University of Zurich[UZH]

Institute of Molecular Life Sciences

Differential expression analyses with the limma package

- Admin: journal club sign-up, project proposals
- In class exercise: how do we know methods work well in practice?
- Preliminaries – quick comments on pre-processing microarrays: background correction, normalization, exploratory analysis
- The philosophy of limma
- limma – INTRO TO linear models for microarray data

Mark D. Robinson, Statistical Genomics, IMLS

Journal club signups → by 18.00 / 31.10.2016

(Ideal: submit a PR to materials/ README.md)

Project proposal: for your team, write 2-3 sentences with the plan. Target: mid/ late-November

24.10.2016	Mark	limma 1		
31.10.2016	Mark	limma 2	x	x
07.11.2016	Hubert	RNA-seq quantification	Reliable detection of subclonal single-nucleotide variants in tumour cell populations {CB,L-WY}	A network-based method to evaluate quality of reproducibility of differential expression in cancer genomics studies {TS, SS}
14.11.2016	Mark	edgeR+friends 1	x	x
21.11.2016	Mark	edgeR+friends 2	x	x
28.11.2016	Hubert	classification	A Method for Checking Genomic Integrity in Cultured Cell Lines from SNP Genotyping Data {SA, FB, PCC}	x
5.12.2016	Mark	epigenomics, DNA methylation	Empirical Bayes Analysis of a Microarray Experiment {GA, IA}	x
12.12.2016	Mark	gene set analysis	x	x
19.12.2016	Mark	single-cell	x	x

From the feed: Terry's IMS Bulletin + "Over-optimism"

We will see a lot of methods in this course – **how do we evaluate what works well in practice ?**

http://bulletin.imstat.org/2012/11/terences-stuff-does-it-work-in-practice/

Gene expression

Advance Access publication June 26, 2010

Over-optimism in bioinformatics: an illustration

Monika Jelizarow[1], Vincent Guillemot[1,2], Arthur Tenenhaus[2], Korbinian Strimmer[3] and Anne-Laure Boulesteix[1,*]

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Associate Editor: John Quackenbush

"if the improvement of a quantitative criterion such as the error rate is the main contribution of a paper, the superiority of new algorithms should always be demonstrated on independent validation data."

In class exercise + discussion

- (5 minutes) Read the excerpt from “Terence’s Stuff” column
- (5-10 minutes; discuss with your neighbour) Answer the following 3 questions:
  1. How do we tell what works in practice?
  2. What problems arise using simulated (synthetic) data?
  3. What problems arise using real data?
  4. What are positive/negative controls?
- Discuss
- If simulation: what metrics could/would/should we use?

