Single Object Profiles Regression Analysis - SOPRA

Project description

Authors: André P. Maeurer, Klaus-Peter Pleissner

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1 Introduction

The analysis of high-content data, such as cell-based feature data derived from image analysis from RNAi screens or FACS data poses an important research field in the genomic area.

The Single Object Profiles Regression Analysis (SOPRA) (Fig.1) workflow enables researchers to identify cell populations with statistically significant changes of normalized frequency distribution profiles (histograms) of measured cellular features based on the regression analysis. The regression-based approach was performed using maSigPro from Bioconductor, an R-package originally applied for time-course microarray analysis. We defined a regression model where the dependent variable was the bin-wise normalized frequency distribution profile and the independent variable was the measured cellular feature within predefined binning intervals. Our experimental design was based on the single series time-course approach of maSigPro. Shortly, maSigPro follows a two steps regression strategy to find genes with significant temporal expression changes and significant differences between experimental groups. The method defines a general regression model for the data where the experimental groups are identified by dummy variables. The procedure first adjusts this global model by the least-squared technique to identify differentially expressed genes and selects significant genes applying false discovery rate control procedures. Secondly, stepwise regression is applied as a variable selection strategy to study differences between experimental groups and to find statistically significant different profiles. The coefficients obtained in this second regression model will be useful to cluster together significant genes with similar expression patterns and to visualize the results. These explanations are given in the maSigPro user's guide.
The workflow can easily be run on analyzed images or FACS data from thousands of cell populations.

2 Description of software

The SOPRA software workflow (Fig.2) is realized as a shiny application with an user interface UI.R and application SERVER.R and mainly consists of four parts, (SOPRA 1 of 4) preprocessing, (SOPRA 2 of 4) data gathering and normalization, (SOPRA 3 of 4) identification of significantly changed cell populations and clustering and (SOPRA 4 of 4) conversion of these significant

findings into genes with their cluster membership.

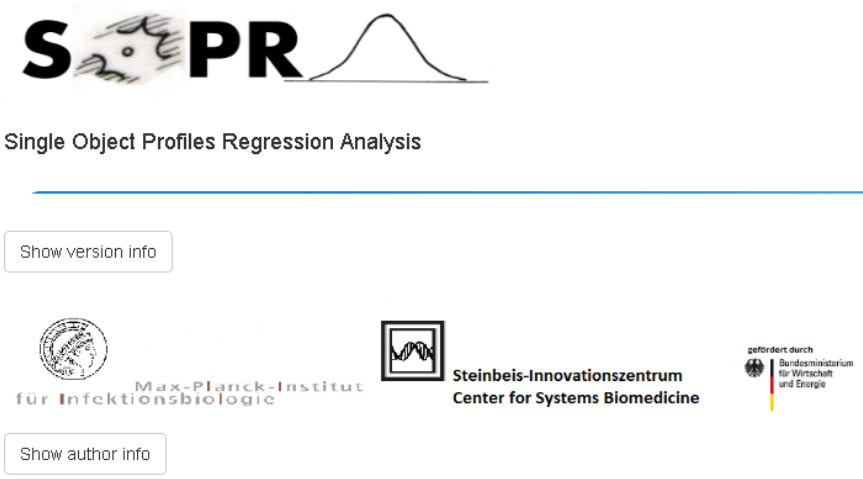


Fig.1 Header of the SOPRA User Interface

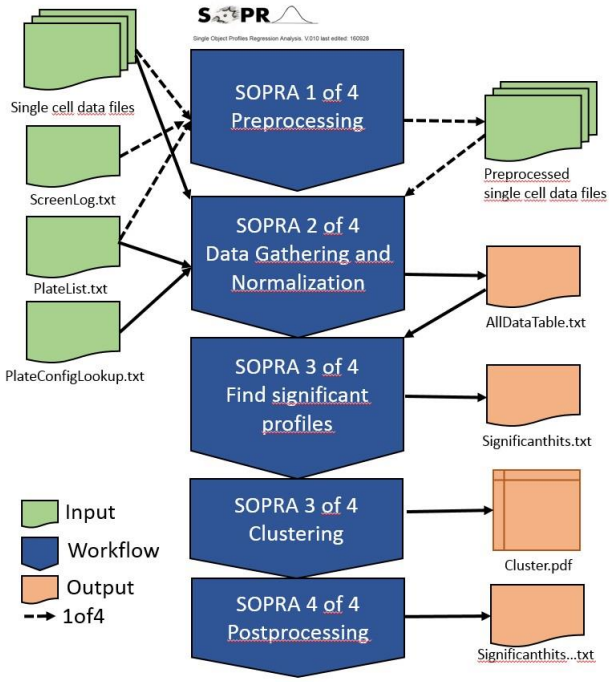


Fig.2 SOPRA workflow

2.1 Preprocessing

Due to technical or other reasons some wells can contain faulty information. Therefore, these wells must be excluded from further analysis. SOPRA 1 of 4 (Preprocessing) performs a preprocessing step, in which all features of the indicated incorrect wells are flagged by NA. The specified wells are listed in the manually-created 'ScreenLog' file (for instance: ScreenLog-384.txt).

2.2 Data Gathering and Normalization

SOPRA 2 of 4 (Data Gathering and Normalization) consists of several steps leading to normalized histograms, in which the original data values within a given interval (bin) are replaced by normalized density distribution (histogram).

First, all data is read according to the file 'PlateList.txt' and annotates all objects (single cell data) with the data from the file 'PlateConf_Lookup.txt'. INF-values are flagged and commas replaced with dotted decimal notation.

Several image analysis (such as SCAN^R) or FACS software programs offer the opportunity to flag objects if they are not in a specific gate. In a next (optional) step we use the flagging information stored in the data files and the 'PlateConf_LookUp.txt' and 'PlateList.txt' file to filter these objects. The 'PlateConf_LookUp.txt' file contains the column 'Filter' with the active filter combinations (e.g. F1 or F1&F2), the 'PlateList.txt' contains the names of the filters (e.g. the column for the Area gating has been given the name 'G_Area41726561' by SCAN^R). After this step, the data is exported as 'Data_rawdata_PlatexReplicatey.txt'.

Next, the quantiles and median raw values of the control wells are calculated and an object-wise median of control (moc) normalization for each plate (called: plate-wise) is performed. After this step, the data is exported as 'Data_nzdata_PlatexReplicatey.txt'.

Then, the binning-(interval)-axis is calculated for the complete data set.

Next, QC blots are exported for the normalized data.

After that, histograms based on the binning-axis are calculated for all sample and control wells, resulting in a certain number of objects (counts) for each bin of a well. For all sample and control wells, the counts of each bin are divided by the median count of the respective bin of the control wells to perform a 'bin-wise' normalization, leading to a log2 normalized frequency distribution (histogram) for all samples and controls. The larger the deviation of a sample from the reference (control) distribution, the more likely the test distribution is statistically significant.

Finally, the data is saved in the file 'AllDataTable.txt'.

2.3 Identify statistically significant frequency distribution profiles

SOPRA 3 of 4 (Find Statistically Significant Profiles) identifies log2 normalized

frequency distributions that are significantly altered compared to the control distributions, using the Bioconductor R-package ‘maSigPro’ in the single series time-course approach (for further details see the maSigPro user’s guide).

2.4 Post-processing

SOPRA 4 of 4 (siRNA to Gene Post Processing) converts the siRNA hit list into a list containing the gene symbols given in the PlateConfLookUp.txt table, the cluster membership, and the frequency in the corresponding cluster. Because post-processing makes always sense it is the final step of SOPRA 3 of 4 software part.

3 Input Sources

The single cell data files and the descriptive files for 384-well plates from our cell cycle screen can be downloaded from MPIIB cloud server [MPIIB cloud server](). .

The single cell data (Fig. 3) is stored in tab-delimited files. Each row contains the data for one cell (object). Each file must contain an ID and the corresponding well and at least one measured feature for each object. Therefore, the objects do not have to be in a chronological order. Optionally, if you want to use the gating filter, it can also contain several gating filters (e.g. 'G_Area41726561'; '1' = object inside gate, '0' = object outside gate).

We programmed an image analysis assay for the 'Scan^R' software (Olympus) to generate single object data. This tab-delimited data files can contain upto 900000 raws (objects) for one replicate of one plate and can be opened by EXCEL. The file structure looks as follows:

Object ID	Total Intensity DAPI	Mean Intensity DAPI	Area	Well	G_Area41726561	G_MeanDAPI4D65616E44415049	G_TotalDAPI546F74616C44415049
0	320989	840.28534	382	1	1	1	1
1	691633	1000.9161	691	1	1	1	1
2	317899	572.79102	555	1	1	1	1
3	820896	987.84113	831	1	1	1	1
4	352608	993.26196	355	1	1	1	1
5	381844	1243.7915	307	1	1	1	1
6	892706.94	739.60809	1207	1	1	1	1
7	361899	1052.032	344	1	1	1	1
8	729447	1146.9292	636	1	1	1	1

Fig.3 File structure for the data files (some columns are

blanked out).

