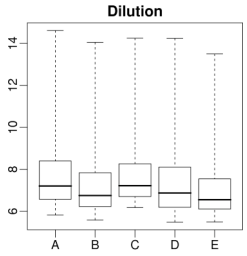
Normalization

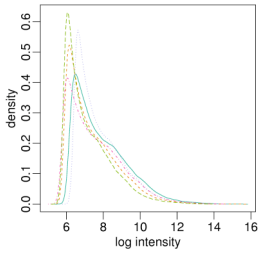
- Normalization is needed to ensure that differences in intensities are indeed due to differential expression, and not some printing, hybridization, or scanning artifact.
- Normalization is necessary before any analysis which involves within or between slides comparisons of intensities, e.g., clustering,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

Example of Replicate Data

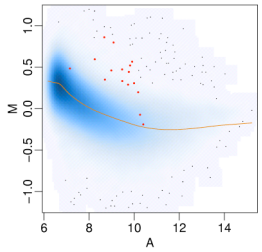


Here different scanners were used

Example of Replicate Data

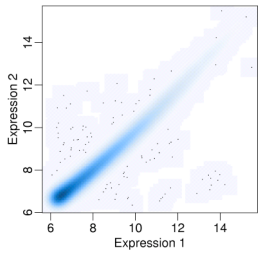


Most Common Problem



Intensity dependent effect: Different background level most likely culprit

Scatter Plot

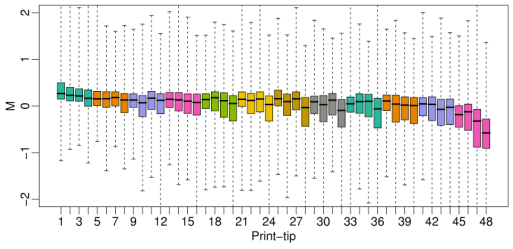


Demonstrates importance of MA plot

Two-color platforms

- **Platforms that use printing robots are prone to many systematic effects:**
  - **Dye**
  - **Print-tip**
  - **Plates**
  - **Print order**
  - **Spatial**
- **Some examples follow**

Print-tip Effect



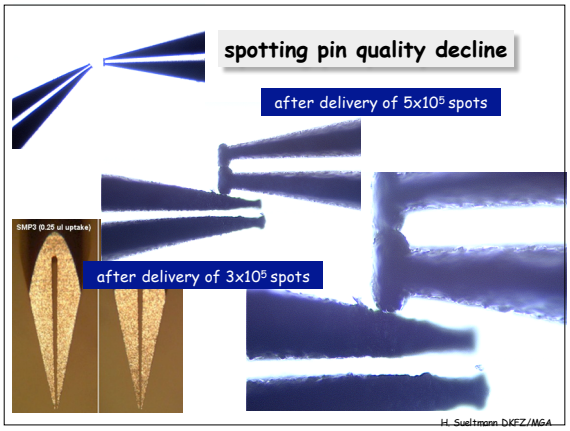
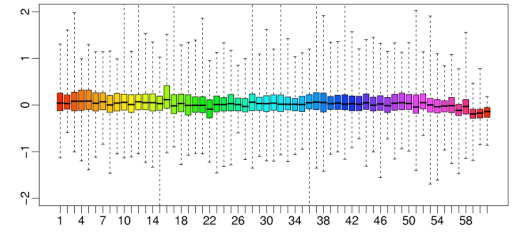
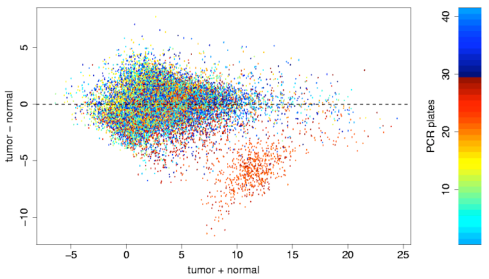


