

Clipper package (Version 1.43.0)

Paolo Martini, Gabriele Sales and Chiara Romualdi

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1 Along signal paths: an empirical gene set approach exploiting pathway topology

1.1 *clipper* approach

Different experimental conditions are usually compared in terms of their gene expression mean differences. In the univariate case, if a gene set changes significantly its multivariate mean expression in one condition with respect to the other, it is said to be differentially expressed. However, the difference in mean expression levels does not necessarily result in a change of the interaction strength among genes. In this case, we will have pathways with significant altered mean expression levels but unaltered biological interactions. On the contrary, if transcripts abundances ratios are altered, we expect a significant alteration not only of their mean, but also of the strength of their connections, resulting in pathways with completely corrupted functionality. Therefore, to look for pathways strongly involved in a biological process, we should look at pathways with both mean and variance significantly altered. *clipper* is based on a two-step approach: 1) it selects pathways with covariance matrices and/or means significantly different between experimental conditions and 2) on such pathways, it identifies the sub-paths mostly associated to the phenotype. This is a very peculiar feature in pathway analysis. To our knowledge this is the first approach able to systematically inspect a pathway deep in its different portions.

1.2 Other approaches on Bioconductor

Currently there are some pathway analysis methods implemented in Bioconductor (probably the most famous is GSEA), but very few of them try to exploit pathway topology. Example of the latter category are SPIA and DEGraph.

In *Martini et al. 2012* is provided a detailed comparison of the performance of non-topological analysis (*GSEA*) and topological analysis (*SPIA* and *clipper*) using both real and simulated data. In the next few words, we are going to highlight the differences of these approaches.

GSEA uses pathway as a list of genes without taking into account the structure of the pathway while *SPIA* takes into account pathway topological information, gene fold-changes and pathway enrichment scores. Then *SPIA* takes as input only the list of differentially expressed genes. So, from a practical point of view *clipper* and *SPIA* test different null hypotheses.

More importantly, *clipper* is able to highlight the portions (sub-path) of the pathway that are mostly involved in the phenotype under study using graph decomposition theory.

For more details please refer to *Martini et al. 2012*.

2 Performing pathway analysis

In this section, we describe how to perform the topological pathway analysis on a whole pathway. As an example we used the gene expression data published by Chiaretti et al. on acute lymphocytic leukemia (ALL) cells associated with known genotypic abnormalities in adult patients. Several distinct genetic mechanisms lead to ALL malignant transformations deriving from distinct lymphoid precursor cells that have been committed to either T-lineage or B-lineage differentiation. Chromosome translocations and molecular rearrangements are common events in B-lineage ALL and reflect distinct mechanisms of transformation. The BCR breakpoint cluster region and the *c-abl* oncogene 1 (BCR/ABL) gene rearrangement occurs in about 25% of cases in adult ALL.

The expression values (available through Bioconductor) deriving from Affymetrix single channel technology, consist of 37 observations from one experimental condition (class 2, n1 37, BCR; presence of BCR/ ABL gene rearrangement) and 42 observations from another experimental condition (class 1, n2 41, NEG; absence of rearrangement). The *clipper* method is based on Gaussian graphical models, therefore it is strongly recommended to use log-transformed data.

In this example, we are going to evidence the differences between BCR/ABL (class 2) and NEG (class 1) though a topological pathway analysis.

We use the *graphite* bioconductor R package as a source of pathway topological information. In our test dataset, given the presence of the BCR/ABL chimera, we expect that all the pathways including BCR and/or ABL1 will be impacted. Here we retrieve, for example, the KEGG "Chronic myeloid leukemia" pathway.

```
> library(graphite)
> kegg <- pathways("hsapiens", "kegg")
> graph <- convertIdentifiers(kegg[["Chronic myeloid leukemia"]], "entrez")
> graph <- pathwayGraph(graph)
> genes <- nodes(graph)
> head(genes)

[1] "ENTREZID:10000" "ENTREZID:1019" "ENTREZID:1021"
[4] "ENTREZID:1026" "ENTREZID:1029" "ENTREZID:1147"
```

