Analysis of RNASeq data

Study of differential gene expression

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Some definitions

- Sequencing: Determine the linear succession of DNA bases A,T,C,G, reading of this sequence allow to study the included biological information
- Next Generation Sequencing (NGS): High throughput sequencing, generation of a high number of sequences simultaneously
- RNA-seq: transcriptome sequencing. Informations about RNAs using the sequencing of complementary DNA (cDNA)
- Re-sequencing: sequencing of a genome that could be compared to a known reference sequence (the genome of the species has been sequenced already)
- *de-novo* sequencing: sequencing of a genome for which there is no reference genome, determination of a unknown sequence

Why using RNA-seq?

Access to sequences of RNA allows to:

- Annotate a genome
- Establish the catalog of expressed genes
- Identify new genes
- Identify alternative transcripts
- Quantify gene expression and compare between different experimental conditions
- Identify small RNAs (regulation of expression, silencing…)

…

Advantages RNA-seq / microarray

- More accurate and sensitive: allows to discover more
- RNA-seq allows detection of alternative splicing
- Possibility to study transcripts that are lowly expressed
- No need reference genome
  (for microarray, it is required to design probes)

Objectives of the trainings

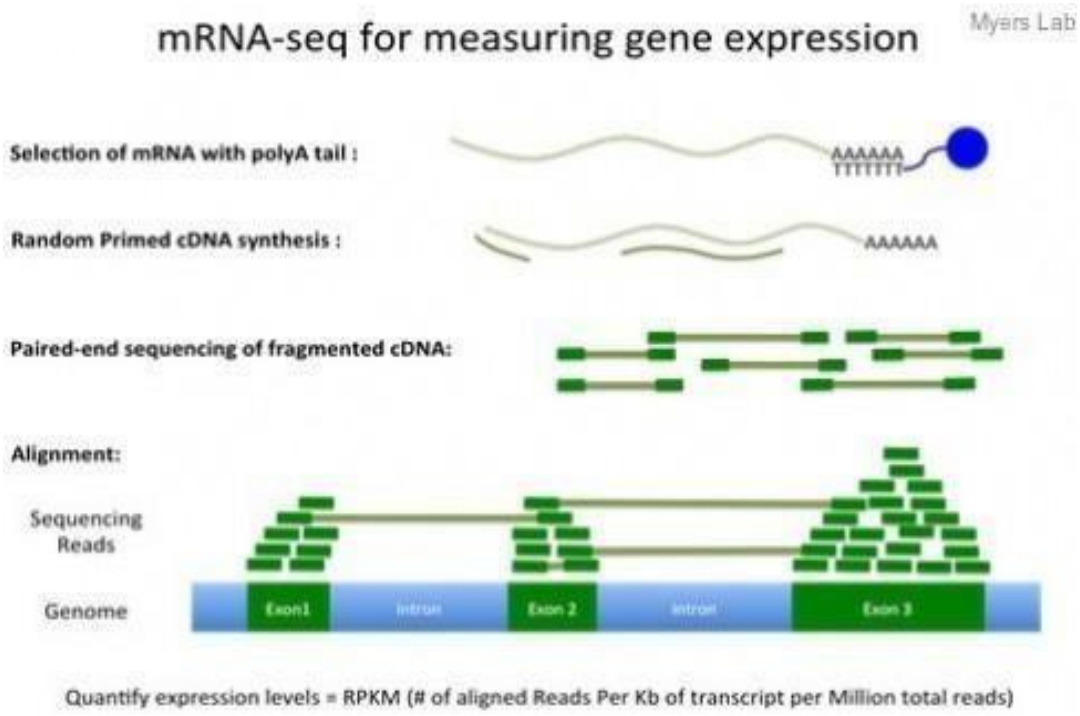
- Know and manipulate packages/tools available for the identification of differentially expressed genes

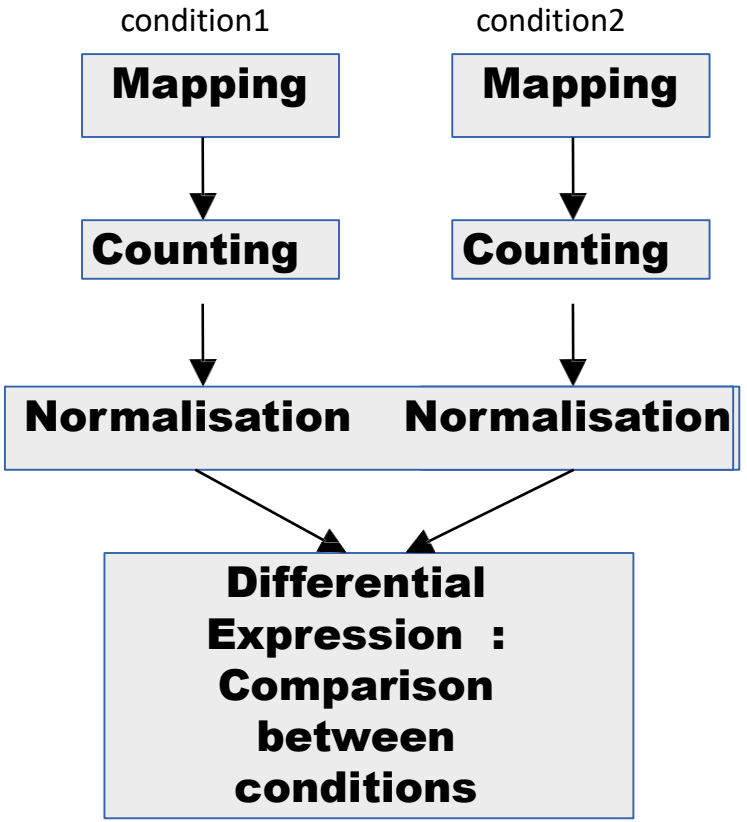
- Think about different techniques of normalization of data

