Background and Normalization:
Investigating the effects of preprocessing on gene expression estimates

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Outline

- “Background” correction methods
- Normalization methods
  - Key results from Bolstad et al (2003)
- Expression Summarization
- Expression Calculation as a 3 step process.
- Case Study: Affymetrix Latin Square
- Conclusions

What is background?

- A measurement of signal intensity caused by auto fluorescence of the array surface and non specific binding.
- Since probes are so densely packed on chip must use probes themselves (rather than region adjacent to probes as in cDNA arrays) to calculate the background.
- In theory the MM should serve as a biological background correction for the PM.

Background correction is:

- A method for removing background noise from signal intensities using information from only one chip

RMA Background

- O = S + N
- O – Observed, S- Signal, N-noise
- Model S by Exponential, N by Normal
- Parameters estimated in ad-hoc way using both PM (and sometimes MM) but worry only about correcting PM
- Background correction is given by E(S|O)

RMA Background Math

- Observed PM intensity (O)
- Model as sum of signal (S) and background (N)
- Assume S is exponential α
- Assume N is Normal μ, σ
- Background corrected values are then

$$E(S \mid O = o) = a + b \frac{\phi\left(\frac{a}{b}\right) - \phi\left(\frac{o-a}{b}\right)}{\Phi\left(\frac{a}{b}\right) + \Phi\left(\frac{o-a}{b}\right) - 1}$$

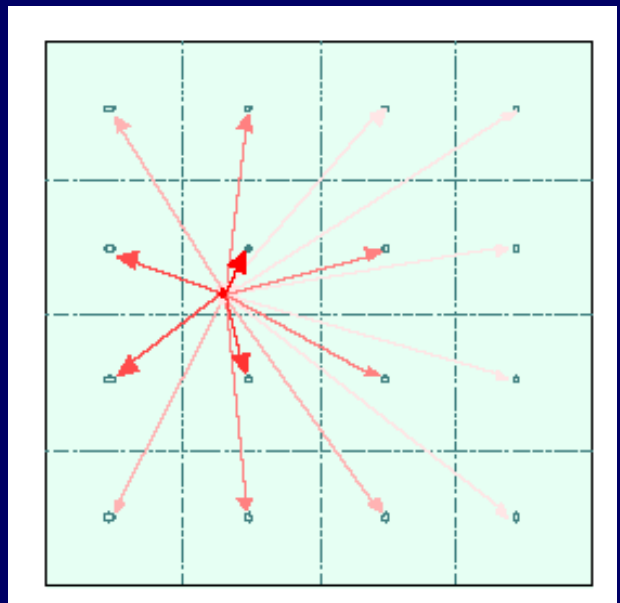
$$a = o - \mu - \sigma^2\alpha, b = \sigma$$

MAS 5.0 Background

- Use the background correction method as described in Affymetrix “Statistical Algorithm Description Document”
- Synopsis:
- Break chip into k (k=1..16) rectangular regions
  - lowest 2% is chosen as background for that region B_k
  - Standard deviation for lowest 2% is chosen as noise for that zone N_k

MAS 5.0 Background (cont)

- The background adjustment to be used for cell at (x,y) is weighted average of the B_k, where the weights depend on the distance between (x,y) and the centroids of the regions. : b(x,y)



MAS 5.0 Background (cont)

- A noise adjustment is computed in a similar way using N_k rather than B_k : n(x,y)
- The Background adjusted intensity is given by
- A(x,y)=max(I(x,y)-b(x,y),NoiseFrac*n(x,y))
  - Where NoiseFrac = 0.5

MAS 5.0 Mismatch correction

- The way Affymetrix make use of MM in MAS5.0
- Define biweight specific background (SB) for probe pair *j* in probeset *I* as
  - *SB_i = T_bi(log_2(PM_i,j) – log_2(MM_i,j):j = 1,…,n_i)*
  - T_bi is Tukey biweight function

MAS 5.0 Mismatch (Cont)

- IM_i,j is the ideal mismatch
- If MM_i,j < PM_i,j
  - IM_i,j = MM_i,j
- If MM_i,j >= PM_i,j and SB_i > contrasttau
  - IM_i,j = PM_i,j/2^(SB_i)
- If MM_ i,j >= PM_i,j and SB_i <= contrasttau
  - IM_i,j = PM_i,j/2^(contrasttau/(1 + (contrasttau – SB_i)/scaletau))
- contrasttau = 0.03, scaletau=10
- Corrected PM_i,j is PM_i,j – MM_i,j

What is normalization?

"Non-biological factors can contribute to the variability of data ... In order to reliably compare data from multiple probe arrays, differences of non-biological origin must be minimized."

- Normalization is a process of reducing unwanted variation across chips, may use information from multiple chips

Quantile Normalization

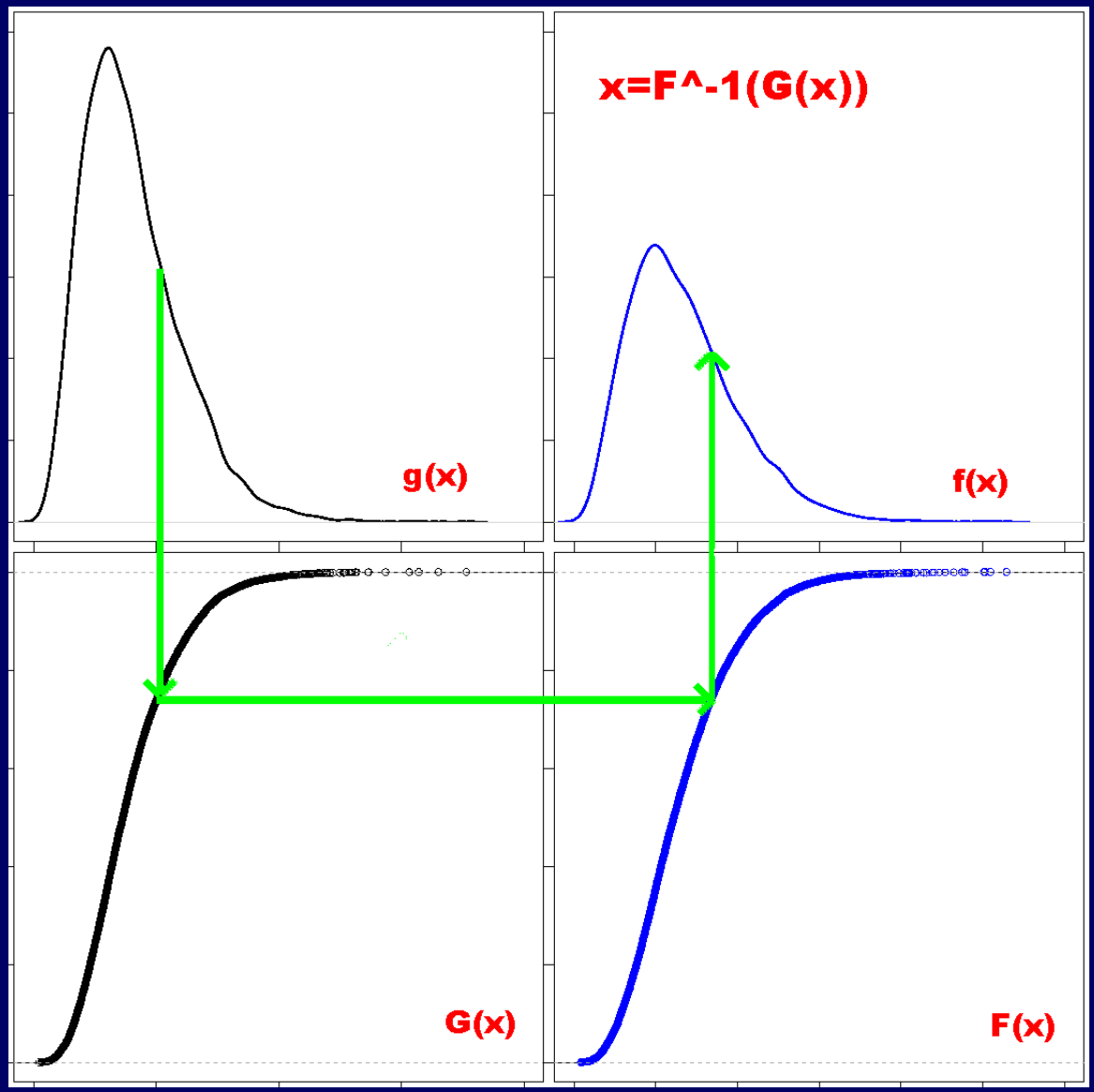
- See Bolstad et al (2002)
- Quantile normalization is a method to make the distribution of probe intensities the same for every chip.
- The normalization distribution is chosen by averaging each quantile across chips.
- The diagram that follows illustrates the transformation.

