First analysis steps

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

Wolfgang Huber

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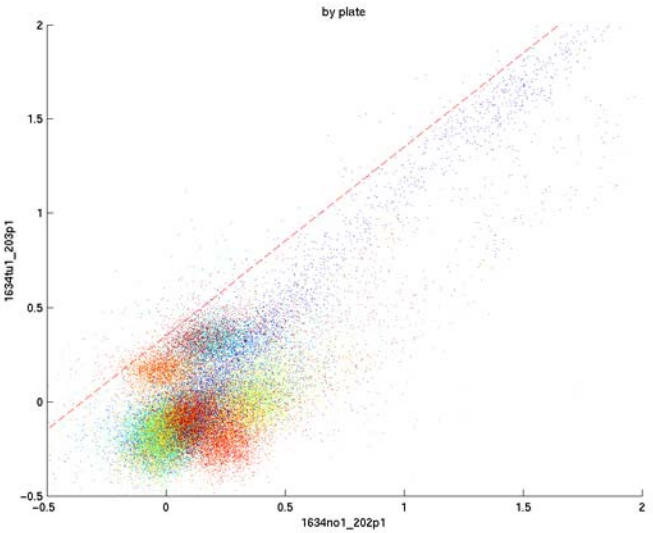

Acknowledgements

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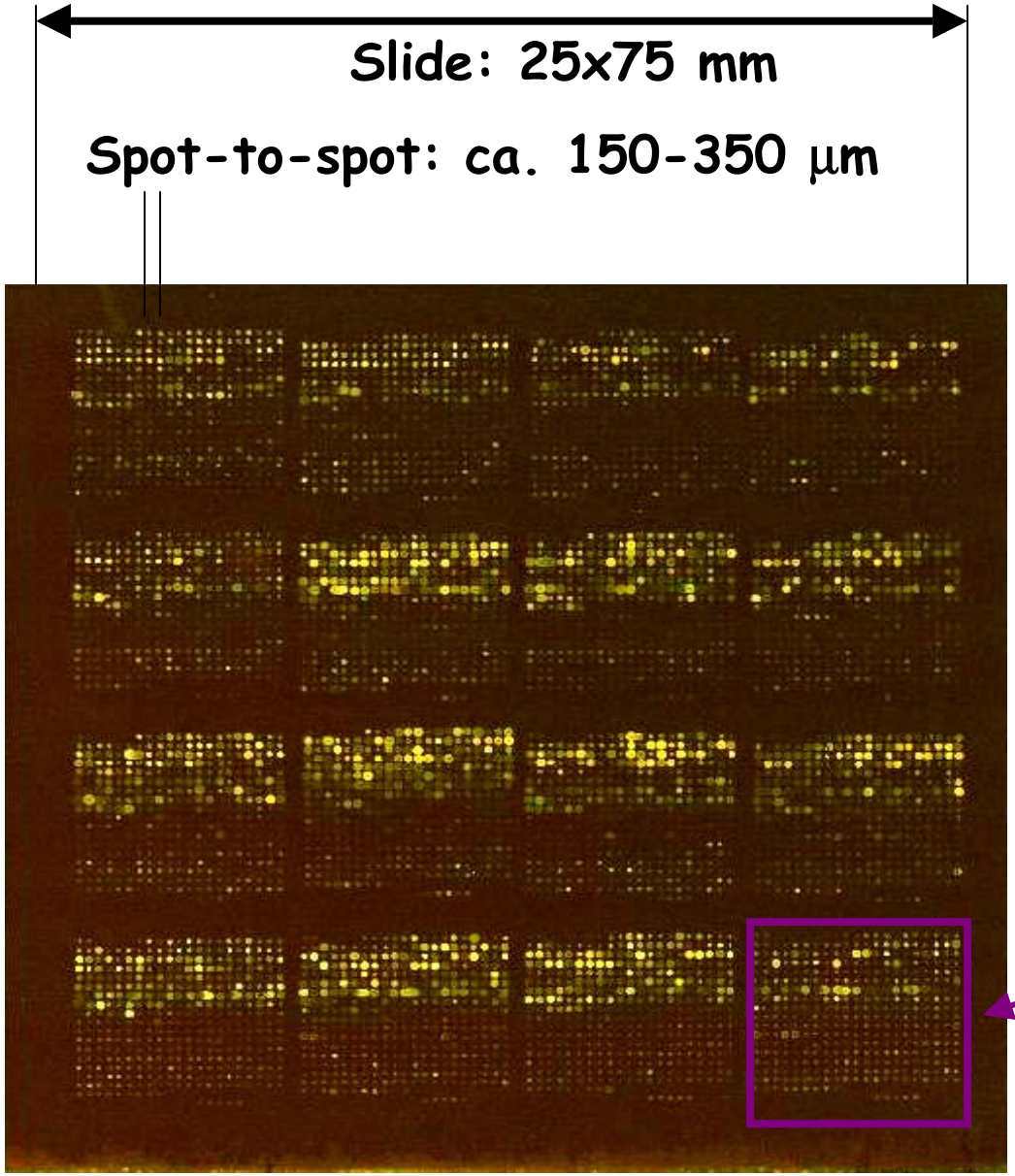
Sandrine Dudoit, Robert Gentleman, Rafael Irizarry and Yee Hwa Yang: Bioconductor short course, summer 2002

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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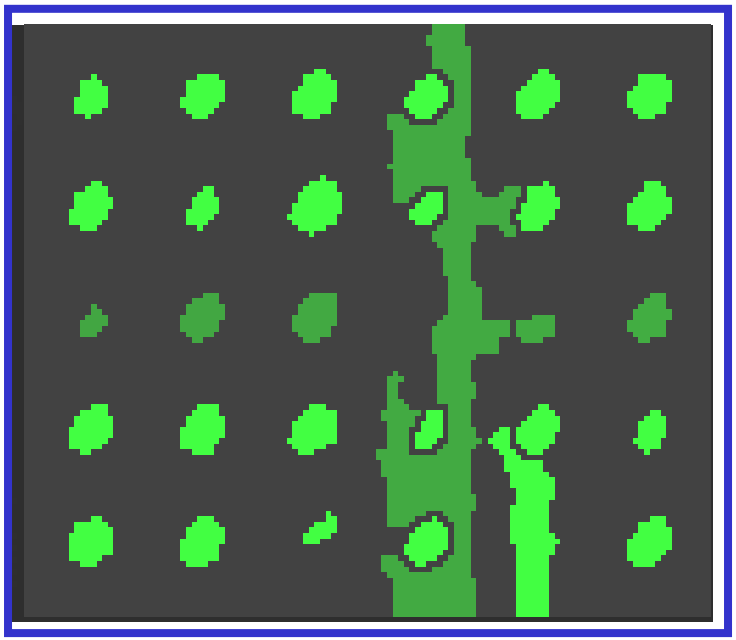
spot intensities

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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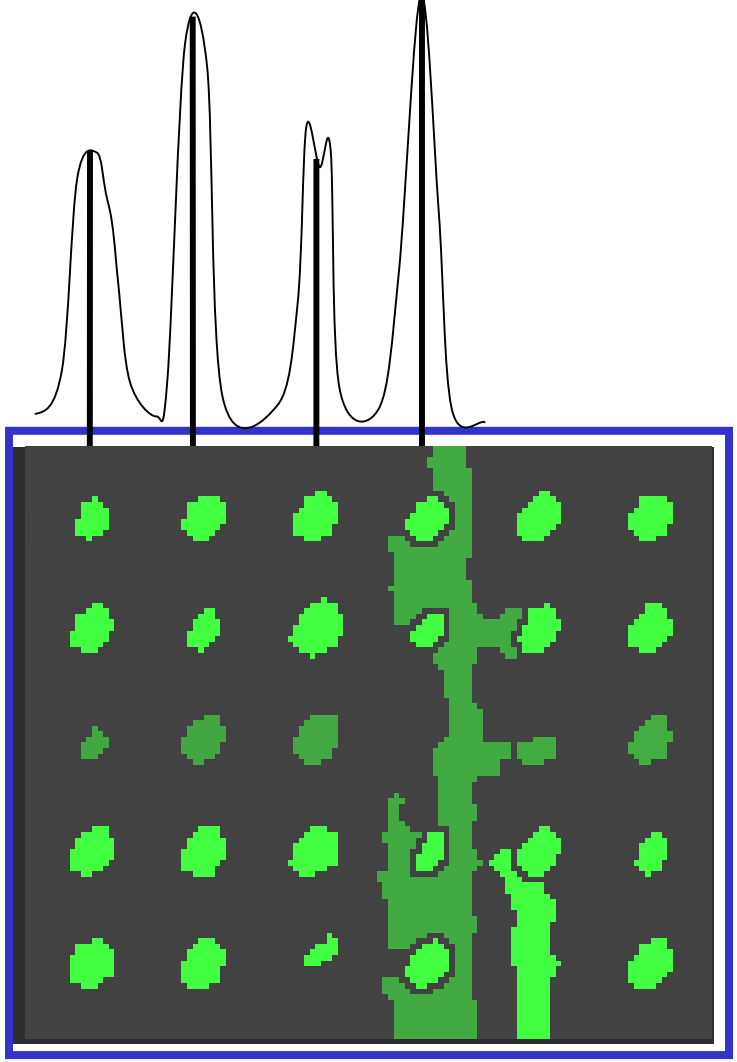

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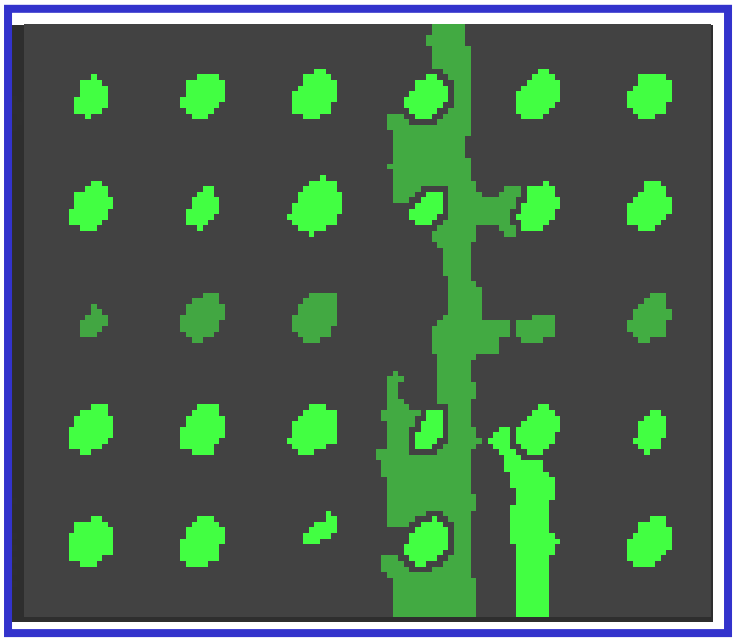

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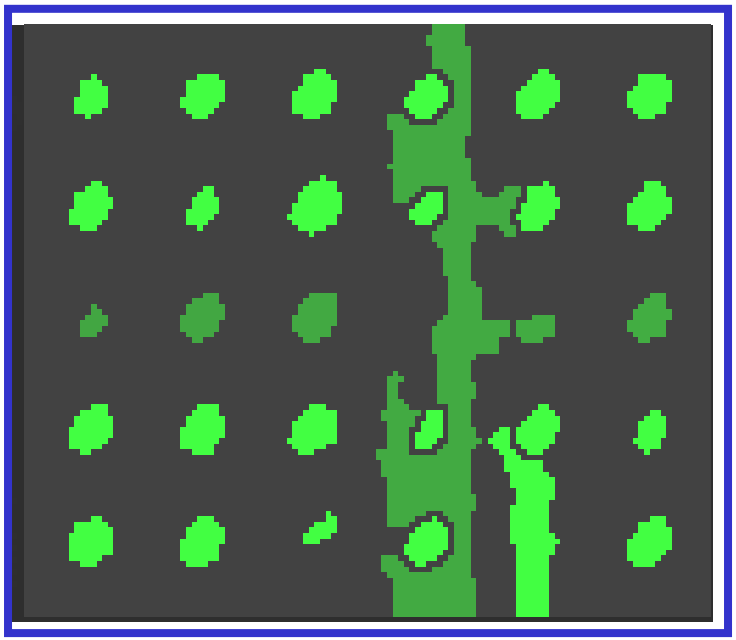

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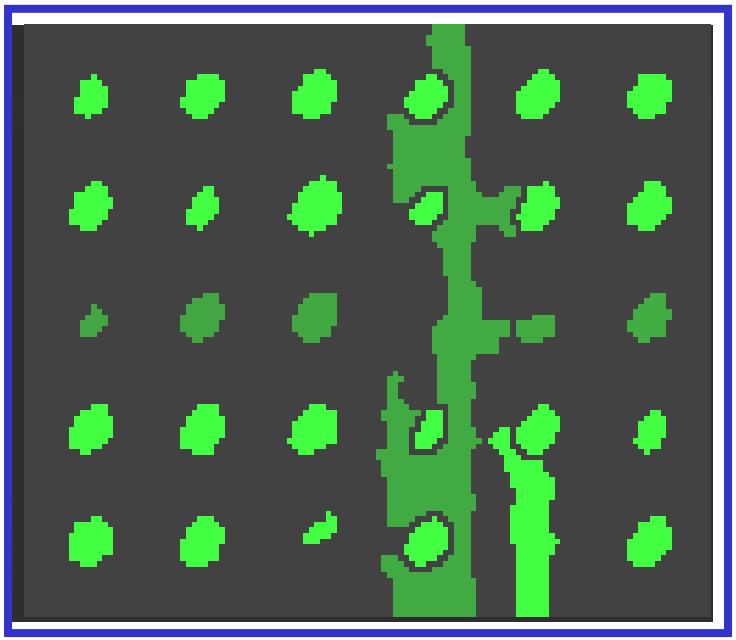
   - foreground intensities;
   - background intensities;
   - quality measures.



