10-810 /02-710
Computational Genomics

Differentially Expressed Genes

Data analysis

- Normalization
- Combining results from replicates
- Identifying differentially expressed genes
- Dealing with missing values
- Static vs. time series

Motivation

- In many cases, this is the goal of the experiment.
- Such genes can be key to understanding what goes wrong / or get fixed under certain condition (cancer, stress etc.).
- In other cases, these genes can be used as ‘features’ for a classifier.
- These genes can also serve as a starting point for a model for the system being studied (e.g. cell cycle, phermone response etc.).

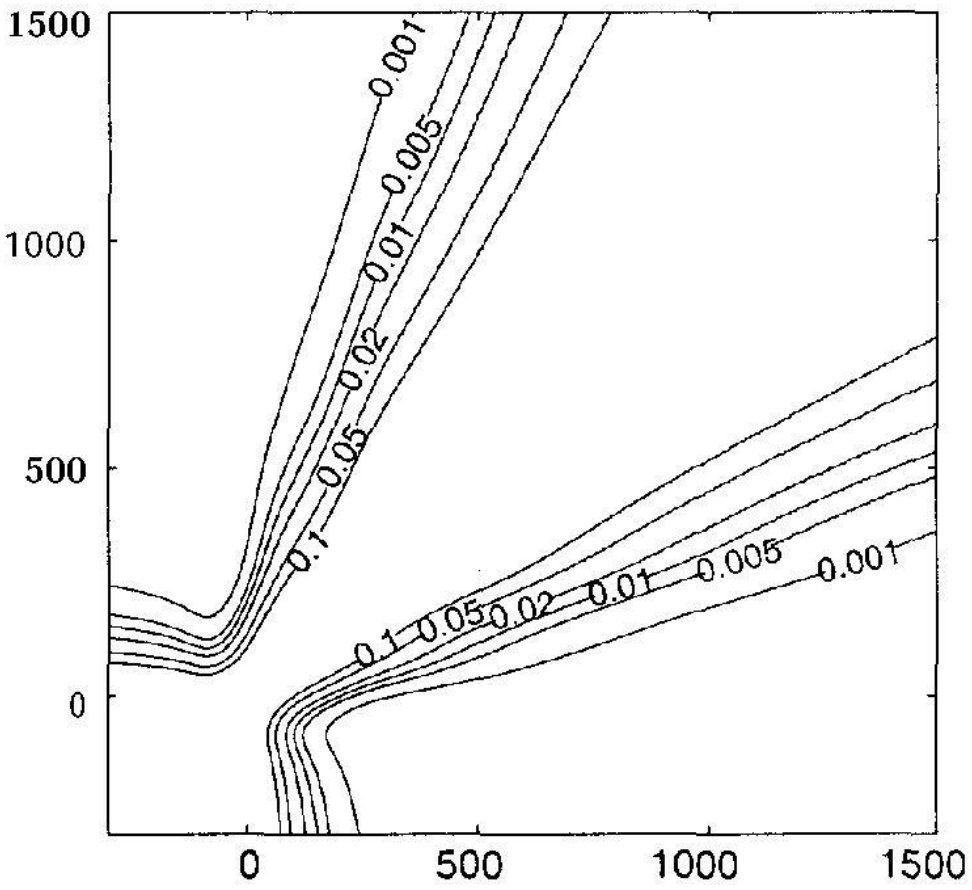
Problems

- As mentioned in previous lectures, differences in expression values can result from many different noise sources.
- Some of these can be related to the technology and so would differ between microarrays and sequencing methods while others are related to biological / experimental variations.
- Our goal is to identify the ‘real’ differences, that is, differences that cannot be explained by the various errors introduced during the experimental phase.
- Need to understand both the experimental protocol and take into account the underlying biology / chemistry

The ‘wrong’ way

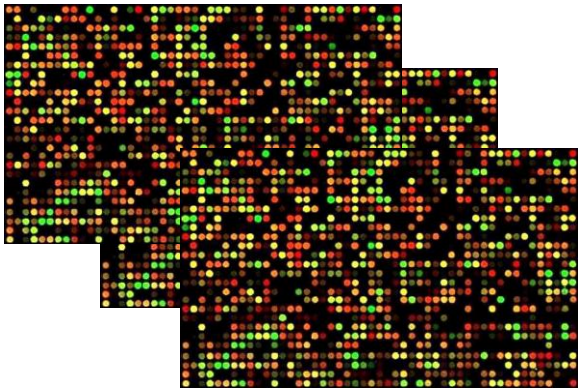
- During the early days (though some continue to do this today) the common method was to select genes based on their fold change between experiments.
- The common value was 2 (or absolute log of 1).
- Obviously this method is not perfect …

Significance bands for Affy arrays

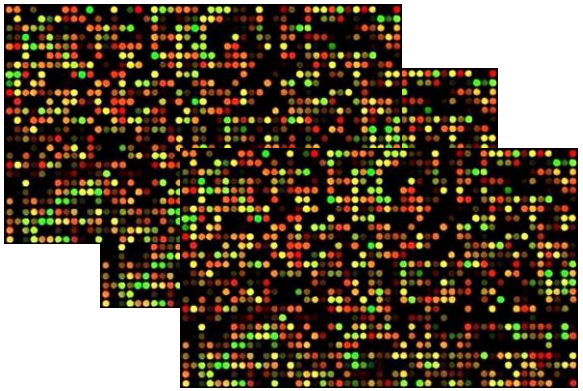


Typical experiment: replicates

healthy



cancer



Dye swap: reverse the color code between arrays

Clinical replicates: samples from different individuals

Hypothesis testing

- A general way of identifying differentially expressed genes is by testing two hypothesis
- Let g_A denote the mean expression of gene *g* under condition *A* (say healthy) and g_B be the mean expression under condition *B* (cancer).
- In this case we can test the following hypotheses:

  $\mathbf{H_0}$ (or the null hypothesis): $g_A = g_B$

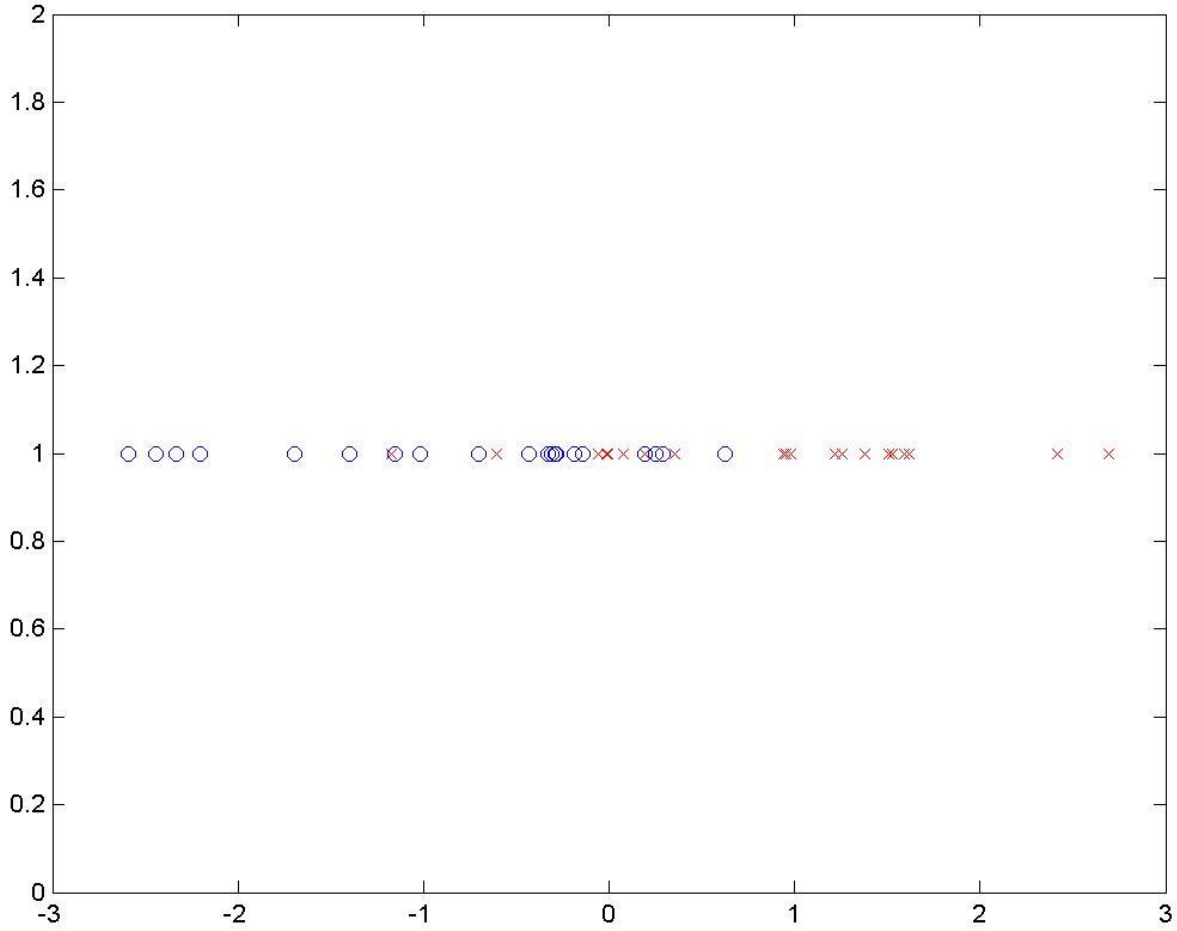
  $\mathbf{H_1}$ (or the alternative hypothesis): $g_A \neq g_B$

- If we *reject* $\mathbf{H_0}$ then gene g has a different mean under the two conditions, and so is *differentially expressed*

P-value

- Using hypothesis testing we need determine our confidence in the resulting decision
- This is done using a *test statistics* which indicates how strongly the data we observe supports our decision
- A p-value (or probability value) measures how likely it is to see the data we observed under the null hypothesis
- Small p-values indicate that it is very unlikely that the data was generated according to the null hypothesis

Example: Measurements for one gene in 40 (20+20) experiments of two conditions



Test statistics

- To determine a p-value we need to chose a test statistic
- There are several possible methods that have been suggested and used for gene expression
  - one sample *t* test
  - two sample *t* test
  - non parametric rank tests
  - etc.
- Each requires certain assumptions and you should make sure you understand what they are before using the test.
- We will discuss one such test that is focused on log likelihood ratios using the χ^2 distribution.

Hypothesis testing: Log likelihood ratio test

- If we have a probabilistic model for gene expression we can compute the likelihood of the data given the model.
- In our case, lets assume that gene expression is normally distributed with different mean under the different conditions and the same variance.
- Thus for the alternative hypothesis (**H_1**) we have:

$$y_A \sim N(\mu_A, \sigma^2) \quad y_B \sim N(\mu_B, \sigma^2)$$

and for the null hypothesis (**H_0**) we have:

$$y_A \sim N(\mu, \sigma^2) \quad y_B \sim N(\mu, \sigma^2)$$

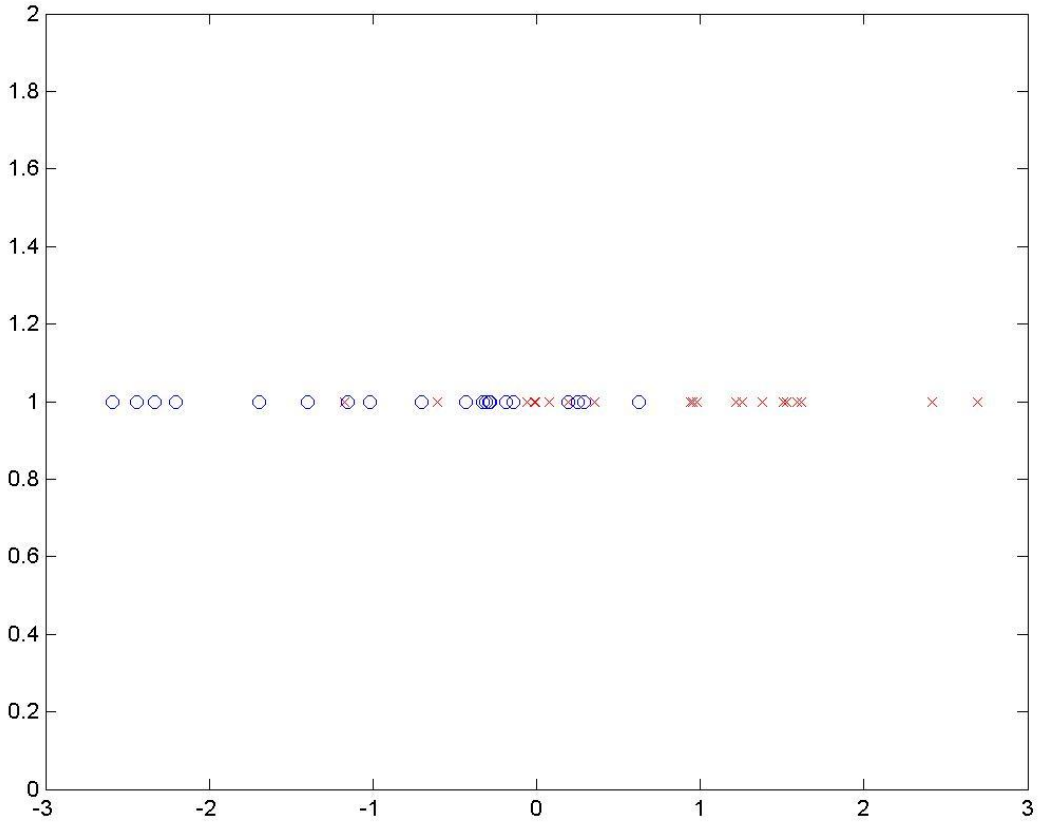
- We can compute the estimated means and variance from the data (and thus we will be using the *sample mean* and *sample variance*)

Example mean

Blue mean: -0.81

Red mean: 0.84

Combined mean: 0.02



Data likelihood

- Given our model, the likelihood of the data under the two hypothesis is:

$$L(0) = \prod_{i \in A} \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(y^i - \mu)^2}{2\sigma^2}} \prod_{i \in B} \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(y^i - \mu)^2}{2\sigma^2}}$$

$$L(1) = \prod_{i \in A} \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(y^i - \mu_A)^2}{2\sigma^2}} \prod_{i \in B} \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(y^i - \mu_B)^2}{2\sigma^2}}$$

- We can also compute the *ratio* of the likelihoods (L(1)/L(0))
- Intuitively, the higher this ratio the *more* likely it is that the data was indeed generated according to the alternative hypothesis (and thus the genes are differentially expressed).

Log likelihood ratio test

- Here we assume the *same* variance for both hypotheses
- We use the *log of the likelihood ratio*, and after using our simplifying assumption arrive it:

$$T = 2\ln\frac{L(1)}{L(0)} = 2\ln e^{\left(\sum_{i\in A}(y^i-\mu)^2+\sum_{i\in B}(y^i-\mu)^2-\sum_{i\in A}(y^i-\mu_A)^2+\sum_{i\in B}(y^i-\mu_B)^2\right)/2\sigma^2}$$

Log likelihood ratio test

- We use the *log of the likelihood ratio*, and after simplifying arrive it:

$$T = 2\ln\frac{L(1)}{L(0)} = \left(\sum_{i\in A}(y^i-\mu)^2 + \sum_{i\in B}(y^i-\mu)^2 - \sum_{i\in A}(y^i-\mu_A)^2 - \sum_{i\in B}(y^i-\mu_B)^2\right)/\sigma^2$$

- *T* is our test statistics, and in this case can be shown to be distributed as χ^2

Log likelihood ratio test

- We use the *log of the likelihood ratio*, and after simplifying arrive it:

$$T = 2\ln\frac{L(1)}{L(0)} = \left(\sum_{i\in A}(y^i-\mu)^2 + \sum_{i\in B}(y^i-\mu)^2 - \sum_{i\in A}(y^i-\mu_A)^2 + \sum_{i\in B}(y^i-\mu_B)^2\right)/\sigma^2$$

Log likelihood ratio is an important test statistics which is very commonly used in hypothesis testing

Degrees of freedom

- We are almost done …
- We still need to determine one more value in order to use the test
- Degrees of freedom for likelihood ratio tests depends on the difference in the number of *free parameters*
- In this case, our free parameters are the mean and variance
- Thus the difference is …