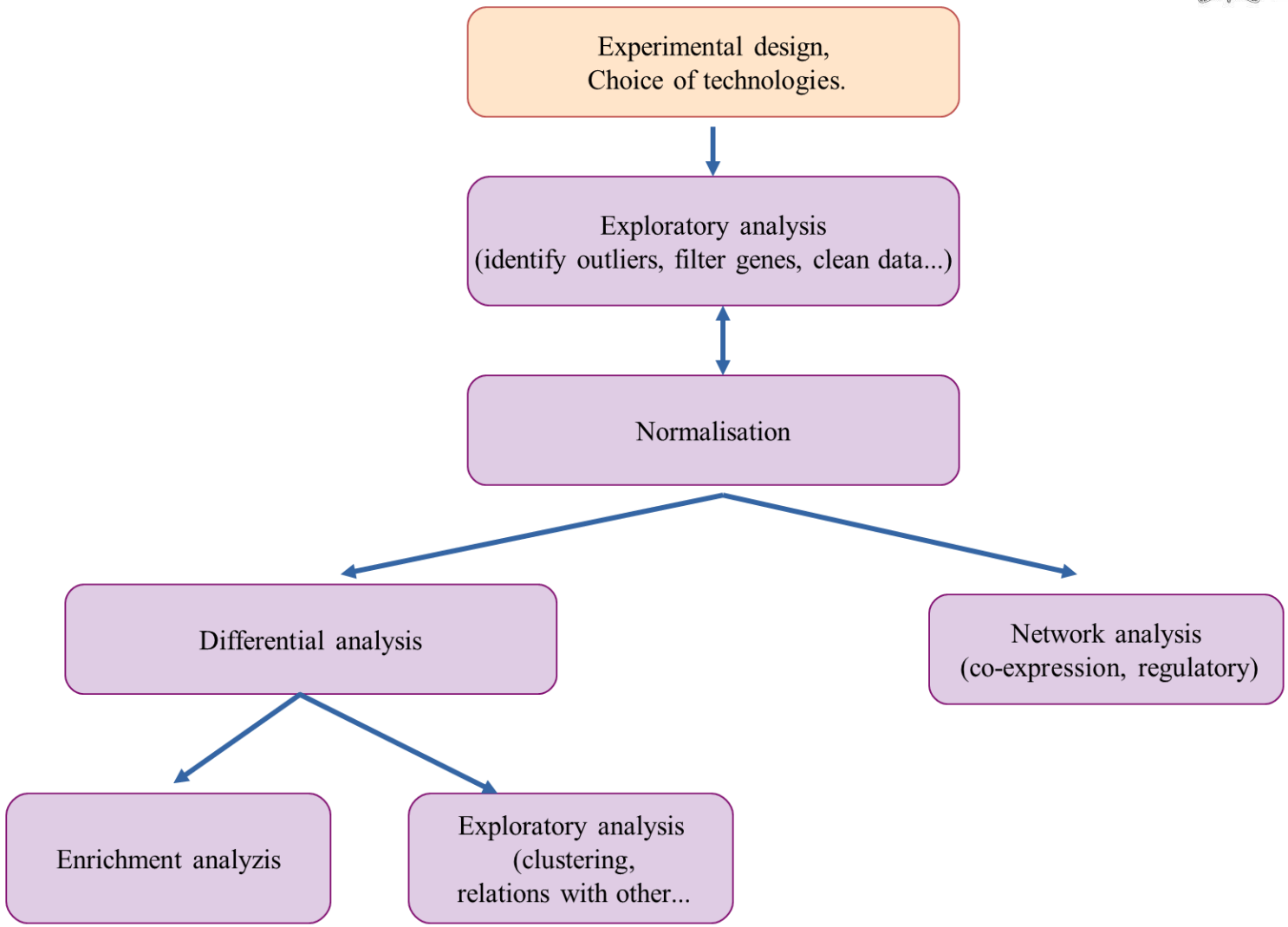
- Detect genes that are differentially expressed between 2 conditions

- Compare results obtained with two different approaches/tools. Understand differences

General principle based on read counting






Experimental design,
Choice of technologies.
Exploratory analysis
(identify outliers, filter genes, clean data...)
Normalisation
Differential analysis
Network analysis
(co-expression, regulatory)
Enrichment analyzis
Exploratory analysis
(clustering,
relations with other...

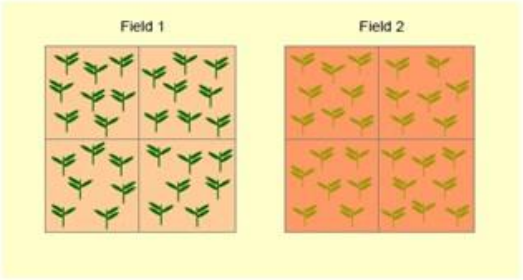
1) Experimental design

Basic experiment : Find differences between conditions control/treated

control group plant

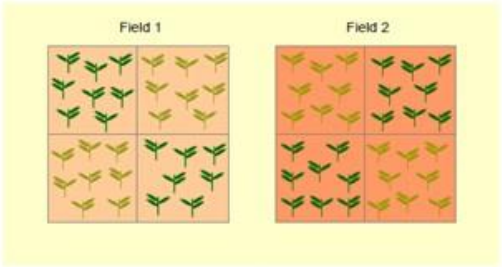
treated group plant

Bad experimental design:
treated plants and control plants are located in 2 different fields



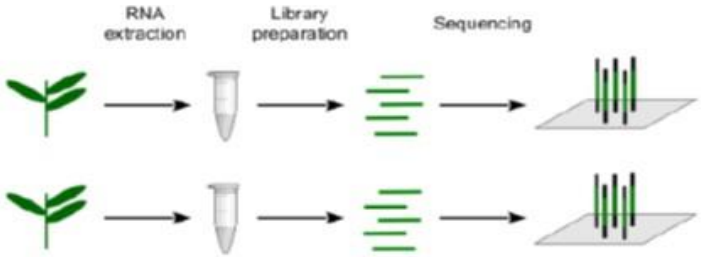
Not possible to differentiate between treatment effect and field effect

Good experimental design:
treated plants and control plants are mixed in the 2 fields



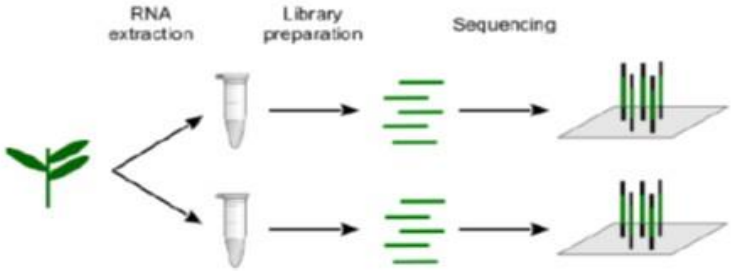
Possible to differentiate between treatment effect and field effect