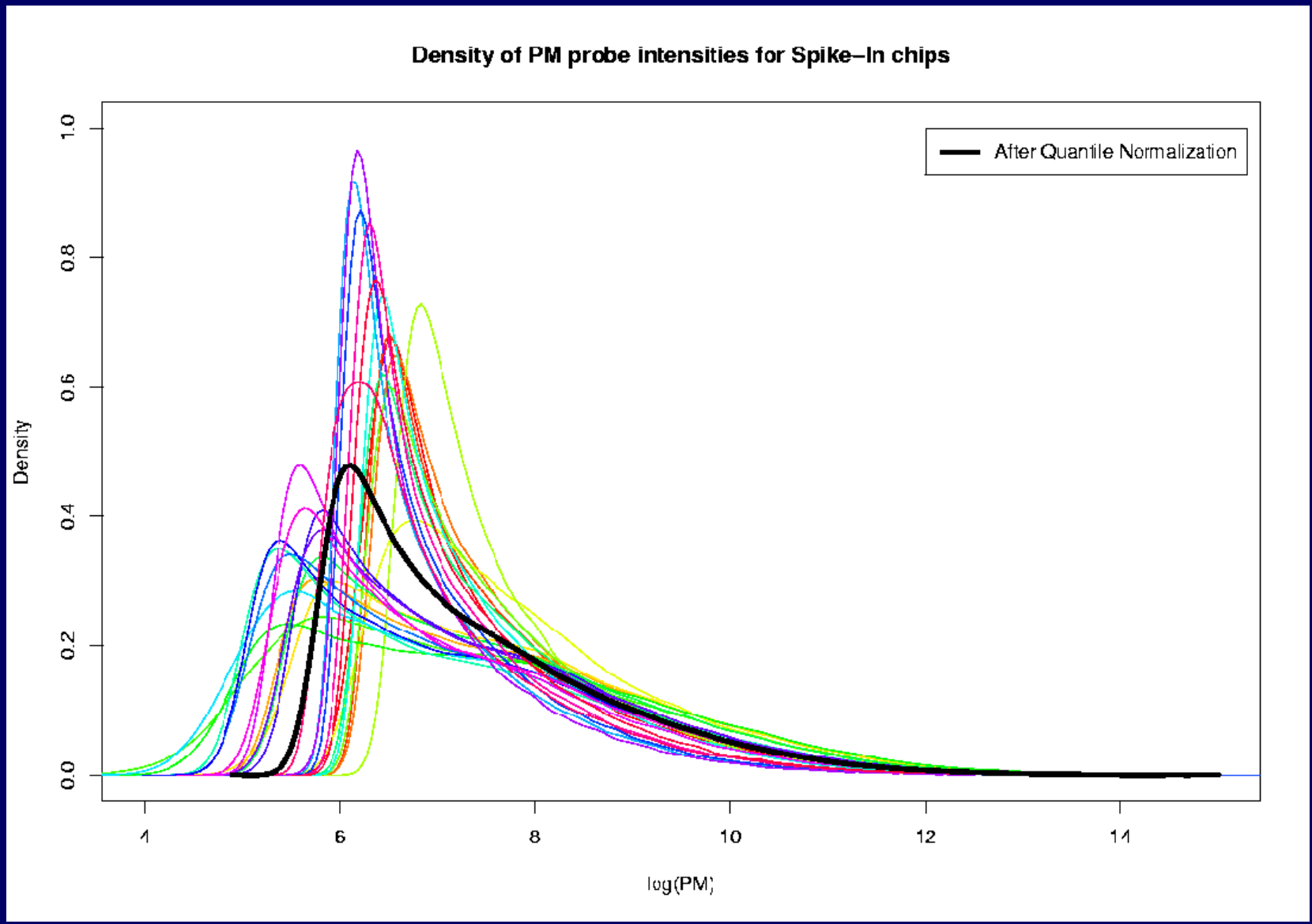
Raw Data

Normalization Distribution

Density Function

Distribution Function




Density of PM probe intensities for Spike–In chips
After Quantile Normalization
Density
0.0
0.2
0.4
0.6
0.8
1.0
4
6
8
10
12
14
log(PM)

Quantile Normalization (cont)

- The two distribution functions are effectively estimated by the sample quantiles.
- Quantile normalization is fast
- After normalization, variability of expression measures across chips reduced

Normalization in MAS

- Compares a collection of experimental array with a baseline array, and normalizes the average intensity of the experimental array to the average intensity of the baseline array during normalization (sometime use a trimmed mean).
- We refer to this method as scaling
- MAS documentation applies normalization after summarization, we will use it before

Other prominent methods

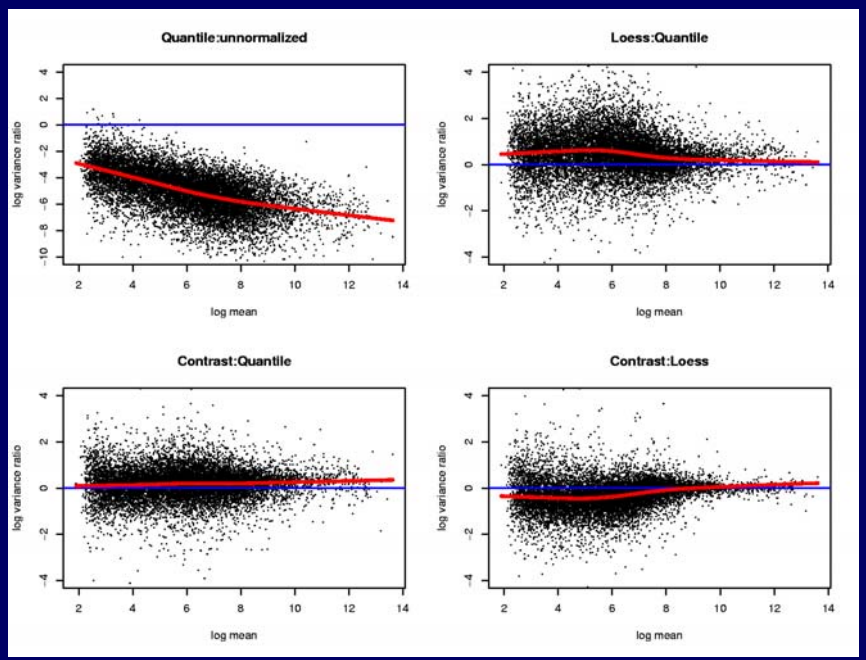
- Nonlinear – method used in dChip
  - pick a baseline chip then fit non linear relations (smoothing spines, running medians) between baseline chips and other chips
- Contrast, Cyclic loess
  - generalized M vs A loess normalization methods

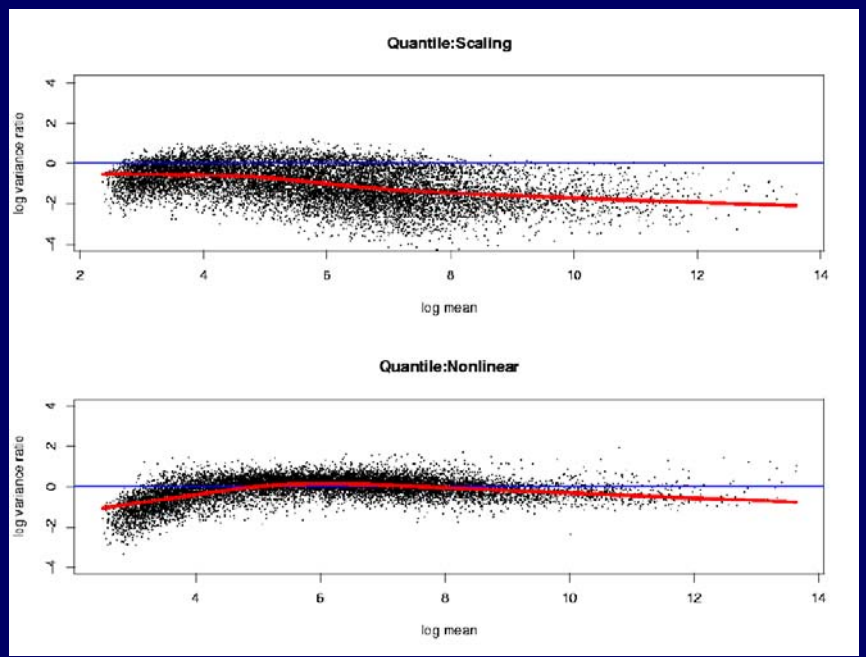
Bolstad et al (2003)

- Compares normalization methods in context of RMA measure
- Classifies normalization methods into two classes:
  - Complete Data Methods
    - Quantile
    - Contrast
    - Cyclic Loess
  - Baseline methods
    - Scaling
    - Non-linear

