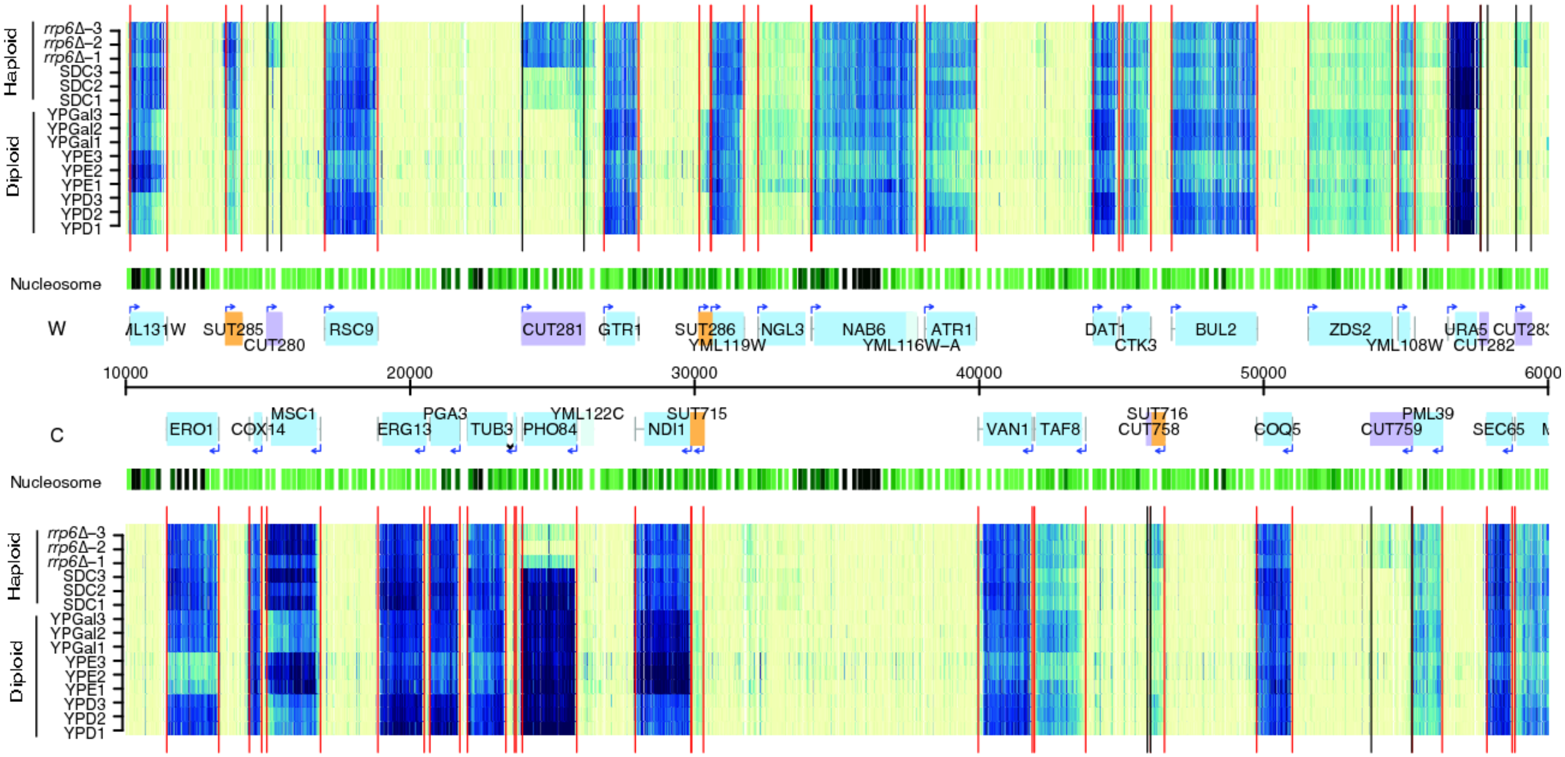
What you still might want to know about microarrays



Brixen 2011

Wolfgang Huber

EMBL

Brief history

Late 1980s: Lennon, Lehrach: cDNAs spotted on nylon membranes

1990s: Affymetrix adapts microchip production technology for in situ oligonucleotide synthesis (commercial, patent-fenced)

1990s: Brown lab in Stanford develops two-colour spotted array technology (open and free)

1998: Yeast cell cycle expression profiling on spotted arrays (Spellmann) and Affymetrix (Cho)

1999: Tumor type discrimination based on mRNA profiles (Golub)

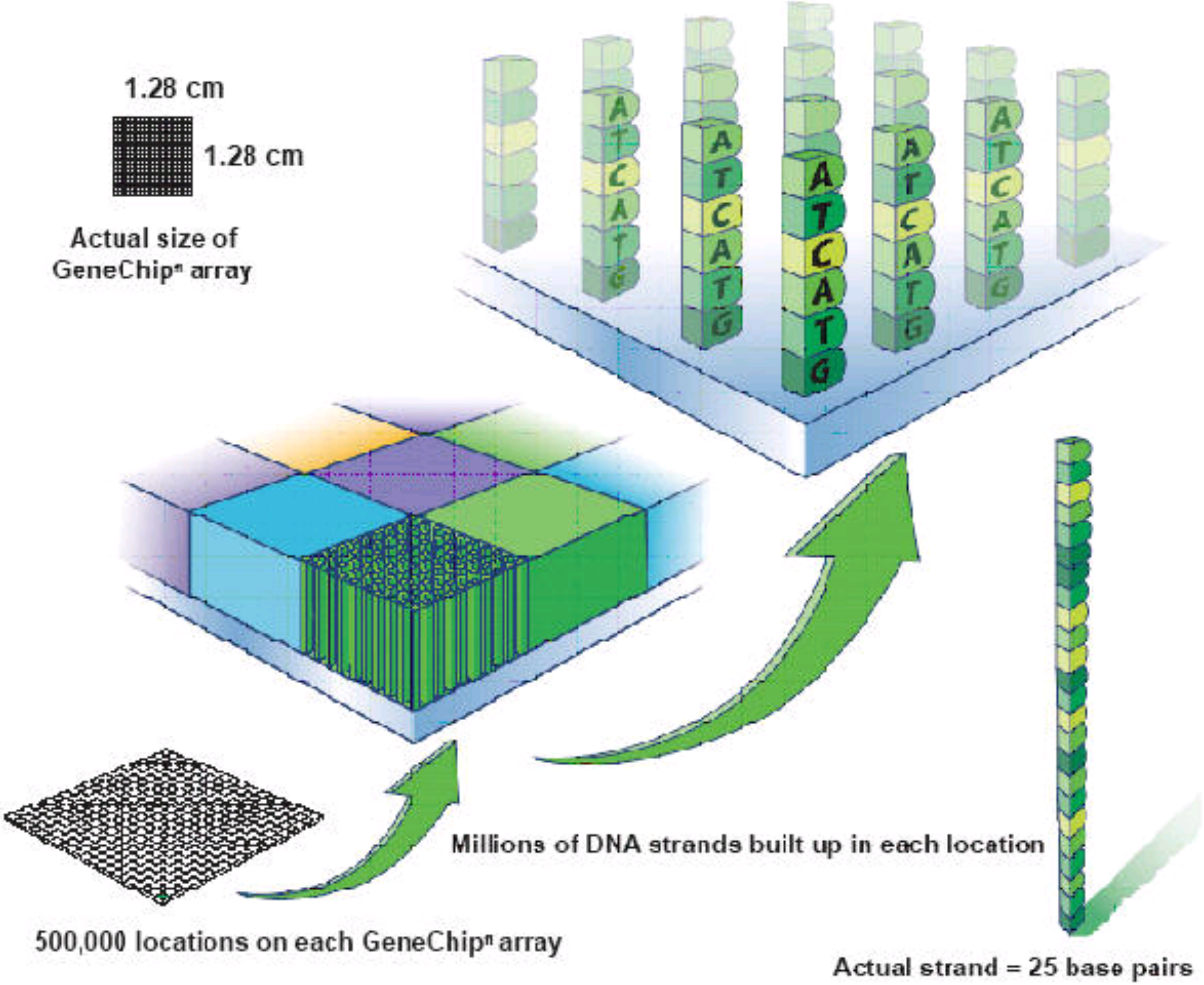
2000-ca. 2004: Affymetrix dominates the microarray market

Since ~2003: Nimblegen, Illumina, Agilent (and many others)

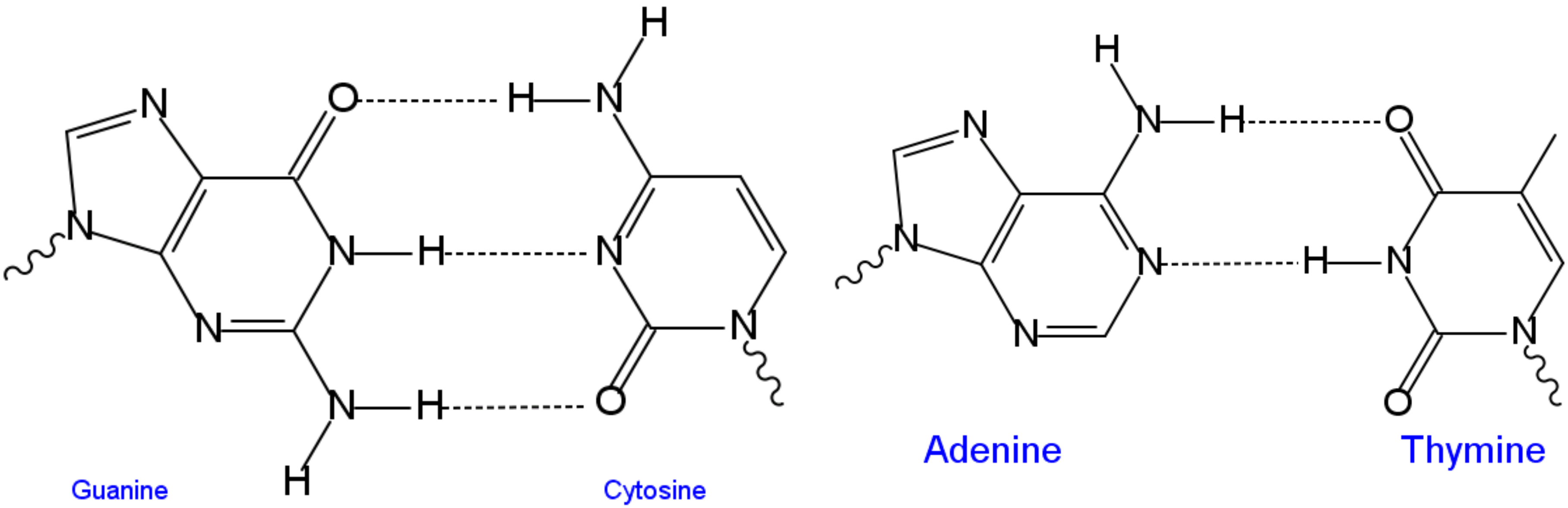
Throughout 2000‘s: CGH, CNVs, SNPs, ChIP, tiling arrays

Since ~2007: Next-generation sequencing (454, Solexa, ABI Solid,...)

Oligonucleotide microarrays

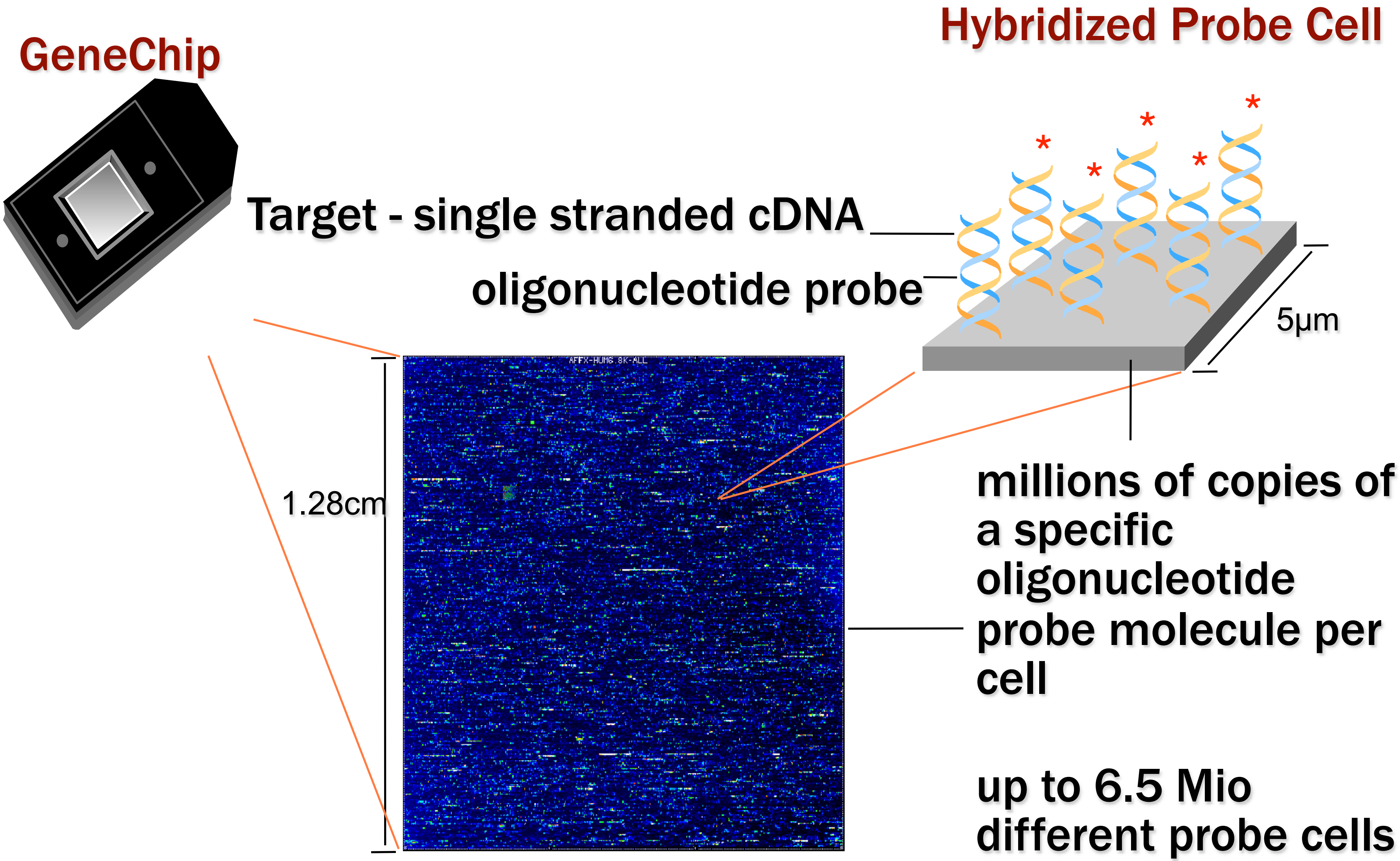

1.28 cm
1.28 cm
Actual size of GeneChip® array
A T C A T G
A T C A T G
A T C A T G
A T C A T G
A T C A T G
Millions of DNA strands built up in each location
500,000 locations on each GeneChip® array
Actual strand = 25 base pairs

Base Pairing



Ability to use hybridisation for constructing specific + sensitive probes at will is unique to DNA (cf. proteins, RNA, metabolites)

