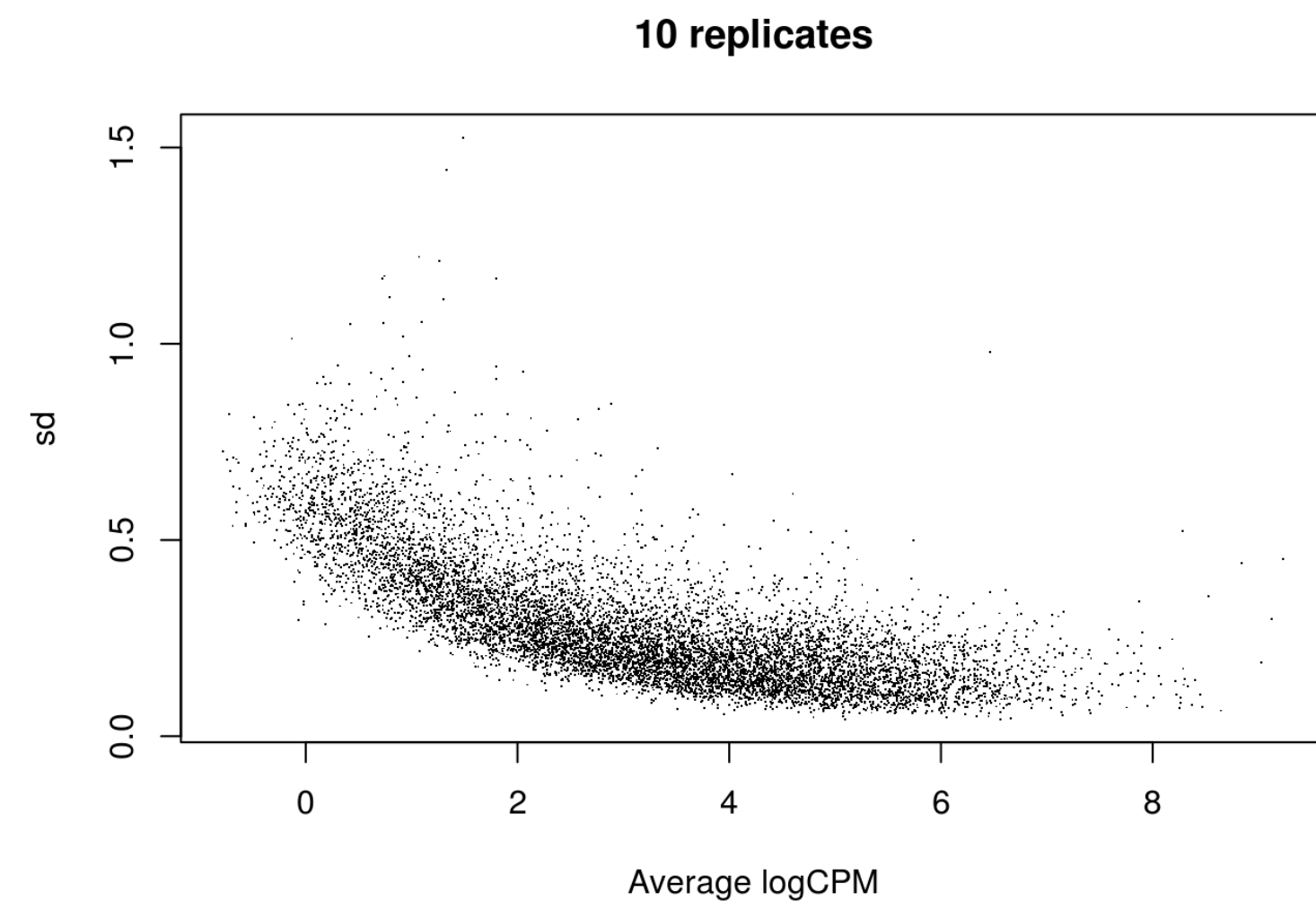
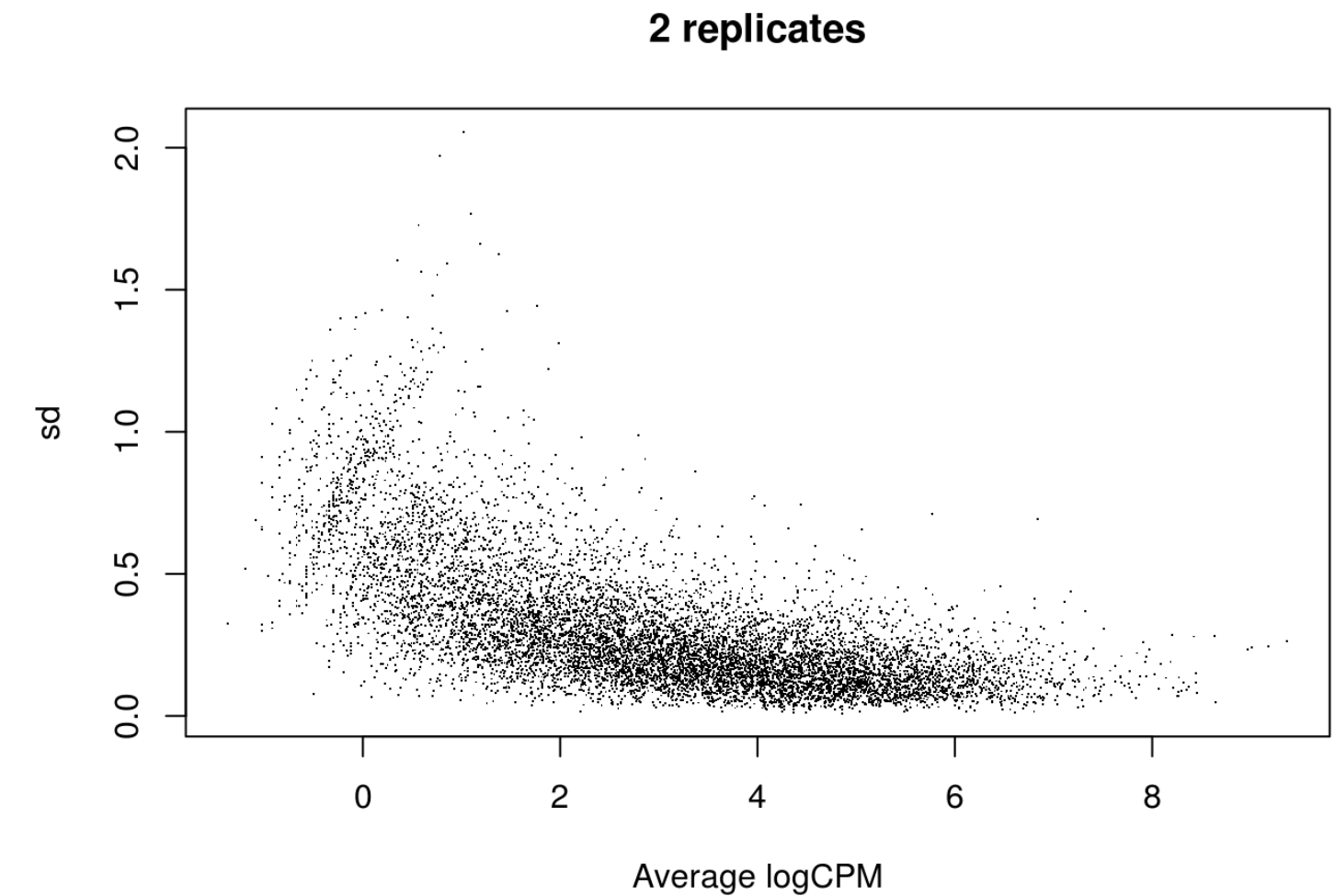
# The need for more replicates

- ▶ Genes with larger average expression have on average larger observed variances across samples, This phenomena of 'having different scatter' is known as data heteroscedasticity.
- ▶ **Mean is not equal to variance**



# Increase the number of replicates...

- ▶ Scatter tends to reduce
- ▶ Standard deviations of averages become smaller
- ▶ With smaller SD, you get more precise estimates of group means, and ultimately greater confidence in the ability to distinguish differences between sample classes



# Counts are modeled using the Negative Binomial (NB) distribution

$$K_{ij} \sim \text{NB}(s_{ij}q_{ij}, \alpha_i)$$

We model read counts  $K_{ij}$ , as following the negative binomial distribution  
with mean  $\mu_i$  and **dispersion**  $\alpha_i$



# Counts are modeled using the Negative Binomial (NB) distribution

raw count for gene  $i$ , sample  $j$



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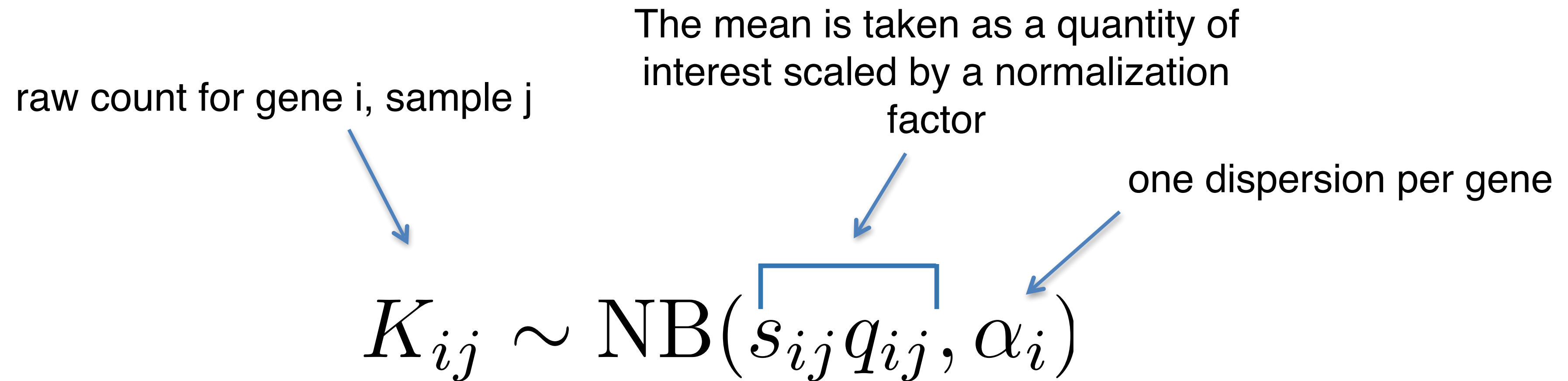
The mean is taken as a quantity of interest scaled by a normalization factor

The diagram shows the equation  $K_{ij} \sim \text{NB}(s_{ij}q_{ij}, \alpha_i)$ . A blue arrow points from the text 'raw count for gene  $i$ , sample  $j$ ' to the variable  $K_{ij}$ . Another blue arrow points from the text 'The mean is taken as a quantity of interest scaled by a normalization factor' to the term  $s_{ij}q_{ij}$ , which is bracketed in the equation.

$$K_{ij} \sim \text{NB}(s_{ij}q_{ij}, \alpha_i)$$

We model read counts  $K_{ij}$ , as following the negative binomial distribution with mean  $\mu_i$  and **dispersion**  $\alpha_i$

# Counts are modeled using the Negative Binomial (NB) distribution



We model read counts  $K_{ij}$ , as following the negative binomial distribution with mean  $\mu_i$  and **dispersion**  $\alpha_i$

