

# Differential analyses with DSS

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### Abstract

This vignette introduces the use of the Bioconductor package DSS (Dispersion Shrinkage for Sequencing data), which is designed for differential analysis based on high-throughput sequencing data. It performs differential expression analyses for RNA-seq, and differential methylation analyses for bisulfite sequencing (BS-seq) data. The core of DSS is a procedure based on Bayesian hierarchical model to estimate and shrink gene- or CpG site-specific dispersions, then conduct Wald tests for detecting differential expression/methylation.

## 1 Introduction

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Recent advances in various high-throughput sequencing technologies have revolutionized genomics research. Among them, RNA-seq is designed to measure the abundance of RNA products, and Bisulfite sequencing (BS-seq) is for measuring DNA methylation. A fundamental question in functional genomics research is whether gene expression or DNA methylation vary under different biological contexts. Thus, identifying differential expression genes (DEGs) or differential methylation loci/regions (DML/DMRs) are key tasks in RNA-seq or BS-seq data analyses.

The differential expression (DE) or differential methylation (DM) analyses are often based on gene- or CpG-specific statistical test. A key limitation in RNA- or BS-seq experiments is that the number of biological replicates is usually limited due to cost constraints. This can lead to unstable estimation of within group variance, and subsequently undesirable results from hypothesis testing. Variance shrinkage methods have been widely applied in DE analyses in microarray data to improve the estimation of gene-specific within group variances. These methods are typically based on a Bayesian hierarchical model, with a prior imposed on the gene-specific variances to provide a basis for information sharing across all genes.

A distinct feature of RNA-seq or BS-seq data is that the measurements are in the form of counts and have to be modeled by discrete distributions. Unlike continuous distributions (such as Gaussian), the variances depend on means in these discrete distributions. This implies that the sample variances do not account for biological variation, and shrinkage cannot be applied on variances directly. In DSS, we assume that the count data are from the Gamma-Poisson (for RNA-seq) or Beta-Binomial (for BS-seq) distribution. We then parameterize the distributions by a mean and a dispersion parameters. The dispersion parameters, which represent the biological variation for replicates within a treatment group, play a central role in the differential analyses.

DSS implements a series of DE/DM detection algorithms based on the dispersion shrinkage method followed by Wald statistical test to test each gene/CpG site for differential expression/methylation. It provides functions for RNA-seq DE analysis for both two group comparison and multi-factor design, BS-seq DM analysis for two group comparison, multi-factor design, and data without biological replicate. Simulation and real data results show that the methods provides excellent performance compared to existing methods, especially when the overall dispersion level is high or the number of replicates is small.

For more details of the data model, the shrinkage method, and test procedures, please read [4] for differential expression from RNA-seq, [1] for differential methylation for two-group comparison from BS-seq, [2] for differential methylation for data without biological replicate, and [3] for differential methylation for general experimental design.

## 2 Using DSS for differential expression analysis

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### 2.1 Input data preparation

DSS requires a count table (a matrix of **integers**) for gene expression values (rows are for genes and columns are for samples). This is different from the isoform expression based analysis such as in cufflink/cuffdiff, where the gene expressions are represented as non-integers values. There are a number of ways to obtain the count table from raw

sequencing data (fastq file), here we provide some example codes using several Bioconductor packages (the codes require installation of `GenomicFeatures`, `Rsamtools`, and `GenomicRanges` packages).

1. Sequence alignment. There are several RNA-seq aligner, for example, `tophat` or `STAR`. Assume the alignment result is saved in a BAM file `data.bam`.
2. Choose a gene annotation. `GenomicFeatures` package provides a convenient way to access different gene annotations. For example, if one wants to use RefSeq annotation for human genome build hg19, one can use following codes:

```
> library(GenomicFeatures)
> txdb = makeTranscriptDbFromUCSC(genom="hg19", tablename="refGene")
> genes = genes(txdb)
```

3. Obtain count table based on the alignment results and gene annotation. This can be done in several steps. First read in the BAM file using the `Rsamtools` package:

```
> bam=scanBam("data.bam")
```

Next, create `GRanges` object for the aligned sequence reads.

```
> IRange.reads=GRanges(seqnames=Rle(bam$rname), ranges=IRanges(bam$pos, width=bam$qwidth))
```

Finally, use the `countOverlaps` function in `GenomicRanges` function to obtain the read counts overlap each gene.

```
> counts = countOverlaps(genes, IRange.reads)
```

There are other ways to obtain the counts, for example, using `QuasR` or `easyRNASeq` Bioconductor package. Please refer to the package vignettes for more details.

