# Estimating DNA methylation levels and finding differentially methylated regions using the charm package

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## 1 Introduction

The Bioconductor package charm can be used to analyze DNA methylation data generated using McrBC fractionation and two-color Nimblegen microarrays. It is customized for use with the from the custom CHARM microarray [1], but can also be applied to many other Nimblegen designs.

Functions include:

- Quality control
- Finding suitable control probes for normalization
- Percentage methylation estimates
- Identification of differentially methylated regions

As input we will need raw Nimblegen data (.xys) files and a corresponding annotation package built with pdInfoBuilder. This vignette uses the following packages:

- charm: contains the analysis functions
- charmData: an example dataset
- pd.charm.hg18.example: the annotation package for the example dataset
- BSgenome.Hsapiens.UCSC.hg18: A BSgenome object containing genomic sequence used for finding non-CpG control probes

Each sample is represented by two xys files corresponding to the untreated (green) and methyl-depleted (red) channels. The 532.xys and 635.xys suffixes indicate the green and red channels respectively.

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# 2 Analyzing data from the custom CHARM microarray

Load the charm package:

```
R> library(charm)
```

# 3 Read in raw data

Get the name of your data directory (in this case, the example data):

```
R> dataDir <- system.file("data", package = "charmData")
R> dataDir
```

```
[1] "/thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data"
```

First we read in the sample description file:

```
R> pd <- read.delim(file.path(dataDir, "phenodata.txt"))
R> pd
```

```
          filename   sampleID tissue
1   136421_532.xys  441_liver  liver
2   136421_635.xys  441_liver  liver
3   136600_532.xys 449_spleen spleen
4   136600_635.xys 449_spleen spleen
5  3788602_532.xys  449_liver  liver
6  3788602_635.xys  449_liver  liver
7  3822402_532.xys 441_spleen spleen
8  3822402_635.xys 441_spleen spleen
9  5739902_532.xys  624_colon  colon
10 5739902_635.xys  624_colon  colon
11 5875602_532.xys  441_colon  colon
12 5875602_635.xys  441_colon  colon
```

A valid sample description file should contain at least the following (arbitrarily named) columns:

- a filename column
- a sample ID column
- a group label column (optional)

The sample ID column is used to pair the methyl-depleted and untreated data files for each sample. The group label column is used when identifying differentially methylated regions between experimental groups.

The `validatePd` function can be used to validate the sample description file. When called with only a sample description data frame and no further options `validatePd` will try to guess the contents of the columns.

```
R> res <- validatePd(pd)

Filenames:
  fileNameColumn not specified. Trying to guess.
  OK - Found in column 1
Sample names:
  sampleNameColumn column not specified. Trying to guess.
  OK - Found in column 2
Group labels:
  groupColumn column not specified. Trying to guess.
  OK - Found in column 3
```

Now we read in the raw data. The `readCharm` command makes the assumption (unless told otherwise) that the two xys files for a sample have the same file name up to the suffixes 532.xys (untreated) and 635.xys (methyl-depleted).

```
R> rawData <- readCharm(files = pd$filename, path = dataDir,
     sampleKey = pd)

Checking designs for each XYS file... Done.
Allocating memory... Done.
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/136421_
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/136600_
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/3788602
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/3822402
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/5739902
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/5875602
Checking designs for each XYS file... Done.
Allocating memory... Done.
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/136421_
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/136600_
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/3788602
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/3822402
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/5739902
Reading /thumper/ctsa/genomicsR/install/R-2.10.0/lib64/R/site-library/charmData/data/5875602

R> rawData

TilingFeatureSet2 (storageMode: lockedEnvironment)
assayData: 243129 features, 6 samples
  element names: channel1, channel2
phenoData
  sampleNames: 136421, 136600, ..., 5875602  (6 total)
  varLabels and varMetadata description:
    sampleID: NA
    tissue: NA
    ...: ...
```

```
    channel2DateTime: date/time from raw files
    (6 total)
  additional varMetadata: channel
featureData
  featureNames: 1, 2, ..., 243129  (243129 total)
  fvarLabels and fvarMetadata description: none
experimentData: use 'experimentData(object)'
Annotation: pd.charm.hg18.example
```

# 4 Array quality assessment

We can calculate array quality scores and generate a pdf report with the `qcReport` command.

