Monday
3. March 08

Microarray: Quality Control, Normalization and Design

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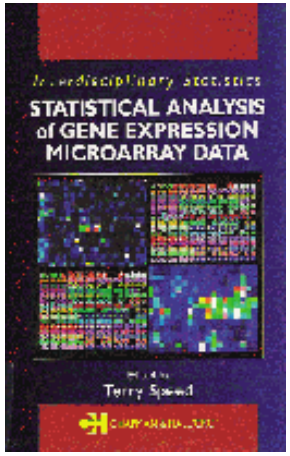
Overview

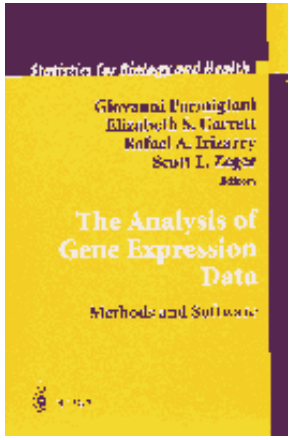
- Introduction to microarray technologies
- Image Processing:
  Spot Identification, Spot/Background quantification, Quality Measures
- Normalization:
  Scaling, Quantile, Lowess, vsn
- Experimental Design:
  Comparison of typical Designs
- Affy Issues

Books

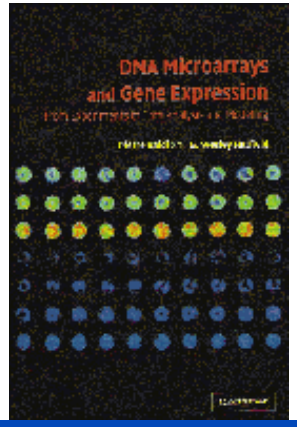
- Terry Speed, „Statistical Analysis of Gene Expression Microarray Data”. Chapman & Hall/CRC



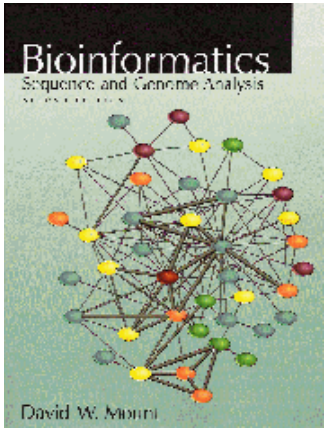
- Giovanni Parmigani et al, „The Analysis of Gene Expression Data“, Springer



- Pierre Baldi & G. Wesley Hatfield, „DNA Microarrays and Gene Expression”, Cambridge

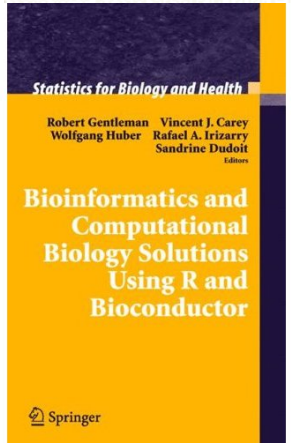


- David W. Mount, „Bioinformatics“, Cold Spring Harbor



- Reinhard Rauhut, „Bioinformatik“, Wiley-VCH

- Gentleman, Carey, Huber, “Bioinformatics and Computational Biology Solutions Using R and Bioconductor”, Springer



Online

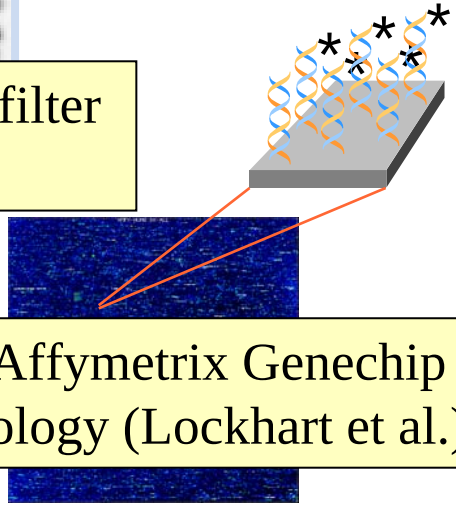
- NGFN Course „Practical DNA Microarray Analysis”: http://compdiag.molgen.mpg.de/lectures.shtml
- Lectures Terry Speed, Berkeley: http://www.stat.berkeley.edu/users/terry/Classes/
- R/Bioconductor Dokumentation (Vignetten): http://www.bioconductor.org
- Google, Pubmed, Wikipedia

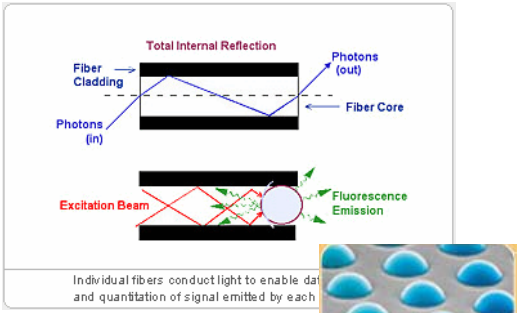
1975: Southern Blotting Technology (Edward Southern)

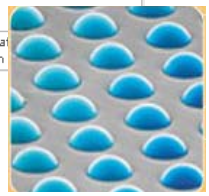
2003: Illumina Bead Arrays

SAGE

1991: First high-density Nylon filter Arrays (Lennon, Lehrach)







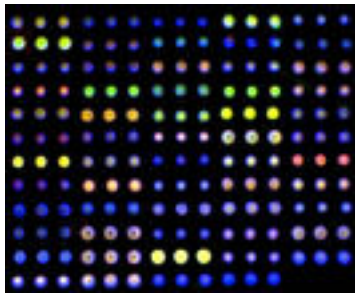
Illumina Bead Array

Different Technolo
for Measuring Ge
Expression

1996: Affymetrix Genechip Technology (Lockhart et al.)

GeneChip Affymetrix

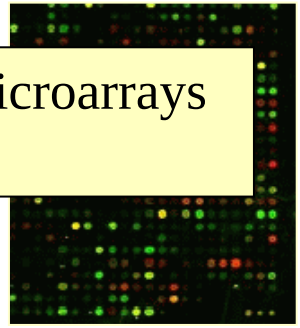
Agilent: Long oligo Ink Jet



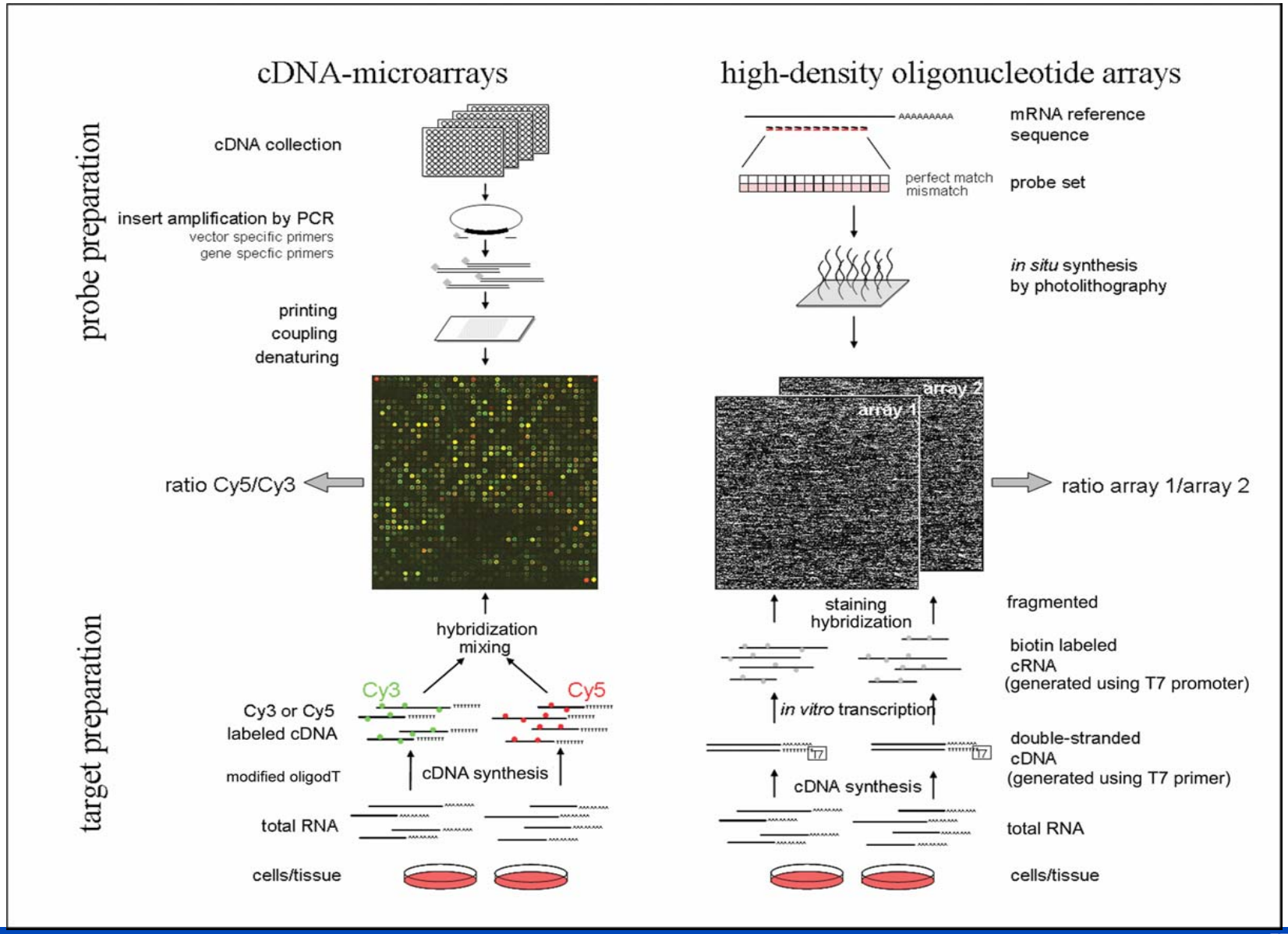
CGH

cDNA microarray

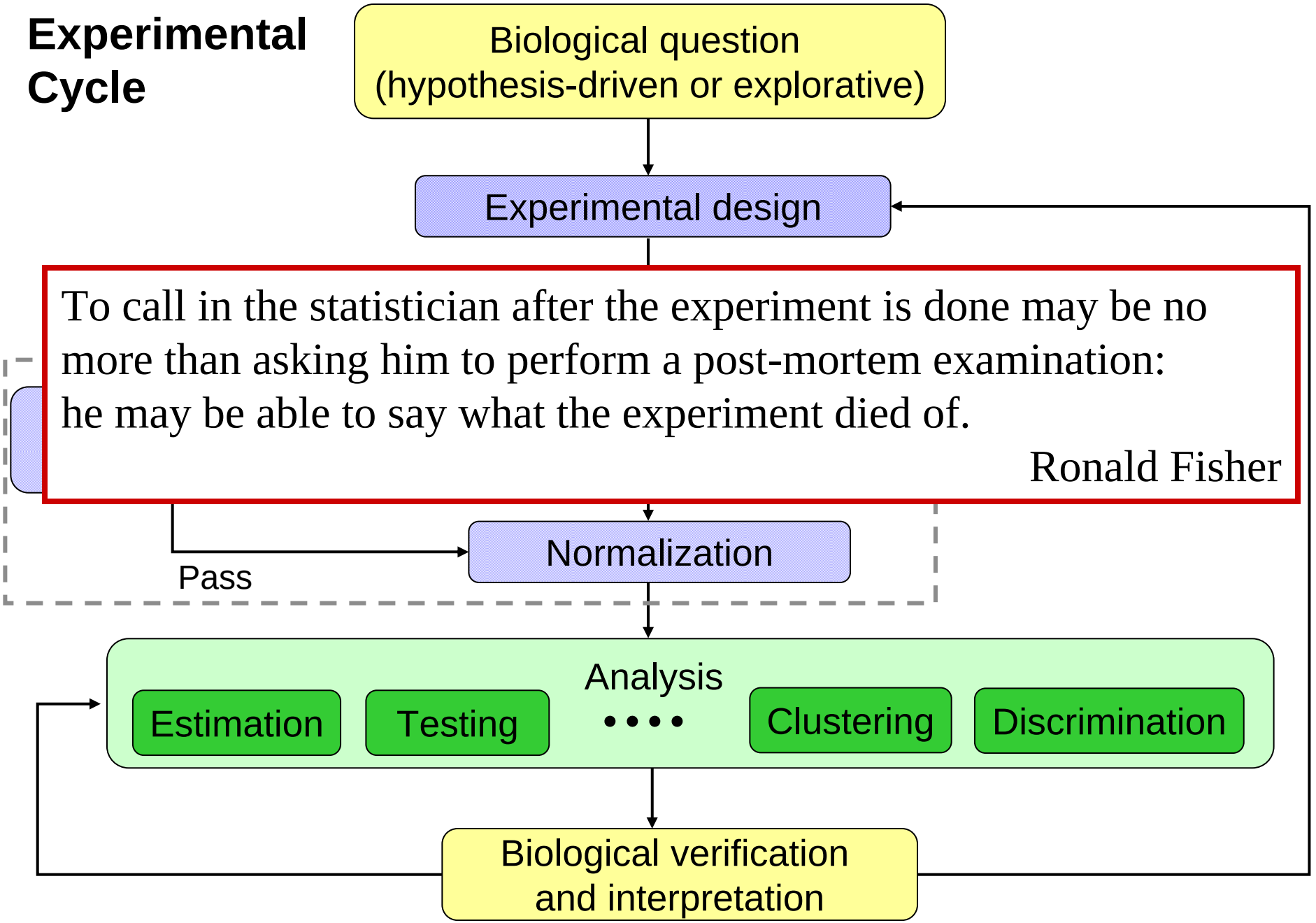
1995: cDNA-Microarrays (Schena et al.)



cDNA and Affymetrix (short, 25 bp) Oligo Technologies. Long Oligos (60-75 bp) are used similar to cDNA.



Experimental Cycle



Gene-expression-data

gene expression-data for **G** genes and **n** hybridiyations.
Genes times arrays data-matrix:

mRNA Samples

Gene	sample1	sample2	sample3	sample4	sample5	...
1	0.46	0.30	0.80	1.51	0.90	...
2	-0.10	0.49	0.24	0.06	0.46	...
3	0.15	0.74	0.04	0.10	0.20	...
4	-0.45	-1.03	-0.79	-0.56	-0.32	...
5	-0.06	1.06	1.35	1.09	-1.09	...

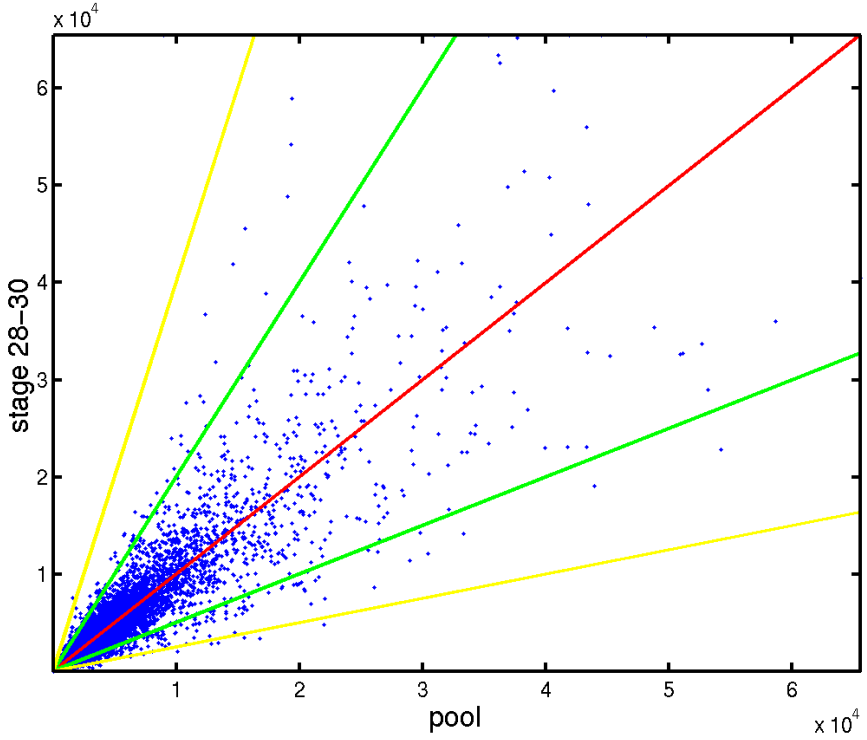
gene-expression level or ratio for gene *i* in mRNA sample *j*

M =
- Log_2(red intensity / green intensity)
- Function (PM, MM) of MAS, dchip or RMA

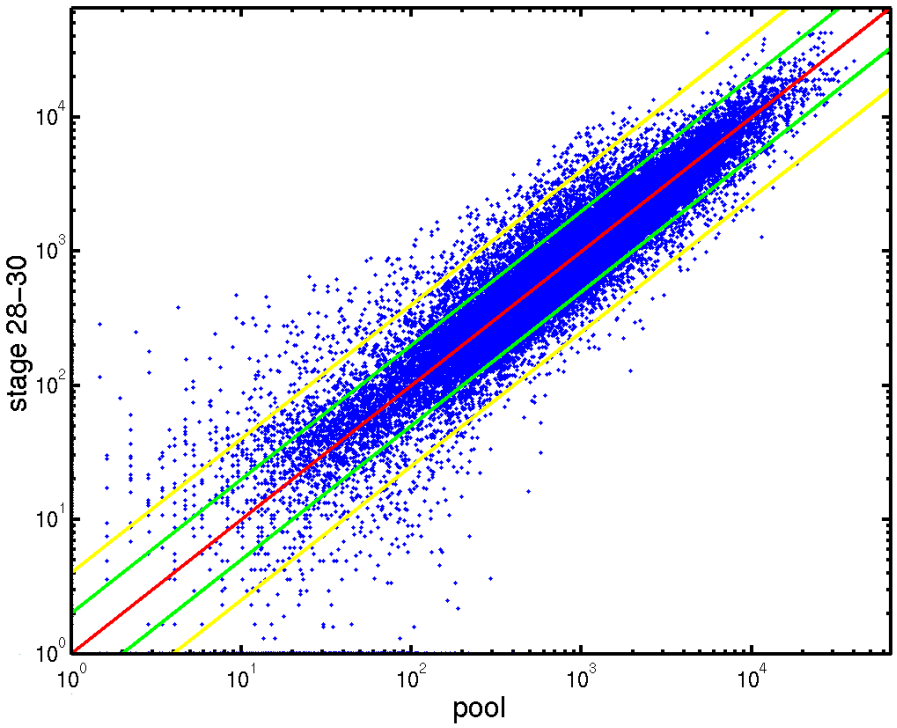
A =
- average: $\log_2$(red intensity), $\log_2$(green intensity)
- Function (PM, MM) of MAS, dchip or RMA

Scatterplot

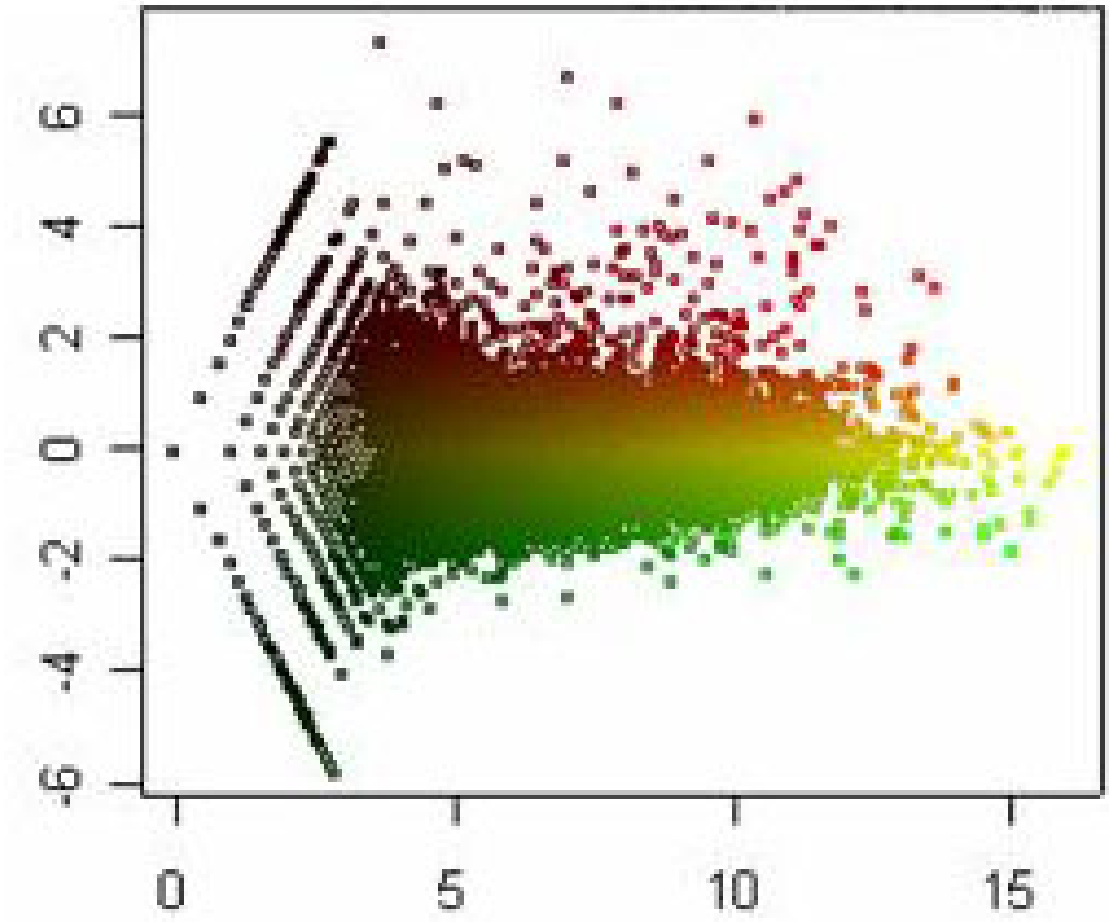
Data



Data (log scale)



MA Plot



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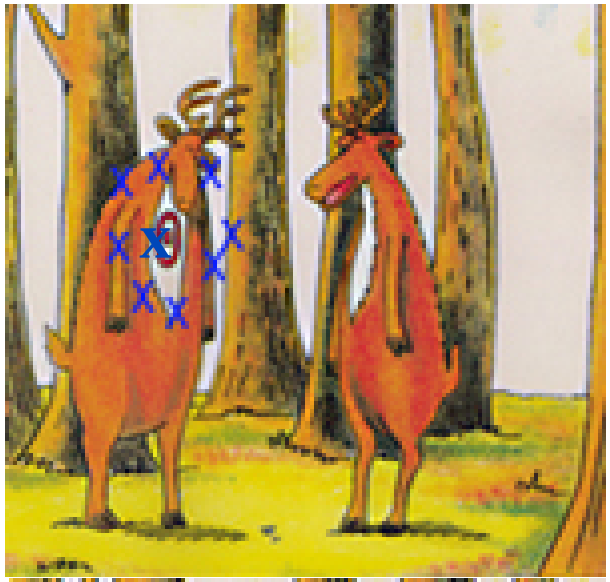
Sources of Variation

- Variance and Bias
- Different Sources of Variation
- Measuring foreground and background signal
- Control quality at different levels

Sources of variation: Bias and Variance

Noisy data

Little noise



“biased”

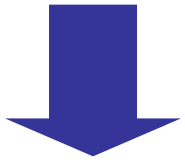
“unbiased”

