The essentials of microarray data analysis

(from a complete novice)

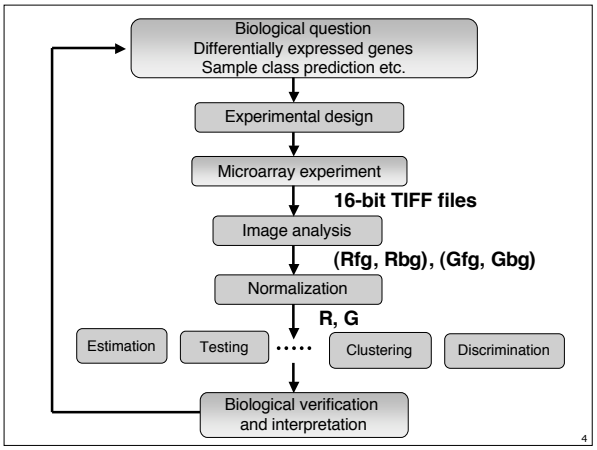
Thanks to Rafael Irizarry for the slides!

Outline

- Experimental design
- Take logs!
- Pre-processing: affy chips and 2-color arrays
- Clustering
- Identifying differentially expressed genes

Resources

- Expressionists working group
  (Search google for "hopkins expressionists")
  "Top ten things to know…" held monthly
- "Stats for gene expression" course (140.688)
  http://www.biostat.jhsph.edu/~ririzarr/Teaching/688



Experimental design

- Choice of platform
- Array design
  - Creation of probes
  - Location on the array
  - Controls
- Target samples

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Experimental design

Proper experimental design is needed to ensure that questions of interest ***can*** be answered and that this can be done **accurately and precisely**, given experimental constraints, such as cost of reagents and availability of mRNA.

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Avoidance of bias

- Conditions of an experiment (mRNA extraction and processing, the reagents, the operators, the scanners and so on) can leave a "global signature" in the resulting expression data.
  - Balance
  - Randomization

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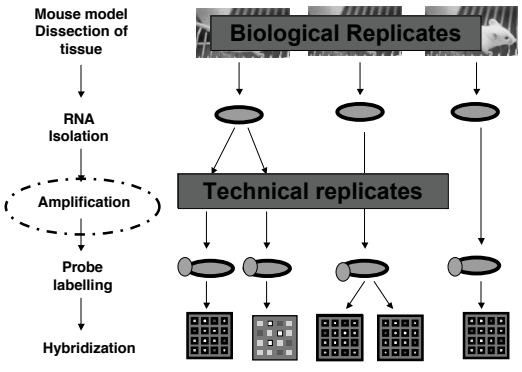
Preparing mRNA samples:



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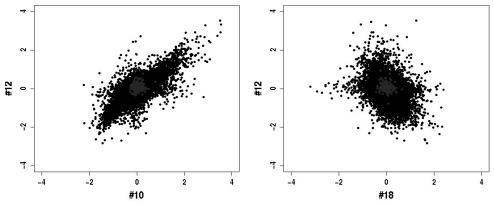
Technical replication - amplification

- 3 sets of two different samples performed on different days
- #10 and #12 were from the same RNA isolation and amplification
- #12 and #18 were from different dissections and amplifications
- All 3 data sets were labeled separately before hybridization



Data provided by
Dave Lin (Cornell)

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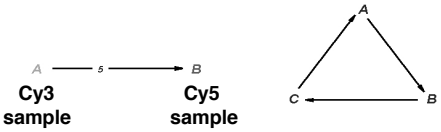
Two-color layouts

For two color platforms it is assumed that the size of the spot/probe effect is too big to trust the absolute intensites. Thus we always use relative measurements

***Vertices*: mRNA samples; *Edges:* hybridization;**
***Direction:* dye assignment.**



(a) *(b)*

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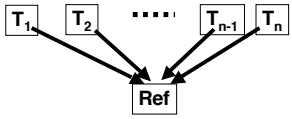
Common reference design



Experiment for which the common reference design is appropriate

Meaningful biological control (C) Identify genes that responded differently / similarly across two or more treatments relative to control.