# Within group variability is accounted for using the dispersion parameter

The **dispersion parameter**  $\alpha_i$ , describes the variance of counts via:

$$\text{Var}(K_{ij}) = \mu_{ij} + \alpha_i \mu_{ij}^2$$



Poisson part:  
sampling fragments



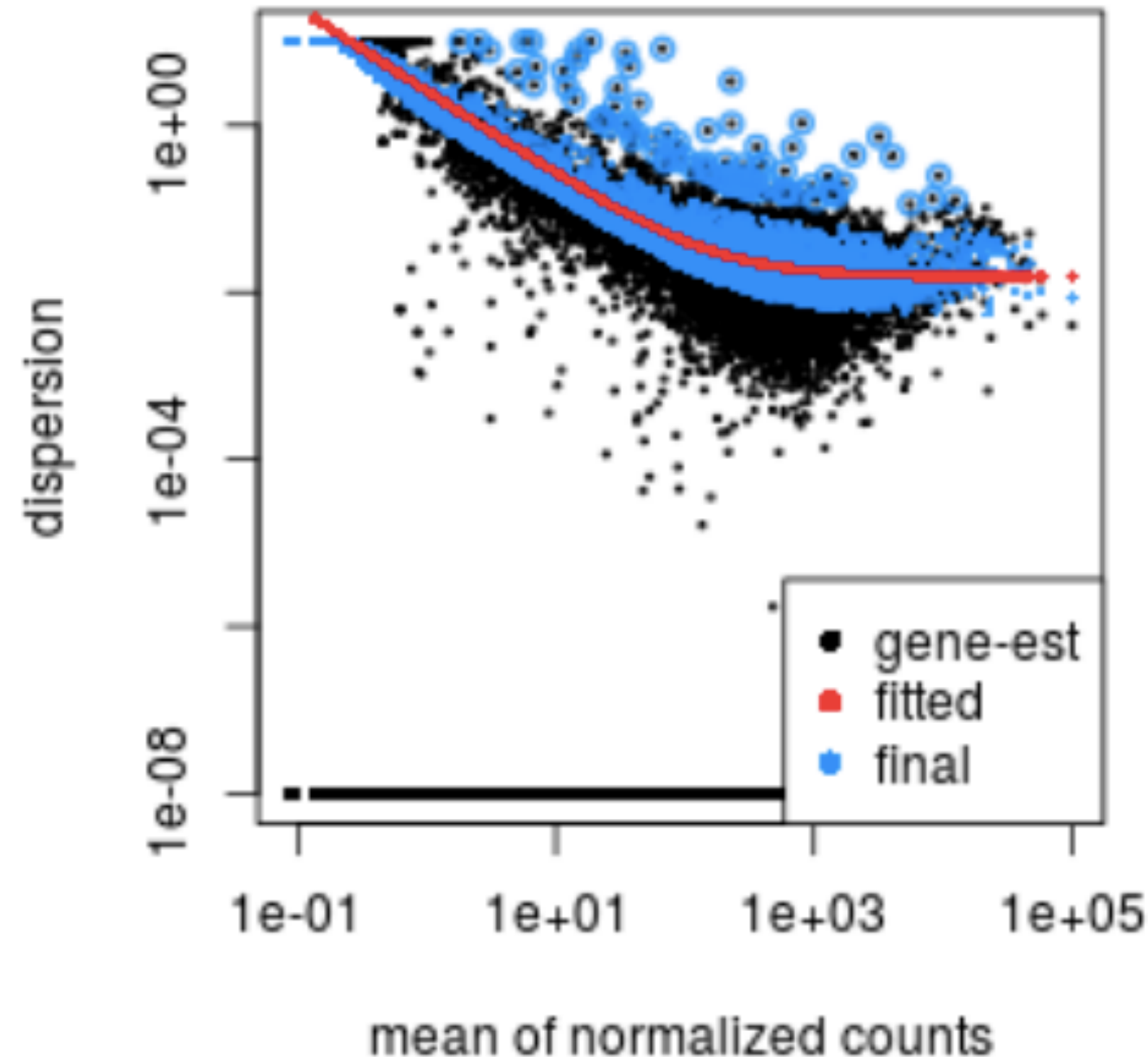
Extra variation  
due to biological variance

# Shrinkage and dispersion

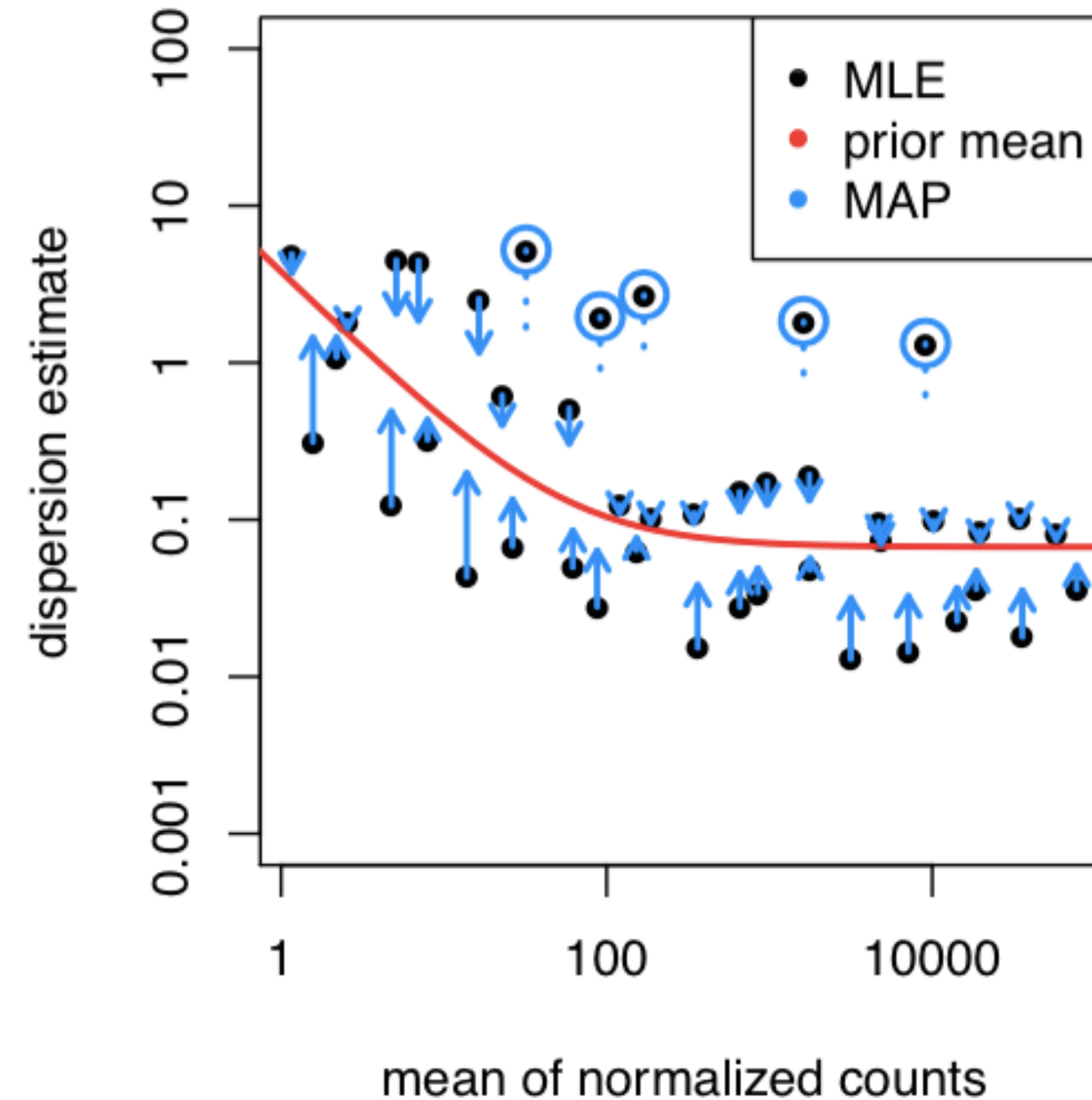
- Different genes naturally have different scales of biological variability
- Over all genes, there will be a distribution of reasonable estimates of dispersion
- With small sample size ( $n=3-5$  replicates per group), we will make *very bad* estimates of gene-wise dispersion unless we **share information across genes**

# Shrinkage of dispersion

all genes (Pasilla)



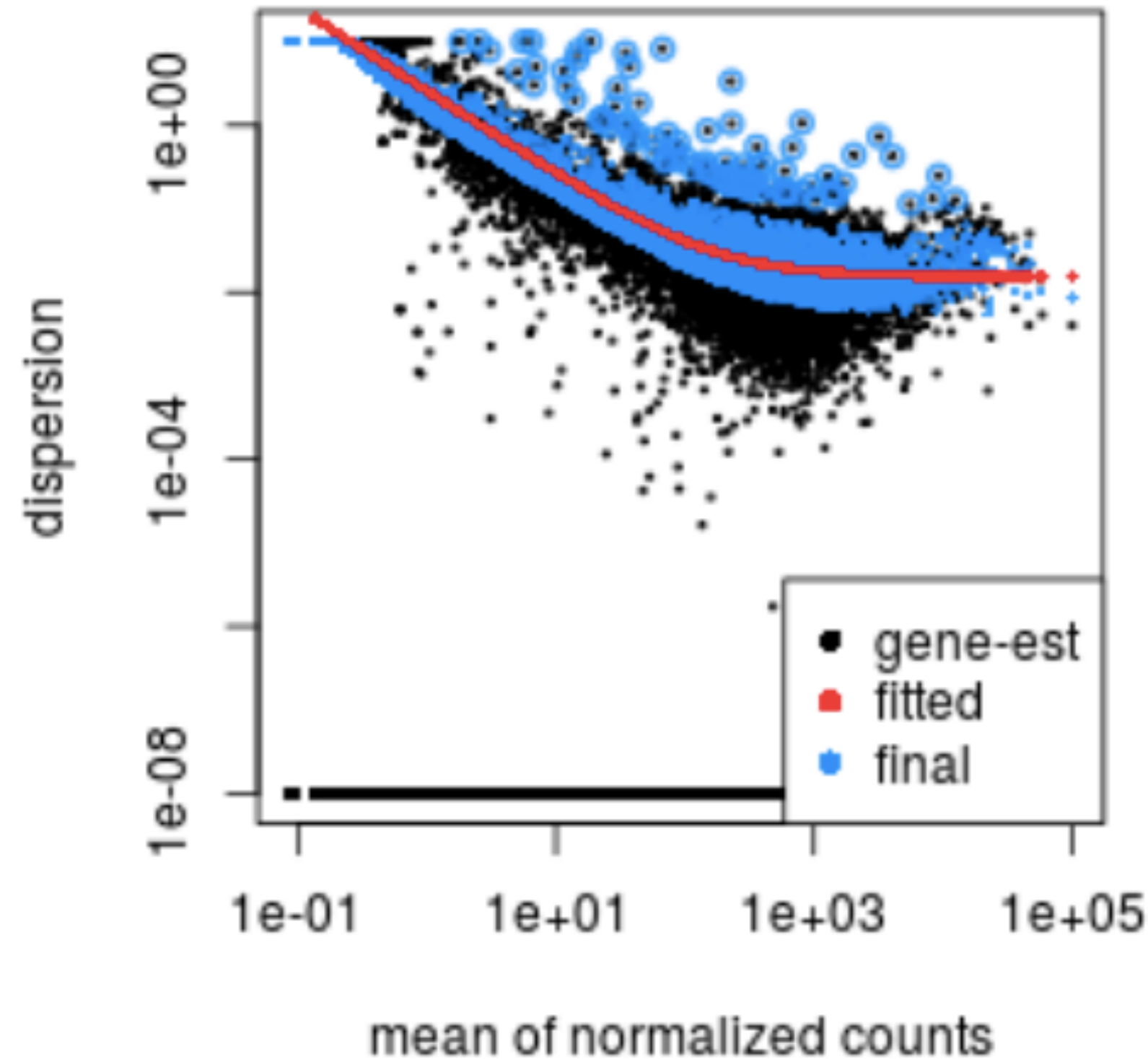
a subset of genes (Pickrell)



