GS01 0163
Analysis of Microarray Data

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Lecture 8: Normalization, Affy, R, and Glass

- Revisiting Normalization in BioConductor
- R manipulations of AffyBatch
- Glass and Project Normal

A Bioconductor Adventure...

Our goal – to reproduce the study of Bolstad et al. (2003) using the data supplied with BioConductor.

First, pull in the Affy functions and get the data

```
> library(affy);
> library(affydata);
> data(Dilution);
> data(affybatch.example);
```


What steps are we trying to follow?

Starting with an AffyBatch object, presumably assembled straight from CEL files, we want to test the effects of different normalization methods on the stability of probeset measurements of the same stuff.

The steps:

Background correction Normalization PM correction Summary Quantification

Monitor as we go!

Which data do we work with?

Eventually, we want to work with `Dilution`, as that's what they used, but there is a key argument for working with `affybatch.example` to begin with: the file is smaller. How much smaller?

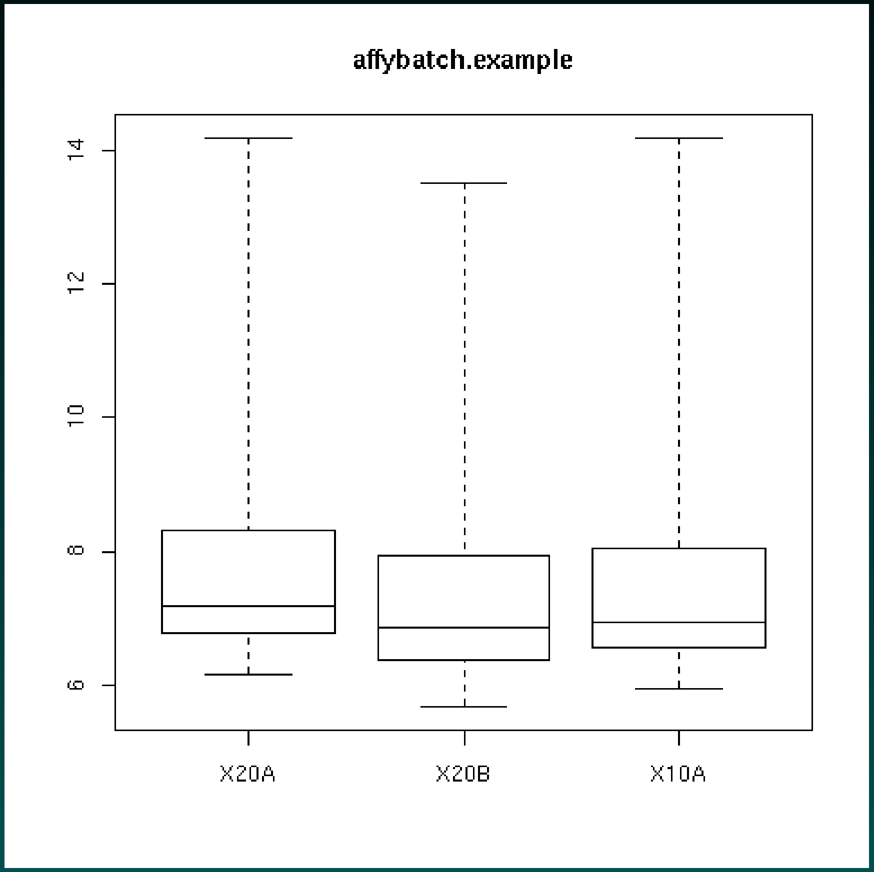
```
> Dilution
AffyBatch object
size of arrays=640x640 features (12805 kb)
cdf=HG_U95Av2 (12625 affyids)
number of samples=4
number of genes=12625
annotation=hgu95av2
```



```
> affybatch.example
AffyBatch object
size of arrays=100x100 features (237 kb)
cdf=cdfenv.example (150 affyids)
number of samples=3
number of genes=150
annotation=
```

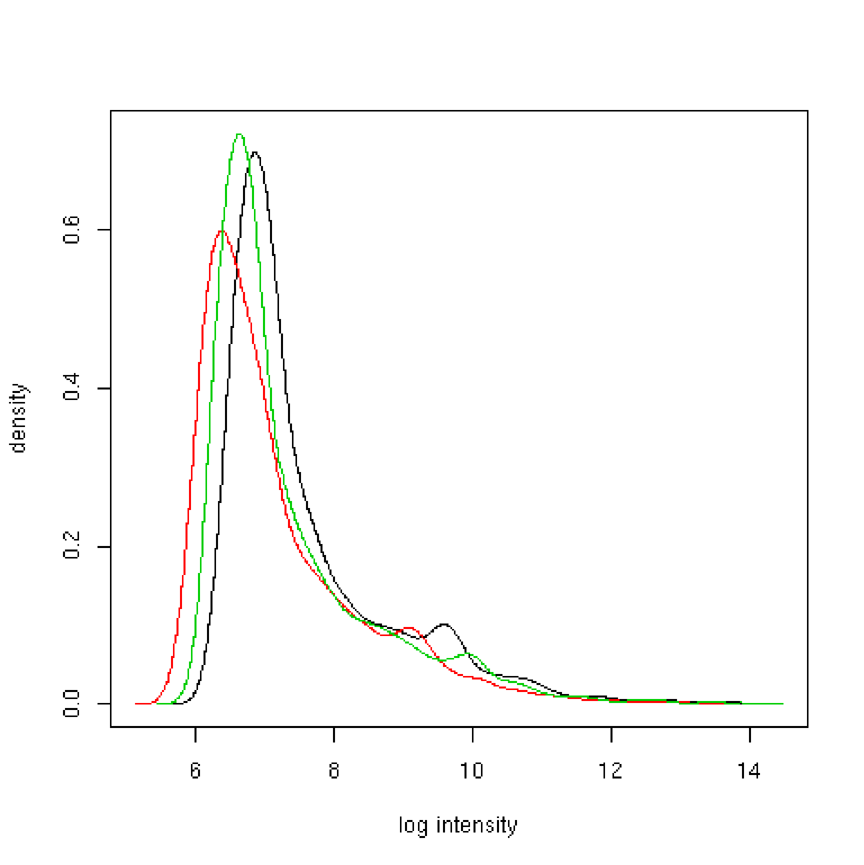
quick check: $237 * 6.4^2 * (4/3) = 12943$

Does this data need normalizing?



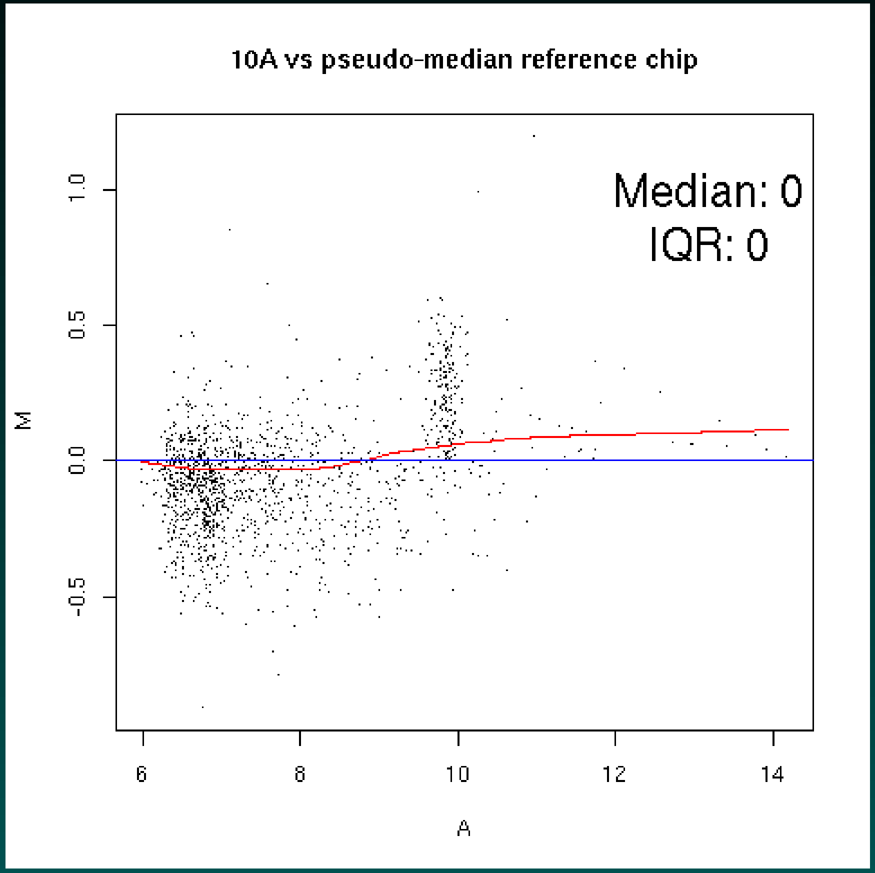

```
boxplot(affybatch.example)
dev.copy(png,file=''boxplot0.png'');
dev.off();
```


What about the densities?




```
hist(affybatch.example,lty=1,col=1:3)
dev.copy(png,file=''hist0.png''); dev.off();
```

and the MA plots?




```
MAplot(affybatch.example);
```

Whoops. Cycles through the pairs too quickly!