A useful quick way of assessing data quality is to examine the untreated channel where we expect every probe to have signal. Very low signal intensities on all or part of an array can indicate problems with hybridization or scanning. The CHARM array and many other designs include background probes that do not match any genomic sequence. Any signal at these background probes can be assumed to be the result of optical noise or cross-hybridization. Since the untreated channel contains total DNA a successful hybridization would have strong signal for all untreated channel genomic probes. The array signal quality score (pmSignal) is calculated as the average percentile rank of the signal robes among these background probes. A score of 100 means all signal probes rank above all background probes (the ideal scenario).

```
R> qual <- qcReport(rawData, file = "qcReport.pdf")
R> qual
```

```
        pmSignal       sd1       sd2
136421  78.56437 0.1950274 0.1932112
136600  81.46541 0.1755225 0.1227921
3788602 83.95419 0.1249030 0.2409803
3822402 81.43751 0.1180708 0.1824810
5739902 82.55727 0.1490854 0.2035761
5875602 79.38069 0.3130266 0.3962373
```

The PDF quality report is shown in Appendix A. Three quality metrics are calculated for each array:

1. Average signal strength: the average percentile rank of untreated channel signal probes among the background (anti-genomic) probes.

2. Untreated channel signal standard deviation. The array is divided into a series of rectangular blocks and the average signal level calculated for each. Since probes are arranged randomly on the array there should be no large differences between blocks. Arrays with spatial artifacts have a larg standard deviation between blocks.

3. Methyl-depleted channel signal standard deviation.

# 5 Percentage methylation estimates and differentially methylated regions (DMRs)

We now calculate probe-level percentage methylation estimates for each sample. As a first step we need to identify a suitable set of unmethylated control probes from CpG-free regions to be used in normalization.

```
R> library(BSgenome.Hsapiens.UCSC.hg18)
R> ctrlIdx <- getControlIndex(rawData, subject = Hsapiens)
```

The minimal code required to estimate methylation would be `p <- methp(rawData, controlIndex=ctrlIdx)`. However, it is often useful to get `methp` to produce a series of diagnostic density plots to help identify non-hybridization quality issues. The `plotDensity` option specifies the name of the output pdf file, and the optional `plotDensityGroups` can be used to give groups different colors.

```
R> grp <- pData(rawData)$tissue
R> p <- methp(rawData, controlIndex = ctrlIdx, plotDensity = "density.pdf",
     plotDensityGroups = grp)
```

```
Spatial normalization
Background removal
Within sample normalization: loess
Between sample normalization: quantile
Estimating percentage methylation
```

```
R> head(p)
```

```
      136421    136600   3788602   3822402   5739902
1 0.17250259 0.3895986 0.3881209 0.5793288 0.3813294
2 0.84184931 0.6773743 0.3465772 0.8999109 0.5788529
3 0.09046145 0.0641674 0.1614183 0.1426585 0.2339750
4 0.77692120 0.4944354 0.4772154 0.4760221 0.3861171
5 0.69668343 0.5593289 0.4191725 0.4469628 0.4110470
6 0.66978815 0.7949903 0.7856860 0.7403811 0.8982202
    5875602
1 0.2708504
2 0.9183155
3 0.7293811
4 0.4904633
5 0.4008233
6 0.8522786
```

The density plots are shown in Appendix B.

We can now identify differentially methylated regions using `dmrFinder`:

```
R> dmr <- dmrFinder(rawData, p = p, groups = grp,
     compare = c("colon", "liver", "colon", "spleen"))

Computing group medians and SDs for 3 groups:
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Done.
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Done.
Finding DMRs for each pairwise comparison.
 colon-liver..
 colon-spleen..
Done
```

