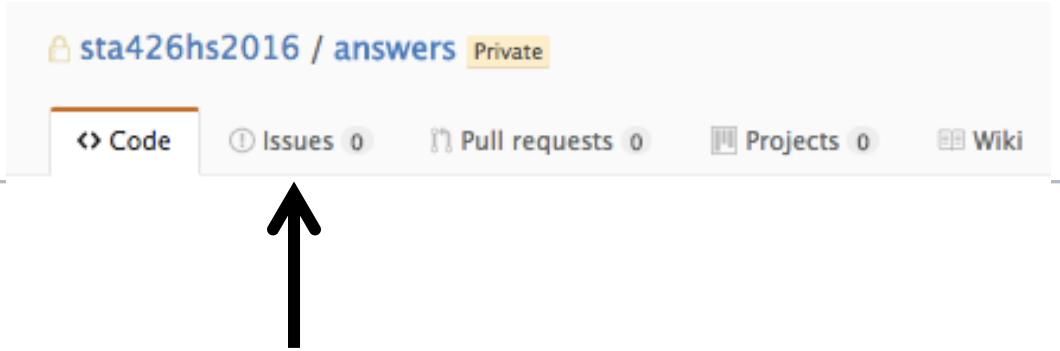
University of Zurich[UZH]

Institute of Molecular Life Sciences

Statistical models for count data analysis

Mark D. Robinson, Institute of Molecular Life Sciences

- real example of SVA
- simple counting (and new alternatives ..)
- why the negative binomial distribution?
- dispersion estimation and information sharing
- normalization considerations
- how about transformations of count data → limma?



Announcements

Plan for project: 28th November (send me a short description of your plan – 3-4 sentences)

Exercises: see comments as Github issues. If you haven't received feedback for week 6/7 exercises, let me know ASAP.

Exercises: solutions posted to 'answers' repo.

Exercises: minimum number needed for full marks: 7.

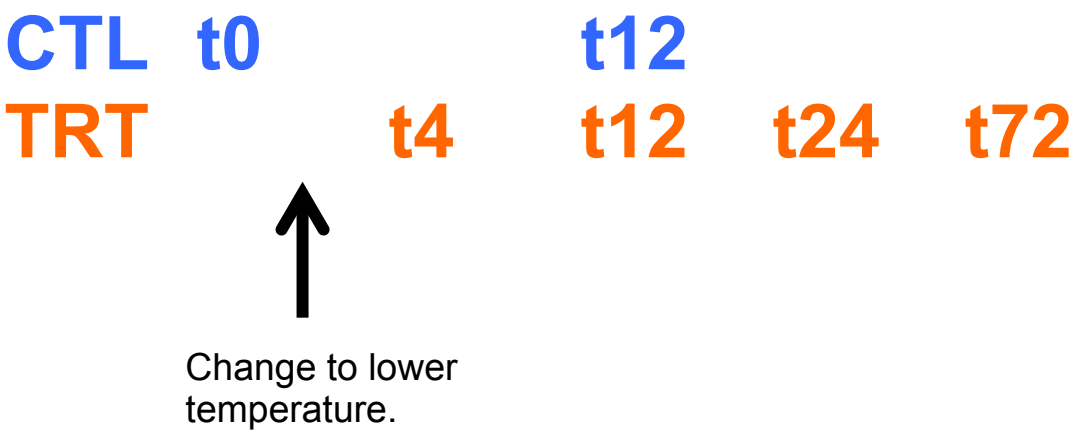
"To consult the statistician after an experiment is finished is often merely to ask him[her] to conduct a post mortem examination. He[She] can perhaps say what the experiment died of." R. A. Fisher

Motivation for exploratory data analysis: Case Study

(from Stefano, a former M.Sc. student in my Institute)

He is studying gene expression in fruitfly and is interested in transcriptional responses following "heat shock".

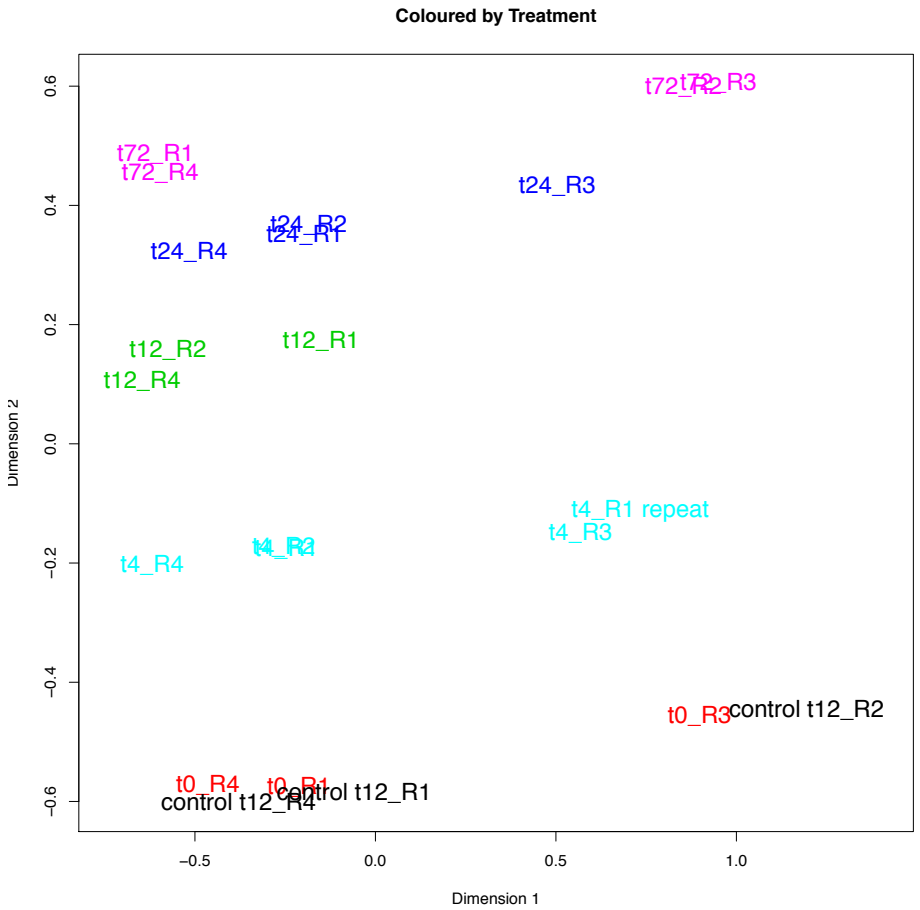
Basic schematic of experiment:

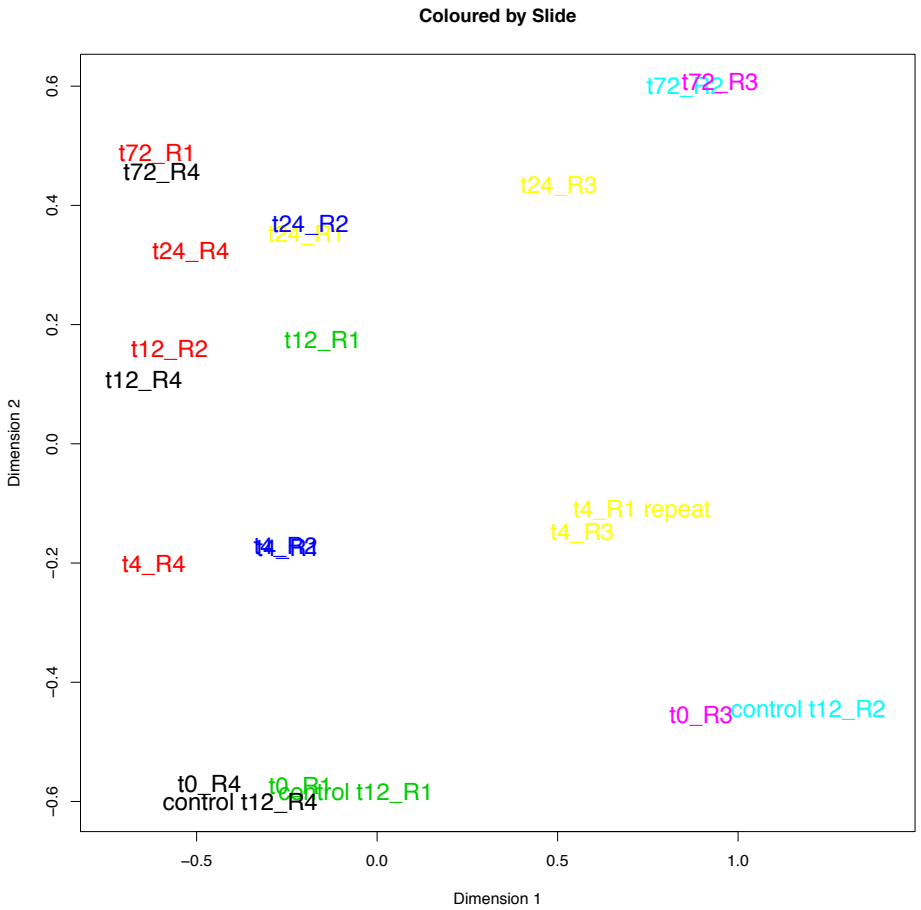


http://en.wikipedia.org/wiki/Multidimensional_scaling

```
library(limma)
plotMDS(d)  # 'd' is a matrix
```

Take a close look at where the replicates are to each other relative to the X- and Y-axes





