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SOFTWARE TOOL ARTICLE

Easy and efficient ensemble gene set testing with EGSEA [version 1; referees: 1 approved, 3 approved with reservations]

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Abstract

Gene set enrichment analysis is a popular approach for prioritising the biological processes perturbed in genomic datasets. The Bioconductor project hosts over 80 software packages capable of gene set analysis. Most of these packages search for enriched signatures amongst differentially regulated genes to reveal higher level biological themes that may be missed when focusing only on evidence from individual genes. With so many different methods on offer, choosing the best algorithm and visualization approach can be challenging. The EGSEA package solves this problem by combining results from up to 12 prominent gene set testing algorithms to obtain a consensus ranking of biologically relevant results.This workflow demonstrates how EGSEA can extend limma-based differential expression analyses for RNA-seq and microarray data using experiments that profile 3 distinct cell populations important for studying the origins of breast cancer. Following data normalization and set-up of an appropriate linear model for differential expression analysis, EGSEA builds gene signature specific indexes that link a wide range of mouse or human gene set collections obtained from MSigDB, GeneSetDB and KEGG to the gene expression data being investigated. EGSEA is then configured and the ensemble enrichment analysis run, returning an object that can be queried using several S4 methods for ranking gene sets and visualizing results via heatmaps, KEGG pathway views, GO graphs, scatter plots and bar plots. Finally, an HTML report that combines these displays can fast-track the sharing of results with collaborators, and thus expedite downstream biological validation. EGSEA is simple to use and can be easily integrated with existing gene expression analysis pipelines for both human and mouse data.

This article is included in the Bioconductor gateway.

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Introduction

Gene set enrichment analysis allows researchers to efficiently extract biological insights from long lists of differentially expressed genes by interrogating them at a systems level. In recent years, there has been a proliferation of gene set enrichment (GSE) analysis methods released through the Bioconductor project[1] together with a steady increase in the number of gene set collections available through online databases such as MSigDB[2], GeneSetDB[3] and KEGG[4]. In an effort to unify these computational methods and knowledge-bases, the **EGSEA** R/Bioconductor package was developed. EGSEA, which stands for *Ensemble of Gene Set Enrichment Analyses*[5] combines the results from multiple algorithms to arrive at a consensus gene set ranking to identify biological themes and pathways perturbed in an experiment. EGSEA calculates seven statistics to combine the individual gene set statistics of base GSE methods to rank biologically relevant gene sets. The current version of the **EGSEA** package[6] utilizes the analysis results of up to twelve prominent GSE algorithms that include: ***ora***[7], ***globaltest***[8], ***plage***[9], ***safe***[10], ***zscore***[11], ***gage***[12], ***ssgsea***[13], ***padog***[14], ***gsva***[15], ***camera***[16], ***roast***[17] and ***fry***[17]. The ***ora***, ***gage***, ***camera*** and ***gsva*** methods depend on a *competitive* null hypothesis which assumes the genes in a set do not have a stronger association with the experimental condition compared to randomly chosen genes outside the set. The remaining eight methods are based on a *self-contained* null hypothesis that only considers genes within a set and again assumes that they have no association with the experimental condition.

EGSEA provides access to a diverse range of gene signature collections through the **EGSEAdata** package that includes more than 25,000 gene sets for human and mouse organised according to their database sources (Table 1). For example, MSigDB[2] includes a number of collections (Hallmark (h) and c1–c7) that explore different biological themes ranging from very broad (h, c2, c5) through to more specialised ones focusing on cancer (c4, c6) and immunology (c7). The other main sources are GeneSetDB[3] and KEGG[4] which have similar collections focusing on different biological characteristics (Table 1). The choice of collection/s in any given analysis should of course be guided by the biological question of interest. The MSigDB c2 and c5 collections are the most widely used in our own analysis practice, spanning a wide range of biological processes and can often reveal new biological insights when applied to a given dataset.

The purpose of this article is to demonstrate the gene set testing workflow available in **EGSEA** on both RNA-seq and microarray data. Each analysis involves four major steps that are summarized in Figure 1: (1) selecting appropriate gene set collections for analysis and building an index that maps between the members of each set and the expression matrix; (2) choosing the base GSE methods to combine and the ranking options; (3) running the EGSEA test and (4) reporting results in various ways to share with collaborators. The **EGSEA** functions involved in each of these steps are introduced with code examples to demonstrate how they can be deployed as part of a limma differential expression analysis to help with the interpretation of results.

Table 1. Summary of the gene set collections available in the EGSEAdata package.

Database	Collection	Description
MSigDB	h Hallmarks	Gene sets representing well-defined biological states or processes that have coherent expression.
	c1 Positional	Gene sets by chromosome and cytogenetic band.
	c2 Curated	Gene sets obtained from a variety of sources, including online pathway databases and the biomedical literature.
	c3 Motif	Gene sets of potential targets regulated by transcription factors or microRNAs.
	c4 Computational	Gene sets defined computationally by mining large collections of cancer-oriented microarray data.
	c5 GO	Gene sets annotated by Gene Ontology (GO) terms.
	c6 Oncogenic	Gene sets of the major cellular pathways disrupted in cancer.
	c7 Immunologic	Gene sets representing different cell types and stimulations relevant to the immune system.
KEGG	Signalling Disease Metabolic	Gene sets obtained from the KEGG database.
GeneSetDB	Pathway Disease Drug Regulation GO Terms	Gene sets obtained from various online databases.

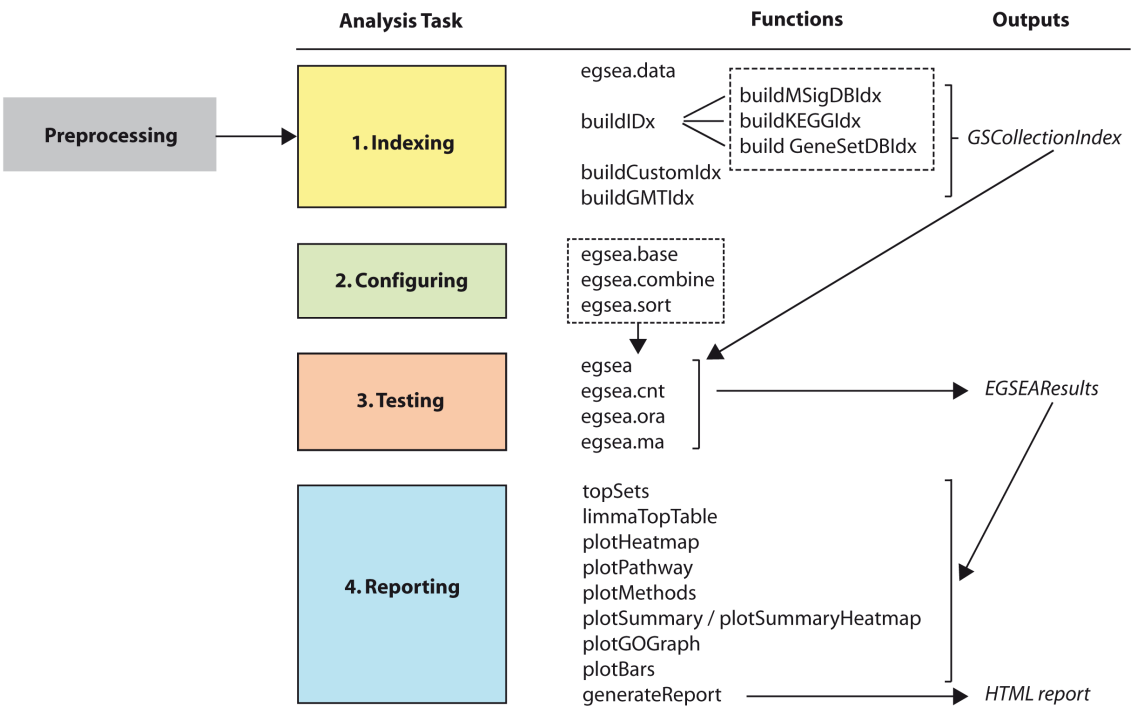


Figure 1. The main steps in an EGSEA analysis and the functions that perform each task.

Gene expression profiling of the mouse mammary gland

The first experiment analysed in this workflow is an RNA-seq dataset from Sheridan *et al.* (2015)[18] that consists of 3 cell populations (Basal, Luminal Progenitor (LP) and Mature Luminal (ML)) sorted from the mammary glands of female virgin mice. Triplicate RNA samples from each population were obtained in 3 batches and sequenced on an Illumina HiSeq 2000 using a 100 base-pair single-ended protocol. Raw sequence reads from the fastq files were aligned to the mouse reference genome (mm10) using the **Rsubread** package[19]. Next, gene-level counts were obtained using `featureCounts`[20] based on **Rsubread's** built-in *mm10* RefSeq-based annotation. The raw data along with further information on experimental design and sample preparation can be downloaded from the Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo/) using GEO Series accession number GSE63310 and will be preprocessed according to the RNA-seq workflow published by Law *et al.* (2016)[21].

The second experiment analysed in this workflow comes from Lim *et al.* (2010)[22] and is the microarray equivalent of the RNA-seq dataset mentioned above. The same 3 populations (Basal (also referred to as "MaSC-enriched"), LP and ML) were sorted from mouse mammary glands via flow cytometry. Total RNA from 5 replicates of each cell population were hybridised onto 3 Illumina MouseWG-6 v2 BeadChips. The intensity files and chip annotation file available in Illumina's proprietary formats (IDAT and BGX respectively) can be downloaded from http://bioinf.wehi.edu.au/EGSEA/arraydata.zip. The raw data from this experiment is also available from GEO under Series accession number GSE19446.

Analysis of RNA-seq data with EGSEA

Our RNA-seq analysis follows on directly from the workflow of Law *et al.* (2016) which performs a differential gene expression analysis on this data set using the Bioconductor packages **edgeR**[23], **limma**[24] and **Glimma**[25] with gene annotation from the `Mus.musculus` package[26]. The **limma** package offers a well-developed suite of statistical methods for dealing with differential expression for both microarray and RNA-seq datasets and will be used in the analyses of both datasets presented in this workflow.

Reading, preprocessing and normalisation of RNA-seq data

To get started with this analysis, download the R data file from http://bioinf.wehi.edu.au/EGSEA/mam.rnaseq.rdata. The code below loads the preprocessed count matrix from Law *et al.* (2016), performs TMM normalisation[27] on the

raw counts, and calculates voom weights for use in comparisons of gene expression between Basal and LP, Basal and ML, and LP and ML populations.

```
> library(limma)
> library(edgeR)
> load("mam.rnaseq.rdata")
> names(mam.rnaseq.data)
[1] "samples" "counts"  "genes"
> dim(mam.rnaseq.data)
[1] 14165     9
> x = calcNormFactors(mam.rnaseq.data, method = "TMM")
> design = model.matrix(~0+x$samples$group+x$samples$lane)
> colnames(design) = gsub("x\\$samples\\$group", "", colnames(design))
> colnames(design) = gsub("x\\$samples\\$lane", "", colnames(design))
> head(design)
  Basal LP ML L006 L008
1     0  1  0    0    0
2     0  0  1    0    0
3     1  0  0    0    0
4     1  0  0    1    0
5     0  0  1    1    0
6     0  1  0    1    0
> contr.matrix = makeContrasts(
+         BasalvsLP = Basal-LP,
+         BasalvsML = Basal - ML,
+         LPvsML = LP - ML,
+         levels = colnames(design))
> head(contr.matrix)
       Contrasts
Levels  BasalvsLP BasalvsML LPvsML
  Basal         1         1      0
  LP           -1         0      1
  ML            0        -1     -1
  L006          0         0      0
  L008          0         0      0
```

The `voom` function[28] from the **limma** package converts counts to log-counts-per-million (log-cpm) and calculates observation-level precision weights. The ***voom*** object (`v`) contains normalized log-cpm values and gene information used by all of the methods in the EGSEA analysis below. The precision `weights` stored within `v` are also used by the ***camera***, ***roast*** and ***fry*** gene set testing methods.

```
> v = voom(x, design, plot=FALSE)
> names(v)
[1] "genes"   "targets" "E"       "weights" "design"
```

For further information on preprocessing see Law *et al.* (2016), as a detailed explanation of these steps is beyond the scope of this article.

Gene set testing

The EGSEA algorithm makes use of the ***voom*** object (`v`), a design matrix (`design`) and an optional contrasts matrix (`contr.matrix`). The design matrix describes how the samples in the experiment relate to the coefficients estimated by the linear model[29]. The contrasts matrix then compares two or more of these coefficients to allow relative assessment of differential expression. Base methods that utilize linear models such as those from **limma** and **GSVA** (***gsva***, ***plage***, ***zscore*** and ***ssgsea***) make use of the design and contrasts matrices directly. For methods that do not support linear models, these two matrices are used to extract the group information for each comparison.

1. Exploring, selecting and indexing gene set collections

The package **EGSEAdata** includes more than 25,000 gene sets organized in collections depending on their database sources. Summary information about the gene set collections available in **EGSEAdata** can be displayed as follows:

```
> library(EGSEAdata)
> egsea.data("mouse")
The following databases are available in EGSEAdata for Mus  musculus:

Database name: KEGG Pathways
Version: NA
Download/update date: 07 March 2017
Data source: gage::kegg.gsets()
Supported species: human, mouse, rat
Gene set collections: Signaling, Metabolism, Disease
Related data objects: kegg.pathways
Number of gene sets in each collection for Mus  musculus :
Signaling: 132
Metabolism: 89
Disease: 67

Database name: Molecular Signatures Database (MSigDB)
Version: 5.2
Download/update date: 07 March 2017
Data source: http://software.broadinstitute.org/gsea
Supported species: human, mouse
Gene set collections: h, c1, c2, c3, c4, c5, c6, c7
Related data objects: msigdb, Mm.H, Mm.c2, Mm.c3, Mm.c4, Mm.c5, Mm.c6, Mm.c7
Number of gene sets in each collection for Mus musculus :
h Hallmark Signatures: 50
c2 Curated Gene Sets: 4729
c3 Motif Gene Sets: 836
c4 Computational Gene Sets: 858
c5 GO Gene Sets: 6166
c6 Oncogenic Signatures: 189
c7 Immunologic Signatures: 4872

Database name: GeneSetDB Database
Version: NA
Download/update date: 15 January 2016
Data source: http://www.genesetdb.auckland.ac.nz/
Supported species: human, mouse, rat
Gene set collections: gsdbdis, gsdbgo, gsdbdrug, gsdbpath, gsdbreg
Related data objects: gsetdb.human, gsetdb.mouse, gsetdb.rat
Number of gene sets in each collection for Mus musculus :
GeneSetDB Drug/Chemical: 6019
GeneSetDB Disease/Phenotype: 5077
GeneSetDB Gene Ontology: 2202
GeneSetDB Pathway: 1444
GeneSetDB Gene Regulation: 201

Type ?<data object name> to get a specific information
about it, e.g., ?kegg.pathways.
```

As the output above suggests, users can obtain help on any of the collections using the standard R help (`?`) command, for instance `?Mm.c2` will return more information on the mouse version of the c2 collection from MSigDB. The above information can be returned as a list:

```
> info = egsea.data("mouse", returnInfo = TRUE)
> names(info)
[1] "kegg"   "msigdb" "gsetdb"
> info$msigdb$info$collections
[1] "h"  "c1" "c2" "c3" "c4" "c5" "c6" "c7"
```