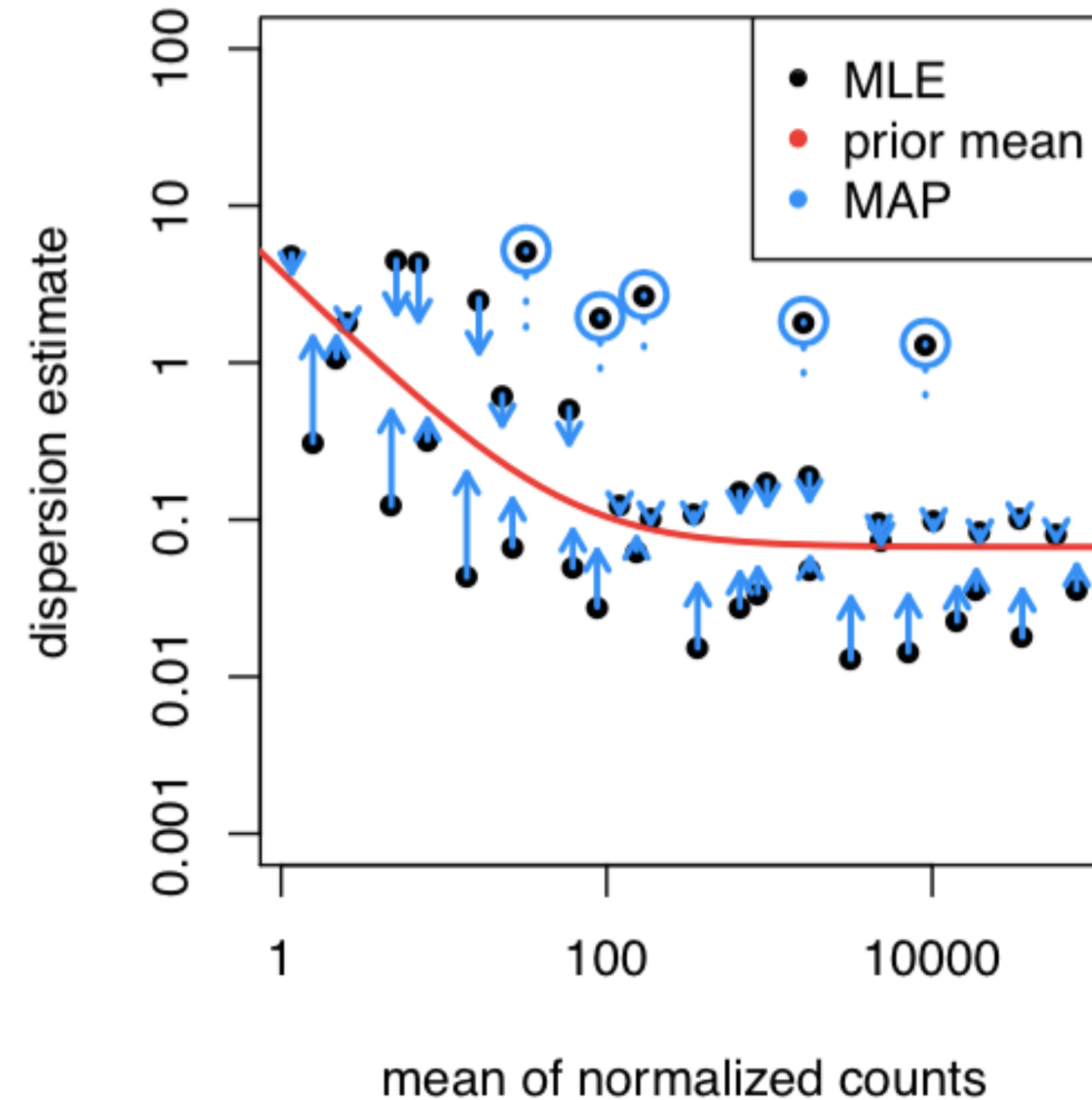
1. Gene-wise estimate = just look at one gene (MLE)
2. Fitted dispersion trend = the middle for the prior
3. Final estimate = posterior, uses shared information (MAP)



all genes (Pasilla)



a subset of genes (Pickrell)



The shrinkage procedure thereby helps avoid potential false positives, which can result from underestimates of dispersion

# Differences across conditions

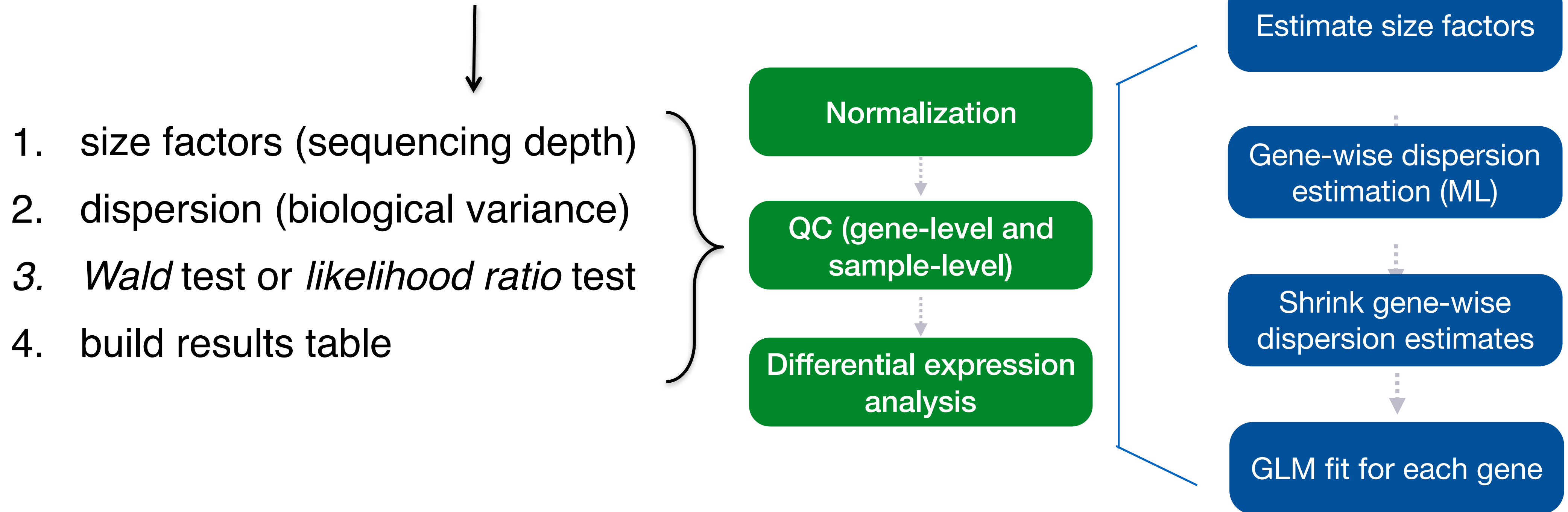


# DESeq2

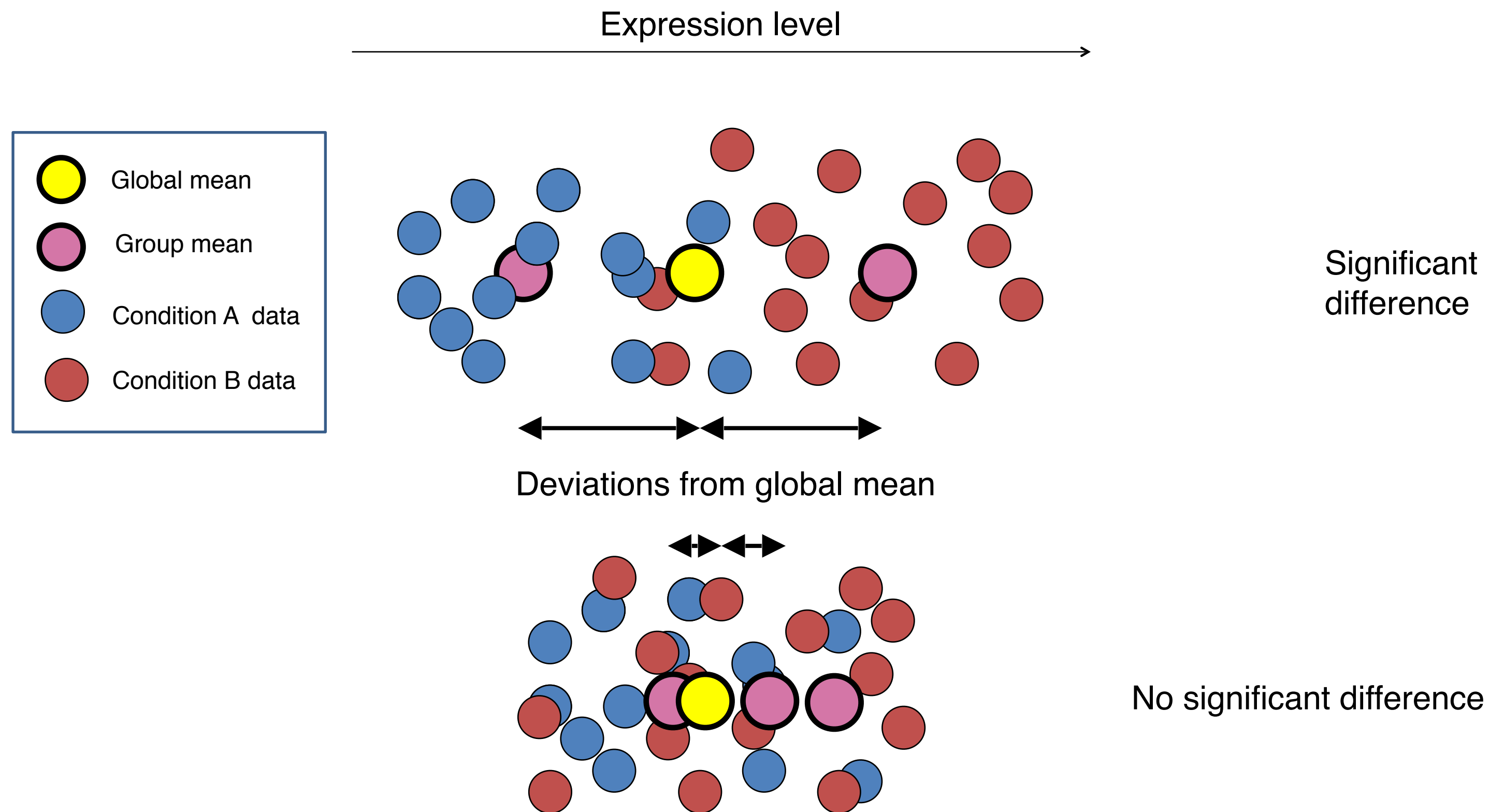
- Available through Bioconductor since 2013
- Publication: Genome Biology, Dec 2014.  
main text written with non-statisticians in mind
- Builds on good ideas for dispersion estimation and use of GLM from the [DSS](#) and [edgeR](#) methods
- See [bioconductor.org/install](http://bioconductor.org/install) for installation
- Note that the latest Bioconductor packages are only available with **latest R version**. Bioconductor and R versions are *linked*

# DESeq2 steps

count matrix (from featureCounts, htseq, tximport, etc.)