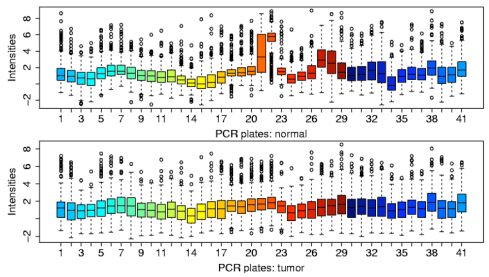
Plate effect



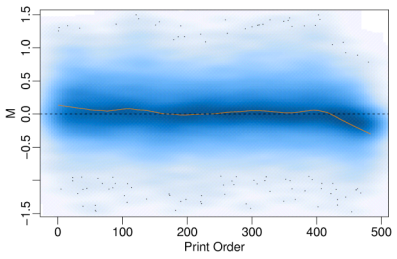
Bad Plate Effect



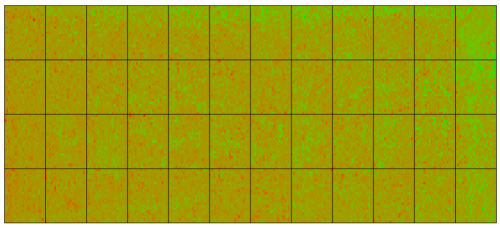
Bad Plate Effect



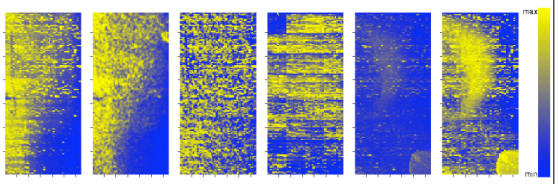
Print Order Effect



Spatial Effect



Spatial Effects



R Rb R-Rb
color scale by rank

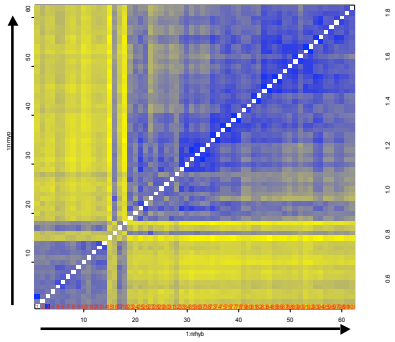
another array: print-tip

color scale ~ log(G)

color scale ~ rank(G)

spotted cDNA arrays, Stanford-type

Batches: array to array differences $d_{ij} = mad_k(h_{ik} - h_{jk})$



arrays i=1…63; roughly sorted by time

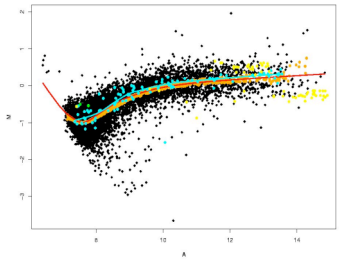
What can we do?

- Throw away the data and start again? Maybe.
- Statistics offers hope:
  - Use control genes to adjust
  - Assume most genes are not differentially expressed
  - Assume distribution of expression are the same

Simplest Idea

- Assume all arrays have the same median log expression or relative log expression
- Subtract median from each array
- In two-color platforms, we typically correct the Ms. Median correction forces the median log ratio to be 0
  - Note: We assume there are as many over-expressed as under-expressed genes)
- For Affymetrix arrays we usually add a constant that takes us back to the original range.
  - It is common to use the median of the medians
  - Typically, we subtract in the log-scale
- Usually this is not enough, e.g. it will not account for intensity dependent bias

