Using lumi, a package processing Illumina Microarray

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1 Overview of lumi

Illumina microarray is becoming a popular microarray platform. The BeadArray technology from Illumina makes its preprocessing and quality control different from other microarray technologies. Unfortunately, until now, most analyses have not taken advantage of the unique properties of the BeadArray system. The lumi Bioconductor package especially designed to process the Illumina microarray data, including Illumina Expression and Methylation microarray data. The lumi package provides an integrated solution for the bead-level Illumina microarray data analysis. The package covers data input, quality control, variance stabilization, normalization and gene annotation.

For details of processing Illumina methylation microarray, especially Infinium methylation microarray, please check another tutorial in lumi package: "Analyze Illumina Infinium methylation microarray data". All following description is focused on processing Illumina expression microarrays.

The lumi package provides unique functions for expression microarray processing. It includes a variance-stabilizing transformation (VST) algorithm [2] that takes advantage of the technical replicates available on every Illumina microarray. A robust spline normalization (RSN), which combines the features of the quantile and loess normalization, and simple scaling normalization (SSN) algorithms are also implemented in this package. Options available in other popular normalization methods are also provided. Multiple quality control plots for expression and control probe data are provided in the package. To better annotate the Illumina data, a new, vendor independent nucleotide universal identifier (nuID) [3] was devised to identify the probes of Illumina microarray. The nuID indexed Illumina annotation packages is compatible with other Bioconductor annotation packages. Mappings from Illumina Target Id or Probe Id to nuID are also included in the annotation packages. The output of lumi processed results can be easily integrated with other microarray data analysis, like differentially expressed gene identification, gene ontology analysis or clustering analysis.

2 Citation

For the people using lumi package, please cite the following papers in your publications.

* For the package:

* For the VST (variance stabilization transformation) algorithm, please cite:

* For nuID annotation packages, please cite:

Thanks for your help!

3 Installation of lumi package

In order to install the lumi package, the user needs to first install R, some related Bioconductor packages. You can easily install them by the following codes.

```r
source("http://bioconductor.org/biocLite.R")
biocLite("lumi")
```

For the users want to install the latest developing version of lumi, which can be downloaded from the developing section of Bioconductor website. Some additional packages may be required to be installed because of the update the Bioconductor. These packages can also be found from the developing section of Bioconductor website. You can also directly install the source packages from the Bioconductor website by specify the developing version number, which can be found at the Bioconductor website. Suppose the developing version is 2.3, to install the latest lumi pakcage in the Bioconductor developing version, you can use the following command:

```r
## replace "xxx" with the Bioconductor version number.
install.packages("lumi",repos="http://www.bioconductor.org/packages/xxx/bioc",type="source")
```

An Illumina benchmark data package `lumiBarnes` can be downloaded from Bioconductor Experiment data website.

4 Object models of major classes

The lumi package has one major class: LumiBatch. LumiBatch is inherited from ExpressionSet class in Bioconductor for better compatibility. Their relations are shown in Figure 1. LumiBatch class includes se.exprs, beadNum and detection in assayData slot for additional informations unique to Illumina microarrays. A controlData slot is used to keep the control probe information, and a QC slot is added for keeping the quality control information. The S4 function plot supports different kinds of plots by specifying the specific plot type of LumiBatch object. See help of plot-methods function for details. The history slot records all the operations made on the LumiBatch object. This provides data provenance. Function getHistory is to retrieve the history slot. Please see the help files of LumiBatch class for more details. A series of functions: lumiR, lumiR.batch, lumiB, lumiT, lumiN and lumiQ were designed for data input, preprocessing and quality control. Function lumiExpresso encapsulates the preprocessing methods for easier usability.

5 Data preprocessing

The first thing is to load the lumi package.

```r
> library(lumi)
```
Figure 1: Object models in lumi package

**class: ExpressionSet**

Slots
- assayData
  - exprs: gene expression (mean of bead replicates)
  - featureData: identifier mapping and annotation
  - phenoData: sample information and experiment design

**class: LumiBatch**

Slots
- assayData
  - se.exprs: expression standard error of bead replicates
  - beadNum: bead replicate number of each gene
  - detection: p-value of expression detectability
- controlData: a data.frame keeping control probe measurements
- QC: a list keeping the quality control information
- history: a data.frame recording previous operation over the object

Major methods
- lumiR, lumiR.batch: read data from BeadStudio output text file
- lumiB: background correction
- lumiT: variance stabilizing transformation
- lumiN: normalization
- lumiQ: quality control evaluation
- lumiExpresso: encapsulate all preprocessing functions
- plot: MAplot, pairs, boxplot, density, sample relation, hist, cv
- summary: summary of the data or QC information
- getHistory: retrieve the previous operation over the object
Other functions dealing with control probe data, annotations

......
5.1 Intelligently read the BeadStudio output file

The \texttt{lumiR} function supports directly reading the Illumina raw data output of the Illumina Bead Studio toolkit from version 1 to version 3. It can automatically detect the BeadStudio output version and format and create a new \texttt{LumiBatch} object for it. An example of the input data format is shown in in Figure\ref{fig:input_data}. For simplicity, only part of the data of first sample is shown. The data in the highlighted columns are kept in the corresponding slots of \texttt{LumiBatch} object, as shown in Figure\ref{fig:input_data}. The \texttt{lumiR} function will automatically determine the starting line of the data. The columns with header including \texttt{AVG\_Signal} and \texttt{BEAD\_STD} are required for the \texttt{LumiBatch} object. By default, the sample IDs and sample labels are extracted from the column names of the data file. For example, based on the column name: \texttt{AVG\_Signal-1304401001\_A}, we will extract "1304401001" as the sample ID and "A" as the sample label (The function assumes the separation of the sample ID and the sample label is "_" if it exists in the column name.). The function will check the uniqueness of sample IDs. If the sample ID is not unique, the entire portion after removing "AVG\_Signal" will be used as a sample ID. The user can suppress this parsing by setting the parameter "parseColumnName" as FALSE.