Oligonucleotide microarrays



GeneChip

Hybridized Probe Cell

Target - single stranded cDNA

oligonucleotide probe

millions of copies of a specific oligonucleotide probe molecule per cell

up to 6.5 Mio different probe cells

Image of array after hybridisation and staining

Probe sets

GeneChip® Expression Array Design

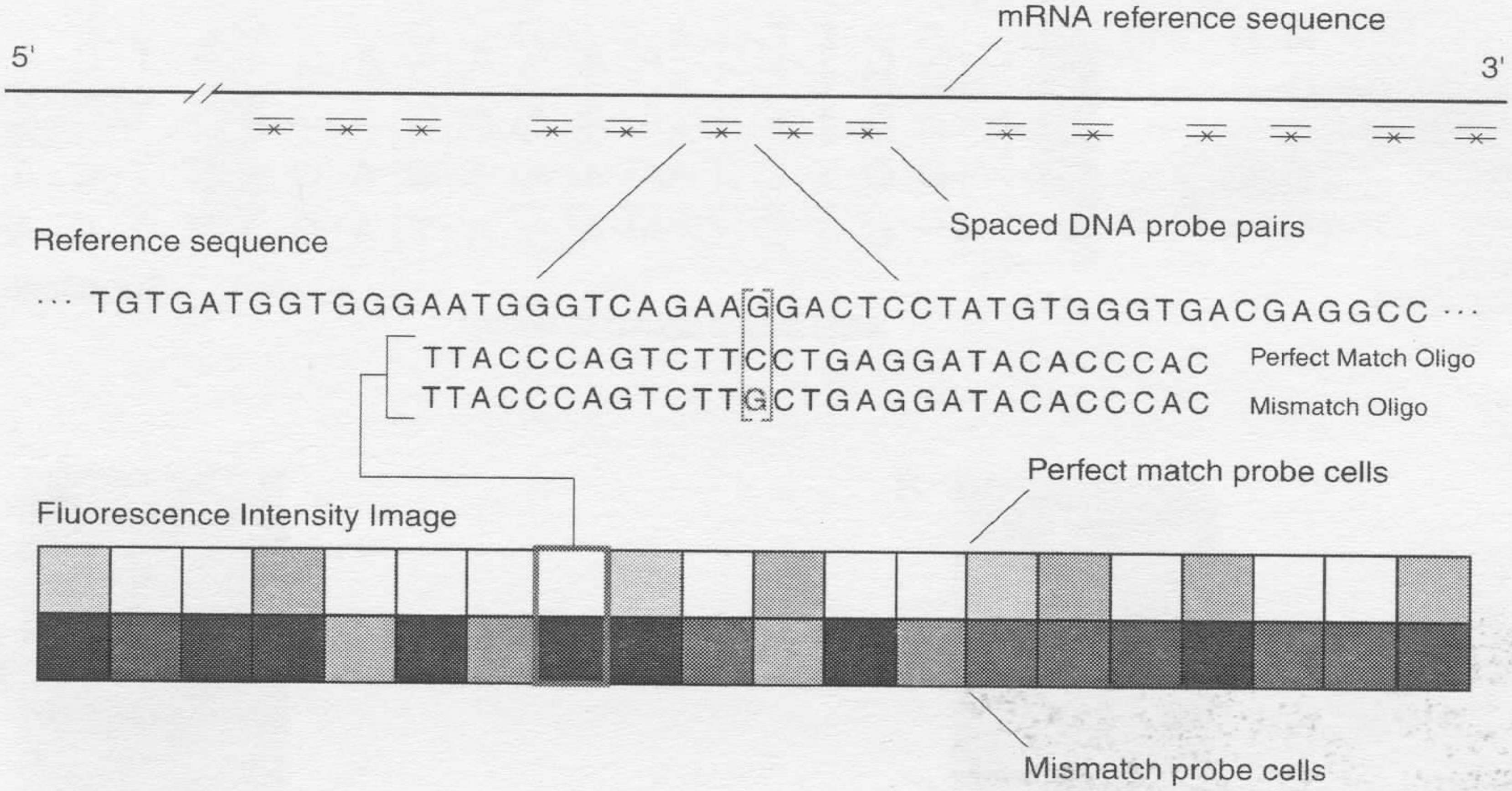

mRNA reference sequence
5'
3'
Spaced DNA probe pairs
Reference sequence
··· TGTGATGGTGGGAATGGGTCAGAAGGACTCCTATGTGGGTGACGAGGCC ···
TTACCCAGTCTTCCTGAGGATACACCCAC
Perfect Match Oligo
TTACCCAGTCTTGCTGAGGATACACCCAC
Mismatch Oligo
Perfect match probe cells
Fluorescence Intensity Image
Mismatch probe cells

Figure 1-3 Expression tiling strategy

Terminology for transcription arrays

Each target molecule (transcript) is represented by several oligonucleotides of (intended) length 25 bases

Probe: one of these 25-mer oligonucleotides

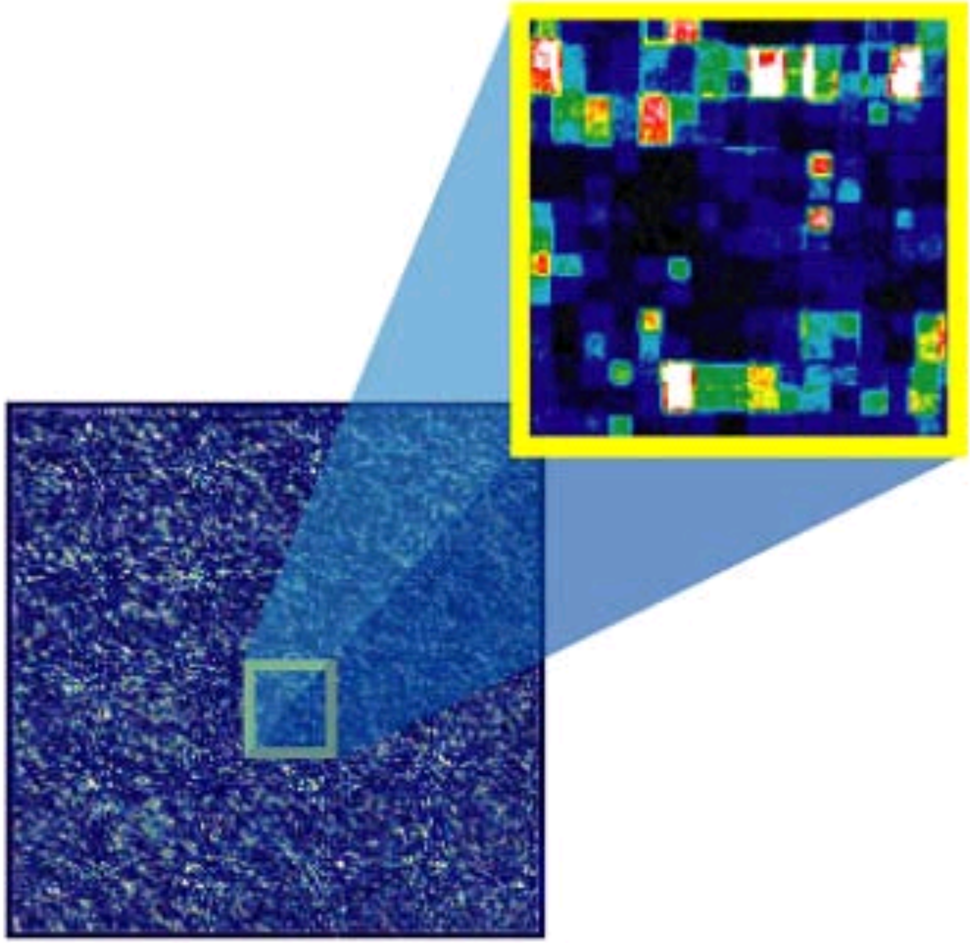
Probe set: a collection of probes (e.g. 11) targeting the same transcript

MGED/MIAME: „probe“ is ambiguous!

Reporter: the sequence

Feature: a physical patch on the array with molecules intended to have the same reporter sequence (one reporter can be represented by multiple features)

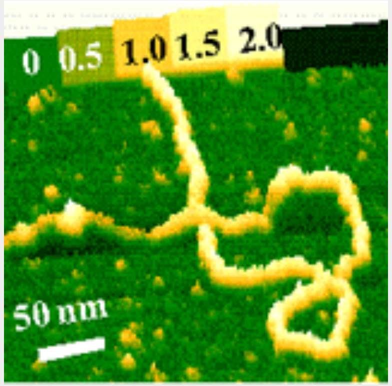
Image analysis



- several dozen pixels per feature
- segmentation
- summarisation into one number representing the intensity level for this feature

→ CEL file

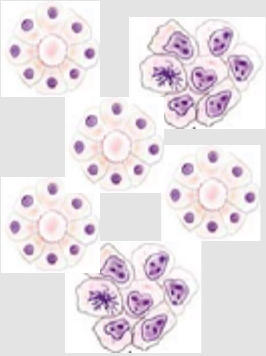
μarray data



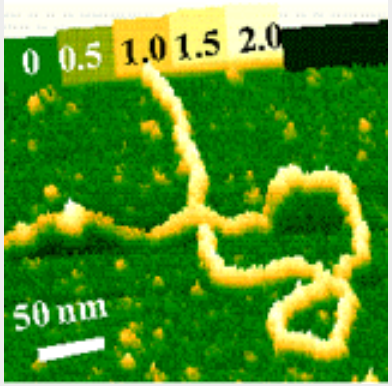
arrays:

probes = gene-specific DNA strands

μarray data



samples:
mRNA from tissue biopsies, cell lines



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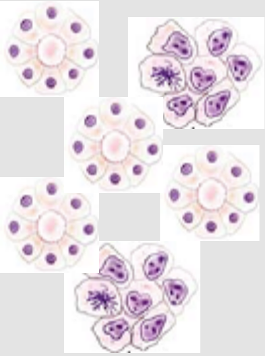

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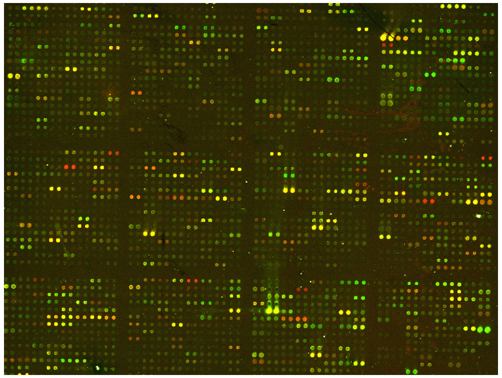

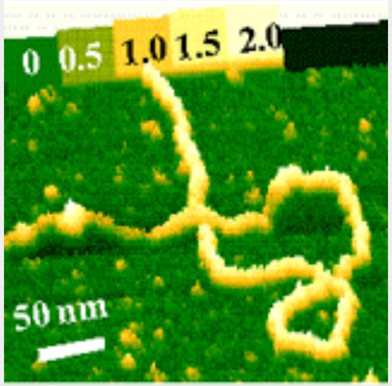

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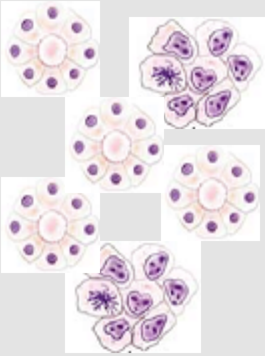

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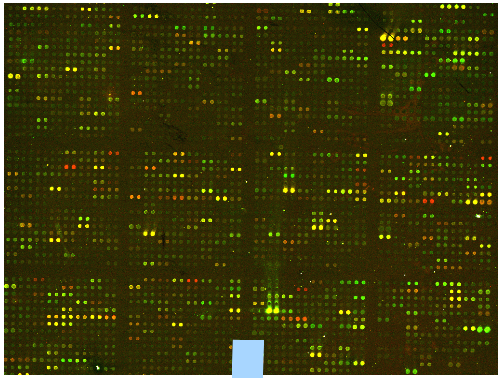
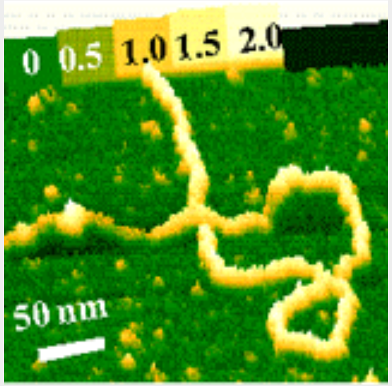


fluorescent detection of the amount of sample-probe binding

	tissue A	tissue B	tissue C
ErbB2	0.02	1.12	2.12
VIM	1.1	5.8	1.8
ALDH4	2.2	0.6	1.0
CASP4	0.01	0.72	0.12
LAMA4	1.32	1.67	0.67
MCAM	4.2	2.93	3.31



arrays:
probes = gene-specific DNA strands

Microarray Infrastructure in Bioconductor

Platform-specific data import and initial processing

Affymetrix 3’ IVT (e.g. Human U133 Plus 2.0, Mouse 430 2.0):

`affy`

Affymetrix Exon (e.g. Human Exon 1.0 ST):

`oligo, exonmap, xps`

Affymetrix SNP arrays:

`oligo`

Nimblegen tiling arrays (e.g. for ChIP-chip):

`Ringo`

Affymetrix tiling arrays (e.g. for ChIP-chip):

`Starr`

Illumina bead arrays:

`beadarray, lumi`

http://www.bioconductor.org/docs/workflows/oligoarrays

Flexible data import

Using generic `R` I/O functions and constructors

`Biobase`

`limma`

Chapter *Two Color Arrays* in the useR-book.

`limma` user guide

Normalisation and quality assessment

preprocessCore

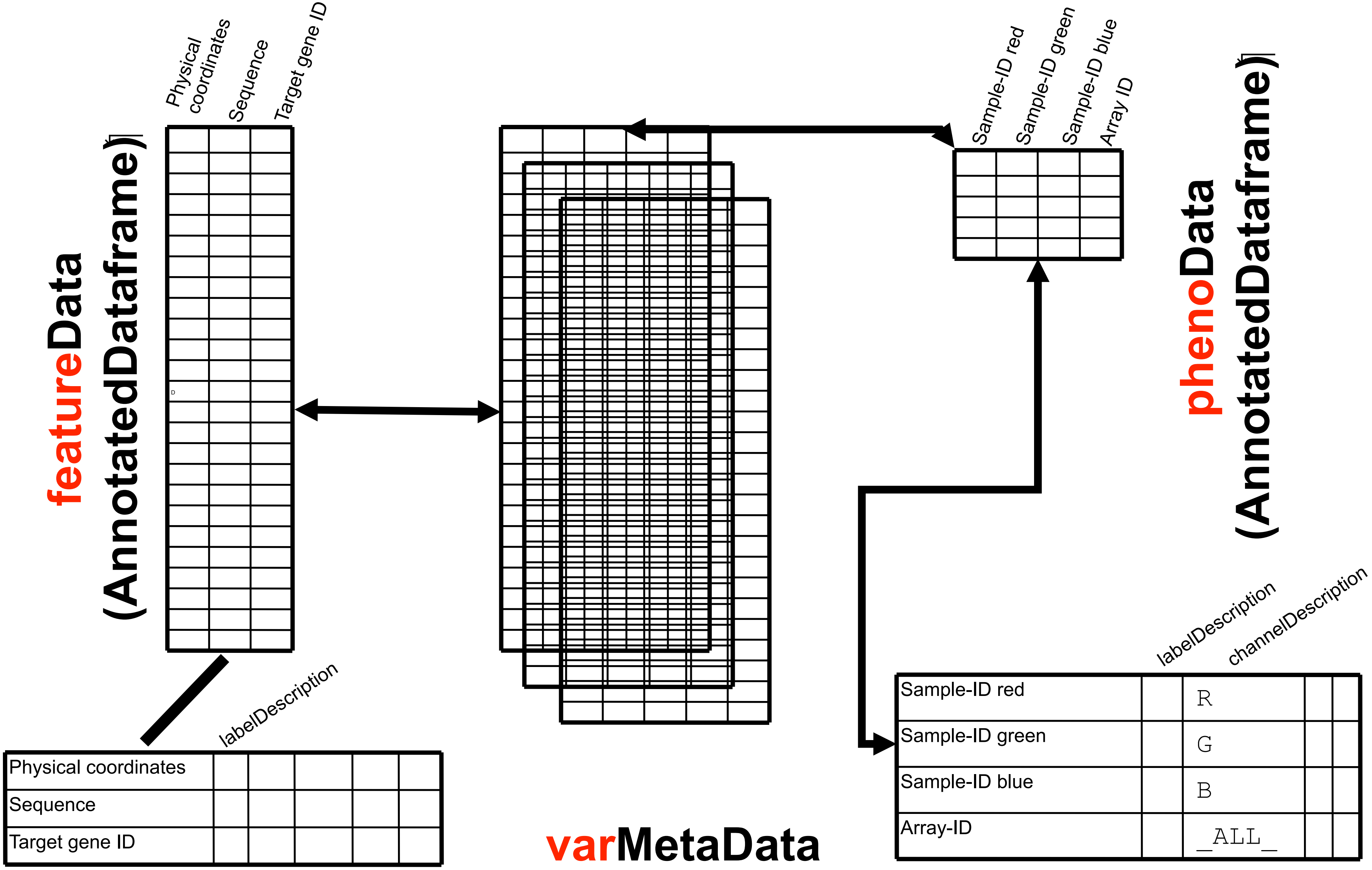
limma

vsn

arrayQualityMetrics

NChannelSet

assayData can contain N=1, 2, ..., matrices of the same size



	labelDescription				
Physical coordinates					
Sequence					
Target gene ID					

	labelDescription	channelDescription		
Sample-ID red		R		
Sample-ID green		G		
Sample-ID blue		B		
Array-ID		_ALL_		

Annotation / Metadata

Keeping data together with the metadata (about reporters, target genes, samples, experimental conditions, ...) is one of the major principles of Bioconductor

- avoid alignment bugs
- facilitate discovery

Often, the same microarray design is used for multiple experiments. Duplicating that metadata every time would be inefficient, and risk versioning mismatches ⇒

instead of `featureData`, just keep a pointer to an annotation package.