## 2.2 Single factor experiment

In single factor RNA-seq experiment, DSS also requires a vector representing experimental designs. The length of the design vector must match the number of columns of the count table. Optionally, normalization factors or additional annotation for genes can be supplied.

The basic data container in the package is `SeqCountSet` class, which is directly inherited from `ExpressionSet` class defined in `Biobase`. An object of the class contains all necessary information for a DE analysis: gene expression values, experimental designs, and additional annotations.

A typical DE analysis contains the following simple steps.

1. Create a `SeqCountSet` object using `newSeqCountSet`.
2. Estimate normalization factor using `estNormFactors`.
3. Estimate and shrink gene-wise dispersion using `estDispersion`
4. Two-group comparison using `waldTest`.

The usage of DSS is demonstrated in the simple simulation below.

1. First load in the library, and make a `SeqCountSet` object from some counts for 2000 genes and 6 samples.

```
> library(DSS)
> counts1=matrix(rnbinom(300, mu=10, size=10), ncol=3)
> counts2=matrix(rnbinom(300, mu=50, size=10), ncol=3)
> X1=cbind(counts1, counts2) ## these are 100 DE genes
```

```
> X2=matrix(rnbinom(11400, mu=10, size=10), ncol=6)
> X=rbind(X1,X2)
> designs=c(0,0,0,1,1,1)
> seqData=newSeqCountSet(X, designs)
> seqData
```

```
SeqCountSet (storageMode: lockedEnvironment)
```

```
assayData: 2000 features, 6 samples
```

```
  element names: exprs
```

```
protocolData: none
```

```
phenoData
```

```
  sampleNames: 1 2 ... 6 (6 total)
```

```
  varLabels: designs
```

```
  varMetadata: labelDescription
```

```
featureData: none
```

```
experimentData: use 'experimentData(object)'
```

```
Annotation:
```

2. Estimate normalization factor.

```
> seqData=estNormFactors(seqData)
```

3. Estimate and shrink gene-wise dispersions

```
> seqData=estDispersion(seqData)
```

4. With the normalization factors and dispersions ready, the two-group comparison can be conducted via a Wald test:

```
> result=waldTest(seqData, 0, 1)
```

```
> head(result,5)
```

	geneIndex	muA	muB	lfc	difExpr	stats	pval	local.fdr
61	61	7.666667	63.54762	-2.059566	-55.88095	-5.538765	3.046126e-08	7.820077e-05
30	30	8.333333	65.72294	-2.014495	-57.38961	-5.515525	3.477399e-08	7.820077e-05
14	14	7.666667	60.32684	-2.007970	-52.66017	-5.491516	3.984974e-08	8.156506e-05
60	60	9.000000	69.23377	-1.993393	-60.23377	-5.481104	4.226790e-08	8.576974e-05
73	73	7.333333	55.92641	-1.974549	-48.59307	-5.430783	5.610727e-08	1.060909e-04

```
  fdr
```

```
61 7.820077e-05
```

```
30 7.820077e-05
```

```
14 7.820077e-05
```

```
60 7.820077e-05
```

```
73 9.378712e-05
```

A higher level wrapper function `DSS.DE` is provided for simple RNA-seq DE analysis in a two-group comparison. User only needs to provide a count matrix and a vector of 0's and 1's representing the design, and get DE test results in one line. A simple example is listed below:

```
> counts = matrix(rpois(600, 10), ncol=6)
```

```
> designs = c(0,0,0,1,1,1)
```

```
> result = DSS.DE(counts, designs)
```

```
> head(result)
```

	geneIndex	muA	muB	lfc	difExpr	stats	pval	local.fdr
45	45	5.500000	11.948718	-0.7298582	-6.448718	-2.520564	0.01171670	0.2080385
86	86	11.361111	6.179487	0.5742239	5.181624	2.025781	0.04278719	0.5008949
38	38	11.305556	6.179487	0.5695290	5.126068	2.007992	0.04464409	0.5008949
64	64	8.138889	13.333333	-0.4708072	-5.194444	-1.812642	0.06988702	0.9363708
61	61	8.111111	13.256410	-0.4684516	-5.145299	-1.800172	0.07183345	0.9433085
23	23	10.944444	6.435897	0.5007940	4.508547	1.773641	0.07612250	0.7647016

	fdr
45	0.2080385
86	0.6872131
38	0.6872131
64	0.5888961
61	0.5888961
23	0.7521542

