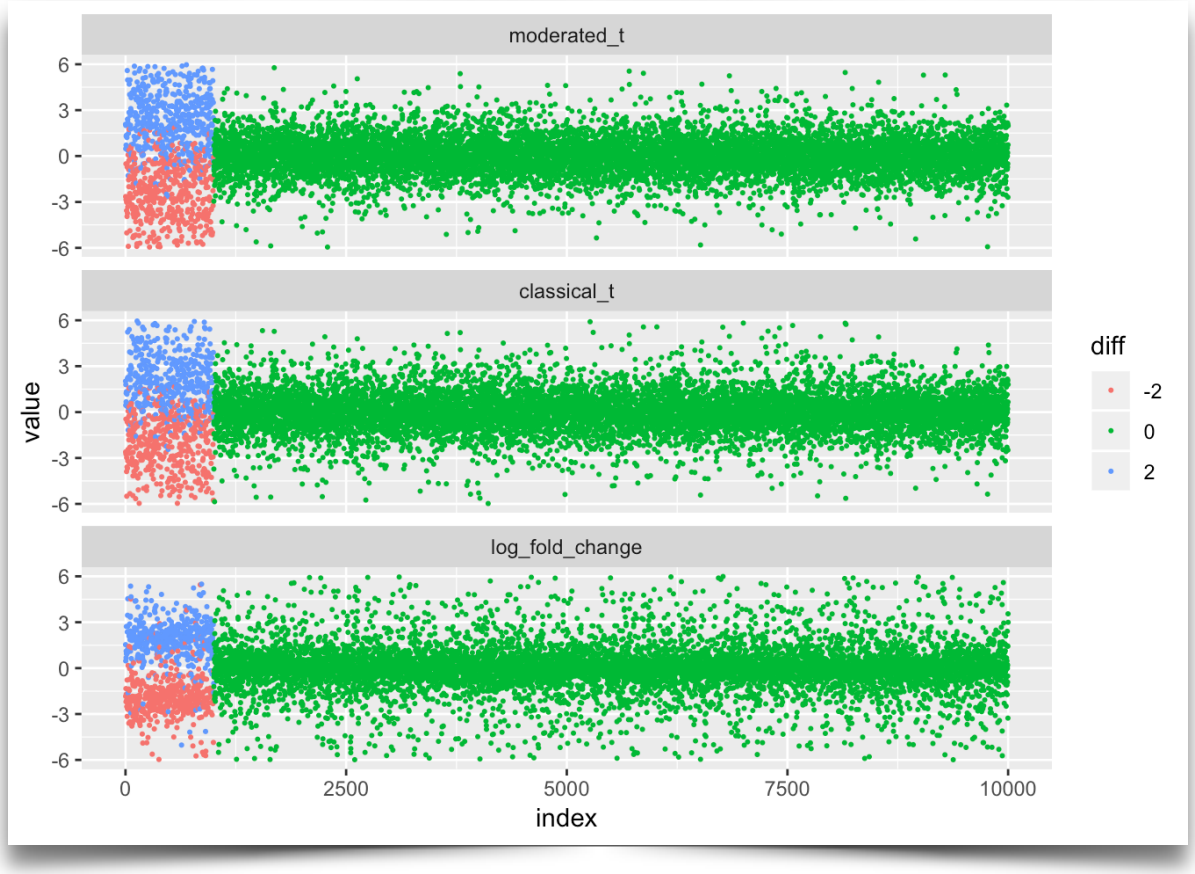
Notes on Exercise 6 (limma) .. and Exercises in general

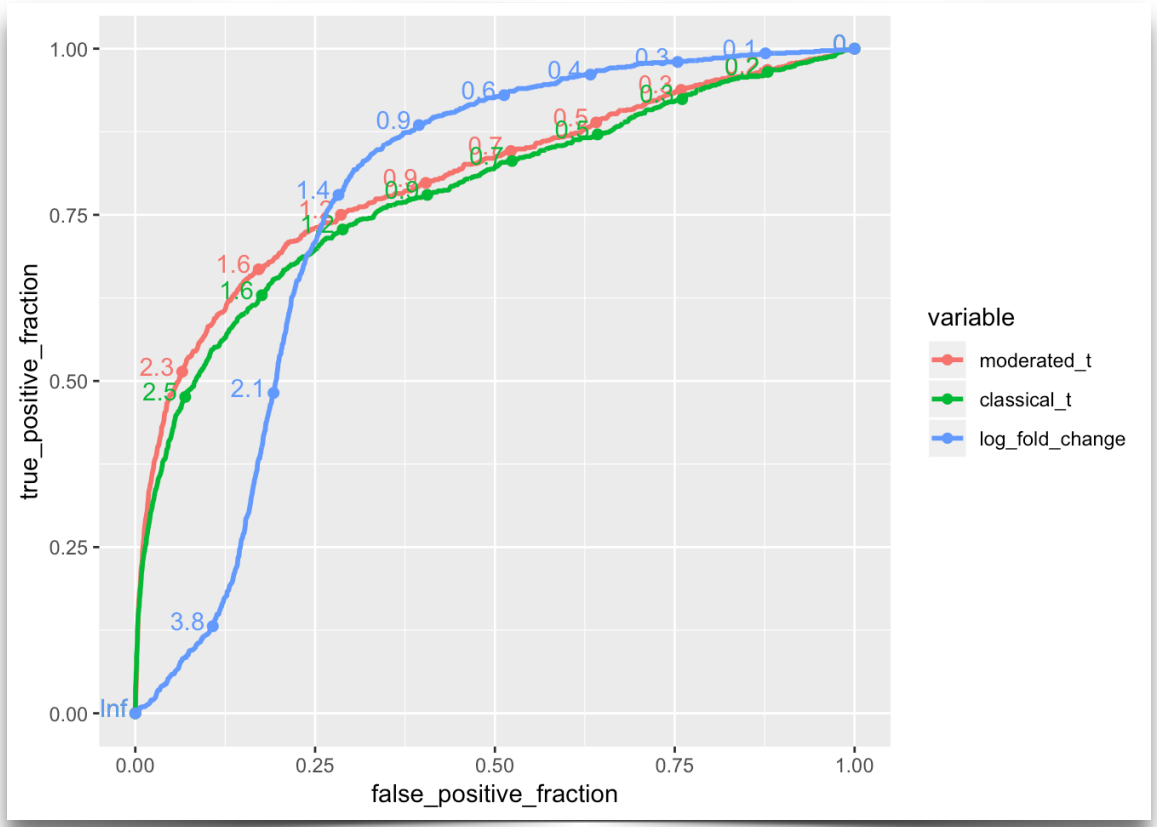
- please don't hide code (```{r show=FALSE} ..)!
- `var.equal` in `t.test` function (Welch's t-test is the default)
- ROC curve: absolute value of score .. or P-value
- make lines thicker
- try to exclude warnings from report, at least for package startup messages (```r my_block, warning=FALSE, message=FALSE} ..)
- write some text to tell me what you are doing

- https://github.com/sta426hs2018/solutions
- Scoring scheme: 3 + [9 best] x 3

Notes on Exercise 6 (limma) .. and Exercises in general



geom_roc in plotROC package

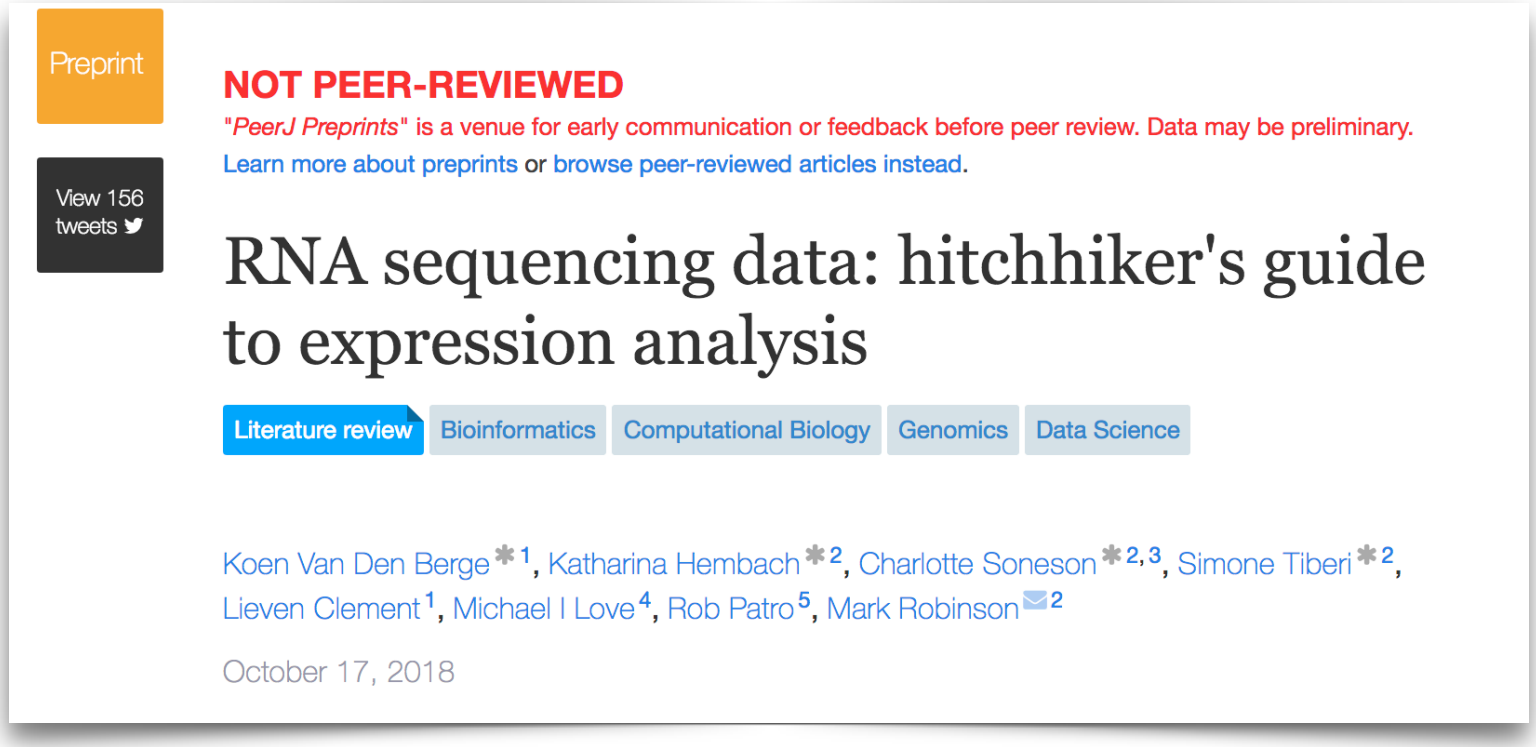


Statistical models for count data analysis (differential expression)