Look at all pairs?

```
mva.pairs(affybatch.example);
```

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```

```
Error in log(x, base) : Non-numeric
  argument to mathematical function
> help(mva.pairs)
```

want to feed this function a matrix, with columns corresponding to arrays. Where are these numbers?

I can never remember...

Objects have slots!

```
> slotNames(affybatch.example)
[1] "cdfName"     "nrow"         "ncol"
[4] "exprs"       "se.exprs"     "phenoData"
[7] "description" "annotation"   "notes"
```

I can never remember...

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I think we want `exprs`.

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```
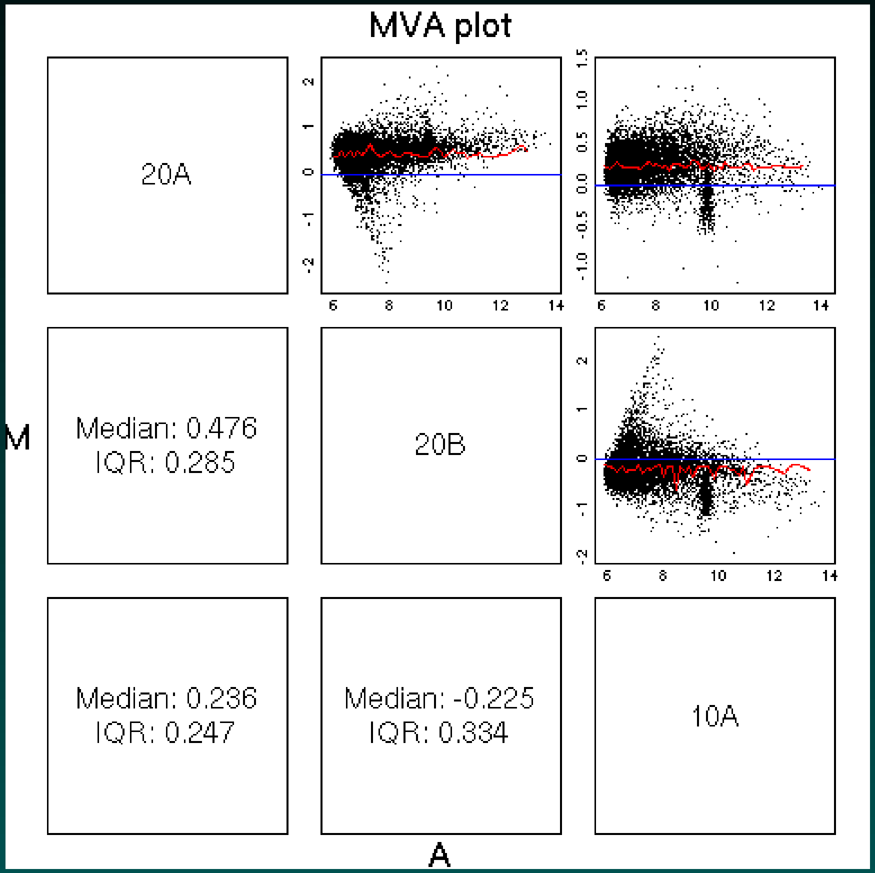

I think we want `exprs`.

```
%> length(exprs(affybatch.example))
%[1] 30000
> dim(exprs(affybatch.example))
```

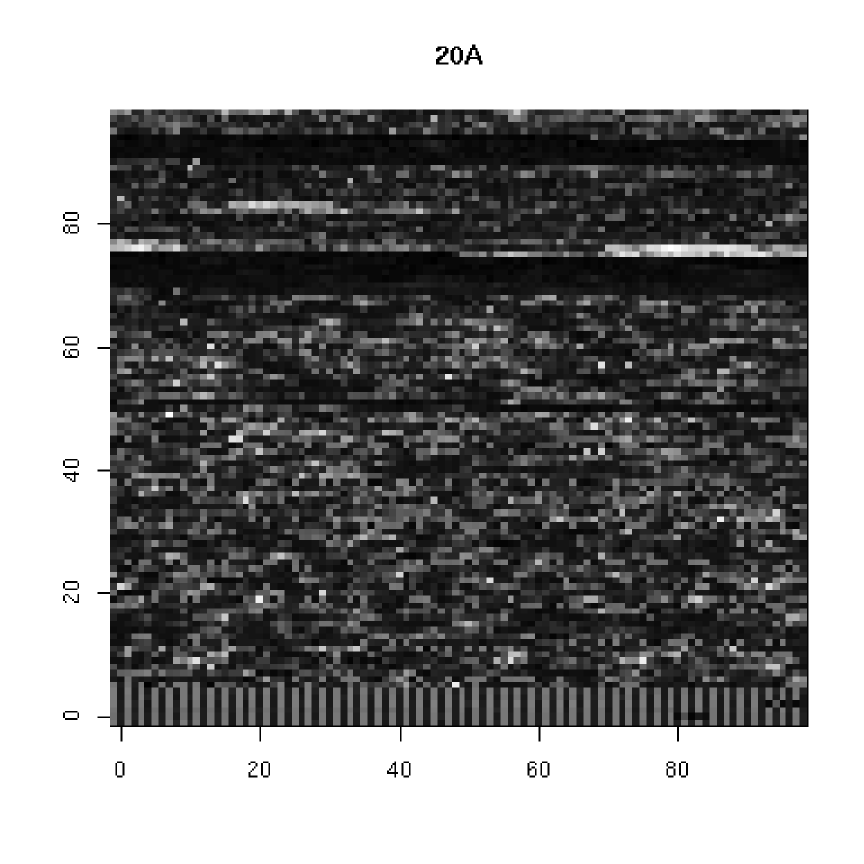
```
[1] 10000 3
```

Back to M vs A




```
mva.pairs(exprs(affybatch.example));
```

Spatial Plots?




```
image(affybatch.example[,1],transfo=log2);
```

Ratios of Spatial Plots?

```
image(matrix(exprs(affybatch.example[,1]),
             nrow=nrow(affybatch.example),
             ncol=ncol(affybatch.example)),
      transfo=log2);
```

Ratios of Spatial Plots?

```
image(matrix(exprs(affybatch.example[,1]),
             nrow=nrow(affybatch.example),
             ncol=ncol(affybatch.example)),
      transfo=log2);
```

parameter “transfo” can’t be set in high-level plot() function.

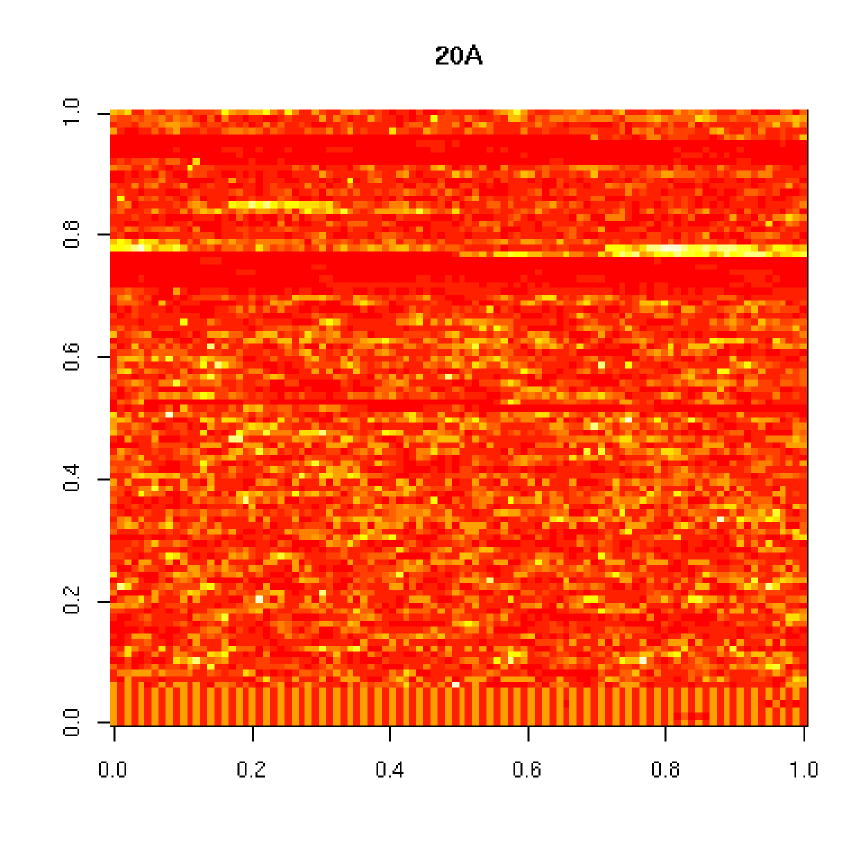
Ratios of Spatial Plots?

```
image(matrix(exprs(affybatch.example[,1]),
             nrow=nrow(affybatch.example),
             ncol=ncol(affybatch.example)),
      transfo=log2);
```

parameter "transfo" can't be set in high-level plot() function.

