

`goseq`: Gene Ontology testing for RNA-seq datasets

Matthew D. Young Nadia Davidson Matthew J. Wakefield
Gordon K. Smyth Alicia Oshlack

8 September 2017

1 Introduction

This document gives an introduction to the use of the `goseq` R Bioconductor package [Young et al., 2010]. This package provides methods for performing Gene Ontology analysis of RNA-seq data, taking length bias into account [Oshlack and Wakefield, 2009]. The methods and software used by `goseq` are equally applicable to other category based test of RNA-seq data, such as KEGG pathway analysis.

Once installed, the `goseq` package can be easily loaded into R using:

```
> library(goseq)
```

In order to perform a GO analysis of your RNA-seq data, `goseq` only requires a simple named vector, which contains two pieces of information.

1. **Measured genes:** all genes for which RNA-seq data was gathered for your experiment. Each element of your vector should be named by a unique gene identifier.
2. **Differentially expressed genes:** each element of your vector should be either a 1 or a 0, where 1 indicates that the gene is differentially expressed and 0 that it is not.

If the organism, gene identifier or category test is currently not natively supported by `goseq`, it will also be necessary to supply additional information regarding the genes length and/or the association between categories and genes.

Bioconductor R packages such as `Rsubread` allow for the summarization of mapped reads into a table of counts, such as reads per gene. From there, several packages exist for performing differential expression analysis on summarized data (eg. `edgeR` [Robinson and Smyth, 2007, 2008, Robinson et al., 2010]). `goseq` will work with any method for determining differential expression and as such differential expression analysis is outside the scope of this document, but in order to facilitate ease of use, we will make use of the `edgeR` package to calculate differentially expressed (DE) genes in all the case studies in this document.

2 Reading data

We assume that the user can use appropriate in-built R functions (such as `read.table` or `scan`) to obtain two vectors, one containing all genes assayed in the RNA-seq experiment, the other containing all genes which are DE. If we assume that the vector of genes being assayed is named `assayed.genes` and the vector of DE genes is named `de.genes` we can construct a named vector suitable for use with `goseq` using the following:

```
> gene.vector=as.integer(assayed.genes%in%de.genes)
> names(gene.vector)=assayed.genes
> head(gene.vector)
```

It may be that the user can already read in a vector in this format, in which case it can then be immediately used by `goseq`.

3 GO testing of RNA-seq data

To begin the analysis, `goseq` first needs to quantify the length bias present in the dataset under consideration. This is done by calculating a Probability Weighting Function or PWF which can be thought of as a function which gives the probability that a gene will be differentially expressed (DE), based on its length alone. The PWF is calculated by fitting a monotonic spline to the binary data series of differential expression (1=DE, 0=Not DE) as a function of gene length. The PWF is used to weight the chance of selecting each gene when forming a null distribution for GO category membership. The fact that the PWF is calculated directly from the dataset under consideration makes this approach robust, only correcting for the length bias present in the data. For example, if `goseq` is run on a microarray dataset, for which no length bias exists, the calculated PWF will be nearly flat and all genes will be weighted equally, resulting in no length bias correction.

In order to account for the length bias inherent to RNA-seq data when performing a GO analysis (or other category based tests), one cannot simply use the hypergeometric distribution as the null distribution for category membership, which is appropriate for data without DE length bias, such as microarray data. GO analysis of RNA-seq data requires the use of random sampling in order to generate a suitable null distribution for GO category membership and calculate each categories significance for over representation amongst DE genes.

However, this random sampling is computationally expensive. In most cases, the Wallenius distribution can be used to approximate the true null distribution, without any significant loss in accuracy. The `goseq` package implements this approximation as its default option. The option to generate the null distribution using random sampling is also included as an option, but users should be aware that the default number of samples generated will not be enough to accurately call enrichment when there are a large number of go terms.

Having established a null distribution, each GO category is then tested for over and under representation amongst the set of differentially expressed genes and the null is used to calculate a p-value for under and over representation.

4 Natively supported Gene Identifiers and category tests

`goseq` needs to know the length of each gene, as well as what GO categories (or other categories of interest) each gene is associated with. `goseq` relies on the UCSC genome browser to provide the length information for each gene. However, because the process of fetching the length of every transcript is slow and bandwidth intensive, `goseq` relies on an offline copy of this information stored in the data package `geneLenDataBase`. To see which genome/gene identifier combinations are in the local database, simply run:

```
> supportedOrganisms()
```

The leftmost columns in the output of this command list the genomes and gene identifiers respectively. If length data exists in the local database it is indicated in the second last column. If your genome/ID combination is not in the local database, it may be downloaded from the UCSC genome browser or taken from a `TxDb` annotation package (if installed). If your genome/ID combination is not found in any database, you will have to manually specify the gene lengths. We encourage all users to manually specify their gene lengths if provided by upstream summarization programs. e.g. `featureCounts`, as these lengths will be more accurate.

In order to link GO categories to genes, `goseq` uses the organism packages from Bioconductor. These packages are named `org.<Genome>.<ID>.db`, where `<Genome>` is a short string identifying the genome and `<ID>` is a short string identifying the gene identifier. Currently, `goseq` will automatically retrieve the mapping between GO categories and genes from the relevant package (as long as it is installed) for commonly used genome/ID combinations. If GO mappings are not automatically available for your genome/ID combination, you will have to manually specify the relationship between genes and categories. Although the Genome/ID naming conventions used by the organism packages differ from the UCSC, `goseq` is able to convert between the two, so the user need only ever specify the UCSC genome/ID in most cases. The final column indicates whether the Genome/ID combination is supported for GO categories.

5 Non-native Gene Identifier or category test

If the organism, Gene Identifier or category test you wish to perform is not in the native `goseq` database, you will have to supply one or all of the following:

- **Length data:** the length of each gene in your gene identifier format.

- **Category mappings:** the mapping (usually many-to-many) between the categories you wish to test for over/under representation amongst DE genes and genes in your gene identifier format.

5.1 Length data format

The length data must be formatted as a numeric vector, of the same length as the main named vector specifying gene names/DE genes. Each entry should give the length of the corresponding gene in bp. If length data is unavailable for some genes, that entry should be set to NA.

5.2 Category mapping format

The mapping between category names and genes should be given as a data frame with two columns. One column should contain the gene IDs and the other the name of an associated category. As the mapping between categories and genes is usually many-to-many, this data frame will usually have multiple rows with the same gene name and category name.

Alternatively, mappings between genes and categories can be given as a list. The names of list entries should be gene IDs and the entries themselves should be a vector of category names to which the gene ID corresponds.

