Pre-processing in DNA microarray experiments

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Bioconductor short course
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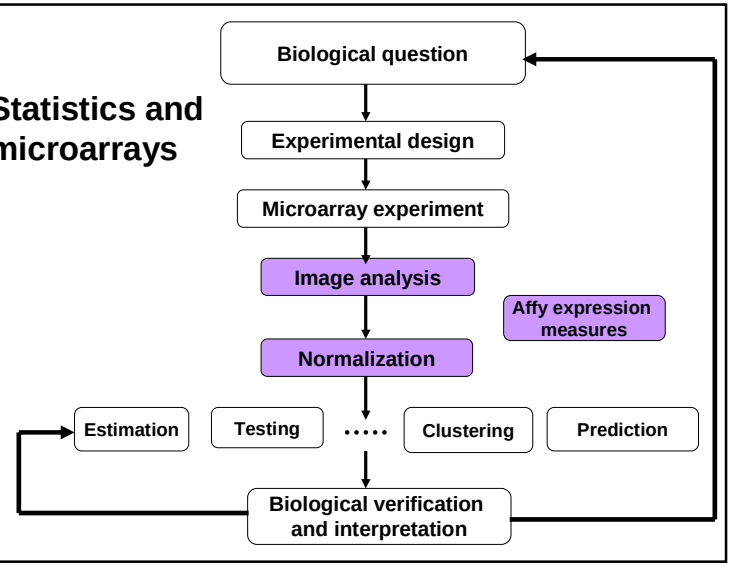
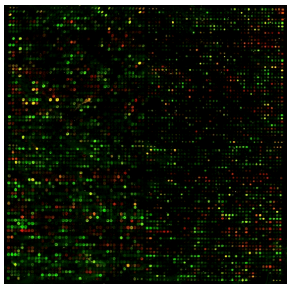
Statistics and microarrays



Outline

- cDNA microarrays
  - Image analysis;
  - Normalization.
- Affymetrix oligonucleotide chips
  - Image analysis;
  - Normalization;
  - Expression measures.

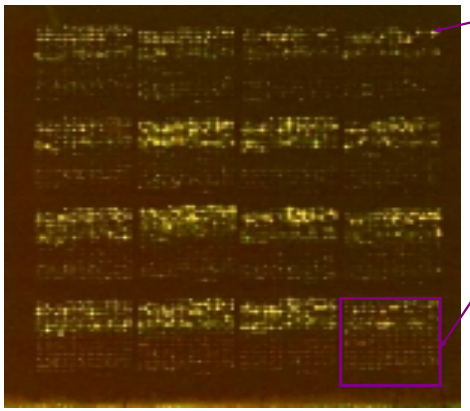
cDNA microarrays



Terminology

- Target: DNA hybridized to the array, mobile substrate.
- Probe: DNA spotted on the array,
  aka. spot, immobile substrate.
- Sector: collection of spots printed using the same print-tip (or pin),
  aka. print-tip-group, pin-group, spot matrix, grid.
- The terms slide and array are often used to refer to the printed microarray.
- Batch: collection of microarrays with the same probe layout.
- Cy3 = Cyanine 3 = green dye.
- Cy5 = Cyanine 5 = red dye.

RGB overlay of Cy3 and Cy5 images



Raw data

E.g. Human cDNA arrays

- ~43K spots;
- 16–bit TIFFs: ~ 20Mb per channel;
- ~ 2,000 x 5,500 pixels per image;
- Spot separation: ~ 136um;
- For a “typical” array, the spot area has
  - mean = 43 pixels,
  - med = 32 pixels,
  - SD = 26 pixels.

Image analysis

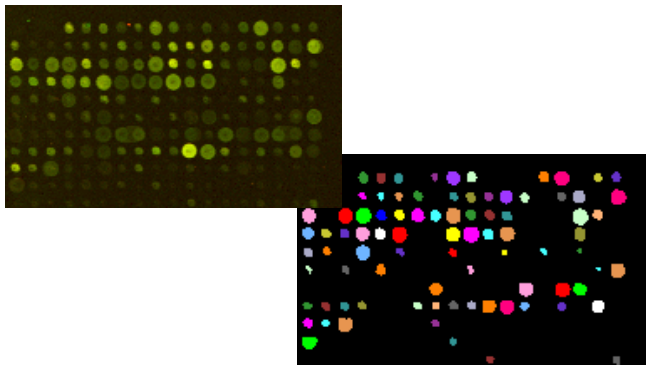
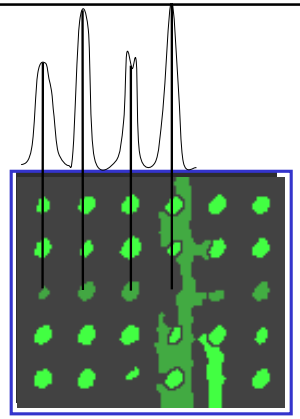