Differential expression, small sample inference

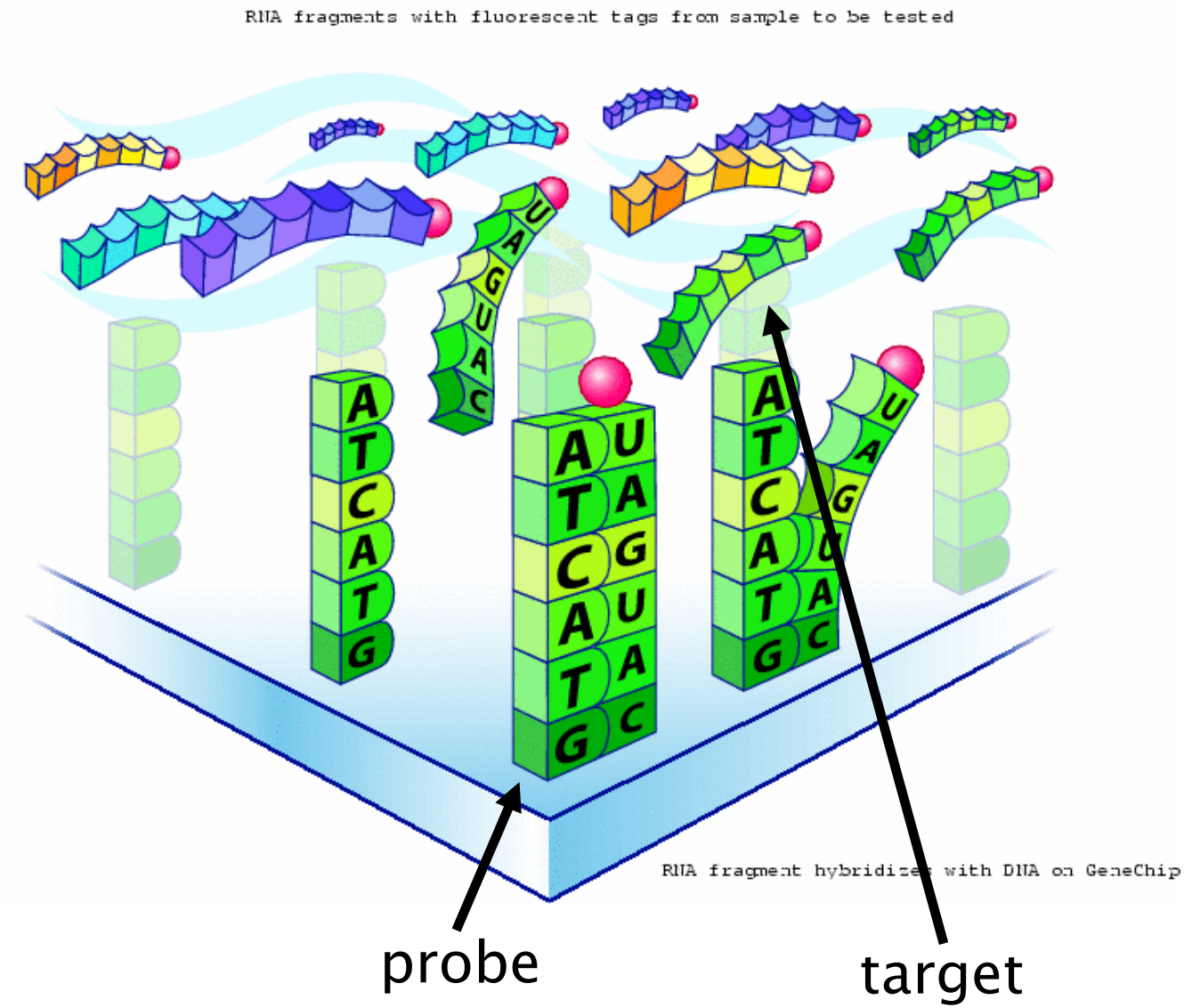
- Table of data (e.g., microarray gene expression data with replicates of each of condition A, condition B)
  - *rows* = features (e.g., genes), *columns* = experimental units (samples)
- Most common problem in statistical bioinformatics: want to infer whether there is a **change in the response** → 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] ?

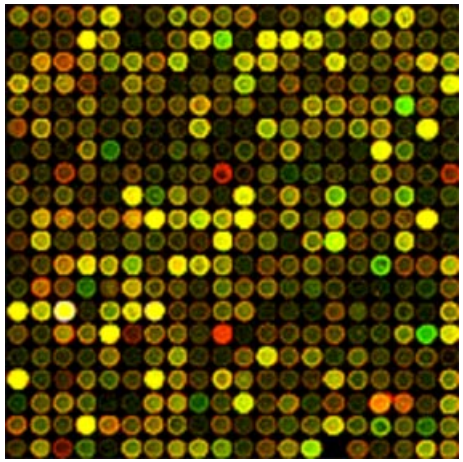
```
> head(y)
        group0     group0      group0     group1     group1      group1
gene1 -0.1874854  0.2584037 -0.05550717 -0.4617966 -0.3563024 -0.03271432
gene2 -3.5418798 -2.4540999  0.11750996 -4.3270442 -5.3462622 -5.54049106
gene3 -0.1226303  0.9354707 -1.10537767 -0.1037990  0.5221678 -1.72360854
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
```