# Differences across two conditions



# Differences across two conditions

- Describe experiment with formula, e.g.: ~ condition
- Per gene the design matrix looks like:

$$\begin{array}{lcl} \log_2 q1 & & 1 \quad 0 \\ \log_2 q2 & & 1 \quad 0 \\ \log_2 q3 & = & 1 \quad 1 \\ \log_2 q4 & & 1 \quad 1 \end{array}$$

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$$\begin{array}{l} \log_2 q1 \\ \log_2 q2 \\ \log_2 q3 \\ \log_2 q4 \end{array} = \begin{array}{|c|c|} \hline 1 & 0 \\ \hline 1 & 0 \\ \hline 1 & 1 \\ \hline 1 & 1 \\ \hline \end{array} \quad \boxed{\text{Intercept}}$$

All samples get an Intercept term

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$$\begin{matrix} \log_2 q1 \\ \log_2 q2 \\ \log_2 q3 \\ \log_2 q4 \end{matrix} = \begin{matrix} \begin{matrix} 1 \\ 1 \\ 1 \\ 1 \end{matrix} & \begin{matrix} 0 \\ 0 \\ 1 \\ 1 \end{matrix} \end{matrix}$$

Intercept
condition B vs A

All samples get an Intercept term

The B condition samples also get a term that accounts for the difference between B and A

# Differences across multiple conditions

- The design matrix now uses a column for each condition

$\log_2 q1$		1	1	0	0	
$\log_2 q2$		1	1	0	0	Intercept
$\log_2 q3$	=	1	0	1	0	conditionA
$\log_2 q4$		1	0	1	0	conditionB
$\log_2 q5$		1	0	0	1	conditionC
$\log_2 q6$		1	0	0	1	

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# GLM: Generalized linear models

- ▶ Extension of linear models to non-normally distributed response data (in our case, negative binomial)
- ▶ Helps address the different mean-variance relationships
- ▶ GLM fit for a gene will return **coefficients indicating the overall expression** strength of the gene for each design matrix element

# Contrasts

- A contrast is the comparison of coefficients we choose to evaluate

$\log_2 q1$		1	1	0	0	
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$\log_2 q5$		1	0	0	1	conditionC
$\log_2 q6$		1	0	0	1	

