RNA-seq II: Differential expression

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STAT/BIOF/GSAT 540 2017

Recap

- RNA-seq data generation
- Preprocessing and QC
- Quantification
- Normalization

Today

- Look more closely at real data
- Motivation for new differential expression methods
- Weighted regression approach (‘limma-voom’)
- Methods specific for count data (EdgeR and DESeq)

Properties of data sets

Study	PMID	Species	Samples	UniqueAlignedReads	ReadsPerSample	Notes
bodymap	22496456	human	19	2,197,622,796	115,664,358	Illumina Human BodyMap 2.0 -- tissue comparison
modencodefly	21179090	fly	30	2,278,788,557	75,959,619	developmental time course
modencodeworm	19181841	worm	46	1,451,119,823	31,546,083	developmental time course
yang	20363980	mouse	1	27,883,862	27,883,862	hybrid cell line, X always inactive
trapnell	20436464	mouse	4	111,376,152	27,844,038	time course
mortazavi	18516045	mouse	3	61,732,881	20,577,627	tissue comparison
cheung	20856902	human	41	834,584,950	20,355,730	HapMap - CEU
hammer	20452967	rat	8	158,178,477	19,772,310	experimental vs. control at 2 time points
bottomly	21455293	mouse	21	343,445,340	16,354,540	2 inbred mouse strains
montgomery+pickrell	20220756	human	60	886,468,054	14,774,468	HapMap - CEU+YRI
wang	18978772	human	22	223,929,919	10,178,633	tissue comparison
gilad	20009012	human	6	41,356,738	6,892,790	liver; males and femlaes
core	19056941	human	2	8,670,342	4,335,171	lung fibroblasts
katz.mouse	21057496	mouse	4	14,368,471	3,592,118	control vs. CUG-BP1 knockdown myoblasts
nagalakshmi	18451266	yeast	4	7,688,602	1,922,151	priming technique comparison
sultan	18599741	human	4	6,573,643	1,643,411	cell type comparison

Modified from http://bowtie-bio.sourceforge.net/recount/; some additions since I made this table

Case study: The gilad data set

Letter

Sex-specific and lineage-specific alternative splicing in primates

Ran Blekhman,[1,4,5] John C. Marioni,[1,4,5] Paul Zumbo,[2] Matthew Stephens,[1,3,5] and Yoav Gilad[1,5]

[1]Department of Human Genetics, University of Chicago, Chicago, Illinois 60637, USA; [2]Keck Biotechnology Laboratory, New Haven, Connecticut 06511, USA; [3]Department of Statistics, University of Chicago, Chicago, Illinois 60637, USA

Genome Res. 2010 Feb;20(2):180-9

- Six human liver samples (3M 3F)
  - Also chimp and macaque, but will not discuss here
- Illumina GAII, two lanes per sample. 35bp
- 13,000 genes detected according to authors
- 627 genes reported as “sexually dimorphic” commonly in all three species.

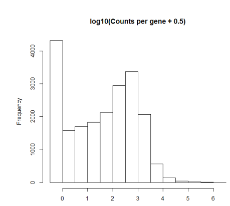
What I got via their supplement table 1: 20689 x 6 matrix with Ensembl gene IDs. (different version available through bowtie web site)

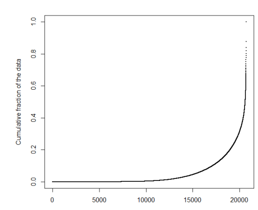
Gilad data set, cont’d

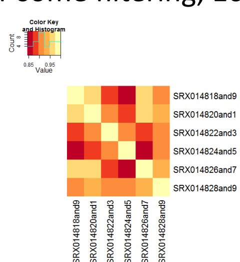
- Total read count: 20,679,864 (2.8 – 4.3 million per sample, mean=3.4 million)
- 4314 genes have 0 counts (total in 6 samples)
- 7599 have less than 10 counts total
- 196 genes have over 10000

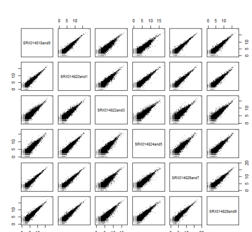
  → 11,527,345 counts for those genes (56%)

  Albumin (12%); complement, Jun, fibrinogen, serpins, APOs
- After some filtering, 10,720 genes.



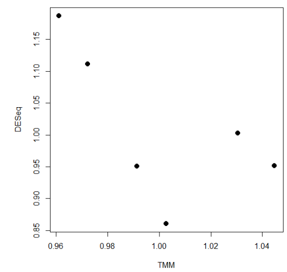






Scale factors for the Gilad data set

lib.size	TMM	DESeq	TMM, unfilt.	DESeq, unfilt.
2096011	1.031	1.002	0.99	1.00
2072827	0.991	0.951	0.92	0.95
1968729	1.045	0.951	1.15	0.963
1862868	1.003	0.866	1.02	0.858
2673491	0.961	1.18	0.92	1.185
2476156	0.972	1.11	0.99	1.115



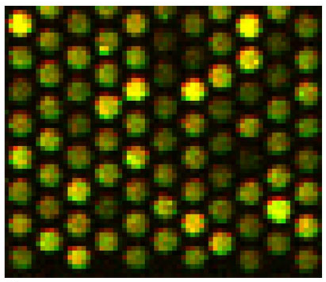
Differential expression: Why we might need new methods

- **Goal**: accurate p-values for our hypothesis tests
- Properties relied upon for inference from *t* statistics shouldn't hold for count data.
- Perhaps most important: Heteroscedasticity
  - Strong mean-variance relationship *expected* with count data.