[1] http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html

DNA microarray: arrays of northern blots

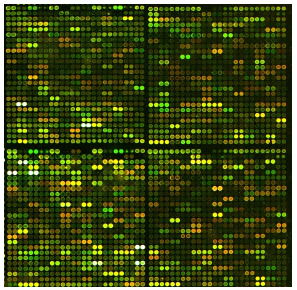


Abundance (of complementary DNA species) measured by flouresence intensity



Microarray expression measures

Two-colour



$$y_{ga} = \log_2(R/G)$$

array

probe or gene

Affymetrix

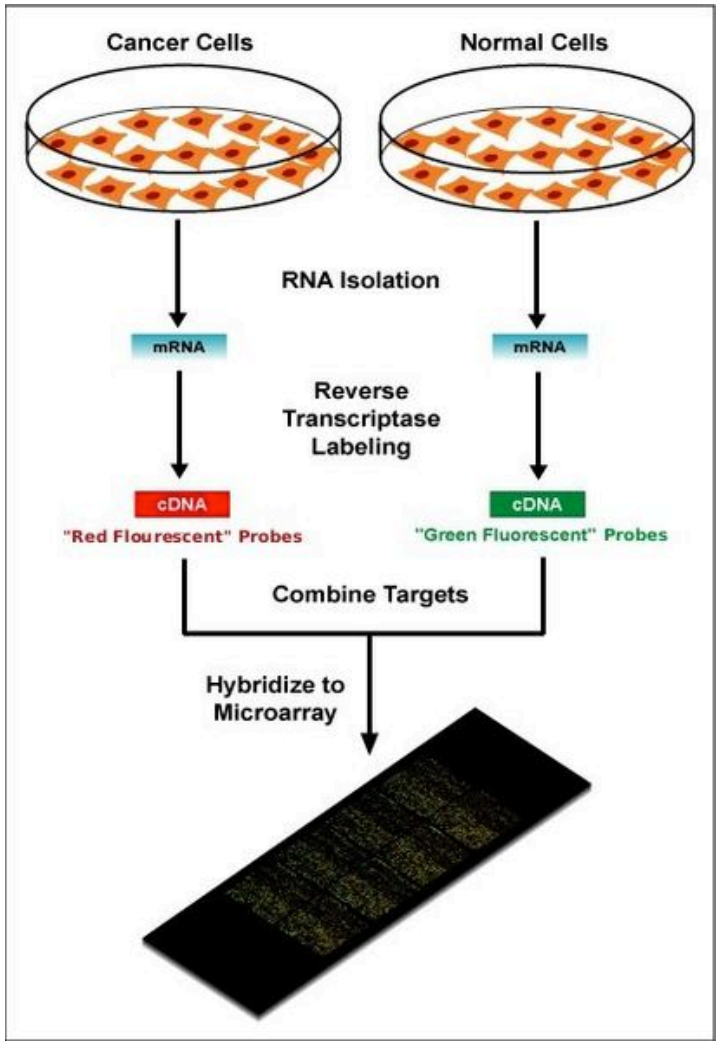
y_{ga} = log-intensity (summarized over probes)

Illumina

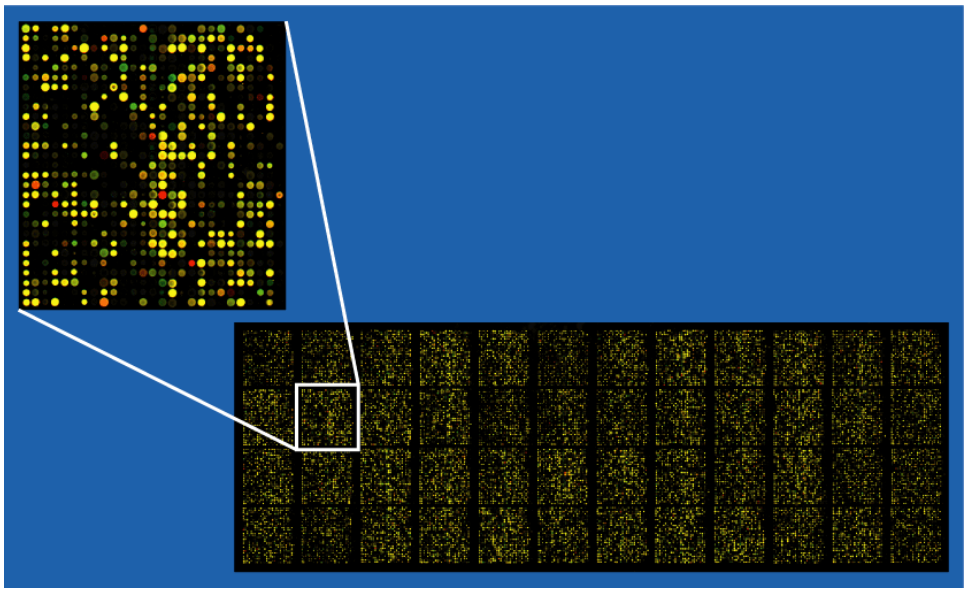
y_{ga} = log-intensity (summarized over beads)