*R> names(dmr)*

```
 [1] "tabs"         "p"            "l"
 [4] "chr"          "pos"          "pns"
 [7] "index"        "controlIndex" "gm"
[10] "groups"       "args"         "comps"
[13] "package"
```

```
R> names(dmr$tabs)

[1] "colon-liver"  "colon-spleen"

R> head(dmr$tabs[[1]])

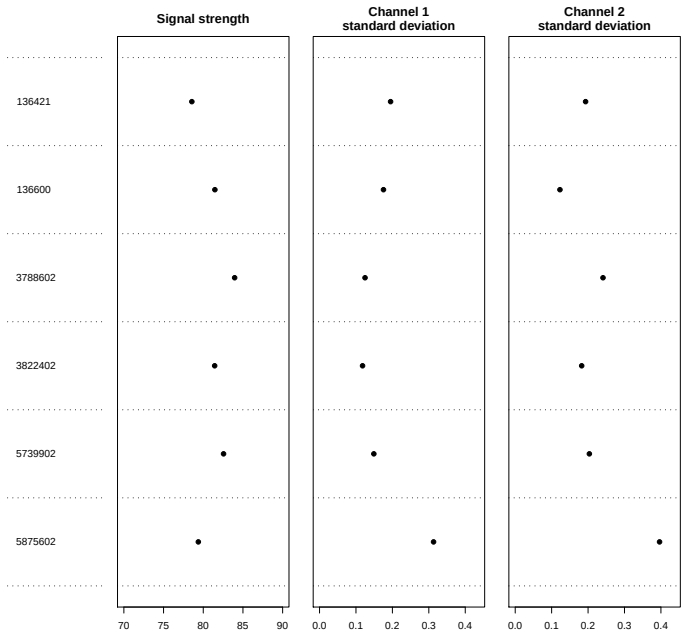
       chr    start      end        p1        p2
447  chr12 88272817 88273811 0.8804792 0.1408243
1773  chr6 52637747 52638747 0.7584872 0.1313882
492  chr13 27090247 27091263 0.8188426 0.1291102
151  chr11 14620645 14621065 0.8799767 0.3324493
643  chr15 58673117 58673711 0.8562095 0.2512823
795  chr17   198791   199552 0.8167333 0.2229383
                 regionName indexStart indexEnd      area
447  chr12:88266873-88274292      41215    41238 17.751716
1773  chr6:52635302-52638967     163819   163843 15.677477
492  chr13:27090144-27095500      46022    46041 13.794648
151  chr11:14620645-14623686      28888    28900  7.117856
643  chr15:58669815-58674073      59008    59024 10.283762
795      chr17:198024-209044      74031    74047 10.094516
       ttarea
447  818.4631
1773 731.6463
492  711.4216
151  478.1902
643  476.9114
795  425.0093
```

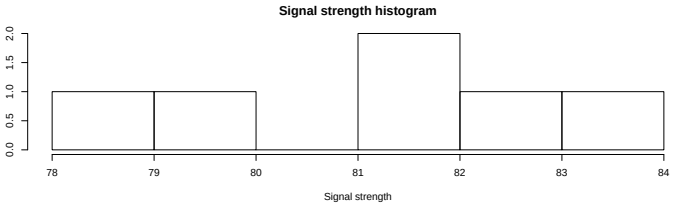
When called without the `compare` option, `dmrFinder` performs all pairwise comparisons between the groups.

# References

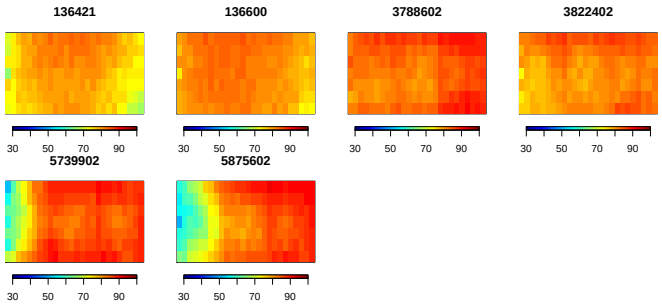
[1] Irizarry et al. Comprehensive high-throughput arrays for relative methylation (charm). *Genome Research*, 18(5):780–790, 2008.

# 6 Appendix A: Quality report

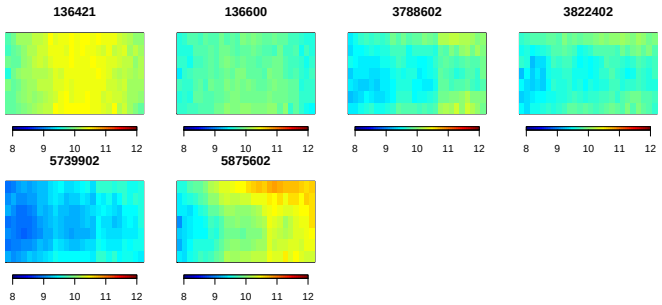

Signal strength
Channel 1
standard deviation
Channel 2
standard deviation
136421
136600
3788602
3822402
5739902
5875602
70
75
80
85
90
0.0
0.1
0.2
0.3
0.4
0.0
0.1
0.2
0.3
0.4


Signal strength histogram
0.0
0.5
1.0
1.5
2.0
78
79
80
81
82
83
84
Signal strength

