Analysis of Bead Summary Data using beadarray

October 2, 2006

Introduction

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. The methods available for processing bead level data are discussed in: Dunning,M.J et al, *Quality Control and Low-level Statistical Analysis of Illumina Beadarrays*, Revstat **4**, 1-30 and in a separate vignette of beadarray.

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. This is the format of the data output by Illumina's BeadStudio application. The methods described within this document are for the analysis of bead summary data which can be obtained using either the BeadChip (6 or 8 arrays on a slide) or SAM (arrays organised in 96 well plates) technologies.

At present, beadarray is for the analysis of Illumina expression data only. For a package to analyse Illumina SNP data, see beadarraySNP.

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 Importing Bead Summary Data

An example data set is included with the beadarray package and can be found as a zipped folder data directory of the beadarray download. Inside this folder you will find three Excel data files and two text files. The Excel files are the raw non-normalised data, a sample sheet and a quality control file for an example experiment. These data were obtained as part of a pilot study into BeadArray technology and comprises of 3 Human-6 BeadChips with 6 different samples, I, MC, MD, MT, P and Norm hybridised. MC, MD, MT and P are all tumours whereas Norm is a normal sample and I is a sample provided by Illumina.

2.1 Description of Files

Reading bead summary data into beadarray requires the three files as given for this example experiment and we now describe these in more detail.

- raw_data.csv - This contains the raw, non-normalised bead summary values as output by BeadStudio and is readable by Excel. Inside the file are several lines of header information followed by a data matrix with some 48,000 rows. Each row is a different gene in the experiment and the columns give different measurements for the gene. For each array, we record the summarised expression level (AVG_Signal), standard error of the bead replicates (BEADSTDEV), Number of beads used (Avg_NBEADS) and a Detection score which estimates the probability of a gene being detected above the background. Note that whilst this data has not been normalised, it has been subjected to local background correction at the bead level prior to summarising.

- raw_data_sample_sheet - Defines the array IDs and samples placed on each array. In order for this information to be read into `beadarray`, we require that the 4th column is a unique identifier for each array in the experiemnt.
- raw_data_qc_info - Gives the summarised expression values for each of the controls that Illumina place on arrays and hence extremely useful for diagnostic purposes. Each row in the file is a different array and the columns give average expression, standard error and detection for various controls on the array. See Illumina documentation for descriptions of control types.

The following code can be used to read the example data into R. Firstly, we have to use the targets.txt file to define the location of the raw data, sample sheet and quality control file. Once this targets information has been read into R we can simply run the function `readBeadSummaryData`. The default parameters for this function will look for the column headings as described above.

```
> targets <- readBeadSummaryTargets("targets.txt")
> targets
> BSData <- readBeadSummaryData(targets)
```

```
> BSData <- readBeadSummaryData(targets)
```

3 The BSData object

BSData is an object of type ExpressionSetIllumina which is an extension of the ExpressionSet class developed by the Biocore team used as a container for high-throughput assays. The data from the the raw_data file has been written to the assayData slot of the object, whereas the phenoData slot contains information from sample_sheet and the QC slot contains the quality control information. For consistency with the definition of other *ExpressionSet* objects, we now refer to the expression values as the *exprs* matrix which can be accessed using `exprs` and subset in the usual manner. The `BeadStDev` matrix can be accessed using `se.exprs`. The rows of `exprs` are named according to the row names of the original raw_data file.

```
> BSData

Instance of ExpressionSetIllumina

assayData
  Storage mode: list
  Dimensions:
         BeadStDev Detection exprs NoBeads
Features     47293     47293 47293   47293
Samples         18        18    18      18

phenoData
  rowNames: I.1, IC.1, IH.2, ..., Norm.2, P42.2 (18 total)
  varLabels and descriptions:
    Sample_Name: Sample_Name
    Sample_Well: Sample_Well
    Sample_Plate: Sample_Plate
    Sample_Group: Sample_Group
    Pool_ID: Pool_ID
    Sentrix_ID: Sentrix_ID
    Sentrix_Position: Sentrix_Position

featureData
  featureNames: GI_10047089-S, GI_10047091-S, GI_10047093-S, ..., thrB, trpF (47293 total)
  varLabels and descriptions:

Experiment data
  Experimenter name:
  Laboratory:
  Contact information:
  Title:
  URL:
  PMIDs:
  No abstract available.

Annotation [1] "Illumina"
QC Information
 Available Slots:  Signal StDev Detection
  featureNames: 1475542110_F, 1475542113_E, 1475542114_A, ..., 1475542113_D, 1475542113_F
  sampleNames: Biotin, cy3_high, cy3_low, ..., pm, negative
```

```
> exprs(BSData)[1:10, 1:2]
```

```
                 I.1   IC.1
GI_10047089-S   87.8  131.8
GI_10047091-S  161.8  130.8
GI_10047093-S  481.2  401.4
GI_10047099-S  633.7  483.8
GI_10047103-S 1535.6 1186.5
GI_10047105-S  247.5  210.2
GI_10047121-S  113.0  101.3
GI_10047123-S  453.9  306.8
GI_10047133-A  103.6  114.5
GI_10047133-I  118.0  123.1
```

```
> se.exprs(BSData)[1:10, 1:2]
```

```
              AVG_Signal.I.1 AVG_Signal.IC.1
GI_10047089-S            5.1             9.5
GI_10047091-S           12.0             7.9
GI_10047093-S           21.7            24.5
GI_10047099-S           21.6            20.9
GI_10047103-S           42.7            34.5
GI_10047105-S           12.7            11.8
GI_10047121-S            6.4             8.1
GI_10047123-S           14.0            13.1
GI_10047133-A            6.8             6.0
GI_10047133-I            5.6             7.2
```

