



Quick note on journal clubs

- Giving feedback to your fellow students is part of your JC mark!
- Feedback is due the following Tuesday (18.00; same as exercises); I recommend to do it synchronously
- Speakers: Post slides to #journal_club channel

How would you rate the presenters' knowledge of the topic? *

Poor
 Fair
 Good
 Very Good
 Excellent

How would you rate the presenters' organization of the subject matter? *

Poor
 Fair
 Good
 Very Good
 Excellent

STA 426 Journal Club feedback

* Required

Select the presentation: *

Choose

Please state your github username (n.b.: this must match the username listed in the markbot reports) *

Your answer

Please write a concise summary of what the article was about (preferably in 2 sentences or less). *

Your answer

Link pinned to #journal_club channel



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

- **Templates!**
- ROC curves
- log-ratios (data is already log-transformed!)



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

- **Templates!**

Exercise 5: ~10 students had no graphics in their documents.

The purpose of this exercise is to simulate some “microarray” data and explore how well different statistical methods perform in a small sample situation. This will introduce you to: i) simulation; ii) metrics to assess statistical power and iii) how to explore differential expression.

Specifically, we will create a synthetic dataset with replicates from 2 experimental conditions and explore how well different statistical summaries can distinguish between those “truly” differential and those not differentially expressed.

Library

Below is some R code to get you started.

```
#BiocManager::install("limma")
library("limma")
```

Warning: Paket 'limma' wurde unter R Version 4.4.2 erstellt

```
library("ggplot2")
```

Warning: Paket 'ggplot2' wurde unter R Version 4.4.3 erstellt

Parameters for the simulation

Next, we set some parameters for the simulation. Modify these to explore alternative situations.

```
nGenes <- 10000                      # number of "features"
nSamples <- 6                           # number of samples (split equal in 2 groups)
pDiff <- .1                            # percent of genes "differential"
grp <- rep(0:1,each=nSamples/2)         # dummy variable for exp. group
trueFC <- 2                            # log-fold-change of truly DE
```

do <- 2



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

- **Templates!**

Load packages

```
library("limma")
library("ggplot2")
library("ROCR")
library("affy")
```

```
Loading required package: BiocGenerics
```

```
Attaching package: 'BiocGenerics'
```

```
The following object is masked from 'package:limma':
```

```
plotMA
```

```
The following objects are masked from 'package:stats':
```

```
IQR, mad, sd, var, xtabs
```

```
The following objects are masked from 'package:base':
```

```
anyDuplicated, aperm, append, as.data.frame, basename, cbind,
colnames, dirname, do.call, duplicated, eval, evalq, Filter, Find,
get, grep, grepl, intersect, is.unsorted, lapply, Map, mapply,
match, mget, order, paste, pmax, pmax.int, pmin, pmin.int,
Position, rank, rbind, Reduce, rownames, sapply, setdiff, table,
```



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

the · [limma paper](#) · and the · [limma user's guide](#); the main details are also given in the lecture.

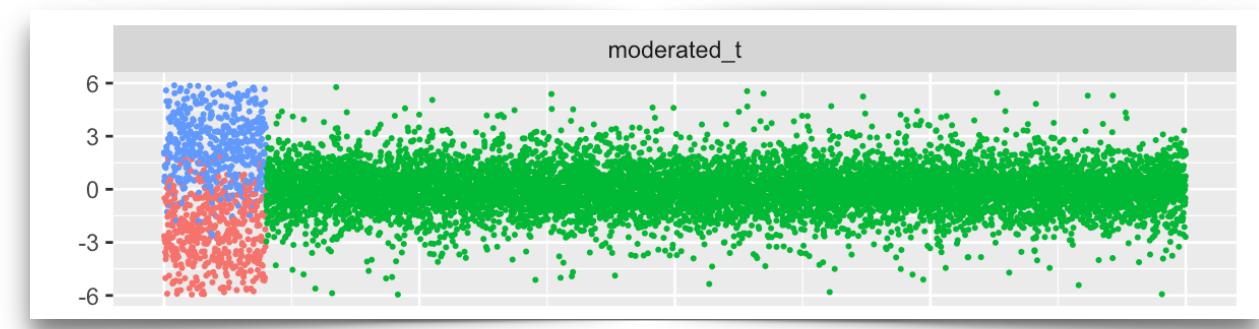
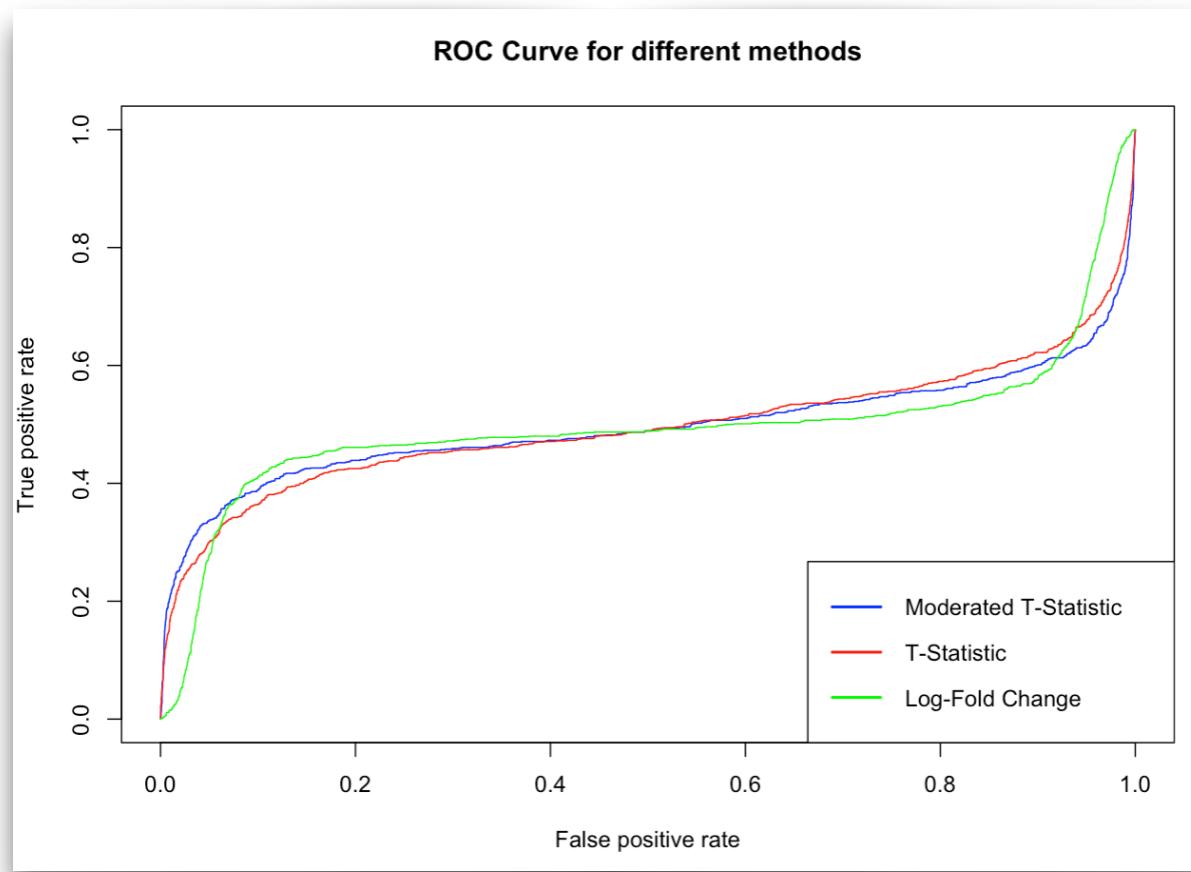
```
{r}
#| include: true
library("affy")
library("preprocessCore")
setwd("~/Documents/courses/STA426/STA426_excersises")
unzip("affy_estrogen.zip")
ddir <- "affy_estrogen"
dir(ddir)
```



I recommend that you don't do this;
Use the 'here' package instead

<https://www.tidyverse.org/blog/2017/12/workflow-vs-script/>
<https://here.r-lib.org/>

Notes on Exercise 3 (limma fundamentals) .. why the weird ROC curve?



