



## Announcements

- Journal clubs: please upload your slides to #papers channel
- Next week: start with 1 journal club (Y27-H-46), then go to computer room (Y01-F-50) for the first ‘workflow’ day, where you get to see all the steps of a computational pipeline (+ exercise)
- led by Dr. Charlotte Soneson: if you want to run on your local computer, some setup is required and Charlotte will share details on Slack



# limma fundamentals

## 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] ?

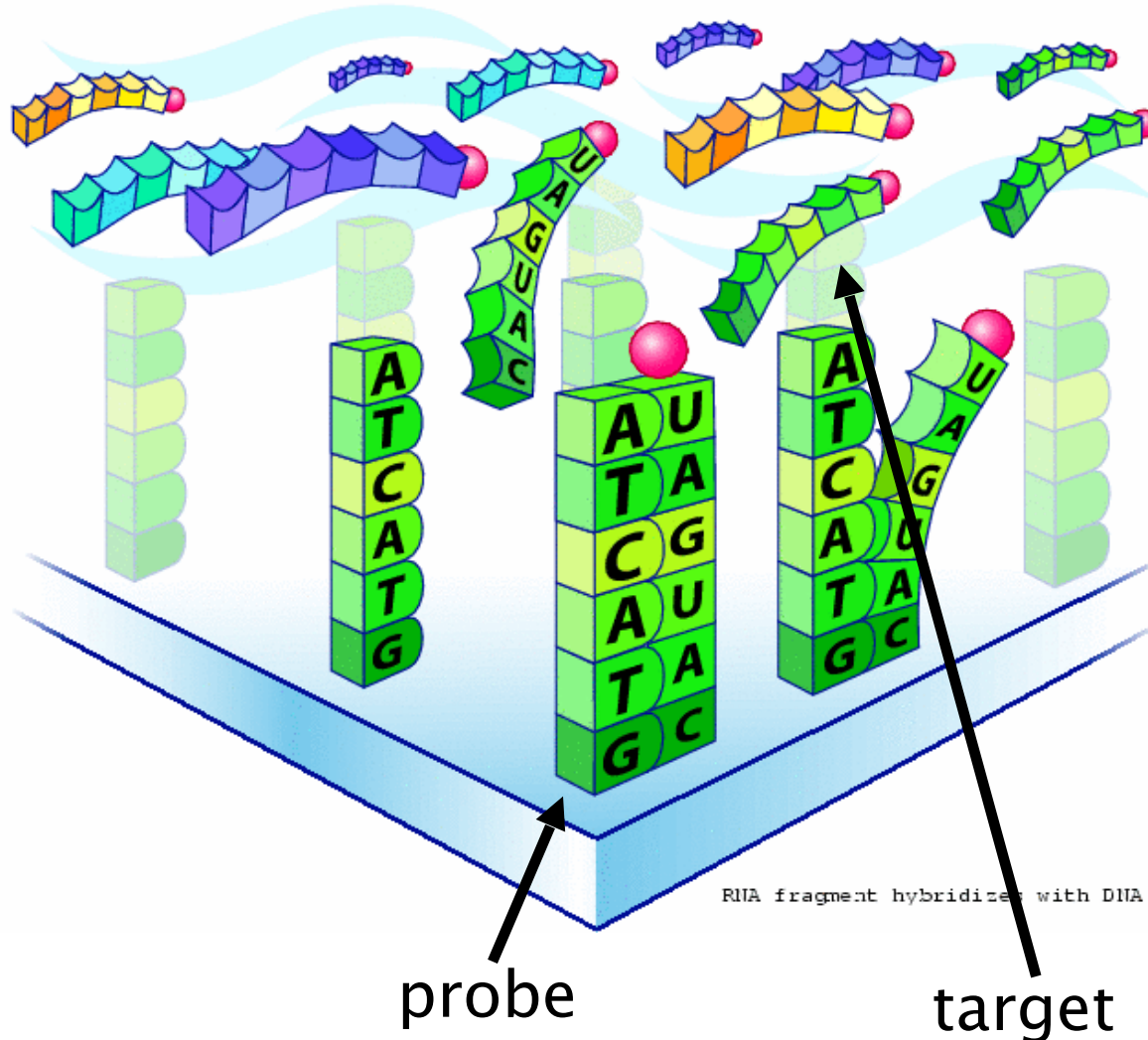
```
> head(y)
      group0      group0      group0      group1      group1      group1
gene1 -0.1874854  0.2584037 -0.05550717 -0.4617966 -0.3563024 -0.03271432
gene2 -3.5418798 -2.4540999  0.11750996 -4.3270442 -5.3462622 -5.54049106
gene3 -0.1226303  0.9354707 -1.10537767 -0.1037990  0.5221678 -1.72360854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

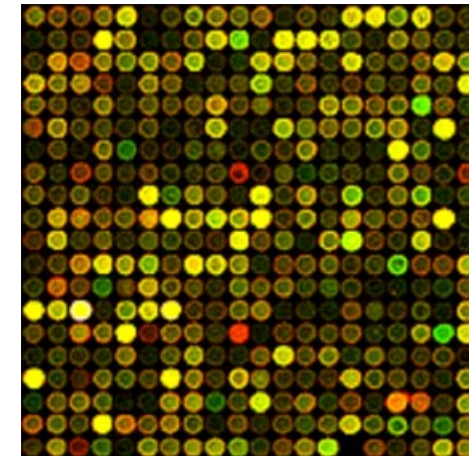


# DNA microarray: arrays of northern blots

RNA fragments with fluorescent tags from sample to be tested



Abundance (of  
complementary DNA  
species) measured by  
fluorescence intensity

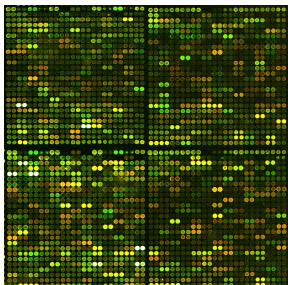


RNA fragment hybridizes with DNA on GeneChip



## Microarray expression measures

Two-colour



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

array

probe or gene

Affymetrix