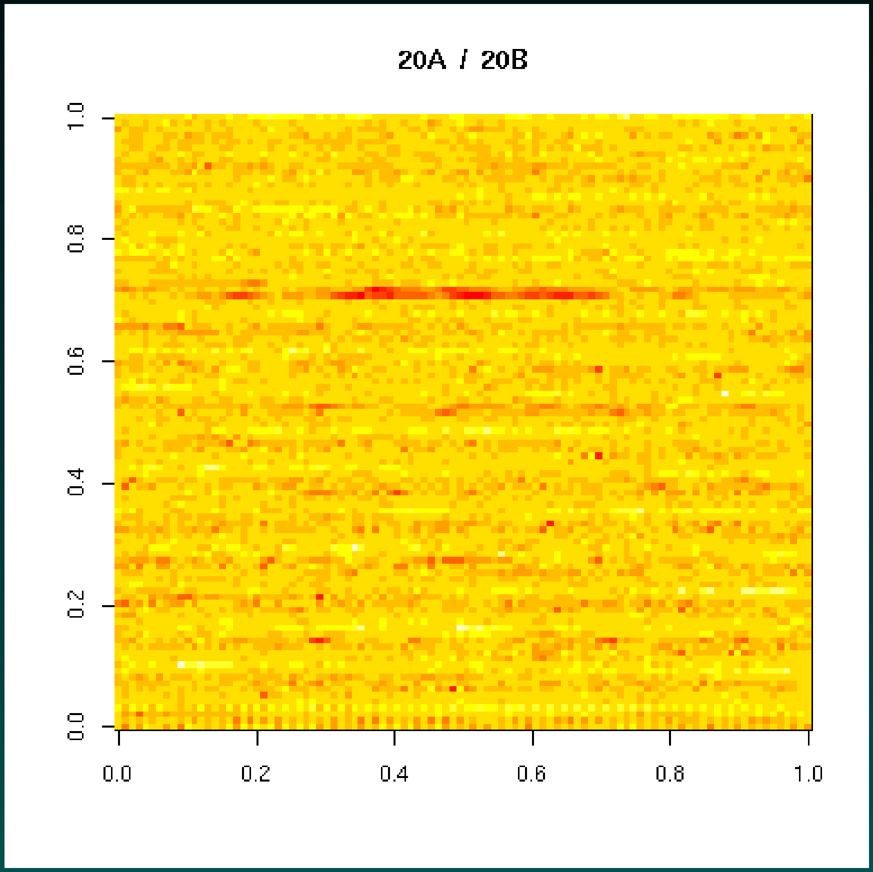
```
image(log2(matrix(
   exprs(affybatch.example[,1]),...
```

Spatial Plot 1




```
image(log2(matrix(exprs(affybatch.example[,1
      main=sampleNames(affybatch.example[,1]
```

Ratio Plot 1 (problem: fake geometry)




```
image(log2(matrix(exprs(affybatch.example[,1
                  exprs(affybatch.example[,2
      main=paste(sampleNames(affybatch.examp
```


Ok, start processing. BG first

```
affybatch.example.bg <-
    bg.correct.rma(affybatch.example)
```

Did this change things?

```
hist(affybatch.example.bg, lty=1, col=1:3)
```

Ok, start processing. BG first

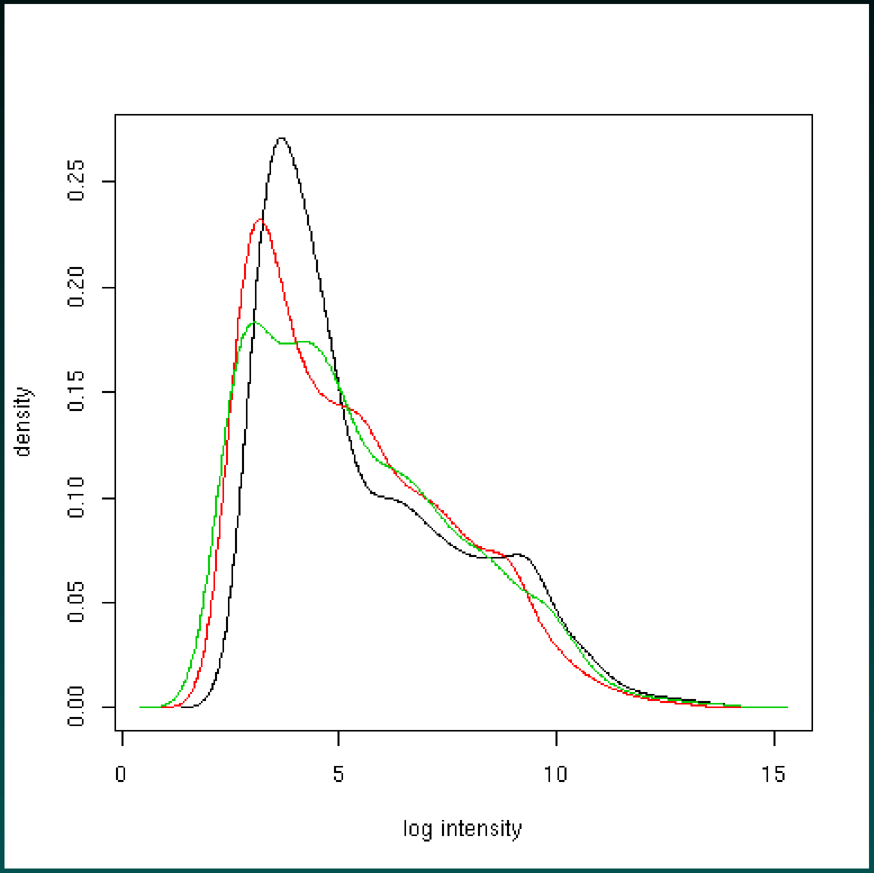
```
affybatch.example.bg <-
    bg.correct.rma(affybatch.example)
```

Did this change things?

```
hist(affybatch.example.bg, lty=1, col=1:3)
```

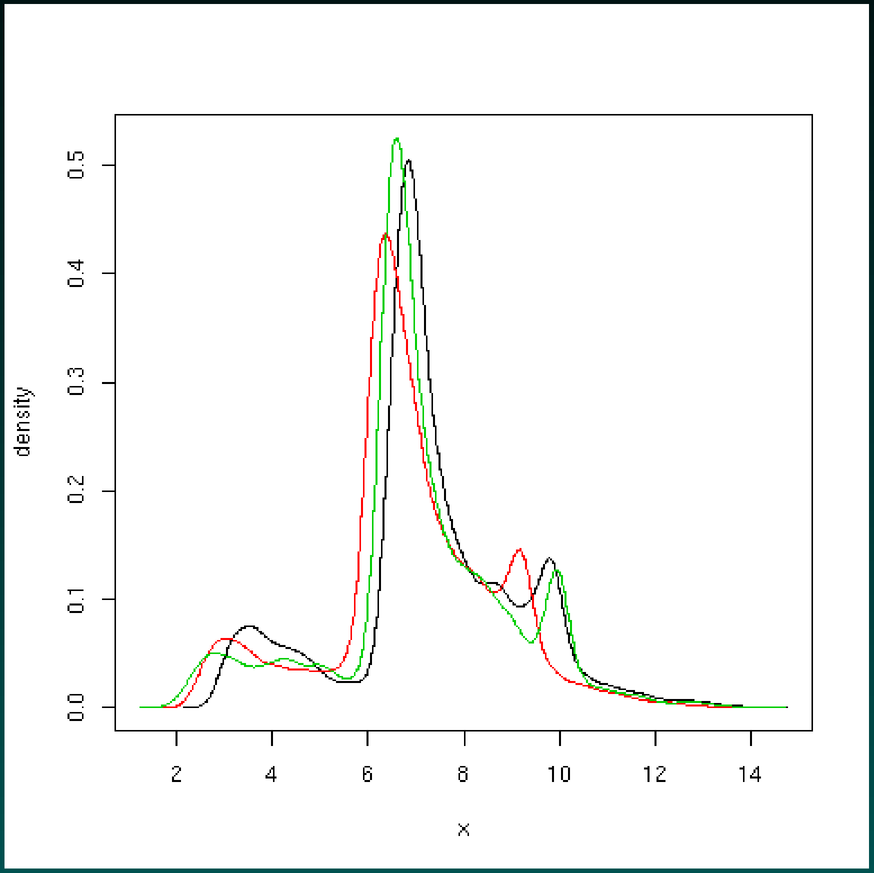
```
plotDensity(log2(exprs(affybatch.example.bg)
```