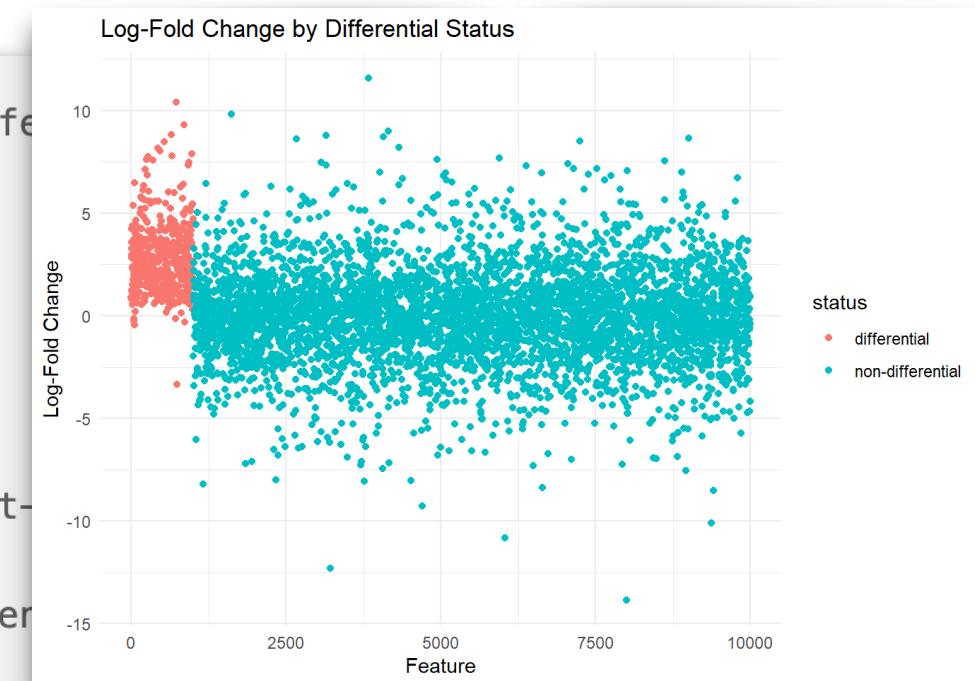
- Use **absolute value** of score .. or P-value

Notes on Exercise 5 (limma fundamentals) .. log-fold-changes

```
# Calculate t-statistics and log-fold changes for each row (feature)
for (i in 1:nrow(y)) {
  group0 <- y[i, grp == 0]                      # Samples in group 0
  group1 <- y[i, grp == 1]                      # Samples in group 1

  # Perform classical 2-sample t-test
  t_test_result <- t.test(group0, group1, var.equal = TRUE)
  t_stat_classical[i] <- t_test_result$statistic # Extract t-statistic

  # Calculate log-fold change (log2 of mean difference between groups)
  log_fc[i] <- log2(mean(group1) / mean(group0))
}
```





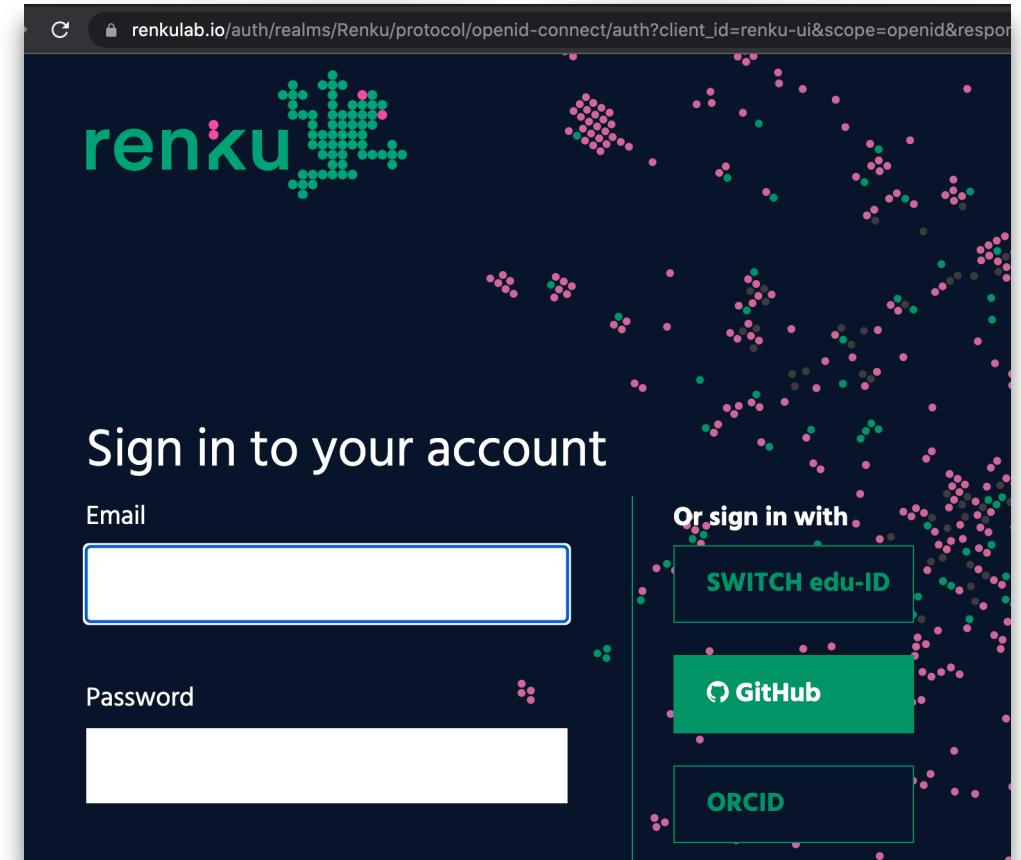
Expectations: **project**

- ~10-15 page report, with R code in line (e.g. **knitr** / **Rmarkdown**)
- Describe the biological setting, statistical analysis, exploratory analysis with publication-quality graphics embedded
- Three possibilities:
 - Comparison of statistical methods (simulation / reference data + metrics)
 - Reproduce an analysis from a paper from the raw data
 - Real collaborative project with FGCZ or a local laboratory
- Be strategic: work on something related to your interests!
- Typically due at end of first working week of January



Info for week 9: Docker!

- First of the hands-on sessions (JCs + short lecture + demonstration); it's recommended to bring a laptop and play along
- Instructions will follow; you will need to install docker on your laptop (8GB RAM + free disk space) or use the cloud (renkulab.io)



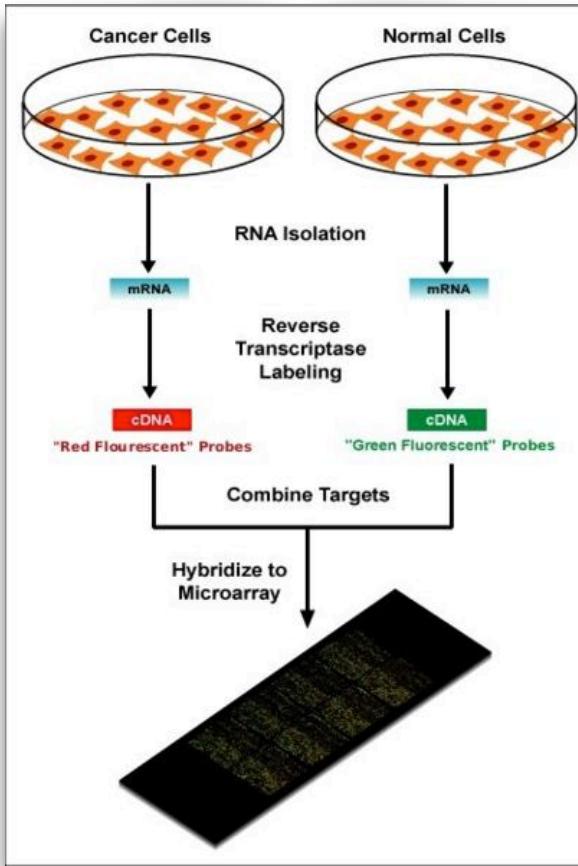


Statistical models for count data analysis (differential expression)

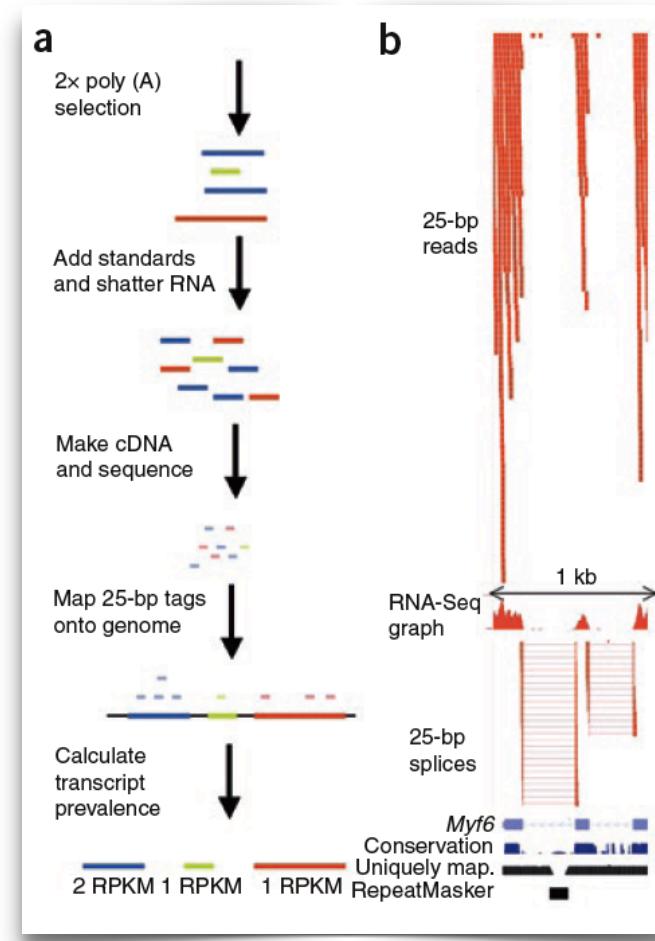
- simple counting (and new alternatives ..)
- edgeR, DESeq/DESeq2 —> why the negative binomial distribution?
- dispersion estimation and information sharing
- normalization considerations
- how about transformations of count data —> use limma for counts?

