

oneChannelGUI Package Vignette

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1 Introduction

This package is an add-on of affyImGUI for *mouse-click* based QC, statistical analysis and data mining for one channel microarray data. It is designed for Bioconductor beginners having limited or no experience in interacting with Bioconductor line commands. OneChannelGUI is a set of functions extending the affyImGUI capabilities, rearranging and extending affyImGUI menus.

This package performs, in a graphical environment, the analysis pipe-line shown in figure 1, green box.

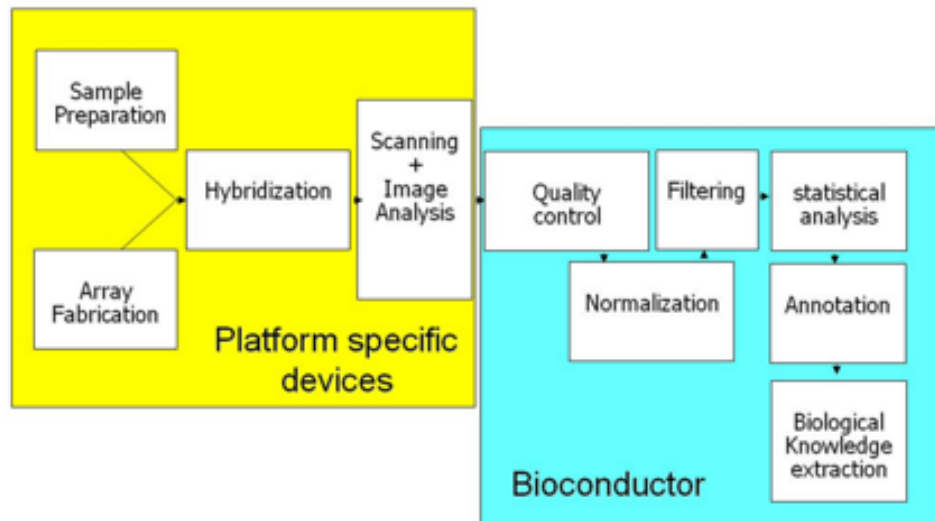


Figure 1: Microarray analysis pipe-line.

This vignette gives a general overview of the graphical interfaces available in oneChannelGUI.

N.B:

All the oneChannelGUI graphical outputs are visualized in the R main window, to reduce RAM consumption, which is a critical issue when Affymetrix array data or large set of data are loaded.

Furthermore, exon data generated with APT tools produce, in the working directory,

a certain amount of temporary files and directories.

A cleanup function is under development.

At the present time, user can manually remove, from the working folder, any file starting with target, elevels, glevels, e.g. target51f81aeb, elevels3e9f6b76, and folders starting with out and outMidas, e.g. out17fb164, outMidas4a31ac4, without affecting the results stored in oneChannelGUI.

2 Installation

For the complete functionality of oneChannelGUI some external softwares and data need to be installed. Please refer to the *install vignette* of oneChannelGUI package.

3 Main graphical window

oneChannelGUI inherits the core functionalities of affylmGUI and its main GUI. In oneChannelGUI some extra topics are available in the main affylmGUI info left frame, e.g. maSigPro results, Normalized Exon data, APT DABG, APT MiDAS, Splice Index, etc. Furthermore, four different menus are automatically exchanged depending on the type of array loaded:

1. .CEL IVT Affymetrix arrays.
2. .CEL exon 1.0 ST arrays uploaded in oneChannelGUI by Affymetrix APT tools or gene/exon data exported from Affymetrix Expression Console.
3. .CEL Gene 1.0 ST arrays uploaded in oneChannelGUI by Affymetrix APT tools.
4. GEO/flat tab delimited expression data file.
5. ILLUMINA output from BeadStudio software version 1, 2 and 3.

Each item in the menus is simply a graphical implementation of a function of a specific Bioconductor library , e.g. ssize: sample size and statistical power estimation. To get more information on those libraries please refer to their specific vignettes, accessible from the *Help menu*.

4 File

This menu allows the loading of .CEL IVT Affymetrix arrays as well as exon arrays, GEO Matrix Series files, tab delimited files containing only expression data and ILLUMINA data produced by BeadStudio software version 1 or 2. In this menu, fig. 2, are given the main functionalities to handle a microarray analysis project.

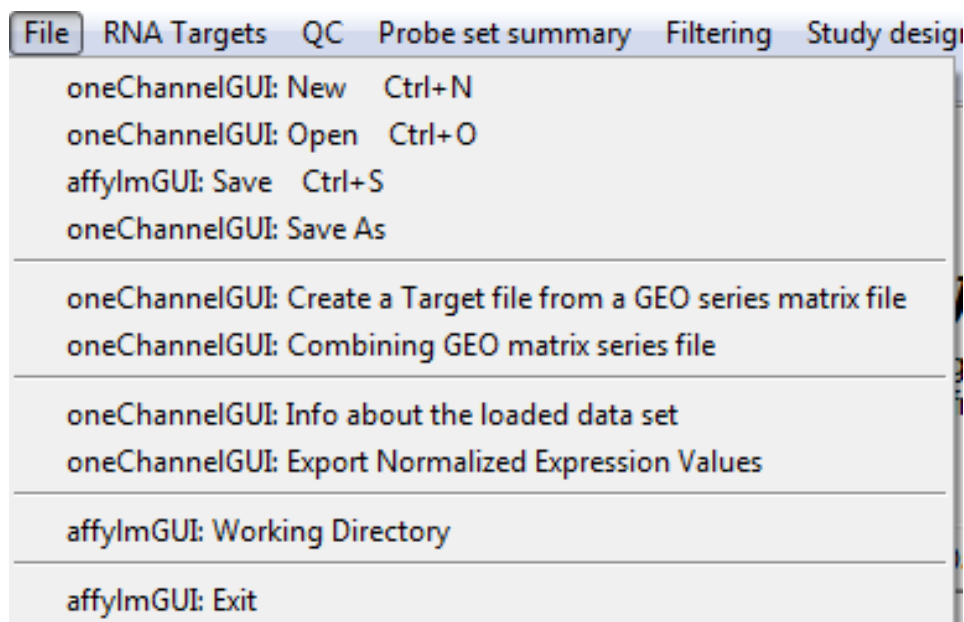


Figure 2: File menu.

4.1 New

The item *New*, fig. 2, allow to load various types of array data, using the sub menu shown in fig. 3,

4.1.1 Target file structure

To load arrays oneChannelGUI uses the information available in a file describing the experimental structure of the data set. This file is called *target file* and it is a tab delimited file with a fixed header structure also used by affylmGUI, fig. 4.

IMPORTANT:

TARGET FILE MUST NOT CONTAIN CHARACTERS LIKE ; , : _ - | \ ! ? + * ^ () [] { }

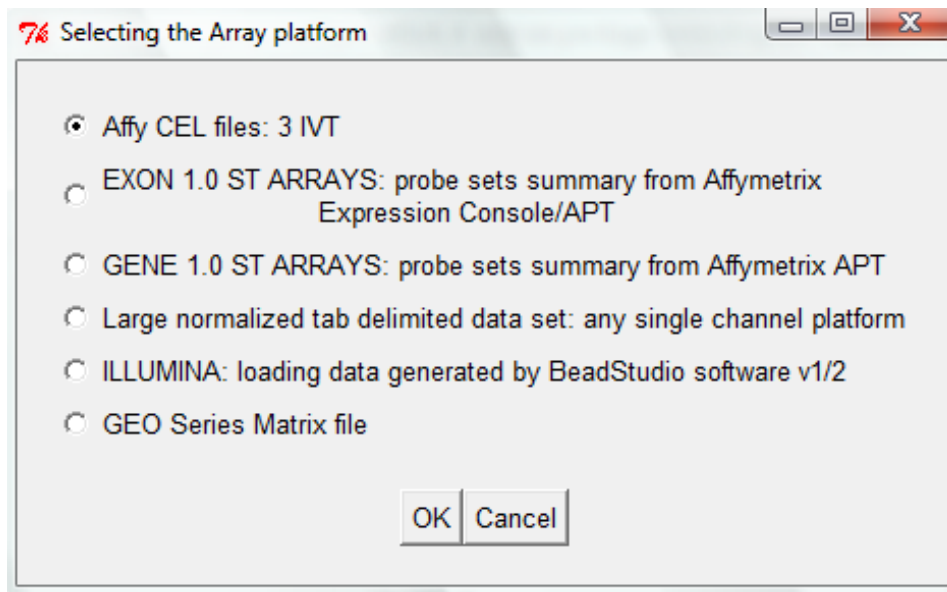


Figure 3: New: array type selection menu.

	A	B	C
1	Name	FileName	Target
2	mC1	M1.CEL	mcf-7ctrl
3	mC2	M4.CEL	mcf-7ctrl
4	mC3	M7.CEL	mcf-7ctrl
5	mE1	M3.CEL	mcf-7E2
6	mE2	M6.CEL	mcf-7E2
7	mE3	M9.CEL	mcf-7E2
8	ml1	M2.CEL	mcf-7IGF
9	ml2	M5.CEL	mcf-7IGF
10	ml3	M8.CEL	mcf-7IGF
11	sC1	S1.CEL	sk-er3ctrl
12	sC2	S4.CEL	sk-er3ctrl
13	sC3	S7.CEL	sk-er3ctrl
14	sE1	S3.CEL	sk-er3E2
15	sE2	S6.CEL	sk-er3E2
16	sE3	S9.CEL	sk-er3E2
17	sl1	S2.CEL	sk-er3IGF
18	sl2	S5.CEL	sk-er3IGF
19	sl3	S8.CEL	sk-er3IGF

7% Targets file
 Selected the "targets" file.
 Then press OK to continue

Please select a filename. No filename is selected at the moment. Press the Select Targets File Button.

Select Targets File

OK Cancel

Targets file is a tab delimited **text file** containing the description of the experiment. It is made of three columns:
Name: the name you want to assign to each array.
FileName: the names of the corresponding .CEL file
Target: the experimental condition associated to the array (e.g. mock, treated, etc). At least two conditions should be present.

Figure 4: Target file structure.

4.1.2 Loading Affy .CEL files

This sub menu, fig. 3, is entirely inherited by affylmGUI and allows to load .CEL files, if a Bioconductor cdf file is available. User will be asked to select the working folder, i.e. the one in which are present the .CEL files and the target file.

4.1.3 Loading EXON/GENE ARRAYS

This sub menu, fig. 3, allows to load exon/gene 1.0 ST arrays starting from .CEL, taking advantage of Affymetrix APT tools (<http://www.affymetrix.com/support/developer/powertools/index.affx>), or flat tab delimited files containing gene/exon level expression data exported from Affymetrix Expression Console (EC, http://www.affymetrix.com/support/technical/software_downloads.affx). If APT tool option is not used (it works only for Exon 1.0 ST data exported from EC), a sub-menu allows to select, for tab delimited data, the organism and the subset of exon data to be evaluated, fig. 5

IMPORTANT:

TO USE APT TOOLS THE DOWNLOAD OF GENE/EXON LIBRARY FILES IS REQUIRED.

THIS CAN BE DONE WITH THE FUNCTION

*oeChannelGUI: Set library folder and install Affy gene/Exon library files
LOCATED IN THE GENERAL TOOLS MENU*

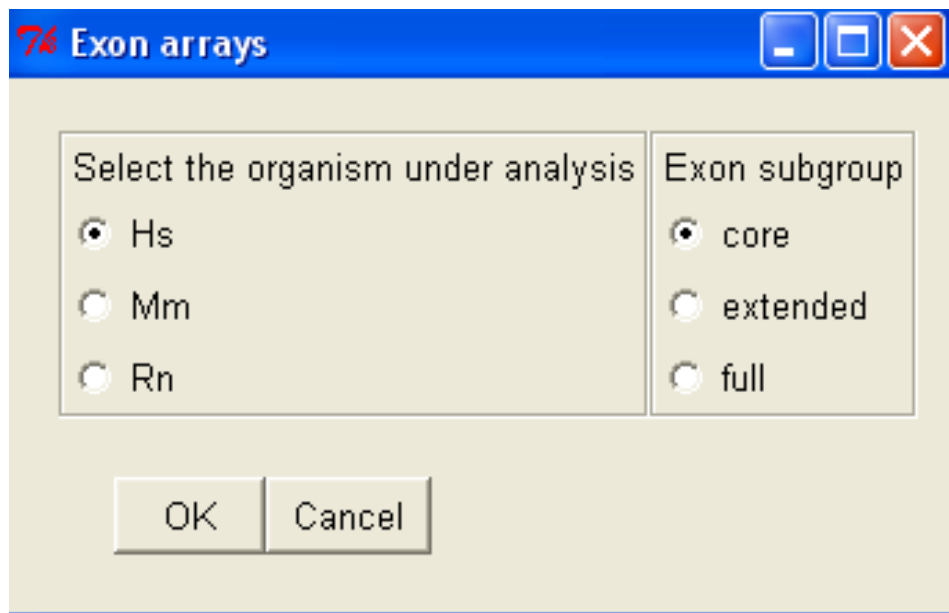


Figure 5: Sub menu to define the organism and the subset of exon data that will be loaded.

Subsequently, the user will select:

1. a working directory, a target file,
2. the flat tab delimited files containing respectively gene-level and exon-level data.

If instead, APT tool option is selected, user will select:

1. the organism and the subset of exon arrays to be evaluated, fig. 5,
2. a working directory,
3. a target file,
4. the type of probe set summary to be applied to gene/exon level data, fig. 6.

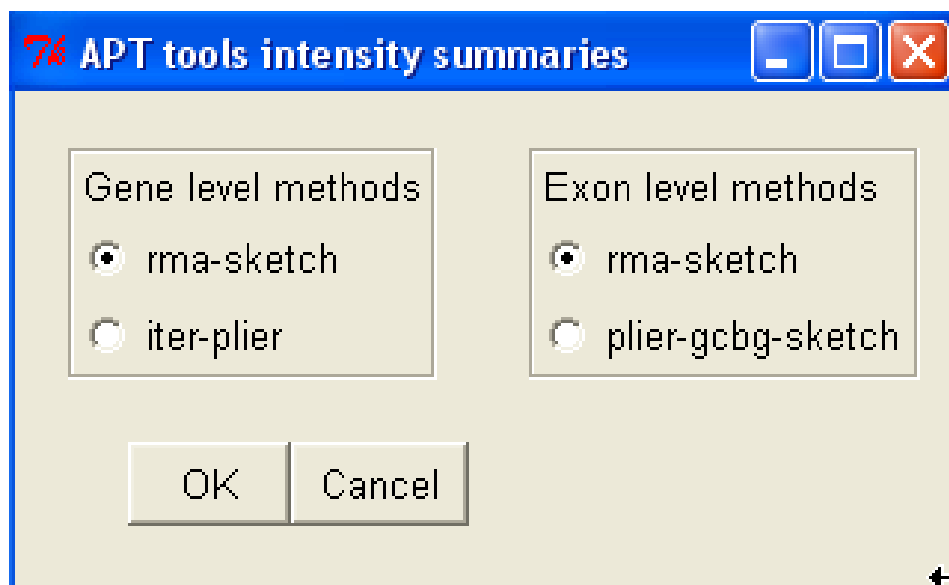


Figure 6: Sub menu to define the type of probe set summary to be applied.

Concerning probe set summary options, fig. 6, since PLIER/RMA are model-based algorithms. Exons that are alternatively spliced in the samples, therefore exhibiting different expression patterns compared to the constitutive exons, will have down-weighted effect in overall gene-level target response values. A better estimation of gene-level signal could be obtained using IterPLIER, which is a variation of PLIER that iteratively discards features (probes) that do not correlate well with the overall gene-level signal and then recalculates the signal estimate to derive a robust estimation of the gene expression value primarily based on the expression levels of the constitutive exons. Concerning exon level expression estimation, most probe sets only have four probes, which is too limited

to be useful with IterPLIER at the individual exon level, therefore it will be better to use PLIER/RMA.

Probe set summary calculation and uploading will take few minutes depending on the number of .CEL to be loaded and the PC in use. Once probe set summary has been calculated, using APT tool, it is also possible to calculate DABG p-values, fig. 7.

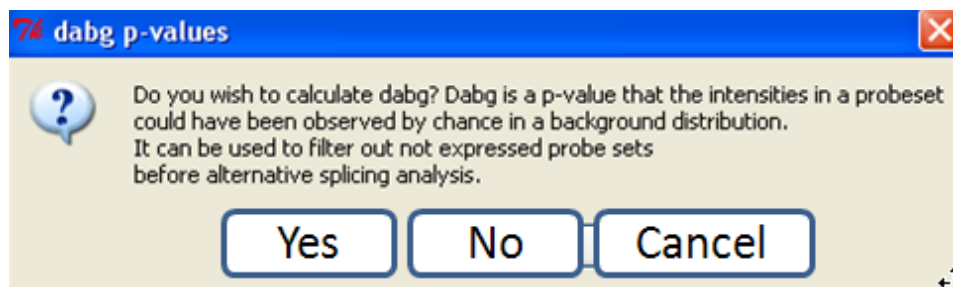


Figure 7: Selecting DABG p-value calculation.

DABG p-values represent *data above background*, it is a p-value similar to that used to derive presence/absence calls in MAS 5.0. DABG p-values could be useful to remove low intensity signals which could produce mis-leading results when alternative splicing events are evaluated using the Splice Index, where signal intensity information is not considered.

The progress of the probe set summary calculation is shown in the main R window.

```
Gene level probe sets summary started
Read 6 cel files from: target3d92750
Opening bgp file: HuEx-1_0-st-v2.r2.antigenomic.bgp
Opening clf file: HuEx-1_0-st-v2.r2.clf
Opening pgf file: HuEx-1_0-st-v2.r2.pgf
Expecting 1 iteration.
Doing iteration: 1
Opening clf file: HuEx-1_0-st-v2.r2.clf
Opening pgf file: HuEx-1_0-st-v2.r2.pgf
Loading 22011 probesets and 908532 probes.
Reading 6 cel files.....Done.
Processing Probesets.....Done.
Cleaning up.
Done.
Run took approximately: 9.56 minutes.

Gene level probe sets summary ended

Gene level probe sets summary ended
```

Exon level probe sets summary started

Exon level probe sets summary started
Read 6 cel files from: target3d92750
Opening bgp file: HuEx-1_0-st-v2.r2.antigenomic.bgp
Opening clf file: HuEx-1_0-st-v2.r2.clf
Opening pgf file: HuEx-1_0-st-v2.r2.pgf
Expecting 1 iteration.
Doing iteration: 1
Opening clf file: HuEx-1_0-st-v2.r2.clf
Opening pgf file: HuEx-1_0-st-v2.r2.pgf
Loading 287329 probesets and 1111849 probes.
Reading 6 cel files.....Done.
Processing Probesets.....Done.
Cleaning up.
Done.
Run took approximately: 6.41 minutes.

Exon level probe sets summary ended

Exon level probe sets summary ended

DABG calculation started
Read 6 cel files from: target3d92750
Opening bgp file: HuEx-1_0-st-v2.r2.antigenomic.bgp
Opening clf file: HuEx-1_0-st-v2.r2.clf
Opening pgf file: HuEx-1_0-st-v2.r2.pgf
Expecting 1 iteration.
Doing iteration: 1
Opening clf file: HuEx-1_0-st-v2.r2.clf
Opening pgf file: HuEx-1_0-st-v2.r2.pgf
Loading 22011 probesets and 908532 probes.
Reading 6 cel files.....Done.
Processing Probesets.....Done.
Cleaning up.
Done.
Run took approximately: 3.55 minutes.

DABG calculation ended

4.1.4 Loading GENE ARRAYS

This sub menu, fig. 3, allows to load gene 1.0 ST arrays starting from .CEL, taking advantage of Affymetrix APT tools (<http://www.affymetrix.com/support/developer/powertools/index.affx>). Subsequently, the user will select:

1. the organism and the subset of exon arrays to be evaluated, fig. 8,
2. a working directory,
3. a target file,
4. the type of probe set summary to be applied to gene/exon level data, fig. 9.

