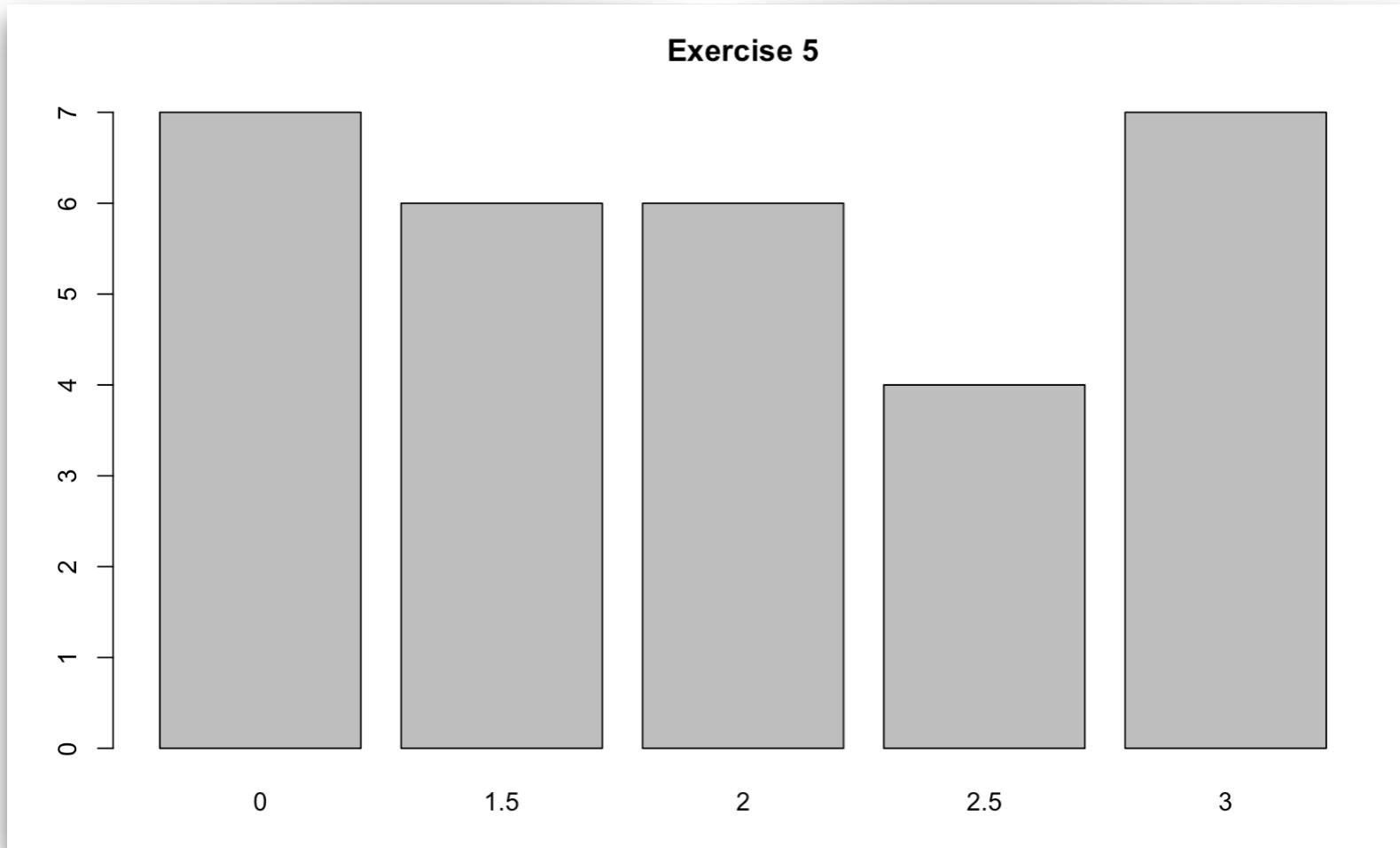
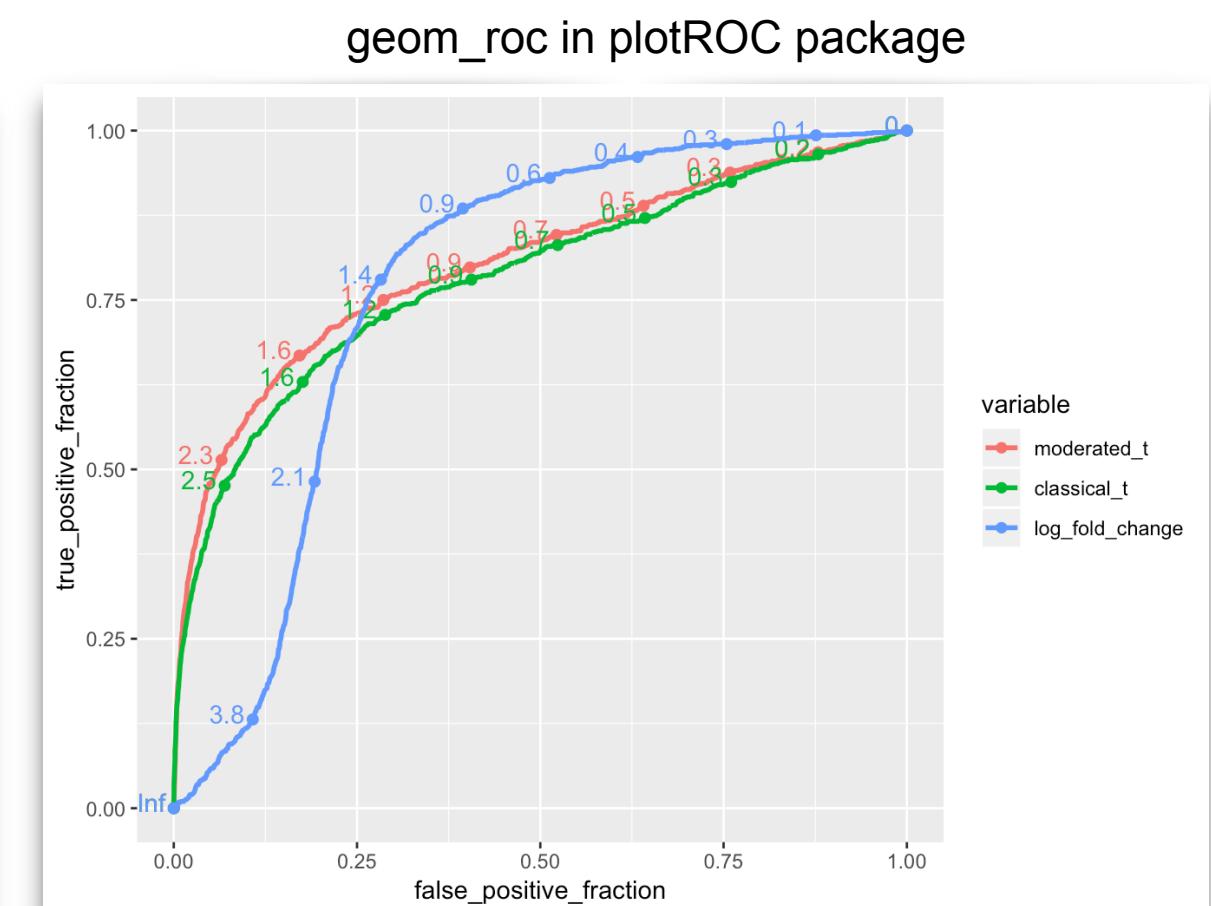
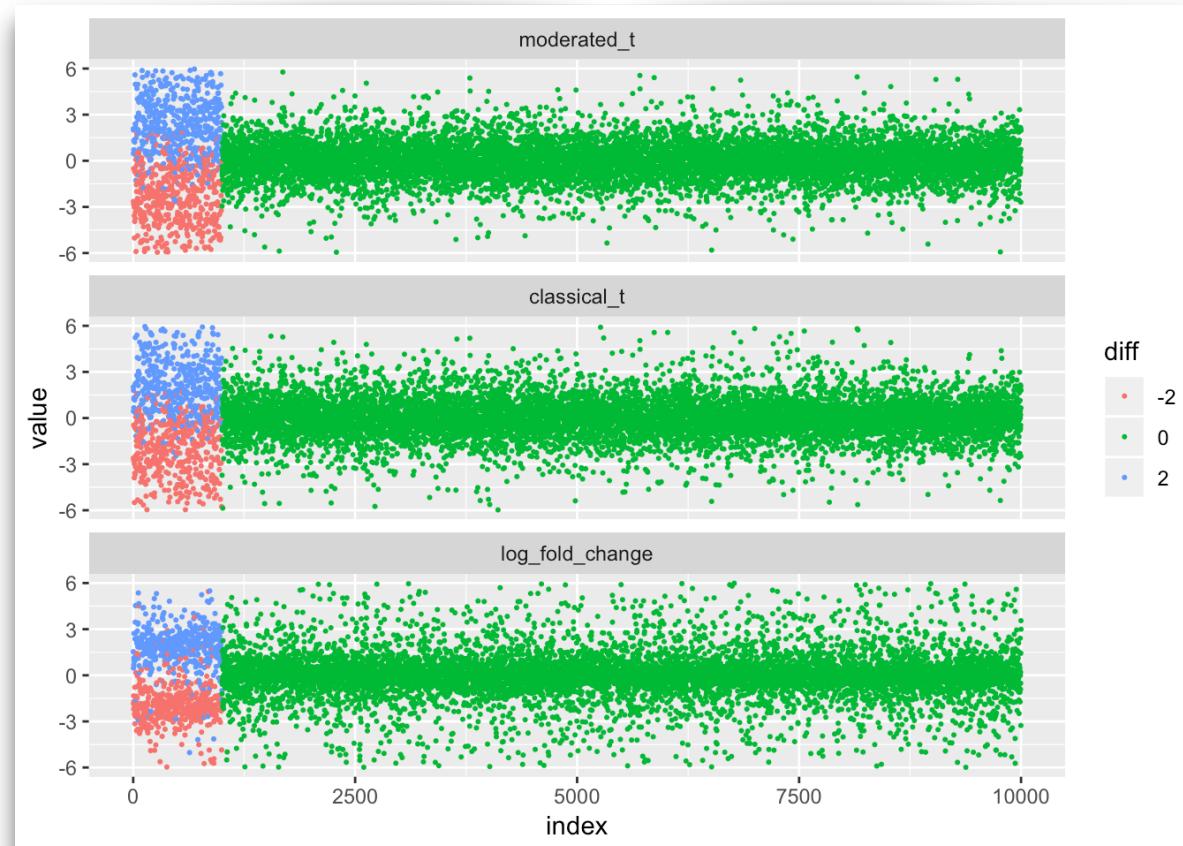


# Exercise 5 (limma fundamentals)



# Notes on Exercise 5 (limma) .. and Exercises in general





# Notes on Exercise 5 (limma fundamentals) .. general things

- please don't hide code (` `` ` { r echo=FALSE } ... ) !
- try to exclude package startup messages from report, at least for package startup messages (```r my\_block,  
message=FALSE} ...)
- please organize your assignments like a report —> -1 for future reports that are hard to follow.