Sources of Variation for Microarray-Data

- amount of RNA in biopsy
- efficiency of:
  - RNA extraction
  - reverse transcription
  - labeling
  - photodetection

Systematic

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



Normalization

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

Stochastic

- Effects, on single spots
- random effects cannot be estimated, „noise“

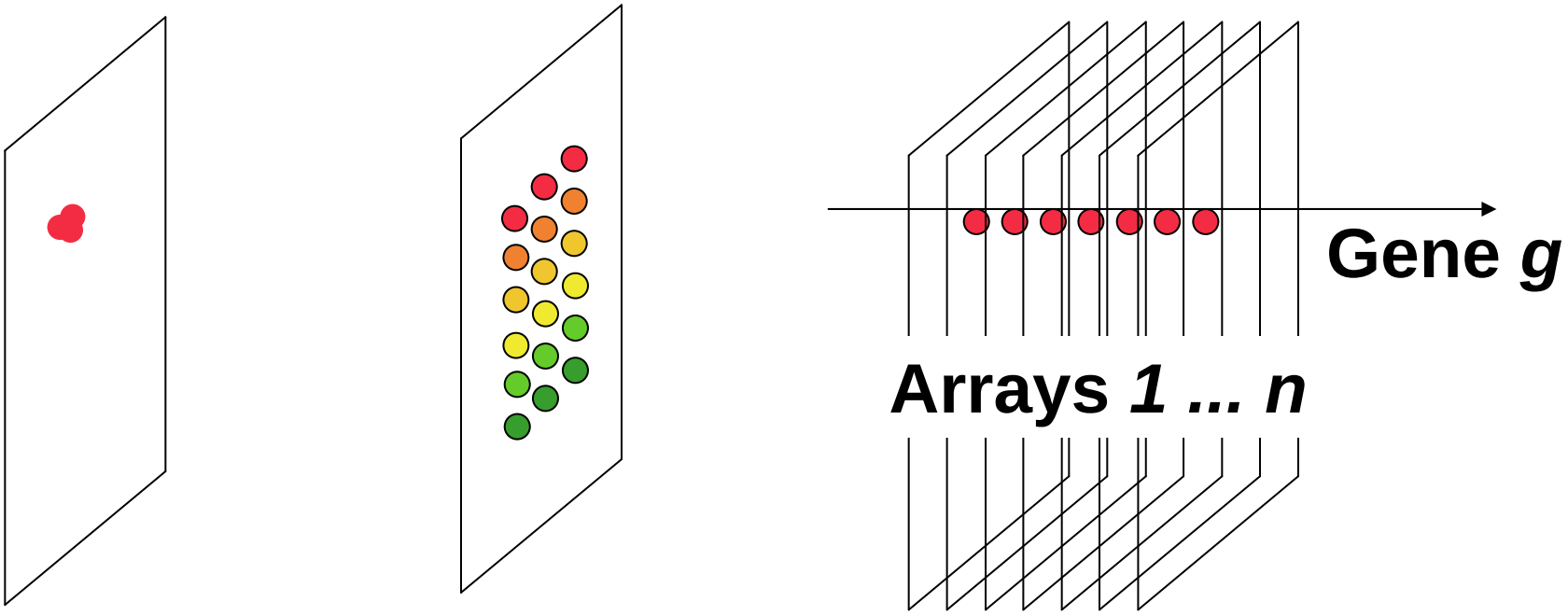
Error model

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

Quality control: Noise and reliable signal

Probe level **Array level** **Gene level**



Probe level: quality of the expression measurement of one spot on one particular array

Array level: quality of the expression measurement on one particular glass slide

Gene level: quality of the expression measurement of one probe across all arrays

Probe-level quality control

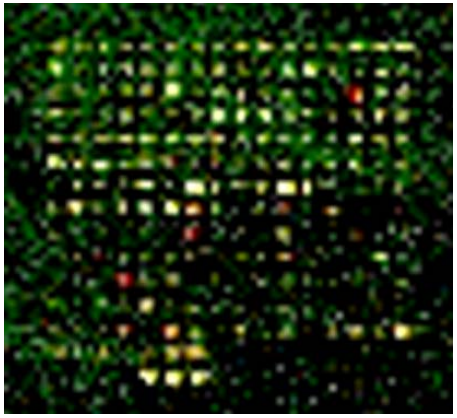
- Individual spots printed on the slide
- Sources:
  - faulty printing, uneven distribution, contamination with debris, magnitude of signal relative to noise, poorly measured spots;
- Visual inspection:
  - hairs, dust, scratches, air bubbles, dark regions, regions with haze
- Spot quality:
  - *Brightness:* foreground/background ratio
  - *Uniformity:* variation in pixel intensities and ratios of intensities within a spot
  - *Morphology:* area, perimeter, circularity.
  - *Spot Size:* number of foreground pixels
- Action:
  - set measurements to NA (missing values)
  - local normalization procedures which account for regional idiosyncrasies.
  - use weights for measurements to indicate reliability in later analysis.

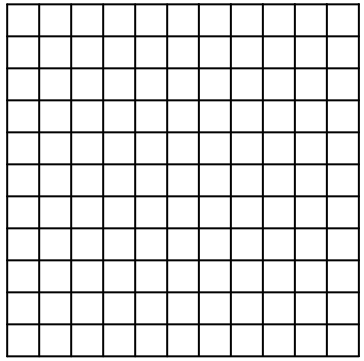
Image Analysis – Spot Identification

- The grid structure is provided by the manufacturer or generated individually for custom-made microarrays (e.g. GAL-files)
- The grid is overlaid by hand or automatically onto the image (beware of column/row displacement errors!)

Columns

Rows

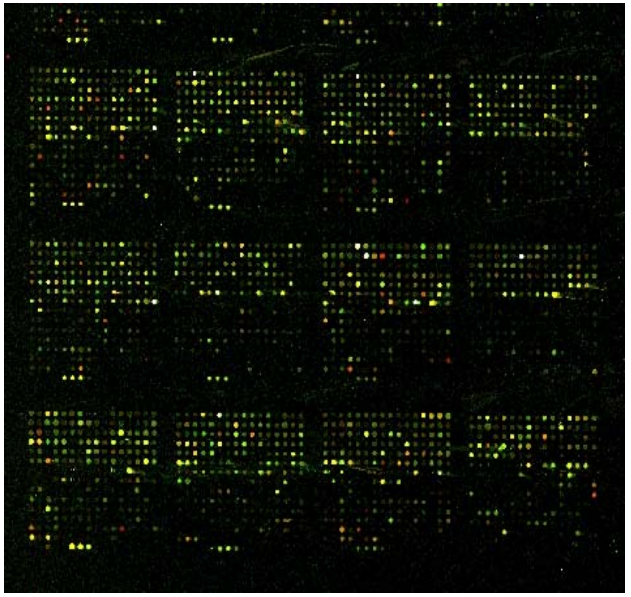




	A	B	C	D	E
1	ATF	1.0			
2	22	5			
3	Type=GenePix ArrayList V1.0				
4	Supplier=Company X				
5	ArrayName=MouseApoptosisProteins 4000				
6	ArrayRevision=2.7				
7	URL=http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Protein&li				
8	BlockCount=16				
9	Block1=100,100,150,24,180,17,180				
10	Block2=4600,100,150,24,180,17,180				
11	Block3=9100,100,150,24,180,17,180				
12	Block4=13600,100,150,24,180,17,180				
13	Block5=100,4600,150,24,180,17,180				
14	Block6=4600,4600,150,24,180,17,180				
15	Block7=9100,4600,150,24,180,17,180				
16	Block8=13600,4600,150,24,180,17,180				
17	Block9=100,9100,150,24,180,17,180				
18	Block10=4600,9100,150,24,180,17,180				
19	Block11=9100,9100,150,24,180,17,180				
20	Block12=13600,9100,150,24,180,17,180				
21	Block13=100,13600,150,24,180,17,180				
22	Block14=4600,13600,150,24,180,17,180				
23	Block15=9100,13600,150,24,180,17,180				
24	Block16=13600,13600,150,24,180,17,180				
25	Block	Column	Row	Name	ID
26	1	1	1	MAP-1	11139671
27	1	2	1	bad protein	1083224
28	1	3	1	bcl2-like	6753170
29	1	4	1	interleukin-1	2137456
30	1	5	1	caspase 6	6753286

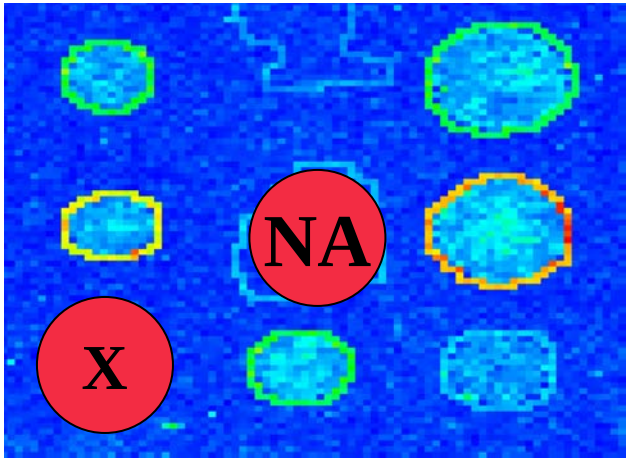
GAL-file contains Clone-IDs and defines their position on the grid

Blocks

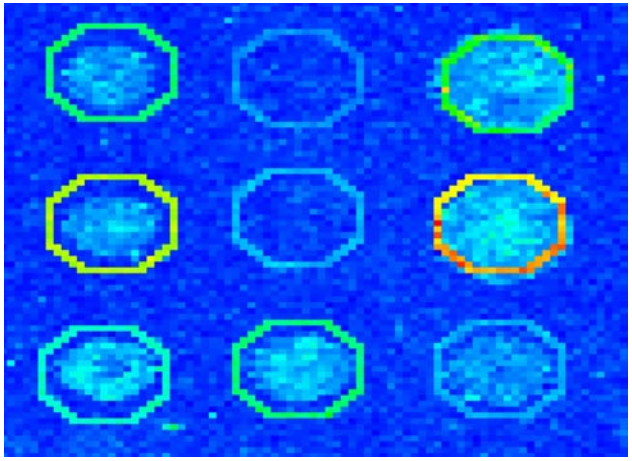


Spot Identification

- Individual spots are recognized, size and shape might be adjusted per spot (automatically fine adjustments by hand).
- Additional manual flagging of bad (X) or non-present (NA) spots



poor spot quality



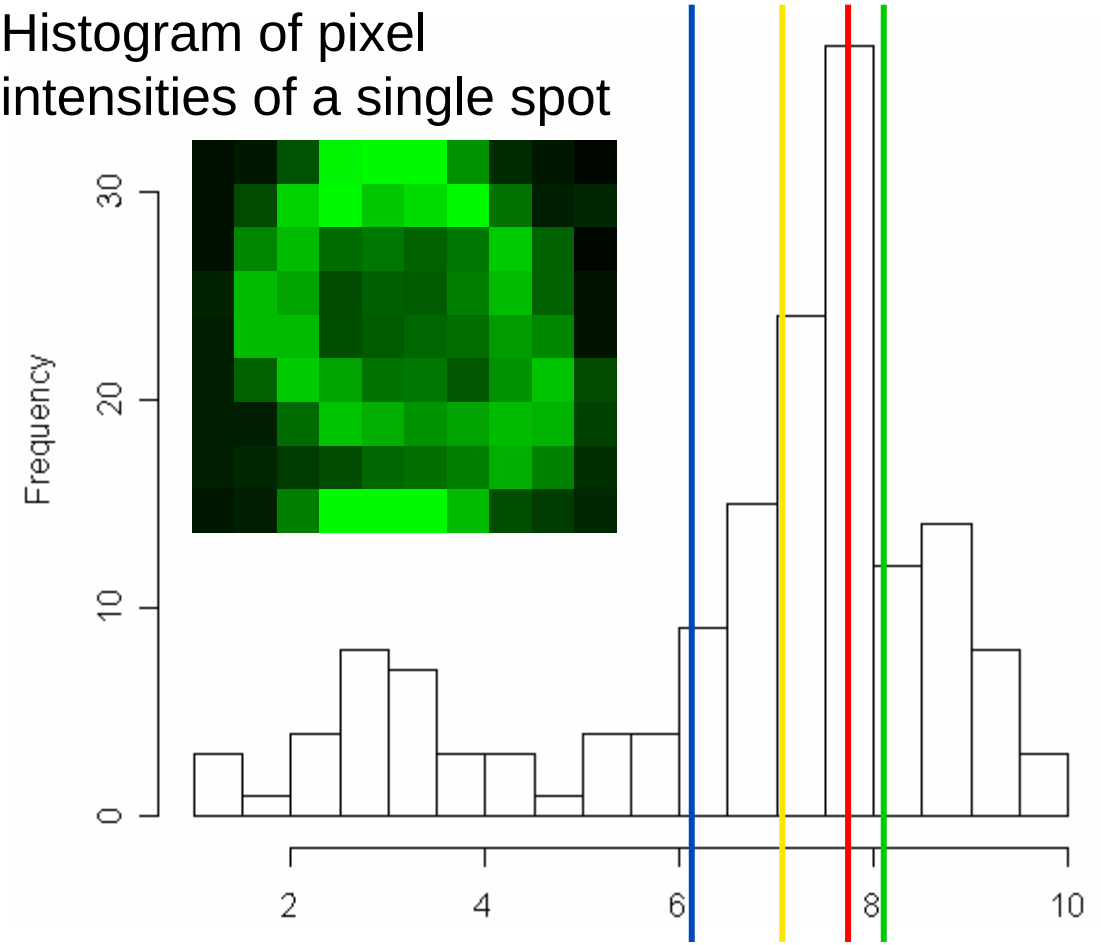
good spot quality

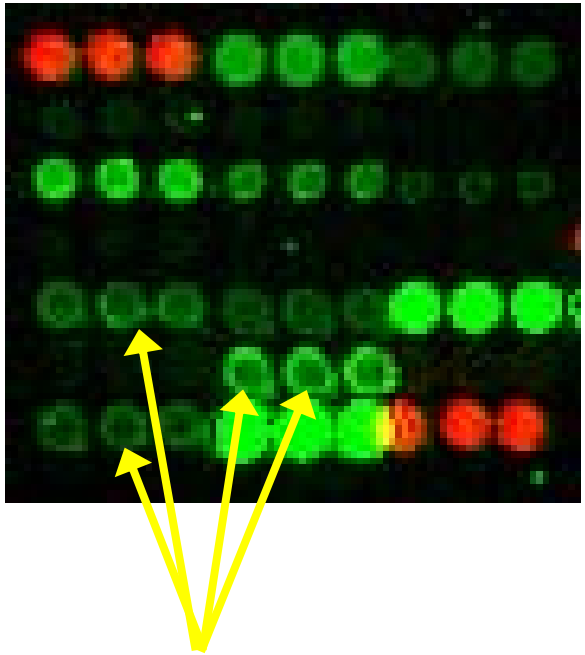
Different Spot identification methods: Fixed circles, circles with variable size, arbitrary spot shape (morphological opening)

Spot identification

- The signal of the spots is quantified.

Histogram of pixel intensities of a single spot





„Donuts“

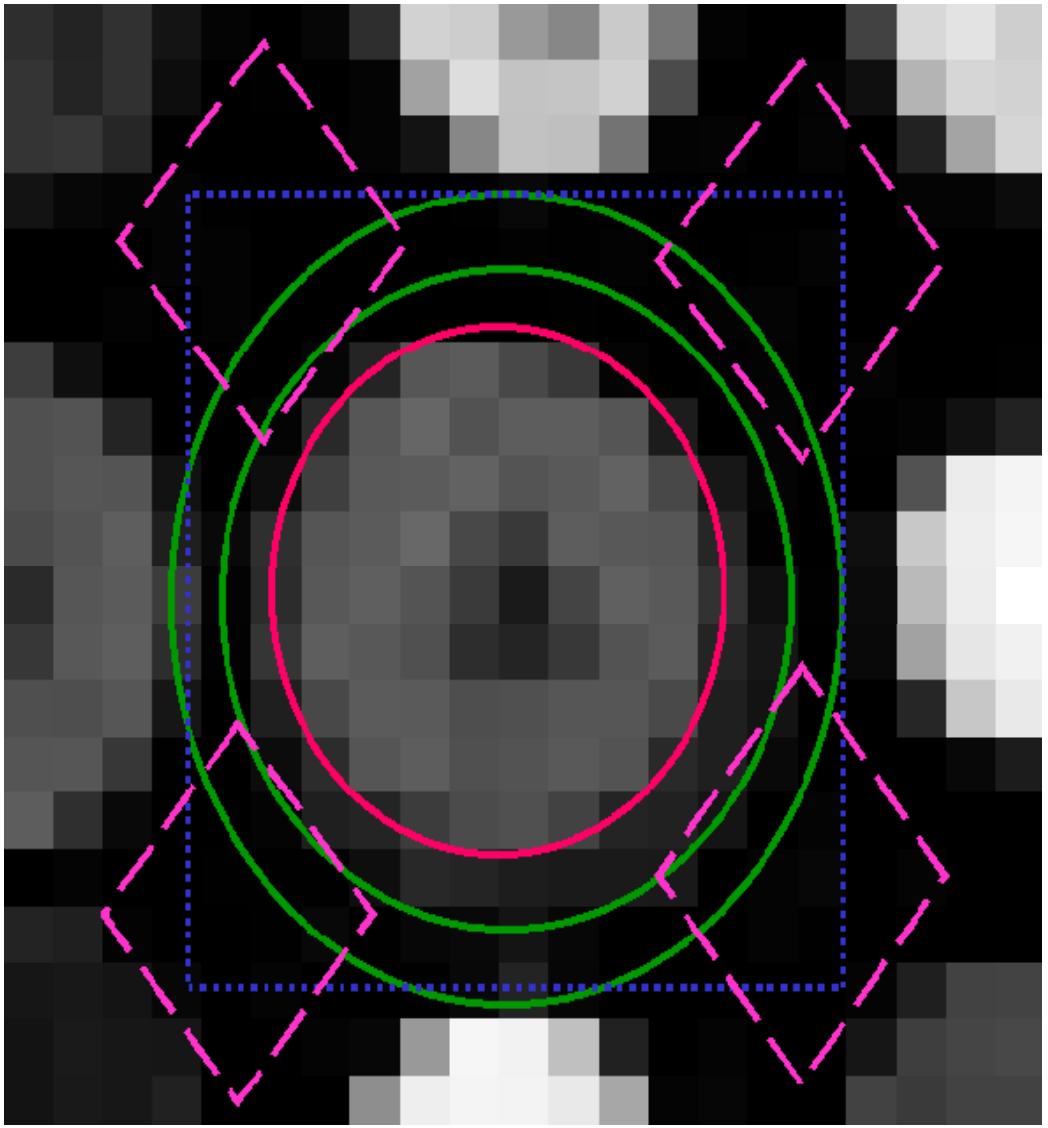
Mean / Median / Mode / 75% quantile

Different Regions around the spot are quantified to measure local background.

GenePix

QuantArray

ScanAlyse

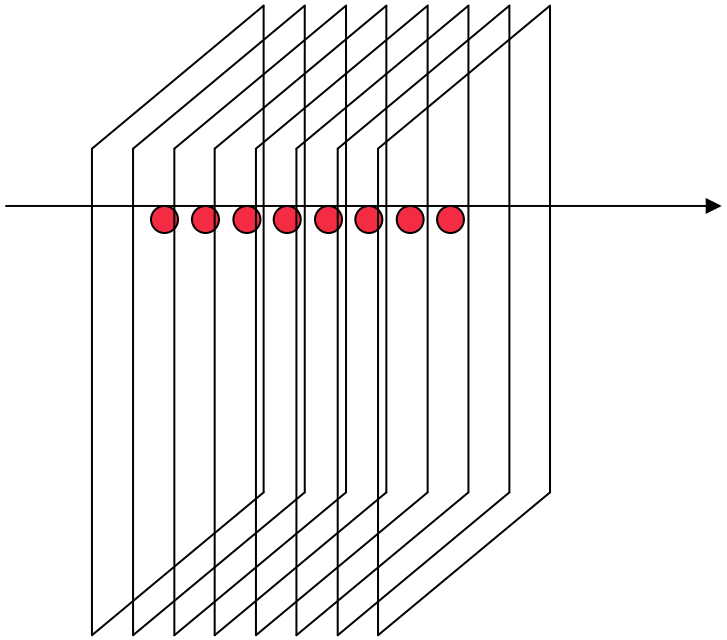


Array-level quality control

- Problems:
  - array fabrication defect
  - problem with RNA extraction
  - failed labeling reaction
  - poor hybridization conditions
  - faulty scanner

- Quality measures:
  - Percentage of spots with no signal (~30% exlcuded spots)
  - Range of intensities
  - (Av. Foreground)/(Av. Background) > 3 in both channels
  - Distribution of spot signal area
  - Amount of adjustment needed: signals have to substantially changed to make slides comparable.

Gene-level quality control



- Poor hybridization in the reference channel may introduce bias on the fold-change

Gene-level quality control:
Poor Hybridization and Printing

- Some probes will not hybridize well to the target RNA
- Printing problems such that all spots of a given inventory well have poor quality.
- A well may be of bad quality – contamination
- Genes with a consistently low signal in the reference channel are suspicious: Median of the background adjusted signal < 200*

**or other appropriate choice*

Gene-level quality control:
Probe quality control based on duplicated spots

- Printing different probes that target the same gene or printing multiple copies of the same probe.

- Mean squared difference of $\log_2$ ratios between spot r and s:

$$\mathrm{MSDLR} = \Sigma(x_{jr} - x_{js})^2/J \quad \text{sum over arrays } j = 1, \ldots, J$$

  recommended threshold to assess disagreement: MSDLR > 1

- Disagreement between copies: printing problems, contamination, mislabeling. Not easy if there are only 2 or 3 slides.

