First analysis steps

- quality control and optimization
- calibration and error modeling
- data transformations

Wolfgang Huber

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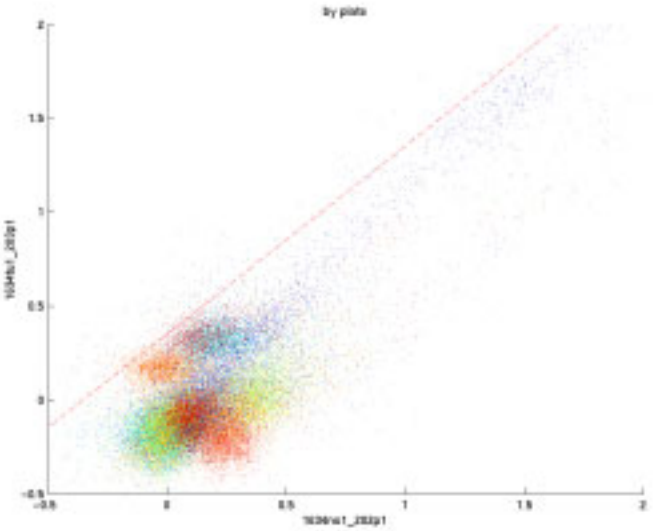


Acknowledgements

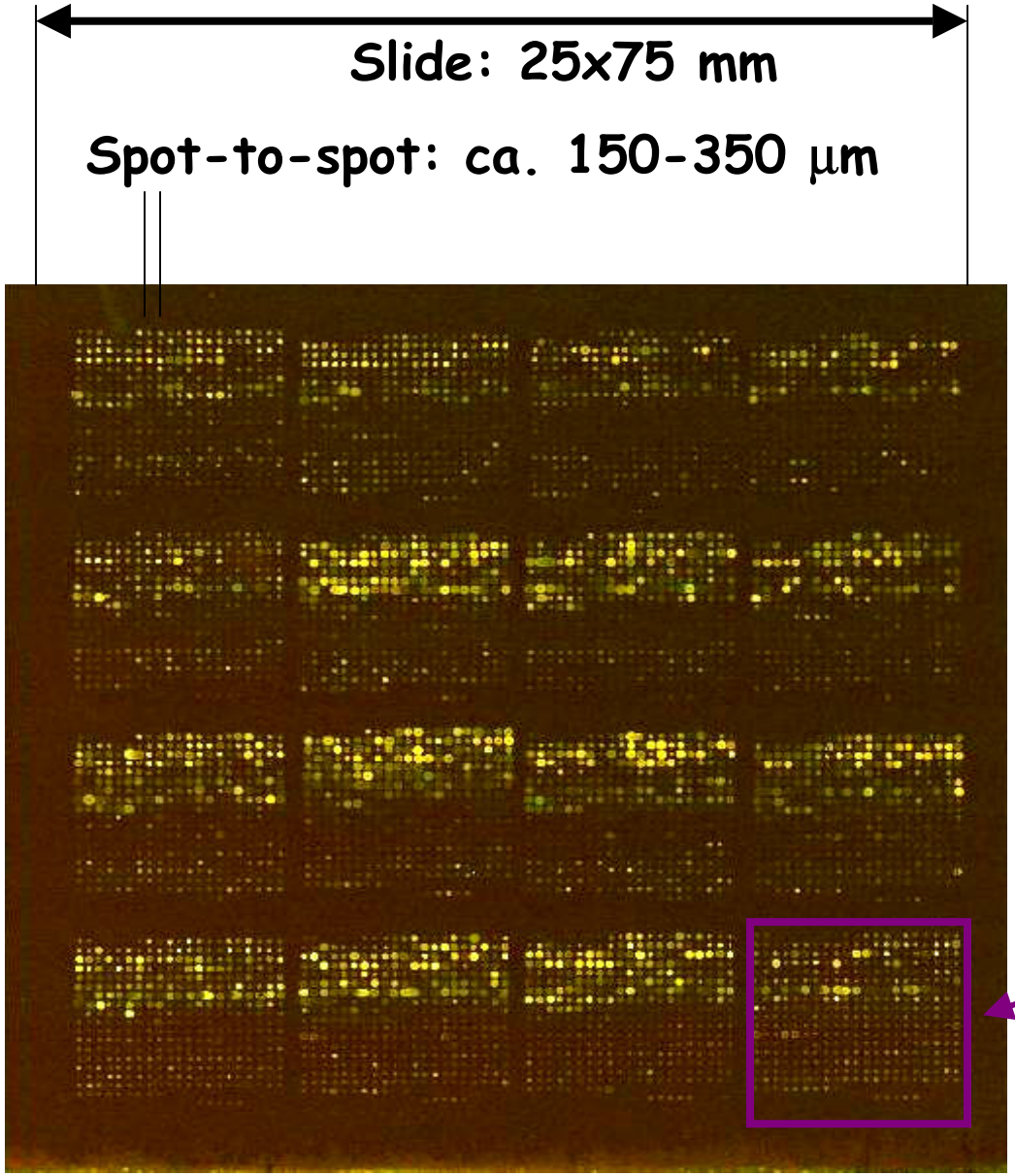
Anja von Heydebreck
Günther Sawitzki

Holger Sültmann, Klaus Steiner, Markus Vogt, Jörg Schneider, Frank Bergmann, Florian Haller, Katharina Finis, Stephanie Süß, Anke Schroth, Friederike Wilmer, Judith Boer, Martin Vingron, Annemarie Poustka

Sandrine Dudoit, Robert Gentleman, Rafael Irizarry and Yee Hwa Yang: Bioconductor short course, summer 2002

and many others

a microarray slide



4 x 4 or 8x4 sectors

17...38 rows and columns per sector

ca. 4600...46000 probes/array

sector: corresponds to one print-tip

Terminology

sample: RNA (cDNA) hybridized to the array, aka target, mobile substrate.

probe: DNA spotted on the array, aka spot, immobile substrate.

sector: rectangular matrix of spots printed using the same print-tip (or pin), aka print-tip-group

plate: set of 384 (768) spots printed with DNA from the same microtitre plate of clones

slide, array

channel: data from one color (Cy3 = cyanine 3 = green, Cy5 = cyanine 5 = red).

batch: collection of microarrays with the same probe layout.

Raw data

scanner signal

resolution:

5 or 10 mm spatial,

16 bit (65536) dynamical per channel

ca. 30-50 pixels per probe (60 μm spot size)

40 MB per array

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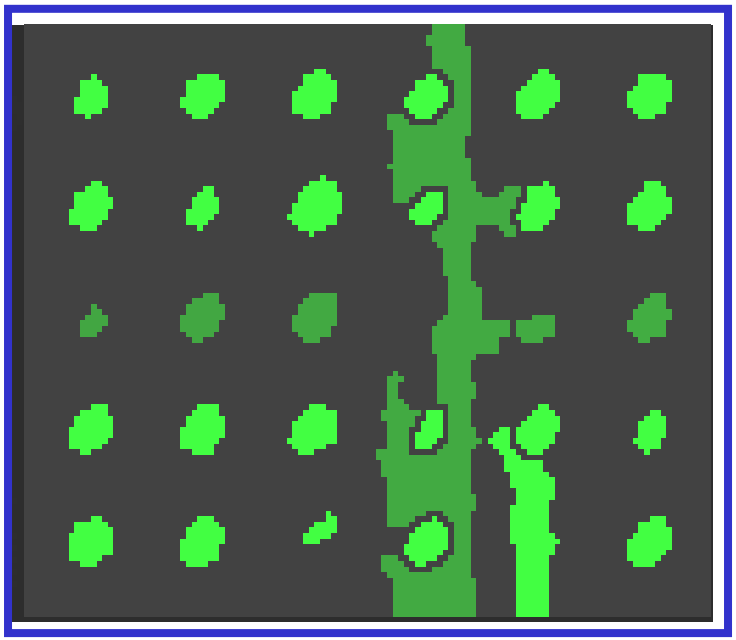
spot intensities

2 numbers per probe (~100-300 kB)

… auxiliaries: background, area, std dev, …

Image analysis

1. Addressing. Estimate location of spot centers.



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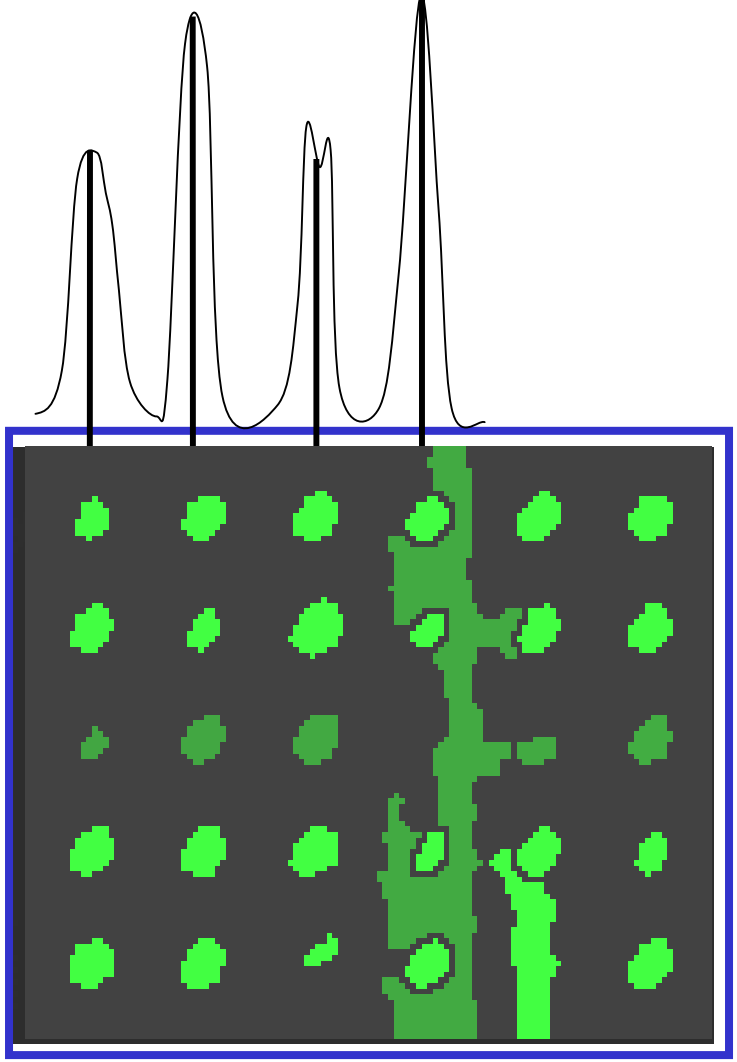

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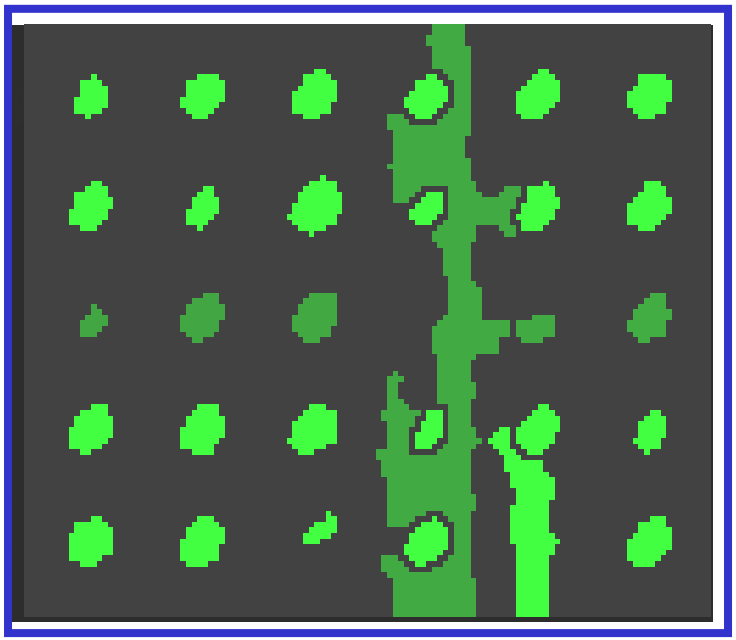

Image analysis

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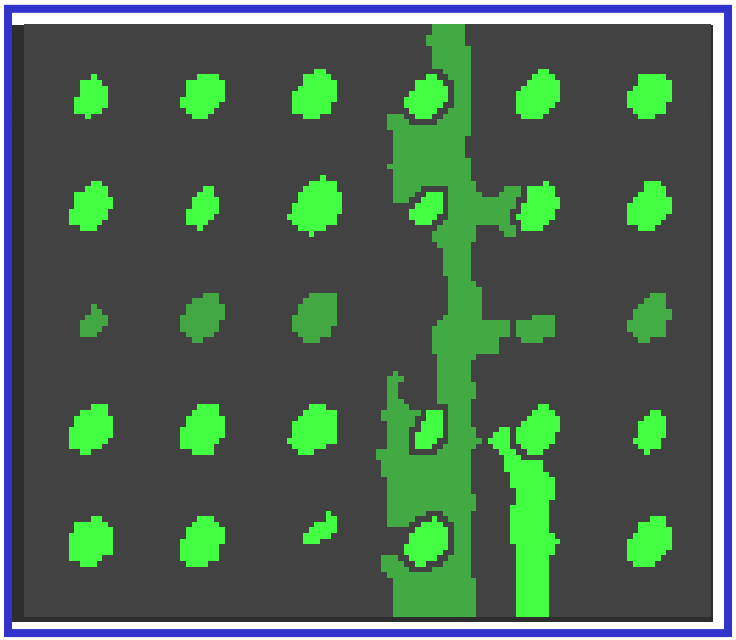
2. Segmentation. Classify pixels as foreground (signal) or background.