The following tab-delimited descriptive files are needed for

the workflow.

- PlateList.txt

The file 'PlateList.txt' (Fig. 4) contains all necessary information about the screening experiment. For each feature to analyze a respective file has to be generated. It must contain the *well range* of the file, the *file name*, the *plate* and *replicate number* of the plate, the *control* and the *feature* to be analyzed (connected to a feature column of the single cell data files).
Optionally, you can use up to three cell gating filters (connected to a gating column of the single cell data files)

Platename	WellRange	ObjectType	Filename	Plate	Replicate	Control	Feature	Filter1	Filter2	Filter3
Plate001_R1	W1-384	Main	Plate001_R1_W1-384_Main.txt	1	1	NeutralControl1	Area	G_Nuclei4E75636C6569	G_Area41726561	NA
Plate001_R2	W1-384	Main	Plate001_R2_W1-384_Main.txt	1	2	NeutralControl1	Area	G_Nuclei4E75636C6569	G_Area41726561	NA
Plate001_R3	W1-384	Main	Plate001_R3_W1-384_Main.txt	1	3	NeutralControl1	Area	G_Nuclei4E75636C6569	G_Area41726561	NA
Plate001_R4	W1-384	Main	Plate001_R4_W1-384_Main.txt	1	4	NeutralControl1	Area	G_Nuclei4E75636C6569	G_Area41726561	NA
Plate002_R1	W1-384	Main	Plate002_R1_W1-384_Main.txt	2	1	NeutralControl1	Area	G_Nuclei4E75636C6569	G_Area41726561	NA
Plate002_R2	W1-384	Main	Plate002_R2_W1-384_Main.txt	2	2	NeutralControl1	Area	G_Nuclei4E75636C6569	G_Area41726561	NA
Plate002_R3	W1-384	Main	Plate002_R3_W1-384_Main.txt	2	3	NeutralControl1	Area	G_Nuclei4E75636C6569	G_Area41726561	NA
Plate002_R4	W1-384	Main	Plate002_R4_W1-384_Main.txt	2	4	NeutralControl1	Area	G_Nuclei4E75636C6569	G_Area41726561	NA

Fig. 4 File structure for the ‘PlateList.txt’ for a 384 well plate

Sometimes, due to the large amount of data generated during the single cell analysis, it is impossible to export all data in one file. Therefore, it is possible to export parts of a complete plate, such as 4 files containing data for 96 wells each, for a 384 well plate. The parts themselves have to be chronological, however the wells inside the files themselves (such as well 1-96) don’t have to be chronological.

For the generation of the manuscript data we used the 1*384 data structure version with Area as selected feature.

- PlateConfLookUp.txt

The file 'PlateConfLookUp.txt' (Fig. 5) has to be prepared once and contains all necessary library information, such as plate number, well annotation, well content and gene symbol.

Also the information on which wells should be used as controls (such as 'NeutralControl1') is derived from the column 'Content'. Which control is used for which plate is stored in the 'PlateList.txt' in the column 'Control'.

Additionally, in the 'Processing' column of the 'PlateConfLookUp.txt' the user can define whether certain wells should be removed from the analysis. This is important for wells that should generally not be part of the analysis, such as toxic controls or outer wells, and are the same across all replicates used. To remove wells that are different between the replicates use the 'ScreenLog.txt' file.

In the column 'Filter' predefined filters can be used (such as F1&F2 for Filter1 and Filter2) for the object gating option. The names of the filters are derived from the 'PlateList.txt' file and only objects that meet the gating criteria and are characterized in the single cell data file as '1' in the respective Filter column are included in the analysis.

Plate	Well_Annotation_1	Well_Annotation_2	Content	GeneSymbol	Processing	Filter
1	A01	1	OuterWell	Mock	FALSE	F1&F2
...						
1	B09	33	Sample	PHB_siRNA-43	TRUE	F1&F2
1	B10	34	Sample	BAG1_siRNA-57	TRUE	F1&F2
1	B11	35	Sample	BAG1_siRNA-57	TRUE	F1&F2
1	B12	36	Sample	BCL10_siRNA-71	TRUE	F1&F2
1	B13	37	Sample	BCL10_siRNA-71	TRUE	F1&F2
1	B14	38	NeutralControl1	Allstars	TRUE	F1&F2
1	B15	39	NeutralControl3	MockI	TRUE	F1&F2
1	B16	40	InhibitorControl1	A1	TRUE	F1&F2
1	B17	41	InhibitorControl2	A2	TRUE	F1&F2
1	B18	42	InhibitorControl5	N1	TRUE	F1&F2
1	B19	43	InhibitorControl6	N2	TRUE	F1&F2
1	B20	44	InhibitorControl3	A3	TRUE	F1&F2
1	B21	45	InhibitorControl4	A4	TRUE	F1&F2
1	B22	46	InhibitorControl7	N3	TRUE	F1&F2
1	B23	47	InhibitorControl8	N4	TRUE	F1&F2
1	B24	48	ToxicControl	Plk 1-1µM	FALSE	F1&F2
1	C01	49	OuterWell	Mock	FALSE	F1&F2
1	C02	50	Sample	CRHSP24_siRNA-2	TRUE	F1&F2
...						
2	P22	382	OuterWell	Mock	FALSE	F1&F2
2	P23	383	OuterWell	Mock	FALSE	F1&F2
2	P24	384	OuterWell	Mock	FALSE	F1&F2

Fig. 5 File structure for the 'PlateConfLookUp.txt'

- ScreenLog.txt (optional)

The file ‘ScreenLog.txt’ (Fig. 6) is optional and contains information about wells that should be dismissed from the analysis but differ among the replicates of a plate. This might be the case if the experimenter notices an error in a certain well (such as a contamination) or certain wells do not meet quality criteria post screening (such as a minimum cell number).
The ‘ScreenLog.txt’ file is only needed if the SOPRA 1 of 4 (Preprocessing) option is used.

Plate	Replicate	Well	WellNo	Flag	Comment	Barcode	Inducer
1	1	K8	248	NA	no siRNA	Plate001_R1_W1-384_Main.txt	none
1	1	K9	249	NA	no siRNA	Plate001_R1_W1-384_Main.txt	none
1	2	K8	248	NA	no siRNA	Plate001_R2_W1-384_Main.txt	none
1	2	K9	249	NA	no siRNA	Plate001_R2_W1-384_Main.txt	none
1	3	K8	248	NA	no siRNA	Plate001_R3_W1-384_Main.txt	none
1	3	K9	249	NA	no siRNA	Plate001_R3_W1-384_Main.txt	none
1	4	K8	248	NA	no siRNA	Plate001_R4_W1-384_Main.txt	none
1	4	K9	249	NA	no siRNA	Plate001_R4_W1-384_Main.txt	none
2	1	O12	348	NA	no siRNA	Plate002_R1_W1-384_Main.txt	none
2	1	O13	349	NA	no siRNA	Plate002_R1_W1-384_Main.txt	none
2	2	O12	348	NA	no siRNA	Plate002_R2_W1-384_Main.txt	none
2	2	O13	349	NA	no siRNA	Plate002_R2_W1-384_Main.txt	none
2	3	O12	348	NA	no siRNA	Plate002_R3_W1-384_Main.txt	none
2	3	O13	349	NA	no siRNA	Plate002_R3_W1-384_Main.txt	none
2	4	O12	348	NA	no siRNA	Plate002_R4_W1-384_Main.txt	none
2	4	O13	349	NA	no siRNA	Plate002_R4_W1-384_Main.txt	none

Fig.6 File structure for the ‘ScreenLog.txt’

/!\ Filter options:

SOPRA offers the possibility to remove wells for all replicates from the analysis using either the column ‘Processing’ in the file ‘PlateConfLookUp.txt’, or for a defined replicate using the file ‘ScreenLog.txt’. Additionally, in wells that are analyzed individual objects can be dismissed from the analysis using the gating filter settings in the single cell data files and the files ‘PlateList.txt’ and ‘PlateConfLookUp.txt’.

4. Analysis

4.1 Install R-Studio and R

First of all install R-Studio (https://www.rstudio.com/) and R (https://www.r-project.org/). We used R-Studio Version 1.3.1093 and R version 4.0.3 for Windows and RStudio-Server with Rstudio 1.1.463 and R-version 3.4.3 for Linux.

