Pre-processing DNA Microarray Data

Sandrine Dudoit, Robert Gentleman, Rafael Irizarry, and Yee Hwa Yang

Bioconductor Short Course

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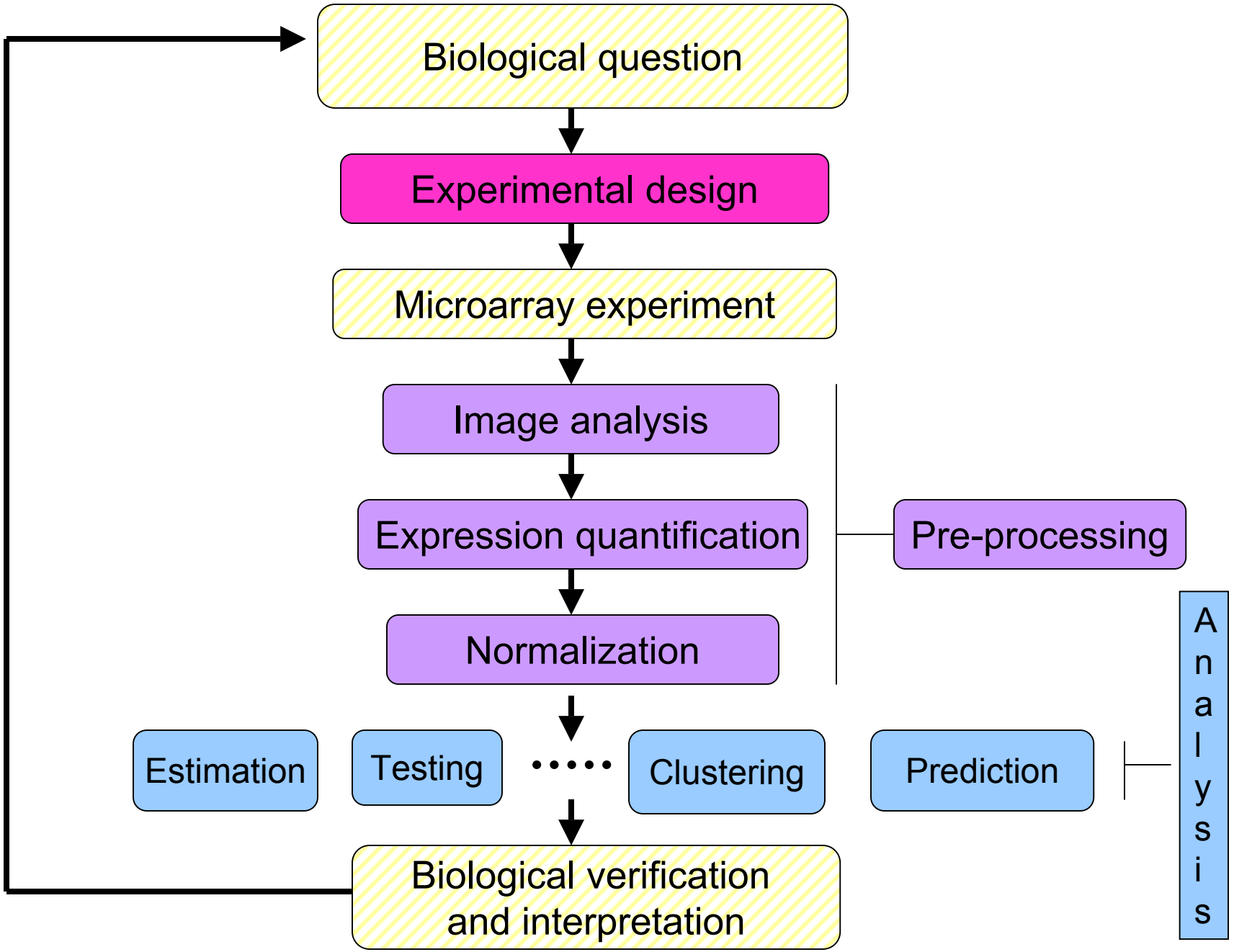
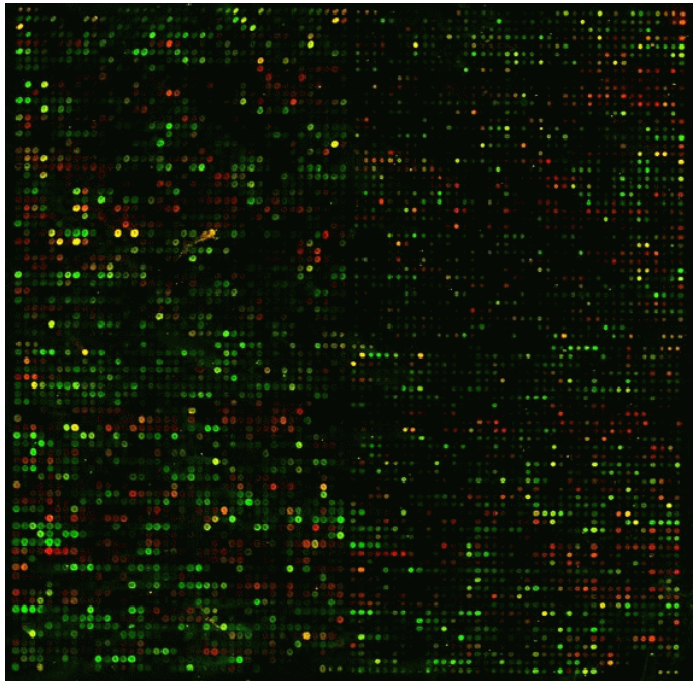

Biological question
Experimental design
Microarray experiment
Image analysis
Expression quantification
Normalization
Pre-processing
Estimation
Testing
.....
Clustering
Prediction
Analysis
Biological verification
and interpretation

Pre-processing

- Spotted DNA microarrays
  - Image analysis;
  - Normalization.

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

Pre-processing: Spotted DNA microarrays



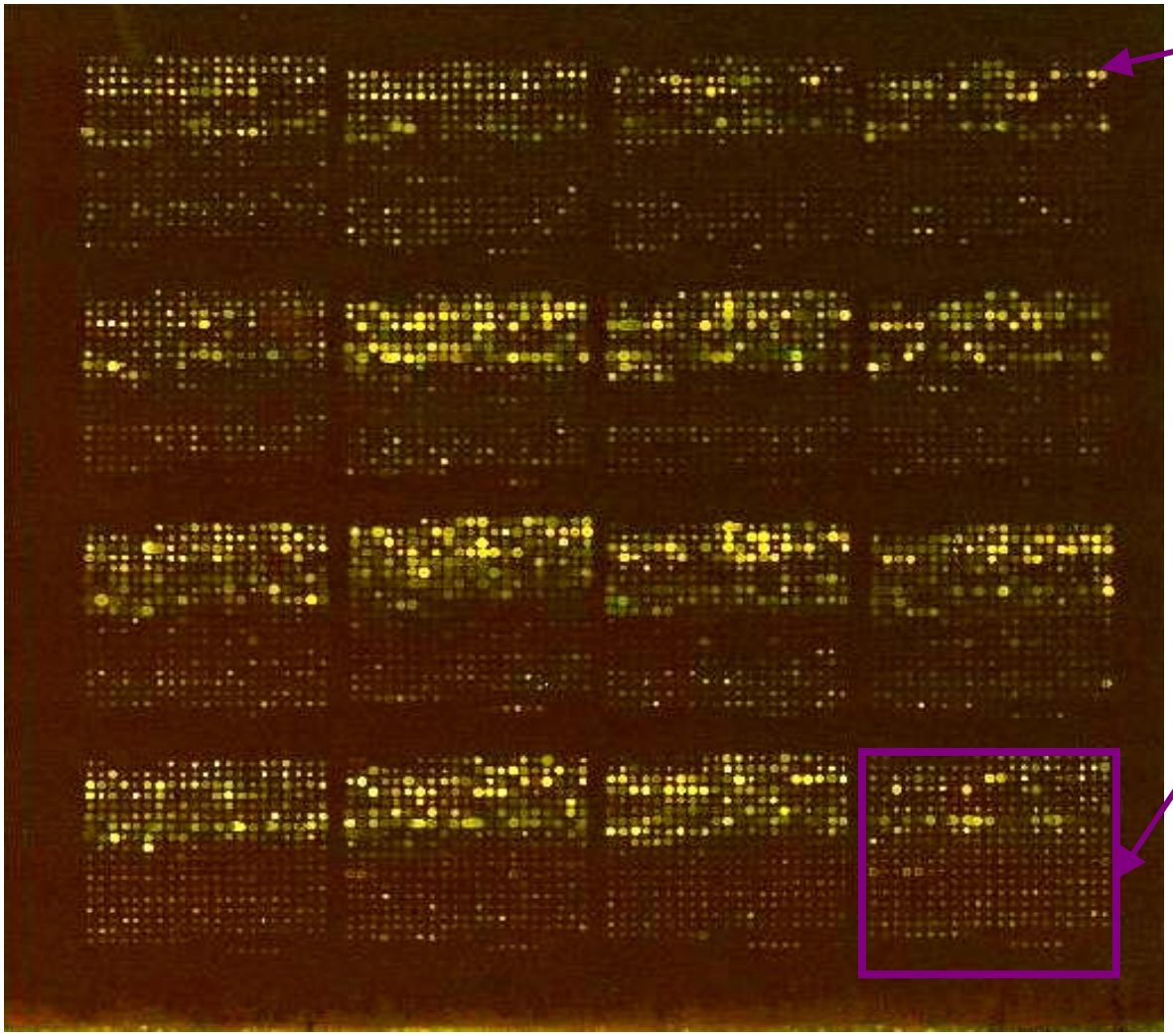
Terminology

- 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

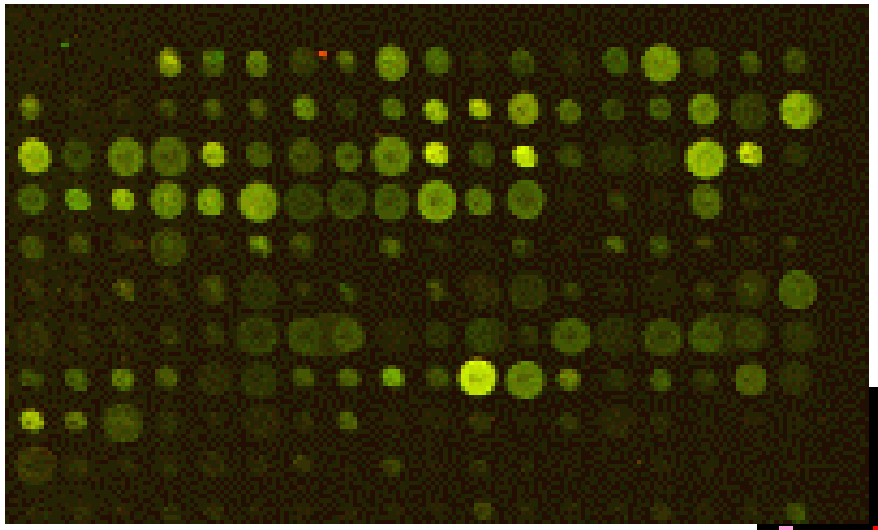


4 x 4 sectors
19 x 21 probes/sector
6,384 probes/array

Raw data

- Pairs of 16–bit TIFFs, one for each dye.
- E.g. Human cDNA arrays:
  - ~43K spots;
  - ~ 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



