

# There is no silver bullet – A guide to Low-level Data Transforms and Normalization Methods for Microarray Data

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## Introduction

Microarray technology provides a powerful tool for large-scale gene expression measurements. Typical application areas include genomics research, quantitative systems biology, and the more traditional screening for candidate genes. Microarray data are the end product of a fairly intricate experimental process,<sup>1-3</sup> and adequate analysis must meet the challenge of separating true biological signals from experimental bias and random fluctuations. A wide range of both commercial and freely available software has been developed to address this need. The choice between the large number of different analysis methods available, however, can be daunting. Unfortunately, there is no ‘silver bullet’: No single ‘catch-all’ method solves the problem of appropriate microarray data analysis. The right path will depend on the biological problem examined, the implemented experimental design (Box 1), and even the microarray type (Box 2).

### Box 1: Experimental Design

Efficiency of data analysis begins with good experimental design. It must be emphasized that it is always advisable to consult both with a statistician and someone with experience conducting similar microarray experiments *before* embarking on sample / data collection. Much effort and resources have been wasted by inappropriately designed experiments, for which an analyst can often only provide a ‘post mortem’ of what has gone wrong in experimental design. Essentially, good design considers all of the following issues:

- A clearly defined biological question / hypothesis much facilitates analysis. For exploratory studies: How will biological patterns be distinguished from technical ones? What independent means of validation are there?
- A sensible experimental arrangement must be chosen. For example, a dye swap design addresses dye bias effects, while the use of a reference sample in one channel aids normalization and comparison across slides.<sup>2,4,5</sup> If it is likely that additional data will be collected, a reference design may be indicated simply by the increased flexibility it gives.
- Can identification and control of systematic experimental errors be aided? This could, for example, mean additional measurements, *e.g.*, of collected sample

amounts, or involve the liberal use of calibration points, such as control probes and exogenous spikes.<sup>6</sup>

- When a large number of probes are expected to differ between samples the use of 'housekeeping genes' and/or exogenous spikes will actually be critical for subsequent analysis (also see discussion in main text). This is likely, *e.g.*, when very different samples are assessed, or when small or 'boutique' arrays are used that feature a set of probes already preselected for their involvement in a particular biological process. A large number of control probes must then be placed randomly across the array to allow normalization and subsequent analysis.
- An optimal number of biological or technical replicate samples<sup>7</sup> must be assessed, as dictated by the experimental situation, observed noise levels (*e.g.*, from pilot experiments), and the available budget.<sup>8</sup> While the optimal replication strategy may strongly vary between different experiments, as a rule of thumb, a minimum of five biological replicates are typically required for robust inference.<sup>9</sup>
- Data collection in compliance with the Minimal Information About a Microarray Experiment (MIAME) is increasingly recommended or required for publication.<sup>10,11</sup>

## Box 2: Too many flavours of chips

There is a variety of microarray platforms available, the technical properties of which affect choices in subsequent data analysis. In the remainder of this article, microarray 'slide' shall mean the support of the microarray probes, no matter whether that is a traditional 'microscope glass slide', or not.

Platforms can be categorized by their probe type. There are three distinct types of microarray probes – cDNA probes, long oligonucleotide probes, and short oligonucleotide probes. Long oligonucleotides (typically 50- to 70-mers) are thought to mimic the properties of cDNA probes offering high sensitivity and good specificity, while giving better probe homogeneity.<sup>12</sup> Oligonucleotide probes can target specific regions of a gene and hence allow the detection of splice variants.

For both cDNA and long oligonucleotide arrays, typically only one probe is designed for each gene that is to be probed (splice variants are an additional complication that will not be addressed here). Short oligonucleotide arrays are typified by the GeneChip™ platform (Affymetrix). The Affymetrix system involves the *in situ* manufacture of short oligos on a glass surface using photolithography. The initial method for doing this was limited to the production of 25-mers and this has meant that Affymetrix has adopted a design approach for their arrays specific to the use of such short probes.<sup>13,14</sup> For each gene, a unique region is identified, then a series of 11–20 complementary probes spanning this region are synthesized. These complementary probes are referred to as 'Perfect Match' probes (PM). Each PM probe is then paired with a 'Mismatch' probe (MM), which has the same sequence as the PM except the central base is replaced with a mismatched nucleotide. The complete set of PM and MM probe pairs for each gene are referred to as a 'Probe Set'.

The process of inferring a single estimate of gene expression from a probe set has recently become an area of very active research.<sup>15,16</sup> Results of a standardized benchmark of competing methods can be found at <http://affycomp.biostat.jhsph.edu/>. Ideally, this process of probe summarization should be done simultaneously with normalization. In practice, for a lack of truly integrated methods, most laboratories normalize across chips before probe summarization.