- Jenssen et al (2002) Nucleic Acid Res, 30: 3235-3244. Theoretical background

Swirl Data

- Experiment to study early development in zebrafish.
- Swirl mutant vs. wild-type zebrafish.
- Two sets of dye-swap experiments.
- Microarray containing 8448 cDNA probes
- 768 control spots (negative, positive, normalization)
- printed using 4x4 print-tips, each grid contains a 22x24 Spot matrix

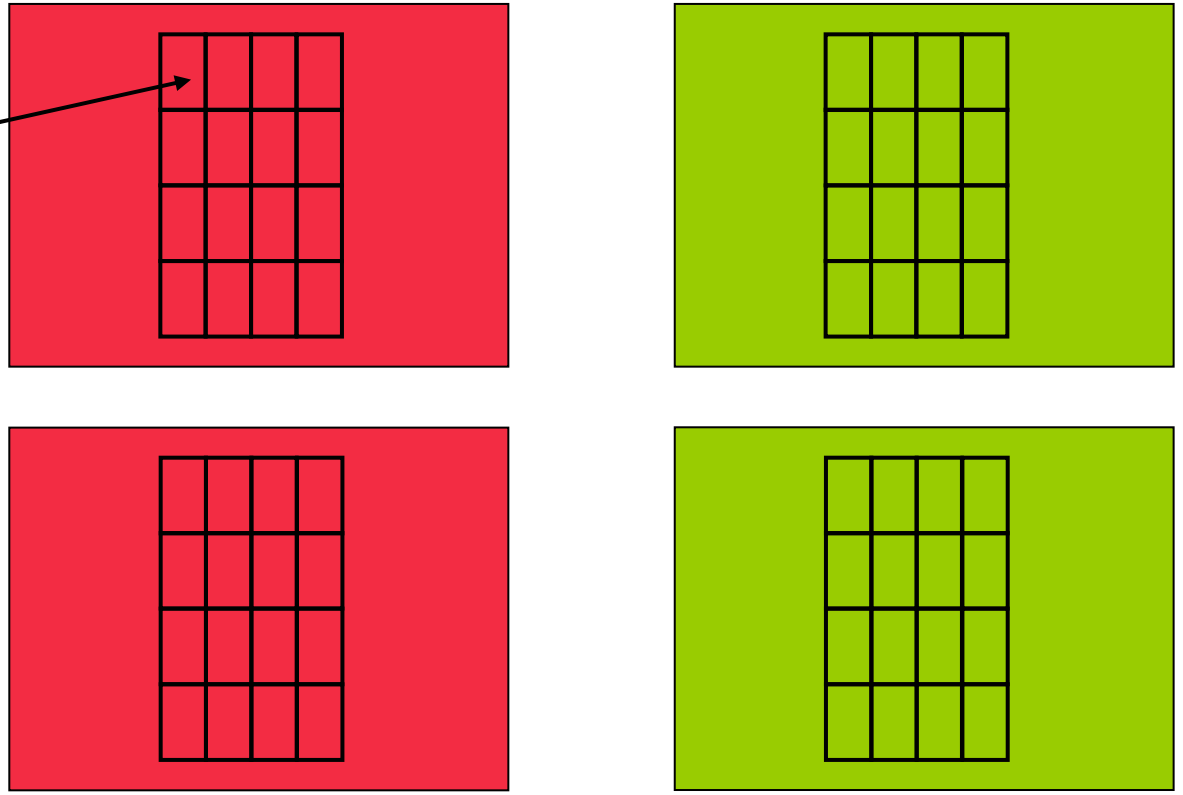
R Console

```
> library(marray)
> data(swirl)
> ll()

   member class     mode   dimension
1  swirl  marrayRaw list   c(8448,4)
```

Swirl Data

MT – R
WT - G

MT – G
WT - R



24 x 22 spots per print-tip

Hybr. I

Hybr. II

technical, biological variability

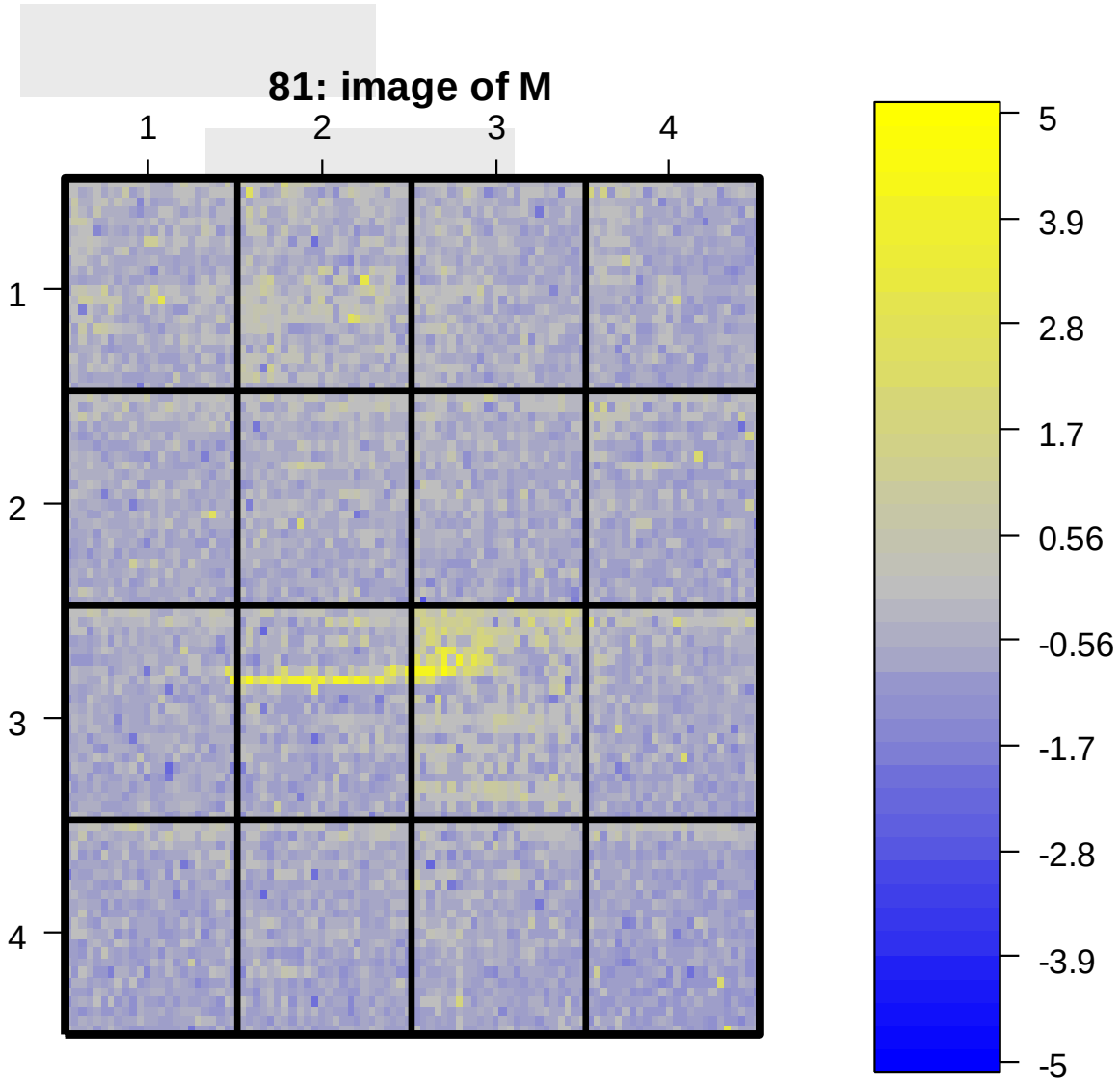
Visual inspection

4 x 4 sectors

Sector:
24 rows
22 columns

8448 spots

Mean signal intensity



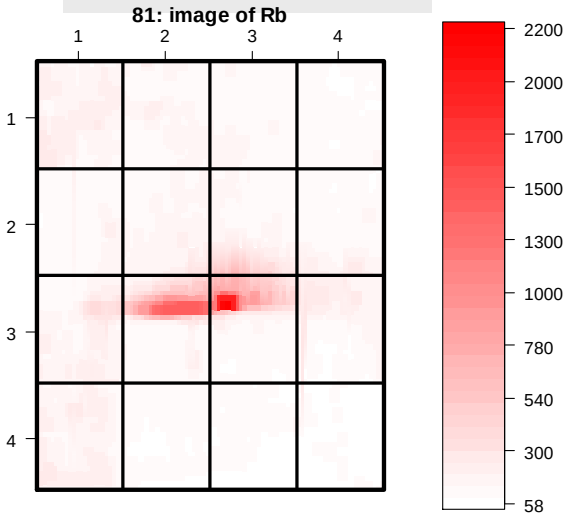
R Console

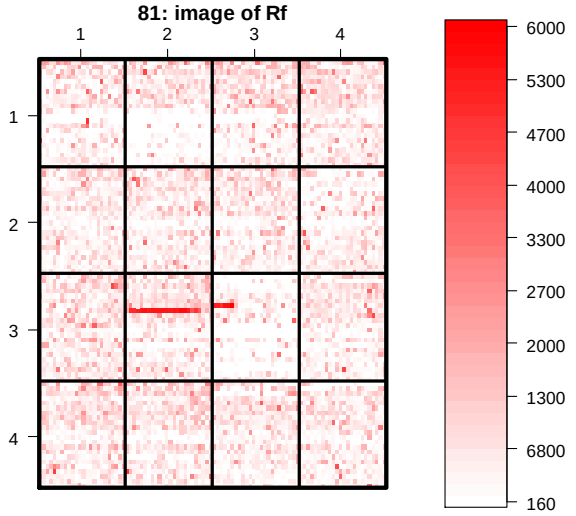
```
> image(swirl[,1])
```

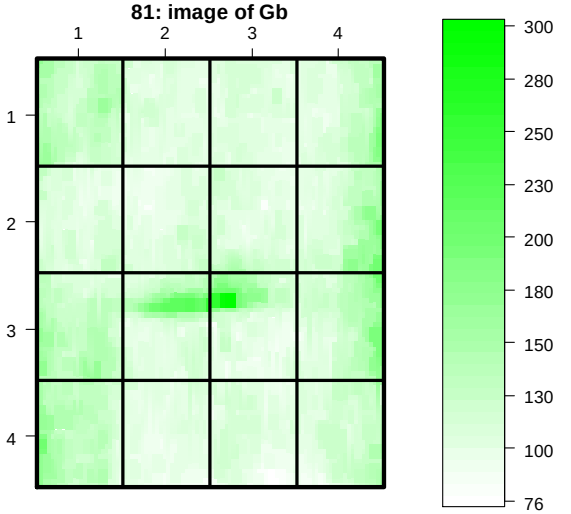
Visual inspection – Foreground and Background intensities

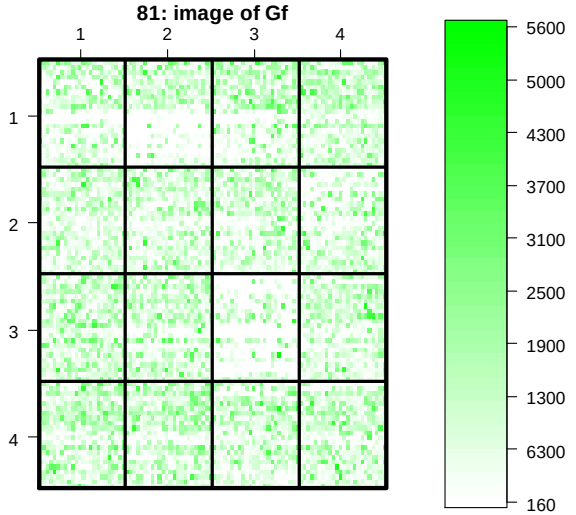
R Console

```
> Gcol <- maPalette(
  low = "white",
  high = "green",
  k = 50)

> Rcol <- maPalette(
  low = "white",
  high = "red",
  k = 50)

> image(swirl[,1]
  xvar="maRb",
  col=Rcol)

> image(swirl[,1]
  xvar="maRf",
  col=Rcol)

> image(swirl[,1]
  xvar="maGb",
  col=Gcol)

> image(swirl[,1]
  xvar="maRf",
  col=Gcol)
```





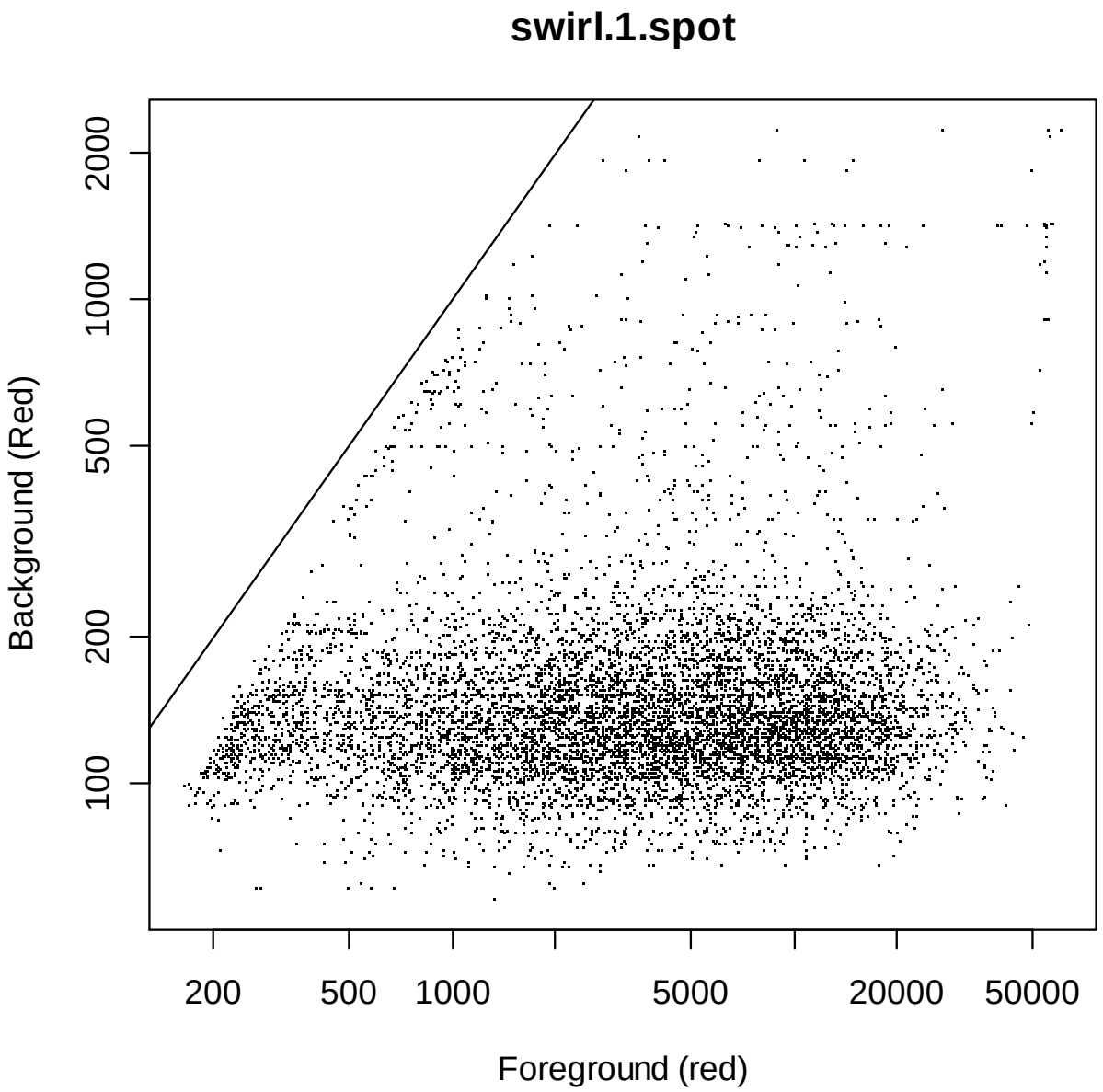




Foreground versus Background intensities

R Console

```
> plot(
  maRf(swirl[,1]),
  maRb(swirl[,1]),
  log="xy")
> abline(0,1)
```



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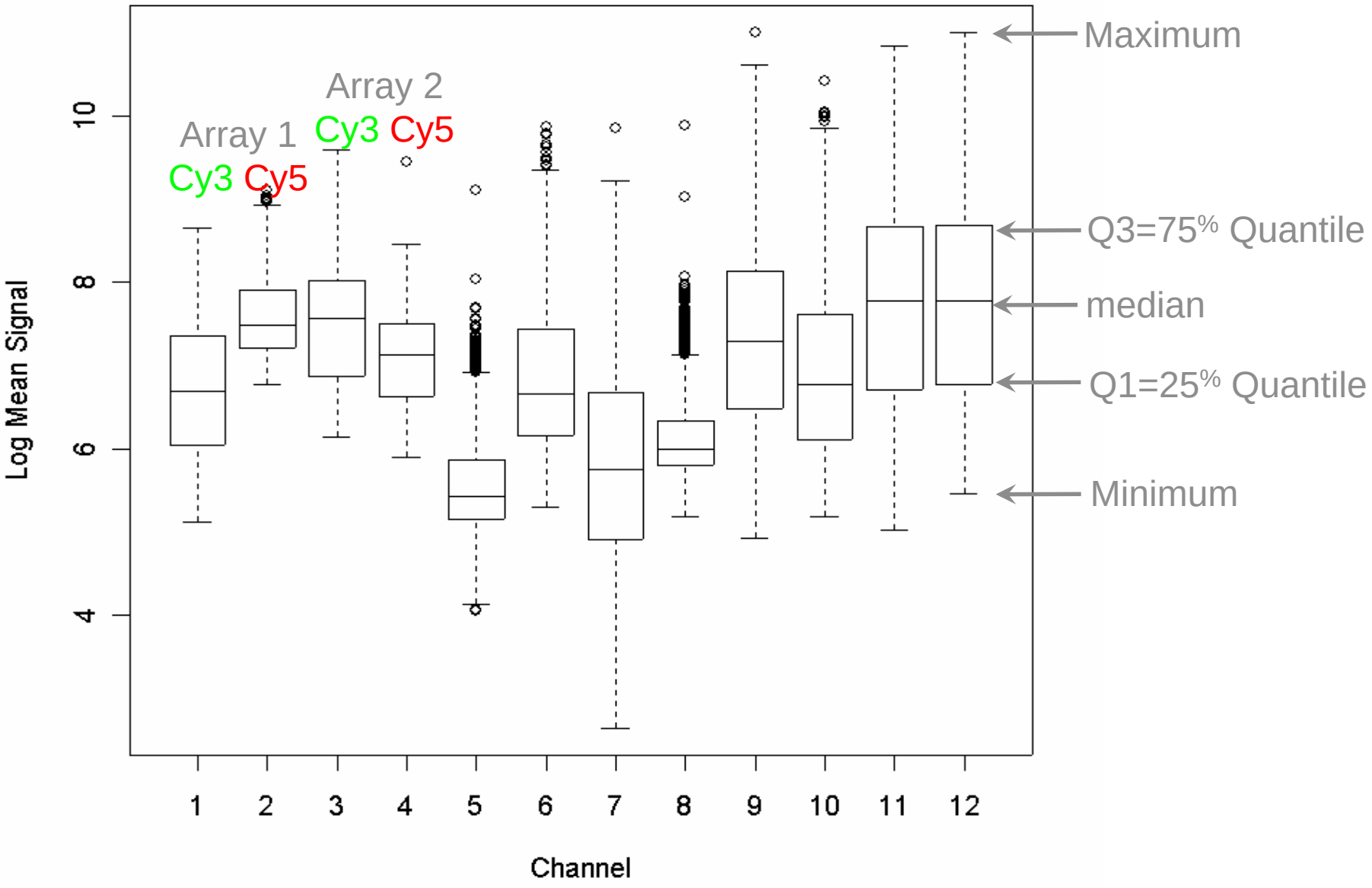
Normalization Methods:

- Scale normalization
- Quantile normalization
- Lowess normalization
- Variance stabilization

Displaying Variability of Microarray-Data



Normalization via rescaling

- Location and scale are basic statistical concepts for data description:

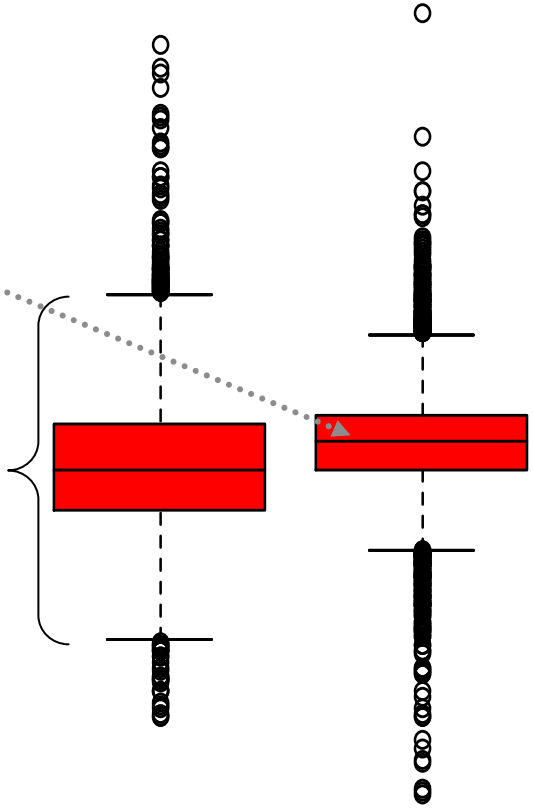
Location
normalization: corrects for spatial or dye bias

Scale
normalization: homogenizes the variability across arrays

Normalized log-intensity ratios are given by

$$M_{norm} = (M\text{-location}) / scale$$

“Location and scale of different MA measurements should be (approximately) the same.“

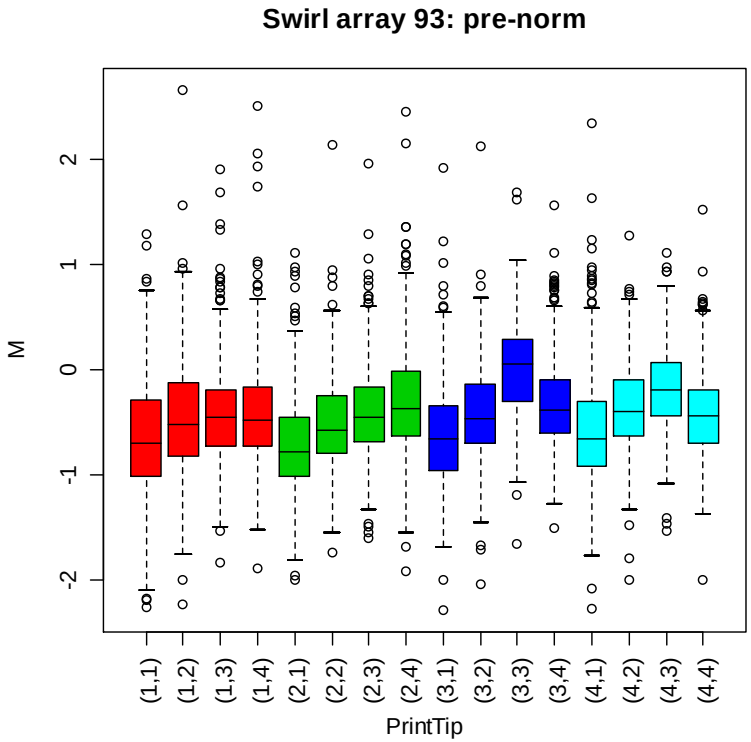


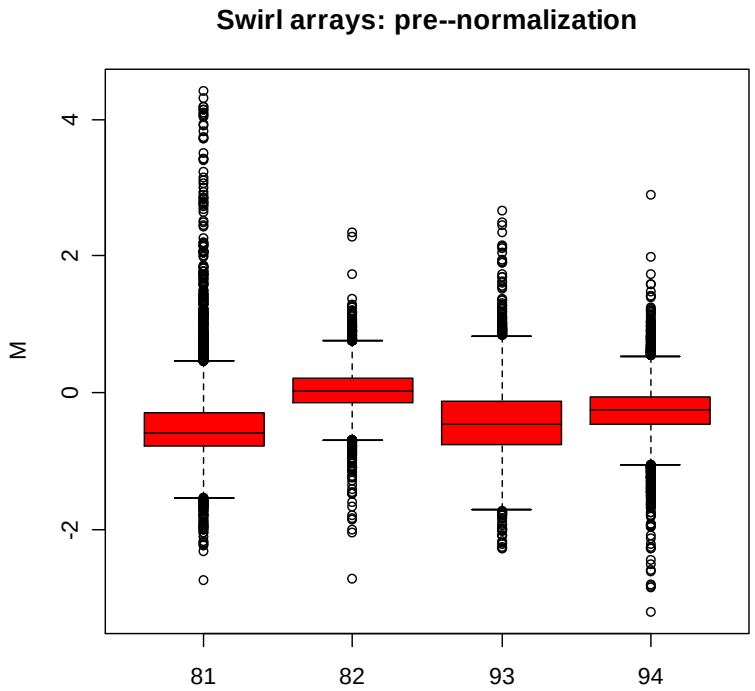
boxplots

Normalizing the Hybridization-Intensities

- Background Correction
  - Local Background → Image Analysis
  - Global Background → e.g. 5% Quantile
- Robust estimation of a “rescaling” Factor, e.g. *Median of Differences* based on
  - the majority of genes
  - Housekeeping genes
  - *Spiked* in control genes
- There are many other normalization methods!
  Other methods:
  - Lowess (aka loess)
  - Quantile
  - VSN