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

Illumina

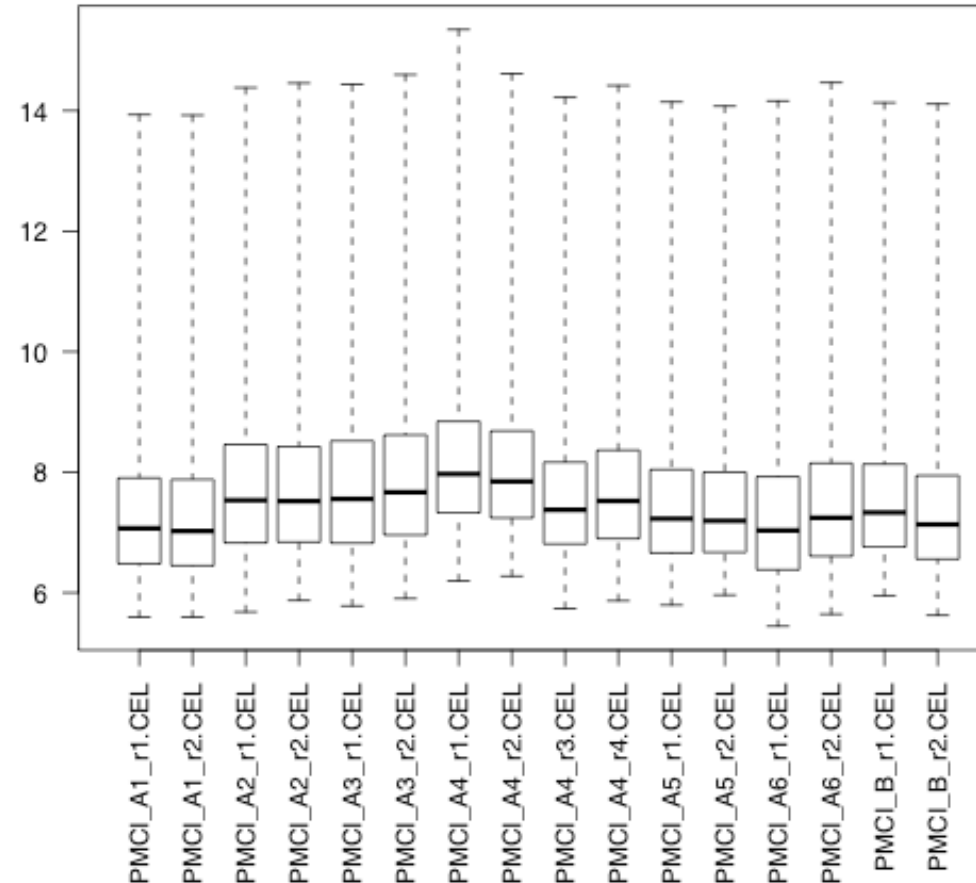


$$y_{ga} = \text{log-intensity (summarized over beads)}$$

## Normalization: one-colour



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





## Preprocessing: additive + multiplicative error model

Observe intensity for one probe on one array

$$\text{Intensity} = \text{background} + \text{signal}$$

$$I = B + S$$

additive  
errors

multiplicative errors

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

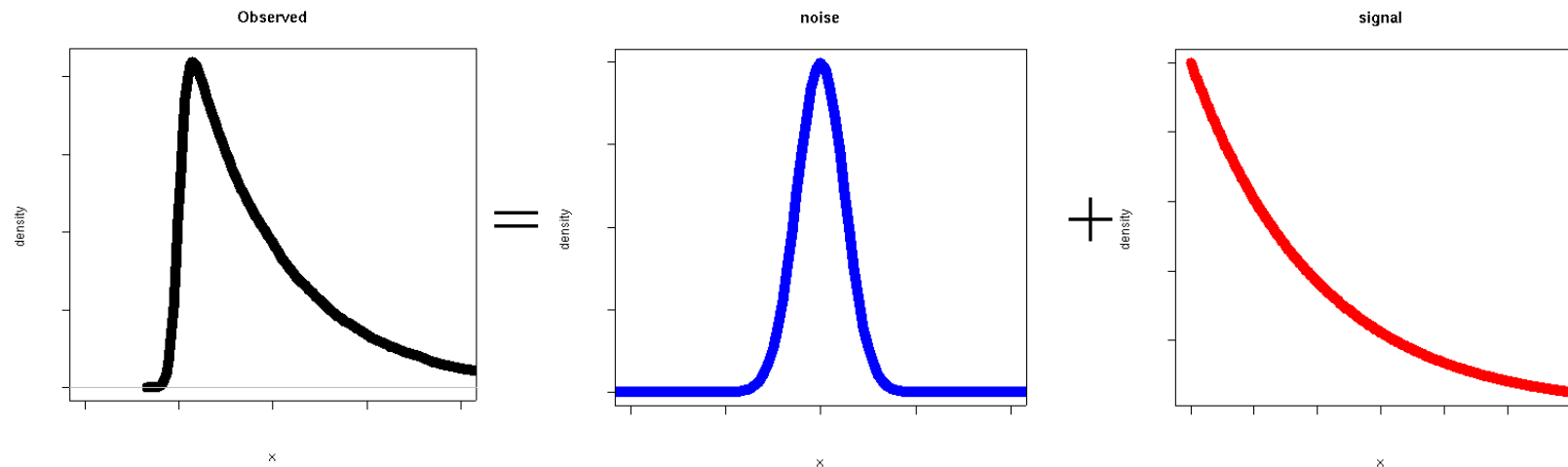


# normexp convolution model

$$\text{Intensity} = \text{Background} + \text{Signal}$$

$N(\mu, \sigma^2)$

Exponential( $\alpha$ )



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

JEREMY D. SILVER

Bioinformatics Division, Walter and Eliza Hall Institute, Parkville 3050, Victoria, Australia and  
Department of Biostatistics, University of Copenhagen, Øster Farimagsgade 5, Entrance B,  
PO Box 2099, DK-1014 Copenhagen K, Denmark  
j.silver@biostat.ku.dk

MATTHEW E. RITCHIE

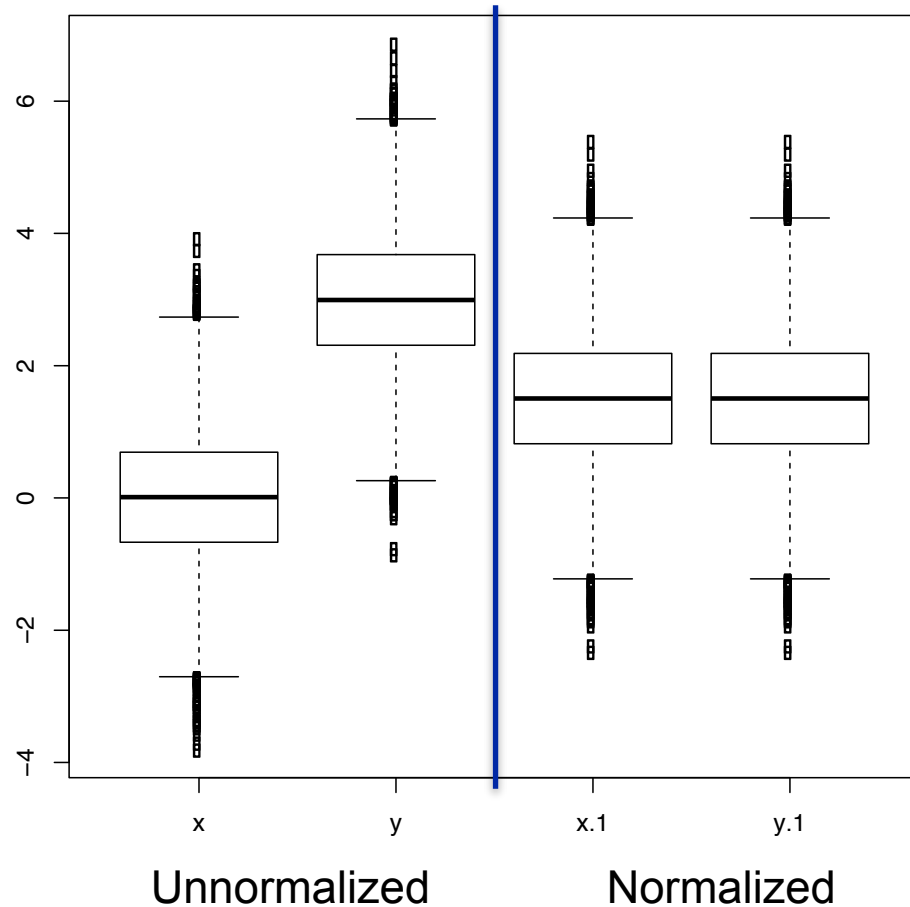
Department of Oncology, University of Cambridge, Cambridge CB2 0RE, UK

GORDON K. SMYTH\*

Bioinformatics Division, Walter and Eliza Hall Institute, Parkville 3050, Victoria, Australia  
smyth@wehi.edu.au



## 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)
```