Image analysis

- The **raw data** from a cDNA microarray experiment consist of pairs of **image files**, 16 bit TIFFs, one for each of the dyes.
- **Image analysis** is required to extract measures of the red and green fluorescence intensities, **R** and **G**, for each spot on the array.

Image analysis



1. Addressing. Estimate location of spot centers.

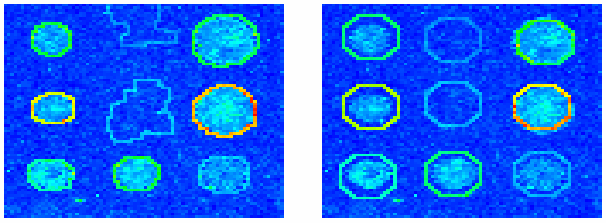
2. Segmentation. Classify pixels as foreground (signal) or background.

3. Information extraction. For each spot on the array and each dye

- foreground intensities;
- background intensities;
- quality measures.

→ **R and G for each spot on the array.**

Segmentation



Adaptive segmentation, SRG **Fixed circle segmentation**

Spots usually vary in size and shape.

Seeded region growing

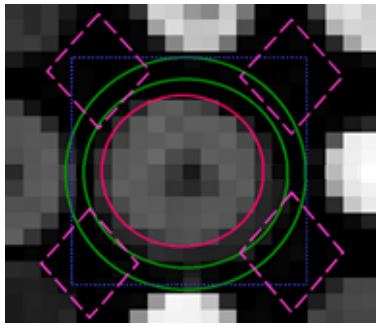
- **Adaptive** segmentation method.
- Requires the input of **seeds**, either individual pixels or groups of pixels, which control the formation of the regions into which the image will be segmented.
  Here, based on fitted foreground and background **grids** from the addressing step.
- The decision to add a pixel to a region is based on the absolute gray-level difference of that pixel's intensity and the average of the pixel values in the neighboring region.
- Done on combined red and green images.
- Ref. Adams & Bischof (1994)

Local background

---- GenePix

---- QuantArray

---- ScanAnalyze

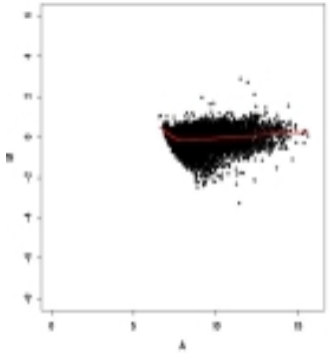


Morphological opening

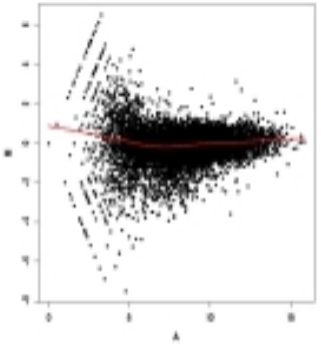
- The image is probed with a **structuring element**, here, a square with side length about twice the spot-to-spot distance.
- **Erosion** (**Dilation**): the eroded (dilated) value at a pixel x is the **minimum** (**maximum**) value of the image in the window defined by the structuring element when its origin is at x.
- **Morphological opening**: **erosion** followed by **dilation**.
- Done separately for the red and green images.
- Produces an image of the estimated background for the entire slide.

Background matters

Morphological opening



Local background



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

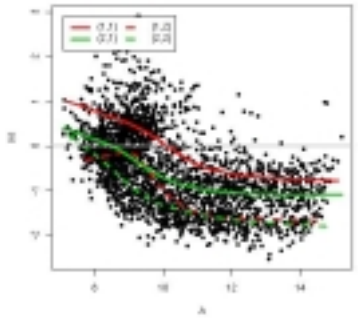
Quality measures

- **Spot quality**
  - **Brightness:** foreground/background ratio;
  - **Uniformity:** variation in pixel intensities and ratios of intensities within a spot;
  - **Morphology:** area, perimeter, circularity.
- **Slide quality**
  - Percentage of spots with no signal;
  - Range of intensities;
  - Distribution of spot signal area, etc.
- How to use quality measures in subsequent analyses?