Picture 1 After BG



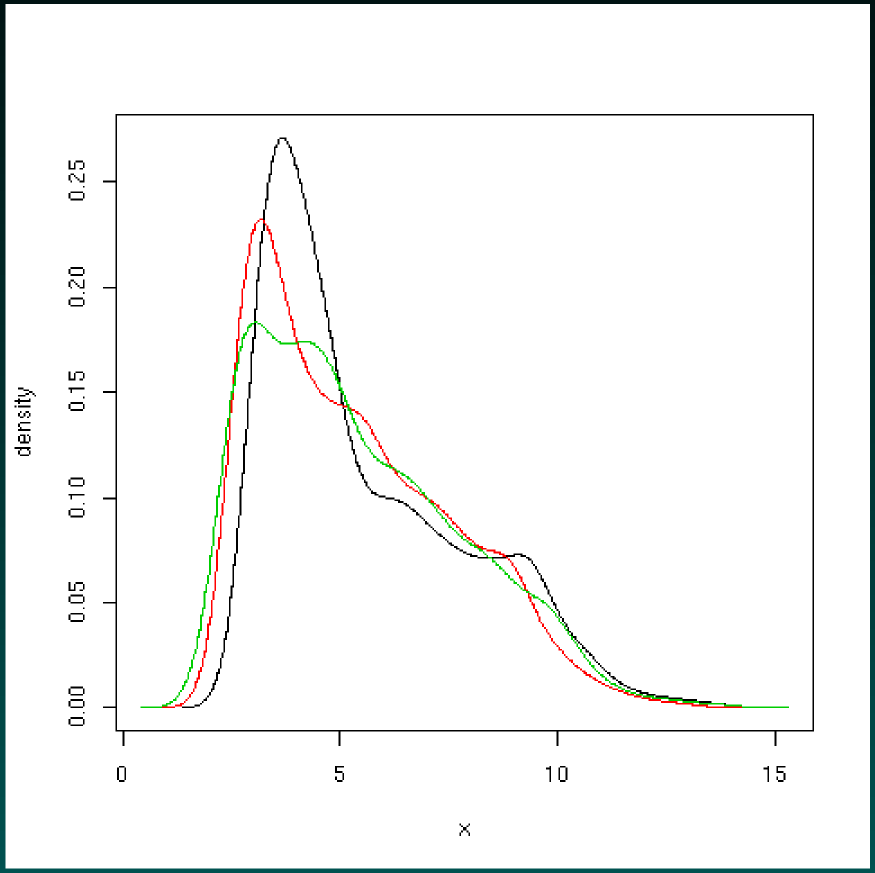

```
hist(affybatch.example.bg, lty=1, col=1:3)
```

Picture 2 After BG




```
plotDensity(log2(exprs(affybatch.example.bg)
    lty=1,col=1:3)
```

Picture 2 (try 2) After BG




```
plotDensity(log2(pm(affybatch.example.bg)),
    lty=1,col=1:3)
```


and now we normalize!

This is where the differences come in. We can invoke

normalize.AffyBatch.constant
normalize.AffyBatch.contrasts
normalize.AffyBatch.invariantset
normalize.AffyBatch.quantiles

or, of course, we can have `expresso`

Expresso, no normalization

```
eset0 <- expresso(affybatch.example,
            bgcorrect.method=''rma'',
            normalize=FALSE,
            pmcorrect.method=''pmonly'',
            summary.method=''medianpolish'');
```

Now at this point, `eset0` is an exprSet object; while it still has slots for `exprs`, the dimensions have changed as we have shifted from features (probes) to probesets.

What do we want?

The mean and variance of the probeset measurements gene by gene, to describe the behavior of this normalization method.

```
> dim(exprs(eset0))
[1] 150 3
```

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The mean and variance of the probeset measurements gene by gene, to describe the behavior of this normalization method.

```
> dim(exprs(eset0))
[1] 150 3
```

```
> eset0.mu <- apply(exprs(eset0),1,''mean'')
> eset0.var <- apply(exprs(eset0),1,''var'')
```

Now we want another method to compare to

Constant normalization: choosing baseline

find the “middle behavior” chip

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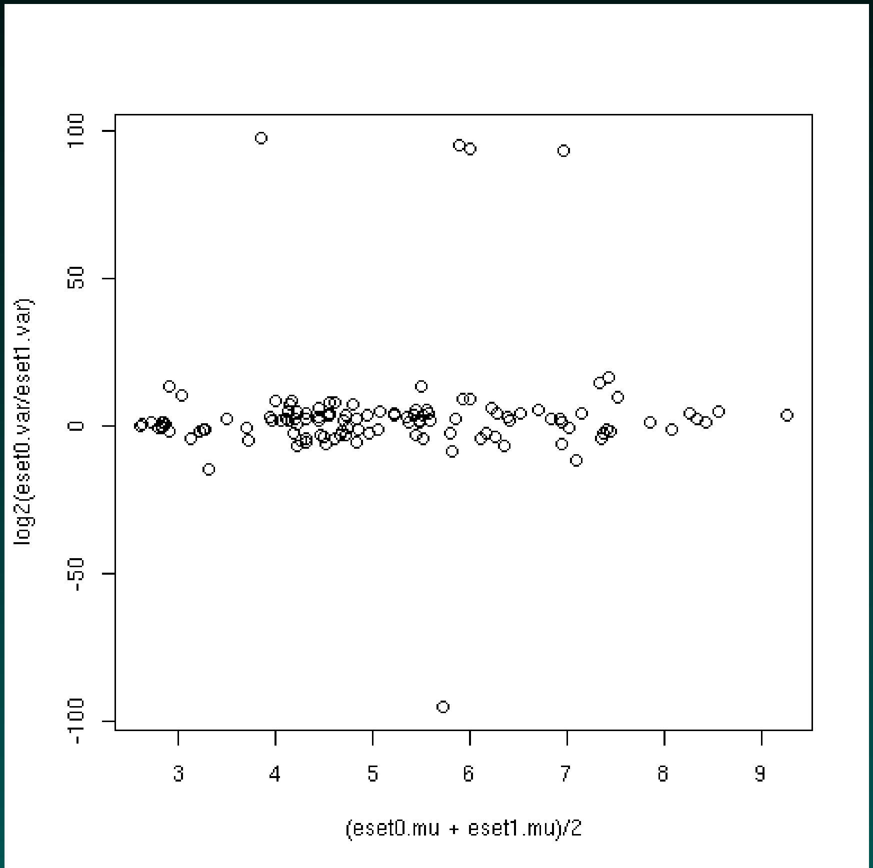
find the “middle behavior” chip

