Introduction to gene expression microarray data analysis

Outline

- Brief introduction:
  - Technology and data.
  - Statistical challenges in data analysis.
- Preprocessing – data normalization and transformation.
- Useful Bioconductor packages.

A short history

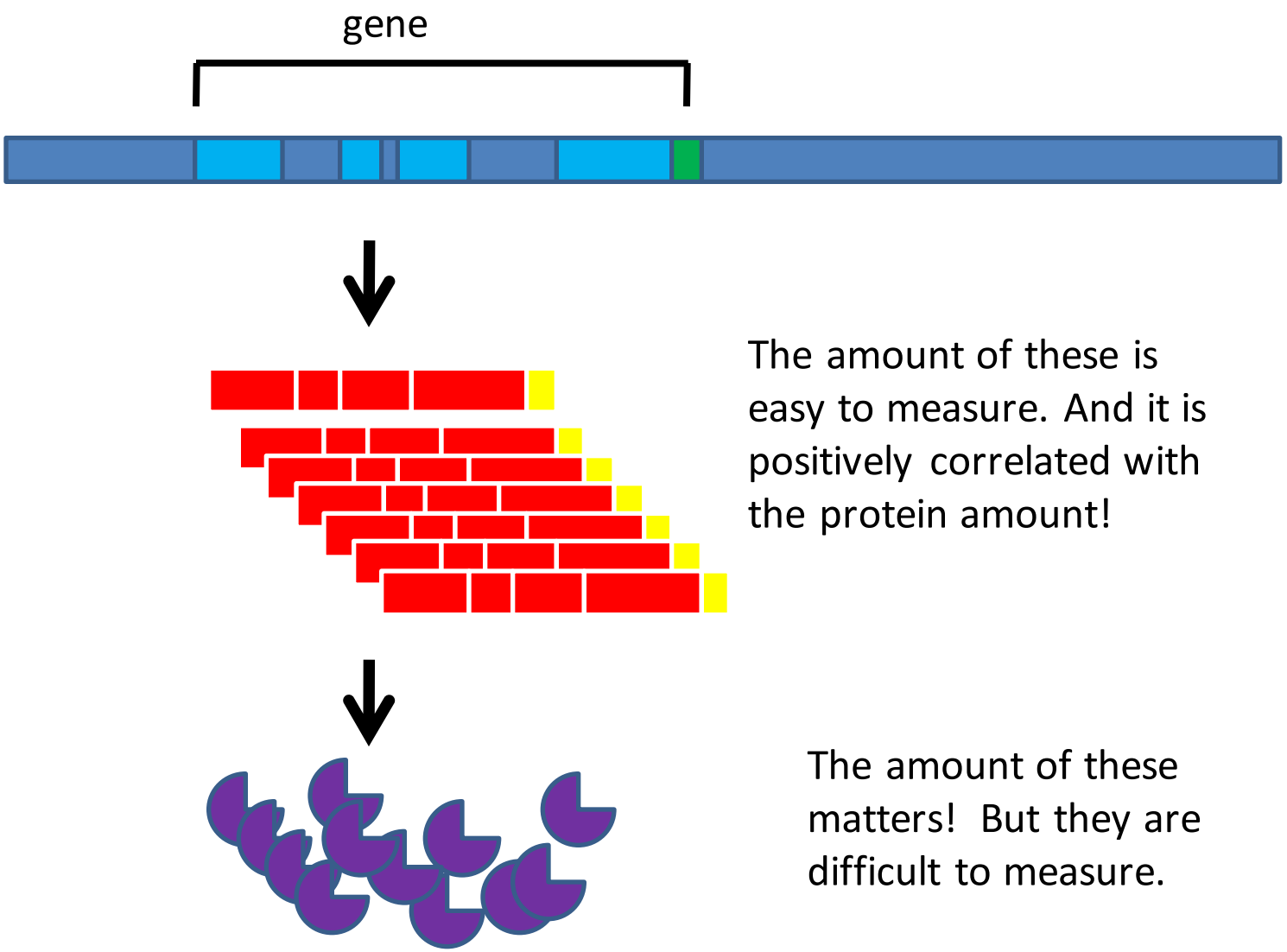
- Evolved from Southern blotting, which is a procedure to detect and quantify a specific DNA sequence.
- Gene expression microarray can be thought as parallelized Southern blotting experiments.
- First influential paper: Schena *et al.* (1995) *Science*.
  - study the expression of 45 Arabidopsis genes.
- Very popular for the past 20 years. Searching "gene expression microarray" on pubmed returns 50,000+ hits.

Still microarray?

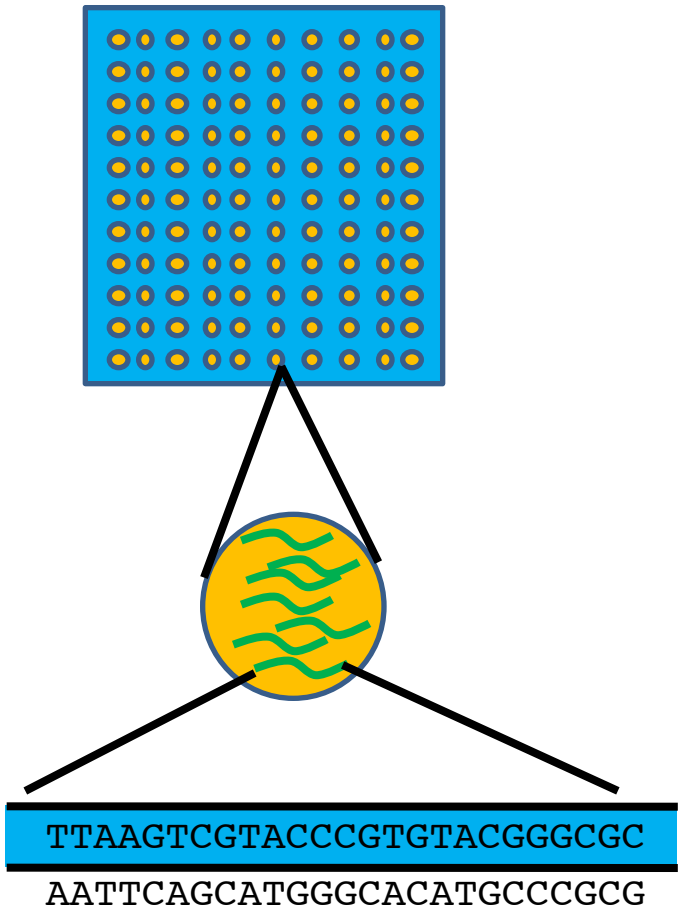
- Microarray is still widely used because of lower costs, easier experimental procedure and more established analysis methods.
  - Still around 4000 publications annually in the past 5 years.
- Similar problems are presented in newer technologies such as RNA-seq, and similar statistical techniques can be borrowed.