To highlight the capabilities of the **EGSEA** package, the KEGG pathways, c2 (curated gene sets) and c5 (Gene Ontology gene sets) collections from the MSigDB database are selected. Next, an index is built for each gene set collection using the EGSEA indexing functions to link the genes in the different gene set collections to the rows of our RNA-seq gene expression matrix. Indexes for the c2 and c5 collections from MSigDB and for the KEGG pathways are built using the `buildIdx` function which relies on Entrez gene IDs as its key. In the **EGSEAdata** gene set collections, Entrez IDs are used as they are widely adopted by the different source databases and tend to be more consistent and robust since there is one identifier per gene in a gene set. It is also relatively easy to convert other gene IDs into Entrez IDs.

```
> library(EGSEA)
> gs.annots = buildIdx(entrezIDs=v$genes$ENTREZID, species="mouse",
+           msigdb.gsets=c("c2", "c5"), go.part = TRUE)
[1] "Loading MSigDB Gene Sets ... "
[1] "Loaded gene sets for the collection c2 ..."
[1] "Indexed the collection c2 ..."
[1] "Created annotation for the collection c2 ..."
[1] "Loaded gene sets for the collection c5 ..."
[1] "Indexed the collection c5 ..."
[1] "Created annotation for the collection c5 ..."
MSigDB c5 gene set collection has been partitioned into
c5BP, c5CC, c5MF
[1] "Building KEGG pathways annotation object ... "
> names(gs.annots)
[1] "c2"   "c5BP" "c5CC" "c5MF" "kegg"
```

To obtain additional information on the gene set collection indexes, including the total number of gene sets, the version number and date of last revision, the methods *summary*, *show* and *getSetByName* (or *getSetByID*) can be invoked on an object of class **GSCollectionIndex**, which stores all of the relevant gene set information, as follows:

```
> class(gs.annots$c2)
[1] "GSCollectionIndex"
attr(,"package")
[1] "EGSEA"
> summary(gs.annots$c2)
c2 Curated Gene Sets (c2): 4726 gene sets - Version: 5.2, Update date: 07 March 2017
> show(gs.annots$c2)
An object of class "GSCollectionIndex"
Number of gene sets: 4726
Annotation columns: ID, GeneSet, BroadUrl, Description, PubMedID, NumGenes, Contributor
Total number of indexing genes: 14165
Species: Mus musculus
Collection name: c2 Curated Gene Sets
Collection unique label: c2
Database version: 5.2
Database update date: 07 March 2017
> s = getSetByName(gs.annots$c2, "SMID_BREAST_CANCER_LUMINAL_A_DN")
ID: M13072
```

```
GeneSet: SMID_BREAST_CANCER_LUMINAL_A_DN
BroadUrl: http://www.broadinstitute.org/gsea/msigdb/cards/SMID_BREAST_CANCER_
            LUMINAL_A_DN.html
Description: Genes down-regulated in the luminal A subtype of breast cancer.
PubMedID: 18451135
NumGenes: 23/24
Contributor: Jessica Robertson
> class(s)
[1] "list"
> names(s)
[1] "SMID_BREAST_CANCER_LUMINAL_A_DN"
> names(s$SMID_BREAST_CANCER_LUMINAL_A_DN)
[1] "ID"          "GeneSet"     "BroadUrl"    "Description" "PubMedID"
[6] "NumGenes"    "Contributor"
```

Objects of class **GSCollectionIndex** store for each gene set the Entrez gene IDs in the slot `original`, the indexes in the slot `idx` and additional annotation for each set in the slot `anno`.

```
> slotNames(gs.annots$c2)
[1] "original"   "idx"       "anno"        "featureIDs" "species"
[6] "name"       "label"     "version"     "date"
```

Other EGSEA functions such as `buildCustomIdx`, `buildGMTIdx`, `buildKEGGIdx`, `buildMSigDBIdx` and `buildGeneSetDBIdx` can be also used to build gene set collection indexes. The functions `buildCustomIdx` and `buildGMTIdx` were written to allow users to run EGSEA on gene set collections that may have been curated within a lab or downloaded from public databases and allow use of gene identifiers other than Entrez IDs. Example databases include, ENCODE Gene Set Hub (available from https://sourceforge.net/projects/encodegenesethub/), which is a growing resource of gene sets derived from high quality ENCODE profiling experiments encompassing hundreds of DNase hypersensitivity, histone modification and transcription factor binding experiments[30]. Other resources include PathwayCommons (http://www.pathwaycommons.org/)[31] and the **KEGGREST**[32] package that provides access to up-to-date KEGG pathways across many species.

2. Configuring EGSEA

Before an EGSEA test is carried out, a few parameters need to be specified. First, a mapping between Entrez IDs and Gene Symbols is created for use by the visualization procedures. This mapping can be extracted from the `genes` data.frame of the ***voom*** object as follows:

```
> colnames(v$genes)
 [1] "ENTREZID" "SYMBOL"   "CHR"
> symbolsMap = v$genes[, c(1, 2)]
> colnames(symbolsMap) = c("FeatureID", "Symbols")
> symbolsMap[, "Symbols"] = as.character(symbolsMap[, "Symbols"])
```

Another important parameter in EGSEA is the list of base GSE methods (`baseMethods` in the code below), which determines the individual algorithms that are used in the ensemble testing. The supported base methods can be listed using the function `egsea.base` as follows:

```
> egsea.base()
 [1] "camera"     "roast"      "safe"       "gage"        "padog"       "plage"
 [7] "zscore"     "gsva"       "ssgsea"     "globaltest"  "ora"         "fry"
```

The ***plage***, ***zscore*** and ***ssgsea*** algorithms are available in the **GSVA** package and ***camera***, ***fry*** and ***roast*** are implemented in the **limma** package[24]. The ***ora*** method is implemented using the `phyper` function from the **stats** package[33], which estimates the hypergeometric distribution for a 2 × 2 contingency table. The remaining algorithms are implemented in Bioconductor packages of the same name. A wrapper function is provided for each individual GSE method to utilize this existing R code and create a universal interface for all methods.

Eleven base methods are selected for our EGSEA analysis: ***camera***, ***safe***, ***gage***, ***padog***, ***plage***, ***zscore***, ***gsva***, ***ssgsea***, ***globaltest***, ***ora*** and ***fry***. ***Fry*** is a fast approximation of ***roast*** that assumes equal gene-wise variances across samples to produce similar *p*-values to a roast analysis run with an infinite number of rotations, and is selected here to save time.

```
> baseMethods = egsea.base()[-2]
> baseMethods
 [1] "camera"     "safe"       "gage"       "padog"      "plage"      "zscore"
 [7] "gsva"       "ssgsea"     "globaltest" "ora"        "fry"
```

Although, different combinations of base methods might produce different results, it has been found via simulation that including more methods gives better performance[5].

Since each base method generates different *p*-values, EGSEA supports six different methods from the **metap** package[34] for combining individual *p*-values (*Wilkinson*[35] is default), which can be listed as follows:

```
> egsea.combine()
[1] "fisher"   "wilkinson" "average"  "logitp"    "sump"      "sumz"
[7] "votep"    "median"
```

Finally, the sorting of EGSEA results plays an essential role in identifying relevant gene sets. Any of EGSEA's combined scores or the rankings from individual base methods can be used for sorting the results.

```
> egsea.sort()
 [1] "p.value"      "p.adj"       "vote.rank"    "avg.rank"       "med.rank"
 [6] "min.pvalue"   "min.rank"    "avg.logfc"    "avg.logfc.dir"  "direction"
[11] "significance" "camera"      "roast"        "safe"           "gage"
[16] "padog"        "plage"       "zscore"       "gsva"           "ssgsea"
[21] "globaltest"   "ora"         "fry"
```

Although `p.adj` is the default option for sorting EGSEA results for convenience, we recommend the use of either `med.rank` or `vote.rank` because they efficiently utilize the rankings of individual methods and tend to produce fewer false positives[5].

3. Ensemble testing with EGSEA

Next, the EGSEA analysis is performed using the `egsea` function that takes a ***voom*** object, a contrasts matrix, collections of gene sets and other run parameters as follows:

```
> gsa = egsea(voom.results=v, contrasts=contr.matrix,
+         gs.annots=gs.annots, symbolsMap=symbolsMap,
+         baseGSEAs=baseMethods, sort.by="med.rank",
+         num.threads = 8, report = FALSE)
EGSEA analysis has started
##------ Fri Jun 16 09:49:11 2017 ------##
Log fold changes are estimated using limma package ...
limma DE analysis is carried out ...
Number of used cores has changed to 3
in order to avoid CPU overloading.
EGSEA is running on the provided data and c2 collection
EGSEA is running on the provided data and c5BP collection
EGSEA is running on the provided data and c5CC collection
EGSEA is running on the provided data and c5MF collection
EGSEA is running on the provided data and kegg collection
##------ Fri Jun 16 09:57:56 2017 ------##
EGSEA analysis took 525.812 seconds.
EGSEA analysis has completed
```

In situations where the design matrix includes an intercept, a vector of integers that specify the columns of the design matrix to test using EGSEA can be passed to the `contrasts` argument. If this parameter is `NULL`, all pairwise comparisons based on `v$targets$group` are created, assuming that `group` is the primary factor in the design matrix. Likewise, all the coefficients of the primary factor are used if the design matrix has an intercept.

EGSEA is implemented with parallel computing features enabled using the **parallel** package[33] at both the method-level and experimental contrast-level. The running time of the EGSEA test depends on the base methods selected and whether report generation is enabled or not. The latter significantly increases the run time, particularly if the argument `display.top` is assigned a large value (> 20) and/or a large number of gene set collections are selected. EGSEA reporting functionality generates set-level plots for the top gene sets as well as collection-level plots.

The **EGSEA** package also has a function named `egsea.cnt`, that can perform the EGSEA test using an RNA-seq count matrix rather than a ***voom*** object, a function named `egsea.ora`, that can perform over-representation analysis with EGSEA reporting capabilities using only a vector of gene IDs, and the `egsea.ma` function that can perform EGSEA testing using a microarray expression matrix as shown later in the workflow.

Classes used to manage the results. The output of the functions `egsea`, `egsea.cnt`, `egsea.ora` and `egsea.ma` is an S4 object of class **EGSEAResults**. Several S4 methods can be invoked to query this object. For example, an overview of the EGSEA analysis can be displayed using the ***show*** method as follows:

```
> show(gsa)
An object of class "EGSEAResults"
Total number of genes: 14165
Total number of samples: 9
Contrasts: BasalvsLP, BasalvsML, LPvsML
Base GSE methods: camera (limma:3.32.2), safe (safe:3.16.0), gage (gage:2.26.0),
  padog (PADOG:1.18.0), plage (GSVA:1.24.1), zscore (GSVA:1.24.1), gsva (GSVA:1.24.1),
  ssgsea (GSVA:1.24.1),
P-values combining method: wilkinson
Sorting statistic: med.rank
Organism: Mus musculus
HTML report generated: No
Tested gene set collections:
c2 Curated Gene Sets (c2): 4726 gene sets - Version: 5.2, Update date: 07 March 2017
c5 GO Gene Sets (BP) (c5BP): 4653 gene sets - Version: 5.2, Update date: 07 March 2017
c5 GO Gene Sets (CC) (c5CC): 584 gene sets - Version: 5.2, Update date: 07 March 2017
c5 GO Gene Sets (MF) (c5MF): 928 gene sets - Version: 5.2, Update date: 07 March 2017
KEGG Pathways (kegg): 287 gene sets - Version: NA, Update date: 07 March 2017
EGSEA version: 1.5.2
EGSEAdata version: 1.4.0
Use summary(object) and topSets(object, ...) to explore this object.
```

This command displays the number of genes and samples that were included in the analysis, the experimental contrasts, base GSE methods, the method used to combine the *p*-values derived from different GSE algorithms, the sorting statistic used and the size of each gene set collection. Note that the gene set collections are identified using the labels that appear in parentheses (e.g. `c2`) in the output of ***show***.