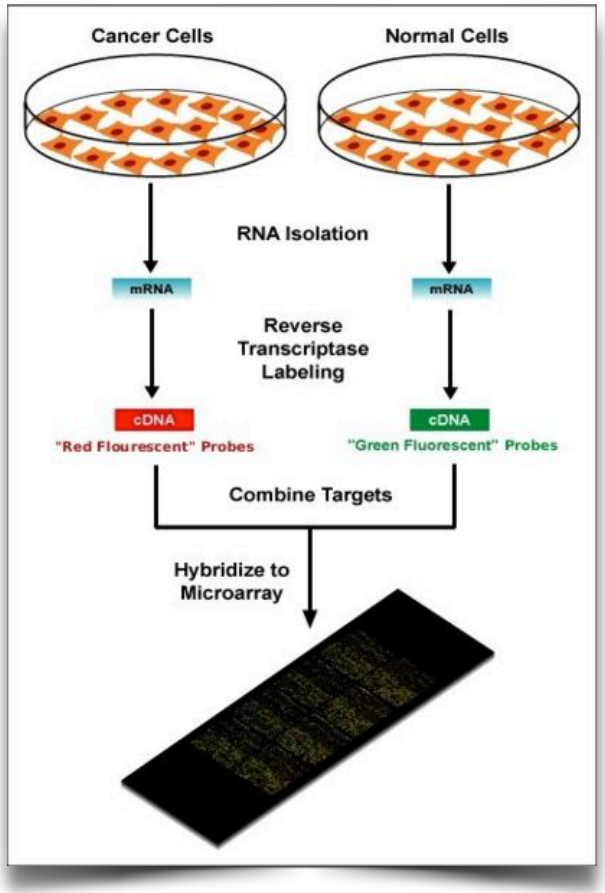
- simple counting (and new alternatives ..)
- edgeR, DESeq/DESeq2 —> why the negative binomial distribution?
- dispersion estimation and information sharing
- normalization considerations
- how about transformations of count data —> limma?