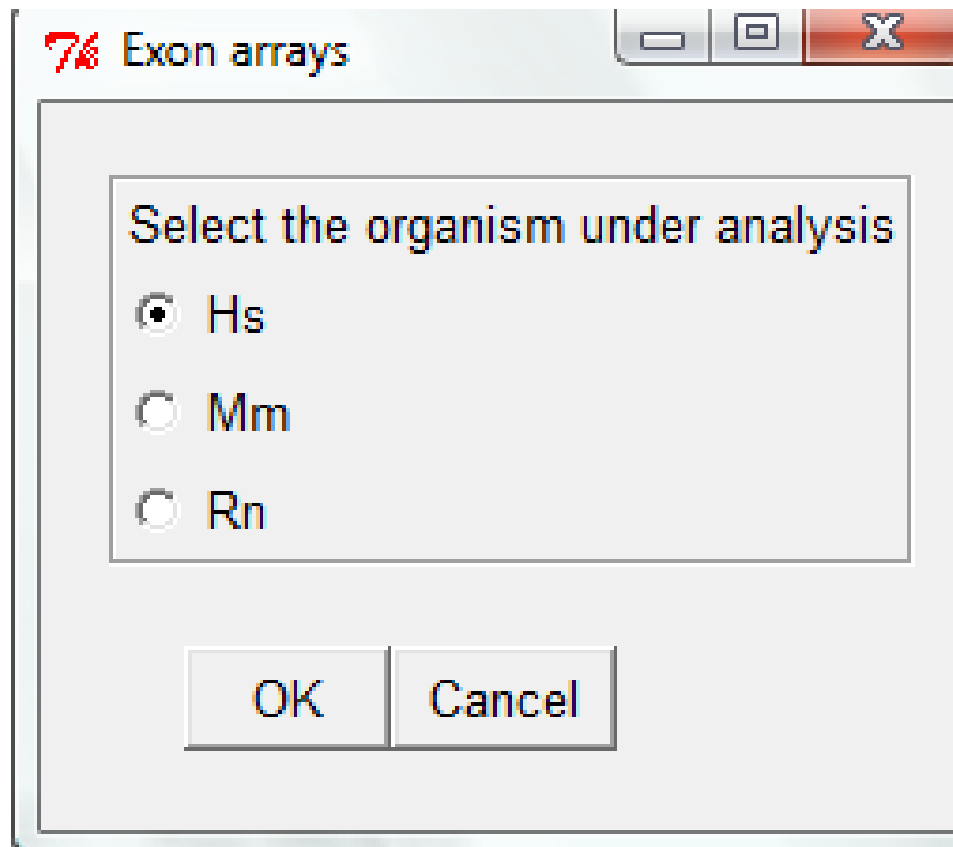


Figure 8: Sub menu to define the organism and the subset of data that will be loaded.

Probe set summary calculation and uploading will take few minutes depending on the number of .CEL to be loaded and the PC in use.

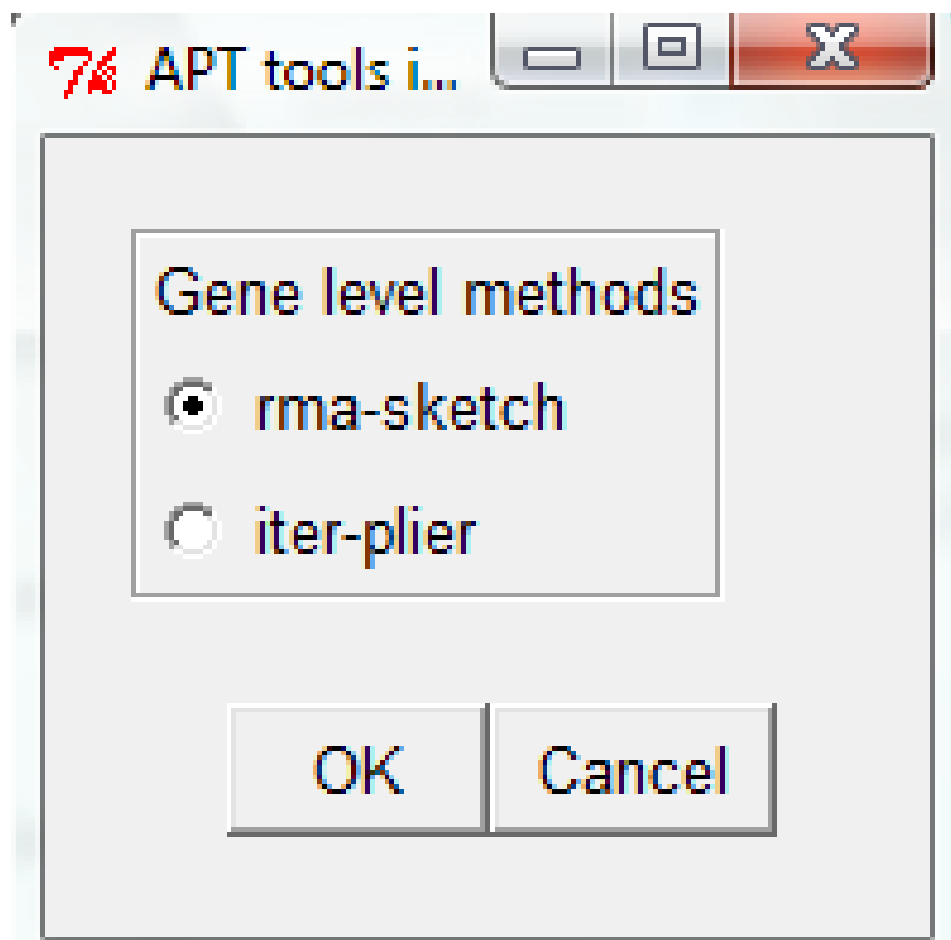


Figure 9: Sub menu to define the type of probe set summary to be applied.

4.1.5 Loading ILLUMINA BeadStudio

This sub menu, fig. 3, allows user to load expression data starting from the output of BeadStudio software. The interface allows to load outputs generated by BeadStudio version 1, 2 and 3. The Bioconductor annotation libraries for illumina arrays are associated to the loaded data. Since output of BeadStudio is not log2 transformed, a popup menu will allow the data modification. Furthermore, if BeadStudio data were not normalized, user could apply various normalization procedures available in the Menu Probe set summary. It is possible to load also data from beadstudio v3. Files should be tab delimited and contain at least Average signal intensity for PROBE LEVEL this is needed to use the annotation library available in Bioconductor. Here are described the steps to generate an export file from BeadStudio v3 compatible with onechannelGUI, figs. 10, 11, 12.

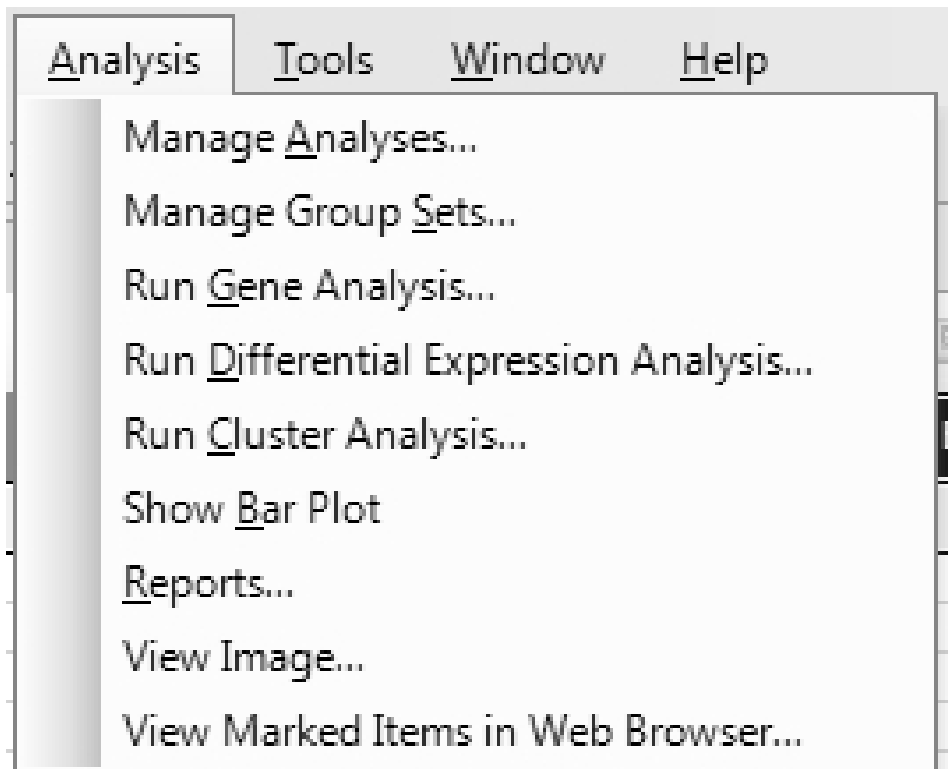


Figure 10: After having generated an analysis in BeadStudio v3 enter in the analysis menu and select reports.

4.1.6 Loading GEO Matrix Series files

This sub menu, fig. 3, allows to load GEO Matrix Series files. To load a GEO Matrix Series file it is only necessary to locate in a specific folder a target file and the Matrix

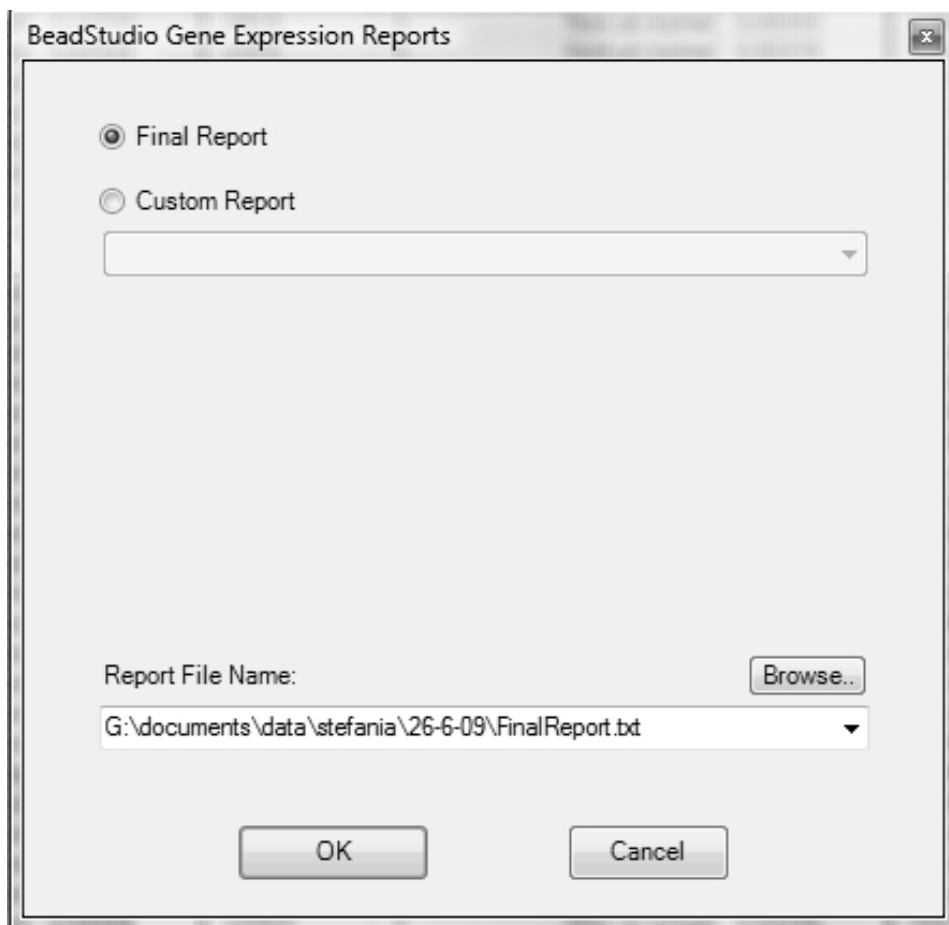


Figure 11: select the name of the final report and press OK.

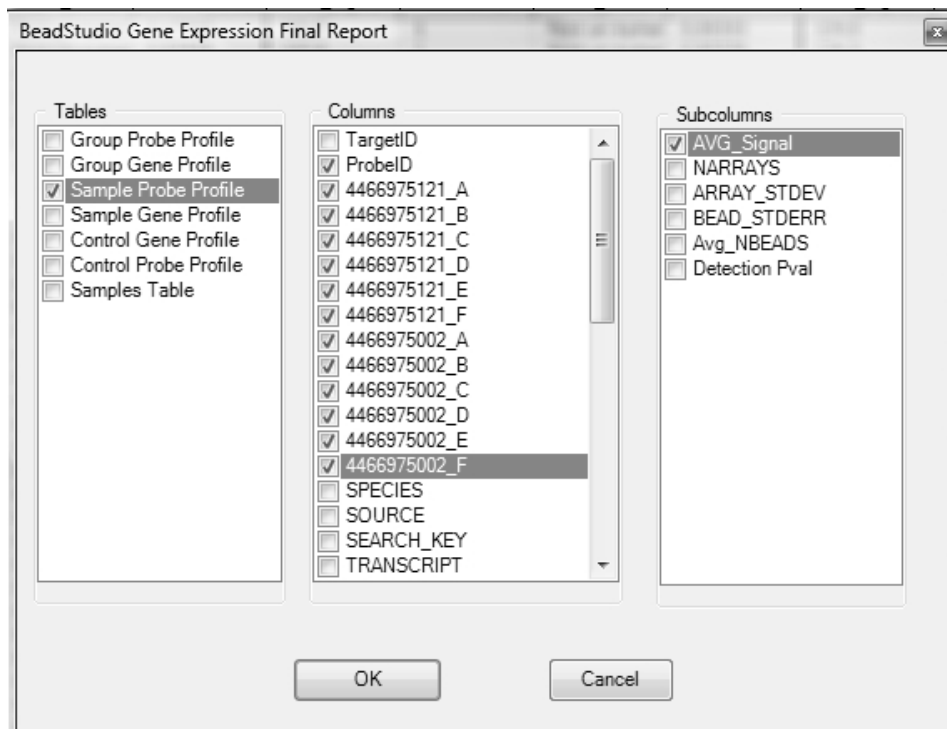


Figure 12: Select sample probe profile, select ProbeID, the array slots you want to export and at least AVG _signal column The output file is now compatible with oneChannelGUI and with illuminaXXXXBeadID.db annotation.

Series file downloaded from GEO database.

NB: In the target file the FileName column must contain exactly the same names present in the header below the row !series_matrix_table_begin in the Matrix Series file. Instead Target column could be derived by the row !Sample_description in the Matrix Series file.

4.1.7 Creating a Target file from GEO matrix series file

To make easier to user the creation of target file for GEO matrix series files. This function, fig. 2 opens the GEO matrix file of interest and creates a data frame with the following columns, Name, FileName, using the informations written in GEO file:

```
Name: !Sample_title
FileName: ID_REF
Target: !Sample_source_name_ch1
```

The data frame is the written in the working directory This target can be further edited and used to load the GEO matrix series file in oneChannelGUI.

N.B. Editing of the target file is frequently neded to correctly organize the Target column, to fullfil the user analysis needs. The Target file could contain a subset of the array data present in the series matrix the GEO matrix series file present in the Target file.

4.1.8 Combining GEO matrix series file

In large GEO experiments, e.g. GSE2109, the experiment is splitted in mutiple Matrix Series Files. The function *Combining GEO matrix series file* ,2, allows to combine the splitted Matrix series Files in a unique ExpressionSet to be used in oneChannelGUI. The user need to prepare a target file for each of the pieces of the experiment to be combined. The function will ask the user the number of GEO matrix series files to be combined and subsequently for each of them will ask for the Target file name and for the corresponding GEO matrix series file to be loaded.

4.1.9 Loading Tab delimited files

This sub menu, fig. 3, allows to load tab delimited file containing expression data only. Also in this case the target and the expression file are the only two files needed to load these data in oneChannelGUI. In the target file the FileName column should contain exactly the same names present in the header of the tab delimited matrix file. Example

of targets are available at <http://www.bioinformatica.unito.it/bioinformatics/DAGEL.II/>. Actually a specialized module to load *processed-data* derived from Array-Express database <http://www.ebi.ac.uk/arrayexpress/> is not available. However, *processed-data*, reorganized in a flat tab delimited file containing only expression values, can be loaded on oneChannelGUI.

4.2 Open, Save, Save as

A project can be saved using the functions *Save as* fig. 2. A microarray project can also be uploaded again in oneChannelGUI with the function *open*.

4.3 Exporting normalized expression values

This function, fig. 2, allows to export, as tab delimited files expression data, loaded in oneChannelGUI. This function is also located in *filtering menu* and in the *exon menu*. If exon arrays are loaded in oneChannelGUI it is possible to extract not only the gene level expression data available but also exon level expression data and any other data generated during exon array analysis: Splice Index, MiDAS p-values, RP alternative splicing data.

4.4 Info about the loaded data set

This function, fig. 2, gives information about the set of data loaded in oneChannelGUI and on the corresponding annotation library, if available.

4.5 Attaching annotation lib info

If a Bioconductor library is available this is attached to the data loaded in oneChannelGUI and it will appear in the output of *Info about the loaded data set*. Using *Attaching annotation lib info* function, after loading expression data as a tab delimited file, it is possible to attach the Bioconductor annotation library associated to it. In case gene-level Affymetrix Whole transcriptome data are loaded as tab delimited file HuEx for human, MoEx for mouse and RaEx for rat annotation need to be attached. This is needed to allow the attachment of annotation information to the data set.

4.5.1 Probe set annotation

The Bioconductor annotation library for IVT Affymetrix arrays or GEO Matrix Series file are directly attached. Concerning Exon 1.0 ST arrays, gene level annotation information are actually embedded in oneChannelGUI, and a stand alone function is provided to use gene-level annotation externally to oneChannelGUI. Exon-level annotation is now provided by three annotation packages: HuExExonProbesetLocation, MoExExonProbesetLocation and RaExExonProbesetLocation . For exon arrays annotation is available

at the gene level for the core subset of Hs/Mm/Rn. As soon as Bioconductor annotation libraries will be available for exon arrays the oneChannelGUI annotation will use them for annotation. Info about the available Affymetrix annotation release can be found in the main R window as part of the oneChannelGUI release major changes. For EXON 1.0 ST arrays, it is possible to link GeneBank accession numbers and EG to the gene-level probe sets using the function *Attaching ACC to Probe set IDs*, present in the Biological Interpretation menu. This function also allows to link EGs to glevel probe sets of a tab delimited file that has in the first column the probe set ids. It is also possible to extract exon-level Probe Selection Region sequences associated to a specific gene-level probe-set using the function *Extracting exon-level PSR sequences associated to one gene-level probeset* in the Biological Interpretation menu.

5 RNA target

The first item in the menu, fig. 13, is inherited from affylmGUI and allows the visualization of the experimental structure described by the target file used to load the expression data.

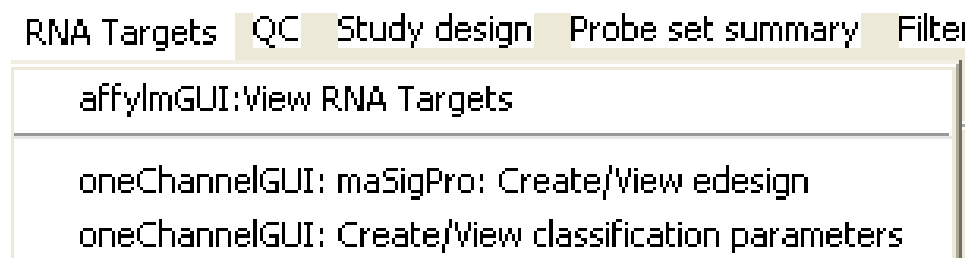


Figure 13: RNA target menu.

The second item, fig. 13, *maSigPro create/view edesign* reorganizes the target file to extract all the information needed to analyse a time course experiment using maSigPro. For time course experiments a specific target file is needed, fig. 14.

Each row of the column named Target, in the target file, describes the array on the basis of the experimental design. Each element needed for the construction of design for time course is separated from the others by an underscore. The first three elements of the row are fixed and represent **Time Replicate Control**, all separated by an underscore:

Time_Replicate_Control

All the other elements refer to various experimental conditions.

Considering two different conditions to be evaluated each row is made of 5 elements:

Time_Replicate_Control_cond1_cond2 all separated by an underscore.

Time Course design for maSigPro

	A	B	C
	Name	FileName	Target
	exp1.01	1539121008.A.CEL	8_1_1_0_0
	exp2.01	1539121006.A.CEL	8_1_1_0_0
	exp3.01	1539121005.A.CEL	8_1_1_0_0
	exp1.03	1539121008.C.CEL	24_2_1_0_0
	exp2.03	1539121006.C.CEL	24_2_1_0_0
	exp3.03	1539121005.C.CEL	24_2_1_0_0
	exp1.05	1539121008.E.CEL	48_3_1_0_0
	exp2.05	1539121006.E.CEL	48_3_1_0_0
	exp3.05	1539121005.E.CEL	48_3_1_0_0
	exp1.07	1539121020.A.CEL	8_4_0_1_0
	exp2.07	1539121009.A.CEL	8_4_0_1_0
	exp3.07	1539121021.A.CEL	8_4_0_1_0
	exp1.09	1539121020.C.CEL	24_5_0_1_0
	exp2.09	1539121009.C.CEL	24_5_0_1_0
	exp3.09	1539121021.C.CEL	24_5_0_1_0
	exp1.11	1539121020.E.CEL	48_6_0_1_0
	exp2.11	1539121009.E.CEL	48_6_0_1_0
	exp3.11	1539121021.E.CEL	48_6_0_1_0
20	exp1.02	1539121008.B.CEL	8_7_0_0_1

Figure 14: Target file for time course analysis.

