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COORDINATORE: Ch.mo. Prof. Antonelli Valerio

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Novel tools for reproducible  
Next Generation Sequencing data analysis and integration

**Relatori**

Ch.mo. Prof. Tagliaferri Roberto  
Ch.ma. Prof. Angelini Claudia

**Candidato**

Righelli Dario  
Matr. 8800800010

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*One pill makes you larger, and one pill makes you small  
And the ones that mother gives you, don't do anything at all  
Go ask Alice, when she's ten feet tall*

*And if you go chasing rabbits, and you know you're going to fall  
Tell 'em a hookah-smoking caterpillar has given you the call  
And call Alice, when she was just small*

*When the men on the chessboard get up and tell you where to go  
And you've just had some kind of mushroom,  
and your mind is moving low  
Go ask Alice, I think she'll know*

*When logic and proportion have fallen sloppy dead  
And the white knight is talking backwards  
And the red queen's off with her head  
Remember what the dormouse said*

*Feed your head, feed your head*





*To the ones who patiently supported and accompanied me  
during this long and winding trip.*



## Abstract

Massive parallel sequencing technologies are producing a vast amount of genome-wide data about cells, tissues and model organisms, useful to understand many of biological mechanisms, like protein-chromatin interactions (e.g. ChIP-Seq), DNA methylation (Methyl-Seq or BS-Seq), chromatin accessibility (e.g. Atac-Seq), global transcriptional and translational activities (e.g. RNA Sequencing (RNA-seq)) and 3-D organisation of chromatin (e.g. Hi-C), giving the possibility to study same individual or experimental condition from many different points of view (transcriptomics, epigenomics, etc.) with a very high resolution. Each type of these omics data explains a different aspect of cellular behaviour. In order to extrapolate relevant information from each omics, it is required to develop specific statistical methodologies for single data analysis and, at the same time, computational methodologies for handling huge amount of data. However, to give a comprehensive view of the cell regulatory mechanisms, it is necessary not only to analyze a single omics but also to develop novel statistical and computational models for integrating different omics types within a unified study.

This thesis is focused on the development of three main computational tools (*Ticorser*, *DEScan2* and *IntegrHO*), allowing data analysis and integration of multiple next-generation sequencing experiments. Additionally, it also contains a fourth tool (*easyReporting*) for reproducible computational research.

*Ticorser* (time course RNA-seq data analyser) is a novel R package aimed to analyse time-course RNA-seq data. It offers multiple methods for differential expression data analysis and provides multiple plots useful to explore and visualize the results at each step of the analysis. Furthermore, it also provides methods for functional integration by annotating genes in pathways and GO-terms.

*DEScan2* (Differential Enriched Scan 2) is a novel R package for ATAC-seq data analysis, one of the emerging techniques for investigating chromatin accessibility. It consists in the following three-step procedure: 1) It identifies candidate regions inside each sample implementing a peak caller; 2) It filters out potential artefacts by aligning the candidate regions between the samples and removing those candidate regions that were not reproducible between samples 3) It produces a count matrix of regions and samples, useful for differential enrichment between multiple conditions and also for integrating this data type with other omics data, such as RNA-seq.

*IntegrHO* (Integration of High-Throughput Omics data) is a Graphical User Interface (GUI), written in R and Shiny, aimed to analyze and integrate multi-omics data types. It provides a friendly interface to the above-mentioned tools and also incorporates a wide selection of methods and other tools available in the literature. This platform, through an easy point-and-click approach, enables the user to analyze and explore single omics data, such as RNA-seq, ChIP-seq and ATAC-seq and, moreover, it offers the possibility to integrate them at different levels, such as gene-peak annotation and functional annotation methods.

Finally, since in last decades the scientific community has experienced a deep crisis known as "irreproducible research", this thesis presents *EasyReporting*, an R package for an automatic report creation, developed to support the reproducibility of scientific research. Thanks to the R6 class paradigm on which is based on, it is easy to use and to extend.

Overall, this work proposes and combines several computational tools for prop-

erly analyzing, visualizing, comparing, integrating and tracing different omics data types. The results are illustrated with downloaded data from the literature or from collaboration projects.

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# Chapter 1

## Introduction

This chapter provides all the needed aspects to understand the context of this thesis work, motivates its contents and describes the main contributions.

In particular, it starts from describing some basic molecular biological aspects and how it is possible to study regulatory cellular mechanisms from multiple points of view, using different sequencing techniques and how to integrate them. Moreover, it discuss the importance of keeping trace of the computational processes involved in the information extraction, known as "reproducible research".

### 1.1 Biological Background

#### 1.1.1 The cell

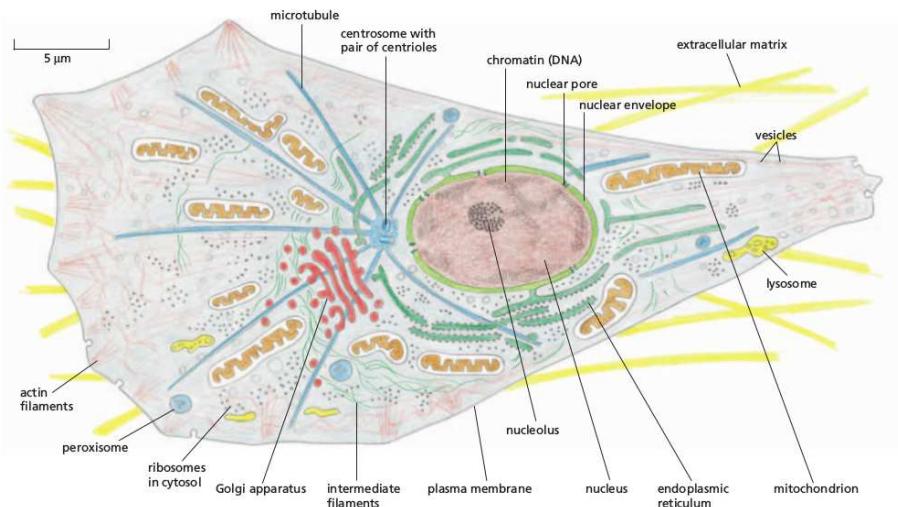
Cells are the fundamental units of every living being, which can be made up of one cell (unicellular) or more (multicellular).

We distinguish between prokaryotic and eukaryotic cells. The first ones are typically unicellular organisms and don't encapsulate the genetic material (DeoxyriboNucleic Acid (DNA)) inside the nucleus. While the second ones protect the genetic material inside a specific organelle called nucleus, protected by membranes.

For any kind of cell, the internal volume is defined by the *cytoplasm*, which is a liquid solution where several insoluble particles stand, such as enzymes, *RNA*

and metabolites.

Moreover, it is possible to distinguish multiple organelles, such as *ribosomes*, the *endoplasmic reticulum*, the *golgi complex*, *lisosomes* and the *nucleus* (figure 1.1.1) for eukaryotic cells.,



**Figure 1.1.1:** Schematic representation of an Eukaryotic Cell. It is surrounded by the plasmatic membrane, which encloses the cytoplasm within multiple organelles. One of these is the nucleus which protects the DNA.

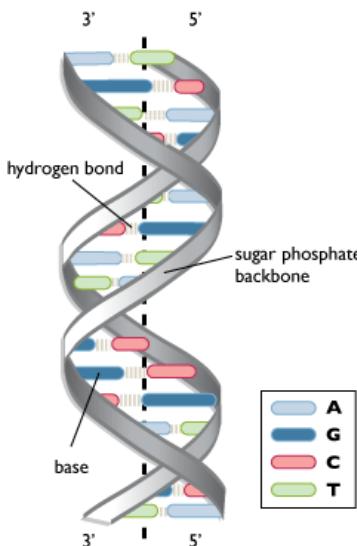
### 1.1.2 The DNA

The DNA has been isolated for the first time by the German doctor Friedrich Miescher in 1869, while in the same decade the English biologist Charles Darwin was publishing *On the Origin of the Species* and the Augustinian friar scientist, Gregor Mendel, was communicating his results on the peas to the Brunn Natural History Society.

Because the isolated substance by Miescher was white, lightly acid and was present only into the cells nuclei, it was been named *Nucleic Acid*. Name modified afterwards into DNA, to distinguish it from another one, very similar, the RiboNucleic Acid (RNA).

These two molecules are constituted by *nucleotides*, formed by a nitrogen base, deoxyribose sugar and a phosphate group. We distinguish two nitrogen bases, purines and pyrimidines. Inside the DNA, we have two *purines*, the *adenine* (*A*) and the *guanine* (*G*), and two *pyrimidines*, the *Cytosine* (*C*) and

the *Thymine* (*T*). Inside the RNA the *Thymine* (*T*) is substituted by the *Uracil* (*U*).

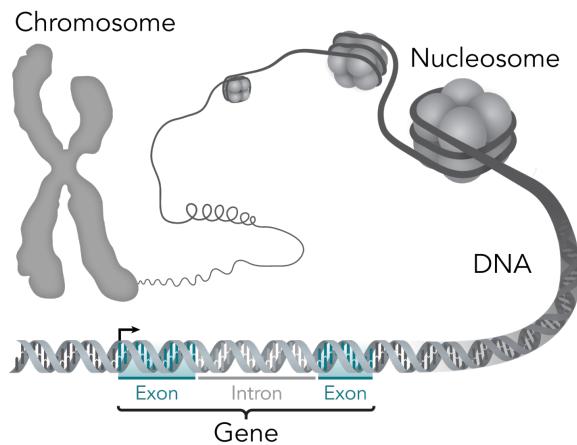


**Figure 1.1.2:** Schematic representation of double-stranded filament structure of the DNA. The legend reports the four nitrogen bases, Adenine (A), Guanine (G), Cytosine (C) and Thymine (T).

The DNA structure (figure 1.1.2) was discovered in the '50s by the American scientist James Watson, the English physicist Francis Crick and the English chemist-physicist Rosalind Franklin. According to their model, the DNA is a double-stranded filament, where Adenines can pair only with Thymines and Guanines only with Cytosines. The four bases constitute the alphabet for the genetic message.

DNA is folded on itself (*DNA packaging*), thanks to specific "beads" called *nucleosomes*, which themselves consist of eight proteins with tails, called *histones*, having the DNA wrapped on them. This mechanism enables to store around 2 meters of chromatin inside a nucleus of a 2-10 micron diameter when referring to Human species.

Moreover, the DNA contains the *genes*, particular sections containing relevant information for building proteins and other fundamental molecules. Each gene is localized on a precise position of a *Chromosome*, which are in a different number for each species. Each chromosome is constituted by DNA within thousands of genes, each one composed by introns and exons (figure 1.1.3).



**Figure 1.1.3:** Representation of the relation between the chromosome and the gene. A gene stands on a portion of a chromosome, which is rolled around a nucleosome. Each gene is composed by introns and exons. When transcribed, the first ones are involved in post-transcriptional cellular mechanisms, while the second ones are destined to be translated into proteins.

It is important to underline that since some decades ago the *Central Dogma of Molecular Biology* was founded on the transcription-translation principle, where *exons* were transcribed into RNAs, which subsequently were translated into proteins.

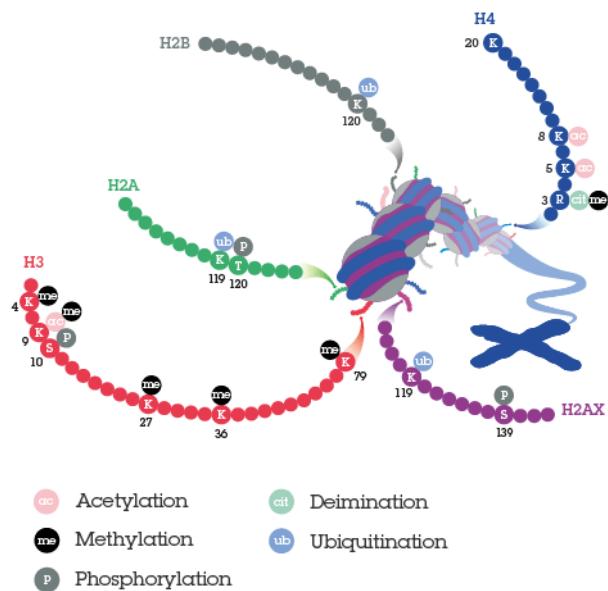
Nowadays, we know that the gene transcription is regulated by several mechanisms, and the translation is not the only process fated for RNA. Indeed, since few years ago, introns were considered junk DNA, but now they have been demonstrated to be involved in regulation mechanisms, such as post-transcriptional processes, while exons are destined to be translated into proteins (figure 1.1.3). Thanks to the alternative splicing different proteins can be generated by the same gene.

### 1.1.3 The Chromatin

In order to fit inside the eukaryotic cells nucleus, the DNA is condensed and wrapped around nuclear proteins (the nucleosomes) forming the chromatin.

Because of chromatin condensed form, for the transcription of a gene there are some requisites to be respected, such as the accessibility of that specific part of the chromatin, or the binding of specific proteins enabling the accession to the gene region, or histone modification processes, such as *Acetylation*, *Methylation*,

*Phosphorylation* among others, which modify the state of a specific histone, influencing gene expression regulation (figure 1.1.4). Additionally the methylation can affect also the DNA, which is basically associated to the non-transcription of the genes in that part of DNA.



**Figure 1.1.4:** Representation of some processes involved in histone modification, influencing gene expression regulation, such as Acetyltyion, Methylation, Phosphorylation, Deimination and Ubiquitination.

In particular, the *Acetylation* mostly involves histones H3 and H4 leading to a lower grade of DNA compaction, subsequently increasing the gene expression. *Phosphorylation* highly involves positions H3S10 and T120 of H2A, which are associated to the chromatin compaction. This seems to be relevant for the chromosomal condensation during cellular division, for DNA damage repair and for transcription regulator. *Methylation* generally involves histones H3 and H4, but the modification effects depend on the affected position, influencing the gene expression/repression. More in detail, acetylation and phosphorylation directly affect the nucleosome structure, while methylation recruits multiprotein complexes involved into the chromatin remodelling.

Each of these modifications affect the DNA structure, varying not only the chromatin compaction but also by promoting, together with proteins and non-coding RNA, the chromatin folding and influencing the gene expression regula-

tion.

## 1.2 Sequencing Techniques

Several sequencing technologies have been developed during the last decades to study multiple aspects of cellular behaviour related to different experimental conditions due to drug treatments, pathologies, diseases, etc.

Starting with Sanger sequencing first and passing through Microarrays technologies then, nowadays with Next Generation Sequencing (NGS) techniques we are able to understand many biological mechanisms, like protein-chromatin interactions (e.g. Chromatin Immunoprecipitation Sequencing (ChIP-seq) [1]), DNA methylation (Methylated DNA immunoprecipitation Sequencing (MeDIP-seq) or Bisulfite Sequencing (BS-seq) [2]), chromatin accessibility (e.g. Assay for Transposase-Accessible Chromatin Sequencing (ATAC-seq) [3]), global transcriptional and translational activities (e.g. RNA Sequencing (RNA-seq) [4]) and 3-D organization of chromatin (e.g. *Hi-C* [5]), giving the possibility to study same individual or experimental conditions from many different points of view (transcriptomics, epigenomics, etc.) with a very high resolution. Each type of these "omics" data explains a different aspect of cellular behaviour. To give a comprehensive view of the cell regulatory mechanisms, it is necessary not only to perform a single level analysis but also to provide novel statistical and computational models for integrating different omics types within a unified study.

The common steps valid for almost every NGS data type starts from a library preparation (depending on the sequencing technique), an amplification of the genomic material and finally the sequencing of the samples.

The typical analysis requires several steps to achieve good quality results, regardless of the specific final aim of the investigation. A standard analysis starts with a quality control assessment of raw sequenced fragments (reads), using software as *FastQC* [6]. After quality control assessment, in general, it is fundamental for the analysis to map the raw reads on a reference genome. But, depending on the sequencing technique, for the reads mapping phase we have to distinguish the aligner to use. For DNA sequencing *Bowtie* [7, 8] and *BWA*

[9, 10] are the most common ones, while for BS-seq there is *Bismark* [11].

For RNA-seq, during the last years, in order to obtain higher accuracy in read mapping (alignment), several software, such as *STAR* [12] and *HISAT2* [13], superseded broadly used tools as *TopHat2* [14]. Usually, an aligner produces Binary Alignment Map (BAM) files<sup>1</sup> [15] where all the reads (mapped and unmapped) are collected together with additional information, relevant for further steps of the analysis. Usually the software used for BAM files processing is *samtools* [15, 16], available also in R programming language with *rsamtools* [17]. From this point on, the analysis steps dependend on the sequencing technique and from the experimental investigation.

Here, we present the basics ideas about the main approaches considered in his thesis.

### 1.2.1 RNA-seq

Since the beginning of modern biology, gene expression has been part of the central dogma of molecular biology. Of course, during the decades some aspects have changed, but the RNA transcripts still have very high relevance. Nowadays, RNA-seq [4, 18–20] is one of the most widely used technologies to understand gene related regulatory mechanisms in response to stress conditions or drug treatments and progressions of several diseases [21].

The main aim of RNA-seq experiments is to highlight which genes are increasingly (*up-regulated*) or decreasingly (*down-regulated*) altered when comparing two or more conditions at a specific instant in time (steady-state experiment) or in subsequent time points (time-course experiment), in order to identify the biological mechanisms regulating such changes.

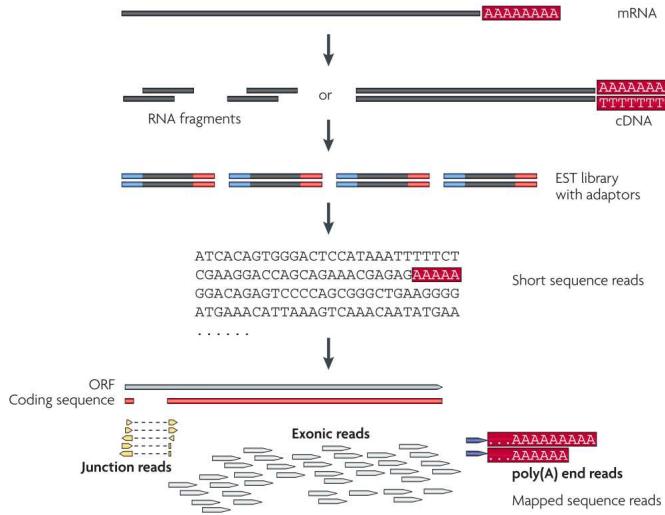
However, RNA-seq also offers the possibility to identify and investigate isoforms abundance [22–25]. Although, such aspect will not be further treated in this thesis.

The general idea underlying the library preparation of an RNA-seq experiment can be viewed as the conversion of long messengers RNA segments in Complementary DNA (cDNA) fragments with RNA or DNA fragmentation. To each sequence, an adapter for the sequencer is added and a short read is

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<sup>1</sup><http://samtools.github.io/hts-specs/SAMv1.pdf>

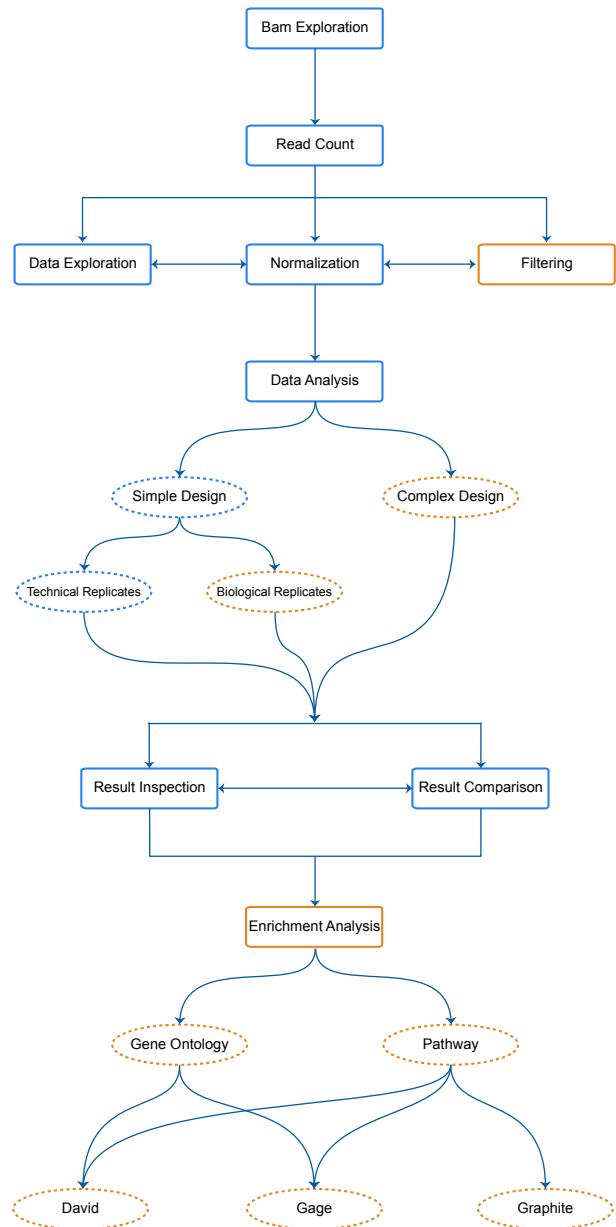
obtained with high-throughput sequencing technology (Figure 1.2.1).



**Figure 1.2.1:** Representation of an RNA-seq experiment. The messenger RNA (mRNA) is converted into complementary DNA (cDNA) or RNA fragments, to be linked to short sequencing adaptors. Producing sequencing reads via high-throughput technology. The resulting reads are aligned to a reference genome or transcriptome, to be classified as exonic, junction or poly(A) end-reads. [4]

Afterwards, the so-obtained reads need to be analyzed with several tools, depending on the particular question the researcher is interested in [26, 27].

In particular, we focused on differential gene expression in case of multiple biological conditions, due to stress, drug treatments, disease-specific, etc. Figure 1.2.2 shows a pipeline we designed for the *RNASeqGUI* [28] a well-known software for RNA-seq data analysis. After the alignment of the reads on a species's reference genome, the software allows for the quantification of mapped reads producing a count matrix  $M \in \mathbb{R}$  of the  $n$  samples (on the columns) and the  $p$  genes related features (on the rows). Typically the identifiers (gene-names) depend on the annotation database used by the analyzer.



**Figure 1.2.2:** A representation of RNA-seq analysis pipeline, as implemented in *RNASEqGUI*, a graphical user interface for RNA-seq data analysis (Image from [29]). BAM files can be explored and their contained reads can be used to quantify the expression of genomic features. Afterwards, it is possible to filter low expressed features and to normalize the resulting ones, in order to lower the noise that can affect further results. Subsequently, it is possible to analyze the data to inspect the differential expression of the genes between multiple conditions. This is relevant to understand how a "treatment" is affecting a specific condition respect to another one. Finally the results can be graphically inspected and/or integrated with Gene Ontology or Pathway databases to obtain a more detailed information about the cellular mechanisms affected by the "treatment".

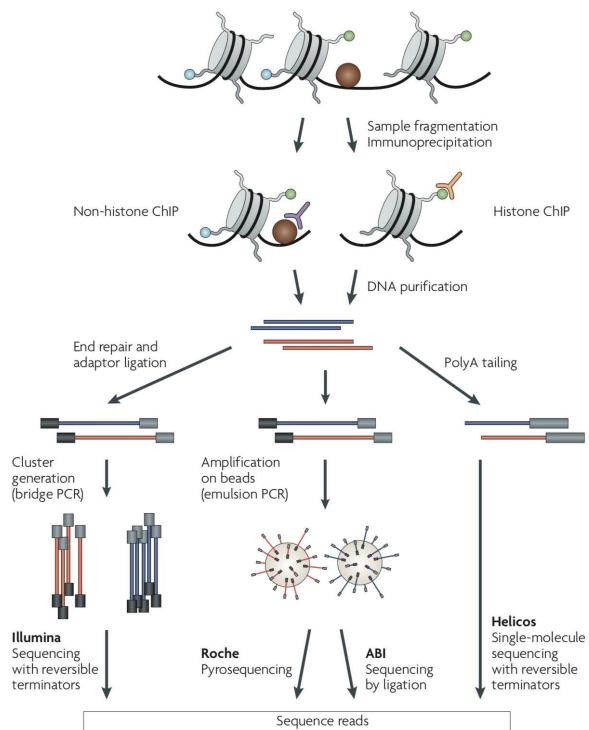
The count matrix needs to be filtered to remove low expressed features and then normalized across the samples, to reduce specific bias for each sample. Then, depending on the experimental design (simple or complex), it is possible to choose between several hypothesis testing algorithms for detecting differentially expressed features (genes) between the conditions (see section 2.2.3). Finally, it is possible to combine the identified features with the annotated information available inside external databases to detect altered pathways and biological functionalities.

### 1.2.2 ChIP-seq

As previously mentioned (section 1.1.3), epigenetic marks are a fundamental aspect of the cellular biological processes. Indeed, their state influences not only gene accession but also the regulation of gene expression.