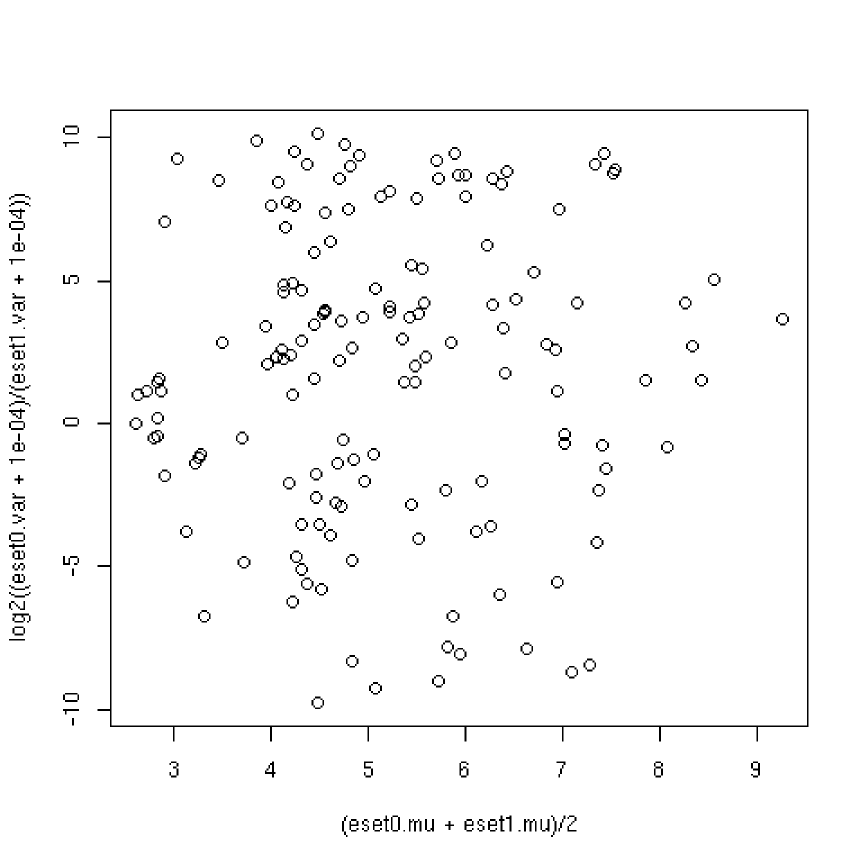
```
> apply(exprs(affybatch.example),2,’’median’
  20A   20B   10A
147.3 118.0 125.0

eset.constant <- expresso(affybatch.example,
    bgcorrect.method=’’rma’’,
    normalize=constant, normalize.param=list
    pmcorrect.method=’’pmonly’’,
    summary.method=’’medianpolish’’);
```

Checking "none" against "scaling"



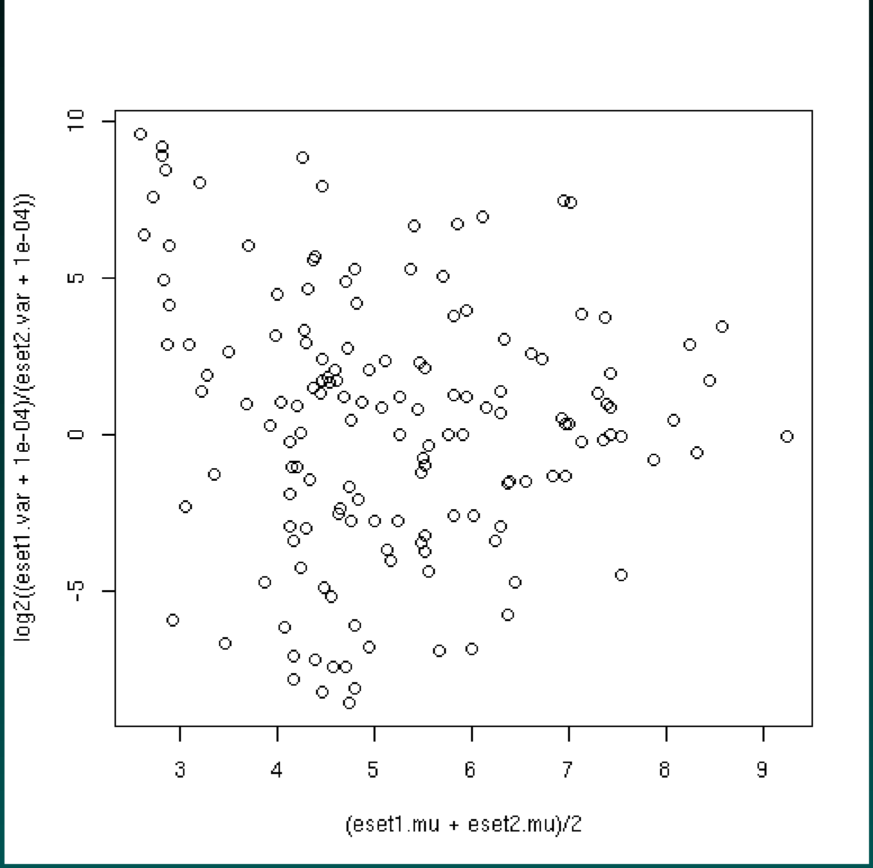
Initial plot is driven by outliers. There are a few observations with variances of 0. Add a small amount and recompute.

Checking “none” against “scaling”



Not that stark – 96 times out of 150, constant scaling gives lower variability. This is a small (fake) array

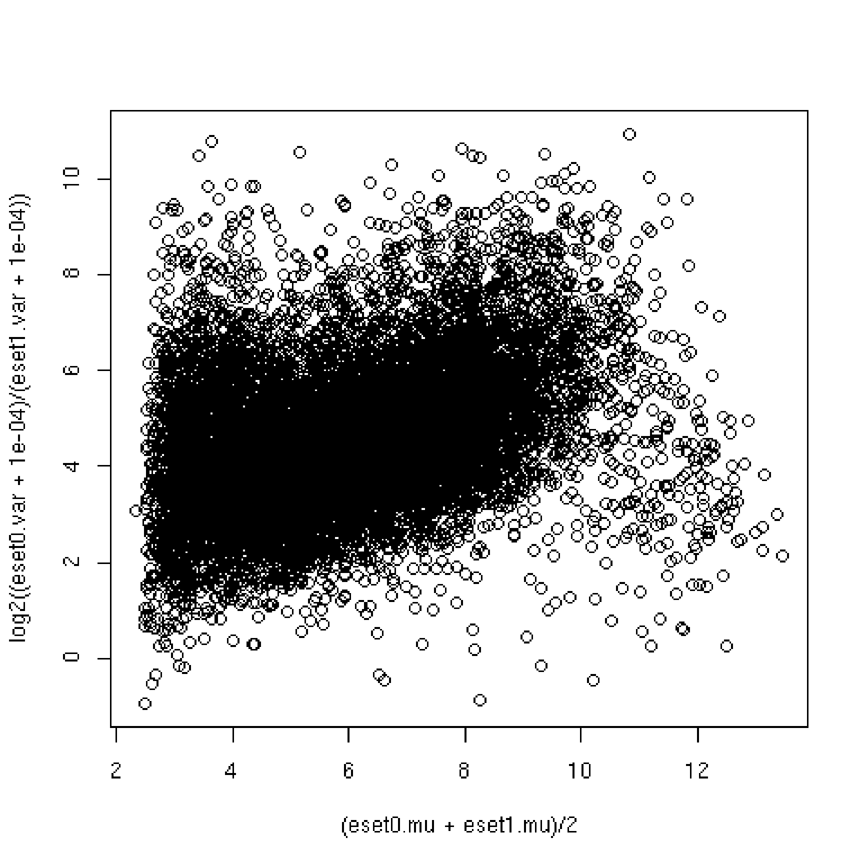
Checking “scaling” against “quantiles”



Not that stark – 83 times out of 150, quantile scaling gives lower variability.

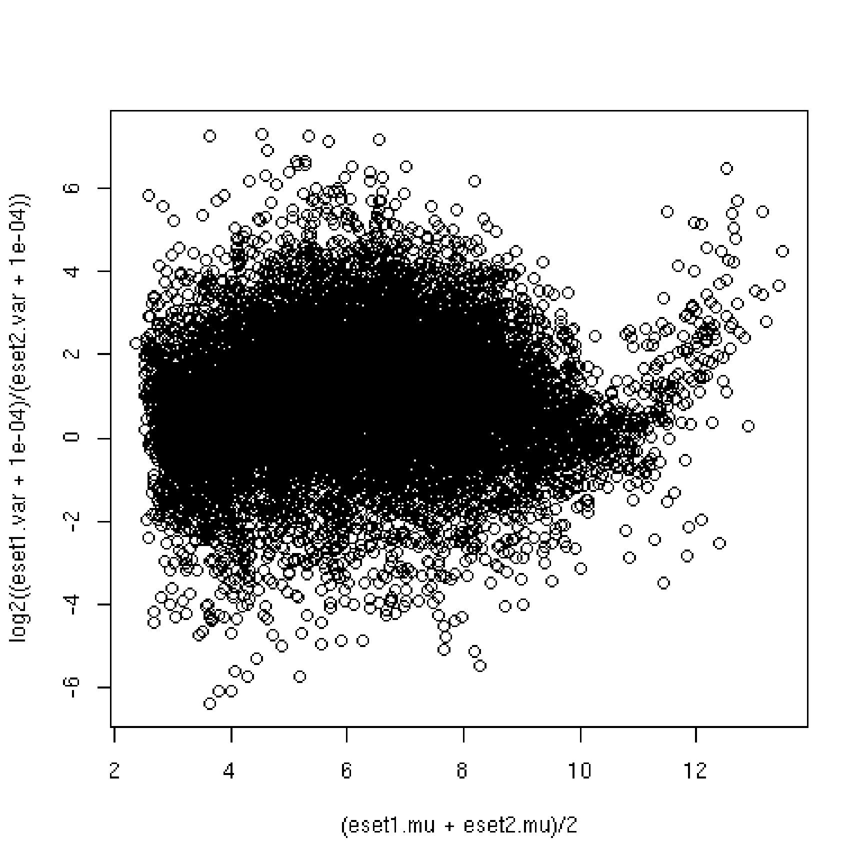
repeat with Dilution, now that we know what we want to do.

