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 25+ years. Searching "gene expression microarray" on PubMed returns 100,000+ hits.

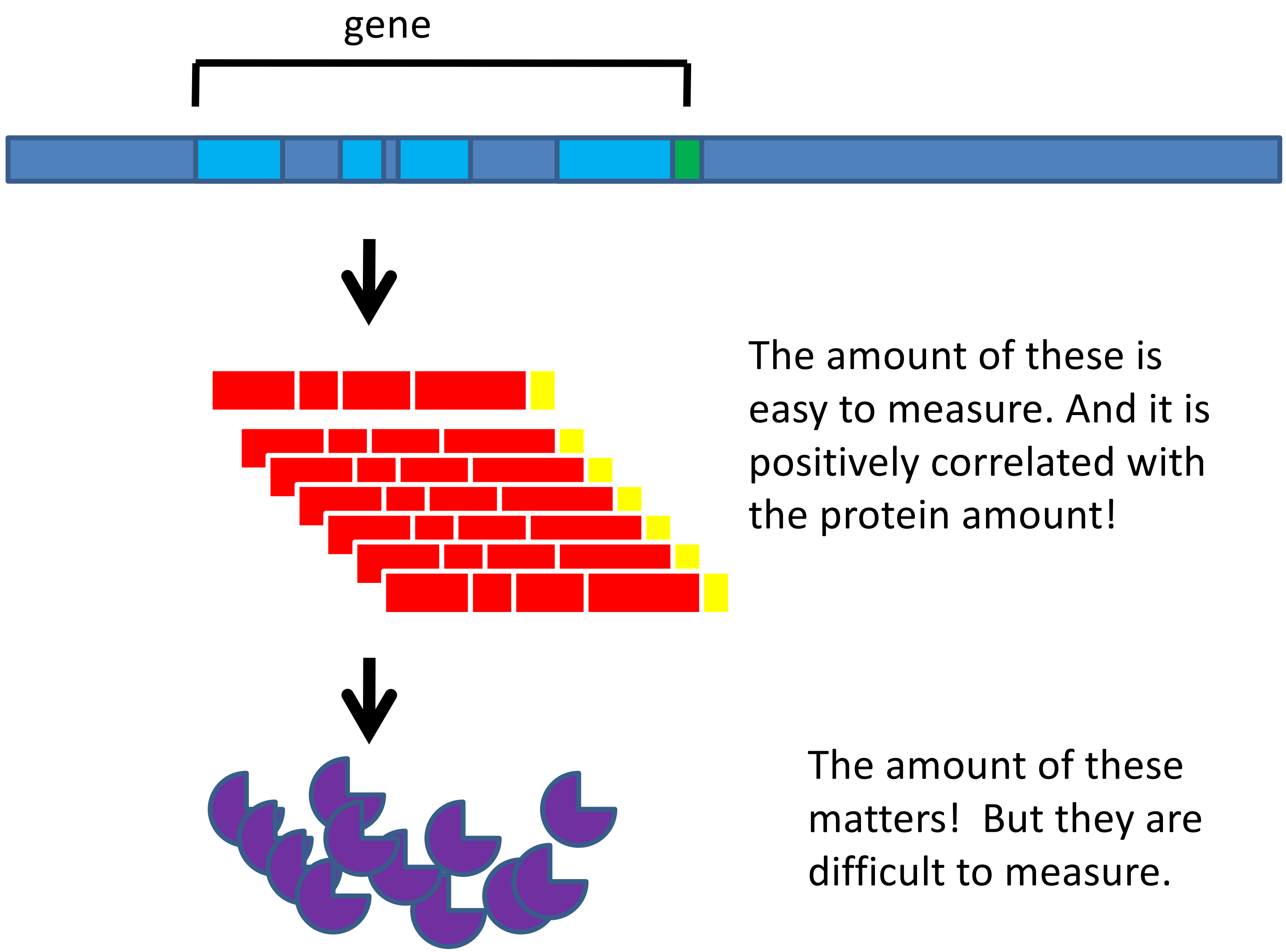
Still microarray?