Large scale comparison. To discover tumor subtypes when you have many different tumor samples.

Advantages:

Ease of interpretation.

Extensibility - extend current study or to compare the results from current study to other array projects.

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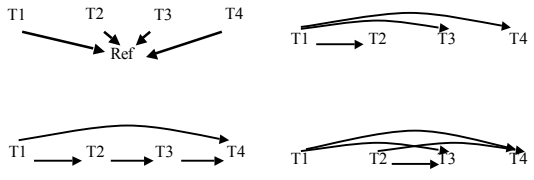
Experiment for which a number of designs are suitable for use

Time Series



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Pooling

- **Should I pool mRNA samples across subjects in an effort to reduce the effect of biological variability?**
- **Notice, in many cases, samples are cheap but arrays are expensive**

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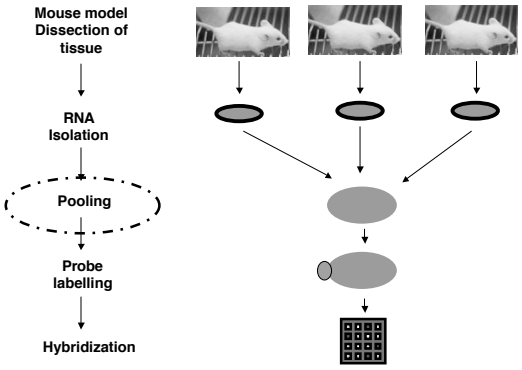
Pooling: looking at very small amount of tissues



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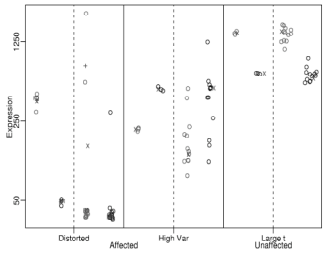
Problem with pooling everything



- You cannot measure variability
- You cannot take logs before "averaging"

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Alternative pooling strategy

- **Instead of pooling everything, how about pooling groups?**
- **For example, will I obtain the same results with 12 individuals on 12 chips as with 12 individuals on 4 chips ?**

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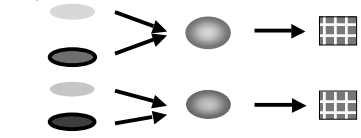
Design 1

Pooled vs Individual samples

Design 2



Taken from Kendziorski etl al (2003)

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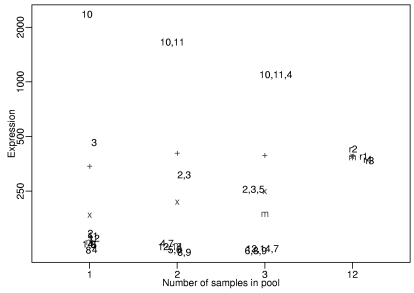
More on Pooling and outliers



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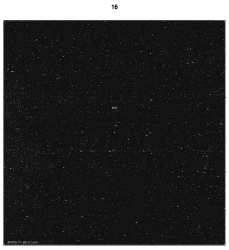
Images of probe level data



This is the raw data

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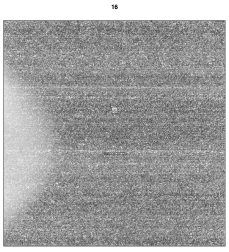
Images of probe level data



Log scale version much more informative

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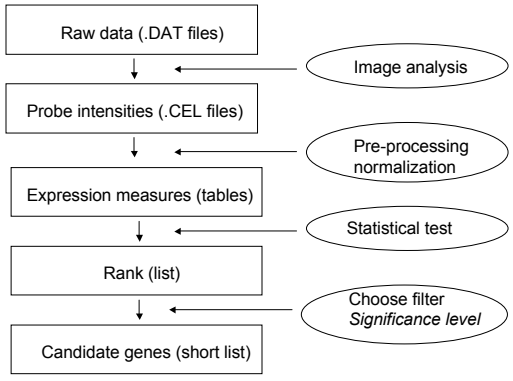
Work flow



