

Analysis of Bead-summary Data using beadarray

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Introduction

The BeadArray technology involves randomly arranged arrays of beads, with beads having the same probe sequence attached colloquially known as a bead-type. BeadArrays are combined in parallel on either a rectangular chip (BeadChip) or a matrix of 8 by 12 hexagonal arrays (Sentrix Array Matrix or SAM). The BeadChip is further divided into strips on the surface known as sections, with each section giving rise to a different image when scanned by BeadScan. These images, and associated text files, comprise the raw data for a beadarray analysis. However, for BeadChips, the number of sections assigned to each biological sample may vary from 1 on HumanHT12 chips, 2 on HumanWG6 chips or sometimes ten or more for SNP chips with large numbers of SNPs being investigated.

This vignette demonstrates the analysis of bead summary data using beadarray. The recommended approach to obtain these data is to start with bead-level data and follow the steps illustrated in the vignette `beadlevel.pdf` distributed with beadarray. If bead-level data are not available, the output of Illumina's BeadStudio or GenomeStudio can be read by beadarray. Example code to do this is provided at the end of this vignette. However, the same object types are produced from either of these routes and the same functionality is available.

To make the most use of the code in this vignette, you will need to install the `beadarrayExampleData` and `illuminaHumanv3.db` packages from Bioconductor.

```
> source("http://www.bioconductor.org/biocLite.R")
> biocLite(c("beadarrayExampleData", "illuminaHumanv3.db"))
```

The code used to produce these example data is given in the vignette of `beadarrayExampleData`, which follow similar steps to those described in the `beadlevel.pdf` vignette of beadarray. The following commands give a basic description of the data.

```
> library("beadarray")
> require(beadarrayExampleData)
> data(exampleSummaryData)
> exampleSummaryData
```

```
ExpressionSetIllumina (storageMode: list)
assayData: 49576 features, 12 samples
  element names: exprs, se.exprs, nObservations
protocolData: none
phenoData
  rowNames: 4613710017_B 4613710052_B ... 4616494005_A (12 total)
  varLabels: sampleID SampleFac
  varMetadata: labelDescription
featureData
  featureNames: ILMN_1802380 ILMN_1893287 ... ILMN_1846115 (49576)
```

```

total)
fvarLabels: ArrayAddressID IlluminaID Status
fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation: Humanv3
QC Information
Available Slots:
QC Items: Date, Matrix, ..., SampleGroup, numBeads
sampleNames: 4613710017_B, 4613710052_B, ..., 4616443136_A, 4616494005_A

```

Summarized data are stored in an object of type *ExpressionSetIllumina* which is an extension of the *ExpressionSet* class developed by the Bioconductor team as a container for data from high-throughput assays. Objects of this type use a series of slots to store the data. For consistency with the definition of other *ExpressionSet* objects, we refer to the expression values as the **exprs** matrix (this stores the probe-specific average intensities) which can be accessed using **exprs** and subset in the usual manner. The **se.exprs** matrix, which stores the probe-specific variability can be accessed using **se.exprs**. You may notice that the expression values have already been transformed to the \log_2 scale, which is an option in the **summarize** function in **beadarray**. Data exported from BeadStudio or GenomeStudio will usually be un-transformed and on the scale 0 to 2^{16} .

```
> exprs(exampleSummaryData)[1:5, 1:5]
```

	G:4613710017_B	G:4613710052_B	G:4613710054_B	G:4616443079_B
ILMN_1802380	8.454468	8.616796	8.523001	8.420796
ILMN_1893287	5.388161	5.419345	5.162849	5.133287
ILMN_1736104	5.268626	5.457679	5.012766	4.988511
ILMN_1792389	6.767519	7.183788	6.947624	7.168571
ILMN_1854015	5.556947	5.721614	5.595413	5.520391
	G:4616443093_B			
ILMN_1802380	8.527748			
ILMN_1893287	5.221987			
ILMN_1736104	5.284026			
ILMN_1792389	7.386435			
ILMN_1854015	5.558717			

```
> se.exprs(exampleSummaryData)[1:5, 1:5]
```

	G:4613710017_B	G:4613710052_B	G:4613710054_B	G:4616443079_B
ILMN_1802380	0.2833023	0.3367157	0.2750020	0.4141796
ILMN_1893287	0.3963681	0.3882834	0.5516421	0.6761106
ILMN_1736104	0.4704854	0.4951260	0.4031143	0.5276266
ILMN_1792389	0.4038533	0.4728013	0.5032908	0.3447242
ILMN_1854015	0.5663066	0.3783570	0.5511991	0.5358812
	G:4616443093_B			
ILMN_1802380	0.3581862			
ILMN_1893287	0.4448673			
ILMN_1736104	0.4864355			
ILMN_1792389	0.3951935			
ILMN_1854015	0.6748219			

feature and pheno data

The `fData` and `pData` functions are useful shortcuts to find more information about the features (rows) and samples (columns) in the summary object. These annotations are created automatically whenever a bead-level data is summarized (see `beadlevel.pdf`) or read from a BeadStudio file. The `fData` will be added to later, but initially contains information on whether each probe is a control or not. In this example the `phenoData` denotes the sample group for each array; either Brain or UHRR (Universal Human Reference RNA).

```
> head(fData(exampleSummaryData))
```

	ArrayAddressID	IlluminaID	Status
ILMN_1802380	10008	ILMN_1802380	regular
ILMN_1893287	10010	ILMN_1893287	regular
ILMN_1736104	10017	ILMN_1736104	regular
ILMN_1792389	10019	ILMN_1792389	regular
ILMN_1854015	10020	ILMN_1854015	regular
ILMN_1904757	10021	ILMN_1904757	regular

```
> table(fData(exampleSummaryData)[, "Status"])
```

	biotin	cy3_hyb
	2	2
cy3_hyb,low_stringency_hyb		housekeeping
	4	7
labeling		low_stringency_hyb
	2	4
negative		regular
	759	48796

```
> pData(exampleSummaryData)
```

	sampleID	SampleFac
4613710017_B	4613710017_B	UHRR
4613710052_B	4613710052_B	UHRR
4613710054_B	4613710054_B	UHRR
4616443079_B	4616443079_B	UHRR
4616443093_B	4616443093_B	UHRR
4616443115_B	4616443115_B	UHRR
4616443081_B	4616443081_B	Brain
4616443081_H	4616443081_H	Brain
4616443092_B	4616443092_B	Brain
4616443107_A	4616443107_A	Brain
4616443136_A	4616443136_A	Brain
4616494005_A	4616494005_A	Brain