marray – Swirl Data: Pre Normalization

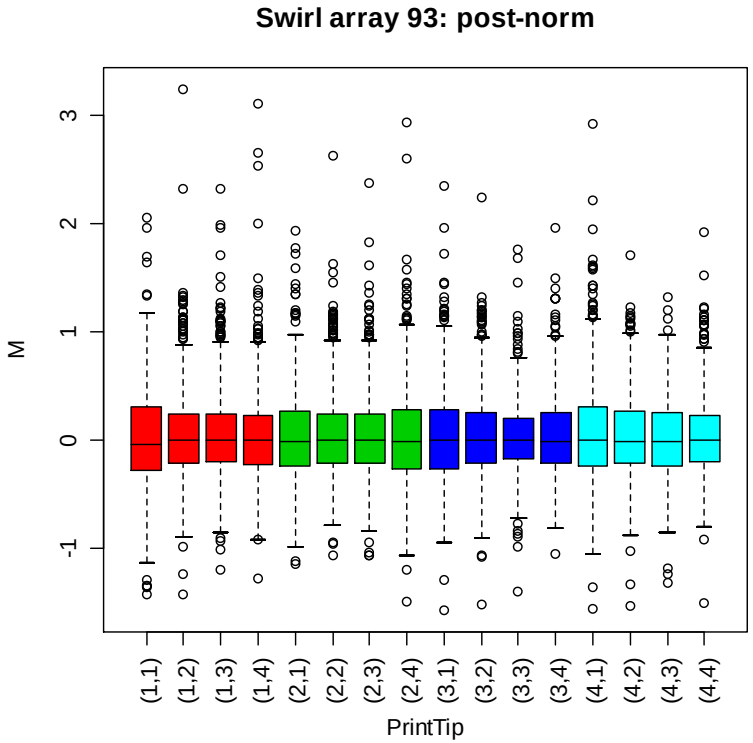


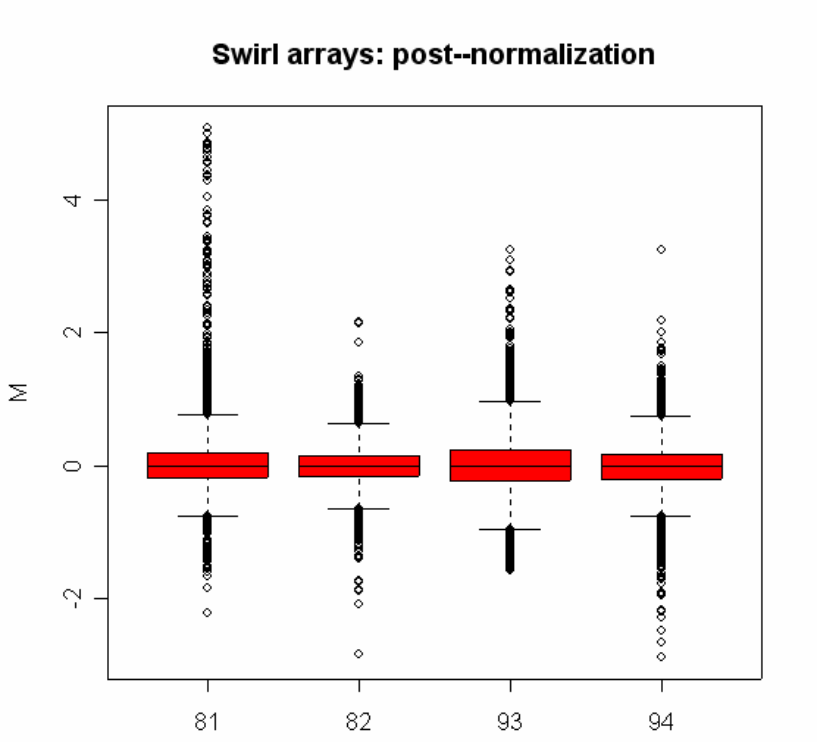


R Console

```
> boxplot(swirl[, 3], xvar = "maPrintTip", yvar = "maM")
> boxplot(swirl, yvar = "maM")
```

marray – Swirl Data: Post Normalization

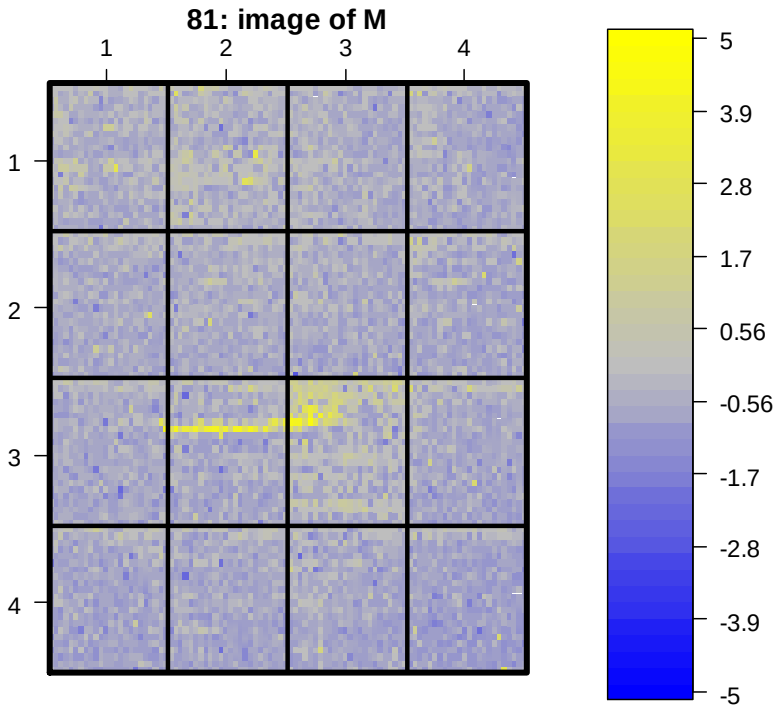


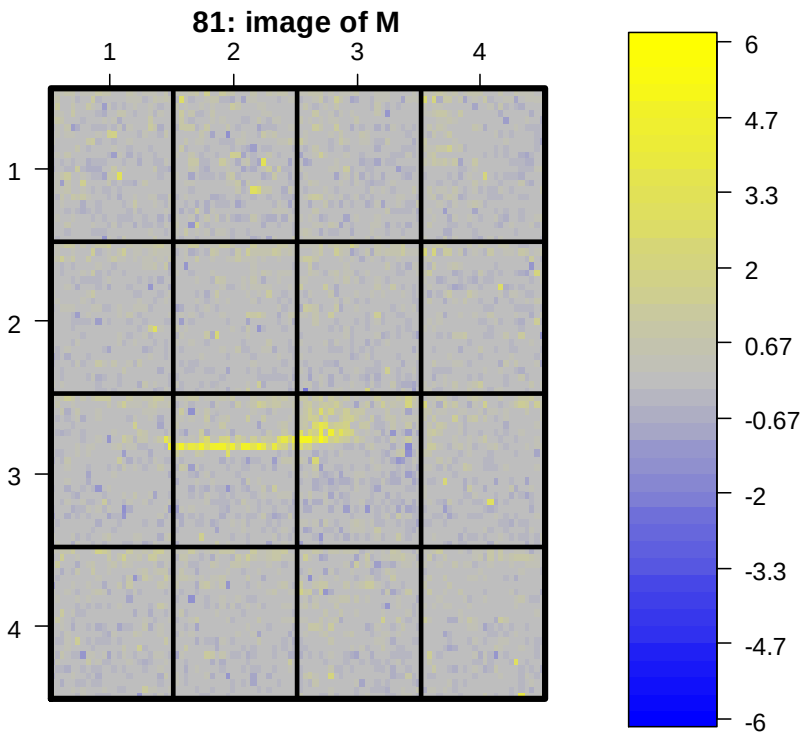


R Console

```
> swirl.norm <- maNorm(swirl, norm = "p")
> boxplot(swirl.norm[, 3], xvar = "maPrintTip", yvar = "maM")
> boxplot(swirl.norm, yvar = "maM")
```

Swirl Data – M values, raw versus normalized





Normalization procedure was not able to remove scratch

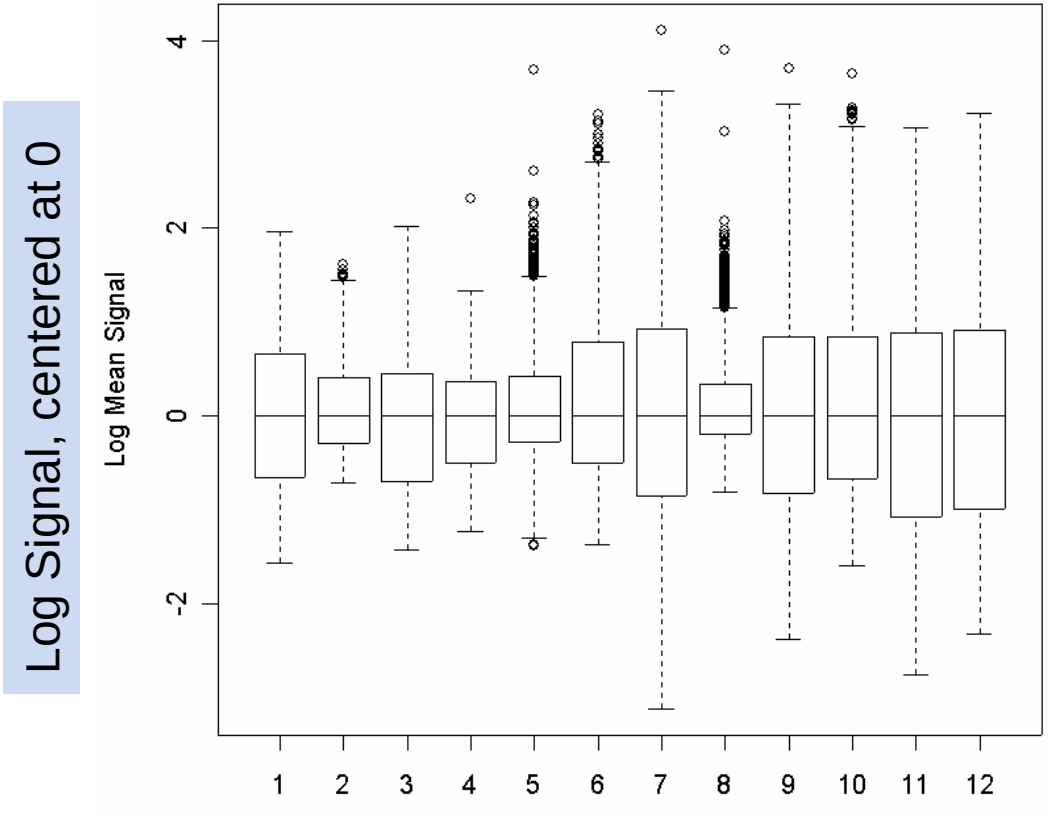
R Console

```
> image(swirl[,1])
> image(swirl.norm[,1])
```

Median-centering

- One of the simplest strategies is to bring all „Centers“ of the array data to the same level.
- Assumption, the majority of genes or the center should not change between conditions.
- the Median is used as a robust measure.

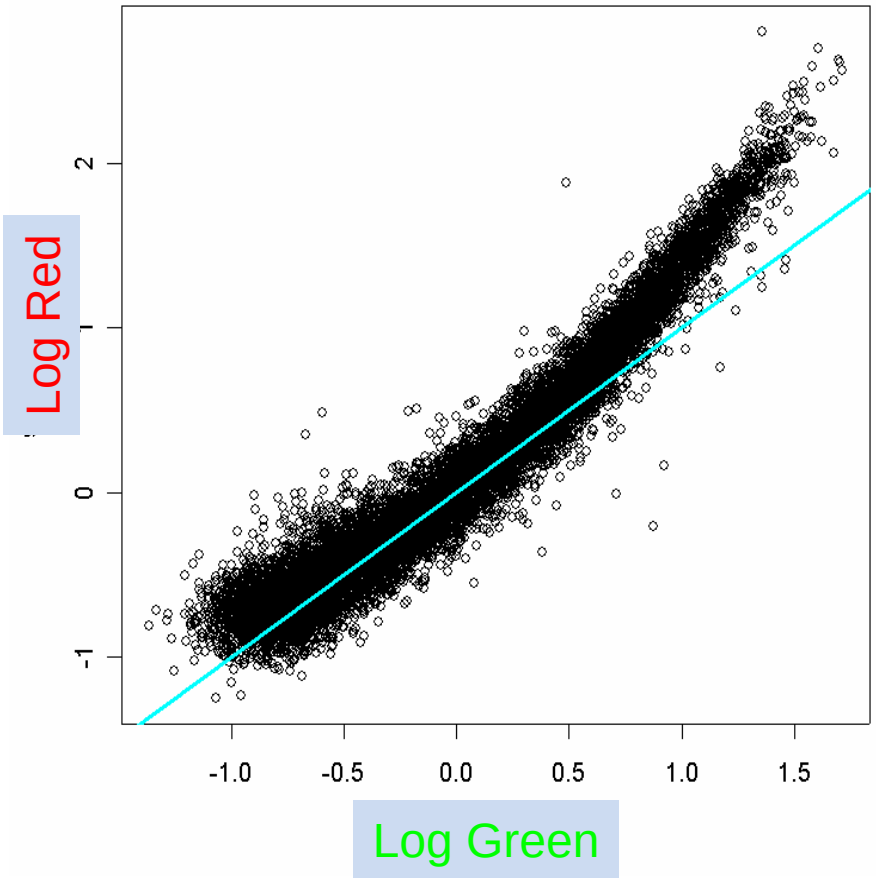
divide all expression measurements of each array by the Median.



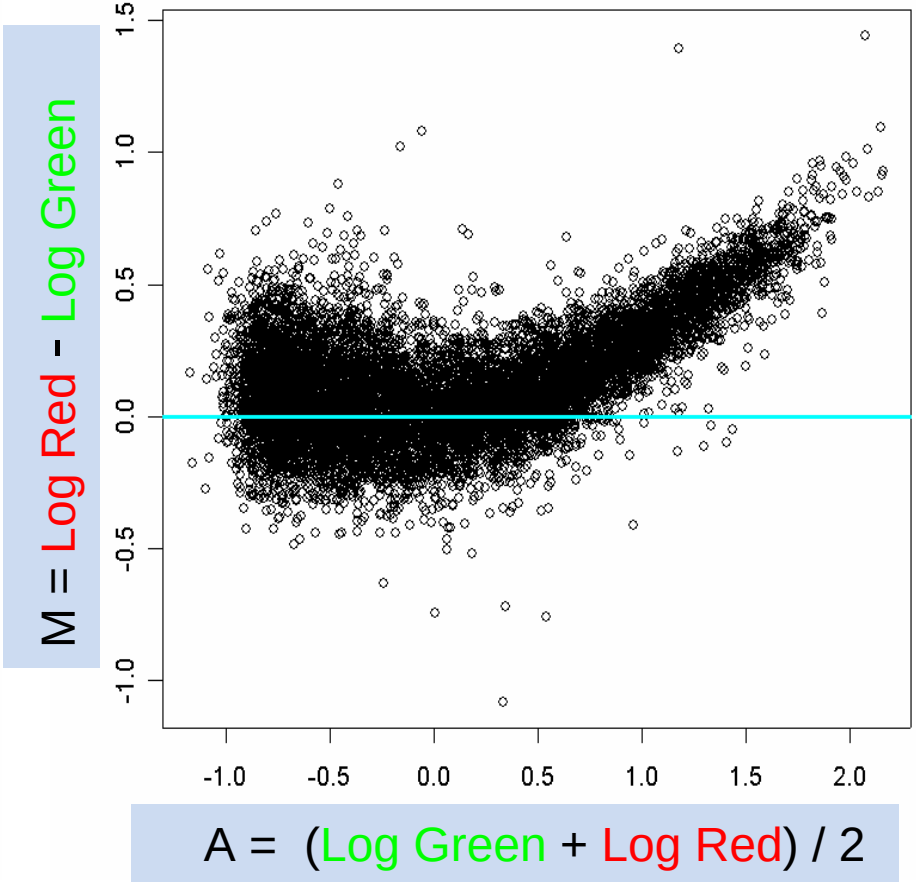
Problems with Median-Centering

Median-Centering is a global Method. It does not adjust for local effects, intensity dependent effects, print-tip effects, etc.

Scatterplot of log-Signals after Median-centering



M-A Plot of the same data

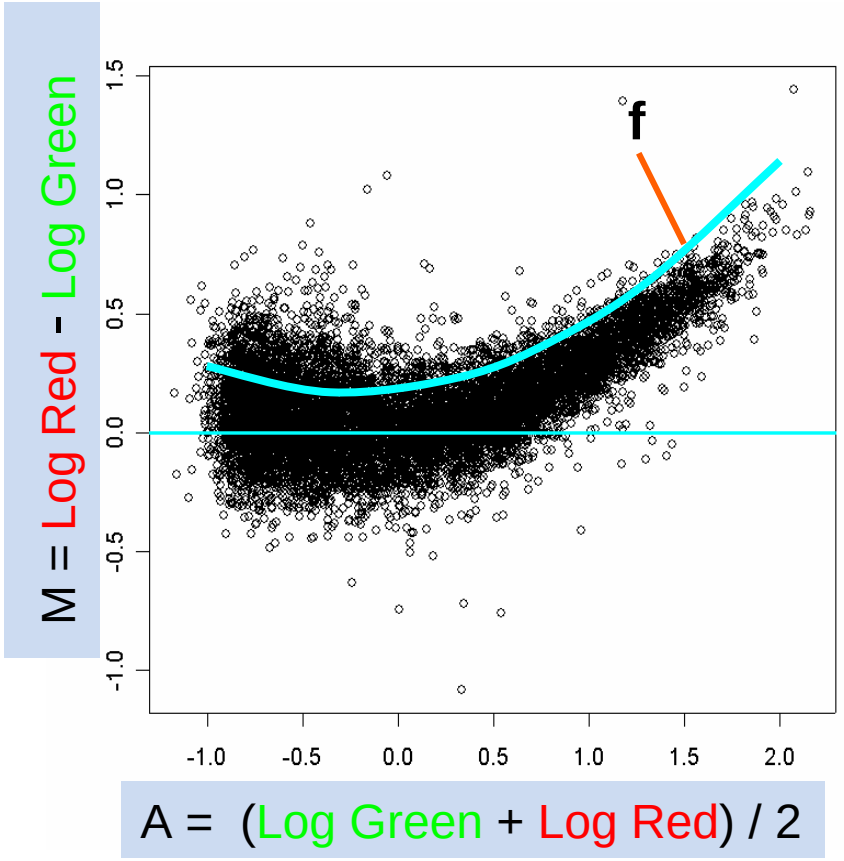


Lo(w)ess Normalization

Assumption: There is an intensity-dependent bias of the fold change,

$$M = f(A)$$

and hence $y_j = f(x_j) + \delta_j$
where δ_j is the "true" log fold change for gene *j*. The true fold change distribution is approximately a zero-symmetric normal distribution.

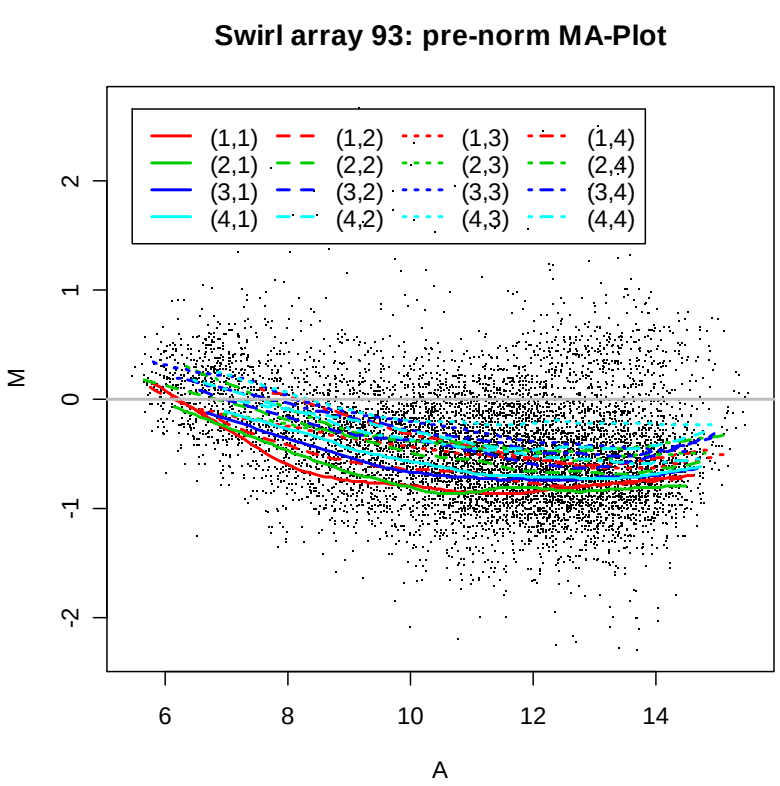


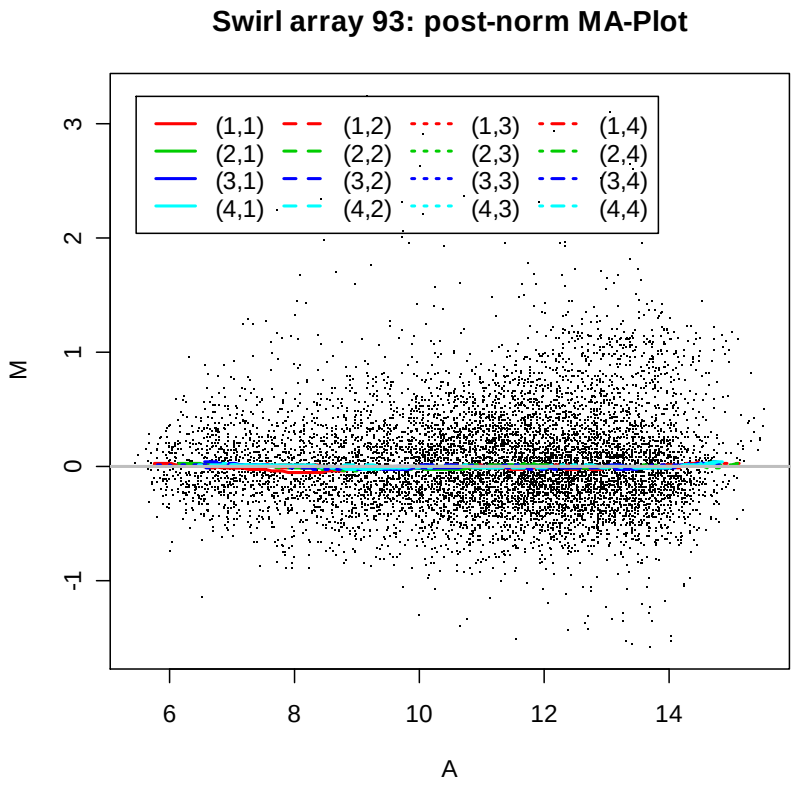
Task: Find *f*, replace y_j by $y_j - f(x_j)$.