# Notes on Exercise 5 (limma fundamentals) .. general things

Feel free to work together, but do not plagiarise code

```
fit_class <- vector("list", length = nrow(y))
t_val <- numeric(nrow(y))
mean_diff <- numeric(nrow(y))
p_val <- numeric(nrow(y))
for(i in 1:nrow(y)){
  myfit <- t.test(y[i, 1:3], y[i, 4:6])
  t_val[i] <- myfit$statistic
  mean_diff[i] <- myfit$estimate[1] - myfit$estimate[2]
  fit_class[i] <- myfit
  p_val[i] <- myfit$p.value
}

plot(t_val)
```

```
## [1] 10000

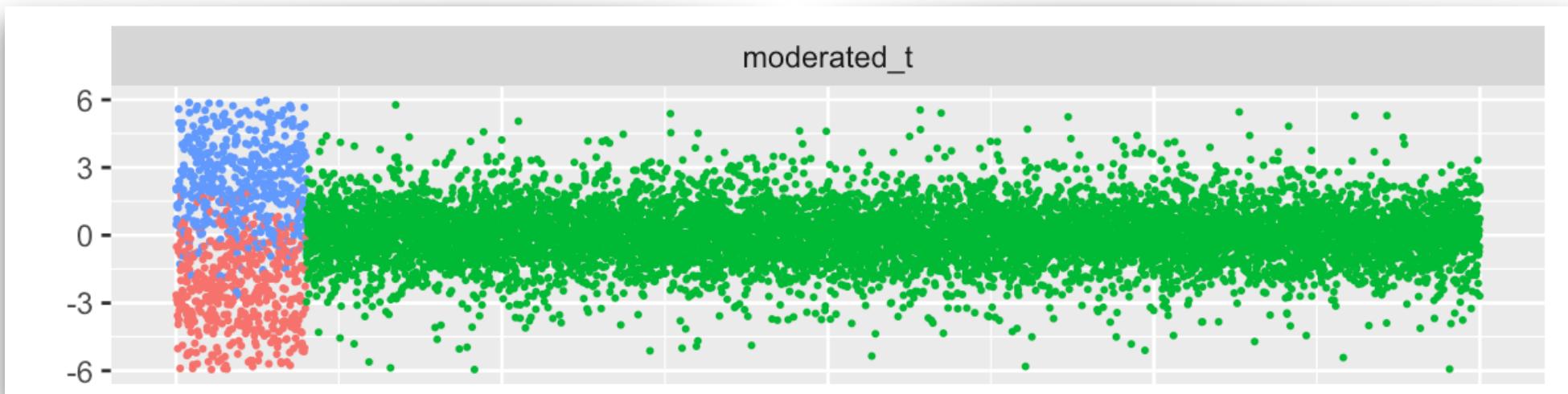
fit_class <- vector("list", length = nrow(y))
t <- numeric(nrow(y))
mean_dif <- numeric(nrow(y))
p <- numeric(nrow(y))

for(i in 1:nrow(y)){
  myfit <- t.test(y[i, 1:3], y[i, 4:6])
  t[i] <- myfit$statistic
  mean_dif[i] <- myfit$estimate[1] - myfit$estimate[2]
  fit_class[i] <- myfit
  p[i] <- myfit$p.value
}

plot(t)
```

# Notes on Exercise 5 (limma fundamentals) .. technical things

- residual variance
- Question asked for: `t.test(..., var.equal=TRUE)`  
(Welch's t-test is the default)
- ROC curve: **absolute value of score** .. or use P-value

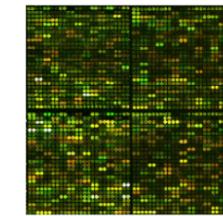


# Notes on Exercise 5 (limma fundamentals) .. technical things

- data are already on log scale .. no need to re-log

## Microarray expression measures

Two-colour



$$y_{ga} = \log_2(R/G)$$

↑  
probe or gene      ↑  
array

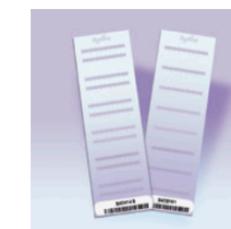
Affymetrix



$$y_{ga} = \text{log-intensity}$$

(summarized over  
probes)

Illumina



$$y_{ga} = \text{log-intensity}$$

(summarized over  
beads)



# Project ideas

Reminder — 3 choices: 1. redo analysis from a paper; 2. do a small comparison of methods; 3. do a real-world analysis (consulting or otherwise)

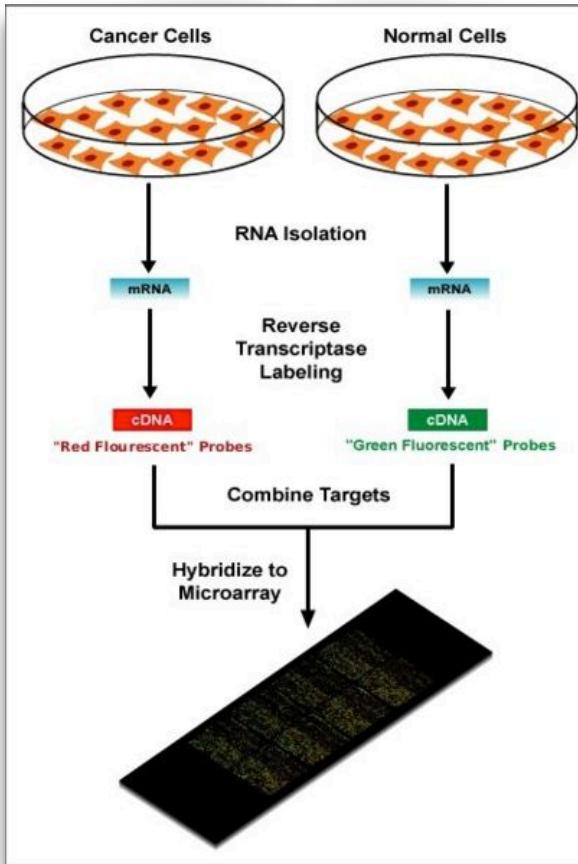
- 1) devise a clustering accuracy metric like ARI, but which takes into account the hierarchical nature of the subpopulations (e.g. that a misclassification mistake from b cell to t cell is worse than one between t-cell subtypes).
- 2) implement a gene correlation structure for simulations
- 3) benchmark measures of gene coexpression for scRNASeq; there are already 2 papers doing that, but they're not amazing and they don't test some fairly obvious things.
- 4) benchmark methods for aggregating signals across features (e.g. across genes of a given pathway, across binding sites of a given TF in single-cell ATAC-seq)
- 5) benchmark the use of sparse/regularized PCA and the likes for scRNASeq clustering (this is probably the simplest, since it could basically be done within pipeComp)



# Statistical models for count data analysis (differential expression)

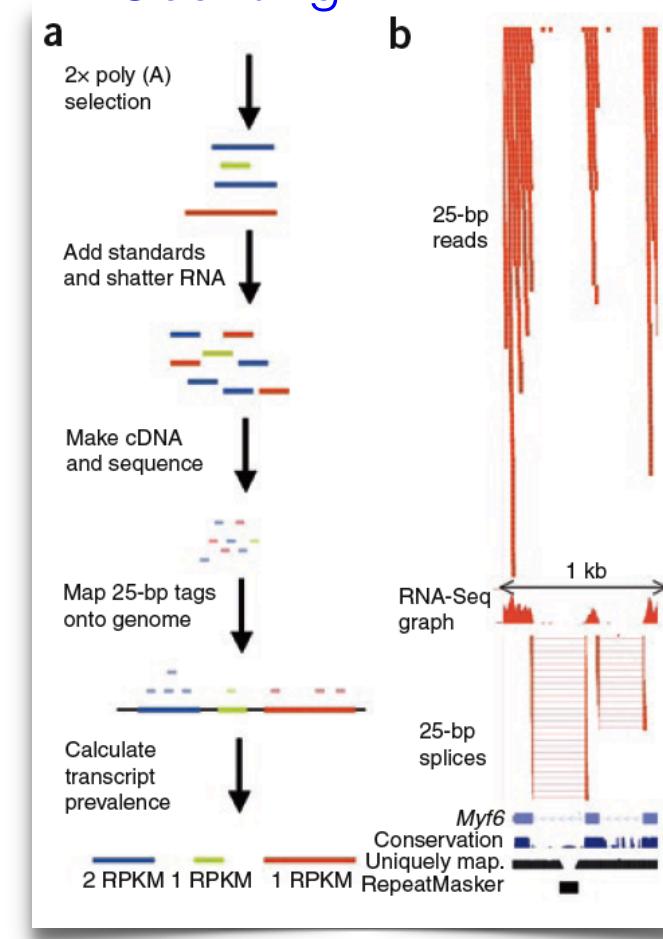
- simple counting (and new alternatives ..)
- edgeR, DESeq/DESeq2 —> why the negative binomial distribution?
- dispersion estimation and information sharing
- normalization considerations
- how about transformations of count data —> limma?

## Abundance by Fluorescence Intensity



[http://en.wikipedia.org/wiki/DNA\\_microarray](http://en.wikipedia.org/wiki/DNA_microarray)