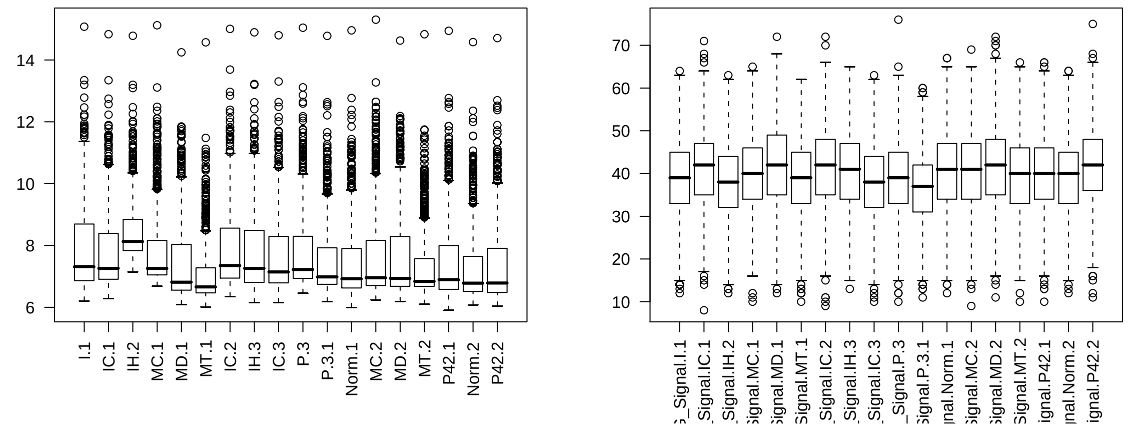
```
> pData(BSData)[, 1:6]

       Sample_Name Sample_Well Sample_Plate Sample_Group Pool_ID Sentrix_ID
I.1             NA          NA           NA         IH-1      NA 1475542114
IC.1            NA          NA           NA         IC-1      NA 1475542114
IH.2            NA          NA           NA         IH-2      NA 1475542114
MC.1            NA          NA           NA         MC-1      NA 1475542114
MD.1            NA          NA           NA         MD-1      NA 1475542114
MT.1            NA          NA           NA         MT-1      NA 1475542114
IC.2            NA          NA           NA         IC-2      NA 1475542110
IH.3            NA          NA           NA         IH-3      NA 1475542110
IC.3            NA          NA           NA         IC-3      NA 1475542110
P.3             NA          NA           NA          P-3      NA 1475542110
P.3.1           NA          NA           NA          P-3      NA 1475542110
Norm.1          NA          NA           NA       Norm-1      NA 1475542110
MC.2            NA          NA           NA         MC-2      NA 1475542113
MD.2            NA          NA           NA         MD-2      NA 1475542113
MT.2            NA          NA           NA         MT-2      NA 1475542113
P42.1           NA          NA           NA          P-1      NA 1475542113
Norm.2          NA          NA           NA       Norm-2      NA 1475542113
P42.2           NA          NA           NA          P-2      NA 1475542113
```

Boxplots of expression may be useful for quality control. Below we show the code to produce boxplots of the log2 intensities of each array in the experiment. Recall that there are 6 arrays per BeadChip and that differences between chips hybridisations on different days may be expected. In this example the differences in intensity between arrays on the same chip and different chips do not seem too large. However, we can see that the first BeadChip seems to be more variable than the others and in particular the third array on the first BeadChip could be an outlier.