Subsetting the data

There are various way to subset an *ExpressionSetIllumina* object, each of which returns an *ExpressionSetIllumina* with the same slots, but different dimensions. When bead-level data are summarized by `beadarray` there is an option to apply different transformation options, and save the results as different channels in the resultant object. For instance, if summarizing two-colour data one might be interested

in summarizing the red and green channels, or some combination of the two, separately. Both \log_2 and un-logged data are stored in the `exampleSummaryData` object and can be accessed by using the `channel` function. Both the rows and columns in the resultant `ExpressionSetIllumina` object are kept in the same order.

```
> channelNames(exampleSummaryData)

[1] "G"      "G.ul"

> exampleSummaryData.log2 <- channel(exampleSummaryData, "G")
> exampleSummaryData.unlogged <- channel(exampleSummaryData, "G.ul")
> sampleNames(exampleSummaryData.log2)

[1] "4613710017_B" "4613710052_B" "4613710054_B" "4616443079_B" "4616443093_B"
[6] "4616443115_B" "4616443081_B" "4616443081_H" "4616443092_B" "4616443107_A"
[11] "4616443136_A" "4616494005_A"

> sampleNames(exampleSummaryData.unlogged)

[1] "4613710017_B" "4613710052_B" "4613710054_B" "4616443079_B" "4616443093_B"
[6] "4616443115_B" "4616443081_B" "4616443081_H" "4616443092_B" "4616443107_A"
[11] "4616443136_A" "4616494005_A"

> exprs(exampleSummaryData.log2)[1:10, 1:3]

      4613710017_B 4613710052_B 4613710054_B
ILMN_1802380      8.454468      8.616796      8.523001
ILMN_1893287      5.388161      5.419345      5.162849
ILMN_1736104      5.268626      5.457679      5.012766
ILMN_1792389      6.767519      7.183788      6.947624
ILMN_1854015      5.556947      5.721614      5.595413
ILMN_1904757      5.421553      5.320500      5.522316
ILMN_1740305      5.417821      5.623998      5.720007
ILMN_1665168      5.321087      5.155455      4.967601
ILMN_2375156      5.894207      6.076418      5.638877
ILMN_1705423      5.426463      4.806624      5.357688

> exprs(exampleSummaryData.unlogged)[1:10, 1:3]

      4613710017_B 4613710052_B 4613710054_B
ILMN_1802380     356.88235     396.46875     367.81481
ILMN_1893287      40.85000      44.29167      38.42105
ILMN_1736104      40.53333      46.50000      33.46154
ILMN_1792389     112.90909     153.17647     122.65000
ILMN_1854015      50.47059      53.26087      51.57143
ILMN_1904757      41.45833      42.10000      49.92593
ILMN_1740305      38.45455      51.50000      46.21429
ILMN_1665168      42.38889      37.95000      30.46154
ILMN_2375156      61.47368      72.73913      52.46154
ILMN_1705423      42.38889      28.14286      38.62500
```

As we have seen, the expression matrix of the `ExpressionSetIllumina` object can be subset by column or row. In fact, the same subset operations can be performed on the `ExpressionSetIllumina` object itself. In the following code, notice how the number of samples and features changes in the output.

```

> exampleSummaryData.log2[, 1:4]

ExpressionSetIllumina (storageMode: list)
assayData: 49576 features, 4 samples
  element names: exprs, se.exprs, nObservations
protocolData: none
phenoData
  rowNames: 4613710017_B 4613710052_B 4613710054_B 4616443079_B
  varLabels: sampleID SampleFac
  varMetadata: labelDescription
featureData
  featureNames: ILMN_1802380 ILMN_1893287 ... ILMN_1846115 (49576
    total)
  fvarLabels: ArrayAddressID IlluminaID Status
  fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation: Humanv3
QC Information
  Available Slots:
    QC Items: Date, Matrix, ..., SampleGroup, numBeads
    sampleNames: 4613710017_B, 4613710052_B, 4613710054_B, 4616443079_B

> exampleSummaryData.log2[1:10, ]

ExpressionSetIllumina (storageMode: list)
assayData: 10 features, 12 samples
  element names: exprs, se.exprs, nObservations
protocolData: none
phenoData
  rowNames: 4613710017_B 4613710052_B ... 4616494005_A (12 total)
  varLabels: sampleID SampleFac
  varMetadata: labelDescription
featureData
  featureNames: ILMN_1802380 ILMN_1893287 ... ILMN_1705423 (10 total)
  fvarLabels: ArrayAddressID IlluminaID Status
  fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation: Humanv3
QC Information
  Available Slots:
    QC Items: Date, Matrix, ..., SampleGroup, numBeads
    sampleNames: 4613710017_B, 4613710052_B, ..., 4616443136_A, 4616494005_A

```

The object can also be subset by a vector of characters which must correspond to the names of features (i.e. row names). Currently, no analogous functions is available to subset by sample.

```

> randIDs <- sample(featureNames(exampleSummaryData), 1000)
> exampleSummaryData[randIDs, ]

```

```

ExpressionSetIllumina (storageMode: list)
assayData: 1000 features, 12 samples
  element names: exprs, se.exprs, nObservations