## 2.3 Multifactor experiment

DSS provides functionalities for dispersion shrinkage for multifactor experimental designs. Downstream model fitting (through generalized linear model) and hypothesis testing can be performed using other packages such as edgeR, with the dispersions estimated from DSS.

Below is an example, based a simple simulation, to illustrate the DE analysis of a crossed design.

1. First simulate data for a 2x2 crossed experiments. Note the counts are randomly generated.

```
> library(DSS)
> library(edgeR)
> counts=matrix(rpois(800, 10), ncol=8)
> design=data.frame(gender=c(rep("M",4), rep("F",4)), strain=rep(c("WT", "Mutant"),4))
> X=model.matrix(~gender+strain, data=design)
```

2. make SeqCountSet, then estimate size factors and dispersion

```
> seqData=newSeqCountSet(counts, as.data.frame(X))
> seqData=estNormFactors(seqData)
> seqData=estDispersion(seqData)
```

3. Using edgeR's function to do glm model fitting, but plugging in the estimated size factors and dispersion from DSS.

```
> fit.edgeR <- glmFit(counts, X, lib.size=normalizationFactor(seqData),
+                     dispersion=dispersion(seqData))
```

4. Using edgeR's function to do hypothesis testing on the second parameter of the model (gender).

```
> lrt.edgeR <- glmLRT(glmfit=fit.edgeR, coef=2)
> head(lrt.edgeR$table)
```

	logFC	logCPM	LR	PValue
--	-------	--------	----	--------

```

1  0.234207110 21.30042 4.598838e-01 0.4976783
2  0.508882118 21.16955 1.897379e+00 0.1683719
3  0.002767047 21.24480 6.118447e-05 0.9937590
4 -0.445160482 21.24126 1.571471e+00 0.2099934
5  0.201479930 20.99076 2.599636e-01 0.6101452
6  0.160690468 21.04128 1.761323e-01 0.6747184

```

### 3 Using DSS for differential methylation analysis

---

#### 3.1 Overview

To detect differential methylation, statistical tests are conducted at each CpG site, and then the differential methylation loci (DML) or differential methylation regions (DMR) are called based on user specified threshold. A rigorous statistical tests should account for biological variations among replicates and the sequencing depth. Most existing methods for DM analysis are based on *ad hoc* methods. For example, using Fisher's exact ignores the biological variations, using t-test on estimated methylation levels ignores the sequencing depth. Sometimes arbitrary filtering are implemented: loci with depth lower than an arbitrary threshold are filtered out, which results in information loss

The DM detection procedure implemented in DSS is based on a rigorous Wald test for beta-binomial distributions. The test statistics depend on the biological variations (characterized by dispersion parameter) as well as the sequencing depth. An important part of the algorithm is the estimation of dispersion parameter, which is achieved through a shrinkage estimator based on a Bayesian hierarchical model [1]. An advantage of DSS is that the test can be performed even when there is no biological replicates. That's because by smoothing, the neighboring CpG sites can be viewed as "pseudo-replicates", and the dispersion can still be estimated with reasonable precision.

DSS also works for general experimental design, based on a beta-binomial regression model with "arcsine" link function. Model fitting is performed on transformed data with generalized least square method, which achieves much improved computational performance compared with methods based on generalized linear model.

DSS depends on bsseq Bioconductor package, which has neat definition of data structures and many useful utility functions. In order to use the DM detection functionalities, bsseq needs to be pre-installed.

#### 3.2 Input data preparation

DSS requires data from each BS-seq experiment to be summarized into following information for each CG position: chromosome number, genomic coordinate, total number of reads, and number of reads showing methylation. For a sample, this information are saved in a simple text file, with each row representing a CpG site. Below shows an example of a small part of such a file:

```

chr    pos      N      X
chr18  3014904  26     2
chr18  3031032  33    12
chr18  3031044  33    13

```

```
chr18    3031065 48      24
```

One can follow below steps to obtain such data from raw sequence file (fastq file), using bismark (version 0.10.0, commands for newer versions could be different) for BS-seq alignment and count extraction. These steps require installation of bowtie or bowtie2, bismark, and the fasta file for reference genome.

