3D RNA-seq App

“Easy-to-use” user manual

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License

Citation

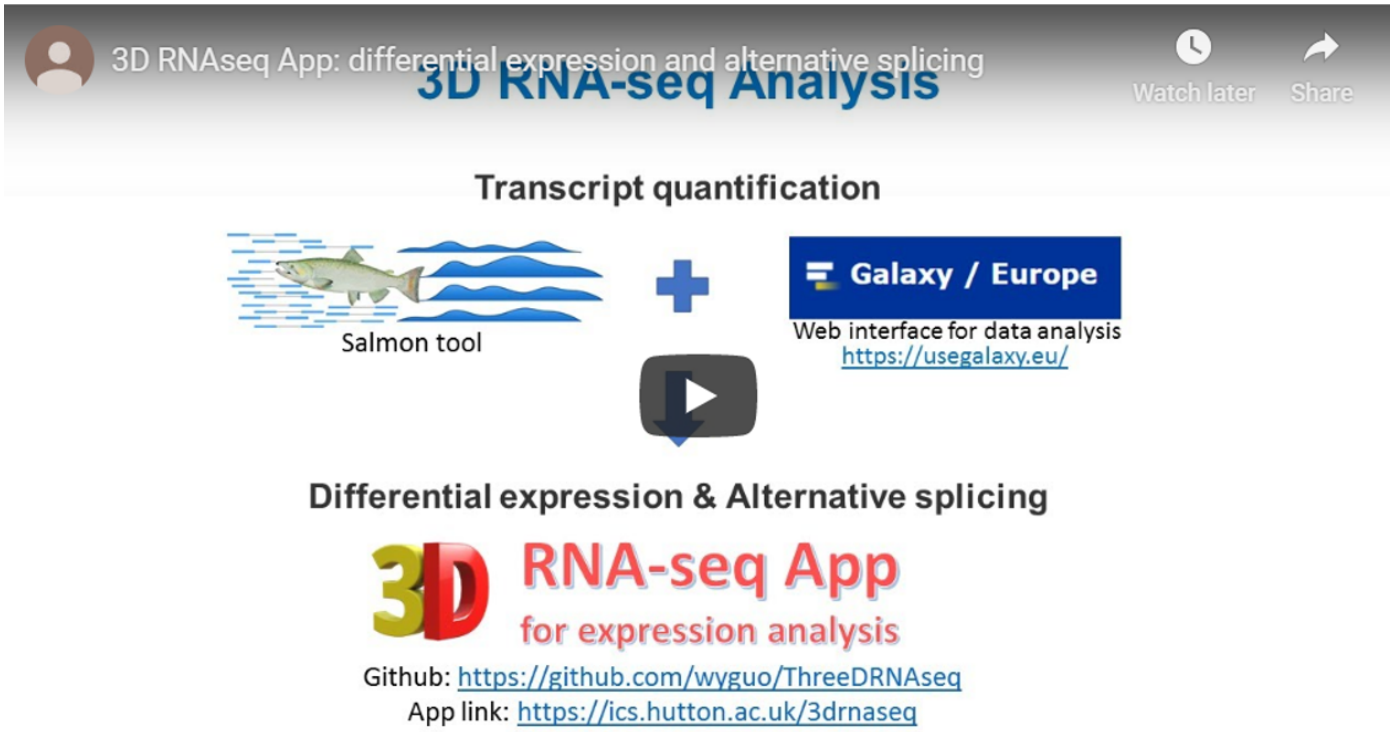
To use our pipeline in your work, please cite:

- Guo,W., Tzioutziou,N., Stephen,G., Milne,I., Calixto,C., Waugh,R., Brown,J.W., and Zhang,R. (2019) 3D RNA-seq - a powerful and flexible tool for rapid and accurate differential expression and alternative splicing analysis of RNA-seq data for biologists. bioRxiv, 656686. doi: https://doi.org/10.1101/656686 (https://doi.org/10.1101/656686).
- Calixto,C.P.G., Guo,W., James,A.B., Tzioutziou,N.A., Entizne,J.C., Panter,P.E., Knight,H., Nimmo,H.G., Zhang,R., and Brown,J.W.S. (2018) Rapid and Dynamic Alternative Splicing Impacts the Arabidopsis Cold Response Transcriptome. Plant Cell, 30, 1424–1444.

Demo video

To watch a demo video, click the screenshot

https://youtu.be/rqeXECX1-T4 (https://youtu.be/rqeXECX1-T4)



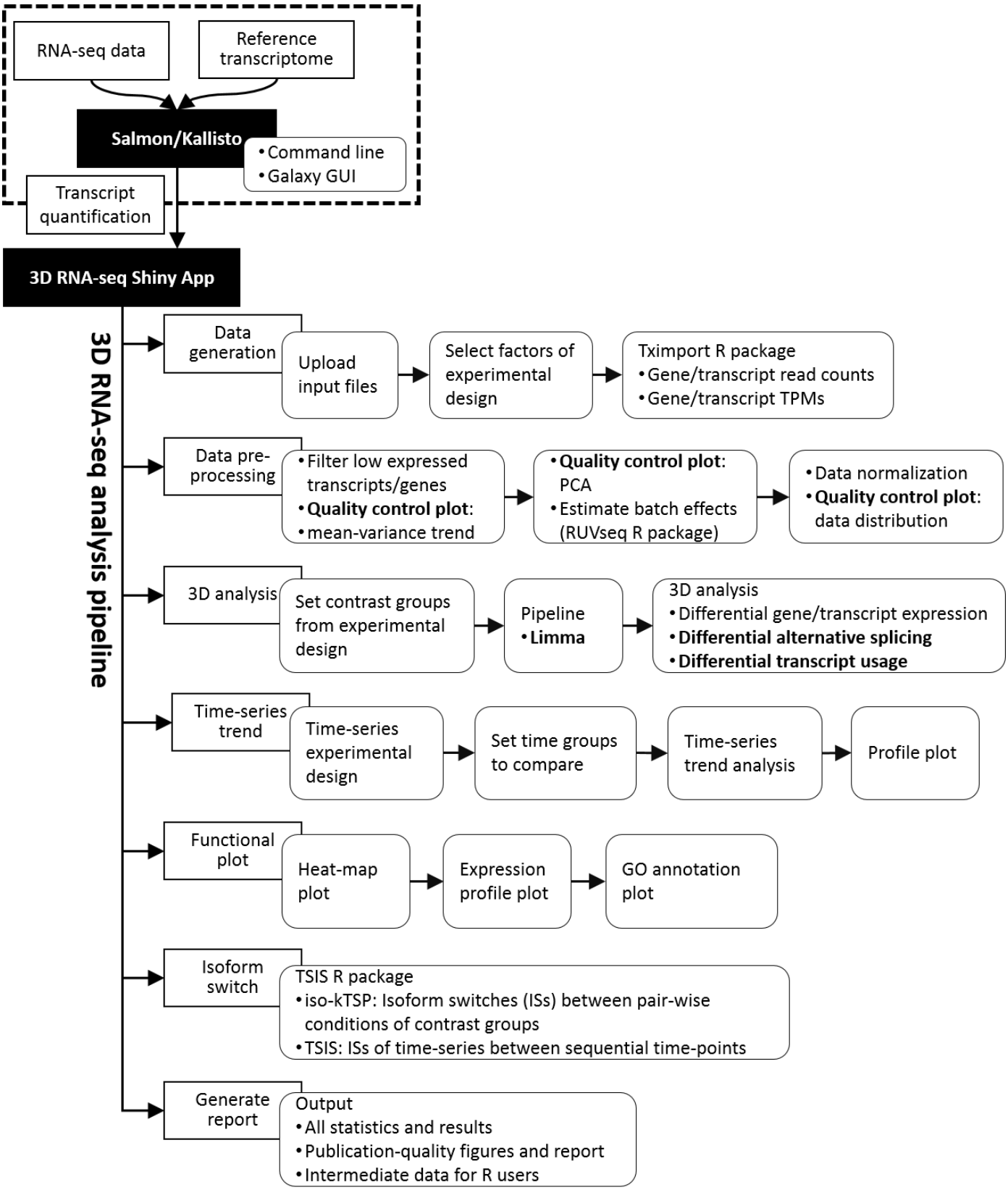
(https://youtu.be/rqeXECX1-T4)

Introduction

The ThreeDRNAseq (3D RNA-seq) R package is designed for use by biologists to analyze their own RNA-seq data (Guo et al., 2019). It provides an interactive graphical user interface (GUI) for differential expression (DE), differential alternative splicing (DAS) and differential transcript usage (DTU) (3D) and changes of time-series trend analyses of RNA-seq data based on the popular pipeline limma (Ritchie et al., 2015). It also integrated transcript isoform switch tools, such as IsoKtsp (Sebestyen et al., 2015) and TSIS (Guo et al., 2017) for an enhanced alternative splicing analysis. The 3D RNA-seq removes all the unnecessary complexities associated with differential expression analysis and enables a complete RNA-seq analysis to be done quickly (3 Days, thus 3D). It allows complex experimental designs such as time-series, developmental series and multiple conditions. It employs state-of-the-art methods/statistics and generates 3D results quickly and accurately.

The 3D RNA-seq App was developed by Dr. Wenbin Guo from research into the analysis of time-series RNA-seq data (Calixto et al., 2018) with help from Dr. Cristiane Calixto and Dr. Nikoleta Tzioutziou and guidance from Prof. John W.S. Brown and Dr. Runxuan Zhang from the University of Dundee - School of Life Sciences and the James Hutton Institute - Information and Computational Sciences. We acknowledge Dr. Iain Milne and Gordon Stephen for technical support.

3D analysis pipeline



Go back to Table of contents

How to get help

User manuals

3D RNA-seq App “easy-to-use” manual:

https://github.com/wyguo/ThreeDRNAseq/blob/master/vignettes/user_manuals/3D_RNA-seq_App_manual.md (https://github.com/wyguo/ThreeDRNAseq/blob/master/vignettes/user_manuals/3D_RNA-seq_App_manual.md)

Tooltips

In the GUI, users can click tooltips  in specific steps for help information.

Contact us

3D RNA-seq App is developed and maintained by Dr. Wenbin Guo from the Plant Sciences Division, School of Life Sciences, University of Dundee. If you have any questions and suggestions, please contact:

- Dr. Wenbin Guo: wenbin.guo@hutton.ac.uk (mailto:wenbin.guo@hutton.ac.uk)
- Dr. Runxuan Zhang: runxuan.zhang@hutton.ac.uk (mailto:runxuan.zhang@hutton.ac.uk)

Go back to Table of contents

Run 3D RNA-seq App

Shiny docker image (no installation)

The 3D RNA-seq App docker image is hosted by the James Hutton Institute server. Open the App by this link: https://ics.hutton.ac.uk/3drnaseq (https://ics.hutton.ac.uk/3drnaseq)

- To perform 3D analysis, no installation is required. Users need to upload input data to our server. All results, reports, figures and intermediate data will be zipped and downloaded in the final step.
- If you are working on RNA-seq data with very big size of transcript quantification (≥ 2GB), it is recommended to remove the redundant files in the Salmon/Kallisto outputs (see Input files) to reduce data size or run the 3D RNA-seq App through RStudio on a local PC.

Shiny App through RStudio (R users, installation required)

To run the 3D RNA-seq App through RStudio on a local PC, users do not need to upload the data to our server and all the outputs will be directly saved to the App working directory. Please run the following command to install ThreeDRNAseq R package and the packages of dependencies. If any other R packages are missing in your PC, please install them.

Install dependency packages

```
################################################################################
## Install packages of dependency
###---> Install packages from Cran
cran.package.list <- c("shiny","shinydashboard","rhandsontable","shinyFiles",
                       "shinyjs","shinyBS","shinyhelper","shinyWidgets",
                       "magrittr","DT","plotly","ggplot2","eulerr",
                       "gridExtra","grid","fastcluster","rmarkdown",
                       "ggrepel","zoo","gtools")
for(i in cran.package.list){
   if(!(i %in% rownames(installed.packages()))){
     message('Installing package: ',i)
     install.packages(i)
   } else next
}

###---> Install packages from Bioconductor
bioconductor.package.list <- c('tximport','edgeR','limma','RUVSeq',
                               'ComplexHeatmap','rhdf5')
for(i in bioconductor.package.list){
  if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")
  if(!(i %in% rownames(installed.packages()))){
    message('Installing package: ',i)
    BiocManager::install(i)
  } else next
}
```

Install ThreeDRNAseq R package

ThreeDRNAseq R package can be installed from Github by using devtools (https://cran.r-project.org/web/packages/devtools/index.html) R package

```
#########################################################################################################
## use devtools R package to install ThreeDRNAseq from Github
###---> If devtools is not installed, please install
if(!requireNamespace("devtools", quietly = TRUE))
  install.packages('devtools',dependencies = TRUE)

###---> Install ThreeDRNAseq
if(!requireNamespace("ThreeDRNAseq", quietly = TRUE))
  devtools::install_github('wyguo/ThreeDRNAseq')
```

Run 3D RNA-seq App

```
library(ThreeDRNAseq)
run3DApp()
```

R command line (advanced R users, installation required)

The ThreeDRNAseq R package can be used as a normal R package. The vignette of command line for 3D analysis can be found in:

https://github.com/wyguo/ThreeDRNAseq/blob/master/vignettes/user_manuals/3D_RNA-seq_command_line_user_manual.md (https://github.com/wyguo/ThreeDRNAseq/blob/master/vignettes/user_manuals/3D_RNA-seq_command_line_user_manual.md)

Go back to Table of contents

Input files

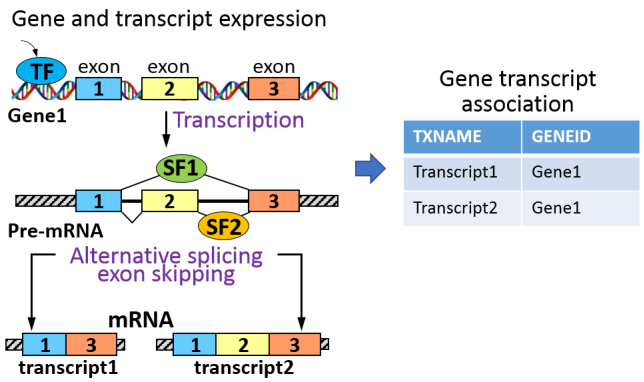
1. Gather the meta-data of the experimental design in a csv spreadsheet, the columns of which must include the following information (Figure 1A):
    - The first row is the header line of the meta-data table.
    - A column of the factors or multiple columns of the factors of the experimental design.
    - A column of the biological replicate labels.
    - A column of the sequencing/technical replicate labels if they exist.
    - A column of the file names of transcript quantifications.

    Note: In the 3D RNA-seq analysis, users can select the experimental design information according to the column names in the header line.

2. A folder that contains the transcript quantification files. Each file contains transcript quantification data of a single sample. Read counts and TPMs for 3D analysis will be generated from (Figure 1B):
    - The “quant.sf” objects if these files are generated by Salmon command line (Patro et al., 2017).
    - The “abundance.tsv” objects if these files are generated by Kallisto (Bray et al., 2016).
    - The “xxx.tabular” objects with file extension “.tabular” if these files are generated by Salmon/Kallisto with Galaxy interface. Please go to the “Transcript quantification using Galaxy” manual for details: https://github.com/wyguo/ThreeDRNAseq/blob/master/vignettes/user_manuals/Transcript_quantification_using_Galaxy.md (https://github.com/wyguo/ThreeDRNAseq/blob/master/vignettes/user_manuals/Transcript_quantification_using_Galaxy.md)

    Note: The 3D analysis is executable in a computer with normal memory and CPU size. If the App is running on our server, it is recommended to reduce the data size to upload. Users can exclude all the files in sub-folders of transcript quantifications, except the files of “quant.sf” from Salmon command line. If the transcript quantifications are generated using Kallisto command line, users can keep “abundance.tsv” in the sub-folders and remove the other files (Figure 1B).

3. Transcript-gene association table. The file can be one of the following formats:



- “csv” spreadsheet with first column of transcript IDs and second column of gene IDs (Figure 1C) (recommended).
- Or the transcriptome sequence “fasta” file that has been used for transcript quantification with Salmon/Kallisto. Transcript names and gene IDs will be extracted the description line starting with “>” in the “fasta” file. However, if the “gene” tag in the discription line is missing, this file is invalid (Figure 1D).
- Or a “gtf” file of the transcriptome. Transcript names and gene IDs will be extracted from the “transcript_id” and “gene_id” tags in the last column, respectively (Figure 1E).

Note: **Transcript-gene mapping in “csv” file is recommended**. Depending on the size, it may take a while to generate the table from a “fasta” or a “gtf” file and any missing tags for transcript name and gene ID extraction in these files may lead to errors.