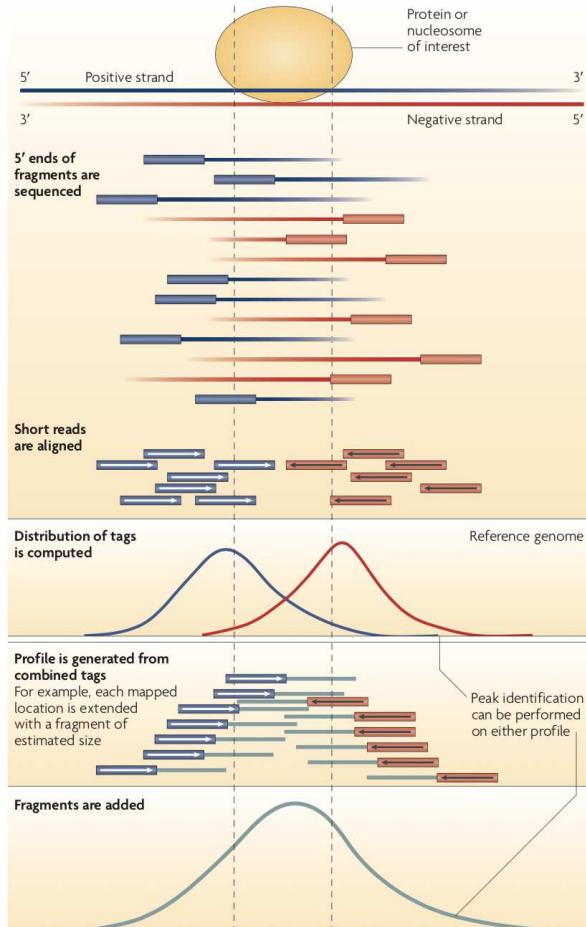
Some relevant aspects are Histone Modifications (HMs) and Transcription Factors (TFs), the first ones are related to transcriptional activation/inactivation, chromosome packaging, and DNA damaging/repairing, the second ones (TFs), also named as sequence-specific DNA-binding factor, are proteins that control the transcription of genetic information by binding specific sequences of DNA.

To investigate these epigenetic aspects, the mostly used sequencing technology is the Chromatin ImmunoPrecipitation sequencing (ChIP-seq) [1]. This technique allows to use antibodies for selected proteins or nucleosomes, which enriches for specific DNA sequences bounded to these proteins/nucleosomes.



**Figure 1.2.3:** ChIP-seq experiments can be used to investigate DNA binding sites such histone modifications (Histone ChIP) and transcription factors (Non-histone ChIP), using specific antibodies for the specific interested protein (immunoprecipitation). After the DNA purification, the genetic material is linked to specific adaptors and then amplified in different manner, depending on the amplification and sequencing type used. [1]

The library preparation consists into locking biological processes with formaldehyde and then cutting the chromatin into small fragments with sonication. Afterwards, a specific antibody for the interested protein is used to immunoprecipitate the DNA-protein complex, in order to be purified and, after amplification, to be sequenced.



**Figure 1.2.4:** The DNA fragments at 5' are sequenced, producing a double profile for each DNA strand in correspondence of the surrounding area of the protein binding site. In such a way it is possible to identificate a unified peak combining the profiles with the reads.[1]

When analyzing the data, it is relevant to distinguish between HM and TF ChIP experiments because, even if the library preparation is similar (it differs only for the antibody used, just because the proteins are different), the data analysis pipeline is different because of the different signal shapes. Indeed, after read mapping on a reference genome, the reads are processed with methodologies for the protein-binding regions detection, known as *Peak Callers*. After the peak calling process it is possible to highlight that the TF signals (peaks) are more narrowed respect to the ones detected for HM, leading to develop different methodologies for investigating further aspects for each one of these ChIP-seq

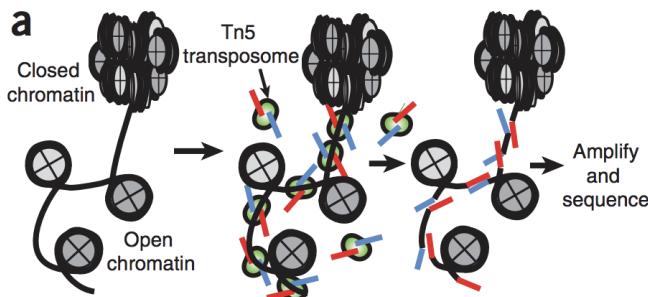
signals, such as *MACS* [30] and *SICER* [31].

Subsequently, after the peak detection, several aspects can be investigated about the signal, such as the identification of peaks related motifs, or the associated genes with detected regions, and, when other omics are available (e.g. RNA-seq), it is interesting to associate the expressed features (e.g. genes) and doing functional enrichment analysis.

### 1.2.3 ATAC-seq

The chromatin packaging of the genome plays a fundamental role in the gene regulation of eukaryotic individuals. To study this aspect of the DNA, several technologies have been developed, such as Formaldehyde-Assisted Isolation of Regulatory Elements Sequencing (FAIRE-seq) [32], DNase I hypersensitive sites Sequencing (DNase-seq) [33], ATAC-seq [3], etc.

ATAC-seq among the others is having a growing interest and diffusion during last years, because it offers comparable results to the DNase-seq with less biological material and less library preparation time.



**Figure 1.2.5:** ATAC-seq library preparation uses *Tn5* transposome natural predisposition to link and cut the DNA. In so doing, it provides DNA sequences that can be easily amplified and sequenced to be mapped and processed [3].

The library preparation adopts a hyperactive *Tn5* transposase, modified with adaptors for high-throughput DNA sequencing, which is able to fragment and tag a genome simultaneously. The technology exploits the *Tn5* capability of integrating itself into active regulatory elements.

After *Tn5* fragmentation, the resulting segments can be amplified and sequenced, producing sequences to map on a reference genome. There is no standard analysis reached for the ATAC-seq analysis, but, inspired by the ChIP-seq

analysis, the resulting reads, typically, are processed with tools (peak callers) for quantifying their amplification, producing, for each detected open chromatin region, a feature: the peak (generally with an associated score) [34].

Depending on the used tool, the peaks can be represented in different data structures, but their representation is typically given by the genomic coordinates; chromosome, starting and ending point of the region, the DNA strand where the region lies, and additional attributes such as a score, a number of samples on which the regions have been detected, etc.

To obtain the first level of integration, the peaks can be annotated with other relevant features of the genome, such as the Transcription Starting Site (TSS) of the genes, Untranslated Regions (UTR), promoters, exons, introns, etc. Then, the annotated genes can be used to enrich for GO terms or pathways, reaching the second level of integration [35–37].

### 1.3 Multi-omics Integration

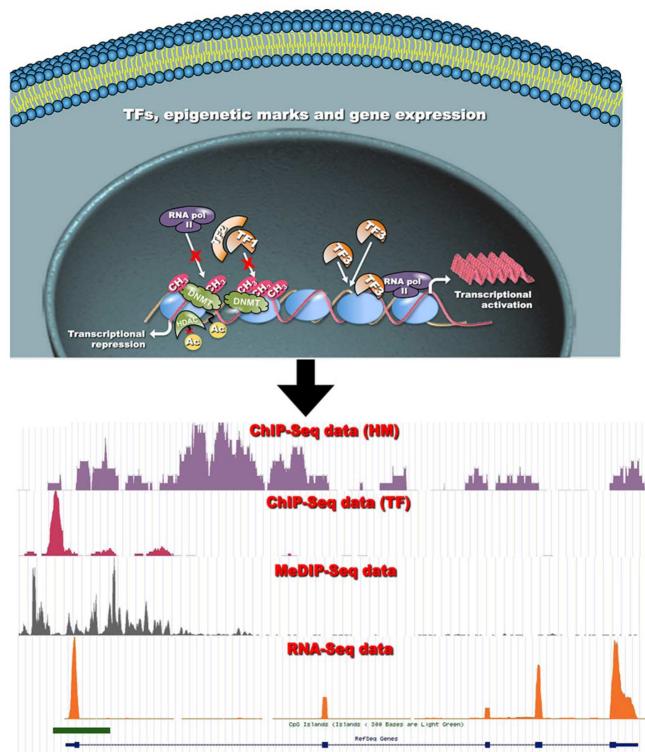
All above-mentioned sequencing techniques are aimed to investigate only one cellular aspect at a time, but in order to obtain a more comprehensive view of the cellular behaviour, it is necessary to look at more than one omics at the same time.

Indeed, we can imagine each omics as a camera in a multi-view camera system pointed on a building from different points of view. Each device takes snapshots of the building from different angles, but the information is still fragmented. In order to reconstruct a 3D model of the building, we need to put all the snapshots coming from different devices together.

The same idea can be adapted to the sequencing techniques, we need to integrate the information coming from different omics in order to reconstruct (and understand) how multiple mechanisms orchestrate the cellular behaviour.

Indeed, we can imagine a typical combination of epigenomics factors affecting the chromatin in order to influence the transcription of one or more genes. An HM (such as *Acetylation*) can have inhibited the accession to bind specific nucleosomes, because already occupied by specific TFs, inhibiting, in such a way, the expression of those genes present in that particular portion of the chromatin

(upper part of figure 1.3.1). To investigate these processes, we can perform multiple experiments of RNA-seq and ChIP-seq (one for the HM and one for the TF) to study singularly each epigenomic factors and the gene expression (lower part of figure 1.3.1).



**Figure 1.3.1:** An example of different omics coming from the same biological experimental conditions. An HMs (such as *Acetylation*) can have inhibited the accession to bind specific nucleosomes, because already occupied by specific TFs, inhibiting, in such a way, the expression of those genes present in that particular portion of the chromatin (upper part). To investigate these processes, we can perform multiple experiments of RNA-seq and ChIP-seq (one for the HM and one for the TF) to study singularly each epigenomics factors and the gene expression (lower part) [38].

As figure 1.3.3 outlines, multi-omics data integration can be made at different levels, by graphical exploration, by functional annotation, by network fusion and by dimensionality reduction. We can distinguish two main approaches; "analyze each omics then integrate the results" and "jointly analyze each omics to improve the power". The first one can be performed also with few samples, where each omics is analyzed standalone and then the results can be combined to obtain a common response (levels 1 and 2 of figure 1.3.3). The second main approach

needs a high number of samples to analyze all the omics together to obtain more power in the response prediction (levels 3 and 4 of figure 1.3.3) [39–42].



**Figure 1.3.2:** An illustrative example of a multi-view camera system pointed on a building. Each omics can be represented by a different camera pointed on the same building. The final snapshots can be integrated to reconstruct the building, which represents the cellular behaviour.

More in general, integration can be performed at different levels (i.e. descriptive, exploratory, inferential, etc.).

With graphical exploration, we can visualize data coming from different sources (e.g. RNA-seq and ChIP-seq) using specific tools designed at this scope, such as *Genome Browser* [43] or Integrative Genomics Viewer (IGV) [44, 45] and looking to the overlapping regions, or where expressed epigenomic markers have correspondence with gene expression sites.

We refer to functional annotation integration when using methods combining analysis results (such as relevant lists of genes) with publicly available databases, like the Gene Ontology<sup>2</sup> and pathway (such as *KEGG*<sup>3</sup> or *Reactome*<sup>4</sup>) based ones, to detect functional responses highly related to the experimental condition under investigation.

It is possible to integrate multiple omics data types by constructing multiple regulation networks, one for each analyzed omics, and then combine these networks with fusion techniques. This integration aspect is used when a high number of data samples is available or when having multiple-omics data types coming from the same patients [46].

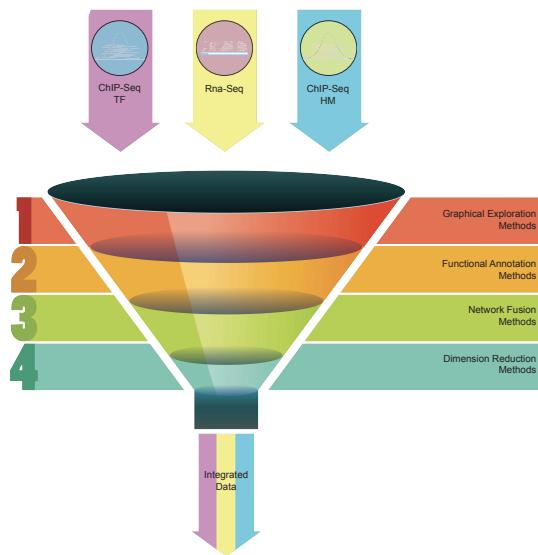
A deeper level of integration is achievable with dimensionality reduction techniques or multitask regression techniques. Also in this case, several samples are needed to be able to obtain relevant and reliable results. Generally speaking,

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<sup>2</sup><http://www.geneontology.org/>

<sup>3</sup><https://www.genome.jp/kegg/>

<sup>4</sup><https://reactome.org/>



**Figure 1.3.3:** A schematic representation of multi-omic data integration levels. Graphical exploration can be used to visualize data coming from different sources (e.g. RNA-seq and ChIP-seq) using specific tools designed at this scope. Functional annotation integration combines analysis results (such as relevant lists of genes) with publicly available databases to detect functional responses. Network fusion techniques are used when a high number of data samples is available or when having multiple-omics data types coming from the same patients. Also dimensionality reduction techniques require several samples to be able to obtain relevant and reliable results.

these methods are able to start from multiple samples coming from different omics and to reduce their dimensionality, enabling to identify common cellular behavioural aspects [39, 40].

## 1.4 Reproducible Research

During last years "irreproducibility" became a general problem, particularly in Medical-Biosciences where high-dimensionality data are collected from so many different omics to investigate the cellular behaviour, in order to propose new drugs and new patient-oriented treatments.

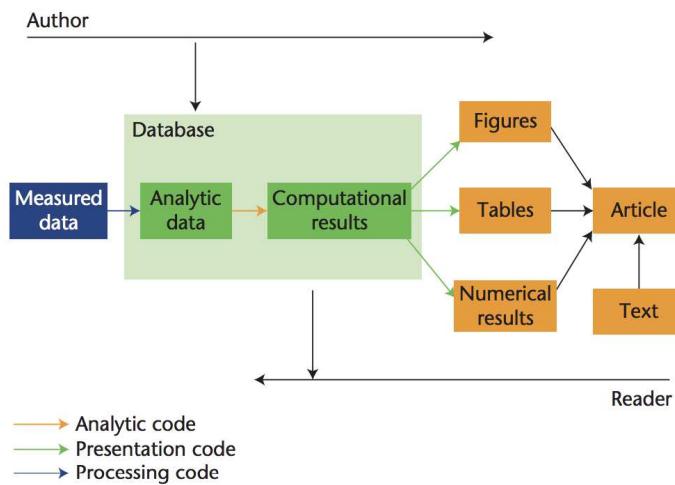
Due to the analysis complexity and to the poorly documented processes, several published papers [47, 48] became a landmark for irreproducibility, leading the scientific community to highlight the weaknesses in reproducing published scientific facts and to promote the Reproducible Research (RR).

The underlying idea of RR [49] is to provide public access to analytic data

and related analysis code (combined with natural language documentation), in order to allow third-parties to entirely reproduce the findings.

In recent years, many efforts have been spent for sensitizing the scientific community on the relevance of RR, such as by producing tools [29] supporting reproducible research, by producing scientific works in reproducible research spirit [50] or by providing an overview of available methodologies for RR [28].

Anyway, to address RR in scientific works many cares are needed during the production of a software or when analyzing data. As figure 1.4.1 [51] shows, the author cannot only make an analysis, but needs to store all the components needed to a third-party user to reproduce the entire analysis. To do so, the author needs to give public access not only to the input/output data but also to the used code to process them, in a "database-like" form, required to maintain the reproducibility across the time, which can bring to different code tool versioning. Moreover, it is fundamental to present the results in the same form as they can be produced with the "database", storing the data and the code.



**Figure 1.4.1:** A scheme of Reproducible Research. The author produces a database within the analytic data and the computational results (green boxes) produced during the analytical phase (yellow arrows), in order to help the reader in reproducing the results presented (green arrows) inside a published article (in yellow). (Image from [51])

Of course there are several disadvantages trying to respect RR constraints, such as the time required not only to analyze the data but also to trace the code and the data in order to give them public access. Moreover, nowadays there are

several tools [52–55] trying to help implementing RR inside the scripts and the tools, but it is not so simple to determine which one works better than another, and this can be due to the problem that a standard has not been reached yet.

Fortunately, some international journals are starting to require proof of transparency for the proposed works, by showing the entire computational analysis. This is advantageous because from one side is leading the scientific community to propose always easier solutions for using tools for RR, and from another side the analysts are "forced" to release their data and code to demonstrate transparency and, consequently, more robust findings.

Unfortunately, despite all efforts of RR community, the Life Science society is still far from a standard for reproducibility [56], showing that the way is still long to be covered.

## 1.5 Aim of the Work

The complexity of NGS data analysis brought the scientific community to develop multiple methodologies and tools for their analysis, reaching in some cases (such as RNA-seq, ChIP-seq, etc.) standard pipelines for their analysis. However, despite such efforts, there are specific context that are much less developed and that still require attention.

Nowadays, the challenge moved on the development of statistical and computational methodologies for the information extrapolation from multiple-omics datasets, in order to produce a unified view of the biological characteristics describing the cellular behaviour.

Motivated by these aspects, we have embarked on a path that goes through the analysis of different omics to flow into multiple projects, each one with the common aim of helping the single omics analysis in order to simplify reaching the goal of integrating them in a unified view.

This thesis is focused on the development of three main computational tools (*Ticorser*, *DEScan2* and *IntegrHO*), firstly approaching single omics data analysis and then on their integration. Additionally, in order to face up the irreproducibility problem in the scientific community, a fourth tool (*easyReporting*) is presented.

Time Course RNA-seq data Analyzer (Ticorser) is a novel R package aimed to analyze time-course RNA-seq data, which still require more attention. It offers multiple methods for differential expression data analysis and provides multiple plots useful to explore and visualize the results at each step of the analysis. Furthermore, it also provides methods for functional integration by annotating genes in pathways and GO-terms.

Differential Enriched Scan 2 (DEScan2) is a novel R package for ATAC-seq data analysis, one of the emerging techniques for investigating chromatin accessibility, for which very few methods are available. It consists in the following three-step procedure: 1) It identifies candidate regions inside each sample implementing a peak caller; 2) It filters out potential artefacts by aligning the candidate regions between the samples and removing those candidate regions that were not reproducible between samples; 3) It produces a count matrix of regions and samples, useful for differential enrichment between multiple conditions and also for integrating this data type with other omics data, such as RNA-seq.

Integration of High-Throghput Omics data (IntegrHO) is a Graphical User Interface (GUI), written in R and Shiny, aimed to analyze and integrate multi-omics data types. It provides a friendly interface to the above-mentioned tools and also incorporates a wide selection of methods and other tools available in the literature. This platform, through an easy point-and-click approach, enables the user to analyse and explore single omic data, such as RNA-seq, ChIP-seq and ATAC-seq and, moreover, it offers the possibility to integrate them at different levels, such as gene-peak annotation and functional annotation methods. Trying to provide a flexible and easy-to-use tool enjoyable also by non-expert analysts, such as biologists, or beginner analysts.

Finally, since in last decades the scientific community has experienced a deep crisis known as "irreproducible research", this thesis presents *EasyReporting*, an R package for an automatic report creation, developed to support the reproducibility of scientific research. Thanks to the R6 class paradigm on which is based on, it is easy to use and to extend.

Overall, this work proposes and combines several computational tools for properly analysing, visualizing, comparing, integrating and tracing different

omics data types. The results are illustrated with downloaded data from the literature or from collaboration projects.



# Chapter 2

## Time Course RNA-seq analyzer: ticorser

When working with RNA Sequencing (RNA-seq) data, there are multiple aspects that can be studied, such as single nucleotide variations, alternative splicing, post-transcriptional alterations, fusion gene detections, etc.

In this chapter we focus on differentian gene expresion in time course experimental design with RNA-seq data using the Time Course RNA-seq data Analyzer (Ticorser), a tool developed for analyzing RNA-seq time-course data.

The tool is available at: <https://github.com/drighelli/ticorser>

### 2.1 Introduction

When working with RNA-seq data it should be interesting to investigate the evolution in time of the gene expression between two or more conditions.

When defining a time-course experiment we refer to muliple samples, taken at several time points, in multiple conditions. If we suppose to want to investigate the cellular gene expression effects of a drug treatment in 24 hours, it could be a good idea to perform multiple sampling (i.e. one per hour) of the treated and the untreated cells. Moreover, to obtain more power to be statistically significant with our results we'll need to have multiple replicates at each sampling. In such a way, we are able to collect multiple replicates at multiple

times (24) for each condition (treated and untreated). By doing sequencing of these samples and inspecting the resulting experimental sequences, we are able to explore with ad-hoc designed statistical and computational tools the differences in time between the conditions.

To afford problematic like this one, we designed Ticorser, an R package for complete and fast analysis of time-dependent RNA-seq data, offering a vast amount of hypothesis tests set up for differential expression between two or more conditions. With aid of *DESeq2*, it offers the possibility to set up the experiment and to obtain differential expression between the experimental biological conditions. The software is developed to assist the user to perform an entire analysis pipeline, from the quantification step, to the functional enrichment analysis, passing through the normalization, filtering and differential expression analysis.

For each step, it offers the possibility of several interactive plots and graphics, such as KEGG-maps and hierarchical GO terms trees.

## 2.2 Methods

Ticorser is a tool fully devoted to the Time Course (TC) RNA-seq data offering features to inspect the data, to normalize them, to capture the differential expression of genes at the static time point and overall time points, supporting different experimental designs. It is also possible to compare the results coming from different analysis and to investigate the most influenced biological functions (i.e. Gene Ontology terms and Pathways [57, 58] ).

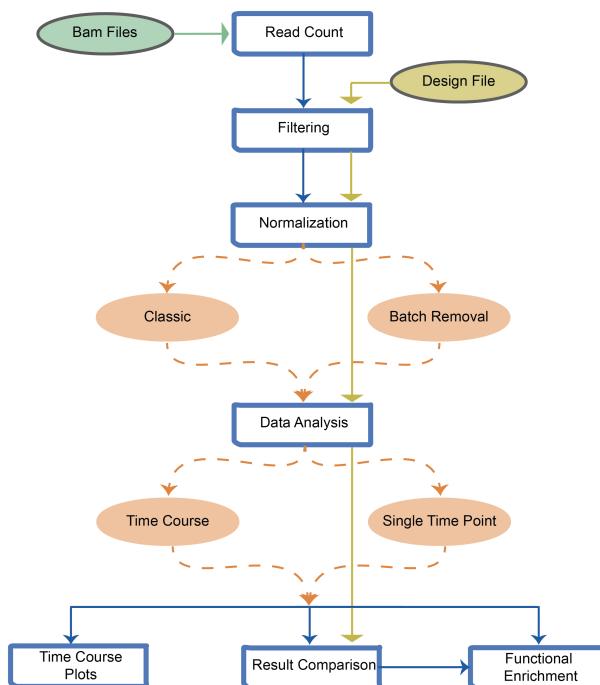
Overall, Ticorser offers the possibility to analyze data using different R-/Bioconductor [54] packages, to compare the results in order to choose the best combination of tools for the user specific problem. Therefore, the software implements a vast amount of exploratory and diagnostic interactive plots to explore data not just at pre-processing step but also during the post-processing phase.

Starting the analysis of TC RNA-seq data from Binary Alignment Map (BAM) files, Ticorser enables RiboNucleic Acid (RNA) expression quantification through `featureCounts` method [59], producing a count matrix of features per samples, which enables the Differentially Expressed Genes (DEGs) detection with hypothesis statistical methods.

As figure 2.2.1 shows, this is a *design file based* tool, where a file, that describes all the samples by several variables, chaperons the count matrix through all the analysis, simplifying, in such a way, the user interaction with all the available instruments.

Particular attention is given to the normalization phase, providing the possibility not only to use several traditional normalization methods but also the methodologies for batch effect removal.

In order to inspect different approaches to analyze TC data, Ticorser offers three different methodologies for analyzing TC RNA-seq data, and four different methods to analyze different biological conditions at single time point level.



**Figure 2.2.1:** Main flow of the ticorser R package. The package takes as input a list of bam files for producing a count matrix of the features (on the rows) and the samples (on the columns). Afterwards, with aid of a design file, Ticorser allows the filtering of low expressed genomic features and their normalization with classic methodologies and with specific ones for batch removal. Once the data are comparable it is possible to perform a differential expression at time-course level and/or at specific time point. The results can be investigated for plotting, comparison or for functional enrichment.

### 2.2.1 Filtering low counts

Low expressed genes in RNA-seq data always affect the detection of DEGs [60] influencing final results.

In order to address this task, Ticorser offers multiple statistical methods for low expressed genes filtering. The `filterLowCounts` method is partially based on the `filtered.data` function of *NOISEq* R/Bioconductor package [61, 62]. The method gives the possibility to apply four different filtering methods, the Counts per Million (CPM), the *proportion* and the *Wilcoxon* tests and an our-defined method named *quantile*.

The CPM filters out all those genes with a mean expression between the samples lesser than the `cpm` parameter threshold and, at the same time, a Coefficient of Variation (COV) higher than the `cv.percentage` parameter in all the samples.

The *proportion* test performs its homonym test on the counts, filtering out all those features with a relative expression equal to  $cpm/10^6$ .

The *Wilcoxon* test filters out all those genes with a median equal to 0.

Finally, the *quantile* method takes as input the `quantile.threshold` argument (default is 1%) and filters out all those genes expressing the between-samples counts mean lower than this threshold.

### 2.2.2 Data Normalization

Normalization is a fundamental aspect in RNA-seq data analysis, especially when a comparison between different biological conditions is aimed to highlight major differences.

For this reason Ticorser is equipped with multiple methodologies to deal with this analysis step. Indeed, through the `normalizeData` function Ticorser provides five different normalization methods, where the user doesn't have to take care of any particular additional information, except for the normalization method. Thanks to the design matrix based system, the method allows to automatically subset the data only to the relevant factors the user want to normalize.

The function offers the possibility to normalize the data with *Full quantile*, *Upper quartile* and *Trimmed Mean of M-values*, setting the `norm.type` parameter to *fqua*, *uqua* or *tmm*.