1. Prepare Bisulfite reference genome. This can be done using the `bismark_genome_preparation` function (details in bismark manual). Example command is:  

```
bismark_genome_preparation -path_to_bowtie /usr/local/bowtie/ -verbose /path/to/refgenomes/
```
2. BS-seq alignment. Example command is:  

```
bismark -q -n 1 -l 50 -path_to_bowtie /path/bowtie/ BS-refGenome reads.fastq
```

This step will produce two text files `reads.fastq_bismark.sam` and `reads.fastq_bismark_SE_report.txt`.
3. Extract methylation counts using `bismark_methylation_extractor` function:  

```
bismark_methylation_extractor -s -bedGraph reads.fastq_bismark.sam.
```

This will create multiple text files to summarize methylation call and cytosine context, a bedGraph file to display methylation percentage, and a coverage file containing counts information. The count file contains following columns: `chr`, `start`, `end`, `methylation%`, `count methylated`, `count unmethylated`. This file can be modified to make the input file for DSS.

A typical DML detection contains two simple steps. First one conducts DM test at each CpG site, then DML/DMR are called based on the test result and user specified threshold.

### 3.3 DML/DMR detection from two-group comparison

Below are the steps to call DML or DMR for BS-seq data in two-group comparison setting.

1. Load in library. Read in text files and create an object of `BSseq` class, which is defined in `bsseq` Bioconductor package. This step requires `bsseq` Bioconductor package. `BSseq` class is defined in that package.

```
> library(DSS)
> require(bsseq)
> path <- file.path(system.file(package="DSS"), "extdata")
> dat1.1 <- read.table(file.path(path, "cond1_1.txt"), header=TRUE)
> dat1.2 <- read.table(file.path(path, "cond1_2.txt"), header=TRUE)
> dat2.1 <- read.table(file.path(path, "cond2_1.txt"), header=TRUE)
> dat2.2 <- read.table(file.path(path, "cond2_2.txt"), header=TRUE)
> BSobj <- makeBSseqData( list(dat1.1, dat1.2, dat2.1, dat2.2),
+   c("C1", "C2", "N1", "N2") )[1:10000,]
> BSobj
```

An object of type 'BSseq' with

10000 methylation loci

4 samples

has not been smoothed

2. Perform statistical test for DML by calling `DMLtest` function. This function basically performs following steps: (1) estimate mean methylation levels for all CpG sites; (2) estimate dispersions at each CpG sites; (3) conduct Wald

test. For the first step, there's an option for smoothing or not. Because the methylation levels show strong spatial correlations, smoothing can help obtain better estimates of mean methylation when the CpG sites are dense in the data (such as from the whole-genome BS-seq). However for data with sparse CpG, such as from RRBS or hydroxyl-methylation, smoothing is not recommended.

To perform DML test without smoothing, do:

```
> dmlTest <- DMLtest(BSobj, group1=c("C1", "C2"), group2=c("N1", "N2"))
Estimating dispersion for each CpG site, this will take a while ...
> head(dmlTest)
```

	chr	pos	mu1	mu2	diff	diff.se	stat	phi1
1	chr18	3014904	0.3850276	0.4623677	-0.07734011	0.24899738	-0.3106061	0.286610813
2	chr18	3031032	0.3384423	0.1416667	0.19677555	0.11402507	1.7257217	0.009525212
3	chr18	3031044	0.3436302	0.3299846	0.01364560	0.12508174	0.1090935	0.010959806
4	chr18	3031065	0.4372540	0.3646882	0.07256585	0.10435917	0.6953471	0.010846613
5	chr18	3031069	0.2939132	0.5397749	-0.24586172	0.13554689	-1.8138499	0.012965376
6	chr18	3031082	0.3529066	0.3903499	-0.03744333	0.08197055	-0.4567900	0.008246131

	phi2	pval	fdr
1	0.01964971	0.75610007	0.9999077
2	0.05282909	0.08439748	0.7175525
3	0.02285714	0.91312835	0.9999077
4	0.01849672	0.48683778	0.9999077
5	0.02658296	0.06970083	0.6313442
6	0.01338446	0.64782202	0.9999077

To perform statistical test for DML with smoothing, do:

```
> dmlTest.sm <- DMLtest(BSobj, group1=c("C1", "C2"), group2=c("N1", "N2"), smoothing=TRUE)
Smoothing ...
```

Estimating dispersion for each CpG site, this will take a while ...