Biological replicates: Different biological samples, repeated several times (at least 3 times)



Technical replicates: Same biological material, repeated several times

- Several extractions from the same sample
- Several sequencing from the same library



2) Mapping

Choice of mapping software

1) If we hold a reference genome

Use of « splice junction mapper »
(ex : TopHat2, CRAC, MapSplice)

1) If we have annotation
=> Optimize alignment by considering GFF annotation
=> Allow to search for new genes

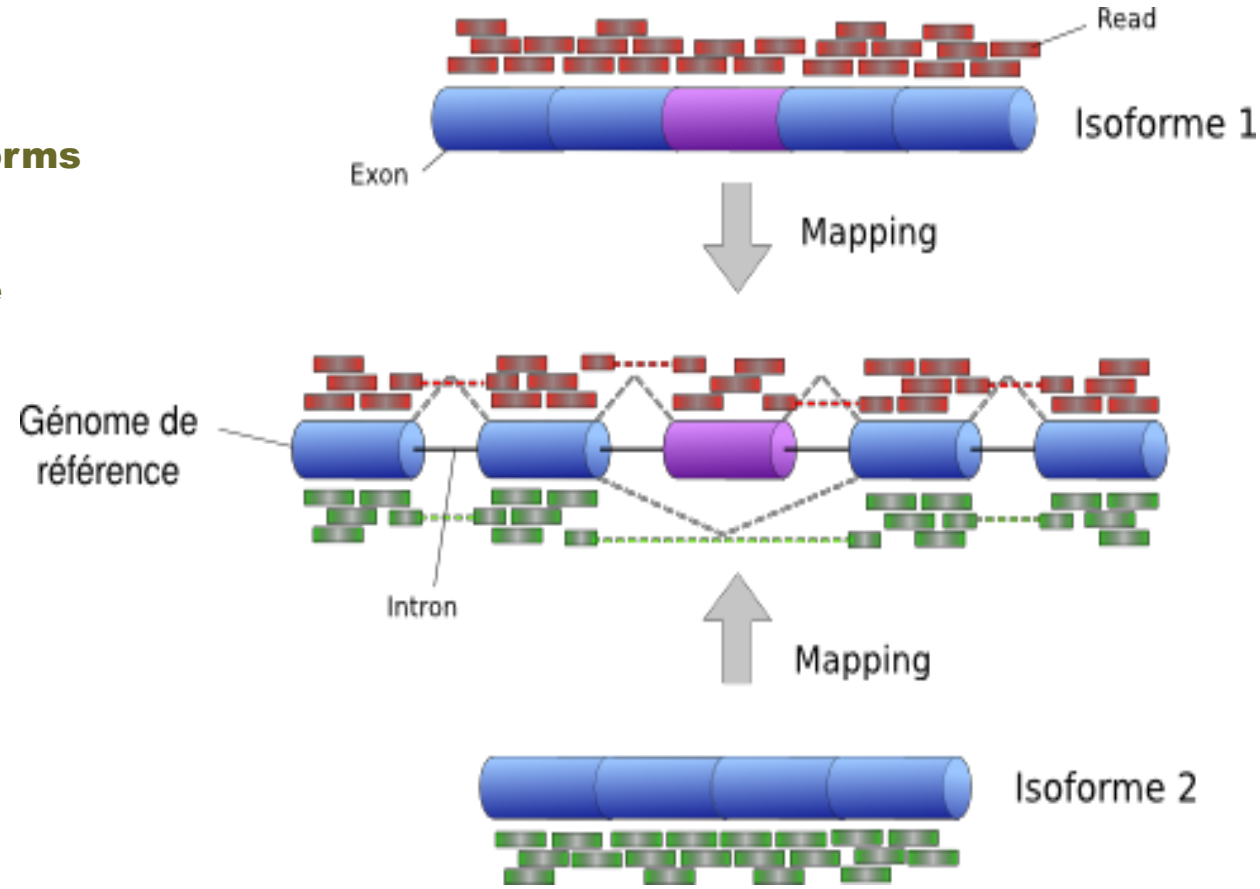
2) If we don't have annotation
=> Help for structural annotation (gene identification)

3) If we hold a reference transcriptome
Use of traditional mapper (ex : BWA, bowtie)

Mapping onto a genomic reference

=> Allow to highlight isoforms

=> Help for the structural annotation of the genome



Suite TopHat / Cufflinks / CummeRbund

(update: HiSAT/StringTie)

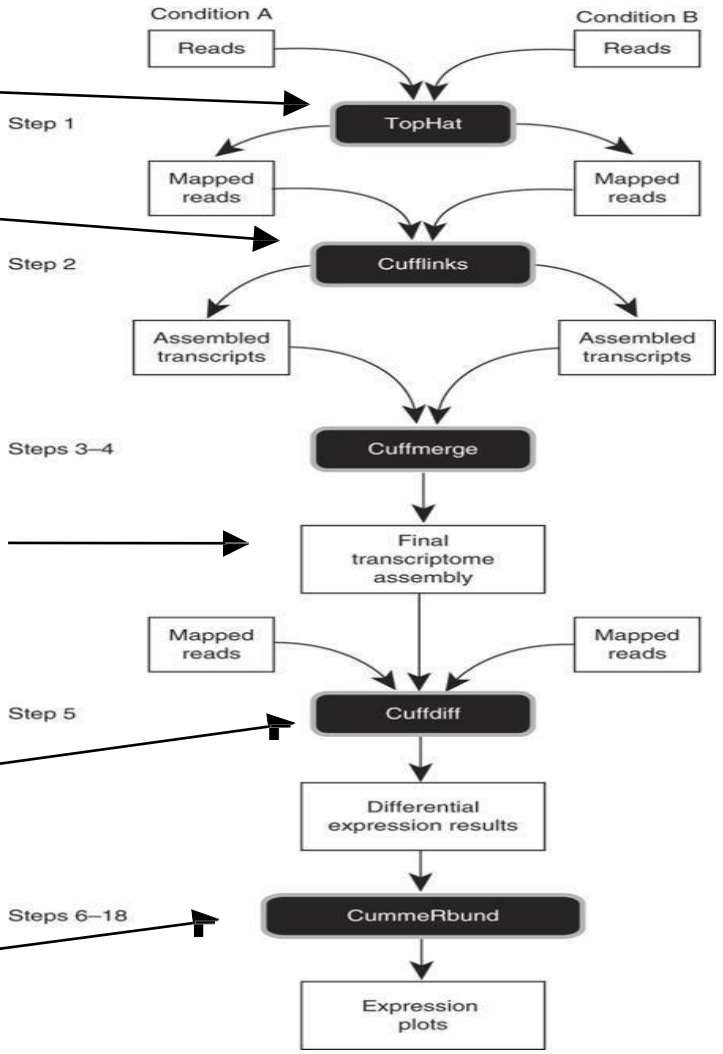
alignment

Assembly (for each condition)