Introduction to GE microarray technology and array designs

Goal: measure mRNA abundance

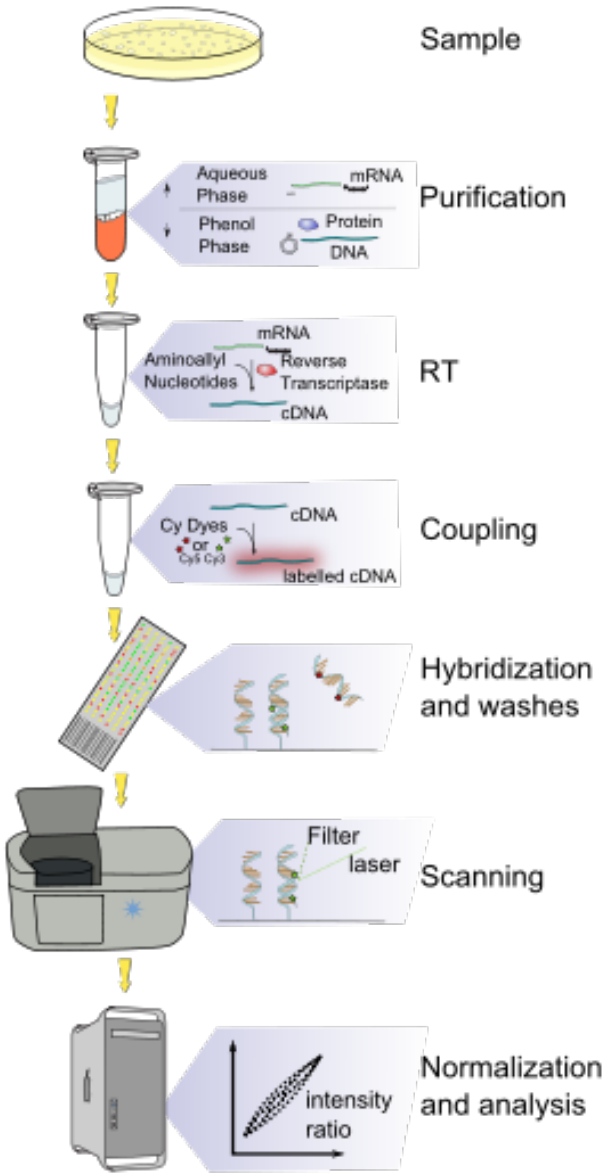


Gene expression microarray design



- A collection of DNA spot on a solid surface.
- Each spot contains many copies of the same DNA sequence (called “probes”).
  - Probe sequences are designed to target specific genes.
- Genes with part of its sequence complementary to a probe will hybridized on (stick to) that probe.
- The amount of hybridization on each probe measures the amount of mRNA for its target gene.

Experimental procedure



wet lab: perform experiment

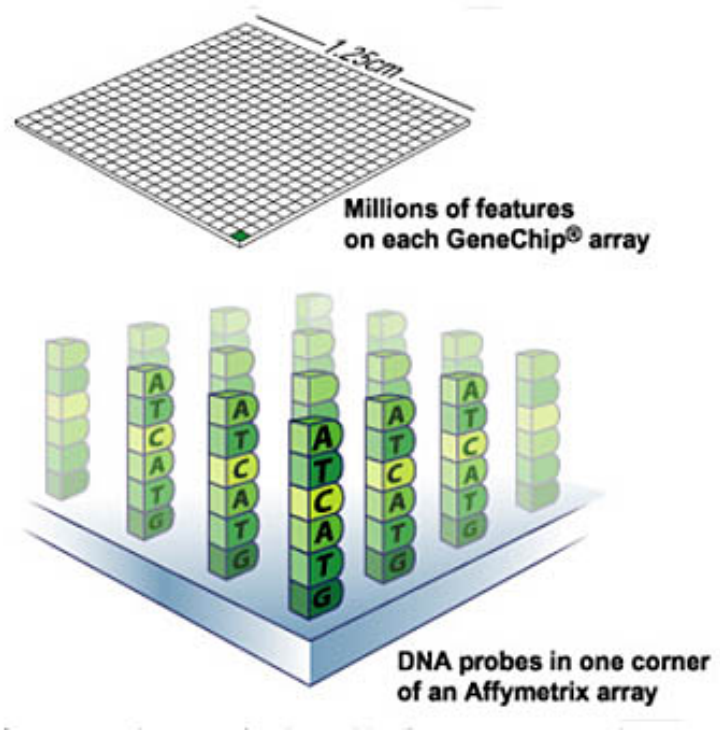
dry lab: data analysis

Available platforms

- Affymetrix
- Agilent
- Nimblegene
- Illumina
- ABI
- Spotted cDNA

Affymetrix Gene expression arrays

The Affymetrix platform is one of the most widely used.

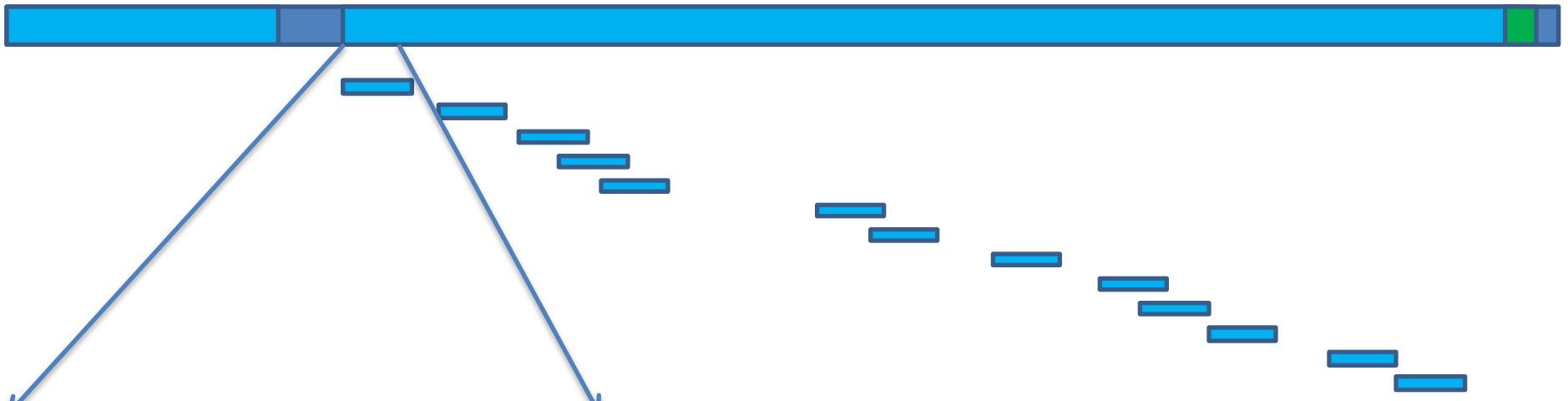


http://www.affymetrix.com/

Affymetrix GeneChip array design

Use U133 system for illustration:

- Around 20 probes per gene.
- Not necessarily evenly spaced: sequence property matters.
- The probes are located at random locations on the chip to average out the effects of the array surface.