## Abundance by Counting

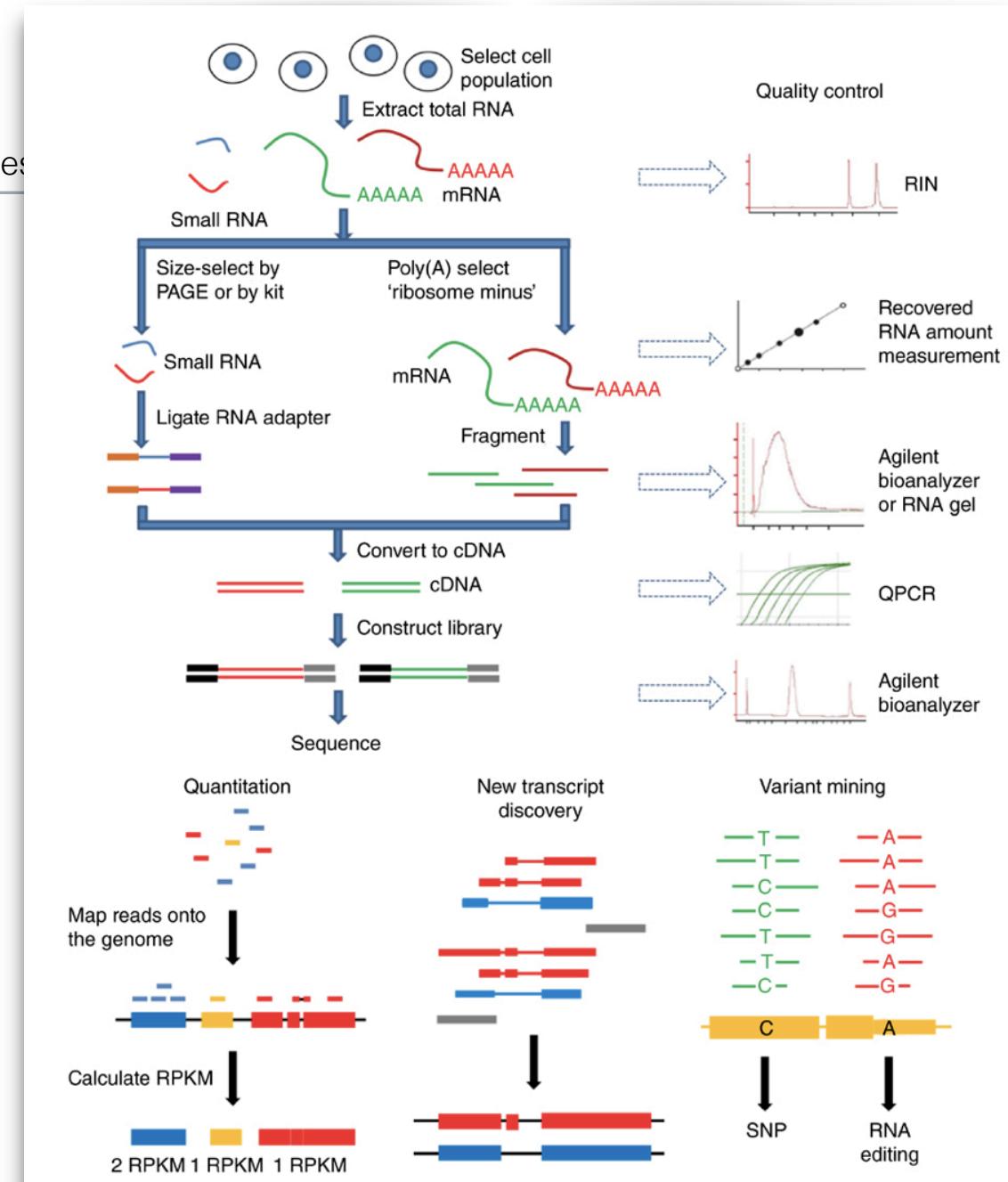


Mortazavi et al., Nature Methods, 2008



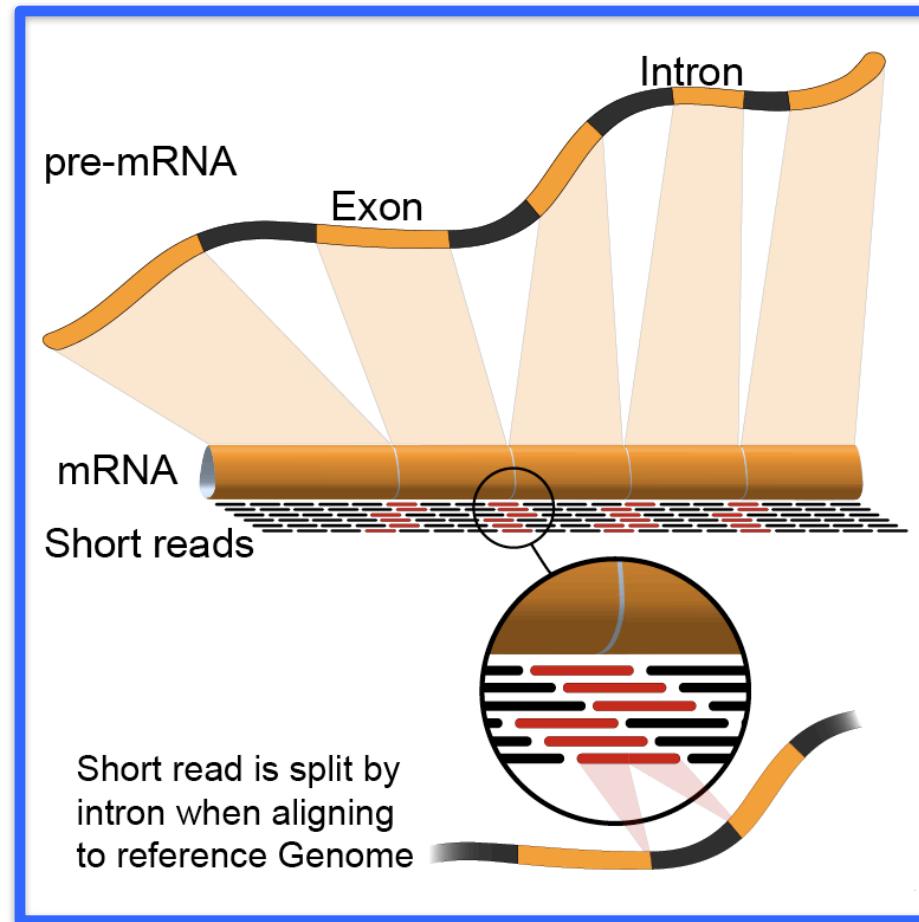
# RNA-seq differential expression analyses

1. Map the reads to reference sequences
2. “Count” reads that map to genes (quantify)
3. Compute DE Statistics

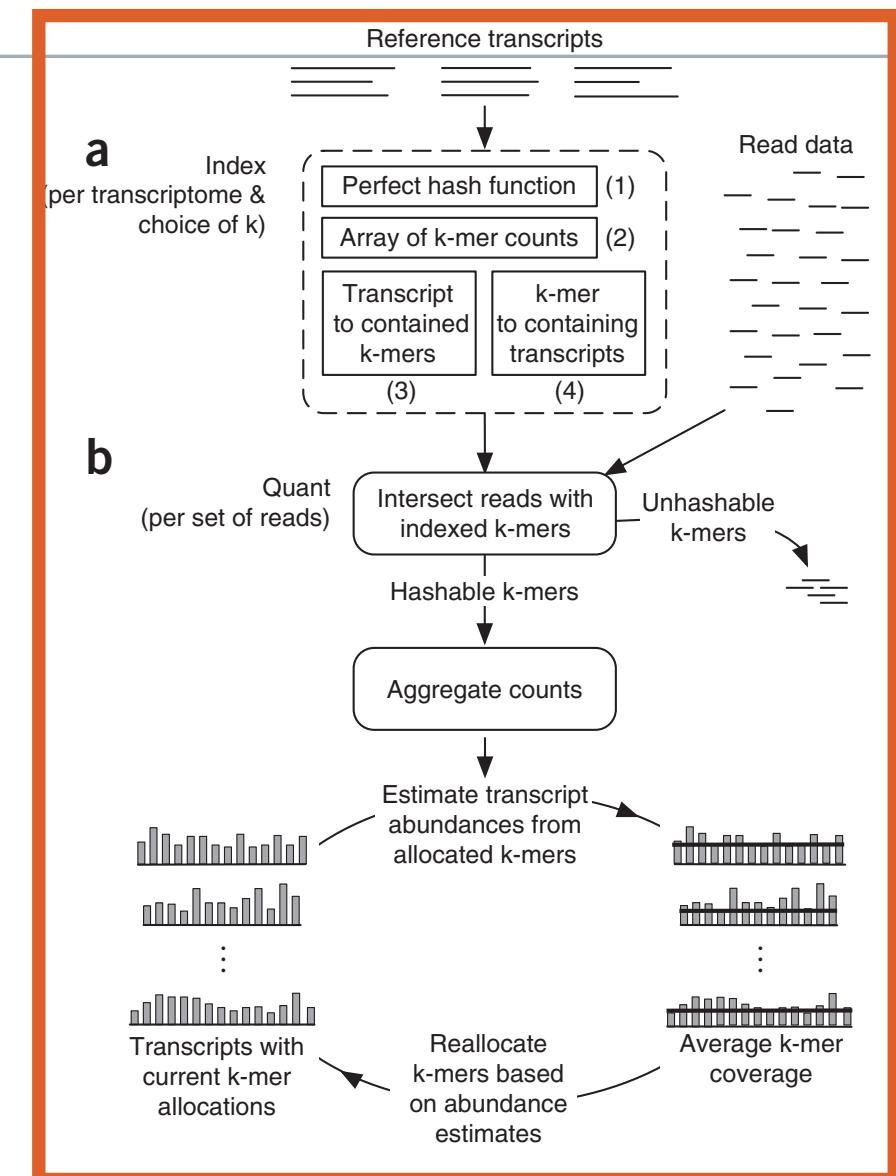


# Alignment versus quasi-alignment

Statistical Bioinformatics // Institute of Molecular Life Sciences



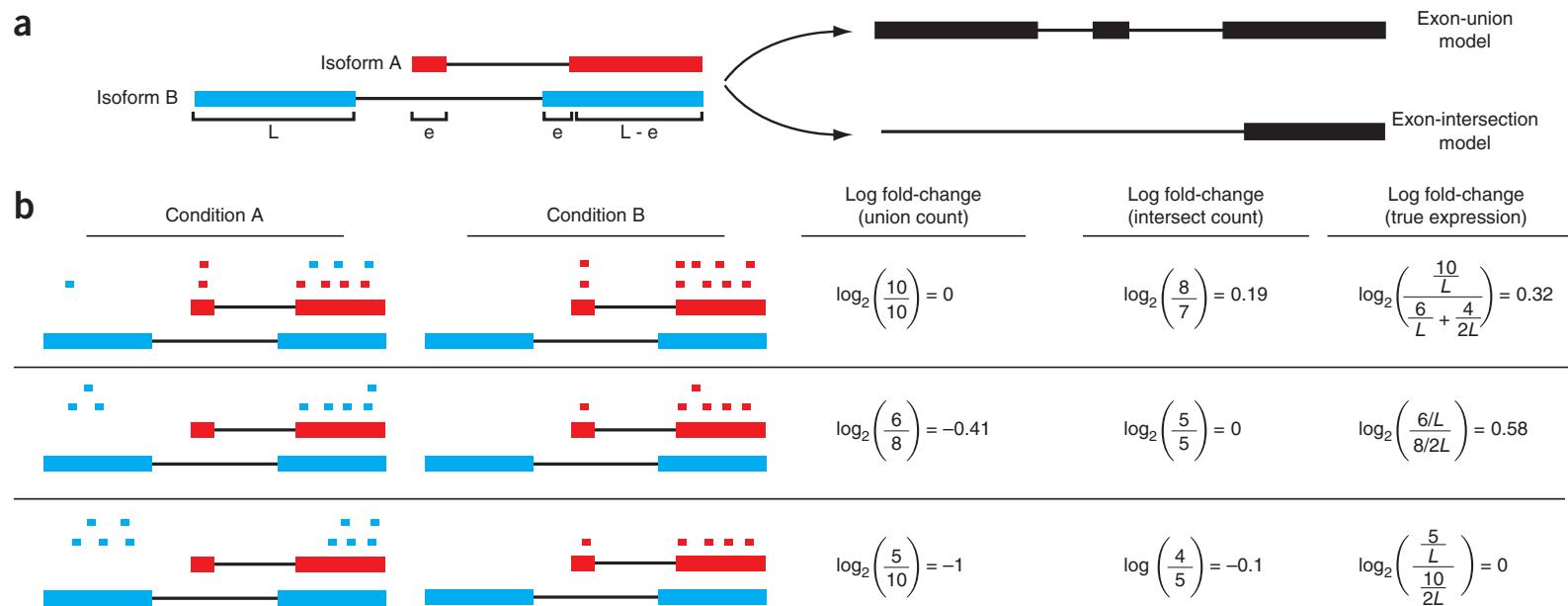
<https://en.wikipedia.org/wiki/RNA-Seq>



sailfish (Patro et al. 2014)

