JOHNS HOPKINS
BLOOMBERG
SCHOOL of PUBLIC HEALTH

BIOINFORMATICS AND COMPUTATIONAL BIOLOGY SOLUTIONS USING R AND BIOCONDUCTOR

Biostatistics 140.688

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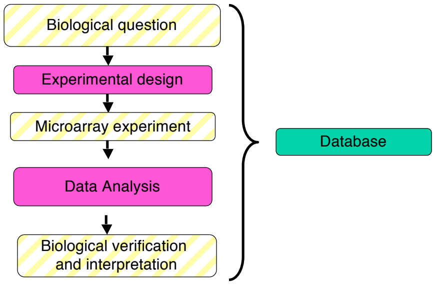
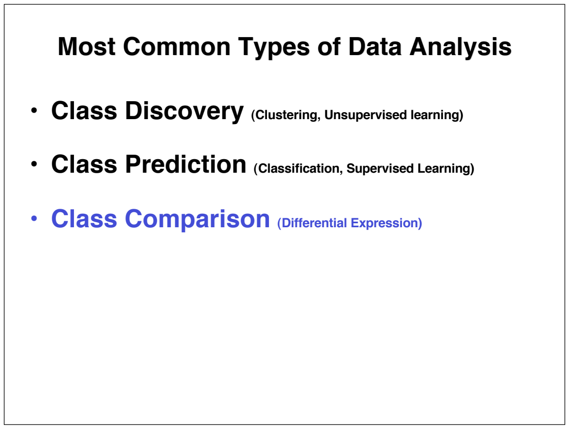
Introduction to Differential Expression Analysis

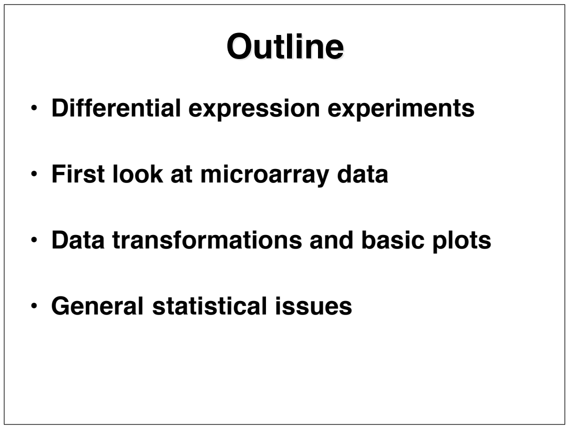
Microarray Experiment Steps





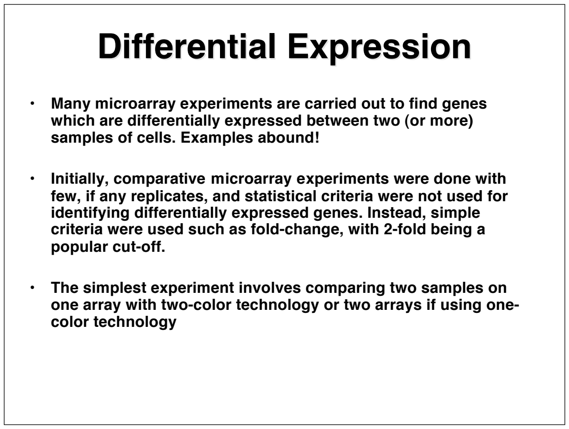
Most Common Types of Data Analysis

- **Class Discovery** (Clustering, Unsupervised learning)
- **Class Prediction** (Classification, Supervised Learning)
- **Class Comparison** (Differential Expression)



Outline

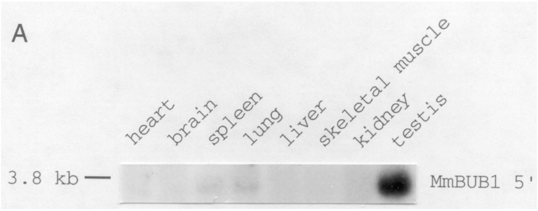
- Differential expression experiments
- First look at microarray data
- Data transformations and basic plots
- General statistical issues



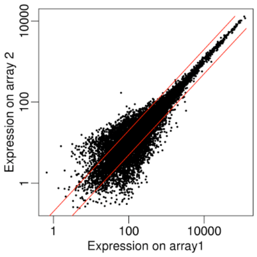
Differential Expression

- Many microarray experiments are carried out to find genes which are differentially expressed between two (or more) samples of cells. Examples abound!
- Initially, comparative microarray experiments were done with few, if any replicates, and statistical criteria were not used for identifying differentially expressed genes. Instead, simple criteria were used such as fold-change, with 2-fold being a popular cut-off.
- The simplest experiment involves comparing two samples on one array with two-color technology or two arrays if using one-color technology

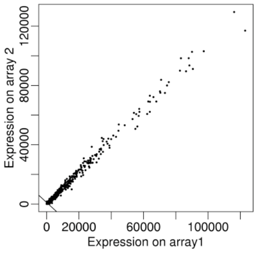
Northern Blot Data



Microarray Data



Why log?



