Summer Institute in Statistical Genetics 2019

Integrative Genomics

2. Dataset normalization

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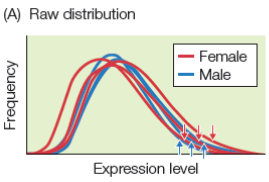
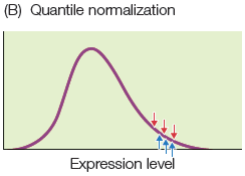
Gene Expression Data is analyzed on the log base 2 scale

1. Log transformation makes the data more normally distributed, minimizing biases due to the common feature that a small number of genes account for over half the transcripts
2. Log base 2 is convenient, because in practice most differential expression is in the range of 1.2x to 8x, depending on the contrast of interest and complexity of the sample.
3. It is also intuitively simple to infer fold changes in a symmetrical manner:
   A difference of -1 unit corresponds to half the abundance, and +1 to twice the abundance
   A difference of -2 units corresponds to a quarter the abundance, and +3 to 8-times the abundance
4. The log scale is insensitive to mean centering, so it is simple to just set the mean or median to 0, preserving the relative abundance above or below the sample average
5. It is sometimes useful to add 1 to all values before taking the log, to avoid "0" returning #NUM! (but not advised with edgeR)

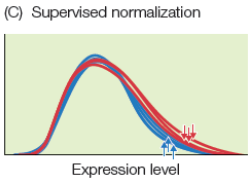
Rank vs Absolute Expression



Raw data:
no effect



Variance transformed:
no effect



Mean centered:
significant effect

Quantile normalization

	S1	S2	S3	R1	R2	R3	Q1	Q2	Q3
Gene A	10.2	11.2	10.9	3	1	4	10.6	11.9	10.3
Gene B	9.6	10.7	8.9	5	2	6	9.6	11.2	9.2
Gene C	12.7	7.8	11.7	1	9	1	11.9	7.6	11.9
Gene D	9.5	10.0	9.9	6	4	5	9.2	10.3	9.6
Gene E	11.3	9.2	11.1	2	7	3	11.2	8.8	10.6
Gene F	7.7	7.7	6.5	9	10	10	7.6	6.7	6.7
Gene G	5.9	9.3	7.8	10	6	8	6.7	9.2	8.1
Gene H	8.8	8.2	8.4	7	8	7	8.8	8.1	8.8
Gene I	10.1	9.4	11.6	4	5	2	10.3	9.6	11.2
Gene J	8.2	10.6	7.2	8	3	9	8.1	10.6	7.6

Step 1: Mean or Median Center Samples

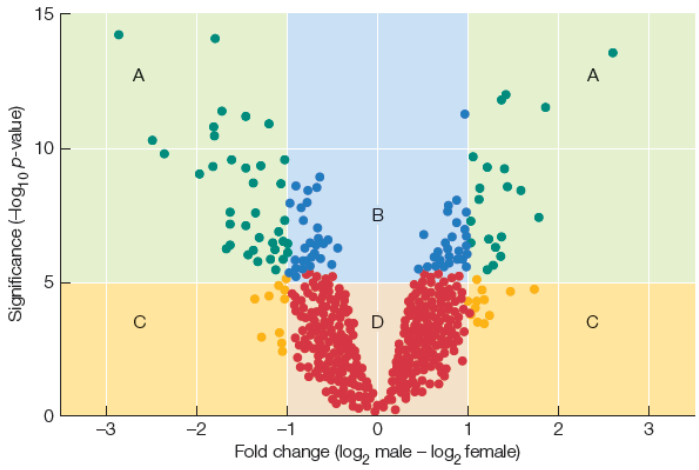
Step 2: Rank each Gene within each Sample

Step 3: Compute average of each Rank

Step 4: Reassign Average Rank to each Gene

	S1	S2	S3	Avg
Rank 1	12.7	11.2	11.7	11.9
Rank 2	11.3	10.7	11.6	11.2
Rank 3	10.2	10.6	11.1	10.6
Rank 4	10.1	10	10.9	10.3
Rank 5	9.6	9.4	9.9	9.6
Rank 6	9.5	9.3	8.9	9.2
Rank 7	8.8	9.2	8.4	8.8
Rank 8	8.2	8.2	7.8	8.1
Rank 9	7.7	7.8	7.2	7.6
Rank 10	5.9	7.7	6.5	6.7

