Analysis of Bead Level Data using beadarray

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Introduction

beadarray is a Bioconductor package for the analysis of expression data dervied using the Illumina BeadArray platform. The package is able to analyse data generated by Illumina's BeadStudio software as well as the raw data created when arrays are scanned.

In this document we will describe how to read raw Bead Level data from a BeadArray experiment. Due to the large files generated by a BeadArray experiment, we are not currently able to offer any example data for download. Also, reading raw data into memory requires at least 1Gb of RAM at the present time.

1 Citing beadarray

If you use *beadarray* for the analysis or pre-processing of BeadArray data please cite:

Dunning M, Smith M, Thorne NP, Tavaré, *beadarray: An R package to Analyse Illumina BeadArrays*, R News, submitted

2 Import

The following code shows how to read the raw data from a BeadChip into R. On this Chip we have 6 arrays where each array is made up 2 strips on the Chip surface. The raw data consists of a tif image scanned from each strip and an csv file which describes the position and identity of each bead on each strip. These csv files are required because of the random nature of BeadArrays which means we cannot rely on each position on the array having the same probe sequence attached. The tif images and csv files are produced by Illumina's BeadScan software. To produce the csv files, BeadScan version 3.1 is required. For more details see

http://www.damtp.cam.ac.uk/user/npt22

The function `readBeadLevelData` implements the image processing steps used by Illumina. However, both the sharpening and background correction steps are optional. We estimate a background for each bead by taking the average of the 5 dimmest pixels in a local area around each bead centre. However, we do not subtract this value automatically.

A targets file is required in order to define the location of the csv and tiff images. We also specify an ID for each array and the sample on that array. Note that `readBeadLevelData` can be used on data generated using either the SAM (96 well plates) or BeadChip technologies. The only difference is the way in which the targets file is defined. In the following example we are reading data from a BeadChip which consists of 12 strips with 24,000 genes on each. There are 6 samples on the chip with each 2 strips comprising each array. The 2 strips for each array have a different set of bead types attached and can therefore be analysed separately at this stage.

In this example data set we have three different samples, three samples supplied by Illumina (I), three tumour samples (P) and three normals (Norm). This BeadChip is part of the same example set

supplied in the BeadSummaryExamples zip file and described in the the Analysis of Bead Summary Data using beadarray vignette.

```
> library(beadarray)
> targets <- readBeadTargets("targets.txt")
> targets
```

```
          ArrayID                 Image1            xyInfo1 SampleID
1  1475542113_A_1 1475542113_A_1_Grn.tif 1475542113_A_1.csv       IC
2  1475542113_A_2 1475542113_A_2_Grn.tif 1475542113_A_2.csv       IC
3  1475542113_B_1 1475542113_B_1_Grn.tif 1475542113_B_1.csv       IH
4  1475542113_B_2 1475542113_B_2_Grn.tif 1475542113_B_2.csv       IH
5  1475542113_C_1 1475542113_C_1_Grn.tif 1475542113_C_1.csv       IC
6  1475542113_C_2 1475542113_C_2_Grn.tif 1475542113_C_2.csv       IC
7  1475542113_D_1 1475542113_D_1_Grn.tif 1475542113_D_1.csv        P
8  1475542113_D_2 1475542113_D_2_Grn.tif 1475542113_D_2.csv        P
9  1475542113_E_1 1475542113_E_1_Grn.tif 1475542113_E_1.csv        P
10 1475542113_E_2 1475542113_E_2_Grn.tif 1475542113_E_2.csv        P
11 1475542113_F_1 1475542113_F_1_Grn.tif 1475542113_F_1.csv     Norm
12 1475542113_F_2 1475542113_F_2_Grn.tif 1475542113_F_2.csv     Norm
```

```
> BLData <- readBeadImages(targets[1:4, ])
```

```
Reading pixels of 1475542113_A_1_Grn.tif
Calculating background
Calculating foregound
Reading pixels of 1475542113_A_2_Grn.tif
Calculating background
Calculating foregound
Reading pixels of 1475542113_B_1_Grn.tif
Calculating background
Calculating foregound
Reading pixels of 1475542113_B_2_Grn.tif
Calculating background
Calculating foregound
```

3 The BLData object

BLData is a type of list object and similar to the *RGList* objects found in limma. The list contains a number of matrices which hold information for each bead over all strips on the BeadChip. Note that due to random placement of beads each row will not always correspond to the same gene, unlike the *RGList* objects of limma, therefore we have to store ProbeID which gives the identity of each bead.