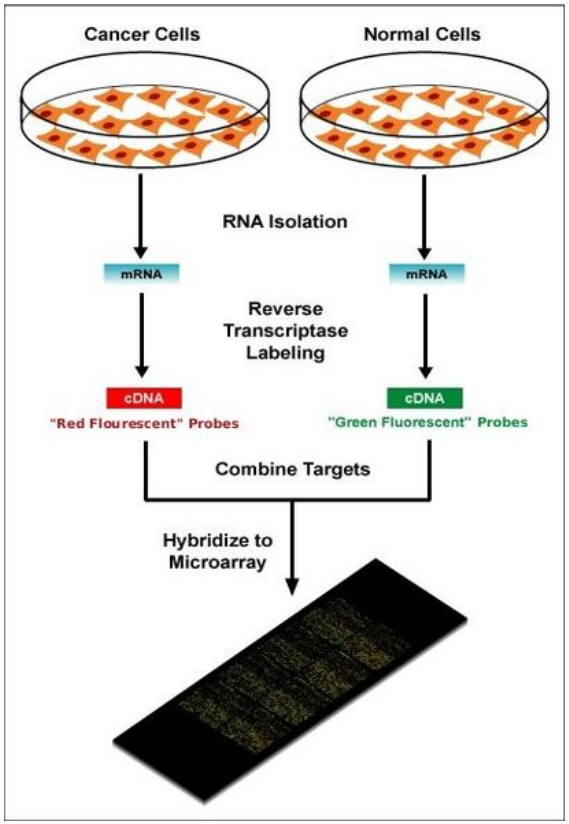


TTAAGTCGTACCCGTGTACGGGCGC Target sequence

AATTCAGCATGGGCACATGCCCGCG Perfect match (PM) probe: measure signals

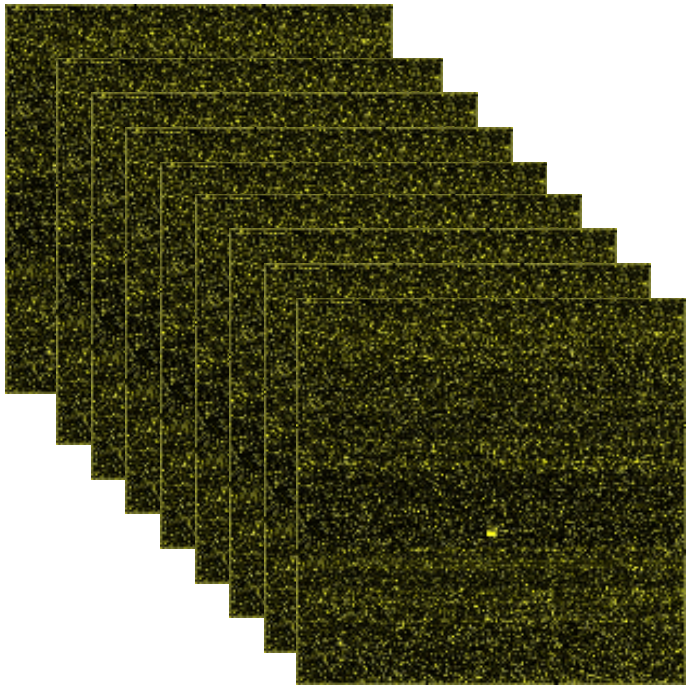
AATTCAGCATGGACACATGCCCGCG Mis-match (MM) probe: measure background

One-color vs. two-color arrays



- Two-color (two-channel) arrays hybridize two samples on the same array with different colors (red and green).
  - Each spot produce two numbers.
  - Agilent, Nimblegen
- One-color (single-channel) arrays hybridize one sample per array.
  - Easier when comparing multiple groups.
  - Have to use twice as many arrays.
  - Affymetrix, Illumina.

Data from microarray



- Data are fluorescent intensities:
  - extracted from the images with artifacts (e.g., cross-talk) removed, which Involves many statistical methods.
  - Final data are stored in a matrix: row for probes, column for samples.
  - For each sample, each probe has one number from one-color arrays and two numbers for two-color arrays.

	sample1	sample2	sample3	sample4
1007_s_at	8.575758	8.915618	9.150667	8.967870
1053_at	6.959002	7.039825	6.898245	7.136316
117_at	7.738714	7.618013	7.499127	7.610726
121_at	10.114529	10.018231	10.003332	9.809068
1255_g_at	5.056204	4.759066	4.629297	4.673458
1294_at	8.009337	7.980694	8.343183	8.025335
1316_at	6.899290	7.045843	6.976185	7.063050
1320_at	7.218898	7.600437	7.433031	7.201984
1405_i_at	6.861933	6.042179	6.165090	6.200671
1431_at	5.073265	5.114023	5.159933	5.063821
...				

Microarray data measure the "relative" levels of mRNA abundance

- Expression levels for different genes on the same array are not directly comparable.
- Expression levels for the same genes from different arrays can be compared, after proper normalization.
- All statistical inferences are for relative expressions, e.g., "the expression of gene X is higher in caner compared to normal".

Statistical challenges

- Data normalization: remove systematic technical artifacts.
  - Within array: variations of probe intensities are caused by:
    - cross-hybridization: probes capture the “wrong” target.
    - probe sequence: some probes are “sticker”.
    - others: spot sizes, smoothness of array surface, etc.
  - Between array: intensity-concentration response curve can be different from different arrays, caused by variations in sample processing, image reader, etc.
- Summarization of gene expressions:
  - summarize values for multiple probes belonging to the same gene into one number.
- Differential expression detection:
  - Find genes that are expressed differently between different experimental conditions, e.g., cases and controls.