## Caveat: simple gene-level counting not perfect, but good first approximation

Trapnell et al. 2013 Nat Biotech



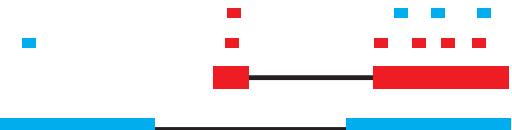
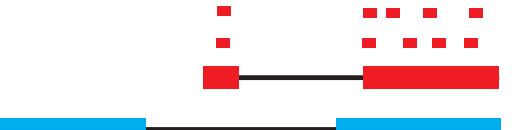
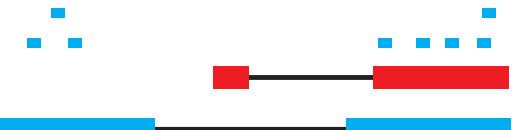
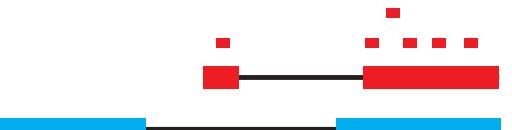
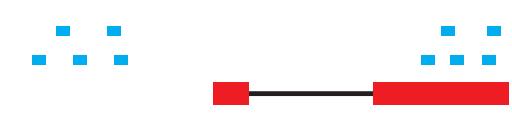
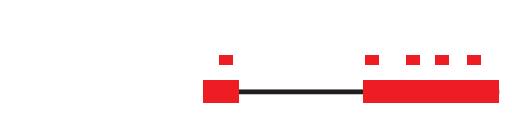
Transcriptome analysis of human tissues and cell lines reveals one dominant transcript per gene

Mar González-Porta<sup>1</sup>, Adam Frankish<sup>2</sup>, Johan Rung<sup>1</sup>, Jennifer Harrow<sup>2</sup> and Alvis Brazma<sup>1\*</sup>

# Counting/Quantification

union counters → simple sum of all reads  
transcript counters → sum of length-normalized reads  
(often unknown which reads map to which transcript → portioning)

**b**

	Condition A	Condition B	Log fold-change (union count)	Log fold-change (intersect count)	Log fold-change (true expression)
			$\log_2\left(\frac{10}{10}\right) = 0$	$\log_2\left(\frac{8}{7}\right) = 0.19$	$\log_2\left(\frac{\frac{10}{L}}{\frac{6}{L} + \frac{4}{2L}}\right) = 0.32$
			$\log_2\left(\frac{6}{8}\right) = -0.41$	$\log_2\left(\frac{5}{5}\right) = 0$	$\log_2\left(\frac{6/L}{8/2L}\right) = 0.58$
			$\log_2\left(\frac{5}{10}\right) = -1$	$\log\left(\frac{4}{5}\right) = -0.1$	$\log_2\left(\frac{\frac{5}{L}}{\frac{10}{2L}}\right) = 0$



# How do all these methods of counting affect DE analyses?

## You've been doing your RNA-Seq all wrong

Posted by: RNA-Seq Blog in Expression and Quantification November 12, 2015 13,162 Views

In recent years, RNA-seq is emerging as a powerful technology in estimation of gene and/or transcript expression, and RPKM (Reads Per Kilobase per Million reads) is widely used to represent the relative abundance of mRNAs for a gene. In general, the methods for gene quantification can be largely divided into two categories: transcript-based approach and 'union exon'-based approach. Transcript-based approach is intrinsically more difficult because different isoforms of the gene typically have a high proportion of genomic overlap. On the other hand, 'union exon'-based approach method is much simpler and thus widely used in RNA-seq gene quantification. Biologically, a gene is expressed in one or more transcript isoforms. Therefore, transcript-based approach is logically more meaningful than 'union exon'-based approach. Despite the fact that gene quantification is a fundamental task in most RNA-seq studies, however, it remains unclear whether 'union exon'-based approach for RNA-seq gene quantification is a good practice or not.

Researchers at [Pfizer Worldwide Research & Development](#) carried out a side-by-side comparison of 'union exon'-based approach and transcript-based method in RNA-seq gene quantification. It was found that the

F1000Research

F1000Research 2016, 4:1521 Last updated: 05 APR 2016



METHOD ARTICLE

**REVISED Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences [version 2; referees: 2 approved]**

Charlotte Soneson<sup>1,2</sup>, Michael I. Love<sup>3,4</sup>, Mark D. Robinson<sup>1,2</sup>

<sup>1</sup>Institute for Molecular Life Sciences, University of Zurich, Zurich, 8057, Switzerland

<sup>2</sup>SIB Swiss Institute of Bioinformatics, University of Zurich, Zurich, 8057, Switzerland

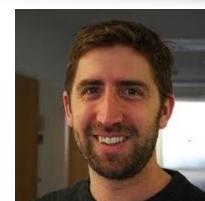
<sup>3</sup>Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA, 02210, USA

<sup>4</sup>Department of Biostatistics, Harvard TH Chan School of Public Health, Boston, MA, 02115, USA

v2 First published: 30 Dec 2015, 4:1521 (doi: [10.12688/f1000research.7563.1](https://doi.org/10.12688/f1000research.7563.1))

Latest published: 29 Feb 2016, 4:1521 (doi: [10.12688/f1000research.7563.2](https://doi.org/10.12688/f1000research.7563.2))

Open Peer Review





## Differential expression: why not use methods developed for microarrays?

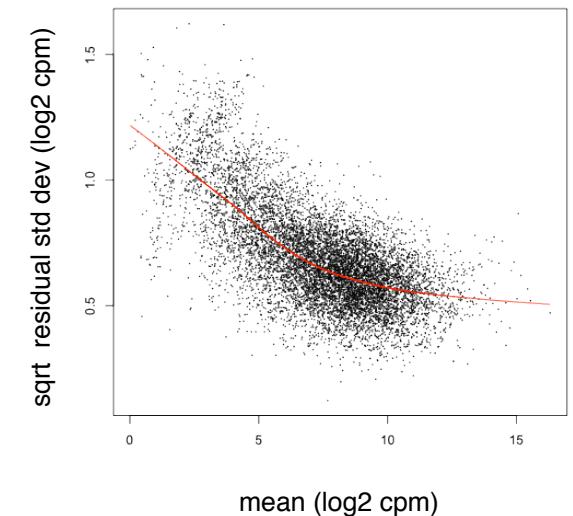
Count data is discrete, not continuous.

Methods designed for microarrays are not directly applicable and suboptimal (**more on this later**)

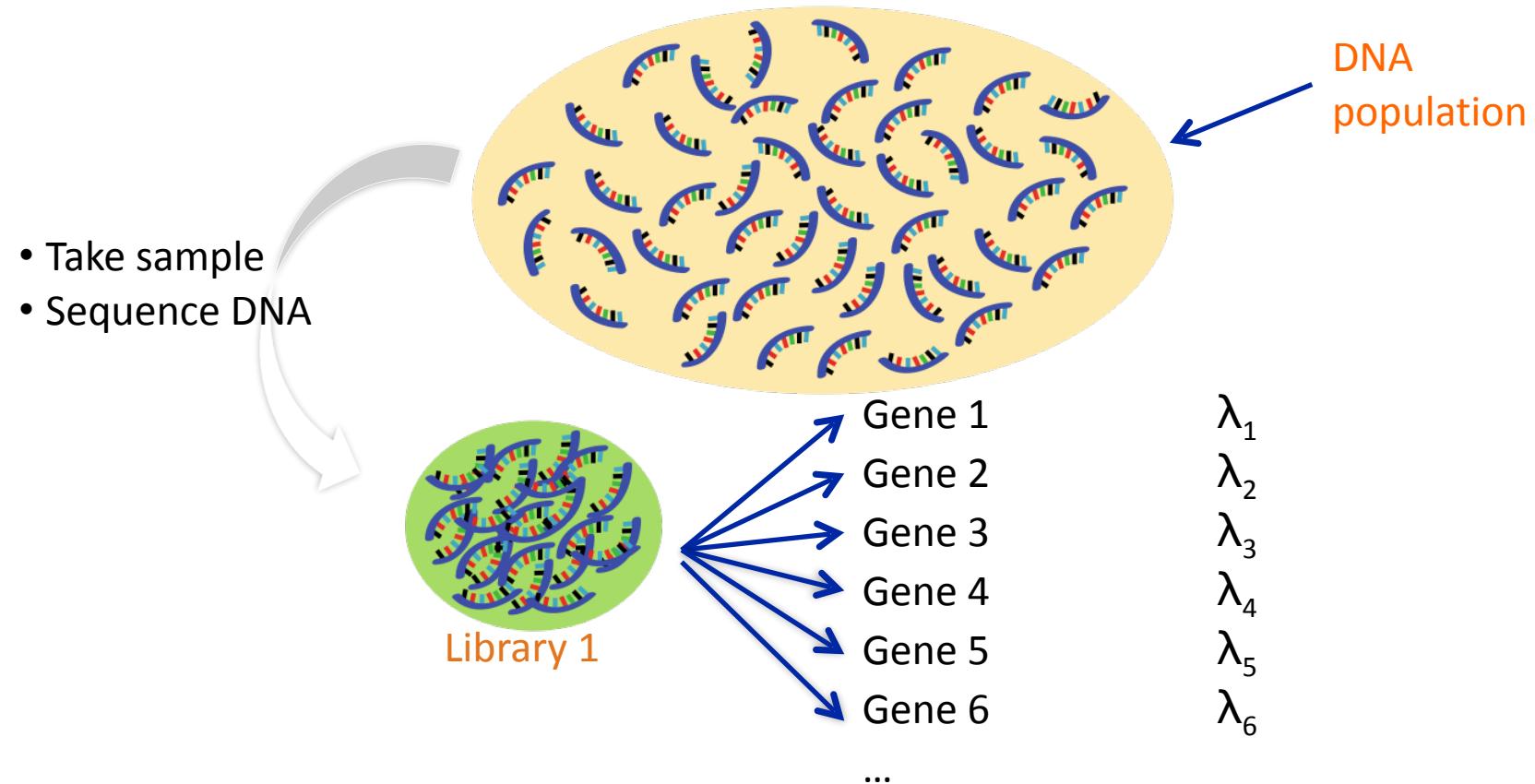
Two options:

Transform count data  
and apply standard  
methodology

Analyze using  
models for count  
data



## Sampling reads from population of DNA fragments is multinomial





# For a single gene, it's a coin toss, i.e. Binomial



$$Y_i \sim \text{Binomial}(M, \lambda_i)$$

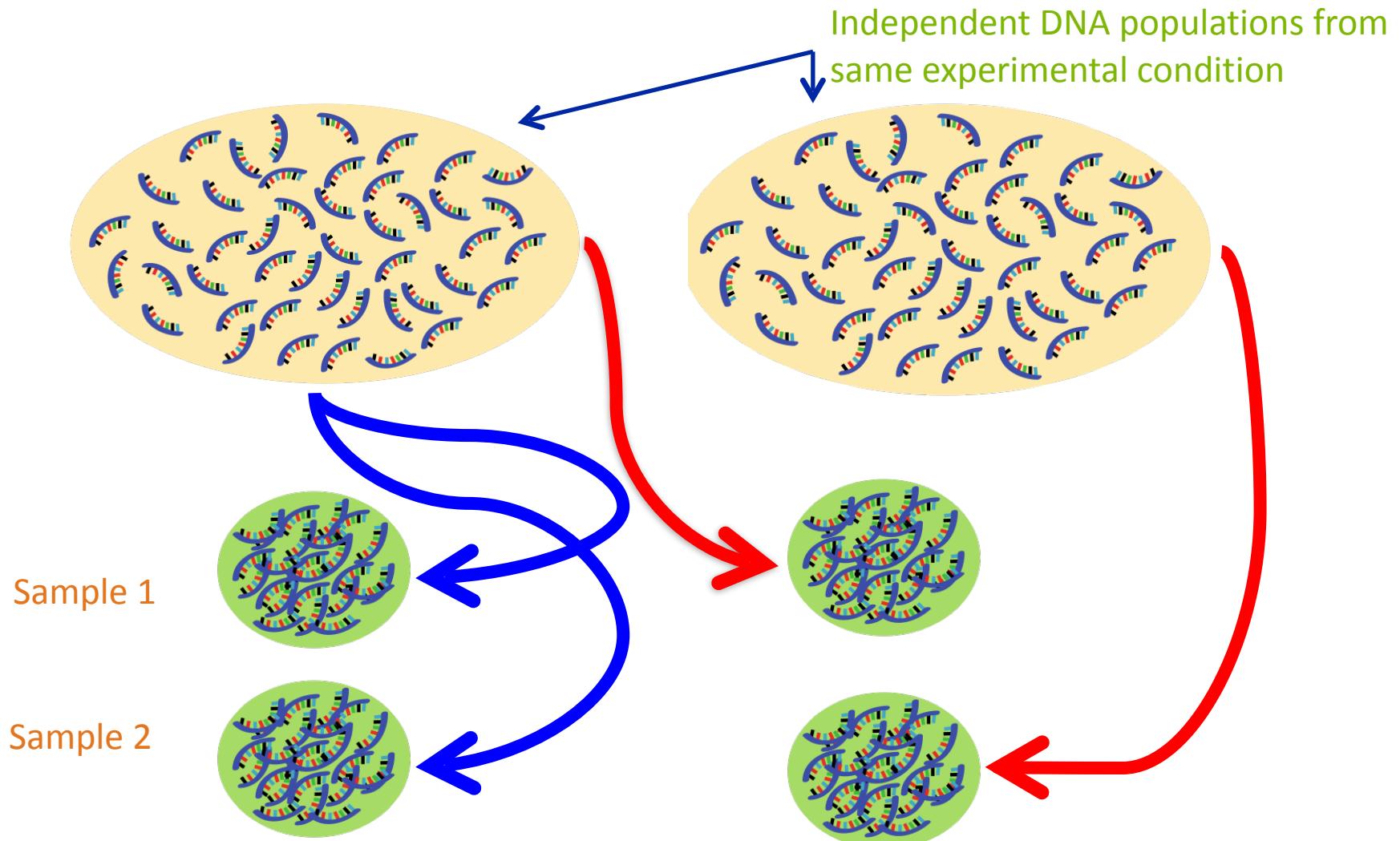
$Y_i$  - observed number of reads for gene  $i$

$M$  - total number of sequences

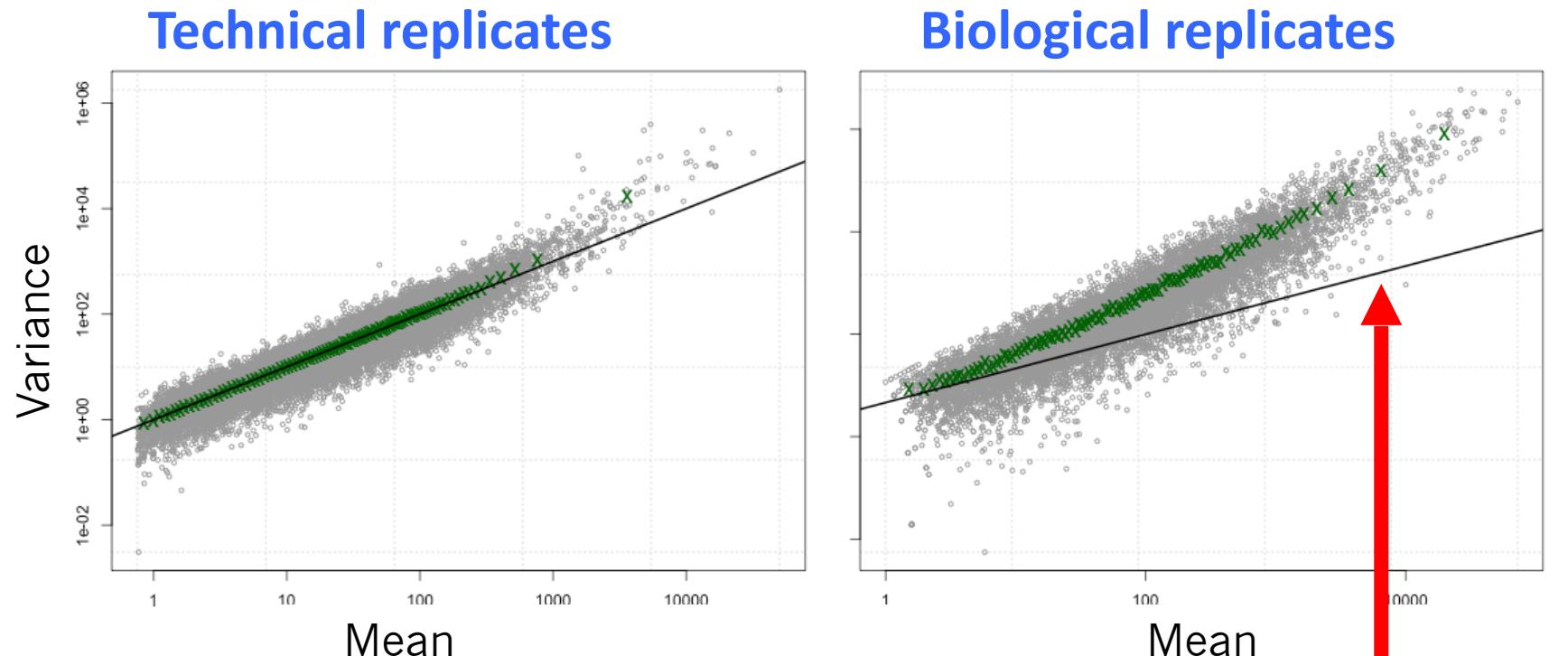
$\lambda_i$  - proportion

Large  $M$ , small  $\lambda_i \rightarrow$  approximated well by Poisson(  $\mu_i = M \cdot \lambda_i$  )

## Technical replication versus biological replication



# Mean-Variance plots: What we see in real data



Data from Marioni et al. *Genome Research* 2008

Data from Parikh et al.  
*Genome Biology* 2010

mean=variance  
(Poisson assumption)



# Count data modeling assumptions

Poisson adequately describes technical variation

$$Y_i \sim \text{Pois}(M * \lambda_i)$$

$$\text{mean}(Y_i) = \text{variance}(Y_i) = M * \lambda_i$$

Negative binomial (gamma-Poisson) model is a natural extension that allows **biological** variability:

$$Y_i \sim \text{NB}(\mu_i = M * \lambda_i, \phi_i)$$

Same mean, variance is quadratic in the mean:

$$\text{variance}(Y_i) = \mu_i (1 + \mu_i \phi_i)$$

$M$  = library size

$\lambda_i$  = relative contribution of gene i



## Similar interpretation

$$Y_i \sim NB(\mu_i = N_i * \lambda_i, \phi_i)$$

$$E(y_{gi}) = \mu_{gi} = N_i \pi_{gi}.$$

(Coefficient of variation = standard deviation/mean)

$$\text{var}(y_{gi}) = E_\pi[\text{var}(y|\pi)] + \text{var}_\pi[E(y|\pi)] = \mu_{gi} + \phi_g \mu_{gi}^2.$$

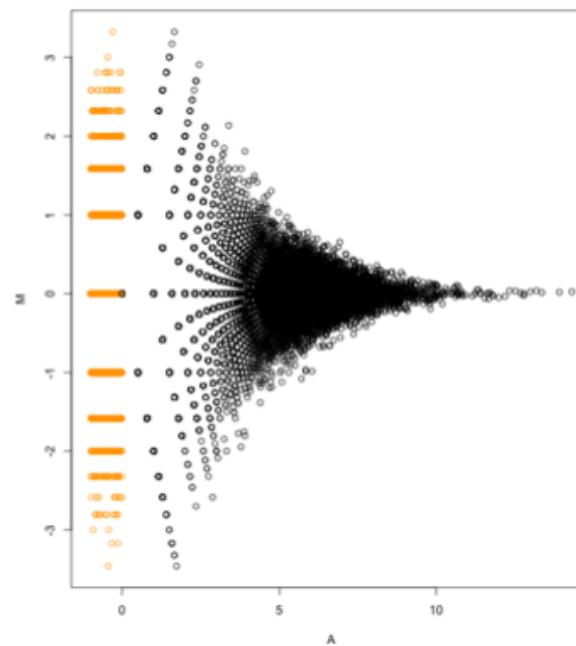
Dividing both sides by  $\mu_{gi}^2$  gives

$$CV^2(y_{gi}) = 1/\mu_{gi} + \phi_g.$$

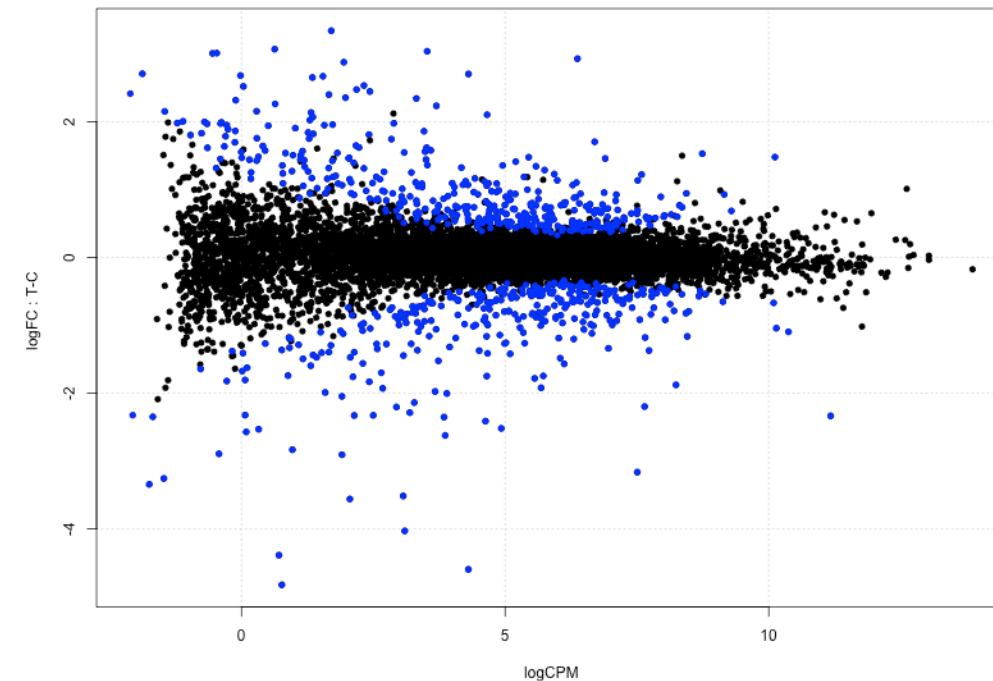
$$\text{CV}^2(\gamma_{gi}) = 1/\mu_{gi} + \phi_g.$$

## A confirmation of what the theory states

Technical replicates  
(~Poisson)



Biological replicates





## Differential expression, small sample inference —> **except now with counts**

- Table of data (e.g., microarray gene expression data with replicates of each of condition A, condition B)
  - rows = features (e.g., genes), columns = experimental units (samples)
- Most common problem in statistical bioinformatics: want to infer whether there is a change in the response  
—> a statistical test for each row of the table.