Feedback welcome

https://peerj.com/preprints/27283/

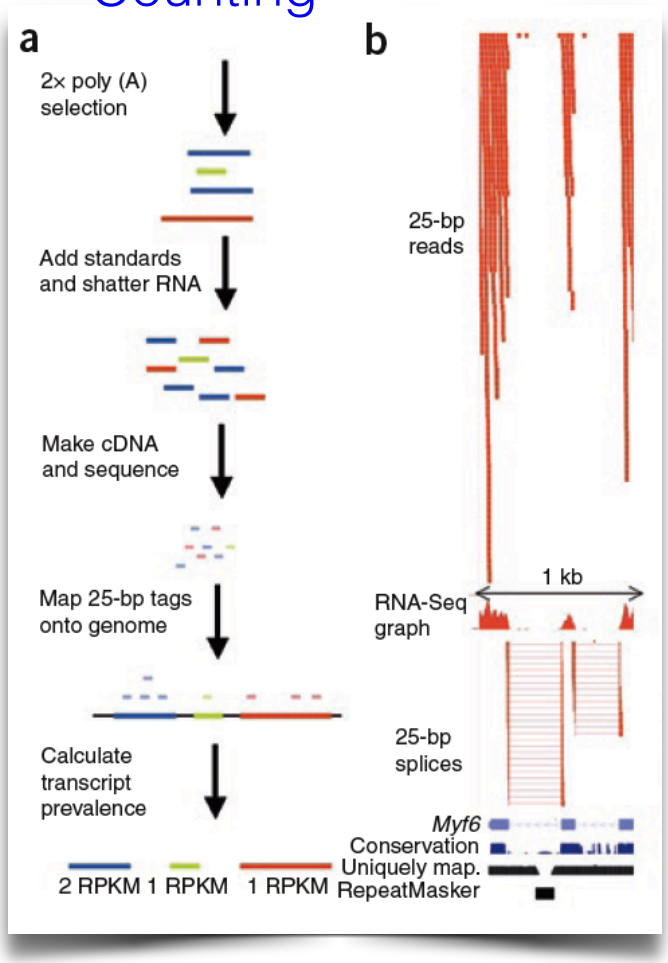


Abundance by Fluorescence Intensity



http://en.wikipedia.org/wiki/DNA_microarray

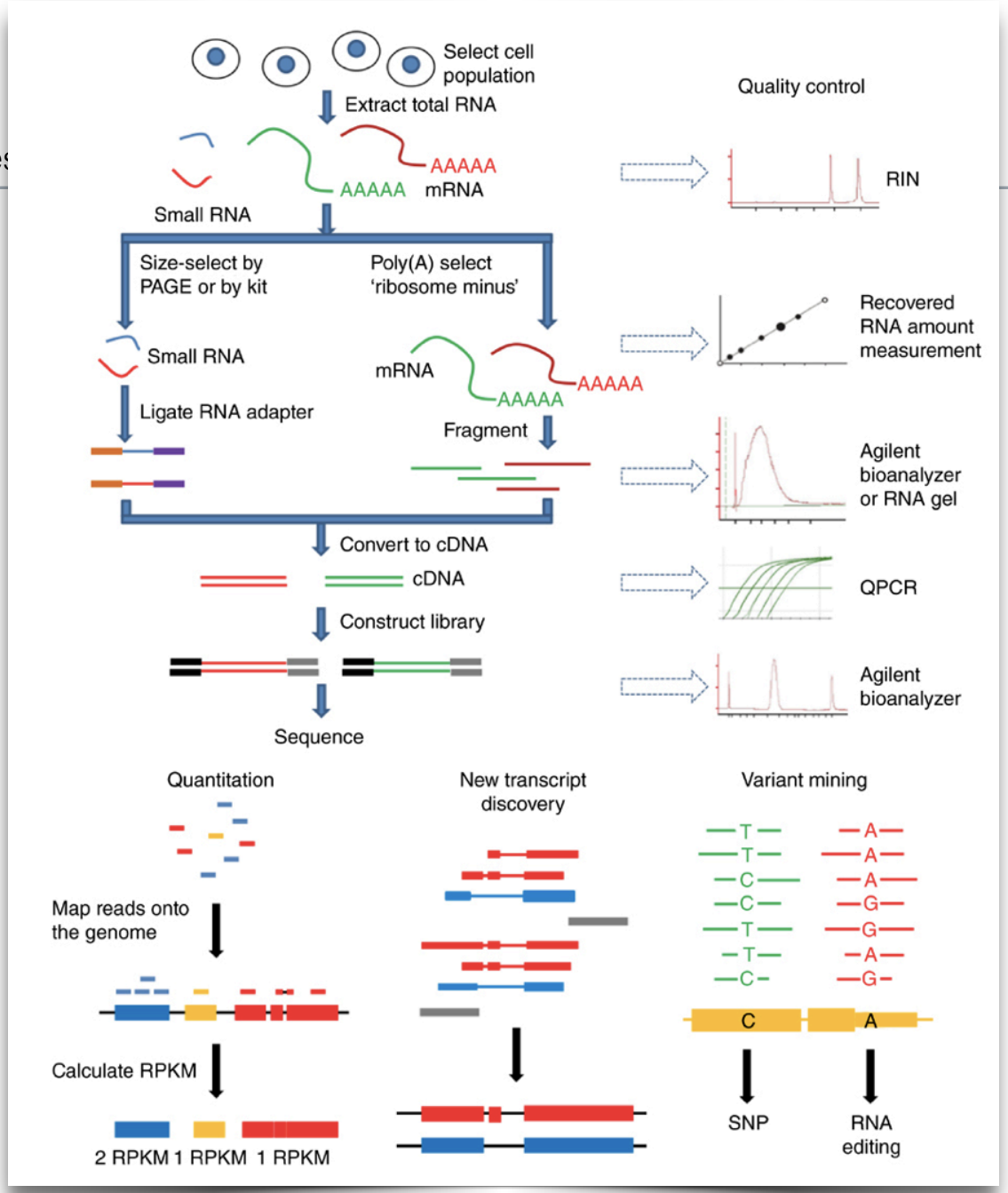
Abundance by Counting



Mortazavi et al., Nature Methods, 2008

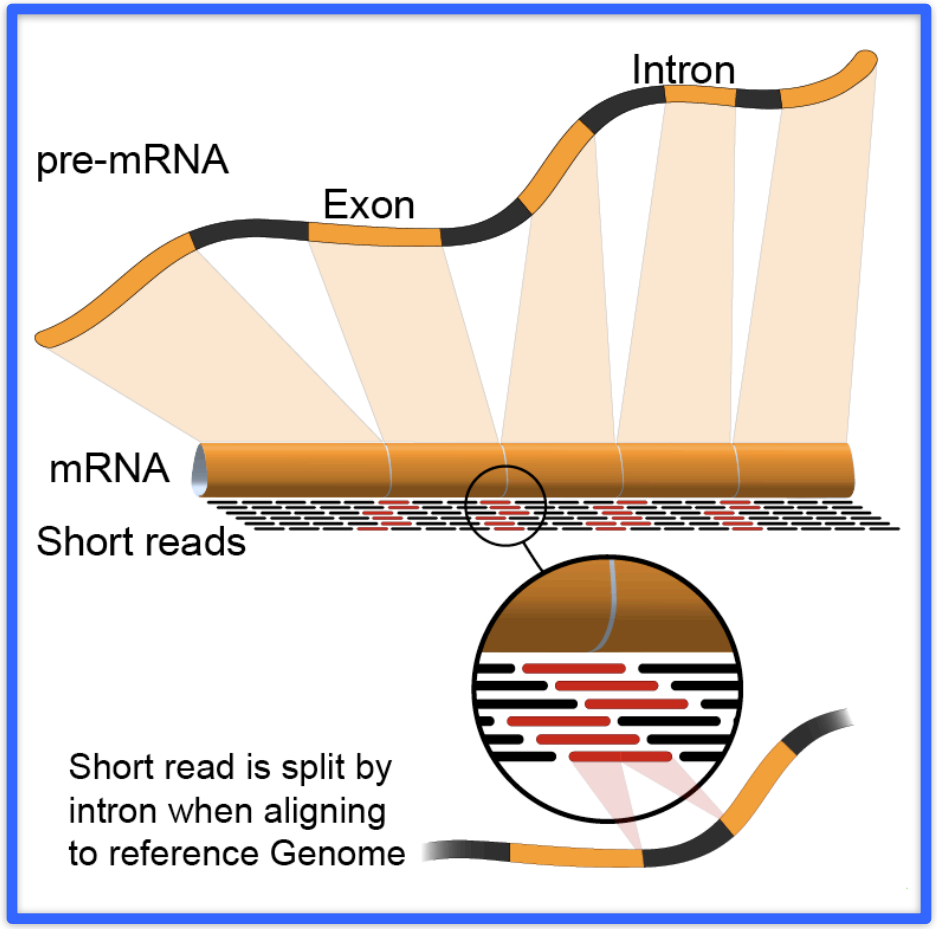
RNA-seq differential expression analyses

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

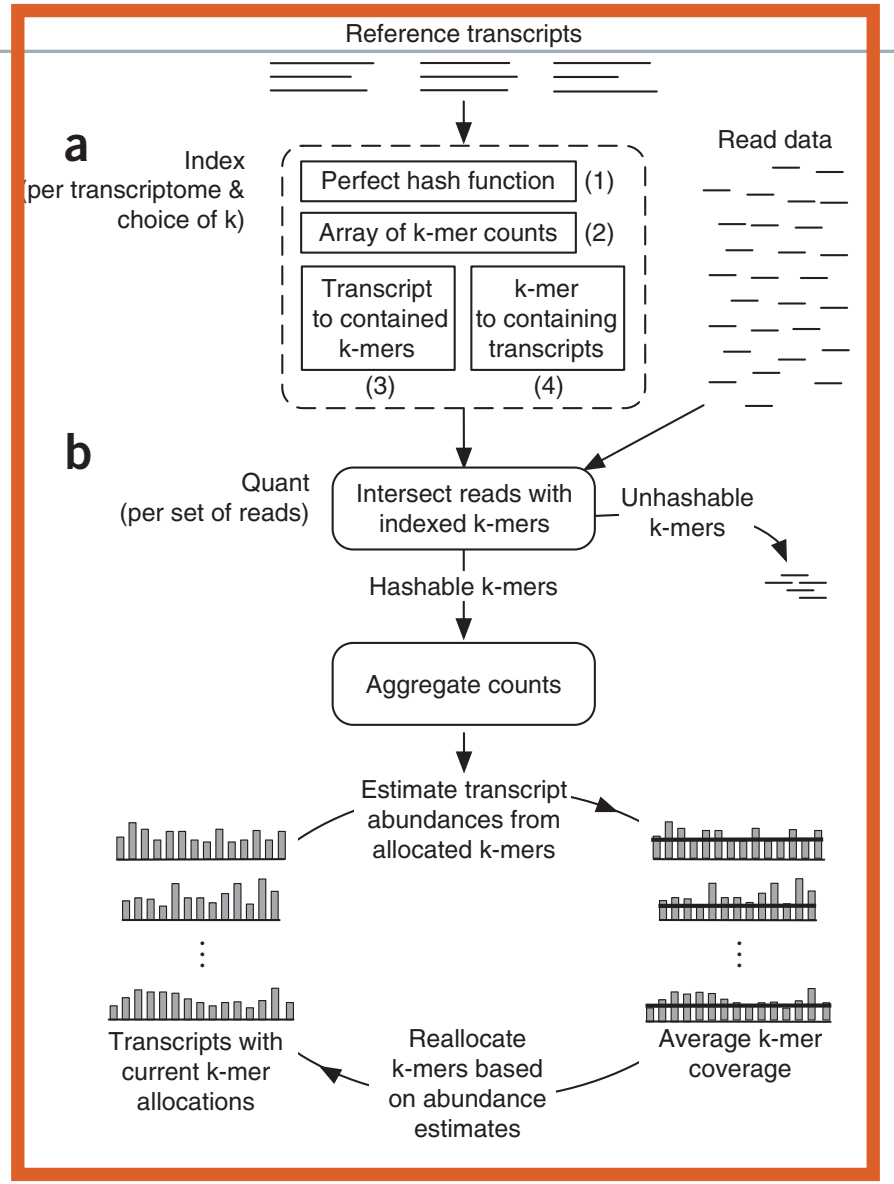


Zeng & Mortazavi, Nature Immunology, 2012

Alignment versus quasi-alignment



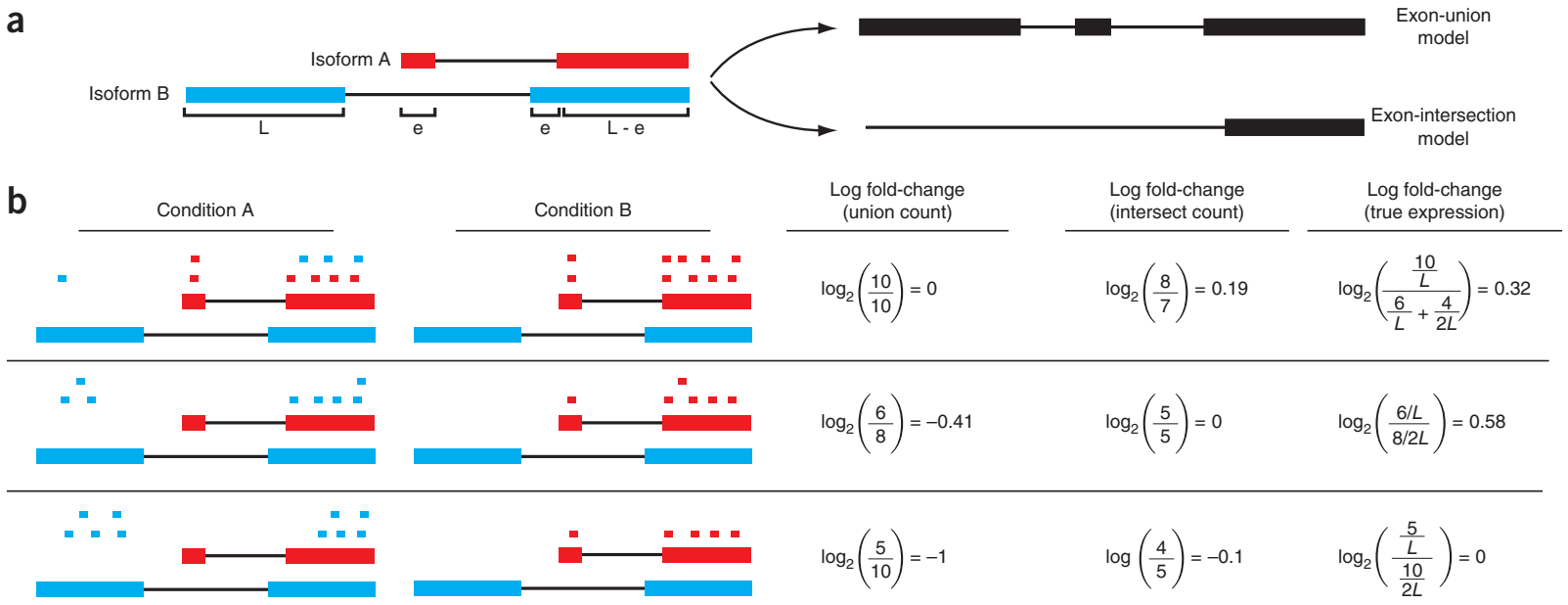
https://en.wikipedia.org/wiki/RNA-Seq



sailfish (Patro et al. 2014)