Gene expression microarray data normalization

Normalization

- Artifacts are introduced at each step of the experiment:
  - Sample preparation: PCR effects.
  - Array itself: array surface effects, printing-tip effects.
  - Hybridization: non-specific binding, GC effects.
  - Scanning: scanner effects.
- Normalization is necessary before any analysis to ensure differences in intensities are due to differential expressions, not artifacts.

Within- and between-array normalization

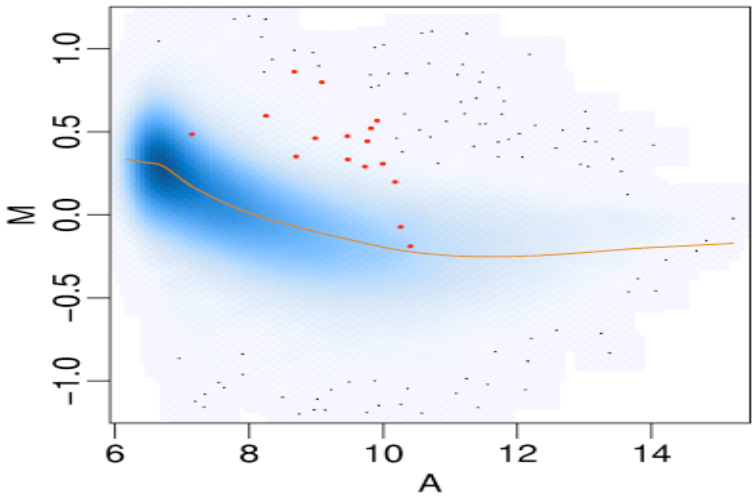
- Within-array: normalization at each array individually to remove array-specific artifacts.
- Between-array: to adjust the values from different arrays and put them at the same baseline, so that numbers are comparable.
- The methods are different for one- and two-color arrays.

Within array normalization, two-color

- Most common problem is intensity dependent effect: log ratios of intensities from two channels depends on the total intensity.
- Most popular: loess normalization.

MA plot

- Widely used diagnostic plot for microarray data (Yang et al. 2002, *Nucleic Acids Research).*
- Also used for sequencing data now.
- For spot i, let R_i and G_i be the intensities, define:
  - $M_i=log_2R_i-log_2G_i$, $A=(log_2R_i+log_2G_i)/2$.
  - M measures relative expression, A measures total expression.
- Visualize relative vs. total expression dependence.

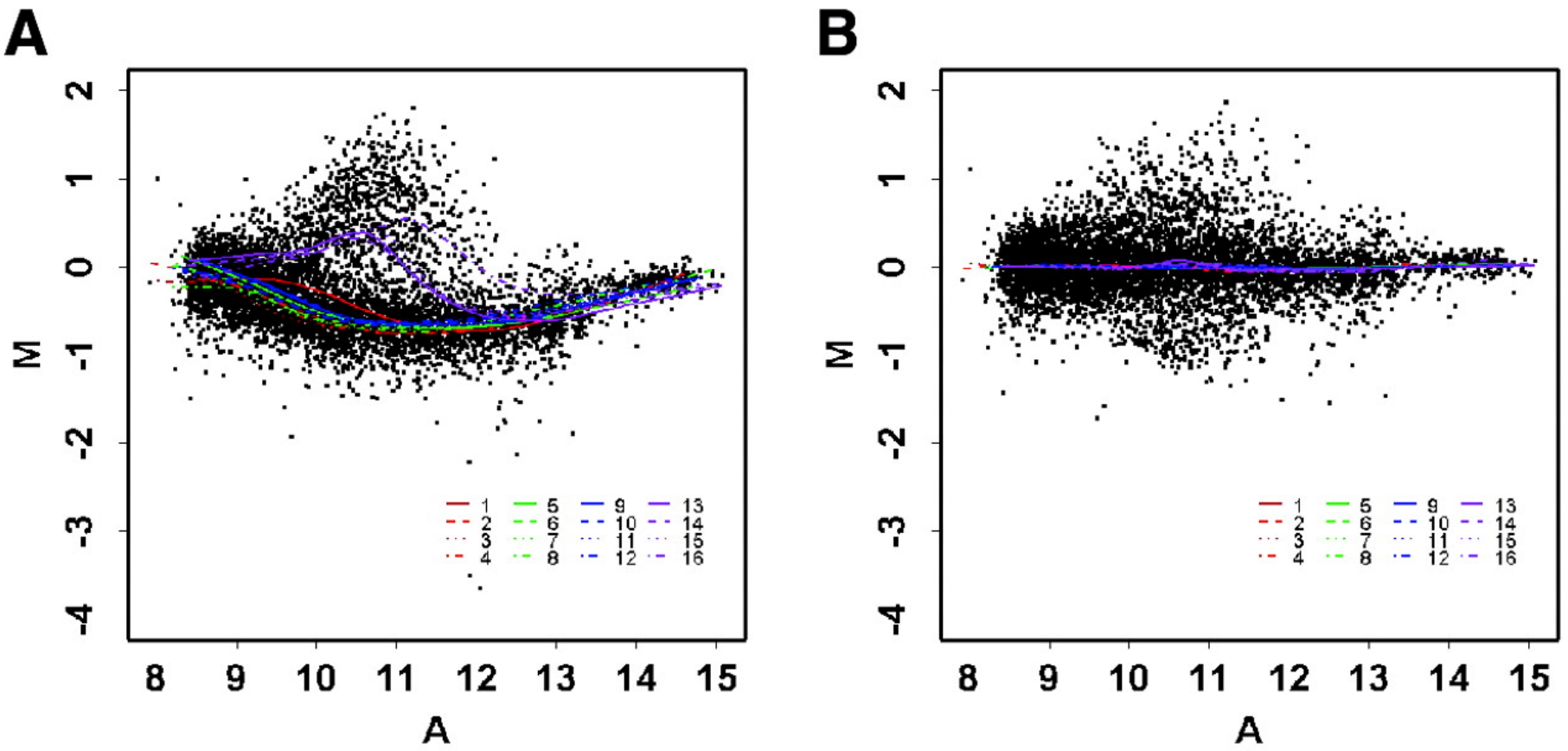


Loess normalization

- Based on the assumptions that: (1) most genes are not DE (with M=0) and (2) M and A are independent, MA plot should be flat and centered at 0.
- Normalization procedure:
  - Fit a smooth curve of M vs. A using loess, e.g., $M=f(A)+\varepsilon$, f(.) is smooth.
  - $M_{norm}=M-f(A)$

– loess (lowess): *locally weighted scatterplot smoothing*.
  – method to fit a smooth curve between two variables .