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

Introduction to GE microarray technology and design

Goal: measure mRNA abundance

DNA
(2 copies)



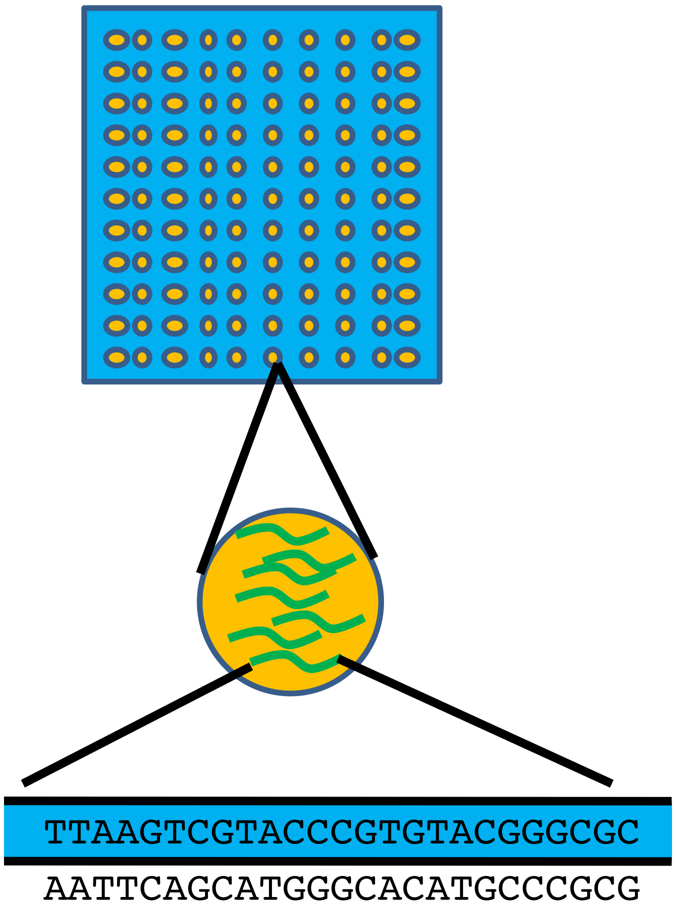
mRNA
(multiple copies)

The amount of these is easy to measure. And it is positively correlated with the protein amount!

Protein
(multiple copies)

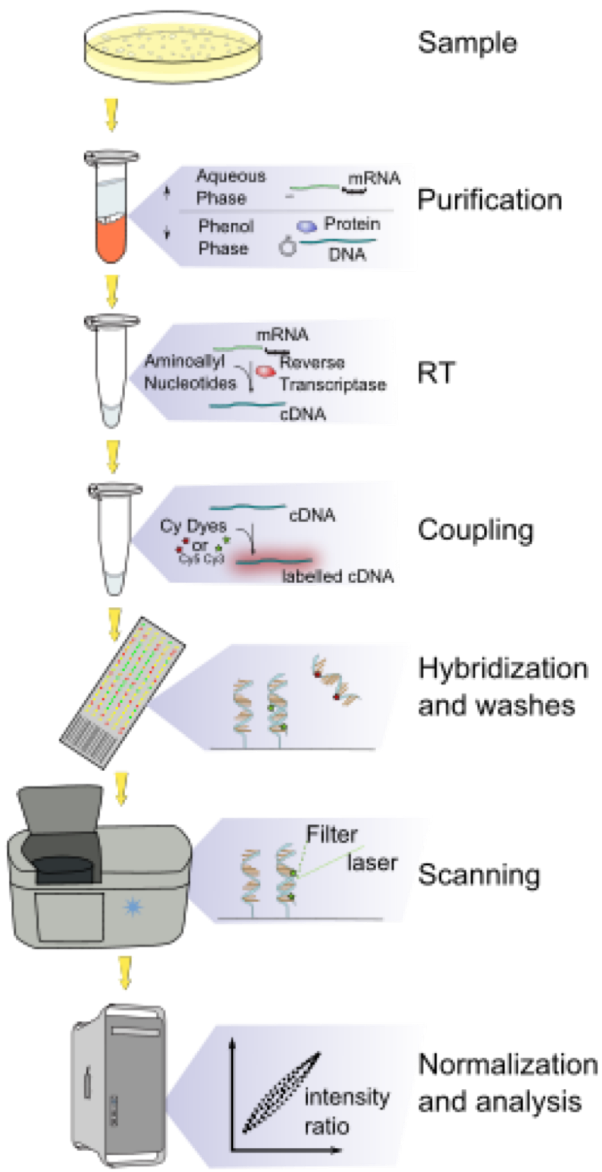
The amount of these matters! But they are difficult to measure.