The \texttt{lumiR} will automatically initialize the QC slot of the \texttt{LumiBatch} object by calling \texttt{lumiQ}. If BeadStudio outputted the control probe data, their information will be kept in the controlData slot of the \texttt{LumiBatch} object. If BeadStudio outputted the sample summary information, which is called [Samples Table] in the output text file, the information will be kept in BeadStudio-Summary within the QC slot of the \texttt{LumiBatch} object.

The BeadStudio can output the gene profile or the probe profile. As the probe profile provides unique mapping from the probe Id to the expression profile, outputting probe profile is preferred. When the probe profile is outputted, as show in Figure\ref{fig:input_data}(B), the ProbeId column will be used as the identifier of \texttt{LumiBatch} object.

We strongly suggest outputting the header information when using BeadStudio, as shown in Figure\ref{fig:input_data}. Please refer to the separate document ("Resolve the Inconsistency of Illumina Identifiers through nuID Annotation") in the lumi package for more details of the changing of BeadStudio output formats.

The recent version of BeadStudio can also output the annotation information together with the expression data. In the users also want to input the annotation information, they can set the parameter "inputAnnotation" as TRUE. At the same time, they can also specify which columns to be inputted by setting parameter "annotationColumn". The BeadStudio annotation columns include: \texttt{SPECIES}, \texttt{TRANSCRIPT}, \texttt{ILMN\_GENE}, \texttt{UNIGENE\_ID}, \texttt{GI}, \texttt{ACCESSION}, \texttt{SYMBOL}, \texttt{PROBE\_ID}, \texttt{ARRAY\_ADDRESS\_ID}, \texttt{PROBE\_TYPE}, \texttt{PROBE\_START}, \texttt{PROBE\_SEQUENCE}, \texttt{CHROMOSOME}, \texttt{PROBE\_CHR\_ORIENTATION}, \texttt{PROBE\_COORDINATES}, \texttt{DEFINITION}, \texttt{ONTOLOGY\_COMPONENT}, \texttt{ONTOLOGY\_PROCESS}, \texttt{ONTOLOGY\_FUNCTION}, \texttt{SYNONYMS}, \texttt{OBsolete\_PROBE\_ID}. As the annotation data is huge, by default, we only input: \texttt{ACCESSION}, \texttt{SYMBOL}, \texttt{PROBE\_START}, \texttt{CHROMOSOME}, \texttt{PROBE\_CHR\_ORIENTATION}, \texttt{PROBE\_COORDINATES}, \texttt{DEFINITION}. As some annotation information may be outdated. We recommend using Bioconductor annotation packages to retrieve the annotation information.

For convenience, another function \texttt{lumiR\_batch} is designed to input files in
Figure 2: An example of the input data format
batch. Basically it combines the output of each file. See the help of lumiR.batch for details. lumiR.batch function also allows users to add sample information in the phenoData slot of the LumiBatch object. This will be useful in the data analysis. The sampleInfoFile parameter is optional. The file is a Tab-separated text file. The first ID column is required. It represents sample ID, which is defined based on the column names of BeadStudio output file. For example, sample ID of column "1881436070_A_STA.AVG_Signal" is "1881436070_A_STA". The sample ID column can also be found in the "Samples Table.txt" file output by BeadStudio. Another "Label" column (if provided) will be used as the sampleNames of LumiBatch object. All information of sampleInfoFile will be directly added in the phenoData slot in LumiBatch object, which can be retrieved by the command like: pData(phenoData(x.lumi)).

```r
> ## specify the file name
> # fileName <- 'Barnes_gene_profile.txt'
> ## load the data
> # x.lumi <- lumiR.batch(fileName, sampleInfoFile='sampleInfo.txt')
# Not Run
```

Here, we just load the pre-saved example data, example.lumi, which is a subset of the experiment data package lumiBarnes in the Bioconductor. The example data includes four samples "A01", "A02", "B01" and "B02". "A" and "B" represent different Illumina slides (8 microarrays on each slide), and "01" and "02" represent different samples. That means "A01" and "B01" are technique replicates at different slides, the same for "A02" and "B02".

```r
> ## load example data (a LumiBatch object)
> data(example.lumi)
> ## summary of the example data
> example.lumi
```

Summary of data information:
Illumina Inc. BeadStudio version 1.4.0.1
Normalization = none
Array Content = 11188230_100CP_MAGE-ML.XML
Error Model = none
DateTime = 2/3/2005 3:21 PM
Local Settings = en-US

Major Operation History:
```
submitted    finished
1 2007-04-22 00:08:36 2007-04-22 00:10:36
2 2007-04-22 00:10:36 2007-04-22 00:10:38
3 2007-04-22 00:13:06 2007-04-22 00:13:10
4 2007-04-22 00:59:20 2007-04-22 00:59:36
```

command lumiVersion
```
1      lumiR("./data/Barnes_gene_profile.txt")  1.1.6
2      lumiQ(x.lumi = x.lumi)                 1.1.6
3      addNuId2lumi(x.lumi = x.lumi, lib = "lumiHumanV1") 1.1.6
4      Subsetting 8000 features and 4 samples. 1.1.6
```
5.2 Quality control of the raw data

The quality control of a LumiBatch object includes a data summary (the mean and standard deviation, sample correlation, detectable probe ratio of each sample (microarray)), different quality control plots, and the control probe information.

BeadStudio will usually separately output (or attached after the expressed data in the same file) the control probe (gene) information, usually named as "Control Probe Profile.txt". The controlData slot in LumiBatch class is designed to keep the control probe (gene) information. The control probe file can be inputted by using function getControlData or directly add it to a LumiBatch object by using function addControlData2lumi. Several functions plotControlData, plotHousekeepingGene and plotStringencyGene are designed to plot control probe data. Please see their help files for more details.

LumiQ function will produce the data summary of a LumiBatch object and organize the results in a QC slot of LumiBatch object. When creating the LumiBatch object, the lumiQ function will be called to initialize the QC slot of the LumiBatch object.