Once the pathway (converted to a graphNEL object) is loaded in the workspace, we need to retrieve the expression matrix and the corresponding sample annotations (2 denoting samples with translocation and 1 denoting samples with no BCR/ABL translocation). We use as an example the ALL Bioconductor package.

```
> library(ALL)
> data(ALL)
```

Fist of all, we should take a look at the phenoData.

```
> head(pData(ALL))

      cod diagnosis sex age BT remission CR   date.cr
01005 1005 5/21/1997  M  53 B2      CR CR  8/6/1997
01010 1010 3/29/2000  M  19 B2      CR CR  6/27/2000
03002 3002 6/24/1998  F  52 B4      CR CR  8/17/1998
04006 4006 7/17/1997  M  38 B1      CR CR  9/8/1997
04007 4007 7/22/1997  M  57 B2      CR CR  9/17/1997
```

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```

04008 4008 7/30/1997 M 17 B1 CR CR 9/27/1997
      t(4;11) t(9;22) cyto.normal citog mol.biol
01005 FALSE TRUE FALSE t(9;22) BCR/ABL
01010 FALSE FALSE FALSE simple alt. NEG
03002 NA NA NA <NA> BCR/ABL
04006 TRUE FALSE FALSE t(4;11) ALL1/AF4
04007 FALSE FALSE FALSE del(6q) NEG
04008 FALSE FALSE FALSE complex alt. NEG
      fusion protein mdr kinet ccr relapse transplant
01005 p210 NEG dyploid FALSE FALSE TRUE
01010 <NA> POS dyploid FALSE TRUE FALSE
03002 p190 NEG dyploid FALSE TRUE FALSE
04006 <NA> NEG dyploid FALSE TRUE FALSE
04007 <NA> NEG dyploid FALSE TRUE FALSE
04008 <NA> NEG hyperd. FALSE TRUE FALSE
      f.u date last seen
01005 BMT / DEATH IN CR <NA>
01010 REL 8/28/2000
03002 REL 10/15/1999
04006 REL 1/23/1998
04007 REL 11/4/1997
04008 REL 12/15/1997

> dim(pData(ALL))
[1] 128 21

```

This data.frame summarized all the phenotypic features of the samples. In our analysis, we are interested in B-cell. This information is hosted in the column called 'BT'.

```

> pData(ALL)$BT
 [1] B2 B2 B4 B1 B2 B1 B1 B1 B2 B2 B3 B3 B3 B2 B3 B B2 B3
[19] B2 B3 B2 B2 B2 B1 B1 B2 B1 B2 B1 B2 B B B2 B2 B2 B1
[37] B2 B2 B2 B2 B2 B4 B4 B2 B2 B2 B4 B2 B1 B2 B2 B3 B4 B3
[55] B3 B3 B4 B3 B3 B1 B1 B1 B1 B3 B3 B3 B3 B3 B3 B3 B1
[73] B3 B1 B4 B2 B2 B1 B3 B4 B4 B2 B2 B3 B4 B4 B4 B1 B2 B2
[91] B2 B1 B2 B B T T3 T2 T2 T3 T2 T T4 T2 T3 T3 T T2
[109] T3 T2 T2 T2 T1 T4 T T2 T3 T2 T2 T2 T2 T3 T3 T3 T2 T3
[127] T2 T
Levels: B B1 B2 B3 B4 T T1 T2 T3 T4

> pAllB <- pData(ALL)[grep("B", pData(ALL)$BT),]
> dim(pAllB)
[1] 95 21

```

After this selection, we are interest in the isolation of sample with translocation from those without translocation. This information is hosted in the column 'mol.biol'.