The **idea of local regression** is that *f* can be estimated locally at a point *x* by a simple (and easy-to-fit) function f_x. For each point *x*, we then estimate *f* by

$$\hat{f}(x) = f_x(x)$$

marray – Swirl Data: Print-tip lowess Normalization





R Console

```
> plot(swirl[, 3], xvar = "maA", yvar = "maM",
  zvar = "maPrintTip")
> plot(swirl.norm[, 3], xvar = "maA", yvar = "maM",
  zvar = "maPrintTip")
```

Non-parametric smoother: loess, lowess, local regression line, generalizes the concept of moving average.

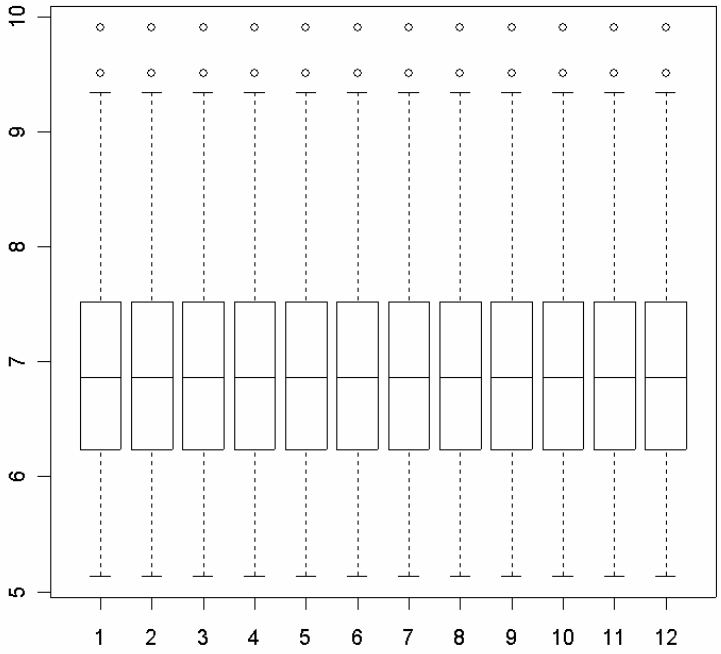
Quantile Normalization

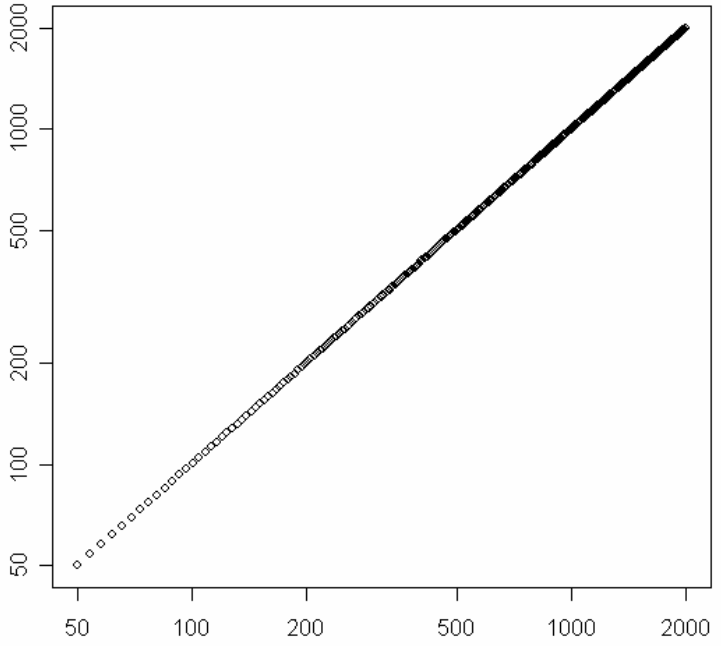
The basic idea of Quantile-Normalization is very simple:

„The Histograms of all Slides are made identical“

Tightens the idea of Median-Centering. Not only the 50%-Quantile is adjusted, but *all* Quantiles.

Boxplot and QQ-plot after Quantile normalization





Quantilenormalization

	Sample A	Sample B	Sample C
Gen 1	100	200	180
Gen 2	10	40	280
Gen 3	100	120	80

Quantilenormalization

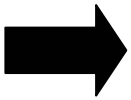
	Sample A	Sample B	Sample C
Gen 1	100	200	180
Gen 2	10	40	280
Gen 3	100	120	80

Quantilenormalization

	Sample A	Sample B	Sample C
	100 Gen 1	200 Gen 1	140 Gen 1
	10 Gen 2	40 Gen 2	270 Gen 2
	100 Gen 3	120 Gen3	70 Gen3

Quantilenormalization

	Sample A	Sample B	Sample C
	10 Gen 2	40 Gen 2	70 Gen 3
	100 Gen 1	120 Gen 3	140 Gen 1
	100 Gen 3	200 Gen 1	270 Gen 2



Mean
(10+40+70) / 3 = 40
(100+120+140) / 3 = 120
(100+200+270) / 3 = 190

Quantilenormalization

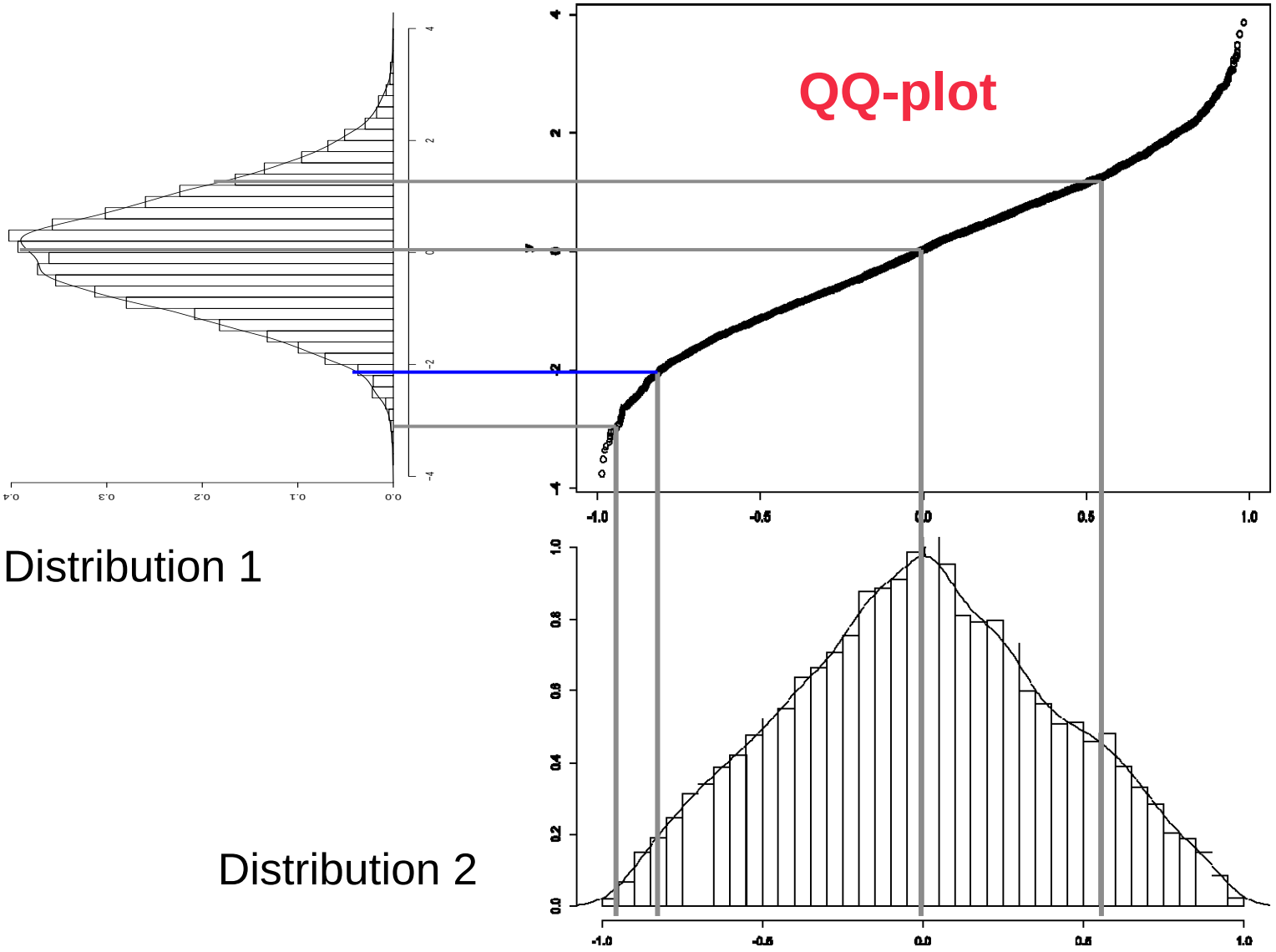
	Sample A	Sample B	Sample C
	40 Gen 2	40 Gen 2	40 Gen 3
	100 Gen 1	120 Gen 3	120 Gen 1
	190 Gen 3	190 Gen 1	190 Gen 2

Mean
(10+40+70) / 3 = 40
(100+120+140) / 3 = 120
(100+200+270) / 3 = 190

Quantilenormalization

	Sample A	Sample B	Sample C
Gen 1	120 (100)	190 (200)	120 (180)
Gen 2	40 (10)	40 (40)	190 (280)
Gen 3	190 (100)	120 (120)	40 (80)

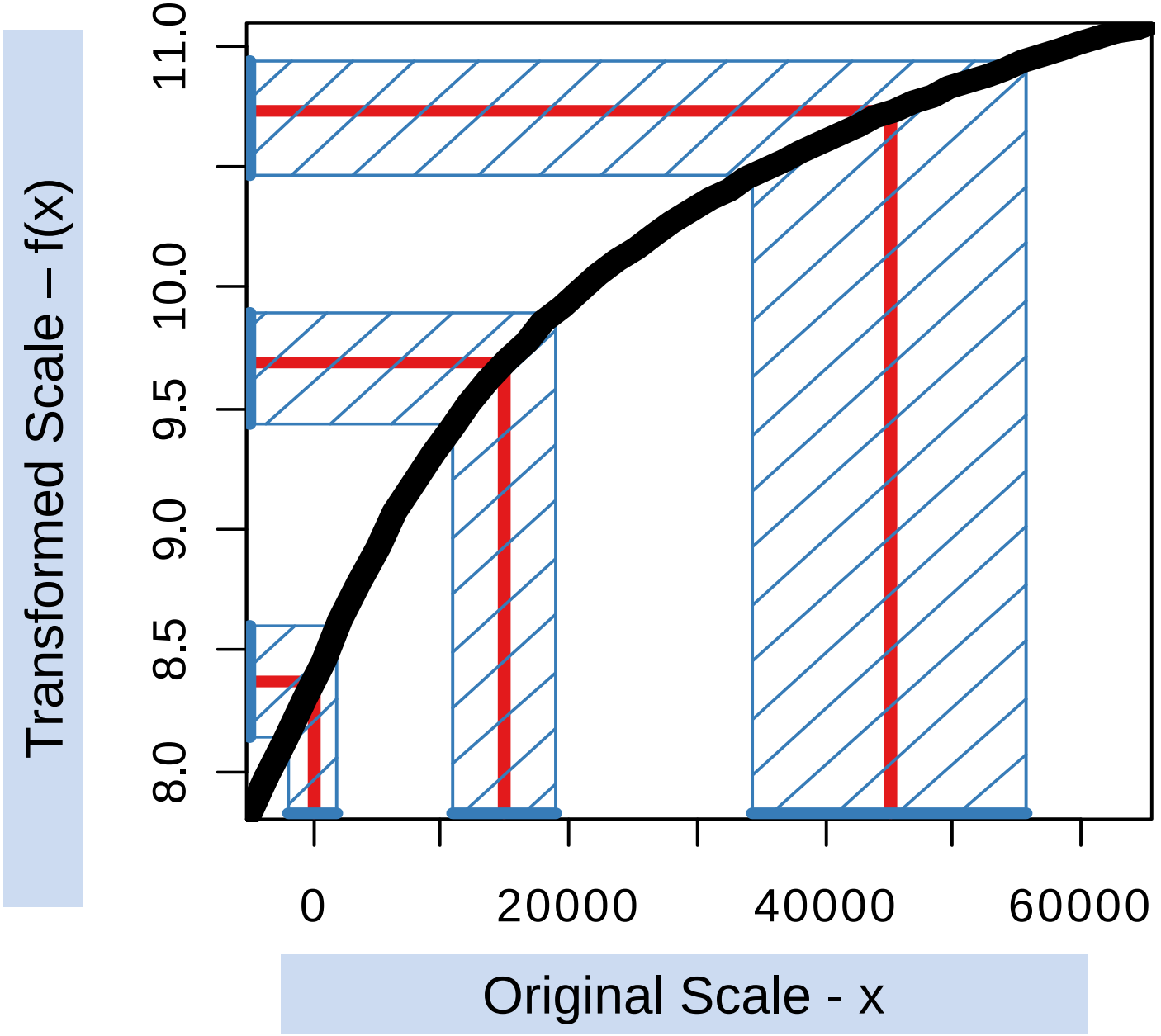
Quantile Normalization



VSN: model and theory

- Huber et al. (2002) Bioinformatics, 18:S96–S104
- Model for measured probe intensity
  Rocke DM, Durbin B (2001) Journal of Computational Biology, 8:557–569
- log-transformation is replaced by a transformation (arcsinh) based on theoretical grounds.
- Estimation of transformation parameters (location, scale) based on ML paradigm and numerically solved by a least trimmed sum of squares regression.
- vsn–normalized data behaves close to the normal distribution

variance stabilizing transformations



variance stabilizing transformations

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

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

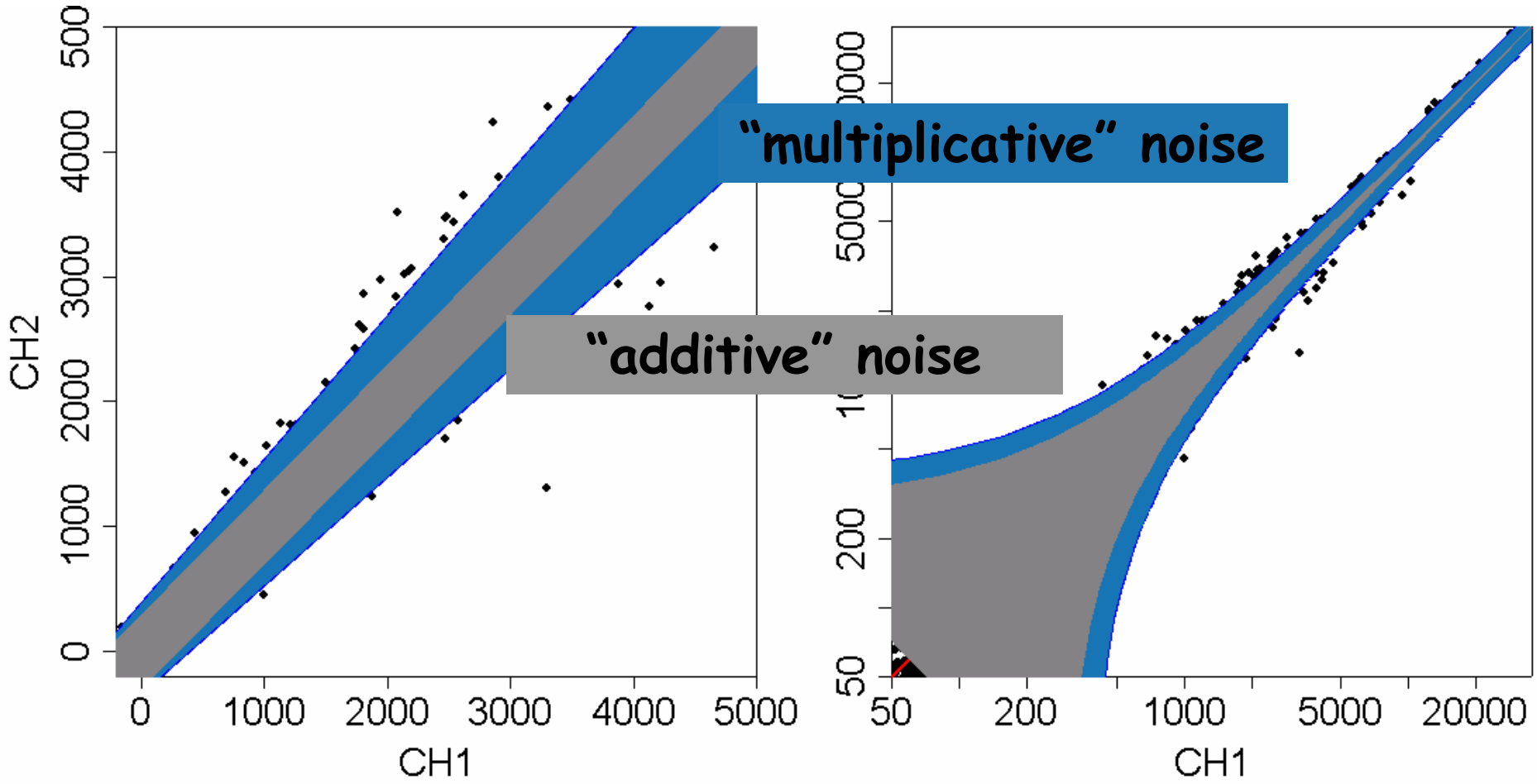
2.) constant CV ('multiplicative') $v(u) \propto u^2 \Rightarrow f \propto \log u$

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

4.) additive and multiplicative

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

The two-component model



raw scale

log scale

B. Durbin, D. Rocke, JCB 2001

Fitting of an error 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

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

"additive noise"

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

b_i per-sample normalization factor

b_k sequence-wise probe efficiency

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

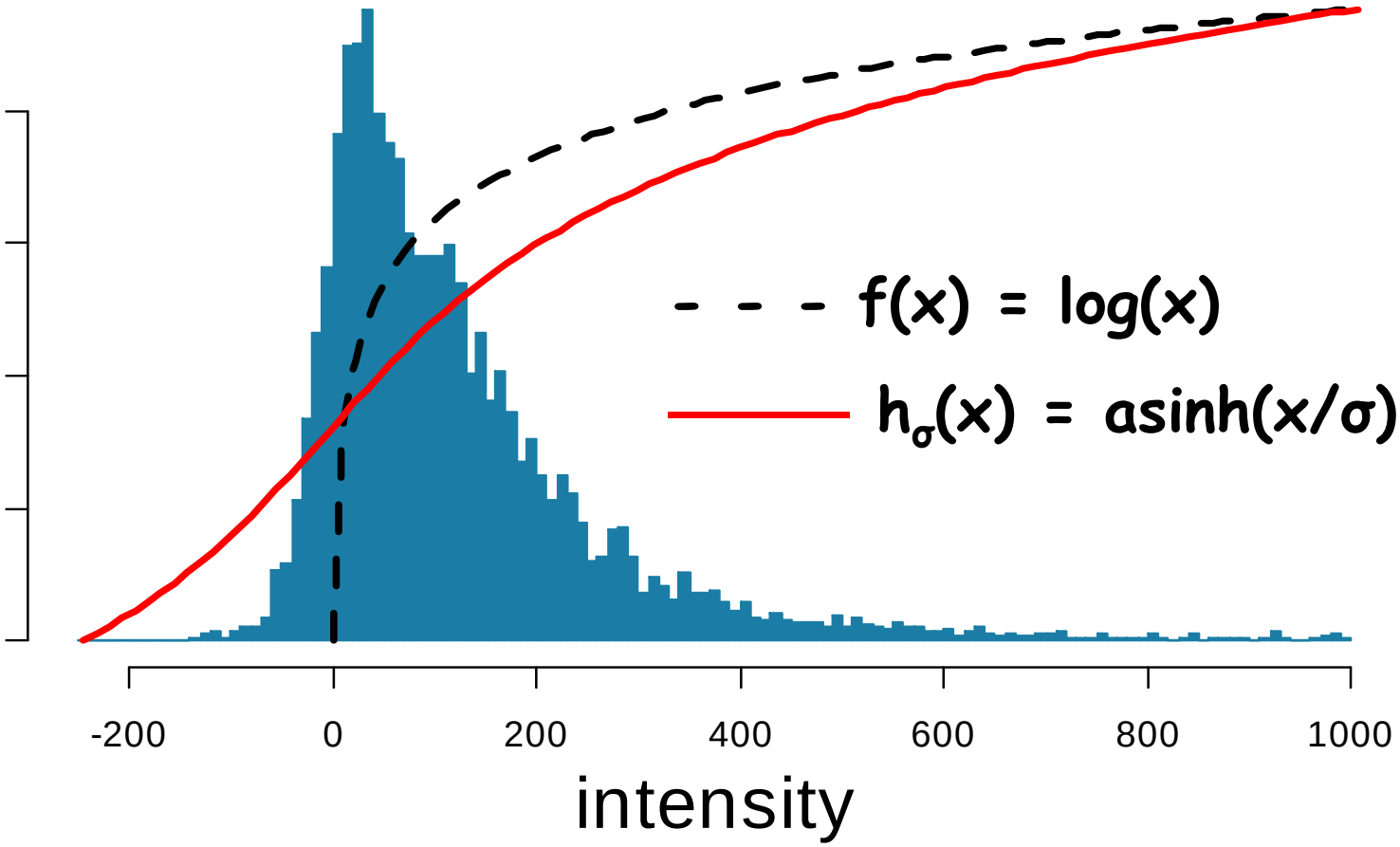
"multiplicative noise"

Parameter-Estimation

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

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

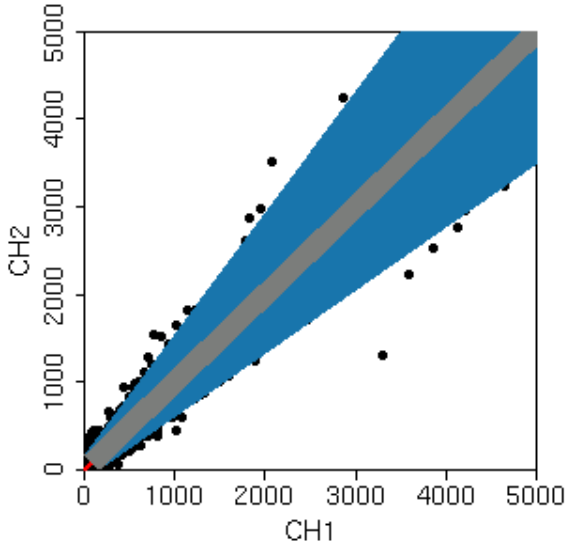
The „glog“-Transformation

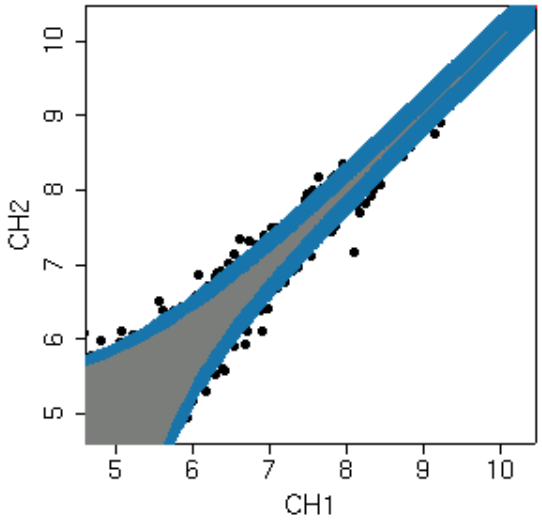


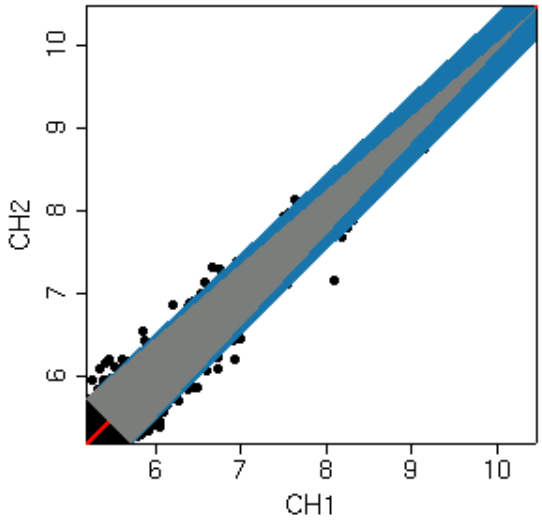
$$\operatorname{arsinh}(x) = \log(x + \sqrt{x^2 + 1})$$

$$\lim_{x \to \infty} (\operatorname{asinh}(x) - \log(x)) \longrightarrow \log(2)$$