(In principle, one could also want to do this for samples.)

Annotation infrastructure for Affymetrix

For `affy`:

`hgu133plus2.db` “all available” information about target genes

`hgu133plus2cdf` maps the physical features on the array to probesets

`hgu133plus2probe` nucleotide sequence of the features (e.g. for `gcrma`)

Genotyping

`crlmm` Genotype Calling (CRLMM) and Copy Number Analysis tool for Affymetrix SNP 5.0 and 6.0 and Illumina arrays.

`snpMatrix`

.... others

See also:
Genome-wide association study of CNVs in 16,000 cases of eight common diseases and 3,000 shared controls, The Wellcome Trust Case Control Consortium, Nature 464, 713-720 (Box 1).

Gene expression analysis with microarrays

Microarray Analysis Tasks

Data import

reformating and setup/curation of the metadata

Normalisation

Quality assessment & control

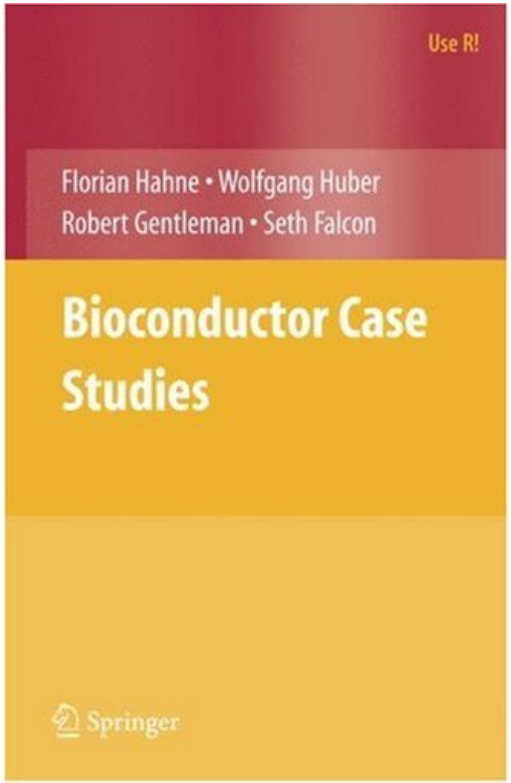
Differential expression

Using gene-level annotation

Gene set enrichment analysis

Clustering & Classification

Integration of other datasets



Use R!

Florian Hahne • Wolfgang Huber
Robert Gentleman • Seth Falcon

Bioconductor Case Studies

Springer

▶ What is wrong with microarray data?

Many data are measured in definite units:

- time in seconds
- lengths in meters
- energy in Joule, etc.

Climb Mount Plose (2465 m) from Brixen (559 m) with weight of 76 kg, working against a gravitation field of strength 9.81 m/s^2 :

$$(2465 - 559) \cdot 76 \cdot 9.81 \;\; \text{m kg m/s}^2$$
$$= 1\,421\,037 \text{ kg m}^2 \text{ s}^{-2}$$
$$= 1\,421.037 \text{ kJ}$$

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$$
\begin{aligned}
&(2465 - 559) \cdot 76 \cdot 9.81 \;\; \mathrm{m\ kg\ m/s^2} \\
&= 1\,421\,037 \ \mathrm{kg\ m^2\ s^{-2}} \\
&= 1\,421.037 \ \mathrm{kJ}
\end{aligned}
$$

A complex measurement process lies between mRNA concentrations and intensities

- RNA degradation
- amplification efficiency
- reverse transcription efficiency
- hybridization efficiency and specificity
- labeling efficiency
- quality of actual probe sequences (vs intended)
- scratches and spatial gradients on the array
- cross-talk across features
- cross-hybridisation
- optical noise
- image segmentation
- signal quantification
- signal "preprocessing"

A complex measurement process lies between mRNA concentrations and intensities

o RNA degradation

o a
eff

o r
tra
eff

o h
eff
specificity

o labeling efficiency

o quality of actual probe sequences

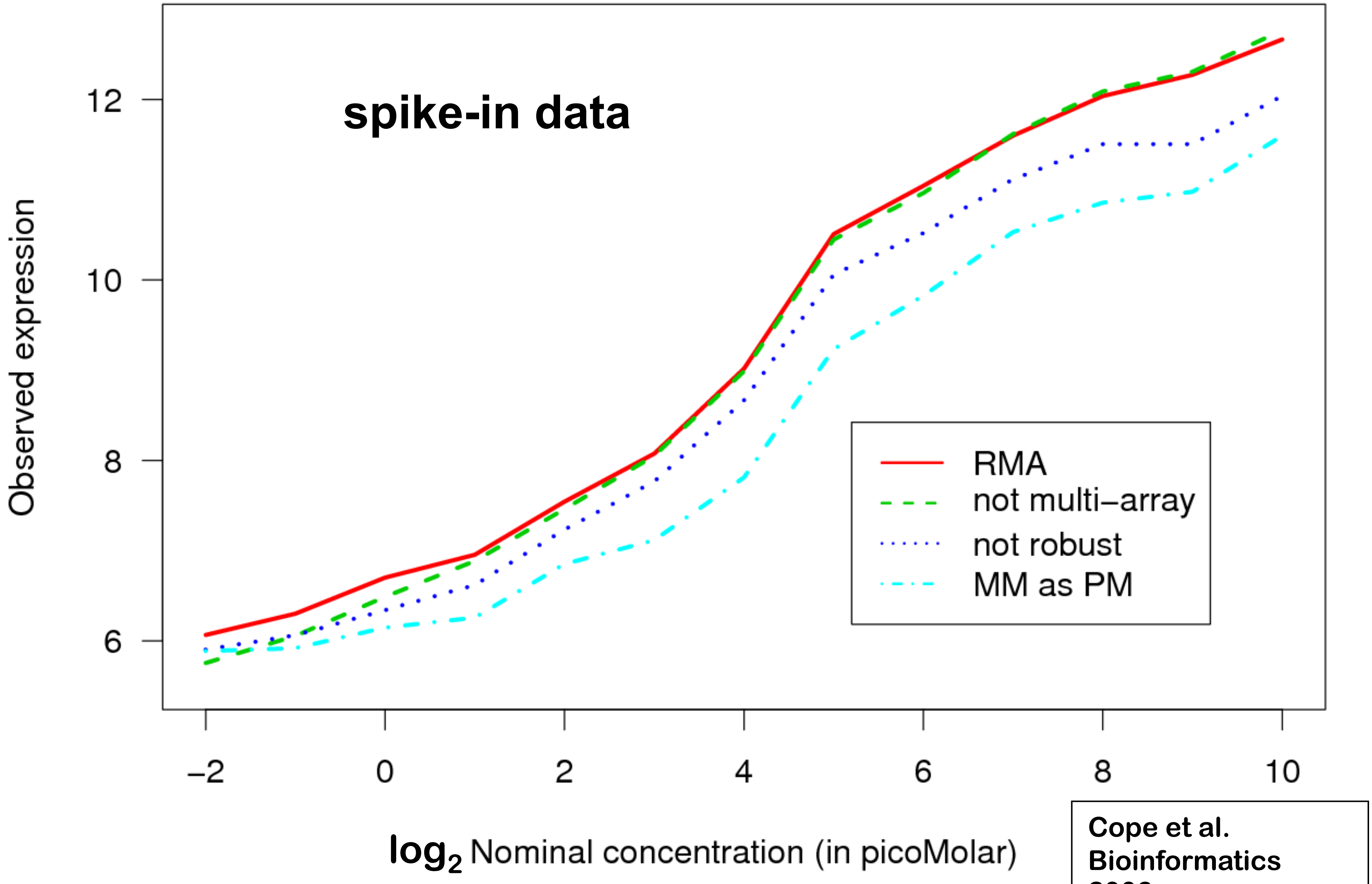
o optical noise

o image segmentation

The problem is less that these steps are ‘not perfect’; it is that they vary from array to array, experiment to experiment.

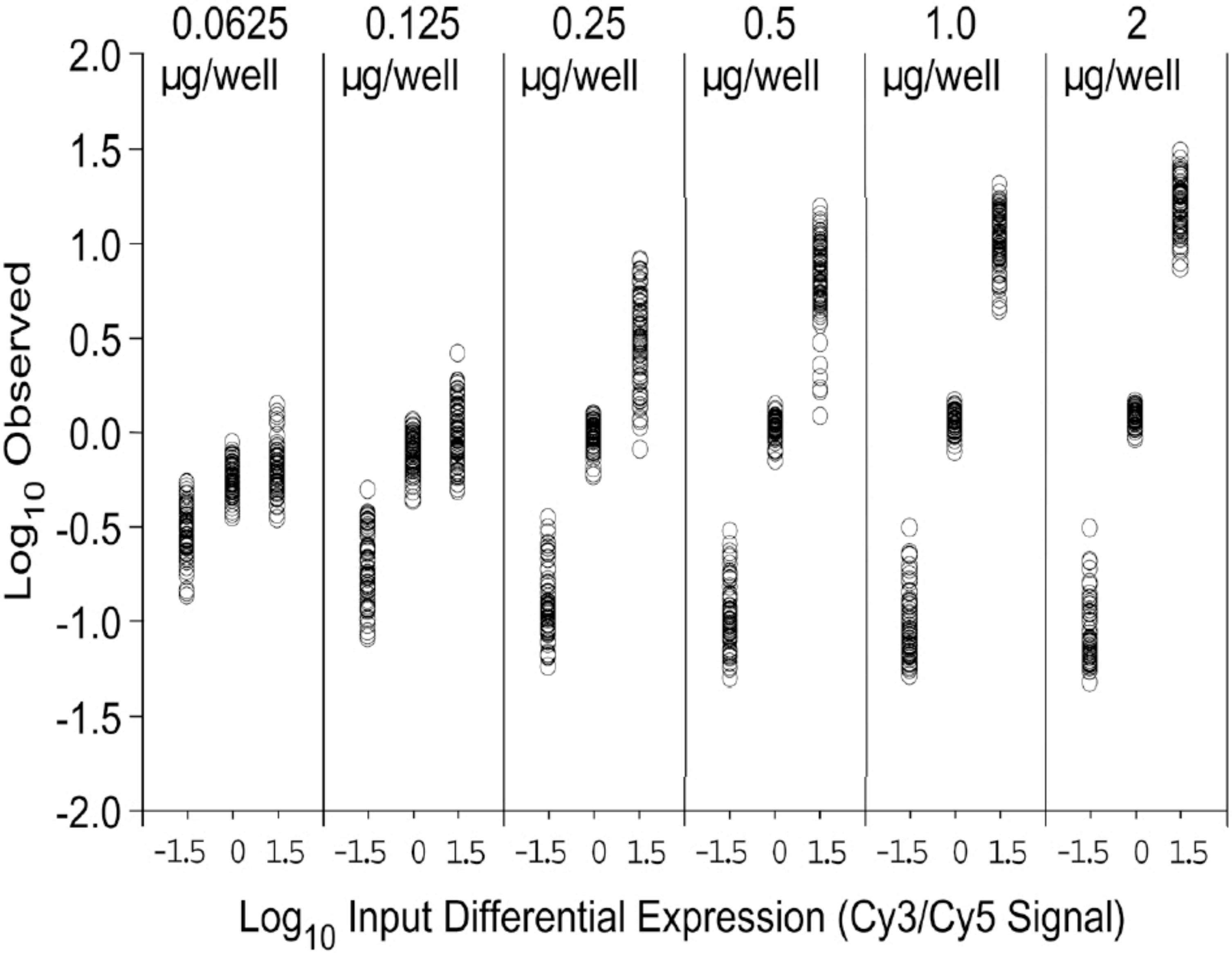
Background signal and non-linearities

“mild” non-linearity



Cope et al.
Bioinformatics
2003

▶ ratio compression



nominal 3:1

nominal 1:1

nominal 1:3

Yue et al., (Incyte Genomics) NAR (2001) 29 e41

Preprocessing Terminology

Calibration, normalisation: adjust for systematic drifts associated with dye, array (and sometimes position within array)

Background correction: adjust for the non-linearity at the lower end of the dynamic range

Transformation: bring data to a scale appropriate for the analysis (e.g. logarithm; variance stabilisation)

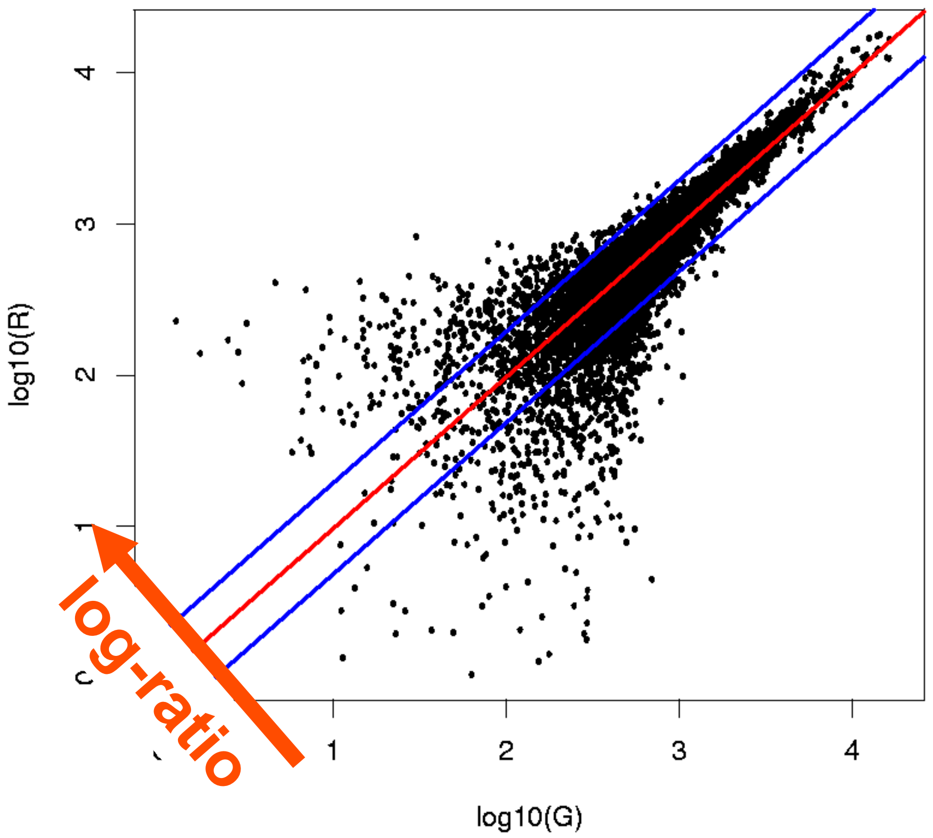
Log-ratio: adjust for unknown scale (units) of the data

Existing approaches differ in the order in which these steps are done, some are exactly stepwise („greedy“), others aim to gain strength by doing things simultaneously.