4. Reporting EGSEA results

Getting top ranked gene sets. A summary of the top 10 gene sets in each collection for each contrast in addition to the EGSEA comparative analysis can be displayed using the S4 method ***summary*** as follows:

```
> summary(gsa)
**** Top 10 gene sets in the c2 Curated Gene Sets collection ****
** Contrast BasalvsLP **
LIM_MAMMARY_STEM_CELL_DN | LIM_MAMMARY_LUMINAL_PROGENITOR_UP
MONTERO_THYROID_CANCER_POOR_SURVIVAL_UP | SMID_BREAST_CANCER_LUMINAL_A_DN
NAKAYAMA_SOFT_TISSUE_TUMORS_PCA2_UP | REACTOME_LATENT_INFECTION_OF_HOMO_SAPIENS...
REACTOME_TRANSFERRIN_ENDOCYTOSIS_AND_RECYCLING | FARMER_BREAST_CANCER_CLUSTER_2
KEGG_EPITHELIAL_CELL_SIGNALING_... | LANDIS_BREAST_CANCER_PROGRESSION_UP
```

```
** Contrast BasalvsML **
LIM_MAMMARY_STEM_CELL_DN | LIM_MAMMARY_STEM_CELL_UP
LIM_MAMMARY_LUMINAL_MATURE_DN | PAPASPYRIDONOS_UNSTABLE_ATEROSCLEROTIC_PLAQUE_DN
NAKAYAMA_SOFT_TISSUE_TUMORS_PCA2_UP | LIM_MAMMARY_LUMINAL_MATURE_UP
CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_UP | RICKMAN_HEAD_AND_NECK_CANCER_A
YAGUE_PRETUMOR_DRUG_RESISTANCE_DN | BERTUCCI_MEDULLARY_VS_DUCTAL_BREAST_CANCER_DN

** Contrast LPvsML **
LIM_MAMMARY_LUMINAL_MATURE_UP | LIM_MAMMARY_LUMINAL_MATURE_DN
PHONG_TNF_RESPONSE_VIA_P38_PARTIAL | WOTTON_RUNX_TARGETS_UP
WANG_MLL_TARGETS | PHONG_TNF_TARGETS_DN
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS | CHIANG_LIVER_CANCER_SUBCLASS_CTNNB1_DN
GERHOLD_RESPONSE_TO_TZD_DN | DURAND_STROMA_S_UP

** Comparison analysis **
LIM_MAMMARY_LUMINAL_MATURE_DN | LIM_MAMMARY_STEM_CELL_DN
NAKAYAMA_SOFT_TISSUE_TUMORS_PCA2_UP | LIM_MAMMARY_LUMINAL_MATURE_UP
COLDREN_GEFITINIB_RESISTANCE_DN | LIM_MAMMARY_STEM_CELL_UP
CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_UP | LIM_MAMMARY_LUMINAL_PROGENITOR_UP
BERTUCCI_MEDULLARY_VS_DUCTAL_BREAST_CANCER_DN | MIKKELSEN_IPS_WITH_HCP_H3K27ME3

**** Top 10 gene sets in the c5 GO Gene Sets (BP) collection ****
** Contrast BasalvsLP **
GO_SYNAPSE_ORGANIZATION | GO_IRON_ION_TRANSPORT
GO_CALCIUM_INDEPENDENT_CELL_CELL_ADHESION_VIA_PLASMA_MEMBRANE_CELL_ADHESION_
  MOLECULES | GO_PH_REDUCTION
GO_HOMOPHILIC_CELL_ADHESION_VIA_PLASMA_MEMBRANE_ADHESION_MOLECULES | GO_VACUOLAR_
  ACIDIFICATION
GO_FERRIC_IRON_TRANSPORT | GO_TRIVALENT_INORGANIC_CATION_TRANSPORT
GO_NEURON_PROJECTION_GUIDANCE | GO_MESONEPHROS_DEVELOPMENT

** Contrast BasalvsML **
GO_FERRIC_IRON_TRANSPORT | GO_TRIVALENT_INORGANIC_CATION_TRANSPORT
GO_IRON_ION_TRANSPORT | GO_NEURON_PROJECTION_GUIDANCE
GO_GLIAL_CELL_MIGRATION | GO_SPINAL_CORD_DEVELOPMENT
GO_REGULATION_OF_SYNAPSE_ORGANIZATION | GO_ACTION_POTENTIAL
GO_MESONEPHROS_DEVELOPMENT | GO_NEGATIVE_REGULATION_OF_SMOOTH_MUSCLE_CELL_MIGRATION

** Contrast LPvsML **
GO_NEGATIVE_REGULATION_OF_NECROTIC_CELL_DEATH | GO_PARTURITION
GO_RESPONSE_TO_VITAMIN_D | GO_GPI_ANCHOR_METABOLIC_PROCESS
GO_REGULATION_OF_BLOOD_PRESSURE | GO_DETECTION_OF_MOLECULE_OF_BACTERIAL_ORIGIN
GO_CELL_SUBSTRATE_ADHESION | GO_PROTEIN_TRANSPORT_ALONG_MICROTUBULE
GO_INTRACILIARY_TRANSPORT | GO_CELLULAR_RESPONSE_TO_VITAMIN

** Comparison analysis **
GO_IRON_ION_TRANSPORT | GO_FERRIC_IRON_TRANSPORT
GO_TRIVALENT_INORGANIC_CATION_TRANSPORT | GO_NEURON_PROJECTION_GUIDANCE
GO_MESONEPHROS_DEVELOPMENT | GO_SYNAPSE_ORGANIZATION
GO_REGULATION_OF_SYNAPSE_ORGANIZATION | GO_MEMBRANE_DEPOLARIZATION_DURING_CARDIAC_
  MUSCLE_CELL_ACTION_POTENTIAL
GO_HOMOPHILIC_CELL_ADHESION_VIA_PLASMA_MEMBRANE_ADHESION_MOLECULES | GO_NEGATIVE_
  REGULATION_OF_SMOOTH_MUSCLE_CELL_MIGRATION

**** Top 10 gene sets in the c5 GO Gene Sets (CC) collection ****
** Contrast BasalvsLP **
GO_PROTON_TRANSPORTING_V_TYPE_ATPASE_COMPLEX | GO_VACUOLAR_PROTON_TRANSPORTING_
  V_TYPE_ATPASE_COMPLEX
```

```
GO_MICROTUBULE_END | GO_MICROTUBULE_PLUS_END
GO_ACTIN_FILAMENT_BUNDLE | GO_CELL_CELL_ADHERENS_JUNCTION
GO_NEUROMUSCULAR_JUNCTION | GO_AP_TYPE_MEMBRANE_COAT_ADAPTOR_COMPLEX
GO_INTERMEDIATE_FILAMENT | GO_CONDENSED_NUCLEAR_CHROMOSOME_CENTROMERIC_REGION

** Contrast BasalvsML **
GO_FILOPODIUM_MEMBRANE | GO_LATE_ENDOSOME_MEMBRANE
GO_PROTON_TRANSPORTING_V_TYPE_ATPASE_COMPLEX | GO_NEUROMUSCULAR_JUNCTION
GO_COATED_MEMBRANE | GO_ACTIN_FILAMENT_BUNDLE
GO_CLATHRIN_COAT | GO_AP_TYPE_MEMBRANE_COAT_ADAPTOR_COMPLEX
GO_CLATHRIN_ADAPTOR_COMPLEX | GO_CONTRACTILE_FIBER

** Contrast LPvsML **
GO_CILIARY_TRANSITION_ZONE | GO_TCTN_B9D_COMPLEX
GO_NUCLEAR_NUCLEOSOME | GO_INTRINSIC_COMPONENT_OF_ORGANELLE_MEMBRANE
GO_ENDOPLASMIC_RETICULUM_QUALITY_CONTROL_COMPARTMENT | GO_KERATIN_FILAMENT
GO_PROTEASOME_COMPLEX | GO_CILIARY_BASAL_BODY
GO_PROTEASOME_CORE_COMPLEX | GO_CORNIFIED_ENVELOPE

** Comparison analysis **
GO_PROTON_TRANSPORTING_V_TYPE_ATPASE_COMPLEX | GO_ACTIN_FILAMENT_BUNDLE
GO_NEUROMUSCULAR_JUNCTION | GO_AP_TYPE_MEMBRANE_COAT_ADAPTOR_COMPLEX
GO_CONTRACTILE_FIBER | GO_INTERMEDIATE_FILAMENT
GO_LATE_ENDOSOME_MEMBRANE | GO_CLATHRIN_VESICLE_COAT
GO_ENDOPLASMIC_RETICULUM_QUALITY_CONTROL_COMPARTMENT | GO_MICROTUBULE_END

**** Top 10 gene sets in the c5 GO Gene Sets (MF) collection ****
** Contrast BasalvsLP **
GO_HYDROGEN_EXPORTING_ATPASE_ACTIVITY | GO_SIGNALING_PATTERN_RECOGNITION_RECEPTOR_
  ACTIVITY
GO_LIPID_TRANSPORTER_ACTIVITY | GO_TRIGLYCERIDE_LIPASE_ACTIVITY
GO_AMINE_BINDING | GO_STRUCTURAL_CONSTITUENT_OF_MUSCLE
GO_NEUROPEPTIDE_RECEPTOR_ACTIVITY | GO_WIDE_PORE_CHANNEL_ACTIVITY
GO_CATION_TRANSPORTING_ATPASE_ACTIVITY | GO_LIPASE_ACTIVITY

** Contrast BasalvsML **
GO_G_PROTEIN_COUPLED_RECEPTOR_ACTIVITY | GO_TRANSMEMBRANE_RECEPTOR_PROTEIN_KINASE_
  ACTIVITY
GO_STRUCTURAL_CONSTITUENT_OF_MUSCLE | GO_VOLTAGE_GATED_SODIUM_CHANNEL_ACTIVITY
GO_CORECEPTOR_ACTIVITY | GO_TRANSMEMBRANE_RECEPTOR_PROTEIN_TYROSINE_KINASE_ACTIVITY
GO_LIPID_TRANSPORTER_ACTIVITY | GO_SULFOTRANSFERASE_ACTIVITY
GO_CATION_TRANSPORTING_ATPASE_ACTIVITY | GO_PEPTIDE_RECEPTOR_ACTIVITY

** Contrast LPvsML **
GO_MANNOSE_BINDING | GO_PHOSPHORIC_DIESTER_HYDROLASE_ACTIVITY
GO_BETA_1_3_GALACTOSYLTRANSFERASE_ACTIVITY | GO_COMPLEMENT_BINDING
GO_ALDEHYDE_DEHYDROGENASE_NAD_ACTIVITY | GO_MANNOSIDASE_ACTIVITY
GO_LIGASE_ACTIVITY_FORMING_CARBON_NITROGEN_BONDS | GO_CARBOHYDRATE_PHOSPHATASE_ACTIVITY
GO_LIPASE_ACTIVITY | GO_PEPTIDE_RECEPTOR_ACTIVITY

** Comparison analysis **
GO_STRUCTURAL_CONSTITUENT_OF_MUSCLE | GO_LIPID_TRANSPORTER_ACTIVITY
GO_CATION_TRANSPORTING_ATPASE_ACTIVITY | GO_CHEMOREPELLENT_ACTIVITY
GO_HEPARAN_SULFATE_PROTEOGLYCAN_BINDING | GO_TRANSMEMBRANE_RECEPTOR_PROTEIN_
  TYROSINE_KINASE_ACTIVITY
GO_LIPASE_ACTIVITY | GO_PEPTIDE_RECEPTOR_ACTIVITY
GO_CORECEPTOR_ACTIVITY | GO_TRANSMEMBRANE_RECEPTOR_PROTEIN_KINASE_ACTIVITY
```

```
**** Top 10 gene sets in the KEGG Pathways collection ****
** Contrast BasalvsLP **
Collecting duct acid secretion | alpha-Linolenic acid metabolism
Synaptic vesicle cycle | Hepatitis C
Vascular smooth muscle contraction | Rheumatoid arthritis
cGMP-PKG signaling pathway | Axon guidance
Progesterone-mediated oocyte maturation | Arrhythmogenic right ventricular
  cardiomyopathy (ARVC)

** Contrast BasalvsML **
Collecting duct acid secretion | Synaptic vesicle cycle
Other glycan degradation | Axon guidance
Arrhythmogenic right ventricular cardiomyopathy (ARVC) | Glycerophospholipid
  metabolism
Lysosome | Vascular smooth muscle contraction
Protein digestion and absorption | Oxytocin signaling pathway

** Contrast LPvsML **
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis | Histidine metabolism
Drug metabolism - cytochrome P450 | PI3K-Akt signaling pathway
Proteasome | Sulfur metabolism
Renin-angiotensin system | Nitrogen metabolism
Tyrosine metabolism | Systemic lupus erythematosus

** Comparison analysis **
Collecting duct acid secretion | Synaptic vesicle cycle
Vascular smooth muscle contraction | Axon guidance
Arrhythmogenic right ventricular cardiomyopathy (ARVC) | Oxytocin signaling pathway
Lysosome | Adrenergic signaling in cardiomyocytes
Linoleic acid metabolism | cGMP-PKG signaling pathway
```

EGSEA's *comparative* analysis allows researchers to estimate the significance of a gene set across multiple experimental contrasts. This analysis helps in the identification of biological processes that are perturbed in multiple experimental conditions simultaneously. This experiment is the RNA-seq equivalent of Lim *et al.* (2010)[22], who used Illumina microarrays to study the same cell populations (see later), so it is reassuring to observe the `LIM` gene signatures derived from this experiment amongst the top ranked c2 gene signatures in both the individual contrasts and comparative results.

Another way of exploring the EGSEA results is to retrieve the top ranked *N* sets in each collection and contrast using the method ***topSets***. For example, the top 10 gene sets in the c2 collection for the comparative analysis can be retrieved as follows:

```
> topSets(gsa, gs.label="c2", contrast = "comparison", names.only=TRUE)
Extracting the top gene sets of the collection
c2 Curated Gene Sets for the contrast comparison
 Sorted by med.rank
 [1] "LIM_MAMMARY_LUMINAL_MATURE_DN"
 [2] "LIM_MAMMARY_STEM_CELL_DN"
 [3] "NAKAYAMA_SOFT_TISSUE_TUMORS_PCA2_UP"
 [4] "LIM_MAMMARY_LUMINAL_MATURE_UP"
 [5] "COLDREN_GEFITINIB_RESISTANCE_DN"
 [6] "LIM_MAMMARY_STEM_CELL_UP"
 [7] "CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_UP"
 [8] "LIM_MAMMARY_LUMINAL_PROGENITOR_UP"
 [9] "BERTUCCI_MEDULLARY_VS_DUCTAL_BREAST_CANCER_DN"
[10] "MIKKELSEN_IPS_WITH_HCP_H3K27ME3"
```

The gene sets are ordered based on their `med.rank` as selected when ***egsea*** was invoked above. When the argument `names.only` is set to `FALSE`, additional information is displayed for each gene set including gene set annotation,

the EGSEA scores and the individual rankings by each base method. As expected, gene sets retrieved by EGSEA included the `LIM` gene sets[22] that were derived from microarray profiles of analagous mammary cell populations (sets 1, 2, 4, 6 and 8) as well as those derived from populations with similar origin (sets 7 and 9) and behaviour or characteristics (sets 5 and 10).

Next, ***topSets*** can be used to search for gene sets of interest based on different EGSEA scores as well as the rankings of individual methods. For example, the ranking of the six `LIM` gene sets from the c2 collection can be displayed based on the `med.rank` as follows:

```
> t = topSets(gsa, contrast = "comparison",
+             names.only=FALSE, number = Inf, verbose = FALSE)
> t[grep("LIM_", rownames(t)), c("p.adj", "Rank", "med.rank", "vote.rank")]
                                          p.adj Rank med.rank vote.rank
LIM_MAMMARY_LUMINAL_MATURE_DN      1.646053e-29    1       36         5
LIM_MAMMARY_STEM_CELL_DN           6.082053e-43    2       37         5
LIM_MAMMARY_LUMINAL_MATURE_UP      2.469061e-22    4       92         5
LIM_MAMMARY_STEM_CELL_UP          3.154132e-103    6      134         5
LIM_MAMMARY_LUMINAL_PROGENITOR_UP  3.871536e-30    8      180         5
LIM_MAMMARY_LUMINAL_PROGENITOR_DN  2.033005e-06  178      636       115
```

While five of the `LIM` gene sets are ranked in the top 10 by EGSEA, the values shown in the median rank (`med.rank`) column indicate that individual methods can assign much lower ranks to these sets. EGSEA's prioritisation of these gene sets demonstrates the benefit of an ensemble approach.

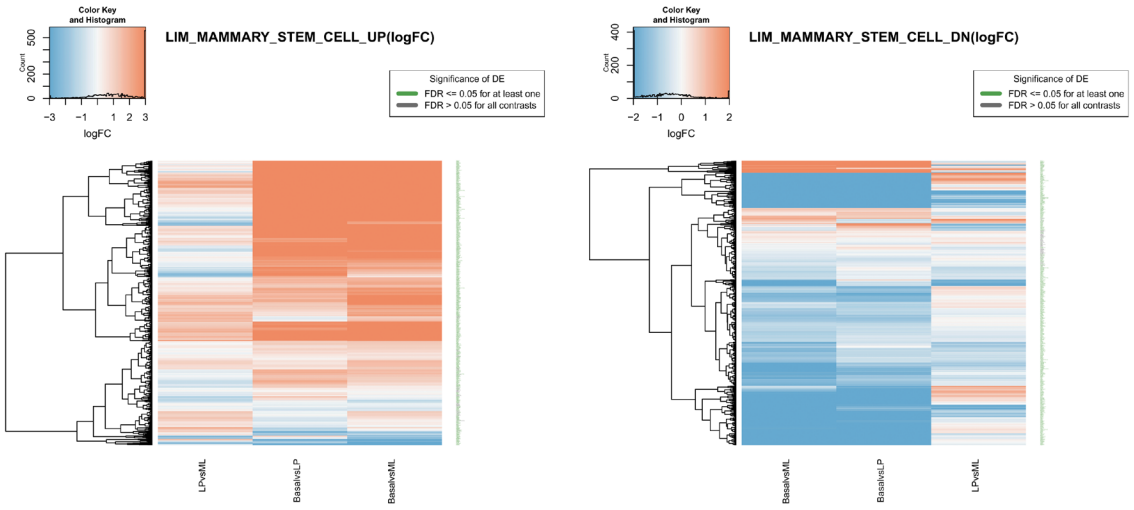
Similarly, we can find the top 10 pathways in the KEGG collection from the ensemble analysis for the Basal versus LP contrast and the comparative analysis as follows:

```
> topSets(gsa, gs.label="kegg", contrast="BasalvsLP", sort.by="med.rank")
Extracting the top gene sets of the collection
KEGG Pathways for the contrast BasalvsLP
 Sorted by med.rank
 [1] "Collecting duct acid secretion"        "alpha-Linolenic acid metabolism"
 [3] "Synaptic vesicle cycle"                "Hepatitis C"
 [5] "Vascular smooth muscle contraction"    "Rheumatoid arthritis"
 [7] "cGMP-PKG signaling pathway"            "Axon guidance"
 [9] "Progesterone-mediated oocyte maturation" "Arrhythmogenic right ventricular
                                                  cardiomyopathy (ARVC)"

> topSets(gsa, gs.label="kegg", contrast="comparison", sort.by="med.rank")
Extracting the top gene sets of the collection
KEGG Pathways for the contrast comparison
 Sorted by med.rank
 [1] "Collecting duct acid secretion"        "Synaptic vesicle cycle"
 [3] "Vascular smooth muscle contraction"    "Axon guidance"
 [5] "Arrhythmogenic right ventricular       "Oxytocin signaling pathway"
        cardiomyopathy (ARVC)"
 [7] "Lysosome"                              "Adrenergic signaling in
                                                 cardiomyocytes"
 [9] "Linoleic acid metabolism"              "cGMP-PKG signaling pathway
```

EGSEA highlights many pathways with known importance in the mammary gland such as those associated with distinct roles in lactation like basal cell contraction (`Vascular smooth muscle contraction` and `Oxytocin signalling pathway`) and milk production and secretion from luminal lineage cells (`Collecting duct acid secretion`, `Synaptic vesicle cycle` and `Lysosome`).