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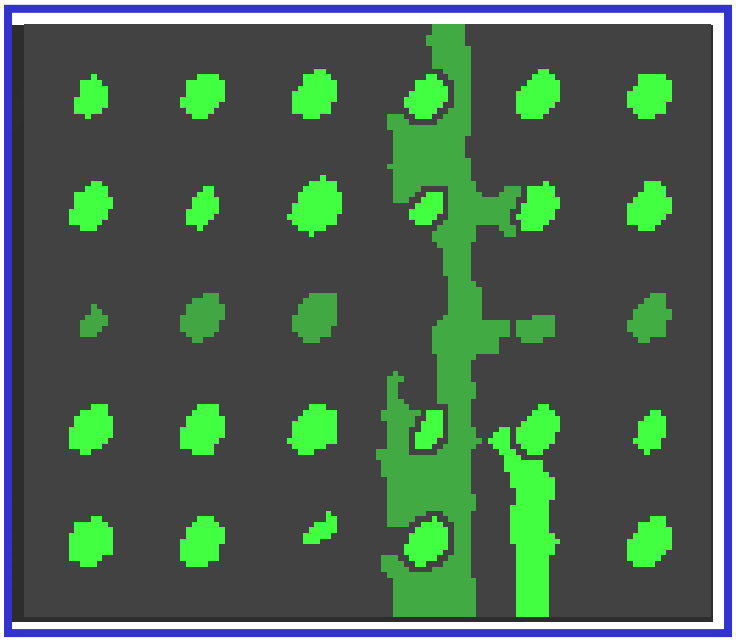
3. Information extraction. For each spot on the array and each dye
   - foreground intensities;
   - background intensities;
   - quality measures.



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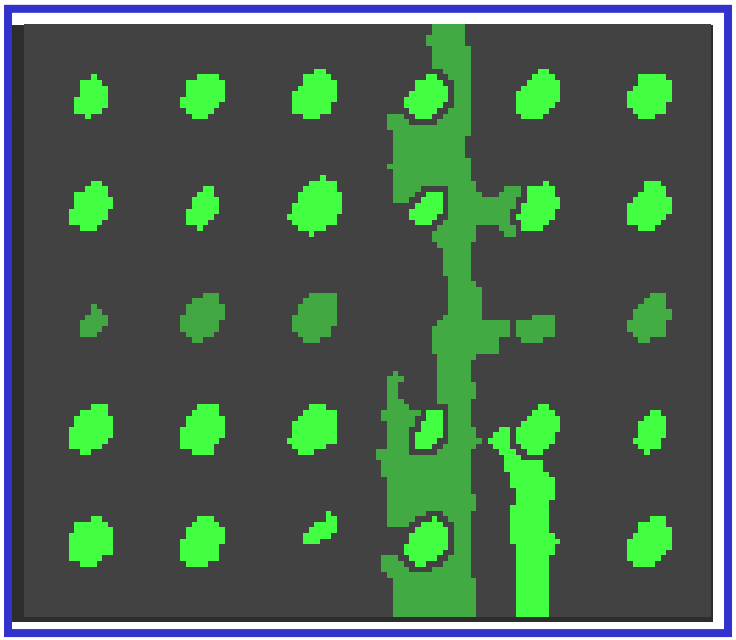
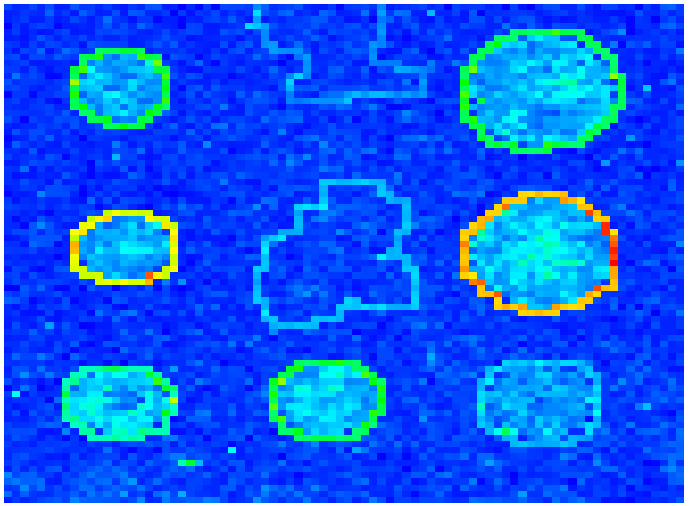
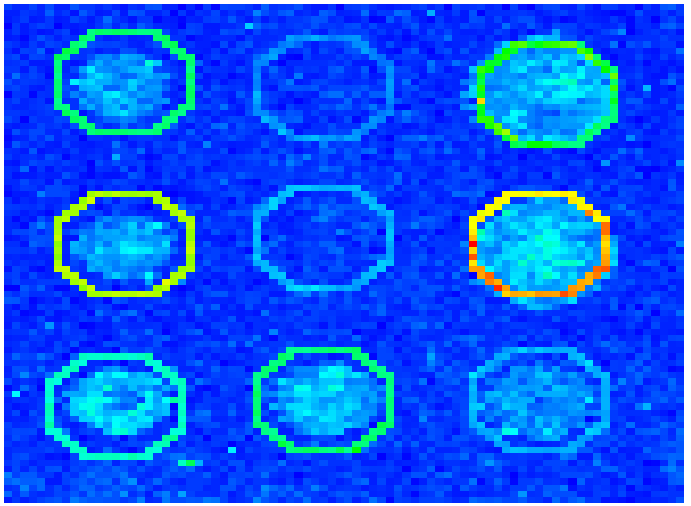


→ R and G for each spot on the array.

Segmentation



adaptive segmentation
seeded region growing



fixed circle segmentation

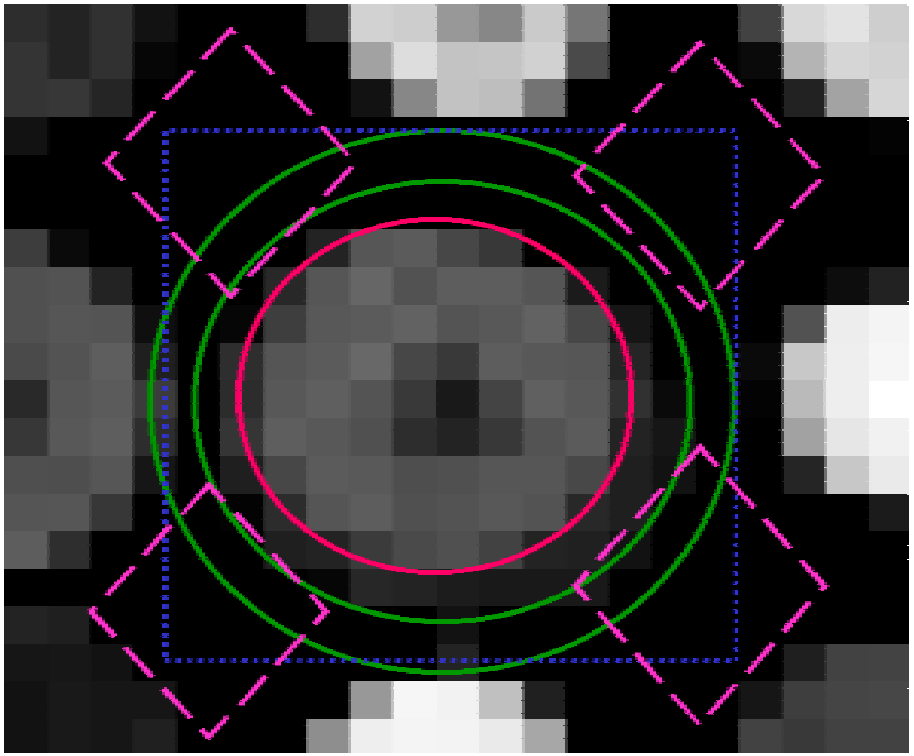
Spots may vary in size and shape.

Local background

---- GenePix

---- QuantArray

---- ScanAlyze



Local background estimation by morphological opening

Image is probed with a window (aka structuring element), eg, a square with side length about twice the spot-to-spot distance.

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$\Rightarrow$ Image of the estimated background

What is (local) background?

usual assumption:

total brightness =

background brightness (adjacent to spot)

\+ brightness from labeled sample cDNA

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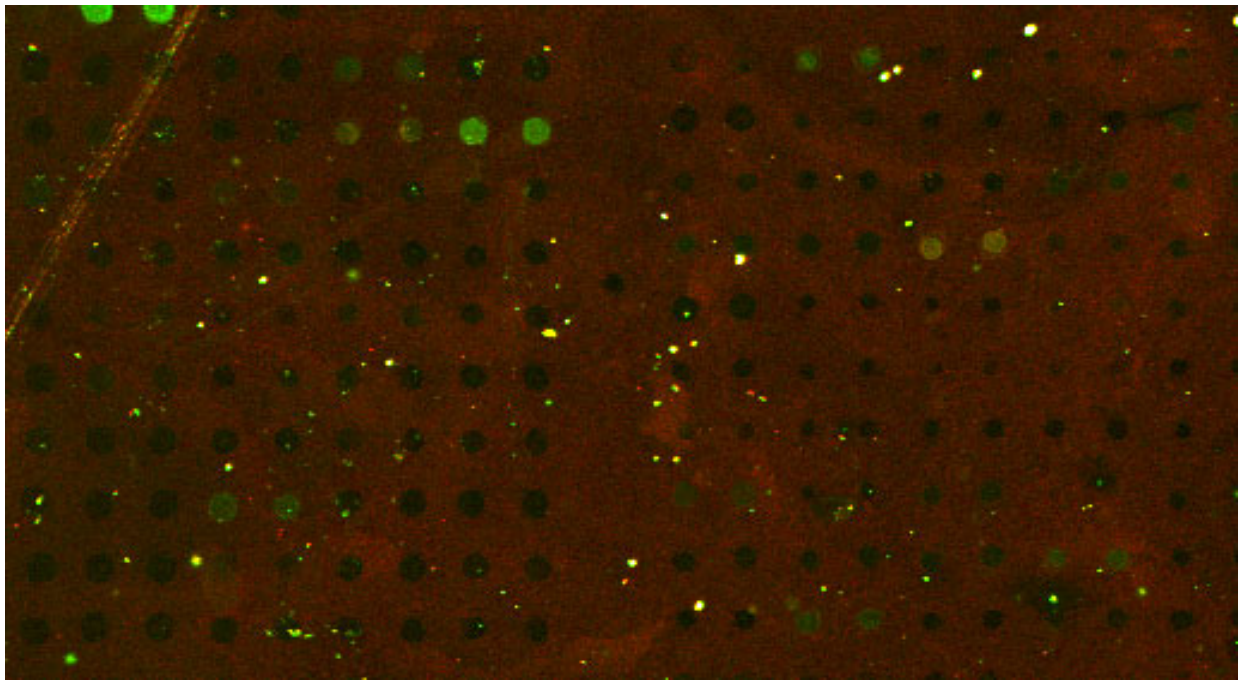


?