What test might you use? Why is this hard? What issues arise? How much statistical power is there [1] ?

> head(y)	group0	group0	group0	group1	group1	group1
gene1	-0.1874854	0.2584037	-0.05550717	-0.4617966	-0.3563024	-0.03271432
gene2	-3.5418798	-2.4540999	0.11750996	-4.3270442	-5.3462622	-5.54049106
gene3	-0.1226303	0.9354707	-1.10537767	-0.1037990	0.5221678	-1.72360854
gene4	-2.3394536	-0.3495697	-3.47742610	-3.2287093	6.1376670	-2.23871974
gene5	-3.7978820	1.4545702	-7.14796503	-4.0500796	4.7235714	10.00033769
gene6	1.4627078	-0.3096070	-0.26230124	-0.7903434	0.8398769	-0.96822312



## What was successful with microarray data: classical/moderated/ shrunken t-tests

$$t_g = \frac{\bar{y}_{\text{mu}} - \bar{y}_{\text{wt}}}{s_g c}$$

Feature-specific

$$\tilde{t}_g = \frac{\bar{y}_{\text{mu}} - \bar{y}_{\text{wt}}}{\tilde{s}_g u}$$

Moderated

$$t_{g,\text{pooled}} = \frac{\bar{y}_{\text{mu}} - \bar{y}_{\text{wt}}}{s_0 c}$$

Common



## Let's try the same strategy with counts

At one extreme, assume all genes have same dispersion (too strong)

At other extreme, estimate dispersion separately/independently for each gene (poor estimates)

Shrink individual estimates toward common/trend (how?)

No hierarchical model (e.g. limma) to do this —> **approximations, weighted likelihood**

No t-distribution theory to formulate statistical tests.



# Count data modeling assumptions

Poisson adequately describes technical variation

$$Y_i \sim \text{Pois}(M * \lambda_i)$$

$$\text{mean}(Y_i) = \text{variance}(Y_i) = M * \lambda_i$$

Negative binomial (gamma-Poisson) model is a natural extension that allows **biological** variability:

$$Y_i \sim \text{NB}(\mu_i = M * \lambda_i, \phi_i)$$

Same mean, variance is quadratic in the mean:

$$\text{variance}(Y_i) = \mu_i (1 + \mu_i \phi_i)$$

$M$  = library size

$\lambda_i$  = relative contribution of gene i



## First challenge: getting good estimates of dispersion in small samples

Several choices here:

- Maximum Likelihood (MLE)
- Pseudo-Likelihood (PL)
- Quasi-Likelihood (QL)
- Conditional Maximum Likelihood (CML)
- Approximate Conditional Inference (Cox-Reid)
- *quantile-adjusted Maximum Likelihood (qCML)*

$$Y_{gij} \sim \text{NegBin}(\mu_{gi} = M_j \lambda_{gi}, \phi)$$

$$(\hat{\lambda}_{MLE}, \hat{\phi}_{MLE}) \big| = \arg \max_{\lambda, \phi} l(\lambda, \phi)$$

$$X^2 = \sum_{gij} \frac{(y_{gij} - \hat{\mu}_{gi})^2}{\hat{\mu}_{gi}(1 + \hat{\phi}_{PL}\hat{\mu}_{gi})} = G(n_1 + n_2 - 2)$$

$$D = 2 \sum_{gij} \left\{ y_{gij} \log \left[ \frac{y_{gij}}{\mu_{gi}} \right] - (y_{gij} + \phi_{QL}^{-1}) \log \left[ \frac{y_{gij} + \phi_{QL}^{-1}}{\mu_{gi} + \phi_{QL}^{-1}} \right] \right\}$$



## Conditional likelihood

Likelihood for single **negative binomial** observation:

$$f(y; \mu, \phi) = P(Y = y) = \frac{\Gamma(y + \phi^{-1})}{\Gamma(\phi^{-1})\Gamma(y + 1)} \left( \frac{1}{1 + \mu\phi} \right)^{\phi^{-1}} \left( \frac{\mu}{\phi^{-1} + \mu} \right)^y$$

If all libraries are the same size (i.e.  $m_i \equiv m$ ), the sum  $Z = Y_1 + \dots + Y_n \sim \text{NB}(nm\lambda, \phi n^{-1})$

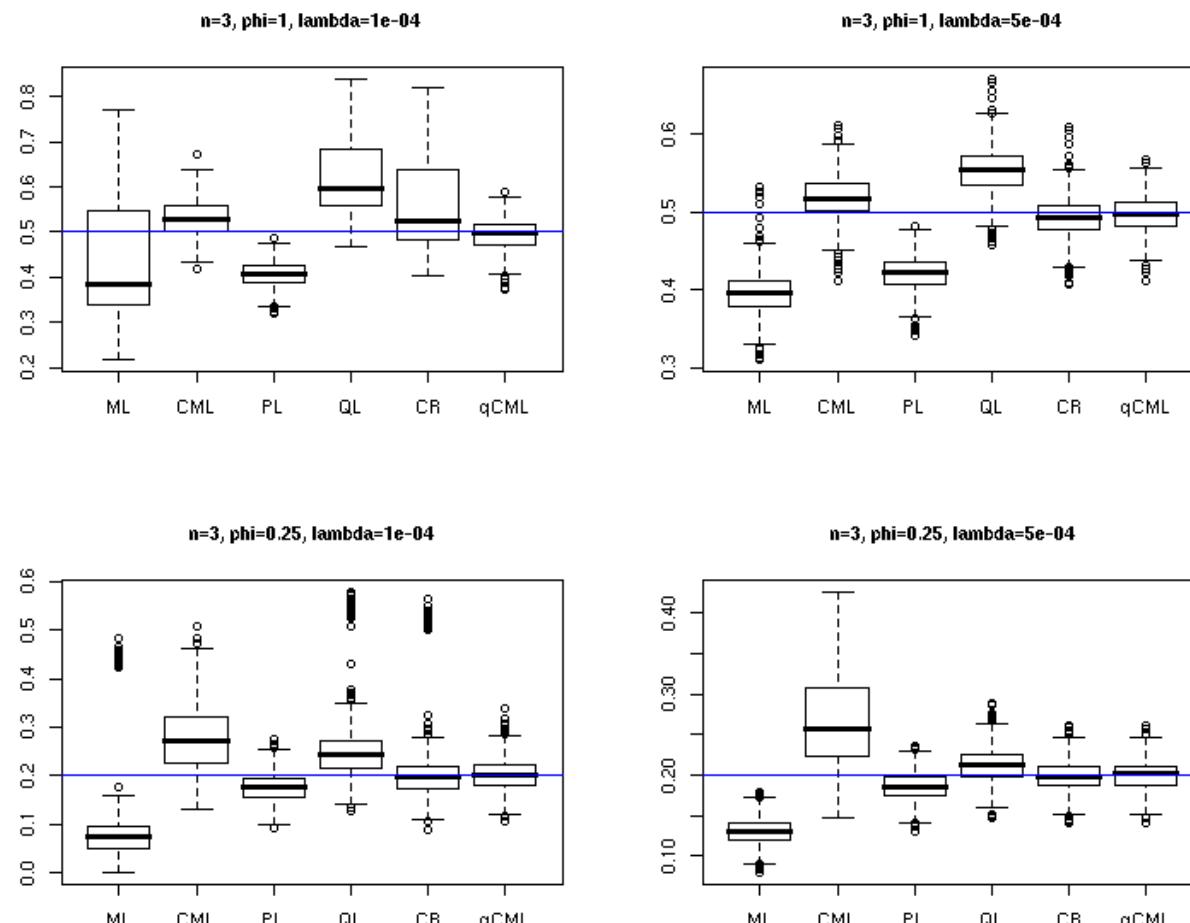
Thus, can form conditional likelihood:

$$l_{Y|Z=z}(\phi) = \left[ \sum_{i=1}^n \log \Gamma(y_i + \phi^{-1}) \right] + \log \Gamma(n\phi^{-1}) - \log \Gamma(z + n\phi^{-1}) - n \log \Gamma(\phi^{-1})$$

## Comparison of Estimators (Common Dispersion)

Horizontal blue line is  
TRUE value.

qCML performs best  
under a wide range of  
conditions.