Visualizing results at the gene set level. Graphical representation of gene expression patterns within and between gene sets is an essential part of communicating the results of an analysis to collaborators and other researchers. **EGSEA** enables users to explore the elements of a gene set via a heatmap using the ***plotHeatmap*** method. Figure 2 shows



(a) LIM_MAMMARY_STEM_CELL_UP (b) LIM_MAMMARY_STEM_CELL_DN

Figure 2. Heatmaps of log-fold-changes for genes in the *LIM_MAMMARY_STEM_CELL_UP* and *LIM_MAMMARY_STEM_CELL_DN* gene sets across the three experimental comparisons (Basal vs LP, Basal vs ML and LP vs ML).

examples for the `LIM_MAMMARY_STEM_CELL_UP` and `LIM_MAMMARY_STEM_CELL_DN` signatures which can be visualized across all contrasts using the code below.

```
> plotHeatmap(gsa, gene.set="LIM_MAMMARY_STEM_CELL_UP", gs.label="c2",
+         contrast = "comparison", file.name = "hm_cmp_LIM_MAMMARY_STEM_CELL_UP")
Generating heatmap for LIM_MAMMARY_STEM_CELL_UP from the collection
c2 Curated Gene Sets and for the contrast comparison
> plotHeatmap(gsa, gene.set="LIM_MAMMARY_STEM_CELL_DN", gs.label="c2",
+         contrast = "comparison", file.name = "hm_cmp_LIM_MAMMARY_STEM_CELL_DN")
Generating heatmap for LIM_MAMMARY_STEM_CELL_DN from the collection
c2 Curated Gene Sets and for the contrast comparison
```

When using ***plotHeatmap***, the `gene.set` value must match the name returned from the ***topSets*** method. The rows of the heatmap represent the genes in the set and the columns represent the experimental contrasts. The heatmap colour-scale ranges from down-regulated (blue) to up-regulated (red) while the row labels (Gene symbols) are coloured in green when the genes are statistically significant in the DE analysis (i.e. FDR ≤ 0.05 in at least one contrast). Heatmaps can be generated for individual comparisons by changing the `contrast` argument of ***plotHeatmap***. The ***plotHeatmap*** method also generates a CSV file that includes the DE analysis results from ***limma::topTable*** for all expressed genes in the selected gene set and for each contrast (in the case of `contrast = "comparison"`). This file can be used to create customised plots using other R/Bioconductor packages.

In addition to heatmaps, pathway maps can be generated for the KEGG gene sets using the ***plotPathway*** method which uses functionality from the **pathview** package[36]. For example, the third KEGG signalling pathway retrieved for the contrast `BasalvsLP` is `Vascular smooth muscle contraction` and can be visualized as follows:

```
> plotPathway(gsa, gene.set = "Vascular smooth muscle contraction",
+             contrast = "BasalvsLP", gs.label = "kegg",
+             file.name = "Vascular_smooth_muscle_contraction")
Generating pathway map for Vascular smooth muscle contraction from the collection
KEGG Pathways and for the contrast BasalvsLP
```

Pathway components are coloured based on the gene-specific log-fold-changes as calculated in the **limma** DE analysis (Figure 3). Similarly, a comparative map can be generated for a given pathway across all contrasts.

```
> plotPathway(gsa, gene.set = "Vascular smooth muscle contraction",
+             contrast = "comparison", gs.label = "kegg",
+             file.name = "Vascular_smooth_muscle_contraction_cmp")
Generating pathway map for Vascular smooth muscle contraction from the collection
KEGG Pathways and for the contrast comparison
```

The comparative pathway map shows the log-fold-changes for each gene in each contrast by dividing the gene nodes on the map into multiple columns, one for each contrast (Figure 4).

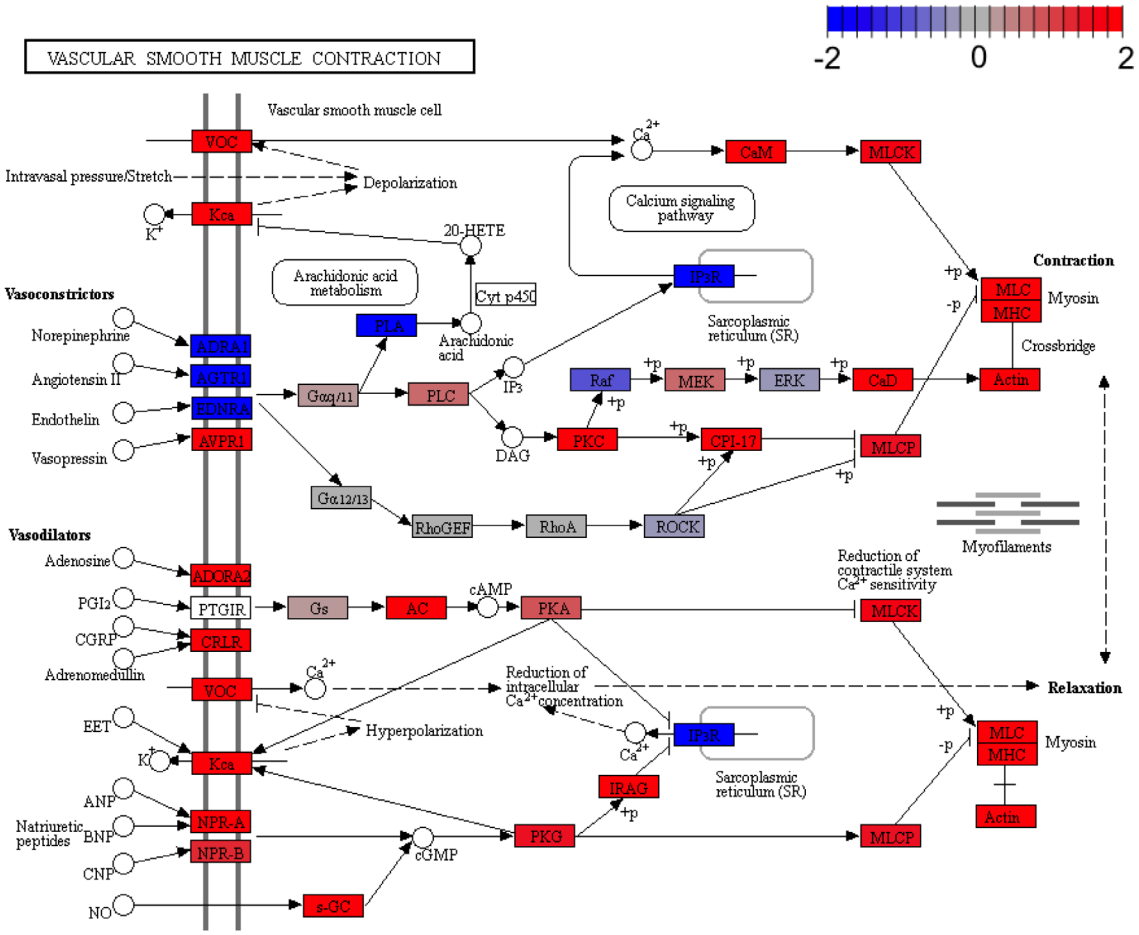


Figure 3. Pathway map for `Vascular smooth muscle contraction` (KEGG pathway mmu04270) with log-fold-changes from the Basal vs LP contrast.

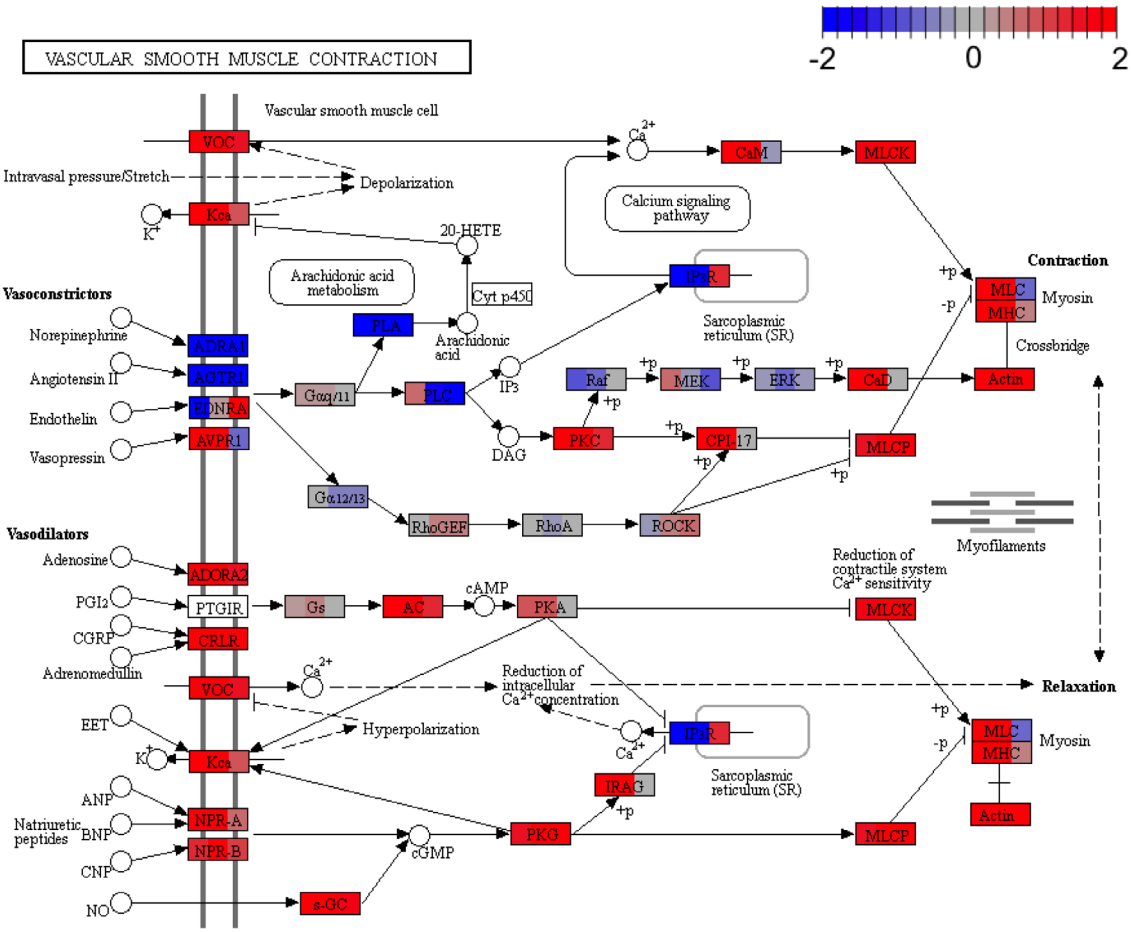
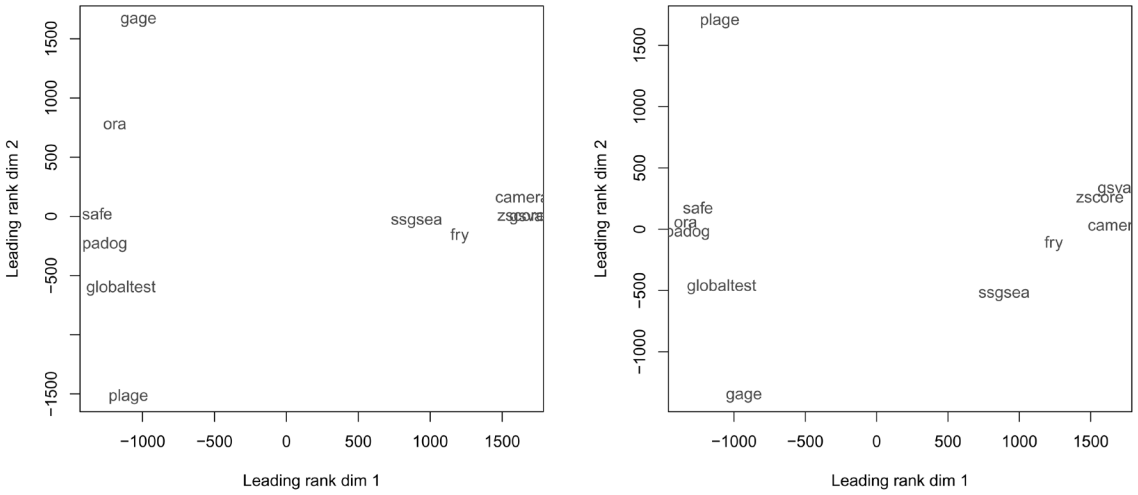


Figure 4. Pathway map for `Vascular smooth muscle contraction` (KEGG pathway mmu04270) with log-fold-changes across three experimental contrasts shown for each gene in the same order left to right that they appear in the contrasts matrix (i.e. Basal vs LP, Basal vs ML and LP vs ML).

Visualizing results at the experiment level. Since **EGSEA** combines the results from multiple gene set testing methods, it can be interesting to compare how different base methods rank a given gene set collection for a selected contrast. The `plotMethods` command generates a multi-dimensional scaling (MDS) plot for the ranking of gene sets across all the base methods used (Figure 5). Methods that rank gene sets similarly will appear closer together in this plot and we see that certain methods consistently cluster together across different gene set collections. The clustering of methods does not necessarily follow the style of null hypothesis tested though (i.e. *self-contained* versus *competitive*).

```
> plotMethods(gsa, gs.label = "c2", contrast = "BasalvsLP",
+         file.name = "mds_c2_BasalvsLP")
Generating MDS plot for the collection
c2 Curated Gene Sets and for the contrast BasalvsLP
> plotMethods(gsa, gs.label = "c5BP", contrast = "BasalvsLP",
+         file.name = "mds_c5_BasalvsLP")
Generating MDS plot for the collection
c5BP GO Gene Sets and for the contrast BasalvsLP
```



(a) c2 MDS **(b) c5 MDS**

Figure 5. Multi-dimensional scaling (MDS) plot showing the relationship between different gene set testing methods based on the rankings of the c2 (**a**) and c5 (**b**) gene sets on the Basal vs LP contrast.