Moreover, it is possible to apply a batch effect removal normalization method, as described in *RUVSeq* R/Bioconductor package [63], using the *RUVg* and *RUVs* normalization methods.

The first one, *RUVg*, can be selected by setting the `norm.type` parameter to `ruvg`, which requires a list of negative control genes with `negative.controls` parameter. If it is not already available from previous studies, this list can be produced by performing a first *differential expression* analysis, and taking the less significative genes.

In order to facilitate this process, we developed a method for creating the negative control genes list from a differential enrichment result matrix. The function `estimateNegativeControlGenesForRUV` takes as input the `de.genes` and the `counts.dataset` dataframes to extrapolate the less significative genes and to redistribute them in groups based on the mean value of the gene counts. By selecting one or two genes per each group, our method is able to produce a list of negative control genes which have equal distribution for each gene counts trend.

Finally, the `normalizeData` function offers the possibility to remove batch effects with *RUVs* normalization, which is more robust to negative controls, giving better results when their estimation is approximated. The method takes care of creating the group samples and also the negative controls list, in case this one is `NULL`.

### 2.2.3 Differential Expression

Depending on the biological questions under investigation, to test differences between multiple conditions, Ticorser offers three different ways of analyzing TC RNA-seq data and three methodologies for analyzing different biological conditions at steady-state.

To do so, we took advantage of some of the most used and well-performing [64] R/Bioconductor packages: *DESeq2* [65]; *edgeR* [66] and *NOISEq* [61, 62].

Exception made for *NOISEq*, the selected methods model the RNA-seq data counts for each gene as independent Negative Binomial distributions, which has been demonstrated [67] to be better suited for this data type. At the same time, each of them differs for the statistical test implemented while approaching the biological question under investigation. In particular, the *NOISEq* method is

based on a non-parametric approach, which implies that it doesn't assume any probability distribution.

In the following sections, we first present the TC methods and then the methods for single time point gene differentiation.

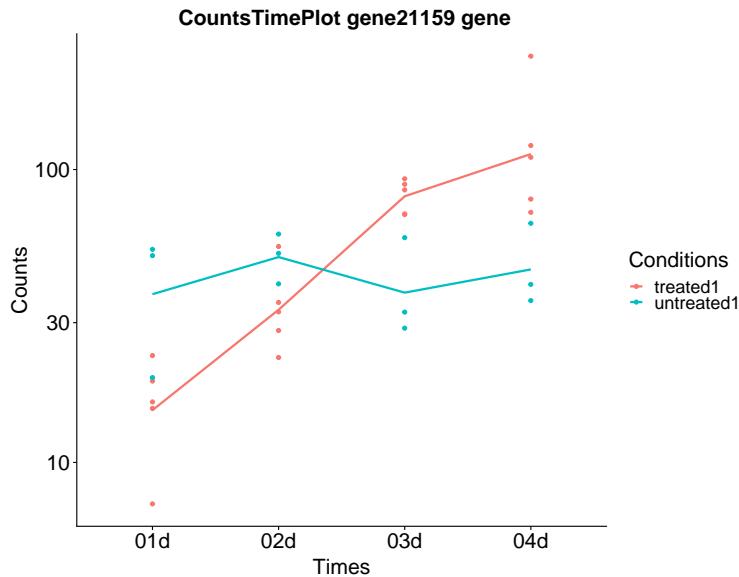
### Time-Course DE Method 1 - *LRT\_TC*

The first method (*LRT\_TC*) uses the Likelihood Ratio Test (LRT) to compare two different models in order to extract all those DEGs that invert their expression between the conditions across all the time points.

Exploiting the LRT, as implemented in *DESeq2* R/Biocnductor package, we compare two different formulas. The first one defines the *full* model where we put together the timepoints, the conditions and an interaction term between these two variables, while the second one is a reduced model where the interaction term has been removed:

$$LRT \sim \frac{times + conditions + times : conditions}{times + conditions}$$

In so doing, we are able to catch all the genes with distinct expression at time 0, and inverting their expression across the conditions along the time-course experiment (figure 2.2.2 provides an example of DEG detected with this method).



**Figure 2.2.2:** A typical trend of a DEG detected with *LRT\_TC* method. The gene trends inverts its expression between the two biological conditions across the experimental time points.

### Time-Course DE Method 2 - *LRT\_T*

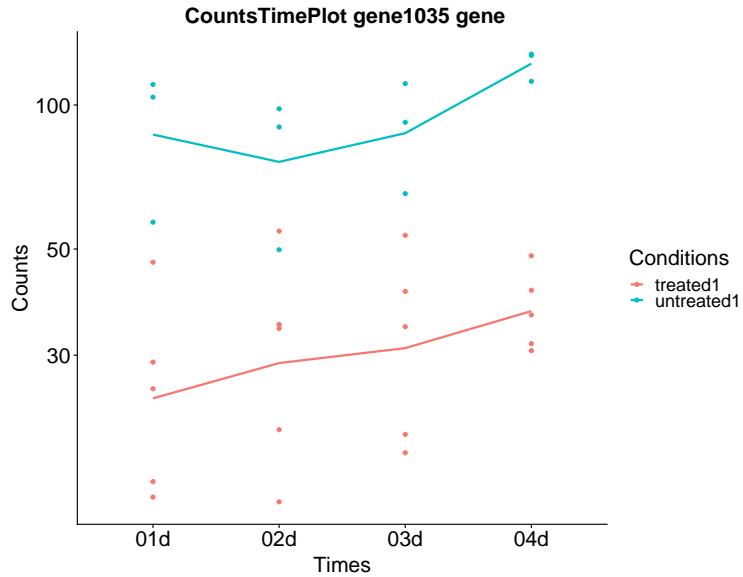
The underlying idea of the second method is the same of the first one, where the difference is to remove from the *reduced* formula, not only the interaction term but also the *conditions* variable.

In such a way, we are able to extract all those DEGs having and preserving different expression profiles between the conditions across all the time points (figure 2.2.3 provides an example of DEG detected with this method).

The first formula here defines the same *full* model of the first method, while the second one is the reduced model where only the times variable appears:

$$LRT \sim \frac{\text{times} + \text{conditions} + \text{times : conditions}}{\text{times}}$$

By loosing the conditions term in the reduced formula, this method is able to catch all the DEGs detected by *LRT\_TC* method, and, additionally, all the genes with different trends across all the time points.



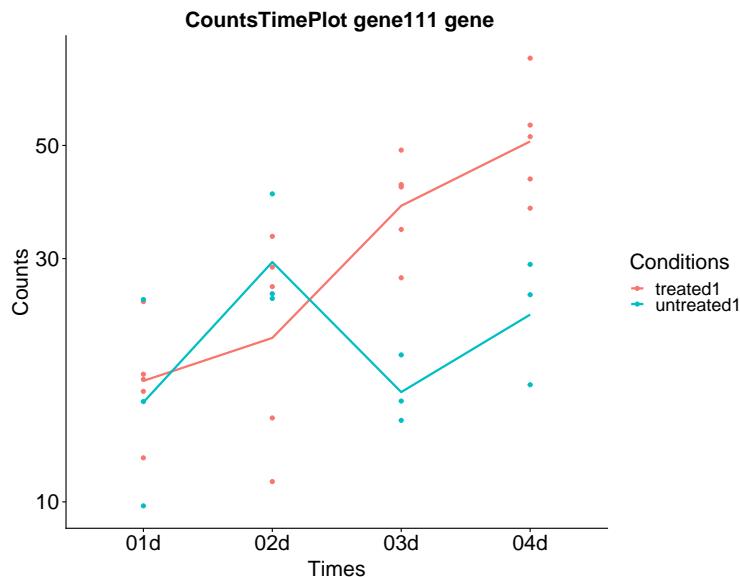
**Figure 2.2.3:** A typical trend of a DEG detected with *LRT\_T* method. The gene has different expression between the biological conditions and maintains this expression across all the experimental time points.

#### Time-Course DE Method 3 - *LRT\_NOInteraction*

Using always the *DESeq2* LRT approach, we defined a third method for the identification of DEGs that have different expression between the conditions across all the time points, but that maintain the same profile in both conditions (figure 2.2.4 provides an example of DEG detected with this method).

Here the *full* model defines the time points and the conditions variables without taking into account the interaction term, while the second the *reduced* model presents only the time point variable:

$$LRT \sim \frac{times + conditions}{times}$$



**Figure 2.2.4:** A typical trend of a DEG detected with *LRT\_NoInteraction* method.

Because of the absence of the interaction term, this method detects the highest number of DEGs expressing difference between the conditions, but not taking into account this difference across the time points. Indeed, it seems to be able to detect several genes in common with other LRT methods, showing an high number of DEGs detected by itself.

#### Time-Course DE Method - *Wald*

After using the LRT test, the *DESeq2* package, thanks to its results data structure, offers the possibility to apply the *Wald* test on the previously computed LRT results, by specifying the contrasts to be tested.

In such a way it is possible to test the differences between the biological conditions at a single time point.

In particular, we designed Ticorser to automatically apply this additional test and return the DEGs expressing differences between the biological conditions at time 0.

From some preliminary tests, this method seems to be able to detect mostly the same DEGs detected with the *LRT\_NOInteraction* method.

### Single DE Methods

To account for fixed time point experiment, we implemented functionalities for helping also the exploration of this aspect, using three different methodologies.

By using the `differentiateConditions` function, it is possible to choose between the `edgeR`, `DESeq2`, `NOISEq` and `NOISEqBio`.

In case of `edgeR` we decided to use the *Quasi-Likelihood* method for the differential expression. While, for this specific case, when using `DESeq2`, we choose the *Wald* test, as suggested by the authors.

The `NOISEq` package offers the possibility to discriminate between *biological* and *technical* replicates, computing a posterior probability in both cases, but applying different hypothesis tests.

In case of *technical* replicates, the `NOISEq` method has to be applied, considering that it requires the full count matrix, within low expressed genes, in order to be able to properly estimate the data distribution. While when biological replicates are available it is more indicated to apply `NOISEqBio`, which requires at least 3 replicates per condition.

#### 2.2.4 Data Visualization

During RNA-seq data analysis, it is almost mandatory to explore the data during each step of the analysis. This is relevant to understand which is the best method to apply at each analysis step and to be more confident with produced results.

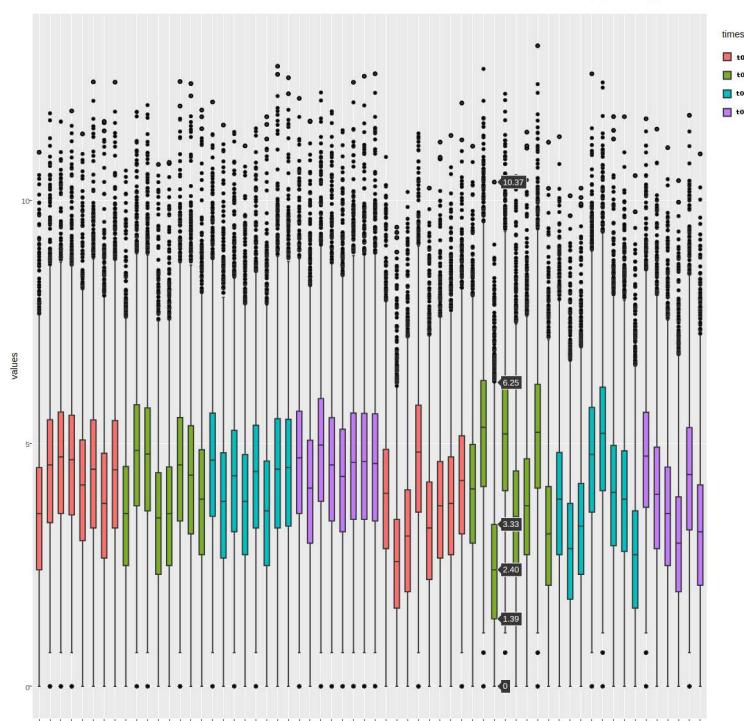
At this scope, we equipped Ticorser of several useful graphics and plots to be used at each analysis step. Each of them, except when otherwise declared, with aid of `plotly` library, enables to convert the plot in an interactive Hypertext Markdown Language (HTML) plot, useful to inspect additional attributes using the mouse pointer.

##### Exploration Plots on Counts

As already mentioned (see section 2.2.2), the normalization phase critically affects the final results in terms of DEGs detected. That's why it is always a good norm to understand how the applied transformations are influencing the features quantification. To address this need we implemented two plots, the

Boxplot and the Principal Component Analysis (PCA).

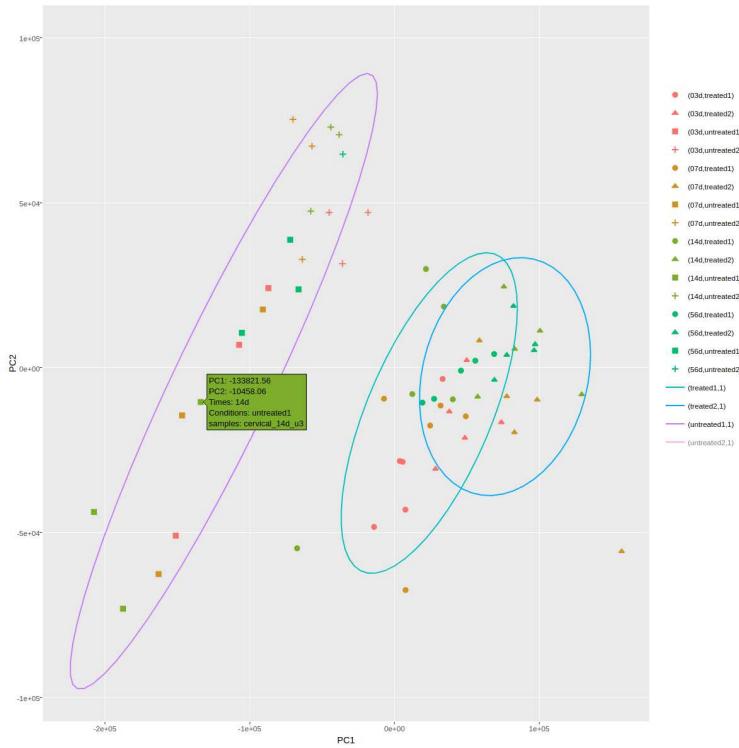
The boxplot is a graphical representation of the distribution of the samples. Our boxplot is organized in group colours accordingly to the time-point each sample belongs to (figure 2.2.5).



**Figure 2.2.5:** An example of interactive boxplot made with Ticorser package. Each box represents a specific sample, while each colour represents a specific time point. When passing the mouse over a boxplot it shows additional information about its quartiles and outliers.

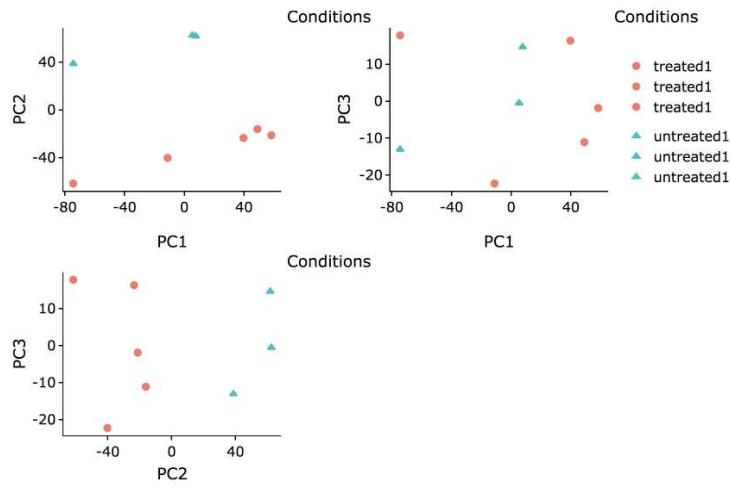
Each box is divided into two main parts, with an outgoing segment from each side. The horizontal upper and lower lines of the box represent the first and third quartile, while the middle horizontal rule represents the median. The upper and lower part of the segments, in case of absence of outliers, represent the minimum and maximum values of the sample distribution.

Our `plotBoxplotPlotly` function is based on the design matrix, describing the data counts, which gives the possibility to select the column to use for colouring the samples thanks to the `colorColname` parameter.



**Figure 2.2.6:** An example of interactive PCA made with Ticorser package. Each dot represents a specific sample, while each colour represents a time point, each symbol represents a biological condition group. When passing the mouse over a dot it shows additional information about the selected sample, while from the legend it's possible to show/hide groups or ellipses.

Due to the very high dimensionality of RNA-seq data, it is widely considered common sense to apply PCA dimensionality reduction technique to visualize the data, limiting their representation to 2 or 3 dimensions. There are several packages allowing to apply a PCA transformation, but we choose to implement it in the `PlotPCAPlotlyFunction` function, by using the `prcomp` from the `stats` package.



**Figure 2.2.7:** An example of interactive matrix PCA made with TicoRser package. Each plot puts in relation two different Principal Components. While each dot represents a specific sample, while each colour represents a time point, each symbol represents a biological condition group. When passing the mouse over a dot it shows additional information about the selected sample, while from the legend it's possible to show/hide groups or ellipses.

The user just needs to give the `counts.data.frame` and the `design.data.frame` by specifying the column name where to find the samples groups. In such a way, the function will compute and plot the PCA, colouring the samples according to the groups identified by the chosen column. It is also possible to specify the *Principal Components* (PCs) to plot with `xPCA` and `yPCA`, where `x` and `y` indicate respectively the x-axis and y-axis. Moreover, by setting the `ellipse.flag` argument to `TRUE` the function will show the ellipses surrounding each sample group, each one describing the variance of the groups.

In order to plot more than one PCs, TicoRser allows to plot more than one component at the same time (figure 2.2.7). By using the `plotPCAMatrixPlotly` function it's possible to compare more than two PCs at the same time, simply by using the `pc.list` argument.

## DE Results Plots

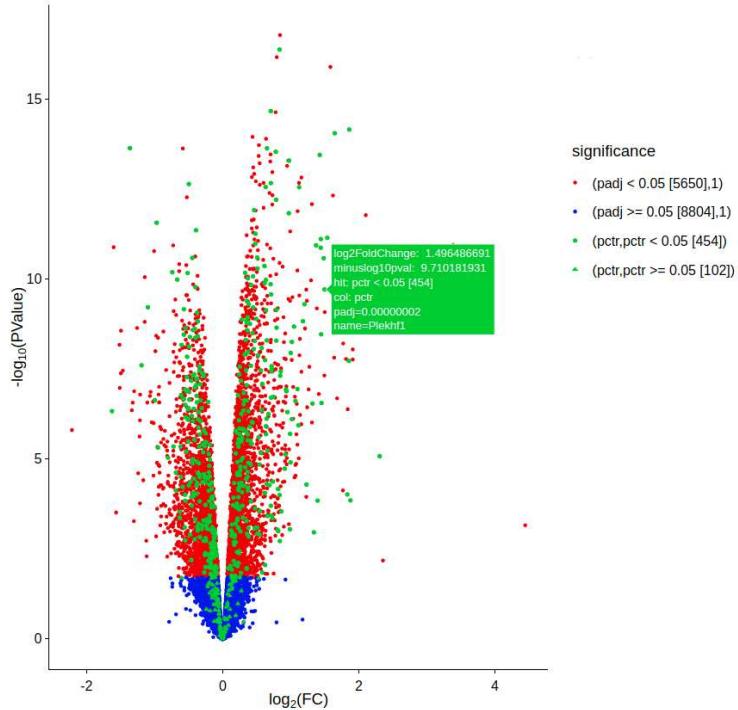
In order to inspect the results produced by DE methods, we implemented two kind of plots, the *VolcanoPlot* and the *MA-Plot*.

Both our implemented methods take as input a DE results data frame, auto-

matically recognizing which method produced it, in order to speed up the user cares during the analysis. Moreover, it gives the possibility to add a list of positive control genes, in order to annotate them on the plot with a third colour (figure 2.2.8).

The volcano plot shows the relation of  $\log_2(FC)$  with  $\log_{10}(p-value)$  of each gene, in order to highlight the significant changes inside the data experiment.

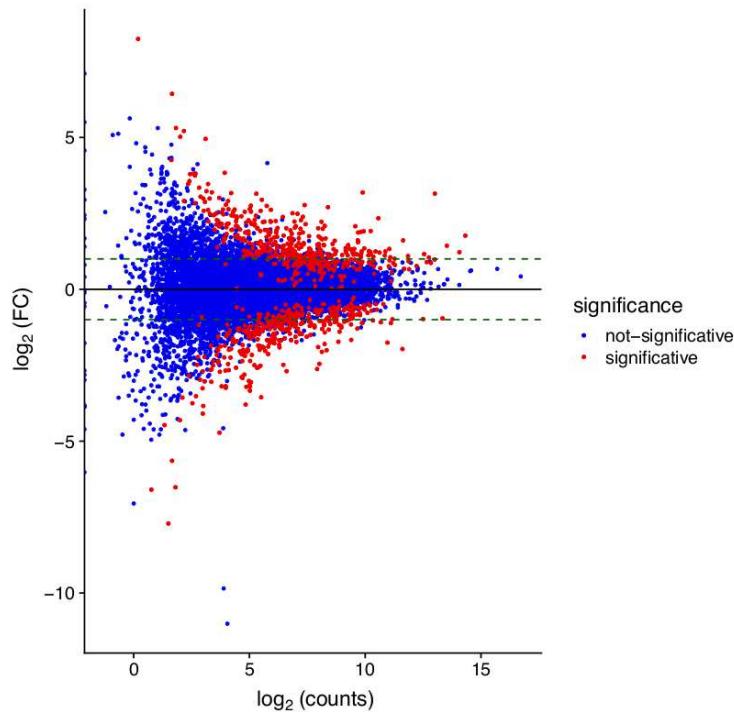
The MA-Plot shows the relation between two quantities useful to understand the differences between the measurements in two conditions. Indeed, on the x-axis, there is represented the  $\log_2(FC)$ , where  $FC$  is the fold change computed as the ratio of the treatment on the reference control. It is not mandatory to have a DE-results dataframe to plot an MA-plot, but it's pretty useful to have it in order to highlight the distribution of the significant genes (figure 2.2.9).



**Figure 2.2.8:** An example of interactive volcano plot made with Ticorser package. Each dot represents a gene, while blue and red colours highlight the significance of the genes. In green, there are those genes coming from the positive control list. When passing the mouse over a dot it shows additional information about the selected gene.

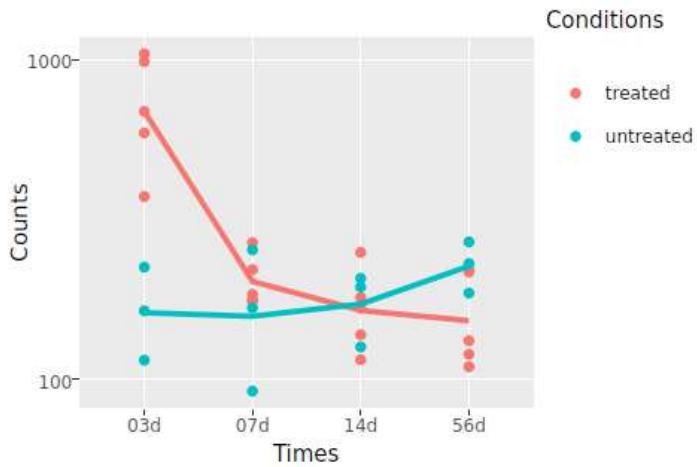
### Gene Profiles plot

When working with time course data experiment, it is really useful to understand the trend of one or more genes across all the time points and the relation between the conditions.



**Figure 2.2.9:** An example of interactive MA-plot made with Ticorser package. Each dot represents a gene, while blue and red colours highlights the significance of the genes. When passing the mouse over a dot it shows additional information about the selected gene.

In order to highlight the expression of a gene across multiple time points and different conditions, we implemented the `plotGeneProfile` function, which takes as input a count matrix, its related design matrix and a gene name (figure 2.4.7).

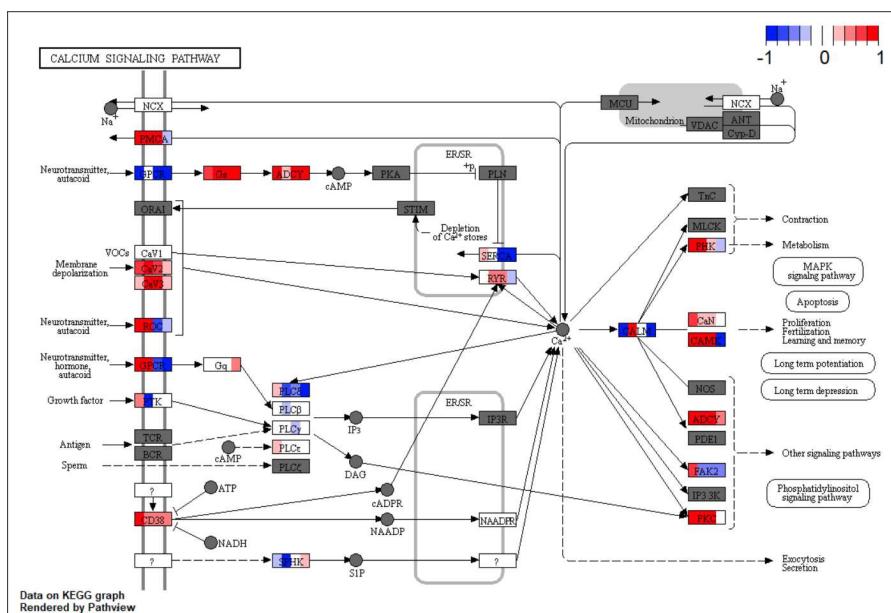


**Figure 2.2.10:** An example of interactive gene profile made with Ticorser package. Each dot represents the counts value of the gene in a sample. Colours identifies the conditions of the samples. The lines represent the gene trend over all time points.