Summary of the quality control information of example.lumi data. If the QC slot of the LumiBatch object is empty, function lumiQ will be automatically called to estimate the quality control information.

```r
> ## summary of the quality control
> summary(example.lumi, 'QC')
```

Data dimension: 8000 genes x 4 samples

Summary of Samples:

<table>
<thead>
<tr>
<th></th>
<th>A01</th>
<th>A02</th>
<th>B01</th>
<th>B02</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>8.3240</td>
<td>8.568</td>
<td>8.2580</td>
<td>8.3470</td>
</tr>
<tr>
<td>standard deviation</td>
<td>1.5580</td>
<td>1.686</td>
<td>1.7230</td>
<td>1.6690</td>
</tr>
<tr>
<td>detection rate(0.01)</td>
<td>0.5432</td>
<td>0.564</td>
<td>0.5774</td>
<td>0.5758</td>
</tr>
</tbody>
</table>
distance to sample mean 76.9500 65.2800 88.3200 49.1100

Major Operation History:

<table>
<thead>
<tr>
<th></th>
<th>submitted</th>
<th>finished</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2007-04-22 00:08:36</td>
<td>2007-04-22 00:10:36</td>
</tr>
<tr>
<td>2</td>
<td>2007-04-22 00:10:36</td>
<td>2007-04-22 00:10:38</td>
</tr>
</tbody>
</table>

command lumiVersion

1 lumiR("./data/Barnes_gene_profile.txt")  1.1.6
2 lumiQ(x.lumi = x.lumi)  1.1.6

The S4 method plot can produce the quality control plots of LumiBatch object. The quality control plots includes: the density plot (Figure 4), box plot (Figure 5), pairwise scatter plot between microarrays (Figure 6) or pair scatter plot with smoothing (Figure 7), pairwise MAplot between microarrays (Figure 8) or MAplot with smoothing (Figure 9), density plot of coefficient of variance, (Figure 10), and the sample relations (Figure 11). More details are in the help of plot,LumiBatch-method function. Most of these plots can also be plotted by the extended general functions: density (for density plot), boxplot, MAplot, pairs and plotSampleRelation.

Figure 4 shows the density plot of the LumiBatch object by using plot or density functions.

```r
> ## plot the density
> plot(example.lumi, what='density')
> ## or
> density(example.lumi)
```

Figure 4 shows the density plot of the LumiBatch object by using plot or density functions.

```r
> ## plot the density
> plot(example.lumi, what='density')
> ## or
> density(example.lumi)
```

Figure 6 shows the pairwise sample correlation of the LumiBatch object by using plot or pairs functions.

```r
> ## plot the pair plot
> plot(example.lumi, what='pair')
> ## or
> pairs(example.lumi)
> ## pairwise scatter plot with smoothing
> pairs(example.lumi, smoothScatter=T)
```
Figure 3: Density plot of Illumina microarrays before normalization

Figure 4: Density plot of Illumina microarrays before normalization
Figure 5: Cumulative Distribution Function (CDF) plot of Illumina microarrays before normalization
Figure 6: Pairwise plot with microarray correlation before normalization
Figure 7: Pairwise plot with microarray correlation before normalization
The density plot of the coefficient of variance of the LumiBatch object. See Figure [10] Figure [10] shows the density plot of the coefficient of variance of the LumiBatch object by using plot function.

Figure [11] shows the sample relations using hierarchical clustering.

Figure [12] shows the sampleRelation using MDS. The color of the sample is based on the sample type, which is "01", "02", "01", "02" for the sample data. Please see the help of plotSampleRelation and plot-methods for more details.
Pairwise MA plots between samples

A01

Median: −0.188
IQR: 0.342

A02

Median: 0.0704
IQR: 0.391

B01

Median: 0.246
IQR: 0.296

B02

Median: 0.225
IQR: 0.236

Median: −0.0293
IQR: 0.323

Figure 9: Pairwise MA plot with smoothing before normalization
> ## density plot of coefficient of variance
> plot(example.lumi, what='cv')

Density plot of coefficient of variance

Figure 10: Density Plot of Coefficient of Varience
> plot(example.lumi, what='sampleRelation')

Sample relations based on 1695 genes with sd/mean > 0.1

Figure 11: Sample relations before normalization
Sample relations based on 1695 genes with sd/mean > 0.1

Figure 12: Sample relations before normalization

```r
> ## plot the sample relations
> plot(example.lumi, what='sampleRelation', method='mds', color=c("01", "02", "01", "02"))
> ## or
> plotSampleRelation(example.lumi, method='mds', color=c("01", "02", "01", "02"))
```

5.3 Background correction

The `lumi` package provides `lumiB` function for background correction. We suppose the BeadStudio output data has been background corrected. Therefore, no background correction used by default. A method `bgAdjust` is designed to approximate what BeadStudio does for background adjustment. In the case when `log2` transform is used in the `lumiT` step, the background correction method (`forcePositive`) will be automatically used, which basically adds an offset (minus minimum value plus one) if there is any negative values to force all expression values to be positive. If users are more interested in the low level background correction, please refer to the package `beadarray` for more details. Users can also provide their own background correction function with a LumiBatch Object as
the first argument and return a LumiBatch Object with background corrected. See lumiB help document for more details.

5.4 Variance stabilizing transform

Variance stabilization is critical for subsequent statistical inference to identify differential genes from microarray data. We devised a variance-stabilizing transformation (VST) by taking advantages of larger number of technical replicates available on the Illumina microarray. Please see [2] for details of the algorithm.

Because the STDEV (or STDERR) columns of the BeadStudio output file is the standard error of the mean of the bead intensities corresponding to the same probe. (Thanks Gordon Smyth kindly provided this information!). As the variance stabilization (see help of vst function) requires the information of the standard deviation instead of the standard error of the mean, the value correction is required. The corrected value will be $x \times \sqrt{N}$, where $x$ is the old value (standard error of the mean), $N$ is the number of beads corresponding to the probe. The parameter `stdCorrection` of lumiT determines whether to do this conversion and is effective only when the `vst` method is selected. By default, the parameter `stdCorrection` is `TRUE`.