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.
- A gene with part of its sequence complementary to a probe will stick to that probe (**hybridization**).
- The amount of hybridization on each probe measures the amount of mRNA for its target gene.

Experimental procedure



wet lab: perform experiment

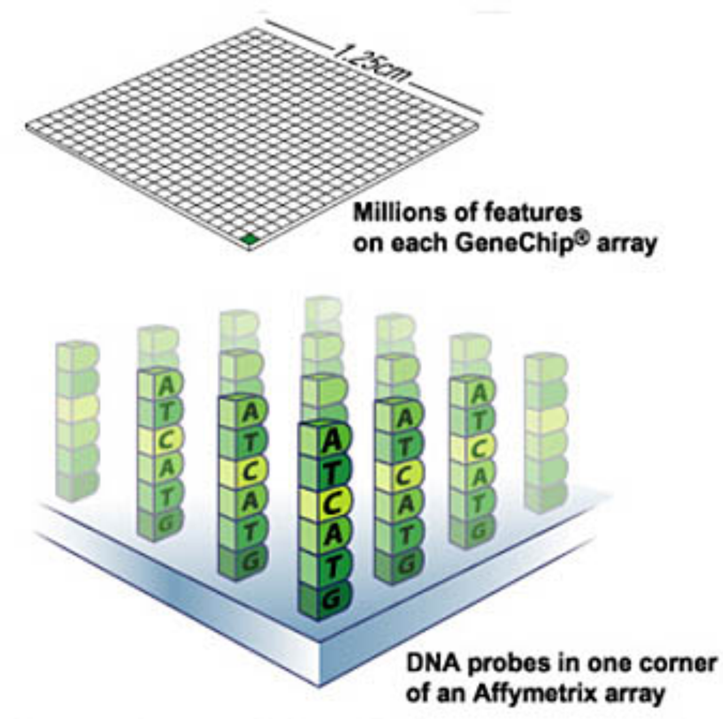
dry lab: data analysis

Available platforms

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

Affymetrix Gene expression arrays

The Affymetrix platform is one of the most widely used.

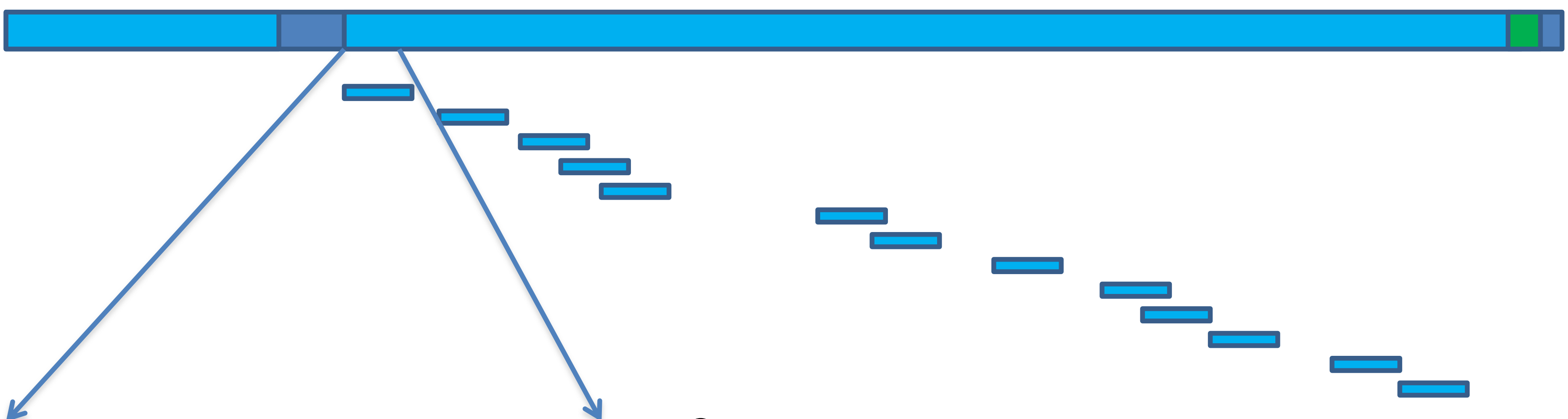


http://www.affymetrix.com/

Affymetrix GeneChip array design

Use U133 system for illustration:

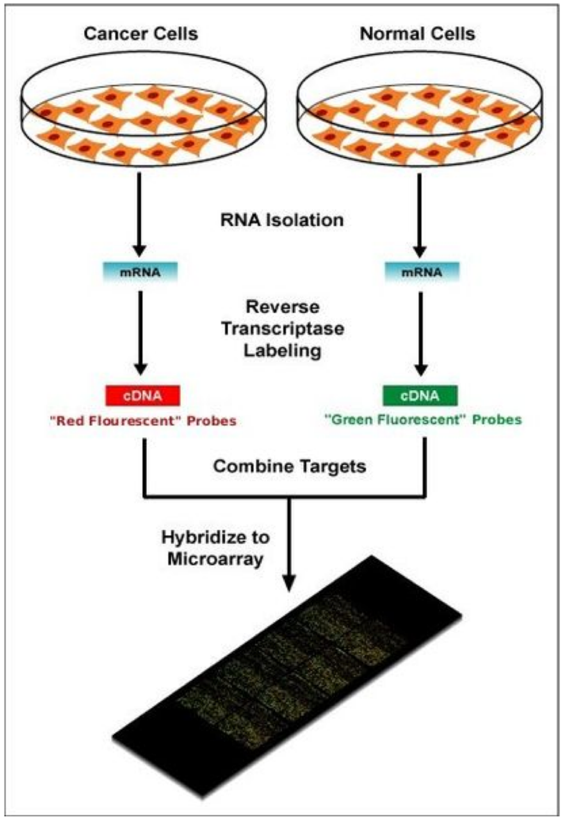
- Around 20 probes per gene.
- Not necessarily evenly spaced: sequence property matters.
- The probes are located at random locations on the array to average out the effects of the surface.



TTAAGTCGTACCCGTGTACGGGCGC Gene sequence

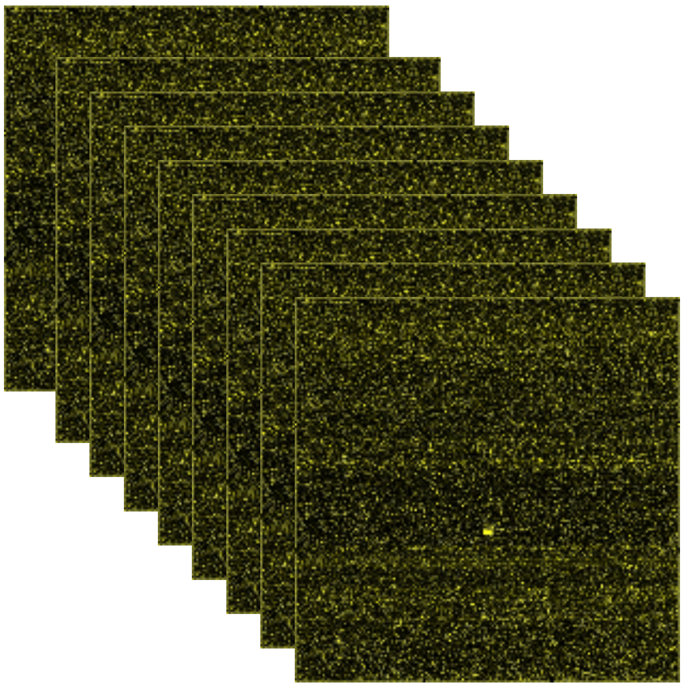
AATTCAGCATGGGCACATGCCCGCG Probe sequence

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 on the same gene to one number.
- Differential expression detection:
  - Find genes 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 expression, not artifacts.