## KEGG-Maps

Once detected the DEGs, it is really important to inspect for functional mechanisms regulated by up and down-regulated genes.

Ticorser offers the possibility to plot *keggmaps* [68] taking into account the  $\log_2(FC)$  of the genes involved in the graphical representation, through all the time points. Indeed, using the `plotKeggmap` function, the software enables to plot a keggmap, using as input the counts and the design matrix, computing the  $\log_2(FC)$  at each time point, and showing it in the gene box inside the *KEGG* map. The color legend in the upper part of the figure indicates color associated to the up and down regulated genes.



**Figure 2.2.11:** A KEGG-map representation for the *Calcium Signaling pathway*. The contributing genes are represented in boxes. Arrows represent the effect of each gene on others. The coloured genes typically come from a differential expression experiment, showing the expression in log Fold Change.

## 2.3 Additional Features

Ticorser offers multiple additional features to support the user during the differential expression analysis of time-course RNA-seq data.

### Gene quantification

A fundamental aspect during RNA-seq analysis is the features (genes) quantification. To account for this, Ticorser give the possibility to quantify the gene expression, starting from BAM files, using the `countBamFilesFeatureCounts` function, in order to guide and facilitate this operation to the user.

The function is based on the `featureCounts` method available through the *Rsubread* R/Bioconductor package [69], hard coding some parameters, such as `useMetafeatures` to `TRUE` and `allowMultiOverlap` to `FALSE`.

In order to be able to quantify for the transcription features, the user has to give as input a list of BAM files and a Gene Transfer Format (GTF) file. The first ones are typically one file per sample, containing all the reads previously

mapped to a reference genome, while the GTF file describes all the features of the specific organism of interest.

Moreover, by properly choosing the `gtf.attr.type` and `gtf.feat.type` the user can define, respectively, the attribute type (i.e. `gene_id`) and the type of feature (i.e. `exon`) to quantify, taking in mind that these values must be equal to the ones present into the GTF file.

At the end of the processing, the `countBamFilesFeatureCounts` function returns a matrix with the *attributes/features* on the rows and the samples on the columns (named with the same name of the BAM files). Each cell of this matrix contains the expression value of the feature for the sample represented by that column.

More formally, each row  $i$  represents a specific feature, while each column  $j$  represents a specific sample. The cell  $c_{ij}$  contains the number of reads assigned to the  $i$ -th feature for the  $j$ -th sample.

### Functional Analysis

In order to obtain a first level of integration, we implemented functions for the functional analysis, a process where lists of DEGs are used to understand the cellular mechanisms affected by the gene expression during the biological experiment.

We selected tools for the functional enrichment using multiple databases for Gene Ontology (GO) and for Pathway analysis. In particular, we chose the *clusterProfiler* and *gProfiler* R/Bioconductor packages [70, 71]. While the choice of *gProfiler* has been made for the updated databases used during the enrichment analysis, the selection of *clusterProfiler* came out for the multiple plots it offers to visualize the results, even if it doesn't have the mostly updated databases for the GO and for the pathways.

The underlying idea of both methods is the same, they take a list of relevant genes (DEGs in our case) and perform an hypergeometric test for GO terms and pathways of publicly available datasets. For the multiple testing correction *clusterPofiler* implements False Discovery Rate (FDR), while the *gProfiler* package implements its own method for the correction.

We designed the `enrichKEGGFunction` and the `enrichGOFFunction` functions

for testing the *KEGG* pathways and the GO with *clusterProfiler*. Moreover, the first function automatically generates the network of pathways, while the second one saves an hierarchical tree of the enriched GO terms. While, for the *gProfiler* package usage, we implemented the `enrichPathwayGProfiler` and the `enrichGOGProfiler` functions for performing functional enrichment on pathways and GO databases.

In order to facilitate the functional enrichment to the user, we designed the `enrichSuited` function, which takes as input the Ticorser `de.gene.list` to perform not only the pathway analysis, but also the GO enrichment both with *clusterProfiler* and *gProfiler*.

### Results Comparison

Usually, during a differential expression analysis, it is pretty common to have the need to compare multiple DEGs results list. In order to facilitate this need we developed three different functions for *Venn* diagrams, `Venn2de`, `Venn3de` and `Venn4de`, respectively for two, three and four lists. During this process, it is often required not just to show the graphical plot, but it is relevant to detect the resulting gene lists from intersections and disjunctions of the Venns. To achieve this objective, these functions take as input the gene lists to compare and automatically store output file lists within the resulting lists of all the areas of the produced Venns.

### Gene Identifiers Conversion

The Ticorser package exports multiple functions for the gene names manipulation. Indeed, it is very common the need to convert DEGs list from a specific identifier to another.

We developed `convertGenesViaMouseDb` and `convertGenesViaBiomart` useful for converting a DEGs list using the *org.Mm.eg.db* [72] for *Mouse* and using the *biomaRt* R/Bioconductor package for *Human*, *Mouse* and *Rat*. Additionally, it is possible to easily attach the resulting list to a *dataframe*, by using the `attachGeneColumnToDf` function, which takes care of adding a new column within the mapped identifiers in the right places of the original *dataframe*.

### Input/Output File Handling

To speed up the reading and writing of input/output files, Ticorser offers two main functions, `readDataFrameFromTSV` and `writeDataFrameAsTSV`.

In the first case, there is only one mandatory parameter, the `file.name.path`, even if gives the possibility to change the classical parameters as `row.names.col`, `headed.flag`, `sep`, `quote.char`.

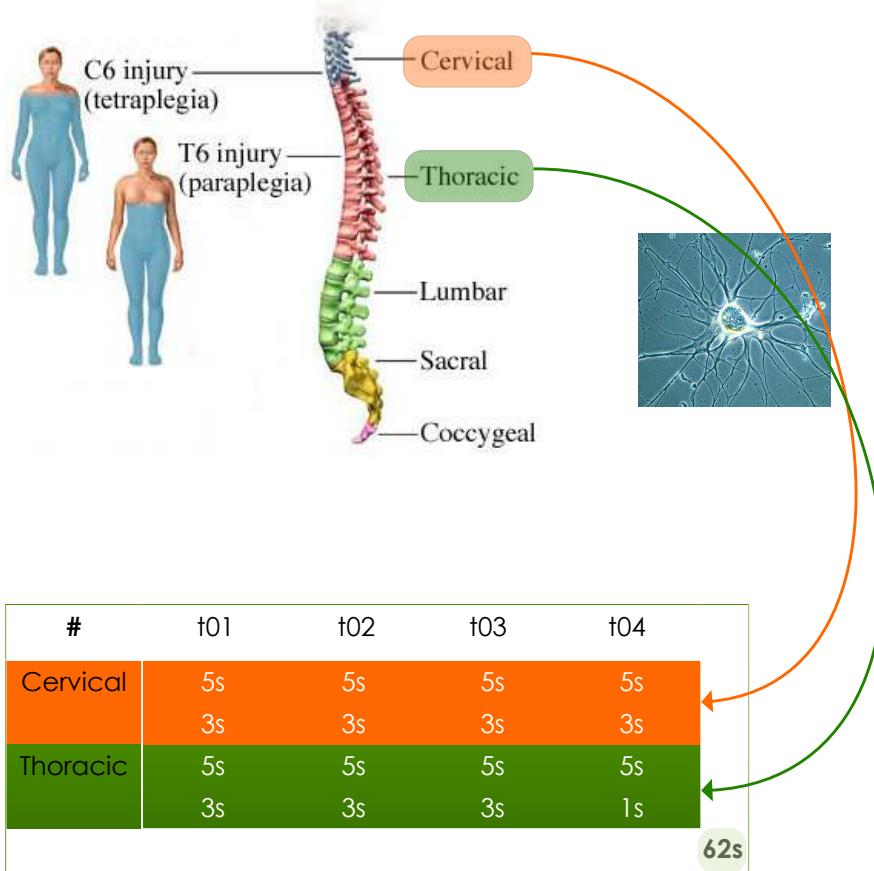
While in the second case the function requires the `data.frame.to.save` and the `file.name.path` where to store the object. Also, in this case, it is possible to set the classical parameters as `col.names` and `row.names`.

Moreover, we implemented a method for creating a folder path in a recursive way. The `updateFolderPath` method takes as input a starting path and a list of strings. It is useful when using a recursive function call or an automated nested function call process. Of course, if a user needs to create a single path by itself, he/she can simply create it using the `dir.create` R base function.

## 2.4 Case Study

For testing our package we selected a not yet published, but very complex dataset, for traumatic Spinal Cord Injury (SCI), that is a neurological condition occurring mainly at the thoracic and cervical levels. The dataset is composed of 62 samples of bulk RNA-seq, divided into groups of two different tissues at four different time points, with treatments and controls at each time point.

The Ticorser pipeline, as described in figure 2.2.1, has been applied for this case study.



**Figure 2.4.1:** An illustrative example of the used dataset. Neurons of Cervical and Thoracic spinal cord injury have been extracted and sequenced at 4 different time points. Inside the table, for each tissue (Cervical/Thoracic) the number of samples for the treatments (upper line) and controls (lower line) are reported at each time point.

Because the dataset is not accessible yet, the genes and the samples have been masked during the analysis and no further details will be provided, but we only use it as an illustrative example (figure 2.4.1 shows the dataset with most relevant details masked).

### Features quantification

Ticorser gives the possibility to quantify the gene expression by using the `featureCounts` method of the `rsubread` R/Bioconductor package, by using the `countBamFiltesFeatureCounts` method with the path of the *BAM* files and a

GTF<sup>1</sup> file within the desired annotation features.

It's really important the choice of the GTF file, in terms of version and release, because it affects the further analysis. For this reason, we suggest to always use the latest version of the GTF of the genome for the under investigation specie<sup>2</sup>.

After the gene quantification, the method produces a count matrix  $M \in \mathbb{R}$  with the features (genes) on the rows and the samples on the columns. Each cell  $c_{ij}$  of the matrix is a discrete value indicating the number of reads quantified for the feature on the  $i$ -th row in the sample represented by the  $j$ -th column.

### The design matrix

From this point afterwards, Ticorser requires a design file illustrating the descriptive characteristics of each sample, in order to speed up the computations and the interactions with the user. In particular, the design matrix must have the first column specifying the sample names, which has to be equal to the column names in the count matrix. Table 2.1 shows an example of a typical design matrix useful to work with Ticorser package.

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<sup>1</sup><https://www.ensembl.org/info/website/upload/gff.html>

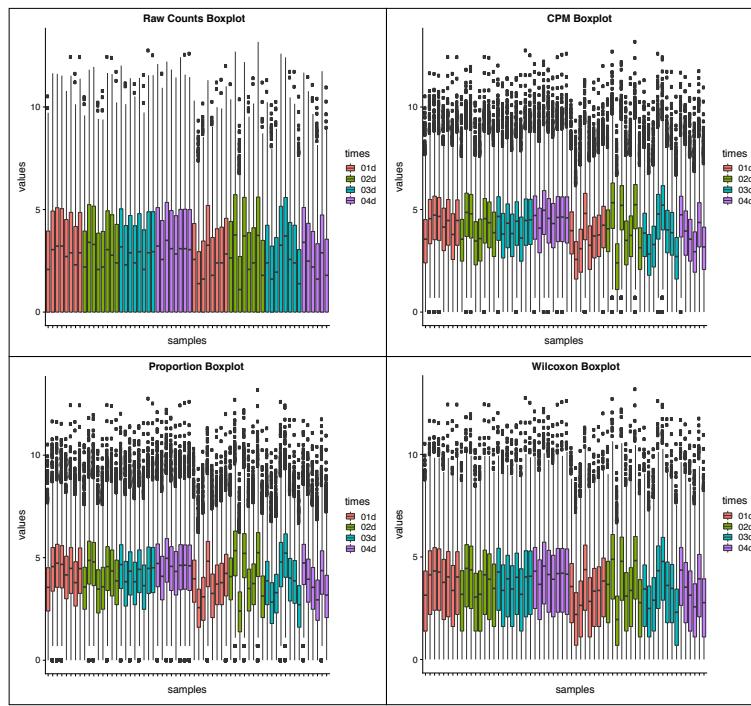
<sup>2</sup>Two main resources for genome download are <https://www.ensembl.org/index.html> and <https://www.ncbi.nlm.nih.gov/grc>

rownames	Times	Conditions	Tissue
s01_t01_t1	01h	treated1	tissue1
s02_t01_t1	01h	treated1	tissue1
s03_t01_t1	01h	treated1	tissue1
s01_t01_u1	01h	untreated1	tissue1
s02_t01_u1	01h	untreated1	tissue1
s03_t01_u1	01h	untreated1	tissue1
s01_t02_t1	02h	treated1	tissue1
s02_t02_t1	02h	treated1	tissue1
s03_t02_t1	02h	treated1	tissue1
s01_t02_u1	02h	untreated1	tissue1
s02_t02_u1	02h	untreated1	tissue1
s03_t02_u1	02h	untreated1	tissue1
s01_t01_t2	01h	treated2	tissue2
s02_t01_t2	01h	treated2	tissue2
s03_t01_t2	01h	treated2	tissue2
s01_t01_u2	01h	untreated2	tissue2
s02_t01_u2	01h	untreated2	tissue2
s03_t01_u2	01h	untreated2	tissue2
s01_t02_t2	02h	treated2	tissue2
s02_t02_t2	02h	treated2	tissue2
s03_t02_t2	02h	treated2	tissue2
s01_t02_u2	02h	untreated2	tissue2
s02_t02_u2	02h	untreated2	tissue2
s03_t02_u2	02h	untreated2	tissue2

**Table 2.1:** An example of design matrix required for the right working of Ticorser package. It provides 3 mandatory columns. The first column describes sample names equal to the column names of the count matrix. The *Times* column indicates the time points for each samples, while the *Conditions* column specifies the experimental conditions. Finally, the *Tissue* column (not mandatory) identifies possible multiple tissues.

## Filtering & Normalization

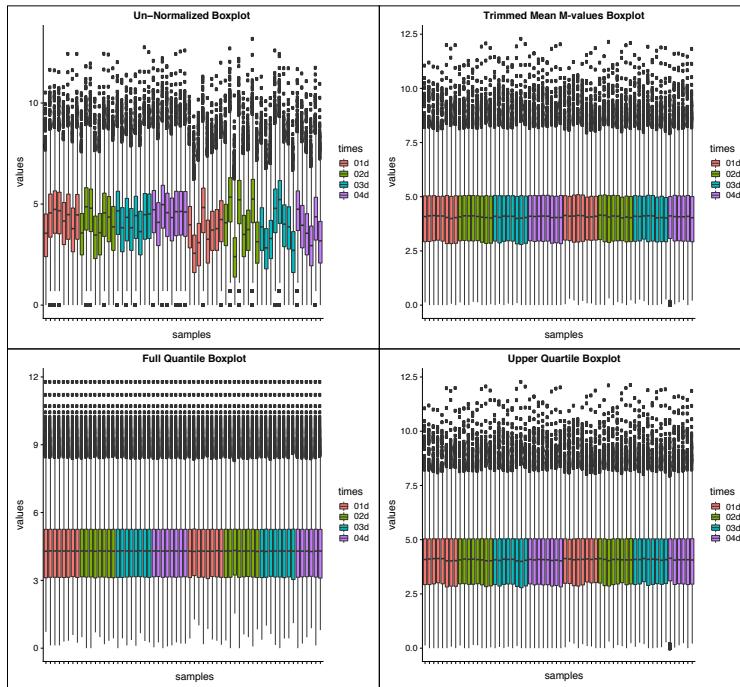
The count matrix obtained from the quantification step, could reflect the effects of one or more bias due to experimental passages during the library preparation or to different sequencing batches. In order to account for this, it is a good practice to normalize data, a step which affects the DEGs detection [73–75]. But before normalizing it, because of the presence of several low expressing features, it might also be useful to filter out them, since they are not relevant and may lead to biased results introducing unwanted noise in the analysis.



**Figure 2.4.2:** A comparison of how the filtering methods available in Ticorser affect the data. The first panel shows the row counts with low expressed genes, while the other boxes show the filtering effect on the samples.

Figure 2.4.2 shows the effects of filtering step on row counts (in upper left corner). What emerges from this comparison is that low expressed features are in a high amount in raw data, while it is quite un-important the method used for filtering them.

Moreover, looking at the *Wilcoxon* test, it is more conservative, because it preserves 17039 features, respect to the *Proportion* test and CPM, that preserves, respectively, 14122 and 14171 features. Because of the high numerosity of our samples but low number of replicates are present for each condition, we decided to use the count matrix filtered with *Proportion* test and *cpm=1*.



**Figure 2.4.3:** A comparison of how the normalization methods available in TICORSER affect the data. The first panel shows the row counts with low expressed genes, while the other boxes show the filtering effect on the samples.

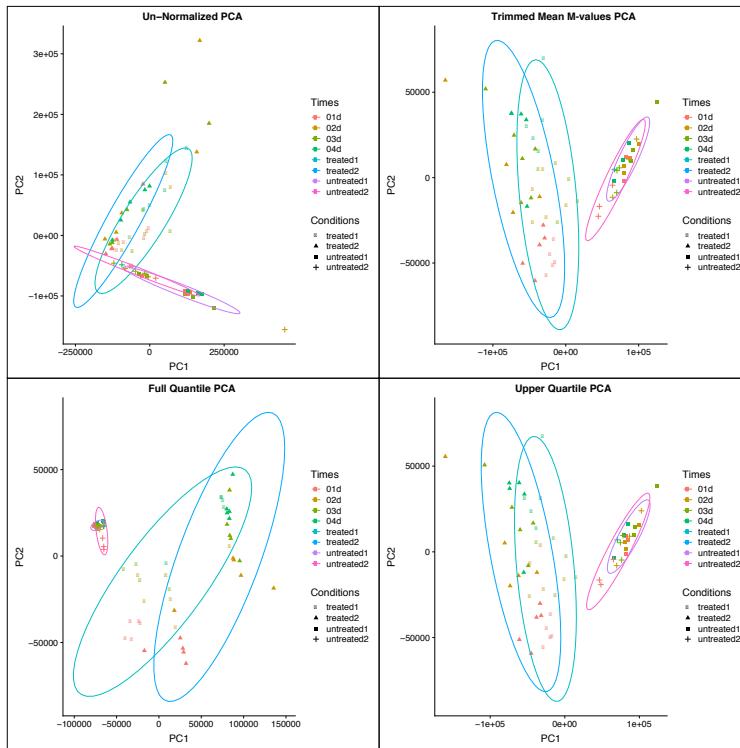
In order to improve the results for the differential expression analysis, it is crucial to correct for between-sample distributional differences in read counts. To compare the differences of normalized data we decided to use not only the boxplots for the samples, as shown in figure 2.4.3, but also the PCA representation, enabling us to understand which normalization better removes the biases from the samples by clustering the samples of the same group (figure 2.4.4).

Figure 2.4.3 perfectly shows different effects of each normalization type on count data. In fact, we can see that while *Upper quartile* aligns all the samples on the third percentile leaving the medians not aligned, the *Full quantile* applies a stronger effect, which totally aligns the samples, not only on the third percentile but also the medians as such as all the upper distributed outliers. At the same time, the *TMM* aligns the samples, but in a not so stringent way, well interpreting and leaving the variability of each sample.

As already mentioned a boxplot data inspection could not be enough to de-

termine which normalization method is better interpreting the data variability. Indeed, when looking at PCAs for normalized counts (figure 2.4.4) the *TMM* and the *Upper quartile* normalizations are able to well discriminate treated and untreated samples, but still preserving some variability inside each group. While *Full quantile*, even if it is able to help to discriminate the treated and untreated groups, it smashes too much the variability inside untreated samples.

Even if the *TMM* and the *Upper quartile* normalizations seem to affect the data in the same manner, we decided to choose for our further analysis the *Upper quartile* normalized count matrix, because, when looking at the PCA, it seems to better discriminate between the conditions.



**Figure 2.4.4:** A comparison of how the normalization methods available in Ticorser affect the data. The first panel shows the clustering of row samples, while the other boxes show the normalization effect on the samples.

### Differential Expression

Once the data are well normalized, in order to be able to well discriminate between the groups, simply by changing our design matrix with only the inter-

esting samples, we can focus on the differential expression step. Here we focus only on half of the total dataset, using only the cervical tissue data, checking differences between treated and untreated samples across experimental time points.

For detecting DEGs across time points and taking into account the conditions, we designed Ticorser with several methods (see section 2.2.3 for further details). Depending on the biological question under investigation, using the `ApplyDeSeq2` function and using the count matrix with the design matrix, the package automatically detects the samples to discriminate for the differential expression. Moreover, depending on the selected method, it is able to detect different types of genes between the samples. When selecting `DeSeqTime_TC` method, it detects all the genes which change their expression across all time points between two conditions in the `Conditions` column of the design matrix, while using `DeSeqTime_T`, it recognizes all the genes which have different expression between the conditions across all the time points. Furthermore, using `DeSeqTime_NoInteraction`, the method is able to detect all those genes demonstrating an oscillating behaviour across the time points in both the conditions.

Additionally, for each of the previously described methods we produce an additional output, applying a *Wald Test* on the LRT results, in order to detect all those genes showing differential expression between the two conditions.

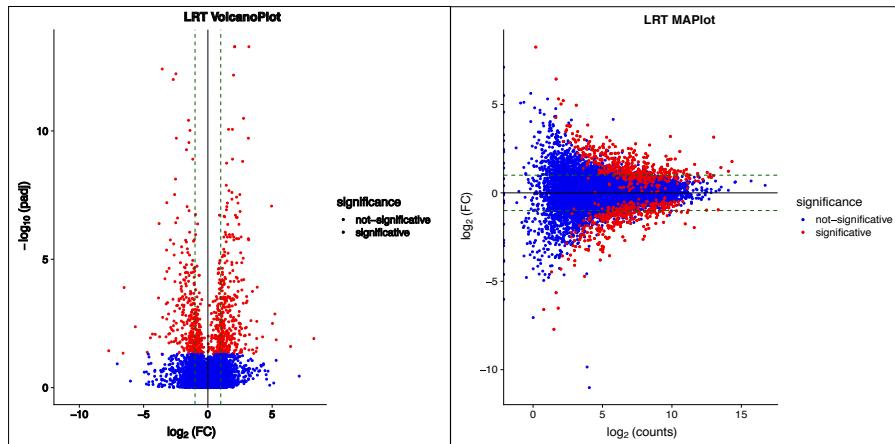
For each method we produce a list of two lists, within the results for the LRT and for the *LRT\_Wald*. Each one organized for all differential expressed genes, only UP genes, only DOWN genes and the results table with all the genes and their statistics. To identify DEGs we selected a threshold of 0.05 on the adjusted p-values, corrected with the FDR method [76].

Table 2.2 illustrates the differences, in terms of DEGs, between the three different methods on the same dataset, highlighting the total number of genes reported, with UP and DOWN regulated. It is relevant to see that, when applying the *Wald Test* on the obtained results by LRT, the number of DEGs for this test is much higher.

	LRT			Wald on LRT		
	Total	UP	DOWN	Total	UP	DOWN
TC	825	454	371	7066	3606	3460
T	8812	4672	4140	7066	3606	3460
No-Int	9560	4893	4667	9567	4900	4667

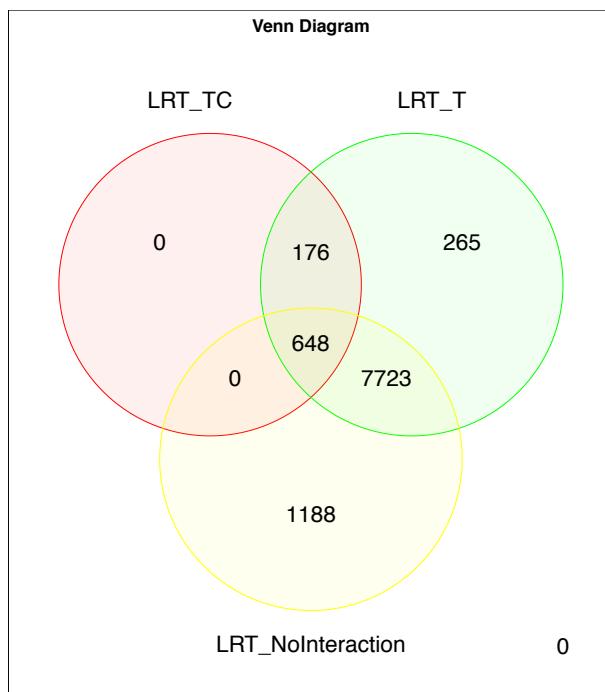
**Table 2.2:** This table illustrates differences in catching DEGs between the three different methods on the same dataset (on the rows), highlighting the total number of genes reported, with UP and DOWN regulated. It is relevant to see that, when applying the *Wald Test* on the results obtained by LRT the DEGs for this test are much higher in number.

It is also possible to inspect the DE results by plotting a *Volcano Plot* or an *MA-Plot*, as figure 2.4.5 shows.