Statistical Bioinformatics // Institute of Molecular Life Sciences

Abundance by Fluorescence Intensity

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

Abundance by Counting

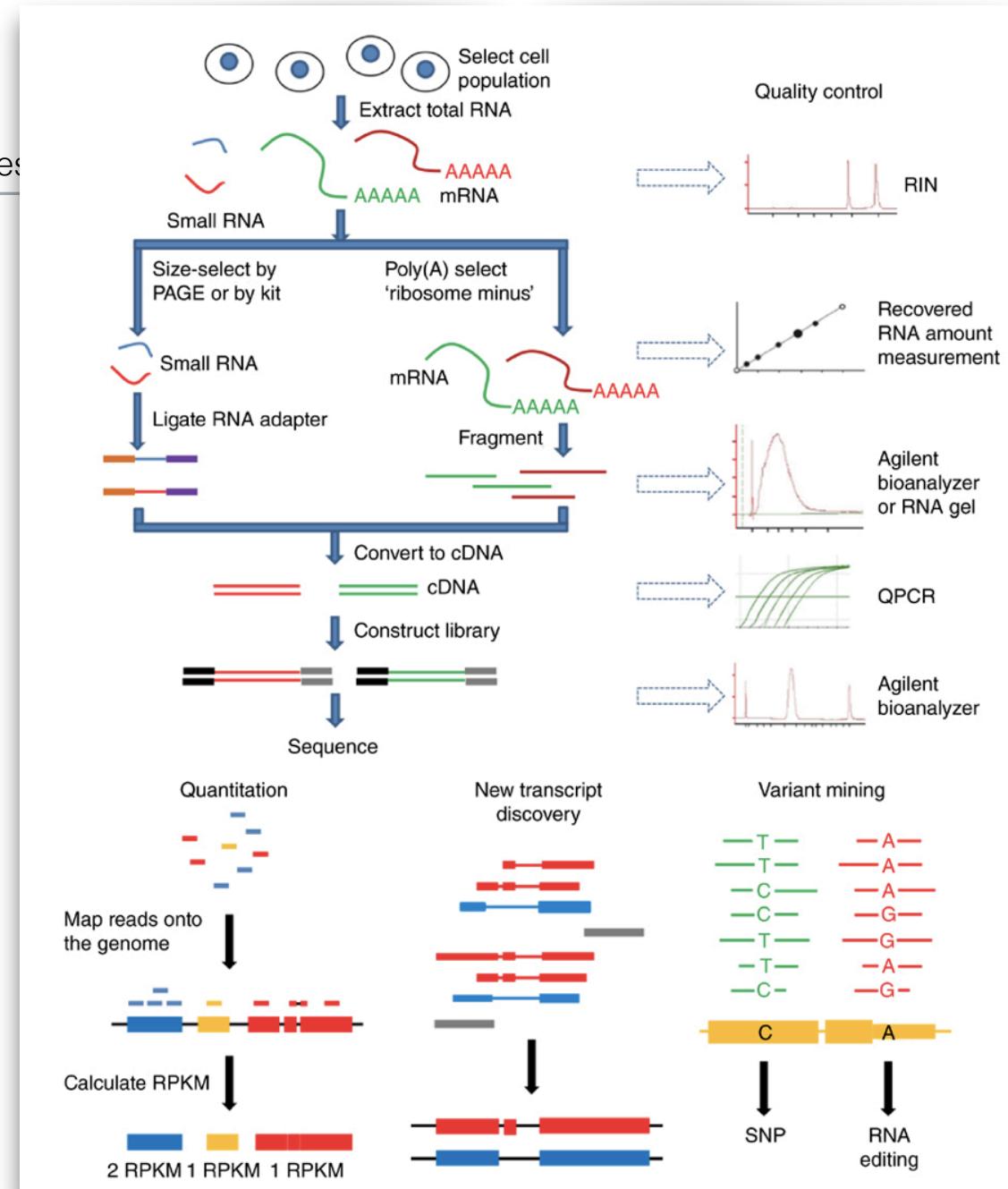


Mortazavi et al., Nature Methods, 2008



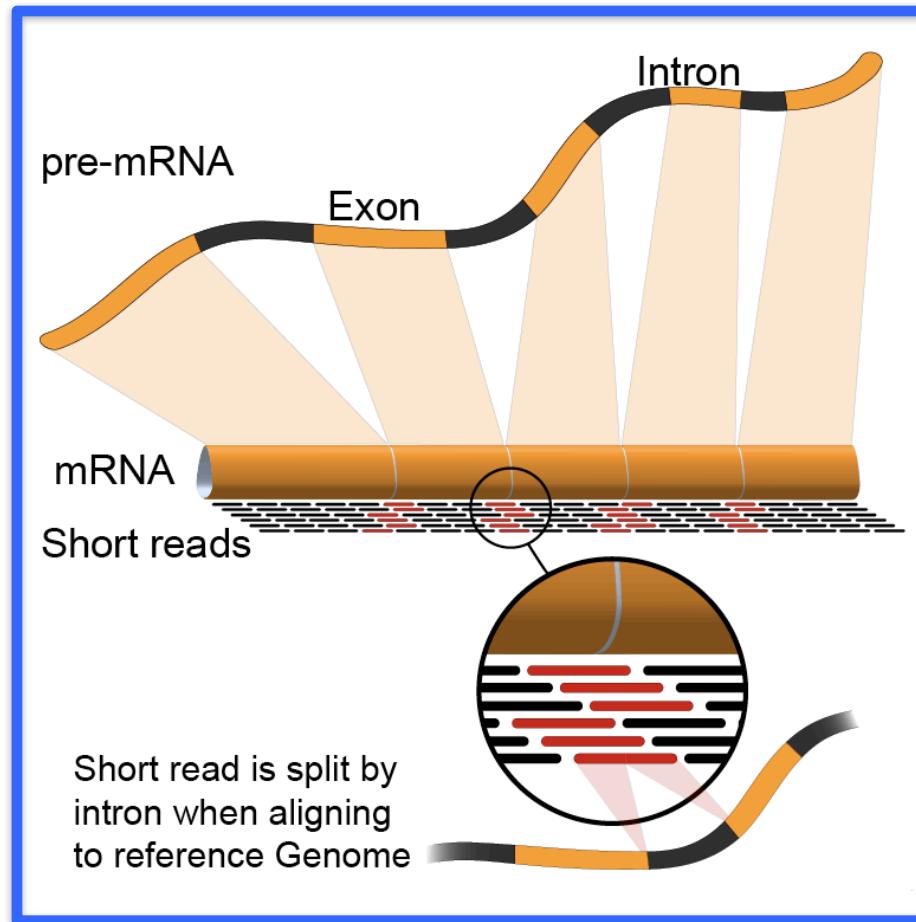
RNA-seq differential expression analyses