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

Trapnell et al. 2013 Nat Biotech

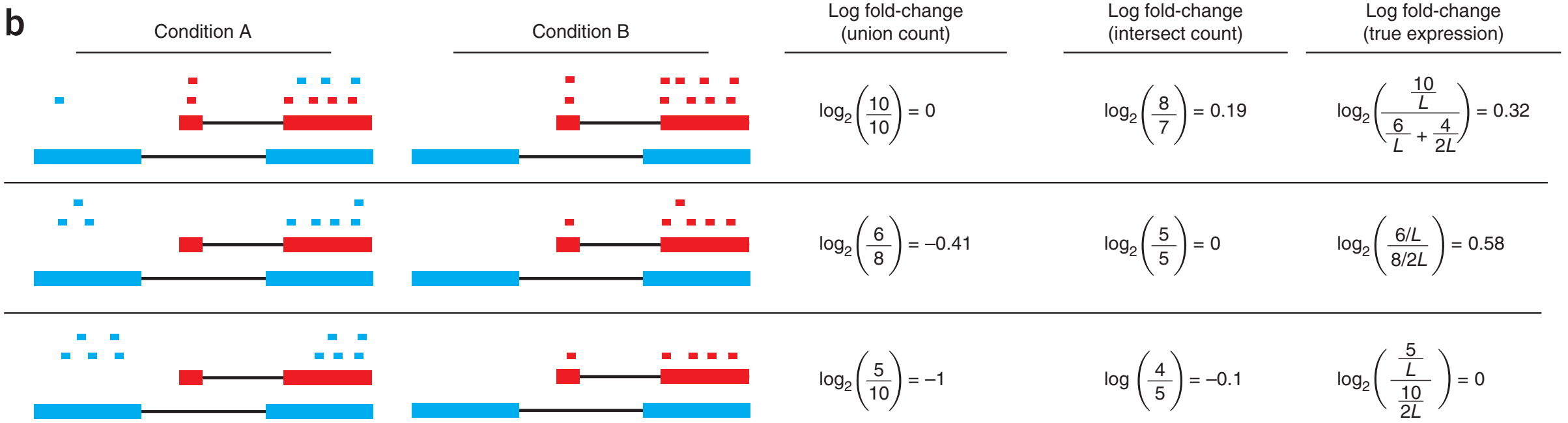


Transcriptome analysis of human tissues and cell lines reveals one dominant transcript per gene

Mar Gonzàlez-Porta[1], Adam Frankish[2], Johan Rung[1], Jennifer Harrow[2] and Alvis Brazma[1*]

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)



adapted from Trapnell et al. 2013 Nat Biotech

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 logistically 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

CrossMark click for updates

METHOD ARTICLE

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

Charlotte Soneson[1,2], Michael I. Love[3,4], Mark D. Robinson[1,2]

[1]Institute for Molecular Life Sciences, University of Zurich, Zurich, 8057, Switzerland
[2]SIB Swiss Institute of Bioinformatics, University of Zurich, Zurich, 8057, Switzerland
[3]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA, 02210, USA
[4]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)
Latest published: 29 Feb 2016, **4**:1521 (doi: 10.12688/f1000research.7563.2)

Open Peer Review

Differential expression: why not use methods developed for microarrays?

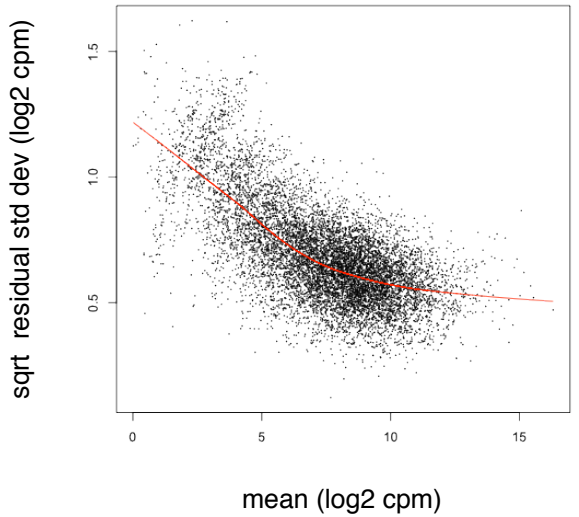
Count data is discrete, not continuous.

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

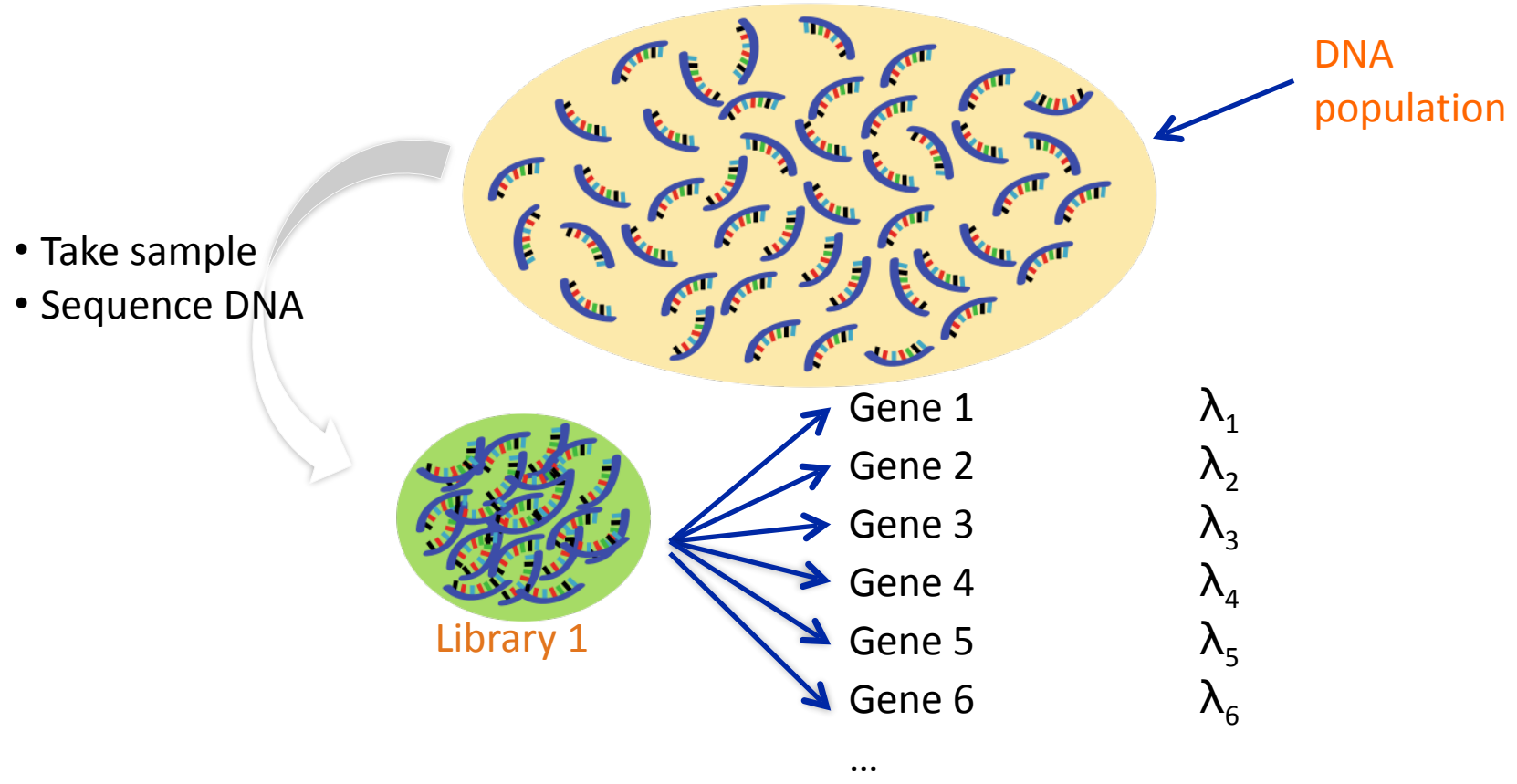
Two options:

Transform count data and apply standard methodology

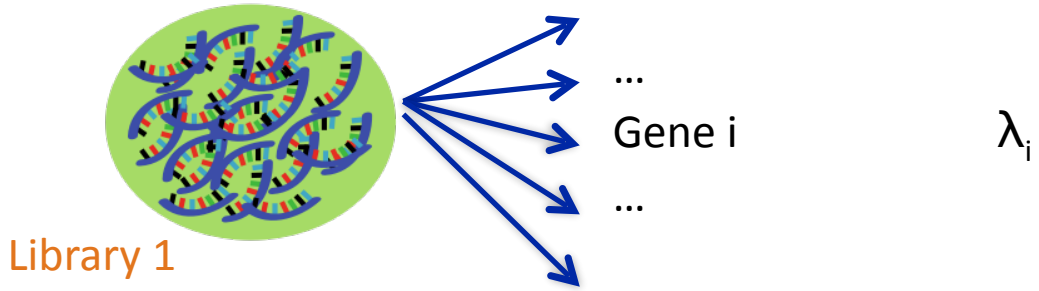
Analyze using models for count data



Sampling reads from population of DNA fragments is multinomial



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



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

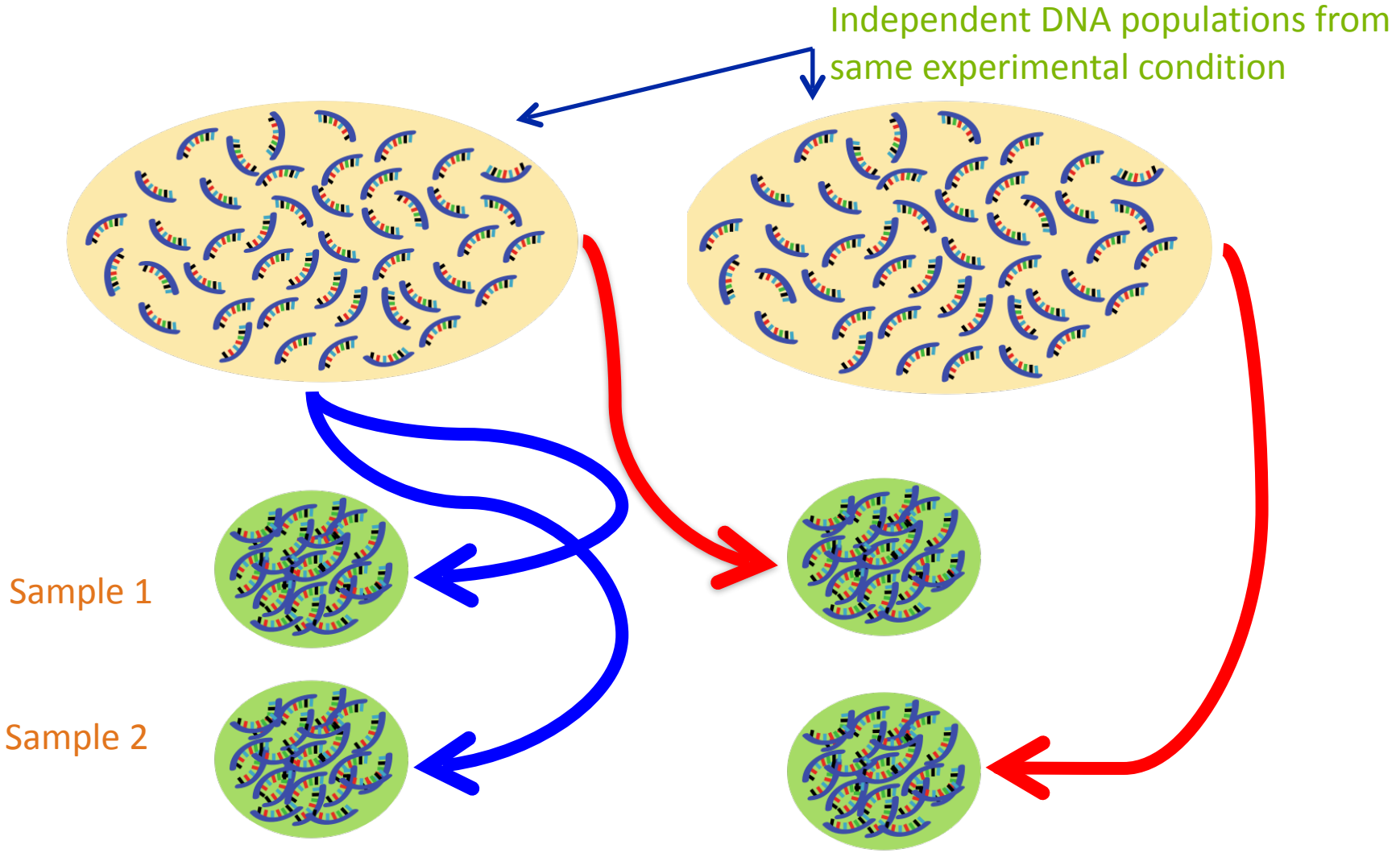
Y_i - observed number of reads for gene i

M - total number of sequences

λ_i - proportion

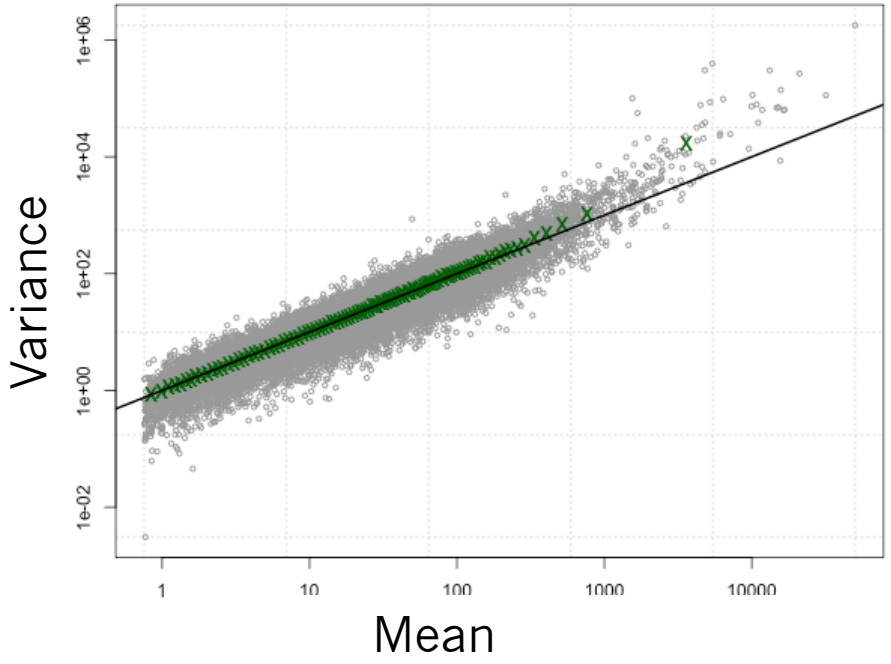
Large M, small λ_i → approximated well by Poisson($\mu_i = M \bullet \lambda_i$)

Technical replication versus biological replication



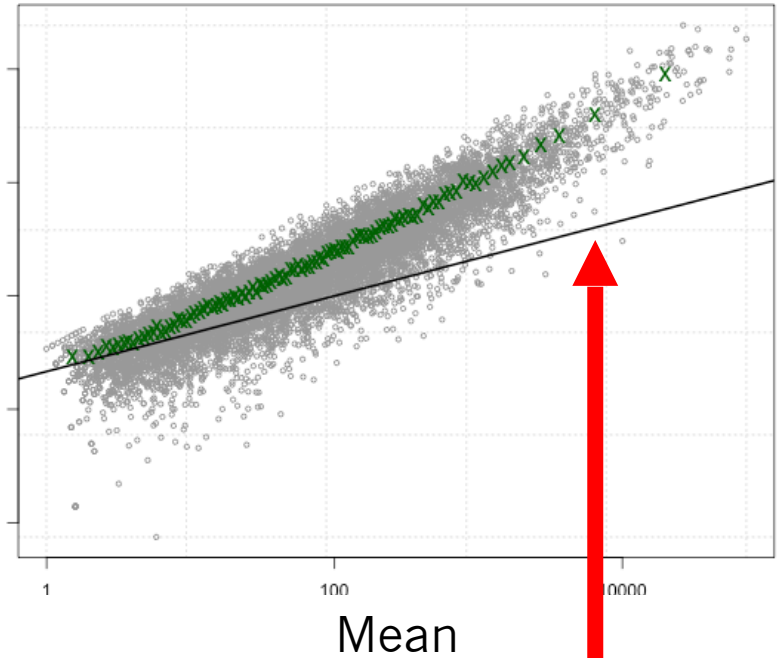
Mean-Variance plots: What we see in real data

Technical replicates



Data from Marioni et al. Genome Research 2008

Biological replicates



Data from Parikh et al. *Genome Biology* 2010

mean=variance
(Poisson assumption)

Count data modeling assumptions

Poisson adequately describes technical variation

$Y_i \sim Pois(M * \lambda_i)$

$mean(Y_i) = variance(Y_i) = M * \lambda_i$

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

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

Same mean, variance is quadratic in the mean:

$variance(Y_i) = \mu_i (1 + \mu_i \phi_i)$

M = library size

λ_i = relative contribution of gene i

Similar interpretation

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

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

(Coefficient of variation = standard deviation/mean)