Having an experiment made of 9 arrays, with two time points, 0h and 24h, in triplicate, and two different experimental conditions to be evaluated, the target file will look like:

<i>Name</i>	<i>FileName</i>	<i>Target</i>
<i>mC1</i>	<i>M1.CEL</i>	<i>0_1_1_0_0</i>
<i>mC2</i>	<i>M4.CEL</i>	<i>0_1_1_0_0</i>
<i>mC3</i>	<i>M7.CEL</i>	<i>0_1_1_0_0</i>
<i>mE1</i>	<i>M3.CEL</i>	<i>24_2_0_1_0</i>
<i>mE2</i>	<i>M6.CEL</i>	<i>24_2_0_1_0</i>
<i>mE3</i>	<i>M9.CEL</i>	<i>24_2_0_1_0</i>
<i>mI1</i>	<i>M2.CEL</i>	<i>24_3_0_0_1</i>
<i>mI2</i>	<i>M5.CEL</i>	<i>24_3_0_0_1</i>
<i>mI3</i>	<i>M8.CEL</i>	<i>24_3_0_0_1</i>

The third item, fig. 13, instead refers to the reorganization of a target file containing the information related to clinical parameters to be used for classification purposes. In this case each clinical parameter is separated from the others by an underscore as in the case of the time course. The absence of a parameter **NEEDS** to be indicated in the Target file by NA. Having an experiment made of 9 arrays with 4 different experimental/clinical parameters the target file will look like:

<i>Name</i>	<i>FileName</i>	<i>Target</i>
<i>mC1</i>	<i>M1.CEL</i>	<i>0_1_pos_0_NA</i>
<i>mC2</i>	<i>M4.CEL</i>	<i>0_1_pos_0_yes</i>
<i>mC3</i>	<i>M7.CEL</i>	<i>0_1_neg_0_no</i>
<i>mE1</i>	<i>M3.CEL</i>	<i>24_2_neg_1_NA</i>
<i>mE2</i>	<i>M6.CEL</i>	<i>24_2_NA_1_yes</i>
<i>mE3</i>	<i>M9.CEL</i>	<i>24_2_neg_1_yes</i>
<i>mI1</i>	<i>M2.CEL</i>	<i>12_3_0_pos_yes</i>
<i>mI2</i>	<i>M5.CEL</i>	<i>12_3_0_pos_no</i>
<i>mI3</i>	<i>M8.CEL</i>	<i>12_3_0_pos_no</i>

Once the target file is reorganized by *create/view classification parameters* function, the user will be requested to select an external file containing the description of the experimental/clinical parameters. In this file, the description of each parameter is separated from the others by a carriage return.

Drug treatment time

Tumor grade

IHC ER

Metastasis within 5 years

Positive lymphonode

This information will be used to select a specific clinical parameter for classification analysis.

6 QC

The functions available in this menu are specific of the type of microarray data set loaded

6.1 QC for IVT arrays loaded starting from .CEL files

This menu, fig. 15, inherits all affyGUI probe/probe set level quality controls, please refer to affyGUI for their usage.

Furthermore, after probe set summary is calculated, samples similarities can be visualized using the *Sample QC: PCA/HCL* function, producing a 2D PCA plot and a hierarchical clustering of the samples, fig. 16.

If exon data are loaded the function *Gene/Exon PCA/HCL* results could be visualized both at gene or exon level. Furthermore, the function *Gene/Exon Intensity Histogram* will show the density plot of the normalized intensities both at gene and at exon level.

6.2 QC for GEO/flat tab delimited files

One's probe set expression data derived by GEO Matrix Series file or an expression tab delimited file *Sample QC: PCA/HCL* function is available as QC. There is also the

QC	Study design	Probe set summary	Filtering	Modelin
affyImGUI: Intensity Histogram affyImGUI: Intensity Density Plot affyImGUI: Raw Intensity Box Plot affyImGUI: RNA Digestion Plot affyImGUI: M A Plot (for two slides) affyImGUI: Image Array Plot(One slide)				
affyImGUI: NUSE-Normalized Unscaled Std.Errors Plot affyImGUI: RLE-Relative Log Expression Plot affyImGUI: Weights pseudo chip Image(s) Plot affyImGUI: Residuals pseudo chip Image(s) Plot				
oneChannelGUI: Samples QC (PCA/HCL)				
affyImGUI: Options				

Figure 15: QC for IVT arrays.

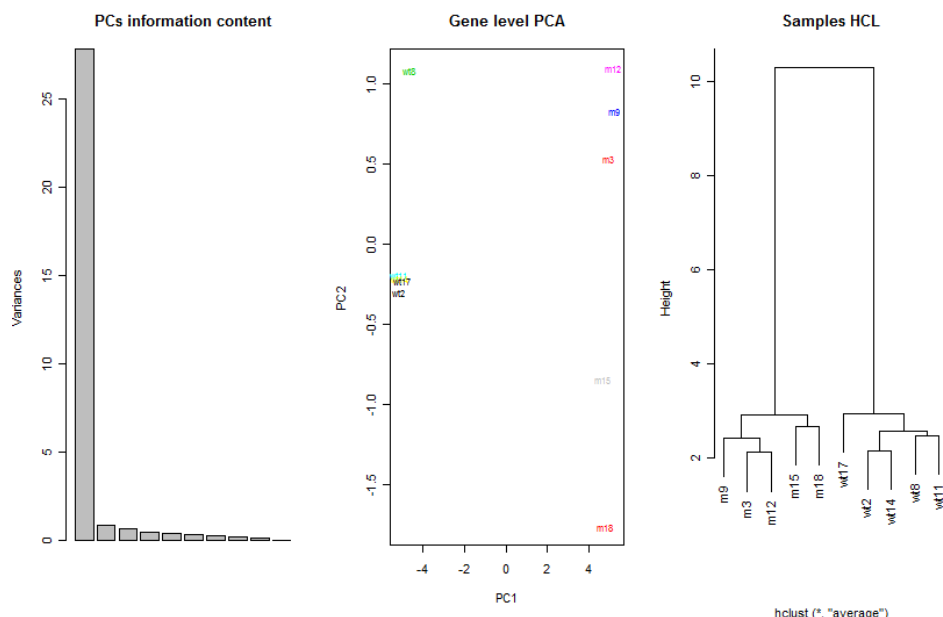


Figure 16: Sample QC: PCA/HCL output for IVT arrays. First panel on the left side contains the information content described by each of the PCs. Panel in the middle PC2 versus PC1 plot. Last panel on the right HCL.

function *Box plot of normalized data* which show the array distribution as box plot 17.

6.3 QC for exon arrays

In the case of exon array the QC menu is slightly different, as shown in fig. 18

Two functions are available:

Sample QC: PCA/HCL This function will produce a PCA/HCL for both gene/exon level data.

Gene/Exon intensity histogram This function will produce a density histogram for gene or exon expression levels.

Controls raw intensity histogram This function will produce a box plot for exon, positive controls, and introns, negative controls, for housekeeping genes. Probe level data are directly extracted from CEL files using APT tools.

It useful, as quality control, to check intensities before normalization.

As it can be seen in fig. 19 normalization masks the fact that a sub set of arrays, i.e. those with a very narrow boxplot 19A, had something wrong in hybridization. This problem is completely masked in the normalized data 19B. For this reason *Controls raw*

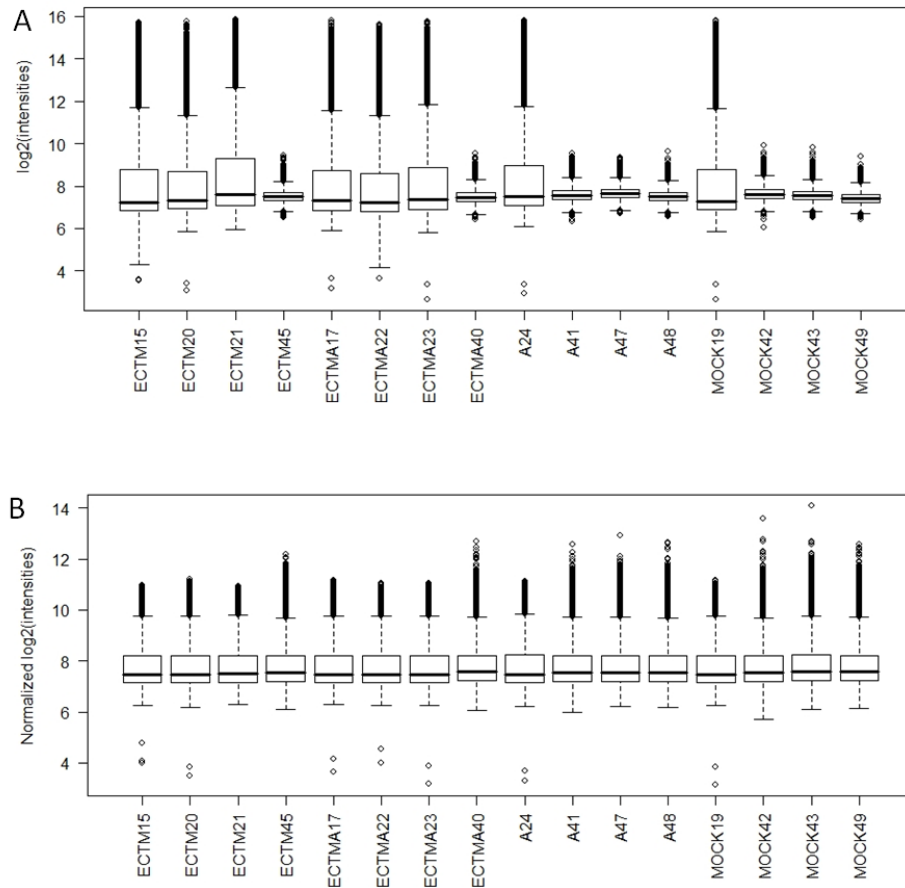


Figure 19: A set of Illumina arrays before and after data normalization.

intensity histogram was written for exon array data since probe sets data are directly uploaded as normalized in oneChannelGUI, via APT tools. This function produce a box plot for exon, positive controls, and introns, negative controls, for housekeeping genes. This box plot gives an idea of signals both at high and low intensity range.

7 Study design

This menu allows to investigate the statistical quality of a microarray study, fig. 20.

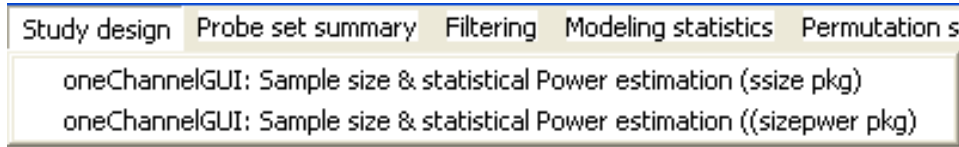


Figure 20: Study design menu.

This menu gives access to two functions, which are graphical implementations of the sizepower Bioconductor library. These functions allow user to determine how many samples are needed to achieve a specified power for a test of whether a gene is differentially expressed or, in reverse, to determine the power of a given sample size.

8 Probe set summary

This menu inherits the affyilmGUI probe set summary methods for IVT arrays. Furthermore, the *expresso* function, which allows the integration of different methods for background correction, normalization, probe specific correction, and summary value computation, is added. This menu is also available for GEO and tab delimited expression data files and it allows to perform the following normalization procedures if a data set without normalization is loaded:

1. Cyclic LOESS.
2. QUANTILE.
3. QSPLINE.

In the case of exon arrays this menu is not available since expression data, for exon arrays are calculated by APT tools using the oneChannelGUI interface or they are loaded as tab delimited files exported by Affymetrix Expression Console.

9 Filtering

A central problem in microarray data analysis is the high dimensionality of gene expression space, which prohibits a comprehensive statistical analysis without focusing on particular aspects of the joint distribution of the gene expression levels. Possible strategies are to perform data-driven nonspecific filtering of genes (von Heydebreck, 2004) before the actual statistical analysis or to filter, making use of biologically relevant a priori knowledge. This menu allows user to apply a variety of filtering procedures, fig. 21

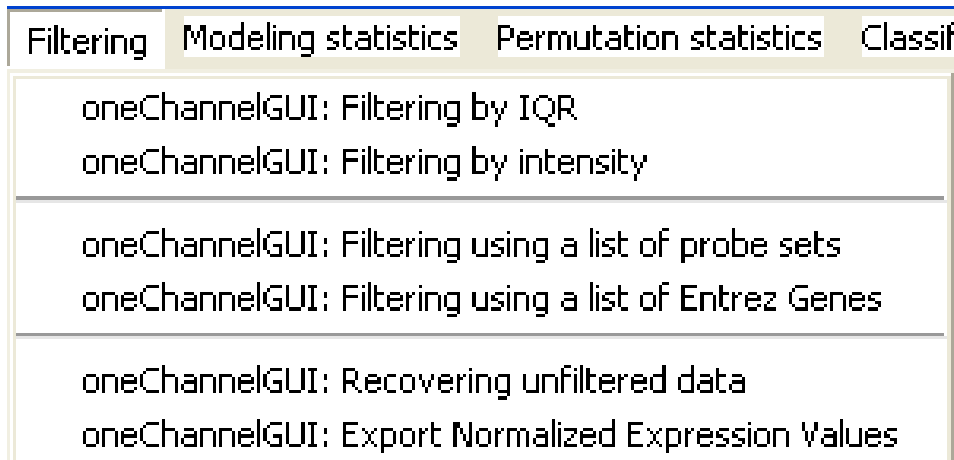


Figure 21: Filtering menu for GEO/Affy IVT arrays.

9.1 Filtering by IQR

The IQR filter will select only those probe sets characterized by a relative large signal distribution. The way the IQR filter is shown in fig. 22

In oneChannelGUI it is possible to select three filtering values:

1. IQR 0.1, weak filter, i.e. only the extreme unchanging probe sets are removed.
2. IQR 0.25, intermediate filter.
3. IQR 0.5, strong filter, i.e. the majority of the unchanged probe sets are removed.

This filtering procedure can be applied to any kind of loaded arrays. However, it seems not to be very effective when it is used to gene level expression data calculated with iterPlier.

How filtering by IQR works?

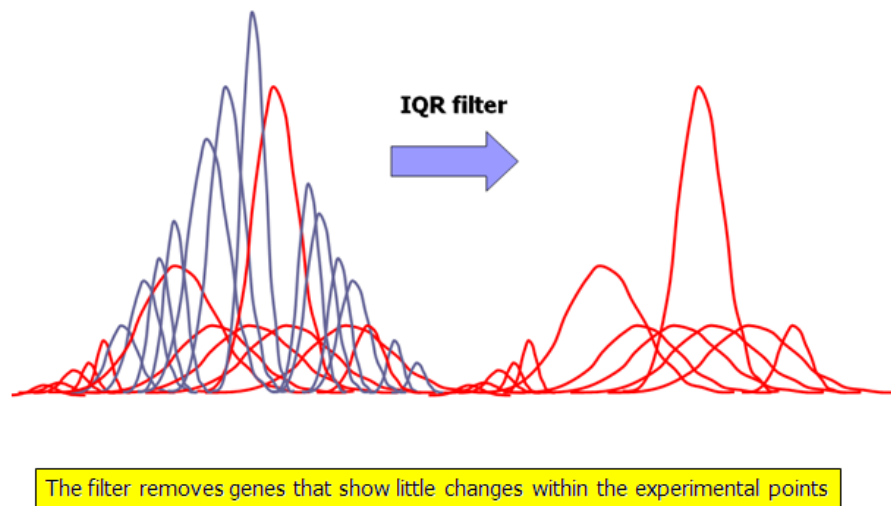


Figure 22: IQR filtering: The distributions of the various probe sets belonging to a data set are shown in red, if they are wide and they are retained by the filter, and in blue, if they are narrow and they are discarded by the filter.

9.2 Filtering by intensity

For IVT/GEO/tab delimited expression data files it is also possible to apply a filtering procedure based on intensity signals, the graphical interface to do it is shown in fig. 23.

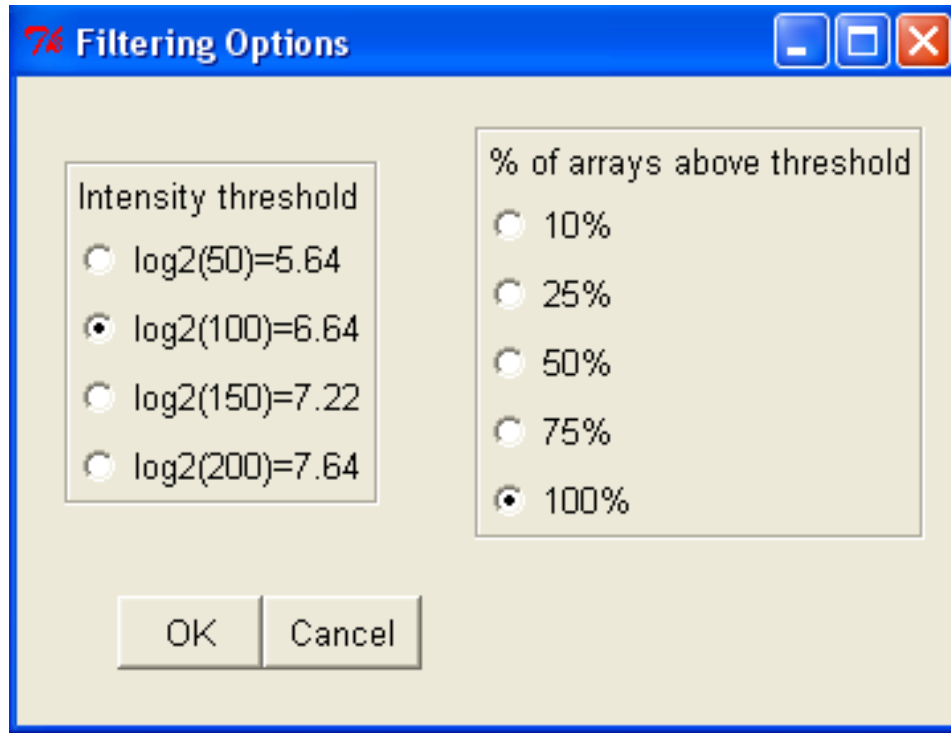


Figure 23: Intensity filtering: This filter will retain a probe set only if a certain fraction of the samples are characterized by an intensity value over a certain user defined threshold.

This filtering approach is quite useful to remove probe sets having very low intensity values.

9.3 Filtering by list of probe sets/EG ids

It is also possible to filter expression data using a text file containing a list of probe set ids separated by carriage return. If the data set is associated to a Bioconductor annotation library the filtering procedure can be also done using a text file containing a list of Entrez gene identifiers separated by carriage return.

9.4 Recovering unfiltered data

It is possible to recover the data before the last filtering using the *Recovering unfiltered data* function.

9.5 Filtering menu: exon data

If exon data are loaded the filtering menu appear slightly different, fig. 24.

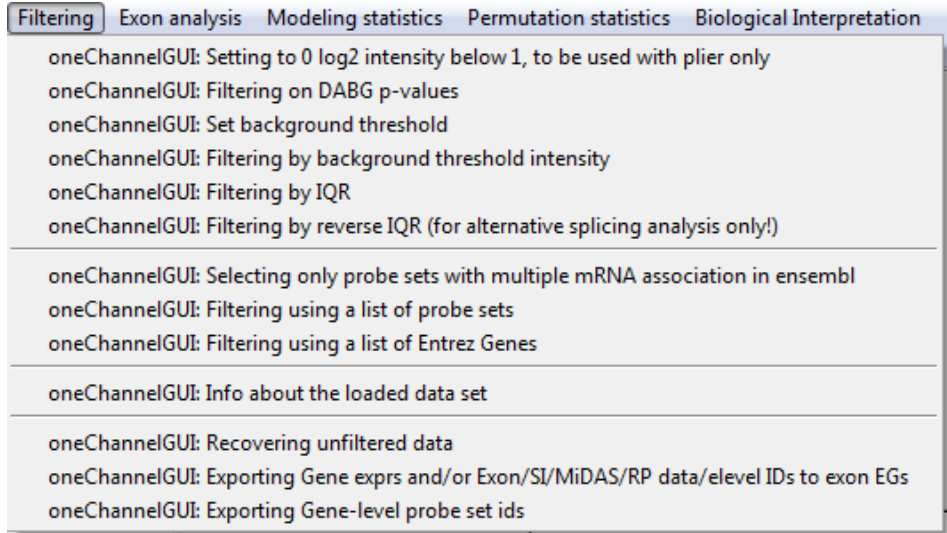


Figure 24: Filtering menu for exon data.

In particular, the function *Set background threshold* collects the exon/intron expression values for a set of housekeeping genes present in the chip within chip quality controls and it offers the opportunity to set a background intensity threshold on the basis of the desired level of intersection between the expression of exons versus introns. RMA intensity calculation is preferred, fig. 25, since, if probe set summaries are calculated with Plier or iterPlier, the differences in expression distribution between exon and introns are not enough wide, fig. 26.