Properties of expression data: counts

Microarray

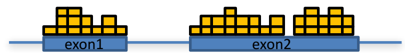
- Signal *is* fundamentally counts (deep down)
- But values are averaged across pixels and counts are high.
- Never really have zero: background ensures that values are not too small and thus "continuous"



http://www.genomics.agilent.com

Sequencing

- Unit of measurement is the read; no such thing as 0.1 read.
- Counts of reads start at 0
- As counts get high, the distinction should diminish



NOTE: We are focused on the distribution of expression values for a gene **across technical or biological replicates**
For this discussion we care less about comparing two genes **within a sample**.

Statistics of counts

- Say RNA for gene g is present "in the cell" at 1 out of 1,000,000 molecules.
  - Abundance $a = 1/1{,}000{,}000$ (1e-6)
- If we randomly pick $R_{lib} = 1{,}000{,}000$ molecules ("reads"), how many gene g RNAs will we see? (R_g)

E(R_g | R_{lib}) = <u>?</u>. But could get 0 or 5 "by chance".

$\rightarrow R_g \sim Binomial(R_{lib}, a)$
Approximately: $R_g \sim Poisson\ (R_{lib} * a)$
As $R_{lib} * a$ gets large, approx: $R_g \sim$Normal($R_{lib} * a$,$R_{lib} * a$)

In all cases, variance is an increasing function of the mean

Options for doing differential expression on counts

Summary of the problem: Count data is expected to violate both normality and equal variance assumptions.

Possibilities for coping:

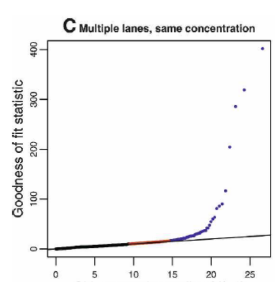
- Use a non-parametric test (e.g. SAM-seq – based on Wilcoxon; larger sample sizes needed, will not discuss further)
- Make adjustments and use standard methodology
- Use a model specific for count data

Some material from Mark Robinson (http://www.fgcz.ch/education/StatMethodsExpression/03_Count_data_analysis.pdf)

Poisson is appropriate for tech rep

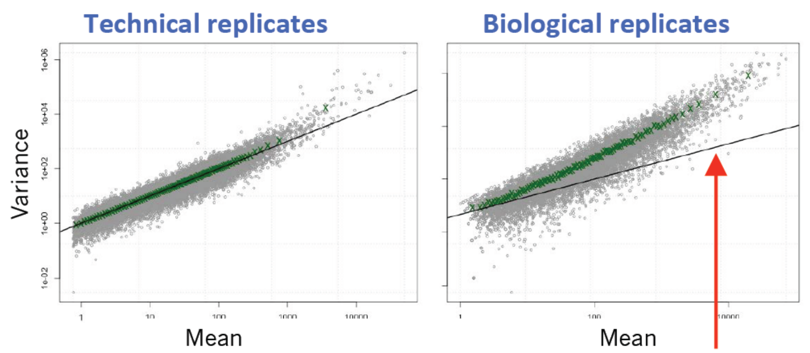
(Marioni et al.)

- Looked for "systematic differences between results for the same sample, sequenced at the same concentration in different lanes, over and above those expected from sampling error"
- Differences reasonably well explained by Poisson statistics, but does not account for biological variation (back to this later)



http://genome.cshlp.org/content/18/9/1509.long

Poisson does not capture biological variability



Data from Marioni et al. Genome Research 2008

Data from Parikh et al. *Genome Biology* 2010

http://www.fgcz.ch/education/StatMethodsExpression/03_Count_data_analysis.pdf

Impact of heteroscedasticity

- OLS: assume all errors have same variance
- If not true: higher variance regions get more weight in minimization of error than they should (since they are less precise)

Standard errors of betas will be poor estimates

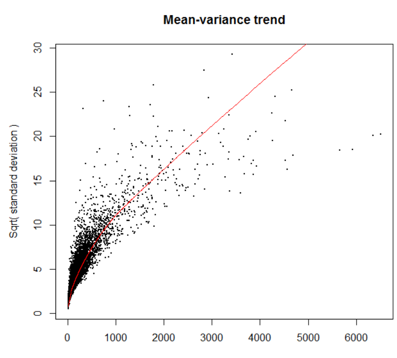
Recall: $t = \hat{\beta}/\hat{\sigma}$

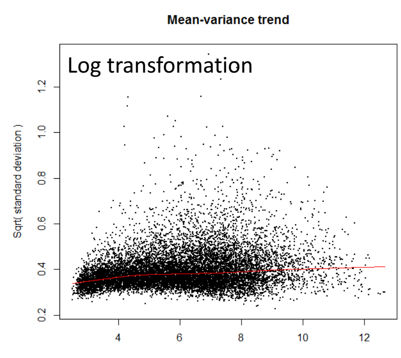
… So p-values will also be wrong; In case of positive relationship, too small.