## Likelihood —> Weighted likelihood

**Likelihood:**  $L(X; \theta) = \prod_i^n f(x_i; \theta)$

**log-likelihood:**

$$l(X; \theta) = \log(L(X; \theta)) = \sum_i^n \log(f(x_i; \theta))$$

**MLE:**  $\hat{\theta} = \arg \max_{\theta} l(X; \theta)$



## Likelihood $\rightarrow$ Weighted likelihood

$$WL(X; \theta) = \prod_i^n f(x_i; \theta)^{w_i}, \text{ where } w_i \text{ is weight.}$$

$$wl(X; \theta) = \log(WL(X; \theta)) = \sum_i^n w_i \log(f(x_i; \theta))$$

$$\hat{\theta} = \arg \max_{\theta} wl(X; \theta)$$

## Second challenge: Moderate dispersion estimate

Weighted likelihood -- individual log-likelihood plus a weighted version of the common log-likelihood:

$$WL(\phi_g) = l_g(\phi_g) + \alpha l_C(\phi_g)$$

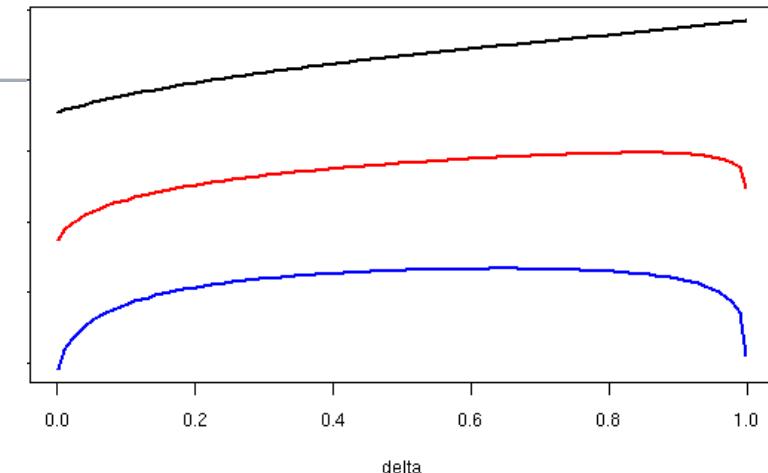
$l_g$  - quantile-adjusted conditional likelihood

Black: single tag

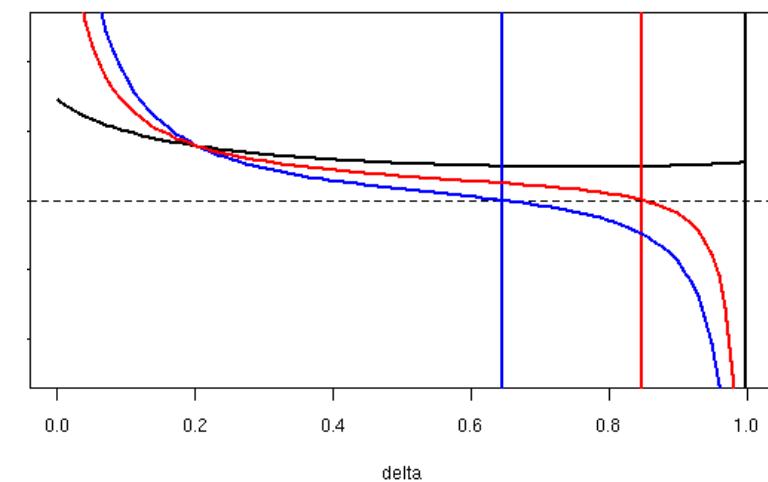
Blue: common dispersion

Red: Linear combination of the two

Log-Likelihood

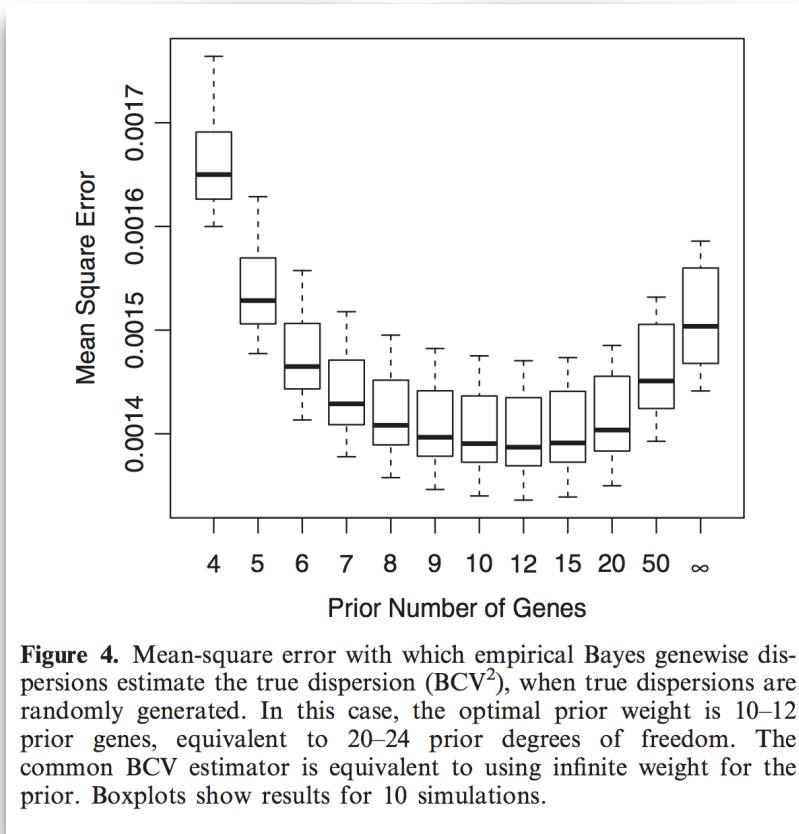


Score (1<sup>st</sup> derivative of LL)



$$\delta = \frac{\phi}{\phi+1}$$

## How much to shrink?



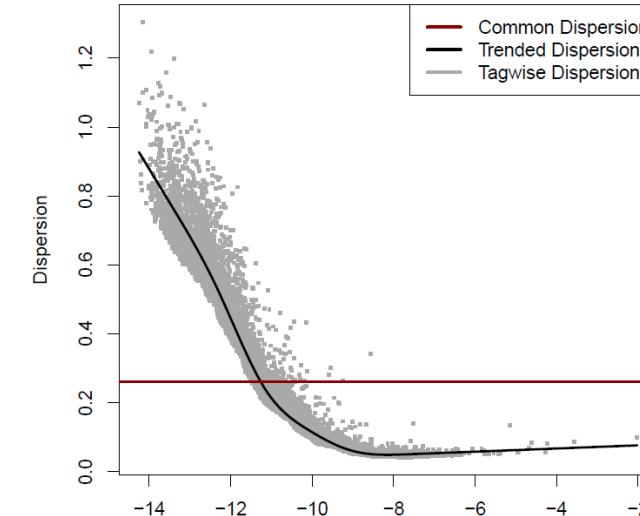
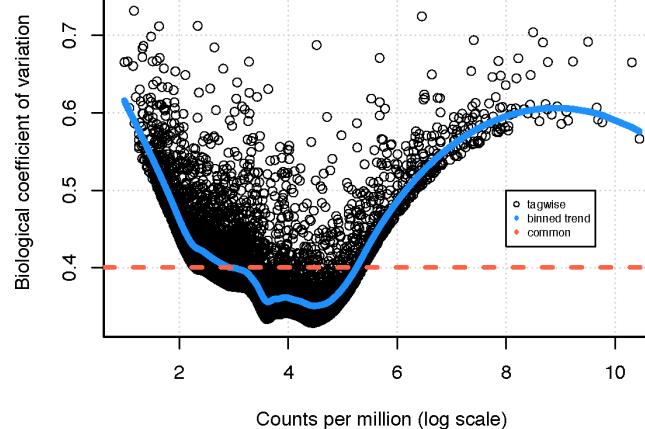
**Figure 4.** Mean-square error with which empirical Bayes genewise dispersions estimate the true dispersion ( $BCV^2$ ), when true dispersions are randomly generated. In this case, the optimal prior weight is 10–12 prior genes, equivalent to 20–24 prior degrees of freedom. The common  $BCV$  estimator is equivalent to using infinite weight for the prior. Boxplots show results for 10 simulations.

Simulations suggest there is an optimal amount to shrink.

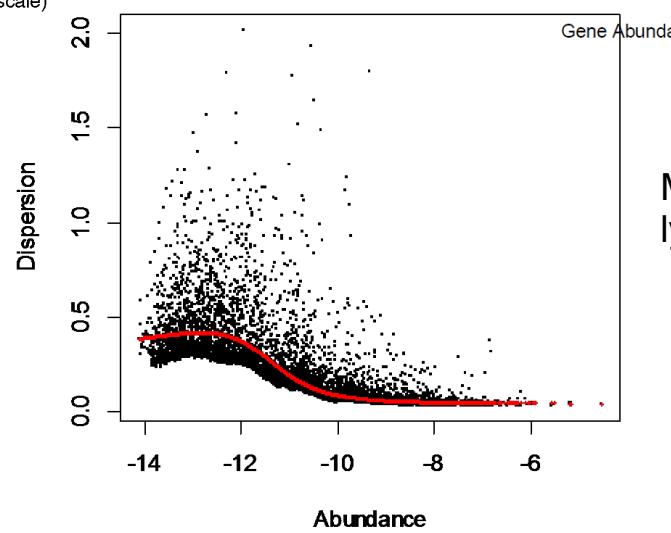
Challenge: choosing/estimating how much

## Dispersion varies with mean: moderate dispersion towards **trend**

Data:  
Tuch et al.,  
2008



Mouse hemopoietic  
stem cells



Mouse  
lymphomas

Advantage: genes are allowed to have their own variance.



*Nature Reviews Genetics* | AOP, published online 18 November 2008; doi:10.1038/nrg2484

**INNOVATION**

## RNA-Seq: a revolutionary tool for transcriptomics

*Zhong Wang, Mark Gerstein and Michael Snyder*

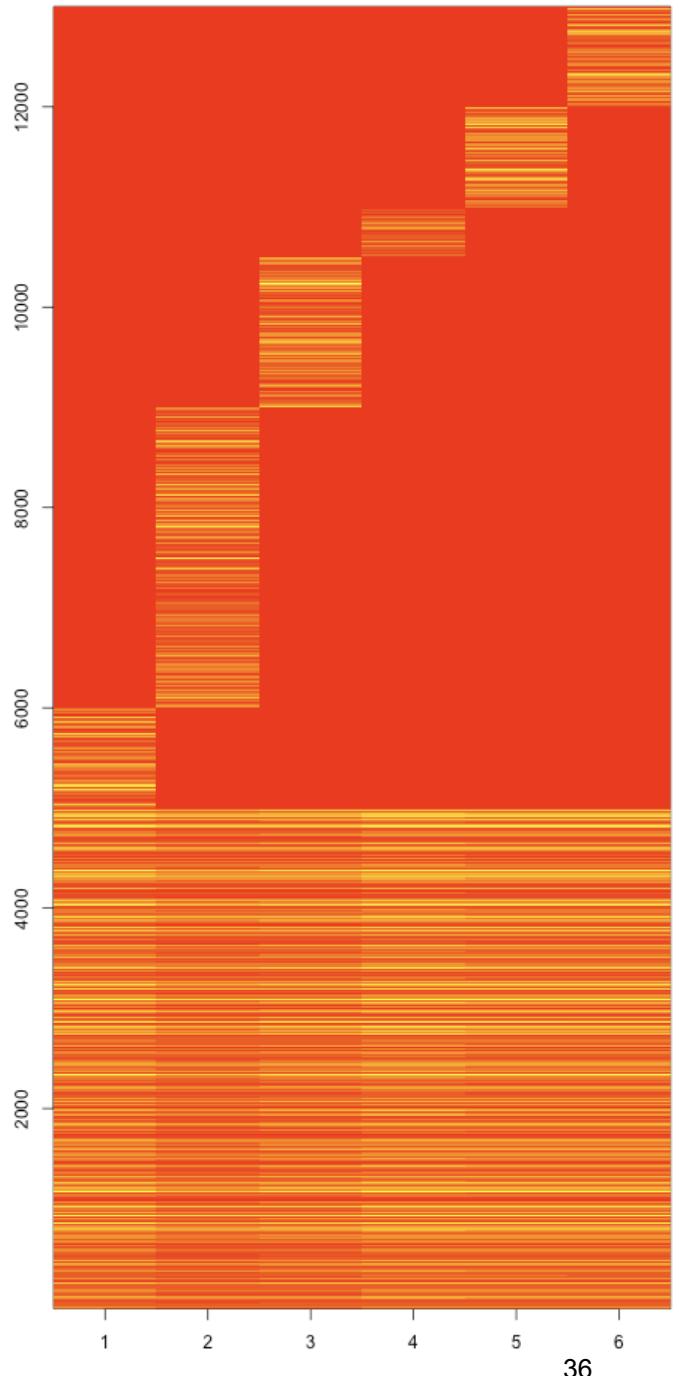
One particularly powerful advantage of RNA-Seq is that it can capture transcriptome dynamics across different tissues or conditions without sophisticated normalization of data sets<sup>19,20,22</sup>.