Within- and between-array normalization

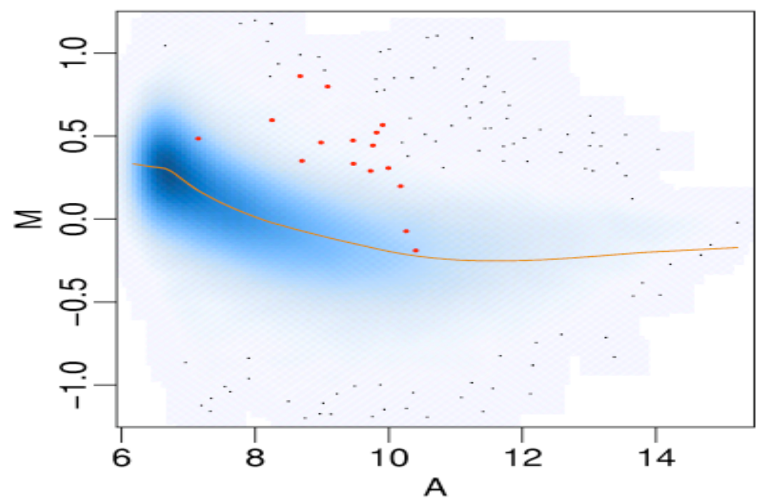
- Within-array: normalization at each array individually to remove array-specific artifacts.
- Between-array: to adjust the values from different arrays and put them at the same baseline, so that numbers are comparable.

Within array normalization, two-color

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

MA plot

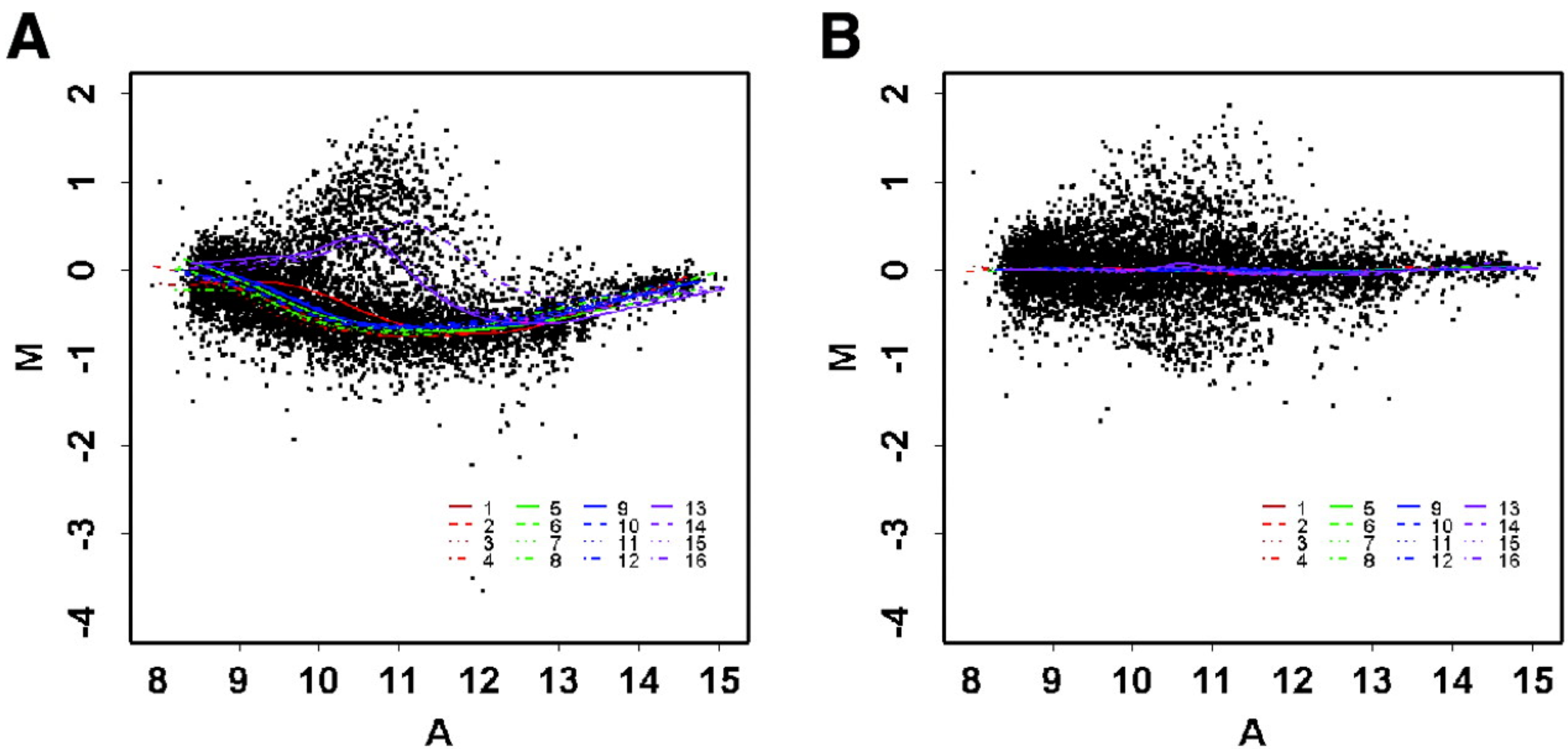
- Widely used diagnostic plot for microarray data (Yang et al. 2002, *Nucleic Acids Research).*
- Also used for different types of sequencing data.
- For spot *i*, let R_i and G_i be the intensities, define:
  - $M_i = log_2 R_i - log_2 G_i$, A=($log_2 R_i + log_2 G_i$)/2.
  - M measures relative expression, 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.