4.2 Start the analysis

1. The software is realized as a shiny application with an user interface ui.R and application server.R within the R-Project SOPRA.Rproj
2. SOPRA shiny application (ui.R, server.R) and single cell data (96-well plates) for testing are accessible from GitHub https://github.com/kppleissner/SOPRA/. Download the entire repository and unzip it locally.
3. 384-well plates for SOPRA are available from MPIIB cloud server https://transfer.mpiib-berlin.mpg.de/s/AibR4AHLCR9xzDB?path=%2F
4. Launch R-Studio
5. Open the project SOPRA.Rproj in RStudio
6. After loading click on the button Run App
7. R-packages that are necesseray are installed automatically. Otherwise install these package manually.
8. Run through the user interface, set input/output folders for data, upload descriptive files and set corresponding parameters
9. Hit ‘Go Analysis’

4.3 Data input and output, uploading Platelist.txt and PlateConfigLookUp.txt

The single cell data files to be read are determined (Select Input Folder) and the files ‘PlateList.txt and ‘PlateConfLookup.txt’ can be uploaded. Additionally you can define where to store the output files (Select Output Folder)-Fig. 7.

Folder selection

Choose the input folder with the ScanR data files

Select Input folder

Following input folder was selected

M:/SOPRAData/SOPRAdata-384/InData384

Choose the output folder for resulting data

Select or create Output folder

Following output folder was selected

M:/Area

File selection

Select Platelist file with information which files should be processed

Choose Platelist-file

Following Platelist was choosen:

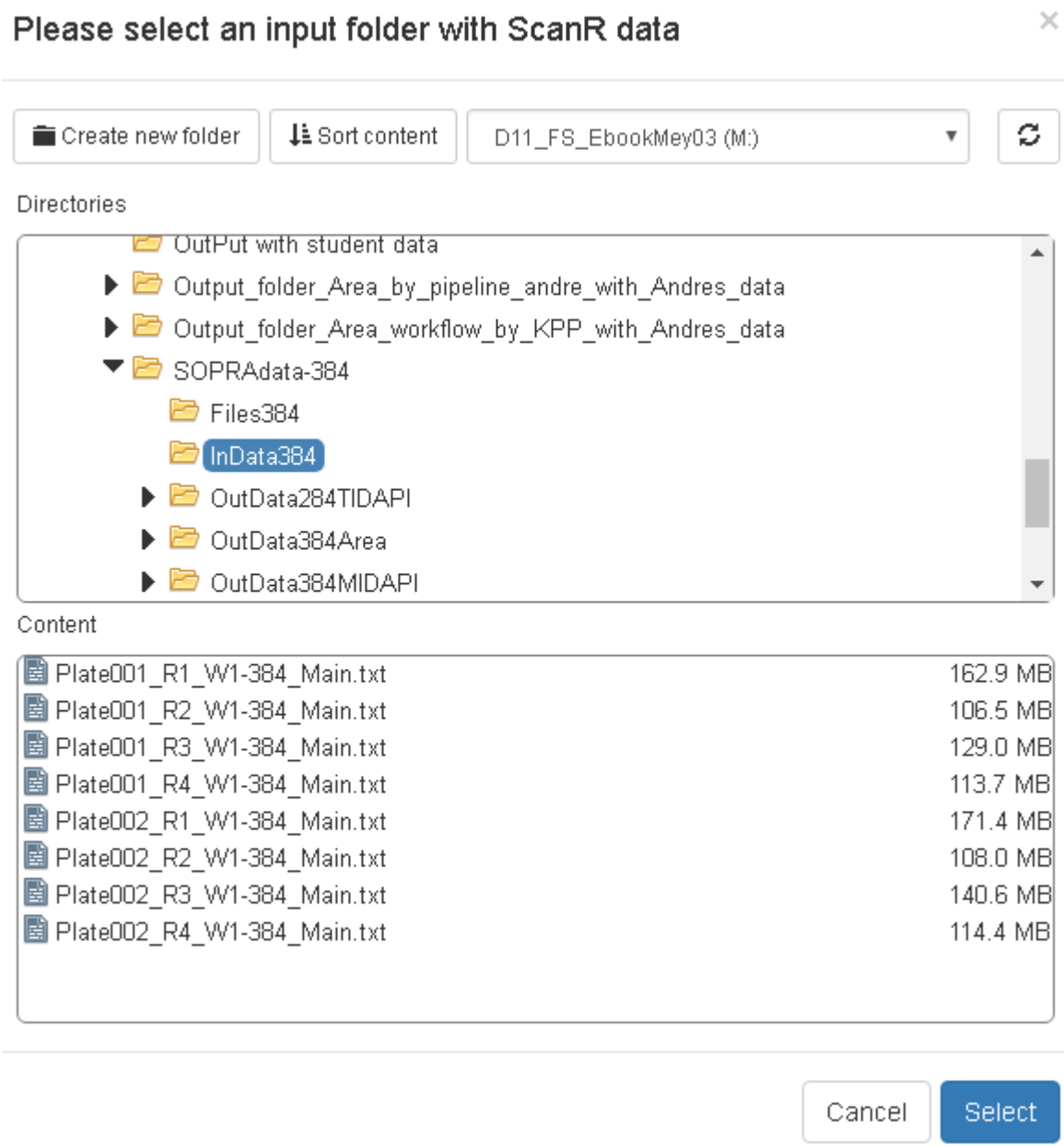
[1] "M:/SOPRAData/SOPRAdata-384/Files384/PlateList-Area-384-with_NeutralC1.txt"

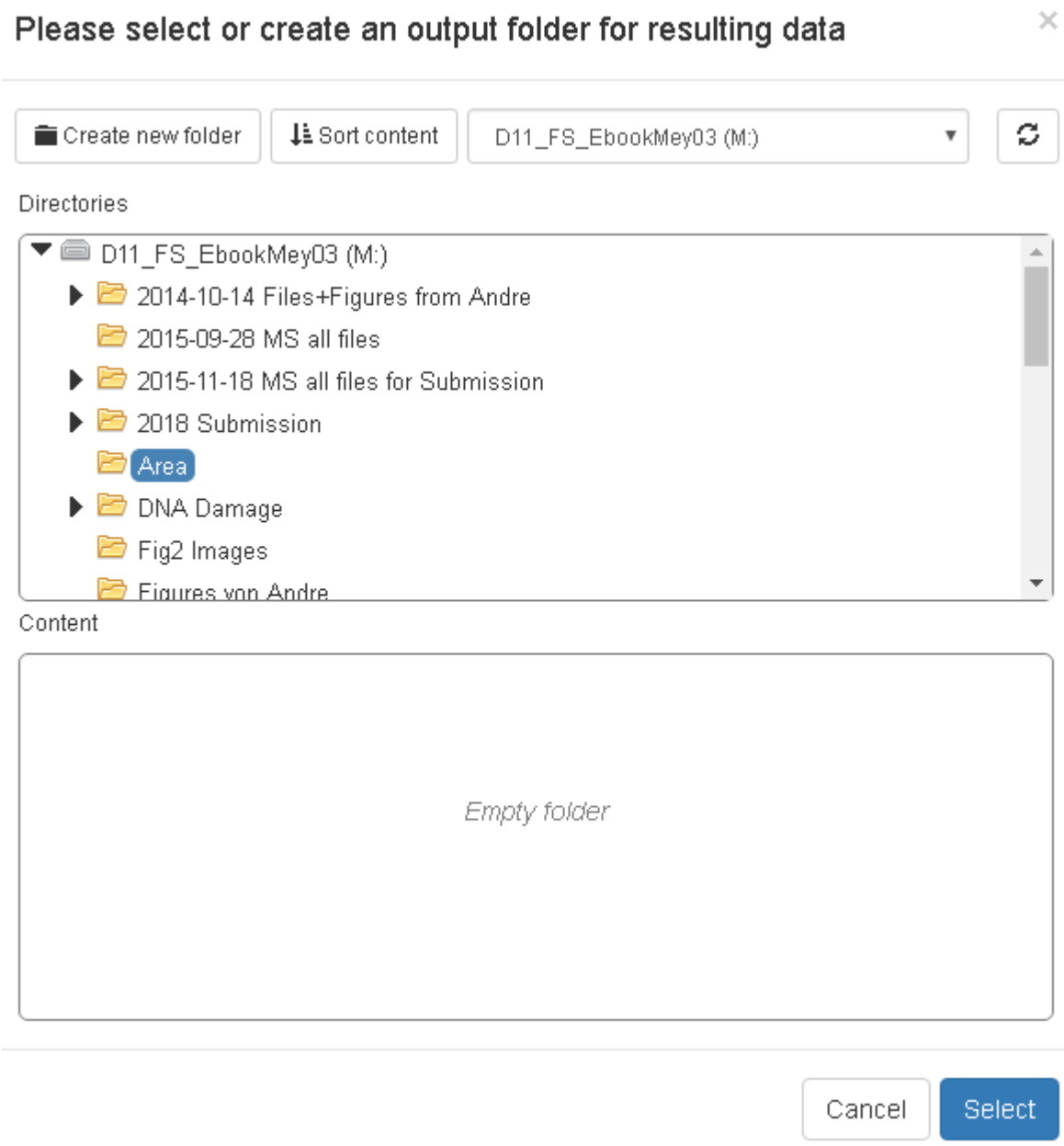
Select PlateConfLookUp file with information on plate configuation