Setting a background threshold using exon/intro distributions for HK genes, it is possible to apply to the full data set an intensity filtering that will remove gene and the corresponding exons on the basis of the selected threshold. The intensity filter for exon arrays works exactly as that for IVT arrays but using a fixed threshold defined as described.

An other filter that allows the removal of low intensity probe sets is based on the DABG p-values. Using the function *Filtering on DABG p-values* it is possible to select the desired level of filtering using a mask, fig. 27.

A threshold of 50% means that only probe sets where in half of the samples over the selected DABG p-value threshold will kept. As can be seen in fig. 28 this filtering also removes low intensity signals very near to zero.

N.B. Recovering the data prior filtering is not implemented for DABG p-value filtering, yet.

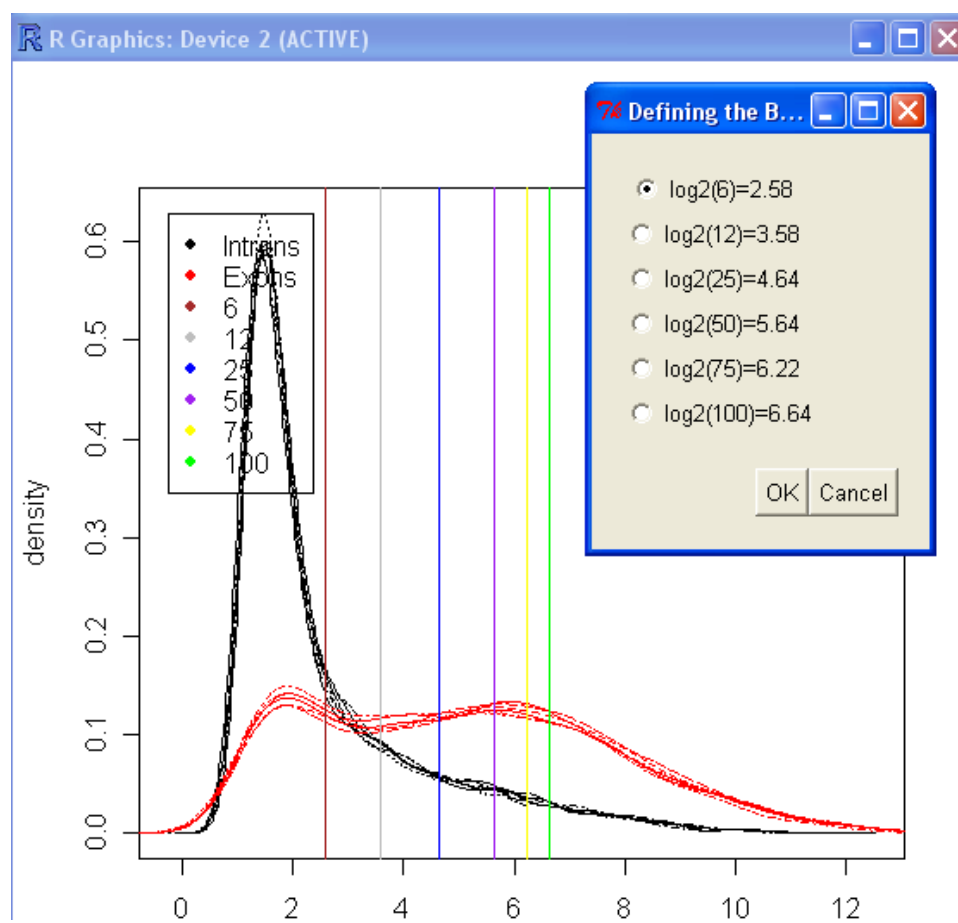


Figure 25: Human exon arrays, probe set summaries were calculated with RMA, exon/intron distribution of HK present in the chip as quality controls.

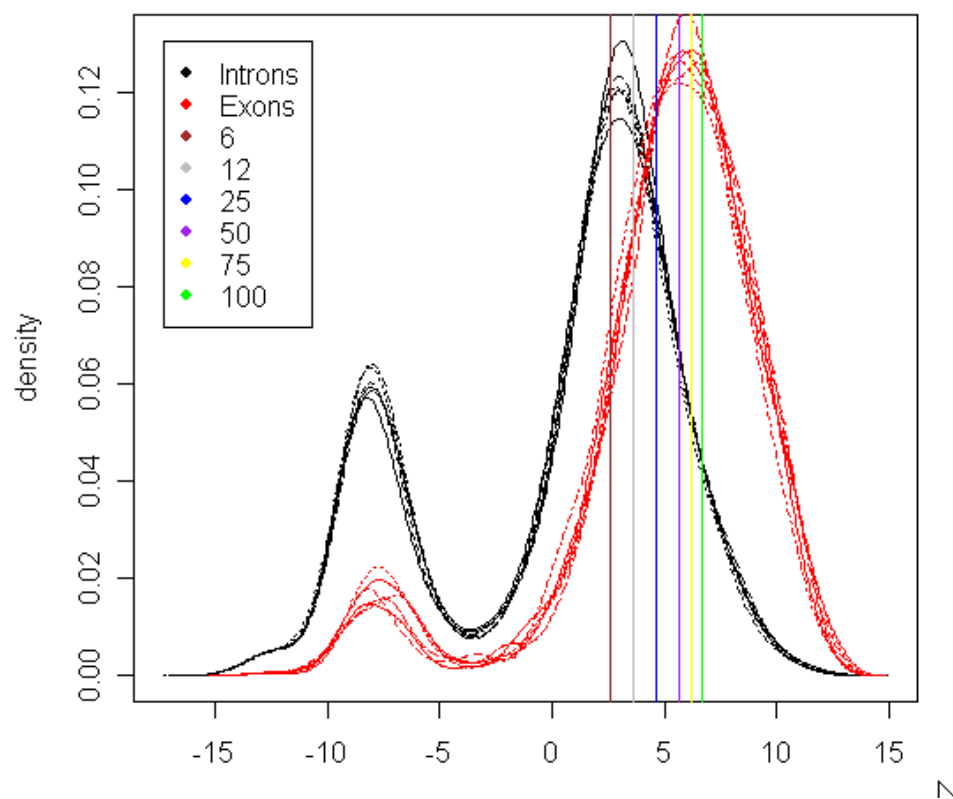


Figure 26: Human exon arrays, probe set summaries were calculated with iterPlier (gene level) and Plier (exon level), exon/intron distribution of HK present in the chip as quality controls.

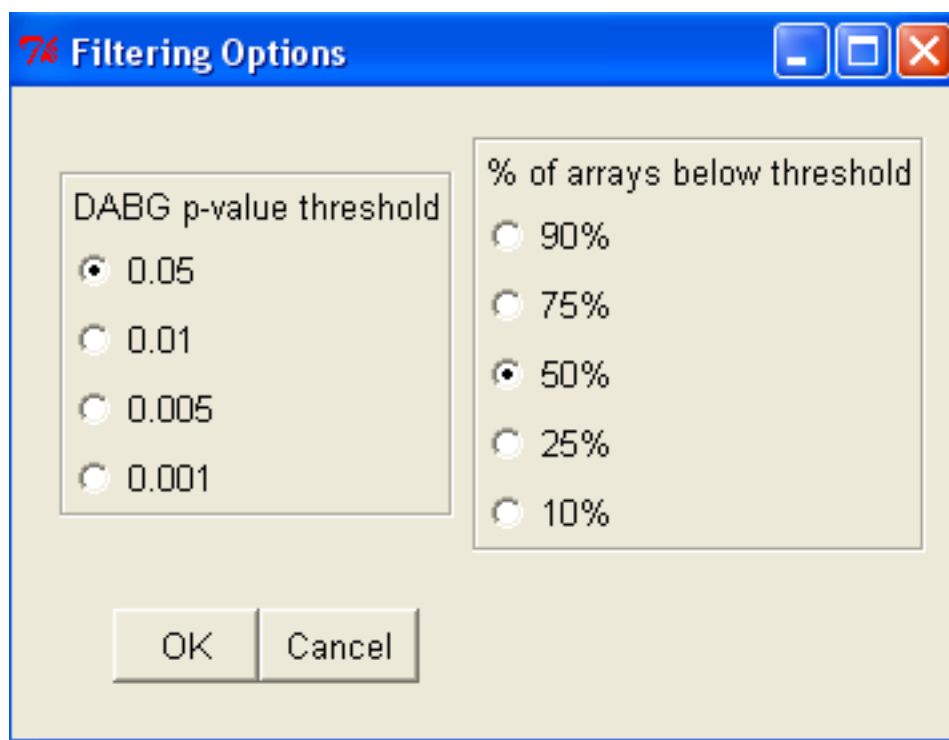


Figure 27: DABG p-value based filtering selection mask.

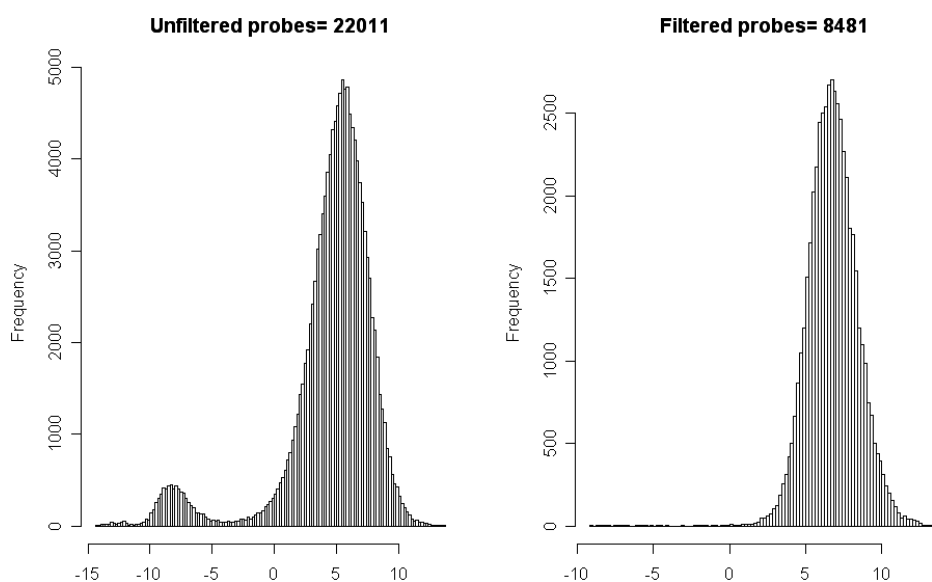


Figure 28: DABG p-value filtering results with parameters: DABG p-value threshold 0.05 and 50% of arrays over the threshold.

Regarding very low intensity probe sets present if iterPlier/Plier are used, the function *Setting to 0 log2 intensity below 1, to be used with plier only* will set them zero.

The function *Selecting only probe sets with multiple mRNA association in ensembl* it is very useful when alternative splicing events are investigated, if the researcher is interested to investigate only those probe sets associated to multiple transcripts annotated on ensembl database. We strongly suggest to apply this filter at least to get an overview of the possible known alternative splicing events that could be collected within the annotated ensembl data. This filter will reduce both the computational time to calculate splice index and type I statistical error, at the level of statistical analysis for alternative splicing detection.

Specifically, this function select at gene-level only those probe sets which are associated to multiple entries on ensembl data base. The filter uses the biomaRt package to collect this information from ensembl database.

The function oneChannelGUI: *Exporting Gene-level probe set ids* is useful to extract the list of probe set ids associated to the gene-level data set loaded on oneChannelGUI.

10 Modelling statistics

This menu allows to perform limma differential expression analysis as well as time course analysis using the maSigPro package, fig. 29.

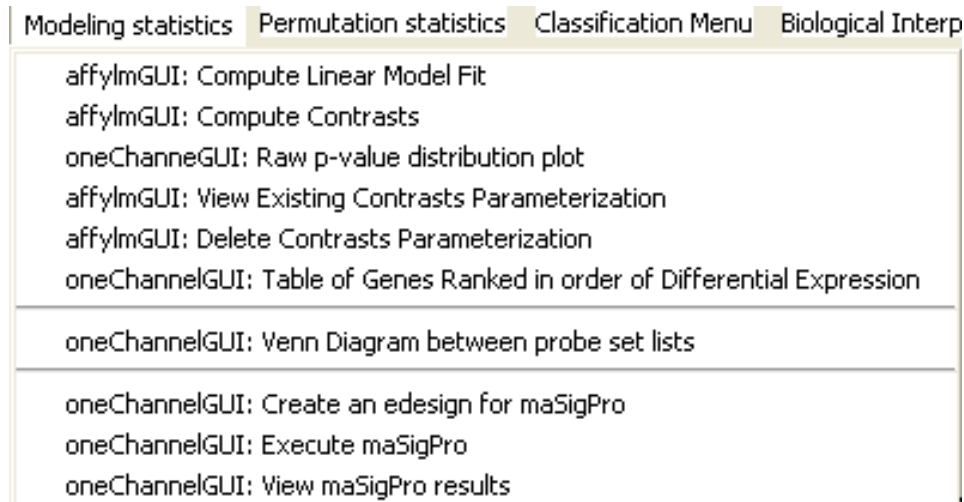


Figure 29: Modelling statistics

10.1 limma

The affyGUI interface to limma is fully inherited, see limma and affyGUI vignettes for usage. The function *raw p-value distribution* is implemented to evaluate if the BH/BY

type I error correction methods could be used. To apply BH correction two conditions should be satisfied:

1. The gene expressions are independent from each other.
2. The raw distribution of p-values should be uniform in the non significant range.

Instead if BY correction is used it is sufficient only the second one, fig. 30.

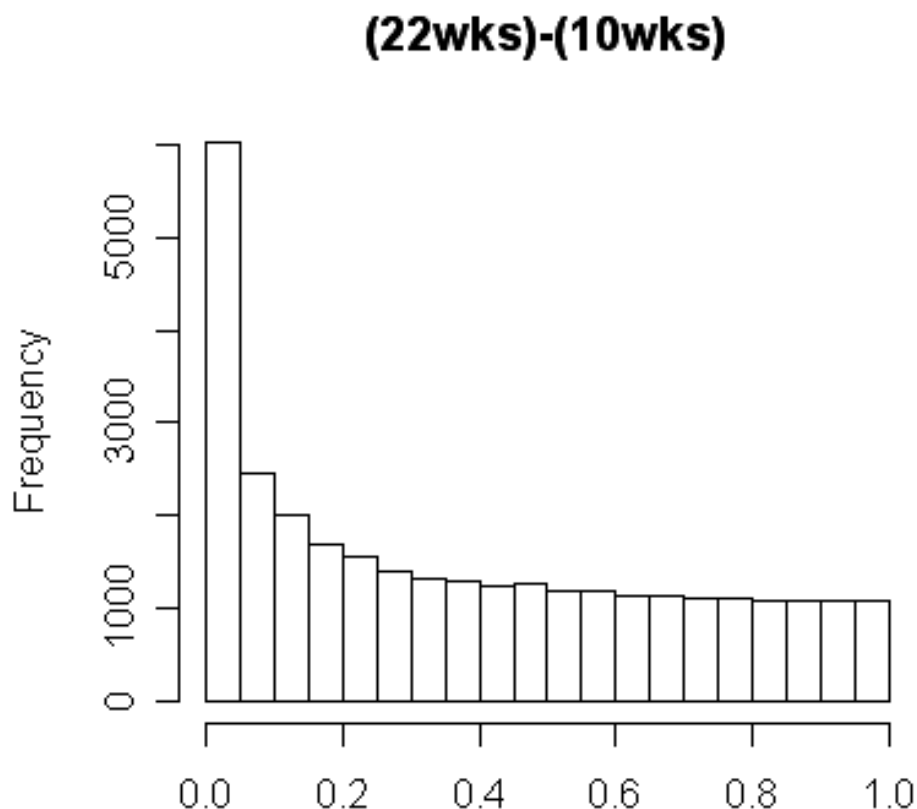


Figure 30: Output of *raw p-value distribution*: The raw distribution of p-values is uniform in the non significant range.

The `affylmGUI` function *Table of genes ranked in order of differential expression* is a modified version of the original found in `affylmGUI` to allow users to check with MA/Volcano plots the set of differentially expressed probe sets before saving the table, fig. 31.

The `limma` analysis, implemented in `affylmGUI`, does not allow the inclusion in the model of batch effect. A new function *Two groups Linear Model with batch effect*, in

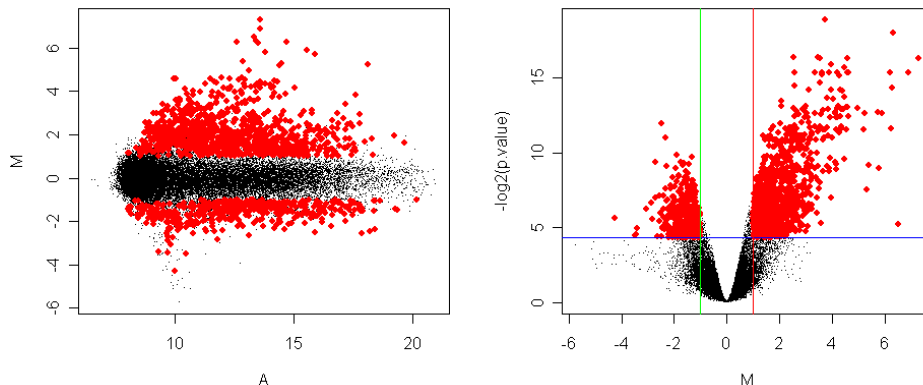


Figure 31: MA and Volcano plots for differentially expressed probe sets, red dots, detected by limma method.

the Modelling menu, integrates in linear model batch effect. This functionality is only available for two sample groups. For this analysis the different origins of the samples are inserted in the Target column of the target file as shown below in the Rank Product section.

10.2 Venn diagrams between probe set list

This function is modified with respect to the original one presents in affylmGUI to allow Venn diagrams using lists of probe sets, saved in text files where each id is separated by the others by carriage return, derived by any of the available statistical methods implemented in oneChannelGUI. Furthermore, if a Bioconductor annotation library is linked to the loaded data set, Venn diagrams can be generated using the Entrez Gene ids associated to the probe sets, removing probe sets redundancy.

10.3 Time course analysis

Time course analysis can be performed on oneChannelGUI using maSigPro package, fig. 29.

maSigPro is a R package for the analysis of single and multiserie time course microarray experiments. maSigPro follows a two steps regression strategy to find genes with significant temporal expression changes and significant differences between experimental groups. To handle the batch effect the target file should be organized as the target file used in the RankProduct section.

The first step, to run maSigPro analysis, is to reorganize the target file using the function *create an edesign for maSigPro*, see also target file paragraph for time course

experiment requirements. Using the function *Execute maSigPro* user will select the parameters needed for maSigPro, fig. 32.

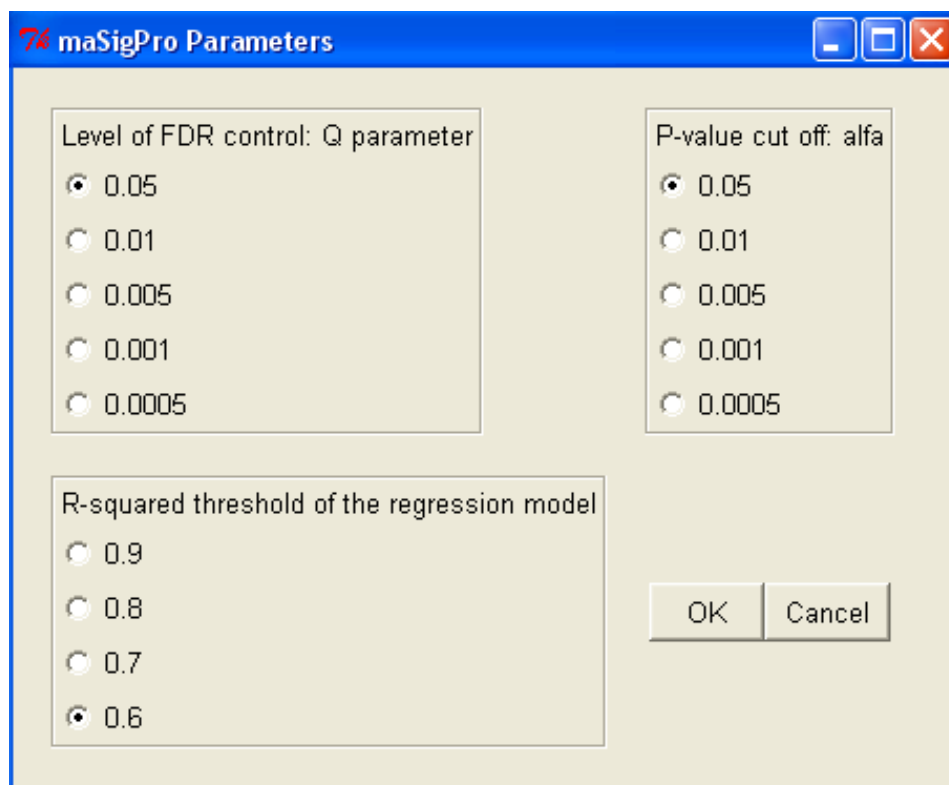


Figure 32: maSigPro parameters setup.