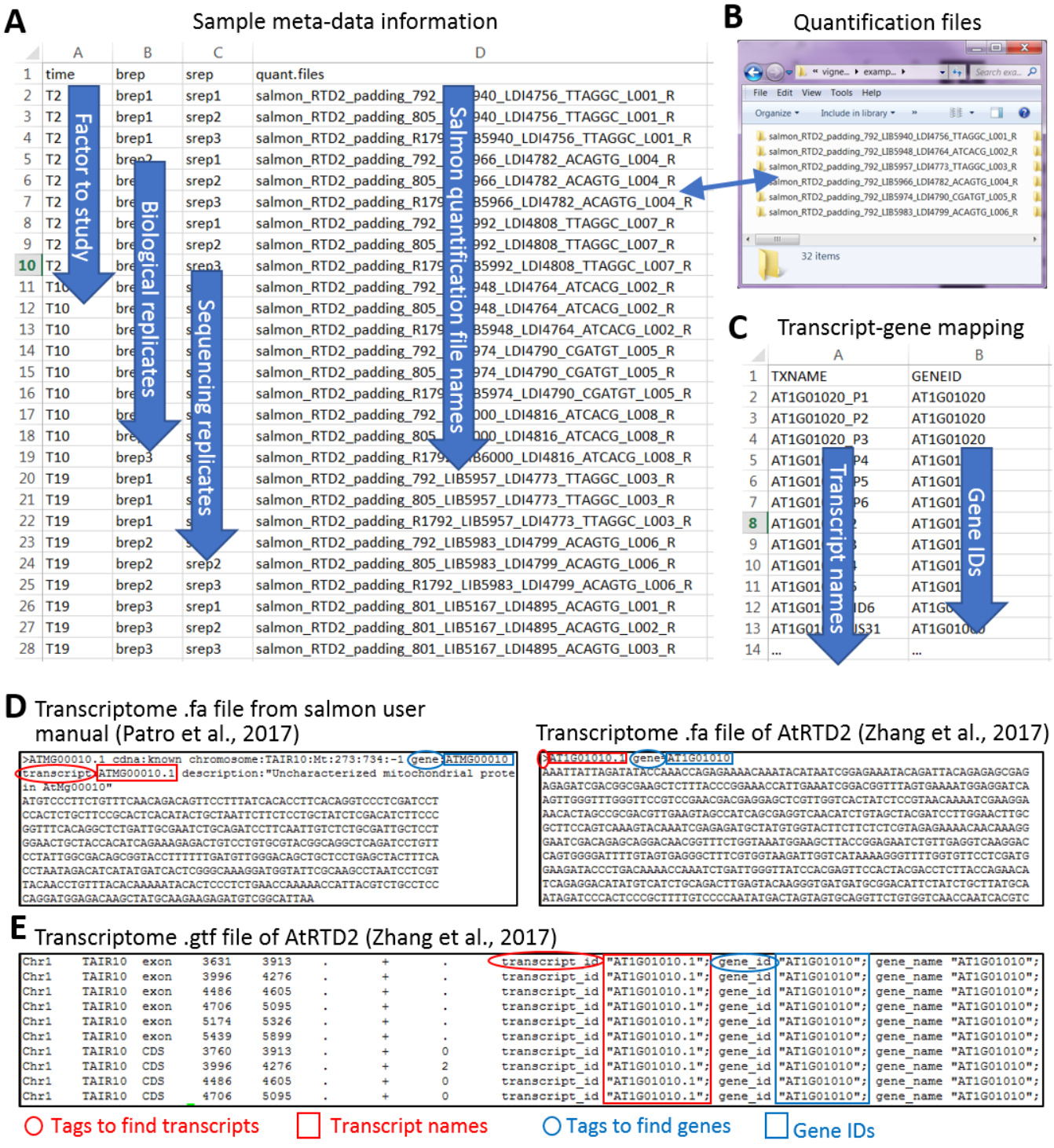


Figure 1: Input files of 3D RNA-seq App. The example is from a RNA-seq study of Arabidopsis in respond to cold (Calixto et al., 2018). (A) Meta-data table of sample information in csv file. (B) The folder contains transcript quantifications. The input of transcript-gene mapping information can be a “csv” spreadsheet with first column of transcript names and second column of gene IDs (C), a “.fa” file (D) or a “.gtf” file (E) of the transcriptome. If a “.fa” or a “.gtf” file is provided, the App will extract transcript-gene association information with specific tags.

Go back to Table of contents

Output files

The results of the 3D RNA-seq analysis are saved in the App working directory in four folders:

1. “report” folder: the final report in html, pdf and word formats will be saved in the “report” folder.
2. “result” folder: the gene lists generated from DE, DAS and DTU analysis will be saved as csv files in the “result” folder.
3. “figure” folder: the figures generated and saves through the analysis will be saved in the “figure” folder.
4. “data” folder: the intermediate datasets of the analysis will be saved in the “data” folder in R data format (.RData). R users can open and process these objects in R software for a further personalized analysis. The detailed descriptions of saved files can be found in “Appendix” at the end of the document.

Example data

Download link: https://www.dropbox.com/s/8fsceneq8jlegwi/3D_RNAseq_example_data.zip?dl=0 (https://www.dropbox.com/s/8vwuz6u2yl7v9qx/3D%20RNA-seq%20App%20example%20data.zip?dl=0)

Description: This example is a sub-dataset from a time-series study of Arabidopsis plants exposed to cold (Calixto et al., 2018). RNA-seq data of 6 time-points were extracted from the whole dataset. The time-points are 3 and 6 hours after dusk at 20^oC, the first day of transition to 4^oC and the fourth day of acclimation to 4^oC (red boxes in Figure 2). Each time-point has 3 biological replicates and each biological replicate has 3 sequencing replicates. Transcript quantifications were generated using Salmon (Patro et al., 2017) and AtRTD2-QUASI (Zhang et al., 2017) as the reference transcriptome. To further reduce the data size, the expression of 8,944 transcripts (from 2,000 genes) were extracted from the Salmon quantification to identify the cold response genes and transcripts at both transcriptional and AS level.

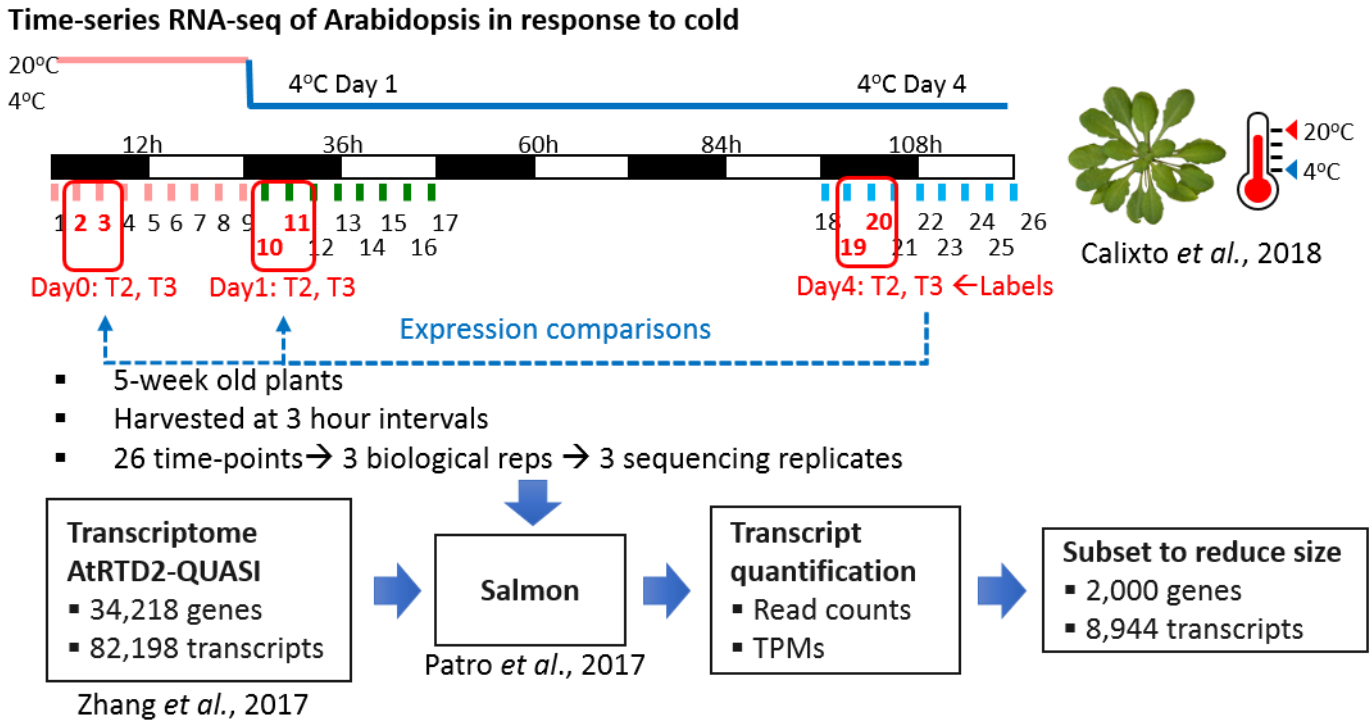


Figure 2: Experimental design of time-series RNA-seq data from study of Arabidopsis cold response. For this example, a subset of samples, genes and transcripts were extracted from the whole dataset to reduce data size.

Go back to Table of contents

Run analysis on 3D App

Basic Workflow

3D RNA-seq App is made of control widgets that users can interact with and send messages to the underlying R code to perform analysis by simple clicking/dragging of the mouse (Figure 3).

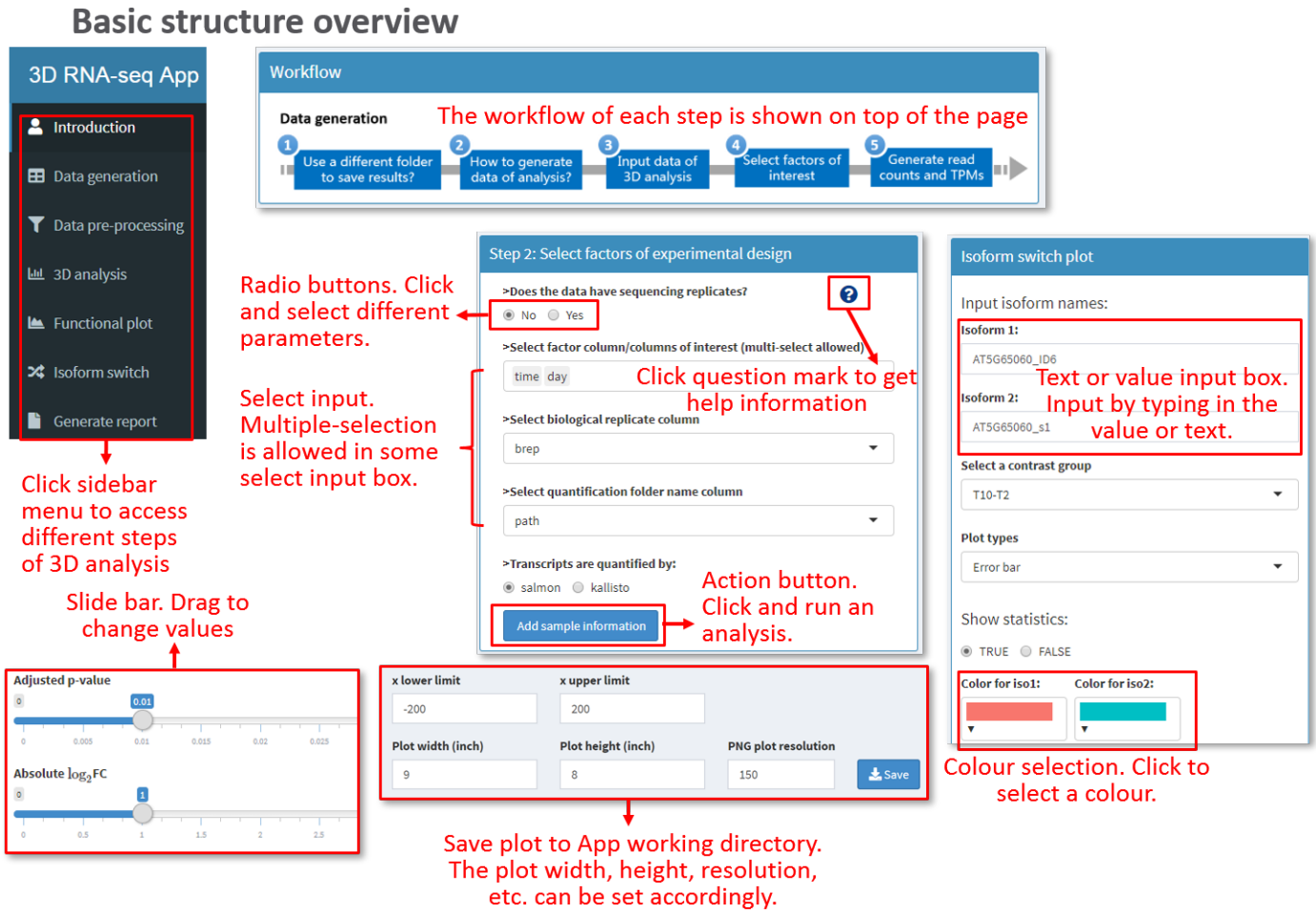


Figure 3: Basic widgets of 3D RNA-seq App.

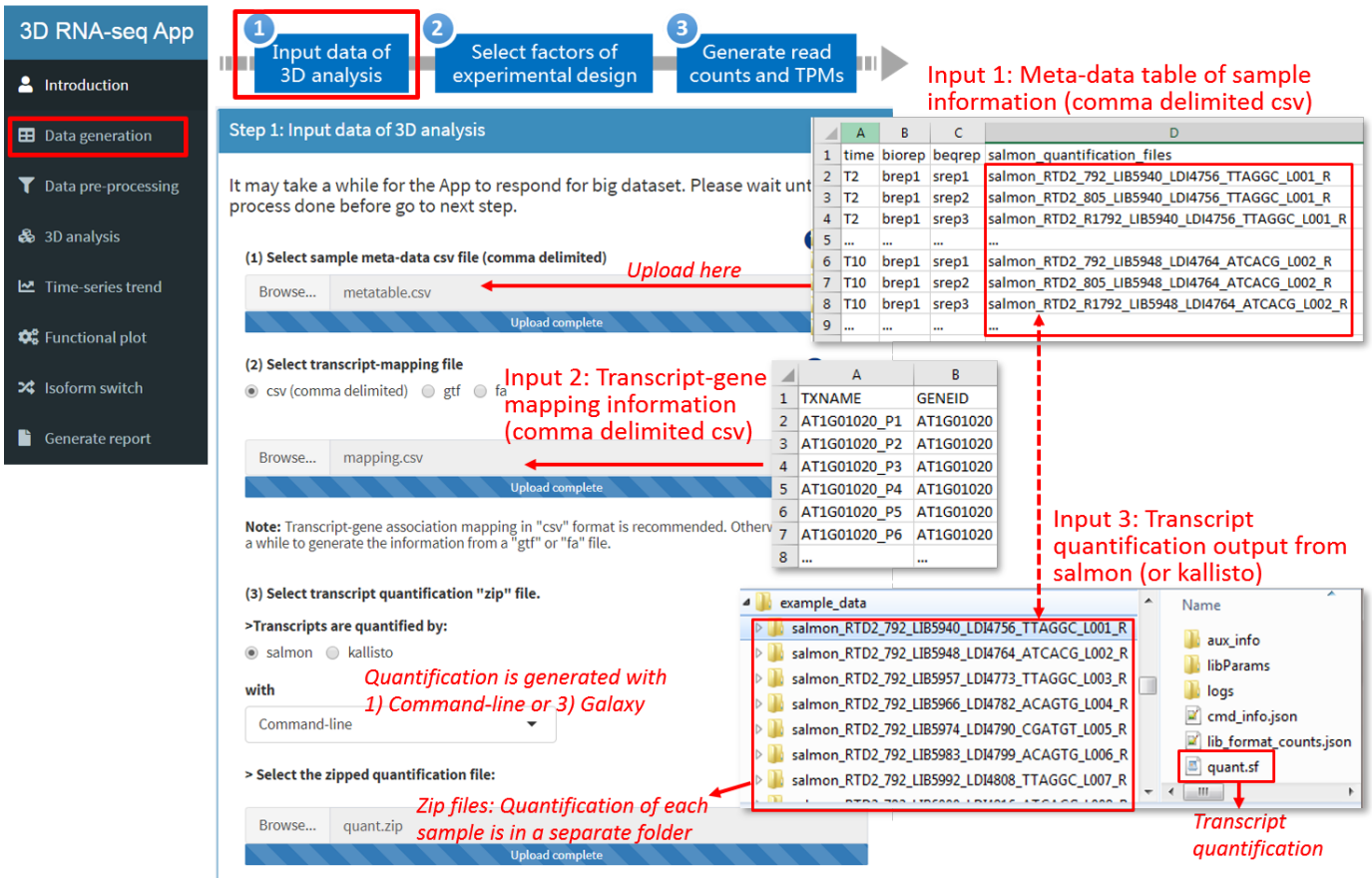
Running 3D RNA-seq just requires users to follow the steps from Tab panel 1 to 7.