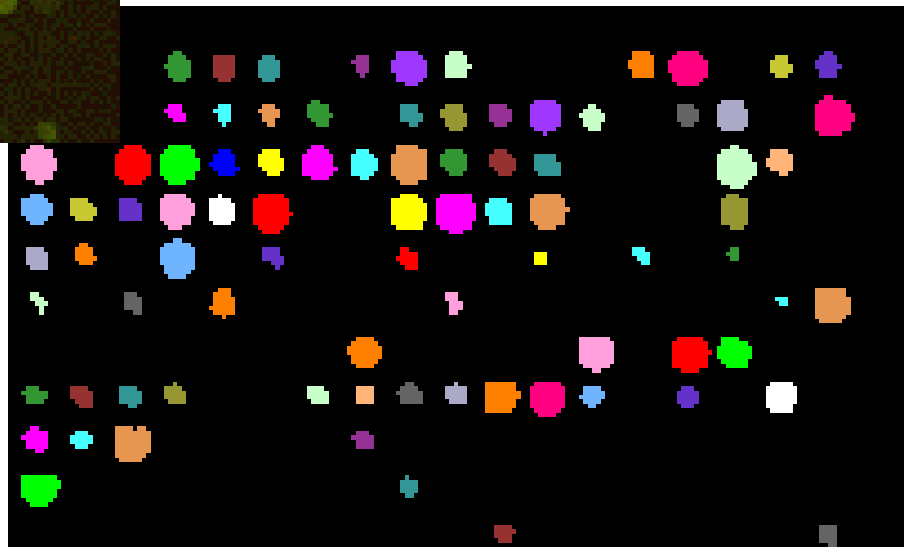
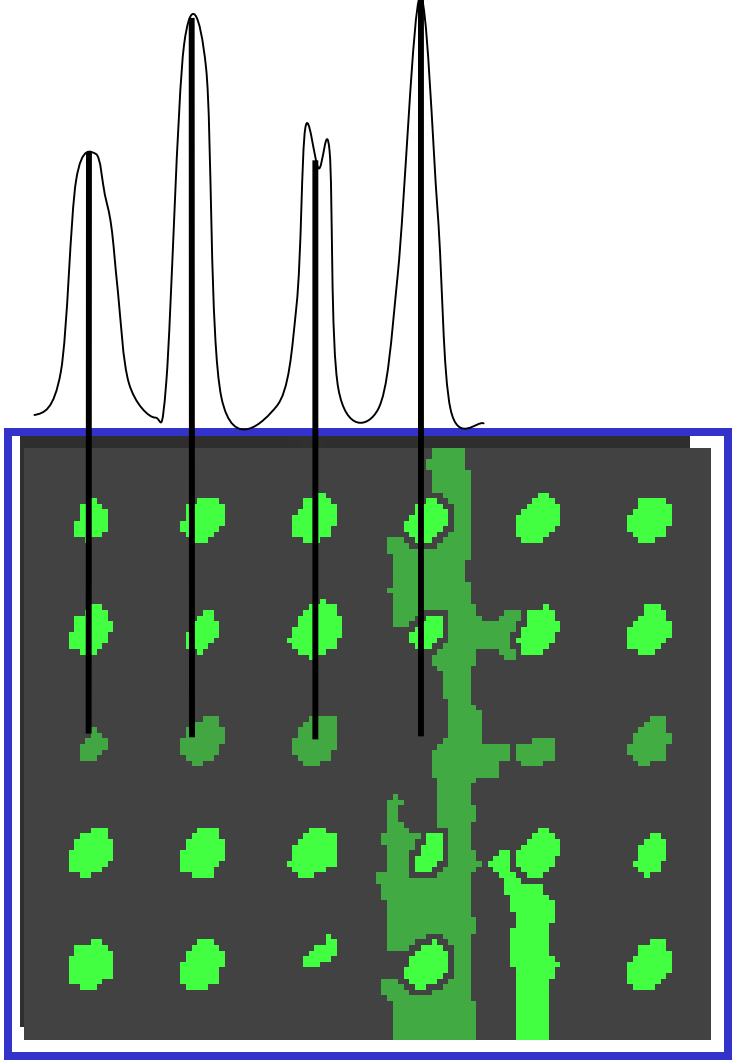


Image analysis

- 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.

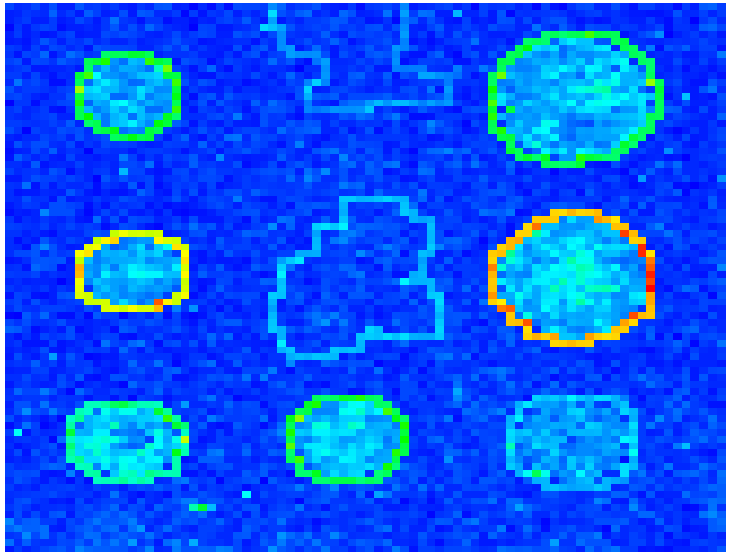
2. Segmentation. Classify pixels as foreground (signal) or background.

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

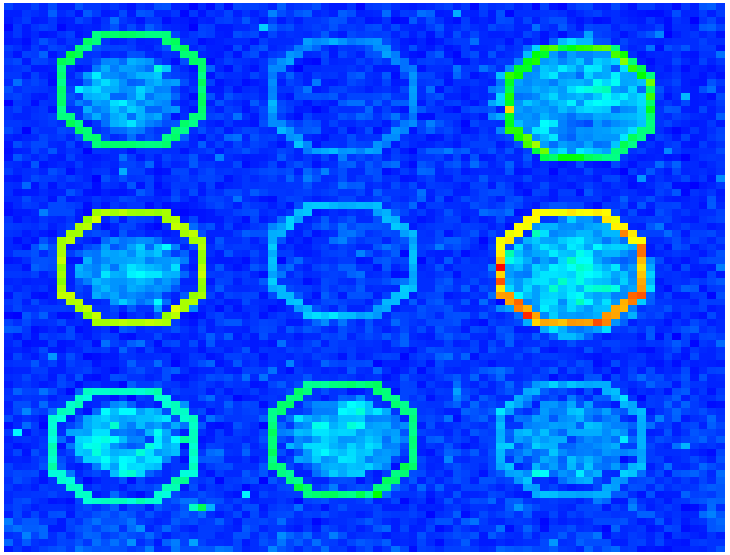
- foreground intensities;
- background intensities;
- quality measures.

→ **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.

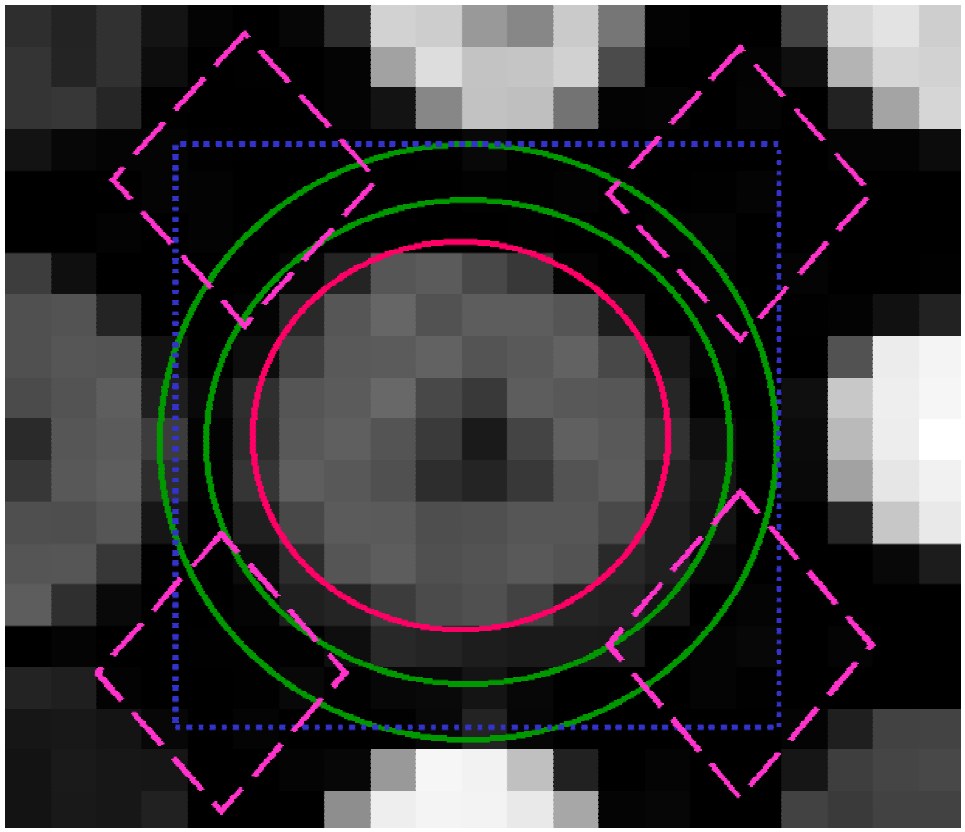
  Here, based on fitted foreground and background grids from the addressing step.
- The decision to add a pixel to a region is based on the absolute gray-level difference of that pixel's intensity and the average of the pixel values in the neighboring region.
- Done on combined red and green images.
- Ref. Adams & Bischof (1994)