10.3.1 Lever of FDR control: Q parameter

The first step is to compute a regression fit for each gene. The p-value associated to the F-Statistic of the model are computed and they are subsequently used to select significant genes. maSigPro corrects this p-value for multiple comparisons by applying false discovery rate (FDR) procedures. The level of FDR control is given by the function parameter Q, fig. 32.

10.3.2 P-value cut off: alfa

maSigPro applies, as second step, a variable selection procedure to find significant variables for each gene. This will ultimately be used to find which are the profile differences between experimental groups. At each regression step the p-value of each variable is computed and variables get in/out the model when this p-value is lower or higher than the given cut-off value alfa, fig. 32.

10.3.3 R-squared threshold of the regression model

The last step in maSigPro analysis is to generate a lists of significant genes. As filtering maSigPro uses the R-squared of the regression model, fig. 32.

maSigPro calculation steps can be followed on the main R window. The end of the maSigPro analysis will be given by a popup message, fig. 33.

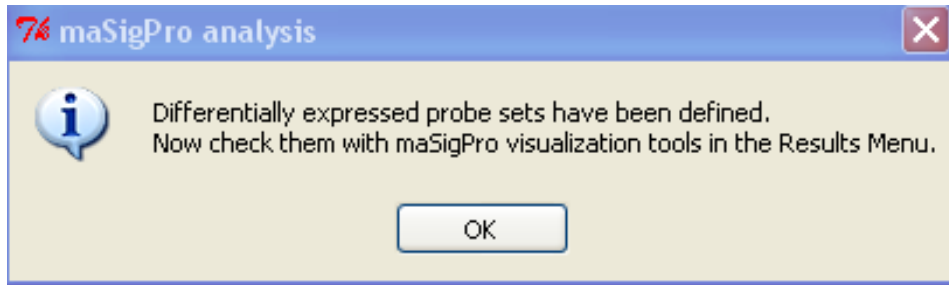


Figure 33: End of maSigPro calculation.

N.B: Multiple test problem is also present in maSigPro analysis. Therefore, before running maSigPro, remember to perform some filter based on functional information or samples distribution.

10.3.4 View maSigPro results

The coefficients obtained in the second regression model will be useful to cluster together significant genes with similar expression patterns and to visualize results. Various visualization options are available:

1. Venn diagrams, fig. 34 .
2. Expression profiles saved in a pdf file, figs. 35, 36.
3. Tab delimited files with the probe sets found differentially expressed in each of the experimental conditions.

11 Permutation statistics

The permutation statistics menu, fig. 37, allows to run two class unpaired SAM analysis implemented in the siggenes package and two class samples analysis using the rank product method implemented in RankProd package.

Results can be visualized as Venn diagrams

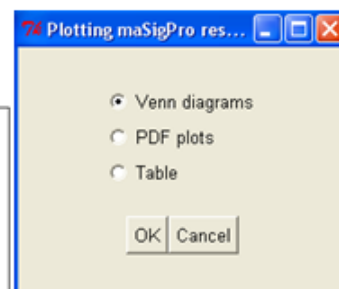
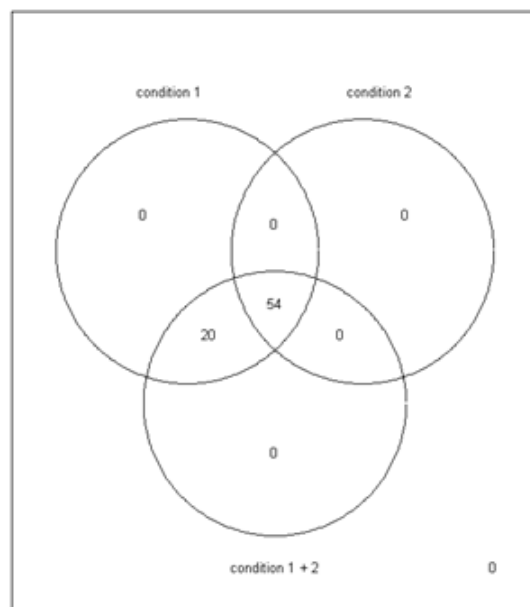


Figure 34: maSigPro Venn diagrams output.

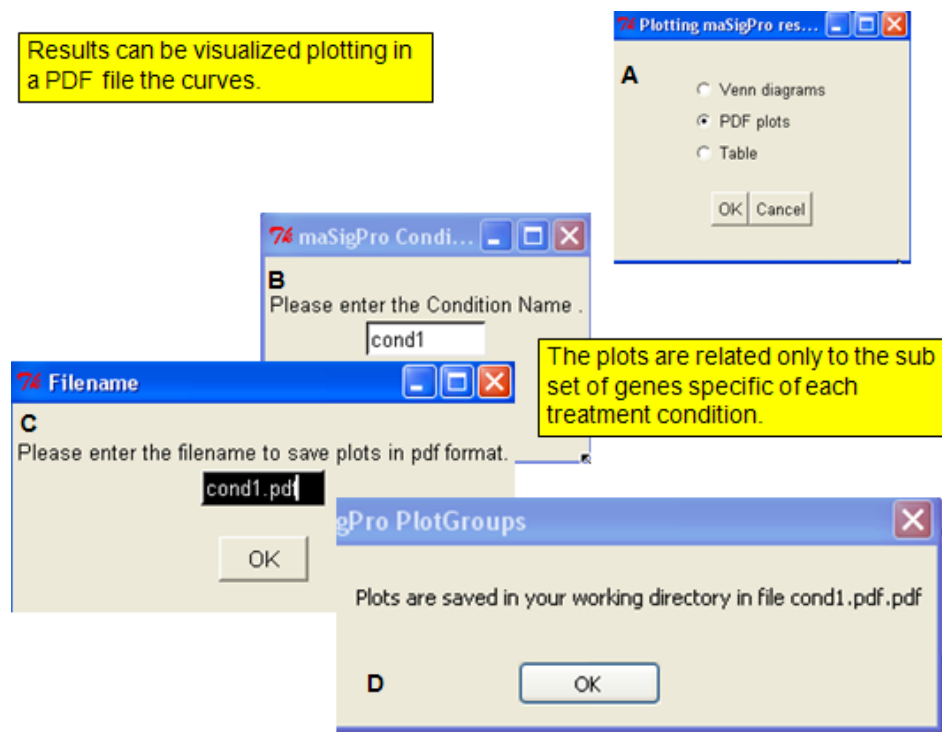


Figure 35: Selecting the experimental condition to be used to profiles plotting.

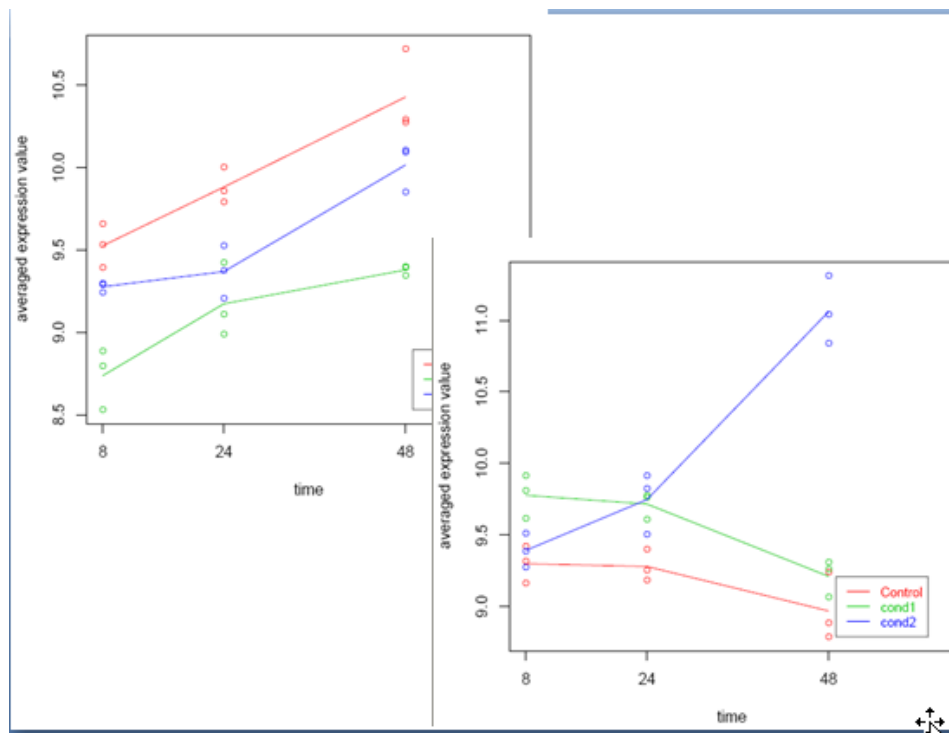


Figure 36: An example of profiles plotting.

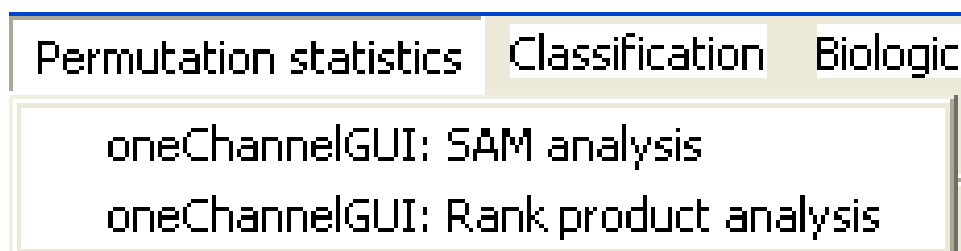
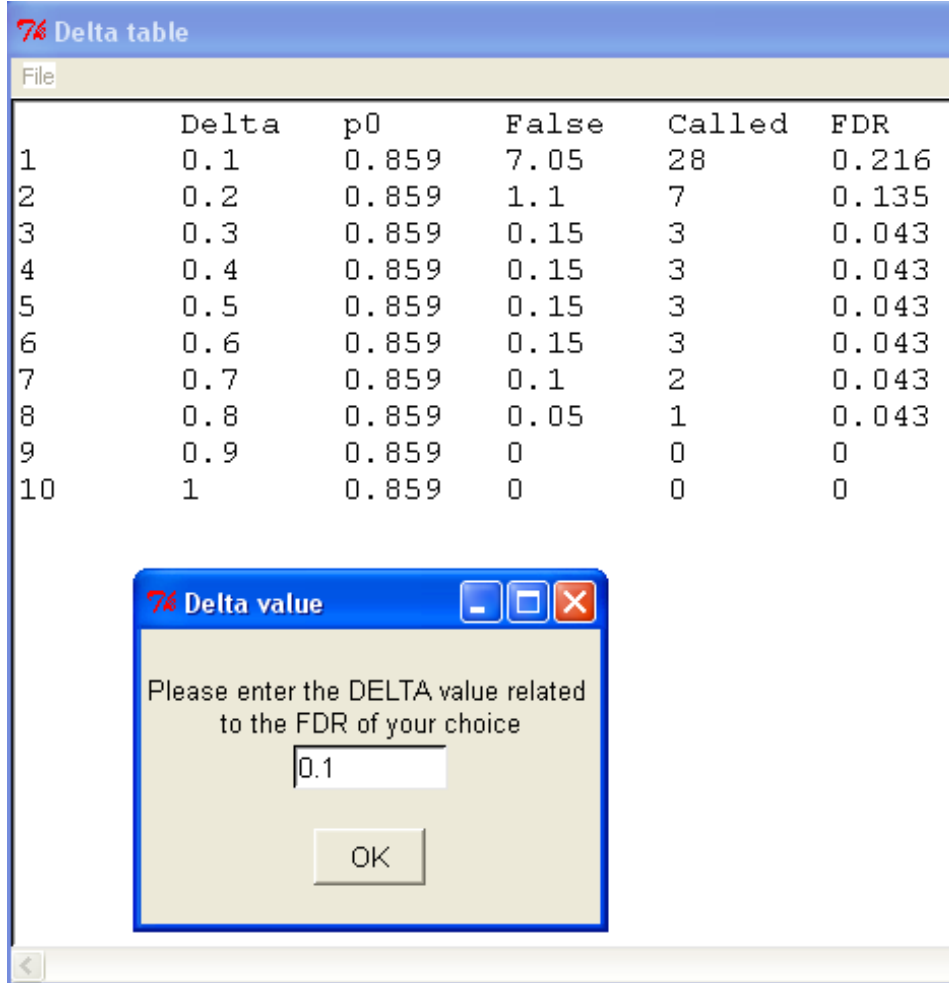


Figure 37: Permutation statistics menu.

11.1 SAM analysis

The module recognizes if a two class unpaired analysis can be performed. Subsequently, a table with DELTA values and FDRs will be shown to the user. Furthermore, user need to select a delta threshold to continue the analysis, fig. 38.



	Delta	p0	False	Called	FDR
1	0.1	0.859	7.05	28	0.216
2	0.2	0.859	1.1	7	0.135
3	0.3	0.859	0.15	3	0.043
4	0.4	0.859	0.15	3	0.043
5	0.5	0.859	0.15	3	0.043
6	0.6	0.859	0.15	3	0.043
7	0.7	0.859	0.1	2	0.043
8	0.8	0.859	0.05	1	0.043
9	0.9	0.859	0	0	0
10	1	0.859	0	0	0

Please enter the DELTA value related to the FDR of your choice

0.1

OK

Figure 38: DELTA table and DELTA value selection module.

Siggenes output for differentially expressed genes, given the selected DELTA value, will be shown in the main R window, fig. 39, together with a absolute $\log_2(\text{FC})$ selection module, fig. 39.

The fold change filters allows the selection, within the SAM significant probe sets, of those greater than a user defined threshold. Subsequently, the differentially expressed genes will be shown, fig. 40, and the user will decide if they should be saved.

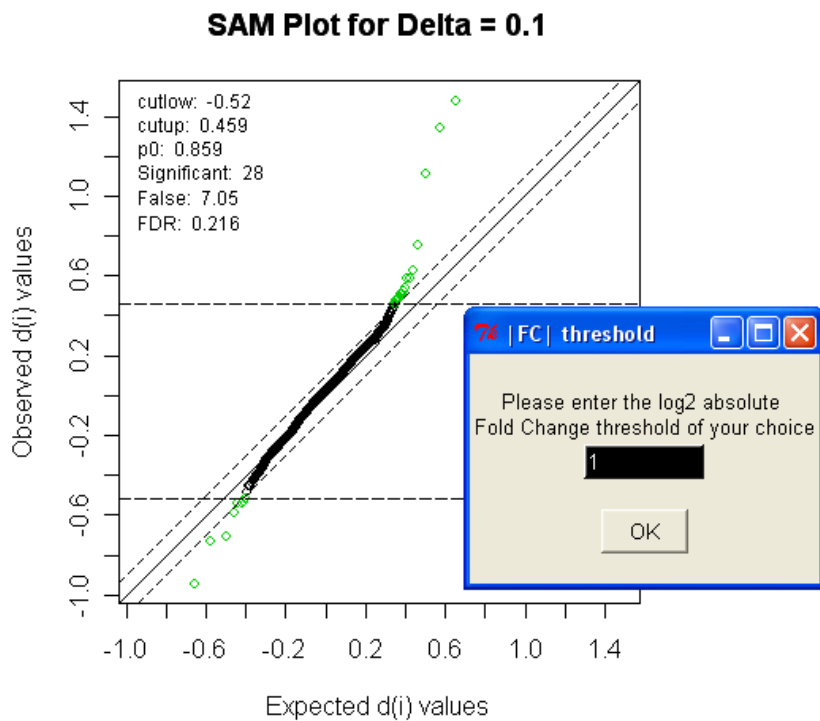


Figure 39: SAM results given at specific user defined DELTA value and the absolute log2(fc) selection mask.

10 genes were found differentially expressed using a delta= 0.1 and a |FC| threshold= 1

File	AffyID	EG	Symbol	d.value	stdev	rawp	q.value	log2.R.fold
	238733_at	1368	CPM	1.5	0.4	1.6e-05	0.086	2.7
	211834_s_at	8626	TP73L	1.3	0.15	3.3e-05	0.086	2.1
	221577_x_at	9518	GDF15	1.1	0.18	4.9e-05	0.086	1.7
	1565483_at	1956	EGFR	-0.94	0.36	0.00011	0.15	-1.7
	202284_s_at	1026	CDKN1A	0.75	0.084	0.00015	0.15	1.1
	1565484_x_at	1956	EGFR	-0.73	0.44	2e-04	0.16	-1.4
	1552701_a_at	114769	COP1	-0.71	0.44	0.00021	0.16	-1.4
	1554400_at	6991	TCTE3	-0.59	0.27	0.00041	0.21	-1
	1555786_s_at	NA	NA	-0.54	0.36	0.00054	0.21	-1
	228697_at	135114	HINT3	0.54	1	0.00057	0.21	2

Figure 40: Differentially expressed probe sets to be saved.

11.2 Rank product analysis

The RankProd module is a graphical interface to the RankProd package functions for the analysis of gene expression microarray data. RankProduct package allows the identification of differentially expressed genes using the so called rank product non-parametric method (Breitling et al., 2004, FEBS Letters 573:83) to identify up-regulated or down-regulated genes under one condition against another condition, e.g. two different treatments, two different tissue types, etc. The user needs only to define the pfp (percentage of false prediction) threshold and the number of permutations to be applied, fig. 41.

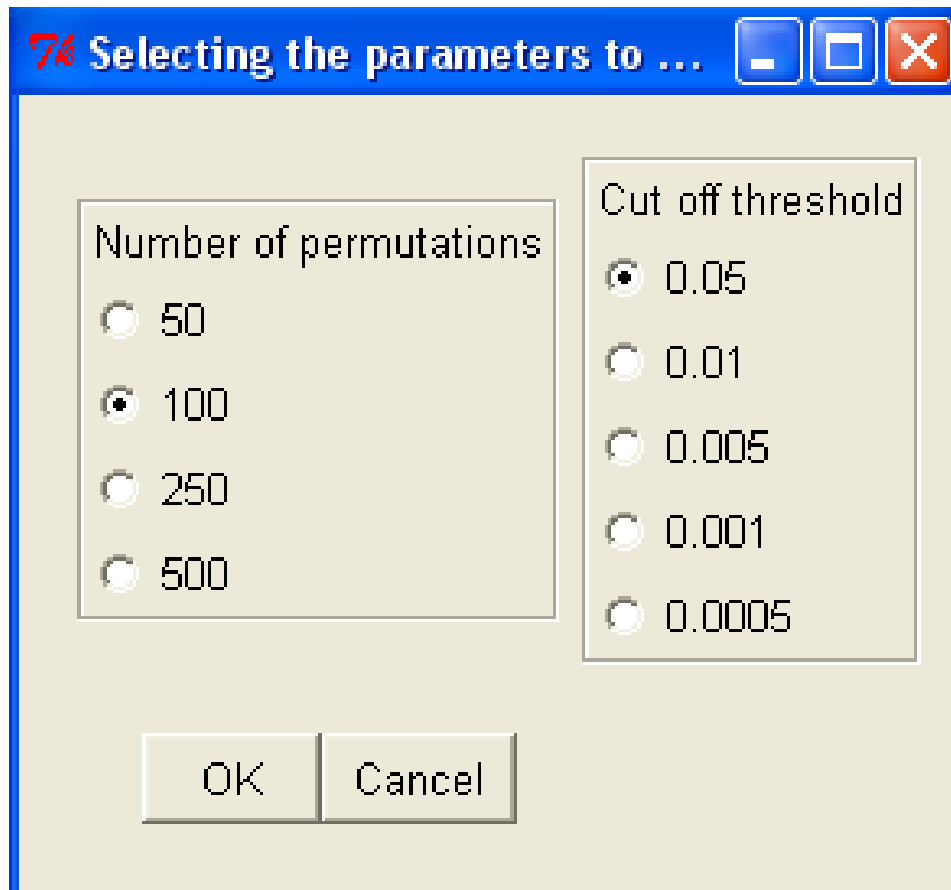


Figure 41: RankProd selection parameters mask.

At the end of the analysis the user will decide if he would like to save the differentially expressed probe sets in a tab delimited file. If a Bioconductor annotation library is available Entrez Gene identifier and Symbols will be added to the saved output.

11.2.1 Target structure

In a rank product analysis for data sets from different origin the structure of the Target column of the target file can contain also an integer describing the data origin.