Why logs?

- For better of worst, fold changes are the preferred quantification of differential expression. Fold changes are basically ratios
- Biologist sometimes use the following weird notation: -2 means 1/2, -3 means 1/3, etc… Note there are no values between -1 and 1!
- Ratios are not symmetric around 1. This makes it problematic to perform statistical operations with ratios. We prefer logs

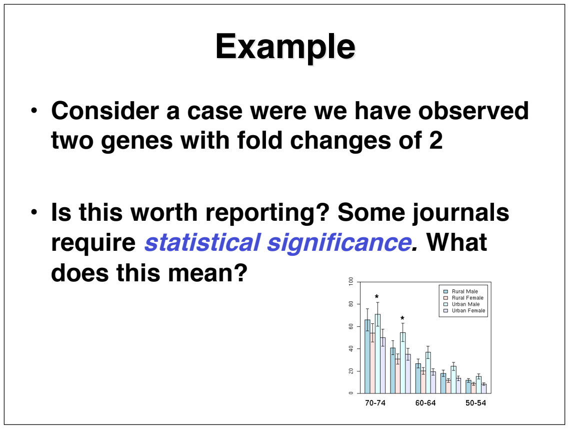
Why logs

- The intensity distribution has a fat right tail
- Log of ratios are symmetric around 0:
  - Average of *1/10* and *10* is about *5*
  - Average of *log(1/10)* and *10* is *0*
  - *Averaging ratios is almost always a bad idea!*

Facts you must remember:

log(1) = 0
log(XY) = log(X) + log(Y)
log(Y/X) = log(Y) - log(X)
log(√X) = 1/2 log(X)

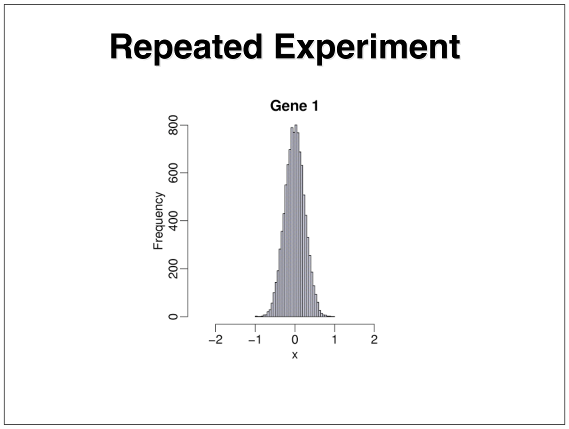
Quantifying differentially expression



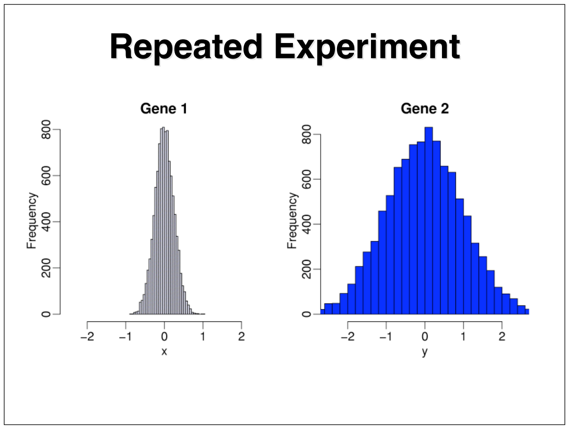
Example

- Consider a case were we have observed two genes with fold changes of 2
- Is this worth reporting? Some journals require ***statistical significance***. What does this mean?

0
20
40
60
80
100
*
*
Rural Male
Rural Female
Urban Male
Urban Female
70-74
60-64
50-54



Repeated Experiment



Repeated Experiment

Review of Statistical Inference

- Let *Y-X* be our measurement representing diferential expression
- What is the typical null hypothesis?
- For simplicity let us assume *Y-X* follows a normal distribution
- *Y-X* may have a different distribution under the null hypothesis for different genes
- More specifically the standard deviation σ of *Y-X* may be different.
- We could consider *(Y-X) /* σ instead
- But we do not know σ!
- What is σ? Why is it not 0?
- How about taking samples and using the t-statistic?

