

# Introduction to OLIN package

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## Contents

<b>1</b>	<b>Overview</b>	<b>1</b>
<b>2</b>	<b>Installation requirements</b>	<b>1</b>
<b>3</b>	<b>Inspection for intensity-dependent and spatial artifacts</b>	<b>2</b>
<b>4</b>	<b>Optimised local intensity-dependent normalisation</b>	<b>7</b>
4.1	OLIN . . . . .	8
4.2	OSLIN . . . . .	11
<b>5</b>	<b>Statistical assessment of efficiency of normalisation</b>	<b>14</b>

## 1 Overview

Microarray measurements are affected by a variety of systematic experimental errors limiting the accuracy of data produced. Two well-known systematic errors for two-colour arrays are the so-called intensity-dependent and spatial (location-dependent) dye bias. Normalisation aims to correct for systematic errors in microarray data.

The common linear (or global) normalisation method often fails to correct for dye bias as this bias is usually not linear. Although non-linear normalisation procedures have been able to reduce the systematic errors, these methods are based on default parameters and leave it to the user to choose “good” parameters. The optimal adjustment of the normalisation models to the data, however, can be crucial for the efficiency of the normalisation process [1].

The OLIN (*Optimised Local Intensity-dependent Normalisation*) R-package includes two normalisation schemes based on iterative local regression and model selection. Both schemes aim to correct for intensity- and location-dependent dye bias in microarray data. For model selection (parameter optimisation), generalized cross-validation (GCV) is used.

Additionally, several procedures to assess the efficiencies of normalisation are implemented in the package.

## 2 Installation requirements

Following software is required to run the OLIN-package:

- R ( $> 1.9.0$ ). For installation of R, refer to <http://www.r-project.org>.
- R-packages: methods, stats, locfit. For installation of these add-on packages, refer to <http://cran.r-project.org>.
- Bioconductor packages: Biobase, marray. Refer to <http://www.bioconductor.org> for installation.

If all requirements are fulfilled, the OLIN add-on R-package can be installed. To see how to install add-on R-packages on your computer system, start *R* and type in *help(INSTALL)*. Optionally, you may use the R-function *install.packages()*. Once the OLIN package is installed, you can load the package by

```
> library(OLIN)
```

### 3 Inspection for intensity-dependent and spatial artifacts

Microarray data often contains many systematic errors. Such errors have to be identified and removed before further data analysis is conducted. The most basic approach for identification is the visual inspection of MA- and MXY-plots. MA-plots display the logged fold change ( $M = \log_2(Ch2) - \log_2(Ch1)$ ) with respect to the average logged spot intensity ( $A = 0.5(\log_2(Ch1) + \log_2(Ch2))$ ). MXY-plots display  $M$  with respect to the corresponding spot location. More stringent, but also computationally more expensive, are statistical tests presented in section 5.

For illustration, we examine a cDNA microarray experiment comparing gene expression in two colon cancer cell lines (SW480/SW620). The SW480 cell line was derived from a primary tumor, whereas the SW620 cell line was cultured from a lymph node metastasis of the same patient. Sharing the same genetic background, these cell lines serve as a model of cancer progression [2]. The comparison was direct i.e. without using a reference sample. cDNA derived from SW480 cells was labeled by Cy3; cDNA derived from SW620 was labeled by Cy5. The SW480/620 experiment consisted of four technical replicates. The data is stored as object of the class **marrayRaw** (see the documentation for the package **marrayClasses**). The average logged spot intensity  $A$  and logged fold changes  $M$  can be accessed by using the slot accessor methods **maA** and **maM**, respectively.

First we want to load the data and inspect the spatial distribution of foreground and background intensities in both channels. This can be done using the function **fgbg** that plots the spatial distribution of fore- and background spot intensities for both channels (figure 1):

```
> data(sw)
> fgbg.visu(sw[, 3])
```

The quantity that we are interested in are the (logged) fold-change  $M$ . To inspect any existing intensity- or location-dependent bias of  $M$ , MA- and MXY-lots can be employed. For MA-lots, the basic **plot** function can be used (figure 2). MXY-lots can be generated by **mxy.plot**(figure 3). Note that the function **mxy.plot** assumes the standard array layout as defined for objects of the **marrayClasses**.

```
> plot(maA(sw[, 3]), maM(sw[, 3]), xlab = "A", ylab = "M")
```

Pause. Press <Enter> to continue...

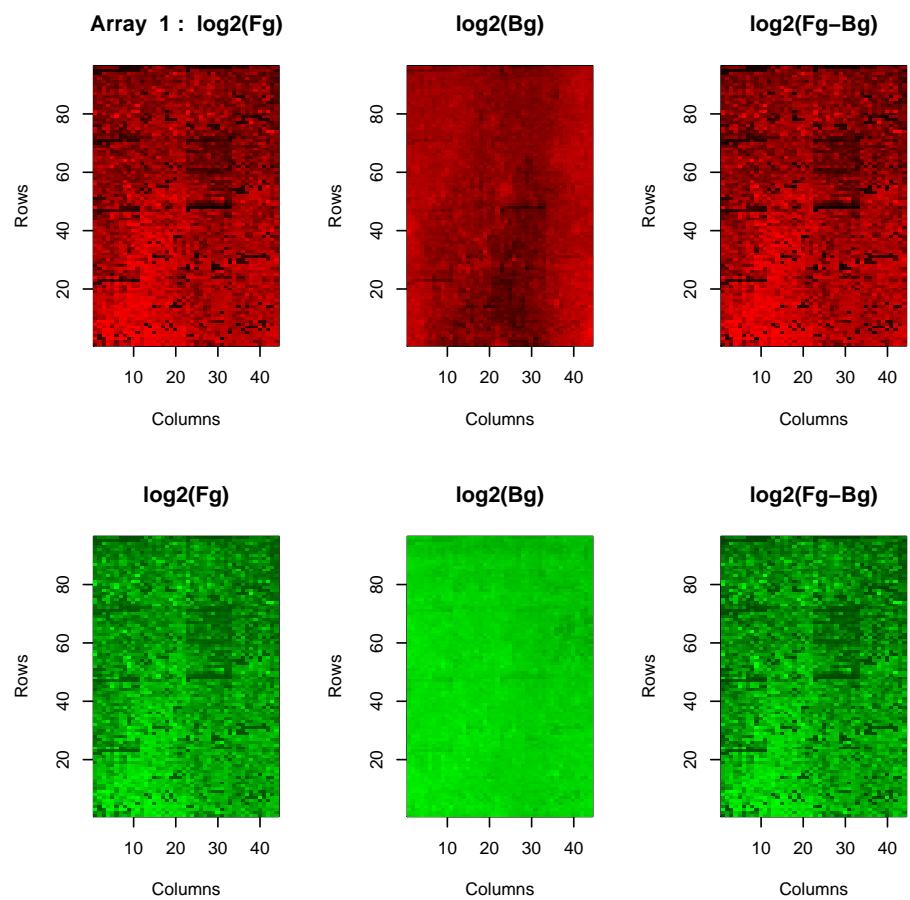


Figure 1: Foreground and background fluorescence intensities for Cy5-channel (top row) and Cy3-channel (bottom) row.