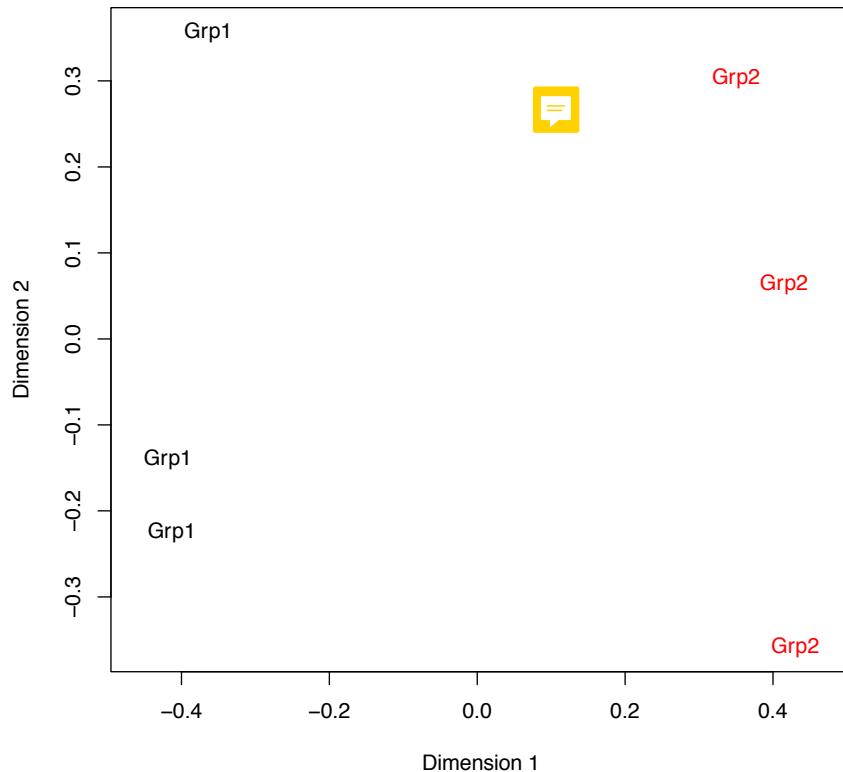
```
# impose ref. distribution by ranks
r <- apply(z,2,rank)
n <- apply(r,2,function(u) sm[u])
```

```
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
```



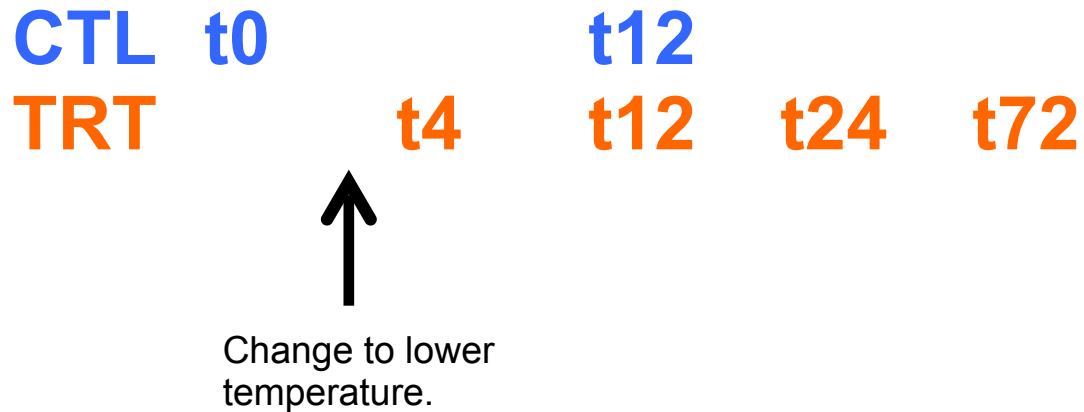
“To consult the statistician after an experiment is finished is often merely to ask him[her] to conduct a post mortem examination. He[She] can perhaps say what the experiment died of.” R. A. Fisher