Microarray data analysis is often, for convenience, split into several distinct steps, such as: (I) image analysis, (II) data preprocessing / normalization, and (III) higher-level analysis and interpretation. While fully-automated microarray image analysis is still an area of active research, most laboratories use the software that came with their scanner, with operators contributing to random error.<sup>8</sup> The myriad of tools available for higher-level analysis reflects the wide range of applications for microarray experiments. Approaches include exploratory techniques, like all forms of clustering,<sup>17,18</sup> algorithms for classification,<sup>19-21</sup> statistical hypothesis tests (*e.g.*, for differential expression,<sup>22</sup> and fitting more complex probabilistic models (Bayesian networks,<sup>23</sup> hierarchical models,<sup>8,24</sup> ...). All analyses though have in common the need to identify and remove experimental bias from measurements before further study. It should be emphasized that correct normalization is essential for meaningful data analysis, as distortions through artefacts can be significantly larger than the biological signals of interest. This tutorial provides an introduction to the underlying concepts of common approaches to this problem, and gives a first guide to choosing between the available microarray data transformation and normalization methods for this task. Where appropriate, we point to corresponding packages from the open-source Bioconductor software repository.<sup>25</sup>

## The challenge of microarray data normalization

### ***Experimental sources of unwanted signal variation***

The experimental process leading to the signals that comprise microarray data is fairly complex. There is a large variety of sources of systematic global differences between measurements that reflect differences in

- sample preparation, *e.g.*, time from surgical removal until cryo-preservation
- RNA extraction efficiency between samples
- overall amplification yield, if an amplification protocol is employed
- overall labelling yield
- overall hybridization efficiency and washing stringency
- incorporation efficiency for different dyes during direct labelling
- fluorescence gain of the excitation/detection system for different dyes

Further sources of variation include specific effects of the manufacturing process, like differences in

- characteristics of individual printing pins
- properties of specific probe source plates
- batch effects (slides, buffers, *etc.*)

More complex distortions can be caused by

- sequence specific label incorporation bias
- spatial variation of hybridization efficiency and washing stringency
- non-linear effects, like non-linear dye fluorescence response and interaction between dyes (cross-talk and quenching) – these can give rise to complex confounding of effects, *e.g.*, bias in the differential signal as a function of the average intensity

Last but not least, it certainly should be mentioned that both the operator and the date of the experiment are significant sources of variation. The general difficulty of combining data from different laboratories is well appreciated. Technical effects often dominate biologically relevant signals in multi-centre studies (Figure 1).

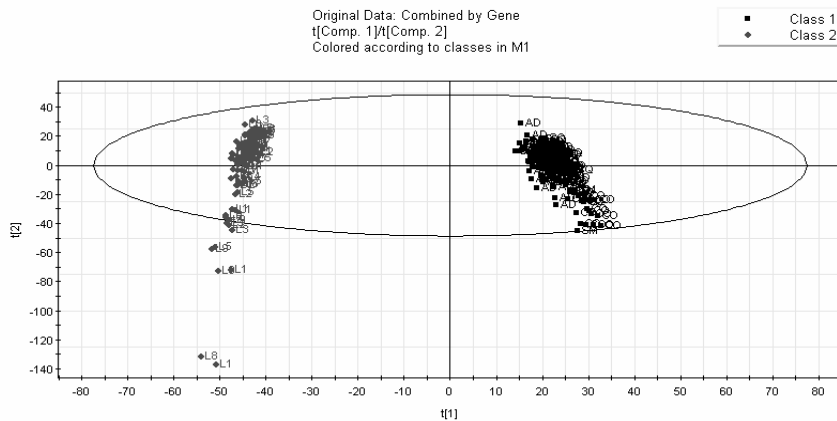


Figure 1: Affymetrix lung cancer data from Harvard (gray) and Michigan (black). The plot in principal components space<sup>26</sup> shows that, despite the laboratories' use of standardized commercial microarray platforms (HGU95a & HuGeneFL chips), the main source of variation maps to the laboratory in which the data have been generated, indicating that differences in sample collection protocols and the microarrays used dominated the biological effect of interest.

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### **Control for technical variation by conservative normalization**

Ideally, the sources of these non-biological variations would be determined and accounted for, or minimized at the experimental stage. Often, this is only partly possible and, to allow comparison of different measurements, the remaining differences need to be controlled for during analysis, a process called *normalization*.