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

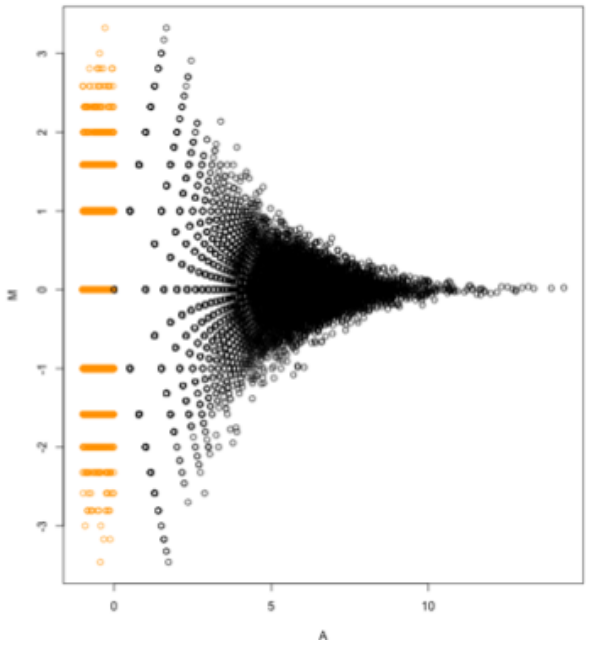
Dividing both sides by μ_{gi}^2 gives

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

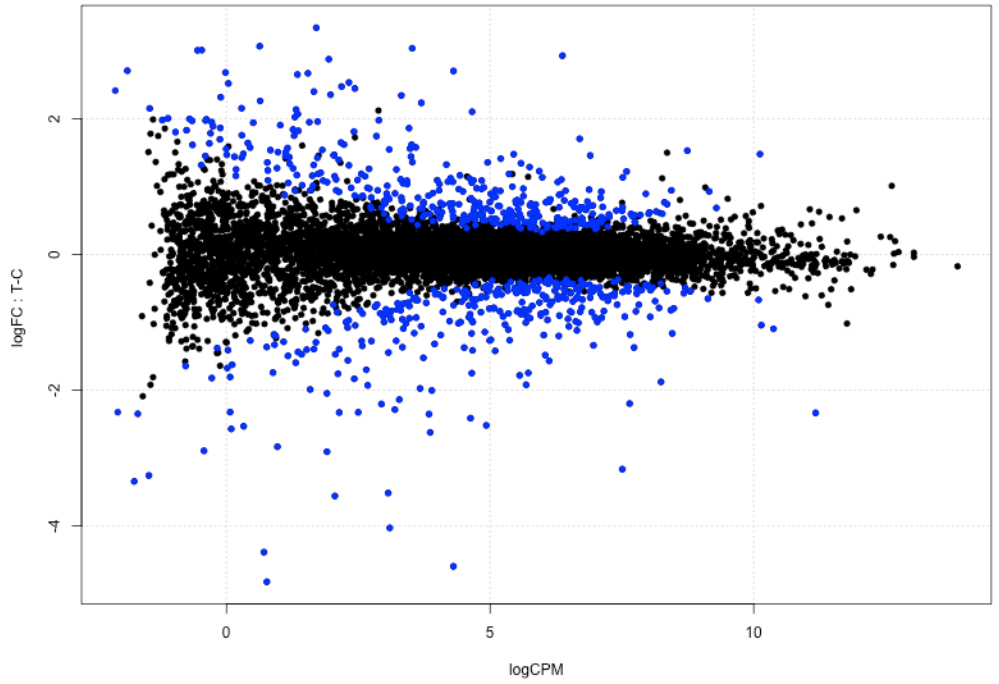
$$\mathrm{CV}^2(y_{gi}) = 1/\mu_{gi} + \phi_g.$$

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

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

Feature-specific

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

Moderated

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

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

$Y_i \sim Pois(M * \lambda_i)$

$mean(Y_i) = variance(Y_i) = M * \lambda_i$

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

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

Same mean, variance is quadratic in the mean:

$variance(Y_i) = \mu_i (1 + \mu_i \phi_i)$

M = library size

λ_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)*

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

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

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

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

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 $Z = Y_1 + \cdots + Y_n \sim \mathrm{NB}(nm\lambda, \phi n^{-1})$

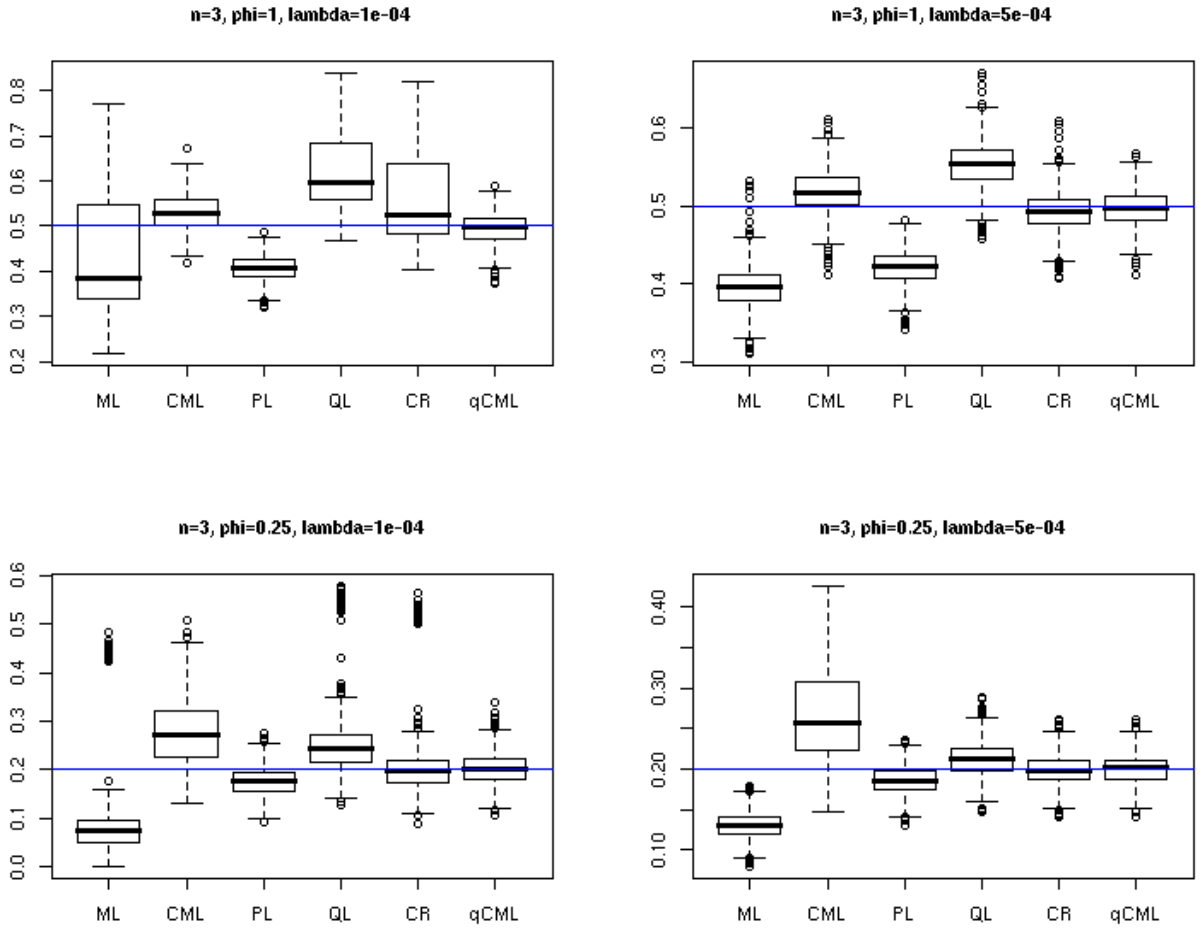
Thus, can form conditional likelihood:

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

Comparison of Estimators (Common Dispersion)

Horizontal blue line is TRUE value.

qCML performs best under a wide range of conditions.



Robinson and Smyth, Biostatistics 2007

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 —> Weighted likelihood

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

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

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

Second challenge: Moderate dispersion estimate

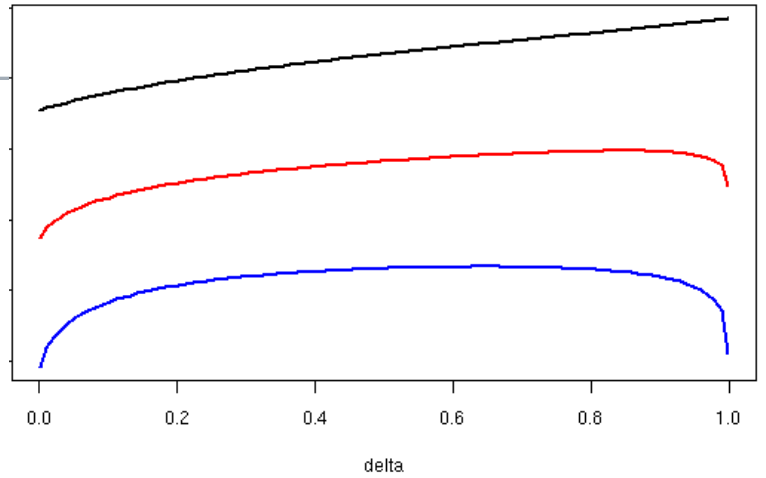
Weighted likelihood -- individual log-likelihood plus a weighted version of the common log-likelihood:

$$\mathrm{WL}(\phi_g) = l_g(\phi_g) + \alpha\, l_C(\phi_g)$$

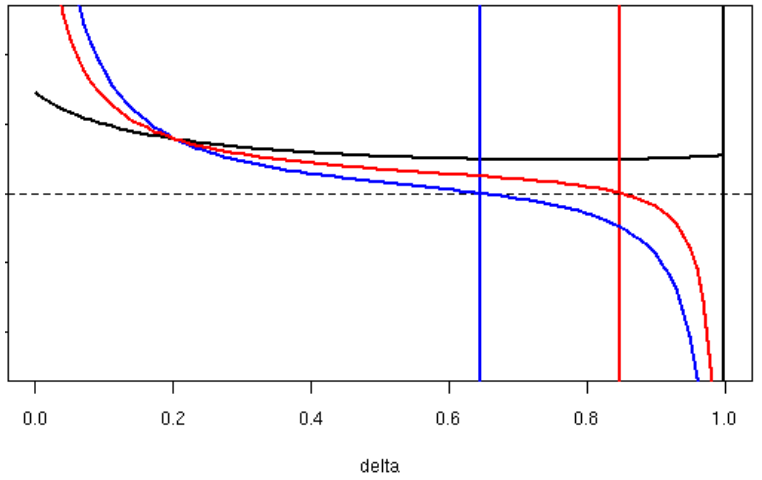
l_g - quantile-adjusted conditional likelihood

Black: single tag
Blue: common dispersion
Red: Linear combination of the two

Log-Likelihood



Score (1st derivative of LL)



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

How much to shrink?