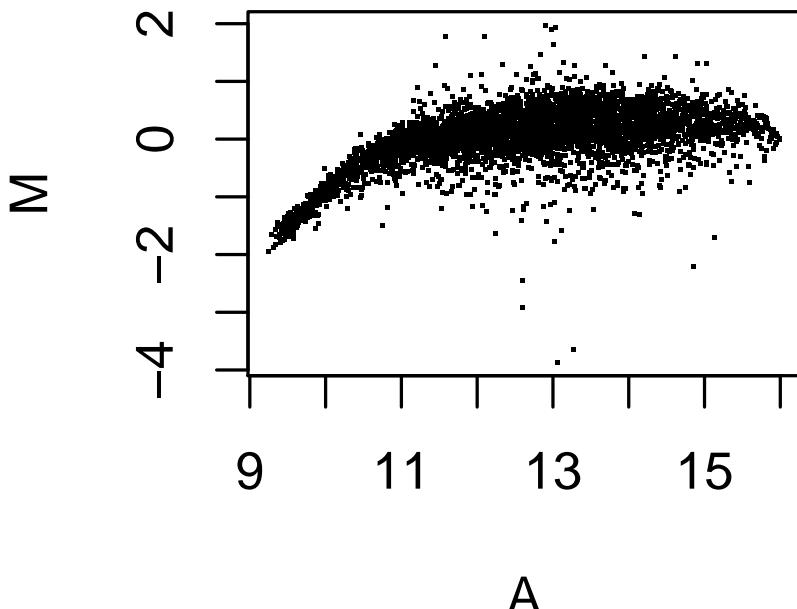


Figure 2: MA-plot of SW480/620 array 3

```
> mxy.plot(maM(sw)[, 3], Ngc = maNgc(sw), Ngr = maNgr(sw), Nsc = maNsc(sw),
+           Nsr = maNsr(sw))
```

Additionally the distribution of absolute values of  $M$  can be displayed. This visualisation may give an indication whether the span of  $M$  is equal across the spatial dimensions of the array (figure 4). To plot  $\text{abs}(M)$ , the function `mxy.abs.plot` can be employed :

```
> data(sw.xy)
> mxy.abs.plot(maM(sw)[, 3], Ngc = maNgc(sw), Ngr = maNgr(sw),
+               Nsc = maNsc(sw), Nsr = maNsr(sw))
```

For the two MXY-plots above, the column and row indices were used as proxies for location of the spot. We can use, however, the physical spot location as determined by the scanner for the MXY plots (if the spots'  $X$ -and  $Y$ -coordinates are available.) Especially, if the gaps between the blocks printed by distinct pins are large, this option may give a better physical representation of the array. Using the function `mxy2.plot`, such plots can be generated (figure 5):

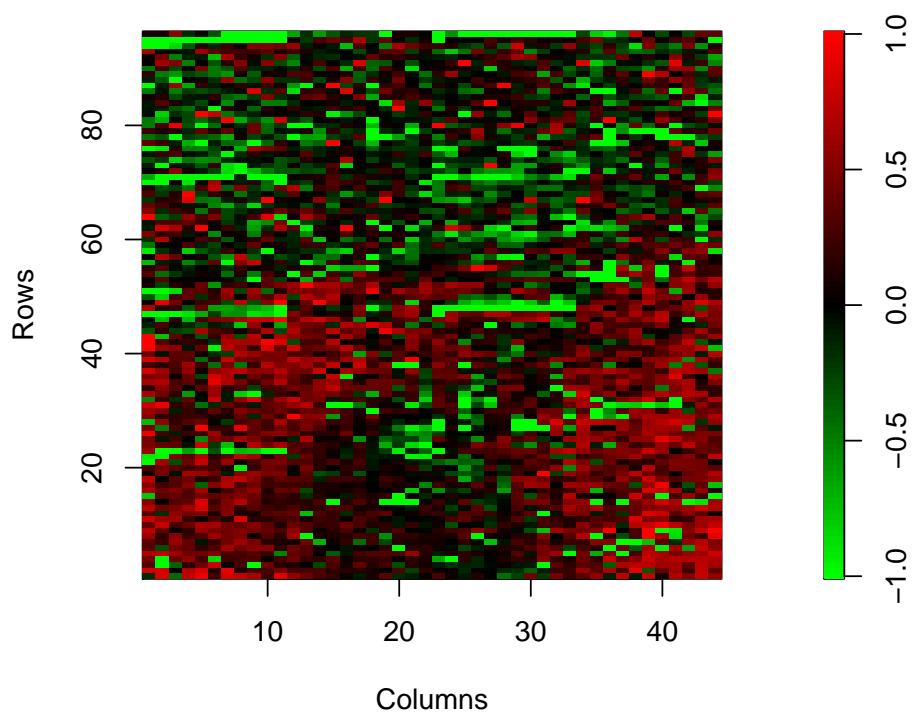


Figure 3: MXY-plot of SW480/620 array 3 with columns and rows as proxies for spatial location