Normalization procedures remove unwanted variances from data by exploiting and enforcing *known or assumed invariances* of the data – in an exact, averaging, or probabilistic manner, and with varying degrees of robustness towards outliers. Considering the complexity of the problem, it is not surprising that new normalization methods are being introduced continuously. Instead of providing a list of the many methods available, we here restrict ourselves to a review of basic concepts. In the next section, several well-known methods and the invariances they require are discussed. The fundamental assumptions of normalization methods differ and are satisfied by real data sets to varying degrees. Consequently, a different normalization method must be chosen appropriate for any specific experiment. Moreover, as the 'correct' result of a microarray experiment is usually not known, it is very difficult to assess the relative performance of normalization methods on realistic data sets. In selecting an appropriate normalization procedure for a particular experiment, a guiding principle is hence the conservative nature of the method – the lower the number of parameters, the less arbitrary. While removal of complex artefacts may require procedures with a higher number of parameters, it is easy to unwillingly introduce novel artefacts by either over-fitting or as a result of failure in separating biological from technical patterns. Satisfying all assumptions of the employed methods, the most conservative approach should hence be chosen that removes the dominant artefacts.

## Normalization Methods – Common approaches

In a discussion of several common normalization approaches, we will highlight situations in which their assumptions are not met, and different methods need to be applied. We start with the most conservative procedures.

### ***Rescaling, robustness, ‘housekeeping genes’, and spike controls***

A very conservative, simple, and popular approach is the rescaling of each sample by a constant. This controls for overall differences in signal strength as, *e.g.*, caused by different extraction or labelling yields. Instead of rescaling by the mean signal intensity, often the median signal intensity is used, giving considerable robustness to outliers. Even this simple method, however, makes a critical assumption, namely, that the median signal intensity will be invariant between samples. A popular variant of this approach assumes invariance of a *subset* of genes (typically called ‘housekeeping genes’), rescaling by their median signal intensity. Although there is good reason to doubt that such a set of invariant genes exists in general (*e.g.* some studies have found the presumed ‘housekeeping’ gene GAPDH to be quite variable<sup>27,28</sup>), there may be sets of genes that are non-differentially expressed across the samples of a particular experiment. Nevertheless, there are cases where true biological differences between samples violate all of the above assumptions. Consider, *e.g.*, the massive generic down-regulation of gene expression in samples of dying tissue. This is a typical example of an experimental situation in which it is very difficult to find an appropriate normalization procedure. It furthermore highlights the value of considering such issues at the stage of experimental design. If known quantities of spike RNA are added to the sample before extraction, standardized sample amounts are ensured, and the slides contain a *high number* of spike probes, then the spike signals can be used for normalization, as these can now be assumed to be invariant. If such spikes are not available, all one can do is hope that the technical variation in signal intensity is smaller than the biological effect examined, and work on the unnormalized data.

### ***Explicit error models, robust fit using iterative trimmed weighted least squares***

For subsequent analysis, it is often desirable to decouple random error and signal intensity. It is very common to transform microarray data to a log-scale before further analysis. A logarithmic transform certainly collapses the original range of the signal, which can span over five orders of magnitude. Moreover, a log-transform decouples a random multiplicative error  $e^\eta$  from the true signal intensity  $\mu$  in the measurement  $y$ :

$$y = \mu e^\eta \quad \implies \quad \log y = \log \mu + \eta \quad (1)$$

It is well known that microarray data variance increases with signal intensity, which is in line with such a model. It is likely that the source of this variance is the hybridization process itself, as it cannot be explained by instrument error (Fig. 2).<sup>29</sup>

A purely multiplicative model, however, unrealistically predicts vanishing measurement error for very small signals, whereas in truth, there will always be some background noise. This can be seen in log–log plots of same–same comparisons (Fig. 3).