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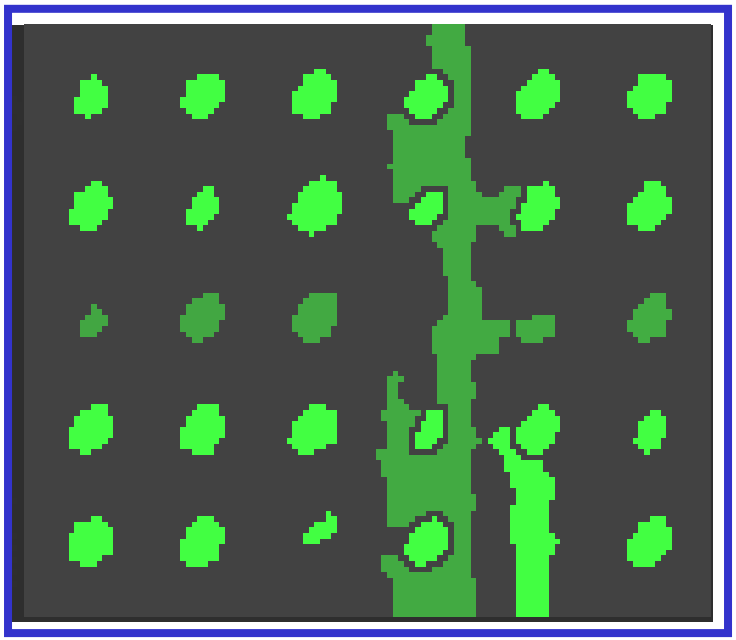
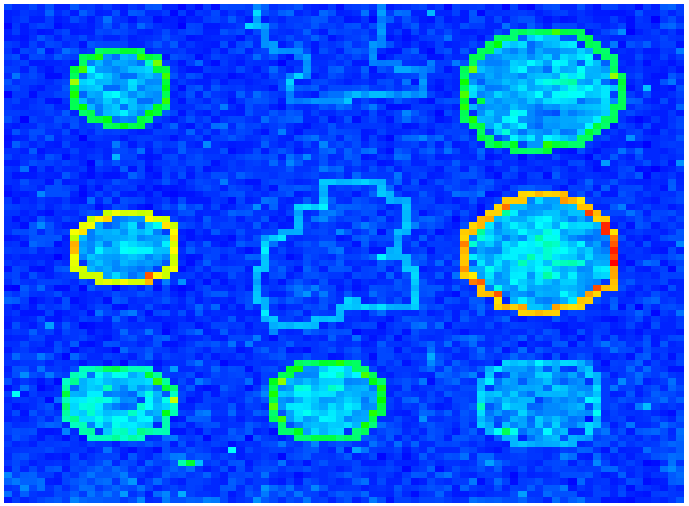
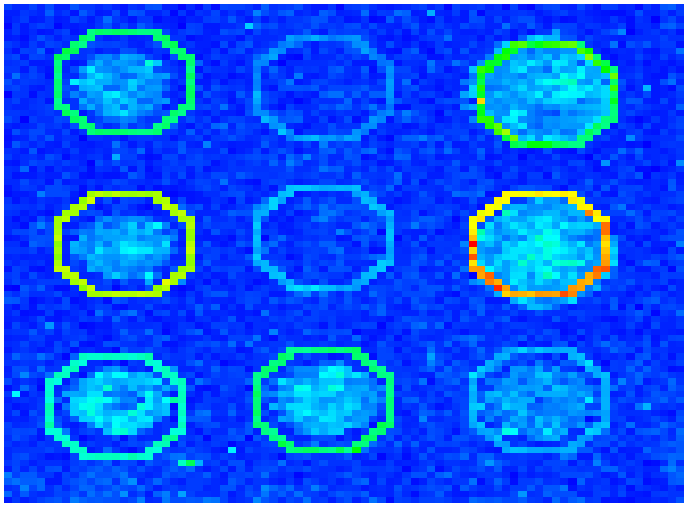


→ R and G for each spot on the array.

Segmentation



adaptive segmentation
seeded region growing



fixed circle segmentation

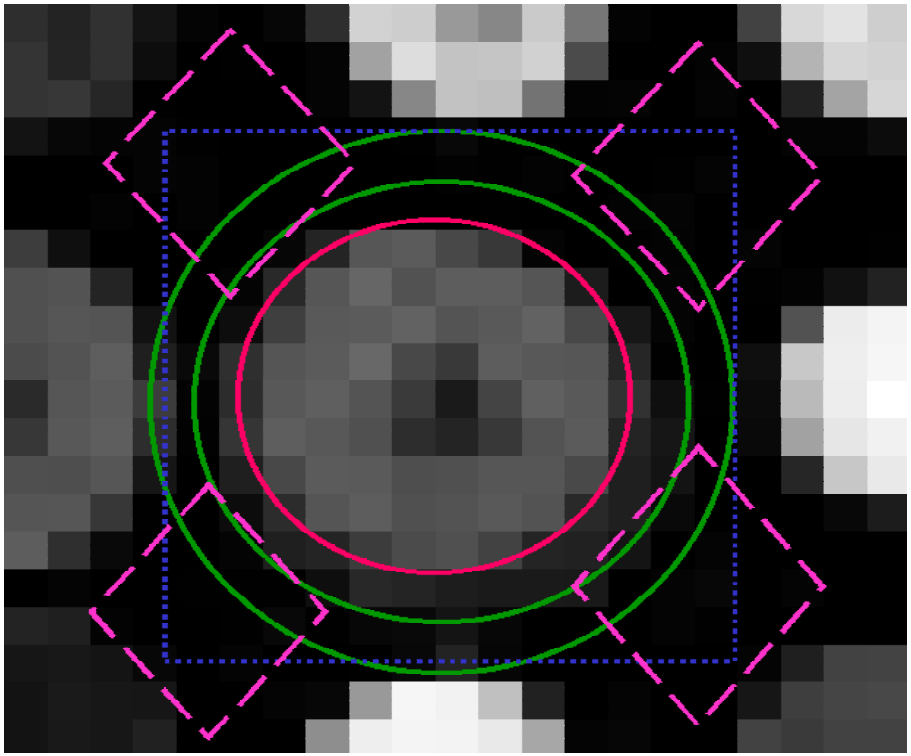
Spots may vary in size and shape.

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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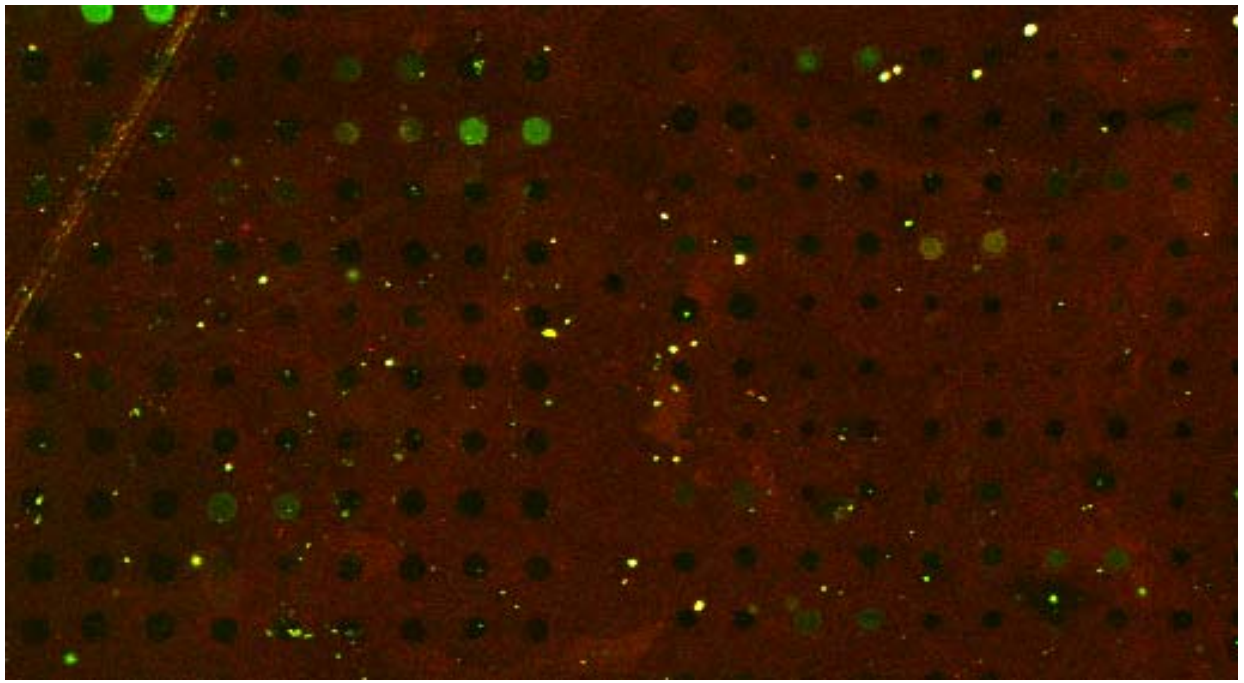


?

