Analysis of Replicated Experiments

Statistical Methods in Microarray Analysis Tutorial
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Some Web Sites

- Technical reports etc
  - http://www.statsci.org/micrarra
  - http://bioinf.wehi.edu.au/marray
  - http://www.stat.berkeley.edu/users/terry/zarray/Html
- Statistical software: R
  - http://www.R-project.org
- Packages within R environment
  - Bioconductor http://www.bioconductor.org
  - LIMMA http://bioinf.wehi.edu.au/limma
  - SPOT (cDNA image analysis) http://experimental.act.cmis.csiro.au/Spot/index.php

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Five Statistical Issues

- Designing gene expression experiments
- Acquiring the raw data: image analysis
- Summarizing and removing artefacts from the data
- Discovering which genes are differentially expressed
- Discovering which genes exhibit interesting expression patterns

For a review see Smyth, Yang and Speed, "Statistical issues in microarray data analysis", In: *Functional Genomics: Methods and Protocols*, Methods in Molecular Biology, Humana Press, March 2003

Lots of other bioinformatics issues …

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Image Analysis

Yang, Buckley, Dudoit and Speed, "Comparison of methods for image analysis on cDNA microarray data", *Journal of Computational and Graphical Statistics*, January 2002.

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Image Analysis Software

- If you're using Affymetrix arrays, the image analysis will be done as part of the Affy system.
- If you're using spotted arrays, you'll scan your arrays to produce TIFF images. The images will be processed by a program such as SPOT, GenePix, Imagene or Quantarray to acquire intensity measurements

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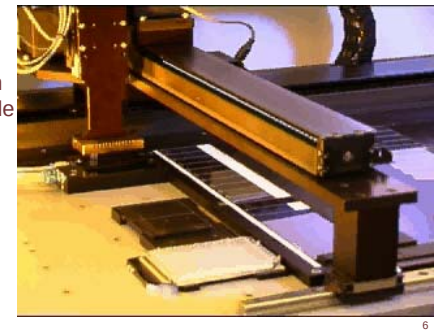
Array Printing

Printing custom library on glass slide



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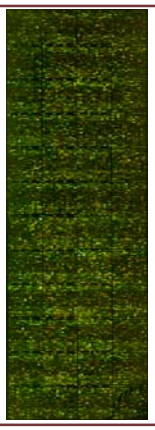

Microarray Image

False-coloured image with two channels overlaid:

- more highly expressed in mutant
- equally expressed
- more highly expressed in normal

AGRF NIA 15k mouse array:

12 x 4 pin groups

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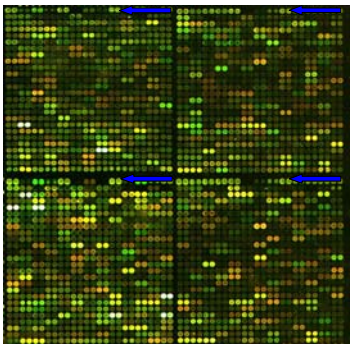

26 x 26 spots in each pin group

Genes printed in duplicate pairs

Arrows indicate print direction

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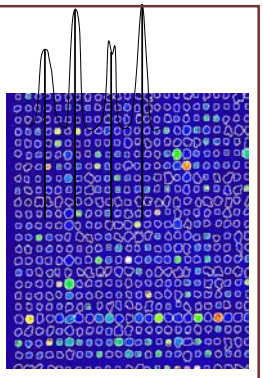
Steps in Image Processing

1. **Addressing**: locate centers

2. **Segmentation**: divide pixels into foreground (part of spot) or background

3. **Information extraction**: calculate signal intensity pairs, background and quality measures for each spot



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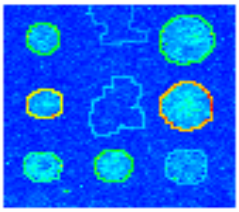
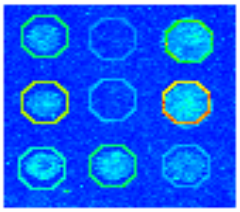
Segmentation



Seeded region growing
SPOT



Fixed circle method
Genepix: adaptive circles

Foreground measured from pixels inside the circle
background measured from ambient intensity around spot

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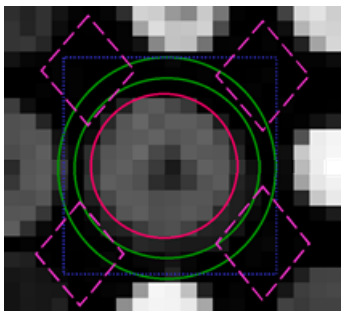
Local Backgrounds Measures