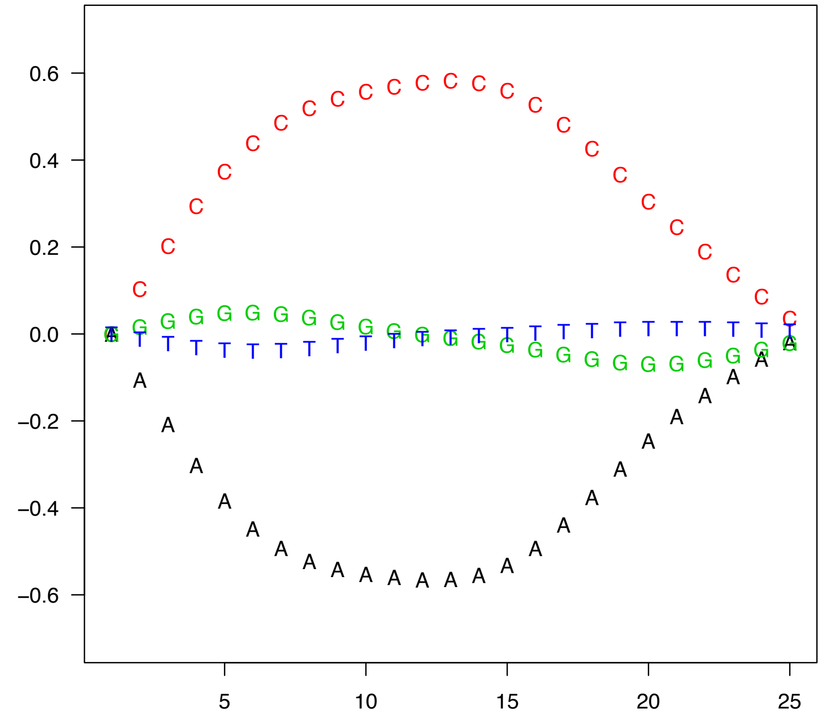
Wu et al. (2005) *JASA*

Probe sequence effects

- Probe affinity is modeled as:

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

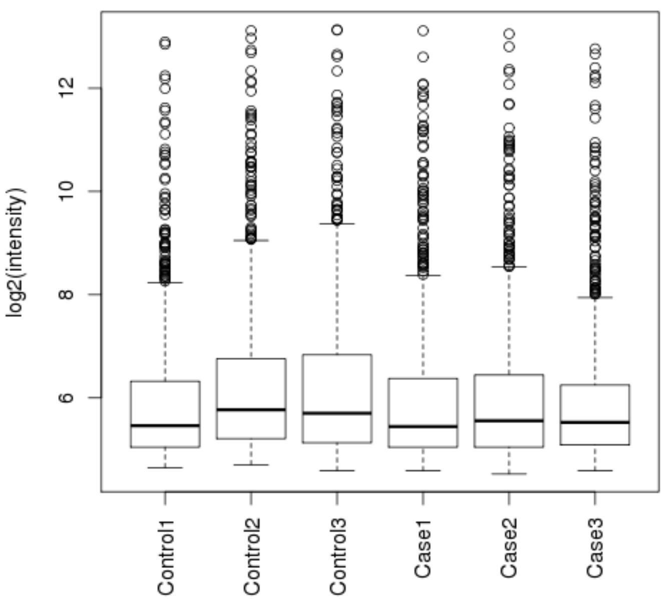
- This kind of modeling is widely used in other microarray and sequencing data!