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

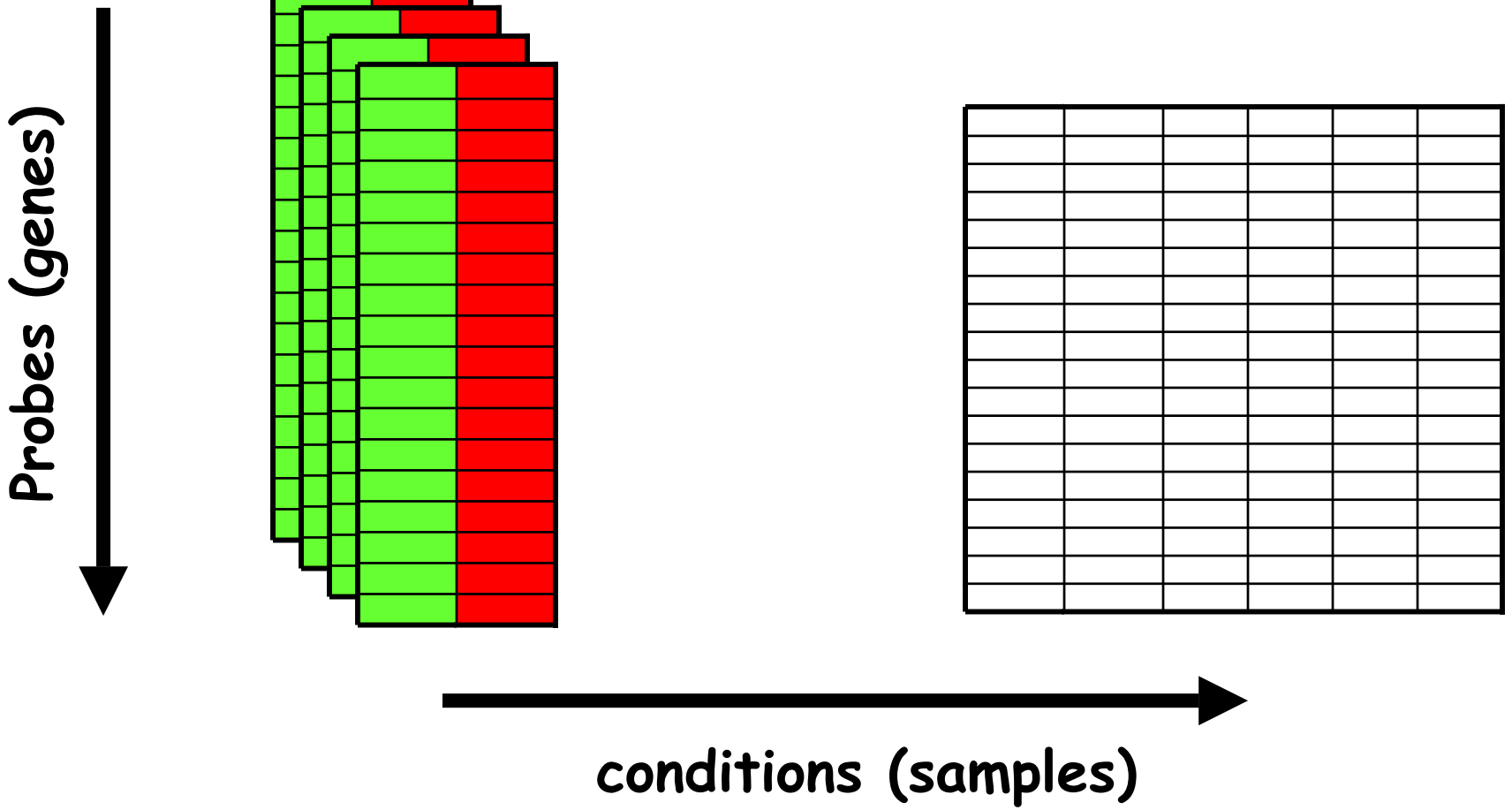
- Percentage of spots with no signal
- Range of intensities
- Distribution of spot signal area, etc.

How to use quality measures in subsequent analyses?

spot intensity data

two-color spotted arrays

n one-color arrays
(Affymetrix, nylon)



Microarrays are measurement instruments

- have limited measurement precision ('error bars')
- need calibration
- involve technology optimization and fine-tuning
- may fail or be mis-operated

Technology is complex and crossdisciplinary
(biotechnology, nanotechnology, chemistry, physics, robotics; plus the application areas: molecular biology, oncology, pathology, medicine…)

Technology and the ways it is applied are still evolving

Computational statistics and data analysis can contribute

Raw data are not mRNA concentrations

- tissue contamination
- RNA degradation
- amplification efficiency
- reverse transcription efficiency
- hybridization efficiency and specificity
- clone identification and mapping
- PCR yield, contamination
- spotting efficiency
- DNA-support binding
- other array manufacturing-related issues
- image segmentation
- signal quantification
- 'background' correction

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- tissue
- clone
- image

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

specificity

related issues

Sources of variation

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

PCR yield
DNA quality
spotting efficiency,
  spot size
cross-/unspecific hybridization
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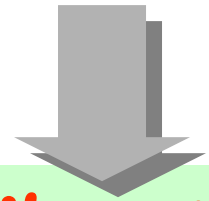

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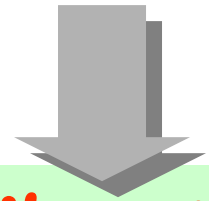

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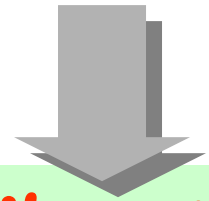
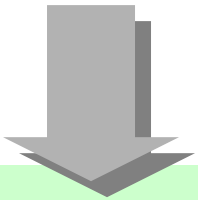
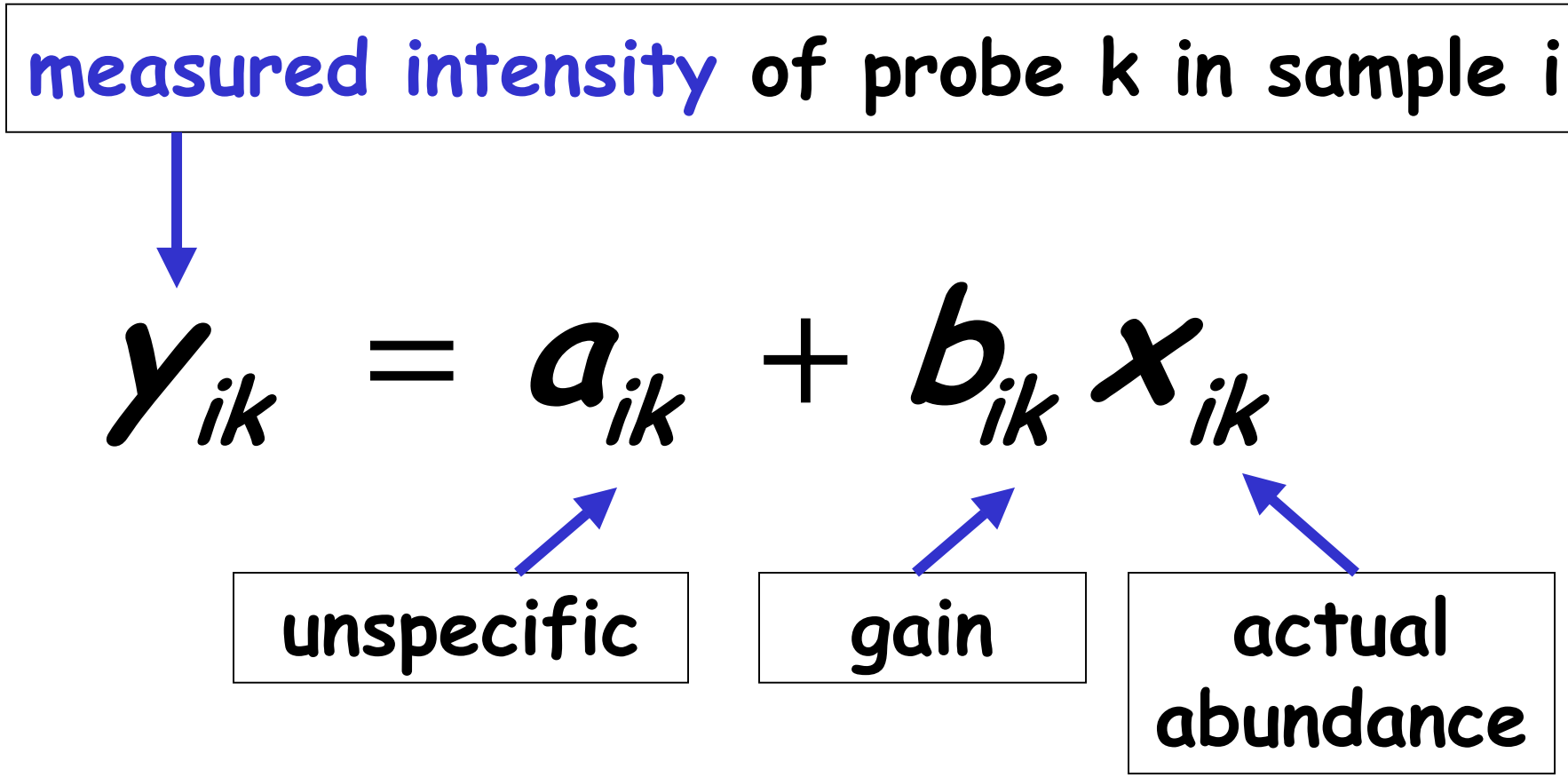




Calibration

Error model


measured intensity of probe k in sample i
$y_{ik} = a_{ik} + b_{ik} x_{ik}$
unspecific
gain
actual abundance

measured intensity of probe k in sample i

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a_{ik}, b_{ik} all unknown: need to approximately determine from data

Implications

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Normalization, calibration: estimate parameters for the data at hand

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$$\text{data} \longrightarrow y_{ik} = a_{ik} + b_{ik} x_{ik} \longleftarrow \text{quantity of interest}$$

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Quality control: verify that assumptions hold for the data at hand
Normalization, calibration: estimate parameters for the data at hand
Error modeling: control the error bars both of measured y_{ik} and of estimated normalization parameters a_{ik}, b_{ik}

A typical set of assumptions

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$$b_{ik} = b_i \; b_k \; \eta_{ik}$$

b_i per-sample normalization factor

b_k sequence-wise labeling efficiency

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

"multiplicative noise"

A typical set of assumptions

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

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

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"

$$b_{ik} = b_i\ b_k\ \eta_{ik}$$

b_i per-sample normalization factor

b_k sequence-wise labeling efficiency

$\log\ \eta_{ik} \sim N(0, s_2^2)$
"multiplicative noise"

Discussion and extensions

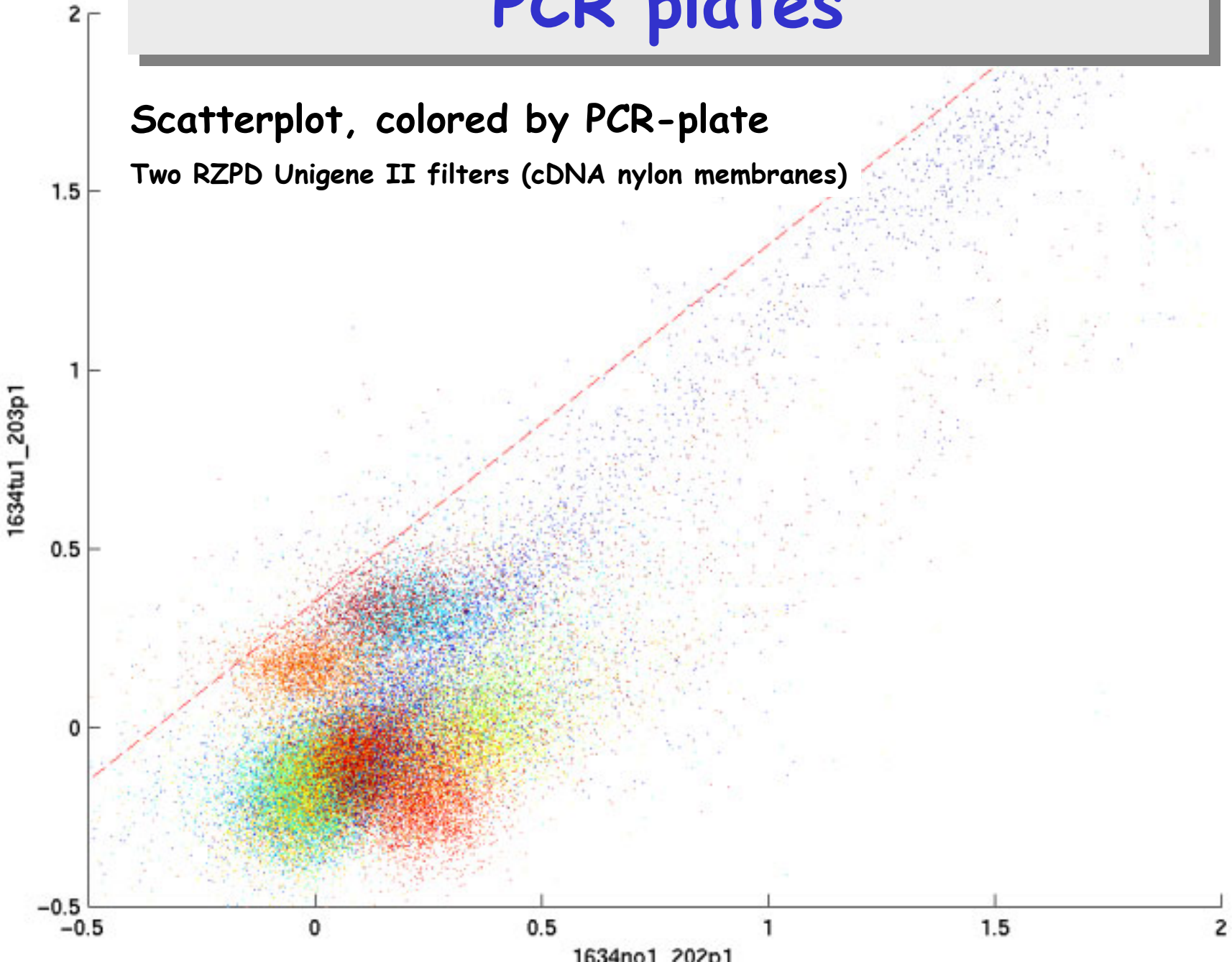
- Sequence-wise factors b_k need not be explicitely determined if only interested in relative expression levels
- The simplifying assumptions bring down number of parameters from 2dn to ~2d - the 'rest' is modeled as stochastic, aka noise.
- Here, array calibration terms a_i, b_i same for all probes on array - can extend this to include print-tip or plate effects
- Here, probe affinities b_k same for all arrays - can extend this to include batch effects