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

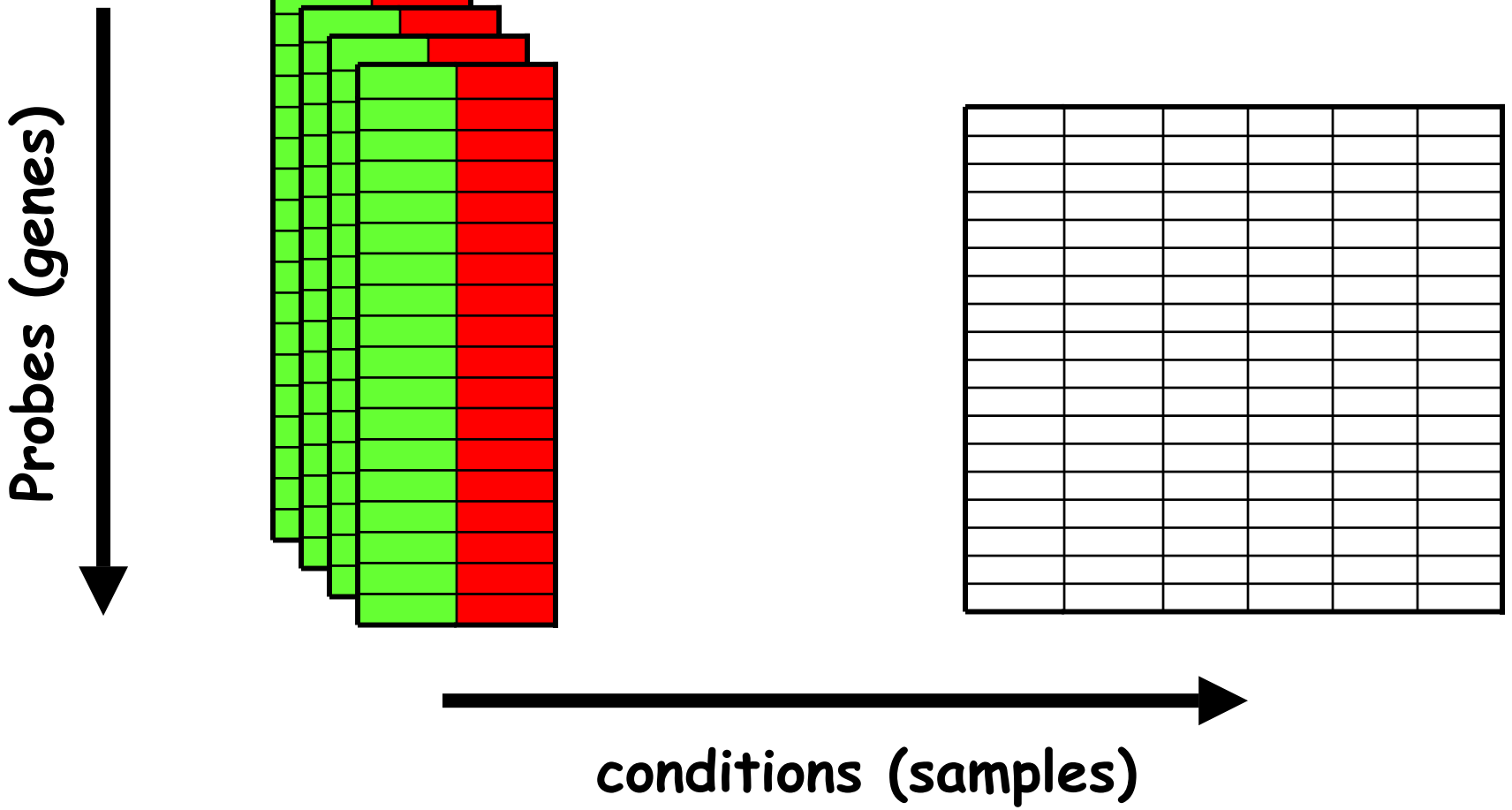
- Percentage of spots with no signal
- Range of intensities
- Distribution of spot signal area, etc.

How to use quality measures in subsequent analyses?

spot intensity data

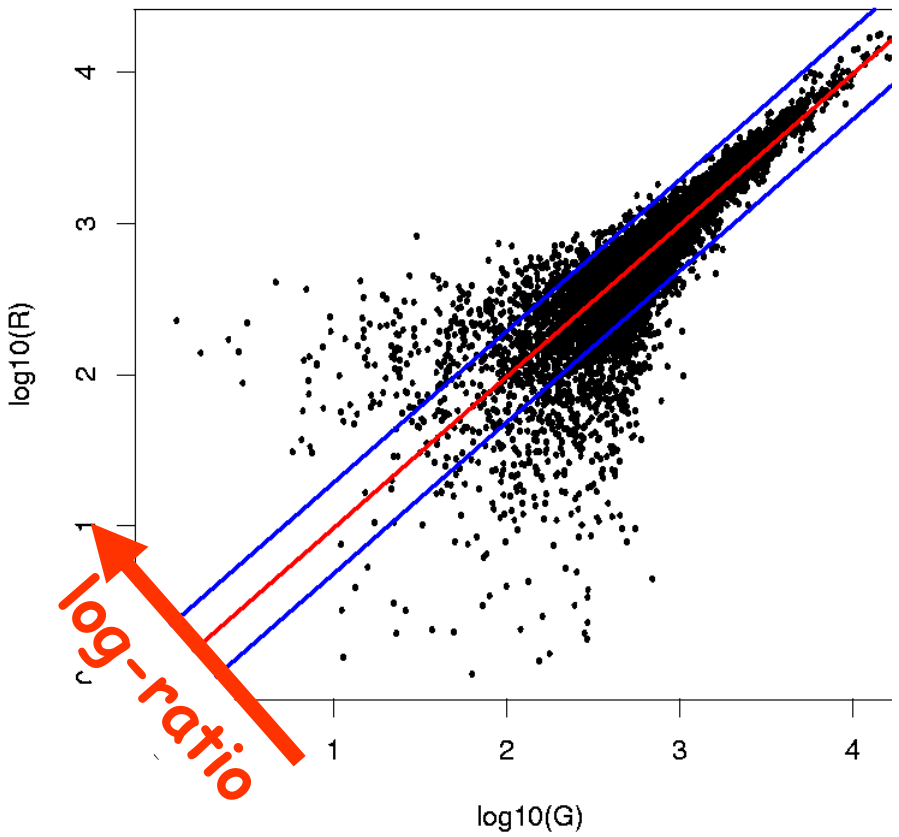
two-color spotted arrays

n one-color arrays
(Affymetrix, nylon)

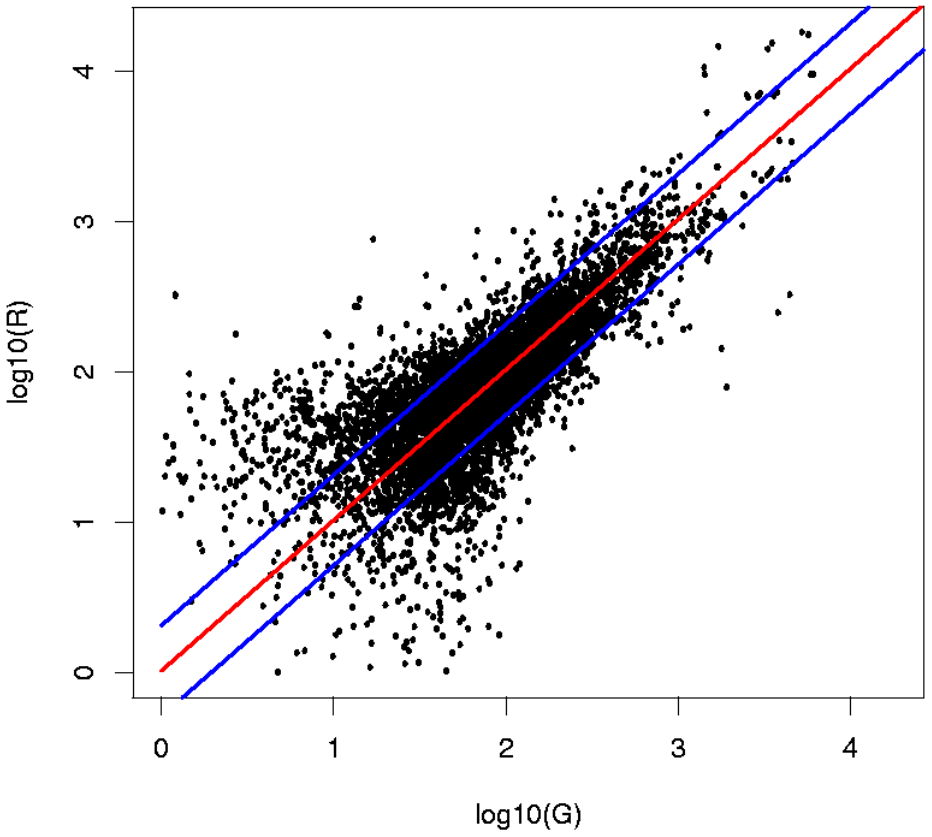


Which genes are differentially transcribed?

same-same



tumor-normal



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

Raw data are not mRNA concentrations

- 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,
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cross-/unspecific hybridization
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- corrections can be estimated from data

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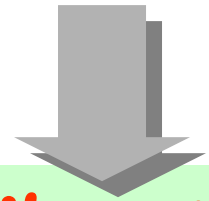

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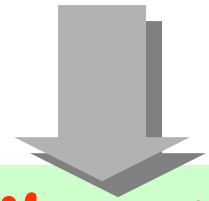

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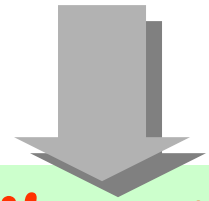

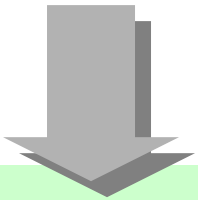

Calibration

Error model

measured intensity = offset + gain * true abundance

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

b_i per-sample normalization factor

b_k sequence-wise labeling efficiency

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

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

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

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"

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

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b_k sequence-wise labeling efficiency

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Calibration ("normalization")

Correct for systematic variations.

To do: fit appropriate "correction parameters" a_i, b_i, and apply to the data.

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Outliers:

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Outliers:

$\Rightarrow$ use a robust method

Ordinary regression

Minimize the sum of squares

$$SoS = \sum_{\text{all } i} (\text{residual } i)^2$$

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

Weighted regression

$$SoS = \sum_{\text{all } i} w_i \times (\text{residual } i)^2$$

If w_i = 1/variance(i), then minimizing SoS produces the maximum-likelihood estimate for a model with normal errors.

$$w(i) = \begin{cases} 1 / \text{variance(i)} & \text{if residual(i)} \le \text{median(residuals)} \\ 0 & \text{otherwise} \end{cases}$$

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Least Median Sum of Squares Regression:

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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.

the variance-mean dependence

model:

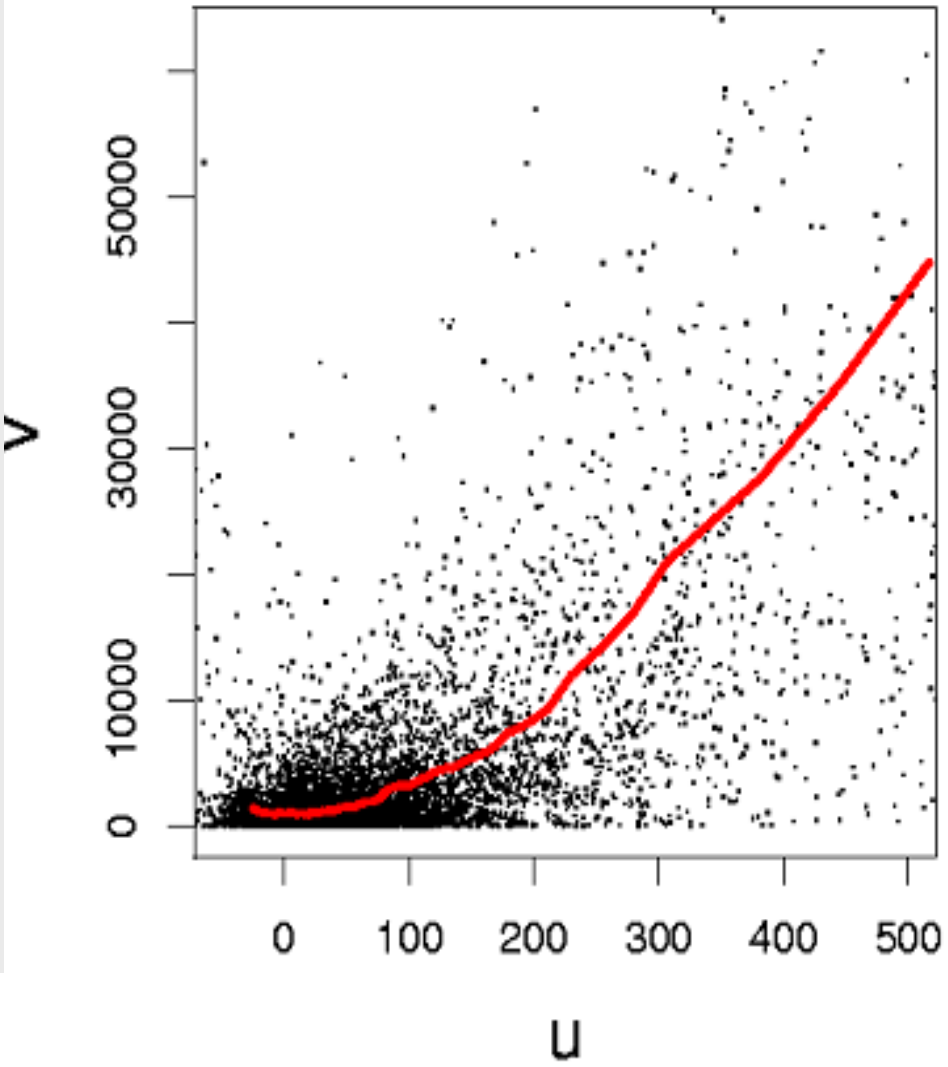
$\Rightarrow$ relation between

$u \equiv E(Y_{ik})$

$v \equiv Var(Y_{ik})$

$$v(u) = c^2(u + u_0)^2 + s^2$$

data (cDNA slide):



variance stabilization

X_u a family of random variables with

$EX_u = u$, $\mathrm{Var} X_u = v(u)$.