Function lumiT performs variance stabilizing transform with both input and output being LumiBatch object.

Do default VST variance stabilizing transform

```r
> ## Do default VST variance stabilizing transform
> lumi.T <- lumiT(example.lumi)
```

Perform vst transformation ...

```
2016-01-12 18:41:09 , processing array 1
2016-01-12 18:41:09 , processing array 2
2016-01-12 18:41:09 , processing array 3
2016-01-12 18:41:09 , processing array 4
```

The plotVST can plot the transformation function of VST, see Figure [13], which is close to log2 at high expression values, see Figure [13]. Function lumiT also provides options to do "log2" or "cubicRoot" transform. See help of lumiT for details.

```r
> ## plot VST transformation
> trans <- plotVST(lumi.T)
> ## compare the log2 and VST transform
> matplot(log2(trans$untransformed), trans$transformed, main='compare VST and log2 transformation',
```

5.5 Data normalization

lumi package provides several normalization method options, which include quantile, SSN (Simple Scaling Normalization), RSN (Robust Spline Normalization), loess normalization and Rank Invariant Normalization.

Comparing with other normalization methods, like quantile and curve-fitting methods, SSN is a more conservative method. The only assumption is that each sample has the same background level and the same scale (if do scaling). It basically make all the samples have the same background level and the same scale
Figure 13: VST transformation
compare VST and log2 transform

Figure 14: Compare VST and log2 transform
comparing to the background (if do scaling). There are three methods (‘density’, ‘mean’ and ‘median’) for background estimation. If bgMethod is ‘none’, then no background adjustment. For the ‘density’ bgMethod, it estimates the background based on the mode of probe intensities based on the assumption that the background level intensity is the most frequent value across all the probes in the chip. For the foreground level estimation, it also provides three methods (‘mean’, ‘density’, ‘median’). For the ‘density’ fgMethod, it assumes the foreground probe levels are symmetrically distributed. The foreground levels were estimated by taking the intensity mean of all other probes except from the background probes. For the ‘mean’ and ‘median’ methods (for both bgMethod and fgMethod), it basically estimates the level based on the mean or median of all probes of the sample. If the fgMethod is the same as bgMethod (except ‘density’ method), no scaling will be performed.

Another normalization method which is unique in the lumi package is the Robust Spline Normalization (RSN) algorithm. RSN combines the features of quantile and loess normalization. The advantages of quantile normalization include computational efficiency and preserving the rank order of genes. However, the intensity transformation of a quantile normalization is discontinuous because the normalization forces the intensity values for different samples (microarrays) having exactly the same distribution. This can cause small differences among intensity values to be lost. In contrast, the loess or spline normalization provides a continuous transformation. However, these methods cannot ensure that the rank of the probes remain unchanged across samples. Moreover, the loess normalization assumes the majority of the genes measured by the probes are non-differentially expressed and their distribution is approximately symmetric, which may not be a good assumption. To address some of these concerns, we developed a Robust Spline Normalization (RSN) method, which combines features from loess and quantile normalization methods. We use a monotonic spline to calibrate one microarray to the reference microarray. To increase the robustness of the spline method, we down-weight the contributions of probes of putatively differentially expressed genes. The probe intensities that are from potentially differentially expressed genes are heuristically determined as follows: First, we run a quantile normalization. Next, we estimate the fold-change of a gene measured by a probe based on the quantile-normalized data. The weighting factor for a probe is calculated based on a Gaussian window function. More details will be shown in a separate manuscript.

The default normalization method used in the Illumina BeadStudio software is Rank Invariant Normalization. In order to support similar functionalities, the lumi package also provides a similar normalization implementation call "rankinvariant" (We thanks Arno Velds implemented this function.). Please check the help of rankinvariant for more details.

By default, function lumiN performs popular quantile normalization. lumiN also provides other options to do "rsn", "ssn", "loess", "vsn", "rankinvariant" normalization. See help of lumiN for details.

Do default quantile between microarray normalization

```r
> ## Do quantile between microarray normalization
> lumi.N <- lumiN(lumi.T)
```

Perform quantile normalization ...
Users can also easily select other normalization method. For example, the following command will run RSN normalization.

```r
> ## Do RSN between microarray normalization
> lumi.N <- lumiN(lumi.T, method='rsn')
```