Gathering assemblies = meta-assembly on which we can calculate the level of expression

Statistics DE

Plots



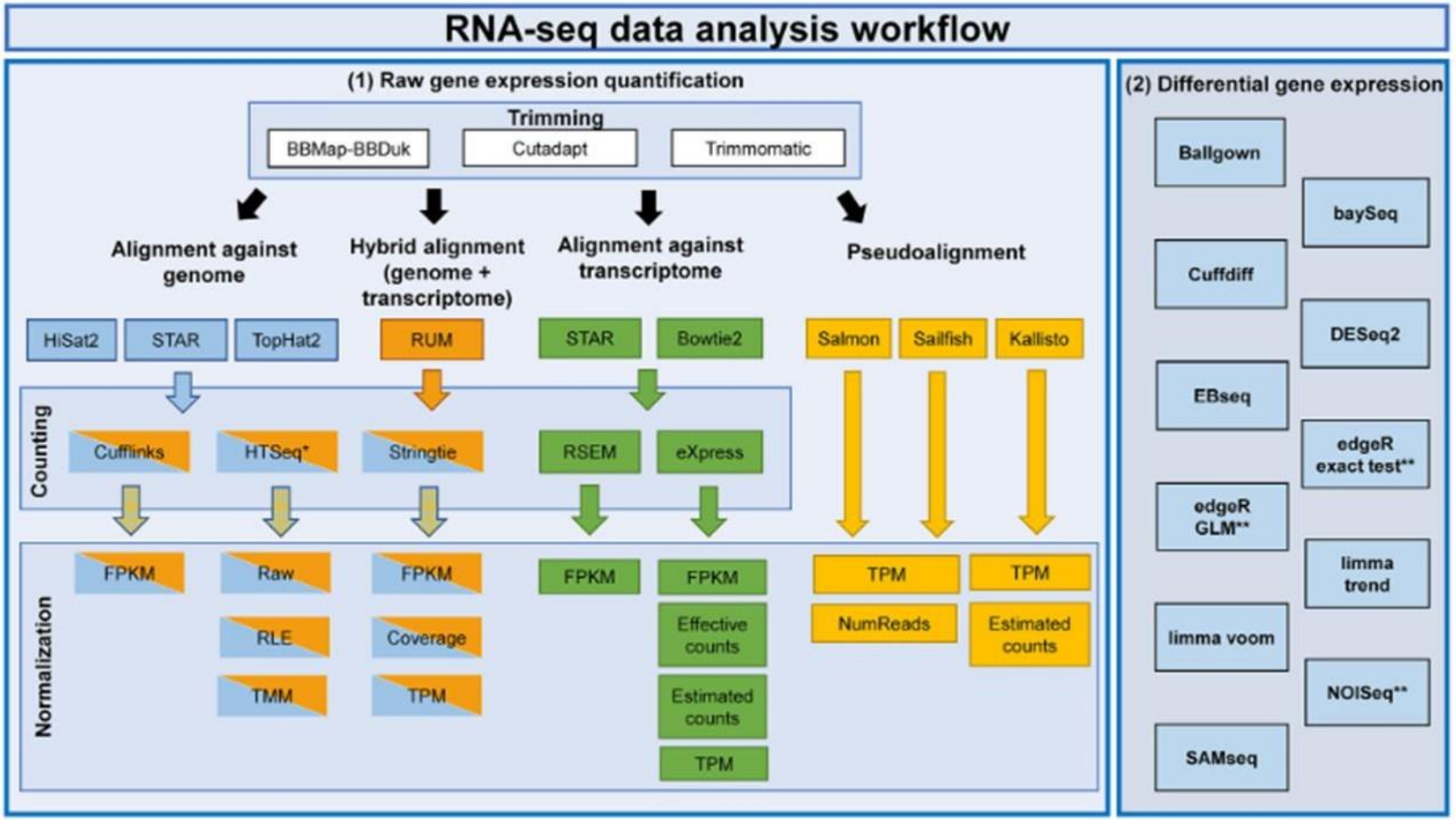


Figure 1. RNA-seq analysis workflow. Left panel (1) represents the raw gene expression quantification workflow. Every box contains the algorithms and methods used for the RNA-seq analysis at trimming, alignment, counting, normalization and pseudoalignment levels. The right panel (2) represents the algorithms used for the differential gene expression quantification. **HTSeq* was performed in two modes: union and intersection-strict. ***EdgeR exact* test, *edgeR GLM* and *NOISeq* have internally three normalization techniques that were evaluated separately.

3) Counting

Choice of the counting software

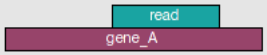
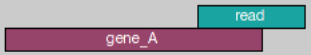
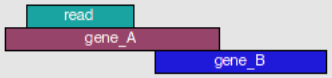
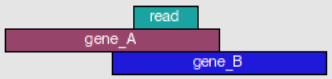
1) If mapping has been performed against an annotated reference genome

=> Use of HTSeq-count (takes as input GFF annotation)

2) If mapping has been performed against reference transcriptome

=> samtools idxstats

=> Kallisto (pseudo-alignment)

	union	intersection_strict	intersection_nonempty
	gene_A	gene_A	gene_A
	gene_A	no_feature	gene_A
	gene_A	no_feature	gene_A
	gene_A	gene_A	gene_A
	gene_A	gene_A	gene_A
	ambiguous	gene_A	gene_A
	ambiguous	ambiguous	ambiguous

4) Data Normalization

Objectives : allows to compare obtained values between different samples

Main biases currently identified :

- Size of the bank (= depth of coverage)
- Gene length
- GC content of genes

Effect of the size of the bank:

For two samples having the same RNA content, we product one bank for each sample

We obtained 2 781 315 reads for bank A and 2 254 901 reads for bank B

=> We have « artificially » 1.2334 times more RNA in bank A although « real » quantity are identical

Effect of gene length:

For the same level of expression, a long transcript will have more chances to be sequenced (and thus more reads) than a shorter transcript

=> More relevant for highlighting DE
=> Need to correct this bias

Methods of normalization :

1) Methods of normalization inter-bank :

Objectives : calculate a scaling factor to be applied to each bank

- Total Count (TC) : we divide every number of reads by the total number of reads (i.e. size of the bank) and we multiply by the average total number of reads across banks

- Upper Quartile (UQ) : same as TC but we replace the total number of reads by the 3rd quartile of counts different to 0
=> normalization less sensitive to extreme values
normalization more robuste, notably in the case where several genes abundant are differentially expressed

- RLE (Relative log expression)

- TMM (Trimmed Means of M-Values)

Methods of normalization :

2) Normalization inter and intra-bank : Reads Per Kilobase per Million (RPKM) :

Objectives : perform a normalization taking into account both size of the bank (using the method Total Count) AND gene length

=> Allows to compare genes between them but not necessarily usefull to compare 2 conditions on a same gene

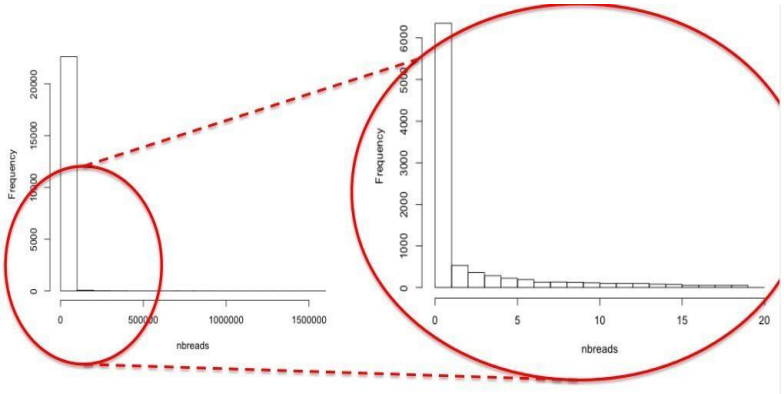
3) Normalization taking into account the bias associated to GC content

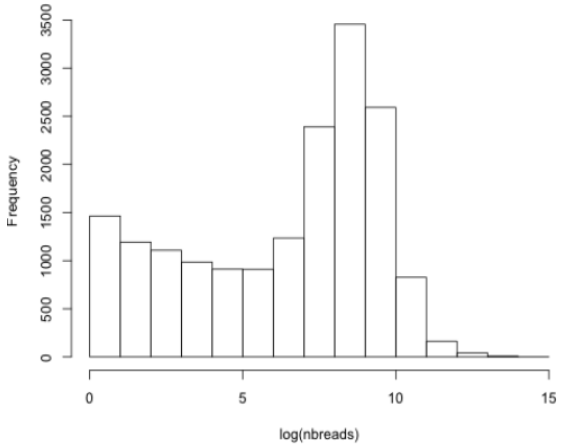
-Total Count method not really efficient (doesn't take into account possible differences in RNA composition between conditions)
- RPKM method not efficient and successfull, is criticized (even for cases where there is bias related to gene length, the use of RPKM doesn't allow to correct it completely)
- More successfull methods to prefer: Upper-Quartile, RLE, TMM

5) Search for differentially expressed genes

Modeling data

- In order to follow a statistics law, use of the log(number reads) instead of the number of reads
+ need to transform « 0 »
=> Negative binomiale distribution



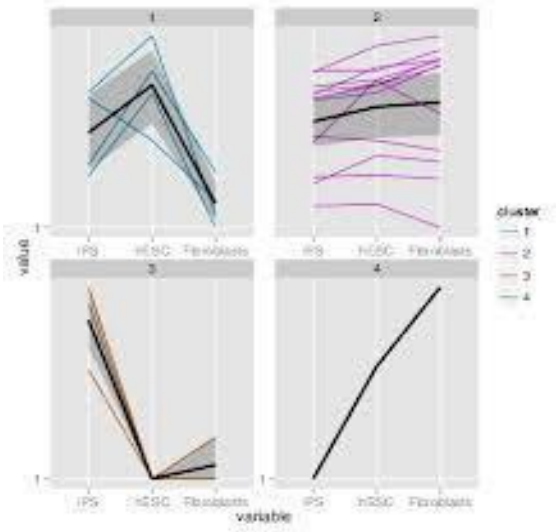


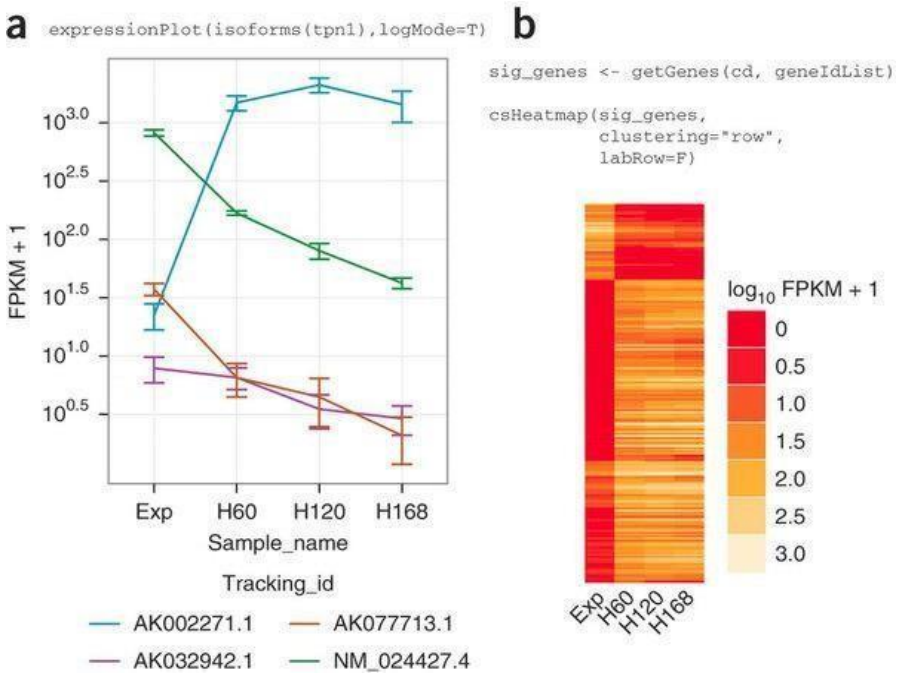
- Use of log(FoldChange)
Fold Change = ratio between 2 expression levels
= ratio final value / initial value

Methods based on RPKM

(Cuffdiff)

Cuffdiff - CummeRbund





Methods based on inter-bank normalization

(RLE, TMM, Upper-Quartile)

(EdgeR et DESeq)

Comparison of softwares DESeq/EdgeR

DESeq uses an estimate of variance that makes it less permissive for high variability between conditions. If at least one of the conditions show a deviation, DESEq doen't trust the gene et will not consider it as differentially expressed, even if there is a high difference between conditions (logFC).