Boxplots of the other slots in BSData can be easily plotted. [1]

```
> par(mfrow = c(1, 2))
> boxplot(log2(exprs(BSData)[1:1000, ]), las = 2)
> boxplot(NoBeads(BSData)[1:1000, ], las = 2)
```



[1] We have restricted the number of points plotted in order to keep the size of this vignettee small.

4 Normalisation and Quality Control

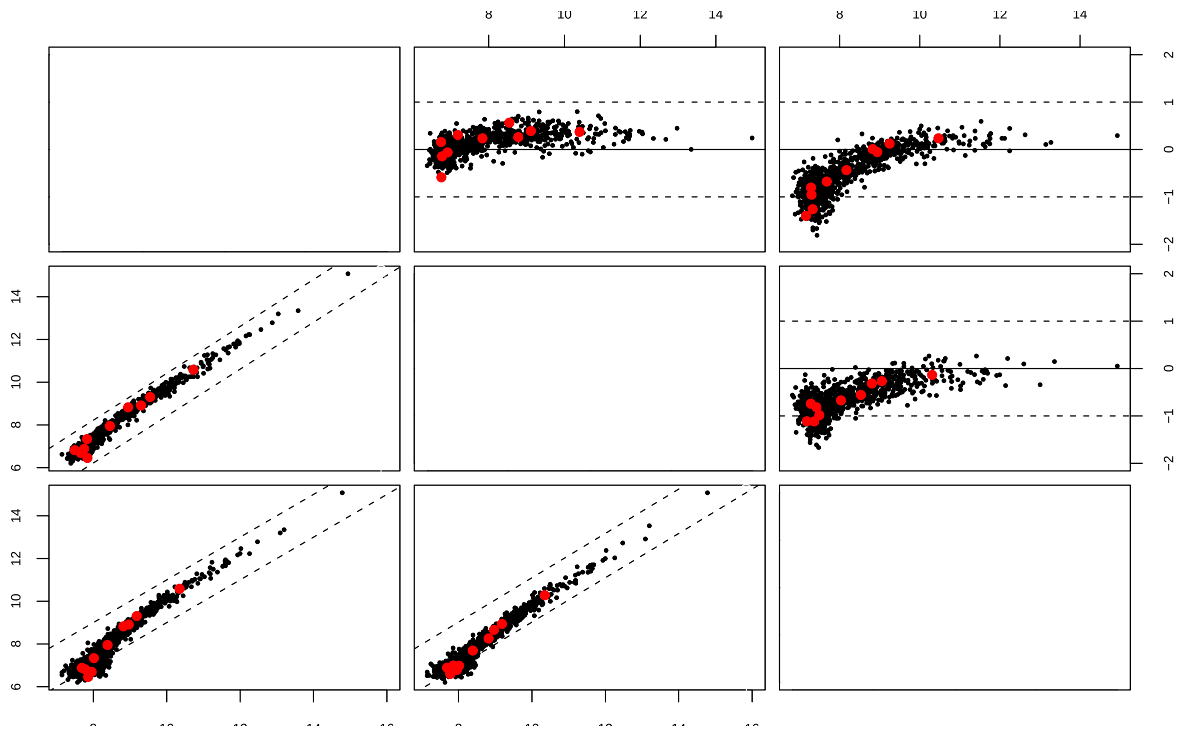
In the expression boxplots we notice that there are differences in expression level across a chip and between chips. Therefore we might want to normalise the arrays in the experiment comparable. We also see the the 3rd array has significantly different intensity. The sample on this array is replicated three times on the first chip, so comparing the MA and XY plots for the replicates of this sample can be informative.