<i>Name</i>	<i>FileName</i>	<i>Target</i>
<i>mC1</i>	<i>M1.CEL</i>	<i>0_1</i>
<i>mC2</i>	<i>M4.CEL</i>	<i>0_1</i>
<i>mC3</i>	<i>M7.CEL</i>	<i>0_1</i>
<i>mE1</i>	<i>M3.CEL</i>	<i>0_2</i>
<i>mE2</i>	<i>M6.CEL</i>	<i>0_2</i>
<i>mE3</i>	<i>M9.CEL</i>	<i>1_1</i>
<i>mI1</i>	<i>M2.CEL</i>	<i>1_1</i>
<i>mI2</i>	<i>M5.CEL</i>	<i>1_2</i>
<i>mI3</i>	<i>M8.CEL</i>	<i>1_2</i>

The oneChannelGUI module will select the RankProd method on the basis of the Target structure.

12 Classification

This module, fig. 42, provides a link to the pamr and pdmclass packages designed to carry out sample classification from gene expression data, respectively by the method of nearest shrunken centroids (Tibshirani, et al., 2002) and by penalized discriminant methods.

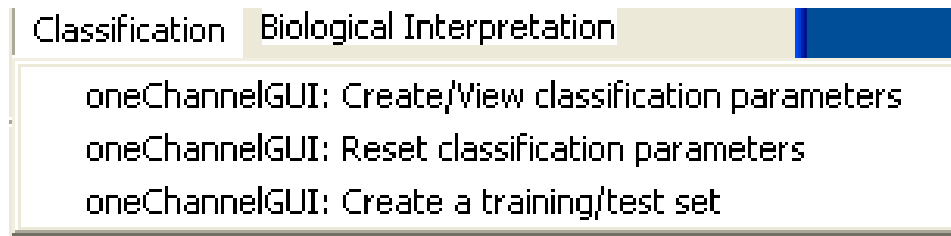


Figure 42: Classification menu.

12.0.2 Create/view/reset classification parameters

The *Create/view classification parameters* function reorganizes the Target columns separating the experimental/clinical parameters. The *Reset classification parameters* function deletes the Targets reorganization and the association to the file containing the names of the parameters present in the Target column of the target file.

12.0.3 Create a training/test set

The first step of this module is the definition of the covariate to be used for the classification analysis. The user will be requested to select, from a table, listing the names clinical parameters, i.e. phenoData covariate names, one of them indicating its row number, fig. 43. Subsequently, the user could decide to divide the data set in a training (2/3) and a

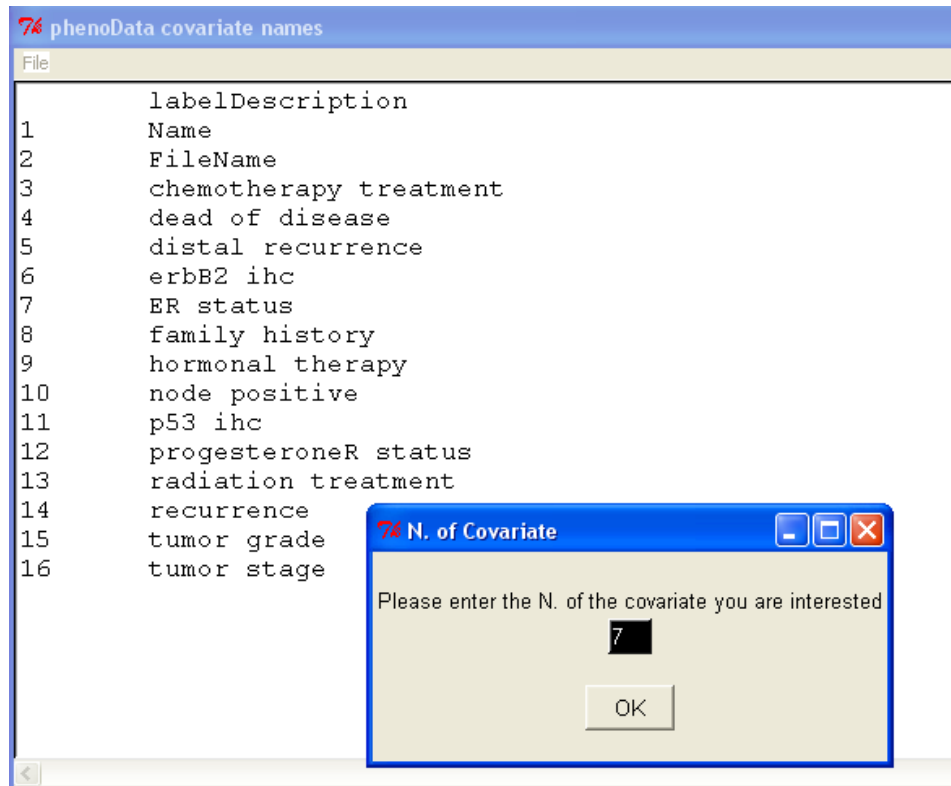


Figure 43: Selecting the classification parameter.

test (1/3) set or use the full data set as training set. All arrays, which are not linked to any of clinical/experimental params, i.e. those marked as NA, will be discarded from the following analyses.

The *Create a training/test set* function then allows the access to PAMR/PDMCLASS classification tools and to a PCA visualization module, fig. 44.

It is also possible to evaluate how the classification performance can be associated to a single subset of probesets or if it is a general characteristic of the data set. Ideally we expect that only a small subset of probe sets should be able to discriminate between groups, if the full data set is able to discriminate in a way independent by the subset of probe sets considered for the classification it might be an indication of some bias that could not be necessarily associated to the biological event under investigation, eg. it could be due to some experimental bias. This functionality is provided if the *Probability*

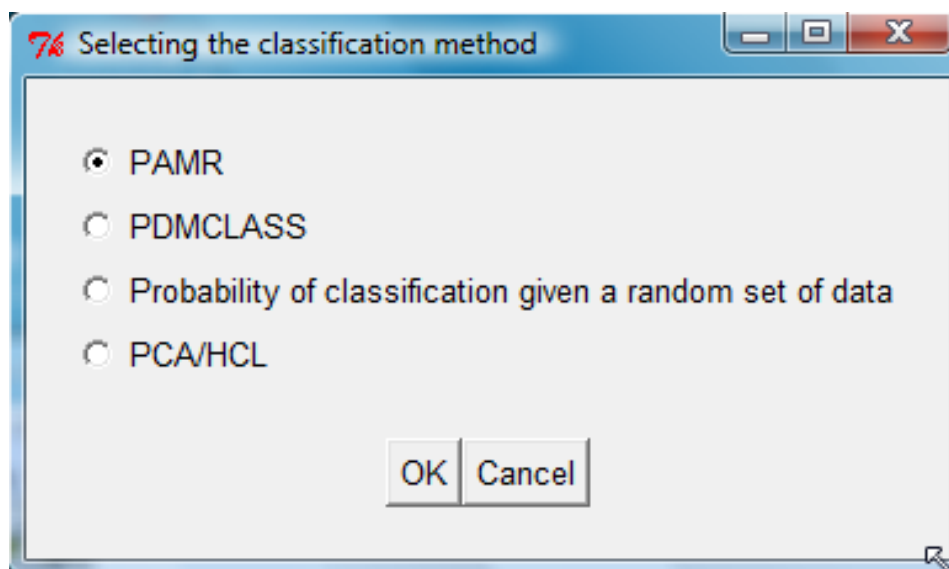


Figure 44: Classification methods selection mask.

of classification given a random set of data option is selected, see fig. 44 Using this function user can select the size of the dandom set to be used for classification, e.g. 100 probesets, and the number of samples retrived form the total data set used to perform this analysis, eg. 1000. Subsequently the user will select a classification method, e.g. PAMR, and the function will calculate the frequence of correct classification of the random set size defined by the user. This funcitn is particularly useful if user is interested to evaluate the specificity of a specific transcription signature.

12.0.4 PAMR

If PAMR method is selected, 2-3 steps are performed and pop-up info messages allow to check the resulting plots. Initially the cross-validated misclassification error curves are calculated, fig. 45, and shown in the main R window. Then, user defines a shrinking threshold and if the number of selected probe sets is below 50 the centroids will be plotted, fig. 46.

Subsequently the classification performance of the selected sub group of probe sets will be shown as plot and as text in the R window , fig. 47.

Results are also available as numerica values in the R window:

	<i>neg</i>	<i>pos</i>	<i>Class</i>	<i>Error rate</i>
<i>neg</i>	23	5		0.1785714
<i>pos</i>	2	48		0.0400000

If the results are satisfying user can save the probe sets defined by this analysis, fig. 48.

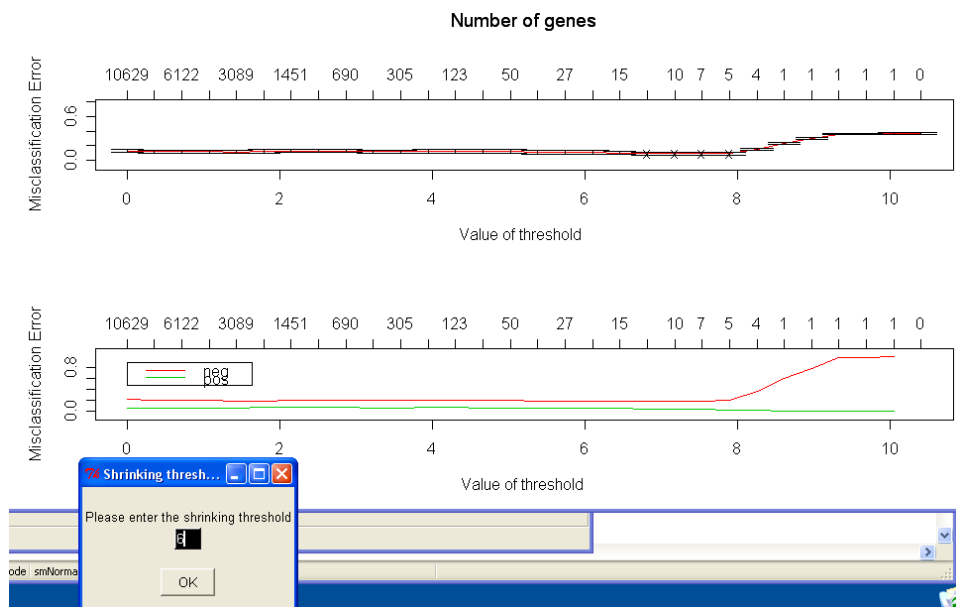


Figure 45: Cross-validated misclassification error curves

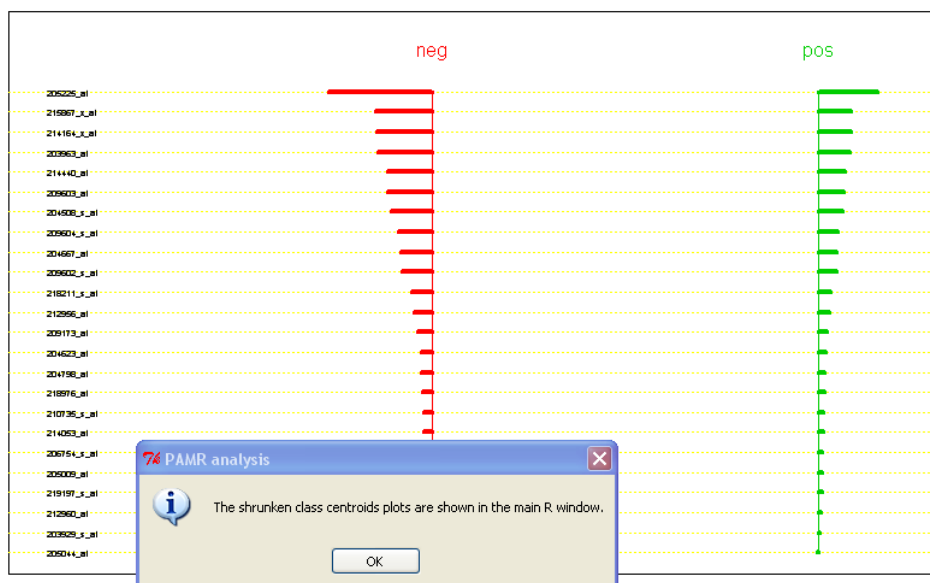


Figure 46: Shrunk class centroids.

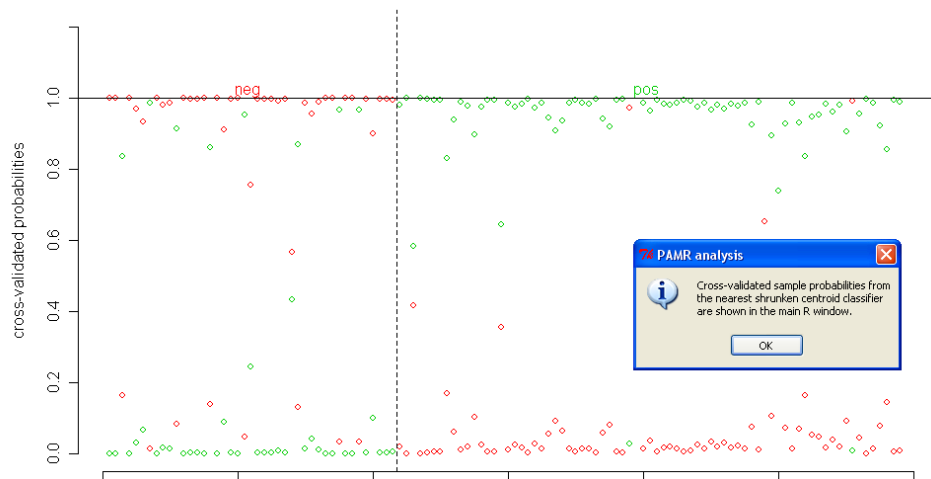


Figure 47: Cross-validated sample probabilities.

7x List of genes that survive the thresholding			
	id	neg-score	pos-score
1	205225_at	-0.5366	0.3077
2	215867_x_at	-0.2947	0.169
3	214164_x_at	-0.2878	0.165
4	203963_at	-0.2822	0.1618
5	214440_at	-0.2335	0.1339
6	209603_at	-0.2302	0.132
7	204508_s_at	-0.2119	0.1215
8	209604_s_at	-0.176	0.1009
9	204667_at	-0.1628	0.0933
10	209602_s_at	-0.1607	0.0921
11	218211_s_at	-0.1075	0.0616
12	212956_at	-0.0968	0.0555
13	209173_at	-0.0799	0.0458
14	204623_at	-0.0595	0.0341
15	204798_at	-0.0567	0.0325
16	218976_at	-0.0488	0.028
17	210735_s at	-0.047	0.027

Figure 48: Probe sets to be use as classifier.

Furthermore, if the test set was created it will be possible to check the ability of the selected sub set of genes to separate the classes under analysis using a hierarchical clustering, fig. 49.

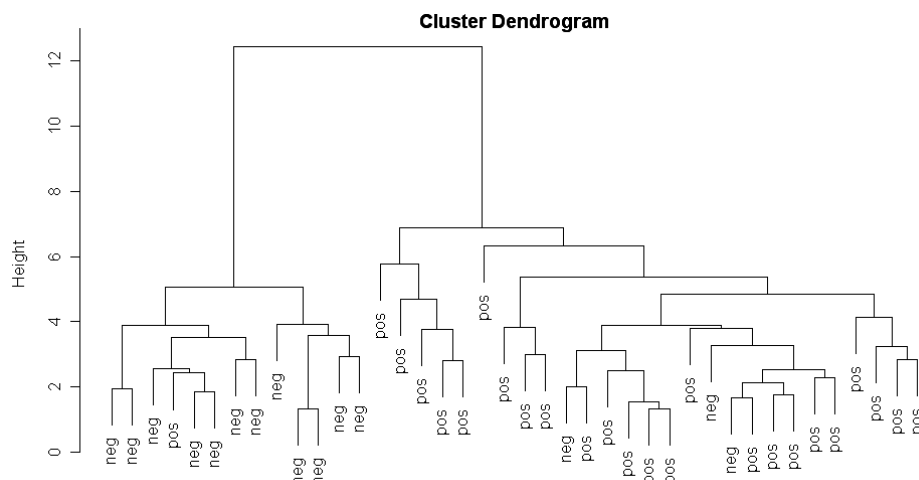


Figure 49: Testing the efficacy of the classifier on the test set by HCL.

12.0.5 PCA

The PCA visualization method offers the possibility to see how the data set can be grouped on the basis of the used clinical/experimental parameter under analysis, fig. 50.

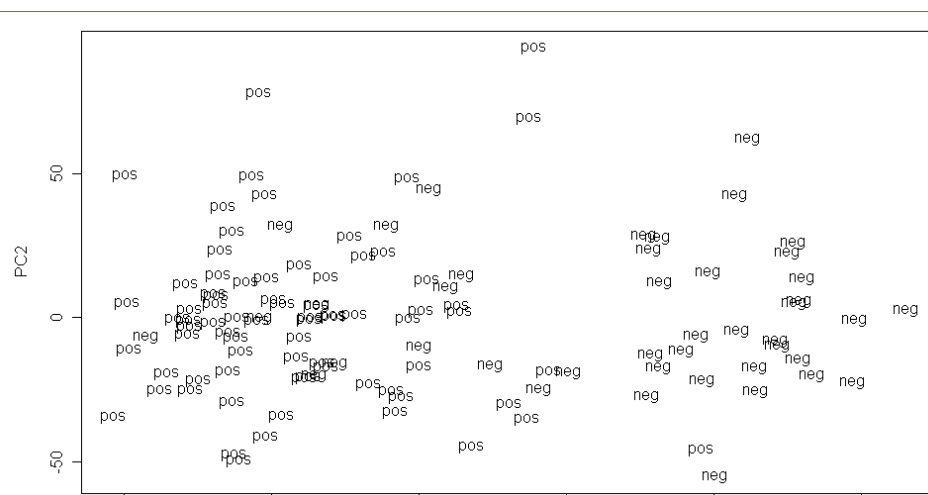


Figure 50: 1st and 2nd principal components space.

12.0.6 PDMCLASS

The PDMCLASS module allows the selection of different type of classification procedures, fig. 51.

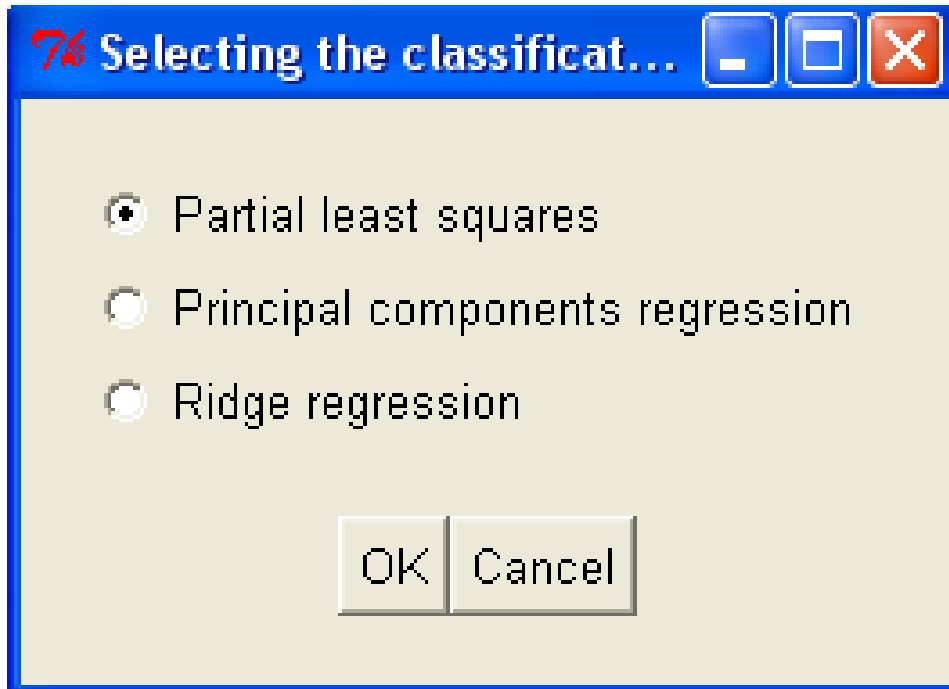


Figure 51: PDMCLASS methods selection mask.

The analysis will produce a numerical output of the efficacy of the dataset as classifier:

```
object neg pos
neg 21 1
pos 22 74
attr(,"error")
[1] 0.1949153
```

It is notable that this part of the analysis could take some minutes depending on the data set under analysis and the machine used for the analysis. Subsequently it will be possible to select the probe sets that have the greatest influence in differentiating sample classes. To do it, user will be requested to select the number of top ranked probe sets and the number of permutations to be used for the cross-validation. Probe set will be shown in a TK/TCL table with their probabilities to be able to discriminate between classes:

	<i>pos vs neg</i>
209604_s_at	1
202088_at	0.92
218807_at	0.8
211430_s_at	0.56
205081_at	0.48
213693_s_at	0.4
209138_x_at	0.44
200670_at	0.32
212099_at	0.44
208682_s_at	0.28

These results could be saved as a tab delimited file.

13 Biological Interpretation

This section gives a graphical interface to the GOstats package and it allows the preparation of template A for IPA analysis on <http://www.ingenuity.com>, fig. 52. It also allows very basic meta-analysis using the metaArray package.