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

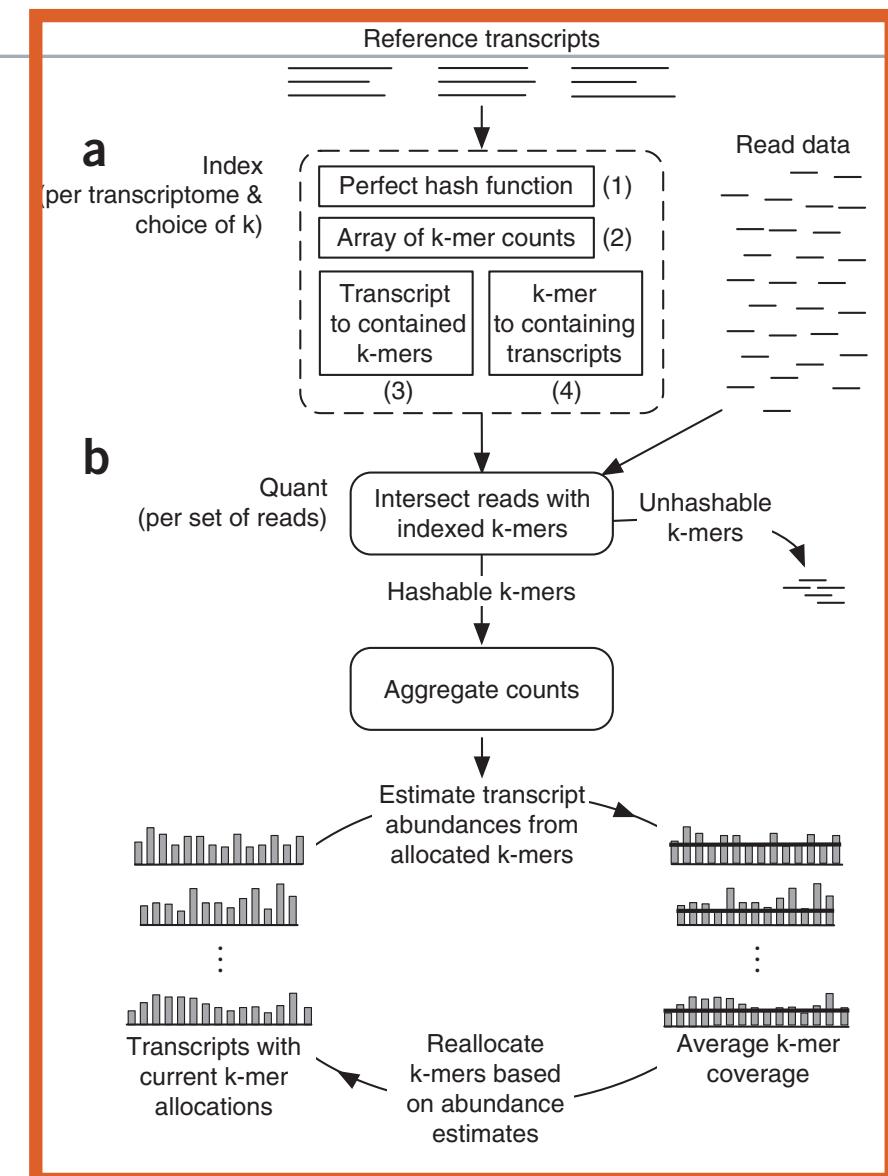


Alignment versus quasi-alignment

Statistical Bioinformatics // Institute of Molecular Life Sciences



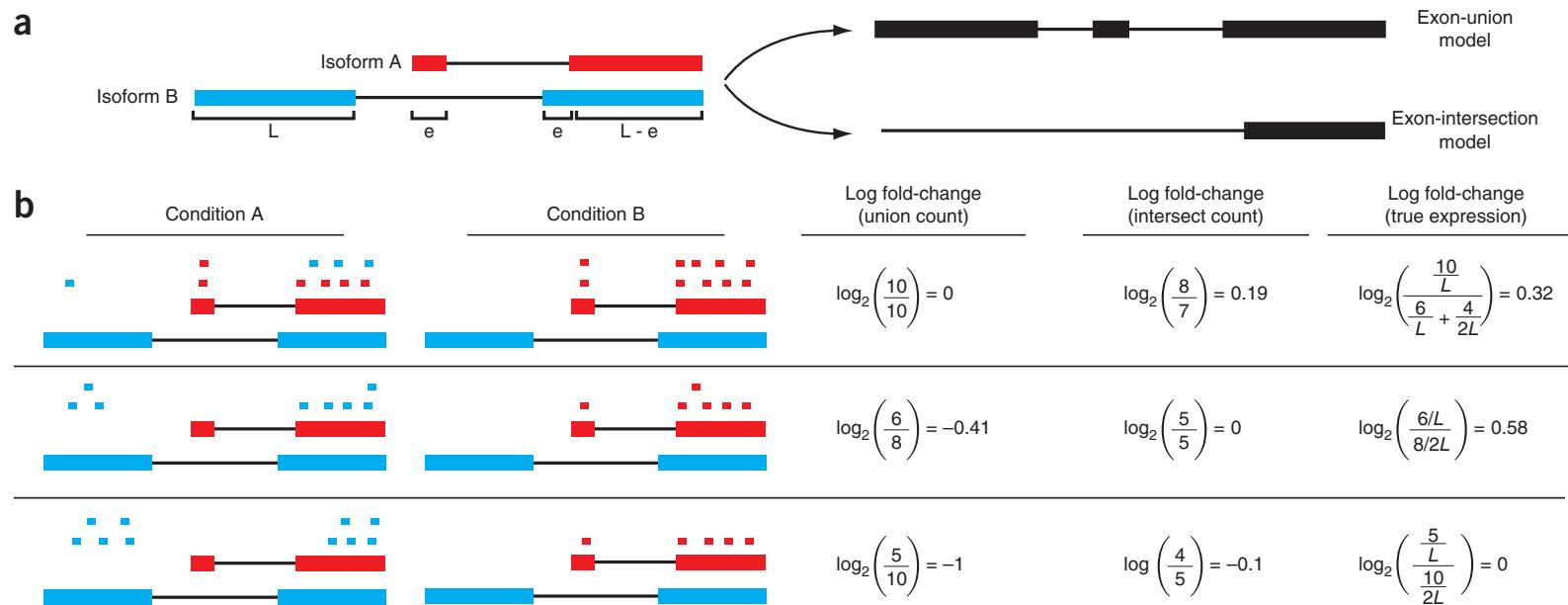
<https://en.wikipedia.org/wiki/RNA-Seq>



sailfish (Patro et al. 2014)

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

Trapnell et al. 2013 Nat Biotech



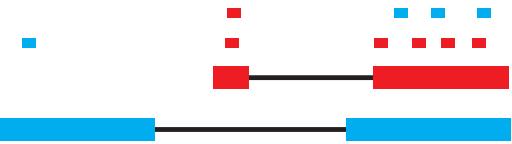
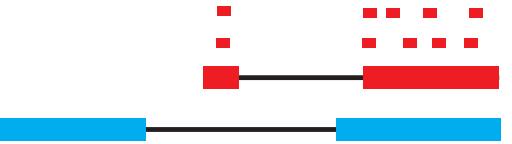
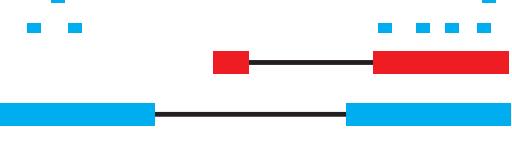
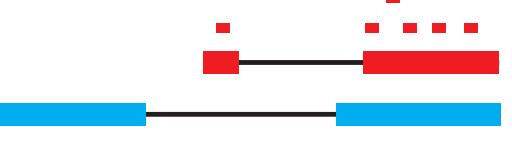
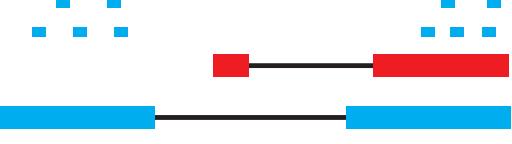
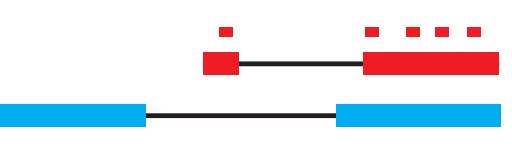
Transcriptome analysis of human tissues and cell lines reveals one dominant transcript per gene

Mar González-Porta¹, Adam Frankish², Johan Rung¹, Jennifer Harrow² 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)

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



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}

¹Institute for Molecular Life Sciences, University of Zurich, Zurich, 8057, Switzerland

²SIB Swiss Institute of Bioinformatics, University of Zurich, Zurich, 8057, Switzerland

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

⁴Department 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?

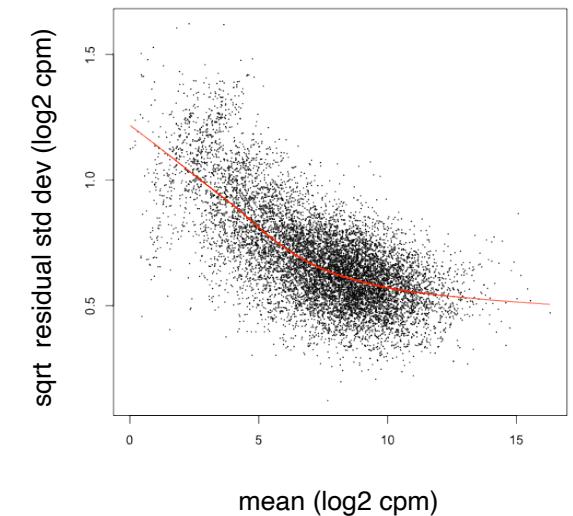
Count data is discrete, not continuous.

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

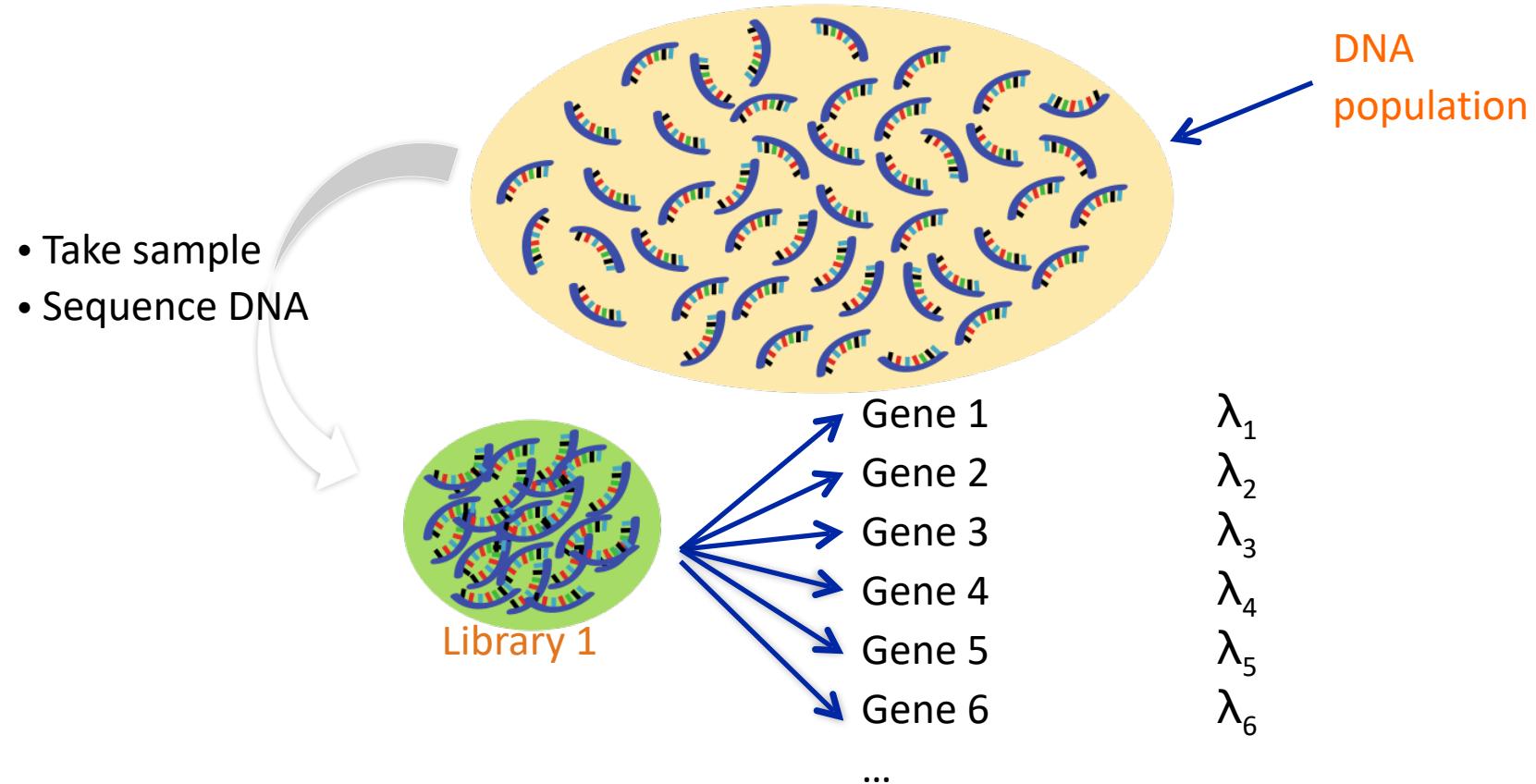
Two options:

Transform count data
and apply standard
methodology

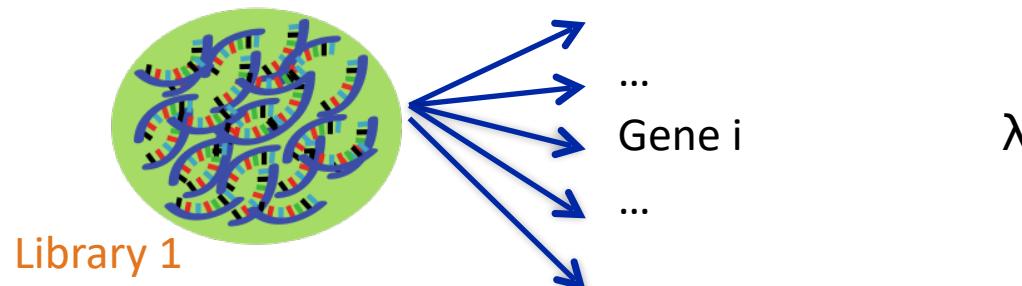
Analyze using
models for count
data



Sampling reads from population of DNA fragments is multinomial



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



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

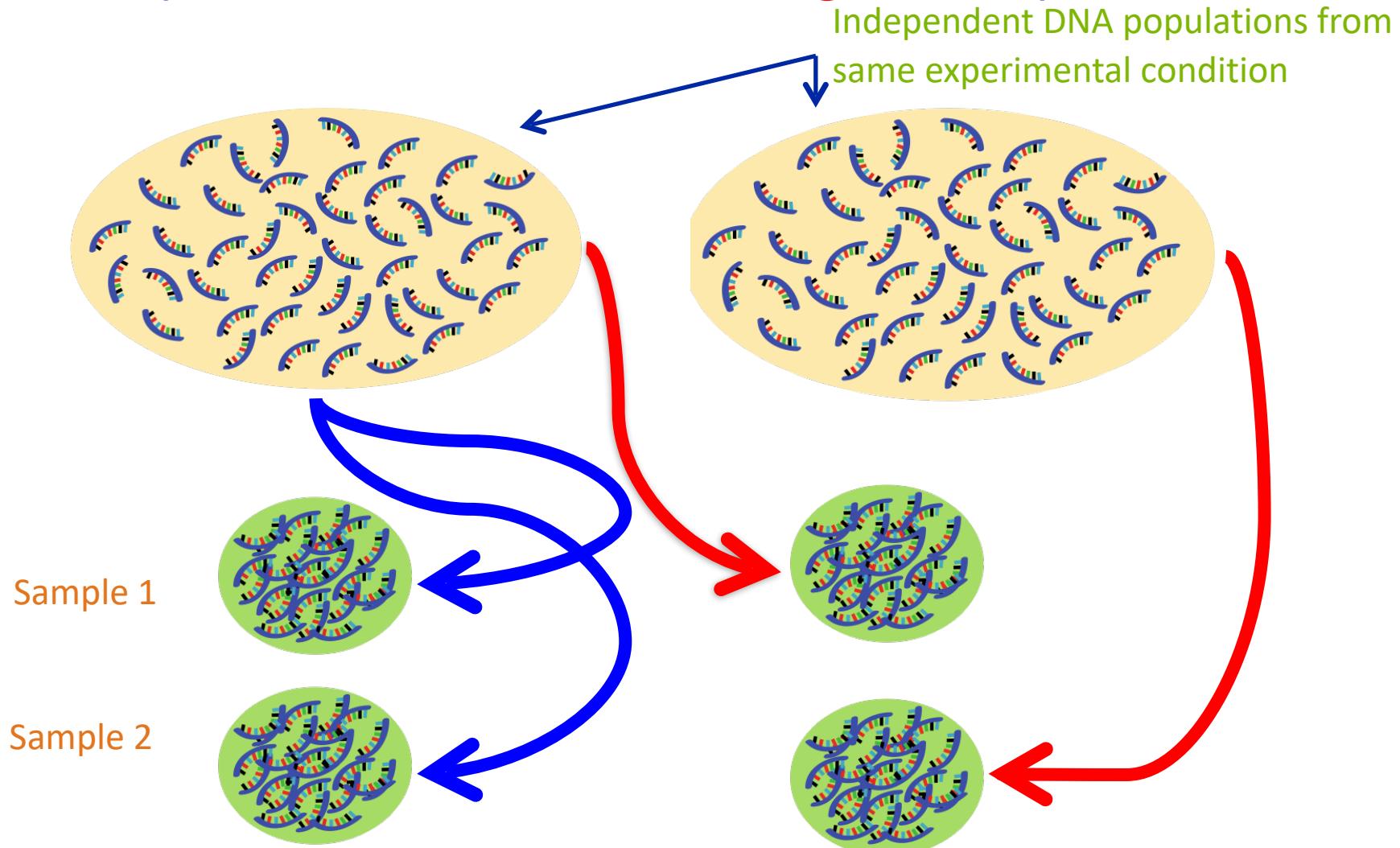
Y_i - observed number of reads for gene i

M - total number of sequences

λ_i - proportion

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

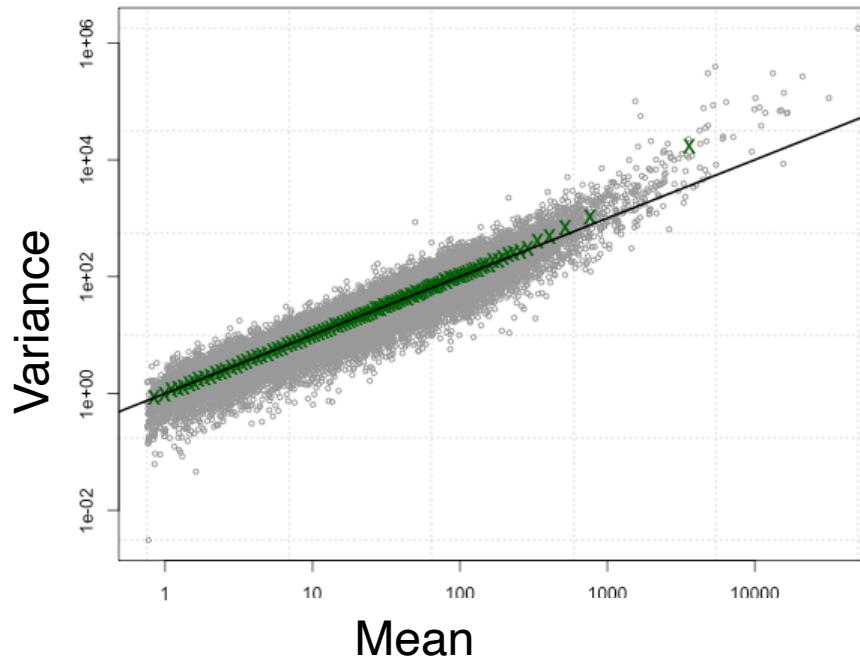
Technical replication versus biological replication





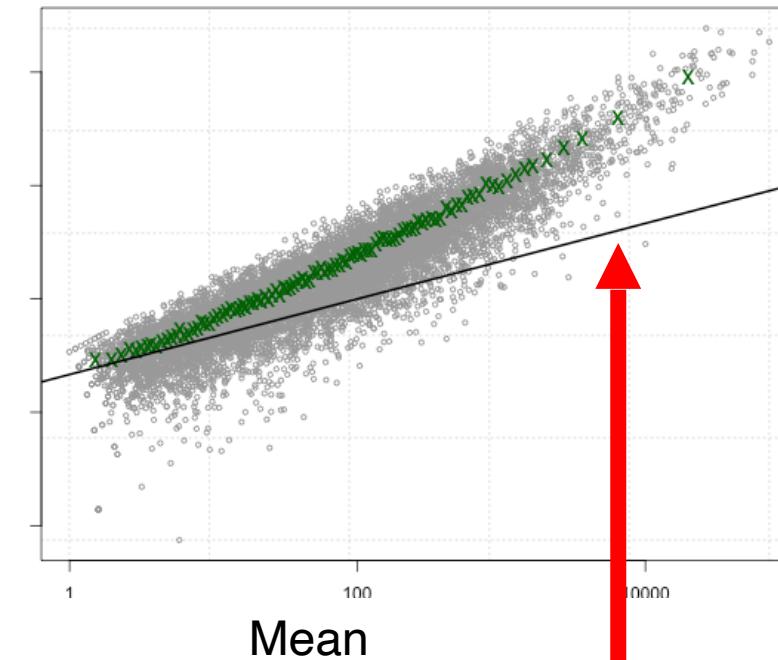
Mean-Variance plots: What we see in real data