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



- Data from arrays (intensity values) represent 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!

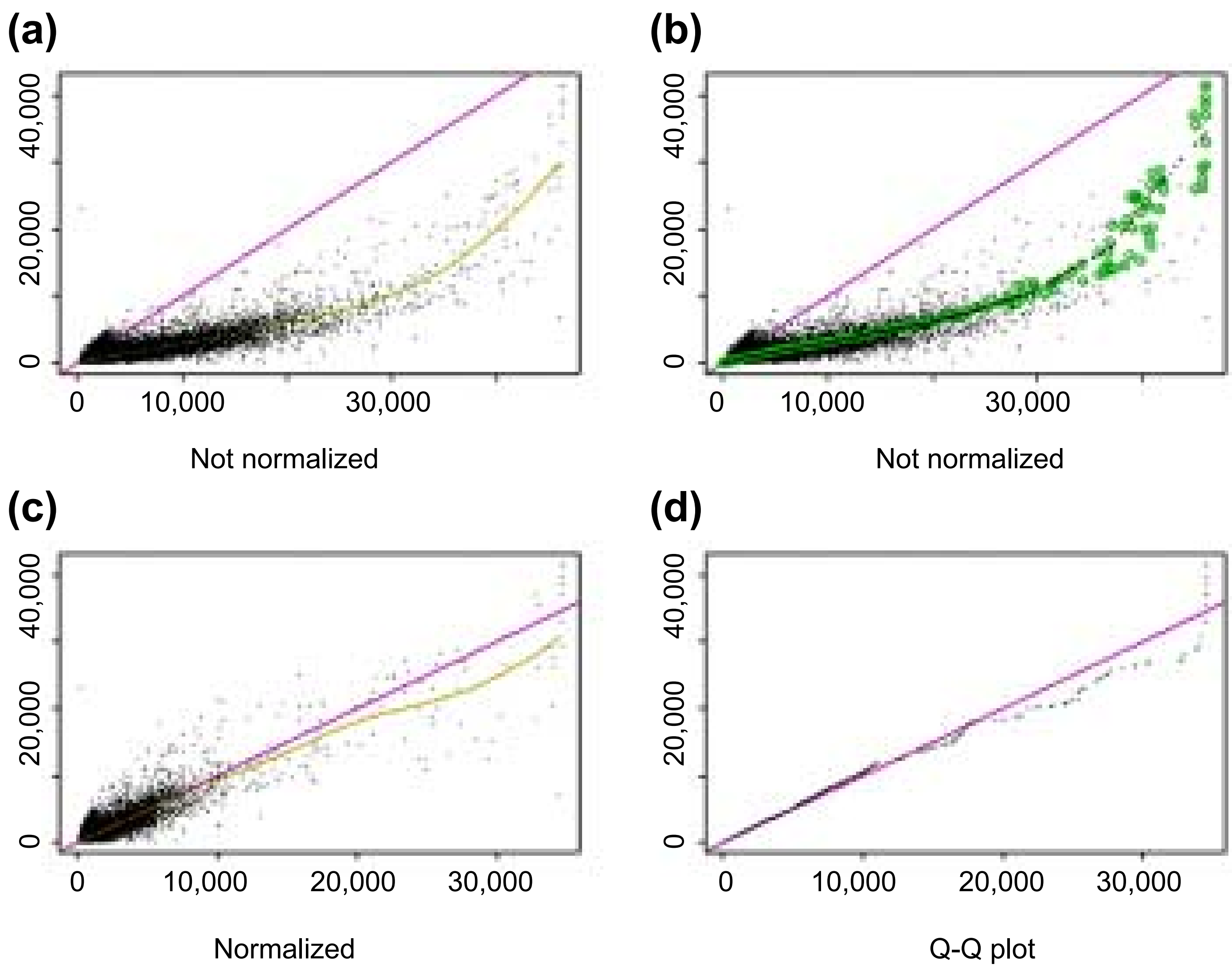
Linear scaling method

- 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 array, 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 the average of 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	$\boxed{3}$	6	$\boxed{5}$	3.25
4	3.25	$\boxed{5}$	3.25	$\boxed{9}$
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

