CMScaller: an R package for consensus molecular subtyping of colorectal cancer pre-clinical models

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- Date: 2017-11-21
- package: CMScaller 0.99.1

1 Introduction

Colorectal cancers (CRCs) can be divided into four gene expression-based biologically distinct consensus molecular subtypes (CMS) [1]. This classification provides prognostic stratification of the patients and presents a potential basis for stratified treatment. The original CMS classifier is dependent on gene expression signals from the immune and stromal compartments and often fails to identify the poor-prognostic CMS4 mesenchymal group in immortalized cell lines and patient-derived organoids. CMScaller uses cancer cell-intrinsic, subtype-specific gene expression markers as features for *Nearest Template Prediction* [2].

2 Input data

`CMScaller` provides robust *cross platform and sample-type* performance given a balanced, homogeneous dataset of >40 unique samples. For less than ~40 samples, sampling variance (by-chance subtype depletion/enrichment) is a concern. Similarly, selection, *e.g.* excluding microsatellite instable (MSI) samples or including only aggressive cancers, would break an underlying assumption and bias the resulting predictions [3].

3 Quick start

3.1 Installation and dependencies

The following packages are required in order to run examples in this vignette.

- Bioconductor[4]: *Biobase*, *limma*

In addition, *edgeR* is needed for specific RNA-sequencing normalization methods and *parallel*, *snow* for `ntp` parallelization.

```
# dependencies: run if not already installed
source("https://bioconductor.org/biocLite.R")
biocLite(c("Biobase", "limma"))
# proper repository to be fixed for publication
install.packages("pathToPackageFile/CMScaller_0.99.0.tar.gz", repos=NULL)
```

3.2 CMS classification

`CMScaller` function requires an expression matrix or ExpressionSet as input (`emat`). Gene names in `rownames(emat)` must be NCBI Entrez, Ensembl or HGNC symbol identifiers. For gene symbols or Ensembl identifiers, parameter `rowNames` must be set to `symbol` or `ensg`, respectively. The code chunk below demonstrates how to perform classification using TCGA primary colorectal cancer example data [5].

- microarray data input should be pre-processed and normalized (often log_2transformed)[1].
- RNA-sequencing counts/RSEM values could be used directly by setting `RNAseq=TRUE` which activates quantile normalization and log_2transform.

```
library(Biobase) # if input is ExpressionSet
library(CMScaller)
# get RNA-seq counts from TCGA example data
counts <- exprs(crcTCGAsubset)
head(counts[,1:3])
##           TCGA-4N-A93T-01A-11R TCGA-4T-AA8H-01A-11R TCGA-5M-AAT4-01A-11R
## 100133144                    9                   13                   18
## 10431                     2450                 1215                 2976
## 57714                      375                  536                  961
## 645851                      43                   18                   29
## 652919                       0                    0                    0
## 729884                       0                    2                    3
# prediction and gene set analysis
par(mfrow=c(1,2))
res <- CMScaller(emat=counts, RNAseq=TRUE, FDR=0.1)
## performing log2-transform and quantile normalization...
## cosine correlation distance
## 92 samples; 4 classes; 82-237 features/class
## serial processing; 1000 permutation(s)...
## predicted samples/class (FDR<0.05)
##
## CMS1 CMS2 CMS3 CMS4 <NA>
##   14   23   15   28   12
## 11/92 samples set to NA
cam <- CMSgsa(emat=crcTCGAsubset, class=res$prediction,RNAseq=TRUE)
## 11 samples with class or batch NA's excluded
```

```
# comparison with true class
table(pred=res$prediction, true=crcTCGAsubset$CMS)
##       true
## pred   CMS1 CMS2 CMS3 CMS4
##   CMS1    8    0    3    0
##   CMS2    0   20    0    0
##   CMS3    0    1    6    0
##   CMS4    0    2    0   19
head(res, n=3)
##                       prediction d.CMS1 d.CMS2 d.CMS3 d.CMS4 p.value    FDR
## TCGA-4N-A93T-01A-11R       <NA>   0.76   0.71   0.72   0.89   0.928 0.9487
## TCGA-4T-AA8H-01A-11R       CMS3   0.76   0.69   0.63   0.90   0.001 0.0014
## TCGA-5M-AAT4-01A-11R       <NA>   0.74   0.69   0.73   0.83   0.802 0.8387
```

- `rownames(res)` equals `colnames(emat)`
- class predictions with `NA` for samples with adjusted-p-value $>$ threshold

[1] Hoshida[2] does not explicitly state whether input should be log_2transformed or not and example data includes both. Such transformation reduces the weight of genes with large deviations and will usually affect results at the margins.