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

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

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

(Coefficient of variation = standard deviation/mean)

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

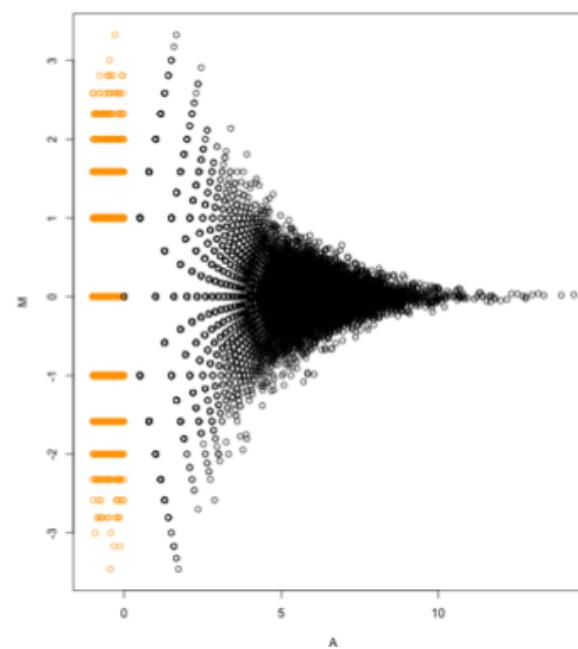
Dividing both sides by μ_{gi}^2 gives

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

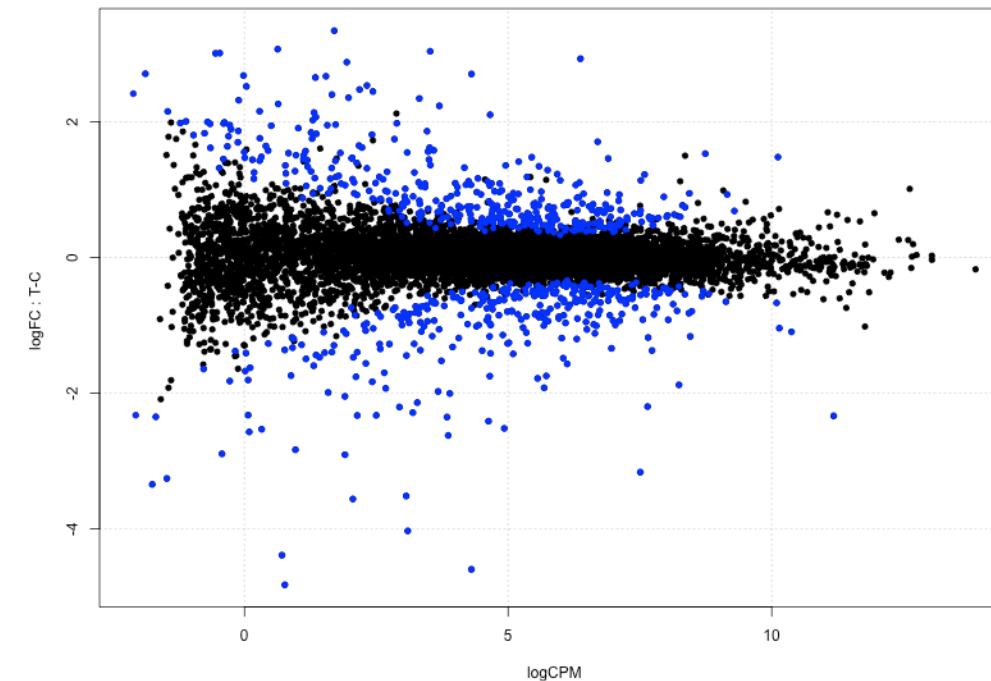
$$\text{CV}^2(\gamma_{gi}) = 1/\mu_{gi} + \phi_g.$$

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
(n.b. variance parameter moderated)

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

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

λ_i = relative contribution of gene i



Given the NB model: what is a good method to estimate parameters (especially dispersion)?

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

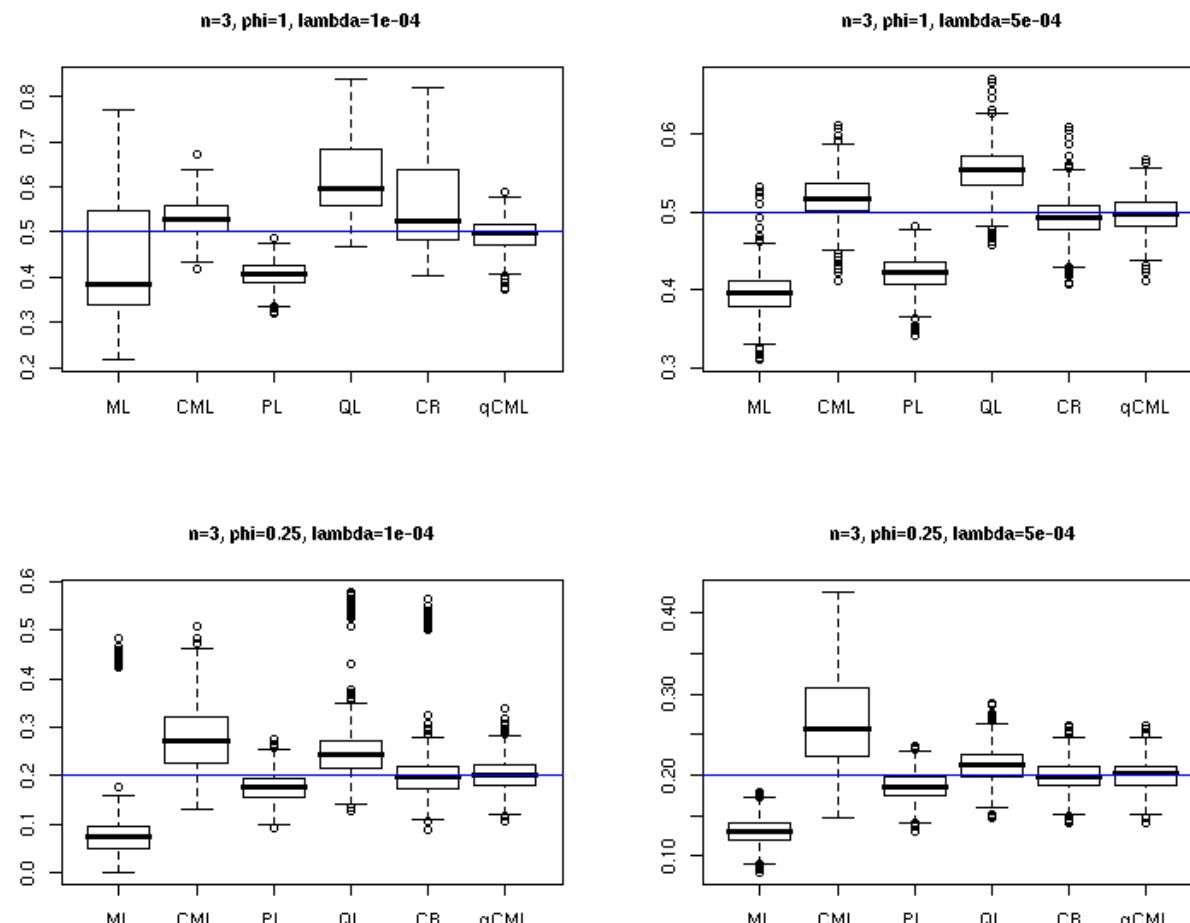
Thus, can form conditional likelihood:

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

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 —> 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:

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

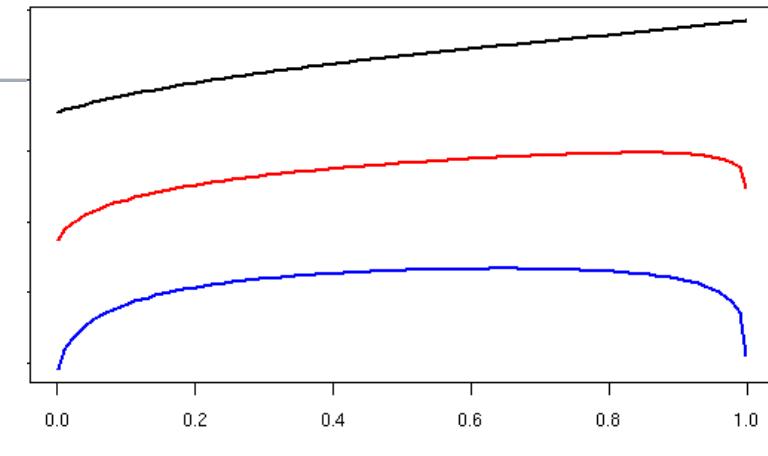
l_g - quantile-adjusted conditional likelihood

Black: single tag

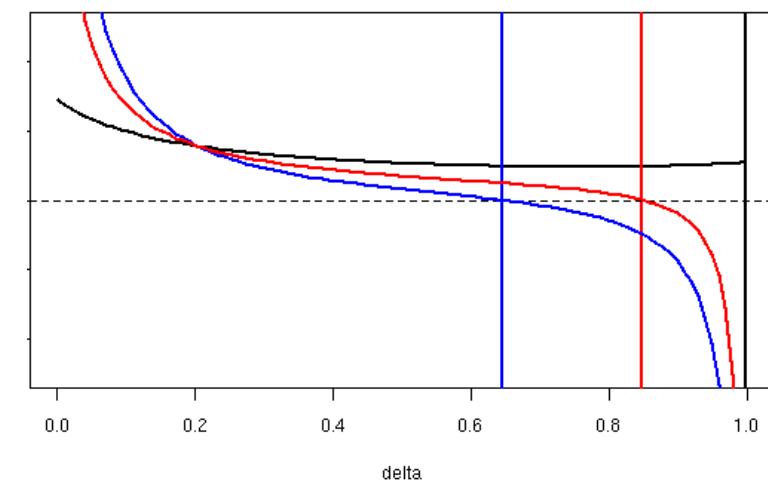
Blue: common dispersion

Red: Linear combination of the two

Log-Likelihood



Score (1st derivative of LL)



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

How much to shrink?