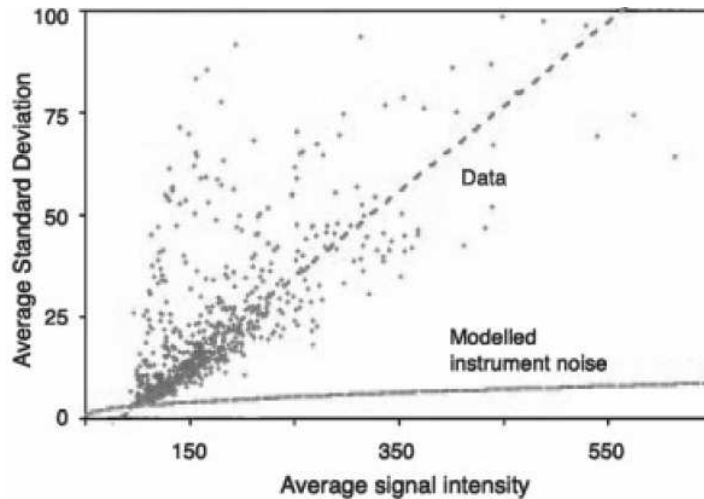


Figure 2: Increasing measurement variance with higher hybridization signal.<sup>29</sup>  
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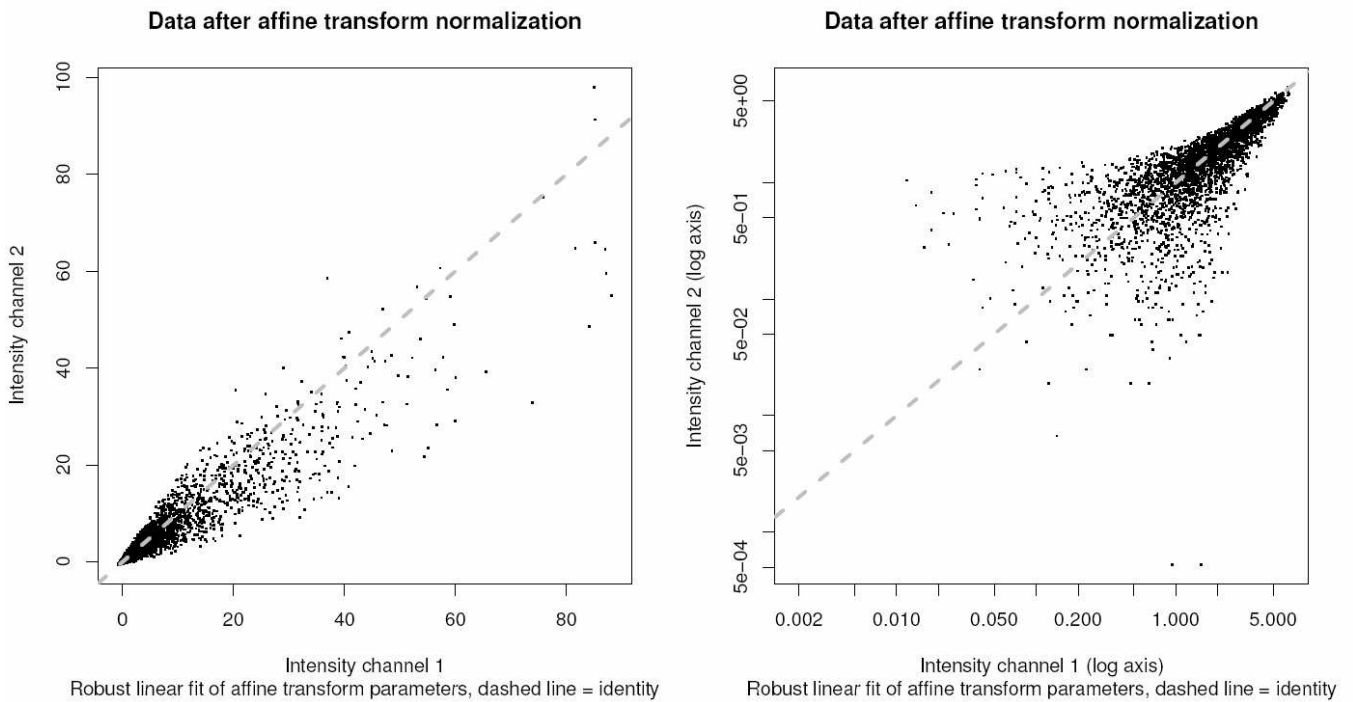


Figure 3: The two panels show the same data, which is from a same–same comparison.

On a linear scale (left panel), the variance increases with signal intensity. On logarithmic scale (right panel), the variance is approximately constant for medium and high signal intensities, in line with the purely multiplicative noise model in (1). The measured variance for low intensity signals, however, is larger than expected.

A more realistic error model (2) allows for both additive and multiplicative error terms:

$$y = a + b\mu e^\eta + \nu \tag{2}$$

Here, the constants  $a$  and  $b$  model a global background and gain, respectively. Just as the log-transforms decouples the true signal  $\mu$  from the error term  $e^n$  in model (1), the asinh transform will decouple the true signal  $\mu$  from the error terms  $e^n$  and  $v$  in (2). As shown in Figure 4, it is approximately linear for small values, for larger ones it is well approximated by a logarithmic transform. Figure 5 shows how this model more successfully decouples variance and signal intensity in real data.