Local background

---- GenePix

---- QuantArray

---- ScanAnalyze

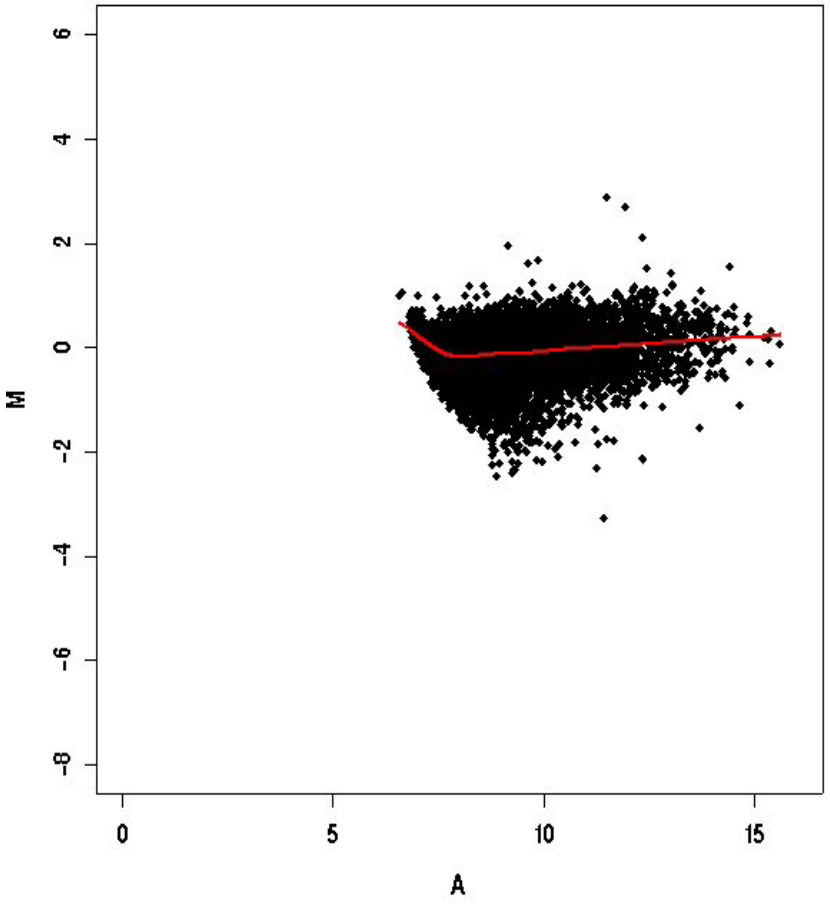


Morphological opening

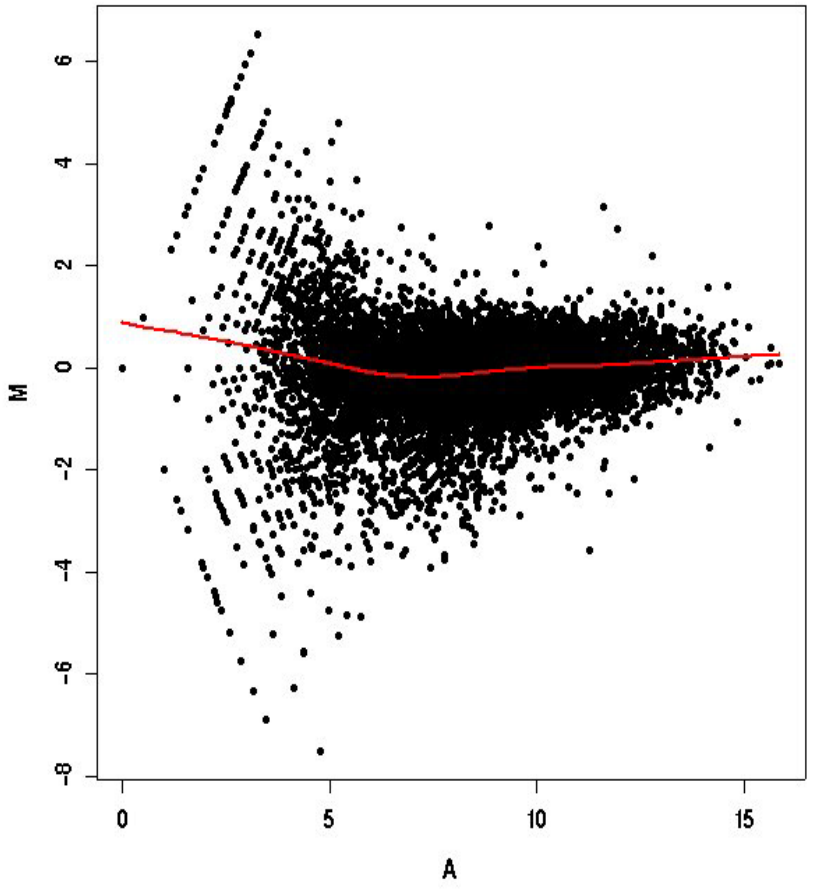
- The image is probed with a structuring element, here, a square with side length about twice the spot-to-spot distance.
- Erosion (Dilation): the eroded (dilated) value at a pixel x is the minimum (maximum) value of the image in the window defined by the structuring element when its origin is at x.
- 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. $A = (\log_2 R + \log_2 G)/2$

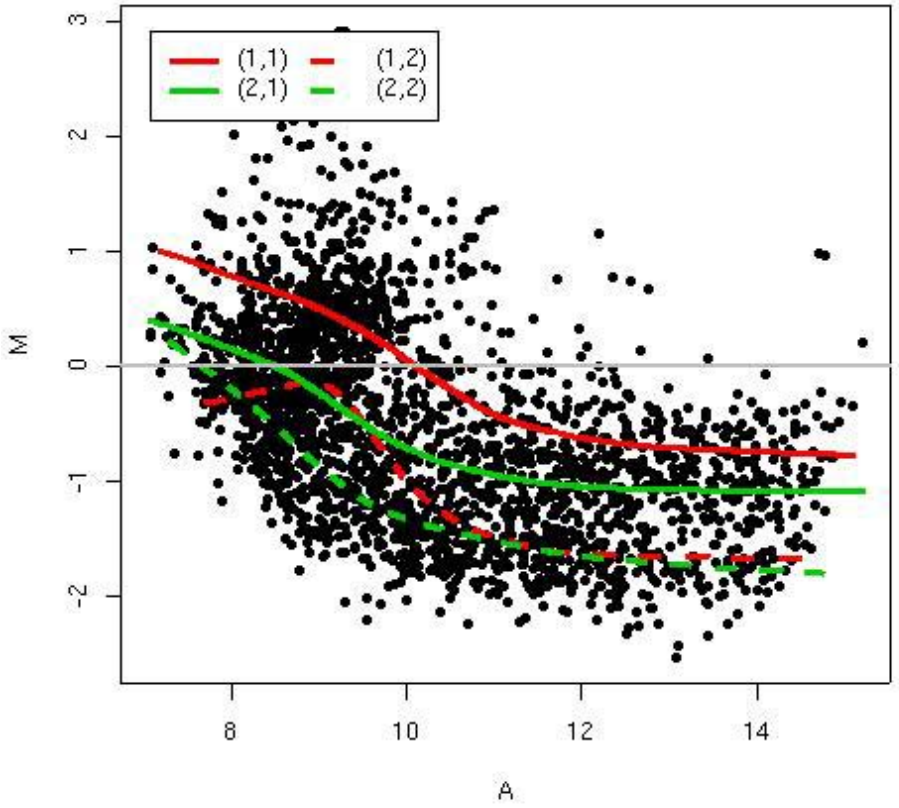
Quality measures

- **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

- After image processing, we have measures of the red and green fluorescence intensities, **R** and **G**, for each spot on the array.
- 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

- 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

- 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

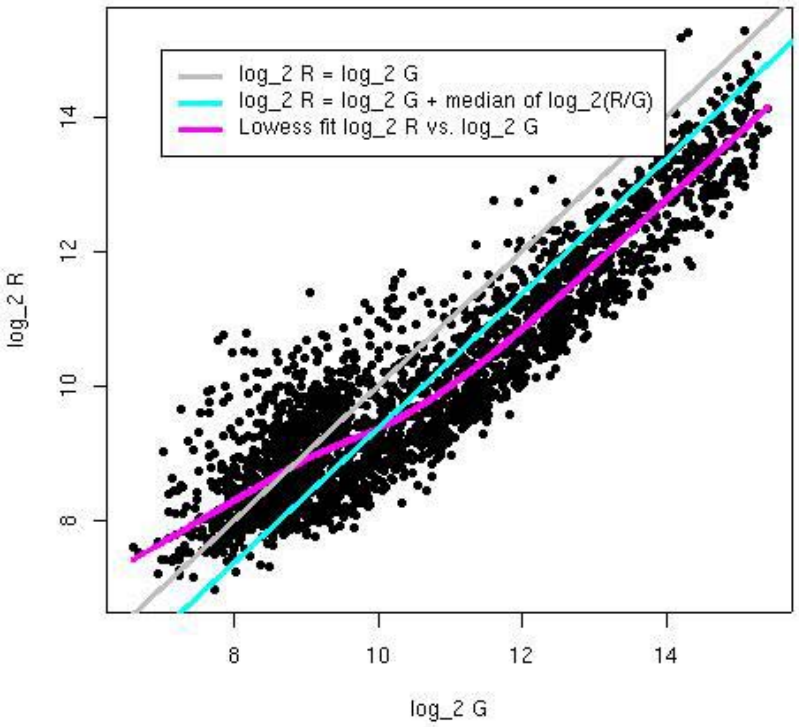
  $M = \log_2 R - \log_2 G$

  vs. **$A = (\log_2 R + \log_2 G)/2$**.
- An MA-plot amounts to a 45^o counterclockwise rotation of a

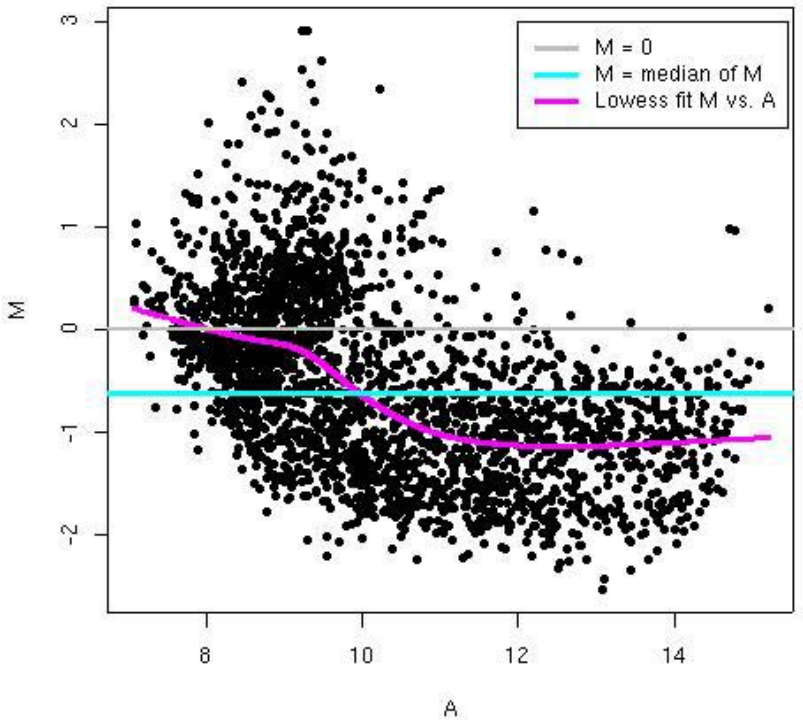
  $\log_2 R$ vs. $\log_2 G$ plot followed by scaling.