Particular genes of interest may be highlighted on the MA and XY plots by using the `genesToLabel` argument which should match up with the row names in `BSData`. The `labelCol` argument can be used to specify a colour for each gene. For simplicity sake we simply highlight the first ten genes in the expression matrix, a possible application might be to highlight control genes on the plot or particular genes of interest.

```
> g = rownames(exprs(BSData))[1:10]
> g
```

```
 [1] "GI_10047089-S" "GI_10047091-S" "GI_10047093-S" "GI_10047099-S"
 [5] "GI_10047103-S" "GI_10047105-S" "GI_10047121-S" "GI_10047123-S"
 [9] "GI_10047133-A" "GI_10047133-I"
```

```
> cols = rainbow(start = 0, end = 5/6, n = 10)
> plotMAXY(exprs(BSData)[1:1000, ], arrays = 1:3, genesToLabel = g,
+     labelCols = cols)
```



In the top right corner we see the MA plots for all pairwise comparisons involving the 3 arrays. On an MA plot, for each gene we plot the average of the expression levels on the two arrays on the x axis and the difference in the measurements on the y axis. For replicate arrays we would expect all genes to be unchanged between the two samples and hence most points on the plot to lie along the line y=0. In the lower left corner of the MAXY plot we see the XY plot and for replicate arrays we would expect to see most points along the diagonal $y = x$. From this MAXY plot it is obvious that the third array is significantly different to the other replicates and requires normalisation.

Both XY and MA plots for a particular comparison of arrays are available separately using `plotXY` and `plotMA`

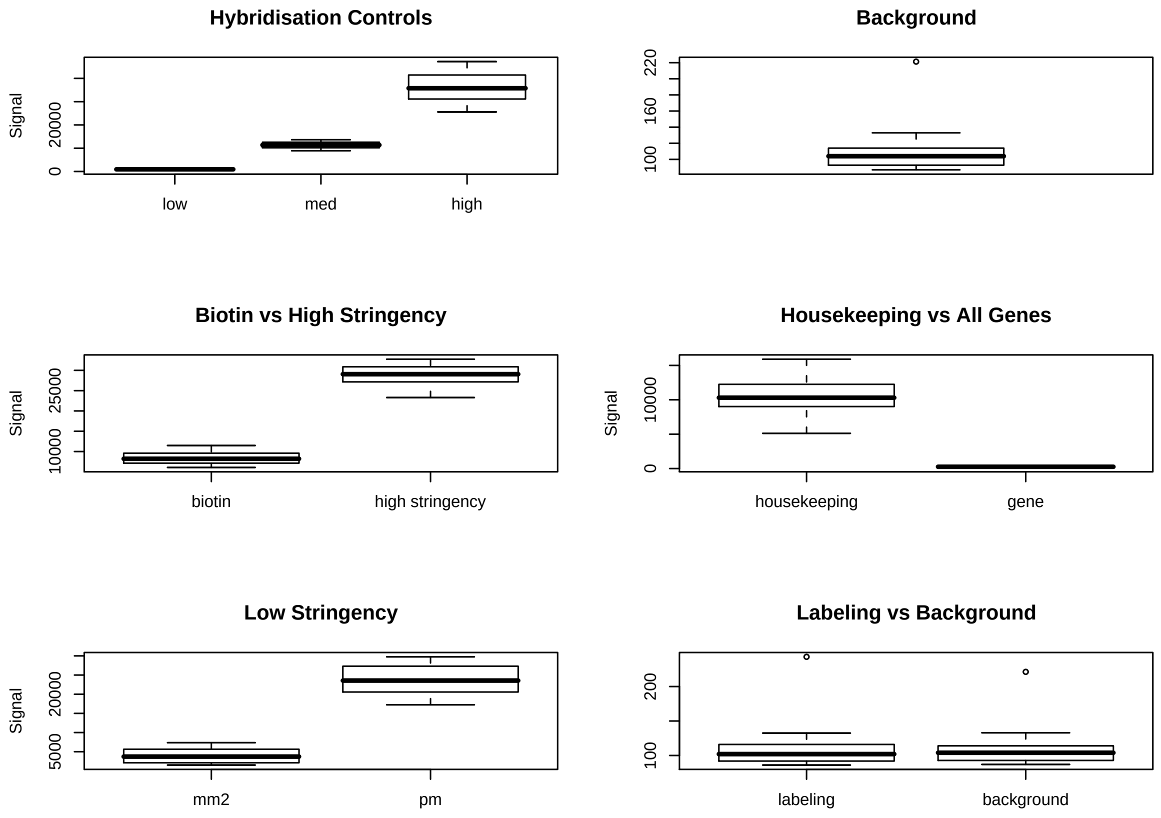
The quality control information which is read in by `readBeadSummaryData` can be plotted to provide useful diagnostic information. To retrieve this quality control data we can use the `QCInfo` function. Alternatively, quality control information can be read using `readQC`.