### 5.6 Quality control after normalization

To make sure the data quality meets our requirement, we do a second round of quality control of normalized data with different QC plots. Compare the plots before and after normalization, we can clearly see the improvements.

```r
> ## Do quality control estimation after normalization
> lumi.N.Q <- lumiQ(lumi.N)

Perform Quality Control assessment of the LumiBatch object ...

> ## summary of the quality control
> summary(lumi.N.Q, 'QC') ## summary of QC

Data dimension: 8000 genes x 4 samples

Summary of Samples:

<table>
<thead>
<tr>
<th></th>
<th>A01</th>
<th>A02</th>
<th>B01</th>
<th>B02</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>8.843</td>
<td>8.843</td>
<td>8.843</td>
<td>8.843</td>
</tr>
<tr>
<td>standard deviation</td>
<td>1.335</td>
<td>1.335</td>
<td>1.335</td>
<td>1.335</td>
</tr>
<tr>
<td>detection rate(0.01)</td>
<td>0.5432</td>
<td>0.564</td>
<td>0.5774</td>
<td>0.5758</td>
</tr>
<tr>
<td>distance to sample mean</td>
<td>15.3300</td>
<td>15.080</td>
<td>15.3200</td>
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Major Operation History:

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</tr>
<tr>
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</tr>
<tr>
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<td>2016-01-12 18:41:09</td>
<td>2016-01-12 18:41:09</td>
</tr>
<tr>
<td>6</td>
<td>2016-01-12 18:41:09</td>
<td>2016-01-12 18:41:09</td>
</tr>
<tr>
<td>7</td>
<td>2016-01-12 18:41:09</td>
<td>2016-01-12 18:41:09</td>
</tr>
</tbody>
</table>

command lumiVersion

1   lumiR("./data/Barnes_gene_profile.txt") 1.1.6
2   lumiQ(x.lumi = x.lumi) 1.1.6
3   addNuId2lumi(x.lumi = x.lumi, lib = "lumiHumanV1") 1.1.6
4   Subsetting 8000 features and 4 samples. 1.1.6
5   lumiT(x.lumi = example.lumi) 2.23.1
6   lumiN(x.lumi = lumi.T) 2.23.1
7   lumiQ(x.lumi = lumi.N) 2.23.1

### 5.7 Encapsulate the processing steps

The `lumiExpresso` function is to encapsulate the major functions of Illumina preprocessing. It is organized in a similar way as the `expresso` function in `affy`
> plot(lumi.N.Q, what='density')  # plot the density

Figure 15: Density plot of Illumina microarrays after normalization
> plot(lumi.N.Q, what='boxplot')  # box plot
> # boxplot(lumi.N.Q)

Figure 16: Density plot of Illumina microarrays after normalization
> plot(lumi.N.Q, what='pair')
## pairwise plots

Figure 17: Pairwise plot with microarray correlation after normalization
> plot(lumi.N.Q, what='MAplot')  ## plot the pairwise MAplot

Pairwise MA plots between samples

A01

Median: 0.0311
IQR: 0.177

A02

Median: −0.00341
IQR: 0.143

B01

Median: 0.0343
IQR: 0.169

B02

Median: 0.00118
IQR: 0.136

Figure 18: Pairwise MAplot after normalization
## plot the sampleRelation using hierarchical clustering

```r
plot(lumi.N.Q, what='sampleRelation')
```

Sample relations based on 3586 genes with sd/mean > 0.1

![Diagram](image)

Figure 19: Sample relations after normalization
## plot the sampleRelation using MDS

```r
plot(lumi.N.Q, what='sampleRelation', method='mds', color=c("01", "02", "01", "02"))
```

Sample relations based on 3586 genes with sd/mean > 0.1

Figure 20: Sample relations after normalization
package. The following code basically did the same processing as the previous multi-steps and produced the same results lumi.N.Q.

```r
> ## Do all the default preprocessing in one step
> lumi.N.Q <- lumiExpresso(example.lumi)
```

Background Correction: bgAdjust
Variance Stabilizing Transform method: vst
Normalization method: quantile

Background correction ...
Perform bgAdjust background correction ...
done.

Variance stabilizing ...
Perform vst transformation ...
2016-01-12 18:41:11 , processing array 1
2016-01-12 18:41:11 , processing array 2
2016-01-12 18:41:11 , processing array 3
2016-01-12 18:41:11 , processing array 4
done.

Normalizing ...
Perform quantile normalization ...
done.

Quality control after preprocessing ...
Perform Quality Control assessment of the LumiBatch object ...
done.

Users can easily customize the processing parameters. For example, if the user wants to do "rsn" normalization, the user can run the following code. For more details, please read the help document of lumiExpresso function.

```r
> ## Do all the preprocessing with customized settings
> # lumi.N.Q <- lumiExpresso(example.lumi, normalize.param=list(method='rsn'))
```

5.8 Inverse VST transform to the raw scale

Figure 14 shows VST is very close to log2 in the high expression range. In convenience, users usually can directly use $2^x$ to approximate the data in raw scale and estimate the fold-change. For the users concern more in the low expression range, we also provide the function inverseVST to resume the data in the raw scale. Need to mention, the inverse transform should be performed after statistical analysis, or else it makes no sense to transform back and forth. The inverseVST function can directly applied to the LumiBatch object after lumiT with VST transform, or VST transform plus RSN normalization (default method of lumiT). For the RSN normalized data, the inverse transform is based on the parameters of the Target Array because the Target Array is the benchmark data and is not changed after normalization. Other normalization methods, like
quantile or loess, will change the values of all the arrays. As a result, no inverse VST transform available for them. Users may use some kind of approximation for the quantile normalized data by themselves. Here we just provide some examples of VST parameters retrieving and inverse VST transform.

> ## Parameters of VST transformed LumiBatch object
> names(attributes(lumi.T))

```
[1] "history"    "controlData"   "QC"
[4] "assayData"  "phenoData"    "featureData"
[7] "experimentData" "annotation" "protocolData"
[10] ".__classVersion__" "class"   "vstParameter"
[13] "transformFun"
```

> ## VST parameters: "vstParameter" and "transformFun"
> attr(lumi.T, 'vstParameter')

```
a   b   g Intercept
A01 0.4944205 0.010158205 1.461128  5.484722
A02 1.4778404 0.009076935 1.502390  5.349984
B01 2.4462693 0.009447733 1.541250  5.006017
B02 2.5012403 0.008940373 1.547270  5.048591
```

> attr(lumi.T, 'transformFun')

```
A01 A02 B01 B02
"asinh" "asinh" "asinh" "asinh"
```

> ## Parameters of VST transformed and RSN normalized LumiBatch object
> names(attributes(lumi.N.Q))

```
[1] "history"    "controlData"   "QC"
[4] "assayData"  "phenoData"    "featureData"
[7] "experimentData" "annotation" "protocolData"
[10] ".__classVersion__" "class"   "vstParameter"
[13] "transformFun"
```

> ## VSN "targetArray" , VST parameters: "vstParameter" and "transformFun"
> attr(lumi.N.Q, 'vstParameter')

```
a   b   g Intercept
2.419753559 0.009512344 1.514310826  5.197110136
```

> attr(lumi.N.Q, 'transformFun')