```

```

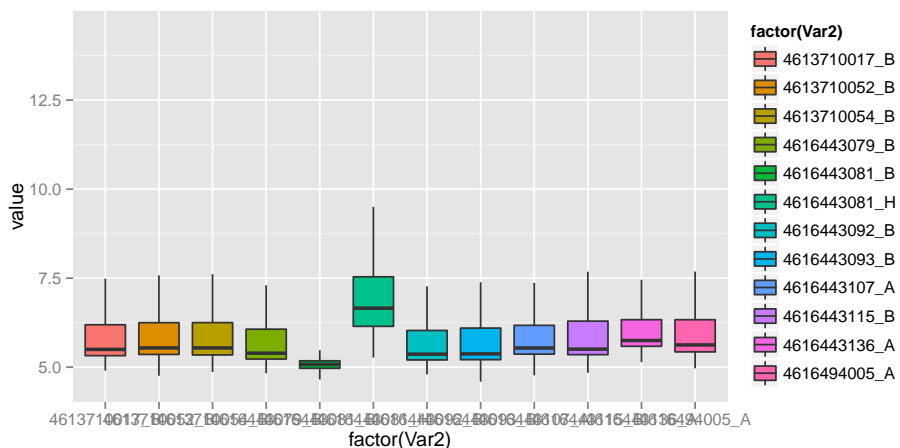
protocolData: none
phenoData
  rowNames: 4613710017_B 4613710052_B ... 4616494005_A (12 total)
  varLabels: sampleID SampleFac
  varMetadata: labelDescription
featureData
  featureNames: ILMN_2140059 ILMN_1897885 ... ILMN_1833889 (1000 total)
  fvarLabels: ArrayAddressID IlluminaID Status
  fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation: Humanv3
QC Information
  Available Slots:
    QC Items: Date, Matrix, ..., SampleGroup, numBeads
    sampleNames: 4613710017_B, 4613710052_B, ..., 4616443136_A, 4616494005_A

```

Exploratory analysis using boxplots

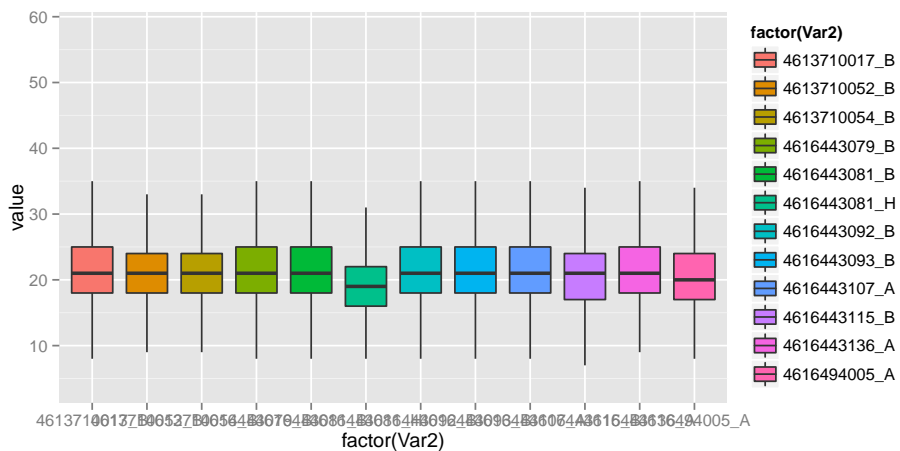
Boxplots of intensity levels and the number of beads are useful for quality assessment purposes. `beadarray` includes a modified version of the `boxplot` function that can take any valid *ExpressionSetIllumina* object and plot the expression matrix by default. For these examples we plot just a subset of the original `exampleSummaryData` object using random row IDs.

```
> boxplot(exampleSummaryData.log2[randIDs, ])
```



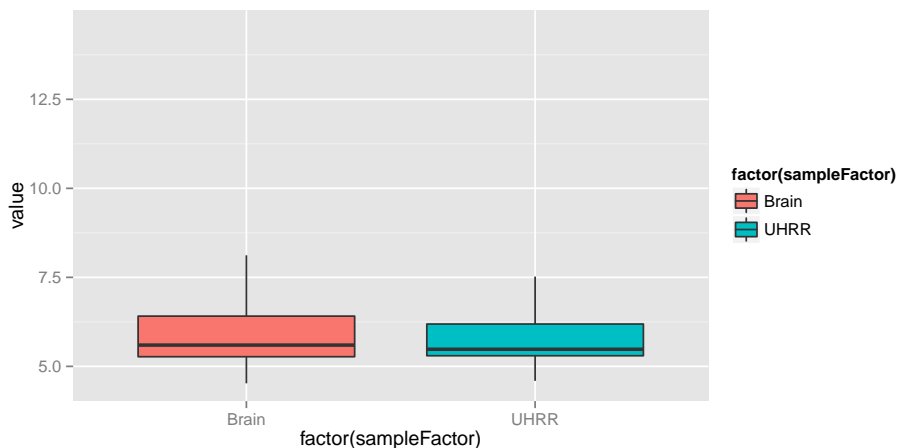
The function can also plot other `assayData` items, such as the number of observations.

```
> boxplot(exampleSummaryData.log2[randIDs, ], what = "nObservations")
```



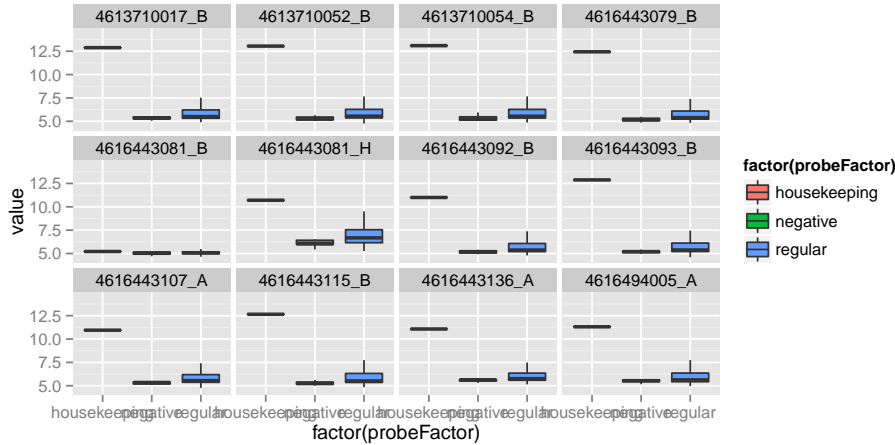
The default boxplot plots a separate box for each array, but often it is beneficial for compare expression levels between different sample groups. If this information is stored in the *phenoData* slot it can be incorporated into the plot. The following compares the overall expression level between UHRR and Brain samples.

```
> boxplot(exampleSummaryData.log2[randIDs, ], sampleFactor = "SampleFac")
```



In a similar manner, we may wish to visualize the differences between sample groups for particular probe groups. As a simple example, we look at the difference between negative controls and regular probes for each array. You should notice that the negative controls are consistently lower (as expected) with the exception of array 4616443081_B.

```
> boxplot(exampleSummaryData.log2[randIDs, ], probeFactor = "Status")
```