The „glog“-Transformation







Variance:

Additive component

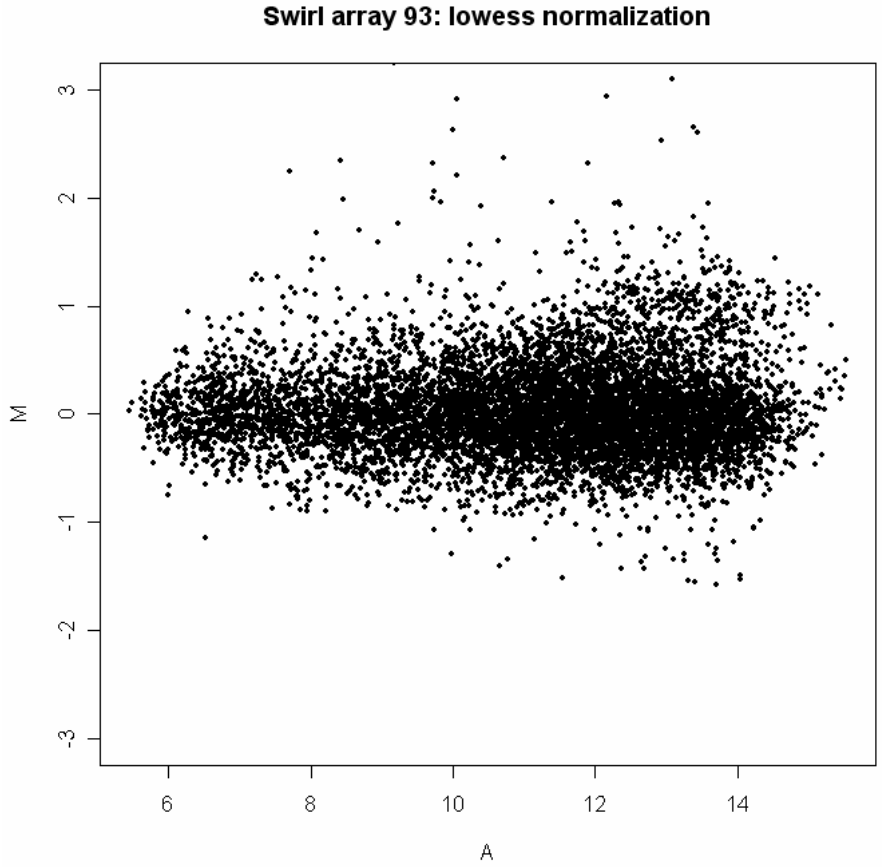
multiplicative component

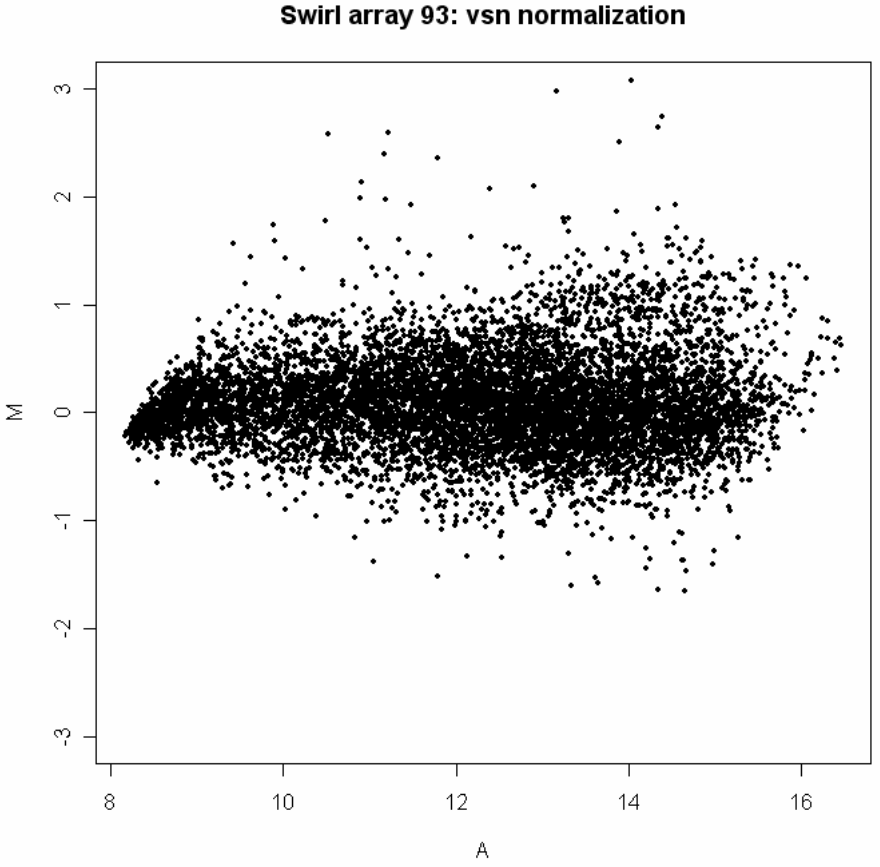
P. Munson, 2001

D. Rocke & B. Durbin, ISMB 2002

W. Huber et al., ISMB 2002

Swirl Data: Lowess versus VSN





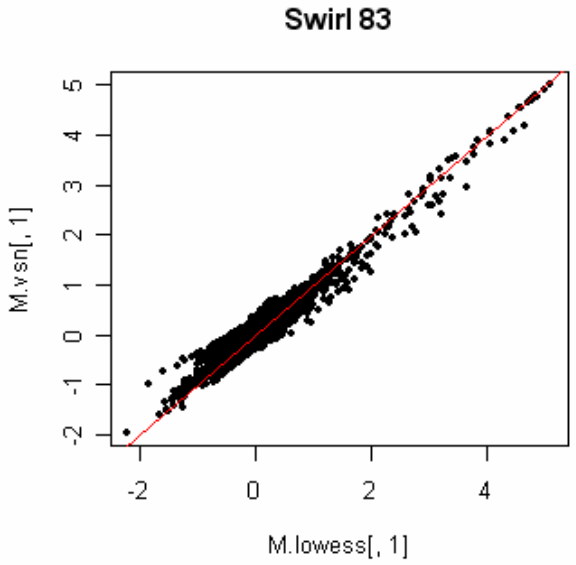
R Console

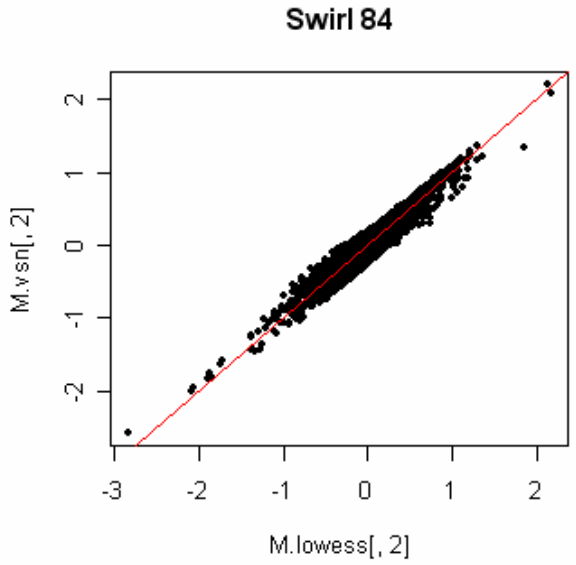
```
> plot(maA(swirl.norm[,3]), maM(swirl.norm[,3]), ylim=c(-3,3))
> library(vsn); library(limma);
> A.vsn<-log2(exp(exprs(swirl.vsn[,6])+exprs(swirl.vsn[,5])))/2
> M.vsn<-log2(exp(exprs(swirl.vsn[,6])-exprs(swirl.vsn[,5])))
> plot(A.vsn, M.vsn, ylim=c(-3,3)
```

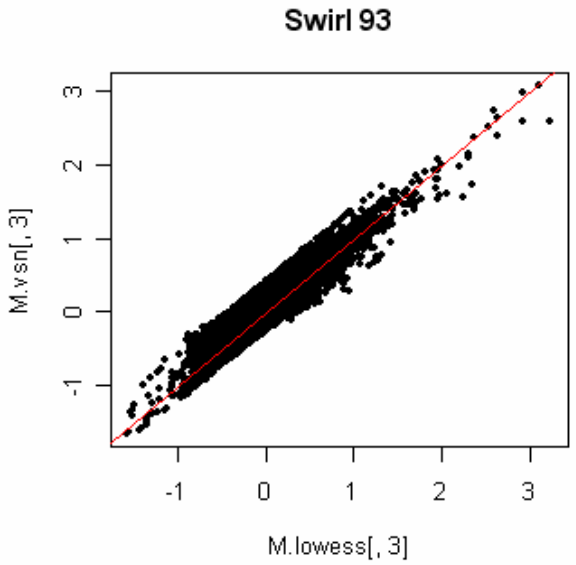
Swirl: LOWESS versus VSN

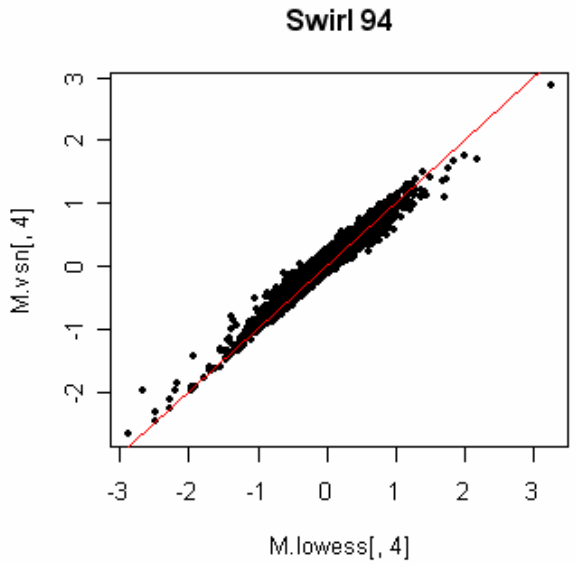
R Console

```
> M.lowess<-maM(swirl.norm)
> M.vsn<-log2(exp(exprs(
  swirl.vsn[,c(2,4,6,8)])-
  exprs(swirl.vsn[,c(1,3,5,7)]
  )))
> par(mfrow=c(2,2))
> plot(M.lowess[,1},
       M.vsn[,1], pch=20)
> abline(0,1, col="red")
> plot(M.lowess[,1},
       M.vsn[,1], pch=20)
> abline(0,1, col="red")
> plot(M.lowess[,1},
       M.vsn[,1], pch=20)
> abline(0,1, col="red")
> plot(M.lowess[,1},
       M.vsn[,1], pch=20)
> abline(0,1, col="red")
```









Summary

- What makes a good measurement: Precision and Unbiasednes
- Need to normalize.
- Normalization is not something trivial, has many practical and theoretical implications which need to be considered.
- What is the best way to normalize?
- How dependent is the result of your analysis from the normalization procedure?

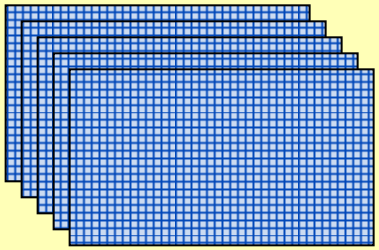
Monday
3. March 08

Experimental Design:

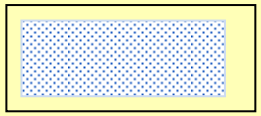
- Different levels of replication
- Pooling vs. non pooling
- different strategies to pair hybridization targets on cDNA arrays
- direct vs. indirect comparisons

Two main aspects of array design

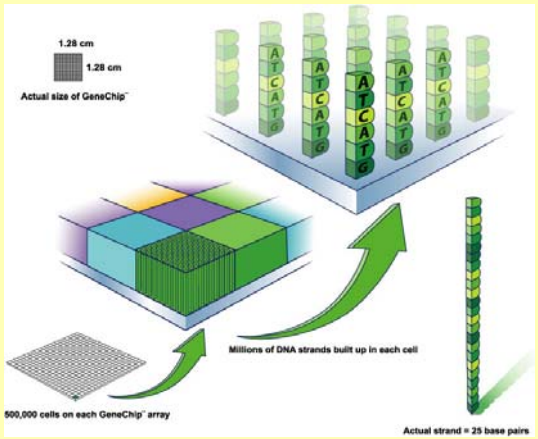
Design of the array



Arrayed Library
(96 or 384-well plates of bacterial glycerol stocks)



Spot as microarray on glass slides

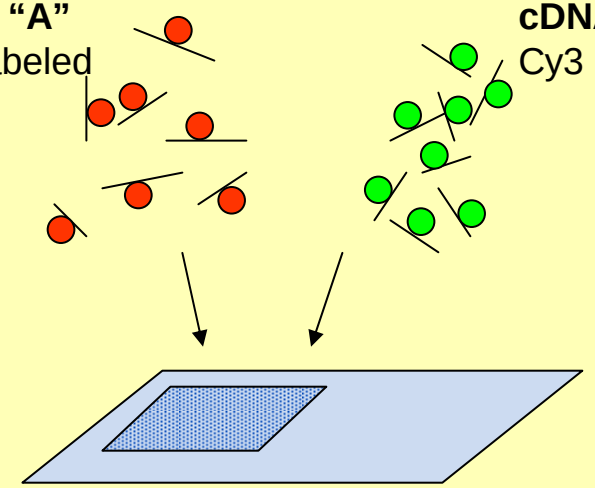


Allocation of mRNA samples to the slides

cDNA

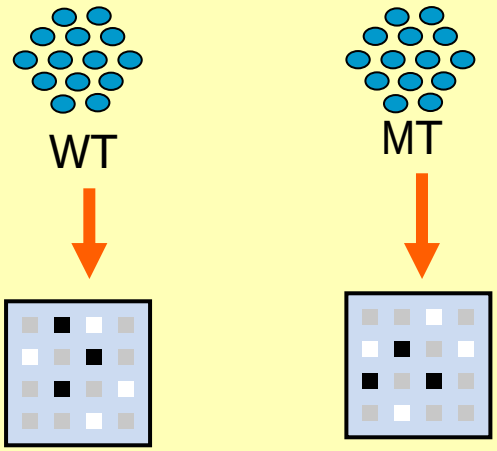
cDNA "A"
Cy5 labeled

cDNA "B"
Cy3 labeled



Hybridization

affy



Some aspects of design

2. Allocation of samples to the slides

A Types of Samples

- Replication – technical, biological.
- Pooled vs individual samples.
- Pooled vs amplification samples.

This relates to both Affymetrix and two color spotted array.

B Different design layout

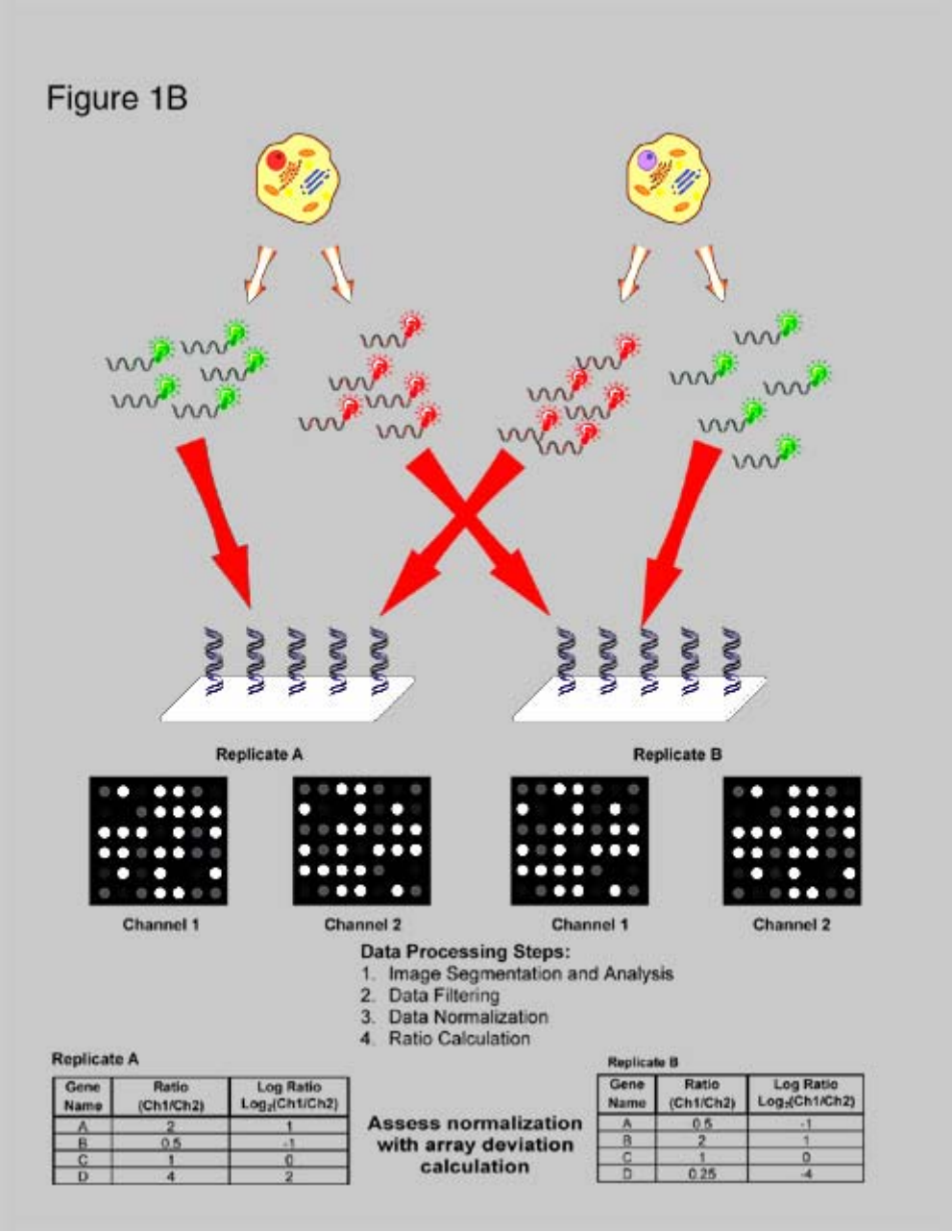
- Scientific aim of the experiment.
- Robustness.
- Extensibility.
- Efficiency.

Taking physical limitations or cost into consideration:

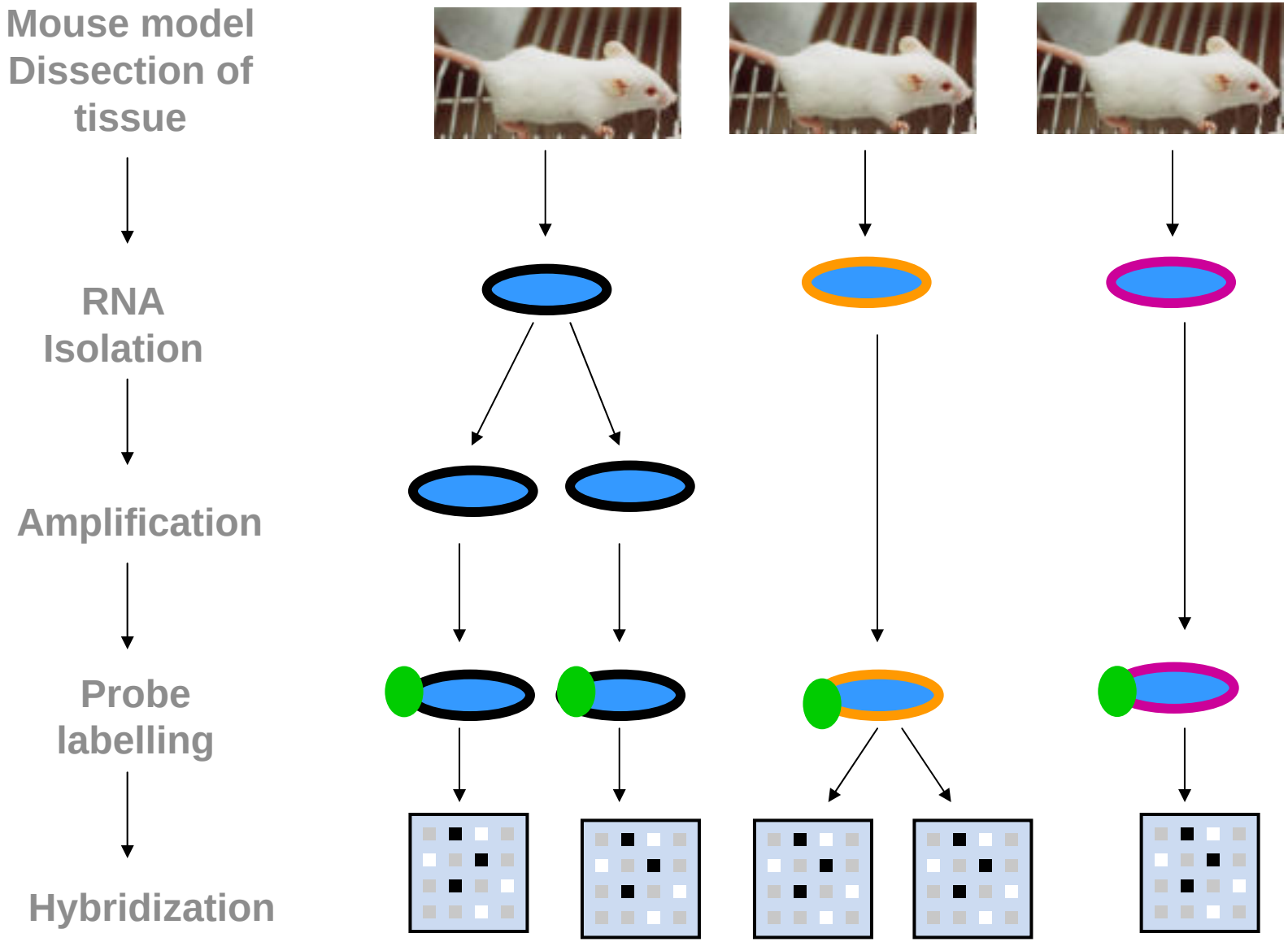
- the number of slides.
- the amount of material.