There are two options for smoothing: a simple moving average, or the BSmooth method implemented in bsseq package. The BSmooth method produces much smoother curve, which is good for visualization purpose. However, it is very computationally intensive, and the results are not very different from moving average in terms of DMR calling. So we recommend using moving average. Smoothing span is an important parameter in smoothing procedure and have non-trivial impact on DMR calling. We use 500 bp as default, and think that it performs well in real data tests.

3. With the test results, one can call DML by using callDML function. The results DMLs are sorted by the significance.

```
> dmls <- callDML(dmlTest, p.threshold=0.001)
> head(dmls)
```

	chr	pos	mu1	mu2	diff	diff.se	stat	phi1
450	chr18	3976129	0.01048590	0.93773387	-0.9272480	0.06785915	-13.664303	0.04319795
451	chr18	3976138	0.01048590	0.93773387	-0.9272480	0.06785915	-13.664303	0.04319795
582	chr18	4340682	0.96365233	0.03162952	0.9320228	0.09947722	9.369209	0.06181184
583	chr18	4340709	0.96365233	0.03162952	0.9320228	0.09947722	9.369209	0.06181184
638	chr18	4431501	0.01325735	0.94195478	-0.9286974	0.09390549	-9.889704	0.04380187
639	chr18	4431511	0.01321013	0.94195478	-0.9287446	0.09387324	-9.893604	0.04377619



```

      phi2 pval fdr postprob.overThreshold
450 0.02756948 0 0 1
451 0.02756948 0 0 1
582 0.05147878 0 0 1
583 0.05147878 0 0 1
638 0.08184949 0 0 1
639 0.08184949 0 0 1

```

By default, the test is based on the null hypothesis that the difference in methylation levels is 0. Alternatively, users can specify a threshold for difference. For example, to detect loci with difference greater than 0.1, do:

```
> dmls2 <- callDML(dmlTest, delta=0.1, p.threshold=0.001)
```

```
> head(dmls2)
```

```

      chr    pos      mu1      mu2      diff      diff.se      stat      phi1
450 chr18 3976129 0.01048590 0.93773387 -0.9272480 0.06785915 -13.664303 0.04319795
451 chr18 3976138 0.01048590 0.93773387 -0.9272480 0.06785915 -13.664303 0.04319795
582 chr18 4340682 0.96365233 0.03162952 0.9320228 0.09947722 9.369209 0.06181184
583 chr18 4340709 0.96365233 0.03162952 0.9320228 0.09947722 9.369209 0.06181184
638 chr18 4431501 0.01325735 0.94195478 -0.9286974 0.09390549 -9.889704 0.04380187
639 chr18 4431511 0.01321013 0.94195478 -0.9287446 0.09387324 -9.893604 0.04377619

```

```

      phi2 pval fdr postprob.overThreshold
450 0.02756948 0 0 1
451 0.02756948 0 0 1
582 0.05147878 0 0 1
583 0.05147878 0 0 1
638 0.08184949 0 0 1
639 0.08184949 0 0 1

```

When delta is specified, the function will compute the posterior probability that the difference of the means is greater than delta. So technically speaking, the threshold for p-value here actually refers to the threshold for 1-posterior probability, or the local FDR. Here we use the same parameter name for the sake of the consistence of function syntax.

- DMR detection is also Based on the DML test results, by calling `callDMR` function. Regions with many statistically significant CpG sites are identified as DMRs. Some restrictions are provided by users, including the minimum length, minimum number of CpG sites, percentage of CpG site being significant in the region, etc. There are some *post hoc* procedures to merge nearby DMRs into longer ones.

```
> dmrs <- callDMR(dmlTest, p.threshold=0.01)
```

```
> head(dmrs)
```

```

      chr    start      end length nCG meanMethy1 meanMethy2 diff.Methy  areaStat
142 chr18 13131705 13131880    176 6 0.9689797 0.06107942 0.9079003 68.741081
26  chr18 4657576 4657639     64 4 0.5084704 0.31870495 0.1897655 14.421382
30  chr18 5027578 5027743    166 4 0.7045195 0.38058436 0.3239352 9.109377

```

Here the DMRs are sorted by "areaStat", which is defined in `bsseq` as the sum of the test statistics of all CpG sites within the DMR.