# Contrasts

- A contrast is the comparison of coefficients we choose to evaluate

$\log_2 q1$		1	1	0	0	
$\log_2 q2$		1	1	0	0	Intercept
$\log_2 q3$	=	1	0	1	0	conditionA
$\log_2 q4$		1	0	1	0	conditionB
$\log_2 q5$		1	0	0	1	conditionC
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```
results(dds, contrast=c("condition", "B", "C"))
```

# Hypothesis testing

## Wald test

- ▶ use the shrunken estimate of the  $\log_2$  fold-change divided by the SE
- ▶ this gives a Z-statistic which is compared to a standard normal distribution
- ▶ allows testing of individual coefficients, or contrasts of coefficients (i.e. two-level comparison)

# Hypothesis testing

## Wald test

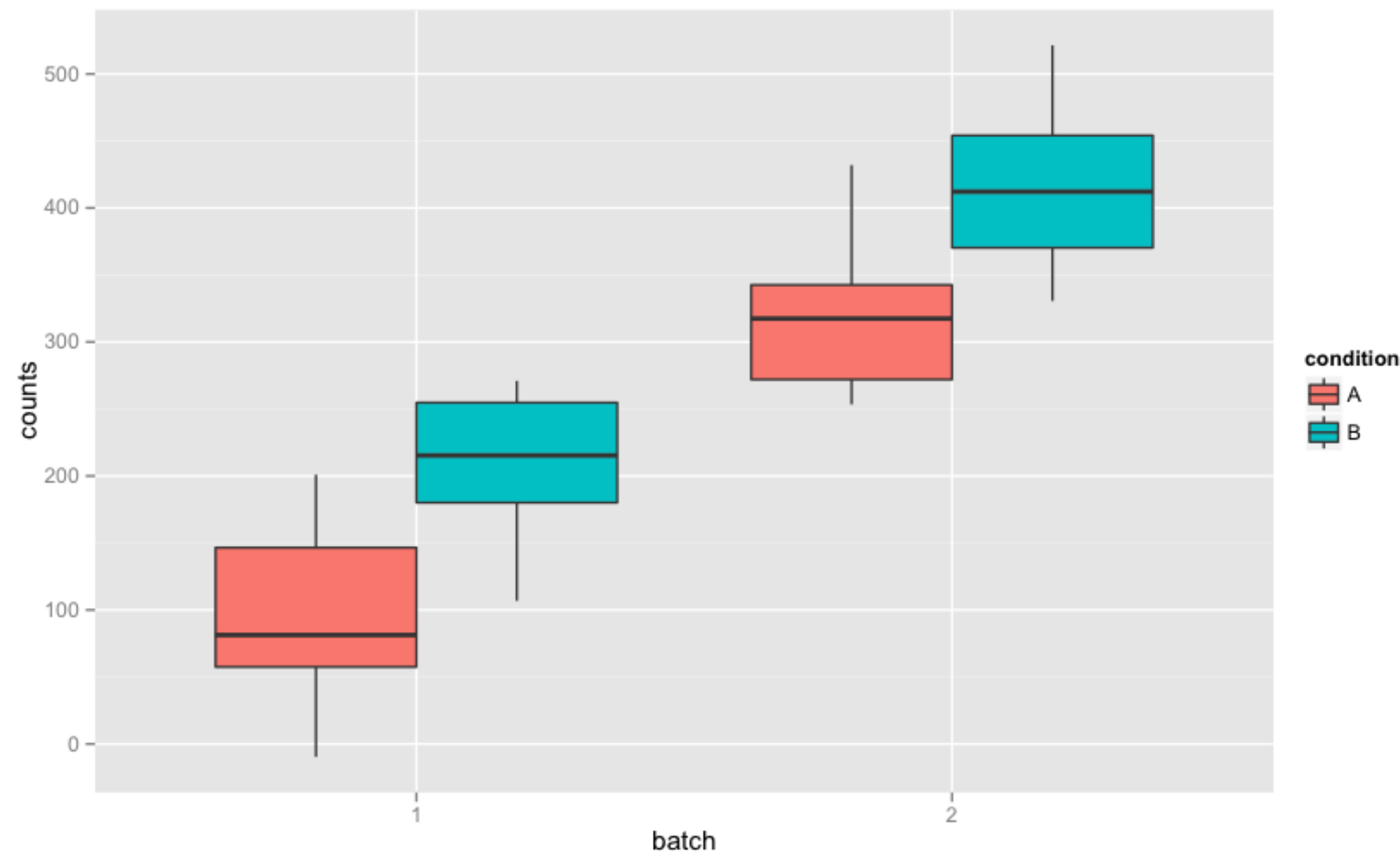
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## Likelihood ratio test (LRT)

- ▶ examines two different models: full and reduced model (with some terms removed)
- ▶ determines if the increased likelihood of the data using the extra terms in the full model is more than expected if those extra terms are truly zero
- ▶ useful for identifying any gene that is changing in expression with respect to the biological factor of interest (useful for 3 or more levels)

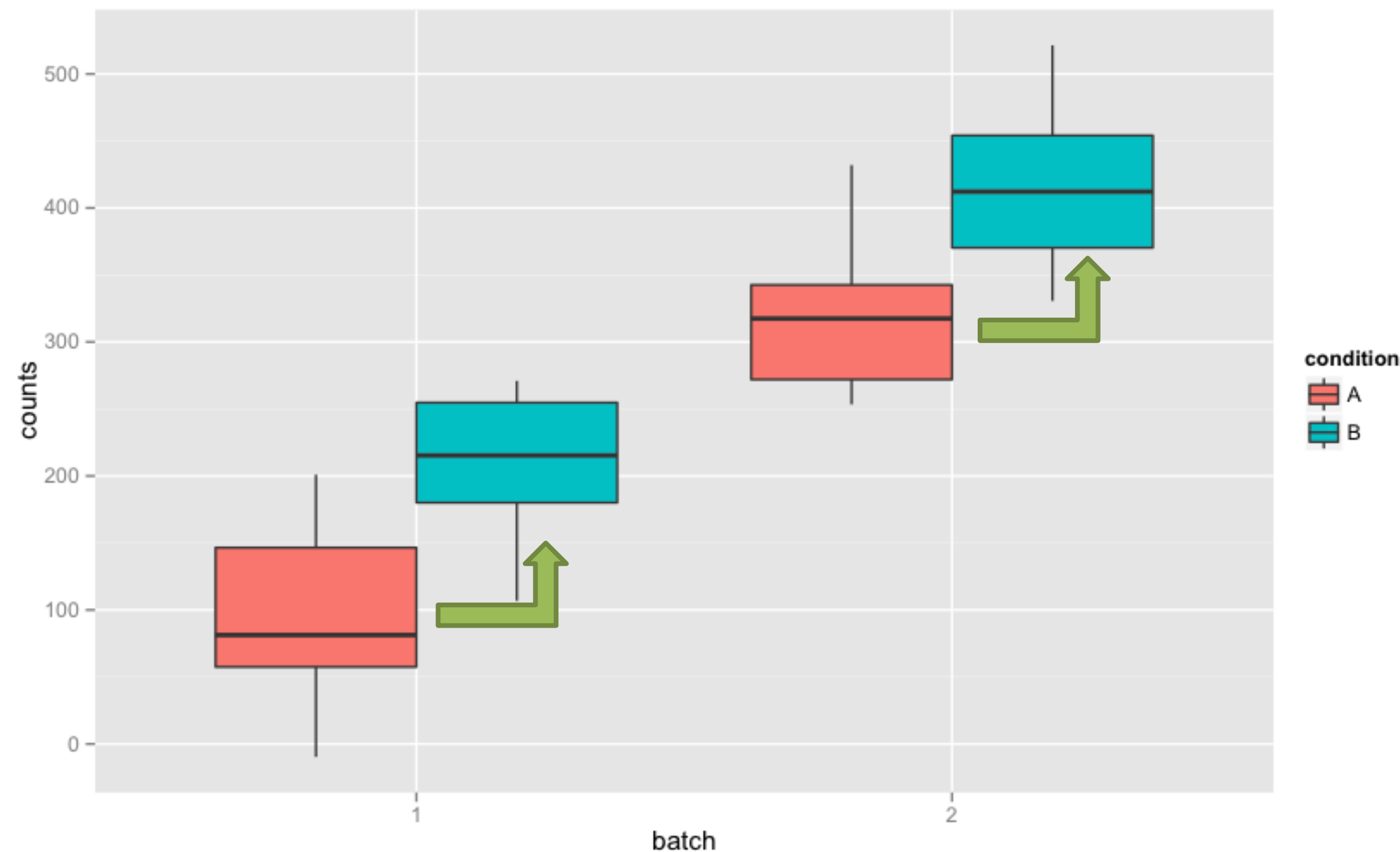
# Controlling for different batches

- Using a design formula:  $\sim \text{batch} + \text{condition}$ , adds terms that control for batch differences