House Keeping Genes



I rarely find house keeping genes useful

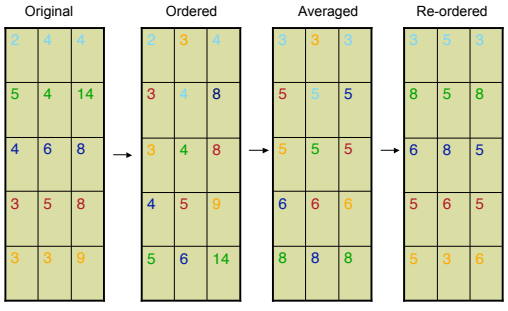
More Elaborate Solutions

- **Proposed solutions**
  - **Force distributions (not just medians) to be the same:**
    - **Amaratunga and Cabrera (2001)**
    - **Bolstad et al. (2003)**
  - **Use curve estimators, e.g. loess, to adjust for the effect:**
    - **Li and Wong (2001) Note: they also use a rank invariant set**
    - **Colantuoni et al (2002)**
    - **Dudoit et al (2002)**
  - **Use adjustments based on additive/multiplicative model:**
    - **Rocke and Durbin (2003)**
    - **Huber et al (2002)**
    - **Cui et al (2003)**

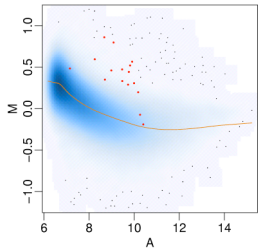
Quantile normalization

- **All these non-linear methods perform similarly**
- **Quantiles is my favorite because its fast and conceptually simple**
- **Basic idea:**
  - **order value in each array**
  - **take average across probes**
  - **Substitute probe intensity with average**
  - **Put in original order**

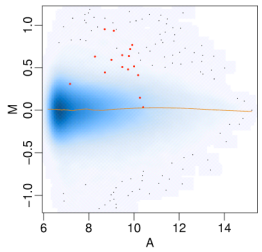
Example of quantile normalization



Before Quantile Normalization



After Quantile Normalization



A worry is that it over corrects

Two-color Platforms

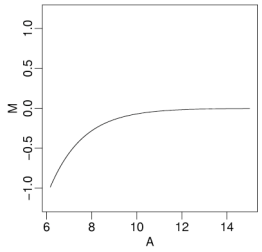
- **Quantile normalization is popular with high-density one channel arrays**
- **With two-color platforms we have many effects to worry about and seems we should take advantage of the paired structure**

ANOVA

- One of the first approaches was to fit ANOVA models to log intensities with a global effect for each Dye
- This does not correct for the non-linear dependence on intensity
- Recent implementations subtract a constant from the original scale to remove the non-linear effect i

For references look at papers by Gary Churchill

Different Background



Above is MA for R=50+S, G=100+S

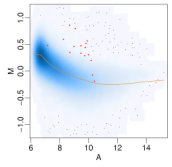
Correcting M approaches

- Most popular approach is to correct M directly
- We assume that we observer M + Bias and that Bias depends on Intensity (A), print-tip, plate, spatial location, etc…
- Idea: Estimate bias and remove it
- For continuous variables we assume the dependence is smooth and use loess to estimtate them
- The normalized M is M - estimated Bias
- Most versatile method

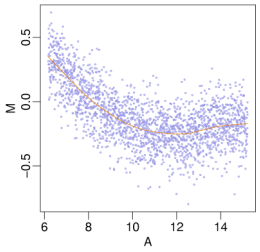
For details look for papers by Terry Speed and Gordon Smyth

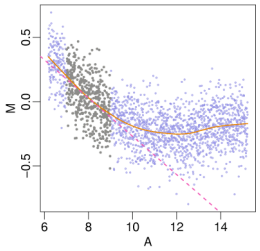
Example: Intensity Effect

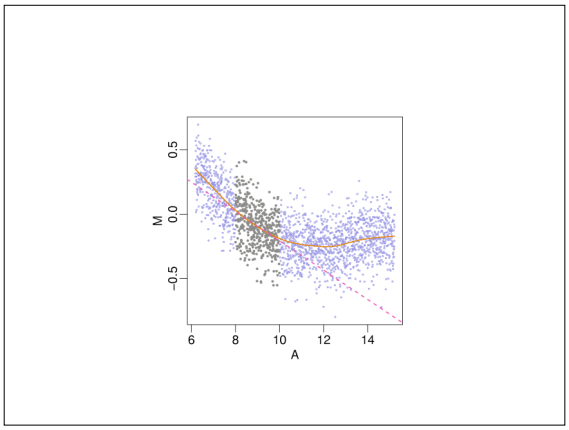
- The most common problem is intensity dependent effects
  - Probably due to different background
- Loess is used to estimate and remove this effects