## 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:

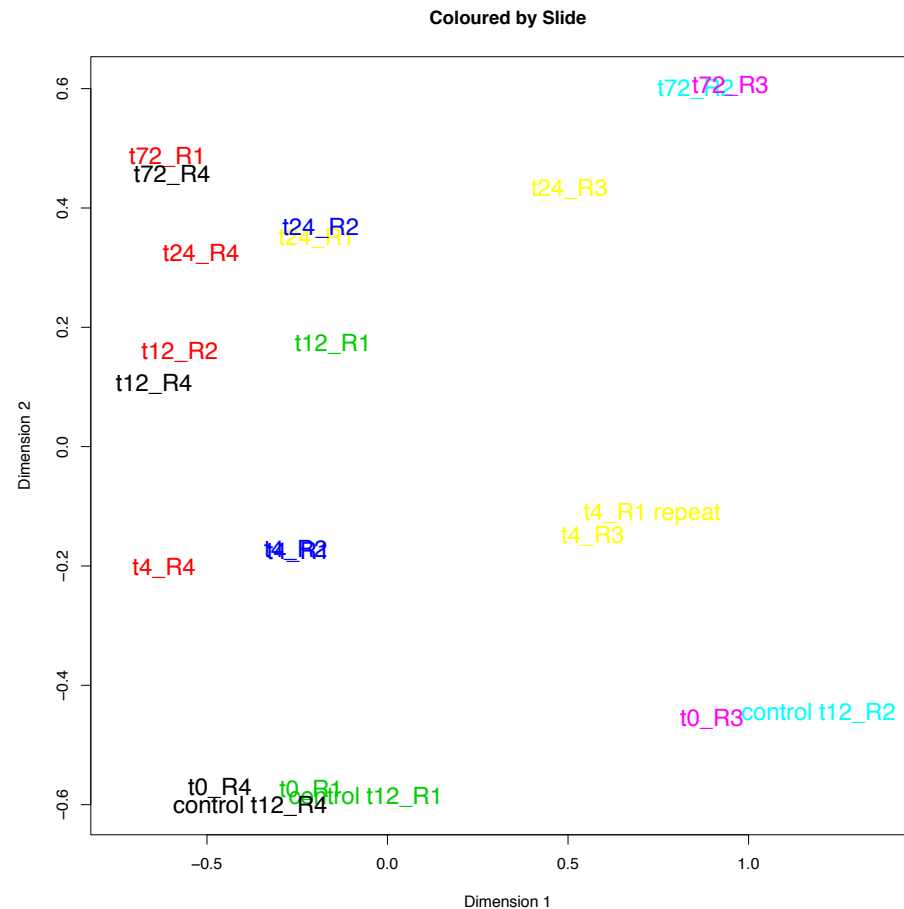
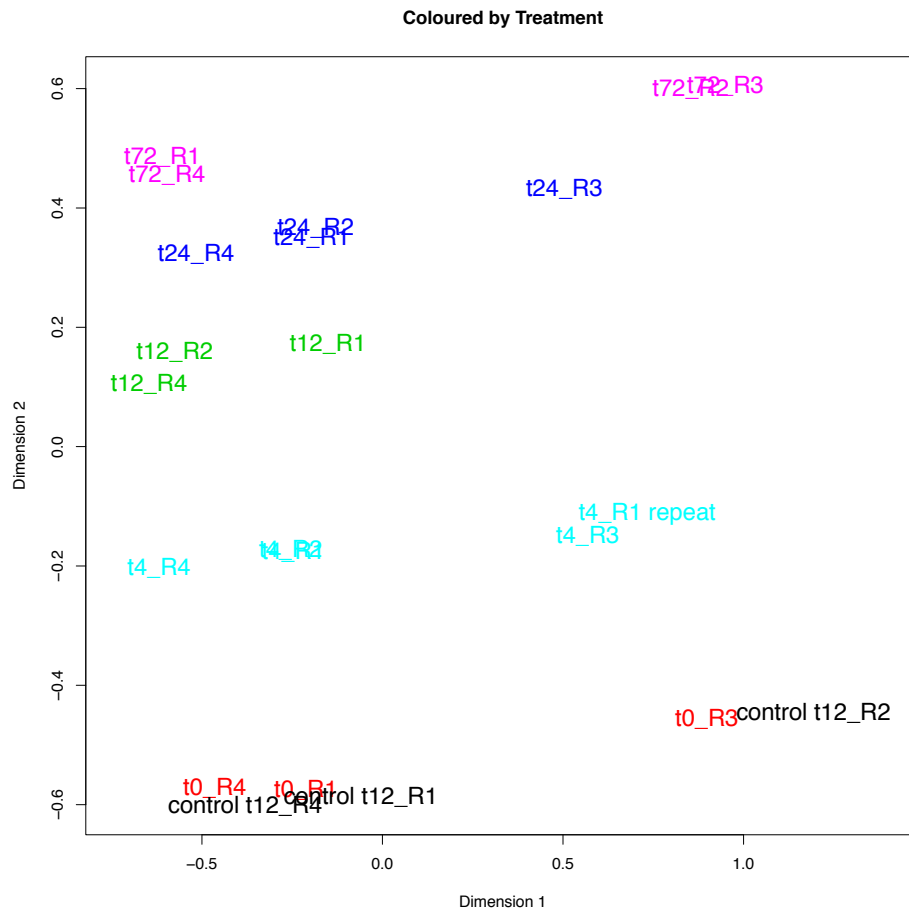