Quality control: diagnostic plots and artifacts

PCR plates

Scatterplot, colored by PCR-plate

Two RZPD Unigene II filters (cDNA nylon membranes)



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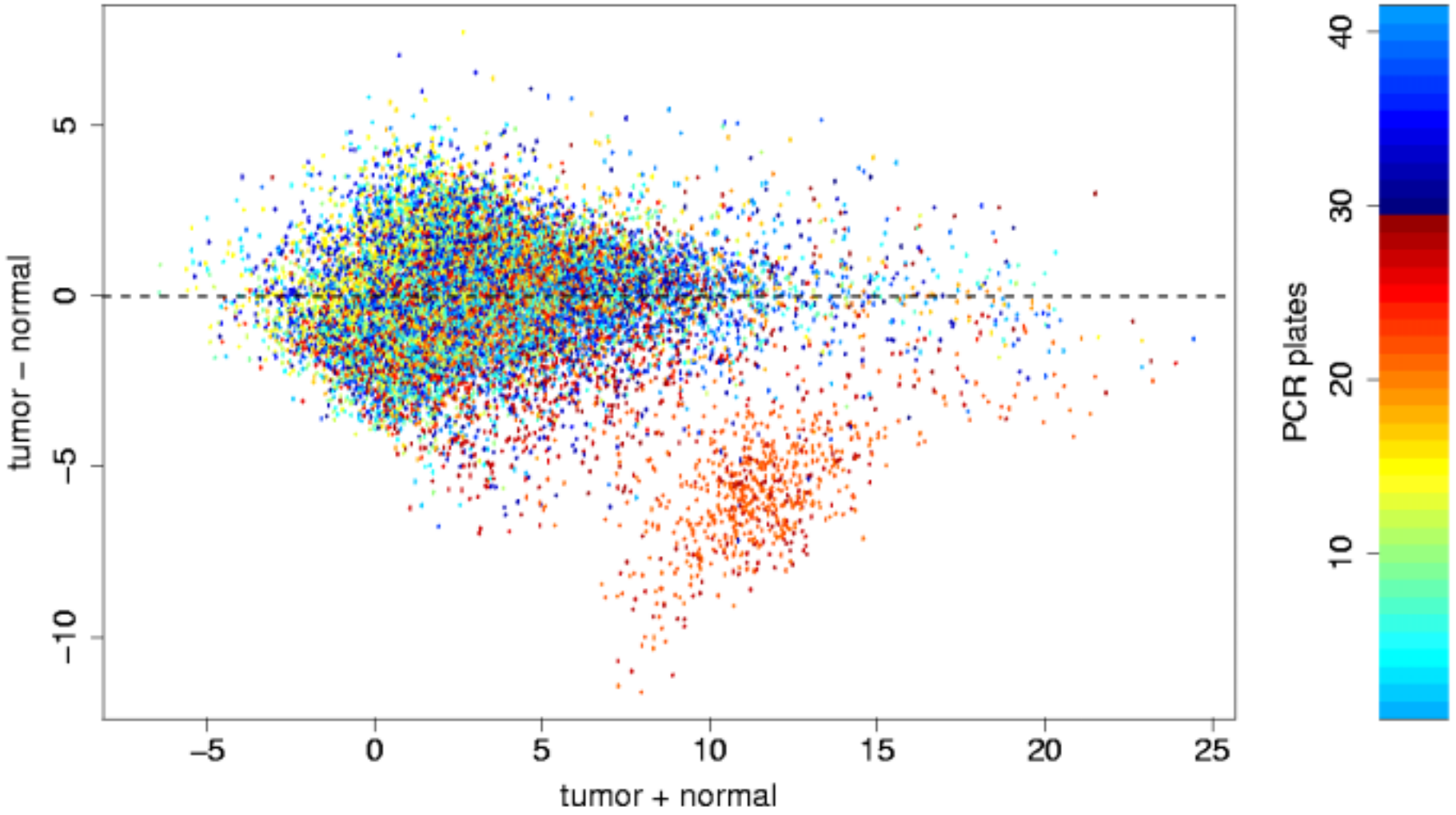
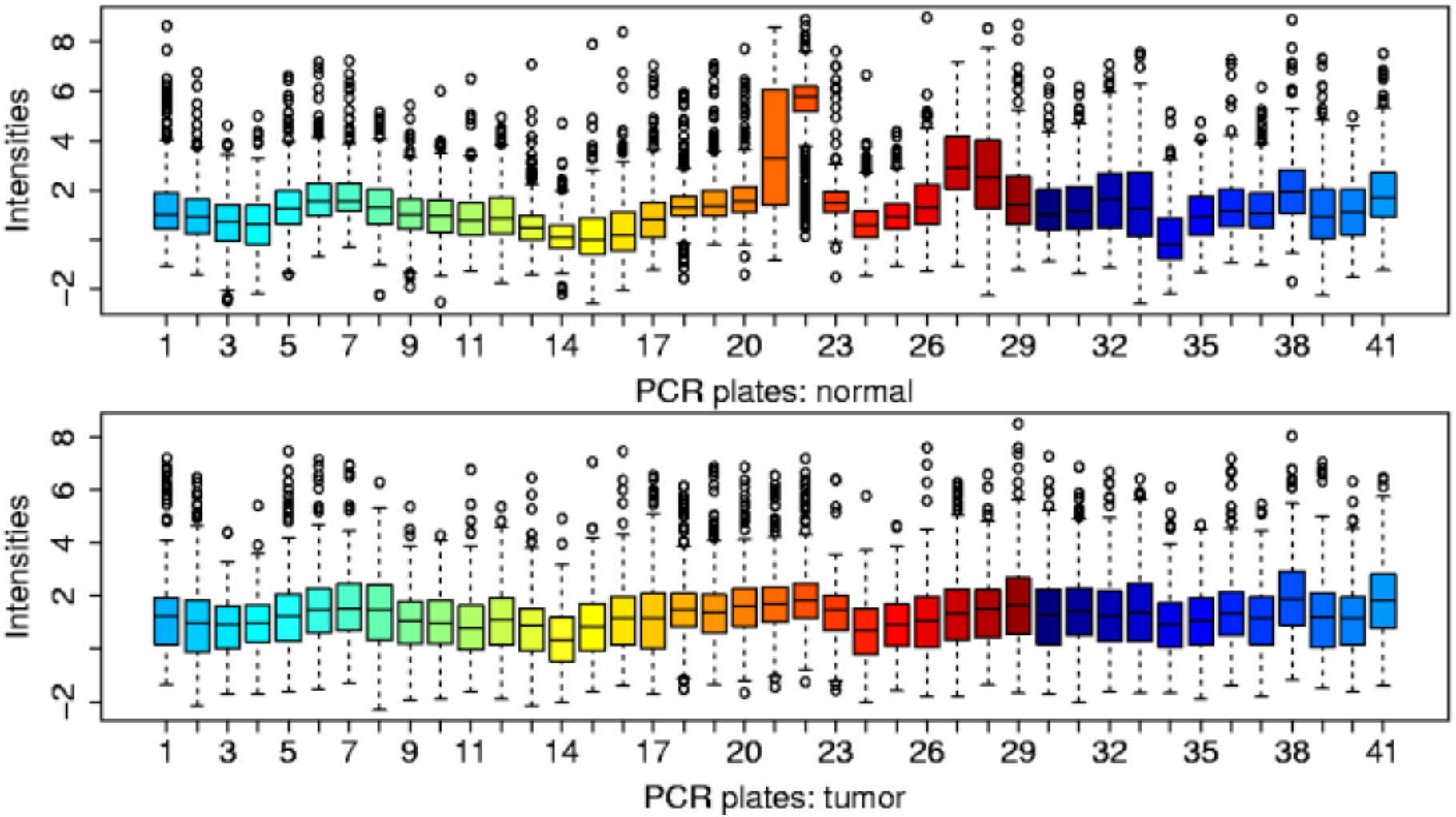
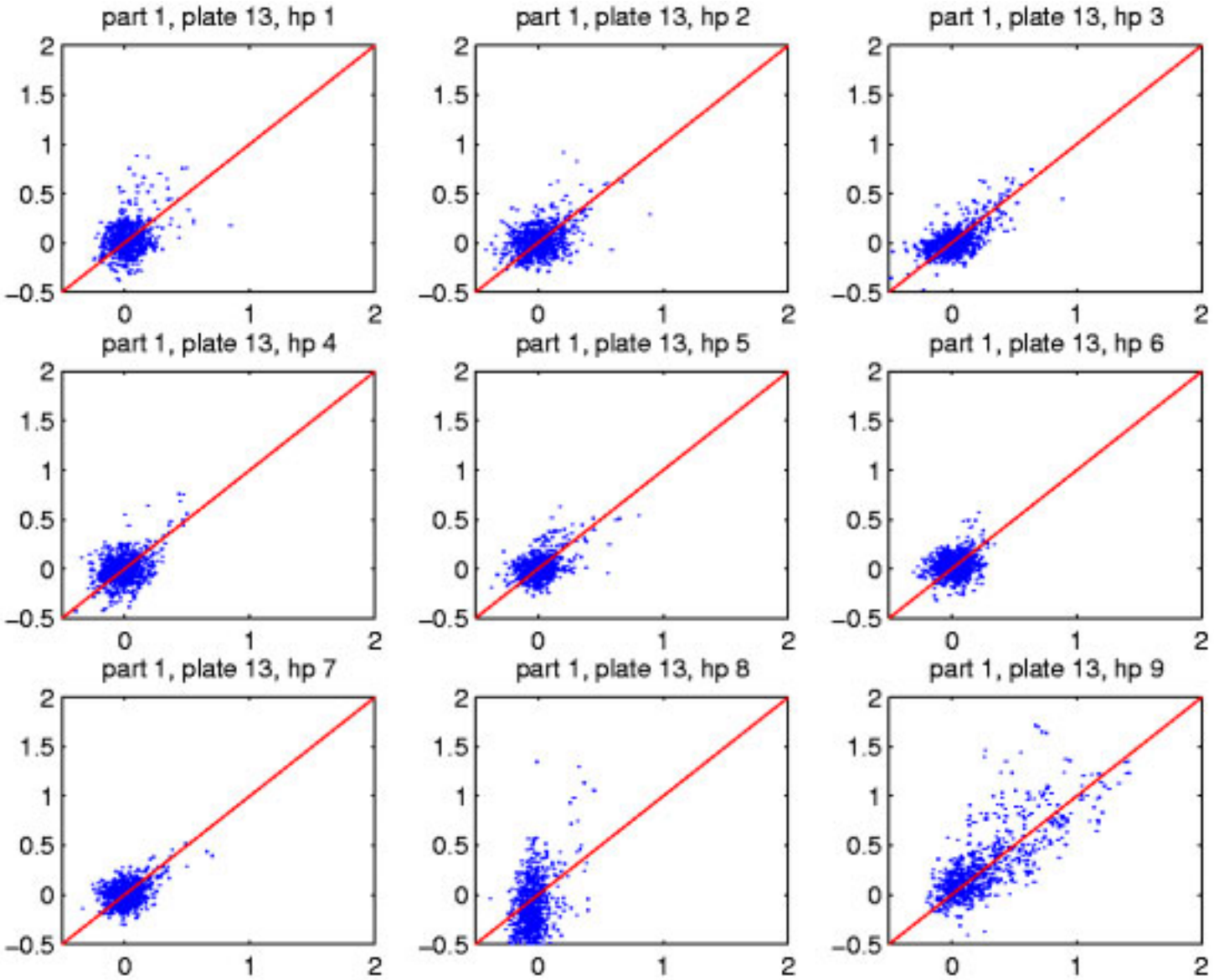

tumor – normal
5
0
−5
−10
−5
0
5
10
15
20
25
tumor + normal
PCR plates
40
30
20
10