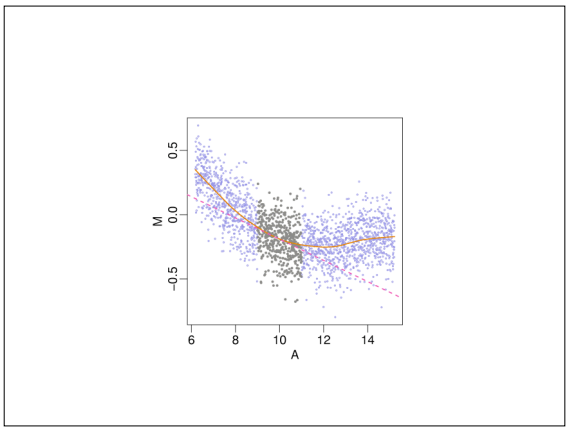


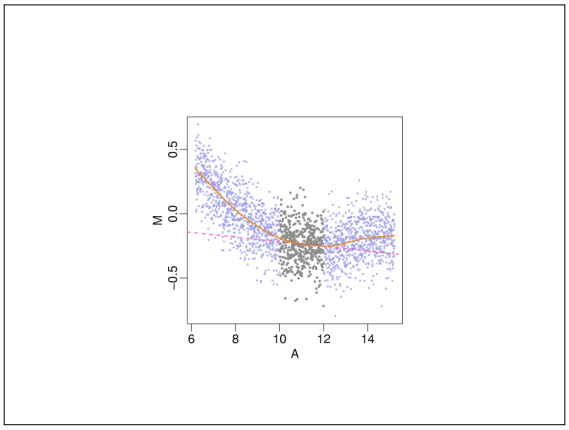
Loess






M
0.5
0.0
−0.5
6
8
10
12
14
A


M
0.5
0.0
−0.5
6
8
10
12
14
A


M
0.5
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−0.5
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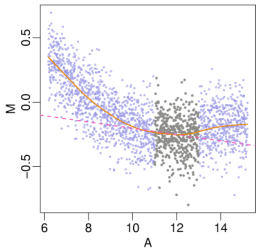
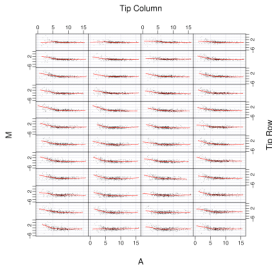
A



Print-tip Loess



Error model approaches

- **Error model approaches describe the need for normalization with an additive background plus stochastic multiplicative error model**
- **From this model an variance stabilizing transformation is obtained**
- **Log ratios are no longer the measure of differential expression**

For details see papers by Wolfgang Huber and David Rocke

Following Slides Provided by Wolfgang Huber

Error models

Describe the possible outcomes of a set of measurements

Outcomes depend on:

-**true value of the measured quantity**
(abundances of specific molecules in biological sample)

-**measurement apparatus**
(cascade of biochemical reactions, optical detection system with laser scanner or CCD camera)

The two component model

measured intensity = offset + gain × true abundance

$$y_{ik} = a_{ik} + b_{ik} x_k$$

$a_{ik} = a_i + \varepsilon_{ik}$

a_i per-sample offset

$e_{ik} \sim N(0, b_i^2 s_1^2)$

"additive noise"