~4 replicates for each condition



```
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



22 samples x  
~20,000 genes

reduced to 22  
samples x 2  
dimensions

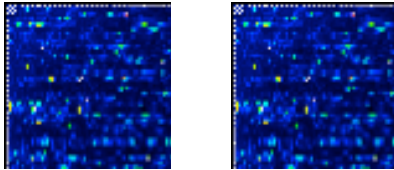


## Limma concept: borrowing information across genes

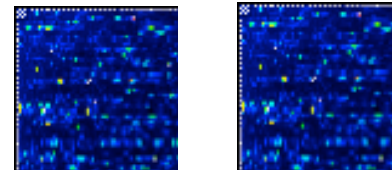
- **Small data sets**: few samples, generally under-powered for 1 gene
- **Curse of dimensionality**: many tests, need to adjust for multiple testing (= loss of power)
- **Benefit of parallelism**: same model is fit for every gene. Can borrow information from one gene to another
  - **Hard**: assume 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)

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

give very high false discovery rates

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

Residual df = 2



## t-tests with common variance

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

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

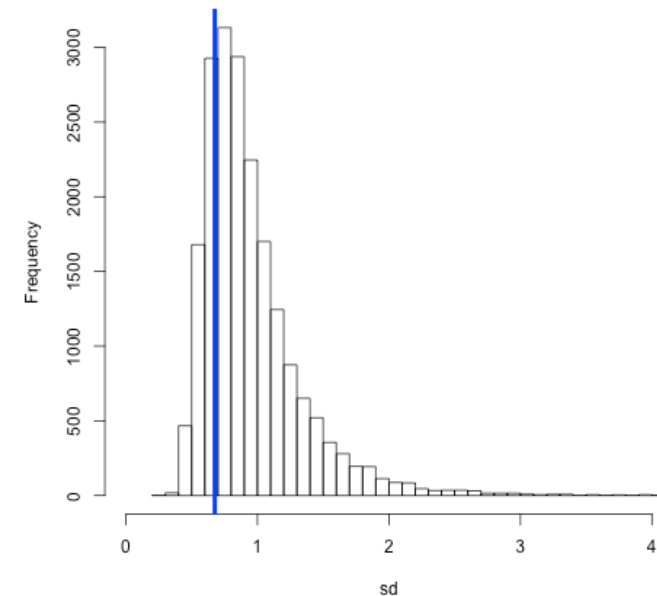
Shrink standard deviations towards common value

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

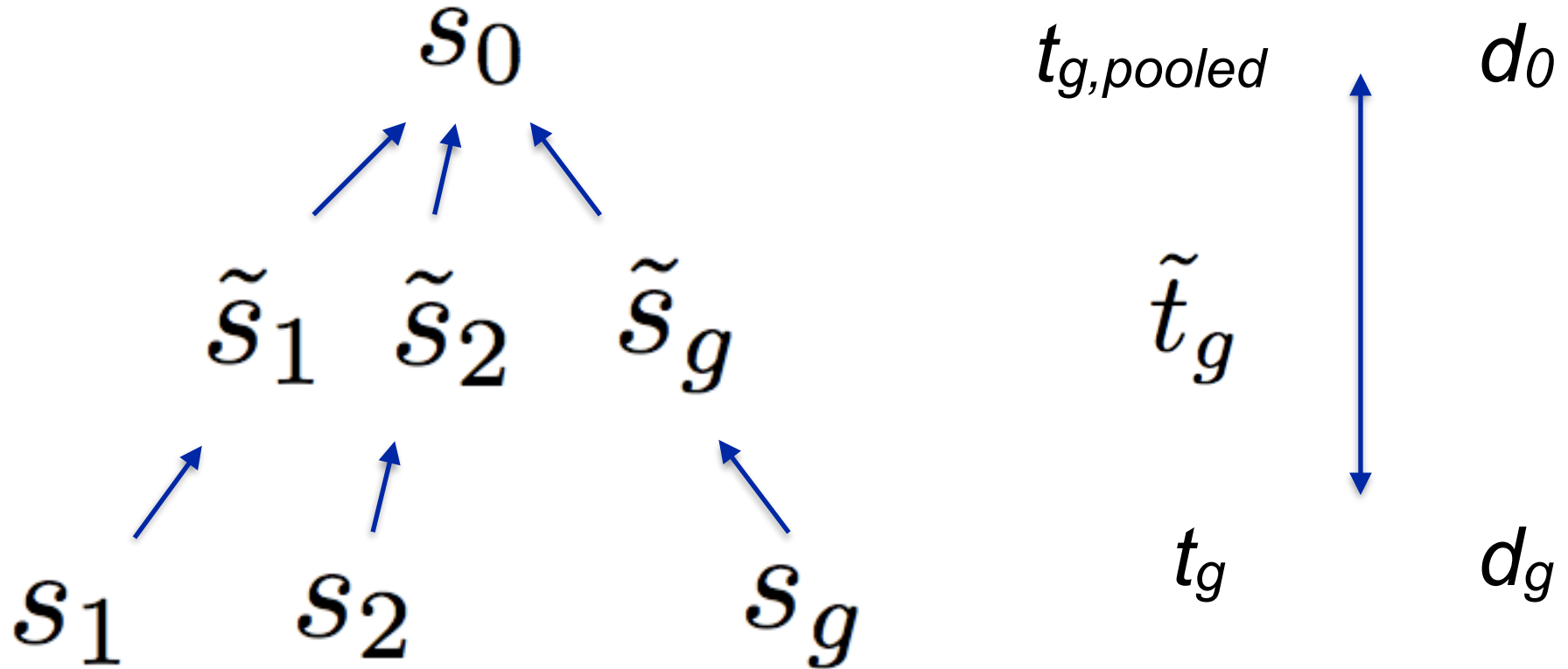
$d$  = degrees of  
freedom

Moderated t-statistics

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



## Shrinkage of standard deviations



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



## 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

$$s_g^2 \sim \sigma_g^2 \frac{\chi_{d_g}^2}{d_g}$$

Prior

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

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

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

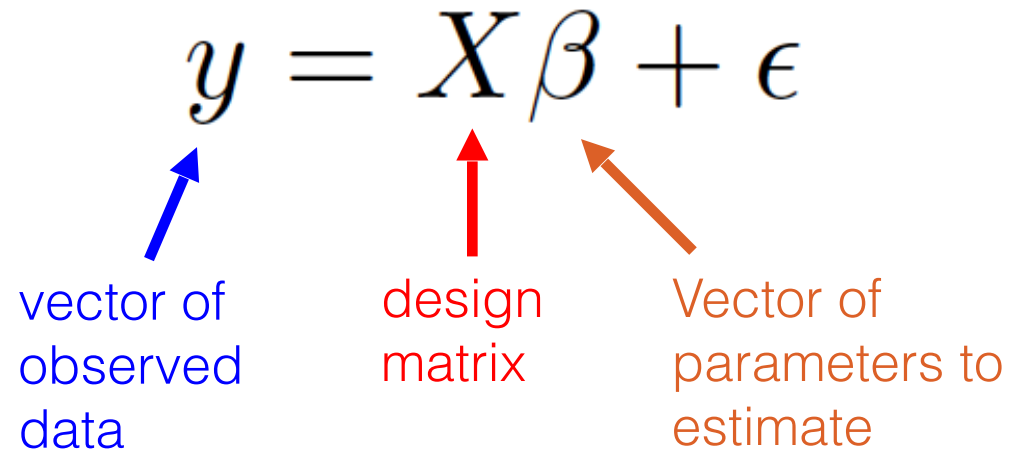
## Multiple testing and adjusted p-values

- Each statistical test has an associated false positive 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:

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


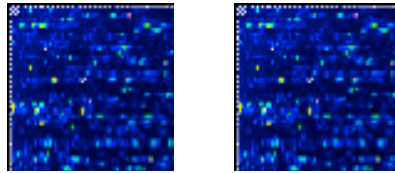
vector of  
observed  
data

design  
matrix

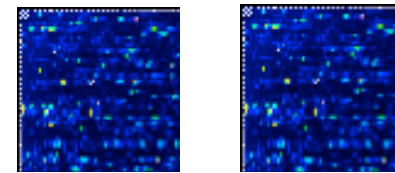
Vector of  
parameters to  
estimate

## Design → Linear models

WT x 2



Mutant x 2



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

$\beta_1$  = wt log-expression

$\beta_2$  = mutant – wt

$$E[y_1] = E[y_2] = \beta_1$$

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



## Layers to add ..

- Where does the moderated variance come from?
- Why the degrees of freedom add:  $d_0 + d$
- empirical Bayes: how to estimate the hyperparameters ( $d_0$  and  $s_0$ )
- Design matrices + contrast matrices in practice

## Unexpected mathematics: Why do degrees of freedom add?

**The construction of the classical t-statistic:**

$$Z = (\bar{X}_n - \mu) \frac{\sqrt{n}}{\sigma}$$
$$V = (n-1) \frac{S_n^2}{\sigma^2}$$
$$T \equiv \frac{Z}{\sqrt{V/\nu}} = (\bar{X}_n - \mu) \frac{\sqrt{n}}{S_n},$$

**Stated another way → Exercise (optional): what are a, b above?**

If  $T$  is distributed as  $(a/b)^{1/2} Z/U$  where  $Z \sim N(0, 1)$  and  $U \sim \chi_\nu$ , then  $T$  has density function

$$p(t) = \frac{a^{\nu/2} b^{1/2}}{B(1/2, \nu/2) (a + bt^2)^{1/2 + \nu/2}}$$

## Optional exercise: Derive the posterior

Data

$$s_g^2 \sim \sigma_g^2 \frac{\chi_{d_g}^2}{d_g}$$

Prior

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

Posterior

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

$$p(\theta|x) = \frac{f(x|\theta)p(\theta)}{\int f(x|\theta)p(\theta)d\theta}$$

Optional exercise

Sketch: i) Let  $x=s^2$ ,  $\theta=\sigma^{-2}$ ; ii) Using the functional form of chi-squared distribution, calculate only the numerator (since denominator does not contain  $\theta$ ); iii) collect terms and see if you can identify the distribution and the parameters of it; iv) What is the mean of this distribution?

## Linear Models

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

$$y = X\alpha + \epsilon$$

vector of  
observed  
data

design  
matrix

Vector of  
parameters to  
estimate

Obtain a linear model for each gene  $g$

$$E(\underline{y}_g) = X\alpha_g$$