One channel grey scale



Genepix: region including pink diamonds

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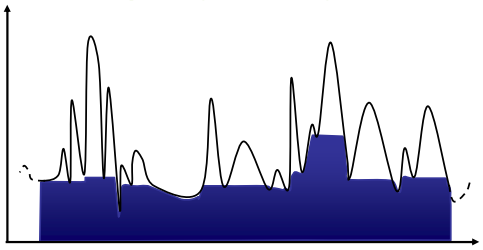
Morphological Background



SPOT estimates background using a nonlinear morphological filter – a lower, less variable measure

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Quantification of Expression: cDNA

- Foreground red, green $Rf, \quad Gf$
- Background red, green $Rb, \quad Gb$
- Background corrected $R = Rf - Rb, \; G = Gf - Gb$
- log-ratio ("Minus") $M = \log_2 R - \log_2 G$
- Average intensity ("Add") $A = (\log_2 R + \log_2 G)/2$

Lots of issues: which bg is best, to subtract bg or not, quality, filtering

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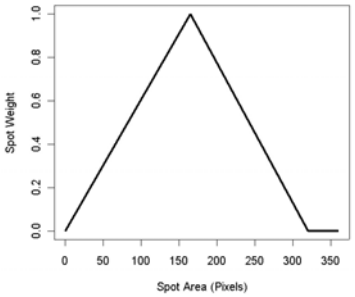
Spot Quality Weights

Example of a quality weighting:

downweight spots smaller or larger than nominal size

Many other possibilities



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Quantification of Expression: Affymetrix

- 12-40,000 probe sets/chip, each consisting of 11-20 Perfect Match (PM) and MisMatch (MM) probe pairs. Consider one set: chips i=1,2,.. probes j=1,2,..
- **Affymetrix summary**:
  log(Signal Intensity) = TukeyBiweight{log(PM_j - MM_j*)}
- **dChip model** (Li, Schadt & Wong):
  $PM_{ij} - MM_{ij} = \theta_i \varphi_j + \varepsilon_{ij}$
- **Robust Multichip Analysis (RMA)**:
  Use median polish or robust regression to fit
  $\log_2(PM\text{-}*BG)_{ij}$ = $chip_i$ effect + $probe_j$ effect + ε_{ij}
  See Bioconductor packages: affy, AffyPLM

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Exploratory Data Display

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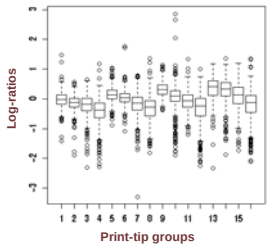
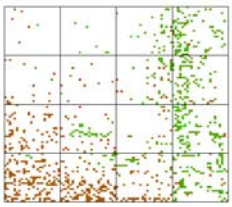
Spatial bias in cDNA arrays





Boxplots of M = $\log_2$R/G by print-tip group (1-16)

Same slide

Locations of spots with extreme 5% M: high, low

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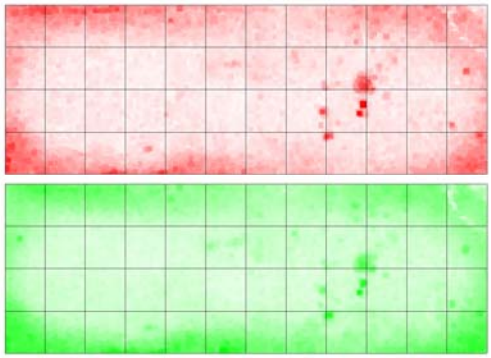
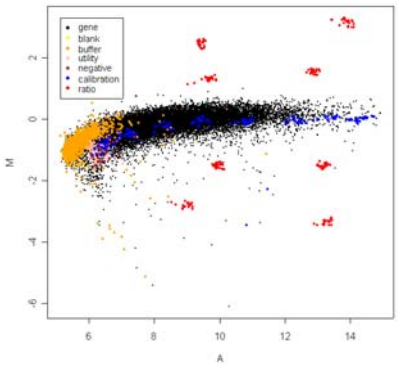
Spatial Plots: Background Intensities



MA Plot



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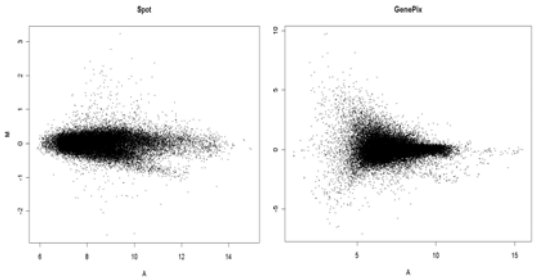
Background Affects Variability



