



Analysis of Microarray Data

Lecture 2:

Differential Expression, Filtering and Clustering

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Outline

- Review
- Measuring differential expression
- Multiple hypothesis testing
- Gene filtering
- Measuring distance between profiles
- Clustering methods

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Review

- Assumption: Expression microarrays measure specific mRNA levels
- Why perform the experiment?
- What best design addresses your goals?
- Normalize to increase power of comparisons.
- Precision doesn't necessarily indicate analysis success.
- Does your analysis pipeline make sense biologically and statistically?

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Caveats and limitations

- Are the probes on the chip for a specific transcript? gene?
- Are mRNA levels correlated with transcription activity?
- Is transcriptional regulation important?
- Are mRNA levels correlated with protein activity?
- Is this the best technology to answer your question(s)?

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Measuring differential expression

- One common goal is to rank all the genes on a chip in order of evidence for differential expression
- Ways to score genes:
 - Fold change
 - T-statistic p-value
 - Another statistic (nonparametric, etc.)
 - A combination of several scores

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Fold change

- Advantage: Fold change makes sense to biologists

$$\text{Fold change} = \frac{\text{expression value in sample 1}}{\text{expression value in sample 2}}$$

- What cutoff should be used?
- Should it be the same for all genes?
- Disadvantages:
 - Only mean values – not variability – are considered
 - Genes with large variances are more likely to make the cutoff just because of noise

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Hypothesis testing

- We want to test ...
 - Is the expression of my gene different in a set in one condition compared to another condition?
 - How big is the difference?
 - Is the mean of one set of values different from the mean of another set of values?
 - If we say “yes”, how much confidence do we have that the means are truly different?
- Assumptions:
 - Data are normally distributed
 - Samples are randomly chosen

Hypothesis testing with the t-test

- Considers mean values and variability
- Equation for the t-statistic in the Welch test:

$$t = \frac{\text{mean}_r - \text{mean}_g}{\sqrt{\frac{s_r^2}{n_r} + \frac{s_g^2}{n_g}}}$$

... and then a p-value is calculated
 r ; g = data sets to compare
 s = standard deviation
 n = no. of measurements

- Disadvantages:
 - Genes with small variances are more likely to make the cutoff
 - Works best with larger data sets than one usually has

Flavors of the t-test

- Are we only considering up-regulated or down-regulated genes, or both?
 - If both, perform a 2-tailed test
- Can we assume that the variance of the gene is similar in both samples?
 - Yes => Homoscedastic (the standard t-test)
 - No => Heteroscedastic (Welch's test)

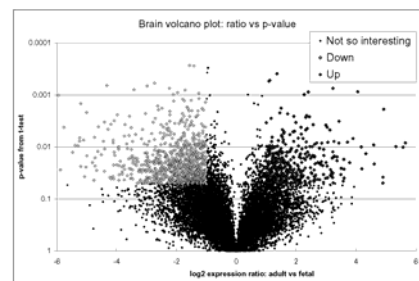
ANOVA

- Analysis of variance – like a multidimensional t-test
- Measure effect of multiple treatments and their interactions
- A thoughtful ANOVA design can help answer several questions with one analysis
- ANOVA can also analyze factors that should be controlled – just to confirm absence of confounding effects
- ANOVA generally identifies genes that are influenced by some factor – but then post-hoc tests must be run to identify the specific nature of the influence
 - Ex: t-tests between all pairs of data

Combining p-values and fold changes

- What's important biologically?
 - How significant is the difference?
 - How large is the difference?
- Both amounts can be used to identify genes.
- What cutoffs to use?
- How many genes should be selected?
- Where are your positive controls?

Volcano plots



Differential expression - summary

- Multiple methods can produce lists of differentially expressed genes
- Which ways make most sense biologically and statistically?
- Be aware of multiple hypothesis testing
- Looking at all the data: volcano plots
- Where do your positive controls fit in?
- There may be no single best way

Multiple hypothesis testing

- We need both sensitivity and specificity:
 - Sensitivity: probability of successfully identifying a real effect
 - Specificity: probability of successfully rejecting a nonexistent effect
- The problem
 - The number of false positives greatly increases as one performs more and more t-tests
 - How seriously do you want to limit false positives?