Choose PlateConfLookUp-file

Following PlateConfLookUp was choosen:

[1] "M:/SOPRAData/SOPRAdata-384/Files384/PlateConf_LookUp.txt"


Please select an input folder with ScanR data
Create new folder
Sort content
D11_FS_EbookMey03 (M:)
Directories
OutPut with student data
Output_folder_Area_by_pipeline_andre_with_Andres_data
Output_folder_Area_workflow_by_KPP_with_Andres_data
SOPRAdata-384
Files384
InData384
OutData284TIDAPI
OutData384Area
OutData384MIDAPI
Content
Plate001_R1_W1-384_Main.txt 162.9 MB
Plate001_R2_W1-384_Main.txt 106.5 MB
Plate001_R3_W1-384_Main.txt 129.0 MB
Plate001_R4_W1-384_Main.txt 113.7 MB
Plate002_R1_W1-384_Main.txt 171.4 MB
Plate002_R2_W1-384_Main.txt 108.0 MB
Plate002_R3_W1-384_Main.txt 140.6 MB
Plate002_R4_W1-384_Main.txt 114.4 MB
Cancel
Select


Please select or create an output folder for resulting data
Create new folder
Sort content
D11_FS_EbookMey03 (M:)
Directories
D11_FS_EbookMey03 (M:)
2014-10-14 Files+Figures from Andre
2015-09-28 MS all files
2015-11-18 MS all files for Submission
2018 Submission
Area
DNA Damage
Fig2 Images
Figures von Andre
Content
Empty folder
Cancel
Select

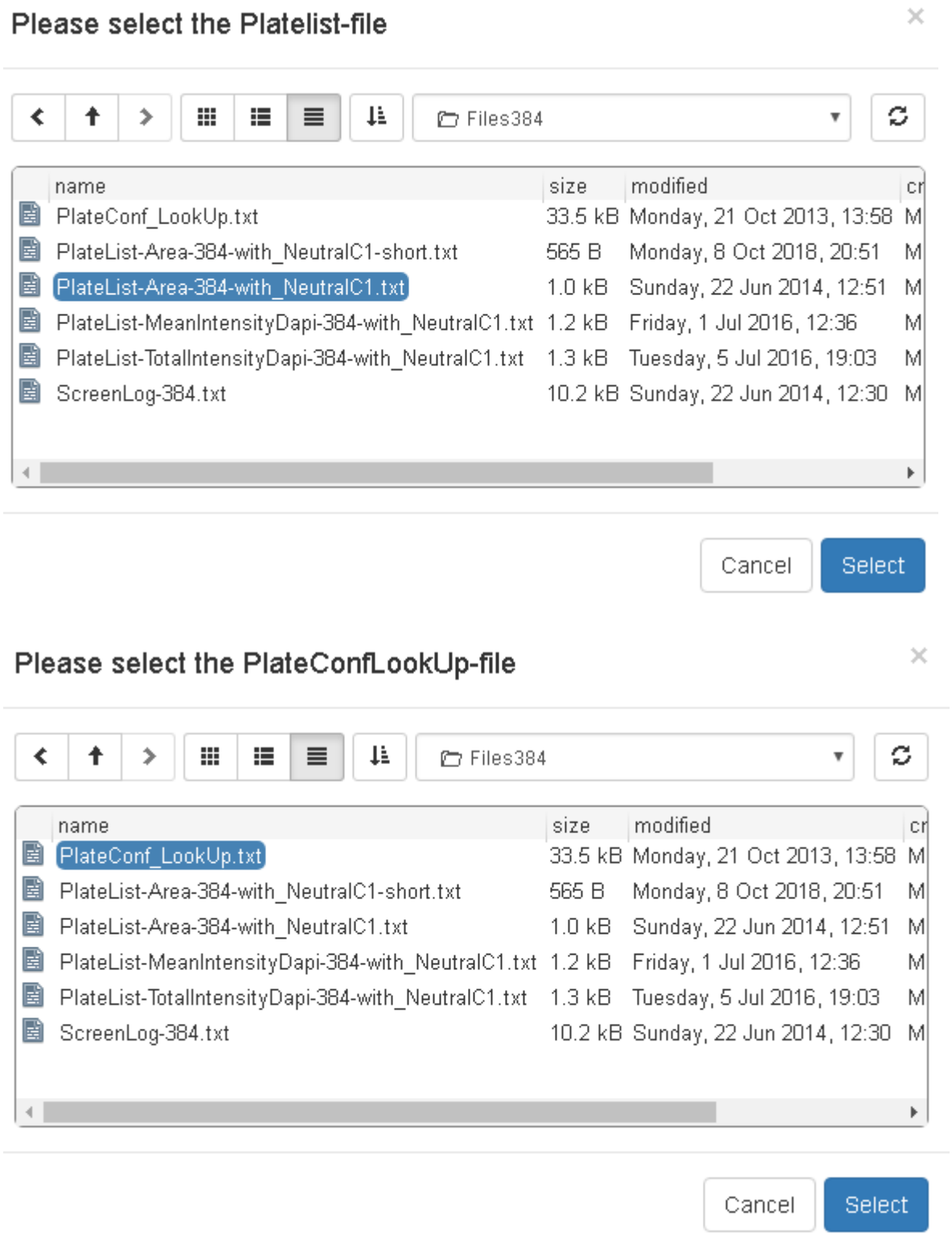
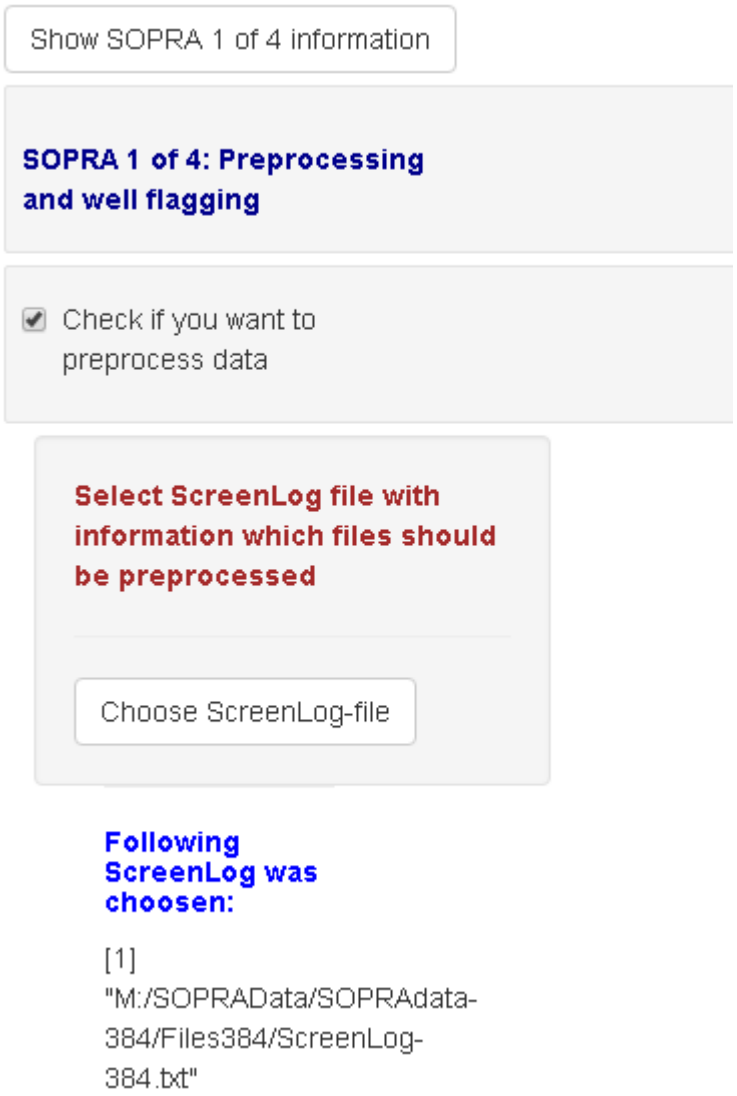


Fig. 7 File upload menu

4.4 SOPRA 1 of 4 (Preprocessing)

The preprocessing is an optional step (Fig. 8) for which the 'ScreenLog.txt' has to be prepared as described above. It can be used for flagging wells that should be removed from the analysis but differ among the replicates of a plate. If the checkbox is activated, the SOPRA workflow generates the subfolder Preprocessing_Results in the dedicated output folder to store the preprocessed files.