The `QC` object contains Signal, StDev and Detection matrices with each row in the matrix being a different array and each column a different control type. An overview of `QC` can be plotted using `plotQC`.

```
> QC = QCInfo(BSData)
> QC$Signal[1:3, ]
```

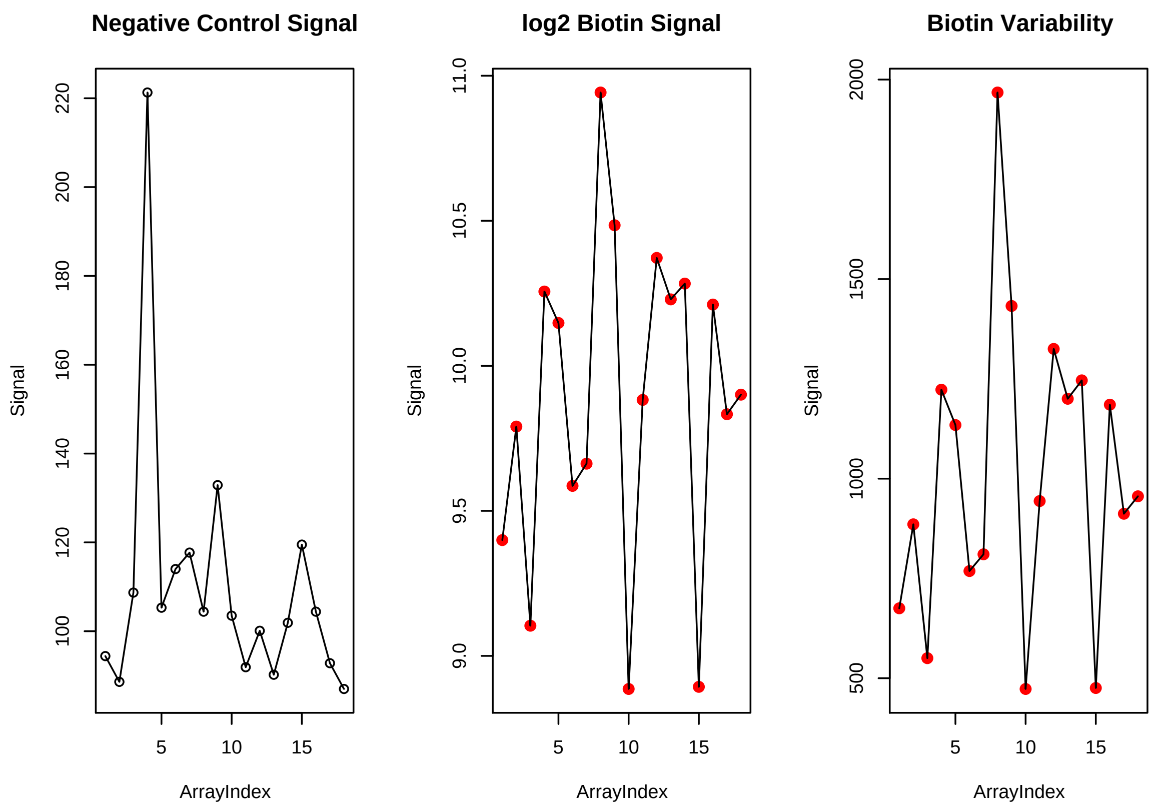
```
               Biotin cy3_high cy3_low cy3_med  gene      hs   house labeling
1475542110_F  7551.0  32436.0   816.6 11178.2 205.8 29498.3  7914.2     92.9
1475542113_E  6137.2  28081.0   739.4  9158.1 176.6 23302.4  6680.7     86.1
1475542114_A 10255.0  41451.7  1040.9 13176.7 320.3 30390.5 15902.3    106.0
                 mm      pm negative
1475542110_F 3584.5 21807.1     94.4
1475542113_E 1516.5 18619.5     88.6
1475542114_A 5738.7 27314.2    108.7
```

```
> plotQC(BSData)
```