Loess normalization: before and after



Within array normalization: one-color

- RMA (Robust Multi-array Average) background model (Irizarry et al. 2003, *Biostatistics*).
- Idea: observed intensity *Y* is composed of the true intensity *S* (exponentially distributed) and a random background noise B (normally distribute).
- For each array, assume:

$$Y = S + B$$

Signal: $S \sim Exp(\lambda)$

Background: $B \sim N(\mu, \sigma^2)$ left-truncated at zero

Simple derivation

- Observed: *Y*; of interest: *S*.
- The idea is to predict S from Y using $E[S \mid Y]$:

$$E[S \mid Y] = \int s\, f(s \mid Y = y) ds = \int s \frac{f(s, Y = y)}{f_Y(y)} ds = \frac{1}{f_Y(y)} \int s\, f(s, Y = y) ds$$

- The joint: $f(s, Y = y) = f(s, B = y - s) = f_S(s) f_B(y - s)$
- Marginal distribution of *Y* $f_Y(y)$ can be derived.

An extension to consider probe sequence effects: GCRMA

$$
\begin{aligned}
Y_{gij} &= O_{gij} + N_{gij} + S_{gij} \\
&= O_{gij} + \exp(\mu_{gij} + \varepsilon_{gij}) + \exp(s_g + \delta_g X_i + a_{gij} + b_i + \xi_{gij}).
\end{aligned}
$$

Here Y_{gij} is the *PM* intensity for the probe j in probeset g on array i, ε_{gij} is a normally distributed error that account for NSB for the same probe behaving differently in different arrays, s_g represents the baseline log expression level for probeset g, a_{gij} represents the signal detecting ability of probe j in gene g on array i, b_i is a term used to describe the need for normalization, ξ_{gij} is a normally distributed term that accounts for the multiplicative error, and δ_g is the expected differential expression for every unit difference in covariate X. Notice δ_g is the parameter of interest. As described by Naef and Magnasco (2003) a_{gj} is a function of α.

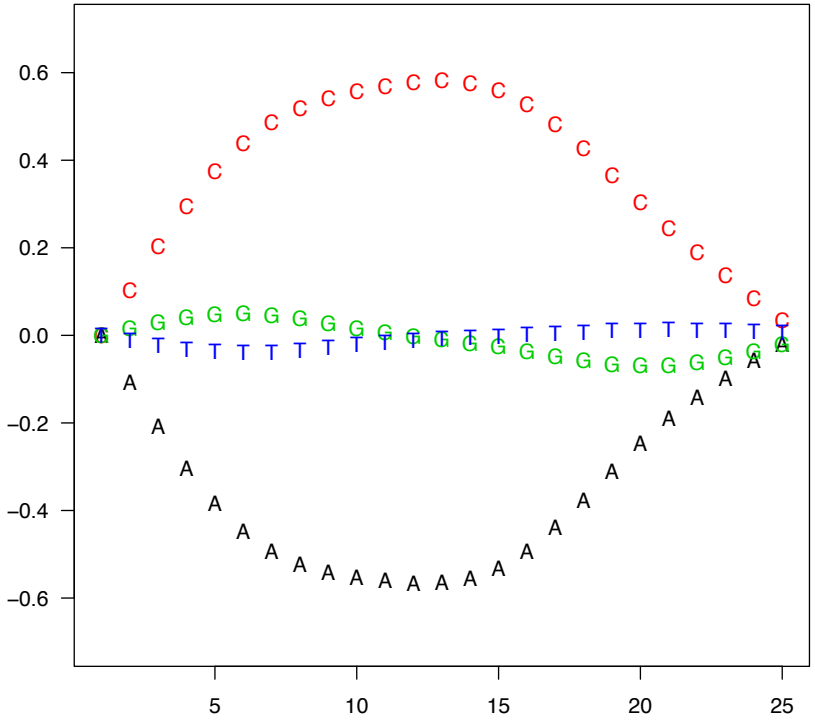
Wu et al. (2005) *JASA*

Probe sequence effects

- Probe affinity is modeled as:

$$\alpha = \sum_{k=1}^{25} \sum_{j \in \{A,T,G,C\}} \mu_{j,k} 1_{b_k = j} \text{ with } \mu_{j,k} = \sum_{l=0}^{3} \beta_{j,l} k^l,$$

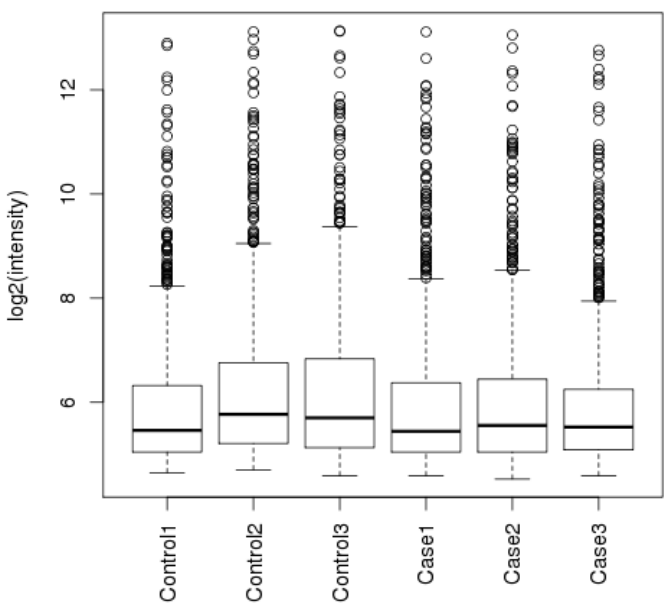
- This kind of modeling is widely used in other microarrays and sequencing data!



Summary: within array normalization

- To remove the unwanted artifacts and obtain true signals.
- Performed at each array individually.
- Both MA-plot based normalization and background error models (eg, RMA) are popular in many other data (other microarrays, ChIP-seq, RNA-seq)
  - Use loess with caution because it assumes most genes are not DE.
  - The error model (additive background, multiplicative error) is very useful.

Between array normalization



- Remember data from arrays (intensity values) estimate mRNA quantities, but the intensity-mRNA quantities response can be different from different arrays. So a number, say, 5, on arrays 1 doesn't mean the same on array 2.
- This could be caused by:
  - Total amount of mRNA used
  - Properties of the agents used.
  - Array properties
  - Settings of laser scanners
  - etc.
- These artifacts cannot be removed by within array normalization.
- Goal: normalize so that data from different arrays are comparable!

