Introduction to Bulk RNAseq data analysis
Gene Set Testing for RNA-seq

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The list of differentially expressed genes is sometimes so long that its interpretation becomes cumbersome and time consuming. It may also be very short while some genes have low p-value yet higher than the given threshold.

A common downstream procedure to combine information across genes is gene set testing. It aims at finding pathways or gene networks the differentially expressed genes play a role in.

Various ways exist to test for enrichment of biological pathways. We will look into over representation and gene set enrichment analyses.

A gene set comprises genes that share a biological function, chromosomal location, or any other relevant criterion.

To save time and effort there are a number of packages that make applying these tests to a large number of gene sets simpler, and which will import gene lists for testing from various sources.

Today we will use clusterProfiler.

Over-representation

Method

This method tests whether genes in a pathway are present in a subset of our data in a higher number than expected by chance (explanations derived from the clusterProfiler manual).

Genes in the experiment are split in two ways:

- annotated to the pathway or not
• differentially expressed or not

We can then create a contingency table with:
• rows: genes in pathway or not
• columns: genes differentially expressed or not

And test for independence of the two variables with the Fisher exact test.

**clusterProfiler**

*clusterProfiler* (Yu et al. 2012) supports direct online access of the current KEGG database (KEGG: Kyoto Encyclopedia of Genes and Genomes), rather than relying on R annotation packages. It also provides some nice visualisation options.

We first search the resource for mouse data:

```r
library(clusterProfiler)
library(tidyverse)
search_kegg_organism('mouse', by='common_name')
```

```r
## kegg_code scientific_name common_name
## 14 mmu Mus musculus mouse
```

We will use the ‘mmu’ ‘kegg_code.’

**KEGG enrichment analysis**

The input for the KEGG enrichment analysis is the list of gene IDs of significant genes.

We now load the R object keeping the outcome of the differential expression analysis for the d11 contrast.

```r
shrink.d11 <- readRDS("RObjects/Shrunk_Results.d11.rds")
```

We will only use genes that have:
• an adjusted p-value (FDR) of less than 0.05
• an absolute fold change greater than 2.

We need to remember to eliminate genes with missing values in the FDR as a result of the independent filtering by DESeq2.

For this tool we need to use Entrez IDs, so we will also need to eliminate genes with a missing Entrez ID (NA values in the ‘Entrez’ column).

```r
sigGenes <- shrink.d11 %>%
  drop_na(Entrez, FDR) %>%
  filter(FDR < 0.05 & abs(logFC) > 1) %>%
  pull(Entrez)

kk <- enrichKEGG(gene = sigGenes, organism = 'mmu')
```

```r
## Reading KEGG annotation online:
##
## Reading KEGG annotation online:

head(kk, n=10) %>% as_tibble()
```
Visualise a pathway in a browser

clusterProfiler has a function browseKegg to view the KEGG pathway in a browser, highlighting the genes we selected as differentially expressed.

We will show one of the top hits: pathway ‘mmu04612’ for ‘Antigen processing and presentation.’

browseKEGG(kk, ‘mmu04612’)

Visualise a pathway as a file

The package pathview (Luo et al. 2013) can be used to generate figures of KEGG pathways.

One advantage over the clusterProfiler browser method browseKegg is that genes can be coloured according to fold change levels in our data. To do this we need to pass pathview a named vector of fold change values (one could in fact colour by any numeric vector, e.g. p-value).

The package plots the KEGG pathway to a png file in the working directory.

```r
library(pathview)
logFC <- shrink.d11$logFC
names(logFC) <- shrink.d11$Entrez
pathview(gene.data = logFC,
         pathway.id = "mmu04612",
         species = "mmu",
         limit = list(gene=20, cpd=1))
```

```
mmu04612.pathview.png
```

Exercise 1

1. Use pathview to export a figure for “mmu04659” or “mmu04658,” but this time only use genes that are statistically significant at FDR < 0.01
Exercise 2 - GO term enrichment analysis

clusterProfiler can also perform over-representation analysis on GO terms using the command enrichGO. Check:

- the help page for the command enrichGO (type ?enrichGO at the console prompt)
- and the instructions in the clusterProfiler book.

1. Run the over-representation analysis for GO terms
   - Use genes that have an adjusted p-value (FDR) of less than 0.01 and an absolute fold change greater than 2.
   - For this analysis you can use Ensembl IDs rather than Entrez
   - You’ll need to provide the background (universe) genes, this should be all the genes in our analysis.
   - The mouse database package is called org.Mm.eg.db. You’ll need to load it using library before running the analysis.
   - As we are using Ensembl IDs, you’ll need to set the keyType parameter in the enrichGO command to indicate this.
   - Only test terms in the “Biological Processes” ontology

2. Use the dotplot function to visualise the results.

# may need devtools::install_github("YuLab-SMU/enrichplot")
# to avoid a 'wrong orderBy parameter' warning.

GSEA analysis

Gene Set Enrichment Analysis (GSEA) identifies gene sets that are related to the difference of interest between samples (Subramanian et al. 2005).