## “Composition” or “Diversity” can affect read depth

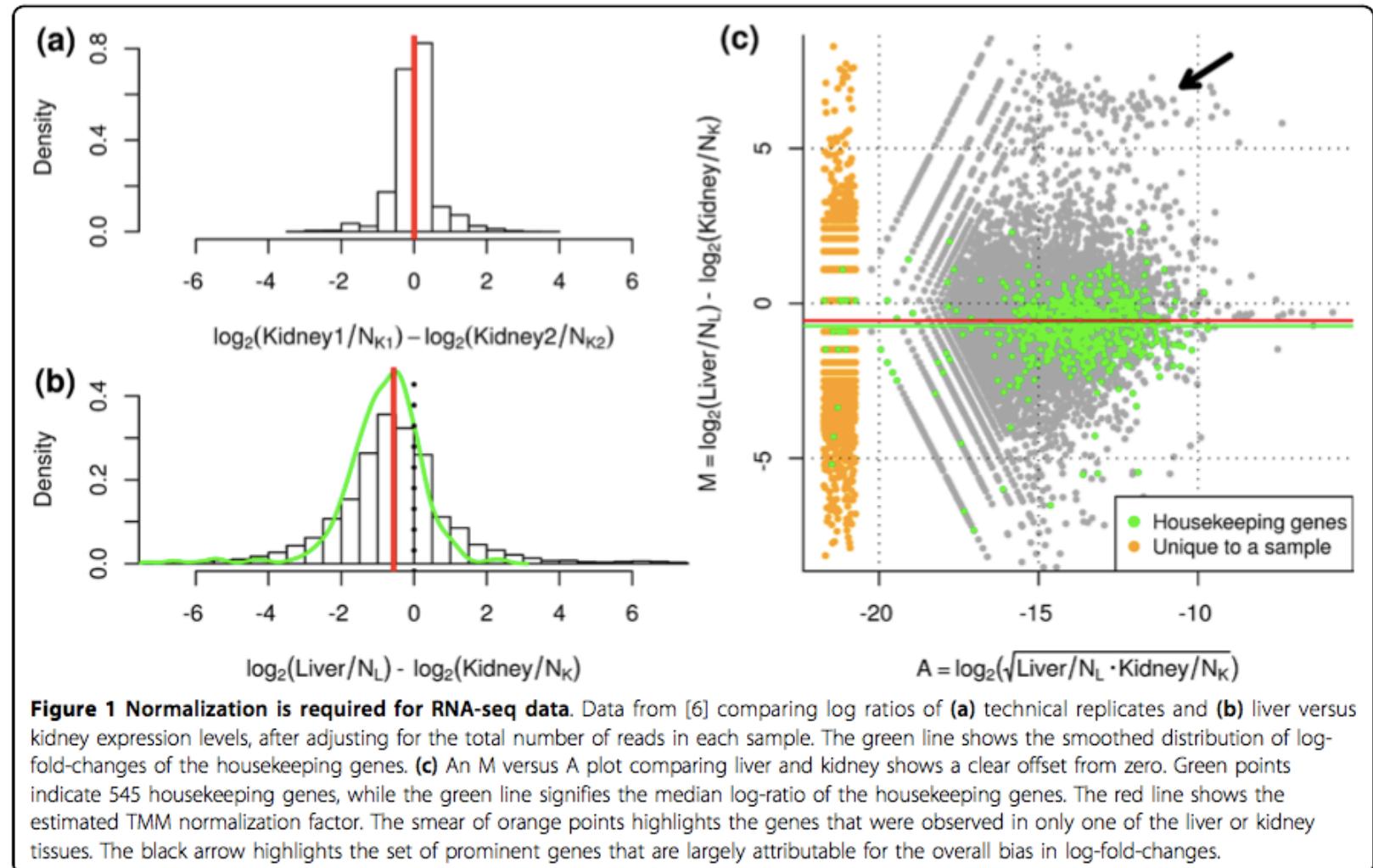
- Hypothetical example: Sequence 6 libraries to the same depth, with varying levels of unique-to-sample counts
- Read depth is affected not only by expression (and length), but also expression levels of other genes
- Composition can induce (sometimes significant) differences in counts

Red=low, goldenyellow=high





# Kidney and Liver RNA have very different composition





## Use scaling factor (“offset”) in statistical model

Assumption: core set of genes/loci that do not change in expression.

Our Pick a reference sample, compute a weighted trimmed mean of M-values (TMM) to reference

Adjustment to statistical analysis:

- Use “effective” library size (edgeR)
- Use additional offset (GLM)

Note: count data is not modified



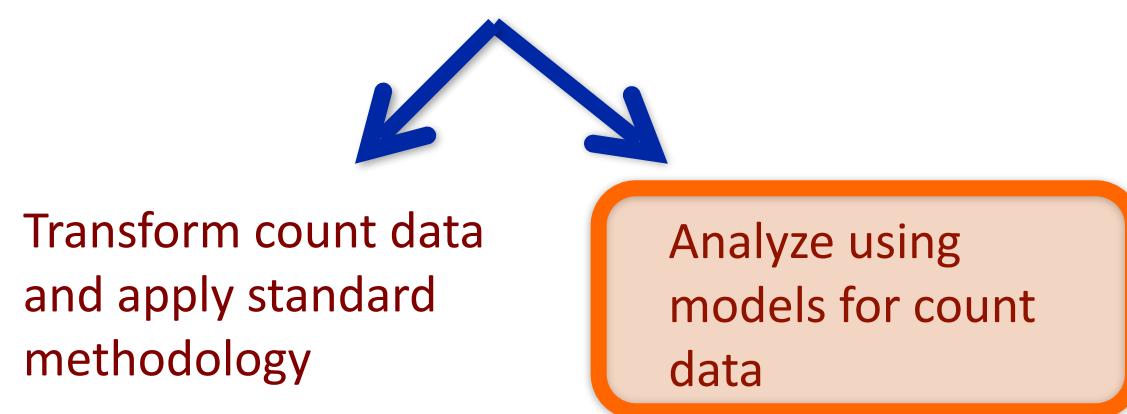
## Differential expression: why not use methods developed for microarrays?

Count data is discrete, not continuous.

Methods designed for microarrays are not directly applicable and suboptimal

Transforming count data with logs, with some special treatment, can give very good results

Two options:





## What does transformation do to M-V relationship?

For Poisson data, square-root should stabilize

Logarithm is too strong – variance decreases to asymptote (Neg Bin) or 0 (Poisson)

How to pick? Doesn't matter —> voom

voom: mean-variance modeling at the observational level

voom

package:limma

R Documentation

Transform RNA-Seq Data Ready for Linear Modelling

Description:

Transform count data to log2-counts per million, estimate the mean-variance relationship and use this to compute appropriate observational-level weights. The data are then ready for linear modeling.



# Model log counts per million

log counts per million:

$$z_{gi} = \log_2 \left( 1e6 \frac{\text{count}_{gi} + 0.5}{\text{libsize}_{gi} + 1.0} \right) = \log_2 \left( 1e6 \frac{y_{gi} + 0.5}{M_{gi} + 1.0} \right)$$

normalize libsize in advance or normalize  $z_{gi}$  as for microarrays.

Linear modelling:

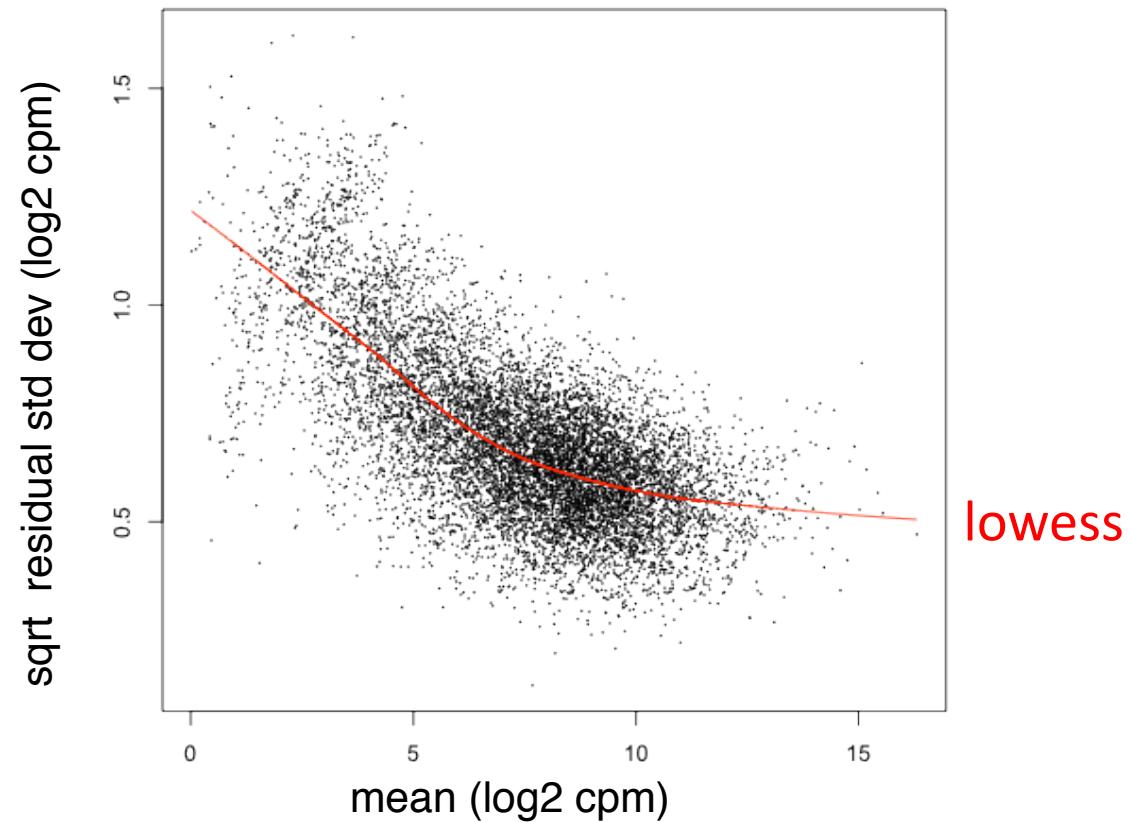
$$E(z_{gi}) = \mu_{gi} = x_i^T \beta_g$$

$$\text{var}(z_{gi}) = s(\mu_{gi}) \sigma_g^2$$

Smooth function of mean



**voom** fits a lowess trend to the mean-variance relationship ...



→ Use weights ( $1/\text{var}$ ) in limma analysis .. i.e., **heteroscedastic regression**