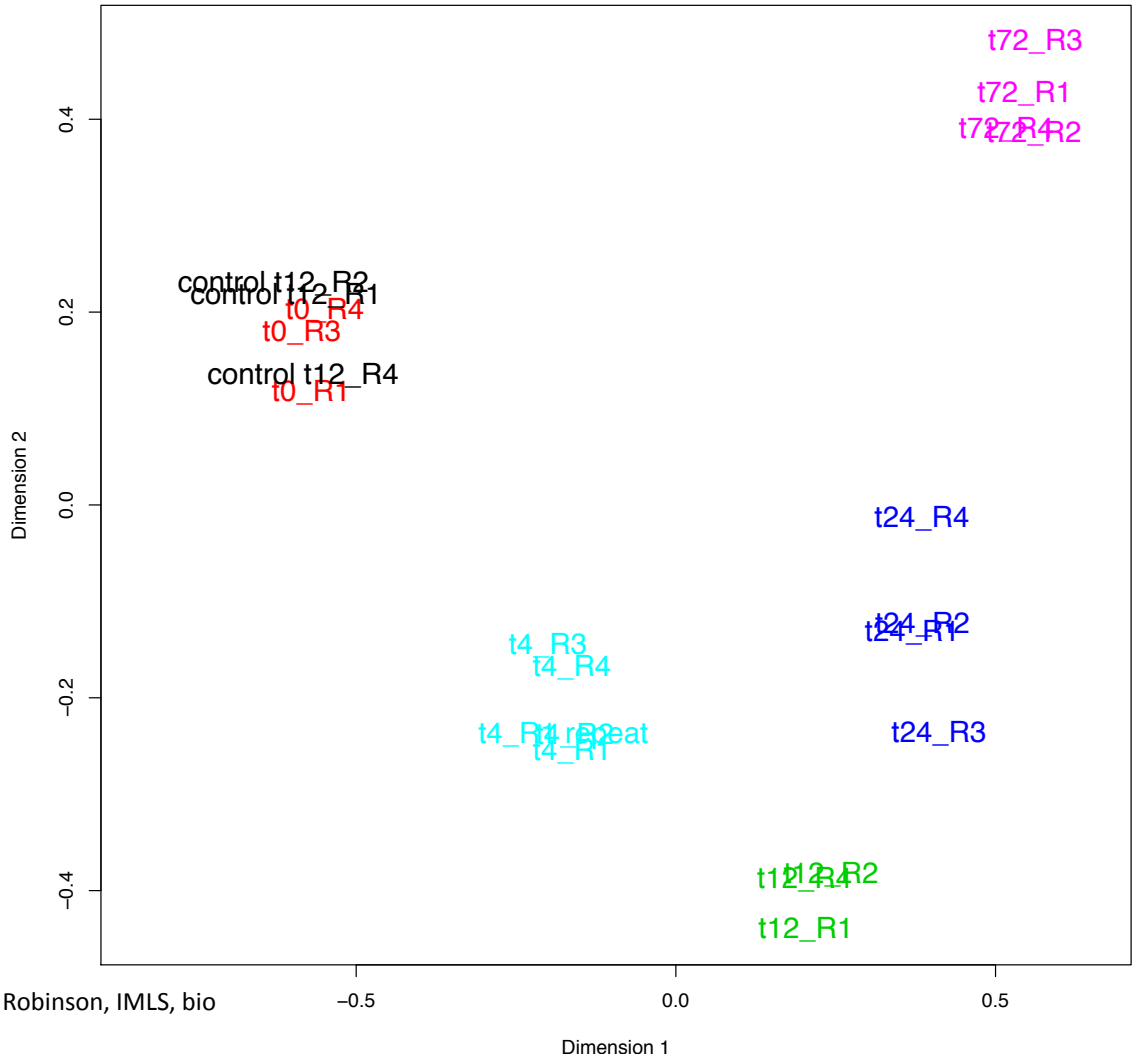
OPEN ACCESS Freely available online PLoS GENETICS

Capturing Heterogeneity in Gene Expression Studies by Surrogate Variable Analysis

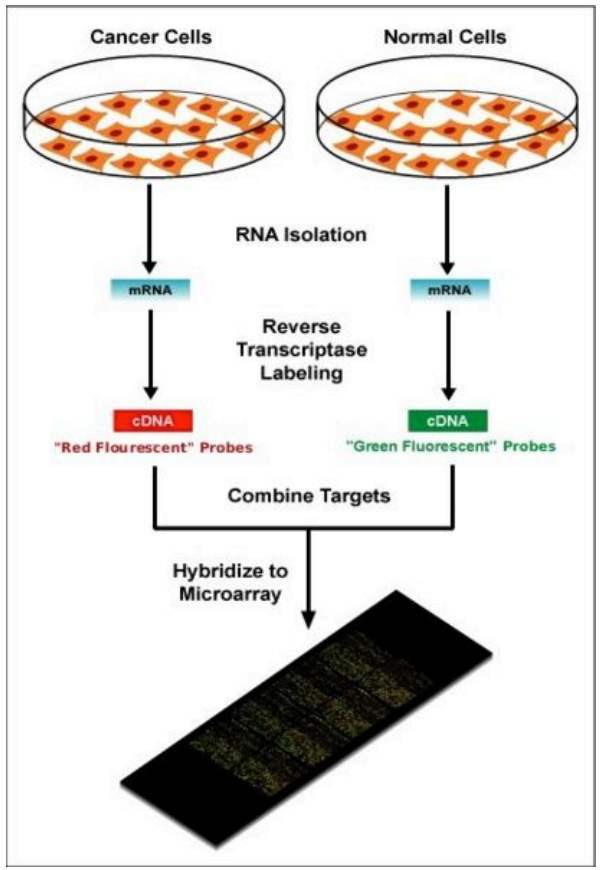
Jeffrey T. Leek[1], John D. Storey[1,2*]

1 Department of Biostatistics, University of Washington, Seattle, Washington, United States of America, 2 Department of Genome Sciences, University of Washington, Seattle, Washington, United States of America