Self-self hybridization

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



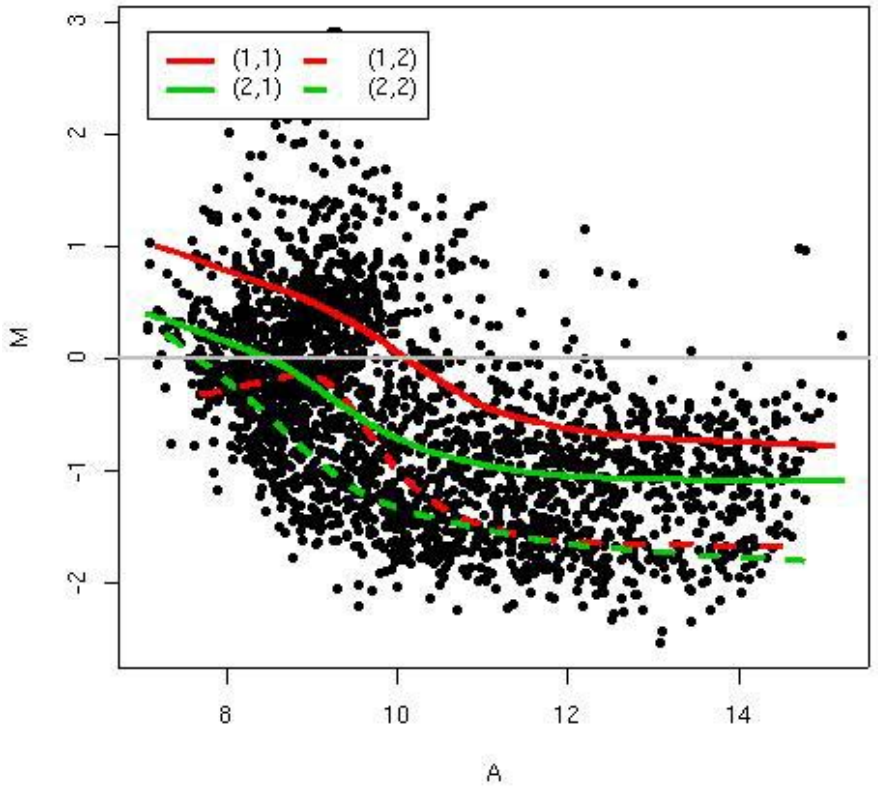
M vs. A



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

Self-self hybridization

M vs. A



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

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

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

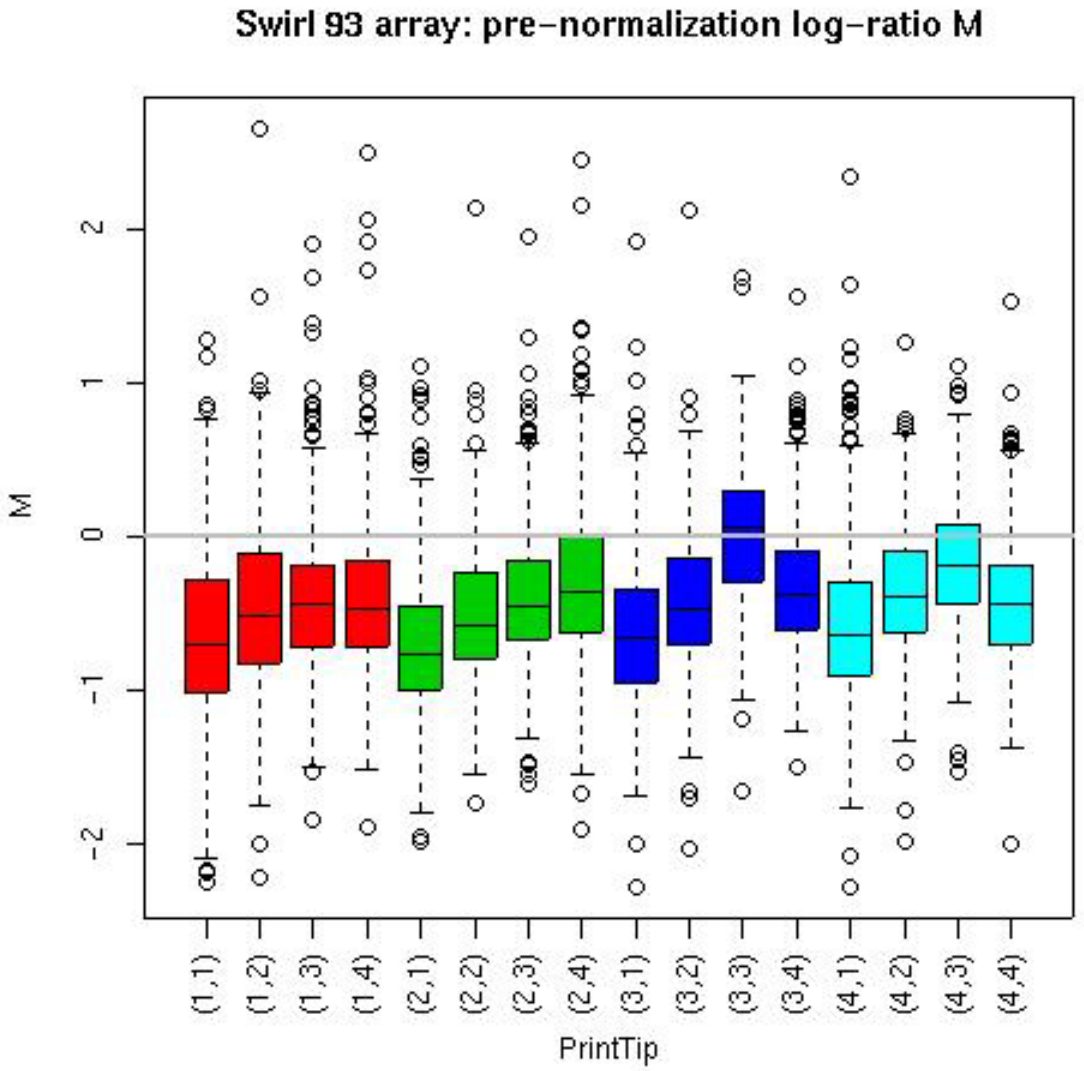
  E.g. red and green log-intensities, intensity log-ratios M, average log-intensities A, spot area.

  – Boxplots;
  – 2D spatial images;
  – Scatter-plots, e.g. MA-plots;
  – Density plots.

- **Stratify** plots according to layout parameters, e.g. print-tip-group, plate.

Boxplots by print-tip-group

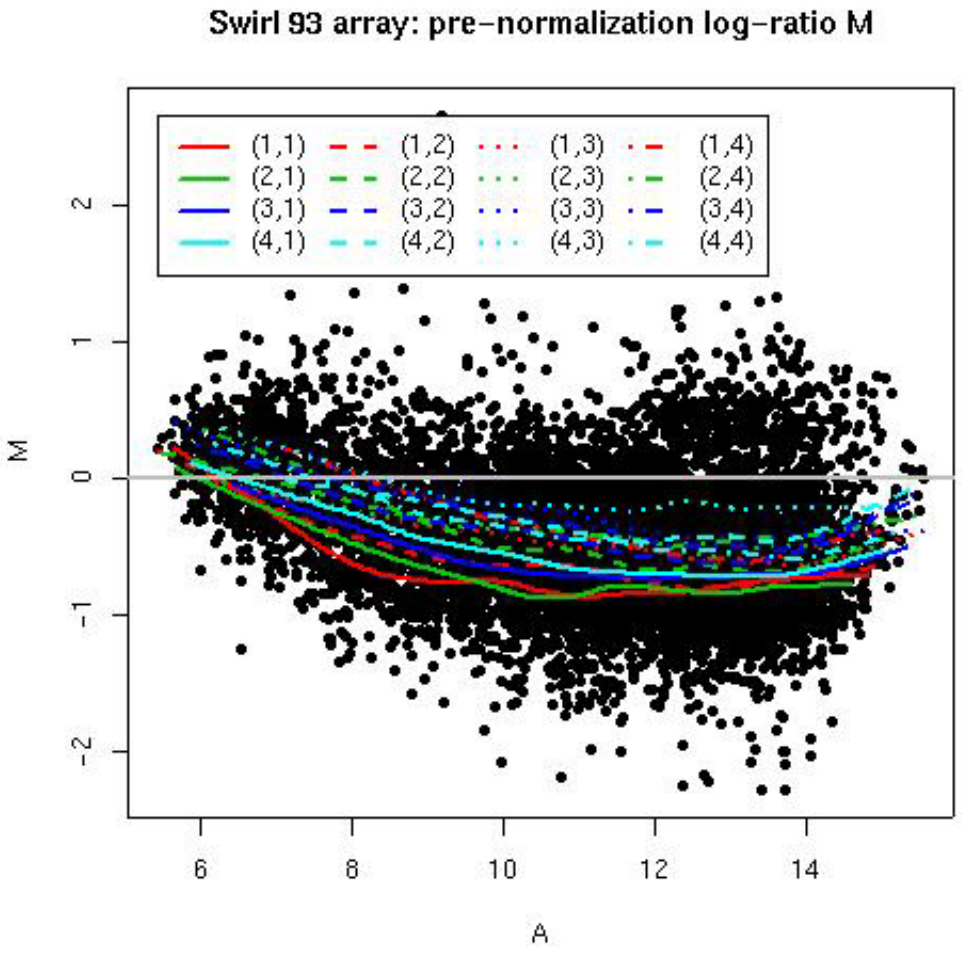
Intensity log-ratio, M



MA-plot by print-tip-group

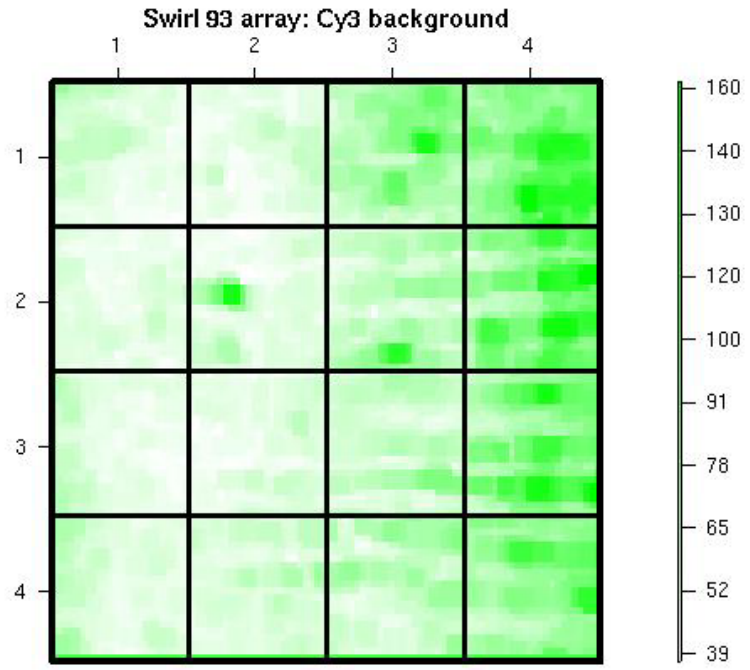
$$M = \log_2 R - \log_2 G, \quad A = (\log_2 R + \log_2 G)/2$$