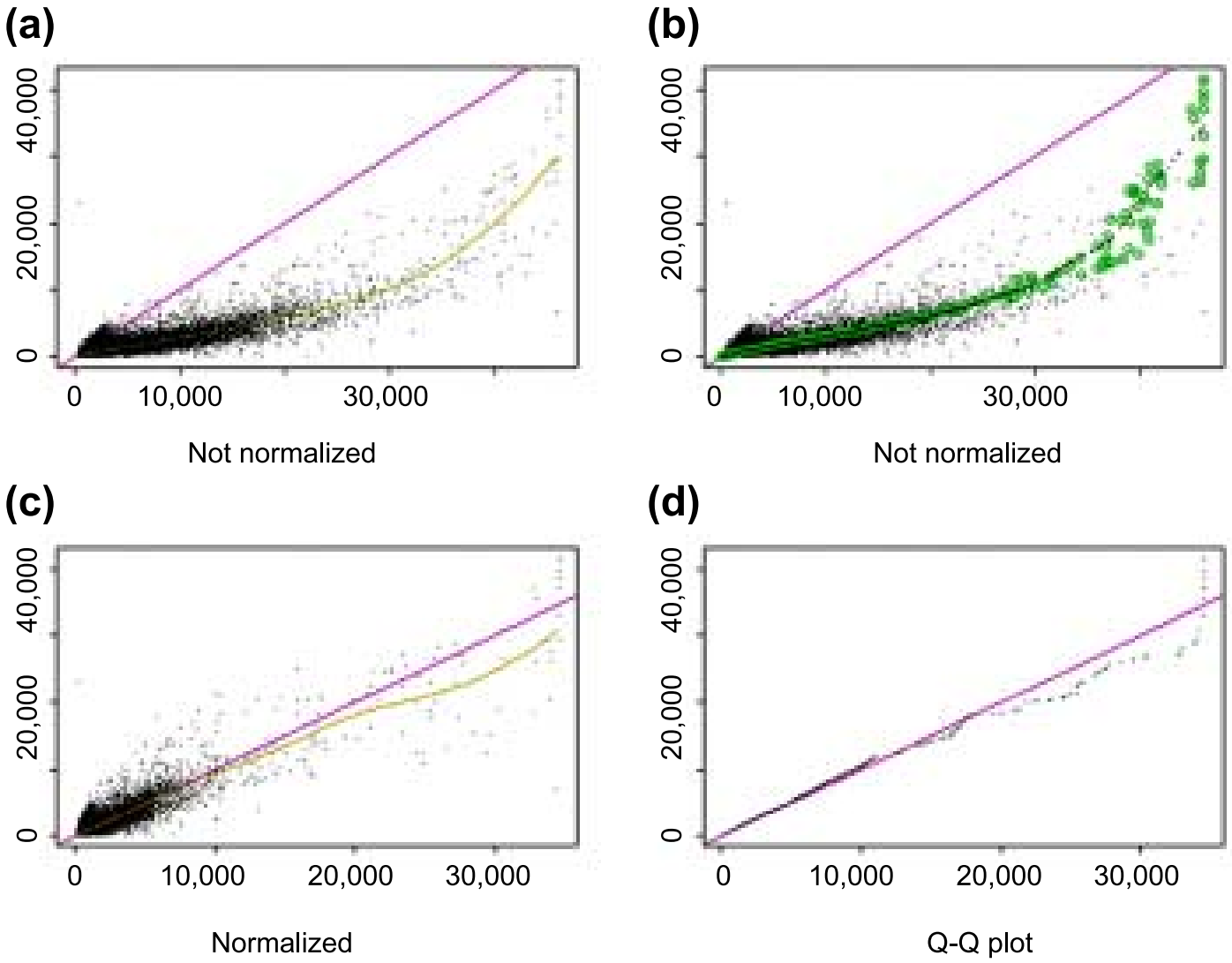
Linear scaling method

- Used in Affymetrix software MAS:
  - Use a number of “housekeeping” genes and assume their expressions are identical across all arrays.
  - Shift and rescale all data so the average expression of these genes are the same across all arrays.

Non-linear smoothing based

- Implemented in dChip (Li and Wong 2001, *Genome Bio*.)
  - Find a set of genes invariant across arrays.
  - Find a “baseline” array
  - For every other arrays fit a smooth curve on expressions of invariant genes
  - Normalize based on the fitted curve.

dChip normalization



Quantile normalization

Proposed in Bolstad *et al.* 2003, *Bioinformatics*:

- Force the distribution of all data from all arrays to be the same, but keep the ranks of the genes.
- Procedures:
  1. Create a target distribution, usually use the average from all arrays.
  2. For each array, match its quantiles to that of the target. To be specific: $x_{norm} = F_2^{-1}(F_1(x))$:
     - x: value in the chip to be normalized
     - F_1: distribution function in the array to be normalized
     - F_2: target distribution function

A simple example for quantile normalization

Gene	sample1	Sample2	Sample3	Sample4
1	8	15	9	13
2	7	2	7	15
3	3	6	5	8
4	1	5	2	9
5	9	13	6	11

1. Find the Smallest Value for each sample

Gene	sample1	Sample2	Sample3	Sample4
1	8	15	9	13
2	7	2	7	15
3	3	6	5	8
4	1	5	2	9
5	9	13	6	11

2. Average them

(1+2+2+8)/4=3.25

3. Replace Each Value by the Average

Gene	sample1	Sample2	Sample3	Sample4
1	8	15	9	13
2	7	3.25	7	15
3	3	6	5	3.25
4	3.25	5	3.25	9
5	9	13	6	11

4. Find the Next Smallest Values, then average

Gene	sample1	Sample2	Sample3	Sample4
1	8	15	9	13
2	7	3.25	7	15
3	3	6	5	3.25
4	3.25	5	3.25	9
5	9	13	6	11

(3+5+5+9)/4=5.5

5. Replace Each Value by the Average

Gene	sample1	sample2	sample3	sample4
1	8	15	9	13
2	7	3.25	7	15
3	5.50	6	5.50	3.25
4	3.25	5.50	3.25	5.50
5	9	13	6	11