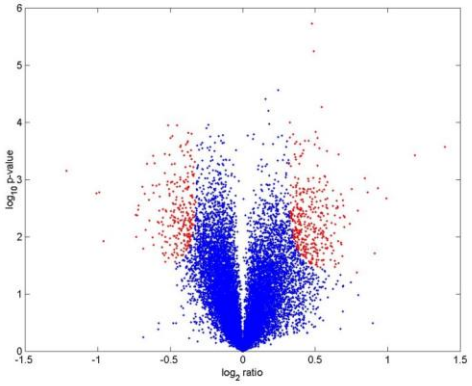
Transcriptome Volcano plots



Russ Wolfinger, Greg Gibson

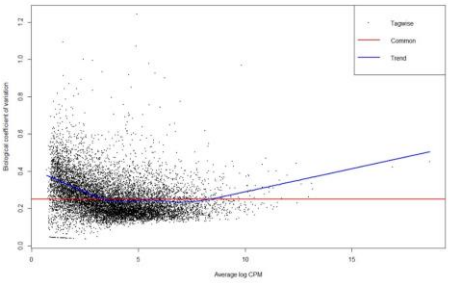
Variance shrinkage

- Gene-specific approach means that the power for each gene varies, but shrinkage can equilibrate the variance
- Permutation approach may be more appropriate where you have many treatments with low replication



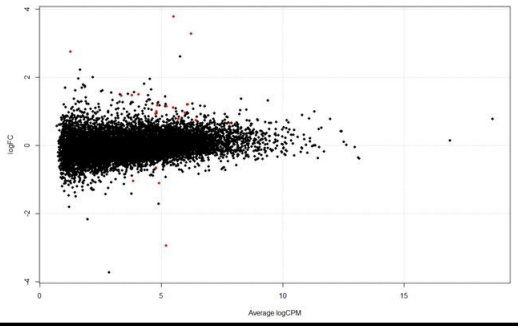
Gary Churchill, Katie Kerr

Plots of Variance and Fold Change against Intensity

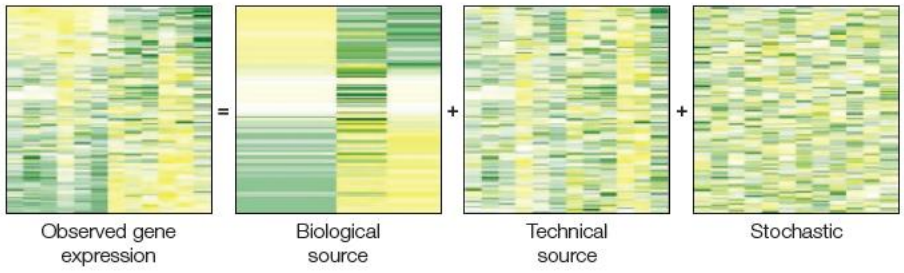


$$M = \log_2(R/G) = \log_2(R) - \log_2(G)$$

$$A = \frac{1}{2}\log_2(RG) = \frac{1}{2}(\log_2(R) + \log_2(G))$$



The normalization challenge



John Storey

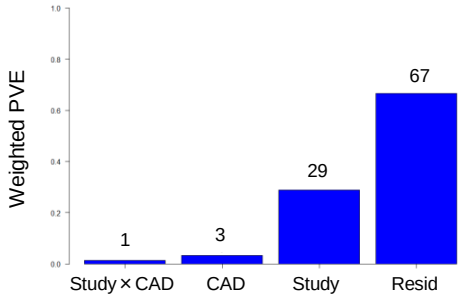
Principal Variance Component Analysis (PVCA)

Step 1: Compute the Principal Components (PC) of the dataset

Step 2: Regress Technical and Biological Parameters on each PC to estimate the proportion of variance explained (PVE)

Step 3: Sum the PVE, weighted by the strength of the PC

This tells you how much of the overall variance is due to each factor.



Surrogate Variable Analysis (SVA)

The idea is to identify components of variation that are due to technical factors (batch effects, RNA quality, other unknown influences) and remove them so that the biological factors of interest are enhanced.

Step 1: Compute the Surrogate Variables

- Fit the biological factors of interest to generate residuals
- Identiffy the PC of the residuals
- SV are weights applied to those genes that correlate with the PC

Step 2: Ask which SV are correlated with Technical factors

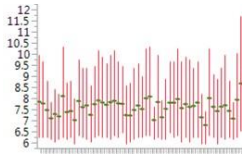
Step 3. Decide which ones to remove or at least adjust for

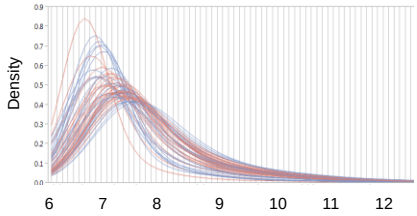
SNM (Supervised Normalization of Microarrays) optimizes this process in an iterative fashion, but has not been revised for RNASeq.