Bolstad et al (2003) cont

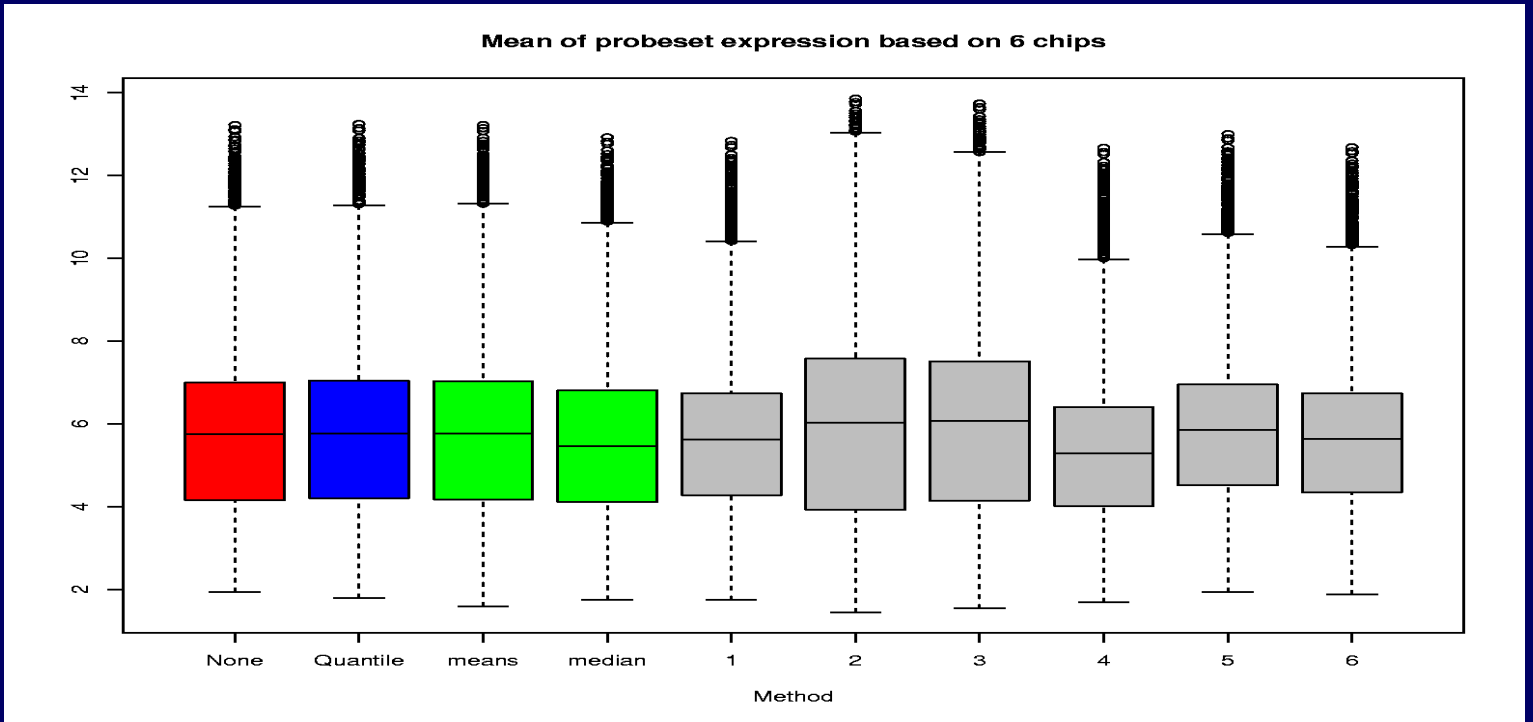
- Quantile normalization reduces between chip variability favorably when compared to other methods





Bolstad et al (2003) cont

- Quantile method also found to perform well on the issue of bias (this was measured by using spike-in data)
- Complete data methods recommended over using a baseline



Expression Summarization

- Given a set of background corrected and normalized PM probe intensities for each probeset compute a single number for that probeset intended to represent gene expression on an array.

RMA: Robust Multichip Average

- Suppose a we have j=1,…,J arrays and i=1,…,I probes for a given probeset
- Fit a robust linear model with probe and chip effects to log transformed data.

$$y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

Where α_i is probe-effect and is β_j chip effect

- Expression is then (on a log scale) and given by

$$\mu + \beta_j$$

RMA continued

- Method is compared with MAS 5.0 and Li-Wong MBEI in Rafael A. Irizarry, Benjamin M. Bolstad, Francois Collin, Leslie M. Cope, Bridget Hobbs, and Terence P. Speed (2002) Summaries of Affymetrix GeneChip Probe Level Data. Accepted to Nucleic Acids Research
- Found to outperform other methods in most regards
- Current implementations use median-polish to fit the linear model, other robust linear model fitting procedures are being explored.

MAS 5.0: “the statistical algorithm”

- Using log-scale data for the probes related to the probeset on single chip.
- Suppose P_i for i=1,…, I are preprocessed probe values
- Then expression is given by

$$E = T_{bi}(P_1, \ldots, P_I)$$

- Where $T_{bi}()$ is the 1 step Tukey Biweight

Other expression measures

- Not explored here
- AvDiff – the old Affymetrix method. Found wanting for a number of reasons
- Li-Wong MBEI (Model Based Expression Index) – implemented in the dChip software

Expression Calculation as a 3 step process.

- Suppose x are probe intensities, then 3 step process is
- Background correct B(x)
- Normalize N(x)
- Transform and Summarize S(log_2(x))
- Put the three together to get S(log_2(N(B(x))))
- In the case of RMA: B(x) is the RMA background correction, N(x) is quantile normalization and S(x) is the robust model fit.
- In the case of MAS 5.0: B(x) is the MAS 5.0 Background followed by IMM subtraction, N(x) leaves the data untouched and S(x) is the tukey bi-weight

Case Study: Affymetrix Latin Square

- Affymetrix has made available the dataset which was used in the development of the MAS 5.0 algorithm.
- The Latin square design for the Human data set consists of 14 spiked-in gene groups in 14 experimental groups. The concentration of the 14 gene groups in the first experiment is 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024pM.
- Total of 59 CEL files.

Case Study: Analysis setup

- We will mix and match the background, normalization and summarization steps, then compare the resulting Gene Expressions.
- In particular we will use:

Background	Normalization	Expression
None	None	RMA - medianpolish
RMA Background	Quantile	Tukey Biweight
MAS5.0 Background		
IMM		
MAS5.0 + IMM		

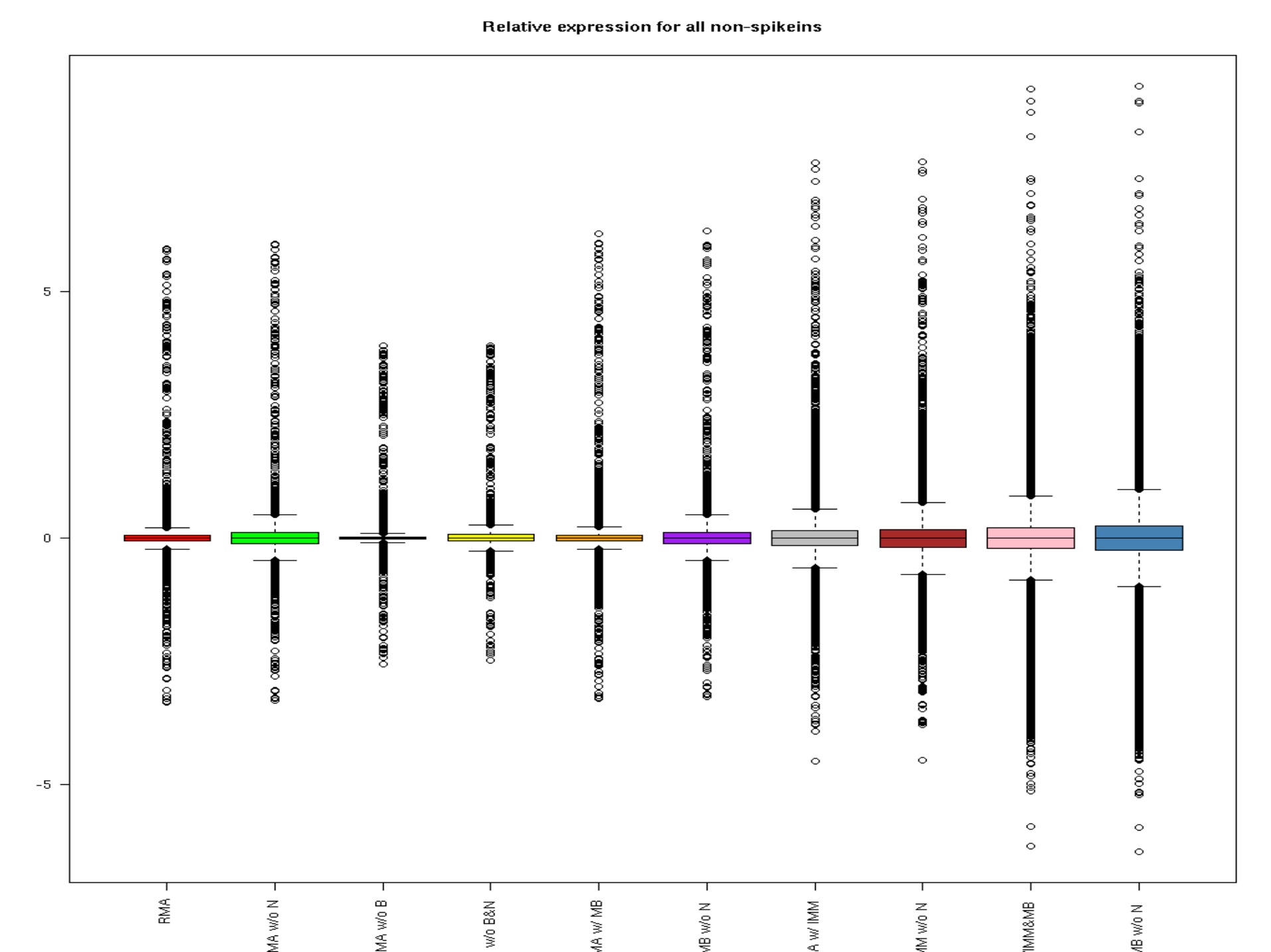
Note MAS 5 implementations are based upon available documentation, may not completely agree with MAS 5.0 software