Questions of Interest

- What genes have changed in expression? (e.g. between disease/normal, affected by treatment) Gene discovery, differential expression
- Is a specified group of genes all up-regulated in a particular condition?
  Gene **set** differential expression
- Can the expression profile predict outcome?
  Class prediction, classification
- Are there tumour sub-types not previously identified? Do my genes group into previously undiscovered pathways?
  Class discovery, clustering



Two colour microarrays



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

Preprocessing: additive + multiplicative error model

Observe intensity for one probe on one array

Intensity = background + signal

$$I = B + S$$

B: additive errors

S: multiplicative errors

This idea underlies variance stabilizing transformations vsn (two colour data) and vst (for Illumina data)

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Microarray background correction: maximum likelihood estimation for the normal–exponential convolution

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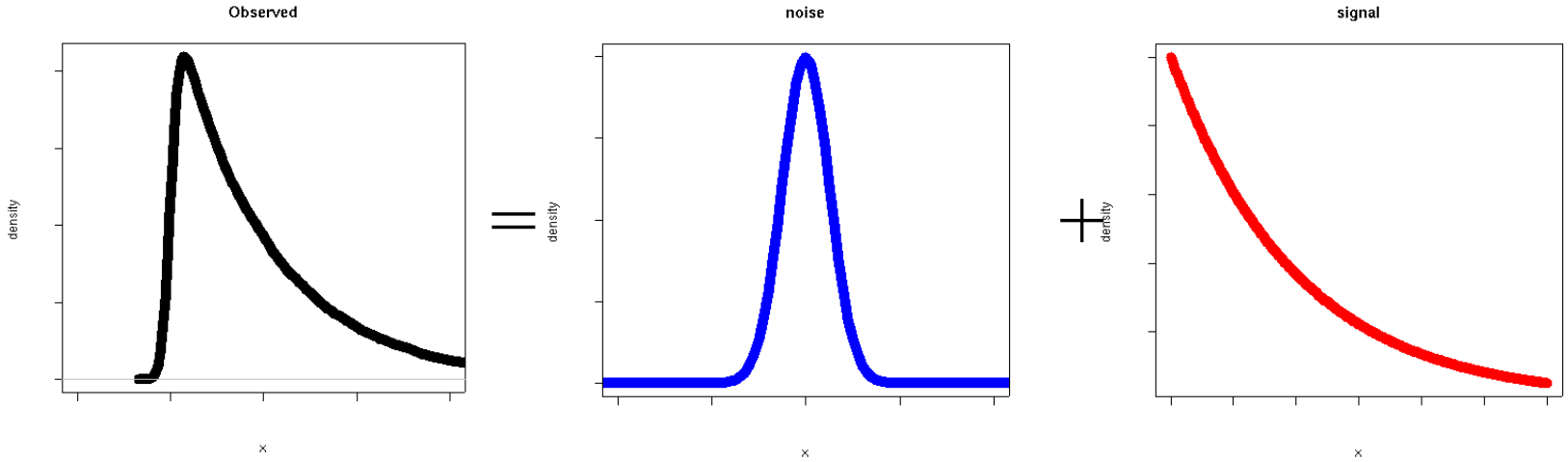
normexp convolution model