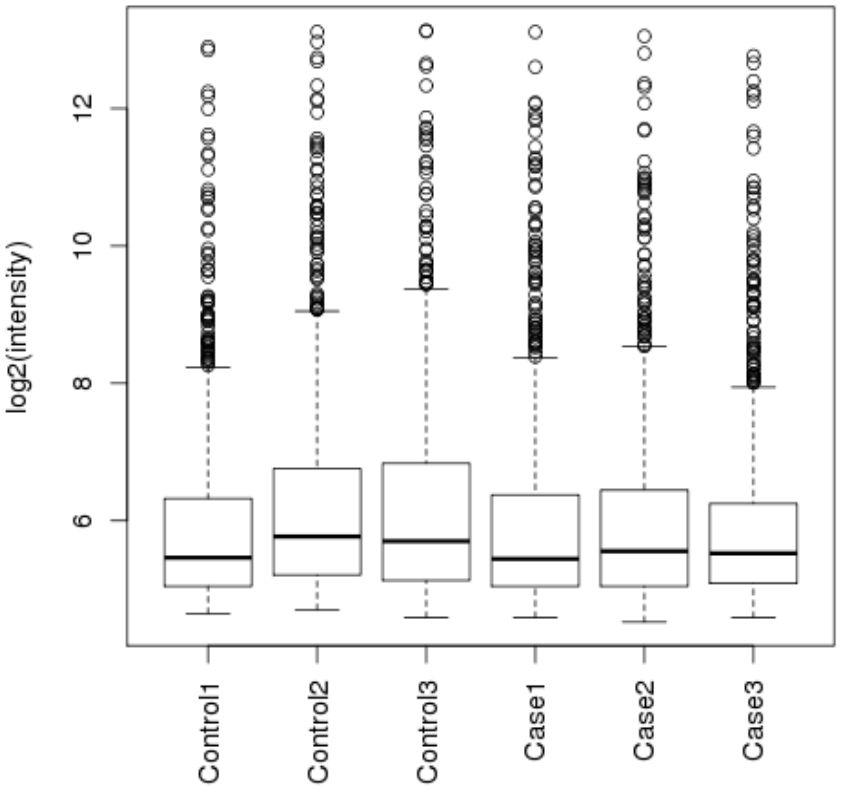
6. Continue the process, we get the following matrix after finishing:

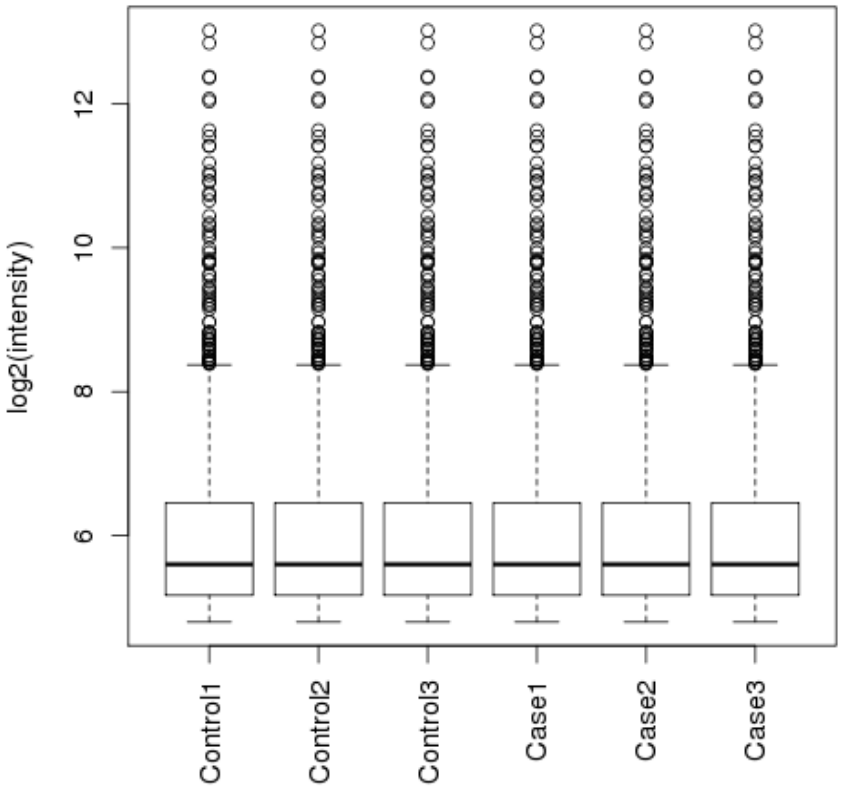
Gene	sample1	sample2	sample3	sample4
1	10.25	12.00	12.00	10.25
2	7.50	3.25	10.25	12.00
3	5.50	7.50	5.50	3.25
4	3.25	5.50	3.25	5.50
5	12.00	10.25	7.50	7.50

The result matrix has following properties:

- The values taken in each column are exactly the same.
- The ranks of genes in each column are the same as before normalization.

Before/after QN boxplot





Summary: between-array normalization

- Must do before comparing different arrays.
- Same problems exist in sequencing data.
- Quantile normalization is too strong and often remove the true signals, use with caution.

Microarray data summarization

- There are usually multiple probes corresponding to a gene. The task is to summarize the readings from these probes into one number to represent the gene expression.
- Naïve methods: mean, median.
- From MAS 5.0: use one-step Tukey Biweight (TBW) to obtain a robust weighted mean that is resistant to outliers.
  - Probes with intensities far away from median will have smaller weights in the average.
- dChip (Li & Wong, 2001): model based on *PM-MM*.

RMA summarization

$$Y_{ijn} = \mu_{in} + \alpha_{jn} + \varepsilon_{ijn}, i = 1, \ldots, I, j = 1, \ldots, J, n = 1, \ldots, n$$

log transformed PM intensities, denoted with Y

μ_i representing the log scale expression level for array i

α_j a probe affinity effect.