Transformation can help

- log, square root, …
- For microarray data, taking logs is often deemed sufficient (but see "VSN" and other methods)
- None of these seem to adequately remove the trends in RNA-seq data

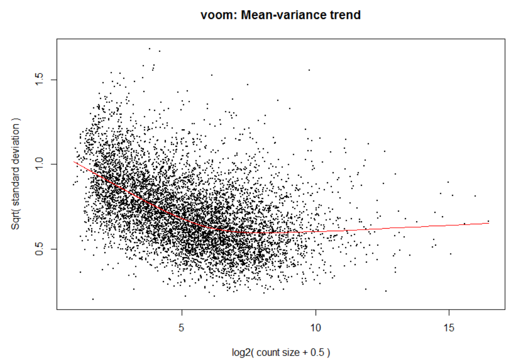
Behaviour of **microarray** data ("photoreceptor" data set)





One point = one gene

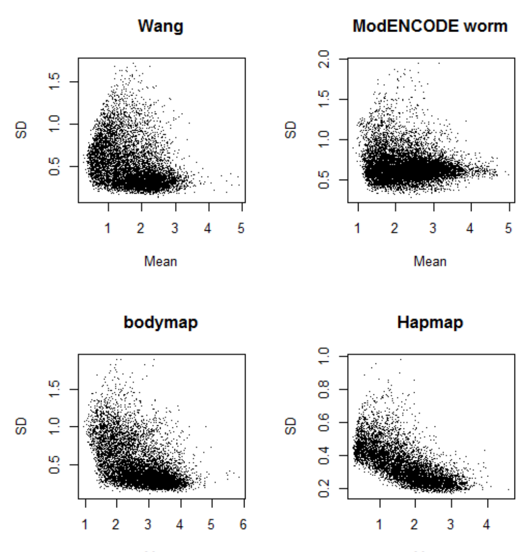
Trend for the 'gilad' data set



Typical for RNA-seq: Log improves but "overcorrects" so now low expression has excess variance; Mean-variance relation is steepest for low log expression. Impact on inference is largest at low expression levels.

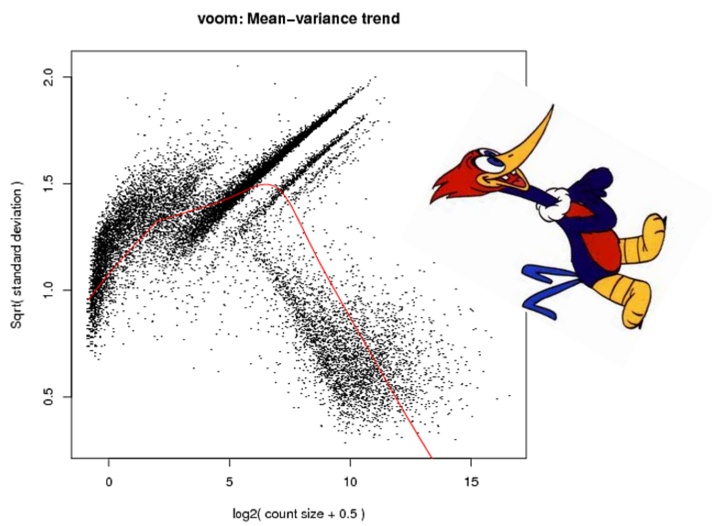
Law et al. (*Genome Biology* 2014, **15**:R29 2014) explain this: biological variability dominates at higher counts, technical (sampling) variability at lower counts.

M-V menagerie



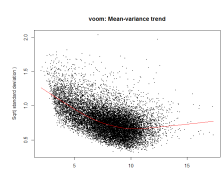
Counts obtained from Bowtie Recount
Data filtered to remove very lowly expressed genes;
$\log_{10}$ transformed.

And then there's this:



http://stats.stackexchange.com/questions/29895/r-limma-voom-function-mean-variance-trend

Voom



Transformation approach to allow use of limma.

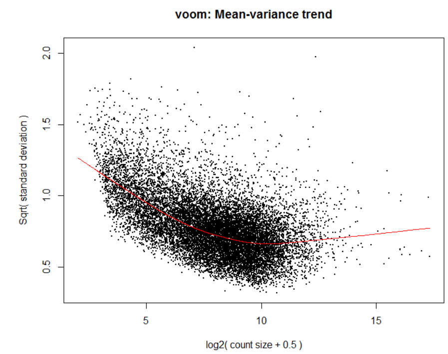
Key idea: Modeling the mean-variance relation is more important than getting the probability distribution exactly right.

Work with log2 counts per million (log-cpm)

Genome Biology 2014, **15**:R29

Rationales

- Why log transform: improves the mean-variance relationship but tends to "over-correct" so now low values are more variable than high values.
- Why quarter-root variance? Makes distribution more symmetric



Voom

"Voom is an acronym for 'mean-variance modelling at the observational level'"

1. Fit your linear model to the data ($\log_2$-transformed cpm)
2. Take the residuals. Their sqrt-stdev (quarter-root variance) per gene usually has a reasonable relationship with the mean; That is, consider

$$\hat{\mu} \sim \sqrt{\widehat{sd}(\varepsilon)}$$

3. Fit a lowess smoother to this relationship (red line in plots)
4. Use the lowess to estimate the variance for each (fitted): get weights

$$w_i = 1/\text{lowessfit}(\widehat{c}_i)^4$$

where $\widehat{c}_i$ the $\log_2$-transformed fitted cpm and lowessfit() provides the predicted sqrt-stdev.