```
[1] "asinh"
```

> ## After doing statistical analysis of the data, users can recover to the raw scale for
> ## Inverse VST to the raw scale
> lumi.N.raw <- inverseVST(lumi.N.Q)
6 Handling large data sets

Several users asked about processing large data set, e.g., over 100 samples. Directly handling such big data set usually will cause "out of memory" error in most computers. In this case, when read the BeadStudio output file, we can ignore the "beadNum" (related columns. The function \texttt{lumiR} provides a parameter called "columnNameGrepPattern". we can set the string grep pattern of "detection" and "beadNum" as NA. You can also ignore "detection" columns. However, the "detection" information is useful for the estimation of present count of each probe and used in the VST parameter estimation. To further save memory, you can suppress the input of annotation data by setting "inputAnnotation" as FALSE.

Here is some example code:

```r
## load the data with empty detection and beadNum slots, and without annotation information
> x.lumi <- lumiR("fileName.txt", columnNameGrepPattern=list(beadNum=NA), inputAnnotation=FALSE)
```

Usually, the large data set is composed of many small data files. In this case, the transformations, like log2 and \texttt{vst}, can be performed right after the input of each data file and some information can be removed in the object after transformation. \texttt{lumi} provides the \texttt{lumiR.batch} function for this purpose.

Here is some example code:

```r
## load the list of data files (a vector of file names) and do VST transformation for each file and combine the results.
> x.lumi <- lumiR.batch(fileList, transform='vst')
```

Another good news is that the normalization, like \texttt{rsn} and \texttt{ssn} in the \texttt{lumi} package, can sequentially process the data and handle such large data set.

The solution can be like this:

1. Read the data file by smaller batches (e.g. 10 or just one by one), and then do the variance stabilization for each data batch using \texttt{lumiR.batch} or \texttt{lumiR} function.
2. Pick one sample as the target array for normalization and then using "RSN" or "SSN" normalization method to normalize all batches of data using the same target array.
3. Combine the normalized data. (In order to save memory, the user can first remove those probes not expressed in all samples.)

In the \texttt{rsn} and \texttt{ssn} functions, there is a parameter called "targetArray", which is the model for other chips to normalize. It can be a column index, a vector or a LumiBatch object with one sample. In our case, we need to use one LumiBatch object with one sample as the "targetArray". The selection of the target array is flexible. We suggest to choose the one most similar to the mean of all samples. For convenience, we can also just select the first sample as "targetArray" (suppose it has no quality problem). The selected target array will also be used for all other data batches. Since different data batches use the same target array as model, the results are comparable and can be combined!

Here is the example code:

```r
## Read in the Batch ith data file, suppose named as "fileName.i.txt"
> x.lumi.i <- lumiR("fileName.i.txt")
## variance stabilization (using vst or log2 transform)
> x.lumiT.i <- lumiT(x.lumi.i)
## select the "targetArray"
## This target array will also be used for other batches of data.
## For convenience, here we just select the first sample as targetArray.
> targetArray <- x.lumiT.i[,1]
## Do RSN normalization
> x.lumiN.i <- lumiN(x.lumiT.i, method='rsn', targetArray=targetArray)

The normalized data batches can be combined by using function `combine(x, y)`.

7 Performance comparison

We have selected the Barnes data set [4], which is a series dilution of two tissues at five different dilutions, to compare different preprocessing methods. In order to better compare the algorithms, we selected the samples with the smallest dilution difference (the most challenging comparison), i.e., the samples with the dilution ratios of 100:0 and 95:5 (each condition has two technical replicates) for comparison. For the Barnes data set, because we do not know which of the signals are coming from ‘true’ differentially expressed genes, we cannot use an ROC curve to compare the performance of different algorithms. Instead, we evaluated the methods based on the concordance of normalized intensity profile and real dilution profile of the selected probes. More detailed evaluations with other criteria and based on other data sets can be found in our paper [2].

Following Barnes et al. (2005)[4], we defined a concordant gene (really a concordant probe) as a signal from a probe with a correlation coefficient larger than 0.8 between the normalized intensity profile and the real dilution profile (five dilution ratios with two replicates at each dilution ratio). If a selected differentially expressed probe is also a concordant one, it is more likely to be truly differentially expressed. Figure 21 shows the percentage of concordant probes among the selected probes, which were selected by ranking the probes’ p-value (calculated based on `limma` package) from low to high. We can see the VST transformed data outperforms the Log2-transformed and VSN processed data. For the normalization methods, RSN and quantile normalization have similar performance for the VST transformed data, and RSN outperforms quantile for the Log transformed data.

Please see another vignette in the lumi package: "lumi_vST_evaluation.pdf" for more details of the evaluation of VST (Variance Stabilizing Transformation).

8 Gene annotation

One challenge of Illumina microarray is the inconsistency and changes of Illumina identifiers across versions, even across different releases. This makes the integration of the Illumina data difficult. In order to resolve these problems, we invented a nuID (nucleotide universal IDentifier) annotation system, released related annotation packages and a website to provide identifier mapping and the latest annotation. Please refer to the separate document ("Resolve the Inconsistency of Illumina Identifiers through nuID Annotation") in the lumi package for more details.
9 A use case: from raw data to functional analysis

Figure 22 shows the data processing flow chart of the use case. Since the classes in \textit{lumi} package are inherited from class \textbf{ExpressionSet}, packages and functions compatible with class \textbf{ExpressionSet} or accepting matrix as input all can be used for \textit{lumi} results. Here we just give two examples: using \textit{limma} to identify differentiated genes and using \textit{GOstats} to annotate the significant genes.

We use the Barnes data set [4] as an example, which has be created as a Bioconductor experiment data package \textit{lumiBarnes}. The Barnes data set measured a dilution series of two human tissues, blood and placenta. It includes six samples with the titration ratio of blood and placenta as 100:0, 95:5, 75:25, 50:50, 25:75 and 0:100. The samples were hybridized on HumanRef-8 BeadChip (Illumina, Inc) in duplicate. We select samples with titration ratio, 100:0 and 95:5 (each has two technique replicates) in this data set to evaluate the detection of differential expressions.