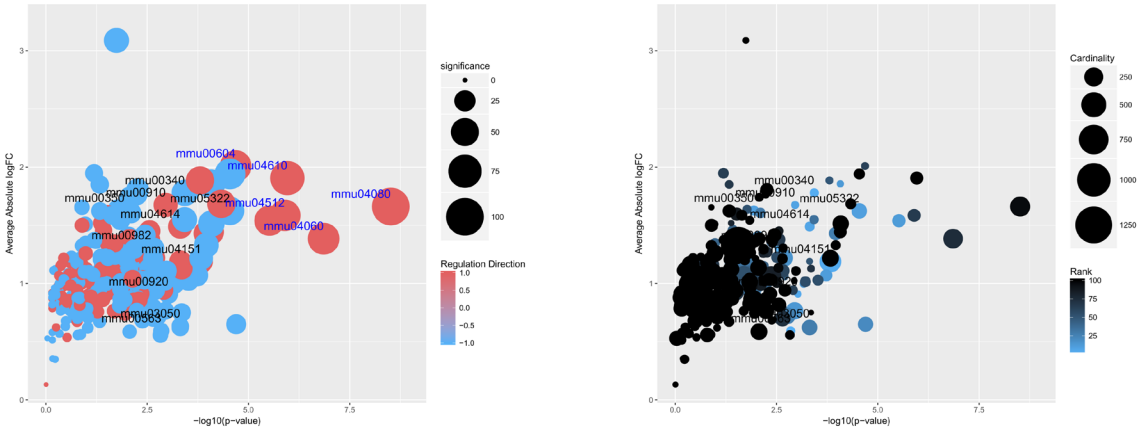
The significance of each gene set in a given collection for a selected contrast can be visualized using EGSEA's `plotSummary` method.

```
> plotSummary(gsa, gs.label = 3, contrast = 3,
+         file.name = "summary_kegg_LPvsML")
Generating Summary plots for the collection
KEGG Pathways and for the contrast LPvsML
```

The summary plot visualizes the gene sets as bubbles based on the $-\log_{10}$ (*p-value*) (X-axis) and the average absolute log fold-change of the set genes (Y-axis). The sets that appear towards the top-right corner of this plot are most likely to be biologically relevant. EGSEA generates two types of summary plots: the directional summary plot (Figure 6a), which colours the bubbles based on the regulation direction of the gene set (the direction of the majority of genes), and the ranking summary plot (Figure 6b), which colours the bubbles based on the gene set ranking in a given collection (according to the `sort.by` argument). The bubble size is based on the EGSEA *significance score* in the former plot and the gene set size in the latter. For example, the summary plots of the KEGG pathways for the LP vs ML contrast show few significant pathways (Figure 6). The blue colour labels on the ranking plot represents gene sets that do not appear in the top 10 gene sets that are selected based on the `sort.by` argument, yet their EGSEA *significance scores* are among the top 5 in the entire collection based on the *significance score*. This is used to identify gene sets with high *significance scores* that were not captured by the `sort.by` score. The gene set IDs and more information about each set can be found in the EGSEA HTML report generated later.

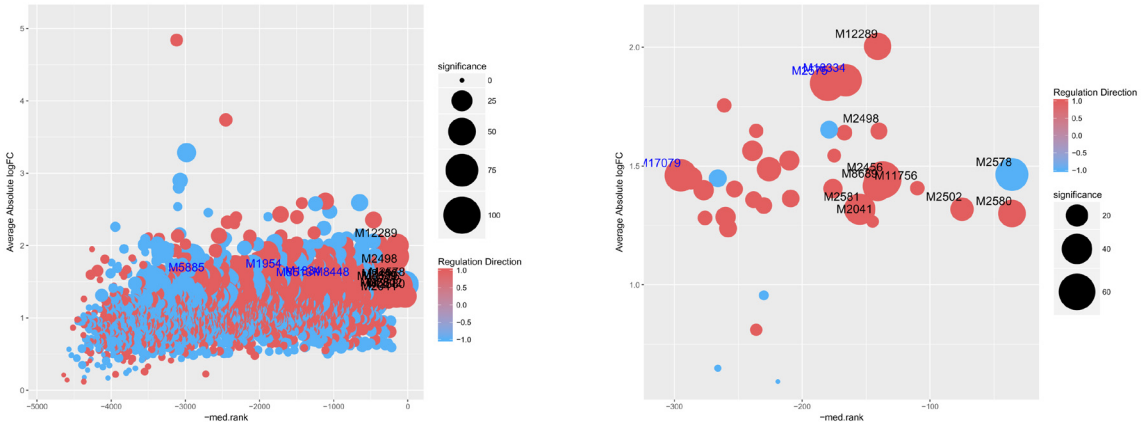
By default, ***plotSummary*** uses a gene set's `p.adj` Score for the X-axis. This behaviour can be easily modified by assigning any of the available ***sort.by*** scores into the parameter `x.axis`, for example, `med.rank` can be used to create an EGSEA summary plot (Figure 7a) as follows:

```
> plotSummary(gsa, gs.label = 1, contrast = 3,
+         file.name = "summary_c2_LPvsML",
+         x.axis = "med.rank")
Generating Summary plots for the collection
c2 Curated Gene Sets and for the contrast LPvsML
```



(a) Directional summary plot (b) Ranking summary plot

Figure 6. Summary plots of the significance of all gene sets in the KEGG collection for the LP vs ML contrast.



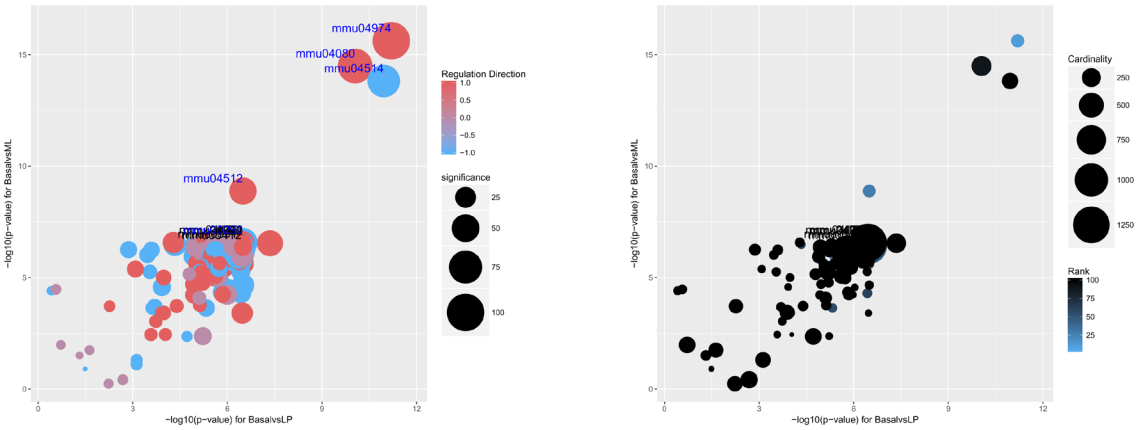
(a) Directional summary plot (b) Filtered directional summary plot

Figure 7. Summary plots of the significance of selected gene sets in the c2 collection for the LP vs ML contrast. The x-axis in each plot is the `med.rank`. A cut-off of 300 was used to select significant gene sets in the filtered plot (**b**).

The summary plot tends to become cluttered when the size of the gene set collection is very large as in Figure 7a. The parameter `x.cutoff` can be used to focus in on the significant gene sets rather than plotting the entire gene set collection, for example (Figure 7b):

```
> plotSummary(gsa, gs.label = 1, contrast = 3,
+          file.name = "summary_sig_c2_LPvsML",
+          x.axis = "med.rank", x.cutoff=300)
Generating Summary plots for the collection
c2 Curated Gene Sets and for the contrast LPvsML
```

Comparative summary plots can be also generated to compare the significance of gene sets between two contrasts, for example, the comparison between Basal vs LP and Basal vs ML (Figure 8a) shows that most of the KEGG



(a) **Directional summary plot** **(b)** **Ranking summary plot**

Figure 8. Comparative summary plots of the significance of all gene sets in the KEGG collection for the comparison of the contrasts: Basal vs LP and Basal vs ML.

pathways are regulated in the same direction with relatively few pathways regulated in opposite directions (purple coloured bubbles in Figure 8a). Such figures can be generated using the *plotSummary* method as follows:

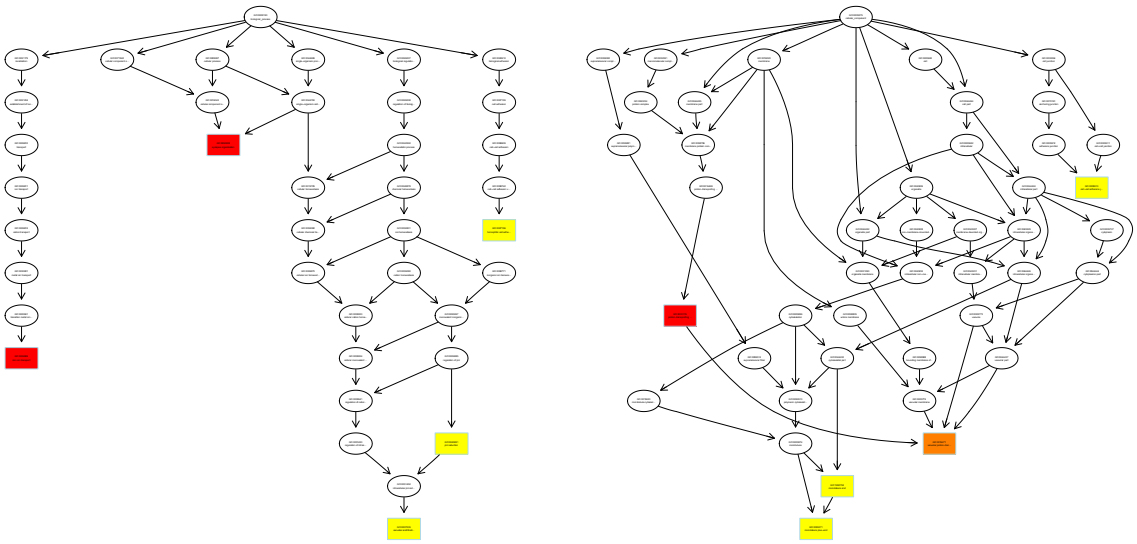
```
> plotSummary(gsa, gs.label = "kegg", contrast = c(1,2),
+          file.name = "summary_kegg_1vs2")
Generating Summary plots for the collection
KEGG Pathways and for the comparison BasalvsLP vs BasalvsML
```

The ***plotSummary*** method has two useful parameters: (i) `use.names` that can be used to display gene set names instead of gene set IDs and (ii) `interactive` that can be used to generate an interactive version of this plot.

The c5 collection of MSigDB and the Gene Ontology collection of GeneSetDB contain Gene Ontology (GO) terms. These collections are meant to be non-redundant, containing only a small subset of the entire GO and visualizing how these terms are related to each other can be informative. **EGSEA** utilizes functionality from the **topGO** package[37] to generate GO graphs for the significant biological processes (BPs), cellular compartments (CCs) and molecular functions (MFs). The ***plotGOGraph*** method can generate such a display (Figure 9) as follows:

```
> plotGOGraph(gsa, gs.label="c5BP", contrast = 1, file.name="BasalvsLP-c5BP-top-")
Generating GO Graphs for the collection c5 GO Gene Sets (BP)
 and for the contrast BasalvsLP based on the med.rank
> plotGOGraph(gsa, gs.label="c5CC", contrast = 1, file.name="BasalvsLP-c5CC-top-")
Generating GO Graphs for the collection c5 GO Gene Sets (CC)
 and for the contrast BasalvsLP based on the med.rank
```

The GO graphs are coloured based on the values of the argument `sort.by`, which in this instance was taken as `med.rank` by default since this was selected when EGSEA was invoked. The top five most significant GO terms are highlighted by default in each GO category (MF, CC or BP). More terms can be displayed by changing the value of the parameter `noSig`. However, this might generate very complicated and unresolved graphs. The colour of the nodes varies between red (most significant) and yellow (least significant). The values of the `sort.by` scoring function are scaled between 0 and 1 to generate these graphs.



(a) GO Biological Processes (b) GO Cellular Compartments

Figure 9. GO graphs of the top significant GO terms from the c5 gene set collection for the contrast Basal vs LP.

Another way to visualize results at the experiment level is via a summary *bar plot*. The method ***plotBars*** can be used to generate a bar plot for the top `N` gene sets in an individual collection for a particular contrast or from a comparative analysis across multiple contrasts. For example, the top 20 gene sets of the comparative analysis carried out on the c2 collection of MSigDB can be visualized in a *bar plot* (Figure 10) as follows:

```
> plotBars(gsa, gs.label = "c2", contrast = "comparison", file.name="comparison-c2-bars")
Generating a bar plot for the collection c2 Curated Gene Sets
 and the contrast comparison
```

The colour of the bars is based on the regulation direction of the gene sets, i.e., red for up-regulated, blue for down-regulated and purple for neutral regulation (in the case of the comparative analysis on experimental contrasts that show opposite behaviours). By default, the $-\log_{10}(p.adj)$ values are plotted for the top 20 gene sets selected and ordered based on the `sort.by` parameter. The parameters `bar.vals`, `number` and `sort.by` of ***plotBars*** can be changed to customize the *bar plot*.

When changes over multiple conditions are of interest, a *summary heatmap* can be a useful visualization. The method ***plotSummaryHeatmaps*** generates a heatmap of the top `N` gene sets in the comparative analysis across all experimental conditions (Figure 11). By default, 20 gene sets are selected based on the `sort.by` parameter and the values plotted are the average log-fold changes at the set level for the genes regulated in the same direction as the set regulation direction, i.e. `avg.logfc.dir`. The parameters `number`, `sort.by` and `hm.vals` of the ***plotSummaryHeatmaps*** can be used to customize the summary heatmap. Additionally, the parameter `show.vals`

can be used to display the values of a specific EGSEA score on the heatmap cells. An example summary heatmap can be generated for the MSigDB c2 collection with the following code:

```
> plotSummaryHeatmap(gsa, gs.label="c2", hm.vals = "avg.logfc.dir",
+         file.name="summary_heatmaps_c2")
Generating summary heatmap for the collection c2 Curated Gene Sets
sort.by: med.rank, hm.vals: avg.logfc.dir, show.vals:
> plotSummaryHeatmap(gsa, gs.label="kegg", hm.vals = "avg.logfc.dir",
+         file.name="summary_heatmaps_kegg")
Generating summary heatmap for the collection KEGG Pathways
sort.by: med.rank, hm.vals: avg.logfc.dir, show.vals:
```