Intuition: points where we are less sure of the actual value (higher variance) get lower weight in the analysis.

Why regress out the model first: Think of it as an iterative process. The first estimate of residuals will be "improved" by the weights computed. Those weights would be very poor estimates if the differential expression is large.

Getting observation-level estimates of variance

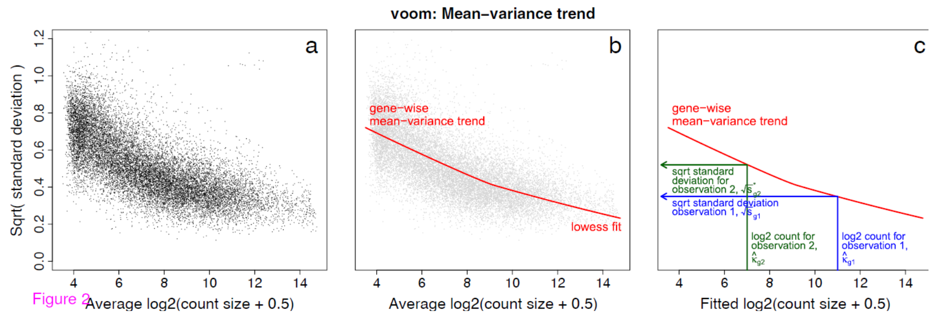


Figure 2 Voom mean-variance modeling. Panel **(a)**, gene-wise square-root residual standard deviations are plotted against average log-count. Panel **(b)**, a functional relationship between gene-wise means and variances is given by a robust lowess fit to the points. Panel **(c)**, the mean-variance trend enables each observation to map to a square-root standard deviation value using its fitted value for log-count.

Genome Biology 2014, **15**:R29

Weighted regression

R & Limma already supported weighted regression, so what it is?

Usual normal equations are

$$\hat{\beta} = (X^T X)^{-1} X^T y$$

Modified to use weights:

$$\hat{\beta} = (X^T W X)^{-1} X^T W y$$

Where W is a diagonal matrix

Intuition: In minimizing the residual, we want to “care less” about data points which are less precise.

$$argmin(\,\hat{\beta}\,) \sum_{i}^{n} w_i (X_i^T \hat{\beta} - y_i)^2$$

Thus the weights are expressed in terms of 1/variance.
Hard part is estimating the variance (we end up treating it as “known”)
But if values are right, assumptions of linear least squares are restored.

More about voom approach

- It does not modify the data. It only modifies the results of the lmFit call: the $\hat{\beta}$ values
- Residual standard error estimates are now (hopefully) better
- limma will further squeeze those:

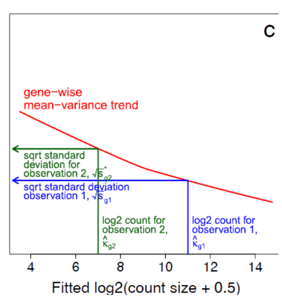
global across all genes, indirect evidence

"raw", gene-specific, direct evidence

$$\text{hybrid} \rightarrow \tilde{s}_g^2 = \frac{d_0 s_0^2 + d_g s_g^2}{d_0 + d_g}$$

Nuances for Voom…

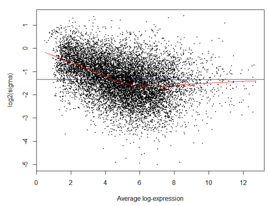
- If M-V relation is flat, it has no effect (but shouldn't hurt; weights in voom will be all equal)
- Small fold-changes only explore a small portion of the M-V distribution, so effects might be minimized; most dramatic for low expression



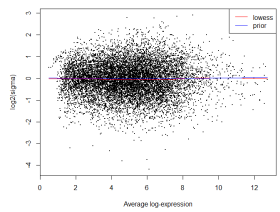
M-V plots from limma (gilad data set)

```
plotSA()
```

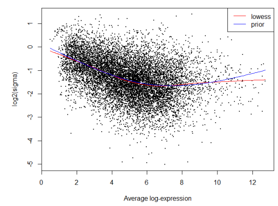
No voom



voom



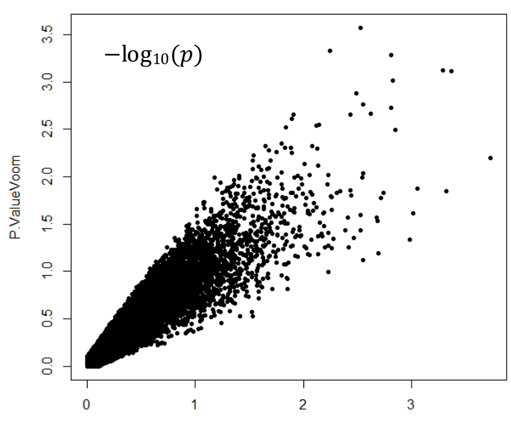
No voom, eBayes(…, trend=TRUE)



eBayes(…, trend=TRUE) should make the prior sensitive to the estimated m-v relationship.

