1 Analysis of bead-level BeadArray data

There are two methods for describing the results of a BeadArray experiment. Firstly, we can use *bead-level data* whereby the position and intensity of each individual bead on an array is known. *Bead summary data* can also be used whereby a summary intensity for each bead type on an array is given. The summarised values for a particular bead type can then be compared between different arrays within an experiment.

Whilst the *beadarray* package includes methods for processing data of both kinds, bead summary data is far more widely available at the present time. Data can be data obtained using either the BeadChip (6 or 8 arrays on a slide) or SAM (arrays organised in 96 well plates) technologies. This document uses a SAM experiment as an example although BeadChip can be read in the same manner.

The purpose of this document is to give an outline of the R functions for analysing bead-level data. Descriptions of how to read and analyse bead summary data are provided in a separate Vignette. Those who are familiar with the R statistical language, and in particular the *limma* package, should be able to adapt easily to our new methods of analysis. Wherever possible we used objects that are similar to those used by *limma*. Example files to read bead-level data are provided at

`http://www.damtp.cam.ac.uk/user/jcm68/beadarray.html`

See References for further reading on BeadArray technology. In particular, the paper by Dunning et al *Quality Control and Low-level Statistical Analysis of Illumina Beadarrays*, Revstat **4**, 1-30, describes the analysis of bead-level data in more detail than this Vignette.

1.1 Reading bead-level data

There are two sets of files that are required by our package in order to create bead-level data.

- TIFF images - These are the raw images scanned directly from each individual array on a 96-well SAM. These are provided by Illumina.
- csv files - These define the location and bead type of each individual bead on a particular array on a 96-well SAM.

Before these files can be read into R, we first convert the TIFF files into PGM files. This can be done be using the *ImageMagick* utility [1]. A batch file is included with this library to convert automatically all the TIFF files in a directory.

If the correct csv and pgm files are available we can read these data into R using `readBeadImages`. This function requires a `beadTargets` object that can be read directly from a beadTargets.txt file. This `beadTargets` object specifies the filename of each image and csv file to be read.

In our example dataset we have two single-channel BeadArray hybridisations. Since the arrays are hybridised with one target only (one-colour), we need only to specify one image file for each array. The beadTargets.txt file, pgm and csv files

[1] available from `www.imagemagick.org` - version 6.2.2 or later is required.

are provided at `http://www.damtp.cam.ac.uk/user/jcm68/beadarray.html`. Once downloaded, these files can be read into R. The R working directory can be set to the folder containing these files using the `setwd` function or the file menu (GUI implementation only). Alternatively the *path* argument in `readBeadImages` can be set to the current directory. The commands to read the data into R are then as follows:

```
> library(beadarray)
> beadTargets = readBeadTargets()
> beadTargets
```

```
                     Image1                xyInfo SAMPLE
1 1269941_R001_C001_Grn.pgm 1269941_R001_C001.csv      6
2 1269941_R001_C002_Grn.pgm 1269941_R001_C002.csv      6
```

```
> BLData = readBeadImages(beadTargets)
```

```
Calculating foreground intensities for 1269941_R001_C001_Grn.pgm
Calculating background intensities.
Calculating foreground intensities for 1269941_R001_C002_Grn.pgm
Calculating background intensities.
```

The default setting for `readBeadImages` is to recreate the foreground and background intensities for each bead in the same way in which they are calculated by Illumina. However, the use of sharpening and local background correction are optional (see Dunning et al for description of sharpening and local background correction). To create unsharpened bead intensities one would use:

```
> BLData.ns = readBeadImages(beadTargets, sharpen = FALSE)
```

```
Calculating foreground intensities for 1269941_R001_C001_Grn.pgm
Calculating background intensities.
Calculating foreground intensities for 1269941_R001_C002_Grn.pgm
Calculating background intensities.
```

Data which does not include the coordinates of bead centres may still be read into the library. It will not be possible to perform any image processing without the bead centre coordinates. Files of this type may not always have the same number of bead present so we have to specify the maximum length of any of the files to be read.

```
> files = dir()
> BLData.nonXY = readNonXYData(files, max_length = 49777)
```