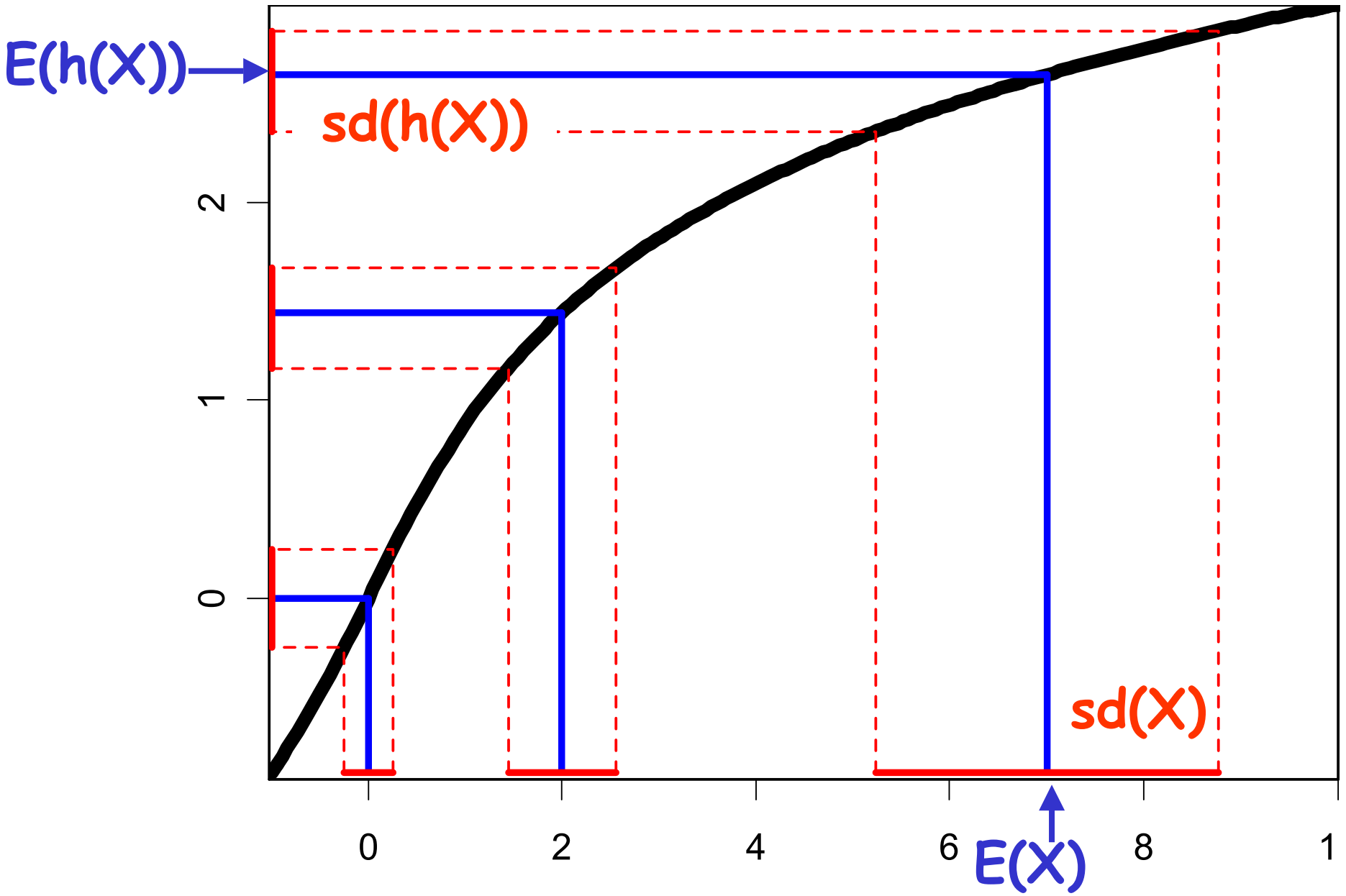
Define

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

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

derivation: linear approximation

variance stabilizing transformation



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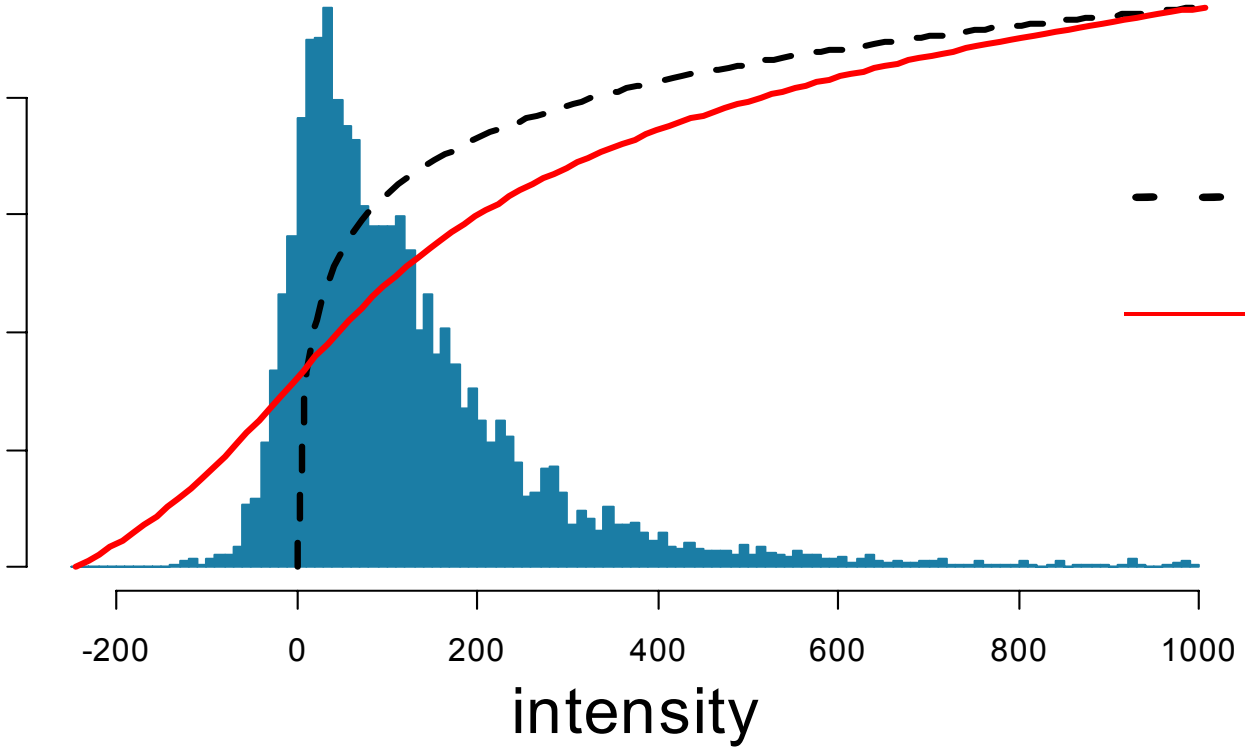
4.) microarray

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

the arsinh transformation



- - - log u

——— arsinh((u+u_0)/c)