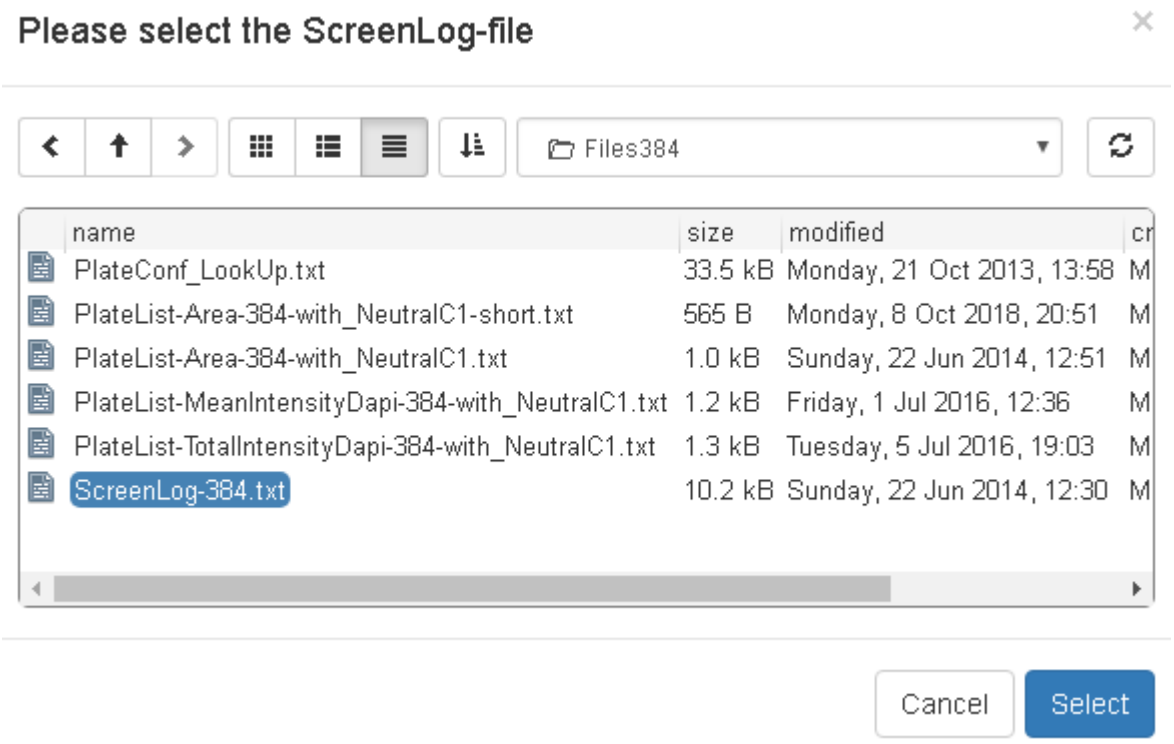


Fig. 8 Menu for SOPRA 1 of 4 (Preprocessing)

4.5 SOPRA 2 of 4 (Data Gathering and Normalization)

For the data gathering and normalization step (Fig. 9), the number of bins for the generation of histograms has to be defined. Additionally the plate format and the left and right quantiles have to be set. The quantile settings offer the option to remove objects from the analysis steps, to avoid that outliers lead to mostly empty bins, which might result in abnormal results. Additionally, objects that have been predefined in the image analysis software to be outside of a certain gating range (see single cell data files) can be removed from further analysis by setting the gating filter options to filter '1-3' (see 'PlateList.txt') or to the predefined filter settings (see 'PlateConfLookUp.txt'). Further, it is possible to define whether the intermediate data files, the QC blots and a file containing the binning parameters should be exported. The external binning file is mandatory if SOPRA 3 of 4 (Find Significant Profiles) and the following steps should be executed without performing SOPRA 2 of 4 (Data Gathering and Normalization) again. All data are exported into the file "AllDataTable.txt" in the output folder.

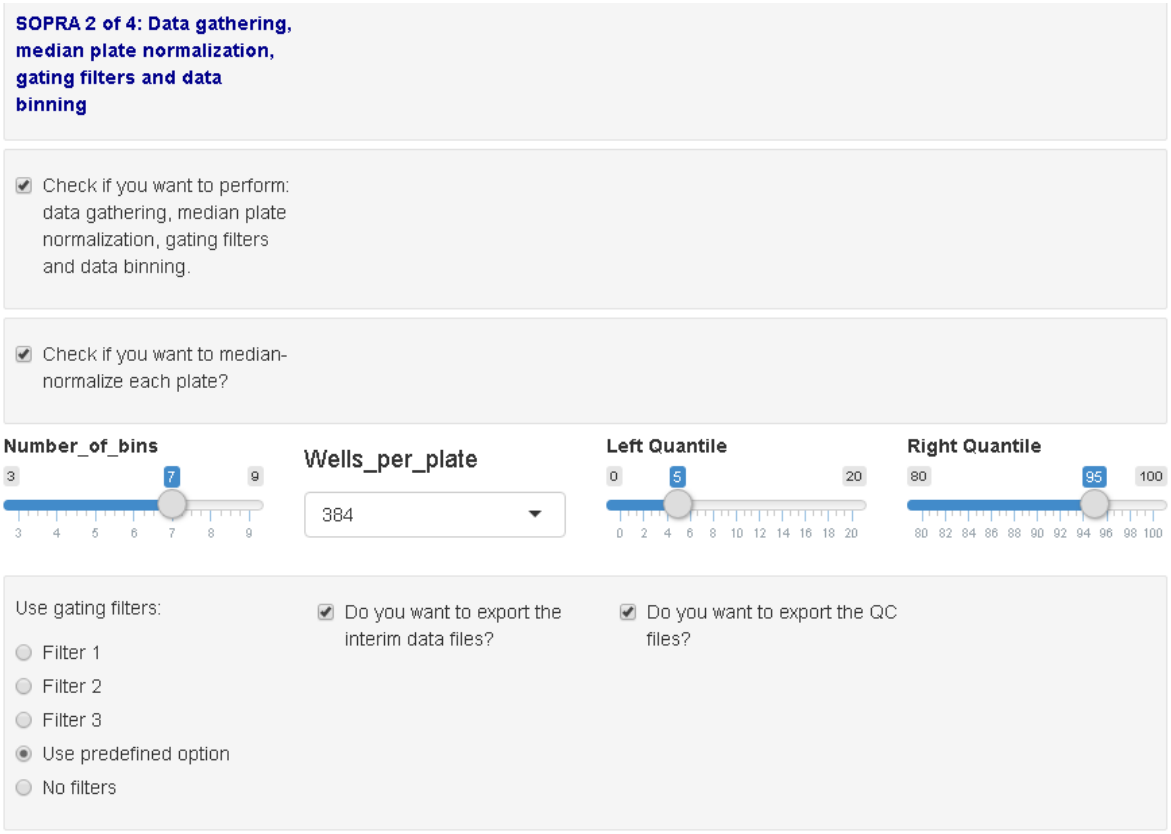


Fig. 9 Menu for SOPRA 2 of 4

4.6 SOPRA 3 of 4 (Find Statistically Significant Profiles- Fig. 10)

As described above, the normalization of the chosen cell population feature leads to density profiles (histograms) for the sample wells relative to the defined control wells. The more distinct a normalized histogram is from a horizontal line, the more significant the change.
The R/Bioconductor package maSigPro is used to perform a regression analysis with a defined false discovery rate, p-value, RSQ-value and degrees of freedom, to identify significantly altered cell population profiles. More information on how the regression analysis is performed can be found in the user's manual of maSigPro at https://www.bioconductor.org/packages/release/bioc/html/maSigPro.html.