1.2 The BeadLevelList Object

The data object (`BLData`) is in fact a list object but behaves like a complex sort of matrix. It can be subsetted or treated like a matrix in lots of ways. We can use the `names` command to see what items can be found in the list. `BLData` is an *BeadLevelList* object and like the *RGList* object in *limma* can contain `R`, `Rb`, `G` and `Gb` objects (i.e. foreground and background intensities of two colour data).

```
> is(BLData)

[1] "BeadLevelList"   "list"            "LargeDataObject" "vector"

> names(BLData)

 [1] "R"                   "Rb"                  "x"
 [4] "y"                   "ProbeID"             "targets"
 [7] "sharpened"           "backgroundSize"      "normalised"
[10] "backgroundCorrected"
```

Individual items in the list can then be accessed by using the $ operator in R. In our example we have the matrices `R` and `Rb` which are the foreground and background intensities for each bead (row) and each array (column). The example shown here is for a single channel experiment, hence we only have a foreground intensity value in the red channel and the green channel is not used. If we had two channel data then `BLData$R` and `BLData$G` would be the red and green channels respectively. The number of rows in the matrix is the same as the number of beads present on the array and the number of columns is the same as the number of arrays. In this example we only read in two arrays, so we only have two columns. In other words, each column of the matrix represents intensities of all beads on the same array. However, due to the random placement of beads on the array, each row of the matrix does not relate to intensities of a bead of the same type (as one might expect having dealt with conventional microarray data).

Since BeadArray technology uses randomly assembled beads it is important to know the location and identity of every bead on the array. Therefore the `BeadLevelList` we are using in this library also contains the x and y co-ordinates for each bead and an identifier (ProbeID) for the bead type of each bead.

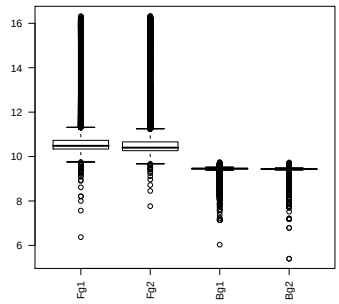
An example *BeadLevelList* is included with the library. However, due to space constraints, the object only contains a single array with 25000 beads on. The object may be loaded at any time with the command.

```
> data(BLData)
```

1.3 Background correction and normalisation

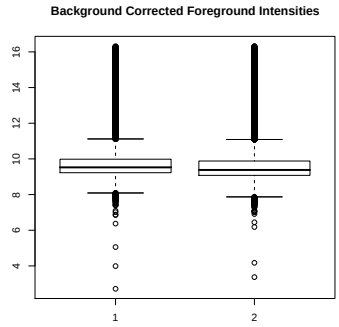
Using the `boxplot` function in R allows boxplots of foreground and background intensities to be compared (see Figure **??**).

```
> boxplot(as.data.frame(cbind(log2(BLData$R), log2(BLData$Rb))),
+     names = c("Fg1", "Fg2", "Bg1", "Bg2"), las = 2)
```



Local background correction can be be performed on the data by:

```
> BLData.c = backgroundCorrectBeads(BLData)
> boxplot(log2(BLData.c$R) ~ col(BLData.c$R), main = "Background Corrected Foreground Intensities")
```



By default, the `backgroundCorrectBeads` function subtracts the values in `BLData$Rb` from `BLData$R` and stores the result in the `R` matrix of the resulting *BeadLevelList* object. Other methods are available such as *minimum* which ensures that no negative values are produced. The only normalisation methods currently supported for bead-level data are median and quantile normalisation. Compatibility with the *limma* and *affy* libraries means that other methods can easily be used.

```
> BLData.med = medianNormalise(BLData)
> BLData.q = quantileNormalise(BLData)
```

1.4 Numbers of beads

We can see which bead types are represented less than 24 times (the 5th percentile for the appropriate Poisson distribution) on the array using `findLowestCounts`. For the first array that we use:

```
> findLowestCounts(BLData, 1)[1:10]
```