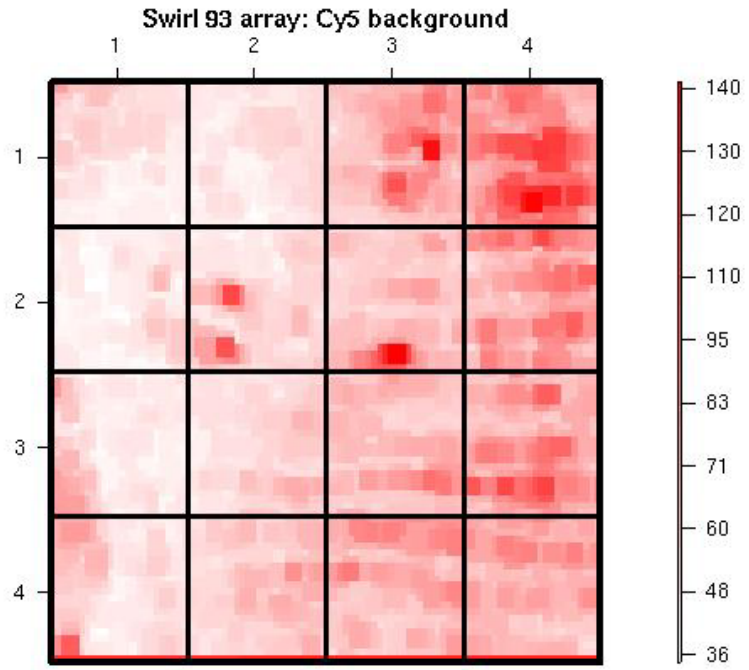
Intensity log-ratio, M



Average log-intensity, A

2D spatial images



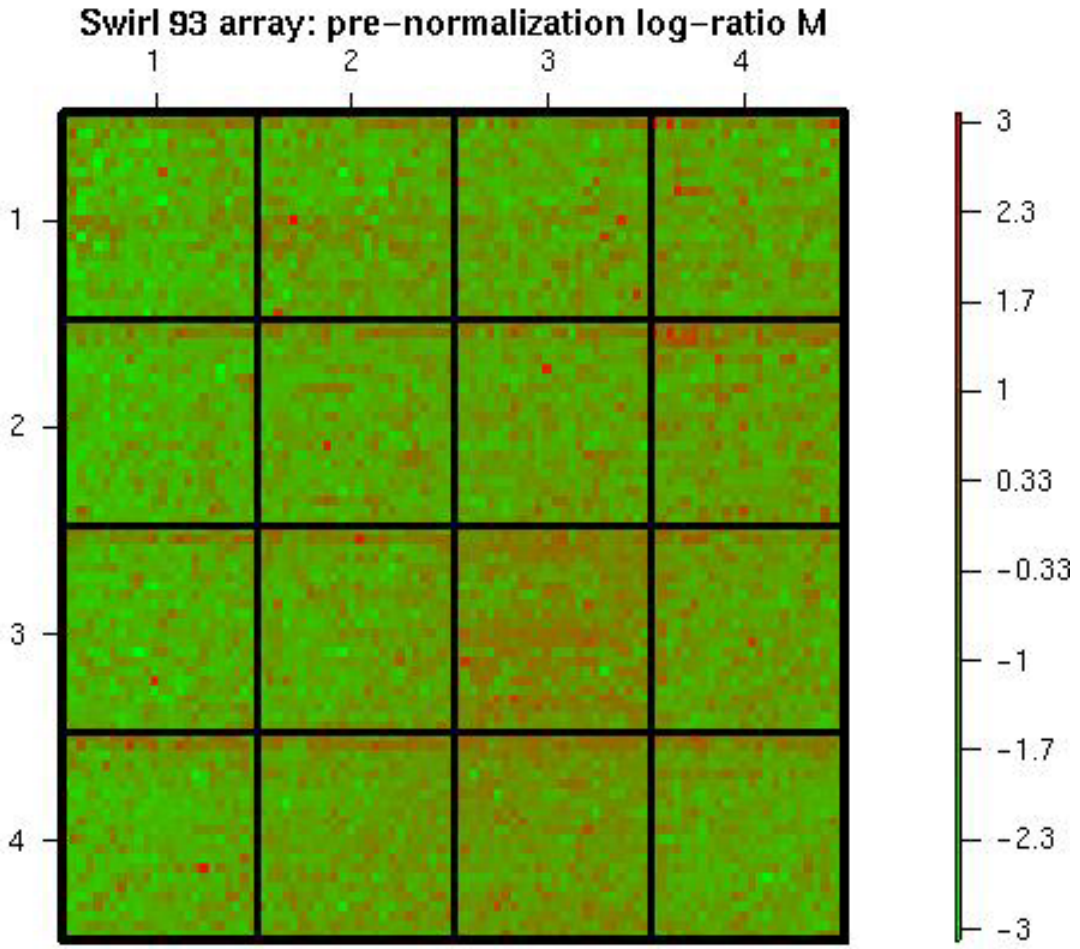


Cy3 background intensity

Cy5 background intensity

2D spatial images

Intensity log-ratio, M



Location normalization

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

- **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.

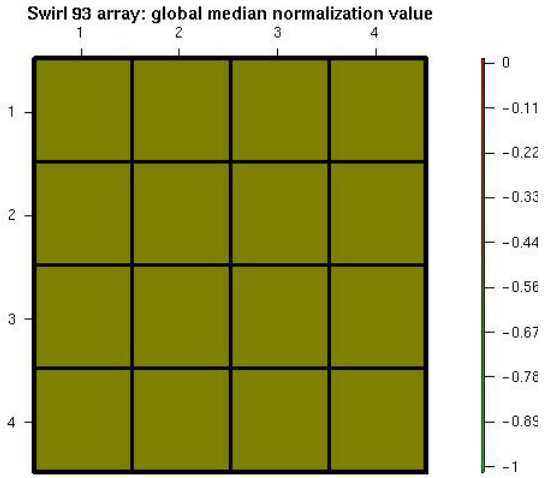
- Regression method: e.g. lowess or loess (Cleveland, 1979; Cleveland & Devlin, 1988).