The slots in BLData can be subset in the usual way.

```
> is(BLData)
```

```
[1] "BeadLevelList"
```

```
> class(BLData)
```

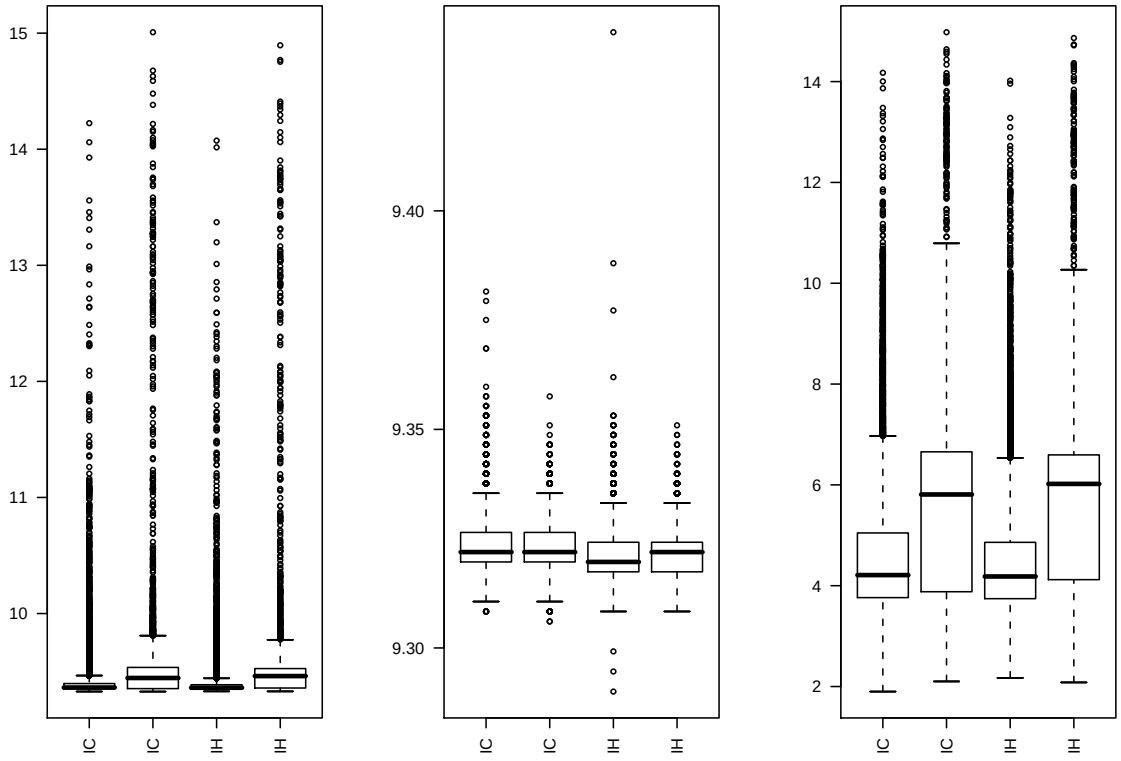
```
[1] "BeadLevelList"
attr(,"package")
[1] "beadarray"
```

```
> slotNames(BLData)

[1] "G"       "Gb"      "GrnY"    "GrnX"    "ProbeID" "targets"

> dim(BLData@G)

[1] 1164798       4

> BLData@G[1:5, ]

          [,1]     [,2]     [,3]     [,4]
[1,]  658.8324 649.9646 742.4284 707.2222
[2,] 1138.5864 646.5080 788.2053 703.6667
[3,] 3726.4027 648.9603 654.2679 718.5514
[4,]  904.4705 654.5395 648.0932 693.5556
[5,]  703.1498 727.2521 745.9367 677.7221
```

Boxplots can be used to compare foreground and background intensities between arrays. In this example we can see very little variation between arrays. Notice that the background level appears to be virtually constant both for beads on the same array and between arrays.