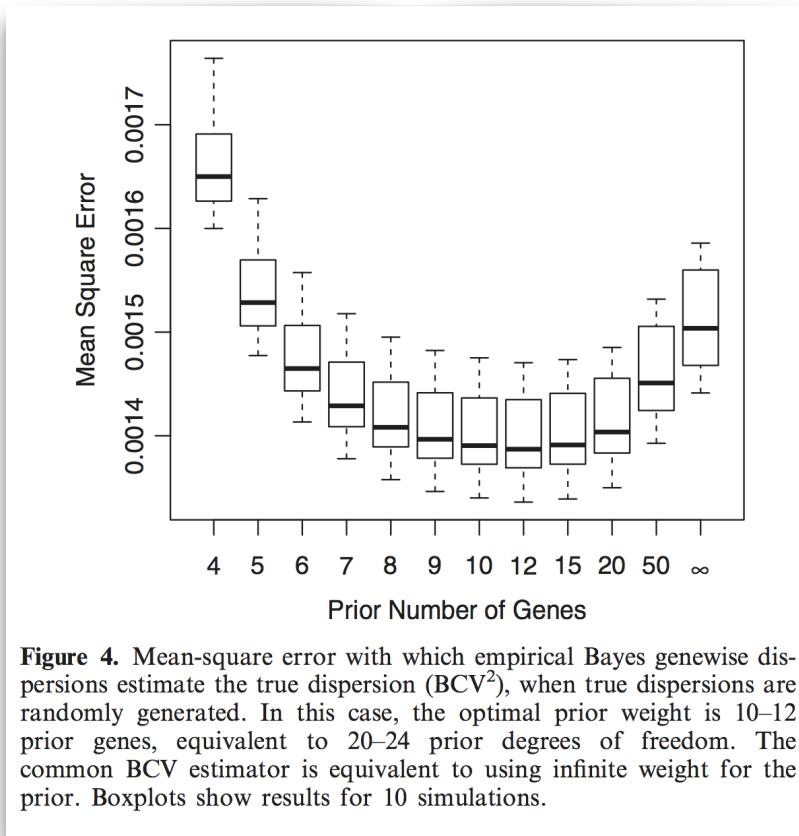


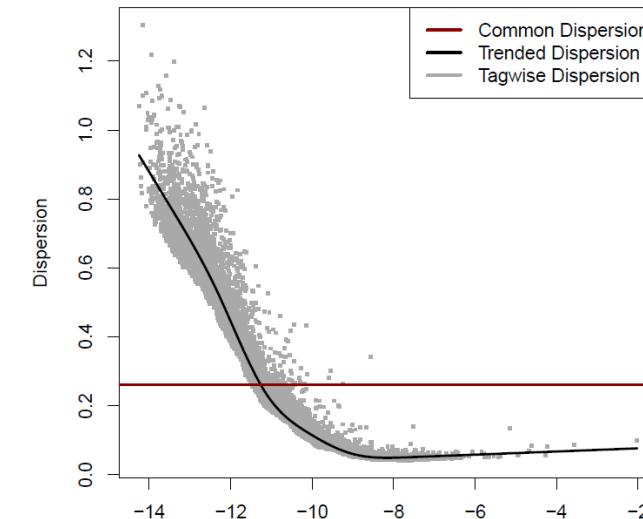
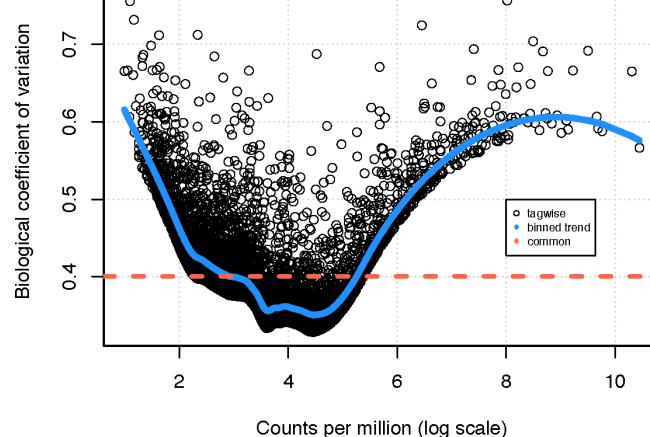
Figure 4. Mean-square error with which empirical Bayes genewise dispersions estimate the true dispersion (BCV^2), when true dispersions are randomly generated. In this case, the optimal prior weight is 10–12 prior genes, equivalent to 20–24 prior degrees of freedom. The common BCV estimator is equivalent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

Simulations suggest there is an optimal amount to shrink.

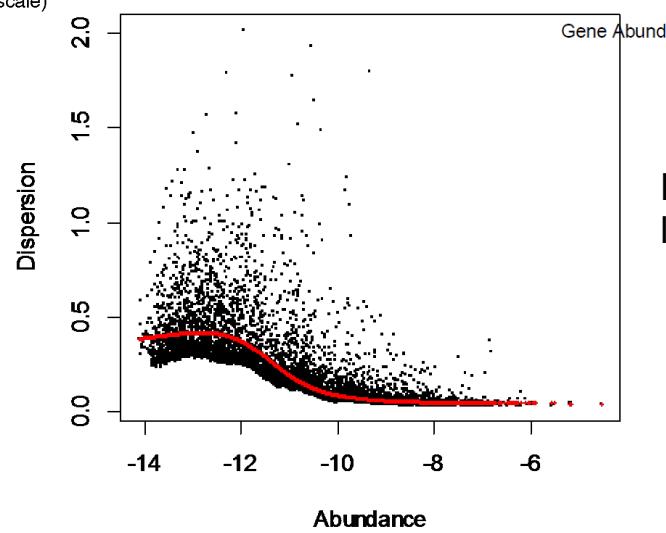
Challenge: choosing/estimating how much

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

Data:
Tuch et al.,
2008



Mouse hematopoietic
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

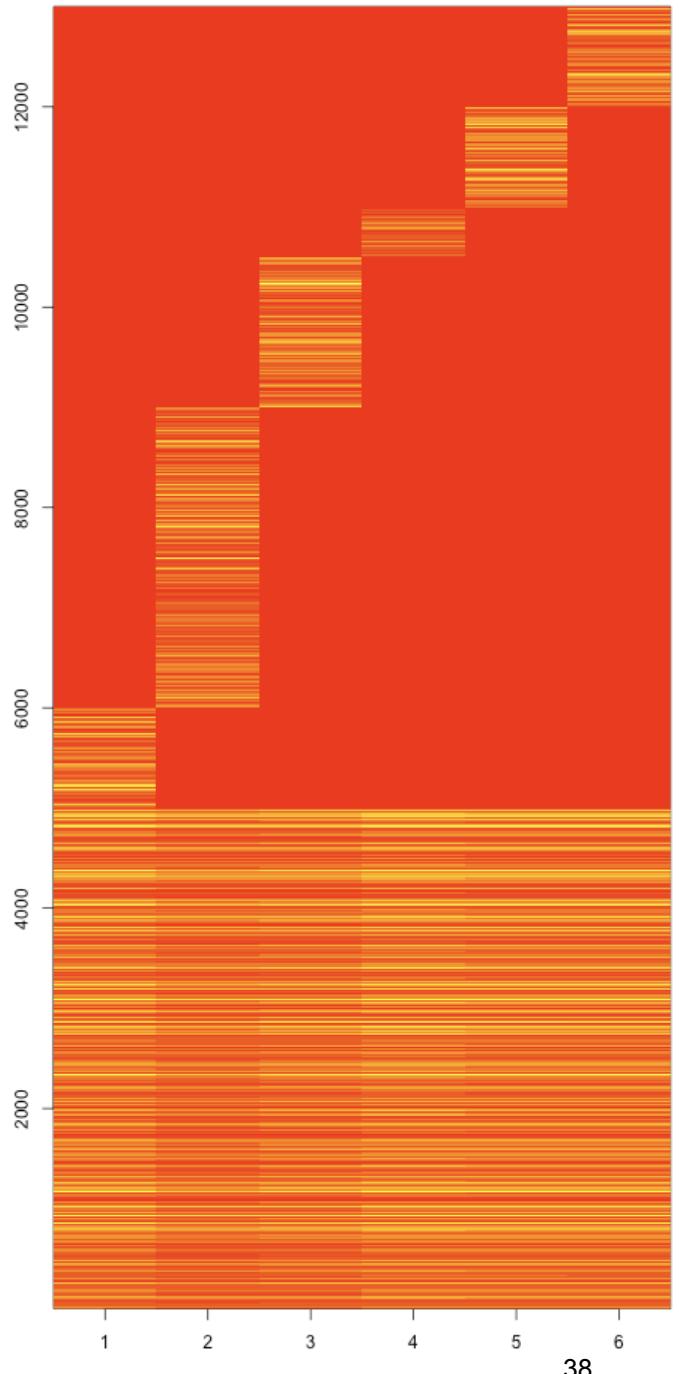
Zhong Wang, Mark Gerstein and Michael Snyder

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}.