The software is distributed by the Broad Institute and is freely available for use by academic and non-profit organisations. The Broad also provide a number of very well curated gene sets for testing against your data - the Molecular Signatures Database (MSigDB). These are collections of human genes. Fortunately, these lists
have been translated to mouse equivalents by the Walter+Eliza Hall Institute Bioinformatics service and made available for download. They are now also available from a recent R package msigdb, which we will use.

Let’s load `msigdb` now.

```r
library(msigdb)
```

**Method**

The analysis is performed by:

1. ranking all genes in the data set
2. identifying in the ranked data set the rank positions of all members of the gene set
3. calculating an enrichment score (ES) that represents the difference between the observed rankings and that which would be expected assuming a random rank distribution.

The article describing the original software is available here, while this commentary on GSEA provides a shorter description.

![Diagram of GSEA analysis](image)

We will use `clusterProfiler`'s `GSEA` package (Yu et al. 2012) that implements the same algorithm in R.

**Rank genes**

We need to provide `GSEA` with a vector containing values for a given gene metric, e.g. log(fold change), sorted in decreasing order.

To start with we will simply use a rank based on their fold change.

We must exclude genes with no Entrez ID.

Also, we should use the shrunk LFC values.
rankedGenes <- shrink.d11 %>%
drop_na(Entrez) %>%
mutate(rank = logFC) %>%
arrange(-rank) %>%
pull(rank, Entrez)

Load pathways

We will load the MSigDB Hallmark gene set with msigdbr, setting the category parameter to ‘H’ for Hallmark gene set. The object created is a tibble with information on each {gene set; gene} pair (one per row). We will only keep the the gene set name, gene Entrez ID and symbol, in mouse.

m_H_t2g <- msigdbr(species = "Mus musculus", category = "H") %>%
dplyr::select(gs_name, entrez_gene, gene_symbol)

Conduct analysis

Arguments passed to GSEA include:

- ranked genes
- pathways
- gene set minimum size
- gene set maximum size

gseaRes <- GSEA(rankedGenes,
TERM2GENE = m_H_t2g[,1:2],
#pvalueCutoff = 0.05,
#pvalueCutoff = 1.00, # to retrieve whole output
minGSSize = 15,
maxGSSize = 500)

## preparing geneSet collections...
## GSEA analysis...
## Warning in fgseaMultilevel(...): For some pathways, in reality P-values are less than 1e-10. You can set the `eps` argument to zero for better estimation.
## leading edge analysis...
## done...

Let’s look at the top 10 results.

# have function to format in scientific notation
format.e1 <- function(x) (sprintf("%.1e", x))
# show table
gseaRes %>%
arrange(desc(abs(NES))) %>%
top_n(10, -p.adjust) %>%
dplyr::select(-core_enrichment) %>%
dplyr::select(-Description) %>%
data.frame() %>%
remove_rownames() %>%
# format
mutate(ES=formatC(enrichmentScore, digits = 3)) %>%
```r
mutate(NES=formatC(NES, digits = 3)) %>%
# format p-values
modify_at(c("pvalue", "p.adjust", "qvalues"),
  format.e1)
DT::datatable(options = list(dom = 't'))
```

## Enrichment score plot

The enrichment score plot displays along the x-axis that represents the decreasing gene rank:

- genes involved in the pathway under scrutiny: one black tick per gene in the pathway (no tick for genes not in the pathway)
- the enrichment score: the green curve shows the difference between the observed rankings and that which would be expected assuming a random rank distribution.

```r
# HALLMARK_INFLAMMATORY_RESPONSE is 4th
topx <- match("HALLMARK_INFLAMMATORY_RESPONSE", data.frame(gseaRes)$ID)
```

Gene log(fold change):

```r
gseaplot(gseaRes, geneSetID = topx, by = "preranked", title = gseaRes$Description[topx])
```

![Enrichment score plot](image)

Running score:
Both the log(fold change) and running score:

```r
gseaplot(gseaRes, geneSetID = topx, title = gseaRes$Description[topx])
```
Exercise 3

Another common way to rank the genes is to order by p-value while sorting so that upregulated genes are at the start and downregulated at the end. You can do this combining the sign of the fold change and the p-value.

1. Rank the genes by statistical significance - you will need to create a new ranking value using $-\log_{10}(\text{p value}) \times \text{sign}(\text{Fold Change})$.
2. Run fgsea using the new ranked genes and the H pathways.
3. Conduct the same analysis for the d33 vs control contrast. Extended: Do results differ between ranking scheme?
   Extended: Do results differ between d11 and d33, with the significance-based ranking scheme?

References