9.1 Preprocess the Illumina data

\begin{verbatim}
> library(lumi)
> ## specify the file name
> # fileNanme <- 'Barnes_gene_profile.txt'   # Not run
> ## load the data if you want to provide the sample Information and map Illumina IDs to nuIDs.
> # example.lumi <- lumiR.batch(fileNanme, lib.mapping='lumiHumanIDMapping', sampleInfoFile=

\end{verbatim}
> ## load saved data
> data(example.lumi)
> ## summary of the data
> example.lumi
> ## summary of quality control information
> summary(example.lumi, 'QC')

> ## preprocessing and quality control after normalization
> lumi.N.Q <- lumiExpresso(example.lumi, QC.evaluation=TRUE)
> ## summary of quality control information after preprocessing
> summary(lumi.N.Q, 'QC')

> ## Output the data as Tab separated text file
> write.exprs(lumi.N.Q, file='processedExampleData.txt')

9.2 Identify differentially expressed genes

Identify the differentiated genes based on moderated t-test using limma.

Retrieve the normalized data

> dataMatrix <- exprs(lumi.N.Q)

To speed up the processing and reduce false positives, remove the unexpressed and un-annotated genes

> presentCount <- detectionCall(example.lumi)
> selDataMatrix <- dataMatrix[presentCount > 0,]
> if (require(lumiHumanAll.db) & require(annotate)) {
+   selDataMatrix <- selDataMatrix[!is.na(getSYMBOL(rownames(selDataMatrix), 'lumiHumanAll'))]
+ }
> probeList <- rownames(selDataMatrix)

> ## Specify the sample type
> sampleType <- c('100:0', '95:5', '100:0', '95:5')
> if (require(limma)) {
+   ## compare '95:5' and '100:0'
+   design <- model.matrix(~ factor(sampleType))
+   colnames(design) <- c('100:0', '95:5-100:0')
+   fit <- lmFit(selDataMatrix, design)
```r
+ fit <- eBayes(fit)
+ ## Add gene symbols to gene properties
+ if (require(lumiHumanAll.db) & require(annotate)) {
+   geneSymbol <- getSYMBOL(probeList, 'lumiHumanAll.db')
+   geneName <- sapply(lookUp(probeList, 'lumiHumanAll.db', 'GENENAME'), function(x) x[1])
+   fit$genes <- data.frame(ID= probeList, geneSymbol=geneSymbol, geneName=geneName, stringsAsFactors=FALSE)
+ }
+ ## print the top 10 genes
+ print(topTable(fit, coef='95:5-100:0', adjust='fdr', number=10))
+ ## get significant gene list with FDR adjusted p.values less than 0.01
+ p.adj <- p.adjust(fit$p.value[,2])
+ sigGene.adj <- probeList[ p.adj < 0.01]
+ ## without FDR adjustment
+ sigGene <- probeList[ fit$p.value[,2] < 0.01]
```
Based on the significant genes identified using limma or t-test, we can do further analysis, like GO analysis (GOstats package) and machine learning (MLInterface package). Next, we will use GO analysis as an example.

9.3 Gene Ontology and other functional analysis

Based on the interested genes identified using limma or other tests, we can further do functional analysis. We can use package GOstats, Category and other packages to do this type of analysis.

There is one important thing need to mention during this type of analysis. As we described in previous section, the lumi annotation packages are nUID indexed and are built by pooling all types of chips of the same species. This makes it different from the traditional Affymetrix annotation packages, which is one package for one type of chip. Because lots of methods in Category/GOstats were originally designed based on the Affymetrix annotation packages, the default setting of these function may not work well for lumi annotation packages. However, this can be solved by first transferring the probe ids as Entrez Ids, and then do analysis at the Entrez Id level instead of the probe Id level. Please see our example for how to transfer Probe Ids as Entrez Ids.

Following is an example of performing Hypergeometric test of Gene Ontology based on the significant gene list (for e. Table 1 shows the significant GO terms of Molecular Function with p-value less than 0.01. Here only show the significant GO terms of BP (Biological Process). For other GO categories MF(Molecular Function) and CC (Cellular Component), it just follows the same procedure.

```r
if (require(GOstats) & require(lumiHumanAll.db)) {
  ## Convert the probe Ids as Entrez Ids and make them unique
  sigLL <- unique(unlist(lookUp(sigGene,'lumiHumanAll.db','ENTREZID')))
  params <- new("GOHyperGParams",
                 geneIds= sigLL,
                 annotation="lumiHumanAll.db",
                 ontology="BP",
                 pvalueCutoff= 0.01,
                 conditional=FALSE,
                 testDirection="over")
}
```
+ hgOver <- hyperGTest(params)
+ ## Get the p-values of the test
+ gGhyp.pv <- pvalues(hgOver)
+ ## Adjust p-values for multiple test (FDR)
+ gGhyp.fdr <- p.adjust(gGhyp.pv, 'fdr')
+ ## select the Go terms with adjusted p-value less than 0.01
+ sigGO.ID <- names(gGhyp.fdr[gGhyp.fdr < 0.01])
+ ## Here only show the significant GO terms of BP (Molecular Function)
+ ## For other categories, just follow the same procedure.
+ sigGO.Term <- getGOTerm(sigGO.ID)["BP"]
+
<table>
<thead>
<tr>
<th>GO ID</th>
<th>Term</th>
<th>p-value</th>
<th>Significant Genes No.</th>
<th>Total Genes No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 GO:0009611</td>
<td>response to wound</td>
<td>8.4244e-06</td>
<td>42</td>
<td>443</td>
</tr>
<tr>
<td>2 GO:0006955</td>
<td>immune response</td>
<td>8.8296e-06</td>
<td>68</td>
<td>859</td>
</tr>
<tr>
<td>3 GO:0006952</td>
<td>defense response</td>
<td>1.7525e-05</td>
<td>72</td>
<td>945</td>
</tr>
<tr>
<td>4 GO:0006950</td>
<td>response to stress</td>
<td>1.9132e-05</td>
<td>81</td>
<td>1103</td>
</tr>
<tr>
<td>5 GO:0009607</td>
<td>response to biotic</td>
<td>5.0811e-05</td>
<td>72</td>
<td>976</td>
</tr>
<tr>
<td>6 GO:0009613</td>
<td>response to pest</td>
<td>7.2813e-05</td>
<td>45</td>
<td>533</td>
</tr>
<tr>
<td>7 GO:0006954</td>
<td>inflammatory response</td>
<td>0.00025402</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>8 GO:0009605</td>
<td>response to external</td>
<td>0.00026005</td>
<td>46</td>
<td>580</td>
</tr>
<tr>
<td>9 GO:0051707</td>
<td>response to other</td>
<td>0.00040553</td>
<td>45</td>
<td>575</td>
</tr>
<tr>
<td>10 GO:0051674</td>
<td>localization of c</td>
<td>0.00082563</td>
<td>30</td>
<td>348</td>
</tr>
<tr>
<td>11 GO:0006928</td>
<td>cell motility</td>
<td>0.00082563</td>
<td>30</td>
<td>348</td>
</tr>
<tr>
<td>12 GO:0040011</td>
<td>locomotion</td>
<td>0.00099205</td>
<td>30</td>
<td>352</td>
</tr>
</tbody>
</table>