```

> pAllB$'mol.biol'
 [1] BCR/ABL NEG BCR/ABL ALL1/AF4 NEG NEG
 [7] NEG NEG NEG BCR/ABL BCR/ABL NEG
[13] E2A/PBX1 NEG BCR/ABL NEG BCR/ABL BCR/ABL

```

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```
[19] BCR/ABL BCR/ABL NEG BCR/ABL BCR/ABL NEG
[25] ALL1/AF4 BCR/ABL ALL1/AF4 NEG ALL1/AF4 BCR/ABL
[31] NEG BCR/ABL NEG BCR/ABL BCR/ABL ALL1/AF4
[37] NEG BCR/ABL BCR/ABL BCR/ABL NEG E2A/PBX1
[43] BCR/ABL NEG NEG NEG BCR/ABL p15/p16
[49] ALL1/AF4 BCR/ABL BCR/ABL NEG E2A/PBX1 NEG
[55] NEG NEG BCR/ABL BCR/ABL NEG NEG
[61] ALL1/AF4 NEG ALL1/AF4 NEG BCR/ABL NEG
[67] NEG NEG NEG NEG BCR/ABL ALL1/AF4
[73] BCR/ABL NEG E2A/PBX1 NEG BCR/ABL BCR/ABL
[79] NEG NEG NEG NEG BCR/ABL NEG
[85] BCR/ABL BCR/ABL BCR/ABL ALL1/AF4 NEG NEG
[91] BCR/ABL NEG BCR/ABL BCR/ABL E2A/PBX1
Levels: ALL1/AF4 BCR/ABL E2A/PBX1 NEG NUP-98 p15/p16
```

```
> NEG <- pAllB$'mol.biol' == "NEG"
> BCR <- pAllB$'mol.biol' == "BCR/ABL"
> pAll <- pAllB[(NEG | BCR),]
```

Now we have to build the vector of classes.

```
> classesUn <- as.character(pAll$'mol.biol')
> classesUn[classesUn=="BCR/ABL"] <- 2
> classesUn[classesUn=="NEG"] <- 1
> classesUn <- as.numeric(classesUn)
> names(classesUn) <- row.names(pAll)
> classes <- sort(classesUn)
```

Now that we have the vector of classes, we can isolate the subset of sample from the original expression set and subsequently we convert affymetrix probe names into entrez gene ids.

```
> library("hgu95av2.db")
> all <- ALL[,names(classes)]
> probesIDS <- row.names(exprs(all))
> featureNames(all@assayData) <- unlist(mget(probesIDS, hgu95av2ENTREZID))
> all <- all[(!is.na(row.names(exprs(all))))]
```

To be compliant with graphite we need to explicit in the row names that the IDs are Entrez-Gene.

```
> all_rnames <- featureNames(all@assayData)
> featureNames(all@assayData) <- paste("ENTREZID", all_rnames, sep = ":")
```

At this point, we compute the intersection between pathway nodes and the genes for which an expression value is available. Thus we obtain a subgraph of the original graph. Moreover, we can extract from the expression set a smaller expression set corresponding to the expression of the genes in the pathway under investigation.

```
> library(graph)
> genes <- intersect(genes, row.names(exprs(all)))
> graph <- subGraph(genes, graph)
> exp <- all[genes, , drop=FALSE]
> exp
```

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```
ExpressionSet (storageMode: lockedEnvironment)
assayData: 72 features, 79 samples
  element names: exprs
protocolData: none
phenoData
  sampleNames: 01010 04007 ... 84004 (79 total)
  varLabels: cod diagnosis ... date last seen (21
    total)
  varMetadata: labelDescription
featureData: none
experimentData: use 'experimentData(object)'
  pubMedIds: 14684422 16243790
Annotation: hgu95av2

> dim(exprs(exp))
[1] 72 79
```

Note that the usage of 'exp' as ExpressionSet or `exprs(exp)` as expression matrix leads exactly at the same result. Here we will use the ExpressionSet but all our function can be used with a simple expression matrix.

The analysis is performed using `pathQ` function as follows:

```
> library(clipper)
> pathwayAnalysis <- pathQ(exp, classes, graph, nperm=100, alphaV=0.05, b=100)
> pathwayAnalysis

$alphaVar
[1] 0.35

$alphaMean
[1] 0
```

The returned list contains the pvalue for the test on the concentration matrices (`alphaVar`) and the pvalue for the test on the means (`alphaMean`).

3 Performing clipper analysis

After a global inspection and identification of the most interesting/impacted pathways (the global analysis we have seen in the previous section), it is important to focus on the genes that drive the differences between the two phenotypes. The following example shows how to identify the sub-paths mostly associated to the phenotype.

```
> clipped <- clipper(exp, classes, graph, "var", trZero=0.01, permute=FALSE)
> clipped[,1:5]

  startIdx endIdx maxIdx length  maxScore
1;4      1      4      1      4 1.07518794226094
1;9      1      9      2      5 3.56236878201274
1;10     1     10      3      6 6.75554607050997
1;17     1     17      7      9 23.5836375910436
1;23     1     23      2     10 1.78118439100637
```

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```
1;30      1      30      2      5 3.56236878201274
```

The analyzed cliques are indexed by the maximum cardinality search (mcs) algorithm and identified hereafter with the index number. The result of the clipper analysis is the matrix described in the following. For each analyzed path (named as <starting clique index>;<ending clique index>) the following information are reported:

- 1 Index of the starting clique
- 2 Index of the ending clique
- 3 Index of the clique where the maximum value is reached
- 4 Length of the path
- 5 Maximum score of the path
- 6 Average score along the path
- 7 Percentage of path activation
- 8 Impact of the path on the entire pathway
- 9 Involved and significant cliques
- 10 Cliques forming the path
- 11 Genes forming the significant cliques
- 12 Genes forming the path

A deeper look at the clipper matrix reveals that many paths overlap. To help users in focusing on the best candidates, we devised a function to prune the paths that are already part of other ones. The pruning process is performed according to a dissimilarity threshold *thr* (if paths dissimilarity value is greater than *thr* they are retained).

```
> clipped <- prunePaths(clipped, thr=0.2)
> clipped[,1:5]
      startIdx endIdx maxIdx length      maxScore
1;17         1     17         7      9 23.5836375910436
```

After the pruning, the results are smaller and much clear to read and interpret.

4 Visualizing clipper results.

WARNING: RCytoscape is no longer support in some Platform so we use RCy3. *clipper* uses the *RCy3* package to connect to Cytoscape and display its results. Cytoscape is a Java based software specifically built to manage biological network complexity and for this reason it is widely used by the biological community.

```
> plotInCytoscape(graph, clipped[1,])
```

After the export of the pathway to Cytoscape, you can choose between different layouts. In figure 1 we show the KEGG "Chronic myeloid leukemia" pathway with an hierarchical layout and with the genes that belong to the most impacted path highlighted in blue.

- [2] Martini P, Sales G, Massa MS, Chiogna M, Romualdi C. **Along signal paths: an empirical gene set approach exploiting pathway topology.** Nucleic Acids Research. 2012.
- [3] Massa MS, Chiogna M, Romualdi C. **Gene set analysis exploiting the topology of a pathway.** BMC System Biol. 2010.
- [4] Sales, G. and Calura, E. and Cavalieri, D. and Romualdi, C. **graphite-a Bioconductor package to convert pathway topology to gene network.** BMC bioinformatics. 2012.
- [5] Laurent J, Pierre N and Dudoit S. **More power via graph-structured tests for differential expression of gene networks** The Annals of Applied Statistics. 2012.
- [6] Tarca AL, Draghici S, Khatri P, Hassan SS, Mittal P, and Kim J, and Kim CJ, Kusanovic JP and Romero R. **A novel signaling pathway impact analysis** Bioinformatics. 2009.
- [7] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, and others. **Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles** Proceedings of the National Academy of Sciences of the United States of America. 2005.