Design of a Dye-Swap Experiment

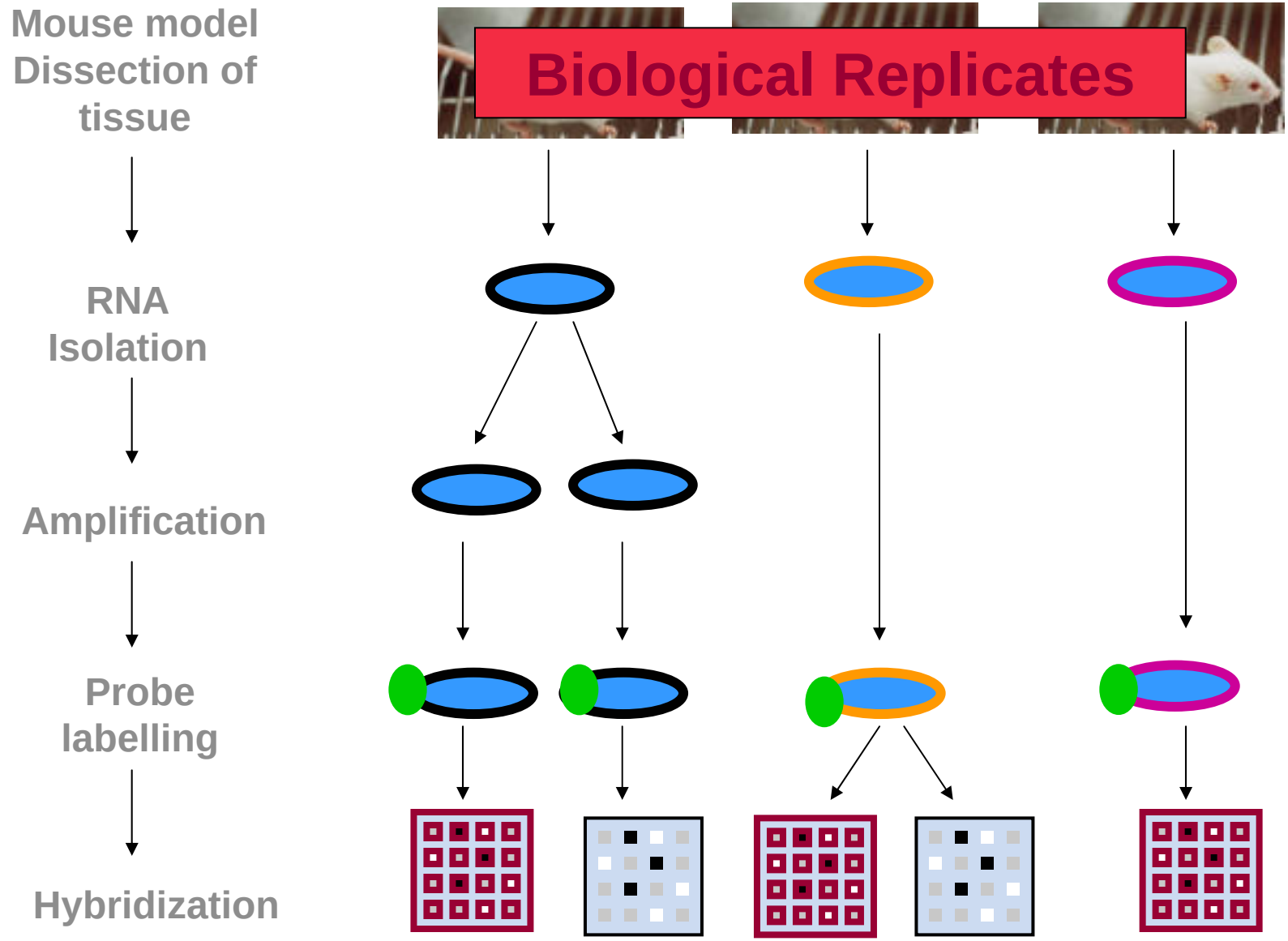
- Repeats are essential to control the quality of an experiment.
- One example for Replicates is the Dye-Swap, i.e. Replicates with the same mRNA Pool but with swapped labels.
- Dye-Swap shows whether there is a dye-bias in the Experiment.



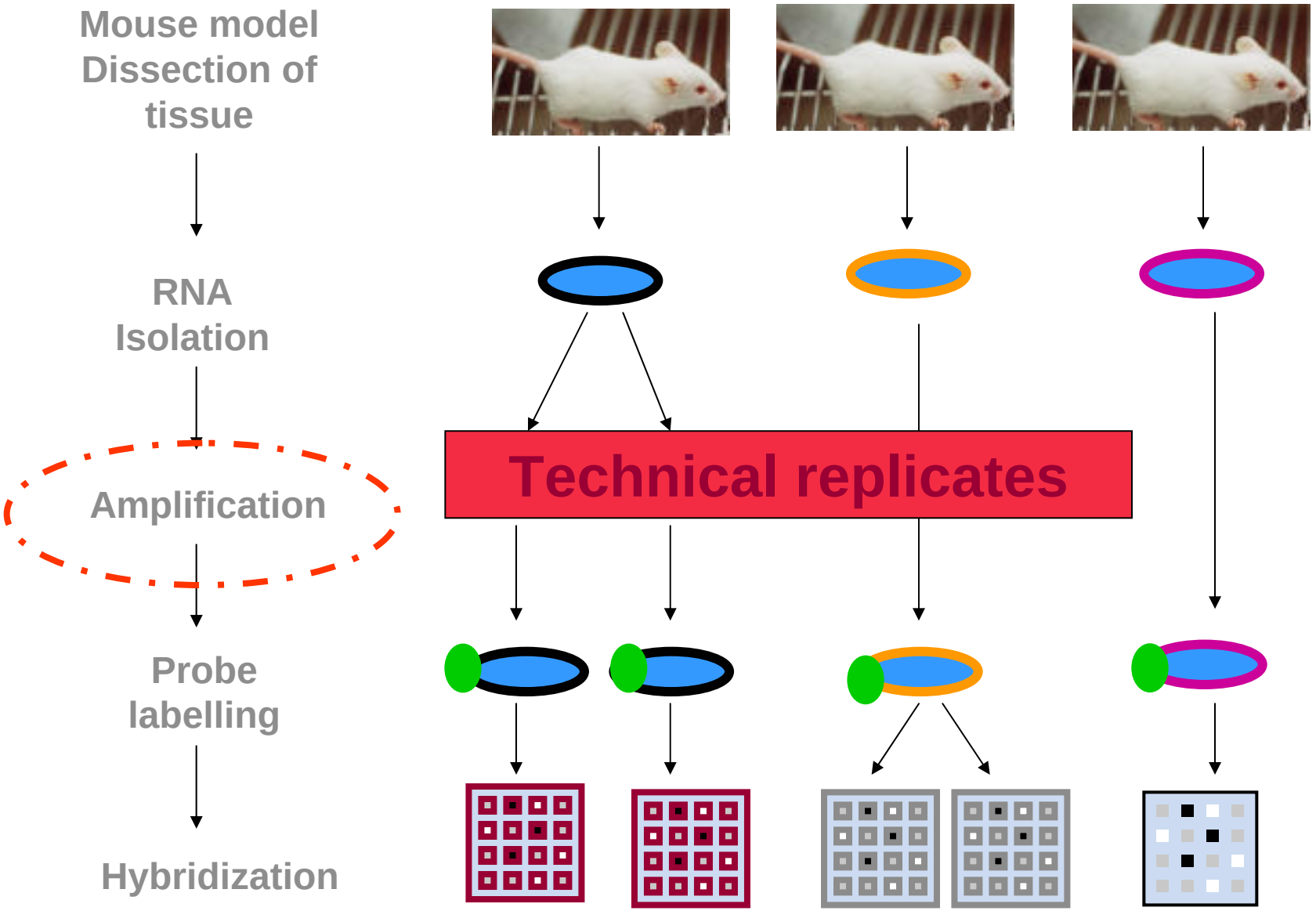
Preparing mRNA samples:



Preparing mRNA samples:



Preparing mRNA samples:



Pooling: looking at very small amount of tissues

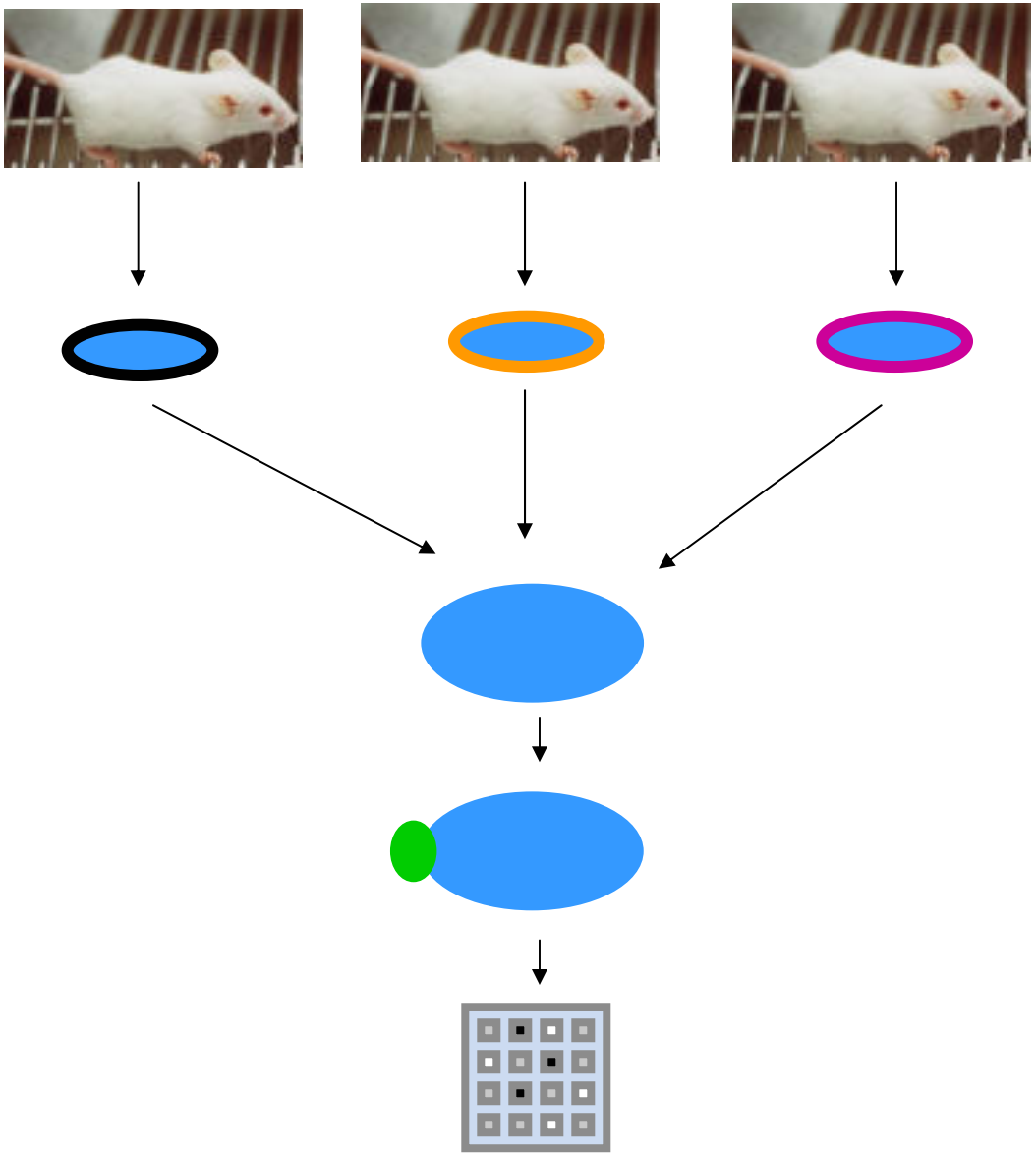
Mouse model
Dissection of tissue

RNA Isolation

Pooling

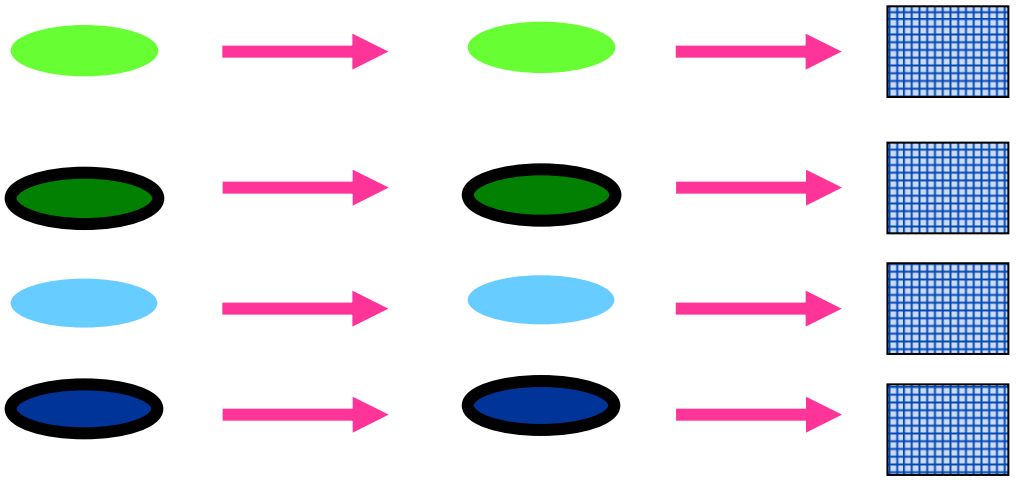
Probe labelling

Hybridization

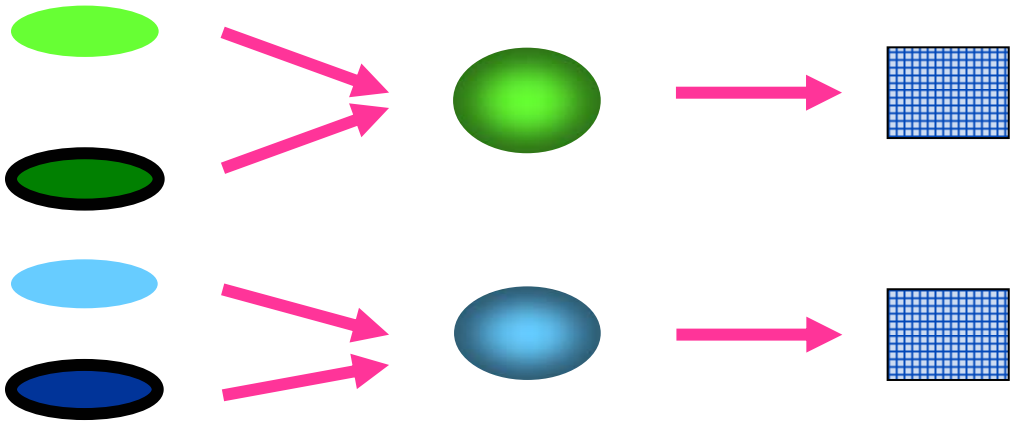


Pooled vs Individual samples

Design 1



Design 2



Taken from
Kendziorski etl al (2003)

Pooled versus Individual samples

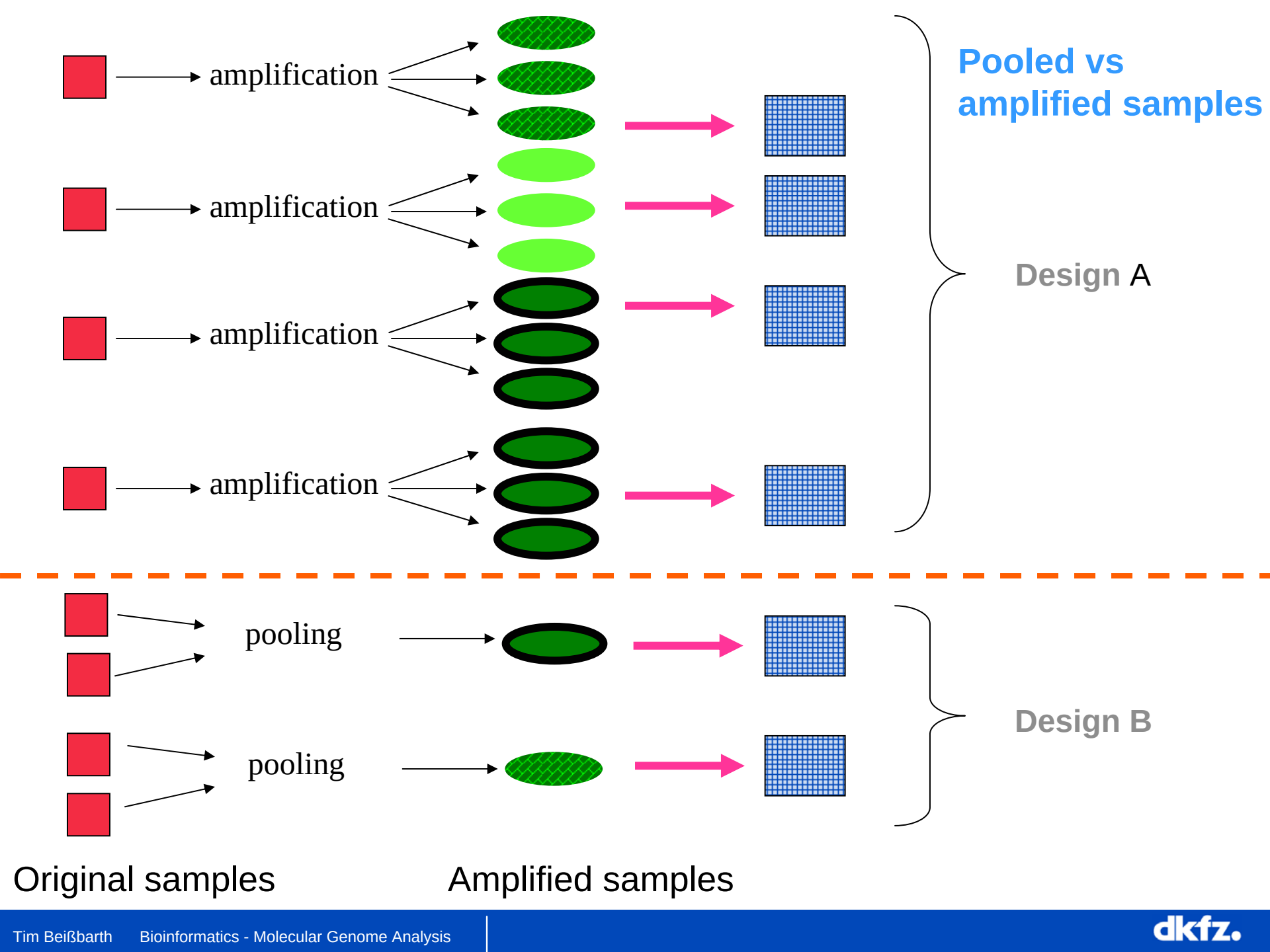
- Pooling is seen as “biological averaging”.

- Trade off between
  - Cost of performing a hybridization.
  - Cost of the mRNA samples.

- Case 1: Cost or mRNA samples << Cost per hybridization
  Pooling can assists reducing the number of hybridization.
- Case 2: Cost or mRNA samples >> Cost per hybridization
  Hybridize every Sample on an individual array to get the maximum amount of information.

- **References:**

  - Han, E.-S., Wu, Y., Bolstad, B., and Speed, T. P. (2003). A study of the effects of pooling on gene expression estimates using high density oligonucleotide array data. Department of Biological Science, University of Tulsa, February 2003.
  - Kendziorski, C.M., Y. Zhang, H. Lan, and A.D. Attie. (2003). The efficiency of mRNA pooling in microarray experiments. *Biostatistics* **4**, 465-477. 7/2003
  - Xuejun Peng, Constance L Wood, Eric M Blalock, Kuey Chu Chen, Philip W Landfield, Arnold J Stromberg (2003). Statistical implications of pooling RNA samples for microarray experiments. *BMC Bioinformatics* 4:26. 6/2003


amplification
amplification
amplification
amplification
Pooled vs amplified samples
Design A
pooling
pooling
Design B
Original samples
Amplified samples

Pooled vs Amplified samples

- In the cases where we do not have enough material from one biological sample to perform one array (chip) hybridizations. Pooling or Amplification are necessary.

- Amplification
  - Introduces more noise.
  - Non-linear amplification (??), different genes amplified at different rate.
  - Able to perform more hybridizations.

- Pooling
  - Less replicates hybridizations.

Some aspects of design

2. Allocation of samples to the slides

A Types of Samples

- Replication – technical, biological.
- Pooled vs individual samples.
- Pooled vs amplification samples.

B Different design layout

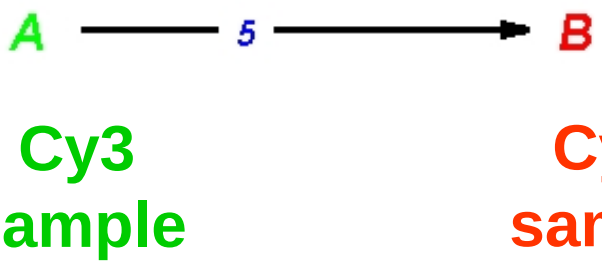
- Scientific aim of the experiment.
- Robustness.
- Extensibility.
- Efficiency.

Taking physical limitation or cost into consideration:

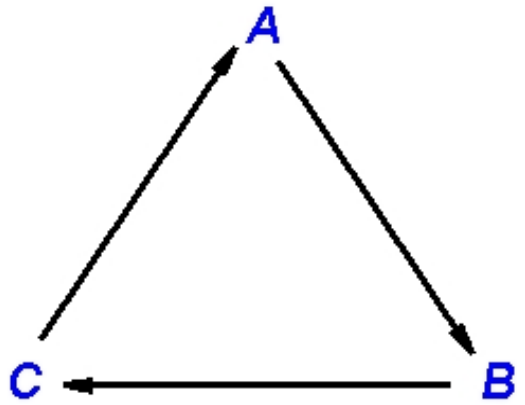
- the number of slides.
- the amount of material.

Graphical representation

Vertices: mRNA samples;
Edges: hybridization;
Direction: dye assignment.



(a)



(b)

Graphical representation

- The structure of the graph determines which effects can be estimated and the precision of the estimates.
  - Two mRNA samples can be compared only if there is a path joining the corresponding two vertices.
  - The precision of the estimated contrast then depends on the number of paths joining the two vertices and is inversely related to the length of the paths.

- Direct comparisons **within slides** yield more precise estimates than indirect ones between slides.

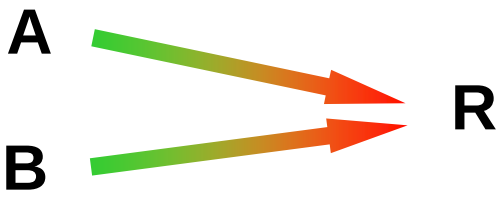
The simplest design question:
Direct versus indirect comparisons

Two samples (A *vs* B)

e.g. KO *vs.* WT or mutant *vs.* WT

Direct

Indirect



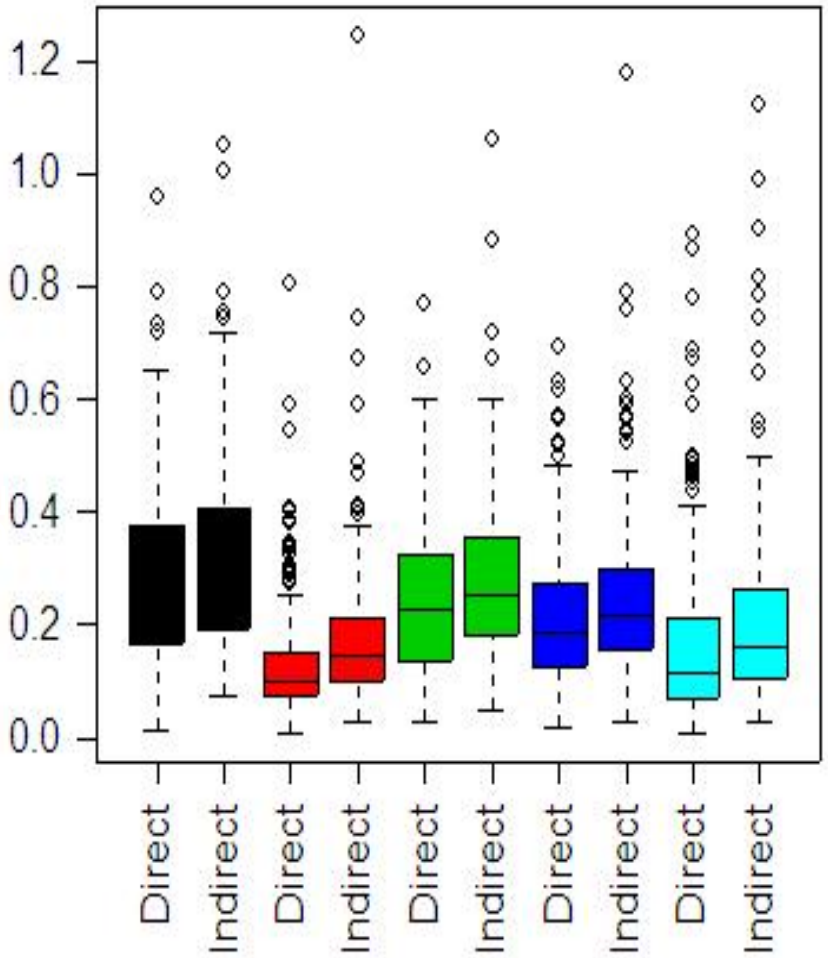
$$\text{average}\,(\log\,(A/B)) \qquad \log\,(A\,/\,R) - \log\,(B\,/\,R\,)$$

$$\sigma^2\,/2 \qquad 2\sigma^2$$

These calculations assume independence of replicates: the reality is not so simple.