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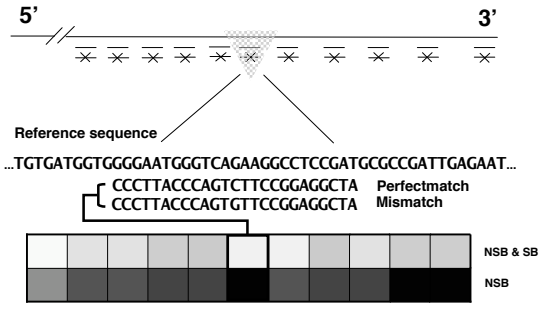
Affymetrix GeneChip Design



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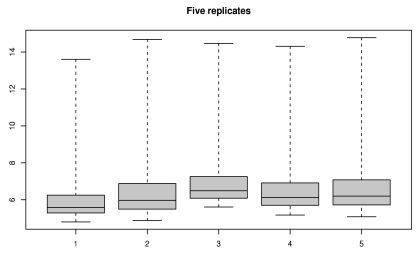
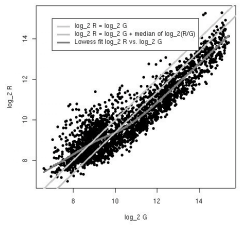
Affy Technical Replicates Boxplot



Different scanners were used.

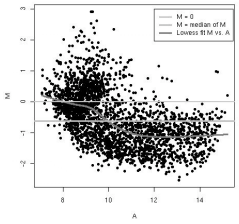
Self-self hybridization

$\log_2$ R vs. $\log_2$ G



Self-self hybridization

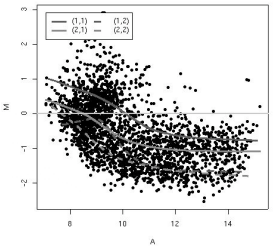
M vs. A



$M = \log_2 R - \log_2 G$, $A = (\log_2 R + \log_2 G)/2$

Self-self hybridization

M vs. A

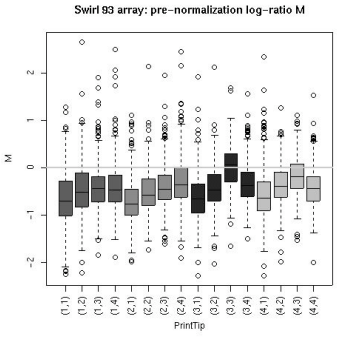


Robust local regression
within sectors
(print-tip-groups)
of intensity log-ratio M
on average log-intensity A.

$M = \log_2 R - \log_2 G$, $A = (\log_2 R + \log_2 G)/2$

Boxplots by print-tip-group

Intensity log-ratio, M



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What can we do?

- Throw away the data and start again? Maybe.
- Statistics offers hope:
  - Use control genes to adjust
  - Assume most genes are not differentially expressed
  - Assume distribution of expression are the same

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Simplest Idea

- Assume all arrays have the same median log expression or relative log expression
- Subtract median from each array
- In cDNA this makes the median log ratio 0 (we assume there are as many over-expressed as under-expressed)
- In Affy we usually add a constant that takes us back to the original range

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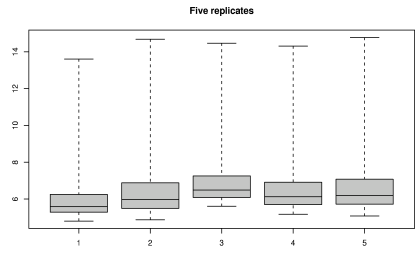
How does it work