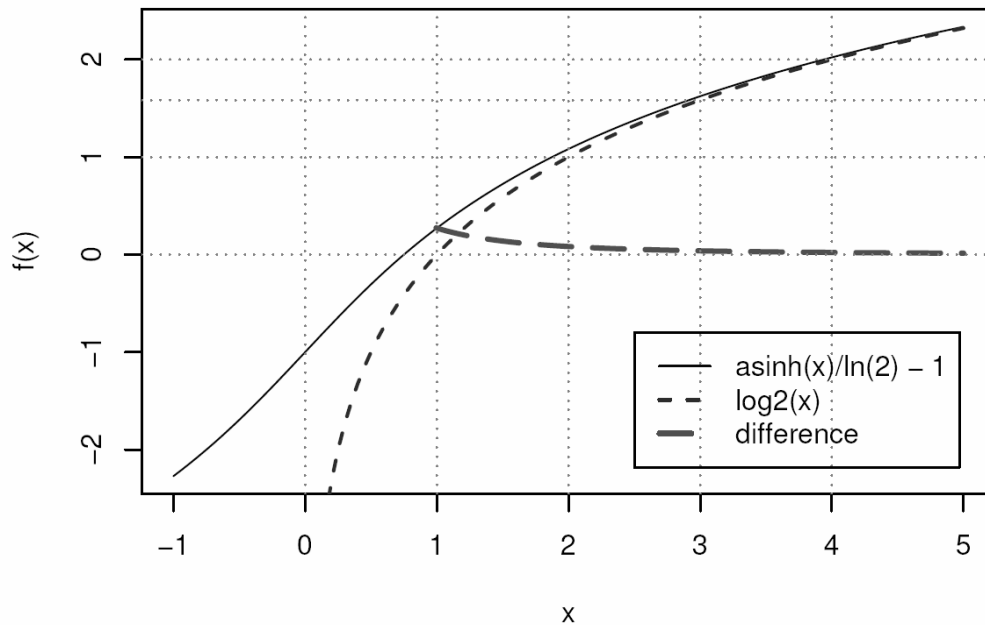
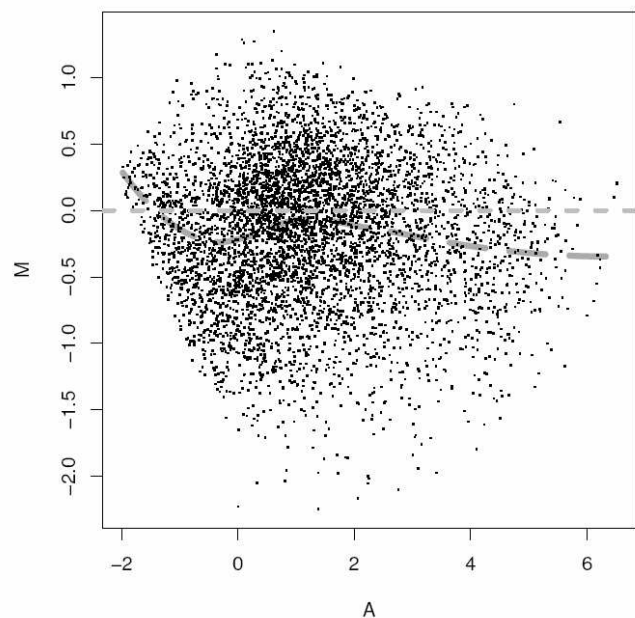
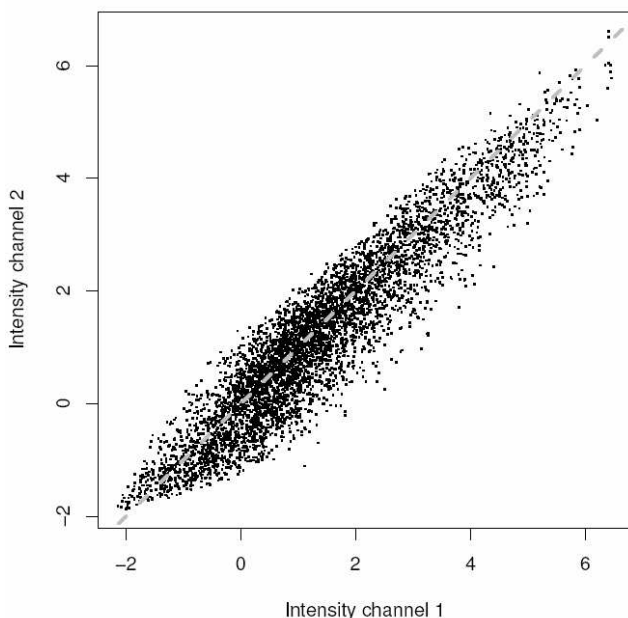


Figure 4: Comparison of an asinh- and a  $\log_2$ -transform. Note the deviation for values close to zero

3. Data after variance stabilizing normalization (vsn)

Data after variance stabilizing normalization (vsn)



Robust linear fit of affine transform; asinh2 transform; dashes = identity

Difference between the channels ( $M$ ) vs the average of the channels ( $A$ )

Figure 5: Both panels: On ‘asinh-scale’ the much-inflated fluctuations at low signal intensities have disappeared. The right panel shows the traditional ‘ $M(A)$ ’-plot, with  $M$  the difference, and  $A$  the mean of the two channels – artefacts are more obvious here. The dark-grey long-dashed line shows a Loess smoother, giving an indication of local trends in the plot. An ‘S’-shaped distortion is clearly seen.