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

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

parameter estimation

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

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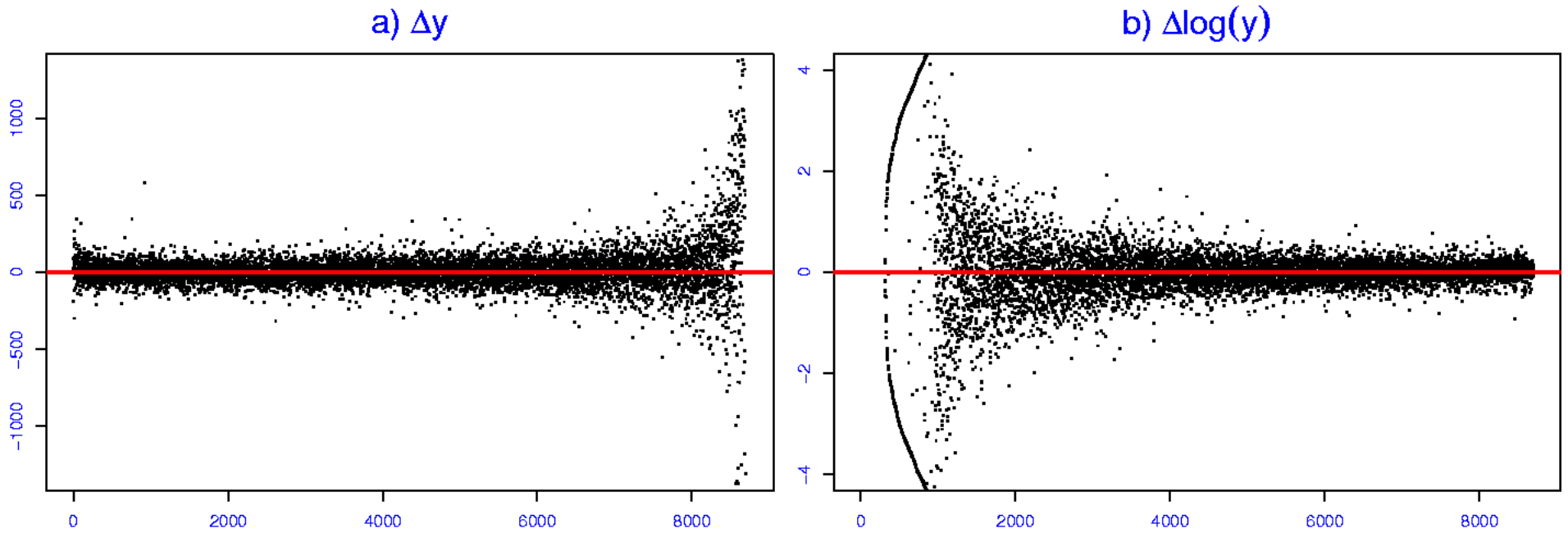
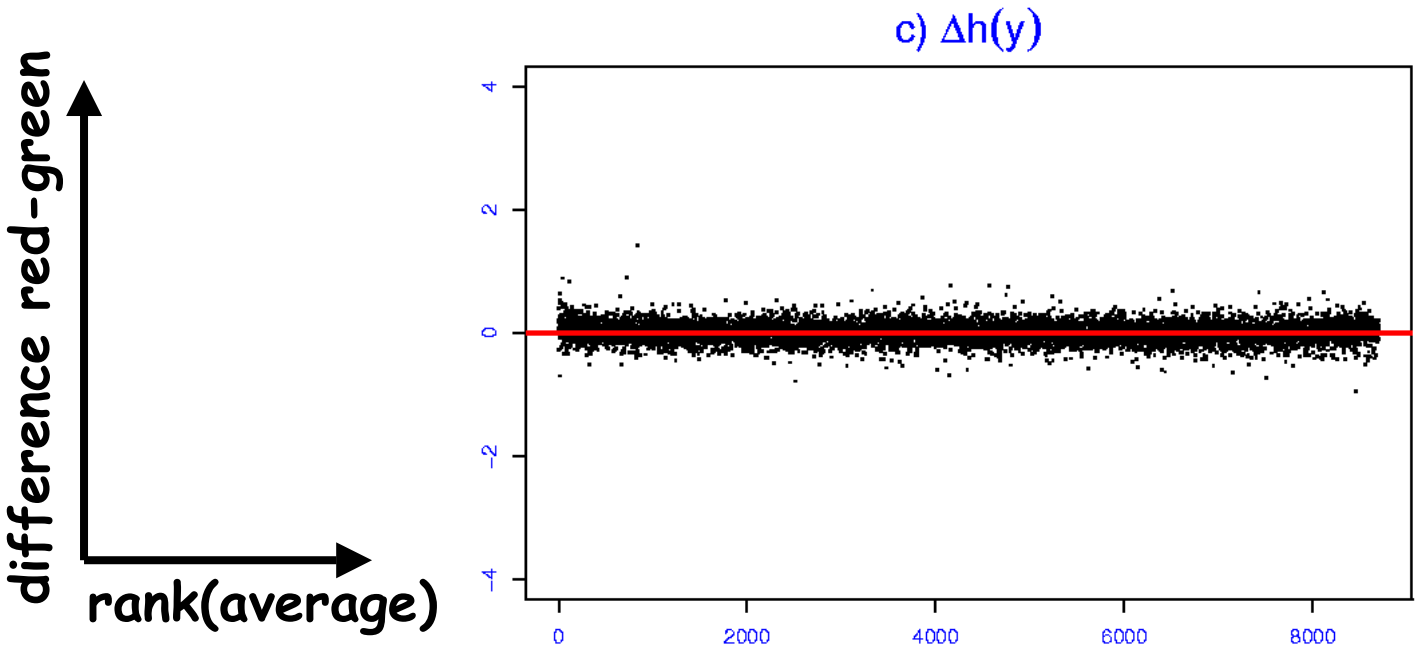
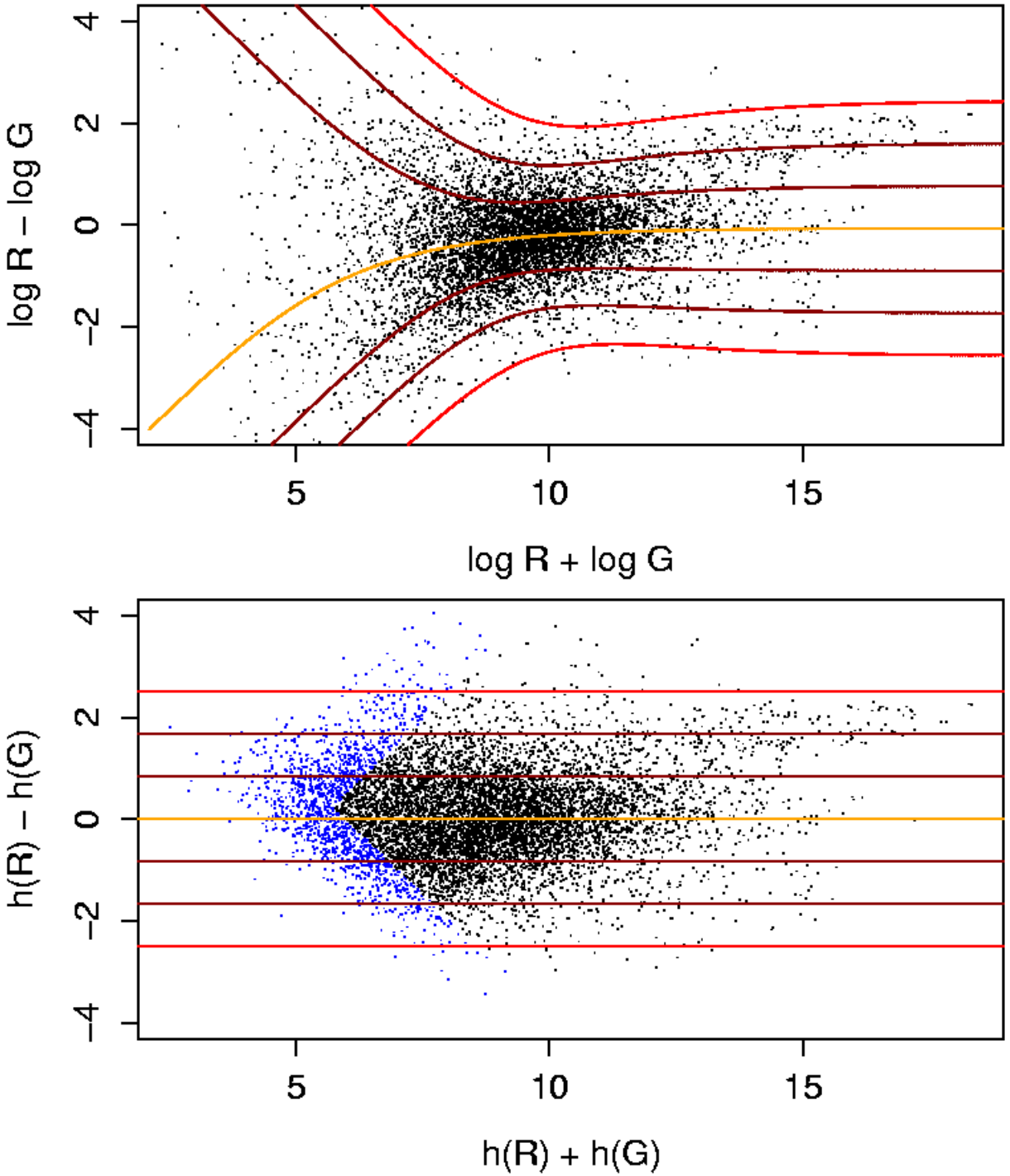
- robust variant of ML estimator, à la *Least Trimmed Sum of Squares* regression.

- works as long as <50% of genes are differentially transcribed

evaluation: effects of different data transformations







Coefficient of variation

cDNA slide:
H. Sueltmann

Summary

log-ratio

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

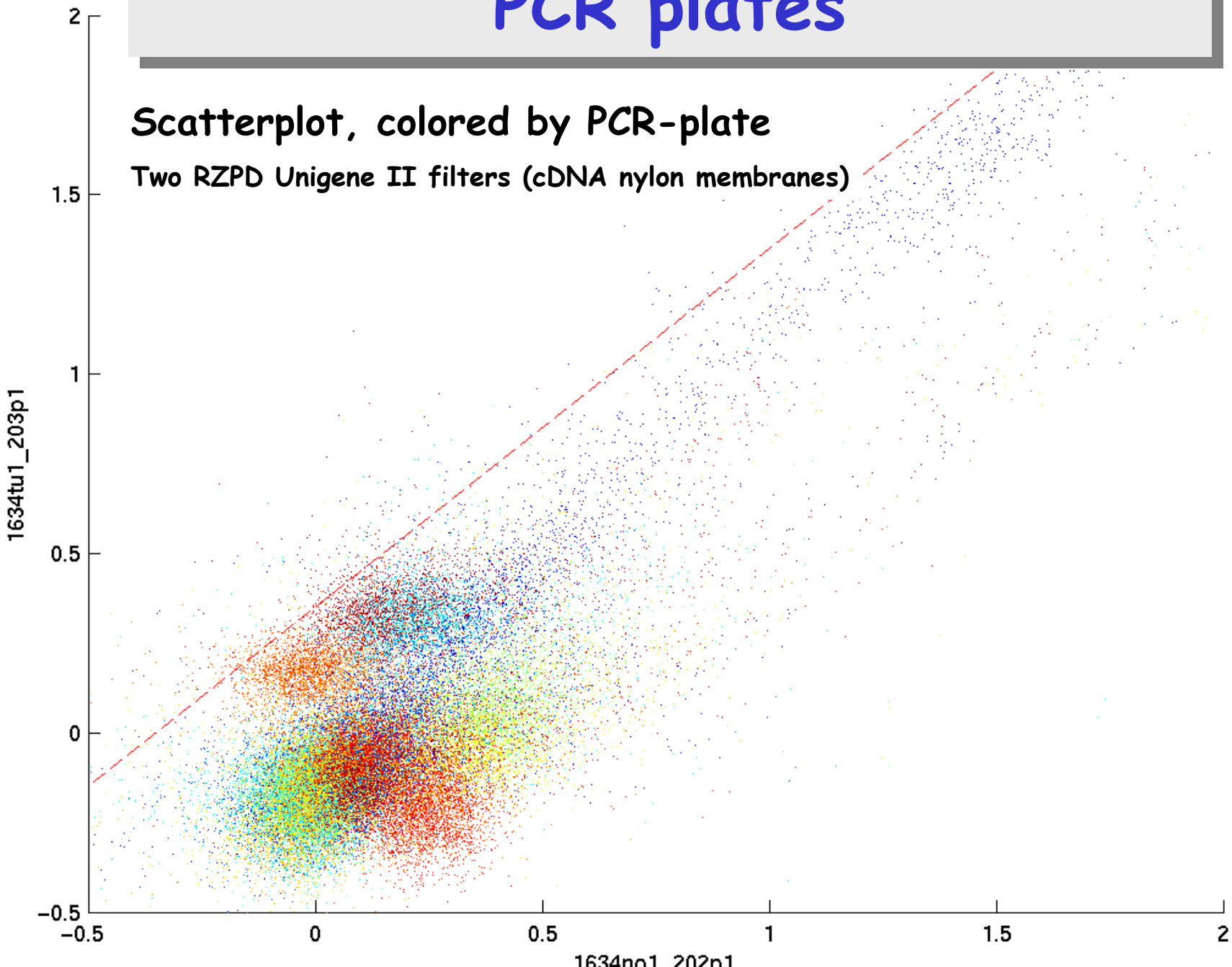
'generalized' log-ratio

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

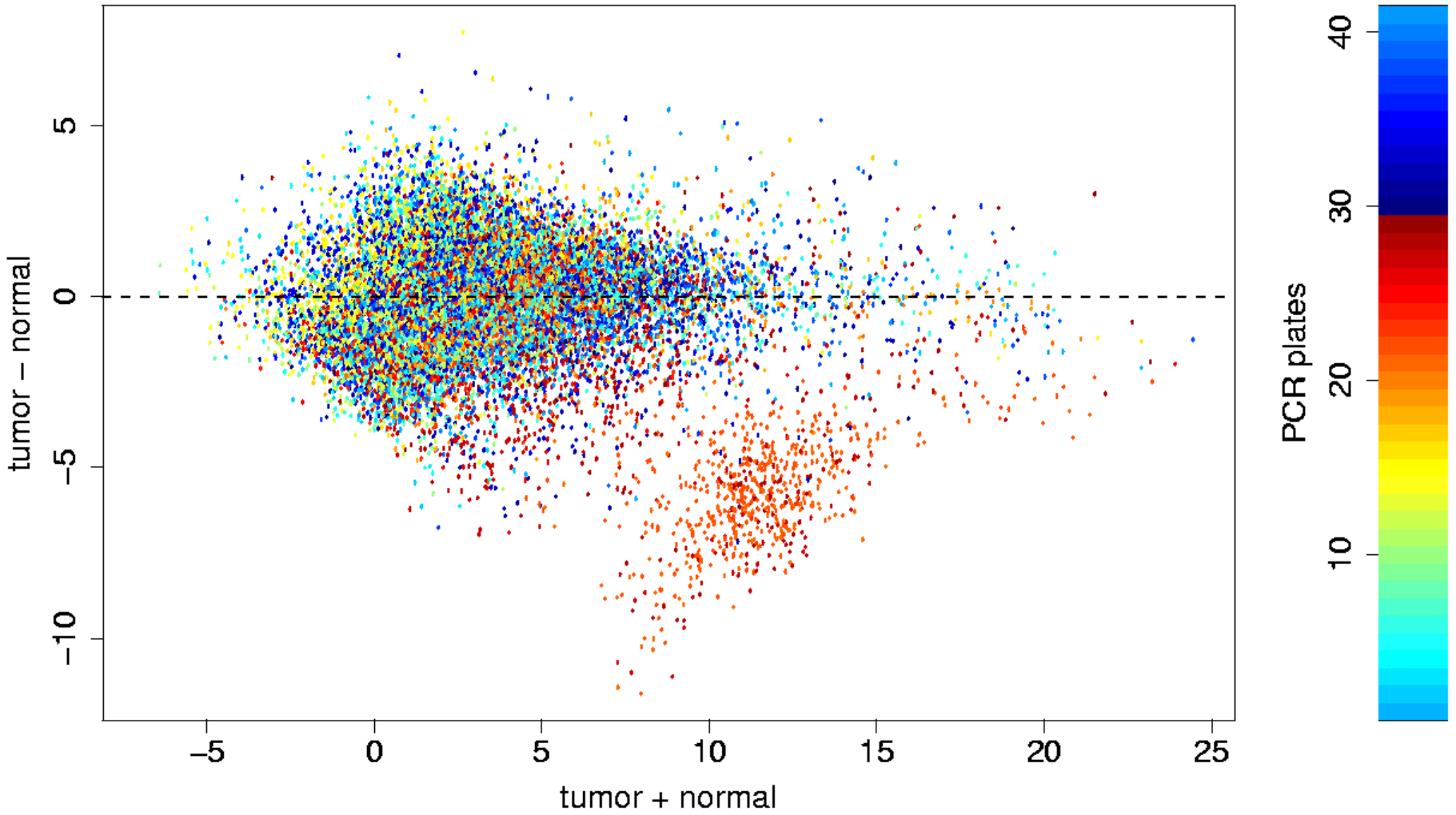
- advantages of variance-stabilizing data-transformation: generally better applicability of statistical methods (hypothesis testing, ANOVA, clustering, classification…)
- R package vsn