5.3 Some additional tips

Any organism for which there is an annotation on either Ensembl or the UCSC, can be easily turned into length data using the GenomicFeatures package. To do this, first create a TranscriptDb object using either `makeTxDbFromBiomart` or `makeTxDbFromUCSC` (see the help in the GenomicFeatures package on using these commands). Once you have a transcriptDb object, you can get a vector named by gene ID containing the median transcript length of each gene simply by using the command.

```
> txsByGene=transcriptsBy(txdb,"gene")  
> lengthData=median(width(txsByGene))
```

The relationship between gene identifier and GO category can usually be obtained from the Gene Ontology website (www.geneontology.org) or from the NCBI. Additionally, the bioconductor AnnotationDbi library has recently added a function "makeOrgPackageFromNCBI", which can be used to create an organism package from within R, using the NCBI data. Once created, this package can then be used to obtain the mapping between genes and gene ontology.

6 Case study: Prostate cancer data

6.1 Introduction

This section provides an analysis of data from an RNA-seq experiment to illustrate the use of `goseq` for GO analysis.

This experiment examined the effects of androgen stimulation on a human prostate cancer cell line, LNCaP. The data set includes more than 17 million short cDNA reads obtained for both the treated and untreated cell line and sequenced on Illumina's 1G genome analyzer.

For each sample we were provided with the raw 35 bp RNA-seq reads from the authors. For the untreated prostate cancer cells (LNCaP cell line) there were 4 lanes totaling 10 million, 35 bp reads. For the treated cells there were 3 lanes totaling 7 million, 35 bp reads. All replicates were technical replicates. Reads were mapped to NCBI version 36.3 of the human genome using `bowtie`. Any read with which mapped to multiple locations was discarded. Using the ENSEMBL 54 annotation from `biomart`, each mapped read was associated with an ENSEMBL gene. This was done by associating any read that overlapped with any part of the gene (not just the exons) with that gene. Reads that did not correspond to genes were discarded.

6.2 Source of the data

The data set used in this case study is taken from [Li et al., 2008] and was made available from the authors upon request.

6.3 Determining the DE genes using `edgeR`

To begin with, we load in the text data and convert it the appropriate `edgeR` `DGEList` object.

```
> library(edgeR)
> table.summary=read.table(system.file("extdata","Li_sum.txt",package='goseq'),
+                           sep='\t',header=TRUE,stringsAsFactors=FALSE)
> counts=table.summary[,-1]
> rownames(counts)=table.summary[,1]
> grp=factor(rep(c("Control","Treated"),times=c(4,3)))
> summarized=DGEList(counts,lib.size=colSums(counts),group=grp)
```

Next, we use `edgeR` to estimate the biological dispersion and calculate differential expression using a negative binomial model.

```
> disp=estimateCommonDisp(summarized)
> disp$common.dispersion
```

```
[1] 0.05688364
```

```
> tested=exactTest(disp)
> topTags(tested)
```

Comparison of groups: Treated-Control

	logFC	logCPM	PValue	FDR
ENSG00000127954	11.557868	6.680748	2.574972e-80	1.274766e-75
ENSG00000151503	5.398963	8.499530	1.781732e-65	4.410322e-61
ENSG00000096060	4.897600	9.446705	7.983756e-60	1.317479e-55
ENSG00000091879	5.737627	6.282646	1.207655e-54	1.494654e-50
ENSG00000132437	-5.880436	7.951910	2.950042e-52	2.920896e-48
ENSG00000166451	4.564246	8.458467	7.126763e-52	5.880292e-48
ENSG00000131016	5.254737	6.607957	1.066807e-51	7.544766e-48
ENSG00000163492	7.085400	5.128514	2.716461e-45	1.681014e-41
ENSG00000113594	4.051053	8.603264	9.272066e-44	5.100255e-40
ENSG00000116285	4.108522	7.864773	6.422468e-43	3.179507e-39

Finally, we Format the DE genes into a vector suitable for use with `goseq`

```
> genes=as.integer(p.adjust(tested$table$PValue[tested$table$logFC!=0],
+ method="BH")<.05)
> names(genes)=row.names(tested$table[tested$table$logFC!=0,])
> table(genes)
```

```
genes
  0    1
19535 3208
```

6.4 Determining Genome and Gene ID

In order to allow for automatic data retrieval, the user has to tell `goseq` what genome and gene ID format were used to summarize the data. In our case we will use the hg19 build of the human genome, we check what code this corresponds to by running:

```
> head(supportedOrganisms())
```

	Genome	Id	Id Description	Lengths in geneLeneDataBase
12	anoCar1	ensGene	Ensembl gene ID	TRUE
13	anoGam1	ensGene	Ensembl gene ID	TRUE
14	apiMel2	ensGene	Ensembl gene ID	TRUE
56	bosTau2	geneSymbol	Gene Symbol	TRUE
15	bosTau3	ensGene	Ensembl gene ID	TRUE
57	bosTau3	geneSymbol	Gene Symbol	TRUE
	GO Annotation Available			

```

12             FALSE
13             TRUE
14             FALSE
56             TRUE
15             TRUE
57             TRUE

```

Which lists the genome codes in the far left column, headed “Genome”. As we are using “hg19” and we also know that we used ENSEMBL Gene ID to summarize our read data, we check what code this corresponds to by running:

```

> supportedOrganisms()[supportedOrganisms()$Genome=="hg19",]

  Genome      Id  Id Description Lengths in geneLeneDataBase
4   hg19 knownGene  Entrez Gene ID                TRUE
36  hg19  ensGene  Ensembl gene ID                TRUE
81  hg19 geneSymbol Gene Symbol                  TRUE
  GO Annotation Available
4                TRUE
36               TRUE
81               TRUE

```

The gene ID codes are listed in the column second from left, titled “Id”. We find that our gene ID code is “ensGene”. We will use these strings whenever we are asked for a genome or id. If the gene ID is missing for your Genome (for example this is the case for hg38), then the genome is not supported in the geneLengthDatabase. Gene lengths will either be automatically fetched from TxDB, UCSC or you will need to provide them manually. Supported Gene IDs to automatically fetch GO terms should usually either be Entrez (“knownGene”), Ensembl (“ensGene”) or gene symbols (“geneSymbol”).