Table 1: GO terms, p-values and counts.

### 9.4 GEO submission of the data

As more and more journals require the microarray data should be submitted to GEO website, we created GEO submission functions for users convenience. The submission file will be in the SOFT format. So users can submit all the data in a batch. There are two major functions users need to use. `produceGEOSampleInfoTemplate` produces a template of sample information with some default fillings. Some fields have been filed in with default settings. Users should fill in or modify the detailed sample descriptions by referring some previous submissions. No blank fields are allowed. Users are also suggested to fill in the "Sample_platform_id" by checking information of the GEO Illumina platform. `produceGEOSubmissionFile` is the main function of produce GEO submission file including both normalized and raw data information in the SOFT format. By default, the R objects, lumiNormalized, lumiRaw and sampleInfo, will be saved in a file named `supplementaryData.Rdata`. (See help information of
Users can include this R data file as a GEO supplementary data file.

## Produce the sample information template
```r
> produceGEOSampleInfoTemplate(lumi.example, lib.mapping = 'lumiHumanIDMapping', fileName = 'GEOsampleInfo.txt')
```

## After editing the 'GEOsampleInfo.txt' by filling in sample information
```r
> produceGEOSubmissionFile(lumi.N.Q, lumi.example, lib='lumiHumanIDMapping', sampleInfo='GEOsampleInfo.txt')
```

### 10 Session Info
```r
> toLatex(sessionInfo())
```

- R Under development (unstable) (2015-12-14 r69775), x86_64-pc-linux-gnu
- Locale: LC_CTYPE=en_US.UTF-8, LC_NUMERIC=C, LC_TIME=en_US.UTF-8, LC_COLLATE=C, LC_MONETARY=en_US.UTF-8, LC_MESSAGES=en_US.UTF-8, LC_PAPER=en_US.UTF-8, LC_NAME=C, LC_ADDRESS=C, LC_TELEPHONE=C, LC_MEASUREMENT=en_US.UTF-8, LC_IDENTIFICATION=C
- Base packages: base, datasets, grDevices, graphics, methods, parallel, stats, stats4, utils
- Other packages: AnnotationDbi 1.33.6, Biobase 2.31.3, BiocGenerics 0.17.2, DBI 0.3.1, IRanges 2.5.20, RSQLite 1.0.0, S4Vectors 0.9.19, XML 3.98-1.3, annotate 1.49.0, limma 3.27.9, lumi 2.23.1, lumiHumanAll.db 1.22.0, lumiHumanIDMapping 1.10.0, org.Hs.eg.db 3.2.3
- Loaded via a namespace (and not attached): BiocInstaller 1.21.2, BiocParallel 1.5.13, Biostrings 2.39.6, GEOquery 2.37.0, GenomeInfoDb 1.7.3, GenomicAlignments 1.7.12, GenomicFeatures 1.23.17, GenomicRanges 1.23.11, KernSmooth 2.23-15, MASS 7.3-45, Matrix 1.2-3, RCcolorBrewer 1.1-2, RCurl 1.95-4.7, Repp 0.12.3, Rsamtools 1.23.1, SummarizedExperiment 1.11.4, XVector 0.11.2, affy 1.49.0, affyio 1.41.0, base64 1.1, beanplot 1.2, biomaRt 2.27.2, bitops 1.0-6, bumphunter 1.9.3, codetools 0.2-14, colorspace 1.2-6, corpcom 1.6.8, digest 0.6.9, doRNG 1.6, ellipse 0.3-8, foreach 1.4.3, genefilter 1.53.0, ggplot2 2.0.0, grid 3.3.0, gtable 0.1.2, igraph 1.0.1, illuminaio 0.13.1, iterators 1.0.8, lattice 0.20-33, locfit 1.5-9.1, magrittr 1.5, matrixStats 0.50.1, mclust 5.1, methylumi 2.17.0, mgcv 1.8-10, minfi 1.17.1, mixOmics 5.2.0, multtest 2.27.0, munsell 0.4.2, nleqslv 2.9.1, nhme 3.1-122, nor1mix 1.2-1, pkgmaker 0.22, plyr 1.8.3, preprocessCore 1.33.0, quadprog 1.5-5, registry 0.3, reshape 0.8.5, rgl 0.95.1441, rngtools 1.2.4, rtracklayer 1.31.4, scales 0.3.0, siggenes 1.45.0, splines 3.3.0, stringi 1.0-1, stringr 1.0.0, survival 2.38-3, tools 3.3.0, xtable 1.8-0, zlibbioc 1.17.0

### 11 Acknowledgments

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12 References