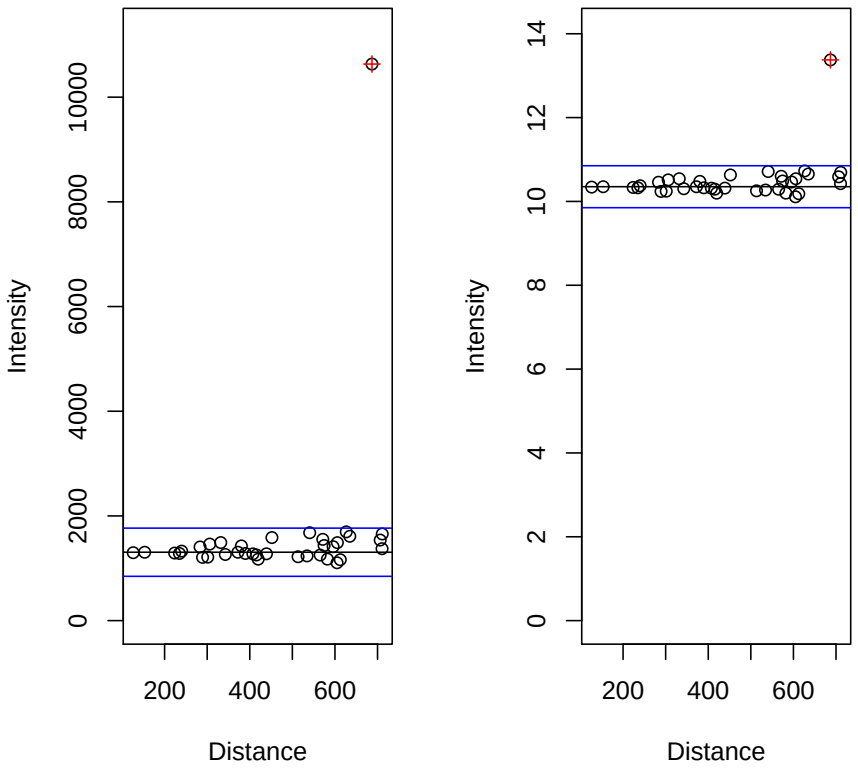
```
 [1]  10  23  30  42  87 119 182 185 585 607
```

The result is a list of ProbeIDs.

1.5 Outliers for each bead type

The `plotBeadIntensities` function can be used to assess variation within a particular bead type. This function shows the intensity of every bead of a particular type against the distance of the bead from the centre of the array. Any outliers which exist for the bead type are marked on the plot by a red cross. As an example we can plot the intensities of all beads with probeID 2 on array 1 and determine outliers using unlogged or $\log_2$ intensities. This function also has the option of changing the number of MADs from the mean used to determine outliers by changing the n parameter.

```
> par(mfrow = c(1, 2))
> plotBeadIntensities(BLData, ProbeIDs = 2, array = 1)
> plotBeadIntensities(BLData, ProbeIDs = 2, array = 1, log = TRUE)
```



We can find all the beads on an array which are outliers for their bead type by using the `findAllOutliers` function. The output of the function is an index which refers to a particular row in the *BeadLevelList* where a bead is located.

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

 [1] 44634  1263  8245   342 46418  6270 31023  2123  9534  4273
```

The length of the list can be easily found (`length`) and compared between different arrays as a diagnostic measure for the quality of the array. Additionally, the location of all the outliers on an array can be plotted using the `plotBeadLocations` function. described below. The function `findBeadStatus` may be used for individual bead types.

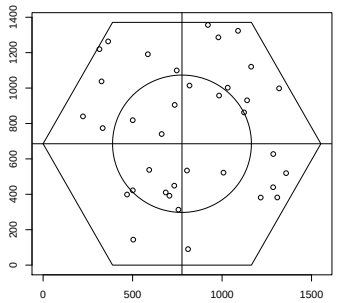
```
> findBeadStatus(BLData, probes = 2, array = 1, outputValid = TRUE)