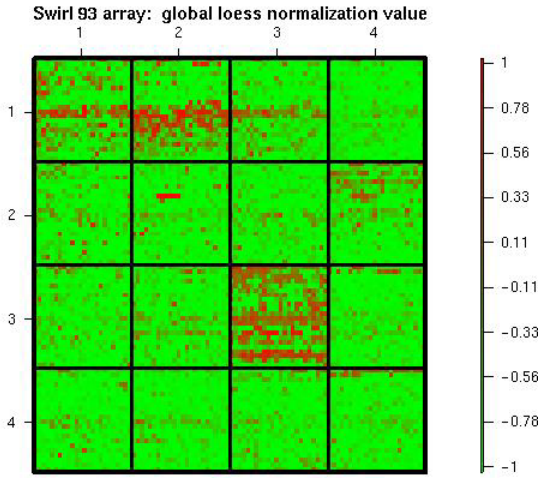
Location normalization

- **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

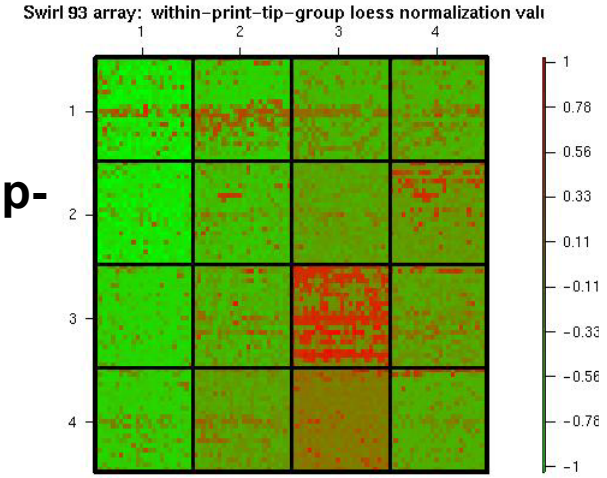
Global median normalization

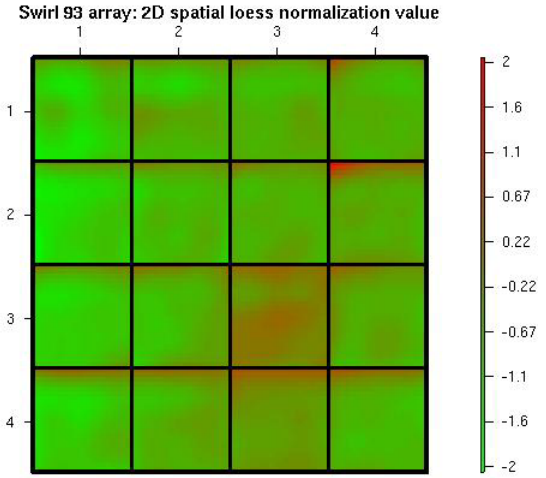




Global loess normalization

Within-print-tip-group loess normalization

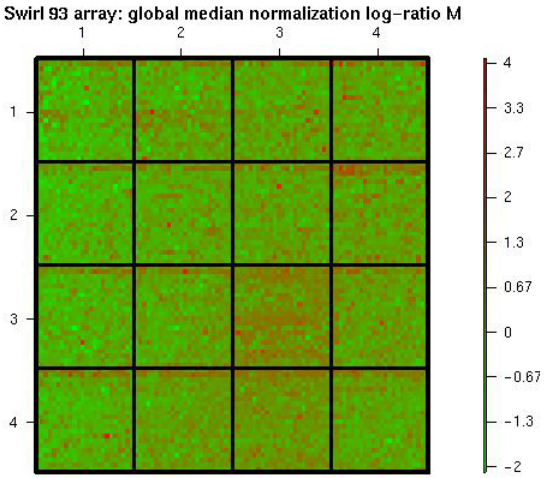


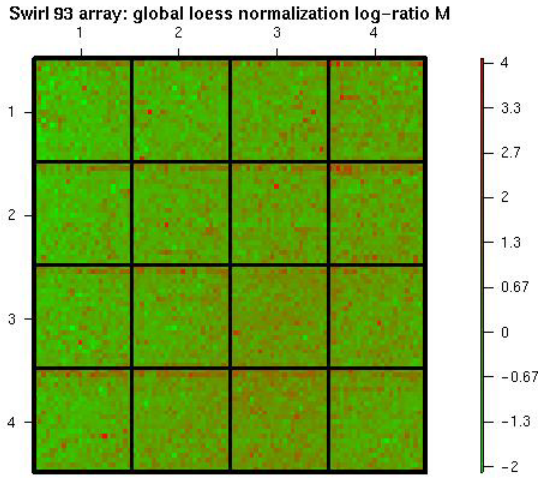


2D spatial normalization

2D images of normalized M-L

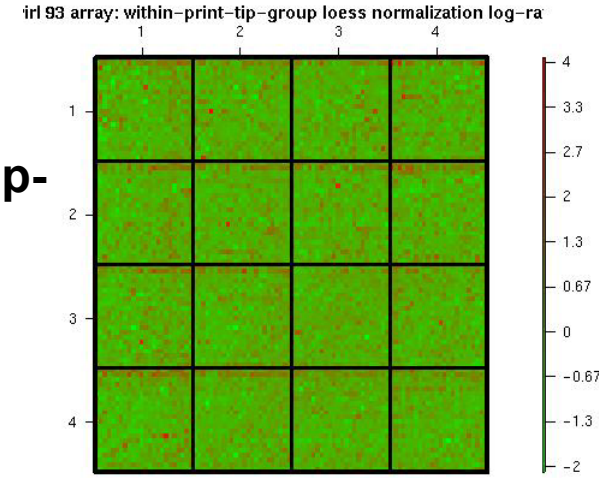
Global median normalization

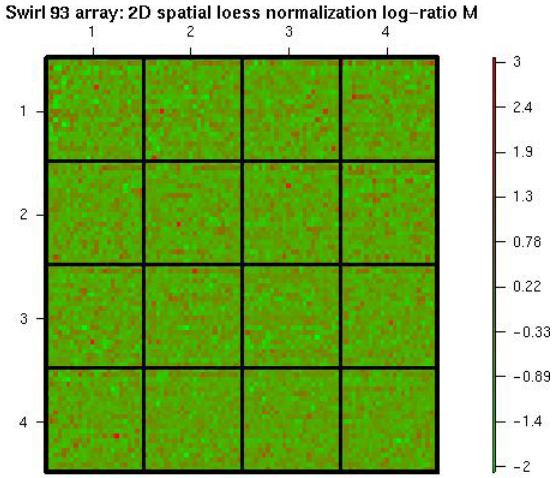




Global loess normalization

Within-print-tip-group loess normalization

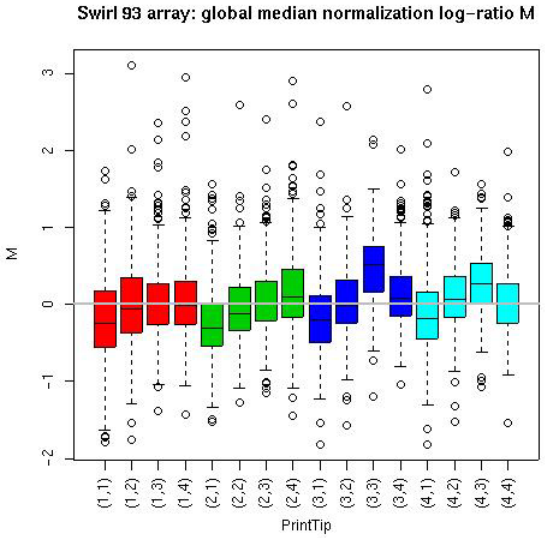


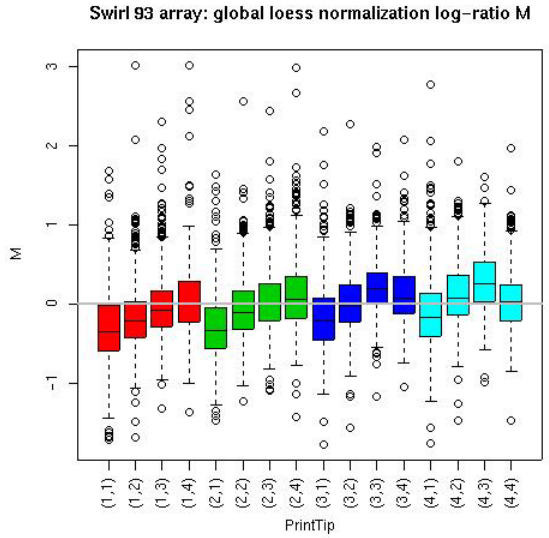


2D spatial normalization

Boxplots of normalized M-L

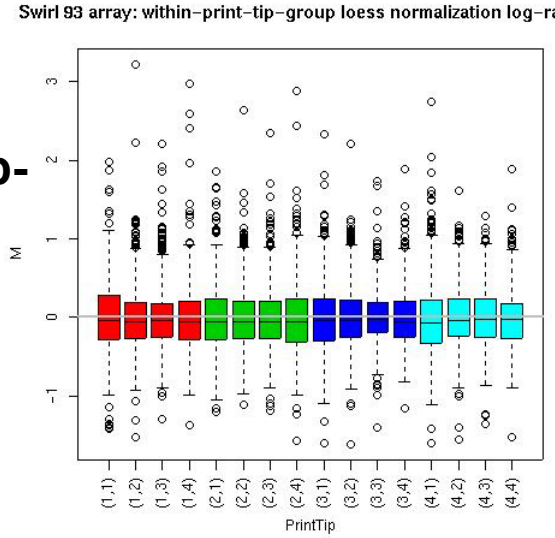
Global median normalization

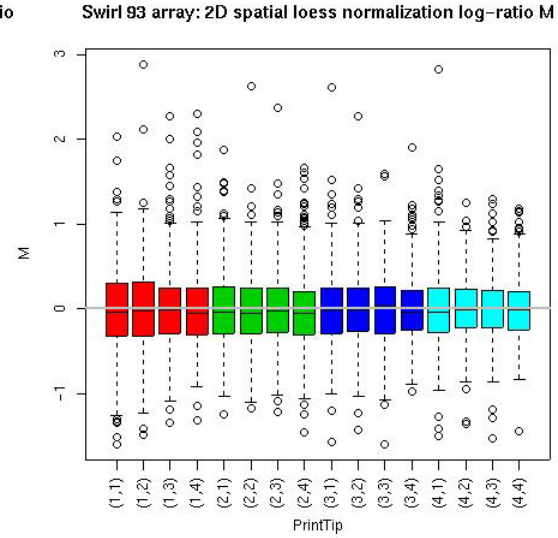




Global loess normalization

Within-print-tip-group loess normalization

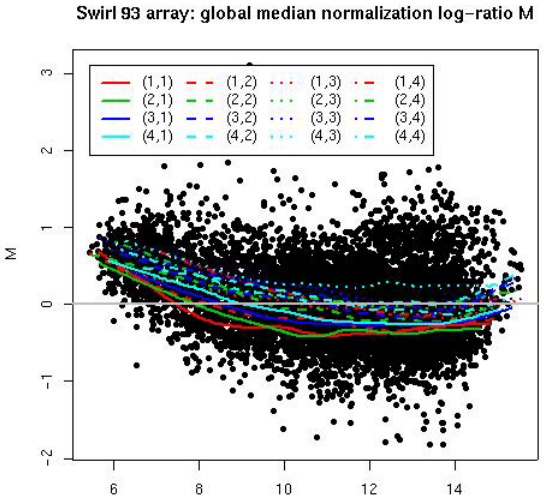


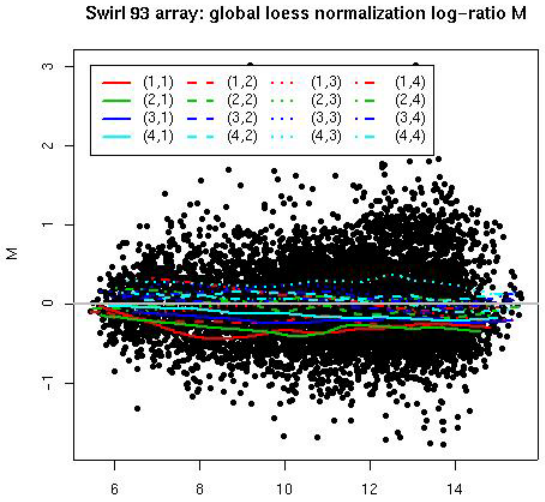


2D spatial normalization

MA-plots of normalized M-L

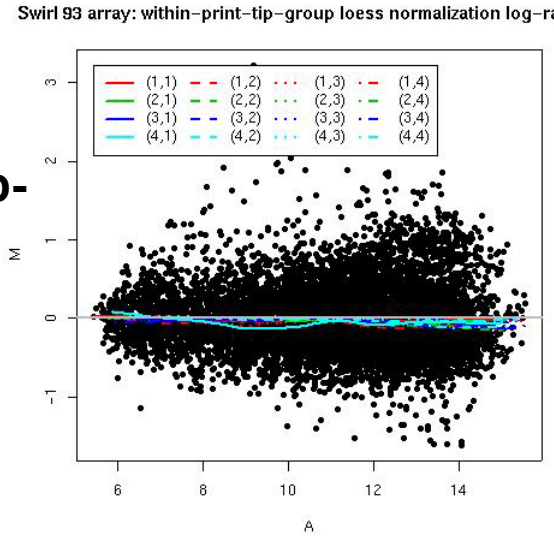
Global median normalization

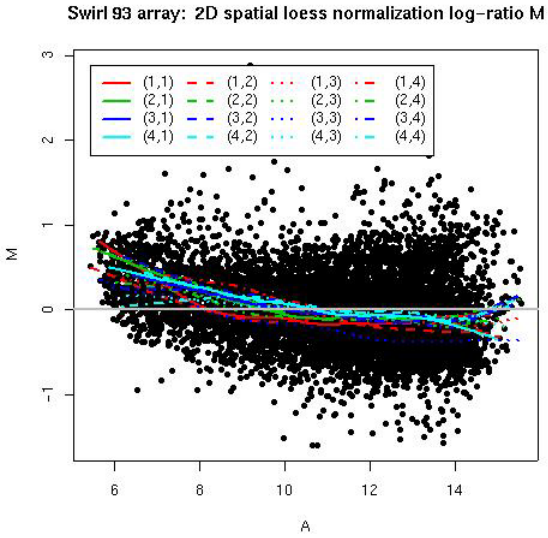




Global loess normalization

Within-print-tip-group loess normalization





2D spatial normalization

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

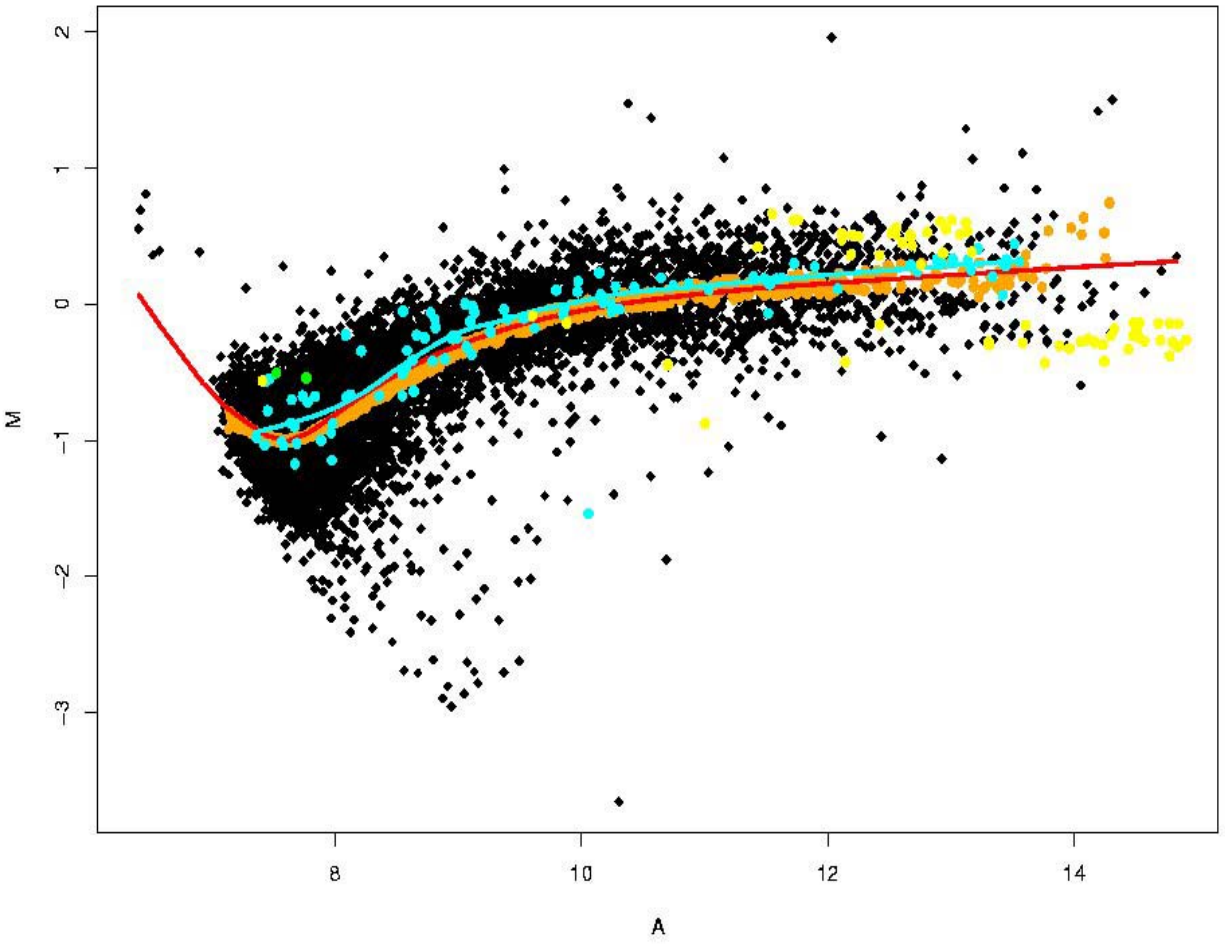
- Microarray Sample Pool, MSP: Control sample for normalization, in particular, when it is not safe to assume most genes are equally expressed in both channels.
- MSP: pooled all 18,816 ESTs from RIKEN release 1 cDNA mouse library.
- Six-step dilution series of the MSP.
- MSP samples were spotted in 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