Untreated Channel: PM probe quality

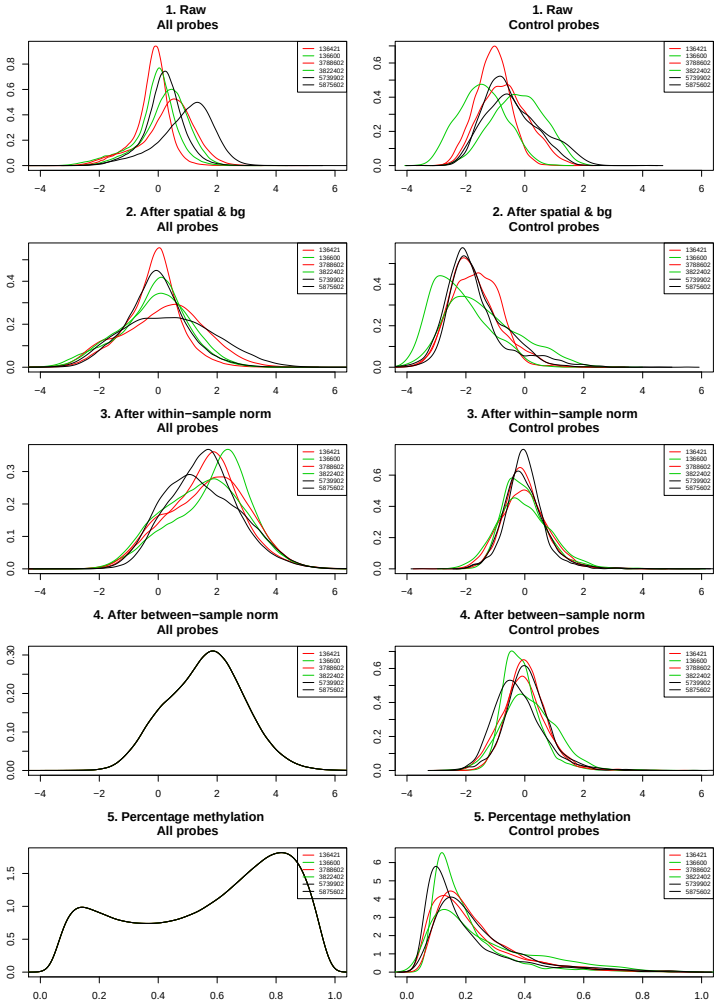


Enriched Channel: PM signal intensity



# 7 Appendix B: Density plots

Each row corresponds to one stage of the normalization process (Raw data, After spatial and background correction, after within-sample normalization, after between-sample normalization, percentage methylation estimates). The left column shows all probes, while the right column shows control probes.


1. Raw
All probes
1. Raw
Control probes
2. After spatial & bg
All probes
2. After spatial & bg
Control probes
3. After within−sample norm
All probes
3. After within−sample norm
Control probes
4. After between−sample norm
All probes
4. After between−sample norm
Control probes
5. Percentage methylation
All probes
5. Percentage methylation
Control probes
136421
136600
3788602
3822402
5739902
5875602

# 8 Details

This document was written using:

```
R> sessionInfo()
```

```
R version 2.10.0 (2009-10-26)
x86_64-unknown-linux-gnu

locale:
 [1] LC_CTYPE=en_US.iso885915
 [2] LC_NUMERIC=C
 [3] LC_TIME=en_US.iso885915
 [4] LC_COLLATE=en_US.iso885915
 [5] LC_MONETARY=C
 [6] LC_MESSAGES=en_US.iso885915
 [7] LC_PAPER=en_US.iso885915
 [8] LC_NAME=C
 [9] LC_ADDRESS=C
[10] LC_TELEPHONE=C
[11] LC_MEASUREMENT=en_US.iso885915
[12] LC_IDENTIFICATION=C

attached base packages:
[1] tools     stats     graphics  grDevices utils
[6] datasets  methods   base

other attached packages:
 [1] BSgenome.Hsapiens.UCSC.hg18_1.3.16
 [2] BSgenome_1.14.2
 [3] Biostrings_2.14.8
 [4] IRanges_1.4.8
 [5] pd.charm.hg18.example_0.9.0
 [6] RSQLite_0.7-3
 [7] DBI_0.2-4
 [8] charm_0.9.34
 [9] SQN_1.0
[10] nor1mix_1.1-1
[11] mclust_3.3.2
[12] fields_6.01
[13] spam_0.15-5
[14] oligo_1.10.2
[15] preprocessCore_1.8.0
[16] oligoClasses_1.8.0
[17] Biobase_2.6.0

loaded via a namespace (and not attached):
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[1] affxparser_1.18.0 affyio_1.14.0     gtools_2.6.1
[4] MASS_7.3-3        multtest_2.2.0    siggenes_1.20.0
[7] spatial_7.3-1     splines_2.10.0    survival_2.35-7
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