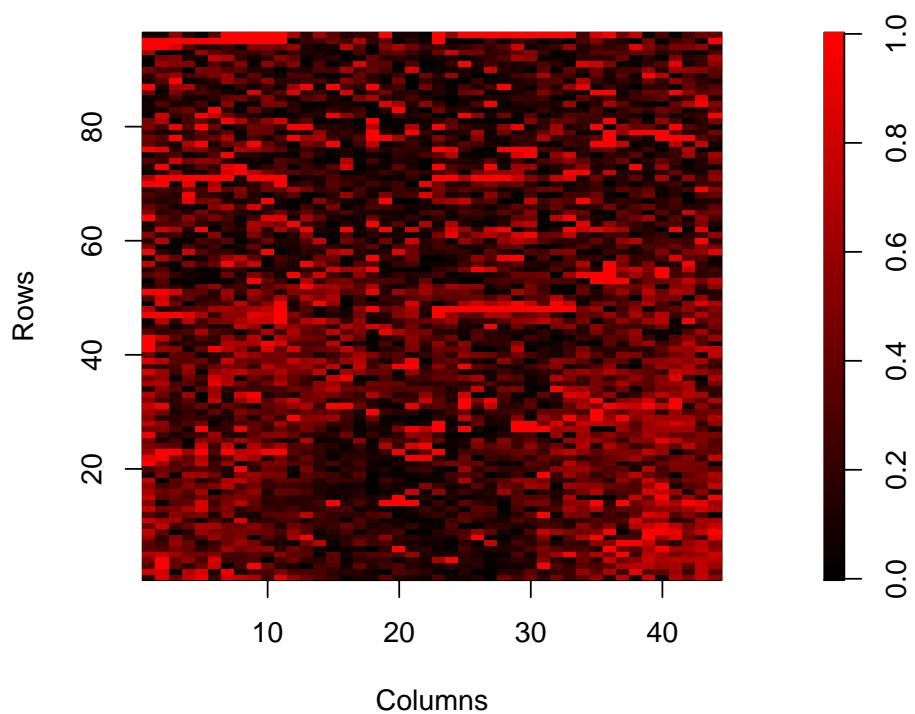


Figure 4: MXY-plot of absolute logged fold changes

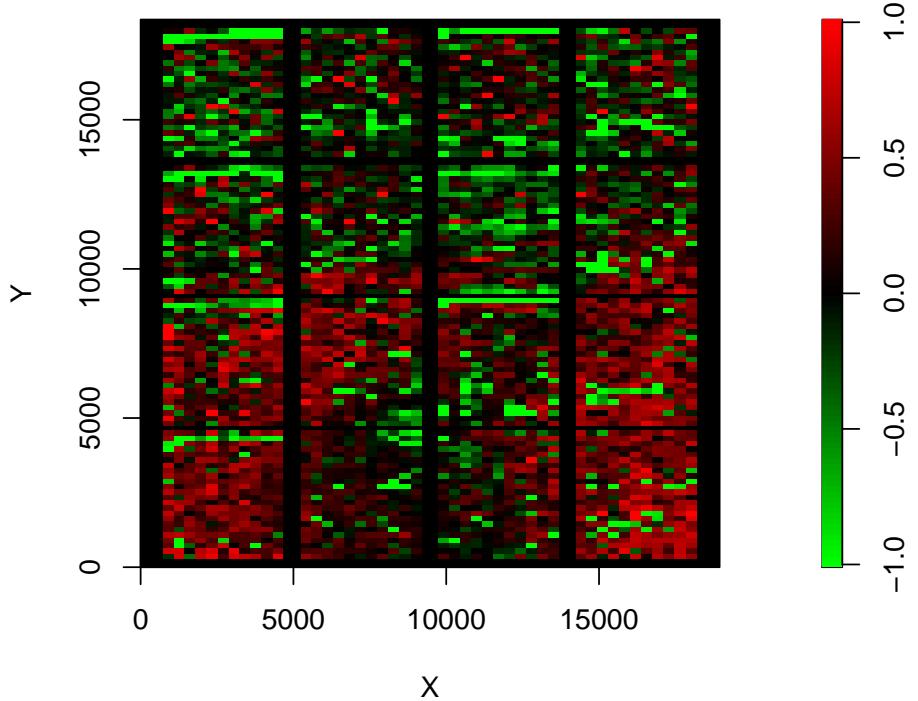


Figure 5: MXY plot of SW480/620 array 1

```
> data(sw.xy)
> mxy2.plot(maM(sw)[, 3], X = sw.xy$X[, 3], Y = sw.xy$Y[, 3], Ngc = maNgc(sw),
+   Ngr = maNgr(sw), Nsc = maNsc(sw), Nsr = maNsr(sw))
```

## 4 Optimised local intensity-dependent normalisation

Inspection of the MA- and MXY-plots in the previous section indicated a intensity- and location-dependent dye bias. Additionally, the plots show clearly the non-linearity of the biases. To correct for these systematic errors, we apply first a local regression of  $M$  with respect to  $A$  and spot location  $X, Y$ . The residuals of the regression are normalised  $M$ . This procedure is motivated by the hybridisation model introduced in reference [1].

The assumption here are: i) Most genes arrayed are not differentially expressed or up-and down-regulation is balanced over the range of  $A$ , ii) the spotting procedure did not generate an spatial accumulation of up- or down-regulated genes in localized areas on the array. The validity of both assumptions should be carefully checked for the data to be normalised.

The local regression is performed using LOCFIT algorithm which is based on the same computational ideas as popular lowess method [3, 4]. Required parameters for LOCFIT are the smoothing parameter  $\alpha$  and the scale parameter(s)  $s$  for multi-dimensional regression. The parameter  $\alpha$  specifies the fraction of points that are included in the neighborhood for local

fitting and can have a value between 0 and 1. Larger values lead to smoother fits. The setting of scale parameters  $s$  is necessary for a local regression with multiple predictor variables. The scale parameters  $s$  determine the amount of smoothing in one direction compared to the other directions.

Choosing accurate regression parameters is crucial for the quality of the normalisation. Too large smoothing parameters, for example, lead to a poor fit where local data features are missed and underfitting occurs. If the smoothing parameter is too small, overfitting is produced and the residuals subsequently underestimated. To optimise the parameter setting, two procedures were developed: OLIN and OSLIN. Both methods are based on iterative local regression and parameter selection by GCV. They aim to correct for systematic errors linked with spot intensity and location. A detailed mathematical description can be found in reference [1].

## 4.1 OLIN

The OLIN (and OSLIN) are implemented in R by the function `olin`:

```
olin(object,X,Y,alpha,iter,scaling,scale)
```

The input arguments are as follows:

**object** Object of class *marrayRaw* or *marrayNorm* containing data of the microarray experiment such as spot intensities and array layout for a batch of microarrays. To generate such an object, see the *marryClasses* documentation.

**X** Matrix with x-coordinates of spots. The location is spots detected by scanner is usually found in the output file program of the scanning program. If X=NA, the spotted columns on the array are used as proxies for the location in x-direction (*default*)

**Y** matrix with y-coordinates of spots. If Y=NA, spotted rows on array are used as proxies for the location in y-direction (*default*).

**alpha** Vector of  $\alpha$  parameters that are tested in the GCV procedure. The  $\alpha$  parameter defines the smoothness of fit. The default vector is `seq(0.05,1,0.1)`.

**iter** Number of iterations in the OLIN or OSLIN procedure. The default value is 3.

**scaling** If scaling=TRUE, a subsequent optimised scaling, and thus OSLIN, is performed. The default value is *FALSE*, i.e. OLIN is performed.

**scale** Vector of scale parameters  $s$  that are tested in a GCV procedure. This allows a different amount of smoothing in Y-direction compared to smoothing in X-direction. The default values of  $s$  are `c(0.05,0.1,0.5,1,2,10,20)`.

For illustration, we apply the OLIN scheme to normalise the third array of the SW480/620 data set.

```
> norm.olin <- olin(sw[, 3], X = sw.xy$X[, 3], Y = sw.xy$Y[, 3])
```

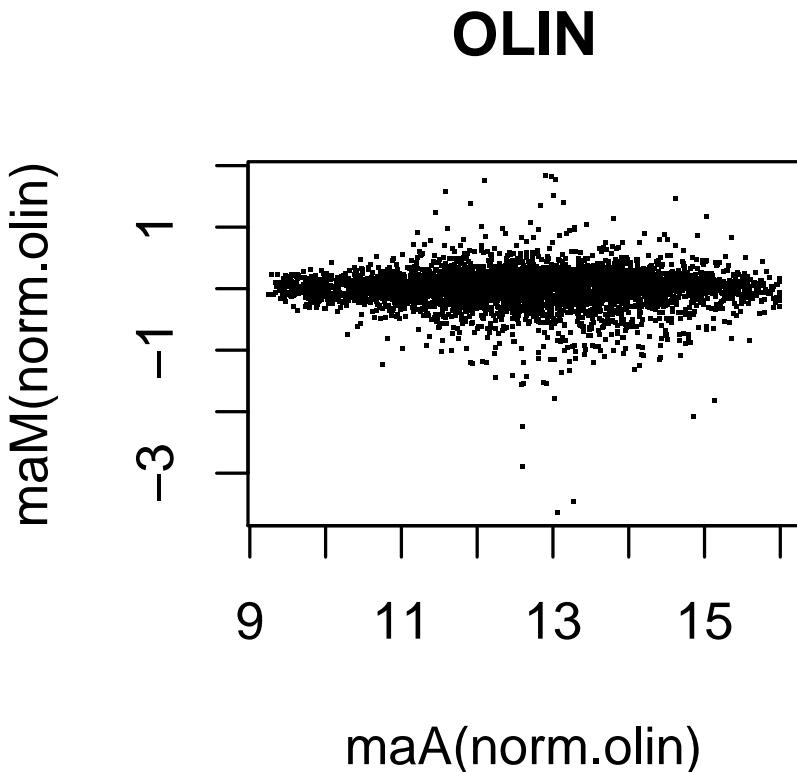


Figure 6: MA-plot of array normalized by OLIN

Inspection of the MA- and MXY-plot indicated that OLIN was able to correct for the intensity- as well as the location-dependent dye bias. The residuals are well balanced around zero in the MA-plot (figure 6). Similarly, spatial bias is no longer apparent (figure 7). Spots with positive and negative log ratio M were evenly distributed across the slide. The statistical tests applied in the next section will confirm these findings.

```
> plot(maA(norm.oln), maM(norm.oln), main = "OLIN", pch = ".")
> mxy.plot(maM(norm.oln), Ngc = maNgc(norm.oln), Ngr = maNgr(norm.oln),
+           Nsc = maNsc(norm.oln), Nsr = maNsr(norm.oln), main = "OLIN")
```