Dilution: “none” against “scaling”



Here, 12615 times out of 12625, constant scaling gives lower variability. Mean log diff: 4.62

Dilution: "scaling" against "quantiles"



Here, 9477 times out of 12625, quantile scaling gives lower variability. Mean log diff: 0.95

Normalizing on Glass?

Main difference is two-color setup

Some general recommendations:

Normalize channels to each other first, then normalize log ratios across chips.

do dye swaps

MA plots, loess fits, and pictures

Project Normal: A Cautionary Tale

Pritchard, Hsu, Delrow and Nelson
Project Normal: Defining Normal Variance in Mouse Gene Expression
PNAS **98** (2001), 13266-13271.

Data set used for the third annual Critical Analysis of Microarray Data (CAMDA 2002)

Their Initial Goals

The goal of many microarray studies is to identify genes that are “differentially expressed”.

Relative to what?

Differences larger in scale than those that would be encountered due to “normal” or technical variation.

Try to assess the fraction of genes exhibiting a large mouse-to-mouse heterogeneity in the absence of structure.

Their Experimental Design

Eighteen Samples

- Six C57BL6 male mice
- Three organs: kidney, liver, testis

Reference Material

- Pool all eighteen mouse organs

Replicate microarray experiments using two-color fluorescence with common reference and dye swaps

- Four experiments per mouse organ, 2 each dye

Their Analysis

Print-tip specific intensity dependent loess normalization

Perform F-tests on log(Exp/Ref) for each gene to see if mouse-to-mouse variance exceeds the array-to-array variance

The Data Supplied

Images

One quantification file each for kidney, liver and testis.

```
CDNA ID, Cluster ID, Title,
Block, Column, Row

F635 Median M1K3_1, B635 Median M1K3_1
F532 Median M1K3_1, B532 Median M1K3_1
```

Mouse 1, Kidney Sample in Cy3 channel, first replicate.

Why We Got Involved

All in all, the analysis described looks pretty good. F-tests on log ratios seem reasonable, and the preprocessing steps they used are fairly standard. Furthermore, the images looked fairly clean.

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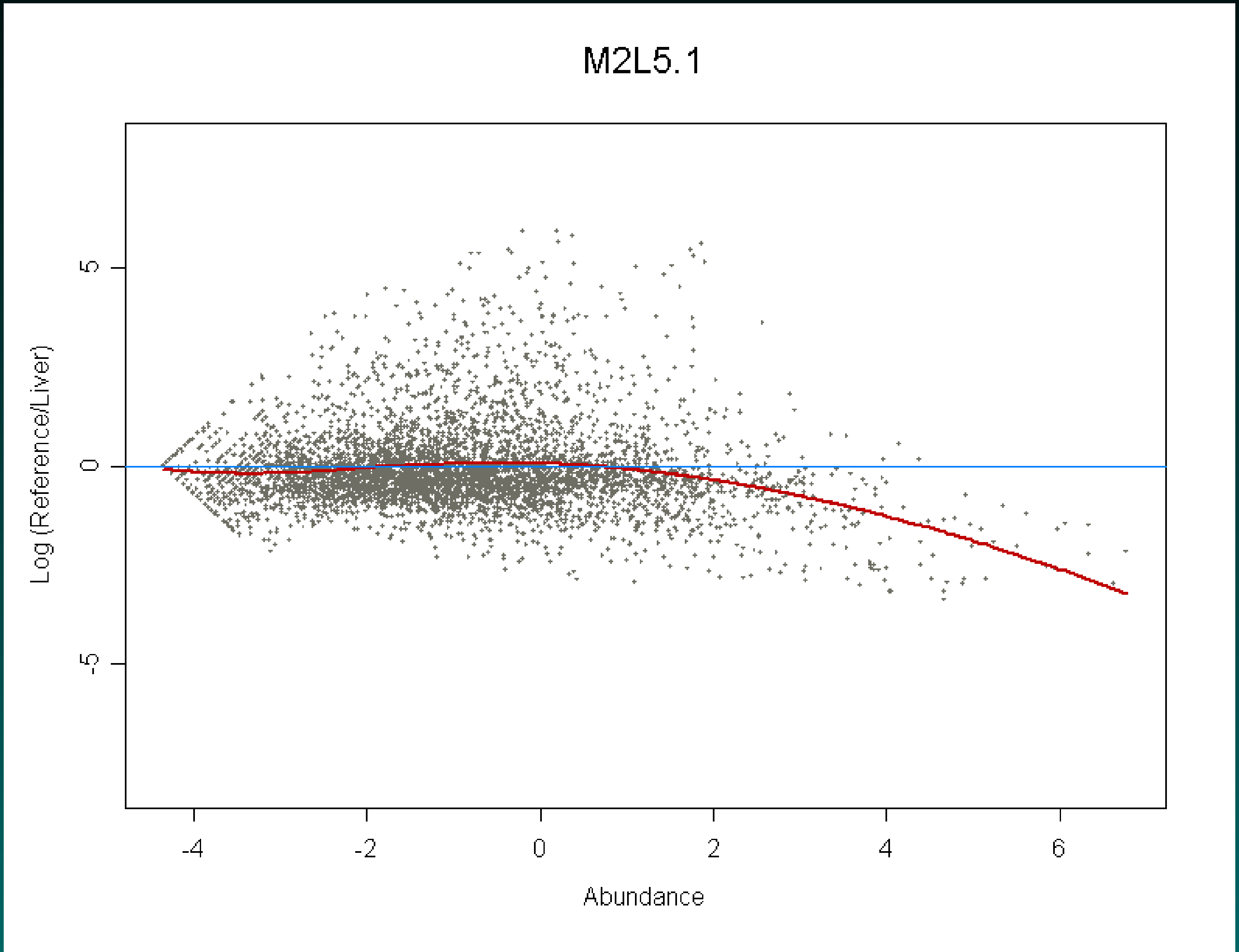
All in all, the analysis described looks pretty good. F-tests on log ratios seem reasonable, and the preprocessing steps they used are fairly standard. Furthermore, the images looked fairly clean.

“Fairly standard” $\neq$ correct

For this data, we think that loess normalization is incorrect.

What Loess Looks Like for 1 Array



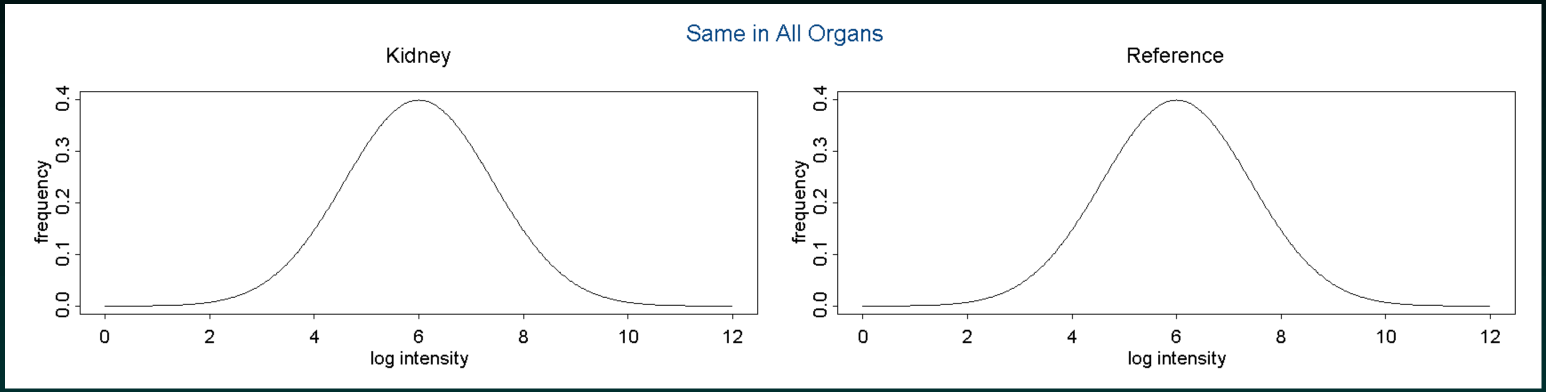
Why Loess Normalization?