Tab panel 1: Data generation

Users can upload input files and experimental design metadata file in this tab panel. Transcript level and gene level read counts and TPMs (transcript per million reads) are generated by using the tximport R package (Soneson et al., 2016).

Step 1: Upload input files to 3D RNA-seq App.

Data generation – Input data of 3D analysis

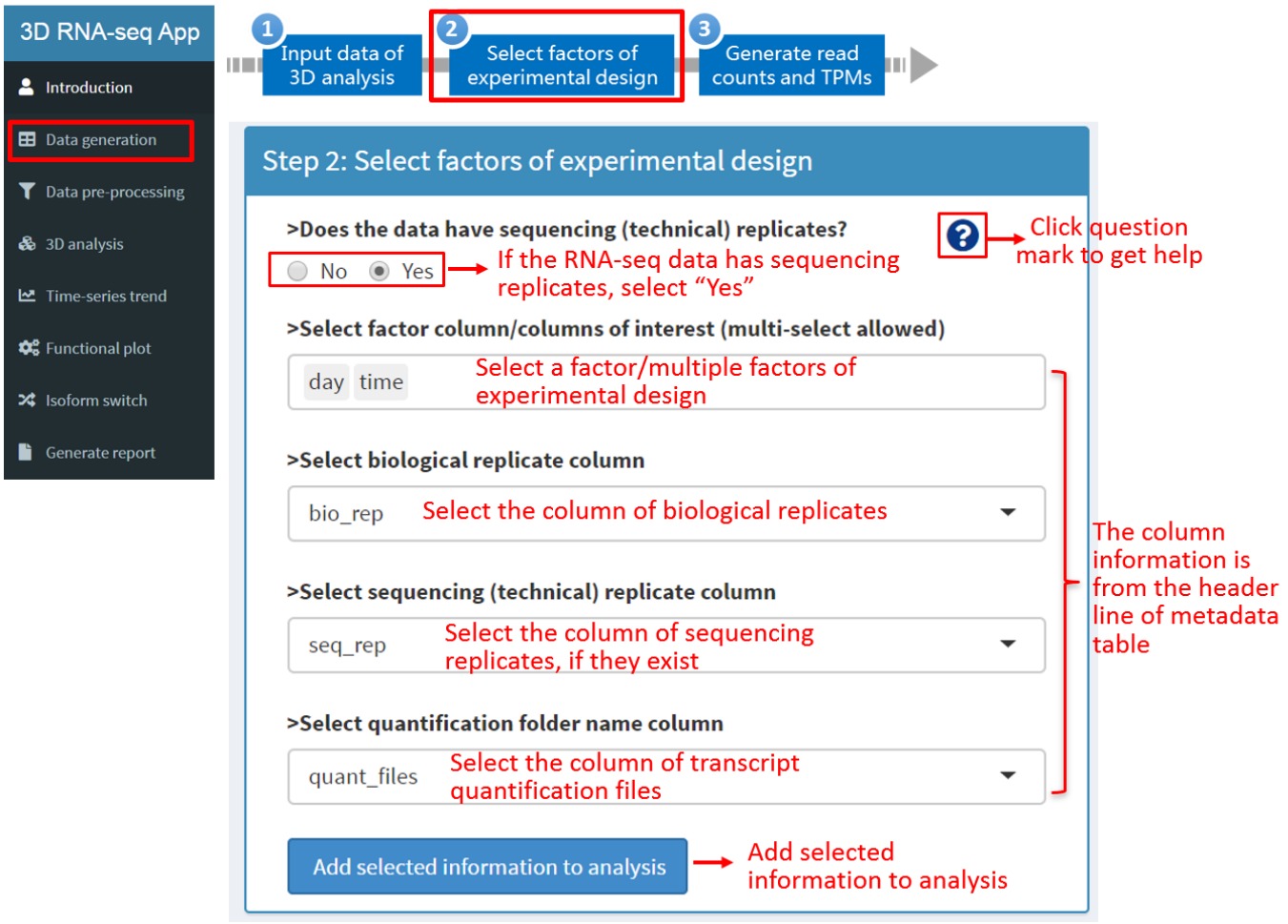


	A	B	C	D
1	time	biorep	beqrep	salmon_quantification_files
2	T2	brep1	srep1	salmon_RTD2_792_LIB5940_LDI4756_TTAGGC_L001_R
3	T2	brep1	srep2	salmon_RTD2_805_LIB5940_LDI4756_TTAGGC_L001_R
4	T2	brep1	srep3	salmon_RTD2_R1792_LIB5940_LDI4756_TTAGGC_L001_R
5	...	...	...	...
6	T10	brep1	srep1	salmon_RTD2_792_LIB5948_LDI4764_ATCACG_L002_R
7	T10	brep1	srep2	salmon_RTD2_805_LIB5948_LDI4764_ATCACG_L002_R
8	T10	brep1	srep3	salmon_RTD2_R1792_LIB5948_LDI4764_ATCACG_L002_R
9	...	...	...	...

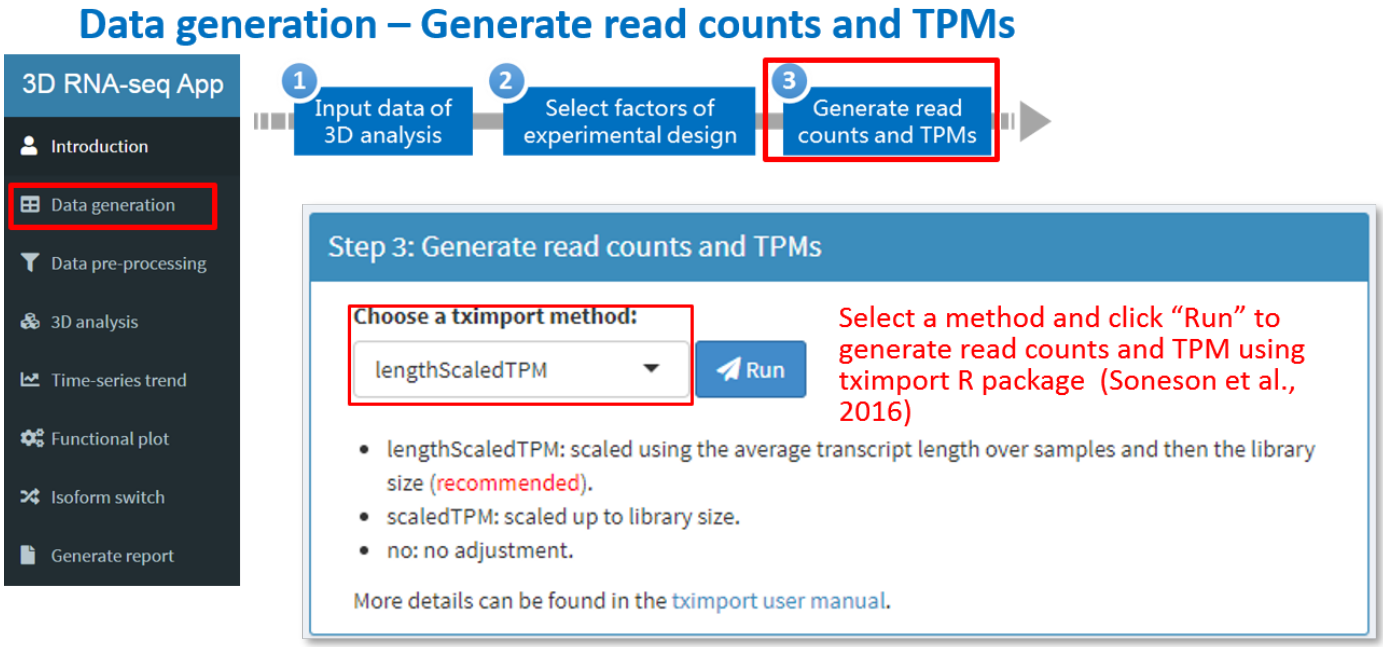
	A	B
1	TXNAME	GENEID
2	AT1G01020_P1	AT1G01020
3	AT1G01020_P2	AT1G01020
4	AT1G01020_P3	AT1G01020
5	AT1G01020_P4	AT1G01020
6	AT1G01020_P5	AT1G01020
7	AT1G01020_P6	AT1G01020
8	...	...

Step 2: Select the columns of factors, labels of replicates and quantification file names from meta-data table for 3D analysis.

Data generation – Select factors of experimental design



Step 3: Generate gene and transcript read counts and TPMs.



Soneson,C., Love,M.I., and Robinson,M.D. (2016) Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Research, 4, 1521.

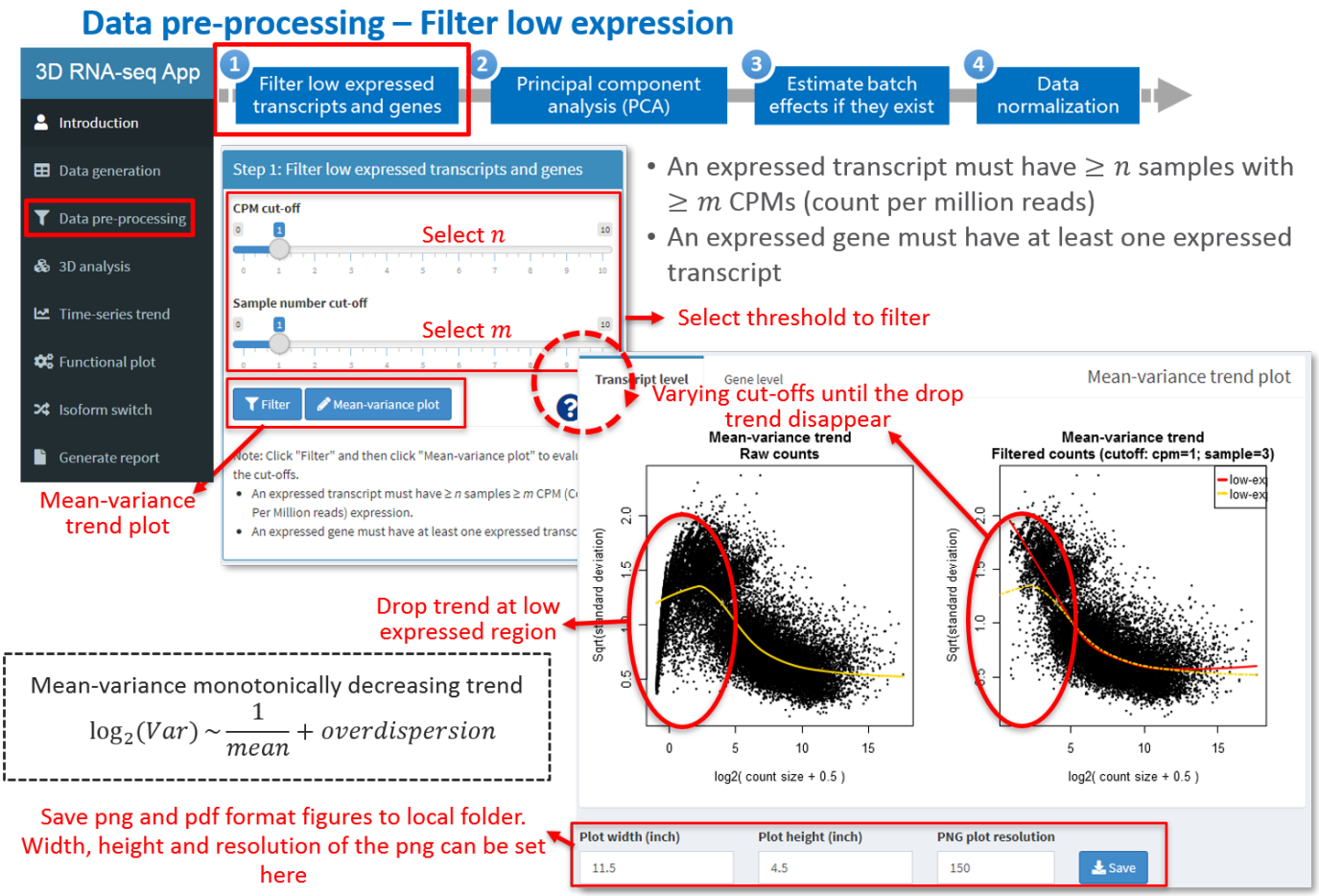
When the button "Run" is clicked, the process currently running will be shown at the lower right corner of the web browser. Once it is done, you can move to Tab panel 2 using the navigation menu on the left and make sure you roll your page to the very top.

Go back to Table of contents

Tab panel 2: Data pre-processing

Once the read counts and TPMs are generated, the data will go through a number of pre-processing steps. In each step, quality control plots are generated to optimise the parameters for pre-processing. If the RNA-seq data has sequencing replicates (seq-reps), they will be merged before 3D analysis according to the seq-rep labels selected by users in the meta-data table to increase sequencing depth, because seq-reps are generated by sequencing the same biological replicate multiple times (e.g. on different sequencing lanes), but they do not add much variability to the biological replicates.

Step 4: Filter low expressed transcripts and genes based on expression mean-variance trend.