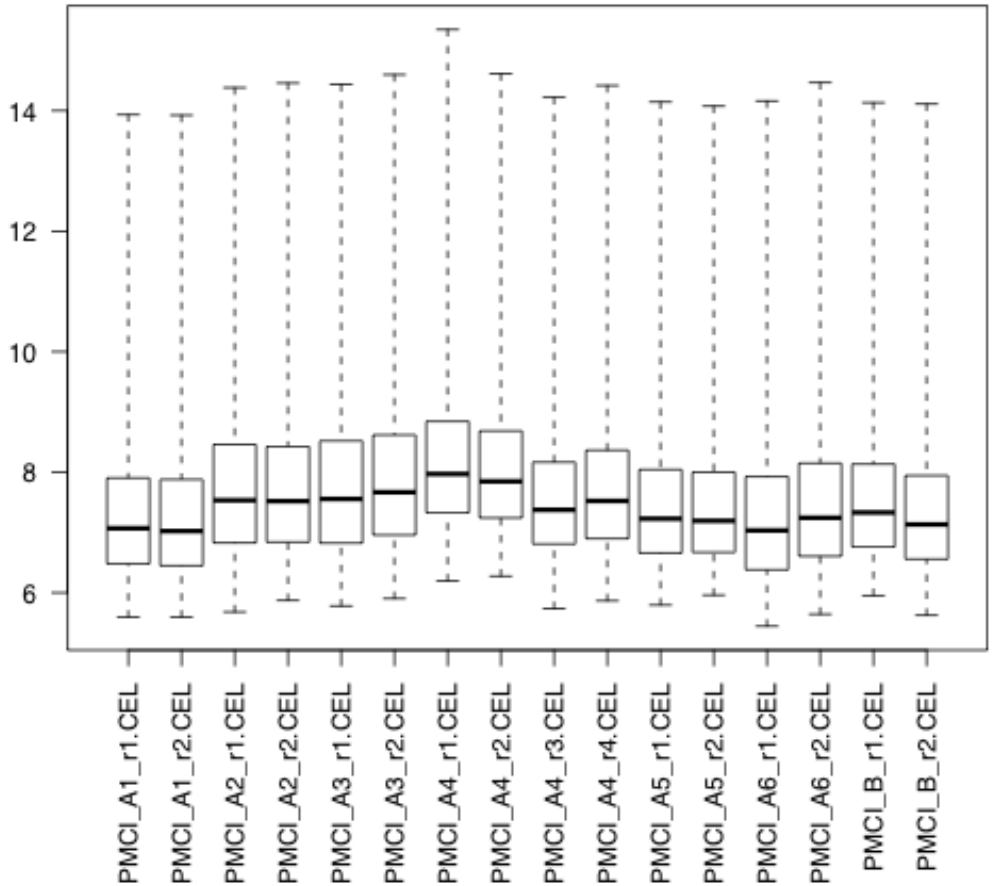
Intensity = Background + Signal

$N(\mu,\sigma^2)$ Exponential(α)

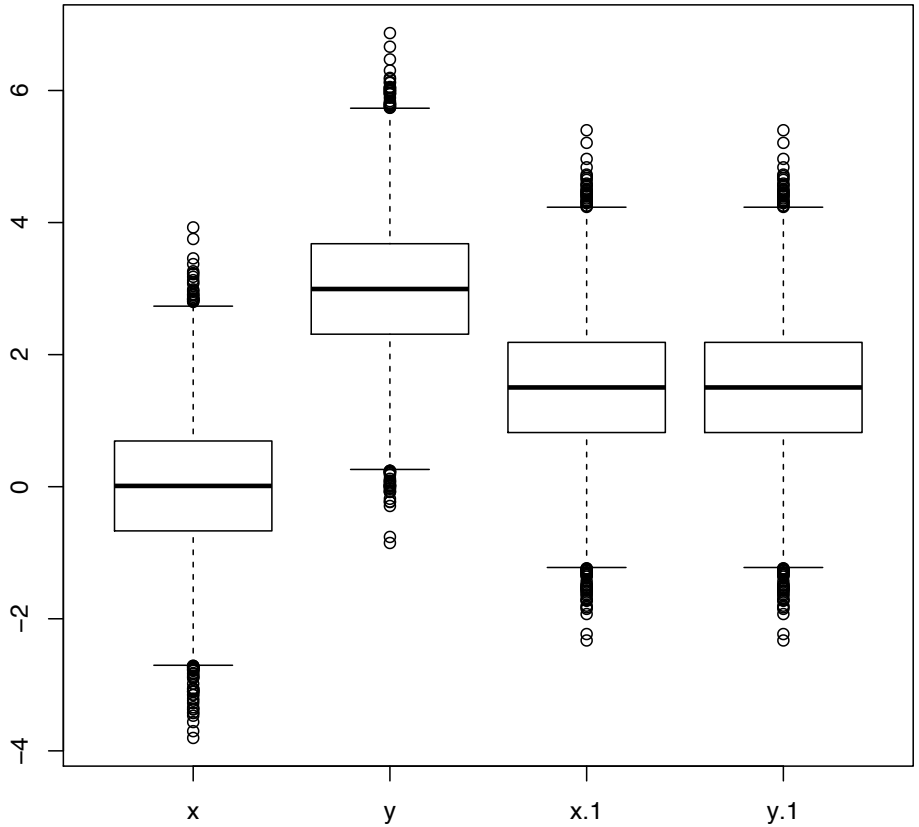


Normalization: one-colour

Similarly for single channel data, adjustments need to be made for all samples to be comparable.