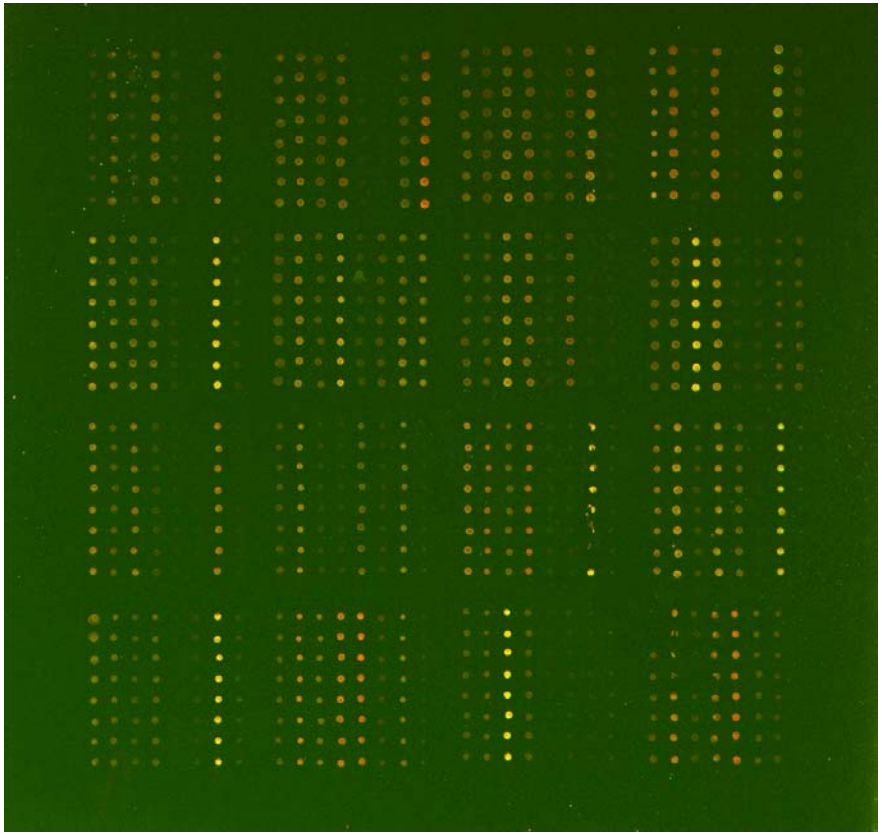
- Probes
  - 50 distinct clones thought to be differentially expressed in apo AI knock-out mice compared to inbred C57Bl/6 control mice (largest absolute t-statistics in a previous experiment).
  - 72 other clones.
- Spot each clone 8 times .

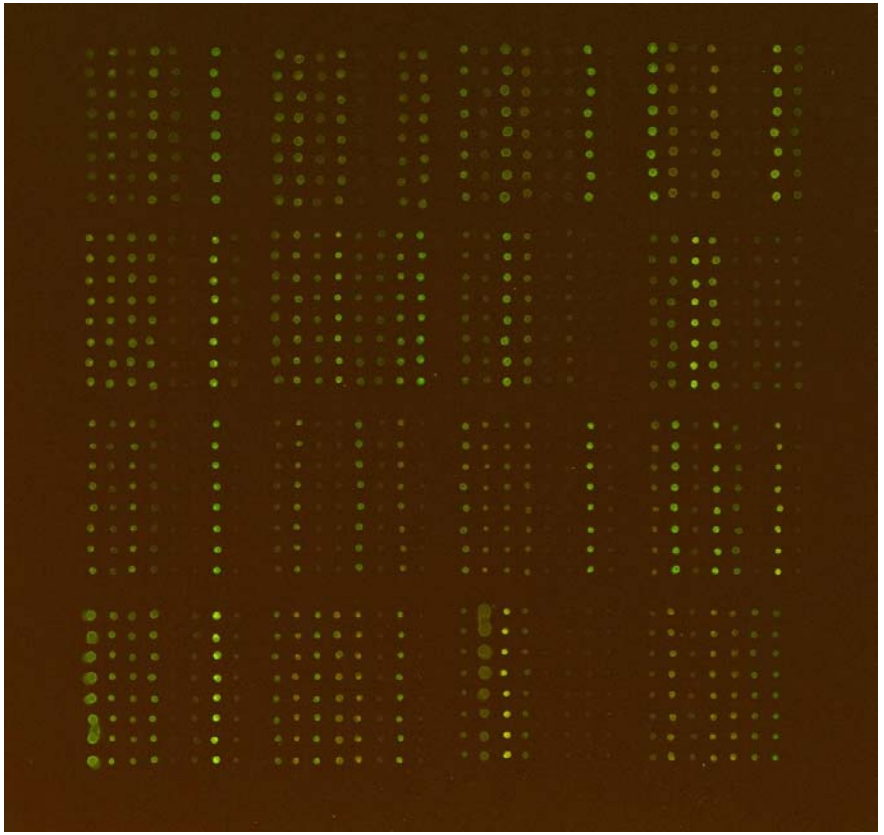
- Two hybridizations with dye-swap:

  Slide 1: trt $\rightarrow$ red, ctl $\rightarrow$ green.

  Slide 2: trt $\rightarrow$ green, ctl $\rightarrow$ 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:

$$M - M'$$

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

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

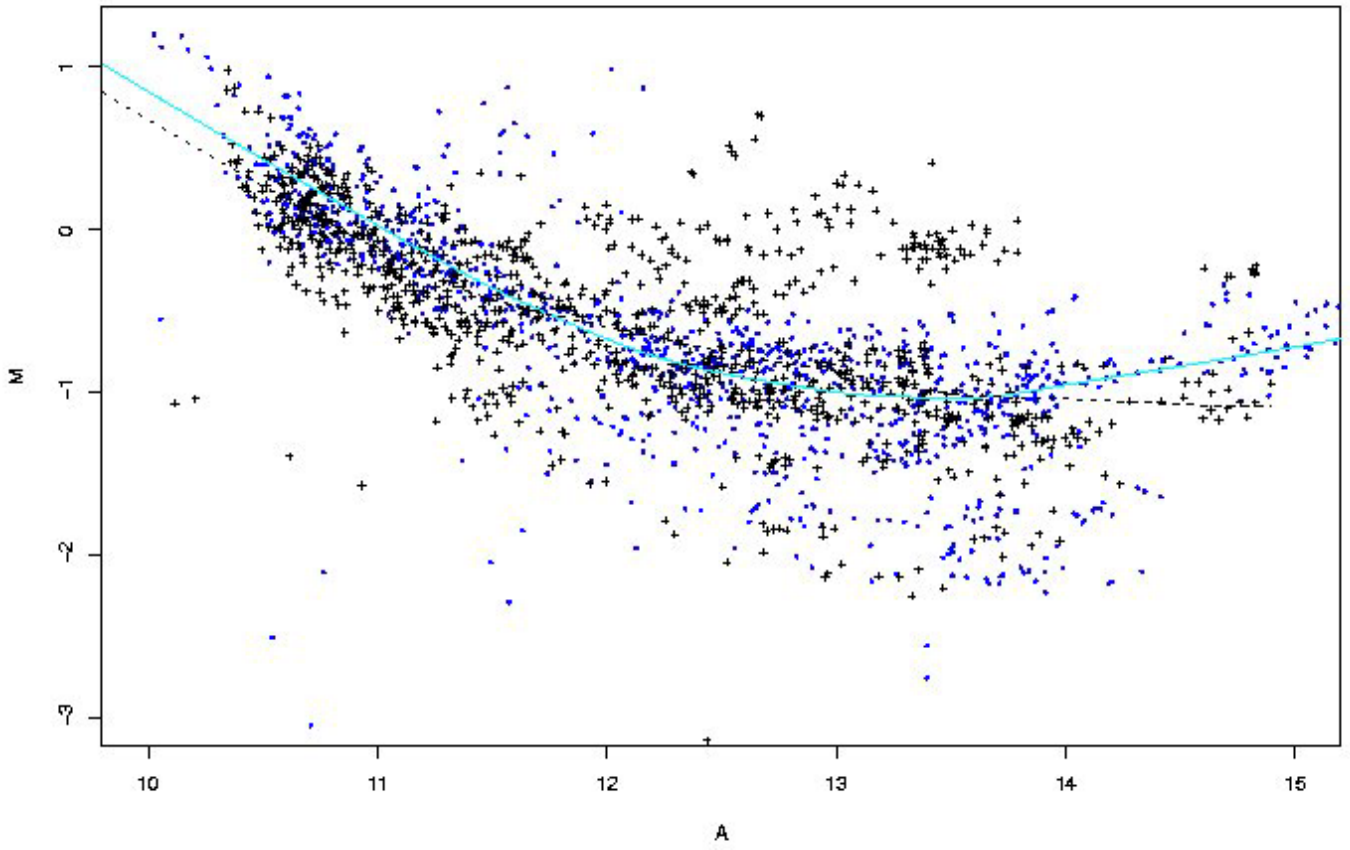
$$\approx [\log_2 (RG'/GR')] / 2$$

provided $L = L'$.