6.5 GO analysis

6.5.1 Fitting the Probability Weighting Function (PWF)

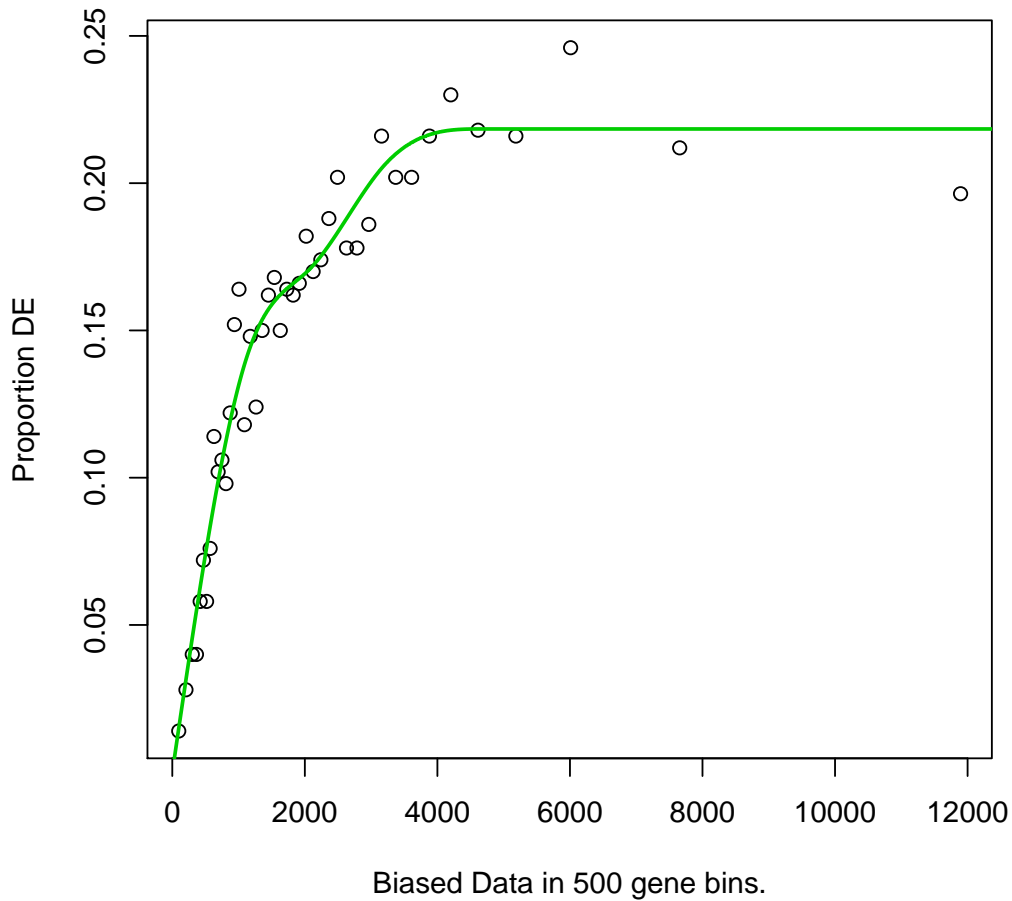
We first need to obtain a weighting for each gene, depending on its length, given by the PWF. As you may have noticed when running supportedGenomes or supportedGeneIDs, length data is available in the local database for our gene ID, “ensGene” and our genome, “hg19”. We will let goseq automatically fetch this data from its databases.

```

> pwf=NULLP(genes, "hg19", "ensGene")
> head(pwf)

```

	DEgenes	bias.data	pwf
ENSG00000230758	0	247	0.03757470
ENSG00000182463	0	3133	0.20436865
ENSG00000124208	0	1978	0.16881769
ENSG00000230753	0	466	0.06927243
ENSG00000224628	0	1510	0.15903532
ENSG00000125835	0	954	0.12711992



`nullp` plots the resulting fit, allowing verification of the goodness of fit before continuing the analysis. Further plotting of the pwf can be performed using the `plotPWF` function.

The output of `nullp` contains all the data used to create the PWF, as well as the PWF itself. It is a data frame with 3 columns, named "DEgenes", "bias.data" and "pwf" with the rownames set to the gene names. Each row corresponds to a gene with the DEgenes column specifying if the gene is DE (1 for DE, 0 for not DE), the bias.data column giving the numeric value of the DE bias

being accounted for (usually the gene length or number of counts) and the pwf column giving the genes value on the probability weighting function.

6.5.2 Using the Wallenius approximation

To start with we will use the default method, to calculate the over and under expressed GO categories among DE genes. Again, we allow `goseq` to fetch data automatically, except this time the data being fetched is the relationship between ENSEMBL gene IDs and GO categories.

```
> GO.wall=goseq(pwf, "hg19", "ensGene")
> head(GO.wall)
```

	category	over_represented_pvalue	under_represented_pvalue	numDEInCat			
2548	GO:0005737	3.792714e-11		1	1962		
11112	GO:0044444	1.247808e-08		1	1682		
2489	GO:0005615	1.641253e-08		1	499		
11096	GO:0044421	1.749055e-08		1	527		
3239	GO:0006793	3.599915e-08		1	620		
3115	GO:0006614	4.732072e-08		1	34		
	numInCat					term	ontology
2548	8974					cytoplasm	CC
11112	7688					cytoplasmic part	CC
2489	2060					extracellular space	CC
11096	2182					extracellular region part	CC
3239	2554					phosphorus metabolic process	BP
3115	95	SRP-dependent cotranslational protein targeting to membrane					BP

The resulting object is ordered by GO category over representation amongst DE genes.

6.5.3 Using random sampling

It may sometimes be desirable to use random sampling to generate the null distribution for category membership. For example, to check consistency against results from the Wallenius approximation. This is easily accomplished by using the `method` option to specify sampling and the `repcnt` option to specify the number of samples to generate:

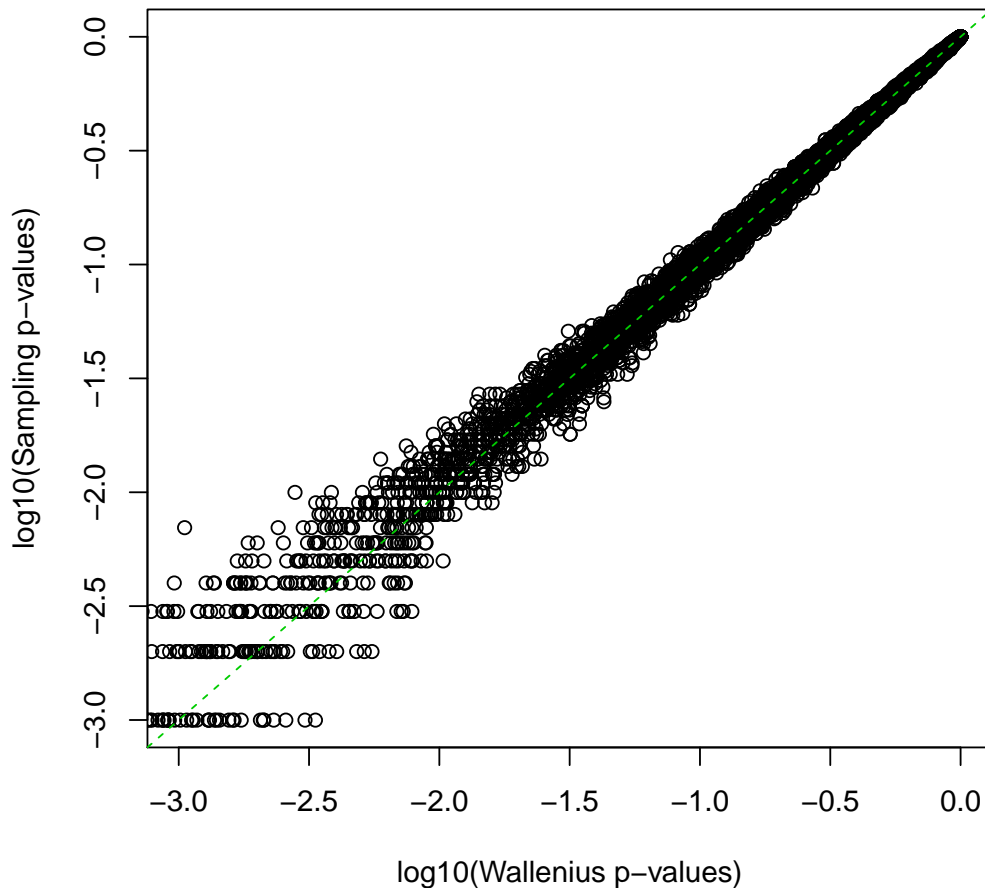
```
> GO.samp=goseq(pwf, "hg19", "ensGene", method="Sampling", repcnt=1000)
> head(GO.samp)
```

	category	over_represented_pvalue	under_represented_pvalue	numDEInCat	numInCat
33	GO:0000070	0.000999001		1	46
92	GO:0000184	0.000999001		1	33
132	GO:0000278	0.000999001		1	243

536	GO:0001667	0.000999001	1	87	284
626	GO:0001816	0.000999001	1	130	482
627	GO:0001817	0.000999001	1	117	439
					term ontology
33		mitotic sister chromatid segregation			BP
92	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay				BP
132		mitotic cell cycle			BP
536		ameboidal-type cell migration			BP
626		cytokine production			BP
627		regulation of cytokine production			BP

You will notice that this takes far longer than the Wallenius approximation. Plotting the p-values against one another, we see that there is little difference between the two methods. However, the accuracy of the sampling method is limited by the number of samples generated, `repnt`, such that very low p-values will not be correctly calculated. Significantly enriched GO terms may then be missed after correcting for multiple testing.

```
> plot(log10(GO.wall[,2]), log10(GO.samp[match(GO.wall[,1],GO.samp[,1]),2]),
+       xlab="log10(Wallenius p-values)",ylab="log10(Sampling p-values)",
+       xlim=c(-3,0))
> abline(0,1,col=3,lty=2)
```



6.5.4 Ignoring length bias

`goseq` also allows for one to perform a GO analysis without correcting for RNA-seq length bias. In practice, this is only useful for assessing the effect of length bias on your results. You should NEVER use this option as your final analysis. If length bias is truly not present in your data, `goseq` will produce a nearly flat PWF and no length bias correction will be applied to your data and all methods will produce the same results.

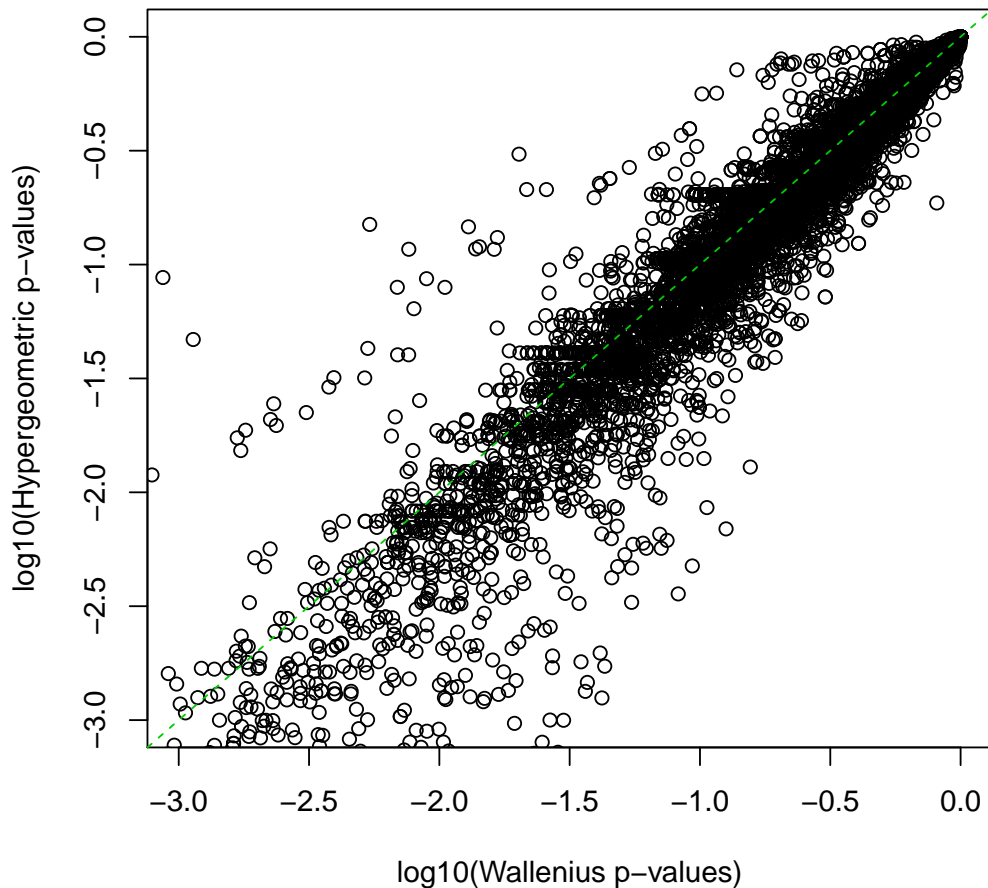
However, if you still wish to ignore length bias in calculating GO category enrichment, this is again accomplished using the `method` option.

```
> GO.nobias=goseq(pwf, "hg19", "ensGene", method="Hypergeometric")
> head(GO.nobias)
```

	category	over_represented_pvalue	under_represented_pvalue	numDEInCat
2548	GO:0005737	6.491023e-12		1962
2651	GO:0005886	1.607529e-09		773
15869	GO:0071944	3.602917e-09		791
8295	GO:0032879	7.421626e-09		482
2439	GO:0005515	7.631647e-09		1939
3239	GO:0006793	1.597993e-08		620
	numInCat	term	ontology	
2548	8974	cytoplasm	CC	
2651	3228	plasma membrane	CC	
15869	3325	cell periphery	CC	
8295	1914	regulation of localization	BP	
2439	8977	protein binding	MF	
3239	2554	phosphorus metabolic process	BP	