PEER factor normalization is a Bayesian version which automatically fits all of the surrogate variables, and is commonly used for cis-eQTL analysis.

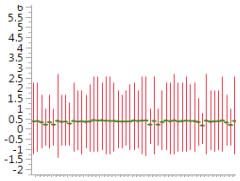
Simple Centering

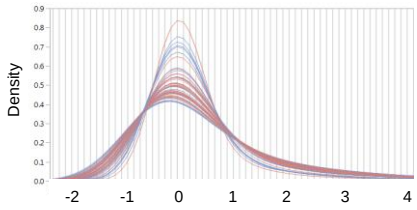
Raw Profiles





Median Transform



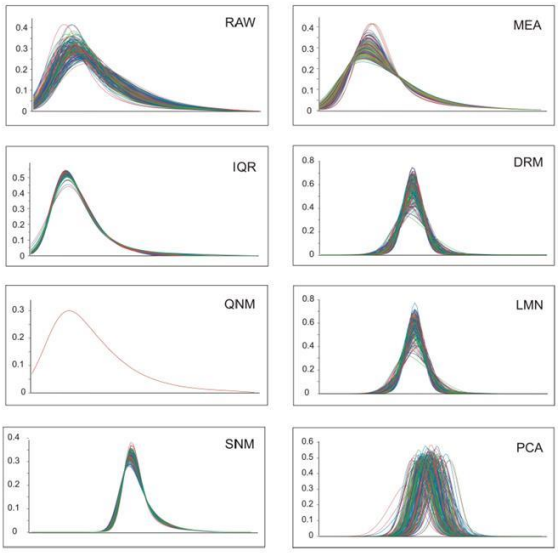


For RNASeq data, CPM essentially does this: cpm = 1,000,000 x reads/total reads

Types of normalization

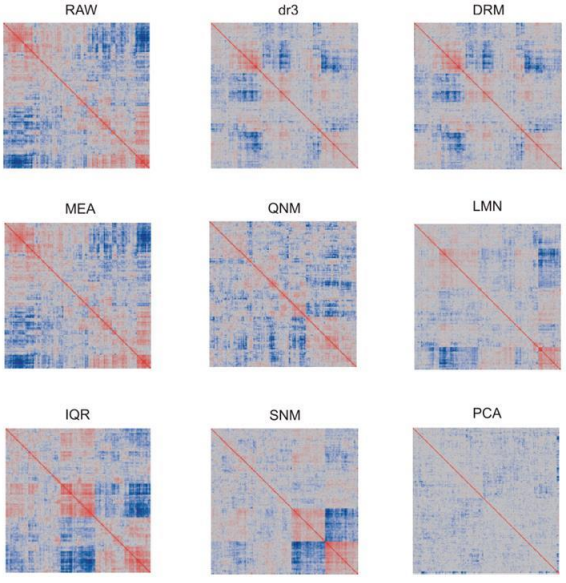
- Mean or Median transform, simply centers the distribution
  - Something like this is essential to control for overall distributional effects (eg RNA concentration)
  - Counts per Million is related, but uses a multiplicative scaling factor
- Variance transforms, such as standardization or inter-quartile range
  - Depends on whether you think the overall distributions should have similar variance
- Quantile normalization
  - Transforms the ranks to the average expression value for each rank
- Gene-level model fitting
  - Remove technical or biological effects before model fitting on the residuals
- Supervised normalization
  - Optimally estimate the biological effect while fitting technical factors across the entire experiment