Spot image analysis software

- Software package **Spot**, built on the **R** language and environment for statistical computing and graphics.
- Batch automatic addressing.
- Segmentation. **Seeded region growing** (Adams & Bischof 1994): **adaptive** segmentation method, no restriction on the size or shape of the spots.
- Information extraction
  - Foreground. Mean of pixel intensities within a spot.
  - Background**. Morphological opening**: non-linear filter which generates an image of the estimated background intensity for the entire slide.
- Spot quality measures.

Normalization



Normalization

- **Purpose.** Identify and remove the effects **of systematic variation** in the measured fluorescence intensities, other than differential expression, for example
  - different labeling efficiencies of the dyes;
  - different amounts of Cy3- and Cy5-labeled mRNA;
  - different scanning parameters;
  - print-tip, spatial, or plate effects, etc.

Normalization

- Normalization is needed to ensure that differences in intensities are indeed due to differential expression, and not some printing, hybridization, or scanning artifact.
- Normalization is necessary before any analysis which involves within or between slides comparisons of intensities, e.g., clustering, testing.

Normalization

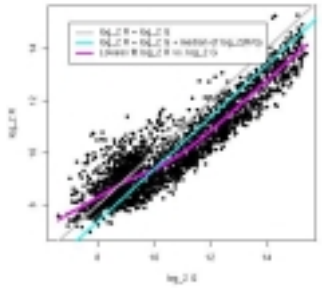
- The need for normalization can be seen most clearly in **self-self hybridizations**, where the same mRNA sample is labeled with the Cy3 and Cy5 dyes.
- The imbalance in the red and green intensities is usually **not constant** across the spots within and between arrays, and can vary according to overall spot intensity, location, plate origin, etc.
- These factors should be considered in the normalization.

Single-slide data display

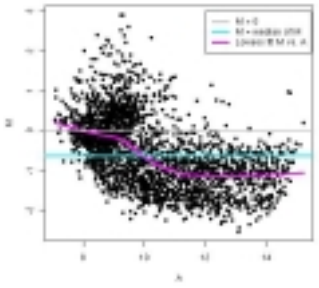
- Usually: R vs. G
  $\log_2 R$ vs. $\log_2 G$.
- Preferred
  $M = \log_2 R - \log_2 G$
  vs. **$A = (\log_2 R + \log_2 G)/2$**.
- An MA plot amounts to a 45° counterclockwise rotation of a $\log_2 R$ vs. $\log_2 G$ plot followed by scaling.

Self-self hybridization

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



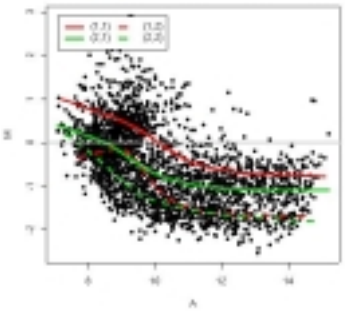
M vs. A



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

Self-self hybridization

M vs. A



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

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

Swirl zebrafish experiment

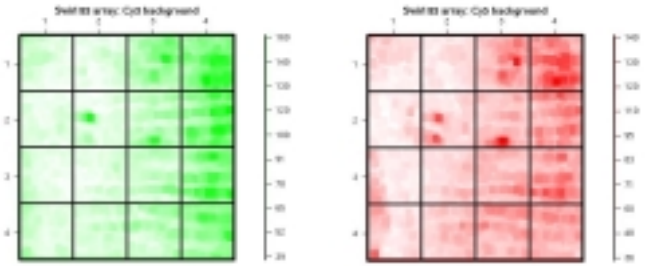
- **Goal**. Identify genes with altered expression in Swirl mutants compared to wild type zebrafish.
- 2 sets of dye swap experiments (n=4).
- Arrays:
  - 8,448 probes (768 controls);
  - 4 x 4 grid matrix;
  - 22 x 24 spot matrices.
- Data available in Bioconductor package `marrayInput`.