Background correction can be performed by the `backgroundCorrect` and the default settings to the function subtract the background estimate for each bead from the foreground. In this example we use the minimum method which stops any negative values from being calculated.

```
> par(mfrow = c(1, 3))
> boxplot(as.data.frame(log2(BLData@G[1:10000, ])), las = 2, names = as.character(BLData@targets[,
+     4]))
> boxplot(as.data.frame(log2(BLData@Gb[1:10000, ])), las = 2, names = as.character(BLData@targets[,
+     4]))
> BLData.bc = backgroundCorrect(BLData, method = "minimum")
> boxplot(as.data.frame(log2(BLData.bc@G[1:10000, ])), las = 2,
+     names = as.character(BLData@targets[, 4]))
```

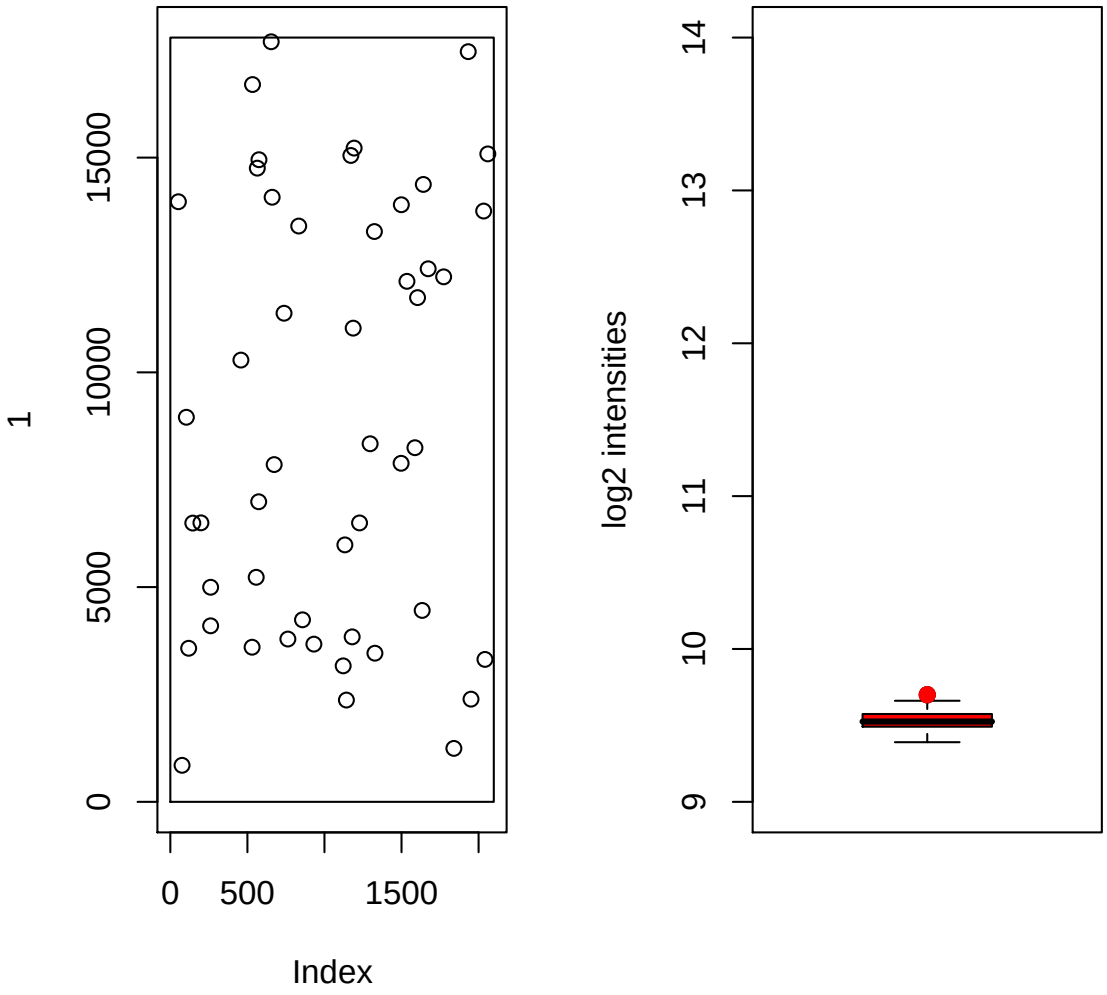


4 Bead Level Analysis

We can plot the position and location of the replicates for a particular bead type using the following code. Each BeadArray is produced using a random sampling mechanism, therefore we would expect the placement of each bead type on an array to be random.