OLIN is based on an iterative procedure. In most cases, we found that two or three iterations are already sufficient for normalisation (figure 8).

```
> norm.oln.1 <- olin(olin.sw[, 3], X = sw.xy$X[, 3], Y = sw.xy$Y[, 
+   3], iter = 1)
> norm.oln.2 <- olin(norm.oln.1, X = sw.xy$X[, 3], Y = sw.xy$Y[, 
+   3], iter = 1)
> norm.oln.3 <- olin(norm.oln.2, X = sw.xy$X[, 3], Y = sw.xy$Y[,
```

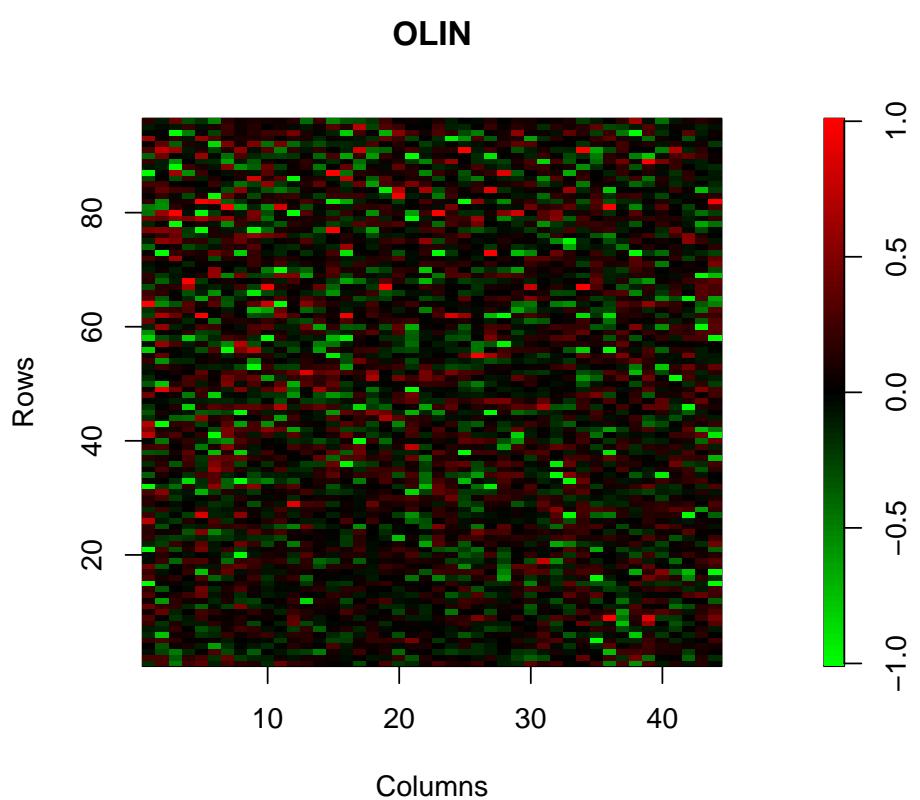


Figure 7: MXY-plot of array normalized by OLIN

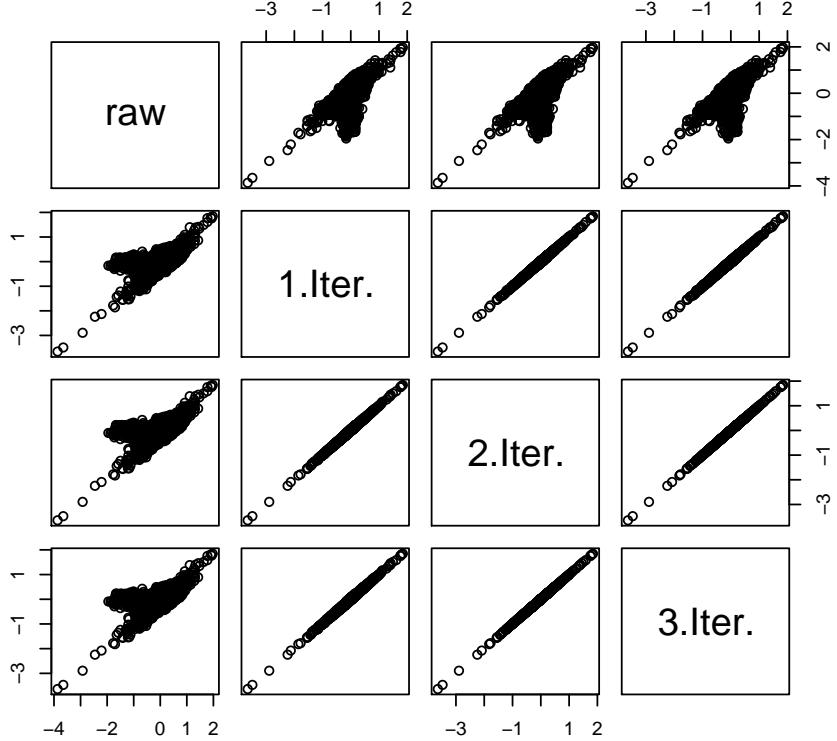


Figure 8: Convergence of iterative normalisation

```

+      3], iter = 1)
> M <- cbind(maM(sw)[, 3], maM(norm.olin.1), maM(norm.olin.2),
+      maM(norm.olin.3))
> pairs(M, labels = c("raw", "1.Iter.", "2.Iter.", "3.Iter."))

```

## 4.2 OSLIN

If we can assume that the variability of log ratios  $M$  should be equal across the array, local scaling of  $M$  can be performed. As in the previous section, the validity of these assumptions has to be carefully checked for each experiment analyzed. The underlying requirement is again random spotting of arrayed genes. To apply OSLIN:

```

> norm.oslin <- olin(sw[, 3], X = sw.xy$X[, 3], Y = sw.xy$Y[, 3],
+      alpha = c(0.1, 1, 0.1), scaling = TRUE)

```

The local scaling factors are derived by optimized local regression of the absolute log ratio  $M$ . The range of regression parameters tested by GCV is [0.1,1] for smoothing parameter. The resulting MA- and MXY-plots for slide 3 are presented in figures 9 and 10.. The variability of log ratios  $M$  appears to be even across the array.