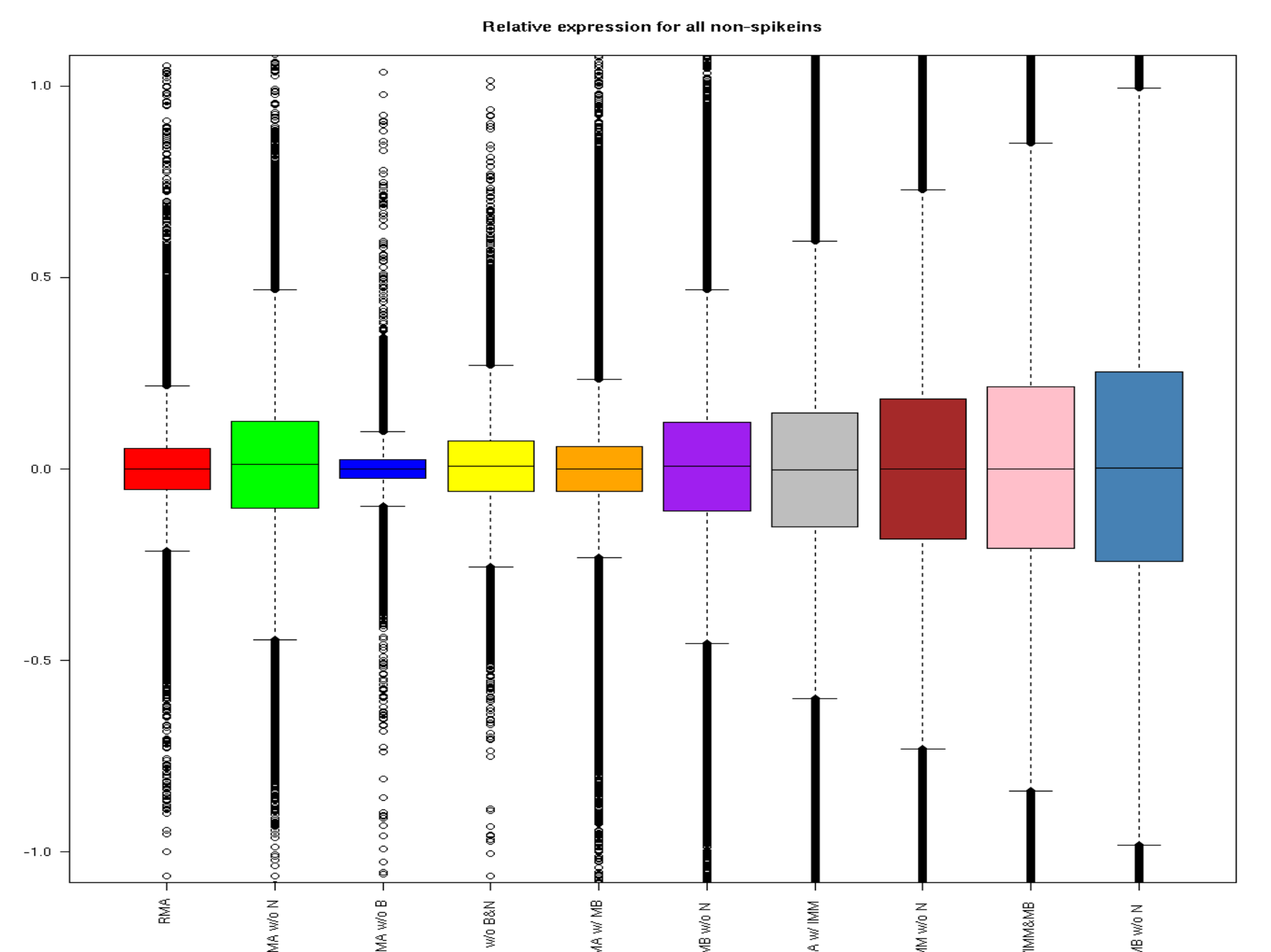
Computing relative expression

- In case of spike-in experiments, average in log –scale across spikein concentration replicates.
- If E_i,j is expression of probeset i in group j, then expression difference between grp 1 and 2 is
  - M_i = E_i,1 – E_i,2

What does preprocessing do to non differential probesets?

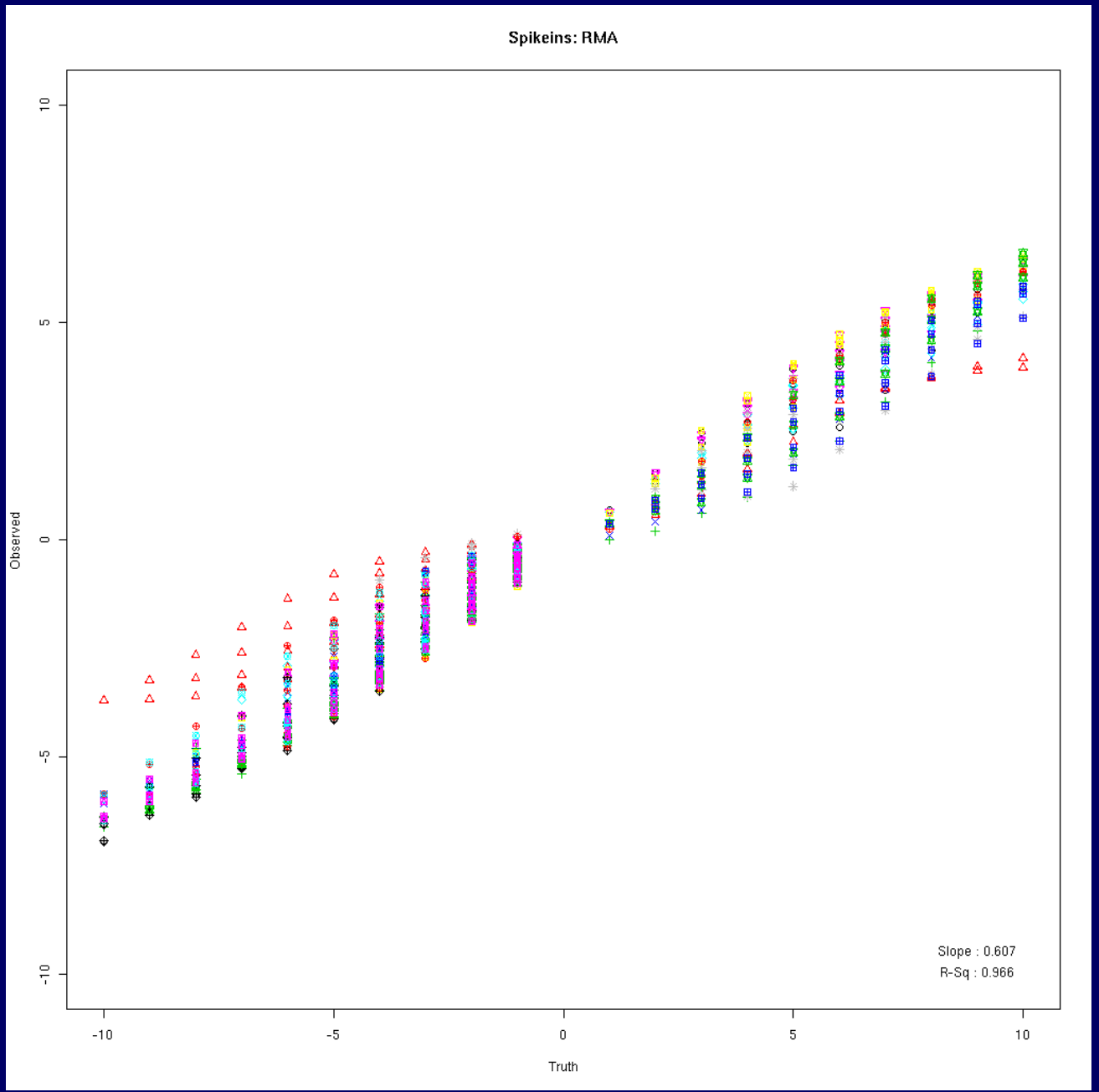
- To answer this we look at the relative expression between groups of non differential probesets.
- For example if there is 14 dilution groups then there is 14*13/2 = 91 different comparisons for each probeset.