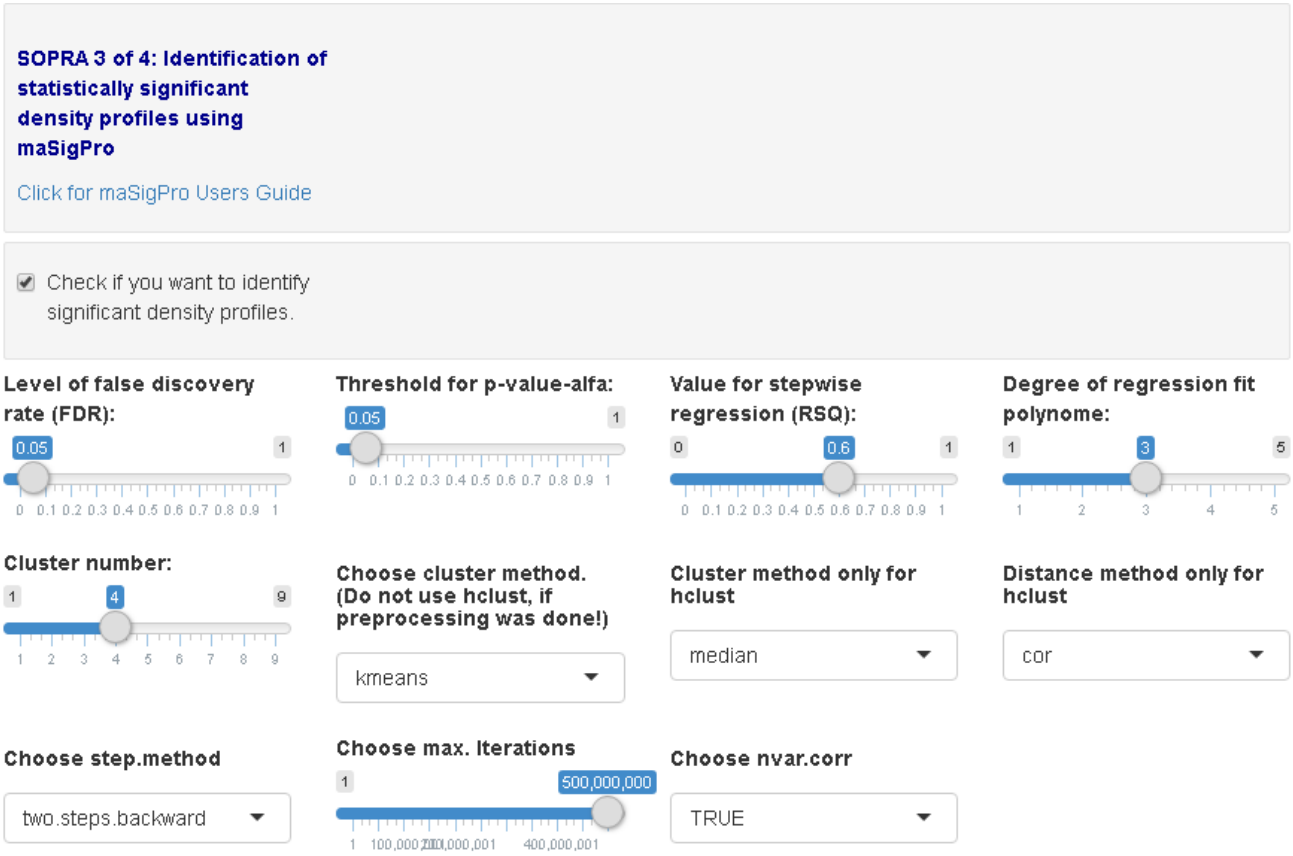


Fig. 10 Menu for SOPRA 3 of 4 (Find statistically significant density profiles)

The result of SOPRA 3 of 4 is exported in the files 'All_p-rsq_values_after_function_tfit_wDegreexQ0.0x_walfa0.0x.txt'.

4.7 SOPRA 3 of 4 (Clustering)

Additionally, the identified statistically significant density profiles can be clustered using a variety of cluster methods (Fig. 11). To read more about the cluster methods, please refer to the maSigPro description.

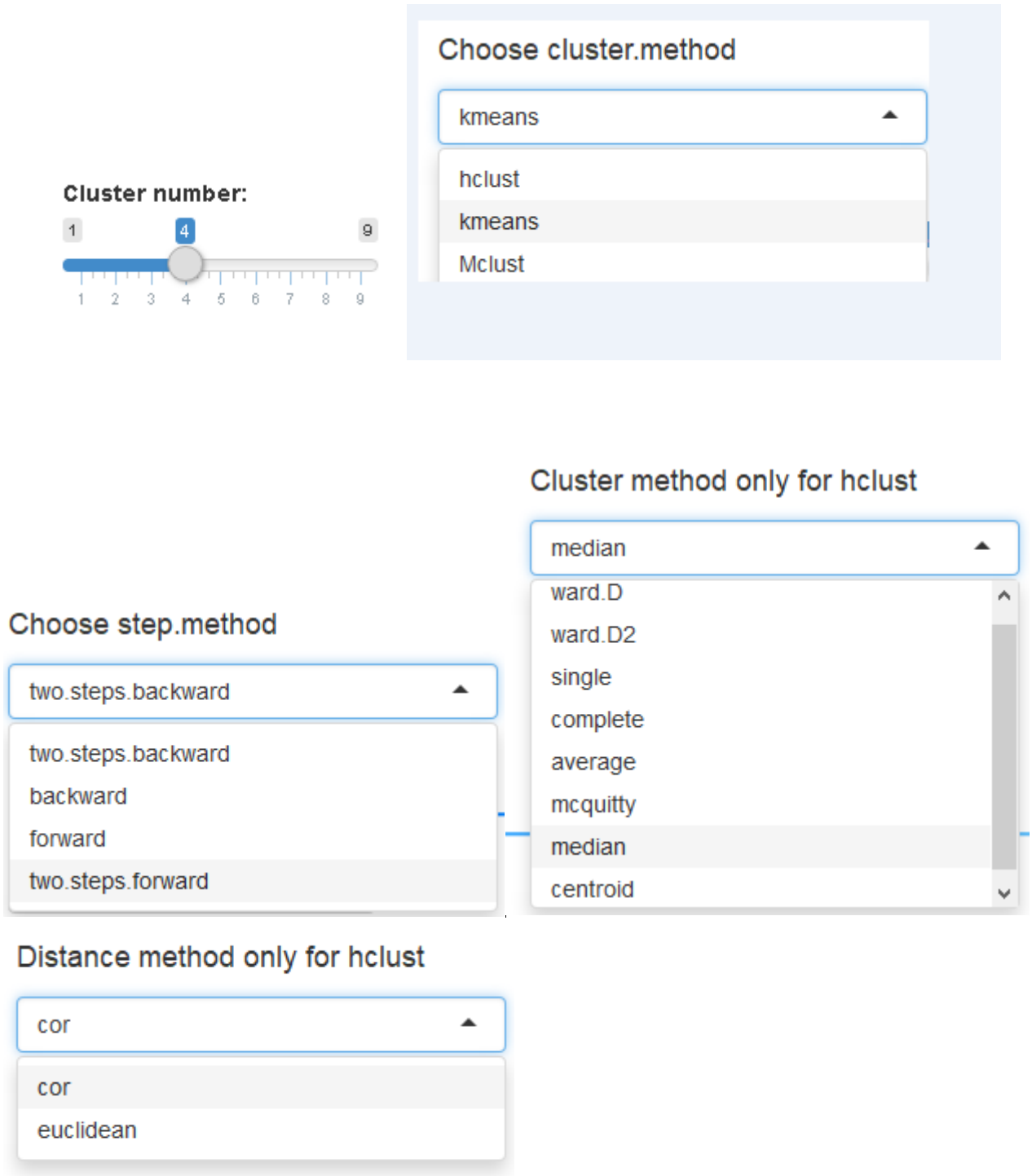


Fig. 11 Selection of cluster parameters for SOPRA 3 of 4

4.8 SOPRA 4 of 4 (Post-processing)

In most siRNA screens multiple siRNA inhibitors are used for the same target gene. Additionally, the same siRNA or chemical compound can be used in several well replicates. The SOPRA workflow uses the following nomenclature to differentiate between multiple siRNAs (or compounds) for the same target gene (or protein) and well replicates: e.g. 'DDX1_siRNA- 1#1#26' for siRNAs and e.g. 'A1#1#88' for chemical compounds.

The first part 'DDX1' or 'A1' is the gene or compound name, the second part 'siRNA-1 'differentiates between different siRNAs used for the same gene. The later part '#1#26' determines the plate and the well location of the inhibitor used.

The SOPRA 4 of 4 (Post-processing- Fig. 12) offers the opportunity to determine which inhibitor was identified in which cluster with which frequency. SOPRA 4 of 4 is running after clustering directly.