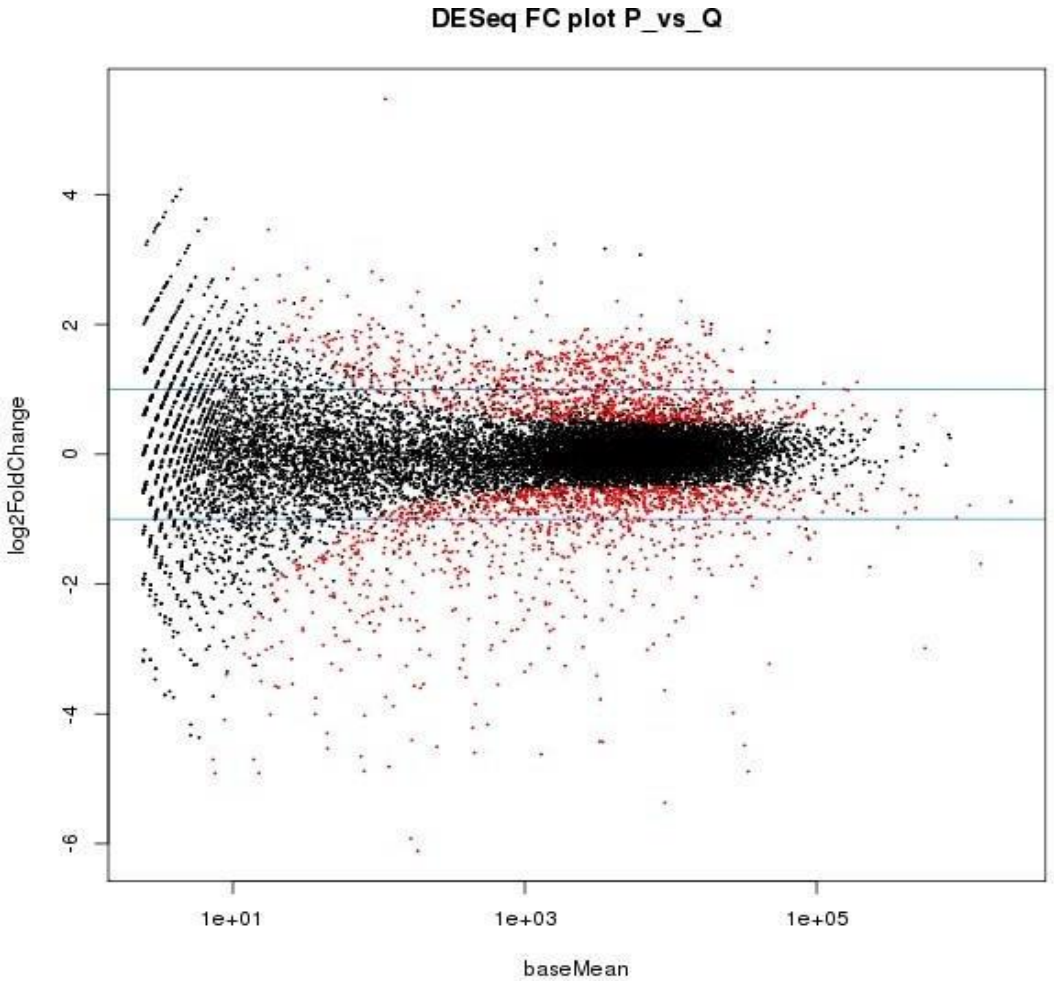
At the opposite, when the variability intra-condition is low, DESeq trust more and may select genes for which fold-Change is low even those discarded by EdgeR.

=> **DESeq is to prefer for experimentations very repeatable**

DESeq2 is more flexible than DESeq, will be less stringent and detect more DE genes