$$\text{var}(\underline{y}_g) = W_g^{-1}\sigma_g^2$$

## Contrasts -- `contrasts.fit()`

A *contrast* is any linear combination of the coefficients  $\alpha_j$  which we want to test equal to zero.

Define contrasts

$$\beta_g = C^T \alpha_g$$

where  $C$  is the contrast matrix.

Want to test

$$H_0 : \beta_{gj} = 0$$

vs

$$H_a : \beta_{gj} \neq 0$$

## Unexpected mathematics: Why do degrees of freedom add?

$$p(\hat{\beta}, s^2 | \beta = 0) = \int p(\hat{\beta} | \sigma^{-2}, \beta = 0) p(s^2 | \sigma^{-2}) p(\sigma^{-2}) d(\sigma^{-2})$$


The integrand is

$$\begin{aligned} & \frac{1}{(2\pi v \sigma^2)^{1/2}} \exp\left(-\frac{\hat{\beta}^2}{2v\sigma^2}\right) \\ & \times \left(\frac{d}{2\sigma^2}\right)^{d/2} \frac{s^{2(d/2-1)}}{\Gamma(d/2)} \exp\left(-\frac{ds^2}{2\sigma^2}\right) \\ & \times \left(\frac{d_0 s_0^2}{2}\right)^{d_0/2} \frac{\sigma^{-2(d_0/2-1)}}{\Gamma(d_0/2)} \exp\left(-\sigma^{-2} \frac{d_0 s_0^2}{2}\right) \\ & = \frac{(d_0 s_0^2/2)^{d_0/2} (d/2)^{d/2} s^{2(d/2-1)}}{(2\pi v)^{1/2} \Gamma(d_0/2) \Gamma(d/2)} \\ & \quad \sigma^{-2(1/2+d_0/2+d/2-1)} \exp\left\{-\sigma^{-2} \left(\frac{\hat{\beta}^2}{2v} + \frac{ds^2}{2} + \frac{d_0 s_0^2}{2}\right)\right\} \end{aligned}$$



## Unexpected mathematics: Why do degrees of freedom add?

$$\begin{aligned}
 p(\hat{\beta}, s^2 \mid \beta = 0) &= \int p(\hat{\beta} \mid \sigma^{-2}, \beta = 0) p(s^2 \mid \sigma^{-2}) p(\sigma^{-2}) d(\sigma^{-2}) \\
 &= \frac{(d_0 s_0^2 / 2)^{d_0/2} (d/2)^{d/2} s^{2(d/2-1)}}{(2\pi v)^{1/2} \Gamma(d_0/2) \Gamma(d/2)} \\
 &\quad \sigma^{-2(1/2+d_0/2+d/2-1)} \exp \left\{ -\sigma^{-2} \left( \frac{\hat{\beta}^2}{2v} + \frac{ds^2}{2} + \frac{d_0 s_0^2}{2} \right) \right\}
 \end{aligned}$$


  
 $\sigma^{-2}$  is chi-squared (or gamma)

$$f(x; k) = \begin{cases} \frac{x^{(k/2)-1} e^{-x/2}}{2^{k/2} \Gamma(\frac{k}{2})}, & x \geq 0; \\ 0, & \text{otherwise.} \end{cases}$$

[http://en.wikipedia.org/wiki/Chi-squared\\_distribution](http://en.wikipedia.org/wiki/Chi-squared_distribution)



## Unexpected mathematics: Why do degrees of freedom add?

$$p(\hat{\beta}, s^2 | \beta = 0) = \int p(\hat{\beta} | \sigma^{-2}, \beta = 0) p(s^2 | \sigma^{-2}) p(\sigma^{-2}) d(\sigma^{-2})$$

$$\begin{aligned} p(\hat{\beta}, s^2 | \beta = 0) \\ = \frac{(1/2v)^{1/2} (d_0 s_0^2/2)^{d_0/2} (d/2)^{d/2} s^{2(d/2-1)}}{D(1/2, d_0/2, d/2)} \left( \frac{\hat{\beta}^2/v + d_0 s_0^2 + d s^2}{2} \right)^{-(1+d_0+d)/2} \end{aligned}$$

## Unexpected mathematics: Why do degrees of freedom add?

$$p(\hat{\beta}, s^2 \mid \beta = 0) = \frac{(1/2v)^{1/2} (d_0 s_0^2/2)^{d_0/2} (d/2)^{d/2} s^{2(d/2-1)}}{D(1/2, d_0/2, d/2)} \left( \frac{\hat{\beta}^2/v + d_0 s_0^2 + d s^2}{2} \right)^{-(1+d_0+d)/2}$$

The null joint distribution of  $\tilde{t}$  and  $s^2$  is

$$p(\tilde{t}, s^2 \mid \beta = 0) = \tilde{s} v^{1/2} p(\hat{\beta}, s^2 \mid \beta = 0)$$

[http://en.wikipedia.org/wiki/Random\\_variable#Distribution\\_functions\\_of\\_random\\_variables](http://en.wikipedia.org/wiki/Random_variable#Distribution_functions_of_random_variables)

$$f_Y(y) = f_X(g^{-1}(y)) \left| \frac{dg^{-1}(y)}{dy} \right|$$

## Unexpected mathematics: Why do degrees of freedom add?

If  $T$  is distributed as  $(a/b)^{1/2}Z/U$  where  $Z \sim N(0, 1)$  and  $U \sim \chi_\nu$ , then  $T$  has density function

$$p(t) = \frac{a^{\nu/2} b^{1/2}}{B(1/2, \nu/2) (a + bt^2)^{1/2 + \nu/2}}$$

$$p(\tilde{t}, s^2 | \beta = 0) = \frac{(d_0 s_0^2)^{d_0/2} d^{d/2} s^{2(d/2-1)}}{B(d/2, d_0/2) (d_0 s_0^2 + ds^2)^{d_0/2 + d/2}} \\ \times \frac{(d_0 + d)^{-1/2}}{B(1/2, d_0/2 + d/2)} \left( 1 + \frac{\tilde{t}^2}{d_0 + d} \right)^{-(1+d_0+d)/2}$$

This shows that  $\tilde{t}$  and  $s^2$  are independent with

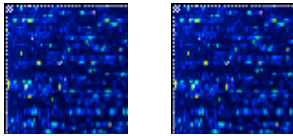
$$s^2 \sim s_0^2 F_{d, d_0}$$

and

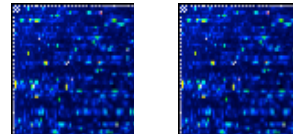
$$\tilde{t} | \beta = 0 \sim t_{d_0+d}.$$

## Analysis of Variance → Linear model

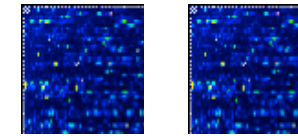
WT x 2



Cond A x 2



Cond B x 2