Statistical issues

▶ Which genes are differentially transcribed?

same-same



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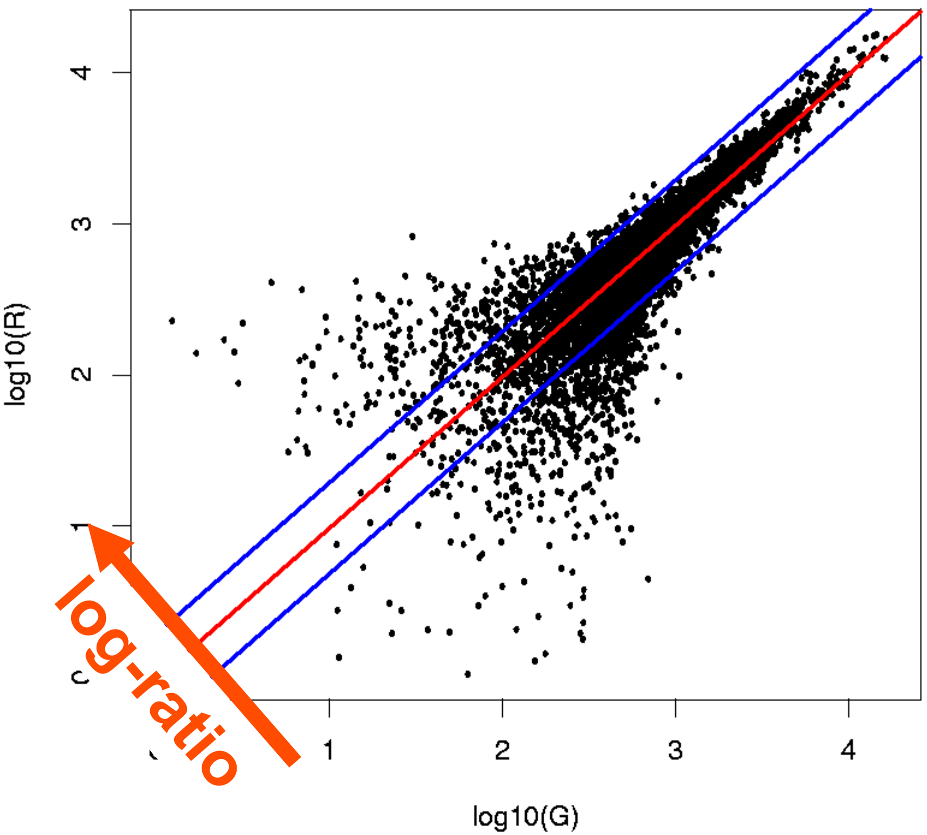
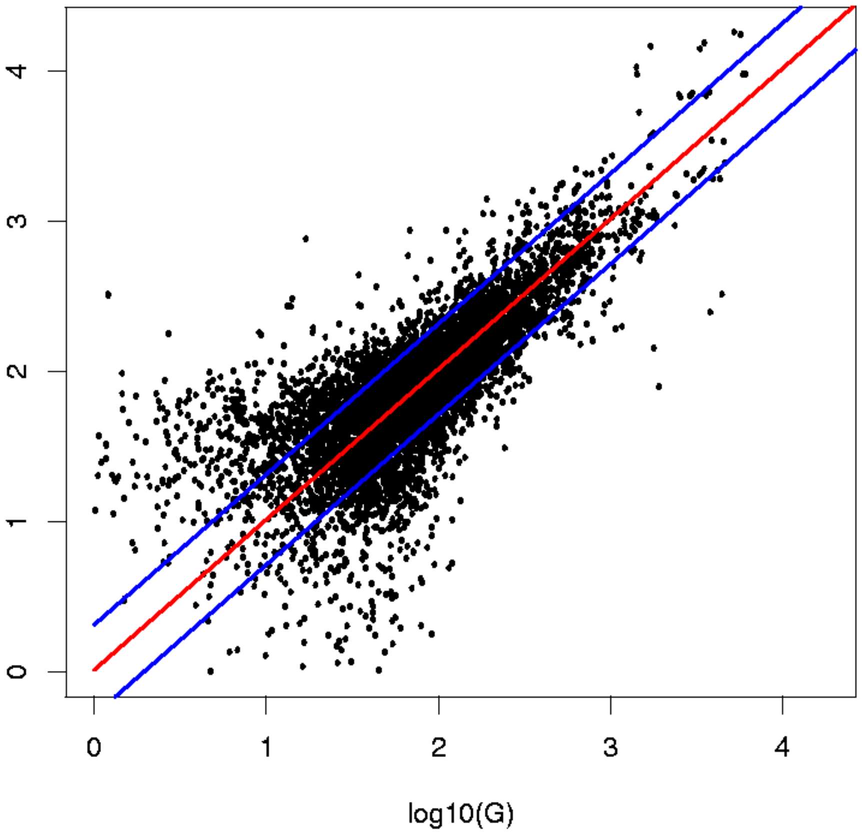


tumor-normal



▶ Sources of variation

amount of RNA in the biopsy
efficiencies of
-RNA extraction
-reverse transcription
-labeling
-fluorescent detection

probe purity and length
distribution
spotting efficiency, spot size
cross-/unspecific hybridization
stray signal

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Systematic

- similar effect on many measurements
- corrections can be estimated from data

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Calibration

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Stochastic

- too random to be explicitely accounted for
- remain as “noise”

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↓

Calibration

Stochastic

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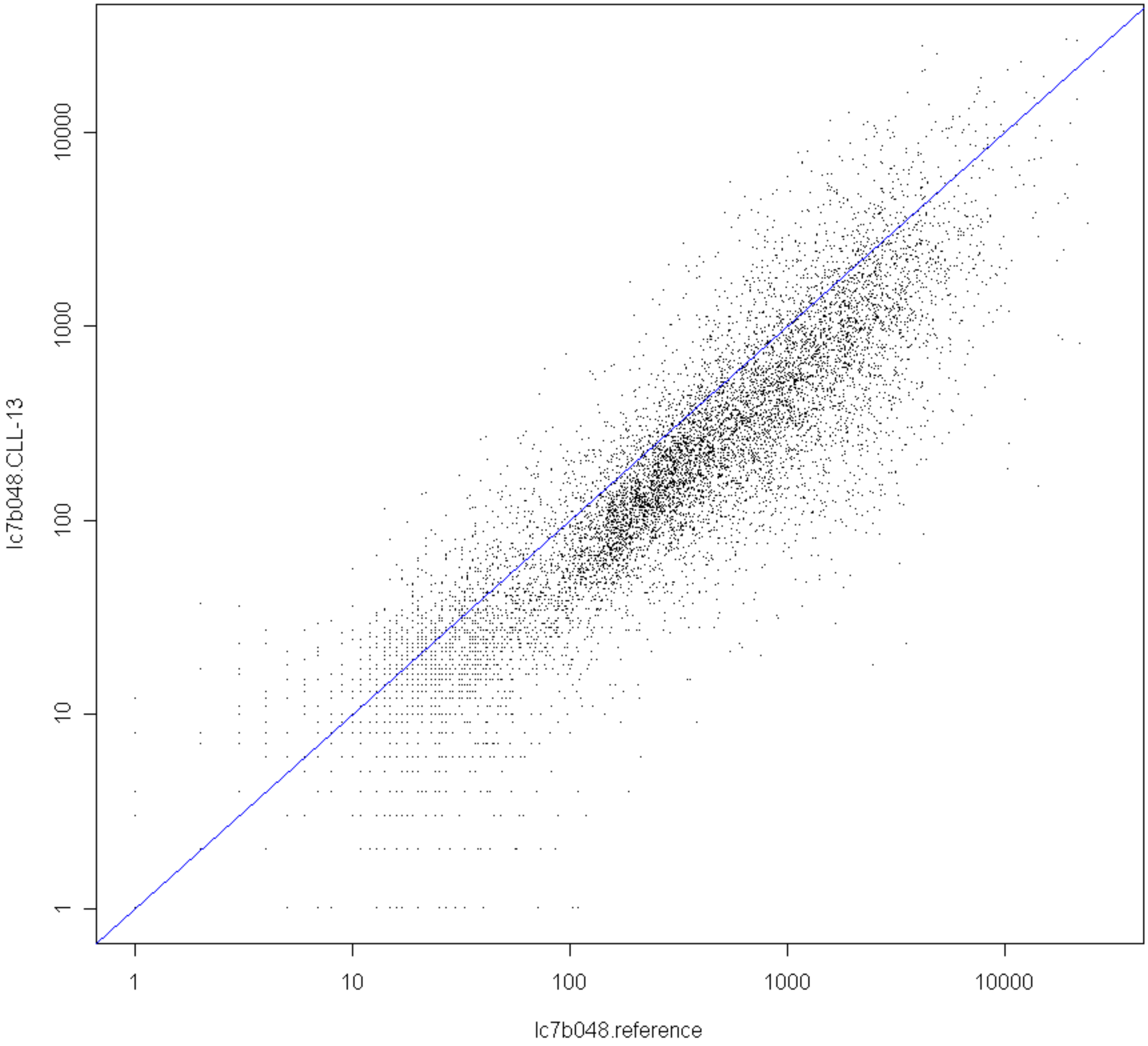
↓

Error model

Why do you need ‘normalisation’ (a.k.a. calibration)?

Systematic drift effects



From: lymphoma dataset

vsn package

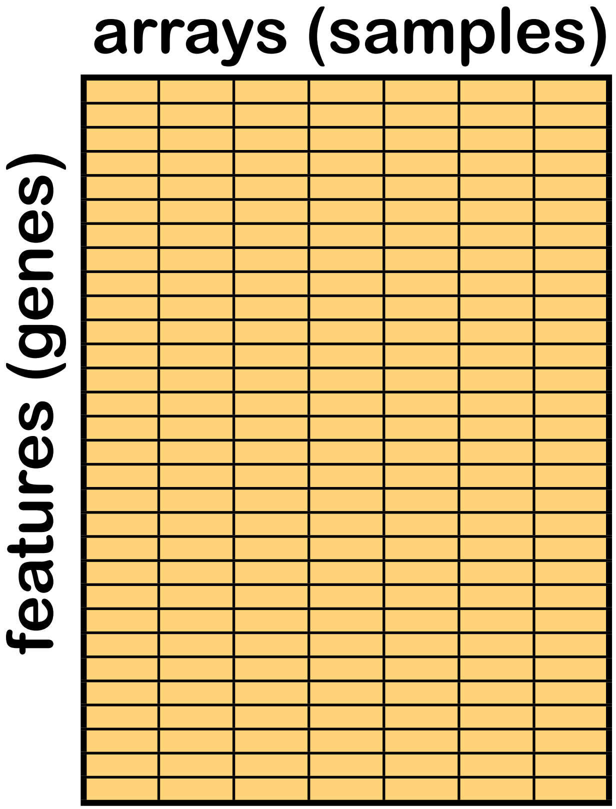
Alizadeh et al., Nature 2000

Quantile normalisation

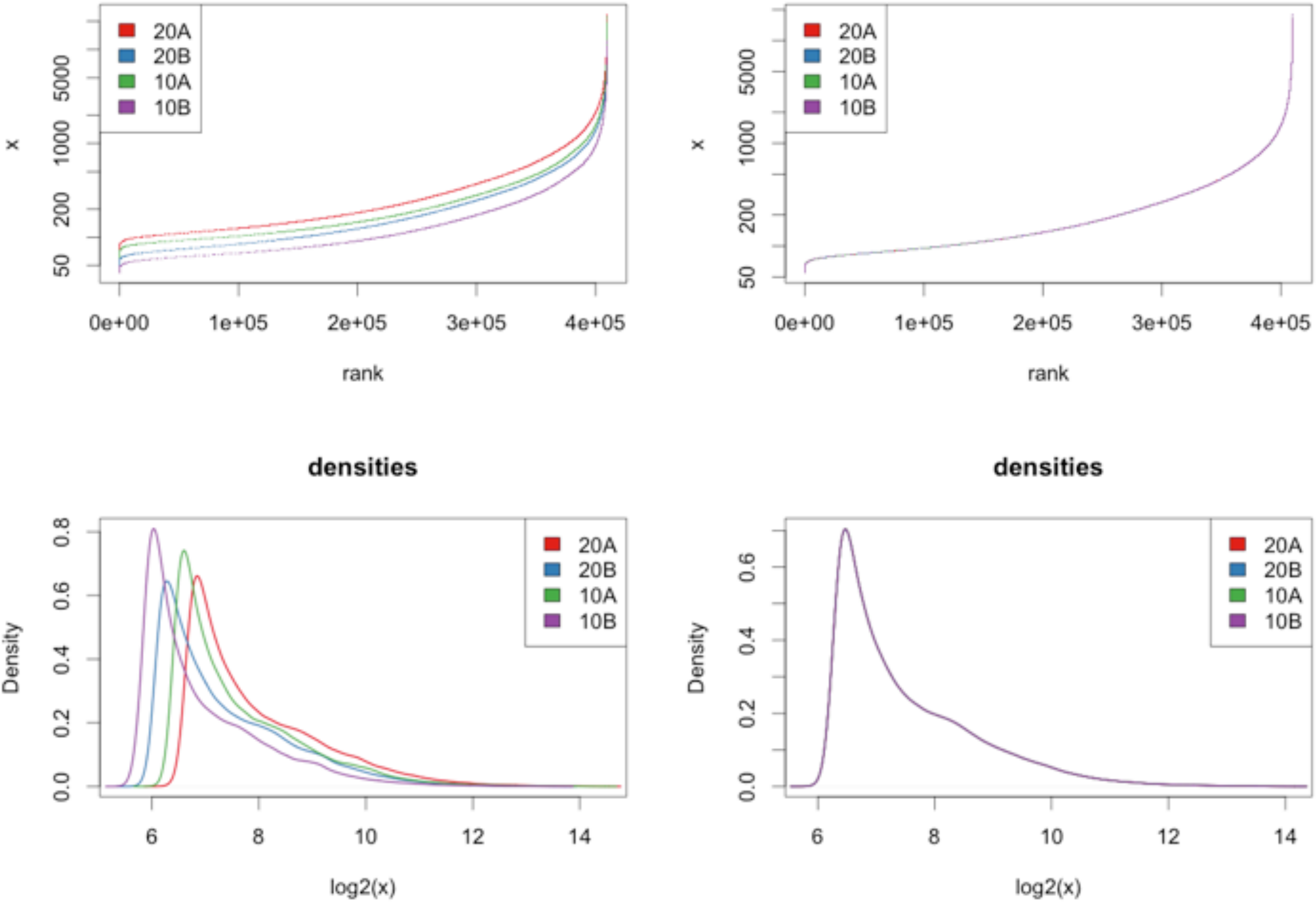
Within each column (array), replace the intensity values by their rank

For each rank, compute the average of the intensities with that rank, across columns (arrays)

Replace the ranks by those averages



Ben Bolstad 2001



20A
20B
10A
10B
x
50
200
1000
5000
0e+00
1e+05
2e+05
3e+05
4e+05
rank
20A
20B
10A
10B
x
50
200
1000
5000
0e+00
1e+05
2e+05
3e+05
4e+05
rank
densities
Density
0.0
0.2
0.4
0.6
0.8
20A
20B
10A
10B
6
8
10
12
14
log2(x)
densities
Density
0.0
0.2
0.4
0.6
20A
20B
10A
10B
6
8
10
12
14
log2(x)

Quantile normalisation

Quantile normalisation

- + Simple, fast, easy to implement

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+ **Simple, fast, easy to implement**
+ **Always works, needs no user interaction / tuning**

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- May be conservative: rank transformation looses information - may yield less power to detect differentially expressed genes

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+ Always works, needs no user interaction / tuning
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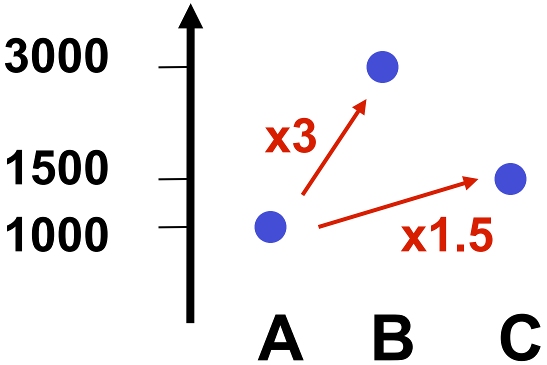
- Always "works", even if data are bad / inappropriate
- May be conservative: rank transformation looses information - may yield less power to detect differentially expressed genes
- Aggressive: if there is an excess of up- (or down) regulated genes, it removes not just technical, but also biological variation

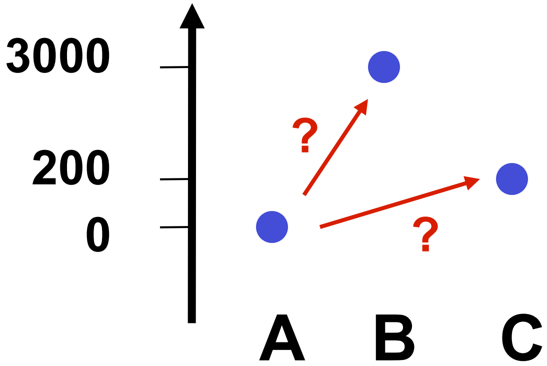
Estimating relative expression (fold-changes)

▶ ratios and fold changes

Fold changes are useful to describe continuous changes in expression



But what if the gene is “off” (below detection limit) in one condition?



ratios and fold changes

The idea of the log-ratio (base 2)

- 0: no change
- +1: up by factor of $2^1 = 2$
- +2: up by factor of $2^2 = 4$
- -1: down by factor of $2^{-1} = 1/2$
- -2: down by factor of 2^{-2} = ¼

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A unit for measuring changes in expression: assumes that a change from 1000 to 2000 units has a similar biological meaning to one from 5000 to 10000.