Most normalization methods assume:

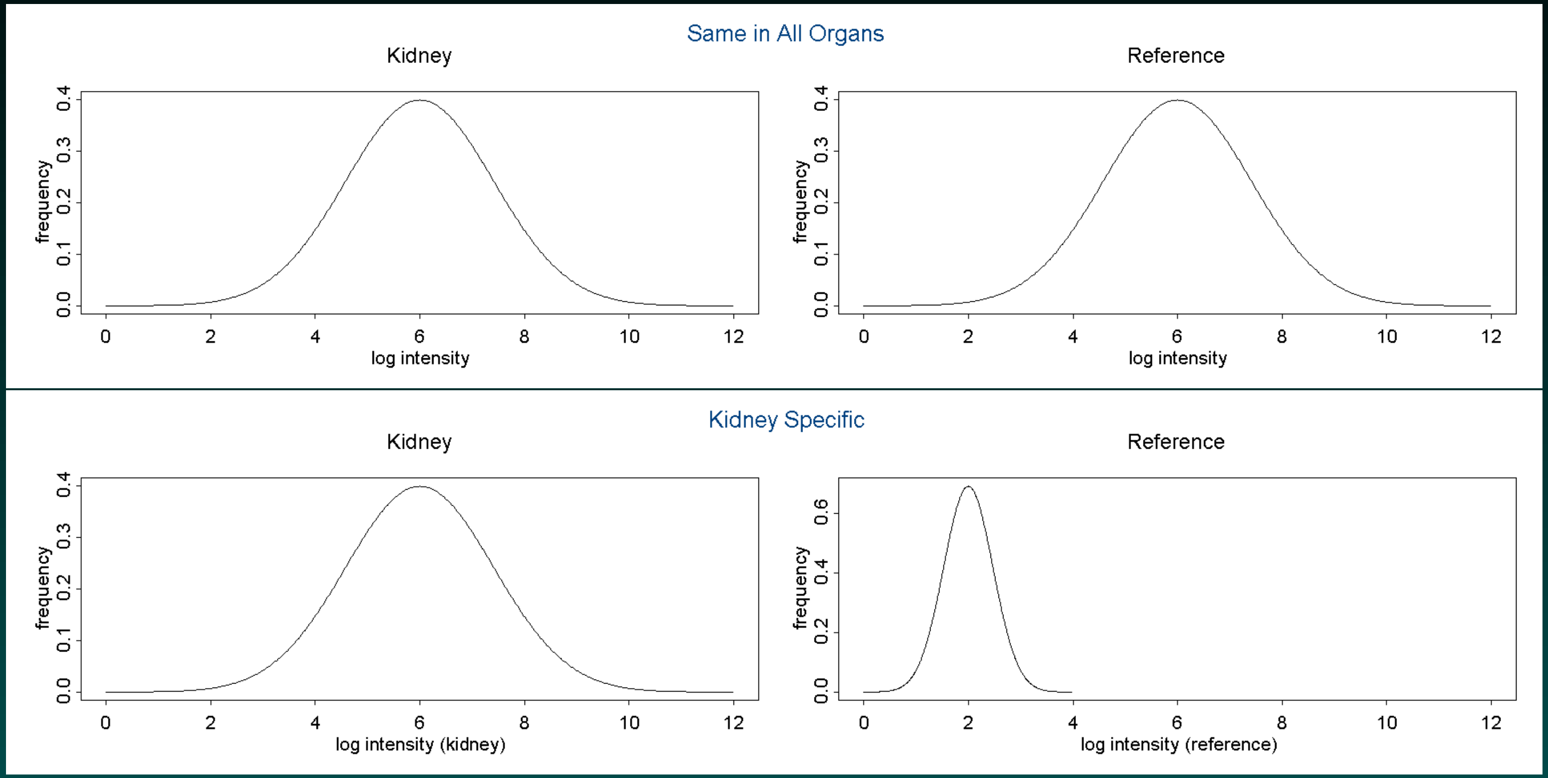
- Distributions of intensities are the same in the two channels
- Most genes do not change expression
- The number of overexpressed genes is about the same as the number of underexpressed genes

Loess normalization tries to force the distributions in the two channels to match, believing that differences are attributable to technology.