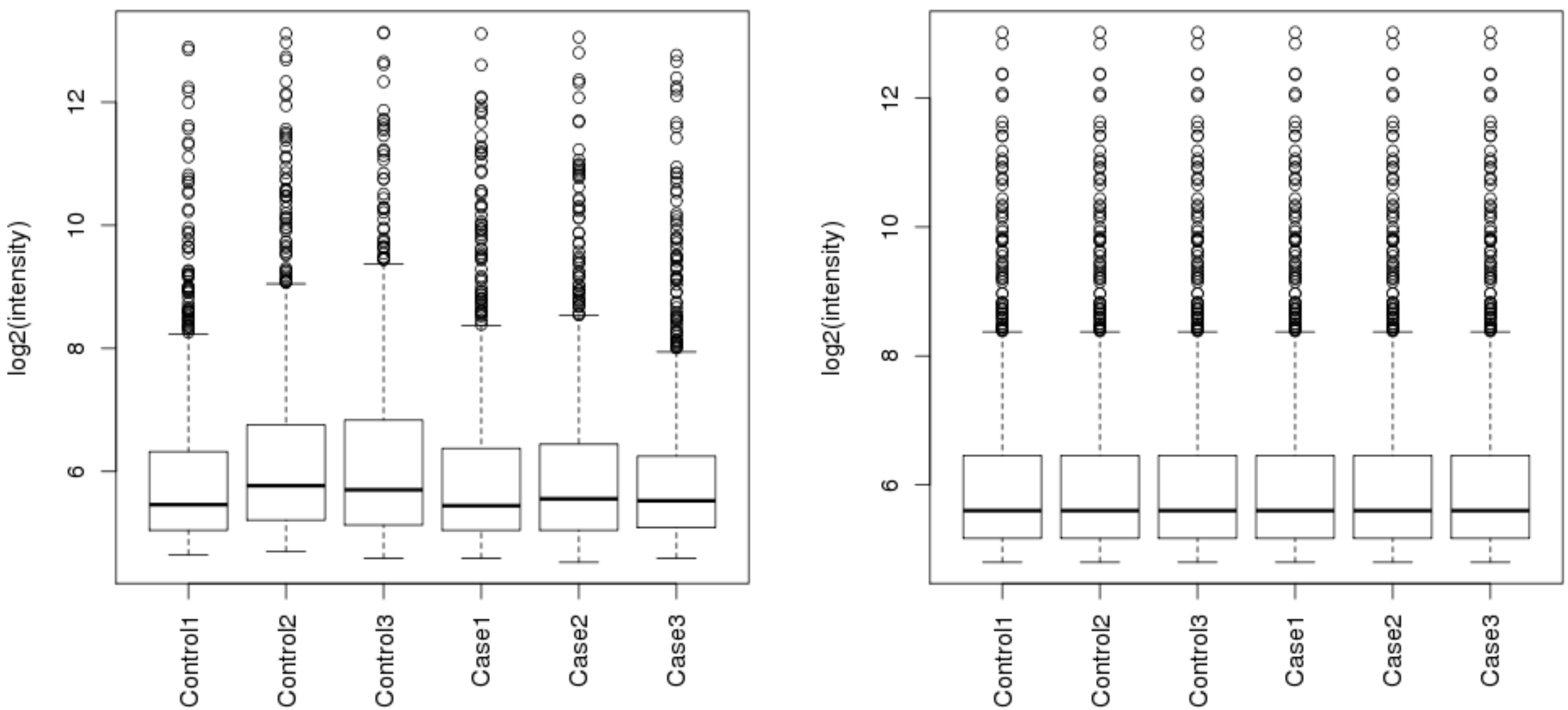
6. Continue the process, we get the following matrix after finishing:

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 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 very strong and could remove the true signals, use with caution.

Microarray data summarization

- There are multiple probes targeting 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*.

Bioconductor for microarray data

- There is a rich collection of Bioconductor packages (hundreds) for microarrays. In fact, Bioconductor started for microarray analysis.
- 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.