**Figure 2.4.5:** An inspection of the results using a volcano plot (on the left) and an MA-Plot (on the right).

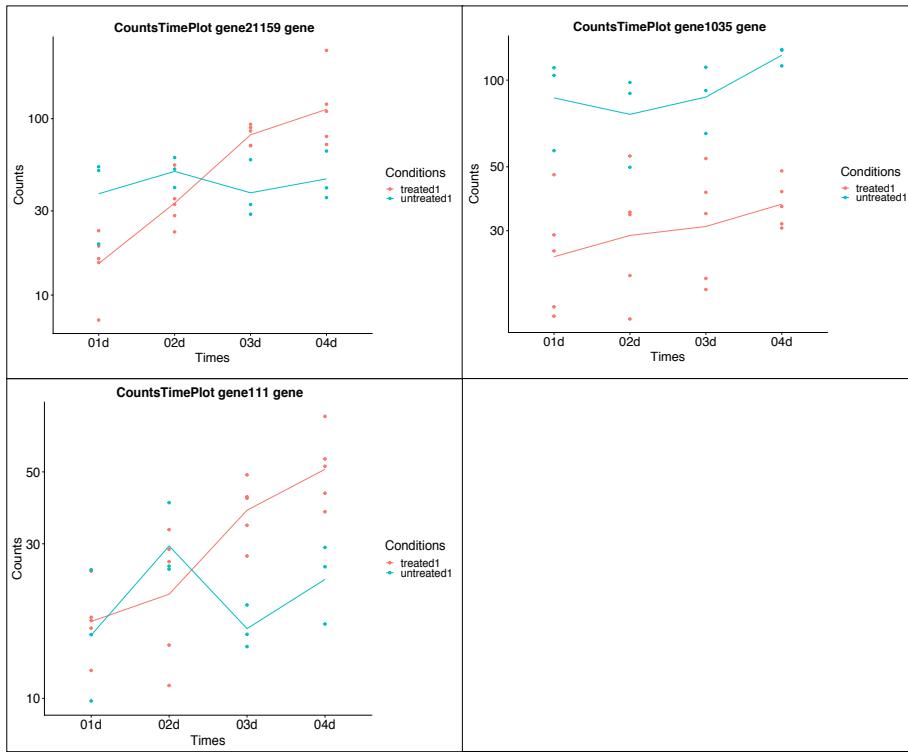
In order to better compare the results coming from different analysis and methodologies approaches we can use one of the VENN diagrams available in Ticorser, which, while intersecting the results, saves all the gene lists coming from the intersections and also from exclusions. Moreover, by setting the `enrich.list.flag` to `TRUE`, the methods automatically start functional enrichment for both pathways (on *Reactome* and *KEGG* databases) and Gene Ontology (on Molecular Function (MF), Cellular Component (CC) and Biological Process (BP)), storing all the results in the specified `output.folder` (refer to the next section for further details on Functional Enrichment Analysis).



**Figure 2.4.6:** A VENN diagram to compare the DEGs detected by the three different TC methods of Ticorser.

Depending on the biological question under investigation, it can be useful to identify the unique genes for each used method during the time course differential expression analysis, or the genes coming from their intersections.

To look at the gene expression, Ticorser has a specific function to explore the trend of a specific gene across the time points between the two conditions. By the usage of the `PlotCountsAlongTimes` and by specifying the name of a gene that is present in the count matrix, we are able to explore its behaviour.



**Figure 2.4.7:** Three different trends examples of genes detected with Ticorser DE TC methods.

For example looking at figure 2.4.7, we identified three different genes, one for each DE method, showing different trends over time points in conditions. The first one is a gene detected by the `DeSeqTime_TC` method, showing a complete inversion of the expression between the conditions during the time course experiment, otherwise, the second gene, detected with `DeSeqTime_T` method, in the right upper corner, shows a difference between the conditions that remain unchanged across the experiment. Finally, using the `DeSeqTime_NoInteraction` method, we detected genes with a peculiar behaviour such as the *gene111* in figure 2.4.7, which totally inverts his expression at each time point between the conditions.

Once inspected the results for the TC experiment analysis, an investigator could be interested in exploring the data on singular time points, here we report an analysis example on the first time point.

For the singular time point analysis, we can choose between four different methodologies, two present in the *NOISEq* package (accessible through the

`ApplyNoiseq` function), the third one, the *Wald test*, present in the *DESeq2* package and, finally, the *Quasi-likelihood* test from the *edgeR* package. It is a good practice to use more than one method while working with transcriptomic data in order to be more confident about the final results. Table 2.3 illustrates the differences between the used methods in terms of detected DEGs.

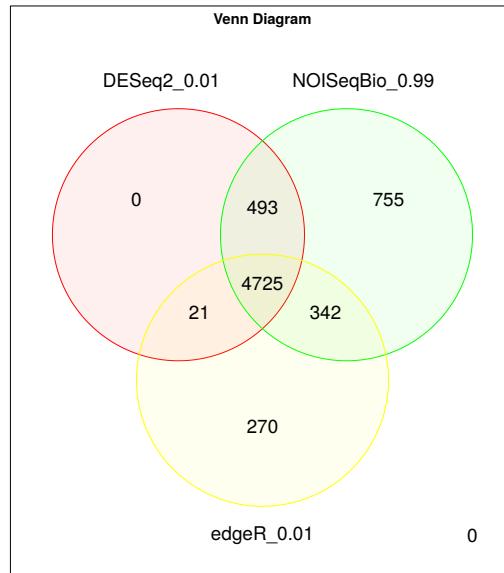
For the mentioned motivations into the section 2.2, we use the *NOISeqBio* method, and not the *NOISeq* method, because we have biological replicates.

	Genes		
	Total	UP	DOWN
DESeq2	5239	2684	2555
edgeR	7095	2935	4170
NOISeqBio	6315	3053	3262

**Table 2.3:** A comparison of the number of DEGs detected with Ticorser at single time point. Here we present the results for the first time point (t01).

Figure 2.4.8 shows differences in the detection of DEGs using the three different methods. In particular, *NOISeqBio* is really useful when working with biological replicates (like in this case), indeed it is able to detect much more DEGs than the other methods. Indeed, when using *NOISeq* on the same data, the DEGs are only 24, while *DESeq2* is able to detect quite the same amount of genes.

It is also possible to use one function, `PerformDEAnalysis`, for applying the preferred method in the differential expression phase. The tool automatically stores the results in the `output.folder` creating an articulate, but intuitive, folder tree with all the results and the plots (Volcano and MA Plots), and performing functional enrichment analysis on the computed results, simply by setting the `enrich.results.flag` to TRUE.

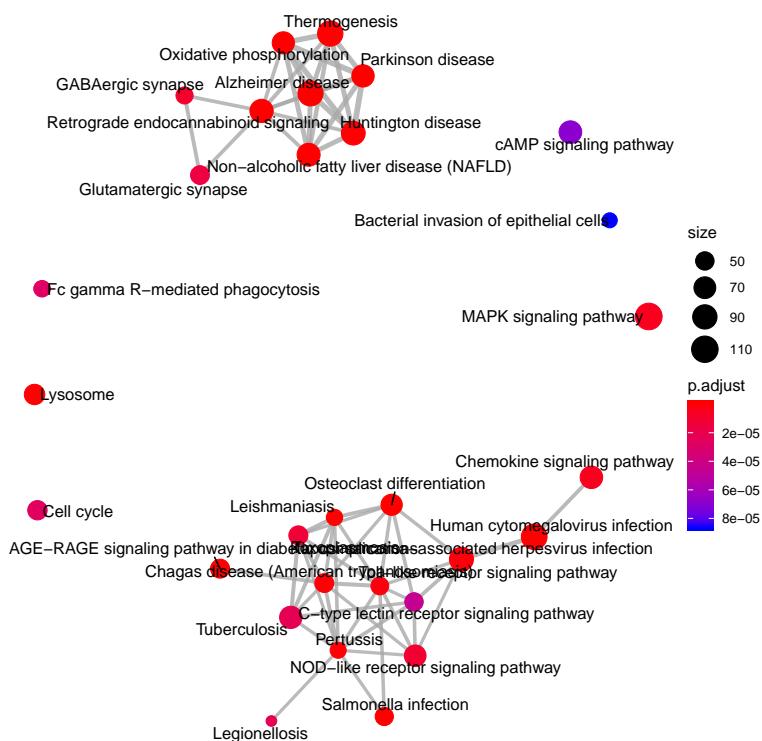


**Figure 2.4.8:** A venn diagram for comparing results coming from different methods used for DEG detection.

### Functional Enrichment Analysis

For the Functional Analysis Ticorser has a set of functions helping to perform it with *GProfiler* and *ClusterProfiler* tools [70, 71].

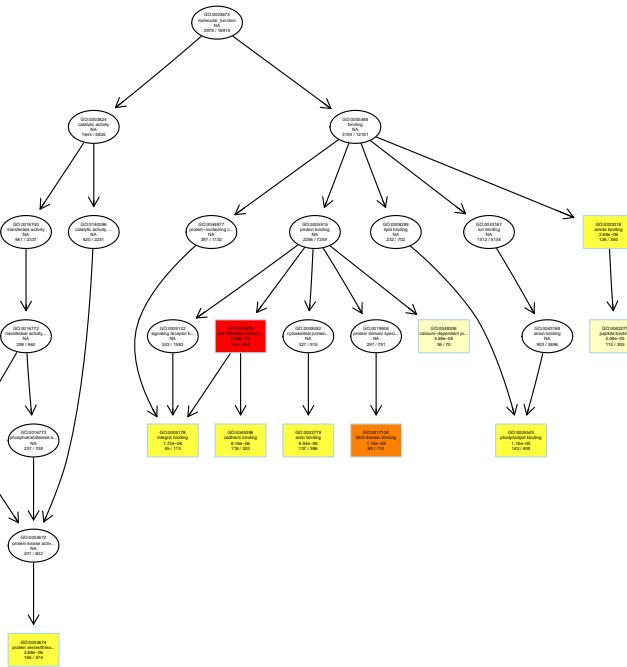
In order to perform pathway analysis with *GProfiler* we used `enrichPathwayGProfiler` function, twice, one for the *Reactome* database and one for the *KEGG* database enrichment. Then, we performed the same analysis with *ClusterProfiler* by using the `enrichKEGGFunction`, which performs only on *KEGG* database but is able to produce a graphical representation of the network of the pathways (see figure 2.4.9).



**Figure 2.4.9:** A network representation of the KEGG results obtained with *clusterProfiler* package, by using Tincer

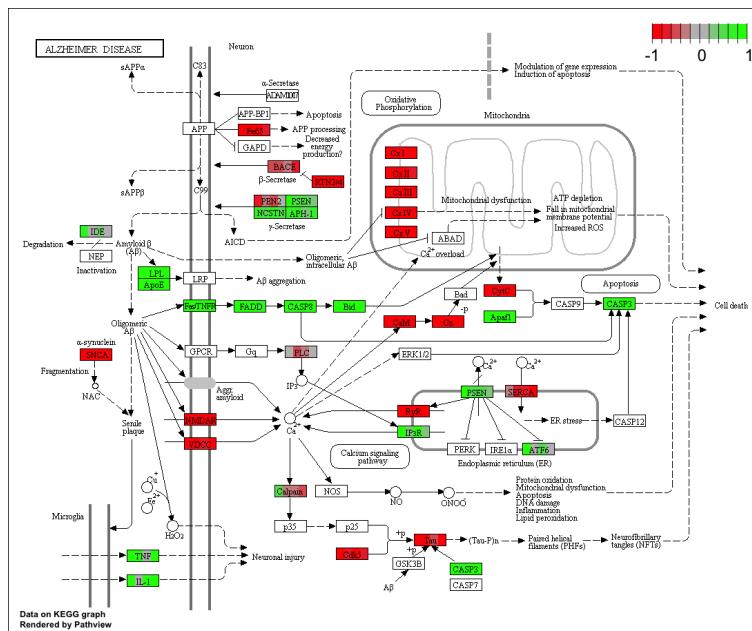
On the other hand, to perform a GO functional enrichment analysis we use `enrichGOProfiler` three times, one for each class of the ontology, `CC` for Cellular Components, `BP` for Biological Processes and `MF` for Molecular Functions. Analogously, we use `enrichGOFunction` for performing the same analysis with *ClusterProfiler*, which produces not only the table of the results but also a tree for the Gene Ontology terms significative in our dataset. (see figure 2.4.10).

Because the data used for this analysis have not been yet published, we do not publish the results obtained with *clusterProfiler* and *gProfiler*, but we show some relevant plots useful for illustrating the obtained results.

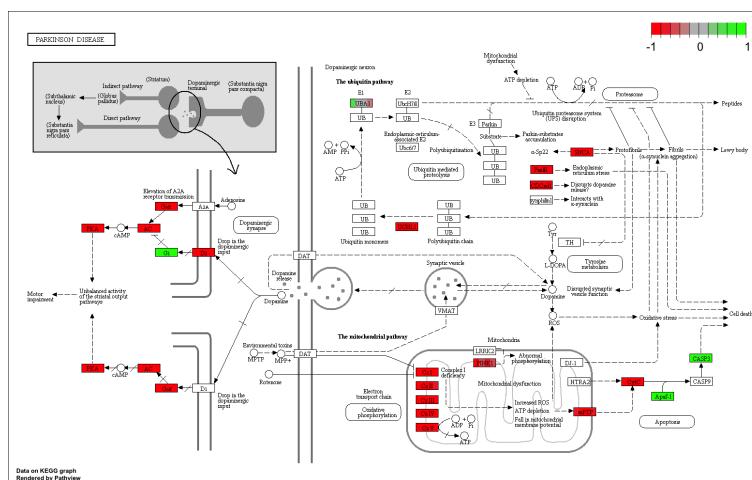


**Figure 2.4.10:** A hierarchical graphical representation of Molecular Function GO-terms obtained with *clusterProfiler*, by using Ticorser. Graphical colours, from red (most significant) to yellow (less significant) indicates the p-value significance of each GO-term. Gray ones, are not significant at all.

Finally, when we identify one or more interesting *KEGG* pathways, we can plot a *KEGG map* representation of it, by using `PlotKeggMapTimeCoursePathview`, which takes as input a data frame of relevant genes expression values for the interested pathway, and the `keggid` identifying the pathway. In such a way a *KEGG* map with the expression values at each time point for those genes will be visualized. Figure 2.4.12 and 2.4.11 graphically shows the *KEGG-maps* of the *Parkinson disease* and *Alzheimer disease* pathways, which both resulted highly significant from our functional analysis, and which are known to be associated to SCI [77, 78].



**Figure 2.4.11:** A keggmap for Alzheimer disease. Each DEG is coloured depending on the  $\log_2(\text{fc})$  of its expression. Moreover, for each highlighted gene, the box is divided in 4 "mini-boxes", one for each time point.



**Figure 2.4.12:** A keggmap for Parkinson disease. Each DEG is coloured depending on the  $\log_2(\text{fc})$  of its expression. Moreover, for each highlighted gene, the box is divided in 4 "mini-boxes", one for each time point.

## 2.5 Discussions

We presented Ticorser, an R command line tool for easy and fast analysis of time course RNA-seq data, presenting a wide range of methods for differential expression data analysis and their visualization.

Our tool offers several already published methods for RNA-seq analysis in order to catch the gene expression differences between multiple biological conditions, focusing on time-course experiments, but also allowing comparisons at single time point. The selected methodologies are some of the most used for time-course RNA-seq differential expression experiments (*DESeq2*, *edgeR*, *NOISEq*), while the choice of filtering methods is due to the lack of methodologies and packages offering multiple approaches to this problem. At the same time, we collected multiple solutions for counts normalization, in order to give the possibility to compare their effects on the data and, consequently, choose the one which better interprets the dataset. Finally, for the functional enrichment analysis we selected two already published tools (*clusterProfiler*, *gProfiler*) which offers the possibility to visualize the results with ad-hoc defined plots.

While the package allows to compare different methodologies to well discriminate between multiple conditions at a steady-state, it would be also good practice to compare the results coming from multiple methodologies when working with time-course data. A good candidate for this aim could be the *next-MASigPro* R/Bioconductor package which takes advantage of the Generalized Linear Model (GLM) with Negative Binomial distribution. This method, despite other implemented ones, allows the detection of all DEGs showing any kind of differences between the conditions across all the time points. And, as suggested by the authors, it is a good norm to cluster the genes to better understand which are their singular behaviours.

*Ticorser* takes in charge of several aspects during the analysis, which are typically left to the final user, even if this one is not so confident with this kind of methodologies. *Ticorser* has been designed in a way that the user can apply several different methodologies for filtering, normalization, differential expression, etc., without taking care about the different input and output data that each of this methodology requires. The main aspect the user need to take care is to well define the experimental design matrix, useful for the proper

working of almost all the Ticorser functions.

Once the design matrix is properly defined and once the final user became familiar with Ticorser instruments, every user can easily perform a complete pipeline in a couple of hours (excluding the quantification step, that can be time demanding).

To conclude, Ticorser, with its ad-hoc designed pipeline, is a useful instrument for analyzing time-course RNA-seq data, offering multiple methodologies for quantification, filtering, normalization, differential expression and functional analysis. Moreover, it offers a set of multiple plots for each step of the analysis, helping the user to inspect the data and the results. Moreover, most of the visualization functionalities allows the user to plot them in interactive mode, giving the possibility to visualize additional information while navigating the plot with the mouse.

At our knowledge, Ticorser is the first pipeline completely dedicated to the analysis of time-course RNA-seq data, giving the possibility to compare multiple methodologies and offering multiple visualization tools for the data exploration. That is why, even if it could be extended with additional features and more methodological comparison, we retain it could be a good candidate for the analysis of this kind of data.



# Chapter 3

## Differential Enriched Scan 2: DEScan2

Epigenetics, as shown in the introduction (section 1.1), is a pretty wide and complex field, and the sequencing technology to adopt depends on the biological question under investigation.

Some studies [36, 79] demonstrated the importance of genome-wide chromatin accessibility of a broad spectrum of chromatin phenomena activation using sequencing techniques as Assay for Transposase-Accessible Chromatin Sequencing (ATAC-seq), Sonication of Cross-linked Chromatin Sequencing (Sono-seq), etc. Even if there are some methods for the analysis of these omic data types, there still is a lack of them, in particular for emerging omics as ATAC-seq.

To address this need, we decided to create a useful instrument for analysing chromatin regions accessibility data (such as ATAC-seq, Sono-seq). Very often the biological questions, to be answered, as for RNA Sequencing (RNA-seq), need the comparison of two or more different biological conditions. Starting from a set of already published [36] scripts, we designed Differential Enriched Scan 2 (DEScan2), a software for the analysis of chromatin accession sequencing data.

In this chapter, we firstly illustrate the developed methodologies and then, with a case study, we will show the obtained results as an application.

### 3.1 Introduction

DEScan2 is an R [80] tool developed for detecting open chromatin regions signal in order to facilitate the differential enrichment of genomic regions between two or more biological conditions.

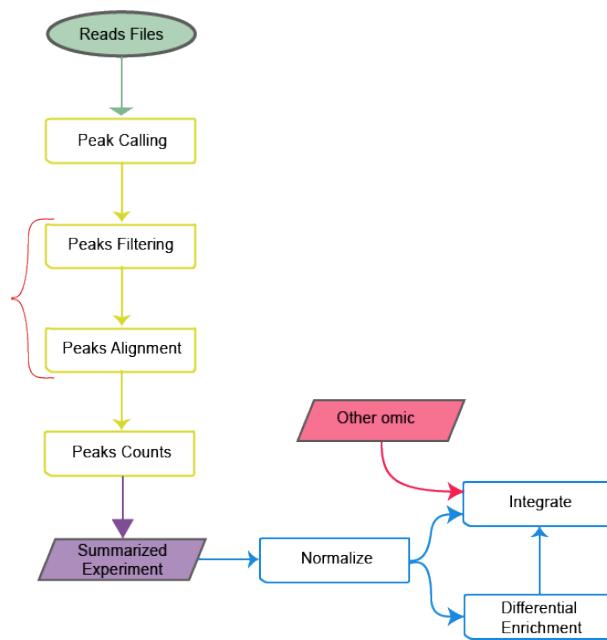
The package has been implemented using Bioconductor [54] data structures and methods, and it is available through the Bioconductor repository since version 3.7.

The tool is organized into three main steps. A peak caller, which is a standard moving scan window that compares the reads coverage signal within a sliding window to the signal in a larger region outside the window. It uses a Maximum Likelihood Estimator of a Poisson Distribution, providing a final score for each detected peak.

The filtering and alignment steps are aimed to determine if a peak is a "true peak" on the basis of its replicability in other samples. These steps are grouped in a single procedure and are based on a double user-defined threshold, one on the peaks scores and one on the number of samples.

The third step produces a count matrix where each column represents a sample and each row a peak. The value of each cell represents the number of reads for the peak in the sample.

The produced count matrix, as illustrated in figure 3.1.1, is useful both for doing differential enrichment between multiple conditions and for integrating the epigenomic data with other -omic data types.



**Figure 3.1.1:** A differential enrichment flow representation. DEScan2 steps are highlighted in yellow.

## 3.2 Methods

The package is organized in three main steps, the peak caller in section 3.2.1, the filtering and alignment of the peaks in section 3.2.2 and the peak counting described in section 3.2.3.

Furthermore, it offers some additional features as described in section 3.2.4.

### 3.2.1 Peak Caller

The Peak Caller (defined by the `findPeaks` function) takes as input a set of alignment files (BAM [15] or BED format) with the code identifier for the reference genome (i.e. *mm10* for Mus Musculus version 10) and several additional parameters, useful for the peak detection setup.

The alignment data are stored as an object of class *GenomicRangesList* [81], where each element represents a file. In order to facilitate the parallelization of the computations over the chromosomes, the list is re-arranged as a chromosome list of *GenomicRangesList*, where each element represents the file containing just the *GenomicRanges* of the specific chromosome (see section 3.2.4 for a detailed

description of this procedure).

For each element of this data structure, the algorithm firstly divides each chromosome into bins of `binSize` parameter length (the default value is 50bp) and then computes the reads coverage of the bins with moving scan windows, spanning from `minWin` to `maxWin` parameters of `binSize` interval.

In order to be able to catch narrow and broad peaks, the algorithm computes the coverage also using windows of two different lengths, that can be defined with `minCompWinWidth` and `maxCompWinWidth` (defaults values are 5000bp and 10000bp) parameters, computing a matrix of  $n$  bins and  $p$  windows.

The coverage matrix is useful to merge contiguous regions and to compute a score for each of them, applying a Maximum Likelihood Extimator (MLE), assuming a *Poisson* distribution of the coverage across the windows.

Formalizing: assuming that each window is distributed as a *Poisson* random variable, we assume to observe the  $n$  coverages as an IID sequence  $X_n$ . Thus, the probability mass function is described as:

$$p(x_i) = \frac{\lambda^{x_i}}{x_i!} \exp(-\lambda)$$

Where the integer nature of the data support the Poisson distribution as the set of non-negative integer number and where  $\lambda$  is the Poisson parameter to estimate with a MLE, described as the estimator:

$$\hat{\lambda}_n = \frac{1}{n} \sum_{i=1}^n x_i$$

Which corresponds to the sample mean of the  $n$  observations in the sample.

In order to define a score for the peaks, we defined a z-score for each bin-window as follow:

$$\hat{z}_n = \frac{x_n - \hat{\lambda}}{\sqrt{\hat{\lambda}}}$$

Subsequently, an iterative algorithm detects the maximum z-score, between all the windows, at each step, and then detects all the contigs bins with a not 0 value, across all the windows. In such a way a peak is defined by the starting base of the "lowest" bin to the last base of the "highest" contiguous bin, assigning

the maximum z-score between all the detected contigs bins.

Additionally, on user request, the function provides as output, for each alignment file, a Tab Separated Value (tsv) file within the regions coordinates and the score of the detected peaks.

### 3.2.2 Peak Filtering and Alignment

In order to filter out false positives peaks, we designed a method (defined in the `finalRegions` function) which firstly filters out low score regions and then aligns the resulting regions between samples, using two different thresholds. One on the peaks score and one on the number of samples.

The filtering step is designed to take as input a list of peaks as *GenomicRangesList*, where each element represents a file. This is the data structure produced by the peak caller, but, we also developed a method to load peaks produced by other software like MACS [30], as described in section 3.2.4.

Firstly, using the threshold on the peaks score (defined by the `zThreshold` parameter), the method filters out the peaks with a score lower than the user-defined threshold value.

Then, for aligning the peaks between the samples, it extends a 200bp window in both directions of remaining regions, computing the overlaps using the `findOverlapsOfPeaks` method (using the `connectedPeaks` parameter set as `merge`), as defined in the *ChIPpeakAnno* [82] R/Bioconductor package.

Based on this idea, the filtering step is developed to filter out those peaks not present in at least a user-defined number of samples, defined by the `minCarriers` parameter. In the light of this, the user can decide the minimum number of samples where each peak has to be detected. In our experience, we suggest setting the samples threshold as a multiple of the number of replicates of the conditions.

### 3.2.3 Counting Peaks

The counting step (`countFinalRegions` method) is designed to take a *GenomicRanges* data structure as input, where for each peak additional attributes are saved, as well as the score and the number of samples. Moreover, to quantify

the peaks given as input, it requires also the path of the alignment files where the reads are stored.

For each region, the method counts the number of reads present in each sample. In so doing, it produces a matrix of counts, where the rows and the columns, respectively, represent the regions and the samples.

In order to keep track of all information associated to the regions, it produces a *SummarizedExperiment* [83] data structure, giving the possibility to retrieve the *GenomicRanges* of associated peaks and the count matrix, respectively, using the `rowRanges` and `assays` methods.

The choice to produce a count matrix is guided by the versatility of this data structure, useful not only for the differential enrichment of the regions between multiple conditions but also for integrating the epigenomic data with other -omics data types, such as RNA-seq.

### 3.2.4 Additional Features

The package offers some additional features for loading data (i.e. peaks) resulting from other sources, and for manipulating *GenomicRanges* data structure.