$b_{ik} = b_i \, b_k \, \exp(\eta_{ik})$

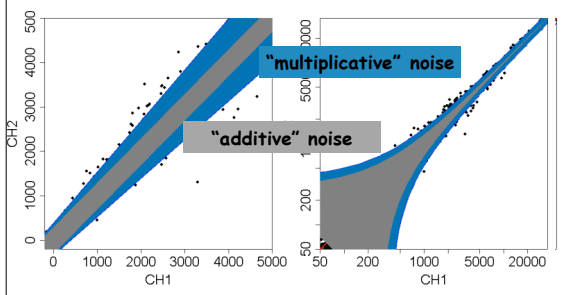
b_i per-sample normalization factor

b_k sequence-wise probe efficiency

$h_{ik} \sim N(0, s_2^2)$

"multiplicative noise"

The two-component model



B. Durbin, D. Rocke, JCB 2001

Parameterization

$$y = a + \varepsilon + b \cdot x \cdot (1 + \eta)$$

$$y = a + \varepsilon + b \cdot x \cdot e^{\eta}$$

two practically equivalent forms (h<<1)

a systematic background	**same for all probes (per array x color)**	**per array x color x print-tip group**
e random background	**iid in whole experiment**	**iid per array**
b systematic gain factor	**per array x color**	**per array x color x print-tip group**
h random gain fluctuations	**iid in whole experiment**	**iid per array**

Important issues for model fitting

Parameterization

variance vs bias

"Heteroskedasticity" (unequal variances)

⇒ weighted regression or variance stabilizing transformation

Outliers

⇒ use a robust method

Algorithm

If likelihood is not quadratic, need non-linear optimization. Local minima / concavity of likelihood?

variance stabilizing transformations

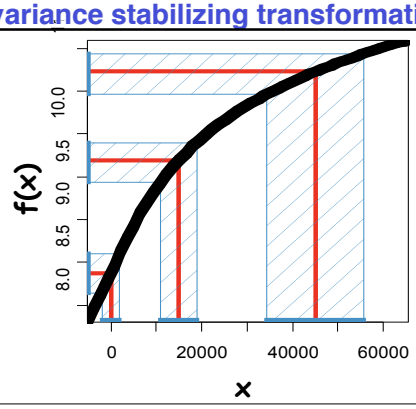
X_u a family of random variables with $EX_u = u$, $\mathrm{Var}X_u = v(u)$. Define

$$f(x) = \int^{x} \frac{1}{\sqrt{v(u)}}\, du$$

derivation: linear approximation

$\Rightarrow$ var $f(X_u) \approx$ independent of u

variance stabilizing transformations



variance stabilizing transformations

$$f(x) = \int^{x} \frac{1}{\sqrt{v(u)}}\, du$$

1.) constant variance ('additive') $v(u) = s^2 \Rightarrow f \propto u$

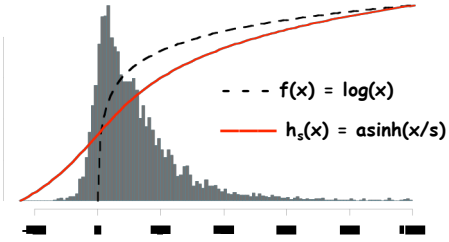
2.) constant CV ('multiplicative') $v(u) \propto u^2 \Rightarrow f \propto \log u$

3.) offset $v(u) \propto (u + u_0)^2 \Rightarrow f \propto \log(u + u_0)$

4.) additive and multiplicative

$$v(u) \propto (u + u_0)^2 + s^2 \Rightarrow f \propto \operatorname{arsinh} \frac{u + u_0}{s}$$

the "glog" transformation

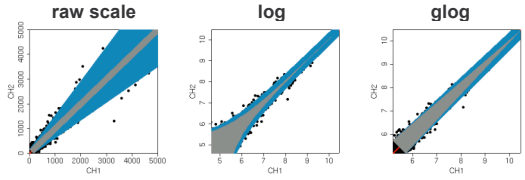


$$\operatorname{arsinh}(x) = \log\left(x + \sqrt{x^2 + 1}\right)$$

$$\lim_{x \to \infty} \left(\operatorname{arsinh} x - \log x - \log 2\right) = 0$$