We can also produce boxplots of bead intensities using `plotBeadIntensities`. This function takes a list of ProbeIDs and arrays as arguments and produces a boxplot for each bead type on each array grouping ProbeIDs on the same array together. Any red dots on a boxplot indicate the outliers for the bead type, these are any beads outside a 3 MAD cut-off from the mean for the bead type and are excluded from analysis. Illumina use the unlogged bead intensities for this outlier removal and this is the default option in beadarray.

```
> par(mfrow = c(1, 2))
> plotBeadLocations(BLData, array = 1, ProbeIDs = 50020, SAM = FALSE)
> plotBeadIntensities(BLData, arrays = 1, ProbeIDs = 50020, ylim = range(9,
+     14))
```

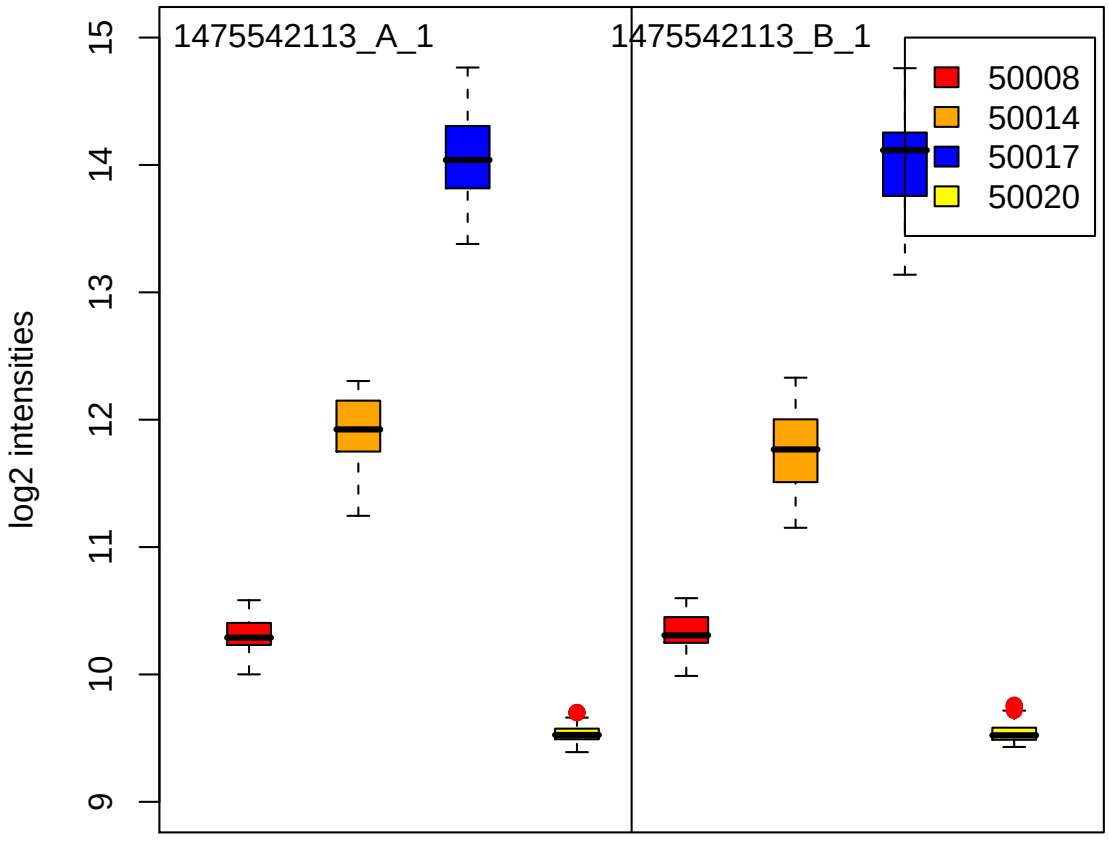