To give the possibility to use our pipeline with external peak callers, the function `readFilesAsGRangesList` takes as input a directory containing BAM or BED data, to load in *GenomicRangesList* format. This data structure is useful to store genomic information, as peaks or mapped reads, produced by other software like *MACS2* or *STAR* and, in case of peaks, it is necessary during the DEScan2 filtering/aligning step. Additionally to `fileType` (BAM, BED, BED.zip) parameter specification, it requires the genome code to use during the file processing. Moreover, when the input files represent peaks the `arePeaks` flag needs to be set to `TRUE`.

Furthermore, DEScan2 provides several functionalities for *GenomicRanges* data structure handling. One example is `fromSamplesToChrsGRangesList`, which gives the possibility to split a *GenomicRangesList* by chromosome. This procedure could be useful for parallelizing computations on the chromosomes, when common operations on them, between multiple samples, are needed. Assigning a single chromosome to a single computing unit. Taken as input a *GenomicRangesList* organized by samples, this method returns a list of chromosomes,

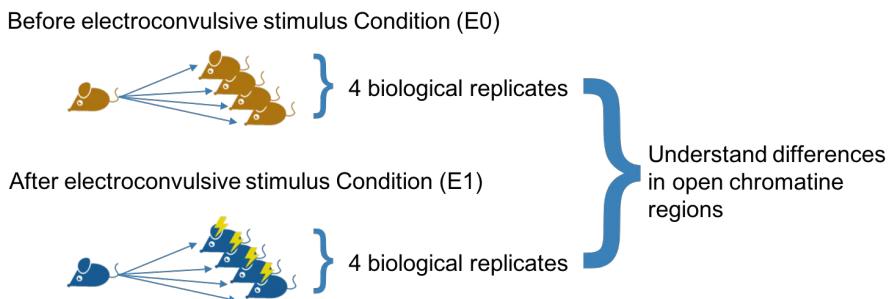
where each element has a *GenomicRangesList* of samples, containing only the regions associated to the single chromosome.

Other useful utilities are `keepRelevantChrs`, that takes a *GenomicRangesList* and a list of chromosomes and return only the interested chromosomes with a cleaned *genomeInfo* assigned; the `saveGRangesAsTsv` function that saves a tab separated value file starting from a *GenomicRanges*; the `saveGRangesAsBed` that save a standard BED file format starting from a *GenomicRanges* data structure; and the `setGRangesGenomeInfo` which, starting from a genome code, sets a specific *genomeInfo* to a *GenomicRanges* object.

### 3.3 Case Study

#### Data Description and Preprocessing

ATAC-seq is an emerging evolved technique which enables to investigate the open chromatin regions at whole genome level. The capability of this technology has been demonstrated in the regulation of mouse brain activity under different conditions [84].



**Figure 3.3.1:** An illustration of our extraction of the GSE82015[84] dataset.

To illustrate the performances of DEScan2 we chose a dataset [84] that describes *in vivo* adult mouse dentate granule neurons before and after synchronous neuronal activation using ATAC-seq and RNA-seq technologies (see sections 1.2.3 and 1.2.1 for a description of these sequencing techniques).

This dataset is organized in 62 samples of ATAC-seq and RNA-seq, extracted at four different time points (0, 1h, 4h, 24h), with four replicates at each time point. We chose to compare the differences between the first two stages, time 0 (E0) and 1 hour after neuronal induction (E1), in order to show a potential ATAC-seq workflow for Differential Enrichment, and how to integrate this data type with RNA-seq. A general illustration of this dataset is represented in figure 3.3.1.

We downloaded the data from Gene Expression Omnibus (GEO) database [85, 86] with accession number GSE82015<sup>1</sup> and mapped the raw data using *STAR* [12] with default parameter on *Mus Musculus* Genome ver.10 (mm10).

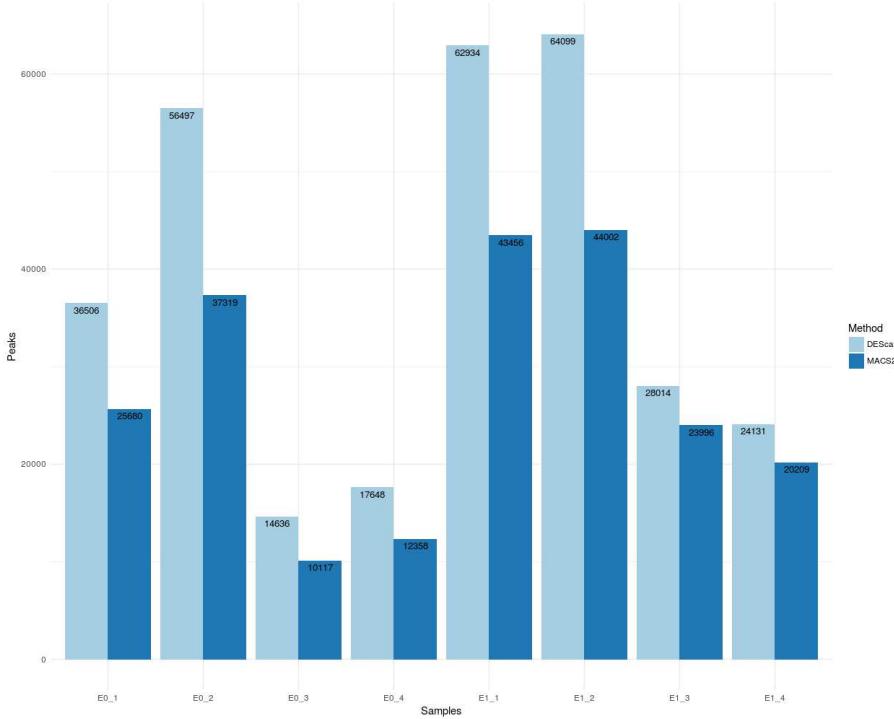
### Peaks Detection

In order to detect open chromatin regions, we run our peak caller, cutting the genome in bins of 50bp and using running windows of minimum 50bp and maximum 1000bp. In this way, we are able to detect not just broad peaks, but also smaller peaks.

To be confident with our results we run DEScan2 and *MACS2* [30] on the same samples, and (as shown in figure 3.3.2) looking to the numbers DEScan2 always find more peaks than *MACS2*. This can be due to a major accuracy gave by *MACS2* on the reliability of the detected peaks, while our method finds more peaks, but, at this stage, still preserving false positive regions.

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<sup>1</sup><https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE82015>

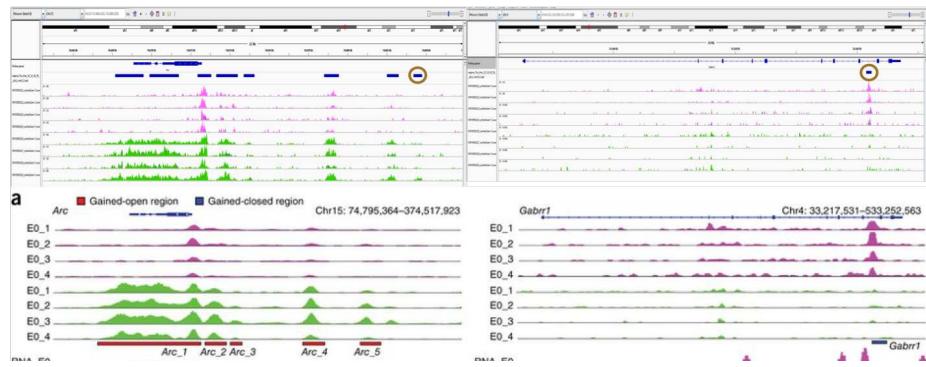


**Figure 3.3.2:** A comparison of DEScan2 and *MACS2* detected peaks for each sample in the dataset.

To be more robust, we compared DEScan2 detected peaks with the same validated regions (*Arc*<sup>2</sup> and *Gabrr1*<sup>3</sup>) of the original work [84]. The lower part of figure 3.3.3 shows the detected and validated regions (in blue and red) resulting differentially enriched between the E0 (in pink) and E1 (in green) conditions, while the upper part shows DEScan2 filtered and aligned peaks (in blue) between the samples, highlighting a capability to catch not only the same regions of the published ones but also (gold circles) to be more accurate in the smaller peaks detection.

<sup>2</sup><https://www.genecards.org/cgi-bin/carddisp.pl?gene=ARC>

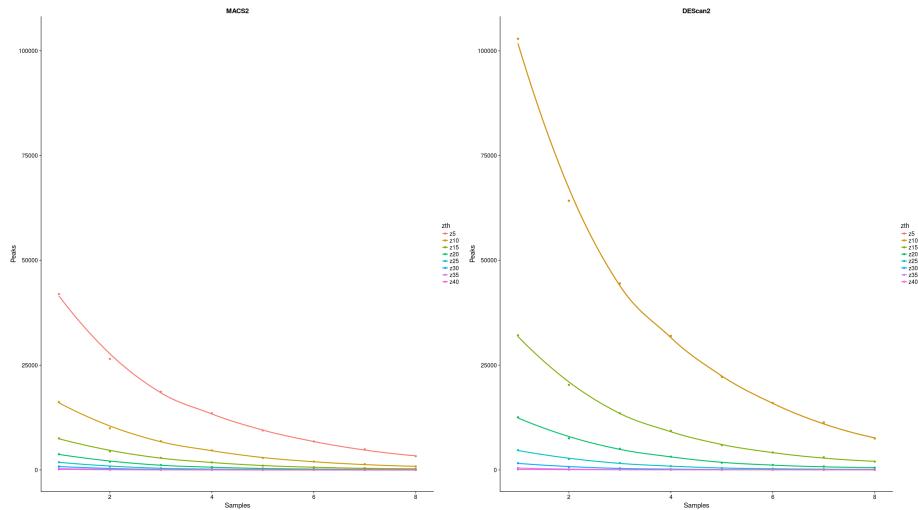
<sup>3</sup><https://www.genecards.org/cgi-bin/carddisp.pl?gene=GABRR1>



**Figure 3.3.3:** A comparison of DEScan2 detected peaks with validated peaks in article [84].

### Removing Unwanted Peaks

While it is very important to detect good peaks with a peak caller, it seems to be more relevant to detect reproducible regions. Indeed, during the filtering/aligning step, the number of peaks depends not only by the peak score but also by the number of replicates designed in the experiment. The figure 3.3.4 puts in relation these two relevant information for both *MACS2* and *DEScan2*. On the x-axis is represented the number of replicates, while on the y-axis is traced the number of peaks, and each curve represents a different threshold on the peaks score, showing that the higher is the thresholds on the scores and the number of replicates, the lower is the number of the detected peaks. Highlighting a inverse relationship between the number of the peaks and the combination of the number of samples and the detected regions score.



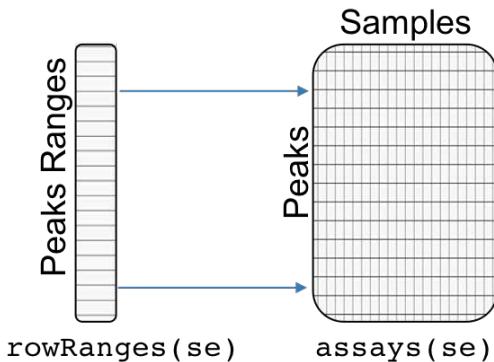
**Figure 3.3.4:** Filtering the detected regions with different thresholds on peak scores between *MACS2* and *DEScan2*.

Moreover, comparing the left and right panels, we notice the high difference in pooling the samples-peaks together with the *DEScan2* filtering/aligning step when using *MACS2* and *DEScan2* peaks. Using *MACS2* peaks the pooling highly reduces the number of detected peaks, even using a threshold as low as 5 on the score, showing that there are many peaks with a score lower than 5. While in the *DEScan2* case the curves representing the threshold equal to 5 and the threshold equal to 10 totally overlap, highlighting that the *DEScan2* peak caller produces scores higher than 10.

Finally, in order to obtain a unique list of peaks, we choose a number of samples equal to 4 for the alignment step, which is a multiple of the total number of samples, in combination with a threshold of 10 for the peaks score filtering.

### Quantifying Peaks

Afterwards, the filtered-in regions can be processed by *DEScan2* in order to obtain a count matrix with samples on the columns and peaks on the rows. This type of data structure is very versatile because it enables to perform several operations, like the Differentially Enriched genomic Regions (DERs) and the integration with other kinds of omics, as RNA-seq.



**Figure 3.3.5:** An illustration of the *SummarizedExperiment* data structure produced by DEScan2.

In order to preserve the information associated to the peaks, DEScan2 produces as output a *SummarizedExperiment* (figure 3.3.5) data structure, which enables to retrieve the count matrix with `assays` method, and to access the peaks information in *GenomicRanges* format with the `rowRanges` method.

### Peaks Normalization

Before detecting DERs, it is a good practice to normalize the data. This is especially needed when working with neuroscience data, where many possible sources of technical and biological noise can confound the analysis [73]. The nature of the data, as discrete numbers, makes it possible to apply several well known RNA-seq normalizations techniques, such as *upper-quartile*, *full-quartile*, *RUVSeq*, etc [63, 87]. To filter out false positives, but preserving enough signal at the same time, we fixed the peaks score threshold to 20.

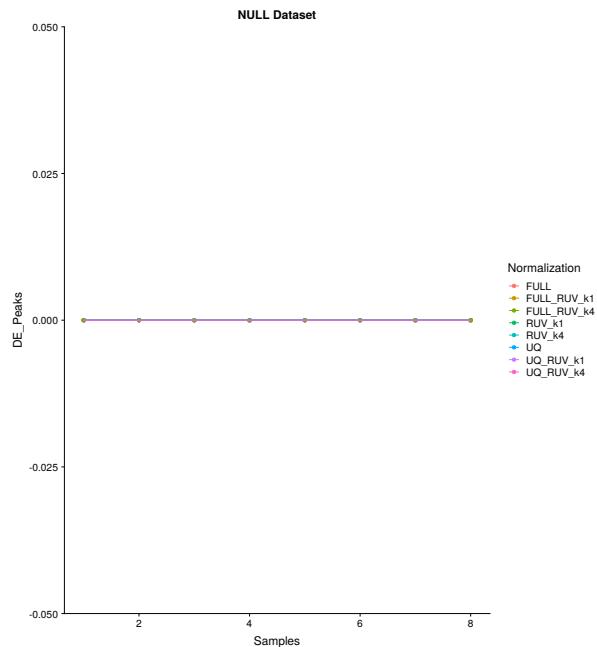
In order to compare the effects of normalizations we had to do differential enrichment of the conditions using `edgeR` (see next section for further details).

While the *upper-quartile* normalization affects the data in a way that makes it impossible to detect DERs, other kinds of normalizations and combinations of them give good results.

Figure 3.3.7 summarizes this concept very well, highlighting a relation between the number of DERs (y-axis) and the minimum number of samples (x-axis) used for aligning the peaks during the DEScan2 filtering/aligning step.

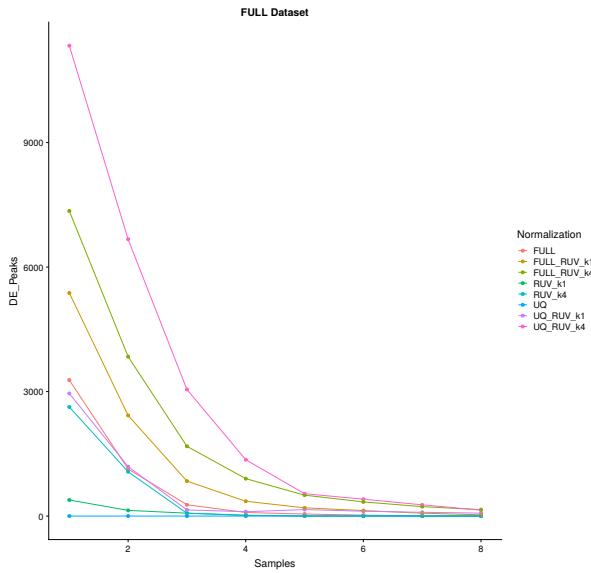
To better compare the normalization effects, we created a *null dataset* of 8

samples, by fixing the conditions in pairs (i.e. s1c1, s2c1, s1c2, s2c2, s3c1, s4c1, s3c2, s4c2). The detection of DERs has been performed on each combination (18) of shuffled samples and then taking the median of the results.



**Figure 3.3.6:** The figure shows the effects of different normalizations on a null dataset of epigenetic regions, putting in relation the DERs with the threshold on the samples used to align peaks.

Figure 3.3.6 shows, as expected, no DER detection. Highlighting that even if a normalization is applied, once reduced the false positives number, we are confident with our results.



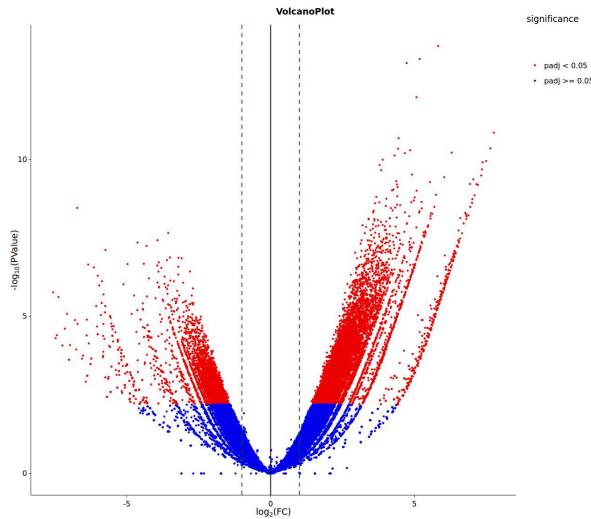
**Figure 3.3.7:** The figure shows the effects of different normalizations on a dataset of epigenetic regions, putting in relation the DERs with the threshold on the samples used to align peaks.

While figure 3.3.7, representing the "full dataset", shows that *upper-quartile*, by itself is not able to linearly detect any amount of DERs. When using *RUVSeq*, the DERs detection depends on the parameter used (figures 3.3.6, 3.3.7 are made using k equal to 1 and 4, for *RUVSeq* normalization). While, the *full-quartile*, even when used alone is able to detect a good amount of DERs. But each normalization, when combined with *RUVSeq*, seems to affect the data in a way that overestimates the number of DERs. In particular, even the *Upper quartile*, which doesn't detect any signal by itself, is able to detect the highest amount of DERs when combined with *RUVSeq*.

Even if these normalization methods show good performances with this type of epigenomics data, our investigations suggest that more testing is required, and maybe an ad-hoc normalization method for these data has to be developed.

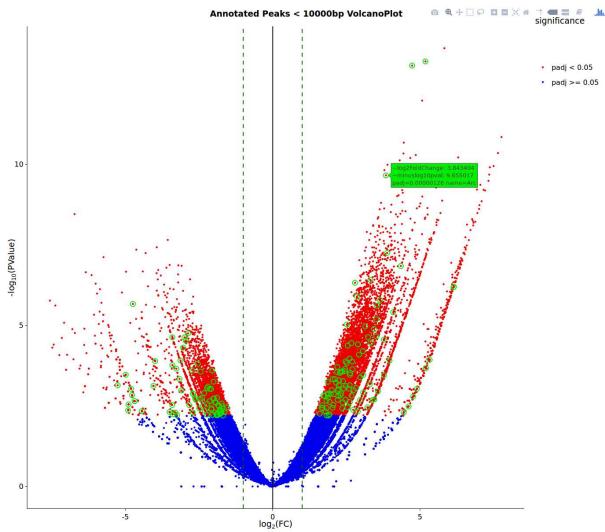
### Differential Enrichment of Peaks

To estimate the DERs, any of the RNA-seq methods can be applied, such as *DESeq2*, *edgeR*, *NOISeq*, etc [61, 62, 66, 88].



**Figure 3.3.8:** A volcano plot of Differential Enriched Regions. Blue dots represent the not significant DERs, while the red ones represent the significant DERs.

In this case, we decided to use *edgeR* package, because of its wide range of available statistical approaches and the possibility to better tune the design of the experiment. Indeed, because we used the *RUVSeq* normalized counts with *k* parameter set to 4, we modelled the experimental design with the *model.matrix* function, adding to our model not only the experimental conditions, but also the *RUVSeq* estimated factors. Then we used the resulted design to estimate the dispersion and fit a Quasi-Likelihood test, as defined in *edgeR*[66].



**Figure 3.3.9:** A volcano plot of DERs. Blue dots represent the not significant DERs, while the red ones represent the significant DERs. Green circles highlights the peaks with a Differentially Expressed Gene (DEG) annotated.

Figure 3.3.8 shows a volcano plot of DERs between E0 and E1 conditions. Red dots highlight the regions with a False Discovery Rate (FDR)[76] lower than 0.05, while blue dots highlight non significant regions.

### Peaks Integration

The next task is to integrate the obtained results with other omic data types, as RNA-seq. Because of the low number of the samples, the easiest way to integrate the data is to annotate the DERs with DEGs resulting from the analysis of RNA-seq.

For the differential expression of the RNA-seq data, we first quantified the signal with the `featureCounts` methods available in the *Rsubread* [69] R/Bioconductor package. Then we filtered lowly expressed genes with the *proportion* test as implemented in *NOISEq* package, and applied the `edgeR` method for differential expression.

We used the resulting significant DEGs (with posterior probability higher than 0.95) to annotate the peaks with `annotatePeakInBatch` method of *ChIP-peakAnno*. Figure 3.3.9 illustrates with green circles the peaks with an annotated gene with distance lower than 10000bp from the gene Transcription Starting Site (TSS), producing a total of 430 annotated peaks. Realizing the plot with `ggplot2`

combined with *plotly* library it is possible to enhance the names of the genes with a tooltip.

Then, to obtain a seconf level of data integration, we used the annotated genes to do functional annotation on Gene Ontology (GO) [57, 58] and Reactome pathways, which showed several interesting results for the neuronal regulation.

### 3.4 Discussions

In the lack of methodologies for open chromatin region detection and their analysis, we developed a novel approach which, compared with very well known tools as *MACS2*, seems to be competitive in the detection of the signal.

DEScan2 demonstrated to be able to catch not only wide signals but also narrow regions across the samples. And with our filtering/aligning step we demonstrated to be able to keep relevant signal producing data structures as *SummarizedExperiment* which are candidates to become standards in the biological data analysis. With our 3-steps analysis, we put our tool at the top of a pipeline for open chromatin regions data analysis, proposing also a possible candidate for a standard analysis of this data type.

The presented case study showed the capability of DEScan2 to be competitive in the detection, definition and filtering signal, but still more comparisons and improvements are needed.

Indeed, in order to validate our obtained results, we plan to compare them with other similar already published approaches, such as *csaw* [89], which peak caller, starting from a set of Binary Alignment Map (BAM) files, constructs a count matrix of the detected peaks in the samples. Moreover, to better test our peak caller, we plan to check if other distributions, as *Negative Binomial*, fit better this kind of data and, additionally, in order to improve our filtering/- aligning step we want to apply additional probabilistic methodologies, taking into account the experimental design.

Despite these improvements, DEScan2, from these preliminary results, already seems to be a powerful and intuitive instrument for ATAC-seq signal detection at the top of a more sophisticated analysis pipeline.



# Chapter 4

## A reproducible research tool: the R6 Class *easyReporting*

It has been claimed that many research findings in omics science are false (or partially false) due to accidental mistakes or mis-usage of methods. To prevent misleading results it is important to be able to inspect and reproduce the entire data analysis carried out in a unified product.

Reproducible Research (RR) consists on making available both the analytic data and the associated code in a manner that other researchers might reproduce the findings.

In this Chapter we illustrate *easyReporting* a novel R package for speeding up the RR implementation when analyzing data or when constructing other packages.

Tool availability at: <https://github.com/drighelli/easyreporting>

### 4.1 The idea of Reproducible Research

During last years, several approaches [28] have been proposed for helping to trace the analysis steps, using different programming languages; such as *Jupyter*<sup>1</sup> [90] in *Python* or *Rmarkdown*<sup>2</sup> in R. Or by building a web environment to

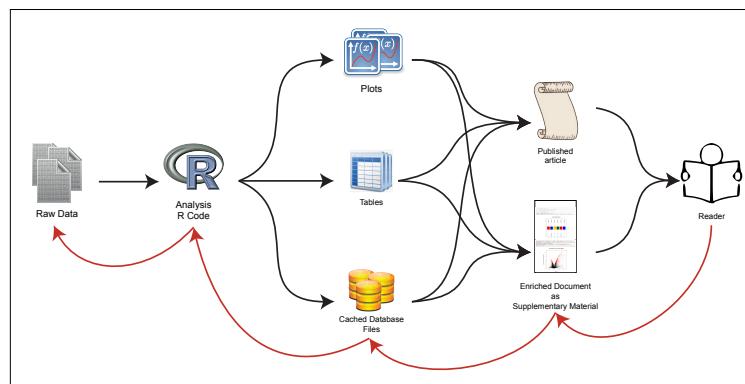
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<sup>1</sup><https://jupyter.org/>

<sup>2</sup><https://rmarkdown.rstudio.com/>

encapsulate several tools made with different programming languages, such as *Galaxy*<sup>3</sup> [91–93].

The common underlying idea of each one of these instruments is to provide a mixture of natural language sentences along with computational language (Code Chunks (CCs)) and visual outputs, in order to produce a unique framework where the CCs and their outputs are explained to the reader, enhancing comprehensibility and reproducibility of the work (Literate Statistical Programming [94]).



**Figure 4.1.1:** A general illustration of Reproducible Research concept. Raw data can be analysed with R code, producing plots, tables and additional caching database files. All together, R code, data and results can be inserted in an enriched document, which can be added as supplementary material to a valuable published article.

Image adapted from [29]

Additionally, it is mandatory to provide the analytic data needful to run through again the entire analysis. In such a way, a mixture of explaining natural language sentences combined with code instructions executed on the analytic data lead to produce a final product that can be easily reused also by a non-expert user.