…. data reduction

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A unit for measuring changes in expression: assumes that a change from 1000 to 2000 units has a similar biological meaning to one from 5000 to 10000.

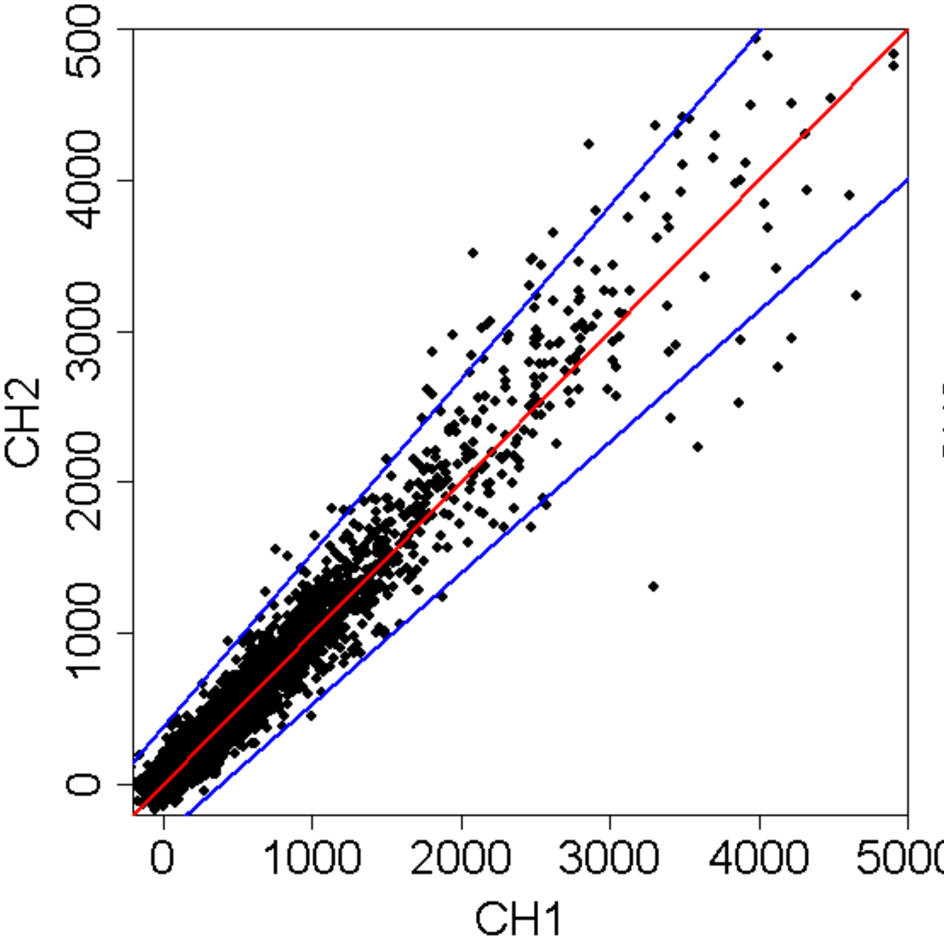
…. data reduction

What about a change from 0 to 500?

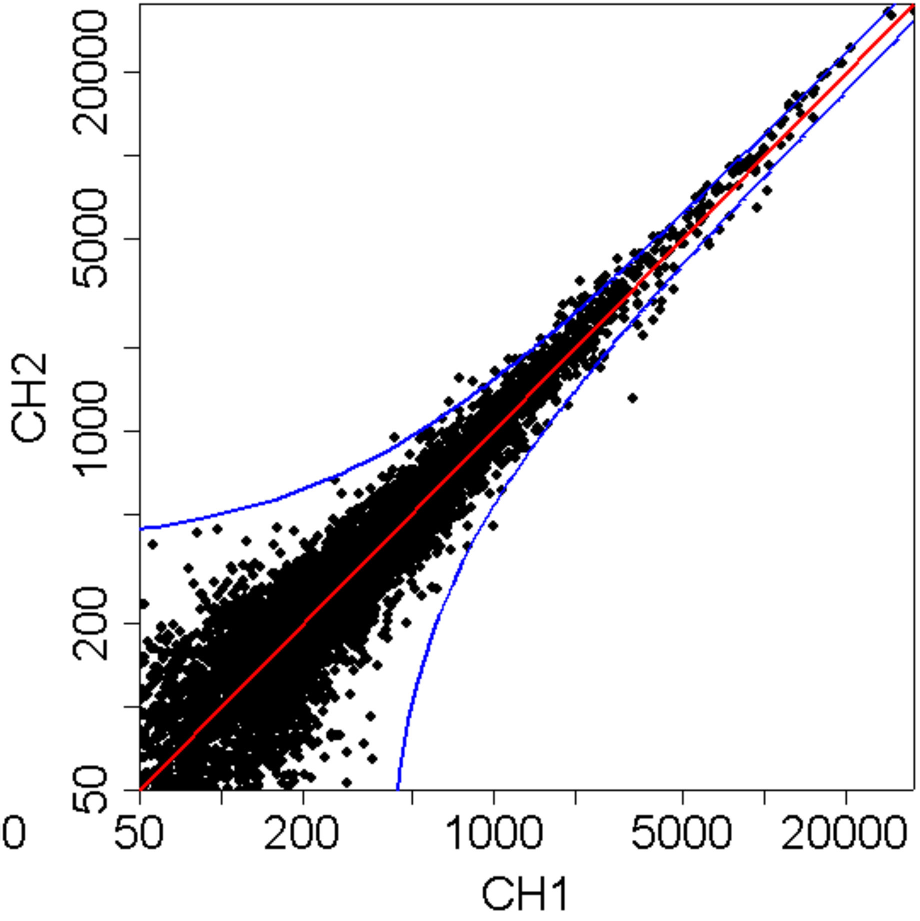
- conceptually
- noise, measurement precision