- If batches are unknown, possible to detect these with other methods: [svaseq](#), [RUVSeq](#)



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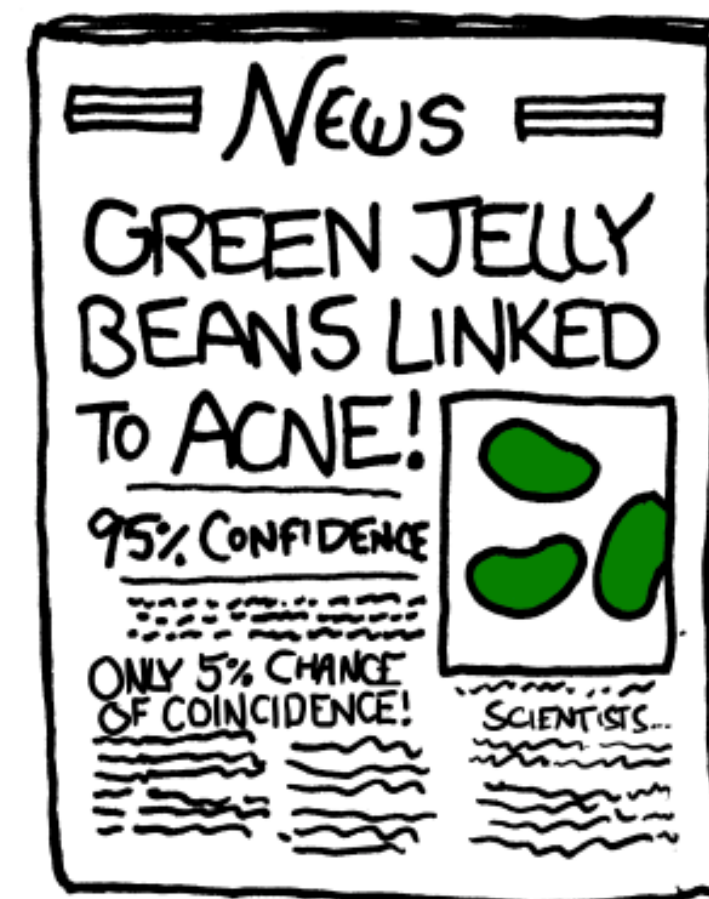
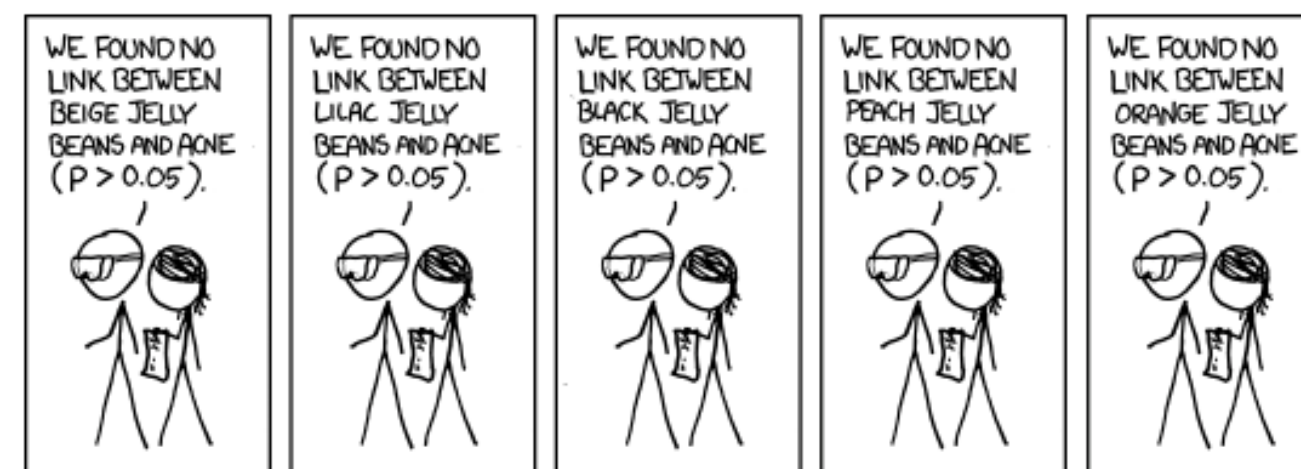
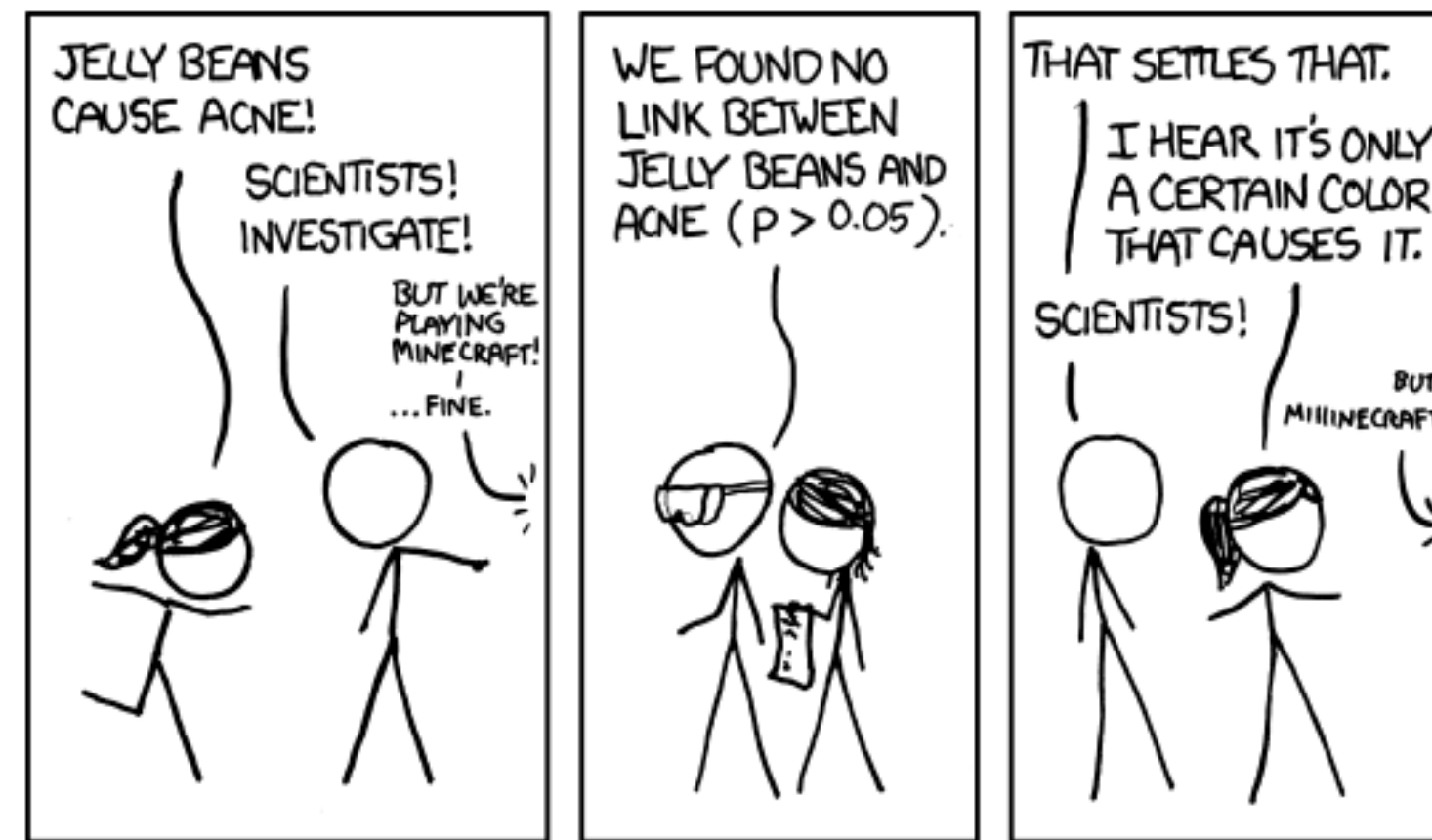
# Complex designs

Want to test: treatment changes for enriched samples over baseline, controlling for individual effects

~individual + enrichment + treatment +  
enrichment:treatment

indiv	.	enrich.	treat.
1	input	control	
1	IP	control	
1	input	treat	
1	IP	treat	
2	input	control	
2	IP	control	
2	input	treat	
2	IP	treat	
...			

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This is the multiple testing problem. The more tests we perform, the more we inflate the number of false positives observed.

# Multiple test correction

## Controlling the FWER

---

- ▶ Control  $\alpha$ , the probability of making an error (false positive)
- ▶ **Bonferroni:** Reject any hypothesis with  $p\text{-value} \leq \alpha/m$ 
  - ▶ Conservative; high probability of false negatives

## Controlling the FDR

---

- ▶ FDR: false discovery rate: the expected percent of false predictions in the set of predictions
- ▶ **Benjamini-Hochberg:** Rank  $j / m$  multiplied by the FDR level
  - ▶ designed to control the FDR