The results are exported into the file

"All_significant_siRNA_in_which_cluster__wd3_Q0.0x_alfa0.0x_rsq0.x_clusterx_Mclusttwo.steps.backwardcor_post_processed.txt"

4.9 Start the SOPRA workflow

Finally, after setting all necessary parameters and options, the SOPRA workflow can be started by clicking the 'Run Analysis!' button - Fig. 12)

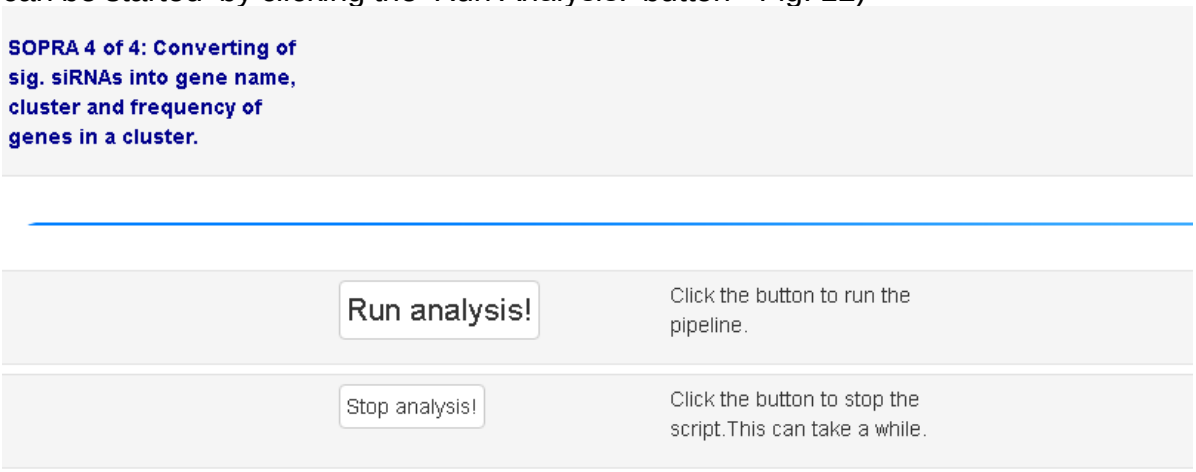


Fig. 12 Post-Processing (SOPRA 4 of 4) and run analysis

After the calculation is finished you can change settings and run the pipeline again. Please note that resulting data files might be overwritten if you use the same output folder. By setting or unsetting the checkboxes one can run the analysis again.

SOPRA 3 of 4 can be rerun independently from SOPRA 1 of 4 and SOPRA 2 of 4 if checkboxes of these steps are unchecked.

The progress of the SOPRA workflow (Fig. 13) can be monitored either by the interactive feedback in the SOPRA window or in the R-Studio console.

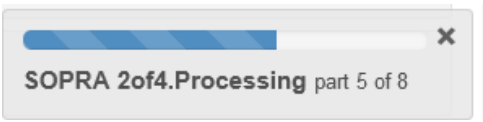


Fig. 13 Interactive feedback in the SOPRA window with progress bar.

If the analysis is finished (Fig. 14) a window pops-up with some explanation how to rerun the workflow under changed conditions or parameters.

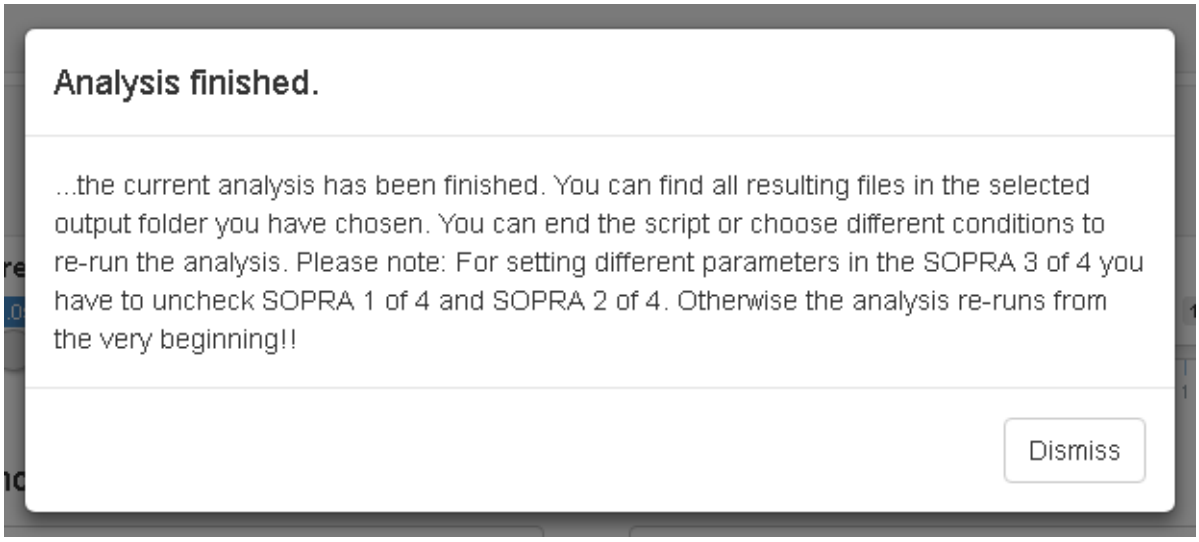


Fig. 14 Analysis finished and some explanations given for re-running the analysis

5 Results

5.1 Result of SOPRA 1 of 4 (Preprocessing)

SOPRA 1 of 4 (Preprocessing) flags all objects for wells that are indicated in the 'ScreenLog.txt' with 'NA'. The preprocessed files are saved in the subfolder 'Preprocessing_Results' in the dedicate output folder.

5.2 Result of SOPRA 2 of 4 (Data Gathering and Normalization)

SOPRA 2 of 4 (Data Gathering and Normalization) results in log2 normalized frequency distributions (histograms) for all samples (and controls). The data is exported in the file 'AllDataTable.txt' including all annotations. The file

'AllDataTable.txt' contains the processed data, with the plates arranged vertically and the replicates arranged horizontally. The file contains the RNA.ID, plate, replicate and well number, the processing condition ('TRUE' or 'FALSE') and the binning data followed by the well content and gene symbol.

Optionally, data generated during the calculation process can be exported. The files 'Data_rawdata_PlateXReplicatey.txt' contain the raw data values for each object for the feature of interest, while the files 'Data_nzdata_PlateXReplicateY.txt' contain the 'plate-wise' normalized values for each object for the feature of interest.
For the binning itself three files can optionally be exported, the file 'Absolute_frequencies_of_PlateXReplicateY.txt' containing the counts per bin, the file 'Relative_frequencies_of_PlateXReplicateY.txt' containing the relative values per bin and the file 'Binwisenz_frequencies of_PlateXReplicateY.txt' containing the normalized control values for each bin.

If the analysis is finished the results are depicted via the shiny user interface by clicking on the corresponding tabs (Fig. 15 - Fig. 21).

<< Click on the tabs to see the results >>

Exp.design | QC plot | clustered profiles of sign. siRNAs | sign. siRNAs with cluster | sign. genes with cluster and frequency | Genecount

Time	Replicate	Bin
46	1	1
46	1	1
46	1	1
46	1	1
72	2	1
72	2	1
72	2	1
72	2	1
98	3	1
98	3	1
98	3	1
98	3	1
124	4	1
124	4	1
124	4	1
124	4	1
150	5	1
150	5	1
150	5	1
150	5	1
176	6	1
176	6	1
176	6	1
176	6	1
202	7	1
202	7	1
202	7	1
202	7	1

Fig. 15 Experimental design table necessary for single series time course experiments in maSigPro

Furthermore, quality control plot (QC boxplot) can be exported containing boxplots for all plates after normalization, as well as the distribution of selected

cell feature per plate.