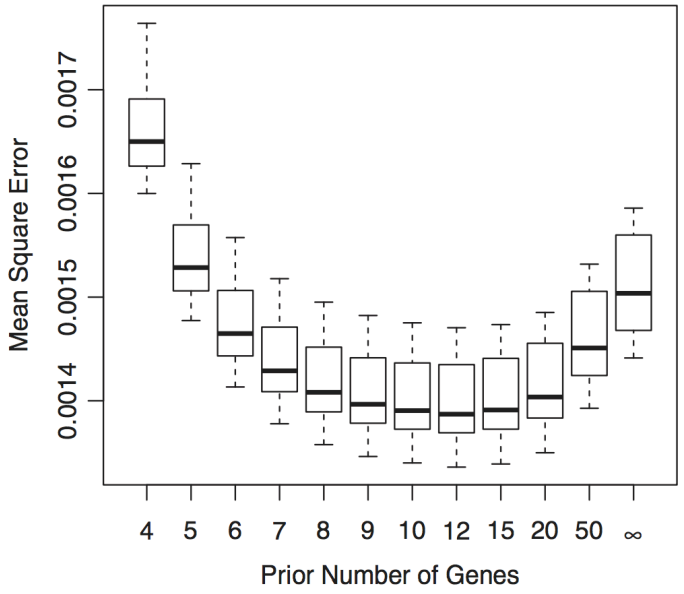


Figure 4. Mean-square error with which empirical Bayes genewise dispersions estimate the true dispersion (BCV^2), when true dispersions are randomly generated. In this case, the optimal prior weight is 10–12 prior genes, equivalent to 20–24 prior degrees of freedom. The common BCV estimator is equivalent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

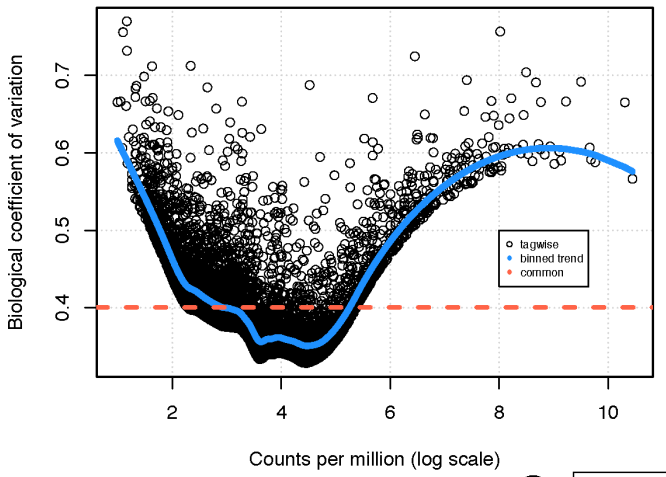
Simulations suggest there is an optimal amount to shrink.

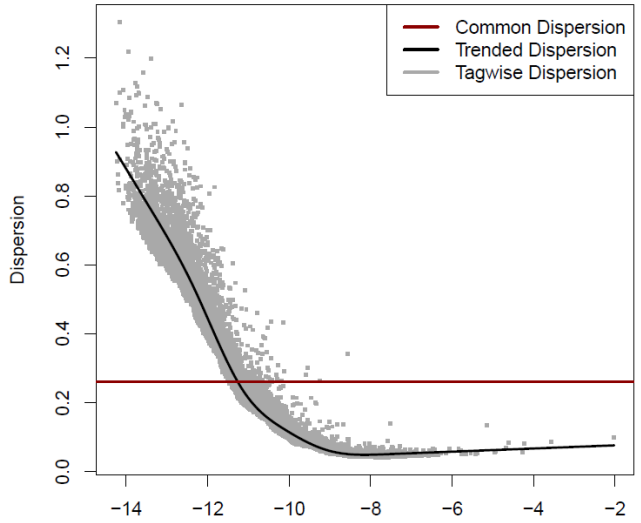
Challenge: choosing/ estimating how much

McCarthy et al. NAR 2012

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

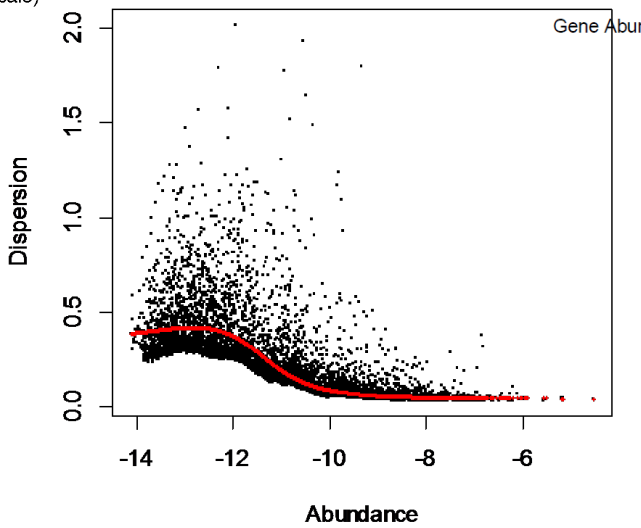
Data:
Tuch et al., 2008





Mouse hemapoeitic stem cells

Advantage: genes are allowed to have their own variance.



Mouse lymphomas

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

INNOVATION

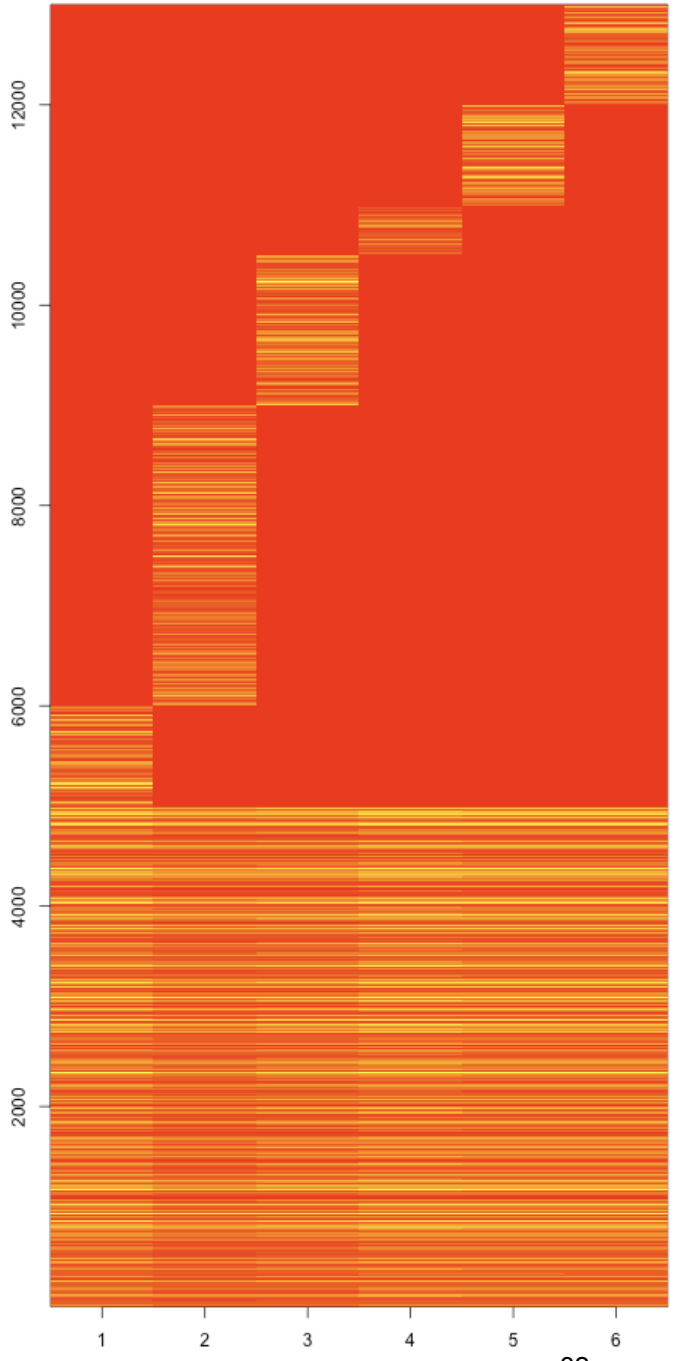
RNA-Seq: a revolutionary tool for transcriptomics

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[19,20,22].