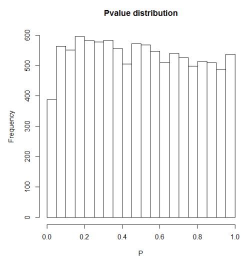
See the voom paper for details; voom worked better

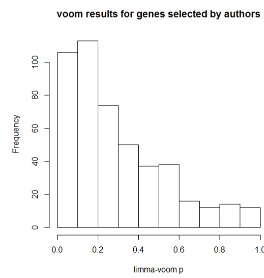
P-values with and without voom

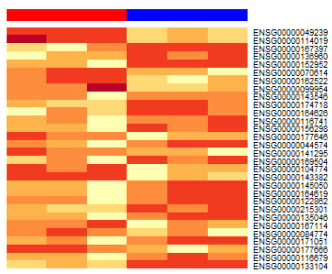


Gilad data set, limma. Your mileage may vary.

Gilad results for limma-voom







Using a model specific for counts

- Implementation: EdgeR, DESeq, baySeq, others
- Some groups used a Poisson model, but field moved to using negative binomial in a generalized linear model framework

- Originally approaches developed with SAGE in mind: small sample sizes, low "library size"

  (>1 million tags would be very unusual. 50-100k typical).
- More recently influenced by RNA-seq data.

EdgeR and DESeq2

- Use negative binomial distribution.
- In addition, both try to address the mean-variance trend in special ways. How they do this is the main difference.
  - Both use NB + GLMs (and offer simpler method if you have a one-way layout)
  - Both use m-v trends to help moderate dispersion estimates.
- At best generate estimates of variance for each gene; voom does this for each observation.
- Caution: peer-reviewed explanations may be out of date, look at user manuals!

Negative binomial distribution

- A gamma mixture of Poisson distributions
  - Count sampling distribution = Poisson
  - Biological sampling means from gamma
    - i.e., distribution of replicates
- No other particular reason to use it – it's (somewhat) convenient.
- "Overdispersed Poisson"
- Has an extra parameter to estimate compared to Poisson: the dispersion.
- Key problem: Estimating the dispersion from small data sets is tricky.

Modeling using negative binomial dist.

$$\sigma^2{}_i = \mu_i(1 + \mu_i \phi_i)$$

where ϕ_i is the dispersion for gene i. With ϕ =0, get Poisson.

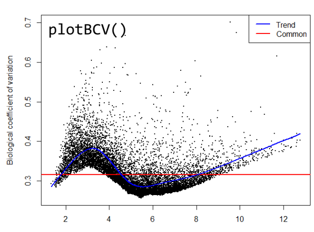
Could estimate directly from the data for gene *i*, but hard to trust data from small samples

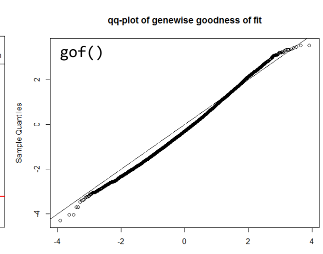
Another option is to make ϕ a parametric function of the mean (e.g. quadratic). But popular methods use more flexible approach:

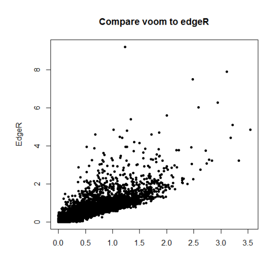
- **edgeR:** ϕ is gene-specific but moderated towards a trend.
  - `estimateGLMTrendedDisp` – fits the trend (bin and fit spline) followed by
  - `estimateGLMTagwiseDisp` – squeezes towards the trend
  - Early versions of edgeR used a common estimate and then squeezing

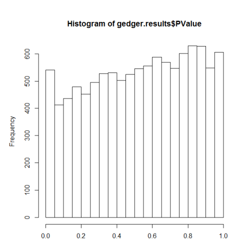
"(DESeq2) sequentially estimate(s) a prior distribution for the true dispersion values around the fit, and then provide the maximum a posteriori (MAP) as the final estimate"
"(DESeq2) differs from the previous implementation of DESeq , which used the maximum of the fitted curve and the gene-wise dispersion estimate as the final estimate and tended to overestimate the dispersions"
"The approach of DESeq2 differs from that of edgeR [3], as DESeq2 estimates the width of the prior distribution from the data and therefore automatically controls the amount of shrinkage based on the observed properties of the data. In contrast, the default steps in edgeR require a user-adjustable parameter, the prior degrees of freedom, which weighs the contribution of the individual gene estimate and edgeR 's dispersion fit." – Law et al.

EdgeR on the gilad data set

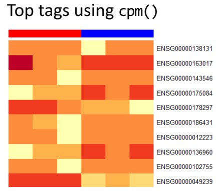




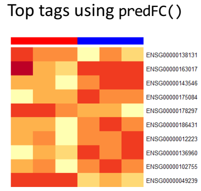




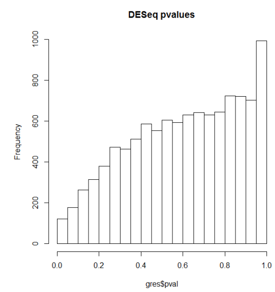
Top tags using cpm()

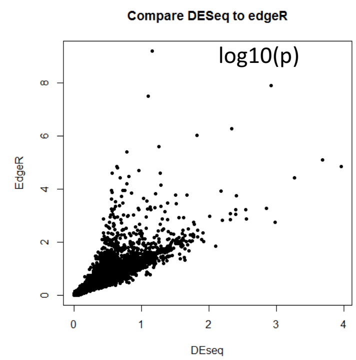


Top tags using predFC()