Magic: Surrogate variable analysis to detect and "remove" batch effects

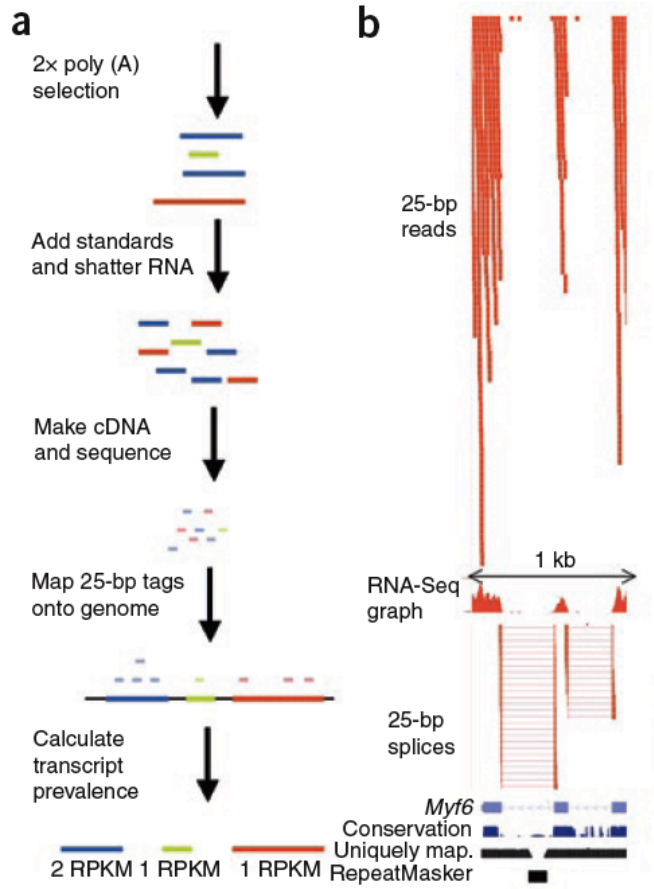


Abundance by Fluorescence Intensity



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

Abundance by Counting



Mortazavi et al., Nature Methods, 2008

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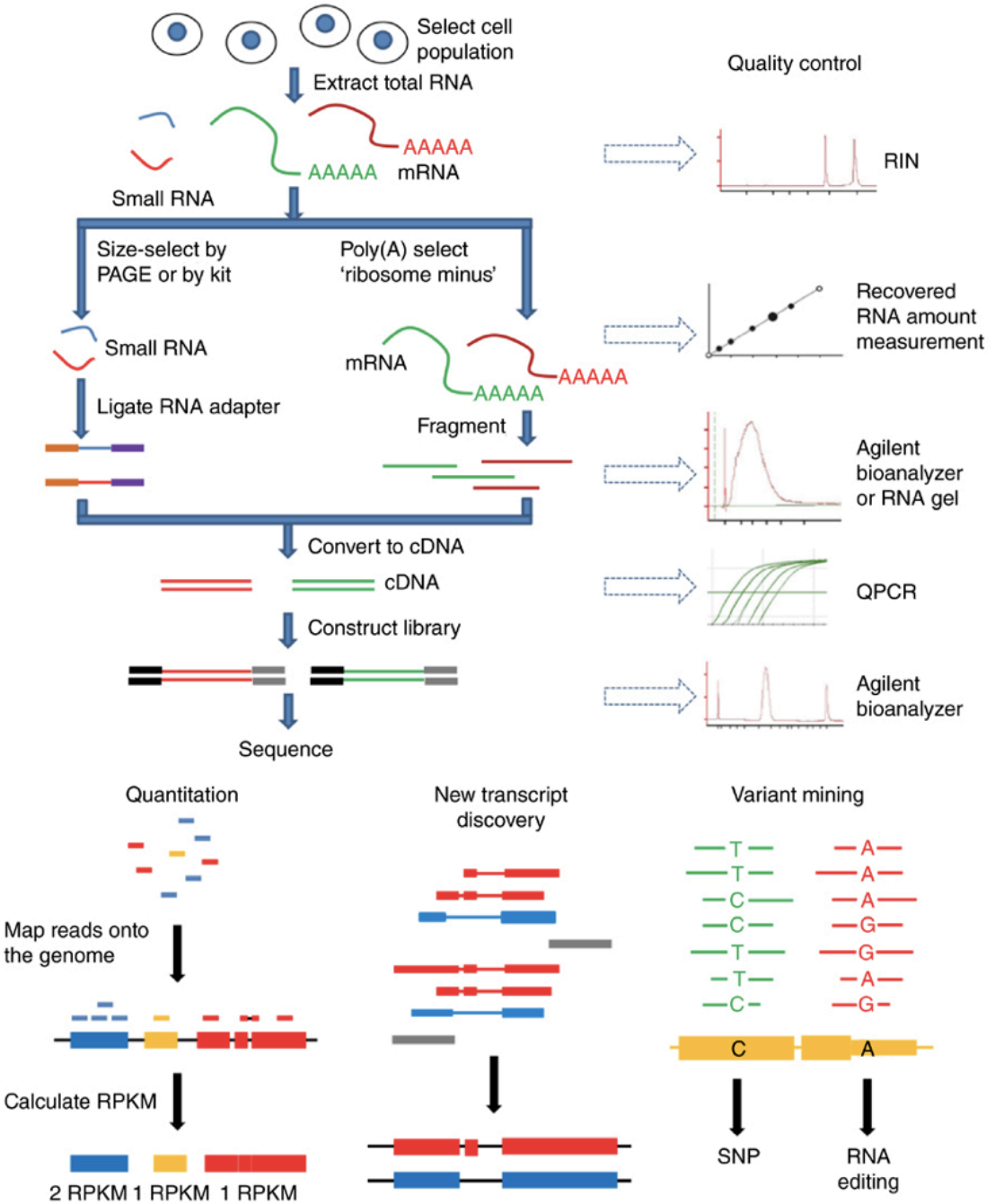
RNA-seq differential expression analyses

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



Zeng & Mortazavi, Nature Immunology, 2012

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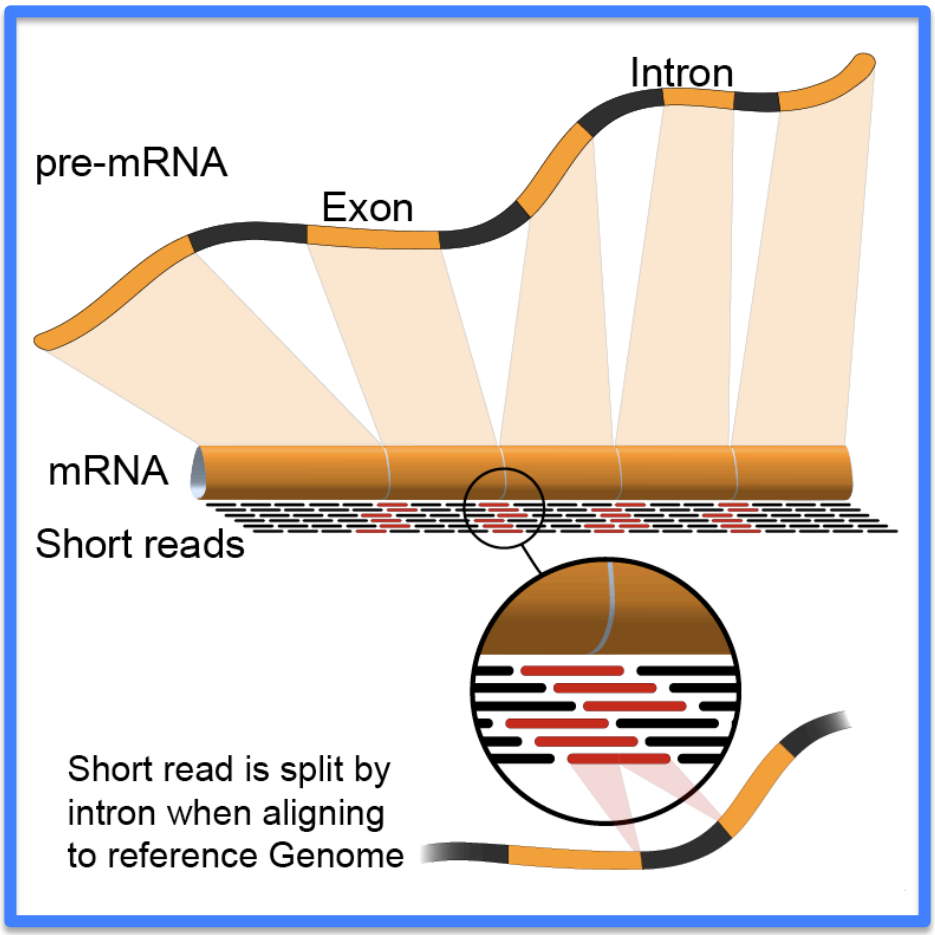
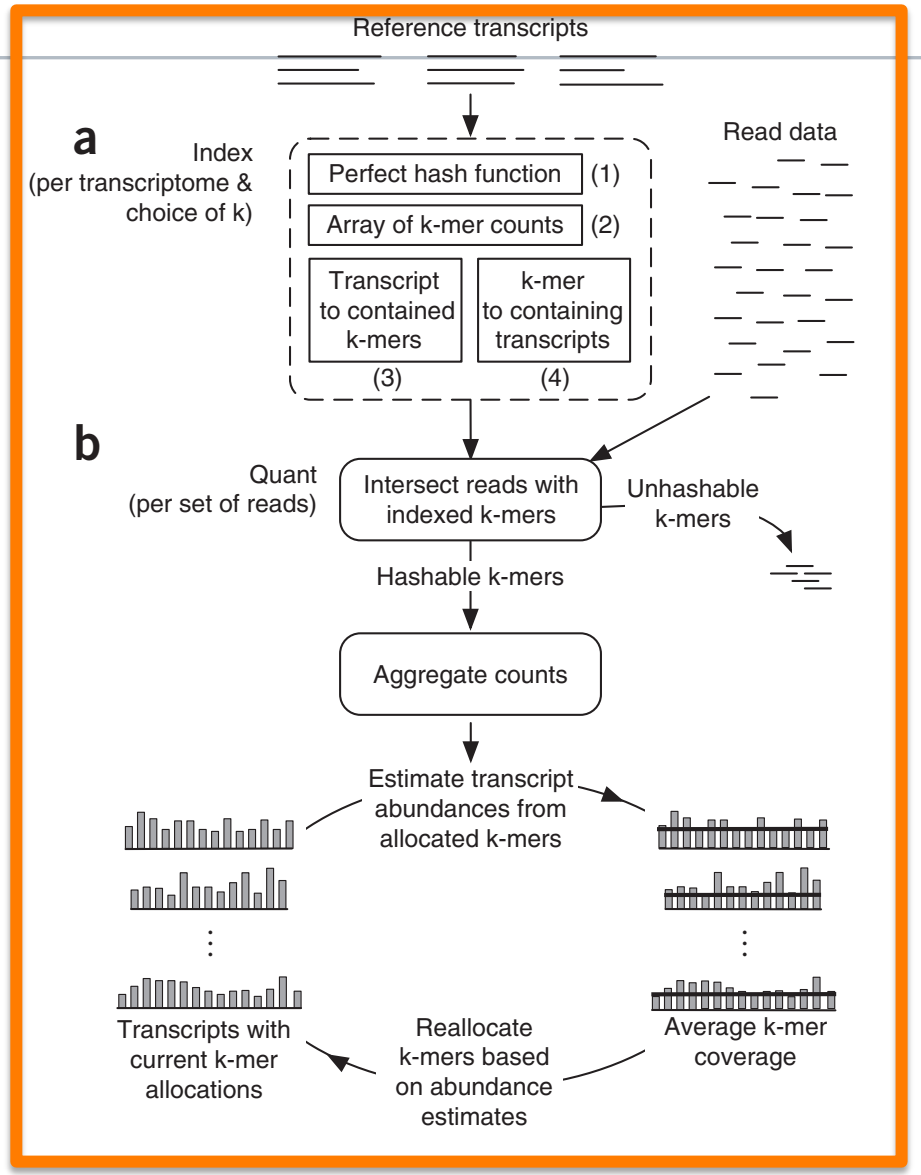
Alignment versus quasi-alignment