- ▶ **Q-value:** The minimum FDR that can be attained when calling that feature significant

# DE vs EDA

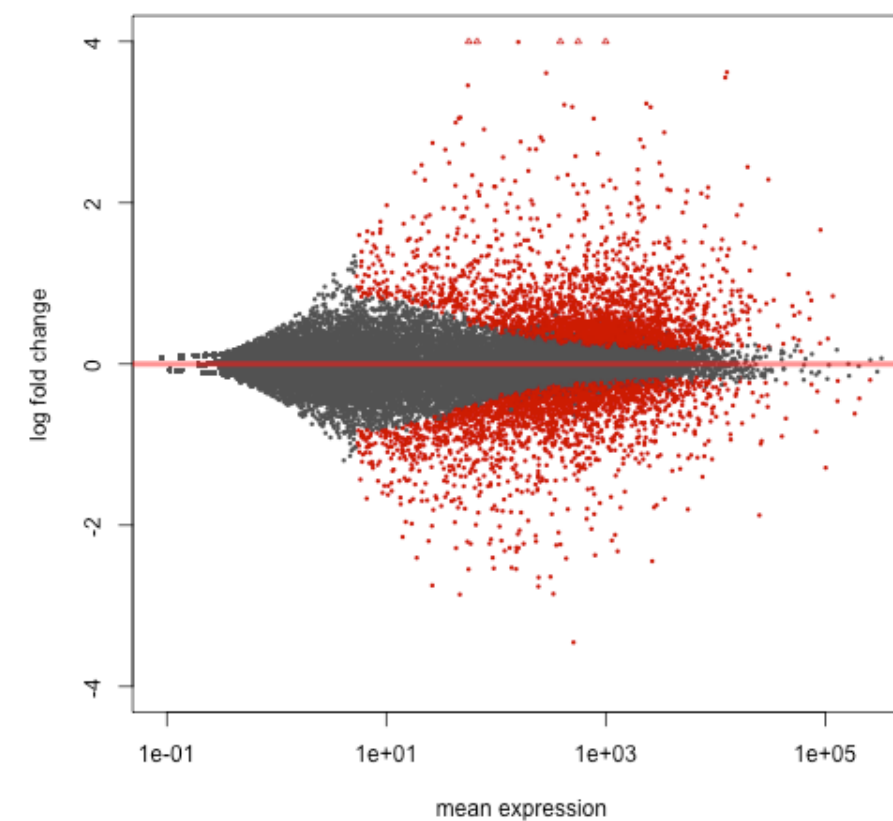


# Two paths in DESeq2

Count matrix

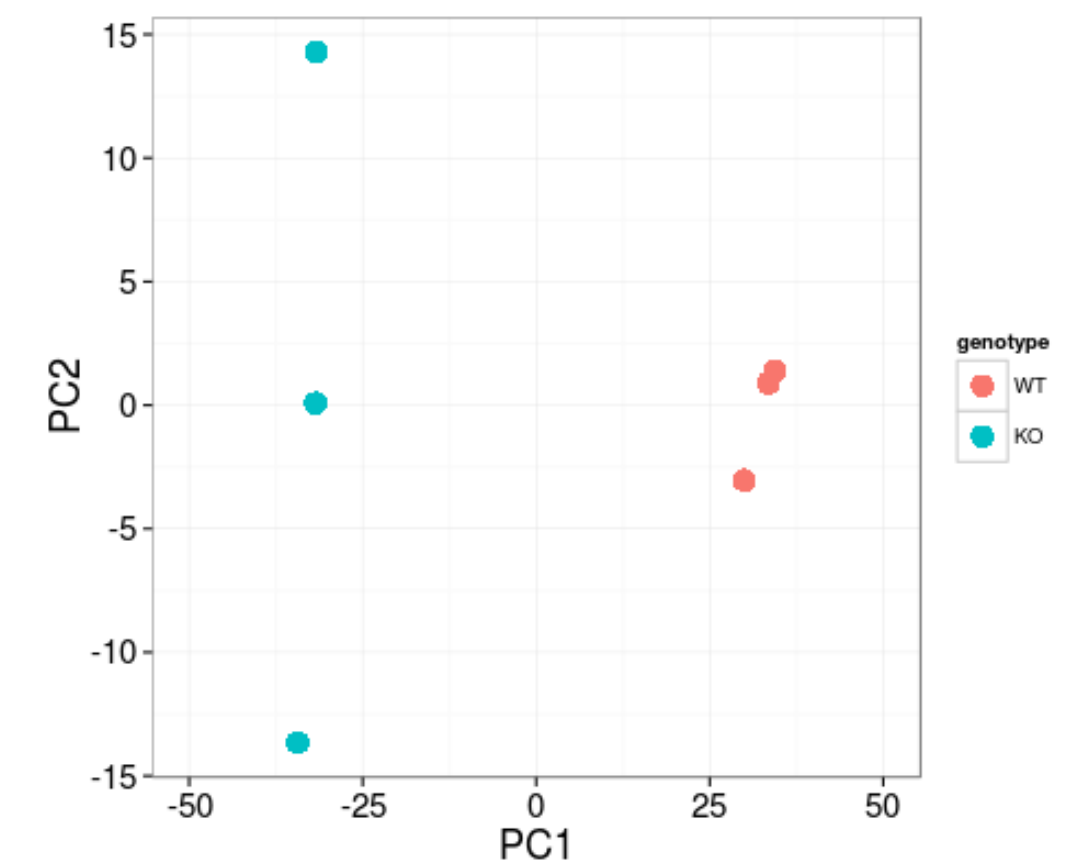
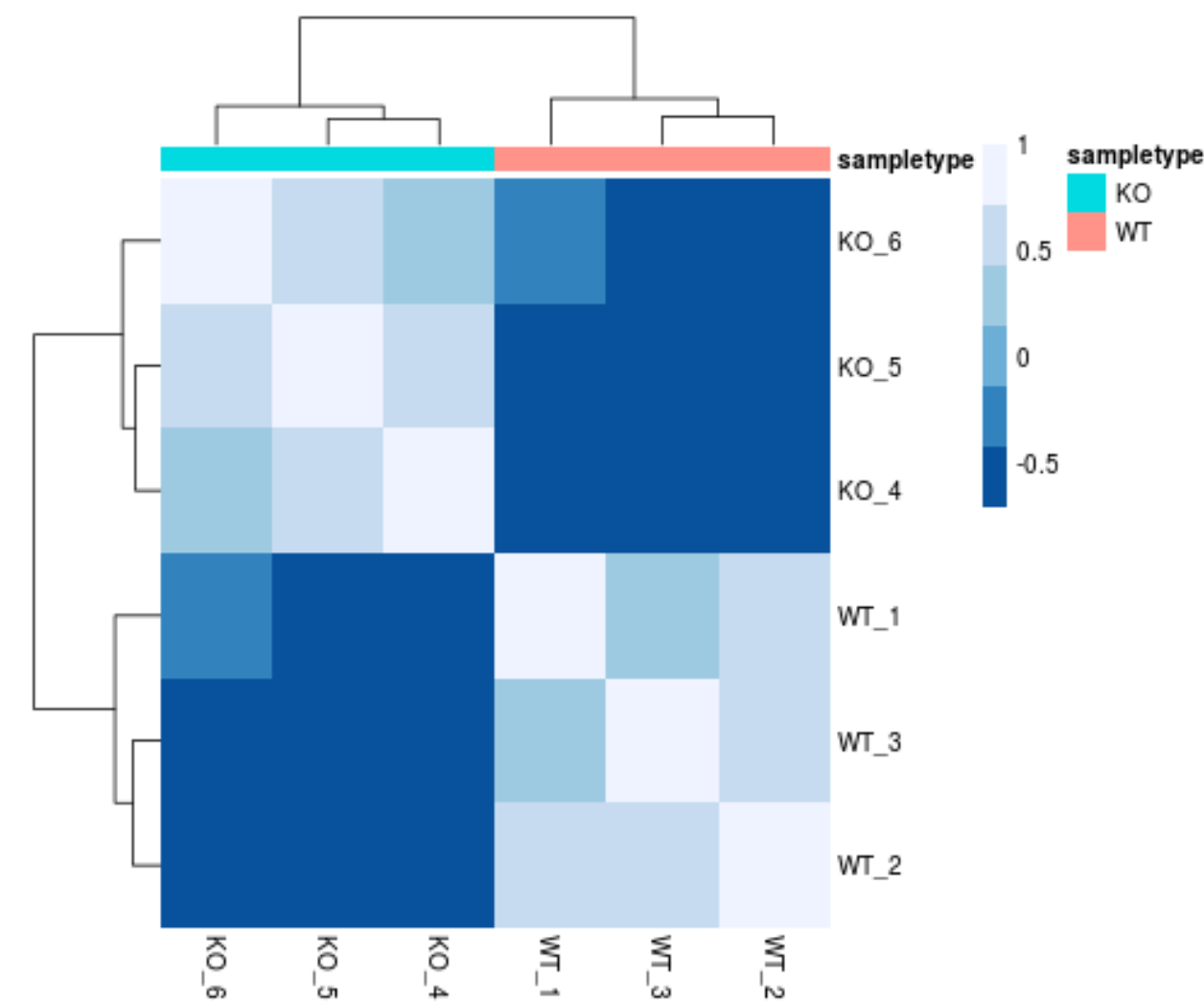
Differential expression

testing, p-values, FDR



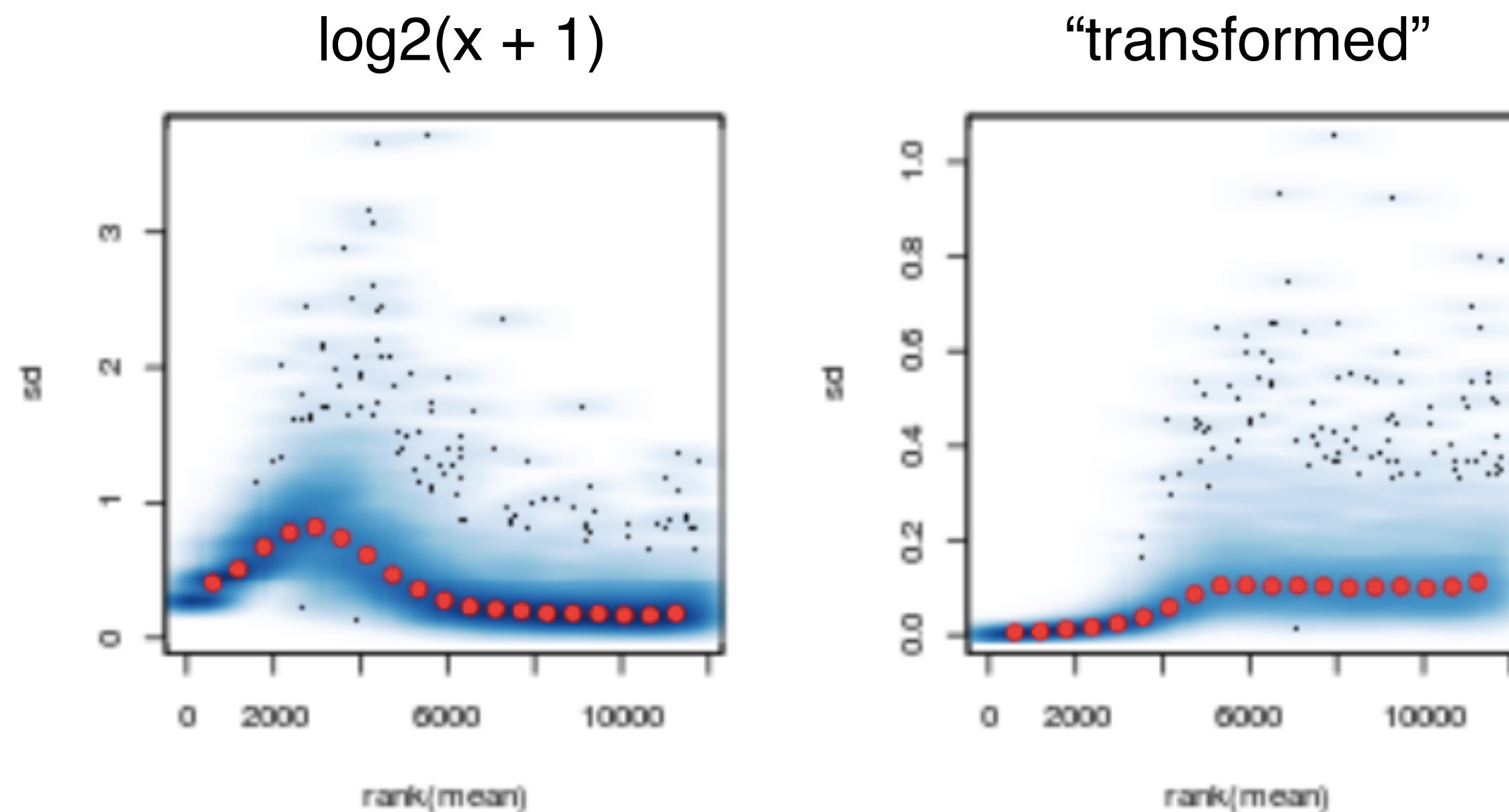
Transformations and  
Exploratory Data Analysis (EDA)

clustering, heatmaps,  
sample-sample distances



# Transformations

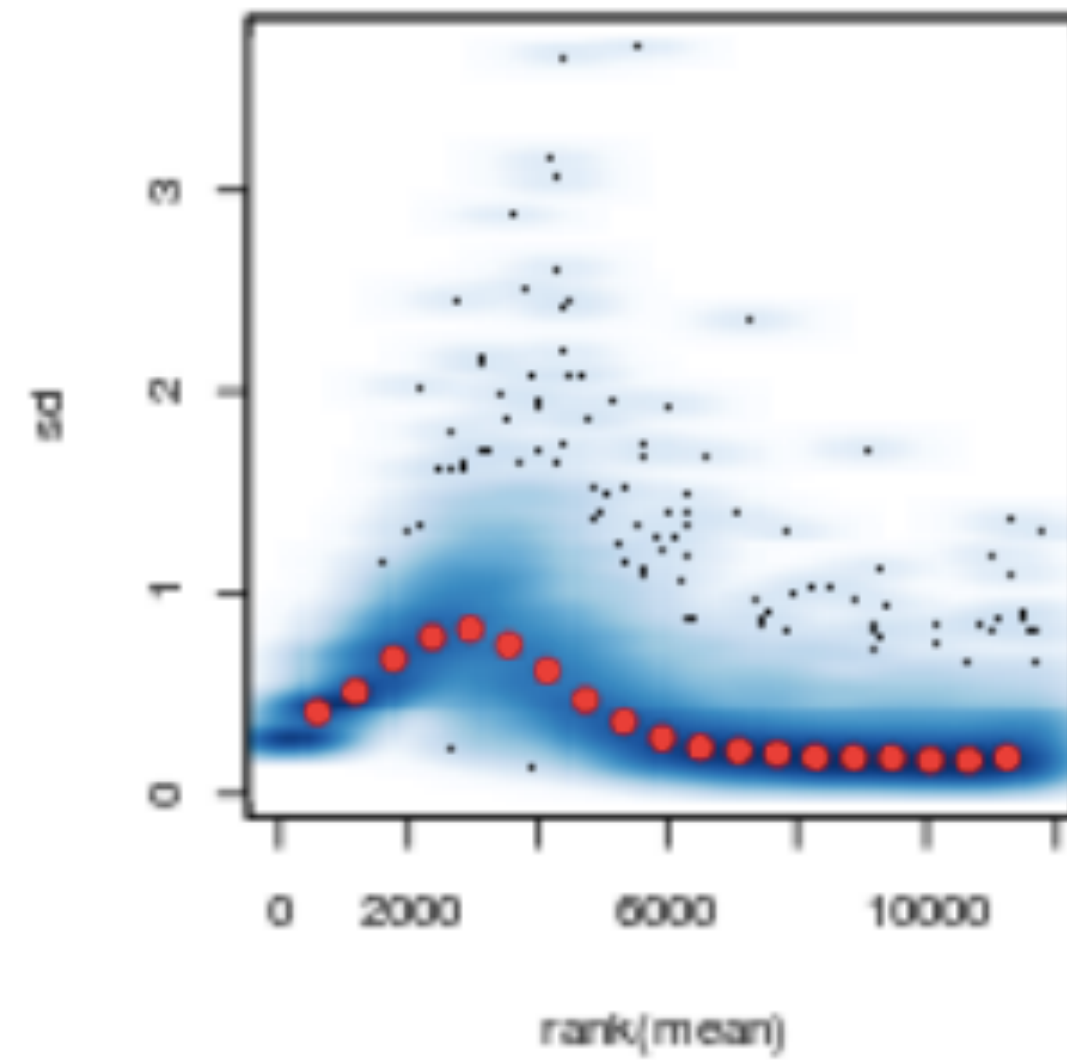
For comparison analyses when using unsupervised techniques, it can be useful to *transform* data. These techniques (VST and rlog) perform better when values have a similar dynamic range



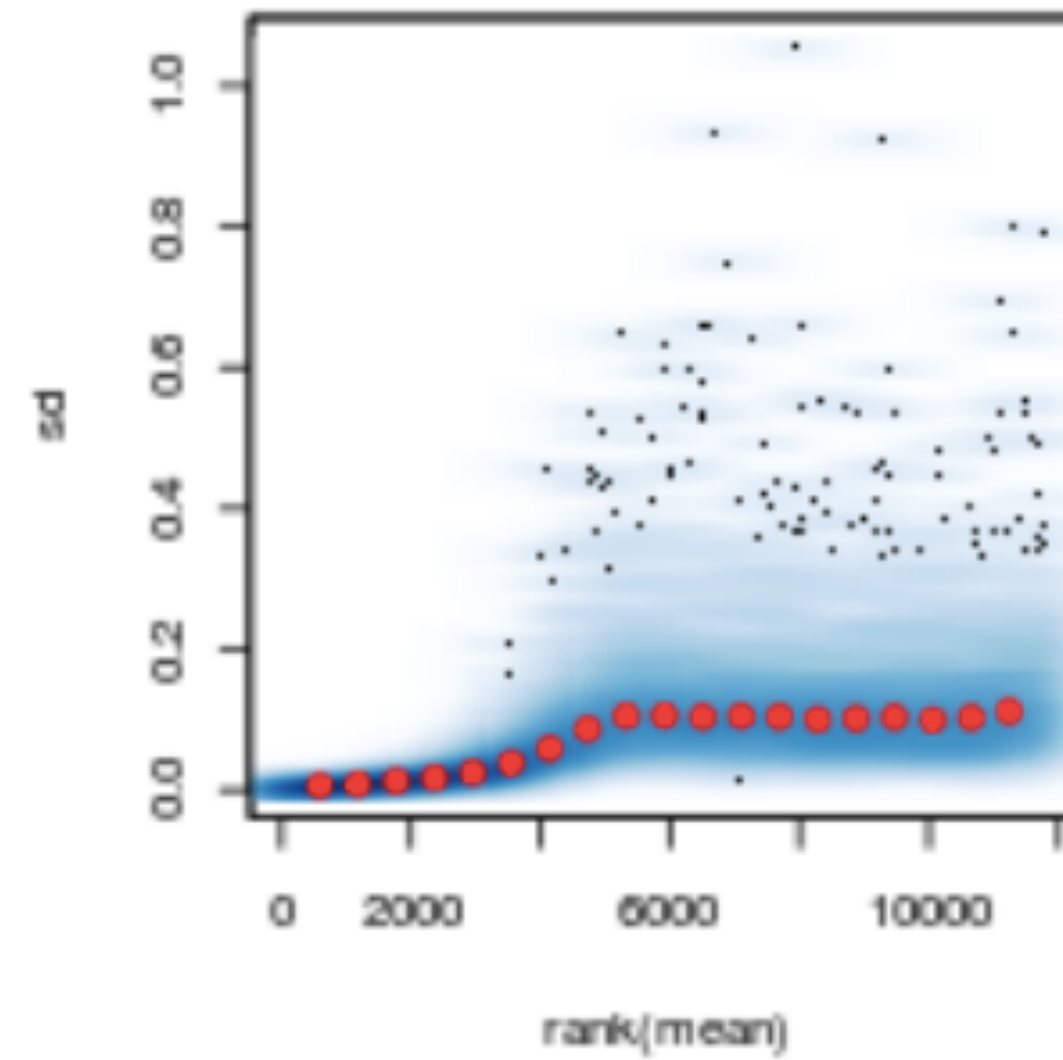
renders data *homoskedastic*  
(variance of the gene is  
stabilized across expression  
levels)

# rlog stabilizes variances along the mean

$\log_2(x + 1)$

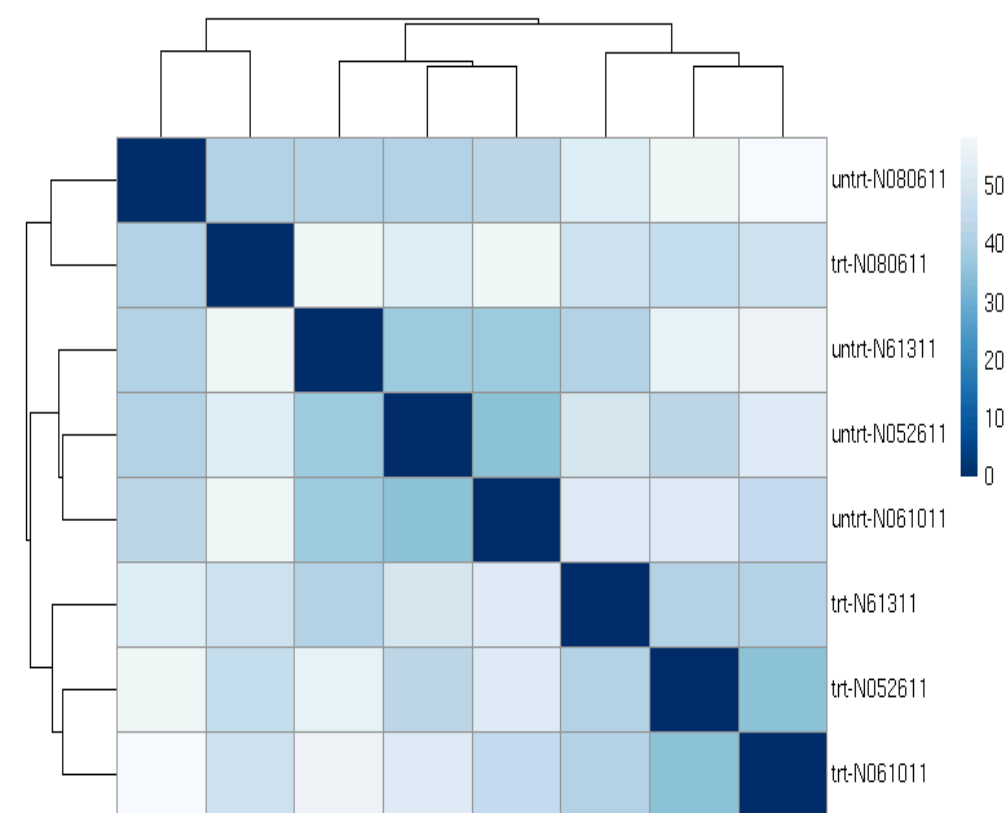
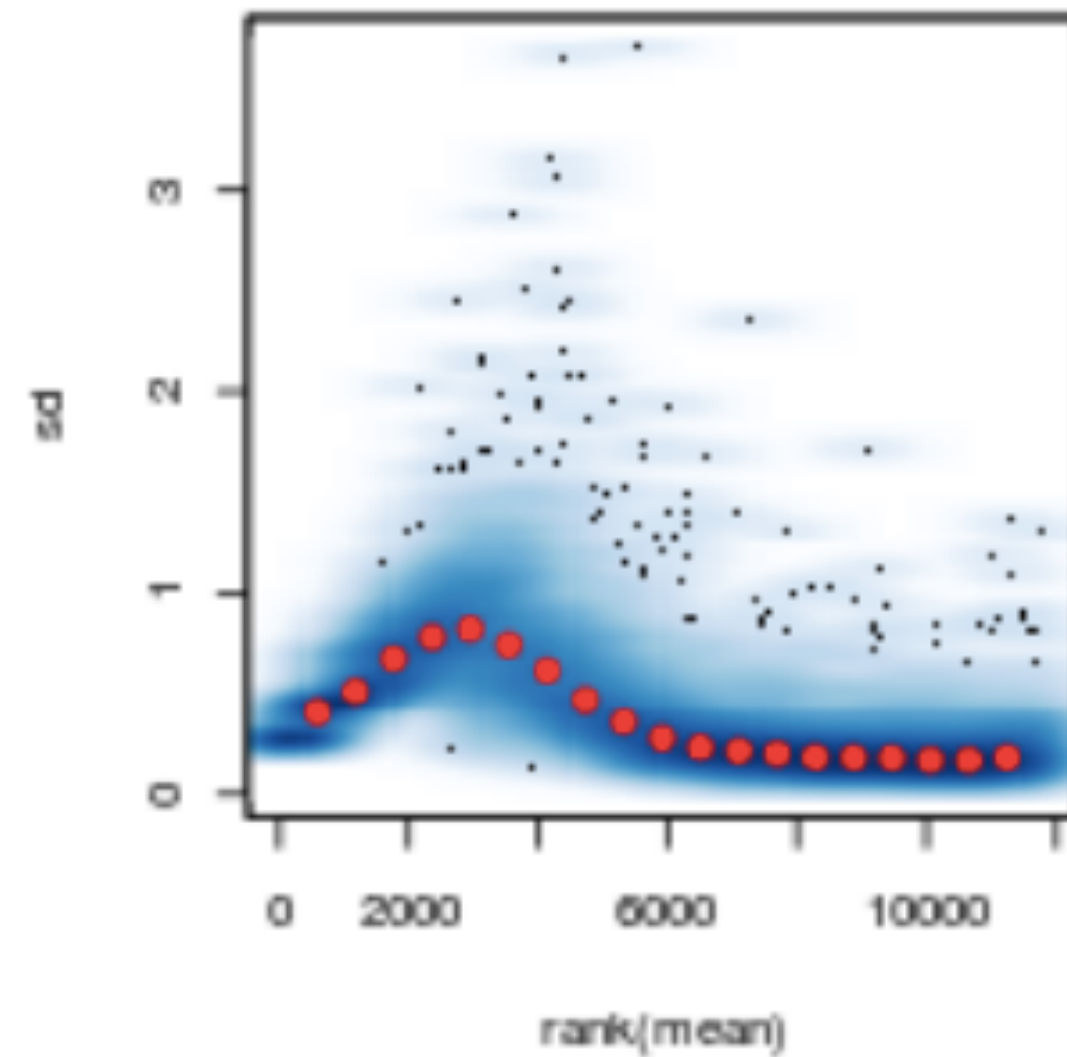


"rlog"

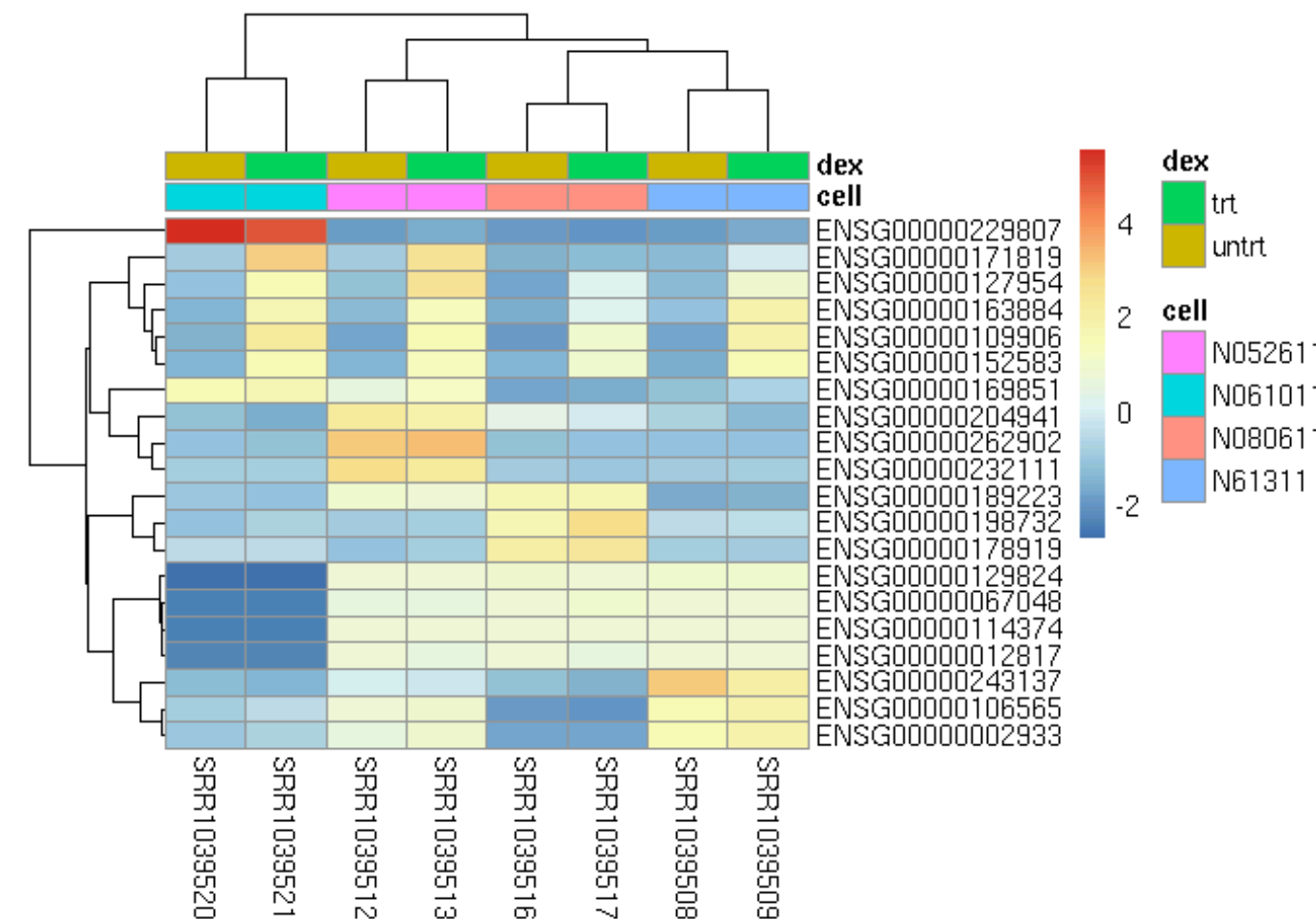
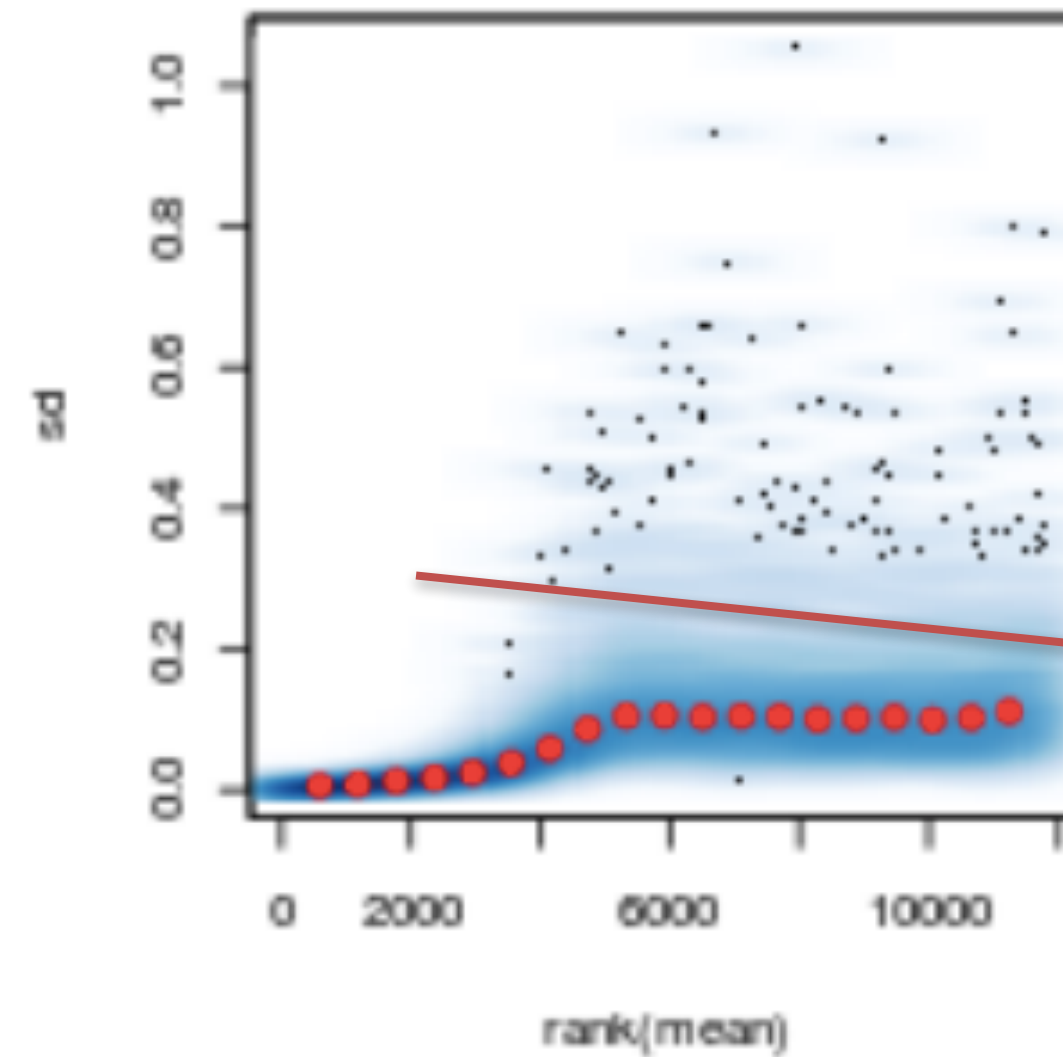


# rlog stabilizes variances along the mean

$\log_2(x + 1)$



"rlog"



Moderating the high variance / low count genes  
Improves distances, clustering, visualizations

# Variance stabilizing transform (VST)

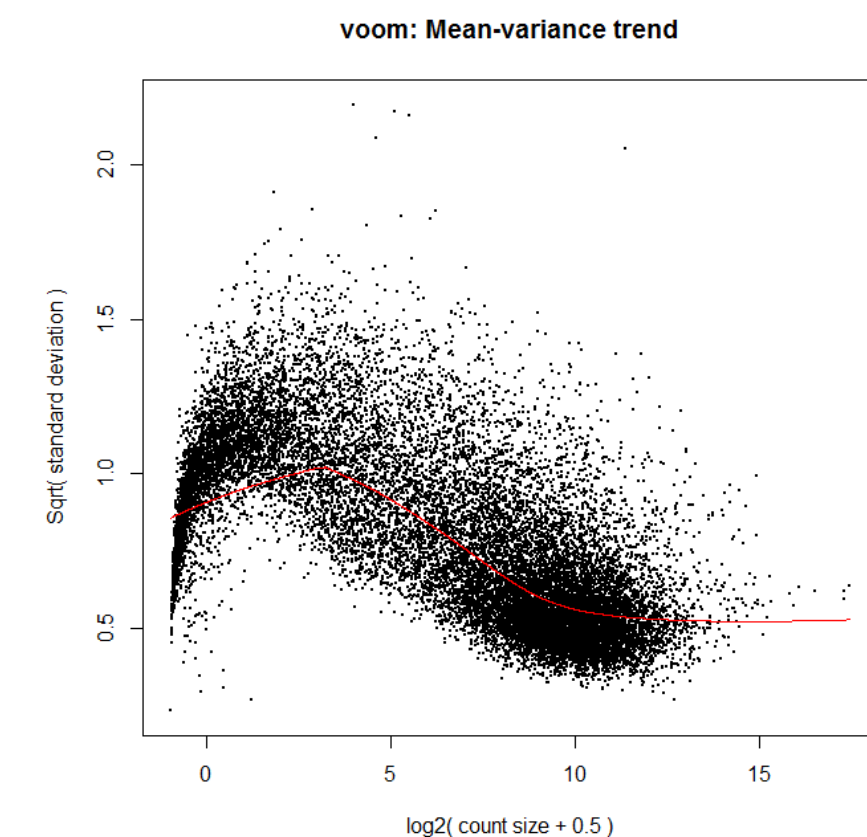