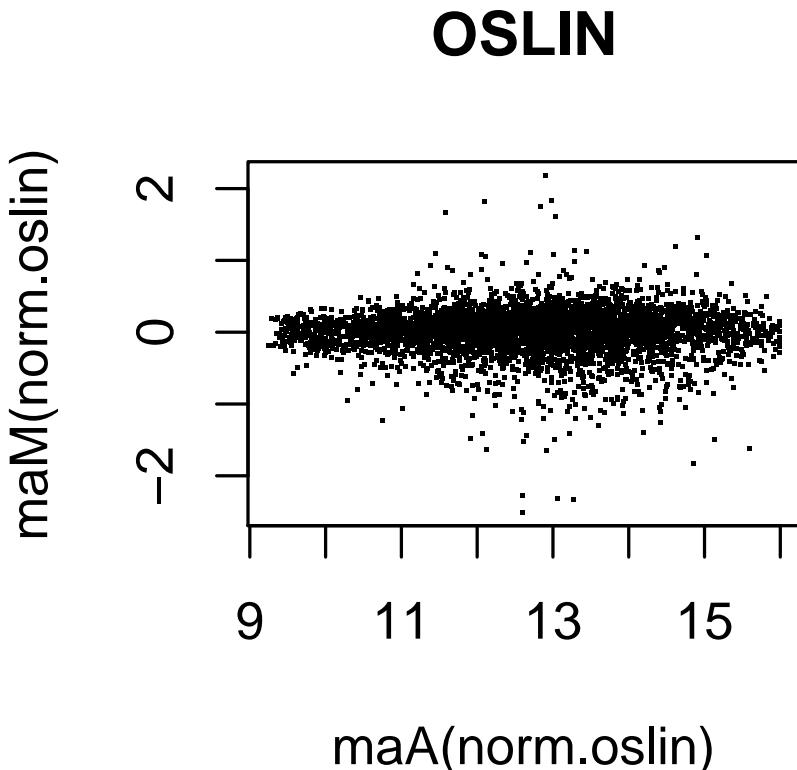


Figure 9: MA-plot of array normalized by OSLIN

```
> plot(maA(norm.oslin), maM(norm.oslin), main = "OSLIN", pch = ".")
> mxy.plot(maM(norm.oslin), Ngc = maNgc(norm.oslin), Ngr = maNgr(norm.oslin),
+   Nsc = maNsc(norm.oslin), Nsr = maNsr(norm.oslin), main = "OSLIN")
```

Note that OLIN and OSLIN are sensitive to violations of the assumptions that most genes are not differentially expressed (or up- and down-regulation is balanced) and that genes are randomly spotted across the array. If these assumptions are not valid, local regression can lead to an underestimation of differential expression. In this context, OSLIN is especially sensitive in its performance. However, the sensitivity can be decreased if the minimal smoothing parameter `alpha` (default value: 0.05) is set to larger values.

It is also important to note that OLIN/OSLIN is fairly efficient in removing intensity- and spatial-dependent dye bias, so that normalised data will look quite "good" after normalisation independently of the true underlying data quality. Normalisation by local regression assumes smoothness of bias. Therefore, localised artifacts such as scratches, edge effects or bubbles should be avoided. Spots of these areas should be flagged (before normalization is applied) to ensure data integrity. To stringently detect artifacts, the OLIN functions `fdr.int`, `fdr.spatial`, `p.int` and `p.spatial` can be used.

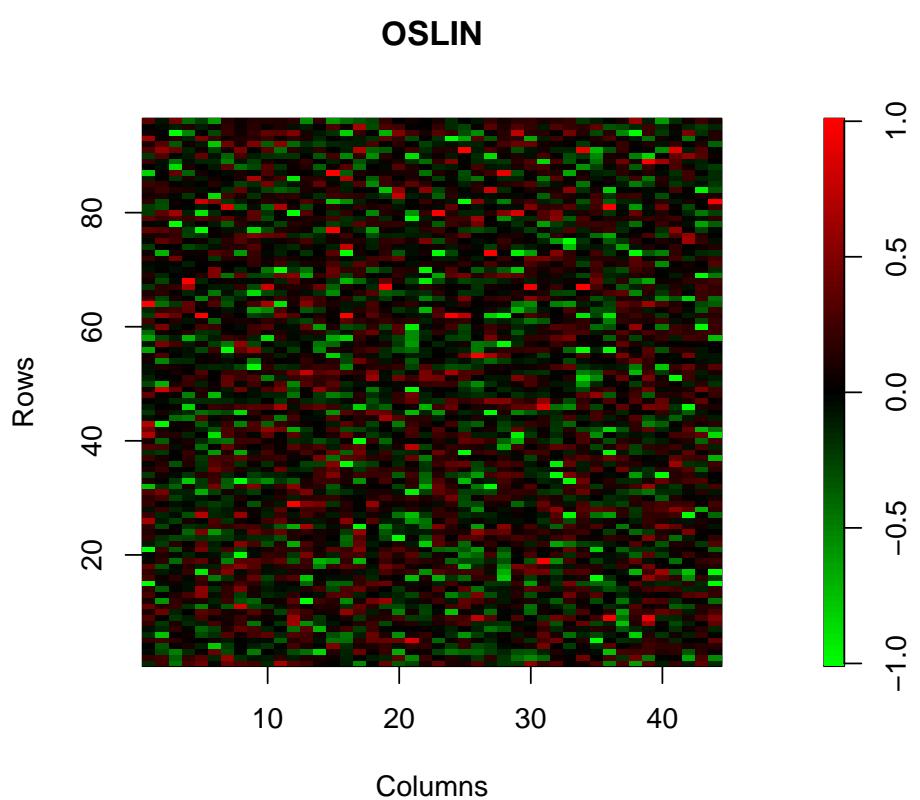


Figure 10: MXY-plot of array normalized by OSLIN

## 5 Statistical assessment of efficiency of normalisation

An important criterion for the quality of normalisation is its efficiency in removing systematic errors. Although visual inspection might readily reveal prominent artifacts in the microarray data (as shown in section 3), it does not allow for their stringent detection. To overcome this limitation, several methods for statistical detection of systematic errors were implemented in the OLIN package. They might be especially valuable for the comparison of the efficiency of different normalisation methods. They also might be helpful for newcomers in the field of microarray data analysis, since they can assist the detection of artifacts and may help to improve the experimental procedures.

A simple method to detect intensity- or location-dependent bias is to calculate the correlation between the log ratio  $M$  of a spot and the average  $M$  in the spot's neighbourhood [5]. A neighbourhood on the intensity scale can be defined by a symmetrical window of size ( $2 * \text{delta} + 1$ ) around the spot. A correlation of zero can be expected assuming the log ratios are uncorrelated. In contrast, a large positive correlation indicates intensity-dependent bias.

```
> A <- maA(sw[, 3])
> M <- maM(sw[, 3])
> Mav <- ma.vector(A, M, av = "median", delta = 50)
> cor(Mav, M, use = "pairwise.complete.obs")

[,1]
[1,] 0.6416701
```

Similarly, the location-dependent bias can be assessed:

```
> MM <- v2m(maM(sw)[, 3], Ngc = maNgc(sw), Ngr = maNgr(sw), Nsc = maNsc(sw),
+   Nsr = maNsr(sw), visu = FALSE)
> MMav <- ma.matrix(MM, av = "median", delta = 2, edgeNA = FALSE)
> Mav <- m2v(MMav, Ngc = maNgc(sw), Ngr = maNgr(sw), Nsc = maNsc(sw),
+   Nsr = maNsr(sw), visu = FALSE)
> cor(Mav, M, use = "pairwise.complete.obs")

[,1]
[1,] 0.4377952
```

Although correlation analysis can be readily applied, it cannot deliver localization of experimental bias in the data. To detect areas of bias in the microarray data, alternative methods can be applied.