Experimental results

- 5 sets of experiments with similar structure.
- Compare (Y axis)
  A) SE for $aveM_{mt}$
  B) SE for $aveM_{mt} - aveM_{wt}$
- Theoretical ratio of (A / B) is 1.6
- Experimental observation is 1.1 to 1.4.



Experimental design

- Create highly correlated reference samples to overcome inefficiency in common reference design.

- Not advocating the use of technical replicates in place of biological replicates for samples of interest.

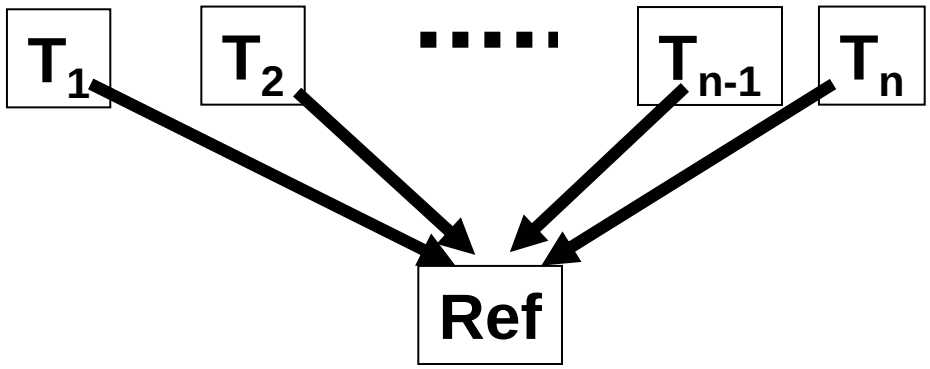
- Efficiency can be measured in terms of different quantities
  - number of slides or hybridizations;
  - units of biological material, e.g. amount of mRNA for one channel.

- In addition to experimental constraints, design decisions should be guided by the knowledge of which effects are of greater interest to the investigator.

  E.g. which main effects, which interactions.

- The experimenter should thus decide on the comparisons for which he wants the most precision and these should be made **within slides** to the extent possible.

Common reference design



- Experiment for which the common reference design is appropriate

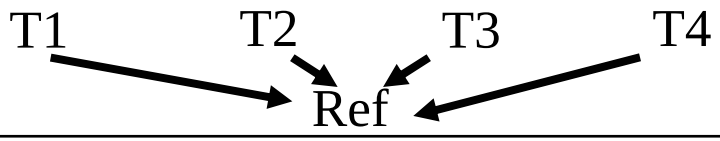
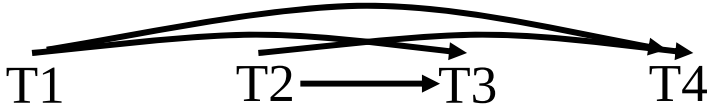
  Meaningful biological control (C) Identify genes that responded differently / similarly across two or more treatments relative to control.

  Large scale comparison. To discover tumor subtypes when you have many different tumor samples.

- Advantages:

  Ease of interpretation.

  Extensibility - extend current study or to compare the results from current study to other array projects.

Design choices in time series		t vs t+1			t vs t+2			
		T1T2	T2T3	T3T4	T1T3	T2T4	T1T4	Ave
N=3	A) T1 as common reference 	1	2	2	1	2	1	1.5
	B) Direct Hybridization 	1	1	1	2	2	3	1.67
N=4	C) Common reference 	2	2	2	2	2	2	2
	D) T1 as common ref + more 	.67	.67	1.67	.67	1.67	1	1.06
	E) Direct hybridization choice 1 	.75	.75	.75	1	1	.75	.83
	F) Direct Hybridization choice 2 	1	.75	1	.75	.75	.75	.83

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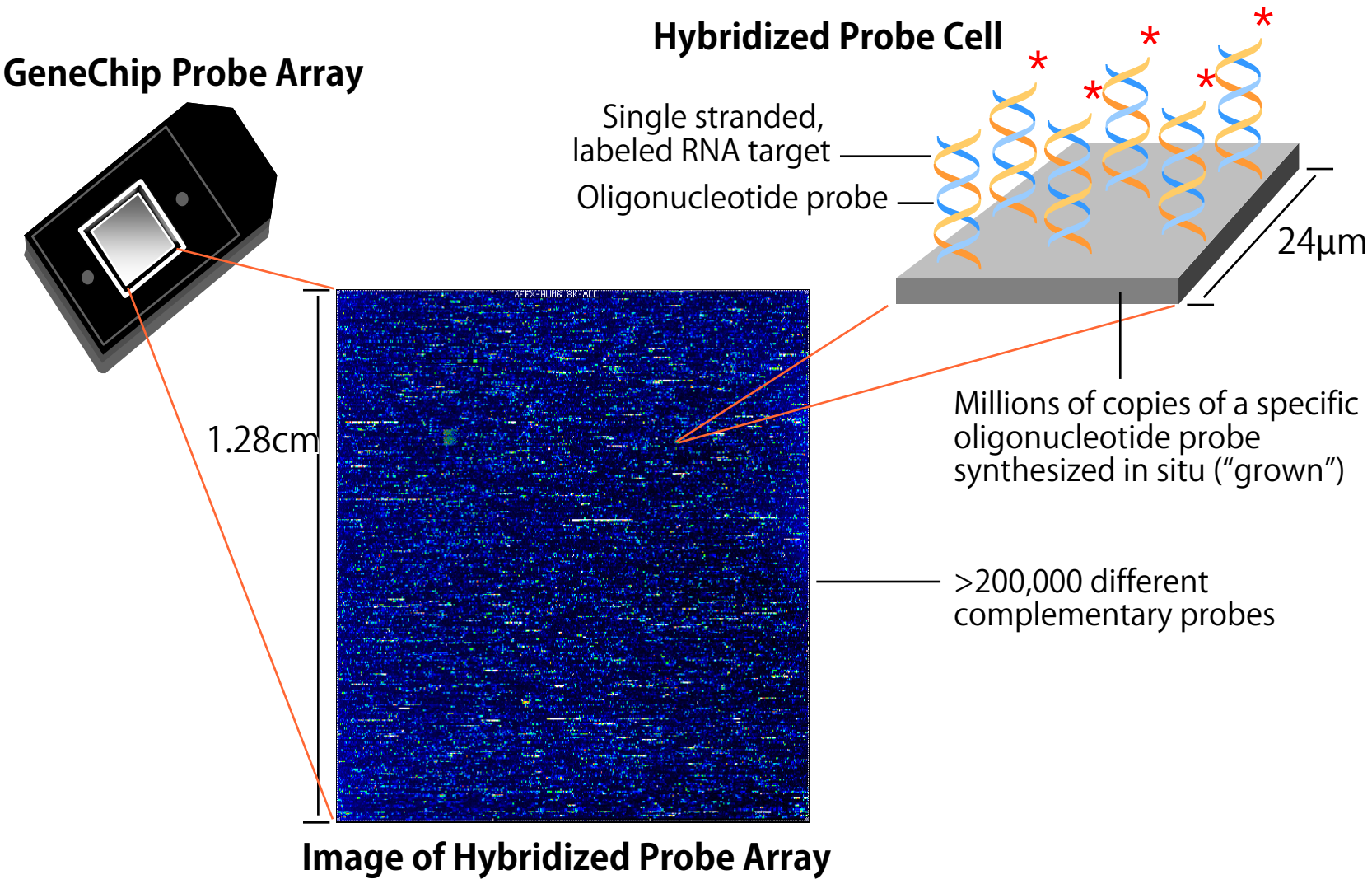
Monday
3. March 08

Affy Chips: PM versus MM and summary information

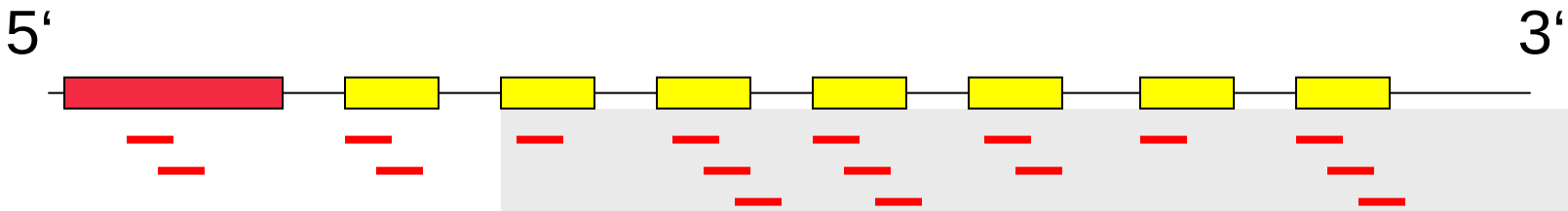
MGA
Molecular Genome Analysis - Bioinformatics and Quantitative Modeling

dkfz.
DEUTSCHES KREBSFORSCHUNGSZENTRUM
IN DER HELMHOLTZ-GEMEINSCHAFT

Affymetrix GeneChips zusammengefasst

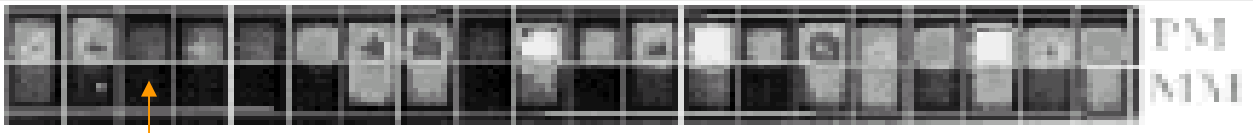


Affymetrix technology



16-20 *probe pairs* per gene

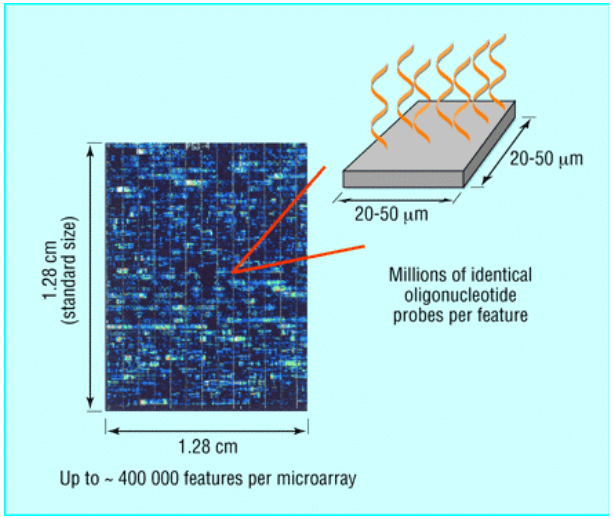
PM: ATGAGCTGTACCAATGCCAACCTGG
MM: ATGAGCTGTACCTATGCCAACCTGG



64 pixels; Signal intensity is upper quartile of the 36 inner pixels

Stored in CEL file

16-20 probe pairs: HG-U95a
11 probe pairs: HG-U133



Affymetrix expression measures

- PM_{ijg}, MM_{ijg} = Intensity for perfect match and mismatch probe j for gene g in chip i.
  - i = 1,…, n one to hundreds of chips
  - j = 1,…, J usually 16 or 20 probe pairs
  - g = 1,…, 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.

Low – level -Analysis

- Preprocessing signals: background correction, normalization, PM-adjustment, summarization.
- Normalization on probe or probe set level?
- Which probes / probe sets used for normalization
- How to treat PM and MM levels?

Normalization – complete data methods

- Quantile normalization:
  Make the distribution of probe intensities the same for all arrays.
  $F_{i,normalised}(x) = F^{-1}_{global}(F_i(x))$ (Q-Q-Plot)
- Robust quantile normalization
- Cyclic loess (MA plots of two arrays for log-transformed signals and loess)
- VSN

What is the best approach? Look at criteria provided by the affycomp procedure.

Cope LM, Irizarry RM, Jaffee H, Wu Z, Speed TP, ***A Benchmark for Affymetrix GeneChip Expression Measures,***
Bioinformatics, 2004, 20:323-31

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

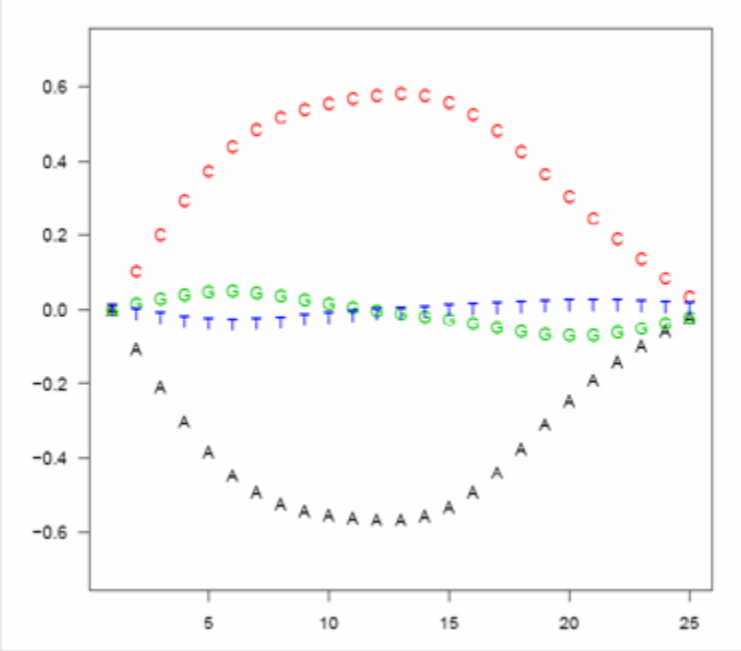
Expression measures
RMA: Irizarry et al. (2002)

- 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.

Expression Measures:
GC-RMA: Wu, Irizarry et al, 2004

- Uses a stochastic model for background adjustment that uses probe sequence information.



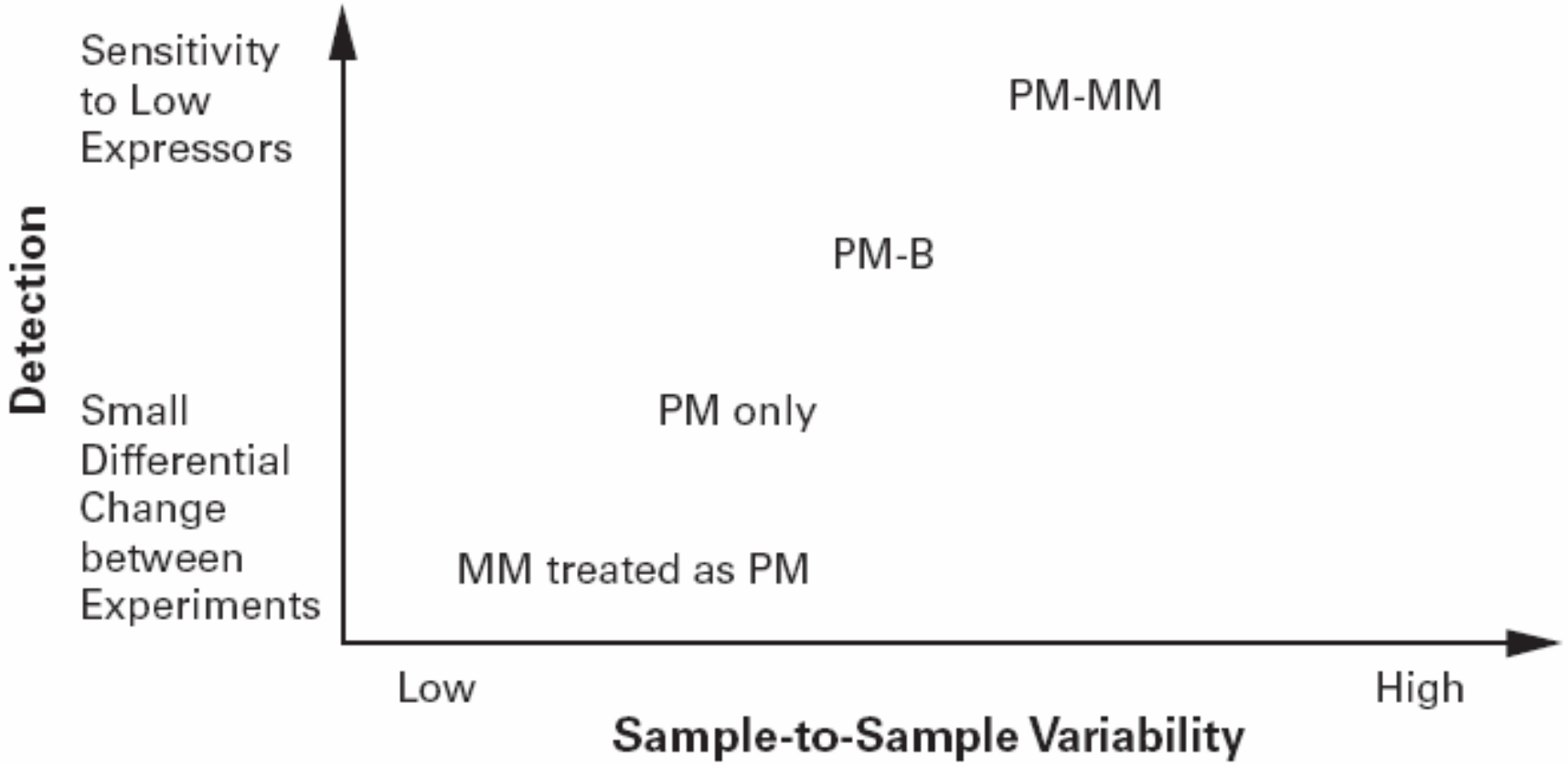
The effect of base *A* in position *k*, $\mu A;k$, is plotted against *k*. Similarly for the other three bases.

$$PM = O_{PM} + N_{PM} + S$$

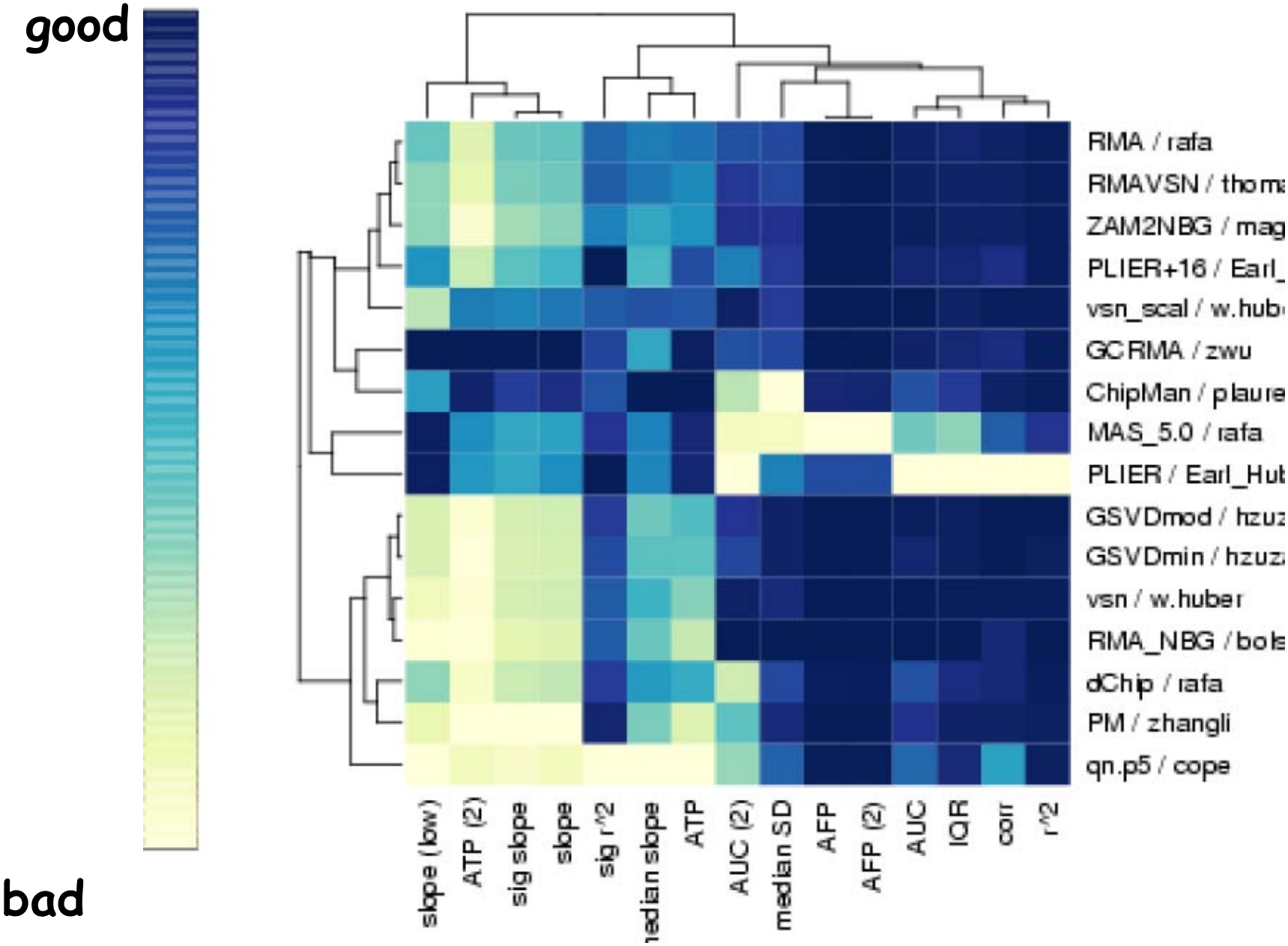
$$MM = O_{MM} + N_{MM} + \phi S.$$

Here *O* represents optical noise, *N* represents NSB noise and *S* is a quantity proportional to RNA expression (the quantity of interest). The parameter $0 < \phi < 1$ accounts for the fact that for some probe-pairs the *MM* detects signal.

Expression Measures:
PLIER - Probe Logarithmic Intensity Error Estimation



affycomp results



Acknowledgements – Slides borrowed from

- **Achim Tresch**
- **Wolfgang Huber**
- **Ulrich Mansmann**
- **Terry Speed**
- **Jean Yang**
- **Benedikt Brors**
- **Anja von Heydebreck**
- **Rainer König**