Correcting for multiple hypothesis testing

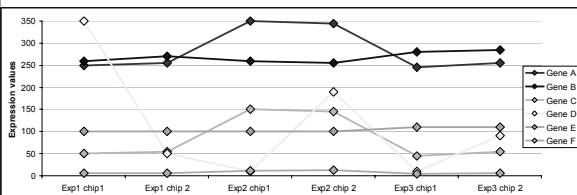
- If false positives are not tolerated
 - Perform Bonferroni correction
 - If you perform 100 t-tests, multiply each p-value by 100 to get corrected (adjusted) values
 $p = 0.0005 \Rightarrow p = 0.05$
- If false positives can be tolerated
 - Use False Discovery Rate (FDR)
 - If you can tolerate 15% false positives, set FDR to 0.15 and calculate what p-value to use
- FDR method is less conservative than Bonferroni and usually more appropriate for microarrays.

Gene filtering

- An infinite number of methods can select “interesting” genes
- Not all genes on the chip need consideration: any meaningful selection is possible
- Filtering by function: using GO or other annotations
- Often the major question: How many genes to choose for further analysis?

Measuring distance between profiles

- Distance metric is most important choice when comparing genes and/or experiments
- What are you trying to do?



Common distance metrics

- Pearson correlation
 - Measures the difference in the shape of two curves
 - Modification: absolute correlation
- Euclidean distance: multidimensional Pythagorean Theorem
 - Measures the distance between two curves
- Nonparametric or Rank Correlation
 - Similar to the Pearson correlation but data values are replaced with their ranks
 - Ex: Spearman Rank, Kendall's Tau
 - More robust (against outliers) than other methods

Clustering and segmenting

- Goal: organize a set of data to show relationships between data elements
- With microarray analysis: genes and/or chips
- Most data does not inherently exist in clusters
- Clustering vs segmenting
- Most effective with optimal quantity of data
- Interpretation of data in obvious clusters: is it filtered?

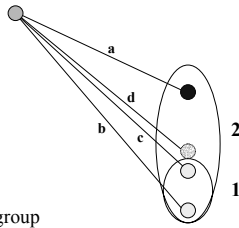
Clustering basics

- How to start:
 - One big cluster (divisive)
 - n clusters for n objects (agglomerative)
 - K clusters, where k is some pre-defined number
- Hierarchical agglomerative clustering
 - Popular method producing a tree showing relationships between objects (genes or chips)
 - Start by creating an all vs. all distance matrix
 - Fuse closest objects, then...

Representing groups of objects during clustering

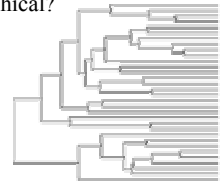
How is distance measured to a cluster of objects?

- Single linkage (a)
 - minimum distance
- Complete linkage (b)
 - maximum distance
- Average linkage (c)
 - average distance
- Centroid linkage (d)
 - distance to “centroid” of group



Representing clustered data

- Hierarchical clustering produces a dendrogram showing relationships between objects
- Are the data really hierarchical?
- Order of leaves 2^{N-2}
- How can objects be partitioned into groups?
 - k-means clustering
 - self-organizing maps
 - How many clusters (k)?
- Original distance matrix may be more informative



Summary

- Determining differential expression:
 - t-test, fold change, etc.
 - methods may be used in combination
- Correcting for multiple hypothesis testing
 - Bonferroni, False Discovery Rate, etc.
- Distance metrics: select carefully
- Clustering/segmentation types and methods
 - hierarchical, k-means, etc.; linkage types
 - Which protocol is best for your experiment?

References

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- Pavlidis P. Using ANOVA for gene selection from microarray studies of the nervous system. *Methods.* 31(4):282-9, 2003.
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- Cui X and Churchill GA. Statistical tests for differential expression in cDNA microarray experiments. *Genome Biology* 4:210, 2003.

Microarray tools

- Course page:
 - <http://jura.wi.mit.edu/bio/education/bioinfo2005/arrays/>
- BaRC analysis tools:
 - <http://jura.wi.mit.edu/bioc/tools/>
- Bioconductor (R statistics package)
 - <http://www.bioconductor.org/>
- Excel
- Many commercial and open source packages
- Cluster 3.0 and Java TreeView

Selecting a large matrix in Excel

1	Select the bottom right cell of the desired matrix	
2	Control - Shift - Up arrow	Select everything above the original cell
3	Control - Shift - Left arrow	Select everything to the left of the original cell
4	Shift - Down arrow	Move down one row
5	Shift - Right arrow	Move to the right one column

Exercise 2: Excel functions

- LOG
- IF
- TTEST
- CONCATENATE
- VLOOKUP

Exercise 2 - To do

- Use t-test to identify differentially expressed genes
- Use the "Absent/Present" calls from the Affymetrix algorithm to flag genes with questionable expression levels
- List all the gene IDs for those that meet your significance threshold (such as $p < 0.05$) and are present in at least one sample.
- Gather expression data for these genes
- Cluster this selected data (multiple methods)
- Visualize clustered data as a heatmap