Similarly, users can specify a threshold for difference. For example, to detect regions with difference greater than

0.1, do:

```
> dmrs2 <- callDMR(dmlTest, delta=0.1, p.threshold=0.05)
> head(dmrs2)
```

	chr	start	end	length	nCG	meanMethy1	meanMethy2	diff.Methy	areaStat
175	chr18	13131705	13131880	176	6	0.9689797	0.06107942	0.90790031	68.74108
29	chr18	4657576	4657639	64	4	0.5084704	0.31870495	0.18976548	14.42138
277	chr18	23100427	23100601	175	4	0.5329967	0.43403678	0.09895989	12.76449
18	chr18	4222533	4222608	76	4	0.7882151	0.36126847	0.42694665	12.66181
33	chr18	5027549	5027743	195	5	0.6696348	0.32605410	0.34358066	11.42014

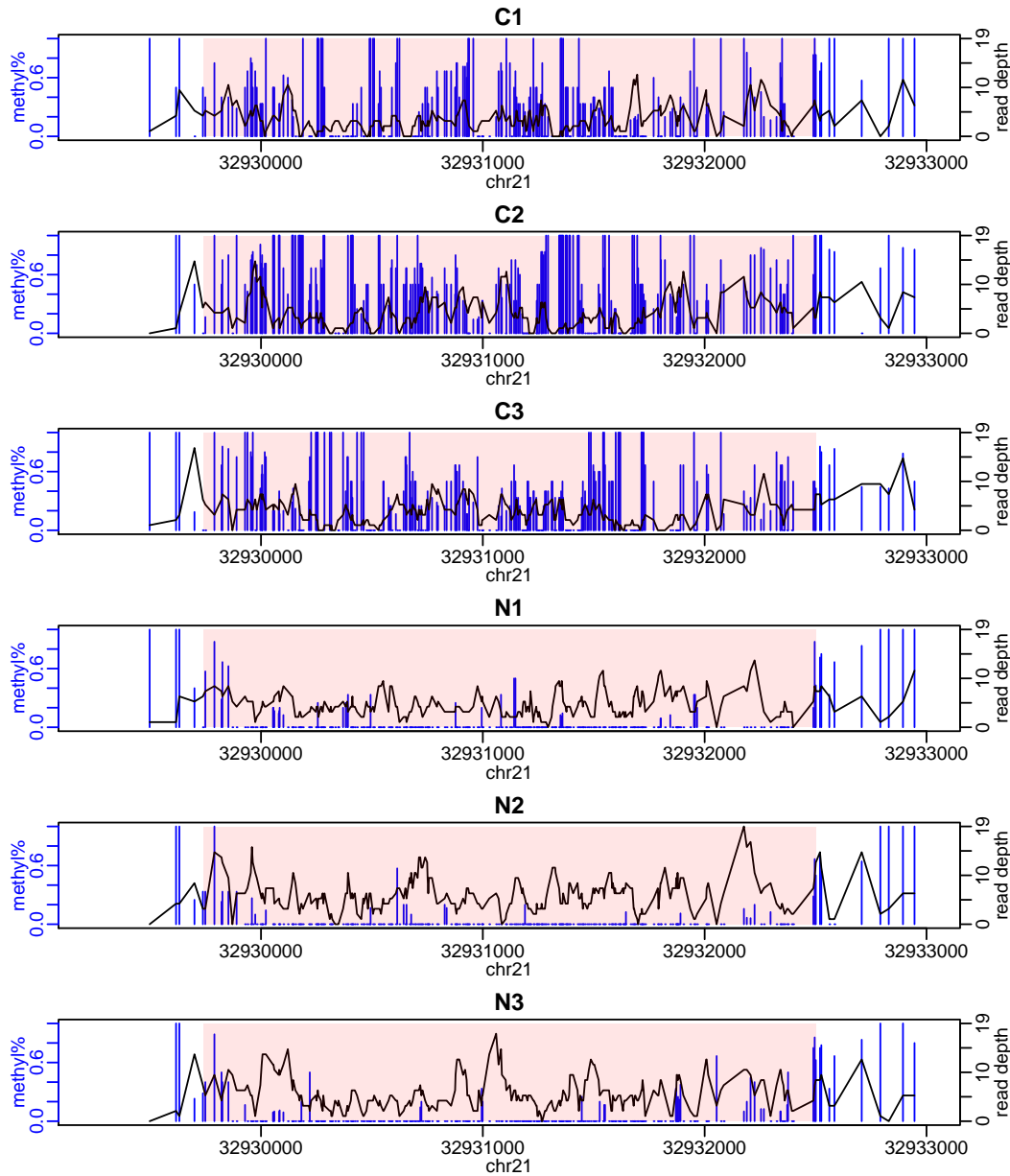
Note that the distribution of test statistics (and p-values) depends on the differences in methylation levels and biological variations, as well as technical factors such as coverage depth. It is very difficult to select a natural and rigorous threshold for defining DMRs. We recommend users try different thresholds in order to obtain satisfactory results.