Extra feature annotation is available from annotation packages in Bioconductor, and `beadarray` includes functionality to extract these data from the annotation packages. The `annotation` of the object must be set in order that the correct annotation package can be loaded. For example, the `exampleSummaryData` object was generated from `Humanv3` data so the `illuminaHumanv3.db` package must be present. The `addFeatureData` function annotates all features of an `ExpressionSetIllumina` object using particular mappings from the `illuminaHumanv3.db` package. To see which mappings are available you can use the `illuminaHumanv3()` function, or equivalent from other packages.

```
> annotation(exampleSummaryData)
```

```
[1] "Humanv3"
```

```
> exampleSummaryData.log2 <- addFeatureData(exampleSummaryData.log2,
+   toAdd = c("SYMBOL", "PROBEQUALITY", "CODINGZONE", "PROBESEQUENCE",
+   "GENOMICLOCATION"))
> head(fData(exampleSummaryData.log2))
```

	Row.names	ArrayAddressID	IlluminaID	Status	SYMBOL
ILMN_1802380	ILMN_1802380	10008	ILMN_1802380	regular	RERE
ILMN_1893287	ILMN_1893287	10010	ILMN_1893287	regular	<NA>
ILMN_1736104	ILMN_1736104	10017	ILMN_1736104	regular	<NA>
ILMN_1792389	ILMN_1792389	10019	ILMN_1792389	regular	RNF165
ILMN_1854015	ILMN_1854015	10020	ILMN_1854015	regular	<NA>
ILMN_1904757	ILMN_1904757	10021	ILMN_1904757	regular	<NA>
	PROBEQUALITY	CODINGZONE			
ILMN_1802380	Perfect	Transcriptomic			
ILMN_1893287	Bad	Transcriptomic?			
ILMN_1736104	Bad	Intergenic			
ILMN_1792389	Perfect	Transcriptomic			
ILMN_1854015	Bad	Intergenic			
ILMN_1904757	Perfect***	Transcriptomic?			
			PROBESEQUENCE		
ILMN_1802380	GCCCTGACCTTCATGGTGTCTTTGAAGCCCAACCACTCGGTTTCCTTCGG				
ILMN_1893287	GGATTTCCTACACTCTCCACTTCTGAATGCTTGGAACACTTGCCATGCT				
ILMN_1736104	TGCCATCTTTGCTCCACTGTGAGAGGCTGCTCACACCACCCCTACATGC				
ILMN_1792389	CTGTAGCAACGTCTGTCAGGCCCCCTTGTGTTTCATCTCCTGCGCGGTA				
ILMN_1854015	GCAGAAAACCATGAGCTGAAATCTCTACAGGAACCACTGCTGGGGTAGGG				


```
ILMN_1904757 AGCTGTACCGTGGGGAGGCTTGGTCCTCTTGCCCCATTGTGTGATGTCT
                GENOMICLOCATION
ILMN_1802380     chr1:8412758:8412807:-
ILMN_1893287     chr9:42489407:42489456:+
ILMN_1736104     chr3:134572184:134572223:-
ILMN_1792389     chr18:44040244:44040293:+
ILMN_1854015     chr3:160827837:160827885:+
ILMN_1904757     chr3:197872267:197872316:+
```

```
> illuminaHumanv3()
```

```
####Mappings based on RefSeqID####
```

```
Quality control information for illuminaHumanv3:
```

This package has the following mappings:

```
illuminaHumanv3ACCCNUM has 31857 mapped keys (of 49576 keys)
illuminaHumanv3ALIAS2PROBE has 61262 mapped keys (of 99696 keys)
illuminaHumanv3CHR has 29705 mapped keys (of 49576 keys)
illuminaHumanv3CHRLNGTHS has 93 mapped keys (of 93 keys)
illuminaHumanv3CHRLOC has 29313 mapped keys (of 49576 keys)
illuminaHumanv3CHRLOCEND has 29313 mapped keys (of 49576 keys)
illuminaHumanv3ENSEMBL has 29186 mapped keys (of 49576 keys)
illuminaHumanv3ENSEMBL2PROBE has 21336 mapped keys (of 27388 keys)
illuminaHumanv3ENTREZID has 29706 mapped keys (of 49576 keys)
illuminaHumanv3ENZYME has 3522 mapped keys (of 49576 keys)
illuminaHumanv3ENZYME2PROBE has 967 mapped keys (of 975 keys)
illuminaHumanv3GENENAME has 29706 mapped keys (of 49576 keys)
illuminaHumanv3GO has 26382 mapped keys (of 49576 keys)
illuminaHumanv3GO2ALLPROBES has 17539 mapped keys (of 17608 keys)
illuminaHumanv3GO2PROBE has 13648 mapped keys (of 13737 keys)
illuminaHumanv3MAP has 29504 mapped keys (of 49576 keys)
illuminaHumanv3OMIM has 21379 mapped keys (of 49576 keys)
illuminaHumanv3PATH has 9172 mapped keys (of 49576 keys)
illuminaHumanv3PATH2PROBE has 229 mapped keys (of 229 keys)
illuminaHumanv3PFAM has 27593 mapped keys (of 49576 keys)
illuminaHumanv3PMID has 29374 mapped keys (of 49576 keys)
illuminaHumanv3PMID2PROBE has 383443 mapped keys (of 394407 keys)
illuminaHumanv3PROSITE has 27593 mapped keys (of 49576 keys)
illuminaHumanv3REFSEQ has 29706 mapped keys (of 49576 keys)
illuminaHumanv3SYMBOL has 29706 mapped keys (of 49576 keys)
illuminaHumanv3UNIGENE has 29521 mapped keys (of 49576 keys)
illuminaHumanv3UNIPROT has 27662 mapped keys (of 49576 keys)
```