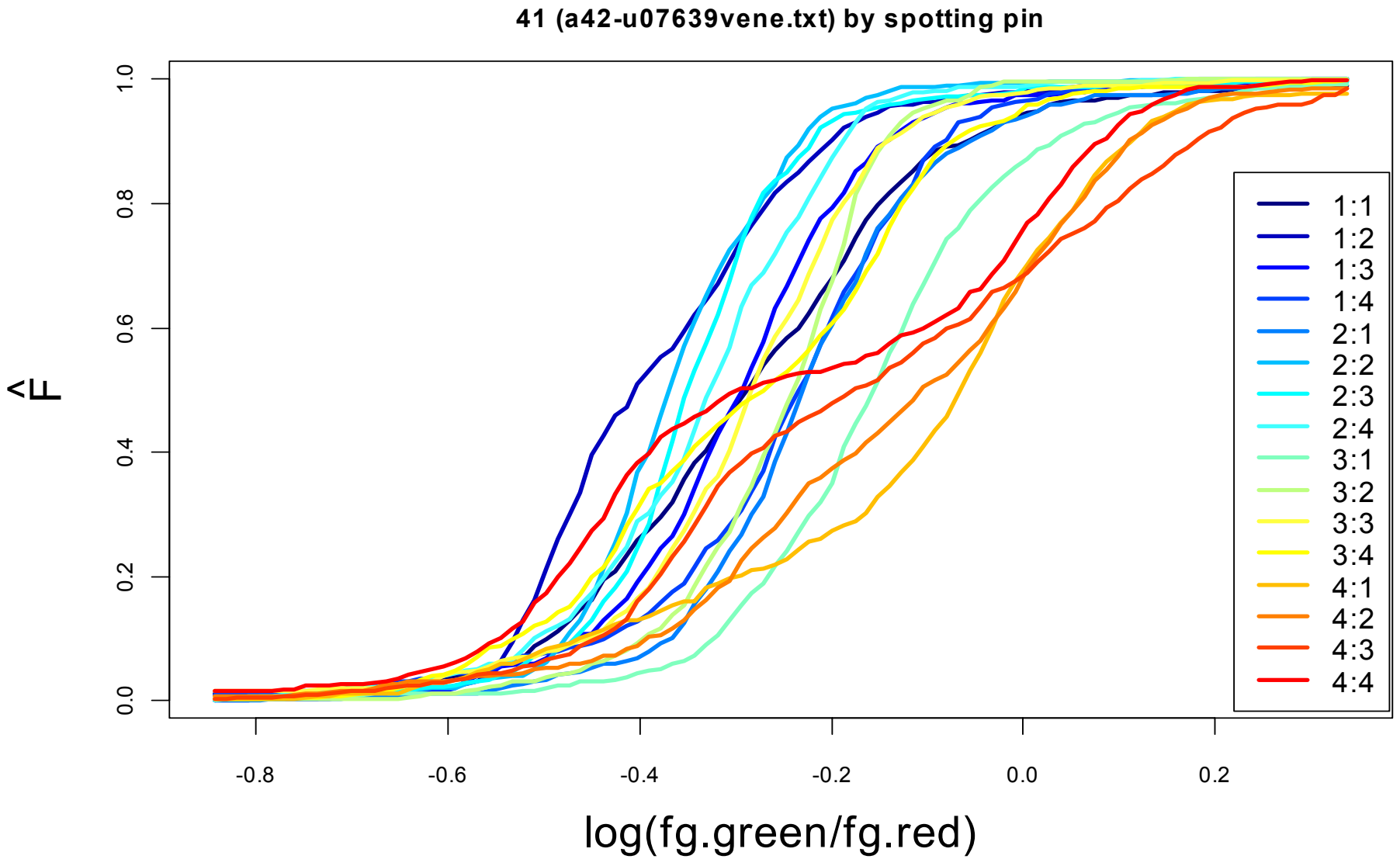
PCR plates: boxplots



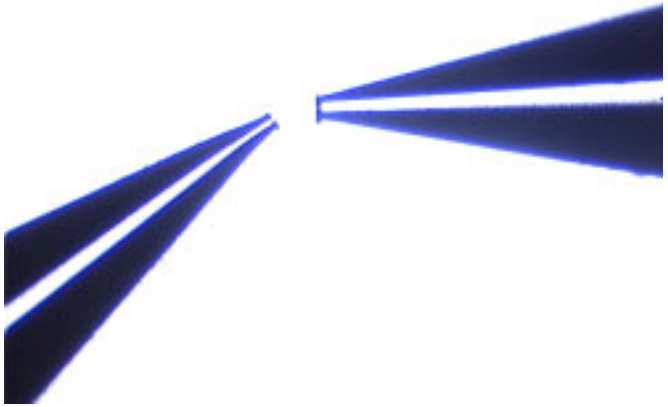
array batches



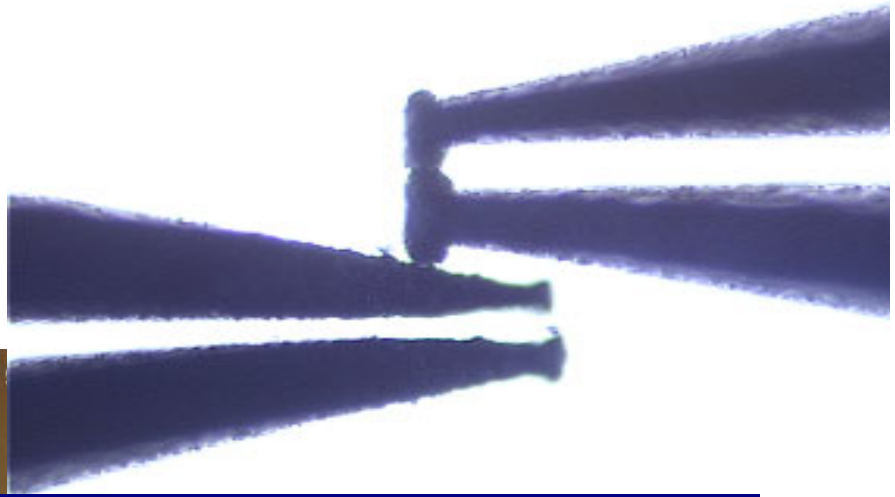
print-tip effects

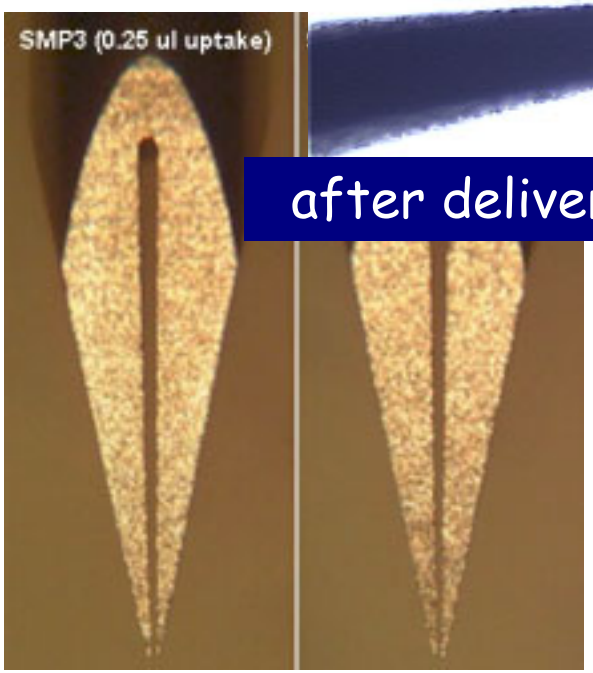


spotting pin quality decline



after delivery of $5x10^5$ spots

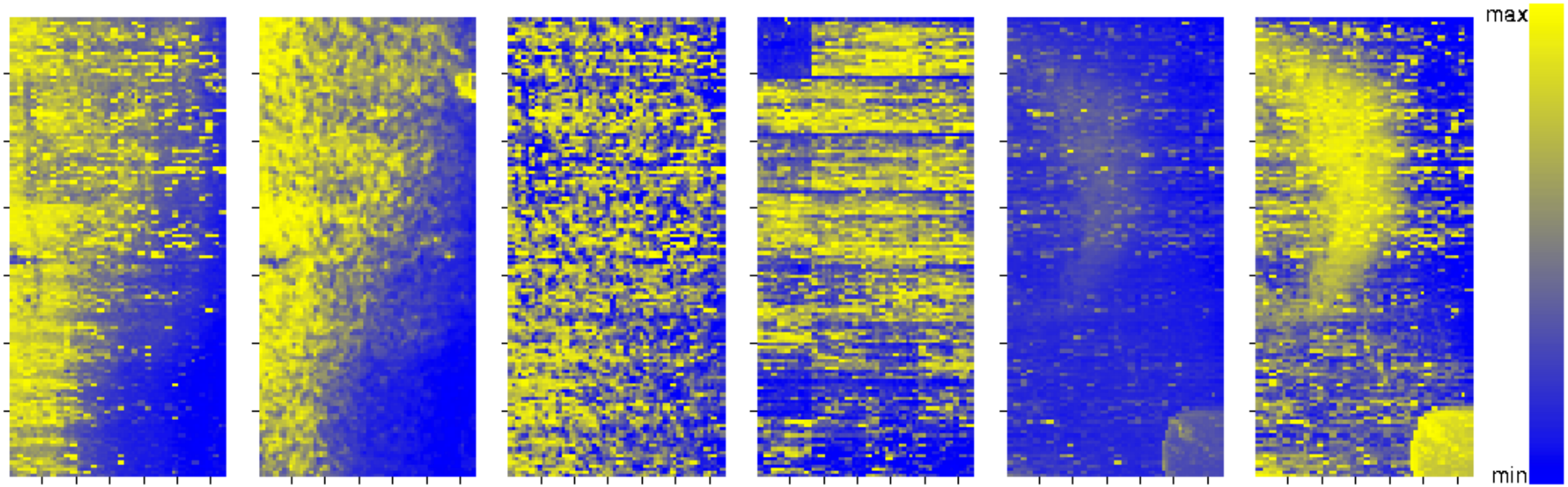




after delivery of $3x10^5$ spots

H. Sueltmann DKFZ/MGA

spatial effects



R Rb R-Rb
color scale by rank

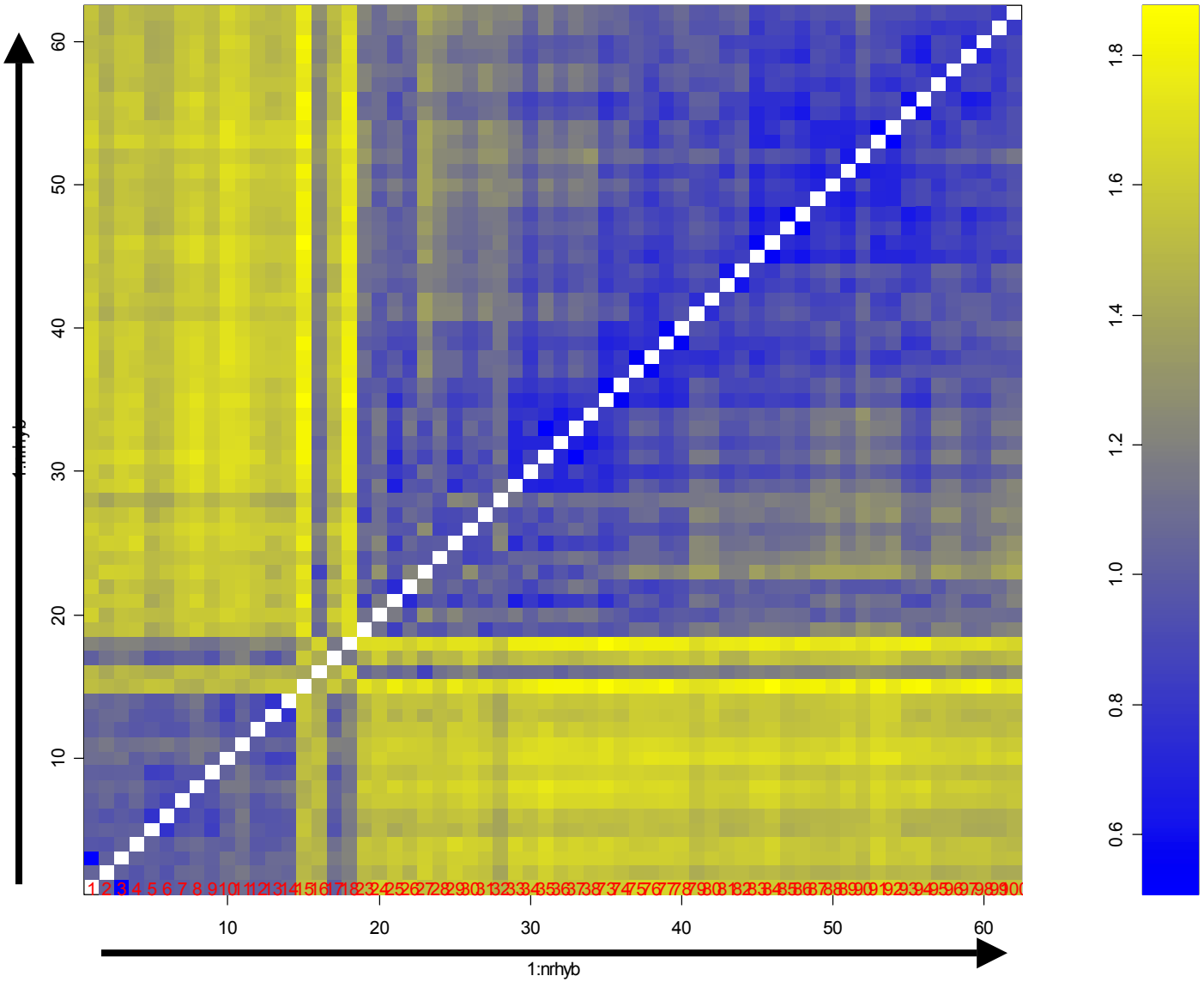
another array: print-tip

color scale ~ log(G)

color scale ~ rank(G)