In the following code we show how to plot the intensities of three different bead types on two separate arrays in the experiment. For this particular example we have to remember that all odd-numbered arrays in the experiment contain RefSeq genes whereas the even-numbered arrays contain Supplemental genes, therefore we plot the intensities of the beads on the first and third arrays.

```
> ids = unique(BLData@ProbeID[, 1])[2:5]
> ids
```

```
[1] 50008 50014 50017 50020
```

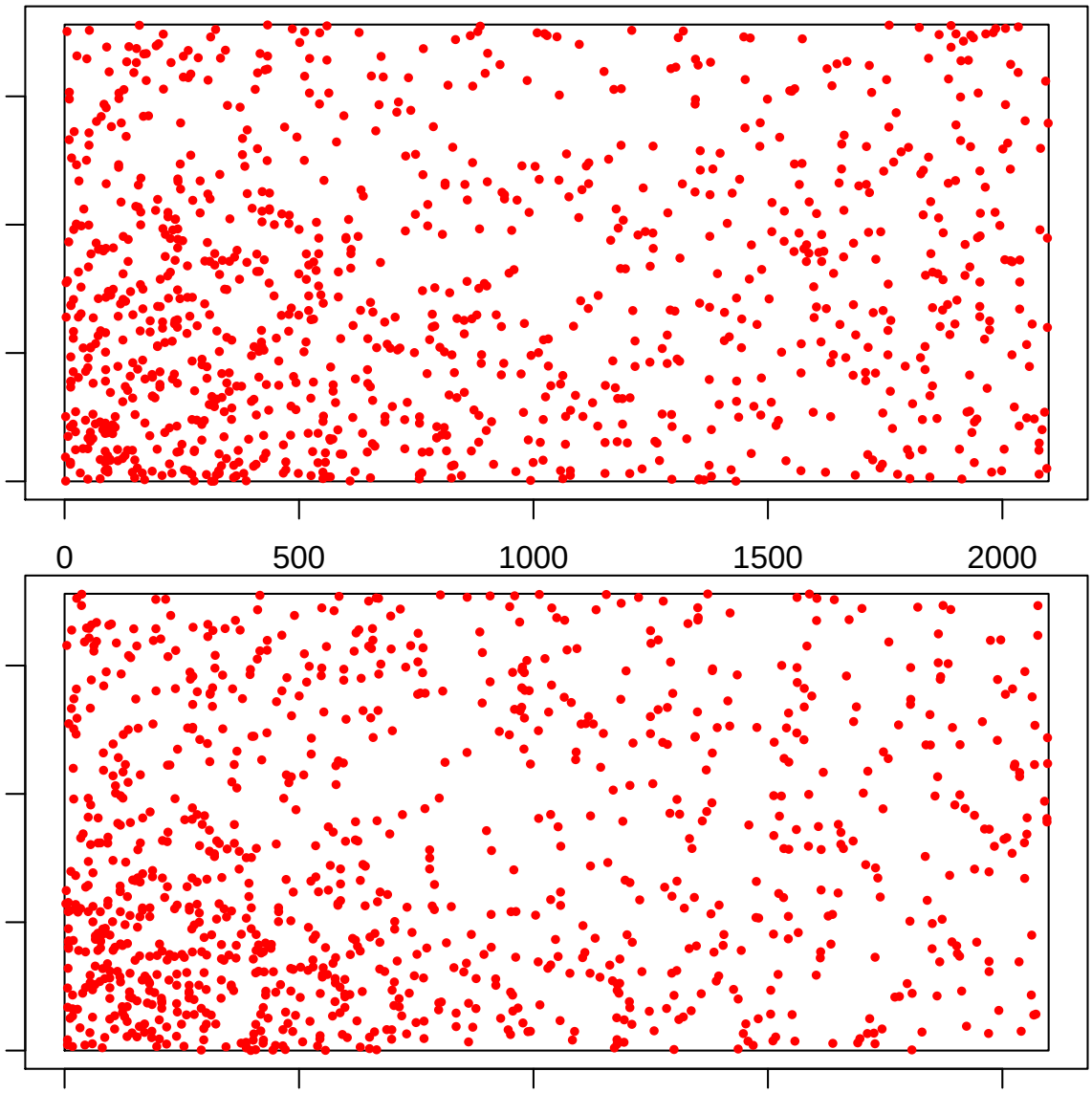
```
> ProbeCols = c("red", "orange", "blue", "yellow")
> plotBeadIntensities(BLData, arrays = c(1, 3), ProbeIDs = ids,
+     ylim = range(9, 15), ProbeCols = ProbeCols)
> legend(6.5, 15, ids, ProbeCols)
> text(1, 15, as.character(targets[1, 1]))
> text(5, 15, as.character(targets[3, 1]))
```