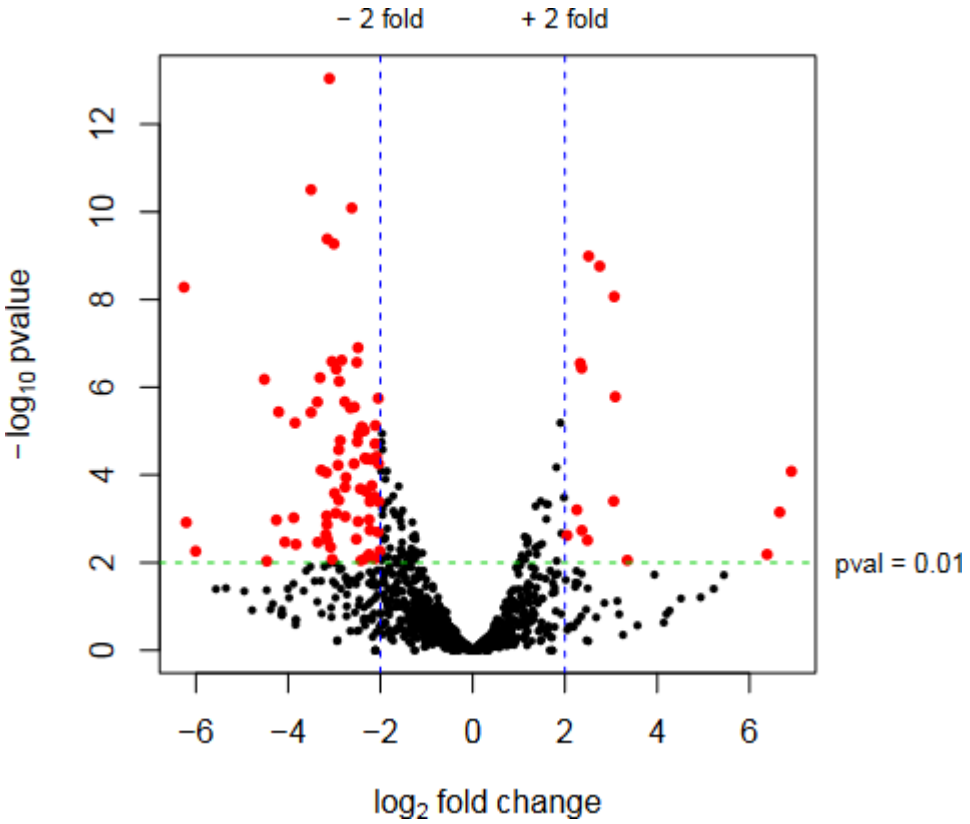
Smear plot / MA plot

Pvalue adj < 0.05

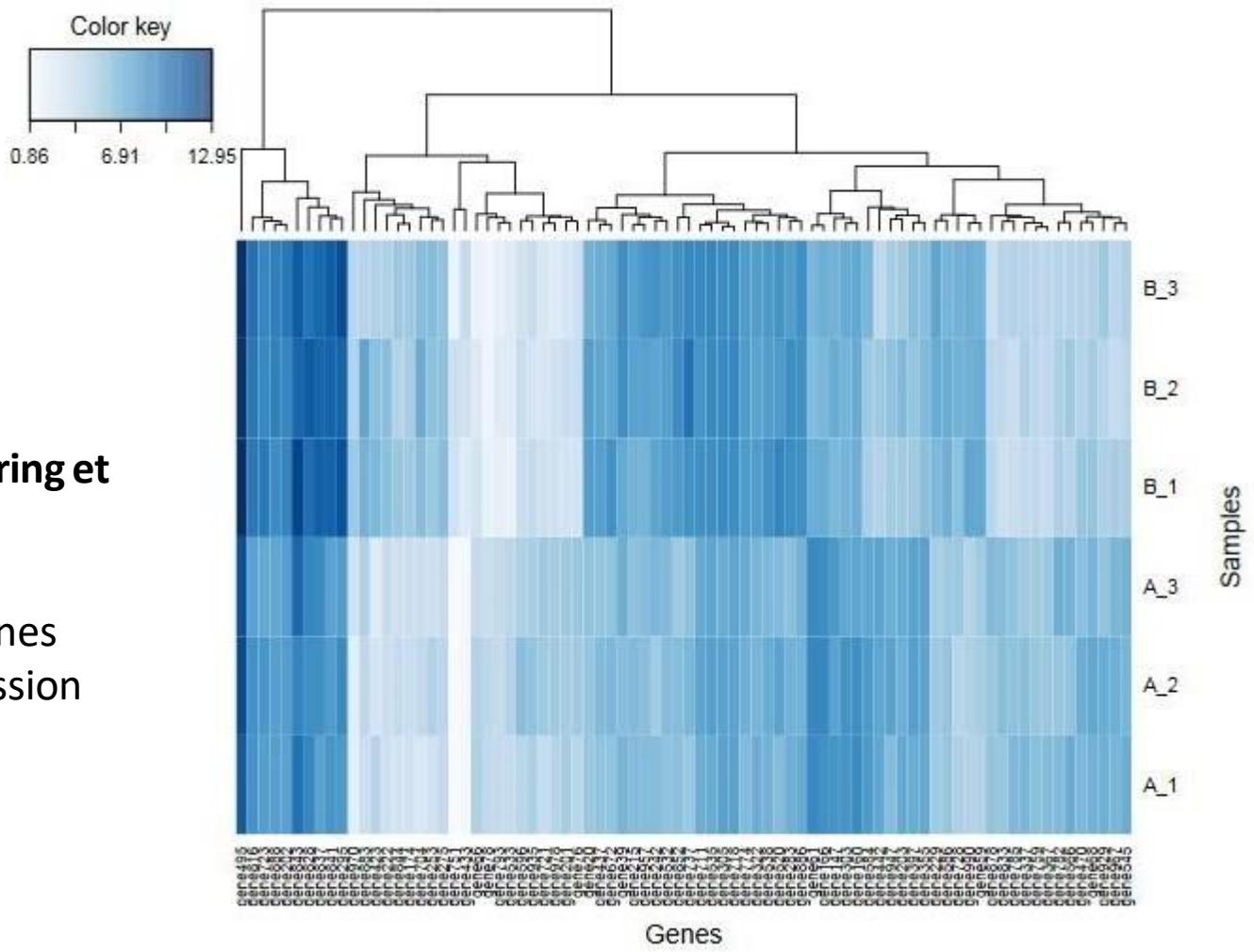


Volcano plot

Pvalue adj < 0.01

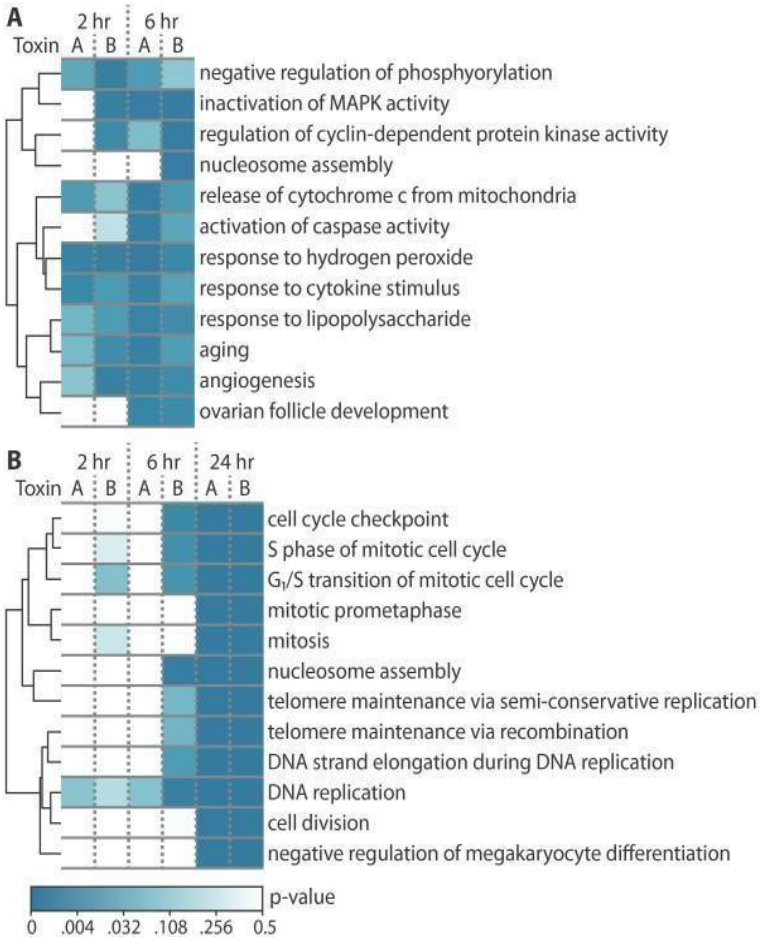


Tutorial: http://www.nathalievilla.org/doc/pdf/tutorial-rnaseq.pdf



Hierarchical Clustering et Heatmap

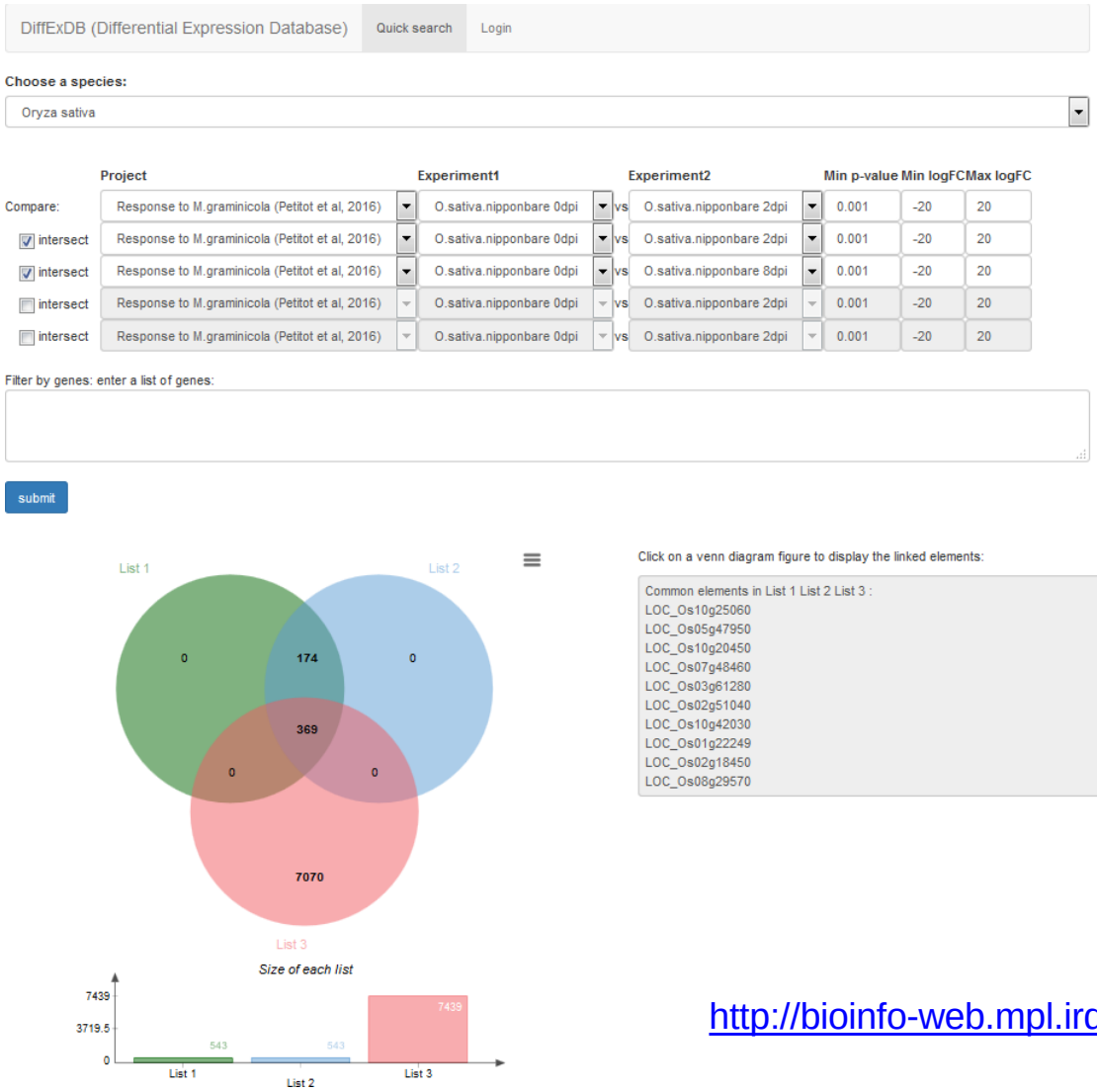
=> Clustering of genes according to expression patterns



TopGO : Study of Gene Ontology terms enrichment

Need to have a GO functional annotation of transcripts

=> Test if it exist significant enrichments of GO functions between DE genes and non-DE genes (between 2 conditions)



DiffExDB

Web application to explore data from differential expression analysis:

- **Overlap between comparisons**
- **Heatmap of expression**

http://bioinfo-web.mpl.ird.fr/cgi-bin2/microarray/public/diffexdb.cgi

ShortStack: Management of small RNA data

BIOINFORMATICS

ShortStack: Comprehensive annotation and quantification of small RNA genes

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ABSTRACT

Small RNA sequencing allows genome-wide discovery, categorization, and quantification of genes producing regulatory small RNAs. Many tools have been described for annotation and quantification of microRNA loci (*MIRNA*s) from small RNA-seq data. However, in many organisms and tissue types, *MIRNA* genes comprise only a small fraction of all small RNA-producing genes. ShortStack is a stand-alone application that analyzes reference-aligned small RNA-seq data and performs comprehensive de novo annotation and quantification of the inferred small RNA genes. ShortStack's output reports multiple parameters of direct relevance to small RNA gene annotation, including RNA size distributions, repetitiveness, strandedness, hairpin-association, *MIRNA* annotation, and phasing. In this study, ShortStack is demonstrated to perform accurate annotations and useful descriptions of diverse small RNA genes from four plants (*Arabidopsis*, tomato, rice, and maize) and three animals (*Drosophila*, mice, and humans). ShortStack efficiently processes very large small RNA-seq data sets using modest computational resources, and its performance compares favorably to previously described tools. Annotation of *MIRNA* loci by ShortStack is highly specific in both plants and animals. ShortStack is freely available under a GNU General Public License.

Keywords: microRNA; small RNA; siRNA; software; bioinformatics; next-generation sequencing