Additional Information about this package:

```
DB schema: HUMANCHIP_DB
DB schema version: 2.1
Organism: Homo sapiens
Date for NCBI data: 2013-Sep12
```

```

Date for GO data: 20130907
Date for KEGG data: 2011-Mar15
Date for Golden Path data: 2010-Mar22
Date for Ensembl data: 2013-Sep3
####Custom Mappings based on probe sequence####
illuminaHumanv3ARRAYADDRESS()
illuminaHumanv3NUID()
illuminaHumanv3PROBEQUALITY()
illuminaHumanv3CODINGZONE()
illuminaHumanv3PROBESEQUENCE()
illuminaHumanv3SECONDMATCHES()
illuminaHumanv3OTHERGENOMICMATCHES()
illuminaHumanv3REPEATMASK()
illuminaHumanv3OVERLAPPINGSNP()
illuminaHumanv3ENTREZREANNOTATED()
illuminaHumanv3GENOMICLOCATION()
illuminaHumanv3SYMBOLREANNOTATED()
illuminaHumanv3REPORTERGROUPNAME()
illuminaHumanv3REPORTERGROUPID()
illuminaHumanv3ENSEMBLREANNOTATED()

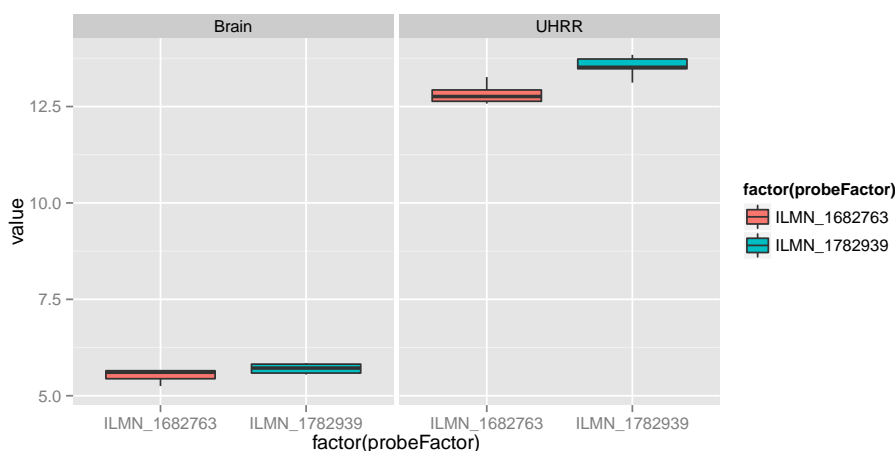
```

If we suspect that a particular gene may be differentially expressed between conditions, we can subset the *ExpressionSetIllumina* object to just include probes that target the gene, and plot the response of these probes against the sample groups. Furthermore, the different probes can be distinguished using the *probeFactor* parameter.

```

> ids <- which(fData(exampleSummaryData.log2)[, "SYMBOL"] == "ALB")
> boxplot(exampleSummaryData.log2[ids, ], sampleFactor = "SampleFac",
+         probeFactor = "IlluminaID")

```



A note about ggplot2

The `boxplot` function in `beadarray` creates graphics using the `ggplot2` package rather than the R base graphics system. Therefore, the standard way of manipulating graphics using `par` and `mfrow` etc will not work with the output of `boxplot`. However, the `ggplot2` package has equivalent functionality and is a more powerful and flexible system. There are numerous tutorials on how to use the `ggplot2` package,

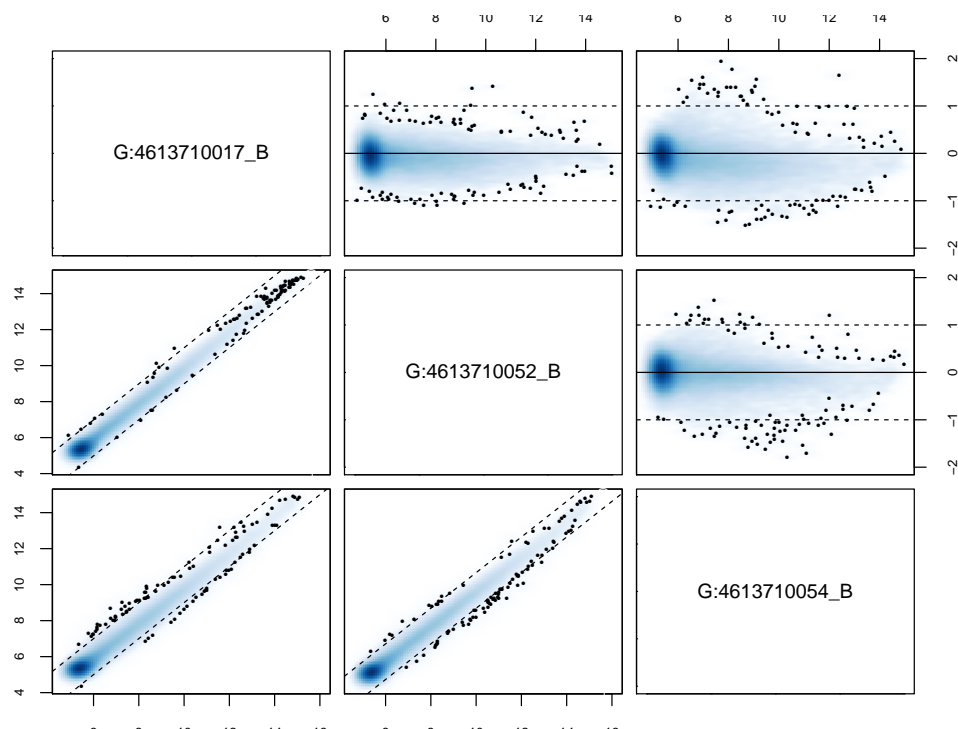
which is beyond the scope of this vignette. In the below code, we assign the results of `boxplot` to objects that we combine using viewports (a concept from the `grid` graphics system). The code also demonstrates how aspects of the plot can be altered programmatically.

```
> require("gridExtra")
> bp1 <- boxplot(exampleSummaryData.log2[ids, ], sampleFactor = "SampleFac",
+   probeFactor = "IlluminaID") + labs(title = "ALB expression level comparison") +
+   xlab("Illumina Probe") + ylab("Log2 Intensity")
> bp2 <- boxplot(exampleSummaryData.log2[randIDs, ], probeFactor = "Status") +
+   labs(title = "Control Probe Comparison")
> print(bp1, vp = viewport(width = 0.5, height = 1, x = 0.25, y = 0.5))
> print(bp2, vp = viewport(width = 0.5, height = 1, x = 0.75, y = 0.5))
```

Other exploratory analysis

Replicate samples can also be compared using the `plotMAXY`.

```
> plotMAXY(exprs(exampleSummaryData), arrays = 1:3, pch = 16, log = FALSE)
```



In the top right corner we see the MA plots for all pairwise comparisons involving the 3 arrays. On an MA plot, for each probe we plot the average of the log₂-intensities from the two arrays on the x-axis and the difference in intensities (log₂-ratios) on the y-axis. For replicate arrays we would expect all probes to be unchanged between the two samples and hence most points on the plot should 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 second array is systematically different to the other replicates and may benefit from normalisation. Both XY and MA plots are available separately for a particular comparison of arrays using `plotXY` and `plotMA`.