each probe set n

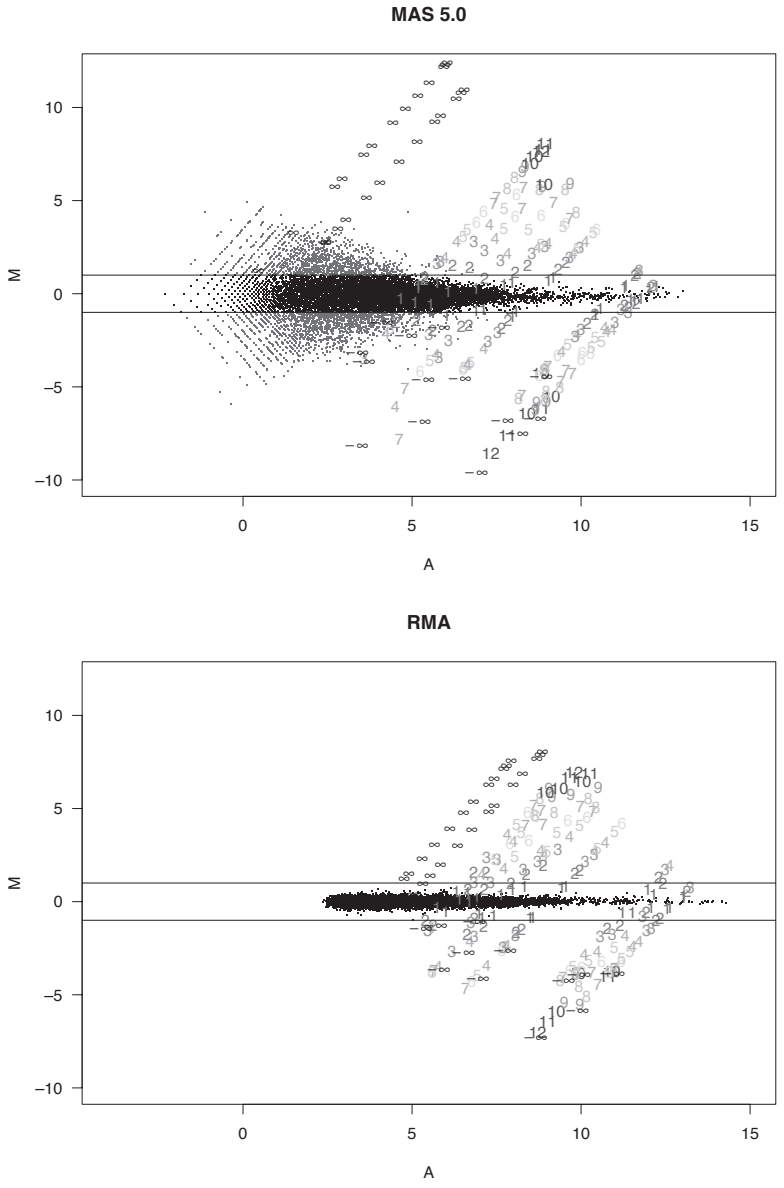
- Borrow information from multiple samples to estimate probe effects.
- Model-fitting: Median Polish (robust against outliers)
  - Iteratively removing the row and column medians until convergence
  - The remainder is the residual;
  - After subtracting the residual, the row medians are the estimates of the expression, and column medians are probe effects.

Irizarry et al. (2003) *Biostatistics*.

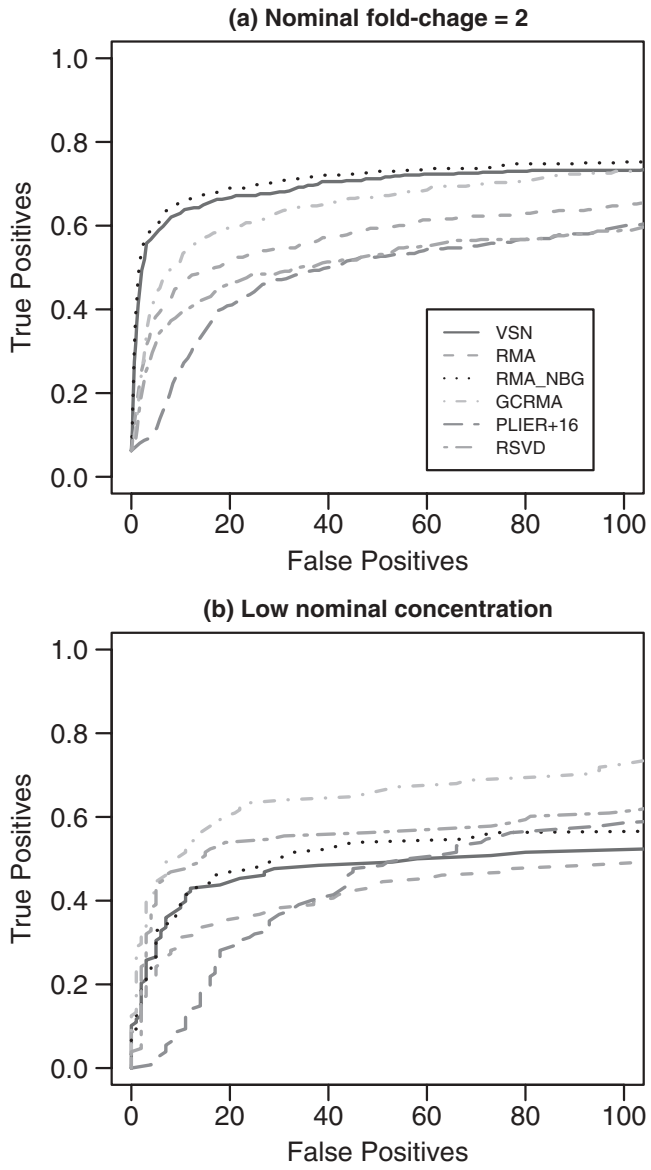
Comparison of different methods

- Some works are done for Affymetrix:
  - Cope et al. (2004) *Bioinformatics*.
  - Irizarry et al. (2006) *Bioinformatics*.
- Gold standard: “spike-in” data in with the true expressions are known.
- Criteria:
  - Accuracy: how close are the estimated values to the truth.
  - Precision: how variable are the estimates.
  - Overall detection ability.

Results: accuracy vs. precision



Results: DE detection ability



Resources

- The `affycomp` R package in Bioconductor.
- Online tools: http://rafalab.rc.fas.harvard.edu/affycomp.

Bioconductor for microarray data

- There is a rich collection of bioc packages for microarrays. In fact, Bioconductor started for microarray analysis.
- There are currently 200+ packages for microarray.
- Important ones include:
  - **affy**: one of the earliest bioc packages. Designed for analyzing data from Affymetrix arrays.
  - **oligo**: preprocessing tools for many types of oligonecleotide arrays. This is designed to replace affy package.
  - **limma** and **siggenes**: DE detection using limma and SAM-t model.
  - Many annotation data package to link probe names to genes.
- Data normalization and summarization can be done using oligo package (details next lecture).

Review

- We have covered microarray analysis, including:
  - Data preprocessing: within and between array normalization.
  - Summarization.
- Next lecture:
  - DE detection for microarray.

To do list

- Review the slides.
- Read the review article on *Nature Reviews Genetics* (link on the class webpage).