We can repeat the outlier analysis shown above for all bead types on an array using `findAllOutliers`. The result of this function is a list of row indices to `BLData` to identify which beads on the given array are outliers. Typically we find that the number of outliers on an array is less than 10% and both the number and location of outliers can be used as a useful diagnostic tool.

```
> o = findAllOutliers(BLData, array = 1)
> o[1:10]

 [1] 81823 81845 81889 81894 81898 81956 81973 82010 82033 82037

> length(o)/nrow(BLData@G)

[1] 0.03751723

> par(mfrow = c(2, 1))
> par(mar = c(1, 1, 1, 1))
```

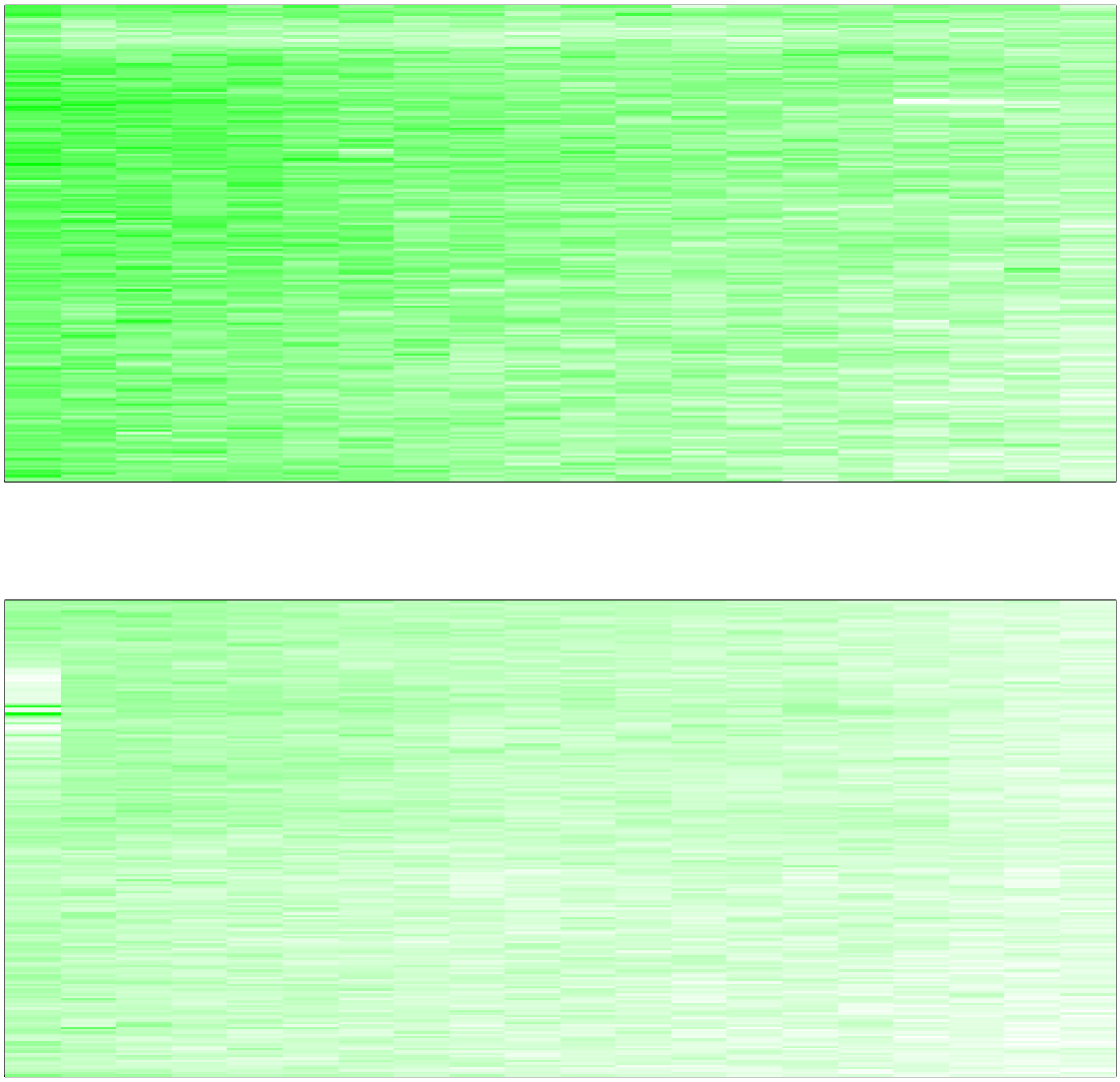
```
> for (i in 1:2) {
+     o = findAllOutliers(BLData, array = i)
+     plotBeadLocations(BLData, BeadIDs = o[1:1000], array = i,
+         SAM = FALSE, cex = 0.5, col = "red", pch = 16)
+ }
```