Ignoring length bias gives very different results from a length bias corrected analysis.

```
> plot(log10(GO.wall[,2]), log10(GO.nobias[match(GO.wall[,1],GO.nobias[,1]),2]),
+       xlab="log10(Wallenius p-values)", ylab="log10(Hypergeometric p-values)",
+       xlim=c(-3,0), ylim=c(-3,0))
> abline(0,1,col=3,lty=2)
```



6.5.5 Limiting GO categories and other category based tests

By default, `goseq` tests all three major Gene Ontology branches; Cellular Components, Biological Processes and Molecular Functions. However, it is possible to limit testing to any combination of the major branches by using the `test.cats` argument to the `goseq` function. This is done by specifying a vector consisting of some combination of the strings “GO:CC”, “GO:BP” and “GO:MF”. For example, to test for only Molecular Function GO categories:

```
> GO.MF=goseq(pwf, "hg19", "ensGene", test.cats=c("GO:MF"))
> head(GO.MF)
```

	category	over_represented_pvalue	under_represented_pvalue	numDEInCat
1152	GO:0005515	5.167307e-07	0.9999998	1939
1196	GO:0008092	3.588065e-05	0.9999752	215

1037	GO:0005198	4.055605e-05	0.9999746	129
242	GO:0003735	7.570650e-05	0.9999665	40
2265	GO:0030215	1.644978e-04	0.9999780	11
1180	GO:0008017	3.447671e-04	0.9998057	68
	numInCat		term ontology	
1152	8977	protein binding	MF	
1196	789	cytoskeletal protein binding	MF	
1037	488	structural molecule activity	MF	
242	152	structural constituent of ribosome	MF	
2265	17	semaphorin receptor binding	MF	
1180	213	microtubule binding	MF	

Native support for other category tests, such as KEGG pathway analysis are also made available via this argument. See the man `goseq` function man page for up to date information on what category tests are natively supported.

6.5.6 Making sense of the results

Having performed the GO analysis, you may now wish to interpret the results. If you wish to identify categories significantly enriched/unenriched below some p-value cutoff, it is necessary to first apply some kind of multiple hypothesis testing correction. For example, GO categories over enriched using a .05 FDR cutoff [Benjamini and Hochberg, 1995] are:

```
> enriched.GO=GO.wall$category[p.adjust(GO.wall$over_represented_pvalue,
+ method="BH")<.05]
> head(enriched.GO)
```

```
[1] "GO:0005737" "GO:0044444" "GO:0005615" "GO:0044421" "GO:0006793" "GO:0006614"
```

Unless you are a machine, GO accession identifiers are probably not very meaningful to you. Information about each term can be obtained from the Gene Ontology website, <http://www.geneontology.org/>, or using the R package `GO.db`.

```
> library(GO.db)
> for(go in enriched.GO[1:10]){
+   print(GOTERM[[go]])
+   cat("-----\n")
+ }
```

```
GOID: GO:0005737
```

```
Term: cytoplasm
```

```
Ontology: CC
```

```
Definition: All of the contents of a cell excluding the plasma membrane
```

and nucleus, but including other subcellular structures.

GOID: GO:0044444

Term: cytoplasmic part

Ontology: CC

Definition: Any constituent part of the cytoplasm, all of the contents of a cell excluding the plasma membrane and nucleus, but including other subcellular structures.

Synonym: cytoplasm component

GOID: GO:0005615

Term: extracellular space

Ontology: CC

Definition: That part of a multicellular organism outside the cells proper, usually taken to be outside the plasma membranes, and occupied by fluid.

Synonym: intercellular space

GOID: GO:0044421

Term: extracellular region part

Ontology: CC

Definition: Any constituent part of the extracellular region, the space external to the outermost structure of a cell. For cells without external protective or external encapsulating structures this refers to space outside of the plasma membrane. This term covers constituent parts of the host cell environment outside an intracellular parasite.

Synonym: extracellular structure

GOID: GO:0006793

Term: phosphorus metabolic process

Ontology: BP

Definition: The chemical reactions and pathways involving the nonmetallic element phosphorus or compounds that contain phosphorus, usually in the form of a phosphate group (PO4).

Synonym: phosphorus metabolism

GOID: GO:0006614

Term: SRP-dependent cotranslational protein targeting to membrane

Ontology: BP

Definition: The targeting of proteins to a membrane that occurs during translation and is dependent upon two key components, the signal-recognition particle (SRP) and the SRP receptor. SRP is a

cytosolic particle that transiently binds to the endoplasmic reticulum (ER) signal sequence in a nascent protein, to the large ribosomal unit, and to the SRP receptor in the ER membrane.

Synonym: ER translocation

Synonym: SRP-dependent cotranslational membrane targeting

Synonym: SRP-dependent cotranslational protein-membrane targeting

GOID: GO:0000278

Term: mitotic cell cycle

Ontology: BP

Definition: Progression through the phases of the mitotic cell cycle, the most common eukaryotic cell cycle, which canonically comprises four successive phases called G1, S, G2, and M and includes replication of the genome and the subsequent segregation of chromosomes into daughter cells. In some variant cell cycles nuclear replication or nuclear division may not be followed by cell division, or G1 and G2 phases may be absent.

Synonym: mitosis

Synonym: GO:0007067

Secondary: GO:0007067

GOID: GO:0006796

Term: phosphate-containing compound metabolic process

Ontology: BP

Definition: The chemical reactions and pathways involving the phosphate group, the anion or salt of any phosphoric acid.

Synonym: phosphate metabolic process

Synonym: phosphate metabolism

GOID: GO:0006613

Term: cotranslational protein targeting to membrane

Ontology: BP

Definition: The targeting of proteins to a membrane that occurs during translation. The transport of most secretory proteins, particularly those with more than 100 amino acids, into the endoplasmic reticulum lumen occurs in this manner, as does the import of some proteins into mitochondria.

Synonym: cotranslational membrane targeting

Synonym: cotranslational protein membrane targeting

Synonym: cotranslational protein-membrane targeting

GOID: GO:0005576

Term: extracellular region

Ontology: CC

Definition: The space external to the outermost structure of a cell. For cells without external protective or external encapsulating structures this refers to space outside of the plasma membrane. This term covers the host cell environment outside an intracellular parasite.