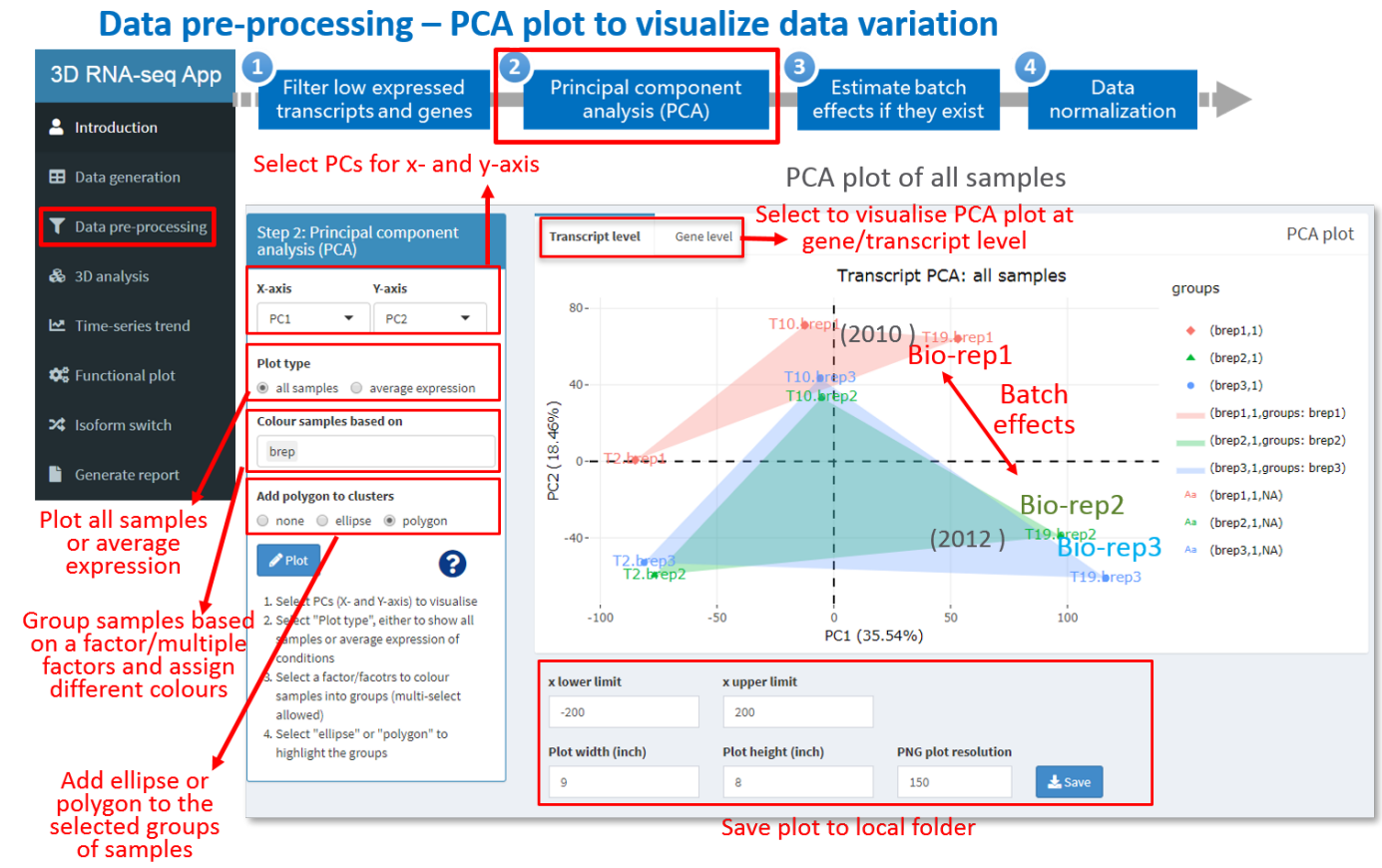


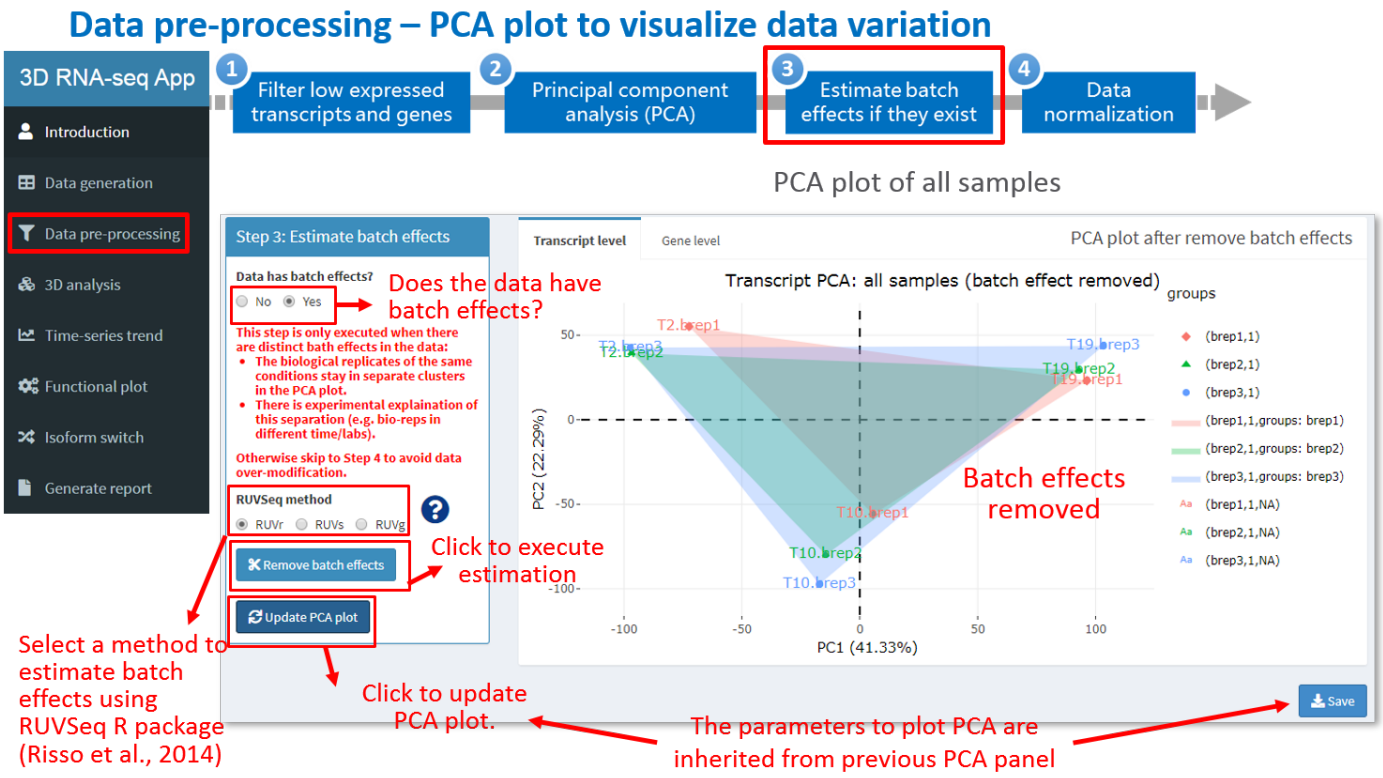
Law,C.W., Chen,Y., Shi,W., and Smyth,G.K. (2014) voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol, 15, R29.

Step 5: PCA plot and removing batch effects.

The PCA plot can be used to visualise the main variations of the expression data and identify whether the RNA-seq data contains batch effects, which are caused by biological replications being prepared in different, for example, laboratory conditions

In this panel, users can select and visualise different PCs based on transcript level or gene level expression of all samples or the average expression of biological replicates. The scatter points can be grouped and coloured according to different factors. Ellipses or polygons can be added to the plots to highlight the grouped clusters.

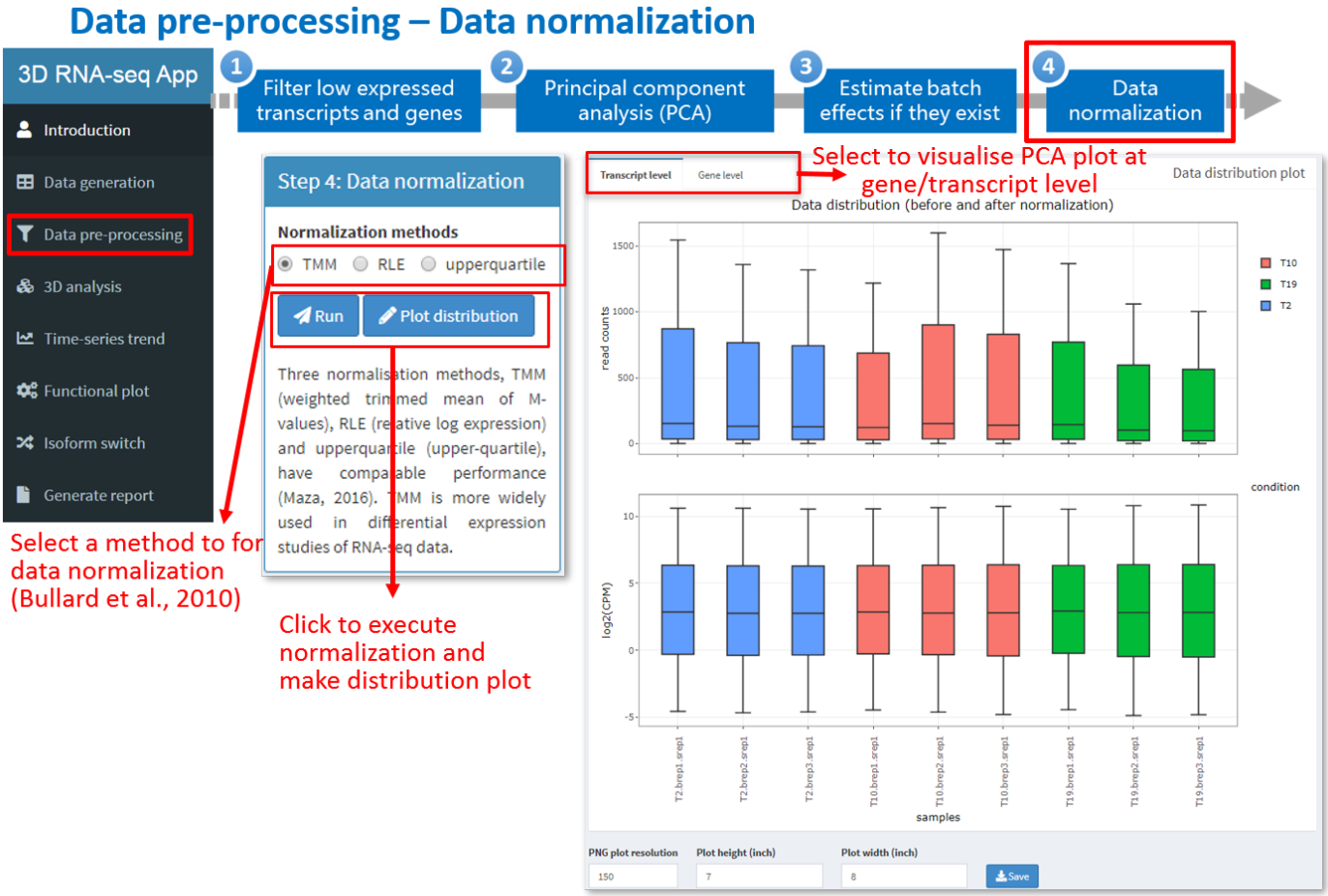




Risso,D., Ngai,J., Speed,T.P., and Dudoit,S. (2014) Normalization of RNA-seq data using factor analysis of control genes or samples. Nat. Biotechnol., 32, 896–902.

Step 6: Data normalisation.

For unbiased comparisons across samples, read counts must be normalised. Normalisation methods such as Trimmed Mean of M-values (TMM), Relative Log Expression (RLE) and upper-quartile can be used to reduce the effect from the systematic technical biases across samples (Bullard et al., 2010). Box plots are used to visualise the expression distribution of raw read counts and normalised expression across samples.



Bullard,J.H., Purdom,E., Hansen,K.D., and Dudoit,S. (2010) Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. *BMC Bioinformatics*, **11**, 94.

Once the normalization is done as shown at the lower right corner on the browser, please proceed to Tab panel 3.

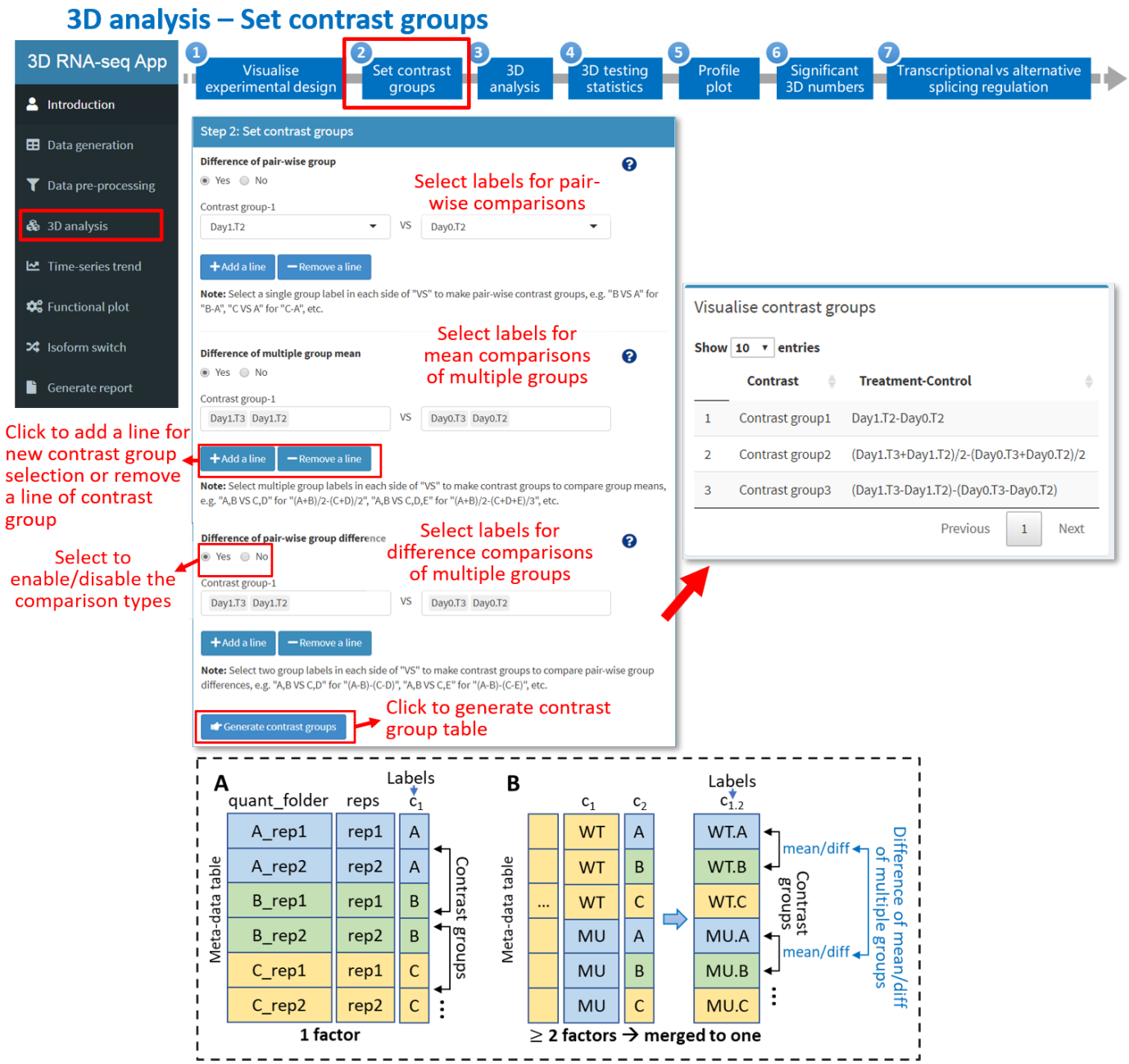
Go back to Table of contents

Tab panel 3: 3D analysis

Step 7: Set contrast groups and perform 3D analysis.

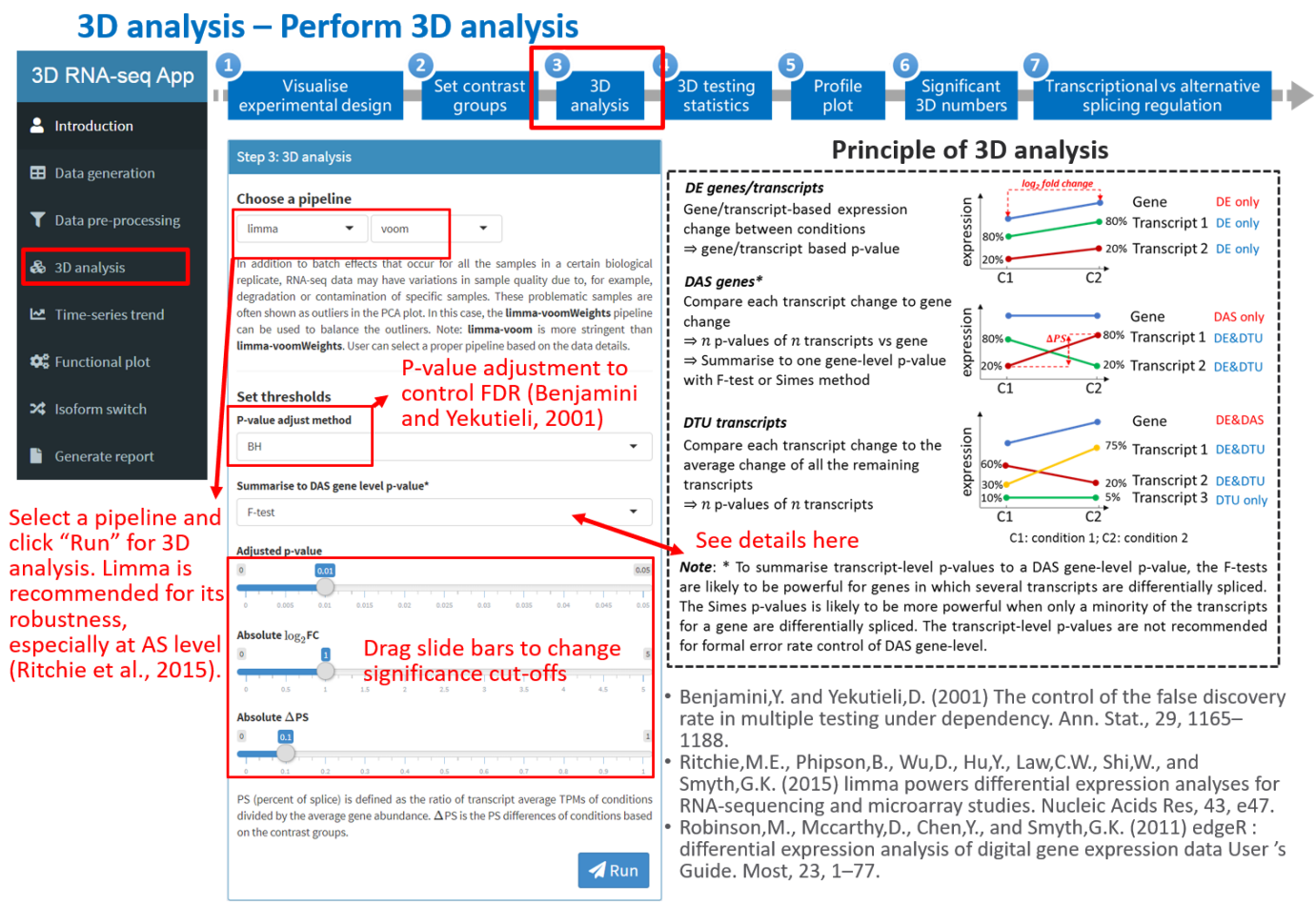
A contrast group is a user-defined comparison between two samples or two groups of samples:

1. Pair-wise comparison of samples: e.g. B-A compares group B to group A; C-B compares group C to group B (Figure A).
2. Compare mean of multiple samples: e.g. (WT.A+WT.B)/2-(MU.A+MU.B)/2 compares the mean of group WT.A and WT.B to the mean of group MU.A and MU.B (Figure B).
3. Compare difference of two differences (interactions): e.g. (WT.A-WT.B)-(MU.A-MU.B) compares the difference (***L_2FC) of group WT.A and WT.B to the difference (L_2FC)*** of group MU.A and MU.B (Figure B).