spotted cDNA arrays, Stanford-type

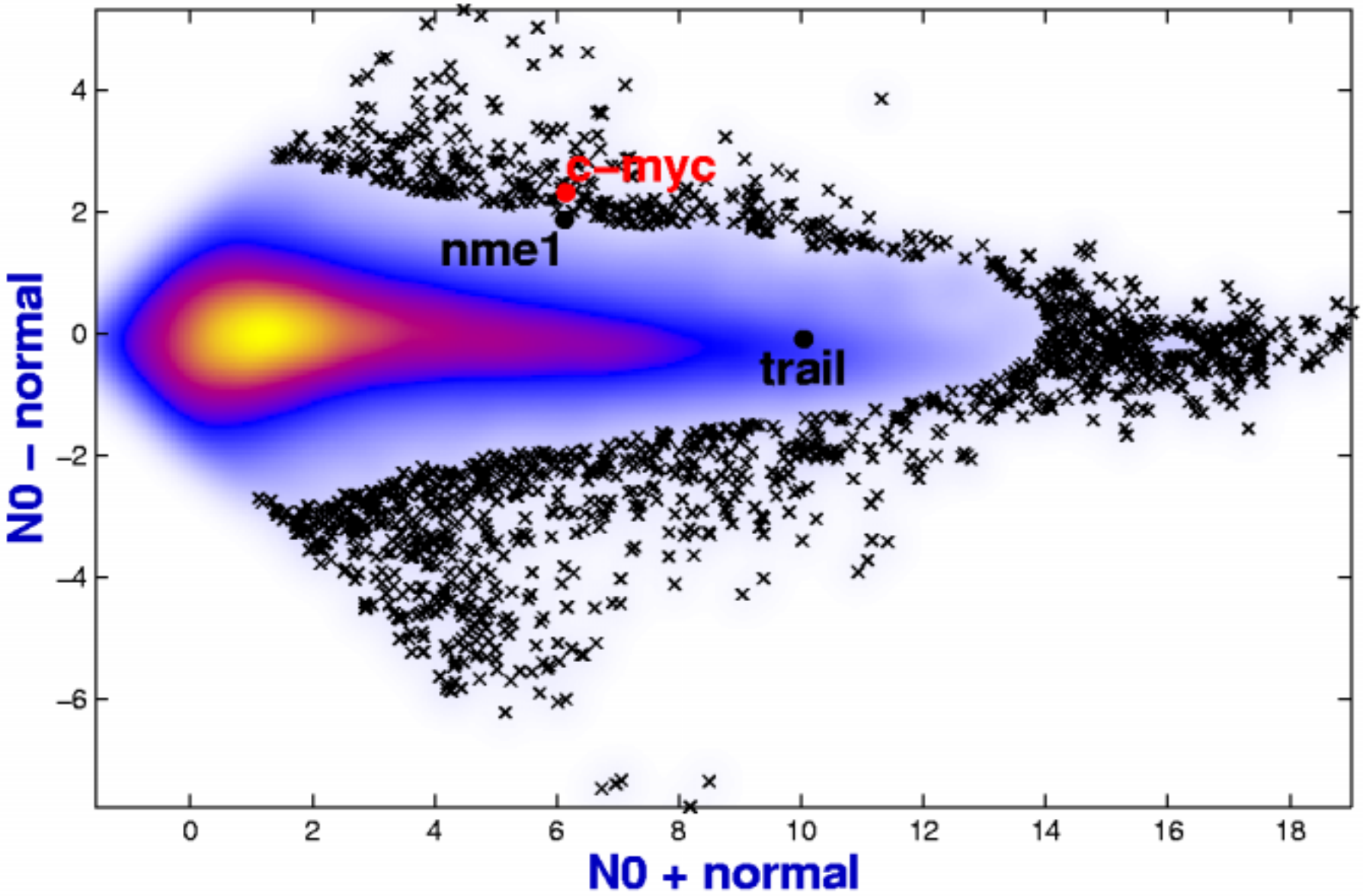
Batches: array to array differences $d_{ij} = mad_k(h_{ik} - h_{jk})$



arrays i=1…63; roughly sorted by time

Density representation of the scatterplot

(76,000 clones, RZPD Unigene-II filters)



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(aka normalization)

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Outliers, long-tailed distributions

Calibration
(aka normalization)

- Correction for systematic variations
  (e.g. dye efficiencies Cy3/Cy5, detector gain, sample mass)
- A parameter estimation task

Raw data is heteroskedastic, i.e. variance is not constant ('different data points have different error bars')
⇒ need for either weighted regression or variance stabilizing data transformation

Outliers, long-tailed distributions
⇒ need for robust methods that do not sensitively depend on e.g. normality

Ordinary regression

Minimize the sum of squares

$$SoS = \sum_{i(slides,channels)} \sum_{k(probes)} \left(\text{residual}_{ik}\right)^2$$

residual: "fit" - "data"

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Solution: weight them accordingly (some weights may be zero)

Weighted regression

$$SoS = \sum_{i(slides,channels)} \sum_{k(probes)} w_{ik} \times \left(residual_{ik}\right)^2$$

If $w_{ik} = 1/var_{ik}$, then minimizing SoS produces the maximum-likelihood estimate for a model with normal errors.

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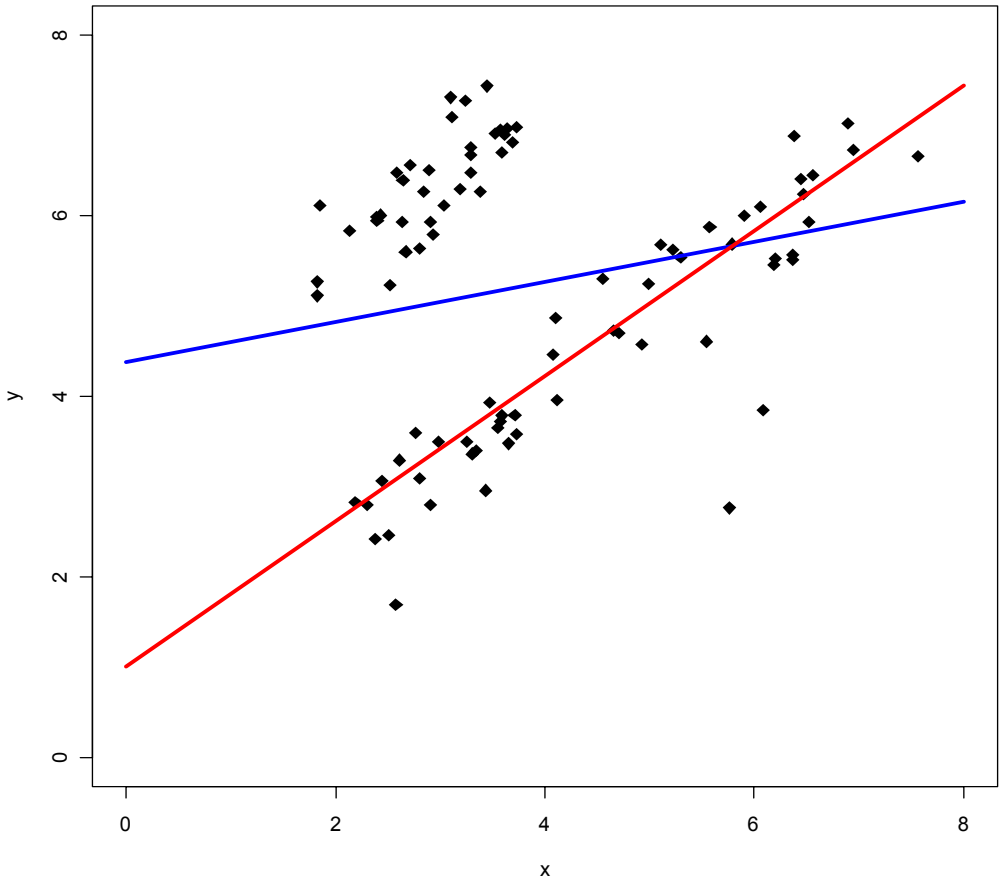
Least Median Sum of Squares Regression:

$$w_{ik} = \begin{cases} 1 / variance_{ik} & \text{if } residual_{ik} \le median(residuals) \\ 0 & \text{otherwise} \end{cases}$$

Least trimmed sum of squares regression



- least sum of squares
- least trimmed sum of squares

minimize

$$\sum_{n}^{\text{"median"}} \left(y_n - f(x_n)\right)^2$$

But what is the variance of a measured spot intensity?

To estimate the variance of an individual probe, need many replicates from biologically identical samples. Often unrealistic.

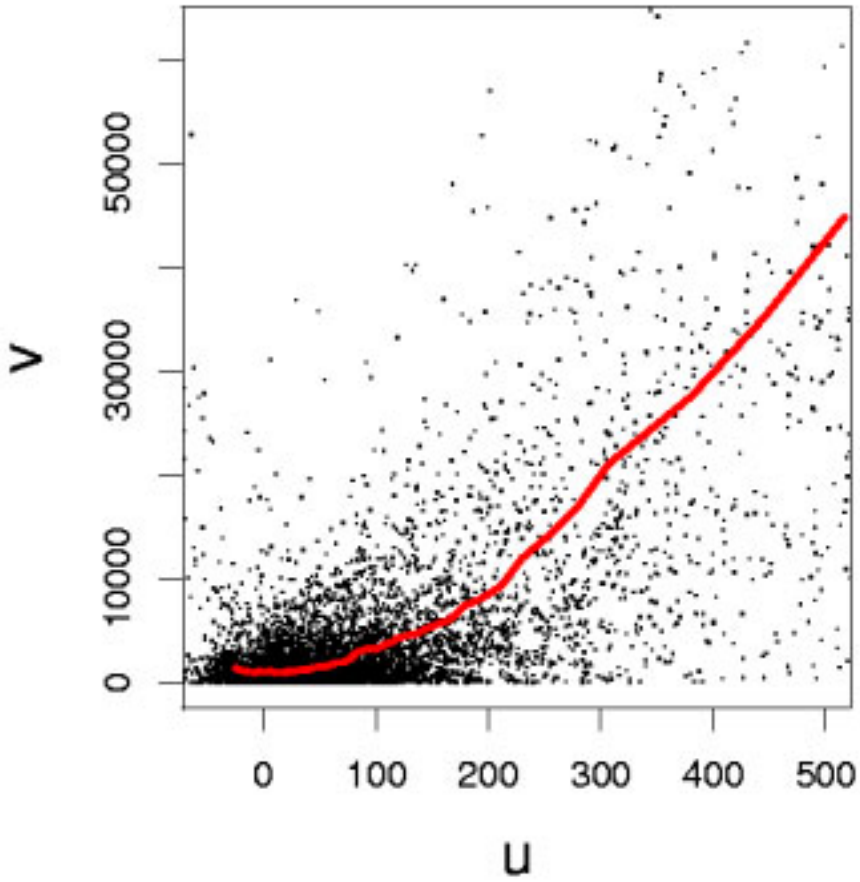
Idea:

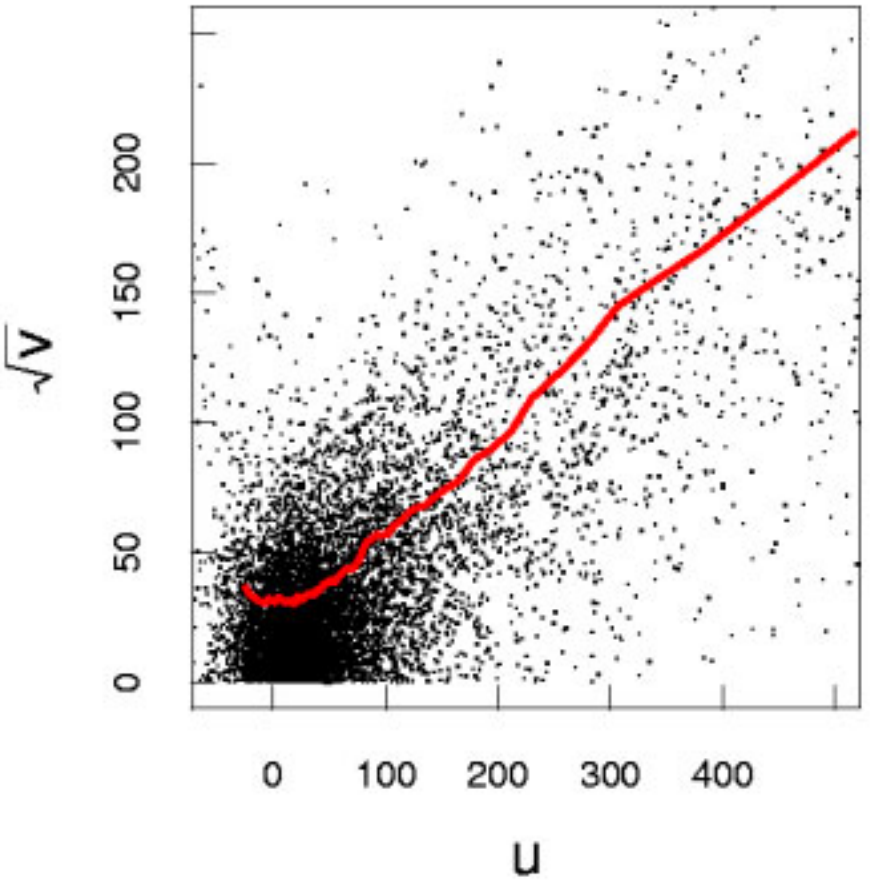
- use pooled estimate from several probes who we expect to have about the same true (unknown) variance

$$\text{var}_{\text{pooled}} = \text{mean}\,(\text{var}_{\text{individual probes}})$$