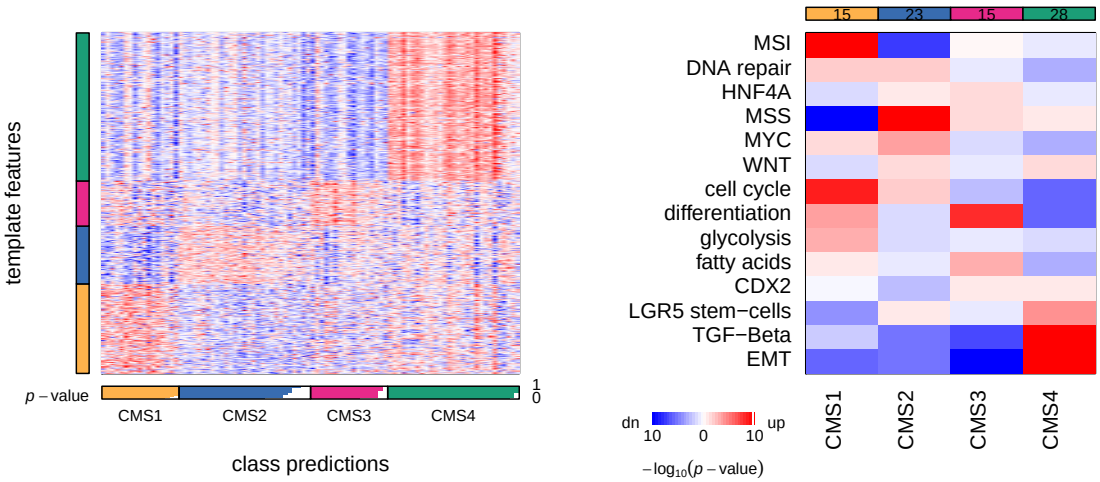


Figure 1: CMScaller graphic output. **Left** heatmap shows relative expression for template genes. Samples (columns) are ordered according to class predictions and confidence. The height of the white bars below gives the unadjusted prediction *p*-values. Genes (rows) are ordered according to class template. Heatmap color saturation indicates magnitude while red and blue indicate genes up and down relative to the sample mean. **Right** heatmap shows results for Camera gene set analysis. Heatmap color saturation indicates statistical significance and red and blue indicates direction of change.

- templates distances
- prediction p-values[2]
- prediction FDR-adjusted p-values

4 Package details

`CMScaller` is basically a wrapper function for `ntp`. Similarly, `CMSgsa` just provides some presets for `subCamera`.

4.1 Preparing custom templates

Templates consists of sets of subtype-specific marker genes. `subDEG` performs *limma* differential expression analysis for identification of such markers. Below, is an example on how to prepare custom templates based on a training set with known class labels. `doVoom=TRUE` enables voom transformation - required for proper *limma* modeling of RNA-sequencing counts [6].

```
emat <- crcTCGAsubset
cms <- emat$CMS.Syn
train <- sample(seq_along(cms), size=length(cms)/(2))
deg <- subDEG(emat[,train], class=cms[train], doVoom=TRUE)
## 16 samples with class or batch NA's excluded
templates <- ntpMakeTemplates(deg, resDEG=TRUE, topN=50)
templates$symbol <- fromTo(templates$probe)
tail(templates,n=3)
##     probe class symbol
## 198  9590  CMS4 AKAP12
```

[2] lowest possible estimate of the p-value is 1/permutations

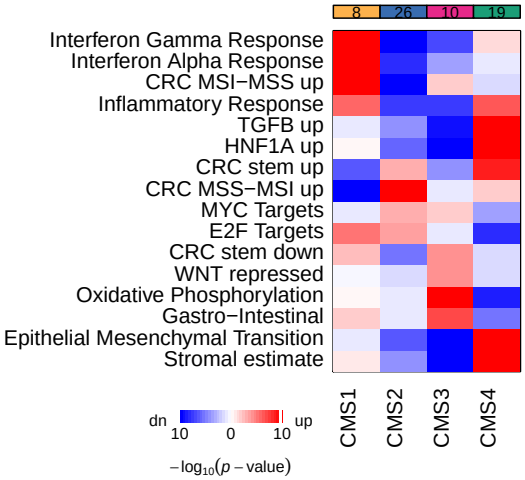


Figure 2: Gene Set Analysis (GSA) shows that CMS are biologically distinct.

```
## 199  4853  CMS4 NOTCH2
## 200 89795  CMS4   NAV3
```

4.2 Gene Set Analysis

subCamera provides gene set analysis and visualization and is a wrapper functions for camera in the *limma* package. camera controls for intra-set gene-wise correlations in order to reduce false-positive rate while retaining statistical power [7, 8]. CMSgsa provides preset gene sets to subCamera.

```
# increase left margins to accomodate gene set names
par.old <- par()
par(mfrow=c(1,1), mar=par.old$mar+c(0,4,0,0))
subCamera(emat, cms, geneList=geneSets.CRC, doVoom=TRUE)
## 29 samples with class or batch NA's excluded
```

```
# restore margins
par(mar=par.old$mar)
```