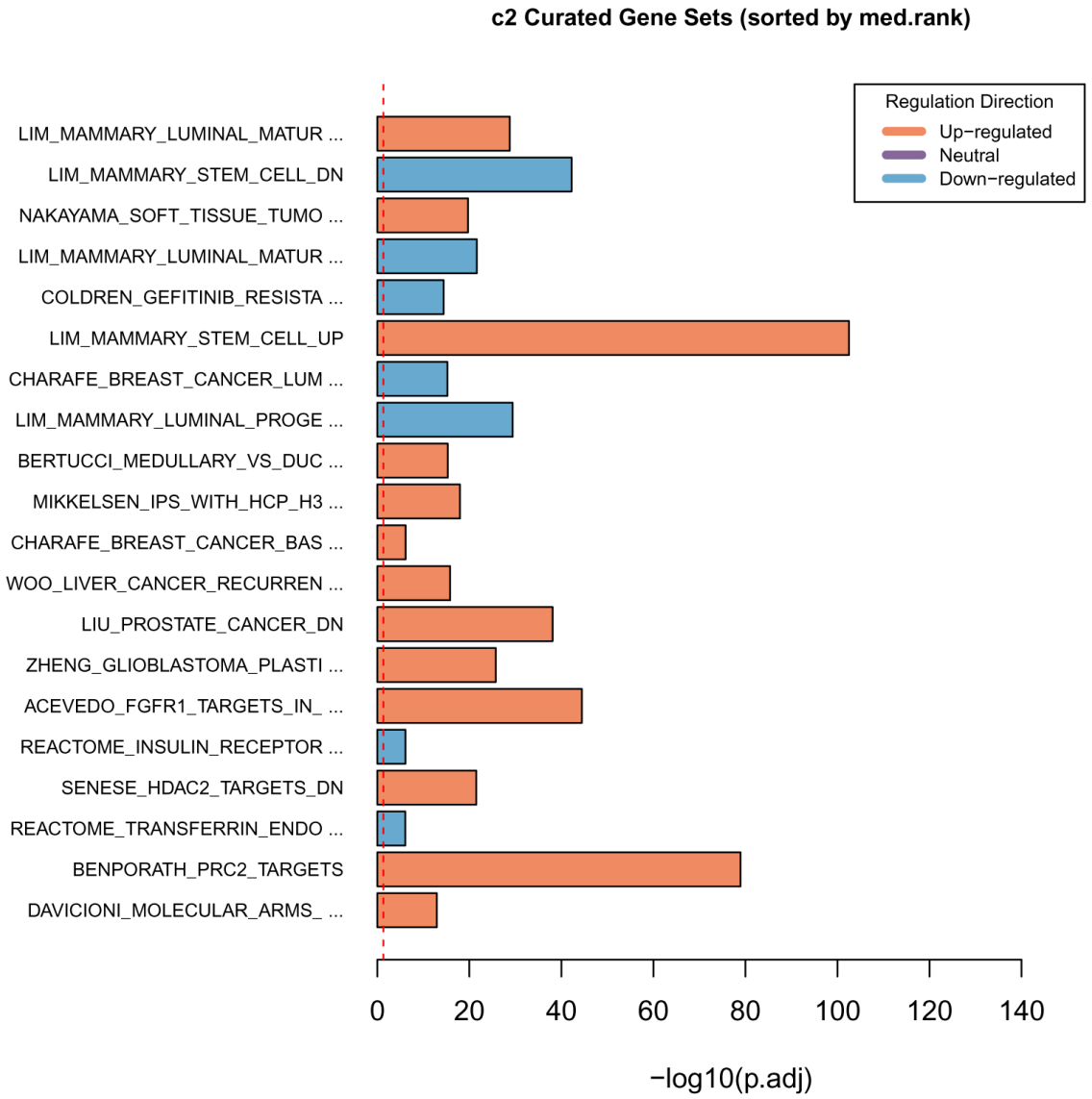
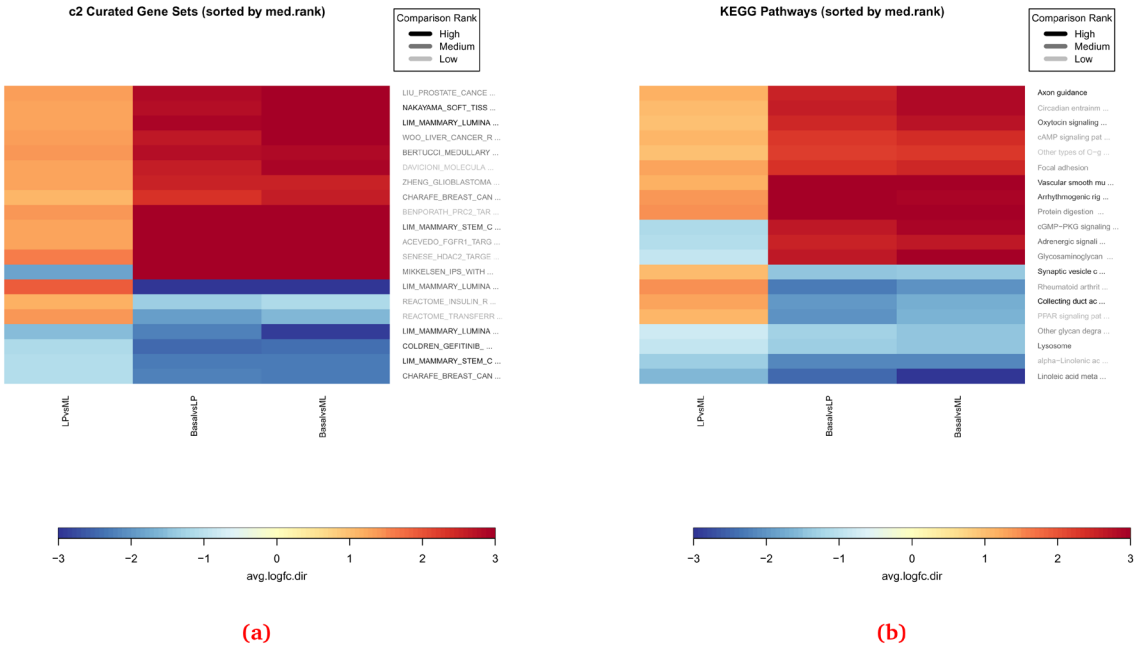


Figure 10. Bar plot of the *-log10(p-value)* of the top 20 gene sets from the comparative analysis of the c2 collection.



(a) **(b)**

Figure 11. Summary heatmaps for the top 20 gene sets from the c2 (**a**) and KEGG (**b**) collections obtained from the EGSEA comparative analysis.

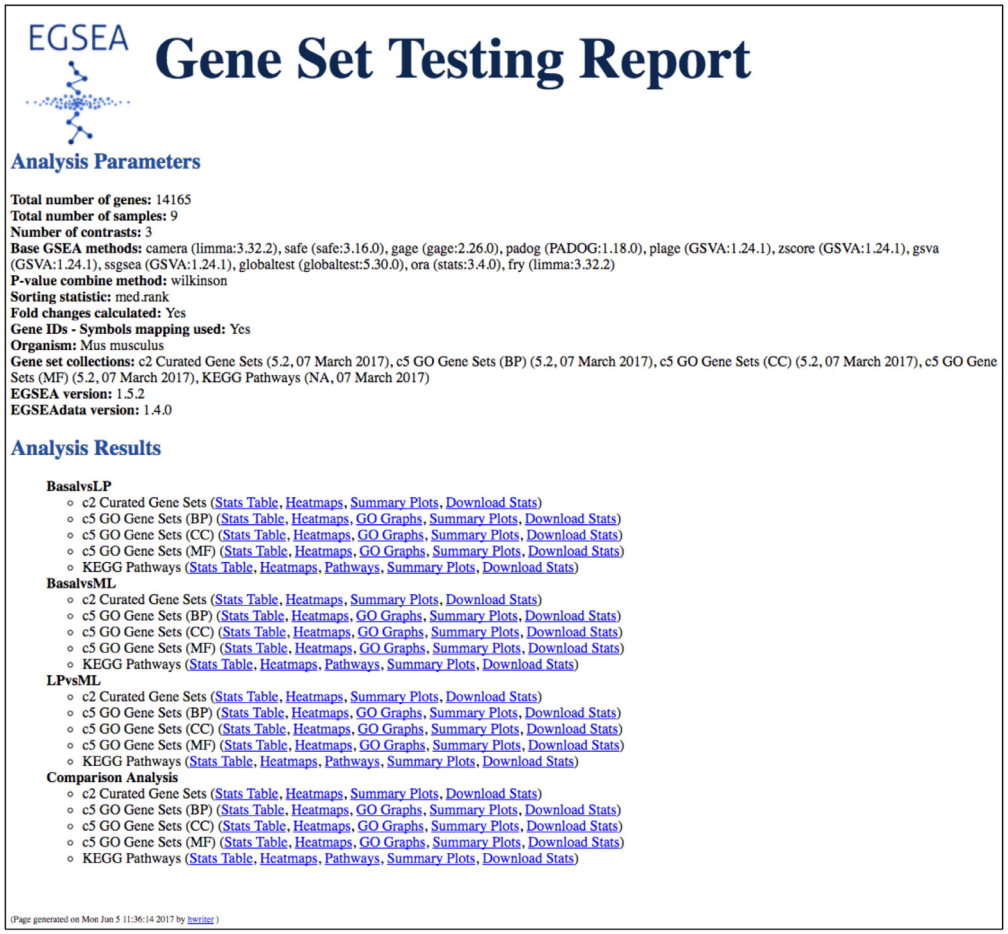
We find the heatmap view at both the gene set and summary level and the summary level bar plots to be useful summaries to include in publications to highlight the gene set testing results. The top differentially expressed genes from each contrast can be accessed from the **EGSEAResults** object using the ***limmaTopTable*** method.

```
> t = limmaTopTable(gsa, contrast=1)
> head(t)
       ENTREZID   SYMBOL CHR logFC AveExpr     t  P.Value adj.P.Val    B
19253     19253   Ptpn18   1 -5.63    4.13 -34.5 5.87e-10  9.62e-07 13.2
16324     16324    Inhbb   1 -4.79    6.46 -33.2 7.99e-10  9.62e-07 13.3
53624     53624    Cldn7  11 -5.51    6.30 -40.2 1.75e-10  9.62e-07 14.5
218518   218518 Marveld2  13 -5.14    4.93 -34.8 5.56e-10  9.62e-07 13.5
12759     12759      Clu  14 -5.44    8.86 -41.0 1.52e-10  9.62e-07 14.7
70350     70350    Basp1  15 -6.07    5.25 -34.3 6.22e-10  9.62e-07 13.3
```

Creating an HTML report of the results. To generate an EGSEA HTML report for this dataset, you can either set `report=TRUE` when you invoke ***egsea*** or use the S4 method ***generateReport*** as follows:

```
> generateReport(gsa, number = 20, report.dir="./mam-rnaseq-egsea-report")
EGSEA HTML report is being generated ...
```

The EGSEA report generated for this dataset is available online at http://bioinf.wehi.edu.au/EGSEA/mam-rnaseq-egsea-report/index.html (Figure 12). The HTML report is a convenient means of organising all of the results generated up to now, from the individual tables to the gene set level heatmaps, pathway maps and summary level plots. It can easily be shared with collaborators to allow them to explore their results more fully. Interactive



EGSEA

Gene Set Testing Report

Analysis Parameters

Total number of genes: 14165
Total number of samples: 9
Number of contrasts: 3
Base GSEA methods: camera (limma:3.32.2), safe (safe:3.16.0), gage (gage:2.26.0), padog (PADOG:1.18.0), plage (GSVA:1.24.1), zscore (GSVA:1.24.1), gsva (GSVA:1.24.1), ssgsea (GSVA:1.24.1), globaltest (globaltest:5.30.0), ora (stats:3.4.0), fry (limma:3.32.2)
P-value combine method: wilkinson
Sorting statistic: med.rank
Fold changes calculated: Yes
Gene IDs - Symbols mapping used: Yes
Organism: Mus musculus
Gene set collections: c2 Curated Gene Sets (5.2, 07 March 2017), c5 GO Gene Sets (BP) (5.2, 07 March 2017), c5 GO Gene Sets (CC) (5.2, 07 March 2017), c5 GO Gene Sets (MF) (5.2, 07 March 2017), KEGG Pathways (NA, 07 March 2017)
EGSEA version: 1.5.2
EGSEAdata version: 1.4.0

Analysis Results

BasalvsLP
- c2 Curated Gene Sets (Stats Table, Heatmaps, Summary Plots, Download Stats)
- c5 GO Gene Sets (BP) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- c5 GO Gene Sets (CC) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- c5 GO Gene Sets (MF) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- KEGG Pathways (Stats Table, Heatmaps, Pathways, Summary Plots, Download Stats)

BasalvsML
- c2 Curated Gene Sets (Stats Table, Heatmaps, Summary Plots, Download Stats)
- c5 GO Gene Sets (BP) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- c5 GO Gene Sets (CC) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- c5 GO Gene Sets (MF) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- KEGG Pathways (Stats Table, Heatmaps, Pathways, Summary Plots, Download Stats)

LPvsML
- c2 Curated Gene Sets (Stats Table, Heatmaps, Summary Plots, Download Stats)
- c5 GO Gene Sets (BP) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- c5 GO Gene Sets (CC) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- c5 GO Gene Sets (MF) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- KEGG Pathways (Stats Table, Heatmaps, Pathways, Summary Plots, Download Stats)

Comparison Analysis
- c2 Curated Gene Sets (Stats Table, Heatmaps, Summary Plots, Download Stats)
- c5 GO Gene Sets (BP) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- c5 GO Gene Sets (CC) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- c5 GO Gene Sets (MF) (Stats Table, Heatmaps, GO Graphs, Summary Plots, Download Stats)
- KEGG Pathways (Stats Table, Heatmaps, Pathways, Summary Plots, Download Stats)

(Page generated on Mon Jun 5 11:36:14 2017 by hwriter)

Figure 12. The EGSEA HTML report main page. This summary page details the analysis parameters (methods combined and ranking options selected) and organises the gene set analysis results by contrast, with further separation by gene set collection. The final section on this page presents results from the comparative analysis. For each contrast and gene set collection analysed, links to tables of results and plots are provided.

tables of results via the **DT** package (https://CRAN.R-project.org/package=DT) and summary plots from `plotly` (https://CRAN.R-project.org/package=plotly) are integrated into the report using **htmlwidgets** (https://CRAN.R-project.org/package=htmlwidgets) and can be added by setting `interactive = TRUE` in the command above. This option significantly increases both the run time and size of the final report due to the large number of gene sets in most collections.

This example completes our overview of EGSEA's gene set testing and plotting capabilities for RNA-seq data. Readers can refer to the EGSEA vignette or individual help pages for further details on each of the above methods and classes.

Analysis of microarray data with EGSEA

The second dataset analysed in this workflow comes from Lim *et al.* (2010)[22] and is the microarray equivalent of the RNA-seq data analysed above. Support for microarray data is a new feature in EGSEA, and in this example, we show an express route for analysis according to the steps shown in Figure 1, from selecting gene sets and building indexes, to configuring EGSEA, testing and reporting the results. First, the data must be appropriately preprocessed for an EGSEA analysis and to do this we make use of functions available in **limma**.