Normalisation

To correct for differences in expression level across a chip and between chips we need to normalise the signal to make the arrays comparable. The normalisation methods available in the `affy` package, or variance-stabilising transformation from the `lumi` package may be applied using the `normaliseIllumina` function. Below we quantile normalise the \log_2 transformed data.

```
> exampleSummaryData.norm <- normaliseIllumina(exampleSummaryData.log2,  
+       method = "quantile", transform = "none")
```

An alternative approach is to combine normal-exponential background correction with quantile normalisation as suggested in the `limma` package. However, this requires data that have not been log-transformed. Note that the control probes are removed from the output object

```
> exampleSummaryData.norm2 <- normaliseIllumina(channel(exampleSummaryData,  
+       "G.ul"), method = "neqc", transform = "none")
```

Filtering

Filtering non-responding probes from further analysis can improve the power to detect differential expression. One way of achieving this is to remove probes whose probe sequence has undesirable properties. Four basic annotation quality categories ('Perfect', 'Good', 'Bad' and 'No match') are defined and have been shown to correlate with expression level and measures of differential expression. We recommend removing probes assigned a 'Bad' or 'No match' quality score after normalization. This approach is similar to the common practice of removing lowly-expressed probes, but with the additional benefit of discarding probes with a high expression level caused by non-specific hybridization. You can verify the relationship between probe quality and intensity by using the `boxplot` function.

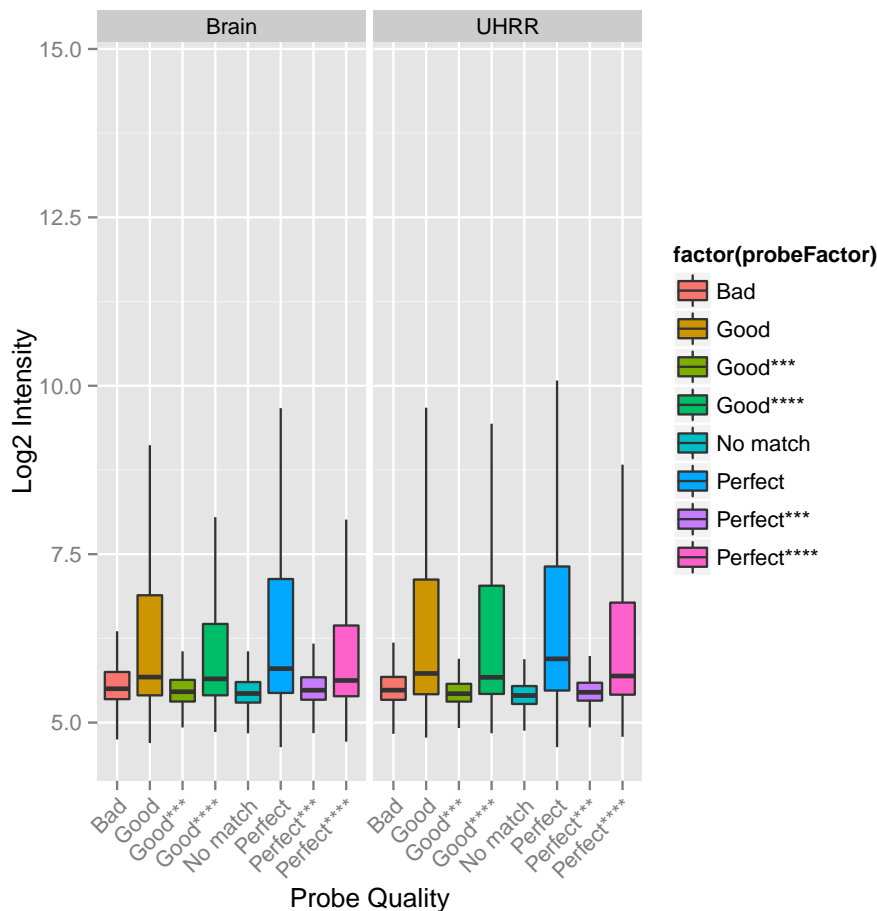
```
> library(illuminaHumanv3.db)  
> ids <- as.character(featureNames(exampleSummaryData.norm))  
> qual <- unlist(mget(ids, illuminaHumanv3PROBEQUALITY, ifnotfound = NA))  
> table(qual)
```

```
qual  
      Bad      Good  Good***  Good****  No match  Perfect  
    13475     925     148      358     1739    24687  
Perfect*** Perfect****  
    6269     1975
```

```
> rem <- qual == "No match" | qual == "Bad" | is.na(qual)  
> exampleSummaryData.filt <- exampleSummaryData.norm[!rem, ]  
> dim(exampleSummaryData.filt)
```

```
Features  Samples Channels  
    34362      12        1
```

```
> boxplot(exampleSummaryData.norm, probeFactor = "PROBEQUALITY",  
+       sampleFactor = "SampleFac") + opts(axis.text.x = theme_text(angle = 45,  
+       hjust = 1)) + xlab("Probe Quality") + ylab("Log2 Intensity")
```



Differential expression

The differential expression methods available in the `limma` package can be used to identify differentially expressed genes. The functions `lmFit` and `eBayes` can be applied to the normalised data. In the example below, we set up a design matrix for the example experiment and fit a linear model to summarise the data from the UHRR and Brain replicates to give one value per condition. We then define contrasts comparing the Brain sample to the UHRR and calculate moderated t -statistics with empirical Bayes shrinkage of the sample variances. In this particular experiment, the Brain and UHRR samples are very different and we would expect to see many differentially expressed genes.

Empirical array quality weights can be used to measure the relative reliability of each array. A variance is estimated for each array by the `arrayWeights` function which measures how well the expression values from each array follow the linear model. These variances are converted to relative weights which can then be used in the linear model to down-weight observations from less reliable arrays which improves power to detect differential expression. You should notice that some arrays have very low weight consistent with their poor QC.