Two component error model and variance stabilisation

The two-component model



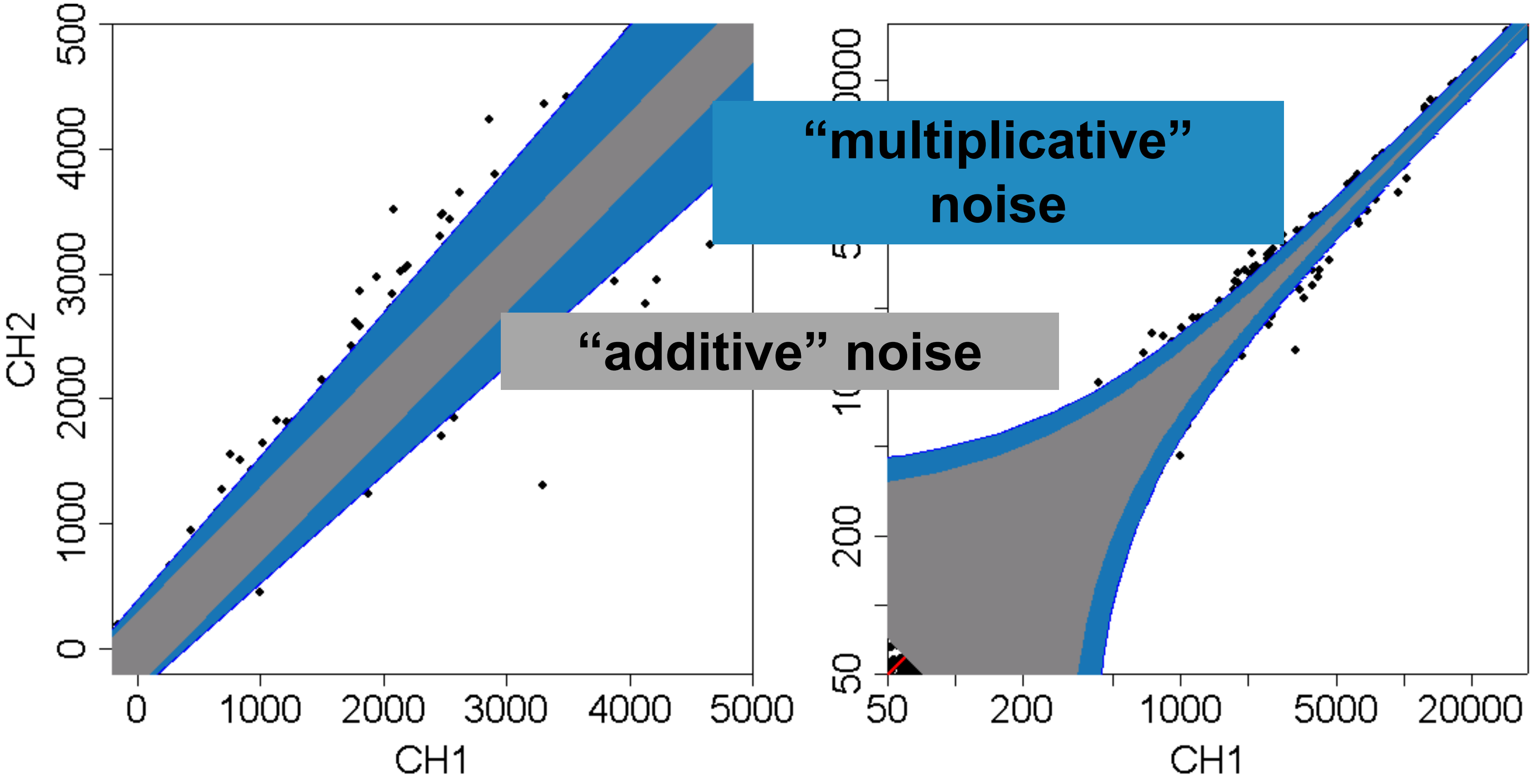
raw scale



log scale

B. Durbin, D. Rocke, JCB 2001

The two-component model



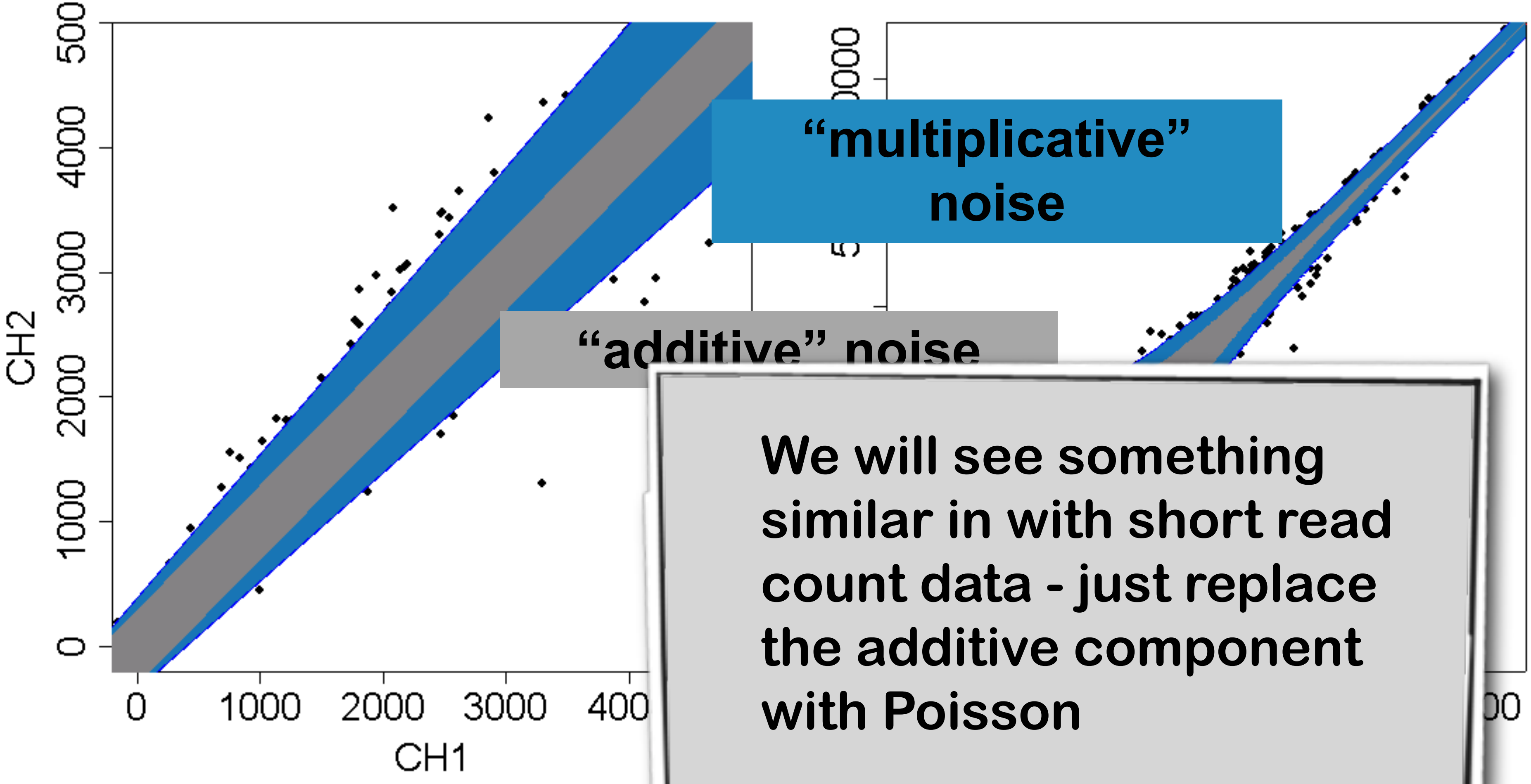
“multiplicative” noise

“additive” noise

raw scale **log scale**

B. Durbin, D. Rocke, JCB 2001

The two-component model



“multiplicative” noise

“additive” noise

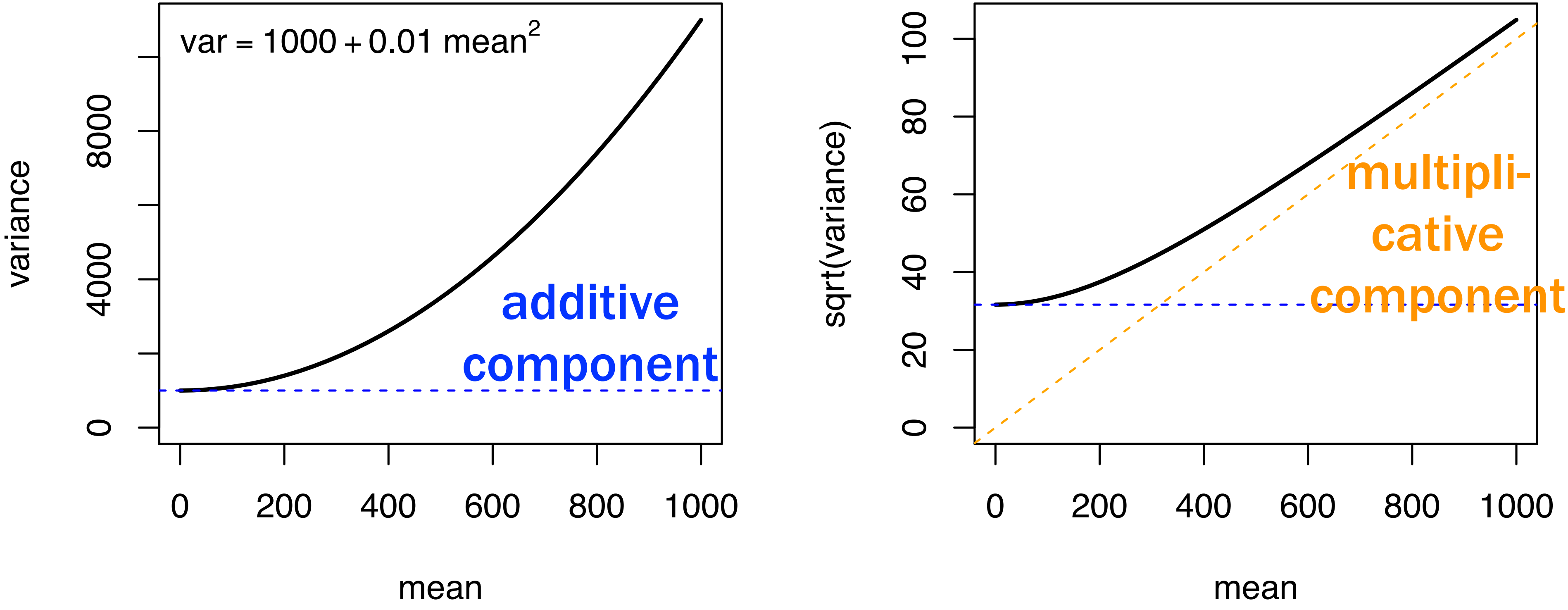
We will see something similar in with short read count data - just replace the additive component with Poisson

raw scale

log scale

B. Durbin, D. Rocke, JCB 2001

The additive-multiplicative error model



The additive-multiplicative error model

Trey Ideker et al.: JCB (2000)

David Rocke and Blythe Durbin: JCB (2001), Bioinformatics (2002)

Use for robust affine regression normalisation: W. Huber, Anja von Heydebreck et al. Bioinformatics (2002).

For background correction in RMA: R. Irizarry et al., Biostatistics (2003).

▶ 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**

ε_{ik} **additive noise**

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

b_i **per-sample gain factor**

b_k **sequence-wise probe efficiency**

η_{ik} **multiplicative noise**

▶ variance stabilizing transformations

X_u a family of random variables with

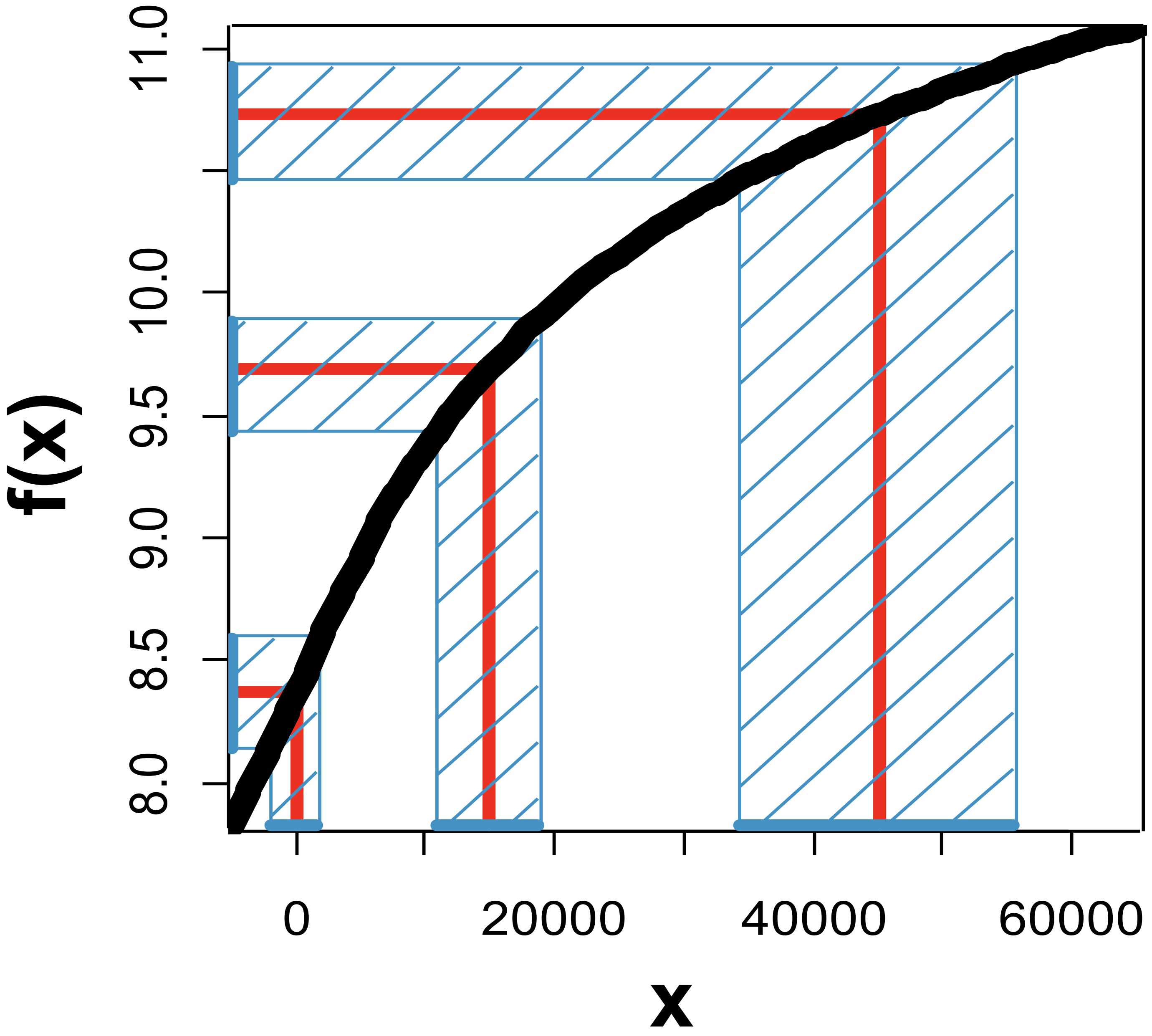
E(X_u) = u and Var(X_u) = $v(u)$. Define

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

Then, var $f(X_u) \approx$ does not depend on u

Derivation: linear approximation,
relies on smoothness of $v(u)$.

▶ variance stabilizing transformation



▶ variance stabilizing transformations

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

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

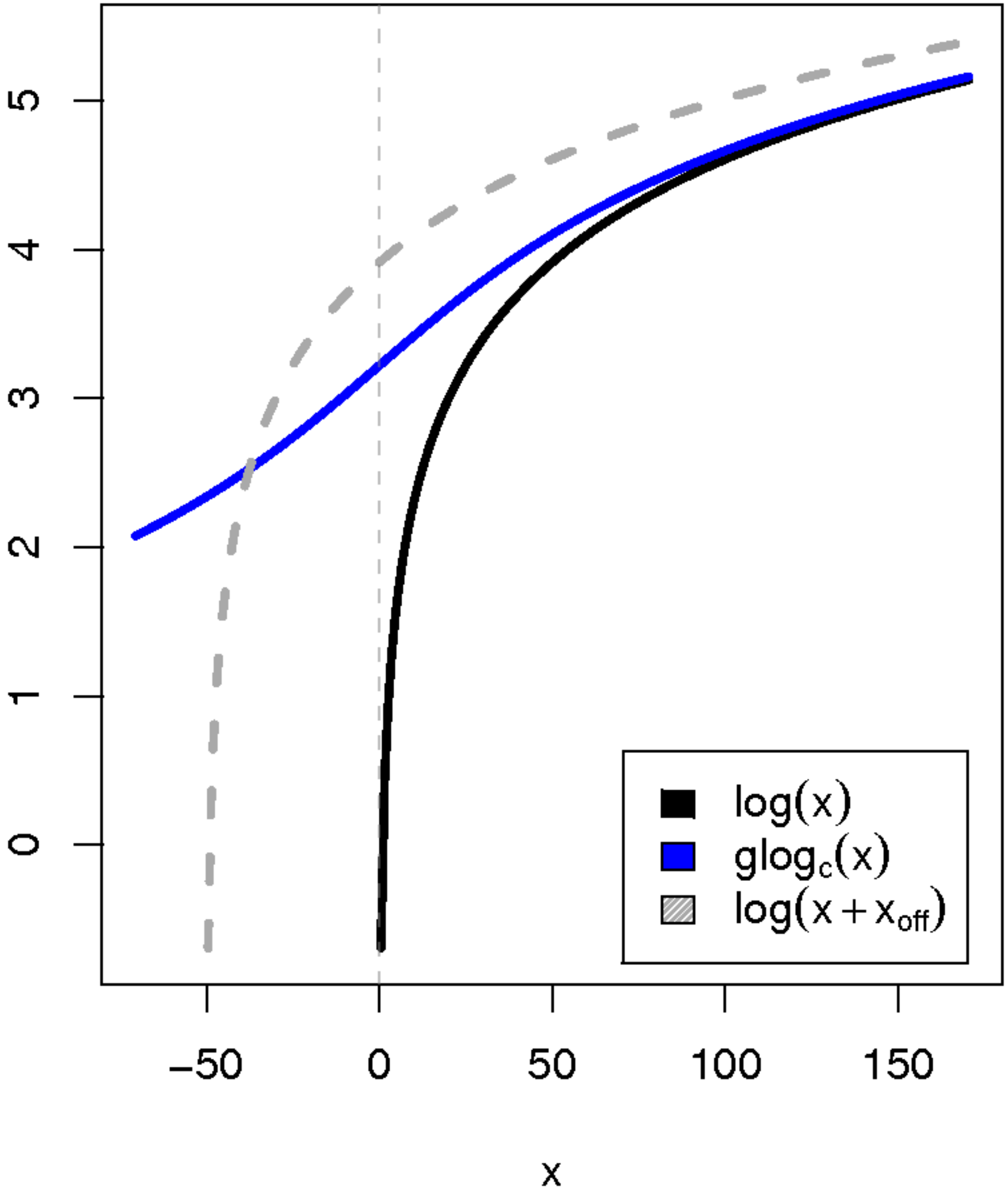
2.) constant CV (‘multiplicative’) $v(u) \propto u^2 \Rightarrow f \propto \log u$

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



$$\mathrm{glog}_2(x,c) = \log_2\left(\frac{x+\sqrt{x^2+c^2}}{2}\right)$$

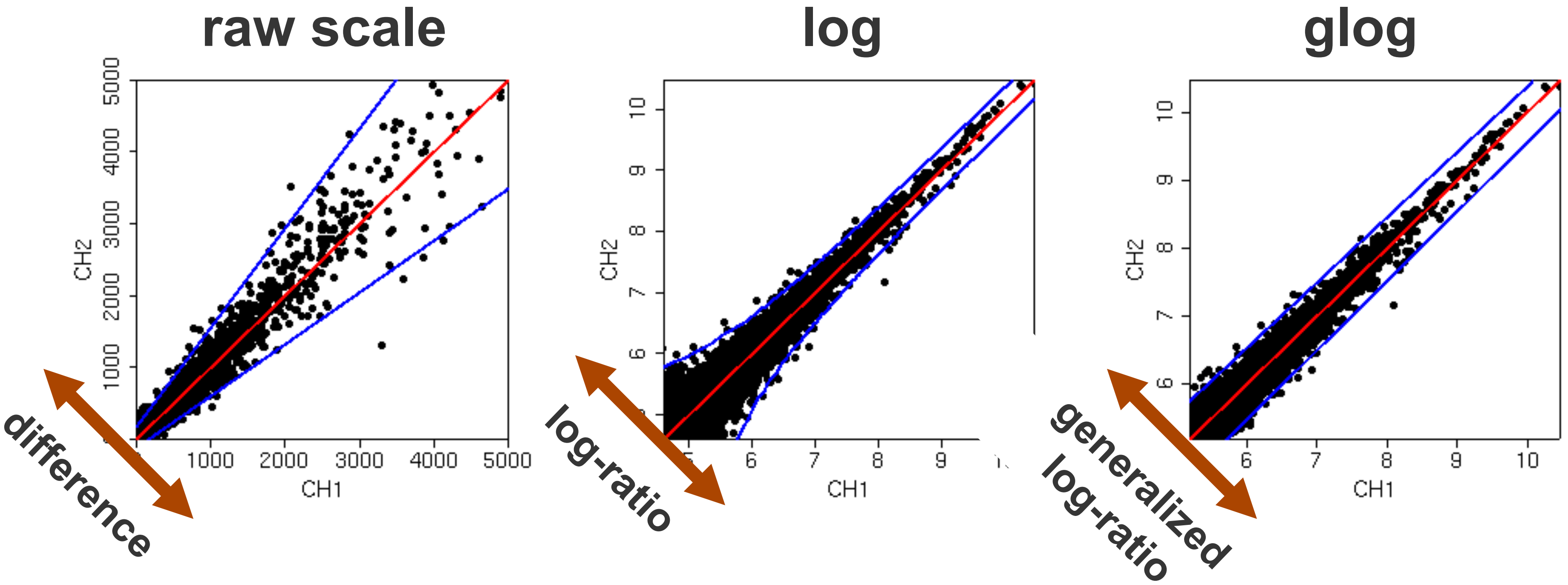
$$\mathrm{glog}_e(x,1) + \log_e 2 = \operatorname{arsinh}(x)$$

P. Munson, 2001

D. Rocke & B. Durbin, ISMB 2002

W. Huber et al., ISMB 2002

glog

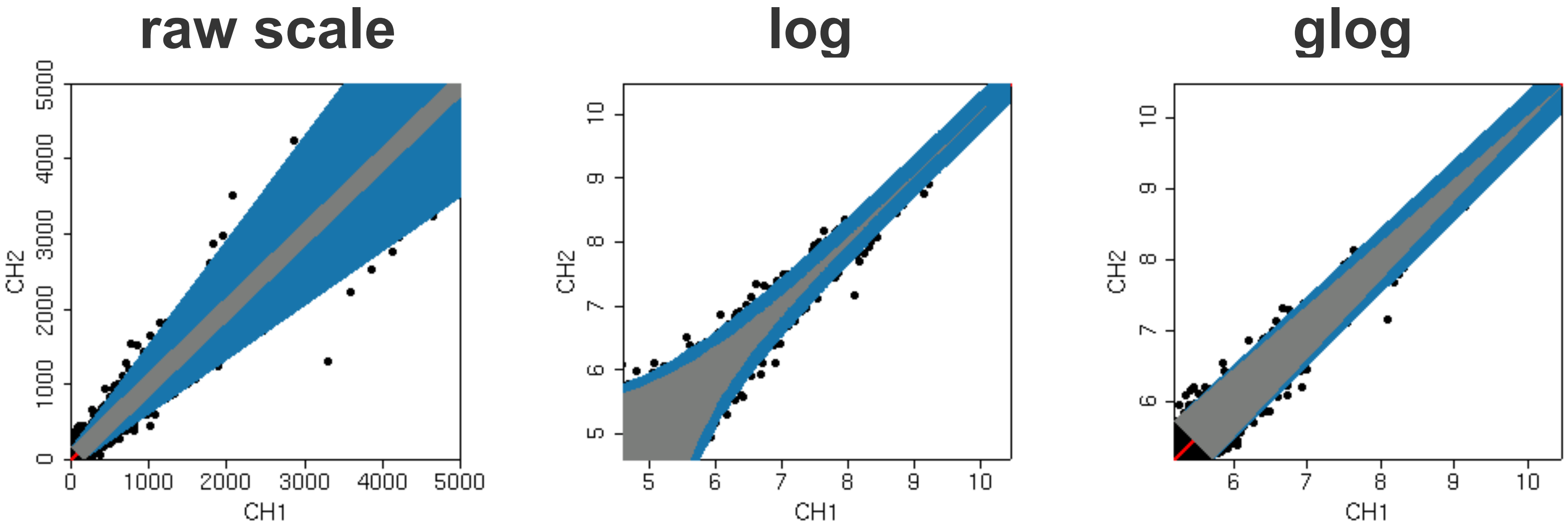


raw scale

log

glog

▶ glog



variance:

▨ **constant part**

🟦 **proportional part**

Parameter estimation

$$\boxed{\operatorname{arsinh}\frac{y_{ki} - a_i}{b_i} = \mu_k + \varepsilon_{ki}, \qquad \varepsilon_{ki} : N(0, c^2)}$$

Parameter estimation

$$\operatorname{arsinh}\frac{y_{ki} - a_i}{b_i} = \mu_k + \varepsilon_{ki}, \qquad \varepsilon_{ki} : N(0, c^2)$$

measured intensity = offset + gain * true abundance

$$y_{ik} = a_{ik} + b_{ik}\, x_{ik}$$

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

a_i per-sample offset

L_{ik} local background provided by image analysis

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

"additive noise"

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

b_i per-sample normalization factor

b_k sequence-wise labeling efficiency

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

"multiplicative noise"

Parameter estimation

$$\operatorname{arsinh}\frac{y_{ki} - a_i}{b_i} = \mu_k + \varepsilon_{ki}, \qquad \varepsilon_{ki} : N(0, c^2)$$

Parameter estimation

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- **maximum likelihood estimator: straightforward – but sensitive to deviations from normality**

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- **model holds for genes that are unchanged; differentially transcribed genes act as outliers.**

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- robust variant of ML estimator, à la Least Trimmed Sum of Squares regression.