Synonym: extracellular

6.5.7 Understanding goseq internals

The situation may arise where it is necessary for the user to perform some of the data processing steps usually performed automatically by `goseq` themselves. With this in mind, it will be useful to step through the preprocessing steps performed automatically by `goseq` to understand what is happening.

To start with, when `nullp` is called, `goseq` uses the genome and gene identifiers supplied to try and retrieve length information for all genes given to the `genes` argument. To do this, it retrieves the data from the database of gene lengths maintained in the package `geneLenDataBase`. This is performed by the `getlength` function in the following way:

```
> len=getlength(names(genes), "hg19", "ensGene")
```

```
> length(len)
```

```
[1] 22743
```

```
> length(genes)
```

```
[1] 22743
```

```
> head(len)
```

```
[1] 247 3133 1978 466 1510 954
```

After some data cleanup, the length data and the DE data is then passed to the `makespline` function to produce the PWF. The `nullp` returns a data frame which has 3 columns, the original DEgenes vector, the length bias data (in a column called `bias.data`) and the PWF itself (in a column named `pwf`). The names of the genes are also kept in this data frame as the names of the rows. If length data could not be obtained for a certain gene the corresponding entries in the `"bias.data"` and `"pwf"` columns are set to NA.

Next we call the `goseq` function to determine over/under representation of GO categories amongst DE genes. When we do this, `goseq` looks for the appropriate organism package and tries to obtain the mapping from genes to GO categories from it. This is done using the `getgo` function as follows:

```
> go=getgo(names(genes), "hg19", "ensGene")
> length(go)
```

```
[1] 22743
```

```
> length(genes)
```

```
[1] 22743
```

```
> head(go)
```

```
$<NA>
```

```
NULL
```

```
$ENSG00000182463
```

```
[1] "GO:0006139" "GO:0006351" "GO:0006355" "GO:0006357" "GO:0006366" "GO:0006725"
[7] "GO:0006807" "GO:0007275" "GO:0008150" "GO:0008152" "GO:0009058" "GO:0009059"
[13] "GO:0009889" "GO:0009987" "GO:0010467" "GO:0010468" "GO:0010556" "GO:0016070"
[19] "GO:0018130" "GO:0019219" "GO:0019222" "GO:0019438" "GO:0031323" "GO:0031326"
[25] "GO:0032501" "GO:0032502" "GO:0032774" "GO:0034641" "GO:0034645" "GO:0034654"
[31] "GO:0043170" "GO:0044237" "GO:0044238" "GO:0044249" "GO:0044260" "GO:0044271"
[37] "GO:0046483" "GO:0048856" "GO:0050789" "GO:0050794" "GO:0051171" "GO:0051252"
[43] "GO:0060255" "GO:0065007" "GO:0071704" "GO:0080090" "GO:0090304" "GO:0097659"
[49] "GO:1901360" "GO:1901362" "GO:1901576" "GO:1903506" "GO:2000112" "GO:2001141"
[55] "GO:0005575" "GO:0005622" "GO:0005623" "GO:0005634" "GO:0043226" "GO:0043227"
[61] "GO:0043229" "GO:0043231" "GO:0044424" "GO:0044464" "GO:0000981" "GO:0003674"
[67] "GO:0003676" "GO:0003677" "GO:0003682" "GO:0003700" "GO:0005488" "GO:0005515"
[73] "GO:0043167" "GO:0043169" "GO:0046872" "GO:0097159" "GO:0140110" "GO:1901363"
```

```
$<NA>
```

```
NULL
```

```
$<NA>
```

```
NULL
```

```
$<NA>
```

```
NULL
```

```
$ENSG00000125835
```

```
[1] "GO:0000375" "GO:0000377" "GO:0000387" "GO:0000398" "GO:0006139" "GO:0006351"
[7] "GO:0006353" "GO:0006366" "GO:0006369" "GO:0006396" "GO:0006397" "GO:0006464"
[13] "GO:0006479" "GO:0006725" "GO:0006807" "GO:0006810" "GO:0006913" "GO:0007275"
[19] "GO:0007399" "GO:0007417" "GO:0007420" "GO:0008150" "GO:0008152" "GO:0008213"
```

```

[25] "GO:0008334" "GO:0008380" "GO:0009058" "GO:0009059" "GO:0009987" "GO:0010467"
[31] "GO:0016043" "GO:0016070" "GO:0016071" "GO:0018130" "GO:0019438" "GO:0019538"
[37] "GO:0022607" "GO:0022613" "GO:0022618" "GO:0032259" "GO:0032501" "GO:0032502"
[43] "GO:0032774" "GO:0034622" "GO:0034641" "GO:0034645" "GO:0034654" "GO:0036211"
[49] "GO:0043170" "GO:0043412" "GO:0043414" "GO:0043933" "GO:0044085" "GO:0044237"
[55] "GO:0044238" "GO:0044249" "GO:0044260" "GO:0044267" "GO:0044271" "GO:0046483"
[61] "GO:0046907" "GO:0048513" "GO:0048731" "GO:0048856" "GO:0051169" "GO:0051170"
[67] "GO:0051179" "GO:0051234" "GO:0051641" "GO:0051649" "GO:0060322" "GO:0065003"
[73] "GO:0071704" "GO:0071826" "GO:0071840" "GO:0090304" "GO:0097659" "GO:1901360"
[79] "GO:1901362" "GO:1901564" "GO:1901576" "GO:0005575" "GO:0005622" "GO:0005623"
[85] "GO:0005634" "GO:0005654" "GO:0005681" "GO:0005682" "GO:0005683" "GO:0005684"
[91] "GO:0005685" "GO:0005686" "GO:0005687" "GO:0005689" "GO:0005697" "GO:0005737"
[97] "GO:0005829" "GO:0030532" "GO:0031974" "GO:0031981" "GO:0032991" "GO:0034708"
[103] "GO:0034709" "GO:0034719" "GO:0043226" "GO:0043227" "GO:0043229" "GO:0043231"
[109] "GO:0043233" "GO:0044422" "GO:0044424" "GO:0044428" "GO:0044444" "GO:0044446"
[115] "GO:0044464" "GO:0046540" "GO:0070013" "GO:0071004" "GO:0071005" "GO:0071007"
[121] "GO:0071010" "GO:0071011" "GO:0071013" "GO:0071204" "GO:0097525" "GO:0097526"
[127] "GO:0120114" "GO:1902494" "GO:1990234" "GO:1990904" "GO:0003674" "GO:0003676"
[133] "GO:0003723" "GO:0005488" "GO:0005515" "GO:0043021" "GO:0044877" "GO:0070034"
[139] "GO:0070990" "GO:0071208" "GO:0097159" "GO:1901363" "GO:1990446" "GO:1990447"

```

Note that some of the gene categories have been returned as "NULL". This means that a GO category could not be found in the database for one of the genes. In the `goseq` command, enrichment will only be calculated using genes with a GO category by default. However, in older versions of `goseq` (below 1.15.2), we counted all genes. i.e. genes with no categories still counted towards the total number of gene outside of any single category. It is possible to switch between these two behaviors using the `use_genes_without_cat` flag in `goseq`.