We then define a contrast comparing UHRR to Brain Reference and calculate moderated t -statistics with empirical Bayes' shrinkage of the sample variances.

```
> library(limma)
```

```

> rna <- factor(pData(exampleSummaryData)[, "SampleFac"])
> design <- model.matrix(~0 + rna)
> colnames(design) <- levels(rna)
> aw <- arrayWeights(exprs(exampleSummaryData.filt), design)
> aw

          1          2          3          4          5          6          7
2.09018696 2.52678943 1.45410355 1.77470959 2.13405097 1.85777235 0.01233139
          8          9         10         11         12
0.11159911 2.45290622 2.04233539 2.08578184 1.28699294

> fit <- lmFit(exprs(exampleSummaryData.filt), design, weights = aw)
> contrasts <- makeContrasts(UHRR - Brain, levels = design)
> contr.fit <- eBayes(contrasts.fit(fit, contrasts))
> topTable(contr.fit, coef = 1)

          logFC AveExpr          t          P.Value      adj.P.Val          B
ILMN_1651358  7.344613 9.202611  87.76990 5.296571e-34 1.660766e-29 67.02204
ILMN_1796678  7.320711 9.608215  85.77897 9.666297e-34 1.660766e-29 66.49238
ILMN_1713458  6.419033 8.954098  78.22104 1.084133e-32 1.210370e-28 64.32882
ILMN_1783832  5.972782 8.323385  77.44244 1.408963e-32 1.210370e-28 64.09107
ILMN_1782939  6.822151 9.310045  76.19989 2.152332e-32 1.479169e-28 63.70543
ILMN_1688543  6.708307 9.032077  73.23368 6.088138e-32 3.123578e-28 62.75274
ILMN_1795679 -7.150689 9.069547 -73.11021 6.363148e-32 3.123578e-28 62.71207
ILMN_2084825  7.980186 9.899318  72.67719 7.433974e-32 3.193078e-28 62.56876
ILMN_1782204  6.275340 8.666149  69.87028 2.084422e-31 7.958322e-28 61.61410
ILMN_1665994  6.693724 8.725046  67.50738 5.127974e-31 1.762074e-27 60.77415

```

Annotation of results

The `topTable` function displays the results of the empirical Bayes analysis alongside the annotation assigned by Illumina to each probe in the linear model fit. Often this will not provide sufficient information to infer biological meaning from the results. Within Bioconductor, annotation packages are available for each of the major Illumina expression array platforms that map the probe sequences designed by Illumina to functional information useful for downstream analysis. As before, the `illuminaHumanv3.db` package can be used for the arrays in this example dataset.

```

> ids2 <- featureNames(exampleSummaryData.filt)
> chr <- mget(ids2, illuminaHumanv3CHR, ifnotfound = NA)
> cytoband <- mget(ids2, illuminaHumanv3MAP, ifnotfound = NA)
> refseq <- mget(ids2, illuminaHumanv3REFSEQ, ifnotfound = NA)
> entrezid <- mget(ids2, illuminaHumanv3ENTREZID, ifnotfound = NA)
> symbol <- mget(ids2, illuminaHumanv3SYMBOL, ifnotfound = NA)
> genename <- mget(ids2, illuminaHumanv3GENENAME, ifnotfound = NA)
> anno <- data.frame(ILL_ID = ids2, Chr = as.character(chr), Cytoband = as.character(cytoband),
+   RefSeq = as.character(refseq), EntrezID = as.numeric(entrezid),
+   Symbol = as.character(symbol), Name = as.character(genename))
> contr.fit$genes <- anno
> topTable(contr.fit)

```

	ILL_ID	Chr	Cytoband
ILMN_1651358	ILMN_1651358	11	11p15.5

```
ILMN_1796678 ILMN_1796678 11 11p15.5
ILMN_1713458 ILMN_1713458 16 16p13.3
ILMN_1783832 ILMN_1783832 X Xp11.4-p11.2
ILMN_1782939 ILMN_1782939 4 4q13.3
ILMN_1688543 ILMN_1688543 1 1q23.3
ILMN_1795679 ILMN_1795679 8 8q21.13
ILMN_2084825 ILMN_2084825 11 11p15.5
ILMN_1782204 ILMN_1782204 4 4q13.3
ILMN_1665994 ILMN_1665994 12 12q13-q14
```

```
ILMN_1651358
ILMN_1796678
ILMN_1713458
ILMN_1783832
ILMN_1782939
ILMN_1688543
ILMN_1795679 c("NM_001199214", "NM_007029", "NP_001186143", "NP_008960", "XM_005251141", "XM_005251142"
ILMN_2084825
ILMN_1782204
ILMN_1665994
```

```
c("NM_001200053", "NM_001
```

	EntrezID	Symbol	Name	logFC	AveExpr	t
ILMN_1651358	3046	HBE1	hemoglobin, epsilon 1	7.344613	9.202611	87.76990
ILMN_1796678	3047	HBG1	hemoglobin, gamma A	7.320711	9.608215	85.77897
ILMN_1713458	3050	HBZ	hemoglobin, zeta	6.419033	8.954098	78.22104
ILMN_1783832	2578	GAGE6	G antigen 6	5.972782	8.323385	77.44244
ILMN_1782939	213	ALB	albumin	6.822151	9.310045	76.19989
ILMN_1688543	336	APOA2	apolipoprotein A-II	6.708307	9.032077	73.23368
ILMN_1795679	11075	STMN2	stathmin-like 2	-7.150689	9.069547	-73.11021
ILMN_2084825	3048	HBG2	hemoglobin, gamma G	7.980186	9.899318	72.67719
ILMN_1782204	174	AFP	alpha-fetoprotein	6.275340	8.666149	69.87028
ILMN_1665994	6490	PMEL	premelanosome protein	6.693724	8.725046	67.50738

	P.Value	adj.P.Val	B
ILMN_1651358	5.296571e-34	1.660766e-29	67.02204
ILMN_1796678	9.666297e-34	1.660766e-29	66.49238
ILMN_1713458	1.084133e-32	1.210370e-28	64.32882
ILMN_1783832	1.408963e-32	1.210370e-28	64.09107
ILMN_1782939	2.152332e-32	1.479169e-28	63.70543
ILMN_1688543	6.088138e-32	3.123578e-28	62.75274
ILMN_1795679	6.363148e-32	3.123578e-28	62.71207
ILMN_2084825	7.433974e-32	3.193078e-28	62.56876
ILMN_1782204	2.084422e-31	7.958322e-28	61.61410
ILMN_1665994	5.127974e-31	1.762074e-27	60.77415

Reading bead summary data into beadarray

BeadStudio/GenomeStudio is Illumina's proprietary software for analyzing data output by the scanning system (BeadScan/iScan). It contains different modules for analyzing data from different platforms. For further information on the software and how to export summarized data, refer to the user's manual. In this section we consider how to read in and analyze output from the gene expression module of BeadStudio/GenomeStudio.