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- **maximum likelihood estimator: straightforward – but sensitive to deviations from normality**

- **model holds for genes that are unchanged; differentially transcribed genes act as outliers.**

- **robust variant of ML estimator, à la Least Trimmed Sum of Squares regression.**

- **works well as long as <50% of genes are differentially transcribed (and may still work otherwise)** “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 and shrinkage estimators

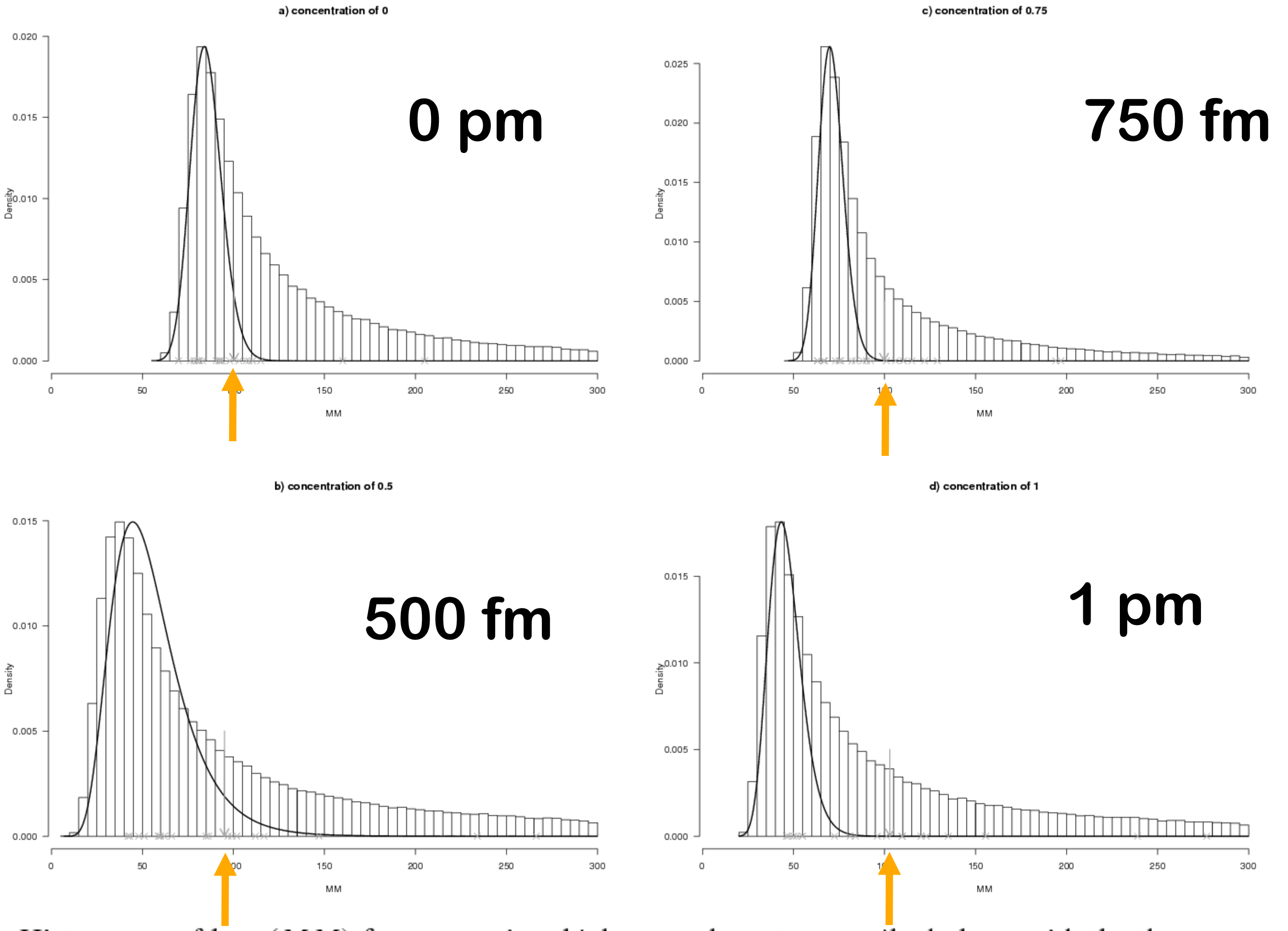
Shrinkage estimators:
a general technology in statistics:
pay a small price in bias for a large decrease of variance, so overall the mean-squared-error (MSE) is reduced.

Particularly useful if you have few replicates.

Generalized log-ratio is a shrinkage estimator for log fold change

other background correction methods

Background correction



0 pm

750 fm

500 fm

1 pm

Fig. 5. Histograms of $\log_2(MM)$ for a array in which no probe-set was spiked along with the three arrays in which BioB-5 was spiked-in at concentrations of 0.5, 0.75, and 1 pM. The observed PM values for the 20 probes associated with BioB-5 are marked with crosses and the average with an arrow. The black curve represents the log normal distribution obtained from left-of-the-mode data.

Irizarry et al.
Biostatistics
2003

RMA Background correction

$PM = B + S$

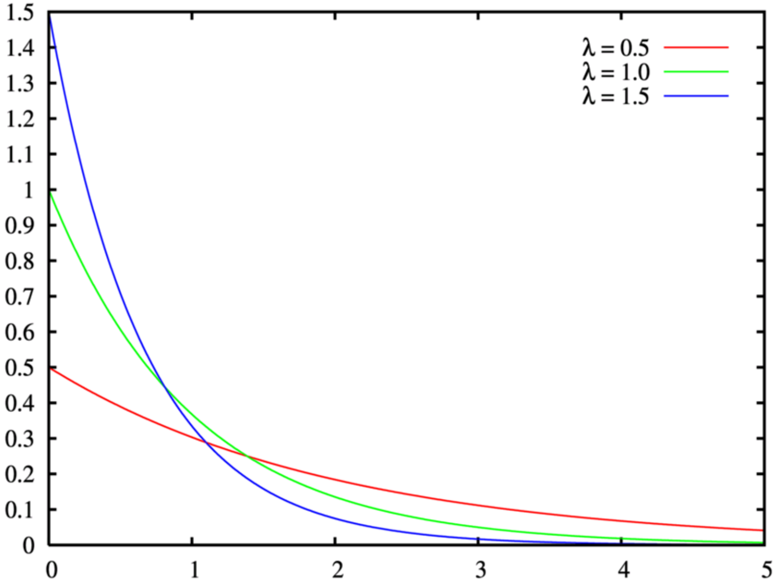
$B \sim$ log-normal with mean and sd read off MM values

$S \sim$ exponential

$\Rightarrow$ closed form expression for $E[S \mid PM]$,

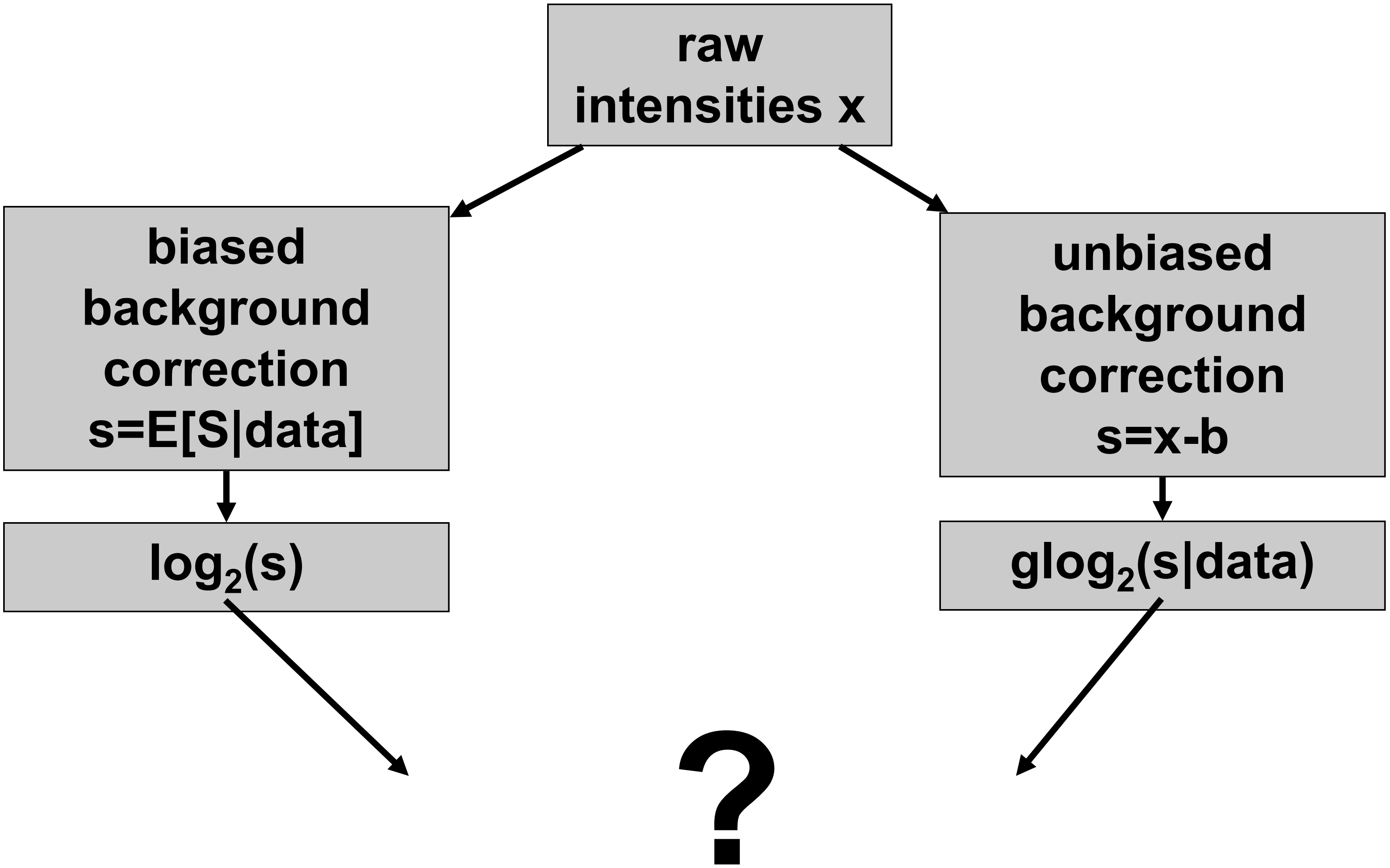
use this as $\hat{s} \quad (> 0)$.

(NB, $P[S > 0] = 1$ is not realistic)



Irizarry et al. (2002)

Background correction:



raw intensities x

biased background correction $s=E[S|data]$

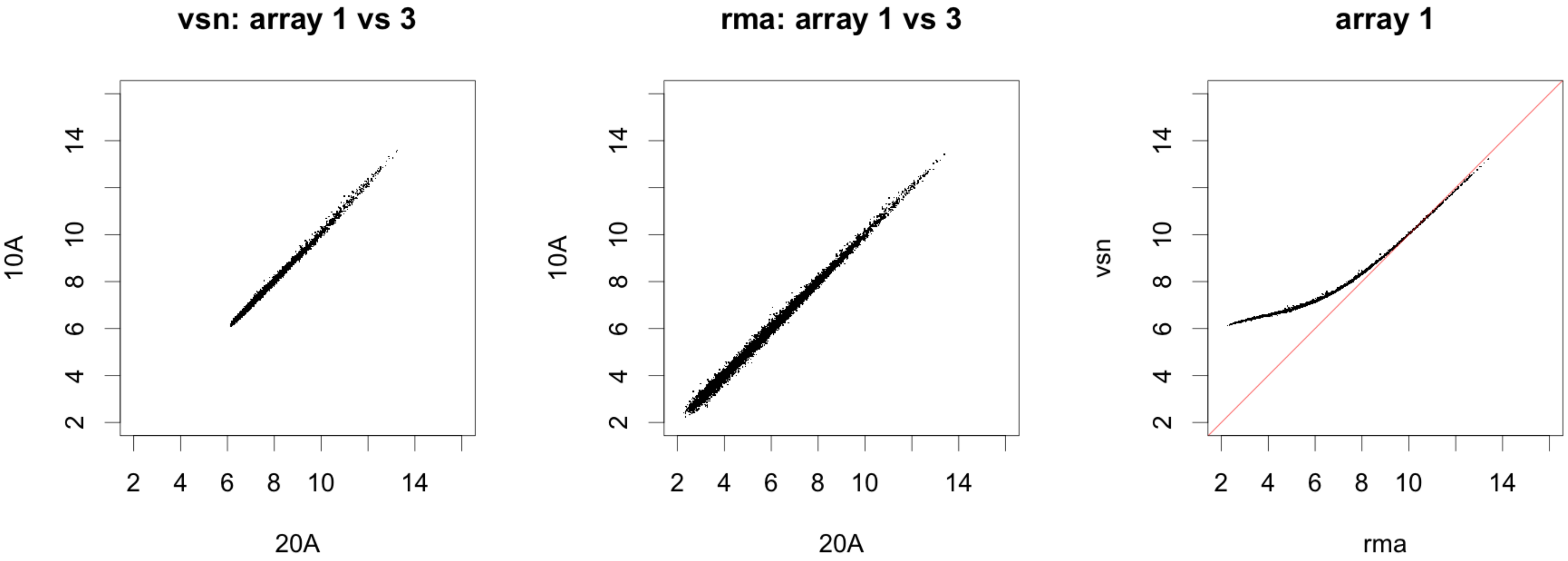
$\log_2(s)$

unbiased background correction $s=x-b$

$\mathrm{glog}_2(s|data)$

?

Comparison between RMA and VSN background correction



vsn package vignette

Summaries for Affymetrix genechip probe sets

Data and notation

PM_{ikg} , MM_{ikg} = Intensities for perfect match and mismatch probe *k* for gene *g* on chip *i*

$i = 1,\ldots, n$	one to hundreds of chips
$k = 1,\ldots, J$	usually 11 probe pairs
$g = 1,\ldots, G$	tens of thousands of probe sets.