“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

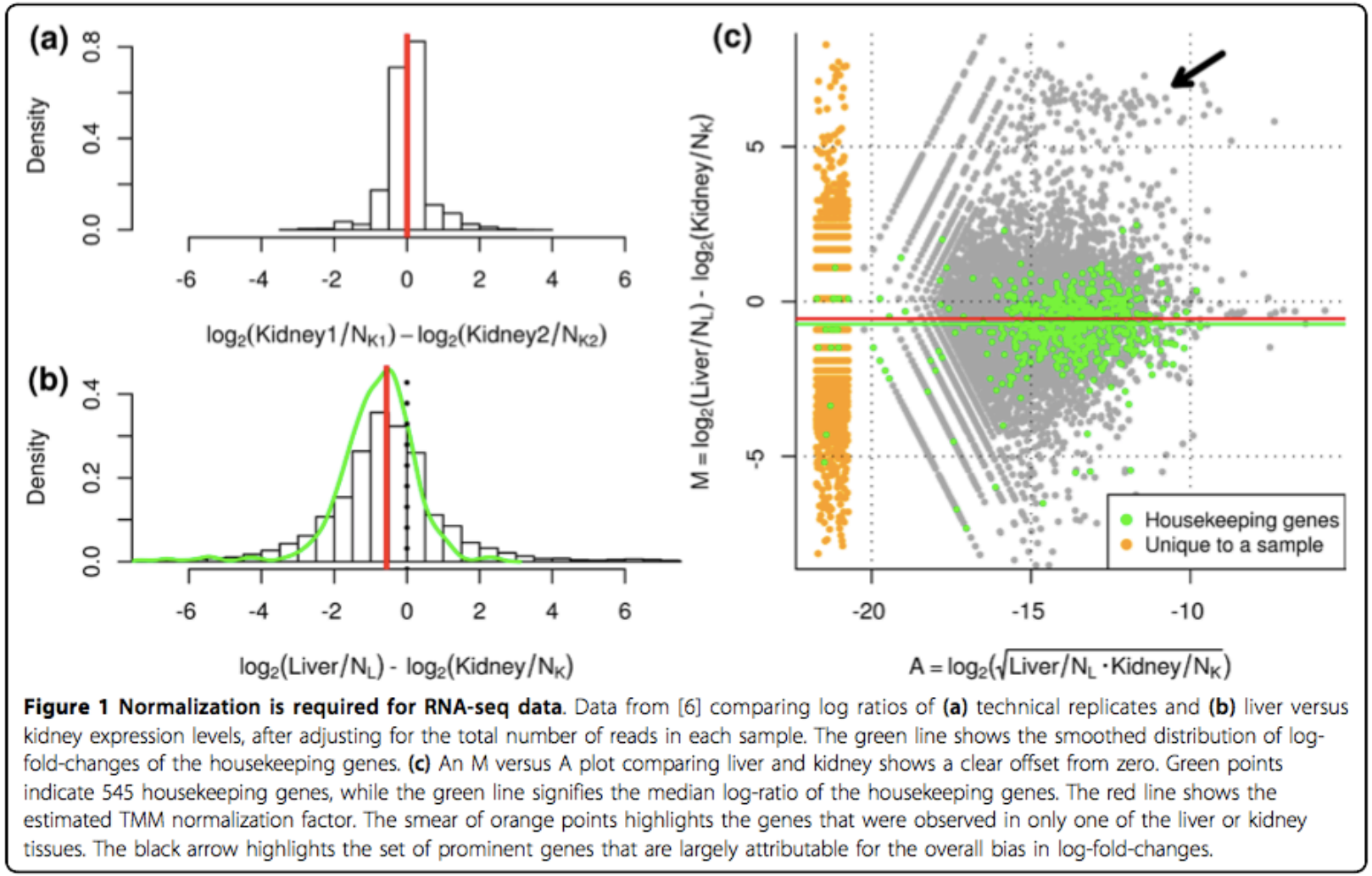


Figure 1 Normalization is required for RNA-seq data. Data from [6] comparing log ratios of **(a)** technical replicates and **(b)** liver versus kidney expression levels, after adjusting for the total number of reads in each sample. The green line shows the smoothed distribution of log-fold-changes of the housekeeping genes. **(c)** An M versus A plot comparing liver and kidney shows a clear offset from zero. Green points indicate 545 housekeeping genes, while the green line signifies the median log-ratio of the housekeeping genes. The red line shows the estimated TMM normalization factor. The smear of orange points highlights the genes that were observed in only one of the liver or kidney tissues. The black arrow highlights the set of prominent genes that are largely attributable for the overall bias in log-fold-changes.

Robinson and Oshlack (2010) Genome Biology

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

Robinson and Oshlack (2010) Genome Biology

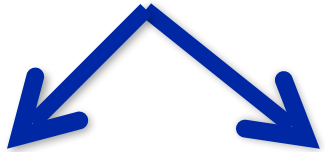
Differential expression: why not use methods developed for microarrays?

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:



Transform count data and apply standard methodology

Analyze using models for count data

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.

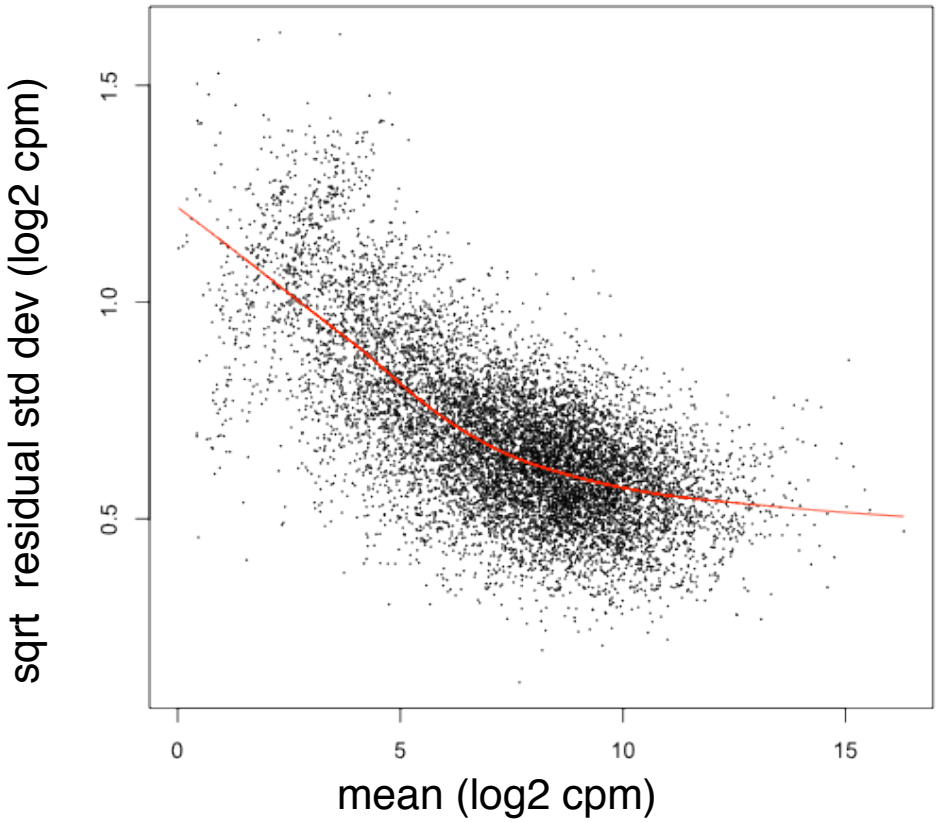
Linear modelling:

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

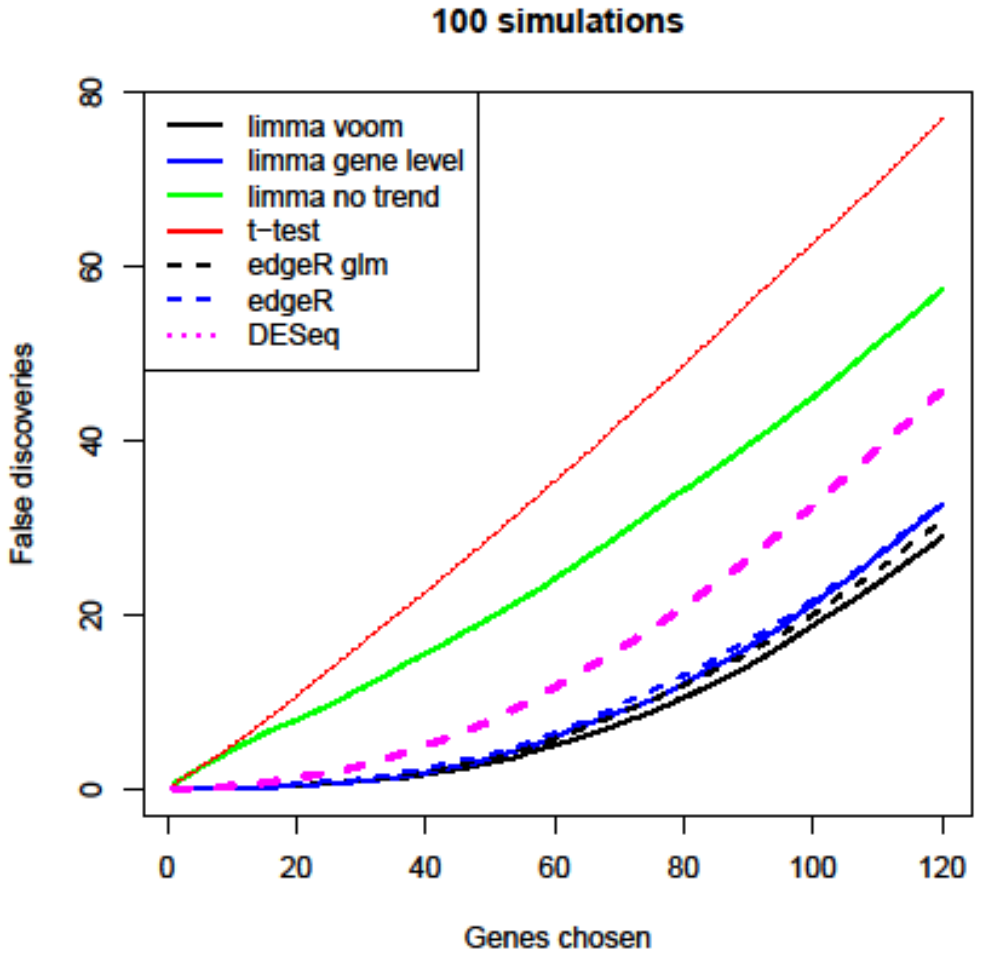
$$\text{var}\left(z_{gi}\right) = s(\mu_{gi})\sigma_g^2$$

Smooth function of mean

voom fits a lowess trend to the mean-variance relationship …



—> Use weights (1/var) in limma analysis .. i.e., **heteroscedastic regression**



Model mean-var relationship

Law et al. 2014 Genome Biology