4.3 Nearest Template Prediction

ntp matches templates$probe against rownames(emat). Missing features and features with NA/NaN's are ignored in the prediction. emat should be row-wise centered and scaled.

```
# loads included emat, scales and centers
emat <- crcTCGAsubset
emat_sc <- ematAdjust(emat, normMethod="quantile")
head(emat_sc[,1:3])
##           TCGA-4N-A93T-01A-11R TCGA-4T-AA8H-01A-11R TCGA-5M-AAT4-01A-11R
## 100133144                -0.30                 0.59                 0.16
## 10431                     0.80                -0.17                 1.05
## 57714                    -0.90                 0.17                 0.11
```

```
## 645851                  1.71                1.39                1.05
## 652919                 -0.99               -0.85               -1.04
## 729884                 -0.77                0.59                0.40
```

`ntp` function requires an expression matrix and templates. Since prediction confidence is estimated from permutations, strict p-value reproducibility requires `set.seed`.

```
# test set prediction
res <- ntp(emat_sc[,-train], templates, nPerm=1000)
res <- subSetNA(res, pValue=.1)
## 7/46 samples set to NA
table(pred=res$prediction, true=cms[-train])
##       true
## pred   CMS1 CMS2 CMS3 CMS4
##   CMS1    2    0    2    0
##   CMS2    0   10    0    0
##   CMS3    1    0    3    0
##   CMS4    1    0    0   11
head(res)
##                      prediction d.CMS1 d.CMS2 d.CMS3 d.CMS4 p.value    FDR
## TCGA-4N-A93T-01A-11R       CMS3   0.72   0.68   0.62   0.90   0.003 0.0037
## TCGA-4T-AA8H-01A-11R       CMS3   0.69   0.76   0.52   0.88   0.001 0.0014
## TCGA-5M-AAT4-01A-11R       <NA>   0.73   0.74   0.68   0.85   0.673 0.6883
## TCGA-A6-2685-01A-01R       CMS4   0.73   0.76   0.70   0.44   0.001 0.0014
## TCGA-A6-5667-01A-21R       CMS2   0.77   0.58   0.85   0.63   0.001 0.0014
## TCGA-A6-6140-01A-11R       CMS2   0.75   0.44   0.76   0.85   0.001 0.0014
```

`ntp` output is a `data.frame` with $3 + K$ columns where K is the number of classes. Rows represent columns in input `emat`.

- `rownames(res)` equals `colnames(emat)`
- class predictions with `levels(res$prediction)` equaling `levels(templats$class)`
- templates distances (defaults to cosine correlation distance)
- prediction p-values
- prediction FDR-adjusted p-values

`subSetNA` function resets predictions with p-value or FDR above some arbitrary threshold to `NA`.

5 Nearest Template Prediction

Nearest template prediction (NTP) was proposed as a classification algorithm by Yujin Hoshida and published in *PLoS ONE* in 2010 [2]. It aims to provide robust single-sample class prediction for high-dimensional, noisy gene expression data. In brief, first, for each subclass, *a template*, a list of genes coherently upregulated is determined. Then, for each sample, *the distance* to each template is calculated and class is assigned based on the smallest distance. Finally, prediction confidence is assessed based on the distance of the null-distribution, estimated from *permutation tests* (feature permutation). The default distance metric selected by Hoshida was a cosine similarity-derived distance (see below). When applied to a reasonably well-balanced homogeneous dataset, row-wise centering and scaling is performed (gene means and standard deviations $\mu = 0$, $\sigma = 1$). In case of single-sample prediction, feature-wise means and standard deviations from a previous sample set are used to perform sample-wise scaling and centering. The key advantages of the NTP algorithm are conceptual simplicity, biological plausibility, ease of implementation and robustness.

Formally, N samples with expression values for P genes divided into K different classes.

- $\mathbf{X}_{[P,N]}$ centered and scaled expression matrix where column vector $x_{[P]}$ is the expression for sample n.

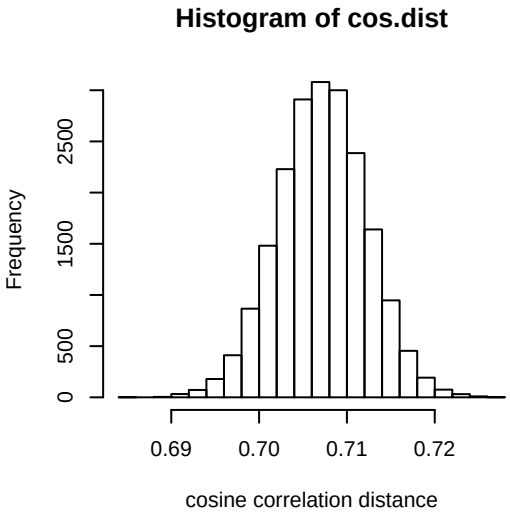
- M is a list of K vectors where each element m is a set of marker features with higher expression in samples belonging to class k as compared to remaining samples. m's may be of uneven length, but are typically $<<P$
- $\mathbf{Y}_{[P,K]}$ template matrix where $y_{[P]} = [p \in m]$ for class k (0 if not marker, 1 otherwise).