NOTE: if the experimental design involves multiple factor levels, e.g. condition A, B and C are performed in wildtype (WT) and mutant (MU), respectively, these factors will be combined to generate group labels

Step 8: Set statistical parameters and perform 3D analysis



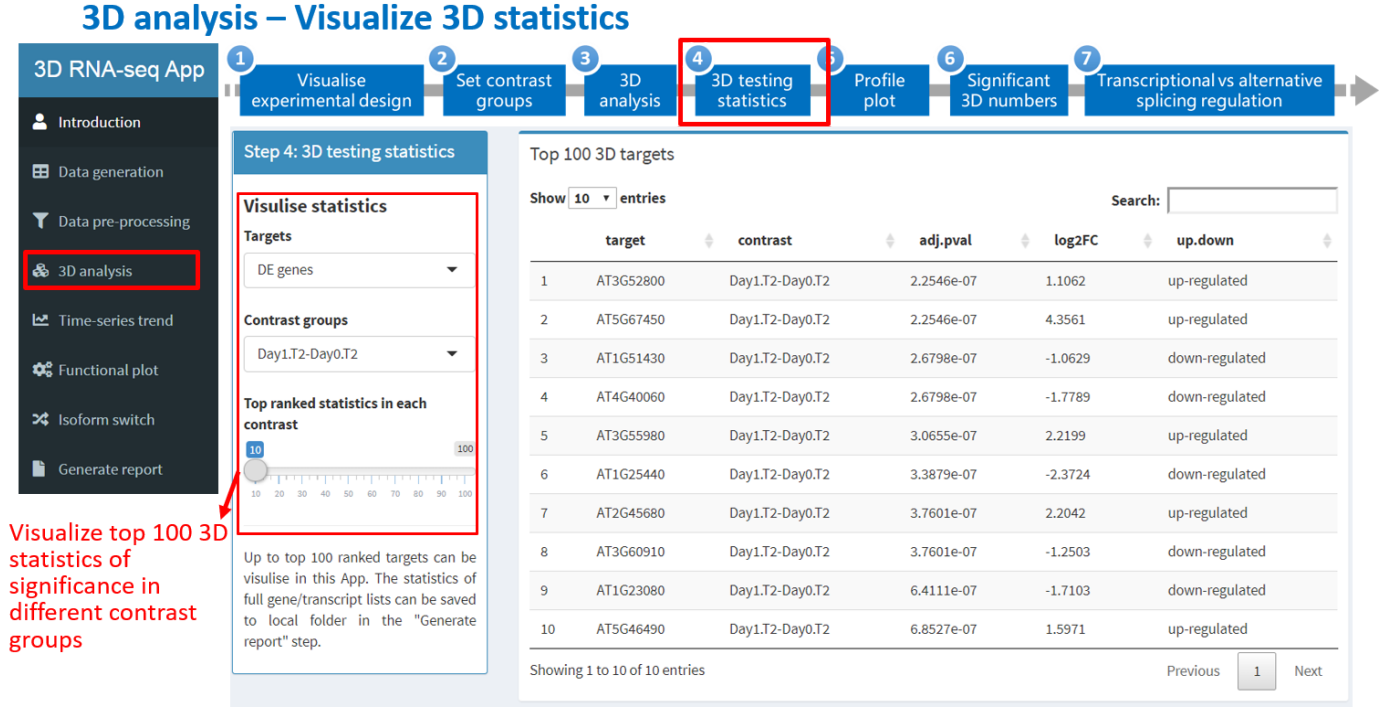
- Benjamini,Y. and Yekutieli,D. (2001) The control of the false discovery rate in multiple testing under dependency. Ann. Stat., 29, 1165–1188.
- Ritchie,M.E., Phipson,B., Wu,D., Hu,Y., Law,C.W., Shi,W., and Smyth,G.K. (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res, 43, e47.
- Robinson,M., Mccarthy,D., Chen,Y., and Smyth,G.K. (2011) edgeR : differential expression analysis of digital gene expression data User 's Guide. Most, 23, 1–77.

Significant result summary

After the the 3D analysis, the following information is summarized and will appear at the bottom of the page:

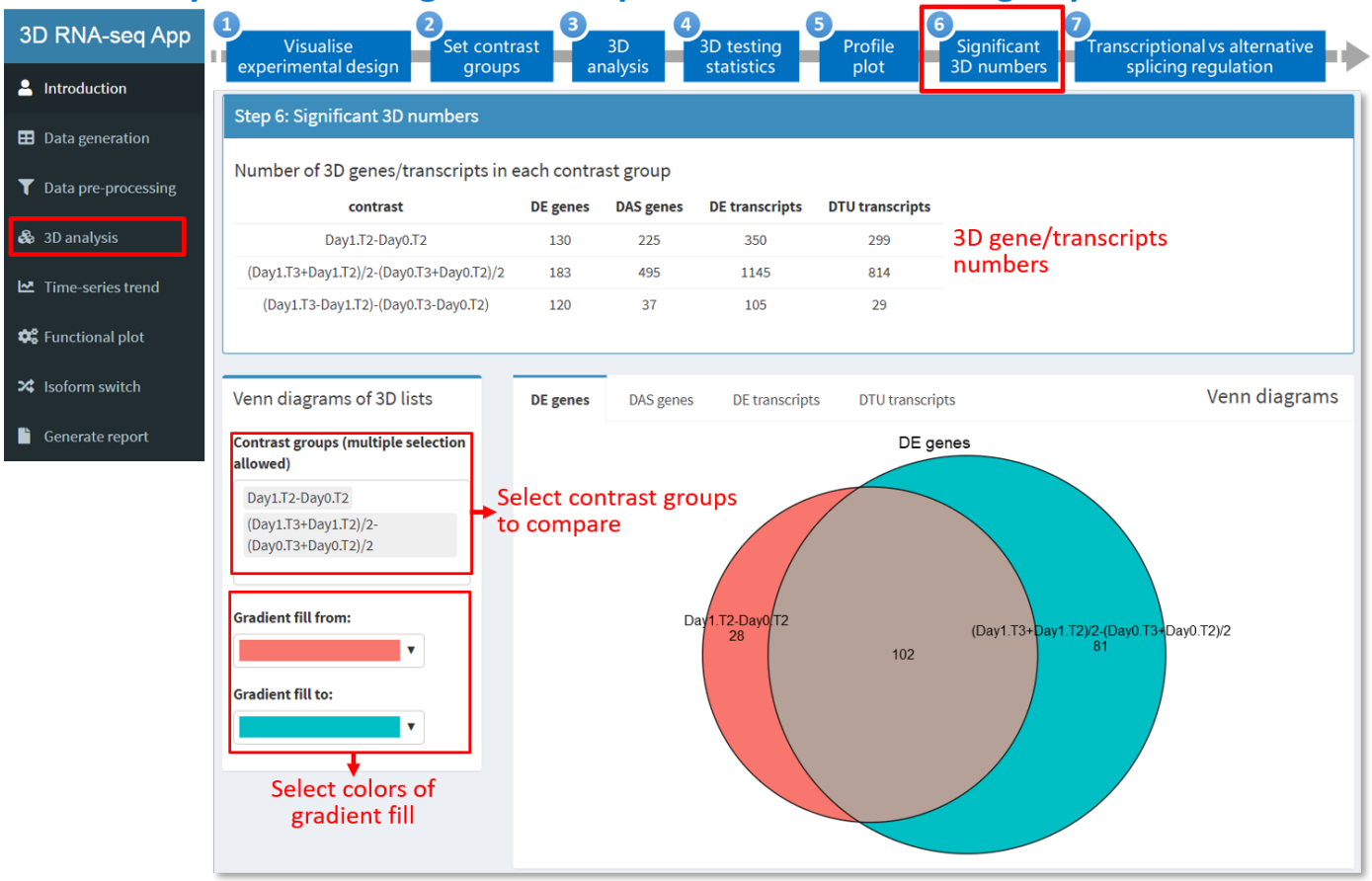
1. The test statistics in different contrast groups, e.g. adjusted p-value and L_2*F**C*.
2. The number of genes and transcripts with significant expression changes in contrast groups.
3. The number of up- and down-regulated DE genes and transcripts.
4. The numbers of genes/transcripts regulated only by transcription (DE), only by alternative splicing (DAS/DTU) and by both transcription and alternative splicing (DE+DAS/DE+DTU).

These summaries can be filtered, customized and the figures can be generated and saved with specified formats and sizes.



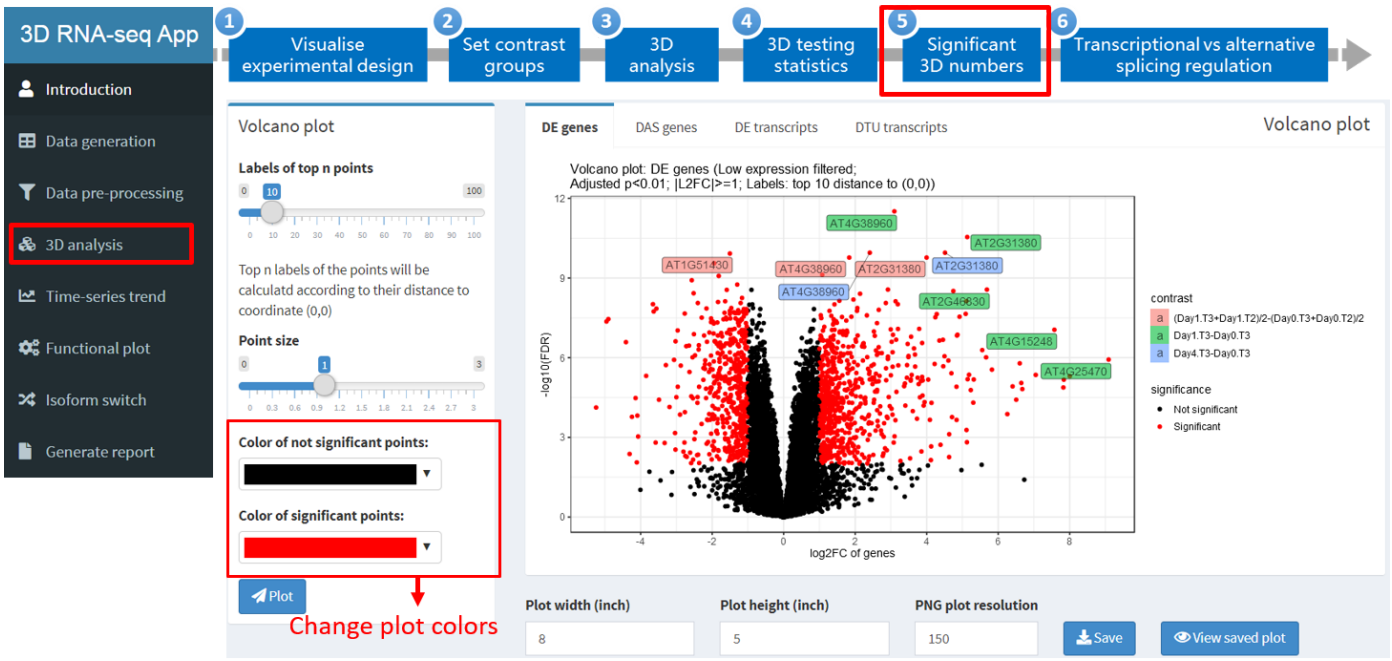
3D analysis – expression profile & percent spliced plots

3D analysis – Venn diagram to compare 3D lists in contrast groups

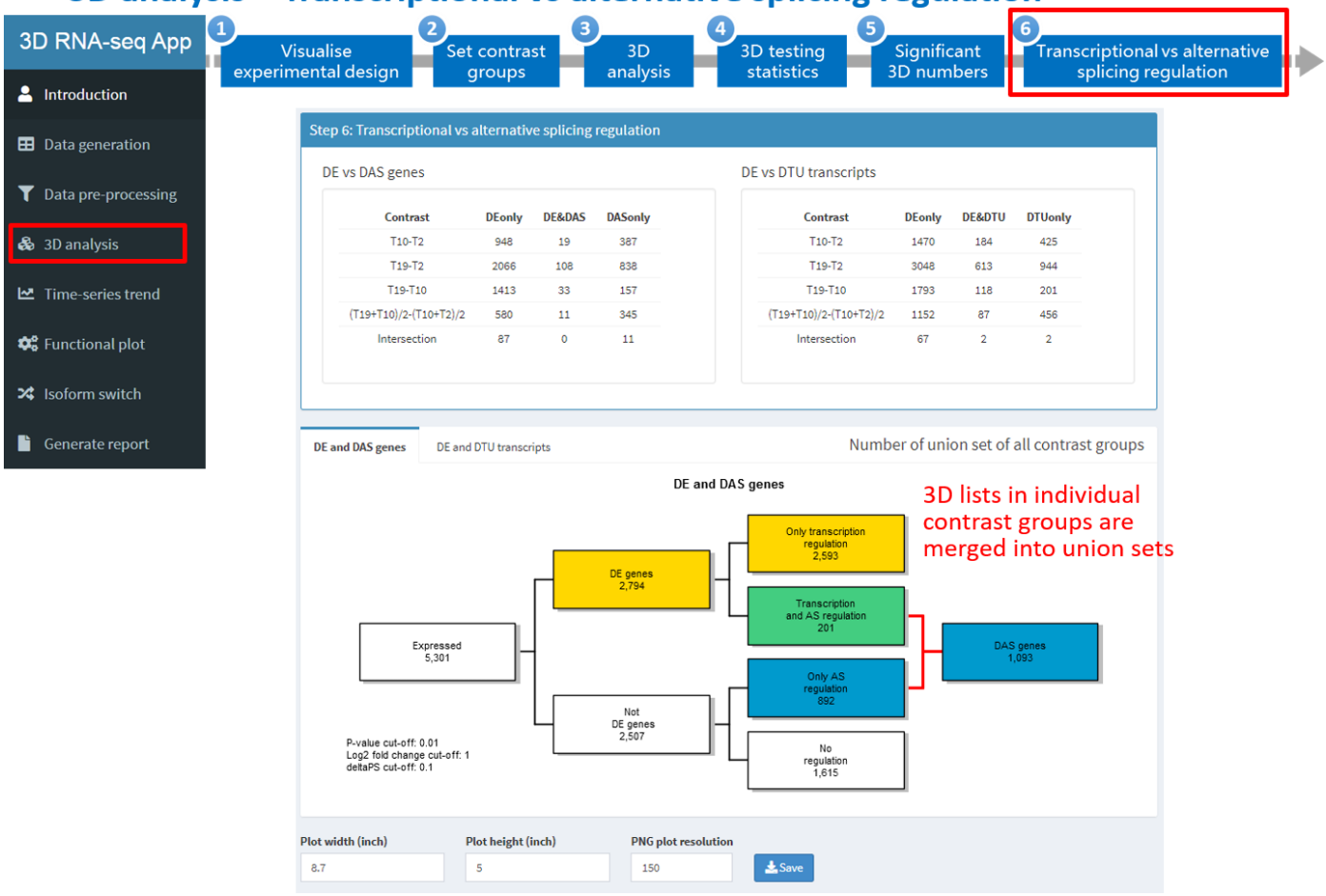


3D analysis – Up- and down-regulation plot

3D analysis – Volcano plot



3D analysis – Transcriptional vs alternative splicing regulation



Contrast	DEonly	DE&DAS	DASonly
T10-T2	948	19	387
T19-T2	2066	108	838
T19-T10	1413	33	157
(T19+T10)/2-(T10+T2)/2	580	11	345
Intersection	87	0	11

Contrast	DEonly	DE&DTU	DTUonly
T10-T2	1470	184	425
T19-T2	3048	613	944
T19-T10	1793	118	201
(T19+T10)/2-(T10+T2)/2	1152	87	456
Intersection	67	2	2