Sample Summaries

Observations: $X_1, \ldots, X_M \quad Y_1, \ldots, Y_N$

Averages: $\bar{X} = \frac{1}{M}\sum_{i=1}^{M} X_i \quad \bar{Y} = \frac{1}{N}\sum_{i=1}^{N} Y_i$

SD^2 or variances:

$$s_X^2 = \frac{1}{M-1}\sum_{i=1}^{M}(X_i - \bar{X})^2 \quad s_Y^2 = \frac{1}{N-1}\sum_{i=1}^{N}(Y_i - \bar{Y})^2$$

The t-statistic

t - statistic:

$$\frac{\bar{Y} - \bar{X}}{\sqrt{\frac{s_Y^2}{N} + \frac{s_X^2}{M}}}$$

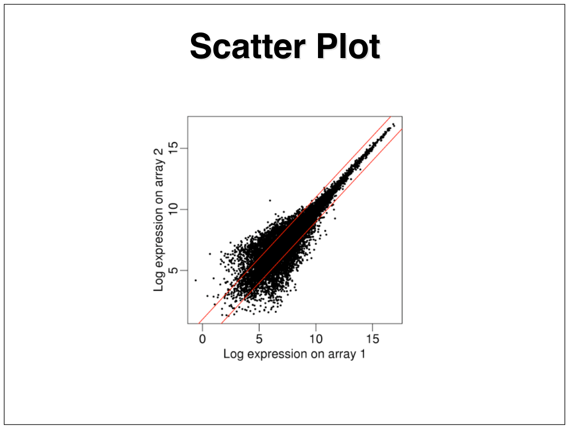
Properties of t-statistic

- If the number of replicates is very large the t-statistic is normally distributed with mean 0 and and SD of 1
- If the observed data is normally distributed then the t-statistic follows a t distribution regardless of sample size
- We can then compute probability that t-statistic is as extreme or more when null hypothesis is true
- Where does probability come from?
- We will see that using the t-statistic is not a good strategy for microarray data when N is small

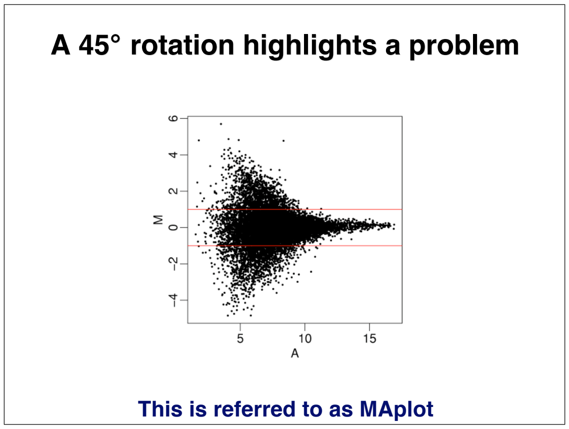
Inference of Ranking

- Are we really interested in inference?
- Sometimes all we are after is a list of candidate genes
- If we are just ranking should we still consider variance?

Some useful plots

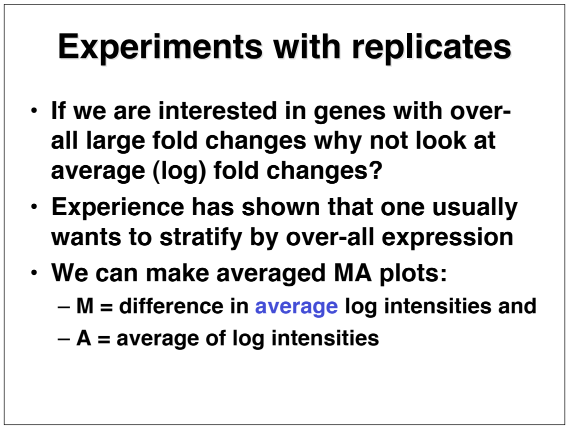


Scatter Plot



A 45° rotation highlights a problem

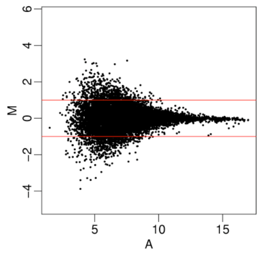
This is referred to as MAplot



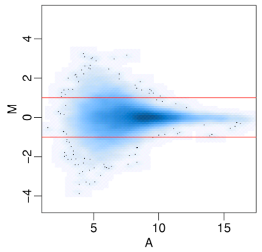
Experiments with replicates

- If we are interested in genes with over-all large fold changes why not look at average (log) fold changes?
- Experience has shown that one usually wants to stratify by over-all expression
- We can make averaged MA plots:
  - M = difference in average log intensities and
  - A = average of log intensities

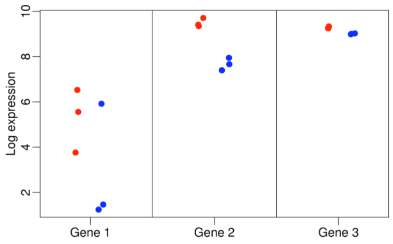
MA plot of average log ratios



Scatter Smooth



Should we consider gene-specific variance?