From Spot: note lack of fanning (not same data)

From GenePix: note fanning out at low intensities

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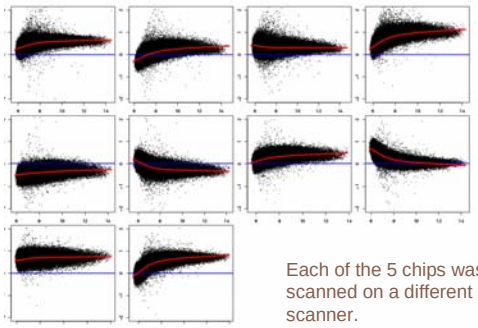
MA plots of 10 Affymetrix replicate chip pairs



Each of the 5 chips was scanned on a different scanner.

Normalization

Smyth and Speed "Normalization of cDNA microarray data", in: *METHODS: Selecting Candidate Genes from DNA Array Screens*, December 2003

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No Absolute Measurement Scale

- The spotted DNA sequences differ in binding efficiency, so absolute intensities are not directly comparable between genes
- The response of the dyes is subject to arbitrary rescaling for each dye on each array – need to normalize the red/green balance for each array (to make M-values unbiased)

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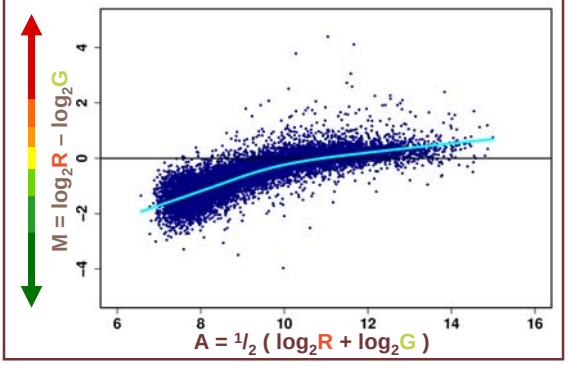
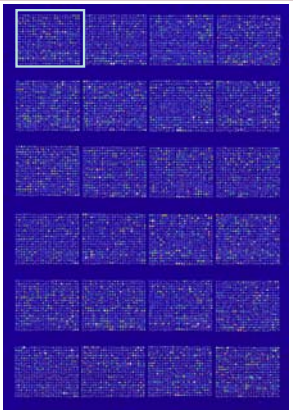
MA – Plot





6 x 4
pin-groups

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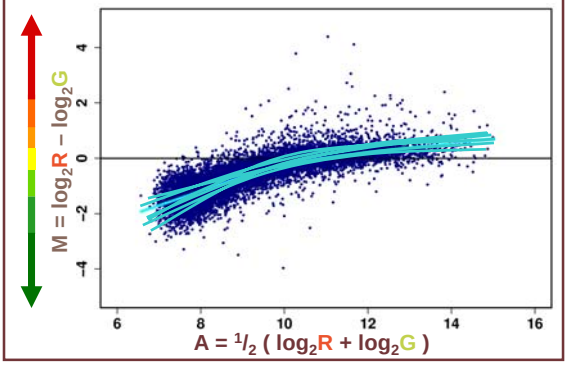
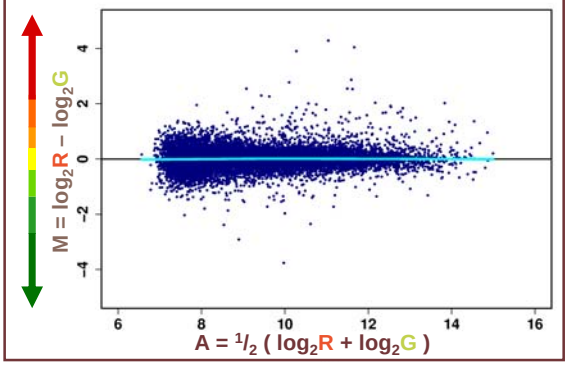
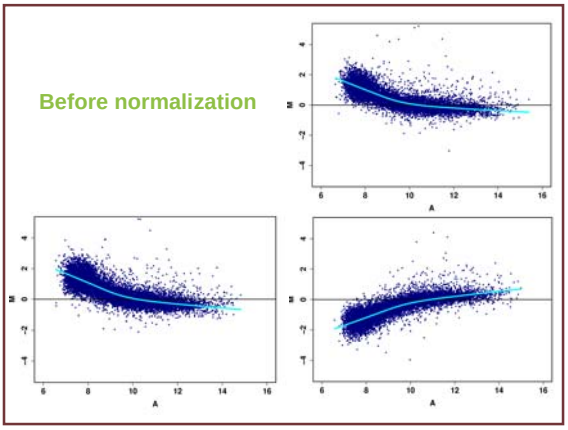
Print-tip-loess Normalization