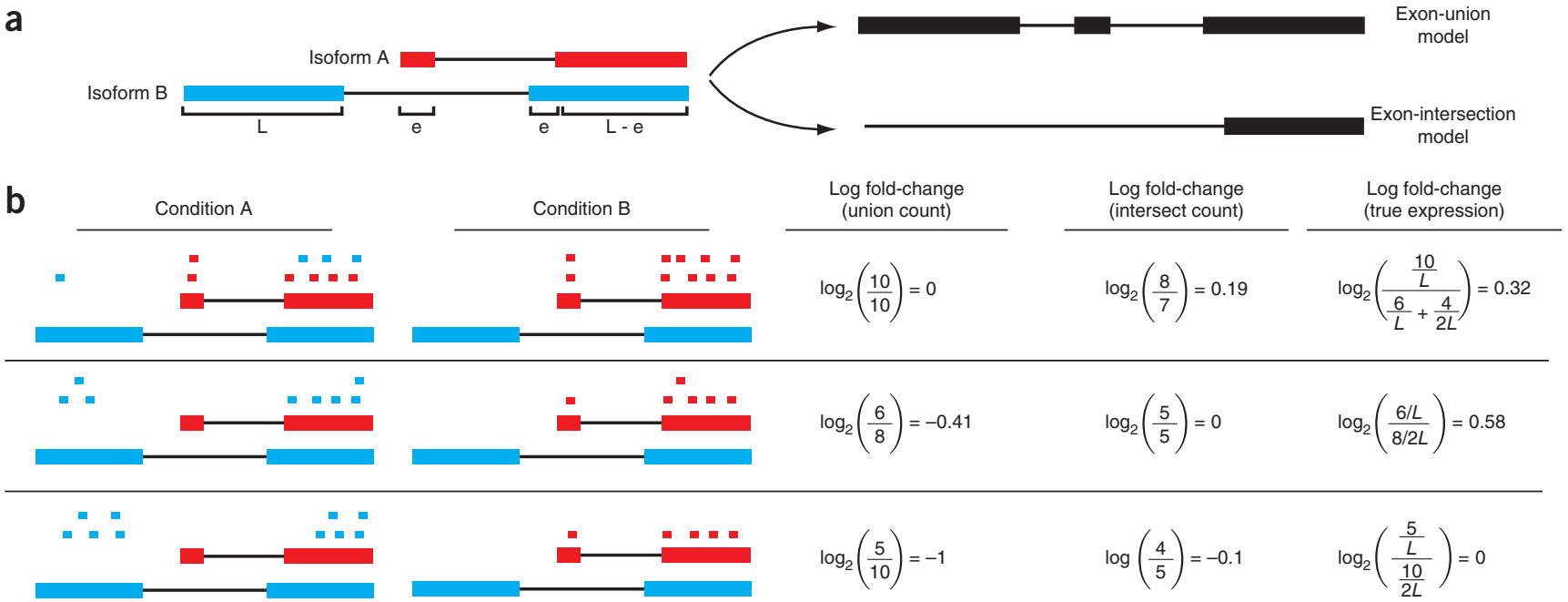
https://en.wikipedia.org/wiki/RNA-Seq



sailfish (Patro et al. 2014)

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

Trapnell et al. 2013 Nat Biotech



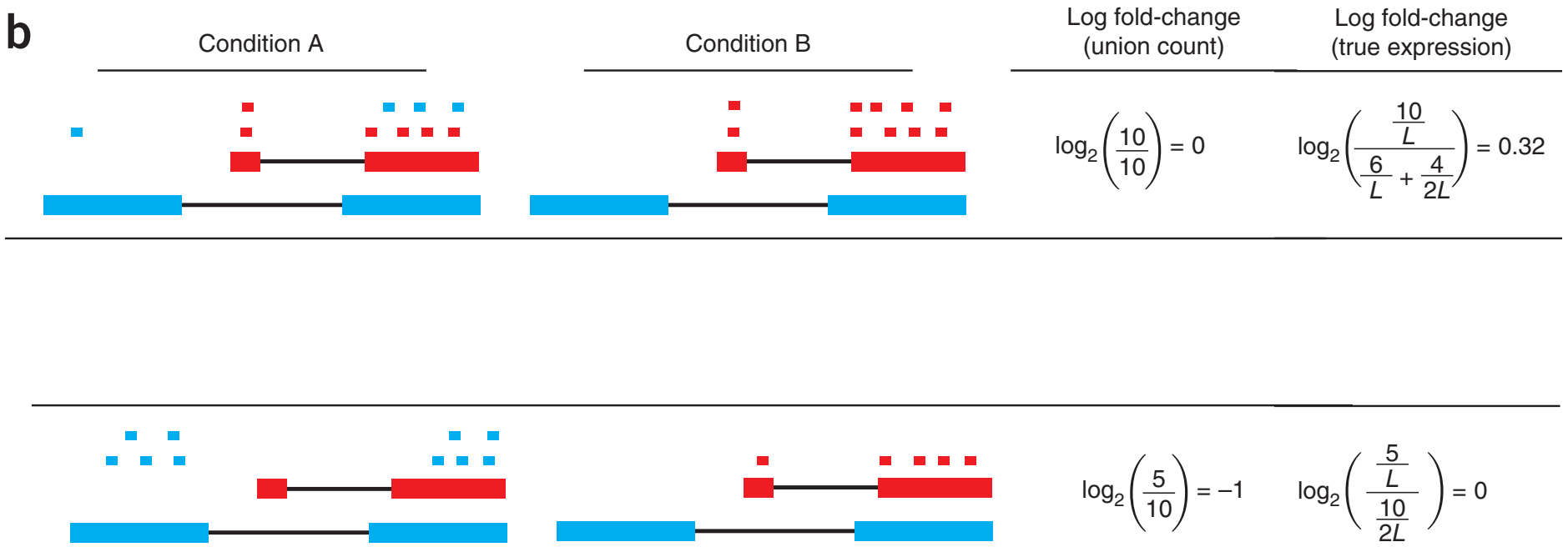
Transcriptome analysis of human tissues and cell lines reveals one dominant transcript per gene

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

Quick plug: open science, PPR

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]

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[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

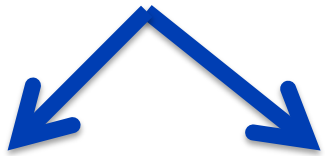
v2 **First published:** 30 Dec 2015, **4**:1521 (doi: 10.12688/f1000research.7563.1)
Latest published: 29 Feb 2016, **4**:1521 (doi: 10.12688/f1000research.7563.2)

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)**

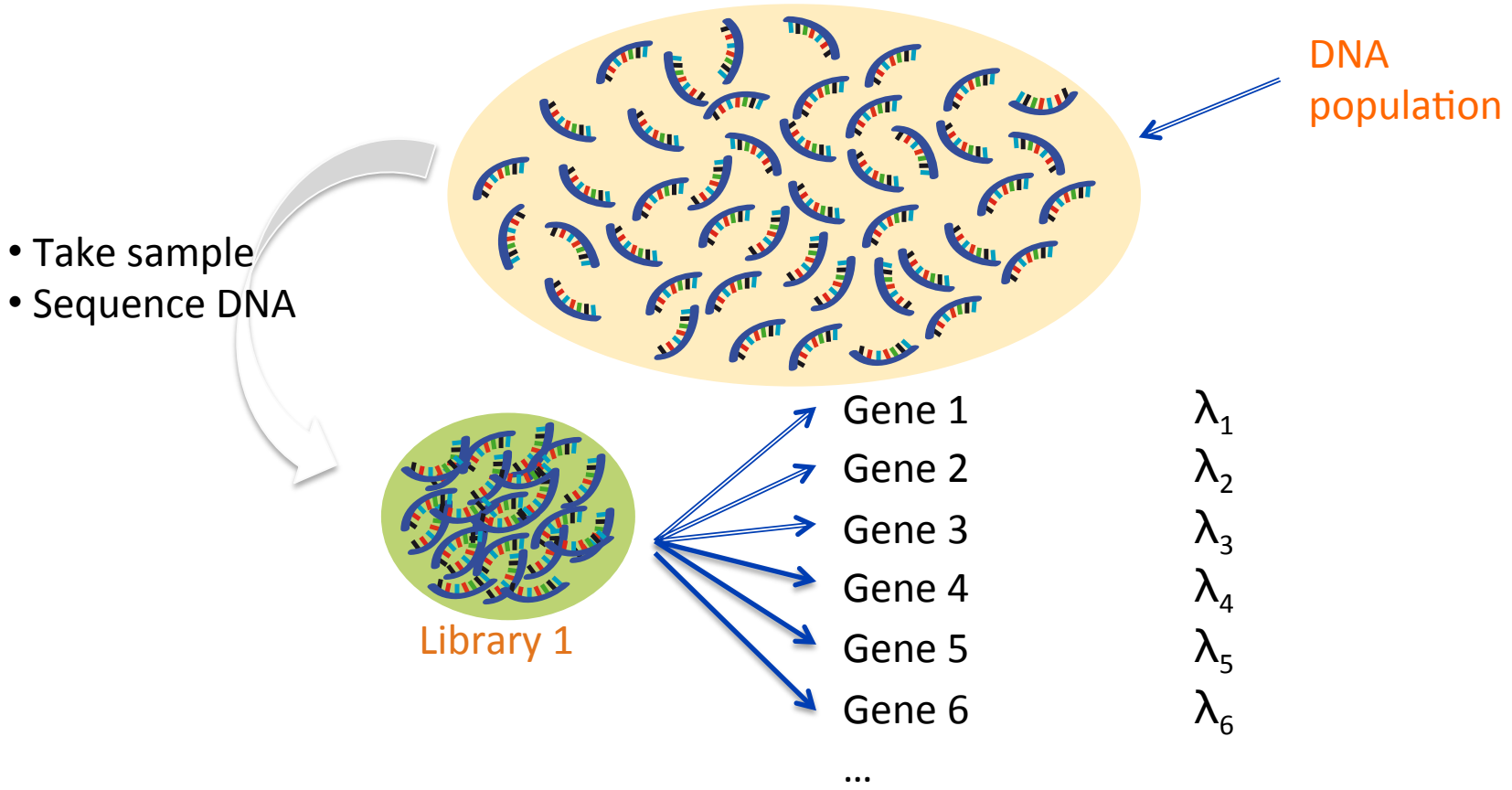
Two options:



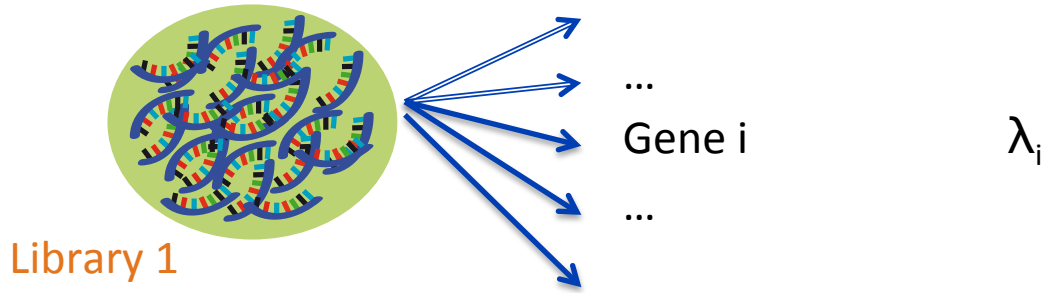
Transform count data and apply standard methodology

Analyze using models for count data

Sampling reads from population of DNA fragment is multinomial



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

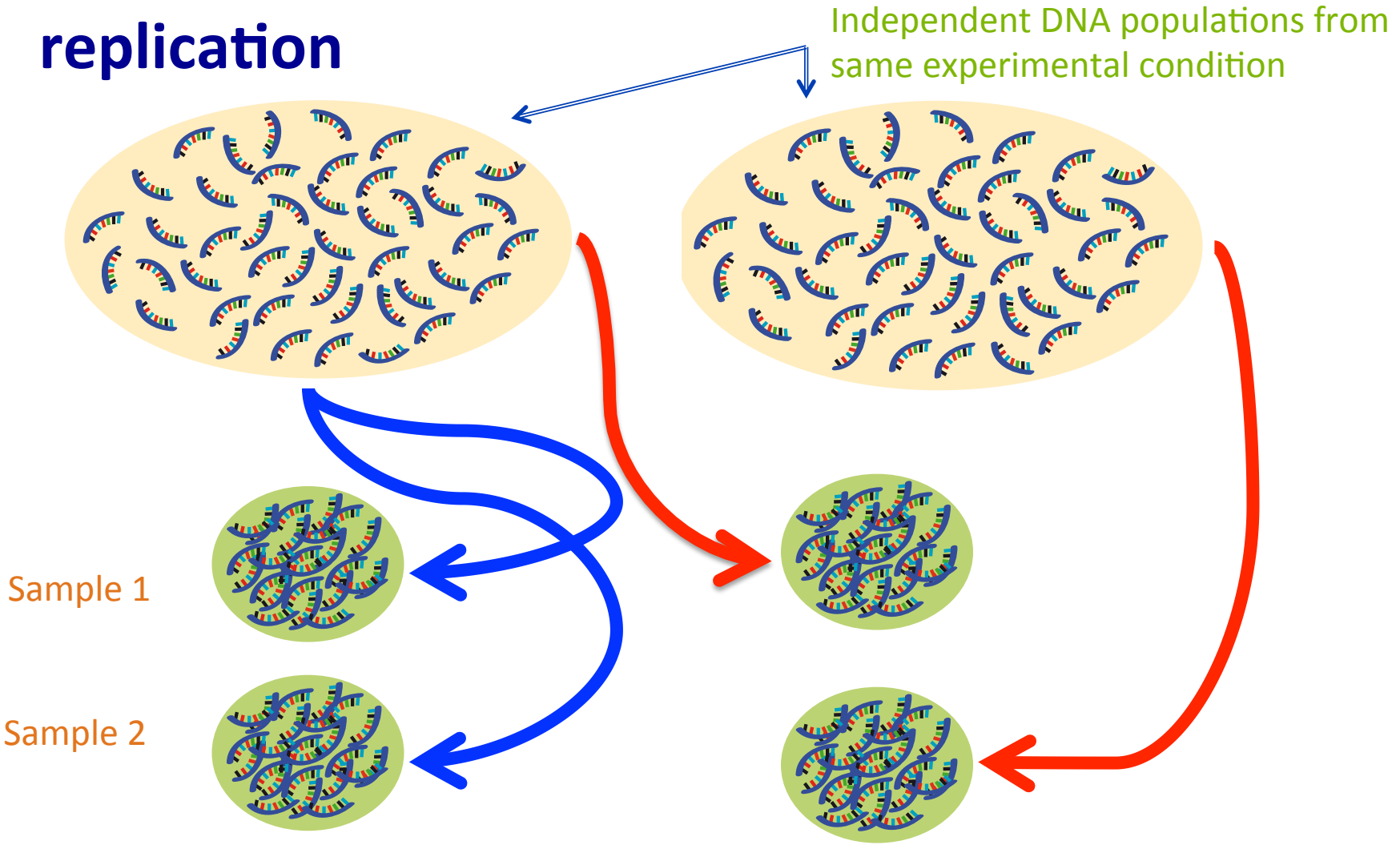


$Y_i \sim \text{Binomial}(M, \lambda_i)$

Y_i - observed number of reads for gene i
M - total number of sequences
λ_i - proportion

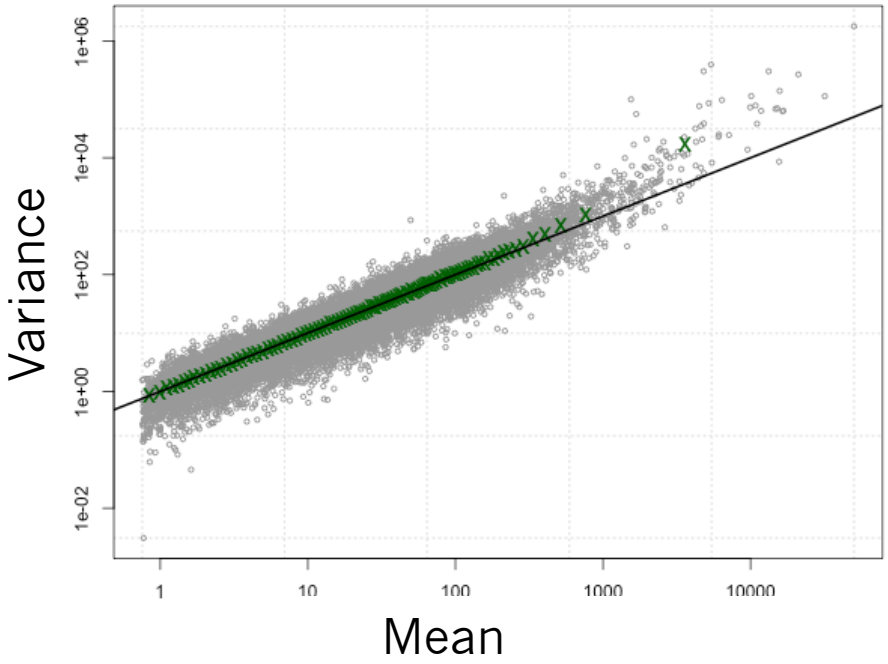
Large M, small λ_i → approximated well by Poisson($\mu_i = M \bullet \lambda_i$)

Technical replication versus biological replication



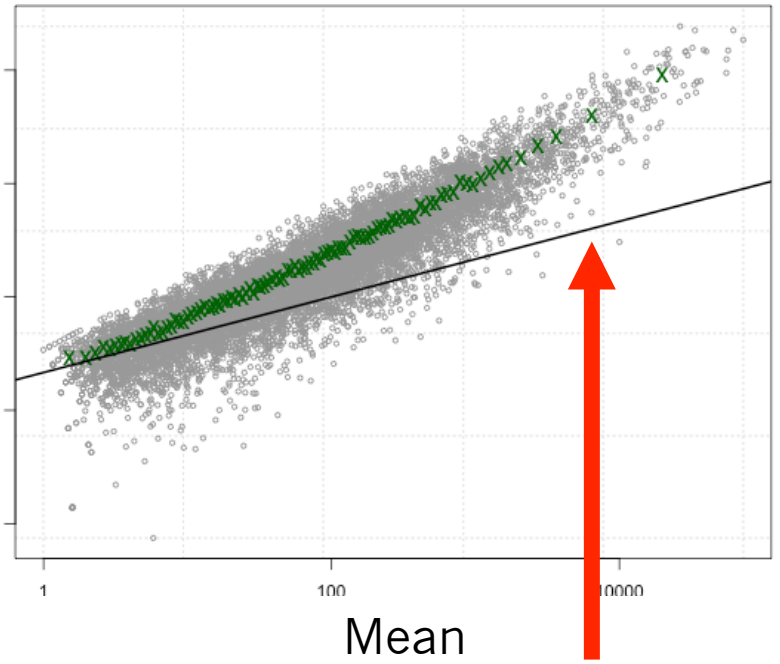
Mean-Variance plots: What we see in real data