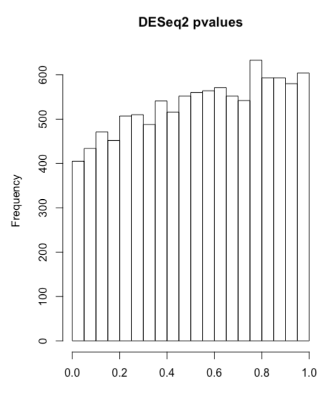


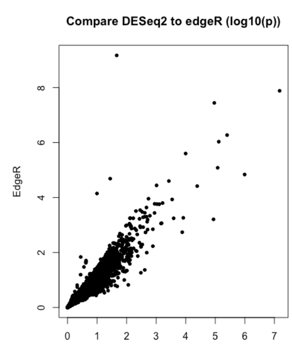
DESeq on the gilad data set





DESeq2 on the gilad data set





Summary of the differences between edgeR and DESeq2

- Dispersion estimation
  - "edgeR uses moderated dispersion (towards trend)"
  - "DESeq use maximum of fitted trend and gene-wise" (conservative) – DESEq2 tries to fix this
  - "edgeR is somewhat sensitive to outliers, but DESeq suffers somewhat in power" – edgeR-robust tries to fix outlier sensitivity.
- Normalization
  - TMM -weighted trimmed mean of M-value
  - DESeq – sample-wise median ratio

Also, GLM features of DESeq are more limited than edgeR. Only provides p-values and some fit statistics; no 'toptable' and no easy facilities for accessing specific contrasts. So for complex designs edgeR is easier.

Quotes from
http://www.fgcz.ch/education/StatMethodsExpression/03_Count_data_analysis.pdf

Voom does well in simulations

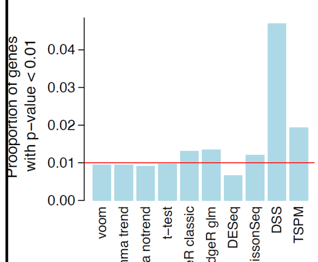


Figure 3

Figure 3 Type I error rates in the absence of true differential expression. The barplots show the proportion of genes with p-value < 0.01 for each method **(a)** when the library sizes are equal and **(b)** when the library sizes are unequal. The red line shows the nominal type I error rate of 0.01. Results are averaged over 100 simulations. Methods that control the type I error at or below the nominal level should lie below the red line.

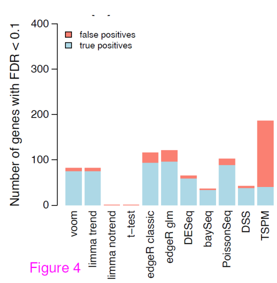


Figure 4

Figure 4 Power to detect true differential expression. Bars show the total number of genes that are detected as statistically significant (FDR < 0.1) **(a)** with equal library sizes and **(b)** with unequal library sizes. The blue segments show the number of true positives while the red segments show false positives. 200 genes are genuinely DE. Results are averaged over 100 simulations. Height of the blue bars shows empirical power. The ratio of the red to blue segments shows empirical FDR.

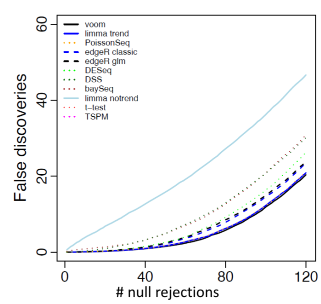


Figure 5 False discovery rates. The number of false discoveries is plotted for each method versus the number of genes selected as DE. Results are averaged over 100 simulations **(a)** with equal library sizes and **(b)** with unequal library sizes. Voom has the lowest FDR at any cutoff in either scenario.

Negative binomial model used to generate the data: should be optimal for edgeR and DESeq

Genome Biology 2014, **15**:R29

Voom has good FDRs on "spike-in" data

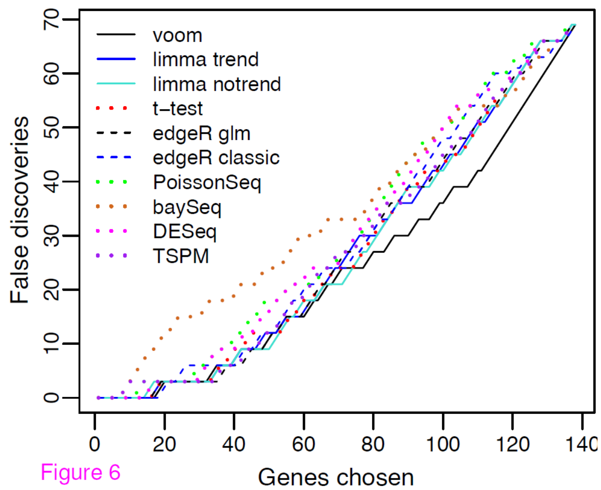


Figure 6

Figure 6 False discovery rates evaluated from SEQC spike-in data. The number of false discoveries is plotted for each method versus the number of genes selected as DE. voom has the lowest FDR overall.