During last decades, RR idea has been improved by the scientific community proposing several approaches in order to provide more accessibility to the analytic data, the code and the results at lower costs in terms of time and efforts. In particular, the R community proposed several solutions, like *sweave* [95, 96], and more recently *knitr* [97] and *rmarkdown*. Due to its easy interactive usage, *rmarkdown* became one of the most used instruments inside the R community, but its usability when developing automated instruments like Graphical User

<sup>3</sup><https://usegalaxy.org/>

Interface (GUI) or packages becomes more difficult, leading developers to give up on implementing it.

In order to facilitate the widespread of RR through the analysts community, in the past we proposed a possible solution with the *RNASeqGUI* [28] project, a graphical interface software for analyzing RNA Sequencing (RNA-seq) data. The software, while the user interacts with the interfaces to analyze the data, traces the code inside an R Markdown (RMD) file and stores the data inside specific caching database files (CDF). At the end of the analysis, the RMD and the CDF contribute together at the compilation of the report, where all the code, the data and the results were presented.

This solution, although very useful, does not allow to insert personal comments at each CC. Indeed, each portion of the code was always automatically accompanied by pre-established comments, requiring some experience of RMD file editing in order to produce a decent final result. However, inserting personal comments for the CC is fundamental for the final knowledge transfer across researchers and lab members across time.

## 4.2 Methods

Here we present *easyReporting*, an R6<sup>4,5</sup> class<sup>6</sup> helping developers to integrate a reproducible research layer inside their software products, as well as lazy analysts to speed up their report production without learning the *rmarkdown* language.

In such a way, thanks to minimal additional efforts of developers, the end user has available an *rmarkdown* file within all the source code generated during the analysis, divided into CCs ready for the compilation.

Once manually edited with comments and descriptions the file can be compiled to produce an enriched document within input data, source code and output results.

A so final document can be attached to the publication of the analysis as supplementary material, helping the interested community to entirely reproduce

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<sup>4</sup><https://adv-r.hadley.nz/r6.html>

<sup>5</sup><https://cran.r-project.org/web/packages/R6/index.html>

<sup>6</sup>[https://en.wikipedia.org/wiki/Class\\_\(computer\\_programming\)](https://en.wikipedia.org/wiki/Class_(computer_programming))

the computational part of work (figure 4.1.1).

### General Description and Initialization

The class can be imagined as a schematic representation of the *rmarkdown* file (report), indeed its attributes represent the report characteristics, which are typically inserted into the header of the file. Additionally, our class methods are useful not only for the attributes manipulations but also for insertion of CCs, comments and section titles inside the report.

As any typical class, before of using it, *easyReporting* requires to be initialized with the `new` command, passing as mandatory arguments the *path* and the name of the file as `filenamepath` and a title as `mainTitle`. Additionally, an `author` and the `documentType` can be specified.

Learn more about chunk options at <a href="http://yihui.name/knitr/options">http://yihui.name/knitr/options</a>		
Chunk options		
option	default value	description
<b>Code evaluation</b>		
<code>child</code>	NULL	A character vector of filenames. Knitr will knit the files and place them into the main document.
<code>code</code>	NULL	Set to R code. Knitr will replace the code in the chunk with the code in the code option.
<code>engine</code>	'R'	Knitr will evaluate the chunk in the named language, e.g. <code>engine = 'python'</code> . Run <code>names(knitr::knit_engines\$get())</code> to see supported languages.
<code>eval</code>	TRUE	If FALSE, knitr will not run the code in the code chunk.
<code>include</code>	TRUE	If FALSE, knitr will run the chunk but not include the chunk in the final document.
<code>purl</code>	TRUE	If FALSE, knitr will not include the chunk when running <code>purl()</code> to extract the source code.
<b>Results</b>		
<code>collapse</code>	FALSE	If TRUE, knitr will collapse all the source and output blocks created by the chunk into a single block.
<code>echo</code>	TRUE	If FALSE, knitr will not display the code in the code chunk above it's results in the final document.
<code>results</code>	'markup'	If 'hide', knitr will not display the code's results in the final document. If 'hold', knitr will delay displaying all output pieces until the end of the chunk. If 'asis', knitr will pass through results without reformatting them (useful if results return raw HTML, etc.)
<code>error</code>	TRUE	If FALSE, knitr will not display any error messages generated by the code.
<code>message</code>	TRUE	If FALSE, knitr will not display any messages generated by the code.
<code>warning</code>	TRUE	If FALSE, knitr will not display any warning messages generated by the code.
<b>Code Decoration</b>		
<code>comment</code>	'##'	A character string. Knitr will append the string to the start of each line of results in the final document.
<code>highlight</code>	TRUE	If TRUE, knitr will highlight the source code in the final output.
<code>prompt</code>	FALSE	If TRUE, knitr will add > to the start of each line of code displayed in the final document.
<code>strip.white</code>	TRUE	If TRUE, knitr will remove white spaces that appear at the beginning or end of a code chunk.
<code>tidy</code>	FALSE	If TRUE, knitr will tidy code chunks for display with the <code>tidy_source()</code> function in the <code>formatR</code> package.

R Studio

Updated 10/30/2014

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**Figure 4.2.1:** A schematic table of options available using knitr and rmarkdown packages.

When initializing, the class automatically creates the report with the entire specified folder tree, setting up its header and declaring the general options for the rmarkdown file. If rmarkdown personal options (see figure 4.2.1 for a list of available options) are required, before creating an instance of the class, it is

possible to use the `makeOptionsList` function, and then assigning the output to the `optionsList` argument of the class `constructor`.

### Class Methods

The class is provided of several methods for *rmarkdown* CC construction.

Once an `easyReporting` instance is available, with `mkdTitle` it is possible to insert six levels of titles, by setting the parameters `title` and `level`. It is also possible to add natural language comments with `mkdGeneralMsg`.

When working with CCs, two main choices are available. The first one gives the possibility to construct a CC as additional steps, by using first the `mkdCodeChunkSt`, then adding variable assignment and/or function calling with `mkdVariableAssignment` or `mkdGeneralMsg`, and finally closing the CC with `mkdCodeChunkEnd`.

In particular, when starting a CC with `mkdCodeChunkSt`, it is possible to assign a specific `optionList` and/or a `source.files.list` to be added to that CC.

Otherwise it is possible to create an entire CC just with `mkdCodeChunkComplete` and assigning the entire function call as a `message`. This way of working is really useful with `function` creation, where inside a developed function a simple recursive call with parameters assignment can be done as a single `message`.

## 4.3 Usage

Here we report an example script where a general illustration of the `easyReporting` is described.

As already mentioned, the class allows to report not only the CC, but also to add personal titles and personal comments, enhancing the knowledge transfer between the "back-side" (the analyst/developer) and the "front-side" (the reader) users. Note that "back-side" and "front-side" can also be the data analyst and the collaborators, respectively.

---

<sup>1</sup> `## Creating report file with default options on global document`

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---

```
2 rd <- easyreporting$new(filenamepath="./project_report",
  title="example_report", author=c("Dario Righelli"))
3
4 rd$mkdTitle("First Level Title")
5
6 rd$mkdGeneralMsg("Here I'm writing a simple paragraph useful
  to describe my code chunk")
7
8 ## Leaving the default options to the code chunk
9 rd$mkdCodeChunkSt()
10
11 ## Adding a variable assignement
12 variable <- 1
13 rd$mkdVariableAssignment("variable", "variable", show=TRUE)
14 rd$mkdCodeChunkEnd()
15
16 ## Or i can create my own options for the chunk
17 rd$mkdTitle("Second Level Title", level=2)
18 optList <- makeOptionsList(includeFlag=TRUE)
19 rd$mkdCodeChunkSt(optionsList=optList)
20 rd$mkdCodeChunkEnd()
21
22 ## Moreover I can add a list of files to source in che code
  chunk
23 rd$mkdCodeChunkSt(optionsList=optList, source.files.list=c(
  "R/cachingFunctions.R", "R/cachingFunctions.R"))
24 rd$mkdCodeChunkEnd()
25
26 rd$mkdCodeChunkComplete(message="a <- 1\nb <- 2\nnc <- a+b\n
  print(c)")
27
28 ## Otherwise I can make a direct call with all the code
  chunk and the comment in one call
29 optList <- makeOptionsList(includeFlag=TRUE, cacheFlag=TRUE)
30
31 rd$mkdCodeChunkCommented(commentMsg="This is the comment of
```

```
the following code chunk", message="a <- 1\nb <- 2\n(c <-
a+b)", optionsList=optList, source.files.list=NULL)
32
33 ## finally I can directly compile my report
34 rd$compile()
```

---

The previous R script leads to automatically produce the following rmarkdown file, where it is possible to see at rows 13 and 23 two titles at different levels. And additionally, starting from row 15, a describing paragraph representing the additional comments that can be added to provide additional explanations to the following CC.

---

```
1 ---
2     title: "example_report"
3     author: "Dario Righelli"
4     date: "‘r Sys.Date()’"
5     output: rmarkdown::html_document
6 ---
7
8 ‘‘'{r global_options, include=FALSE}
9 knitr::opts_chunk$set(eval=TRUE, echo=TRUE, warning=FALSE,
10   message=FALSE, include=TRUE, cache=TRUE)
11 ‘‘‘
12 # First Level Title
13 Here I'm writing a simple paragraph useful to describe my
14   code chunk
15 ‘‘'{r eval=TRUE, echo=TRUE, warning=FALSE, message=FALSE,
16   include=TRUE, cache=TRUE}
17 variable <- 'variable'
18 print(variable)
19 ‘‘‘
20 ## Second Level Title
21 ‘‘'{r eval=TRUE, echo=TRUE, warning=FALSE, message=FALSE,
```

```
    include=TRUE, cache=TRUE}

22  """
23
24  """{r eval=TRUE, echo=TRUE, warning=FALSE, message=FALSE,
25   include=TRUE, cache=TRUE}
25  source("/Users/inzirio/Desktop/gDrive/works/coding/
26       easyreporting/R/cachingFunctions.R")
26  source("/Users/inzirio/Desktop/gDrive/works/coding/
27       easyreporting/R/cachingFunctions.R")
27  """
28
29  """{r eval=TRUE, echo=TRUE, warning=FALSE, message=FALSE,
30   include=TRUE, cache=TRUE}
30  a <- 1
31  b <- 2
32  c <- a+b
33  print(c)
34  """
35
36 This is the comment of the following code chunk
37
38 """{r eval=TRUE, echo=TRUE, warning=FALSE, message=FALSE,
39   include=TRUE, cache=TRUE}
39  a <- 1
40  b <- 2
41  (c <- a+b)
42  """
```

---

Thanks to the `rd$compile()` command inside the first script, it automatically produces the final Hypertext Markdown Language (HTML) report illustrated in figure 4.3.1.

Finally, the report in HTML format provides a human readable document, where all the CC are highlighted in grey, and the results in white. Titles and comments are in plain text, highlighting the natural language format of a self-explaining document.

## example\_report

Dario Righelli

2018-12-06

### First Level Title

Here I'm writing a simple paragraph useful to describe my code chunk

```
variable <- `variable`  
print(variable)
```

```
## [1] 1
```

### Second Level Title

```
source("/Users/inzirio/Desktop/gDrive/works/coding/easyreporting/R/cachingFunctions.R")  
source("/Users/inzirio/Desktop/gDrive/works/coding/easyreporting/R/cachingFunctions.R")
```

```
a <- 1  
b <- 2  
c <- a+b  
print(c)
```

```
## [1] 3
```

This is the comment of the following code chunk

```
a <- 1  
b <- 2  
(c <- a+b)
```

```
## [1] 3
```

**Figure 4.3.1:** An example of HTML report produced with *easyReporting* R package.

## 4.4 Discussions

We presented *easyReporting*, an R package enabling the implementation of RR using rmarkdown language scripting, without any knowledge of it.

Despite other previously proposed solutions [98, 99], that always require too much commitment by the final user, potentially bringing him/her to totally renounce to include RR inside the scripts, our approach is versatile and easy to be implemented, leaving maximum freedom to the final developer/analyzer, automatically creating and storing an *rmarkdown* document, and providing also methods for its compilation.

It gives the possibility to obtain reproducible results and to publish better findings, increasing the scientific publications transparency by improving the knowledge transfer, and to support the methodologies integrity also into the scientific labs, where people can change position through the years.

On the other hand, the *easyReporting* package, even if useful and easy to handle, can be improved with several additional functionalities. While it is

really important to generate an rmarkdown file, it could be useful to introduce methods for its file editing. Indeed, if an analyst or a final user, wants to correct an analysis, by changing an already inserted CC, he/she could have the possibility to do this, by editing that specific CC or by overwriting it. A possible approach to do this could be to trace the CCs, with a dedicated data structure, while inserting them, and give the possibility to the user to access them with specific class methods.

At the same time, even if the rmarkdown options enable the user to store the input/output data with `cache` option, it could be better to provide caching methods, in order to give higher manageability of the data, storing them inside caching database files, as also reported in figure 4.1.1. Additionally, a caching store system (such as `BiocFileCache`<sup>7</sup>) could produce caching database files, easily shareable through the Internet or as supplementary data of a publication.

Finally, in order to provide a graphical representation of an entire analysis, it could be useful to equip *easyReporting* with methods for graph construction. In such a way, the final report could be, not only read by third-party users, but also graphically impress the final reader.

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<sup>7</sup><https://bioconductor.org/packages/release/bioc/html/BiocFileCache.html>

## Integration of High-Throughput Omics data: IntegrHO

In previous chapters, we described some tools (Time Course RNA-seq data Analyzer (Ticorser), Differential Enriched Scan 2 (DEScan2)) for the analysis of specific experimental conditions. However, the practical usage of such tools requires a certain level of programming language from a user.

Moreover, it is clear that as soon as the analysis becomes more complex, and different types of integration have to be performed, data analysis becomes more consuming and requires higher computational and statistical knowledge.

To face such limits, we noticed the growing interest by part of the scientific community in using interactive and user friendly software. This interest is led by multiple motivations, such as the need to analyze data very fast or the lack of time in learning programming languages and terminal-line based tools, by different types of data analysts, that could be either expert bioinformaticians or experimental biologists who wants to explore their own data to get novel insights about a cellular mechanism.

Several interface-based tools [100] have been proposed during last years but too often they were oriented to analyze singular-omics (such as *RNASEqGUI* [28]). However, in many cases such tools are organized as rigid pipelines, rather than interactive environments.

In this chapter we introduce Integration of High-Throughput Omics data (In-

tegrHO), our web-based platform for multi-omics data analysis and integration, built in a Reproducible Research (RR) spirit.

## 5.1 Introduction

In order to deeply understand and reconstruct cellular mechanisms influenced by drug treatments, pathologies or diseases, it is fundamental to look at multiple omics data types at the same time.

Previous chapters already described some tools for multiple omics data analysis, integration and visualization using command line tools. Although, the command line approach is quite common inside the bioinformatics community, it is not well suited for every scientist who is not so confident with programming languages or with the terminal. Moreover, when working with multiple omics data, there is an overabundance of available tools for each sequencing that can bewilder a beginner, up to the point to renounce approaching the analysis problem. Additionally justified if we think about the technical knowledge required when working with multiple different input/output data structures required for each tool.

Furthermore, even if the bioinformatics community has massively moved on the development of novel statistical and computational methods for multi-omics data integration, part of the scientific community is still anchored to the single-omics analysis side. Indeed, without entering into the merit of the obtained scientific results, it is still very common to read published papers based on single-omics data analysis without taking into account possible integrated solution with other omics data types.

Nowadays, it is more easily achievable to afford for multi-omics experiments because of the lowering costs of them. Additionally, there are international big projects for collecting biological data and providing public access to their biological data banks, such as Gene Expression Omnibus (GEO)<sup>1</sup> [101] or The Cancer Genome Atlas (TCGA)<sup>2</sup> [102], where it is possible to retrieve as many datasets as needed.

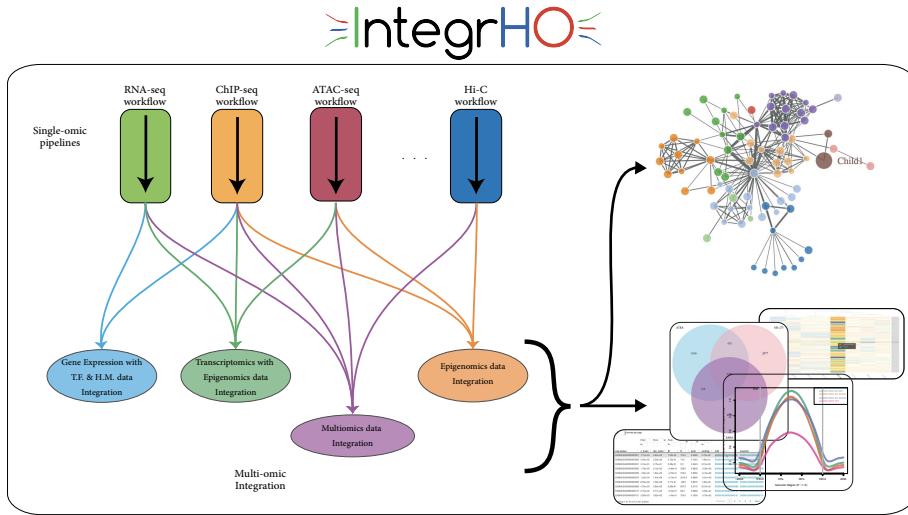
On the other side, multi-omics data integration is an emerging research field,

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<sup>1</sup><https://www.ncbi.nlm.nih.gov/geo/>

<sup>2</sup><https://cancergenome.nih.gov/>

that still needs to become a standard of biological data analysis.



**Figure 5.1.1:** A schematic representation of IntegrHO underlying idea. Single-omics analysis methods are proposed in order to facilitate their multiple integration. This integration can lead to produce graphical results, in case of low dimension datasets, or to more sophisticated integration model such as regulation networks, in case of high-dimension datasets.

Based on the above mentioned considerations and in order to promote the multi-omics data integration, we are developing IntegrHO, a novel easy-to-use instrument which not only gives the possibility to analyze single-omics data types but also guides the user through multiple ways of integrating multi-omics data types (figure 5.1.1 gives an underlying idea of IntegrHO).

## 5.2 Methods

Roughly speaking, when working with multiple omics data, the integration phase requires that the data used for this step are "connected" between different omics.

After some preprocessing (alignment, quantification, etc.) the results are combined in specific omics matrices  $X_k$  with  $k = 1, \dots, m$ , where  $m$  denotes the number of omics, such that  $\dim[X_k] = [n_k, p_k]$ , where  $n_k$  is the number of samples (such as patients, tissues, etc.) and  $p_k$  the number of observed variables (features) on the specific omics. In particular, for each of the  $m$  matrices it is possible to investigate different biological questions, depending on the nature

and availability of the data. We can distinguish between the variable based analysis, where each matrix has the same variables and differs on the samples, and the samples based analysis, where for the same samples across multiple omics we have different features. In the first case we can answer relevant common biological questions on the features (such as genes, genomic regions, etc.) across multiple omics, while in the second case we can answer biological questions related to the samples observing different variables at each omics level.

Once we collected all the required samples, we can either start analyzing each omics individually and/or integrating them to better understand the cellular behaviours involved in the studied disease. Depending on the number of collected samples, we can choose from different methodologies to apply. If we have few numbers of samples, we can decide to work on single omics analysis and then to integrate the results. Otherwise, when having a high number of samples we can use deeper statistical methodologies, such as network fusion techniques for constructing multiple levels regulatory networks [38–40].

In order to account for these kind of studies, inside IntegrHO we are implementing several aspects and interfaces to help and guide the user to achieve integrated results.

### 5.2.1 The main interface

IntegrHO is a web platform fully developed in R with aid of Shiny libraries, combining the power of the R statistical instrument with HTML5/javascript flexibility.

Shiny apps are typically designed for small applications, allowing a very easy and versatile way for developing and releasing them. The basic structure of a shiny app is based on two main entities, the Shiny User Interface (SUI) and the Shiny Server (SS). The first one includes all the aesthetic components which the user interacts with, while the SS processes all the computations.

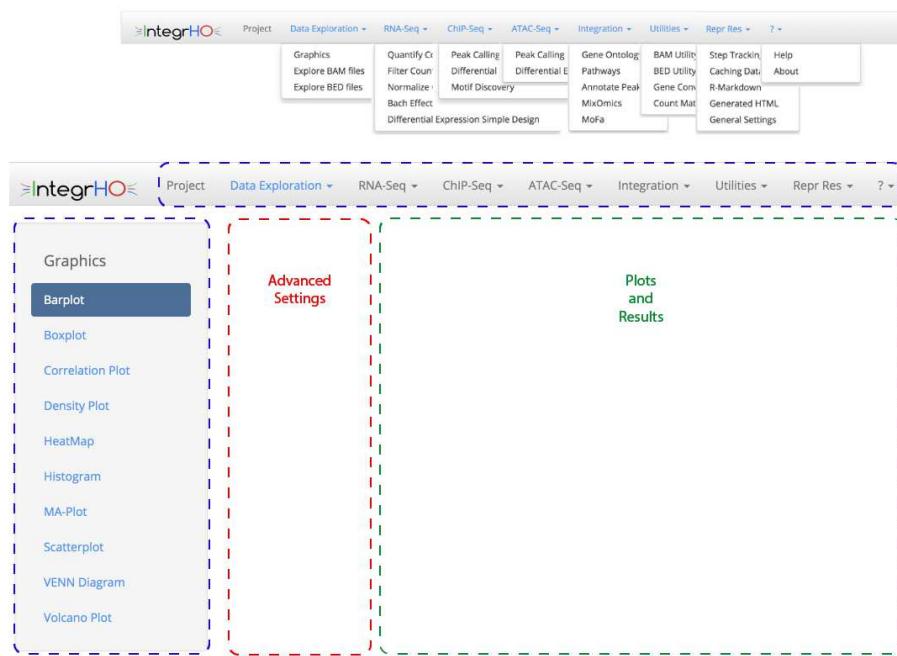
Natively, shiny apps support only one server, limiting the implementation features when more sophisticated analysis are required, such those in multi-omics data analysis and integration. Indeed, to account this problematic, Shiny libraries have been recently extended with the shiny modules technology<sup>3</sup>, al-

---

<sup>3</sup><https://shiny.rstudio.com/articles/modules.html>

lowing us to build IntegrHO as a combination of self-containing modules. In such a way, the main shiny app can be shredded into multiple "mini apps", each one with its own SUI and SS. This approach is totally invisible to the final user, but helps the developer for the maintainability and the extensibility of the entire tool. Indeed, when future needs arise for the implementation of novel functionalities, it will be only necessary to add a novel module.

The upper part of IntegrHO interface displays a menu bar, which organizes the analytics features in main topics. Each topic contains a sub-menu within broad categories of functionalities. By accessing a specific category, the list of available functions is displayed on a left side menu. In order to well set up the parameters for each functionality, an additional side menu is presented with the parameters and their possible values for the right setup (figure 5.2.1 shows a general representation of the main interface).



**Figure 5.2.1:** IntegrHO main interface description. A main menu in the upper part is presented with all the available main functionalities, and a left side menu is presented with additional functionalities (in blue). Red part indicates the settings for each functionality within the parameter setup. While in green results in table or graphical form are presented.

Once the user set up the required parameters and pressed the section button,

the parameters are processed and the results are shown in graphical or table format in the main part of the interface.

Before to proceed to the data analysis, it is mandatory for the user to set up the project with a dedicated interface.

One of the key concepts in the implementation of IntegrHO is the "experimental design file", which the user has to upload and, if needed, to manually edit directly from the interface (figure 5.2.2). In such file the user needs to specify several information describing each data file describing the under investigation experiment. This is useful for defining samples belonging to the same condition and to the same omics data type. Moreover, some additional describing variables are mandatory, such as the filename (with path) of the Binary Alignment Map (BAM) files and the conditions of each sample, while others are optional as the tissue or the run id. The design file information will be used during all the following steps of the analysis.

The choice of using a mandatory variable describing the path of BAM filenames is due to a *Shiny* library limitation. Because of its "UI-server" structure, shiny applications, when using a `fileInput` widget, make a temporary copy of the selected file(s). But this aspect becomes really problematic when a file can reach several gigabytes of memory on disk, as one or more BAM files can. Even if there are alternative packages, such as *shinyFiles* [103], offering the possibility to solve the problem, they are not very well tested over multiple platform architectures.

Using the project interface, IntegrHO creates inside the working directory (returned by the `getwd()` function) a dedicated folder with all the required sub-folders and stores all the basic information of the project into an ad-hoc designed `R6ProjectClass`, which is re-used during the whole session to speed up the configuration of each step of the analysis (Figure 5.2.3 shows a representation of a typical folder tree produced for a project).

In particular, we designed a `R6ProjectClass`, which stores all the paths represented inside the figure 5.2.3. This allows fast access to the files stored in each folder, that will be processed specifically for each selected interface.

	path	File.type	experiment	paired	label_ID	treatment	platform.opt.	run.opt.
1	Path/to/file_1.bam	bam	rna-seq	no	1	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2	Path/to/file_2.bam	bam	rna-seq	no	1	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3	Path/to/file_3.bam	bam	rna-seq	no	2	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4	Path/to/file_4.bam	bam	rna-seq	no yes	2	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5	Path/to/file_5.bed	bed	chip-seq	no	3	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
6	Path/to/file_6.bed	bed	chip-seq	yes	3	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

**Figure 5.2.2:** The project design IntegrHO interface. The user has to insert a name for the project and to load a design file. The design file can be edited through the interface itself.