Why We Think It's Wrong



Why We Think It's Wrong



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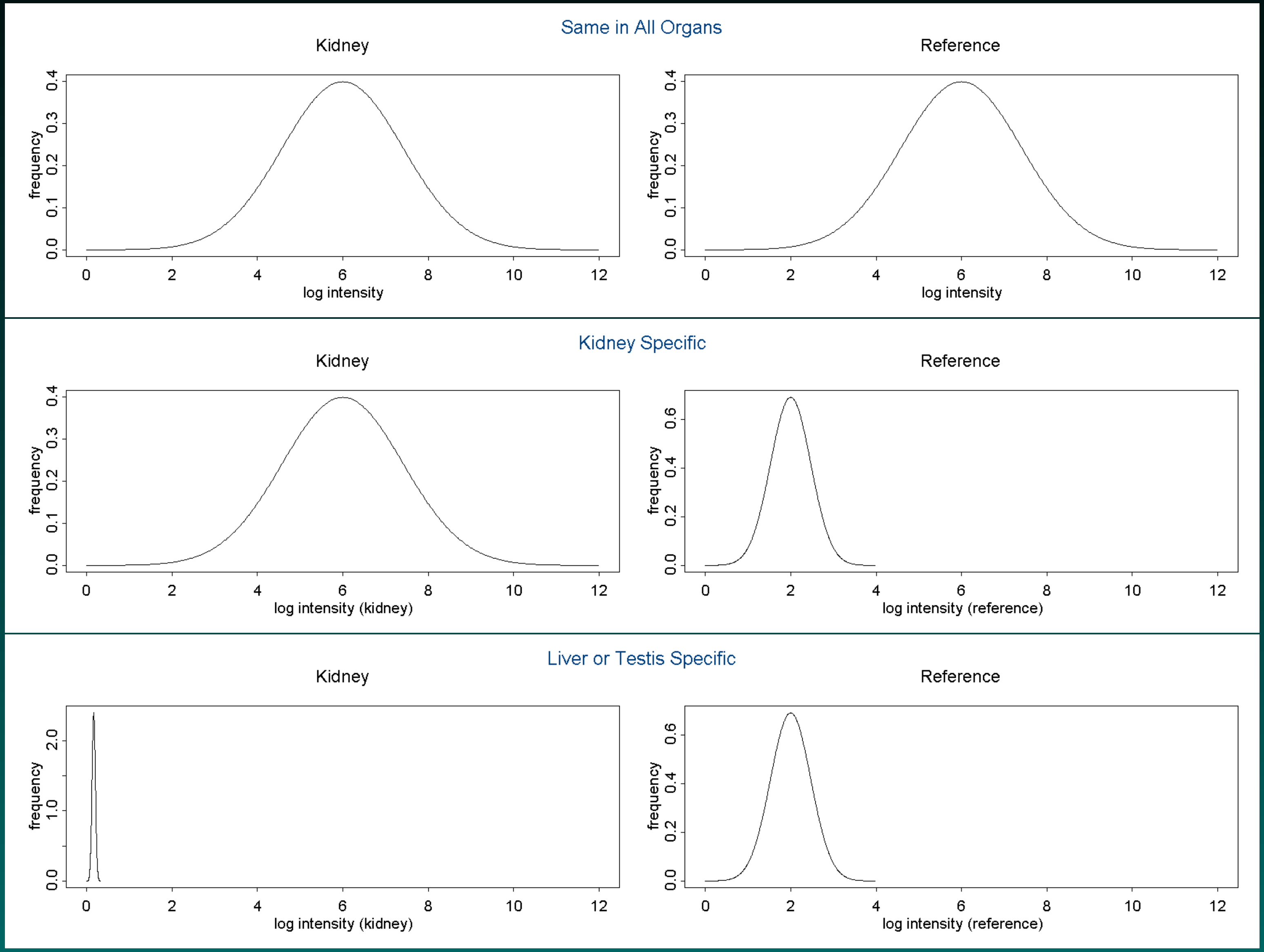
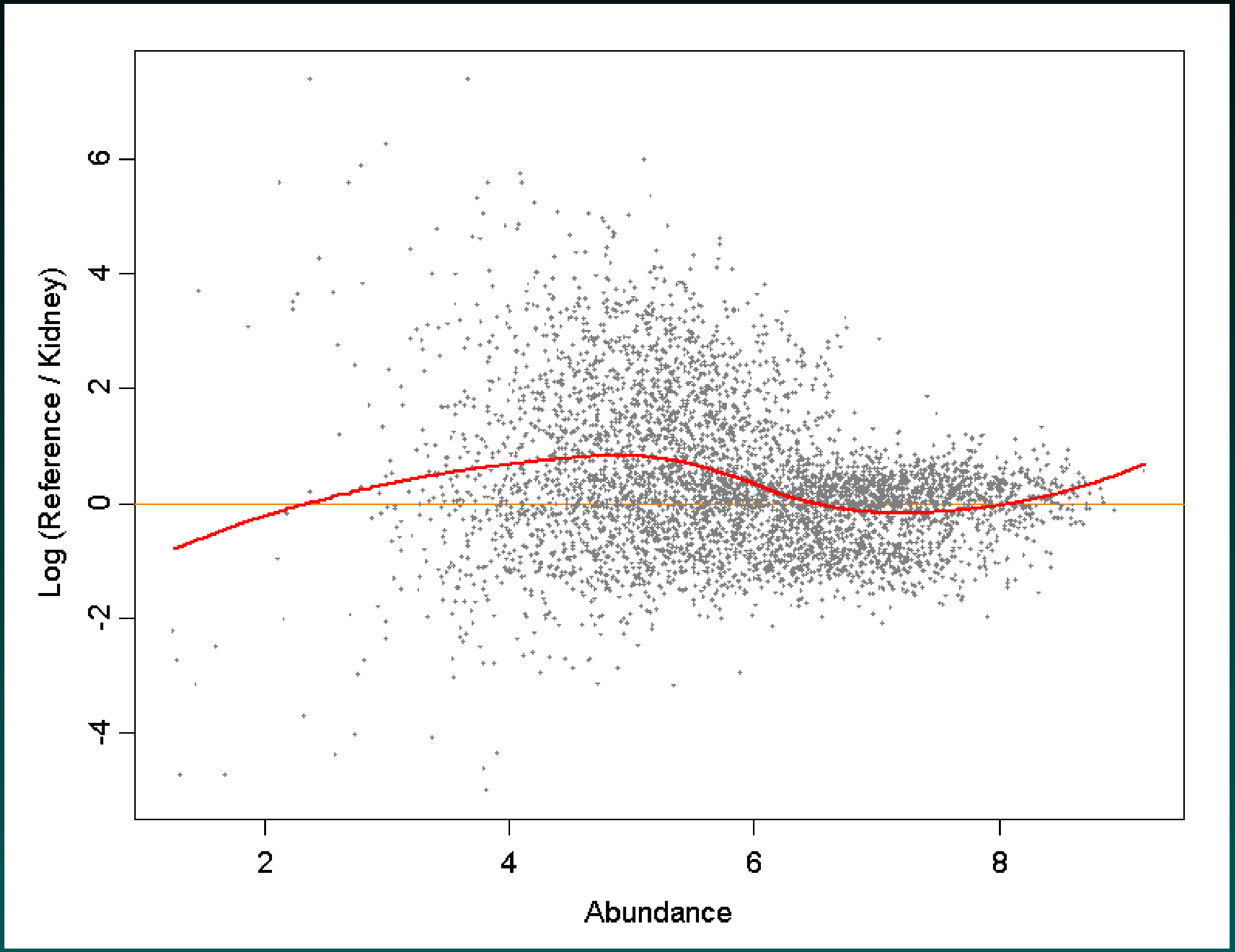
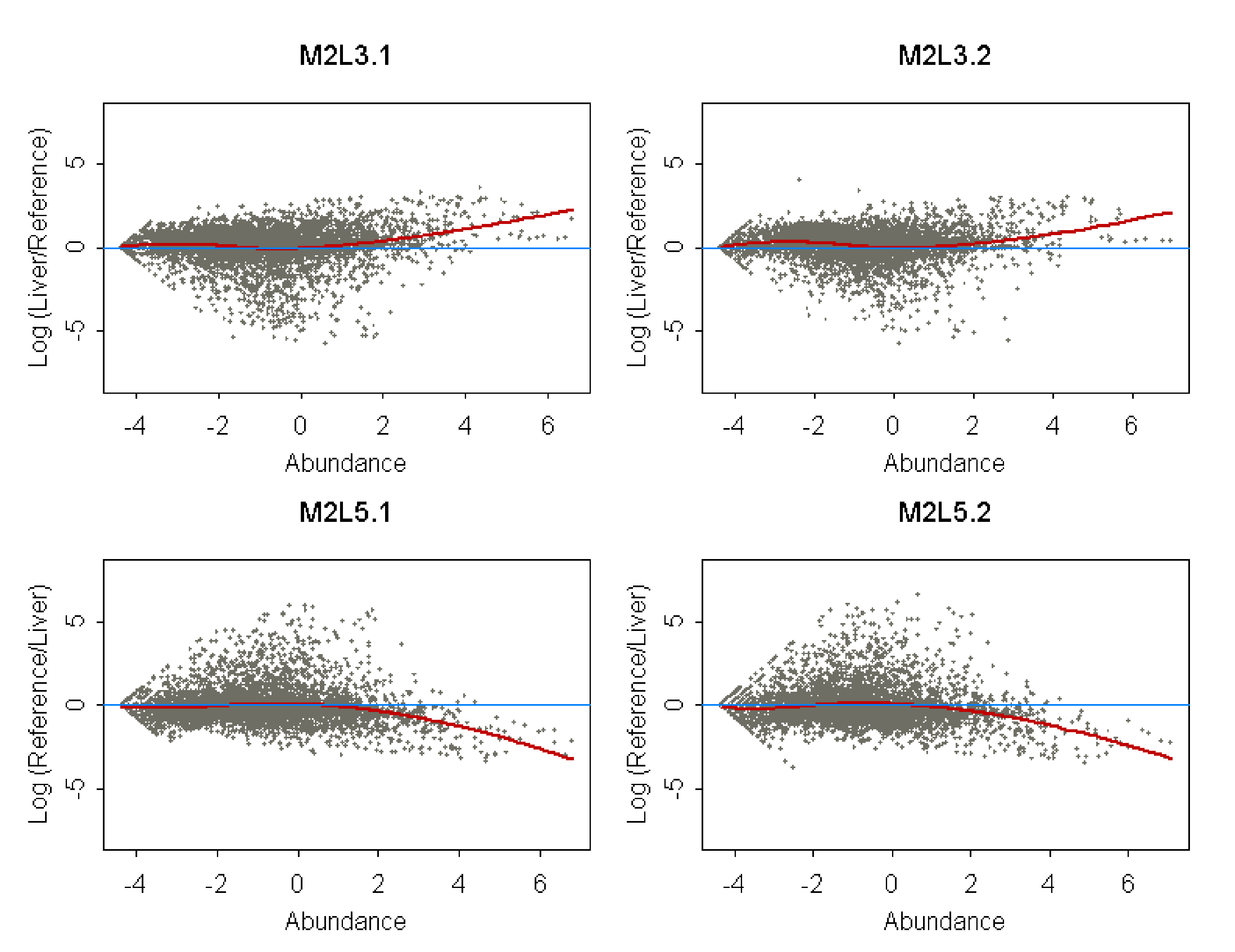


Simulated Data Using Our Approach



Are We Right? Checking the Dye Swaps



Interpretation

- Distributions of intensities are different in the two channels
- Difference is NOT caused by arrays, dyes, or technology
- Difference is inherent in the choice of reference material

So, How Do We Normalize This Data?

Normalize channels separately

Divide by 75^{th} percentile (magic)

Multiply by 10 (arbitrary, equalizes scale)

Set threshold at 0.5 (more magic)

Log transform