P. Munson, 2001
D. Rocke & B. Durbin, ISMB 2002
W. Huber et al., ISMB 2002

glog



variance:
constant part
proportional part

the transformed model

$$\operatorname{arsinh}\frac{Y_{ki} - a_{si}}{b_{si}} = \mu_k + \varepsilon_{ki}$$

$$\varepsilon_{ki} : \; N(0, c^2)$$

i: arrays
k: probes
s: probe strata (e.g. print-tip, region)

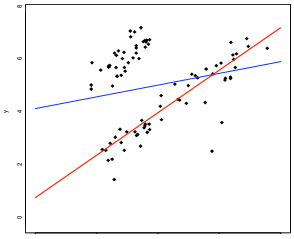
profile log-likelihood

$$pll(a,b) = \sup_{c,\mu} ll(a,b,c,\mu)$$

Here:

$$\begin{aligned}
&\mathrm{pll}(a_1, b_1, \ldots, a_d, b_d) = \\
&= -nd \log \hat{\sigma} + \sum_{k=1}^{n} \sum_{i=1}^{d} \log h_i'(y_{ki}) \\
&= -\frac{nd}{2} \log \left(\sum_{k=1}^{n} \sum_{i=1}^{d} (h_i(y_{ki}) - \hat{\mu}_k)^2 \right) + \sum_{k=1}^{n} \sum_{i=1}^{d} \log h_i'(y_{ki})
\end{aligned}$$

Least trimmed sum of squares regression



minimize

$$\sum_{i=1}^{n/2} \left(y_{(i)} - f(x_{(i)}) \right)^2$$

P. Rousseeuw, 1980s

- least sum of squares
- least trimmed sum of squares

"usual" log-ratio

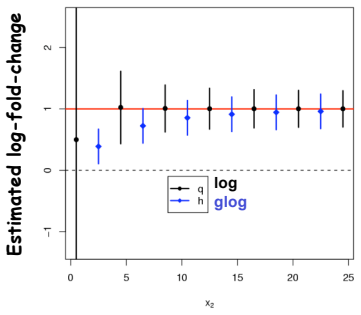
$$\log \frac{x_1}{x_2}$$

'glog' (generalized log-ratio)

$$\log \frac{x_1 + \sqrt{x_1^2 + c_1^2}}{x_2 + \sqrt{x_2^2 + c_2^2}}$$

c_1, c_2 are experiment specific parameters (~level of background noise)

Variance Bias Trade-Off



Variance-bias trade-off and shrinkage estimators

Shrinkage estimators:
pay a small price in bias for a large decrease of variance, so overall the mean-squared-error (MSE) is reduced.

Particularly useful if you have few replicates.

Generalized log-ratio:
= a shrinkage estimator for fold change

There are many possible choices, we chose "variance-stabilization":
+ interpretable even in cases where genes are off in some conditions
+ can subsequently use standard statistical methods (hypothesis testing, ANOVA, clustering, classification…) without the worries about low-level variability that are often warranted on the log-scale

"Single color normalization"

n red-green arrays (R_1, G_1, R_2, G_2,… R_n, G_n)

within/between slides

for (i=1:n)

calculate $M_i = \log(R_i/G_i)$, $A_i = \frac{1}{2} \log(R_i * G_i)$

normalize M_i vs A_i

normalize $M_1 \ldots M_n$

all at once

normalize the matrix of (R, G)

then calculate log-ratios or any other contrast you like

Concluding Remarks

- Notice Normalization and background correction are related
- Current procedures are based on assumptions
- Many new problems clearly violate these assumptions
- We will discuss this problem in another lecture