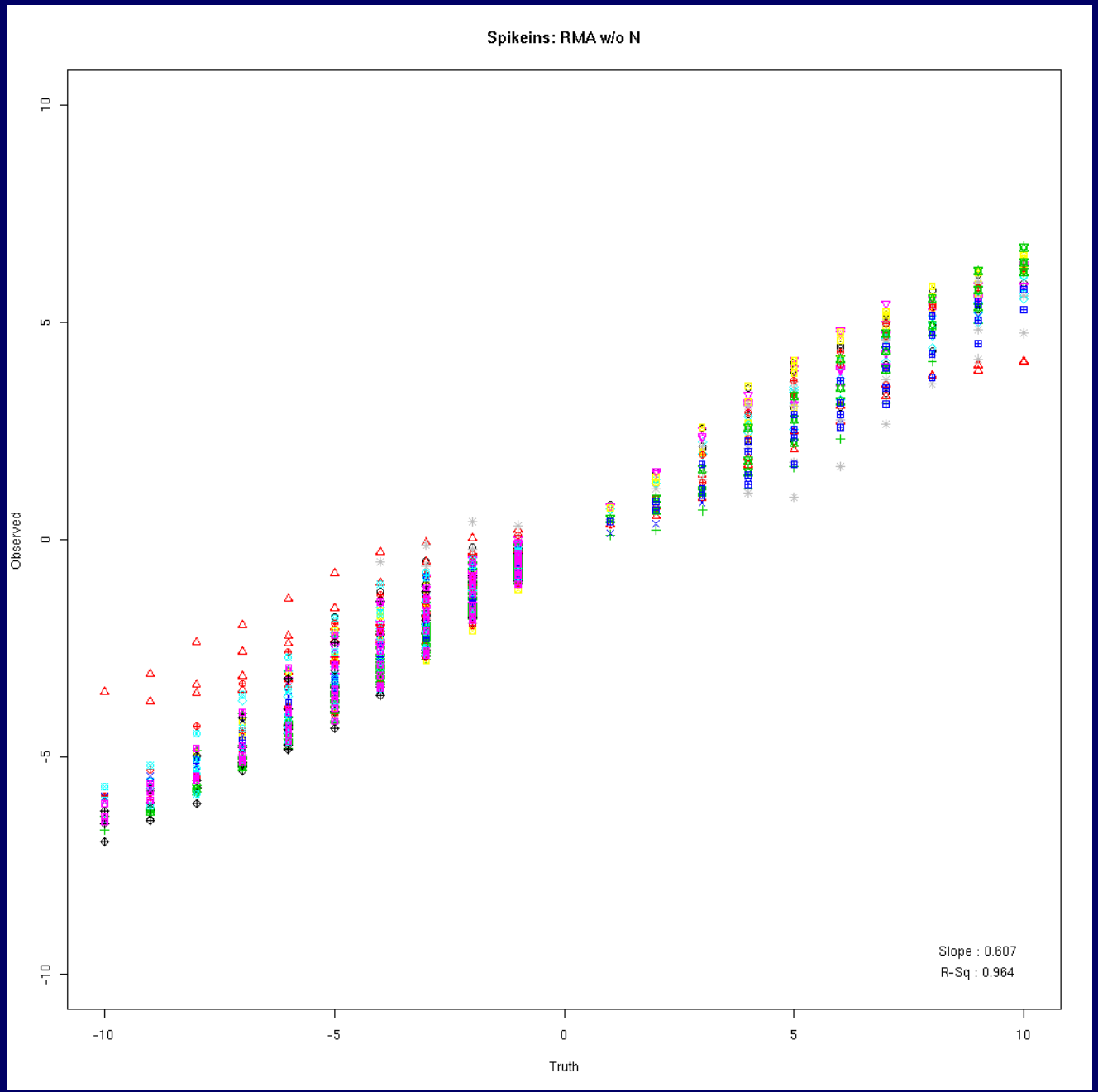

Relative expression for all non-spikeins
5
0
-5
RMA
MA w/o N
MA w/o B
w/o B&N
MA w/ MB
MB w/o N
A w/ IMM
IM w/o N
IMM&MB
MB w/o N

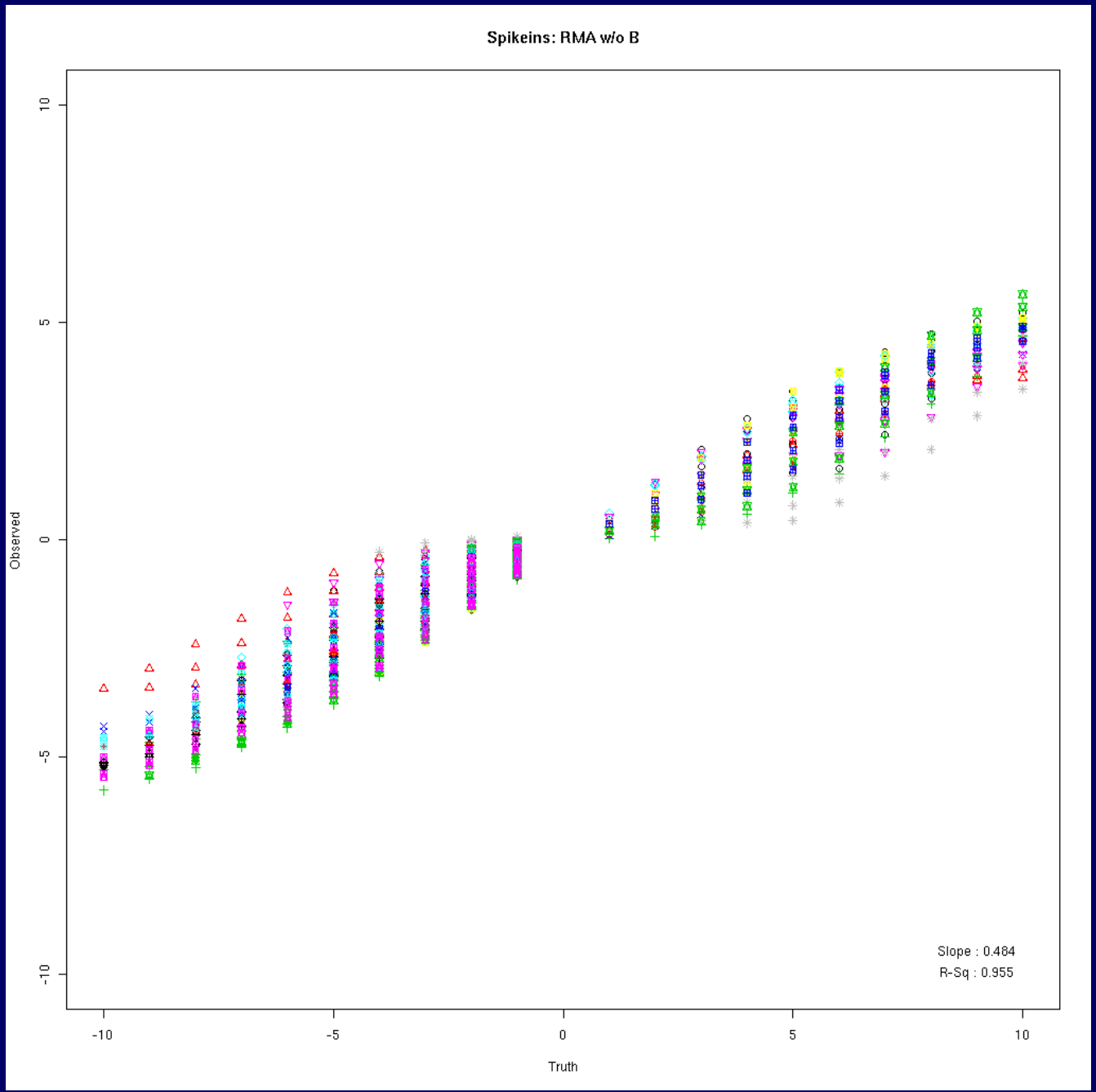

Relative expression for all non-spikeins
1.0
0.5
0.0
-0.5
-1.0
RMA
MA w/o N
MA w/o B
w/o B&N
A w/ MB
vIB w/o N
A w/ IMM
IM w/o N
IMM&MB
IB w/o N

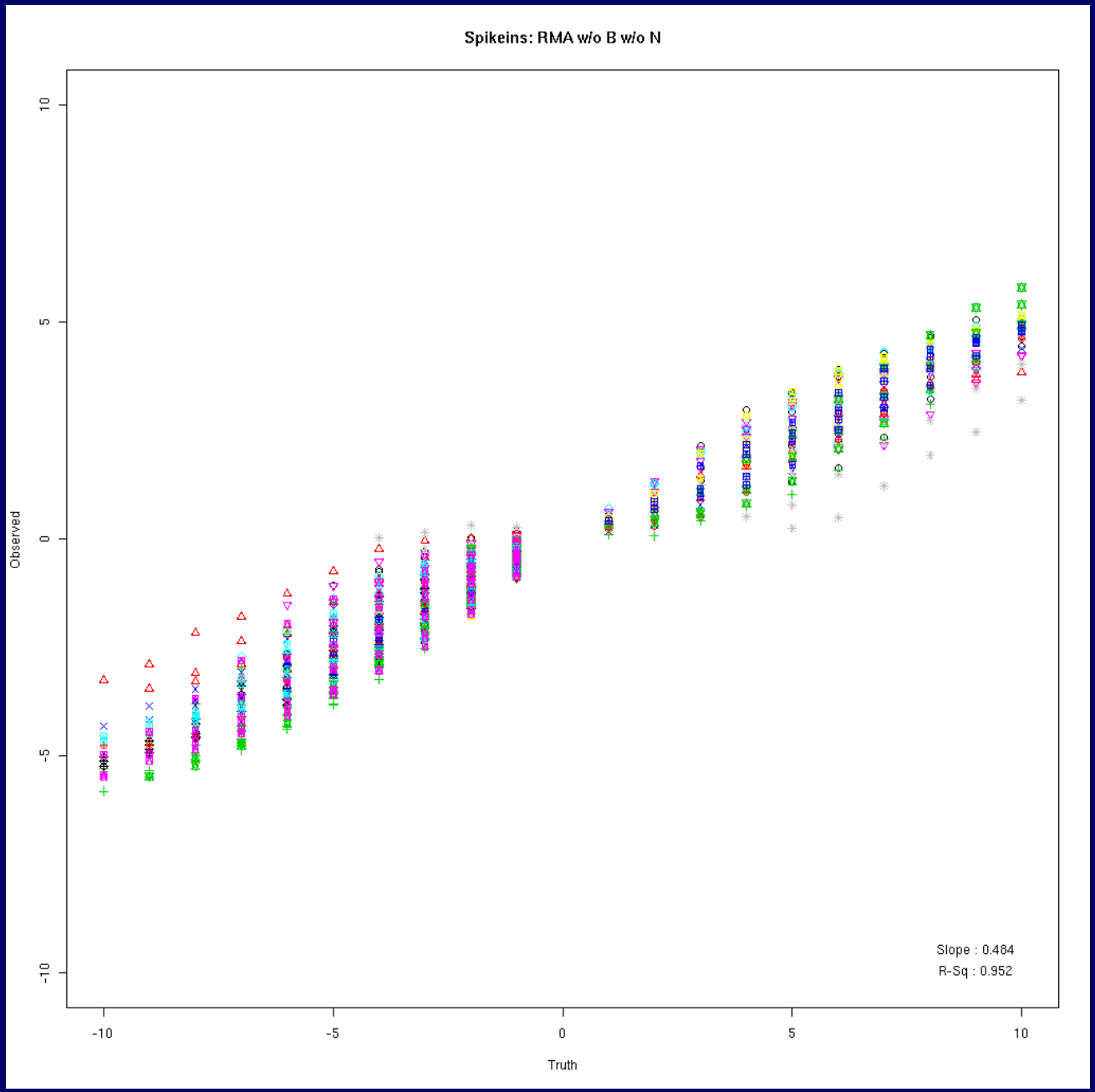
What about the Spike-ins?