To apply model (2) for normalization, the parameters  $a$  and  $b$  need to be obtained by fitting the model to the data. The measurements for all the genes of a sample share the same parameters in this model. Once the parameters have been obtained for each sample, the differences in global background and gain can be corrected for. Obviously, differentially expressed genes or technical outlier measurements should not affect this fit. The iterative trimmed least-squares fit implemented in the `vsN` package<sup>30</sup> (available from the Bioconductor repository) will identify a subset of non-differentially expressed non-outlier genes with a high breakdown point of 50%. This means that up to 50% of genes can be differentially expressed or technical outliers without this affecting the fit arbitrarily much, making the result *robust*. In contrast to many other methods, this procedure even works reliably if the differentially expressed genes introduce a strong asymmetry, *e.g.*, if they are mostly up-regulated (rather than having an even mix of up- and down-regulation).

It should be emphasized that this still is a very conservative approach, using only two parameters per sample. Alternatives to global, constant background and gain terms could either fit a slowly varying function as background or gain terms or attempt to measure these directly. Modelling spatial variation without additional measurements has the disadvantage of greatly increasing the number of parameters of the model. Hence, there has been a strong interest in obtaining approximate measurements for ‘local’ background intensities, *e.g.*, by measuring the fluorescence intensity in the slide area between the spotted probes. Unfortunately, there are two sources of background fluorescence observed on microarrays: (a) smears which cover both the probes and the area between probes, and (b) non-specific binding of fluorescent material to the area between the spotted probes, only. If the former dominates, then estimation of local background intensities by measuring fluorescence between probes makes a lot of sense, and correcting for this local background will improve data quality. On the other hand, if non-specific binding to only the area between spots dominates, such a correction is inappropriate and can create artefacts, *e.g.*, ‘negative intensities’. Many laboratories find that this is indeed the case, and measurements suggest that the background obtained from fitting a model like (2) actually corresponds to the self-fluorescence of the substrate slide before hybridization.<sup>29</sup>

The  $M(A)$ -plot of Figure 5 clearly shows an ‘S’-shaped technical artefact which has not been removed by this transformation. The methods discussed next can sometimes be used to remove such complex distortions.

#### ***Detrending of the signal difference as a function of the signal average***

‘Smile’- or ‘S’-shaped trends in the difference  $M$  between samples as a function of the average signal intensity  $A$  are commonly observed in microarray data (Fig. 5). A possible cause for such trends may be differences in the non-linear fluorescence response of dyes. In a comparison of similar samples, ideally, there should be no such distortions. A very flexible but rather aggressive approach hence enforces this invariance  $M(A)=0$  by computing an ‘average trend’ in order to subtract it, *e.g.*, by means of a Loess smoother, which is available from the `loess` function in the statistics environment R. While most bias in the trend of  $M(A)$  can indeed be removed this way, this normalization procedure is not conservative, having a large number of parameters to be obtained from the data, as well as an arbitrary parameter setting the ‘window length’ for the smoothing algorithm. This flexibility of the transform can become a problem when a researcher fails to detect that the assumption  $M(A)=0$  does not hold for biological reasons. This can, *e.g.*, be the case if a large number of genes

are asymmetrically differentially expressed (Fig. 6). Simulations by Coombes *et al.*<sup>31</sup> have shown that such observed non-linear trends in  $M(A)$  can be explained if 20% of genes are specifically expressed in only one of the samples, which is, *e.g.*, easily perceivable for comparisons of rather different tissue types. Dye swap experiments can help detect such situations (Fig. 6) to avoid indiscriminate application of, *e.g.*, a loess smoother to detrend  $M(A)$ . Obviously, inappropriate detrending would both remove biological signal *and* create further technical artefacts.