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

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

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

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

λ_i = relative contribution of gene i

Similar interpretation

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

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

Coefficient of variation = standard deviation/mean

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

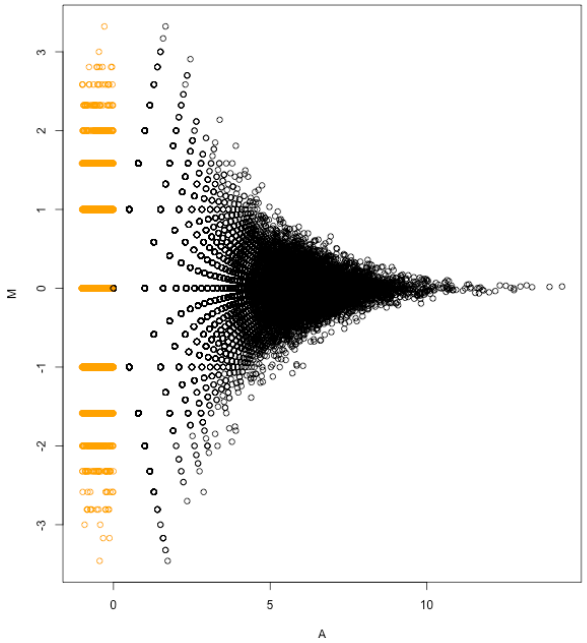
Dividing both sides by μ_{gi}^2 gives

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

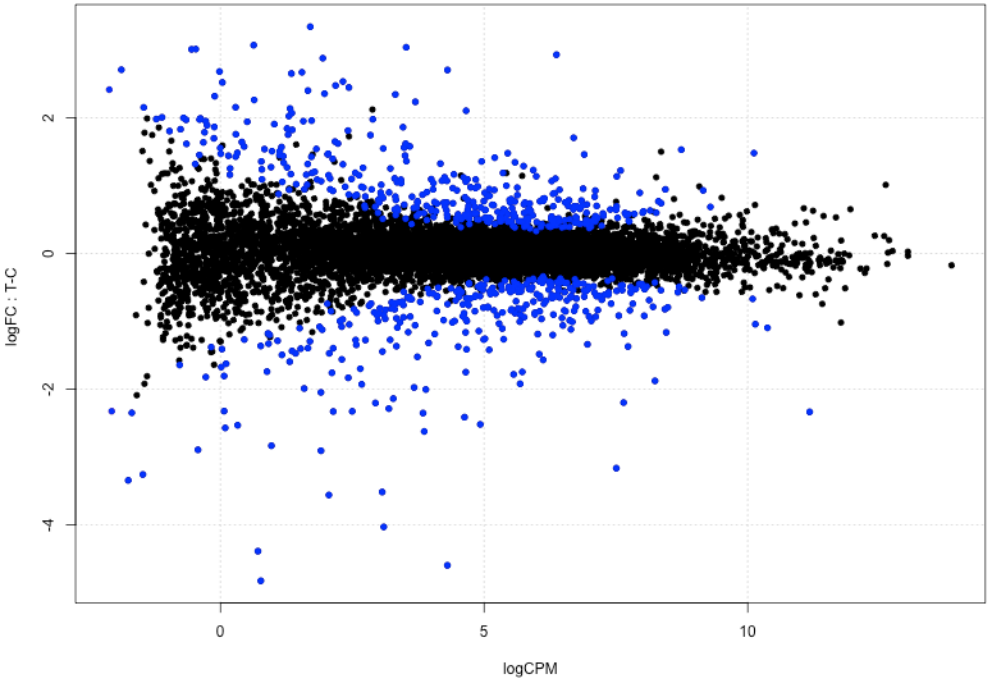
$$\mathrm{CV}^2(y_{gi}) = 1/\mu_{gi} + \phi_g.$$

A confirmation of what the theory states

Technical replicates (~Poisson)



Biological replicates



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

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

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

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

λ_i = relative contribution of gene i

First challenge: getting good estimates of dispersion in small samples

Several choices here:

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

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

- Maximum Likelihood (MLE)
- Pseudo-Likelihood (PL)

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

- Quasi-Likelihood (QL)

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

- Conditional Maximum Likelihood (CML)
- Approximate Conditional Inference (Cox-Reid)
- *quantile-adjusted Maximum Likelihood (qCML)*

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

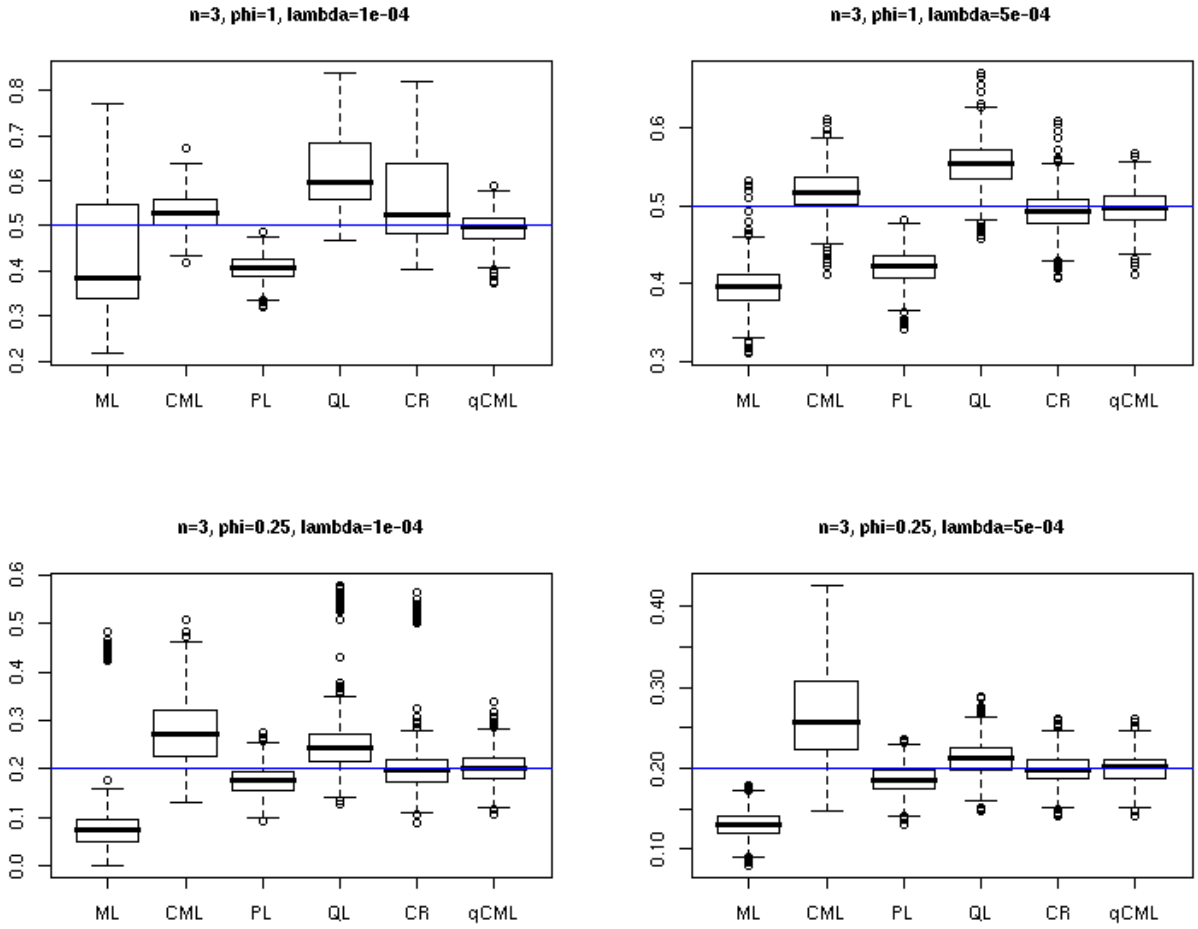
Can form conditional likelihood:

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

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

Maximum Likelihood Estimation (MLE)

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

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:

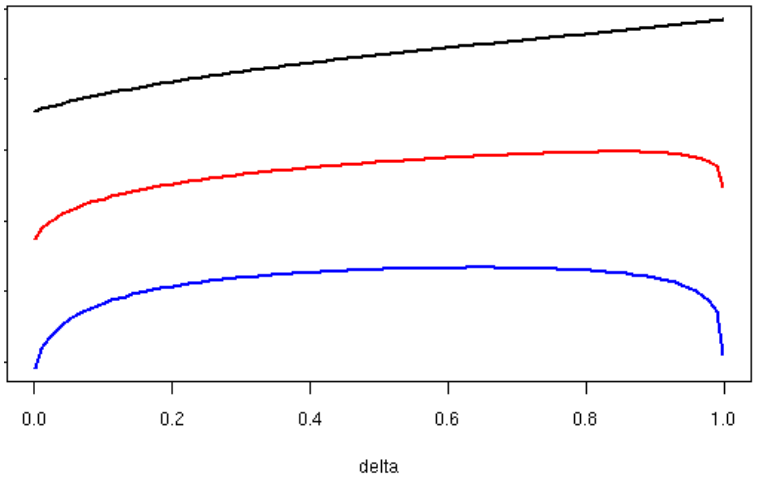
$$\mathrm{WL}(\phi_g) = l_g(\phi_g) + \alpha\, l_C(\phi_g)$$

$(1-\alpha)$

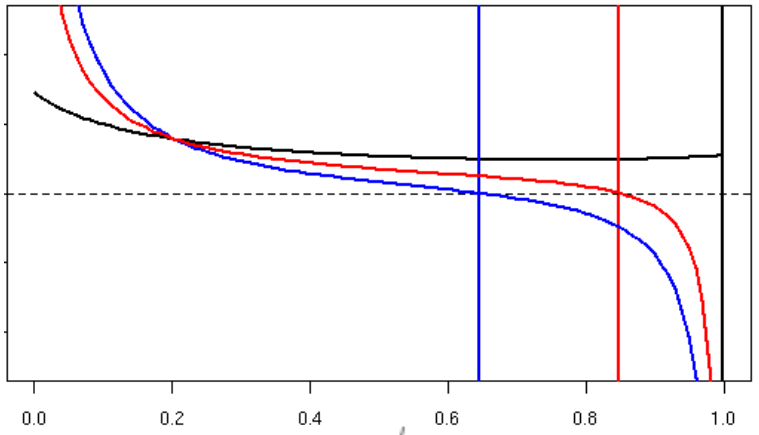
L_g - quantile-adjusted conditional likelihood

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?