The `singleQCPlot` function allows a particular control type to be plotted across all samples. The type argument must match one of the column names of QC$Signal and the `what` argument selects which of the Signal, StDev and Detection slots to plot. Additional plotting arguments such as a title for the plot, plotting character etc can also be passed to the function. We can also choose to plot on the $\log_2$ scale.

```
> par(mfrow = c(1, 3))
> singleQCPlot(BSData, type = "negative", main = "Negative Control Signal",
+     what = "Signal")
> singleQCPlot(BSData, type = "Biotin", log = TRUE, pch = 16, col = "red",
+     lwd = 2, lty = 2, what = "StDev", main = "log2 Biotin Signal")
> singleQCPlot(BSData, type = "Biotin", pch = 16, col = "red",
+     lwd = 2, lty = 2, what = "StDev", main = "Biotin Variability")
```



Illumina also use this quality control information to normalise bead summary data. In a procedure known as background normalisation, the averaged values of all negative controls on a particular array are subtracted from the summarised expression of each gene. This normalisation can be repeated by the function `backgroundNormalise`. The intended effect of this normalisation is to remove the effects of non-specific binding from the expression values. This effect is more noticable for genes with low expression level and hence can produce negative values.

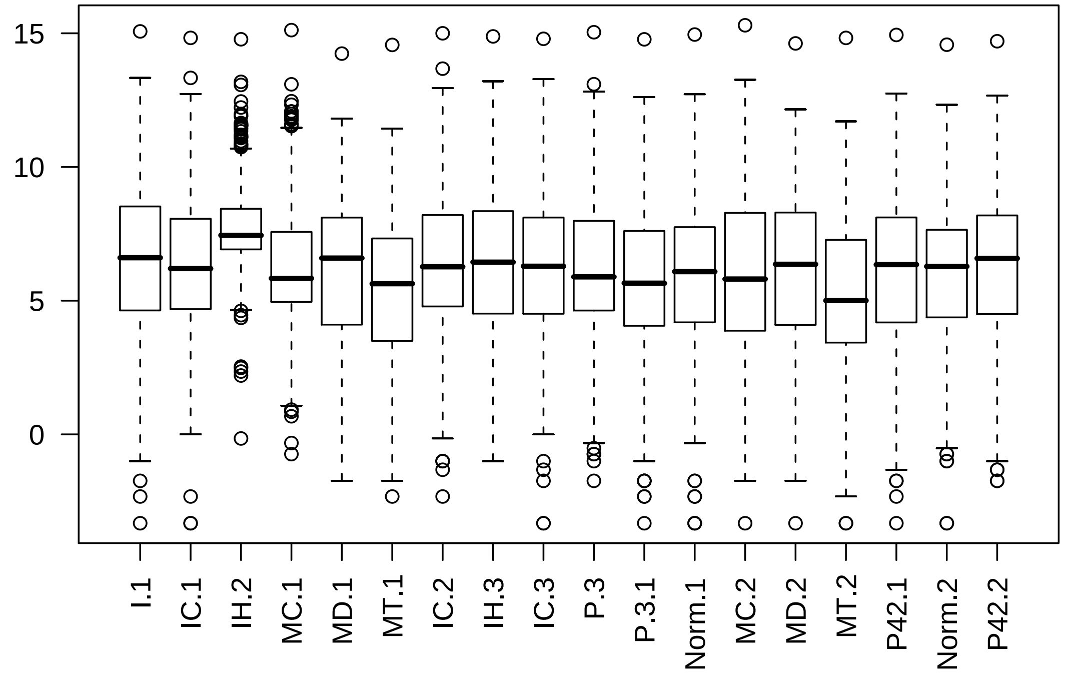
```
> BSData.bgnorm = backgroundNormalise(BSData)
> range(exprs(BSData)[, 1])
```

```
[1]    65.9 45311.7
```

```
> range(exprs(BSData.bgnorm)[, 1])
```

```
[1]  -142.7 45221.5
```

```
> boxplot(log2(exprs(BSData.bgnorm)[1:1000, ]), las = 2)
```



It is possible to use the normalisation methods available in the affy such as quantile, qspline or others. The method of rank invariant normalisation recommended by Illumina may also be applied once a suitable target distribution has been defined. In the following example we define this to be the mean of each row before using the `normalize.invariantset` to find a set of invariant genes and define a normalising curve using this set and the target distribution.

```
> library(affy)
> BSData.quantile = assayDataElementReplace(BSData, "exprs", normalize.quantiles(as.matrix(exprs(BSData))))
> BSData.qspline = assayDataElementReplace(BSData, "exprs", normalize.qspline(as.matrix(exprs(BSData))))
> T = apply(exprs(BSData.bgnorm), 1, mean)
> BSData.rankinv = assayDataElementReplace(BSData.bgnorm, "exprs",
+     rankInvariantNormalise(exprs(BSData.bgnorm), T))
```

5 Differential Expression

Research into the best method for detecting differential expression for BeadArray data is still work in progress. In the meantime, users are able to use the `lmFit` and `eBayes` functions from limma on the matrix `exprs(BSdata)` with a $\log_2$ transformation applied.