Arrays with a high proportion of outliers might be explained by areas of an array with unusually high background or foreground. Such regions can also be investigated by using image plots. To produce these plots we divide the array up into rectangles with a defined number of rows of columns. On the plot, the colour of the rectangle is the average of all beads lying inside that rectangle.

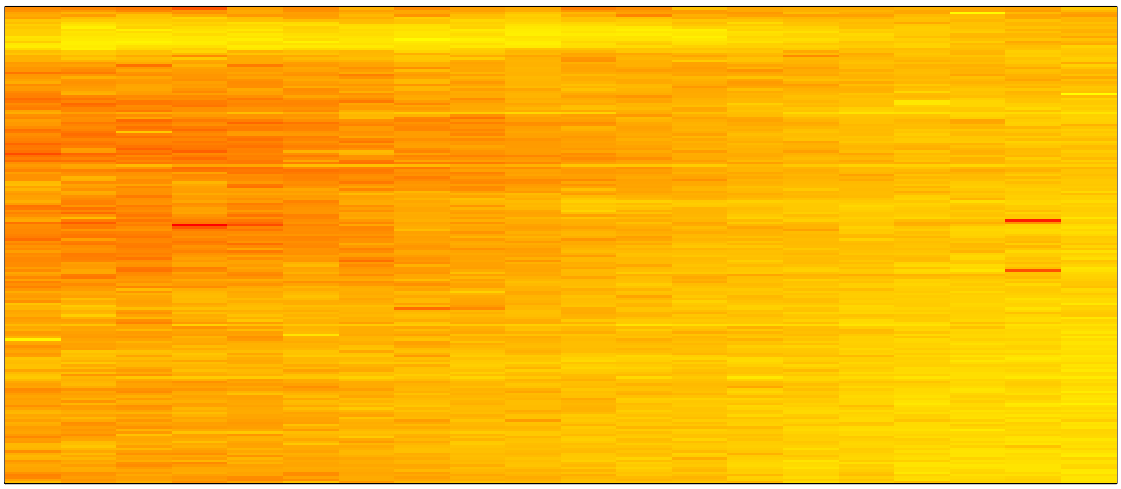
```
> par(mfrow = c(2, 1))
> for (i in 1:2) {
+     imageplot(BLData, array = i, nrow = 200, ncol = 20, zlim = range(9,
+         11))
```