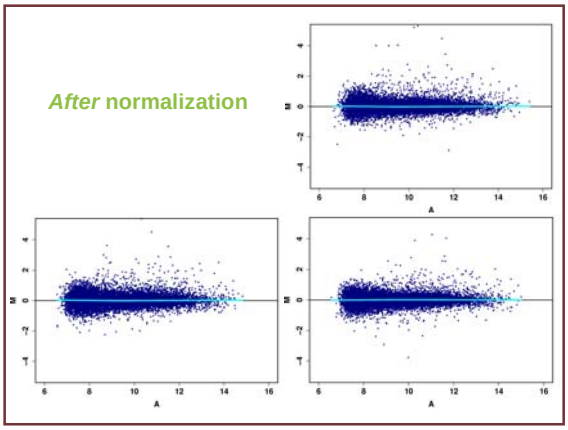
After Normalization



Before normalization



After normalization



Which Genes are Differentially Expressed?

Smyth, "Linear models and empirical Bayes methods for assessing differential expression in microarray experiments", July 2003

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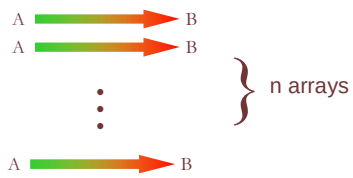
Replicate Arrays

Need replication to do statistical analysis of differential expression. Most basic possible is series of arrays all comparing the same two RNA sources:



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Gene-wise Summaries

- Each gene gives a series of log-ratios
- Summarize log-ratios by average and standard deviation for each gene, or robust versions of these:

$$M_1, \ldots, M_n$$

$$\bar{M} = \text{ave}\, M \qquad s = \text{std.dev}\, M$$

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Ranking Criteria

- Average log **fold change**. $\bar{M}_g$
  Problem: non DE genes with **large variances** have too much chance of being selected.
- **t-statistics** $t_g = \dfrac{\bar{M}_g}{s_g / \sqrt{n}}$
  Problem: genes with very **small sample variances** are suspect
- **Moderated t-statistics**. We use a happy compromise between the two $\tilde{t}_g = \dfrac{\bar{M}_g}{\tilde{s}_g / \sqrt{n}}$

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Moderated t-Statistics

Shrunk standard deviations

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

Moderated t-statistics

$$\tilde{t}_g = \frac{\bar{M}_g}{\tilde{s}_g \sqrt{c_g}}$$

Eliminates large t-statistics merely from very small *s*

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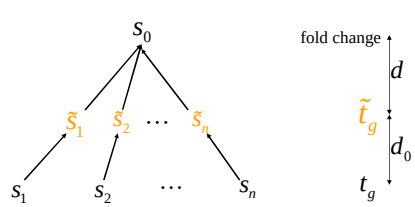
Shrinkage of Standard Deviations



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

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Posterior Odds

Posterior probability of differential expression for any gene is

$$\frac{p(\beta \neq 0 \mid \bar{M}, s^2)}{p(\beta = 0 \mid \bar{M}, s^2)} = \frac{p}{1-p}\left(\frac{c}{c+c_0}\right)^{1/2}\left\{\frac{\tilde{t}^2 + d + d_0}{\tilde{t}^2 \dfrac{c}{c+c_0} + d + d_0}\right\}^{\frac{1+d+d_0}{2}}$$

Monotonic function of $\tilde{t}^2$ for constant *d*

Reparametrization of Lönnstedt and Speed 2002

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Estimating Hyper-Parameters

Closed form estimators with good properties are available:

- for s_0 and d_0 in terms of the first two moments of log s^2
- for c_0 in terms of quantiles of the $|\tilde{t}_g|$

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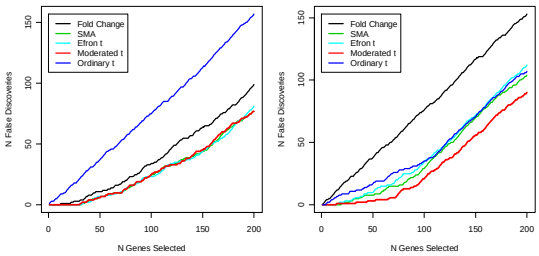
Simulations



σ^2 similar

σ^2 very different

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How many genes are differentially expressed?