Reading, preprocessing and normalisation of microarray data

To analyse this dataset, we begin by unzipping the files downloaded from http://bioinf.wehi.edu.au/EGSEA/arraydata.zip into the current working directory. Illumina BeadArray data can be read in directly using the `readIDAT` and `readBGX` functions from the **illuminaio** package[38]. However, a more convenient way is via the `read.idat` function in **limma** which uses these **illuminaio** functions and outputs the data as an **EListRaw** object for further processing.

```
> library(limma)
> targets = read.delim("targets.txt", header=TRUE, sep=" ")
> data = read.idat(as.character(targets$File),
+                  bgxfile="GPL6887_MouseWG-6_V2_0_R0_11278593_A.bgx",
+                  annotation=c("Entrez_Gene_ID","Symbol", "Chromosome"))
Reading manifest file GPL6887_MouseWG-6_V2_0_R0_11278593_A.bgx ... Done
 4481850214_B_Grn.idat ... Done
 4481850214_C_Grn.idat ... Done
 4481850214_D_Grn.idat ... Done
 4481850214_F_Grn.idat ... Done
 4481850187_A_Grn.idat ... Done
 4481850187_B_Grn.idat ... Done
 4481850187_D_Grn.idat ... Done
 4481850187_E_Grn.idat ... Done
 4481850187_F_Grn.idat ... Done
 4466975058_A_Grn.idat ... Done
 4466975058_B_Grn.idat ... Done
 4466975058_C_Grn.idat ... Done
 4466975058_D_Grn.idat ... Done
 4466975058_E_Grn.idat ... Done
 4466975058_F_Grn.idat ... Done
Finished reading data.
> data$other$Detection = detectionPValues(data)
> data$targets = targets
> colnames(data) = targets$Sample
```

Next the `neqc` function in **limma** is used to carry out *normexp* background correction and quantile normalisation on the raw intensity values using negative control probes[39]. This is followed by $\log_2$-transformation of the normalised intensity values and removal of the control probes.

```
> data = neqc(data)
```

We then filter out probes that are consistently non-expressed or lowly expressed throughout all samples as they are uninformative in downstream analysis. Our threshold for expression requires probes to have a detection *p*-value of less than 0.05 in at least 5 samples (the number of samples within each group). We next remove genes without a valid Entrez ID and in cases where there are multiple probes targeting different isoforms of the same gene, select the probe with highest average expression as the representative one to use in the EGSEA analysis. This leaves 7,123 probes for further analysis.

```
> table(targets$Celltype)
Basal    LP    ML
    5     5     5
> keep.exprs = rowSums(data$other$Detection<0.05)>=5
> table(keep.exprs)
keep.exprs
FALSE  TRUE
23638 21643
> data = data[keep.exprs,]
> dim(data)
[1] 21643    15
```

```
> head(data$genes)
      Probe_Id Array_Address_Id Entrez_Gene_ID        Symbol Chromosome
3 ILMN_1219601          2030280           <NA> C920011N12Rik
4 ILMN_1252621          1980164         101142 2700050P07Rik          6
6 ILMN_3162407          6220026           <NA>         Zfp36
7 ILMN_2514723          2030072           <NA> 1110067B18Rik
8 ILMN_2692952          6040743         329831 4833436C18Rik          4
9 ILMN_1257952          7160091           <NA> B930060K05Rik
> sum(is.na(data$genes$Entrez_Gene_ID))
[1] 11535
> data1 = data[!is.na(data$genes$Entrez_Gene_ID), ]
> dim(data1)
[1] 10108    15
> ord = order(lmFit(data1)$Amean, decreasing=TRUE)
> ids2keep = data1$genes$Array_Address_Id[ord][!duplicated(data1$genes$Entrez_
    Gene_ID[ord])]
> data1 = data1[match(ids2keep, data1$genes$Array_Address_Id),]
> dim(data1)
[1] 7123   15
> expr = data1$E
> group = as.factor(data1$targets$Celltype)
> probe.annot = data1$genes[, 2:4]
> head(probe.annot)
> head(probe.annot)
      Array_Address_Id Entrez_Gene_ID   Symbol
39513          4120224          20102    Rps4x
9062           2260576          22143   Tuba1b
15308          5720202          12192  Zfp36l1
39894          1470600          11947    Atp5b
24709          2710477          20088    Rps24
9872           1580471         228033   Atp5g3
```

Setting up the linear model for EGSEA testing

As before, we need to set up an appropriate linear model[29] and contrasts matrix to look for differences between the Basal and LP, Basal and ML and LP and ML populations. A batch term is included in the linear model to account for differences in expression that are attributable to the day the experiment was run.

```
> head(data1$targets)
                     File Sample Celltype Time Experiment
2-2 4481850214_B_Grn.idat    2-2       ML  At1          1
3-3 4481850214_C_Grn.idat    3-3       LP  At1          1
4-4 4481850214_D_Grn.idat    4-4    Basal  At1          1
6-7 4481850214_F_Grn.idat    6-7       ML  At2          1
7-8 4481850187_A_Grn.idat    7-8       LP  At2          1
8-9 4481850187_B_Grn.idat    8-9    Basal  At2          1
> experiment = as.character(data1$targets$Experiment)
> design = model.matrix(~0 + group + experiment)
> colnames(design) = gsub("group", "", colnames(design))
> design
   Basal LP ML experiment2
1      0  0  1           0
2      0  1  0           0
3      1  0  0           0
4      0  0  1           0
5      0  1  0           0
6      1  0  0           0
7      0  0  1           0
8      0  1  0           0
```

```
9      1  0  0           0
10     0  0  1           1
11     0  1  0           1
12     1  0  0           1
13     1  0  0           1
14     0  0  1           1
15     0  1  0           1
attr(,"assign")
[1] 1 1 1 2
attr(,"contrasts")
attr(,"contrasts")$group
[1] "contr.treatment"

attr(,"contrasts")$experiment
[1] "contr.treatment"

> contr.matrix = makeContrasts(
+         BasalvsLP = Basal-LP,
+         BasalvsML = Basal-ML,
+         LPvsML = LP-ML,
+         levels = colnames(design))
> contr.matrix
             Contrasts
Levels        BasalvsLP BasalvsML LPvsML
  Basal               1         1      0
  LP                 -1         0      1
  ML                  0        -1     -1
  experiment2         0         0      0
```

1. Creating gene set collection indexes

We next extract the mouse c2, c5 and KEGG gene signature collections from the **EGSEAdata** package and build indexes based on Entrez IDs that link between the genes in each signature and the rows of our expression matrix.

```
> library(EGSEA)
> library(EGSEAdata)
> gs.annots = buildIdx(entrezIDs=probe.annot[, 2],
+             species="mouse",
+             msigdb.gsets=c("c2", "c5"), go.part = TRUE)
[1] "Loading MSigDB Gene Sets ... "
[1] "Loaded gene sets for the collection c2 ..."
[1] "Indexed the collection c2 ..."
[1] "Created annotation for the collection c2 ..."
[1] "Loaded gene sets for the collection c5 ..."
[1] "Indexed the collection c5 ..."
[1] "Created annotation for the collection c5 ..."
MSigDB c5 gene set collection has been partitioned into
c5BP, c5CC, c5MF
[1] "Building KEGG pathways annotation object ... "
> names(gs.annots)
[1] "c2"   "c5BP" "c5CC" "c5MF" "kegg"
```

2. Configuring and 3. Testing with EGSEA

The same 11 base methods used previously in the RNA-seq analysis were selected for the ensemble testing of the microarray data using the function `egsea.ma`. Gene sets were again prioritised by their median rank across the 11 methods.

```
> baseMethods = egsea.base()[-2]
> baseMethods
 [1] "camera"     "safe"       "gage"       "padog"       "plage"    "zscore"
 [7] "gsva"       "ssgsea"     "globaltest" "ora"         "fry"
>
> gsam = egsea.ma(expr=expr, group=group,
+     probe.annot = probe.annot,
+     design = design,
+         contrasts=contr.matrix,
+         gs.annots=gs.annots,
+         baseGSEAs=baseMethods, sort.by="med.rank",
+         num.threads = 8, report = FALSE)
EGSEA analysis has started
##------ Tue Jun 20 14:27:32 2017 ------##
Log fold changes are estimated using limma package ...
limma DE analysis is carried out ...
Number of used cores has changed to 3
in order to avoid CPU overloading.
EGSEA is running on the provided data and c2 collection

EGSEA is running on the provided data and c5BP collection

EGSEA is running on the provided data and c5CC collection

EGSEA is running on the provided data and c5MF collection

EGSEA is running on the provided data and kegg collection

##------ Tue Jun 20 14:33:37 2017 ------##
EGSEA analysis took 365.359 seconds.
EGSEA analysis has completed
```

4. Reporting EGSEA results

An HTML report that includes each of the gene set level and summary level plots shown individually for the RNA-seq analysis was then created using the `generateReport` function. We complete our analysis by displaying the top ranked sets for the c2 collection from a comparative analysis across all contrasts.

```
> generateReport(gsam, number = 20, report.dir="./mam-ma-egsea-report")
EGSEA HTML report is being generated ...
> topSets(gsam, gs.label="c2", contrast = "comparison", names.only=TRUE, number=5)
 Sorted by med.rank
 [1] "LIM_MAMMARY_STEM_CELL_UP"
 [2] "LIM_MAMMARY_LUMINAL_MATURE_DN"
 [3] "LIM_MAMMARY_STEM_CELL_DN"
 [4] "CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_DN"
 [5] "LIU_PROSTATE_CANCER_DN"
```

The EGSEA report generated for this dataset is available online at http://bioinf.wehi.edu.au/EGSEA/mam-ma-egsea-report/index.html. Reanalysis of this data retrieves similar c2 gene sets to those identified by analysis of RNA-seq data. These included the `LIM` gene signatures (sets 1, 2 and 3) as well as those derived from populations with similar cellular origin (set 4).

Discussion

In this workflow article, we have demonstrated how to use the **EGSEA** package to combine the results obtained from different gene signature databases across multiple GSE methods to find an ensemble solution. A key benefit of an EGSEA analysis is the detailed and comprehensive HTML report that can be shared with collaborators to help them interpret their data. This report includes tables prioritising gene signatures according to the user specified analysis options, and both gene set specific and summary graphics, each of which can be

generated individually using specific R commands. The approach taken by EGSEA is facilitated by the diverse range of gene set testing algorithms and plotting capabilities available within Bioconductor. EGSEA has been tailored to suit a limma-based differential expression analysis which continues to be a very popular and flexible platform for transcriptomic data. Analysts who choose an individual GSE algorithm to prioritise their results rather than an ensemble solution can still benefit from EGSEA's comprehensive reporting capability.

Software availability

Code to perform this analysis can be found in the **EGSEA123** workflow package available from Bioconductor: https://www.bioconductor.org/help/workflows/EGSEA123.

Latest source code is available at: https://github.com/mritchie/EGSEA123.

Archived source code as at the time of publication is available at: https://doi.org/10.5281/zenodo.1043436[40].

Software license: Artistic License 2.0.

Competing interests

MA and MN are employees of CSL Limited.

Grant information

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References

1. Huber W, Carey VJ, Gentleman R, *et al.*: **Orchestrating high-throughput genomic analysis with Bioconductor.** *Nat Methods.* 2015; **12**(2): 115–21.
PubMed Abstract | Publisher Full Text | Free Full Text
2. Subramanian A, Tamayo P, Mootha VK, *et al.*: **Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.** *Proc Natl Acad Sci U S A.* 2005; **102**(43): 15545–50.
PubMed Abstract | Publisher Full Text | Free Full Text
3. Araki H, Knapp C, Tsai P, *et al.*: **GeneSetDB: A comprehensive meta-database, statistical and visualisation framework for gene set analysis.** *FEBS Open Bio.* 2012; **2**: 76–82.
PubMed Abstract | Publisher Full Text | Free Full Text
4. Kanehisa M, Goto S: **KEGG: kyoto encyclopedia of genes and genomes.** *Nucleic Acids Res.* 2000; **28**(1): 27–30.
PubMed Abstract | Publisher Full Text | Free Full Text
5. Alhamdoosh M, Ng M, Wilson NJ, *et al.*: **Combining multiple tools outperforms individual methods in gene set enrichment analyses.** *Bioinformatics.* 2017; **33**(3): 414–424.
PubMed Abstract | Publisher Full Text | Free Full Text
6. Alhamdoosh M, Ng M, Ritchie ME: **EGSEA: Ensemble of Gene Set Enrichment Analyses.** R package version 1.5.2. 2017.
7. Tavazoie S, Hughes JD, Campbell MJ, *et al.*: **Systematic determination of genetic network architecture.** *Nat Genet.* 1999; **22**(3): 281–5.
PubMed Abstract | Publisher Full Text
8. Goeman JJ, van de Geer SA, de Kort F, *et al.*: **A global test for groups of genes: testing association with a clinical outcome.** *Bioinformatics.* 2004; **20**(1): 93–9.
PubMed Abstract | Publisher Full Text
9. Tomfohr J, Lu J, Kepler TB: **Pathway level analysis of gene expression using singular value decomposition.** *BMC Bioinformatics.* 2005; **6**: 225.
PubMed Abstract | Publisher Full Text | Free Full Text
10. Barry WT, Nobel AB, Wright FA: **Significance analysis of functional categories in gene expression studies: a structured permutation approach.** *Bioinformatics.* 2005; **21**(9): 1943–9.
PubMed Abstract | Publisher Full Text
11. Lee E, Chuang HY, Kim JW, *et al.*: **Inferring pathway activity toward precise disease classification.** *PLoS Comput Biol.* 2008; **4**(11): e1000217.
PubMed Abstract | Publisher Full Text | Free Full Text
12. Luo W, Friedman MS, Shedden K, *et al.*: **GAGE: generally applicable gene set enrichment for pathway analysis.** *BMC Bioinformatics.* 2009; **10**: 161.
PubMed Abstract | Publisher Full Text | Free Full Text
13. Barbie DA, Tamayo P, Boehm JS, *et al.*: **Systematic RNA interference reveals that oncogenic *KRAS*-driven cancers require**

TBK1. *Nature.* 2009; **462**(7269): 108–12.
PubMed Abstract | Publisher Full Text | Free Full Text

14. Tarca AL, Draghici S, Bhatti G, *et al.*: **Down-weighting overlapping genes improves gene set analysis.** *BMC Bioinformatics.* 2012; **13**: 136.
PubMed Abstract | Publisher Full Text | Free Full Text

15. Hänzelmann S, Castelo R, Guinney J: **GSVA: gene set variation analysis for microarray and RNA-seq data.** *BMC Bioinformatics.* 2013; **14**: 7.
PubMed Abstract | Publisher Full Text | Free Full Text

16. Wu D, Smyth GK: **Camera: a competitive gene set test accounting for inter-gene correlation.** *Nucleic Acids Res.* 2012; **40**(17): e133.
PubMed Abstract | Publisher Full Text | Free Full Text

17. Wu D, Lim E, Vaillant F, *et al.*: **ROAST: rotation gene set tests for complex microarray experiments.** *Bioinformatics.* 2010; **26**(17): 2176–82.
PubMed Abstract | Publisher Full Text | Free Full Text

18. Sheridan JM, Ritchie ME, Best SA, *et al.*: **A pooled shRNA screen for regulators of primary mammary stem and progenitor cells identifies roles for *Asap1* and *Prox1*.** *BMC Cancer.* 2015; **15**(1): 221.
PubMed Abstract | Publisher Full Text | Free Full Text

19. Liao Y, Smyth GK, Shi W: **The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote.** *Nucleic Acids Res.* 2013; **41**(10): e108.
PubMed Abstract | Publisher Full Text | Free Full Text

20. Liao Y, Smyth GK, Shi W: **featureCounts: an efficient general purpose program for assigning sequence reads to genomic features.** *Bioinformatics.* 2014; **30**(7): 923–30.
PubMed Abstract | Publisher Full Text

21. Law CW, Alhamdoosh M, Su S, *et al.*: **RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR [version 2; referees: 3 approved].** *F1000Res.* 2016; **5**: 1408.
PubMed Abstract | Publisher Full Text | Free Full Text

22. Lim E, Wu D, Pal B, *et al.*: **Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways.** *Breast Cancer Res.* 2010; **12**(2): R21.
PubMed Abstract | Publisher Full Text | Free Full Text

23. Robinson MD, McCarthy DJ, Smyth GK: **edgeR: a Bioconductor package for differential expression analysis of digital gene expression data.** *Bioinformatics.* 2010; **26**(1): 139–40.
PubMed Abstract | Publisher Full Text | Free Full Text

24. Ritchie ME, Phipson B, Wu D, *et al.*: ***limma* powers differential expression analyses for RNA-sequencing and microarray studies.** *Nucleic Acids Res.* 2015; **43**(7): e47.
PubMed Abstract | Publisher Full Text | Free Full Text

25. Su S, Law CW, Ah-Cann C, *et al.*: **Glimma: interactive graphics for gene expression analysis.** *Bioinformatics.* 2017; **33**(13): 2050–2.
PubMed Abstract | Publisher Full Text