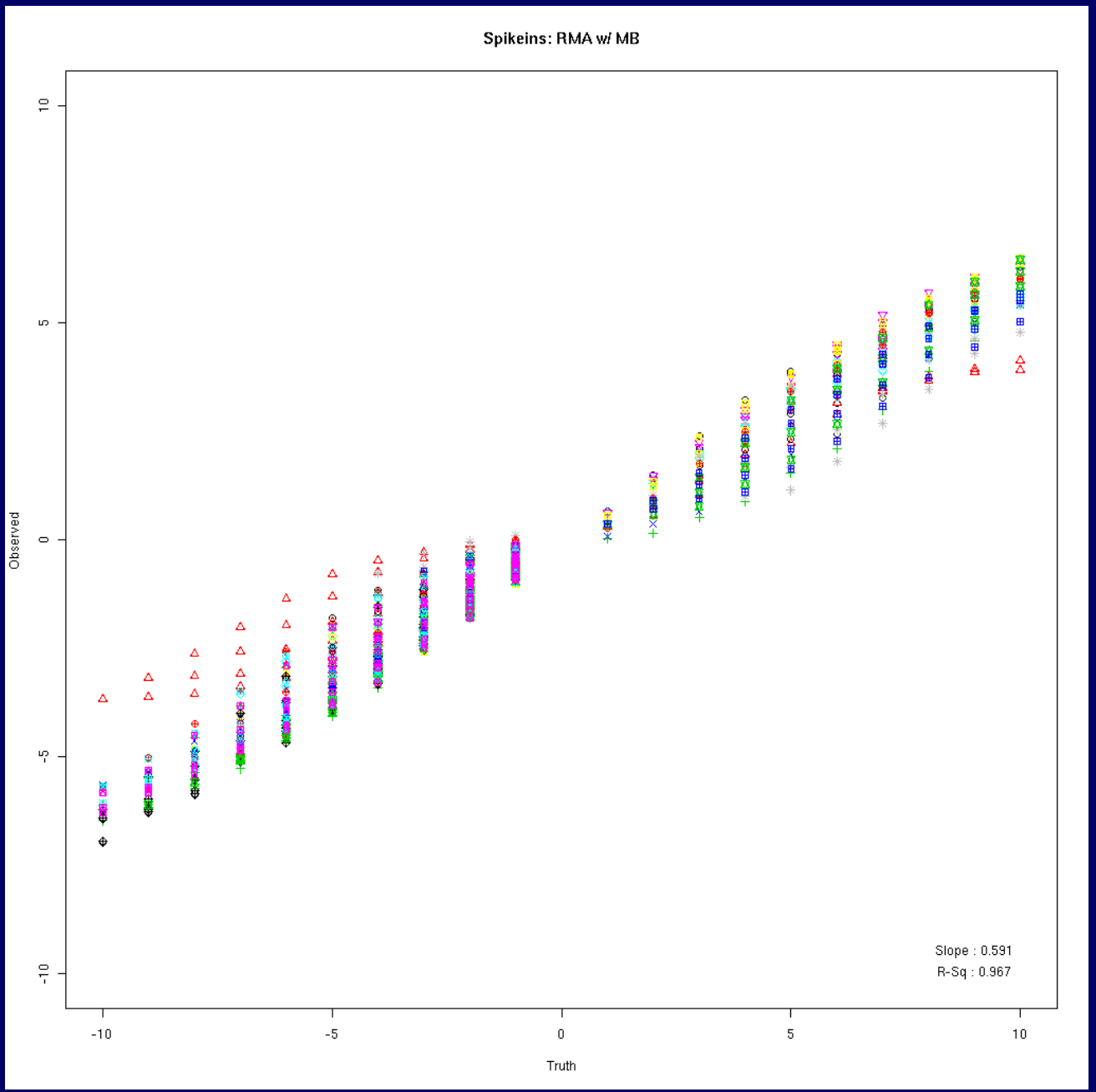
- Plot Observed versus Truth in relative expression.
- Also fit linear regression of Observed on truth

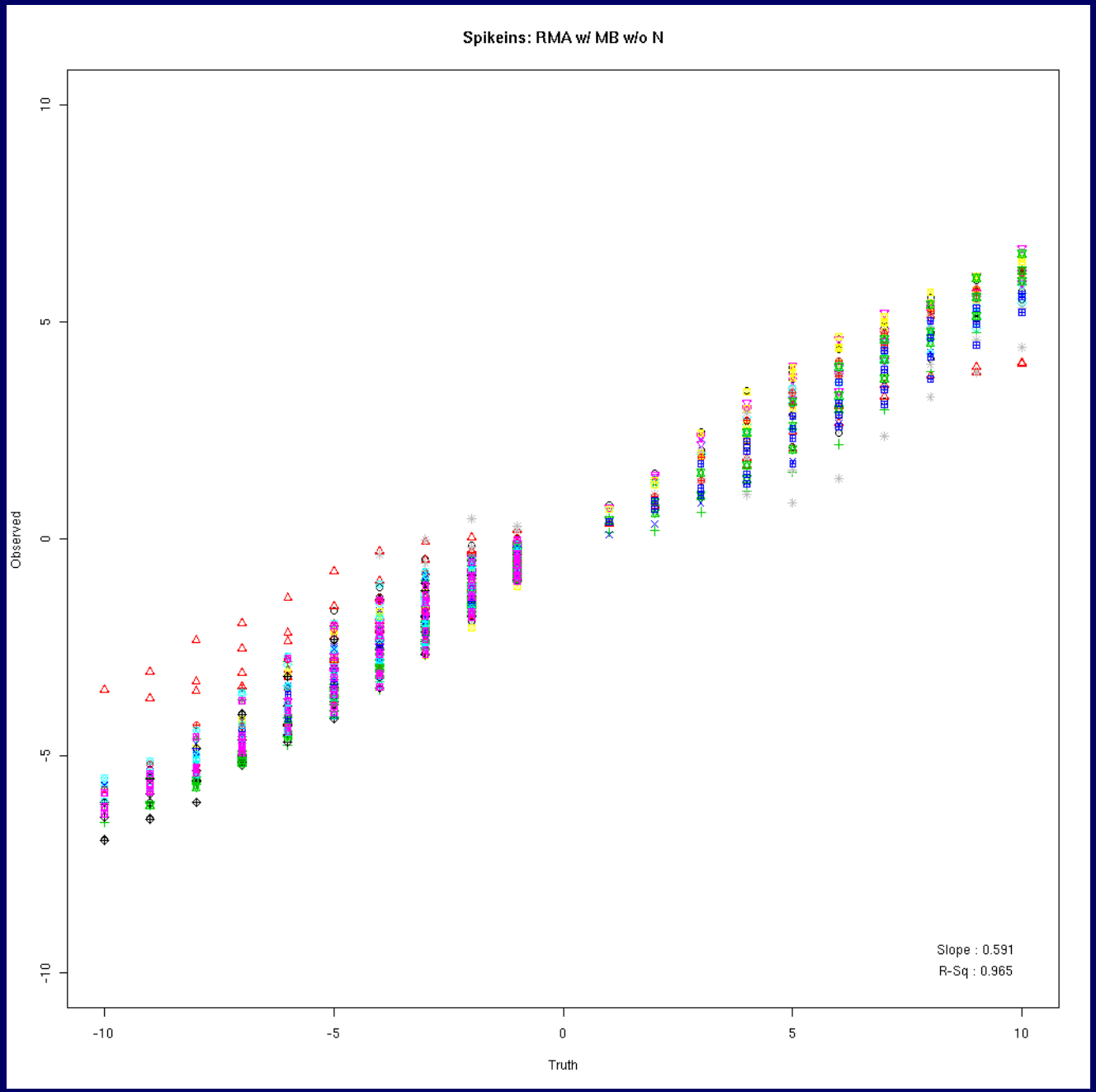

Spikeins: RMA
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.607
R-Sq : 0.966