Assigning absolute significance levels on the basis of probability models is problematic:

- Log-ratios don't appear to be normally distributed, hard to check
- Log-ratios for different genes are correlated in unknown way
- High level of multiple testing means that very small p-values are required – distributional assumptions must hold in extreme tail
- Little opportunity for usual CLT results to apply

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Ranking Easier Than Testing

- If there was only one gene, a **t-test** would give a reliable P-value for judging whether the true log-ratio was zero
- With many genes, computed **P-values** cannot be trusted (unless have > 16 arrays)
- It is more realistic to **rank** the genes in order of evidence for differential expression

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In Search of Truth

- We treat moderated t and posterior odds as ranking criteria rather than as providers of absolute significance or posterior odds
- To rigorously estimate type I or type II error rates or to compare competing analysis methods, need to construct microarray data with known *truth*

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Spike-Ins

- Spike-in artificial RNA cocktail to induce known fold changes in control genes
- Spike-ins can give an objective basis for choosing cut-off for differential expression

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Spike-In Controls

- Print artificial genes on microarray, then add spike-ins of corresponding RNA in known quantities to sample RNA before labelling and hybridization
- ScoreCard and SpotReport are commercial systems in use at the AGRF

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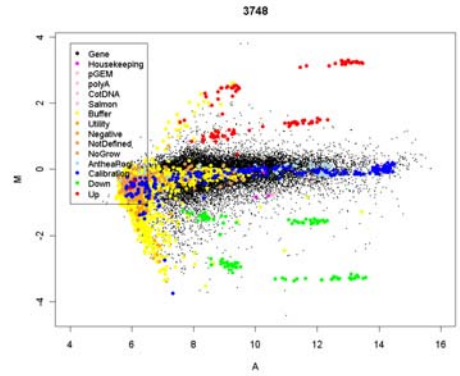
MA-Plot with spike-in controls highlighted



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How well do moderated t-statistics distinguish ratio (DE) controls from calibration (non-DE) controls?

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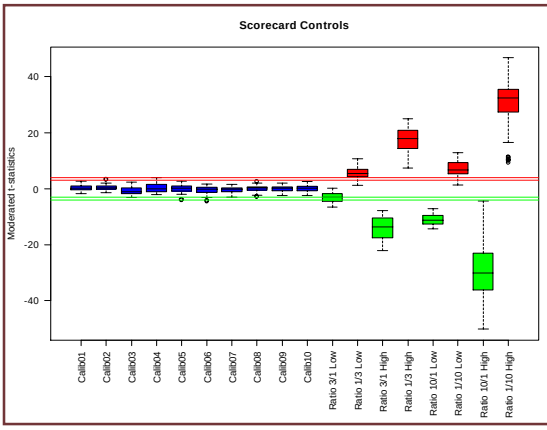

Empirical Cut Off

$$|\tilde{t}_g| > 4$$

appears a conservative rule for differential expression with very small false discovery rate for this data

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Other Data with Constructed Truth

Assessing Precision:

- Replicate arrays
- Same vs same hybridizations
- Whole library pool titration series – dynamic range but not differentially expressed

Assessing Bias:

- Independent measurement of differential expression – PCR, independent arrays
- Spike-in ratio and calibration controls
- Mixture and dilution experiments

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LIMMA Package for R

- Linear models for microarray data. A software package for the R programming environment. Focus is differential expression including
  - moderated t-statistics
  - methods for duplicate spots
  - classifying F-tests
  - stemmed heat diagrams

  Available from www.bioconductor.org

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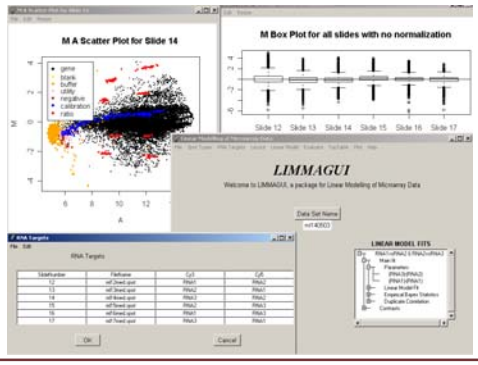
limma GUI …



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- Mirielle Lahoud
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Berkeley
- Jean Yang
- Speed Lab
- Ngai Lab

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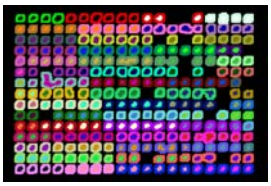
When the chips are done …



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