$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 \\ 1 & 0 & 0 \\ 1 & 1 & 0 \\ 1 & 1 & 0 \\ 1 & 0 & 1 \\ 1 & 0 & 1 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} + \begin{bmatrix} \epsilon_1 \\ \epsilon_2 \\ \epsilon_3 \\ \epsilon_4 \\ \epsilon_5 \\ \epsilon_6 \end{bmatrix}$$

$\alpha_1$  = wt log-expression

$\alpha_2$  = Cond A - wt

$\alpha_3$  = Cond B - wt

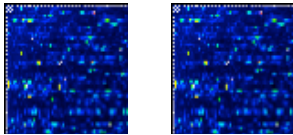
$$E[y_1] = E[y_2] = \alpha_1$$

$$E[y_3] = E[y_4] = \alpha_1 + \alpha_2$$

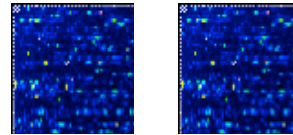
$$E[y_5] = E[y_6] = \alpha_1 + \alpha_3$$

## Analysis of Variance → Linear model, alternative parameterization

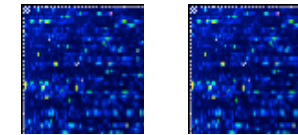
WT x 2



Cond A x 2



Cond B x 2



$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \\ 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} + \begin{bmatrix} \epsilon_1 \\ \epsilon_2 \\ \epsilon_3 \\ \epsilon_4 \\ \epsilon_5 \\ \epsilon_6 \end{bmatrix}$$

$\alpha_1 = \text{wt log-expression}$   
 $\alpha_2 = \text{Cond A log-expression}$   
 $\alpha_3 = \text{Cond B log-expression}$

$$E[y_1] = E[y_2] = \alpha_1$$

$$E[y_3] = E[y_4] = \alpha_2$$

$$E[y_5] = E[y_6] = \alpha_3$$

## An example use of design and contrast matrices

**design matrix**

$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \\ 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} + \begin{bmatrix} \epsilon_1 \\ \epsilon_2 \\ \epsilon_3 \\ \epsilon_4 \\ \epsilon_5 \\ \epsilon_6 \end{bmatrix}$$

$$\begin{aligned} E[y_1] &= E[y_2] = \alpha_1 \\ E[y_3] &= E[y_4] = \alpha_2 \\ E[y_5] &= E[y_6] = \alpha_3 \end{aligned}$$

**contrast matrix**

$$\beta = C\alpha = \begin{bmatrix} -1 & 1 & 0 \\ 0 & -1 & 1 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} = \begin{bmatrix} \alpha_2 - \alpha_1 \\ \alpha_3 - \alpha_2 \end{bmatrix}$$

## Contrasts -- `contrasts.fit()`

A *contrast* is any linear combination of the coefficients  $\alpha_j$  which we want to test equal to zero.

Define contrasts

$$\beta_g = C^T \alpha_g$$

where  $C$  is the contrast matrix.

Want to test

$$H_0 : \beta_{gj} = 0$$

vs

$$H_a : \beta_{gj} \neq 0$$



## Limma / Analysis of Variance

$$F = \frac{\text{variance between treatments}}{\text{variance within treatments}}$$

$$F = \frac{MS_{\text{Treatments}}}{MS_{\text{Error}}} = \frac{SS_{\text{Treatments}}/(I - 1)}{SS_{\text{Error}}/(n_T - I)}$$

The moderated  $t$ -statistics also lead naturally to moderated  $F$ -statistics which can be used to test hypotheses about any set of contrasts simultaneously. Appropriate quadratic forms of moderated  $t$ -statistics follow  $F$ -distributions just as do quadratic forms of ordinary  $t$ -statistics. Suppose that we wish to test all contrasts for a given gene equal to zero, i.e.,  $H_0 : \beta_g = 0$ . The correlation matrix of  $\hat{\beta}_g$  is  $R_g = U_g^{-1}C^T V_g C U_g^{-1}$  where  $U_g$  is the diagonal matrix with unscaled standard deviations  $(v_{gj})^{1/2}$  on the diagonal. Let  $r$  be the column rank of  $C$ . Let  $Q_g$  be such that  $Q_g^T R_g Q_g = I_r$  and let  $\mathbf{q}_g = Q_g^T \mathbf{t}_g$ . Then

$$F_g = \mathbf{q}_g^T \mathbf{q}_g / r = \mathbf{t}_g^T Q_g Q_g^T \mathbf{t}_g / r \sim F_{r, d_0 + d_g}$$

## Aside: Marginal Distributions to calculate

Fun fact: 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

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

Thus, the set of  $s_g$  can be used to estimate  $d_0$  and  $s_0$

Section 6.2 limma paper: other tricks, such as Fisher's z distribution to estimate  $d_0$  and  $s_0$



## Relate to limma objects

$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \\ 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} + \begin{bmatrix} \epsilon_1 \\ \epsilon_2 \\ \epsilon_3 \\ \epsilon_4 \\ \epsilon_5 \\ \epsilon_6 \end{bmatrix}$$

$$E[y_1] = E[y_2] = \alpha_1$$

$$E[y_3] = E[y_4] = \alpha_2$$

$$E[y_5] = E[y_6] = \alpha_3$$

$$\beta = C\alpha = \begin{bmatrix} -1 & 1 & 0 \\ 0 & -1 & 1 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} = \begin{bmatrix} \alpha_2 - \alpha_1 \\ \alpha_3 - \alpha_2 \end{bmatrix}$$