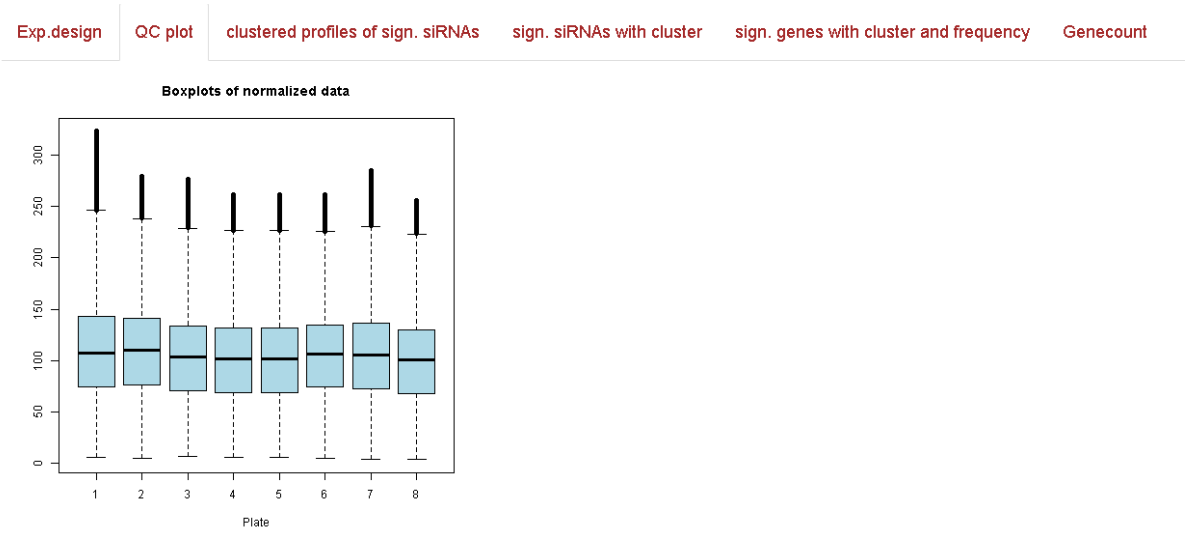


Fig. 16 QC boxplot. It shows the number of plates that were processed with the distribution of selected feature per plate after normalization.

5.3 Result of SOPRA 3 of 4 (Find Statistically Significant Profiles)

For SOPRA 3 of 4 the file ‘All_prsq_values_after_function_tfit_wDegreeXQY_walfaZ.txt’ is exported into the output folder.This file contains all calculated p-values and rsq-values for each processed probe.

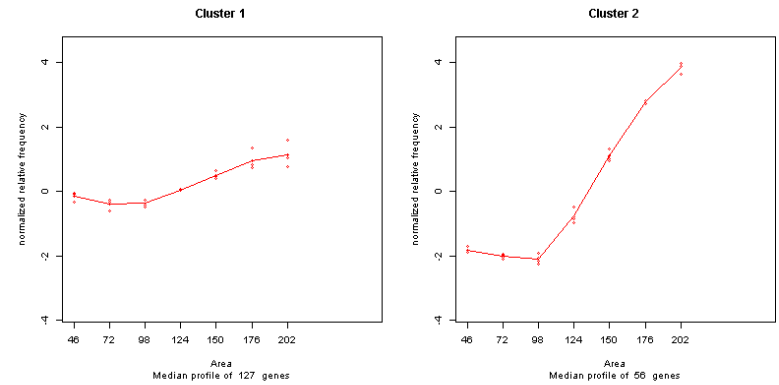
	p-value	R-squared
DDX1_siRNA-1#1#26	6.25E-11	0.873674209
DDX1_siRNA-1#1#27	5.56E-08	0.776545053
HNRPM_siRNA-15#1#28	3.84E-06	0.68043179
HNRPM_siRNA-15#1#29	1.65E-05	0.638235816
PSMC5_siRNA-29#1#30	0.001282625	0.333834073
PSMC5_siRNA-29#1#31	4.61E-08	0.689372425
PHB_siRNA-43#1#32	0.006574113	0.33099868
PHB_siRNA-43#1#33	0.008269675	0.318604791
BAG1_siRNA-57#1#34	4.45E-06	0.561593992
BAG1_siRNA-57#1#35	4.43E-08	0.690277432
BCL10_siRNA-71#1#36	0.000547369	0.451644245
BCL10_siRNA-71#1#37	0.008062588	0.31998584
Allstars#1#38	0.000770622	0.357990056
A1#1#40	2.14E-08	0.756513428
A2#1#41	1.63E-22	0.986381108
N1#1#42	6.50E-16	0.95160789
N2#1#43	2.66E-14	0.934009892
A3#1#44	2.01E-14	0.935551082
A4#1#45	1.01E-14	0.939138851
N3#1#46	4.54E-07	0.689147363
N4#1#47	1.68E-08	0.797926353

Fig. 17 Result file for SOPRA 3 of 4 with siRNA, p-value and R-squared value

5.4 Result for SOPRA 3 of 4 (Clustering)

Depending on the user's choice, clustering is performed and the results are exported as file “All_significant_siRNA_in_which_cluster__wd3_Q0.05_alfa0.05_rsq0.6_cluster4_hclusttwo.steps.backwardcorgray.txt” and as a png file ‘Clustered_Sign_Samples_w….png’. The file name contains hints with which parameters maSigPro was carried out. The clustered profiles of significant siRNAs are depicted (Fig. 18).

Exp.design | QC plot | clustered profiles of sign. siRNAs | sign. siRNAs with cluster | sign. genes with cluster and frequency | Genecount



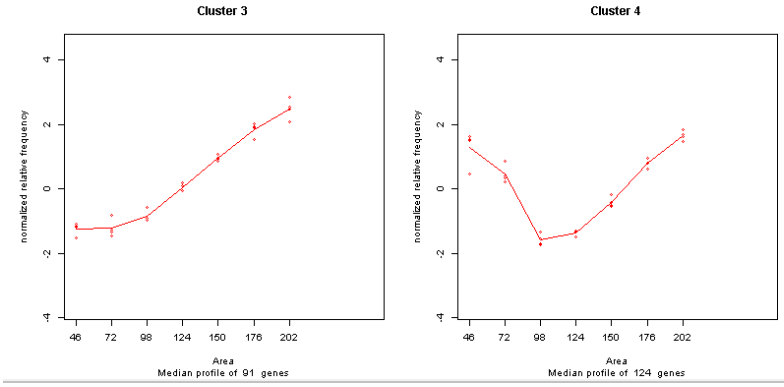


Fig. 18 Statistically significant, normalized frequency density profiles after clustering. Each replicate per plate is vertically depicted as a dot over a binning interval , i.e. here 4 replicates per plate were processed. The x-axis is labeled with the selected cellular feature given in the PlateList.txt

Exp.design | QC plot | clustered profiles of sign. siRNAs | sign. siRNAs with cluster | sign. genes with cluster and frequency | Genecount

siRNA	Cluster
DDX1_siRNA-1#1#26	2
DDX1_siRNA-1#1#27	2
HNRPM_siRNA-15#1#28	2
HNRPM_siRNA-15#1#29	2
PSMC5_siRNA-29#1#31	2
BAG1_siRNA-57#1#35	2
A1#1#40	4
A2#1#41	4
N1#1#42	3
N2#1#43	3
A3#1#44	1
A4#1#45	1
N3#1#46	3
N4#1#47	3
BARD1_siRNA-16#1#52	2
BARD1_siRNA-16#1#53	2
PSMC4_siRNA-30#1#54	2
PSMC4_siRNA-30#1#55	2
PSMA3_siRNA-44#1#56	1
PSMA3_siRNA-44#1#57	1
BCL2L11_siRNA-72#1#61	2
A1#1#64	4
A2#1#65	4

Fig. 19 Part of statistically significant siRNAs with its cluster membership.

5.5 Result for SOPRA 4 of 4 (Post-processing)

SOPRA 4 of 4 (Post-processing) generates the file 'All_significant_siRNA_in_which_cluster__post_processed.txt' with gene, cluster membership of gene and frequency of gene in the corresponding cluster (Fig. 20).

Exp.design | QC plot | clustered profiles of sign. siRNAs | sign. siRNAs with cluster | sign. genes with cluster and frequency | Genecount

Gene	Cluster	Frequency
A1	4	28
A2	4	28
N1	3	28
N2	3	28
A3	1	28
A4	1	28
N4	3	28
N3	3	25
PSMA3	1	4
CSK	2	4
MYC	2	4
RPLP0	3	4
BET1L	2	4
SF3A1	1	4
BCL2L1	2	4
BCL2L11	2	3
BIRC5	3	3
BCL2	2	3
APAF1	2	3

Fig. 20 Result of post-processing showing the gene name, cluster and frequency of gene in the corresponding cluster

Exp.design | QC plot | clustered profiles of sign. siRNAs | sign. siRNAs with cluster | sign. genes with cluster and frequency | Genecount

Fig. 21 GeneCount (Wordcloud) shows greater prominence to genes that appear more frequently.

6 Software

The software has been implemented in R (https://www.r-project.org) with corresponding R-packages and using RStudio (https://www.rstudio.com) as integrated development environment (IDE).

If SOPRA workflow is used to generate data, please cite:

"Single Object Profiles Regression Analysis (SOPRA): A novel method for analyzing high content cell-based screens" Rajendra Kumar Gurumurthy, Klaus-Peter Pleissner, Cindrilla Chumduri, Thomas F. Meyer, Andre P. Maeurer