Go back to Table of contents

Tab panel 4: Time-series trend analysis

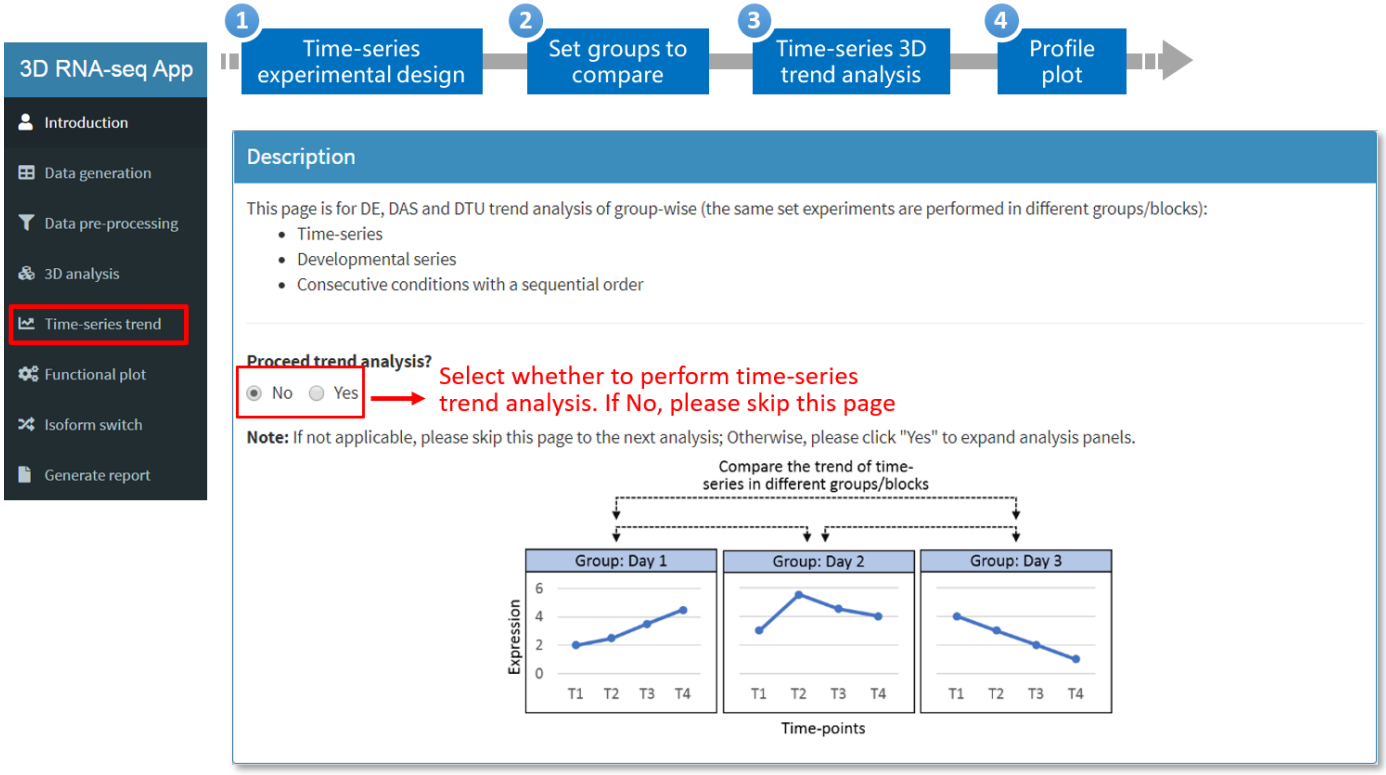
Time-series trend analysis aims to study an experiment with many time-points in each group (see the following table). When there are many time-points, it is recommended to analyse on smoothed expression changes over time instead of discrete comparisons of individual time-points in each group.

Day	Time
Day1	T1

Day	Time
Day1	T2
Day1	T3
Day1	T4
Day2	T1
Day2	T2
Day2	T3
Day2	T4

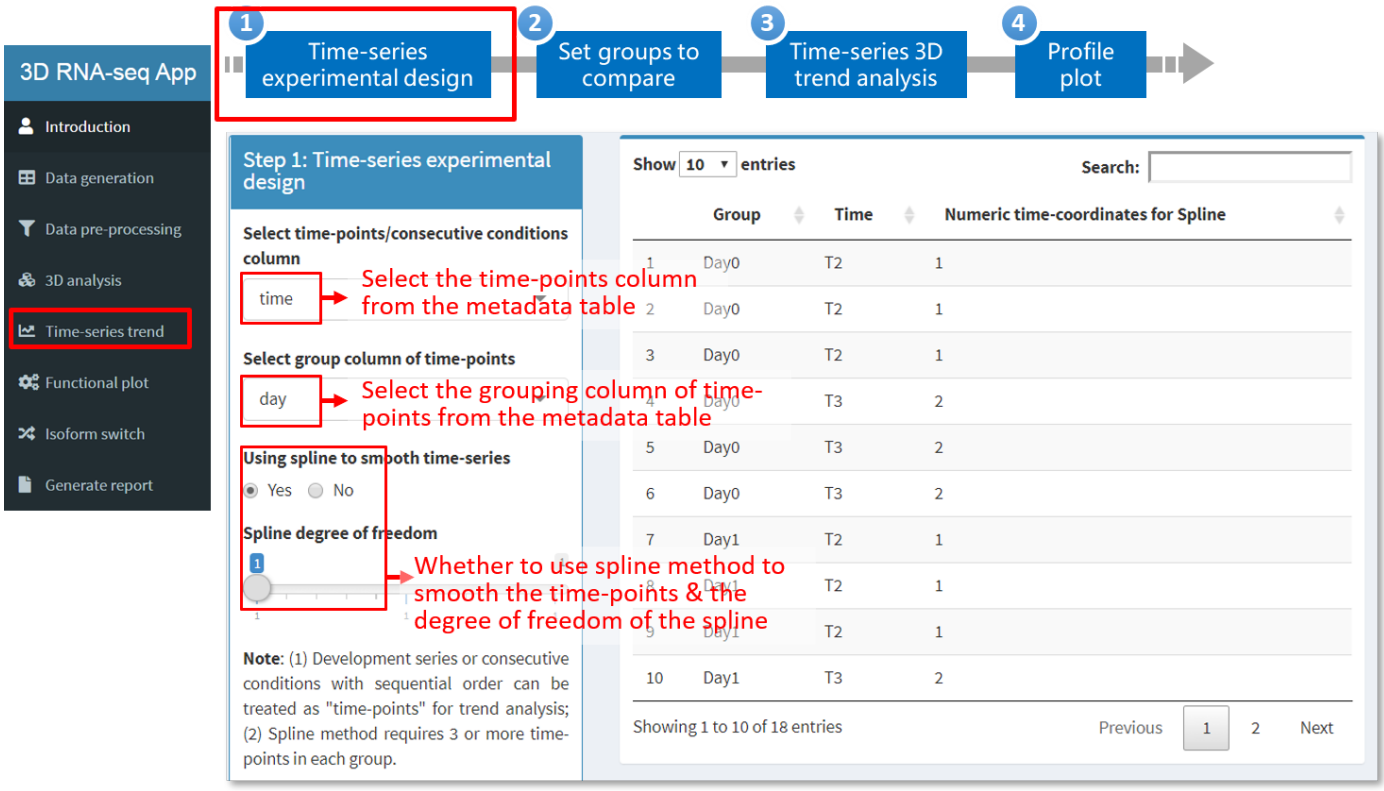
Step 9: Whether to perform time-series trend analysis.

Select whether to perform time-series trend analysis. If “No”, please skip this panel.



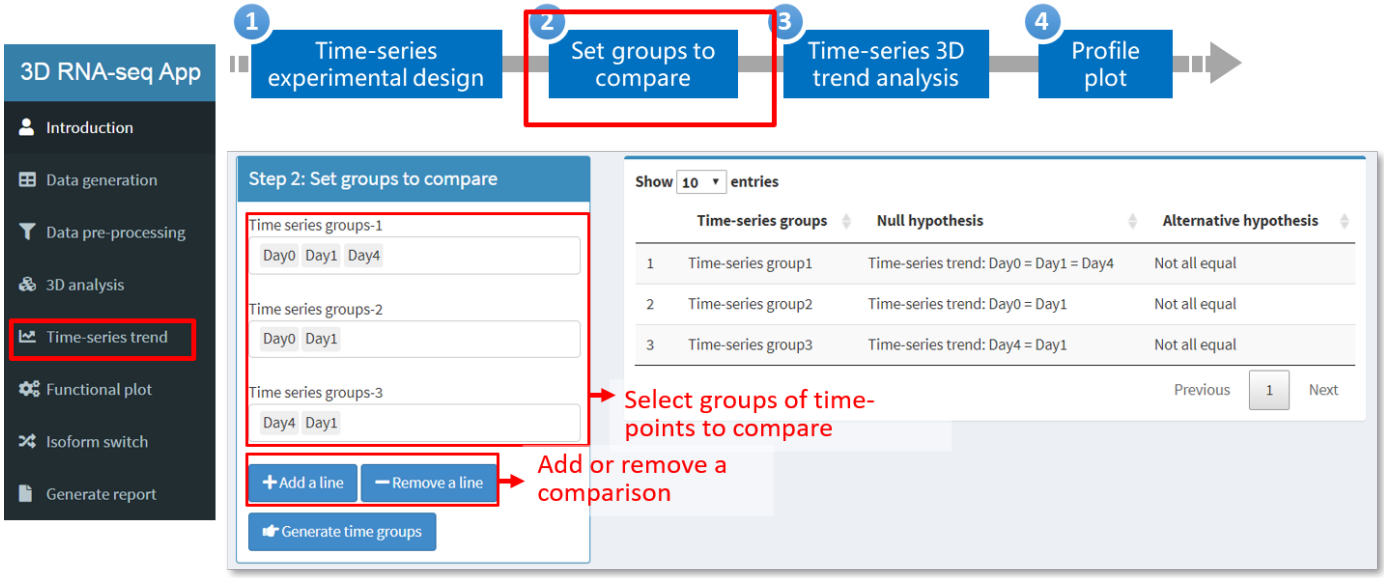
Step 10: Time-series experimental design.

1. Select the columns of time-points and grouping of time-points according to the header information of the metadata table.
2. Select whether to use spline method to smooth the expression changes over time-points and the degree to use for the spline ((https://en.wikipedia.org/wiki/Spline_(mathematics))https://en.wikipedia.org/wiki/Spline_(mathematics) (https://en.wikipedia.org/wiki/Spline_(mathematics))>). . If “No” is selected, trend will be compared in the way of discrete jumping from one to another time-point in each group.

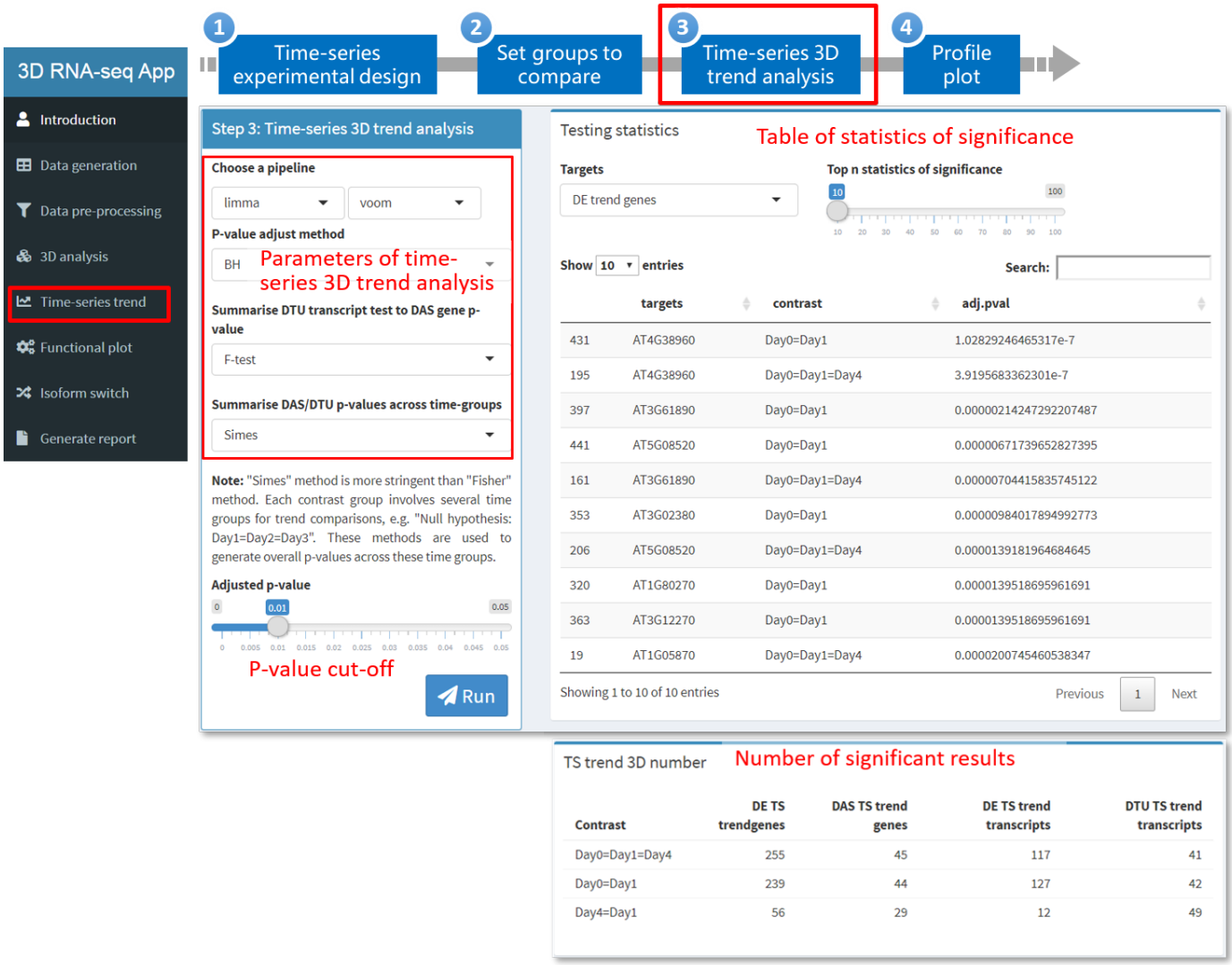


Step 11: Set groups to compare

Select multiple groups for comparisons of time-series expression change trends between these groups. User can add a line to activate a new comparison.



Step 12: 3D analysis of time-series trend

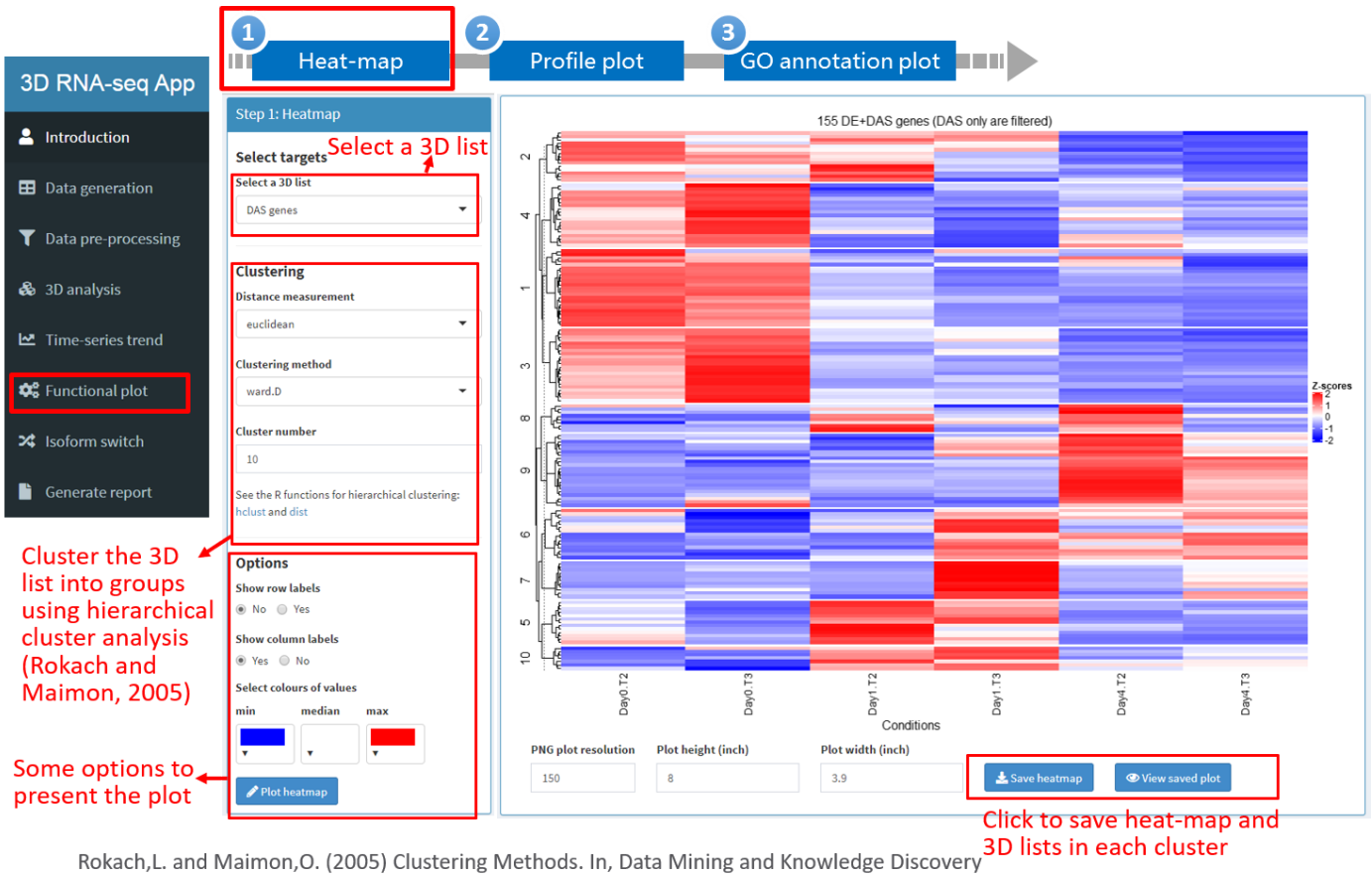


Go back to Table of contents

Tab panel 5: Functional plot

Step 13: Generating heat-maps.