Quality control: diagnostic plots and artifacts

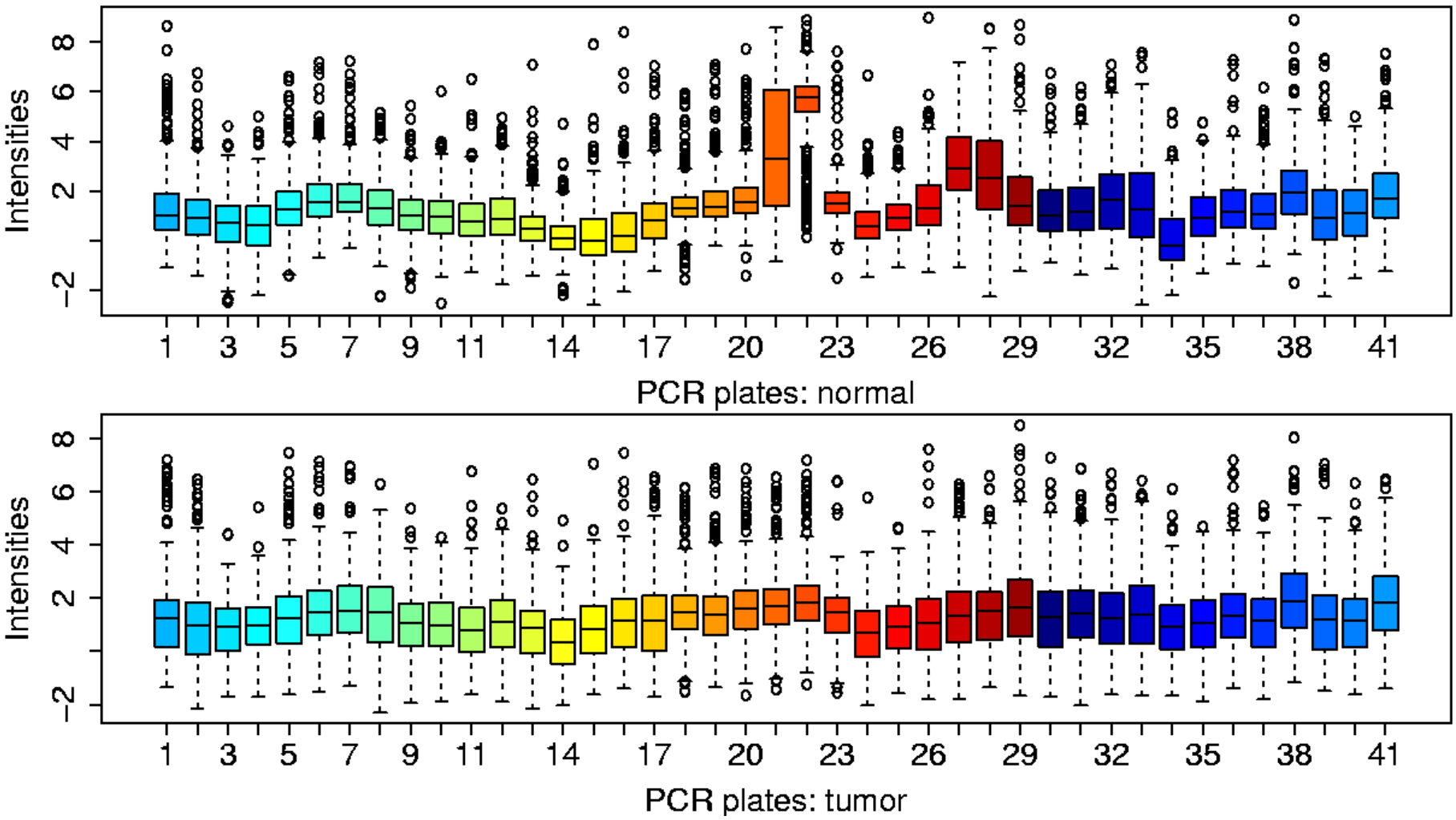
PCR plates


Scatterplot, colored by PCR-plate
Two RZPD Unigene II filters (cDNA nylon membranes)
1634tu1_203p1
1634no1_202p1
2
1.5
1
0.5
0
−0.5
−0.5
0
0.5
1
1.5
2