- there is an obvious dependence of the variance on the mean intensity, hence stratify (group) probes by that.

dependence of variance on mean





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

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

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"

$$b_{ik} = b_i\ b_k\ \eta_{ik}$$

b_i per-sample normalization factor

b_k sequence-wise labeling efficiency

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

"multiplicative noise"

Decomposition:

signal =

(true concentration

x gain factor

x multiplicative noise)

+ additive offset

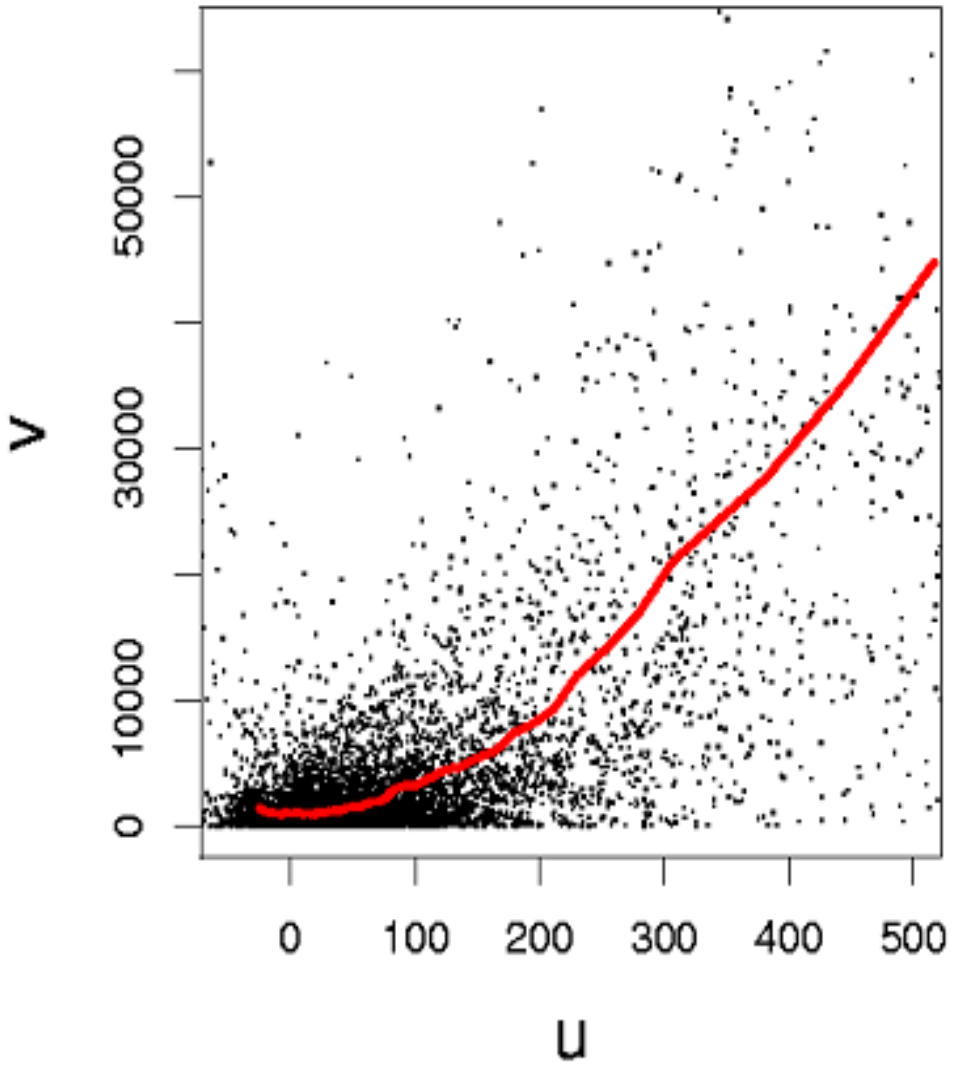
+ additive noise

"additive noise"

"multiplicative noise"

variance-vs-mean dependence

data (cDNA slide)



model $\Rightarrow$

quadratic dependence of $v \equiv \mathrm{Var}(Y_{ik})$ on $u \equiv \mathrm{E}(Y_{ik})$

$$v(u) = c^2u^2 + s^2$$

variance stabilization

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

Define

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

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

"stabilized"

variance stabilizing transformations

y = true value + add. noise	y
y = true value + add. noise + offset	$y - b$
y = true value x mult. noise	$\log \frac{y}{c}$
y = true value x mult. noise + offset	$\log \frac{y - b}{c}$
y = true value x mult. noise + add. noise + offset	$\operatorname{arsinh} \frac{y - b}{c}$

variance stabilizing transformations

y

y

y

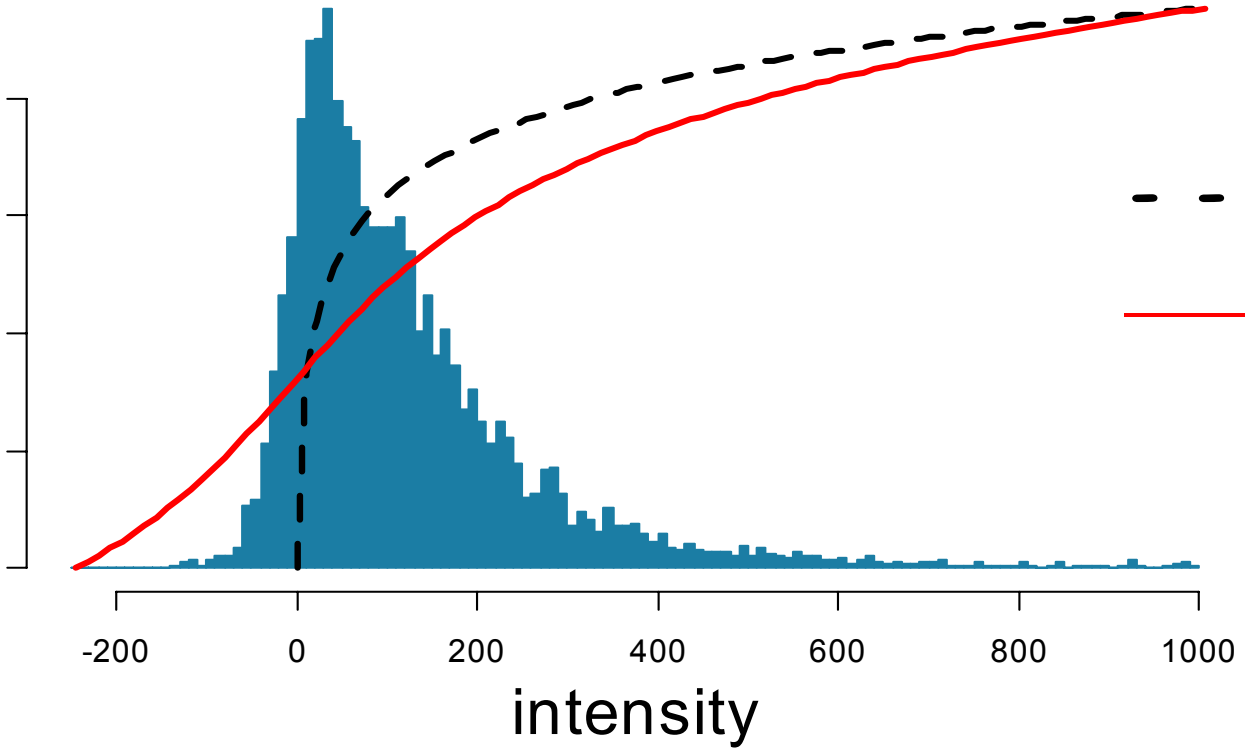
y

y

Advantage:

eliminates the need to explicitely account for the variance-mean dependence through weighted regression (cumbersome) - on the transformed scale, all data have the same weight

the arsinh transformation



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

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

the transformed model: estimation of parameters from data

$$\operatorname{arsinh}\frac{Y_{ki} - a_i}{b_i} = \mu_{ki} + \varepsilon_{ki}, \qquad \varepsilon_{ki} \sim N(0, c^2)$$

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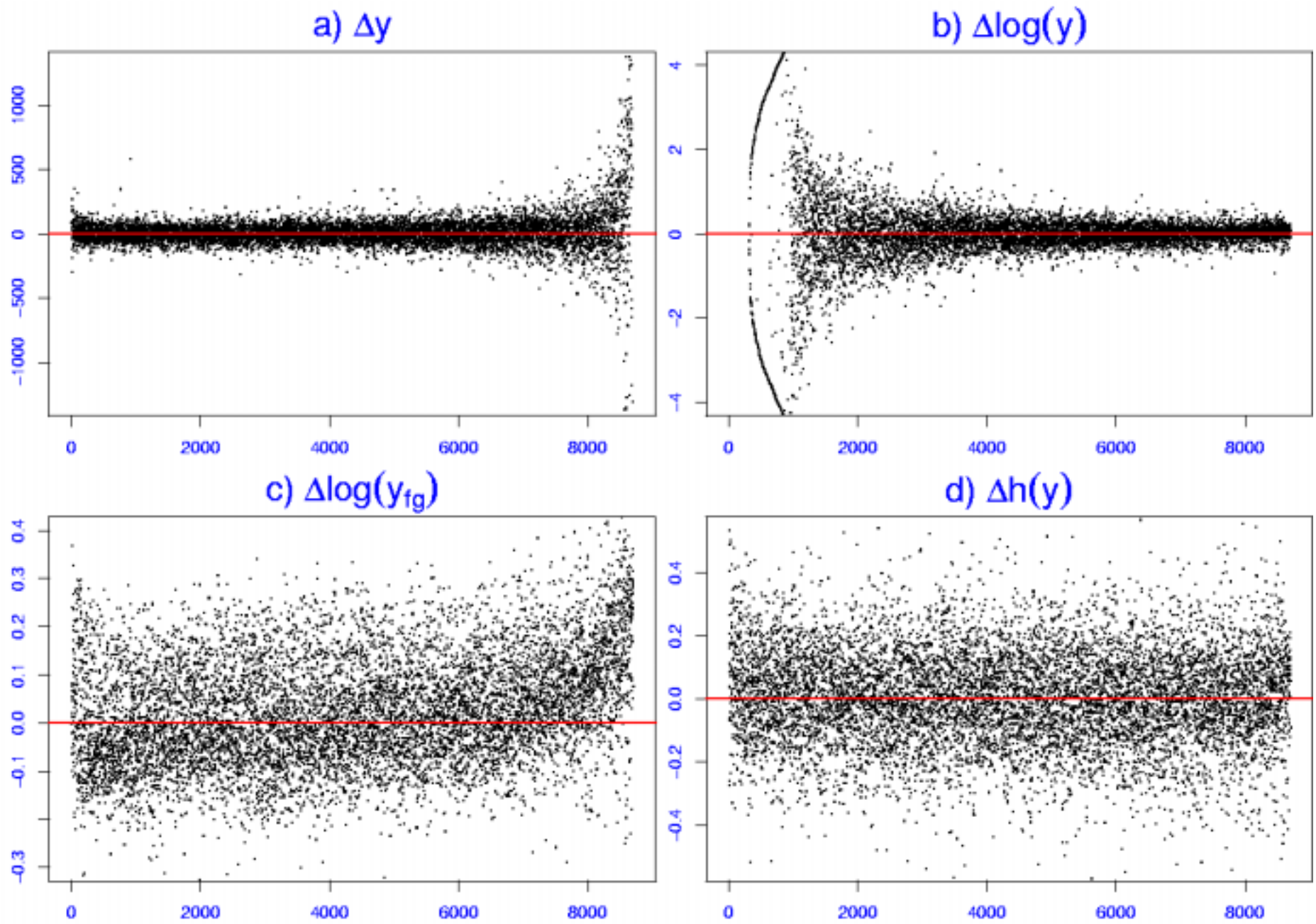
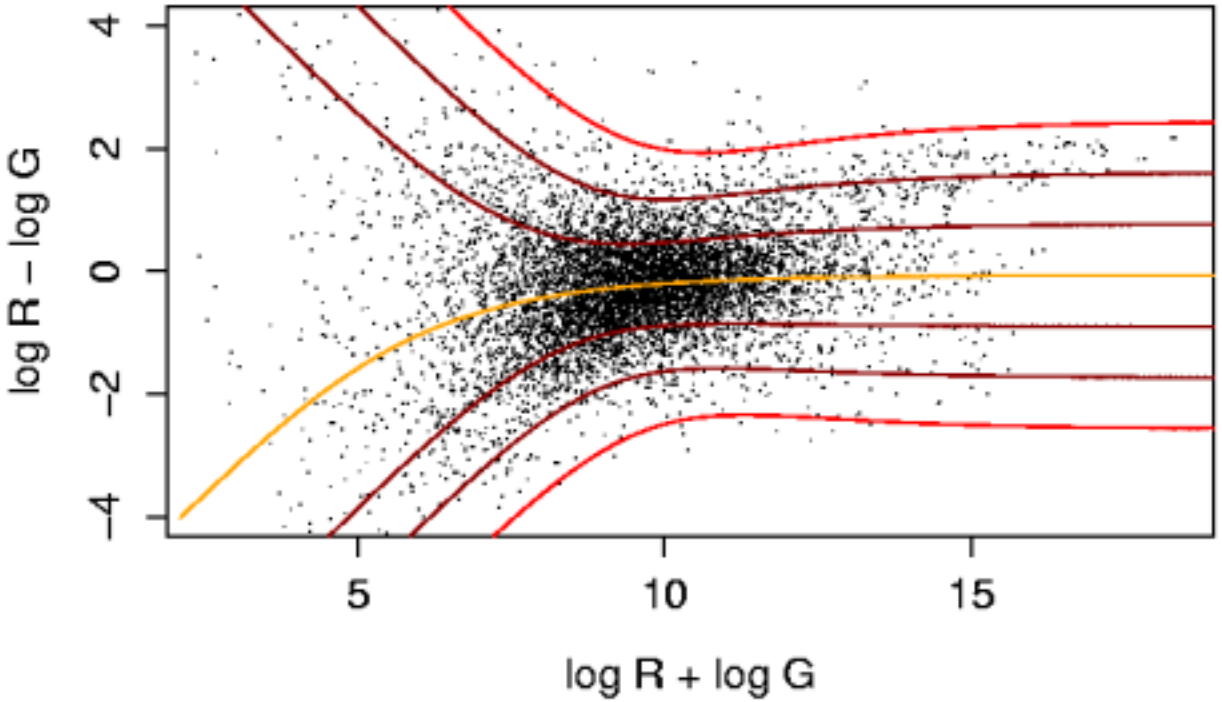
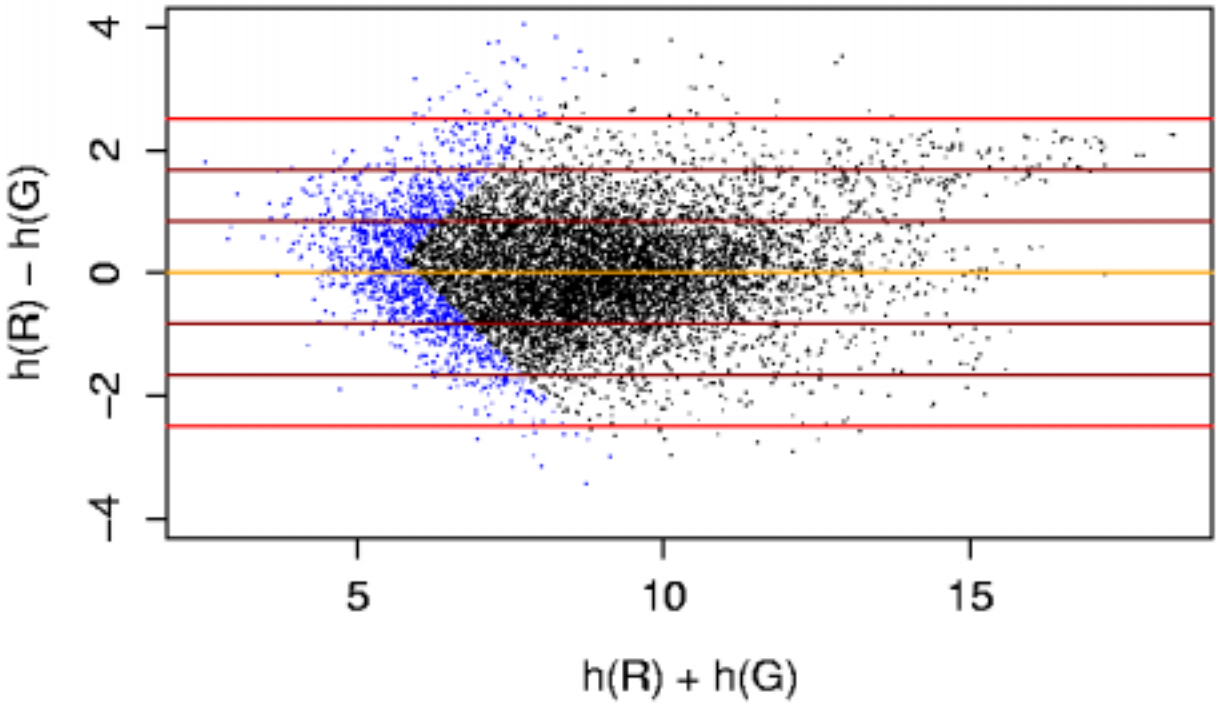
- solution: if most (more than half) genes are unchanged, that's almost as good
- minority: act as outliers. Use robust variant of ML estimator, à la *Least Trimmed Sum of Squares* regression.