26. Bioconductor Core Team: **Mus.musculus: Annotation package for the Mus.musculus object.** R package version 1.3.1. 2015.
Publisher Full Text

27. Robinson MD, Oshlack A: **A scaling normalization method for differential expression analysis of RNA-seq data.** *Genome Biol.* 2010; **11**(3): R25.
PubMed Abstract | Publisher Full Text | Free Full Text

28. Law CW, Chen Y, Shi W, *et al.*: **Voom: precision weights unlock linear model analysis tools for RNA-seq read counts.** *Genome Biol.* 2014; **15**(2): R29.
PubMed Abstract | Publisher Full Text | Free Full Text

29. Smyth GK: **Linear models and empirical Bayes methods for assessing differential expression in microarray experiments.** *Stat Appl Genet Mol Biol.* 2004; **3**(1): Article 3.
PubMed Abstract | Publisher Full Text

30. Ziemann M, Kaspi A, Rafehi H, *et al.*: **The ENCODE Gene Set Hub.** *Lorne Genome Conference.* 2017.
Publisher Full Text

31. Cerami EG, Gross BE, Demir E, *et al.*: **Pathway commons, a web resource for biological pathway data.** *Nucleic Acids Res.* 2011; **39**(Database issue): D685–D690.
PubMed Abstract | Publisher Full Text | Free Full Text

32. Tenenbaum D: **KEGGREST: Client-side REST access to KEGG.** R package version 1.16.0. 2017.

33. R Core Team: **R: A Language and Environment for Statistical Computing.** R Foundation for Statistical Computing, Vienna, Austria, 2017.
Reference Source

34. Dewey M: **metap: meta-analysis of significance values.** R package version 0.8. 2017.
Reference Source

35. Wilkinson B: **A statistical consideration in psychological research.** *Psychol Bull.* 1951; **48**(3): 156–8.
PubMed Abstract | Publisher Full Text

36. Luo W, Brouwer C: **Pathview: an R/Bioconductor package for pathway-based data integration and visualization.** *Bioinformatics.* 2013; **29**(14): 1830–1.
PubMed Abstract | Publisher Full Text | Free Full Text

37. Alexa A, Rahnenfuhrer J: **topGO: Enrichment Analysis for Gene Ontology.** R package version. 2016.
Publisher Full Text

38. Smith ML, Baggerly KA, Bengtsson H, *et al.*: ***illuminaio*: an open source idat parsing tool for Illumina microarrays [version 1; referees: 2 approved].** *F1000Res.* 2013; **2**: 264.
PubMed Abstract | Publisher Full Text | Free Full Text

39. Shi W, Oshlack A, Smyth GK: **Optimizing the noise versus bias trade-off for Illumina Whole Genome Expression Beadchips.** *Nucleic Acids Res.* 2010; **38**(22): e204.
PubMed Abstract | Publisher Full Text | Free Full Text

40. mritchie: **mritchie/EGSEA123: F1000 Research article version 1 (Version v1).** *Zenodo.* 2017.
Data Source

Open Peer Review

Current Referee Status: ? ? ? ✔

Version 1

Referee Report 20 December 2017

doi:10.5256/f1000research.13583.r27967

✔ **Pekka Kohonen** [1], **Roland Grafström** [2]
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GSEA analysis methods do not all produce same results. Score-based gene set analysis methods like the Broad Institute GSEA tool are considered to perform better than normal Fisher's exact test (overrepresentation analysis). But analysts often use methods they know to be less than ideal in order to reduce complexity and save time. So it is good to have a unified interface for GSEA analyses with R – it helps save programming time and reduces complexity. In addition EGSEA is a unique method that combines up to 12 gene set analysis methods into a single score. Independent test also corroborate that the tool using the 12 has more specificity and good sensitivity compared to using some of the tests alone.

The EGSEA 1-2-3 workflow is easy to use and generate good-quality figures with the ggplot2 R package. So9me of the figures are novel compared to other packages e.g., scatter plots designed to compare different contrasts. It is also very useful that the tool can be applied to multiple contrasts at a time, although if there are too many contrasts then the number of plots becomes unwieldly (increases combinatorically).

Some more technical comments:
The results object is very complicated for retrieving individual method analysis results (although summaries are readily available). I quite like the "biobroom" Bioconductor package that does "tidy" data frames from limma results objects.

All in all a very useful package both for automating the running of lots of methods at the same time and of course for the "ensemble" method. It is recommended to be considered to be part of a standard bioinformatics workflow.

Is the rationale for developing the new software tool clearly explained?
Yes

Is the description of the software tool technically sound?
Yes

Are sufficient details of the code, methods and analysis (if applicable) provided to allow replication of the software development and its use by others?
Yes

Is sufficient information provided to allow interpretation of the expected output datasets and any results generated using the tool?
Yes

Are the conclusions about the tool and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 13 December 2017

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EGSEA is a new gene set analysis tool that combines results from multiple individual tools in R as to yield better results. The authors have published EGSEA methodology previously. This paper focuses on the practical analysis workflow based on EGSEA with specific examples. As EGSEA is a compound and complicated analysis procedure, this work serves as a valuable guidance for the users to make full use of this tool. I've gone through the workflow line by line, it seems to work well. However, authors can improve their work by addressing the following issues.

1. There should be an R code script which includes all source code and concise comments like the one in company with the vignette in any Bioconductor package. It would be much easy for the users/reviewers to try the example code. It is not convenient to follow the code in this manuscript, the code need to be edit to remove the prompt symbols (> or +) at each line when copying/pasting.

2. It takes too long to run the egsea analysis example on modest machine. It is advisable to show a lesser example in the workflow with only one gene set collection like kegg and just a few base methods like:

```
gsa = egsea(voom.results=v, contrasts=contr.matrix,
      gs.annots=gs.annots$kegg, symbolsMap=symbolsMap,
      baseGSEAs=baseMethods[1:4], sort.by="med.rank",
      num.threads = 3, report = FALSE)
```

3. The rank of the gsa results shown following the t = topSets(..) line is confusing. The p.adj for the top 1 gene set is not the smallest, actually much bigger than top 2, 6 and 8. Presumably, the gene sets are ranked by med.rank instead of p.adj here. However, the opposite was described in the text above near the egsea.sort() line: "Although p.adj is the default option for sorting EGSEA results for convenience, ..."

4. In addition, there is big difference between the final rank and med.rank (e.g. 1 vs 36). This may suggest inconsistent results came from different base methods. This may also be due to the large number of gene sets being tested. Again, using a smaller gene set collection and a few base methods could make the ranking more consistent.

5. All visualization functions, i.e. plotHeatmap, plotPathway, plotGOGraph, plotMethods, plotSummary and plotBars share largely the same set of arguments, they can have a unified wrapper function like plot.gsa() with an extra argument type to specify the plot type.

6. Functions plotPathway, plotGOGraph are wrapper functions for those in the pathview and topGO package as the author noted in the text. It would be good to explicit show some message like "calling plotting function from pathview or topGO package etc", just like the message when running egsea().

7. HTML report of the results is a very valuable feature for the users. However, the code can run a long time, it would be helpful to add some progress reminder message to generateReport() function like egsea(). BTW, the KEGG Pathway graphs are not shown properly in the report example at http://bioinf.wehi.edu.au/EGSEA/mam-rnaseq-egsea-report/index.html.

Is the rationale for developing the new software tool clearly explained?
Yes

Is the description of the software tool technically sound?
Yes

Are sufficient details of the code, methods and analysis (if applicable) provided to allow replication of the software development and its use by others?
Yes

Is sufficient information provided to allow interpretation of the expected output datasets and any results generated using the tool?
Partly

Are the conclusions about the tool and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 05 December 2017

doi:10.5256/f1000research.13583.r27974

?

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This F1000 software tool article describes the EGSEA package that incorporates many different gene set testing methods from various packages and also allows access to a wide array of gene sets from different databases through the accompanying EGSEAdata package. These packages will enable researchers to conveniently test many different methods and incorporate their results to get more robust biological insights[1], and this article gives a well-written walk-through of how to use the packages.

The biggest limitation I see it that EGSEA is focused only on human and mouse data (and rat? The article does not list rat but the help page for buildIdx() lists rat as one of the species). I understand that many of the gene set collections like MSigDB and GeneSetDB are only available for human/mouse, but KEGG currently lists 429 Eukaryotic organisms (http://www.genome.jp/kegg/catalog/org_list.html) and GO terms are readily available for 19 species using BioC's pre-built OrgDB packages and hundreds of other through AnnotationHub. It is unclear whether EGSEA functions buildCustomIdx and buildGMTIdx that were "written to allow users to run EGSEA on gene set collections that may have been curated within a lab or downloaded from public databases and allow use of gene identifiers other than Entrez IDs" can be used to run EGSEA on additional species. If so, this should be clearly stated in both the Abstract and in the body of the article, plus an example given on how to use buildCustomIdx for another species. If there is some reason that EGSEA cannot currently extend to other species, this should be acknowledged as a limitation and future versions should strive to allow this (although not required before approval).

Other issues to address before approval:

1. I am unable to create the html report on my Windows machine, getting the following error:

   Build GO DAG topology .....

   There are no adj nodes for node: GO:0061857
   Error in switch(type, isa = 0, partof = 1, -1) :
   EXPR must be a length 1 vector

   However, I reported this error to the support site (https://support.bioconductor.org/p/103640/#103748) and got a speedy reply from the author. It hopefully will be resolved soon, although there is a concern of why the error was not found on another Windows machine.

2. I am concerned that as demonstrated in this paper, EGSEA seems to take the place of standard limma differential expression analysis, in that the model fitting takes place within the egsea() function. Certain gene set testing functions do need the individual expression values and not just the fitted values in an MArrayLM object but given the computational time (8 min as shown in the article code block and 19 min on my own computer) you should never run egsea() without first assessing the model fit on your own! Ideally the egsea function could be written to accept MArrayLM, or at least the article should clearly state that users should have first assessed the validity of the model fit through the usual workflow of Law *et al.* (2016)[2] prior to running EGSEA.

3. I also wonder why there are different interfaces for voom-based analysis and microarray data given that both use EList objects. I understand that the voom weights need to be used internally, but limma's lmFit function handles both without trouble, although it was originally coded for microarray data and the voom functionality came later. Even if there needs to be a separate

function egsea.ma() for non-voom, non-count data, it should still accept an EList object so that the user does not have to pull out the expression data and the grouping info.

4. Back to the computational time required, there are several vague references to removing the roast method "to save time" and that the report generation "significantly" increases run time. it would be nice to have an example of the time required to run roast and the report generation for the computational architecture that created the article.

References

1. Alhamdoosh M, Ng M, Wilson N, Sheridan J, Huynh H, Wilson M, Ritchie M: Combining multiple tools outperforms individual methods in gene set enrichment analyses. *Bioinformatics*. 2016. Publisher Full Text
2. Law CW, Alhamdoosh M, Su S, Smyth GK, Ritchie ME: RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR.*F1000Res*. 2016; **5**: 1408 PubMed Abstract | Publisher Full Text

Is the rationale for developing the new software tool clearly explained?
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Are sufficient details of the code, methods and analysis (if applicable) provided to allow replication of the software development and its use by others?
Partly

Is sufficient information provided to allow interpretation of the expected output datasets and any results generated using the tool?
Partly

Are the conclusions about the tool and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 27 November 2017

doi:10.5256/f1000research.13583.r27972

Robert Castelo
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This article describes a gene set enrichment analysis (GSEA) workflow for the "Ensembl of GSEA" (EGSEA) R/Bioconductor software package. EGSEA is an ensemble-like method recently published[1] by the authors of this workflow that allows the user to simultaneously apply different GSEA algorithms on a high-throughput molecular profiling data set, by combining p-values associated with each algorithm using classical meta-analysis approaches such as the Fisher's method.

Because the statistical methodology is already described in detail in the corresponding publication, the present software tool article focuses on showing a step-by-step workflow with EGSEA. However, the vignette of the software package already provides a very detailed description about how to use EGSEA through its 39 pages. Therefore, it would be useful for the interested reader to find upfront when he/she should be consulting the vignette and when he/she should be consulting this workflow. Besides this introductory aspects, the following issues should be addressed before approval:

1. The code given in the article breaks, at least in my computer, more concretely, at this line:

```
gsa = egsea(voom.results=v, contrasts=contr.matrix,
        gs.annots=gs.annots, symbolsMap=symbolsMap,
        baseGSEAs=baseMethods, sort.by="med.rank",
        num.threads = 8, report = FALSE)
EGSEA analysis has started
##------ Mon Nov 27 12:37:42 2017 ------##
Log fold changes are estimated using limma package ...
limma DE analysis is carried out ...
Number of used cores has changed to 4
in order to avoid CPU overloading.
EGSEA is running on the provided data and c2 collection
.......camera*....safe*...gage*.padog*....gsva*..fry*...plage*...globaltest*...zscore*...ora*...ssgsea*
  Error in temp.results[[baseGSEA]][[i]][names(gs.annot@idx), ] :
  incorrect number of dimensions
```

   while running it with the latest release version 1.6.0. This is strange since the package builds and runs the vignette without problems. So, this might be related to the different sample data sets. A possible hint may come from the fact that the 'buildIdx()' call is not returning the expected class of object, according to the workflow:

```
class(gs.annots$s2)
## [1] "NULL"
summary(gs.annots$s2)
## Length  Class   Mode
##      0   NULL   NULL
```

2. The workflow contains a rather high amount of code, often with a non-trivial use of externally instantiated objects and nested calls to functions. It would be helpful for the interested reader to be able to easily copy and paste the instructions, but the fact that R commands are given with the R shell '>' and '+' symbols makes it less easy. A non-expert user may even copy those characters and get an error. I would recommend removing those characters from the illustrated code, just as it happens with the vignette.

3. The workflow assumes that the user has a 'DGEList' object with gene metadata including the mapping between Entrez identifiers' and HGNC symbols. This is a rather unrealistic assumption and I would recommend that the workflow starts building that object from scratch and showing how to build that table of gene metadata.

Below I also describe other issues that I would recommend to be considered in future versions of the software but which I do not consider them to be required for approval of this article:

1. The so-called "summary plot" shows the -log10 p-value on the x-axis and average absolute log fold-change of the set genes on the y-axis. Because this is in a way analogous to a rotated volcano plot, I would suggest to use the same arrangement of axes as in the volcano plot, which is a rather standardized display of significance and magnitude of the effects of interest.

2. One of the key features of the Bioconductor project, to which the EGSEA package is contributing to, is enabling software interoperability through sharing the use of common data structures across different software packages. Using specialized data structures, where analogous ones have been already designed by the Bioconductor core team or by a wider community of developers, locks the user into that package and limits the possibilities of using it as a building block in other more complex workflows. I'm making this comment because I have the impression that the EGSEA package would benefit of using the infrastructure provided by the Bioconductor GSEABase package, in which data structures are defined to store and access gene sets and collections of gene sets of different kinds. A salient feature of that infrastructure is the possibility to seamlessly map gene identifiers of different kinds. This would simplify and improve the user experience of EGSEA since mapping between genes coded with a particular kind of identifier, and gene sets defined with another kind, is one of the most common tasks in a GSEA-like analysis.

References

1. Alhamdoosh M, Ng M, Wilson N, Sheridan J, Huynh H, Wilson M, Ritchie M: Combining multiple tools outperforms individual methods in gene set enrichment analyses. *Bioinformatics*. 2016. Publisher Full Text

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Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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