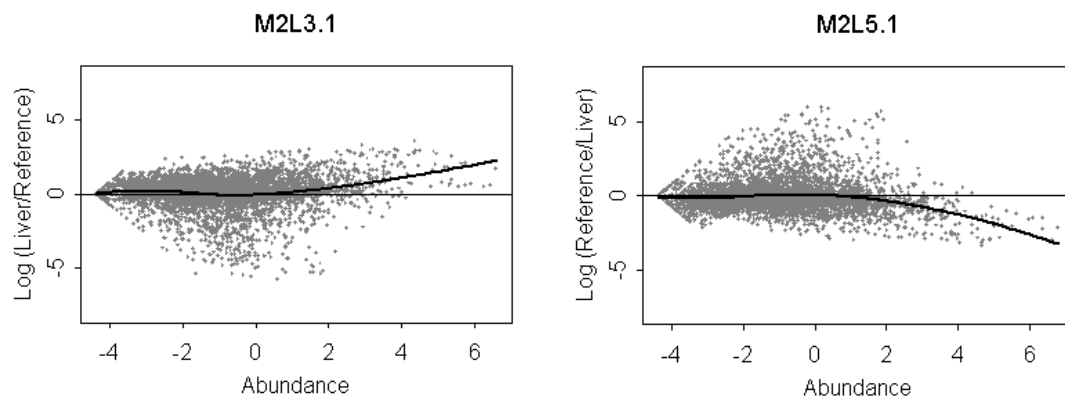


Figure 6: The left panel shows the  $M(A)$  plot for a microarray experiment comparing liver tissue with a reference sample comprising a mix of liver, kidney, and testis tissue<sup>31</sup>. The trend, shown by the solid line (a loess smoother), reverses under dye swap (right panel). Two replicate experiments each confirmed this picture. [Figure courtesy K. Coombes.]

#### **Equalization of signal distributions**

While the  $M(A)$  detrending described above can deal with a large variety of distortions, it is computationally expensive and its focus on expression difference does not lend itself well to situations with large numbers of (unpaired) samples<sup>32</sup>. A different approach, that is similarly generic, deals directly with the signal distributions of individual samples. For sufficiently similar samples, or if there are no strong asymmetries expected in differential expression, it is valid to also expect similar signal distributions for all samples, and to enforce this assumption by a normalization transform. This process can be depicted by quantile–quantile (Q–Q) plots (Fig. 7) – data are normalized by projection so that their Q–Q plot to a reference distribution gives a straight line. The reference distribution can be that of an arbitrary array, or a constructed / artificial one. A particularly efficient implementation<sup>33</sup> has made this approach very popular as part of the `affy` package for the analysis of Affymetrix slides in Bioconductor. This direct mapping, however, is not robust in the tails of distributions, where data tend to be sparse. An approach fitting natural cubic splines to quantile data<sup>32</sup> seems to resolve this issue in a computationally efficient way.

Like the transform discussed in the previous section, quantile normalization is not a conservative transform and should not be used in situations, where the signal distributions of samples are expected to differ, *e.g.*, when asymmetric differential expression of many genes. Figure 8 shows that the signal distribution in male and female fruit-flies differs, reflecting true biological differences. Quantile normalization of these samples would not only remove this biological signal but also introduce further technical artefacts.

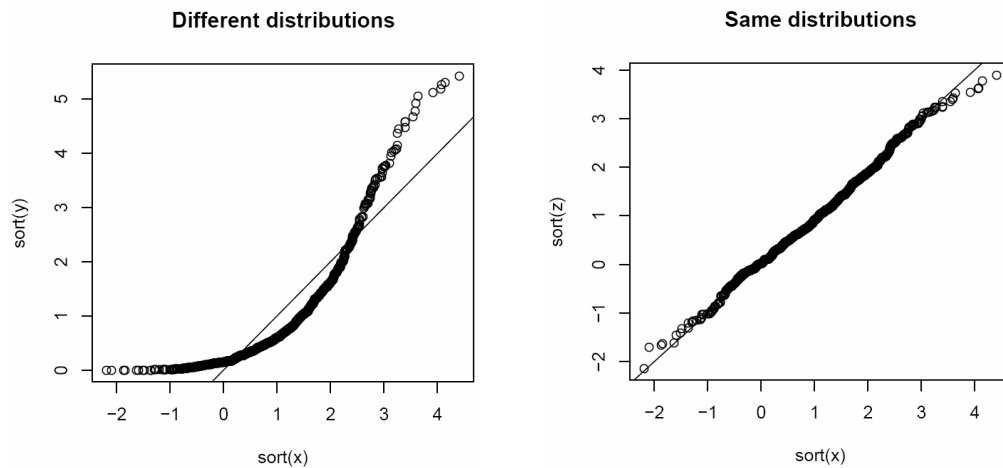


Figure 7: Quantile–quantile (Q–Q) plots for rather different distributions (left panel) and for similar distributions (right panel). In Q–Q plots, the quantiles of one sample are graphed as a function of the same quantiles of the other, allowing direct comparison of, *e.g.*, the 25<sup>th</sup> percentile of the two samples (which marks the value which is larger than 25% of the data in each sample, respectively). Identical distributions will hence give a straight line in Q–Q plots. For samples of equal size, a Q–Q plot can easily be generated as scatter plot of the sorted samples to be compared.