Notice subtracting in the original scale won't work well

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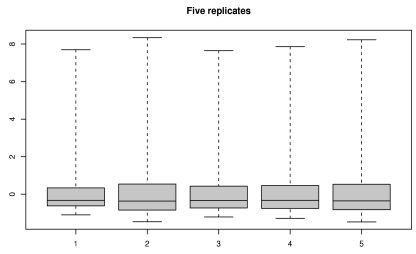
How does it work



The medians match but there are some discrepancies

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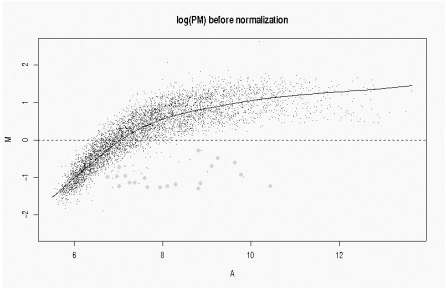
What are the consequences



These are two technical replicates with spike-ins.

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Quantile normalization

- All these non-linear methods perform similarly
- Quantiles is my favorite because its fast
- Basic idea:
  - order value in each array
  - take average across probes
  - Substitute probe intensity with average
  - Put in original order

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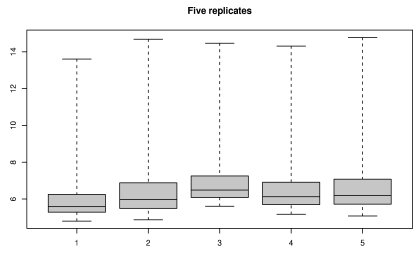
How does it work



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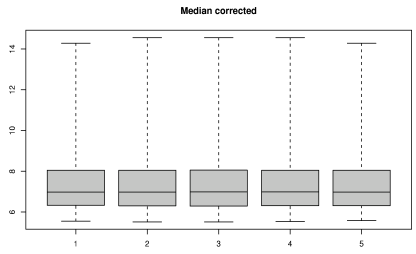
How does it work



Does it wash away real differential expression?

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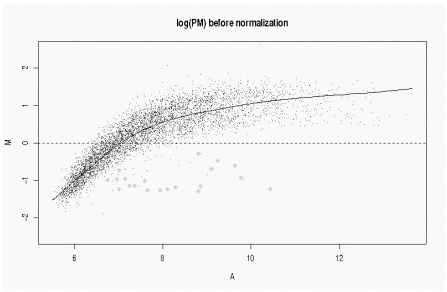
Before and



These are two technical replicates. Only the red are differentially expressed

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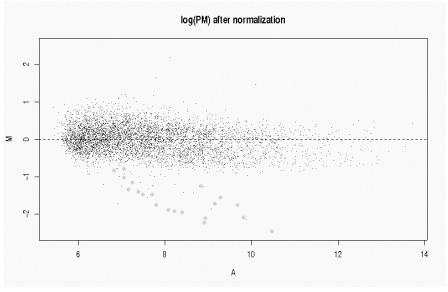
After quantile normalization



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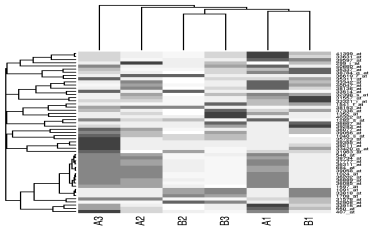
Clustering is not a universally good tool



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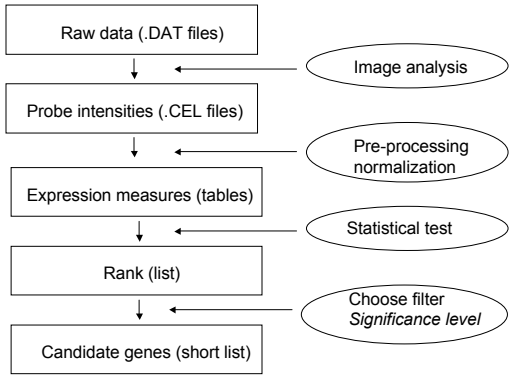

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Differential gene expression

- Identify genes whose expression levels are associate with a response or covariate of interest
  - clinical outcome (e.g. survival, response to treatment, tumor class)
  - covariate such as treatment, dose, time.
- Estimation: In a statistical framework, assigning a score can be viewed as estimating an effects of interest (e.g. difference in means, slope, interaction). We can also take the variability of these estimates into account.
- Testing: In a statistical framework, deciding on a cut-off can be viewed as an assessment of the statistical significance of the observed associations.