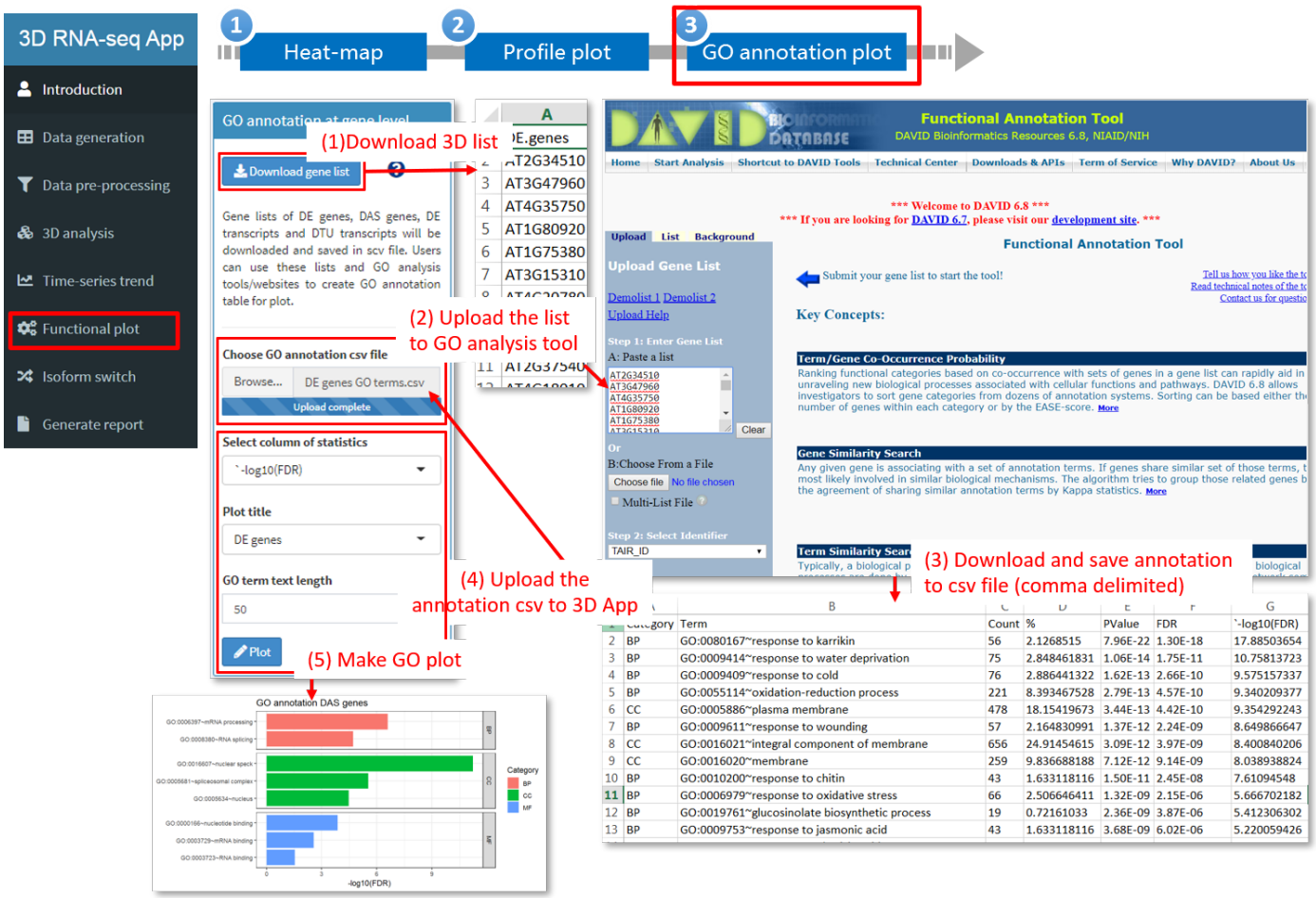
Users can make heat-maps of significant DE genes, DAS genes, DE transcripts and DTU transcripts identified from the analysis. The heat-maps and the gene/transcript list in each cluster of the heat-maps can be saved to local folder.



Rokach,L. and Maimon,O. (2005) Clustering Methods. In, Data Mining and Knowledge Discovery Handbook. Springer-Verlag, New York, pp. 321–352.

Step 14: Generating GO enrichment plot.

Users can generate gene lists of DE genes, DAS genes, DE transcripts and DTU transcripts by clicking “Download gene list” button. These gene lists can be uploaded to Gene Ontology (GO) analysis tools/databases (e.g. DAVID and agriGO) to generate GO annotation. A csv file with GO annotation information is required to generate the annotation plot. The file includes a column of “Category” of CC (cellular component), BP (biological process) and MF (molecular function), a column of “Term” of GO annotation and the rest columns of statistics to report the annotation enrichment, e.g. count, -log10(FDR), etc.

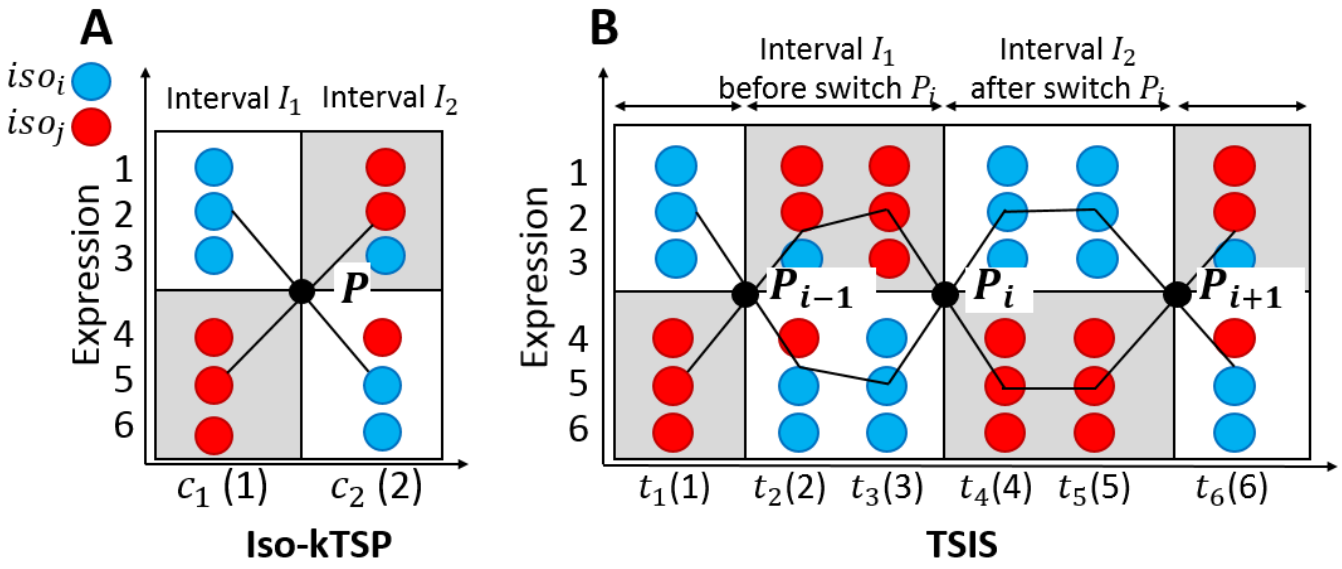


	Category	Term	Count	%	PValue	FDR	`-log10(FDR)
2	BP	GO:0080167~response to karrikin	56	2.1268515	7.96E-22	1.30E-18	17.88503654
3	BP	GO:0009414~response to water deprivation	75	2.848461831	1.06E-14	1.75E-11	10.75813723
4	BP	GO:0009409~response to cold	76	2.886441322	1.62E-13	2.66E-10	9.575157337
5	BP	GO:0055114~oxidation-reduction process	221	8.393467528	2.79E-13	4.57E-10	9.340209377
6	CC	GO:0005886~plasma membrane	478	18.15419673	3.44E-13	4.42E-10	9.354292243
7	BP	GO:0009611~response to wounding	57	2.164830991	1.37E-12	2.24E-09	8.649866647
8	CC	GO:0016021~integral component of membrane	656	24.91454615	3.09E-12	3.97E-09	8.400840206
9	CC	GO:0016020~membrane	259	9.836688188	7.12E-12	9.14E-09	8.038938824
10	BP	GO:0010200~response to chitin	43	1.633118116	1.50E-11	2.45E-08	7.61094548
11	BP	GO:0006979~response to oxidative stress	66	2.506646411	1.32E-09	2.15E-06	5.666702182
12	BP	GO:0019761~glucosinolate biosynthetic process	19	0.72161033	2.36E-09	3.87E-06	5.412306302
13	BP	GO:0009753~response to jasmonic acid	43	1.633118116	3.68E-09	6.02E-06	5.220059426

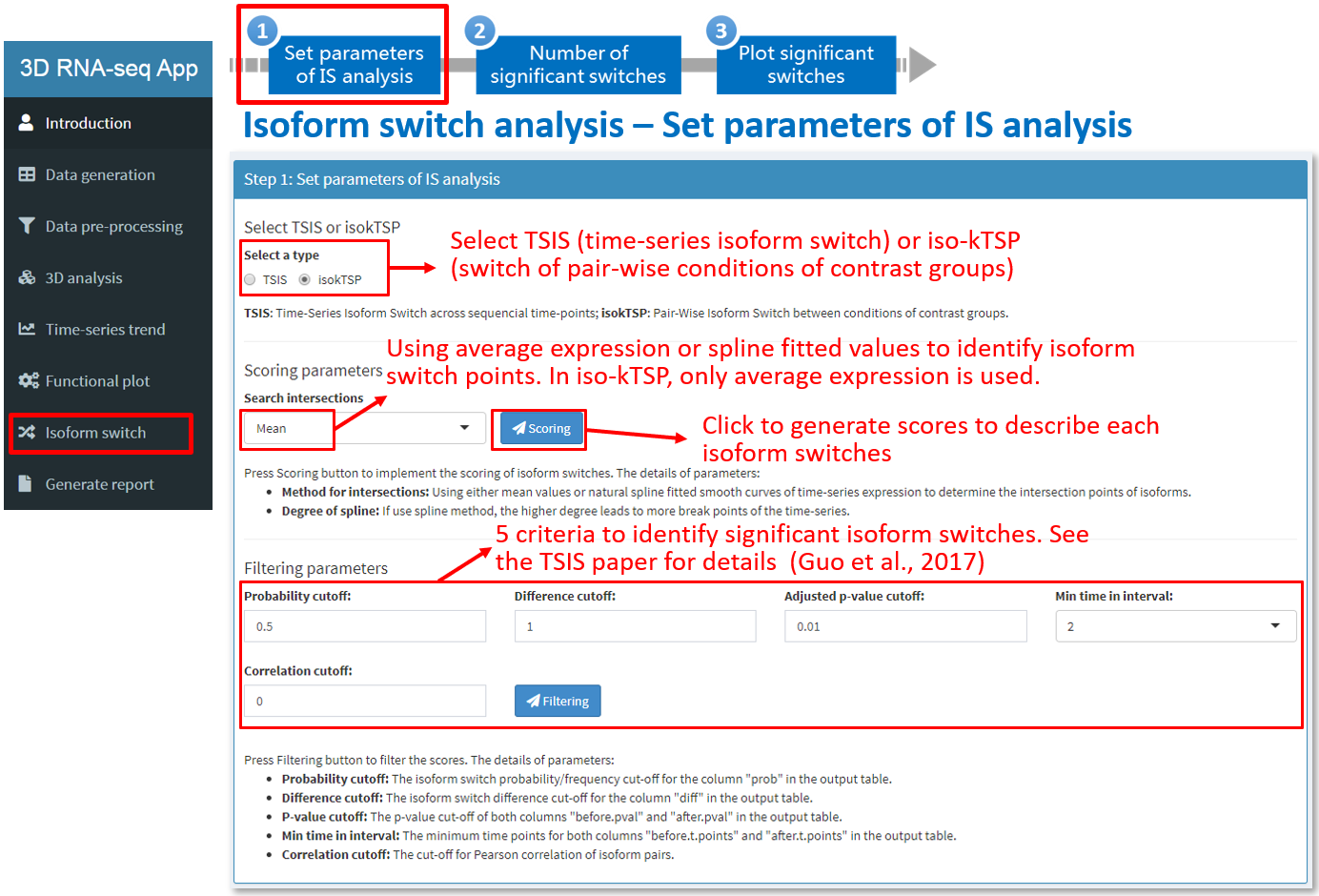
Go back to Table of contents

Tab panel 6: Isoform switch

Transcript isoform switches (ISs) occur when within a gen a pair of alternatively spliced isoforms reverse the order of their relative expression levels. **IsokTSP** is a method to detect transcript ISs between pair-wise conditions (Sebestyen et al., 2015) while **TSIS** (time-series IS) is used to identify ISs in time-series data (Guo et al., 2017).