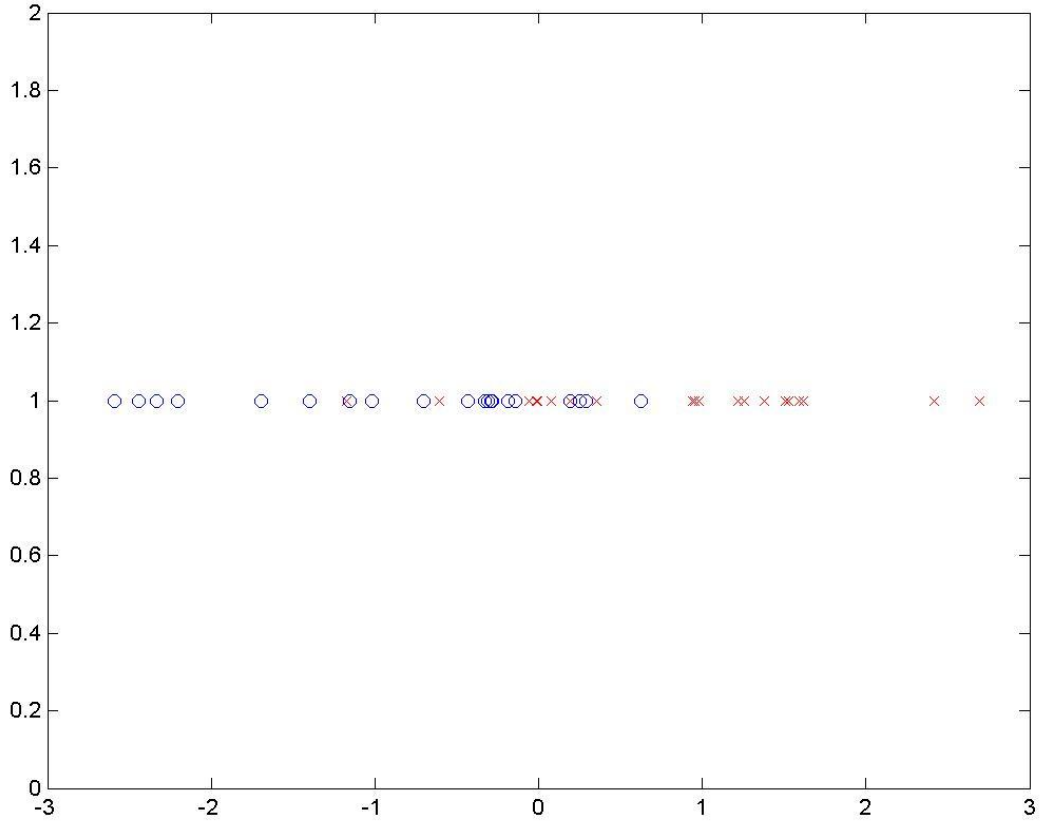
- In this case, the difference is 1 (two means vs. one)

Example: Log likelihood ratio

T = 2*(64.3/37.1) = 3.46

D.O.F = 1

P-value = 0.06



Limitations

- We assumed a specific probabilistic model (Gaussian noise) which may not actually capture the true noise factors
- We may need many replicates to derive significant results
- Multiple hypothesis testing

Multiple hypothesis testing

- A p-value is meaningful when one test is carried out
- However, when thousands of tests are being carried out, it is hard to determine the real significance of the results based on the p-value alone.
- Consider the following two cases:

we test **100** genes we find **10** to be differentially expressed with a p-value **< .01**	we test **1000** genes we find **10** to be differentially expressed with a p-value **< .01**

- We need to correct for the multiple tests we are carrying out!

Bonferroni Correction

- Bonferroni Correction is a simple and widely used method to correct for multiple hypothesis testing
- Using this approach, the significance value obtained is divided by the number of tests carried out.
- For example, if we are testing 1000 genes and are interested in a (gene specific) p-value of 0.05 we will only select genes with a p-value of $0.05/1000 = 0.00005 = 5*10^{-5}$
- Motivation: If

$$p(specific \quad T_i \quad passes \mid H_0) < \frac{\alpha}{n}$$

- Then

$$p(some \quad T_i \quad passes \mid H_0) < \alpha$$

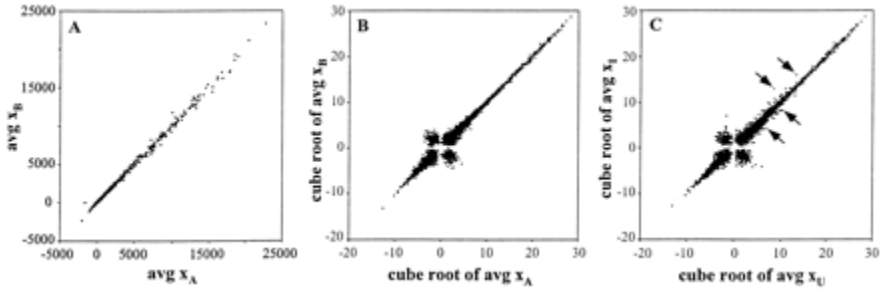
Bonferroni Correction

- The Bonferroni Correction is very conservative
- Using it may lead to missing important genes
- An alternative is to use randomization methods
- Other methods rely on the false discovery rate (FDR) as we discuss for SAM

SAM – Significance Analysis of Microarray

- Relies on repeats.
- Avoid using fold change alone.
- Use permutations to determine the false discovery rate.

Data



- Many genes were assigned negative values
- Many genes expressed at low levels
- Noise is larger for genes expressed at low levels.