### 5.2.2 Available Methodologies

Unlike tools as Galaxy [104] and Taverna [105], focused on analysis workflows, IntegrHO gives to the user high freedom of interaction, reporting all performed steps in a human-readable Hypertext Markup Language (HTML) report.

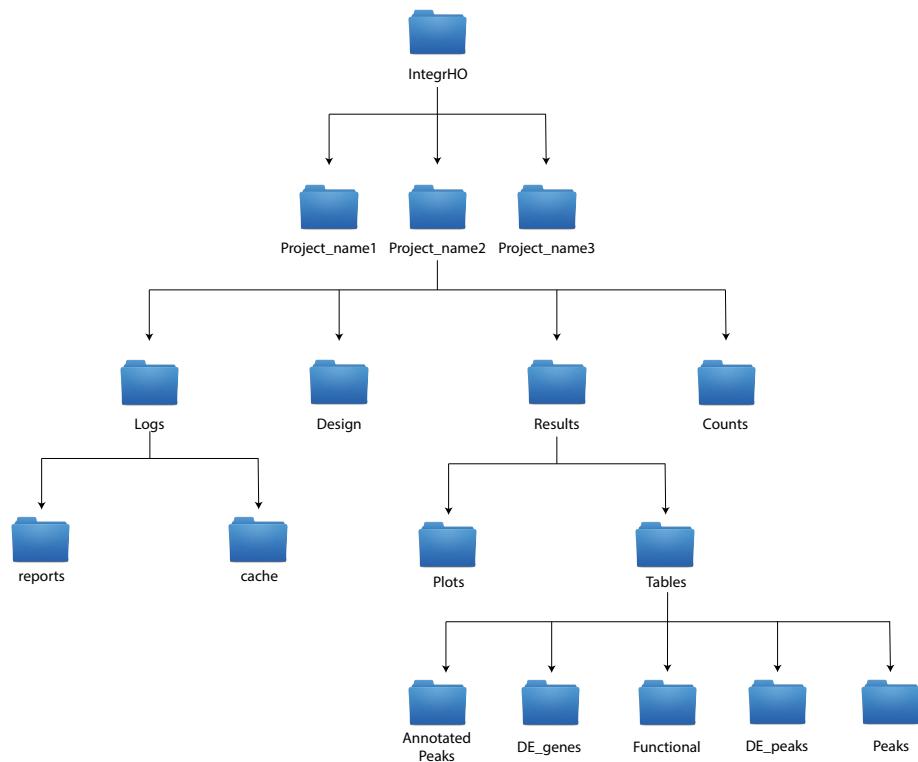
Actually, IntegrHO methodologies have been organized in a way that the user can analyze every single omics, and afterwards, combine the results to obtain an integrated view of them.

In particular, we defined specific interfaces for RNA Sequencing (RNA-seq), Chromatin Immunoprecipitation Sequencing (ChIP-seq) and Assay for Transposase-Accessible Chromatin Sequencing (ATAC-seq) data, providing additional interfaces for their integration, such as *functional enrichment* and *gene-peaks annotations*.

Moreover, through the `graphics` interface the user has the possibility to choose between a great variability of graphics, useful to explore data and results, both in pre-processing and post-processing phase, such as *barplots*, *correlation plots*, *heatmap*, *scatterplots*, *keggmap*, etc.

IntegrHO starts from already mapped samples, starting the omics analysis from already available BAM files for every sample the user wants to investigate.

Figure 5.2.4 shows a schematic representation of implemented packages inside IntegrHO. The upper part shows the single-omics packages used for the analysis of RNA-seq, ChIP-seq and ATAC-seq, while the lower part refers to



**Figure 5.2.3:** A schematical representation of IntegrHO project folder tree.

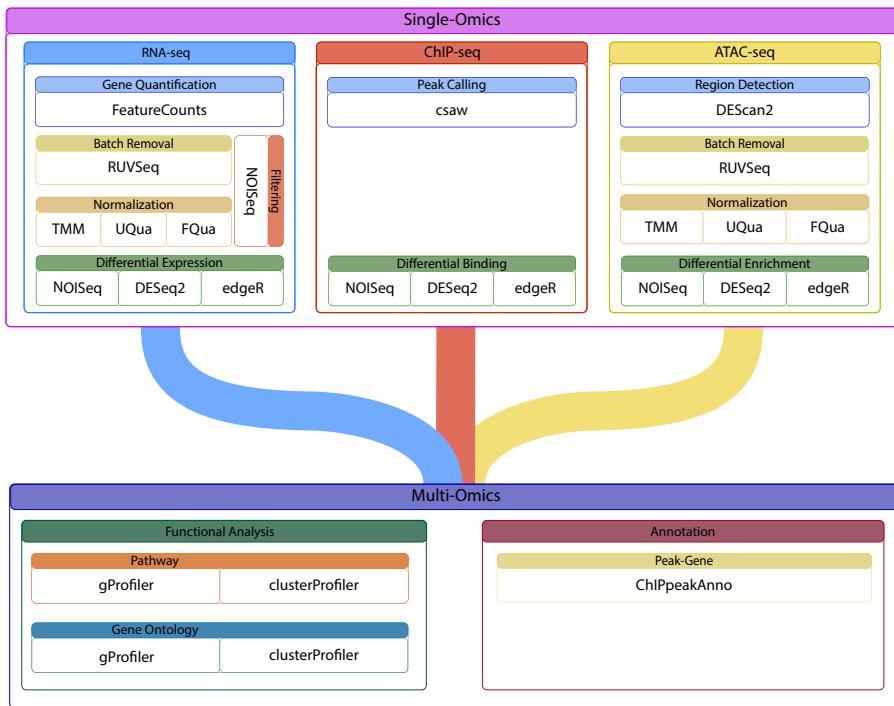
the multi-omics integration, showing the packages used for *functional analysis* and *peak-gene annotations*.

### RNA-seq

For RNA-seq data analysis section we chose multiples methodologies in order to allow the user to have a good overview of the entire *Differential Expression* analysis steps. In such a way, the user has several methodologies for accounting a complete RNA-seq pipeline (refer to 1.2.1 section for a typical RNA-seq pipeline description).

When working with RNA-seq data it is really important to quantify the reads contained inside BAM files associating them to genomic features. To do that, it is necessary a file with the genomic features description, typically a Gene Transfer Format (GTF) file (defined in 2.4 section) describing the genomic coordinates for each feature (genes, transcripts etc.).

To account for genomic feature quantification, the user has to select the



**Figure 5.2.4:** A schematical representation of packages included inside IntegrHO web-platform. The upper part shows the packages for the the single-omics analysis, while the lower part shows the packages for the multi-omics analysis.

Quantify Counts interface, designed for `featureCounts` of `Rsubread` R/Bioconductor package function [59] appears. The BAM file paths for the RNA-seq data are automatically selected from the design matrix loaded during the project creation phase.

We choose proper widgets for selecting the reference genome and its annotation, it is also possible to configure the `featureType` to use, while the `attributeType` is set by default to `gene_id`. Additionally, parameters can be selected for `allowMultiOverlap` and to select if the reads are `paired`. Finally, the number of threads to use can be set from 1 to 8.

At the end of the processing, the resulting count matrix is presented in the main part of the interface as a `data.table` format, and automatically stored inside the `IntegrHO/project_name/counts` folder.

When working with RNA-seq data, several features (genes) are lowly expressed, affecting the further analysis and leading to biased results. When selecting the `Filter Counts` the designed interface automatically loads the counts files

inside the `IntegrHO/project_name/counts`, otherwise allowing, with a `fileInput` widget, to chose another count matrix file. It is possible to switch between three different filtering methods (*Proportion Test*, *Wilcoxon Test*, *CPM*), as defined inside the `filtered.data` function of *NOISeq* R/Bioconductor package [61, 62].

The filtered-in features need to be normalized in order to be compared across multiple conditions. Also in this case, a not proper data normalization can lead to misleading results.

The same type of interface built for the filtering step is loaded when the `Normalize Counts` menu voice is selected. Three different normalization methods can be selected, the *Trimmed Mean of M-values* [106], the *Upper Quartile* and the *Full Quantile*.

Additionally, a main aspect of RNA-seq data analysis is to account for "unwanted factors" (batch effects) that, very often, affect the data. This noises are part of the data because reflecting batch, library preparation, and other nuisance effects.

When selecting the `Batch Effect` section there will be the possibility to apply the *RUVs* or *RUVg* methods from *RUVSeq* R/Bioconductor package [63]. Moreover, when selecting one of this methods, an additional file can be loaded, in order to upload an optional list of negative control genes.

To graphically inspect the normalized data two different plots will be presented into the main interface, the Principal Component Analysis (PCA) and the *boxplots* for before and after normalization of the samples. This comparison is dictated by the need of inspecting the effects of noise removal from the data.

Finally, at the end of execution the filtered/normalized features, as Tab Separated Value (tsv) file format, are stored inside the `IntegrHO/project_name/counts` folder, adding at the end of the file name the "transformation" applied.

Finally, when the data are well normalized, the user can address for differential expression inspection of the samples.

In order to detect Differentially Expressed Genes (DEGs), we designed the `Differential Expression Simple Design`, where it is possible to choose between four different methods.

The available count matrix files are automatically loaded from the `IntegrHO/project_name/counts` folder. Otherwise, the user can additionally load another

count matrix file using the apposite file selector.

In order to address for the factors to analyze, the interface automatically gives the possibility to select the design matrix column name where to find them. Additionally, two `selectInput` widgets appear where to select the two factors to contrast.

We selected four different methods implemented inside *edgeR*, *DESeq2*, *NOISEq* and *NOISEqBio* R/Bioconductor packages [Tarazona2012, 65, 66].

In case of *edgeR* we decided to use the *Quasi-Likelihood* method for the differential expression. While when using *DESeq2* for this specific case we choose the *Wald* test, as the authors suggest. The *NOISEq* package offers the possibility to discriminate between *biological* and *technical* replicates, computing a posterior probability in both cases, but applying different hypothesis tests.

The dedicated interface automatically shows a `tabsetPanel` of 3 different tabs, within, respectively, a *VolcanoPlot*, an *MA-Plot* and the table of the DEG results.

### ChIP-seq

ChIP-seq is a very common technique to investigate multiple epigenetic factors, the sequencing technique by itself identifies chromatin regions where specific proteins bind on. Considering to have multiple BAM samples of ChIP-seq data, we constructed specific interfaces for peak calling and Differential Binding Sites (DBSs) detection. Also in this case, we are focusing on the problem of detecting how the signal differs between multiple biological conditions. We refer to DBSs because of the nature of the biological data, coming it from proteins binding specific regions of the chromatin.

Assuming that the samples are in BAM format, we assume that the user needs to reconstruct the signal of the chromatin regions binded by the interested protein. We underline that a ChIP-seq experiment is "protein specific", because it involves using an antibody that is specific for a known protein.

For the **peak calling**, because of the lack of specific methods starting from BAM files in R, we implemented a dedicated interface for *csaw* [89] peak caller, which allows quantification for both broad and narrow peaks, typically resulting from Histone Modification (HM) and Transcription Factor (TF) ChIP-seq data.

Firstly, it is possible to select the design matrix (stored inside `IntegrHO/project_name/design` folder) and the column specifying the experiment. In such a way, IntegrHO is able to pick up the BAM filenames associated to the ChIP-seq experiments by itself.

Afterwards, the interface enables the specification of several parameters useful for the `windowCounts` method of `csaw` R/Bioconductor package. The method builds a count matrix where the rows indicate the detected windows of length `window width` and the columns indicate samples.

Additional parameters selected for the analysis with the `csaw` method are the `paired end reads`, a flag for ignoring duplicated reads with `ignore duplicates`, and the possibility to make the analysis only on a list of chromosomes, using the `chromosomes list` implemented with a `selectizeInput`. The possibility to make parallel computing is implemented with the `core number` widget (from 1 to 8).

At the end of the processing, the count matrix is shown into the main part of the interface, and it is saved as tsv format, into the `IntegrHO/project_name/results/tables/peaks` folder. In order to distinguish the produced output from others, the results file is saved by adding the method name at the end of the filename.

Once constructed a count matrix of genomic windows (features) on the rows and samples on the columns, in order to detect DBSs across the conditions, we are able to apply the same methodologies designed for RNA-seq data (see section 5.2.2 for further details). For this reason, we used the same methods as defined for RNA-seq, showing the results table as output.

Other methodologies for differential binding have been developed in R, such as the package `DiffBind` [107], but at the moment we didn't implemented them yet.

Moreover, it is relevant for ChIP-seq data analysis to account for the "right normalization" to use. The count matrix form allows to apply the same normalization methodologies developed for RNA-seq data, but at the moment we didn't implemented this aspect. Additionally, it is still an open issue to study for the "correctness" of applied normalization on ChIP-seq data, even if some approaches have already been presented [108].

## ATAC-seq

ATAC-seq is a sequencing technology for investigating chromatin accessible regions, which are typically associated on the identification of the instrumental epigenetic changes responsible for differential gene expression, cell proliferation, functional diversification and disease development (see 1.2.3 for further details).

Thanks to the work made with DEScan2 (chapter 3), we had the possibility to deeply investigate ATAC-seq data, presenting a specific analysis workflow. We tried to give access to the same methodologies, defined for DEScan2, herein IntegrHO, in order to simplify and spread the proposed work.

As for the ChIP-seq, the user needs to reconstruct the signal across the entire genome by detecting the genomic regions.

In particular, to account for the regions detection, we built a specific interface for the peak caller defined in DEScan2 (see section 3.2.1).

As for the *csaw*, the user can select the design matrix (stored inside `IntegrHO/project_name/design` folder) and the column associated with the experiment definition. Additional parameter selection is required for the selection of the `genome name`, the `bin size` for the genome binning, and for the `max window` and `min window` lengths. Also, in this case, we gave the possibility to select for a multi-core computation with the `core number` sliderInput widget.

The user can also select the `peak score` threshold for filtering out the regions with a lower score than the threshold, and the `samples threshold` for the alignment phase (see section 3.2.2).

In order to better compare DEScan2 results with similar methods, such as *csaw*, we preferred to include also the DEScan2 filtering method inside the *region detection* interface. In such a way, the presented output is a count matrix of detected regions (peaks) on the rows and samples on the columns.

After the reconstruction of the signal, it is a good norm to normalize the data, in order to remove unwanted factors biasing the data leading to misleading results. Also in this case, we took advantages of the count matrix form to apply the same methodologies developed for RNA-seq, and doing the same for the detection of Differentially Enriched genomic Regions (DERs) (section 5.2.2).

## Integration

The previously studied omics, even if not so many, already give the possibility to construct an integrated view of multiple cellular aspects.

For example, if we are able to collect samples of ChIP-seq for an HM involved into the chromatin accessibility, and ATAC-seq samples with RNA-seq samples, we already are able to understand which regions of the genome are influenced by that specific HM, obtaining a crossed inspection with resulting region of ATAC-seq. Moreover, we can look at how the gene expression has been influenced by these chromatin accession mechanisms.

To account even for simple integration problems like this one, we implemented functionalities for the **peak-gene annotation** of DERs/DBSs with DEGs using *ChIPpeakAnno*.

The interface allows for the selection of a `peaks` file (DER/DBS), automatically loaded from `IntegrHO/project_name/results/tables/peaks`. Additionally, an `annotation file` is required to retrieve the genomic features to annotate the peaks. In alternative, a list of DEGs can be selected. Other relevant parameters, such as the gene `feature` or the `max distance` between the peaks and the genes, are needed for the right working of `annotatePeakInBatch` function, defined inside the `ChIPpeakAnno` R/Bioconductor package.

When the results are computed by the server side, they are showed with two plots organized in two panels of a `tabsetPanel` widget. A *pie chart* showing the percentage of detected features organized on their distance, and a table of annotated regions with corresponding genes.

Finally, a `tsv` file, within annotated peaks, is saved inside the `IntegrHO/project_name/results/tables/annotated_peaks` folder, while the volcano plot, in HTML format, is saved into the `IntegrHO/project_name/results/plot` folder.

Another kind of integration is given by **functional annotation** analysis, where list of genes (annotated or resulting from differential expression analysis) can be used to detect cellular functional behaviours, thanks to databases born to annotate multiple gene contributing to specific cellular mechanisms (called pathways or gene ontology terms).

In order to interrogate these databases, several methodologies for functional analysis born [70, 71, 109–111]. The underlying idea of them is to use a list of

genes to see which of them mostly *up-regulate*/*down-regulate* annotated cellular functional mechanisms

To account for this integration aspect we developed one interface for allowing the user to choose between two methods *gProfiler* [71] and *clusterProfiler* [70]. Both methodologies allow to investigate multiple databases, for pathways *KEGG* and *REACTOME*, while for Gene Ontology we give the possibility to choose between Biological Process (BP), Molecular Function (MF) and Cellular Component (CC).

The interface automatically loads the file inside the `IntegrHO/project_name/results/tables/annotated_peaks` and `IntegrHO/project_name/results/tables/DE_genes` folders, giving the possibility to select the number corresponding to the column within the gene list.

In order to choose the method to apply, a `selectInput` shiny widget gives the possibility to choose between the *clusterProfiler* and the *gProfiler* methods. Moreover, the user has to select the type of functional annotation to do (pathway or Gene Ontology) and the database to use.

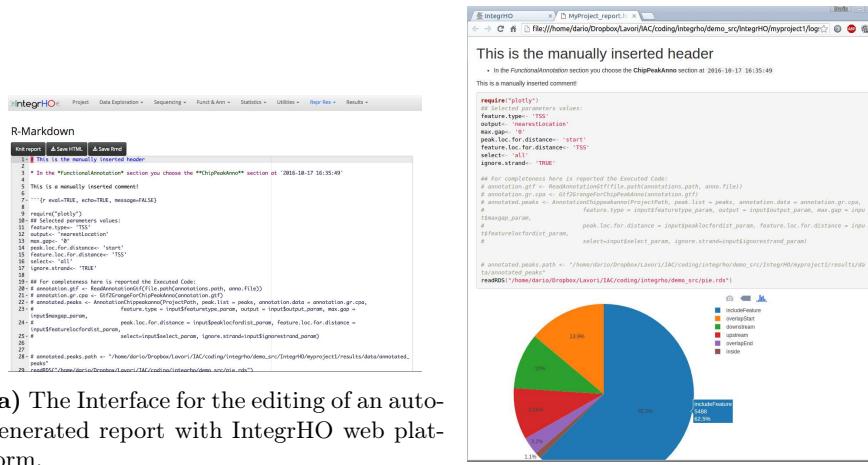
At the end of computations, IntegrHO will present the table of the results in the main interface and will store them into the `IntegrHO/project_name/results/tables/functional` folder.

### 5.3 Reproducible Computational Research

The most difficult part while using a Graphical User Interface (GUI) is keeping track the executed functionalities during the analysis. To face this need we equipped IntegrHO of a RR hidden layer (with aid of *easyReporting* package) able to trace all executed code by the user.

In combination with a system of caching database files (CDF), IntegrHO stores code chunks and the input/output data of each analysis step inside an R Markdown (RMD) file.

The auto-produced RMD file as it is, it's not usable to be showed to third-parties, because it needs to be enriched with natural language comments. To account for this need, we built a specific interface enabling the user to edit the automatically produced RMD file and to compile it on the fly (see sub-figure



**Figure 5.3.1:** The reproducible research with IntegrHO.

### 5.3.1a).

The enriched output report can be produced in HTML (sub-figure 5.3.1b) or Portable Document Format (PDF) formats, in order to be easily attached as supplementary material of a published article, facilitating the reproducibility of the analysis to third-party users.

Furthermore, thanks to the CDF system, each code chunk is not dependent on previous ones, enabling the final user to add/remove multiple code chunks.

## 5.4 Discussions

We presented IntegrHO, a web-platform for multi-omics data analysis, integration and visualization, tracing each performed step by the user by storing produced input/output data and saving the executed data inside an RMD file.

IntegrHO takes advantage of several already-published tools for single omics data analysis and their integration, making it difficult to find a unified way to work with so many different data types and tools for their processing. Indeed, even if some approaches have been proposed in last years, a global standard has not been reached yet, leaving the developers to operate as they better prefer.

At the same time, working with R statistical programming language makes

it difficult the choise of a good library for graphical interfaces. Shiny seems to be a good choise at the beginning, when working with small applications, but once the application grows, it become always more difficult to maintain the modularity and to pass the data across multiple modules.

In order to maintain the modularity, IntegrHO implements the Shiny modules, allowing it to be extended with additional functionalities. At the moment, we are focusing on the implementation of methodologies for high-dimensionality samples integration methods, such as *mixOmics* [39] and *MoFa* [40].

Moreover, to allow the user to account for better quality analysis of ChIP-seq data, we are working on novel interfaces for the normalization and on methodologies for accounting for the right normalization to use [108].

Additionally, IntegrHO traces the code and stores the input/output data into caching database files, in order to construct an RMD file, which is live editable from a dedicated interface. To improve IntegrHO RR layer we would like to implement additional methodologies, such as the graph construction of executed modules, in such a way the user will have also a graphical representation of the final enriched document, that can be presented as a graphical abstract of the analysis.

In general, at the moment IntegrHO allows to deeply investigate single-omics data and, at the same time, it allows for an integration at functional analysis and a peak-gene annotation levels, offering various modules for data visualization. But, in order to promote a wider overview of integration methodologies, it still needs some work to do.



# Chapter 6

## Conclusions

Multiple omics data information extraction became always more complex with the evolution of sequencing techniques and with the consequent development of methodologies for their analysis and integration. With this thesis work, we focused on the development of novel strategies for several problems related to multiple omics sequencing data information extraction.

Starting from approaching the most studied problem of genomic, the transcriptomic, we focused on time-course RNA Sequencing (RNA-seq) data. Time-course RNA-seq data experiments are very common when investigating multiple biological conditions across the time, in order to explore the most affected biological phenomena due to an evolution of drug treatment, or to a tissue injury, etc. Despite the wide use of this kind of experiments, even if several methods have been proposed for their analysis, there still are too few tools fully dedicated to these specific omic data experiments. Guided by our experience in time-course RNA-seq data analysis, we tried to implement, into a unique solution named *ticorser*, multiple approaches and features useful to investigate this omic data type. Indeed, with this instrument we propose a complete pipeline for time-course RNA-seq data analysis, presenting also ad-hoc designed approaches for their visualization at different levels. Additionally, allowing a first integration level with functional annotation and also for their visualization. Indeed, thanks to the proposed case study it is possible to understand multiple difficulties that typically are encountered during a typical time-course RNA-seq analysis.

Afterwards, when moved on the Assay for Transposase-Accessible Chromatin Sequencing (ATAC-seq) technology, an emerging technique for open chromatin region detection, we noticed that this omic still needs appropriate solutions for its analysis. To address the lack of methodologies with this data, we implemented *DEScan2*, an R/Bioconductor package allowing the detection of open chromatin regions. In order to give an overview of a possible analysis workflow for the detection of Differentially Enriched genomic Regions (DERs) and their integration with RNA-seq data, we proposed a case study with an already published dataset, offering ATAC-seq and RNA-seq data. Indeed, we showed how our approach can detect *DNA* open regions signal across multiple samples in different conditions, exploring how to treat the resulting count matrix by applying different normalization methodologies and how obtain differentially enriched regions between multiple conditions. Moreover, we showed how this regions can be integrated with other omics, such as RNA-seq, in order to produce a double level of integration by annotating the common results with functional approaches.

While working with omics we noticed the difficulties of keeping track all the processes involved into the analysis, in particular when the results have to be shared with collaborators and/or to be published into a scientific work. We generally refer to any type of solutions to this problem with Reproducible Research. To help with this, even during single omic analysis, we presented *easyReporting*, a possible approach to the reproducible research inside R. This tool is in the form of an R6 class which helps not only software developers to include reproducible research layers inside their tools, but also non-expert R analysts to easily produce reports for their analysis.

Even with the most simplified instruments, analysing omics data can be very difficult and time demanding, in particular for non-expert users. In order to speed up and help also novice users to analyze multiple omics data, we presented Integration of High-Throghput Omics data (IntegrHO), a web graphical user interface for the analysis of omics data. Combining the flexibility of a point-and-click approach with the power of R/Bioconductor statistical approaches for the omics data analysis we proposed a novel graphical user interface for multiple omics data analysis and integration. Despite other approaches for the multiple omics data analysis, IntegrHO is not pipeline oriented, leaving

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to the user maximum flexibility during the analysis and integration processes, enabling him/her to spread across the functionalities. In order to keep track of the performed analysis steps, IntegrHO has been equipped of a Reproducible Research layer which registers all the code and the processed data. Moreover, it enables to live edit the final report with aid of dedicated interface.

By developing so many different solutions taking advantage of so many already published tools, it emerges that each already published approach for the data analysis proposes its own data structures and methodologies, making it difficult to process the same data across multiple tools. Indeed, it is not for everyone to work with multiple already published bioinformatics instruments, implemented by so many different authors. Each tool has its own data structures needing dedicated data preprocessing and subsequently assembling, even if the starting data type is the same (such as a count matrix). The same idea has to be imagined with the final output of each step. For example, when using a method for testing the differences between multiple biological conditions, independently by the used method, it starts by a count matrix and produces a differential expression matrix (or a dataframe). But, at the moment, each tool produces its own final data structure, with its own variables that are related to the adopted statistics to obtain the final results.

With our instruments, we tried not only to facilitate the use of so many different instruments but also to mask different implementation problems due to different authors, by producing each time the same typology of data structure. In such a way, the final user, whatever is his/her background should be able to easily interpret the results.

Despite the efforts of the bioinformatics community, a global standard has not been reached yet, highlighting that the road is still long to be covered in order to allow as many people as possible to work and integrate multiple omics data and discover novel biological facts.



## Chapter 7

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