Diagnostic plots

- **Diagnostics plots** of spot statistics

  E.g. red and green log-intensities, intensity log-ratios M, average log-intensities A, spot area.
  - Boxplots;
  - 2D spatial images;
  - Scatter-plots, e.g. MA-plots;
  - Density plots.
- **Stratify** plots according to layout parameters, e.g. print tip group, plate.

2D spatial images

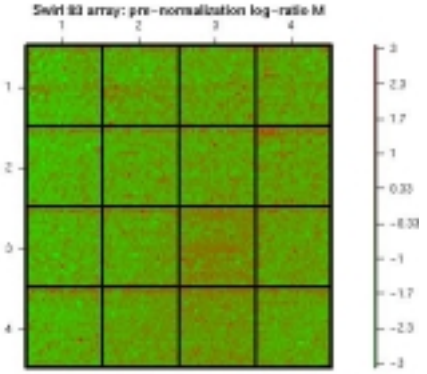


Cy3 background intensity

Cy5 background intensity

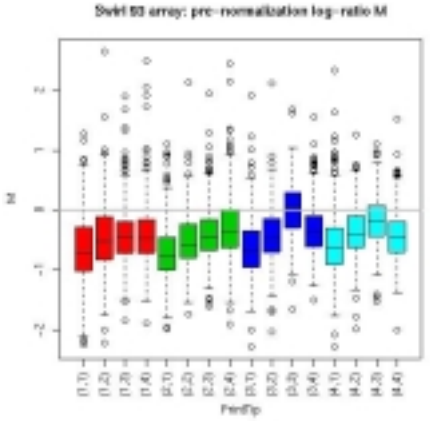
2D spatial images

Intensity log-ratio, M



Boxplots by print-tip-group

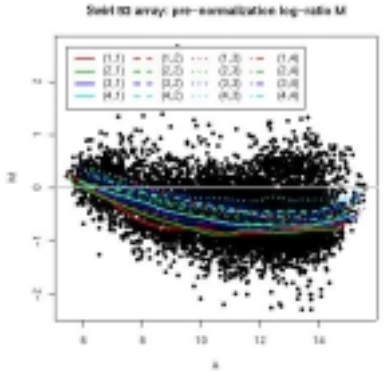
Intensity log-ratio, M



MA-plot by print-tip-group

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

Intensity log-ratio, M



Average log-intensity, A

Location normalization

$\log_2 R/G \leftarrow \log_2 R/G - L(\text{intensity, sector, …})$

- **Constant normalization.** Normalization function L is **constant** across the spots, e.g. mean or median of the log-ratios M.
- **Adaptive normalization.** Normalization function L depends on a number of **predictor variables**, such as spot intensity A, sector, plate origin.

Location normalization

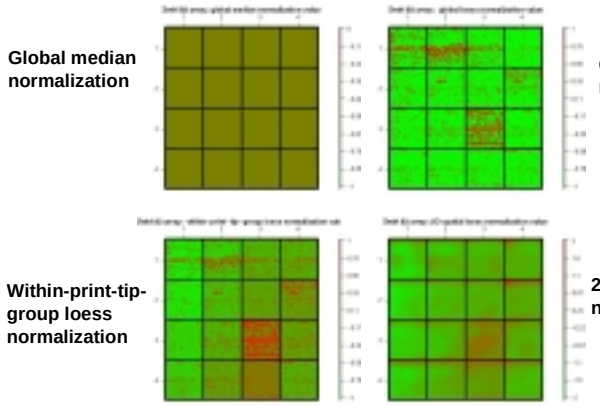
- The normalization function can be obtained by **robust locally weighted regression** of the log ratios M on predictor variables.

  E.g. regression of M on A within sector.
- Regression method: e.g. lowess or loess (Cleveland, 1979; Cleveland & Devlin, 1988).