The following code shows how to set up a design matrix for the example experiment combining the I, MC, MD, MT, P and Normal samples together. We then define contrasts comparing the I samples to the P samples and I to Normal and perform an empirical bayes shrinkage. In this particular experiment, the I and P samples are completely different so we would expect to see plenty of differentially expressed genes.

For more information about `lmFit` and `eBayes` please see the comprehensive limma documentation.

```
> design = matrix(nrow = 18, ncol = 6, 0)
> colnames(design) = c("I", "MC", "MD", "MT", "P", "Norm")
> design[which(strtrim(colnames(exprs(BSData)), 1) == "I"), 1] = 1
> design[which(strtrim(colnames(exprs(BSData)), 2) == "MC"), 2] = 1
> design[which(strtrim(colnames(exprs(BSData)), 2) == "MD"), 3] = 1
> design[which(strtrim(colnames(exprs(BSData)), 2) == "MT"), 4] = 1
> design[which(strtrim(colnames(exprs(BSData)), 1) == "P"), 5] = 1
> design[which(strtrim(colnames(exprs(BSData)), 1) == "N"), 6] = 1
> design
```

```
      I MC MD MT P Norm
 [1,] 1  0  0  0 0    0
 [2,] 1  0  0  0 0    0
 [3,] 1  0  0  0 0    0
 [4,] 0  1  0  0 0    0
 [5,] 0  0  1  0 0    0
 [6,] 0  0  0  1 0    0
 [7,] 1  0  0  0 0    0
 [8,] 1  0  0  0 0    0
 [9,] 1  0  0  0 0    0
[10,] 0  0  0  0 1    0
[11,] 0  0  0  0 1    0
[12,] 0  0  0  0 0    1
[13,] 0  1  0  0 0    0
[14,] 0  0  1  0 0    0
[15,] 0  0  0  1 0    0
[16,] 0  0  0  0 1    0
[17,] 0  0  0  0 0    1
[18,] 0  0  0  0 1    0
```

```
> fit = lmFit(log2(exprs(BSData)), design)
> cont.matrix = makeContrasts(IvsP = I - P, IvsNorm = I - Norm,
+     PvsNorm = P - Norm, levels = design)
> fit = contrasts.fit(fit, cont.matrix)
> ebFit = eBayes(fit)
> topTable(ebFit)
```

```
            ProbeID      IvsP   IvsNorm      PvsNorm        F       P.Value
9259   GI_28302130-S  7.499572  7.361939 -0.137632534 937.0935  0.000000e+00
9260   GI_28302132-S  7.694362  7.544722 -0.149640427 985.6984  0.000000e+00
9258   GI_28302129-S  6.402578  6.512401  0.109823045 699.3457 1.896739e-304
24442   GI_6633805-S  6.288645  6.355043  0.066398690 671.6779 1.967835e-292
25430   GI_8392890-S  6.512861  5.830264 -0.682596858 669.7313 1.378516e-291
21840   GI_4501988-S  6.067112  5.813525 -0.253586595 604.0850 4.458776e-263
31186    Hs.449602-S  6.605590  1.619840 -4.985750409 598.4842 1.206731e-260
19978 GI_42542384-S  5.726441  5.629671 -0.096770366 546.8808 3.109321e-238
2128  GI_15149480-S -5.669899 -5.671313 -0.001414323 542.2465 3.201302e-236
22143   GI_4503886-S  5.617491  5.751247  0.133756180 540.7956 1.366055e-235
           adj.P.Val
9259    0.000000e+00
9260    0.000000e+00
```

```
9258  2.990083e-300
24442 2.326621e-288
25430 1.303883e-287
21840 3.514482e-259
31186 8.152844e-257
19978 1.838114e-234
2128  1.682213e-232
22143 6.460483e-232
```

The algorithm for the Illumina method is implemented in the function `DiffScore` although it not completely accurate at present. To compare array 1 in the experiment to array 10 (ie comparing an I sample to a P) we would use the following code. At present, the DiffScore is only able to make pairwise comparisons between arrays. Notice that some genes appear in both the top lists of the empirical bayes methods and the Illumina method.