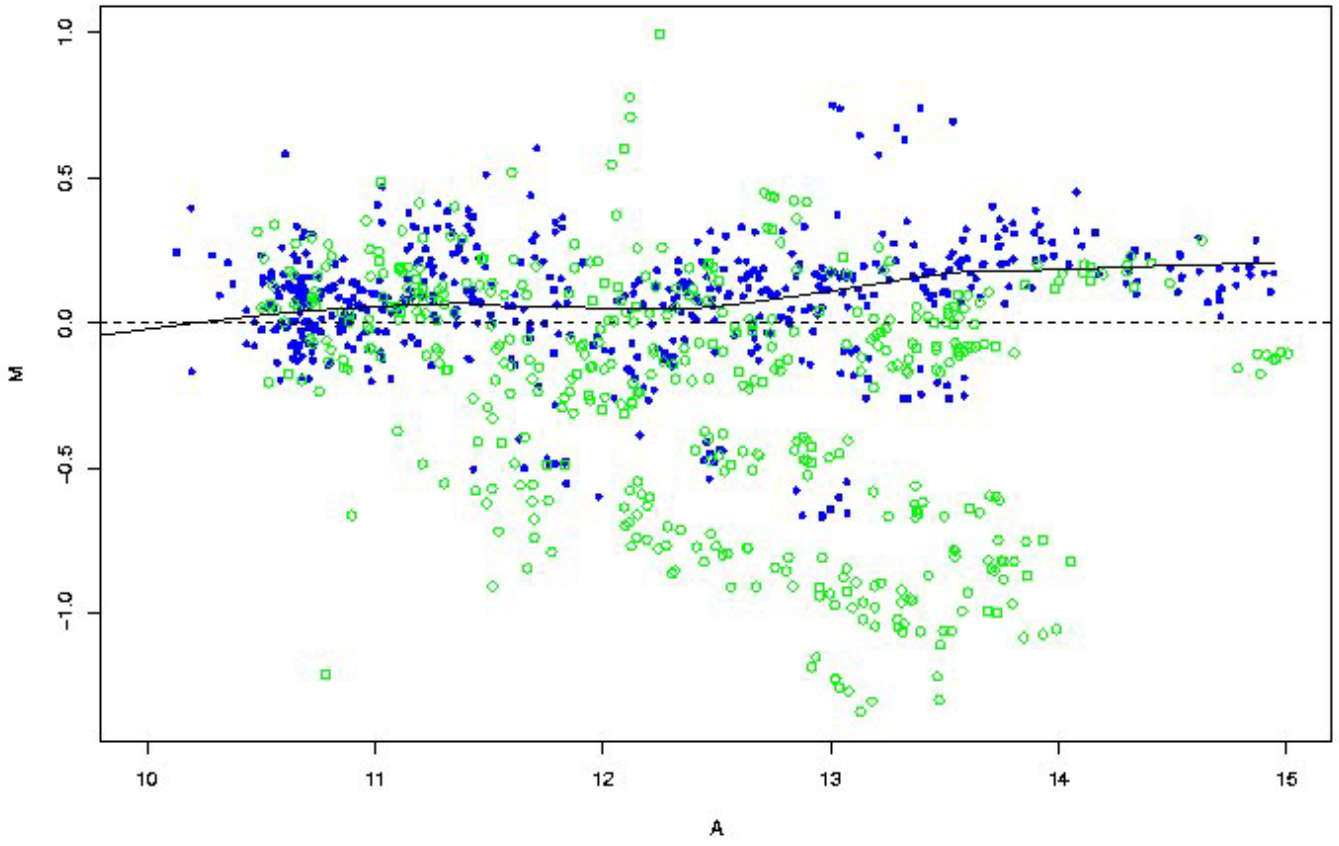
Assumption: the normalization functions are the same for the two slides.

Checking the assumption

MA-plot for slides 1 and 2



Result of self-normalization



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.

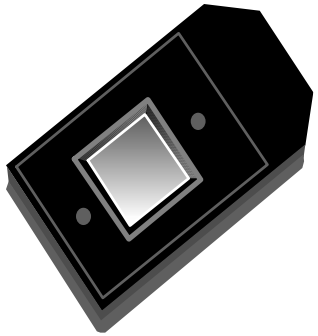
Between-slides

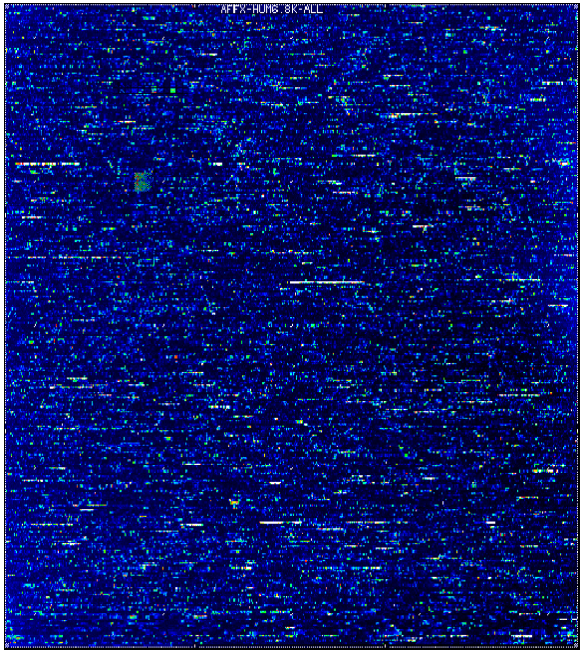
- An extension of within-slide scale normalization.

Case 2. Many genes are expected to change.

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

Pre-processing: Oligonucleotide chips





Spotted vs. Affymetrix arrays

Spotted arrays	Affymetrix arrays
One probe per gene	16 – 20 probe-pairs per gene
Probes of varying length	Probes are 25-mers
Two target samples per array	One target sample per array

Terminology

- Each gene or portion of a gene is represented by 16 to 20 oligonucleotides of 25 base-pairs.

- Probe: an oligonucleotide of 25 base-pairs, i.e., a 25-mer.
- Perfect match (PM): A 25-mer complementary to a reference sequence of interest (e.g., part of a gene).
- Mismatch (MM): same as PM but with a single homomeric base change for the middle (13^{th}) base (transversion purine <-> pyrimidine, G <->C, A <->T) .
- Probe-pair: a (PM,MM) pair.
- Probe-pair set: a collection of probe-pairs (16 to 20) related to a common gene or fraction of a gene.
- Affy ID: an identifier for a probe-pair set.
- The purpose of the MM probe design is to measure non-specific binding and background noise.

Probe-pair set

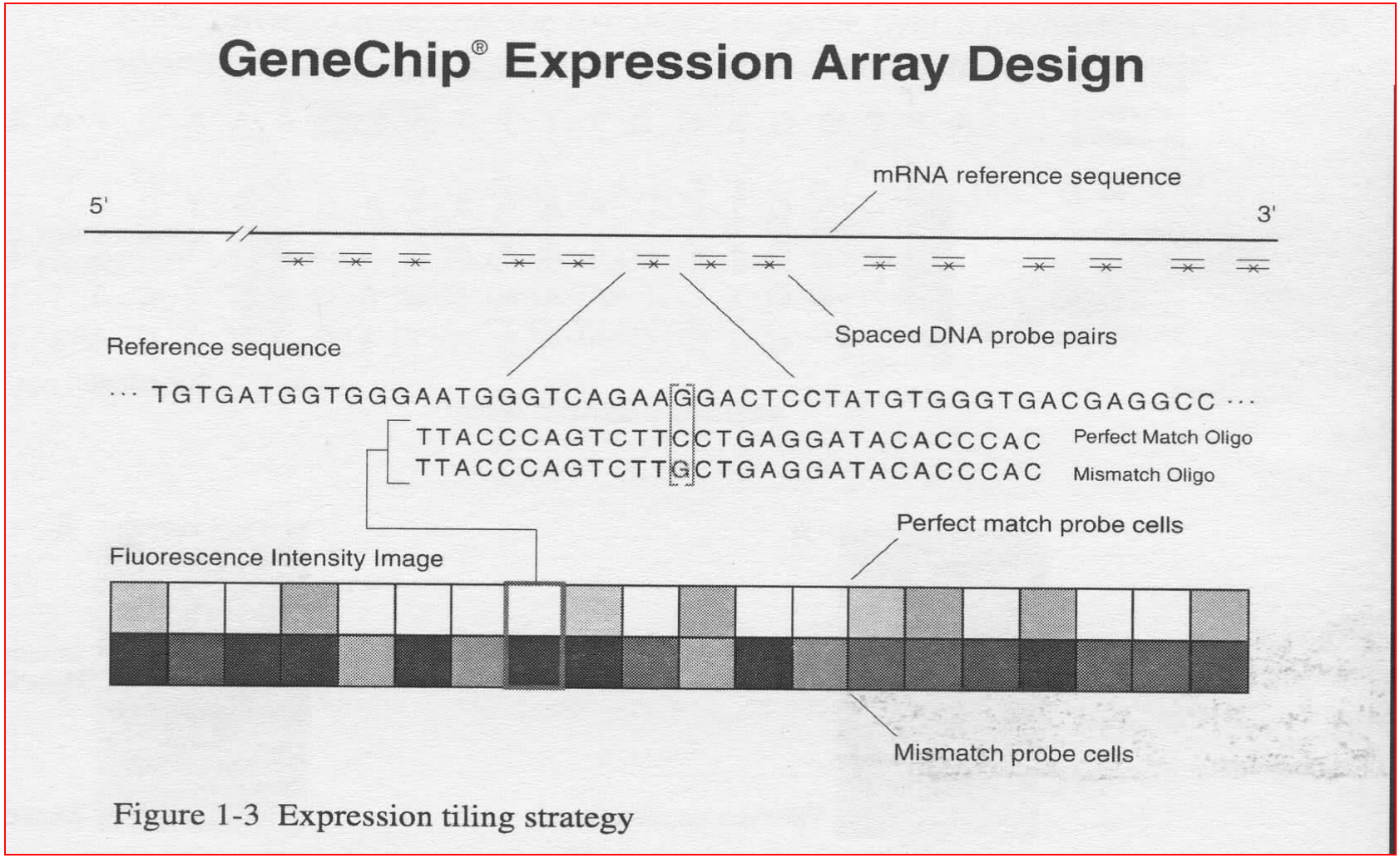


Figure 1-3 Expression tiling strategy

Affymetrix files

- Main software from Affymetrix company *MicroArray Suite* - *MAS*, 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 and the location of probe-pair 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,…, n* -- from one to hundreds of chips;
  - *j = 1,…, J* -- usually 16 or 20 probe pairs;
  - *g = 1,…, 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. (2003).

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-array Average, 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

- Bolstad et al. (2002). 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

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

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.

Summary

- 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. (2003).

`marray`: Pre-processing spotted DNA microarray data

- **`marrayClasses`**:
  - class definitions for cDNA microarray data (MIAME);
  - basic methods for manipulating microarray objects: printing, plotting, subsetting, class conversions, etc.
- **`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.

`affy`: Pre-processing Affymetrix data

- Basic classes and methods for probe-level data.
- Widgets for data input.
- Diagnostic plots: 2D spatial images, boxplots, MA-plots, etc.
- Background estimation.
- Probe-level normalization: quantile and curve-fitting normalization (Bolstad et al., 2002).
- Expression measures: MAS 4.0 AvDiff, MAS 5.0 Signal, MBEI (Li & Wong, 2001), RMA (Irizarry et al., 2003).
- 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