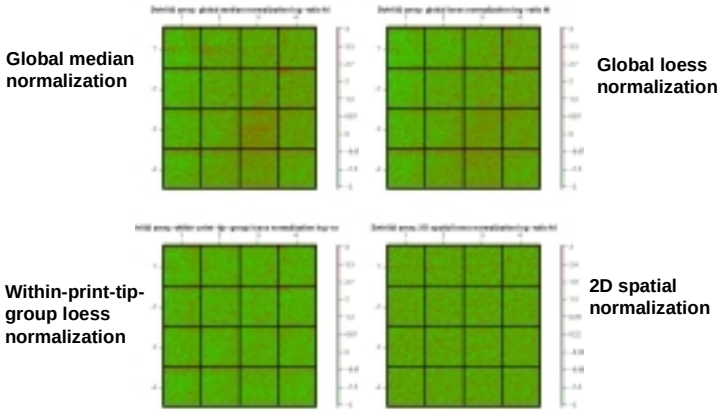
Location normalization

- **Intensity-dependent normalization.**
  Regression of M on A (*global loess*).
- **Intensity and sector-dependent normalization**.
  Same as above, for each sector separately (*within-print-tip-group loess*).
- **2D spatial normalization**.
  Regression of M on 2D-coordinates.
- Other variables: time of printing, plate, etc.
- **Composite normalization**. Weighted average of several normalization functions.

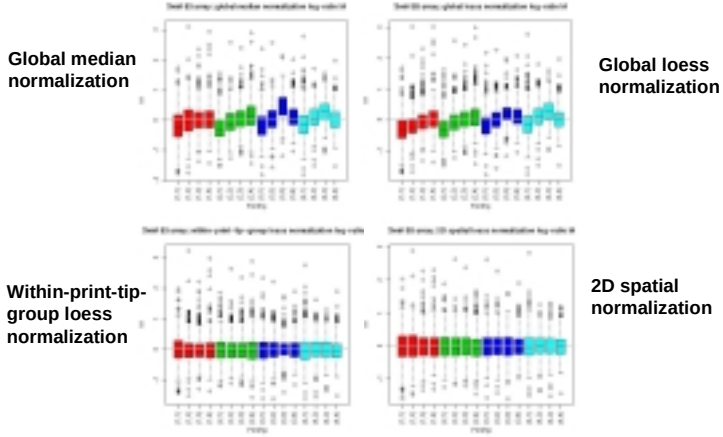
2D images of L values



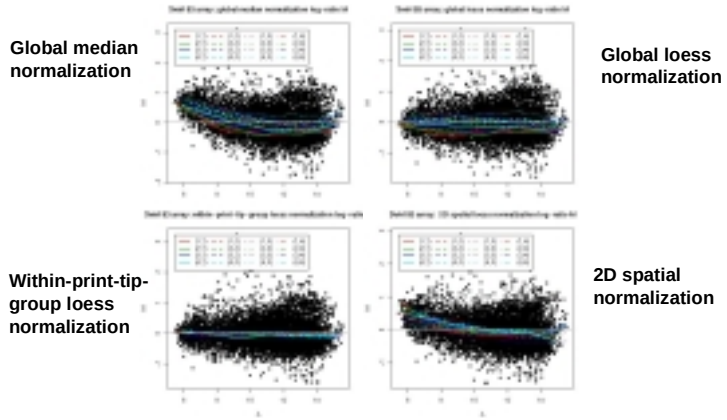
2D images of normalized M-L



Boxplots of normalized M-L



MA-plots of normalized M-L



Normalization

- Within-slide
  - **Location** normalization - additive on log-scale.
  - **Scale** normalization - multiplicative on log-scale.
  - **Which spots** to use?
- Paired slides (dye swap experiments)
  - Self-normalization.
- Between-slides.

Scale normalization

- The log-ratios M from different sectors, plates, or arrays may exhibit different spreads and some **scale** adjustment may be necessary.

$$\log_2 R/G \leftarrow (\log_2 R/G - L)/S$$

- Can use a robust estimate of scale such as the **median absolute deviation (MAD)**

  MAD = median | M – median(M) |.

Scale normalization

- For print-tip-group scale normalization, assume all print-tip-groups have the same spread in M.
- Denote ***true*** and ***observed*** log-ratio by μ_{ij} and M_{ij}, resp., where $M_{ij} = a_i \mu_{ij}$, and i indexes print-tip-groups and j spots. Robust estimate of a_i is

$$\hat{a}_i = \frac{MAD_i}{\sqrt[I]{\prod_{i=1}^{I} MAD_i}}$$

  where MAD_i is MAD of M_{ij} in print-tip-group i.
- Similarly for between-slides scale normalization.

Which genes to use?