Tasks:

calibrate (normalize) the measurements from different chips (samples)

summarize for each probe set the probe level data, i.e., 11 PM and MM pairs, into a single expression measure.

compare between chips (samples) for detecting differential expression.

Expression measures: MAS 4.0

Affymetrix GeneChip MAS 4.0 software used AvDiff, a trimmed mean:

$$AvDiff = \frac{1}{\# K} \sum_{k \in K} (PM_k - MM_k)$$

- **sort $d_k = PM_k - MM_k$**
- **exclude highest and lowest value**
- **K := those pairs within 3 standard deviations of the average**

Expression measures
MAS 5.0

Instead of MM, use "repaired" version CT

CT = MM if *MM<PM*

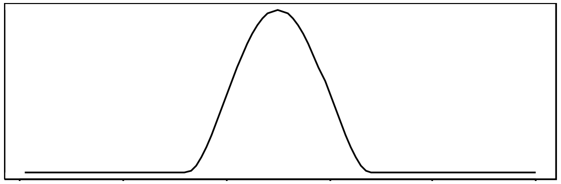
= PM / "typical log-ratio" if *MM>=PM*

Signal = Weighted mean of the values log(PM-CT)

weights follow Tukey Biweight function

(location = data median,

scale a fixed multiple of MAD)



Expression measures: Li & Wong

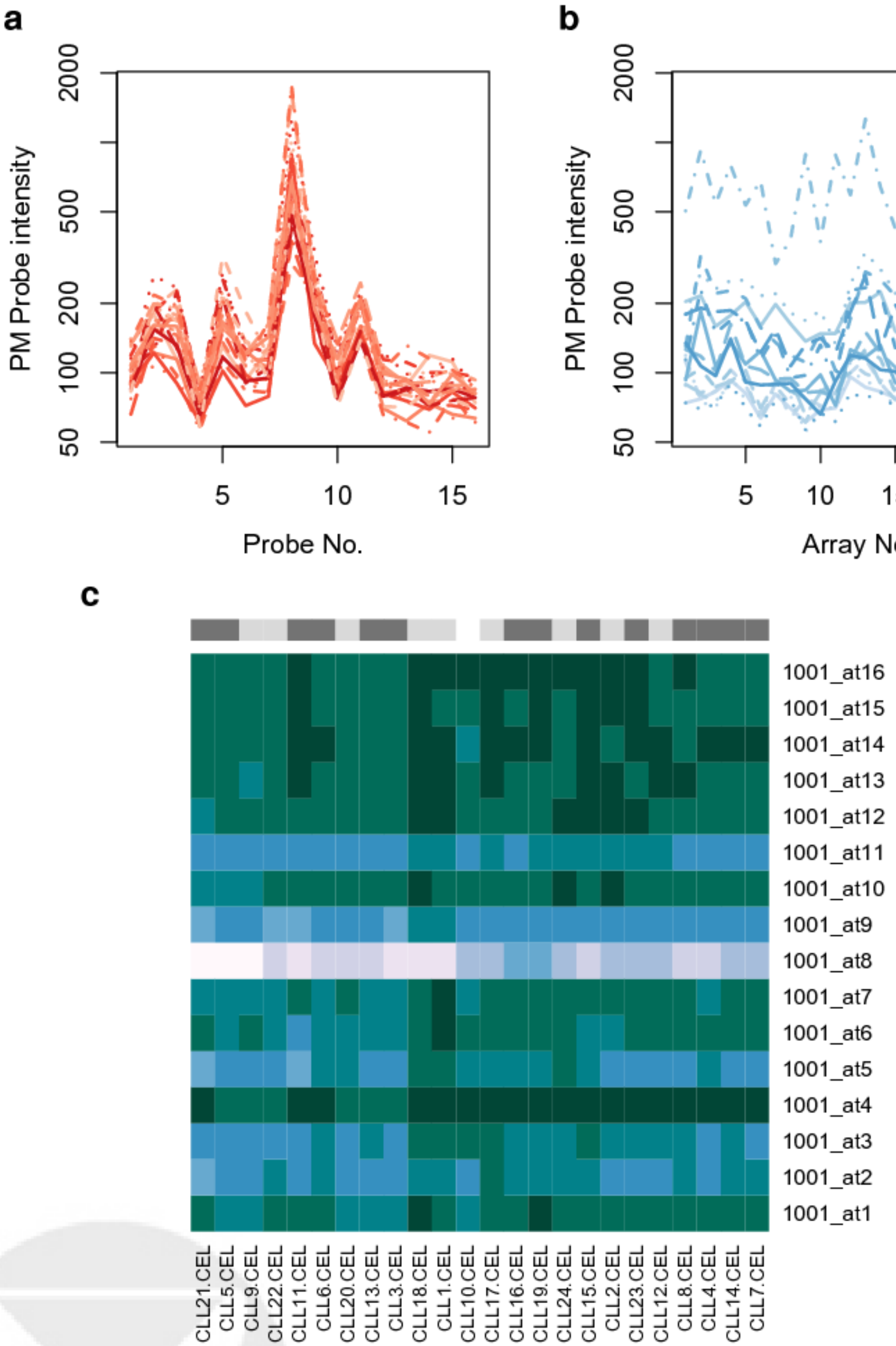
dChip **fits a model for each gene**

$$PM_{ki} - MM_{ki} = \theta_k \phi_i + \varepsilon_{ki}, \qquad \varepsilon_{ki} \propto N(0,\sigma^2)$$

where

- ϕ_i : **expression measure** **for the gene in sample *i***
- θ_k : **probe effect**

ϕ_i **is estimated by maximum likelihood**


a
PM Probe intensity
2000
500
200
100
50
5
10
15
Probe No.
b
PM Probe intensity
2000
500
200
100
50
5
10
15
20
Array No.
c
1001_at16
1001_at15
1001_at14
1001_at13
1001_at12
1001_at11
1001_at10
1001_at9
1001_at8
1001_at7
1001_at6
1001_at5
1001_at4
1001_at3
1001_at2
1001_at1
CLL21.CEL
CLL5.CEL
CLL9.CEL
CLL22.CEL
CLL11.CEL
CLL6.CEL
CLL20.CEL
CLL13.CEL
CLL3.CEL
CLL18.CEL
CLL1.CEL
CLL10.CEL
CLL17.CEL
CLL16.CEL
CLL19.CEL
CLL24.CEL
CLL15.CEL
CLL2.CEL
CLL23.CEL
CLL12.CEL
CLL8.CEL
CLL4.CEL
CLL14.CEL
CLL7.CEL

Expression measures
RMA: Irizarry et al. (2002)

dChip

$$Y_{ki} = \theta_k \phi_i + \varepsilon_{ki}, \qquad \varepsilon_{ki} \propto N(0, \sigma^2)$$

RMA

$$\log_2 Y_{ki} = a_k + b_i + \varepsilon_{ki}$$

***b*$_i$ is estimated using the robust method median polish (successively remove row and column medians, accumulate terms, until convergence).**

Quality assessment

Quality assessment

arrayQualityMetrics
example quality report

▶ References

Bioinformatics and computational biology solutions using R and Bioconductor, R. Gentleman, V. Carey, W. Huber, R. Irizarry, S. Dudoit, Springer (2005).

Variance stabilization applied to microarray data calibration and to the quantification of differential expression. W. Huber, A. von Heydebreck, H. Sültmann, A. Poustka, M. Vingron. Bioinformatics 18 suppl. 1 (2002), S96-S104.

Exploration, Normalization, and Summaries of High Density Oligonucleotide Array Probe Level Data. R. Irizarry, B. Hobbs, F. Collins, …, T. Speed. Biostatistics 4 (2003) 249-264.

Error models for microarray intensities. W. Huber, A. von Heydebreck, and M. Vingron. Encyclopedia of Genomics, Proteomics and Bioinformatics. John Wiley & sons (2005).

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A Benchmark for Affymetrix GeneChip Expression Measures. L.M. Cope, R.A. Irizarry, H. A. Jaffee, Z. Wu, T.P. Speed. Bioinformatics (2003).

….many, many more…

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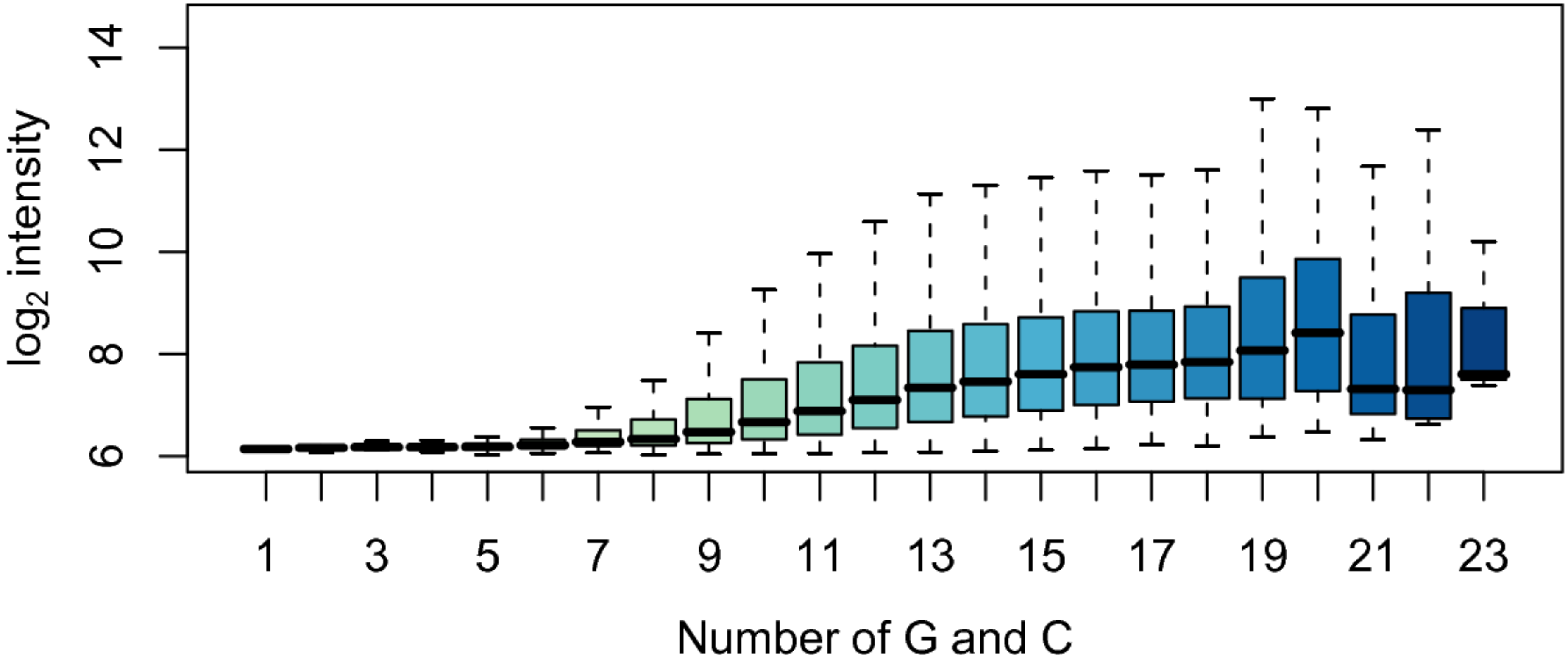

log$_2$ intensity
6
8
10
12
14
1
3
5
7
9
11
13
15
17
19
21
23
Number of G and C

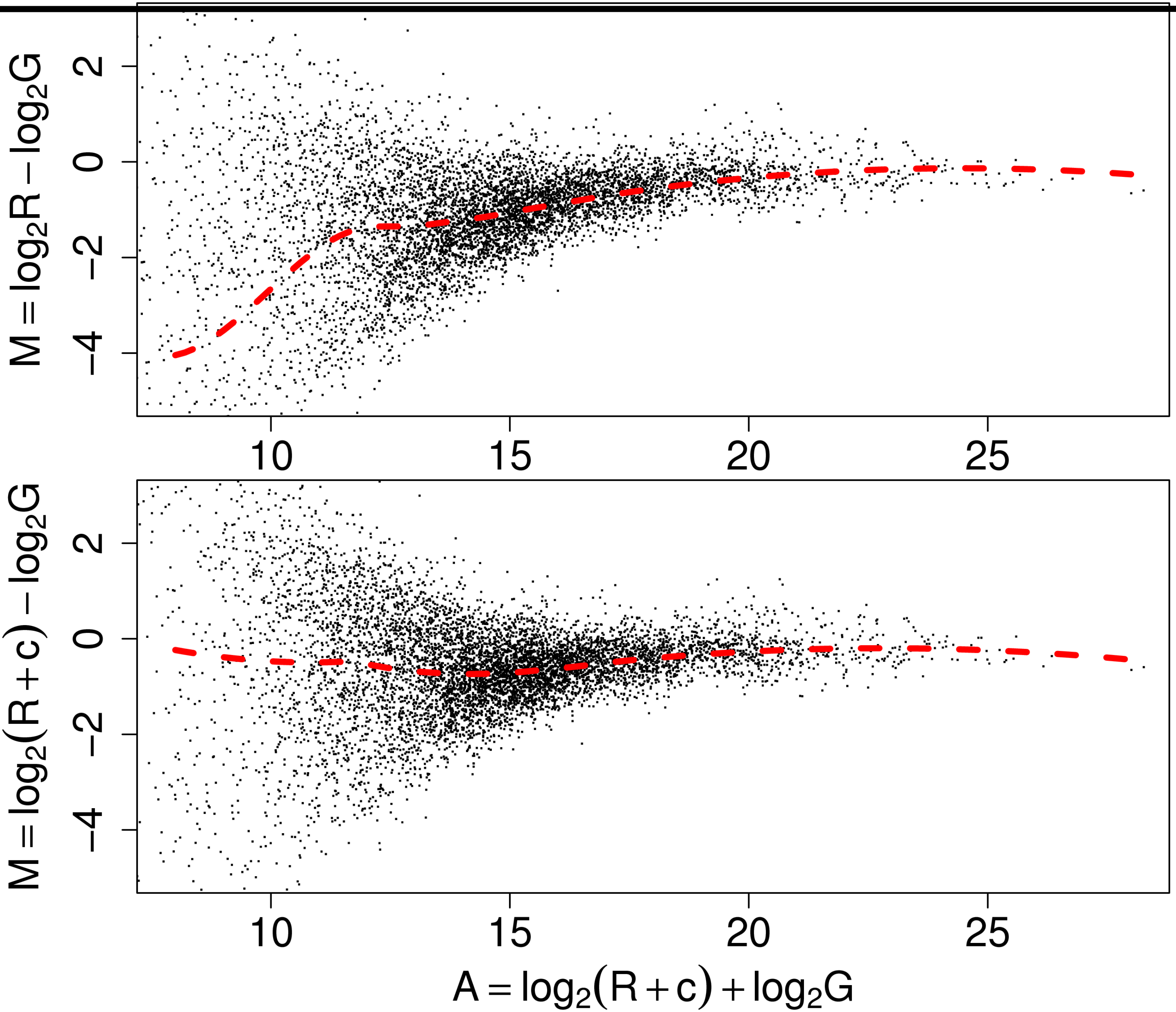
▶ What about non-linear effects

- Microarrays can be operated in a linear regime, where fluorescence intensity increases proportionally to target abundance (see e.g. Affymetrix dilution series)

Two reasons for non-linearity:

- At the high intensity end: saturation/quenching. This can (and should) be avoided experimentally - loss of data!

- At the low intensity end: background offsets, instead of $y=k\cdot x$ we have $y=k\cdot x+x_0$, and in the log-log plot this can look curvilinear. But this is an affine-linear effect and can be correct by affine normalization. Local polynomial regression may be OK, but tends to be less efficient.


$M = \log_2 R - \log_2 G$
2
0
−2
−4
10
15
20
25
$M = \log_2(R + c) - \log_2 G$
2
0
−2
−4
10
15
20
25
$A = \log_2(R + c) + \log_2 G$