The OLIN package contains two ANOVA models. The first model (`anovaint`) can be used to assess intensity-dependent bias. For this task, the A-scale is divided into  $N$  intervals containing equal number of spots. The null hypothesis tested is the equality of the means of  $M$  for the different intervals. The input argument `index` indicates which array stored in object `sw` should be examined. The function `anovaint` is a wrapper around the core function `lm`. The output of `anovaint` equals `summery(lm)`.

```
> print(anovaint(sw, index = 3, N = 10))
```

```

Call:
lm(formula = Mo ~ intensityint - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-4.06150 -0.18971  0.01900  0.23877  1.77781 

Coefficients:
            Estimate Std. Error t value Pr(>|t|)    
intensityint1 -0.86456   0.01856 -46.578 < 2e-16 ***
intensityint2 -0.11228   0.01856 -6.049 1.58e-09 ***
intensityint3  0.01868   0.01856  1.006 0.314355  
intensityint4  0.06458   0.01856  3.479 0.000508 *** 
intensityint5  0.16762   0.01856  9.030 < 2e-16 ***
intensityint6  0.19606   0.01856 10.563 < 2e-16 ***
intensityint7  0.25546   0.01856 13.763 < 2e-16 ***
intensityint8  0.21835   0.01856 11.763 < 2e-16 ***
intensityint9  0.24910   0.01856 13.420 < 2e-16 ***
intensityint10 0.21253   0.01869 11.369 < 2e-16 *** 
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

```

Residual standard error: 0.3818 on 4214 degrees of freedom  
Multiple R-Squared: 0.4198,           Adjusted R-squared: 0.4185  
F-statistic: 304.9 on 10 and 4214 DF, p-value: < 2.2e-16

```

> data(sw.olm)
> print(anovaint(sw.olm, index = 3, N = 10))

```

```

Call:
lm(formula = Mo ~ intensityint - 1)

```

```

Residuals:
    Min      1Q  Median      3Q     Max 
-3.52250 -0.12509  0.02538  0.16351  1.85153 

Coefficients:
            Estimate Std. Error t value Pr(>|t|)    
intensityint1 -0.003941   0.015189 -0.259   0.795  
intensityint2  0.004405   0.015189  0.290   0.772  
intensityint3  0.006523   0.015189  0.429   0.668  
intensityint4  0.010283   0.015189  0.677   0.498  
intensityint5  0.003360   0.015189  0.221   0.825  
intensityint6 -0.004773   0.015189 -0.314   0.753  
intensityint7 -0.011006   0.015189 -0.725   0.469  
intensityint8 -0.004075   0.015189 -0.268   0.789  
intensityint9  0.005160   0.015189  0.340   0.734  

```

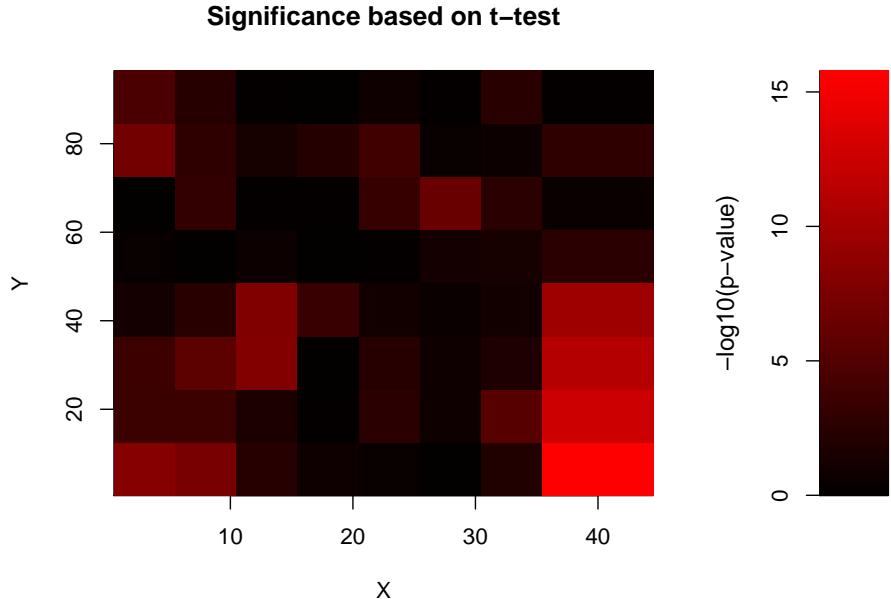


Figure 11: ANOVA for raw data

```
intensity int10 -0.008787 0.015298 -0.574 0.566
```

```
Residual standard error: 0.3124 on 4214 degrees of freedom
Multiple R-Squared: 0.0004707, Adjusted R-squared: -0.001901
F-statistic: 0.1984 on 10 and 4214 DF, p-value: 0.9964
```

Similarly, the location-dependent bias can be examined by an ANOVA model implemented as `anovaspacial`. The array is divided into  $(xN \times yN)$  rectangular blocks. The null hypothesis tested is the equality of the means of  $M$  for the different blocks. The function `anovaspacial` is a wrapper around `functipn lm`. The output is the summary of `lm` (which is suppressed in following examples). Additionally, `anovaspacial` allows for visualisation of the results (see figure 11 and 12.) The figures display the log10-transformed p-values as derived in the block-wise  $t$ -tests. Note that the differences in scales.

```
> anovaspacial(sw, index = 3, xN = 8, yN = 8, visu = TRUE)
> anovaspacial(sw.olm, index = 3, xN = 8, yN = 8, visu = TRUE)
```

ANOVA methods assume normality of the analysed data. This, however, may not be the general case for microarray data. To relax this restriction, permutation tests can be applied. Permutation (or randomization) tests have the advantage that a particular data distribution is not assumed. They rely solely on the observed data examples and can be applied with a variety of test statistics. A major restriction, however, is that permutation tests are computationally very intensive. Generally, such tests are not used in interactive mode but are performed in batch-mode.