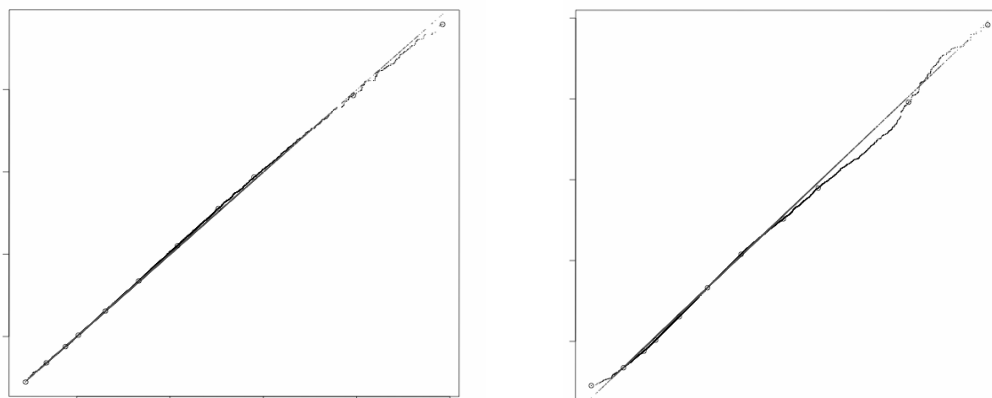


Figure 8: Q–Q plots of two female fruitfly samples (left) show similar signal distribution, while a comparison of samples from a male and a female fly consistently show distinct differences (right).

### **Spatial detrending**

Well-designed microarrays have replicate probes distributed in a random order across the slide surface to allow separation of spatial artefacts from biological signal. Unfortunately, many early arrays (from both academic and commercial providers) have not followed this design criterion for reasons of manufacturing convenience. For these older-generation slides – which are still widely employed – computational approaches are prone to fail separating spatial artefacts and biological signal. Where possible, one may try to alleviate this problem by hybridizing every other replicate slide rotated by 180°. The most stringent designs are currently implemented in the

most recent high-density arrays with multiple redundant probes per gene randomly distributed across the slide (e.g., as available from Affymetrix or Nimblegen).

In modern microarrays, spatial trends in signal intensity are usually a sign of technical artefacts, such as caused by unequal hybridization conditions or washing. For example, one can sometimes clearly identify the areas beneath the probe inlet and outlet holes of an Affymetrix slide (Fig. 9). Traditionally, Loess smoothers are computed in order to estimate and subtract spatial trends. While this can reduce variance between replicates, recent calibration experiments suggest that spatial variation of *unspecific* binding cannot be corrected this way (manuscript in preparation).

The original design of a two-channel DNA microarray is actually quite ingenious, as it already provides approximate self-normalization for spatial variation of hybridization and washing efficiency by performing the measurements of samples to be directly compared in a single probe that is subject to the same local reaction conditions. To extend the sensitivity of screens, however, replicate slides are needed. In addition, more complex experiments require the joint analysis of multiple samples. In either case, modern work needs normalization between slides, not just between channels. It hence seems natural to expect future microarray designs to feature novel experimental designs that support between slide normalization directly, rather than rely on purely computational approaches.

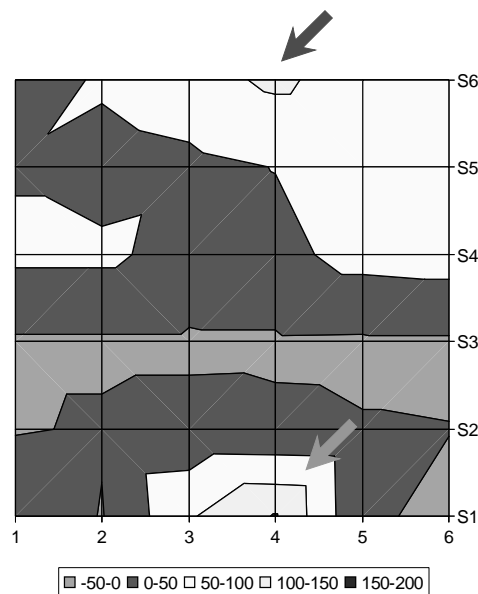


Figure 9: Spatial trends in the signal intensities measured on an Affymetrix slide. The brighter areas beneath the probe inlet and outlet holes (arrows) can be clearly identified.<sup>34</sup> [Figure courtesy of A. Ptitsyn.]

## Summary and Outlook

We have explained how modern microarray experiments, which require the combination of data from multiple slides, rely on removing unwanted technical variation between sample measurements, a process called normalization. We have then given an introduction to common concepts and principles employed by current normalization methods, including the assumptions that they exploit and which they rely on. It is essential that a normalization / data-transformation method appropriate to the properties of the experiment in question is chosen. We have discussed the

strengths and limitations of several popular approaches, providing guidance for their use. The normalization of microarray data is still a very active area of research, with frequent novel interactions between microarray design and analysis methods (*cf.* probe summarization, Box 2). Just as the original design of two-channel microarrays had technical features reducing the impact of unwanted experimental variance, it can be expected that future microarray designs together with advanced models will further improve the ability to normalize data across samples. Such progress will allow an even more quantitative and sensitive approach to modern applications in the clinical and biological sciences.

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