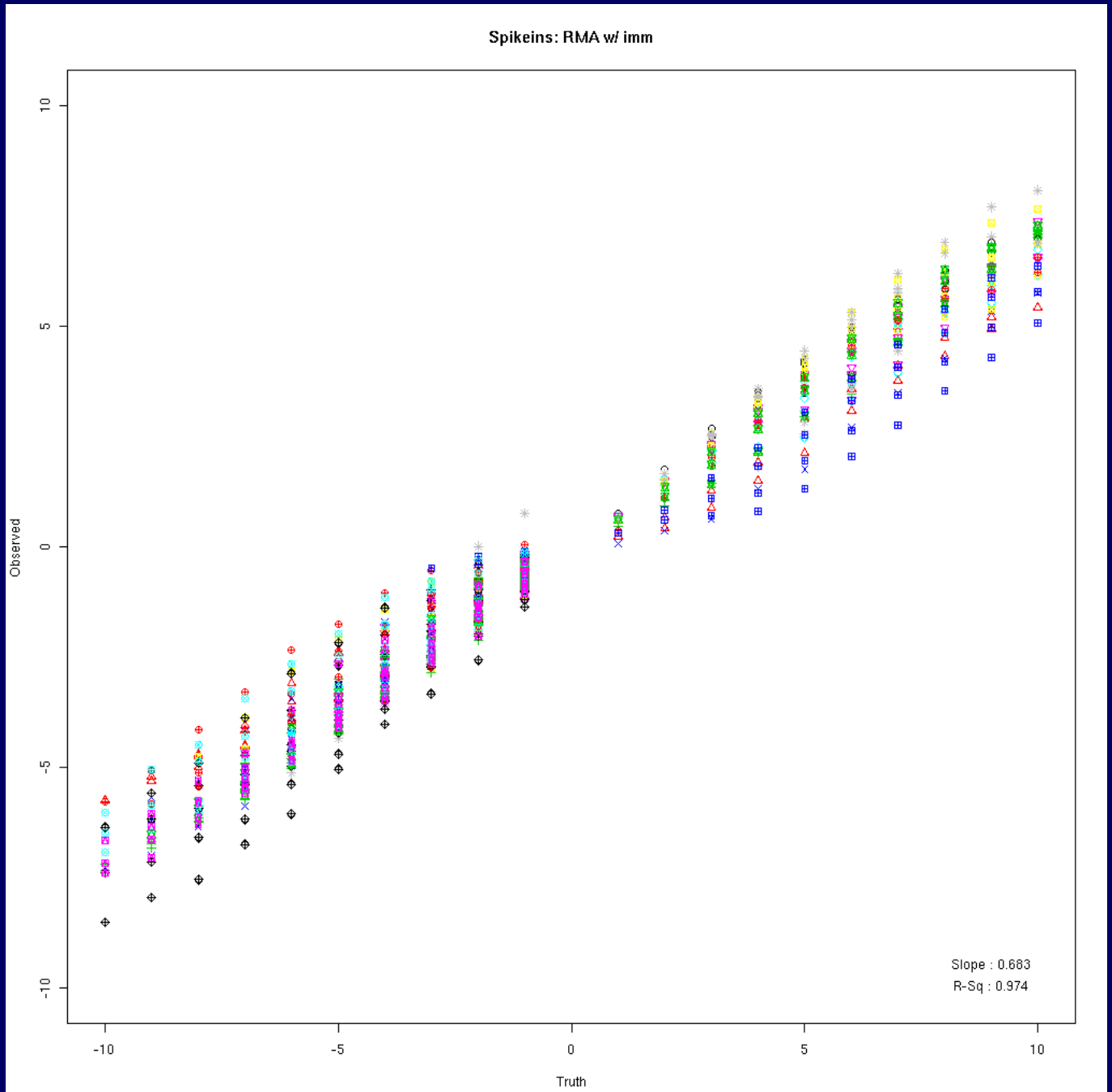

Spikeins: RMA w/o N
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.607
R-Sq : 0.964

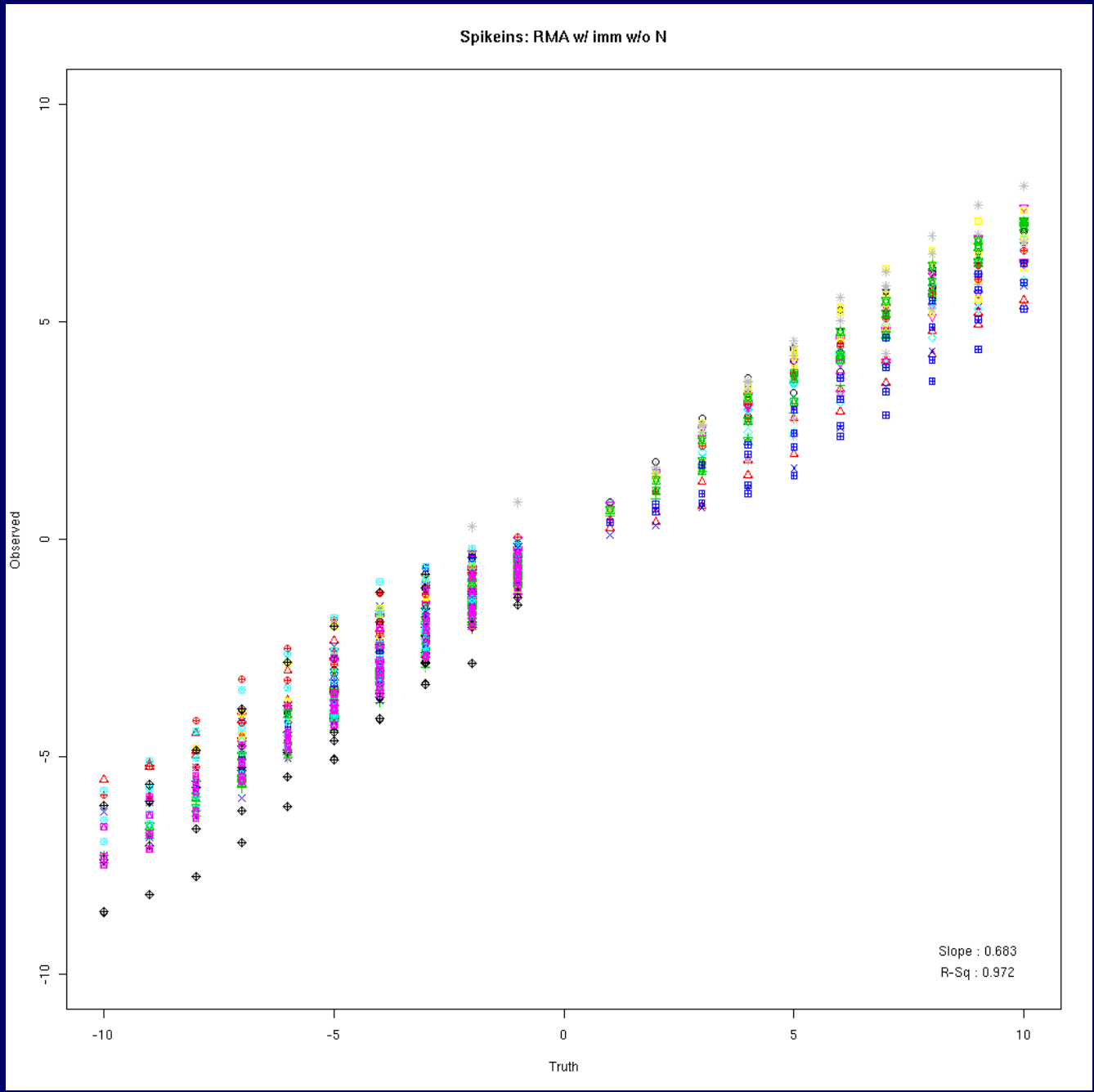

Spikeins: RMA w/o B
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.484
R-Sq : 0.955