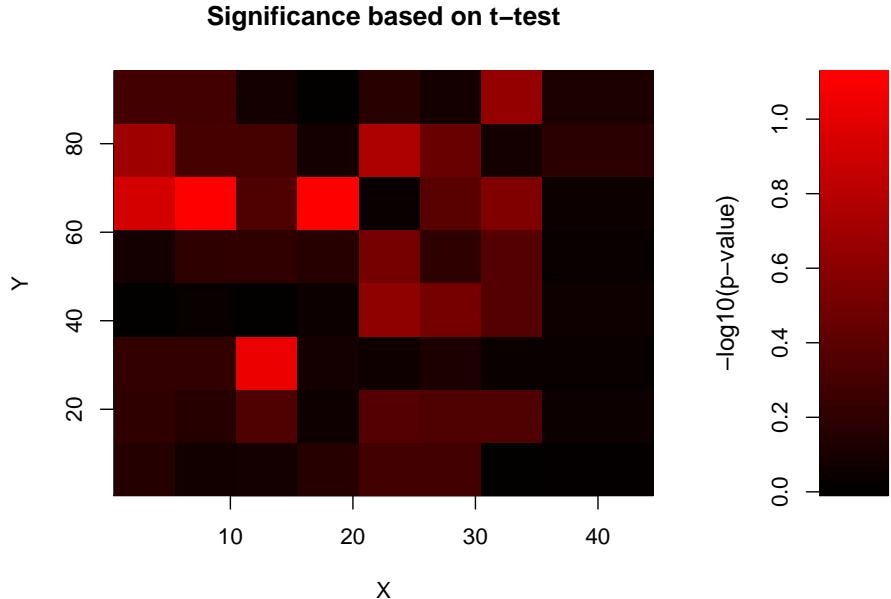


Figure 12: ANOVA for normalised data

Four permutation tests procedures were implemented in the OLIN package. The functions `fdr.int` and `p.int` assess intensity-dependent bias. The functions `fdr.spatial` and `p.spatial` assess location-dependent bias. The basic procedure is similar for all four functions. First, a (intensity or location) neighbourhood of spots is defined similarly to the procedure we used for the correlation analysis . Next, a test statistic is constructed by calculating the *median* or *mean* of  $M$  the spot's neighbourhood of chosen size. An empirical distribution of the test statistic  $\bar{M}$  is then produced based on random permutations of the data. Comparing  $\bar{M}$  of the original data with the empirical distribution, the significance of observing  $\bar{M}$  is derived. (Note that a rather low number of random permutations was chosen to avoid time-consuming calculations here. Generally, however, a larger number should be chosen.)

The functions `fdr.int`, `p.int`, `fdr.spatial` and `p.spatial` perform two one-sided random permutation tests. The result can be visualised by the plotting functions `sigint.plot` and `sigxy.plot`. The significance of a spot neighbourhood with large positive deviations of  $\bar{M}$  is displayed in red along the A-scale or across the spatial dimensions of the array. Correspondingly, the significance of spot neighbourhood with large negative deviations of  $\bar{M}$  are displayed in green.

```
> FDR <- fdr.int(maA(sw)[, 3], maM(sw)[, 3], delta = 50, N = 10,
+      av = "median")
> sigint.plot(maA(sw)[, 3], maM(sw)[, 3], FDR$FDRp, FDR$FDRn, c(-5,
+      -5))

> data(sw.olin)
> FDR <- fdr.int(maA(sw.olin)[, 3], maM(sw.olin)[, 3], delta = 50,
```