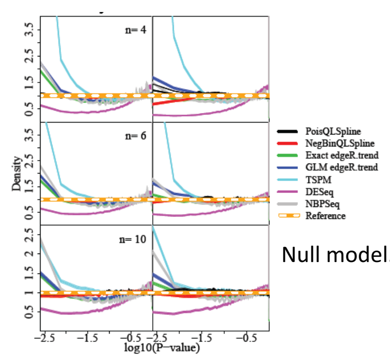
Spike-ins models 1.5-4-fold changes, ~6 million reads.

Only the spike-ins analyzed

Another (older) evaluation

"Although the negative binomial distribution provides flexibility in modeling variances, **existing popular methods based on this distribution fail to adequately account for uncertainty in parameter estimates**. A simulation study described in Section 4 demonstrates that most of these methods produce an over-abundance of small p-values for tests with true null hypotheses, relative to a uniform distribution, even for data simulated from negative binomial distributions."

"Although it ignores uncertainty in its estimated dispersion parameters, **DESeq** (Anders and Huber, 2010) produces too few small null p-values because its estimation procedure **systematically overestimates negative binomial dispersion parameters**. The resulting non-uniform distributions of null p-values obtained from these methods are shown to produce q-values that inaccurately estimate false discovery rates."



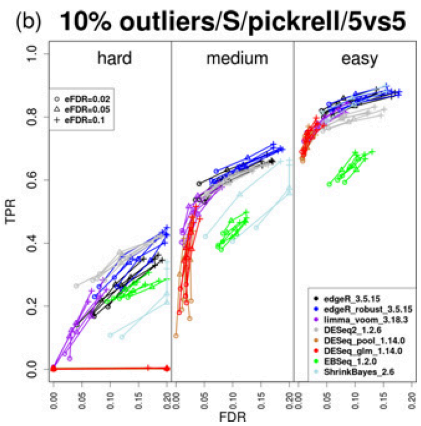
Null model.

Figure 10: Histograms of p-values for EE genes in negative binomial simulations based on fly embryo (left) and Arabidopsis (right) data sets with $n = 4$ (top), $n = 6$ (middle) and $n = 10$ (bottom).

Lund et al. Stat Appl Genet Mol Biol. 2012 Oct 22;11
Out of date due to software updates…

More evaluation

(From edgeR authors – edgeR-robust)



Power-to-achieved-FDR across hard (foldDiff ∈ [2, 2.2]), medium (foldDiff ∈ [3, 3.3]) and easy (foldDiff ∈ [6, 6.6]) simulation settings. (**a**) No outliers; (**b**) 10% outliers. Y-axis shows TP rate and X-axis shows FD rate. Five simulations are shown for each method and each setting. Points are taken according to each method's FDR cutoffs at 0.02, 0.05 and 0.1.

"In all cases, limma-voom controls FDR well and maintains power"

Nucleic Acids Research, 2014, Vol. 42, No. 11

EdgeR can be sensitive to outliers

- Because it squeezes the dispersion quite strongly to the trend estimate, this can yield overly-optimistic adjustments.
  - Adjust by dialing down prior.df from default of 20
- DESeq uses a more pessimistic (conservative) estimate of dispersion by default. Thus p-values are probably inflated (even in a null data set)
- Suggestions for checking and coping from Smyth https://stat.ethz.ch/pipermail/bioconductor/2012-January/043187.html
  - "If none of this solves your problems, you might try the voom() function in the limma package instead."
- See also:
  https://stat.ethz.ch/pipermail/bioconductor/2012-January/043168.html
  https://stat.ethz.ch/pipermail/bioconductor/2012-May/045562.html

How do we choose a method?

- There is no great gold standard to use. Simulations somewhat unsatisfying, spike-ins not completely realistic
- EdgeR and DESeq2 are very similar in design.
- **Limma-voom** has emerged as a sound choice
  - Performs as well or better than NB
  - Familiar to limma users
  - Flexible, fast
  - Might not do as well when sample size is very small – but nobody should be doing N=2 experiments.

Selected bibliography

Marioni et al. 2008 Genome Research 18:1509-1517. Shows that count distributions for technical replicates fit Poisson. Also show comparison to microarrays.

Mortazavi et al. 2008 Nature Methods 5:621-628. Another important paper introducing RNA-seq.

Robinson and Smyth, 2008 Biostatistics 9:321-332. Introduces NB model, common dispersion estimate; qCML libSizes, exact test for diff ex. from NB.

Robinson and Smyth, 2007 Bioinformatics. Adds EB moderation of common dispersion estimate (gene-wise) to edgeR – Published "out of order"?

Zhou et al., 2014 Nucleic Acids Research - doi: 10.1093/nar/gku310 – Describes edgeR-robust

*Robinson and Oshlack 2010 Genome Biology 11:R25. Library space concept and TMM normalization.

Oshlack et al. 2010 Genome Biology 11:220 Useful review, but already out of date.

Bullard et al. 2010 BMC Bioinformatics 11:94. Evaluation of Fisher's test, Poisson GLM and t-test. Proposes "gold standard" based on MAQC data.

Auer and Doerge 2010 Genetics 185:405-416. Proposes Poisson GLM with overdispersion.

*Anders and Huber 2010 Genome Biology 11:R106. Introduces DESeq, trended dispersion estimate, normalization method; and a diff ex method for one-way layouts.

Love et al. 2014 Genome Biology http://genomebiology.com/2014/15/12/550/abstract Describes DESeq2

Blekhman et al. 2010 Genome Research 20(2):180-9. "Sex-specific and lineage-specific alternative splicing in primates" Source of the 'gilad' data set.