Effect of Normalization on Distributions

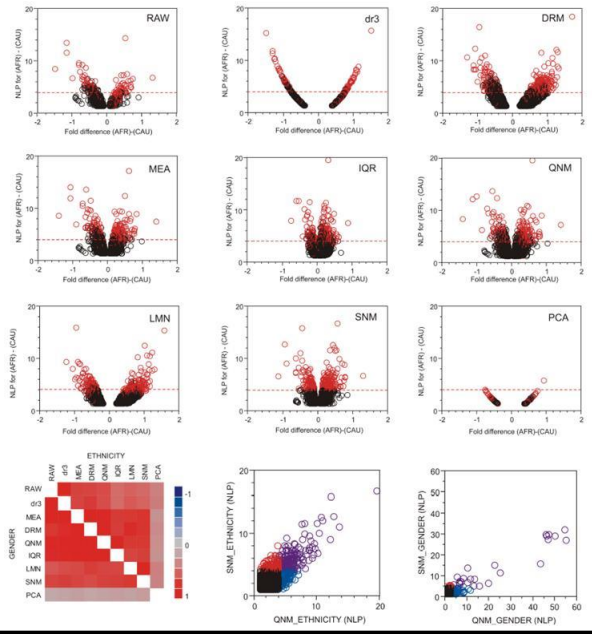


Qin et al, 2013. Frontiers in Genetics **3**: 160

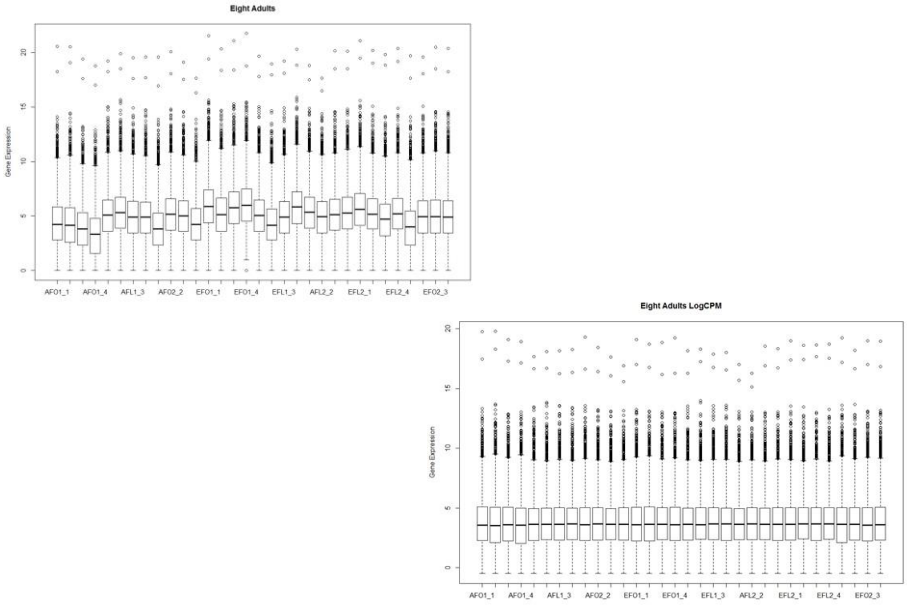
Effect of Normalization on Covariance



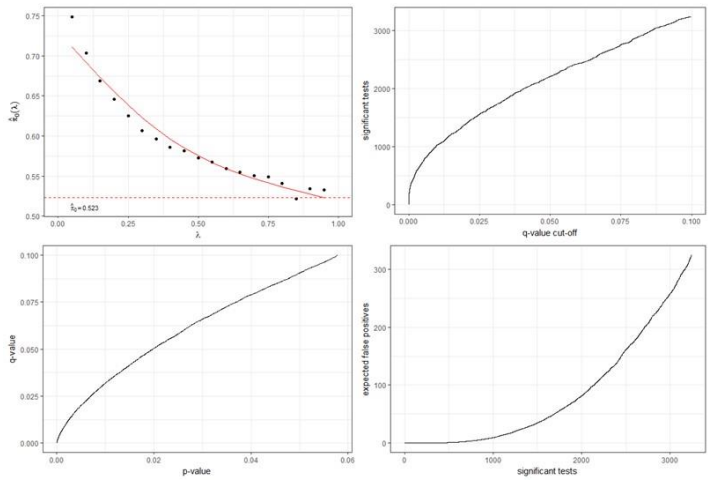
Effect of Normalization on Significance



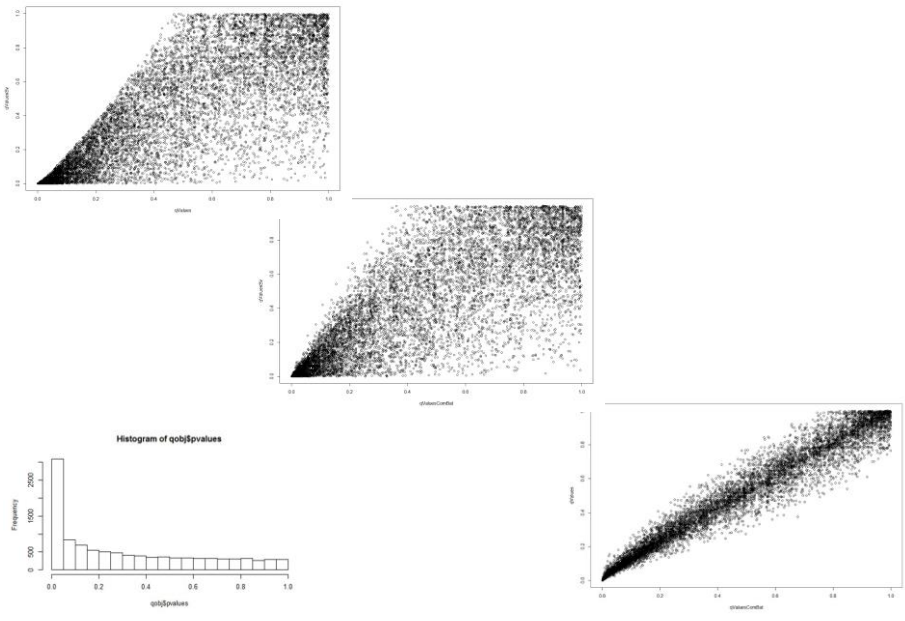
Raw vs Normalized Boxplots



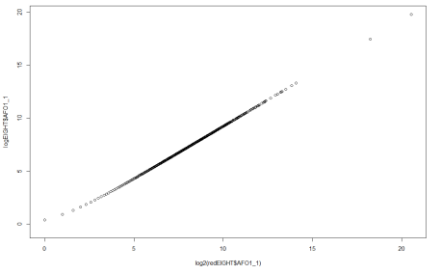
qValue Plots

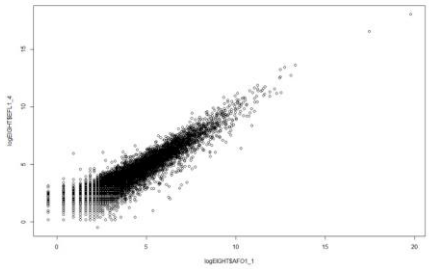


Raw vs SVA adjusted vs COMBAT ethnicity adjusted qvalues



Effect of Negative Binomial Adjustment

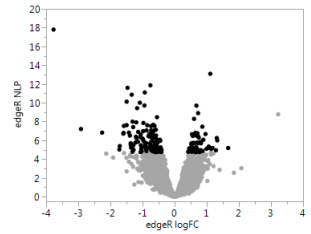


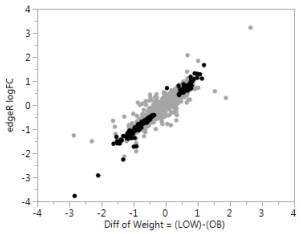


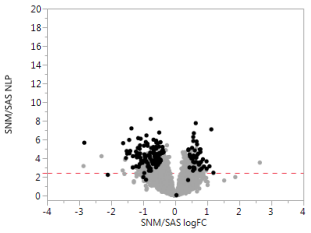
ANOVA of Surrogate Variables

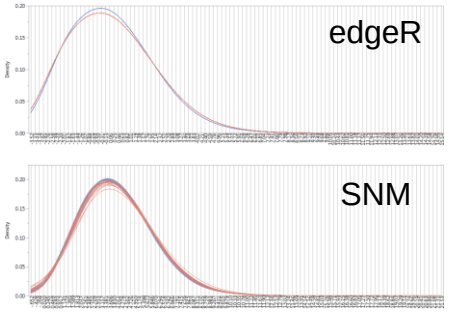
```
> summary(fit2)
            Df Sum Sq Mean Sq F value   Pr(>F)
ethn         1 0.0070 0.00699   0.708    0.409
weight       1 0.0015 0.00153   0.155    0.698
visit        3 0.0175 0.00585   0.593    0.626
person       5 0.7668 0.15335  15.545 1.92e-06 ***
Residuals   21 0.2072 0.00987
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> fit3 <- aov(SV3 ~ ethn + weight + visit + person, data=phEIGHT)
> summary(fit3)
            Df Sum Sq Mean Sq F value   Pr(>F)
ethn         1 0.5516  0.5516  74.451  2.4e-08 ***
weight       1 0.0005  0.0005   0.061 0.806947
visit        3 0.0188  0.0063   0.846 0.483938
person       5 0.2735  0.0547   7.383 0.000392 ***
Residuals   21 0.1556  0.0074
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Comparison of SNM/SAS vs edgeR in a small expt









Recommended analytical strategy

1. Normalize the samples

2. Extract the Principal components of gene expression

3. Ask whether the major PC are correlated with technical covariates such as Batch or RNA quality; or with Biological variables of interest

4. If they are, renormalize to remove those effects

   (PEER factor normalization is a Bayesian approach to fitting Surrogate Variables;
   SVA is a linear modeling approach often performed with COMBAT;
   SNM is a supervised approach that allows you to retain Biological factors while fitting or removing technical ones)

5. As much as possible, analyze the dataset in several different ways to (i) confirm that the findings are not sensitive to your analytical choice, and (ii) gain insight into what may cause differences, eg find confounding factors