- **All spots on the array**:
  - Problem when many genes are differentially expressed.
- **Housekeeping genes**: Genes that are thought to be constantly expressed across a wide range of biological samples (e.g. tubulin, GAPDH). Problems:
  - sample specific biases (genes are actually regulated),
  - do not cover intensity range.

Which genes to use?

- **Genomic DNA titration series**:
  - fine in yeast,
  - but weak signal for higher organisms with high intron/exon ratio (e.g. mouse, human).
- **Rank invariant set** (Schadt et al., 1999; Tseng et al., 2001): genes with same rank in both channels. Problems: set can be small.

Microarray sample pool

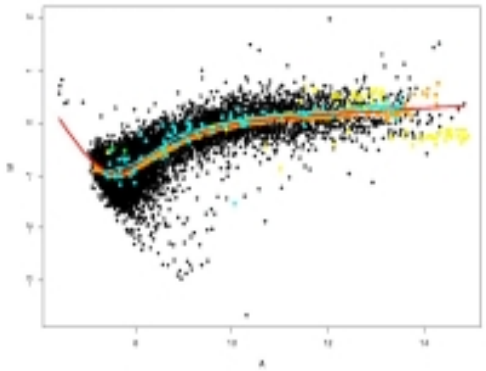
- **Microarray Sample Pool**, **MSP**: Control sample for normalization, in particular, when it is not safe to assume most genes are equally expressed in both channels.
- MSP: **pooled** all 18,816 ESTs from RIKEN release 1 cDNA mouse library.
- Six-step **dilution series** of the MSP.
- MSP samples were spotted in middle of first and last row of each sector.
- Ref. Yang et al. (2002).

Microarray sample pool

MSP control spots

- provide potential probes for every target sequence;
- are constantly expressed across a wide range of biological samples;
- cover the intensity range;
- are similar to genomic DNA, but without intron sequences → better signal than genomic DNA in organisms with high intron/exon ratio;
- can be used in composite normalization.

Microarray sample pool

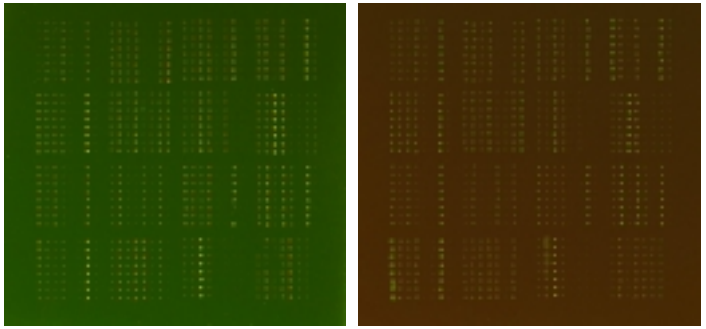


MSP
Rank invariant
Housekeeping
Tubulin, GAPDH

Dye-swap experiment

- Probes
  - 50 distinct clones thought to be differentially expressed in apo AI knock-out mice compared to inbred C57Bl/6 control mice (largest absolute t-statistics in a previous experiment).
  - 72 other clones.
- Spot each clone 8 times .
- Two hybridizations with dye-swap:
  Slide 1: trt → red, ctl → green.
  Slide 2: trt → green, ctl → red.

Dye-swap experiment



Self-normalization

- Slide 1, $M = \log_2 (R/G) - L$
- Slide 2, $M' = \log_2 (R'/G') - L'$

Combine by **subtracting** the normalized log-ratios:

$M - M'$

$= [(\log_2 (R/G) - L) - (\log_2 (R'/G') - L')] / 2$

$\approx [\log_2 (R/G) + \log_2 (G'/R')] / 2$

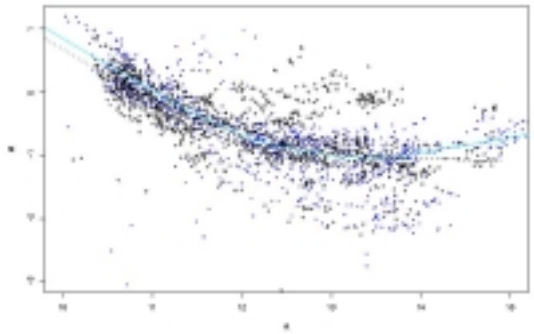
$\approx [\log_2 (RG'/GR')] / 2$

provided L= L'.

Assumption: the normalization functions are the same for the two slides.