5. The DMRs can be visualized using `showOneDMR` function. This function provides more information than the `plotRegion` function in `bsseq`. It plots the methylation percentages as well as the coverage depths at each CpG sites, instead of just the smoothed curve. So the coverage depth information will be available in the figure.

To use the function, do

```
> showOneDMR(dmrs[1,], BSobj)
```

The result figure looks like the following. **Note that the figure below is not generated from the above example. The example data are from RRBS experiment so the DMRs are much shorter.**



### 3.4 DML/DMR detection from general experimental design

In DSS, BS-seq data from a general experimental design (such as crossed experiment, or experiment with covariates) is modeled through a generalized linear model framework. We use “arcsine” link function instead of the typical logit link for it better deals with data at boundaries (methylation levels close to 0 or 1). Linear model fitting is done through ordinary least square on transformed methylation levels. Standard errors for the estimates are derived with consideration of count data distribution and transformation. A Wald test is applied to perform hypothesis testing.

1. Load in data distributed with DSS. This is a small portion of a set of RRBS experiments. There are 5000 CpG sites and 16 samples. The experiment is a 2 design (2 cases and 2 cell types). There are 4 replicates in each case:cell combination.

```
> data(RRBS)
> RRBS
An object of type 'BSseq' with
  5000 methylation loci
  16 samples
has not been smoothed
> design
```

```
  case cell
1    HC   rN
2    HC   rN
3    HC   rN
4   SLE  aN
5   SLE  rN
6   SLE  aN
7   SLE  rN
8   SLE  aN
9   SLE  rN
10  SLE  aN
11  SLE  rN
12  HC   aN
13  HC   aN
14  HC   aN
15  HC   aN
16  HC   rN
```

2. Fit a linear model using `DMLfit.multiFactor` function, include case, cell, and case:cell interaction.

```
> DMLfit = DMLfit.multiFactor(RRBS, design=design, formula=~case+cell+case:cell)
Fitting DML model for CpG site:
```

3. Use `DMLtest.multiFactor` function to test the cell effect. It is important to note that the `coef` parameter is the index of the coefficient to be tested for being 0. Because the model (as specified by formula in `DMLfit.multiFactor`) include intercept, the cell effect is the 3rd column in the design matrix, so we use `coef=3` here.

```
> DMLtest.cell = DMLtest.multiFactor(DMLfit, coef=3)
```

Result from this step is a data frame with chromosome number, CpG site position, test statistics, p-values (from normal distribution), and FDR. Rows are sorted by chromosome/position of the CpG sites. To obtain top ranked CpG sites, one can sort the data frame using following codes:

```
> ix=sort(DMLtest.cell[, "pvals"], index.return=TRUE)$ix
> head(DMLtest.cell[ix,])
```