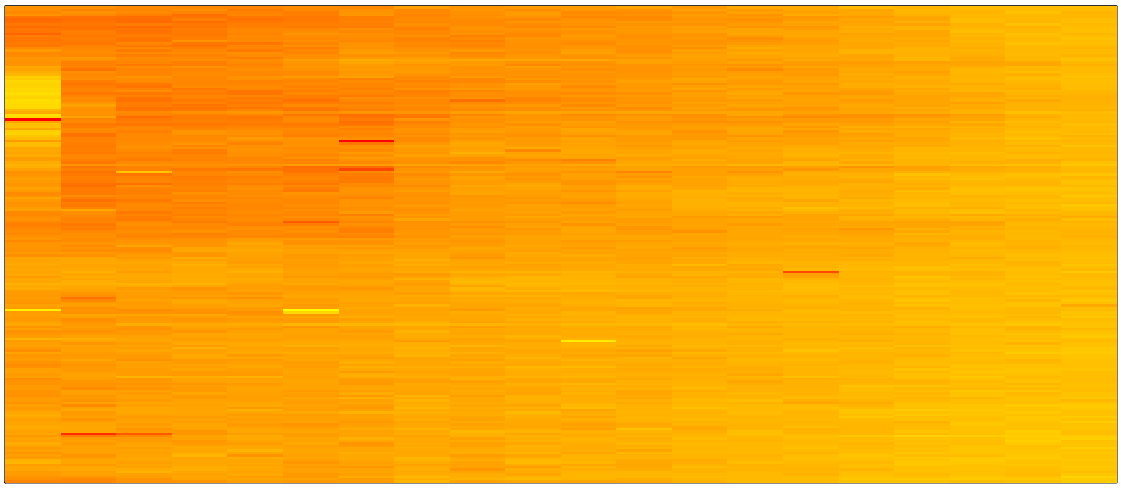
```
+ }
```



The default for options for `BLImagePlot` plot average over the foreground intensities inside each rectangle. By using the `whatToPlot` argument we can also plot background or values contained in each other matrix in `BLData`. The colours used to plot low and high values can be changed by `low` and `high` respectively whereas `zlim` specifies which values to associate with these colours. Setting `zlim` to the same value for a series of plots allows imageplots to be compared more easily.

```
> par(mfrow = c(2, 1))
> for (i in 1:2) {
+     imageplot(BLData, array = i, nrow = 200, ncol = 20, what = "Gb",
+         low = "yellow", high = "red", zlim = range(9, 10))
+ }
```





The `createBeadSummaryData` function can be used to summarise the values for each probe. Unlike Affymetrix technoloy, each replicate of a bead contains the same probe sequence and therefore using an average of the replicates should be valid. Outliers are removed using a cut-off of 3 MADS and the mean of the remaining beads is used as the summary value. At this point we combine the two strips for each array by using the `imagesPerArray` argument, leaving us with 6 columns now instead of 12. The `createBeadSummaryData` function can be used to summarise the values for each probe. Outliers are removed using a cut-off of 3 MADS and the mean of the remaining beads is used as the summary value. At this point we combine the two strips for each array by using the `imagesPerArray` argument, leaving us with 6 columns now instead of 12.

```
> BSData = createBeadSummaryData(BLData, imagesPerArray = 2)
```

The default settings for `createBeadSummaryData` assume that the same probes are to be found on each array in the experiment as this will be true in general. At present, `createBeadSummaryData` is a memory intensive operation and requires at least 1Gb of RAM.

The `BSData` can be analysed using functionality described in the Analysis of Bead Summary Data vignette.

5 Normalisation

As noted previously, BeadArrays are able to produce large data sets which are difficult to handle without large amounts of RAM available. Ideally, for normalisation we would like to the full bead level data to correct for effects between and within arrays. The `BeadLevelNormalise` function combines the data reading, normalisation and probe summary steps into one function. Each array is read in turn, normalised according to some target distribution and then summarised. Therefore one only need to read one array into memory at a time. The target distribution used for normalisation can be defined by the user, otherwise it is calculated to be the mean quantiles across all arrays. Note that quality control steps should be performed prior to normalisation and any defective arrays should be removed from the targets file.

```
> BSData.BLNorm = BeadLevelNormalise(targets, backgroundCorrect = "none")
```