Standard test statistics

$$d(i) = \frac{\hat{x}_1(i) - \hat{x}_2(i)}{s(i)}$$

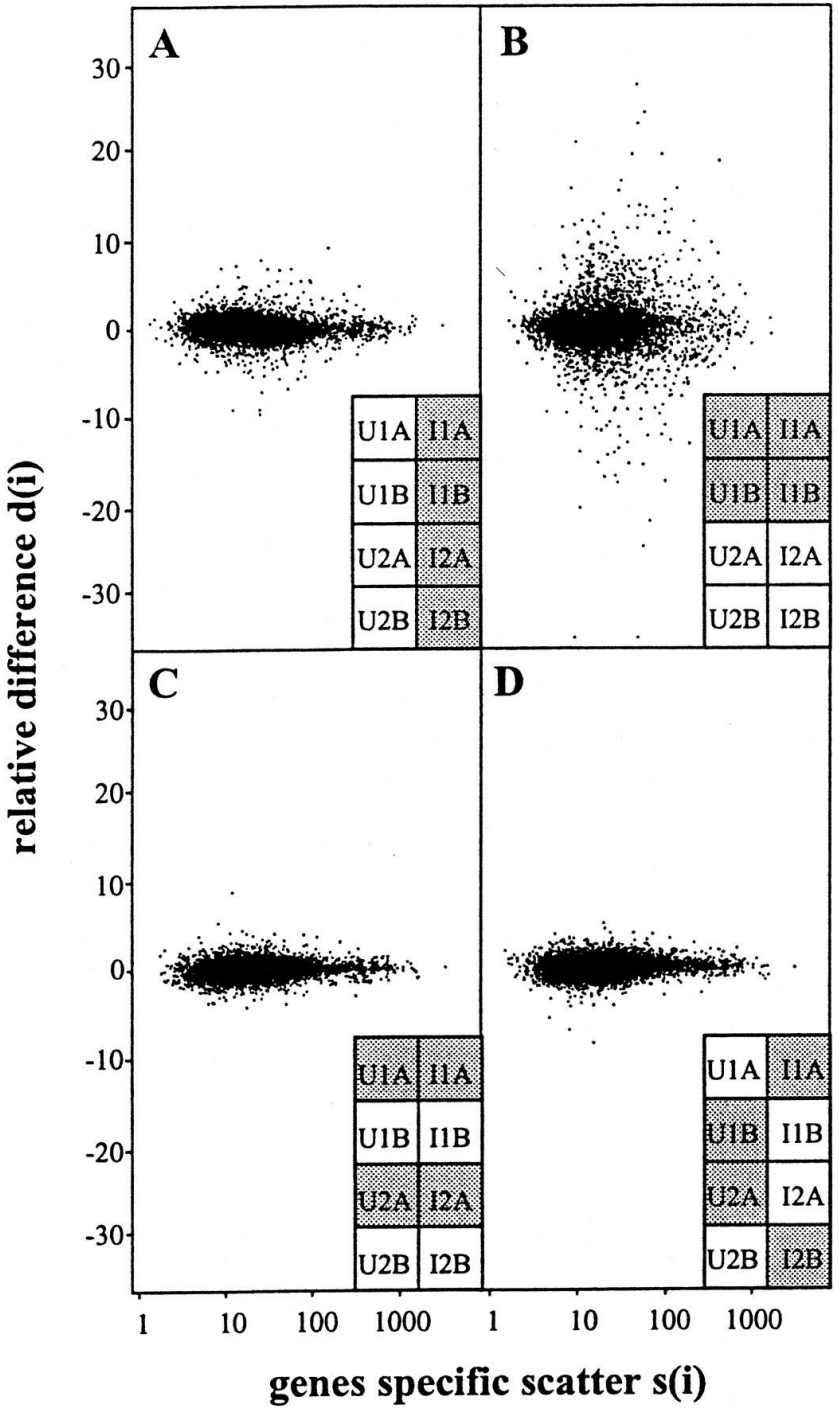
• Where x_1 and x_2 are the observed means and *s(i)* is the observed standard deviation.

Revised statistics: Relative difference

$$d(i) = \frac{\hat{x}_1(i) - \hat{x}_2(i)}{s(i) + s_0}$$

- Where x_1 and x_2 are the observed means and *s(i)* is the observed standard deviation.
- S_0 is chosen so that *d(i)* is consistent across the different expression levels.

Different comparisons of repeated experiments.



Identifying differentially expressed genes

- Using the normalized *d(i)* we can detect differentially expressed genes by selecting a cutoff above (or below for negative values) which we will declare this gene to be differentially expressed.
- However selecting the cutoff is still a hard problem.
- Solution: use the False Discovery Rate (FDR) to choose the best cutoff.

False Discovery Rate

- Percentage of genes wrongly identifies / total gene identified.
- How can we determine this using the p-value ?

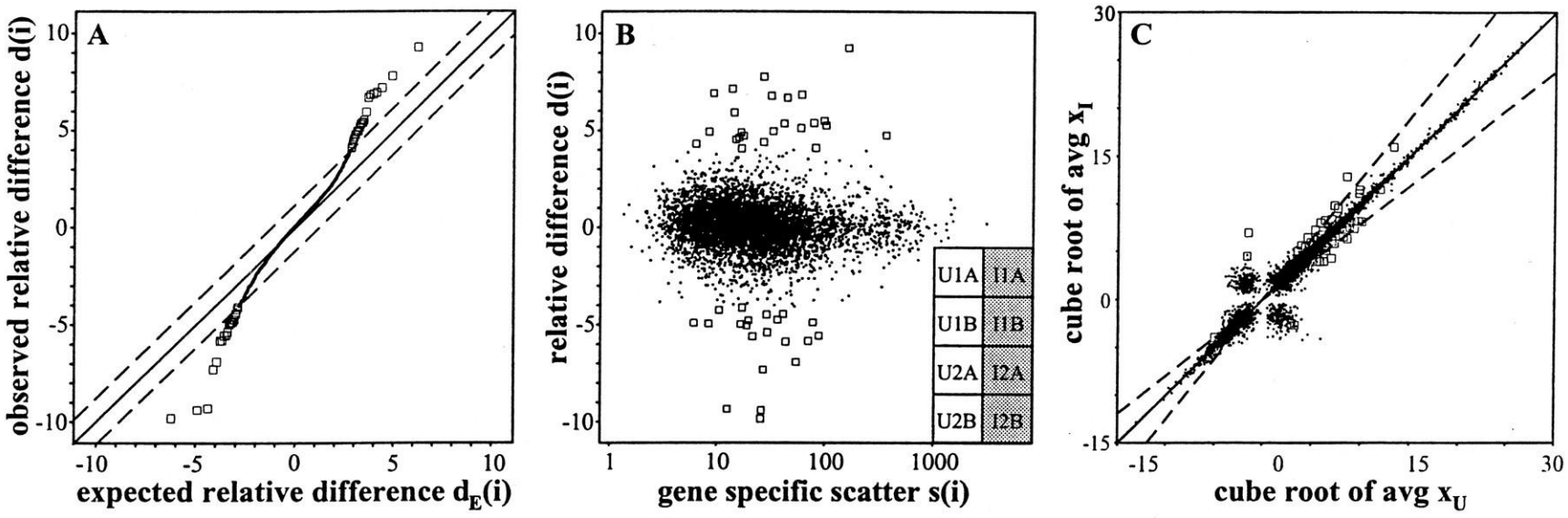
Recall: P-value - probability under the null hypothesis for observing this value

- Given a p-value, the total number of genes tested and the number of genes identified as differentially expression, the FDR can be computed directly.