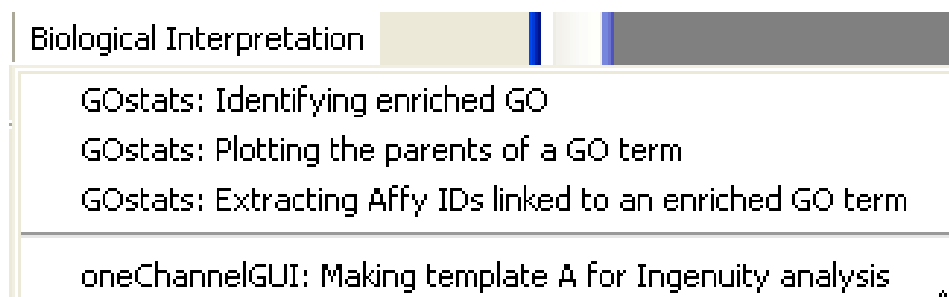


Figure 52: Biological interpretation menu.

13.1 Identifying enriched GO terms and related issues

This function is also available for gene level exon array analysis. Specific annotation libraries are not available for exon arrays, yet. Therefore, to perform this analysis we use the annotation informations embedded in oneChannelGUI and link the accession ids available in this annotation to Entrez Gene ids using the humanLLMappings, mouseLLMappings and ratLLMappings available in Bioconductor. The function *oneChannelGUI: Identifying enriched GO terms* searches for the presence of enriched GO terms within a set of differentially expressed probe sets, given a certain probe set universe, i.e. the array data available in Normalized Affy Data. For more information

about GO enrichment please refer to the GOstats vignette in the oneChannelGUI help menu. The user needs to select some parameters using a selection mask, fig. 53.

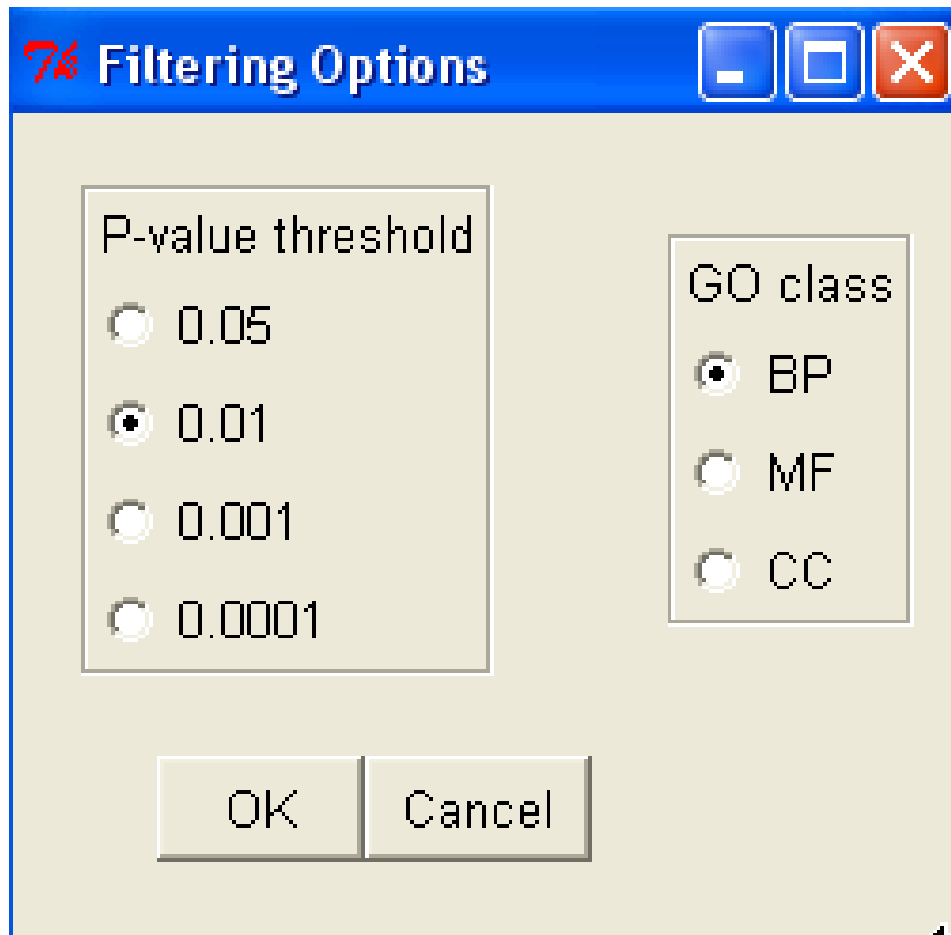


Figure 53: GO terms enrichment parameters selection mask.

Subsequently, the user will be requested to select a list of differentially expressed probe sets, saved in a txt file. The file should contain only a list of probe set separated by carriage return, without header:

```
1452968_at
1448228_at
1418028_at
1439113_at
1424338_at
1416503_at
1416371_at
1437165_a_at
```

1451047_at
1434005_at
1421916_at
1457012_at
1443823_s_at
1429379_at
1416168_at
1429974_at
1416121_at
1421917_at
1416405_at

The analysis could require quite a lot of RAM and when it is finished a message summarizing the results pops up, fig. 54.

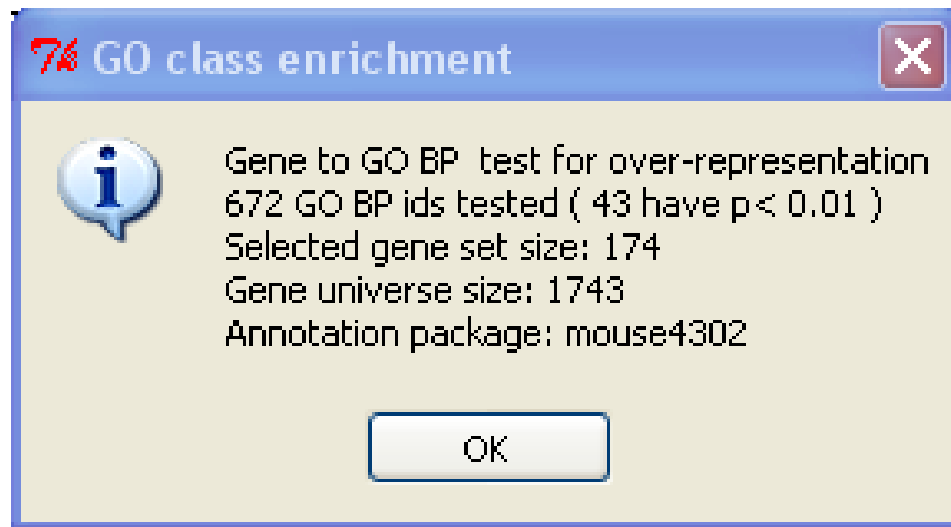


Figure 54: GO enrichment results summary message.

A table with the enriched GO terms will be then shown and it could be saved as tab delimited file, fig. 55.

In the main R window it will be possible to see a plot summarizing the GO terms relations existing between the enriched GO terms, fig. 56. Plotting results depends on the availability in your system of graphviz software and Rgraphviz package. Their installation can be quite critical for windows users, please contact the oneChannelGUI maintainer if you get problem in their installation or search over the Bioconductor mailing list.

It is also possible to highlight parents of a specific GO term using the function *Plotting parents of a GO term*. In this case a dialog will be used to pass to the function the GO

74 GO enriched classes using p-value= 0.01 annotation lib= mouse4302 GO class= BP

GOBPID	Pvalue	OddsRatio	ExpCount	Count
GO:0006817	GO:0006817	1e-09	21	13
GO:0006820	GO:0006820	2.2e-08	9.8	15
GO:0015698	GO:0015698	1.2e-07	9.1	14
GO:0007155	GO:0007155	7.8e-07	3.4	29
GO:0048513	GO:0048513	2.6e-05	2.5	35
GO:0006811	GO:0006811	2.8e-05	3.2	22
GO:0001568	GO:0001568	0.00012	4.3	13
GO:0009607	GO:0009607	0.00014	3.7	15
GO:0001944	GO:0001944	0.00016	4.1	13
GO:0006952	GO:0006952	0.00034	3.5	14
GO:0051707	GO:0051707			
GO:0001525	GO:0001525			
GO:0030334	GO:0030334			
GO:0045765	GO:0045765			
GO:0009611	GO:0009611			
GO:0048514	GO:0048514			
GO:0006955	GO:0006955			
GO:0007596	GO:0007596	0.0016	9.3	5
GO:0007599	GO:0007599	0.0016	9.3	5
GO:0050878	GO:0050878	0.0016	9.3	5
GO:0050817	GO:0050817	0.0016	9.3	5
GO:0051270	GO:0051270	0.0016	5.4	7

74 Plotting GO parents

Plot of GO term parents will be displayed on the main R window

OK

Figure 55: Enriched GO terms table.

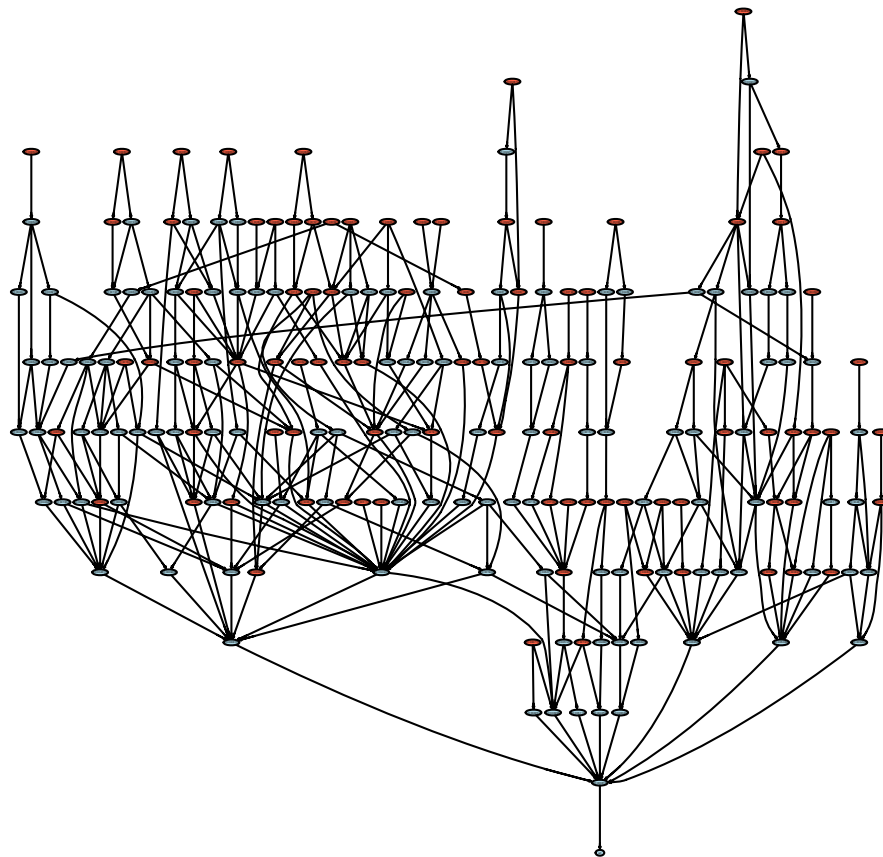


Figure 56: Relations between enriched GO terms. Enriched GO terms, red, others, light blue.

term, e.g. GO:0001525. Subsequently after selecting the GO class, i.e. BP, MF or CC, the results will be available in the main R window, fig. 57.

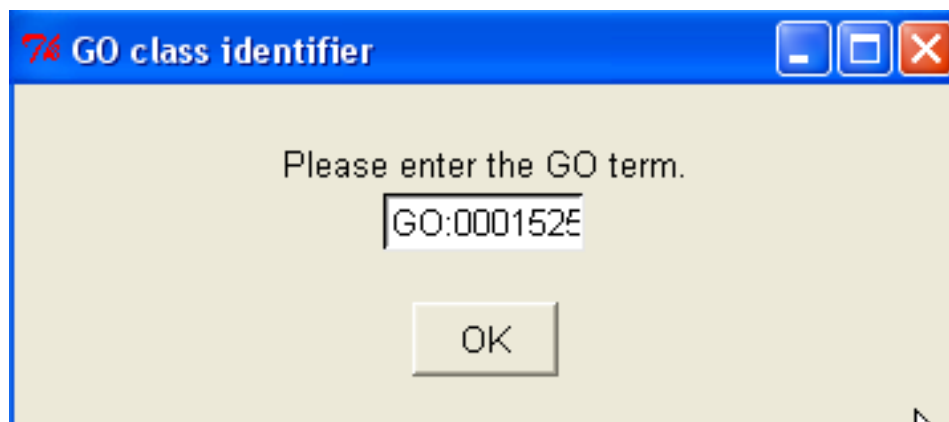


Figure 57: Plotting GO term parents.

It is also possible to annotate and save, in an html file, the subset of differentially expressed probe sets associated to a specific enriched GO term using the function *oneChannelGUI: Extracting Affy IDs linked to an enriched GO term*. In the case exon arrays are used with *oneChannelGUI: Extracting Affy IDs linked to an enriched GO term* function the output file is a tab delimited file with the available annotations instead of an HTML file. The user will be requested to select the GO term of interest, fig. 56, and subsequently to open the file list of differentially expressed probe sets used for the GO enrichment analysis. A pop-up message will indicate when the annotation table will be ready to be saved in an HTML file, fig. 58. The output for exon arrays will be instead a tab delimited file.

13.2 Biological Interpretation

13.2.1 Exon arrays

In case EXON 1.0 arrays are loaded into Biological Interpretation menu contains the following functions: *oneChannelGUI: Attaching ACC and Entrez Gene IDs to Probe set IDs (EXON 1.0 ST)* which allows to associate EG ids to gene-level probe sets.

13.2.2 meta-analysis

This menu gives also access to some meta-analysis tools, fig. 59.

It is possible to merge to the NormalizedAffyData up to 3 other data sets characterized by having the same ids and the same order of the NormalizedAffyData ids. To merge the data sets it is necessary a tab delimited file and a target for each data set. Integrative correlation (Parmignani et al. 2004), implemented in the metaArray

Differentially expressed probe sets in GO:0001525

Probe	Symbol	Description	Function	Chromosome	Chromosome Location	GenBank	LocusLink	Cytoband	UniGene	PubMed	Gene Ontology
1416238_at	Tie1	tyrosine kinase receptor 1		4	-117969122	NM_011587	21846	4 D2.1 4 50.0 cM	Mm.4345	62	nucleotide binding protein kinase activity protein serine/threonine kinase activity protein-tyrosine kinase activity receptor activity ATP binding extracellular space protein amino acid phosphorylation membrane integral to membrane kinase activity negative regulation of angiogenesis transferase activity negative regulation of cell migration

Figure 58: Annotation file for a subset of differentially expressed probe sets linked to GO:0001525 BP enriched term.

Biological Interpretation
oneChannelGUI: Identifying enriched GO terms
oneChannelGUI: Plotting the parents of a GO term
oneChannelGUI: Extracting Affy IDs linked to an enriched GO term
oneChannelGUI: Making template A for Ingenuity analysis
oneChannelGUI: Merging the same set of probe sets from different data sets
oneChannelGUI: Mining similarities/dissimilarities between merged data sets (IC)

Figure 59: Biological Interpretation DEVEL version.

package, can be accessed with the function *Mining similarities/dissimilarities between merged data sets (IC)*. The function produces an histogram of the various comparisons and it saves, in tab delimited file, the IC values for the various comparisons.

14 General tools

This section allows the use of some functions which are not part of a specific Bioconductor package but could be of general use. The function *oneChannelGUI: Update all Bioconductor libraries* allows to run a on-line update of all the Bioconductor libraries present in the system. This function is very useful to keep updated for bugs correction during the 6 months life of a Bioconductor release. The function *oneChannelGUI: Extract a column from a tab delimited file* allows the extraction of any of the columns of a tab delimited file. This function is particularly useful to generate probeset ids list to be used for Venn diagram representation. The function *oneChannelGUI: Filtering a tab delimited file* allows to subset a tab delimited file given a list of values, e.g. values, symbols, probe sets, etc., present in a file where each value is separated from the others by carriage return. The tab delimited file subsetting is performed on the basis of the column, fig. 60 yellow, sharing the same header of the list of values, fig. 60.

	A	B	C	D	E	F	G	H	
1	AffyID	EG	Symbol	M	A	t	P.Value	B	
2	1452968_e	68588	Cthrc1	2.94535	6.280509	29.36407	3.73E-09	19.09287	
3	1448228_e	16948	Lox	3.362383	5.198592	28.75112	3.73E-09	18.86776	
4	1418028_e	13190	Dct	-3.03019	7.065521	-26.5272	6.42E-09	17.99636	
5	1439113_e	77114	6030426L1	2.046895	6.247831	24.33392	1.08E-08	17.04304	
6	1424338_e	14412	Slc6a13	3.278416	5.598225	24.11259	1.08E-08	16.94107	
7	1416503_e	17035	Lxn	2.411422	9.176315	23.92397	1.08E-08	16.85329	
8	1416371_e	11815	Apod	2.266252	7.631505	23.40282	1.20E-08	16.6064	
9	1437165_e	18542	Pcolce	2.490832	5.3099	21.41889	2.58E-08	15.60399	
10	1451047_e	16431	Itm2a	1.576752	9.02295	21.18512	2.58E-08	15.47887	
11	1434005_e	56878	Rbms1	1.716531	6.752608	21.16227	2.58E-08	15.46656	
12	1421916_e	18595	Pdgfra	2.348468	5.370426	20.90527	2.58E-08	15.32701	
13	1457012_e	13172	Dbx1	3.548188	6.274709	20.81448	2.58E-08	15.27724	
14	1443823_s	98660	Atp1a2	1.588681	8.180743	20.75586	2.58E-08	15.24499	
15	1429379_e	114332	Xlkd1	3.989934	4.626859	20.65556	2.58E-08	15.18955	
16	1416168_e	20317	Serpinf1	3.893176	5.396654	20.42969	2.74E-08	15.06359	
17	1429974_e	76365	Tbx18	3.946038	5.021675	19.77173	3.76E-08	14.68763	
18	1416121_e	16948	Lox	3.663294	5.733764	19.57311	3.86E-08	14.57141	
19	1421917_e	18595	Pdgfra	3.345095	6.658905	19.44049	3.86E-08	14.49308	
20	1416405_e	12111	Bgn	1.910941	7.617915	19.437	3.86E-08	14.49101	
21	1449368_e	13179	Dcn	3.441702	8.380267	19.2456	4.11E-08	14.3769	
22	1416431_e	67951	Tubb6	1.365443	6.948872	18.92398	4.76E-08	14.18233	

Figure 60: Sub setting a tab delimited file by a list of symbols.

The function *oneChannelGUI: Downloading Gene/Exon library files* allows to download all the library files needed to use APT tools for probe set summaries for Gene and Exon 1.0 ST arrays. The function *oneChannelGUI: Set Affymetrix apt tools folder and*

download Reference Sequences allows the user to define a folder where apt tools were installed and to download in the subdir blast of the apt dir the reference sequences from NCBI repository. Those file are compressed and have the gz extension. They can be unpacked manually by the user or via oneChannelGUI. This option is available at the end of the downloading but takes quite a long time.

The function *oneChannelGUI: deleteLocalData* will reset the folders defined by *oneChannelGUI: Downloading Gene/Exon library files* and *oneChannelGUI: Downloading Gene/Exon library files*. Data present in the two folders will not be deleted! The function *oneChannelGUI: buildingLocalAnnotation* allows to update the internal oneChannelGUI gene/exon-level annotations quiering netaffx database using the affyCompatible library. Annotation files are saved in .rda format in the subdir data in located in the oneChannelGUI folder. Windows users need to drag those .rda files in the Rdata.zip file present in the data dir. A file called netaffxUpdates.txt in the etc subdir kept tracks of annotation file updating. The function *oneChannelGUI: variantExons* allows to update the internal oneChannelGUI gene/exon-level annotations that links exon-level probesets to exons which are associated only to a subset of transcript isoforms. The table is generated integrating the exon-level annotation derived by the core subset of Affymetrix exon level annotation and a USCS table mapping the location of variant exons on the genome. The function *Installing meV clustering software* will install the meV clustering tool. A java application very powerful for microarray data clustering. meV software can be started using the function *Starting meV clustering software* in the Biological Interpretation menu. The function *Information on external software and data connected to oneChannelGUI* provides information on external software installed and connected to oneChannelGUI

15 Help

This menu allows to acces to the vignettes of the Bioconductor packages implemented in oneChannelGUI and to this oneChannelGUI vignette.

16 Exon analysis and data mining

Exon analysis menu allows a certain number of functions to identify and visualize alternative splicing events. The part related to loading gene/exon level data is described in the File menu chapter. If APT tools are used to calculate probe set intensities in oneChannelGUI will be available gene level expression data in Normalized Affy Data, exon level expression data in Normalized Exon data and, if selected, DABG p-values.