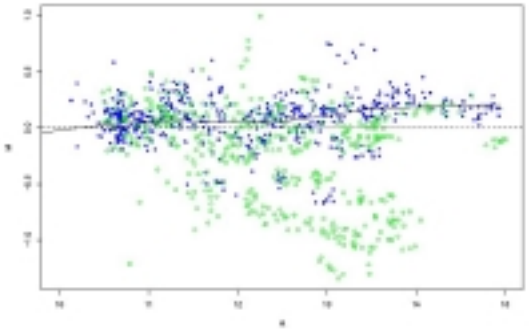
Checking the assumption

MA-plot for slides 1 and 2



Result of self-normalization

(M - M')/2 vs. (A + A')/2



Summary

Case 1. Only a few genes are expected to change.

Within-slide

- Location: intensity + sector-dependent normalization.
- Scale: for each sector, scale by MAD.

Between-slides

- An extension of within-slide scale normalization.

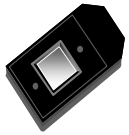
Case 2. Many genes are expected to change.

- Paired-slides: Self-normalization.
- Use of controls or known information, e.g. MSP.
- Composite normalization.

R software for normalization cDNA microarrays

- Bioconductor R packages
  - **`marrayClasses`**:
    - class definitions for microarray data objects;
    - basic methods for manipulation of microarray objects.
  - **`marrayInput`**:
    - reading in intensity data and textual data describing probes and targets;
    - automatic generation of microarray data objects;
    - widgets for point & click interface.
  - **`marrayPlots`**: diagnostic plots.
  - **`marrayNorm`**: robust adaptive location and scale normalization procedures.

Oligonucleotide chips



Affymetrix files

- Main software from Affymetrix company MAS- MicroArray Suite, now version 5.
- **DAT** file: Image file, ~10^7 pixels, ~50 MB.
- **CEL** file: Cell intensity file, probe level PM and MM values.
- **CDF** file: Chip Description File. Describes which probes go in which probe sets (genes, gene fragments, ESTs).

Image analysis

- Raw data, **DAT image files ➔ CEL files**
- Each probe cell: 10x10 pixels.
- **Gridding**: estimate location of probe cell centers.
- **Signal**:
  - Remove outer 36 pixels ➔ 8x8 pixels.
  - The probe cell signal, PM or MM, is the 75th percentile of the 8x8 pixel values.
- **Background**: Average of the lowest 2% probe cell values is taken as the background value and subtracted.
- Compute also quality measures.

Data and notation

- PM_{ijg} , MM_{ijg} = Intensity for perfect match and mismatch probe in cell *j* for gene *g* in chip *i*.
  - $i = 1,\ldots, n$ -- from one to hundreds of chips,
  - $j = 1,\ldots, J$ -- usually 16 or 20 probe pairs,
  - $g = 1,\ldots, G$ -- between 8,000 and 20,000 probe sets.
- Task: summarize for each probe set the probe level data, i.e., 20 PM and MM pairs, into a single **expression measure**.
- Expression measures may then be compared within or between chips for detecting differential expression.

Expression measures MAS 4.0

- GeneChip® MAS 4.0 software uses **AvDiff**

$$AvDiff = \frac{1}{|\mathrm{A}|}\sum_{j\in \mathrm{A}}(PM_j - MM_j)$$

where A is a set of "suitable" pairs, e.g. pairs with $d_j = PM_j - MM_j$ within 3 SDs of the average of $d_{(2)}, \ldots, d_{(J-1)}$.

- Log-ratio version is also used: average of log(PM/MM).

Expression measures MAS 5.0

- GeneChip® MAS 5.0 software uses **Signal**

$$signal = \text{Tukey Biweight}\{\log(PM_j - MM_j^*)\}$$

with MM* a new version of MM that is never larger than PM.

- If MM < PM, MM* = MM.
- If MM >= PM,
  - SB = Tukey Biweight (log(PM)-log(MM)) (log-ratio).
  - log(MM*) = log(PM)-log(max(SB, +ve)).
- Tukey Biweight: B(x) = (1 – (x/c)^2)^2 if |x|<c, 0 ow.