Determining the FDR

- A permutation based method.
- Use all 36 permutations (why 36 ?).
- For each one compute the $d_p(i)$ for all genes.
- Scatter plot observed *d(i)* vs. expected *d(i).*

Selecting differentially expressed genes



Extensions

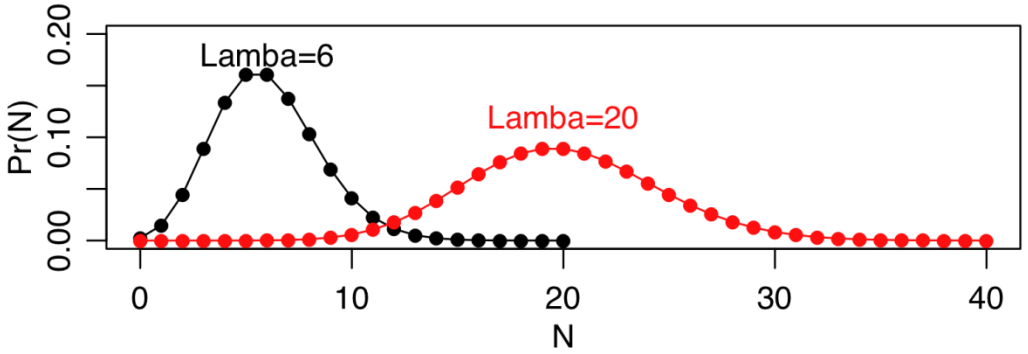
- Can be extended to multiple labels.
- Compute average for each label.
- Compute difference between specific class average and global average and corresponding variance.
- As before, adjust variance to correct for low / high level of expression.

RNA-Seq

Methods Based on Counts

- For microarrays (continuous values) Gaussian-based methods are most common
- Because sequencing data uses discrete counts, other statistical distributions may be more appropriate
- Relevant distributions are
  - Binomial distribution
  - Poisson distribution
  - Negative binomial distribution

Poisson Distribution

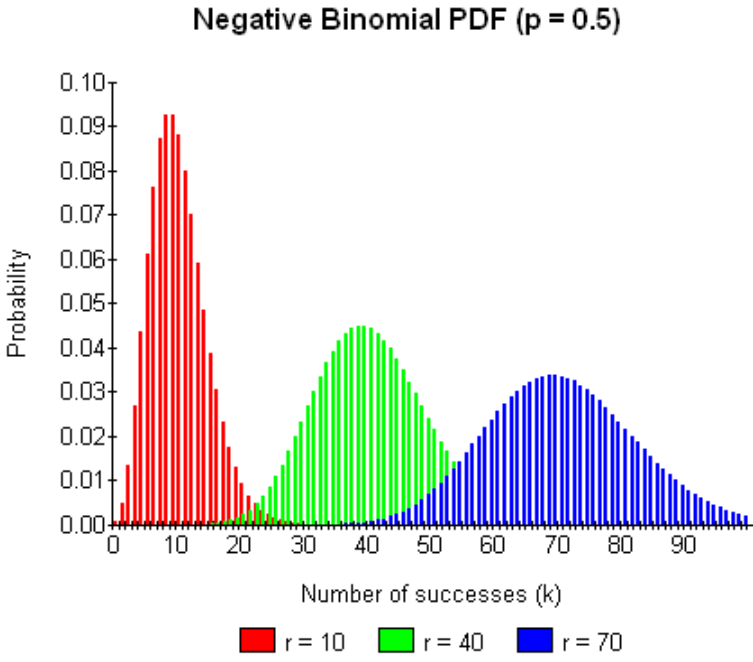


- The Poisson probability mass function is

  $Pr(N)=\exp(-\lambda)\lambda^N/N!$, for rate parameter λ
- The mean and variance of a Poisson random variable is the same: λ
- The consensus is that this model is appropriate for technical replicates but that biological replicates have extra variability.

Negative Binomial Distribution

- The negative binomial distribution is common when count data has variance significantly greater than its mean (overdispersed)
- This is a discrete probability distribution of the number of successes in a sequence of Bernoulli trials before a specified number of events *r* occurs. For example, if we flip a coin until we see three heads (r = 3), then the probability distribution of the number of tails will be negative binomial.
- The NB distribution has mean λ and variance λ + φλ; as φ goes to 0 it goes to a Poisson
- Appropriate for modeling biological replicates

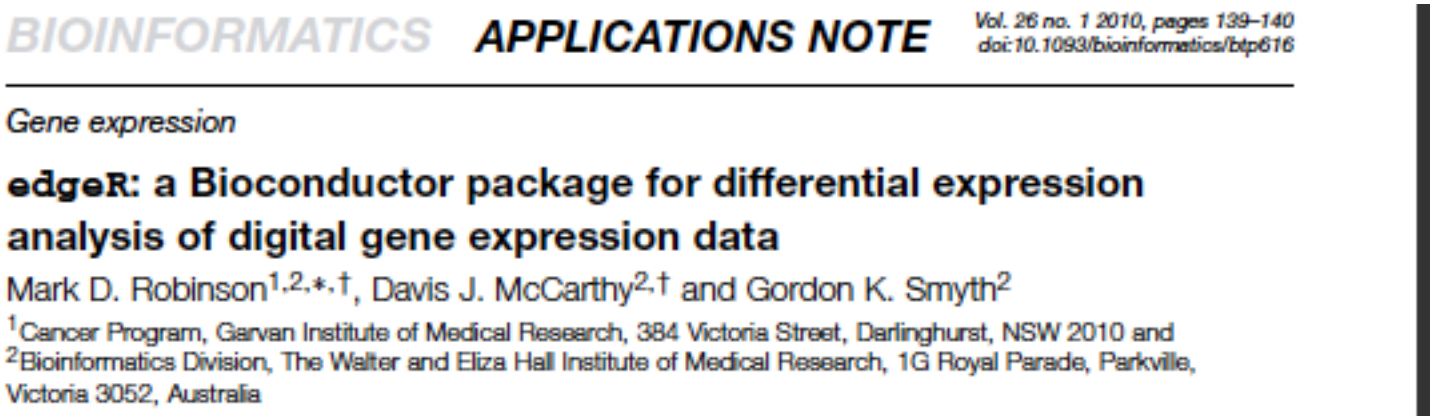


Negative Binomial Methods

- Different dispersion (φ) for every gene – not enough data to estimates this
- Common dispersion (Robinson and Smyth, *Biostatistics,* 2008) – good, but does not include any gene level variability
- Moderated dispersion (Robinson and Smyth, *Bioinformatics*, 2007) – best, but hard to weight gene level vs common dispersion