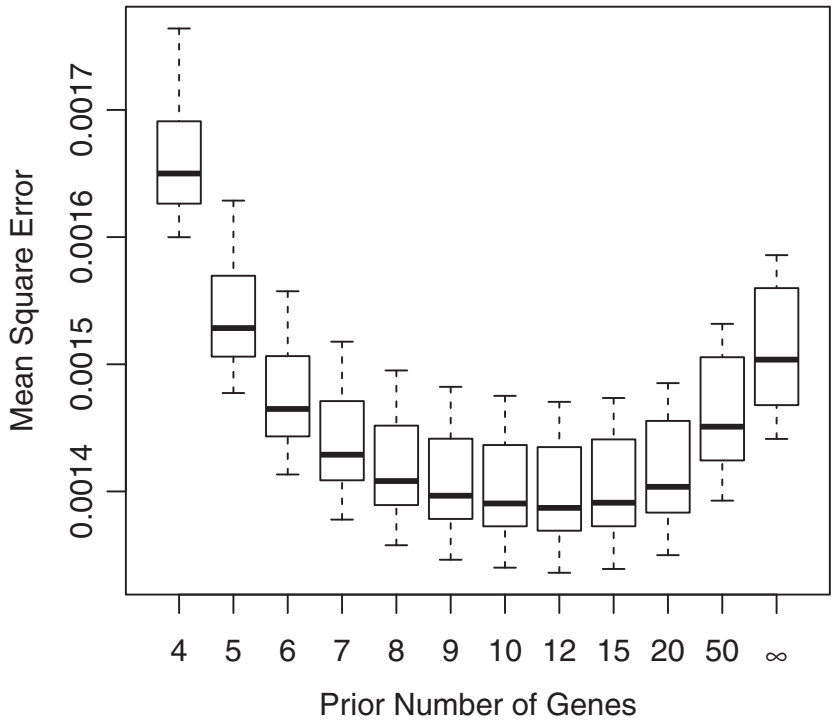


Figure 4. Mean-square error with which empirical Bayes genewise dispersions estimate the true dispersion (BCV^2), when true dispersions are randomly generated. In this case, the optimal prior weight is 10–12 prior genes, equivalent to 20–24 prior degrees of freedom. The common BCV estimator is equivalent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

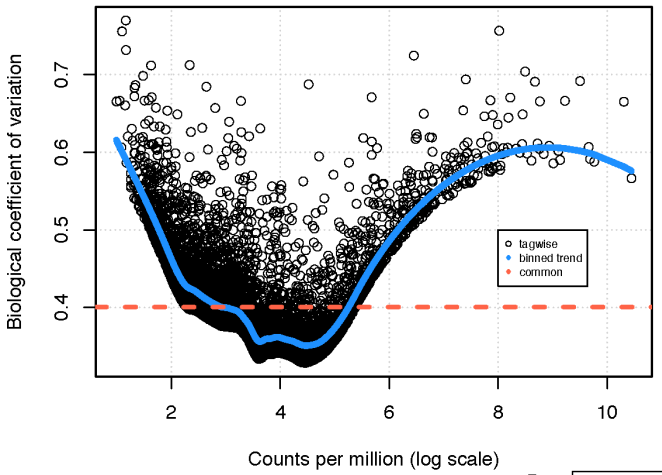
Simulations suggest there is an optimal amount to shrink.

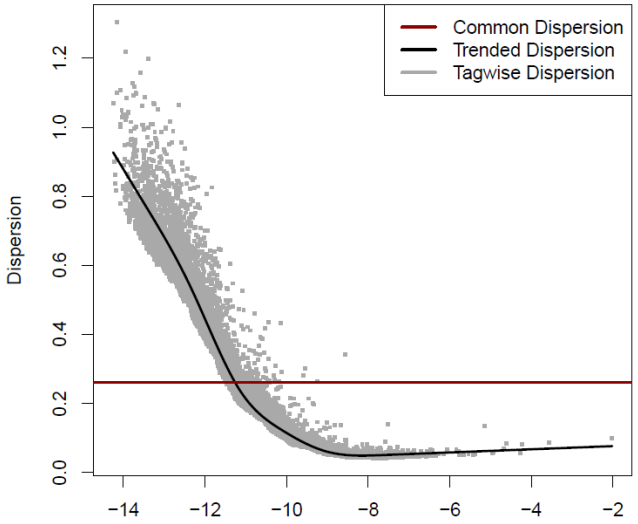
Challenge: choosing/ estimating how much

McCarthy et al. NAR 2012

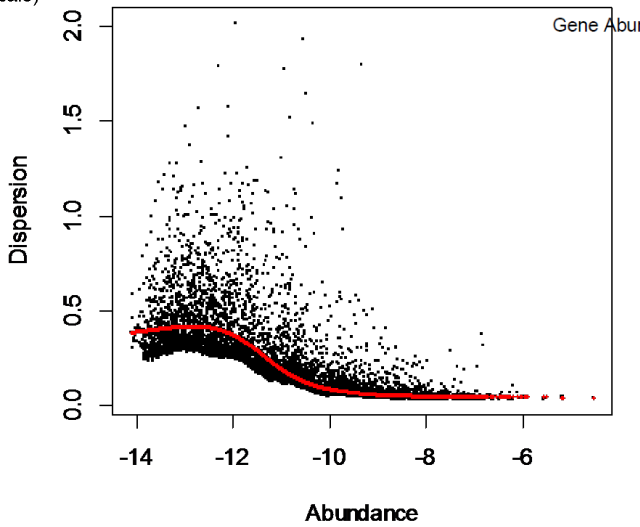
Dispersion varies with mean: moderate dispersion towards trend

Data:
Tuch et al., 2008





Mouse hemapoeitic stem cells



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

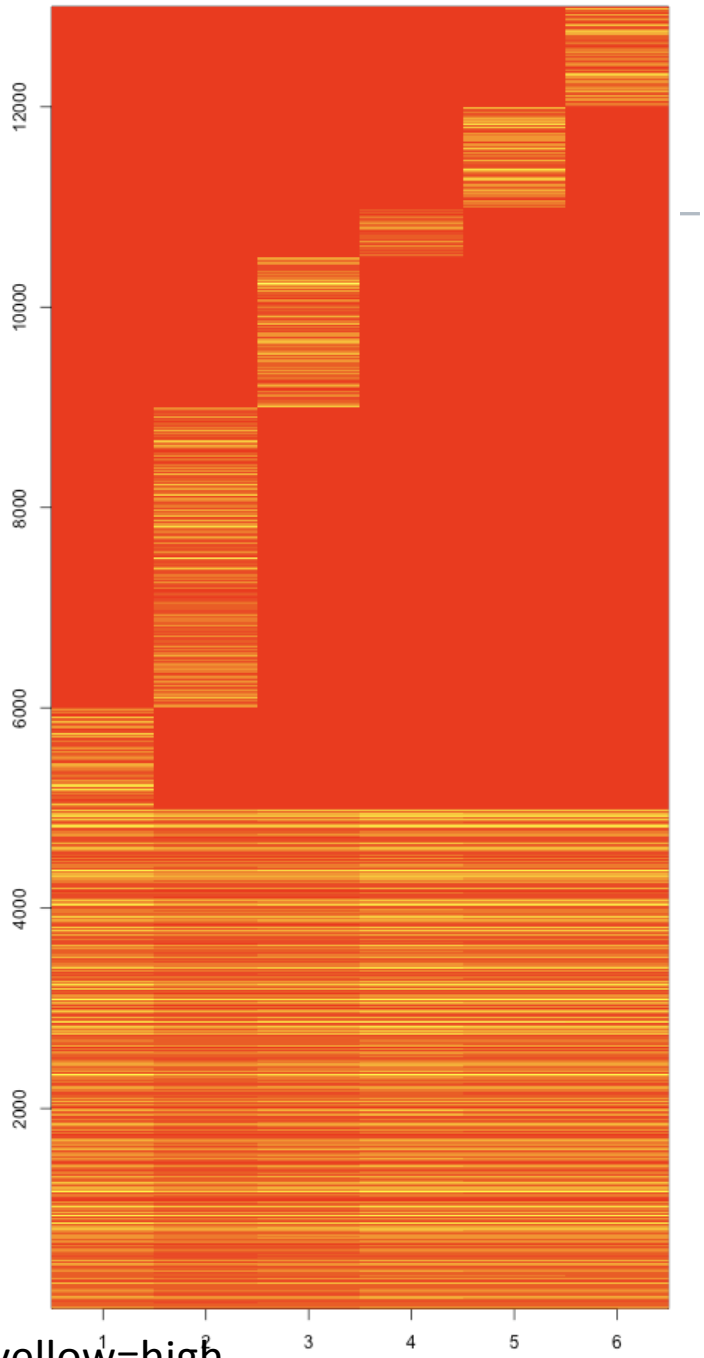
RNA-Seq: a revolutionary tool for transcriptomics

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[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