Expression measures Li & Wong

- Li & Wong (2001) fit a model for each probe set, i.e., gene

$$PM_{ij} - MM_{ij} = \theta_i\phi_j + \varepsilon_{ij},\ \varepsilon_{ij} \propto N(0,\sigma^2)$$

where
  - θ_i: **model based expression index** (MBEI),
  - ϕ_j: probe sensitivity index.
- Maximun likelihood estimate of MBEI is used as expression measure for the gene in chip *i*.
- Need at least 10 or 20 chips.
- Current version works with PMs only.

Expression measures

- Most expression measures are based on **PM-MM**, with the intention of correcting for non-specific binding and background noise.
- Problems:
  - MMs are PMs for some genes,
  - removing the middle base does not make a difference for some probes .
- Why not simply average PM or log PM? Not good enough, still need to adjust for background.
- Also need to normalize.

Expression measures RMA

Irizarry et al. (2002).

1. Estimate **background** BG and use only background corrected PM: $\log_2(PM - BG)$.
2. Probe level **normalization** of $\log_2(PM - BG)$ for suitable set of chips.
3. **Robust Multi-chip Analysis**, **RMA**, of $\log_2(PM - BG)$.

RMA background, I

Simple background estimation

- Estimate $\log_2(BG)$ as the mode of the $\log_2(MM)$ distribution for a given chip (kernel density estimate).
- Quick fix when PM <= BG: use half of the minimum of $\log_2(PM - BG)$ for PM > BG over all chips and probes.

RMA background, II

More refined background estimation

- Model observed PM as the sum of a signal intensity SG and a background intensity BG

$$PM = SG + BG,$$

  where it is assumed that SG is *Exponential* (α), BG is *Normal* (μ, σ^2), and SG and BG are independent.
- Background adjusted PM values are then **E(SG|PM)**.

Quantile normalization

- **Probe level quantile normalization** (Bolstad et al., 2002).
- Co-normalize probe level intensities, e.g. PM-BG or just PM or MM, for *n* chips by averaging each quantile across chips.
- Assumption: same probe level intensity distribution across chips.
- No need to choose a baseline or work in a pairwise manner.
- Deals with non-linearity.

Curve-fitting normalization

- Astrand (2001). Generalization of M vs. A robust local regression normalization for cDNA arrays.
- For *n* chips, regress orthonormal contrasts of probe level statistics on the average of the statistics across chips.

RMA expression measures, I

Simple measure

$$\text{RMA} = \frac{1}{|\text{A}|}\sum_{j \in A} \log_2(PM_j - BG_j)$$

with A a set of "suitable" pairs.

RMA expression measures, II

- **Robust regression method** to estimate expression measure and SE from PM-BG values.
- Assume additive model

$$\log_2(PM_{ij} - BG) = a_i + b_j + \varepsilon_{ij}$$

- Estimate RMA = a_i for chip *i* using robust method, such as **median polish** (fit iteratively, successively removing row and column medians, and accumulating the terms, until the process stabilizes).
- Fine with *n=2* or more chips.

Conclusions

- Don't use MM.
- "Background correct" PM. Even global background improves on probe-specific MM.
- Take logs: probe effect is additive on log scale.
- PMs need to be normalized (e.g. quantile normalization).
- RMA is arguably the best summary in terms of bias, variance, and model fit. Comparison study in Irizarry et al. (2002).

R software for pre-processing of Affymetrix data

- Bioconductor R package **`affy`**.
- Background estimation.
- Probe level normalization: quantile, curve fitting.
- Expression measures: AvDiff, Signal, Li & Wong (2001), RMA.
- Two main functions: **`ReadAffy`**, **`express`**.

Combining data across slides

Data on *G* genes for *n* hybridizations

→ *G x n* genes-by-arrays data matrix

Arrays

Genes	Array1	Array2	Array3	Array4	Array5	...
Gene1	0.46	0.30	0.80	1.51	0.90	...
Gene2	-0.10	0.49	0.24	0.06	0.46	...
Gene3	0.15	0.74	0.04	0.10	0.20	...
Gene4	-0.45	-1.03	-0.79	-0.56	-0.32	...
Gene5	-0.06	1.06	1.35	1.09	-1.09	...
...	...	...	...	...	...	

M = $\log_2$(Red intensity / Green intensity)
expression measure, e.g, RMA

Combining data across slides

… but columns have **structure**

How can we design experiments and combine data across slides to provide accurate estimates of the effects of interest?

Experimental design
Regression analysis