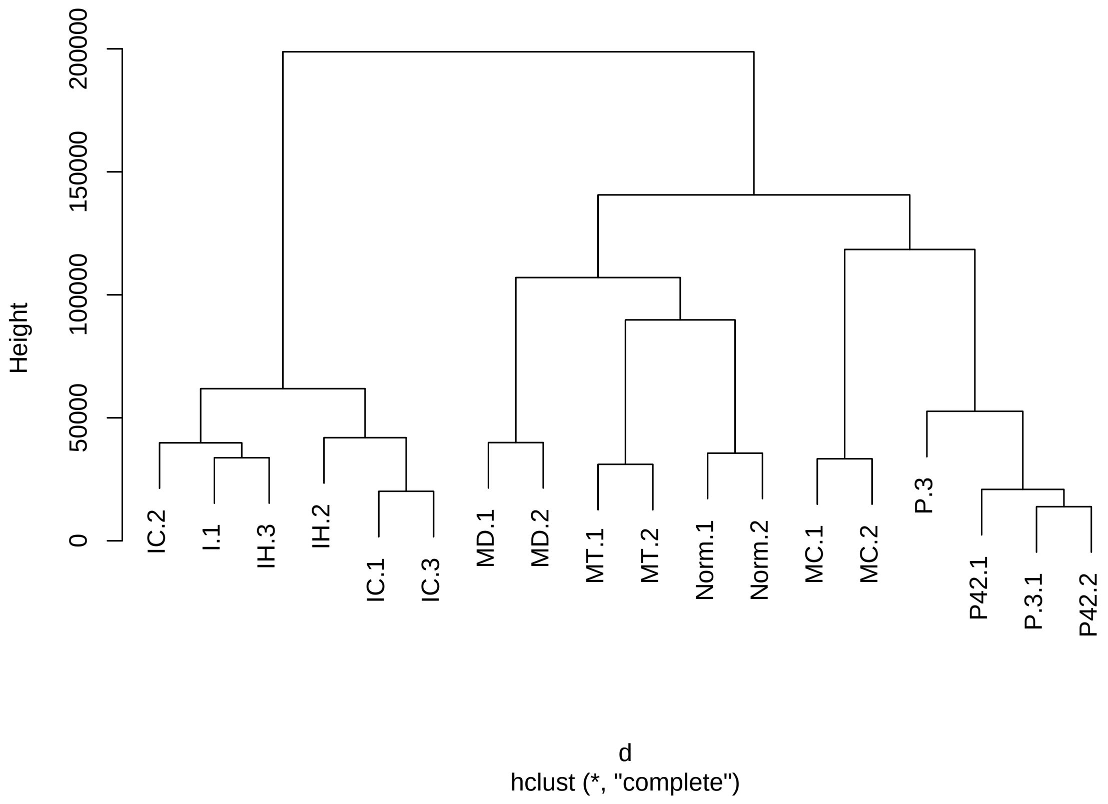
```
> df = DiffScore(BSData, QC, cond = 10, ref = 1)
> o = order(abs(df), decreasing = TRUE)[1:50]
> o[1:20]
```

```
 [1]  9259  9260 31186 25430  9258 24442 21840 21844  5569 22143 19978 21864
[13] 25150 12104 22144 22269 21865 23042  5821  7612
```

6 Further Analysis

The clustering functionality available in BeadStudio can be easily performed through `R` using the `hlcust` once a distance matrix has been defined. In this example we see that the clusters correspond well to the different sample types. The `heatmap` function could also be used in a similar manner and principal components analysis is possible using `princomp`.

```
> d = dist(t(exprs(BSData)))
> plclust(hclust(d), labels = rownames(pData(BSData)))
```


Height
0
50000
100000
150000
200000
IC.2
I.1
IH.3
IH.2
IC.1
IC.3
MD.1
MD.2
MT.1
MT.2
Norm.1
Norm.2
MC.1
MC.2
P.3
P42.1
P.3.1
P42.2
d
hclust (*, "complete")