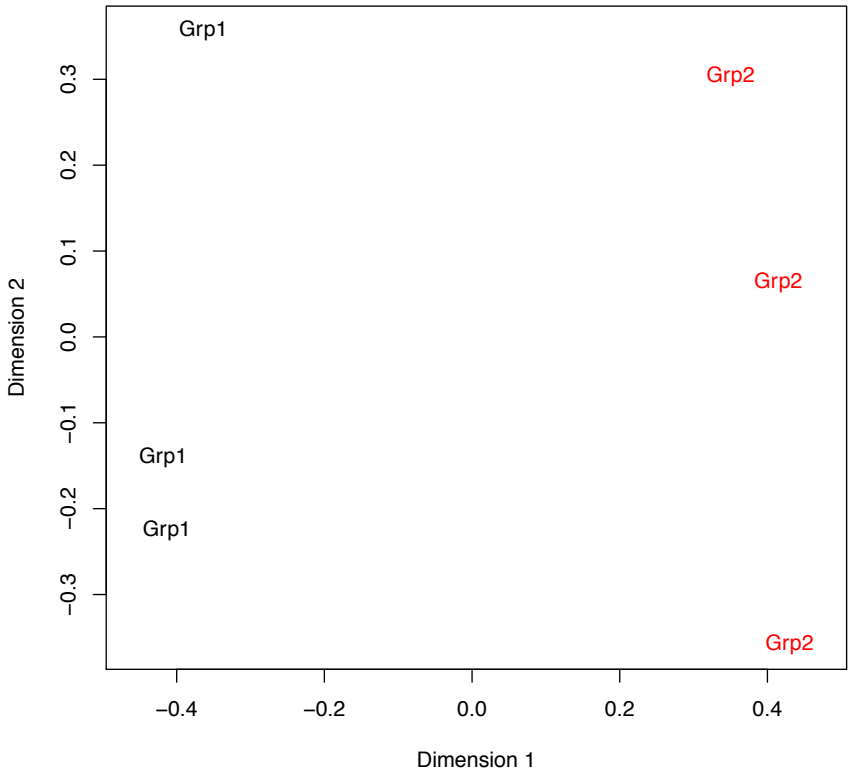
Quantile normalization




```
x <- rnorm(10000, mean=0, sd=1)
y <- rnorm(10000, mean=3)
z <- cbind(x,y)

# create "reference" distribution
s <- apply(z,2,sort)
sm <- rowMeans(s)

# impose ref. distribution by ranks
r <- apply(z,2,rank)
n <- apply(r,2,function(u) sm[u])

boxplot( data.frame(x=x,y=y,n) )

#> library(limma)
#> zn <- normalizeQuantiles(z)
#> all(zn==n)
#[1] TRUE
```

Quality assessments

Multidimensional scaling plot



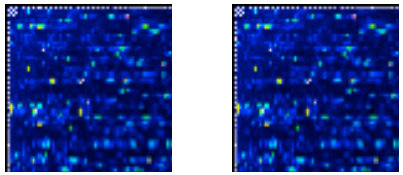

```
sd <- 0.3*sqrt(4/rchisq(1000,df=4))
x <- matrix(rnorm(1000*6,sd=sd),1000,6)
x[1:50,4:6] <- x[1:50,4:6] + 2

mds <- plotMDS(x)

> round(mds$distance.matrix,3)
      [,1]  [,2]  [,3]  [,4] [,5] [,6]
[1,] 0.000 0.000 0.000 0.000 0.00    0
[2,] 0.835 0.000 0.000 0.000 0.00    0
[3,] 0.850 0.793 0.000 0.000 0.00    0
[4,] 1.089 1.068 1.058 0.000 0.00    0
[5,] 1.050 1.058 1.072 0.863 0.00    0
[6,] 0.991 1.047 1.046 0.865 0.85    0
```