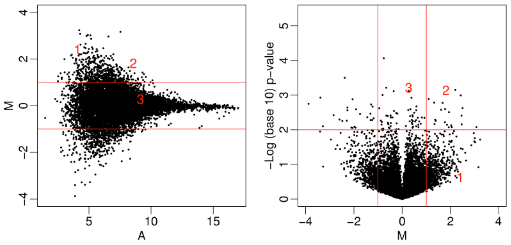
How do we summarize?

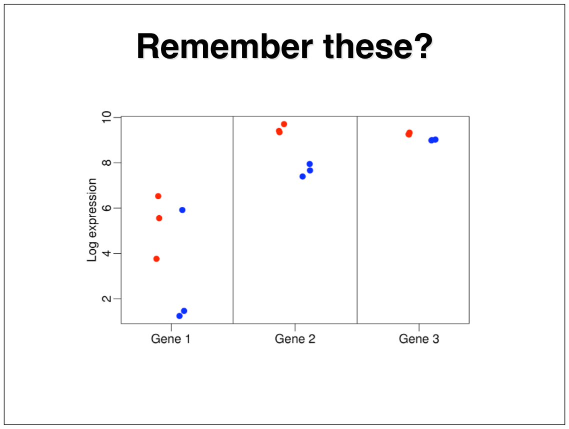
- Seems that we should consider variance even if not interested in inference
- The t-test is the most used summary of effect size and within population variation

Another useful plot

- The volcano plot shows, for a particular test, negative log p-value against the effect size (M)

MA and volcano





Remember these?

Estimating the variance

- If different genes (or probes) have different variation then it is not a good idea to use average log ratios even if we do not care about significance
- Under a random model we need to estimate the SE
- The t-test divides by SE
- But with few replicates, estimates of SE are not stable
- This explains why t-test is not powerful
- There are many proposals for estimating variation
- Many *borrow strength* across genes
- Empirical Bayesian Approaches are popular
- SAM, an ad-hoc procedure, is even more popular
- Many are what some call “moderated” t-tests
- More in later lecture

One final problem

- Say we are interested in statistical inference, we need to define statistical significance. If we are ranking we may need to define a cut-off that defines *interesting enough*
- The naïve answer to determinig a cut-off is the p-values. Are they appropriate?
- Test for each gene null hypothesis: no differential expression.
- Notice that if you have look at 10,000 genes for which the null is true you expect to see 500 attain p-values of 0.05
- This is called the multiple comparison problem. Statisticians fight about it. But not about the above.
- Main message: p-values can’t be interpreted in the usual way
- A popular solution is to report FDR instead.

The Multiple Comparison Problem

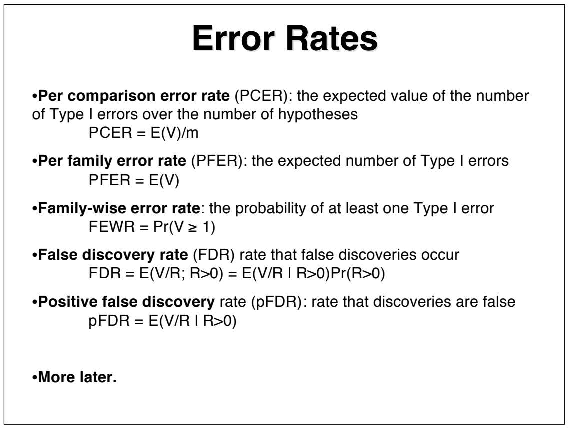
What do we do?

- Adjusted p-values
- List of genes along with FDR
- Bayesian inference
- Forget about inference: use EDA
- We may talk about this in detail in another lecture

Multiple Hypothesis Testing

- What happens if we call all genes significant with p-values ≤ 0.05, for example?

	Called Significant	Not Called Significant	Total
Null True	V	$m_0 - V$	m_0
Altern.True	S	$m_1 - S$	m_1
Total	R	$m - R$	m



Error Rates

- **Per comparison error rate** (PCER): the expected value of the number of Type I errors over the number of hypotheses
  PCER = E(V)/m
- **Per family error rate** (PFER): the expected number of Type I errors
  PFER = E(V)
- **Family-wise error rate**: the probability of at least one Type I error
  FEWR = Pr(V ≥ 1)
- **False discovery rate** (FDR) rate that false discoveries occur
  FDR = E(V/R; R>0) = E(V/R | R>0)Pr(R>0)
- **Positive false discovery** rate (pFDR): rate that discoveries are false
  pFDR = E(V/R | R>0)

- **More later.**