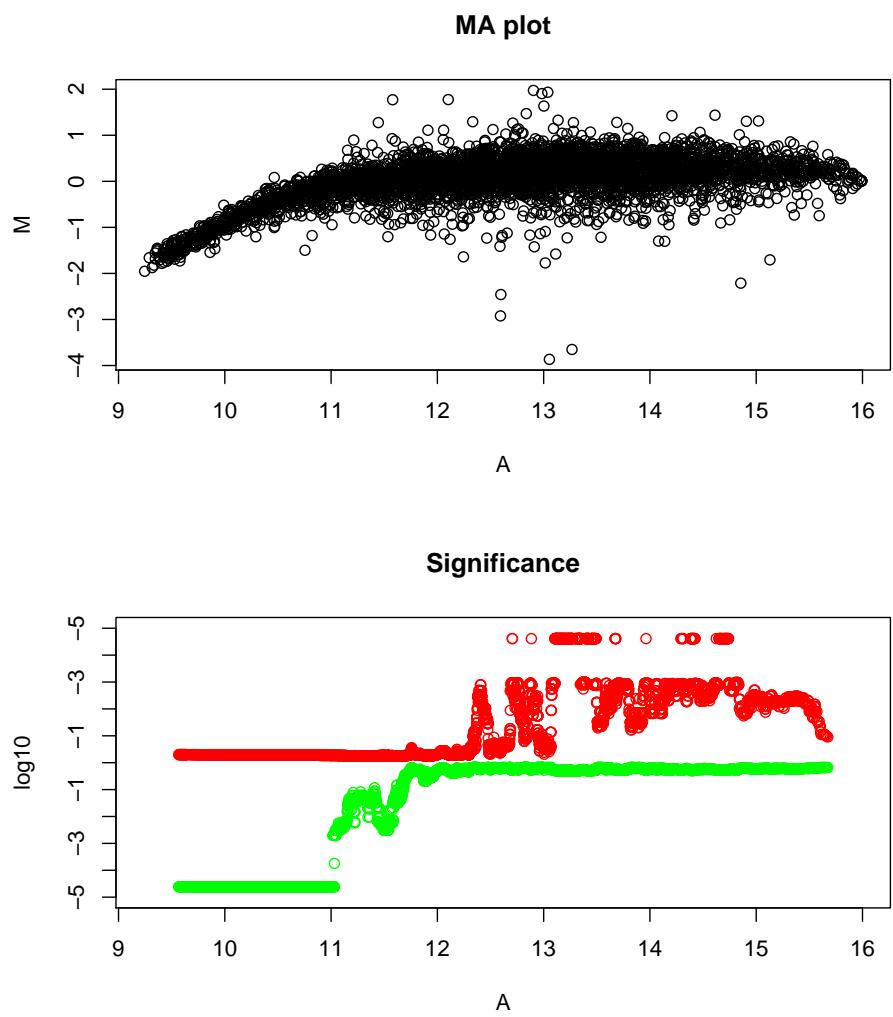


Figure 13: MA-plot and FDRs of intensity-dependent bias for raw data

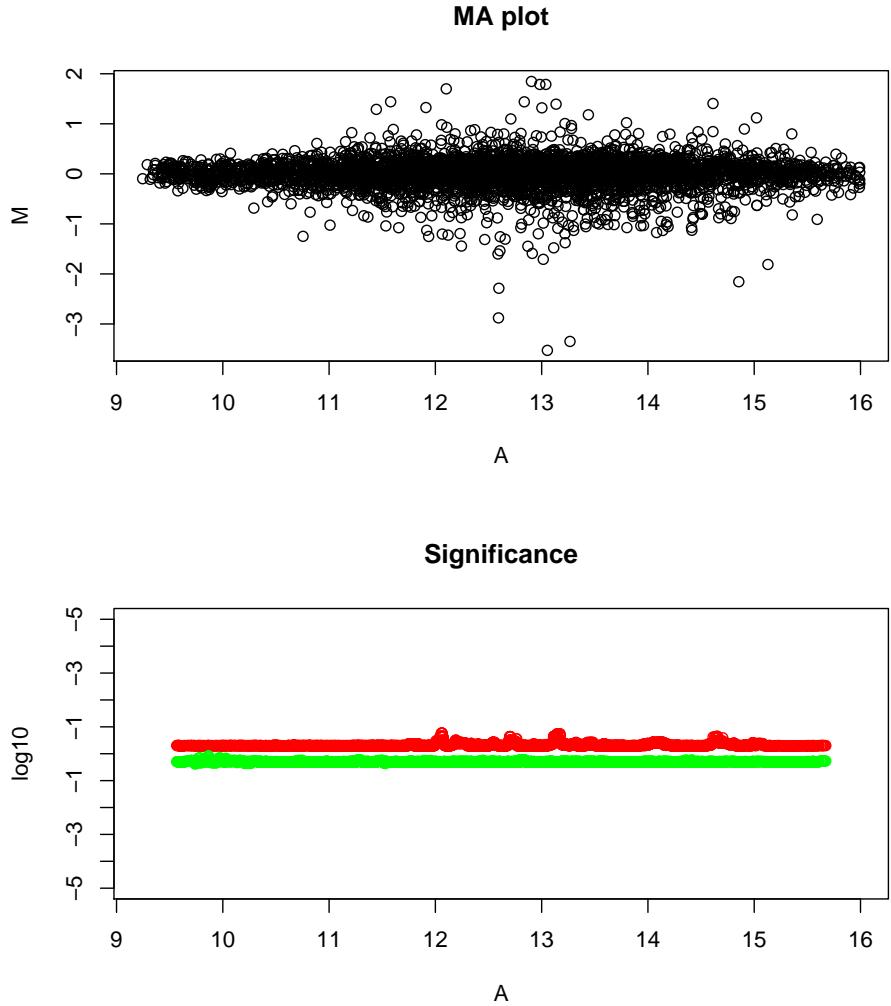


Figure 14: MA-plot and FDRs of intensity-dependent bias for data normalised by OLIN

```
+      N = 10, av = "median")
> sigint.plot(maA(sw.olm)[, 3], maM(sw.olm)[, 3], FDR$FDRp, FDR$FDRn,
+      c(-5, -5))
```

For slide 3, a significant intensity-dependent bias towards channel 2 (Cy3) was detected for low spot intensities, whereas high-intensity spots are biased towards channel 2 (figure 13). After OLIN normalisation, no significant intensity-dependent bias is apparent (figure 14). A similar result can be found for the removal of location-dependent bias by OLIN (figure 15 and 16).

```
> M <- v2m(maM(sw)[, 3], Ngc = maNgc(sw), Ngr = maNgr(sw), Nsc = maNsc(sw),
+      Nsr = maNsr(sw), main = "MXY plot of SW-array 1")
> FDR <- fdr.spatial(M, delta = 2, N = 10, av = "median", edgeNA = TRUE)
> sigxy.plot(FDR$FDRp, FDR$FDRn, color.lim = c(-5, 5), main = "FDR")
```

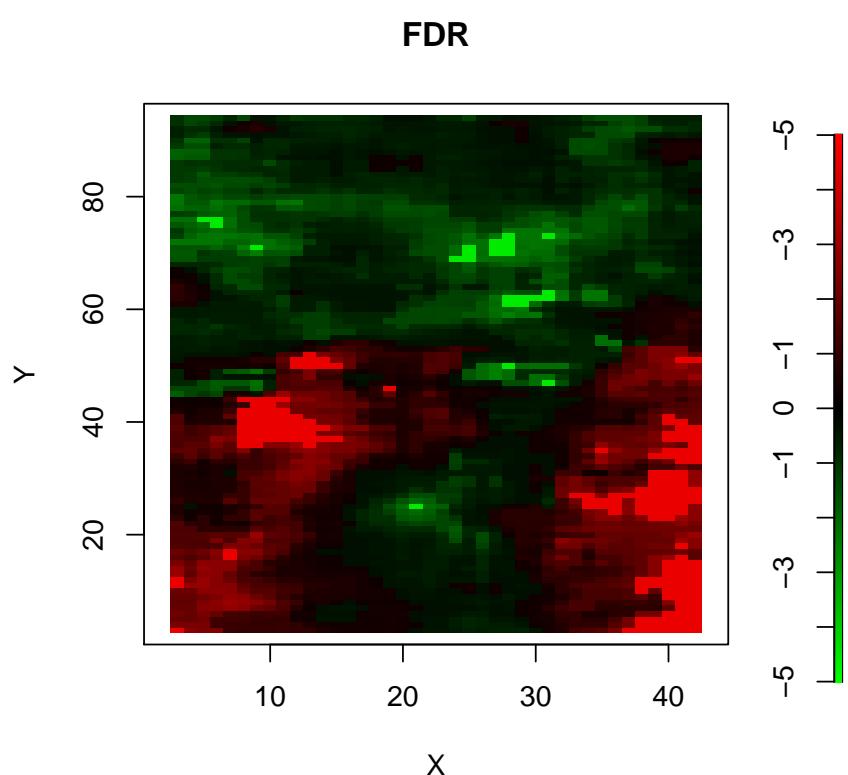


Figure 15: FDR of location-dependent bias for raw data

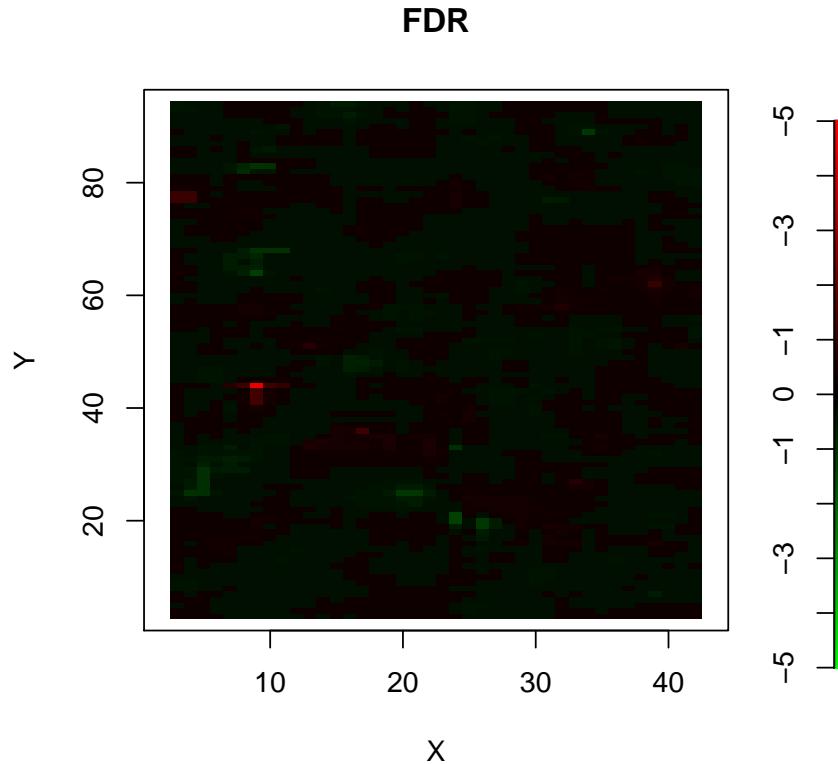


Figure 16: FDR of location-dependent bias for data normalised by OLIN

```
> M <- v2m(maM(sw.olin)[, 3], Ngc = maNgc(sw.olin), Ngr = maNgr(sw.olin),
+   Nsc = maNsc(sw.olin), Nsr = maNsr(sw.olin), main = "MXY plot of SW-array 1")
> FDR <- fdr.spatial(M, delta = 2, N = 10, av = "median", edgeNA = TRUE)
> sigxy.plot(FDR$FDRp, FDR$FDRn, color.lim = c(-5, 5), main = "FDR")
```

Fold changes should be treated with care if the corresponding spots have significantly biased neighborhoods even after normalisation.

The functions `fdr.int` and `fdr.spatial` assess the significance of dye bias using the false discovery rate whereas the related functions `p.int` and `p.spatial` produce (adjusted) p-values. Another difference between the functions is the procedure to generate random permutation. The function `fdr.int` and `fdr.spatial` are based on re-sampling without replacement, whereas `p.int` and `p.spatial` are based on re-sampling with replacement. A more detailed description of the permutation tests can be found in the *help*-pages of these functions.

## References

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