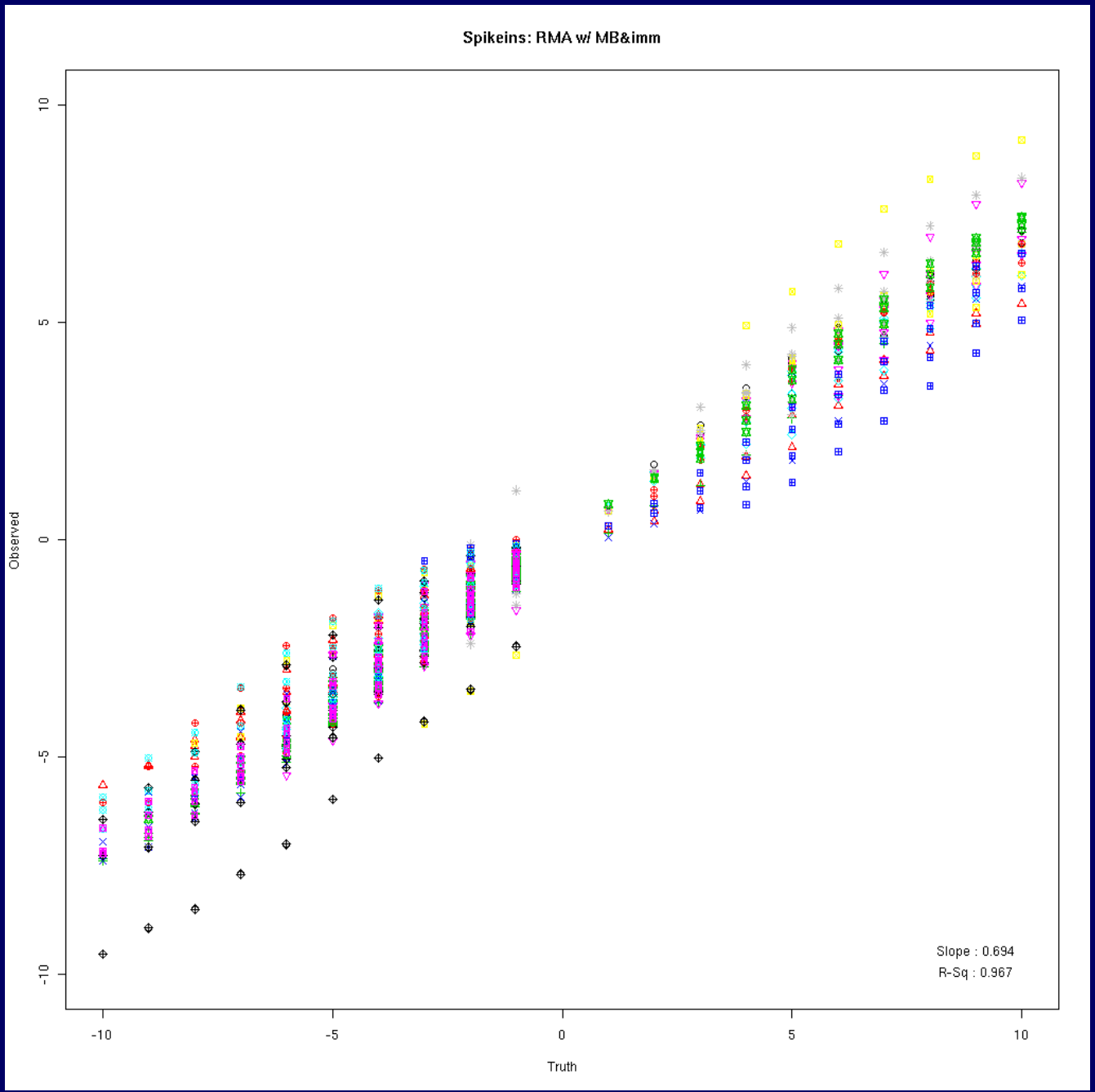

Spikeins: RMA w/o B w/o N
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.484
R-Sq : 0.952

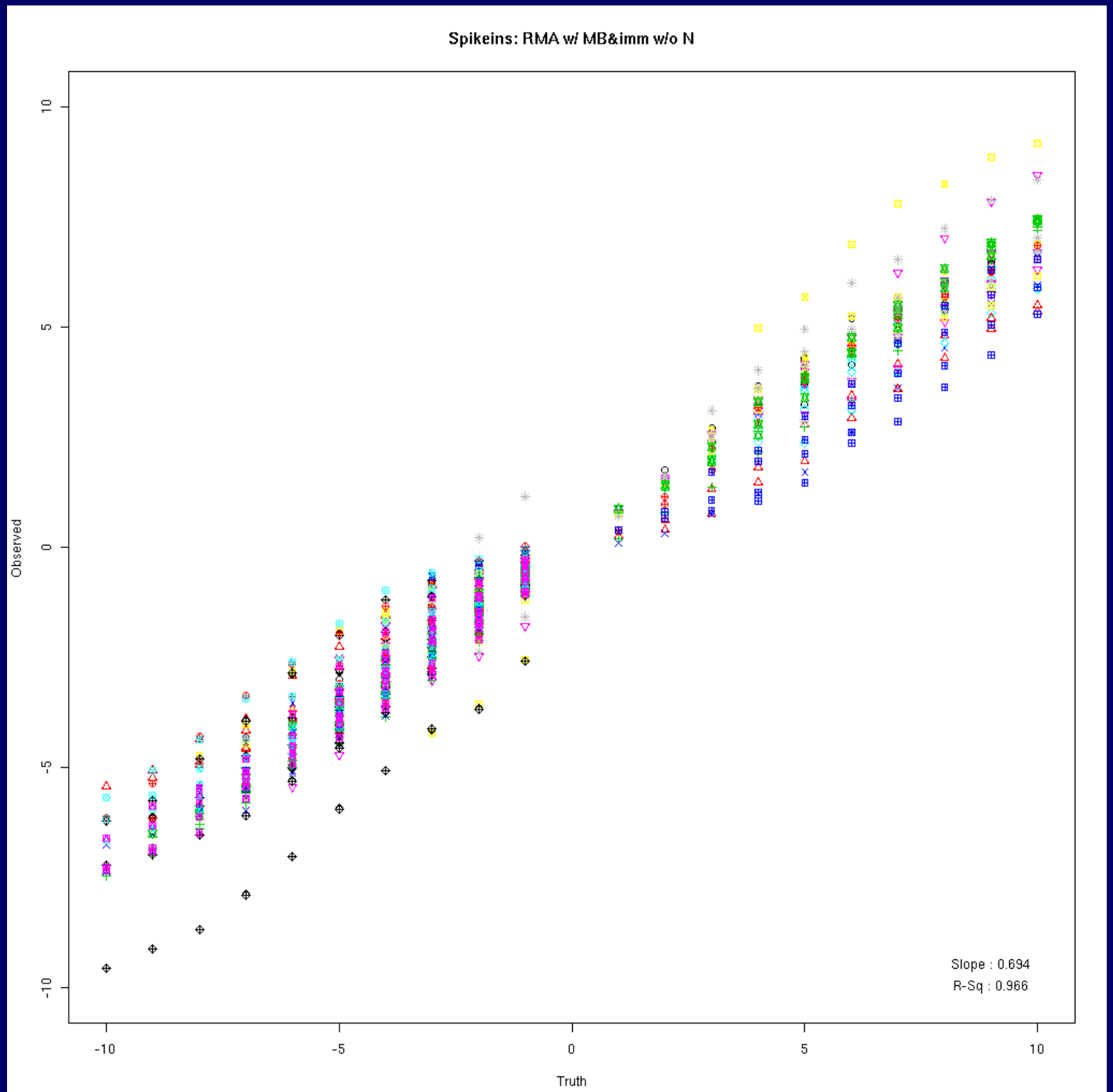

Spikeins: RMA w/ MB
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.591
R-Sq : 0.967


Spikeins: RMA w/ MB w/o N
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.591
R-Sq : 0.965


Spikeins: RMA w/ imm
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.683
R-Sq : 0.974


Spikeins: RMA w/ imm w/o N
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.683
R-Sq : 0.972


Spikeins: RMA w/ MB&imm
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.694
R-Sq : 0.967


Spikeins: RMA w/ MB&imm w/o N
Observed
Truth
10
5
0
-5
-10
-10
-5
0
5
10
Slope : 0.694
R-Sq : 0.966

Reconciling Results

- We look at how many spike-in relative expressions lie outside various quantiles of all non spike in relative expressions.
- Probably a little conservative, an improved method would be to see how many spikeins are outside non-spikeins on each group to group comparison

	100%	98%	95%	50%
RMA	326	1237	1249	1269
w/o N	325	1161	1191	1253
w/o B	418	1240	1255	1269
w/o B&N	424	1153	1171	1254
w/ MB	287	1226	1239	1270
w/MB -	287	1153	1191	1259
imm	232	1073	1142	1267
imm-	231	1063	1130	1265
mb/imm	84	904	995	1260
mb/imm-	76	902	985	1258

What about using Tukey Bi-weight?

- For brevity similar plots not shown, but conclusions about effects of preprocessing similar

Conclusions

- Preprocessing can help you, but it can also harm you.
- RMA does pretty good, the MAS IMM helps predict true fold change, but reduces sensitivity in detecting outliers.

Useful Data Sets

- Affymetrix
  - "Affymetrix® Latin Square Data for Expression Algorithm Assessment"

    http://www.affymetrix.com/analysis/download_center2.affx
- Genelogic
  - Spike in and dilution datasets

    http://qolotus02.genelogic.com/DataSets.nsf

Useful software

- The R “affy” package which is a component of the bioconductor project http://www.bioconductor.org
  - Provides
    - Framework for doing low level analysis and expression computation of GeneChip data.
    - Fast RMA expression computation (as of version 1.1)
    - Ability to mix and match background, normalization and expression summary methods.

Some Papers

- Bolstad, B.M., Irizarry R. A., Astrand, M., and Speed, T.P. (2003), **A Comparison of Normalization Methods for High Density Oligonucleotide Array Data Based on Bias and Variance**. *To appear in Bioinformatics*
- Irizarry, RA, Hobbs, B, Collin, F, Beazer-Barclay, YD, Antonellis, KJ, Scherf, U, Speed, TP (2003) **Exploration, Normalization, and Summaries of High Density Oligonucleotide Array Probe Level Data.** *To appear in Biostatistics.*
- Rafael A. Irizarry, Benjamin M. Bolstad, Francois Collin, Leslie M. Cope, Bridget Hobbs, and Terence P. Speed (2002) **Summaries of Affymetrix GeneChip Probe Level Data.** Accepted to Nucleic Acids Research

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