$outliers
[1] 44634

$valid
 [1]   728  2828  6180  7591  7728  7943  8481  9417 10258 10799 12545 14432
[13] 14892 16443 17017 17261 19282 19885 21691 25684 27546 30345 30707 32370
[25] 33540 33736 35006 38074 39685 39939 40978 43626 43685 44915 45680

$nextStart
[1] 1072
```

Here, the result is a list of beads with ProbeID=2 which are outliers for this bead type, along with beads which are valid beads.

1.6 Spatial plots

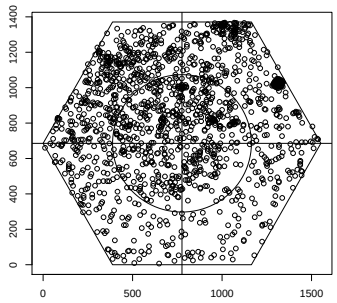
The `plotBeadLocations` function can be used to plot the location of a set of beads on an array. Beads can be specified by ProbeIDs or by a list of row indices as returned by `findBeadStatus`.

```
> plotBeadLocations(BLData, ProbeIDs = 2, array = 1)
```



The function plots all beads on the first array with ProbeID 2. By using the `o` object created above we can also plot the location of all outliers on the first array.

```
> plotBeadLocations(BLData, beadIDs = o, array = 1)
```



The `plotBeadLocations` provides a quick diagnostic check for the distribution of a set of beads. As described in Dunning et al., we have also implemented a χ^2 statistic to quantify the non-randomness of bead distributions. This χ^2 test can be applied to all bead types on an array and the probeID of those with the highest value can be returned by:

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

 [1]  213  278  606  658  791  800  936  960  961 1071
```

Shown above are the ProbeIDs for the first 10 bead types with a χ^2 statistic greater than 14 (chosen because this is the 5th percentile of the appropriate χ^2 distribution). Any of these bead types can be investigated further by using the `plotBeadLocations` function.

Any regions on an array found to have a high proportion of outliers can be investigated further by the `displayTIFFImage` function. See Figure 3 of Dunning et al.

```
> displayTIFFImage(BLData, array = 1, a = 1000:1400, b = 1200:1400)

[1] "1269941_R001_C001_Grn.pgm"
[1] "finding the outliers...."
[1] "<mean 14"
[1] ">mean 80"
[1] "negative intensity 1"
```

The example above loads the original image for array 1 (the name of which is stored in the targets object) and displays the intensities of pixels with x in the range from $1000 : 1400$ and y in the range $1200 : 1400$.

The intensity of every pixel in the plot is represented by a shade of green, with brighter colours indicating a higher value. The blue and red spots indicate the position of outliers in the particular region with blue indicating beads with intensity higher than the mean for that bead type and red being beads with intensity lower than the average for their bead type. Yellow spots on the picture represent beads which have been calculated to have a negative foreground intensity. The black crosses show where the bead centres are located. Any beads which failed the decoding process can also be highlighted by setting the *showUnregistered* parameter.

The plot can also be made interactive by setting the *locateBeads* parameter to TRUE. We can then click on any bead centre and display the foreground and background intensities for this bead as well as a measure of the raw intensity.

We feel that `displayTIFFImage` gives more useful information about the raw images than the equivalent function included in BeadStudio. In BeadStudio, the user can explore the TIFF images and see the intensity of each individual pixel. However, the identity of each bead on the image is not given and there is no information about outliers.

1.7 Creating bead summary data

Bead summary data can be created using the bead level data. In producing these summaries we must first remove outliers for each bead type as described in Dunning et al. This averaging is done by the `createBeadSummaryData` function and the method of detecting outliers can be specified by changing the *log* (for unlogged or logged parameters) and n (number of MADs) parameters.

```
> BSData = createBeadSummaryData(BLData)
```

The structure of the resulting object is described in greater detail in the Vignette *Analysis of bead summary data with beadarray* supplied with *beadarray*.