Limma concept: borrowing information across genes

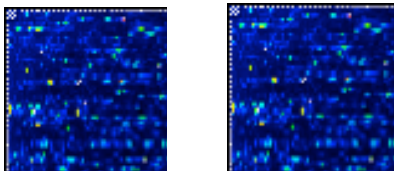
- **Small data sets**: few samples, generally under-powered for 1 gene
- **Curse of dimensionality**: many tests, need to adjust for multiple testing (= loss of power)
- **Benefit of parallelism**: same model is fit for every gene. Can *borrow information* from one gene to another
  - **Hard**: assume parameters are constant across genes
  - **Soft**: smooth genewise parameters towards a common value in a graduated way, e.g., Bayes, empirical Bayes, Stein shrinkage …

A very common experiment (1-colour)

Mutant x 2



WT x 2



Gene X

Which genes are differentially expressed?

$n_1 = n_2 = 2$ Affymetrix arrays

~30,000 probe-sets

Ordinary t-tests (1-colour)

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

give very high false discovery rates

$$c = \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

Residual df = 2

t-tests with common variance

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

with residual standard deviation s_0 pooled across genes

More stable, but ignores gene-specific variability

$$c = \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

A better compromise

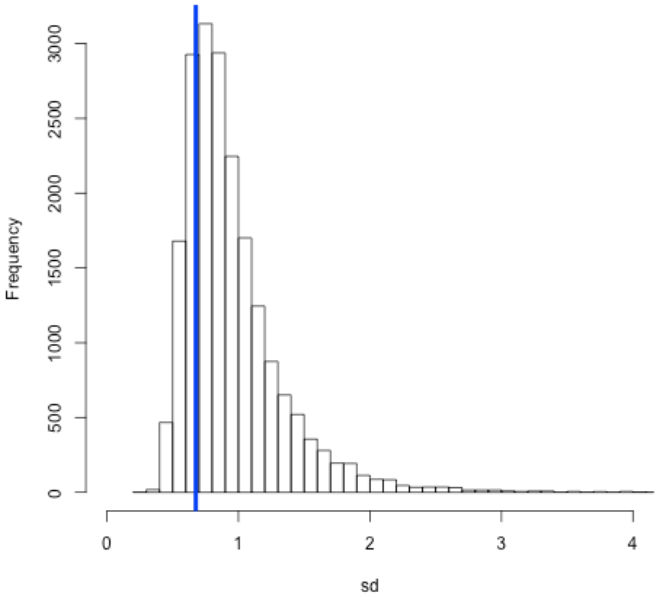
Shrink standard deviations towards common value