Mardis 2011 Nature 470: 198-203 – Good review of sequencing technology but already out of date.

Di et al. Stat. Appl. in Genetics Mol. Bio. 2011 vol10. Introduction is a useful review of statistical approaches.

McCarthy et al., 2012 NAR : Extension of edgeR to GLM; Decomposition of TCV and BCV; adds trended dispersion.

Lund et al. Stat. Appl. in Genetics Mol. Bio. 2012 11:5. McCarthy and Smyth are coauthors on this paper that shows that EdgeR and DESeq do not give accurate p-values. Proposes another NB method using quasi-likelihood to address the problems.

* Law et al. Voom: precision weights unlock linear model analysis tools for RNA-seq read Counts Genome Biology 2014, **15**:R29. Paper from Smyth formally describing Voom and evaluation of its performance.
* Soneson -2013 A comparison of methods for differential expression analysis of RNA-seq data http://www.biomedcentral.com/1471-2105/14/91

* Conesa et al. 2016 – A survey of best practices for RNA-seq data analysis Genome Biology (2016) 17:13

Also
DESeq, EdgeR and limma user manuals
*Mark Robinson lecture slides: http://www.fgcz.ch/education/StatMethodsExpression, lectures 3 and 4 – very useful!
Davis McCarthy 2009 Thesis
Bioconductor forums
SeqAnswers.org
https://www.youtube.com/watch?v=dvozWzolVI8 - Mark Robinson on transcript vs gene-level analysis 2016

Conesa *et al. Genome Biology* (2016) 17:13
DOI 10.1186/s13059-016-0881-8

Genome Biology

REVIEW Open Access

A survey of best practices for RNA-seq data analysis

Ana Conesa[1,2*], Pedro Madrigal[3,4*], Sonia Tarazona[2,5], David Gomez-Cabrero[6,7,8,9], Alejandra Cervera[10], Andrew McPherson[11], Michał Wojciech Szcześniak[12], Daniel J. Gaffney[3], Laura L. Elo[13], Xuegong Zhang[14,15] and Ali Mortazavi[16,17*]

A comprehensive assessment of RNA-seq accuracy, reproducibility and information content by the Sequencing Quality Control Consortium

SEQC/MAQC-III Consortium*

Soneson and Delorenzi *BMC Bioinformatics* 2013, **14**:91
http://www.biomedcentral.com/1471-2105/14/91

BMC Bioinformatics

RESEARCH ARTICLE Open Access

A comparison of methods for differential expression analysis of RNA-seq data

Charlotte Soneson[1*] and Mauro Delorenzi[1,2]

Differential expression using Fisher's exact test

- Appropriate for Poisson assumption. Add counts across replicates, test for equality of proportions.
- Limited to one-way layouts (no "two-way ANOVA")
- Original EdgeR (exactTest) and DESeq (nbinomTest) use similar approach but adapted to negative binomial distribution (requires work to make library sizes equal).

	group A	group B	total
counts for gene X	65	25	90
counts for remaining genes	897455	901665	1799120
total	897520	901690	1799210

```
> b<-matrix(c(65, 897455, 25, 901655),2,2)
> fisher.test(b)$p.value
[1] 1.956e-05
```

Analogy to *t* statistics

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

Feature-specific

Student's t
Limma with prior.df = 0

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

Moderated

Limma et al.

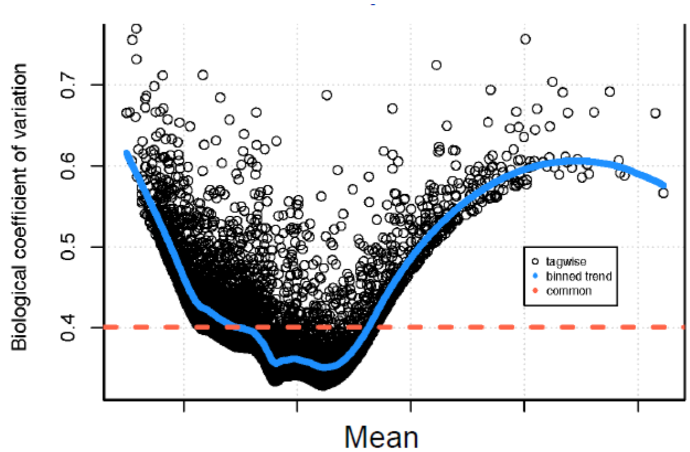
$$t_{g,\text{pooled}} = \frac{\overline{y}_{\text{mu}} - \overline{y}_{\text{wt}}}{s_0\, c}$$

Common

Ignore all gene dependency
Limma with prior.df = Inf

http://www.fgcz.ch/education/StatMethodsExpression/03_Count_data_analysis.pdf

Estimation approaches (edgeR)



http://www.fgcz.ch/education/StatMethodsExpression/03_Count_data_analysis.pdf

Generalized linear models

- Extension of linear models to non-normal response data (in this case, negative binomial)
- One motivation is dealing with different mean-variance relationships
- Handle complex models as per standard linear modeling
- Fitting requires iterations – slow
  - McCarthy et al. 2012 describe a way to speed it up.
- Hypothesis testing in edgeR and DESeq: likelihood ratio tests