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Example: Two populations

- A common problem is to find genes that are differentially expressed in two populations.
- Many method papers appear in both statistical and molecular biology literature.
- The proposed scores range from:
  - ad-hoc summaries of fold-change
  - variantes on the t-test
  - and posterior means obtained from Bayesian or empircal Bayes methods.
- What's the difference? Mainly the way in which the variation within population is incorporated

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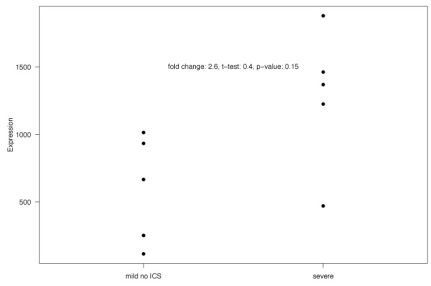
Should we consider variability?



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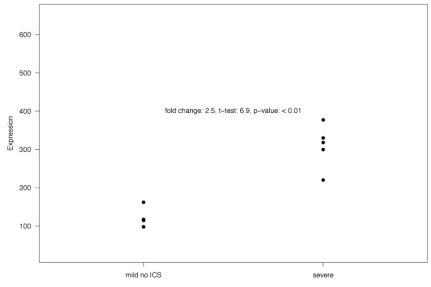

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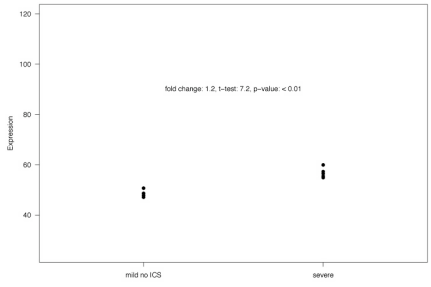
Should we consider variability?



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Useful Plots

- The MA plot shows M=log fold change, plotted against A=average log intensity
- If we have various replicates in each population we can plot M=difference in log averages in two populations
- The volcano plot shows, for a particular test, - log p-value against the effect size (M)

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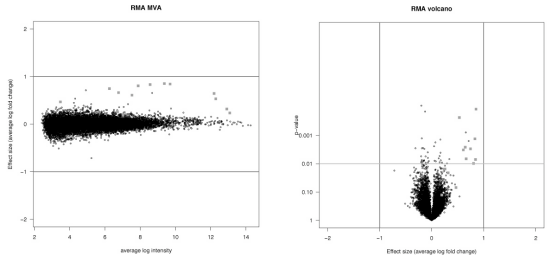
Example



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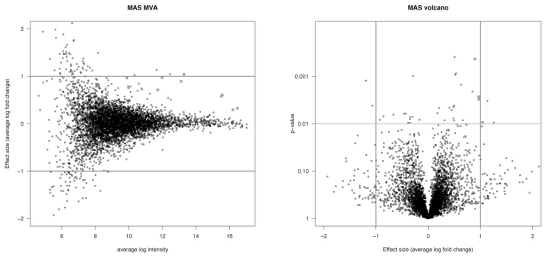
If you insist on using MAS 5.0 it really helps



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Summary

- Experimental design is critical
  - Biological vs technical replicates
- Pre-processing (calibration) can have an enormous effect on the end results
- Analysis should be guided not by what others have done, but by what you want to learn.
- Regarding multiple testing: view this process as exploratory, and don't get hung up on adjustments for multiple testing.

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