```
> design
  alpha1 alpha2 alpha3
1      1      0      0
2      1      0      0
3      0      1      0
4      0      1      0
5      0      0      1
6      0      0      1
> cont.matrix <- makeContrasts(beta1="alpha2-alpha1",
                               beta2="alpha3-alpha2", levels=design)
> cont.matrix
      Contrasts
Levels  beta1 beta2
alpha1   -1     0
alpha2    1    -1
alpha3    0     1

fit <- lmFit(y, design)

fit.c <- contrasts.fit(fit, cont.matrix)
fit.c <- eBayes(fit.c)

> head(round(y, 2), 3)
      [,1] [,2] [,3] [,4] [,5] [,6]
[1,] -1.62  1.49  2.50  1.57 -0.71  0.38
[2,] -4.50 -4.95 -3.66 -7.83 -1.59  6.94
[3,] -10.17 -21.90 14.03  3.66 -12.21 -15.26

> head(round(fit$coef, 2), 3)
      alpha1 alpha2 alpha3
[1,]  -0.07   2.03  -0.16
[2,]  -4.73  -5.75   2.67
[3,] -16.04   8.85 -13.74

> head(round(fit.c$coef, 2), 3)
      Contrasts
      beta1  beta2
[1,]   2.10  -2.20
[2,]  -1.02   8.42
[3,]  24.89 -22.59
```