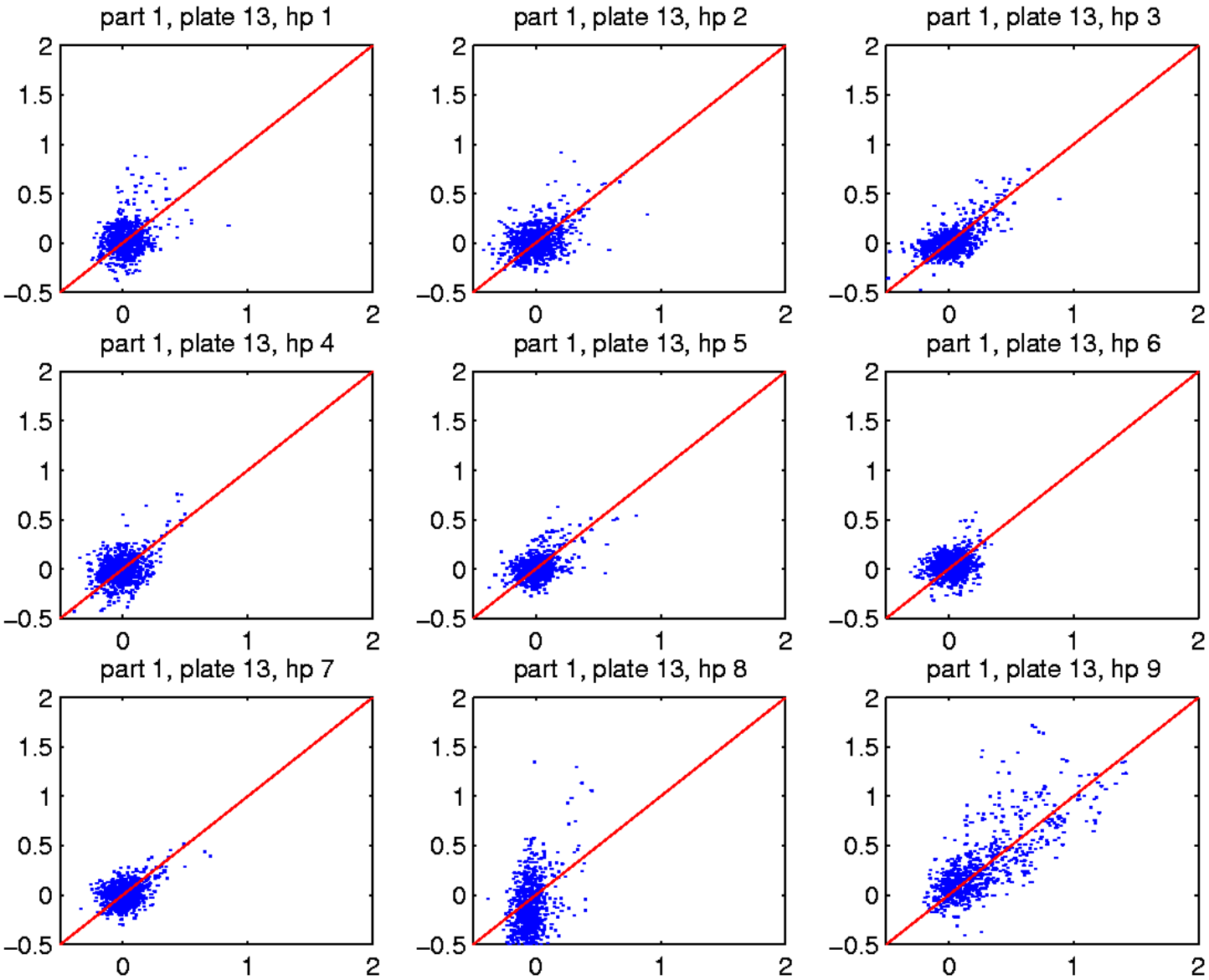
PCR plates


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

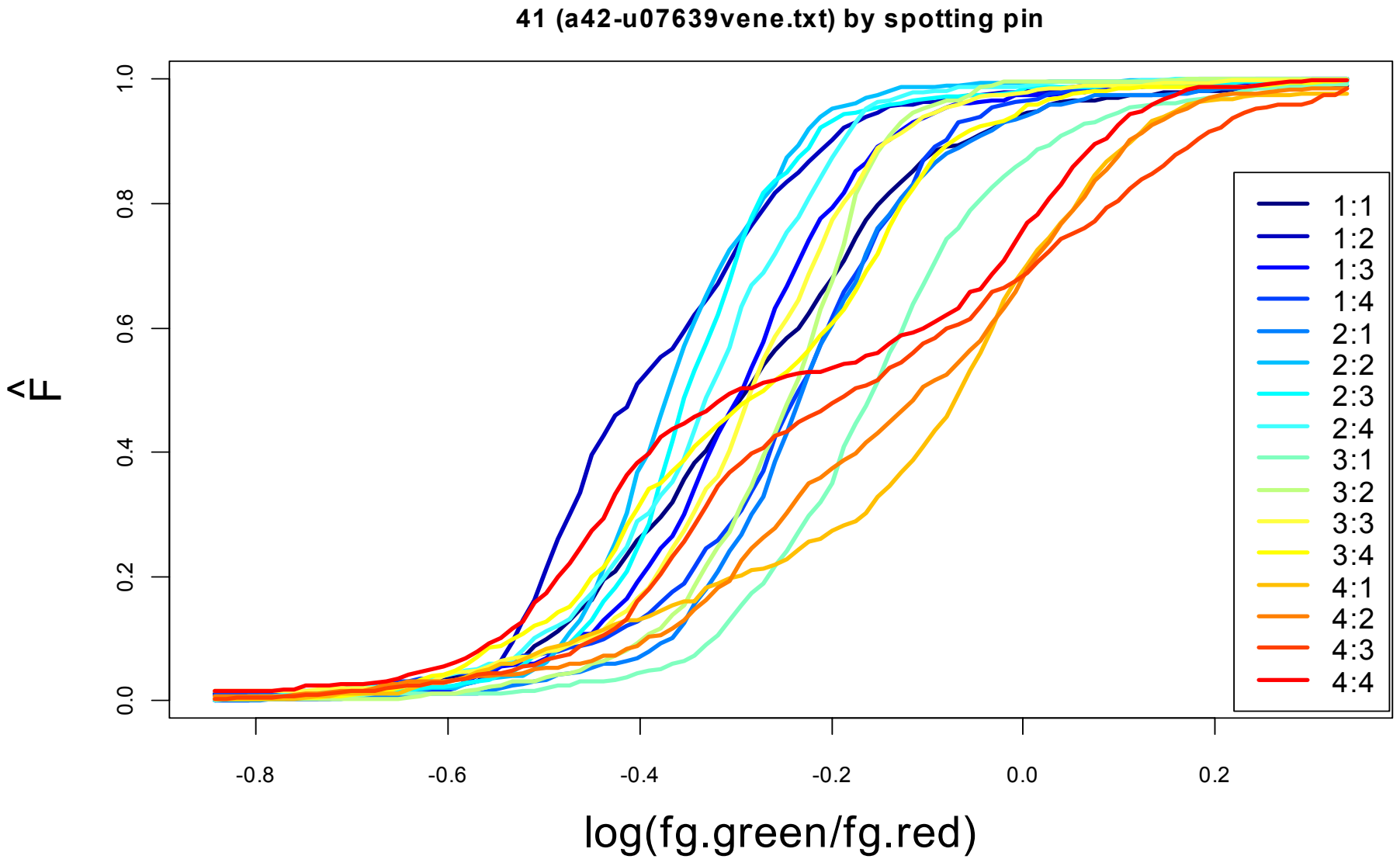
PCR plates: boxplots



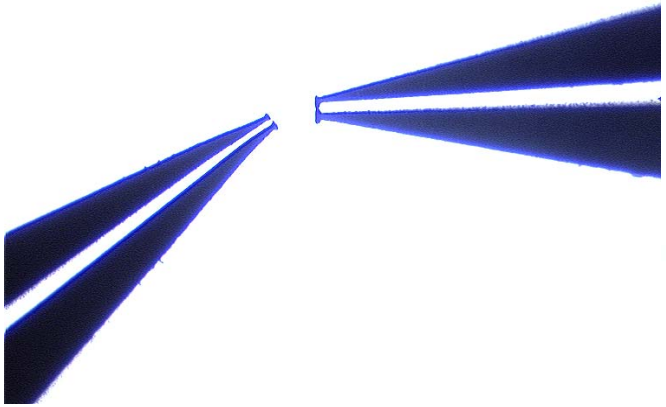
array batches



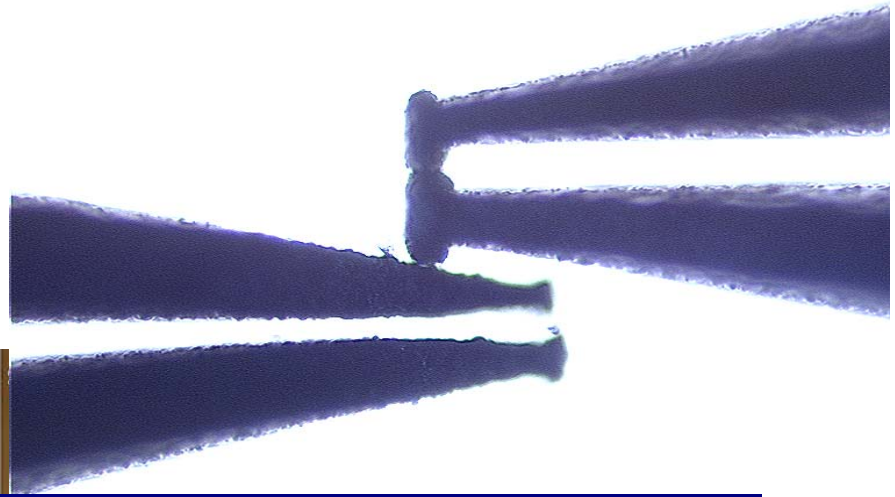
print-tip effects

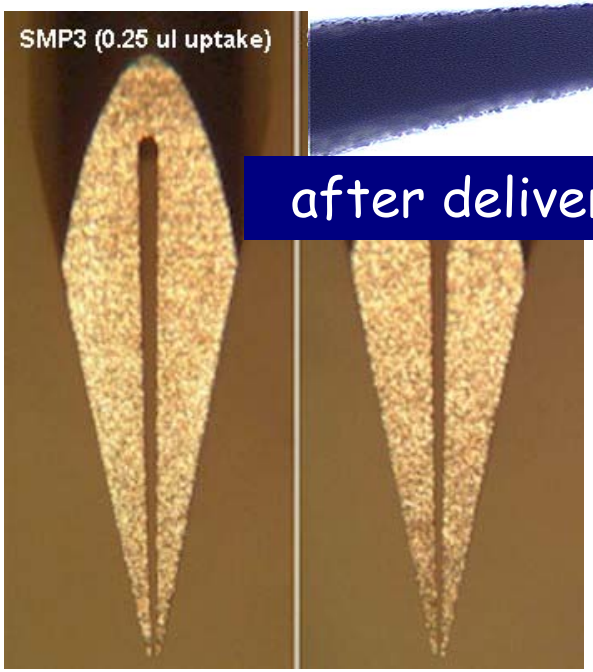


spotting pin quality decline

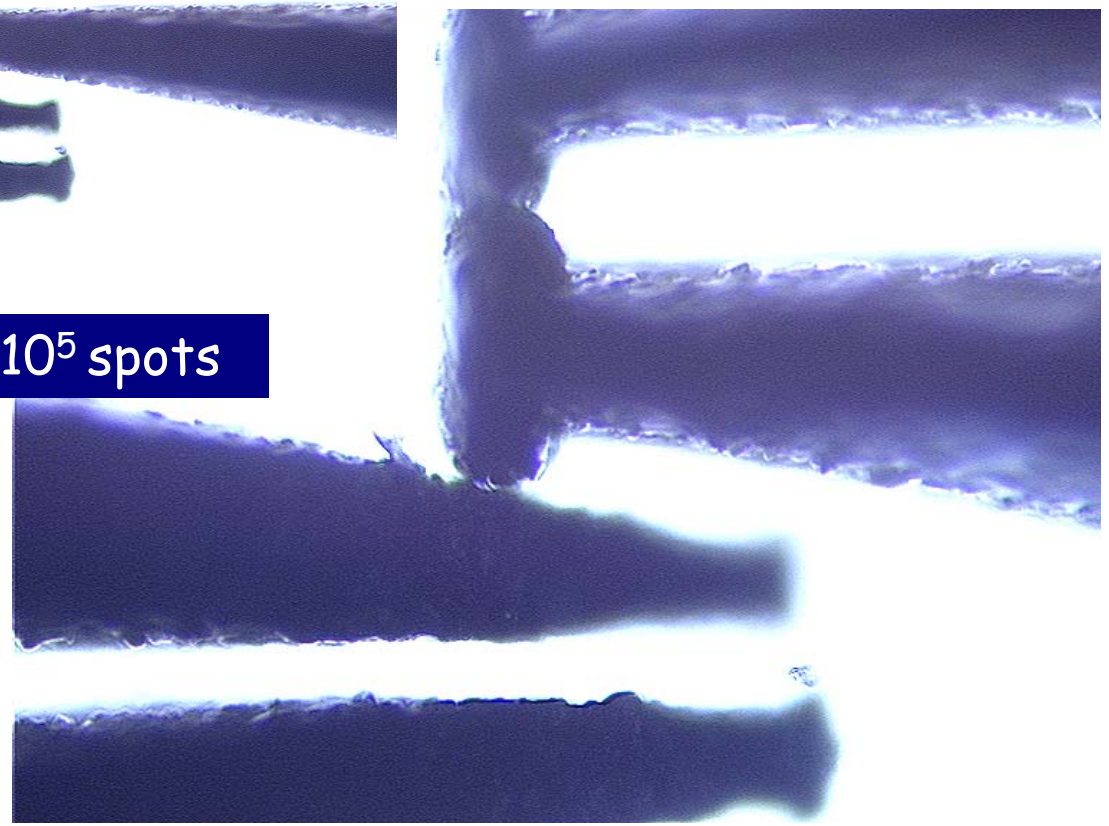


after delivery of $5x10^5$ spots



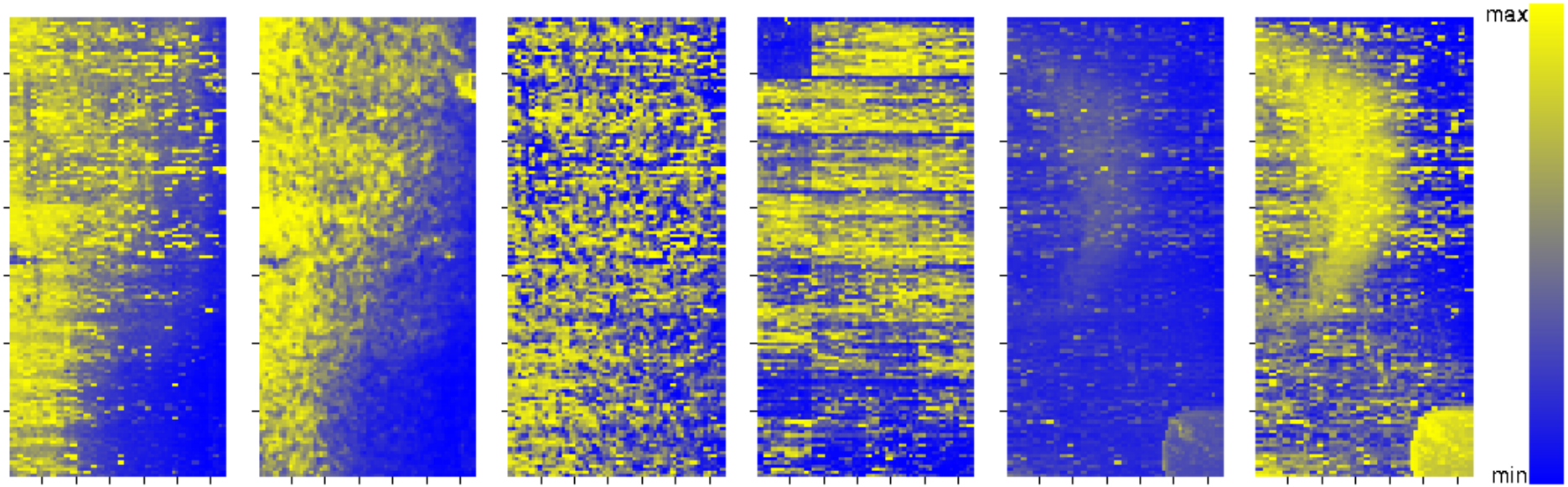


after delivery of $3x10^5$ spots



H. Sueltmann DKFZ/MGA

spatial effects



R Rb R-Rb
color scale by rank

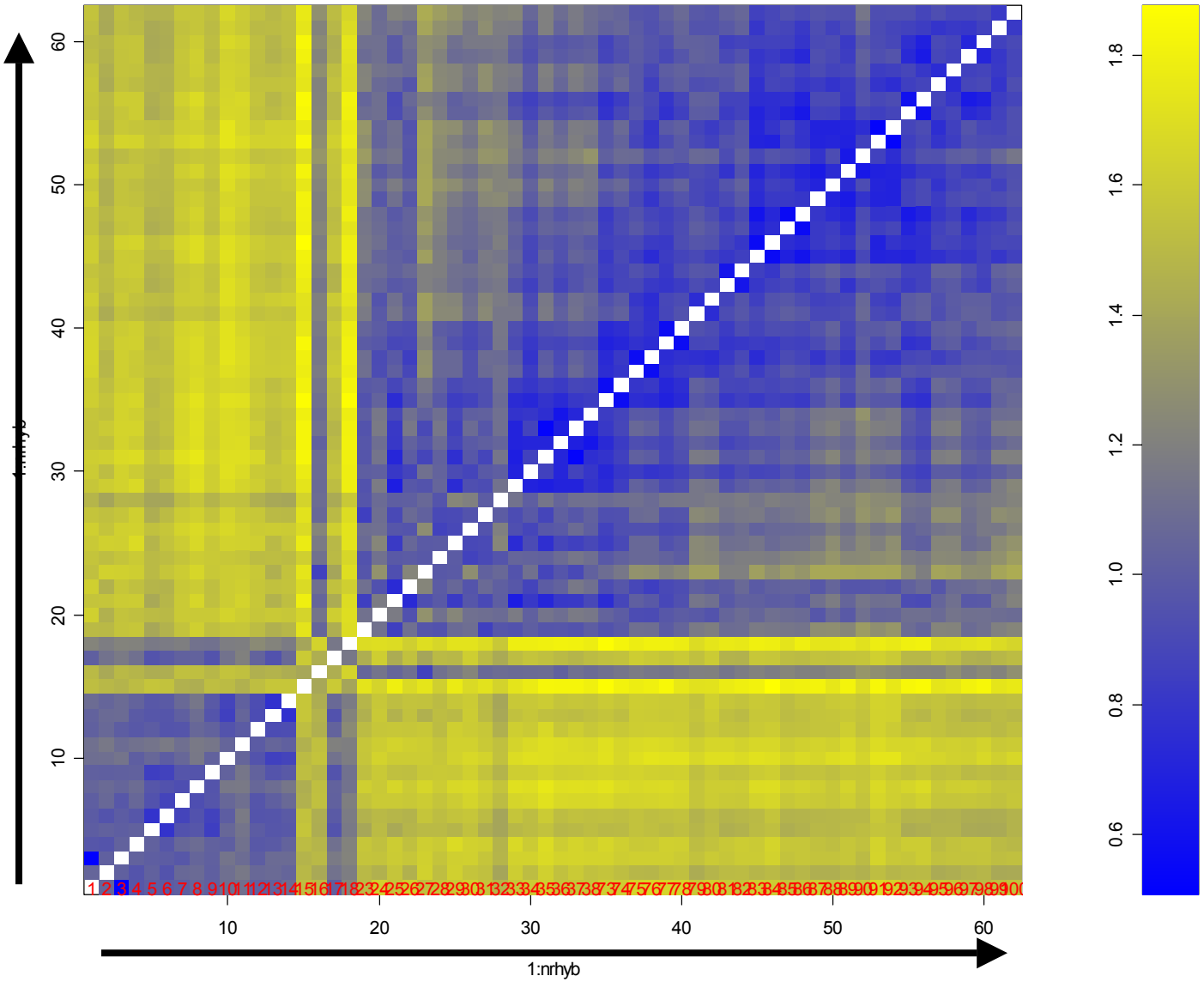
another array: print-tip

color scale ~ log(G)

color scale ~ rank(G)

spotted cDNA arrays, Stanford-type

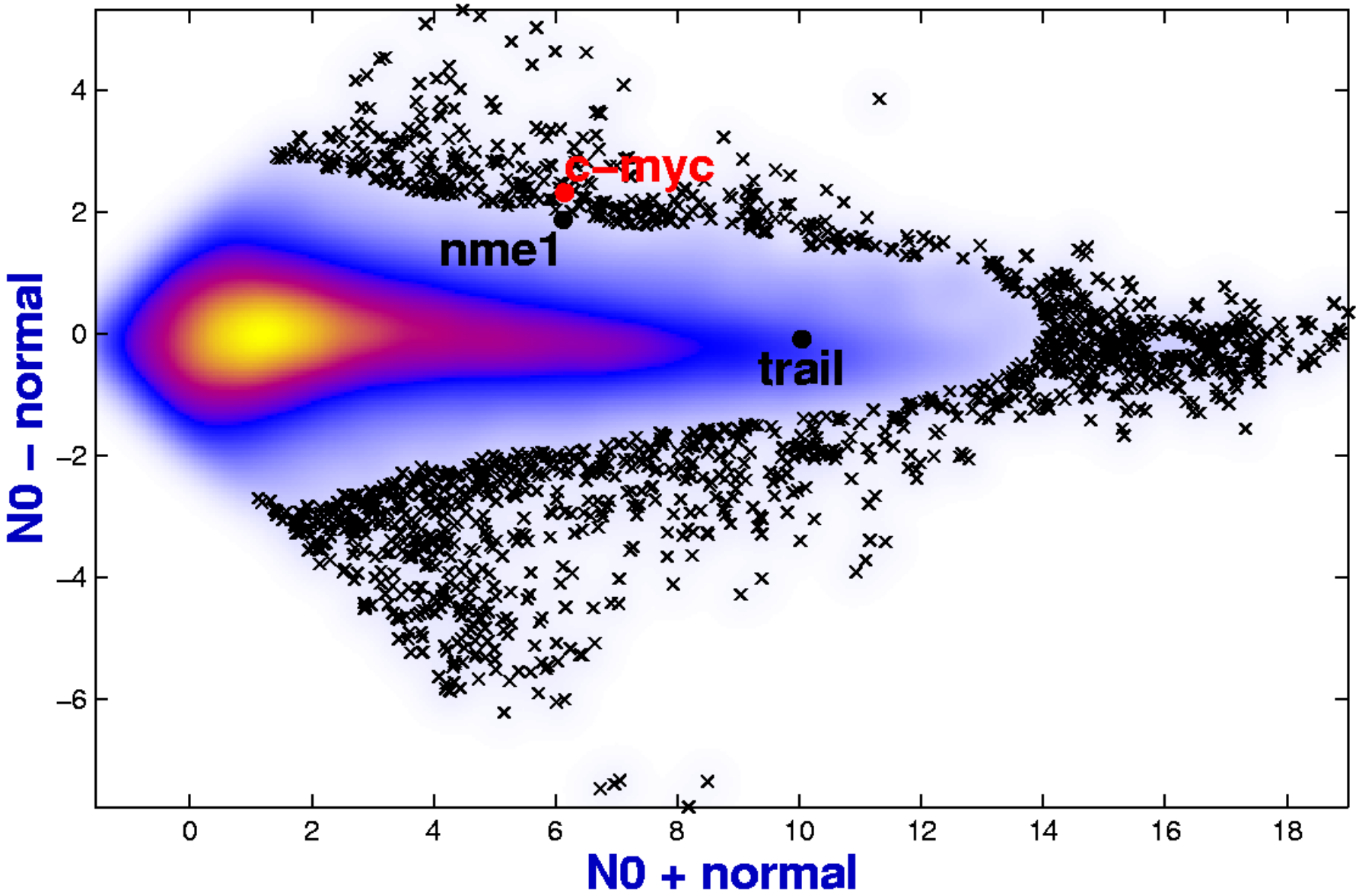
Batches: array to array differences $d_{ij} = mad_k(h_{ik} - h_{jk})$



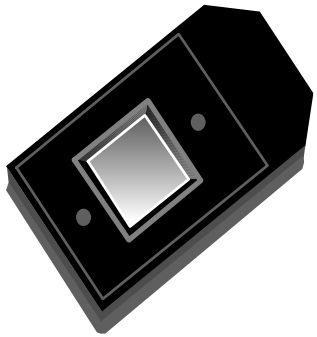
arrays i=1…63; roughly sorted by time

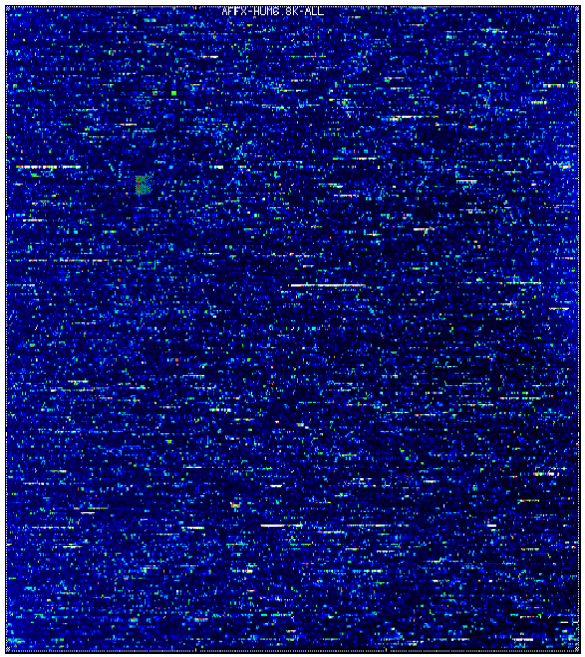
Density representation of the scatterplot

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



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