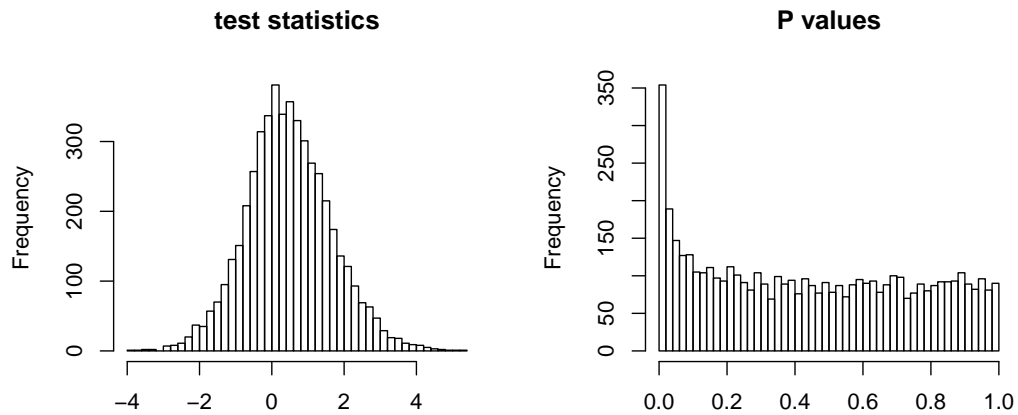
	chr	pos	stat	pvals	fdrs
1273	chr1	2930315	5.280301	1.289720e-07	0.0006448599
4706	chr1	3321251	5.037839	4.708164e-07	0.0011770409
3276	chr1	3143987	4.910412	9.088510e-07	0.0015147517
2547	chr1	3069876	4.754812	1.986316e-06	0.0024828953

```
3061 chr1 3121473 4.675736 2.929010e-06 0.0029290097
```

```
527 chr1 2817715 4.441198 8.945925e-06 0.0065858325
```

Below is a figure showing the distributions of test statistics and p-values from this example dataset

```
> par(mfrow=c(1,2))  
> hist(DMLtest.cell$stat, 50, main="test statistics", xlab="")  
> hist(DMLtest.cell$pvals, 50, main="P values", xlab="")
```



These procedures are computationally very efficient. For a typical RRBS dataset with 4 million CpG sites, it takes around 20 minutes. In comparison, other methods such as RADMeth or BiSeq takes at least 10 times longer.

## 4 Session Info

---

```
> sessionInfo()

R version 3.3.0 RC (2016-04-25 r70549)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows Server 2008 R2 x64 (build 7601) Service Pack 1

locale:
[1] LC_COLLATE=C                      LC_CTYPE=English_United States.1252
[3] LC_MONETARY=English_United States.1252 LC_NUMERIC=C
[5] LC_TIME=English_United States.1252

attached base packages:
[1] splines      stats4      parallel     stats        graphics    grDevices    utils        datasets
[9] methods     base

other attached packages:
[1] edgeR_3.14.0          DSS_2.12.0          bsseq_1.8.0
[4] limma_3.28.0          SummarizedExperiment_1.2.0 GenomicRanges_1.24.0
[7] GenomeInfoDb_1.8.0    IRanges_2.6.0        S4Vectors_0.10.0
[10] Biobase_2.32.0        BiocGenerics_0.18.0

loaded via a namespace (and not attached):
[1] Rcpp_0.12.4.5      XVector_0.12.0      zlibbioc_1.18.0     munsell_0.4.3
[5] colorspace_1.2-6   lattice_0.20-33     plyr_1.8.3          tools_3.3.0
[9] grid_3.3.0         data.table_1.9.6    R.oo_1.20.0         gtools_3.5.0
[13] matrixStats_0.50.2 permute_0.9-0       R.utils_2.3.0       scales_0.4.0
[17] R.methodsS3_1.7.1  locfit_1.5-9.1     BiocStyle_2.0.0     chron_2.3-47
```

## References

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- [2] Hao Wu, Tianlei Xu, Hao Feng, Li Chen, Ben Li, Bing Yao, Zhaohui Qin, Peng Jin and Karen N. Conneely. (2015). Detection of differentially methylated regions from whole-genome bisulfite sequencing data without replicates. *Nucleic Acids Research*. doi: 10.1093/nar/gkv715.
- [3] Yongseok Park, Hao Wu. (2016). Differential methylation analysis for BS-seq data under general experimental design. *Bioinformatics*. doi:10.1093/bioinformatics/btw026.

- [4] Hao Wu, Chi Wang and Zhijing Wu. (2013). A new shrinkage estimator for dispersion improves differential expression detection in RNA-seq data. *Biostatistics*. **14**(2), 232–243.