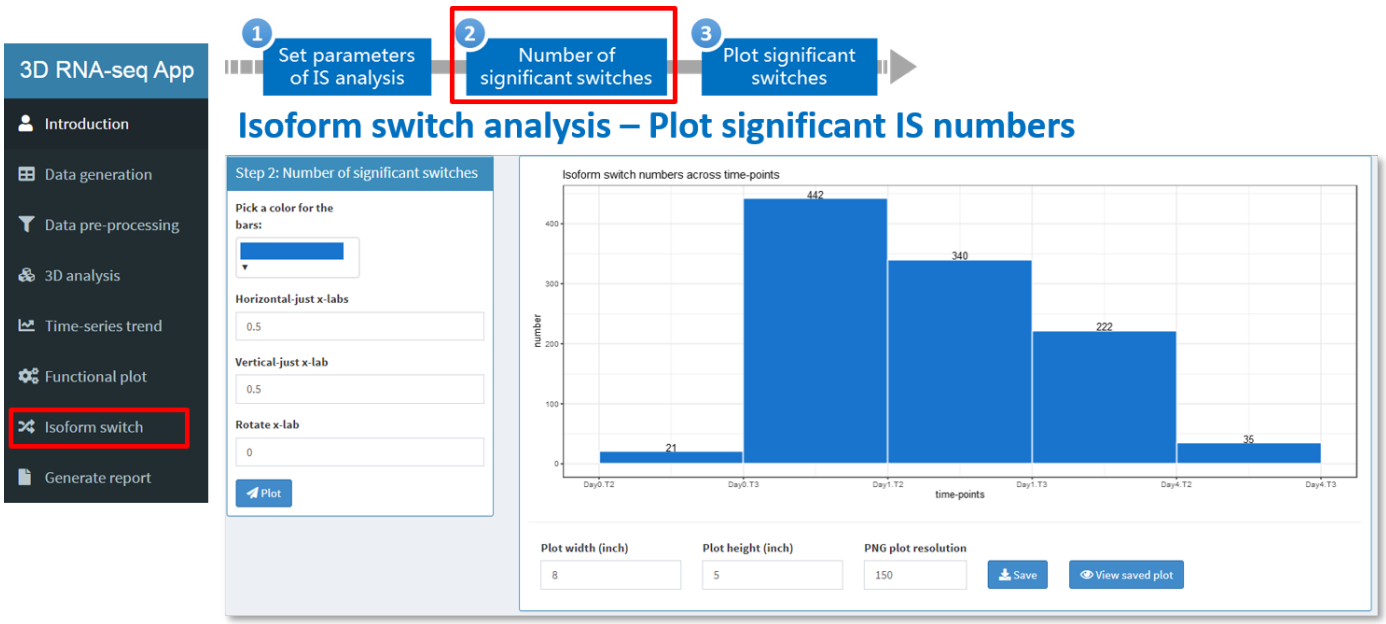


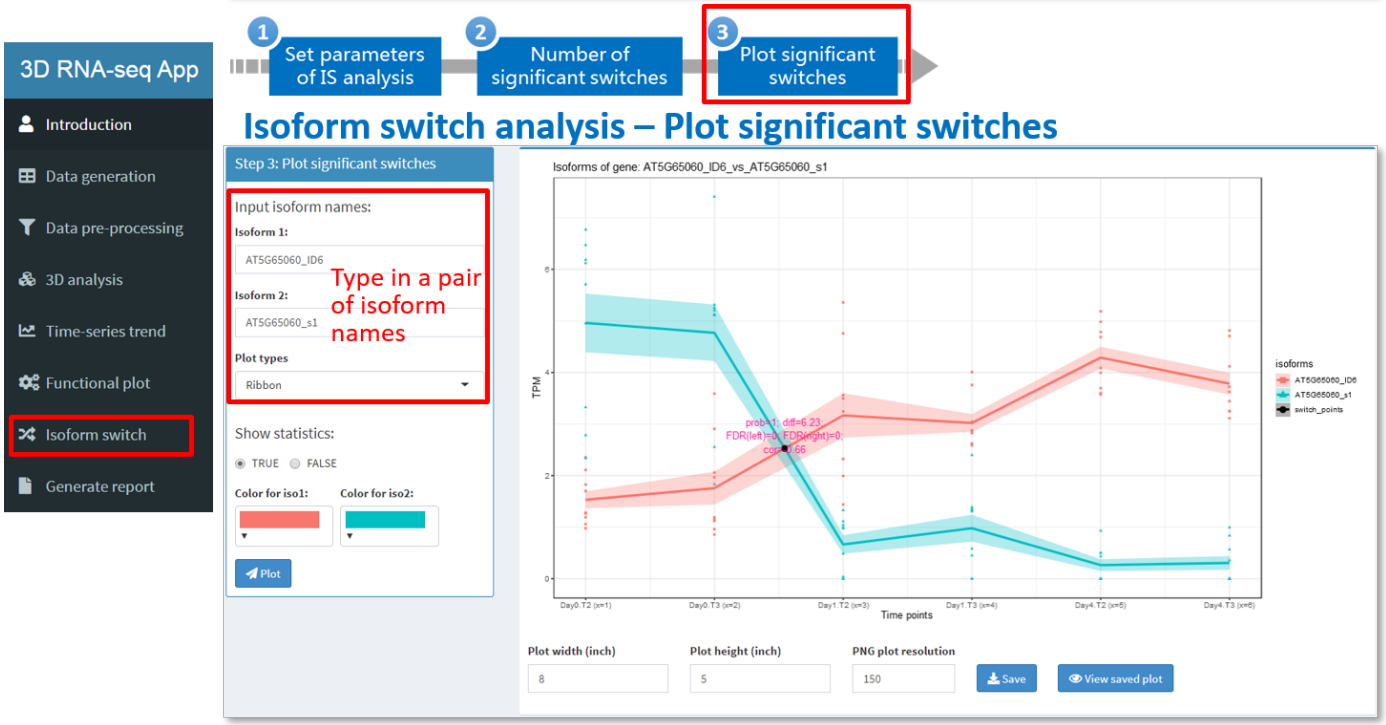
Step 15: Perform isoform switch analysis.



Guo,W., Calixto,C.P.G., Brown,J.W.S., and Zhang,R. (2017) TSIS: An R package to infer alternative splicing isoform switches for time-series data. Bioinformatics, 33, 3308–3310.

After the analysis is done, a number of plots will be generated automatically to visualize the results.



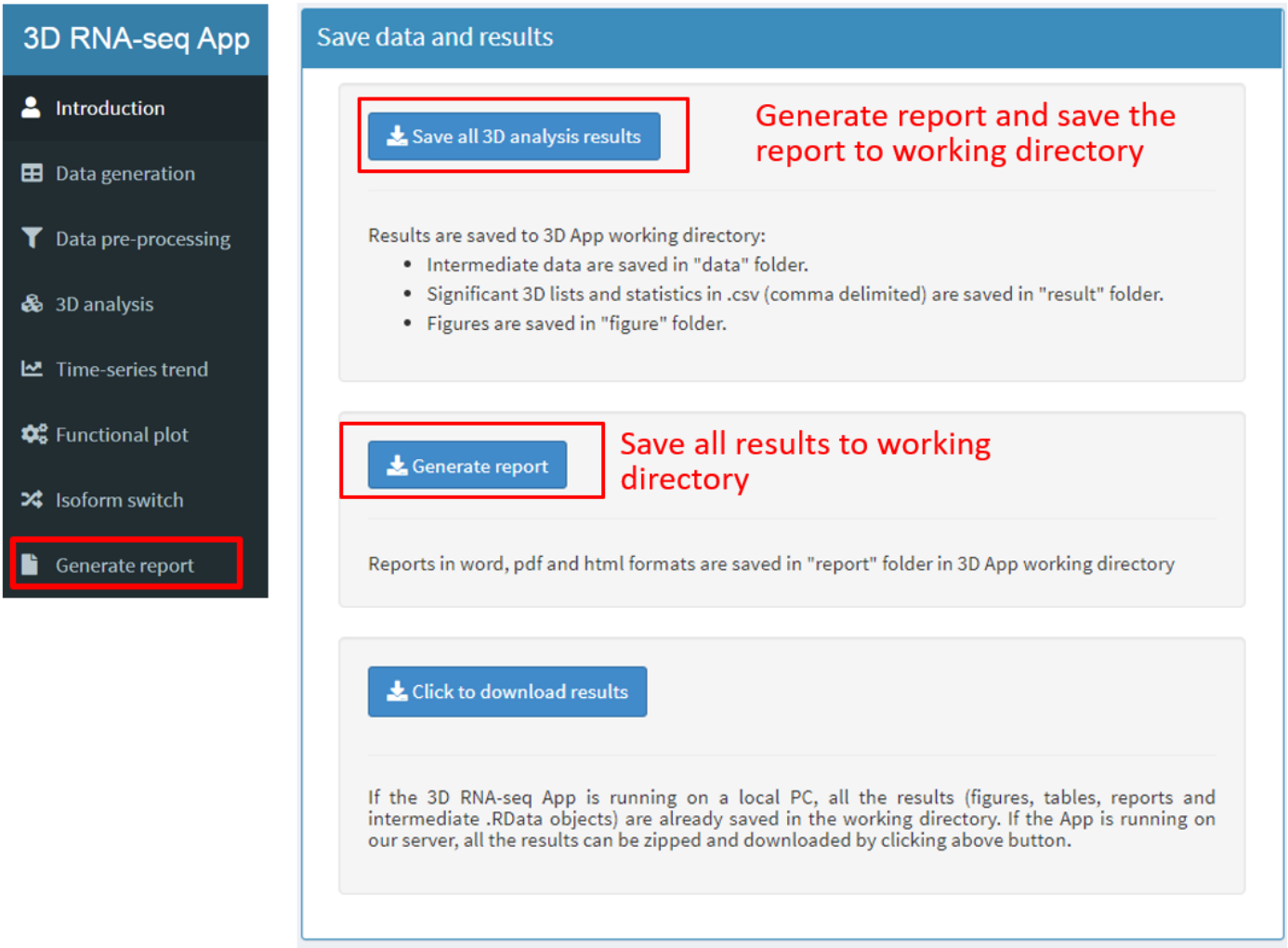


Tab panel 7: Generate report

Step 16: generate report and download all the results.

Publication-quality reports will generated and together with figures, results and statistics of significance can be downloaded in a zipped file.

Generate report



Go back to Table of contents

Appendix

Files in report folder

Reports are saved in report folder.

File name	Description
3D_report.pdf/html/doc	Report of 3D analysis in pdf, html and doc format

Files in figure folder

File names (alphabetical)	Description
DAS genes euler plot across contrast*.pdf/.png	Euler diagram to compare DAS genes in different contrast groups
DAS genes GO annotation plot.pdf/.png	DAS genes GO annotation plot
DE genes euler plot across contrast*.pdf/.png	Euler diagram to compare DE genes in different contrast groups
DE genes GO annotation plot.pdf/.png	DE genes GO annotation plot
DE genes up and down regulation numbers.pdf/.png	DE genes up and down regulation numbers
DE transcripts euler plot across contrast*.pdf/.png	Euler diagram to compare DE transcripts in different contrast groups
DE transcripts up and down regulation numbers.pdf/.png	DE transcripts up and down regulation numbers

File names (alphabetical)	Description
DE vs DAS gene euler plot in contrast*.pdf/.png	Euler diagram to compare DE and DAS genes in different contrast groups
DE vs DTU transcript euler plot in contrast*.pdf/.png	Euler diagram to compare DE and DTU transcripts in different contrast groups
DTU transcripts euler plot across contrast*.pdf/.png	Euler diagram to compare DTU transcripts in different contrast groups
Gene expression distribution.pdf/.png	Gene expression distribution
Gene mean-variance trend.pdf/.png	Gene mean-variance trend plot
Gene PCA all samples*.pdf/.png	Gene PCA plot of all samples
Gene PCA average expression.pdf/.png	Gene PCA plot of average expression
Gene PCA batch effect removed all samples*.pdf/.png	Gene PCA plot of all samples after removing batch effects
Heatmap DAS genes.pdf/.png	Heat-map of DAS genes
Heatmap DE genes.pdf/.png	Heat-map of DE genes
Heatmap DE transcripts.pdf/.png	Heat-map of DE transcripts
Heatmap DTU transcripts.pdf/.png	Heat-map of DTU transcripts
Isoform switch number.png/.pdf	Number of significant isoform switch numbers
Profile/Abundance/PS plots	Folders contain gene/transcript profile plots
Transcript expression distribution.pdf/.png	Transcript expression distribution
Transcript mean-variance trend.pdf/.png	Transcript mean-variance trend plot
Transcript PCA all samples*.pdf/.png	Transcript PCA plot of all samples
Transcript PCA average expression.pdf/.png	Transcript PCA plot of average expression
Transcript PCA batch effect removed all samples*.pdf/.png	Transcript PCA plot of all samples after removing batch effects
Union set DE genes vs DAS genes.pdf/.png	Flow chart -Union set DE genes vs DAS genes
Union set DE transcripts vs DTU transcripts.pdf/.png	Flow chart -Union set DE transcripts vs DTU transcripts

Files in result folder

Important results are saved in csv (comma delimited) files.

File names (alphabetical)	Description
contrast.csv	Contrast groups used for 3D analysis.
DDD genes and transcript lists across all contrast groups.csv	List of DE genes, DAS genes, DE transcripts and DTU transcripts, which are the union sets across all contrast groups.
DDD numbers.csv	DE/DAS/DTU genes/transcript numbers in each contrast group.
DE genes/DAS genes/DE transcripts/DTU transcripts testing statistics.csv	DAS genes/DE genes/DE transcripts/DTU transcripts test statistics, including not significant results.
DEvsDAS/DEvsDTU results.csv	Number of DE vs DAS genes/DE vs DTU transcripts.
Gene read counts.csv	Raw read counts of genes before data pre-processing.
Gene TPM.csv	Raw TPM of genes before data pre-processing.
Raw isoform switch scores.csv	Statistics of all possible isoform switches, including not significant results.
RNAseq info.csv	RNA-seq data information before and after pre-processing.
samples.csv	Meta-data table of sample information.
Significant DE genes/DAS genes/DE transcripts/DTU transcripts list and statistics.csv	Significant DE genes/DAS genes/DE transcripts/DTU transcripts test statistics; not significant results are filtered.

File names (alphabetical)	Description
Significant isoform switch scores.csv	Statistics of significant isoform switches.
Significant TS DAS trend gene/DE trend gene/DE trend transcript/DTU trend transcript list and statistics.csv	Significant time-series DE trend genes/DAS trend genes/DE trend transcripts/DTU trend transcripts test statistics; not significant results are filtered.
Target in each cluster heatmap DE genes.csv	DE gene lists of individual clusters of the heatmap
Target in each cluster heatmap DE transcripts.csv	DE transcript lists of individual clusters of the heatmap
Target in each cluster heatmap DE+DAS genes (DAS only are filtered).csv	DE+DAS gene lists of individual clusters of the heatmap; DAS only are filtered
Target in each cluster heatmap DE+DTU transcripts (DTU only are filtered).csv	DE+DTU transcript lists of individual clusters of the heatmap; DAS only are filtered
Target in each cluster heatmap TS DAS trend genes in contrast All.csv	TS DAS trend gene lists of individual clusters of the heatmap
Target in each cluster heatmap TS DE trend genes in contrast All.csv	TS DE trend gene lists of individual clusters of the heatmap
Target in each cluster heatmap TS DE trend transcripts in contrast All.csv	TS DE trend transcript lists of individual clusters of the heatmap
Target in each cluster heatmap TS DTU trend transcripts in contrast All.csv	TS DTU trend transcript lists of individual clusters of the heatmap
Transcript and gene mapping.csv	Transcript-gene association table.
Transcript read counts.csv	Raw read counts of transcripts before data pre-processing.
Transcript TPM.csv	Raw TPM of transcripts before data pre-processing.
TS DE trend genes/TS DAS trend genes/TS DE trend transcripts/TS DTU trend transcripts testing adjusted p-values.csv	Adjusted p-values of time-series 3D trend testing, including not significant results.

Files in data folder

Intermediate data in .RData for 3D RAN-seq analysis are saved in the data folder. There are three .RData objects: 1) txi_trans.RData and 2) txi_genes.RData are transcript and gene level read count and TPM outputs from the tximport R package (Soneson et al., 2016). All the intermediate data generated in the process of 3D analysis is saved in the list object intermediate_data.RData. R users can access to the data using command line.

List object	Elements in list object	Element type	Description
intermediate_data.RData	conditions	character	Labels of conditions to study
	contrast	character	Contrast groups
	DAS_genes	data.frame	Statistics of significant DTU transcripts
	DDD_numbers	data.frame	Number of DE/DAS/DTU genes/transcripts in contrast groups
	DE_genes	data.frame	Statistics of significant DE genes
	DE_trans	data.frame	Statistics of significant DE transcripts
	deltaPS	data.frame	Delta PS based on contrast groups
	DEvsDAS_results	data.frame	Number of DE vs DAS genes
	DEvsDTU_results	data.frame	Number of DE vs DTU transcripts
	DTU_trans	data.frame	Statistics of significant DTU transcripts
	genes_3D_stat	list	All the raw results of linear regression and statistics of DE genes

List object	Elements in list object	Element type	Description
	genes_batch	list	Estimated gene level batch effects, if they exist. 1) W: matrix, estimated batch effect term, which can be added to design matrix of linear regression; 2) normalizedCounts: matrix, read counts where batch effects are removed; 3) method: a string, method used to estimate batch effects.
	genes_counts	data.frame	Read counts of genes. Seq-reps are merged if exist.
	genes_log2FC	matrix	log2-CPM of genes
	genes_TPM	matrix	TPMs of genes
	mapping	data.frame	Transcript-gene mapping
	params_list	list	Parameters used for the 3D analysis
	PS	matrix	Percent spliced (PS) of expressed transcripts
	RNAseq_info	data.frame	RNA-seq data information before and after pre-processing
	samples	data.frame	Sample information.
	samples_new	data.frame	Sample information after merging sequencing replicates (seq-reps, if exist).
	scores	data.frame	Statistics of isoform switches
	scores_filtered	data.frame	Statistics of significant isoform switches
	target_high	list	1. trans_high: character, expressed transcripts; 2) genes_high: character, expressed genes; 3) mapping_high: data.frame, expressed transcript-gene mapping
	trans_3D_stat	list	All the raw results of linear regression and statistics of DAS genes, DE and DTU transcripts
	trans_batch	list	Estimated transcript level batch effects, if they exist. 1) W: matrix, estimated batch effect term, which can be added to design matrix of linear regression; 2) normalizedCounts: matrix, read counts where batch effects are removed; 3) method: string, method used to estimate batch effects.
	trans_counts	data.frame	Read counts of transcripts. Seq-reps are merged if exist.
	trans_log2FC	matrix	log2-CPM of transcripts.
	trans_TPM	matrix	TPMs of transcripts.
	Other elements		The list object may include other elements.
txi_genes.Rdata and txi_trans.Rdata	abundance	matrix	TPMs of genes/transcripts
	counts	matrix	Read counts of genes/transcripts
	countsFromAbundance	character	Method used to generate read counts and TPMs
	length	matrix	Length of genes/transcripts

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Session information

```
## R version 3.5.1 (2018-07-02)
## Platform: x86_64-w64-mingw32/x64 (64-bit)
## Running under: Windows 10 x64 (build 17763)
##
## Matrix products: default
##
## locale:
## [1] LC_COLLATE=English_United Kingdom.1252
## [2] LC_CTYPE=English_United Kingdom.1252
## [3] LC_MONETARY=English_United Kingdom.1252
## [4] LC_NUMERIC=C
## [5] LC_TIME=English_United Kingdom.1252
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## loaded via a namespace (and not attached):
##  [1] compiler_3.5.1  magrittr_1.5    tools_3.5.1     htmltools_0.3.6
##  [5] yaml_2.2.0      Rcpp_1.0.2      stringi_1.4.3   rmarkdown_1.15
##  [9] knitr_1.25      stringr_1.4.0   xfun_0.9        digest_0.6.20
## [13] evaluate_0.14
```