The first thing the `getgo` function does is to convert the UCSC genome/ID namings into the naming convention used by the organism packages. This is done using two hard coded conversion vectors that are included in the `goseq` package but usually hidden from the user.

```
> goseq:::ID_MAP
```

knownGene	refGene	ensGene	geneSymbol	sgd	plasmo	tair
"eg"	"eg"	"ENSEMBL"	"SYMBOL"	"sgd"	"plasmo"	"tair"

```
> goseq:::ORG_PACKAGES
```

anoGam	Arabidopsis	bosTau	ce
"org.Ag.eg"	"org.At.tair"	"org.Bt.eg"	"org.Ce.eg"
canFam	dm	danRer	E. coli K12
"org.Cf.eg"	"org.Dm.eg"	"org.Dr.eg"	"org.EcK12.eg"
E. coli Sakai	galGal	hg	mm

```

"org.EcSakai.eg"      "org.Gg.eg"      "org.Hs.eg"      "org.Mm.eg"
      rheMac          Malaria          panTro           rn
"org.Mmu.eg"  "org.Pf.plasmo"  "org.Pt.eg"      "org.Rn.eg"
      sacCer          susScr          xenTro
"org.Sc.sgd"      "org.Ss.eg"      "org.Xl.eg"

```

It is just as valid to run the length and GO category fetching as separate steps and then pass the result to the `nullp` and `goseq` functions using the `bias.data` and `gene2cat` arguments. Thus the following two blocks of code are equivalent:

```

> pwf=nullp(genes, "hg19", "ensGene")
> go=goseq(pwf, "hg19", "ensGene")

```

and

```

> gene_lengths=getlength(names(genes), "hg19", "ensGene")
> pwf=nullp(genes, bias.data=gene_lengths)
> go_map=getgo(names(genes), "hg19", "ensGene")
> go=goseq(pwf, "hg19", "ensGene", gene2cat=go_map)

```

6.6 KEGG pathway analysis

In order to illustrate performing a category test not present in the `goseq` database, we perform a KEGG pathway analysis. For human, the mapping from KEGG pathways to genes are stored in the package `org.Hs.eg.db`, in the object `org.Hs.egPATH`. In order to test for KEGG pathway over representation amongst DE genes, we need to extract this information and put it in a format that `goseq` understands. Unfortunately, the `org.Hs.eg.db` package does not contain direct mappings between ENSEMBL gene ID and KEGG pathway. Therefore, we have to construct this map by combining the ENSEMBL <-> Entrez and Entrez <-> KEGG mappings. This can be done using the following code:

```

> # Get the mapping from ENSEMBL 2 Entrez
> en2eg=as.list(org.Hs.egENSEMBL2EG)
> # Get the mapping from Entrez 2 KEGG
> eg2kegg=as.list(org.Hs.egPATH)
> # Define a function which gets all unique KEGG IDs
> # associated with a set of Entrez IDs
> grepKEGG=function(id,mapkeys){unique(unlist(mapkeys[id],use.names=FALSE))}
> # Apply this function to every entry in the mapping from
> # ENSEMBL 2 Entrez to combine the two maps
> kegg=lapply(en2eg,grepKEGG,eg2kegg)
> head(kegg)

```

Note that this step is quite time consuming. The code written here is not the most efficient way of producing this result, but the logic is much clearer than faster algorithms. The source code for `getgo` contains a more efficient routine.

We produce the PWF as before. Then, to perform a KEGG analysis, we simply make use of the `gene2cat` option in `goseq`:

```
> pwf=nullp(genes, "hg19", "ensGene")
> KEGG=goseq(pwf, gene2cat=kegg)
> head(KEGG)
```

Note that we do not have to tell the `goseq` function what organism and gene ID we are using as we are manually supplying the mapping between genes and categories.

KEGG analysis is shown as an illustration of how to supply your own mapping between gene ID and category, KEGG analysis is actually natively supported by `GOseq` and we could have performed it with the following code.

```
> pwf=nullp(genes, 'hg19', 'ensGene')
> KEGG=goseq(pwf, 'hg19', 'ensGene', test.cats="KEGG")
> head(KEGG)
```

	category	over_represented_pvalue	under_represented_pvalue	numDEInCat	numInCat
88	03010	6.331426e-06	0.9999980	29	87
77	00900	2.393680e-04	0.9999710	10	15
113	04115	8.178449e-04	0.9996829	26	64
175	04964	2.152488e-03	0.9995921	10	17
27	00330	3.673147e-03	0.9986576	18	44
20	00250	5.204967e-03	0.9984341	13	28

Noting that this time it was necessary to tell the `goseq` function that we are using HG19 and ENSEMBL gene ID, as the function needs this information to automatically construct the mapping from `geneid` to KEGG pathway.

6.7 Extracting mappings from organism packages

If you know that the information mapping gene ID to your categories of interest is contained in the organism packages, but `goseq` fails to fetch it automatically, you may want to extract it yourself and then pass it to the `goseq` function using the `gene2cat` argument. This is done in exactly the same way as extracting the KEGG to ENSEMBL mappings in the section “KEGG pathway analysis” above. This example is actually the worst case, where it is necessary to combine two mappings to get the desired list. If we had instead wanted the association between Entrez gene IDs and KEGG pathways, the following code would have been sufficient:

```

> kegg=as.list(org.Hs.egPATH)
> head(kegg)

$`1`
[1] NA

$`2`
[1] "04610"

$`3`
[1] NA

$`9`
[1] "00232" "00983" "01100"

$`10`
[1] "00232" "00983" "01100"

$`11`
[1] NA

```

A note on fetching GO mappings from the organism. The data structure of GO is a directed acyclic graph. This means that in addition to each GO category being associated with a set of genes, it may also have children that are associated to other genes. It is important to use the `org.Hs.egGO2ALLEGS` and NOT the `org.Hs.egGO` object to create the mapping between GO categories and gene identifiers, as the latter does not include the links to genes arising from "child" GO categories. Thank you to Christopher Fjell for pointing this out.

6.8 Correcting for other biases

It is possible that in some circumstances you will wish to correct not just for length bias, but for the total number of counts. This can make sense because power to detect DE depends on the total number of counts a gene receives, which is the product of gene length and gene expression. So correcting for read count bias will compensate for all biases, known and unknown, in power to detect DE. On the other hand, it will also remove bias resulting from differences in expression level, which may not be desirable.