EdgeR-Robinson's Methods

- R Package
- Normalization
- DE



BIOINFORMATICS APPLICATIONS NOTE

Vol. 26 no. 1 2010, pages 139–140
doi:10.1093/bioinformatics/btp616

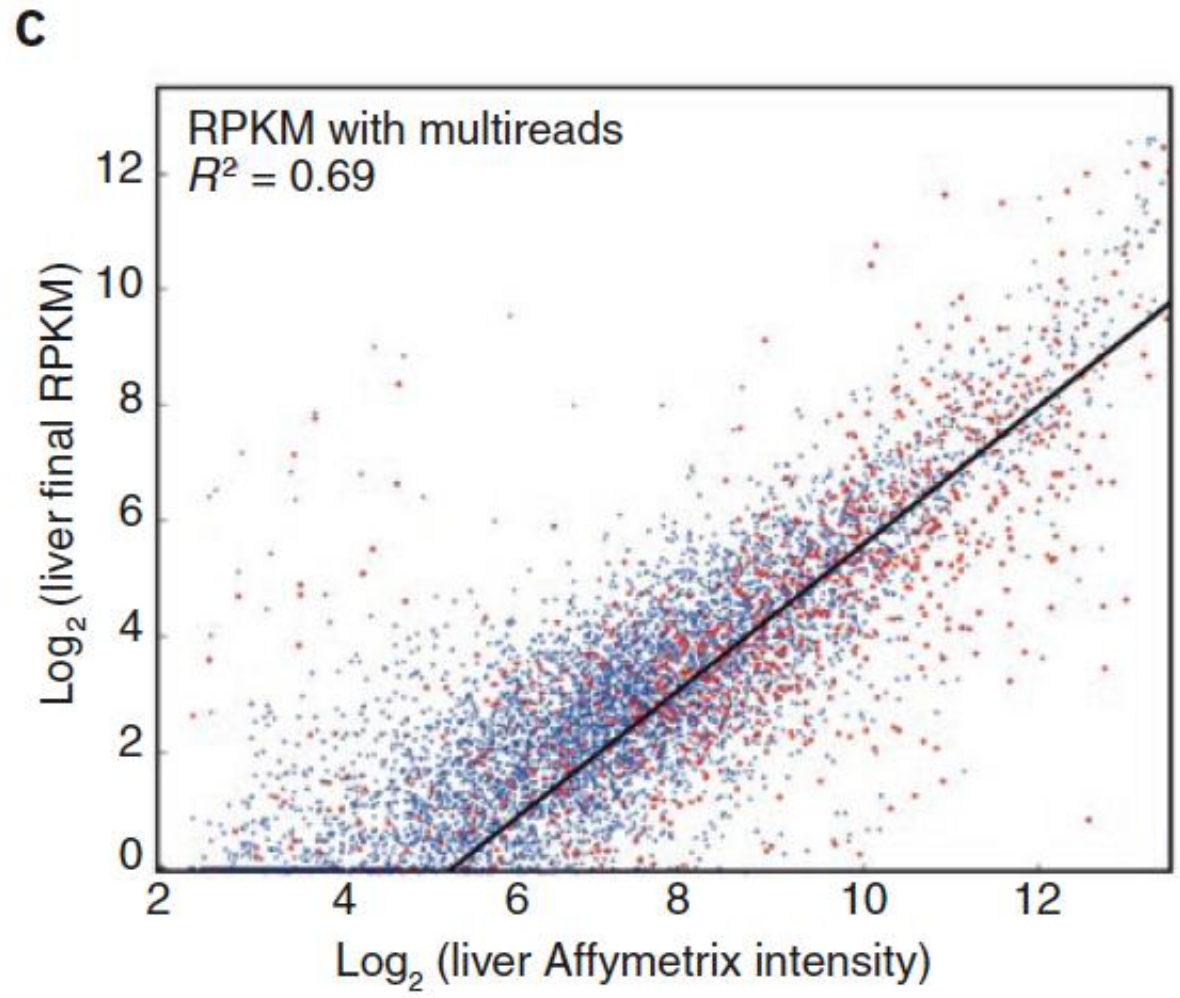
Gene expression

edgeR: a Bioconductor package for differential expression analysis of digital gene expression data

Mark D. Robinson[1,2,*,†], Davis J. McCarthy[2,†] and Gordon K. Smyth[2]

[1]Cancer Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010 and [2]Bioinformatics Division, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia