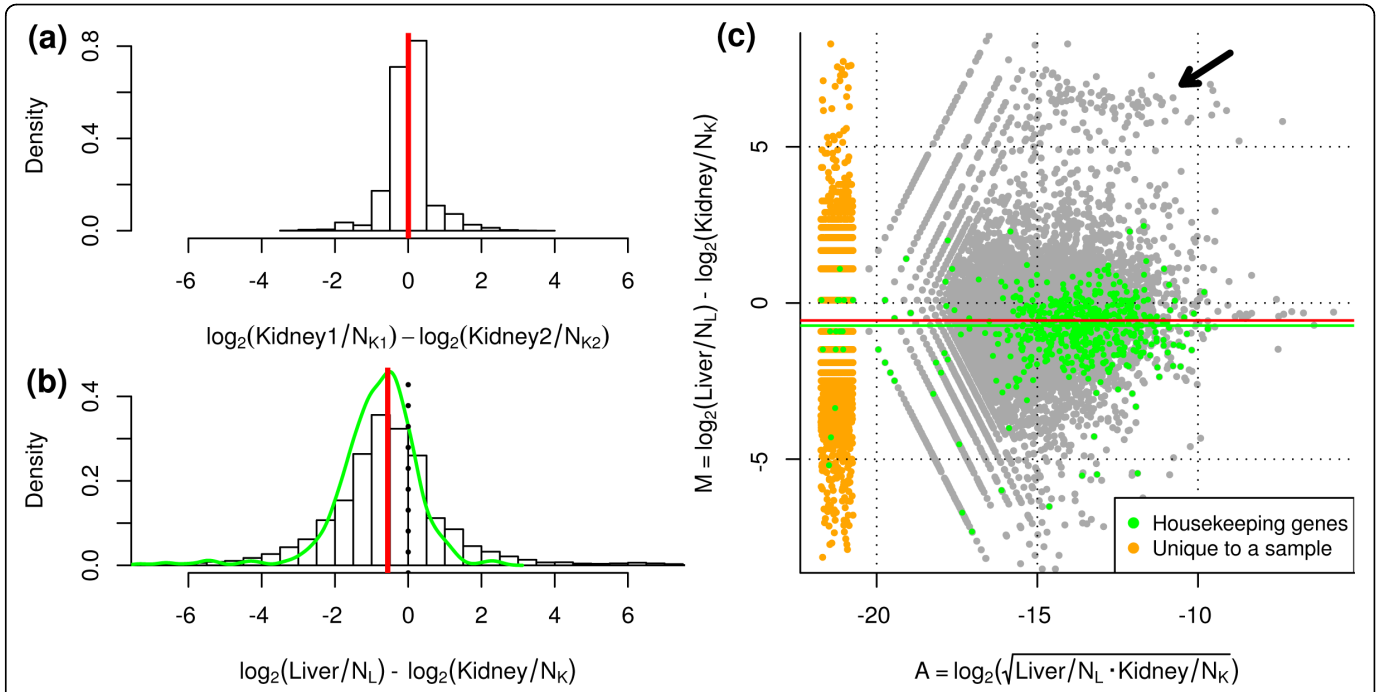


Figure 1 Normalization is required for RNA-seq data. Data from [6] comparing log ratios of **(a)** technical replicates and **(b)** liver versus kidney expression levels, after adjusting for the total number of reads in each sample. The green line shows the smoothed distribution of log-fold-changes of 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

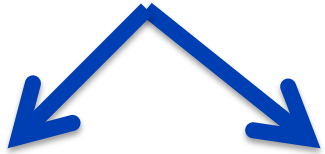
Differential expression: why not use methods developed for microarrays?

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.

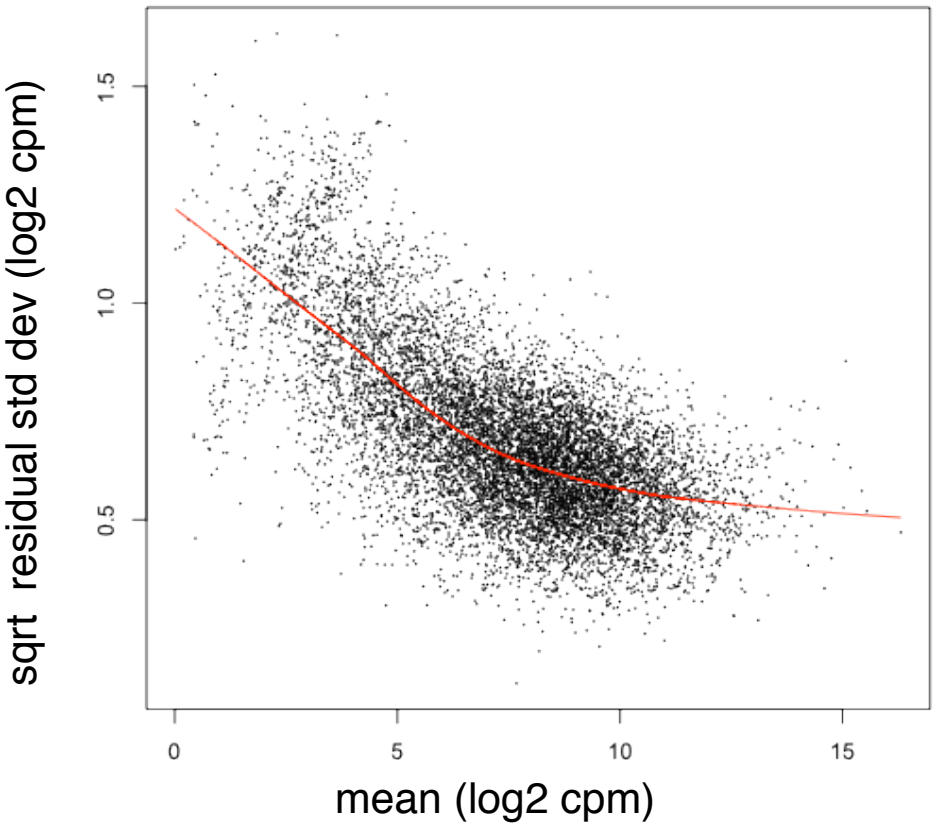
Linear modelling:

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

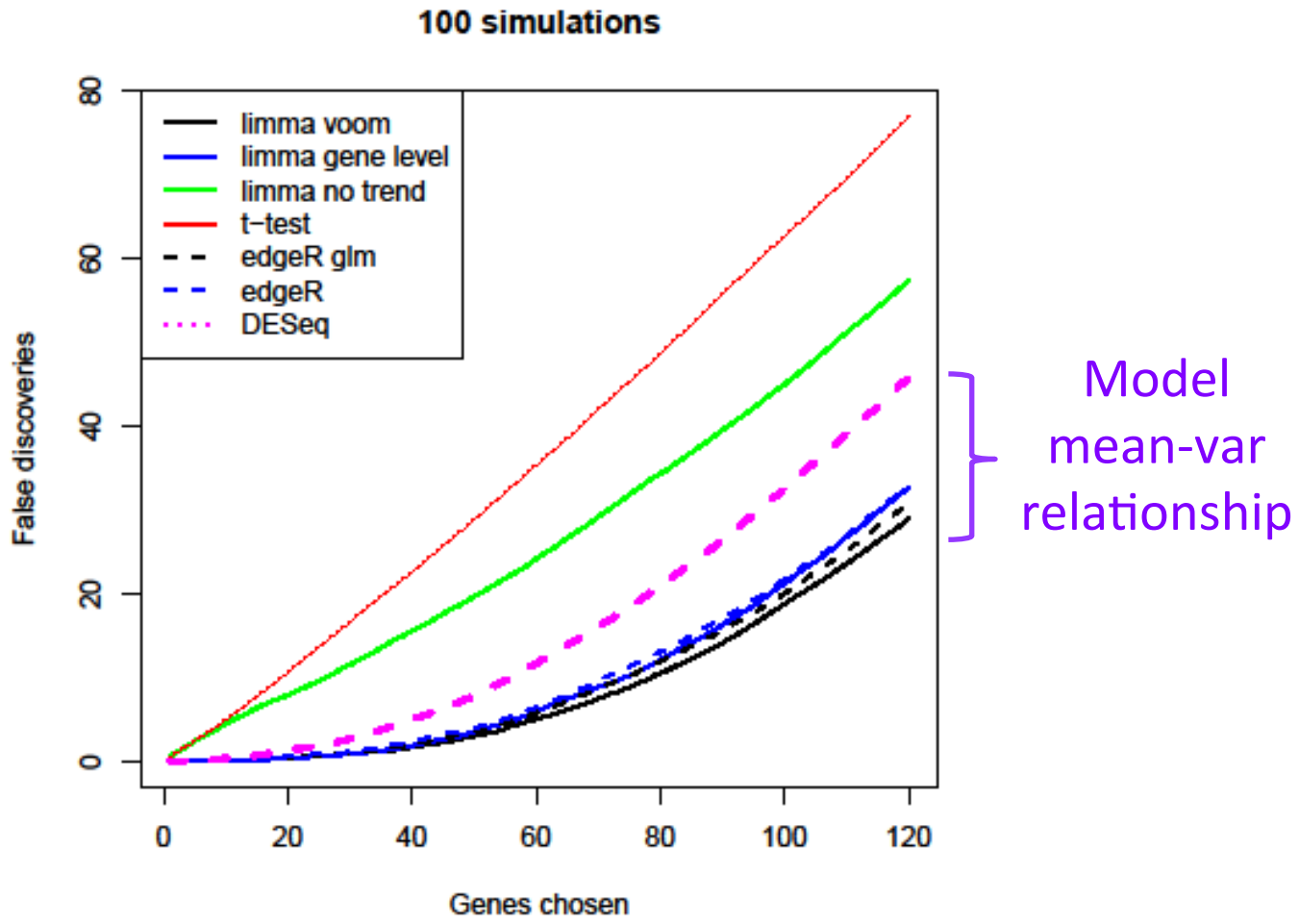
$$\text{var}\left(z_{gi}\right) = s(\mu_{gi})\sigma_g^2$$

Smooth function of mean

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



→ Use weights (1/var) in limma analysis



Law et al. 2014 Genome Biology – also a good candidate for journal club.