effects of different data transformations





log



generalized log (variance stabilizing)

cDNA slide data from H. Sueltmann

Summary

log-ratio

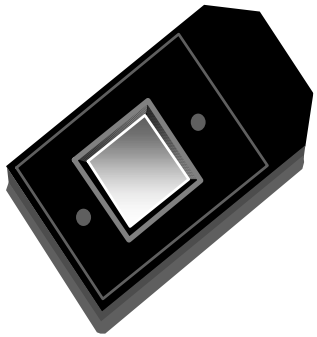
$$\log \frac{y_{k1} - a_1}{b_1} - \log \frac{y_{k2} - a_2}{b_2}$$

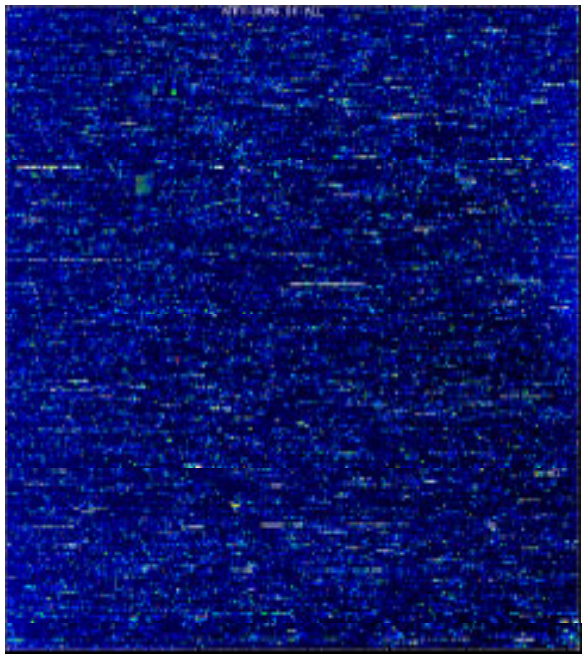
'generalized' log-ratio

$$\operatorname{arsinh} \frac{y_{k1} - a_1}{b_1} - \operatorname{arsinh} \frac{y_{k2} - a_2}{b_2}$$

- advantages of variance-stabilizing data-transformation: generally better applicability of statistical methods (hypothesis testing, ANOVA, clustering, classification…)
- R package vsn

Oligonucleotide chips





Affymetrix files

Main software from Affymetrix:

MAS - MicroArray Suite.

DAT file: Image file, ~10^7 pixels, ~50 MB.

CEL file: probe intensities, ~400000 numbers

CDF file: Chip Description File. Describes which probes go in which probe sets (genes, gene fragments, ESTs).

Image analysis

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 cells is taken as the background value and subtracted.

Compute also quality values.

Data and notation

PM_{ijg} , MM_{ijg} = Intensity for perfect match and mismatch probe j for gene g in chip i.

$i = 1,\ldots, n$ one to hundreds of chips

$j = 1,\ldots, J$ usually 16 or 20 probe pairs

$g = 1,\ldots, G$ 8…20,000 probe sets.

Tasks:

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

summarize for each probe set the probe level data, i.e., 20 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 uses AvDiff, a trimmed mean:

$$AvDiff = \frac{1}{\#J} \sum_{j \in J} (PM_j - MM_j)$$

- sort $d_j = PM_j - MM_j$
- exclude highest and lowest value
- J := 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*

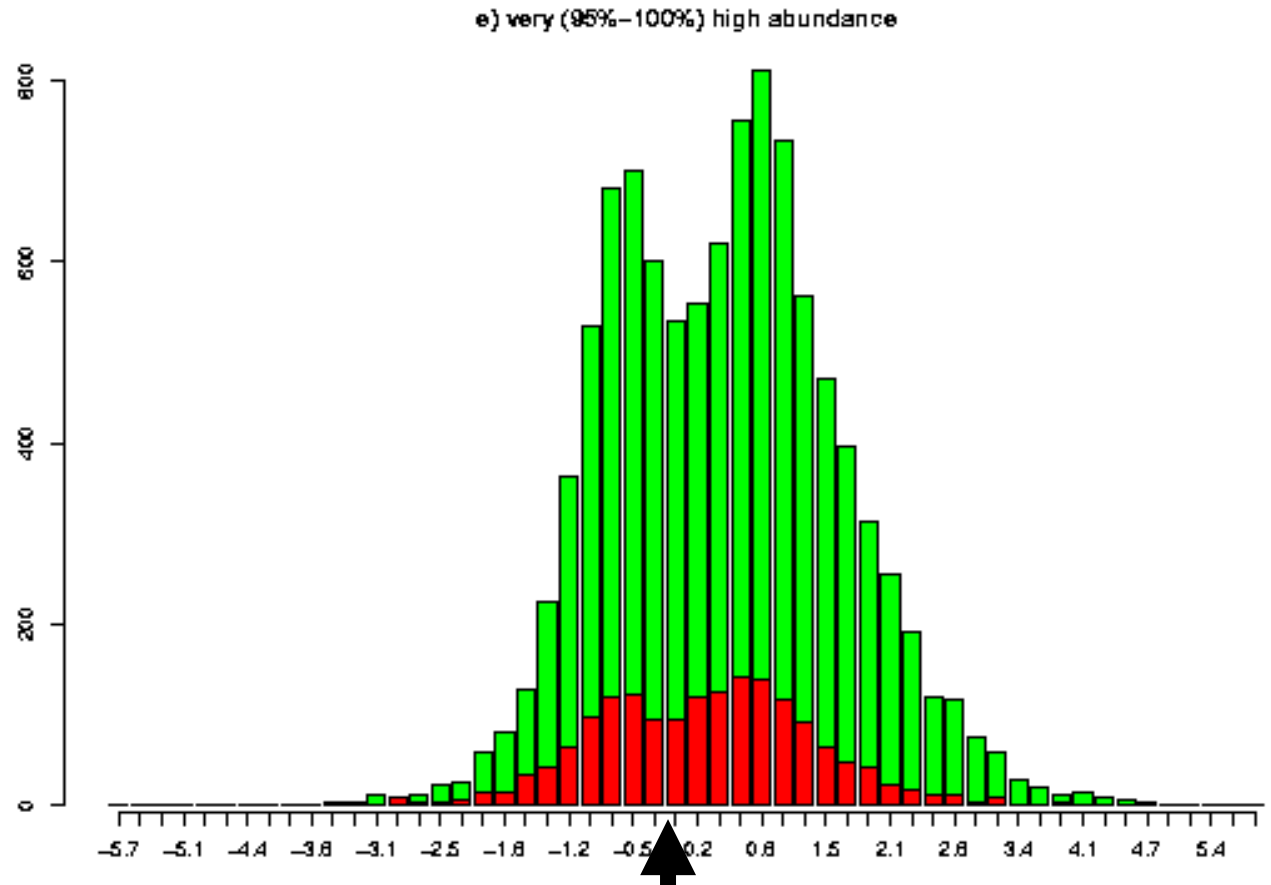
"Signal" =

Tukey.Biweight (log(PM-CT))

(... ≈median)

Tukey Biweight: B(x) = (1 – (x/c)^2)^2 if |x|<c, 0 otherwise

Affymetrix: $I_{PM} = I_{MM} + I_{specific}$?



0

log(PM/MM)

From: R. Irizarry et al.,
Biostatistics 2002

Expression measures: Li & Wong

dChip fits a model for each gene

$$PM_{ij} - MM_{ij} = \theta_i \phi_j + \varepsilon_{ij}, \qquad \varepsilon_{ij} \propto N(0, \sigma^2)$$

where

- θ_i: expression index for gene i
- ϕ_j: probe sensitivity

Maximum likelihood estimate of MBEI is used as expression measure of the gene in chip *i*.

Need at least 10 or 20 chips.

Current version works with PMs only.

Expression measures
RMA: Irizarry et al. (2002)

- Estimate one global background value b=mode(MM). No probe-specific background!
- Assume: $PM = s_{true} + b$

  Estimate $s \geq 0$ from PM and b as a conditional expectation $E[s_{true}|PM, b]$.
- Use $\log_2(s)$.
- Nonparametric nonlinear calibration ('quantile normalization') across a set of 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 methods** 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.

Software for pre-processing of Affymetrix data

- Bioconductor R package affy.
- Background estimation.
- Probe-level normalization: quantile, curve- fitting.
- Expression measures: AvDiff, Signal, Li & Wong (2001), RMA.
- Two main functions: ReadAffy, express.

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A more complete list of references is in:

Elementary analysis of microarray gene expression data. W. Huber, A. von Heydebreck, M. Vingron, manuscript.

http://www.dkfz-heidelberg.de/abt0840/whuber/