Thought experiment: “Composition” or “Diversity” can affect read depth

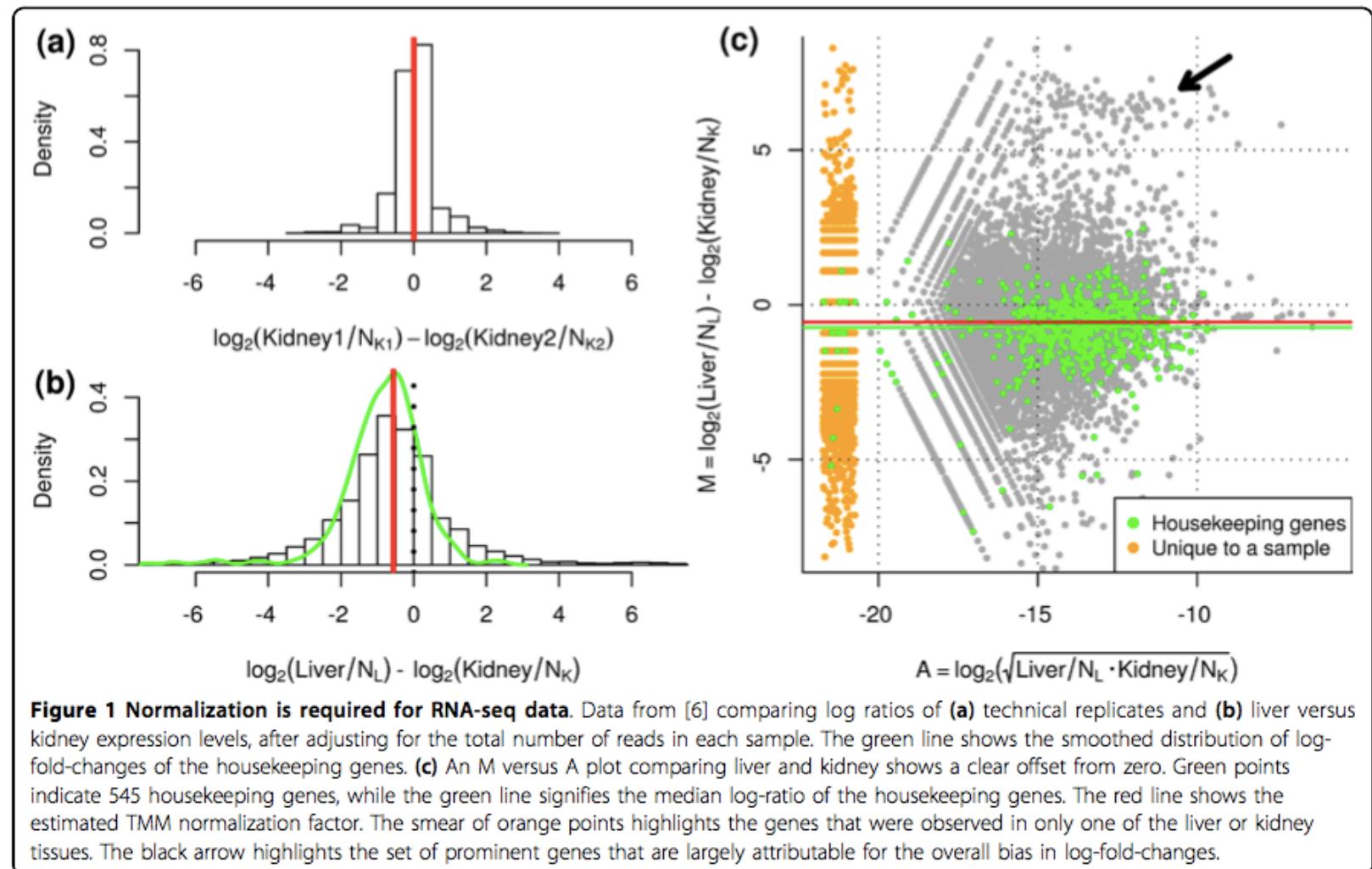
- Hypothetical example: Sequence 6 libraries to the same depth, with varying levels of unique-to-sample counts
- Read depth is affected not only by expression (and length), but also expression levels of other genes
- Composition can induce (sometimes significant) differences in counts

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); size factors (DESeq2)
- Use additional offset (GLM)

Note: count data is not modified



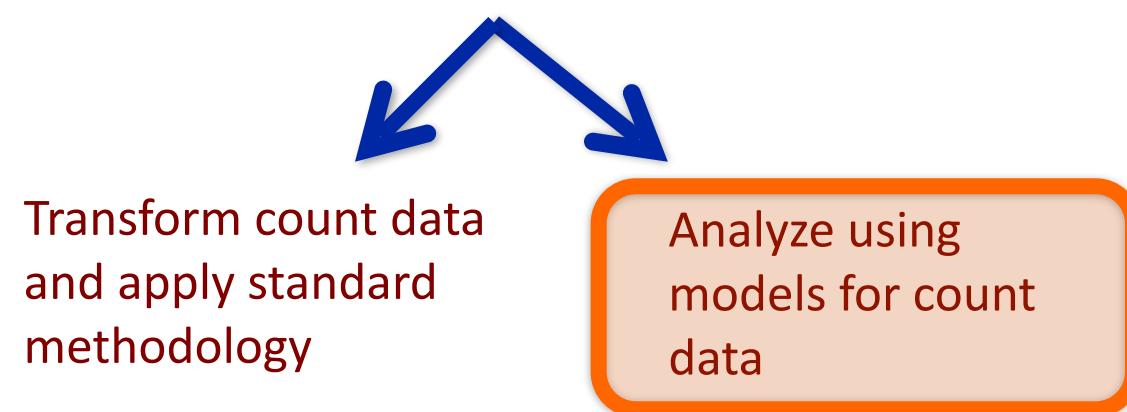
Differential expression: why not use methods developed for microarrays?

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:

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

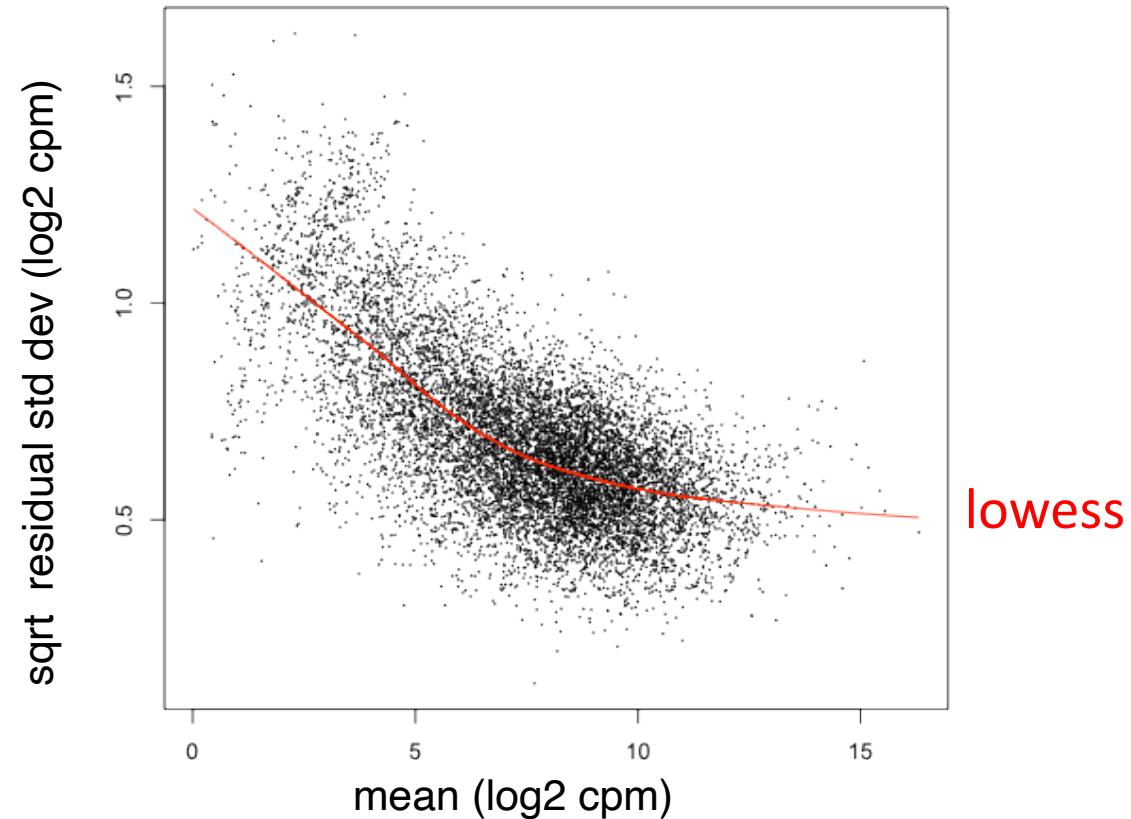
$$\text{var}(z_{gi}) = s(\mu_{gi}) \sigma_g^2$$



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**