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

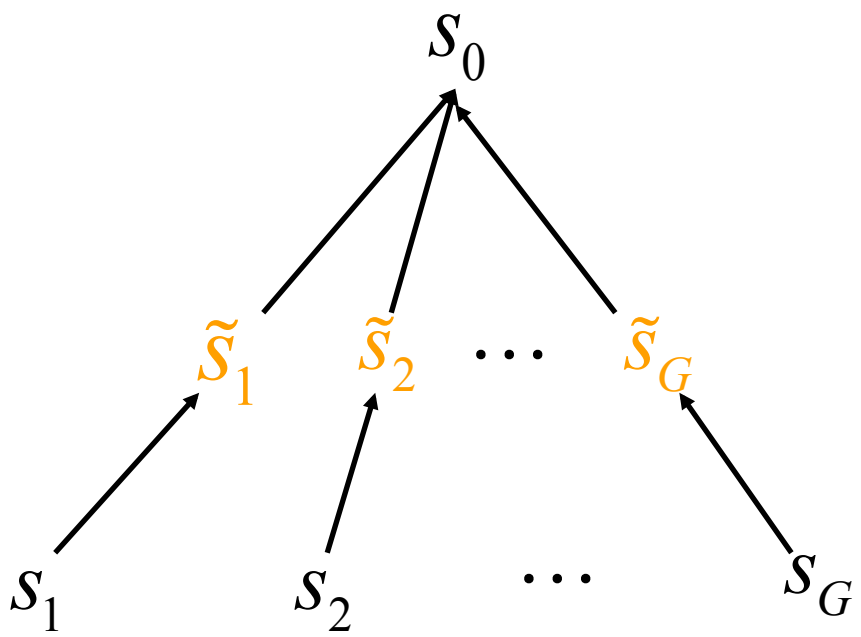
d = degrees of freedom

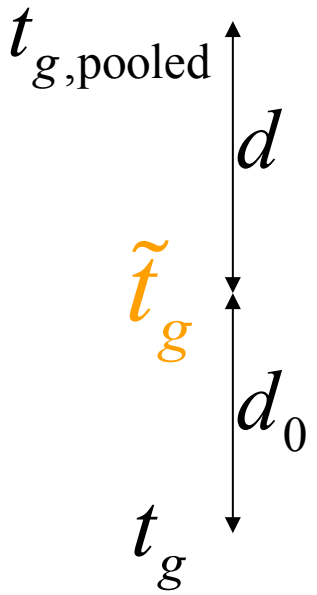
Moderated t-statistics

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



Shrinkage of standard deviations





The **data decides** whether $\tilde{t}_g$ should be closer to $t_{g,\text{pooled}}$ or to t_g

Why does it work?

- We learn what is the typical variability level by looking at all genes, but allow some flexibility from this for individual genes
- Adaptive – data (through hyperparameter estimates, d_0 and s_0) suggests how much to "squeeze" toward common value

Hierarchical model for variances

Data

$$s_g^2 \sim \sigma_g^2 \frac{\chi^2_{d_g}}{d_g}$$

Prior

$$\frac{1}{\sigma_g^2} \sim s_0^2 \frac{\chi^2_{d_0}}{d_0}$$

Posterior

$$E\left(\frac{1}{\sigma_g^2} \mid s_g^2\right) = \frac{d_0 + d_g}{s_0^2 d_0 + s_g^2 d_g}$$

Posterior Statistics

Posterior variance estimators

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

Moderated t-statistics

$$\tilde{t}_{gj} = \frac{\hat{\beta}_{gj}}{\tilde{s}_g \sqrt{c_{gj}}}$$

Baldi & Long 2001, Wright & Simon 2003, Smyth 2004

Exact distribution for moderated t

An **unexpected piece of mathematics** shows that, under the null hypothesis,

$$\tilde{t}_g \sim t_{d_0+d_g}$$

The degrees of freedom add!

The Bayes prior in effect adds d_0 extra arrays for estimating the variance.

Wright and Simon 2003, Smyth 2004

Aside: Marginal Distributions to calculate

Under usual likelihood model, s_g is independent of the estimated coefficients.

Under the hierarchical model, s_g is independent of the moderated t-statistics instead

$$s_g^2 \sim s_0^2 F_{d,d_0}$$

Multiple testing and adjusted p-values

- Each statistical test has an associated false error rate
- Traditional method in statistics is to control family wise error rate, e.g., by Bonferroni.
- Controlling the false discovery rate (FDR) is more **appropriate** in microarray studies
- Benjamini and Hochberg method controls expected FDR for independent or weakly dependent test statistics. Simulation studies support use for genomic data.
- All methods can be implemented in terms of adjusted p-values.

Linear Models

- In general, need to specify:
  - Dependent variable
  - Explanatory variables (experimental design, covariates, etc.)
- More generally:

$$y = X\beta + \epsilon$$

vector of observed data

design matrix

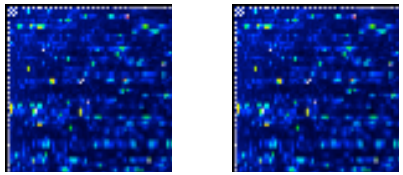
Vector of parameters to estimate

Linear Models for microarrays

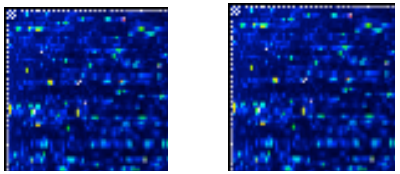
- Analyse all arrays together combining information in optimal way
- Combined estimation of precision
- Extensible to arbitrarily complicated experiments
- **Design matrix**: specifies RNA targets used on arrays
- **Contrast matrix**: specifies which comparisons are of interest

Design → Linear models

WT x 2



Mutant x 2



$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ 1 & 0 \\ 1 & 1 \\ 1 & 1 \end{bmatrix} \begin{bmatrix} \beta_1 \\ \beta_2 \end{bmatrix}$$

β_1 = wt log-expression

β_2 = mutant − wt

$E[y_1]=E[y_2]=\beta_1$ $E[y_3]=E[y_4]=\beta_1+\beta_2$