For the sample and template vectors x and y, a proper distance metric, $d_{x,y}$ for the similarity function $f(x,y)$ is given by $d = \sqrt{\frac{1}{2}(1 - f(x,y))}$ [9]. Here f is either cosine, Kendall, Pearson or Spearman correlation. Cosine similarity, the angle between two Euclidean vectors is given by

$$f(x,y) = \cos(\theta) = \frac{\sum xy}{\sqrt{\sum x^2}\sqrt{\sum y^2}}$$

The following code chunks demonstrate NTP in code.

```
# random centered/scaled expression matrix and templates
set.seed(42)
N <- 5000;P <- 5000;K <- 4;nPerm <- 1000;n <- 1
X <- matrix(rnorm(P*N, mean=0, sd=1), ncol=N)
Y <- matrix(rbinom(P*K, size = 1, prob=.01), ncol=K)
# sample-template correlations (implemented in corCosine)
cos.sim <- crossprod(X,Y) / outer(
               sqrt(apply(X, 2, crossprod)),
               sqrt(apply(Y, 2, crossprod)))
# sample-template distances (vectorized)
simToDist <- function(cos.sim) sqrt(1/2 * (1-cos.sim))
cos.dist <- simToDist(cos.sim)
hist(cos.dist, xlab="cosine correlation distance")
```



For centered, scaled and uncorrelated data, the cosine correlation distance is $\sqrt{0.5 \times (1-0)} \approx 0.707$.

Resulting distances is ranked among distances of permutated samples and used to estimate prediction confidence. The lowest possible p-value estimate is therefore $1/permutations$

```
# estimate prediction confidence
pred.class <- apply(cos.dist, 1, which.min)
pred.dists <- apply(cos.dist, 1, min)
null.dist <- replicate(nPerm, min(simToDist(corCosine(sample(X[,n]), Y))))
p <- rank(c(pred.dists[n], null.dist))[1]/(length(null.dist))
```

Code below is not evaluated for this vignette, but illustrates the uniform p-value distribution for centered and scaled uncorrelated input[3].

```
# rearrange matrix and templates for ntp input
rownames(X) <- make.names(seq_len(P))
templates <- lapply(seq_len(K), function(k) rownames(X)[Y[,k]==1])
names(templates) <- paste("k", seq_len(K))
templates <- ntpMakeTemplates(templates, resDEG = FALSE)
# takes a couple of minutes: 5000 samples and 1000 permutations
res <- ntp(X, templates, nCores=4L, nPerm=nPerm, doPlot=TRUE)
# expect uniform distribution
hist(res$p.value)
```

6 Session

```
## R version 3.4.2 (2017-09-28)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: Ubuntu 16.04.3 LTS
##
## Matrix products: default
## BLAS: /usr/lib/libblas/libblas.so.3.6.0
## LAPACK: /usr/lib/lapack/liblapack.so.3.6.0
##
## locale:
##  [1] LC_CTYPE=en_US.UTF-8       LC_NUMERIC=C
##  [3] LC_TIME=nb_NO.UTF-8        LC_COLLATE=en_US.UTF-8
##  [5] LC_MONETARY=nb_NO.UTF-8    LC_MESSAGES=en_US.UTF-8
##  [7] LC_PAPER=nb_NO.UTF-8       LC_NAME=C
##  [9] LC_ADDRESS=C               LC_TELEPHONE=C
## [11] LC_MEASUREMENT=nb_NO.UTF-8 LC_IDENTIFICATION=C
##
## attached base packages:
## [1] parallel  stats     graphics  grDevices utils     datasets  methods
## [8] base
##
## other attached packages:
## [1] CMScaller_0.99.1    Biobase_2.36.2      BiocGenerics_0.22.0
## [4] BiocStyle_2.4.1
##
## loaded via a namespace (and not attached):
##  [1] Rcpp_0.12.13    digest_0.6.12   rprojroot_1.2   backports_1.1.0
##  [5] magrittr_1.5    evaluate_0.10.1 highr_0.6       stringi_1.1.5
##  [9] limma_3.32.5    rmarkdown_1.6   tools_3.4.2     stringr_1.2.0
## [13] yaml_2.1.14     compiler_3.4.2  htmltools_0.3.6 knitr_1.17
```

References

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