The functions actually available for exon analysis are summarised in fig. 61.

Splice Index (SI), which represents the exon expression normalized with respect to the transcript expression, can be calculated with *oneChannelGUI: Calculating splice index*. Before SI calculation it is possible to correct gene/exon-levels intensity signals for probe sequence effect: *oneChannelGUI: Correcting intensity signals using COSIE*

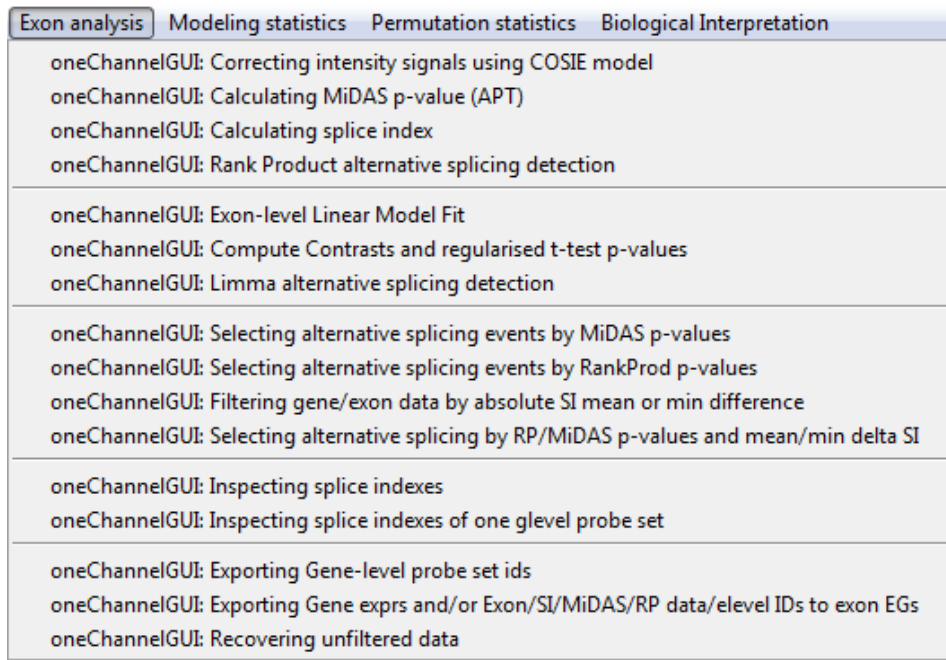


Figure 61: Exon menu.

model. This function is a wrapper to the function developed by Gaidatzis et al. (Nucleic Acids Research, 2009). They have analyzed exon array data from many different human and mouse tissues and have uncovered a systematic relationship between transcript fold change and alternative splicing as reported by the splicing index. Evidence from dilution experiments and deep sequencing suggest that this effect is of technical rather than biological origin and that it is driven by sequence features of the probes. This effect is substantial and results in a 12-fold overestimation of alternative splicing events in genes that are differentially expressed. They have developed a R function called COSIE (Corrected Splicing Indices for Exon arrays) that for any given set of new exon array experiments, core and full exons set for human and mouse, corrects for the observed bias and improves the detection of alternative splicing.

Starting from the work of Shah and Pallas work BMC Bioinformatics. 2009 Jan 20;10:26. Limma routines available for gene-level analysis were also implemented at exon-level to detect alternative splicing events. It is notable that it is possible in many cases to apply BH type I error correction at exon-level. The limma analysis is performed at intensity level, therefore it will not discriminate between exon-level probe sets alternative spliced or belonging to a differentially expressed gene. Although this limitation is present we did not applied limma analysis as Shah and Pallas to SI because there are no evidences that SI variance will be independent by the gene it belong to. The analysis steps are very similar to gene level: *oneChannelGUI: Exon-level Linear Model Fit* will fit the linear model. Then *oneChannelGUI: Compute Contrasts and regularised*

t-test p-values will compute the contrasts and the regularised t-test p-values. Then *oneChannelGUI: Limma alternative splicing detection* will produce a file containing the exon-level ids of the alternative spliced exons and will filter the gene and exon-level data to retain only the genes affected by alternative splicing events.

For a two group experiment the function *oneChannelGUI: Calculating MiDAS p-value (APT)* uses APT tools to calculate MiDAS p-values for the difference between SIs in the two conditions, i.e. alternative splicing events. It is possible to subset gene/exon level data on the basis of a MiDAS p-value threshold using the function *oneChannelGUI: Selecting alternative splicing events by MiDAS p-values*. We have also applied the rank product method (RankProd package) *oneChannelGUI: Rank Product alternative splicing detection (devel)* to detect significant differences between SI or exon-level $\log_2(\text{intensities})$ in two experimental conditions, i.e. alternative splicing events. Rank Product is a non-parametric statistic that detects items that are consistently highly ranked in a number of lists. It is based on the assumption that under the null hypothesis that the order of all items is random the probability of finding a specific item among the top r of n items in a list is $p = \frac{r}{n}$. Multiplying these probabilities leads to the definition of the rank product $RP = \prod_i \frac{r_i}{n_i}$, where r_i is the rank of the item in the i -th list and n_i is the total number of items in the i -th list. The smaller the RP value, the smaller the probability that the observed placement of the item at the top of the lists is due to chance. Due to performance reasons on windows based computers, the number of random permutations is fixed to 100, a menu to select the number of permutations will be implemented soon. At the end of the analysis p-values of class 1 < class2 and p-values of class 1 > class2 and average SI difference histograms are shown in the main R window.

IMPORTANT All filtering functions devoted to the selection of alternative spliced events produce a flat file containing only the ids of the detected spliced exons.

It is possible to subset gene/exon level data on the basis of rank product results using the function *oneChannelGUI: Selecting alternative splicing events by RankProd p-values*. It is also possible to filter data on the basis of the average mean or min SI difference with the function *oneChannelGUI: Filtering gene/exon data by absolute SI mean or min difference*

It is also possible to filter exon data integrating midas p-values with RP p-values and average mean SI difference. This option is given by the function *oneChannelGUI: Selecting alternative splicing by RP/MiDAS p-values/average mean SI difference* Since no correction for statistical type I error is given for MiDAS we decided to use the integration of two statistical tests based on different approaches to reduce statistical type I errors. Furthermore, in this filter is integrated also the possibility to subset data on the basis of a average mean SI difference threshold. Visualization of the splicing events using one gene-level probe set at a time is possible with the function

In the Biological Interpretation menu, fig. 62, the function *oneChannelGUI: Associating alternative spliced exon-level probe set to variant exons* a set of spliced probe sets can be associated on the basis of the variant exons mapped on the UCSC genome

browser.

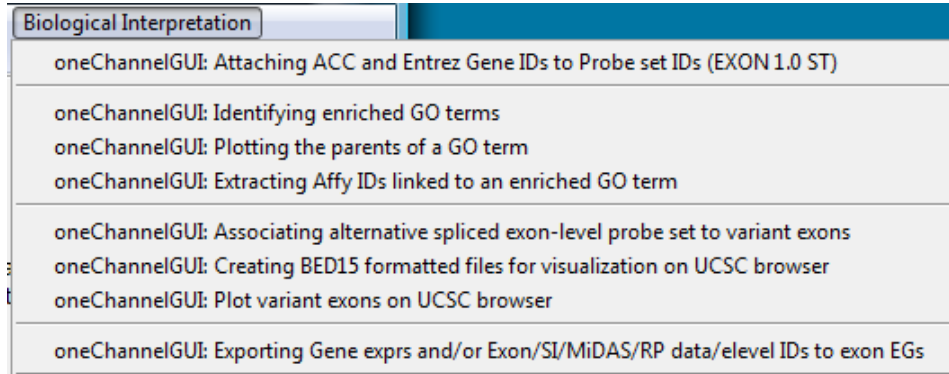


Figure 62: Biological Interpretation menu for exon arrays

Variant exons are those exons that are associated only to a subgroup of the available isoforms mapped on a gene. This association is very useful if researcher is not interested to select splicing events associated to exons conserved over all isoforms associated to a specific gene. The structure of the file is shown in fig. fig. 63

The tab delimited file produced by this filtering can be visualized on the UCSC genome browser, using two functions: *oneChannelGUI: Creating BED15 formatted files for visualization on UCSC browser* creates for each chromosome a BED15 formatted file, which allows the visualization of microarray data on the UCSC genome browser. Files need to be uploaded on the browser as shown in fig. 64,65

Exon-level feature are visualized as red colour in case of insertion and in green colour in case of skipping. Colour range is limited ranges between 3 and -3 deltaSI. Therefore colour for a deltaSI of 3 and 4 will be the same but the true deltaSI is also inserted as part of exon-level probeset name.

oneChannelGUI: Plot variant exons on UCSC browser takes advantage of the rtrack-layer and plots directly on the UCSC genome browser from R. The output might take some time to be plotted on the default web browser.

The behaviour of exon-level probe sets, within a gene characterized by the presence of alternative splicing event can be inspected using two functions: *oneChannelGUI: Inspecting splice indexes of one glevel probe set* and *oneChannelGUI: Inspecting splice indexes*. The first function produces a pdf as the second one allows the analysis of gene-level probeset at a time. The structure of the output of these function is shown in fig. 66,

The function *oneChannelGUI: Inspecting splice indexes*, fig. 61. produces a pdf and txt file as output containing gene level probeset id and exon level probeset ids for all spliced exon in the following format:

```
"glevel id/exon level ids "  
"3899173/3899229"
```

C	D	E	F	G	H	I	J	K	L	M
affyend	affywidth	affystrand	vspname	vspstart	vspend	vspwidth	vspstrand	chr	genome	exon.fc
65658967	62	+	NST0000037104	65658901	65659011	111	+	1	=Human&vers	0.476112
65658967	62	+	NST0000037104	65658957	65659011	55	+	1	=Human&vers	0.476112
67222999	95	+	NST0000040104	67222842	67223955	1114	+	1	=Human&vers	0.450294
67222999	95	+	NST0000035769	67222842	67226302	3461	+	1	=Human&vers	0.450294
90265838	338	+	NST0000037044	90265500	90266685	1186	+	1	=Human&vers	0.910732
168031362	162	+	NST0000028603	168031173	168031748	576	+	1	=Human&vers	0.354134
226615373	126	+	NST0000028454	226613920	226615573	1654	+	1	=Human&vers	0.93415
15723282	131	-	NST0000037585	15723150	15723527	378	-	1	=Human&vers	0.472062
56817402	40	-	NST0000037125	56817138	56817845	708	-	1	=Human&vers	0.931792
150222342	36	-	NST0000036881	150222010	150222424	415	-	1	=Human&vers	0.400616
150222342	36	-	NST0000036881	150222017	150222424	408	-	1	=Human&vers	0.400616
154936307	235	-	NST0000036822	154936033	154936492	460	-	1	=Human&vers	1.424034
154936307	235	-	NST0000036822	154936171	154936492	322	-	1	=Human&vers	1.424034
154941905	27	-	NST0000036822	154941792	154941919	128	-	1	=Human&vers	0.858686
154941905	27	-	NST0000036822	154941792	154941962	171	-	1	=Human&vers	0.858686
154941905	27	-	NST0000036822	154941792	154941999	208	-	1	=Human&vers	0.858686

Figure 63: The columns present in the tab delimited file are the following: affyname: exon-level probe set, affystart: start position of PSR from Affymetrix annotation, affyend: end position of PSR from Affymetrix annotation, affywidth: width of the PSR, affystrand: stand of the PSR, vspname: ESEMBL transcript overlapping to the PSR, vspstart: start of the ESEMBL transcript, vspend: end of the ESEMBL transcript, vspwidth: width of the ESEMBL transcript, vspstrand: strand of the ESEMBL transcript, chr: chromosome location, genome: genome release used for the mapping, exon.fc: delta Splice Index, i.e. the fold change variation between the expression of the two experimental conditions for the signals normalized for the gene expression level.

"3210737/3358127"
"3358112/3234972"
"2587961/3267416"
"3644510/3357399/3357446"
"3415109/2759224"
"3611625/3308001/3308013/3308031"
"3234760/3308001/3308013/3308031"
"3267382/3308001/3308013/3308031"
"3357397/2455983/2455993/2456013"
"2759205/2455983/2455993/2456013"
"3307939/3733603/3733609"

Exon-level data can be saved using the function *oneChannelGUI: Exporting Gene exprs and/or Exon/SI/MiDAS/RP data/elevel IDs to exon EGs*

16.1 Plotting single splicing event

It is possible to plot average intensity signals over the genes and transcripts structure to identify a specific splicing event. This can be done using the function *oneChannelGUI: Plotting a splicing event* located in the exon menu. User has to pass to the function the

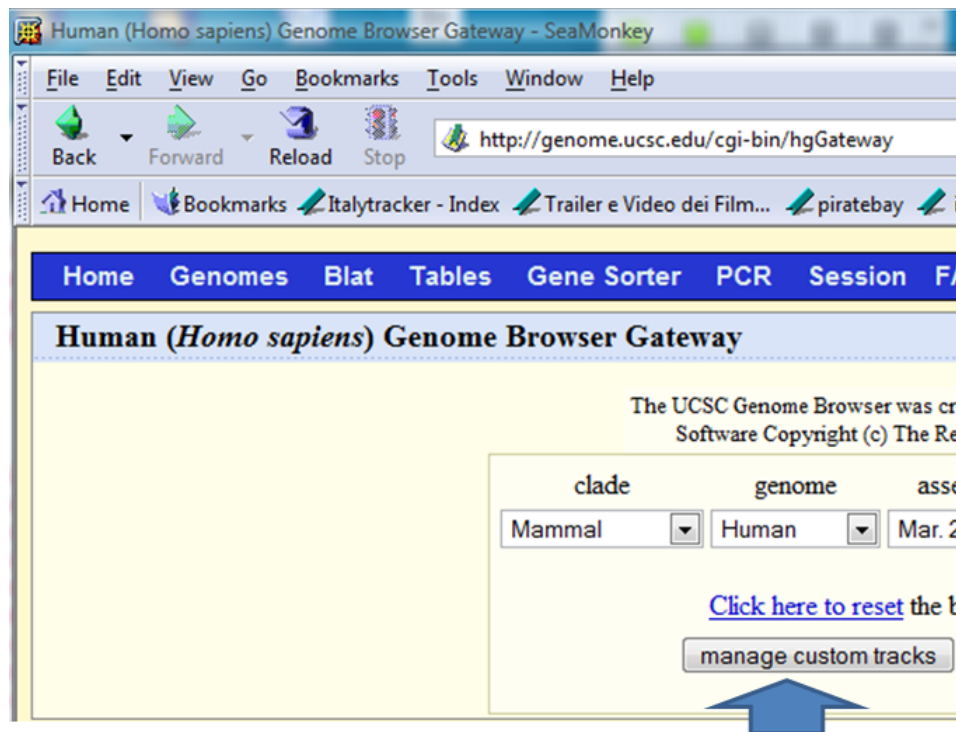


Figure 64: How to handle BED15 formatted files on UCSC genome browser, first step.

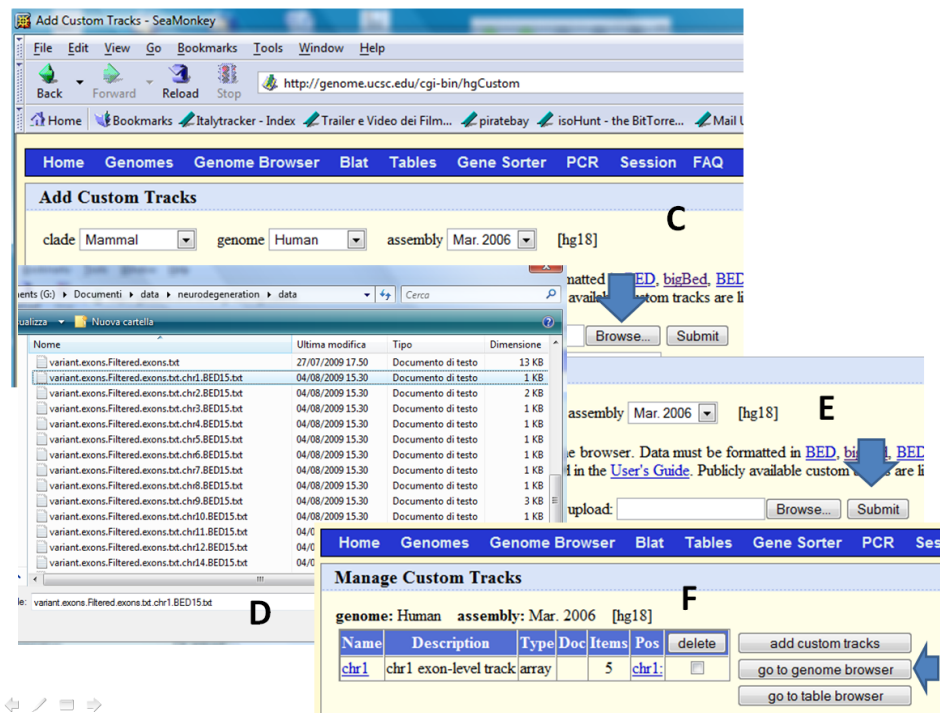


Figure 65: How to handle BED15 formatted files on UCSC genome browser, second step.

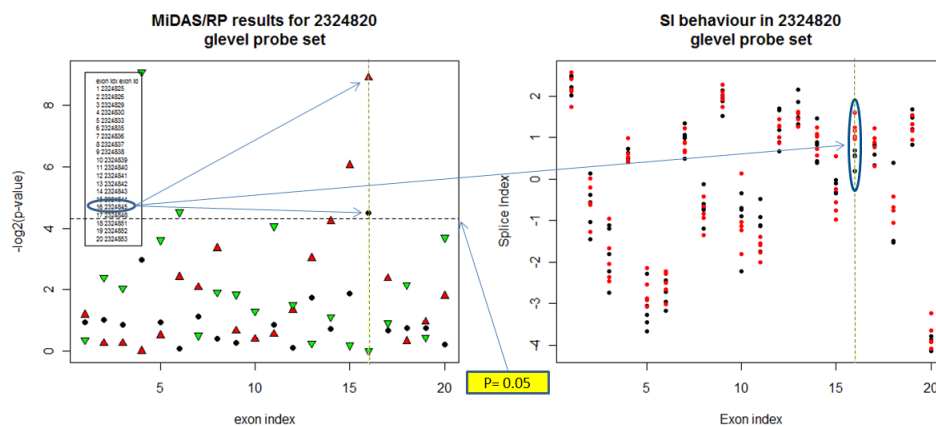


Figure 66: Example of the output of the putative alternative splicing inspection. The output is made of a tab delimited file where glevelevel probe sets associated to elevel probe sets and of a pdf file where each page is made of a plot of MiDAS/RP p-values with respect to exon index (black dot MiDAS, red triangle and green triangle RP). The horizontal black dashed line indicates a p-value of 0.05. The vertical yellow dashed line indicates a condition in which both MiDAS and RP p-values are below 0.05 value. In the second plot, it is shown the behaviour of splice indexes with respect to exon indexes. The vertical yellow dashed lines indicate those exon-level $\log_2(\text{intensity})/\text{SI}$ associated to MiDAS and RP p-values below 0.05 value.

exon-level probeset id for the spliced exon. This is used to retrieve gene-level information as well as the other exon-levels data. Using the package GenomeGraphs gene and transcript chromosomal structures are retrieved from ENSEMBL. In case the chromosome location data are associated to obsolete exon-level probeset data. These data can be provided externally using a file with the structure shown in fig. 67

EPROBESETID	GPROBESETID	CHR	START	STOP	STRAND	ANNLEVEL	SCORE
3804147	3804143	chr18	33569794	33571808	-	core	1000
3804148	3804143	chr18	33569794	33571808	-	core	1000
3804149	3804143	chr18	33569794	33571808	-	core	1000
3804150	3804143	chr18	33569794	33571808	-	core	1000
3804151	3804143	chr18	33571818	33573263	-	core	1000
3804152	3804143	chr18	33571818	33573263	-	core	1000
3804153	3804143	chr18	33571818	33573263	-	core	1000
3804170	3804143	chr18	33605561	33606838	-	core	1000
3804172	3804143	chr18	33606863	33607038	-	core	1000
3804173	3804143	chr18	33607147	33608143	-	core	1000
3804176	3804143	chr18	33610771	33610857	-	core	1000
3804177	3804143	chr18	33610954	33611060	-	core	1000
3804178	3804143	chr18	33613671	33613800	-	core	1000
3804180	3804143	chr18	33620770	33620812	-	core	1000
3804188	3804143	chr18	33647166	33647442	-	core	1000
3804189	3804143	chr18	33647166	33647442	-	core	1000

Figure 67: Example of the file structure describing chromosomal information for exon-level probesets. A file with this structure can be provided to the *oneChannelGUI: Plotting a splicing event* when user does not want to use annotation data available internally in oneChannelGUI.