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

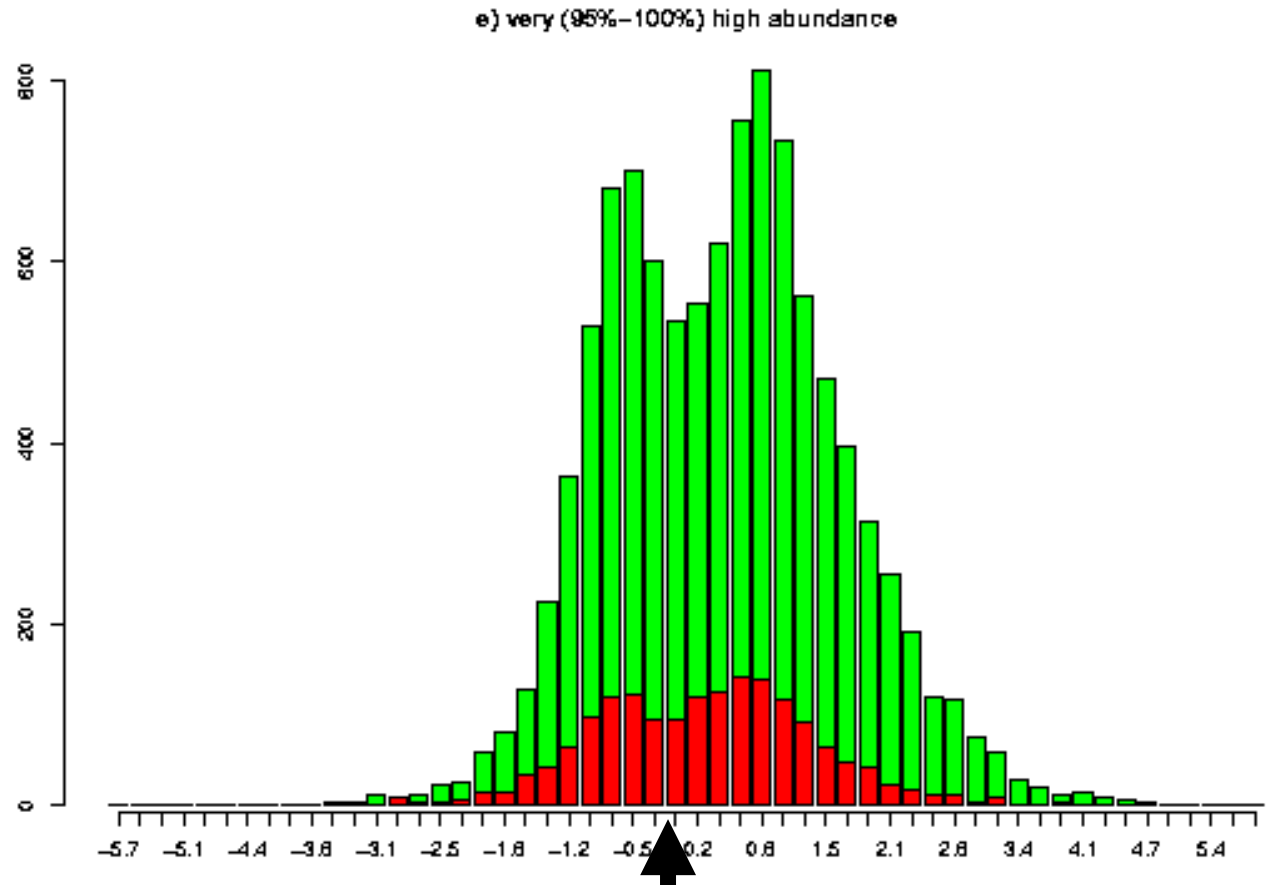
- θ_i: expression index for gene i
- ϕ_j: probe sensitivity

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.

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



0

log(PM/MM)

From: R. Irizarry et al.,
Biostatistics 2002

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.

Robust expression measures
RMA: Irizarry et al. (2002)

AvDiff-like

$$RMA = \frac{1}{|A|}\sum_{j \in A} \log_2(PM_j - BG_j)$$

with A a set of "suitable" pairs.

Li-Wong-like: additive model

$$\log_2(PM_{ij} - BG) = a_i + b_j + \varepsilon_{ij}$$

Estimate RMA = a_i for chip *i* using robust method median polish (successively remove row and column medians, accumulate terms, until convergence). Works with d>=2

Software for pre-processing of Affymetrix data

- Bioconductor R package affy.
- Background estimation.
- Probe-level normalization.
- Expression measures
- Two main functions: ReadAffy, expresso.
- Can use vsn as a normalization method for expresso.

References

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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/