RNA Seq vs Microarrays

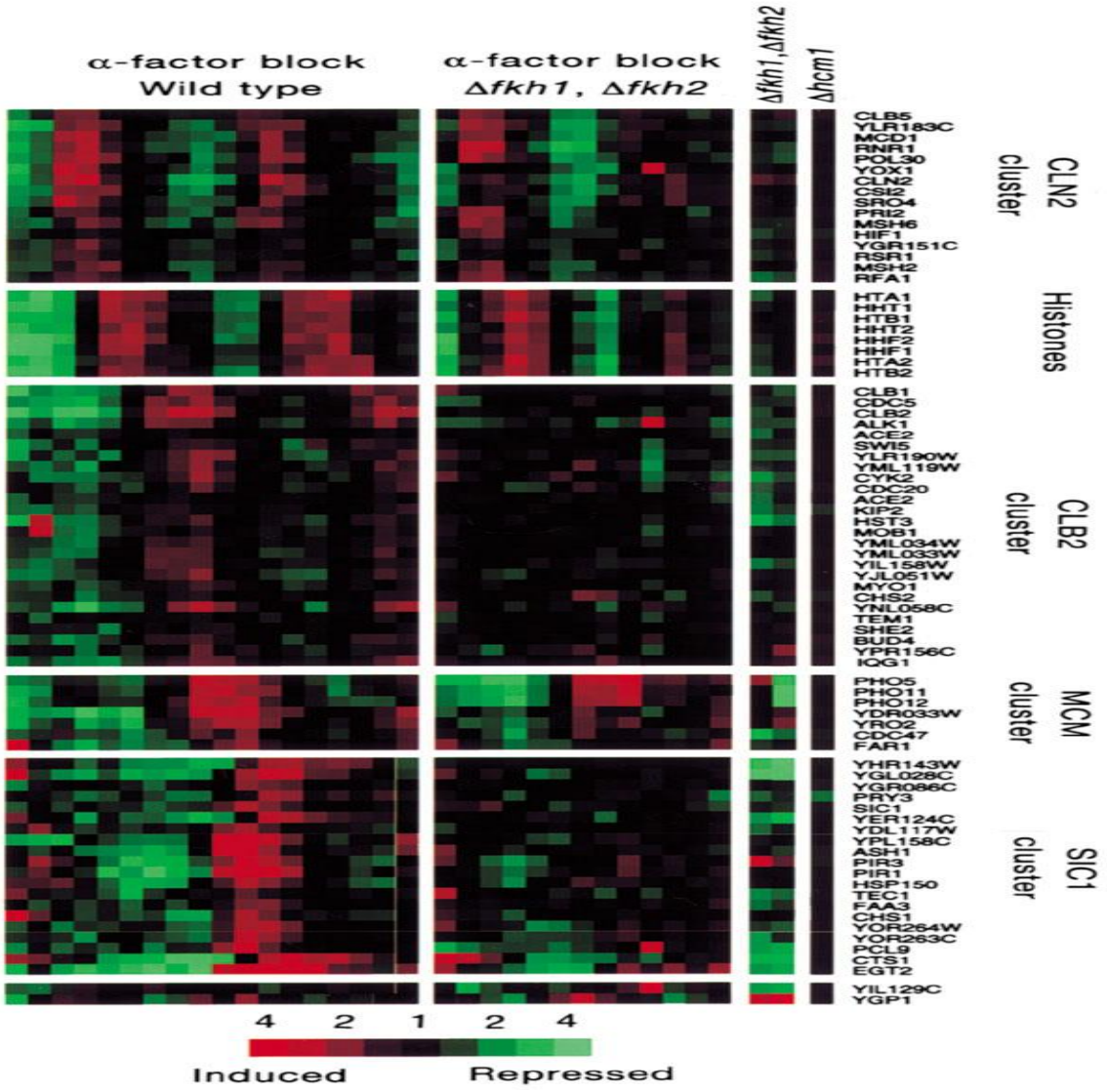


Mortazavi et al., *Nature Methods*, 2008

What about time series ?

- Comparing time points is not always possible (different sampling rates).
- Even if sampling rates are the same, there are differences in the *timing* of the system under different conditions.
- Another problem is lack of repeats.

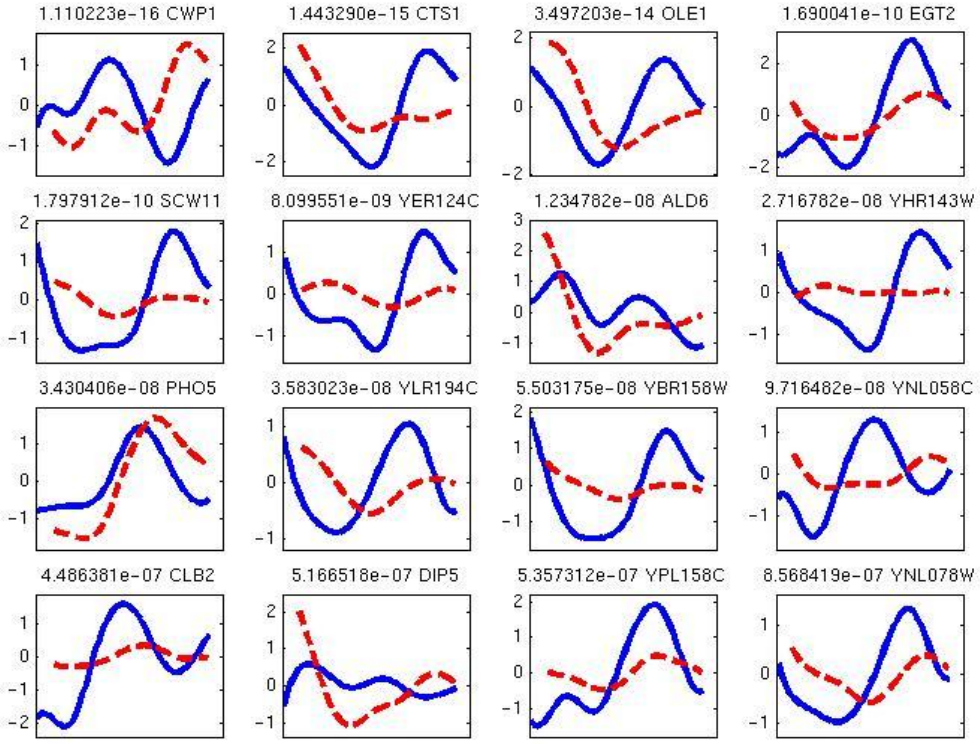
Time series comparison

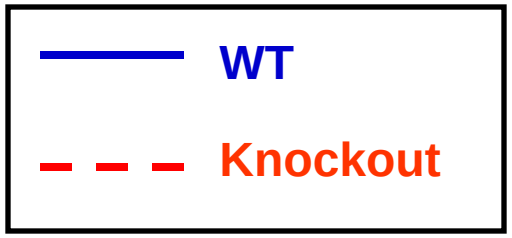


knockout = deletion of gene(s) from the sequence

Zhu *et al*, Nature 2000

Results for the Fkh1/2 Knockout





What you should know

- Statistical hypothesis testing
- Log likelihood ratio test
- Why SAM is successful:
  - No need to model expression distribution
  - Handles Excel data well