Correcting for count bias will produce a different PWF. Therefore, we need to tell `goseq` about the data on which the fraction DE depends when calculating the PWF using the `nullp` function. We then simply pass the result to `goseq` as usual.

So, in order to tell `goseq` to correct for read count bias instead of length bias, all you need to do is supply a numeric vector, containing the number of counts for each gene to `nullp`.

```
> countbias=rowSums(counts)[rowSums(counts)!=0]
> length(countbias)
```

```
[1] 22743
```

```
> length(genes)
```

```
[1] 22743
```

To use the count bias when doing GO analysis, simply pass this vector to `nullp` using the `bias.data` option. Note that we have to supply "hg19" and "ensGene" to `goseq` as it is not used by `nullp` and hence not in the `pwf.counts` object.

```
> pwf.counts=nullp(genes,bias.data=countbias)
> GO.counts=goseq(pwf.counts,"hg19","ensGene")
> head(GO.counts)
```

	category	over_represented_pvalue	under_represented_pvalue	numDEInCat
2651	GO:0005886	3.325305e-15		1
15869	GO:0071944	2.227417e-14		1
5399	GO:0016021	5.206973e-14		1
11126	GO:0044459	8.161624e-14		1
7381	GO:0031224	1.243555e-13		1
11099	GO:0044425	1.154449e-12		1

	numInCat	term	ontology
2651	3228	plasma membrane	CC
15869	3325	cell periphery	CC
5399	3093	integral component of membrane	CC
11126	1698	plasma membrane part	CC
7381	3175	intrinsic component of membrane	CC
11099	4218	membrane part	CC

Note that if you want to correct for length bias, but your organism/gene identifier is not natively supported, then you need to follow the same procedure as above, only the numeric vector supplied will contain each gene's length instead of its number of reads.

7 Setup

This vignette was built on:

```
> sessionInfo()
```

R version 3.6.1 (2019-07-05)
Platform: x86_64-pc-linux-gnu (64-bit)
Running under: Ubuntu 18.04.3 LTS

Matrix products: default
BLAS: /home/biocbuild/bbs-3.10-bioc/R/lib/libRblas.so
LAPACK: /home/biocbuild/bbs-3.10-bioc/R/lib/libRlapack.so

locale:
[1] LC_CTYPE=en_US.UTF-8 LC_NUMERIC=C
[3] LC_TIME=en_US.UTF-8 LC_COLLATE=C
[5] LC_MONETARY=en_US.UTF-8 LC_MESSAGES=en_US.UTF-8
[7] LC_PAPER=en_US.UTF-8 LC_NAME=C
[9] LC_ADDRESS=C LC_TELEPHONE=C
[11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C

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

other attached packages:
[1] GO.db_3.10.0 org.Hs.eg.db_3.10.0 AnnotationDbi_1.48.0
[4] Biobase_2.46.0 rtracklayer_1.46.0 GenomicRanges_1.38.0
[7] GenomeInfoDb_1.22.0 IRanges_2.20.0 S4Vectors_0.24.0
[10] BiocGenerics_0.32.0 edgeR_3.28.0 limma_3.42.0
[13] goseq_1.38.0 geneLenDataBase_1.21.0 BiasedUrn_1.07

loaded via a namespace (and not attached):
[1] locfit_1.5-9.1 Rcpp_1.0.2
[3] lattice_0.20-38 prettyunits_1.0.2
[5] Rsamtools_2.2.0 Biostrings_2.54.0
[7] assertthat_0.2.1 zeallot_0.1.0
[9] digest_0.6.22 BiocFileCache_1.10.0
[11] R6_2.4.0 backports_1.1.5
[13] RSQLite_2.1.2 httr_1.4.1
[15] pillar_1.4.2 zlibbioc_1.32.0
[17] rlang_0.4.1 GenomicFeatures_1.38.0
[19] progress_1.2.2 curl_4.2
[21] blob_1.2.0 Matrix_1.2-17
[23] splines_3.6.1 BiocParallel_1.20.0
[25] stringr_1.4.0 RCurl_1.95-4.12
[27] bit_1.1-14 biomaRt_2.42.0

[29] DelayedArray_0.12.0	compiler_3.6.1
[31] pkgconfig_2.0.3	askpass_1.1
[33] mgcv_1.8-30	openssl_1.4.1
[35] tidyselect_0.2.5	SummarizedExperiment_1.16.0
[37] tibble_2.1.3	GenomeInfoDbData_1.2.2
[39] matrixStats_0.55.0	XML_3.98-1.20
[41] crayon_1.3.4	dplyr_0.8.3
[43] dbplyr_1.4.2	GenomicAlignments_1.22.0
[45] bitops_1.0-6	rappdirs_0.3.1
[47] grid_3.6.1	nlme_3.1-141
[49] DBI_1.0.0	magrittr_1.5
[51] stringi_1.4.3	XVector_0.26.0
[53] vctrs_0.2.0	tools_3.6.1
[55] bit64_0.9-7	glue_1.3.1
[57] purrr_0.3.3	hms_0.5.1
[59] memoise_1.1.0	

8 Acknowledgments

Christopher Fjell for a series of bug fixes and pointing out the difference between the egGO and egGO2ALLEGS objects in the organism packages.

References

- Y. Benjamini and Y. Hochberg. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B*, 57:289–300, 1995.
- Hairi Li, Michael T Lovci, Young-Soo Kwon, Michael G Rosenfeld, Xiang-Dong Fu, and Gene W Yeo. Determination of tag density required for digital transcriptome analysis: application to an androgen-sensitive prostate cancer model. *Proc Natl Acad Sci USA*, 105(51):20179–84, Dec 2008. doi: 10.1073/pnas.0807121105.
- Alicia Oshlack and Matthew J Wakefield. Transcript length bias in RNA-seq data confounds systems biology. *Biol Direct*, 4:14, Jan 2009. doi: 10.1186/1745-6150-4-14.
- M. D. Robinson and G. K. Smyth. Moderated statistical tests for assessing differences in tag abundance. *Bioinformatics*, 23(21):2881–2887, 2007.
- M. D. Robinson and G. K. Smyth. Small-sample estimation of negative binomial dispersion, with applications to sage data. *Biostatistics*, 9(2):321–332, 2008.

M. D. Robinson, D. J. McCarthy, and G. K. Smyth. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1):139–40, Jan 2010. doi: 10.1093/bioinformatics/btp616.

M. D. Young, M. J. Wakefield, G. K. Smyth, and A. Oshlack. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology*, 11:R14, Feb 2010.