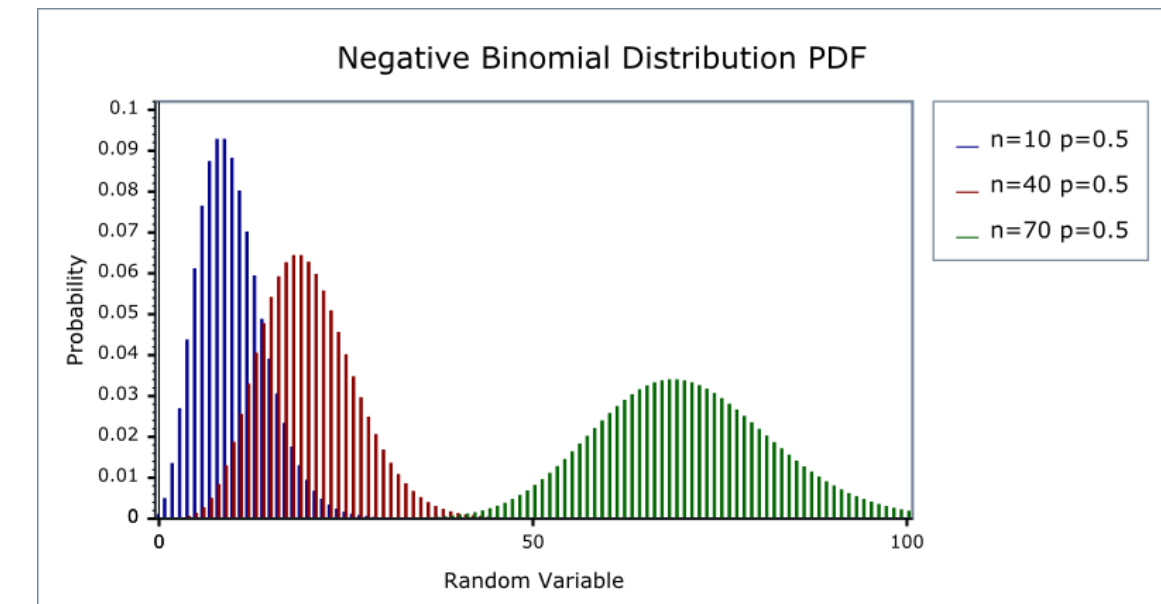
- The variance stabilizing transformation is an earlier approach (from original DESeq) for transforming counts.
- Uses a function which is log-like but doesn't go to  $-\text{Inf}$  at  $x=0$ .
- VST doesn't use size factors so is **better for data that has consistent sequence depth across samples**
- VST is closed form, so can be **better for large datasets ( $n > 50$ )** due to speed.

# Comparison of methods



# Count model vs linear model

- **DESeq2** and **edgeR** similar approach, similar results
  - very sensitive, may sometimes underestimate FDR
- **limma+voom** uses a linear model, weights determined by variance over mean
  - strong control of FDR, may be less sensitive for small sample size
  - recommended when number of biological replicates per group grows large (e.g.  $> 20$ )



# Credits

## *RNA-seq statistical analysis and gene-level differential expression*

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Dept. of Biostatistics and Computational Biology

Dana-Farber Cancer Institute & Harvard TH Chan School of Public Health

### Acknowledgements:

DESeq2/DEXSeq:

Wolfgang Huber  
Simon Anders  
Alejandro Reyes

Work supported by:

Rafael Irizarry

Dana-Farber Cancer  
Institute

Harvard TH Chan School  
of Public Health

NIH Training Grant