The example dataset used in this section consists of an experiment with one Human WG-6 version 2 BeadChip. These arrays were hybridized with the control RNA samples used in the MAQC project (3 replicates of UHRR and 3 replicates of Brain Reference RNA).

The non-normalized data for regular and control probes was output by BeadStudio/GenomeStudio.

The example BeadStudio output used in this section is available in the file `AsuragenMAQC_BeadStudioOutput.zip` which can be downloaded from <http://www.switchtoil.com/datasets.ilmn>.

You will need to download and unzip the contents of this file to the current R working directory. Inside this zip file you will find several files including summarized, non-normalized data and a file containing control information. We give a more detailed description of each of the particular files we will make use of below.

- Sample probe profile (`AsuragenMAQC-probe-raw.txt`) (*required*) - text file which contains the non-normalized summary values as output by BeadStudio. Inside the file is a data matrix with some 48,000 rows. In newer versions of the software, these data are preceded by several lines of header information. Each row is a different probe in the experiment and the columns give different measurements for the gene. For each array, we record the summarized expression level (`AVG_Signal`), standard error of the bead replicates (`BEAD_STDERR`), number of beads (`Avg_NBEADS`) and a detection *p*-value (`Detection Pval`) which estimates the probability of a gene being detected above the background level. When exporting this file from BeadStudio, the user is able to choose which columns to export.
- Control probe profile (`AsuragenMAQC-controls.txt`) (*recommended*) - text file which contains the summarized data for each of the controls on each array, which may be useful for diagnostic and calibration purposes. Refer to the Illumina documentation for information on what each control measures.
- targets file (*optional*) - text file created by the user specifying which sample is hybridized to each array. No such file is provided for this dataset, however we can extract sample annotation information from the column headings in the sample probe profile.

Files with normalized intensities (those with `avg` in the name), as well as files with one intensity value per gene (files with `gene` in the name) instead of separate intensities for different probes targeting the same transcript, are also available in this download. We recommend users work with the non-normalized probe-specific data in their analysis where possible. Illumina's background correction step, which subtracts the intensities of the negative control probes from the intensities of the regular probes, should also be avoided.

```
> library(beadarray)
> dataFile = "AsuragenMAQC-probe-raw.txt"
> qcFile = "AsuragenMAQC-controls.txt"
> BSData = readBeadSummaryData(dataFile = dataFile, qcFile = qcFile,
+   controlID = "ProbeID", skip = 0, qc.skip = 0, qc.columns = list(exprs = "AVG_Signal",
+   Detection = "Detection Pval"))
```

The arguments of `readBeadSummaryData` can be modified to suit data from versions 1, 2 or 3 of BeadStudio. The current default settings should work for version 3 output. Users may need to change the argument `sep`, which specifies if the `dataFile` is comma or tab delimited and the `skip` argument which specifies the number of lines of header information at the top of the file. Possible skip arguments of 0, 7 and 8 have been observed, depending on the version of BeadStudio or way in which the data was exported. The `columns` argument is used to specify which column headings to read from `dataFile` and store in various matrices. Note that the naming of the columns containing the standard

errors changed between versions of BeadStudio (earlier versions used BEAD STDEV in place of BEAD STDERR - be sure to check that the columns argument is appropriate for your data). Equivalent arguments (qc.sep, qc.skip and qc.columns) are used to read the data from qcFile. See the help page (?readBeadSummaryData) for a complete description of each argument to the function.

Citing beadarray

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

Dunning MJ, Smith ML, Ritchie ME, Tavaré S, **beadarray: R classes and methods for Illumina bead-based data**, *Bioinformatics*, **23**(16):2183-2184

1 Asking for help on beadarray

Wherever possible, questions about beadarray should be sent to the Bioconductor mailing list¹. This way, all problems and solutions will be kept in a searchable archive. When posting to this mailing list, please first consult the *posting guide*. In particular, state the version of beadarray and R that you are using², and try to provide a reproducible example of your problem. This will help us to diagnose the problem.

This vignette was built with the following versions of R and

```
> sessionInfo()
```

```
R version 3.0.2 (2013-09-25)
```

```
Platform: i386-w64-mingw32/i386 (32-bit)
```

```
locale:
```

```
[1] LC_COLLATE=C
```

```
[2] LC_CTYPE=English_United States.1252
```

```
[3] LC_MONETARY=English_United States.1252
```

```
[4] LC_NUMERIC=C
```

```
[5] LC_TIME=English_United States.1252
```

```
attached base packages:
```

```
[1] grid      parallel  stats      graphics  grDevices  utils      datasets
```

```
[8] methods  base
```

```
other attached packages:
```

```
[1] limma_3.18.0          gridExtra_0.9.1
```

```
[3] illuminaHumanv3.db_1.20.0 org.Hs.eg.db_2.10.1
```

```
[5] RSQLite_0.11.4        DBI_0.2-7
```

```
[7] AnnotationDbi_1.24.0   beadarrayExampleData_1.0.6
```

```
[9] beadarray_2.12.0       ggplot2_0.9.3.1
```

```
[11] Biobase_2.22.0         BiocGenerics_0.8.0
```

```
loaded via a namespace (and not attached):
```

```
[1] AnnotationForge_1.4.0 BeadDataPackR_1.14.0 IRanges_1.20.0
```

```
[4] KernSmooth_2.23-10    MASS_7.3-29          RColorBrewer_1.0-5
```

```
[7] colorspace_1.2-4      dichromat_2.0-0      digest_0.6.3
```

¹<http://www.bioconductor.org>

²This can be done by pasting the output of running the function sessionInfo().

[10]	gtable_0.1.2	labeling_0.2	munsell_0.4.2
[13]	plyr_1.8	proto_0.3-10	reshape2_1.2.2
[16]	scales_0.2.3	stats4_3.0.2	stringr_0.6.2
[19]	tools_3.0.2		